A study of root aphid *Aploneura lentisci* Pass. biology and root aphid-host interactions with perennial ryegrass/endophyte associations in New Zealand

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ABSTRACT

The root aphid *Aploneura lentisci* Pass. is an underestimated, under-researched pasture pest likely to become more problematic in New Zealand if the environmental temperature and the frequency of water deficit stress increase, as predicted. The research presented here aimed at gaining first insights into its biology and interaction with plants and endophytes to promote future pest management research. For this purpose, root aphids were observed in model systems (in climate chambers, glasshouse or insectary; in empty microcentrifuge tubes or on diploid perennial ryegrass *Lolium perenne* L. plants grown on nutrient-enriched agar, with or without endophy *Epichloë festucae* var. *lolii* [Latch, M.J. Chr. & Samuels] C.W. Bacon & Schard of the AR1, AR37 or common-toxic CT strains).

Apterous neonate offspring, the presumed main dispersal stage of *A. lentisci*, survived up to four weeks without food (median survival: 8 days). On endophyte-free, mature ryegrass kept at 17 to 21 °C, neonates developed to adults within three to four weeks and lived about two months, feeding mainly on young roots of first and second branching order. Taking into account lower outdoor temperatures, root aphids are thus likely to complete six to nine generations per year in the field. Adults produced 39 to 70 offspring over their lifetime. Presuming a similar nymphal mortality in the field as in the experiments, outdoor root aphid populations could theoretically multiply 23- to 45-fold at each generation.

Root aphids raised on endophyte-infected, mature plants were shorter-lived than peers raised on endophyte-free plants. Most aphids on AR37-infected plants did not even reach reproductive maturity. The response to CT-infection was dependent on the plant genotype. Why AR1-infected plants frequently support larger root aphid populations than endophyte-free plants in the field could not be explained by the data collected, however.

Root aphid feeding affected the root biomass but not the shoot biomass of perennial ryegrass in the experimental environment. This finding differed from previous reports. Furthermore, colour analyses suggested root aphid feeding could modify some leaf properties. More research will be required to confirm these findings and assess whether irrigation or fertilisation could mitigate root aphid yield losses in the field.

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Equation 2.2 Initial root/shoot ratio

$$\text{Root/Shoot}_{\text{Start}}\ i\ [\%] = \frac{\text{Root DM}_{\text{Start}}\ j\ [\text{g}]}{\text{Shoot DM}_{\text{Start}}\ j\ [\text{g}]} \cdot 100$$

Equation 2.3 Initial dry root (or shoot) biomass

$$\text{Root DM}_{\text{Start}}\ i\ [\text{g}] = \frac{\text{Root DM}_{\text{Start}}\ j\ [\text{g}] \cdot \text{Tiller FW}_{\text{Start}}\ i\ [\text{g}]}{\text{Tiller FW}_{\text{Start}}\ j\ [\text{g}]}$$

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$$\text{Age of aphid at event } x \text{ [days]} = \text{Date of event x – date of day 1 + 1}$$

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$$\text{Aphid occupation [days]} = \text{Date of aphid harvest – date of aphid placement + 1}$$

Equation 2.17 Reproductive rate

$$RR\ [\text{offspring/day}] = \frac{\text{Number of offspring collected}}{\text{Reproductive time [days]}}$$

Equation 3.1 Instar on day *i*

$$\text{Instar } (i) = \text{Number of exuviae removed up to day } i + 1$$

Equation 3.2 Instar on day *i* (mature aphids only)

$$\text{Instar } (i) = \text{5- Number of exuviae found after day } i$$

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$$\text{Size (Age) [mm or mm}^2\text{]} = \frac{\mathbf{d} - \mathbf{c}}{\left(1 + \mathbf{e}^{\left(\mathbf{b} \cdot \left(\log_e(\mathbf{Age[days]}) - \log_e(\mathbf{f})\right)\right)}\right)^{\mathbf{g}}}$$

Equation 3.4 Reproductive lifespan

$$RLS_i\ [\text{days}] = \text{Age at exit [days]}_i - (\text{Age at reproduction [days]}_i - 3)$$

Equation 3.5 Average general reproductive rate

$$aRR\ [\text{offspring/day}] = \frac{1}{n} \cdot \sum_{i=1}^{n} \left(\frac{\textbf{Total number of offspring produced by } \boldsymbol{i}}{\textbf{RLS } \boldsymbol{i}} \right)$$

Equation 3.6	Average lifetime fecundity	Average lifetime fecundity [offspring/established aphid] = $\mathbf{aRR} \cdot \frac{\mathbf{1}}{\boldsymbol{m}} \cdot \sum_{\boldsymbol{k=1}}^{\boldsymbol{m}} (\mathbf{RLS}\boldsymbol{k})$
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$$\frac{}{(\text{AgePlant}_{\text{Tx}}\, i - \text{AgePlant}_{\text{T1}}\, i)}$$

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$$\text{CF}i = \text{G}i \cdot (\text{AgePlant}_{\text{AphidCollection}}\, i - \text{AgePlant}_{\text{Tx}}\, i)$$

Equation A11.2.3 Time-adjusted parameter

$$\text{Parameter}_{\text{Adj.}}i = \text{Parameter}_{\text{Tx}}\, i + \text{CF}i$$

LIST OF ABBREVIATIONS

ABO	Average branching order
aE	Endophyte status of the plant the ancestor (i.e. the mother of the observed root aphid) lived upon
AgeM	Age of root aphid at maturity (when it has produced its first offspring)
AgePAP	Age of plant at aphid placement
AIC	Akaike information criterion
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
aPG	Plant genotype of the plant the ancestor (i.e. the mother of the observed root aphid) lived upon
AphP	Aphid placement
BO	Branching order of a root
BS	Blocking solution
CT	Common-toxic strain of endophyte (sometimes referred to as "wild-type" or "standard" endophyte; AR93 in clone-plants)
DM	Dry matter, i.e. dry weight
$\mathbf{DM_{Harv}}$	Dry matter (i.e. dry biomass) at harvest
$\mathbf{DMref_{Start}}$	Dry matter content of one or several reference tiller(s) at the beginning of an experiment.
$\mathbf{DM_{Start}}$	Dry matter (i.e. dry biomass) at the beginning of an experiment (after tiller trim)
$\mathbf{E^-}$	Endophyte-free plant
$\mathbf{E^+}$	Endophyte-containing plant

ELISA	Enzyme-linked immunosorbent assay
EP	Ellipsoid body projection
EPadult	Ellipsoid body projection of a root aphid as adult (size as adult [mm^2], estimated as body length/2 ·abdominal width/2·π)
EPneo	Ellipsoid body projection of a root aphid as neonate (size as neonate[mm^2], estimated as body length/2 ·abdominal width/2·π)
FW_{Harv}	Fresh weight at harvest
FW_{Start}	Fresh weight at start, just after a tiller has been trimmed for experimental purposes
G ratio	Green ratio; Colour measurement coefficient, calculated from reflectance measurements in the RGB colour model space as follows: G/(R+G+B), with R, G and B being the red, green and blue reflectance measurements in the RGB colour space.
GL	Number of green leaves (GLi: number of green leaves at aphid placement; GLf: number of green leaves at final harvest)
GSA (T1, T2)	Green shoot area [mm^2]; green blade and sheath surface visible on a two-dimensional projection of a plant (i.e. on a photograph taken at a first time point T1 or a later time point T2; Section 2.4.2)
HRM	High resolution melting
KW	Kruskal-Wallis test; non-parametrical test for groups with > 2 levels
L	Body length of root aphids [mm]
Ladult	Body length of a root aphid as adult [mm]
Lneo	Body length of a root aphid as neonate [mm]
LFA	Long-term feeding aphids. Number of adult and older immature aphids found on a plant at harvest; all aphids minus first instars.
LME	Linear mixed-effects models
L_{Ref}	Light reference value, i.e. the average light intensity measured outside during daylight hours next to the Watchdog weather station;

	This value was used as 100%, to compare the differences in light intensity between various locations (glasshouses, insectary, climate chambers; Appendix 2)
MANCOVA	Multivariate analysis of covariance
MANOVA	Multivariate analysis of variance
MBM agar	Modified Bollard's medium [(Bollard, 1966); Appendix 7]
MC tube	Microcentrifuge tube (1.5 to 2.0 mL tube with lid)
MWU	Mann-Whitney-U non-parametrical test for groups with exactly 2 levels
N-AR1	A clone of a perennial ryegrass genotype (named N) of the cultivar 'Grasslands Nui', hosting the AR1 endophyte strain
N-AR37	A clone of a perennial ryegrass genotype (named N) of the cultivar 'Grasslands Nui', hosting the AR37 endophyte strain
NCM	Nitrocellulose membrane
N-CT	A clone of a perennial ryegrass genotype (named N) of the cultivar 'Grasslands Nui', hosting the common-toxic endophyte strain AR93
Nil or NIL	Endophyte-free plant; synonym: E^-
NIRS	Near infrared reflectance spectroscopy
nm	Nanometer
N-NIL	An endophyte-free clone of a perennial ryegrass genotype (named N) of the cultivar 'Grasslands Nui'
nRBd	Normalised red (R)-blue (B) difference; colour measurement coefficient, calculated from reflectance measurements in the RGB colour model space as follows: nRBd = (R-B)/(R+B)
PA	Plant age period (see Table A12.2.1.1)
PAR	Photosynthetically active radiation (μmol of photons$\cdot m^{-2}\cdot s^{-1}$)
PD	Petri dish

PG-E	Plant genotype-endophyte status combination; describes groups
R^2c	Conditional R^2; Variance explained by the fixed and random factors in a mixed-effects model, as calculated by the 'MuMIn' R package (Barton, 2018)
relAgePAP	Age of plant at aphid placement, in relation to the first day an aphid was placed during the Biology II experiment (date of placement - 14/11/2013)
RO-water	Water purified by reverse osmosis
S&D	Senescing and dead
S&DL	Number of senescing and dead leaves (S&DLi: number of senescing and dead leaves at aphid placement; S&DLf; number of senescing and dead leaves at the final harvest)
S-AR1	A clone of a perennial ryegrass genotype (named S) of the cultivar 'Grasslands Samson', hosting the AR1 endophyte strain
S-AR37	A clone of a perennial ryegrass genotype (named S) of the cultivar 'Grasslands Samson', hosting the AR37 endophyte strain
S-CT	A clone of a perennial ryegrass genotype (named S) of the cultivar 'Grasslands Samson', hosting the common-toxic endophyte strain AR93
SD	Standard deviation
S-NIL	An endophyte-free clone of a perennial ryegrass genotype (named S) of the cultivar 'Grasslands Samson'
W	Abdominal width measurement, dimension of aphid at the widest point of the abdomen [mm]
Wadult	Abdominal width measurement of a root aphid as adult [mm]
Wneo	Abdominal width measurement of a root aphid as neonate [mm]

CHAPTER 1: LITERATURE REVIEW

1.1. Introduction

Grasslands are an important resource for New Zealand's economy. Dairy cows, cattle, sheep and deer generate over 70% of the gross agricultural production in New Zealand. Furthermore, grazing animals generate about 43% of New Zealand's total exports (The Treasury, 2016). Preserving the productivity of New Zealand grasslands is, therefore, a matter of national importance.

Among a range of pasture pests, the root aphid *Aploneura lentisci* Pass. deserves closer attention. *A. lentisci* uses diverse grasses as hosts, reproducing asexually on them while feeding from the phloem of their roots (Blackman & Eastop, 1994; Wool & Kurzfeld-Zexer, 2008; Cottier, 1953). *A. lentisci* is a known pest of wheat and barley (McDonald *et al.*, 2011; Mustafa & Akkawi, 1987; Wool & Kurzfeld-Zexer, 2008). Its impact on forage grasses and its potential as a pasture pest in Australia and New Zealand have probably been underestimated in the past, however (Clement, 2009; Popay & Gerard, 2007). According to recent estimates, *A. lentisci* may reduce the foliar growth and persistence of potted perennial ryegrass plants by up to 27% and 35%, respectively (Popay & Cox, 2016).

In New Zealand's grassland ecosystems, microscopic fungi known as asexual *Epichloë* endophytes [formerly *Acremonium* or *Neotyphodium* (Leuchtmann *et al.*, 2014)] play an important role. Fungi of this group live asymptomatically within the tissues of living plants (Popay, 2004; Popay & Thom, 2009) and obtain assimilates from them without causing host cell death (Cheplick, 2004). In exchange, they confer a certain degree of protection against herbivory and may improve the plants' tolerance to heat and drought stress (Grasslanz Technology Ltd, 2010a; Hume & Cosgrove, 2005). In the last century, endophytes were discovered to be a source of problems, causing ryegrass staggers and heat stress in livestock (Appendix 2). Currently, selected endophytes that do not harm cattle and sheep but deter and inhibit the development of insect pests are widely used to protect pastures from insect damage throughout New Zealand (Grasslanz Technology Ltd, 2010b; Popay & Thom, 2009; Card *et al.*, 2011; Ruppert *et al.*, 2017). Some of these endophyte strains are known to influence *A. lentisci* [e.g. Popay & Cox (2016), Pennell *et al.* (2005)]. The mechanisms behind such influences are not well

understood, however. In particular, plants infected with the strain known as AR1 often appear more susceptible to root aphids than even endophyte-free plants (Popay & Hume, 2011).

The research reported in this thesis aimed to provide first insights into *A. lentisci*'s biology and interactions with plants and endophytes to assist future research about this pest (Section 1.7, Table 1.6 for more detailed objectives). Achieving this goal in a full field ecosystem poses practical difficulties, however. The relationship between plants and endophytes alone is complex. Besides plant genetics and endophyte genetics, plant-endophyte interactions and environmental factors such as season, climate, nutrient availability, etc., can influence the outcome of an experiment (Popay, 2004; Rasmussen *et al.*, 2008a). Environmental interactions can also interfere with actual interspecific genotype-by-genotype interactions (Zytynska & Weisser, 2016) between insects, plants and endophytes. The insects' feeding activity may further change the plant and the biotic and abiotic surroundings of the system (Popay & Cox, 2016). Feeding root aphids, for example, release various organic compounds such as wax or honeydew when they feed (Wool & Kurzfeld-Zexer, 2008). Such secretions can boost the microbial population or the microbial activity in the soil, which may in return have an impact on the nutrient availability for the roots (Dixon, 1985; Hoffmann, 2016; Katayama *et al.*, 2014). A simplified model system was thus chosen for experimental purposes. It consisted of perennial ryegrass *Lolium perenne* L., the most widely sown species in New Zealand pastures (Easton *et al.*, 2001), without an endophytic symbiont ('endophyte-free', E^-) or infected (E^+) with one of the economically relevant *Epichloë festucae* var. *lolii* endophyte strains AR1, AR37 or common-toxic (CT), grown in nutrient-enriched agar.

The results of five main experiments and one observation on living model plants, two follow-up experiments *ex planta* and four pre-trials are reported in this thesis (Section 1.7, Table 1.6). An experimental overview and a general description of the material and methods used for this research are reported in Chapter 2. To explain the methodology of Chapter 2 and provide a foundation for discussion of results in Chapters 3 to 6, the following sections will summarise what is known about (root) aphids (Section 1.2), perennial ryegrass *L. perenne* (Section 1.3), endophyte *E. festucae* var. *lolii* (Section 1.4), and the interactions between these three component and abiotic and biotic grassland elements (Sections 1.5 and 1.6). Finally, the most pressing gaps in knowledge, the aims of this thesis and its structure are recapitulated in Section 1.7.

1.2. Biology and ecology of *A. lentisci*

1.2.1. *A. lentisci* in New Zealand: history and significance

Aploneura lentisci Passerini [*Tetraneura lentisci* Passerini, 1856 (Eastop, 1966)] is native to the Mediterranean area, but was likely introduced to New Zealand more than 80 years ago (Cottier, 1953; Popay, 2008). Formally identified specimens were collected in Palmerston North in 1937 (Cottier, 1953). Cottier (1953) suspects *A. lentisci* was observed in New Zealand as early as 1904, however, but was mistakenly recorded under a wrong species name at that time. Today, *A. lentisci* is common throughout New Zealand (Jensen & Popay, 2007).

A. lentisci has not been studied sufficiently to allow a comprehensive estimation of its economic significance in New Zealand (Ferguson *et al.*, 2018). Cottier (1953) stated that *A. lentisci* is of no economic importance in the country. This opinion has been challenged recently, however, when experiments with endophyte-infected plants suggested *A. lentisci* could significantly contribute to ryegrass pasture failure (Figure 1.1) and reduced herbage yields (Agricom & PGG Wrightson Seeds, 2015; Popay, 2008; Popay & Cox, 2016). Such symptoms are very general and multifactorial, however. Besides endophyte presence, other factors such as environmental conditions and other plant stresses can modulate the impact of an aphid infestation (Cosgrove *et al.*, 2018; McDonald *et al.*, 2011; Miles, 1989b). While *A. lentisci* is now recognised as a possible problem, more research is needed to quantify its New Zealand-wide pest potential (Ferguson *et al.*, 2018; McDonald *et al.*, 2011).

Figure 1.1. Plots of tetraploid ryegrass with AR1, AR37 or Endo5 (AR5) endophyte strains at Ballarat (Australia) [Reproduced from Agricom & PGG Wrightson Seeds (2015), with permission from PGG Wrightson Seeds]. The trial was sown in April 2008 and the picture taken in February 2010, after a period of summer drought with concomitant infestation by *A. lentisci*.

1.2.2. Identification

Aploneura lentisci belongs to the family Aphididae [Order Hemiptera, Sub-order Sternorrhyncha (de Jong *et al.*, 2014; Nieto Nafria, 2018)], a group with specific body structures (Figure 1.2). Through parthenogenetic reproduction, it gives birth to small, elongate and light yellow first instar nymphs ['crawlers' (Wool & Kurzfeld-Zexer, 2008)]. These nymphs develop over several moults to either alate (winged) or apterous morphs (wingless) with adult characteristics as described in Table 1.1. The apterous, asexually reproducing morph of *A. lentisci* commonly found on grass roots in New Zealand (Popay & Cox, 2016) is easily distinguished from other aphid species by the following characters: (a) short antennae with a short terminal process on the last antennal segment (less than half the length of the base of the same segment), (b) no siphunculi on the abdomen, and (c) a broadly spindle-shaped body that is wax-covered in life (Blackman & Eastop, 1984; Blackman & Eastop, 2000; Eastop, 1966).

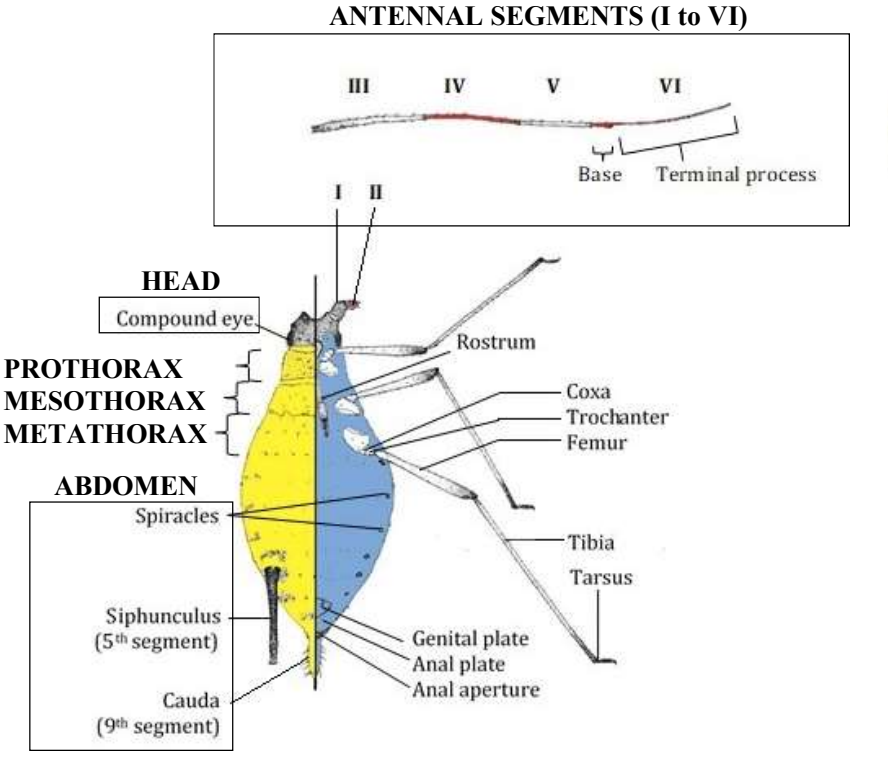


Figure 1.2. Aphid terminology, illustrated on an example of an apterous vivipara of *Macrosiphum rosae* seen from above (left side, yellow) vs. below (right side, blue). Adapted from Eastop (1966), with permission from CSIRO Publishing.

Table 1.1. Identification and anatomic description of adult apterous and alate *A. lentisci* morphs. (Blackman & Eastop, 1984; Cottier, 1953; Eastop, 1966; Mustafa & Akkawi, 1987)

	Apterous morph	Alate morph
	 	 
Antennae	Short, 4 to 5-segmented; segment II as long as segment I; sub-circular sensoria on segment IV (one, medium-sized) and V only (one large- and 1-3 small-sized; all primary rhinaria, no secondary rhinaria)	Short, 6-segmented, with one large sub-circular sensorium per segment on segments III to VI (primary rhinaria on segments V and VI, secondary rhinaria on segments III and IV)
Head	Darker coloured than the body	
	A pair of dark red to almost black eyes with only 3 facets	3 ocelli, in addition to a pair of compound eyes
	Ultimate rostral segment approx. same length, as 2nd tarsal segment of hind leg	Ultimate rostral segment little more than ½ length of 2nd tarsal segment of hind leg
Body	1.1-3.0 mm long; about 10-19x length of antenna, pale yellow, yellow or light green	1.3-2.3 mm long, 4-5x length of antenna, light brown
	Anal plate and cauda approximately the same size	Anal plate and cauda approximately the same size
Thorax	Three clearly defined segments	Three segments, with fused meso- and metathoracic segments
	One pair of legs attached to each segment	One pair of legs attached to each segment
	No wings	Four membranous and transparent wings attached to the fused segments, reposing flat on the back (venation pattern shown in Section 1.2.4.5).

1.2.3. Life cycle and ontogeny

Aphids are hemimetabolous insects with complex life cycles (Podsiadlowski, 2016). A complete life cycle in this insect family describes all generations leading up to the production of a new genotype through sexual reproduction. It can include several events of asexual reproduction ('parthenogenesis') and aphids of different phenotypes (Mukherjee & Baudach, 2016; Podsiadlowski, 2016). Although of a same genotype, the nymphs produced over a life cycle may indeed develop into various adult morphs in response to diverse extrinsic and intrinsic signals such as temperature, photoperiod, air humidity, quality of host plants, crowding during sensitive periods, maternal effects and alternating developmental mechanisms, for example ('multiple, environment-dependent polymorphism/polyphenism'; Dixon, 1973; Dixon, 1985; Lambers, 1966; Moran, 1992, Mukherjee & Baudach, 2016). Which signals trigger the production of a specific morph vary by aphid species and morph (Dixon, 1973; Lambers, 1966; Moran, 1992).

The aphids produced in the course of a life cycle each pass through a number of nymphal stages (or 'larval instars') before achieving their adult morph (Lambers, 1966). This individual development of one particular aphid will be referred to hereafter as 'ontogeny'and is to distinguish from a genotype's life cycle.

In its native range in the Mediterranean region, *Aploneura lentisci* has a life cycle of two years with host change (Figure 1.3). A fertilized egg that has successfully overwintered on the primary host *Pistacia lentiscus* L. hatches as a new genetic entity ('fundatrix'). This morph moves to a new shoot where it induces a leaflet to form a kidney-shaped gall (Wool & Kurzfeld-Zexer, 2008). Within this closed, protected structure, the fundatrix develops and produces offspring parthenogenetically, amplifying the number of aphids in a gall within ≥ 2 generations from the one founding individual to several hundred. The last generation produced in autumn is alate ('migrantes'). Each of these alatae leaves the gall with seven to eight embryos in the abdomen, ready for deposition, to establish on the roots of a secondary host (various grasses; Wool & Manheim, 1986). The wingless generation ('apterous virginoparae') viviposited on the roots by these alatae develops and reproduces parthenogenetically for several more generations while spending the winter below ground in waxy colonies. Alate virginoparae morphs may be produced for dispersal at this stage. Alate sexuparae may develop in the apterous colonies in the spring of the second year. These fly back to the primary host

where they give birth to sexuales, i.e. males and oviparae (egg-producing females). Males and oviparae mate. One egg develops and overwinters in the shrivelled female skin to start a new cycle in the following spring (Blackman & Eastop, 1984; Wool, 1990; Wool & Manheim, 1986).

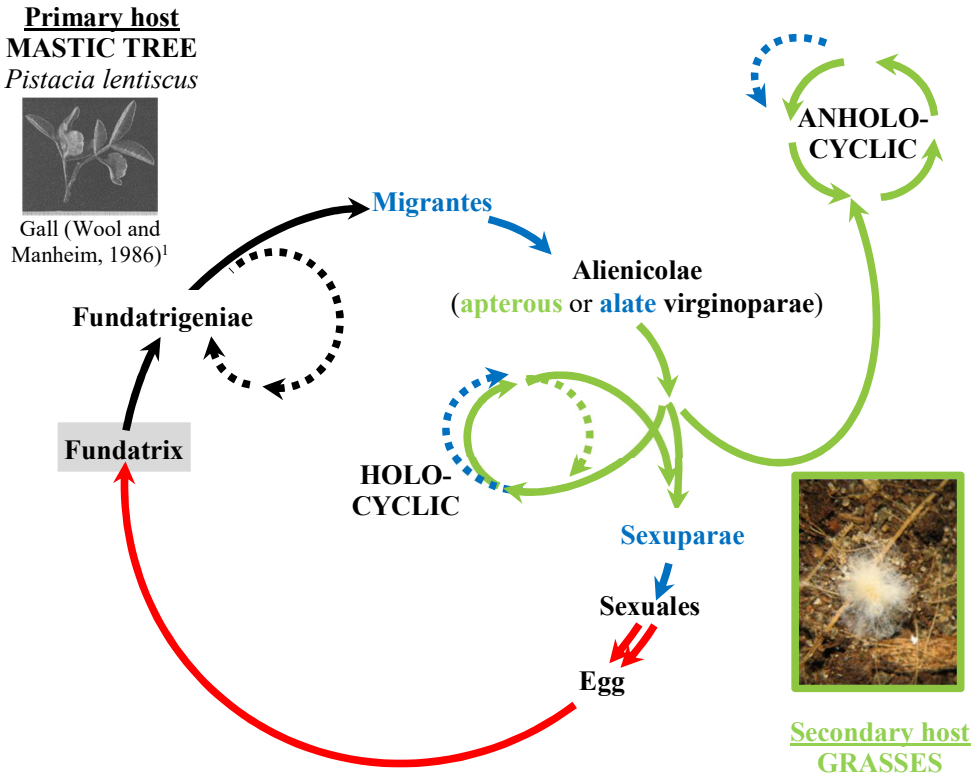


Figure 1.3. Stylised life cycle of *A. lentisci*, for a given aphid genotype. It starts with a hatching fundatrix and is completed within two years in areas where the primary host *P. lentiscus* is present. Blue arrows/text represent winged morphs. The other morphs are wingless. Steps represented by black arrows mandatorily happen on *P. lentiscus*. Steps shown in green occur on the roots of a secondary host. Steps shown in red may possibly take place elsewhere. Dashed arrows symbolize presumed but not yet reported development steps. Sources: Cottier (1953); Wool (1990); Wool and Manheim (1986); Wool and Sulami (2001); [1]Reprinted by permission from Springer Nature, Wool and Manheim (1986) "Population ecology of the gall-forming aphid *Aploneura lentisci* (Pass.) in Israel", *Researches on Population Ecology*, 28, 151-162 [Figure 1, p 152].

A. lentisci is unlikely to complete a full life cycle in New Zealand since the primary host *P. lentiscus* is not present in the country [personal communication of Alexandra McCabe, Plant and Forestry Import & Export (Plants) Group, Ministry for Primary Industries]. Large flights of sexuparae have been observed in New Zealand in late summer, however (Blackman & Eastop, 2000), suggesting New Zealand *A. lentisci* populations have not yet lost all abilities to reproduce sexually. Whether alate virginoparae are produced in significant numbers in New Zealand grasslands is not known.

Information on the individual ontogeny of *A. lentisci* is sparse too. An average generation interval of less than a month or 8 to 18 days from colonisation to reproduction has been reported for viviparous (asexually reproducing) colonies of Israeli *A. lentisci* kept at 19 to 20 °C, under permanent illumination, on wheat or barley seedlings (Wool & Kurzfeld-Zexer, 2008; Wool & Sulami, 2001). Presumably, *A. lentisci* moults four times in that time interval [most aphid species undergo four moults in their development from first instar to adult according to Dixon (1973)]. Observations are needed to confirm these presumed patterns for New Zealand *A. lentisci* populations, however.

1.2.4. Anatomy and physiology

Understanding interactions and population dynamics in root aphids (Sections 1.2.6 and 1.2.7), handling aphids (Section 2.3) and analysing aphid data appropriately (Sections 2.4, 3.2.2, and 5.2) requires knowledge of aphid anatomy and physiology. Sections 1.2.4.1 to 1.2.4.6 provide, therefore, a general review about feeding and nutrition-related features, growth, reproduction, sensory organs, wing development and wax production in aphids.

1.2.4.1. Feeding and nutrition

Aphids feed on phloem sap using piercing-sucking mouthparts composed of an inner pair of maxillary stylets and an outer pair of mandibular stylets. In resting position, these stylets are surrounded by a protective sheath (‘proboscis’ or ‘rostrum’; Miyazaki, 1987). When an aphid inserts its stylets into a plant to feed (Figure 1.4), its maxillary stylets are locked together to form a salivary canal and a food canal. The second segment

of the proboscis telescopes within the basal first segment, unsheathing up to 66% of the total stylet length in the process (Dixon, 1973; Dixon & Logan, 1973; Miyazaki, 1987).

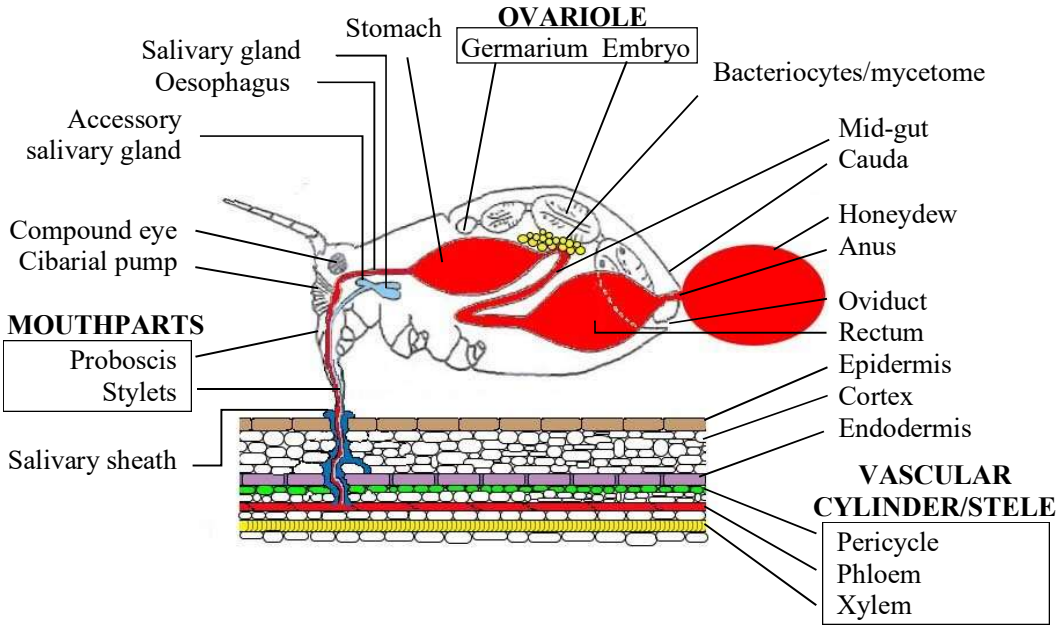


Figure 1.4. Diagram of a root aphid feeding on root phloem. Lateral view of an adult *A. lentisci* virginopara on a grass root; Adapted from Dixon (1973), [Figure 1.4, p 4].

How an aphid's mouthparts progress within plant tissues is fairly well documented. How sieve-tubes are found remains unclear, however. The stylets follow a mainly intercellular path. They likely pass through the weaker primary lamellae (the softer layer between the harder cell walls of two adjacent plant cells) and probe cell vacuoles along the path (Hewer *et al.*, 2011). Saliva secretions assist the stylets' progression: while salivary enzymes (pectinases, cellulases) break down the bonding between cells, a watery component probably prevents or reduces the intensity of a normal wound response in the plant (Dixon, 1985; Hewer *et al.*, 2011), and some components harden immediately to form a rigid lining along the feeding track ('salivary sheath'; Urbańska, 2010). This lining is likely allowing the stylets to control their direction and anchoring them when they pierce through cell walls (Dixon, 1985). It may also seal puncture holes and avoid leakage of cell contents that could interfere with the orientation of the stylet or trigger the synthesis of poisonous compounds (Hewer *et al.*, 2011). The stylets may progress in a pre-programmed radial orientation and re-orient when encountering fortified cell walls or

when they are exposed to specific stimuli (e.g. low concentrations of sucrose and low pH in the cells probed along the way; Hewer *et al.*, 2011). In any case, the stylets end up following a branched path from the plant surface to the sieve-tubes (Urbańska, 2010). That may preclude a rapid stylet withdrawal (Fisher & Frame, 1984).

The actual feeding starts once the phloem is reached, about 25 minutes to 1 h after the insertion of stylets has begun (Rosell *et al.*, 2003). Food may be driven passively by the hydrostatic pressure inside the sieve tubes, but at least one other control mechanism must be involved in the ingestion (e.g. cibarial pump; Figure 1.4) since aphids are able to feed on artificial diets that are not under pressure and do not excrete honeydew or swell up if anaesthetised while feeding (Dixon, 1985). During the feeding process, watery saliva is secreted in regular intervals and mixed with the sieve tube sap. The mix is then taken up. This procedure may prevent wound responses by the plant (e.g. call formation), avoid food canal occlusion by proteins, degrade plant protease inhibitors and/or detoxify problematic phytochemicals (Will, 2016). The phloem-saliva mix moves through the oesophagus into the stomach, intestine, hindgut and rectum (Ponsen, 1987). The digested mix is finally excreted through the anus as honeydew (Dixon, 1973). Unlike other insects, aphids have no Malpighian tubules (urinary system) and excrete nitrogenous waste as ammonia through the gut (Dixon, 1973; Ponsen, 1987).

Phloem sap is a rather unbalanced diet for aphids: it is rich in sugar beyond aphid needs (90% of the energy ingested is excreted), has a high osmotic pressure (2 to 4 times greater, than that of aphid body fluids), a high ratio of sugars:amino acids, a low ratio of essential:non-essential amino acids (1:4 to 1:20, when aphid body protein has a ratio of 1:1) and low lipid levels (Dixon, 1985; Douglas, 2003; Douglas, 2006; Sandström & Moran, 1999). To convert it into aphid biomass, various digestive processes and endosymbiotic bacteria have to intervene. Sucrose, the phloem's main sugar, is digested to fructose and glucose in the alimentary tract. While the fructose is absorbed with high efficiency and used to cover the energy demands of aphid cells and synthesize lipids, the glucose is mainly incorporated into oligosaccharides and excreted in the honeydew (Douglas, 2003; Douglas, 2006; Hoffmann, 2016). Both processes reduce the concentration of sugars from 1 M in phloem sap to 0.2 to 0.3 M in honeydew, thereby decreasing the osmotic pressure in the gut (Douglas, 2006). Further osmoregulation may occur through water transfer from the hindgut to the stomach (stomach content dilution) and through periodical xylem drinking (Douglas, 2006; Pompon *et al.*, 2010; Shakesby

et al., 2009). The unbalanced profile of the free amino acids, the main source of nitrogen in phloem sap, is remediated in most aphid species by endosymbiotic *Buchnera* spp. bacteria (Podsiadlowski, 2016; Sandström & Moran, 1999).

Buchnera bacteria are *E. coli*-related, Gram-negative bacteria also known as 'primary' or 'obligate' endosymbionts. They live in the body cavity of aphids, within special aphid cells ('bacteriocytes') aggregated in a structure called a 'mycetome' or 'bacteriosome' (Martinez-Torres *et al.*, 2001; Vilcinskas, 2016). *Buchnera* are thought to have evolved in a close relationship with aphids for 100 to 200 million years. They are transmitted from a mother aphid to her offspring and cannot be cultivated outside of their aphid host (Martinez-Torres *et al.*, 2001; Skaljac, 2016). They are essential for survival on a phloem diet: aphid nymphs deprived of their *Buchnera* symbionts by antibiotic treatment grow slowly and do not reproduce on plants (Dixon, 1985; Douglas, 2006; Martinez-Torres *et al.*, 2001). Besides supplying a large proportion of the essential amino acid requirements (e.g. 90% in *Acyrthosiphon pisum* Harris), *Buchnera* bacteria may also provide vitamins and sterols to the aphids (Douglas, 2006; Hoffmann, 2016; Martinez-Torres *et al.*, 2001).

Other non-essential ('secondary') endosymbionts of various families that do not reside in a specialized organ may also be nutritionally relevant for aphids. *Serratia symbiotica*, for example, supplies nutrients and could thereby modify its host aphid's range of colonisable plants (Skaljac, 2016). The function of secondary endosymbionts is not limited to the mitigation of nutritional challenges, however. Heat tolerance and enhanced resistance to pathogens and parasites have also been reported (Enders & Miller, 2016; Skaljac, 2016).

Whether the nutritional anatomy and physiology of *A. lentisci* differ significantly from that of other aphids is not known. However, in the light of the information above, it appears nevertheless advisable to handle feeding aphids patiently and to include maternal effects into statistical analyses whenever possible, to account for the influence of maternally-transmitted endosymbionts.

1.2.4.2. Growth

Size (weight or body dimensions) has multiple consequences for an aphid's biology and ecology. Weight is positively correlated with aphid fitness (Bastias *et al.*, 2017).

Offspring with a large initial body weight develop faster to maturity, have a higher survival rate and a longer reproductive period than smaller peers (Traicevski & Ward, 2002). Large adults tend to produce larger offspring and be more fecund than smaller aphids (Dixon, 1985; Honěk, 1993; Traicevski & Ward, 2002). Body fat increases with aphid size, suggesting that aphids of a large body stature should be more resistant to starvation than smaller individuals (Dixon, 1984). Aphids with a high body weight, a long body and long legs may also walk faster and disperse farther than smaller younger aphids of the same species and other aphid species not posessing such attributes (Ben-Ari *et al.*, 2015; Tokunaga & Suzuki, 2008). Finally, Dixon (1985) reports also a correlation between aphid size and depth of phloem fed upon. A larger body could, therefore, allow the use of resources inaccessible to aphids of smaller size and offer some foraging advantages.

Aphid growth has two components: a more or less continuous growth of mass and, generally, four events of episodic growth of the exoskeleton known as 'ecdyses' (Nijhout, 2013; Dixon, 1973). Both forms of growth are interrelated as the amount of growth at a moult depends upon the biomass accumulated in the time since the last moult (Nijhout, 2013). Various factors such as temperature, crowding and host plant quality can influence this biomass accumulation. Aphids achieve a smaller adult size at high temperatures than at low temperatures, for example, an observation explained by increasing catabolic reactions by elevated temperatures. Aphids developing in crowded conditions are smaller than aphids raised in isolation (Dixon, 1985). Poor hosts (e.g. mature plants, plants expressing antibiotic traits) can also result in a decreased body size (Dixon, 1985; Smith, 1989). Therefore, comparative size analyses may give valuable information on plant suitability as an aphid habitat.

1.2.4.3. Reproduction

Little is known about the reproduction patterns of *A. lentisci* in New Zealand. Therefore, this section will review what is known about aphid reproduction in general.

Aphids are diploid insects (2n = 16 for *A. lentisci*; Blackman & Eastop, 1984) with the potential to multiply both asexually or sexually (Dixon, 1973). By asexual reproduction ('parthenogenesis' or 'virginopary'), aphids give birth to live, genetically identical offspring ('clones'; Skaljac, 2016). Neither meiosis nor fecundation is involved

in this process (Le Trionnaire *et al.*, 2008). By sexual reproduction, on the contrary, special morphs known as males and oviparae ('sexuales') are produced and combine haploid gametes through mating. The oviparae then lay eggs that each contain a new, distinct genetic entity (Dixon, 1973). The ability to reproduce sexually is apparently a matter of monofactorial intermediate inheritance (Table 1.2) in species such as *Myzus persicae* Sulzer (Dixon, 1985) and *Rhopalosiphum padi* L. (Simon et al., 1994). Genotypes able to produce sexuales generate males through a specialised meiosis procedure in which only the X-chromosomes undergo a reduction division. The resulting males are thus diploid for all chromosomes but the X chromosome [XX/X0 sex-determination system (Dixon, 1985)]. Special mechanisms eliminate spermatocytes without the X chromosome in the gonads of these males, ensuring that all eggs fertilised with their sperm will develop into diploid XX virginoparous aphids (Dixon, 1985; Moran, 1992).

Table 1.2. Inheritance of the ability to reproduce sexually, as reported for *Myzus persicae* Sulzer (Dixon, 1985) and *Rhopalosiphum padi* L. (Simon *et al.*, 1994). H: allele for holocycly (ability to reproduce sexually), a: allele for anholocycly (inability to reproduce sexually).

Genotype		**HH**	**Ha, aH**	**aa**
Phenotype	Life cycle type	holocyclic	androcyclic	anholocyclic
	Sexual reproduction	possible	(possible)	no
	Offspring – asexual	males and oviparae	males	-
	Offspring – sexual	virginoparae	virginoparae	virginoparae

The gonads of both asexually and sexually reproducing females are composed of two ovaries with four to six ovarioles each. They differ strongly in their anatomy and physiology, however (Figure 1.5). In virginoparae (asexual pathway), the germarium, a structure at the distal end of each ovariole, produces diploid embryos that develop continuously within the maternal body. At term, a fully active first instar offspring is born ('vivipary'), rear end first, and already carrying the developing embryos of the next generation (and their granddaughter's generation) in their abdomen ['telescoping generations' (Dixon, 1973; Dixon, 1985; Kindlmann & Dixon, 1989; Le Trionnaire *et al.*, 2008)]. In oviparae (sexual pathway), the germarium produces oocytes blocked in metaphase I of a meiotic division. These oocytes complete the meiosis process when they

are fertilized and are oviposited. The further development of the embryo proceeds in the egg, outside of the maternal body. It may be arrested for a time [‘diapause’ (Le Trionnaire *et al.*, 2008)].

Asexual and sexual reproductive strategies both have strengths and weaknesses. In asexual reproduction, a high concurrence amongst clones and lack of selection against recessive, deleterious or non-functional alleles may pose a problem (Dixon, 1985). However, asexual reproduction is less costly than sexual reproduction for a genotype: parthenogenesis doesn’t require resource allocation to winged sexuparae (Section 1.2.4.5) or (non-egg-laying) males, and yields more offspring per ovariole, than sexual reproduction (e.g. 6 to 10 embryos/vivipara ovariole, compared to 1 to 2 eggs/ovipara ovariole in *A. pisum*; Le Trionnaire *et al.*, 2008; Moran, 1992). There are also differences in reproductive output between asexual morphs, though: asexually produced winged morphs are generally less fecund than asexually produced apterous morphs [Section 1.2.4.5; Dixon, 1973; Moran, 1992]. A medium brood size of 4 offspring (with a significantly female-biased sex ratio in broods of > 5 offspring only) was reported for Israeli *A. lentisci* sexuparae raised in laboratory conditions, while apterous virginoparae produced 9 ± 1.3 and alate autumn migrants about 6 ± 0.2 virginoparous offspring per mother on average (Wool & Kurzfeld-Zexer, 2008; Wool & Sulami, 2001). Another benefit of asexual reproduction is its rapidity: parthenogenetically produced embryos develop faster than sexually produced embryos, and telescoping generations allow aphids to reproduce as soon as they reach adulthood (Kindlmann & Dixon, 1989; Le Trionnaire *et al.*, 2008). The generation interval is thus considerably reduced compared to sexual reproduction, which can be an advantage in the colonisation of new habitats and the escape from localised extinction by natural enemies (Dixon, 1985; Kindlmann & Dixon, 1989). However, telescoping of generation also means that the environment experienced by an aphid may have an influence over several generations. The maternal environment can, for example, influence the number of ovarioles allocated to the asexually produced offspring (Stadler, 1992). Later changes in the environment experienced by the offspring themselves may correct this to some extent, though. Under adverse conditions, the youngest embryos are selectively resorbed while under favourable conditions, extra embryo ovulation may occur (Dixon, 1985; Enders & Miller, 2016). The eggs produced by sexual reproduction usually survive cold periods (e.g. temperatures below -40 °C) better than other aphid stages. They may also persist through desiccation and times of

very poor feed quality, when other aphid stages may perish (Dixon, 1985; Moran, 1992). As a wider range of genotypes is produced through random fertilisation, there may be less competition between sexually produced siblings, than asexually produced clones (Dixon, 1985). Besides the higher costs and the longer generation interval (see above), the long, discontinuous embryogenesis process in sexual reproduction is also a disadvantage: it increases the risks of severe malformations (e.g. by heat exposure; Le Trionnaire *et al.*, 2008).

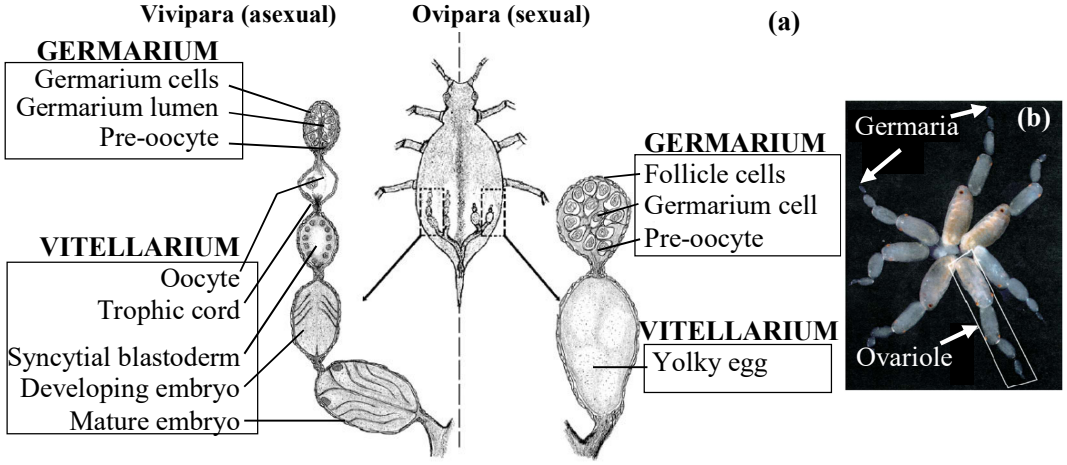


Figure 1.5. Aphid reproductive system. (a) Diagrammatic ovarioles for parthenogenetic viviparous (left) and oviparous females (right); reproduced from Le Trionnaire *et al.* (2008), Figure 3, p 444; Original credits: Blackman (1987) [Figure 3.8 © R. L. Blackman]; Lees (1983), [Figure V-5-2, © Alan R. Liss] **(b)** Dissected *A. pisum* ovary; reproduced from Miura *et al.* (2003) [Figure 3, p 64].

In many aphid species, the length of the scotophase is known as the most important external trigger for sexual reproduction, but temperature, intrinsic factors and nutritional signals from host plants are thought to interfere with it (Kawada, 1987; Le Trionnaire *et al.*, 2008). What induces the sexual pathway for *A. lentisci* is not known. As sexuparae of this species generally appear in spring (Section 1.2.3), decreasing night length and rising temperatures would be conceivable triggers. Sexuparae are also induced in laboratory experiments by permanent illumination, however. It is thus possible that crowding, alone or in combination with low temperatures would suffice (Wool & Sulami, 2001).

1.2.4.4. Sensory organs

Sensory organs help aphids to find their host plant and feeding sites (Dixon, 1985; Manheim & Wool, 2003). By transferring information about the environment, they may also modulate an aphid's behaviour and physiology [e.g. phototaxis and reproductive behaviour in response to light or day/night length changes (Hajong & Varman, 2002; Le Trionnaire *et al.*, 2008)]. Thus, understanding these in the case of *A. lentisci* could potentially assist with the development of future plant protection strategies.

Aphids have a multitude of sensory organs with various functions. Hairlike sensilla on the body may act as mechanoreceptors, with additional gustatory functions for sensilla on the legs (Anderson & Bromley, 1987). Chemoreceptors and tactile receptors at the tip of the proboscis may help find an appropriate site and analyse products derived from the interaction between saliva and plant epidermis when aphids are searching for a host or feeding site (Dixon, 1985). The membrane-covered, circular openings on the antennae containing receptor neurons ('rhinaria') are likely used in close-range chemoreception/gustation and are believed to reduce landing mistakes in alates (Boullis & Verheggen, 2016; Dixon, 1985; Manheim & Wool, 2003). In the dorsal wall of the food canal, at the base of the stylets, an epipharyngeal organ with the typical structure of a contact chemoreceptor possibly detects specific substances in the ingested sap (Dixon, 1985). Finally, three structures may act as receptors for light: (i) extraocular photoreceptors, (ii) ocelli, and (iii) compound eyes [primary compound eyes or 'triommatidia' and secondary compound eyes (Anderson & Bromley, 1987; Dixon, 1973; Döring & Chittka, 2007; Le Trionnaire *et al.*, 2008)]. The extraocular photoreceptors are located in the mid-dorsal region of the head of aphids, likely in the brain, below a semi-transparent part of the head capsule. They are very sensitive to blue light of 450 to 470 nm wavelength (Le Trionnaire *et al.*, 2008). They may help regulate circadian rhythms (Döring & Chittka, 2007). Ocelli are found on alatae and some male morphs only, one between the antennae and one above each secondary compound eye. They have an unpigmented cornea and a simple structure. They are probably capable of detecting changes in light intensity (Anderson & Bromley, 1987). Compound eyes are composed of a few to many ommatidia (visual units), with various types of photoreceptors (e.g. green, blue, and UV photoreceptors with maximum sensitivities at 530 nm, 440 to 480 nm and 320 to 330 nm, respectively, in the green peach aphid *Myzus persicae* Sulzer). They probably detect movements and allow a form of colour vision differing from the

human colour vision (Anderson & Bromley, 1987; Döring & Chittka, 2007). The newborn of some aphid groups may lack secondary compound eyes. All instars possess primary compound eyes, however, i.e. units of three ommatidia of similar, but simpler structure than secondary compound eyes, placed on stalks or tubercles behind these (Anderson & Bromley, 1987; Dixon, 1985).

Published literature does not report many specifics on the senses of *A. lentisci* or subterranean aphids. *A. lentisci* alates possess fewer rhinaria (Manheim & Wool, 2003) and show lower host selection capabilities than several other aphid species living on *Pistacia* spp. (Wool *et al.*, 1994). Whether this observation may be extended to apterous virginoparae living below ground is not known, however. The simple, 3-facetted eyes of virginoparae (Cottier, 1953) suggest that, like other underground aphids, root aphids are probably sensitive to light, however (Adams & van Emden, 1972). Verifying both points and whether *A. lentisci* can also sense disturbance and changes in soil moisture (Adams & van Emden, 1972) will require more research.

1.2.4.5. Wings

The following section focusses on virginoparous alatae born on grass roots because, of the three possible winged *A. lentisci* morphs (virginoparous alatae, sexuparae and migrantes; Figure 1.3), they are the morph most relevant for New Zealand's grasslands. Winged *A. lentisci* sexuparae have been reported in New Zealand (Blackman & Eastop, 2000). They are unlikely to significantly influence *A. lentisci*'s dispersal and population dynamics since the eggs produced by the sexuparae's offspring cannot be deposited on a *Pistacia lentiscus* tree (primary host) on which to complete their development (Section 1.2.3). In absence of *P. lentiscus*, migrantes should be a rare sight in New Zealand.

The flight apparatus of aphids is composed of three types of indirect flight muscles and two pairs of wings with species-specific venation pattern (Figure 1.6). It is functional in adults only (Klowden, 2013). Whether an aphid is winged as an adult is decided at two developmental switch points according to research on *Acyrthosipon pisum* (Ogawa & Miura, 2013). The always apterous fundatrices and oviparae are born without wing and flight muscle primordia [embryonic development switch point (Ogawa & Miura, 2013)]. By contrast, virginoparae (which may or may not become alates as adults) possess wing and flight muscle primordia at birth. These primordia are lysed and disappear around the

first moult in nymphs destined to aptery (post-embryonic switch point). In nymphs destined to become alate, the primordia develop further over each moult (Ogawa & Miura, 2013).

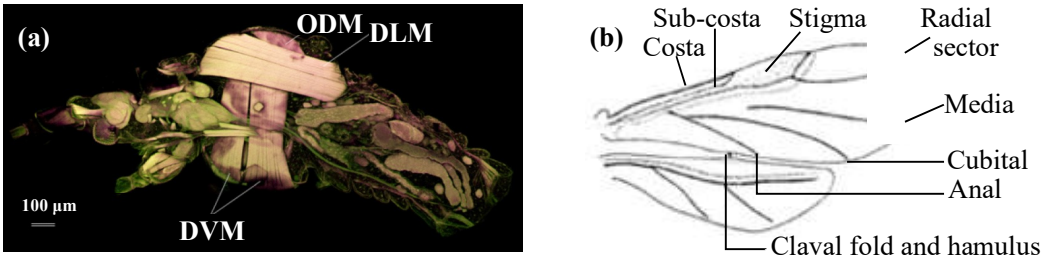


Figure 1.6. Flight apparatus of aphids. (a) Indirect flight muscles of a winged *A. pisum* male, confocal laser scanning microscope image; DLM: dorsal longitudinal muscle, DVM: dorsoventral muscle, ODM: oblique dorsal muscle. Reproduced from Ogawa and Miura (2013) [Figure 3a, p 4], originally published by BioMed Central Ltd. **(b)** *A. lentisci* wing venation pattern. Reproduced from Eastop (1966) [Figure 176, p 585], with permission from CSIRO Publishing.

Whether an apterous or alate pathway is followed at the post-embryonic switch point may be influenced by various factors such as light/photoperiod, temperature, air humidity, nutritional signals and tactile stimulation from crowding (of the mother, the offspring, or of both, mother and offspring) in certain sensitive periods (Dixon, 1973; Lambers, 1966; Wool & Kurzfeld-Zexer, 2008). Crowding on plants of low or decreasing nutritional quality, in particular, is often reported to result in a higher percentage of alate colony members (e.g. Dixon, 1973; Lambers, 1966). Release of alarm pheromones and tactile stimulation by avoidance behaviour in presence of natural enemies may also stimulate the alate production (Mukherjee & Baudach, 2016). Ant-tending, on the contrary, may impair alate production in some aphid species (Hoffmann, 2016). Finally, maternal effects may play a role: alate mothers usually give birth to apterous offspring, even in crowded conditions (Moran, 1992). Ant-attendance is unlikely to play a role in the case of the myrmecoxenous *A. lentisci* (Wright & McManus, 1998). Whether any of the other factor(s) may act as a specific trigger for alate virginoparae production in this species still requires research.

Winged morphs may disperse over long distances (Ben-Ari *et al.*, 2015) and seek new resources farther away than apterae, but these abilities come at considerable cost (Ogawa & Miura, 2013). Nymphs developing to alates may need more time to reach

maturity than nymphs developing to wingless adults (e.g. about 8 to 26% more time; Dixon, 1985). Furthermore, flight and maintenance of flight muscles has an energy cost, which may reduce the fecundity [e.g. 24% less offspring in *Drepanosiphum dixoni* Hille Ris Lambers with fully functional wings, compared to *D. dixoni* with non-functional wings (Dixon, 1973, 1985)]. The percentage of winged morphs produced in an aphid population is thus likely to significantly shape its population dynamics. Whether winged virginoparous *A. lentisci* represent an important aspect of New Zealand root aphid population dynamics is not known, though, and will require further study.

1.2.4.6. Wax

Feeding *A. lentisci* aphids surround themselves with copious amounts of wax (Popay & Cox, 2016; Wool & Kurzfeld-Zexer, 2008). Most of the hydrocarbons in this wax are synthesised by the insects themselves. That requires considerable resources and metabolic investments (Smith, 1999). Wax must therefore fulfil crucial functions for *A. lentisci*, such as perhaps (i) depository for honeydew to protect aphids from being "glued" to the vegetation or each other, (ii) protection against fungal attack, (iii) protection from parasites, (iv) cuticular transpiration control, (v) protection against humidity or rain and inoculative freezing, and/or (vi) protection against solar radiation, heat or ultraviolet radiation (Pretorius *et al.*, 2016; Smith, 1999).

Notwithstanding its function, wax provides information on plant quality "from an aphid's perspective", so to speak. Presence of wax confirms the aphid's acceptance of the plant as a host (see above). Furthermore, the plant's quality influences the quantity and quality of the wax produced (Dunn, 1974). The aphid *Adelges abietis* L. produces more wax on susceptible Norway spruce trees than on resistant Norway spruce trees, for example. The wax cover, in that case, is also correlated with maternal size, suggesting possible links between wax cover and fecundity (Björkman, 2000; Section 1.2.4.2). Wax measurements on *A. lentisci* should thus be considered for a complete picture of plant quality.

1.2.5. Influences of the abiotic environment on root aphids

Abiotic factors such as temperature, light, and humidity or drought can influence aphids both directly and indirectly, by interfering with the aphid's behaviour, development and reproductive performance, or by modifying a host plant's sap flow or composition. The following sections consider the known, direct abiotic effects.

Temperature is a central factor for aphid physiology, population growth and species distribution (Enders & Miller, 2016). It affects birth size, nymphal development, adult size, adult longevity, dispersal behaviour (flight), and morph production (Dixon & Kindlmann, 1994; Gange *et al.*, 1999; Manheim & Wool, 2003; Wool & Sulami, 2001). Temperature thresholds for survival (upper and lower), minimal temperature for development and thermal optimum vary by species, origin, instar and experienced rearing temperatures (Blackman, 1987; Hazell *et al.*, 2010; McCornack *et al.*, 2005; Skaljac, 2016). Furthermore, such thresholds are not absolute: temperature exposure pattern may also matter. For example, aphids acclimatised to mild cold or heat stress beforehand and aphids exposed to extreme temperatures in short bursts may better survive extreme temperatures than aphids suddenly exposed to a prolonged temperature stress (Ghaedi & Andrew, 2016; Jeffs & Leather, 2014). As a general rule, the egg stage is the most resilient towards cold. In some cases, eggs can survive temperatures below -40 °C (Dixon, 1985; Moran, 1992). Older developmental stages are more susceptible to chill injuries, but there are differences by stages. Nymphs, for example, show slightly higher freezing tolerance than adult aphids in various species (McCornack *et al.*, 2005). Development *per se* starts at temperatures above 0 to 6 °C in most aphid species (Brabec *et al.*, 2014). Higher temperatures accelerate the biological processes, shortening the development time to maturity and reducing the (asexual) generation interval (Dixon, 1985). The fecundity increases up to an optimal temperature (Dixon, 1973). However, the body size decreases with rising temperatures, a fact presumably explained by the balance between catabolic and anabolic reactions [more catabolism at higher temperatures may limit the energy available for anabolic processes such as growth (Dixon, 1985)]. At very high temperatures, aphid mobility, fitness and survival are reduced (Enders & Miller, 2016; Skaljac, 2016; Wool & Kurzfeld-Zexer, 2008). For many nymphs and adults, a critical temperature is reached around 38 to 40 °C (Enders & Miller, 2016; Skaljac, 2016). In some species, temperatures above 25 to 28 °C may already impair the fitness, however

(Skaljac, 2016). Besides direct effects on aphids, high temperatures have a negative impact on the obligate *Buchnera* endosymbionts (Section 1.2.4.1) and the number of bacteriocytes (Skaljac, 2016). Specific secondary endosymbionts may increase the heat tolerance of their host, however. *Hamiltonella defensa* and *Serratia symbiotica*, for example, allow heat-stressed nymphs to retain more of their bacteriocytes and heat-stressed adult aphids to maintain some reproductive activity (Skaljac, 2016).

Light can directly affect aphid populations by influencing the dispersal [e.g. positive phototaxis (Hajong & Varman, 2002)], or by influencing the aphid morph produced. Long days in combination with high temperatures may inhibit the production of alatae, for example (Dixon, 1973). Changes in photoperiod can furthermore influence the reproductive activity (Mukherjee & Baudach, 2016): day length changes (or more precisely, night length changes) can be a signal for sexuparae production (Le Trionnaire *et al.*, 2008; Moran, 1992). Finally, development time from birth to maturity may also respond to photoperiod in some aphid species [e.g. in *Uroleucon jaceae* L. (Stadler, 1992)].

Below-ground aphids are likely sensitive to changes in soil moisture (Adams & van Emden, 1972). Dry soil conditions are known to result in rapid population growth in various other root aphid species (Kindler *et al.*, 2004; Mustafa & Akkawi, 1987; Pretorius *et al.*, 2016). An influence of either humidity or aridity appears therefore likely. Whether humidity triggers the production of winged morphs as hypothesized by some sources (Lambers, 1966), affects the aphid behaviour and biology, or is just relevant in other ways, is still unknown, however.

How any of the above-mentioned factors affects New Zealand *A. lentisci* and their population dynamics has yet to be experimentally demonstrated.

1.2.6. Aphid-host relations

1.2.6.1. Host plants

The fundatrix of *A. lentisci* develops exclusively on *Pistacia lentiscus* L. (Wool & Manheim, 1986). *A. lentisci* is less limited in its options for secondary hosts, however. It has been occasionally recorded on the dicotyledonous *Ranunculus* sp. and *Veronica* sp.,

but is generally found on the roots of *Poaceae* such as *Bromus catharticus* Vahl, *Echinochloa crus-galli* (L.) Beauv., *Poa pratensis* L., *Poa annua* L., *Lolium perenne* L., *Cynodon dactylon* (L.) Pers., *Dactylis glomerata* L., and *Festuca sp* (Blackman & Eastop, 1984; Cottier, 1953; Eastop, 1966; Popay, 2004). It is able to develop on wheat (*Triticum aestivum* L.) and barley seedlings (*Hordeum vulgare* L.) but fails to colonise oat seedlings (*Avena sativa* L.) in a laboratory environment (Mustafa & Akkawi, 1987; Wool & Kurzfeld-Zexer, 2008). Which secondary host species *A. lentisci* would prefer in a situation of choice is not known. At any rate, species (and genotype) preferences are not mandatorily linked with the performance on the plant (Zytynska & Weisser, 2016). Sometimes, aphids show a preference for the host plant they developed on (Ben-Ari *et al.*, 2015). Secondary symbionts and their interaction with plant genotype and aphid genotype may also matter (Skaljac, 2016).

1.2.6.2. Host plant finding

Finding a suitable host plant is essential for aphids when migrantes have to move to a secondary host, when sexuparae have to get back to the primary host or when unfavourable conditions such as overcrowding, predators or plant quality deterioration force some alate and apterous virginoparae to relocate. Even under good conditions, a certain proportion of individuals may spontaneously leave their colonies in quest of a new host (Ben-Ari *et al.*, 2015).

Relocating aphids find new plants either by long-distance or short-distance dispersal (Dixon, 1985). Only winged morphs (migrantes, mature sexuparae or alate virginoparae) are thought to disperse long-distance, i.e. over a few meters to up to thousands of kilometres (Ben-Ari *et al.*, 2015; Dixon, 1985). Alates usually take off for their long-distance flight after their last moult and before reproduction. The start is more likely to happen at low host quality, high light intensities, high temperatures and/or lower wind speeds, with species-specific thresholds for most of these factors (Dixon, 1985). Once in the air, aphids are thought to ride turbulent convection currents as do dust and certain plant spores. The convection currents carry them over 30 m above the ground, where the local winds determine the speed and direction of the journey. The aphids control the flight distance to some extent, however, by actively flying downwards and settling. Visual cues such as long wavelength light reflected from the ground and vegetation may be important signals for this movement (Dixon, 1973). Olfactory cues

may also intervene in many aphid species (Webster, 2012). Once landed, the aphids assess the surface of the plant by walking over it and briefly testing it with the antennae and the mouthparts, leaving some saliva on the surface. This process takes less than 60 seconds. The stylets are unlikely to penetrate anything other than epidermal cells in that time. Features such as the nature of the surface waxes, the hairiness of the surface and some specific secondary plant metabolites must, therefore, provide the necessary information to the aphids (Dixon, 1973, 1985). A threshold of suitability is believed to apply at this point, i.e. aphids leave the plant only if it is found unsuitable (Zytynska & Weisser, 2016). The likelihood of taking off again decreases at each flight, and may eventually not be possible anymore. The aphid's indirect wing muscles may indeed be autolysed as aphids settle and start reproducing (Dixon, 1973, 1985).

By contrast, all active morphs and instars may disperse over a short distance, through ground dispersal or walking directly from one plant to a neighbouring one (Dixon, 1985). Recent research suggests aphids may move much farther this way, than previously thought, e.g. up to 13.5 m within 7 h in a meadow for *A. pisum* (Ben-Ari *et al.*, 2015). Beyond the dispersal distance aspect, the host finding process is likely similar to the one described for long-distance dispersal, however. Olfaction mediates the process at least partly since aphids walk actively towards host plant odours (Webster, 2012). Visual cues also may intervene since apterous morphs of some species show colour preferences on synthetic diets (Srivastava, 1987).

Although alate morphs have been reported (Blackman and Eastop, 2000; Lowe, 1966, 1968), *A. lentisci* is believed to mainly disperse as highly mobile wind-blown first instar nymphs in New Zealand (Blackman and Eastop, 2000; Popay, 2004). Dispersal with water would also be conceivable as *A. lentisci* first-instar nymphs float and may be carried away on the water by the virtue of their wax (Salt *et al.*, 1996). More research will have to confirm this and assess what this means in term of dispersal potential, however. In absence of concrete knowledge of *A. lentisci*'s sensory abilities as an apterous nymph (Section 1.2.4.4) and published studies about its host finding behaviour, it is difficult to assess what points of general knowledge reported above might apply in this species.

1.2.6.3. Feeding site choice

To tap new resources requires a considerable energy investment (Section 1.2.4.1). An inappropriate choice may also put an aphid's life at risk. It is thus not surprising that 30 minutes to several hours may be spent on selecting a suitable feeding site (Will, 2016). Gravity, light and features of the plant may be used for site location (Hopkins & Dixon, 2000). Above-ground aphids are known to colonize the nutritionally richest parts of a host plant, i.e. young, growing tissues or senescing tissues remobilising nutrients (Dixon, 1985; Reece *et al.*, 2011). Besides nutritional quality, physical or chemical plant defences may play a role (Dixon, 1985). External structures such as hairs, for example, may either limit the access to the plant and hurt aphids, or instead protect young instars from predators (Dixon, 1985; Tomczak & Müller, 2018; Zytynska & Weisser, 2016). A specific site can be used only as long, as the rate of phloem removal does not trigger a wounding reaction in the plant (Dixon & Kindlmann, 1994). The size of sieve elements and the flow in them are therefore important. Moreover, the depth of veins and the lignification of the tissues surrounding them may restrict the usability of some phloem vessels: aphid stylets have physical length limitations and appear unable to penetrate fortified sclerenchyma cell walls (Dixon, 1985; Dixon & Logan, 1973; Hewer *et al.*, 2011; Volkl, 1990).

How this translates to roots, and how root aphids choose their feeding sites on a host plant is not yet clear. The only fact reported so far is that young *A. lentisci* nymphs are more often found on new roots of plants with good growth (Popay & Cox, 2016).

1.2.6.4. Food quality

Although food quality may have little influence on the developmental time in aphids (no to sub-proportional effect), it can have a large impact on aphid fitness by affecting the birth size, adult size, offspring number and offspring fecundity (Dixon, 1985, 1987; Dixon & Kindlmann, 1994; Traicevski & Ward, 2002). In interaction with other factors such as crowding, for example, food quality may further influence the morph produced (Dixon, 1985).

Food quality in aphids is determined by the chemical composition of the phloem sap (Tomczak & Müller, 2018). Phloem sap is generally a sterile and rich food source lacking toxins and feeding deterrents [exceptions: glucosinolates in Capparales,

cardenolides in Asclepiadaceae and pyrrolizidine alkaloids in various plant groups (Dixon & Kindlmann, 1994; Douglas, 2006; Hoffmann, 2016; Vilcinskas, 2016)]. Its composition differs between plant species, changes with plant age, varies in diurnal and seasonal rhythms, and reacts to environmental changes such as an elevation in atmospheric CO_2 or nitrogen fertiliser application, however (Hoffmann, 2016; Tomczak & Müller, 2018; Weibull, 1987). It may also be manipulated by some aphids to their advantage, for example, to re-allocate nitrogen towards aphid feeding sites (Dardeau *et al.*, 2015; Dixon, 1985; Giordanengo *et al.*, 2010). Central phloem quality aspects for aphids are, in particular, the water-soluble carbohydrate concentration (sucrose) and the soluble nitrogen concentration, or more specifically, the amino acid content and amino acid profile (Popay & Cox, 2016; Zytynska & Weisser, 2016). Sucrose, the main solute of phloem, helps aphids find the sieve tubes and stimulates feeding (Kuroli *et al.*, 1998). It is detrimental to aphids at high concentrations, however, possibly because it causes osmotic stress in the aphid gut (Section 1.2.4.1; Zytynska & Weisser, 2016). A higher nitrogen content in the host plant part is usually associated with higher population growth rates in aphids (Zytynska & Weisser, 2016). However, such a statement is problematic as it is the total amino acid concentration in the phloem and the amino acid profile that matter to aphids (Srivastava, 1987). Furthermore, the carbon:nitrogen content of a leaf may not be well correlated with the leaf's phloem sucrose:amino acid ratio (Douglas, 2003). What constitutes a high-quality amino acid profile is not easy to define either; even aphid populations of a same species may differ in their requirements for specific amino acids (Srivastava, 1987). Primary and secondary endosymbionts (Section 1.2.4.1) may have a part in the determination of diet suitability and quality: while the former may partly balance out a phloem's high ratio of non-essential:essential amino acid (Douglas, 2006; Vilcinskas, 2016), the latter may either help an aphid cope with, or, in some cases, reduce the aphid's performance on plants with low amounts of dietary nitrogen (Skaljac, 2016). Defining food quality for New Zealand *A. lentisci* will, therefore, require specific feeding experiments.

1.2.6.5. Effect on host plant

Unlike chewing insects, aphids do not generally destroy their food plant (Miles, 1989a). Under specific circumstances, a moderate aphid infestation may even benefit the plants. For example, plant growth may be improved when aphid feeding stimulates the

nutrient uptake in roots or leads to increased chlorophyll concentrations in the leaves (Dixon, 1973; Miles, 1989a). However, negative impacts through tissue damage (chlorosis, necrosis), interference with plant metabolism, nutrients and energy drain or virus transmission are more frequently reported (Dixon, 1973, 1985; Saheed *et al.*, 2007). Aphid feeding can damage tissues both directly, i.e. by wounding cells when the stylets probe them (Section 1.2.4.1) or indirectly, by triggering a defence response in the plant or via reduction in the functionality of vascular tissues (Klingler *et al*., 2009; Saheed *et al.*, 2007). For example, one genotype of *Medicago truncatula* Gaertn. responds to the feeding of aphid *Acyrthosiphon kondoi* Shinji with programmed cell death at the point of attack ('hypersensitive response'; Klingler *et al*., 2009). The occlusion of sieve elements and xylem vessels triggered by *Diuraphis noxia* Mordvilko feeding on wheat (*Triticum aestivum* L.) may cause water, nutrient and photosynthetic stress, which may result in chlorosis, necrosis, leaf streaking and leaf rolling (Saheed *et al.*, 2007). Such effects can further reduce the photosynthetic capacities of the plant. Local or systemic interference in a plant's metabolism can be observed as changes in anatomy and physiology [e.g. galls, stem thickening, abnormal root and shoot growth in reaction to feeding (Dardeau *et al.*, 2015; Dixon, 1973)], changes in nutrient allocation dynamics [mobilisation and transport of nutrients from other plant parts to the feeding site (Miles, 1989a)], early leaf senescence (Dixon, 1985), and impaired plant defence (Hoffmann, 2016). Such changes may exacerbate the energy and nutrient drain already imposed by feeding aphids. Since remobilisation from older leaves supplies a part of the nitrogen for new leaf growth (Irving, 2015), repeated early leaf fall as reaction to aphid feeding (Dixon, 1985), for example, can deprive a plant of valuable nitrogen resources. The energy and nutrient drain can result in reduced plant growth (e.g. shorter twigs, shorter roots, and smaller leaves), which may, in turn, reduce the plant's ability to photosynthesise and acquire nutrients. Under conditions of extreme stress from heavy aphid infestations, plant death is also possible (Dixon, 1973, 1985; Pretorius *et al.*, 2016).

The severity of the damage depends on the aphid and plant genetics involved, their interaction under given environmental conditions, and the viruses that may be present (Dixon, 1973; Zytynska & Weisser, 2016). Particularly important parameters are the size and number of aphids, their consumption efficiency (e.g. the amount of carbohydrates potentially lost to the plant through honeydew excretion), the nature of their feeding process (e.g. whether toxic or hormonal chemicals are transferred between aphids and

plants), the tissues attacked, the importance of the affected tissues to the plant, and the ability of the plant to recover from the feeding (Gavloski & Lamb, 2000). If viruses are present, the severity of the damage will further depend upon aphid behaviour, virus impact on plants and virus type (Dixon, 1973, Brault *et al.*, 2010). Even in small numbers, aphids that frequently change their host plant may cause serious losses through virus transmission (Dixon, 1973; Heathcote, 1972). How viruses spread depends also on their mode of transmission, however ['non-circulative', i.e. quickly acquired but briefly infective, vs. 'circulative', i.e. acquired through feeding for a period of time and generally persisting in the aphid's body up to its death (Brault *et al.*, 2010; Dáder *et al.*, 2012; Dixon, 1973)].

Root rotting, chlorosis, plant distortions and drastic yield reductions are reported for *A. lentisci* on wheat (Mustafa & Akkawi, 1987). The symptoms described in New Zealand grasslands relate mainly to nutrient drain, however (poor vigour, yield, and persistence, particulary in conjunction with environmental stress; McDonald *et al.*, 2011; Popay and Cox, 2016). *A. lentisci* appears able to transmit at least one non-circulative virus (*Potyvirus Y* on tobacco plants; Boukhris-Bouhachem & Souissi, 2012). Whether *A. lentisci* has a part in transmitting some of the important grassland viruses such as the barley yellow dwarf viruses (Delmiglio *et al.*, 2010) and whether it significantly contributes to virus-related yield losses has not yet been examined, however.

1.2.6.6. Plant resistance to aphids

Three main mechanisms of resistance to aphids are reported in plants: antibiosis, antixenosis, and tolerance (de Wet & Botha, 2007; Smith, 1989; Smith & Clement, 2012). Plants with antibiotic resistance affect the aphid life-history negatively, i.e. increase the mortality, and/or reduce the growth, longevity, and fecundity of aphids feeding on them (Koch *et al.*, 2016; Smith & Clement, 2012). Plants that produce chemical defences in phloem and tissues (insecticides, phytoalexins or secondary plant compounds) or plants that bear glandular hairs with entangling secretions belong to this group. Plants with an antixenotic or non-preference resistance affect the aphid behaviour adversely. They delay or deter aphid settling and colonisation, e.g. by releasing aphid alarm pheromones that possibly repel new aphids and disrupt ongoing feeding (Boullis & Verheggen, 2016; de Wet & Botha, 2007; Smith & Clement, 2012). Finally, tolerant plants are able to

withstand aphid feeding or recover from aphid damage (Smith & Clement, 2012). The ability to produce molecules that break down aphid-induced obstructions in conducting elements qualifies as a tolerance trait, for example (de Wet & Botha, 2007).

At the present time, plant resistance to *A. lentisci* is poorly understood (Podmore, 2015). Cases of specific endophyte-linked resistances have been reported. On what these are based is not clear, however (Section 1.5). It is likely that more resistance traits will be identified through studies of *A. lentisci*'s biology, perception (Section 1.2.4.4), host/feeding site selection behaviour (Sections 1.2.6.2 and 1.2.6.3), and its damage in grasslands (Section 1.2.6.5).

1.2.7. Colonies and population dynamics

Aphids have gregarious instincts (Shaposhnikov, 1987). They live in groups ('colonies'). This behaviour appears to benefit them: individuals raised in a group may develop faster and be more fecund than individuals raised in isolation, a fact explained either by the physicochemical changes induced in the plant by a group of feeding aphids (Section 1.2.6.5) or by the sink effect of cooperative feeding (Michaud *et al.*, 2006; Miles, 1989a; Shaposhnikov, 1987). The plant may indeed treat the feeding site like a bud, i.e. re-allocate resources from other parts towards it, if the nutrient drain is sufficiently strong and localised (Miles, 1989a). Furthermore, colonies seem to offer a defensive advantage against predators (Shaposhnikov, 1987; Turchin & Kareiva, 1989). Where colony size is too large, such benefits are likely gradually replaced by negative, competition-related effects, however (Michaud *et al.*, 2006).

Aphid populations have particular development dynamics. The number of aphids in a colony increases first rapidly to high numbers, before declining equally rapidly to colony extinction. This colony development is not caused by natural enemies (predators, parasites) even if these may contribute to it (Kindlmann & Dixon, 2010), and is not fully explained by host plant quality deterioration either (Dixon, 1973). The decline appears, rather, to result from aphids reacting to their own density ('intra-specific' mechanisms): crowded aphid colonies produce more winged morphs which disperse, and small adults with low fecundity (Dixon, 1973; Kindlmann & Dixon, 2010). Host plant quality fluctuations over the seasons may, however, explain differences in within-year population dynamics. As a general rule, aphid carrying capacity of annual crop plants tends to

increase up to plant maturity and decrease very rapidly thereafter (Kindlmann & Dixon, 2010). Developing grass stands are thus likely to support larger aphid populations than mature grass stands.

By periodical addition of new seedlings under constant laboratory conditions, Israeli *A. lentisci* colonies were found to persist up to 223 days on wheat, and 160 days on barley (Wool & Kurzfeld-Zexer, 2008). Whether New Zealand *A. lentisci* colonies develop similarly and what this would mean for population dynamics in New Zealand grasslands is still not known, however.

1.3. Biology and ecology of *Lolium perenne*

Perennial ryegrass is the most widely sown grass species in New Zealand (Easton et al., 2001). It covers about 5.8 million ha (ca. 22% of New Zealand's land area; Ministry for the Environment and Statistics New Zealand, 2015b; Wakelin et al., 2015). This dominance is not surprising as *L. perenne* has many positive attributes. It is a highly digestible forage plant of good quality and palatability that can produce about 14 to 20 t of dry herbage per hectare and year in New Zealand grasslands under good conditions and appropriate management. It establishes fast, is robust enough for direct livestock grazing, is easy to manage and may persist for more than ten years under favourable environmental conditions and low biotic pressure (Dairy NZ, 2018; Hannaway et al., 1999).

1.3.1. Anatomy and physiology

Perennial ryegrass *Lolium perenne* L. (Figure 1.7) is a naturally diploid (2n = 14), self-incompatible, and cross-pollinated grass of the *Poaceae* family (Jung *et al.*, 1996). It is densely tufted, grows up to heights of 30 to 100 cm and has a shallow root system [> 70% of the roots in the top 20 cm of the soil (Hannaway *et al.*, 1999; Hatier *et al.*, 2014; Reed, 2008)]. Mature perennial ryegrass plants consist of a group of interconnected but distinct shoots ('tillers'), each with its own roots, about three living, fully expanded leaves, a fourth growing leaf, and axillary buds able to produce daughter tillers under

appropriate conditions (Hunt & Field, 1979; McCarthy *et al.*, 2015). Although assimilates and nitrogen may be translocated between tillers by partial foliage removal (Forde, 1966; Robin, 2011), each tiller is independent. A newly developing daughter tiller is able to survive on its own from the moment it reaches ca. 25 mg dry mass and has developed its own adventitious roots (Danckwerts & Gordon, 1987).

Each tiller is built up modularly, i.e. in individual units connected to each other by vascular plexuses ('phytomers'; Robin, 2011). These units are developed by the apical meristem, a tissue of dividing cells residing in the centre of the tiller, in the cylinder formed by the previously developed leaf sheaths (Robin, 2011). In a vegetative tiller, the apical meristem initiates a new phytomer by generating a leaf primordium. Over time, each phytomer develops (i) a complete leaf with ligule, leaf blade and sheath, (ii) an axillary bud with potential for daughter tiller formation, (iii) an internode, and eventually (iv) roots that grow, branch increasingly (Table 1.3) and may develop a sclerified cortical parenchyma of one to two cells thickness around the endodermis before dying (Figure 1.8; Robin, 2011; Silsbury, 1970; Soper, 1959). The formation of new vegetative phytomers and the further development of phytomers are continuous processes. They may proceed at varying speeds, however. Some of the later development steps represented in Figure 1.8 may not be isochronous with the ongoing leaf primordia appearance interval ('plastochron') on the same tiller (Robin, 2011). In the field, new leaf production is faster in late summer -early autumn, for example, while bursts of biomass deposition in the roots are observed after autumn rains and in late winter, about one month before the shoots start resuming intense leaf production (Matthew *et al.*, 2016; Robin, 2011).

In a reproductive tiller, the apical meristem forms an inflorescence and the tiller becomes henceforth incapable of any further leaf or tiller initiation (Hunt & Field, 1979).

Through its development, a phytomer fulfils various functions and undergoes various changes. The green leaf blade supplies the rest of the plant with carbohydrates. Its net photosynthetic rate is maximal at its youngest, fully expanded stage and declines quickly later on (Robin, 2011). The sheath base of the living leaf stores carbohydrates that can be used for leaf regrowth after defoliation (Amiard *et al.*, 2004; Danckwerts & Gordon, 1987; Fulkerson and Donaghy, 2001). Through nitrogen remobilisation, senescing leaves may become a significant source of nitrogen for newly developing leaves and roots (Irving, 2015; Robin, 2011). Finally, roots supply the plant with water

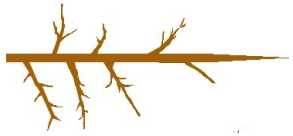
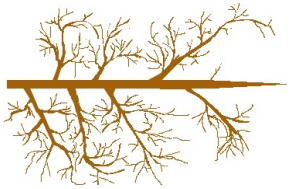
and inorganic nutrients and anchor it in the ground (Reece *et al.*, 2011). Occasionally, roots may also serve as a source of nitrogen (nitrogen remobilisation by repeated defoliation; Thornton & Millard, 1996). The roots depend on the shoots for assimilates by this division of functions. The total root biomass deposition amounts to about 15% of the biomass harvested above-ground in a sward (Matthew *et al.*, 2016). The deposition rate is not equal for all root-bearing phytomers, however. The newest roots (connected to the tiller axis closest to the photosynthetic leaves) get the largest share of assimilates. The investments in older roots decrease gradually with phytomer age (Table 1.3; Robin, 2011). Roots of 3rd and 4th branching order may even have to re-allocate resources internally to form new lateral branches (Robin, 2011).

Figure 1.7. Vegetative and reproductive characteristics of perennial ryegrass. (a) Tiller bases/leaf sheaths: reddish-purple and pale green, without hairs; **(b)** Leaves: folded at emergence, hairless, sharply taper-pointed, prominently ridged on the upper surface with a deep V-shaped groove at mid-rib and a smooth and shiny lower surface, with no/small auricles (not visible here: thin-membranous ligule, rounded or toothed at the tip); **(c)**, **(d)** Inflorescence: awnless spikes, 20 cm long, with alternately arranged spikelets of 4 to 8 florets; **(e)** Adventitious root system: fibrous, shallow; **(f)** Seeds: 6 mm long, 1.92 g/1000 seeds (Hannaway *et al.*, 1999; Massey University, 2000; Reed, 2008)

Morphological stage	Time [Phytomer number]	Events
Leaf development	0	Apical meristem
	1	Formation of leaf primordium
	2	
	3	
	4	
	5	
	6	
	7	Axillary bud appearance and leaf tip formation
Photosynthetically active leaf	8	Ligule formation, leaf emergence
	9	Leaf elongation
	10	Leaf sheath elongation
	11	Internode extension, final maturation of leaf tissues
Senescing leaf	12	Leaf senescence (Root initiation [BO: 0], axillary tiller development)
Roots	13	Leaf senescence Root initiation and elongation (BO: 0), axillary tiller development
	14	Start of root branching (BO: 1)
	15	Root elongation Start of 1 to 2° root branching (BO: 1-2)
	16	
	17	Start of 2 to 3° root branching (BO: 2-3)
	18	
	19	Cessation of root dry matter deposition
	20	
	21	
	22	
	23	Start of 4° root branching (BO: 4)
	24	Root dying
	25-30	

Figure 1.8. Phytomer development diagram for perennial ryegrass. This representation is based on Yang *et al.* (1998) and literature and experimental results of Robin (2011). Time is expressed in number of phytomers produced since the formation of the considered phytomer (biological time scale). This scale cannot be directly converted into days as the timing of the events can vary with season, environmental conditions and plant genetics (Langer, 1979; Robin, 2011). Grey text: possible earlier or later start of events. BO: branching order (see Table 1.3).

Table 1.3. Root development and characteristics by root branching order (BO) [Robin 2011].

Root phase	Diagram	Description	Root age at start[1]	Carbon expenditures
Unbranched root		Only main axis elongation	Limited to first 10 days Pr 1-2	24-55 µmol C $Pr^{-1} \cdot d^{-1}$
1st BO root		Main axis elongation: still occurring Branching occurring on the main axis	10-20 days Pr 2-4	20-40 µmol C $Pr^{-1} \cdot d^{-1}$
2nd BO root		Main axis elongation: still in progress Branching on lateral branches (2°), ongoing branching on the main axis	25 -30 days Pr 2-4 in Spring Pr 4-6 in Autumn	18-36 µmol C $Pr^{-1} \cdot d^{-1}$
3rd BO root		Tertiary root branching phase (3°), i.e. branching of 2° branches Main axis elongation has ceased	After 40 days Pr 5-7 in Spring Pr 8 in Autumn	8-24 µmol C $Pr^{-1} \cdot d^{-1}$
4th BO root		Quaternary root branching phase (Only produced in some plant genotypes)	Approximately 50 days (if at all) After Pr 10	< 8.0 µmol C $Pr^{-1} \cdot d^{-1}$

[1] Robin (2011) estimated the age of the roots in a hydroponic system placed in a glasshouse (natural photoperiod), at average temperatures of 11.6 and 18.6 °C in spring and autumn, respectively, on the commercial *L. perenne* cultivars 'Alto' and 'AberDart'. BO: branching order; Pr: root phytomer number, i.e. root-bearing tiller segment, with Pr1 being the segment with the most recently grown root(s); d^{-1}: per day

1.3.2. Environmental requirements and biotic influences

Perennial ryegrass is able to adjust to a wide range of environmental conditions. It is fairly resistant towards cold and frost (Reed, 2008), starts developing when the soil temperature rises above 2.1 °C (base temperature; Moot *et al.*, 2000) and achieves maximal growth performances at temperatures around 20 to 25 °C but suffers production losses by high temperatures [daytime temperatures above 31 °C or night-time temperatures above 25 °C (Hannaway *et al.*, 1999)]. It may have a root system that is less effective at intercepting nitrate than the root systems of other grasses (Popay & Crush, 2010) and is known to require a relatively high soil fertility to perform well (Massey University, 2000). It therefore grows best on fertile, well-drained soils with average pH values of 5.5 to 7.5 (Hannaway *et al.*, 1999). Nevertheless, it tolerates soil pHs in the range of 5.1 to 8.4 and can survive heavy soils, waterlogged grounds and longer periods of flooding at moderate temperatures. It is moderately drought tolerant (i.e. limited to areas with > 450 to 700 mm annual rainfall), however, and may perform poorly during short-term summer droughts (Hannaway *et al.*, 1999; Hatier *et al.*, 2014; He *et al.*, 2017; Massey University, 2000; Reed, 2008). Last but not least, sufficient light is important for maximal yields. Light saturation for *L. perenne* roots and shoots is achieved at levels of 100 $W \cdot m^{-2}$ and 200 $W \cdot m^{-2}$ in a controlled experimental environment at 20 °C, respectively (Hunt & Field, 1979; i.e. approx. 457 and 914 μmol photons$\cdot m^{-2} \cdot s^{-1}$)[1], but very poor light conditions likely affect the roots more than the shoots: while the shoot biomass was reduced five-fold, the root biomass was decreased 30-fold when the grass *Bromus inermis* Leyss was grown at 1.7 Klux instead of 30.5 Klux (32 instead of 580 μmol photons$\cdot m^{-2} \cdot s^{-1}$), for example (Langer, 1979).

Various biotic agents may influence the amount, quality and palatability of the biomass produced by *L. perenne* in the field. Their effects may be both, direct or indirect, and depend on a complex network of interactions between these agents' genetics, the plant's genetics, the genetics of other biotic agents, environmental influences such as temperature and humidity, and grassland management decisions. The rhizosphere's macrofauna and microbiome (e.g. earthworms, bacteria) can influence the roots' environment through changes in the soil structure and mineralisation of nutrients (Scheu *et al.*, 1999; Section 1.6). Symbiotic microorganisms such as mycorrhizae can improve

[1] Source of conversion factors in this paragraph: Environmental Growth Chambers (2015)

the water and mineral absorption of a plant but cost it assimilates (Eissenstat & Volder, 2005; Reece *et al.*, 2011). Specific foliar *Epichloë* endophytes can help reduce herbivore damage but can also affect the plant growth and its suitability as forage (Section 1.4). Especially during humid summers, fungal diseases such as crown and stem rusts may decrease the photosynthetic efficiency and the herbage quality in plants of susceptible cultivars (Charlton & Stewart, 1999; Hannaway *et al.*, 1999; Hunt & Easton, 1989). Barley yellow dwarf viruses, which are widespread in older New Zealand *L. perenne* pastures (> 2 years of age), may reduce the yields by more than 20% (Hunt & Easton, 1989). Heavy infestations of insect pests such as root aphid *A. lentisci*, Argentine stem weevil larvae (*Listronotus bonariensis* Kuschel), pasture mealy bugs (*Balanococcus* spp), adult African black beetles (*Heteronychus arator* Fabricius), porina (*Wiseana* spp) and grass grubs (*Costelytra zealandica* White) can cause fatal damage to perennial ryegrass plants in pastures, particularly during a period of drought stress (Grasslanz Technology Ltd, 2010b; Popay & Thom, 2009; Tozer *et al.*, 2017). Livestock influences strongly depend on grazing management. Rotationally grazed pastures yield generally more biomass than permanently used pastures (set stocked pastures; Brock *et al.*, 1996). If *L. perenne* plants cannot recover long enough to replenish the water-soluble carbohydrate reserves in their leaf sheath, their recovery after a new grazing event may be retarded and the persistence of the pasture reduced, however (Fulkerson & Donaghy, 2001).

1.4. Biology and ecology of endophyte *E. festucae* var. *lolii*

1.4.1. Characteristics and life cycle

Asexual *Epichloë* endophytes are microscopic, biotrophic fungi of the family Clavicipitaceae [phylum Ascomycota – class Sordariomycetes – order Hypocreales (Mycobank, 2014)] living in a systematic and controlled way between the cells of a grass host and obtaining nutrients from it without causing host cell death (Cheplick, 2004). In exchange, they confer their host some protection against herbivory and environmental stress (Cheplick, 2004; Grasslanz Technology Ltd, 2010a).

Epichloë endophytes were first identified as hazardous for livestock in the late 1970s – early 1980s, when their presence in plants was associated with fescue foot, heat

stress symptoms and ryegrass staggers (Hume & Cosgrove, 2005; Omacini *et al.*, 2012). Their protective effect against insect pests was soon recognised too, however [e.g. by Prestidge *et al.* (1982)]. As toxicity to livestock and protection against insects appeared to have different chemical origin (Clay, 1988), strains of *Epichloë coenophiala* Morgan-Jones & W. Gams and *Epichloë festucae* var. *lolii* (Latch, M.J.Chr. & Samuels) C.W.Bacon & Schardl that protect their host plant against New Zealand grasslands pests without major impact on livestock health could be identified and inoculated into proprietary tall fescue (*Festuca arundinacea* Schreb.) and perennial ryegrass (*Lolium perenne* L.) cultivars, respectively (Johnson *et al.*, 2013). Such endophyte-grass symbionts have been successfully commercialised for more than 17 years (Johnson *et al.*, 2013) and are now considered necessary for pasture persistence in various regions of New Zealand (Dairy NZ, 2018; Popay & Cox, 2016).

E. festucae var. *lolii* and *E. coenophiala* are asexual, develop inside of their grass host and are completely dependent on it. Although many strains may be isolated onto artificial media and cultured in the laboratory (Johnson *et al.*, 2013), they do not have any stage outside of plants in the field (Card *et al.*, 2016; Popay & Cox, 2016). They lack propagation and initial infection mechanisms (Card *et al.*, 2016), and are transmitted vertically, from a mother plant to her seeds (Johnson *et al.*, 2013; Popay & Cox, 2016). Transmission by physical contact appears to succeed under very specific conditions only [inoculation of very young seedlings, deposition of fungal mycelium in a meristematic region (Liu *et al.*, 2011a; Simpson *et al.*, 1997)]. Experimental infection of mature tillers fails, and the rate of success of axillary bud infection is below 1% in the laboratory (Christensen & Voisey, 2007; Latch & Christensen, 1985; Liu *et al.*, 2011a; Simpson *et al.*, 1997). Thus, the life cycle of an endophyte in a new plant begins generally when hyphae grow inside of a developing plant inflorescence (before fertilisation) and colonise an ovary. At seed maturity, the hyphae are found between aleurone and pericarp layers and in the embryo. Only the hyphae in the embryo are involved in the actual endophyte transmission, however (Zhang *et al.*, 2017). These endophyte hyphae may die within the seed under unfavourable storage conditions [temperature > 5 to 10 °C, seed moisture > 11%, relative humidity > 50% (Easton *et al.*, 2001)], or fail to establish within the newly developing tillers once the seed starts germinating [e.g. if the seedling is exposed to weekly fungicide treatments (Card *et al.*, 2011)]. A successfully established endophyte proceeds to colonise leaf primordia and auxiliary buds, however, spreading systemically

throughout the above-ground parts. Eventually, hyphae are found in all meristematic tissues of the shoot (stem apex, leaf primordia, and axillary buds), in the inflorescence primordia, and in the leaves (Card *et al.*, 2014b; May *et al.*, 2008). In the latter, they run orderly, seldom branched, parallel to the longitudinal leaf axis (Figure 1.9), in greater abundance in the leaf sheaths than in the leaf blades (Christensen & Voisey, 2007; May *et al.*, 2008). Root tissues are not colonised although some hyphae are found in the meristem at the tip of the main root axis (Christensen & Voisey, 2007). The hyphae in the shoot develop in the intercellular spaces, tightly attached to host's cell walls in a highly regulated manner and in synchronisation with the grass development, switching from a proliferative tip growth in the meristem to intercalary division and extension growth in the expanding leaf (Christensen *et al.*, 2008; Johnson *et al.*, 2013). The hyphae stop growing when the leaf they live in does but remain metabolically active (Tan *et al.*, 2001). In the mature endophyte-infected plant, the hyphae represent about 0.5 to 2.0% of the association in terms of the total amount of DNA (Rasmussen *et al.*, 2009). Colonisation extent and hyphal biomasses are influenced by host genotype and various environmental factors, however. For example, endophyte concentrations in the leaves are reduced in some high sugar grass cultivars, by high phosphate availability, cold weather (winter), and in presence of mycorrhiza (di Menna & Waller, 1986; Liu *et al.*, 2011a).

Figure 1.9. *Epichloë* endophyte in perennial ryegrass. (a) Hyphae in lower epidermis of leaf sheath, stained with aniline blue © Mike Christensen; **(b)** Hyphae (green) on transverse section of a tiller as revealed by fluorescence microscopy; reproduced from Christensen and Voisey (2007) [Figure 27, p 129] with the permission of the New Zealand Grassland Association; B: emerging leaf blade of main tiller, DT: daughter tiller grown from an axillary bud, S: leaf sheaths.

1.4.2. Impact on plants

Endophytes can modulate the (re)growth, morphology, nitrogen assimilation, resource allocation and mineral uptake, gene transcription, metabolite profiles and metabolite concentrations of their host plant (Rasmussen *et al.*, 2009; Spiering *et al.*, 2006). Meta-analyses report that endophyte presence reduces the root biomass of a plant by an average of 9%, decreases arbuscular-mycorrhizal colonisation by 31%, and stimulates root exudation by 9%, but has no consistent effect on soil fauna, microbial respiration and aerial litter decomposition (Omacini *et al.*, 2012). However, such general summaries do not consider the specific effects of various endophyte genetics (Table 1.4), the unique biological outcome of specific endophyte-plant genotype or endophyte-cultivar interactions (Wakelin *et al.*, 2015), or the influence of the environment on the interaction (Sections 1.4.1 and 1.5). The nature of the relationship of an endophyte with its host is likely to vary from antagonistic to mutualistic depending on parameters such as the availability of nutrients in the soil and the complexity of the food web (Saari *et al.*, 2010). *E. festucae* var. *lolii* endophytes are detrimental to *L. perenne* in nutrient-poor, low light environments, for example (reduced root/shoot ratio, reduced photosynthetic shoot fraction; Cheplick, 2007). Endophytes can confer significant fitness advantages to their grass host in the field, however, by producing a strain-specific spectrum of toxins (Table 1.5) which may deter some herbivores, reduce their growth, or even kill them (Cheplick, 2004; Cheplick & Cho, 2003; Rasmussen *et al.*, 2007). Endophytes can also fundamentally modify the primary metabolism of plants (Johnson *et al.*, 2013). Increased drought tolerance by means of osmoregulation and stomatal regulation has been reported in some instances (Singh *et al.*, 2011).

Besides the above-mentioned, the age of the symbiosis may also be very relevant when assessing endophyte impact. Although leaf growth and hyphal extension are synchronised in a mature plant (Tan *et al.*, 2001), an endophyte grows into a seedling and/or develops at least some of its metabolic influences only gradually after germination (Ruppert *et al.*, 2017). *De novo* synthesis of some alkaloids such as peramine and lolitrems, for example, begins ca. 30 to 50 days after sowing (Rowan & Latch, 1994). Thus, while very young endophyte-infected *L. perenne* seedlings may still be protected from insect herbivory through alkaloids translocated from seeds into seedlings, older seedlings of 10-20 to 43-56 days of age may temporarily be more vulnerable to, and more damaged by herbivores than older plants (Ruppert, 2016; Ruppert *et al.*, 2017).

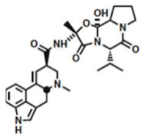
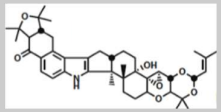
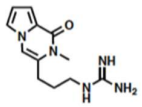
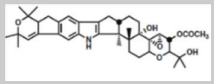
Table 1.4. Characteristics of three important *E. festucae* var. *lolii* endophyte strains (AR1, AR37 and common-toxic CT) and summary of their impact on perennial ryegrass plants and herbivores.

	Common-toxic (CT)	AR1	AR37
History	• Naturalised in New Zealand (formerly known as 'wild-type') • Caused ryegrass staggers and heat stress in sheep	• From Italy • Selected to control Argentine stem weevil (ASW) without staggers; commercially released in 2001	• From France • Selected to control more pests although staggers were occasionally observed; commercially released in 2007
Characteristics	Alkaloid profile: Peramine, Ergovaline, Lolitrem B Endophyte presence: • Hyphal density: higher than AR1, lower than AR37 • Seed infection rate: high (93%) Pest control: Argentine stem weevil, pasture mealybug, black beetle adults (not larvae), *Schizaphis graminum*, (*Aploneura. lentisci*) Side-effects: • Ryegrass staggers • Reduced live weight gain	Alkaloid profile: Peramine Endophyte presence: • Hyphal density: Lowest • Seed infection rate: high (96%) Pest control: Argentine stem weevil, (pasture mealy bug) Side-effects: • No negative ones known for livestock • + 9% annual milk production in large scale dairy trials • Plant sometimes more susceptible to *A. lentisci*	Alkaloid profile: Epoxy-janthitrem complexes Endophyte presence: • Hyphal density: highest • Seed infection rate: low (75%) Pest control: Most insects pests, i.e. Argentine stem weevil, pasture mealybug, black beetle, porina, *A. lentisci* Side-effects: • Ryegrass staggers (less frequent, less severe and shorter than by CT containing plants)
Effect on plants[1] (without stress)	Generally: • Growth similar or occasionally exceeding that of AR37 by low herbivory, but only in large soil volume Roots: • Higher overall root weight, than AR1 and NIL • Root diameter: Fewer small roots than AR1: 78% < 0.5 mm diameter (75% for NIL) [S1] • Higher concentrations of K (and P) than NIL (difference not always significant) • Higher root dry weight than AR1 or NIL	Generally: • Higher nutrients contents in plants (more N, P, K in roots and shoots) Roots: • Lower overall root weight and lower root/shoot ratio, than CT, AR37 and NIL • Specific root length higher than AR37, similar to CT • Higher main root dry weight than CT, AR37 or NIL plant • Root diameter: o Highest % of roots of small diameter (80% < 0.5mm diameter) [S1] o Smaller than in AR37, CT and NIL plants [S1] o Thicker roots in top 0-10 cm, than AR37 [S2] • Higher concentrations of K (and P) than NIL (difference not always significant) • Higher levels of N than CT, NIL and sometimes AR37	Generally: • Plant phenology very different o Lower investment in root growth in summer o Rel. large investment in roots in autumn-early winter o % of N in roots increasing during summer • Root/shoot ratio clearly lower than by CT and AR1 Roots: • Higher overall root weight, than AR1, not significantly higher, than NIL • Root diameter: o Fewer small roots, than AR1: 77% < 0.5mm diameter (75% for NIL) [S1] o Thinner roots in top 0-10 cm compared to AR1 [S2] • Higher concentrations of K (and P) than NIL (difference not always significant) • Specific root length shorter, than by AR1, CT • Less cumulative root outgrowth
Effect on plants under insect stress[1]	• Tends to better protect from insects, than NIL plants (lower tiller mortality) • Total length of main roots decreased	• Plant suffers the most by herbivory, high(er) tiller mortality • Total length of main roots decreased • Low persistence in areas with black beetle	• High(er) root/shoot ratio, root growth and shoot growth • High(er) persistence (lower tiller mortality)

Main sources: [S1] Popay (2004), [S2] Popay and Crush (2010); Other sources: Grasslanz Technology Ltd (2010a); Johnson *et al.* (2013); Popay and Gerard (2007); Popay and Hume (2011); Popay and Thom (2009); Ruppert (2016). NIL: endophyte-free plants; N: nitrogen; P: phosphorus; K: potassium

[1] The effects reported here refer to experiments with perennial ryegrass of cultivar 'Grasslands Samson', and may not be reproducible or generalisable (plant genotype-endophyte strain interactions).

Table 1.5. Endophytic alkaloid toxins and their properties, with focus on perennial ryegrass as host plant.

Toxins	Chemical structure	Producer	Effect	Concentrations *in planta*[1]	Note
Ergovaline (Ergots)	Ergopeptines 	*E. festucae* var. *lolii* in ryegrass *E. coenophiala* in tall fescue	• Affects insects (deterrent and/or toxic for ASW; confers resistance to black beetle) **Eff. conc.:** ASW deterrence at 0.1 µg/g diet • Toxic to livestock: vasoconstrictor, causes heat stress, fescue toxicosis and reduced live weight gain (LWG) **Eff. conc.:** LWG: no threshold, linear effect Fescue foot: ≥ 0.05 µg/g DM Heat stress: 0.4-0.8 µg/g DM	• Primarily concentrated at the base of the shoot • High concentrations in seed heads and stems • Concentration in roots and pseudostems may be similar • Strong seasonal and environmental variations • Values: In seed: 21.5-30.9 µg/g DM In leaf sheaths: 0.71-1.38 µg/g DM In shoot: 1.0-14 µg/g DM possible In roots: 0 – 0.15 µg/g DM	Hydrophobic Is detected in guttation fluid of E^+ tall fescue
Lolitrem B	Indole-diterpenes 	*E. festucae* var. *lolii*	• Reduces development and growth, increases mortality of ASW larvae **Eff. conc.:** growth reduced at 5 µg/g diet • Affects primarily vertebrates • Toxic to livestock: causes ryegrass staggers (tremorgenic) **Eff. conc.:** ≥ 2 µg/g DM	• Primarily concentrated at the base of the shoot • Concentrations lower in young plant tissues • Seasonal peak: highest late December – late autumn • Values: In seed: 2.6-17.1 µg/g DM In shoot: 0.2-2 µg/g DM typical In leaf sheaths: 1.15-6.0 µg/g DM In roots: 0-0.17 µg/g DM	Hydrophobic
Peramine	Pyrrolopyrazine alkaloid 	*E. festucae* var. *lolii* in ryegrass *E. coenophiala* in tall fescue	• Affects ASW adults (deterrent, reduced oviposition) & larvae (deterrent, development) **Eff. conc.:** ASW adults, lab: 1-10 µg/g diet (detected at 0.1 µg/g diet) ASW larvae, lab: 2 µg/g diet ASW in field: 15-20 µg/g DM proposed • No known effect on livestock health	• Freely translocated in above-ground parts • Leaf sheath > leaf blade, reduced in senescing leaf sheaths • Values: In seed: 31.4-38.2 µg/g DM In shoot: 10-50 µg/g DM In leaf sheaths: 20.1 – 38.0 µg/g DM In roots: 0-3.9 µg/g DM (low, only sporadic)	Water soluble
Epoxy-janthitrems (5 compounds)	Indole-diterpenoids 	*E. festucae* var. *lolii* strain AR37	• Strong effect, antifeedant and probably toxic to porina and possibly other invertebrates • Toxic to livestock, but less than lolitrem B	• High concentrations in reproductive tillers • Pseudostems/sheaths > blades • High concentrations during summer-autumn • Values: In seed: 63.5 µg/g DM In shoot: 83.9 µg/g DM at 20 °C (7.4 at 7 °C) In blades: 30.6 µg/g DM at 20 °C (0.67 at 7 °C) In roots: 0- 0.24 µg/g DM in some parts	Hydrophobic Is related to lolitrem B
Lolines	Pyrrolizidine lolines 	*E. coenophiala* in tall fescue *Epichloë uncinata* in meadow fescue *Epichloë occultans*	• Affects primarily invertebrates (insecticidal and feeding deterrent, broad activity spectrum) • No known toxic effects on livestock	• Relatively uniformly distributed throughout the shoot in grasses where it is expressed	No/low levels if endophyte is transferred into *L. perenne*

Sources: Bultman *et al.* (2009); Charlton and Stewart (1999); Clement (2009); Fuchs *et al.* (2013); Hennessy *et al.* (2016); Hume and Cosgrove (2005); Johnson *et al.* (2013); Popay (2004); Popay and Hume (2011); Popay and Wyatt (1995); Popay *et al.* (1990); Prestidge & Gallagher (1985); Rowan & Latch (1994); Ruppert (2016); Figures: Johnson *et al.* (2013) [Figure 2, p 175]. ASW: Argentine stem weevil; DM: dry matter; Livestock: sheep, cattle; E^+: endophyte-infected; Eff. conc.: effective concentration

[1] The values reported here refer to measurements on specific plant genotypes-endophyte associations and should only be seen as indicative.

1.5. Interactions between *A. lentisci*, *L. perenne*, and *E. festucae* var. *lolii*

The interactions between *A. lentisci*, its endophyte-infected host plant and the abiotic and biotic macro- and micro-environment are multiple and complex (Figure 1.10). They become even more complex if perennial ryegrass is considered in a sward, competing with other grasses for resources such as light and nutrients.

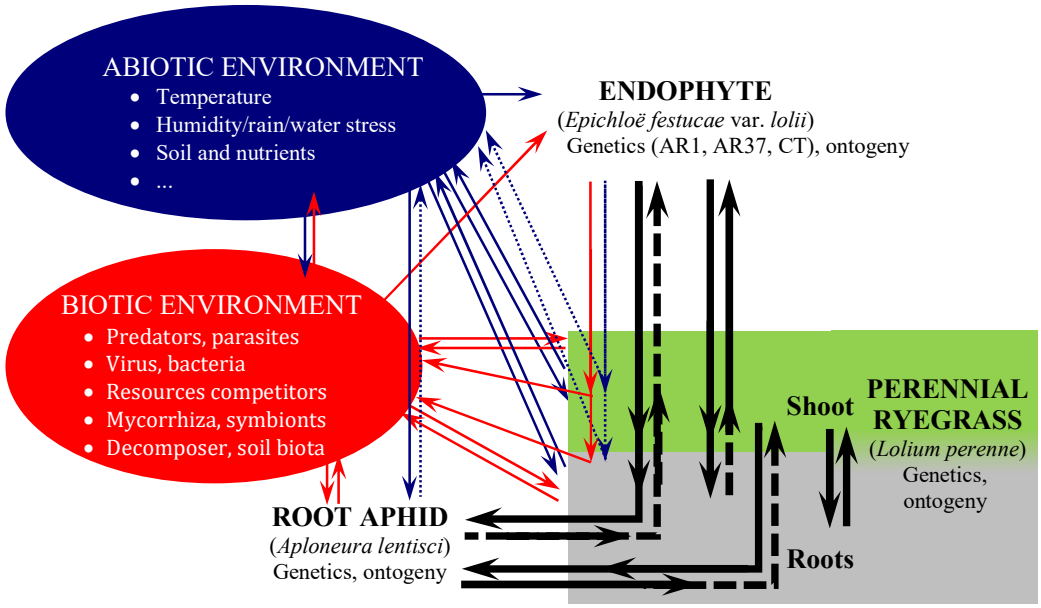


Figure 1.10. Diagram of the interactions between root aphids, perennial ryegrass, endophyte, and environment. Plain arrows represent reported interactions and influences, dashed arrows indicate postulated interactions yet to be demonstrated.

The effects of various *E. festucae* var. *lolii* strains on perennial ryegrasses have been studied extensively [e.g. Bryant *et al.* (2009); Popay (2004); Rasmussen *et al.* (2008a); Simpson *et al.* (2012); Spiering *et al.* (2006); Section 1.4.2]. Although the response to an endophyte infection depends both on the host genotype and environment (Cheplick, 1998), some general observations can be reported. Plant fecundity, seed germination and seedling vigour may be increased in endophyte-infected (E^+) plants, but the endophyte may not unequivocally stimulate the growth of the plant (Cheplick & Cho, 2003). Under certain environmental conditions, it can cost *L. perenne* assimilates (Majidi & Mirlohi, 2016), for example, in nutrient-poor substrates (Cheplick, 2007). Unlike what is reported

for endophyte-infected tall fescue (Bultman & Bell, 2003), perennial ryegrass - *E. festucae* var. *lolii* symbioses do not become more tolerant towards drought stress than endophyte-free perennial ryegrass plants (Johnson *et al.*, 2013). The endophytes mainly benefit *L. perenne* by increasing its resistance to various insects in New Zealand (Table 1.4; Hume & Cosgrove, 2005; Rasmussen *et al.*, 2007) but are probably disadvantageous when insect herbivores are controlled by other means (He, 2016). He (2016) demonstrated that E^+ plants had lower herbage yields than E^- plants when the plants were treated with insecticides, for example.

How perennial ryegrass influences an endophyte is less well understood (Faville *et al.*, 2015). There is evidence that the host genotype may modulate the fungal biomass (Tian *et al.*, 2013) and its expression (Johnson *et al.*, 2013). Endophyte biomass is lower in cultivars with a high content of water-soluble carbohydrates (high sugar grasses), for example (Liu *et al.*, 2011a; Rasmussen *et al.*, 2008a)] and the leaf lamina of tetraploid E^+ ryegrasses have significantly lower peramine concentrations than the ones of diploid E^+ ryegrasses (Johnson *et al.*, 2013). As season, nutrients or other environmental factors can affect both the plants and the endophyte (Sections 1.3.2 and 1.4.1), they are also likely to modify the outcome of the plant's influence on its endophyte.

General influences of plant quality on aphid biology and physiology have been discussed earlier (Section 1.2.6.4). Plants with good growth and many new roots may be advantageous for young root aphids (Popay & Cox, 2016). What traits further modulate the development and fitness of *A. lentisci* on *L. perenne* beyond this will require more research, however.

Root aphid influences on plants are just beginning to be investigated. Popay and Cox (2016) estimated root aphid feeding can reduce the tiller survival of perennial ryegrass by 35% and decrease the foliar biomass by 16 to 27% in an experiment with potted plants. Root aphids may also reduce the root biomass (up to -33%) but this influence is not always significant (Popay & Cox, 2016). However, the environment is, again, bound to have a strong influence on the outcome. Root aphid populations are known to build up more rapidly in dry than in moist soils (Pretorius *et al.*, 2016), for example. Feeding damages by *A. lentisci* are likely more severe in conjunction with concomitant stresses such as drought or heavy grazing (McDonald *et al.*, 2011).

Experiments in controlled environments will have to reveal how such effects develop and how else *A. lentisci* may modify its host plant's anatomy or physiology.

How various endophyte strains influence *A. lentisci* is generally assessed by comparing the number of root aphids found on endophyte-infected plants to the numbers on endophyte-free plants of the same cultivar and/or by directly observing the root aphid behaviour. Several experiments confirm that plants with AR37 endophyte are not colonised, or only poorly colonised by root aphids, an effect that seems due to some antibiotic rather than antixenotic effects of E^+ plants (Popay & Cox, 2016; Popay & Gerard, 2007; Thom *et al.*, 2014). Symbioses with common-toxic (CT) strains have generally transient negative effects on aphid numbers, while plants with AR1 frequently support as many or even more root aphids, than endophyte-free plants (Popay & Cox, 2016; Popay & Gerard, 2007). The mechanisms of resistance to root aphids that underlie these observations are not well understood, however (Podmore, 2015). Alkaloids are traditionally considered the basis of insect resistance (Clay, 1988). Although these candidate molecules appear in low overall quantities in the roots (Table 1.5), they may have higher concentrations in the phloem consumed by the aphids (Popay, 2004). Their concentrations in perennial ryegrass could partly explain the reported observations. Plant-endophyte associations producing epoxy-janthitrems, lolines or ergovaline appear indeed to control root aphids up to a certain extent, while symbioses producing peramine and/or lolitrem B do not (Popay & Gerard, 2007). The variations in alkaloid concentrations because to environmental changes (e.g, temperature) or host plant genotype influences (Christensen & Voisey, 2007; Hennessy *et al.*, 2016; Popay & Cox, 2016) could also explain some of the effect variability by CT-infections. However, as wider ranges of metabolites are examined, there is the growing suspicion that alkaloids might not be the only substances involved in pest protection (Rasmussen *et al.*, 2008b). Root aphids could not be persuaded to feed on artificial diets (personal communication, Dr Sarah Finch, AgResearch). Thus, it is still unclear which specific molecular structures could be feeding deterrent or antibiotic for *A. lentisci*.

Whether root aphids have an influence on the amount of endophytic mycelium in the shoot they live upon is unknown. Interactions between plant symbionts or aphids and plant symbionts have been reported. *Glomus* mycorrhizae in roots reduced the above-ground endophyte colonisation (Liu *et al.*, 2011a), for example, and pea aphids feeding on the shoots of broad beans decreased the mycorrhizal colonisation in the roots

(Babikova *et al.*, 2014). *Rhopalosiphum padi* L. aphids feeding on the shoots of perennial ryegrass had no significant influence on endophyte DNA or alkaloid concentrations, however (Fuchs *et al.*, 2017a). Thus, research will have to consider this point.

1.6. Interactions with other biological grassland elements

Potential natural enemies, resource competitors and other grassland biota are all likely to influence the development of aphid populations in the field (Figure 1.10). There is little information on such biotic interactions for *A. lentisci* in grasslands, however. *A. lentisci* is not ant-attended (Manheim & Wool, 2003) but may be consumed by ants on occasions [e.g. nuptial flight time; Wright and McManus (1998)]. To what extent ants, other aphidivores such as birds, ladybird beetles (Coccinellidae), larval lacewings (Chrysopidae), larval hoverflies (Syrphidae), predatory cecidomyiid fly species (Cecidomyiidae) or generalist predators of herbivore pests such as Carabidae or Staphylinidae beetles, spiders (e.g. Lycosidae), centipedes and mites (Dixon, 1973; Hoffmann, 2016; Keiser *et al.*, 2013; Kergunteuil *et al.*, 2016; Klimaszewski *et al.*, 1996) may control root aphid populations in New Zealand grasslands is not known. As intensively grazed areas without native vegetation are associated with a decline in diversity and abundance of predators (Tomasetto *et al.*, 2017), their effect is likely modest. Whether any entomopathogenic nematodes, entomopathogenic fungi, and bacterial or viral antagonists attack *A. lentisci* and significantly influence aphid numbers in pastures is not known either.

Competing herbivores such as leaf-chewing insects and vertebrates may reduce the resources available to root aphids as herbage removal reduces the leaf area and the root growth in grasses (White, 1973). Furthermore, they may negatively influence aphid populations by triggering systemic defensive pathways in the plants they feed upon (Enders & Miller, 2016). How root aphids are affected by these and various grazing regimes has not yet been reported, however.

The impact of mycorrhizae on aphid life-history traits can range from positive to none, or negative (Tomczak & Müller, 2018). As mycorrhizae are known to improve the nutrient uptake (phosphorus, in particular; van der Heijden *et al.*, 1998), the positive effects reported in some instances (e.g. increased weight and fecundity in adult aphids)

may be explained by alterations of plant nutrient status, plant quality as food, and/or plant resistance to herbivores (Gange *et al*., 1999). Aphid feeding can reduce mycorrhizal colonisation in return, however (Babikova *et al.*, 2014). Furthermore, endophytes in the shoot can also decrease the mycorrhizal colonisation in some cultivars, and mycorrhizal presence can itself lead to a decrease in concentration of foliar endophyte (Liu *et al.*, 2011a) Thus, understanding the interactions of *A. lentisci* with mycorrhizae will likely need in-depth studies considering the growth conditions offered to the roots, the mycorrhiza genotype, the endophyte genotype, the plant cultivar and the interactions between all these elements in presence vs. absence of root aphids.

Finally, endophyte-induced root exudates (Omacini *et al.*, 2012) and the copious amounts of honeydew and wax released in the environment by feeding aphids (Wool & Kurzfeld-Zexer, 2008) are likely to change the soil processes in the rhizosphere around the roots, in ways that may not be easy to predict or comprehend. Collembola, for example, can increase in numbers in parallel with root aphids (Popay, 2004). How this interspecies syngergy impacts the rest of the ecosystem is difficult to say since the (fungivore) collembola may have a variable influence on the biomasses and nitrogen concentration of the tissues of the plants they live next to (Scheu *et al.*, 1999).

1.7. Knowledge gaps and thesis overview

There is a major gap of knowledge about individual *A. lentisci* life history traits (establishment/colonisation success, life span, development, reproductive potential, survival by starvation), plant use (root use, colony dynamics) and impact on plants in New Zealand grasslands. The available information on root aphid interaction with endophytes is limited to short bio-assays and occasional monitoring of populations in grasslands or potted plants (Jensen & Popay, 2007; Moate *et al.*, 2012; Popay & Cox, 2016). As it is difficult in such settings to control the aphid infestation levels, repeatedly monitor specific root aphids without disturbing them and/or the root environment, and avoid random abiotic and biotic influences (Sections 1.5 and 1.6), this knowledge does not yet allow definition of research hypotheses on population dynamics, plant resistance mechanisms, damage thresholds and possible damage mitigation strategies. This thesis aimed to acquire some of the as yet unreported information, using a simplified model system (perennial ryegrass grown in nutrient-enriched agar, infected with or free of the

commercially relevant AR1, AR37 or CT endophyte strains), in a reasonably controlled environment. How this was achieved and is reported is summarised in Table 1.6.

Table 1.6. Thesis structure

	Contents	Experiments
Chapter 1	**Literature review**	-
Chapter 2	**General description of material and methods**	-
Chapter 3	**Biology of apterous morphs of *A. lentisci*** **Objective:** Acquire reference data on root aphid biology (establishment success, colonisation success, size, development time, longevity, fecundity, offspring fitness) on perennial ryegrass, in the absence, and in the presence of the economically relevant AR1, AR37 and CT endophyte strains.	• Biology I experiment • Biology II experiment, *root aphid biology data* • Biology II follow-up experiment • Mature plant experiment, *root aphid reproduction data* • Mature plant follow-up experiment
Chapter 4	**Influences of root aphids on perennial ryegrass plants** **Objective:** Quantify the effects of root aphid numbers on young seedlings and mature plants in perennial ryegrass, in the presence, and in the absence of AR1, AR37 and CT endophyte strains.	• Mature plant experiment, *plant aspects* • Seedling experiment
Chapter 5	**Colonisation ecology of root aphids** **Objectives:** • Analyse the root use of root aphids in the experimental system • Identify possible key elements for root aphid establishment and colonisation • Identify colony development patterns in the experimental system	• Biology II experiment, *root aphid behaviour and root use data* • Wax observations • Population experiment
Chapter 6	**General discussion**	-
Chapter 7	**References**	-
Appendices	**Additional material, r codes used for analyses**	-

CHAPTER 2: GENERAL MATERIALS AND METHODS

This chapter gives a general overview of the materials and methods of the pre-trials and experiments performed for this thesis (Section 2.1). It further describes the facilities used for experiments and production of plant material (Section 2.2), the origins and handling of all biological material (plants, aphids and endophytes; Section 2.3), and the methods used for data collection (Section 2.4). General aspects of the statistical analyses are reported in Section 2.5. Specific statistical information by experiment and data are found in the respective material and methods sections of Chapters 3, 4 and 5.

2.1. Experimental overview

This thesis reports the results of four pre-trials (Table 2.1), four experiments and one observation on mature plants, one experiment on seedlings and two follow-up experiments *ex planta* (Table 2.2). The overviews present the experiments in their execution order. To ease comparisons and discussions, the information collected during these experiments was grouped by topic into the three experimental chapters, however. All aspects related to aphid biology, plant impact and ecology or plant use were reported in Chapters 3, 4, and 5, and some experiments with multiple foci (e.g. Biology II experiment) are consequently mentioned in more than one of these chapters.

Table 2.1. Overview of the pre-trials reported in the appendices.

Time	Pre-trial	Focus (Result section)	Experimental specifics (***Location***[1], conditions)	Plants[1] endophyte statuses	Aphids
June 2012	**Calibration trial I**	Body size changes by preservation (Appendix 9.2)	**-** processed in laboratory	-	Root aphids of all ages collected outdoors on endophyte-free *L. perenne* plants of unknown cultivar
January 2013	**Viviposition trial**	Reproduction and survival of adults *ex planta* (Appendix 7)	***Climate chamber 1*** 17-20 ºC, 14 h light/day REPEATED CHECKS[2] In empty glass tubes	*ex planta*	Adult root aphids collected from colonies living in the glasshouse, on endophyte-free *L. perenne* plants of unknown cultivar
April – May 2016	**Instar measurements**	Size of various instars (Appendix 9.1)	***Climate chamber 1*** 17 °C, 14 h light/day	Clone-plants of genotype N and S AR1, AR37, CT or NIL	• Up to 10 neonates per Petri dish (i.e. plant) • Source: mothers kept on clone-plants at climate chamber conditions (17 ºC, 14 h light/day) for ≥ 6 generations
June 2016	**Calibration trial II**	Relation between body size and weight (Appendix 9.3)	**-** processed in laboratory	-	Adult root aphids collected in the glasshouse, from AR1-infected *L. perenne* plants (unnamed cultivar)

[1] See Sections 2.2 (location) and 2.3.1 (plants) for more specifics; AR1, AR37, CT: infected with AR1, AR37 or common-toxic endophyte strain, respectively; NIL: endophyte-free (Section 2.3.3)

[2] REPEATED CHECKS: aphids repeatedly monitored (if not mentioned, aphids were observed once only, at harvest)

Table 2.2. Overview of the experiments reported in Chapters 3, 4, 5.

Time	Experiment	Focus (Result section)	Experimental specifics[1] (***location***, conditions)	Plants[1] endophyte statuses	Aphids
(Aug – Oct 2012)	**Biology I**	Root aphid biology (3.3.1)	***Glasshouse 9, cubicle 12*** 21 ºC, natural photoperiod, in Petri dishes, REPEATED CHECKS	**Source plants** (16 genotypes) NIL	• 1 neonate per Petri dish (i.e. plant) • Source: mothers from colonies kept in glasshouse 9 on random source plants for ≥ 2 generations
(Sep 2013 – Jan 2014)	**Population**	Population development (5.3.4)	***Conviron climate chamber 1*** 18 ºC, 14 h light/day, in Petri dishes	**Clone-plants** (2 genotypes, N and S) AR1, AR37, CT or NIL	• 0, 1 or 5 neonates per Petri dish (i.e. plant) • Source: mothers from colonies kept in climate chamber on clone-plants for ≥ 2 generations
(Oct 2013 – Jul 2014)	**Biology II**	Root aphid biology (3.3.2) Root aphid behaviour (5.3.1) Root aphid root use (5.3.2)	***Conviron climate chamber 2*** 18 ºC, 14 h light/day, in Petri dishes, REPEATED CHECKS	**Clone-plants** (2 genotypes, N and S) AR1, AR37, CT or NIL	• 1 or 5 neonates per Petri dish (i.e. plant) • Source: mothers from Population experiment and colonies kept in climate chamber on clone-plants for ≥ 3 generations
	Biology II follow-up	Offspring survival (3.3.4)	***Conviron climate chamber 1*** 18 ºC, no light, in microcentrifuge tubes, REPEATED CHECKS	*Ex planta*	• Cohorts of neonates (siblings of 2-3 days) • Source: mothers of the Biology II experiment
(Jun – Oct 2015)	**Mature plant**	Plant reaction to root aphids (4.3.2) Root aphid reproduction *ex planta* (3.3.3)	***Insectary*** 10 ºC, natural photoperiod, in Petri dishes	**Clone-plants** (2 genotypes, N and S) AR1, AR37, CT or NIL	• 0 or 10 neonates per Petri dish (i.e. plant) • Source: mothers from colonies kept in insectary on clone-plants for 1 generation
	Mature plant follow-up	Offspring survival (3.3.4)	***Laboratory*** 20 ºC, no light, in microcentrifuge tubes, REPEATED CHECKS	*Ex planta*	• Cohorts of neonates (siblings of 1 day) • Source: mothers of the Mature plant experiment
(Sep – Oct 2015)	**Colony wax observations**	Colony wax description (5.3.3) Colony description (5.3.3) Root aphid root use (5.3.3)	***Conviron climate chamber 2*** 18 ºC, 14 h light/day, in Petri dishes	**Clone-plants** (2 genotypes, N and S) AR1, AR37, CT or NIL	• 10 neonates per Petri dish (i.e. plant) • Source: mothers from colonies kept in climate chamber on clone-plants for ≥ 2 generations
(Dec 2015 – Jan 2016)	**Seedling**	Plant response to root aphids (4.3.1)	***Percival climate chamber*** 19 ºC, 12 h light/day, in open 50 mL centrifuge tubes	64 genotypes-AR1 75 genotypes-AR37 53 genotypes-CT 51 genotypes-NIL	• 0 or 3 neonates per tube (i.e. seedling) • Source: mothers collected on endophyte-free *L. perenne* plants in the outdoor area (Section 2.2.1)

[1] See Sections 2.2 (location) and 2.3.1 (plants) for more specifics; AR1, AR37, CT: infected with AR1, AR37 or common-toxic endophyte strain, respectively; NIL: endophyte-free (Section 2.3.3);

[2] REPEATED CHECKS: aphids repeatedly monitored (if not mentioned, aphids were observed/counted once only, at harvest)

2.2. Facilities

Seven distinct locations at the Grasslands Research Centre, AgResearch Limited, Palmerston North, New Zealand, were used to carry out experiments, keep plants and maintain aphid colonies for the research reported in thesis: (i) an outdoor area within the Grasslands Research Centre's nursery area, (ii) an insectary in the plant nursery, (iii) two glasshouses (glasshouse 18 and cubicle 12 of glasshouse 9), and (iv) three climate chambers (Conviron climate chambers 1 and 2, Percival climate chamber; Figure 2.1). The general settings and peculiarities of each location are summarised in Sections 2.2.1 to 2.2.4. Their use in various experiments is reported in Tables 2.1 and 2.2 (Section 2.1). In the unshaded centre of the plant nursery, a reference weather station (Section 2.4.1) was installed. This station was used to characterise the climatic conditions in the outdoors sites (Appendix 3). This station was also used in combination with a mobile light measuring device (Section 2.4.1) to estimate and compare the light intensities in all location (Appendix 4).

2.2.1. Outdoor area

This location was used to store a backup of source plants and clone-plants (Section 2.3.1). The plants in this location were naturally colonised by root aphids and served therefore as a source of root aphids for the Seedling experiment and for the setting up of colonies. The outdoor area consisted of a concrete pad within the plant nursery (Figure 2.1a). As it was partly shaded by a hedge, the light intensity in this location was slightly lower than the light intensity measured at the reference weather station (96% of the average reference value of 614 µmoles photons·m^{-2}·s^{-1} [L_{Ref}]; Appendix 4). The other weather parameters were similar, however (local weather conditions, Appendix 3). To avoid water stress, the plants were watered daily from the top via a sprinkler. No pesticide was ever applied to the plants kept in this location.

Figure 2.1. Research locations. (**a**) Outdoor area; (**b**) Insectary (outdoors, covered with insect screen); (**c**) Glasshouse 18; (**d**) Glasshouse 9; (**e**) Conviron climate chamber 2 with top illumination; (**f**) Percival climate chamber with top and side illumination.

2.2.2. Insectary

The insectary was used to perform the Mature plant experiment (Sections 3.2.1.3 and 4.2.2). It consisted of a walk-in pollination cage (2.8 m in length × 1.9 m in width × 1.85 m in height covered by dark insect screen with fine apertures of 1.3 × 1.1 mm; Figure 2.1b) fixed to the concrete pad of the plant nursery, close to a hedge. The plants were kept therein 79 cm above the ground, on temporary benches. The average light intensity in the insectary was approximately 21% of L_{Ref} (the solar radiation measured outside at the same time; Appendix 4). There were probably slight differences in the air temperature and humidity measured inside and outside (reference weather station; Appendix 4) of the insectary, but these differences were unlikely to have had any biological consequences for experiments. The plants were neither watered nor sprayed with any pesticide in this location.

2.2.3. Glasshouses

Glasshouse 18 (ca. 18 m in length × 11 m in width with 6.4 m height at its summit; with twin skin polythene cladding; Figure 2.1c) was used to raise source plants from March 2012 to August 2012 and to maintain aphid-free clone-plants from March 2012 to January 2015 (Section 2.3.1). The plants were kept 0.9 m above ground, on a bench with a capillary mat (Transportect Ltd, Auckland, New Zealand) covered by a black woven weed control mat (Cosio Industries Ltd, Auckland, New Zealand). Light deflection by the cladding and shadows cast by surrounding structures resulted in the plants being exposed to a natural photoperiod length (Appendix 3) at reduced light intensity in this location (56% of the outdoors measured L_{Ref} intensity, Appendix 4). Water was provided by an automatic watering system embedded in the capillary mat and additional watering from the top with a hose as necessary. The climate within the glasshouse was regulated by a general control system in response to air temperature and humidity conditions monitored within the glasshouse. At plant level, the mean relative humidity was 76 ± 17.2% and mean temperature of 23 ± 6.4 °C from 16/01/2013 to 25/02/2013. From the 22/06/2012 onwards, all plants in this glasshouse were subject to a biweekly pest control spraying programme of either Orthene® (Arysta Life Science Ltd., Cary, NC, U.S.A.; 0.8 g/L), Nuvos® (Orion AgriScience Ltd, Christchurch, New Zealand; 0.6 mL/L) or Mavrik® aquaflo (ADAMA New Zealand Ltd, Nelson, New Zealand; 0.4 mL/L). These products

were mixed with DC-Tron® mineral oil (Caltex Australia Petroleum Pty Ltd, Sydney, Australia; 10 mL/L) to enhance the products' adherence to plant surfaces. Occasionally, other compounds and preparations were also used (Appendix 5). Treated plants were not used to set up any experiments or colonies for at least double of each pesticide's recommended withholding period after an application.

Glasshouse 9 was a small glasshouse clad by twin skin polythene and subdivided into small cubicles of 2.5 m in width × 3.96 m in length × 2.16 m (outer side) to 3.65 m (inner side) in height, equipped with 90 cm high benches (Figure 2.1d). Its cubicle 12 was used to grow source and clone-plants (Section 2.3.1), keep aphid colonies and perform the Biology I experiment (Section 3.2.1.1). This cubicle was oriented eastward and provided the plants with a natural photoperiod. The average light intensity in it was about 28% of the L_{Ref}, however. A general climate control system was set to maintain average air temperatures of 10 to 25 °C and an average relative humidity over 60%, but the measured temperature and relative humidity on the benches at plant level varied between 4.0 and 39.2 °C and 17.5 to 100%, respectively. The plants grown in potting mix (Appendix 5) in this cubicle were also kept on a water holding mat covered with a black woven weed mat (see above) and were watered from the top every other day with a hand-held hose. Mavrik® aquaflo (0.4 mL/L)/DC-Tron® (10 mL/L), Orthene® (0.8 g/L)/DC-Tron® (10 mL/L) or Nuvos® (0.6 mL/L)/DC-Tron® (10 mL/L) mixture were sprayed for pest control as required without compromising experiments (treated plants were not used in experiments or as colony plants for at least double the recommended withholding period). In two instances, Diazinon® 20G (Nufarm Ltd., Auckland, New Zealand) granules were also sprinkled over the potted plants to control porina caterpillars, at rates of approximately 10 to 15 granules/pot.

2.2.4. Climate chambers

Three different climate chambers of two distinct types (Conviron and Percival) were used. The two Conviron growth cabinets (Conviron® CMP 3023, Winnipeg, Canada; e.g. Figure 2.1e) were used to maintain aphid colonies and to conduct several aphid biology experiments (e.g. population and Biology II experiments, Sections 5.2.3 and 3.2.1.2). Illumination was provided by eight Philips cool daylight triphosphor tubes 36W/865 and four Philips Softone 100 W incandescent lamps installed in the ceiling of

each chamber, which provided a light of approx. 230 µmoles photons·m^{-2}·s^{-1}, i.e. about 38% of the average intensity measured outdoors during daytime hours (Appendix 4). Both chambers were set to maintain reference conditions of 17°C [the optimal growth temperature for perennial ryegrass roots according to Robin (2011)] and 14 h of light per day [14:10 h light/dark, a photoperiod expected to maximize shoot growth under artificial light; personal communication by Alison Popay]. The relative air humidity in the chambers was approximately 60%.

The Percival climate chamber (Model I-35LLVL, Boone, U.S.A.; Figure 2.1f) was used for the Seedling experiment only (Section 4.2.1). Illumination was provided by two short cool daylight tubes (Philips TLD 18W/865) fixed horizontally to the chamber's ceiling, and four cool daylight tubes (Philips TLD 36W/865) arranged laterally (from the ceiling to the floor of the chamber), which provided a total light intensity of 70 µmoles photons·m^{-2}·s^{-1} on the top shelf (11% of L_{Ref}, the average intensity measured outdoors). Following recommendations for seedling germination tests (Ellis *et al.*, 1985), this chamber was set to maintain a photoperiod of 12 h light per day and temperatures of 19 to 20 °C. A relative humidity of 88.0 ± 4.6% was measured in it during the Seedling experiment.

As the plants kept in the climate chambers were rooting in agar, they were not watered unless the agar was close to drying out (see Biology II and Population experiments, Sections 3.2.1.2 and 5.2.3 for specifics in this case). No pesticide was ever applied to any plant in the climate chambers.

2.3. Origin and handling of plants, aphids and endophytes

2.3.1. Plants

2.3.1.1. Plant material

Three types of plant material were used: (i) mature 'clone-plants' (the most commonly used, model plants), (ii) mature 'source plants' (used in the Biology I experiment and as occasional colony plants), and (iii) seedlings (used in the Seedling experiment only).

The 'clone-plants' were eight mature, long-standing perennial ryegrass-endophyte associations of two distinct plant genotypes (N and S) each available in one of four

endophyte status each (namely, endophyte-free or infected with the AR1, AR37 or common-toxic strain of *Epichloë festucae* var. *lolii*; Table 2.3). These plant-endophyte associations were created through inoculations of clone ramets in a previous project (Simpson *et al.*, 1997), and maintained since then in the mycology laboratory of AgResearch. As these plants offered the unique possibility to examine the effects of plant and endophyte genetics in a fully factorial design (2 plant genotypes x 4 endophyte status), they were used as a model system in most experiments. A group of tillers was split off from each mother plant in March 2012 and re-potted into a PB1½ planter bag (Caranz Ltd, Drury, New Zealand) filled with approx. 0.9 L of potting mix which included a 8-9 month slow release fertiliser (Appendix 5). These plants were left to recover and then re-split and re-potted in the same way to generate several plants of each plant-endophyte association and guarantee thereby enough tillers for larger experiments.

Table 2.3. Clone-plant nomenclature.

Name[1]	Plant genotype (Cultivar)[2]	Endophyte[3]
N-AR1	Genotype N (Grasslands Nui)	AR1
N-AR37	Genotype N (Grasslands Nui)	AR37
N-CT	Genotype N (Grasslands Nui)	Common-toxic (AR93)
N-NIL	Genotype N (Grasslands Nui)	No endophyte
S-AR1	Genotype S (Grasslands Samson)	AR1
S-AR37	Genotype S (Grasslands Samson)	AR37
S-CT	Genotype S (Grasslands Samson)	Common-toxic (AR93)
S-NIL	Genotype S (Grasslands Samson)	No endophyte

[1] These names will be used henceforth to refer to the respective plant-endophyte association
[2] The two genotypes available were merely random individuals of the respective cultivars. Their response to various treatments should not be considered as representative for these cultivars.
[3] *E. festucae* var. *lolii* strains inoculated into the plants (Simpson *et al.*, 1997)

To get a wider spectrum of plant genetics, 192 seeds of each accession listed in Table 2.4 were sown into seedling trays (hygiene trays of 42 cm in length × 30 cm in width × 5 cm in depth, filled with standard potting mix with a three to four month slow release fertiliser; Appendix 5) in March 2012. The endophyte status of each developing

seedling was confirmed by immunoblot of one tiller/plantlet ≥ 4 weeks after sowing. Sixty qualifying plantlets per accession ('source plants') were then randomly selected, re-potted into PB¾ planter bags filled with 0.45 L potting mix with eight to nine month slow release fertiliser (Appendix 5) and left to recover in glasshouse 18 (Section 2.2.3). Eighteen of these plants from the endophyte-free accession were used for the Biology I experiment in August 2012, and ramets from several plants of the endophyte-free accession were also used to keep aphid colonies on in 2012 and 2013.

In December 2015, the source plants accessions (Table 2.4) were used again to produce seedlings for the Seedling experiment (Section 4.2.1).

Table 2.4. Perennial ryegrass accessions used for the Biology I and Seedling experiments.

Cultivar	Endophyte strain	Accession	Additional information
Grasslands Samson	None	A11104	Harvested in 2000
Grasslands Samson	AR1	A14559	Harvested in 2003
Grasslands Samson	AR37	A16863	Harvested in 2011[1]
Grasslands Samson	Common-toxic (CT)	A12421	Harvested in 2005

These accessions were provided by the Margot Forde Germplasm Centre, AgResearch Ltd., Grasslands Research Centre, Palmerston North, New Zealand

[1] Field dressed accession (all other accessions were machine dressed)

2.3.1.2. General plant handling

Seedling handling is described in length in Section 4.2.1 (Seedling experiment). The plants supplying mature tillers for trials were grown in potting mix with a eight to nine month slow release fertiliser (Appendix 5), initially in PB¾ and PB1½ planter bags (Caranz Ltd, Drury, New Zealand), then in rigid, square tube forestry pots of 9 × 9 cm at the top, 6.5 × 6.5 cm at the bottom and 15 or 18 cm in depth for bottomless pots and olive pots, respectively, with 0.8 to 0.97 L content (Daltons™, Matamata, New Zealand). The plants needed for experiments were kept in glasshouses (Section 2.2.3), watered as required to avoid water stress, and periodically trimmed back to approximately 3 cm tiller length, to stimulate plant growth and tillering. Once the nutrients from the initial potting mix were depleted, the plants were either supplied 6 g controlled-release fertiliser beads

[SK Cote™ precise with 17% N, 2% P, 10.5% K, 2% Mg +Trace elements from S K Specialists Sdn BhD, Sibu, Sarawak (Malaysia)] or re-potted by splitting off 5 to 10 tillers and cutting them back to 3 cm shoot length before planting them into new potting mix. Insecticides were applied according to a spraying plan in Glasshouse 18 or as needed in Glasshouse 9 (Section 2.2.3; Appendix 5). All plants were left to recover for at least two weeks after trimming, re-potting or pesticide treatment, before being used to set up new colonies or experiments.

2.3.1.3. Plant mounting

An experimental setup for plants for this research: (i) needed to provide a healthy, developing/growing plant for the root aphids to feed on, (ii) had to reliably contain all aphid instars placed in it, and (iii) had to allow for repeated aphid observation with a minimal amount of disturbance for both aphids and plant. As solidified nutrient solutions worked best for plant survival and aphid monitoring in pre-trials (data not shown), nutrient-enriched agar was chosen to supply perennial ryegrass roots with water and nutrients in all the *in planta* experiments presented in this thesis. Nutrient-enriched agar was first made by mixing 10 g agar with 1 L of Thrive™ fertiliser solution (0.2 g/L; Yates New Zealand, Auckland, New Zealand; Biology I experiment). This solution was low in nutrients and the agar lacked firmness, however. It was replaced by a modified Bollard (1966) medium solution agar with 15 g agar/L in 2013 (MBM agar, Appendix 6). In both cases, the nutrient-agar mix was autoclaved for ≥ 20 minutes at ≥ 117 °C (Burns&Ferral Steam Generator, Burns & Ferral, Auckland, New Zealand) and left to cool down to ≤ 80 °C before being poured into the final containers (Petri dishes or GLAD® BBQ trays, see below). Once the agar had fully cooled down, the agar-filled containers were sealed with GLAD® wrap (GLAD® Products Australia, Padstow, Australia), sterilised by ≥ 15 minutes exposure to UV light and kept in a fridge at 5 °C until needed for mounting the plants.

Plants for root aphid colonies were initially kept in standard AgResearch potting mix (with three to four month slow release fertiliser; Appendix 5). As this substrate required re-watering in 3- to 5-day-intervals, it was replaced by MBM agar from March 2013 onwards.

Two methods were used to prepare colony plants for root aphid colony rejuvenation and mount plants for experiments: (i) Petri dish embedding (Figure 2.2) and (ii) tube embedding (Figure 2.3). Petri dish embedding (Figure 2.2) was developed on the basic concept of notched Petri dishes described by Popay (2004). It was used to embed mature tillers. The tillers to embed were split off their mother plants, washed clean under running tap water and necrotic tissue removed. Tillers for colony plants were then cut back 4 cm above the shoot base and immediately embedded (without root trimming). Tillers for experiments were initially (Biology I experiment) cut back to 4 cm shoot length and 2 cm root length, weighed and immediately embedded. This trimming resulted in tillers with very different initial root/shoot ratios (Figure 2.4b) and did not allow to screen out tillers that had been damaged during the splitting of the mother plant, however. Consequently, tillers for later experiments were trimmed to 4 cm shoot length, 0.3 cm root length and any remaining side roots removed (Figure 2.4c) before being weighed and left to recover for several days in a Conviron climate chamber, in glass tubes (7.6 cm in length × 2.4 cm in diameter) filled with approx. 20 to 30 mL tap water. The water in these tubes was changed every 24 to 48 h until embedding. At the embedding, Petri dishes (9.0 cm in diameter × 1.5 cm in height, LabServ®, Thermo Fisher Scientific New Zealand Ltd) loaded with either 90 mL potting mix (90 mL; 2012 colony plants) or 40 to 60 g nutrient-enriched agar (2013-2016 colony and experimental plants) were notched in the side wall with a scorching hole puncher (0.6 cm diameter). The previously trimmed tillers were threaded through this hole and fixed with BluTack™ (Bostik Australia Pty. Ltd., Victoria, Australia) with the shoot outside and roots inside of the Petri dish as represented in Figure 2.2(a), at rates of three tillers per Petri dish for colony plants, and one tiller per Petri dish for experimental plants. In early experiments (Biology I and II), an autoclaved, moistened cotton ball was also placed onto the roots to prevent them from dessicating until they had grown into the substrate The Petri dishes were then sealed with 2-cm wide strips of GLAD® wrap and individually wrapped into a black polyethylene sheet (35 × 35 cm, 80 microns thick; Cosio Industries Ltd., Auckland, New Zealand) with star-shaped slit (Figure 2.2e). As this wrapping alone led to a heating up of the root area when the mounted plants were exposed to light (Biology I experiment), the Petri dishes were further wrapped in a second, outer white sheet of the same size ('panda film', 125 microns thick; Cosio Industries Ltd., Auckland, New Zealand). The wrapped experimental units (Figure 2.2c) were then stored vertically in hygiene trays (42 cm in length × 30 cm in width × 5

cm in depth, up to 48 samples/tray; Figure 2.2d) in their respective experimental location. On impending water stress, all samples were either re-watered by spraying approx. 0.8 ml sterilised tap water on the agar every three ot seven days (2012-2014), or by adding solidified MBM agar at room temperature into the Petri dishes (2015-2016).

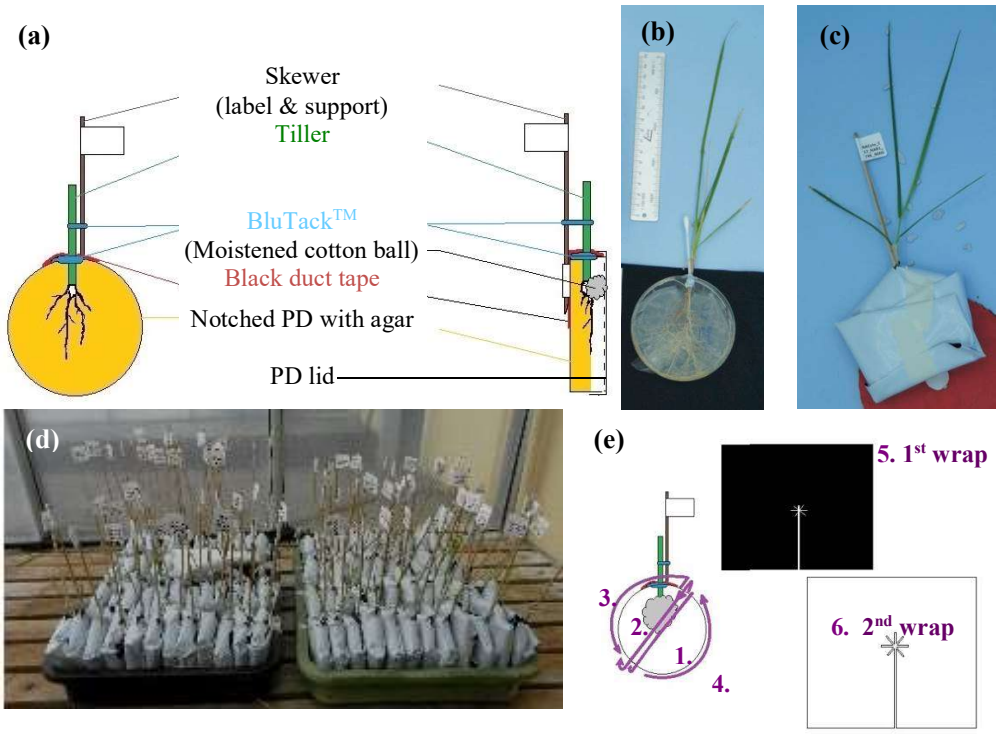


Figure 2.2. Petri dish (PD) embedding. (a) Mounting diagram from above (left; open PD without cotton ball) and side (right; closed PD with cotton ball, not sealed yet); **(b)** Mounting and recovering tiller in PD, unwrapped and open; **(c)** Recovering tiller in closed, fully wrapped PD; **(d)** Hygiene trays with closed, fully wrapped PDs in Glasshouse 9, and **(e)** PD closing and wrapping diagram, with wrapping steps for a tight sealing of PDs (purple arrows/numbers).

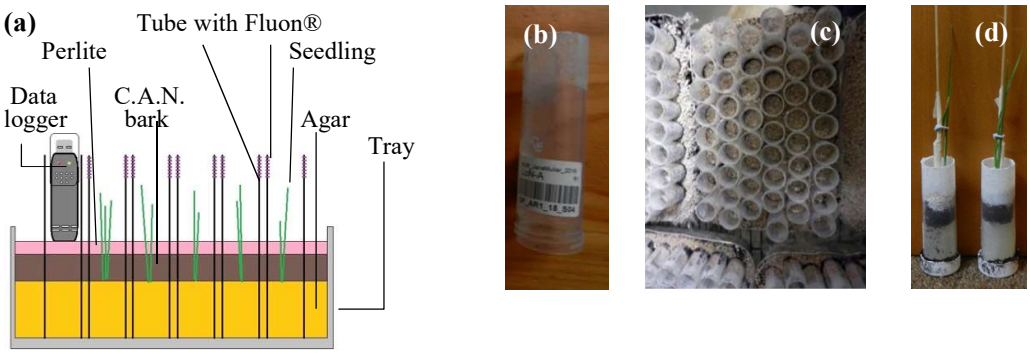


Figure 2.3. Tube embedding. (a) Diagram of embedded tubes, **(b)** Prepared tube before mounting with label, **(c)** and **(d)** mounted tubes.

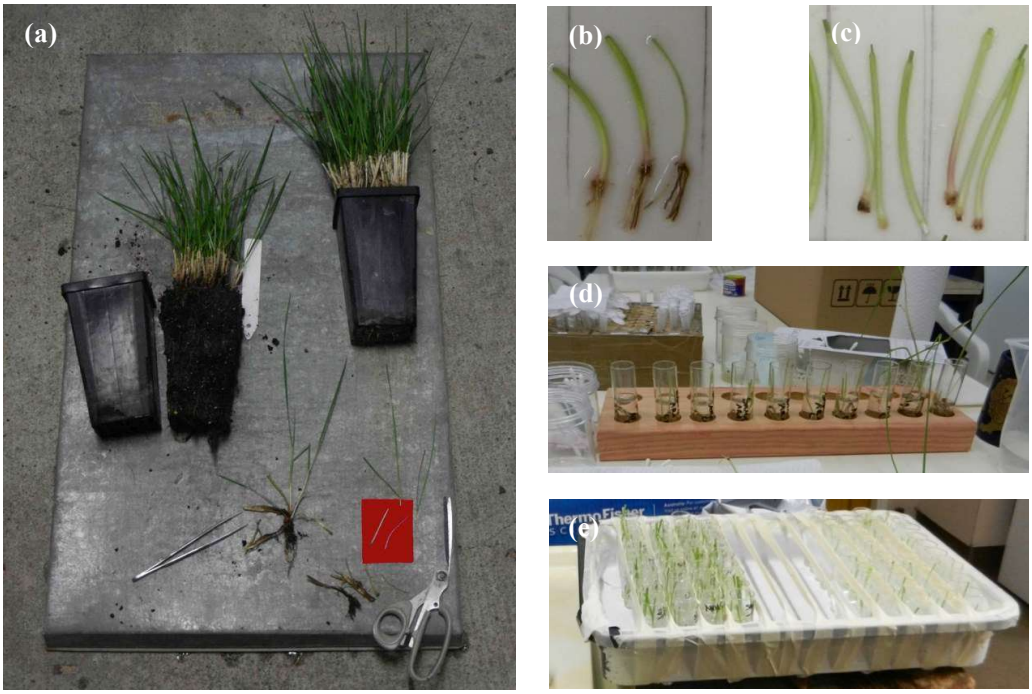


Figure 2.4. Trimming perennial ryegrass tillers. (a) Steps from plant (top) to trimmed tiller for experiments (bottom, red square); **(b)** Tillers trimmed to 2 cm root and 4 cm shoot length (Biology I experiment) and **(c)** tiller trimmed later on to 0.3 cm root length and 4 cm shoot length (from September 2013/Population experiment onwards); **(d)** Trimmed tillers in 20 to 30 mL water (seven tubes left) and tillers yet to be trimmed (three tubes at the right); **(e)** Tillers recovering in glass tubes, before embedding.

Tube embedding (Figure 2.3) was specifically developed for this thesis because, unlike mature tillers, seedlings did not survive an embedding procedure as described above. Two litres of autoclaved modified Bollard (1966) medium agar (Appendix 6) were poured into cling wrap lined GLAD® BBQ trays (29 cm in length × 19 cm in width × 5 cm in height, filled to approx. 4 cm height). The MBM agar was left to solidify before being wrapped in cling wrap and exposed for 30 minutes to UV light in a fume hood for surface sterilisation. Centrifuge tubes (50 mL; LabServ®, Thermo Fisher Scientific New Zealand Ltd) were prepared by cutting them open at the pointed end and dipping that end 1 to 2 cm deep into fluon (Insect-a-Slip Insect Barrier - Fluon®; BioQuipProducts, Inc., Rancho Dominguez, California, U.S.A.) to prevent aphids from escaping during the experiment. The other end of the tubes was exposed to UV light for 30 minutes for sterilisation. Germinating seedlings (Section 4.2.1) were deposited on the solidified agar (Figure 2.3a), 3 cm apart of each other into notches, and were isolated from each other by pushing the UV-treated side of one prepared tube around each seedling into the agar, to the bottom of the tray. Autoclaved, screened fine bark shreds (C.A.N. bark, Daltons Ltd., Matamata, New Zealand; Appendix 5) was poured over the agar and seedlings to a height of 1.2 to 1.5 cm to cover the root area. Additionally, a thin layer of autoclaved perlite (grade C500; Industrial Processors Ltd., Auckland, New Zealand) was powdered over the bark layer to avoid excessive heating of the substrate when exposed to light (Figure 2.3).

2.3.2. Aphids

It is particularly difficult to use first instar root aphids for inoculation because of their small size and wandering behaviour (Campbell & Hutchison, 1995). To achieve the aims of this thesis, a best practice method had to be developed. This was done empirically from March 2012 to March 2013.

Work on root aphids was performed under stereo microscopes (Stemi SV6 Zeiss, Carl Zeiss AG, Oberkochen, Germany or Wild M7A, Heerbrugg, Switzerland) at a magnification of 8 to 60×. The identity of *A. lentisci* was confirmed using the criteria reported in Section 1.2.2. Before March 2013 (Biology I experiment, Viviposition trial, early colony rejuvenations), the aphid handling was performed in daylight. Colonies were rejuvenated by directly transferring 10 to 40 first instar root aphids with a paintbrush from

an old colony plant to a new plant. To obtain nymphs for experiments, roots infested with adult root aphids were harvested (Figure 2.5a) either from colony plants kept in glasshouse 9, cubicle 12 (Section 2.2.3) or from source plants (Section 2.3.1) kept in the outdoor area of the plant nursery (Section 2.2.1). These adults were transferred in groups onto viviposition plants [endophyte-free (E^-) perennial ryegrass plants embedded in Petri dishes laid out with moistened root germination cardboard (Appendix 10, Section A10.1)], and left there for up to 24 h. The offspring produced in that time were directly transferred onto the experimental plants with a fine paintbrush. Later checks on feeding aphids (e.g. for size analyses; Section 3.2.1.1) were done by carefully brushing the wax away from the aphids with a fine paintbrush. This approach was very time consuming, however, did frequently result in wounded root aphids, was not able to satisfactorily contain wandering first instars, exposed the aphids to a lot of unusual and varying environmental stimuli (e.g. changing daylight; Section 1.2.4.4), and did not allow records to be kept on the individual mother of each neonate nymph. As the environmental effects experienced by a mother can influence her offspring's biology for several generations (telescoping generations; Sections 1.2.4.3), this procedure appeared particularly problematic.

The handling after March 2013 addressed the afore mentioned issues in various ways. Work on aphids was performed in a dark room illuminated by a 6-Watt white LED light only (Austrabeam/stylelux, model L3-857391/PLU7080; Mercator Lighting Pty Ltd, Coolaroo, Australia). When nymphs were needed for a placement, roots infested with *A. lentisci* (colony plants or roots cut off E^- perennial ryegrass plants from the outdoor area in the plant nursery; Figure 2.5a) were brought into the dark room and sprayed with tap water. This removed the wax, accelerated the voluntary stylet withdrawal (Figure 2.6a and b), and increased locomotion in both, adult and first instar *A. lentisci*. If colonies had to be rejuvenated, walking early first instar nymphs were directly transferred from the root pieces onto the new plants with a fine paintbrush. However, if individuals of known age were required, wandering adult root aphids were collected instead, and maintained either (i) individually in 1.5 or 2-mL microcentrifuge tubes (Eppendorf Quality™, Eppendorf AG, Hamburg, Germany or LabServ®, Thermo Fisher Scientific New Zealand Ltd) or (ii) in groups of up to 100 aphids in small glass Petri dishes (3.6 or 4.5 cm diameter) sealed with one square of Parafilm® M (Bemis Company, Inc.; Oshkosh, USA; Figure 2.5b). The aphid-loaded tubes and glass Petri dishes were double wrapped in a

black polyethylene sheet (35 × 35 cm^2) or brown paper bag (Figure 2.5b) surrounded by a piece of panda film (white side out) to prevent further exposure to light, and were maintained at controlled conditions for 12 to 24 hours, in climate chambers or in a laboratory (for the specifics of each experiment, see Chapters 3, 4 and 5). The offspring viviposited by the end of this period were transferred into microcentrifuge tubes to contain them until placement. The aphid mothers were moved into new tubes or glass Petri dishes and re-used in the same way for up to three more reproduction rounds of 12 h or one more round of 24 h since previous experience suggested maternal reproductive potential and survival of offspring were fairly similar over that time frame (Appendix 7). Before being placed onto new plants, the neonate nymphs of known age were tapped out of the microcentrifuge tube onto a glass Petri dish, into the middle of a water ring to prevent them from walking away (Figure 2.6). The exact number needed was transferred from there into a new microcentrifuge tube with a fine paintbrush. These neonates were then released above their new plant's roots all at once, by turning the open tube over them and tapping it with a large marker pen. The success of this operation was monitored in Petri dish-embedded plants by re-counting the number of aphids on the roots under a stereo microscope before closing, sealing and wrapping the Petri dishes as represented in Figure 2.2. Autoclaved tap water at room temperature was sprayed in a fine mist onto the wax to gently remove it when established aphids had to be observed, counted or photographed (Section 2.4.3).

For *ex planta* experiments, root aphids were placed with a fine paintbrush either into a glass vial (Appendix 7) or a microcentrifuge tube (see Chapter 3 for specifics by experiment) and checked upon periodically. When the aphids were not monitored, the containers were wrapped light-proof and kept in a specific location at constant conditions.

Figure 2.5. Root aphid handling. (a) *A. lentisci* infected root harvest (roots surrounded by white wax on the plant); **(b)** Adult collection (left), transfer of adults into microcentrifuge tubes or onto glass Petri dishes (centre), and packing of loaded glass Petri dishes into a primary wrapping (right); **(c)** Microscope and LED lamp used when handling root aphids beyond March 2013.

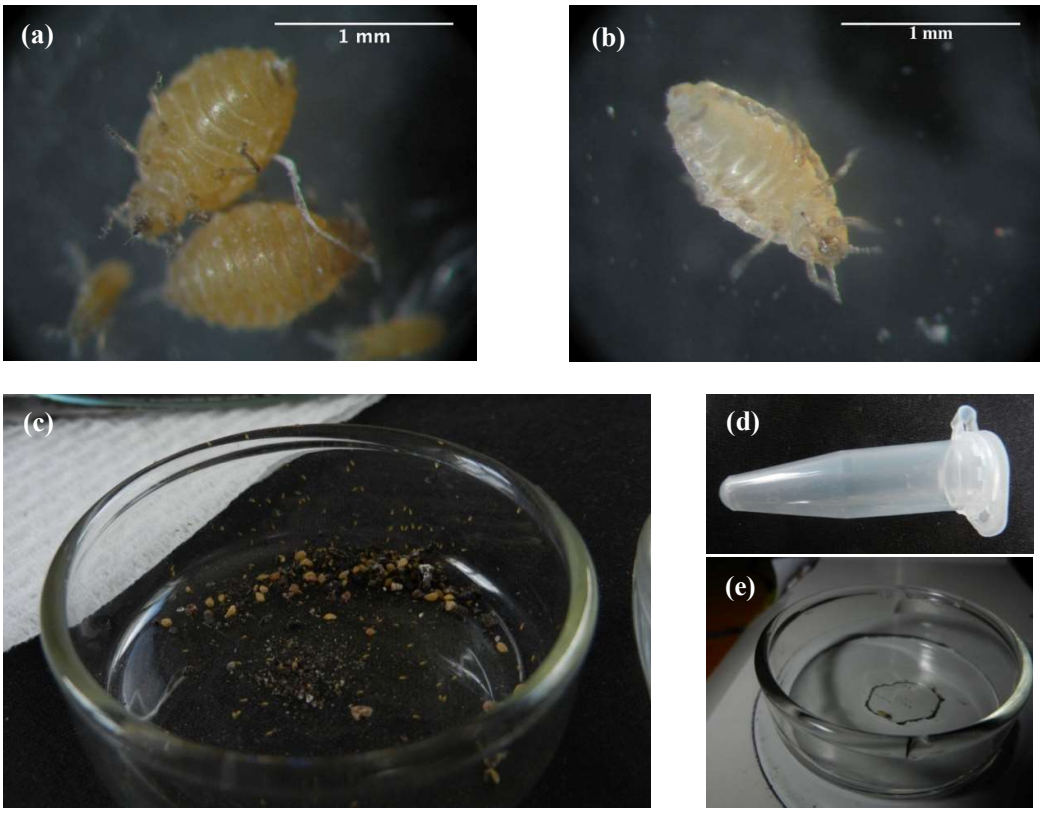


Figure 2.6. Root aphid handling issues and solutions. (a) Injured aphid (forcefully removed stylets), unsuitable for later use; **(b)** Apparently adult aphid having had the time to voluntarily withdraw its stylets (rostrum in resting position, stylets sheathed); **(c)** Mobile first instar nymphs climbing out of a glass Petri dish; **(d)** First instar nymphs contained in microcentrifuge tubes and **(e)** first instars trapped in the middle of a water circle in a glass Petri dish.

2.3.3. Endophytes

Four *Epichloë festucae* var. *lolii* strains were represented in the plant material used for this research: AR1, AR37 and two common-toxic endophyte strains [AR93 in clone-plants (Table 2.3) and an unknown common-toxic strain in plants of the A12421 accession (Table 2.4)]. Although the exact molecular nature of the two common-toxic strains may have been slightly different, they were considered to have an equivalent alkaloid profile (personal communication, David Hume). Both were therefore referred to as 'CT strain' in this thesis. The genetic identity of the endophytes in all mature plants used over the course of this thesis was confirmed on the basal (1 cm) piece of two tillers per plant, by one of two methods:

(i) **Simple sequence repeat** (SSR, also known as 'microsatellites') analyses were performed by the team of the molecular marker laboratory of the Forage Science group of AgResearch (Palmerston North, New Zealand) on one set of clone-plants. Thereby, selected fungal DNA segments were amplified and analysed by electrophoresis according to a standard protocol (Card *et al.*, 2014a), using the two markers ans25 and egs02 to discriminate between the strains.

(ii) **High resolution melting** (HRM) analyses were performed by SlipStream Automation (Palmerston North, New Zealand) on all mature E^+ clone-plants and source plants. Strain identification by HRM is also based on differences in selected DNA segments but uses small differences in the dissociation behaviour of well-known, polymorphic DNA sequences when exposed to increasing temperatures to discriminate between endophyte strains (Applied Biosystems, 2009). Although this method is not able to discriminate between all known endophyte strains, it is able to reliably distinguish AR1, AR37 and CT strains (personal communication, Mike Cook, SlipStream Automation).

Epichloë festucae var. *lolii* is maternally transmitted, i.e. from a plant to its seeds (Popay & Hume, 2011). Transfer by pollen or by physical contact during normal plant maintenance work has not been observed to date (Christensen & Voisey, 2007). Artificial inoculation attempts on mature plants generally fail (Liu *et al.*, 2011a). Empirical evidence suggests that only targeted laboratory work on axillary buds has ever succeeded in establishing endophytic symbionts into mature, endophyte-free tillers and that even then, the success rate was very low (Simpson *et al.*, 1997). An endophyte-free (E^-) plant

could, therefore, be expected to remain so under standard handling procedures. Endophyte-infected (E^+) plants, however, may occasionally produce E^- tillers when the meristematic zone of an axillary bud has not been colonised by the fungus before its separation from the meristematic zone of its mother plant (Christensen & Voisey, 2007). Endophyte presence needed, therefore, to be confirmed in every tiller used for experimental work. Two methods described in Simpson *et al.* (2012) were used for that in this thesis: (i) direct microscopy (also known as 'staining') and (ii) immunodetection or immunoblotting ('blot', 'blotting'). Direct microscopy supplied immediate results and did not require sacrificing the tiller of interest. The protocol for this method was to first remove all dirt and necrotic leaf sheaths from the tiller (Figure 2.7a), then peel off the outermost of the living sheaths and cut off an approx. 0.5-cm-long piece of the lower leaf sheath with a sharp scalpel (Figure 2.7b). That piece was then opened by a vertical cut in the middle and transferred to a glass slide, in a drop of aniline blue solution (50% glycerol, 25% lactic acid, 24.95% water, 0.05% aniline blue) with the adaxial epidermis facing upwards (Figure 2.7c). A cover slip was placed over it before the slide was held over a naked flame until the aniline solution started to boil, enabling the stain to better penetrate the tissues (Figure 2.7d). Once the slide had cooled, it was examined for blue-stained fungal hyphae with a compound microscope (Carl Zeiss AG, Oberkochen, Germany), at magnifications of 100 to 400x (Figure 2.7f). Endophytes were easily distinguished from saprophytes and other fungi when using this method, by the characteristic, cell-parallel and rarely branched arrangement of their hyphae. This method did not detect endophytes which had colonised the tiller meristem but had failed to establish in leaves, however.

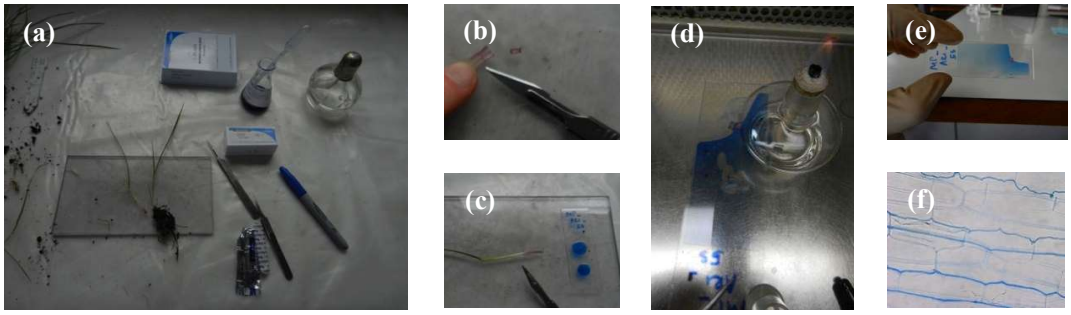


Figure 2.7. Detecting endophyte presence by direct microscopy. (a) A green leaf is removed from a tiller; **(b)** Ca. 5 mm of the inner epidermis or **(c)** Ca. 5 mm of the full leaf sheath is cut off and spread in a drop of aniline blue, inner side upwards; **(d, e)** The slide is covered and the solution heated over a flame for the stain to better penetrate the tissues; and **(f)** Stained hyphae as observed at 400× magnification. © Mike Christensen.

Immunodetection was a more practicable method when large numbers of tillers from a same plant had to be screened, but could not be performed without killing the screened tiller. The protocol for this method was to rid the tiller to test of dirt and necrotic material, severe it at the very base of the shoot, get a clean cross section of the shoot by a second cut with a sharp, clean scalpel a few mm above the first cut and press this cross-section onto a nitrocellulose membrane ('NCM', Amersham™ Protran™ 0.45 NC, Global Science & Technology Ltd, Auckland, New Zealand). Plant and fungal (if the tiller was E^+) proteins were deposited on the membrane by this process. The NCM was then developed as described by Simpson *et al.* (2012) in the following steps:

1) The remaining surfaces on the NCM were blocked by immersion in a milk protein blocking solution (BS) [2.42 g Tris(hydroxymethyl)methylamine, 2.92 g NaCl, 5 g non-fat milk powder, 10 mL of 1M HCl, made up to 1 L with RO water (i.e. water filtered by a reverse osmosis process) and adjusted to pH 7.5]. The NCM was shaken for ≥ 2 h in this solution on an orbital shaker at room temperature.
2) The old BS was decanted off the NCM and the NCM rinsed twice with fresh BS before being shaken for 15 minutes in a solution of primary antibody (rabbit anti-endophyte; produced at AgResearch in collaboration with the Massey University Small Animal Production Unit) and BS (1:1000 dilution) and left incubating in it overnight at 4°C.
3) The NCM was rinsed twice in fresh BS to remove any unbound primary antibody.
4) The NCM was shaken at room temperature for 15 minutes in a solution of secondary antibody (goat anti-rabbit IgG-AP, sc-2034, Santa Cruz Biotechnology, Dallas, TX, U.S.A.) and BS (1:4000), and left incubating in this solution for 5 h at 4°C.
5) Excess antibodies were removed by decanting and rinsing the NCM twice in BS.
6) Two separate chromogen solutions were prepared in amounts adjusted to the NCM surface area to be developed. For any 10 cm^2 NCM, 20 mg Fast Red TR and 12.5 mg of naphthol AS-MX phosphate (F-2768 and Sigma N4875, respectively, both products of Sigma-Aldrich New Zealand Ltd, Auckland, New Zealand) were dissolved in 12.5 ml of Tris buffer each [24.2 g

Tris(hydroxymethyl)methylamine in 1 L RO water, adjusted to pH 8.2]. The NCM was then immersed into a combination of these two chromogen solutions and shaken at room temperature for ca. 15 minutes until tillers known as E^+ (or positive reference tiller blots added to the NCM for this purpose) had developed a bright red colour (Figure 2.8)

7) Finally, the NCM was rinsed three times in RO water.

Immunodetection occasionally resulted in falsely positive (E^+) records, e.g. when the NCM was old and/or got being contaminated with soil during the handling (David Hume, personal communication) or when fungal pathogens or saprophytes were present in the tiller and left fungal protein on the NCM. Endophytes present in the base of the tiller, but having failed to colonise the leaves were revealed too, however.

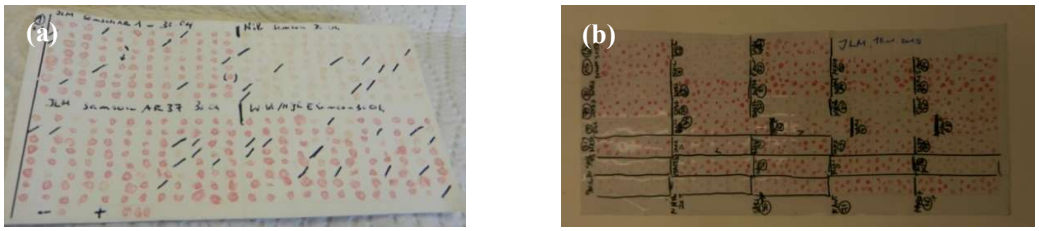


Figure 2.8. Revealing endophyte presence by immunodetection, blotting results. (**a**) Developed immunoblot, dry; (**b**) Developed immunoblot, humidified for better discrimination of negative (pale pink) and positive results (bright red).

2.4. Data collection and processing

This section gives a general overview of all material and methods ever applied to gather environmental (Section 2.4.1), plant-related (2.4.2) and root aphid-related data (section 2.4.3) for this thesis. Which parameters were selected for a given experiment and how they were analysed is described in greater length for each experiment, in the respective material and methods sections of Chapters 3, 4 and 5.

2.4.1. Environmental conditions recording

The climatic parameters likely to most influence the growth and development of plants and aphids during this research were considered to be (i) the temperature experienced by the aphids and roots and (ii) the light available to the shoot for

photosynthesis (photoperiod and light intensity). As scientific equipment was in limited supply, these parameters were preferentially measured over other parameters (e.g. air temperature and relative air humidity in close vicinity of the shoot). Three types of devices provided information on environmental conditions, namely (i) Digitech data loggers, (ii) an outdoor Watchdog weather station, and (iii) a LI-250 light meter.

Digitech QP-6013 data loggers (Jaycar Electronics, Auckland, New Zealand; Figure 2.9) monitored air temperature and relative humidity at 10-minute intervals in selected locations. When set up to log the conditions experienced by root and root aphids, these loggers were wrapped in the same way as the experimental units to monitor. When they measured the ambient air conditions amongst plants in the glasshouse or outdoors, the loggers were placed unwrapped at plant level but were shielded from water or rain as represented in Figure 2.9(c).

The WatchDog 2900ET Weather Station (Spectrum Technologies, Inc.; Aurora, IL, U.S.A.) was installed outdoors, on a pole in the plant nursery area, approx. 95 cm above the ground (Figure 2.10). It logged air temperature, relative humidity, rainfall, solar radiation, wind direction, wind speed, wind gusts and dew point at 15-minute intervals (Appendix 3). The solar radiation measurements of the weather station were combined with synchronised light intensity measurements obtained with a LI-250 light meter (see below) in glasshouses, insectary, or the outdoor area to estimate the total light intensity experienced by the plants in these locations (Appendix 4).

Figure 2.9. Digitech data logger. (**a**) Fully wrapped as placed amongst experimental plants, (**b**) unwrapped, and **(c)** at plant level in Glasshouse 9, measuring the ambient air conditions.

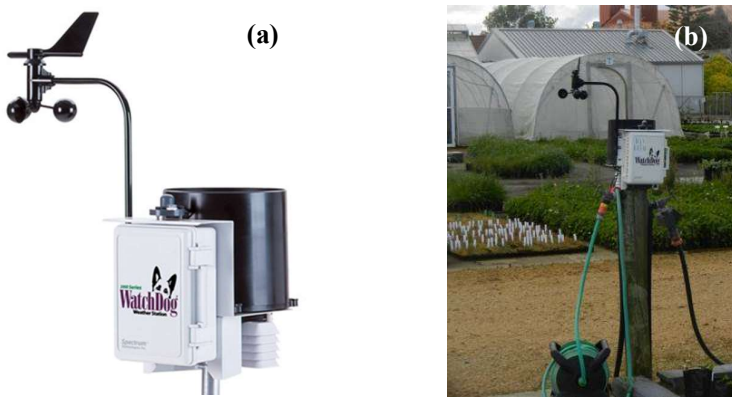


Figure 2.10. Watchdog weather station. (**a**) Close-up picture retrieved from http://www.specmeters.com/weather-monitoring/weather-stations/2000-full-stations/watchdog2900et/ on 20/09/2017, reproduced with the permission of Parkland Products & Spectrum Technologies; (**b**) Mounted weather station in the AgResearch plant nursery.

The LI-250 light meter was equipped with a sensor model 'Quantum' (LI-COR Environmental; Lincoln, U.S.A.; Figure 2.11). It was used to measure the light intensity (15-second averages) just above the plant canopy [on the top shelf level in the Percival climate chamber (Section 2.2.4) and 35 cm above shelf/ground level in all other experimental locations] in all experimental locations used in this research. These measurements and synchronised Watchdog weather station measurements allowed a correction factor for locally modified light conditions to be calculated (resulting, for example, from glasshouse cladding and construction shading) and to estimate the total photosynthetically active radiation (PAR) available to the plants in each experiment (Appendix 4).

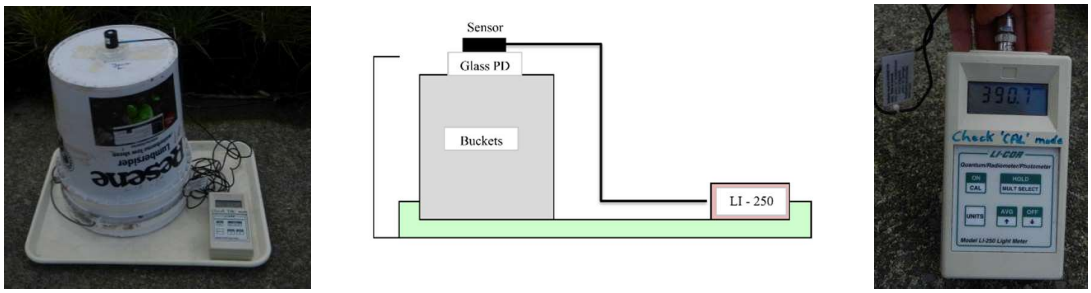


Figure 2.11. LI-COR measuring device, mounted as for light measurements. The 35 cm high stand allowed the sensor to measure the incident light levels just above plant canopy height.

2.4.2. Plant parameters

The various plant measurements collected aimed at quantifying differences in initial plant material, describing plant growth and documenting plant responses to aphids. They included three types of parameters: (i) Initial parameters such as initial tiller dry matter, initial tiller diameter and regrowth 24 h after initial trim, (ii) intermediary parameters that could be measured at any time between initial trim and final harvest, and be repeated at any time, e.g. number of specific plant organs (leaves, tillers, roots), green leaf area and green leaf colour, and (iii) final parameters requiring the destructive harvest of the plant such as final biomass of roots, green shoot and senescing and dead leaves, dry matter content of green shoot and final root branching pattern. The following section describes these parameters and the process of their collection and analysis:

(i) The **initial tiller dry weight** (Tiller DM_{Start}) **[**g] was determined by weighing all freshly trimmed tillers (Tiller FW_{Start}) and drying a representative sub-sample to calculate the dry matter content of the tillers at the time of trimming ($DMref_{Start}$). The initial dry weight of a living tiller *i* was then calculated by Equation 2.1.

$$\text{Tiller } DM_{Start}\ i\ [g] = \text{Tiller } FW_{Start}\ i\ [g] \cdot DMref_{Start} \qquad \textbf{Equation 2.1}$$

(ii) The **initial root/shoot ratio** ($Root/Shoot_{Start}$) of a tiller *i* was estimated directly after the initial trim, by dissecting another comparable trimmed tiller *j* into roots and shoot (cut at the shoot base), drying it, weighing the dry shoot (Shoot DM_{Start}) and roots (Root DM_{Start}) and applying Equation 2.2:

$$Root/Shoot_{Start}\ i\ [\%] = \frac{\text{Root } DM_{Start}\ j\ [g]}{\text{Shoot } DM_{Start}\ j\ [g]} \cdot 100 \qquad \textbf{Equation 2.2}$$

(iii) The **initial dry root (or shoot) biomass** (Root $DM_{Start,}$ Shoot DM_{Start}) [g] of a tiller *i* was estimated using the dry root/fresh tiller weight (Tiller FW_{Start}) ratio of a comparable, trimmed, dissected and dried tiller *j* as described by Equation 2.3

$$\text{Root } DM_{Start}\ i\ [g] = \frac{\text{Root } DM_{Start}\ j\ [g] \cdot \text{Tiller } FW_{Start}\ i\ [g]}{\text{Tiller } FW_{Start}\ j\ [g]} \qquad \textbf{Equation 2.3}$$

(iv) Twenty-four hours after a tiller had been trimmed back to 4 cm shoot length, a calibrated photograph of it was taken either with a Nikon COOLPIX S9100

(Nikon Corporation, Tokyo, Japan) or a Sony SLT-A35 camera (Sony Electronics Inc., San Diego, CA, U.S.A). This photograph was analysed with ImageJ version 1.46r (Rasband, 1997-2016) to measure the **regrowth 24 h after the initial trim** (Regrowth 24h [cm]; Figure 2.12). This measurement was expected to quantify the growth potential of the tiller since the rate of elongation of a tiller's youngest leaf is said to be a relatively accurate proxy for the dry weight gain of the whole plant (Silsbury, 1970)

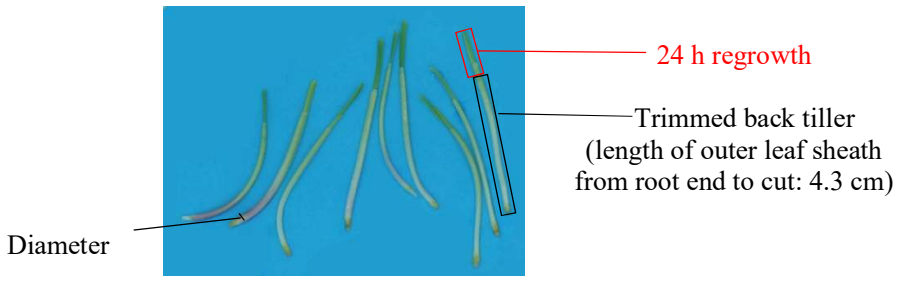


Figure 2.12. Measuring of tiller diameter and regrowth 24 h after the initial trim (Regrowth 24h), on a photograph taken 24 h after the shoot had been trimmed.

(v) The **initial tiller diameter** [mm] was measured with ImageJ on the same picture as the 24 h regrowth, at the point of transition between root and shoot (the largest point overall; Figure 2.12). As grasses store carbohydrates in their leaf sheaths (Section 1.3.1), this measurement was also expected to give information on the (re)growth potential of the tiller.

(vi) **Plant anatomy parameters:** Plant anatomy parameters could help to quantify a plant's fitness, its response to the environment, and the degree of stress. Early leaf senescence has been observed in some plants when subjected to a massive aphid infestation, for example (Dixon, 1973). Therefore, the number of tillers, green leaves, senescing or dead leaves and new or old roots were recorded before root aphids were placed on to experimental plants (precondition), after the aphids had lived on the plant for some time (2nd growth assessment) and/or at the end of most experiments (harvest). All visible leaves were counted. A yellowish leaf was only recorded as 'senescing or dead' if more than half of its sheath and blade was no longer green.

(vii) The **green shoot area** (GSA [cm^2]) was considered a proxy for photosynthetic capabilities. It was measured on photographs of the tillers taken either 24 h after the initial trim (GSA 24h), before aphid placement (GSA T1) and/or after aphid infestation (GSA T2; see Sections 4.2.2 and 5.2.3 for specifics). These photographs were taken within a blue painted imaging chamber, by 12V LED strip lighting, with a remote-controlled digital Sony SLT-A35 camera (Sony Electronics Inc., San Diego, CA, U.S.A) positioned on a permanent mount inside the chamber (Appendix 8, Figure A8.1). The camera was equipped with a Sony DT 18-55 mm F3.5-5.6 SAM zoom lens kit and an external TFT display. The tillers and leaves of the plant to photograph were spread out on the floor of the imaging chamber so as to not overlap each other, and were maintained in that position by BluTack™. A size and colour calibration target (red with a black zone and a grey zone) was also included on each photograph. The photographs taken 24 h after trim were analysed with ImageJ, by manually measuring length and width of both tillers and leaves. The GSA 24 h was then calculated with Equation 2.4, whereby *i* was a given piece of leaf or tiller and n the total number of distinct green plant pieces in the photograph.

$$\text{GSA 24h } [cm^2] = \sum_{i=1}^{n} \text{length}_i\,[cm] \cdot \frac{(\text{width}_i \text{ at base } [cm] + \text{width}_i \text{ at top } [cm])}{2} \quad \textbf{Equation 2.4}$$

Photographs taken before the aphid placement and after infestation were first manually processed in Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, U.S.A) and cleared of undesirable elements (dead leaves, senescing leaf parts, labels, etc.; Figure 2.13) before being analysed by a program written by Chris Hunt in MATHWORKS© MATLAB R2013A (MathWorks, Inc., Natick, MA, U.S.A.; Appendix 8).

(viii) The **colour of the green shoot area** (colour of the GSA) was recorded as a proxy for the chlorophyll content of the leaves in full plants, before aphid placement and after aphid infestation (see Sections 4.2.2 and 5.2.3 for specifics). It was measured on the same processed photographs, as the GSA T1 and the GSA T2, by the same MATLAB program (see also Appendix 8). Colour was reported as three measurements in the RGB colour model. The absolute values after aphid infestation, the difference in values measured before aphid placement and after

infestation were analysed for each of the three dimensions [red (R), green (G), and blue (B)]. Furthermore, the ratio between green and the sum of all colour measurements (Green ratio 'G ratio'; Equation 2.5) and the normalised red-blue difference ('nRBd'; Equation 2.6) were calculated and analysed. These ratios correlate well with chlorophyll and total N contents in several grass species such as wheat, rye (Ali *et al.*, 2012; Jia *et al.*, 2004; Kawashima & Nakatani, 1998), and perennial ryegrass (G ratio in particular; Chris Hunt, personal communication).

$$\text{G ratio} = \frac{G}{(R+G+B)} \qquad \textbf{Equation 2.5}$$

$$\text{nRBd} = \frac{(R-B)}{(R+B)} \qquad \textbf{Equation 2.6}$$

(ix) Because the newest leaves were expected to be more affected by stress than the older ones, the **colour of the last fully extended leaf** and the **size of the last fully extended leaf** were also measured by the same MATLAB program as the colour of the GSA, on further Adobe Photoshop-processed photographs that showed only the last fully extended leaf of each tiller (Figure 2.13). Colour measurements were processed as described above for the colour of the GSA.

(x) The **branching order** of individual roots used for feeding (Section 1.3.1, Table 1.3) and the **full branching profile or pattern** of a root system were occasionally recorded. The latter described how many roots of each branching order X ($nRx_{\circ}$) were present on a plant, for branching order X = 0 to 4. Full root system branching profiles were only established for young plants before aphid placement (few new roots) and for harvested plants (whose roots could be spread out for a clear view in a water-filled tray). The information was used to introduce a comparative root development parameter into some analyses [**Average branching order (ABO)**; Equation 2.7].

$$\text{ABO} = \frac{(1 \cdot nR_{0^{\circ}} + 2 \cdot nR_{1^{\circ}} + 3 \cdot nR_{2^{\circ}} + 4 \cdot nR_{3^{\circ}} + 5 \cdot nR_{4^{\circ}})}{\text{Total number of roots}} \qquad \textbf{Equation 2.7}$$

Whereby $nR_{0^{\circ}} + nR_{1^{\circ}} + nR_{2^{\circ}} + nR_{3^{\circ}} + nR_{4^{\circ}}$ = Total number of roots.

(xi) **Tiller, green shoot, senescing and dead shoot, and root biomasses at harvest** [g] were measured at the end of most experiments. The plants were first cleaned

of any foreign material (agar, potting mix, BluTack^{TM}, etc.) and dissected into three fractions, namely roots (everything below the cut at the base of the shoot), green shoot and senescing and dead (S&D) shoot. All these fractions were immediately weighed to determine their fresh weight (FW_{Harv}) and were then either frozen or preserved in 50% ethanol (root fraction in Population, Biology II and Mature plants experiments)[2]. The samples were dried in an air-draught oven for ≥ 3 days at 60°C (before March 2013) or a freeze-dryer at standard settings for ≥ 48h (after March 2013) before being re-weighed for measurements of dry matter (dry biomass DM_{Harv}). These records were used to calculate the dry tiller weight at harvest (Tiller DM_{Harv}), the root/shoot ratio at harvest ($Root/Shoot_{Harv}$), the net growth of a plant part *x* (green shoot, S&D shoot, roots or full tiller) and the percentage of dry matter of the green shoot (DM content of green shoot) as described in Equation 2.8, Equation 2.9, Equation 2.10, and Equation 2.11.

$$\text{Tiller } DM_{Harv}\,[g] = \text{Root } DM_{Harv}\,[g] + \text{Green shoot } DM_{Harv}\,[g] + \text{S\&D shoot } DM_{Harv}\,[g] \qquad \textbf{Equation 2.8}$$

$$\text{Root/shoot ratio}_{Harv} = \frac{\text{Root } DM_{Harv}\,[g]}{\text{Green shoot } DM_{Harv}\,[g]} \qquad \textbf{Equation 2.9}$$

$$\text{Net growth of plant part } x\,[g] = x\ DM_{Harv}\,[g] - x\ DM_{Start}\,[g] \qquad \textbf{Equation 2.10}$$

Where $x\ DM_{Start}$ was the dry weight of plant part *x* after the initial tiller trim.

$$\text{DM content of green shoot } [\%] = \frac{\text{Green shoot } DM_{Harv}\,[g]}{\text{Green shoot } FW_{Harv}\,[g]} \cdot 100 \qquad \textbf{Equation 2.11}$$

In addition to all the above-mentioned plant parameters, confounding variables such as (i) plant age since trim or (ii) plant age at aphid placement and (iii) agar left in the Petri dishes at a check or at the end of an experiment (a proxy for water stress) were also recorded as needed.

[2] Ethanol preserving was used to keep root samples in a stable condition for scanning at a later date (planned, not realised). Ethanol-preserved roots were rinsed twice with tap water to get rid of the ethanol before being freeze-dried.

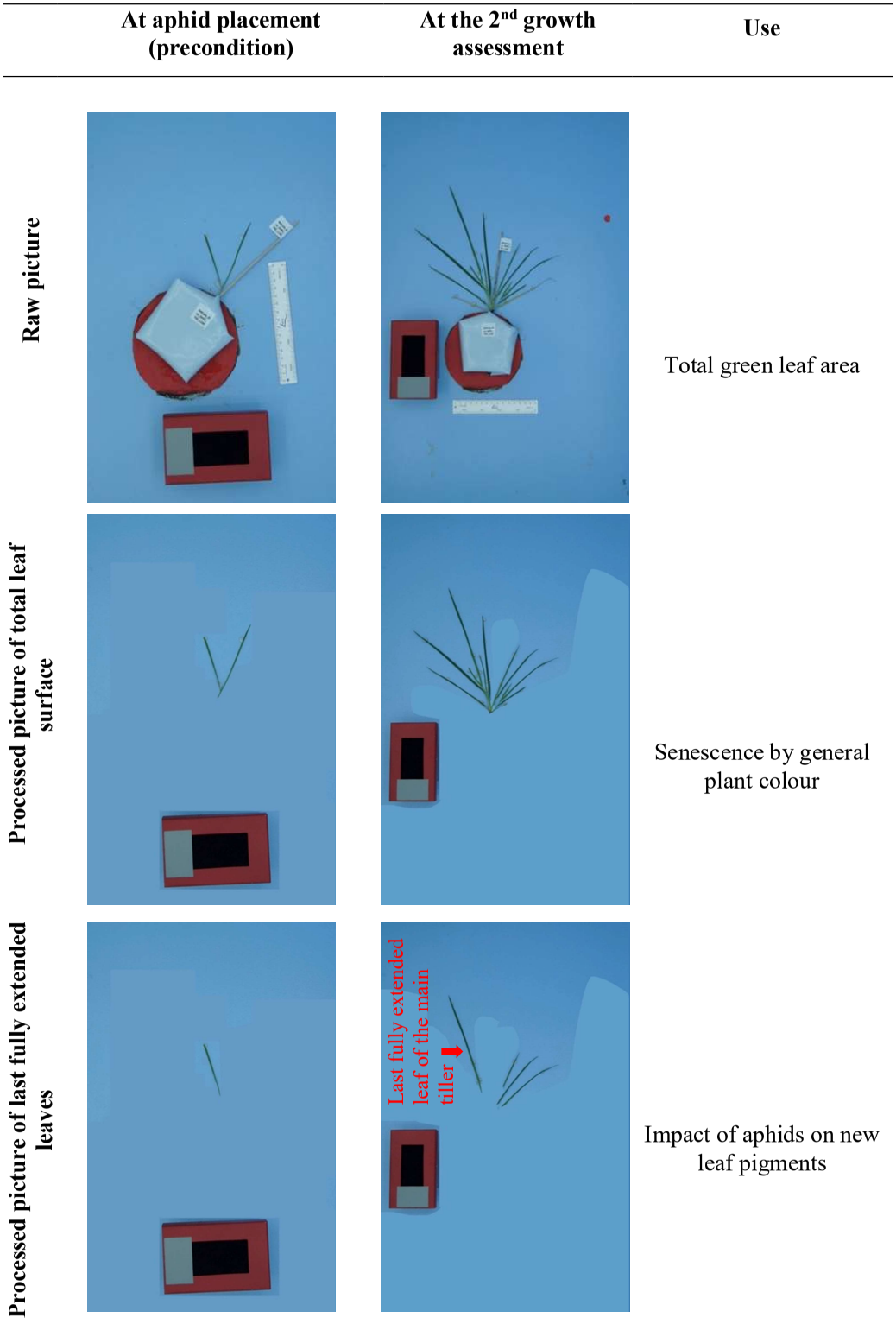


Figure 2.13. Refining raw photographs into a suitable form for computerized image analysis. The red box represented in every photograph is the colour (and size) reference necessary to standardize all photographs.

2.4.3. Aphid and aphid feeding-related parameters

The type of data that was collected on aphids differed by experiment (Table 2.5). The general methodology is described below. Trial-specific changes in methodology are mentioned in the respective sections of Chapters 3 to 5 and Appendices.

To perform size measurements, living root aphids were photographed three times in a short sequence while peacefully feeding, resting or unhurriedly walking on a plant or in the water-surrounded area of a glass Petri dish (Figure 2.6e). Any wax obstructing the view was removed prior, either by brushing it away with a fine paintbrush or spraying a fine mist of autoclaved tap water over it. The photographs were realised with a digital camera (Canon PowerShot A620, Canon Inc., Tokyo, Japan) adjusted to a stereo microscope (Stemi SV6 Zeiss, Carl Zeiss AG, Oberkochen, Germany) through a digital camera adapter d30 (M37/52×0.75, Carl Zeiss AG, Oberkochen, Germany) at the following settings: ocular magnification 10×, lateral zoom 5×, objective Plan S 1.0×, camera set on full zoom (focal length 29.2, F number 4.1, exposure time 1/8), no flash. Image size calibration was obtained by a 2 mm calibration slide (Ernst Leitz Wetzlar GmBH, Wetzlar, Germany) photographed at the same settings as the root aphids. Body length (L), abdomen width (W), thoracic width (tW) and length of rostrum (Figure 2.14) were measured on each of the three photographs (if sufficiently visible) with the program ImageJ 1.46r. The three repeated measurements for each parameter were compared to establish whether any sudden aphid movements could have been biasing some of them (outlier check). A mean value for each parameter (L, W, tW and rostrum length) was calculated with all unbiased measurements, and the body surface of the aphid (ellipsoid body projection, EP; a measurement that was less variable, than individual width or length measurements for mature aphids in pre-trials) was then calculated as described in Equation 2.12.

$$EP\ [mm^2] = \frac{L\ [mm]}{2} \cdot \frac{W\ [mm]}{2} \cdot \pi \quad \textbf{Equation 2.12}$$

In a few cases in which measuring living aphids was not practical, specimens were killed and preserved in 1.5 to 2 mL 80% ethanol, in microcentrifuge tubes stored in a dark laboratory room until further processing. When these aphids were measured later on, they were taken out of the ethanol, deposited on a glass slide and photographed once only.

Table 2.5. Aphid-related parameters collected, by experiment.

Parameters	Aphid biology experiments					Plant physiology experiments		Other experiments	
	Calibration II	Viviposition	Biology I	Biology II	Instar measurements	Mature plant	Seedling	Wax obs.	Population
Maternal[1] aphid size	YES	-	YES	YES	YES	-	-	-	-
Offspring size	-	-	-	YES	-	-	-	-	-
Maternal establishment success	-	-	(YES)	(YES)	-	-	-	-	-
Colonisation success	-	-	(YES)	(YES)	-	YES	(YES)	-	(YES)
Age at ecdyses	-	-	YES	-	(YES)	-	-	-	-
Age at maturity	-	-	YES	YES	-	-	-	-	-
Maternal longevity	-	-	(YES)	YES	-	-	-	-	-
Maternal survival *ex planta*[2]	-	YES	-	-	-	-	-	-	-
Offspring survival *ex planta*	-	YES	-	YES	-	YES	-	-	-
Duration of aphid occupation	-	-	YES	YES	YES	YES	YES	YES	YES
Colony/population composition	-	-	-	-	-	YES	YES	(YES)	YES
Maternal lifetime fecundity	-	-	YES	YES	-	-	-	-	-
Maternal reproduction rate *in planta*	-	-	YES	YES	-	-	-	-	-
ex planta	-	YES	-	-	-	YES	-	-	-
Ecological lifetime fecundity	-	-	(YES)	(YES)	-	-	-	-	-
Feeding-related behaviour	-	-	(YES) repeated	YES repeated	-	-	-	YES once	-
Feeding site properties	-	-	(YES)	YES	-	-	-	(YES)	-

YES: data collected; (YES): data collected but only conditionally interpretable; - data that was not or could not be collected.

[1] "maternal" refers to the aphids studied, "offspring" stands for the descendants produced by the aphid studied during the experiment/observation.

[2] *Ex planta*: aphids locked into an air-tight container without roots to feed upon. Other parameters were measured on aphids kept on plants.

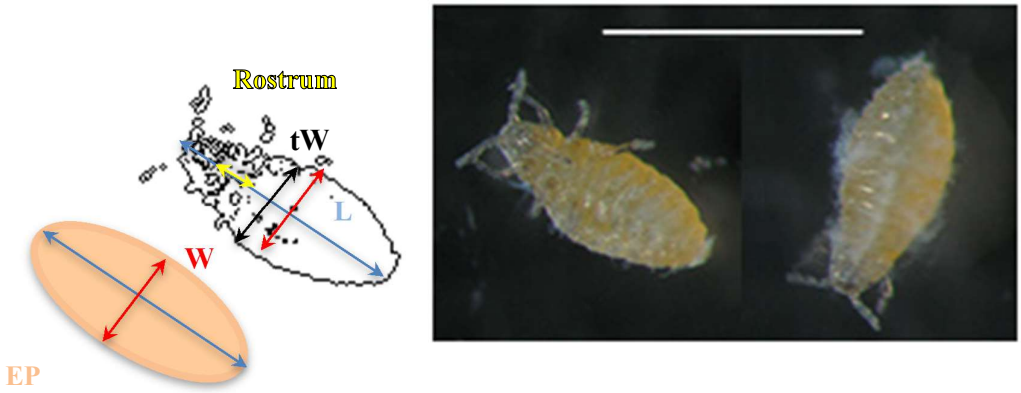


Figure 2.14. Size measurements on root aphids. Drawing and photographs of a second instar, from below (left) and above (right). The white scale bar represents 1 mm. The arrows illustrate the measurements, whereby L = body length from front to cauda, W = abdomen width, tW = thorax width. EP, the ellipsoid body projection was calculated as $EP = L/2 \cdot W/2 \cdot \pi$.

Aphids were considered successfully established in aphid biology experiments if they were found alive feeding (stylets inserted in a root) and/or if there was evidence of them having fed such as wax deposits on a root or if they presented an enlarged abdomen. Establishment success was calculated with Equation 2.13.

$$\text{Establishment success [\%]} = \frac{\text{Number of established aphids}}{\text{Number of aphids placed}} \cdot 100 \quad \textbf{Equation 2.13}$$

Colonisation success (Equation 2.14) was preferred for experiments in which the early development of aphids could not be followed (Mature plant experiment, Seedling experiment).

$$\text{Colonisation success [\%]} = \frac{\text{Number mature aphids}}{\text{Number of aphids placed}} \cdot 100 \quad \textbf{Equation 2.14}$$

The number of mature aphids was thereby estimated by counting both the living adults and the adult-sized dead aphids.

The age of aphids at critical events such as ecdyses, maturity and death ('longevity') could only be estimated during aphid biology experiments that involved repeated checking. The recorded dates of the respective events and the known date of birth of each aphid (day 1 of life) were used for these age calculations (Equation 2.15).

Age of aphid at event *x* [days] = Date of event *x* – date of day 1 + 1 **Equation 2.15**

Survival in *ex planta* experiments was assessed by checking daily (Viviposition trial) or every other day (both follow-up experiments) whether the contained aphid(s) were still moving (walking or twitching in any way) under the microscope. The date on which the aphids were placed in the microcentrifuge tubes was recorded as day 1. The age of aphids at death was calculated by Equation 2.15 too.

Duration of aphid occupation was defined as the time span between aphid placement and aphid harvest, and calculated as by Equation 2.16:

Aphid occupation [days] = Date of aphid harvest – date of aphid placement + 1 **Equation 2.16**

In agreement with Salt *et al.* (1996) and in order to ease the interpretation of data, three age groups only were distinguished when the colony composition of a sample had to be described: (i) '(early) first instars', the dispersing individuals, clearly recognisable by their straight, not convex abdomen; (ii) 'older immatures', i.e. individuals that were living and feeding on a plant for some time for sure; and (iii) 'adults', the reproducing individuals, clearly recognisable by a genital papilla on the ventral side. Heathcote (1972)'s recommendation to avoid any random influences on numbers by ongoing reproduction during the harvesting and counting processes was also followed. While other sources may report the total number of aphids found on a plant, this thesis reported and analysed generally the number of '(long-term) feeding aphids' only (i.e. older immatures and adults).

Maternal lifetime fecundity was estimated by harvesting all offspring found in Petri dishes in 48- to 72-h-intervals and counting them twice. The reproduction rate (RR) was deduced from these records by Equation 2.17.

$$\text{RR [offspring/day]} = \frac{\text{Number of offspring collected}}{\text{Reproductive time [days]}} \qquad \textbf{Equation 2.17}$$

With reproductive time being the time difference between the last check before reproduction and aphid death. The RR *ex planta* was obtained by the same formula, by summing up the number of offspring produced by a mother every day and dividing the total by the length of the observation.

The activity of the aphid in a Petri dish was observed just at the Petri dish opening, and recorded as either "feed", "walk", "stand" (remaining on a root without having the stylets inserted), "ecdysis" and "unknown". The records of the two first groups were then analysed by binary models (feed vs. walk).

The feeding site properties included:

(i) Root branching order (unbranched, 1st BO, 2nd BO, 3rd BO, 4th BO). This parameter was considered a proxy for general root age and metabolic activity (Table 1.3, Section 1.3.1)

(ii) Position on root (on main axis, lateral 1, i.e. on a lateral branch of the main axis; vs. on lateral 2, i.e. on a lateral branch of a lateral 1 branch, etc.; Section 5.2.1, Figure 5.1)

(iii) Colour of the root at the feeding site (white, pale brown, and brown/dark brown). This visually assessed parameter was considered a proxy for site age since white roots are associated with new growth (Popay, 2004) and may take up to six to nine weeks to change colour (Brock *et al.*, 1996)

(iv) Root diameter at the feeding site, measured with ImageJ on the photographs taken for feeding aphid size measurements (if the root was visible next to the head).

More specifics on the analyses of these parameters are reported in Chapter 5, Section 5.2.1

2.5. Statistics

All data were recorded on Microsoft Excel sheets and graphically examined for tendencies, outliers and data entry errors with both Excel and the statistics program R 3.4.3 (R Core Team, 2017). R was also used to analyse and present the data. All parameters deemed important were set in an initial model, and this model was then

reduced by removing stepwise the redundant parameters until reaching a minimal AIC value by a normal residuals distribution. Plant age was always included as a first confounding co-variable in any model. A significance level of $\alpha = 0.05$ was adopted for all analyses, and a Benjamini-Hochberg correction used when making multiple comparisons. When data with zero values had to be log-transformed for analyses, the constant term added to each measurement before the transformation was the second smallest, non-zero measurement of the respective response variable. Specifics about each data analysis are given by experiment in the corresponding sections of Chapters 3 to 5.

CHAPTER 3: BIOLOGY OF APTEROUS MORPHS OF *APLONEURA LENTISCI*

3.1. Introduction

Alate *Aploneura lentisci* Pass. morphs do occasionally appear in New Zealand aphid monitoring records (Blackman & Eastop, 1984; Lowe, 1966, 1968). The dominant and most problematic morph for New Zealand grasslands, however, is the apterous, parthenogenetic female that lives in wax-surrounded colonies on the roots of various grass species (Jensen & Popay, 2007; McDonald *et al.*, 2011; Popay & Cox, 2016). Evidence for the impact of this morph on grassland productivity is accumulating (Agricom & PGG Wrightson Seeds, 2015; Popay & Cox, 2016). It is sensitive to the presence of specific ryegrass endophyte strains. Root aphid populations are generally less numerous on perennial ryegrass with *Epichloë festucae* var. *lolii* AR37 or CT endophyte symbionts than on endophyte-free (NIL) plants, for example. AR1-symbioses, however, are reported to be more susceptible to *A. lentisci*, supporting significantly larger populations than NIL plants (Pennell *et al.*, 2005; Popay, 2004; Popay & Cox, 2016; Popay & Easton, 2006; Popay & Gerard, 2007). Yet, little is known about the biology of root aphids (e.g. size, developmental time, fecundity, offspring fitness) in presence or absence of these endophyte strains. Consequently, this chapter considered the following questions:

1) How successful are root aphids in establishing on perennial ryegrass and colonising it?
2) What is the average generation interval of root aphids on endophyte-free *Lolium perenne* plants?
3) What lifetime fecundity, reproduction rate and offspring fitness can be expected from root aphids raised on the roots of endophyte-free *L. perenne* plants?
4) How do various plant characteristics influence the biological traits of *A. lentisci*?
5) What influences do various endophyte strains have on the biology of *A. lentisci*?

3.2. Materials and methods

3.2.1. Experimental designs

Five experiments are reported in this chapter: (i) two main longitudinal studies in which the development and reproductive performances of aphids individually confined to perennial ryegrass roots were repeatedly monitored from birth to death [Biology I and II experiments]; (ii) a trial recording the colonisation success of a cohort of undisturbed aphids and their *ex planta* reproductive rates [Mature plant experiment]; (iii) two follow-up experiments in which the survival of offspring collected during the Biology II and Mature plant experiments was assessed *ex planta* [Biology II follow-up experiment and Mature plant follow-up experiment].

3.2.1.1. Biology I experiment

The Biology I experiment was carried out in cubicle 12, glasshouse 9 (Section 2.2.3) from 17/08/2012 to 2/11/2012, under natural photoperiod and semi-controlled temperature conditions [mean and standard deviation of 20.7 ± 6.0 °C inside Petri dishes wrapped in a black polyethylene sheet]. For this trial, 18 genotypically different plants were randomly selected from amongst endophyte-free, five-month-old source plants (Section 2.3.1.1; perennial ryegrass of cultivar Grasslands Samson, accession A11104). Six individual tillers were split each plant, trimmed back to 2 cm root and 4 cm shoot length, weighed and grouped into three pairs of similar-sized tillers. Each pair was allocated to one of three checking groups (G3, G4 and G5). One tiller of each pair was then dissected into roots and shoots, by cutting the shoot off at its base, dried at 60 °C for three days and re-weighed to estimate the initial dry matter content and the root/shoot ratio. The other tiller was immediately embedded as described in Section 2.3.1.3 into a Petri dish filled with ca. 45 g of nutrient-enriched agar (Thrive™ all-purpose soluble fertiliser, 0.2 g/L; Yates New Zealand, Auckland, New Zealand), with a moist, previously autoclaved cotton ball on the roots. The Petri dishes were sealed with GLAD® wrap, wrapped into a black polyethylene sheet, placed upwards in a hygiene tray filled with potting mix and left to recover in the glasshouse for three weeks. Eighteen to 21 days after trimming, the cotton ball was removed from the Petri dish. The number of tillers, leaves and roots of each branching order was recorded (plant precondition; Section 2.4.2). Under a stereo microscope (10 to 63x magnification), one neonate root aphid (‘starter

aphid', < 24 h old, produced on one of ten viviposition plants; Appendix 10, Section A10.1) was placed onto plant roots in each Petri dish. The Petri dishes were then re-closed, wrapped and maintained as before. Aphid monitoring ("checks") was performed on the second day after placement and at three-day-intervals thereafter, in a schedule defined by the checking group (day 3, 6, 9, etc. of aphid life for G3 plants, day 4, 7 10, etc. for G4 and day 5, 8, 11, etc. for G5). At each check, the exuviae and offspring present in each Petri dish were counted and removed under a stereo microscope at 40 to 50x magnification, leaving only the starter aphid in the Petri dish. The wax surrounding the starter aphid was then gently brushed away with a fine paintbrush. If the aphid was still moving, it was reported as "alive" and photographed for size measurements. Starter aphids lost or killed within 10 days of the first placement (5/09/2012) were replaced by new neonates, which resulted in the placement of 59 aphids during the Biology I experiment. Water was sprayed onto the agar in each Petri dish (approx. 0.8 mL/check at every check) to prevent water stress from the 31st day after plant trim onwards. Seventy-four to 75 days after the initial trim, the plants were destructively harvested and dissected into green shoot material, senescing and dead (S&D) shoot material and roots as described in Section 2.4.2. The roots were washed to remove residual agar. All three biomass fractions were weighed, dried for ≥ 3 days in an air-draught oven at 60 °C and re-weighed.

3.2.1.2. Biology II experiment

This trial was conducted on plants of two distinct plant genotypes (N and S) and one of four endophyte statuses [in symbiosis with one of three endophyte strains (AR1, AR37 or common-toxic CT), or endophyte-free (NIL)] ('clone-plants'; Section 2.3.1.1) as represented in Figure 3.1. In five batches of 6 tillers/batch, a total of 30 tillers per plant genotype-endophyte status (PG-E) combination was trimmed back to 4 cm shoot length and 0.3 cm root length without adventitious side roots. The trimmed tillers were weighed and one tiller from each batch sacrificed to estimate the dry matter content of the others. Each of the remaining five tillers of a batch was set into 20 to 30 mL tap water (in a glass vial of 7.6 cm length and 2.4 cm diameter) and left to recover for two weeks in the Conviron® climate chamber 2 (Section 2.2.4) set to a photoperiod of 14 h light/10 h dark and an air temperature of 17 °C. The water in the tubes was changed every 24 to 48 h. The recovered plants were individually embedded in unvented Petri dishes filled with ca.

45 to 50 g modified Bollard medium (MBM) and a moist, autoclaved cotton ball was placed on the tiller base and roots (Section 2.3.1.3). These Petri dishes were sealed with GLAD® wrap, each one wrapped into one black polyethylene sheet and a panda sheet (white side out), tied together in groups of two plants and placed in an upright position on a hygiene tray in the climate chamber, in a random order. To further standardise the conditions over the experiment, all plants were systematically rotated, moving them once a day by four positions within the trays. The plants were left in the climate chamber until the plant precondition assessment, immediately before aphid placement (≥ 30 days after the initial plant trim), returned after placement, and removed periodically for checks on the root aphids until aphid death (see below). The temperature in the wrapped root area averaged 17.5 ± 2.4 °C (mean ± standard deviation).

The genitors of the neonates that were to be placed into the Petri dishes ('starter aphids') had been kept for at least 3 generations in conditions similar to what was just described (climate chambers set to 14 h light/10 h dark, air temperature of 17 to 18 °C, on clone-plants embedded in MBM agar) and were, whenever possible, collected from colonies on plants of the same PG-E group as the one they were transferred to. They were maintained in the climate chamber for approx. 12 to 18 h, in 1.5 to 2 mL microcentrifuge tubes in light-proof wrapping (Section 2.3.2). The offspring viviposited in that time were transferred to a new microcentrifuge tube until placement (> 6 h after transfer, but before the end of the neonates' third day of life). The neonate starter aphids were photographed before being moved with a fine paintbrush onto the plants' roots, at rates of one or five neonates per Petri dish ('solitary' vs. 'group' setting; n = 5 to 13 Petri dishes per PG-E setting). Solitary starter aphids that died before maturity or were killed by handling were replaced by one new neonate. This resulted in the placement of 206 solitary starter aphids in 82 Petri dishes over the complete Biology II experiment.

Aphid handling was carried out in a dark room, under a 6-W white LED light (Austrabeam/stylelux, model L3-857391/PLU7080; Mercator Lighting Pty Ltd, Coolaroo, Australia) and a Zeiss stereo microscope (Stemi SV6; Carl Zeiss AG, Oberkochen, Germany) at 50x magnification. Photographs were taken with a digital camera (Canon PowerShot A620, Canon Inc., Tokyo, Japan) fitted to the microscope, with the flash function turned off.

Aphid monitoring after placement was performed in two steps:

(i) First two weeks: checks once a week (aphid age: 7 to 9 and 14 to 15 days, respectively). Survival and activity at the check were recorded with a site description if the aphid was feeding (see Chapter 5). A fine mist of previously autoclaved tap water at room temperature was sprayed over the aphids to remove the wax. Living aphids were triply photographed.

(ii) Subsequently: the aphids were monitored every second day up to death, recording their survival, activity (Section 5.2.1), a site description if feeding (Section 5.2.1) and the number of offspring they had produced since last checked. Three photographs were taken at the appearance of the first offspring to determine the mature adult size, and several more times thereafter if possible. All offspring were removed from the Petri dishes, counted, and triply photographed. First instar aphids were then placed together in one microcentrifuge tube per Petri dish (= a cohort) and used for follow-up experiments (Section 3.2.1.4). Older instars were discarded.

Approx. 0.8 g water was sprayed onto the agar to prevent water stress at each check if the agar filled ≤ ¼ of the Petri dish. Once an aphid had completed a full life span (i.e. achieved maturity and died a natural death on its plant), the plant it lived on was destructively harvested. The plant was divided into green shoots, senescing and dead shoots, and roots, weighed fresh, freeze-dried for ≥ 48 h and finally re-weighed for dry matter measurement. All green tillers were blotted for confirmation of endophyte presence before freeze-drying (Section 2.3.3).

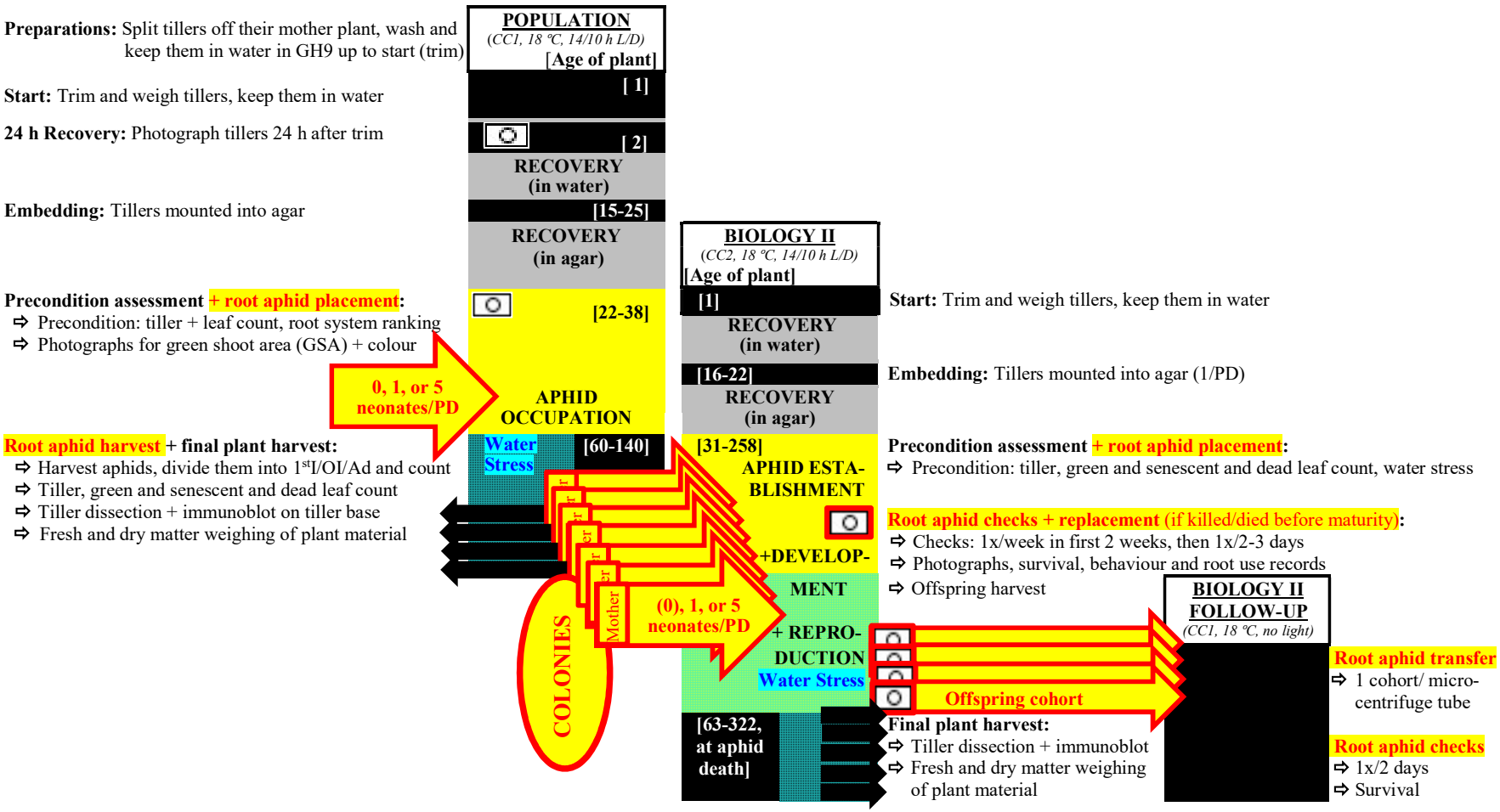


Figure 3.1. Diagram of experimental set up for Population experiment, Biology II experiment and Biology II follow-up experiment. 1stI/OI/Ad: first instar, older immatures, adults; The age of a plant [Age of plant] at the events is given in days; CC1, CC2: climate chamber 1, 2 (Section 2.2.4); GH9: glasshouse 9 (Section 2.2.3); L:D: light/dark; PD: Petri dish (i.e. plant sample); photographs taken.

3.2.1.3. Mature plant experiment

This experiment was performed outdoors on 'clone-plants' (eight plant genotype-endophyte status [PG-E] combinations of two genotypes [N or S] either endophyte-free (NIL) or infected with one of three endophyte strains [*E. festucae* var. *lolii* strain AR1, AR37 or common-toxic]; Section 2.3.1.1), from 22/06/2015 to 21/10/2015, in an insectary (Section 2.2.2), at an average temperature of 10.1 ± 4.6 °C [-2.4 to 27.3 °C]. Twenty tillers of each PG-E combination were trimmed to 4 cm shoot and 0.3 cm root length without any side roots (Section 2.3.1.3). The leftover shoot (shoot 4 cm above the tiller base) was blotted for endophyte presence confirmation. Five of the trimmed tillers were used to estimate the initial tiller dry weight (Section 2.4.2). The fifteen remaining tillers were embedded into Petri dishes filled with 55 to 60 g modified Bollard medium agar (MBM agar, Appendix 6), maintained outdoors and monitored for plant growth as described in Chapter 4, Section 4.2.2. Thirty to 39 days after the initial trim, a cohort of ten root aphids in their 2nd to 3rd day of life ('starter aphids') were introduced to each of five to seven plants per PG-E group. These neonates were provided by adult aphids reared in the insectary, on plants of the same PG-E group. The adults were individually detained in microcentrifuge tubes for a maximum of two viviposition periods of 24 h to produce the starter aphid cohorts. The colonies that developed from the starter aphids were harvested 9 to 12 weeks later, before the first offspring of the starter aphids reached maturity. The number of living and dead adult aphids found in each Petri dish (i.e. the number of former starter aphids that achieved reproductive maturity on each plant) was recorded to estimate the colonisation success. The living adults from each Petri dish were then individually placed into 1.5 mL microcentrifuge tubes, wrapped light tight and left in a dark room at 19.6 ± 2.0 °C for 24 h. The offspring produced in that time were counted and returned to the microcentrifuge tubes, while each mother was moved into a new microcentrifuge tube for another viviposition period of 24 h. The offspring collected at the end of both viviposition periods were used for a follow-up experiment (Section 3.2.1.4).

3.2.1.4. Follow-up experiments: offspring survival *ex planta*

The neonate nymphs collected during the Biology II and the Mature plant experiment (Sections 3.2.1.2 and 3.2.1.3, respectively) were used in two *ex planta* follow-

up experiments to determine offspring survival. These nymphs had been transferred into one microcentrifuge tube per collection date and Petri dish (Biology II; Figure 3.1) or mother (Mature plant experiment). That resulted in 1597 microcentrifuge tubes with 7.2 ± 5.2 [1 to 27] one- to two-day-old offspring from one to five mothers/microcentrifuge tube for the Biology II follow-up experiment, and 234 microcentrifuge tubes with 8.9 ± 4.1 [1 to 24] one-day-old offspring from one mother per microcentrifuge tube for the Mature plant follow-up experiment, respectively. These microcentrifuge tubes were kept wrapped in the Conviron climate chamber 1 at 17.6 ± 2.5 °C (Biology II follow-up experiment), or in a dark laboratory room at 19.6 ± 2.0 °C (Mature plant follow-up experiment). The number of live aphids in each microcentrifuge tube was counted every other day under the stereo microscope at 50x magnification, in a dark room by a 6 W LED light, until all aphids died.

3.2.2. Data processing and statistical analysis

Several technical failures and initial handling difficulties occurred during the trials. To avoid bias due to these without heavily reducing the number of observations, the raw data were edited by response variable, removing the cases that were problematic for the specific variables from the data prior to analysis. The following sections describe more precisely how this was done for each trial and variable. The genotype S and the endophyte status NIL or the S-NIL group were set as a baseline/reference group in Cox models and mixed-effects models. Full models were set up with all variables and interactions considered, before reducing them stepwise, removing non-significant terms ($p > 0.05$ for the likelihood ratio between models with vs. without the term) to achieve the smallest Akaike information criterion (AIC) value possible by acceptable residuals distribution. If not stated otherwise, the assumptions for each model were verified graphically, with a Shapiro-Wilk normality test (normality assumptions) or Levene's test (homogeneity of variance between groups) ['stats' (R Core Team, 2017),'car' (Fox & Weisberg, 2011)]. Whenever possible, the 'predictmeans' package (Luo *et al.*, 2014) was employed to assess the residual distribution and perform post hoc tests at average covariate values, with Benjamini Hochberg *p*-value adjustment for multiple comparisons. Where this was not possible, alternative residual plots were generated, and multiple comparisons at average covariate values were calculated with 'multcomp' (Hothorn *et al.*, 2008) using Tukey's

procedure. In Cox proportional hazard models for age at events (ecdyses, reproduction, death), the proportional hazard assumption was tested with the 'survival' package (Therneau, 2015a; Therneau & Grambsch, 2000), and Martingale residuals were plotted. 'MuMIn' (Barton, 2018) was used to estimate conditional R^2 values (R^2c: proportion of variance explained by fixed and random effects together) for mixed-effects models or likelihood-ratio based pseudo-R^2 (adjusted R^2) for non-linear regression models.

3.2.2.1. Biology I experiment

Contaminated Petri dishes, i.e. Petri dishes in which an unexpected, second aphid was found, were excluded from the analyses. Therefore, the establishment success was evaluated on a total of 59 neonates (placed or re-placed in a total of 43 Petri dishes), with Equation 2.13. An aphid was considered successfully established if it showed evidence of feeding (stylets inserted in a root, wax production, abdominal width increase). Colonisation success was assessed with Equation 2.14.

The nymph instar on a checking day was determined in three ways:

(1) By counting the number of exuviae removed from a Petri dish up to checking date *i* and calculating the instar with Equation 3.1:

$$\text{Instar } (i) = \text{Number of exuviae removed up to day } i + 1 \qquad \textbf{Equation 3.1}$$

(2) By reverse counting of exuviae in Petri dishes in which offspring were produced (Equation 3.2) assuming the imago was the 5th instar (Section 3.3.1.1)

$$\text{Instar } (i) = 5 - \text{number of exuviae found after day } i \qquad \textbf{Equation 3.2}$$

(3) In cases of missing or belatedly found exuviae where aphids did not reach reproductive maturity: by size, screening the pictures chronologically for discrete size changes. The size measurements on verified instars (see below) and further instar size data (Appendix 9, Section A9.1) were used to support the instar attribution by this method.

Body length (L) and abdominal width (W) were measured on all useable photographs and the derived ellipsoid body projection value (EP) was calculated as

described in Section 2.4.3, Equation 2.12. However, instar size analyses were realised on pictures of verified instars only, i.e. pictures for which the instar determination did not require applying a method as in (3) above. By these standards, 11 to 65 specimens could be used for individual instar description (Section 3.3.1.1). By repeated measurements of a given instar (e.g. a first instar aphid photographed on day 1, 3 and 6 before the first ecdysis), a mean size for this specimen in that specific instar was calculated for each size parameter. Data on instar size were then analysed by multiple pairwise t-tests using the 'stats' package (R Core Team, 2017) with Benjamini-Hochberg *p*-value adjustment.

In general size analyses (instar-unrelated), all clear measurements were included, which resulted in n = 299, 227 and 222 measurements of 67 distinct specimens for L, W and EP, respectively. The size analyses focussed on three aspects: (i) neonate size, (ii) size development, and (iii) mature adult size. Neonate size was examined to determine the maternal effects by viviposition plant (Appendix 10, Section A10.1) with a Kruskal-Wallis test and post hoc Dunn test with Benjamin-Hochberg *p*-values adjustment for multiple comparisons. For size development, all specimens supplying ≥ 2 EP measurements were assigned to one of two size groups based on an EP development plot: individuals whose EP exceeded 1.0 mm^2 at least once in their life were classified as 'Large'. Two specimens with a steep, 'Large' group-like size development were further classified as 'Large' too, despite not having reached the 1.0 mm^2 limit before their untimely death. All others aphids were assigned to a default 'Small' size group. One five-parameter log-logistic model of the type described in Equation 3.3 was then fitted per size group to each size variable (L, W, EP) ['drc' package (Ritz *et al.*, 2015)]:

$$\text{Size}(\text{Age}) = \frac{\text{d}-\text{c}}{\left(1+\text{e}^{\left(\text{b}\cdot\left(\log_e(\text{Age})-\log_e(\text{f})\right)\right)}\right)^{\text{g}}} \qquad \textbf{Equation 3.3}$$

where c was the minimum asymptote (size at birth), d the maximum size achieved, and parameters b, f and g model-specific constants. Differences between the 'Large' and the 'Small' models were assessed by comparing the estimates for each parameter (b, c, d, f, g): if the estimates of the 'Large' group were not within the 95% confidence interval of the "Small" group's estimates, they were considered "significantly different".

Adult size was defined as the size at first reproduction (Schuett *et al.*, 2015). It was square root transformed and tentatively analysed in several mixed-effect models ['nlme' package (Pinheiro *et al.*, 2017)] with plant genotype or maternal identity (viviposition

plant) as random grouping factors, and various combinations of the variables of Table 3.1 as fixed effects. The final model explained square root transformed adult size (n = 16) by maternal identity (random grouping factor), initial root biomass and reproductive rate only (fixed effects).

Data on lifespan were analysed by a Cox proportional hazard model stratified by checking group. Aphid age at death or disappearance was used as the time variable (Equation 2.15). A binary variable describing whether the aphids died of natural causes ("TRUE") or were killed/lost ("FALSE") served as a censoring variable. An aphid was considered killed by handling if it was damaged during an observation, or if its ultimate record suggested it could have been (e.g. if it was touched during the wax removal and responded to it with sudden body contractions indicating possible injury, or if it was moved with a paintbrush the last time it was seen alive). The initial model included neonate size and all plant characteristics of Table 3.1 as covariates, with n = 48 data points (all established aphids – one record for which the day of birth was unclear). No final model developed this way was better than the null model, however (Section 3.3.1.3).

The timing of the five development steps, ecdyses 1 to 4 and reproductive maturity, was analysed in a similar way to lifespan (Cox proportional hazard models with stratification by checking group). As various environmental conditions likely to affect plant biomass covariates (e.g. heat and possible water stress) occurred only after the starter aphids had developed, two approaches were used: (1) drawing null models with stratification by checking group, to estimate the median age of aphids at each step (Kaplan-Meier curves, observations/"raw data"); and (2) examining the possible significance of neonate size and plant characteristics (Table 3.1) with a stratified model for each development step, but without median prediction. The age at each event (or alternatively, the age at aphid exit if the event did not happen) was used as a time variable. It was calculated with Equation 2.15 too, using the days of exuviae collection (or the day of a distinct EP size change in Petri dishes with unverified instars) as dates of ecdysis completion, and the date offspring of the starter aphid was first found in a Petri dish as date of reproductive maturity. The censoring variable for these development steps recorded whether the event occurred ("TRUE") or whether the aphid was lost, killed or died before achieving it ("FALSE").

Table 3.1. Covariates recorded and used in analyses during the Biology I experiment.

Group	Time	Type	Covariate
Plant characteristics	Initial	C	Initial dry root biomass [g]
	Initial	C	Initial dry shoot biomass [g]
	Initial	C	Number of old roots (trimmed at start)
	Aphid placement	C	Number of new roots
	Aphid placement	C	Average branching order of (new) roots
	Harvest	C	Number of new roots at harvest
	Harvest	C	Average branching order of (new) roots at harvest
	Harvest	C	Dry matter content of green shoot at harvest [%]
	Harvest	C	Dead and senescing shoot biomass [g]
	Harvest	C	Green shoot biomass at harvest [g] *A: net growth of green shoot [g]*
	Harvest	C	Root biomass at harvest [g] *A: net growth of root [g]*
Aphid traits	Aphid placement	C, R	EP size of starter aphid as neonate [mm^2]
	Reproduction	C, R	EP size of starter aphid as adult, i.e. at 1st reproduction [mm^2]
	Reproduction-death	C,R	Fecundity, i.e. general reproductive rate [offspring/day]

A: alternative form of a variable, used instead of that variable in some models; C: covariate; EP: ellipsoid body projection (aphid size parameter); R: response variable; For more parameter specifics, see Sections 2.4.2 (plants) and 2.4.3 (aphids).

All offspring (living and dead) found in a Petri dish were recorded, from the day the starter aphid was placed to the final harvest of the plants. Two types of lifetime fecundity were estimated from the data: (1) the biological lifetime fecundity, i.e. the aphid's potential to reproduce when reaching maturity and (2) the ecological lifetime fecundity, i.e. the average reproduction success of an aphid in a population, which also included aspects of pre-reproductive mortality. As standard methods (1a) and (2a) [see below] did not account for handling-caused deaths, alternative calculations were also considered. The biological lifetime fecundity [offspring/reproducing mother] was estimated using:

(1a) Average number of offspring from starter aphids that reproduced (n = 26).

(1b) Medians of censored observations (n = 26): a Kaplan-Meier type of plot was drawn using the total number of offspring produced by every mature aphid up to its death and a censoring variable used to account for mothers killed

or lost before achieving their lifetime offspring production potential. The median lifetime production at death was graphically established.

(1c) Reproductive rate extrapolation (Equations 3.4, 3.5 and 3.6):

$$\text{Reproductive lifespan } RLS_i = \text{Age at exit}_i - (\text{Age at reproduction}_i - 3) \quad \textbf{Equation 3.4}$$

$$\text{Average reproductive rate aRR} = \frac{1}{n} \cdot \sum_{i=1}^{n} \left(\frac{\textbf{Total number of offspring produced by } \boldsymbol{i}}{\text{RLS } i} \right) \quad \textbf{Equation 3.5}$$

$$\text{Average lifetime fecundity} = aRR \cdot \frac{1}{m} \cdot \sum_{k=1}^{m} (RLS_k) \quad \textbf{Equation 3.6}$$

with n = 26 reproducing aphids *i* and m = 8 mature aphids *k* dying naturally. $(\text{Age at exit})_i$ represented the age of aphid *i* at death or at disappearance, $(\text{Age at reproduction-3})_i$ the age of aphid *i* at the last check before offspring was found in its Petri dish and Offspring_i the number of offspring produced by *i* over its entire reproductive life.

The ecological lifetime fecundity [offspring/established starter aphid] was estimated by:

(2a) Net reproduction rate R0 (Birch, 1948) [Equation 3.7]:

$$R_0 \text{ [offspring/established aphid]} = \sum_{x=1}^{q} (l_x \cdot m_x) \quad \textbf{Equation 3.7}$$

with l_x being the age *x*-specific survival rate of all aphids that established (n = 49), m_x the age *x*-specific fecundity rate of all established aphids alive at age *x* and *q* the age in days of the longest surviving aphid. R_0 is the rate of multiplication in one generation, i.e. the average number of female offspring produced per female (Birch, 1948).

(2b) Censored observations as described in (2a), applied to all aphids that established (n = 49).

(2c) Mean calculation using the data of all established aphids that died of natural causes in their Petri dish (aphids with complete life span; n = 15).

The influence of various aphid and plant parameters (Table 3.1) on the starter aphid's reproductive performance was assessed by a mixed-effects model for the general

reproductive rate (i.e. the number of offspring produced divided by the reproductive lifespan; Equation 2.17), which included plant genotype as a random grouping effect, and adult aphid size as an explanatory covariate (n = 20) after reduction to the most important variables.

3.2.2.2. Biology II experiment

Only data from Petri dishes with one aphid (solitary aphids; Section 3.2.1.2) were used for development and reproductive analyses. The processing was similar to the one described for the Biology I experiment, with a few differences. No attempt was made to attribute any size measurements to a specific instar. All offspring found in a Petri dish were counted as the reproductive output of the starter aphid living in this Petri dish. Final plant biomass records were not used. They were considered to be a poor indication of the feed on offer during the aphid development considering the unexpectedly long lifespans in the Biology II experiment. The plant parameters presented in Table 3.2 were used instead. The two aphids that achieved reproductive maturity on AR37-infected plants did not provide enough data for analyses. Adult aphid trait analyses considered therefore only the three other endophyte statuses (AR1, CT and NIL).

A generalised linear model of binomial type was fitted to the binary establishing variable using 'stats' (R Core Team, 2017), with plant characteristics and neonate EP size as initial parameters (Table 3.2). The final model included the age of the plant at aphid placement, the number of green leaves at aphid placement, the neonate EP size, the endophyte status and the interaction between neonate EP size and endophyte status only.

The correlations between L, W and EP were calculated with Spearman's ρ rank correlation coefficient ['Hmisc' (Harrell, 2018)]. Further size analyses in the Biology II experiment focussed on EP, and examined four aspects:

(1) Size of the neonate starter aphids (n = 166). Only neonates which had at least 1 measureable sibling (≥ 2 measurements/ancestor identity) were included in a mixed-effects analysis with ancestor identity as random factor and RelAgePAP (Table 3.2), PG-E group and their interaction as fixed effects.

(2) Growth of the starter aphids (n = 219 measurements): 95 specimens supplying ≥ 2 EP measurements were allocated to one of two size groups ('Large' or

'Small') as described for the Biology I experiment (Section 3.2.2.1). Gompertz growth models based on Equation 3.8 were then fitted to each PG-E-size group using the 'drc' and 'stats' packages (R Core Team, 2017; Ritz *et al.*, 2015).

$$\text{Size}(\text{Age}) = d \cdot e^{(-e^{b\cdot(\text{Age}-c)})} \qquad \textbf{Equation 3.8}$$

where d was the final size of the adult aphid or the size it was predicted to reach, and b and c model constants. Significant differences between growth models were assessed by parameter (b, c, d), considering whether the parameter estimates for the 'Larger' model were within the 95% confidence interval of the 'Small' model within each PG-E group. Differences between PG-E groups were assessed similarly, comparing means and confidence intervals pairwise within each size group.

(3) Adult size (n = 32): none of the mixed-effects models fitted to the data was better than a null model. A canonical discriminant analysis ['MASS' package (Venables & Ripley, 2002)] was therefore performed on all plant parameters and aphid width and length measurements (Table 3.2) in order to assess what combination of various properties could discriminate between 'Large' and 'Small' size specimen. Compliance with the multivariate normality assumption was assessed graphically (Mahalanobis distance).

(4) Neonate offspring size (n = 1104, from 45 mothers): only unfed early first instar nymphs were included in this analysis (aphids that had fed were observed to have a convex-shaped abdomen). Data of the AR37 endophyte group were also excluded from the analysis (too few). A mixed-effects model ['nlme' (Pinheiro *et al.*, 2017)] was developed with covariates as given in Table 3.2. The final model to explain the log-transformed ellipsoid neonate body projection (EP) included GLi, ancestral PG-E group, Age, AgeM, RR and the interaction term ancestral PG-E×Age as fixed effects. The random effects by starter mother identity (grouping factor) comprised a random intercept and a random slope for maternal age (Age).

Analyses of lifespan and time to reproductive maturity excluded data of starter aphids that disappeared from Petri dishes in their first week of life and those with more aphids in the first two weeks than the one starter aphid placed. As for the Biology I experiment, a Meier-Kaplan curve of the raw data was drawn for each PG-E group for the time point of reproduction and death, to determine the median age at these events. The possible influences of EPneo and plant parameters (Table 3.2) were then analysed by fitting Cox proportional hazard models to all data on death (n = 146), and to a subset of 98 individuals for reproduction (excluding the aphids on plants with AR37 symbiont). Besides the above-mentioned covariates, the model for death initially included the main factor PG (2 levels), E (4 levels), the interaction term PG×E and interactions between the main factors and covariates. For reproduction, the initial model included the mentioned covariates, the main factor PG-E (6 levels: N-AR1, N-CT, N-NIL, S-AR1, S-CT, S-NIL) and interactions between covariates and PG-E. The models were reduced to GLi, E and GLi×E interaction terms for the aphid age at death and AgePAP, S&DLi, PG-E, neonate size and AgePAP×PG-E interaction terms for the age at reproduction.

The reproductive performance of starter aphids was estimated by the same six methods described in Section 3.2.2.1 for the Biology I experiment. The influence of plant precondition parameters and aphid traits (Table 3.2), ancestor plant genotype ('aPG'), ancestor endophyte status ('aE'; AR1, CT and NIL only) and their interactions ('aPG×aE') on the reproduction were examined by analysis of covariance of the log-transformed, general reproductive rates of specimens that lived on AR1 symbioses, CT symbioses and NIL plants.

Table 3.2. Plant and aphid parameters recorded and used as covariates during the Biology II experiment.

Time	Name	Covariate description
Plant characteristics		
Aphid placement	AgePAP, *A: relAgePAP*	Age of plant at aphid placement *Age of plant at aphid placement, in relation to the first day aphids, were placed during the Biology II experiment, i.e. date of placement - 14/11/2013*
Aphid placement	GLi	Number of green leaves at aphid placement
Aphid placement	S&DLi	Number of dead leaves at aphid placement
Final	GLf	Number of green leaves at aphid death/harvest
Final	S&DLf	Number of dead leaves at aphid death/harvest
Aphid characteristics		
Aphid placement	EPneo *A: Lneo* *A:Wneo*	Ellipsoid body projection size as neonate [mm^2] *Body length of neonate [mm]* *W size of neonate [mm]*
Reproduction	EPadult *A: Ladult* *A: Wadult*	EP size as adult [mm^2] *Body length as adult [mm]* *Abdominal width size as adult [mm]*
-	Age *A: repAge*	Age of starter aphid at check or offspring collection [days] *Reproductive age length, i.e. Age at death – AgeM [days]*
Reproduction	AgeM	Age of starter aphid by first offspring in PD [days]
Reproduction-death	RR	General reproductive rate, i.e. offspring divided by reproductive lifespan [offspring/day reproduction]

A: alternative variable, used instead of the main variable in specific analyses.

3.2.2.3. Mature plant experiment

Differences in colonisation success between PG-E groups were assessed by Kruskal-Wallis test and post hoc Dunn test with Benjamini-Hochberg adjustment. Each Petri dish was further allocated to one of three colonisation groups [no (0), few (1-4) or many successfully colonising starter aphids (> 4)], to perform a canonical discriminant analysis on (i) initial parameters (tiller weight, dry matter content, tiller diameter and leaf regrowth 24 h after trim; all log-transformed), (ii) plant development parameters at aphid placement (number of roots, square root transformed; green leaf surface, colour ratio in the green shoot [G ratio = G/(R+G+B)]; Section 2.4.2), and (iii) further plant growth and development parameters (root appearance rate and leaf appearance rate after aphid placement [i.e. number of new roots or leaves produced per plant and day], both log-transformed; green leaf area growth rate to week 12, and green shoot growth, dead shoot biomass and root biomass in 116-day-old plants.

The reproductive rate was only assessed on mothers that survived both collection periods (n = 127). For reasons described in more depth in Section 3.3.3, the nymphs produced in each collection period (0 to 24 h and 24 to 48 h) were added together and divided by 2 to calculate an average daily reproductive rate. The reproductive rate was analysed with a linear mixed-effects model with Petri dish identity (Petri dish identity, n = 35) as the random grouping factor. All parameters mentioned for the colonisation analysis [(i), (ii), and (iii) above; all untransformed], age of mother (i.e. starter aphid) at harvest, water stress and PG-E groups were initially considered as fixed effects. The final model included only leaf appearance rate and PG-E groups as fixed effects, and Petri dish identity as a random effect.

3.2.2.4. Follow-up experiments on offspring survival

As this experiment aimed at predicting the survival of neonate root aphids *ex planta*, the offspring survival records (number of living offspring at each check in a tube) were converted into individual age-at-death records. Specific individual survival records were excluded from the data analyses if one of the criteria (1) to (5) below was fulfilled for offspring collected during the Biology II experiment, and if one of (4) to (6) was fulfilled for offspring collected in the Mature plant experiment, respectively:

(1) The aphid was clearly older than 1st instar.

(2) The aphid was maintained in the Conviron climate chamber 1 on the 4/02/2014 (likely heat stressed due to a climate chamber failure; exposed for > 3 h to temperatures above 35 °C.

(3) The aphid was harvested from an AR37 symbiosis (too few records).

(4) The aphid died within the first two days after being placed in a microcentrifuge tube (killed by handling; see Appendix 7)

(5) The aphid had no observable siblings (necessary for determining stable maternal effects).

(6) The mother of the offspring was wounded

A Cox proportional hazard model with mixed-effects was fitted to each experiment using the 'coxme' package (Therneau, 2015a; Therneau & Grambsch, 2000) and the parameters of Table 3.3. (n = 2331 and 1292 for the Biology I and Mature plant experiments, respectively).

Table 3.3. Parameters initially (all) and finally (grey) included in the Biology II and mature plant follow-up experiments to analyse offspring survival.

Variable type	Biology II experiment	Mature plant experiment
Fixed effects		
Plant covariates	• Age of plant at aphid placement (AgePAP) • GL at aphid placement • S&DL at aphid placement	• Tiller dry weight at start • Tiller dry matter content at start • Tiller diameter 24 h after initial trim • Leaf length regrowth 24 h after initial trim • Number of roots at aphid placement • Average root branching order at aphid placement • Green shoot area 35 days after trim (GSA35D) • Colour ratio G/(R+G+B) of GSA35D • Age of plant at aphid placement (AgePAP) • Green shoot area 80 days after trim (GSA80D) • Colour ratio G/(R+G+B) of GSA80D • Green shoot dry weight 116 days after trim • S&D shoot dry weight 116 days after trim • Root dry weight 116 days after trim
Aphid covariates	• Age of mother at offspring collection (Age) • General reproductive rate (RR) • Recent reproductive rate (24 h; RR24h) • Age of mother at first reproduction • Maternal socialisation (1 vs. > 1/PD)	• Age of mother at mother harvest • General reproductive rate (RR) • Recent reproductive rate (RR24h)
Methodical effects	• Number of offspring in MC tube	• Water stress (yes vs. no) • Number of offspring in MC tube • Collection period (0-24 h vs. 24-48 h)
Main effects	• PG-E group	• PG-E group
Interactions	• PG-E×Age • PG-E×RR	• PG-E×RR24h
Random effects		
Grouping variables	• Mother identity	• Petri dish identity (PDID) • Mother identity (nested in PDID)
Random slopes	• Age • Recent reproductive rate (24 h; RR24h)	
Other	• Intercept	• Intercept

MC tube: microcentrifuge tube in which the live offspring of one cohort was kept in the follow-up experiments; PD: Petri dish; PG-E: plant genotype-endophyte status grouping variable; GL: number of green leaves; S&DL: number of senescing and dead leaves.

3.3. Results

3.3.1. Biology I experiment

3.3.1.1. Instars

Most starter aphids moulted four times before achieving reproductive maturity (four exuviae collected in 62% of all Petri dishes with offspring; Table 3.4), suggesting *A. lentisci* has four nymphal instars and one adult stage (Figure 3.2). Nine exuviae were not found [2·(4-2) + 5·(4-3); 9% of all exuviae produced, assuming all reproductive adults moulted four times; Table 3.4], probably because of the 2 cm long roots that obstructed the observations. The additional exuvia found in three Petri dishes (Table 3.4) could be explained by a failure to completely retrieve an exuviae at one check (handling issues around the feeding aphid, and re-counting of an exuviae piece at the subsequent check) or by offspring that managed to hide for one check and moulted amongst the roots.

Table 3.4. Exuviae collection in Petri Dishes (PD).

		Number of exuviae found						
		0	**1**	**2**	**3**	**4**	**5**	**Total**
Number of PD	Overall	4	4	4	9	19	3	43
	PD with mature adults (≥ 1 offspring)	0	0	2	5	16	3	26

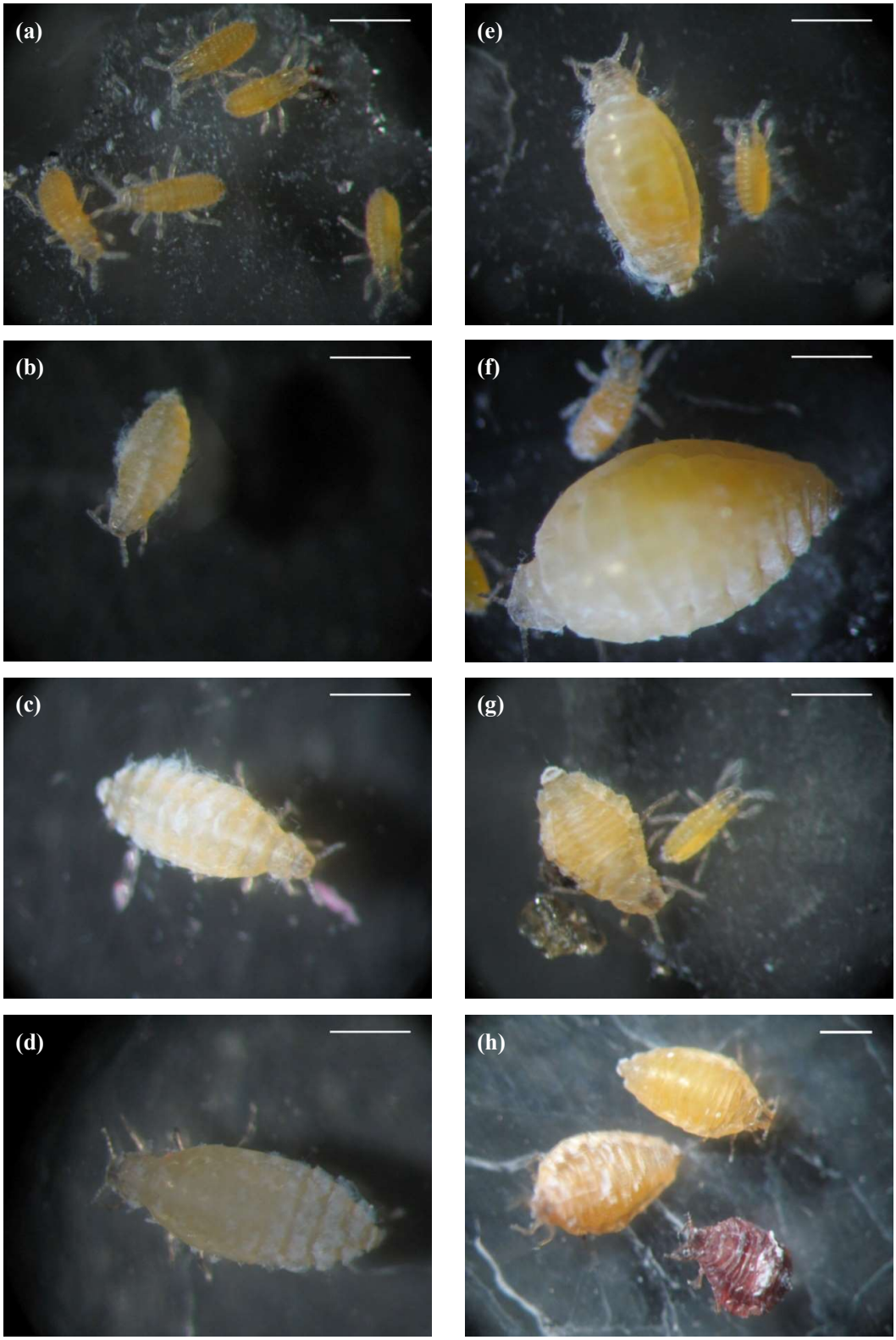


Figure 3.2. Development of *A. lentisci* on ryegrass roots. (a) 1st instars, (b) 2nd instar, (c) 3rd instar, (d) 4th instar; (e, f, g) adults with offspring, (h) Adults of various ages (top to bottom: youngest to oldest). The white scale bar represents 0.5 mm.

3.3.1.2. Aphid body size

Root aphid dimension ranged from 0.51 to 1.88 mm for body length L, 0.17 to 1.14 mm for abdominal width W and 0.07 to 1.45 mm^2 for EP. Although individual aphids increased discretely in size from one instar to the next (models not shown), it was not possible to distinguish between 3rd and 4th instars on the basis of the measurements considered (Table 3.5).

Table 3.5. Mean body length [mm], abdominal width [mm] and ellipsoid body projection EP [mm^2] of different instars (± standard deviation).

	Body length		Abdominal width		EP	
	n	Size	n	Size	n	Size
Instar 1	65	0.61 ± 0.052 [a]	59	0.22 ± 0.023 [a]	59	0.11 ± 0.022 [a]
Instar 2	19	0.88 ± 0.157 [b]	15	0.38 ± 0.065 [b]	15	0.27 ± 0.093 [b]
Instar 3	20	1.17 ± 0.149 [c]	17	0.52 ± 0.074 [c]	17	0.47 ± 0.127 [c]
Instar 4	19	1.27 ± 0.231 [cd]	11	0.60 ± 0.130 [c]	11	0.60 ± 0.213 [c]
Instar 5	23	1.39 ± 0.248 [d]	22	0.72 ± 0.154 [d]	22	0.83 ± 0.258 [d]

n: number of specimens; Where several measurements were available for one specimen and instar, the data were pooled to one value. Means with the same letter within a column were not significantly different ($p > 0.05$).

The viviposition plant (and thus the group of mothers) had a significant effect on the size of neonate starter aphids [$\chi^2_{(9)} = 24.64$, $p = 0.003$ for L; $\chi^2_{(9)} = 23.42$, $p = 0.005$ for W; $\chi^2_{(9)} = 24.29$, $p = 0.004$ for EP; Appendix 10, Section A10.1]. The EP size of a neonate starter aphid was not correlated with its own adult EP size, however [$r_{(15)} = 0.16$, $p = 0.533$].

Individual body lengths L and abdominal widths W correlated [$\rho_{spearman(217)} = 0.96$, $p < 0.001$ for all size measurements]. The general development pattern of L and W resembled that of EP, but with more variation from one check to the next, however (Appendix 10, Section A10.2.2; no further analyses). Two size groups with distinct development were apparent in the EP measurements (Table 3.6, Figure 3.3): 'Large' (EP ≥ 1.0 mm^2) and 'Small' (EP < 1.0 mm^2). Aphids of the 'Large' group gained more size initially but maintained or slightly decreased in size in their late life. Aphids of the 'Small' group developed more slowly but continued growth after maturity at a similar rate (Figure 3.3).

Table 3.6. Estimated parameters (± standard error) of the log-logistic EP size growth models for aphids of the 'Small' and 'Large' size group in the Biology I experiment.

Parameters	Small size		Large size		
	Estimate ± SE	*p*-value	Estimate ± SE	*p*-value	Difference[1]
b	-1.051 ± 0.346	*0.003*	-36.049 ±33.876	*0.292*	Significant
c	0.093 ± 0.014	*< 0.001*	0.096 ± 0.033	*0.006*	n.s.
d	1.319 ± 0.332	*< 0.001*	1.165 ± 0.024	*< 0.001*	n.s.
f	2.997 ± 5.716	*0.601*	18.097 ± 0.633	*< 0.001*	Significant
g	6.971 ±10.980	*0.527*	0.047 ± 0.046	*0.311*	n.s.
Residual SE	0.087		0.107		
Degrees of freedom	129		60		

Models: EP (Age) = c + (d - c)/(1 +exp(b·(log$_e$(Age)-log$_e$(f))))^g.

[1] A parameter of the 'Large' group was significantly different if its estimate lay outside the 95% confidence interval of the estimate in 'Small' size for the same parameter; n.s.: not significant

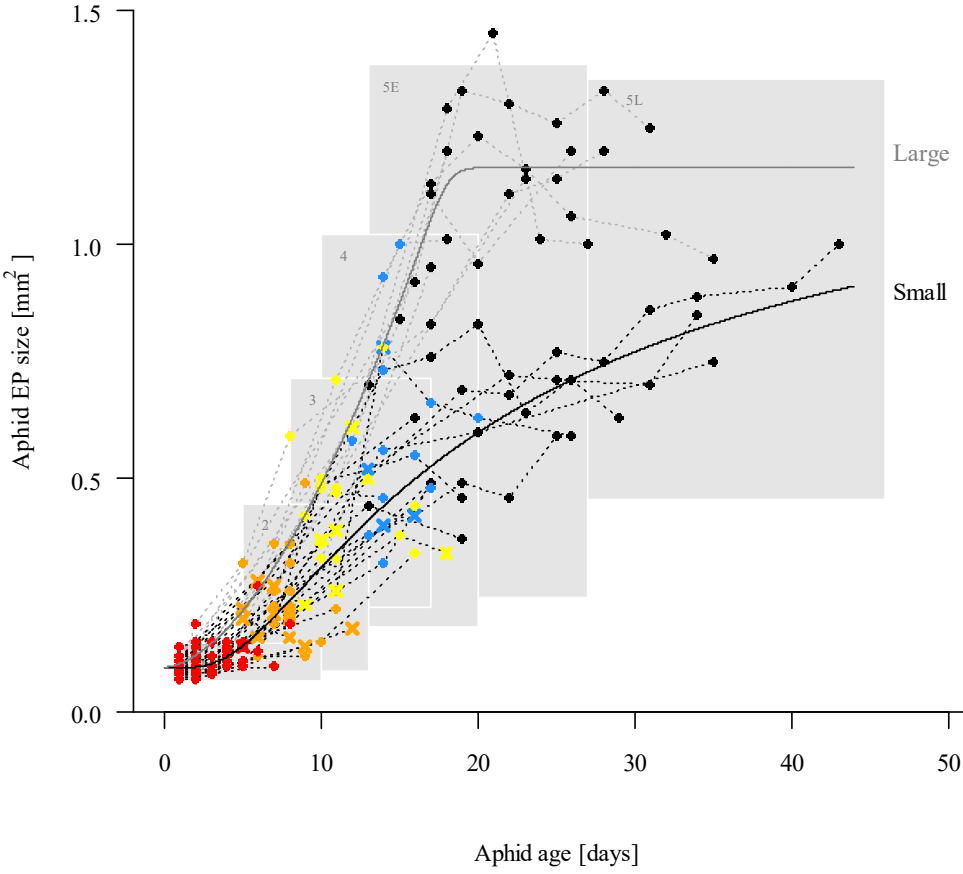


Figure 3.3. Aphid EP size development over the Biology I experiment. Instars 1, 2, 3, 4 and 5 are represented in red, orange, yellow, blue and black, respectively. Dots: measurements on aphids of verified instar; Crosses: measurements on aphids of presumed instar (unconfirmed). Shaded areas indicate the 95% confidence interval of size for verified instar measurements (5E: 5th instar size at or before first offspring birth; 5L: later measurements).

Neither plant genotype (not included in the chosen model) nor maternal identity significantly affected adult size (likelihood ratio for random maternal effects: $\chi^2_{[1]} = 0.00$, $p = 1.000$). The reproduction rate RR explained most adult size variance: the square-root transformed adult EP size increased by 0.23 ± 0.05 units for each offspring produced per day ($p < 0.001$; log-likelihood ratio: $\chi^2_{[1]} = 17.10$, $p < 0.001$; Conditional R^2 for the model: $R^2c = 0.661$). Initial root biomass was the only other plant parameter of possible influence on adult size, with a marginally not significant increase in square-root transformed adult size of 0.08 ± 0.04, $p = 0.091$ per 10 mg initial root biomass ($\chi^2_{[1]} = 3.80$, $p = 0.051$).

3.3.1.3. Aphid establishment, development and longevity

Approximately 85% of the 59 starter aphids placed established, and 44% achieved reproductive maturity (52% of the established aphids). Since most of the failures to establish during this experiment were likely due to handling, no attempt was made to explain the establishment success with any plant properties modelling. The median established aphid had completed its first ecdysis by its 7th to 9th day of life (Figure 3.4). Ecdyses 2, 3 and 4 had taken place by day 10 to 15, 13 to 15 and 17 to 19, respectively. Offspring appeared as early as day 15, but the median age at reproduction was 20 to 21 days. In 16 of 26 cases, the last exuviae and the first offspring were found simultaneously. The neonate size of a starter aphid had no significant effect on its development in this experiment (Table 3.7). Shoot growth and root architecture significantly influenced development time. High initial shoot biomasses were associated with an early ecdysis 1, but aphids on plants with high final green shoot weight gain moulted (ecdysis 2, 3 and 4) and reproduced later, than aphids on plants with less green growth at harvest (Table 3.7). Dead shoot biomass was associated with a delayed development too, but only for ecdysis 3. A large final root biomass significantly delayed ecdysis 1. However, aphids on plants with many old roots developed faster from neonate to 2nd instar (ecdysis 1), to 3rd instar (ecdysis 2) and to reproduction whereas plants with many new roots at precondition were associated with late ecdysis 2 and ecdysis 3 (Table 3.7).

The longevity in this experiment could not be explained by any variable (no model was better than the null model). The median aphid was dead by day 33 in the only group with enough natural deaths for a median estimation [G3; Figure 3.4 a)]. The aphid with the longest lifespan was killed by handling at 49 days.

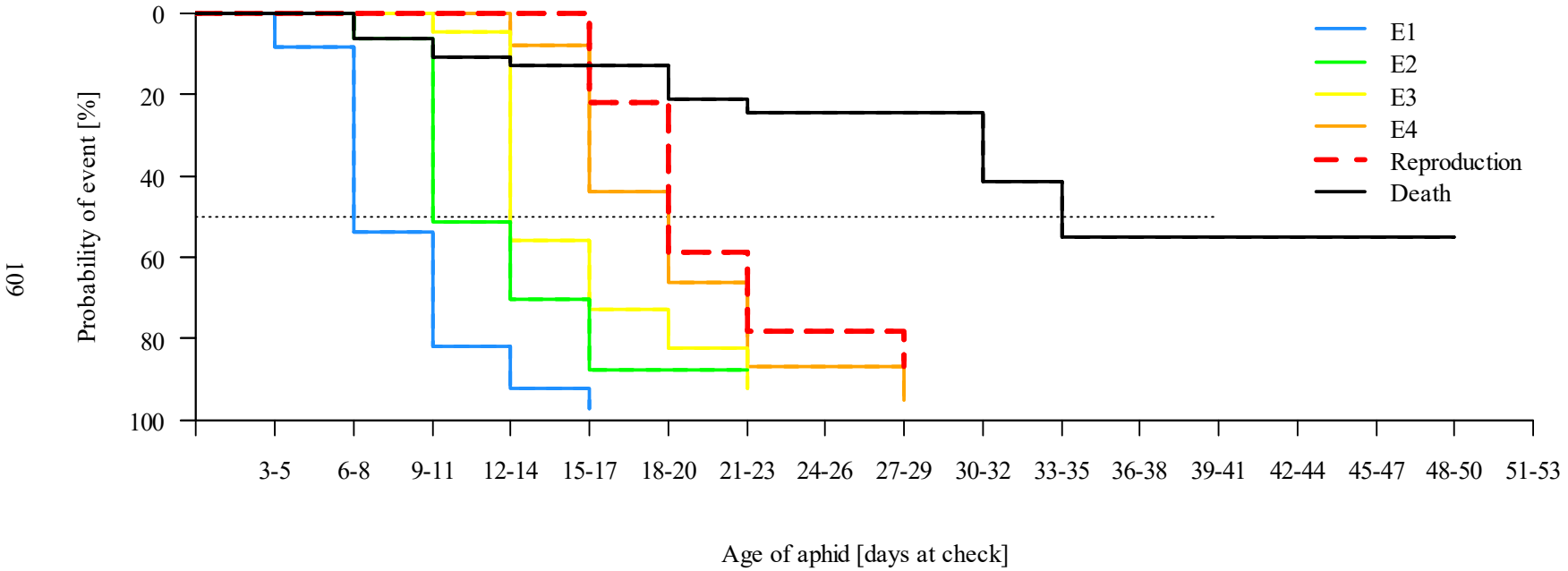


Figure 3.4. Aphid age at ecdyses 1 to 4 (E1, E2, E3 and E4), reproductive maturity (Reproduction) and death during the Biology I experiment. The raw data were pooled by check for this graphical representation. Thereby, the first check was performed on day 3, 4 and 5 (3-5), the second on day 6, 7 and 8 (6-8), etc. in group 3, 4 and 5, respectively.

Table 3.7. Influence of aphid and plant characteristics on age at moults (ecdysis 1 to 4) and reproduction (Repro) in the Biology I experiment.

Event	Ecdysis 1	Ecdysis 2	Ecdysis 3	Ecdysis 4	Repro
Model statistics					
R^2	0.297	0.306	0.305	0.127	0.230
p-value (Likelihood ratio)	*< 0.001*	*< 0.001*	*< 0.001*	*0.011*	*0.002*
p-value (Wald test)	*0.002*	*0.001*	*0.002*	*0.011*	*0.003*
PH assumption, global *p*-value	*0.878*	*0.396*	*0.627*	*0.994*	*0.691*
Coefficients p-values and sign					
Aphid characteristics					
Aphid neonate size (EP)					
Initial plant characteristics					
Number of old roots (at trim)	*< 0.001* +	*0.039* +			*0.028* +
Number of new roots (at placement)		*0.014* -	*0.016* -		
ABO of new roots (at placement)					
Shoot biomass (at trim)	*0.024* +				
Root biomass (at trim)					
Root/shoot ratio (at trim)					
Final plant characteristics					
Number of new roots					
ABO of new roots					
Dead shoot biomass			*0.030* -		
Green shoot biomass, net growth		*0.002* -	*< 0.001* -	*0.011* -	*0.009* -
Roots biomass, net growth	*0.003*[1] -				
Root/shoot ratio					

Final Cox proportional hazard models. Parameters with positive sign (+) increased the hazard of the event (i.e. reduced the age at the event), parameters with negative sign (-) decreased the hazard. Initial characteristics referred either to plant properties assessed on the freshly trimmed tiller or on the plant before aphid placement 19 to 30 days after trim. Final plant characteristics were measured 75 to 76 days after trim, at plant harvest. ABO: average branching order (see Section 2.4.2); EP: ellipsoid body projection (see Section 2.4.3). PH: proportional hazard.

[1] Biomass at harvest (dry matter, [g]) was used for analyses instead of net growth (dry matter, [g])

3.3.1.4. Aphid fecundity

Of the aphids that established, 52% produced offspring (44% of the aphids placed). The censoring method suggested this might have been as high as 79% in absence of handling-caused deaths (data not shown). The maximum lifetime fecundity observed was 34 offspring. A reproducing mother viviposited a mean of 12.9 ± 11.3 offspring. Alternative predictive methods that considered handling bias estimated the biological

lifetime fecundity was in the range of 9 to 34 offspring per reproducing mother (Section 3.3.2.3, Table 3.9). The ecological lifetime fecundity calculated by the method of Birch (1948) predicted a reproduction rate of 6.8 offspring per established, neonate starter aphid. The alternative methods considering handling bias (Section 3.2.2.1) estimated it to be 6 to 29 offspring per established neonate.

The random factor 'plant genotype' was not significant for the reproductive rate *per se* (log likelihood ratio: $\chi^2_{[1]} = 1.28$, $p = 0.258$). Adult aphid size, however, significantly explained about 33% of the variance of the reproductive rate (log-likelihood ratio for adult size: $\chi^2_{[1]} = 11.26$, $p < 0.001$; Model $R^2c = 0.695$), with 1.04 ± 0.28 offspring/day per mm^2 adult size ($p < 0.001$).

3.3.2. Biology II experiment

3.3.2.1. Aphid size

Maternal identity significantly influenced the size of neonate starter aphids ($\chi^2_{[1]} = 6.85$, $p = 0.009$ for the random effect). None of the fixed effects was significant, however ($\chi^2_{[7]} = 9.44$, $p = 0.223$ for the interaction term PG-E×RelAgePAP; log-likelihood of PG-E and RelAgePAP in a simplified model without interactions: $\chi^2_{[7]} = 7.07$, $p = 0.421$ and $\chi^2_{[1]} = 1.90$, $p = 0.168$, respectively; model $R^2_c = 0.334$).

As in the Biology I experiment (Section 3.3.1.2), maximum EP sizes were not achieved at reproductive maturity (Figure 3.5; Appendix 10, Section A10.4.2), and in all PG-E groups except S-CT, two size groups were observed (Figure 3.6). The 'Large' growth curves of all PG-E groups were not significantly different (predicted final size: 1.33 to 2.14 mm^2). Aphids of the 'Small' S-CT group developed to a significantly smaller final EP (0.59 ± 0.07 mm^2) than aphids of every other 'Small' PG-E group except N-AR1 (0.99 ± 0.41 mm^2). On S-AR1 plants, 'Small' aphids tended towards a significantly larger final size (1.19 ± 0.22 mm^2), than 'Small' aphids on S-NIL (0.74 ± 0.06 mm^2, $p < 0.05$).

A large adult abdominal width, a late placement (i.e. a placement on old plants, which included also an effect of using other mother aphids as source of neonates), and good plant preconditions (many green and few dead leaves at the time point of aphid placement) were common to the 'Large' adult size. These criteria together discriminated the aphids of the 'Large' group best from aphids of the 'Small' group (Canonical discriminant analysis; Appendix 10, Section A10.4.3).

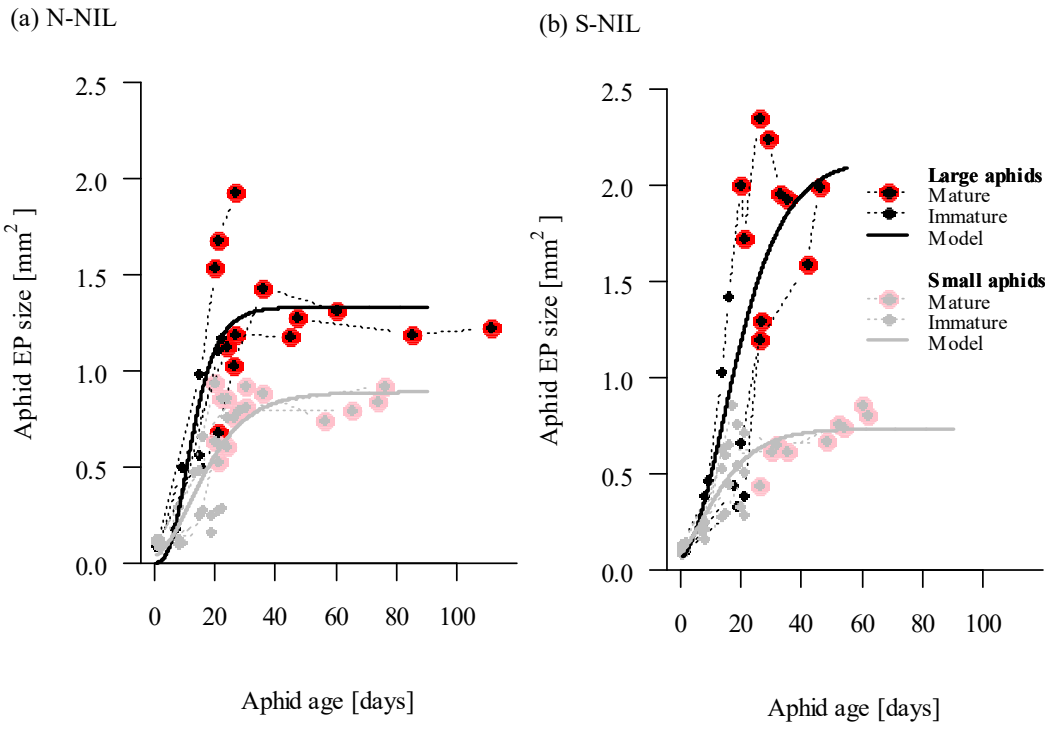


Figure 3.5. Size development of individual root aphids living on endophyte-free perennial ryegrass tillers of two different genotypes N (a) and S (b). EP, ellipsoid body projection**.**

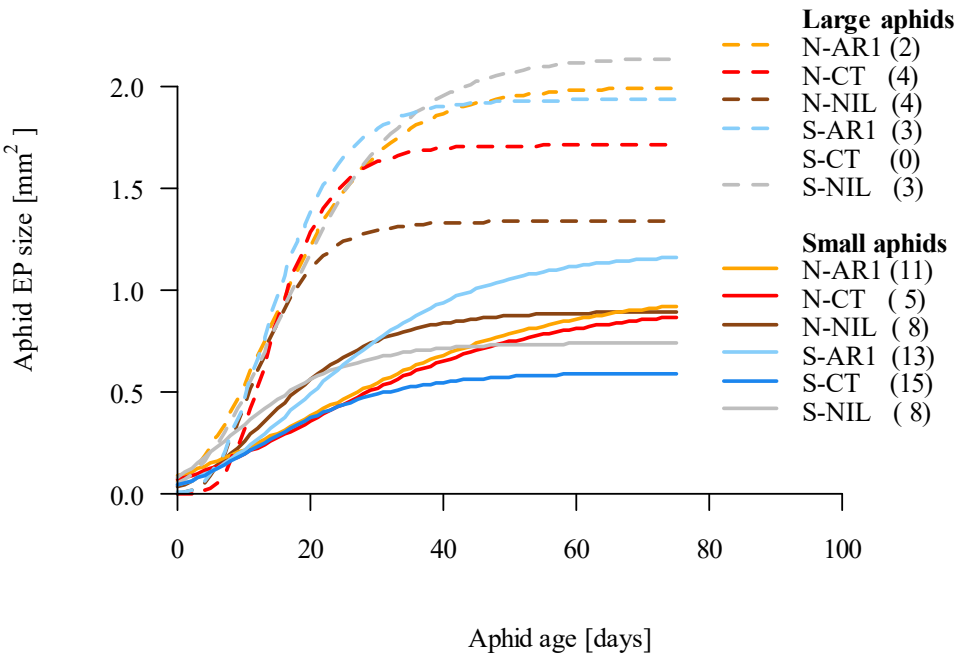


Figure 3.6. Growth of aphid size of large and small groups on two perennial ryegrass plant genotypes (N, S) infected with endophyte *E. festucae* var. *lolii* strain AR1 or CT, or without endophyte (NIL). Numbers in brackets: number of specimens assigned to the respective group. Model: $\mathbf{Size(Age)} = \mathbf{d} \cdot \mathbf{e}^{(-\mathbf{e}^{\mathbf{b}\cdot(\mathbf{Age}-\mathbf{c})})}$.

The mean neonate offspring size in each PG-E group varied between 0.09 ± 0.01 (aS-CT) and 0.13 ± 0.02 mm^2 (aS-AR1) but was not significantly different by average covariate values. Random maternal identity effects (starter aphid identity) and random, mother-specific age effects (starter aphid age at offspring collection) improved the model significantly (Table 3.8). The number of green leaves of a plant at maternal placement improved the model marginally although the coefficient was not significant. Offspring of more fecund mothers were larger than those of less fecund ones ($p < 0.001$). Significant interactions between maternal age and ancestor groups were observed. When these effects were summed by PG-E group, mothers of all groups except the reference (aS-NIL) tended to produce smaller offspring with advancing age.

Table 3.8. Coefficients and log-likelihood of factors affecting the log-transformed neonate offspring size.

	Linear coefficients					L ratio[2]	
	Value	**SE**	**df**	**t-value**	***p*-value**	x^2	***p*-value**
Fixed effects							
(Intercept)	-2.580	0.101	1053	-25.526	0.000		
Number of green leaves at AphP	-0.017	0.009	36	-1.839	0.074	4.19	0.041
Ancestor group (aPG-E)[1]:						-	-
aN-AR1	0.182	0.099	36	1.833	0.075		
aN-CT	0.204	0.093	36	2.202	0.034		
aN-NIL	0.136	0.073	36	1.871	0.070		
aS-AR1	0.224	0.080	36	2.789	0.008		
aS-CT	0.185	0.125	36	1.482	0.147		
Age of mother (Age)[3]	0.004	0.002	1053	2.549	0.011	-	-
General reproductive rate (RR)	0.120	0.016	36	7.384	< 0.001	37.15	< 0.001
Maternal maturity age (AgeM)[3]	0.006	0.003	36	1.901	0.065	4.62	0.032
Interaction aPG-E×Age:						14.97	0.010
aN-AR1×Age	-0.006	0.003	1053	-1.871	0.062		
aN-CT×Age	-0.008	0.003	1053	-2.611	0.009		
aN-NIL×Age	-0.005	0.002	1053	-2.437	0.015		
aS-AR1×Age	-0.007	0.002	1053	-3.088	0.002		
aS-CT×Age	-0.009	0.004	1053	-2.322	0.020		
Random effect of mother identity						**86.15**	**< 0.001[4]**
(Intercept)						10.37	0.006
Random slope for Age						19.36	< 0.001

Mixed effect model, $R^2_c = 0.564$ [conditional R^2, variance explained by fixed and random factors (Barton, 2018)]; SE: standard error; AphP: aphid placement

[1]With 2 exceptions, aPG-E and PG-E were identic (aS-AR1 used for a S-NIL and a N-AR1 plant)

[2] Log-likelihood ratios were tested comparing the full model to a model without the term; -: main effects cannot be tested by interactions as both terms would have to be removed.

[3]'Age': mothers' age on the day of offspring collection; 'AgeM': age at first viviposition.

[4]Full model compared to one without random effects.

3.3.2.2. Aphid establishment, colonisation success, development and longevity

Only 106 of the 206 aphids introduced to 82 plants established. Various experimental aspects significantly influenced the establishment success: late placement (i.e. placement on old plants, possibly combined with some aphid source effects as new mothers had to be used for later placement) marginally reduced the odds (-1% for an additional day, $p = 0.073$; log-likelihood ratio of the effect: $\chi^2_{[1]} = 3.36$, $p = 0.067$), while the number of green leaves of a plant at aphid placement significantly increased it (+154% for an additional green leaf, $p = 0.005$; $\chi^2_{[1]} = 10.72$, $p = 0.001$). Plant genotype was of no significance when these confounding variables were included, and could be removed from the model without consequences. Endophyte status, neonate EP size and their interaction significantly improved the model (likelihood ratio of the interaction: $\chi^2_{[3]} = 8.01$, $p = 0.046$), even if the coefficients for neonate EP size, all endophyte statuses and the interactions were not significant *per se* ($p > 0.05$; adjusted R^2 for the model: 0.608). For placements on 124-day-old plants with two to three green leaves, however, neonates of 0.10 mm^2 established significantly less frequently on plants with AR37 symbionts than on plants with other symbionts. At these average covariate values, the probability for establishing on AR37 infected plants was 23 to 43%, against 88 to 99% in the other groups. The colonisation success ranged between 2-3 % for aphids placed on N-AR37 and S-AR37 plants, compared to 21 to 71% for aphids on other PG-E groups (this equates to 14 to 17% and 38 to 77% of the established aphids, respectively; Appendix 10, Section A10.5). As handling effects likely interfered with these results, these data were not analysed further.

The median age of aphids at reproduction varied between 23 and 36 days depending on the PG-E group (Figure 3.7). The apparent differences between PG-E groups were partly explained by experimental and plant growth-related covariates. Large neonates reached maturity significantly earlier than smaller ones (hazard multiplication coefficient = $1.9 \cdot 10^{15}$, $p = 0.028$). A development delay was associated (marginally not significant) with a large number of dead leaves (S&DLi; hazard multiplication coefficient = 0.81 [i.e. a decrease of the risk by -19%], $p = 0.061$). Plant age at placement (AgePAP) *per se* did not significantly shorten the time to maturity (hazard increase of + 4%, or a coefficient of 1.04, $p = 0.072$). However, the interaction between some PG-E groups and AgePAP was significant. The N-CT group, in particular, developed faster to maturity than the S-NIL group (hazard multiplication coefficient = 53.05, $p = 0.007$) but the developmental time

increased in aphids placed on N-CT at a later time (i.e. on older plants; hazard multiplication coefficient 0.95 or risk decrease by –5%, $p = 0.005$; note that this factor may also have included some aphid source effects, since new mothers from other colony plants had to be used to produce neonates at a later time point). Delayed development to maturity by increasing AgePAP was also observed in the other groups (exception: S-NIL) but these effects were not significant ($p > 0.05$). By average covariates, aphids on S-CT symbionts achieved maturity significantly later than aphids on S-NIL or S-AR1 plants ($p < 0.05$). Furthermore, aphids on N-AR1 plants also took significantly longer to mature than aphids on S-NIL plants. The differences between other groups were not significant.

The median age of natural death was 47 and 43 days for aphids on N-AR1 and S-AR1 plants, 15 and 8 days on N-AR37 and S-AR37, 29 and 40 days on N-CT and S-CT, and at 63 and 70 days on endophyte-free plants of N and S genotypes, respectively. Only endophyte status and its interaction with the number of green leaves available at aphid placement had an influence on survival. The lifespan of aphids on plants with AR1 endophyte was not significantly shorter than that of aphids on NIL plants (risk of death: 3.75 times that of NIL, $p = 0.070$). On AR37 and CT symbioses, aphids had significantly higher risks of death than those living on endophyte-free plants (154 and 10 times that of NIL, $p < 0.001$ and $p = 0.003$, respectively), which translated into a significantly shorter lifespan (Figure 3.7). On AR37 and CT infected plants with a large number of green leaves (GLi), the death risk of aphids was significantly reduced (-60%, $p < 0.001$ and -46%, $p = 0.022$ for each additional green leaf at aphid placement, respectively). GLi had no significant effect on aphids living on AR1 or NIL plants. At an average number of GLi (2.5), aphids on NIL plants lived significantly longer ($p < 0.05$) than aphids on endophyte-infected plants. Aphids on AR1 and CT plants had similar lifespans, while those on AR37 had the shortest lifespan of all ($p < 0.05$).

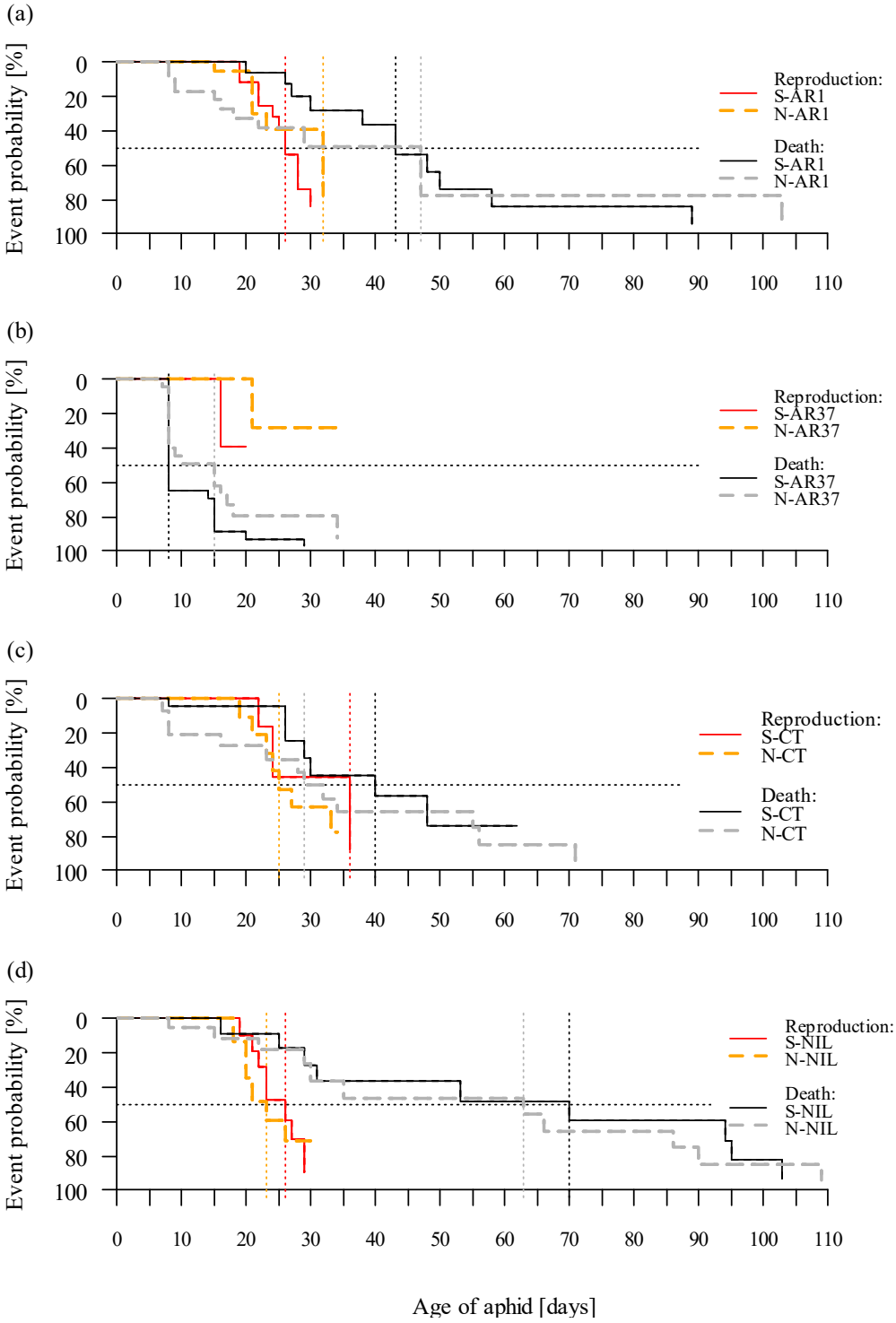


Figure 3.7. Probability of reproduction and mortality of root aphids on perennial ryegrass of two genotypes (N and S) with AR1 (a), AR37 (b), or common-toxic CT (c) endophyte symbiont, or without any endophyte [NIL(d)]. Vertical dashed lines indicate median values.

3.3.2.3. Aphid fecundity

Reproductive performances of over 100 offspring/mother were recorded (Table 3.9, Max; Appendix 10, Figure A10.6.1) in the Biology II experiment. The reproducing root aphids viviposited an average of 45 offspring per mother on endophyte-free plants. By the nymphal mortality observed in the laboratory, that corresponded to an average of 23 to 45 offspring on endophyte-free plants per established starter aphid (Table 3.9, ecological lifetime fecundity). It is presumed that the aphid handling in this experiment was suboptimal, though, and that the actual reproductive potential is likely higher [50 to 70 and 39 to 54 offspring per reproductive mother in N-NIL and S-NIL plants, respectively (Table 3.9, biological lifetime fecundity)].

Root aphids living on AR1- and CT-infected plants appeared to have a lower lifetime fecundity than root aphids living on endophyte-free plants, but the differences were not significant (Table 3.9). That endophytes had an influence on the reproduction became obvious when the average reproductive rate (RR) was analysed, however (Table 3.10): root aphids living on CT-infected plants had a significantly lower log-transformed RR (-0.621 ± 0.214, $p = 0.007$) than NIL peers. At average covariate values (i.e. when comparing mothers of similar size [0.10 mm^2 neonate size, 0.86 mm^2 adult size] that had been placed on 98-day-old plants), the root aphids on CT-infected plants produced half as many offspring, as the root aphids on endophyte-free plants (0.66 offspring·$mother^{-1}$·day^{-1} compared to 1.23 for NIL). Aphids on AR1 symbioses had intermediate fecundity (0.88 offspring·$mother^{-1}$· day^{-1}; $p > 0.05$ for comparisons with both CT and NIL). This endophyte effect did not include any endophyte-related effects on aphid size (Section 3.3.2.1) in the chosen model. Possible additional endophyte influences through aphid size were indeed accounted for with the adult body size parameter [3.2 more offspring/day for every additional 1 mm^2 adult body size; $p < 0.001$]. The differences in initial body size of these adults when they were neonates had no significant effect ($p = 0.161$).

Root aphids placed on old plants had a significantly lower reproductive rate than root aphids placed on younger plants (coefficient for age of plant at aphid placement = -0.003 ± 0.002 log-transformed RR per day).

Table 3.9. Maximum reproductive performance (Max), average lifetime fecundity of adult root aphids (biological fecundity) and average lifetime fecundity of a root aphid populations (ecological fecundity), as estimated by various methods for the Biology I and Biology II experiments.

Trial	Plant geno-type	Endo-phyte	Max	Biological lifetime fecundity												Ecological lifetime fecundity				
				Average			Censored O.			Reproductive rate extrapolation						R_0	Censored O.	CLC average		
				n	mean	CI	n	mdn	CI	n_{RR}	RR	n_{RLS}	RLS	mean	CI[1]	mean	mdn[2]	n	mean	CI
I[3]	Pooled	NIL	34	26	**13**	[0- 36]	28	**34**	[29-n.a.]	26	0.9±0.6	8	11±6.0	**9**	[0- 21]	**7**	**29**	15	**6**	[0- 28]
II	N	AR1	75	6	**21**	[0- 75]	6	**21**	[4 -n.a.]	6	0.7±0.4	4	36±32.5	**26**	[0- 56]	**6**	**21**	12	**8**	[0- 51]
		AR37	(4)[4]	1	**(4)**		1			1	1.0	0				**0**		16	**0**	[0]
		CT	34	7	**18**	[0- 39]	7	**16**	[12-n.a.]	7	1.2±1.0	6	21±15.9	**25**	[0- 64]	**9**	**14**	12	**8**	[0- 29]
		NIL	110	9	**45**	[0-115]	9	**50**	[36-n.a.]	9	1.3±0.9	6	55±26.6	**70**	[0-162]	**23**	**45**	11	**31**	[0-108]
	S	AR1	117	11	**34**	[0-114]	11	**29**	[11-n.a.]	11	1.1±0.7	8	28±17.8	**31**	[0- 70]	**18**	**24**	10	**35**	[0-120]
		AR37	2	1	**2**		1	**2**	[2 -n.a.]	1	0.1	1	14	**2**		**0**	**2**	21	**0**	[0- 1]
		CT	10	7	**6**	[0- 12]	7	**7**	[2 -n.a.]	7	0.5±0.3	5	8±4.3	**4**	[0- 8]	**2**	**7**	8	**3**	[0- 9]
		NIL	122	7	**45**	[0-143]	7	**39**	[7 -n.a.]	7	1.2±0.8	7	46±32.6	**54**	[0-128]	**45**	**35**	8	**39**	[0-135]

Censored O.: censored observations (Section 3.2.2.1); CI: 95% confidence interval; CLC: complete life cycle, i.e. only aphids that died a natural death were included in the calculation of the mean; mdn: median; n: number of specimens offering useable data for analyses; n.a.: no estimate available; R_0: net reproduction rate calculated by Birch (1948); RLS: reproductive lifespan, i.e. time elapsed between last check before reproductive maturity and death; RR: general reproductive rate [offspring·day^{-1}·mother^{-1}].

[1] The CI for reproductive life was estimated as = RLS·(RR ± 1.96·standard deviation of RR).

[2] The CI for this parameter was [0 - n.a.] for all groups because many aphids did not die of natural causes (lost or killed by handling).

[3] The fecundity of root aphids was likely underestimated in this experiment because of high temperatures in the glasshouse (Appendix 10, Figure A10.3.1)

[4] The only specimen developing to reproductive maturity was killed by handling

Table 3.10. Analysis of covariance table for the log-transformed reproductive rate.

Variables	df	SS	MS	F value	*p*-value
Age of plant at aphid placement	1	2.278	2.278	10.263	0.003
Neonate size of aphid (EP)	1	0.646	0.646	2.911	0.099
Adult size of aphid (EP)	1	8.686	8.686	39.140	< 0.001
Endophyte status of the ancestor[1]	2	1.902	0.951	4.286	0.023
Residuals	29	6.436	0.222		

$F_{[5,29]} = 12.18$, $p < 0.001$, $R^2 = 0.6217$; EP: body projection; MS: mean squares; SS: sum of squares

[1] With one exception, the mothers (ancestors) of the observed aphid lived on plants of the same endophyte status. A model with the endophyte status of the observed aphids' plant came to the same conclusions but with less well-distributed residuals. That was interpreted as a long-lasting food quality effect.

3.3.3. Mature plant experiment

Nine to ten weeks after placement, root aphids had colonised S-AR1 and N-CT symbionts significantly better than S-AR37 symbionts ($\chi^2_{[7]} = 16.94$, $p = 0.0195$; Figure 3.8). A combination of low final root biomass and high green shoot area growth differentiated colonised plants with > 1 aphid from plants that were not colonised across all PG-E groups (Canonical discriminant analysis; Appendix 10, Section A10.7). While the lower root biomass was more likely a consequence of aphid feeding on successfully colonised plants (Section 4.3.2), the fast-growing green shoot area appeared to be a good predictor for higher colonisation success.

The fecundity of a mother in her first 24 h (D1) was positively correlated with her next 24 h (D2; $\rho_{spearman\ [125]} = 0.49$, $p < 0.001$), with a relationship of $D2 = 3.54 + 0.36 \cdot D1$ ($R^2 = 0.175$, $p < 0.001$). The relationship was not always significant when the groups were considered individually (Table 3.11). Aphids collected from two Petri dishes with S-NIL plants appeared to delay viviposition by a day and to produce twice as many offspring in the second viviposition period as the other PG-E groups (Table 3.11). Handling was the suspected cause of this observation as the aphid harvest in these two Petri dishes was an unusually lengthy process. To avoid an over-interpretation of this experimental issue, an average 24-h-reproductive rate was therefore preferred to two repeated, individual rates for analyses. Starter aphids that developed on plants with a high leaf appearance rate per plant were more fertile, than peers on plants with lower leaf

appearance rates (+3.95 ± 1.11 more offspring·day^{-1} for 0.10 leaf·day^{-1}, $p = 0.001$; log-likelihood of the effect: $\chi^2_{[1]} = 13.63$, $p < 0.001$). The PG-E groups explained a significant part of the variance too (likelihood ratio PG-E $\chi^2_{[7]} = 18.32$, $p = 0.011$; model $R^2_c = 0.493$). Post hoc tests indicated that aphids raised on S-CT symbioses produced significantly fewer offspring per day than aphids that developed on N-CT plants with the same, average leaf appearance rate (0.16 leaves·day^{-1}·plant^{-1}; Table 3.11).

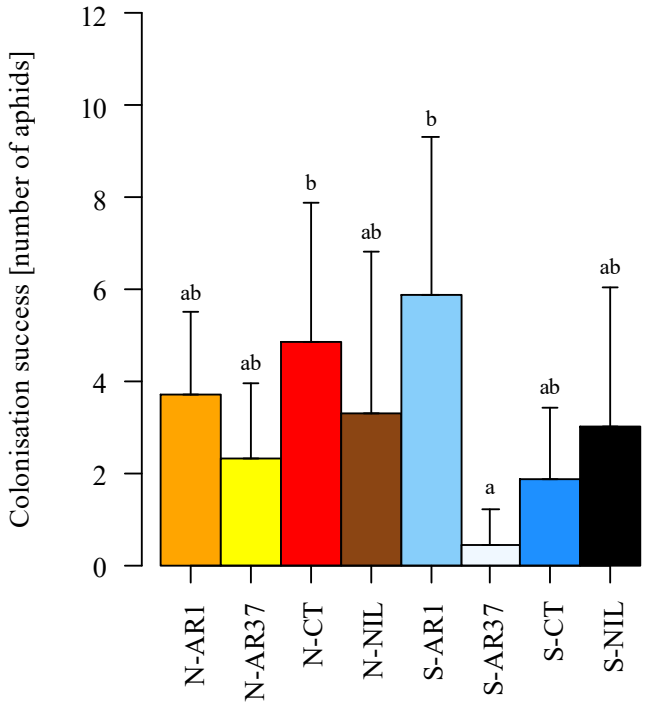


Figure 3.8. Colonisation success (mean + SD) of *A. lentisci* neonates on perennial ryegrass plants with different plant genotype (N or S) and endophyte status (AR1, AR37, CT or NIL) in the Mature plant experiment, as assessed 9 to 12 weeks after aphid placement. Groups with the same letters were not significantly different ($p > 0.05$).

Table 3.11. *Ex planta* reproductive rate of starter aphids from the Mature plant experiment raised on two plant genotypes (N or S) without endophyte [NIL] or with AR1, AR37 or CT endophytic partner.

Plant genotype	Endo-phyte	n	RAW DATA				MODEL
			Daily rate[1]		Correlation D1 - D2		Average daily rate[2]
			D1	D2	$\rho_{Spearman}$	(*p*-value)	Mean ± SD
N	AR1	19	7 [ab]	7 [ab]	0.62	(*0.005*)	6.43 ± 3.79 [ab]
	AR37	7	8 [ab]	3 [ab]	-0.04	(*0.005*)	7.62 ± 3.48 [ab]
	CT	27	8 [b]	7 [b]	0.56	(*0.002*)	8.15 ± 4.46 [b]
	NIL	16	4 [ab]	5 [ab]	0.56	(*0.026*)	6.55 ± 4.50 [ab]
S	AR1	30	8 [b]	6 [ab]	0.66	(*< 0.001*)	5.22 ± 4.85 [ab]
	AR37	2	5 [ab]	4 [ab]	-	-	3.91 ± 2.92 [ab]
	CT	9	3 [a]	3 [a]	0.19	(*0.623*)	2.40 ± 3.50 [a]
	NIL	17	4 [a]	10 [b]	0.19	(*0.455*)	5.62 ± 4.68 [ab]

n: number of mothers observed (starter aphids);

[1] Median number of offspring produced by starter aphids 0 to 24 h (D1) and 24 to 48 h (D2) after being placed in microcentrifuge tubes; daily rates with the same letter were not significantly different ($p > 0.05$; Dunn test with Benjamini-Hochberg *p*-value adjustment)

[2] Mixed-effects model predictions on plants developing one new leaf every 6 days, on average; average daily rates with a same letter were not significantly different

3.3.4. Offspring survival in follow-up experiments

At 17 to 20 °C, the median offspring survived for about one week without food. A few lived for more than three weeks (Figure 3.9). There were too many confounding variables in the Biology II follow-up experiment to determine whether plant genotype-endophyte (PG-E) groups had *per se* an effect on survival, even if significant interaction effects were observed between PG-E and maternal age, or PG-E and reproductive rate RR (Table 3.12). The Mature plant follow-up experiment suggested that PG-E effects may exist under more standardised conditions (Table 3.13): offspring of mothers raised on S-NIL plants survived significantly longer than offspring from most other groups, for example. The difference between the S-NIL and the S-AR37 group was the only statistically not significant difference (Figure 3.9), an observation presumably due to the small number of mothers and offspring in the S-AR37 group, and the variability in the offspring survival in this group.

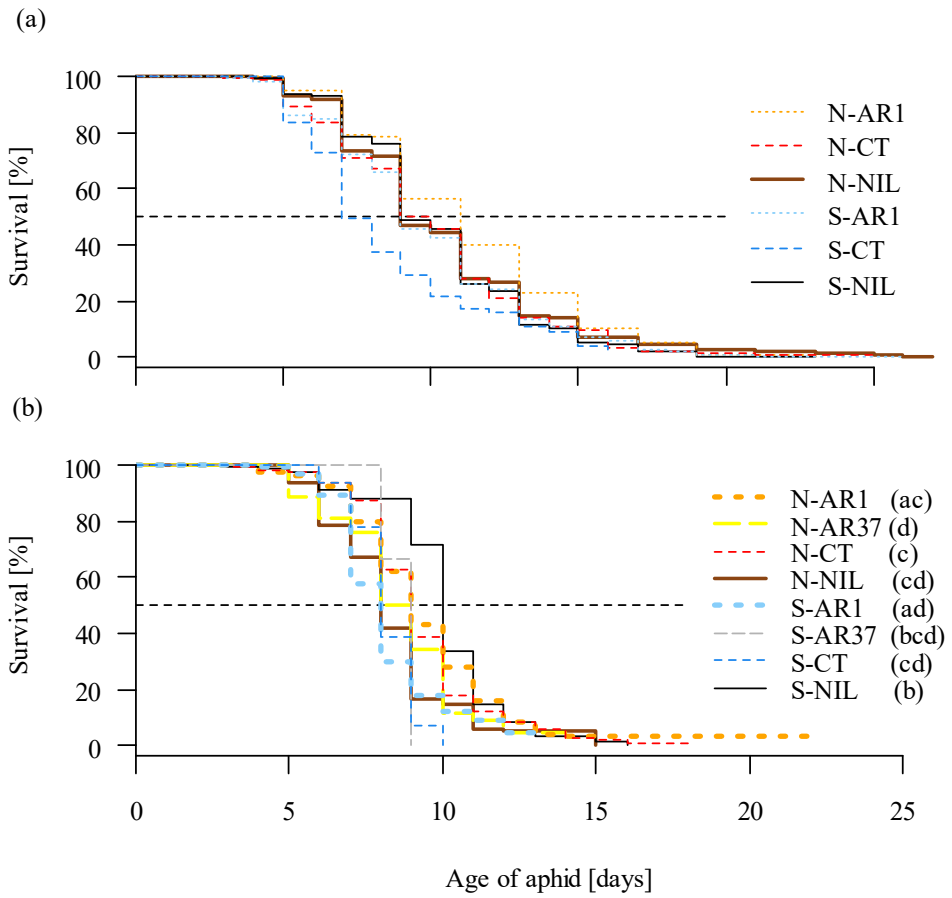


Figure 3.9. Offspring survival in the Biology II follow-up experiment (a) and the Mature plant follow-up experiment (b). Groups with the same letters in brackets were not significantly different at average covariate values ($p > 0.05$).

In both follow-up experiments, offspring survival was strongly influenced by various maternal characteristics and the quality of the maternal plant. In the Biology II follow-up experiment, offspring survival decreased significantly with the age of the mother in the S-AR1 and S-CT groups, for example (Table 3.12). In the S-AR1 and N-AR1 group, the offspring of fecund mothers lived significantly longer than the offspring of less fecund mothers. Offspring from mothers that were raised on old plants died significantly earlier than peers from mothers raised on young plants ($p = 0.008$, Table 3.12). In the Mature plant follow-up experiment, fecund mothers also produced longer-lived offspring than less fecund mothers ($p < 0.001$; Table 3.13). Some of this effect may be due to a cohort size effect. As all live offspring of a cohort were placed in one microcentrifuge tube, tubes from fecund mothers contained generally higher neonate densities per tube. Models with maternal fecundity were always better, than models

including the number of offspring per microcentrifuge tube (Table 3.3), however, suggesting that fecundity had an influence on offspring survival nevertheless. Mothers that developed on vigorously growing plants (plants with a fast leaf regrowth 24 h after the initial trim; Table 3.12) produced significantly longer-lived offspring, than mothers on plants with less leaf regrowth 24h after the initial trim.

Table 3.12. Factors affecting offspring survival in the Biology II follow-up experiment.

Variables		**coef**[1]	**se(coef)**	**z**	**p-value**	**L ratio**	
						χ^2	**p-value**
Number of green leaves at aphid placement		0.003	0.064	0.04	0.970	0.00	0.970
Age of plant at aphid placement		0.005	0.002	2.67	0.008	6.92	0.009
Age of mother at collection (Age)		0.006	0.006	0.99	0.320	-	-[2]
General reproductive rate (RR)		-0.116	0.230	-0.51	0.610	-	-
PG-E						-	-
	N-AR1	1.752	1.033	1.70	0.090		
	N-CT	0.929	0.850	1.09	0.270		
	N-NIL	-0.088	0.576	-0.15	0.880		
	S-AR1	0.979	0.677	1.45	0.150		
	S-CT	-1.623	1.219	-1.33	0.180		
Interaction Age×PG-E						12.3	0.031
	N-AR1	0.000	0.012	0.02	0.980		
	N-CT	-0.006	0.016	-0.35	0.730		
	N-NIL	-0.001	0.009	-0.08	0.940		
	S-AR1	0.034	0.012	2.83	0.005		
	S-CT	0.051	0.025	2.05	0.040		
Interaction RR×PG-E						15.6	0.008
	N-AR1	-1.795	0.878	-2.05	0.041		
	N-CT	-0.190	0.382	-0.50	0.620		
	N-NIL	0.109	0.332	0.33	0.740		
	S-AR1	-1.114	0.388	-2.87	0.004		
	S-CT	1.358	1.200	1.13	0.260		
Random effects (Mother identity)	Intercept					37.7	< 0.001
	Slope (Age)					19.8	< 0.001

coe: model coefficient; se(coef): standard error of model coefficient; L ratio: log-likelihood ratio; PG-E: plant genotype (N or S)-endophyte status (AR1, AR37, CT, NIL) of the maternal plant.

[1] A positive coefficient indicates that the variable reduced the survival, a negative that the variable increased the survival.

[2] The likelihood ratio of main terms with interactions cannot be estimated as they cannot be removed from the model without removing the interaction term too.

Table 3.13. Factors affecting offspring survival in the Mature plant follow-up experiment[1].

Variables		coef[1]	se(coef)	z	*p*-value	L ratio	
						χ^2	*p*-value
Regrowth 24h		-0.624	0.275	-2.27	0.023	4.98	0.026
Recent reproductive rate		-0.048	0.014	-3.50	0.000	11.19	< 0.001
Collection period	from 24-48 h	0.965	0.069	14.0	0.000	195.00	< 0.001
PG-E group						19.28	0.007
	N-AR1	0.968	0.342	2.83	0.005		
	N-AR37	1.827	0.468	3.90	0.000		
	N-CT	0.928	0.340	2.73	0.006		
	N-NIL	1.483	0.424	3.50	0.000		
	S-AR1	1.540	0.334	4.61	0.000		
	S-AR37	0.410	0.708	0.58	0.560		
	S-CT	1.249	0.424	2.94	0.003		
Random effects:	Plant identity					5.12	0.024
	Mother identity					105.70	< 0.001

coe: model coefficient; se(coef): standard error of model coefficient; L ratio: log-likelihood ratio; PG-E: plant genotype (N or S)-endophyte status (AR1, AR37, CT or NIL) group; Regrowth 24h: length of the first leaf regrown in the first 24 h after the initial tiller trim.

[1] A positive coefficient indicates that the variable reduced the survival, a negative coefficient that the variable increased the survival.

An unexpected, interesting experimental influence was observed in the Mature plant follow-up experiment: root aphids born to a mother during the second collection period (24 to 48 h) died significantly earlier than their siblings born in the first 24h ($p < 0.001$, Table 3.13).

3.4. Discussion

Establishment and colonisation success, growth and development, reproductive performance and offspring fitness of root aphid *A. lentisci* on perennial ryegrass roots without and with endophytes were examined in this chapter, considering the possible impact of plant genotype and various plant biomass parameters.

The establishment success of > 75% achieved on endophyte-free perennial ryegrass in both the Biology I and II experiments was high, compared to the < 20% previously reported for *A. lentisci* neonates on barley and wheat seedlings (Wool & Kurzfeld-Zexer, 2008). It is possible that mature perennial ryegrass roots were more favourable in terms of root anatomy and/or physiology for establishment than the roots of young crop seedlings (see root usage and size discussion in Chapter 5, for example). Alternatively, other experimental aspects could have helped the starter nymphs of the Biology I and II experiments to establish, such as the comparatively drier environment of the agar-embedded roots. Condensation droplets were observed more than once to be a deadly trap for neonate root aphids (Appendix 7). Based on their observation of root age preferences in immature root aphids Popay and Cox (2016) hypothesised that actively growing plants would likely promote the development of root aphid populations. The positive effect of a large number of green leaves on the aphid establishment success during the Biology II experiment supported this view. Whether the two plant genotypes observed had an influence on the establishment success will need assessing in an experimental context with fewer confounding variables, in particular without large variations in plant age at aphid placement. The observed endophyte effect that aphids were significantly less likely to establish on AR37 symbionts, than on the alternatives (Biology II experiment), was consistent with previous observations by Popay and Cox (2016).

The colonisation successes of 30 to 71% achieved on endophyte-free plants in the Biology I, Biology II and Mature plant experiments corroborate what has been reported for *A. lentisci* too (Wool & Kurzfeld-Zexer, 2008). The reasons were likely the same as for the establishment success. Significant interactions by plant genotype-endophyte status group were detected for this variable in the Mature plant experiment. Aphids placed on S-AR37 symbioses were less successful in developing to colonies, than aphids on S-AR1 and N-CT plants in particular. Toxins produced in AR37 symbioses may partly explain the reduced colonisation success (Popay & Cox, 2016). However, biomass aspects may

also have had an effect. S-AR1 and N-CT symbioses had marginally faster growing shoots than S-AR37, and may thus have provided more food to the root aphids, considering that green shoot area growth and final root biomass were significantly correlated in the Mature plant experiment.

As with most aphid species (Dixon, 1973), *A. lentisci* moulted four times to maturity. Although the size of individual aphids increased at each ecdysis, the body length, abdominal width and their derived EP measurement did not differentiate between late instars (Table 3.5). These measurements are thus only conditionally useful when a classification has to be undertaken. If sizes in the field can be presumed similar to the ones reported during the Biology I experiment, some common criteria to separate mature from immature aphids could need reconsideration. The criterion 'mature if > 1 mm body length' (Popay & Cox, 2016) would indeed over-estimate the reproductive population of a sample considering that 21, 85 and 85% of all 2nd, 3rd and 4th instar specimen observed exceeded this critical body length.

Two aspects complicated adult root aphid size analyses and results interpretation. Firstly, many root aphids grew further after the final moult and the first viviposition (Figure 3.3, Figure 3.5). Secondly, two clearly defined groups of adult size, 'Large' and 'Small', were observed in both Biology I and Biology II experiments. 'Large' aphids grew faster to a wider body frame than 'Small' aphids but receded in size towards the end of their adult life. Their predicted adult body length (Appendix 10, Tables A10.2.2.1. and A10.4.2.1) lay within the range reported in the literature for *A. lentisci* [e.g. 1.6 ± 1.1 mm (Podmore, 2015), 1.1 to 3.0 mm (Blackman & Eastop, 2000)]. By contrast, adults in the Small group maintained or slightly expanded their size up to their death. Their final adult length was below the typical range reported by Blackman and Eastop (2000). A figure by Schuett *et al.* (2015) [Figure 4; Schuett *et al.*, 2015] suggested that the observed growth dichotomy may also exist in the pea aphid *Acyrthosiphon pisum* Harris. Further investigations will be necessary to gain insights into this phenomenon and its biological consequences. A link to aphid fecundity was obvious in both trials considering aphid size, however. As fecundity increases with body size or/and weight in many insect species (Honěk, 1993), this observation could simply confirm that 'Large' aphids were generally more fecund than 'Small' ones. A part of an aphid's reproductive potential is determined

before its birth, however (ovariole number; Section 1.2.4.3), and developing embryos are already present in an aphid long before it achieves maturity [telescoping generations (Kindlmann & Dixon, 1989)]. Thus, if 'Large' sizes were at least partly the result of the presence of many embryos, the two distinct growth patterns could have a "mechanical" explanation. Alternative explanations could involve possible modifications in abdominal structures with advancing age.

Approximately 90% of the live adult size measurements conformed to the literature ranges for dead, mounted and thus possibly somewhat extended *A. lentisci* specimens (Blackman & Eastop, 1984; Cottier, 1953). Smaller adult sizes in the biology experiment I compared to the Biology II experiment (no aphid over 1.5 mm^2 EP, marginally lower average body size; Appendix 10, Section A10.4.4) may have been the result of high temperatures in the glasshouse (Dixon, 1984), or lower feed quality as the agar used in the Biology I experiment contained only a fraction of the nutrients provided to plants in the Biology II experiment (Section 2.3.1.3). That 'Large' aphids were associated with healthier plants in the Biology II experiment (Section 3.3.2.1) supports the latter explanation. The smaller adult size and the absence of large specimens in S-CT plants (Figure 3.6) may be explained by plant genotype-endophyte interactions. *Rhopalosiphum padi* L., for example, developed smaller adults on *Lolium multiflorum* Lam. with endophyte *Epichloe occultans* Schardl., than on NIL *L. multiflorum* plants (Bastias *et al.*, 2017). On *L. perenne* with *E. festucae* var. *lolii* symbiont, *R. padi* showed no significant reaction in this regard, however (Meister *et al.*, 2006). More research is needed to confirm this first report of a reduced growth response of root aphids in the presence of specific endophyte symbionts in their host plant.

The three to four weeks developmental time required by neonates to achieve reproductive maturity fell into the range reported by Wool and Sulami (2001) (< 1 month), but was longer than that recorded by Wool and Kurzfeld-Zexer (2008). While the checking schedule of the Biology I experiment likely masked any neonate size effects, the findings in the Biology II experiment agreed with the general observation that larger neonate aphids mature significantly earlier than smaller ones (Dixon, 1984). Plant characteristics influenced the development time too. However, an interpretation of the observed effects is challenging, considering various experimental and environmental

effects that likely interfered with aphid development [e.g. heat, possible water and nutrient stress for the green shoot biomass in the Biology I experiment; possible water stress, root lignification and various maternal origins of the neonates for the plant age at placement during the Biology II experiment]. In contrast to the observations by Meister *et al.* (2006) for *Metopolophium dirhodum* Walker and *R. padi* L on perennial ryegrass infected with a common-toxic strain of *E. festucae* var. *lolii*, time to reproductive maturity in the Biology II experiment was delayed in presence of some endophytes. The effect depended on the genotype of the plant the endophyte was associated with, however, and would need to be confirmed on a large number of aphids.

Long-lived aphids and the plants they dwelled on were exposed to heat stress towards the end of the Biology I experiment, when the temperatures in the glasshouse reached 38.0 to 50.2 °C for 0.2 to 3.7 $h \cdot day^{-1}$ on 14 occasions (Enders & Miller, 2016; Hannaway *et al.*, 1999; Skaljac, 2016). Handling was also a frequent cause of death. While this may explain the failure to fit an explanatory model to the mortality data of the Biology I experiment, it also suggested the longevity observed in the Biology II experiment was the better estimate for the potential lifespan of *A. lentisci*. With a median longevity of over two months at 17 to 18 °C (Figure 3.7, d) for an aphid on endophyte-free plants, *A. lentisci* was clearly a longer-lived aphid species than other root aphids and in general, above-ground feeding aphids (Kuo *et al.*, 2006; National Pesticide Information Center, 2017; Tsai & Liu, 1998). The longevity of *A. lentisci* decreased significantly in the presence of endophytes during the Biology II experiment, a finding in line with observations for *R. padi* on *L. perenne-E. festucae* var. *lolii* (Meister *et al.*, 2006) and *L. multiflorum-E. occultans* symbioses (Bastias *et al.*, 2017). The data of the Biology II experiment indicated that the effects of AR37 and CT endophytes were possibly mitigated on plants with a larger number of green leaves. Because this plant trait is influenced by many factors (plant genotype [Chapter 4], plant age, water stress etc.) and influences, in turn, many other aspects (e.g. alkaloid production and distribution), its significance will need closer examination in further trials.

The average aphid lifetime fecundity recorded in the Biology I experiment matched that reported in the literature (Wool & Kurzfeld-Zexer, 2008) but was likely lower, than it could have been due to the mothers dying early through handling and heat stress. The

nutrient stress experienced by the plants during the Biology I experiment could also have limited the reproductive potential of the root aphids. As for the longevity, the findings of the Biology II experiment were therefore considered a more accurate estimate of *A. lentisci*'s fecundity potential. These were in the range observed for aphids in general (National Pesticide Information Center, 2017). Experimental bias rendered any analysis of the lifetime fecundity difficult. Besides, the total lifetime fecundity of an aphid could be less relevant in the field than its reproductive rate in the first days, considering that natural agents are likely to result in an untimely death for many aphids (Adams & van Emden, 1972). The reproductive rate appeared, therefore, a better response variable to analyse in the present thesis. With average daily rates of 2 to > 8 offspring·mother^{-1}·day^{-1}, the mothers kept *ex planta* (Mature plant experiment; Table 3.11) were significantly more fecund than the ones left to reproduce on plant roots (Biology I and II experiments, ~ 1 offspring·mother^{-1}·day^{-1}; Table 3.9). This difference could have been the result of a large body size developed in the cold outdoor conditions (Dixon, 1985). Large body sizes are known to be positively linked to daily reproductive rates in root aphids (Biology II experiment; Table 3.10) as well as in many other aphid and insect species (Adams & van Emden, 1972; Honěk, 1993; Schuett *et al.*, 2015; Traicevski & Ward, 2002). The sudden exposure to a warmer laboratory environment (19.6 ± 2.0 °C) could have caused or contributed to the higher reproduction rate, by accelerating all biological processes, embryo development and viviposition included. It is further possible that this was simply a reaction to starving. Accelerated maturation of the largest embryos has been observed in several aphid species when starved [e.g. *Megoura viciae* Buckton, *Aphis craccivora* Koch (Traicevski & Ward, 2002; Ward & Dixon, 1982). Actual starvation experiments on *A. lentisci* with imago dissection will be needed to confirm this explanation, however. Root aphids developing on young, strongly growing plants are likely more fecund as adults than aphids on plants of poorer quality, considering the decreasing reproductive rates on ageing plants and the positive effect of plants with high leaf appearance rates observed in the Biology II and Mature plant experiments, respectively. Which plant traits would *per se* be pivotal for root aphids could not be assessed from the data at hand, however. The reproductive response to specific endophytes can vary by aphid species (Meister *et al.*, 2006). *A. lentisci* appeared to respond similarly to *R. padi* (Bastias *et al.*, 2017; Meister *et al.*, 2006), with smaller reproductive rates in S-CT endophyte symbioses in particular (Table 3.10, Table 3.11). The difference between S-CT and other plant

genotype-endophyte strain combinations was not always significant, however, an observation that in the specific case of the Mature plant experiment could have been related to seasonal endophyte development and activity patterns. During winter, endophyte hyphae numbers and alkaloid concentrations are indeed known to decrease (Christensen & Voisey, 2007; Hennessy *et al.*, 2016; Hume & Cosgrove, 2005).

Offspring of more fecund mothers were larger (Table 3.8) and lived longer (Table 3.12, Table 3.13) than offspring of mothers with a low reproductive rate, supporting previous findings on aphids (Dixon, 1985, 1987; Traicevski & Ward, 2002). Maternal age also appeared significant for offspring fitness in the Biology II experiment only. Traicevski and Ward (2002) reported that nymphs produced late in a mother's life tended to be larger than earlier-born offspring in several aphid species. This pattern was observed in the S-NIL group of the Biology II experiment only, however. In all other groups, the opposite effect prevailed ['Lansing effect' (Zehnder *et al.*, 2007)]. These size changes could possibly explain the reduced offspring survival observed in neonates born to old mothers on S-CT and S-AR1 plants during the Biology II follow-up experiment. Decreasing plant quality during the experiment or maternal age effects (Zehnder *et al.*, 2007) could also have had an effect. Young plants with good growth were beneficial for offspring fitness (Age effect for Biology II offspring in Table 3.12, 24-h-leaf regrowth for offspring on mature plants in Table 3.13). Plant genotype-endophyte groups appeared generally not to affect offspring survival (i.e. fitness) in the Biology II follow-up experiment. Only offspring of fecund mothers on AR1-infected plants lived longer than offspring of fecund S-NIL mothers. S-NIL-born neonates during the mature plant follow-up experiment were exceptionally long-lived compared to all other groups, however. Why this was so is unclear.

During the mature plant follow-up experiment, an unexpected variable of experimental bias was evident, the 'collection period'. Aphids born in the second viviposition period were clearly shorter-lived than aphids born in the first one (Table 3.13). That could be an effect of maternal food deprivation, a phenomenon that has not yet been documented in root aphids. Whether such an effect would be observed in the field too by food withdrawal [e.g. when a herbicide is sprayed to eliminate an old sward before sowing a new pasture (Trafford & Trafford, 2011)] could be worth investigating.

3.5. Conclusion

The root aphid *A. lentisci* completes one generation in three to four weeks on the roots of endophyte-free *L. perenne* plants at average temperatures of 17 to 21 °C. Adults can live for over 3 months and produce an average of 40 to 70 offspring up to their death. Plant genotypes and endophytes, either alone or in associations (PG-E groups), affect aphid parameters in various ways, such as establishment and colonisation success, aphid growth, body size, survival and fecundity. AR37 causes significant mortality of the first instar nymphs but its effect on adult size and fecundity is as yet uncertain, for want of specimens to examine. Aphids living on S-CT symbioses develop more slowly, die earlier and are less fecund than those fed on S-NIL plants. Not all of these effects were observed when the endophyte was associated with another plant genotype (N-CT). Aphids raised on plants with AR1 endophyte showed no enhancement of any biological trait, with the possible exceptions of an improved colonisation success (S-AR1) and offspring fitness in fecund mothers. In a nutrient-sufficient context, strong shoot growth and healthy young plants have a generally positive effect on aphid establishment, development and reproduction. A larger number of plant genotypes need examining under more "standardised" environmental conditions to determine the significance of these findings for the development of pest management programmes.

CHAPTER 4: EFFECTS OF ROOT APHIDS ON PERENNIAL RYEGRASS PLANTS

4.1. Introduction

Aphids can impair plants both directly, by damaging vascular tissues and causing chloroses and necroses on infested plants, and indirectly through transmission of viruses and reducing the energy and nutrients available to the plant (Dixon, 1973; Saheed *et al.*, 2007). In a natural ecosystem, aphid effects may be even more complex. Aphid infestation can, for example, modify the amino acid composition of plant exudates (Hoffmann, 2016). This may boost the microbial activity in the rhizosphere, resulting in increased mineralisation and a locally higher nutrient availability (de Parseval *et al.*, 2017). Aphid feeding and exogenous application of honeydew may furthermore interfere with the plant's general defences against herbivores (Hoffmann, 2016).

Large populations of root aphid *Aploneura lentisci* Pass. have been associated with small, weak, pale plants with dead material around the base (McDonald *et al.*, 2011). In comparisons between perennial ryegrass plants (*Lolium perenne* L.) protected from root aphids by the AR37 endophyte strain of *Epichloë festucae* var. *lolii* and unprotected plants, Popay and Cox (2016) found that root aphids reduce the survival of tillers by approximately 35% and the foliar growth by 16 to 27%. A quantification of the damage to plants of the same genotype with vs. without root aphids, or an estimation of *A. lentisci*'s damage in relation to aphid numbers has not yet been reported, however. Although genetic effects of plants on root aphids have been observed in AR1-infected plants, in particular (Popay & Cox, 2016), it is not known whether a reverse pattern exists too [i.e. whether some plant genotypes, endophytes or plant genotype-endophyte symbioses respond differently than others when fed upon by a controlled number of root aphids]. The ability for a plant to cope with the demand imposed by root aphid feeding [tolerance; de Wet and Botha (2007)] is a desirable trait in plants and plant-endophyte associations because it raises the level at which economic injury occurs (Smith & Chuang, 2014). Tolerance traits are further believed to elicit pest resistances to a lesser degree than antibiotic or antixenotic (non-preference) plant traits as they do not interfere with the physiology or behaviour of a pest (Koch *et al.*, 2016; Smith & Chuang, 2014). Because the interaction between the plant and endophyte changes over time and may be influenced

by plant age (Fuchs *et al.*, 2017b), two experiments were performed, one on seedlings (Seedling experiment, in a climate chamber) and one on mature tillers of 'clone-plants' (Section 2.3.1.1; Mature plant experiment). In each experiment, half of the plants were supplied with a group of young root aphids, which were left on the plants, while the other half remained aphid-free. The plant parameters recorded on both aphid-free and aphid-infested plants were then compared, with allowances being made for the effect of plant precondition. The following questions were considered:

1) Which plant parameters are affected by root aphid feeding?
2) How large is the effect on the respective plant parameters?
3) How do specific plant genotypes and fungal endophytes influence the aphid-plant interaction?

4.2. Materials and methods

4.2.1. Seedling experiment

Perennial ryegrass seeds of four accessions of cultivar 'Grasslands Samson' [Section 2.3.1.1, Table 2.4; three with endophyte (E^+; A14559, A16863, A12421 with AR1, AR37 and CT endophyte strains, respectively) and without endophyte (E^- or NIL; A11104)] were germinated by placing 4 × 25 seeds/accession in unvented Petri dishes (9.0 cm diameter × 1.5 cm height; Thermo Fisher Scientific New Zealand Ltd., Auckland, New Zealand) on three layers of sterilised filter paper (LabServ®, 90 mm diameter, Thermo Fisher Scientific New Zealand Ltd., Auckland, New Zealand) moistened with 6 ml of sterile RO-water. The seed-loaded Petri dishes were incubated in a Percival climate chamber at 19.0 ± 0.5 °C and 12 h light per day (Section 2.2.4). They were checked daily for seed germination and re-watered with 1 ml RO-water as needed. Seedling groups of up to twenty seedlings from each germination Petri dish were transferred onto a 4.5 cm-thick layer of modified Bollard's medium (MBM agar; Appendix 6) on the day they germinated, and were embedded there as described in Section 2.3.1.3 (Tube embedding; Figure 2.3), i.e. by placing the seedlings individually in a row behind each other in 3 cm interspaced notches and isolating them from each other by pressing both-end opened centrifuge tubes [50 mL; LabServ®, Thermo Fisher Scientific New Zealand Ltd; prepared with Insect-a-Slip – Fluon® (BioQuipProducts, Inc., Rancho Dominguez, California, U.S.A)] into the agar to the bottom of the tray (Figure 4.1a). A total of 64, 75, 53 and 51 seedlings (i.e. genotypes) were mounted in this way for accessions A14559, A16863, A12421 and A11104, respectively, in 19 randomly arranged seedling groups. The seedlings were then covered with a 1.5 cm-high layer of autoclaved C.A.N. bark dusted with perlite (Figure 4.1a; Appendix 5).

Four to seven days after the seedling transfer, three newborn aphids (≤ 1 day old, born in glass Petri dishes to mothers collected outdoors, Section 2.3.2) were placed on a spoon with a fine brush and introduced onto every other seedling from the top by gently tapping the spoon over the plant (every second seedling was left aphid-free). Aphids and seedlings were harvested 23 to 33 days later, by transferring the tubes and their content into a black plastic tray (Figure 4.1c), spraying tap water onto the roots (Figure 4.1b) and collecting all floating aphids. The washing water was poured into a larger container (Figure 4.1c, square blue container) and more tap water was sprayed onto it to separate

the remaining root aphids from agar, bark and perlite. The process was repeated a third time in a larger container (Figure 4.1c, white container) before the water was discarded. The collected aphids were divided into three age groups (early 1st instar, older instar, and adult) under a stereo microscope at magnification 60× and counted. Seedlings were recorded as water stressed if the agar cone left in the tube was small and difficult to separate from the roots at the harvest. The survival of each seedling ("dead" in absence of green shoot parts, "alive" otherwise), and its numbers of tillers, leaves and roots were further recorded before severing all tillers at their base and blotting them to confirm the endophyte presence (Section 2.3.3). Directly after blotting, shoot and roots of a seedling were wiped dry, weighed and frozen. After freeze-drying (approx. 60 h in a Labconco Freezone Plus model 7960011, Labconco Corporation, Kansas City, MO, USA), the dry biomass of the seedling was re-weighed.

Figure 4.1. Seedling experiment. (**a**) Mounted seedlings; (**b**) Spray bottle with water used to separate aphids from the medium at final harvest; (**c**) final harvest, washing out trays.

4.2.2. Mature plant experiment

This trial was conducted in an insectary, on a bench 79 cm above ground, in the plant nursery of AgResearch Ltd., Grasslands Research Centre, Palmerston North (Section 2.2.2) during winter to early spring 2015 (approximately 11 h daylight/day and 10.1 ± 4.6 °C mean temperature). The plants used were mature perennial ryegrass plants generated in a previous project by Simpson *et al.* (1997), vegetatively propagated since then and kept in a glasshouse for more than 2 years before the experiment (Section 2.2.3). Two ryegrass genotypes ('N', from cultivar Grasslands Nui and 'S', from cultivar

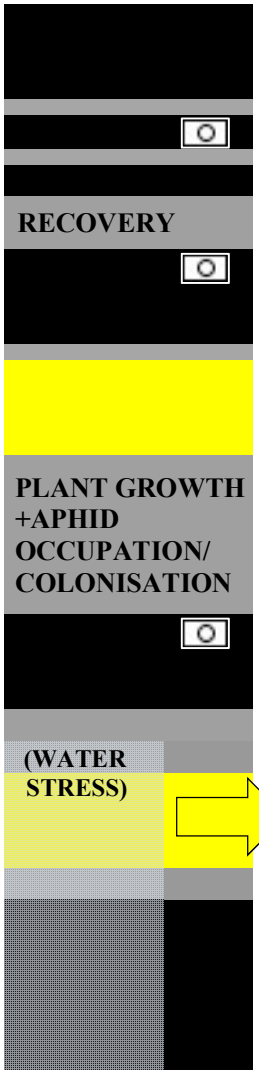
Grasslands Samson; Section 2.3.1) with one of three endophyte strains of *E. festucae* var. *lolii* (AR1, AR37, or CT), or without endophyte (NIL) [four endophyte statuses] were used, i.e. eight plant genotype-endophyte (PG-E) treatments: N-AR1, N-AR37, N-CT, N-NIL, S-AR1, S-AR37, S-CT and S-NIL. Handling of plants and data collection were performed as described in Figure 4.2. For each PG-E treatment, 20 tillers were split off 1-3 mother plants, a fact accounted for in statistical analyses with the random grouping factor for maternal plant identity. The tillers were cut back to 4 cm shoot length and 0.3 cm root length. Adventitious roots were severed at their base (initial plant trim; Chapter 2, Figure 2.4a and c). Five of these tillers were weighed fresh, freeze-dried and re-weighed dry to estimate the dry matter content of the plant tissues at the beginning of the experiment. The 15 remaining tillers of each PG-E group were placed in a climate chamber at 17 to 18 °C, in 20 to 30 mL tap water for 24 h, photographed (Figure 4.2), embedded in unvented Petri dishes filled with 55 to 60 g MBM agar (Section 2.3.1.3; Figure 2.2), and maintained henceforward in the insectary.

Adult root aphids were obtained from 80- to 108-day-old colonies on a set of plants, identical to the Mature plant experiment plants, that had been set-up three to four months earlier. These adults were individually held for 12 to 24 h in 1.5 mL microcentrifuge tubes to produce the neonates to place on the Mature plant experiment plants. Cohorts of 10 neonates in their 2nd to 3rd day of life were placed on 7 experimental plants of the same PG-E group as their mothers (one cohort of 10 per plant), 31 to 40 days after the initial plant trim. The seven to eight other experimental plants of each PG-E group were left aphid-free. All root aphids were harvested 63 to 79 days later (plant age 98 to 108 days), with a fine paintbrush, under a stereo microscope at magnification 40×. The aphids were classified as either 'adult', 'older immature instar' or 'early 1st instar' and counted. MBM agar was added as needed (0 to 45 g) before re-closing the Petri dishes and placing the plants again into the insectary until final plant harvest (Section 2.4.2). If the agar layer within the Petri dish was < 2 mm high at aphid collection or at final harvest, the plants were recorded as having possibly been water stressed.

A Sony digital TTL camera (α35, DT18-55mm F3.5-5.6 SAM) with external TFT display in an imaging chamber with 12V LED strip lighting was used to photograph plants (Section 2.4.2). The 24-h-recovery pictures were used to measure regrowth length of the extending leaf (Regrowth 24h) and initial tiller diameter [ImageJ 1.46r (Rasband, 1997-

2016); Section 2.4.2]. Pre-condition and 2nd growth assessment photographs were processed in Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, U.S.A.) as described in Section 2.4.2., and analysed by Chris Hunt's MATHWORKS© MATLAB R2013A program (MathWorks, Inc., Natick, MA, U.S.A.; Appendix 8) to obtain the green shoot area (GSA) and colour measurements (R, G and B values). After the final plant dissection, the shoots were stored at -18°C and the roots preserved in 50% ethanol in the laboratory at room temperature until final freeze-drying and consecutive dry biomass weighing.

Endophyte presence/absence was confirmed by immunoblot at start (initial trim) and at final dissection, 4 and 0 cm above the tiller base, respectively (Section 2.3.3).



Start
⇨ Trim and weigh tillers, keep them in water in the climate chamber
⇨ Immunoblot of remnant tiller piece

24-h-recovery: photographs of tillers 24 h after trim

Embedding: tillers mounted into agar

Precondition assessment (Plant age: 28-31 days)
⇨ Take photographs for green shoot area (GSA T1) and colour
⇨ Precondition for shoot: Tillers + leaves count

Aphid placement (Plant age: 31-40 days)
⇨ Precondition for roots: root count
⇨ 10 aphids in 2-3rd day of life placed per PD, on 50% of the plants

2nd growth assessment (Plant age: 78-84 days)
⇨ 2nd set of photographs for green shoot area (GSA T2) and colour
⇨ After-condition: tiller and leaf count

Aphid harvest (Plant age: 98-108 days)
⇨ Collection and counting of aphids in PD (microscope)
⇨ Agar addition (if needed)

Final plant harvest and plant dissection (Plant age: 112-121 days)
⇨ Final condition: tiller, leaf and root count
⇨ Dissection to green shoot, senescing/dead shoot (S&D), roots
⇨ Immunoblot on tiller base
⇨ Fresh and dry matter weighing of green shoot, S&D and roots

Figure 4.2. Schematic representation, diverse data collection time points and water stress in the Mature plant experiment. Plant photographs: ; PD: Petri dish

4.2.3. Data analysis and statistics

All data were analysed and graphically represented with the statistics program R 3.3.3 (R Core Team, 2017). Two types of variables were considered to analyse the aphid effect: a binary aphid exposure factor ('aphid presence'; "yes" if at least one adult or older immature aphid was harvested off the plant at harvest time, "no" otherwise) and aphid number covariates (either the number of adult aphids at harvest time or the number of feeding aphids, i.e. adults and older instar nymphs at harvest time). Model fitting was performed including all covariates, factors and their interactions into an initial model, and then removing stepwise all terms that did not significantly improve the model ($p > 0.05$ for the log-likelihood ratio when a model with the term was compared to a model without it) to achieve minimal AIC scores by an acceptable residual distribution. The residual distribution was assessed graphically with the package 'predictmeans' (Luo *et al.*, 2014). Normality assumptions were verified by a Shapiro-Wilk test ['stats' R package (R Core Team, 2017)] and homoscedasticity assumptions by Levene's test ['car' R package (Fox & Weisberg, 2011)]. Analyses of co-variance were performed using type III sum of squares ('car' R package). Post-hoc tests on final models were performed with the 'predictmeans' package, adjusting the *p*-value with the Benjamini-Hochberg procedure by multiple comparisons. R^2 values were calculated by the 'stats', 'MuMIn' and 'rsq' packages (Barton, 2018; R Core Team, 2017; Zhang, 2018).

The germination rates of different accessions in the Seedling experiment were compared by Fisher's exact test ['stats' R package (R Core Team, 2017)] summing the counts of the four Petri dishes of each accession. The survival of seedlings in presence vs. absence of aphids (aphid treatment, 3 vs. 0 neonates placed) was analysed by a Cochran-Mantel-Haenszel test with continuity correction, stratifying the data by accession ['stats' R package (R Core Team, 2017)]. A post-hoc test with Benjamini-Hochberg *p*-values adjustments was performed with 'rcompanion' (Mangiafico, 2018), and differences in plant survival between accessions were then analysed by a Fisher's exact test and a post-hoc pairwise test with the same package, by pooling both aphid treatments within each accession. Only surviving plants were considered for endophyte status compliance, aphid occupation success, plant morphology traits and biomasses. Endophyte status compliance was evaluated on the blotting results using a Fisher's exact test. Seedlings of the A11104 accession (endophyte-free accession) with E^+ blots were discarded from further analyses, while endophyte-free seedlings of the other three E^+

accessions were considered henceforth as an additional endophyte treatment group [i.e. 'secondarily endophyte-free plants' (NIL2), Table 4.3]. Aphid occupation (or colonisation) success was assessed by a Fisher's exact test for count data. As plant morphology parameters (tiller, leaf and root counts) did not follow any normal or (quasi-)poisson distribution, the effects of water stress (yes/no) and aphid presence (yes/no) on them were estimated by Mann-Whitney-U tests. The effects of the age of the seedlings at harvest and endophyte status (AR1/AR37/CT/NIL/NIL2) were analysed by Kruskal-Wallis tests (KW) and Dunn post-hoc tests with Benjamini-Hochberg *p*-value adjustments. Whether plant genotypes or endophyte groups differed in their response to root aphids was assessed by performing a Mann-Whitney-U test on the effect of root aphids within each plant genotype, endophyte or plant genotype-endophyte group. The dry shoot biomass and the dry matter content of the shoot were analysed by linear mixed-effects models (LME) with seedling group as random effect, and seedling age (days), aphid numbers (adults and older immature nymphs), endophyte status (AR1/AR37/CT/NIL/NIL2), water stress (yes/no) and all dual interactions between the first three variables as initial fixed effects. The initial LME model for root biomass was similar to the one for shoot biomass, but included the additional covariate 'shoot biomass' and its interactions with age, aphid number and endophyte status. The square-root-transformed dry matter content of roots at harvest was assessed by an analysis of covariance (ANCOVA) using the same explanatory variables as the root biomass model. The final models are presented in Section 4.3.1.

The immunoblot made for plants of the Mature plant experiment at the initial trim confirmed that all plants had the expected endophyte status. Therefore, no further analysis was performed for this parameter. Plant survival was not analysed either in the Mature plant experiment as no plants died in the time between aphid placement and aphid harvest. Other response variables were analysed using the factors and covariates listed in Table 4.1 as initial explanatory variables. The number of tillers, green leaves and senescing and dead leaves at precondition and at the 2nd growth check were analysed by a Mann-Whitney-U/Kruskal-Wallis test. The number of new roots at final harvest was analysed by a generalised linear model (GLM) with a quasi-Poisson distribution. Data on biomasses were analysed by multivariate analysis of covariance using the 'stats' package [MANCOVA with green shoot (square-root transformed), senescing and dead shoot and root dry matter at harvest]. The model so defined was then applied to green shoot, dead

shoot and root biomass separately for further analyses (ANCOVA). Univariate and multivariate normality of the response variables were assessed graphically. Red (R), green (G) and blue (B) colour measurements were combined into two chlorophyll content-related variables, green ratio [G ratio: G/(R+G+B)] and normalised red-blue difference [nRBd: (R-B)/(R+B)] (Ali *et al.*, 2012) for both the measurements performed on the full green shoot area and the measurements realised on the last fully extended leaf. G ratio, nRBd and all other continuous response variables were analysed either by linear mixed-effects models (LME) or by analysis of covariance (ANCOVA). Specifics of the final models are given in Section 4.3.2.

Table 4.1. Variables used for analyses in the Mature plant experiment

Type [Time]	Variable	Group	Description
Structural [-]	Plant identity	Rand	Identity of plant from which the tiller was collected (17 levels overall)
	Plant genotype	F	2 levels: N or S genotype
	Endophyte status	F	4 levels: AR1, AR37, CT or NIL
	Water stress at AH	F	Water stress of the plant at aphid harvest (agar layer in PD < 2 mm); 2 levels: Yes/No
	(A: water stress to end)	F	Water stress of the plant up to the final harvest; 2 levels: Yes/No
	Age of plant	F, Cov	Age of plant at measurement
Plant initial condition [Trim -24 h]	Tiller diameter	Cov	Measured on pictures 24 h after trim
	Regrowth 24h	Cov	Length of leaf regrowth 24 h after trim, on pictures
	GSA 24h	Cov	Green shoot surface 24 h after trim, calculated from Tiller diameter and Regrowth 24 h
	Initial tiller dry weight	Cov	Dry weight of trimmed tillers, estimated from the tiller's fresh weight and the matter content of destructively harvested tillers
Plant precondition [28-31 days]	GSA T1	Cov	Green shoot area before aphid placement (precondition)
	nRBdA T1	Cov	Normalised red-blue difference; RGB colour quotient of the GSA T1; calculation: (R-B)/(R+B)
	G ratioA T1	Cov	RGB colour quotient measured on the GSA T1; calculation: G/(R+G+B)
	LEL T1	Cov	Green area of the last fully extended leaf at precondition
	nRBdL T1	Cov	Normalised red-blue difference at precondition; RGB colour quotient of the LEL T1; calculation: (R-B)/(R+B)
	G ratioL T1	Cov	RGB colour quotient measured on the LEL T1; calculation: G/(R+G+B)
Later plant condition [78-84 days]	GSA T2	Cov	Green shoot area at 2nd growth assessment
	LEL T2	Cov	Green area of the last fully extended leaf at the 2nd growth assessment
Aphid effect [98-108 days]	Aphid presence	F	2 levels: Yes/No
	(A: number of adults)	Cov	Number of adult aphids at aphid harvest (dead or alive)
	(A: feeding aphids)	Cov	Number of feeding aphids (adults and older immatures, dead or alive) harvested on a plant

(A:): alternative variables (used instead of the variable above them in some models); AH: aphid harvest; Cov: covariate; F: factor; PD: Petri dish; Rand: random factor. Time information in the first column [days] refers to the plant age at the time of collection, with day 1 being the day the tiller was trimmed at the beginning of the experiment.

4.3. Results

4.3.1. Seedling experiment

The seedlings of the AR37 ryegrass accession had a significantly higher mortality than the seedlings of the NIL and CT accessions (Table 4.2; Cochran-Mantel-Hanszel test: $\chi^2_{[3,\ N = 218]} = 16.51$, $p < 0.001$). Root aphids had no effect on the death rate of seedlings (similar mean rates of seedling mortality for seedlings with or without root aphids; Cochran-Mantel-Hanszel test: $\chi^2_{[1,\ N = 218]} = 0.00$, $p = 0.990$; Table 4.2).

The differences in root aphid occupation (or colonisation) success were not significant between endophyte status groups (Fisher's test: $p = 0.0814$) even if the absolute numbers suggested that the root aphids had struggled somewhat to colonise the seedlings of the AR37 accession (Table 4.2).

Table 4.2. Seedling mortality in *L. perenne* by root aphid treatment ('No aphids' vs. 'Aphids') and further characteristics of the accessions.

Accession	Endophyte status	Germination rate [%]	Endophyte prevalence [%]	Seedling mortality [counts (dead/total)] No aphids	Seedling mortality [counts (dead/total)] Aphids[2]	Seedling mortality [%] Pooled	Aphid occupation success [%][3]
A14559	AR1 (E^+)	99	93.8	5 / 29	3 / 27	14.3 [bc]	74
A16863	AR37 (E^+)	97	89.1	7 / 30	7 / 31	23.0 [c]	55
A12421	CT (E^+)	94	86.8	0 / 27	0 / 26	0.0 [a]	90
A11104[1]	NIL (E^-)	95	2.3	0 / 24	3 / 24	6.3 [ab]	70

Means with a same letter were not significantly different ($p > 0.05$).

[1] Low-endophyte accession, referred to as "endophyte-free" (E^- or NIL) accession for simplicity.

[2] Aphid treatment: 0 vs. 3 neonate *A. lentisci* placed on a seedling in its 1st week of life.

[3] Determined at 28 to 38 days (harvest), on living seedlings of the expected endophyte status only. The colonisation success on the 15 endophyte-free seedlings produced by A14559, A16863 or A12421 (NIL2) was 78%.

Seedling traits were influenced by various parameters. The age of seedlings at harvest was significant for most traits except the dry matter content of root tissues, for example (Table 4.3; Appendix 11, Sections A11.1.2, A11.1.3 and A11.1.4). Between day 28 and 38, the seedlings developed from 1 tiller with 4 leaves and 9 roots to plantlets of 2 tillers, 7 leaves and 12 to 15 roots (median values). The green shoot biomass and the

dry matter content of the shoot tissues increased daily by +2.98 ± 0.38 mg ($p < 0.001$) and 0.14 ± 0.03% ($p < 0.001$), respectively. Root biomass increased significantly with plant age too, but this increase was dependent on the shoot biomass (Table 4.3; Appendix 11, Section A11.1.4). Seedlings classified as "water stressed" were larger (i.e. had more tillers, leaves and roots) and had higher biomass measurements than non-water stressed seedlings (Table 4.3; Appendix 11, Section A11.1.2). That suggested that water stress was more likely a result of fast growing plants rather than an external condition that influenced the seedlings. The endophyte group had an influence on the root biomass in interaction with the shoot biomass only: for every additional mg shoot biomass, plants of the CT group developed 0.06 ± 0.03 mg more root biomass than plants of the NIL group ($p = 0.020$; Appendix 11, Section A11.1.4).

In this context, root aphid feeding did not significantly affect shoot morphology or shoot biomass ($p > 0.05$; Table 4.3). It had a significant effect on the roots, however (Table 4.3). While the dry matter content of root tissues increased slightly but significantly with the number of aphids feeding on it ($p = 0.003$), the total root biomass of a seedling was reduced by -0.05 ± 0.01 mg for every root aphid feeding on a plant after four to five weeks of aphid occupation ($p < 0.001$). There were no significant differences in the response of various endophyte groups to root aphid feeding, however (no interaction between root aphid numbers and endophyte status).

Table 4.3. Seedling characteristics at final harvest (means ± SD) and significance of selected variables (*p*-values).

		Plant age [days]	Tillers [counts]	Leaves [counts]	Roots [counts]	DM content shoot [%]	DM content roots [%]	Shoot biomass [mg DM]	Root biomass [mg DM]
Endophyte group	AR1	32 ± 3	1.6 ± 0.6	5.0 ± 1.7	10.0 ± 3.0	11 ± 0.9	14 ± 2.8 [a]	36 ± 15.1	8 ± 4.2
	AR37	33 ± 3	1.7 ± 0.8	5.8 ± 1.7	11.6 ± 3.6	12 ± 1.4	14 ± 3.1 [ab]	41 ± 16.5	9 ± 4.2
	CT (Common-toxic)	32 ± 4	1.6 ± 0.7	5.1 ± 1.6	10.4 ± 2.3	11 ± 0.9	14 ± 3.0 [ab]	36 ± 16.9	10 ± 5.6
	NIL	33 ± 3	1.5 ± 0.6	4.8 ± 1.3	10.7 ± 2.6	12 ± 1.0	14 ± 2.7 [ab]	36 ± 14.3	8 ± 3.4
	NIL2	32 ± 4	1.8 ± 0.8	5.7 ± 1.7	10.7 ± 2.9	11 ± 1.0	15 ± 2.5 [b]	40 ± 16.9	10 ± 5.2
Analysis	Model (n)	KW(185)	KW(185)	KW(185)	KW(185)	LME(173)	AOV(173)	LME(182)	LME(184)
	R^2	-	-	-	-	0.349	0.275	0.477	0.877
	Data transformation	None	None	None	None	None	Square-root	None	None
Variables (*p*-values)	Plant age at harvest (AgeP)		< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001	(no est.)[1]
	Shoot biomass at harvest (SBM)					-	< 0.001		(no est.)
	Aphid effect: Exposure (A)	0.970	0.792	0.793	0.055	-	-	-	-
	Number (NbA)					-	0.003	-	< 0.001
	Endophyte group (E)	0.474	0.527	0.066	0.186	-	-	-	(no est.)
	Water stress	0.053	0.002	< 0.001	0.050	-	0.027	0.002	0.044
	Interactions: AgeP×NbA					-	-	-	-
	AgeP×E					-	-	-	-
	E×NbA					-	-	-	-
	E×SBM					-	-		0.012
	AgeP×SBM					-	-		0.032
	Random effect: Seedling group					0.005		0.066	0.273

Means with different letters were significantly different (post hoc test $p < 0.05$). -: effects tested but removed because not significant. AOV: analyse of co-variance; KW: Mann-Whitney-U test (by 2 groups) vs. Kruskal-Wallis test (if > 2 groups); LME: linear mixed-effects model; n: number of observations used for analyses; DM: dry matter.

[1] Effect significance for LME are calculated as log-likelihood ratio of full model vs. model without the effect; the log-likelihood of the main terms of an interaction can thus not be estimated (no est.).

4.3.2. Mature plant experiment

The morphology and physiology of the two plant genotypes observed in this trial differed significantly (Table 4.4, Table 4.5). Tillers of the N-genotype had consistently higher dry matter contents than those of the S-genotype (+3.93 ± 0.86% and +3.31 ± 0.88 % for the green shoot at the beginning and end of the experiment, respectively). Plants of the N-genotype had one fewer tiller and one to two fewer leaves than those of the S-genotype by the time of the 2nd growth assessment (Table 4.4; Appendix 11, Section A11.2.2). Seventy-eight to 84 days after the initial trim, N-genotype plants also differed in the shoot colour (significantly higher G ratio for the colour of the full green shoot area; Table 4.5). Despite having more new roots (Table 4.4), the N-genotype tillers had a smaller root biomass at the final harvest (-0.07 ± 0.02 g dry matter/plant; Table 4.6), and a significantly lower root/shoot ratio than the S-genotype plants (Table 4.5; Appendix 11, Sections A11.2.2 and A11.2.8).

Endophyte status had less influence on plant traits than plant genotypes. The initial weight of trimmed tillers was significantly greater in endophyte-free plants (NIL) than in plants with AR1, AR37 or CT endophyte (Table 4.4). Plants with AR1 symbiont in particular had more green leaves than endophyte-free (NIL) plants or AR37 symbioses 78-84 days after trimming, and had more roots at the final harvest than NIL plants (Table 4.4; Appendix 11, Sections A11.2.2 and A11.2.3). The green shoot area was larger in AR1 plants at precondition compared to the NIL reference group (+118.31 ± 39.74, p = 0.011; Appendix A11.2.6) but became similar by the 2nd growth assessment. Plants with AR1 endophyte produced more senescing and dead leaf biomass than NIL or CT plants. At average covariate values, the difference was significant within the N-genotype only (Figure 4.3).

The plants classified as possibly water stressed were slightly larger than the plants of the non-stressed group before water stress occured (e.g. more green leaves and tillers at the 2nd growth assessment; Table 4.4). Whether the additional root biomass and the higher root/green shoot biomass observed in the water stressed group were a response to water stress or a consequence of a higher growth rate, is therefore not clear (Table 4.6, Table 4.5). Overall, however, water stress was apparently short or mild enough to have no significant impact on the multivariate biomass analysis ($F_{[1, 98]}$ = 1.62, p = 0.191; Appendix 11, Section A11.2.9). This factor was, therefore, not further analysed.

Root aphid feeding had no significant effect on morphological characteristics of plants such as the numbers of tillers, green leaves, senescing and dead leaves, the green shoot area at the 2nd growth assessment or the number of roots at final harvest (Table 4.4 and Table 4.5). Aphid feeding did, however, significantly influence the biomass response of plants (multivariate analysis: $F_{[1, 98]} = 3.19$, $p = 0.027$; Appendix 11, Section A11.2.9), particularly the final root biomass (Table 4.6). Although the *p*-value for this effect was marginally not significant in a univariate model for root biomass (Table 4.6, $p = 0.052$), the reduction in root/green shoot ratio on plants colonised by aphids supported the view that roots were affected (Table 4.5). While shoot biomass showed no significant response to root aphid feeding, some small effects on shoots were nevertheless observed. The last fully extended leaf of plants infested by root aphids had a significantly lower nRBd colour quotient at the 2nd growth assessment than such leaves had on root aphid-free plants (-0.02 ± 0.01, $p = 0.049$; Table 4.5; Appendix A11.2.7), a fact due to lower reflectance values for red in particular (R-values measurements; data not shown).

Despite the differences between plant genotypes and between the endophyte statuses, the plant response to root aphids was generally not different in either plant genotype, all four endophyte statuses, or all eight plant genotype-endophyte groups (PG×E): only the number of senescing and dead leaves at the 2nd growth assessment and the nRBd measurement of the last fully extended leaf at precondition (nRBd T1; Table 4.5) showed some significant interactions. The number of senescing or dead leaves per plant was significantly higher on root aphid-free plants than on aphid-infested plants in the AR37 endophyte group at the 2nd growth assessment (Median: 2 vs. 1 leaf; Mann-Whitney-U test: 103.5, $p = 0.044$). There were no such differences for the other three endophyte levels. While in most PG×E groups, the nRBd values at precondition were comparable for plants assigned to the aphid infestation treatment and plants kept aphid-free, N-NIL plants assigned to aphid infestation presented higher nRBd values and S-AR37 plants lower nRBd values than the equivalent plants of the aphid-free treatment. As no aphids had been placed on the plants at that time, this was not an effect attributable to a diverging PG×E response to root aphids, however, but merely a result of the random distribution.

Table 4.4. Morphological characteristics of two perennial ryegrass genotypes (N, S) with endophyte *E. festucae* var. *lolii* symbiont (AR1, AR37 or common-toxic CT strain) or without endophyte (NIL), and influence of various statistical parameters.

		Tiller DM T0 [%]	Tiller BM T0 [mg]	Leaf regrowth 24h T0 [cm]	Tillers T2 [counts]	Green leaves T2 [counts]	Dead leaves T2 [counts]	Roots FH [counts]
Means ± std deviation	N - AR1	13.0 ± 2.6 ^{b}	9.1 ± 2.5 ^{b}	1.2 ± 0.4 ^{a}	3.0 ± 0.8 ^{acd}	9.3 ± 2.1 ^{bc}	0.6 ± 0.5 ^{ab}	46.1 ± 8.5 ^{d}
	N - AR37	12.1 ± 2.2 ^{b}	8.1 ± 1.6 ^{b}	1.4 ± 0.4 ^{a}	2.3 ± 0.8 ^{a}	5.9 ± 2.9 ^{a}	1.2 ± 0.7 ^{bc}	38.8 ± 9.0 ^{c}
	N - CT	11.8 ± 1.9 ^{b}	9.8 ± 2.5 ^{b}	1.3 ± 0.3 ^{a}	2.7 ± 0.7 ^{ad}	8.3 ± 1.6 ^{ac}	0.4 ± 0.6 ^{a}	47.7 ±13.8 ^{cd}
	N - NIL	15.0 ± 2.3 ^{b}	12.5 ± 4.5 ^{c}	1.2 ± 0.4 ^{a}	2.4 ± 0.8 ^{a}	7.1 ± 1.8 ^{a}	0.8 ± 0.8 ^{ab}	39.0 ±10.2 ^{c}
	S - AR1	9.1 ± 1.2 ^{a}	6.9 ± 1.4 ^{a}	1.3 ± 0.4 ^{a}	3.9 ± 0.6 ^{b}	10.3 ± 1.4 ^{b}	1.8 ± 0.6 ^{c}	32.6 ± 9.1 ^{b}
	S - AR37	9.1 ± 1.3 ^{a}	6.8 ± 1.8 ^{a}	0.9 ± 0.2 ^{a}	3.3 ± 0.7 ^{bcd}	8.3 ± 1.9 ^{ac}	1.6 ± 0.6 ^{c}	28.2 ± 8.6 ^{a}
	S - CT	7.8 ± 0.7 ^{a}	6.3 ± 1.5 ^{a}	0.8 ± 0.4 ^{a}	3.5 ± 0.5 ^{bc}	9.0 ± 1.1 ^{bc}	1.8 ± 0.7 ^{c}	30.0 ± 6.7 ^{ab}
	S - NIL	10.1 ± 0.8 ^{a}	8.5 ± 2.2 ^{b}	0.9 ± 0.2 ^{a}	3.5 ± 1.0 ^{bcd}	9.1 ± 2.1 ^{bc}	1.7 ± 0.7 ^{c}	31.1 ± 7.1 ^{a}
Analysis	Model (n)	LME(39)[1]	AOV(115)	LME(115)[1]	KW(115)	KW(115)	KW(115)	GLM(112)
	R^2	0.780	0.361	0.455				0.600
	Data transformation	None	Log_e	Log_e	None	None	None	None
***p*-values**								
Covariates	Tiller diameter			< 0.001				
	Plant age (Age)				0.520	0.818	0.477	< 0.001
Main factors	Plant genotype (PG)	< 0.001	< 0.001	(no est.)	< 0.001	< 0.001	< 0.001	< 0.001
	Endophyte group (E)	-	< 0.001	(no est.)	0.054	< 0.001	0.435	0.009
	Aphid exposure (Aph)		-	-	0.943	0.228	0.262	-
	Water stress [future]				[0.020]	[0.006]	[0.351]	-
Interactions	PG×E	-	-	0.015	< 0.001	< 0.001	< 0.001	-
	PGxExAph	-	-	-	-	-	0.044	-
Random effects	Plant identity	0.002	-	0.086				

T0: 0-24 h after initial trim; T1: precondition (28-31 days after trim); T2: 2nd growth assessment (78-84 days); FH: final harvest (112-121 days after trim); AOV: analysis of covariance; DM: dry matter content; BM: dry biomass; GLM: generalised linear model assuming a quasi-Poisson distribution; KW: Kruskal-Wallis test (by > 2 groups) and Mann-Whitney-U test (by 2 groups); LME: linear mixed-effects model; Means with same letters were not different in post hoc test ($p > 0.05$); n: number of useable samples; -: non-significant variables removed from the models ($p > 0.05$);

[1] Effect significance is calculated as log-likelihood ratio of full model and model without the effect for LME and GLM. By interactions, main terms cannot be estimated (no est.).

Table 4.5. Green shoot and biomass-related characteristics of two distinct perennial ryegrass genotypes (N, S) living either in symbiosis with one of three strains of the endophyte *E. festucae* var. *lolii* (AR1, AR37 or common-toxic CT) or without any symbiotic partner (NIL).

		Green shoot area (GSA)		GSA colour	Last extended leaf colour		Root/green shoot	DM green
		T1 [cm^2]	T2 [cm^2]	G ratio T2 [%]	nRBd T1	nRBb T2	ratio FH [%]	shoot FH [%]
Means ± SD	N-AR1	3.0 ± 1.2 [a]	26.3 ± 6.0	47.7 ± 0.3 [c]	-0.11 ± 0.04 [a]	-0.17 ± 0.04	42 ± 8 [b]	24.2 ± 4.2
	N-AR37	2.0 ± 0.9 [a]	17.3 ± 6.7	47.7 ± 0.4 [cd]	-0.06 ± 0.04 [a]	-0.18 ± 0.05	32 ±11 [a]	25.7 ± 3.4
	N-CT	2.6 ± 1.0 [a]	22.7 ± 5.7	48.0 ± 0.3 [d]	-0.06 ± 0.06 [a]	-0.15 ± 0.03	49 ±13 [b]	23.3 ± 4.6
	N-NIL	2.0 ± 0.8 [a]	22.8 ±13.5	47.6 ± 0.4 [c]	-0.09 ± 0.05 [a]	-0.19 ± 0.03	36 ± 9 [a]	23.4 ± 2.5
	S-AR1	2.7 ± 0.7 [a]	23.8 ± 3.4	47.3 ± 0.3 [b]	-0.10 ± 0.04 [a]	-0.16 ± 0.05	69 ±21 [c]	21.7 ± 4.4
	S-AR37	2.2 ± 0.6 [a]	17.8 ± 4.9	46.9 ± 0.2 [a]	-0.08 ± 0.05 [a]	-0.19 ± 0.04	69 ±17 [c]	21.0 ± 2.5
	S-CT	2.1 ± 0.8 [a]	18.5 ± 4.8	47.0 ± 0.4 [ab]	-0.06 ± 0.05 [a]	-0.18 ± 0.04	75 ±26 [c]	21.6 ± 1.8
	S-NIL	2.3 ± 1.0 [a]	20.8 ± 5.4	47.1 ± 0.3 [ab]	-0.10 ± 0.05 [a]	-0.18 ± 0.04	72 ±17 [c]	20.1 ± 2.3
Analysis	Data transformation	None	None	None	None	None	Log_e	Log_e
	Model (number of observation)	LME(110)	LME(114)	AOV(113)	LME(109)	AOV(106)	AOV(114)	LME(114)
	R^2	0.564	0.376	0.621	0.585	0.201	0.681	0.379
Covariates	GSA at T1		-	-	-	-		
(***p*-value**)	Leaf surface at T1				< 0.001	0.234		
	GSA at T2			0.009		-		
	Leaf surface at T2					0.005		
	Colour at T1 (G ratio or nRBd)			-		0.009		
	Plant age (AgeP)	0.001	0.211	-	0.033	-	< 0.001	0.004
	Tiller diameter	-	< 0.001	-	-	-		
	Initial tiller weight [g dry matter]	< 0.001	-	-	-	0.008		
Main effects	Plant genotype (PG)	-	-	< 0.001	(no est.)[1]	-	< 0.001	< 0.001
(***p*-value**)	Endophyte group (E)	0.013	-	0.202	(no est.)	-	0.069	-
	Water stress						0.040	-
	Aphid presence (Aph)	-	-	-	(no est.)	0.049	0.019	-
Interactions	PG×E	-	-	0.004	(no est.)	-	0.014	-
(***p*-value**)	AgeP×E	-	-		-	-	0.066	-
	PG×Aph, E×Aph	-	-		(no est.)	-	-	-
	PG×E×Aph		-		0.015	-	-	-
	Colour at T1×Leaf surface at T1					0.010		
Random effects	Plant identity	0.001	< 0.001		0.003			0.038

FH: at final harvest (112-121 days after initial trim); T1: precondition (28-31 days); T2: 2nd growth assessment (78-84 days); DM: dry matter content; AOV: analyse of covariance. LME: linear mixed-effects model. Means with same letters were not significantly different by post hoc test ($p > 0.05$). -: effects tested but not significant and removed from the final models. G ratio: G/(R+G+B) in the RGB colour model space; nRBd: (R-B)/(R+B) in the RGB colour model space.

[1] Effect significance is calculated as log-likelihood ratio of full model vs. model without the effect in LME and GLM. By interactions, main terms cannot be estimated (no est.) .

Table 4.6. Multivariate analysis of covariance and parameter estimates for plant biomass [g dry matter] in mature perennial ryegrass tillers of two distinct genotypes (N, S) in symbiosis with one of three endophyte *E. festucae* var. *lolii* strains (AR1, AR37 or common-toxic CT) or without any symbiotic partner (NIL), 112 to 121 days after the initial trim.

		Green shoot dry matter (sqrt)				Senescing and dead shoot dry matter				Roots dry matter			
Analysis of variance: effects	**Df**	**SS**	**MS**	**F value**	***p*-value**	**SS**	**MS**	**F value**	***p*-value**	**SS**	**MS**	**F value**	***p*-value**
Initial tiller weight, dry matter [g]	1	0.043	0.043	10.87	*0.001*	0.024	0.024	38.51	*< 0.001*	0.011	0.011	6.35	*0.013*
Plant age at harvest [days]	1	0.052	0.052	13.26	*< 0.001*	0.005	0.005	7.44	*0.008*	0.100	0.100	55.40	*< 0.001*
Plant genotype	1	0.010	0.010	2.50	*0.117*	0.001	0.001	1.48	*0.226*	0.042	0.042	23.52	*< 0.001*
Endophyte status	3	0.059	0.020	4.97	*0.003*	0.002	0.001	0.81	*0.489*	0.005	0.002	0.95	*0.420*
Water stress	1	0.004	0.004	0.96	*0.329*	0.000	0.000	0.00	*0.988*	0.008	0.008	4.35	*0.040*
Aphid presence	1	0.000	0.000	0.04	*0.833*	0.001	0.001	2.00	*0.161*	0.007	0.007	3.87	*0.052*
Plant age × Endophyte	3	0.058	0.019	4.95	*0.003*	0.001	0.000	0.77	*0.514*	0.005	0.002	0.91	*0.440*
Plant genotype × Endophyte	3	0.009	0.003	0.78	*0.507*	0.003	0.001	1.43	*0.240*	0.016	0.005	2.88	*0.040*
Residuals	98	0.385	0.004			0.061	0.001			0.177	0.002		
Coefficients of fitted linear models		**coef**	**se(coef**	**t value**	***p*-value**	**coef**	**se(coef)**	**t value**	***p*-value**	**coef**	**se(coef)**	**t value**	***p*-value**
(Intercept)		-1.759	0.607	-2.90	*0.005*	-0.605	0.242	-2.50	*0.014*	-1.768	0.411	-4.30	*< 0.001*
Initial tiller weight, dry matter		8.411	2.551	3.30	*0.001*	6.307	1.016	6.21	*< 0.001*	4.355	1.728	2.52	*0.013*
Plant age at harvest (AgeP)		0.019	0.005	3.64	*< 0.001*	0.006	0.002	2.73	*0.008*	0.016	0.003	4.71	*< 0.001*
Plant genotype N		0.040	0.025	1.58	*0.117*	-0.012	0.010	-1.22	*0.226*	-0.072	0.017	-4.18	*< 0.001*
Endophyte AR1		2.600	0.817	3.18	*0.002*	0.382	0.325	1.17	*0.244*	0.635	0.553	1.15	*0.254*
Endophyte AR37		-0.006	0.826	-0.01	*0.995*	0.099	0.329	0.30	*0.765*	0.609	0.560	1.09	*0.280*
Endophyte CT		1.197	0.802	1.49	*0.139*	0.402	0.319	1.26	*0.211*	0.876	0.543	1.61	*0.110*
Water stress Yes		0.013	0.013	0.98	*0.329*	0.000	0.005	-0.02	*0.988*	0.019	0.009	2.09	*0.040*
Aphid presence Yes		0.003	0.013	0.21	*0.833*	0.007	0.005	1.41	*0.161*	-0.017	0.009	-1.97	*0.052*
AgeP × Endophyte AR1		-0.022	0.007	-3.18	*0.002*	-0.003	0.003	-1.11	*0.270*	-0.006	0.005	-1.17	*0.247*
AgeP × Endophyte AR37		0.000	0.007	0.02	*0.986*	-0.001	0.003	-0.27	*0.786*	-0.005	0.005	-1.11	*0.272*
AgeP × Endophyte CT		-0.010	0.007	-1.50	*0.136*	-0.003	0.003	-1.24	*0.220*	-0.008	0.005	-1.62	*0.109*
Plant genotype N × Endophyte AR1		0.045	0.034	1.35	*0.179*	0.017	0.013	1.27	*0.206*	0.059	0.023	2.60	*0.011*
Plant genotype N × Endophyte AR37		0.046	0.036	1.29	*0.200*	0.022	0.014	1.52	*0.131*	0.009	0.024	0.38	*0.708*
Plant genotype N × Endophyte CT		0.030	0.035	0.87	*0.385*	-0.001	0.014	-0.09	*0.929*	0.039	0.023	1.69	*0.095*
R^2				**0.543**	*< 0.001*			**0.449**	*< 0.001*			**0.531**	*< 0.001*

MS: mean squares; SS: sum of squares; coe: model coefficient; se(coef): standard error of model coefficient; Sqrt: square root-transformed.

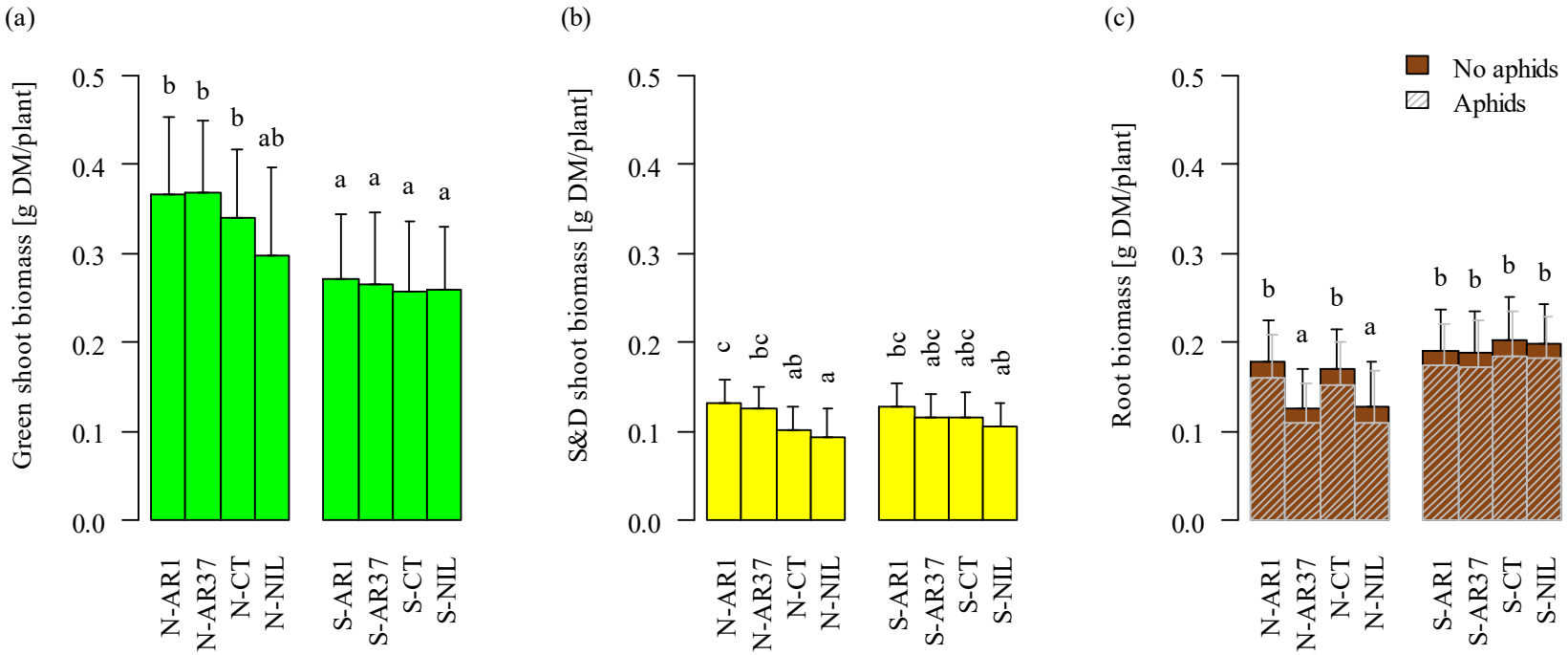


Figure 4.3. Biomasses of green shoot (a), senescing and dead shoot (b), and root (c) at the end of the Mature plant experiment, as determined by the MANCOVA model by average covariate values (86 mg initial tiller weight after trim, harvest 116 to 117 days after the initial trim), in presence or absence of root aphids for roots ($p = 0.052$). The bars represent the means ± SD for each plant genotype-endophyte group (PG-E). PG-E groups with a same letter were not significantly different ($p > 0.05$ in a post hoc test).

4.4. Discussion

High root aphid populations in pastures have been associated with reduced plant survival and small, pale and weak plants carrying dead material around their base (McDonald *et al.*, 2011). Damage quantification approaches in experiments with potted perennial ryegrass reported that plants that are not protected from root aphid feeding by the AR37 endophytic symbiont survive less well (-35%) and produce less above-ground biomass [between 16 to 27% less foliar growth (Popay & Cox, 2016)]. Root biomass is either unaffected or lower in AR1 symbioses and endophyte-free plants infested by root aphids, compared to plants with AR37 symbiont (Popay & Cox, 2016). However, endophytes can profoundly modify other parameters besides the plant's resistance towards insects, for example, their morphology, physiology and interaction with the abiotic and biotic environment [e.g. higher root weight in perennial ryegrass-CT symbioses, modified phenology of perennial ryegrass-AR37 symbioses; Chapter 1, Table 1.4]. Differentiating between root aphid effects, endophyte effects, plant genotype effects and environmental influences may be difficult under such circumstances. In particular, information on the plant's response to root aphid feeding is lacking. The experiments reported in this chapter aimed at filling this gap in knowledge by comparing plants of identical genetics and endophyte status exposed to vs. protected from root aphid feeding.

Root aphids had no effect on plant mortality in either of the two experiments presented here. While the root biomass was the most affected by root aphid feeding (Table 4.3, Table 4.6), the shoots showed only minor responses to root aphid infestations such as colour changes in the newest, fully extended leaf. This lack of response in shoot biomass in particular was atypical, considering what has been reported for *A. lentisci* (Popay & Cox, 2016) or for other root aphids such as the rice root aphid on wheat (Kindler *et al.*, 2004). In the case of the Mature plant experiment, the effects of aphid feeding may have been attenuated through plant recovery in the time interval between aphid harvest and plant harvest. This explanation is not applicable for the Seedling experiment, however, in which the seedlings were harvested at the same time as the root aphids. It is possible that the number of aphids was too low or that the length of the exposure to root aphid feeding was too short to result in a significant effect. The former explanation is unlikely, considering the median loads in the experiments reported here (60 to 286 feeding aphids per gram root for plants with CT and AR1 endophyte in the Mature plant experiment; 635 to 4086 feeding aphids per gram root for seedlings with AR37- and AR1-

endophyte during the Seedling experiment, respectively). These values were similar to or higher than the ones reported on plants displaying aphid-associated biomass losses in Popay and Cox (2016). The absence of additional environmental stress, good nutrient availability or/and some plant compensation strategies (see leaf colour below) could offer some alternative explanations for the unusual absence of shoot response in the Mature plant and Seedling experiments. There was no additional heat or insect stress in the laboratory, for example. Another explanation could be that a reduction in root biomass is likely to decrease the plant's water and nutrient uptake capacities (Catherali & Parry, 1987). That may be critical in the field or in potting mix in which nutrients may be heterogeneously distributed, and may limit the plant's growth and survival (Tozer *et al.*, 2017). In the homogenous, water-abundant, nutrient-enriched agar solution used during the trials (modified Bollard medium; Appendix 6), the loss of smaller amounts of root biomass may have been irrelevant or compensated for by an increase in the absorption capacity of the remaining roots (Eissenstat & Volder, 2005). While this would suggest appropriate fertilisation and irrigation could help to alleviate root aphid damage in the field, this hypothesis will need a practical examination. Nitrogen fertilisation may indeed also increase the incidence of root aphids (Cosgrove *et al.*, 2018).

Leaf colour is a good proxy for chlorophyll and nitrogen (N) contents in leaves (Ali *et al.*, 2012; Jia *et al.*, 2004; Kawashima & Nakatani, 1998). It may be used to examine plant health since stress can reduce chlorophyll concentrations in leaves (Carter & Knapp, 2001). Insect feeding may also influence leaf colour (Carter & Knapp, 2001). Infestation by Russian wheat aphid (*Diuraphis noxia* Kurdjumov), for example, resulted in plants reflecting more light as photosynthetic pigments were degraded (Mirik *et al.*, 2014). Root aphids appeared to have the contrary effect on plants in the Mature plant experiment (Table 4.5). An increased absorption of red light (data not shown) resulted in a significantly lower nRBd colour coefficient in the last fully extended leaf, which suggested an increase in the photosynthetic pigment content of the newest leaves. It is possible that the change was an artefact resulting from the short period of aphid feeding or other experimental parameters associated with endophyte. After all, reflectance measurements are also sensitive to leaf characteristics such as waxes, for example, and some endophyte species can modify the total chlorophyll content of plants (Sanchez-Azofeifa *et al.*, 2012). As higher chlorophyll contents caused by aphid feeding have been observed in some cases [e.g. +25% chlorophyll in leaves of aphid-infested oak trees

(Dixon, 1973)], this observation could also be the first evidence for root aphid-induced photosynthesis facilitation, and explain how the shoots might have compensated for root aphid feeding. More research is needed to examine this aspect.

None of the plant genotypes and plant genotype-endophyte associations studied in this thesis were significantly more tolerant of root aphid feeding than the others. While a short exposure to root aphid feeding may again be invoked as an explanation (see above), plant age and experimental set up should also be considered. Some metabolic functions of endophytes develop only gradually after germination (Ruppert *et al.*, 2017). By the time the Seedling experiment started, the endophyte-plant relationship might not have been fully functional in the seedlings. The Mature plant experiment was performed outdoors during the winter-early spring, on two plant genotypes that may well have a somewhat similar response to root aphids when low temperatures reduce all biological functions. Endophyte concentrations in perennial ryegrass plants, too, was likely lower during the Mature plant experiment, than it would have been at other times of the year [lower concentrations of hyphae in the plants in winter (Christensen & Voisey, 2007)]. Alternatively, it is also possible that the endophyte strains observed have mainly antibiotic and/or antixenotic effects on root aphids, but do not confer their host any tolerance benefits. Future experiments will need to monitor more plant genotypes, in conditions that more closely resemble those that occur in the field and over several seasons to gain more insights.

4.5. Conclusion

The plant genotypes and the endophyte strains considered in this chapter did not display any significant differences in their response to root aphids. Root aphid feeding appeared to first reduce the root biomass of the *L. perenne* plants they lived upon without any effect on the overall shoot biomass of both seedlings and mature tillers in the conditions studied here. These findings suggest that the detrimental effect on grass yields frequently reported in the literature could be a consequence of a diminished root system. Whether appropriate fertilisation and/or irrigation could compensate for the lost root biomass in the field will require more research. While data suggested perennial ryegrass might adjust to root aphid feeding by increasing the photosynthetic pigments in newly grown leaves, more evidence will have to be gathered to confirm these first observations.

CHAPTER 5: ESTABLISHMENT, COLONISATION, AND POPULATION ECOLOGY OF ROOT APHIDS

5.1. Introduction

A good understanding of population dynamics is important for crop protection (Kindlmann & Dixon, 2010). There is not much information in this regard for root aphid *Aploneura lentisci* Pass. on its secondary hosts. It is merely known to aggregate in sometimes large, wax-coated colonies on the roots of grasses (Popay & Cox, 2016). Even if the absolute number of root aphids on a perennial ryegrass (*Lolium perenne* L.) plant tended to be highest in autumn in past research, aphid populations (and, in particular, aphid loads) did not change in any clear pattern over time or season (Popay, 2004; Popay & Cox, 2016). The populations of other aphid species fluctuate in the field, however, with rapid decline in population size after an initial, dramatic increase and perhaps a phase of population stability (Campbell & Hutchison, 1995; Dixon, 1973; Kindlmann & Dixon, 2010). Self-regulation processes such as the production of migrants in response to population density play a major role in such population declines. The deterioration of host plant quality and natural enemies (predators, parasites) appear to only contribute to, rather than to cause, such population fluctuations (Dixon, 1973; Kindlmann & Dixon, 2010). Whether such population regulation mechanisms appear also in *A. lentisci* populations on perennial ryegrass, and if so, at which thresholds, has yet to be examined. Annual crops are believed to have an increasing aphid carrying capacity through the season until plant maturity (Kindlmann & Dixon, 2010).

Understanding a root aphid's root use pattern and what promotes its establishment or colonisation is an important first step when studying population dynamics since (i) establishment and colonisation are prerequisites for successful population development and (ii) large differences in fitness may result from the selection of a host, plant genotype, microhabitat and feeding site (Keiser *et al.*, 2013; Lowe, 1967; Zytynska & Weisser, 2016). As "plant characters governing aphid host selection often dictate eventual plant resistance or susceptibility to aphid herbivory" (Smith & Chuang, 2014), a better understanding of *A. lentisci*'s root use is also likely to facilitate the development of

effective root aphid control strategies (Kindlmann & Dixon, 2010). Aphid age needs to be considered in root use research since behavioural traits such as activity and exploration are observed to change with their ontogeny (Tomczak & Müller, 2018). Most literature on *A. lentisci* has focussed on general population observations at plant (pot trials) or field levels, however (e.g. Moate *et al.*, 2012; Pennell *et al.*, 2005; Popay & Cox, 2016), and the only feeding site information available to date relates to the more frequent presence of immature nymphs on new roots (Popay, 2004; Popay & Cox, 2016).

Based on the premises that a feeding site used is probably suitable enough for the root aphid to invest energy into using it, and that some differences in plant traits at aphid placement may influence success of establishment and colonisation, this chapter used data from two experiments (Biology II and Population experiments) and one observational study (Colony wax measurements) to get first insights into the topic. The following questions were examined:

1) What characterises a suitable feeding site for *A. lentisci*?
2) How does *A. lentisci* use suitable feeding sites?
3) What plant characteristics favour successful colonisation by *A. lentisci*?
4) Do *A. lentisci* populations display any specific population dynamics on the roots of perennial ryegrass (in laboratory conditions)?

5.2. Materials and methods

5.2.1. Biology II experiment

This experiment offered behavioural and feeding site-related observations for aphids of known age on perennial ryegrass tillers of two distinct genotypes (N and S) and four endophyte statuses (NIL, AR1, AR37 and CT, for endophyte-free plants, plants with AR1, AR37 and a common-toxic endophyte strain, respectively), at standard climate chamber conditions (17.5 ± 2.40 °C, 14 h light per day). The plants were prepared, mounted in modified Bollard medium agar and supplied as described in Section 3.2.1.2 (see also Figure 3.1) with 0, 1 or 5 neonate root aphids ('no', 'solitary' or 'group' aphid treatment, respectively). The data used here for behaviour and feeding site analyses were collected by repeated checks on the solitary root aphid treatment group only (i.e. n = 5 to 11 aphids per PG-E group). Information on both aspects was obtained once a week for the first two weeks after aphid placement, and then once every second day up to aphid death. At each check, the aphid was first located within the Petri dish, and wax that was blocking the view was removed by spraying a fine mist of tap water over it or, occasionally, by brushing the wax away with a fine paintbrush. Observations were carried out under a stereo microscope at 50× magnification. The aphid behaviour was recorded as 'feeding', 'walking', 'other' (if in ecdysis, or standing without feeding) or 'unknown' (if not found). The root and the aphid feeding on it were photographed at the first two checks, at the production of the first offspring and, later, at several random time points, with a Canon PowerShot A620 camera attached to the stereo microscope (Section 2.4.3). The feeding site was described at each check by the following information, as far as this could be established without damaging the feeding aphid:

(1) Root branching order (BO): unbranched, 1st BO, 2nd BO, 3rd BO or 4th BO, see Table 1.3, Section 1.3.1 of Chapter 1)

(2) Feeding position of the aphid on the root system: 'main', 'lateral 1' or 'lateral 2', if the aphid fed on the main root axis, a lateral branch of the main axis, or a lateral branch of the lateral branch, respectively (Figure 5.1).

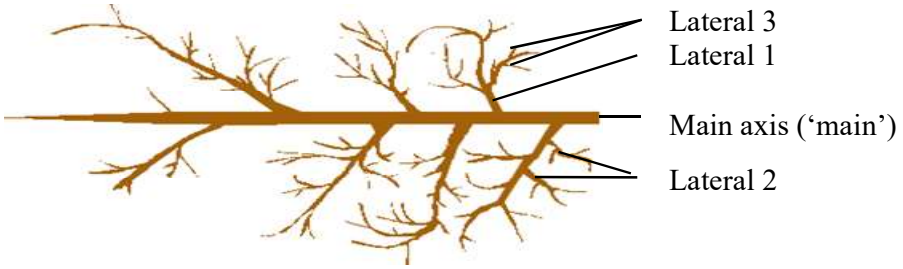


Figure 5.1. Position terminology for a perennial ryegrass root of 3rd branching order (BO).

(3) Root colour at the feeding site: white, pale brown or dark brown.

(4) Root diameter at feeding site: the diameter of the root used was measured next to the head of the aphid on all photographs of sufficient quality, using ImageJ version 1.46r (Rasband, 1997-2016). If visible, aphid body length and abdominal width were also measured on these photographs and the ellipsoid body projection was calculated as reported by Equation 2.12 (Section 2.4.3).

Site fidelity (whether the aphid remained on a given root) was assessed by comparing root and site specifications on two consecutive checks and verifying whether there were no other wax deposits on the rest of the root system. Site fidelity assessments were validated with photographs where possible. After recording this information, the agar in each Petri dish was rehydrated by spraying autoclaved tap water at room temperature (approx. 0.7 mL/check) on it if it filled less than ¼ of the Petri dish. The Petri dish was then re-closed and again light-proofed by wrapping it as described in Chapter 3. To take into account the effects of the handling and observations on behaviour, potentially confounding variables 'rewatering' (no/yes), 'light exposure' (checking time in minutes) and 'disturbance' ("yes" if the aphid was wounded at wax removal and/or left its initial feeding position, "no" otherwise) were also recorded at each check, and used to explain the behavioural pattern at the next check. Aphid age at check was calculated with Equation 2.15 (Section 2.4.3). The plant age at check was obtained using the same equation, with the day of initial trim as day one. At aphid death, the plant was harvested as described in Section 2.4.2 (plant age: 63 to 322 days). The number of roots of each branching order was counted on the finally processed, re-hydrated root samples spread in a white, water-filled tray for a clearer view.

Behavioural analyses focussed on records of known activities. As there were only nine records of the 'other' category (< 1%), the behaviour was reduced to the two main patterns for statistical analyses: 'feed' (0) and 'walk' (1). A mixed-effects logistic regression model was fitted to this binary response variable ('aphid restlessness') using the 'lme4' package (Bates *et al.*, 2015). The independent variables included the precondition proxies (plant age, number of green leaves and number of dead leaves when the aphid was introduced), aphid age (grouped into a categorical variable with 13 levels: week 01&02, 03, 04, 05, 06, 07, 09, 10, 11, 12, 13, 14, and 15+; week 08 had to be left out of the analysis for lack of variance by 100% feeding records), the confounding variables 'rewatering', 'light exposure' and 'disturbance at last check' (see above), plant genotype and endophyte status of the respective plant (applied as fixed explanatory variables). Aphid identity was added as a random grouping effect. The model was then reduced stepwise to the essential components, assessing the significance of each variable with a type II Wald χ^2 test ['car' (Fox & Weisberg, 2011)]. Multiple comparisons with Benjamini-Hochberg *p*-value adjustment were calculated post hoc with the 'predictmeans' package (Luo *et al.*, 2014). A likelihood-ratio based pseudo-R-squared was provided by 'MuMIn' (Barton, 2018).

A total of 745 feeding observations at 331 different feeding sites provided at least partial information on the characteristics of roots used for feeding. Analyses were performed by trait (branching order of the root used, position on root, root colour and root diameter), on all available records ('use analysis'). This approach gave more weight to feeding sites that were used more than once but acknowledged the fact that aphids obviously chose to re-use some sites several times (see Section 5.3.2). Information on analyses by feeding site (where each site was only counted once) is reported in the appendices (Appendix 12, Section A12.2). The root use records were classified into records collected on either 'immature' root aphids if the observed aphid had not yet started reproduction, or on 'mature' aphids if the aphid had already produced at least one offspring by the time of the check.

The branching pattern of a plant could not be established while an aphid was still living on it. It was computed after a plant's final harvest only, by counting the number of roots of each branching order (BO) and calculating the relative proportion of roots of each BO in the full root system. This information was used to predict a theoretical BO use per

plant genotype-endophyte (PG-E) group and plant age with Equation 5.1, simplifying the plant age to a factor with nine levels (plant age period I to IX: < 35, 36 to 65, 66 to 85, 86 to 107, 108 to 135, 136 to 170, 171 to 220, 221 to 260 and ≥ 261 days of age at final harvest).

$$\text{Theoretical } BO_x \text{ use}\,(PG\text{-}Ei, t) = n(PG\text{-}Ei, t) \cdot pBO_x(PG\text{-}Ei, t) \qquad \textbf{Equation 5.1}$$

Thereby, BO_x use(PG-Ei, t) was the use of roots of branching order x in the plant genotype-endophyte group i during plant age period t; n(PG-Ei, t) was the number of aphid records reported for the same plant age period on the plant genotype-endophyte group i (BO use records); pBO_x(PG-Ei, t) was the average proportion of roots of branching order x in the plants of the PG-E group i harvested during plant age period t. The predicted root use was compared to the observed BO use by aphids of all ages with one Cochran-Mantel-Haenszel test for each plant age period, stratifying the data by PG-E group. The effect of aphid age (immature vs. adult) on BO use, position use, and root colour use were analysed with the same test. In absence of aphid age effects (e.g. by the position), the data were pooled and Fisher's exact test for count data used to analyse (i) the difference between PG-E groups within each plant age period, and (ii) the differences between plant age periods within each PG-E group, respectively. By post hoc analyses, p-values were adjusted for multiple comparisons with the Benjamini-Hochberg procedure.

Kruskal-Wallis/Mann-Whitney-U tests and post hoc Dunn tests with Benjamini-Hochberg p-value adjustments for multiple comparisons were used to compare root diameter measurements. Spearman's ρ rank correlation coefficients were computed to assess the relationship between plant age, diameter of the used roots, aphid population age and aphid size measurements. The r-packages 'doBy' (Højsgaard & Halekoh, 2016), 'FSA' (Ogle, 2017), 'stats' (R Core Team, 2017), 'rcompanion' (Mangiafico, 2018) and 'Hmisc' (Harrell, 2018) were used for these analyses.

5.2.2. Colony wax observations

The purpose of specific colony wax observations was to provide corroborating evidences for root use hypotheses developed in the Biology II experiment but in a less strongly and frequently disturbed system. In addition, they provided more insights into

the early ontogenesis of colonies. The observations were performed on clone-plants [plants of genotype N and S without endophyte (NIL) or with an *Epichloë festucae* var. *lolii* endophyte symbiont of strain AR1, AR37 or common-toxic (CT); Table 2.3, Section 2.3.1.1)]. Four tillers of each plant genotype-endophyte combination ('PG-E group') were removed from their mother plant, washed carefully under tap water, trimmed to 4 cm shoot length (leaving the roots on), and embedded in Petri dishes filled with nutrient-enriched agar (55 to 60 g modified Bollard medium agar [MBM agar]; Appendix 6) as described in Section 2.3.1.3. The embedded plants were then kept at 18.1 ± 1.15°C and 14 h light·day^{-1} in the Conviron climate chamber 2 (Section 2.2.4). At the end of the third week of recovery, the agar in the root area was topped up for each plant to prevent water stress. Eighteen to 26 days after the initial trim, a cohort of ten neonate root aphids[3] was placed on each plant. The plants were then re-wrapped as before and returned to the climate chamber. Forty-four to 49 days after the initial trim (after 19 to 31 days of aphid occupation), each Petri dish was opened once to photograph every wax deposition visible to the naked eye on the roots ('colony') with a digital Canon PowerShot A620 camera (Canon Inc., Tokyo, Japan) adjusted to a stereo microscope (Stemi SV6 Zeiss, Carl Zeiss AG, Oberkochen, Germany; magnification 21×). Colonies that could not be photographed in a plane parallel to the camera were left out, to avoid measurement distortions. This procedure resulted in a total of 38 colony photographs on 20 plants (3, 3, 6, 6, 5, 3, 3 and 9 colonies from 3, 1, 4, 2, 3, 2, 2 and 3 N-AR1, N-AR37, N-CT, N-NIL, S-AR1, S-AR37, S-CT and S-NIL plants, respectively). Whether colonies covered only one or several roots (single vs. multiple roots) was recorded. For single root colonies, the root branching order and colony position on the root were also reported. The colonies were quickly excised from the root system after photographing, severing all roots with embroidery scissors and depositing them into microcentrifuge tubes filled with 1.5 mL 80% ethanol. Besides dissolving the wax around them, this procedure preserved the aphids and roots for later processing. The preserved aphids were subdivided into two age categories: 'young' aphids if < 1 mm body length and presenting an elongate rod shape, and 'old' if their length appeared to exceed 1 mm and if the body was already oval to pear-shaped. The number of aphids of each age group and the number of exuviae present in each tube were

[3] These neonates were produced over a 24-h-viviposition period in a glass Petri dish (Chapter 2, Section 2.3.2), by mothers collected from root aphid colonies kept for ≥ 2 generations in a Conviron climate chamber at the same conditions as the experimental plants [approximately 18°C and 14 h light·day^{-1}, on agar-embedded 'clone-plants' of the same PG-E group].

then counted. The aphids' activity at the time point of harvest/preserving was assessed as follows: individuals attached to roots, or in feeding position (i.e. with rostrum perpendicular to the body, and/or unsheathed stylets) were classified as 'feeding', aphids with sheathed stylets and rostrum parallel to the body as 'resting or walking'.

The photographs of colonies before preservation were used to subjectively classify the wax density (Figure 5.2, Table 5.1) and identify a primary root (the root with the densest wax sheath). The total surface covered by wax ('wax area'), the length of the primary root covered with wax ('root length') and the average diameter of the primary root ('root diameter') were measured in ImageJ (Rasband, 1997-2016) as represented in Figure 5.2. Correlations between various numerical parameters were calculated by Spearman's method. The total number of aphids and the number of exuviae were analysed by fitting generalised linear models with quasi-Poisson distribution to the data, with all respectively meaningful variables of Table 5.1 at the start. After a stepwise reduction of the variables, only root diameter remained as an explanatory variable in both models. The difference in feeding frequency between young and old aphids was estimated with a Wilcoxon test for paired samples (signed rank test). Wax area and wax-covered root length were analysed by linear mixed-effects models with plant identity as a random grouping factor. The final fixed effects for wax area comprised root diameter, wax-covered root length, wax density and PG-E, the ones for the wax-covered root length only PG-E (Table 5.1). The 'doBy', 'Hmisc', 'predictmeans', 'rsq' and 'stats' packages were used for these analyses (Harrell, 2018; Højsgaard & Halekoh, 2016; Luo *et al.*, 2014; R Core Team, 2017; Zhang, 2018).

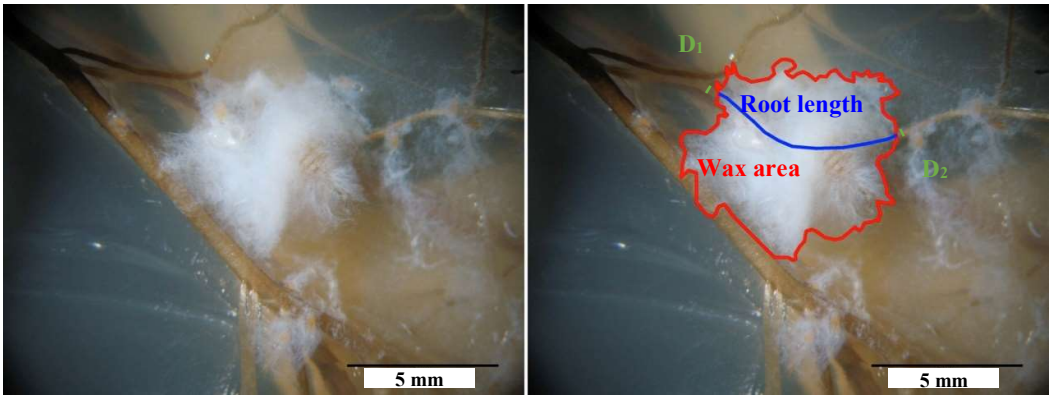


Figure 5.2. *A. lentisci* colony, unprocessed photograph (left) and photograph with wax measurements (root length, wax area and root diameters D_1 and D_2; right). Average root diameter D = $(D_1+D_2)/2$. This example was classified as a colony with medium wax density.

Table 5.1. Explanatory variables used for colony wax measurement analyses.

Variables	Type	Description
Aphid traits		
Duration of aphid occupation	Co	Time since placement, i.e. maximal age of a colony [days], calculated as date of aphid harvest (day of photograph) - date at aphid placement + 1.
Total number of aphids	Co, R	Total number of aphids found in a colony [counts]
Feeding old aphids	Co, R	Number of feeding old aphids [counts]
Feeding young aphids	Co, R	Number of feeding young aphids [counts]
Number of exuviae	Co, R	Number of exuviae found in the colony at harvest [counts]; proxy for colony use time.
Aphid age group	F	Old vs. young (used only for the proportion of feeding aphids)
Plant and colony properties		
Root diameter	Co	Average root diameter (Figure 5.2)
Root length	Co, R	Length of primary root covered by wax (Figure 5.2)
Wax density	F	Subjective wax classification, 3 levels: • **Low:** root and aphid(s) are visible, their shape is clearly seen through the wax • **Medium:** root and aphid(s) are partly visible, but their shape is not fully recognizable • **High:** neither aphid nor root is visible
Plant genotype (PG)	F	Factor with 2 levels: N or S
Endophyte (E)	F	Factor with 4 levels: AR1, AR37, CT or NIL
Plant genotype-endophyte status (PG-E)	F	Factor with 8 levels (N-AR1, N-AR37, N-CT, N-NIL, S-AR1, S-AR37, S-CT, S-NIL), interaction term of PG and E.

Co: covariate; F: factor; R: response variable

5.2.3. Population experiment

Many plant precondition parameters were recorded during the Population experiment, which made it valuable for studying the possible effect of various plant characteristics on colonisation success. The harvest of replicates over an extended harvest period also allowed observations of population development for several weeks.

Two sets of 'clone-plants' (Section 2.3.1.1), i.e. two plants of each of the eight plant genotype-endophyte status combinations N-AR1, N-AR37, N-CT, N-NIL, S-AR1, S-AR37, S-CT, S-NIL (2 genotypes [N, S] x 4 endophyte statuses [endophyte-free NIL, or with *Epichloë festucae* var. *lolii* strains AR1, AR37 or common-toxic CT]) were split into individual tillers for the Population experiment. These tillers were thoroughly washed and held in cubicle 12 of glasshouse 9 (Chapter 2, Section 2.2.3.), in transparent plastic containers (7.8 cm diameter, 9.5 cm height; LabServ®, Thermo Fisher Scientific New Zealand Ltd, Auckland, New Zealand) filled with tap water that was changed daily. Within 14 days of the initial plant splitting, seven batches of five of these tillers per PG-E group were processed as represented in Figure 3.1; the tillers in each batch were trimmed to a length of 4 cm shoot and 0.3 cm root without side roots and then weighed. One of the trimmed tillers per PG-E group was freeze-dried to determine the initial dry matter content of all others. The other four were placed into one small glass vial each (7.6 cm length, 2.4 cm diameter), in approximately 20 ml tap water changed once every 24 to 48 h and were left to recover in the Conviron climate chamber 1 at 17.7 ± 2.4 °C and 14 h light·day^{-1} (Section 2.2.4). Twenty-four hours after the initial trim, the plants were spread on a platter and photographed for initial tiller measurements (regrowth after 24 h, initial tiller diameter; Section 2.4.2), before being returned to the climate chamber for recovery. The tillers were embedded in Petri dishes filled with approximately 45 g modified Bollard medium (Appendix 6) as described in Section 2.3.1.3, 15 to 25 days after the initial trim. In the second week after embedding, the surviving plants of each PG-E group and batch were randomly assigned to one aphid treatment (0, 1 or 5 aphids per plant). That resulted in a total of five to nine replicates of various batches for each PG-E group and aphid treatment. The plant precondition was then recorded counting the number of tillers, green leaves and senescing and dead leaves, and photographing the plants to estimate the initial green shoot area (GSA) and its colour (see Chapter 2, Section 2.4.2). The root system was further ranked into one of three categories depending on whether it was composed of only a few, short or very fine roots ("slender", 1), several

roots longer than 4 cm ("average", 2) or many long roots ("profuse", 3). Within three days of the pre-condition assessment, the pre-assigned number of early first instar *A. lentisci* nymphs was placed onto the roots of experimental plants. The nymphs used for this purpose had been transferred from acclimatised colonies on clone-plants of the same PG-E group (maintained for ≥ 2 generations at standard climate chamber conditions) into microcentrifuge tubes before being placed. As there were not enough nymphs for N-AR37 plants, 12 of the N-AR37 Petri dishes were supplied nymphs produced on S-AR37 plants. The experimental plants were returned to the climate chamber after aphid placement and left there for populations to develop (in a random order alternating aphid treatments and PG-E groups). To prevent water stress, the Petri dishes were opened once a week from the fifth week after aphid placement onwards and re-watered by spraying a fine mist of previously autoclaved tap water on the agar (approximately 0.8 mL, at room temperature at the time of application). If the agar layer left in the Petri dish at the root aphid harvest was < 2 mm thick despite this treatment, the plants were recorded as "possibly water stressed". Root aphid harvesting started 38 days after aphid placement, under a stereo microscope at magnification 50× (Stemi SV 6, Carl Zeiss AG, Oberkochen, Germany). All root aphids present were collected, classified into two types (alates vs. apterous), two conditions (alive vs. dead) and in three age categories (early first instars, older immature and adults; Section 2.4.3) and counted. After the aphid harvest, the plants were photographed again (end condition) before being dissected into green shoot, senescing and dead (S&D) material and roots, weighed, freeze-dried for approximately 60 h and re-weighed to get the final dry matter weights (see Chapter 2, Section 2.4.2). The green shoots were blotted before being freeze-dried to confirm the plants' endophyte status (E^+ or E^-; Section 2.3.3).

Plants of the no-aphid-treatment served to estimate the amount of migration between Petri dishes in this experiment. Only plants of the one- and five-aphid treatments ('aphid-exposed plants') were included in the colonisation and population analyses. The photographs of these plants taken 24 h after trim and at precondition were processed as reported in Section 2.4.2 to estimate the regrowth 24 h after the initial trim, the initial tiller diameter, the green shoot area of the plant (GSA) at precondition and the colour of the GSA at precondition. Aphid-exposed plants were considered to have been successfully colonised if wax depositions, dead or live adult root aphids and live offspring were found on them at harvest. The proportion of Petri dishes successfully colonised was

analysed using a Cochran-Mantel-Haenszel test stratifying the data by plant genotype-endophyte status (PG-E) group for the effect of aphid treatment ['stats' (R Core Team, 2017)]. Plant genotype effects were analysed in the same way, stratifying by aphid treatment and endophyte status. Plant genotypes were then pooled, and endophyte status analysed with a stratification for aphid treatment only. A Woolf test was performed in all cases to verify the assumption of similar log odds ratios in all strata ['vcd' package (Meyer *et al.*, 2017)]. PG-E group comparisons and post hoc pairwise comparisons were performed within each aphid treatment with Fisher's exact test for count data, adjusting the *p*-values for multiple comparisons through Benjamini-Hochberg's procedure ['rcompanion' (Mangiafico, 2018)]. One canonical discriminant analysis (CDA) for colonisation success was performed for each of the three groups S-CT, N-AR37 and S-AR37 [CDA; 'MASS' package (Venables & Ripley, 2002)]. Initial number of aphids placed, regrowth rate in the first 24 h after trim, initial tiller biomass (dry matter), root system score at precondition, green shoot area (GSA) at precondition and the green ratio of the GSA (G ratio, colour parameter; Chapter 2, Section 2.4.2, Equation 2.5) were used as explanatory variables for these CDA. The multivariate normality of the combined variables was verified graphically plotting the Mahalanobis distance. A Mann-Whitney-U test was applied when the values of these variables were considered in successfully vs. unsuccessfully colonised plants.

Alates were found in endophyte-free plants and plants with the AR1 symbiont. The frequency with which they appeared was estimated in those two groups only, with a generalised linear model of binomial type (binary model) including the number of live feeding aphids and duration of aphid occupation (Equation 2.16) as explanatory variables. The R^2 of this model was estimated using the 'MuMIn' package (Barton, 2018). Due to the large differences in duration of aphid occupation at harvest (38 to 111 days), the possible influences of water stress on plants and the generally small number of replicates per PG-E-aphid treatment group, a graphical, descriptive approach was used in preference to a numerical analysis for further population analyses. Besides the total number of alate nymphs and adults found in each Petri dish, these analyses considered: (i) the population structure at harvest [i.e. the number of live 'long-term feeding aphids' (adults and older immatures), the number of live first instar offspring, and the number of dead aphids], (ii) the dead/living ratio for all aphids found and (iii) the immature ratio, i.e. the proportion of living, older immatures in the live long-term feeding aphids. The data were plotted

once by population size pooling both aphid treatments. Thereby, 'population size' was defined as the number of living and dead older immatures and adult aphids. First instars were not included in the count to circumvent a possible influence of the harvesting procedure on the population size (Heathcote, 1972)]. In a second step, the data were plotted by duration of aphid occupation in days, distinguishing between data from the one- and five-aphid treatment group. To simplify the graphical representation, all data were pooled in categories of 20 aphids (0 to 20, 21 to 40, 41 to 60 etc.) for the graphs of population size and in five-day-means for the graphs of duration of aphid occupation. The biomass composition of the plant in percentage of root, green shoot and senescing and dead shoot at final plant harvest [dry weight] was also added to the graphs, as information on plant quality.

5.3. Results

5.3.1. Biology II experiment, behaviour

A total of 1105 records was provided by 18, 10, 11, 16, 20, 9, 19 and 11 aphids on N-AR1, N-AR37, N-CT, N-NIL, S-AR1, S-AR37, S-CT and S-NIL plants respectively, with 1 to 49 records/aphid. Aphid activity was unknown for 6% of the records. These records were excluded from the analyses. The aphids of all PG-E groups were mainly feeding (50 to 91% of all observations; Appendix 12, Section A12.1). Significant differences in the frequency of walking were observed amongst endophyte statuses and aphid ages [Figure 5.3; $\chi^2_{(3, N = 982)} = 12.88$, $p = 0.005$ and $\chi^2_{(12 , N = 982)} = 36.82$, $p < 0.001$, respectively; $R^2 = 0.135$]. Aphids living on plants with AR37 and CT symbionts were significantly more restless than aphids living on endophyte-free plants (probability multiplied by 6.04, $p < 0.001$ and 2.24, $p = 0.038$, respectively). Very young (< 15 days) and very old (≥ 85 days) root aphids seemed more restless than older nymphs and young adults (Figure 5.3). Aphids in their third and fifth week of life fed more frequently than one- to two-week-old aphids ($p = 0.003$ and $p = 0.015$, respectively). Disturbance during a check multiplied the probability of walking at the next check by a factor of 2.40 [$\chi^2_{(1, N = 982)} = 7.23$, $p = 0.007$].

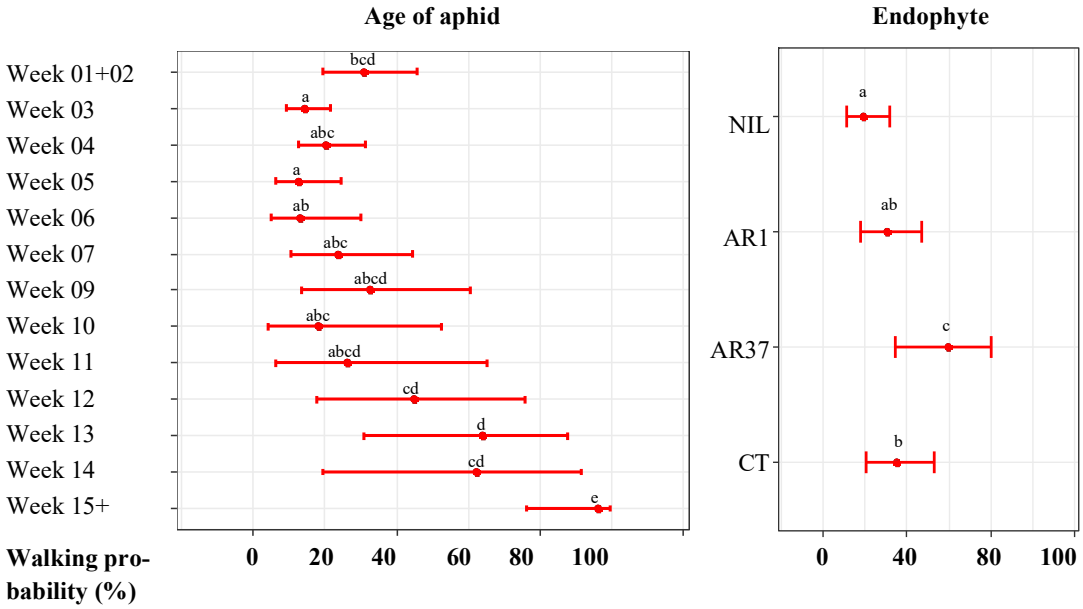


Figure 5.3. Effects of aphid age and endophyte status on aphid restlessness (back-transformed means with 95% confidence intervals). Means with the same superscript letter were not significantly different ($p > 0.05$). The graphs were produced by the package 'predictmeans' (Luo *et al.*, 2014).

5.3.2. Biology II experiment, feeding site characterisation

Although most sites where aphids fed were used only once, more than 14% were used for more than four days, with a maximum site use time of 72 days in one case. Defining the concept of a 'feeding site' was more difficult than initially anticipated, however. Some site identities could not be validated with photographs as distinctive marks or structures were lacking. Enlargements of photographs of several distinctive, long-stay sites (e.g. Figure 5.4) also showed that at least some of the aphids observed in the Biology II experiment changed the exact stylets insertion point over time while remaining in the same general location. These aphids thus "re-chose" periodically the same general site, without re-using exactly the same feeding point. For this reason, feeding site property data were analysed by observations (i.e. using all feeding site records) and not by feeding site (i.e. counting each feeding site only once) in the following.

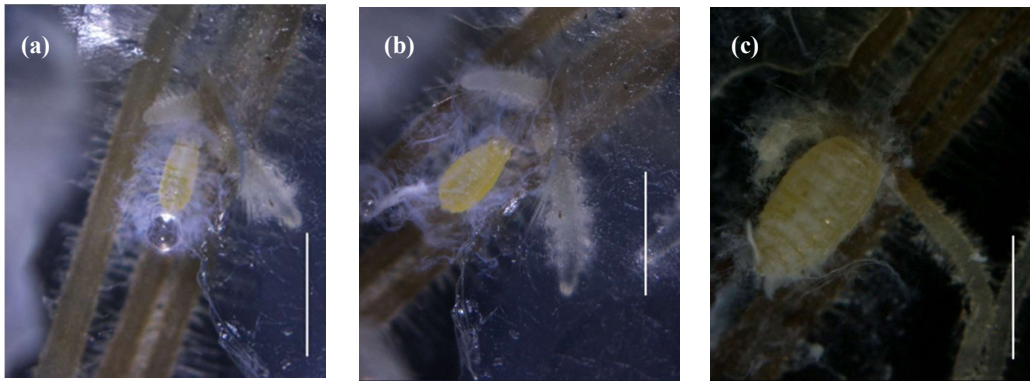


Figure 5.4. An aphid at the same site on its (a) 2nd, (b) 4th and (c) 13th day of life. The white scale bar represents 1 mm

Unbranched roots and roots of the 1st and 2nd branching order (BO) were the most common root type in plants of the Biology II experiment (Figures 5.5 and 5.6). *A. lentisci* fed mainly on branched roots of 1st to 2nd BO (35 and 63% of 522 checks providing branching order information, or 39 and 57% of the 238 feeding sites; Figures 5.5 and 5.6). Unbranched roots were hardly ever used for feeding. The difference between the theoretical BO availability and the BO use by aphids was generally significant ($p < 0.05$; exception: plant age periods II and VIII [36 to 65 and 221to 260 days]; Appendix 12, Section A12.2).

Immature and adult aphids used roots of similar BO on plants of a given age and PG-E group ($p > 0.100$ in all cases except for plants that were 171 to 220 days old; on plants of this age, the immature nymphs used 2nd BO roots more frequently than adults did; Appendix 12, Section A12.2). There were non-systematic, significant differences in BO use over the various plant age periods ($p < 0.05$ in all PG-E groups except N-AR37 and S-AR37; see Appendix 12, Section A12.2.1). BO use on plants of various PG-E groups was similar in young plants. Differences between PG-E groups became significant on plants older than 135 days at the checks only ($p < 0.05$; Appendix 12, Section A12.2.1).

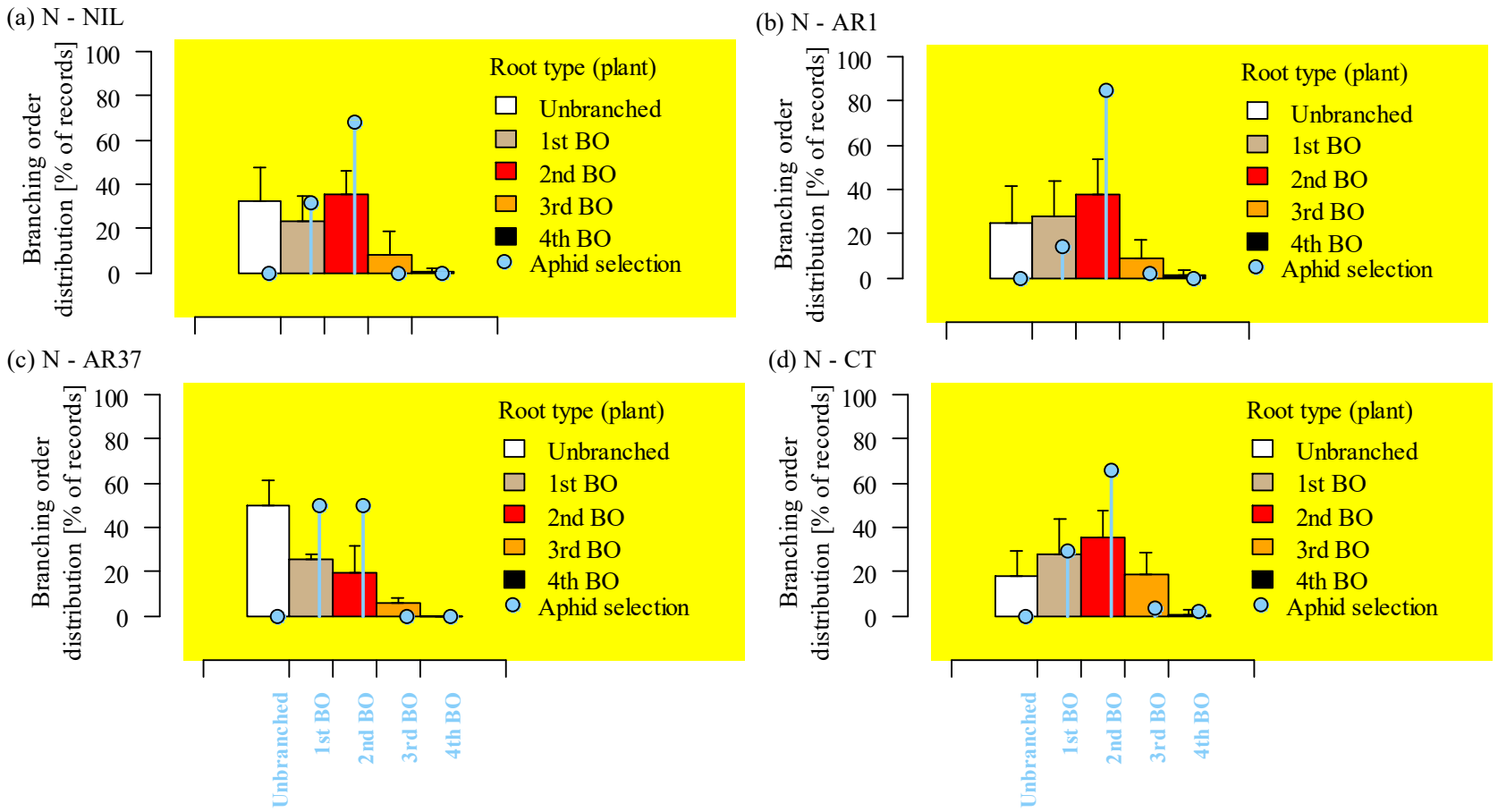


Figure 5.5. Root branching pattern of *L. perenne* plants of genotype N at final harvest (bars; means ± SD) for endophyte-free plants (a) or symbioses with AR1 (b), AR37 (c) or common-toxic CT (d), and usage frequency of each root type by *A. lentisci* (dots; percent of all records on the respective BO). The data for all plant age periods were pooled together for this representation.

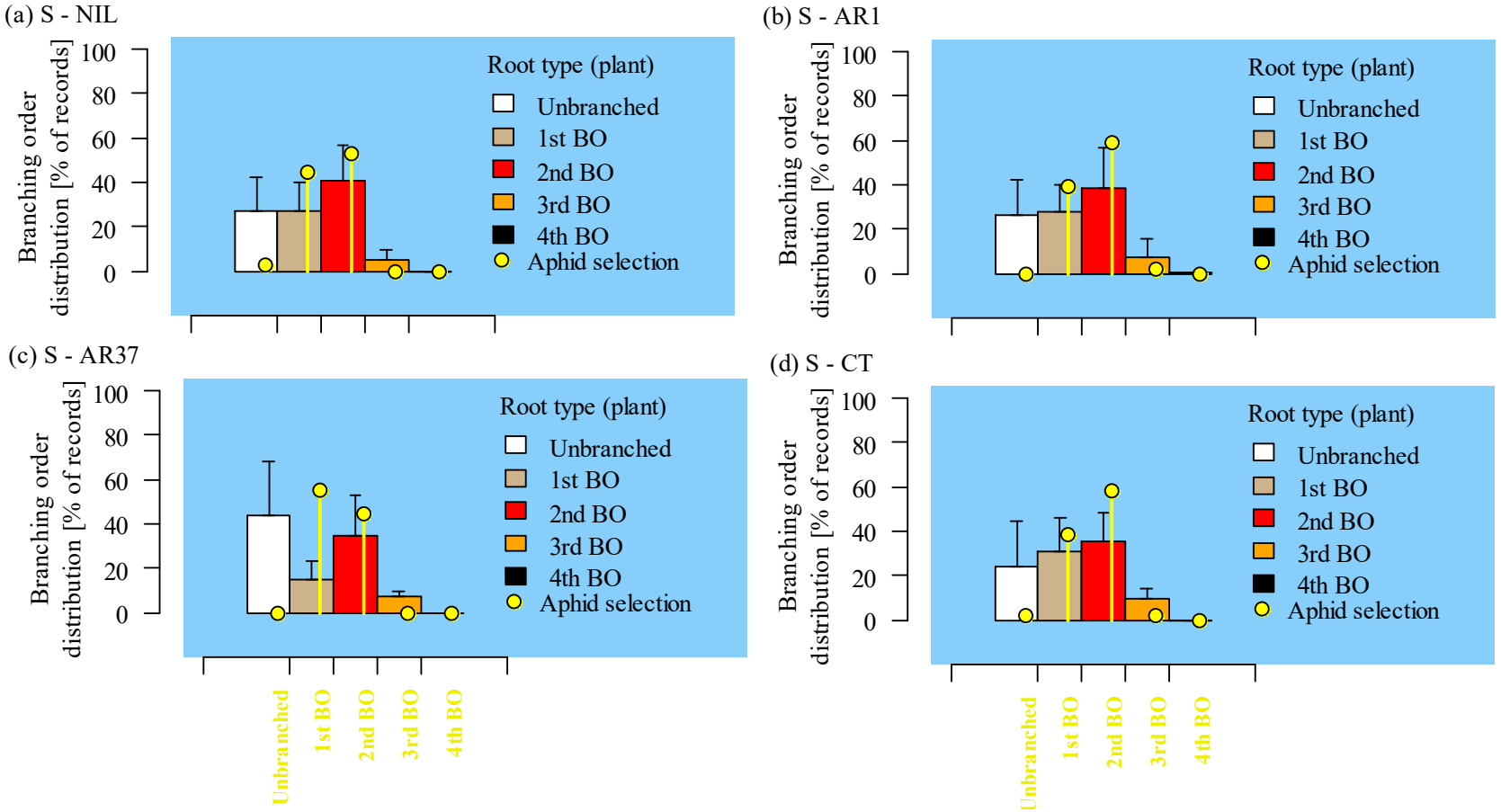


Figure 5.6. Root branching pattern of *L. perenne* plants of genotype S at final harvest (bars; means ± SD) for endophyte-free plants (a) or symbioses with AR1 (b), AR37 (c) or common-toxic CT endophyte (d), and usage frequency of each root type by *A. lentisci* (dots; percent of records on the respective BO). The data for all plant age periods were pooled together for this representation.

Root aphids primarily used the main axis and lateral branches of first branching order to feed upon (> 70 and > 20% of 562 useable records, respectively). Immatures and adults used similar positions ($p > 0.05$ in all PG-E groups and plant age periods except on N-CT plants of 136 to 170 days of age). Significant differences in position by PG-E group and changes in position with advancing plant age were observed ($p < 0.05$ for PG-E difference in all plant age periods except 66 to 85 and 221 to 260 days; $p < 0.05$ for the time effect within each PG-E group except N-AR37, S-AR37 and S-NIL; Appendix 12, Section A12.2.2). Lateral positions were more frequently used on plants of genotype N than on plants of genotype S of the same endophyte status and age [$\chi^2_{(2, N = 478)} = 29.98$, $p < 0.001$]. The use of lateral roots tended to increase in plants of the genotype N as the plants aged (Appendix 12, Section A12.2.2, Figure A12.2.2.1).

Immatures were more frequently found on white roots and less frequently on dark roots than adults (Figure 5.7 ; differences in root use patterns significant in three plant age periods, $p < 0.05$; Appendix 12, Section A12.2.3, Table A12.2.3.4). Dark root usage generally increased with plant age [Figure 5.7 (a) and (b); $p < 0.05$ in mature aphids of all PG-E groups, and immature aphids living on the roots of N-CT, S-AR1 and S-NIL plants], an observation presumably related to a reduced development of new, paler roots in old plants. Significant PG-E group effects were also observed ($p < 0.05$ in all plant age periods; Appendix 12, Section A12.2.3, Tables A12.2.3.2 and A12.2.3.3). Adults on N-AR1 plants and immatures on S-CT plants, in particular, were less frequently found on dark brown roots than their peers on plants of other PG-E groups (Figure 5.7).

The diameters of roots used by aphids ranged between 0.09 and 0.73 mm, with an average diameter of 0.32 ± 0.14 mm overall. Although aphids with a long body tended to feed on roots of large diameter (marginal, but significant correlation, Table 5.2), immature nymphs and adults, in general, used roots of comparable diameters ($p > 0.05$ in all PG-E-plant age periods except in 108 to 135 day-old S-NIL plants; Appendix 12, Section A12.2.4). Aphid age and root diameter were not correlated (Table 5.2). There were no significant effects of the plants' PG-E group within each plant age period. The average diameter of the roots used tended to decrease with the plant age however [Spearman's $\rho_{(264)} = -0.26$, $p < 0.001$]. The lateral roots used were significantly smaller than main roots [median diameters of 0.14, 0.21 and 0.38 mm for lateral 2, lateral 1 and main axis, respectively; $H_{(2)} = 32.82$, $p < 0.001$]. Differences within a PG-E group were not always significant, however (Appendix 12, Section A12.2.4 for individual values).

The diameter of roots of various colours did not differ significantly in most PG-E groups ($p > 0.05$ in all except S-NIL, in which the root diameters for dark brown feeding sites were significantly larger than the ones for pale brown or white roots [$H_{(2)} = 13.34$, $p = 0.001$; Appendix 12, Section A12.2.4, Table A12.2.4.2]).

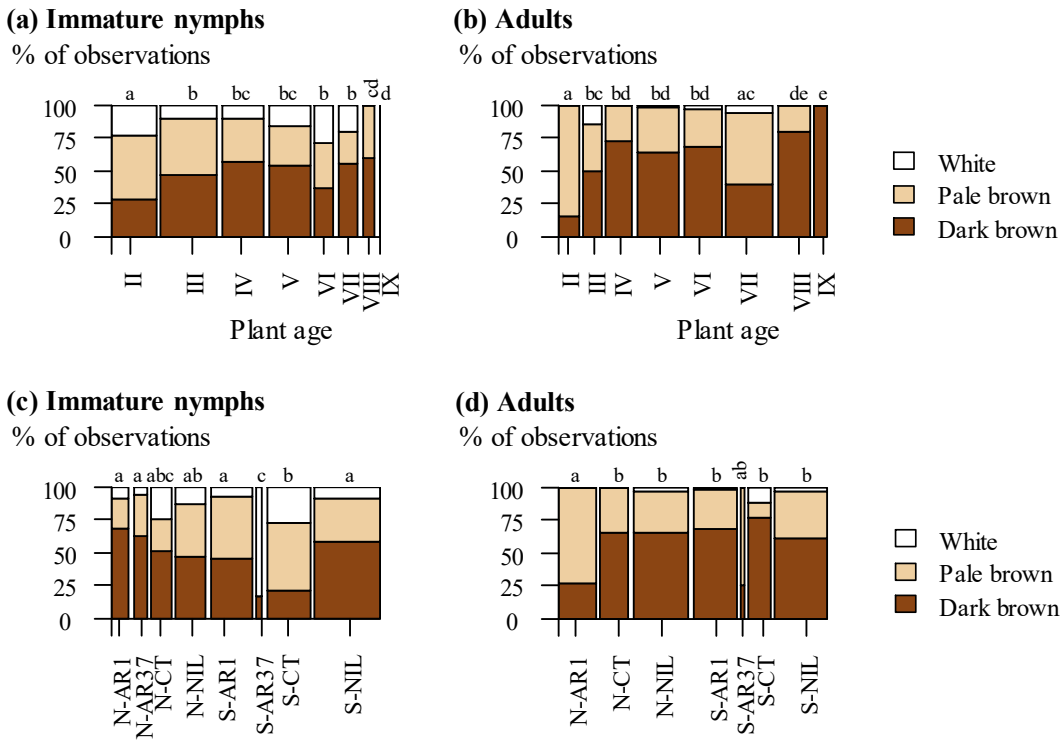


Figure 5.7. Colour of roots used by immature nymphs and adult *A. lentisci* aphids on perennial ryegrass of increasing plant age [plant age periods] (a, b), and plants of various genotypes (N or S) and endophyte statuses (AR1, AR37, CT or NIL) (c, d). Bar width: % of records in the respective category, with 298 as 100% records for (a) and (c), and 294 for (b) and (d), respectively. Bars with a same letter were not significantly different. Plant age: 36-65, 66-85, 86-107, 108-135, 136-170, 171-220, 221-260, and ≥ 261 days since initial trim for plant age period II to IX, respectively.

Table 5.2. Spearman's rank correlation coefficient (Spearman's ρ) for the relationship between the average root diameter and various aphid traits in the Biology II experiment.

Aphid traits	Spearman's ρ	Degree of freedom	*p*-value
Aphid age at check	0.10	264	*0.113*
Aphid length (L)	0.14	208	*0.043*
Aphid width (W)	0.08	141	*0.336*
Aphid body projection (L/2 ·W/2 · π)	0.11	138	*0.197*

5.3.3. Wax colony observations

The colonies contained an average of 4.16 ± 3.89 root aphids, 19 to 31 days after aphid placement. Plant genotype and endophyte status had no significant influence on the number of root aphids in a wax colony (Appendix 12, Table A12.3.1; these factors were not included in the model since $p > 0.05$ for them when the model was reduced). Only the diameter of the primary root explained some of that variance [effect coefficient: $e^{(1.71 \pm 0.52)} = 5.53$ aphids per additional mm root diameter, $p = 0.003$; residual model deviance 37.78 with 24 degrees of freedom; $R^2 = 0.772$]. Approximately 40 ± 31% of the aphids found in the colonies were classified as 'old' aphids. Younger aphids fed significantly less frequently than older aphids (median: 82% and 100%, respectively; Wilcoxon's signed rank test $Z = 141$, $p = 0.002$). The proportion of young aphids feeding increased with root diameter [Spearman's $\rho_{(19)} = 0.55$, $p = 0.009$]. Most colonies (92%) comprised at least one exuviae (Figure 5.8) and about half of them had more than three. The average number of exuviae per aphid was more than one in nearly 60% of all colonies. This supported previous observations that once selected, feeding sites were used for a long period of time (Section 5.3.2). As for the total number of aphids, the root diameter was the only significant parameter explaining the number of exuviae per colony (coefficient: $e^{1.57 \pm 0.57} = 4.78$ exuviae more for each additional mm root diameter, $p = 0.011$; residual deviance of the model: 37.17 on 24 degrees of freedom; $R^2 = 0.478$). There was a weak positive correlation between the number of exuviae and the wax area [Spearman's $\rho_{(35)} = 0.48$, $p = 0.002$].

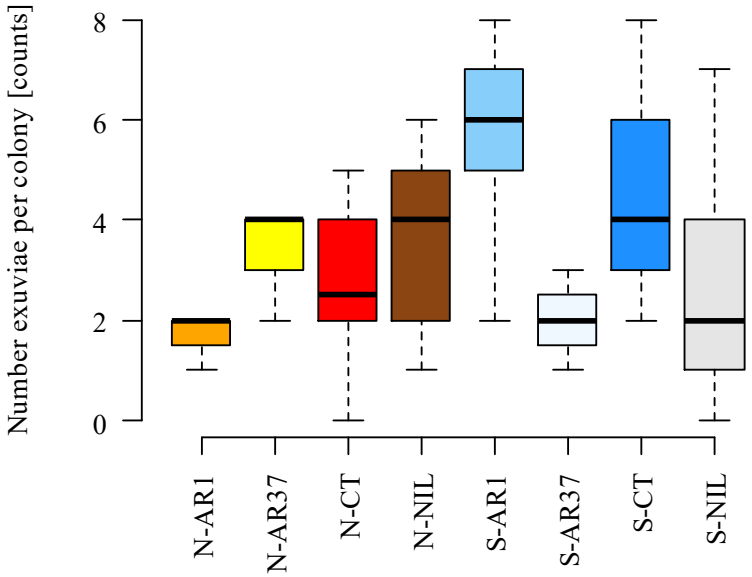


Figure 5.8. Number of exuviae in a colony, represented as boxplot. The colonies were collected on perennial ryegrass plants of either genotype N or S, without (NIL) or with endophyte symbiont of an AR1, AR37 or common-toxic (CT) strain, 19 to 31 days after aphid placement.

About one-third of the colonies observed encased more than one root. As in the Biology II experiment, the colonies on single roots were found on roots of 1st and 2nd BO only (58 and 42%, respectively), on the main axis mainly (69%). These colonies covered 1.47 to 14.23 mm root length (5.76 ± 3.10 mm). Plant genotype-endophyte status groups provided the only significant explanation for variances in wax length measurements (n = 28, log-likelihood ratio: 22.19, p = 0.002; R^2 = 0.600). These differences were not sufficient for significance by post hoc comparisons with Benjamini-Hochberg adjustment, however ($p > 0.05$). Wax length and aphid numbers were not correlated [Spearman's $\rho_{(33)} = 0.26$, $p = 0.174$]. The average wax area of the colonies was of 21.25 ± 17.01 mm^2, and colonies with 'medium' wax density were the most common (46%). Wax area increased with both root diameter and length of root covered by wax (Table 5.3). Colonies with low wax density were significantly smaller than colonies of medium wax density (Table 5.3). PG-E groups appeared significant overall too, but no pairwise post hoc comparison was significant ($p > 0.05$). The total number of aphids in a colony and the colony's wax area were correlated [Spearman's $\rho_{(33)} = 0.53$, $p < 0.001$].

Table 5.3. Coefficients of the linear mixed-effects model describing the wax area.

Variables	coef	se(coef)	z	*(p-value)*	L ratio	
					χ^2	*p*-value
(Intercept)	2.31	4.84	0.48	*(0.645)*		
Root diameter	22.86	6.16	3.71	*(0.008)*	17.56	*< 0.001*
Wax-coated root length	1.72	0.83	2.06	*(0.078)*	6.74	*0.009*
Wax density					18.54	*< 0.001*
High	-9.49	6.41	-1.48	*(0.182)*		
Low	-13.58	3.62	-3.76	*(0.007)*		
PG-E					15.95	*0.026*
N-AR1	-0.88	8.78	-0.10	*(0.923)*		
N-AR37	0.00	5.99	0.00	*(1.000)*		
N-CT	-4.00	8.65	-0.46	*(0.656)*		
N-NIL	11.37	5.68	2.00	*(0.081)*		
S-AR1	5.50	5.55	0.99	*(0.351)*		
S-AR37	-4.89	7.35	-0.66	*(0.525)*		
S-CT	-12.02	8.89	-1.35	*(0.213)*		
Random effects (plant identity)						
Intercept					0.00	*1.000*

coe: model coefficient; se(coef): standard error of model coefficient; L ratio: log-likelihood ratio; PG-E: plant genotype (N or S)-endophyte status (with AR1, AR37 or CT endophyte strain, or without any endophyte [NIL]). Number of observations: 27, model $R^2 = 0.768$.

5.3.4. Population experiment

Although six Petri dishes of the no-aphid treatment contained immigrant root aphids, the immigrant numbers were generally low (one to three neonate root aphids/Petri dish, and 25 in one exceptional case, only). By the definition given in Section 5.2.3., this equated to less than 4% of all no-aphid Petri dishes being successfully colonised. Contamination was thus a minor problem in this experiment.

Unsurprisingly, plants supplied with five first instar nymphs were more frequently colonised than plants supplied with one nymph only [Table 5.4; overall 84% vs. 68% for plants supplied five and one nymph(s), respectively; $\chi^2_{(1, N=108)} = 4.05$, $p = 0.044$]. While no difference was observed for plant genotype [$\chi^2_{(1, N=108)} = 2.95$, $p = 0.086$], successful colonisation was significantly less frequent on plants with AR37 endophyte [$\chi^2_{(3, N=108)} = 40.43$, $p < 0.001$] than on plants of all other endophyte statuses. The difference between

AR37 and CT plants was not statistically significant in the five-aphid-treatment group, however. There may also have been a significant plant genotype-endophyte status (PG-E) interaction for plant supplied with one aphid: N-CT plants were significantly better colonised than N-AR37 plants (Table 5.4; $p = 0.044$) but S-CT plants had a similar colonisation success to S-AR37 plants, and only S-AR1 plants were better colonised than S-AR37 ($p = 0.044$). Although Fisher's test found significant differences between PG-E groups for plants supplied with 5 aphids ($p < 0.001$), these differences were too small to be significant by post hoc tests corrected for multiple comparisons (Table 5.4).

Full discrimination between colonised and non-colonised plants was achieved in the canonical discriminant analyses for S-AR37 and N-AR37 plants only (Figure 5.9, Table 5.5). As only one plant of each aphid treatment was successfully colonised in the S-AR37 group, the analyses of this group may not be meaningful to interpret (these two samples could have been special cases). Successfully colonised plants of the N-AR37 group were supplied 5 starter aphids and developed from larger tillers with a better initial regrowth after 24 h and a more developed root system at aphid placement (Table 5.5). In the S-CT group, the G ratio (colour index) of the green shoot area at precondition was significantly higher in colonised than in non-colonised plants (Table 5.5; $p = 0.022$). This was generally due to lower R-, G- and B-value measurements in successfully colonised S-CT plants compared to not-colonised S-CT plants. The difference was significant for the B-value only, however ($p = 0.035$; data not shown).

Table 5.4. Effect of plant genotype (N, S)-endophyte status (AR1, AR37, CT and SNIL) combinations and number of root aphids placed (1 vs. 5 first instar nymphs) on the colonisation success.

Plant genotype	Endophyte status	1 aphid placed			5 aphids placed		
		Colonised		Success	Colonised plants		Success
		Yes	No	[%]	Yes	No	[%]
N	AR1	7	1	88 $^{abc\,(B)}$	7	0	100 $^{a\,(B)}$
	AR37	1	6	14 $^{a\,(A)}$	4	2	67 $^{a\,(AB)}$
	CT	6	0	100 $^{bc\,(B)}$	7	0	100 $^{a\,(B)}$
	NIL	6	1	86 $^{abc\,(B)}$	7	0	100 $^{a\,(B)}$
S	AR1	7	0	100 $^{c\,(B)}$	7	0	100 $^{a\,(B)}$
	AR37	1	5	17 $^{ab\,(A)}$	1	4	20 $^{a\,(A)}$
	CT	3	4	43 $^{abc\,(AB)}$	4	2	67 $^{a\,(AB)}$
	NIL	8	1	89 $^{abc\,(B)}$	6	0	100 $^{a\,(B)}$

Colonisation was considered successful if there was at least one adult aphid in the Petri dish (dead or alive), offspring and significant amounts of wax at final harvest.
Groups with different lowercase superscript letters were significantly different ($p < 0.05$) according to Fisher's exact test for count data with Benjamini-Hochberg adjustment for multiple comparisons; Groups with different (uppercase superscript letters) were significantly different according to Fisher's exact test without *p*-value adjustments.

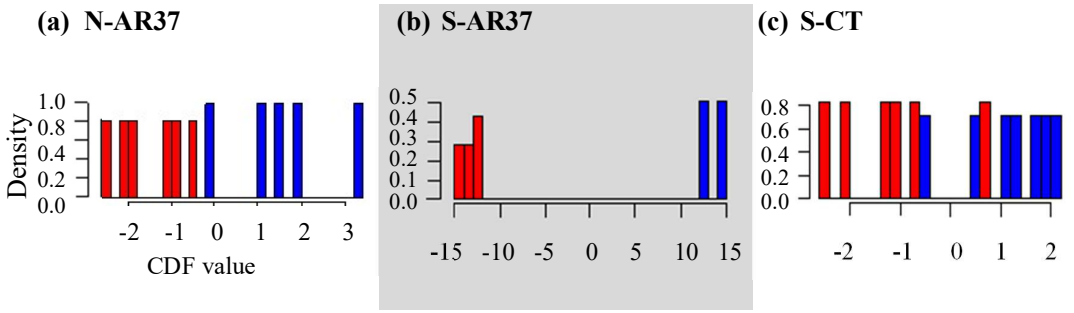


Figure 5.9. Separation achieved by the canonical discriminant functions (CDF) of Table 5.5. A value was calculated for each sample using a linear function of the variables considered with CDF coefficients. A density plot of the results was drawn marking the values from successfully colonised plants in blue and the ones of plants on which colonisation failed in red.

Table 5.5. Standardised canonical discriminant analysis coefficients for colonisation success analyses in plants of various plant genotype (N, S) and endophyte status (AR37 or a common-toxic CT endophyte).

Variables	N-AR37	S-AR37	S-CT
Number of aphids placed	0.843	-6.715	0.735
Regrowth 24 h after trim [mm]	1.091	6.502	-0.394
Tiller biomass after trim [g dry matter]	0.79	4.16	-0.264
Root system score at precondition	0.501	-8.568	0.027
Green shoot area at precondition [mm^2]	0.313	2.354	0.516
Colour of green shoot area at precondition (G ratio)	-0.137	11.925	1.124

Each column represents the result of one distinct canonical discriminant analysis; the coefficients of the most influential variables are shaded. G ratio: green ratio, Section 2.4.2.

Root aphid populations did not show any signs of decline on colonised plants for up to 60 days after the placement of the first neonates (Figure 5.10 and Figure 5.11). The populations harvested before that time point were exclusively apterous, had stable population structures of approximately 88 ± 5% older immature aphids and 12% adults (e.g. Figure 5.10 b), and contained very few dead aphids (e.g. Figure 5.10 a). Up to that time, the total number of living aphids had increased (e.g. Figure 5.10 c). Clear signs of decline happened only beyond this time point (or after 80 days for all parameters but alatae presence on plants on which five starter aphids had been placed; Figure 5.11). Beyond 60 days, Figure 5.10 (c) suggested further a pattern of population increase and decrease in 20 to 30 days intervals. This time interval would match the generation time reported for *A. lentisci* in Chapter 3. However, it is also possible that this apparent pattern resulted from pooling all PG-E groups by a small number of samples.

Whether the observed colony decline was of an aphid-biological nature or was the result of decreasing plant quality in the model system used for this research is unclear. The proportion of green shoot biomass in experimental plants started to decline severely around 70 to 80 days too (Figure 5.10 d, Figure 5.11 d). Figure 5.12 suggests, however, that there was no obvious link between the population size (number of established root aphids that spent some time feeding on a plant) and typical population decline signs such as increased mortality or decreasing immature/mature ratio.

Migrating morphs in the form of nymphs with wing buds (third and fourth instar) and winged adults were sighted in 9 Petri dishes or 14 and 21% of the endophyte-free

plants and plants with AR1 symbiont, respectively, with between 1 and 25 immature and adult alatae per plant. No direct maternal effects explained their appearance as the Petri dishes with alates had different mothers. Their appearance was more likely on plants with a long aphid occupation (probability multiplied with $e^{0.0669 \pm 0.0252} = 1.07$ per day, $p = 0.008$) and a larger number of live long-term feeding aphids (probability multiplied with $e^{0.0205 \pm 0.0085} = 1.02$ per live aphid, $p = 0.016$; model residual deviance: 34.96 by 52 degrees of freedom; $R^2 = 0.525$). In this experiment, that meant alatae appeared in populations older than 60 days, on plants with more than 50 live or dead older immature and adult aphids ('long-term feeding aphids'), or at loads of > 27 live long-term feeding aphids/cm^2 green shoot area, > 1730 live long-term feeding aphids/g green shoot dry matter or > 430 live long-term feeding aphids/g root dry matter at final harvest (Appendix 12, Figures A12.4.1, A12.4.2, A12.4.3).

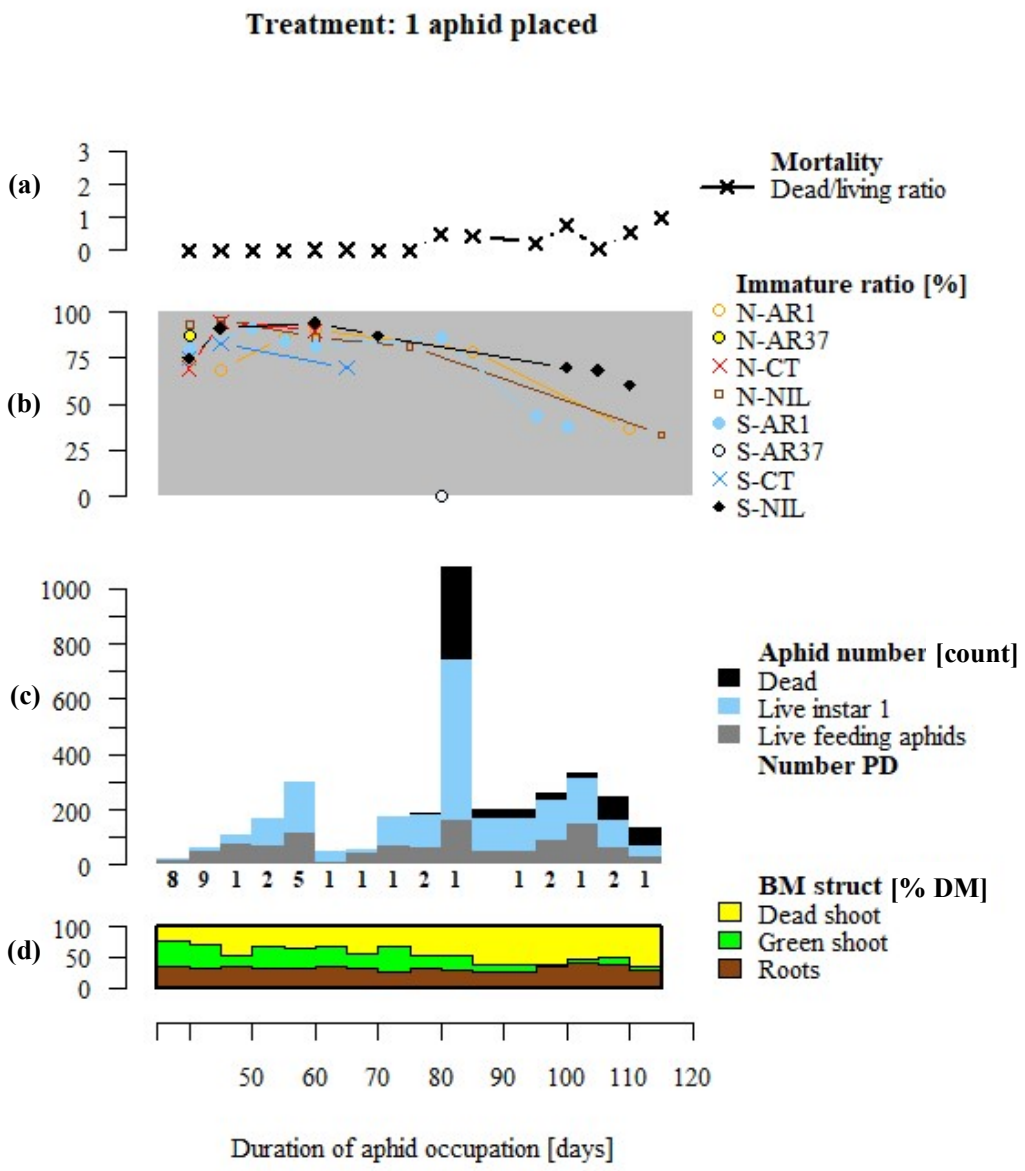


Figure 5.10. Relationship between duration of aphid occupation, aphid population and plant parameters at harvest in the Population experiment, on plants supplied with one starter aphid. The experiment was conducted on plants of genotype N or S without endophyte (NIL) or with one of three endophyte strains (AR1, AR37 or CT). The data were pooled in five-day-means. Parameters: **(a)** Dead/living ratio, **(b)** Proportion of immatures in the long-term feeding aphid population (presented by plant genotype-endophyte group), **(c)** Number of aphids collected, **(d)** Plant biomass distribution; DM: dry matter; PD: Petri dish, i.e. plant sample

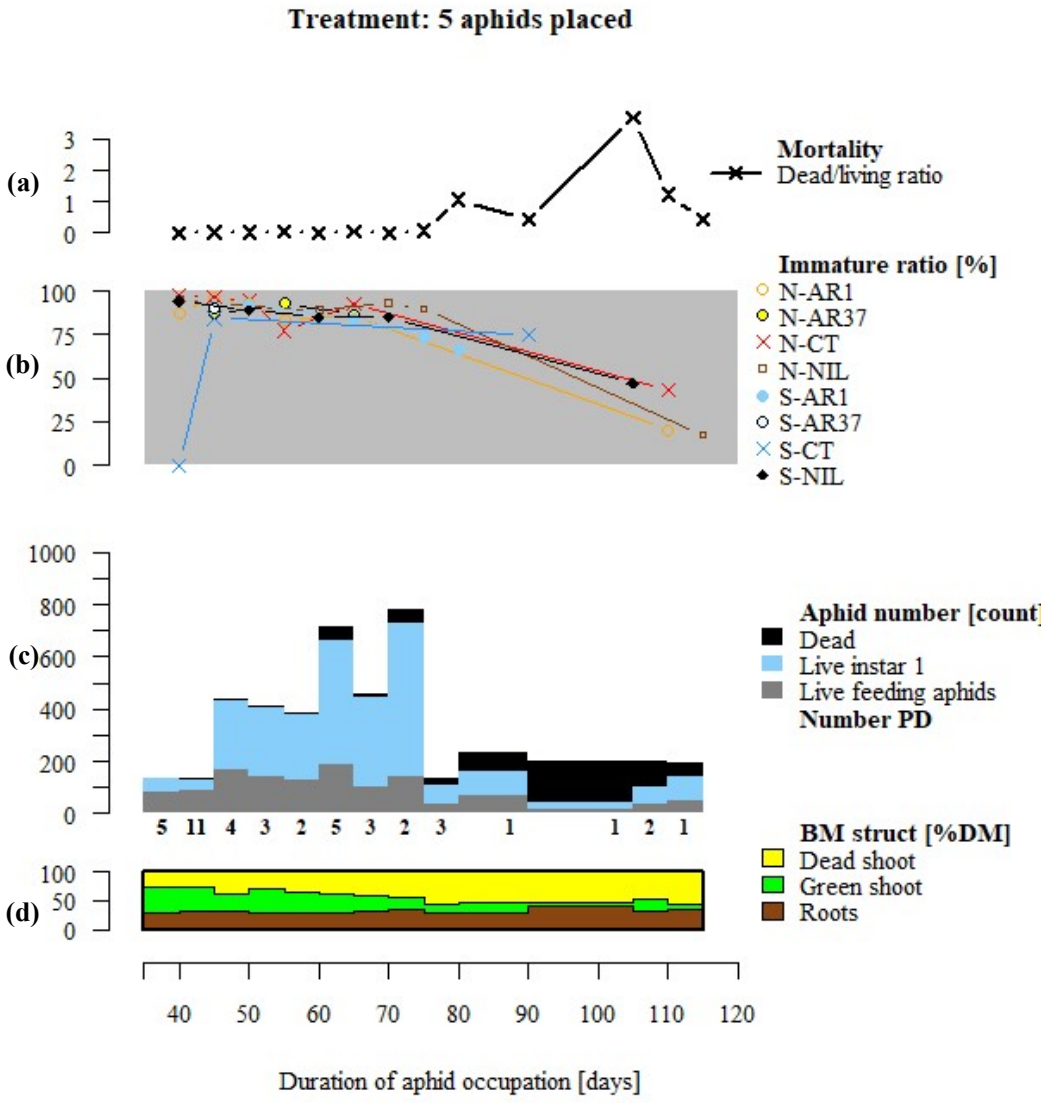


Figure 5.11. Relationship between duration of aphid occupation, aphid population and plant parameters at harvest in the Population experiment, on plants supplied with five starter aphids. The experiment was conducted on plants of genotype N or S without endophyte (NIL) or with one of three endophyte strains (AR1, AR37 or CT), i.e. on plants of eight PG-E groups. The data were pooled in five-day-means (and over all PG-E groups for all parameters except the immature ratio). Parameters: **(a)** Dead/living ratio, **(b)** Proportion of immature in the long-term feeding aphid population (presented by plant genotype-endophyte group), **(c)** Number of aphids collected, **(d)** Plant biomass distribution; DM: dry matter; PD: Petri dish, i.e. plant sample.

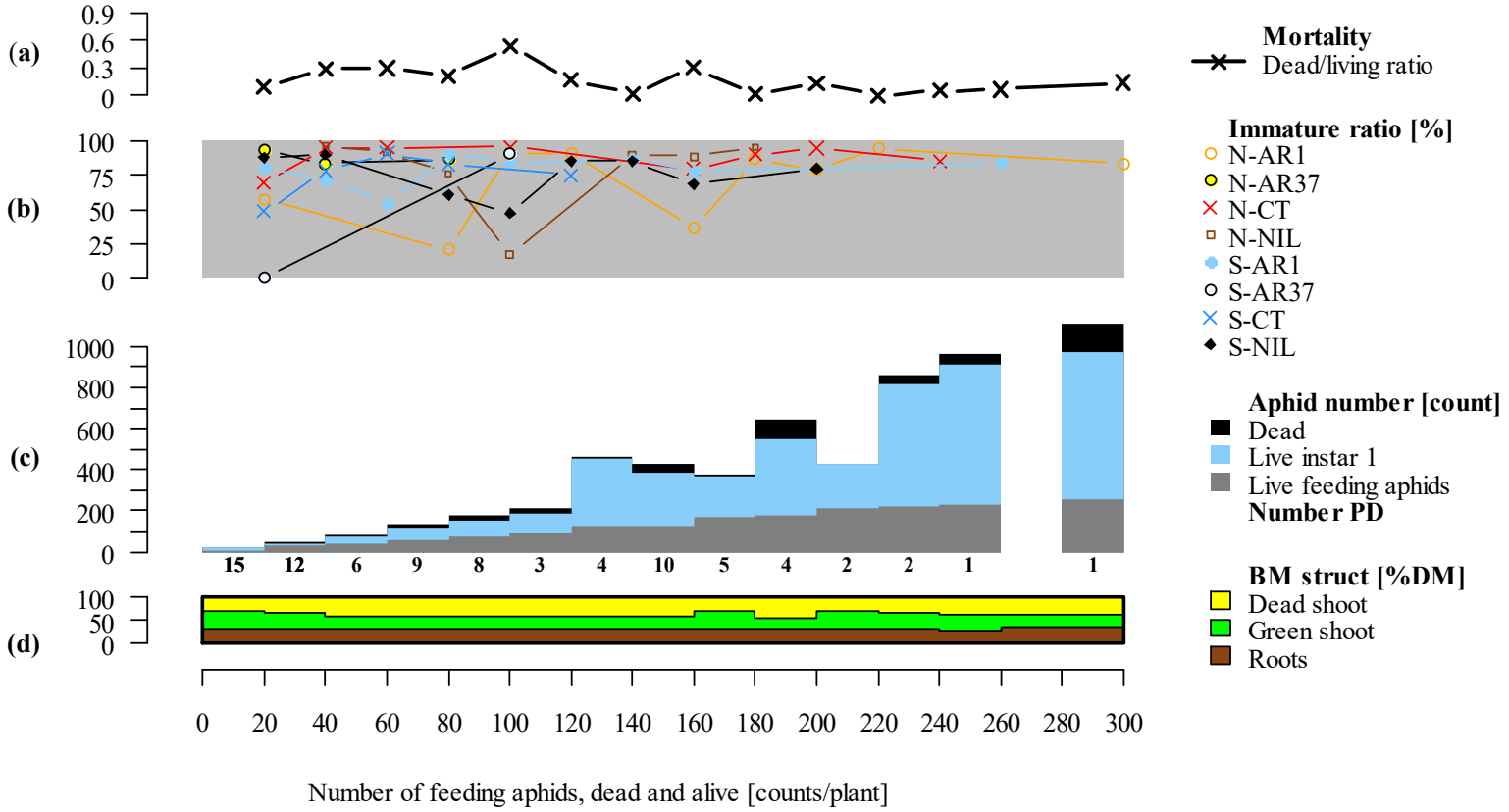


Figure 5.12. Population and plant parameters in relation to the total number of feeding aphids, as recorded at the harvest of the Population experiment. The experiment was conducted on plants of genotype N or S without endophyte (NIL) or with one of three endophyte strains (AR1, AR37 or CT). The data were pooled in categories of 20 aphids. Parameters: **(a)** Dead/living ratio, **(b)** Proportion of immature in the long-term feeding aphid population (presented by plant genotype-endophyte group), **(c)** Number of aphids collected, **(d)** Plant biomass distribution; DM: dry matter; PD: Petri dish, i.e. plant sample.

5.4. Discussion

The three experiments presented in this chapter have provided further insights into the establishment, colonisation and population ecology of the root aphid *Aploneura lentisci*. This was realised by examining feeding site usage on perennial ryegrass, plant traits favourable for colonisation, and more general information on population dynamics under controlled environment conditions. It is possible that the roots observed differed somewhat in their anatomy and physiology from roots grown in the field. Roots developing in nutrient solutions are known to be less branched than roots growing in a soil environment, for example (James & Hutto, 1972). The findings discussed here may, therefore, be useful to plan experiments in comparable laboratory conditions, but should be used with care when developing hypotheses for experimental work in field conditions.

Two conclusions were drawn from the observations of *A. lentisci*'s root use in perennial ryegrass: (i) the root aphid uses relatively young, still growing roots, and (ii) it does so in a relatively sustainable way since it is using feeding sites for some length of time. That immature root aphids are found more frequently on young roots has already been reported by Popay and Cox (2016). That the developmental stage or age of a root as described by its branching order (BO) could be an important criterion for root aphids of all ages, was a new finding, however. Virtually no roots other than those of 1st and 2nd BO were fed upon (Section 5.3.2; Figure 5.13). It may be argued that some of the plants in the Biology II experiment were quite old when the last starter aphids were placed on them, possibly of low health, and, thus, not the best model for a statement on root use. Physiological factors such as an increasing lignification, for example, could have made it more difficult for root aphids to probe and use the oldest roots in this experiment (Popay & Cox, 2016). Since the same BO use was also observed in all other experiments in which the branching order was recorded (colony wax observations, for example), these findings are still considered to be robust, however. A plant's allocations of resources to its roots could explain the observed absence of roots of 3rd and 4th BO in *A. lentisci*'s diet. While shoots direct significant amounts of assimilates into unbranched and branching roots of 1st and 2nd BO, comparatively little assimilate is allocated to roots when they start producing tertiary lateral branches, i.e. when they become roots of 3rd BO (Chapter 1, Table 1.3, carbon expenditures). Robin (2011) speculates that re-allocations within a root to fuel further branching and root respiration occurs in aging roots beyond the 3rd BO. It

is thus likely that, in addition to lignification issues, little high quality phloem transits through the vascular bundles of the main axis and the lateral 1 branches in roots of 3rd and 4th BO, thereby inhibiting their use by the aphids. Why unbranched roots were also hardly ever used by feeding root aphids (Figure 5.13, Figure 5.5, Figure 5.6) is less clear, however. A larger root diameter in newly produced, unbranched roots (Robin, 2011) could offer an explanation, given that the length of an aphid's stylets limits its access to phloem tissues (Manheim & Wool, 2003). Assuming a stele-to-root diameter ratio of 0.41 for perennial ryegrass plants (Soper, 1959), the minimum distance from a root's surface to its vascular bundles is likely to be at least 0.59 · (root diameter)/2. Measurements on living specimens (Appendix 9, Section A9.1) established that the average rostrum length in *A. lentisci* was 0.27 to 0.32 mm. Useable roots would, therefore, have to measure less than 0.91 to 1.08 mm diameter, or 0.60 to 0.72 mm if roots aphids were behaving similarly to sycamore aphids, i.e. limiting their phloem usage depth to 66% of their total stylets' length (Dixon & Logan, 1973). This prediction agreed with average and maximum root diameters measured respectively in the Biology II experiment (0.32 and 0.73 mm) and during the Colony wax observations (0.5 and 1.06 mm). The diameters of the few unbranched roots used during the Biology II experiment were similar to the root diameters measured in branched roots, which appears to support this explanation (Appendix 12, Section A12.2.4, Table A12.2.4.2). Alternatively, physical access to a feeding site may have simply been limited (Dixon, 1985) on the new roots by the root hairs found immediately behind the elongation zone in unbranched roots.

The cause of the observed position of feeding sites (on main axis and lateral 1 branches, close to the root's attachment point to the tiller; Section 5.3.2) is more speculative. It could be a way to maximise access to the resources. Indeed, the sugar concentration is higher close to the source of production in plants (Reece *et al.*, 2011) and it is possible that this applies also to amino acid concentrations. Furthermore, being upstream of all sinks (root tips) may also give root aphids an advantage. The position use pattern may again be governed by root diameter, however, with stylet length limiting access to phloem in larger root (Manheim & Wool, 2003). Low root diameters may also pose a problem to root aphids, though. Individual root aphids and root aphid colonies have to avoid a wounding reaction from the plant to be able to use a feeding site for some time (Dixon & Kindlmann, 1994) and can, therefore, remove only small volumes of phloem from small roots. Since branching results in roots of increasingly finer diameter

(Robin, 2011), lateral 3 and lateral 4 branches (i.e. tertiary or quarternary branches) may simply have been too small for root aphids to use over a longer time. This would also explain the positive effects of an increasing root diameter on various colony traits such as the number of root aphids in a colony, the percentage of feeding immatures, and the number of exuviae (a proxy for the length of time a site was used; Section 5.3.3)

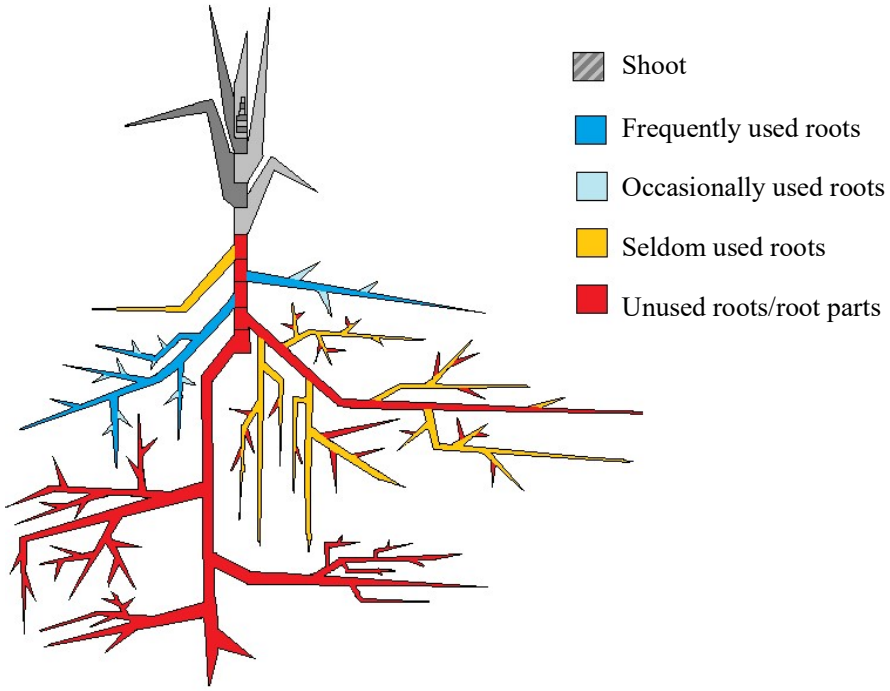


Figure 5.13. Graphical representation of root use in root aphids on perennial ryegrass grown in nutrient-enriched agar. (Use definition by percent of records: > 5%, 2-5%, 1%, and 0% for frequently, occasionally, seldom and unused roots or root parts, respectively)

The root aphid *A. lentisci* is reported to be largely sedentary as an adult (Popay & Cox, 2016). This view was confirmed in the experimental setup by the large amount of time spent feeding rather than moving (Section 5.3.1) and the site fidelity/root re-use during the Biology II experiment (Section 5.3.2). Furthermore, it was indirectly confirmed by the number of exuviae embedded in the wax surrounding colonies (Figure

5.8). Reasons for this root use pattern could include possible benefits from a wax cover [e.g. protection against fungal attacks, humidity and UV, or reduction of the cuticular transpiration rate (Pope, 1983; Smith, 1999)]. Site re-use could also offer the advantages of a faster access to the phloem (re-use of stylet sheaths) and enhanced feed quality (systemic throughout the leaf and not limited to a single feeding site; Morris & Foster, 2008). Previous aphid feeding was found to enhance the levels of essential amino acids for later feeding by *Diuraphis noxia* Kurdjumov aphids on susceptible wheat plants, for example (Morris & Foster, 2008). As *A. lentisci* displayed slight changes in exact stylet insertion point on a same site, enhanced feed quality and protection by wax would appear more likely explanations than faster access, however.

The greater activity of young nymphs and the more settled behaviour of adults (usually feeding, Figure 5.3; percentage of feeding aphids in Section 5.3.3.) is consistent with reports about root aphids on ryegrass (Popay & Cox, 2016) and about other aphid species in general (Tomczak & Müller, 2018). The reduced feeding activity and a (non-significant) tendency towards higher mobility in root aphids on endophyte-infected plants compared to endophyte-free plants have also been reported in the past on other plants (e.g. tall fescue; Jensen & Popay, 2007). That mature aphids beyond their 11th week of life would become increasingly restless again was unexpected, however. It is possible that this was a response to the disturbance by light, as the aphids were exposed to several minutes of light at two-day-intervals while offspring were collected during the Biology II experiment (Chapter 3, Section 3.2.1.2). Light sensitivity is, a fairly common trait in underground aphid species (Adams & van Emden, 1972). The increased restlessness could also have been a reaction to decreasing host suitability (Klingauf, 1987), however, since the plants aged together with the root aphid.

The symbiotic relationship between ryegrass and endophyte strain AR37 or a combination of plant genotype S and the common-toxic endophyte (S-CT) were primary causes for colonisation failure in the Population experiment. This observation was mostly consistent with previous experiments in this thesis (Biology II experiment) and findings in the field (Agricom & PGG Wrightson Seeds, 2015; Popay & Cox, 2016). The negative effect of AR37, however, appeared to be mitigated in the N genotype by strong plant growth. Successfully colonised plants in this group all had a large initial leaf regrowth rate 24 h after trim and a well-developed root system by the time of root aphid placement (Table 5.5). Plant growth parameters could also have influenced the colonisation by root

aphids in the S-CT group. Since a loss of chlorophyll results in an increase in visible light reflectance (Carter & Knapp, 2001), the lower blue reflectance (B-values) in the few successfully colonised S-CT plants could, on the contrary, express higher photosynthetic capabilities and higher growth potential. These observations would support Popay and Cox (2016)'s view that plants with a better (root) growth are likely to stimulate root aphid population development. It is possible that the parameters monitored during the Population experiment were only insignificant traits, however, changing in parallel with more significant parameters such as the concentrations of endophyte alkaloids. More research will also have to demonstrate the causality effect of plant growth on colonisation success.

Intrinsic factors such as size, fecundity, mortality, and migration rate can be the cause of extreme oscillations in aphid numbers in the field (Kindlmann & Dixon, 2010). Understanding these mechanisms is an important step towards better aphid population forecasting systems (Dixon, 1973; Kindlmann & Dixon, 2010). Populations are known to initially increase dramatically (Kindlmann & Dixon, 2010) before reaching a stable state (Campbell & Hutchison, 1995). Up to a certain level, group feeding can benefit the aphids in this phase: a group of aphids can act as a stronger sink for assimilates and can cause the plant it lives upon to react to it as if it was a plant bud and re-allocate more nutrients towards the aphids (Michaud *et al.*, 2006; Miles, 1989a). Beyond a certain population size, however, the high performance on preferred plants or sites may reduce plant or site quality (Zytynska & Weisser, 2016). Under the influence of crowding, smaller adults with lower fecundity may be produced (Dixon, 1973). Under high population stress and/or declining host suitability, aphids colonies tend to also produce higher percentages of alates (Dixon, 1973) and to leave the host plant (Klingauf, 1987). As migration and other population limitation processes begin, a steep decline in abundance may follow the initial population increase (Kindlmann & Dixon, 2010). On the agar-embedded perennial ryegrass plants of the Population experiment, a development as described above did occur. Most neonates placed on roots in this trial developed within 5 to 6 weeks to a stable population, similar in its composition to the one reported for a stable sugar-beet root aphid (*Pemphigus betae* Doane) population in a hydroponic system (Campbell & Hutchison, 1995). The first clear signs of population reaction to density stress and/or plant quality decline were recorded around eight to nine weeks, at aphid loadings of approximately 430 living aphids/g root or 50 individuals per plant of one to three tillers in that system

(Section 5.3.4). Whether these thresholds would be relevant in the field will have to be examined more closely. The aphid carrying capacity of plants in the field is, perhaps, not as limited, as it was in the experimental system (in which the plants may have suffered from nutrient and water stress). Aphid carrying capacity is thought to increase with the season until plant maturity (Kindlmann & Dixon, 2010) but this may be quite different in perennial grasslands that are grazed and well fertilised. The findings by Popay and Gerard (2007) supported this view, by reporting healthy colonies with average aphid loadings that surpassed the 430 aphids/g root dry matter on potted AR1 plants. Lags in alatae appearance also need to be considered. Density stress leading to alatae production is certain to have commenced some time before alatae were observed. Whether *Acyrthosiphon pisum* Harris virginoparae maintain the ability to produce wings is already established around the first moult, for example (Section 1.2.4.5; Ogawa & Miura, 2013), i.e. at an instar at which the future alata cannot be microscopically distinguished from a future aptera. More knowledge of the factors triggering alatae production and the stage or generation responding to crowding in *A. lentisci* is required to establish a critical density threshold. Nevertheless, the observations in the Population experiment support the view that *A. lentisci* dynamics are likely to be similar to what is known for other aphid species which can thus provide basic information for future studies on root aphid population dynamics.

5.5. Conclusion

A. lentisci appeared to principally feed on the main root axis and lateral 1 branches of 1st and 2nd branching order roots of perennial ryegrass with average diameters of 0.3 to 0.55 mm. This use may be explained by the plant's nutrient supply to growing roots, the necessity of aphids to divert resources before they reach other sinks (root tips) and other feeding limitations. Observations and exuviae counts in colonies confirmed that root aphids re-use specific sites over a long period of time. Behavioural records suggested that relocations may be more common in early and late aphid life rather than in the main phase of development and early adulthood. The endophyte status of the plants may influence this root use pattern. Root aphids on plants with the AR37 symbiont, in particular, were more restless than aphids on plants of another endophyte status.

Plants with AR37 endophyte were less easily colonised by root aphids than plants with another endophyte status. Good plant growth could perhaps mitigate the endophyte's effects on colonisation success, however.

Perennial ryegrass growing in Petri dishes filled with nutrient-enriched agar provided good conditions for root aphid populations to develop for up to two months. The population structure tended towards a ratio of seven to eight older immature nymphs per living adult aphid when in a stable, growing state. First alates, a possible indication of colony decline, appeared only after two months, in colonies with > 430 feeding root aphids/g root [dry matter]. Changes in the immature/mature ratio, another possible sign of declining populations, also began around 60 to 70 days after aphid introduction. These thresholds and population structure observations will require confirmation under field conditions, however, and should therefore be considered as preliminary information for planning new experiments.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

Root aphid *Aploneura lentisci* Pass. is a common and important plant pest in New Zealand grasslands (Jensen & Popay, 2007; McDonald *et al.*, 2011). Despite this, little is known about its biology on the roots of perennial ryegrass (*Lolium perenne* L.). This chapter summarises and discusses this thesis' key findings about the biology of *A. lentisci* (Section 6.1), its potential to cause damage to *L. perenne* (Section 6.2) and its interactions with the perennial ryegrass endophyte, *Epichloë festucae* var. *lolii* (Latch, M.J.Chr. & Samuels) C.W.Bacon & Schardl (Section 6.3). Section 6.4 considers the most pressing, unanswered questions and research needs around *A. lentisci.* Finally, Section 6.5 discusses possible approaches for prospective *A. lentisci* pest management options.

6.1. Root aphid biology, behaviour and life history

Based on observations from the Biology I and II experiments at 20.7 ± 6.0 °C and 17.5 ± 2.4 °C, respectively, apterous root aphids take about two to four weeks to develop to reproductive maturity on endophyte-free perennial ryegrass (median age at reproduction: 20 to 26 days) and die at an age of 33 to 70 days (Table 6.1). An adult can produce up to 122 offspring in its lifetime (median biological fecundity: 39 to 70 offspring per adult, depending on the estimation method; Table 3.9). By the mortalities observed in the laboratory, a net reproduction rate of 23 to 45 offspring per aphid and generation can be expected (ecological fecundity). Median neonate offspring survive about one week without food but the fittest offspring can live up to four weeks. The survival of offspring appears compromised, however, if their mother has been starved before their birth. Further research would be advisable before making broad generalisations from these results as the experiments on aphid biology presented in this thesis were undertaken in model systems with limitations. All data were acquired in specific conditions, either on plants rooted in nutrient-enriched agar or in empty microcentrifuge tubes. Furthermore,

many data were obtained from root aphids reared individually on cloned copies of just two plant genotypes (genotypes N and S, 'clone-plants'; Section 2.3.1).

The conditions during the experiments might have influenced the aphid biology both directly and indirectly. Rising temperatures up to a species-specific physiological optimum, for example, are likely to accelerate the development of an aphid and increase its fecundity (Dixon, 1973; Enders & Miller, 2016), but extreme temperatures may reduce aphid survival, reproduction and dispersal (Enders & Miller, 2016). Growth stops below 1.7 to 8.3 °C and death from chill injuries is possible at sub-zero temperatures (Blackman, 1987; McCornack *et al.*, 2005). At temperatures above 38 to 40 °C, most aphids suffer from heat stress and may die (Enders & Miller, 2016; Skaljac, 2016). The optimal development temperature for *A. lentisci* is not known yet. However, seven to eight months per year, the average temperature between the surface and 20 cm depth [i.e. the horizon with most *L. perenne* roots (Hatier *et al.*, 2014)] is below the 17 to 21 °C maintained in the experiments (Appendix 4). Although the data reported above would suggest 8 to 14 generations may be produced per year, six to nine generations appear thus more likely for root aphids in the field [estimates based on growing-degree-day-like calculations (Hutchinson *et al.*, 2000); not shown]. While root aphids may compensate for temperature changes by adjusting their position in the ground (Lu *et al.*, 2017), the temperature may still have an indirect effect on them by modulating the plant growth and thus the quality and quantity of the phloem available (Dixon, 1984; Popay & Cox, 2016; Zehnder *et al.*, 2007). Low temperatures impair metabolic processes in plants and high temperature may lead to denaturation and aggreagation of cellular proteins that may lead to cell death, for example (Singh *et al.*, 2011). *L. perenne* roots kept at 27 °C have a higher mortality and develop shorter root lengths than roots maintained at 15 °C (Robin, 2011). Besides temperature, other environmental factors such as light intensity may be important. In grasslands, root growth and carbon input to the soil are known to be more closely related to radiation fluxes than to temperature (Fitter *et al.*, 1998). This suggests the light intensity provided to the plants in the experiments could have influenced the root aphid biology at least indirectly, via effects on plants. Since aphids are known to respond to cues heralding seasonal food quality trends (Dixon, 1987), more complex seasonal patterns may also have shaped the root aphid development and reproduction in the field. How root aphid biology responds to such pattern as the rapid root biomass development reported in

perennial ryegrass in late winter and following autumn rains, about one month before the corresponding shoot development (Matthew *et al.*, 2016), has not been studied yet. Finally, nutrient-enriched agar solutions such as the ones used as root growing media in the experiments can influence the root system developed by a plant. The roots of plants growing in a culture solution are generally less profusely branched than those of plants grown in soil (James & Hutto, 1972). Roots also adjust their diameter and branching patterns in response to their environment (Care, 1999). To understand what impacts such root architecture changes may have on root aphids, further investigations will be necessary.

Because specific combinations of host plant genotype and aphid genetics (interspecific genotype-by-genotype interactions) can strongly determine the performance of an aphid (Zytynska and Weisser, 2016), further experiments are needed on a wider range of plant genotypes to validate the aphid biological measurements reported for the Population, Biology II and Mature plant experiments. As aphids in colonies may grow faster than isolated individuals and have other advantages through being members of a group (Miles, 1989a), further research may also need to consider working with several aphids instead of individual aphids.

Table 6.1. Summary of life-history and biological traits observed in *A. lentisci* root aphids reared on endophyte-free perennial ryegrass (*L. perenne*).

Parameters	Experiments (Experimental temperature)	Calibration II -	Viviposition 17 - 20 °C	Biology I 21 °C	Biology II 18 °C	Instar measurements 17 °C	Mature plant 10 °C	Seedling 19 °C	Population 18 °C
Maternal aphid size, adult[1] EP [mm^2], raw		(1.52 ± 0.43)[2]	-	0.84 ± 0.31	0.95 ± 0.44	0.84 ± 0.27	-	-	-
	model range	-	-	1.16 - 1.32	0.74 - 2.14	-	-	-	-
adult[1] L [mm]		(1.96 ± 0.31)[2]	-	1.40 ± 0.30	1.42 ± 0.32	1.51 ± 0.28	-	-	-
Offspring size, first instar EP [mm^2]		-	-	0.11 ± 0.02	0.10 ± 0.02	0.10 ± 0.01	-	-	-
first instar L [mm]		-	-	0.61 ± 0.05	0.65 ± 0.07	0.65 ± 0.08	-	-	-
Maternal establishment success [%]		-	-	(85)[4]	(76 - 93)[4]	-	-	-	-
Colonisation success [% of placed neonates]		-	-	(44)[4]	(43 - 71)[4]	-	30	(38)[4]	-
[% of samples colonised]		-	-	-	-	-	51 - 71	70	86 - 100
Age at ecdyses (E) [days]		-	-	E1: 7 - 9 E2: 10 - 15 E3: 13 - 15 E4: 17 - 19	-	-	-	-	-
Age at maturity [days], median		-	-	20 - 21	23 - 26	-	-	-	-
Maternal longevity[5] [days], median		-	-	(33)[3]	63 - 70	-	-	-	-
maximal				(49)[3]	109				
Maternal survival[5] *ex planta* [days], median		-	9	-	-	-	-	-	-
maximal		-	12	-	-	-	-	-	-
Offspring survival[5] *ex planta* [days], median		-	5	-	8	-	7 - 9	-	-
maximal		-	(7)[4]	-	27	-	21		
Biological lifetime fecundity [offspring/reproducing mother], median		-	-	(13)[3,4]	(45)[4]	-	-	-	-
	model median	-	-	9 - 34	39 - 70	-	-	-	-
Reproductive rate *in planta* [offspring/mother·day]		-	-	(0.9 ± 0.6)[3,4]	1.2 ± 0.8	-	-	-	-
ex planta		-	2.8 ± 1.4	-	-	-	5.6 - 6.5	-	-
Ecological lifetime fecundity [offspring/established aphid], median		-	-	(7)[3,4]	(23 - 45)[4]	-	-	-	-
	model median	-	-	6 - 29	31 - 45	-	-	-	-

Data labelled as 'model' are estimated based on models (model predictions), all other data are raw means ± standard deviation or median values; ( ): conditionally interpretable data; - data that was not collected; EP: ellipsoid projection, EP = L/2·W/2·π; L= body length, W = abdominal width; *ex planta*: aphids kept in air-tight containers without roots to feed upon.

[1] Adult size was defined as the size at the appearance of first offspring for the Biology I and II experiments, and size at the day of measurement (various age) for all the other experiments

[2] These root aphids developed on AR1-infected perennial ryegrass plants.

[3] These values were likely underestimated because of hot weather conditions towards the end of the Biology I experiment.

[4] These values were likely underestimated because of experimental handling issues.

[5] While longevity reports the age of the root aphid at death, survival is reported here as age at death -1 day.

6.2. Effects of root aphids on perennial ryegrass plants

Aphids can have positive effects on plants. In low densities, for example, they may stimulate the nutrient uptake of a plant (Miles, 1989a). In some cases, leaf chlorophyll content and CO_2 assimilation may increase (Dixon, 1973; Miles, 1989a). However, direct damage such as chloroses and necroses, and indirect damage through virus transmission or nutrient and energy drain (growth reductions) are more frequently reported [e.g. Saheed *et al.* (2007); Dixon (1973)]. Aphid feeding can furthermore interfere with plant defence responses (Hoffmann, 2016).

While the leaf colour analyses performed during the Mature plant experiment (Section 4.3.2) indicate that there might be some positive effects of *A. lentisci* feeding on *L. perenne*, more evidence on a larger selection of plant genotypes occupied for a longer time by root aphids is needed to confirm this relationship. The fact that root aphids used feeding sites for a few days (Sections 5.3.2 and 5.3.3) and the absence of necroses or swellings on the roots suggest that feeding by *A. lentisci* causes no direct damage. Some root aphids may be vectors for yellow dwarf viruses [see the bioassays on *Rhopalosiphum rufiabdominale* Sasaki by Hadi *et al.* (2011)] but whether that applies to *A. lentisci* and whether that could be the cause of significant yield losses in New Zealand pastures is not known. Although most literature reports shoot damage, and only occasionally root biomass reduction in the presence of large root aphid populations (McDonald *et al.*, 2011; Popay & Cox, 2016), root aphid feeding in these experiments first limited root biomass, without having any significant effect on shoot biomass (Mature plant and Seedling experiments, Chapter 4). A higher dependence on roots in a heterogeneous environment such as grassland soils or potting mix could explain this difference. As long, branched roots help plants to access nutrient-rich patches, aphid-induced reduction in root biomasses is likely to result in a significantly lower uptake of both water and minerals (Catherali & Parry, 1987; Langer, 1979), which may in turn limit the shoot growth in the field. To what extent irrigation and various fertilisers would alleviate the problem has to be examined, however. While fertilisation with less mobile nutrients such as phosphorus could perhaps mitigate the reduced access issue, fertilisation with nitrogen was shown to increase the incidence of root aphids (Cosgrove *et al.*, 2018). Whether this effect on the aphid population would negate any possible, positive effects on the yields is not known, however. Since fertiliser use may have further undesirable side effects in perennial

ryegrass swards [e.g. nitrate leaching and reduced endophyte and fungal alkaloid concentrations with possible impact on the control of other pests by nitrogen fertilisation (Popay & Crush, 2010; Rasmussen *et al.*, 2008b)], future research would have to consider the question in a wide agronomic perspective.

6.3. Root aphid-*Epichloë* endophyte interactions

Aphid feeding and exogenous honeydew application are reported to induce an accumulation of the defence hormone salicylic acid within plant tissues (Hoffmann, 2016). Applying salicylic acid to *Lolium multiflorum-Epichloë occultans* symbioses results in reduced concentrations of fungal alkaloids and a decreased resistance towards subsequent aphid feeding (Bastías *et al.*, 2018). It would thus be conceivable that, besides their nutrient and energy drain on their host plants, root aphids could interfere with the endophyte and its ability to protect the plant from other insects in the field. To what extent root aphids influence the endophytes is a question that could not be addressed during this thesis in absence of alkaloid analyses and endophyte quantification, however. Hence, this section will focus instead on the effects of *Epichloë* endophytes on root aphids.

The genotype of a host plant is possibly less relevant for root aphid performance than the endophyte strain it is host to and the plant genotype-endophyte interaction (Popay & Cox, 2016). Factors such as plant age, temperature/season and nutrient supply also influence the endophyte and interaction effects, if observations in alkaloid concentrations can be considered as representative of the endophyte-plant relationship. Alkaloid production is indeed known to start with some delay in seedlings and to develop further over time (Fuchs *et al.*, 2013; Ruppert *et al.*, 2017). Both alkaloids and hyphal concentrations in the shoot decline with high nitrogen supply (Rasmussen *et al.*, 2008b) and over winter with colder temperatures, with some delay in the response to the latter for some alkaloids such as epoxy-janthitrems (Christensen & Voisey, 2007; Hennessy *et al.*, 2016). These changes could explain the absence of strong endophyte effects in the Seedling and Mature plant experiments. Alternatively, the strong endophyte effects observed in other experiments could have been themselves a result of experimental conditions such as water stress. For example, the levels of ergovaline, an alkaloid believed

to contribute to a reduction in root aphid numbers on some plant-endophyte associations (Popay & Gerard, 2007), can increase in endophyte-infected ryegrasses under water stress (Rowan & Latch, 1994).

Various researchers have monitored the effect of the presence of specific endophytes on root aphid numbers in older plants and over several seasons (Pennell *et al.*, 2005; Popay, 2004; Popay & Cox, 2016; Popay & Gerard, 2007). Apart from some information on population development by Popay and Cox (2016), there is little information on endophyte influence on root aphid biological parameters helping to explain these observations, however. In the above-mentioned literature, plants with AR37 endophyte exert a strong control over root aphid populations regardless of the plant genotype. In the various experiments included in this thesis, root aphids generally failed to establish and had a significantly higher early mortality on AR37-infected plants than aphids on plants with another endophyte status. As only a few individuals achieved maturity on plants with AR37 in the Biology II experiment, the effect of this endophyte on adult size and fecundity could not be examined (Section 3.2.1.2). This study indicated that root aphids living on plants with CT symbiont have diverging biological reactions depending on the plant genotype with which this endophyte is associated. Root aphids on the S-CT symbioses developed more slowly and died earlier with smaller body size and lower fecundity than those on S-NIL plants, for example. The root aphids on N-CT plants, too, had a shorter longevity than those on N-NIL plants, but were similar to them for the other parameters. These results agree with Popay and Cox (2016). Individuals raised on plants with AR1 endophyte had reduced longevity compared to those on endophyte-free plants, and the endophyte presence did not significantly enhance any other biological trait on either of the two plant genotypes considered in this thesis. Why AR1 symbionts frequently support more root aphids in the field than endophyte-free plants (Popay & Cox, 2016; Popay & Easton, 2006) can therefore not be explained by the data reported here.

What triggers the biological effects generally observed in the case of each endophyte remains somewhat speculative. The deterrent or toxic, protective effects of endophytes in regard to insect herbivory have traditionally been related to the alkaloid profile and the concentration of alkaloids in the plant part consumed by specific insects (Fuchs *et al.*, 2017a, 2017b). Based on observations of several endophyte strains with varying alkaloid patterns, Popay and Gerard (2007) suggest the hypothesis that epoxy-

janthitrems, lolines and ergovaline could possibly be important agents for root aphid resistance in *Epichloë festucae* var. *lolii* AR37 symbioses, in *Epichloë coenophiala* or *Epichloë uncinata* associations, and in *E. festucae* var. *lolii* CT symbioses, respectively. As endophyte strains producing peramine or lolitrem B do not limit *A. lentisci* numbers (Popay & Gerard, 2007), these alkaloids are presumably either not detrimental, or not released into the root phloem in amounts critical to root aphids. However, because the grass-endophyte relationship affects the metabolic profile of the symbionts in ways beyond the alkaloid profile (Rasmussen *et al.*, 2008a; Rasmussen *et al.*, 2008b), the effects associated with alkaloids have yet to be confirmed in root aphid feeding experiments. Besides metabolic changes, colonisation by endophytes can also result in physical changes for the plants (Section 1.4.2, Table 1.4). Although there is genotypic variance in the response of host grasses to the endophyte infection too (Cheplick & Cho, 2003), plants with AR1, AR37 or CT symbiont are reported to sometimes have significantly higher root potassium (K) and phosphorus (P) concentrations than NIL plants (Popay, 2004). The AR37 strain appears also to modify plant phenology, delaying investments into root biomass to a later time point in the year compared to NIL plants (Popay, 2004). CT-infected perennial ryegrass plants are found to build up more root biomass than NIL plants (Popay, 2004), an observation consistent with what was observed on plant genotype N in the Mature plant experiment (Table 4.5). Perennial ryegrass plants of cultivar 'Grasslands Samson'-AR1 symbioses infested by aphids have higher nutrient concentrations in the shoots, lower root biomasses, lower root/shoot ratio and smaller root diameters compared to NIL plants (Popay, 2004) but larger root diameters in the top 10 cm soil than similar plants with AR37 symbiont (Popay & Crush, 2010). Such changes or related plant root structure differences could also explain the large root aphid populations frequently reported by the literature on AR1 plants.

6.4. Gaps in knowledge and future research

The knowledge gathered during this thesis provides first insights into root aphid biology in a laboratory context. To achieve a better understanding of population dynamics in the field and to better protect grasslands, more research on *A. lentisci*'s root selection

and establishment behaviour, dispersal behaviour, response to climatic factors (temperature, precipitations) and response to farming practices (e.g. mowing, grazing, fertilising) is needed. To investigate these questions on an appropriate field scale, a high-throughput method to quantify root aphid infestation in the field may first have to be developed, however.

As we now know which roots and positions to focus on from experiments in the laboratory (Section 5.4; Figure 5.13), understanding an aphid's decision-making behaviour at the selection of a feeding site and its establishing behaviour may be easier. The root diameter or more specifically the distance from the root surface to the phloem (i.e. the width of cortex and endodermis) is likely a central element, considering there is a correlation between aphid size and the depth of the phloem elements that can be fed upon (Dixon, 1985). How root hairs density, or root or phloem water content, water soluble carbohydrate content, and nitrogen content influence a root aphid's choice and establishment success may also be worth considering as these are known influential factors for aphid performance and/or varietal resistance in plants (Harper, 1964; Popay & Cox, 2016; Zytynska & Weisser, 2016). Besides possibly providing some explanations for the larger numbers of root aphids frequently observed on AR1 symbioses (Section 6.3), such knowledge may help in breeding root aphid-resistant plants (Section 1.2.6.6).

The dispersal patterns of pests in agroecosystems have a significant impact on their damage potential (Ben-Ari *et al.*, 2015), and can be used for pest control purposes. The knowledge that slugs come out at night to feed on leaves, for example, has been used to successfully control this pest with overnight mob stocking [1500 sheep/ha (Trafford & Trafford, 2011)]. Dispersal is also one of the main intrinsic factors responsible for variations in aphid numbers in the field (Kindlmann & Dixon, 2010). Understanding the patterns for *A. lentisci* may thus help both adequately monitoring populations and developing control strategies for root aphids on endophyte-free and AR1-infected plants. Written records of winged *A. lentisci* (alates) observations in New Zealand exist but are rather sparse [e.g. Blackman & Eastop, 2000; Lowe (1966), Lowe (1968)], and alates rarely developed in the laboratory over the course of this study. Furthermore, only apterous, generally first instar root aphids were trapped in both aerial and walk-in sticky traps placed outdoors amongst perennial ryegrass plants (data not shown). This supports the reports that dispersal happens mainly through walking wingless morphs, likely with

some wind assistance in the case of young nymphs climbing on the foliage of plants (Blackman & Eastop, 2000; Popay & Cox, 2016; Rasmussen *et al.*, 2008b). A focus on research that can provide a better knowledge of the causes and the timing of such first instar nymph migration may thus be rewarding for root aphid control.

External factors such as weather and temperature can cause wild oscillations in aphid numbers (Kindlmann & Dixon, 2010). While the experiments reported in this thesis demonstrated that some New Zealand root aphids were apparently able to survive short exposures to sub-zero temperatures [0.1 to 11.5 h below 0 °C, minimal temperature recorded: -2.4 °C (Mature plant experiment, Section 3.2.1.3)] and heat stress [0.1 to 3.2 h above 40 °C during the Biology I experiment, maximal temperature: 45 °C (Biology I experiment, Section 3.2.1.1; Appendix 10, Figure A10.3.1)], they did not allow for any statements on the development and other biological processes at temperatures below or above 17 to 21 °C (i.e. summer temperatures at the research site; Appendix 4). To predict how root aphid populations are likely to develop in other seasons will require more experiments using varying climatic conditions. Particularly important for modelling purposes may be an estimate of the lowest temperature for root aphid development to allow meaningful growing-degree-days calculations (Ministry for the Environment and Statistics New Zealand, 2015a).

Neonate root aphids died prematurely when trapped in condensation water (Appendix 7) or failed to establish in various pre-trials using very humid root environments (data not shown). Dry, friable soil conditions are known to result in larger populations of other root aphid species such as, e.g. *P. betae* and *Rhopalosiphum rufiabdominale* Sasaki (Kindler *et al.*, 2004; Pretorius *et al.*, 2016). Considering droughts will become more frequent in the North Island and the eastern areas of the South Island with climate change (Hatier *et al.*, 2013; He *et al.*, 2017), it could be worth investigating what effect soil humidity has on root aphids, and whether it may be used in the field for controlling *A. lentisci*. Maintaining a sufficient humidity is a recommended strategy to keep sugar-beet root aphid *Pemphigus betae* Doane populations low (Pretorius *et al.*, 2016). However, heavy precipitation events after a drought fail to dramatically reduce numbers (Kindler *et al.*, 2004). Precipitation or irrigation may even increase the dispersal of root aphids if they carry away the young nymphs [which float on water (Pretorius *et al.*, 2016; Salt *et al.*, 1996)]. That suggests soil humidity control could be a key element

for root aphid dispersal and establishment control before massive population build up. To manage this environmental parameter adequately, more knowledge on the migration behaviour and a good method for monitoring aphid populations in the field would be necessary, however.

On several occasions while conducting the research for this thesis, adult root aphids and long-established root aphid colonies disappeared almost completely from perennial ryegrass plants after shoot trimming. Since root aphids are likely to be penalised by herbage removal [reduced root growth when the shoot has to re-build (Langer, 1979; White, 1973)], this observation could have been a biological response to the dwindling food supply or to a reduced food quality as nitrogen was remobilised and carried away from roots into the reconstructing shoot to some extent (Thornton & Millard, 1996). If root aphids could be driven to leave their feeding position in the field by herbage removal, mowing or mob stocking could become valuable tools for root aphid control even in situations with a large aphid population. The response itself needs to be better understood if it is to be used, however. It is particularly important to know whether the behaviour is a short-term response when assimilates and nitrogen (total amino acids, nitrate) concentrations decline in the phloem in the first hours following defoliation (Amiard *et al.*, 2004), or whether it happens more progressively. If a mass-migration is to happen after a mowing event, procedures to kill aphids (drowning with water, pesticides) can be effective ways to reduce aphid numbers. As frequent defoliations may have undesirable side effects on pastures such as a decreasing ground cover, increasing winter mortality of plants (White, 1973), or increasing endophyte toxin levels in E^+ grasses (Fuchs *et al.*, 2017a), any research aiming at using such a method in the field will have to use a multidisciplinary approach. The benefits or possible problems of fertiliser application after a clipping may also have to be considered thereby, since fertiliser application may be necessary for plants to cope with frequent herbage removal but could be counterproductive for aphid control (see Section 6. 2).

Quantifying root aphids in a large aphid population is challenging in the laboratory but can be even more so in the field. The most common methods reported in root aphid research involve removing standardised volumes of plant roots with soil from the study site, as a first step. The presence of root aphids in the sample may then be reported as frequency (aphid present vs. absent) with or without additional scoring for the intensity

of the infestation [e.g. Moate *et al.* (2012); Thom *et al.* (2014)] or as number of colonies counted, with or without allowances for colony size (Bryant *et al.*, 2009; McDonald *et al.*, 2011; Popay *et al.*, 2012). If precise information on numbers is needed, the root aphids are extracted, either by hand sorting (Pennell *et al.*, 2005), by washing and wet sieving (Pennell *et al.*, 2005; Popay & Cox, 2016), by flotation in water with or without adjuvants such as $CaCl_2$ (Heathcote, 1972; Liu *et al.*, 2011b; Salt *et al.*, 1996) or with self-extraction devices such as Berlese-Tullgren funnels and Winkler sacks (García-Martínez *et al.*, 2017; Liu *et al.*, 2011b; Salt *et al.*, 1996). The number of aphids in the sample or a representative subset of it can then be counted. Counting requires a lot of time [for example, up to 50 h of intensive microscopy work to count aphids for 200 samples, washing and sieving not included (Podmore, 2015)]. That time requirement may severely restrict the number of samples and/or the frequency of monitoring in some projects, a problem, considering the heterogeneous distribution of aphids in the field (Murdie, 1972). There are reports that first instar *A. lentisci* are found in aerial suction traps (personal communication, Dr David Teulon, Plant and Food Research), but what such measurements may say about the populations in the ground is not yet known. Quantifying root aphid numbers by molecular methods using DNA extracts from a blended sample of soil and root aphids is technically feasible [amplification of the mitochondrial gene region CO1 (cytochrome *c* oxidase subunit 1) by real time quantitative polymerase chain reaction], but has not yet been validated for field use and could be unsuitable as a monitoring tool for low aphid concentrations [< 60 adult aphids/10 g soil (Podmore, 2015)]. An alternative molecular biology monitoring method possibly worth investigating could involve wax. Root aphid wax is produced in large quantities by feeding root aphids (Popay & Cox, 2016). Its chemical composition is unrelated to that of the host plant (Pike *et al.*, 2002) but its amount may be influenced by the quality of the plant as feed source (Dunn, 1974) and appears proportional to the number of root aphids present (weak but significant correlation in Chapter 5, Section 5.3.3). Because of the hydrophobic nature of the wax (Pike *et al.*, 2002), its harvest should be much easier than an aphid harvest. Another, possibly faster and less destructive option could involve hyperspectral imagery (Mendiguren *et al.*, 2015; Mirik *et al.*, 2014) on the youngest, fully extended leaves of perennial ryegrass plants. This technique requires special sensors collecting reflectance information in continuous, narrow spectral channels (< 20 nm wide bands) throughout

the visible to thermal infrared wavelengths regions of the electromagnetic spectrum (250 nm to 15 µm) (Mirik *et al.*, 2014; SphereOptics GmbH, 2017). Unlike the simplistic colour analyses presented in Section 4.2.2 (Mature plant experiment), these measurements may be able to give enough information to discriminate between various sources of stress by colour changes in grasses, such as water stress and aphid feeding, for example (Mendiguren *et al.*, 2015). Development of such a method for root aphid monitoring purposes would likely require some time, but could allow a fast, non-intrusive data collection once calibrated. Finally, if emigrant root aphids on herbage (Rasmussen *et al.*, 2008b) are representative of the below-ground population, monitoring their numbers on the herbage could also be worth considering as possible quantification technique. As mentioned earlier, however, more knowledge on migration patterns would be necessary to develop this approach.

6.5. Prospective pest management options for *A. lentisci*

Extensive use of chemical insecticides is expensive, can be detrimental for human health and environment, may rapidly result in resistant target pests and may not be as effective against root aphids in the ground as against aphids living on the shoots (McDonald *et al.*, 2011; Tomasetto *et al.*, 2017). Mature perennial ryegrass with AR37 endophyte controls root aphids reliably, from an early stage of the aphid colonisation onwards. Newly sown pastures with AR37 endophyte may establish poorly, however, if the number of living root aphids cannot be minimised for one to two months after sowing. The metabolic functions of the endophyte are still developing and not offering full protection to a seedling in this period (Ruppert *et al.*, 2017). The common practice of eliminating the existing, old sward with non-residual herbicides (Trafford & Trafford, 2011) may contribute to root aphid control by starving the local root aphid population (fitness loss in offspring of starved mothers, Section 3.3.4). However, how this effect could best be used will require some research. While the average first instar nymphs of non-fasting mothers died after one week without food at 17 to 21 °C (Chapter 3, Section 3.3.4), the offspring of starving mothers in the field at lower and/or more variable conditions may present a diverging mortality pattern.

Fully relying on the AR37 endophyte to control *A. lentisci* may not be wise in the long-term, as this may encourage the build-up of resistance. Despite being unable to complete a full sexual reproduction cycle in absence of its primary host, *A. lentisci* has more than enough potential for adaptation. Resistant individuals may be generated via epigenetics (Mukherjee & Baudach, 2016), immigration (Phillips *et al.*, 2006), mutation (Loxdale, 2009) and association with helpful secondary endosymbionts (Skaljac, 2016). Epigenetic regulation mechanisms for complex traits such as development, reproduction and immunity allow the asexually produced aphid clones a certain amount of phenotypic adjustment (Mukherjee & Baudach, 2016). Migration from Asia or Australia has been documented for the wheat aphid *Sitobion miscanthi* Takahashi in New Zealand (Phillips *et al.*, 2006; Wilson *et al.*, 1999). As *P. lentiscus* has been present in Australia since the 19th century (Mills, 2010; Sandhu, 2011), migration could also bring in various new root aphid genotypes. Considering *A. lentisci*'s annual number of generations and reproduction potential (4.7×10^{4} to 7.6×10^{14} offspring per year; Section 6.1), a large amount of mutations can also be expected, even assuming a conservative mutation rate [for example 10^{-7} mutations per gene and generation (Loxdale, 2009)]. Some of these mutations may have a significant effect, as with the proven development of resistance to organophosphate insecticides in spotted Alfalfa aphids *Therioaphis trifolii* form *maculata* Buckton (Loxdale, 2009). Finally, specific secondary symbionts (Section 1.2.4.1) can enhance their aphid host's fitness, improving its resistance towards pathogens and its ability to handle plant defences (Enders & Miller, 2016; Skaljac, 2016). Such symbionts may be horizontally transferred between individuals of the same species as well as between un-related insect species, likely through commonly shared predators or parasitoids, through mating or in rare cases through shared host plants (Skaljac, 2016). There is therefore a considerable potential for adaptation and resistance development towards AR37 or AR37-toxins in *A. lentisci*.

Alternative or complementary approaches to an AR37-dominated root aphid control could involve: breeding root aphid tolerant or resistant plants in association with the AR1 endophyte in particular (Popay & Easton, 2006), alternative bio-agents such as predators, entomopathogenic fungi and entomopathogenic nematodes (Card *et al.*, 2016; Kergunteuil *et al.*, 2016), and possibly beneficial management practices. As it is not yet known which insects may be significant predators of *A. lentisci* in New Zealand, a control

system using predators may take time to investigate and implement. Whether the other bio-agents could significantly contribute to keeping *A. lentisci* numbers low in pastures is yet to be demonstrated. Entomopathogenic fungi such as *Beauveria bassiana* and entomopathogenic nematodes are already commercially available in New Zealand for use on aphids and root aphids (Bioforce Ltd, 2012; Biological Solutions Ltd, 2018). However, as nematodes are sensitive towards pesticides and abiotic stresses [drought, low/high pH, soil salinity and UV (Kergunteuil *et al.*, 2016)], they may be of limited use in non-irrigated grasslands. Among the possible management practices, irrigation may be important. Regardless of whether an increased soil humidity succeeds in killing root aphids and/or handicapping first instar dispersal (Section 6.4), supplying sufficient moisture for the plants to maintain root growth and avoid a water-stress-caused reduction in photosynthesis will likely help compensating at least partly for root aphid feeding damages (Singh *et al.*, 2011; Tozer *et al.*, 2017). Whether mowing, grazing (preferably mob stocking) or fertilising may be used as tools for root aphid population manipulation still needs to be examined (Section 6.4).

6.6. Conclusions

The root aphid *A. lentisci* develops to maturity within three to four weeks at 17 to 21 °C and may survive over two months. Allowing for cooler temperatures outdoors during autumn, winter and spring, this aphid is thus likely to complete six to nine generations per year. An adult can parthenogenetically produce 39 to 70 offspring over its lifetime on endophyte-free perennial ryegrass (biological reproductive potential). A population is likely to multiply by a factor 23 to 45× per generation (ecological reproductive potential at 17 to 21 °C), assuming natality and mortality rates similar to those observed in the experiments reported here. The offspring are very mobile in their first days of life and can survive more than a week without food.

Root aphids generally feed on a plant's youngest branched roots (first and second branching order; main axis and lateral 1 branches). They first affect the root biomass of plants grown in agar in the laboratory. This suggests that the shoot yield reductions observed in the field could be a secondary result of insufficient nutrients and/or water

supply. A tendency for shoots to display an increase in photosynthetic pigments in newly developed leaves, in presence of root aphids, was also noted. The significance of this effect in the field and its possible benefits for the plant have yet to be confirmed, however.

The general patterns of interaction between *A. lentisci* and endophytic plant symbionts *E. festucae* var. *lolii* of various strains observed during this thesis were in line with the responses observed in the field for these endophytes. The AR37 strain controls the establishment of root aphids and their survival to maturity so strongly that there are not enough survivors to assess its effect on other parameters. The effect of the common-toxic (CT) strain depends upon the plant genotype it is associated with. In the experiments presented here, AR1 had no significant effect on root aphid biological traits except reducing the aphids' life span. Whether this would be different on other plant genotypes, or whether the more numerous populations frequently observed on AR1 plants in the field could possibly be explained by establishment success needs to be experimentally verified.

Because of the possible mechanisms for *A. lentisci* to adjust to particular factors in its growth environment and the lack of alkaloid protection in the early developmental stages of seedlings with AR37 endophyte, it may be wise to not rely exclusively on the protection by AR37 to control root aphids in the future. To this end, research may consider investigating the effect of various cultural techniques (e.g. control of ground humidity, mowing/grazing, fertilising) and alternative bio-agents (predators, entomopathogenic fungi and entomopathogenic nematodes) on root aphid populations and perennial ryegrass yields. Breeding root aphid resistant perennial ryegrass-AR1 endophyte associations may be a further option worth exploring. The best use of each option will need to be established considering root aphid behaviour, population development and plant response in the field. To achieve the first two of those outcomes future research will first have to concentrate on developing tools for root aphid quantification, however. Wax analyses and hyperspectral imaging of leaf reflectance could both be promising approaches for this. A better understanding of root aphid migration patterns and aerial trapping could also be an option to consider.

REFERENCES

Adams, J. B., & van Emden, H. F. (1972). The biological properties of aphids and their host plant relationships. In H. F. van Emden (Ed.), *Aphid technology* (pp. 47-104). London: Academic Press.

Agricom & PGG Wrightson Seeds. (2015). The key to better pasture persistence and productivity - AR37. Retrieved from http://www.ar37.com.au/assets/AR37Brochure.pdf on 29/07/2018.

Ali, M. M., Al-Ani, A., Eamus, D., & Tan, D. K. Y. (2012). A new image processing based technique to determine chlorophyll in plants. *American-Eurasian Journal of Agricultural & Environmental Sciences, 12*(10), 1323-1328.

Amiard, V., Morvan-Bertrand, A., Cliquet, J.-B., Billard, J.-P., Huault, C., Sandström, J. P., & Prud'homme, M.-P. (2004). Carbohydrate and amino acid composition in phloem sap of *Lolium perenne* L. before and after defoliation. *Canadian Journal of Botany, 82*(11), 1594-1601.

Anderson, M., & Bromley, A. K. (1987). Sensory system. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 153-162). Amsterdam: Elsevier Science Publishers B.V.

Applied Biosystems. (2009). A guide to high resolution melting (HRM) analysis. Retrieved from http://www.gene-quantification.de/ab-hrm-guide.pdf on 29/07/2018.

Babikova, Z., Gilbert, L., Bruce, T., Dewhirst, S. Y., Pickett, J. A., & Johnson, D. (2014). Arbuscular mycorrhizal fungi and aphids interact by changing host plant quality and volatile emission. *Functional Ecology, 28*(2), 375-385.

Barton, K. (2018). MuMIn: multi-model inference. *R package version 1.40.4.* Retrieved from https://CRAN.R-project.org/package=MuMIn on 9/03/2018.

Bastías, D. A., Martínez-Ghersa, M. A., Newman, J. A., Card, S. D., Mace, W. J., & Gundel, P. E. (2018). The plant hormone salicylic acid interacts with the mechanism of anti-herbivory conferred by fungal endophytes in grasses. *Plant, Cell and Environment, 41*(2), 395-405.

Bastias, D. A., Ueno, A. C., Assefh, C. R. M., Alvarez, A. E., Young, C. A., & Gundel, P. E. (2017). Metabolism or behavior: explaining the performance of aphids on alkaloid-producing fungal endophytes in annual ryegrass (*Lolium multiflorum*). *Oecologia, 185*(2), 245-256.

Bates, D., Maechler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software, 67*(1), 1-48.

Ben-Ari, M., Gish, M., & Inbar, M. (2015). Walking aphids can partake in within-field dispersal to distant plants. *Basic and Applied Ecology, 16*(2), 162-171.

Bioforce Ltd. (2012). Nemaplus - Steinernema feltiae. Retrieved from http://www.bioforce.co.nz/products/nemaplus.html on 29/07/2018.

Biological Solutions Ltd. (2018). ContegoBB. *Plant protection and nutrition.* Retrieved from http://www.biosolutions.co.nz/uploads/2/1/4/8/21483126/contegobblabela5_print.pdf on 29/07/2018.

Birch, L. C. (1948). The intrinsic rate of natural increase of an insect population. *Journal of Animal Ecology, 17*(1), 15-26.

Björkman, C. (2000). Interactive effects of host resistance and drought stress on the performance of a gall-making aphid living on Norway spruce. *Oecologia, 123*(2), 223-231.

Blackman, R. L. (1987). Reproduction, cytogenetics and development. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 163-196). Amsterdam: Elsevier Science Publishers B.V.

Blackman, R. L., & Eastop, V. F. (1984). *Aphids on the world's crops: an identification and information guide*. Chichester [West Sussex], United Kingdom: Wiley.

Blackman, R. L., & Eastop, V. F. (1994). *Aphids on the world's trees: an identification and information guide*. Wallingford [Oxfordshire], United Kingdom: CAB International in association with the Natural History Museum.

Blackman, R. L., & Eastop, V. F. (2000). *Aphids on the world's crops: an identification and information guide* (2nd ed.). Chichester [West Sussex], United Kingdom: Wiley.

Bollard, E. G. (1966). A comparative study of the ability of organic nitrogenous compounds to serve as sole source of nitrogen for the growth of plants. *Plant and Soil, 25*(2), 153-166.

Boukhris-Bouhachem, S., & Souissi, R. (2012). *Aploneura lentisci* (Hemiptera: Aphididae) a new vector of PVY^{NTN} in Tunisia. Poster presented at *Entomology 2012, Entomological Society of America's 60th annual meeting.* Retrieved from www.researchgate.net/publication/267526273_Aploneura_lentisci_Hemiptera_Aphididae_a_new_vector_of_PVY_in_Tunisia on 24/03/2019.

Boullis, A., & Verheggen, F. J. (2016). Chemical Ecology of Aphids (Hemiptera: Aphididae). In A. Vilcinskas (Ed.), *Biology and Ecology of Aphids* (pp. 171-198). Boca Raton: CRC Press.

Brabec, M., Honěk, A., Pekár, S., & Martinková, Z. (2014). Population dynamics of aphids on cereals: digging in the time-series data to reveal population regulation caused by temperature. *Plos One, 9*(9), e106228. doi: 10.1371/journal.pone.0106228

Brault, V., Uzest, M., Monsion, B., Jacquot, E., & Blanc, S. (2010). Aphids as transport devices for plant viruses. *Comptes Rendus Biologies, 333*(6-7), 524-538.

Brock, J. L., Hume, D. E., & Fletcher, R. H. (1996). Seasonal variation in the morphology of perennial ryegrass (*Lolium perenne*) and cocksfoot (*Dactylis glomerata*) plants and populations in pastures under intensive sheep grazing. *Journal of Agricultural Science, 126*, 37-51.

Bryant, R. H., Parsons, A. J., Rasmussen, S., & Edwards, G. R. (2009). Pasture production and botanical composition of high sugar and control ryegrasses with or without endophyte under irrigation in Canterbury. *Proceedings of the New Zealand Grassland Association* 71, 177-185. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_85.pdf on 31/07/2018.

Bultman, T. L., & Bell, G. D. (2003). Interaction between fungal endophytes and environmental stressors influences plant resistance to insects. *Oikos, 103*(1), 182-190.

Bultman, T. L., Rodstrom, J. L., Radabaugh, K. R., VanDop, J. D., Librizzi, J. M., Longwell, L. L., Pulas, C., Grant, L., & Sullivan, T. J. (2009). Influence of genetic variation in the fungal endophyte of a grass on an herbivore and its parasitoid. *Entomologia Experimentalis Et Applicata, 130*(2), 173-180.

Campbell, C. D., & Hutchison, W. D. (1995). Rearing methods and demographic statistics for a subterranean morph of the sugarbeet root aphid (Homoptera, Aphididae). *Canadian Entomologist, 127*(1), 65-77.

Card, S. D., Faville, M. J., Simpson, W. R., Johnson, R. D., Voisey, C. R., de Bonth, A. C., & Hume, D. E. (2014a). Mutualistic fungal endophytes in the Triticeae - survey and description. *FEMS Microbiology Ecology, 88*(1), 94-106.

Card, S., Johnson, L., Teasdale, S., & Caradus, J. (2016). Deciphering endophyte behaviour: the link between endophyte biology and efficacious biological control agents. *FEMS Microbiology Ecology, 92*(8), fiw114. doi: 10.1093/femsec/fiw114.

Card, S. D., Rolston, M. P., Lloyd-West, C., & Hume, D. E. (2014b). Novel perennial ryegrass-*Neotyphodium* endophyte associations: relationships between seed weight, seedling vigour and endophyte presence. *Symbiosis, 62*(1), 51-62.

Card, S. D., Rolston, M. P., Park, Z., Cox, N., & Hume, D. E. (2011). Fungal endophyte detection in pasture grass seed utilising the infection layer and comparison to other detection techniques. *Seed Science & Technology, 39*(3), 581-592.

Care, D. A. (1999). *Effect of some external factors on root hair demography in Trifolium repens L. and Lolium perenne L.* (PhD Thesis), University of Auckland, Auckland.

Carter, G. A., & Knapp, A. K. (2001). Leaf optical properties in higher plants: linking spectral characteristics to stress and chlorophyll concentration. *American Journal of Botany, 88*(4), 677-684.

Catherali, P. L., & Parry, A. L. (1987). Effects of barley yellow dwarf virus on some varieties of italian, hybrid and perennial ryegrasses and their implication for grass breeders. *Plant Pathology, 36*(2), 148-153.

Charlton, J. F. L., & Stewart, A. V. (1999). Pasture species and cultivars used in New Zealand - a list. *Proceedings of the New Zealand Grassland Association 61*, 147-166. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_510.pdf on 31/07/2018.

Cheplick, G. P. (1998). Genotypic variation in the regrowth of *Lolium perenne* following clipping: effects of nutrients and endophytic fungi. *Functional Ecology, 12*(2), 176-184.

Cheplick, G. P. (2004). Recovery from drought stress in *Lolium perenne* (Poaceae): are fungal endophytes detrimental? *American Journal of Botany, 91*(12), 1960-1968.

Cheplick, G. P. (2007). Costs of fungal endophyte infection in *Lolium perenne* genotypes from Eurasia and North Africa under extreme resource limitation. *Environmental and Experimental Botany, 60*(2), 202-210.

Cheplick, G. P., & Cho, R. (2003). Interactive effects of fungal endophyte infection and host genotype on growth and storage in *Lolium perenne*. *New Phytologist, 158*(1), 183-191.

Christensen, M. J., Bennett, R. J., Ansari, H. A., Koga, H., Johnson, R. D., Bryan, G. T., Simpson, W. R., Koolaard, J. P., Nickless, E. M., & Voisey, C. R. (2008). *Epichloë* endophytes grow by intercalary hyphal extension in elongating grass leaves. *Fungal Genetics and Biology, 45*(2), 84-93.

Christensen, M. J., & Voisey, C. R. (2007). The biology of the endophyte/grass partnership. In A. J. Popay & E. R. Thom (Eds.), *Proceedings of the 6th International Symposium on Fungal Endophtye of Grasses* (pp. 123-133), Dunedin, New Zealand: New Zealand Grassland Association. Retrieved from: http://www.grassland.org.nz/publications/nzgrassland_publication_2405.pdf on 31/07/2018.

Clay, K. (1988). Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology, 69*(1), 10-16.

Clement, S. L. (2009). Variable effects of fungal endophyte-infected grasses on the performance of pestiferous aphids. *Redia, 92*, 205-209.

Cosgrove, G. P., Popay, A. J., Taylor, P. S., Wilson, D. J., Aalders, L. T., & Bell, N. L. (2018). Implications of grass-clover interactions in dairy pastures for forage indexing systems. 3. Manawatu. *New Zealand Journal of Agricultural Research, 61*(2), 174-203.

Cottier, W. (1953) Aphids of New Zealand. *New Zealand Department of Scientific and Industrial Research Bulletin 106*. Wellington, New Zealand: New Zealand DSIR.

Dáder, B., Moreno, A., Viñuela, E., & Fereres, A. (2012). Spatio-temporal dynamics of viruses are differentially affected by parasitoids depending on the mode of transmission. *Viruses, 4*(11), 3069-3089.

Dairy NZ. (2018). Ryegrass. Retrieved from http://www.dairynz.co.nz/feed/pasture-renewal/select-pasture-species/ryegrass/ on 30/07/2018.

Danckwerts, J. E., & Gordon, A. J. (1987). Long-term partitioning, storage and re-mobilization of ^{14}C assimilated by *Lolium perenne* (cv. Melle) *Annals of Botany, 59*(1), 55-66.

Dardeau, F., Body, M., Berthier, A., Miard, F., Christidès, J. P., Feinard-Duranceau, M., Brignolas, F., Giron, D., Lieutier, F., & Sallé, A. (2015). Effects of fertilisation on amino acid mobilisation by a plant-manipulating insect. *Ecological Entomology, 40*(6), 814-822.

de Jong, Y., Verbeek, M., Michelsen, V., de Place Bjørn, P., Los, W., Steeman, F., Bailly, N., Basire, C., Chylarecki, P., Stloukal, E., Hagedorn, G., Wetzel, F., Glöckler, F., Kroupa, A., Korb, G., Hoffmann, A., Häuser, C., Kohlbecker, A., Müller, A., Güntsch, A., Stoev, P., & Penev, L. (2014). Fauna Europaea - all European animal species on the web. *Biodiversity Data Journal 2: e4034*, doi: 10.3897/BDJ.3892.e4034.

de Parseval, H., Barot, S., Gignoux, J., Lata, J. C., & Raynaud, X. (2017). Modelling facilitation or competition within a root system: importance of the overlap of root depletion and accumulation zones. *Plant and Soil, 419*(1-2), 97-111.

de Wet, L. R., & Botha, C. E. J. (2007). Resistance or tolerance: an examination of aphid (*Sitobion yakini*) phloem feeding on Betta and Betta-Dn wheat (*Triticum aestivum*). *South African Journal of Botany, 73*(1), 35-39.

Delmiglio, C., Pearson, M. N., Lister, R. A., & Guy, P. L. (2010). Incidence of cereal and pasture viruses in New Zealand's native grasses. *Annals of Applied Biology, 157*(1), 25-36.

di Menna, M. E., & Waller, J. E. (1986). Visual assessment of seasonal changes in amount of mycelium of *Acremonium loliae* in leaf sheaths of perennial ryegrass. *New Zealand Journal of Agricultural Research, 29*(1), 111-116.

Dixon, A. F. G. (1973). *Biology of aphids*. London, United Kingdom: Edward Arnold.

Dixon, A. F. G. (1984). *Aphid ecology: an optimization approach*. Glasgow, United Kingdom: Blackie & Son.

Dixon, A. F. G. (1985). *Aphid ecology*. Glasgow, United Kingdom: Blackie & Son.

Dixon, A. F. G. (1987). Parthenogenetic reproduction and the rate of increase in aphids. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 269-287). Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Dixon, A. F. G., & Kindlmann, P. (1994). Optimum body size in aphids. *Ecological Entomology, 19*(2), 121-126.

Dixon, A. F. G., & Logan, M. (1973). Leaf size and availability of space to the sycamore aphid *Drepanosiphum platanoides*. *Oikos, 24*(1), 58-63.

Döring, T. F., & Chittka, L. (2007). Visual ecology of aphids-a critical review on the role of colours in host finding. *Arthropod-Plant Interactions, 1*(1), 3-16.

Douglas, A. E. (2003). The nutritional physiology of aphids. *Advances in Insect Physiology, 31*, 73-140.

Douglas, A. E. (2006). Phloem-sap feeding by animals: problems and solutions. *Journal of Experimental Botany, 57*(4), 747-754.

Dunn, J. A. (1974). The influence of host plant on the production of sexuparae in the aphid *Pemphigus bursarius*. *Entomologia Experimentalis Et Applicata, 17*(3), 445-457.

Easton, H. S., Christensen, M. J., Eerens, J. P. J., Fletcher, L. R., Hume, D. E., Keogh, R. G., Lane, G. A., Latch, G. C. M., Pennell, C. G. L., Popay, A. J., Rolston, M. P., Sutherland, B. L., & Tapper, B. A. (2001). Ryegrass endophyte: a New Zealand grassland success story. *Proceedings of the New Zealand Grassland Association 63*, 37-46. Retrieved from: www.grassland.org.nz/publications/nzgrassland_publication_261.pdf on 31/07/2018.

Eastop, V. F. (1966). A taxonomic study of Australian Aphidoidea (Homoptera). *Australian Journal of Zoology, 14*(3), 399-592.

Eissenstat, D. M., & Volder, A. (2005). The efficiency of nutrient acquisition over the life of a root. In H. BassiriRad (Ed.), *Nutrient acquisition by plants: ecological perspective. Ecological studies-analysis and synthesis, Vol. 181* (pp. 185-220). Berlin: Springer-Verlag Berlin.

Ellis, R. H., Hong, T. D., & Roberts, E. H. (1985). *Handbook of seed technology for genebanks Vol 2: compendium of specific germination information and test recommendations*. Rome: International Board for Plant Genetic Resources.

Enders, L. S., & Miller, N. J. (2016). Aphid molecular stress biology. In A. Vilcinskas (Ed.), *Biology and ecology of aphids* (pp. 135-151). Boca Raton: CRC Press.

Environmental Growth Chambers. (2015). Lighting radiation conversion. Retrieved from http://www.egc.com/useful_info_lighting.php on 30/07/2018.

Faville, M. J., Briggs, L., Cao, M., Koulman, A., Jahufer, M. Z., Koolaard, J., & Hume, D. E. (2015). A QTL analysis of host plant effects on fungal endophyte biomass

and alkaloid expression in perennial ryegrass. *Molecular Breeding, 35*(8), 161. doi: 10.1007/s11032-015-0350-1.

Ferguson, C. M., Barratt, B. I. P., Bell, N., Goldson, S. L., Hardwick, S., Jackson, M., Jackson, T. A., Phillips, C. B., Popay, A. J., Rennie, G., Sinclair, S., Townsend, R., & Wilson, M. (2018). Quantifying the economic cost of invertebrate pests to New Zealand's pastoral industry. *New Zealand Journal of Agricultural Research*, 1-62.

Fisher, D. B., & Frame, J. M. (1984). A guide to the use of the exuding-stylet technique in phloem physiology. *Planta, 161*(5), 385-393.

Fitter, A. H., Graves, J. D., Self, G. K., Brown, T. K., Bogie, D. S., & Taylor, K. (1998). Root production, turnover and respiration under two grassland types along an altitudinal gradient: influence of temperature and solar radiation. *Oecologia, 114*(1), 20-30.

Forde, B. J. (1966). Translocation in grasses. 2. Perennial ryegrass and couch grass. *New Zealand Journal of Botany, 4*(4), 496-514.

Fox, J., & Weisberg, S. (2011). *An {R} companion to applied regression* (2nd ed.). Thousand Oaks, CA: SAGE Publications.

Fuchs, B., Krischke, M., Mueller, M. J., & Krauss, J. (2013). Peramine and lolitrem B from endophyte-grass associations cascade up the food chain. *Journal of Chemical Ecology, 39*(11-12), 1385-1389.

Fuchs, B., Krischke, M., Mueller, M. J., & Krauss, J. (2017a). Herbivore-specific induction of defence metabolites in a grass-endophyte association. *Functional Ecology, 31*(2), 318-324.

Fuchs, B., Krischke, M., Mueller, M. J., & Krauss, J. (2017b). Plant age and seasonal timing determine endophyte growth and alkaloid biosynthesis. *Fungal Ecology, 29*, 52-58.

Fulkerson, W. J., & Donaghy, D. J. (2001). Plant-soluble carbohydrate reserves and senescence - key criteria for developing an effective grazing management system for ryegrass-based pastures: a review. *Australian Journal of Experimental Agriculture, 41*(2), 261-275.

Gange, A. C., Bower, E., & Brown, V. K. (1999). Positive effects of an arbuscular mycorrhizal fungus on aphid life history traits. *Oecologia, 120*(1), 123-131.

García-Martínez, M. A., Valenzuela-González, J. E., Escobar-Sarria, F., López-Barrera, F., & Castaño-Meneses, G. (2017). The surrounding landscape influences the diversity of leaf-litter ants in riparian cloud forest remnants. *Plos One, 12*(2), e0172464. doi: 10.1371/journal.pone.0172464.

Gavloski, J. E., & Lamb, R. J. (2000). Specific impacts of herbivores: Comparing diverse insect species on young plants. *Environmental Entomology, 29*(1), 1-7.

Ghaedi, B., & Andrew, N. R. (2016). The physiological consequences of varied heat exposure events in adult *Myzus persicae*: a single prolonged exposure compared to repeated shorter exposures. *PeerJ, 4*.

Giordanengo, P., Brunissen, L., Rusterucci, C., Vincent, C., van Bel, A., Dinant, S., Girousse, C., Faucher, M., & Bonnemain, J. L. (2010). Compatible plant-aphid interactions: how aphids manipulate plant responses. *Comptes Rendus Biologies, 333*(6-7), 516-523.

Grasslanz Technology Ltd. (2010a). Novel endophyte technologies. *Understanding the Science.* Retrieved from http://www.grasslanz.com/UnderstandingtheScience/novelendophytetechnologies.aspx on 30/07/2018.

Grasslanz Technology Ltd. (2010b). AR37 endophyte. *Understanding the Science.* Retrieved from http://www.grasslanz.com/understandingthescience/ar37endophyte.aspx on 30/07/2018.

Hadi, B. A. R., Flanders, K. L., Bowen, K. I., Murphy, J. F., & Halbert, S. E. (2011). Species composition of aphid vectors (Hemiptera: Aphididae) of barley yellow dwarf virus and cereal yellow dwarf virus in Alabama and Western Florida. *Journal of Economic Entomology, 104*(4), 1167-1173.

Hajong, S. R., & Varman, A. R. (2002). A report on positive phototaxis exhibited by polymorphic forms of an aphid. *Journal of Insect Behavior, 15*(2), 295-298.

Hannaway, D., Fransen, S., Cropper, J., Teel, M., Chaney, M., Griggs, T., Halse, R., Hart, J., Cheeke, P., Hansen, D., Klinger, R., & Lane, W. (1999). Perennial ryegrass (*Lolium perenne* L.). *Pacific Northwest Extension Publications, PNW-503.* Retrieved from http://content.libraries.wsu.edu/index.php/utils/getfile/collection/cahnrs-arch/id/487/filename/91035182432004_PNW503.pdf on 30/07/2018.

Harper, A. M. (1964). Varietal resistance of sugar beets to sugar-beet root aphid *Pemphigus betae* Doane (Homoptera - Aphididae). *Canadian Entomologist, 96*(3), 520-522.

Harrell, F. E., Jr. with contributions from Charles Dupont and many others. (2018). Hmisc: Harrell miscellaneous. *R package version 4.1-1.* Retrieved from https://CRAN.R-project.org/package=Hmisc on 9/03/2018.

Hatier, J.-H. B., Faville, M. J., Hickey, M. J., Koolaard, J. P., Schmidt, J., Carey, B.-L., & Jones, C. S. (2014). Plant vigour at establishment and following defoliation are both associated with responses to drought in perennial ryegrass (*Lolium perenne* L.). *Journal of Experimental Botany, 65*(20), 5823-5834.

Hatier, J.-H. B., He, L., Faville, M. J., Hickey, M. J., Jones, C. S., Jahufer, M. Z. Z., & Matthew, C. (2013). Dissecting drought-response strategies of perennial ryegrass (*Lolium perenne* L.). In D. L. Michalk, G. D. Millar, W. B. Badgery, & K. M. Broadfoot (Eds.), *Revitalising grasslands to sustain our communities: Proceedings of the 22nd International Grassland Congress* (pp. 133-134), Orange, Australia: New South Wales Department of Primary Industry. Retrieved from: https://www.researchgate.net/profile/Chris_Jones23/publication/264971104_Dissecting_drought-response_strategies_of_perennial_ryegrass_Lolium_perenne_L/links/5641ab9b08aeacfd89377c22/Dissecting-drought-response-strategies-of-perennial-ryegrass-Lolium-perenne-L.pdf on 31/07/2018.

Hazell, S. P., Groutides, C., Neve, B. P., Blackburn, T. M., & Bale, J. S. (2010). A comparison of low temperature tolerance traits between closely related aphids from the tropics, temperate zone, and Arctic. *Journal of Insect Physiology, 56*(2), 115-122.

He, L. (2016). *Drought tolerance of perennial ryegrass (Lolium perenne L.) and the role of Epichloë endophyte.* A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Plant Science at Massey University, Manawatu, Palmerston North, New Zealand. Retrieved from https://mro.massey.ac.nz/handle/10179/10960 on 13/04/2019.

He, L., Hatier, J.-H. B., & Matthew, C. (2017). Drought tolerance of two perennial ryegrass cultivars with and without AR37 endophyte. *New Zealand Journal of Agricultural Research, 60*(2), 173-188.

Heathcote, G. D. (1972). Evaluating aphid populations on plants. In H. F. van Emden (Ed.), *Aphid technology* (pp. 105-145). London: Academic Press.

Hennessy, L. M., Popay, A. J., Finch, S. C., Clearwater, M. J., & Cave, V. M. (2016). Temperature and plant genotype alter alkaloid concentrations in ryegrass infected

with an *Epichloe* endophyte and this affects an insect herbivore. *Frontiers in Plant Science, 7*, 1097. doi: 10.3389/fpls.2016.01097.

Hewer, A., Becker, A., & van Bel, A. J. (2011). An aphid's odyssey-the cortical quest for the vascular bundle. *The Journal of Experimental Biology, 214*, 3868-3879.

Hoffmann, K. H. (2016). Aphid honeydew: rubbish or signaler. In A. Vilcinskas (Ed.), *Biology and ecology of aphids* (pp. 199-220). Boca Raton: CRC Press.

Højsgaard, S., & Halekoh, U. (2016). doBy: groupwise statistics, LSmeans, linear contrasts, utilities. *R package version 4.5-15.* Retrieved from https://CRAN.R-project.org/package=doBy on 09/03/2018.

Honěk, A. (1993). Intraspecific variation in body size and fecundity in insects - a general relationship. *Oikos, 66*(3), 483-492.

Hopkins, G. W., & Dixon, A. F. G. (2000). Feeding site location in birch aphids (Sternorrhyncha: Aphididae): the simplicity and reliability of cues. *European Journal of Entomology, 97*(2), 279-280.

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal, 50*(3), 346-363.

Hume, D. E., & Cosgrove, G. P. (2005). Endophyte - what is it and its significance in New Zealand pastoral agriculture. In H. L. Davies (Ed.), *Proceedings of the 20th Annual Conference of the Grassland Society of NSW inc.* (pp. 31-36), Orange, Australia: Grassland Society of NSW inc. Retrieved from: http://grasslandnsw.com.au/news/wp-content/uploads/2011/10/Hume-Cosgrove-2005.pdf on 31/07/3018.

Hunt, W. F., & Easton, H. S. (1989). Fifty years of ryegrass research in New Zealand. *Proceedings of the New Zealand Grassland Association 50*, 11-23. Retrieved from:

https://www.grassland.org.nz/publications/nzgrassland_publication_999.pdf on 31/07/2018.

Hunt, W. F., & Field, T. R. O. (1979). Growth characteristics of perennial ryegrass. *Proceedings of the New Zealand Grassland Association 40*, 104-113. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_1373.pdf on 31/07/2018.

Hutchinson, G. K., Richards, K., & Risk, W. H. (2000). Aspects of accumulated heat patterns (growing degree-days) and pasture growth in Southland. *Proceedings of the New Zealand Grassland Association 62*, 81-85. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_2200.pdf on 31/07/2018.

Irving, L. J. (2015). Carbon assimilation, biomass partitioning and productivity in grasses. *Agriculture, 5*(4), 1116-1134.

James, D. B., & Hutto, J. M. (1972). Effects of tiller separation and root pruning on the growth of *Lolium perenne* L. *Annals of Botany, 36*(3), 485-495.

Jeffs, C. T., & Leather, S. R. (2014). Effects of extreme, fluctuating temperature events on life history traits of the grain aphid, *Sitobion avenae*. *Entomologia Experimentalis Et Applicata, 150*(3), 240-249.

Jensen, J. G., & Popay, A. J. (2007). Reductions in root aphid populations by non-toxic endophyte strains in tall fescue. *New Zealand Grassland Association: Endophyte Symposium*, 341-344.

Jia, L. L., Chen, X. P., Zhang, F., Buerkert, A., & Römheld, V. (2004). Use of digital camera to assess nitrogen status of winter wheat in the northern China plain. *Journal of Plant Nutrition, 27*(3), 441-450.

Johnson, L. J., de Bonth, A. C. M., Briggs, L. R., Caradus, J. R., Finch, S. C., Fleetwood, D. J., Fletcher, L. R., Hume, D. E., Johnson, R. D., Popay, A. J., Tapper, B. A., Simpson, W. R., Voisey, C. R., & Card, S. D. (2013). The exploitation of epichloae endophytes for agricultural benefit. *Fungal Diversity, 60*(1), 171-188.

Jung, G. A., van Wijk, A. J. P., Hunt, W. F., & Watson, C. E. (1996). Ryegrasses. In L. E. Moser, D. R. Buxton, & M. D. Casler (Eds.), *Cool-season forage grasses* (pp. 605-642). Madison, WI: ASA, CSSA, SSSA.

Katayama, N., Silva, A. O., Kishida, O., Ushio, M., Kita, S., & Ohgushi, T. (2014). Herbivorous insect decreases plant nutrient uptake: the role of soil nutrient availability and association of below-ground symbionts. *Ecological Entomology, 39*(4), 511-518.

Kawada, K. (1987). Polymorphism and morph determination. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 255-268). Amsterdam: Elsevier Science Publishers B.V.

Kawashima, S., & Nakatani, M. (1998). An algorithm for estimating chlorophyll content in leaves using a video camera. *Annals of Botany, 81*(1), 49-54.

Keiser, C. N., Sheeks, L. E., & Mondor, E. B. (2013). The effect of microhabitat feeding site selection on aphid foraging and predation risk. *Arthropod-Plant Interactions, 7*(6), 633-641.

Kergunteuil, A., Bakhtiari, M., Formenti, L., Xiao, Z., Defossez, E., & Rasmann, S. (2016). Biological control beneath the feet: a review of crop protection against insect root herbivores. *Insects, 7*(4).

Kindler, D., Hesler, L., Elliott, N., Royer, T., & Giles, K. (2004). Seasonal abundance of rice root aphid in wheat and its effect on forage and grain yields. *Southwestern Entomologist, 29*(4), 245-252.

Kindlmann, P., & Dixon, A. F. G. (1989). Developmental constraints in the evolution of reproductive strategies: telescoping of generations in parthenogenetic aphids. *Functional Ecology, 3*(5), 531-537.

Kindlmann, P., & Dixon, A. F. G. (2010). Modelling population dynamics of aphids and their natural enemies. In P. Kindlmann, A. F. G. Dixon, & J. P. Michaud (Eds.), *Aphid biodiversity under environmental change: patterns and processes* (pp. 1-20). Berlin: Springer-Verlag Berlin.

Klimaszewski, J., Newton, A. F., & Thayer, M. K. (1996). A review of the New Zealand rove beetles (Coleoptera: Staphylinidae). *New Zealand Journal of Zoology, 23*(2), 143-160.

Klingauf, F. A. (1987). Host plant finding and acceptance. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 209-224). Amsterdam: Elsevier Science Publishers B.V.

Klingler, J. P., Nair, R. M., Edwards, O. R., & Singh, K. B. (2009). A single gene, *AIN*, in *Medicago truncatula* mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid. *Journal of Experimental Botany, 60*(14), 4115-4127

Klowden, M. J. (2013). *Physiological systems in insects* (Third ed.). San Diego: Academic Press.

Koch, K. G., Chapman, K., Louis, J., Heng-Moss, T., & Sarath, G. (2016). Plant tolerance: a unique approach to control hemipteran pests. *Frontiers in Plant Science, 7*, 1-12 (Article number: 1363).

Kuo, M.-H., Lu, W.-N., Chiu, M. C., Kuo, Y.-H., & Hwang, S.-H. (2006). Temperature-dependent development and population growth of *Tetraneura nigriabdominalis* (Homoptera: Pemphigidae) on three host plants. *Journal of Economic Entomology, 99*(4), 1209-1213.

Kuroli, G., Németh, L., & Pocsai, K. (1998). Effect of primary metabolites on the number of aphids. In *Proceedings of the the 50th International Symposium on Crop Protection* (pp. 339-344). Ghent, Belgium: Universiteit Gent.

Lambers, D. H. R. (1966). Polymorphism in Aphididae. *Annual Review of Entomology, 11*, 47-78.

Langer, R. H. M. (1979). *How grasses grow* (2nd ed.). London: Edward Arnold Publishers.

Latch, G. C. M., & Christensen, M. J. (1985). Artificial infection of grasses with endophytes. *Annals of Applied Biology, 107*(1), 17-24.

Le Trionnaire, G., Hardie, J., Jaubert-Possamai, S., Simon, J.-C., & Tagu, D. (2008). Shifting from clonal to sexual reproduction in aphids: physiological and developmental aspects. *Biology of the Cell, 100*(8), 441-451.

Lees, A. D. (1983). The endocrine control of polymorphism in aphids. In R. G. H. Downer & N. Laufer (Eds.), *Endocrinology of Insects* (pp. 369-377): Alan R. Liss.

Leuchtmann, A., Bacon, C. W., Schardl, C. L., White, J. F. J., & Tadych, M. (2014). Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. *Mycologia, 106*(2), 202-215.

Liu, Q., Parsons, A. J., Xue, H., Fraser, K., Ryan, G. D., Newman, J. A., & Rasmussen, S. (2011a). Competition between foliar *Neotyphodium lolii* endophytes and mycorrhizal *Glomus* spp. fungi in *Lolium perenne* depends on resource supply and host carbohydrate content. *Functional Ecology, 25*(4), 910-920.

Liu, T.-X., Zhang, Y., & Yue, B. (2011b). Extraction from soil of apterous *Pemphigus populitransversus* (Hemiptera: Pemphigidae) feeding on cruciferous vegetable roots. *Journal of Economic Entomology, 104*(3), 1116-1119.

Lowe, A. D. (1966). Aphids trapped at three sites in Canterbury, New Zealand, over four years, with flight patterns for nine main species. *New Zealand Journal of Agricultural Research, 9*(3), 771-807.

Lowe, H. J. B. (1967). Interspecific differences in biology of aphids (Homoptera - Aphididae), on leaves of *Vicia faba*. 2. Growth and excretion. *Entomologia Experimentalis Et Applicata, 10*(3-4), 413-420.

Lowe, A. D. (1968). Alate aphids trapped over 8 years at two sites in Canterbury, New Zealand. *New Zealand Journal of Agricultural Research, 11*(4), 829-848.

Loxdale, H. D. (2009). What's in a clone: the rapid evolution of aphid asexual lineages in relation to geography, host plant adaptation and resistance to pesticides. In I. Schön, K. Martens, & P. Dijk (Eds.), *Lost sex: the evolutionary biology of parthenogenesis* (pp. 535-557). Dordrecht: Springer Netherlands.

Lu, J.-J., Dong, J.-M., Ren, M.-F., Li, X., Wu, Y.-P., Li, D.-Q., & Ma, E.-B. (2017). Migration patterns of subterranean pest insects in the soil of winter wheat-summer corn rotation fields in Linfen, Shanxi. *Acta Entomologica Sinica, 60*(9), 1046-1059.

Luo, D., Ganesh, S., & Koolaard, J. (2014). Predictmeans: calculate predicted means for linear models. *R package version 0.99.* Retrieved from https://CRAN.R-project.org/package=predictmeans on 25/04/2017.

Majidi, M. M., & Mirlohi, A. (2016). Impact of endophytic fungi on seed and seedling characteristics in tall and meadow fescues. *International Journal of Plant Production, 10*(4), 469-478.

Mangiafico, S. (2018). rcompanion: functions to support extension education program evaluation. *R package version 1.11.3.* Retrieved from https://CRAN.R-project.org/package=rcompanion on 9/03/2018.

Manheim, O., & Wool, D. (2003). Differential response of genotypes to alternative environments: a comparative morphological study of gall-inducing aphids (Homoptera: Pemphigidae: Fordinae). *Israel Journal of Zoology, 49*(4), 287-305.

Martinez-Torres, D., Buades, C., Latorre, A., & Moya, A. (2001). Molecular systematics of aphids and their primary endosymbionts. *Molecular Phylogenetics and Evolution, 20*(3), 437-449.

Massey University. (2000). Perennial ryegrass (*Lolium perenne*). Retrieved from http://pastureinfo.massey.ac.nz/grasspages/gperennialryegrass.html on 30/07/2018.

Matthew, C., Mackay, A. D., & Robin, A. H. K. (2016). Do phytomer turnover models of plant morphology describe perennial ryegrass root data from field swards? *Agriculture, 6*(3), 28.

May, K. J., Bryant, M. K., Zhang, X., Ambrose, B., & Scott, B. (2008). Patterns of expression of a lolitrem biosynthetic gene in the *Epichloe festucae*-perennial ryegrass symbiosis. *Molecular Plant-Microbe Interactions, 21*(2), 188-197.

McCarthy, S., Wims, C., Lee, J., & Donaghy, D. (2015). Perennial ryegrass grazing management in spring. Retrieved from https://www.dairynz.co.nz/media/2634153/perennial-ryegrass-grazing-guide-web.pdf on 30/07/2018.

McCornack, B. P., Carrillo, M. A., Venette, R. C., & Ragsdale, D. W. (2005). Physiological constraints on the overwintering potential of the soybean aphid (Homoptera: Aphididae). *Environmental Entomology, 34*(2), 235-240.

McDonald, G., Govender, A., & Umina, P. (2011). PestFact Issue no 2 - Pasture root aphids. *PestFacts South-Eastern (10/05/2011).* Cesar pty ltd. Retrieved from http://cesaraustralia.com/sustainable-agriculture/pestfacts-south-eastern/past-

issues/2011/pestfacts-issue-no-2-10th-may-2011/pasture-root-aphids/ on 30/07/2018.

Meister, B., Krauss, J., Härri, S. A., Schneider, M. V., & Müller, C. B. (2006). Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. *Basic and Applied Ecology, 7*(3), 244-252.

Mendiguren, G., Martín, M. P., Nieto, H., Pacheco-Labrador, J., & Jurdao, S. (2015). Seasonal variation in grass water content estimated from proximal sensing and MODIS time series in a Mediterranean Fluxnet site. *Biogeosciences, 12*(18), 5523-5535.

Meyer, D., Zeileis, A., & Hornik, K. (2017). vcd: visualizing categorical data. *R package version 1.4-4.*

Michaud, J. P., Jyoti, J. L., & Qureshi, J. A. (2006). Positive correlation of fitness with group size in two biotypes of Russian wheat aphid (Homoptera: Aphididae). *Journal of Economic Entomology, 99*(4), 1214-1224.

Miles, P. W. (1989a). The responses of plants to the feeding of Aphidoidea: principles. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. C, pp. 1-21). Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Miles, P. W. (1989b). Specific responses and damage caused by Aphidoidea. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. C, pp. 23-47). Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Mills, C. (2010). *Pistacia lentiscus* L. In Hortus Camdenensis: an illustrated catalogue of plants grown by Sir William MacArthur at Camden Park, NSW, Australia between c.1820 and 1861. Retrieved from http://hortuscamden.com/plants/view/pistacia-lentiscus-l on 30/07/2018.

Ministry for the Environment and Statistics New Zealand. (2015a). Growing degree days. *New Zealand's Environmental Reporting Series: Environmental indicators Te taiao Aotearoa.* Retrieved from http://www.stats.govt.nz/browse_for_stats/environment/environmental-reporting-series/environmental-indicators/home/atmosphere-and-climate/growing-degree-days.aspx on 30/07/2018.

Ministry for the Environment and Statistics New Zealand. (2015b). Land cover. *New Zealand's Environmental Reporting Series: Environmental indicators.* Retrieved from http://www.stats.govt.nz/browse_for_stats/environment/environmental-reporting-series/environmental-indicators/Home/Land/land-cover.aspx on 30/07/2018.

Mirik, M., Ansley, R. J., Steddom, K., Rush, C. M., Michels, G. J., Workneh, F., Cui, S., & Elliott, N. C. (2014). High spectral and spatial resolution hyperspectral imagery for quantifying Russian wheat aphid infestation in wheat using the constrained energy minimization classifier. *Journal of Applied Remote Sensing, 8*. doi: 10.1117/1.jrs.8.083661.

Miura, T., Braendle, C., Shingleton, A., Sisk, G., Kambhampati, S., & Stern, D. L. (2003). A comparison of parthenogenetic and sexual embryogenesis of the pea aphid *Acyrthosiphon pisum* (Hemiptera: Aphidoidea). *Journal of experimental Zoology Part B-Molecular and Developmental Evolution, 295B*, 59-81.

Miyazaki, M. (1987). Morphology of aphids. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 1-26). Amsterdam: Elsevier Science Publishers B.V.

Moate, P. J., Williams, S. R. O., Grainger, C., Hannah, M. C., Mapleson, D., Auldist, M. J., Greenwood, J. S., Popay, A. J., Hume, D. E., Mace, W. J., & Wales, W. J. (2012). Effects of wild-type, AR1 and AR37 endophyte-infected perennial ryegrass on dairy production in Victoria, Australia. *Animal Production Science, 52*(12), 1117-1130.

Moot, D. J., Scott, W. R., Roy, A. M., & Nicholls, A. C. (2000). Base temperature and thermal time requirements for germination and emergence of temperate pasture species. *New Zealand Journal of Agricultural Research, 43*(1), 15-25.

Moran, N. A. (1992). The evolution of aphid life cycles. *Annual Review of Entomology, 37*, 321-348.

Morris, G., & Foster, W. A. (2008). Duelling aphids: electrical penetration graphs reveal the value of fighting for a feeding site. *Journal of Experimental Biology, 211*(9), 1490-1494.

Mukherjee, K., & Baudach, A. F. (2016). Epigenetic control of polyphenism in aphids. In A. Vilcinskas (Ed.), *Biology and Ecology of Aphids* (pp. 89-99). Boca Raton: CRC Press.

Murdie, G. (1972). Problems of data analysis. In H. F. van Emden (Ed.), *Aphid technology* (pp. 296-318). London: Academic Press.

Mustafa, T. M., & Akkawi, M. (1987). The occurrence, economic importance and control of wheat root aphid (*Aploneura lentisci* Passerini) on wheat in Jordan. *Dirasat, XIV*(2), 83-88.

Mycobank. (2014). *Epichloë festucae* var. *lolii*. Retrieved from http://www.mycobank.org/BioloMICS.aspx?Table=Mycobank&Rec=519420&Fields=All on 31/07/2018

National Pesticide Information Center. (2017). Aphids. Retrieved from http://npic.orst.edu/pest/aphid.html on 31/07/2018.

Nieto Nafria, J. M. (2018). Fauna Europaea: Hemiptera, Aphidoidea, Aphididae, *Aploneura lentisci*. *Fauna Europaea version 2018.07.* Retrieved from http://www.faunaeur.org/full_results.php?id=54706 on 31/07/2018.

Nijhout, H. F. (2013). Arthropod developmental endocrinology. In A. Minelli, G. Boxshall, & G. Fusco (Eds.), *Arthropod biology and evolution* (pp. 123-148). Berlin Heidelberg: Springer.

Ogawa, K., & Miura, T. (2013). Two developmental switch points for the wing polymorphisms in the pea aphid Acyrthosiphon pisum. *EvoDevo, 4*, 30. doi: 10.1186/2041-9139-4-30.

Ogle, D. H. (2017). FSA: fisheries stock analysis. *R package version 0.8.17*.

Omacini, M., Semmartin, M., Pérez, L. I., & Gundel, P. E. (2012). Grass-endophyte symbiosis: a neglected aboveground interaction with multiple belowground consequences. *Applied Soil Ecology, 61*, 273-279.

Pennell, C. G. L., Popay, A. J., Ball, O. J. P., Hume, D. E., & Baird, D. B. (2005). Occurrence and impact of pasture mealybug (*Balanococcus poae*) and root aphid (*Aploneura lentisci*) on ryegrass (*Lolium* spp.) with and without infection by *Neotyphodium* fungal endophytes. *New Zealand Journal of Agricultural Research, 48*(3), 329-337.

Phillips, C., Townsend, H., & Vink, C. (2006, August 2006). Blow in the wind or border slippage? What natural dispersal of exotic species to New Zealand has to do with biosecurity. *Biosecurity, 69.* Retrieved from http://www.nzffa.org.nz/farm-forestry-model/the-essentials/forest-health-pests-and-diseases/biosecurity/blown-in-the-wind-or-border-slippage/ on 31/07/2018.

Pike, N., Richard, D., Foster, W., & Mahadevan, L. (2002). How aphids lose their marbles. *Proceedings of the Royal Society B: Biological Sciences, 269*(1497), 1211-1215.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., & R Core Team. (2017). Nlme: linear and nonlinear mixed effects models. *R package version 3.1-131.* Retrieved from https://CRAN.R-project.org/package=nlme on 9/03/2018.

Podmore, C. (2015). *Assessing mitochondrial DNA (CO1) barcodes for measuring diversity of Schizaphis spp. and abundance of Aploneura lentisci.* A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science in Biological Sciences at The University of Waikato, Hamilton, New Zealand. Retrieved from http://hdl.handle.net/10289/9886 on 31/07/2018.

Podsiadlowski, L. (2016). Phylogeny of the aphids. In A. Vilcinskas (Ed.), *Biology and Ecology of Aphids* (pp. 1-13). Boca Raton: CRC Press.

Pompon, J., Quiring, D., Giordanengo, P., & Pelletier, Y. (2010). Role of xylem consumption on osmoregulation in *Macrosiphum euphorbiae* (Thomas). *Journal of Insect Physiology, 56*(6), 610-615.

Ponsen, M. B. (1987). Alimentary tract. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 79-97). Amsterdam: Elsevier Science Publishers B.V.

Popay, A. J. (2004). *Aspects of the root ecology of Neotyphodium endophytes in Lolium perenne*. (Unpublished doctoral dissertation). University of Waikato, Hamilton, New Zealand.

Popay, A. J. (2008, 24.11.2008). Insect pests of crops, pasture and forestry - Introduced pests of pasture roots and foliage. *Te Ara - the encyclopedia of New Zealand.* Retrieved from http://www.TeAra.govt.nz/en/insect-pests-of-crops-pasture-and-forestry/page-3 on 31/07/2018.

Popay, A. J., Cotching, B., Moorhead, A., & Ferguson, C. M. (2012). AR37 endophyte effects on porina and root aphid populations and ryegrass damage in the field. In *Proceedings of the New Zealand Grassland Association 74*, 165-170. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_2287.pdf on 31/07/2018.

Popay, A. J., & Cox, N. R. (2016). *Aploneura lentisci* (Homoptera: Aphididae) and its interactions with fungal endophytes in perennial ryegrass (*Lolium perenne*). *Frontiers in Plant Science, 7*, 1395. doi: 10.3389/fpls.2016.01395.

Popay, A. J., & Crush, J. R. (2010). Influence of different forage grasses on nitrate capture and leaching loss from a pumice soil. *Grass and Forage Science, 65*(1), 28-37.

Popay, A. J., & Easton, H. S. (2006). Interactions between host plant genotype and *Neotyphodium* fungal endophytes affects insects. In C. F. Mercer (Ed.), *Breeding for Success: Diversity in Action - Proceedings of the 13th Australasian Plant Breeding Conference* (pp. 561-567), Dunedin, New Zealand: New Zealand Grassland Association. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_2468.pdf on 31/07/2018.

Popay, A. J., & Gerard, P. J. (2007). Cultivar and endophyte effects on a root aphid, *Aploneura lentisci*, in perennial ryegrass. *New Zealand Plant Protection, 60*, 223-227.

Popay, A. J., & Hume, D. E. (2011). Endophytes improve ryegrass persistence by controlling insects. *Pasture Persistence – Grassland Research and Practice Series 15* (pp. 149-156). Dunedin, New Zealand: New Zealand Grassland Association. Retrieved from https://www.grassland.org.nz/publications/nzgrassland_publication_2247.pdf on 31/07/2018.

Popay, A. J., Prestidge, R. A., Rowan, D. D., & Dymock, J. J. (1990). The role of *Acremonium lolii* mycotoxins in insect resistance of perennial ryegrass (*Lolium perenne*). In S. S. Quisenberry & R. E. Joost (Eds.), *Proceedings of the 1st international symposium on Acremonium/grass interactions* (pp. 44-47). Baton Rouge, LA: Louisiana Agricultural Experiment Station.

Popay, A. J., & Thom, E. R. (2009). Endophyte effects on major insect pests in Waikato dairy pasture. In *Proceedings of the New Zealand Grassland Association Conference Vol.* 71, (pp. 121-126), Dunedin, New Zealand: New Zealand Grassland Association. Retrieved from: https://www.grassland.org.nz/publications/nzgrassland_publication_77.pdf on 31/07/2018.

Popay, A. J., & Wyatt, R. T. (1995). Resistance to Argentine stem weevil in perennial ryegrass infected with endophytes producing different alkaloids. In A. J. Popay (Ed.), *Proceedings of the 48th New Zealand Plant Protection Conference* (pp. 229-236), Rotorua: New Zealand Plant Protection Society. Retrieved from: https://www.cabdirect.org/cabdirect/abstract/19961106228 on 31/07/3018.

Pope, R. D. (1983). Some aphid waxes, their form and function (Homoptera: Aphididae). *Journal of Natural History, 17*(4), 489-506.

Prestidge, R. A., & Gallagher, R. T. (1985). Lolitrem B - a stem weevil toxin isolated from *Acremonium*-infected ryegrass. In M. J. Hartley (Ed.), *Proceedings of the 38th New Zealand Weed and Pest Control Conference* (pp. 38-40). Rotorua, New Zealand: The New Zealand Weed and Pest Control Society.

Prestidge, R. A., Pottinger, R. P., & Barker, G. M. (1982). An association of *Lolium* endophyte with ryegrass resistance to Argentine stem weevil. In M. J. Hartley (Ed.), *Proceedings of the 35th New Zealand Weed and Pest Control Conference* (pp. 119-122). Hamilton: New Zealand Weed and Pest Control Society.

Pretorius, R. J., Hein, G. L., & Bradshaw, J. D. (2016). Ecology and management of *Pemphigus betae* (Hemiptera: Aphididae) in sugar beet. *Journal of Integrated Pest Management, 7*(1), 1-9.

R Core Team. (2017). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org/ on 9/03/2018.

Rasband, W. S. (1997-2016). ImageJ. Bethesda, Maryland, USA: U. S. National Institutes of Health. Retrieved from http://imagej.nih.gov/ij/ on 11/09/2014.

Rasmussen, S., Parsons, A. J., Bassett, S., Christensen, M. J., Hume, D. E., Johnson, L. J., Johnson, R. D., Simpson, W. R., Stacke, C., Voisey, C. R., Xue, H., & Newman, J. A. (2007). High nitrogen supply and carbohydrate content reduce fungal endophyte and alkaloid concentration in *Lolium perenne*. *New Phytologist, 173*(4), 787–797.

Rasmussen, S., Parsons, A. J., Fraser, K., Xue, H., & Newman, J. A. (2008a). Metabolic profiles of *Lolium perenne* are differentially affected by nitrogen supply, carbohydrate content, and fungal endophyte infection. *Plant Physiology, 146*(3), 1440-1453.

Rasmussen, S., Parsons, A. J., & Newman, J. A. (2009). Metabolomics analysis of the *Lolium perenne–Neotyphodium lolii* symbiosis: more than just alkaloids? *Phytochemistry Reviews, 8*(3), 535-550.

Rasmussen, S., Parsons, A. J., Popay, A., Xue, H., & Newman, J. A. (2008b). Plant-endophyte-herbivore interactions. *Plant Signaling & Behavior, 3*(11), 974-977.

Reece, J. B., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., & Jackson, R. B. (2011). *Campbell biology* (B. Wilbur Ed. 9th global ed.). San Francisco, CA: Pearson Education.

Reed, K. F. M. (2008). Fact sheet - Perennial ryegrass. *Pastures Australia.* 7/08/2008. Retrieved from http://keys.lucidcentral.org/keys/v3/pastures/Html/Perennial_ryegrass.htm on 20/08/2017.

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose-response analysis using R. *Plos One, 10*(12), e0146021. doi: 10.1371/journal.pone.0146021.

Robin, A. H. K. (2011). *Segmental morphology of perennial ryegrass (Lolium perenne L.): a study of functional implications of plant architecture.* A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy (PhD) in Plant Science, Institute of Natural Resources, College of Sciences, Massey University, Manawatu, Palmerston North, New Zealand. Retrieved from https://mro.massey.ac.nz/handle/10179/2783 on 31/07/2018.

Rosell, R. C., Davidson, E. W., Jancovich, J. K., Hendrix, D. L., & Brown, J. K. (2003). Size limitations in the filter chamber and digestive tract of nymphal and adult *Bemisia tabaci* whiteflies (Hemiptera: Aleyrodidae). *Annals of the Entomological Society of America, 96*(4), 544-552.

Rowan, D. D., & Latch, G. C. M. (1994). Utilization of endophyte-infected perennial ryegrasses for increased insect resistance. In C. W. Bacon & J. F. J. White (Eds.), *Biotechnology of endophytic fungi of grasses* (pp. 169-183). Boca Raton: CRC Press.

Ruppert, K. G. (2016). *Fungal alkaloid occurrence during seedling establishment and early growth in Lolium perenne seedlings infected with Epichloë festucae var. lolii and the influence of adult Argentine stem weevil (Listronotus bonariensis) feeding on alkaloid concentrations.* (Master's thesis Thesis), Massey University, Palmerston North, New Zealand. Retrieved from https://mro.massey.ac.nz/handle/10179/11134 on 31/07/2018.

Ruppert, K. G., Matthew, C., McKenzie, C. M., & Popay, A. J. (2017). Impact of *Epichloë* endophytes on adult Argentine stem weevil damage to perennial ryegrass seedlings. *Entomologia Experimentalis Et Applicata, 163*(3), 328-337.

Saari, S., Helander, M., Faeth, S. H., & Saikkonen, K. (2010). The effects of endophytes on seed production and seed predation of tall fescue and meadow fescue. *Microbial Ecology, 60*, 928-934.

Saheed, S. A., Liu, L., Jonsson, L., & Botha, C. E. J. (2007). Xylem – as well as phloem – sustains severe damage due to feeding by the Russian wheat aphid. *South African Journal of Botany, 73*(4), 593-599.

Salt, D. T., Major, E., & Whittaker, J. B. (1996). Population dynamics of root aphids feeding on Sitka spruce in two commercial plantations. *Pedobiologia, 40*(1), 1-11.

Sanchez-Azofeifa, A., Oki, Y., Fernandes, G. W., Ball, R. A., & Gamon, J. (2012). Relationships between endophyte diversity and leaf optical properties. *Trees, 26*(2), 291-299.

Sandhu, D. (2011). *Pistachia* L. Anacardiaceae. *The friends of the Waite arboretum Inc, Newsletter No. 66, Summer 2011.* Retrieved from http://www.adelaide.edu.au/waite-historic/friends/arboretum/newsletters/fwa_newsletter_66%20summer_2011.pdf on 31/07/2018.

Sandström, J., & Moran, N. (1999). How nutritionally imbalanced is phloem sap for aphids? *Entomologia Experimentalis Et Applicata, 91*(1), 203-210.

Scheu, S., Theenhaus, A., & Jones, T. H. (1999). Links between the detritivore and the herbivore system: effects of earthworms and Collembola on plant growth and aphid development. *Oecologia, 119*(4), 541-551.

Schuett, W., Dall, S. R. X., Kloesener, M. H., Baeumer, J., Beinlich, F., & Eggers, T. (2015). Life-history trade-offs mediate 'personality' variation in two colour morphs of the pea aphid, *Acyrthosiphon pisum*. *Journal of Animal Ecology, 84*(1), 90-101.

Shakesby, A. J., Wallace, I. S., Isaacs, H. V., Pritchard, J., Roberts, D. M., & Douglas, A. E. (2009). A water-specific aquaporin involved in aphid osmoregulation. *Insect Biochemistry and Molecular Biology, 39*(1), 1-10.

Shaposhnikov, G. C. (1987). Organization (structure) of populations and species, and speciation. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 415-430). Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Silsbury, J. H. (1970). Leaf growth in pasture grasses. *Tropical Grasslands, 4*(1), 17-36.

Simon, J. C., le Gallic, J. F., Bonhomme, J., & Dedryver, C. A. (1994). Breeding system complex in the cereal aphid *Rhopalosiphum padi* L. (Homoptera, Aphididae) and its influences on population biology and dynamics. *Bulletin OILB/SROP, 17*(4), 11-15.

Simpson, W. R., Christensen, M. J., & Hume, D. E. (1997). An appraisal of the use of axillary buds of grasses as clonal material for inoculation with *Neotyphodium* endophytes. In C. W. Bacon & N. S. Hill (Eds.), *3rd International Symposium on Neotyphodium/Grass Interactions*(pp. 275-277), New York and London: Plenum Press. Retrieved from: https://link.springer.com/chapter/10.1007/978-1-4899-0271-9_51 on 31/07/2018.

Simpson, W. R., Schmid, J., Singh, J., Faville, M. J., & Johnson, R. D. (2012). A morphological change in the fungal symbiont *Neotyphodium lolii* induces dwarfing in its host plant *Lolium perenne*. *Fungal Biology, 116*(2), 234-240.

Singh, L. P., Gill, S. S., & Tuteja, N. (2011). Unraveling the role of fungal symbionts in plant abiotic stress tolerance. *Plant Signaling & Behavior, 6*(2), 175-191.

Skaljac, M. (2016). Bacterial symbionts of aphids (Hemiptera: Aphididae). In A. Vilcinskas (Ed.), *Biology and Ecology of Aphids* (pp. 100-125). Boca Raton: CRC Press.

Smith, C. M. (1989). *Plant resistance to insects: a fundamental approach*. New York: John Wiley & Sons.

Smith, C. M., & Chuang, W. P. (2014). Plant resistance to aphid feeding: behavioral, physiological, genetic and molecular cues regulate aphid host selection and feeding. *Pest Management Science, 70*(4), 528-540.

Smith, C. M., & Clement, S. L. (2012). Molecular bases of plant resistance to arthropods. *Annual Review of Entomology, 57*, 309-328.

Smith, R. G. (1999). Wax glands, wax production and the functional significance of wax use in three aphid species (Homoptera: Aphididae). *Journal of Natural History, 33*(4), 513-530.

Soper, K. (1959). Root anatomy of grasses and clovers. *New Zealand Journal of Agricultural Research, 2*(2), 329-341.

SphereOptics GmbH. (2017). Remote sensing and proximal sensing. Retrieved from http://sphereoptics.de/en/application/remote-sensing-proximal-sensing/ on 31/07/2018.

Spiering, M. J., Greer, D. H., & Schmid, J. (2006). Effects of the fungal endophyte, *Neotyphodium lolii,* on net photosynthesis and growth rates of perennial ryegrass (*Lolium perenne*) are independent of *in planta* endopyte concentration. *Annals of Botany, 98*(2), 379-387.

Srivastava, P. N. (1987). Nutritional physiology. In A. K. Minks & P. Harrewijn (Eds.), *Aphids: their biology, natural enemies and control* (Vol. A, pp. 99-121). Amsterdam: Elsevier Science Publishers B.V.

Stadler, B. (1992). Physiological responses of *Uroleucon jaceae* (L.) to seasonal changes in the quality of its host plant C*entaurea jacea* L.: multilevel control of adaptations to the life cycle of the host. *Oecologia, 91*(2), 273-280.

Tan, Y. Y., Spiering, M. J., Scott, V., Lane, G. A., Christensen, M. J., & Schmid, J. (2001). In planta regulation of extension of an endophytic fungus and maintenance

of high metabolic rates in its mycelium in the absence of apical extension. *Applied and Environmental Microbiology, 67*(12), 5377-5383.

The Treasury. (2016). New Zealand economic and financial overview 2016. Retrieved from http://www.treasury.govt.nz/economy/overview/2016/nzefo-16.pdf on 3/08/2017.

Therneau, T. (2015a). A package for survival analysis in S. *R package version 2.38.* Retrieved from https://CRAN.R-project.org/package=survival on 9/03/2018.

Therneau, T. M., & Grambsch, P. M. (2000). *Modeling survival data: extending the cox model*. New York: Springer.

Thom, E. R., Popay, A. J., Waugh, C. D., & Minneé, E. M. K. (2014). Impact of novel endophytes in perennial ryegrass on herbage production and insect pests from pastures under dairy cow grazing in northern New Zealand. *Grass and Forage Science, 69*(1), 191-204.

Thornton, B., & Millard, P. (1996). Effects of severity of defoliation on root functioning in grasses. *Journal of Range Management, 49*(5), 443-447.

Tian, P., Le, T.-N., Ludlow, E. J., Smith, K. F., Forster, J. W., Guthridge, K. M., & Spangenberg, G. C. (2013). Characterisation of novel perennial ryegrass host-Neotyphodium endophyte associations. *Crop & Pasture Science, 64*(7), 716-725.

Tokunaga, E., & Suzuki, N. (2008). Colony growth and dispersal in the ant-tended aphid, *Aphis craccivora* Koch, and the non-ant-tended aphid, *Acyrthosiphon pisum* Harris, under the absence of predators and ants. *Population Ecology, 50*(1), 45-52.

Tomasetto, F., Tylianakis, J. M., Reale, M., Wratten, S., & Goldson, S. L. (2017). Intensified agriculture favors evolved resistance to biological control.

Proceedings of the National Academy of Sciences of the United States of America 114(15), 3885-3890.

Tomczak, V. V., & Müller, C. (2018). Plant species, mycorrhiza, and aphid age influence the performance and behaviour of a generalist. *Ecological Entomology, 43*(1), 37-46.

Tozer, K. N., Carswell, K., Griffiths, W. M., Crush, J. R., Cameron, C. A., Chapman, D. F., Popay, A., & King, W. (2017). Growth responses of diploid and tetraploid perennial ryegrass (*Lolium perenne*) to soil-moisture deficit, defoliation and a root-feeding invertebrate. *Crop & Pasture Science, 68*(7), 632-642.

Trafford, G., & Trafford, S. (Eds.). (2011). *Farm technical manual*. Lincoln, New Zealand: Faculty of Commerce, Lincoln University.

Traicevski, V., & Ward, S. A. (2002). The effect of past and current hosts on reproductive investment by the adult cowpea aphid *Aphis craccivora*. *Ecological Entomology, 27*(5), 601-607.

Tsai, J. H., & Liu, Y.-H. (1998). Effect of temperature on development, survivorship, and reproduction of rice root aphid (Homoptera: Aphididae). *Environmental Entomology, 27*(3), 662-666.

Turchin, P., & Kareiva, P. (1989). Aggregation in *Aphis varians*: an effective strategy for reducing predation risk. *Ecology, 70*(4), 1008-1016.

Urbańska, A. (2010). Histochemical analysis of aphid saliva in plant tissue. *Electronic Journal of Polish Agricultural Universities.* Retrieved from http://www.ejpau.media.pl/volume13/issue4/art-26.html on 13/04/2019.

van der Heijden, M. G. A., Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., & Sanders, I. R. (1998). Mycorrhizal fungal diversity

determines plant biodiversity, ecosystem variability and productivity. *Nature, 396*(6706), 69-72.

Venables, W. N., & Ripley, B. D. (Eds.). (2002). *Modern applied statistics with S* (Fourth ed.). New York, NY: Springer

Vilcinskas, A. (2016). Aphid immunity. In A. Vilcinskas (Ed.), *Biology and Ecology of Aphids* (pp. 126-134). Boca Raton: CRC Press.

Volkl, W. (1990). Seasonal changes in the feeding sites of *Capitophorus carduinus* (Walker)- Relation to plant morphology. *Acta Phytopathologica Et Entomologica Hungarica, 25*(1-4), 197-202.

Wakelin, S., Harrison, S., Mander, C., Dignam, B., Rasmussen, S., Monk, S., Fraser, K., & O'Callaghan, M. (2015). Impacts of endophyte infection of ryegrass on rhizosphere metabolome and microbial community. *Crop & Pasture Science, 66*(10), 1049-1057.

Ward, S. A., & Dixon, A. F. G. (1982). Selective resorption of aphid embryos and habitat changes relative to life-span. *Journal of Animal Ecology, 51*(3), 859-864.

Webster, B. (2012). The role of olfaction in aphid host location. *Physiological Entomology, 37*(1), 10-18.

Weibull, J. (1987). Seasonal changes in the free amino-acids of oat and barley phloem sap in relation to plant growth stage and growth of *Rhopalosiphum padi*. *Annals of Applied Biology, 111*(3), 729-737.

White, L. M. (1973). Carbohydrate reserves of grasses: a review. *Journal of Range Management, 26*(1), 13-18.

Will, T. (2016). Function of aphid saliva in aphid-plant interaction. In A. Vilcinskas (Ed.), *Biology and ecology of aphids* (pp. 221-237). Boca Raton: CRC Press.

Wilson, A. C. C., Sunnucks, P., & Hales, D. F. (1999). Microevolution, low clonal diversity and genetic affinities of parthenogenetic *Sitobion* aphids in New Zealand. *Molecular Ecology, 8*(10), 1655-1666.

Wool, D. (1990). Regular alternation of high and low population size of gall-forming aphids: analysis of ten years of data. *Oikos, 57*(1), 73-79.

Wool, D., & Kurzfeld-Zexer, L. (2008). Life underground: exposing the subterranean stage in the holocycle of galling aphids (Homoptera: Pemphigidae: Fordinae) on *Pistacia* (Anacardiaceae). *Israel Journal of Entomology, 38*, 1-18.

Wool, D., & Manheim, O. (1986). Population ecology of the gall-forming aphid, *Aploneura lentisci* (Pass.) in Israel. *Researches on Population Ecology, 28*, 151-162.

Wool, D., Manheim, O., Burstein, M., & Levi, T. (1994). Dynamics of re-migration of sexuparae to their primary hosts in the gall-forming Fordinae (Homoptera: Aphidoidea: Pemphigidae). *European Journal of Entomology, 91*(1), 103-108.

Wool, D., & Sulami, Z. (2001). Induction of alate sexuparae in root-cage colonies, and female-biased sex ratios in the galling aphid, *Aploneura lentisci*. *Entomologia Experimentalis Et Applicata, 101*, 299-303.

Wright, P. J., & McManus, C. E. L. (1998). Root aphids and ants in a Bedfordshire grassland. *The Entomologist's Monthly Magazine, 134*, 75-76.

Yang, J. Z., Matthew, C., & Rowland, R. E. (1998). Tiller axis observations for perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea*): Number of active phytomers, probability of tiller appearance, and frequency of root appearance per phytomer for three cutting heights. *New Zealand Journal of Agricultural Research, 41*(1), 11-17.

Zehnder, C. B., Parris, M. A., & Hunter, M. D. (2007). Effects of maternal age and environment on offspring vital rates in the Oleander aphid (Hemiptera: Aphididae). *Environmental Entomology, 36*(4), 910-917.

Zhang, D. (2018). rsq: R-squared and related measures. *R package version 1.0.1*.

Zhang, W., Card, S. D., Mace, W. J., Christensen, M. J., McGill, C. R., & Matthew, C. (2017). Defining the pathways of symbiotic *Epichloë* colonization in grass embryos with confocal microscopy. *Mycologia, 109*(1), 153-161.

Zytynska, S. A., & Weisser, W. (2016). The effect of plant within-species variation on aphid ecology. In A. Vilcinskas (Ed.), *Biology and ecology of aphids* (pp. 152-170). Boca Raton: CRC Press.

A study of root aphid *Aploneura lentisci* Pass. biology and root aphid-host interactions with perennial ryegrass/endophyte associations in New Zealand

A dissertation presented in partial fulfilment of the requirements for the degree of

Doctor in Entomology

at **Massey University, New Zealand**

APPENDICES

Jana Leonie Muller

2019

APPENDIX 1: Declaration of content

Declaration confirming content of digital version of thesis

I confirm that the content of the digital version of this thesis

A study of root aphid *Aploneura lentisci* Pass. biology and root aphid-host interactions with perennial ryegrass/endophyte associations in New Zealand

is the final amended version following the examination process and is identical to this hard-bound paper copy.

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Student's signature: 

Date: 12.05.2019

APPENDIX 2: Glossary

Accession A collection of plant material from a particular location (Aubry *et al.*, 2005).

Adventitious root Root developed from a lower tiller node, i.e. every root that did not develop from the seed (Langer, 1979; Hannaway *et al.*, 1999).

Alkaloids Very diverse chemicals and biomolecules that are secondary compounds derived from amino acids or from transamination processes (Aniszewski, 2015).

Base temperature Threshold temperature below which a plant does not develop (Moot *et al.*, 2000)

Blot, blotting Immuno-detection technique to reveal the presence of endophytes. The method bases on the application of a transversal, basal tiller cut to a nitrocellulose membrane (blot), and a consecutive revealing of fungal protein via antibodies (Card *et al.*, 2011; Simpson *et al.*, 2012).

BS A milk protein blocking solution used to develop blots and prepared as follows: 2.42 g Tris(hydroxymethyl)methylamine, 2.92 g NaCL, 5 g non/fat milk powder, 10 mL of 1M HCl, made up to 1 L with RO water and adjusted to pH 7.5 (Simpson *et al.*, 2012).

Conditional R^2 Variance explained by both fixed and random factors in a mixed effects model, as calculated by the 'MuMIn' R package (Bartoń, 2016); see also marginal R^2.

Culm Flowering grass shoot (vegetative grass shoots are called 'tillers').

ELISA Enzyme/linked immunosorbent assay; Technique that allows the quantification of fungal DNA in a sample.

Endodermis	Layer of selectively permeable cells surrounding the vascular bundles (phloem, xylem and pericycle) in the roots. The endodermis controls what enters the vascular system of a plant (Reece *et al.*, 2011).
Exuvium (*pl.* exuviae)	Shed exoskeleton left behind at moulting.
Fescue toxicosis/ fescue foot	Condition of strong restricted blood flow to feet and tail that may lead to tissue death and gangrene (Ruppert, 2016).
Heat stress	Symptom of ergot toxins consumption; Elevation of body temperature and respiration rate as a result of vasoconstriction
Hemimetabolous	Adjective used to describe insects that develop from nymphs to adults without a pupal stage.
HRM analysis	High resolution melting analysis.
Hypha (hyphae)	One of many interconnected filaments that together constitute the "body" of a fungus (mycelium) (Reece *et al.*, 2011).
Imago	Adult
Marginal R^2	Variance explained by fixed factors in a mixed effects model (see also conditional R^2). Pseudo-R^2 calculated by the 'MuMIn' R package (Bartoń, 2016).
Meristem	Group of actively dividing cells that forms leaves, shoots, roots and inflorescences.
Morph	Adult phenotype of a species that differs from another phenotype of the same species by its morphology and/or its reproduction (Lambers, 1966).
Mycelium	Physical body of a fungus; The totality of all hyphae belonging to one individual (Reece *et al.*, 2011).

NCM	Nitrocellulose membrane. This membrane is able to bind proteins and is used for immuno/detection of endophyte presence in plant tillers.
Net reproduction rate	Rate of multiplication in one generation (Birch, 1984); number of female offspring per mother.
Nymph	Immature stage.
Phyllochron	Interval between the appearance of successive leaf tips.
Plastochron	Interval between the appearance of successive leaf primordia; often used as a biological time scale in physiological experiments (Langer, 1979).
Pseudostem	Above-ground plant part that looks like a stem, but is composed by leaf sheaths.
Regrowth 24h	Length of the leaf blade regrown by a trimmed tiller 24 h after the initial trim (Section 2.4.2)
Rhinaria	Sensory organs found on the antennae of aphids. Circular openings covered by a membrane and containing receptor neurons that allow aphids to detect volatile compounds. Primary rhinaria are constituted by an assembly of several sensillum types and appear involved in host plant location. Secondary rhinaria are made out of sensilla placoidea that contains neurons with receptors perceiving aphid sex pheromone constituents (and plant volatiles and alarm pheromones in asexual morphs that do not react to sexual stimuli) (Boullis & Verheggen, 2016).
RO water	Water purified by the process of reverse osmosis.
Ryegrass staggers	Neuromuscular disorder caused by the consumption of common-toxic endophyte-infected perennial ryegrass forage. Severe muscular spasms leading to poor coordination and hypersensitivity to external stimulations occur thereby. Live weight gains and milk production may be reduced in affected animals. Endophytes producing lolitrem

B are the most frequent cause for the disease. Epoxy-janthitrems producing AR37-infected perennial ryegrass may cause staggers too. Frequency and severity of outbreaks are lower in endophyte/plant symbioses producing these alkaloids, though (Ruppert, 2016).

Seminal root Seed root, root emerging from the seed.

Stolon Creeping stem that roots at the nodes.

Tiller Vegetative grass shoot (flowering shoots are called 'culm').

References:

Aniszewski, T. (2015). Definition, typology, and occurrence of alkaloids. *Alkaloids* (second ed., pp. 1-97). Boston: Elsevier.

Aubry, C., Shoal, R., & Erickson, V. (2005). Grass cultivars: their origins, development, and use on national forests and grasslands in the Pacific Northwest. *USDA Forest Service*. Portland, Oregon.

Bartoń, K. (2016). MuMIn: multi-model inference. *R package version 1.15.6.*

Birch, L. C. (1948). The intrinsic rate of natural increase of an insect population. *Journal of Animal Ecology, 17*(1), 15-26. doi:10.2307/1605.

Boullis, A., & Verheggen, F. J. (2016). Chemical Ecology of Aphids (Hemiptera: Aphididae). In A. Vilcinskas (Ed.), *Biology and Ecology of Aphids* (pp. 171-198). Boca Raton: CRC Press.

Card, S. D., Rolston, M. P., Park, Z., Cox, N., & Hume, D. E. (2011). Fungal endophyte detection in pasture grass seed utilising the infection layer and comparison to other detection techniques. *Seed Science & Technology, 39*(3), 581-592.

Hannaway, D., Fransen, S., Cropper, J., Teel, M., Chaney, M., Griggs, T., Halse, R., Hart, J., Cheeke, P., Hansen, D., Klinger, R., & Lane, W. (1999). Perennial ryegrass (*Lolium perenne* L.). *Pacific Northwest Extension Publications, PNW-503.*

Lambers, H. R. (1966). Polymorphism in aphididae. *Annual Review of Entomology, 11*, 47-78.

Langer, R. H. M. (1979). *How grasses grow* (2nd ed. Vol. 34). London: Edward Arnold.

Moot, D. J., Scott, W. R., Roy, A. M., & Nicholls, A. C. (2000). Base temperature and thermal time requirements for germination and emergence of temperate pasture species. *New Zealand Journal of Agricultural Research, 43*(1), 15-25.

Reece, J. B., Urry, L. A., Cain, M. L., Wasserman, S. A., Minorsky, P. V., & Jackson, R. B. (2011). *Campbell biology* (B. Wilbur Ed. Global edition, 9th ed.). San Francisco, CA: Pearson Education.

Ruppert, K. G. (2016). *Fungal alkaloid occurrence during seedling establishment and early growth in Lolium perenne seedlings infected with Epichloë festucae var. lolii and the influence of adult Argentine stem weevil (Listronotus bonariensis) feeding on alkaloid concentrations.* Master of Science (MSc) Thesis, Massey University, Palmerston North, New Zealand.

Simpson, W. R., Schmid, J., Singh, J., Faville, M. J., & Johnson, R. D. (2012). A morphological change in the fungal symbiont *Neotyphodium lolii* induces dwarfing in its host plant *Lolium perenne*. *Fungal Biology, 116*(2), 234-240.

APPENDIX 3: Meteorological data for Palmerston North and the research site

PN reference weather station (Met report): Palmerston North Ews NIWA weather station; Agent Number 21963, Network Number EO536D; coordinates: Latitude 40.38195 S, Longitude 175.60915 E

On-site weather station: WatchDog 2900ET Weather Station (Spectrum Technologies, Inc., Aurora, Illinois, U.S.A.), on a permanent stand in the outdoor nursery plot of the Grasslands Research Centre, AgResearch Ltd, Palmerston North; July 2015-June 2016, logs in 15 minutes intervals.

Table A3.1. Temperatures in Palmerston North (average of monthly means for years 1987 to 2016, as recorded by the PN reference weather station).

Month	Season	Maximum air [°C]	Minimum air [°C]	Average air [°C]	Minimum grass [°C]	10 cm depth [°C]	20 cm depth [°C]	30 cm depth [°C]	1 m depth [°C]	Number frost days
January	Summer	22.7	12.6	17.7	10.5	18.4	19.7	19.7	19.0	0.2
February	Summer	23.1	13.3	18.2	11.0	18.5	19.8	19.9	19.8	0.1
March	Autumn	21.4	11.5	16.4	8.7	16.2	17.5	18.2	18.9	0.6
April	Autumn	18.6	8.9	13.7	6.1	13.2	14.4	15.3	16.9	2.9
May	Autumn	15.9	7.1	11.5	4.3	10.7	11.8	12.6	14.6	6.5
June	Winter	13.4	5.1	9.3	2.3	8.2	9.3	10.0	12.3	10.4
July	Winter	12.8	4.4	8.6	1.5	7.1	8.1	8.8	10.7	11.9
August	Winter	13.7	5.1	9.4	2.1	8.0	9.0	9.4	10.7	9.8
September	Spring	15.2	6.7	10.9	4.0	10.2	10.8	11.2	11.8	5.5
October	Spring	16.7	8.2	12.5	5.8	12.3	13.1	13.5	13.4	3.2
November	Spring	18.2	9.6	13.9	7.3	14.6	15.4	16.0	15.3	1.1
December	Summer	20.8	11.7	16.3	9.7	17.1	17.9	18.1	17.3	0.2
Mean		**17.7**	**8.7**	**13.2**	**6.1**	**12.9**	**13.9**	**14.4**	**15.1**	
Total										**52.4**

The variance between monthly means laid in all cases between 0.45 and 1.70 [°C]. Frost days: days for which the minimal air temperature was below 0 °C (Ministry for the Environment and Statistics New Zealand, 2015). PN reference weather station; Palmerston North Ews NIWA weather station, Latitude 40.38195 S, Longitude 175.60915 E

Table A3.2. Astronomical day length calculated by the U.S. Naval Observatory (2013) (daylight hours), and sunshine, rainfall, rain days, pan evaporation, and wind run in Palmerston North (average of monthly means for years 1987 to 2016, as recorded by the PN reference weather station).

Month	Season	Daylight hours (astronomical average) [h/day]	Sunshine [h/month]	Wind run [km/day]	Rainfall [mm/month]	Rain days [number/month]	Pan evaporation [mm/month]	Relative humidity air [%]
January	Summer	14:39	213 ± 32.1	299 ± 60.0	60 ± 30.1	10 ± 2.9	165 ± 21.0	76 ± 3.5
February	Summer	13:38	192 ± 34.4	278 ± 58.6	71 ± 65.2	10 ± 4.2	134 ± 16.7	78 ± 3.8
March	Autumn	12:20	182 ± 30.1	257 ± 54.4	63 ± 38.8	11 ± 3.4	106 ± 15.6	78 ± 3.6
April	Autumn	11:01	151 ± 24.5	222 ± 47.7	80 ± 42.0	12 ± 4.2	65 ± 11.2	80 ± 3.3
May	Autumn	09:54	115 ± 22.3	217 ± 56.3	88 ± 43.2	16 ± 3.8	39 ± 7.3	85 ± 2.1
June	Winter	09:20	89 ± 18.3	212 ± 57.9	100 ± 32.5	17 ± 3.1	23 ± 4.7	88 ± 2.3
July	Winter	09:36	111 ± 25.0	219 ± 62.4	84 ± 42.8	17 ± 4.6	25 ± 4.9	87 ± 1.6
August	Winter	10:34	129 ± 25.7	224 ± 47.2	83 ± 31.2	17 ± 4.3	39 ± 5.1	84 ± 2.0
September	Spring	11:49	131 ± 26.1	272 ± 65.0	86 ± 49.5	16 ± 4.6	61 ± 10.2	79 ± 2.5
October	Spring	13:09	141 ± 25.5	302 ± 73.4	101 ± 39.6	17 ± 3.1	90 ± 12.8	78 ± 4.5
November	Spring	14:21	165 ± 35.0	322 ± 61.8	84 ± 40.7	14 ± 4.1	121 ± 15.1	76 ± 3.2
December	Summer	14:59	170 ± 31.1	303 ± 55.6	86 ± 34.4	15 ± 3.8	150 ± 18.9	76 ± 4.4
Daily average		**12:07**		**261**				**80**
Yearly total			**1790**		**985**	**171**	**1017**	

PN reference weather station; Palmerston North Ews NIWA weather station, Latitude 40.38195 S, Longitude 175.60915 E

Table A3.3. Wind in the outdoor nursery area of the Grasslands Research Centre, AgResearch Ltd, Palmerston North.

Month	Season	Average wind speed overall [km/h]	Frequency [% of records > 0 km/h]	Cardinal wind direction [% of records > 0 km/h]							
				N	NE	E	SE	S	SW	W	NW
July 2015	Winter	1.0 ± 2.25	31	11	14	11	12	11	5	7	**29**
August 2015	Winter	1.6 ± 2.63	40	11	**20**	**21**	8	5	4	8	**22**
September 2015	Spring	2.8 ± 3.65	58	7	**16**	**22**	15	11	5	7	**16**
October 2015	Spring	2.8 ± 3.17	64	12	10	12	7	11	9	14	**25**
November 2015	Spring	2.7 ± 3.05	63	9	13	18	13	12	10	9	**17**
December 2015	Summer	2.5 ± 3.16	59	9	**14**	**17**	10	15	10	9	**16**
January 2016	Summer	2.7 ± 3.27	58	7	**21**	**22**	11	**18**	8	6	8
February 2016	Summer	2.2 ± 3.17	51	7	**20**	**18**	10	**19**	8	6	12
March 2016	Autumn	3.0 ± 3.95	57	7	**25**	**28**	10	10	4	5	12
April 2016	Autumn	1.8 ± 2.70	45	6	**23**	**25**	7	5	5	9	**21**
May 2016	Autumn	1.8 ± 2.56	48	6	6	4	4	5	10	21	**43**
June 2016	Winter	1.5 ± 2.57	39	8	16	10	7	4	3	16	**35**

The data was logged by a WatchDog 2900ET Weather Station, and records reported in 15 minutes intervals. The average wind speed was calculated over all available records. The most frequent cardinal directions were highlighted in yellow. E: East, N: North, S: South, W: West.

Table A3.4. Wind gusts (short episodes of wind increase that lasted only for a few seconds) in the outdoor nursery area of the Grasslands Research Centre, AgResearch Ltd, Palmerston North.

Month	Season	Frequency [% of records > 0 km/h]	Average gust speed [km/h]	Gust cardinal direction [% of records > 0 km/h]							
				N	NE	E	SE	S	SW	W	NW
July 2015	Winter	73	6.6 ± 5.51	13	15	14	9	10	6	7	**24**
August 2015	Winter	76	8.2 ± 6.08	13	**20**	**18**	7	6	6	9	**23**
September 2015	Spring	85	11.3 ± 7.47	9	**16**	**19**	12	10	6	8	**20**
October 2015	Spring	85	11.8 ± 6.41	12	10	11	6	13	11	13	**24**
November 2015	Spring	92	10.9 ± 6.36	9	12	**15**	10	13	11	12	**18**
December 2015	Summer	88	10.6 ± 6.37	11	**14**	**13**	8	**15**	10	10	**18**
January 2016	Summer	82	11.3 ± 6.55	8	**17**	**18**	10	**18**	9	9	12
February 2016	Summer	74	10.5 ± 6.25	8	**16**	**15**	9	**18**	10	8	**16**
March 2016	Autumn	79	11.9 ± 7.80	8	**23**	**22**	9	11	5	7	**15**
April 2016	Autumn	69	9.6 ± 5.87	9	**21**	**18**	5	5	8	12	**22**
May 2016	Autumn	80	8.9 ± 5.67	8	11	5	3	7	11	20	**35**
June 2016	Winter	71	8.3 ± 6.02	13	19	8	5	5	5	14	**30**

The data were logged by a WatchDog 2900ET Weather Station, and records reported in 15 minutes intervals. The average gust speed was calculated as the mean of all records > 0 km/h. The most frequent cardinal directions are highlighted in yellow. E: East, N: North, S: South, W: West.

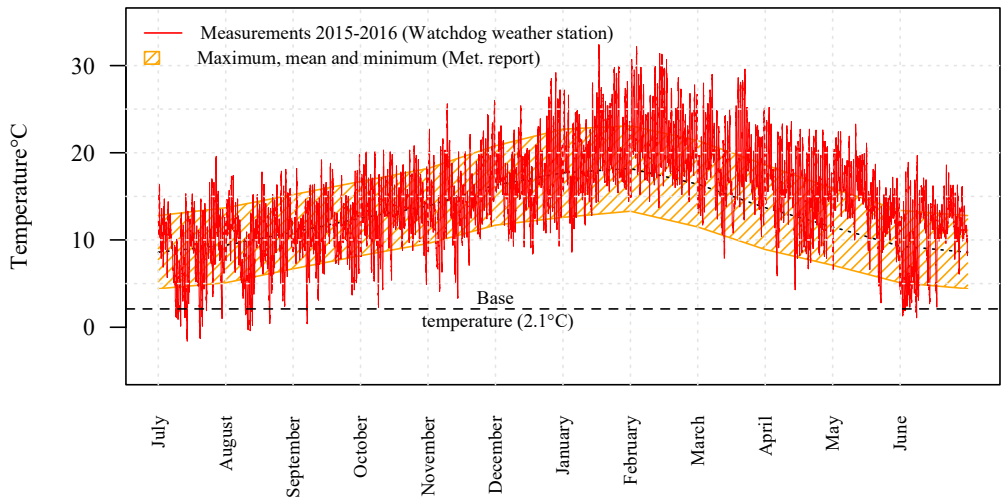


Figure A3.1. Air temperature in the outdoor nursery plot of the Grasslands Research Centre, AgResearch Ltd, Palmerston North. The data measured in 15 minutes intervals from July 2015 to June 2016 (red line) by the Watchdog weather station were laid over the long-term mean of monthly air temperatures (striped orange area), as recorded by the reference weather station over the last 30 years. Base temperature: minimal temperature for perennial ryegrass development (Matthew *et al.*, 2016)

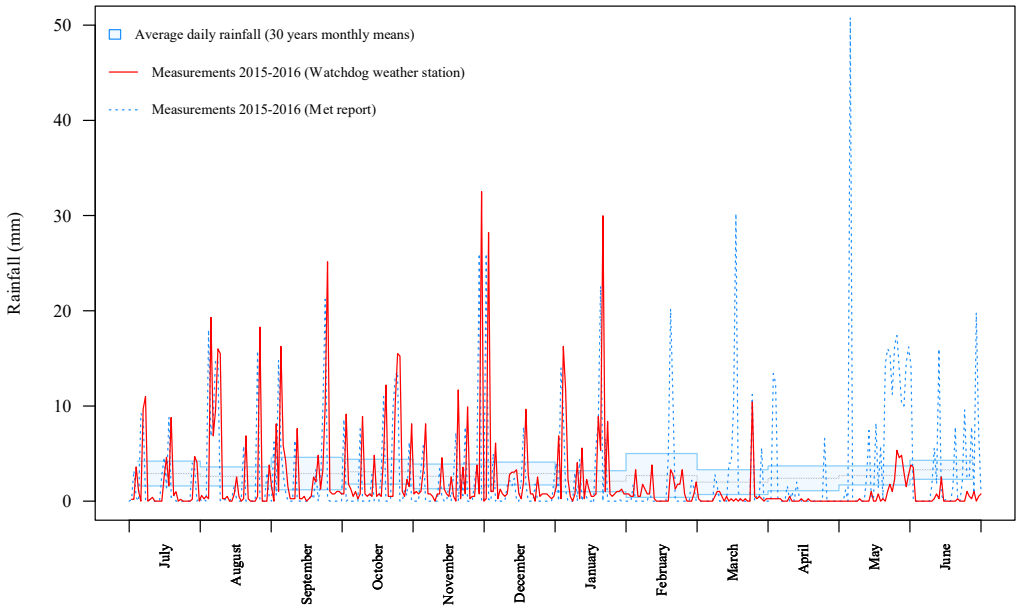


Figure A3.2. Daily rainfall at the research site from July 2015 to June 2016, as estimated, by the average of records from two independent Palmerston North weather stations. For comparison, the monthly averages of the last 30 years were divided by the number of days in the respective month and represented as daily mean ± standard deviation in the background. A malfunction of the rain gauge of the Watchdog weather station seemed to have happened from February/March 2016 onwards, as the reference station and Watchdog station started to disagree from that point onwards only.

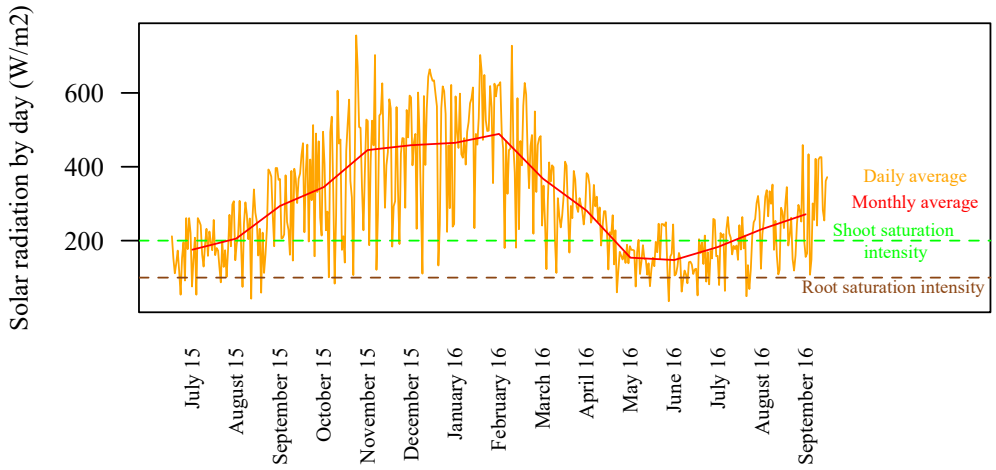


Figure A3.3. Daily solar radiation at the research site during daylight in Palmerston North, as measured from July 2015 to September 2016 by a Watchdog weather station at the site of the Grasslands Research Centre, AgResearch Ltd. The 30 years daily average for each month as measured by the reference station was overlaid to the measurements for comparison (monthly average). The light saturation threshold for perennial root and shoot marked here were estimated by Hunt and Field (1979).

References

Hunt, W. F., & Field, T. R. O. (1979). Growth characteristics of perennial ryegrass. *Proceedings of the New Zealand Grassland Association, 40*, 104-114.

Matthew, C., Mackay, A. D., & Robin, A. H. K. (2016). Do phytomer turnover models of plant morphology describe perennial ryegrass root data from field swards? *Agriculture, 6*(3), 28.

Ministry for the Environment and Statistics New Zealand. (2015). Frost and warm days. *New Zealand's Environmental Reporting Series: Environmental indicators.* Retrieved 16/03/2018, from http://www.stats.govt.nz/browse_for_stats/environment/environmental-reporting-series/environmental-indicators/Home/Atmosphere-and-climate/frost-warm-days.aspx.

U.S. Naval Observatory (2013). Duration of daylight for 2013. Retrieved 16/03/2018 http://aa.usno.navy.mil/cgi-bin/aa_durtablew.pl?form=2&year=2013&task=-1&place=&lon_sign=1&lon_deg=175&lon_min=35&lat_sign=-1&lat_deg=40&lat_min=21&tz=&tz_sign=1

APPENDIX 4: Light measurements in experimental locations

Aims:

1) Quantify the methodological variance by light intensity measurements with the mobile device (repeatability)
2) Quantify the variance in light intensity within each location (homogeneity of light within a location)
3) Compare locations in terms of light intensity experienced during the photoperiod, and light available for growth (over a one year period)

Measuring devices:

Mobile device: LI-250 light meter equipped with a Quantum sensor (LI-COR Environmental, Lincoln, Nebraska, U.S.A.); light intensity in μmol photons·m^{-2}·s^{-1}, measured over 15 seconds period when manually started.

Permanent outdoor logger: WatchDog 2900ET Weather Station (Spectrum Technologies, Inc., Aurora, Illinois, U.S.A.), fixed on a permanent stand in an open area of the outdoor nursery plot at the Grasslands Research Centre, AgResearch Ltd, Palmerston North; light intensity recorded in W·m^{-2}, once every 15 minutes, for 1 minute.

Methodology:

The measurements with the LI-250 light meter were undertaken in from 2015 to 2016, in the frame of the Mature plant experiment, and extended to all locations in which plants were kept. Figure A4.1 stylises how the data were collected and processed. The following procedures and analyses were applied to assess the methodological variances presented in Table A4.1:

1) In all experimental locations except the Percival climate chamber, the light intensity was measured 35 cm above the shelf/surface on which the plants were kept, i.e. just above the highest leaves of Petri Dishes mounted, or potted plants (plant canopy height). Three positions were selected within each location. Data collection and processing were undertaken as represented in the upper part of Figure A4.1. The light intensity was measured in each position for an average of 15 seconds. This process was repeated two more times immediately after the first set of measurements. For each location, ≥ 3 such repeated measurements were performed whereby the first measurement was synchronised with outdoors measurements of the Watchdog weather station. If cloud movements interfered with the measurements, the corresponding time points were removed from the data to analyse. An analysis of variance (ANOVA) was realised by location with the program R 3.0.0 (R Core Team, 2013), using the model described in Equation A4.1. Variance (VAR) within a position vs. between positions was calculated for each location by Equations A4.2 and A4.3, respectively.

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \qquad \textbf{Equation A4.1}$$

$$VAR_{Measures} = MS_{Error} \qquad \textbf{Equation A4.2}$$

$$VAR_{Position} = \frac{(MS_{\beta} - MS_{\alpha\beta})}{(3 \cdot n)} \qquad \textbf{Equation A4.3}$$

whereby y_{ijk} is light intensity, α_i the time point i, and β_j the position *j*. MS were the main squares calculated by the ANOVA, and n the number of time points with repeated measurements recorded for the location considered.

2) In the Percival climate chamber, the light intensity was measured on the level of the top shelf, in seven positions, on two occasions only. As the differences were minimal, the measurements were not repeated. The two time points were considered as repeated measurements in a same position and analysed by one-way ANOVA (factor: position).

3) Next to the Watchdog weather station, light intensity was measured on weather station level, three times in a row at 18 distinct time points, without further repeats. The data were analysed using a one-way ANOVA (factor: time point). The methodological variance was the main square of the error of this ANOVA.

To calculate correlations and regression relationships between light intensities in locations exposed to daylight (outdoor area, glasshouses, insectary) and outdoor logs, the first measurement at each position of the data mentioned above was re-used. Furthermore, as many additional synchronised measurements as time permitted were collected in each location, with one measurement in each of the three positions per time point (Figure A4.1). The records in each position were combined to a mean for the location at a given time point, and paired with the synchronous outdoor log from the weather station. A Pearson correlation coefficient was calculated from this data in R 3.0.0. A correction factor was furthermore estimated on a graphical basis by MS Excel (trend line). The assumptions thereby were (i) that there was a linear relationship between measurements of the outdoor weather station and measurements by the light meter for locations exposed to natural day light, and (ii) that no light would be measured by the mobile device when it is dark, i.e. the intercept of a trend line describing the relationship between weather station and light meter should always be equal to zero. The light intensity within climate chambers was assumed to be constant and independent of outdoor light intensities.

With all measurements, corrections factor and a year's data loggings form the weather station (July 2015-June 2016), it was possible to estimate a total yearly light supply and average day light hours light intensities for all locations. The weather station's logs [$W \cdot m^{-2}$], converted by the weather station's own correction factor into μmol photons$\cdot m^{-2} \cdot s^{-1}$ [LI-COR light meter unit] were set as 100% light intensity outdoors and used as a baseline for comparisons.

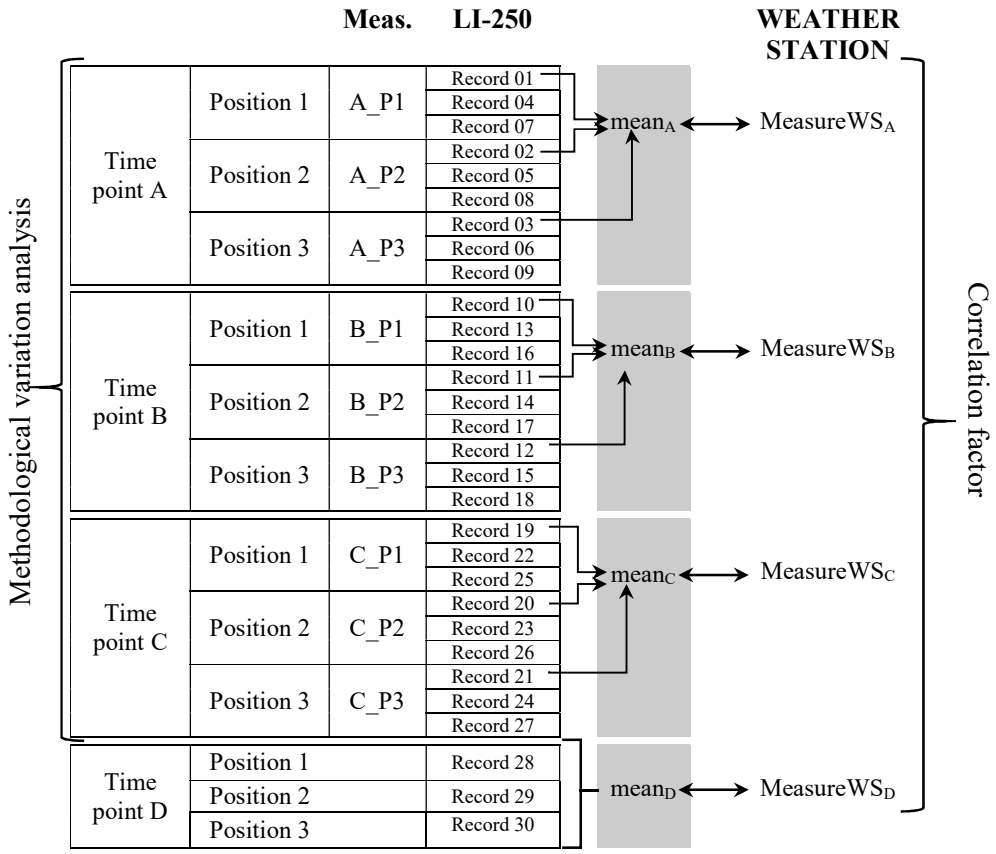


Figure A4.1. Diagram of data collection and processing in one location. Meas: measurement; WS: weather station

Table A4.1. Summary of light measurements, variance analysis and estimated correction factors by experimental location. The data were collected from 2015 to 2017.

Location	Methodological variance [µmol photons·m^{-2}·s^{-1}]^2			Correlation WWS - LM		Correction factor [µmol photons·W^{-1}·s^{-1}]	Daytime intensity [µmol photons·m^{-2}·s^{-1}]	estimated PAR [mol photons·m^{-2}·year^{-1}]	Note
	n	Measures	Between pos. (*p-value*)	r (*p-value*)	$n_{co\,r}$	(R^2)	Absolute [Relative]	Absolute [Relative]	
Outdoors, next to WWS (reference)	18	3403	-	0.9725 (*< 0.000*)	18	1.8079·x (0.9711)	614 [100%]	9,925 [100%]	Served for LM calibration; natural PhP
Outdoor area (nursery)	9	5927	2005 (*< 0.000*)†	0.9391 (*< 0.000*)	17	1.7291·x (0.7981)	587 [96%]	9,493 [96%]	Source plants, aphid habitat; natural PhP
Insectary	14	339	78 (*< 0.000*)†	0.8273 (*< 0.000*)	15	0.3793·x (0.7647)	129 [21%]	2,082 [21%]	Mature plant experiment; natural PhP
Climate chamber Conv. 1 (before December 2015)	3	5	1189 (*< 0.000*)	-	-	-	230 [37%]	4,235 [43%]	Population experiment, colonies; 14h PhP
Climate chamber Conv. 1 (after December 2015)	3	7	1310 (*< 0.000*)	-	-	-	339 [55%]	6,249 [63%]	After light servicing;14h PhP
Climate chamber Conv. 2	7	57	275 (*< 0.000*)	-	-	-	233 [38%]	4,303 [43%]	Biology II experiment, colonies; 14h PhP
Climate chamber Percival	2	6	1 (*0.344*)	-	-	-	70 [11%]	1,106 [11%]	Seedling experiment; 12h PhP
Glasshouse 9, cubicle 04	38	4800	76 (*0.092*)†	0.9199 (*< 0.000*)	38	0.7747·x (0.8313)	263 [43%]	4,253 [43%]	Colonies; natural PhP
Glasshouse 9, cubicle 12	9	16	570 (*< 0.000*)†	0.7073 (*0.010*)	12	0.5038·x (0.4994)	171 [28%]	2,766 [28%]	Storage area for plants, Biology I and recovery experiments; natural PhP
Glasshouse 18 ('Bubble')	11	485	12427 (*< 0.000*)†	0.7168 (*0.009*)	12	1.0211·x (0.1032)	347 [56%]	5,606 [56%]	Storage area for plants; natural PhP

Conv.: Conviron; LM: LI-250 light meter; PAR = photosynthetically active radiation, i.e. light available for plant growth; PhP: photoperiod, x: solar radiation as measured by the weather station [W^{-1}·s^{-1}]; WWS: Watchdog weather station; †: results to handle with care as standard assumptions of ANOVA were not fulfilled or/and residuals not normally distributed.

References:

R Core Team. (2013). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from http://www.R-project.org/.

APPENDIX 5: Nursery media and chemicals

1) AgResearch standard potting mix

Raw material supplier:
Midland horticulture, 160 Napier Road, RD 10 Palmerston North, NZ

Composition:

- Peat: 60%,
- Washed river sand: 40%
- Fertilizer:
    - Superphosphate: 0.95 kg/m3
    - Dolomite: 2.875 kg/m3
    - NPK and trace elements:
        a) For 8-9 months: Nutricote[4] Total® 18N-2.6P-6.6 K+TE, 2.25 kg/m3
        b) For 3-4 months: Nutricote Total® 13N-5.7P-10.8K+TE, 1.425 kg/m3

2) C.A.N. bark (screened fine bark)

Properties of the substrate, analysis report as e-mailed by Kevin Timms, territory manager at Daltons Ltd on 12/02/2016

COMPOSTING MATERIALS	pH Finish	EC mS/cm Finish	MOISTURE % Finish	AFP %	WHC %
Screened CAN Fines	4.5-5.5	0.1-0.5	45-65	5-15	50-60

COMPOSTING MATERIALS	Particle size range(% retained)											
	Fines mm	2mm mm	3mm mm	4mm mm	5mm mm	6mm mm	7mm mm	8mm mm	9mm mm	10mm mm	11mm mm	12mm mm
Screened CAN Fines	40-55	10-35	10-35	10-35	0-10							

Figure A5.1. Properties of the C.A.N. bark produced by Daltons Ltd. AFP: air filled porosity; EC: electrical conductivity; WHC: water holding capacity.

3) Perlite

Industrial Processors Ltd, Auckland, New Zealand; grade: C500

[4] Yates, DuluxGroup (New Zealand) Pty Ltd, Lower Hutt, New Zealand

4) Pest control

Table A5.1. Chemicals used for pest control in the glasshouses.

Product	Producer	Application rate
Frequently used		
Orthene®	Arysta Life Science Ltd., Cary, NC, U.S.A.	0.8 g/L
Nuvos®	Orion AgriScience Ltd, Christchurch, New Zealand	0.6 mL/L
Mavrik® aquaflo	ADAMA New Zealand Ltd, Nelson, New Zealand	0.4 mL/L
Occasionally used		
Attack®	Nufarm Ltd, Auckland, New Zealand	1 mL/L
Carbaryl Insect Control -Omite® 30W	Kiwicare Corporation Limited, Christchurch, New Zealand Nufarm Ltd, Auckland, New Zealand	1.5 g 2 g/L
Carbaryl Insect Control -Peropal®	Kiwicare Corporation Limited, Christchurch, New Zealand Bayer New Zealand Ltd, Auckland, New Zealand	0.8 g/L 1 g/L
Chess WG -Peropal®	Syngenta Crop Protection Ltd, Auckland, New Zealand Bayer New Zealand Ltd, Auckland, New Zealand	0.4 mL/L 1 g/L
Confidor®	Bayer New Zealand Ltd, Auckland, New Zealand	2 mL/L
Crown® 225SL	Everris Australia Pty Ltd, Bella Vista, Australia	0.2 mL/L
Diazinon® 20G	Nufarm Ltd., Auckland, New Zealand	10-15 granules/plant
Garlic & Pyrethrum	Multicrop (Aust). Pty Ltd, Melbourne, Australia	12 mL/L
Orthene® -Peropal®	Arysta Life Science Ltd., Cary, NC, U.S.A. Bayer New Zealand Ltd, Auckland, New Zealand	0.8 g/L 1 g/L

These products were generally mixed with DC-Tron® mineral oil (Caltex Australia Petroleum Pty Ltd, Sydney, Australia; 10 mL/L) to enhance the products' adherence to plant surfaces.

APPENDIX 6: Agar preparation

Credits

The basic recipe for the agar used in most trials was provided by Dr James Crush, Agresearch Ltd. Agresearch collaborators of the Ruakura team in Hamilton adapted the original recipe by Bollard (1966) to suit perennial ryegrass. Slight modifications were undertaken by the student to make the agar more suitable for long-term use in a Petri dish system (higher agar concentration, addition of a micronutrient solution).

Preparation protocol for modified Bollard medium (MBM agar)

1) The nutrient solution is prepared adding the nutrients stock solutions (Table A6.1, grey shaded) into about 0.4 L RO water
2) Calcium sulphate and iron-EDTA are added and the solution is well mixed through. As calcium sulphate tended to clog, this seemed to be the only way to avoid larger, hard to dissolve pieces on the ground of the mixing bottle.
3) The solution is completed to 1 L with RO water
4) 15 g agar/L is added (10 g in the original AgResearch recipe)
5) The agar-nutrients solution is autoclaved for ≥ 20 minutes at ≥ 117 °C (Autoclave: Burns&Ferral Steam Generator, Burns & Ferral, Auckland, New Zealand)
6) If poured into Petri Dishes: the agar is left to cool down to 80 °C, and then poured into the Petri dishes in amounts of either 40-45 g or 55-60 g per Petri dish, with frequent mixing of the agar bottle's content. The agar is left to cool and solidify. The Petri dishes are then sealed with Glad® wrap and exposed to UV light for at least 16 hours. They are then wrapped together in an additional Glad® wrap layer, re-exposed in this packaging 15 minutes to UV for quick sterilisation of the wrapping, and then stored in a fridge up to the moment of their use.

Table A6.1. Nutrient mix

Main reagents		Concentration of the solution	Quantity for 1 L	
Ammonium nitrate	NH_4NO3	1M	8	mL
Di-potassium hydrogen orthophosphate	K_2HPO_4	0.2M	5	mL
Potassium sulphate	K_2SO_4	0.25M	4	mL
Magnesium sulphate	$MgSO_4$	1M	2	mL
Micro-elements solution (Table A6.2)	Mix		1	mL
Calcium sulphate	$CaSO4 *1/2H_2O$		0.3625	g
Iron -Ethylenediaminetetraacetic acid	Fe-EDTA		20	mg

M: mol

Table A6.2. Microelement solution recipe, as prepared in July 2012 with RO water.

Reagents		g for 1 L (1000x solution)
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.1645
Boric Acid	H_3BO_3	0.7419
Copper sulphate	$CuSO_4.5H_2O$	0.1017
Manganese sulphate	$MnSO_4.4H_2O$	2.6265
Zinc sulphate	$ZnSO_4.7H_2O$	0.2913

The solution based on values known to work for aquaponics provided by Dr Stuart Card, AgResearch Ltd. RO: water purified by reverse osmosis

Properties of the prepared agar

The electrical conductivity of the solution prepared ranged between 2.0-2.2 Millisiemen·cm^{-2} before autoclaving (recommended optimum for plants in hydroponics systems: 1.5-2.0 mS·cm^{-3}; personal communication, Stephen Odering, nursery manager at AgResearch). The pH of the produced agar was measured with a waterproof handheld H160 Hach portable pH meter (Hach Company, Loveland, CO, U.S.A) designed for measuring the pH of semi-solid samples. Measurements were first made on samples collected at pouring, before being measured on three to four agar samples collected from random Petri dishes with a hole puncher of 0.8 cm diameter. The pH of the agar prepared as described was moderately acidic to neutral (i.e. generally between 6.0 -7.1).

References

Bollard, E. G. (1966). A comparative study of the ability of organic nitrogenous compounds to serve as sole source of nitrogen for the growth of plants. *Plant and Soil, 25*(2), 153-166.

APPENDIX 7: Viviposition trial

Preparing viviposition plants is time-consuming. Any plant rooting system presents a few hiding spots for first instars, which makes their re-use for a second viviposition period problematic when neonate aphids of known age are required. Besides, the quality of the viviposition plant can influence the morph production in aphids. Mothers kept isolated *ex planta* in a glass or plastic container are therefore likely to produce offspring of more homogenous quality. Besides this, offspring produced in such a system would remain naïve until placed on a trial plant, another aspect likely to improve the standardisation of the aphid inoculum by experiments. It is not known whether *A. lentisci* is able to viviposit under such circumstances, however. What reproduction rates can be expected and how long mother and offspring may decently survive in such conditions are other points that need clarification. A methodical mini-trial was therefore set up to answer to the following questions:

1) Are adult root aphids vivipositing *ex planta*?
2) How many offspring will be produced *ex planta*?
3) How long do adult aphids and their offspring survive in a container without feed?
4) Does high environmental humidity improve the longevity of mothers and/or offspring?

Ten mature, apparently unhurt aphids of a second generation acclimated to climate chamber conditions (17 to 20ºC, 14h photoperiod) were placed into glass vials (7.6 cm length, 2.4 cm diameter), one per vial. Half of these vials contained a piece of tap water humidified toilet paper (high humidity). The vials were closed tightly, wrapped into a black polyethylene sheet, kept in the climate chamber and daily checked under a stereo microscope (Stemi DRC [1-6.3x objective, 10x/25 glasses ocular], ZEISS Germany) to assess (i) the survival of the mature aphid, (ii) the number of offspring, distinguishing alive and dead ones. As individual fates could not be followed in such a system, it was assumed that the oldest offspring was the first to die when the records were converted to survival data. Non-parametric tests (Mann-Whitney-U/Kruskal-Wallis) were applied to analyse the difference between the various levels of the factors humidity (2 levels, low

vs. high) in mother survival, and humidity, birth day [i.e. maternal time in vial; 10 levels, day 1 to 10] and the interaction of these two aspects for offspring. Post-hoc Dunn's tests with Benjamini-Hochberg correction for multiple comparisons was performed by significant Kruskal-Wallis test results. A cox proportional hazard model was finally fitted to the survival of offspring born in the first two days in the vials, to predict the median age at death of undisturbed, early produced offspring.

Viviposition happened for all but one aphid. Average offspring numbers of 2.9 ± 1.60, 3.2 ± 1.75, 2.5 ± 1.58, 4.1 ± 2.08, 3.8 ± 2.25, 1.7 ± 1.50, 2.7 ± 2.36, 0.9 ± 1.27 were observed on day 1, 2, 3, 4, 5, 6, 7 and ≥ 8 after mothers had been placed into the vials, respectively (Figure A7.1). No significant difference in viviposition rate was observed between mothers kept in humid vs. dry conditions when assessed within a given birth day level. The number of offspring tended to decrease when the mother was kept longer than a week in a vial, but only individuals that survived more than 7 days were significantly less fecund, than individuals in their first days (Dunn's test of multiple comparison: p = 0.075, 0.041, 0.164, 0.005, 0.016, 0.543 and 0.199 for contrasts between ≥ 8 and 1, 2, 3, 4, 5, 6 or 7, respectively). The adult aphids all survived at least five days, with no significant difference between dry and moist vials (Figure A7.2). Offspring, however, survived significantly less time in the moist vials, in which they were often found sticking to water droplets (Figure A7.2). In dry vials, the survival seemed further to decrease with the length of maternal captivity. While the median aphid born in the first two days died on 6th to 7th day of life only (100% alive by day 4), the ones born on day 7 or later were dead by the third to fourth day (Figure A7.2, Figure A7.3).

First instar aphids of known age can therefore be produced *ex planta*, but some points are to be given attention. The containers used for offspring production should be dry, the adults used should not have been kept fasting therein for more than 2-3 days. Offspring produced under such conditions can then be expected to survive at least three days by careful handling.

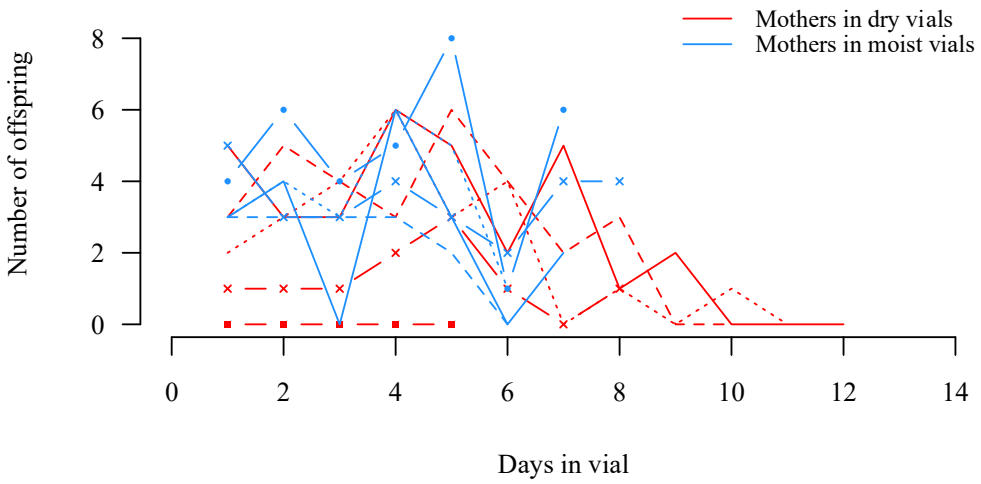


Figure A7.1. Reproductive performance of mothers kept in vials with low vs. high humidity.

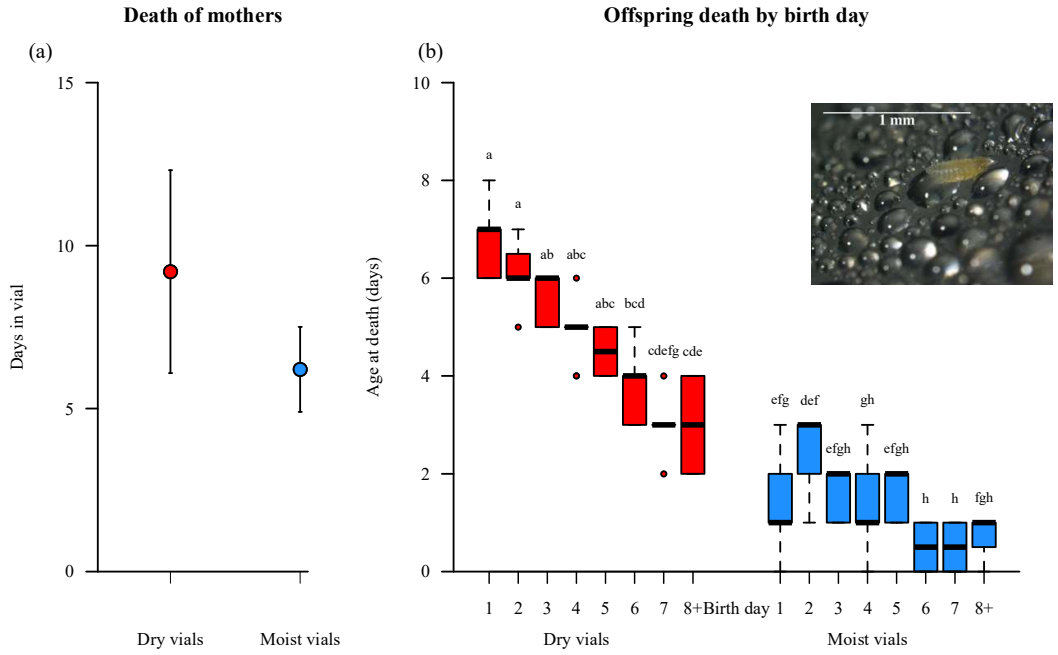


Figure A7.2. Survival in vials. (**a**) Days spent by mothers in vials unto death, by daily monitoring over a period of 14 days. (**b**) Age at death of offspring by humidity and number of days the mother was already in the vials by aphid birth (birth day). Inlay picture: immature *A. lentisci* trapped on water droplets. Moist vials: vials with a piece of humid tissue in the bottom.

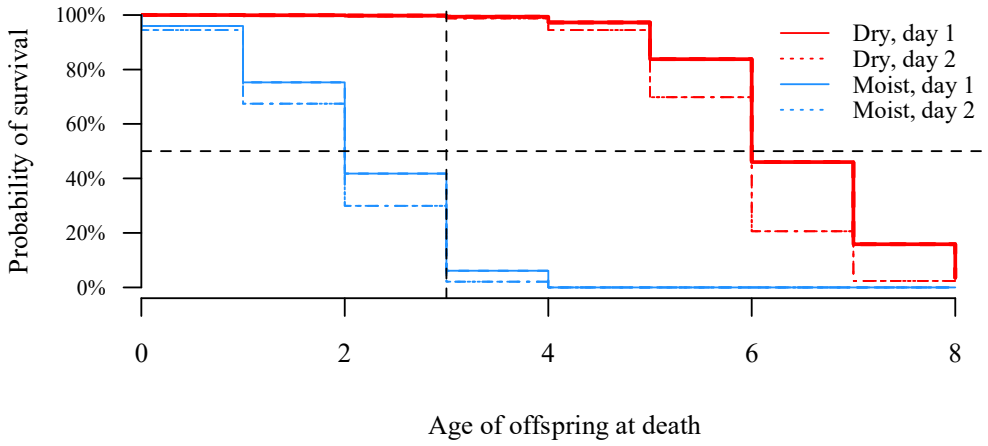


Figure A7.3. Survival of aphids born during the first two days of maternal captivity in a glass vial, by dry and moist conditions. Moist: offspring in tubes with a piece of humid tissue. Dry: offspring in an empty, dry tube.

APPENDIX 8: Image analysis steps

Author: Chris Hunt, scientist at AgResearch

The **customised image analysis procedures** are developed in MATHWORKS© MATLAB R2013A using the signal processing tool box and the image processing toolbox. The image processing procedure is implemented on the raw images as follows:

1. Identification/segmentation of black target zone
2. Determination of area calibration coefficient, (Kc[mm/pixel]) from known dimensions (area) of black target zone and enumeration of black zone pixels.
3. Identification of grey target zone
4. Assessment of black and grey zone mean colour characteristics.
5. Colour calibration of raw image by scaling image RGB based on black and grey colour values.
6. Identification and segmentation of leaf material.
7. Selection of leaf segments on basis of minimal size discriminator (200 pixels).
8. Assessment of mean colour attributes of each selected leaf segment.
9. Calibration of true leaf segment area using area calibration coefficient (calculated in step 4).
10. Individual image and segment parameters stored in EXCEL spreadsheet for further analysis.

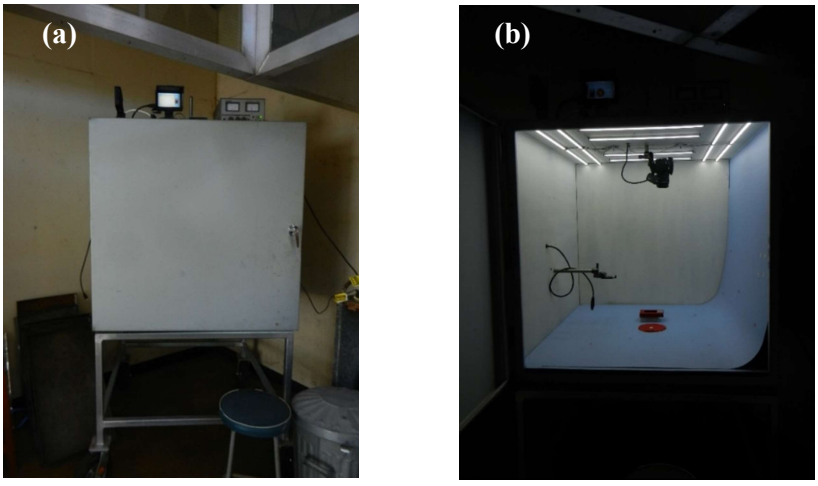


Figure A8.1. Photographic chamber used for image acquisition, from (a) outside and (b) inside with mounted camera and colour referential (red box with grey rectangle). Dimensions: 100 cm width × 103 cm depth × 94 cm height. The camera was suspenspended approximately 82 cm above the chamber floor

APPENDIX 9: Root aphid live size measurements

This section describes three methodical pre-trials supplying additional information on root aphid live size measurements (Section 2.4.3). Section A9.1 reports average live size measurements in aphids of known instar. Section A9.2 compares live size measurements with size measurements on dead individuals preserved in various ethanol solutions, to allow comparisons with literature values. Section A9.3 sets size in relation to weight in adult root aphids.

A9.1. Instar measurements (Live size of various instars)

Root aphid length, width at abdomen, or thorax and ellipsoid body projection (EP) were frequent parameters in Chapter 3. The size measurements collected during the two biology experiments could not always be unambiguously related to a specific instar, however, and may also have been influenced by maternal effects (Chapter 3). This experiment aimed at supplying information for discrimination and comparison.

Aphid colonies on clone-plants (Section 2.3.1.1) embedded in modified Bollard agar were used to raise aphids of known instar under standard climate chamber conditions (17.4 ± 2.4 °C, 14h light per day, Conviron® climate chamber n°1, April-May 2016). The colonies plants were set up by trimming four tillers of each of the eight plant genotype-endophyte status combinations to 4 cm shoot length and 0.3 cm root length without side roots. The trimmed tillers were left to recover in tap water before being embedded in agar-filled Petri Dishes (Section 2.3.1.3) at the end of the third week. Four days later, up to ten[5] neonate aphids (first to second day of life) of colonies acclimated to standard climate chamber conditions for ≥ 6 generations were dyed with magenta powder (JS-MG3018, Radiant Color Company, Richmond, CA, U.S.A.). The aphids were placed with a small amount of the powder into a 1.5 mL Eppendorf Tube® 3810X (Eppendorf AG, Hamburg, Germany), which was then closed and turned gently three times upside down. The dyed aphids (Figure A9.1.1) were then deposited onto the roots of the prepared, new colony plants, and checked at three day intervals for individuals that had moulted. Three to six

[5] As available from earlier colonies.

intact individuals were collected amongst the earliest moulting aphids and photographed alive thrice from above (dorsal, Figure A9.1.2a), and thrice from below (ventral, i.e. aphids laying on their back, Figure A9.1.2b), before being preserved in 80% ethanol for possible later analyses. All other moulted aphids were marked with sunset orange dye powder (JS-SO3019 Radiant Color Company, Richmond, CA, U.S.A.), dragging a loop made by a hair through the powder before dragging it over the aphids' back. Collection and new marking were performed in the same way at the next moult, using again magenta to mark the freshly moulted 3rd instars, and sunset orange for the 4th instar. As initial numbers and young aphid survival were limited in this setting, colonies had to be allowed to recover before mature adults (5th instar) could be collected too. Only 47 days after neonate placement were 28 adults (recognizable by the genital plate and aperture at the distal abdomen end) and nine neonate first instar offspring (distinguishable by their linear body shape) harvested and photographed to complete the data. The photographs were analysed as described in Section 2.4.3, measuring the parameters (i) body length, (ii) width at the widest point of the thorax, (iii) width at the widest point of the abdomen, and (iv) rostrum length from tip to the frontal attachment point whenever picture quality allowed it. Further, the ellipsoid body projection (EP) parameter was calculated from each (i) and (iii). Repeated measurements of a same parameter were then averaged to one mean measurement per aphid and side (ventral vs. dorsal).

The data were analysed with R 3.3.1 (R Core Team, 2017). The difference in measurements between dorsal and ventral measurements was assessed by parameter, with a Wilcoxon-signed rank test. The effects of plant genotype (N or S) and endophyte status of the colony plants were analysed for instar 1 and 5 only (separately), by a Mann-Whitney-U test for plant genotype, and a Kruskal-Wallis with subsequent post-hoc Dunn test with Benjamini-Hochberg correction for multiple comparisons for endophyte status. A Kruskal-Wallis test and subsequent post-hoc Dunn test were applied to all measurements and derived parameters to assess the significance of differences between instars too. Finally, a canonical discriminant analysis established which measured parameters could best help to discriminate aphid instars, and how successful this discrimination would likely be.

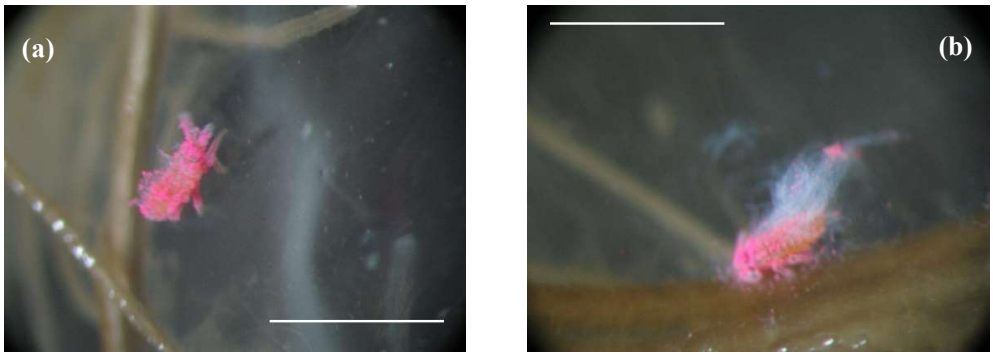


Figure A9.1.1. First instar root aphids marked with magenta powder dye for live size measurements by instar (a) just after marking and (b) several days later. White scale bar: 1 mm

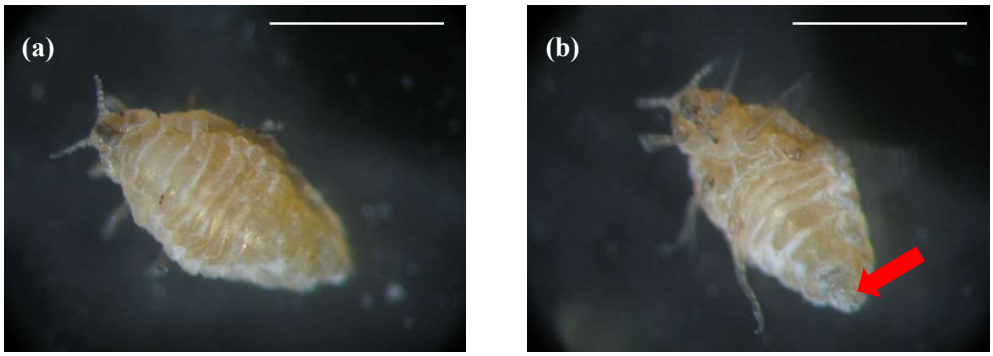


Figure A9.1.2. Dorsal (a) and ventral (b) photographs of an adult aphid (5th instar), as used for size measurements. Genital plate and aperture are clearly visible on the rear abdomen end of the ventral picture (red arrow). White scale bar: 1 mm

The body length was often shorter when measured on ventral pictures $V_{(47)} = 986$, $p < 0.001$, with an average of 6% and up to 25% size difference to the length measured dorsally, presumably because aphids bent in the effort to turn on themselves to regain walking position. There was no systematic difference in size measurements between dorsal and ventral pictures for both abdominal and thoracic width measurements [$V_{(47)} = 675$, $p = 0.240$ and $V_{(47)} = 496$, $p = 0.479$, respectively].

Figure A9.1.3 and Table A9.1.1 summarise the mean body length, abdominal width and thoracic width of each instar as measured on dorsal photographs, the mean rostrum length as measured on ventral photographs, and the mean value of derived parameters calculated from these measurements. The genotype of the plants lived on did not influence

any of the measurements or the derived parameters in first instars and adults ($p > 0.10$). As far as detectable with the data at hand, a plant's endophyte status had no effect on size either, with an exception for the rostrum length in the fifth instar [$\chi^2_{(2)} = 6.5551$, $p = 0.0377$]. Adult aphids collected from plants with common-toxic endophyte had indeed a significantly longer rostrum, than adults from plants without endophyte [$m_{CT(5)} = 0.34$ mm and $m_{NIL(15)} = 0.31$ mm]. Rostrum size in aphids from plants with AR1 endophyte laid in between both groups [$m_{AR1(5)} = 0.32$ mm]. As the only adult aphid collected on AR37 did not supply useable rostrum pictures, there is no information for this endophyte treatment.

Although the Kruskal-Wallis tests found significant differences between instars overall for every parameter ($p = 0.005$ for rostrum length, $p < 0.001$ for all other measurements and derived parameters), none of the measurements allowed an unequivocal identification of each instar. Width measurements (in particular thoracic width), derived measurements and combinations of these allowed the best discrimination (Tables A9.1.1 and A9.1.2). Nevertheless, the fourth and fifth instar could not be distinguished by this information (Figure A9.1.4).

The general applicability of these analyses needs to be confirmed by more measurements. The most important aspects that may nevertheless be deduced from the data at hand are likely:

(i) Dorsal pictures are to be preferred to ventral pictures when assessing body length.

(ii) The 1 mm length threshold reported sometimes in the literature as an easy way to discriminate mature root aphids from immature ones appears not appropriate for the root aphid population observed. The majority of third instars (> 79%) and almost all fourth instars meet this target too.

(iii) Abdominal and thoracic widths could be the best parameters to discriminate between instars, but do not suffice for that purpose.

Practical experience has further taught us that it may be difficult to accurately measure width on a live photograph of feeding aphids, in particular, if an abundant wax coat is available and if aphids cannot be rid of it without damages. Besides, contraction movements in feeding aphids disturbed by observations can increase the measured dorsal

width. Repeated measurements on a same picture could occasionally result in differences of up to 0.01 mm, i.e. 8 to 154% of the standard deviation of the width of fifth and first instars respectively. Multiplied by the average length, that difference represents only 11 to 89% of the standard deviation of fifth and first instar EP, respectively. For all these reasons, EP was considered a more advisable and stable size parameter for analyses in Chapter 3.

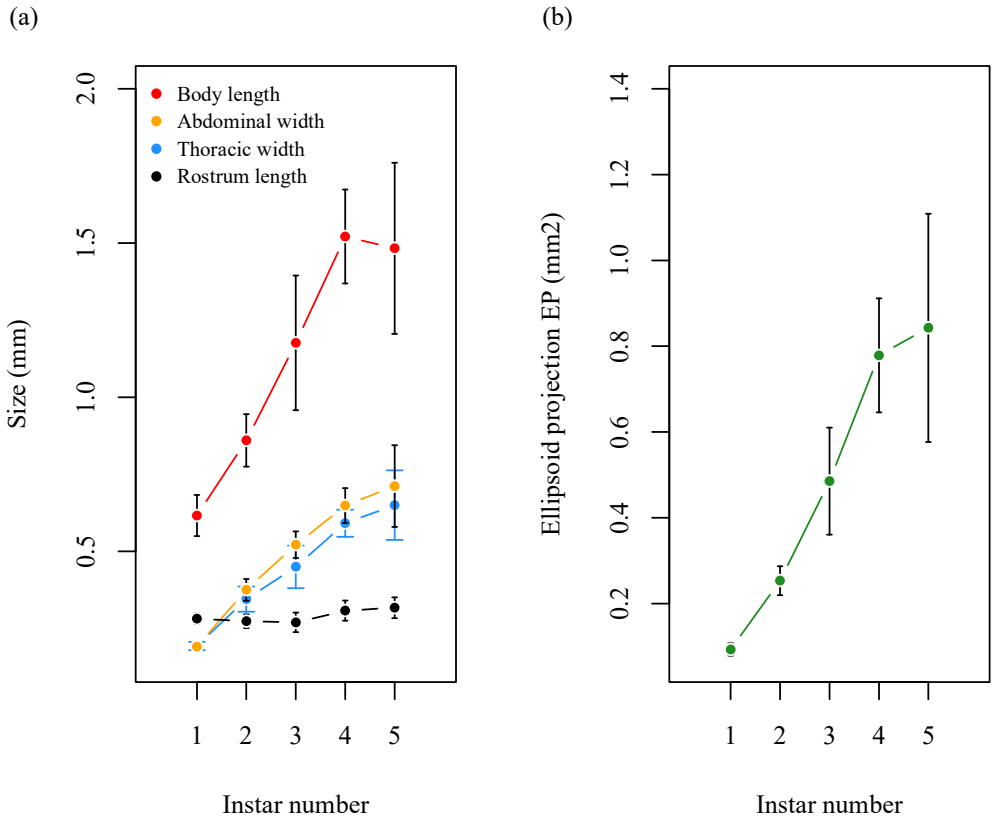


Figure A9.1.3. Size measurements by instar, measurements in mean ± standard deviation (a) and derived parameter ellipsoid body projection, calculated as EP = Length/2·abdominal width/2·π (b). The apterous root aphids observed during the thesis were born as first instars. The fifth instar is the imago (adult, reproducing morph).

Table A9.1.1. Size measurements of living apterous root aphids of known instar (clear section) and derived parameters (shaded section).

	N		Length	Width		Rostrum	Body projection	W/L	Rostrum/L	tW/W
	Total	(Rostrum)	[mm]	Thoracic [mm]	Abdominal [mm]	[mm]	EP [mm²]	[%]	[%]	[%]
Instar 1	9	(8)	0.62 ± 0.067[a]	0.19 ± 0.013[a]	0.19 ± 0.013[a]	0.28 ± 0.018[a]	0.09 ±0.014[a]	31 ±2.8[a]	46 ± 3.3[a]	101 ± 5.5[a]
Instar 2	4	(4)	0.86 ± 0.085[a]	0.35 ± 0.041[ab]	0.38 ± 0.035[ab]	0.27 ± 0.022[a]	0.25 ±0.034[ab]	44 ±6.7[ab]	32 ± 3.5[ab]	92 ± 3.2[b]
Instar 3	3	(3)	1.18 ± 0.218[ab]	0.45 ± 0.069[ac]	0.52 ± 0.043[ac]	0.27 ± 0.032[a]	0.49 ±0.125[ac]	45 ±6.6[ab]	23 ± 1.9[bc]	86 ± 7.0[b]
Instar 4	6	(4)	1.52 ± 0.152[b]	0.59 ± 0.044[bc]	0.65 ± 0.056[bc]	0.31 ± 0.033[a]	0.78 ±0.133[bc]	43 ±3.1[ab]	20 ± 2.6[c]	91 ± 2.6[b]
Instar 5	28	(26)	1.48 ± 0.278[b]	0.65 ± 0.112[c]	0.71 ± 0.132[c]	0.32 ± 0.034[a]	0.84 ±0.266[c]	49 ±9.8[b]	22 ± 4.1[c]	92 ± 4.8[b]

N: number of specimens. The ellipsoid body projection (EP) is defined as length/2·abdominal width/2·π, W/L is the abdominal width-to-length ratio, Rostrum/L the rostrum-to-length ratio and tW/W the ratio of thoracic and abdominal widths. Means with different letters in a column differed significantly in a Dunn post hoc test with *p*-values adjusted for multiple comparisons using the Benjamini-Hochberg method.

Table A9.1.2. Canonical discriminant analysis results: coefficients of linear discriminants and proportion of variance explained by each linear dimension (trace).

		LD1	LD2	LD3	LD4
Proportion of trace		**92.83%**	**5.67%**	**1.33%**	**0.16%**
Coefficients[1]:	Body length	-0.2146	1.5465	1.0170	-0.0148
	Abdominal width	-0.5626	2.5641	-0.2913	2.6355
	Thoracic width	1.6802	-3.2139	-0.5487	-2.5586
	Rostrum length	-0.0274	-0.5594	0.5245	0.7700

[1]Note that variables with large negative of positive coefficients have more weight in a linear dimension than variables with lower absolute value.

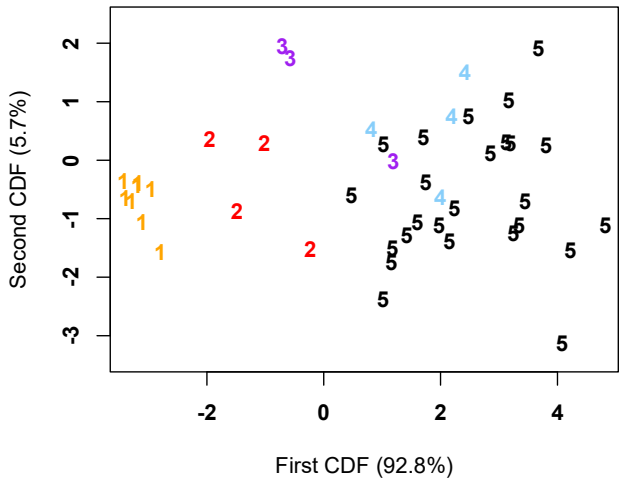


Figure A9.1.4. Instar discrimination by canonical discriminant analysis. CDF: canonical discriminant function; 1, 2, 3 ... : first, second, third, etc. instars, respectively.

A9.2. Calibration trial I (Preserved vs. live size calibration)

Many measurements on aphids in the literature are performed on samples preserved in 80 to 90% ethanol [Cottier (1953) or Blackman and Eastop (1994), e.g.], but also 70% ethanol is encountered as preserving solution [Manheim and Wool (2003), e.g.]. To be able to compare the live size measurements performed in this thesis with literature records, models predicting the preserved size in function of the ethanol concentration and the live size were therefore needed. A total of 60 aphids of mixed ages were collected from endophyte-free outdoor plants in June 2012 for this purpose. The living aphids were photographed thrice from above (dorsal live size measurements) and were then preserved in either 70%, 80%, or 90% ethanol for two days, before being re-photographed thrice once more (dead size measurements). The measurements comprised the three parameters body length (L), abdominal width (W) and thoracic width (tW), and were measured on each picture with the program ImageJ 1.46r (Schneider *et al.*, 2012) as described in Section 2.4.3. The additional parameter ellipsoid body projection (EP) was calculated from these records with Equation 2.12 (Section 2.4.3). The means of the triple measured values were computed discarding individual live size measurements biased by sudden aphid movements. Records from obviously wounded aphids (broken stylets visible on live pictures, e.g.) were also excluded from further analyses. A total of 45 aphids offered useable records by this selection procedure. They were assigned to one of two age groups according to their live body length (early immature if < 1mm and older instars if ≥ 1mm). The correlation between the live and dead size measurements was then realised with the statistic program R 3.3.1 by parameter and age group. Older instar data were analysed with Equation A9.2.1 were fitted to each parameter and age group. Thereby, Size MX_D and Size MX_L were the respective dead and live size measurement of parameter X (L, tW, W or EP) measured on aphid *i*, γ_i the main effect of the ethanol concentration of the solution the aphid was killed and preserved in (70%, 80% or 90%), β_i (Size MX_{Lij} – $\overline{\text{Size } MX_{Li.}}$) the interaction term of the live measurement and the ethanol concentration and ε_{ij} the residual error. Early immature data were analyzed by Equation A9.2.2 as the interaction term appeared not significant in this group. Ethanol concentration was further removed as main effect from the model describing tW in early immature, as its removal did not significantly affect the model.

$$\text{Size MX}_{Dij} = \mu + \gamma_i + \beta_i \cdot (\text{Size MX}_{Lij} - \overline{\text{Size MX}_{Li.}}) + \varepsilon_{ij} \quad \textbf{Equation A9.2.1}$$

$$\text{Size MX}_{Dij} = \mu + \gamma_i + \beta \cdot (\text{Size MX}_{Lij} - \overline{\text{Size MX}_{Li.}}) + \varepsilon_{ij} \quad \textbf{Equation A9.2.2}$$

Overall, live and dead size measurements were correlated, with a prominent exception for length in young immature aphids (Table A9.2.1). Meaningful linear regression could, therefore, be fitted to most parameters (Figures A9.2.1 and A9.2.2). In older instars, the ethanol concentration of the preserving solution seemed to affect all parameters except the thoracic width measurements ($F(2, 25) = 2.753$, $p = 0.0831$). While specimens kept in 70 to 80% ethanol behaved quite similarly, specimens preserved in 90% ethanol appeared often shorter. For abdominal width and EP, an interaction effect with the live size measurement was also observed ($F(2, 23) = 6.91$, $p = 0.005$ and ($F(2, 24) = 4.33$, $p = 0.025$, respectively). Larger-sized individuals, in particular, appeared to shrink more in 90% ethanol solution than smaller-sized specimens.

In early instars, adding the ethanol concentration improved the models, but differences between 70%, 80%, and 90% were significant for abdominal width only (Figure A9.2.1; $F(2,9) = 10.59$, $p = 0.004$).

The samples were measured shortly after killing and preservation. It is possible that this may have influenced the results as the ethanol may not have had enough time to fully penetrate all aphid tissues. The interpretation of these results is also delicate given the small number of samples available for the early immature instars group in particular. Overall, however, ethanol preservation seemed to decrease the body length and EP of root aphids, and more so at high ethanol concentration (90%). Thoracic width appeared unaffected, but abdomen width increased slightly at killing, whereby the size increase was dependent on both, ethanol concentration and original abdominal width in most (older) instars.

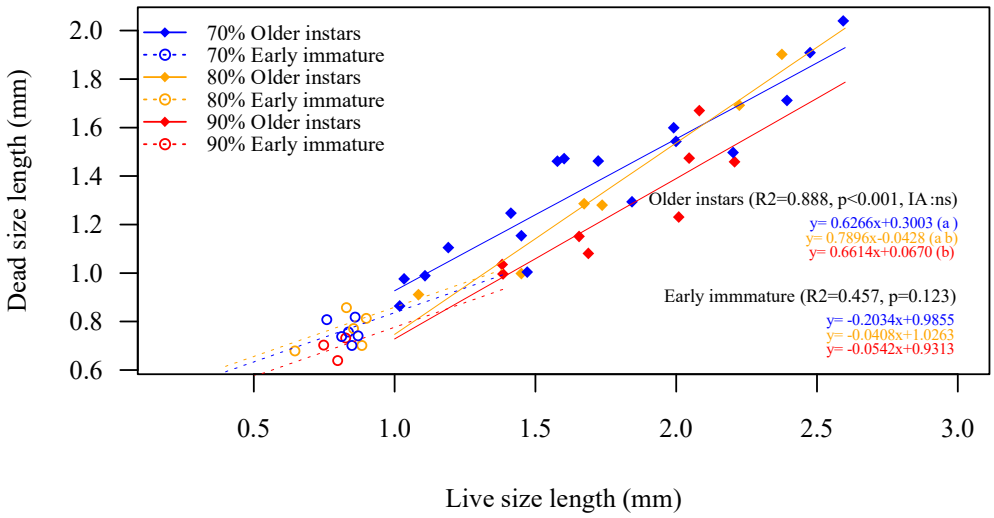
The linear regressions presented in on Figures A9.2.1 and A9.2.2 may help to estimate the dead dimensions of aphids if live measurements are to be compared with dead size measurements. If earlier instars (first to second instar, see Section A9.1) are to be assessed, these equations may not be very accurate, however.

Table A9.2.1 Pearson's correlation coefficients and *p*-values for live vs. dead measurement

Age group	EtOH conc.	n	Body length		Thoracic width		Abdo. width		EP	
			r	*p*-value	r	*p*-value	r	*p*-value	r	*p*-value
Early instars (< 1 mm length)	pooled	14	0.36	*0.200*	0.58	*0.030*	0.77	*0.001*	0.74	*0.002*
Older instars	pooled	31	0.91	*< 0.001*	0.98	*< 0.001*	0.98	*< 0.001*	0.97[1]	*< 0.001*
	70%	17	0.94	*< 0.001*	0.98	*< 0.001*	0.98	*< 0.001*	0.98	*< 0.001*
	80%	6	0.98	*< 0.001*	0.99	*< 0.001*	0.99	*< 0.001*	0.99	*< 0.001*
	90%	8	0.87	*< 0.005*	0.99	*< 0.001*	0.98	*< 0.001*	0.98	*< 0.001*

[1]Data not normally distributed; Abdo.: abdominal; EP: ellipsoid body projection [EP = (Body length/2)·(Abdo. width/2)·π]; EtOH conc.: ethanol concentration; n: number of samples.

(a)



(b)

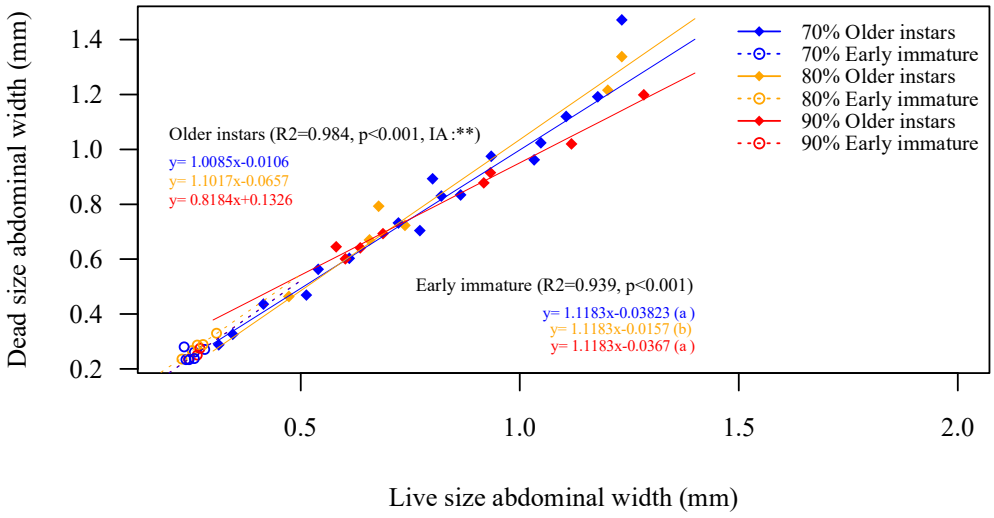
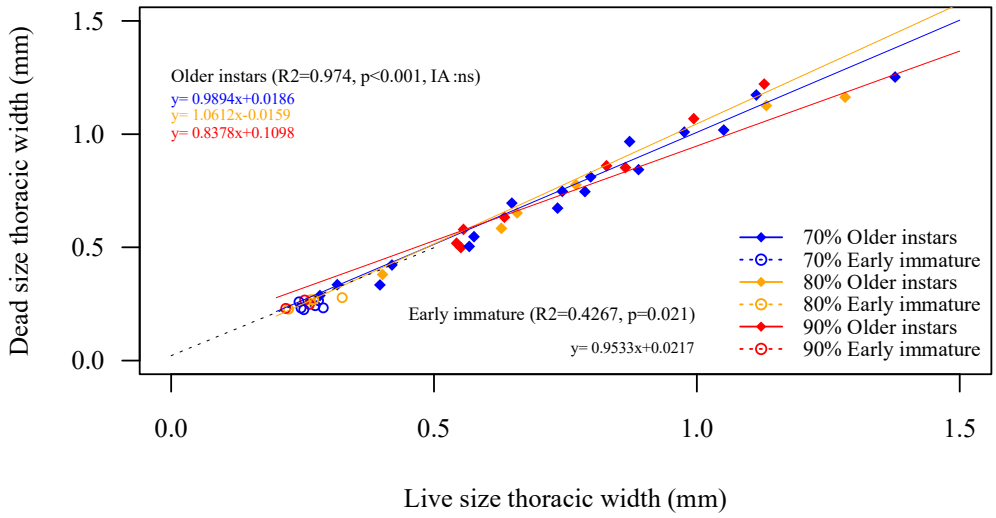


Figure A9.2.1. Relationship between live and dead size measurements, for (a) body length and (b) abdominal width, in early immature (< 1 mm length) and older instars (> 1 mm length) of root aphid *A. lentisci* , before and after being killed by and preserved in 70%, 80%, or 90% ethanol solution for two days. Regression equations with different letters mark groups that were found to differ significantly different on $\alpha = 0.05$ level (with Benjamin-Hochberg adjustment for multiple comparisons).

(a)



(b)

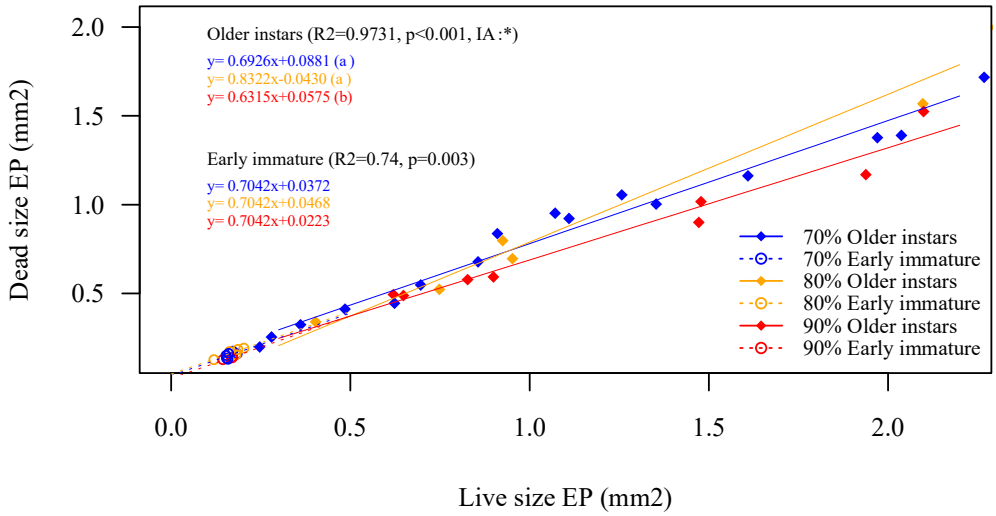


Figure A9.2.2. Relationship between live and dead size measurements, for (a) thoracic width and (b) ellipsoid body projection EP, in early immature (< 1 mm length) and older instars (> 1 mm length) of root aphid *A. lentisci*, before and after being killed by and preserved in 70%, 80%, or 90% ethanol solution for two days. Regression equations with different letters mark groups that were found to differ significantly different on $\alpha = 0.05$ level (with Benjamin-Hochberg adjustment for multiple comparisons). EP = Body length/2·Abdominal width/2·π

A9.3. Calibration trial II (Weight vs. live size calibration)

Weight is sometimes reported as aphid dimension [Dixon & Kindlmann (1994) or Podmore (2015), e.g.]. To allow a comparison between such literature data and the thesis' measurements, calibration records were collected on 60 mature root aphids in September 2017. The aphids were harvested from 11-months-old perennial ryegrass plants of a new, not yet named proprietary cultivar that had been inoculated with endophyte AR1 just after germination and had been growing from that point onwards in glasshouse conditions at AgResearch Ltd., Palmerston North. The aphids were placed individually into 1.5 mL microcentrifuge tubes (Eppendorf Tubes® 3810X, Eppendorf AG, Hamburg, Germany) and stored overnight in a 5 °C fridge. The 56 aphids that did not appear damaged by the transfer were weighed on the next day to a precision of 0.00001 g (AG135 DualRange, Mettler-Toledo, Columbus, Ohio, U.S.A.), and subsequently photographed three times by a magnification of approx. 50×. Body length, abdominal width and thoracic width were measured on these photographs and the ellipsoid projection was calculated from these measurements as described in Section 2.4.3. Correlation factors between the logarithmised weight measurements and each size measurement were then calculated in R.

All measurements were significantly correlated to the logarithmised aphid weight, and meaningful regressions could be fitted in all cases ($p < 0.001$; Figure A9.3.1). Thoracic width and ellipsoid projection had the strongest correlation with the logarithmised weight, with values of $r(56) = 0.92$ both, and the best fitting regressions of all measurements. A slightly lower correlation factor applied for length and abdominal width ($r = 0.78$ and 0.89, respectively). Thoracic width is not always easy to measure as the passage from thorax to abdomen may be unclear. The ellipsoid projection of the body, i.e. its surface as estimated by length and abdominal width, may therefore also be the more recommendable measurement to estimate the weight of adult aphids from live pictures.

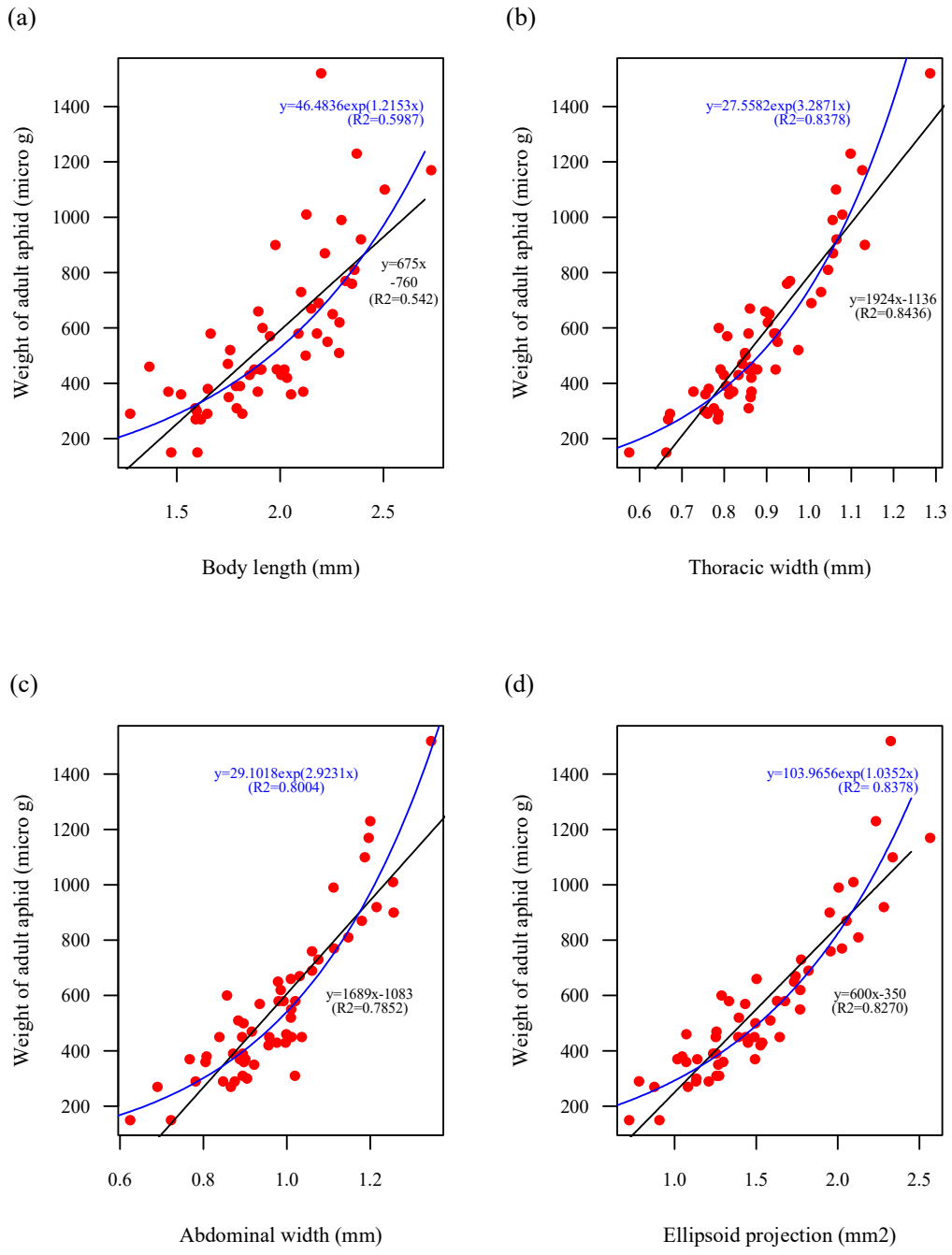


Figure A9.3.1. Relationship between adult weight and (a) length, body width at the thorax, (c) body weight at the abdomen, or (d) ellipsoid body projection (EP). EP = (length/2)·(width at abdomen/2)·π

References

Blackman, R.L., & Eastop, V.F. (1994). *Aphids on the world's trees: an identification and information guide*. Wallingford, Oxfordshire, United Kingdom: CAB International in association with the Natural History Museum.

Cottier, W. (1953). Aphids of New Zealand. *New Zealand Department of Scientific and Industrial Research bulletin 106*. Wellington, New Zealand.

Dixon, A. F. G., & Kindlmann, P. (1994). Optimum body size in aphids. *Ecological Entomology, 19*, 121-126.

Manheim, O., & Wool, D. (2003). Differential response of genotypes to alternative environments: a comparative morphological study of gall-inducing aphids (Homoptera: Pemphigidae: Fordinae). *Israel Journal of Zoology, 49*, 287-305.

Podmore, C. (2015). *Assessing mitochondrial DNA (CO1) barcodes for measuring diversity of Schizaphis spp. and abundance of Aploneura lentisci* A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science in Biological Sciences at The University of Waikato, Hamilton, New Zealand. Retrieved from http://hdl.handle.net/10289/9886 on 31/07/2018.

R Core Team. (2017). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.R-project.org/ on 9/03/2018.

Schneider, C.A., Rasband, W.S., & Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature methods, 9*(7), 671-675.

APPENDIX 10: Chapter 3 addendum

A10.1. Biology I experiment, viviposition plants

Viviposition plants consisted of 2 to 3 tillers of endophyte-free (E^-) perennial ryegrass that had been preliminarily washed and embedded in Petri dishes laid out with humid root germination cardboard (Figure A10.1.1). Twenty-eight to 40 aphids were placed on the roots of such plants, to obtain first instar nymphs of known age for the Biology I experiment (Table A10.1.1).

Figure A10.1.1. Root germination cardboard (a), used for embedding plants (b)

Table A10.1.1. Viviposition plants statistics, number of offspring collected (N) and size of offspring on their first day of life.

Viviposition plant	Day set up	N	Mean neonate offspring size (± SD)		
			Body length [mm]	Abdom. width [mm]	EP [mm^2]
LP05	05-09-12	3	0.62 ± 0.09 ^{ab}	0.23 ± 0.01 ^{a}	0.12 ± 0.03 ^{ab}
LP06	05-09-12	23	0.63 ± 0.05 ^{b}	0.22 ± 0.02 ^{a}	0.11 ± 0.02 ^{b}
LP08	06-09-12	5	0.60 ± 0.06 ^{ab}	0.21 ± 0.00 ^{a}	0.09 ± 0.01 ^{ab}
LP09	07-09-12	19	0.57 ± 0.03 ^{a}	0.20 ± 0.01 ^{a}	0.09 ± 0.01 ^{a}
LP11	08-09-12	8	0.55 ± 0.02 ^{a}	0.20 ± 0.02 ^{a}	0.08 ± 0.01 ^{a}
LP12	10-09-12	2	0.53 ± 0.01 ^{ab}	0.20 ± 0.00 ^{a}	0.08 ± 0.00 ^{ab}
LP13	11-09-12	3	0.54 ± 0.06 ^{ab}	0.19 ± 0.01 ^{a}	0.08 ± 0.01 ^{ab}
LP14	12-09-12	2	0.58 ± 0.04 ^{ab}	0.25 ^{a}	0.12 ^{ab}
LP15	14-09-12	1			
LP16	15-09-12	1	0.64 ^{ab}	0.23 ^{a}	0.11 ^{ab}
Total		**67**	**0.59 ± 0.05**	**0.21 ± 0.02**	**0.10 ± 0.02**

EP: ellipsoid body projection [mm^2], calculated as EP = Body length/2·abdominal width/2·π .
Means within a column with a same superscript letter are not significantly different on an α = 0.05 level.

A10.2. Biology I experiment, size

A10.2.1. Neonate size

```
> ###### ---------------------------------------------------------------------------------------
> # NEONATE SIZE: PLANT GENOTYPE
> kruskal.test(x=ACSizeMCNoNA$EPday1,g=ACSizeMCNoNA$PlantGenotype,method="BH")

        Kruskal-Wallis rank sum test

data:  ACSizeMCNoNA$EPday1 and ACSizeMCNoNA$PlantGenotype
Kruskal-Wallis chi-squared = 13.36, df = 17, p-value = 0.7118

> kruskal.test(x=ACSizeMCNoNAaW$EPday1,g=ACSizeMCNoNAaW$PlantGenotype,method="BH")

        Kruskal-Wallis rank sum test

data:  ACSizeMCNoNAaW$EPday1 and ACSizeMCNoNAaW$PlantGenotype
Kruskal-Wallis chi-squared = 13.36, df = 17, p-value = 0.7118

> kruskal.test(x=ACSizeMCNoNAEP$EPday1,g=ACSizeMCNoNAEP$PlantGenotype,method="BH")

        Kruskal-Wallis rank sum test

data:  ACSizeMCNoNAEP$EPday1 and ACSizeMCNoNAEP$PlantGenotype
Kruskal-Wallis chi-squared = 12.974, df = 17, p-value = 0.7379
```

A10.2.2. Growth

Table A10.2.2.1. Estimated parameters (± standard error) for the log-logistic length growth models for aphids of different size groups in the Biology I experiment.

		Small size		Large size		
Model parameters		Estimate ± SE	*p*-value	Estimate ± SE	*p*-value	Difference[1]
b		-1.506 ± 0.642	0.020	-15.637 ± 17.879	0.384	Significant
c	Birth size	0.583 ± 0.020	< 0.001	0.566 ± 0.061	< 0.001	n.s.
d	Maximal size	1.530 ± 0.149	< 0.001	1.619 ± 0.030	< 0.001	n.s.
f		4.543 ± 7.837	0.563	15.953 ± 1.272	< 0.001	n.s.
g		3.652 ± 7.281	0.617	0.078 ± 0.097	0.426	n.s.
Residual std. error		0.130		0.155		
Degrees of freedom		165		82		

Model type: Length (Age) = c + (d - c)/(1 +exp(b·(log$_e$(Age)-log$_e$(f))))^g ; std.: standard

[1] Parameters of the large-size group were considered significantly different if their estimate laid outside of the 95% confidence interval of the small-size model parameters.

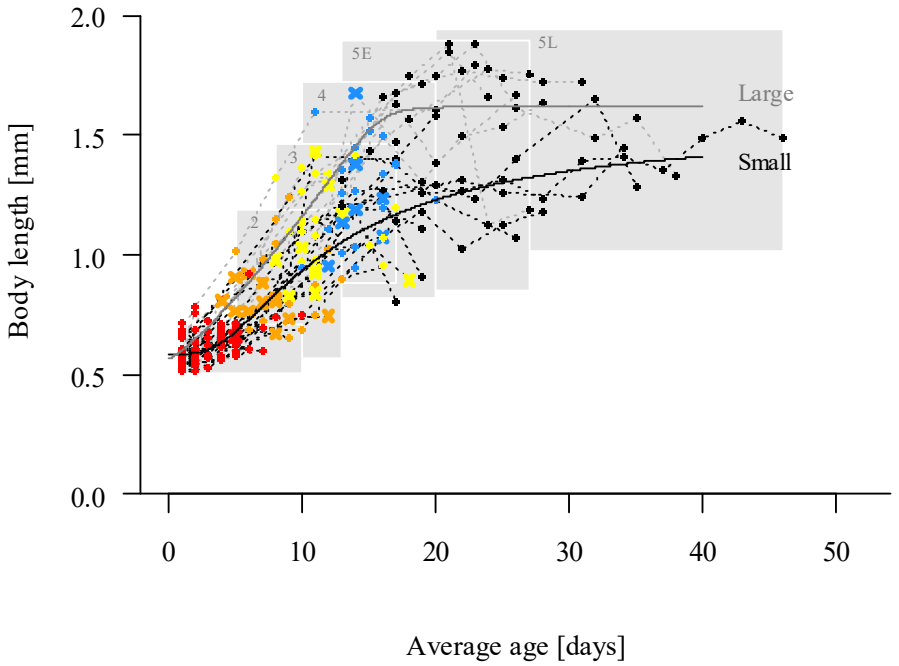


Figure A10.2.2.1. Aphid length development during the Biology I experiment. Instars 1, 2, 3, 4 and 5 are represented in red, orange, yellow, blue and black respectively. Dots: measurements on aphids of verified instar; Crosses: measurements on aphids of presumed instar (unconfirmed). Shaded areas indicate the 95% confidence interval of size for verified instar measurements (5E: size before or at first offspring birth; 5L: later measurements).

Table A10.2.2.2. Estimated parameters (± standard error) for the log-logistic abdominal width growth models for aphids of different size groups in the Biology I experiment.

		Small size		Large size		
Model parameters		Estimate ± SE	*p*-value	Estimate ± SE	*p*-value	Difference[1]
b		-1.517 ± 0.764	0.049	-45.188 ±34.840	0.200	Significant
c	Starting size	0.206 ± 0.011	< 0.001	0.196 ± 0.029	< 0.001	n.s.
d	Final size	0.896 ± 0.122	< 0.001	0.913 ± 0.016	< 0.001	n.s.
f		7.790 ±11.504	0.499	18.203 ± 0.654	< 0.001	n.s.
g		2.367 ± 4.059	0.561	0.028 ± 0.022	0.202	n.s.
Residual std error		0.058		0.075		
Degrees of freedom		133		60		

Model type: Abdominal width (Age) = $c+(d-c)/(1+\exp(b\cdot(\log_e(Age)-\log_e(f))))^g$; std.: standard

[1] Parameters of the large-size group were considered significantly different if their mean value laid outside of the 95% confidence interval of the small-size model parameters.

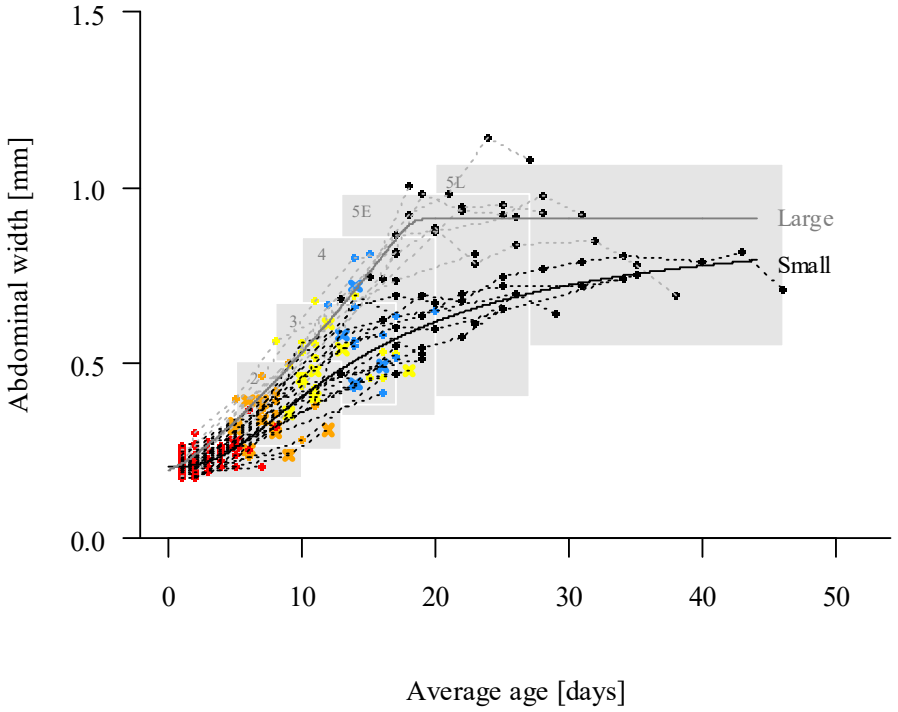


Figure A10.2.2.2. Size development of *A. lentisci* during Biology I experiment, abdominal width. Instars 1, 2, 3, 4 and 5 are represented in red, orange, yellow, blue and black respectively. Dots: measurements on aphids of verified instar; Crosses: measurements on aphids of presumed instar (unconfirmed). Shaded areas indicate the 95% confidence interval of size for verified instar measurements (5E: size before or at first offspring birth; 5L: later measurements).

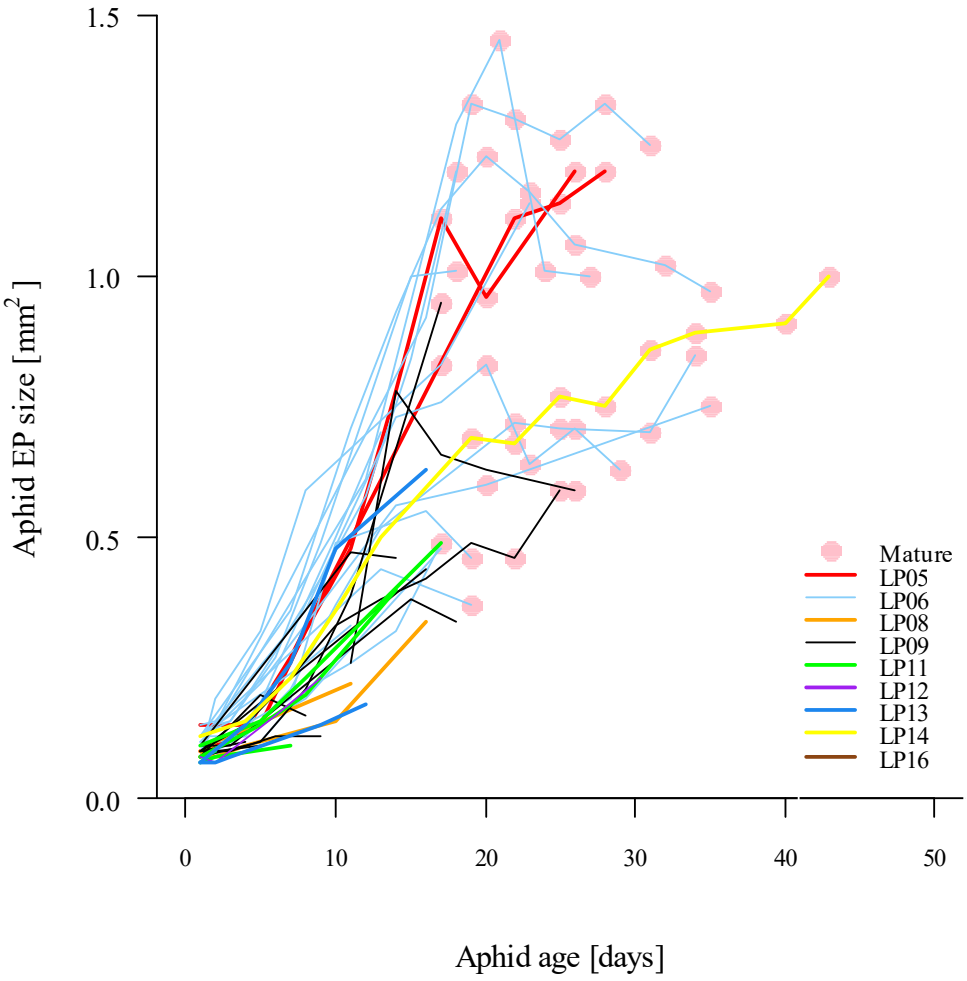


Figure A10.2.2.3. Maternal effects in aphid size development during the Biology I experiment, with information on reproductive maturity (pink circles). LP: viviposition plant name.

A10.2.3. Size

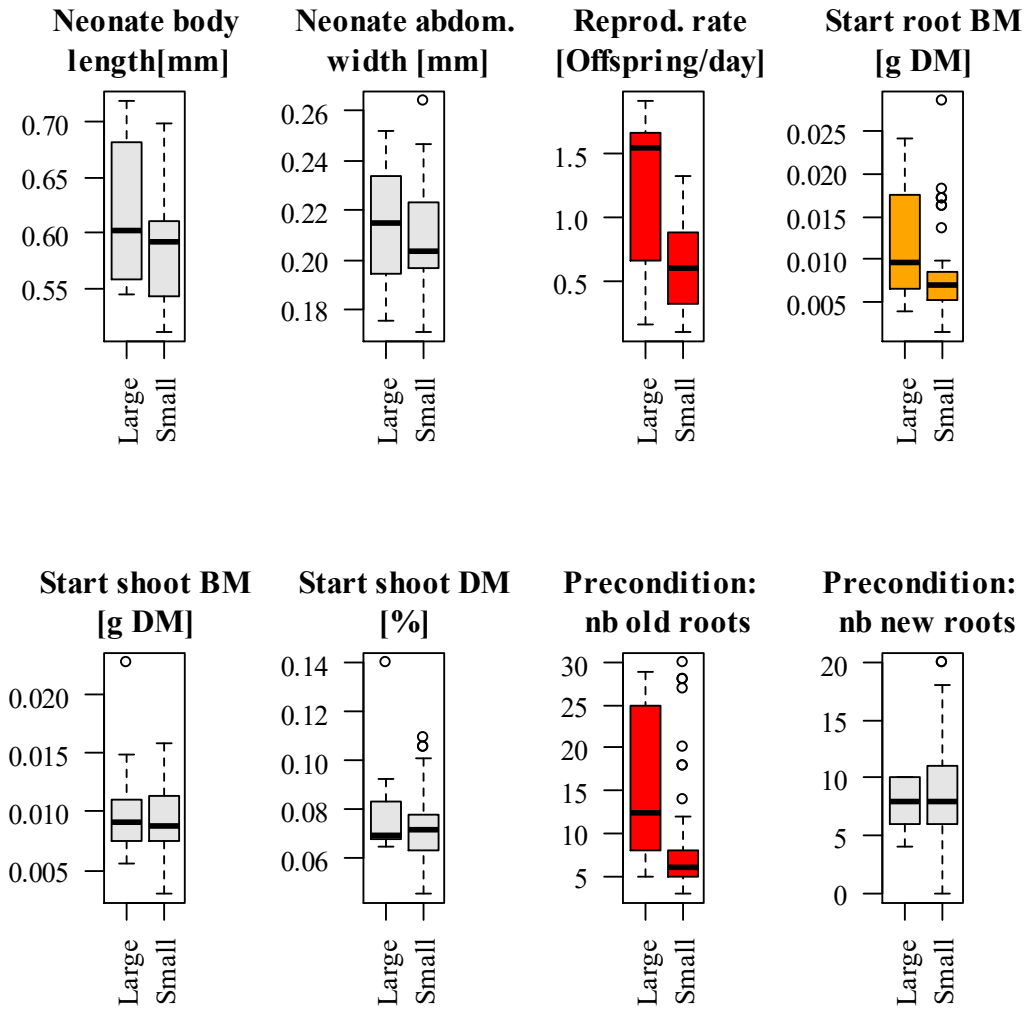


Figure A10.2.3.1. Aphid- and plant-related characteristics associated with large vs. small size development during the Biology I experiment. Body length of neonate aphid, abdominal width of neonate aphid, reproduction rate, root biomass (BM) at initial trim, and shoot biomass after initial trim, both in g dry matter (DM) per plant, number of old roots at the start, number of new roots at precondition. Red variables: significant difference between the 'Large' and the 'Small' group ($p \leq 0.05$; Wilcoxon rank test); Orange: marginally different ($0.05 < p \leq 0.1$); Grey: difference not significant.

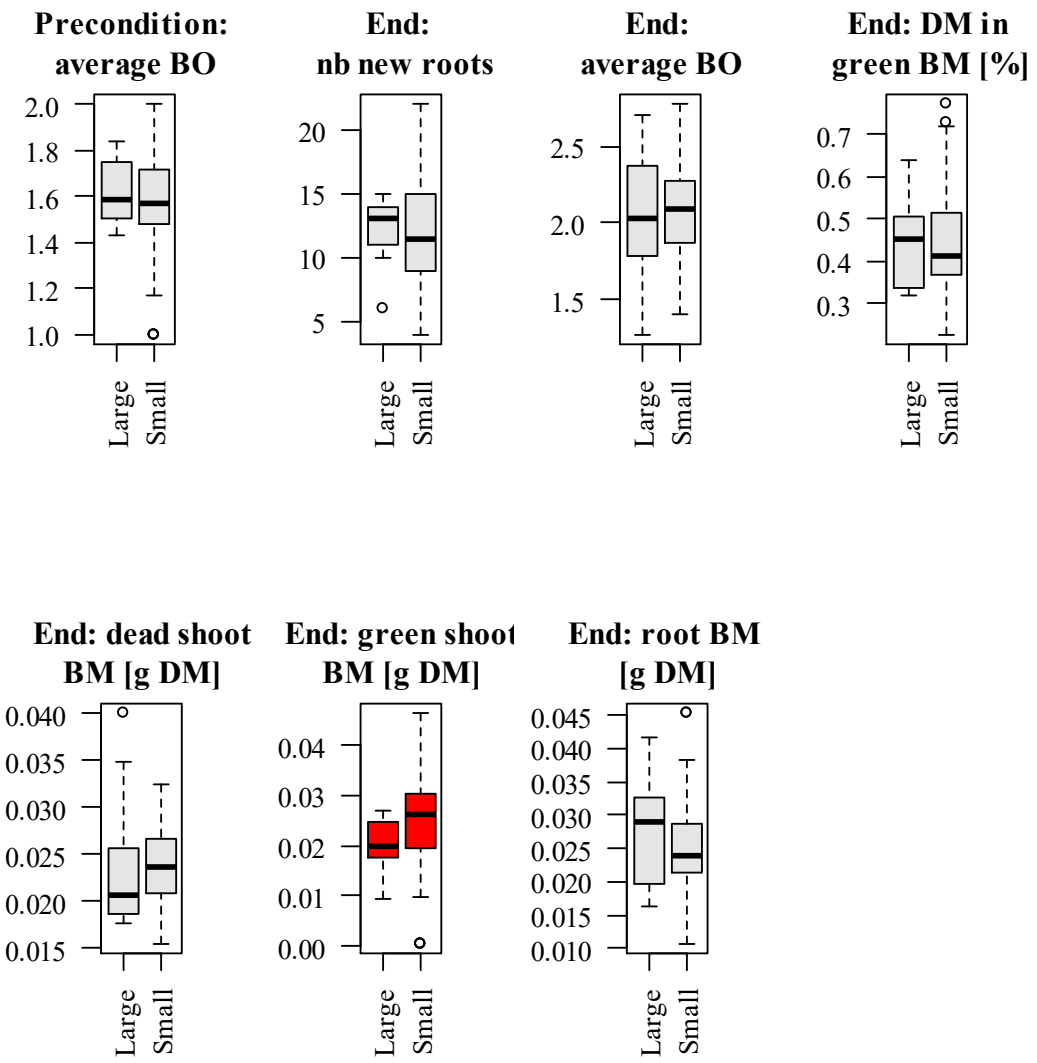


Figure A10.2.3.2. Plant-related characteristics associated with large vs. small size development during the Biology I experiment. Average branching order (BO) of plants at precondition, number of new roots at the final harvest (End), average branching order at the final harvest, dry matter content of green shoot, at final harvest, dead shoot biomass at final harvest, green shoot biomass at final harvest and root biomass at final harvest. Red variables: significant difference between the 'Large' and the 'Small' group ($p \leq 0.05$; Wilcoxon rank test); Orange: marginally different ($0.05 < p \leq 0.1$); Grey: difference not significant.

A10.3. Biology I experiment, reproduction

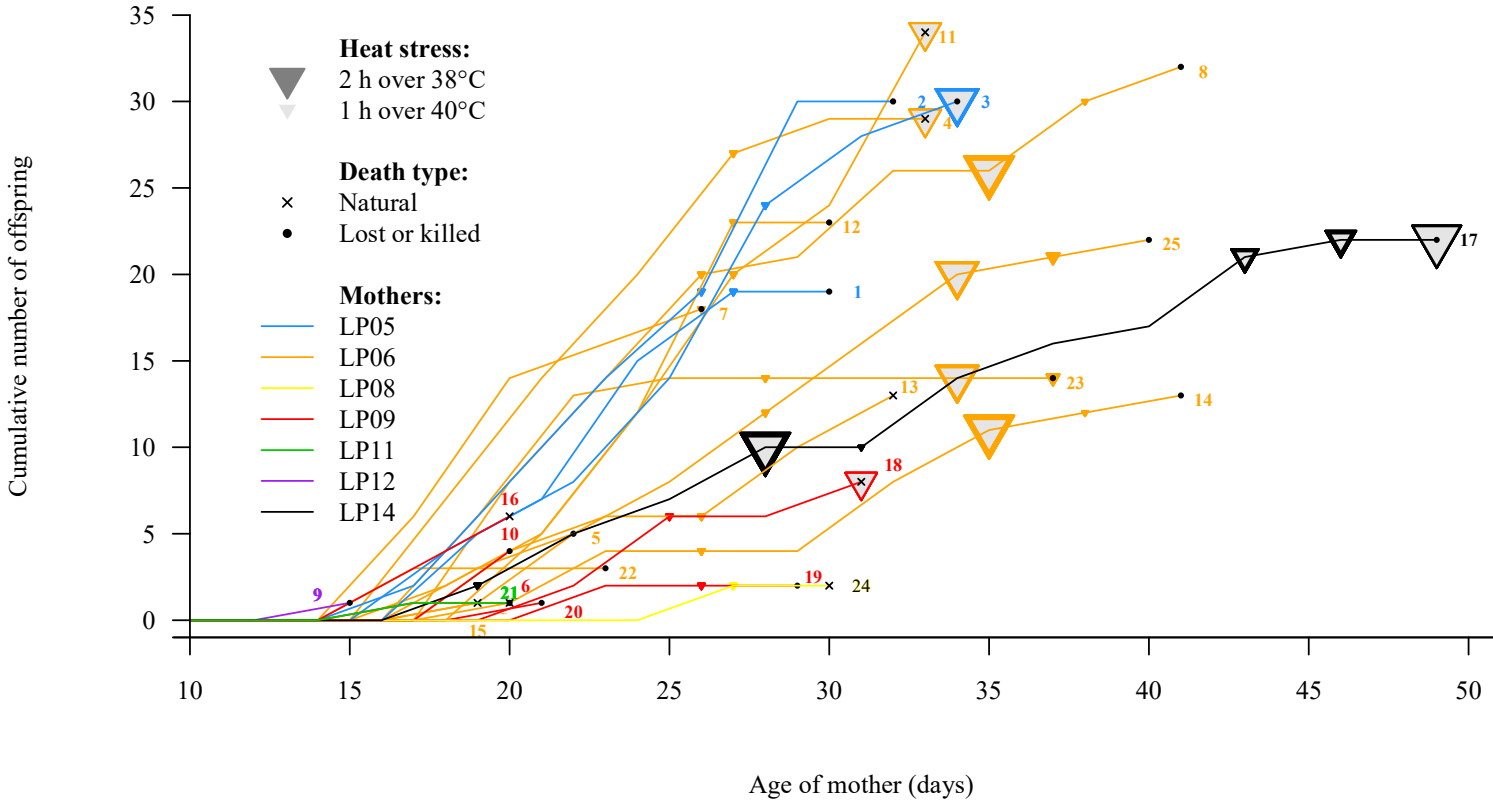


Figure A10.3.1. Maternal influences on cumulative offspring production in the Biology I experiment, with heat stress exposure information. The total number of hours above 38 and 40 °C were calculated from 10 minute interval logger records for the three days before each check, and the total length represented on the day of check, by triangle size (coloured ones for h > 38 °C, grey triangles for h above 40 °C, respectively). An aphid was declared dead of natural causes if there was no evidence for experimental handling stress (aphid moving or heavy disturbance) at the previous check. LP: name of viviposition plant/group

A10.4. Biology II experiment, size statistics

A10.4.1. Neonate size

Figure A10.4.1. illustrates the size of neonate starter aphids placed on plants in the Biology II experiment, from 14/11/2014 (day 0) onwards. Early aphids were placed onto target plants of same plant genotype/endophyte status group. As time went on, the colonies available could not supply sufficient offspring for that.

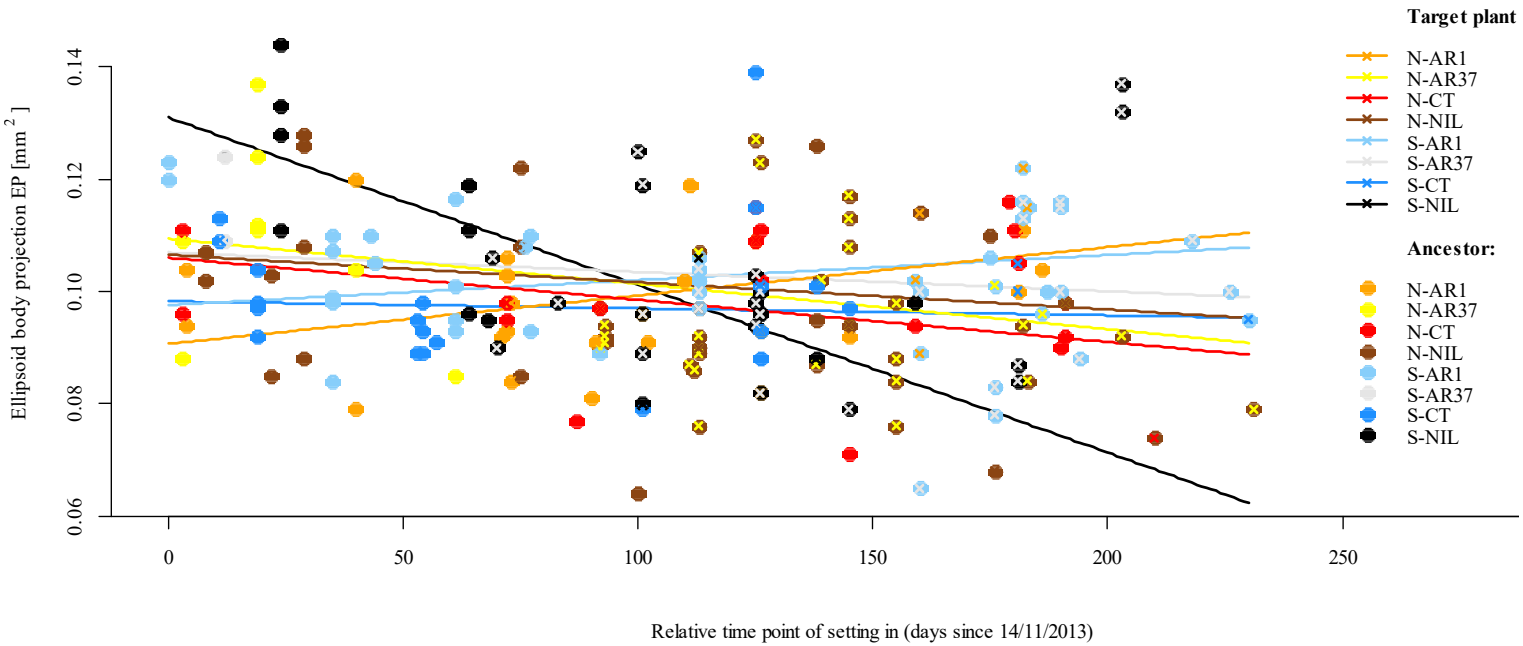


Figure A10.4.1. Size and size trends for neonate starter aphids placed onto plants of genotype N or S with one of four possible endophyte statuses (with endophytes AR1, AR37 or CT, or without endophyte, i.e. NIL), with information of the plant on which the mother (Ancestor; circles) lived. Setting in: time of placement of the aphid. EP = Body length/2·Abdominal width/2·π

A10.4.2. Growth curves

Table A10.4.2.1. Gompertz growth models parameters for aphids of the 'Large' and 'Small' groups, and comparison between 'Small' and 'Large' for model parameters by plant genotype-endophyte status group (PG-E).

PG-E	Parameter values for 'Large' groups			Parameter values for 'Small' groups			'Large' vs. 'Small'[1]			% 'Large'
	b	c	d	b	c	d	b	c	d	
N-AR1	-0.10 ± 0.03	12.78± 2.63	1.99 ± 0.40	-0.05 ± 0.02 [b]	19.07 ±10.36 [abc]	0.99 ± 0.41 [abc]	L < S	L = S	L > S	15
N-CT	-0.18 ± 0.07	12.95± 1.27	1.71 ± 0.27	-0.05 ± 0.02 [b]	18.95 ± 4.60 [bc]	0.92 ± 0.17 [bc]	L < S	L = S	L > S	44
N-NIL	-0.18 ± 0.07	10.70± 1.89	1.33 ± 0.11	-0.10 ± 0.02 [a]	12.25 ± 1.76 [bc]	0.89 ± 0.08 [bc]	L < S	L = S	L > S	33
S-AR1	-0.15 ± 0.08	12.49± 2.83	1.93 ± 0.35	-0.07 ± 0.02 [ab]	18.11 ± 3.35 [c]	1.19 ± 0.22 [c]	L < S	L = S	L > S	19
S-CT				-0.09 ± 0.02 [ab]	11.33 ± 2.20 [ab]	0.59 ± 0.07 [a]				0
S-NIL	-0.09 ± 0.05	14.33± 3.75	2.14 ± 0.52	-0.10 ± 0.02 [a]	7.63 ± 1.58 [a]	0.74 ± 0.06 [b]	L = S	L > S	L > S	27

Model: $\text{Size}(\text{Age}) = d \cdot e^{(-e^{b\cdot(\text{Age}-c)})}$, with b, c and d being constant model parameters; Same letters in a column: differences are not significant based on the 95% confidence interval. PG: plant genotype (N or S); E: endophyte status (with endophyte strain AR1, AR37 or CT, or without endophyte NIL)

[1] A parameter was not significantly different (L = S) if its estimate for the 'Large' group was within the 95% confidence interval of the 'Small' group's estimate for the same parameter.

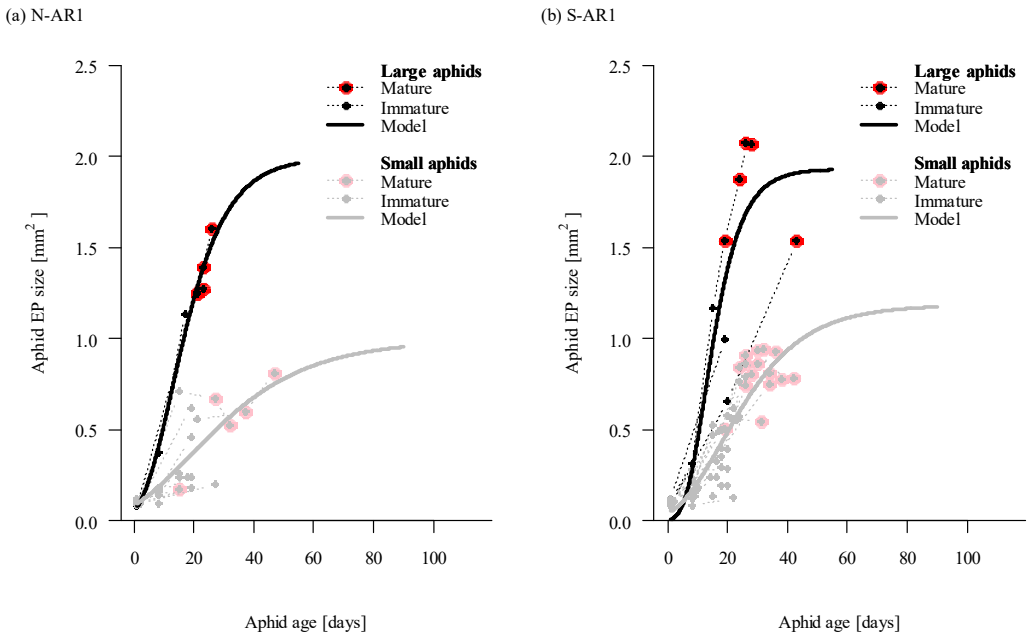


Figure A10.4.2.1. Size development of root aphids living on symbionts of endophyte AR1-perennial ryegrass of two different genotypes N (a) and S (b). EP: ellipsoid body projection**.**

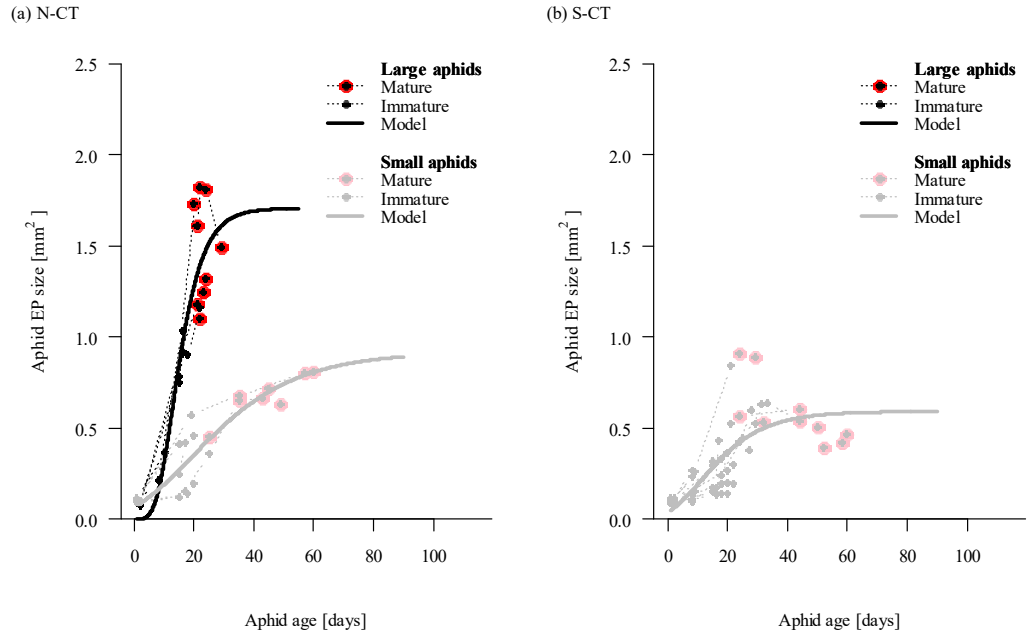


Figure A10.4.2.2. Size development of root aphids living on symbionts of endophyte CT-perennial ryegrass of two different genotypes N (a) and S (b). EP: ellipsoid body projection**.**

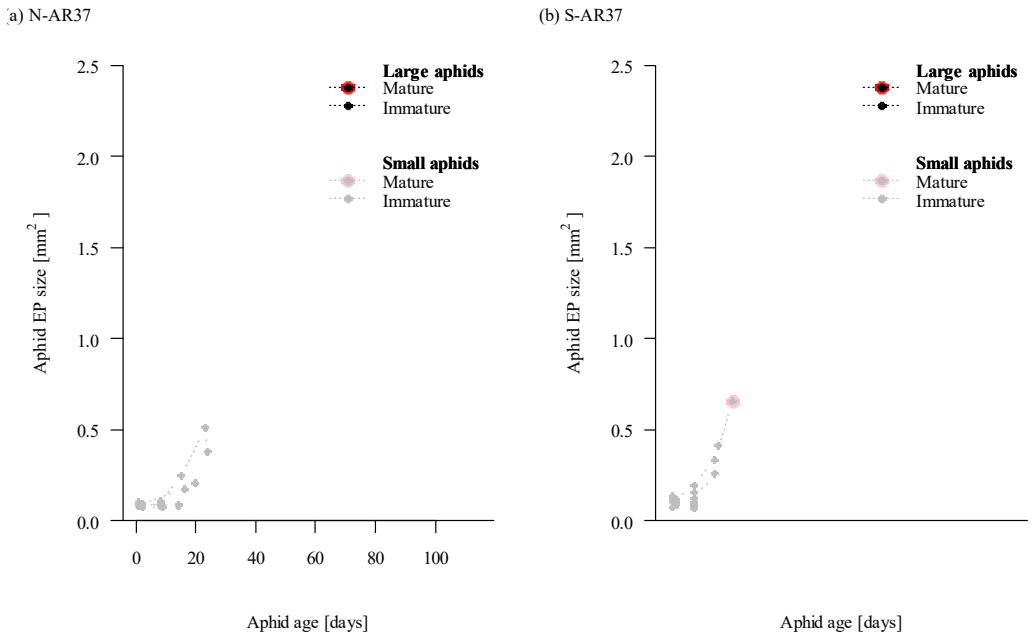


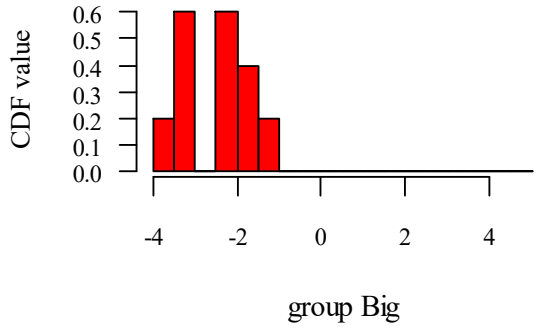
Figure A10.4.2.3. Size development of root aphids living on symbionts of endophyte AR37-perennial ryegrass of two different genotypes N (a) and S (b). EP: ellipsoid body projection**.**

A10.4.3. Canonical discriminant analysis (CDA) for size groups

Program adjusted from a template provided by Dr Siva Ganesh; a variable was considered important for a canonical discriminant function if the absolute value of its standardised coefficient was ≥ ½ the absolute value of the largest coefficients.

```
> ###### ------------------------------------------------------------------------------------
> # CDA for adult size
> IDSize.lda<-lda(SizeCode2x~AgePlantSI+PREC_NbSDL+PREC_NbGL+END_NbSDL+END_NbGL+LengthNeo+aWidthN
eo+LifeSpan+OffspRateTotal+Age_1stOff+L_1Ad+aW_1Ad,data=IDadCDA)
> print(IDSize.lda,digits=4)
Call:
lda(SizeCode2x ~ AgePlantSI + PREC_NbSDL + PREC_NbGL + END_NbSDL +
    END_NbGL + LengthNeo + aWidthNeo + LifeSpan + OffspRateTotal +
    Age_1stOff + L_1Ad + aW_1Ad, data = IDadCDA)

Prior probabilities of groups:
   Big  Small
0.3125 0.6875

Group means:
      AgePlantSI PREC_NbSDL PREC_NbGL END_NbSDL END_NbGL LengthNeo aWidthNeo
Big        84.80      7.900     3.900     13.60     2.600    0.6569    0.1980
Small      96.68      7.773     2.909     11.64     2.318    0.6630    0.2065
      LifeSpan OffspRateTotal Age_1stOff L_1Ad aW_1Ad
Big      62.60         2.0046      21.80 1.663  1.046
Small    56.91         0.7709      24.18 1.297  0.682

Coefficients of linear discriminants:
                    LD1
```

```
AgePlantSI      -0.026979
PREC_NbSDL       0.261902
PREC_NbGL       -0.767562
END_NbSDL       -0.063010
END_NbGL        -0.042537
LengthNeo       -2.833861
aWidthNeo       51.135369
LifeSpan        -0.011401
OffspRateTotal -0.552734
Age_1stOff      -0.001853
L_1Ad           -0.544737
aW_1Ad          -8.614533

> # Standardised coefficients
> IDSize.maov <- manova(cbind(AgePlantSI,PREC_NbSDL,PREC_NbGL,END_NbSDL,END_NbGL,LengthNeo,aWidth
Neo,LifeSpan,OffspRateTotal,Age_1stOff,L_1Ad,aW_1Ad)~SizeCode2x,data=IDadCDA)
> Wi= diag(summary.manova(IDSize.maov)$SS$Residuals)
> std.coefi <- (IDSize.lda$scaling)*sqrt(Wi/IDSize.maov$df.residual)
> round(std.coefi,digits=4)
                 LD1
AgePlantSI     -1.4802
PREC_NbSDL      1.8388
PREC_NbGL      -1.0175
END_NbSDL      -0.4398
END_NbGL       -0.0498
LengthNeo      -0.1175
aWidthNeo       0.6736
LifeSpan       -0.2926
OffspRateTotal -0.3420
Age_1stOff     -0.0079
L_1Ad          -0.1135
aW_1Ad         -1.0424
```



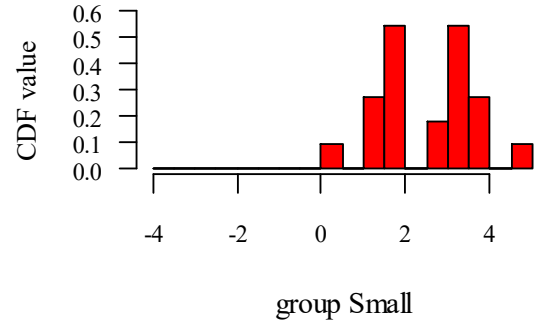


Figure A10.4.3.1. Canonical discriminant analysis plot (CDF: canonical discriminant function) for adult ellipsoid body projection (EP) size group.

A10.4.4. Adult size

```
> ##### ----------------------------------------------------------------------------------------------------
> # ADULT SIZE:
> # Difference between size groups abdominal width/length ratio
> wilcox.test(IDAdS$RatioaWtoL_1Ad~IDAdS$SizeCode2x)

    Wilcoxon rank sum test

data:  IDAdS$RatioaWtoL_1Ad by IDAdS$SizeCode2x
W = 269, p-value = 0.0006659
alternative hypothesis: true location shift is not equal to 0

> summaryBy(RatioaWtoL_1Ad~SizeCode2x,data=IDAdS,FUN=c(mean,sd),na.rm=T)
  SizeCode2x RatioaWtoL_1Ad.mean RatioaWtoL_1Ad.sd
1        Big           0.6403172        0.11641925
2      Small           0.5259329        0.08592577
```

Table A10.4.4.1. Mean and standard deviation (SD) of adult size measurements for *Aploneura lentisci* individuals raised on plants of N or S genotype without (NIL) or with endophyte *Epichloë festucae* var. *lolii* of strain AR1 or common/toxic (CT).

Trial	Plant genotype	Endo-phyte		EP [mm²]		Body length [mm]		Abdominal width [mm]	
			n	mean ± SD	E^+/E^-	mean ± SD	E^+/E^-	mean ± SD	E^+/E^-
Bio. I	pooled	NIL	23	0.84 ± 0.313		1.40 ± 0.300		0.73 ± 0.167	
Bio. II	N	AR1	5	0.79 ± 0.463	84%	1.27 ± 0.324	88%	0.73 ± 0.326	89%
	N	CT	5	0.94 ± 0.517	100%	1.40 ± 0.346	97%	0.81 ± 0.243	99%
	N	NIL	7	0.94 ± 0.311		1.44 ± 0.180		0.82 ± 0.174	
	S	AR1	9	0.92 ± 0.377	96%	1.43 ± 0.243	105%	0.79 ± 0.187	93%
	S	CT	4	0.64 ± 0.182	67%	1.29 ± 0.095	95%	0.62 ± 0.126	73%
	S	NIL	7	0.96 ± 0.569		1.36 ± 0.439		0.85 ± 0.281	

Bio.: biology experiment

EP: ellipsoid body projection, calculated as body length/2·abdominal width/2·π;

E^+/E^-: mean value for the symbiont/mean value for the endophyte-free plant of the same genotype; the differences between adult sizes in all groups were not significant (Kruskal-Wallis rank sum test $p > 0.05$)

A10.5. Biology II experiment, establishment and development

Table A10.5.1. Number and proportion of aphids that established and achieved maturity during the Biology II experiment on plants of two distinct genotypes (N, S) without endophyte (NIL), or with AR1, AR37 or common-toxic (CT) endophyte.

Plant genotype	Endophyte status	PD	Placed aphids	Establishment (E)		Maturity/ Colonisation		
		n	n	n	[% placed]	n	[% placed]	[%E]
N	AR1	9	29	16	55	6	21	38
	AR37	13	42	7	17	1	2	14
	CT	11	18	10	56	7	39	70
	NIL	10	21	16	76	9	43	56
S	AR1	9	21	20	95	11	52	55
	AR37	11	36	6	17	1	3	17
	CT	11	25	18	72	7	28	39
	NIL	8	14	13	93	10	71	77

The table was summarised from raw data that did not take into account differences in plant age and precondition at aphid placement; Establishment was considered successful if the aphids showed evidence of feeding (stylets inserted in a root, wax production, abdominal width increase). Aphids that disappeared, were killed or died before maturity were replaced by new neonates until a specimen completed a full cycle on the plant or until the plant died. n: absolute numbers; PD: Petri dishes.

```
> ##### ------------------------------------------------------------------------------------------------------------
> # ID ESTABLISHING: binary model for establishing success
> # Terms in initial models: AgePlantSI, PREC_NbGL, PREC_NbSDL, PlantGenotype, Endophyte, AncestorAdaptation2, EPavNeo, and
interactions between PlantGenotype or Endophyte and other variables
> summary(EstSuccEE13.glm<-
glm(Establishing2~AgePlantSI+PREC_NbGL+Endophyte+EPavNeo+Endophyte:EPavNeo,data=LCdataDbb,family=binomial()))

Call:
glm(formula = Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte +
    EPavNeo + Endophyte:EPavNeo, family = binomial(), data = LCdataDbb)

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-3.0546  -0.4905   0.1666   0.4977   2.0148

Coefficients:
                       Estimate Std. Error z value Pr(>|z|)
(Intercept)            7.403253   9.970545   0.743  0.45778
AgePlantSI            -0.009805   0.005468  -1.793  0.07297 .
PREC_NbGL              0.933029   0.334056   2.793  0.00522 **
EndophyteAR1          -4.305875  11.037738  -0.390  0.69646
EndophyteAR37         -1.770795  10.067419  -0.176  0.86038
EndophyteCT           -14.205386  11.313467  -1.256  0.20925
EPavNeo               -46.875341  89.208924  -0.525  0.59927
EndophyteAR1:EPavNeo   29.275528 101.451002   0.289  0.77291
EndophyteAR37:EPavNeo -28.271929  91.610373  -0.309  0.75762
EndophyteCT:EPavNeo   129.874150 105.922808   1.226  0.22015
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

(Dispersion parameter for binomial family taken to be 1)

    Null deviance: 172.703  on 139  degrees of freedom
Residual deviance:  93.703  on 130  degrees of freedom
AIC: 113.7

Number of Fisher Scoring iterations: 6
```

```
> # Effects:
> EstSuccEE13a.glm<-glm(Establishing2~AgePlantSI+PREC_NbGL+Endophyte+EPavNeo,data=LCdataDbb,family=binomial())
> anova(EstSuccEE13.glm,EstSuccEE13a.glm, test="Chisq")
Analysis of Deviance Table

Model 1: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte + EPavNeo +
    Endophyte:EPavNeo
Model 2: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte + EPavNeo
  Resid. Df Resid. Dev Df Deviance Pr(>Chi)
1       130     93.703
2       133    101.711 -3  -8.0076  0.04585 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> EstSuccEE13b.glm<-glm(Establishing2~AgePlantSI+PREC_NbGL+Endophyte,data=LCdataDbb,family=binomial())
> anova(EstSuccEE13a.glm,EstSuccEE13b.glm, test="Chisq")
Analysis of Deviance Table

Model 1: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte + EPavNeo
Model 2: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte
  Resid. Df Resid. Dev Df Deviance Pr(>Chi)
1       133     101.71
2       134     103.81 -1  -2.1016   0.1471
> EstSuccEE13c.glm<-glm(Establishing2~AgePlantSI+PREC_NbGL+EPavNeo,data=LCdataDbb,family=binomial())
> anova(EstSuccEE13a.glm,EstSuccEE13c.glm, test="Chisq")
Analysis of Deviance Table

Model 1: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte + EPavNeo
Model 2: Establishing2 ~ AgePlantSI + PREC_NbGL + EPavNeo
  Resid. Df Resid. Dev Df Deviance       Pr(>Chi)
1       133     101.71
2       136     142.69 -3   -40.98 0.000000006603 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> EstSuccEE13d.glm<-glm(Establishing2~AgePlantSI+Endophyte+EPavNeo+Endophyte:EPavNeo,data=LCdataDbb,family=binomial())
> anova(EstSuccEE13.glm,EstSuccEE13d.glm, test="Chisq")
Analysis of Deviance Table

Model 1: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte + EPavNeo +
    Endophyte:EPavNeo
Model 2: Establishing2 ~ AgePlantSI + Endophyte + EPavNeo + Endophyte:EPavNeo
  Resid. Df Resid. Dev Df Deviance Pr(>Chi)
1       130     93.703
2       131    104.427 -1  -10.723 0.001058 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
> EstSuccEE13e.glm<-glm(Establishing2~PREC_NbGL+Endophyte+EPavNeo+Endophyte:EPavNeo,data=LCdataDbb,family=binomial())
> anova(EstSuccEE13.glm,EstSuccEE13e.glm, test="Chisq")
Analysis of Deviance Table

Model 1: Establishing2 ~ AgePlantSI + PREC_NbGL + Endophyte + EPavNeo +
    Endophyte:EPavNeo
Model 2: Establishing2 ~ PREC_NbGL + Endophyte + EPavNeo + Endophyte:EPavNeo
  Resid. Df Resid. Dev Df Deviance Pr(>Chi)
1       130     93.703
2       131     97.067 -1  -3.3642  0.06663 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
>
```

```
> ##### ------------------------------------------------------------------------------------------------------------
> # ID development:
> # Reproduction
> summary(IDReproR3.coxph<-coxph(Surv(AgeAphid,Event)~AgePlantSI+PREC_NbSDL+PGxE+EPavNeo+AgePlantSI:PGxE,data=LCdataR3r))
Call:
coxph(formula = Surv(AgeAphid, Event) ~ AgePlantSI + PREC_NbSDL +
    PGxE + EPavNeo + AgePlantSI:PGxE, data = LCdataR3r)

  n= 98, number of events= 45

                          coef  exp(coef)   se(coef)      z Pr(>|z|)
AgePlantSI           3.877e-02  1.040e+00  2.153e-02  1.800  0.07178 .
PREC_NbSDL          -2.076e-01  8.125e-01  1.108e-01 -1.874  0.06100 .
PGxENAR1             4.987e-01  1.647e+00  2.123e+00  0.235  0.81432
PGxENCT              3.971e+00  5.305e+01  1.482e+00  2.680  0.00736 **
PGxENNIL             2.423e+00  1.127e+01  1.435e+00  1.688  0.09136 .
PGxESAR1             5.181e-01  1.679e+00  1.270e+00  0.408  0.68325
PGxESCT              6.400e-02  1.066e+00  1.472e+00  0.043  0.96531
EPavNeo              3.516e+01  1.869e+15  1.605e+01  2.191  0.02843 *
AgePlantSI:PGxENAR1 -2.096e-02  9.793e-01  2.006e-02 -1.045  0.29622
AgePlantSI:PGxENCT  -4.976e-02  9.515e-01  1.763e-02 -2.822  0.00477 **
AgePlantSI:PGxENNIL -3.209e-02  9.684e-01  1.696e-02 -1.892  0.05854 .
AgePlantSI:PGxESAR1 -9.824e-03  9.902e-01  1.251e-02 -0.786  0.43211
AgePlantSI:PGxESCT  -2.077e-02  9.794e-01  1.618e-02 -1.284  0.19907
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

                    exp(coef) exp(-coef) lower .95 upper .95
AgePlantSI          1.040e+00  9.620e-01   0.99657 1.084e+00
PREC_NbSDL          8.125e-01  1.231e+00   0.65390 1.010e+00
PGxENAR1            1.647e+00  6.073e-01   0.02566 1.057e+02
PGxENCT             5.305e+01  1.885e-02   2.90625 9.685e+02
PGxENNIL            1.127e+01  8.869e-02   0.67713 1.877e+02
PGxESAR1            1.679e+00  5.957e-01   0.13938 2.022e+01
PGxESCT             1.066e+00  9.380e-01   0.05957 1.908e+01
EPavNeo             1.869e+15  5.351e-16  40.97731 8.523e+28
AgePlantSI:PGxENAR1 9.793e-01  1.021e+00   0.94150 1.019e+00
AgePlantSI:PGxENCT  9.515e-01  1.051e+00   0.91914 9.849e-01
AgePlantSI:PGxENNIL 9.684e-01  1.033e+00   0.93675 1.001e+00
AgePlantSI:PGxESAR1 9.902e-01  1.010e+00   0.96625 1.015e+00
AgePlantSI:PGxESCT  9.794e-01  1.021e+00   0.94887 1.011e+00

Concordance= 0.718  (se = 0.053 )
Rsquare= 0.23   (max possible= 0.956 )
Likelihood ratio test= 25.67  on 13 df,   p=0.01885
Wald test            = 25.02  on 13 df,   p=0.02297
Score (logrank) test = 26.9  on 13 df,   p=0.01283

> cox.zph(IDReproR3.coxph)
                      rho   chisq     p
AgePlantSI          -0.0766  0.3732 0.541
PREC_NbSDL           0.1344  1.1895 0.275
PGxENAR1             0.0948  0.6123 0.434
PGxENCT             -0.0246  0.0303 0.862
PGxENNIL             0.0374  0.1345 0.714
PGxESAR1            -0.0185  0.0171 0.896
PGxESCT             -0.1422  1.3186 0.251
EPavNeo              0.0652  0.2694 0.604
AgePlantSI:PGxENAR1 -0.0631  0.2832 0.595
AgePlantSI:PGxENCT   0.0484  0.1393 0.709
AgePlantSI:PGxENNIL -0.0667  0.4627 0.496
AgePlantSI:PGxESAR1  0.0441  0.1041 0.747
AgePlantSI:PGxESCT   0.1070  0.8093 0.368
GLOBAL                   NA 13.9881 0.375
> survfit(IDReproR3.coxph)
Call: survfit(formula = IDReproR3.coxph)

      n  events  median 0.95LCL 0.95UCL
     98      45      26      25      33


> ReproID<-glht(IDReproR3.coxph, linfct = mcp(PGxE="Tukey",interaction_average = TRUE, covariate_
average = TRUE))
> mcsReproID<-summary(ReproID,test=adjusted("none"))
> cld(mcsReproID,level=0.05,decreasing=F)
aSNIL  NAR1   NCT  NNIL  SAR1   SCT
  "c"  "ab"  "bc"  "bc"  "ac"   "b"


> # ID_NA37_02: AgePlantSI= 50, PREC_NbSDL= 1 , EPavNeo= 0.111
>
survfit(IDReproR3.coxph,newdata=data.frame(AgePlantSI=rep(50,each=6),PREC_NbSDL=rep(c(1),each=6),EPavNeo=rep(c(0.111),each=6),P
GxE=c("aSNIL","NAR1","NCT","NNIL","SAR1","SCT")))
Call: survfit(formula = IDReproR3.coxph, newdata = data.frame(AgePlantSI = rep(50,
    each = 6), PREC_NbSDL = rep(c(1), each = 6), EPavNeo = rep(c(0.111),
    each = 6), PGxE = c("aSNIL", "NAR1", "NCT", "NNIL", "SAR1",
    "SCT")))

   n events median 0.95LCL 0.95UCL
1 98     45     23      21      NA
2 98     45     24      21      NA
3 98     45     20      19      NA
4 98     45     21      20      NA
5 98     45     23      21      NA
6 98     45     26      23      NA
```

```
> # ID_SA37_03: AgePlantSI= 43, PREC_NbSDL= 2 , EPavNeo= 0.109
>
survfit(IDReproR3.coxph,newdata=data.frame(AgePlantSI=rep(43,each=6),PREC_NbSDL=rep(c(2),each=6),EPavNeo=rep(c(0.109),each=6),P
GxE=c("aSNIL","NAR1","NCT","NNIL","SAR1","SCT")))
Call: survfit(formula = IDReproR3.coxph, newdata = data.frame(AgePlantSI = rep(43,
    each = 6), PREC_NbSDL = rep(c(2), each = 6), EPavNeo = rep(c(0.109),
    each = 6), PGxE = c("aSNIL", "NAR1", "NCT", "NNIL", "SAR1",
    "SCT")))

   n events median 0.95LCL 0.95UCL
1 98     45     24      22      NA
2 98     45     26      22      NA
3 98     45     20      19      NA
4 98     45     22      20      NA
5 98     45     24      22      NA
6 98     45     29      24      NA
>
> # Death time point
> summary(IDDeath3.coxph<-coxph(Surv(AgeAphid,Event)~PREC_NbGL+Endophyte+PREC_NbGL:Endophyte,data=LCdataD3))
Call:
coxph(formula = Surv(AgeAphid, Event) ~ PREC_NbGL + Endophyte +
    PREC_NbGL:Endophyte, data = LCdataD3)

  n= 146, number of events= 99

                          coef exp(coef) se(coef)      z       Pr(>|z|)
PREC_NbGL                0.1991    1.2203   0.1575  1.264       0.206126
EndophyteAR1             1.3212    3.7478   0.7297  1.811       0.070216 .
EndophyteAR37            5.0342  153.5808   0.7855  6.409 0.000000000146 ***
EndophyteCT              2.3461   10.4444   0.8014  2.928       0.003416 **
PREC_NbGL:EndophyteAR1  -0.2561    0.7741   0.2185 -1.172       0.241141
PREC_NbGL:EndophyteAR37 -0.9026    0.4055   0.2488 -3.627       0.000287 ***
PREC_NbGL:EndophyteCT   -0.6187    0.5386   0.2709 -2.284       0.022383 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

                        exp(coef) exp(-coef) lower .95 upper .95
PREC_NbGL                  1.2203   0.819463    0.8962    1.6616
EndophyteAR1               3.7478   0.266826    0.8967   15.6641
EndophyteAR37            153.5808   0.006511   32.9423  716.0116
EndophyteCT               10.4444   0.095745    2.1715   50.2358
PREC_NbGL:EndophyteAR1     0.7741   1.291858    0.5045    1.1878
PREC_NbGL:EndophyteAR37    0.4055   2.465899    0.2490    0.6604
PREC_NbGL:EndophyteCT      0.5386   1.856501    0.3167    0.9160

Concordance= 0.792  (se = 0.039 )
Rsquare= 0.455   (max possible= 0.995 )
Likelihood ratio test= 88.63  on 7 df,   p=2.22e-16
Wald test            = 84.86  on 7 df,   p=1.443e-15
Score (logrank) test = 128.6  on 7 df,   p=0

> cox.zph(IDDeath3.coxph)
                          rho     chisq     p
PREC_NbGL              0.094867 0.8653968 0.352
EndophyteAR1           -0.027248 0.0839209 0.772
EndophyteAR37           0.074802 0.6142673 0.433
EndophyteCT            -0.000435 0.0000223 0.996
PREC_NbGL:EndophyteAR1  0.044045 0.2427699 0.622
PREC_NbGL:EndophyteAR37 -0.068316 0.5019974 0.479
PREC_NbGL:EndophyteCT   0.038542 0.1958386 0.658
GLOBAL                        NA 5.0664350 0.652



> DeathID<-glht(IDDeath3.coxph, linfct = mcp(Endophyte="Tukey", interaction_average = TRUE, covar
iate_average = TRUE))
> mcsDeathID<-summary(DeathID,test=adjusted("none"))
> cld(mcsDeathID,level=0.05,decreasing=F)
aaNIL   AR1  AR37    CT
  "c"   "b"   "a"   "b"
```

A10.6. Biology II experiment, reproduction

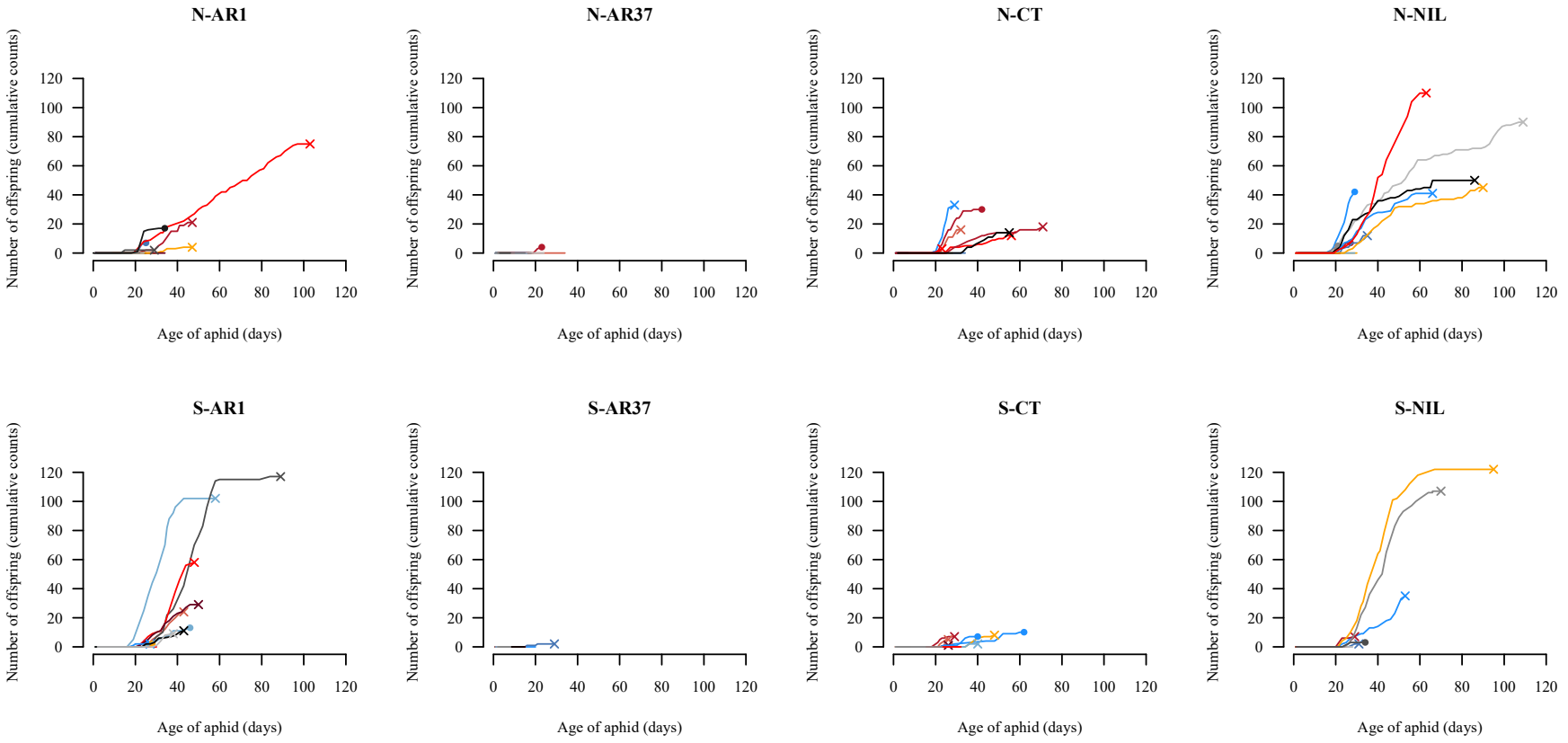


Figure A10.6.1. Cumulative offspring production of root aphids raised on plants with various genotypes (N or S) and endophyte statuses [in symbiosis with strain AR37, AR1, or CT or without any symbiont (NIL)]. Each line represents the offspring production of a particular aphid up to the considered day and is ended by a dot if the aphid disappeared or was killed, and by a cross if the death appeared to have happened without external influences ("natural death").

A10.7. Mature plant experiment, colonisation success

Kruskal-Wallis test for differences between PG-E groups

```
> ###### ------------------------------------------------------------------------------------
> # OUT ESTABLISHING
> # Kruskal-Wallis test on living adult aphids at final harvest
> kruskal.test(Aphids_LivAd~Group,data=OutSurvSI)

        Kruskal-Wallis rank sum test

data:  Aphids_LivAd by Group
Kruskal-Wallis chi-squared = 16.547, df = 7, p-value = 0.02056

> DunnTest(Aphids_LivAd~Group,data=OutSurvSI,method="BH",out.list=FALSE)

 Dunn's test of multiple comparisons using rank sums : BH

      aaSNIL NAR1  NAR37 NCT   NNIL  SAR1  SAR37
NAR1  0.627  -     -     -     -     -     -
NAR37 0.759  0.398 -     -     -     -     -
NCT   0.436  0.774 0.312 -     -     -     -
NNIL  0.945  0.611 0.774 0.416 -     -     -
SAR1  0.312  0.627 0.271 0.774 0.312 -     -
SAR37 0.280  0.072 0.398 0.049 0.280 0.017 -
SCT   0.728  0.384 0.945 0.312 0.754 0.222 0.398
```

Canonical discriminant analysis calculation

```
> # CDA with transformed variables (Basic codes provided by Dr. Siva Ganesh)
> print(OutData22.lda<-lda(EstablishedCode3~Diameter24h+Regrowth24h+LOGStart_TillerDM+percDMstart
+All_Start_Area+All_Start_GtoAllRatio+RAW_Pre_RootsNb+LOGLeafAPPrateBA+LOGRootAPPrateBH+Harv_H2OG
reen+ADJ116GLDMNet+ADJ116SLDM+ADJ116RootsDM+All_End_AreaGrowthRateSE+All_End_GtoAllRatio,data=Out
SurvSI),digits=4)
Call:
lda(EstablishedCode3 ~ Diameter24h + Regrowth24h + LOGStart_TillerDM +
    percDMstart + All_Start_Area + All_Start_GtoAllRatio + RAW_Pre_RootsNb +
    LOGLeafAPPrateBA + LOGRootAPPrateBH + Harv_H2OGreen + ADJ116GLDMNet +
    ADJ116SLDM + ADJ116RootsDM + All_End_AreaGrowthRateSE + All_End_GtoAllRatio,
    data = OutSurvSI)

Prior probabilities of groups:
   1-4     >4      0
0.5098 0.2353 0.2549

Group means:
     Diameter24h Regrowth24h LOGStart_TillerDM percDMstart All_Start_Area
 1-4      0.1894       1.133            -4.823      0.1070          241.9
>4        0.1833       1.341            -4.696      0.1205          257.2
0         0.1807       1.017            -4.819      0.1068          209.7
     All_Start_GtoAllRatio RAW_Pre_RootsNb LOGLeafAPPrateBA LOGRootAPPrateBH
 1-4                0.4836           4.038            1.905           1.0030
>4                  0.4850           4.667            1.895           0.9393
0                   0.4832           2.923            2.039           1.1281
     Harv_H2OGreen ADJ116GLDMNet ADJ116SLDM ADJ116RootsDM
 1-4        0.2367        0.3068    0.12285        0.1564
>4          0.2326        0.3284    0.12603        0.1489
0           0.2214        0.2539    0.09559        0.1472
     All_End_AreaGrowthRateSE All_End_GtoAllRatio
 1-4                    27.63              0.4742
>4                      28.46              0.4758
0                       20.49              0.4717

Coefficients of linear discriminants:
                             LD1       LD2
Diameter24h              -27.037195  31.188487
Regrowth24h               -0.816583  -1.672846
LOGStart_TillerDM          1.773798  -2.373687
percDMstart                1.221489  -6.533891
All_Start_Area             0.006933  -0.001553
All_Start_GtoAllRatio     73.405992  -4.778336
RAW_Pre_RootsNb           -0.042052  -0.160441
LOGLeafAPPrateBA          -0.221586   1.835648
LOGRootAPPrateBH           0.160807   0.062955
Harv_H2OGreen             11.580342   4.468149

ADJ116GLDMNet              -2.838727   3.864596
ADJ116SLDM                -19.919650  14.025475
ADJ116RootsDM              17.415167  -7.163579
All_End_AreaGrowthRateSE   -0.208074   0.037032
All_End_GtoAllRatio         8.391168 -50.667057

Proportion of trace:
   LD1    LD2
0.8286 0.1714
```

Canonical discriminant analysis output (standardised coefficients and graph):

```
> OutData22.maov <- manova(cbind(Diameter24h,Regrowth24h,LOGStart_TillerDM,percDMstart,All_Start_
Area,All_Start_GtoAllRatio,RAW_Pre_RootsNb,LOGLeafAPPrateBA,LOGRootAPPrateBH,Harv_H2OGreen,ADJ116
GLDMNet,ADJ116SLDM,ADJ116RootsDM,All_End_AreaGrowthRateSE,All_End_GtoAllRatio)~EstablishedCode3,d
ata=OutSurvSI)
> W22=diag(summary.manova(OutData22.maov)$SS$Residuals)
> std.coef22<-(OutData22.lda$scaling)*sqrt(W22/OutData22.maov$df.residual)
> round(std.coef22,digits=4)
                              LD1     LD2
Diameter24h               -0.6547  0.7552
Regrowth24h               -0.3034 -0.6215
LOGStart_TillerDM          0.5645 -0.7554
percDMstart                0.0357 -0.1911
All_Start_Area             0.5860 -0.1313
All_Start_GtoAllRatio      0.6490 -0.0422
RAW_Pre_RootsNb           -0.0694 -0.2646
LOGLeafAPPrateBA          -0.0617  0.5113
LOGRootAPPrateBH           0.0545  0.0213
Harv_H2OGreen              0.3750  0.1447
ADJ116GLDMNet             -0.2953  0.4021
ADJ116SLDM                -0.6205  0.4369
ADJ116RootsDM              1.0241 -0.4213
All_End_AreaGrowthRateSE -1.4935  0.2658
All_End_GtoAllRatio        0.0434 -0.2621
```

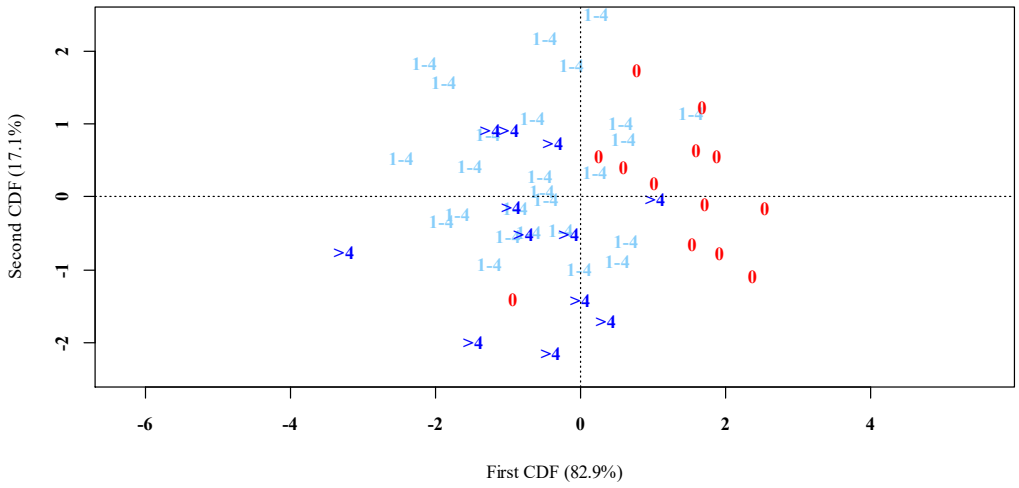


Figure A10.7.1. Canonical discriminant analysis functions (CDF) for establishment success during the Mature plant experiment.

APPENDIX 11: Chapter 4 addendum

A11.1. Seedling experiment statistics

A11.1.1. Aphid numbers

Table A11.1.1. Root aphid numbers and loads per g dry matter on the seedlings successfully colonised in the Seedling experiment (mean ± standard deviation).

Accession	Endophyte group	n	Number of aphids per seedling			Aphid loads [LFA/g DM]	
			Adult	Older immatures	1st instar	Green shoot	Root
A14559	AR1	18	1.7 ± 1.0	23 ± 17	14 ± 13	820 ± 611	4119 ± 3286
A16863	AR37	11	1.2 ± 0.8	15 ± 16	4 ± 6	316 ± 301	1360 ± 1455
A12421	CT	19	1.7 ± 1.1	13 ± 18	13 ± 10	349 ± 322	1580 ± 1559
A11104	NIL	17	1.4 ± 1.1	15 ± 19	12 ± 12	435 ± 245	1943 ± 1133
Pooled	NIL2	7	1.3 ± 0.8	22 ± 8	15 ± 10	782 ± 711	3853 ± 4316
Overall	**range**	**72**	**[0 - 4]**	**[0 - 67]**	**[0 - 41]**	**[15 - 2625]**	**[61-18133]**
	pooled		**1.5 ± 1.0**	**18 ± 17**	**12 ± 11**	**572 ± 547**	**2753 ± 3050**

DM: dry matter (dry weight) of plants at aphid harvest; LFA: long-term feeding aphids, i.e. total number of adults and older immatures; n: number of seedlings successfully colonised.

A11.1.2. Plant morphology criteria

Median, mean values and standard deviations (sd) by group for plant age (AgePlantHarvest), water stress (DroughtStress2), endophyte status (EndoGroup), and aphid presence (AphPres2).
Variables considered: age of plant at harvest (AgePlantHarvest), number of tillers (TillerNb), number of green leaves (LeafNb) and number of roots at harvest (RootsNb)

```
> ###### ------------------------------------------------------------------------------------
> # PLANT AGE STATISTICS
> summaryBy(AgePlantHarvest~DroughtStress2,data=SeedR,FUN=list(median,mean,sd))
  DroughtStress2 AgePlantHarvest.median AgePlantHarvest.mean AgePlantHarvest.sd
1             No                    32             32.22137           3.18238
2       Possible                    32             33.31481           3.55986
> summaryBy(AgePlantHarvest~EndoGroup,data=SeedR,FUN=list(median,mean,sd))
  EndoGroup AgePlantHarvest.median AgePlantHarvest.mean AgePlantHarvest.sd
1       AR1                    32             32.31111           3.013773
2      AR37                    34             33.34146           3.252766
3        CT                    33             32.16667           3.900698
4   FalseE-                    33             31.86667           3.522715
5       NIL                    31             32.61905           3.011785
> summaryBy(AgePlantHarvest~AphPres2,data=SeedR,FUN=list(median,mean,sd))
  AphPres2 AgePlantHarvest.median AgePlantHarvest.mean AgePlantHarvest.sd
1       No                    32             32.53982           3.259698
2      Yes                    32             32.54167           3.447545
```

```
> # TILLERS NUMBERS STATISTICS ----------------------------------
> summaryBy(TillerNb~AgePlantHarvest2,data=SeedR,FUN=list(median,mean,sd))
   AgePlantHarvest2 TillerNb.median TillerNb.mean TillerNb.sd
1                28               1      1.375000   0.5757792
2                29               1      1.346154   0.5615911
3                30               1      1.250000   0.4522670
4                31               1      1.285714   0.4688072
5                32               2      1.608696   0.6563764
6                33               2      1.750000   0.6831301
7                34               1      1.461538   0.5188745
8                35               2      2.400000   0.5477226
9                36               2      1.800000   0.7637626
10               37               2      2.142857   0.6900656
11               38               2      1.950000   0.6863327

> summaryBy(TillerNb~EndoGroup,data=SeedR,FUN=list(median,mean,sd))
  EndoGroup TillerNb.median TillerNb.mean TillerNb.sd
1       AR1               2      1.622222   0.6497863
2      AR37               2      1.682927   0.7562746
3        CT               1      1.571429   0.6678271
4   FalseE-               2      1.800000   0.7745967
5       NIL               1      1.452381   0.5500502
> summaryBy(TillerNb~DroughtStress2,data=SeedR,FUN=list(median,mean,sd))
  DroughtStress2 TillerNb.median TillerNb.mean TillerNb.sd
1             No               1      1.503817   0.6369887
2       Possible               2      1.833333   0.6936368
> summaryBy(TillerNb~AphPres2,data=SeedR,FUN=list(median,mean,sd))
  AphPres2 TillerNb.median TillerNb.mean TillerNb.sd
1       No               2      1.610619   0.6739228
2      Yes               1      1.583333   0.6660796
>
> # LEAVES NUMBERS STATISTICS-----------------------------------
> summaryBy(LeafNb~AgePlantHarvest2,data=SeedR,FUN=list(median,mean,sd))
   AgePlantHarvest2 LeafNb.median LeafNb.mean LeafNb.sd
1                28              4    4.458333 0.8836272
2                29              4    4.346154 1.1293293
3                30              4    4.500000 0.6741999
4                31              4    4.142857 1.2314559
5                32              5    4.913043 0.9960396
6                33              5    5.375000 1.8211718
7                34              5    5.153846 0.8006408
8                35              7    7.200000 0.4472136
9                36              6    6.240000 2.1463147
10               37              7    6.857143 1.6761634
11               38              7    6.450000 1.4680815
> summaryBy(LeafNb~DroughtStress2,data=SeedR,FUN=list(median,mean,sd))
  DroughtStress2 LeafNb.median LeafNb.mean LeafNb.sd
1             No             5    4.946565  1.405007
2       Possible             6    5.907407  1.865966
> summaryBy(LeafNb~EndoGroup,data=SeedR,FUN=list(median,mean,sd))
  EndoGroup LeafNb.median LeafNb.mean LeafNb.sd
1       AR1             5    5.044444  1.678142
2      AR37             5    5.780488  1.695762
3        CT             5    5.142857  1.555030
4   FalseE-             5    5.666667  1.718249
5       NIL             4    4.809524  1.329553
> summaryBy(LeafNb~AphPres2,data=SeedR,FUN=list(median,mean,sd))
  AphPres2 LeafNb.median LeafNb.mean LeafNb.sd
1       No             5    5.203540  1.642946
2      Yes             5    5.263889  1.565410
>
> # ROOT NUMBERS STATISTICS ----------------------------------
> summaryBy(RootsNb~AgePlantHarvest2,data=SeedR,FUN=list(median,mean,sd))
   AgePlantHarvest2 RootsNb.median RootsNb.mean RootsNb.sd
1                28            9.0     9.041667   1.706233
2                29            9.5     9.384615   2.499231
3                30           11.0    10.583333   2.020726
4                31           10.0     9.714286   2.920184
5                32           10.0    10.826087   1.722899
6                33           10.0     9.750000   2.294922
7                34           13.0    12.230769   2.278664
8                35           15.0    12.200000   6.300794
9                36           11.0    11.160000   3.847943
10               37           14.0    13.714286   2.870208
11               38           12.0    12.200000   2.440880
> summaryBy(RootsNb~DroughtStress2,data=SeedR,FUN=list(median,mean,sd))
  DroughtStress2 RootsNb.median RootsNb.mean RootsNb.sd
1             No            10     10.35878   2.877148
2       Possible            11     11.31481   2.899734

> summaryBy(RootsNb~EndoGroup,data=SeedR,FUN=list(median,mean,sd))
  EndoGroup RootsNb.median RootsNb.mean RootsNb.sd
1       AR1            10     10.00000   2.961879
2      AR37            12     11.51220   3.627134
3        CT            10     10.42857   2.253916
4   FalseE-            10     10.66667   2.894987
5       NIL            11     10.66667   2.553493
> summaryBy(RootsNb~AphPres2,data=SeedR,FUN=list(median,mean,sd))
  AphPres2 RootsNb.median RootsNb.mean RootsNb.sd
1       No           11.0     10.61947   3.306699
2      Yes           10.5     10.66667   2.162419
```

A11.1.3. Dry matter content

```
> ###### ---------------------------------------------------------------------------------------
> # SHOOT DRY MATTER [%]
> summary(ShootDMPerc10.lme<-lme(fixed=LeafDM_NoSmall~AgePlantHarvest,random=~1|TraySubGroups,dat
a=SeedR[c(-16,-31),],method=c("REML"),na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: SeedR[c(-16, -31), ]
       AIC       BIC   logLik
  -1147.385 -1134.818 577.6923

Random effects:
 Formula: ~1 | TraySubGroups
        (Intercept)    Residual
StdDev: 0.003038587 0.007626034

Fixed effects: LeafDM_NoSmall ~ AgePlantHarvest
                    Value   Std.Error  DF  t-value p-value
(Intercept)     0.06881434 0.009185346 153 7.491752       0
AgePlantHarvest 0.00140178 0.000279221 153 5.020326       0
 Correlation:
                (Intr)
AgePlantHarvest -0.995

Standardized Within-Group Residuals:
       Min          Q1         Med          Q3         Max
-2.47022625 -0.60129629  0.01533887  0.65066194  2.79858516

Number of Observations: 173
Number of Groups: 19
> r.squaredGLMM(ShootDMPerc10.lme)
      R2m       R2c
0.2460580 0.3493554
> shapiro.test(ShootDMPerc10.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  ShootDMPerc10.lme$residuals[, 1]
W = 0.99163, p-value = 0.4132

> shapiro.test(ShootDMPerc10.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  ShootDMPerc10.lme$residuals[, 2]
W = 0.9955, p-value = 0.8844

> residplot(ShootDMPerc10.lme)
Error in data.frame(x = tmp$x, y = tmp$y, lab = rownames(mf)) :
  arguments imply differing number of rows: 173, 183
> ShootDMPerc11.lme<-lme(fixed=LeafDM_NoSmall~1,random=~1|TraySubGroups,data=SeedR[c(-16,-31),],m
ethod=c("REML"),na.action=na.omit)
> anova(ShootDMPerc11.lmeM<-update(ShootDMPerc11.lme,method="ML"),ShootDMPerc10.lmeM<-update(Shoo
tDMPerc10.lme,method="ML"))
                                                          Model df
ShootDMPerc11.lmeM <- update(ShootDMPerc11.lme, method = "ML")     1  3
ShootDMPerc10.lmeM <- update(ShootDMPerc10.lme, method = "ML")     2  4
                                                             AIC
ShootDMPerc11.lmeM <- update(ShootDMPerc11.lme, method = "ML") -1158.864
ShootDMPerc10.lmeM <- update(ShootDMPerc10.lme, method = "ML") -1174.145
                                                             BIC
ShootDMPerc11.lmeM <- update(ShootDMPerc11.lme, method = "ML") -1149.405
ShootDMPerc10.lmeM <- update(ShootDMPerc10.lme, method = "ML") -1161.532
                                                           logLik   Test
ShootDMPerc11.lmeM <- update(ShootDMPerc11.lme, method = "ML") 582.4323
ShootDMPerc10.lmeM <- update(ShootDMPerc10.lme, method = "ML") 591.0725 1 vs 2
                                                            L.Ratio p-value
ShootDMPerc11.lmeM <- update(ShootDMPerc11.lme, method = "ML")
ShootDMPerc10.lmeM <- update(ShootDMPerc10.lme, method = "ML") 17.28039  <.0001
> ShootDMPerc10.gls<-gls(LeafDM_NoSmall~AgePlantHarvest,data=SeedR[c(-16,-31),],na.action=na.omit
)
  anova(ShootDMPerc10.lme,ShootDMPerc10.gls)
                   Model df       AIC       BIC   logLik   Test  L.Ratio p-value
ShootDMPerc10.lme     1  4 -1147.384 -1134.818 577.6923
ShootDMPerc10.gls     2  3 -1141.635 -1132.210 573.8176 1 vs 2 7.749359  0.0054
```

```
> ###### ------------------------------------------------------------------------------------------
> ## SEED: ROOT DRY MATTER [%]
> # Initial model: SQRTRootDMPercB.aov<-aov(SQRTRootDM_NoSmall~AgePlantHarvest+Leaf_dBM_Corr+Drou
ghtStress2+EndoGroup+AphAd.Av+AgePlantHarvest:EndoGroup+AgePlantHarvest:AphAd.Av+EndoGroup:AphAd.
Av+AgePlantHarvest:EndoGroup:AphAd.Av+Leaf_dBM_Corr:AgePlantHarvest+Leaf_dBM_Corr:AphAd.Av,data=S
eedR)
> summary(SQRTRootDMPercB2.aov<-aov(SQRTRootDM_NoSmall~Leaf_dBM_Corr+DroughtStress2+AphAd.Av,data
=SeedR))
               Df  Sum Sq Mean Sq F value   Pr(>F)
Leaf_dBM_Corr   1 0.04825 0.04825  48.980 5.62e-11 ***
DroughtStress2  1 0.00700 0.00700   7.108  0.00841 **
AphAd.Av        1 0.00865 0.00865   8.779  0.00348 **
Residuals     171 0.16845 0.00099
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
10 observations deleted due to missingness
> summary.lm(SQRTRootDMPercB2.aov)

Call:
aov(formula = SQRTRootDM_NoSmall ~ Leaf_dBM_Corr + DroughtStress2 +
    AphAd.Av, data = SeedR)

Residuals:
      Min        1Q    Median        3Q       Max
-0.077466 -0.018194 -0.000335  0.018885  0.091192

Coefficients:
                       Estimate Std. Error t value Pr(>|t|)
(Intercept)           0.3277533  0.0063341  51.744  < 2e-16 ***
Leaf_dBM_Corr         0.9180848  0.1607494   5.711 4.87e-08 ***
DroughtStress2Possible 0.0122373  0.0054698   2.237  0.02656 *
AphAd.Av              0.0004907  0.0001656   2.963  0.00348 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.03139 on 171 degrees of freedom
  (10 observations deleted due to missingness)
Multiple R-squared:  0.275,     Adjusted R-squared:  0.2623
F-statistic: 21.62 on 3 and 171 DF,  p-value: 6.378e-12

> shapiro.test(residuals(SQRTRootDMPercB2.aov))

        Shapiro-Wilk normality test

data:  residuals(SQRTRootDMPercB2.aov)
W = 0.98882, p-value = 0.1827
```

A11.1.4. Biomasses

```
> ###### ------------------------------------------------------------------------------------
> # SEED TRIAL: SHOOT BIOMASS (mg)
> # Initial: ShootBMdN.lme<-lme(fixed=ShootMG~AgePlantHarvest+EndoGroup2+AphAd.Av+DroughtStress2+
AgePlantHarvest:EndoGroup+AgePlantHarvest:AphAd.Av+EndoGroup:AphAd.Av+AgePlantHarvest:EndoGroup:A
phAd.Av,random=~1|AgarTray/TrayGroups/TraySubGroups,data=SeedR[c(-12,-164,-169),],method=c("REML"
),na.action=na.omit)
> summary(ShootBMdNb38.lme<-lme(fixed=ShootMG~AgePlantHarvest+DroughtStress2,random=~1|TraySubGro
ups,data=SeedR[c(-12,-164,-169),],method=c("REML"),na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: SeedR[c(-12, -164, -169), ]
      AIC      BIC    logLik
  1435.301 1451.238 -712.6504

Random effects:
 Formula: ~1 | TraySubGroups
        (Intercept) Residual
StdDev:    3.612618 12.05226

Fixed effects: ShootMG ~ AgePlantHarvest + DroughtStress2
                       Value Std.Error  DF   t-value p-value
(Intercept)          -62.21438 12.464722 161 -4.991237  0.0000
AgePlantHarvest        2.98069  0.381716 161  7.808671  0.0000
DroughtStress2Possible  7.33521  2.166820 161  3.385241  0.0009
 Correlation:
                      (Intr) AgPlnH
AgePlantHarvest       -0.993
DroughtStress2Possible  0.067 -0.120

Standardized Within-Group Residuals:
         Min           Q1          Med           Q3          Max
-2.972659055 -0.568270459  0.007954901  0.565565012  3.199932301

Number of Observations: 182
Number of Groups: 19
> r.squaredGLMM(ShootBMdNb38.lme)
      R2m       R2c
0.4300230 0.4770123
> shapiro.test(ShootBMdNb38.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  ShootBMdNb38.lme$residuals[, 1]
W = 0.98981, p-value = 0.2201


> shapiro.test(ShootBMdNb38.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  ShootBMdNb38.lme$residuals[, 2]
W = 0.99137, p-value = 0.3481

> ShootBMdNb38a.lme<-lme(fixed=ShootMG~DroughtStress2,random=~1|TraySubGroups,data=SeedR[c(-12,-1
64,-169),],method=c("REML"),na.action=na.omit)
> anova(ShootBMdNb38.lmeM<-update(ShootBMdNb38.lme,method="ML"),ShootBMdNb38a.lmeM<-update(ShootB
MdNb38a.lme,method="ML"))
                                                      Model df
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")      1  5
ShootBMdNb38a.lmeM <- update(ShootBMdNb38a.lme, method = "ML")    2  4
                                                       AIC
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")   1440.719
ShootBMdNb38a.lmeM <- update(ShootBMdNb38a.lme, method = "ML") 1466.572
                                                       BIC
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")   1456.739
ShootBMdNb38a.lmeM <- update(ShootBMdNb38a.lme, method = "ML") 1479.388
                                                      logLik   Test
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")   -715.3597
ShootBMdNb38a.lmeM <- update(ShootBMdNb38a.lme, method = "ML") -729.2861 1 vs 2
                                                     L.Ratio p-value
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")
ShootBMdNb38a.lmeM <- update(ShootBMdNb38a.lme, method = "ML") 27.85282  <.0001

> ShootBMdNb38b.lme<-lme(fixed=ShootMG~AgePlantHarvest,random=~1|TraySubGroups,data=SeedR[c(-12,-
164,-169),],method=c("REML"),na.action=na.omit)
> anova(ShootBMdNb38.lmeM<-update(ShootBMdNb38.lme,method="ML"),ShootBMdNb38b.lmeM<-update(ShootB
MdNb38b.lme,method="ML"))
                                                      Model df
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")      1  5
ShootBMdNb38b.lmeM <- update(ShootBMdNb38b.lme, method = "ML")    2  4
                                                       AIC
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")   1440.719
ShootBMdNb38b.lmeM <- update(ShootBMdNb38b.lme, method = "ML") 1448.624
                                                       BIC
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")   1456.739
ShootBMdNb38b.lmeM <- update(ShootBMdNb38b.lme, method = "ML") 1461.440
                                                      logLik   Test
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")   -715.3597
ShootBMdNb38b.lmeM <- update(ShootBMdNb38b.lme, method = "ML") -720.3121 1 vs 2
                                                     L.Ratio p-value
ShootBMdNb38.lmeM <- update(ShootBMdNb38.lme, method = "ML")
ShootBMdNb38b.lmeM <- update(ShootBMdNb38b.lme, method = "ML") 9.904812  0.0016
> ShootBMdNb38.gls<-gls(ShootMG~AgePlantHarvest+DroughtStress2,data=SeedR[c(-12,-164,-169),],na.a
ction=na.omit)
> anova(ShootBMdNb38.lme,ShootBMdNb38.gls)
               Model df      AIC      BIC    logLik   Test  L.Ratio p-value
ShootBMdNb38.lme     1  5 1435.301 1451.238 -712.6504
ShootBMdNb38.gls     2  4 1436.687 1449.436 -714.3433 1 vs 2 3.385766  0.0658
>
```

```
> ###### ------------------------------------------------------------------------------------
> # ROOT BM
> # Initial: RootBML.lme<-lme(fixed=RootMG~AgePlantHarvest+ShootMG+EndoGroup2+AphAd.Av+DroughtStr
ess2+AgePlantHarvest:ShootMG+AgePlantHarvest:EndoGroup2+AgePlantHarvest:AphAd.Av+EndoGroup2:AphAd
.Av+ShootMG:EndoGroup2+AgePlantHarvest:EndoGroup2:AphAd.Av,random=~1|TraySubGroups,data=SeedR[-19
,],method=c("REML"),na.action=na.omit)
> summary(RootBML6.lme<-lme(fixed=RootMG~AgePlantHarvest+ShootMG+EndoGroup2+AphAd.Av+DroughtStres
s2+AgePlantHarvest:ShootMG+ShootMG:EndoGroup2,random=~1|TraySubGroups,data=SeedR[-19,],method=c("
REML"),na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: SeedR[-19, ]
       AIC      BIC    logLik
  790.0849 840.2576 -379.0424

Random effects:
 Formula: ~1 | TraySubGroups
        (Intercept) Residual
StdDev:   0.4337041 1.661898

Fixed effects: RootMG ~ AgePlantHarvest + ShootMG + EndoGroup2 + AphAd.Av +      DroughtStress2 +
 AgePlantHarvest:ShootMG + ShootMG:EndoGroup2
                              Value Std.Error  DF   t-value p-value
(Intercept)                3.528235  3.846866 152  0.917171  0.3605
AgePlantHarvest           -0.087134  0.115993 152 -0.751202  0.4537
ShootMG                    0.008142  0.100654 152  0.080892  0.9356
EndoGroup2AR1              0.187482  1.007037 152  0.186172  0.8526
EndoGroup2AR37             0.302922  1.083154 152  0.279666  0.7801
EndoGroup2CT              -0.934019  1.038340 152 -0.899531  0.3698
EndoGroup2FalseE-         -0.954737  1.412308 152 -0.676011  0.5001
AphAd.Av                  -0.046148  0.009170 152 -5.032462  0.0000
DroughtStress2Possible     0.766834  0.332131 152  2.308829  0.0223
AgePlantHarvest:ShootMG    0.006175  0.002896 152  2.132393  0.0346
ShootMG:EndoGroup2AR1     -0.000084  0.025080 152 -0.003364  0.9973
ShootMG:EndoGroup2AR37    -0.003000  0.025509 152 -0.117606  0.9065
ShootMG:EndoGroup2CT       0.061675  0.026218 152  2.352428  0.0199
ShootMG:EndoGroup2FalseE-  0.049192  0.033175 152  1.482817  0.1402
 Correlation:
                           (Intr) AgPlnH ShotMG EG2AR1 EG2AR3 EnG2CT EG2FE-
AgePlantHarvest            -0.979
ShootMG                    -0.872  0.828
EndoGroup2AR1              -0.119 -0.036  0.120
EndoGroup2AR37             -0.066 -0.085  0.096  0.558
EndoGroup2CT               -0.166  0.017  0.117  0.580  0.551

EndoGroup2FalseE-          -0.155  0.052  0.102  0.441  0.395  0.461
AphAd.Av                   -0.004 -0.024  0.075 -0.035  0.104  0.082 -0.035
DroughtStress2Possible     -0.178  0.127  0.136  0.251  0.222  0.270  0.181
AgePlantHarvest:ShootMG     0.879 -0.870 -0.981  0.008  0.027  0.002 -0.017
ShootMG:EndoGroup2AR1       0.100  0.041 -0.135 -0.881 -0.507 -0.525 -0.394
ShootMG:EndoGroup2AR37      0.075  0.066 -0.134 -0.522 -0.889 -0.510 -0.368
ShootMG:EndoGroup2CT        0.110  0.025 -0.098 -0.528 -0.502 -0.892 -0.408
ShootMG:EndoGroup2FalseE-   0.128 -0.028 -0.115 -0.411 -0.361 -0.423 -0.912
                           AphA.A DrgS2P APH:SM SMG:EG2AR1 SMG:EG2AR3 SMG:EG2C
AgePlantHarvest
ShootMG
EndoGroup2AR1
EndoGroup2AR37
EndoGroup2CT
EndoGroup2FalseE-
AphAd.Av
DroughtStress2Possible     -0.111
AgePlantHarvest:ShootMG    -0.066 -0.127
ShootMG:EndoGroup2AR1       0.028 -0.202 -0.013
ShootMG:EndoGroup2AR37     -0.080 -0.141 -0.013  0.593
ShootMG:EndoGroup2CT       -0.070 -0.244 -0.040  0.592       0.576
ShootMG:EndoGroup2FalseE-   0.034 -0.127  0.009  0.462       0.437       0.470

Standardized Within-Group Residuals:
         Min           Q1          Med           Q3          Max
-2.549453698 -0.569109286 -0.004335905  0.547919473  2.612503804

Number of Observations: 184
Number of Groups: 19
> r.squaredGLMM(RootBML6.lme)
      R2m       R2c
0.8681775 0.8765828
> shapiro.test(RootBML6.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  RootBML6.lme$residuals[, 1]
W = 0.99176, p-value = 0.3798

> shapiro.test(RootBML6.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  RootBML6.lme$residuals[, 2]
W = 0.98804, p-value = 0.1228
```

```
data:  RootBML6.lme$residuals[, 1]
W = 0.99176, p-value = 0.3798

> shapiro.test(RootBML6.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  RootBML6.lme$residuals[, 2]
W = 0.98804, p-value = 0.1228

> RootBML6a.lme<-lme(fixed=RootMG~AgePlantHarvest+ShootMG+EndoGroup2+AphAd.Av+DroughtStress2+AgeP
lantHarvest:ShootMG,random=~1|TraySubGroups,data=SeedR[-19,],method=c("REML"),na.action=na.omit)
> anova(RootBML6a.lmeM<-update(RootBML6a.lme,method="ML"),RootBML6.lmeM<-update(RootBML6.lme,meth
od="ML"))
                                                    Model df      AIC
RootBML6a.lmeM <- update(RootBML6a.lme, method = "ML")     1 12 738.9493
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")       2 16 734.0828
                                                     BIC    logLik
RootBML6a.lmeM <- update(RootBML6a.lme, method = "ML") 777.5285 -357.4747
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   785.5218 -351.0414
                                                       Test L.Ratio p-value
RootBML6a.lmeM <- update(RootBML6a.lme, method = "ML")
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   1 vs 2 12.8665  0.0119
> RootBML6b.lme<-lme(fixed=RootMG~AgePlantHarvest+ShootMG+EndoGroup2+AphAd.Av+DroughtStress2+Shoo
tMG:EndoGroup2,random=~1|TraySubGroups,data=SeedR[-19,],method=c("REML"),na.action=na.omit)
> anova(RootBML6b.lmeM<-update(RootBML6b.lme,method="ML"),RootBML6.lmeM<-update(RootBML6.lme,meth
od="ML"))
                                                    Model df      AIC
RootBML6b.lmeM <- update(RootBML6b.lme, method = "ML")     1 15 736.6882
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")       2 16 734.0828
                                                     BIC    logLik
RootBML6b.lmeM <- update(RootBML6b.lme, method = "ML") 784.9122 -353.3441
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   785.5218 -351.0414
                                                       Test  L.Ratio p-value

RootBML6b.lmeM <- update(RootBML6b.lme, method = "ML")
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   1 vs 2 4.605386  0.0319
> RootBML6c.lme<-lme(fixed=RootMG~AgePlantHarvest+ShootMG+EndoGroup2+AphAd.Av+AgePlantHarvest:Sho
otMG+ShootMG:EndoGroup2,random=~1|TraySubGroups,data=SeedR[-19,],method=c("REML"),na.action=na.om
it)
> anova(RootBML6c.lmeM<-update(RootBML6c.lme,method="ML"),RootBML6.lmeM<-update(RootBML6.lme,meth
od="ML"))
                                                    Model df      AIC
RootBML6c.lmeM <- update(RootBML6c.lme, method = "ML")     1 15 736.1231
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")       2 16 734.0828
                                                     BIC    logLik
RootBML6c.lmeM <- update(RootBML6c.lme, method = "ML") 784.3472 -353.0616
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   785.5218 -351.0414
                                                       Test  L.Ratio p-value
RootBML6c.lmeM <- update(RootBML6c.lme, method = "ML")
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   1 vs 2 4.040322  0.0444
> RootBML6d.lme<-lme(fixed=RootMG~AgePlantHarvest+ShootMG+EndoGroup2+DroughtStress2+AgePlantHarve
st:ShootMG+ShootMG:EndoGroup2,random=~1|TraySubGroups,data=SeedR[-19,],method=c("REML"),na.action
=na.omit)
> anova(RootBML6d.lmeM<-update(RootBML6d.lme,method="ML"),RootBML6.lmeM<-update(RootBML6.lme,meth
od="ML"))
                                                    Model df      AIC
RootBML6d.lmeM <- update(RootBML6d.lme, method = "ML")     1 15 756.7426
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")       2 16 734.0828
                                                     BIC    logLik
RootBML6d.lmeM <- update(RootBML6d.lme, method = "ML") 804.9667 -363.3713
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   785.5218 -351.0414
                                                       Test  L.Ratio p-value
RootBML6d.lmeM <- update(RootBML6d.lme, method = "ML")
RootBML6.lmeM <- update(RootBML6.lme, method = "ML")   1 vs 2 24.65985  <.0001
> RootBML6.gls<-gls(RootMG~AgePlantHarvest+ShootMG+EndoGroup2+AphAd.Av+DroughtStress2+AgePlantHar
vest:ShootMG+ShootMG:EndoGroup2,data=SeedR[-19,],na.action=na.omit)
> anova(RootBML6.gls,RootBML6.lme)
            Model df      AIC      BIC    logLik   Test L.Ratio p-value
RootBML6.gls     1 15 789.2876 836.3246 -379.6438
RootBML6.lme     2 16 790.0849 840.2576 -379.0424 1 vs 2 1.20272  0.2728
```

```
> predictmeans(RootBML6.lme,"EndoGroup2",adj="BH",pairwise=T,df)

 The predicted means are estimated at

AgePlantHarvest         ShootMG        AphAd.Av
        32.5217         36.2485          7.5326

$`Predicted Means`
EndoGroup2
  aaNIL     AR1    AR37      CT FalseE-
 8.3052  8.4896  8.4994  9.6068  9.1336

$`Standard Error of Means`
EndoGroup2
  aaNIL     AR1    AR37      CT FalseE-
0.34907 0.35402 0.37678 0.35186 0.49025

$`Standard Error of Differences`
  Max.SED   Min.SED  Aveg.SED
0.5841468 0.4640589 0.5110071

$LSD
 Max.LSD  Min.LSD Aveg.LSD
 1.15410  0.91684  1.00959
attr(,"Significant level")
[1] 0.05
attr(,"Degree of freedom")
EndoGroup2
       152

$`Pairwise p-value`
         aaNIL     AR1    AR37      CT FalseE-
aaNIL   0.0000 -0.3870 -0.3896 -2.7663 -1.4181
AR1     0.7770  0.0000 -0.0202 -2.4074 -1.1569

AR37    0.7770  0.9839  0.0000 -2.3015 -1.1355
CT      0.0637  0.0757  0.0757  0.0000  0.8806
FalseE- 0.3955  0.4299  0.4299  0.5428  0.0000
attr(,"Degree of freedom")
EndoGroup2
       152
attr(,"Note")
[1] "The matrix has t-value above the diagonal, p-value (adjusted by 'BH' method) below the diago
nal"
attr(,"Letter-based representation of pairwise comparisons at significant level '0.05'")
        Group
aaNIL       A
AR1         A
AR37        A
CT          A
FalseE-     A
```

A11.2. Mature plant experiment, statistics

A11.2.1. Aphid loadings during the Mature plant experiment

Aphids per unit biomass or green shoot area (GSA) were calculated dividing the number of long-term feeding aphids (LFA, i.e. adults and older immatures) collected at aphid harvest on a plant by the approximate biomass or GSA at that time point, as estimated by equations A11.2.1, A11.2.2 and A11.2.3:

Growth rate of plant *i*: $$Gi = \frac{(\text{Parameter}_{\text{Tx}}\ i - \text{Parameter}_{\text{T1}}\ i)}{(\text{AgePlant}_{\text{Tx}}\ i - \text{AgePlant}_{\text{T1}}\ i)}$$ **Equation A11.2.1**

Correction factor for plant *i:* $$CFi = Gi \cdot (\text{AgePlant}_{\text{AphidCollection}}\ i - \text{AgePlant}_{\text{Tx}}\ i)$$ **Equation A11.2.2**

Time-adjusted parameter: $$\text{Parameter}_{\text{Adj.}}i = \text{Parameter}_{\text{Tx}}\ i + CFi$$ **Equation A11.2.3**

The specifics for each type of aphid loading calculation are given in Table A11.2.1.

Table A11.2.1. Specifics for aphid loading calculations

	LFA/GSA [aphids/cm^2]	**LFA/Green shoot DM [aphids/g]**	**LFA/Root DM [aphids/g]**
T_1	24h after trim (approx. day 1)	Day of trim (Day 1)	Day of trim (Day 1)
T_x	2nd growth assessment	Final harvest	Final harvest
AgePlant $_{T1}$	1	1	1
AgePlant $_{Tx}$	Age of plant at 2nd growth assessment	Age of plant at final harvest	Age of plant at final harvest
Parameter $_{T1}$	Green shoot area at beginning (24 h after trim), as measured on pictures	Initial tiller dry weight (full trimmed tiller)	0
Parameter $_{Tx}$	Green shoot area at time x, as measured on pictures	Green shoot biomass at final harvest	Root biomass at final harvest
Note	Because of some missing precondition pictures, that approach was preferred		The dry root part of the trimmed tiller was too small, to be weighed in the appropriate way

DM: dry matter; GSA: green shoot area; LFA: long-term feeding aphids, i.e. adult and older immatures found at harvest on a plant. T1: first time point; Tx: later time point.

Table A11.2.2. Total number of dead and live aphids at final harvest (98 to 108 days after initial plant trim, i.e. 63 to 79 days after aphid placement; means ± standard deviation) and estimated plant loadings on two distinct perennial ryegrass plant genotypes (N, S) living in symbiosis with one of three endophyte *E. festucae* var. *lolii* strains (AR1, AR37 or common-toxic CT) or without endophyte (NIL).

Plant genotype-endophyte status	n	Colonisation success [%]	LFA /plant	1st instars/plant	LFA/GSA [aphids/cm^2]	LFA/Shoot BM [aphids/g DM]	LFA/Green shoot BM [aphids/g DM]	LFA/Root BM [aphids/g DM]
N-AR1	7	37.1 ± 18.0	50 ± 37	33 ± 32	1.6 ± 1.2	107 ± 76	144 ± 100	385 ± 258
N-AR37	5	28.0 ± 13.0	31 ± 23	15 ± 7	1.2 ± 0.9	72 ± 52	102 ± 69	352 ± 246
N-CT	7	51.4 ± 21.2	45 ± 48	49 ± 41	1.7 ± 2.0	99 ± 108	124 ± 131	323 ± 410
N-NIL	4	57.5 ± 25.0	55 ± 68	40 ± 43	2.5 ± 3.0	133 ± 156	186 ± 214	534 ± 574
S-AR1	7	58.6 ± 34.4	76 ± 89	70 ± 59	2.6 ± 2.9	235 ± 306	335 ± 437	561 ± 612
S-AR37	2	15.0 ± 7.1	20 ± 27	10 ± 6	0.8 ± 1.0	47 ± 63	65 ± 88	92 ± 121
S-CT	6	21.7 ± 14.7	7 ± 6	7 ± 9	0.3 ± 0.2	23 ± 16	32 ± 23	55 ± 52
S-NIL	6	35.0 ± 30.2	33 ± 39	32 ± 45	1.1 ± 1.3	93 ± 109	130 ± 153	203 ± 225
Overall range		**[0 - 100]**	**[1 - 230]**	**[0 - 128]**	**[0.2-7.3]**	**[2 - 849]**	**[3 - 1215]**	**[4 - 1576]**
Mean ± SD	**44**	**40 ± 26**	**42 ± 51**	**35 ± 41**	**1.5 ± 1.9**	**108 ± 152**	**150 ± 213**	**330 ± 386**

Only the plants colonised by aphids were included in the calculation of the means; BM: dry biomass weight (estimated from the weights of plants initially on day 1, and at final harvest 112-121 days after initial trim, presuming an approximately linear growth in that time); DM: dry matter; GSA: Green shoot area; LFA: long-term feeding aphids, i.e. adults and older instars, as opposed to early first instar; n: number of Petri Dishes (i.e. plants) colonised

A11.2.2. Tillers, green leaves, dead leaves and root count at final harvest

```
> ###### ------------------------------------------------------------------------------------
> ## OUT: TILLERS, GREEN LEAF AND DEAD LEAF COUNTS
> summaryBy(After_Tillers~AgePlant_After,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  AgePlant_After After_Tillers.median After_Tillers.mean After_Tillers.sd
1             79                  3.0           3.000000                NA
2             80                  3.0           3.000000         0.8991722
3             81                  3.0           3.208333         0.9215697
4             82                  3.5           3.437500         0.8139410
5             84                  3.0           3.000000                NA
> summaryBy(After_Tillers~DroughtStressAfter,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  DroughtStressAfter After_Tillers.median After_Tillers.mean After_Tillers.sd
1                 No                    3           3.034483         0.9332149
2                Yes                    3           3.518519         0.6427328
> summaryBy(After_Tillers~PlantGenotype,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  PlantGenotype After_Tillers.median After_Tillers.mean After_Tillers.sd
1            aS                    4           3.603448         0.7478544
2             N                    3           2.678571         0.7887192
> summaryBy(After_Tillers~Endophyte,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  Endophyte After_Tillers.median After_Tillers.mean After_Tillers.sd
1     aaNIL                    3           2.966667         1.0661996
2       AR1                    4           3.517241         0.8289705
3      AR37                    3           2.888889         0.8918558
4        CT                    3           3.214286         0.6299408
> summaryBy(After_Tillers~AphidPresence2,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  AphidPresence2 After_Tillers.median After_Tillers.mean After_Tillers.sd
1             No                    3           3.140845         0.8667802
2            Yes                    3           3.162791         0.9494418
>
> summaryBy(After_GL~AgePlant_After,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  AgePlant_After After_GL.median After_GL.mean After_GL.sd
1             79               9        9.0000          NA
2             80               9        8.3125    2.001662
3             81               9        8.7500    2.365533
4             82               9        9.1250    1.857418
5             84               9        9.0000          NA
> summaryBy(After_GL~DroughtStressAfter,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  DroughtStressAfter After_GL.median After_GL.mean After_GL.sd
1                 No               8       8.344828    2.245552
2                Yes               9       9.518519    1.396985
> summaryBy(After_GL~PlantGenotype,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  PlantGenotype After_GL.median After_GL.mean After_GL.sd
1            aS               9       9.310345    1.827729
2             N               8       7.910714    2.201461
> summaryBy(After_GL~Endophyte,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  Endophyte After_GL.median After_GL.mean After_GL.sd
1     aaNIL               8       8.200000    2.187740
2       AR1              10       9.931034    1.850310
3      AR37               8       7.444444    2.342473
4        CT               9       8.857143    1.238706
> summaryBy(After_GL~AphidPresence2,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  AphidPresence2 After_GL.median After_GL.mean After_GL.sd
1             No               9       8.380282    2.193277
2            Yes               9       9.023256    1.981923
>
> summaryBy(After_SDL~AgePlant_After,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  AgePlant_After After_SDL.median After_SDL.mean After_SDL.sd
1             79               0.0        0.000000          NA
2             80               1.0        1.187500   0.8418887
3             81               1.0        1.285714   0.8660254
4             82               1.5        1.500000   0.8944272
5             84               1.0        1.000000          NA
> summaryBy(After_SDL~DroughtStressAfter,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  DroughtStressAfter After_SDL.median After_SDL.mean After_SDL.sd
1                 No                1       1.298455   0.8187331
2                Yes                1       1.148148   0.9885383
> summaryBy(After_SDL~PlantGenotype,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  PlantGenotype After_SDL.median After_SDL.mean After_SDL.sd
1            aS                2       1.7758621   0.6500500
2             N                1       0.7368421   0.7202756
> summaryBy(After_SDL~Endophyte,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  Endophyte After_SDL.median After_SDL.mean After_SDL.sd
1     aaNIL                1       1.266667   0.8683450
2       AR1                1       1.275862   0.8407714
3      AR37                1       1.444444   0.6979824
4        CT                1       1.068966   0.9975339
> summaryBy(After_SDL~AphidPresence2,data=TREdata,FUN=c(median,mean,sd),na.rm=T)
  AphidPresence2 After_SDL.median After_SDL.mean After_SDL.sd
1             No                1       1.338028   0.8773932
2            Yes                1       1.136364   0.8238003
```

```
> ###### ---------------------------------------------------------------------------------------
> ## OUT: ROOT COUNTS AT FINAL HARVEST
> # Initial model: Roots.glm<-glm(Harv_Roots~AgePlantHarvest+AphidPresence2*PlantGenotype*Endophy
te+DroughtStressEnd,data=TREdata,family=poisson())
> summary(Roots2.glm<-glm(Harv_Roots~AgePlantHarvest+PlantGenotype+Endophyte,data=TREdata[c(-3,-4
1),],family=quasipoisson()))

Call:
glm(formula = Harv_Roots ~ AgePlantHarvest + PlantGenotype +
    Endophyte, family = quasipoisson(), data = TREdata[c(-3,


    -41), ])

Deviance Residuals:
    Min       1Q   Median       3Q      Max
-2.2737  -0.9524  -0.1134   0.6519   2.8294

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)     -3.341853   0.869772  -3.842 0.000207 ***
AgePlantHarvest  0.057599   0.007448   7.733 6.11e-12 ***
PlantGenotypeN   0.357695   0.038333   9.331 1.67e-15 ***
EndophyteAR1     0.128553   0.052273   2.459 0.015525 *
EndophyteAR37   -0.045802   0.056133  -0.816 0.416330
EndophyteCT      0.059435   0.053208   1.117 0.266482
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


Number of Fisher Scoring iterations: 4

> shapiro.test(residuals(Roots2.glm))

        Shapiro-Wilk normality test

data:  residuals(Roots2.glm)
W = 0.98335, p-value = 0.1739

> rsq(Roots2.glm)
[1] 0.5995388
```

A11.2.3. Tiller diameter at start and initial tiller weight

```
> ###### ---------------------------------------------------------------------------------------
> ## TILLER DIAMETER
> # Initial model: Diam.lme<-lme(fixed=X24hDiam~PlantGenotype*Endophyte*AphidPresence2,random=~1|
PlantID,data=TREdata,method="REML",na.action=na.omit)
> summary(Diam8.lme<-lme(fixed=X24hDiam~AphidPresence2,random=~1|PlantID,data=TREdata,method="REM
L",na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: TREdata
       AIC       BIC   logLik
  -494.0092 -483.0996 251.0046

Random effects:
 Formula: ~1 | PlantID
        (Intercept)   Residual
StdDev: 0.007938768 0.02445311

Fixed effects: X24hDiam ~ AphidPresence2
                     Value   Std.Error DF  t-value p-value
(Intercept)       0.17842006 0.003616609 97 49.33352  0.0000
AphidPresence2Yes 0.00999364 0.004786561 97  2.08785  0.0394
 Correlation:
                  (Intr)
AphidPresence2Yes -0.504

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-2.2742990 -0.6001134 -0.1202597  0.5689293  2.4961761

       of Observations: 115
Number of Groups: 17

> shapiro.test(Diam8.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  Diam8.lme$residuals[, 1]
W = 0.98879, p-value = 0.4642

> shapiro.test(Diam8.lme$residuals[,2])

        Shapiro-Wilk normality test
```

```
> r.squaredGLMM(Diam8.lme)
       R2m        R2c
0.03475412 0.12678990
>
> Diam9.lme<-lme(fixed=X24hDiam~1,random=~1|PlantID,data=TREdata,method="REML",na.action=na.omit)
> anova(Diam9.lmeM<-update(Diam9.lme,method="ML"),Diam8.lmeM<-update(Diam8.lme,method="ML"))
                                      Model df       AIC       BIC
Diam9.lmeM <- update(Diam9.lme, method = "ML")     1  3 -510.3821 -502.1473
Diam8.lmeM <- update(Diam8.lme, method = "ML")     2  4 -512.6080 -501.6283
                                       logLik   Test  L.Ratio p-value
Diam9.lmeM <- update(Diam9.lme, method = "ML") 258.191
Diam8.lmeM <- update(Diam8.lme, method = "ML") 260.304 1 vs 2 4.225959  0.0398
>
> Diam9.gls<-gls(X24hDiam~AphidPresence2,data=TREdata,na.action=na.omit)
> anova(Diam8.lme,Diam9.gls)
          Model df       AIC       BIC   logLik   Test  L.Ratio p-value
Diam8.lme     1  4 -494.0092 -483.0996 251.0046
Diam9.gls     2  3 -494.2500 -486.0679 250.1250 1 vs 2 1.759179  0.1847
```

```
> ###### ------------------------------------------------------------------------------------
> ## OUT: TILLER AT START, BIOMASS
> # Initial model: BM.aov<-aov(LOGTillerDMstart~PlantGenotype*Endophyte*AphidPresence2,data=TREda
ta)
> summary.lm(BM2.aov<-aov(LOGTillerDMstart~PlantGenotype+Endophyte,data=TREdata))

Call:
aov(formula = LOGTillerDMstart ~ PlantGenotype + Endophyte, data = TREdata)

Residuals:
     Min       1Q   Median       3Q      Max
-0.59190 -0.17741 -0.00355  0.18550  0.67427

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)    -4.77711    0.05486 -87.077  < 2e-16 ***
PlantGenotypeN  0.31317    0.04993   6.273 7.17e-09 ***
EndophyteAR1   -0.24457    0.06969  -3.510 0.000652 ***
EndophyteAR37  -0.30487    0.07099  -4.295 3.79e-05 ***
EndophyteCT    -0.24593    0.06969  -3.529 0.000610 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.2676 on 110 degrees of freedom
Multiple R-squared:  0.3619,    Adjusted R-squared:  0.3387
F-statistic:  15.6 on 4 and 110 DF,  p-value: 3.886e-10
```

A11.2.4. Dry matter content of shoot

```
> ###### ------------------------------------------------------------------------------------
> ##  OUT: DRY MATTER AT START
> # Initial model: PercGreen.lme<-lme(fixed=PercGreen~PlantGenotype*Endophyte3,random=~1|PlantID,
data=TREgreenPrec,method="REML")
> summary(PercGreen3.lme<-lme(fixed=PercGreen~PlantGenotype,random=~1|PlantID,data=TREgreenPrec,m
ethod="REML"))
Linear mixed-effects model fit by REML
 Data: TREgreenPrec
       AIC       BIC   logLik
  -182.9745 -176.5308 95.48726

Random effects:
 Formula: ~1 | PlantID
        (Intercept)    Residual
StdDev:   0.0147329 0.01315386

Fixed effects: PercGreen ~ PlantGenotype
                   Value   Std.Error DF   t-value p-value
(Intercept)    0.08727060 0.006209442 22 14.054501  0.0000
PlantGenotypeN 0.03934016 0.008605912 15  4.571295  0.0004
 Correlation:
               (Intr)
PlantGenotypeN -0.722

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-1.6802308 -0.5348755  0.1800962  0.5253342  1.9043629

Number of Observations: 39
Number of Groups: 17
> r.squaredGLMM(PercGreen3.lme)
      R2m       R2c
0.5042891 0.7801236
> shapiro.test(PercGreen3.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  PercGreen3.lme$residuals[, 1]
W = 0.97615, p-value = 0.5655


 > shapiro.test(PercGreen3.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  PercGreen3.lme$residuals[, 2]
W = 0.96484, p-value = 0.2576


> ###### ------------------------------------------------------------------------------------
> ## OUT: GREEN SHOOT DRY MATTER AT THE END

> # Initial: FinalGreenC.lme<-lme(fixed=GreenPerc~AgePlantHarvest*PlantGenotype*Endophyte*AphidPr
esence2+DroughtStressAfter,random=~1|PlantID,data=TREdata,method="REML",na.action=na.omit)
> summary(FinalGreen15.lme<-lme(fixed=GreenPerc~AgePlantHarvest+PlantGenotype,random=~1|PlantID,d
ata=TREdata,method="REML",na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: TREdata
       AIC       BIC   logLik
  -430.0717 -416.5241 220.0359

Random effects:
 Formula: ~1 | PlantID
        (Intercept)    Residual
StdDev:  0.01304303 0.02968477

Fixed effects: GreenPerc ~ AgePlantHarvest + PlantGenotype
                     Value  Std.Error DF   t-value p-value
(Intercept)      0.6237437 0.14392966 96  4.333670  0.0000
AgePlantHarvest -0.0035527 0.00123148 96 -2.884935  0.0048
PlantGenotypeN   0.0331322 0.00883661 15  3.749422  0.0019
 Correlation:
                (Intr) AgPlnH
AgePlantHarvest -0.999
PlantGenotypeN  -0.024 -0.007

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.050687176 -0.769630005  0.002157131  0.747846097  2.139839550

Number of Observations: 114
Number of Groups: 17
> r.squaredGLMM(FinalGreen15.lme)
      R2m       R2c
0.2594152 0.3792555
```

A11.2.5. Regrowth 24h

```
> ###### ------------------------------------------------------------------------------------
> ## OUT: 24h LEAF REGROWTH
> # Initial model:LogMaxRegr24i.lme<-lme(fixed=LOG24h_Regrowth~X24hDiam+PlantGenotype*Endophyte*A
phidPresence2,random=~1|PlantID,data=TREdata,method="REML",na.action=na.omit)
> summary(LogMaxRegr24.lme<-lme(fixed=LOG24h_Regrowth~X24hDiam+PlantGenotype*Endophyte,random=~1|
PlantID,data=TREdata,method="REML",na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: TREdata
       AIC      BIC    logLik
  88.14365 117.4415 -33.07182

Random effects:
 Formula: ~1 | PlantID
        (Intercept)  Residual
StdDev:   0.1461455 0.2932481

Fixed effects: LOG24h_Regrowth ~ X24hDiam + PlantGenotype * Endophyte
                                Value Std.Error DF   t-value p-value
(Intercept)                 -0.897995 0.2630132 97 -3.414259  0.0009
X24hDiam                     4.035392 1.1413726 97  3.535561  0.0006
PlantGenotypeN               0.246139 0.2128496  9  1.156401  0.2773
EndophyteAR1                 0.328073 0.2127468  9  1.542082  0.1574
EndophyteAR37                0.030875 0.2133594  9  0.144709  0.8881
EndophyteCT                 -0.180332 0.2016652  9 -0.894217  0.3945
PlantGenotypeN:EndophyteAR1 -0.271355 0.2861012  9 -0.948458  0.3677
PlantGenotypeN:EndophyteAR37  0.161145 0.2790046  9  0.577570  0.5777
PlantGenotypeN:EndophyteCT    0.307964 0.2774017  9  1.110175  0.2957
 Correlation:
                            (Intr) X24hDm PlntGN EndAR1 EnAR37 EndpCT PGN:EAR1
X24hDiam                    -0.780
PlantGenotypeN              -0.459 -0.032
EndophyteAR1                -0.477 -0.009  0.599
EndophyteAR37               -0.489  0.008  0.596  0.597
EndophyteCT                 -0.482 -0.036  0.632  0.632  0.629
PlantGenotypeN:EndophyteAR1  0.356  0.006 -0.743 -0.744 -0.444 -0.470
PlantGenotypeN:EndophyteAR37  0.336  0.043 -0.763 -0.457 -0.764 -0.483  0.567
PlantGenotypeN:EndophyteCT    0.365  0.008 -0.767 -0.459 -0.458 -0.726  0.570
                            PGN:EAR3
X24hDiam
PlantGenotypeN
EndophyteAR1
EndophyteAR37
EndophyteCT
PlantGenotypeN:EndophyteAR1
PlantGenotypeN:EndophyteAR37
PlantGenotypeN:EndophyteCT    0.585

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.51506824 -0.56517274 -0.01700524  0.78232486  2.15175108

Number of Observations: 115
Number of Groups: 17
> r.squaredGLMM(LogMaxRegr24.lme)
      R2m       R2c
0.3199111 0.4552188
> shapiro.test(LogMaxRegr24.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  LogMaxRegr24.lme$residuals[, 1]
W = 0.99356, p-value = 0.8749

> shapiro.test(LogMaxRegr24.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  LogMaxRegr24.lme$residuals[, 2]
W = 0.98695, p-value = 0.3342
```

A11.2.6. Green shoot area

```
> ###### ------------------------------------------------------------------------------------
> ## GREEN SHOOT AREA AT PRECONDITION (28-31 DAYS)
> # Initial model: GSAStartC.lme<-lme(fixed=All_Start_Area~X24hDiam+Start_Tiller_DMe+AgePlant_Pre
c*PlantGenotype*Endophyte+DroughtStressEnd-AgePlant_Prec:PlantGenotype:Endophyte,random=~1|PlantI
D,data=TREdata,method="REML",na.action=na.omit)
> summary(GSAStartC7.lme<-lme(fixed=All_Start_Area~Start_Tiller_DMe+AgePlant_Prec+Endophyte,rando
m=~1|PlantID,data=TREdata,method="REML",na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: TREdata
       AIC      BIC    logLik
  1216.043 1237.198 -600.0216

Random effects:
 Formula: ~1 | PlantID
        (Intercept) Residual
StdDev:    42.61852 69.07426

Fixed effects: All_Start_Area ~ Start_Tiller_DMe + AgePlant_Prec + Endophyte
                     Value Std.Error DF   t-value p-value
(Intercept)       -917.695  314.5451 91 -2.917530  0.0044
Start_Tiller_DMe 16706.698 2677.4169 91  6.239857  0.0000
AgePlant_Prec       32.090   10.3767 91  3.092536  0.0026
EndophyteAR1       118.314   39.7440 13  2.976899  0.0107
EndophyteAR37       29.061   38.6915 13  0.751094  0.4660
EndophyteCT         39.862   37.9539 13  1.050283  0.3127
 Correlation:
                  (Intr) S_T_DM AgPl_P EndAR1 EnAR37
Start_Tiller_DMe -0.112
AgePlant_Prec    -0.992  0.022
EndophyteAR1      0.023  0.181 -0.107
EndophyteAR37    -0.082  0.219 -0.006  0.581
EndophyteCT      -0.067  0.182 -0.019  0.586  0.606

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.517022471 -0.697009572  0.009303757  0.579373568  2.302376612

Number of Observations: 110
Number of Groups: 17
> r.squaredGLMM(GSAStartC7.lme)
      R2m       R2c
0.3976728 0.5637471
> ###### ------------------------------------------------------------------------------------
> ## GREEN SHOOT AREA AT 2nd GROWTH CHECK (78-84 DAYS)
> # Initial model: PSAEndDiamterAd.lme<-lme(fixed=All_End_Area~All_Start_Area+X24hDiam+AgePlant_A
fter+PlantGenotype*Endophyte*AphidPresence2,random=~1|PlantID,TREdata,na.action=na.omit,method="R
EML")
> summary(PSAEndDiamterAd10.lme<-lme(fixed=All_End_Area~X24hDiam+AgePlant_After,random=~1|PlantID
,TREdata,na.action=na.omit,method="REML"))
Linear mixed-effects model fit by REML
 Data: TREdata
       AIC      BIC    logLik
  1727.658 1741.205 -858.8289

Random effects:
 Formula: ~1 | PlantID
        (Intercept) Residual
StdDev:    314.9778 495.3267

Fixed effects: All_End_Area ~ X24hDiam + AgePlant_After
                    Value Std.Error DF   t-value p-value
(Intercept)    -6441.915  5714.964 95 -1.127201  0.2625
X24hDiam        8028.549  1944.304 95  4.129266  0.0001
AgePlant_After    87.559    70.925 95  1.234539  0.2200
 Correlation:
                (Intr) X24hDm
X24hDiam       -0.004
AgePlant_After -0.998 -0.058

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.69675169 -0.63334999  0.01055698  0.60878027  2.06381051

Number of Observations: 114
Number of Groups: 17
> r.squaredGLMM(PSAEndDiamterAd10.lme)
      R2m       R2c
0.1236915 0.3760120
> shapiro.test(PSAEndDiamterAd10.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  PSAEndDiamterAd10.lme$residuals[, 1]
W = 0.98757, p-value = 0.3807

> shapiro.test(PSAEndDiamterAd10.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  PSAEndDiamterAd10.lme$residuals[, 2]
W = 0.9922, p-value = 0.7696
```

A11.2.7. Green shoot colour

Table A11.2.7.1. Average colour measurements ± standard deviation (R: red, G: green, B: blue) during the Mature plant experiment and correlation factors (Spearman's ρ) between the various R, G and B measurements.

	nPD	Green shoot area at precondition GSA T1 (n = 109)			Green shoot area at 2nd assessment GSA T2 (n = 113)			Last extended leaf at precondition LEL T1 (n = 109)			Last extended leaf at 2nd assessment LEL T2 (n = 112)		
		R-values	G-values	B-values	R-values	G-values	B-values	R-values	G-values	B-values	R-values	G-values	B-values
N-AR1	14	46.4 ±7.4	95.7 ± 9.5	55.4 ±6.4	39.6 ±2.4	84.9 ±4.4	53.4 ±2.9	43.1 ±6.6	92.9 ± 9.6	53.2 ±5.4	33.4 ±2.8	77.8 ± 5.1	47.1 ±2.9
N-AR37	12	49.7 ±3.7	100.5 ± 5.9	57.1 ±4.8	39.4 ±2.2	84.6 ±3.5	53.3 ±2.6	49.8 ±6.6	102.1 ± 9.3	56.0 ±5.4	32.5 ±3.6	76.1 ± 6.4	47.1 ±2.3
N-CT	15	51.2 ±9.3	102.4 ±10.6	57.2 ±5.3	40.1 ±3.7	85.5 ±5.7	52.4 ±2.5	48.7 ±9.9	100.7 ±12.0	54.5 ±5.7	34.4 ±3.1	78.9 ± 5.6	46.5 ±2.4
N-NIL	15	49.1 ±7.5	99.9 ± 9.3	58.2 ±7.0	39.4 ±3.1	84.2 ±5.0	53.4 ±3.7	47.7 ±7.9	99.6 ±11.2	56.6 ±7.0	32.4 ±2.9	75.7 ± 5.3	47.6 ±3.9
S-AR1	15	44.5 ±3.0	92.5 ± 5.3	54.4 ±3.5	42.0 ±2.7	88.2 ±4.8	56.3 ±2.8	41.6 ±3.6	89.8 ± 6.6	50.9 ±3.4	37.7 ±5.2	83.9 ± 8.7	51.4 ±2.6
S-AR37	14	48.9 ±3.4	99.0 ± 5.6	57.4 ±4.4	41.0 ±3.0	85.8 ±4.3	56.3 ±2.0	47.2 ±4.6	98.0 ± 6.8	55.2 ±4.9	34.1 ±3.6	77.1 ± 5.8	50.2 ±2.4
S-CT	14	51.6 ±6.4	101.5 ± 8.8	58.6 ±5.6	42.7 ±4.6	88.2 ±7.0	56.9 ±3.8	48.8 ±7.8	99.6 ±11.3	54.5 ±6.0	37.5 ±8.9	82.3 ±13.2	51.2 ±4.7
S-NIL	15	47.8 ±4.4	95.7 ± 6.5	57.5 ±4.1	40.4 ±2.5	85.1 ±4.0	55.2 ±2.5	44.6 ±5.7	93.5 ± 8.6	54.3 ±2.7	34.6 ±4.1	78.4 ± 7.3	50.0 ±3.3
$R_{GSA\ T1}$		-	0.96 *(< 0.001)*	0.79 *(< 0.001)*	0.00 *(0.985)*	-	-	0.84 *(< 0.001)*	-	-			
$G_{GSA\ T1}$		-	-	0.80 *(< 0.001)*	-	-0.04 *(0.668)*	-	-	0.90 *(< 0.001)*	-			
$B_{GSA\ T1}$		-	-	-	-	-	0.04 *(0.679)*	-	-	0.83 *(< 0.001)*			
$R_{GSA\ T2}$					-	0.97 *(< 0.001)*	0.85 *(< 0.001)*				0.70 *(< 0.001)*	-	-
$G_{GSA\ T2}$					-	-	0.84 *(< 0.001)*				-	0.73 *(< 0.001)*	-
$B_{GSA\ T2}$					-	-	-				-	-	0.86 *(< 0.001)*
$R_{LEL\ T1}$								-	0.98 *(< 0.001)*	0.71 *(< 0.001)*	-0.11 *(0.267)*	-	-
$G_{LEL\ T1}$								-	-	0.76 *(< 0.001)*	-	-0.14 *(0.135)*	-
$B_{LEL\ T1}$								-	-	-	-	-	-0.11 *(0.251)*
$R_{LEL\ T2}$											-	0.98 *(< 0.001)*	0.74 *(< 0.001)*
$G_{LEL\ T2}$											-	-	0.74 *(< 0.001)*

Precondition (T1): at 28-31 days after trim; 2nd growth assessment (T2): at 78-84 days after trim. n: number of useable measurements; nPD: number of Petri dishes (plants)

```
> ###### ------------------------------------------------------------------------------------
> ## COLOUR
> # G RATIO (G/(R+G+B))OF FULL GREEN SHOOT AT PRECONDITION
> # Initial model:GratioStart.lme<-lme(fixed=GratioAll_Start~Start_Tiller_DMe+All_Start_Area+Plan
tGenotype*Endophyte+AphidPresence2,random=~1|PlantID,data=TREdataF,method="REML",na.action=na.omi
t)
> summary(GratioStart5.lme<-lme(fixed=GratioAll_Start~Start_Tiller_DMe+All_Start_Area,random=~1|P
lantID,data=TREdataF,method="REML",na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: TREdataF
       AIC       BIC   logLik
  -718.0473 -704.7775 364.0237

Random effects:
 Formula: ~1 | PlantID
        (Intercept)    Residual
StdDev: 0.002871425 0.006802242

Fixed effects: GratioAll_Start ~ Start_Tiller_DMe + All_Start_Area
                    Value  Std.Error DF   t-value p-value
(Intercept)      0.4676401 0.00250845 89 186.42559  0.0000
Start_Tiller_DMe 0.7381613 0.27382610 89   2.69573  0.0084
All_Start_Area   0.0000374 0.00000856 89   4.36681  0.0000
 Correlation:
                 (Intr) S_T_DM
Start_Tiller_DMe -0.550
All_Start_Area   -0.413 -0.444

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-2.61982090 -0.63454291 -0.07324101  0.51140786  2.89432560

> shapiro.test(GratioStart5.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  GratioStart5.lme$residuals[, 1]
W = 0.99319, p-value = 0.8726

> shapiro.test(GratioStart5.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  GratioStart5.lme$residuals[, 2]
W = 0.98401, p-value = 0.223

> r.squaredGLMM(GratioStart5.lme)
      R2m       R2c
0.2918279 0.3989338



> ###### ------------------------------------------------------------------------------------
> #  G RATIO (G/(R+G+B))OF FULL GREEN SHOOT AT 78-84 DAYS
> # Initial model: GratioBI.aov<-aov(GratioAll_End~GratioAll_Start+AgePlant_After+AgePlantDiffGSA
+All_End_Area+X24hDiam+Start_Tiller_DMe+PlantGenotype*Endophyte*AphidPresence2,data=TREdata)
> summary.lm(GratioB.aov)

Call:
aov(formula = GratioAll_End ~ All_End_Area + PlantGenotype *
    Endophyte, data = TREdata[-3, ])

Residuals:
      Min        1Q    Median        3Q       Max
-0.009539 -0.002140 -0.000122  0.002133  0.008090

Coefficients:
                              Estimate    Std. Error t value  Pr(>|t|)
(Intercept)                 0.4677423321  0.0014199711 329.403   < 2e-16 ***
All_End_Area                0.0000015622  0.0000005757   2.713    0.0078 **
PlantGenotypeN              0.0052393056  0.0011528307   4.545 0.0000149 ***
EndophyteAR1                0.0014176278  0.0011652020   1.217    0.2265
EndophyteAR37              -0.0019054957  0.0011734780  -1.624    0.1074
EndophyteCT                -0.0007597856  0.0011941301  -0.636    0.5260
PlantGenotypeN:EndophyteAR1 -0.0013878981  0.0016571973  -0.837    0.4042
PlantGenotypeN:EndophyteAR37  0.0029829852  0.0016924548   1.763    0.0809 .
PlantGenotypeN:EndophyteCT    0.0041950992  0.0016902377   2.482    0.0147 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.003141 on 104 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.6207,    Adjusted R-squared:  0.5916
F-statistic: 21.28 on 8 and 104 DF,  p-value: < 2.2e-16
> shapiro.test(residuals(GratioB.aov))

        Shapiro-Wilk normality test

data:  residuals(GratioB.aov)
W = 0.99172, p-value = 0.7329
```

```
> ###### ---------------------------------------------------------------------------------
> # OUT: NORMALISED RB DIFFERENCE OF LAST ELONGATED LEAF AT PRECONDITION (28-31 days)
> # Initial model: RB1AL.lme<-lme(fixed=RmBtoRpB1AL_Start~X1AL_Start_Area+X24hDiam+Start_Tiller_D
Me+AgePlant_Prec+PlantGenotype*Endophyte*AphidPresence2,random=~1|PlantID,data=TREdata[-3,],metho
d="REML",na.action=na.omit)
> summary(RB1AL3.lme<-lme(fixed=RmBtoRpB1AL_Start~X1AL_Start_Area+AgePlant_Prec+PlantGenotype*End
ophyte*AphidPresence2,random=~1|PlantID,data=TREdata[-3,],method="REML",na.action=na.omit))
Linear mixed-effects model fit by REML
 Data: TREdata[-3, ]
       AIC       BIC   logLik
  -242.168 -191.9508 141.084

Random effects:
 Formula: ~1 | PlantID
        (Intercept)   Residual
StdDev:  0.03195963 0.03732688

Fixed effects: RmBtoRpB1AL_Start ~ X1AL_Start_Area + AgePlant_Prec + PlantGenotype *      Endophy
te * AphidPresence2
                                                 Value  Std.Error DF
(Intercept)                                  0.3798613 0.18681332 82
X1AL_Start_Area                             -0.0003070 0.00007701 82
AgePlant_Prec                               -0.0147926 0.00610326 82
PlantGenotypeN                               0.0026614 0.04296879  9
EndophyteAR1                                -0.0061016 0.04378740  9
EndophyteAR37                                0.0224666 0.04399673  9
EndophyteCT                                  0.0320811 0.04137908  9
AphidPresence2Yes                           -0.0091384 0.02263934 82
PlantGenotypeN:EndophyteAR1                  0.0398411 0.05764398  9
PlantGenotypeN:EndophyteAR37                 0.0038432 0.05617448  9
PlantGenotypeN:EndophyteCT                  -0.0019925 0.05600026  9
PlantGenotypeN:AphidPresence2Yes             0.0640780 0.03192924 82
EndophyteAR1:AphidPresence2Yes               0.0168468 0.03019177 82
EndophyteAR37:AphidPresence2Yes             -0.0547293 0.03834584 82
EndophyteCT:AphidPresence2Yes                0.0369621 0.03065105 82
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes -0.0964473 0.04286818 82
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes  0.0166744 0.05013224 82
PlantGenotypeN:EndophyteCT:AphidPresence2Yes -0.0680893 0.04295442 82
                                               t-value p-value
(Intercept)                                   2.033374  0.0452
X1AL_Start_Area                              -3.985814  0.0001
AgePlant_Prec                                -2.423716  0.0176
PlantGenotypeN                                0.061938  0.9520
EndophyteAR1                                 -0.139346  0.8922
EndophyteAR37                                 0.510643  0.6219
EndophyteCT                                   0.775298  0.4580
AphidPresence2Yes                            -0.403649  0.6875
PlantGenotypeN:EndophyteAR1                   0.691158  0.5069
PlantGenotypeN:EndophyteAR37                  0.068416  0.9470
PlantGenotypeN:EndophyteCT                   -0.035581  0.9724
PlantGenotypeN:AphidPresence2Yes              2.006877  0.0481
EndophyteAR1:AphidPresence2Yes                0.557993  0.5784
EndophyteAR37:AphidPresence2Yes              -1.427255  0.1573
EndophyteCT:AphidPresence2Yes                 1.205899  0.2313
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes -2.249858  0.0271
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes  0.332607  0.7403
PlantGenotypeN:EndophyteCT:AphidPresence2Yes  -1.585153  0.1168
 Correlation:
                                              (Intr) X1AL_S AgPl_P PlntGN
X1AL_Start_Area                               -0.005
AgePlant_Prec                                 -0.982 -0.039
PlantGenotypeN                                -0.142 -0.064 -0.001
EndophyteAR1                                  -0.124 -0.069 -0.017  0.630
EndophyteAR37                                 -0.147 -0.035  0.006  0.624
EndophyteCT                                   -0.145 -0.017 -0.007  0.663
AphidPresence2Yes                             -0.132 -0.094  0.101  0.165
PlantGenotypeN:EndophyteAR1                    0.187  0.012 -0.080 -0.743
PlantGenotypeN:EndophyteAR37                   0.090  0.069  0.019 -0.766
PlantGenotypeN:EndophyteCT                     0.108 -0.060  0.007 -0.760
PlantGenotypeN:AphidPresence2Yes              -0.044 -0.002  0.071 -0.183
EndophyteAR1:AphidPresence2Yes                 0.010  0.022  0.017 -0.121
EndophyteAR37:AphidPresence2Yes                0.065  0.120 -0.049 -0.101
EndophyteCT:AphidPresence2Yes                  0.099  0.067 -0.076 -0.122
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes  0.045  0.005 -0.066  0.136
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes  0.059 -0.095 -0.072  0.123
PlantGenotypeN:EndophyteCT:AphidPresence2Yes  -0.007  0.099 -0.017  0.130
                                              EndAR1 EnAR37 EndpCT AphP2Y
X1AL_Start_Area
AgePlant_Prec
PlantGenotypeN
EndophyteAR1
EndophyteAR37                                  0.613
EndophyteCT                                    0.650  0.647
AphidPresence2Yes                              0.161  0.159  0.166
PlantGenotypeN:EndophyteAR1                   -0.755 -0.465 -0.493 -0.127
PlantGenotypeN:EndophyteAR37                  -0.483 -0.785 -0.507 -0.126
PlantGenotypeN:EndophyteCT                    -0.476 -0.475 -0.738 -0.116
PlantGenotypeN:AphidPresence2Yes              -0.112 -0.110 -0.118 -0.689
EndophyteAR1:AphidPresence2Yes                -0.259 -0.117 -0.124 -0.736
EndophyteAR37:AphidPresence2Yes               -0.100 -0.242 -0.099 -0.595
EndophyteCT:AphidPresence2Yes                 -0.118 -0.117 -0.276 -0.738
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes  0.182  0.082  0.088  0.512
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes  0.079  0.185  0.077  0.445
PlantGenotypeN:EndophyteCT:AphidPresence2Yes   0.076  0.078  0.195  0.507
```

```
                                          P1GN:EAR1 P1GN:EAR37 P1GN:ECT
X1AL_Start_Area
AgePlant_Prec
PlantGenotypeN
EndophyteAR1
EndophyteAR37
EndophyteCT
AphidPresence2Yes
PlantGenotypeN:EndophyteAR1
PlantGenotypeN:EndophyteAR37                0.567
PlantGenotypeN:EndophyteCT                  0.569     0.580
PlantGenotypeN:AphidPresence2Yes            0.131     0.142     0.141
EndophyteAR1:AphidPresence2Yes              0.194     0.093     0.090
EndophyteAR37:AphidPresence2Yes             0.075     0.194     0.065
EndophyteCT:AphidPresence2Yes               0.094     0.093     0.199
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes -0.246   -0.105    -0.105
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes -0.082  -0.259    -0.084
PlantGenotypeN:EndophyteCT:AphidPresence2Yes  -0.099   -0.098    -0.276
                                          PGN:AP EAR1:A EAR37: ECT:AP
X1AL_Start_Area
AgePlant_Prec
PlantGenotypeN
EndophyteAR1
EndophyteAR37
EndophyteCT
AphidPresence2Yes
PlantGenotypeN:EndophyteAR1
PlantGenotypeN:EndophyteAR37
PlantGenotypeN:EndophyteCT
PlantGenotypeN:AphidPresence2Yes
EndophyteAR1:AphidPresence2Yes              0.523
EndophyteAR37:AphidPresence2Yes             0.408  0.436
EndophyteCT:AphidPresence2Yes               0.509  0.544  0.439
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes -0.746 -0.705 -0.303 -0.378
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes -0.639 -0.336 -0.760 -0.328
PlantGenotypeN:EndophyteCT:AphidPresence2Yes  -0.741 -0.386 -0.293 -0.699
                                           PGN:EAR1: PGN:EAR37:
X1AL_Start_Area
AgePlant_Prec
PlantGenotypeN
EndophyteAR1
EndophyteAR37
EndophyteCT
AphidPresence2Yes
PlantGenotypeN:EndophyteAR1
PlantGenotypeN:EndophyteAR37
PlantGenotypeN:EndophyteCT
PlantGenotypeN:AphidPresence2Yes
EndophyteAR1:AphidPresence2Yes
EndophyteAR37:AphidPresence2Yes
EndophyteCT:AphidPresence2Yes
PlantGenotypeN:EndophyteAR1:AphidPresence2Yes
PlantGenotypeN:EndophyteAR37:AphidPresence2Yes  0.477
PlantGenotypeN:EndophyteCT:AphidPresence2Yes    0.552     0.463

Standardized Within-Group Residuals:
         Min           Q1          Med           Q3          Max
-2.144401425 -0.535805677  0.008061676  0.536567288  2.520926930

Number of Observations: 109
Number of Groups: 17
> r.squaredGLMM(RB1AL3.lme)
      R2m       R2c
0.2810404 0.5851585
> shapiro.test(RB1AL3.lme$residuals[,1])

        Shapiro-Wilk normality test

data:  RB1AL3.lme$residuals[, 1]
W = 0.98541, p-value = 0.2824

> shapiro.test(RB1AL3.lme$residuals[,2])

        Shapiro-Wilk normality test

data:  RB1AL3.lme$residuals[, 2]
W = 0.98949, p-value = 0.5614
```

```
> ###### ------------------------------------------------------------------------------------
> ## OUT: NORMALISED RB DIFFERENCE OF LAST ELONGATED LEAF AT 78-84 DAYS
> # Initial model: RBMLai.aov<-aov(RmB1MLEnd~X1ML_End_Area+All_End_Area+RmBtoRpB1AL_Start*X1AL_St
art_Area+All_Start_Area+X24hDiam+Start_Tiller_DMe+AgePlant_After+AgePlantDiffGSA+PlantGenotype*En
dophyte*AphidPresence2,data=TREdata[c(-3,-88),])
> summary.lm(RBMLa2.aov)

Call:
aov(formula = RmB1MLEnd ~ X1ML_End_Area + RmBtoRpB1AL_Start +
    X1AL_Start_Area + Start_Tiller_DMe + AphidPresence2 + RmBtoRpB1AL_Start:X1AL_Start_Area,
    data = TREdata[c(-3, -88), ])

Residuals:
      Min        1Q    Median        3Q       Max
-0.096252 -0.027173 -0.004504  0.023174  0.088566

Coefficients:
                                  Estimate  Std. Error t value
(Intercept)                     -0.16346731  0.02295502  -7.121
X1ML_End_Area                    0.00009508  0.00003311   2.871
RmBtoRpB1AL_Start                0.58452098  0.21957854   2.662
X1AL_Start_Area                 -0.00018263  0.00015255  -1.197
Start_Tiller_DMe                -3.79453006  1.40759792  -2.696
AphidPresence2Yes               -0.01559035  0.00783244  -1.990
RmBtoRpB1AL_Start:X1AL_Start_Area -0.00424503  0.00162002  -2.620
                                      Pr(>|t|)
(Intercept)                     0.000000000173 ***
X1ML_End_Area                         0.00500 **
RmBtoRpB1AL_Start                     0.00907 **
X1AL_Start_Area                       0.23408
Start_Tiller_DMe                      0.00825 **
AphidPresence2Yes                     0.04929 *
RmBtoRpB1AL_Start:X1AL_Start_Area     0.01017 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.03869 on 99 degrees of freedom
  (7 observations deleted due to missingness)
Multiple R-squared:  0.2012,    Adjusted R-squared:  0.1528
F-statistic: 4.156 on 6 and 99 DF,  p-value: 0.0009113

> shapiro.test(residuals(RBMLa2.aov))

        Shapiro-Wilk normality test

data:  residuals(RBMLa2.aov)
W = 0.98817, p-value = 0.478



> # Assumption
> shapiro.test(TREdata[c(-3,-88),]$RmB1MLEnd)

        Shapiro-Wilk normality test

data:  TREdata[c(-3, -88), ]$RmB1MLEnd
W = 0.98687, p-value = 0.3536

> leveneTest(TREdata[c(-3,-88),]$RmB1MLEnd~TREdata[c(-3,-88),]$PGxE)
Levene's Test for Homogeneity of Variance (center = median)
       Df F value Pr(>F)
group   7  1.2956 0.2599
      103
> leveneTest(TREdata[c(-3,-88),]$RmB1MLEnd~TREdata[c(-3,-88),]$Endophyte)
Levene's Test for Homogeneity of Variance (center = median)
       Df F value Pr(>F)
group   3  1.4912 0.2211
      107
> leveneTest(TREdata[c(-3,-88),]$RmB1MLEnd~TREdata[c(-3,-88),]$PlantGenotype)
Levene's Test for Homogeneity of Variance (center = median)
       Df F value Pr(>F)
group   1  0.0995  0.753
      109
> leveneTest(TREdata[c(-3,-88),]$RmB1MLEnd~TREdata[c(-3,-88),]$AphidPresence2)
Levene's Test for Homogeneity of Variance (center = median)
       Df F value Pr(>F)
group   1  0.6388 0.4259

      109
```

A11.2.8. Root/shoot ratio

```
> ###### ------------------------------------------------------------------------------------
> ## OUT: ROOT/GREEN SHOOT RATIO
> # Initial: RtogS.aov<-aov(LOGRootsToGreen~AgePlantHarvest+PlantGenotype+Endophyte+DroughtStress
End+AphidPresence2+AgePlantHarvest:PlantGenotype+AgePlantHarvest:Endophyte+PlantGenotype:Endophyt
e+AgePlantHarvest:AphidPresence2+PlantGenotype:AphidPresence2+Endophyte:AphidPresence2+AgePlantHa
rvest:PlantGenotype:Endophyte+AgePlantHarvest:PlantGenotype:AphidPresence2+AgePlantHarvest:Endoph
yte:AphidPresence2+PlantGenotype:Endophyte:AphidPresence2+AgePlantHarvest:PlantGenotype:Endophyte
:AphidPresence2,data=TREdata)
> summary.lm(RtogS2.aov<-aov(LOGRootsToGreen~AgePlantHarvest+PlantGenotype+Endophyte+DroughtStres
sEnd+AphidPresence2+AgePlantHarvest:Endophyte+PlantGenotype:Endophyte,data=TREdata))

Call:
```

```
aov(formula = LOGRootsToGreen ~ AgePlantHarvest + PlantGenotype +
    Endophyte + DroughtStressEnd + AphidPresence2 + AgePlantHarvest:Endophyte +
    PlantGenotype:Endophyte, data = TREdata)

Residuals:
     Min       1Q   Median       3Q      Max
-0.61089 -0.17090  0.04336  0.15872  0.56747

Coefficients:
                              Estimate Std. Error t value Pr(>|t|)
(Intercept)                  -5.01294    2.34865  -2.134   0.0353 *
AgePlantHarvest               0.04011    0.02011   1.995   0.0488 *
PlantGenotypeN               -0.68217    0.09189  -7.424 3.85e-11 ***
EndophyteAR1                 -4.19006    3.19239  -1.313   0.1924
EndophyteAR37                 3.39951    3.22799   1.053   0.2948
EndophyteCT                   2.40118    3.07794   0.780   0.4372
DroughtStressEndYes           0.10904    0.05233   2.083   0.0398 *
AphidPresence2Yes            -0.11886    0.04981  -2.386   0.0189 *
AgePlantHarvest:EndophyteAR1  0.03543    0.02738   1.294   0.1987
AgePlantHarvest:EndophyteAR37 -0.02963    0.02773  -1.068   0.2879
AgePlantHarvest:EndophyteCT   -0.02048    0.02634  -0.777   0.4388
PlantGenotypeN:EndophyteAR1    0.28970    0.13095   2.212   0.0292 *
PlantGenotypeN:EndophyteAR37  -0.08552    0.13818  -0.619   0.5374
PlantGenotypeN:EndophyteCT     0.24174    0.13430   1.800   0.0749 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.2468 on 100 degrees of freedom
  (1 observation deleted due to missingness)
Multiple R-squared:  0.6805,    Adjusted R-squared:  0.639
F-statistic: 16.38 on 13 and 100 DF,  p-value: < 2.2e-16

> shapiro.test(RtogS2.aov$residuals)

        Shapiro-Wilk normality test

data:  RtogS2.aov$residuals
W = 0.9878, p-value = 0.3969

> predictmeans(RtogS2.aov,"Endophyte",adj="BH",pairwise=T)

 The predicted means are estimated at

AgePlantHarvest
       116.4609
```

A11.2.9. Biomass analyses

Table A11.2.9.1. Plant biomass at harvest (raw means, with vs. without aphids pooled)

Plant genotype	Endo-phyte	n	Green shoot biomass [mg DM]	Dead shoot biomass [mg DM]	Root biomass [mg DM]
N	AR1	14	378 ± 64.9 [c]	134 ± 24.3 [d]	158 ± 47 [b]
N	AR37	13	375 ± 119.6 [bc]	123 ± 36.4 [acd]	119 ± 37 [a]
N	CT	15	366 ± 105.6 [bc]	111 ± 25.7 [bc]	178 ± 40 [b]
N	NIL	15	336 ± 108.4 [ab]	115 ± 39.2 [b]	127 ± 64 [a]
S	AR1	15	258 ± 39.2 [a]	118 ± 28.2 [ad]	175 ± 46 [b]
S	AR37	14	246 ± 82.3 [a]	99 ± 29.7 [abcd]	166 ± 56 [b]
S	CT	14	246 ± 51.9 [a]	103 ± 30.8 [abcd]	184 ± 73 [b]
S	NIL	15	265 ± 49.8 [a]	107 ± 28.6 [abc]	193 ± 57 [b]

Means with a same letter in a column were not significantly different by average covariate values ($p > 0.05$ in post hoc tests). n: number of plants.

```
> ###### ------------------------------------------------------------------------------------
> ## OUT: BIOMASSES, MANCOVA
> # Initial model:Biomass.maov<-manova(cbind(SQRTDry_GreenBM,Dry_S.DBM,Dry_RootsBM)~Start_Tiller_
DMe+AgePlantHarvest+PlantGenotype+Endophyte+DroughtStressEnd+AphidPresence2+AgePlantHarvest:Plant
Genotype+AgePlantHarvest:Endophyte+PlantGenotype:Endophyte+AgePlantHarvest:AphidPresence2+PlantGe
notype:AphidPresence2+Endophyte:AphidPresence2+AgePlantHarvest:PlantGenotype:Endophyte+AgePlantHa
rvest:PlantGenotype:AphidPresence2+AgePlantHarvest:Endophyte:AphidPresence2+PlantGenotype:Endophy
te:AphidPresence2+AgePlantHarvest:PlantGenotype:Endophyte:AphidPresence2,data=TREdataF)
> Biomass11.maov<-lm(cbind(SQRTDry_GreenBM,Dry_S.DBM,Dry_RootsBM)~Start_Tiller_DMe+AgePlantHarves
t+PlantGenotype+Endophyte+DroughtStressEnd+AphidPresence2+AgePlantHarvest:Endophyte+PlantGenotype
:Endophyte,data=TREdataF)
> options(contrasts = c("contr.sum", "contr.poly"))
> Manova(Biomass11.maov,type=3,test="Wilks")

Type III MANOVA Tests: Wilks test statistic
                       Df test stat approx F num Df den Df    Pr(>F)
(Intercept)             1   0.67400  15.4774      3  96.00 2.740e-08 ***
Start_Tiller_DMe        1   0.70466  13.4119      3  96.00 2.212e-07 ***
AgePlantHarvest         1   0.62526  19.1787      3  96.00 7.970e-10 ***
PlantGenotype           1   0.50362  31.5403      3  96.00 2.816e-14 ***
Endophyte               3   0.82799   2.0948      9 233.79   0.03080 *
DroughtStressEnd        1   0.95192   1.6161      3  96.00   0.19070
AphidPresence2          1   0.90942   3.1873      3  96.00   0.02721 *
AgePlantHarvest:Endophyte  3   0.82884   2.0831      9 233.79   0.03184 *
PlantGenotype:Endophyte    3   0.86125   1.6443      9 233.79   0.10370
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> Manova(Biomass11.maov,type=3,test="Pillai")

Type III MANOVA Tests: Pillai test statistic
                       Df test stat approx F num Df den Df    Pr(>F)
(Intercept)             1   0.32600  15.4774      3     96 2.740e-08 ***
Start_Tiller_DMe        1   0.29534  13.4119      3     96 2.212e-07 ***
AgePlantHarvest         1   0.37474  19.1787      3     96 7.970e-10 ***
PlantGenotype           1   0.49638  31.5403      3     96 2.816e-14 ***
Endophyte               3   0.17626   2.0391      9    294   0.03504 *
DroughtStressEnd        1   0.04808   1.6161      3     96   0.19070
AphidPresence2          1   0.09058   3.1873      3     96   0.02721 *
AgePlantHarvest:Endophyte  3   0.17541   2.0287      9    294   0.03608 *
PlantGenotype:Endophyte    3   0.14363   1.6426      9    294   0.10269
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

APPENDIX 12: Chapter 5 addendum

A12.1. Biology II experiment, behaviour

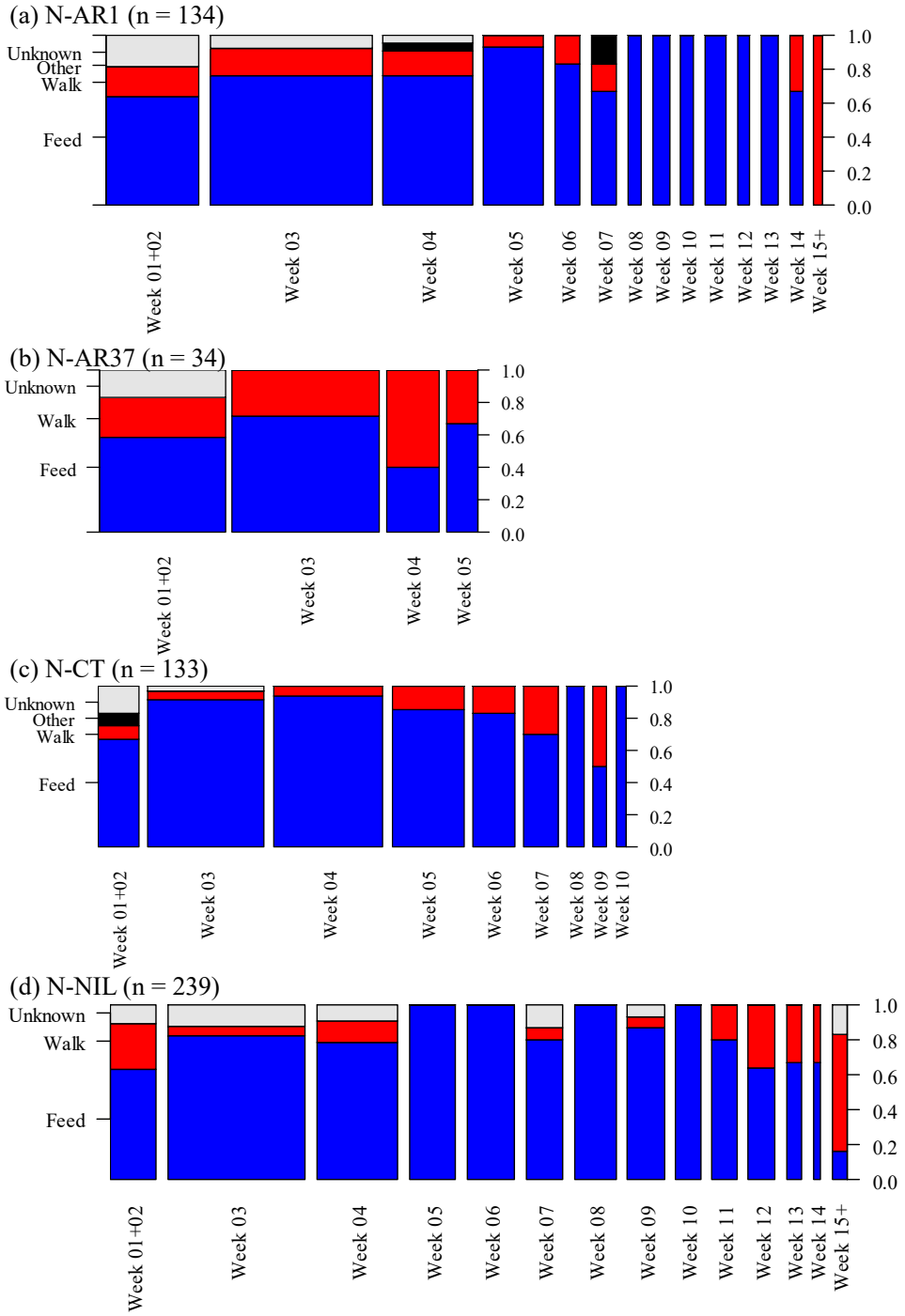


Figure A12.1.1. Behaviour of root aphids during the Biology II experiment, for specimens living on plants of N-AR37 (a), N-AR1 (b), N-CT (c) and N-NIL (d) plant genotype-endophyte status. The width of each column was proportional to the number of records in the corresponding week. n: number of observations

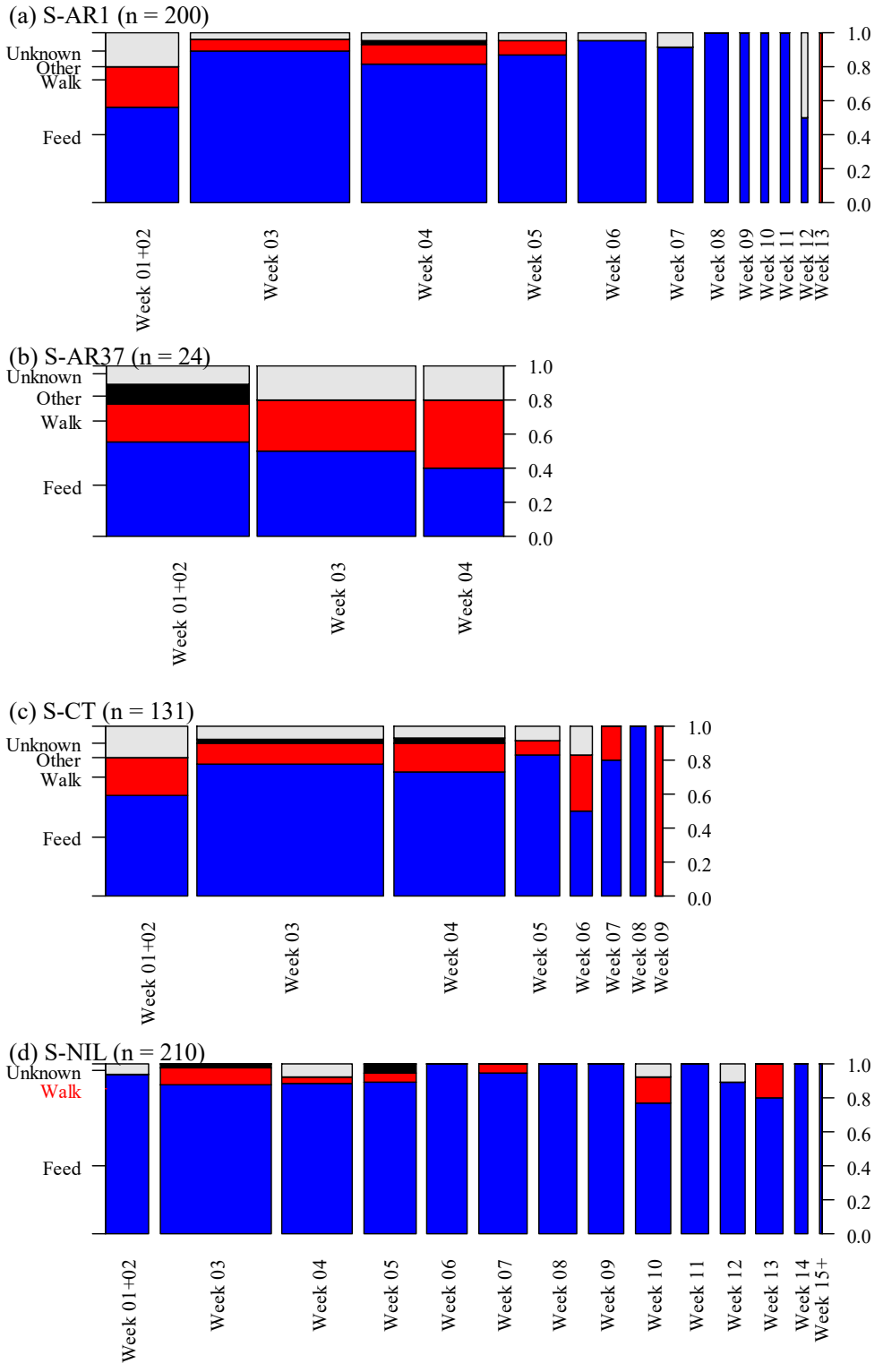
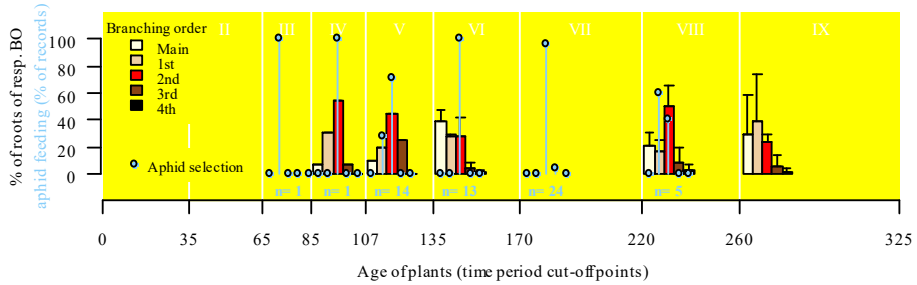


Figure A12.1.2. Behaviour of root aphids during the Biology II experiment, for specimens living on plants of S-AR37 (a), S-AR1 (b), S-CT (c), S-NIL (d) plant genotype-endophyte status. The width of each column was proportional to the number of records in the corresponding week. n: number of observations

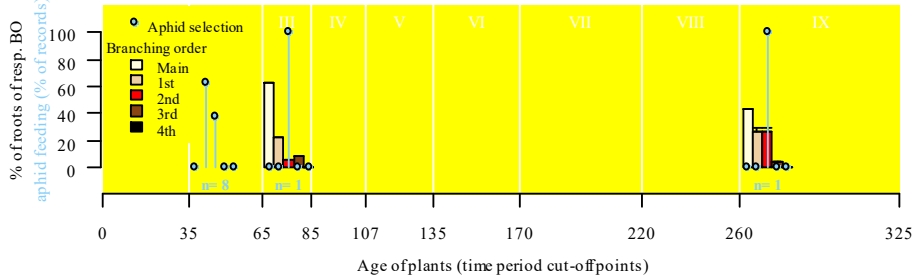
A12.2. Biology II experiment feeding site characteristics

A12.2.1. Branching order

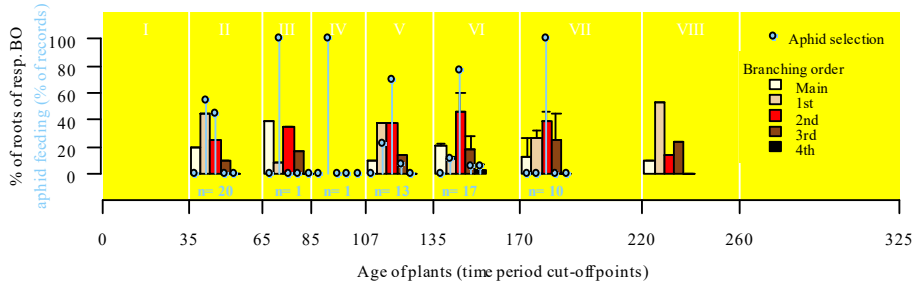
(a) Genotype N - AR1



(b) Genotype N - AR37



(c) Genotype N - common-toxic (CT)



(d) Genotype N - no endophyte

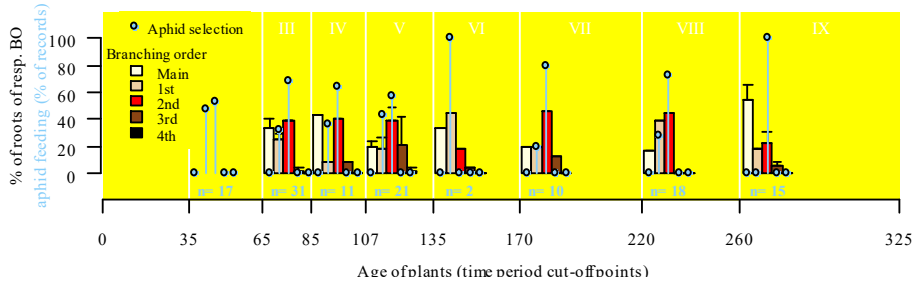


Figure A12.2.1.1. *L. perenne* root branching (BO) pattern at final dissection (barplots) and *A. lentisci* root use (blue points) on plants of genotype N in absence (d) or presence of the endophyte strains AR1 (a), AR37 (b) or common-toxic (c). Nine age intervals (roman numerals), by age of the plant at the data collection (aphid data) or at final harvest (plant data; mean % roots of the corresponding BO +SD when > 1 plant dissected). Note that the two data sets were collected on slightly different sample subsets, as a full assessment of the root BO pattern of a plant required untangling the roots, and was only possible when a plant was harvested.

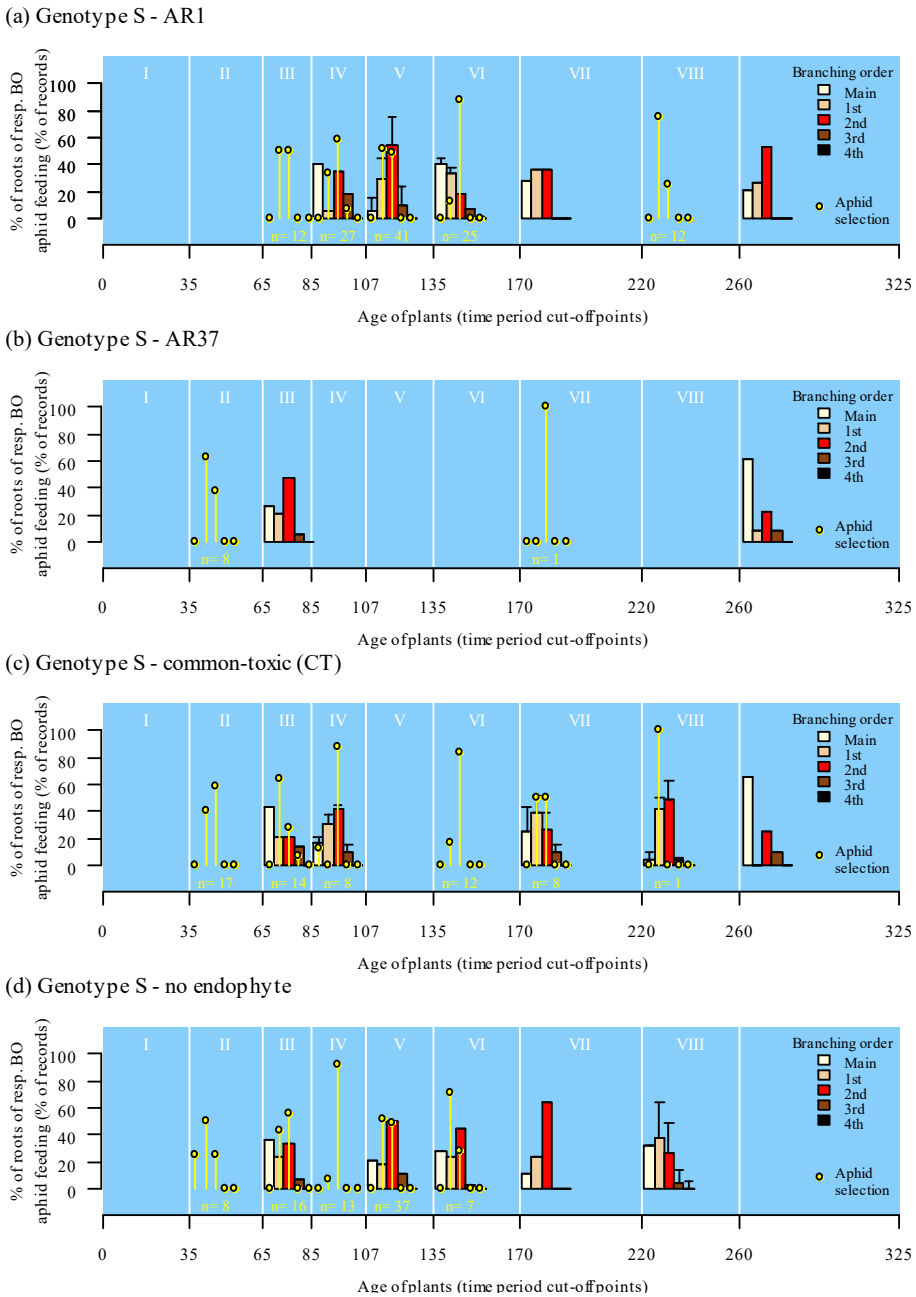


Figure A12.2.1.2. *L. perenne* root branching (BO) pattern at final dissection (barplots) and *A. lentisci* root use (yellow points) on plants of genotype S in absence (d) or presence of the endophyte strains AR1 (a), AR37 (b) or common-toxic (c). Nine age intervals (roman numerals), by age of the plant at the data collection (aphid data) or at final harvest (plant data; mean % roots of the corresponding branching order +SD when > 1 plant dissected). Note that the two data sets were collected on slightly different sample subsets, as a full assessment of the root BO pattern of a plant required untangling the roots, and was only possible when a plant was harvested.

Table A12.2.1.1. Number of records collected for branching order use, by plant genotype (N, S)-endophyte (endophyte-free NIL, with AR1, AR37 or common-toxic CT endophyte) and plant age group (plant age period).

Plant age [period]	Plant age [days]	NAR1	NAR37	NCT	NNIL	SAR1	SAR37	SCT	SNIL
II	36 - 65		8	20	17		8	17	8
III	66 - 85	1	1	1	31	12		14	16
IV	86 - 107	1		1	11	27		8	13
V	108 - 135	14		13	21	41			37
VI	136 - 170	13		17	2	25		12	7
VII	171 - 220	24		10	10		1	8	
VIII	221 - 260	5			18	12		1	
IX	≥ 261		1		15				

Table A12.2.1.2. Plant age effect on branching order selection in *A. lentisci* of all ages on various plant genotype (N, S)-endophyte (endophyte-free NIL, with AR1, AR37 or common-toxic CT endophyte) symbionts. Plant age periods (II to IX): see table A12.2.1.1.

	N-AR1	N-AR37	N-CT	N-NIL	S-AR1	S-AR37	S-CT	S-NIL
Statistics								
Fisher's test *p*-value	*< 0.001*	*0.444*	*0.004*	*0.034*	*< 0.001*	*0.444*	*0.009*	*0.003*
Number of checks (Feeding sites)	58 (23)	10 (6)	62 (27)	125 (46)	117 (57)	9 (6)	60 (34)	81 (39)
Multiple comparisons for plant age periods, p-values of post-hoc tests								
II : III		0.666	1.000	0.632			0.390	0.173
II : IV			1.000	0.858			0.174	0.025
II : V			0.309	1.000				0.062
II : VI			0.082	0.781			0.390	0.676
II : VII			0.061	0.495		0.444	1.000	
II : VIII				0.569			0.605	
II : IX		0.666		0.064				
III : IV	1.000		1.000	1.000	0.416		0.046	0.089
III : V	0.624		0.536	0.824	1.000			0.766
III : VI	0.240		0.521	0.389	0.074		0.075	0.530
III : VII	0.240		0.309	0.858			0.969	
III : VIII	1.000			1.000	0.444		1.000	
III : IX		1.000		0.124				
IV : V	1.000		0.536	1.000	0.074			0.025
IV : VI	1.000		0.521	0.489	0.252		0.453	0.025
IV : VII	1.000		0.309	0.858			0.288	
IV : VIII	1.000			0.858	0.054		0.390	
IV : IX				0.124				
V : VI	0.244		1.000	0.495	0.007			0.535
V : VII	0.068		0.521	0.524				
V : VIII	0.624			0.784	0.276			
V : IX				0.064				
VI : VII	1.000		1.000	0.364			0.390	
VI : VIII	0.068			0.388	0.003		0.390	
VI : IX				0.069				
VII : VIII	0.041			1.000			1.000	
VII : IX				0.420				
VIII : IX				0.228				

Table A12.2.1.3. Effect of plant genotype (N, S)-endophyte status (with AR1, AR37 or common-toxic CT endophyte, or without endophyte NIL) combination on root branching order use by *A. lentisci* of all ages.

Plant age at observation [days]	36 - 65	66 - 85	86 - 107	108 - 135	136 - 170	171 - 220	221 - 260	≥ 261
Fisher's test *p*-value	0.343	0.137	*0.153*	*0.1995*	*< 0.001*	*0.005*	*0.033*	-
Number observations [sites]	78 [47]	76 [41]	61 [33]	126 [65]	76 [24]	53 [18]	36 [9]	16[1]
Pairwise comparisons *p*-value								
NAR1 - NAR37		1.000						
NAR1 - NCT		1.000	1.000	1.000	0.671	1.000		
NAR1 - NNIL		1.000	1.000	0.747	0.038	0.267	0.632	
NAR1 - SAR1		1.000	1.000	0.524	0.673		0.900	
NAR1 - SAR37						1.000		
NAR1 - SCT		1.000	1.000		0.367	0.020	1.000	
NAR1 - SNIL		1.000	1.000	0.524	0.020			
NAR37 - NCT	1.000	1.000						
NAR37 - NNIL	1.000	1.000						
NAR37 - SAR1		1.000						
NAR37 - SAR37	1.000							
NAR37 - SCT	1.000	1.000						
NAR37 - SNIL	1.000	1.000						
NCT - NNIL	1.000	1.000	0.782	0.524	0.164	0.948		
NCT - SAR1		1.000	0.765	0.315	0.588			
NCT - SAR37	1.000					1.000		
NCT - SCT	1.000	1.000	0.590		1.000	0.114		
NCT - SNIL	0.625	1.000	0.536	0.315	0.038			
NNIL - SAR1		1.000	1.000	0.747	0.086		0.142	
NNIL - SAR37	1.000					1.000		
NNIL - SCT	1.000	0.582	0.536		0.141	0.802	0.632	
NNIL - SNIL	0.625	1.000	0.536	0.747	1.000			
SAR1 - SCT		1.000	0.536		1.000		1.000	
SAR1 - SNIL		1.000	0.590	1.000	0.036			
SAR37 - SCT	1.000					1.000		
SAR37 - SNIL	1.000							
SCT - SNIL	0.625	1.000	1.000		0.112			

Table A12.2.1.4. Effect of aphid age (immature vs. adult) on root branching order use, by observations

Plant age [days] period		**36 - 65 II**	**66 - 85 III**	**86 - 107 IV**	**108 - 135 V**	**136 - 170 VI**	**171 - 220 VII**	**221 - 260 VIII**	**≥ 261 IX**
CMH test, χ^2 (*p*-value)		0.51 *(0.477)*	4.19 *(0.123)*	4.27 *(0.119)*	2.79 *(0.247)*	5.65 *(0.130)*	11.19 (*0.004)*	0.42 *(0.516)*	-
df		1	2	2	2	3	2	1	-
Number observations[1]		62	57	40	126	62	52	35	-
[Number of sites]		[34]	[26]	[19]	[65]	[22]	[15]	[-]	[-]
Fisher tests by group[2]									
	N-AR1				*1.000*	*1.000*	*0.083*	*1.000*	
	N-AR37								
	N-CT	*0.617*			*1.000*	*0.002*	*1.000*		
	N-NIL	*0.637*	*1.000*		*0.660*		*1.000*	*1.000*	
	S-AR1		*1.000*	*0.096*	*0.454*	*1.000*		*0.546*	
	S-AR37	*0.196*							
	S-CT	*0.412*	*0.005*				*1.000*		
	S-NIL			*1.000*	*0.003*	*0.286*			

[1] Only records of groups with both, immature and adult records could be used for these analyses. CMH test: Cochran-Mantel-Haenszel test; df: degree of freedom.

[2] N, S: plant genotype; AR1, AR37, CT, or NIL: endophyte status, i.e. plants infected with the *Epichloë festucae* var. *lolii* endophyte strain AR1, AR37, common-toxic CT, or endophyte-free, respectively.

A12.2.2. Position on root

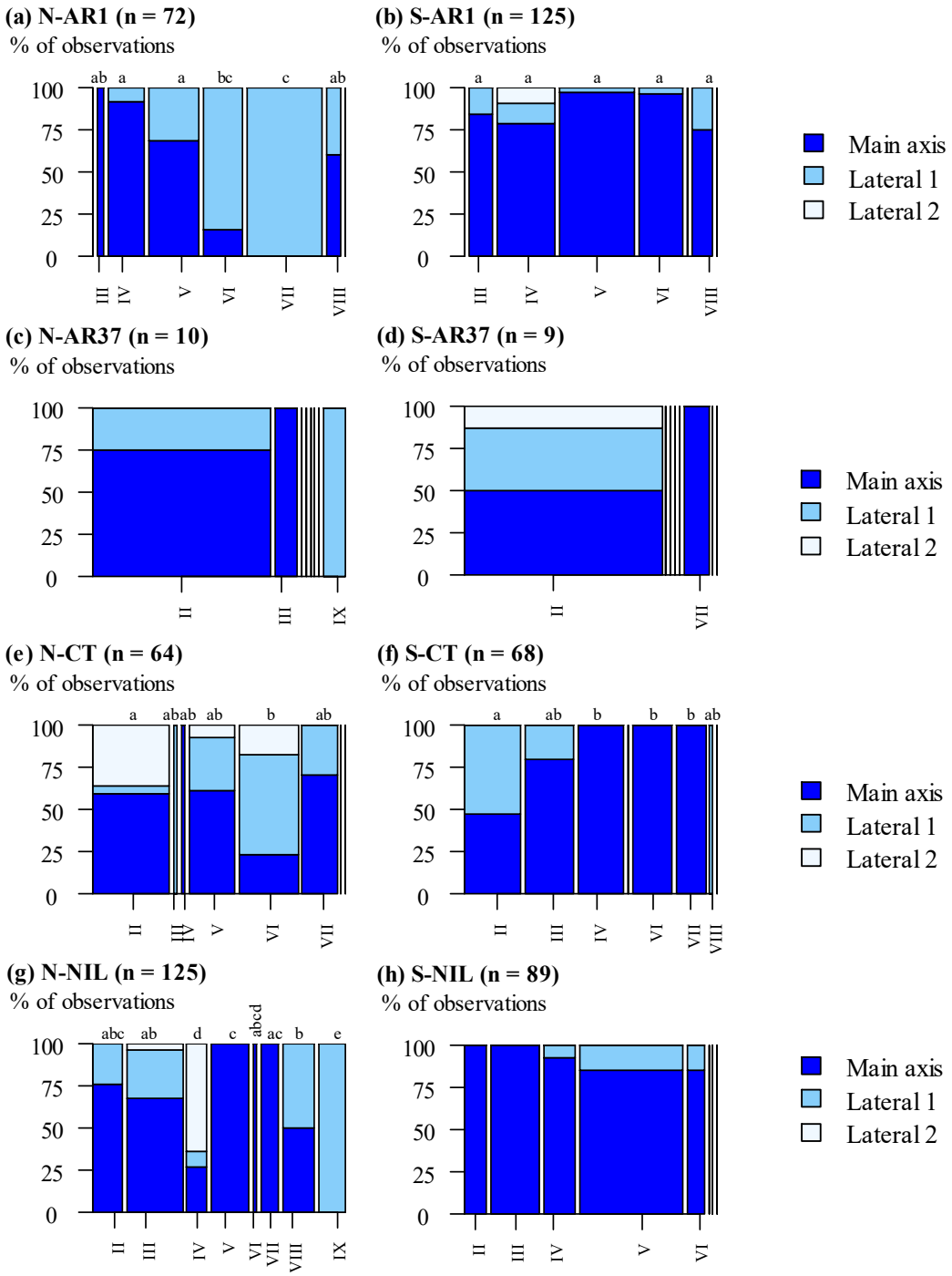


Figure A12.2.2.1. Position of *A. lentisci* on the roots of perennial ryegrass of plant genotype N or S with AR1, AR37 or common-toxic (CT) endophyte, or without endophyte (NIL), at various plant ages. Plant age: 36-65, 66-85, 86-107, 108-135, 136-170, 171-220, 221-260, and ≥ 261 days since the initial trim for plant age period II to IX, respectively; n: total number of records analysed. Bar width: % of records in the respective plant age period, with n = 100%. Bars with a same letter were not significantly different ($p > 0.05$ in post hoc test for plant groups with significant time effect).

Table A12.2.2.1. Plant age effect on position used by root aphids of all ages when feeding on various plant genotype (N, S)-endophyte (endophyte-free NIL, with AR1, AR37 or common-toxic CT endophyte) symbioses.

	N-AR1	N-AR37	N-CT	N-NIL	S-AR1	S-AR37	S-CT	S-NIL
BY OBSERVATIONS								
Tests	Fisher	Fisher	Fisher	X^2	X^2	Fisher	Fisher	Fisher
Sample size	72	10	64	125	125	9	68	89
(p-values)	*(< 0.001)*	*(0.533)*	*(0.002)*	*(< 0.001)*	*(0.032)*	*(1.000)*	*(< 0.001)*	*(0.321)*
Post-hoc multiple comparisons (*p* -values)								
II : III		*1.000*	*0.261*	*1.000*			*0.192*	*1.000*
II : IV			*1.000*	*0.001*			*0.020*	*1.000*
II : V			*0.156*	*0.056*				*1.000*
II : VI			*0.018*	*1.000*			*0.025*	*1.000*
II : VII			*0.142*	*0.376*		*1.000*	*0.047*	
II : VIII				*0.255*			*1.000*	
II : IX		*0.999*		*0.000*				
III : IV	*1.000*		*1.000*	*0.001*	*0.772*		*0.363*	*1.000*
III : V	*1.000*		*0.740*	*0.015*	*0.340*			*1.000*
III : VI	*0.122*		*1.000*	*1.000*	*0.442*		*0.363*	*1.000*
III : VII	*0.009*		*0.740*	*0.168*			*0.363*	
III : VIII	*1.000*			*0.376*	*0.772*		*0.363*	
III : IX		*1.000*		*0.000*				
IV : V	*0.267*		*1.000*	*0.000*	*0.154*			*1.000*
IV : VI	*0.001*		*0.740*	*0.376*	*0.426*		*1.000*	*1.000*
IV : VII	*0.000*		*1.000*	*0.004*			*1.000*	
IV : VIII	*0.267*			*0.001*	*0.680*		*0.192*	
IV : IX				*0.000*				
V : VI	*0.020*		*0.298*	*1.000*	*1.000*			*1.000*
V : VII	*0.000*		*1.000*	*1.000*				
V : VIII	*1.000*			*0.001*	*0.154*			
V : IX				*0.000*				
VI : VII	*0.195*		*0.261*	*1.000*			*1.000*	
VI : VIII	*0.185*			*0.610*	*0.303*		*0.192*	
VI : IX				*0.015*				
VII : VIII	*0.009*			*0.018*			*0.214*	
VII : IX				*0.000*				
VIII : IX				*0.004*				
BY FEEDING SITES								
Tests	Fisher	Fisher	Fisher	Fisher	Fisher	Fisher	Fisher	Fisher
Sample size	26	6	28	46	61	6	39	43
(p-values)	*(0.061)*	*(1.000)*	*(0.731)*	*(0.078)*	*(0.044)*	*(1.000)*	*(0.003)*	*(0.259)*

Plant age periods (II to IX): See table A12.2.1.1. Aphid age groups were pooled. Fisher: Fisher's exact test for count data; X^2: Chisquare test, used instead of Fisher's test by large numbers.

Table A12.2.2.2. Effect of plant genotype (N, S)-endophyte status (with AR1, AR37 or common-toxic CT endophyte, or without endophyte NIL) combination on position used by mature *A. lentisci.*

Plant age at observation [days]	**36 - 65**	**66 - 85**	**86 - 107**	**108 - 135**	**136 - 170**	**171 - 220**	**221 - 260**
Period	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
BY OBSERVATIONS							
Test (*p*-value)	X^2 *(< 0.001)*	Fisher *(0.077)*	Fisher *(< 0.001)*	Fisher *(< 0.001)*	X^2 *(< 0.001)*	Fisher *(< 0.001)*	Fisher *(0.339)*
Number observations	81	82	84	133	76	54	36
Post-hoc comparisons, *p*-values							
NAR1 - NAR37		1.000					
NAR1 - NCT		0.903	1.000	0.920	0.443	< 0.001	
NAR1 - NNIL		1.000	0.004	0.025	0.122	< 0.001	1.000
NAR1 - SAR1		1.000	1.000	0.016	< 0.001		1.000
NAR1 - SAR37						0.100	
NAR1 - SCT		1.000	0.902		< 0.001	< 0.001	1.000
NAR1 - SNIL		1.000	1.000	0.325	0.013		
NAR37 - NCT	0.169	1.000					
NAR37 - NNIL	1.000	1.000					
NAR37 - SAR1		1.000					
NAR37 - SAR37	0.651						
NAR37 - SCT	0.319	1.000					
NAR37 - SNIL	0.319	1.000					
NCT - NNIL	0.037	0.903	0.902	0.016	0.197	0.352	
NCT - SAR1		0.875	1.000	0.016	< 0.001		
NCT - SAR37	0.198					1.000	
NCT - SCT	0.004	0.875	1.000		< 0.001	0.352	
NCT - SNIL	0.169	0.525	1.000	0.096	0.075		
NNIL - SAR1		1.000	0.004	1.000	1.000		0.924
NNIL - SAR37	0.319					1.000	
NNIL - SCT	0.294	1.000	0.002		1.000	1.000	1.000
NNIL - SNIL	0.329	0.248	0.003	0.125	1.000		
SAR1 - SAR37							
SAR1 - SCT		1.000	0.768		1.000		0.924
SAR1 - SNIL		0.824	1.000	0.096	0.539		
SAR37 - SCT	0.520					1.000	
SAR37 - SNIL	0.110						
SCT - SNIL	0.047	0.532	0.902		0.539		

Table A12.2.2.2 (continued). Effect of plant genotype (N, S)-endophyte status (with AR1, AR37 or common-toxic CT endophyte, or without endophyte NIL) combination on position used by mature *A. lentisci*.

Plant age at observation [days]	**36 - 65**	**66 - 85**	**86 - 107**	**108 - 135**	**136 - 170**	**171 - 220**	**221 - 260**
Period	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
BY FEEDING SITE							
Test (*p*-value)	Fisher *(0.062)*	Fisher *(0.007)*	Fisher *(0.197)*	Fisher *(0.064)*	Fisher *(0.053)*	Fisher *(0.015)*	Fisher *(0.571)*
Number of sites	49	46	38	69	24	19	9

Fisher: Fisher's exact test for count data, used by small numbers; X^2: Chisquare test, used alternatively

Table A12.2.2.3. Effect of aphid age (immature vs. adult *A. lentisci*) on root position when feeding on various plant genotype (N, S)-endophyte (endophyte-free NIL, with AR1, AR37 or common-toxic CT endophyte) symbioses.

Plant age at observation [days]	36 - 65	66 - 85	86 - 107	108 - 135	136 - 170	171 - 220	221 - 260	≥ 261
BY OBSERVATIONS								
CMH test, χ^2	3.58	2.74	*3.90*	0.51	*13.15*	0.16	0.42	-
(*p*-value)	*(0.167)*	*(0.253)*	*(0.142)*	*(0.773)*	*(0.001)*	(0.688*)*	*(0.516)*	-
df	2	2	2	2	2	1[2]	1[2]	-
Number observations[1]	64	59	59	133	76	53	17	16
Fisher's test by group (*p*-value)								
N-AR1				*0.214*	*0.077*	*1.000*	*1.000*	
N-AR37								
N-CT	*1.000*			*1.000*	*< 0.001*	*0.500*		
N-NIL	*0.082*	*0.168*	*0.333*	*1.000*		*1.000*		
S-AR1		*1.000*	*0.381*	*0.214*	*0.080*		*0.182*	
S-AR37	*0.143*							
S-CT	*1.000*	*1.000*				*1.000*		
S-NIL			*1.000*	*1.000*	*1.000*			
BY FEEDING SITES								
CMH test χ2	1.65	3.38	2.56	0.122	0.88	-	-	-
(p-value)	*(0.439)*	*(0.185)*	*(0.277)*	*(0.940)*	*(0.643)*	-	-	-
df	2	2	2	2	2	-		
Number sites[1]	35	29	30	69	22	16	-	-

[1] Only records of groups with both, immature and adult records could be used for these analyses, which resulted in a lower total number of records, than by other analyses. CMH: Cochran-Mantel-Haenszel test; df: degree of freedom

[2] Lateral roots pooled to one group, i.e. 2 position categories.

A12.2.3. Colour of root

Table A12.2.3.1. Plant age effect on root colour (3 colour categories: dark brown, pale brown and white roots). Plant age periods (II to IX): see table A12.2.1.1

	N-AR1	N-AR37	N-CT	N-NIL	S-AR1	S-AR37	S-CT	S-NIL[4]
BY FEEDING SITE								
CMH test, stratified by aphid age[1]								
X^2	13.16	-	11.20	15.55	11.27	5.00	6.35	18.83
(p-value)	*(0.215)*	-	*(0.048)*	*(0.213)*	*(0.337)*	*(0.083)*	*(0.785)*	*(0.016)*
df	10	-	5[2]	12	10	3[2]	10	8
Number	21	-	29	36	56	7	49	51
BY OBSERVATIONS								
CMH test, stratified by aphid age[1]								
X^2	80.23	-	26.24	93.63	24.79	8.00	21.29	20.00
(p-value)	(< 0.001)	-	(< 0.001)	(< 0.001)	(0.006)	(0.018)	(0.019)	(0.010)
df	14		5[2]	14	10	2	10	8
Number records	67	-	60	106	105	10	81	105
Fisher's test for immature aphids								
Test (*p*-value)	*0.265*	*0.677*	*0.142*	*0.239*	*< 0.001*	*0.167*	*0.070*	*0.001*[3]
Number records	22	16	25	38	53	6	55	83
Multiple comparisons (p-values)								
II : III		1.000		0.537	1.000		0.083	0.292
II : IV			0.728		1.000		1.000	0.288
II : V			0.728	0.537	0.273			0.292
II : VI		1.000	0.740	0.537	1.000		1.000	0.332
II : VII		1.000	0.476			0.167	1.000	0.588
II : VIII		1.000		0.537	1.000		0.750	
II : IX		1.000						
III : IV	1.000				0.396		0.081	0.691
III : V	1.000			0.537	0.000			0.025
III : VI	1.000	1.000		0.537	0.275		0.171	0.025
III : VII	1.000	1.000					0.021	0.308
III : VIII	1.000	1.000		0.537	0.398		1.000	
III : IX		1.000						
IV : V	1.000		1.000		0.026			0.021
IV : VI	1.000		1.000		0.273		1.000	0.025
IV : VII	1.000		1.000				1.000	0.166
IV : VIII	1.000				1.000		0.542	
IV : IX								
V : VI	1.000		1.000	0.537	0.358			0.509
V : VII	1.000		0.585					0.819
V : VIII	1.000			0.537	0.179			
V : IX								
VI : VII	1.000	1.000	0.728				1.000	0.390
VI : VIII	1.000	1.000		0.537	0.536		0.750	
VI : IX		1.000						
VII : VIII	1.000	1.000					0.231	
VII : IX		1.000						
VIII : IX		1.000						

[1] Only records of groups with both, immature and adult records could be used for these analyses.

[2] Pale brown and white roots pooled to one category

[3] Chisquare test

[4] N, S: plant genotype; AR1, AR37, CT, or NIL: endophyte status, i.e. plants infected with the *Epichloë festucae* var. *lolii* endophyte strain AR1, AR37, common-toxic CT, or endophyte-free, respectively.

Table A12.2.3.1. (continued). Plant age effect on root colour (3 colour categories). Plant age periods (II to IX): see table A12.2.1.1.

	N-AR1	N-AR37	N-CT	N-NIL	S-AR1	S-AR37	S-CT	S-NIL[4]
Fisher's test for mature aphids								
Test (*p*-value)	*< 0.001*		*< 0.001*	*< 0.001*	*< 0.001*	*0.250*	*0.039*	*< 0.001*
Number records	45		35	68	52	4	26	64
Multiple comparisons								
II : III			1.000	0.031		0.250	1.000	
II : IV			1.000	1.000			1.000	
II : V			0.858	0.003				
II : VI			0.005				1.000	
II : VII			0.023	0.166			1.000	
II : VIII				0.000				
II : IX				0.000				
III : IV			1.000	0.031	1.000		0.888	
III : V			1.000	0.205	1.000			0.011
III : VI			0.536		1.000		0.278	0.068
III : VII			0.536	0.205			0.888	0.011
III : VIII				0.005	0.286			0.022
III : IX				0.006				
IV : V	1.000		1.000	0.003	0.468			
IV : VI	0.000		1.000		1.000		1.000	
IV : VII	0.000		1.000	0.166			0.238	
IV : VIII	0.130			0.000	0.002			
IV : IX				0.000				
V : VI	0.037		1.000		0.286			0.379
V : VII	0.012		1.000	0.206				0.379
V : VIII	0.584			1.000	0.023			0.011
V : IX				1.000				
VI : VII	1.000		1.000				0.238	0.173
VI : VIII	0.128				0.002			0.011
VI : IX								
VII : VIII	0.037			0.100				0.031
VII : IX				0.103				
VIII : IX				1.000				

[1] Only records of groups with both, immature and adult records could be used for these analyses.

[2] Pale brown and white roots pooled to one category

[3] Chisquare test

[4] N, S: plant genotype; AR1, AR37, CT, or NIL: endophyte status, i.e. plants infected with the *Epichloë festucae* var. *lolii* endophyte strain AR1, AR37, common-toxic CT, or endophyte-free, respectively.

Table A12.2.3.2. Effect of plant genotype (N, S)-endophyte status (with AR1, AR37 or common-toxic CT endophyte, or without endophyte NIL) combination on the colour of roots used by mature *A. lentisci* (3 colour categories: dark brown, pale brown and white roots).

Plant age at observation [days] **Period**	**36 - 65** **II**	**66 - 85** **III**	**86 - 107** **IV**	**108 - 135** **V**	**136 - 170** **VI**	**171 - 220** **VII**	**221 - 260** **VIII**
BY OBSERVATION							
Fisher's test (*p*-value)	*(0. 103)*	*(0.003)*	*(< 0.001)*	*(0.306)*	*(< 0.001)*	*(< 0.001)*	*(< 0.001)*
Number observations [sites]	25	22	33	53	47	58	40
Post-hoc comparisons, *p*-values							
NAR1 - NCT			0.000	1.000	0.000	0.000	
NAR1 - NNIL				1.000		0.062	0.052
NAR1 - SAR1			1.000	1.000	0.000		0.160
NAR1 - SAR37							
NAR1 - SCT			1.000		0.000	0.005	
NAR1 - SNIL				1.000	0.510	0.000	0.075
NCT - NNIL	0.429	1.000		1.000		0.161	
NCT - SAR1		1.000		1.000	1.000		
NCT - SAR37	1.000	0.640					
NCT - SCT	0.429	0.002			0.871	0.062	
NCT - SNIL		0.782		1.000	0.012	0.161	
NNIL - SAR1		1.000	0.000	1.000			0.000
NNIL - SAR37	1.000	1.000					
NNIL - SCT	0.429	0.429	0.002			1.000	
NNIL - SNIL		1.000		0.613		0.062	1.000
SAR1 - SAR37		1.000					
SAR1 - SCT		0.429	1.000		1.000		
SAR1 - SNIL		1.000		1.000	0.012		0.000
SAR37 - SCT	0.429	0.158					
SAR37 - SNIL		1.000					
SCT - SNIL		0.416			0.007	0.037	
BY FEEDING SITE							
Fisher's test *p*-value	*(0.250)*	*(1.000)*	*(0.200)*	*(0.583)*	*(0.600)*	*(0.886)*	-
Number sites	8	11	11	20	7	7	1

Fisher: Fisher's exact test for count data, used by small numbers; X^2: Chisquare test, used alternatively

Table A12.2.3.3. Effect of plant genotype (N, S)-endophyte status (with AR1, AR37 or common-toxic CT endophyte, or without endophyte NIL) combination on the colour of the roots used by immature *A. lentisci* (3 colour categories: dark brown, pale brown and white roots).

Plant age at observation [days] **Period**	**36 - 65** **II**	**66 - 85** **III**	**86 - 107** **IV**	**108 - 135** **V**	**136 - 170** **VI**	**171 - 220** **VII**	**221 - 260** **VIII**
BY OBSERVATIONS							
Fisher's test (*p*-value)	*(0. 021)*	*(< 0.001)*	*(0.002)*	*(0.002)*	*(0.223)*	*(0.507)*	*(0.078)*
Number observations [sites]	56	71	54	52	24	25	15
Post-hoc comparisons, *p*-values							
NAR1 - NAR37		*1.000*			*1.000*	*1.000*	*1.000*
NAR1 - NCT			*1.000*	*0.177*	*1.000*	*1.000*	
NAR1 - NNIL		*1.000*		*1.000*	*1.000*		*1.000*
NAR1 - SAR1		*0.702*	*0.236*	*0.173*	*1.000*		*1.000*
NAR1 - SAR37						*1.000*	
NAR1 - SCT		*0.444*	*0.085*	*0.169*	*1.000*	*1.000*	*0.773*
NAR1 - SNIL		*0.702*	*1.000*		*0.467*	*1.000*	
NAR37 - NCT	*1.000*				*1.000*	*1.000*	
NAR37 - NNIL	*1.000*	*1.000*			*0.999*		*1.000*
NAR37 - SAR1	*1.000*	*0.585*			*1.000*		*0.773*
NAR37 - SAR37	*0.028*					*1.000*	
NAR37 - SCT	*1.000*	*0.300*			*1.000*	*1.000*	*0.500*
NAR37 - SNIL	*1.000*	*1.000*			*0.467*	*1.000*	
NCT - NNIL	*1.000*			*0.177*	*1.000*		
NCT - SAR1	*1.000*		*0.714*	*0.054*	*1.000*		
NCT - SAR37	*0.200*					*1.000*	
NCT - SCT	*1.000*		*0.455*	*0.377*	*1.000*	*1.000*	
NCT - SNIL	*1.000*		*1.000*		*0.601*	*1.000*	
NNIL - SAR1	*1.000*	*0.189*		*0.108*	*1.000*		*0.773*
NNIL - SAR37	*0.028*						
NNIL - SCT	*1.000*	*0.009*		*0.184*	*1.000*		*0.500*
NNIL - SNIL	*1.000*	*0.444*			*0.999*		
SAR1 - SAR37	*0.501*						
SAR1 - SCT	*1.000*	*0.628*	*0.206*	*0.005*	*1.000*		*0.773*
SAR1 - SNIL	*1.000*	*0.002*	*0.098*		*0.467*		
SAR37 - SCT	*0.154*					*1.000*	
SAR37 - SNIL	*0.021*					*1.000*	
SCT - SNIL	*0.399*	*0.000*	*0.009*		*0.601*	*1.000*	

Table A12.2.3.3. (continued). Effect of plant genotype (N, S)-endophyte status (with AR1, AR37 or common-toxic CT endophyte, or without endophyte NIL) combination on the colour of the roots used by immature *A. lentisci* (frequency of dark brown, pale brown and white roots).

Plant age at observation [days] **Period**	**36 - 65** **II**	**66 - 85** **III**	**86 - 107** **IV**	**108 - 135** **V**	**136 - 170** **VI**	**171 - 220** **VII**	**221 - 260** **VIII**
BY FEEDING SITE							
Fisher's test *p*-value	*(0.066)*	*(0.398)*	*(0.193)*	*(0.047)*	*(0.875)*	*(0.378)*	*(0.357)*
Number sites	45	38	25	39	19	19	9

Table A12.2.3.4. Effect of aphid age (immature vs. adult *A. lentisci*) on root colour (frequency of dark brown, pale brown and white roots).

Plant age [days] **period**	**36 - 65** **II**	**66 - 85** **III**	**86 - 107** **IV**	**108 - 135** **V**	**136 - 170** **VI**	**171 - 220** **VII**	**221 - 260** **VIII**	**≥ 261** **IX**
BY OBSERVATIONS								
CMH test χ^2	7.47	1.89	14.93	4.72	13.42	0.37	2.30	-
(p-value)	*(0.024)*	*(0.389)*	*(< 0.001)*	*(0.094)*	*(0.001)*	*(0.831)*	*(0.130)*	-
Df	2	2	2	2	2	2	1	
Number observations[1]	61	57	78	105	67	80	38	-
Fisher tests by group (*p-values*, not adjusted)								
N-AR1			*1.000*	*1.000*	*0.001*	*0.083*	*1.000*	
N-AR37								
N-CT	*1.000*			*1.000*	*0.025*	*1.000*		
N-NIL	*0.137*	*0.765*		*0.221*			*1.000*	
S-AR1		*0.312*	*0.013*	*0.098*	*0.200*		*0.182*	
S-AR37	*0.018*							
S-CT	*0.600*	*0.027*	*0.014*		*0.093*	*1.000*		
S-NIL		*0.012*	*1.000*	*0.0263*	*1.000*	*0.149*		
BY FEEDING SITES								
CMH test $\chi 2$	*3.56*	*3.16*	*11.23*	*2.06*	*2.42*	*0.58*	-	-
(p-value)	*(0.168)*	*(0.206)*	*(0.004)*	*(0.356)*	*(0.298)*	*(0.749)*	*(1.000)*	-
df	2	2	2	2	2	2	1	
Number of sites	36	26	29	59	21	21	2	-

[1] Only records of groups with both, immature and adult records could be used for these analyses

A12.2.4. Root diameter at feeding sites

Table A12.2.4.1. Root diameter used by immature and mature root aphids on plants of various genotype (N, S) and endophyte status (in symbiosis with an AR1, AR37, common-toxic CT endophyte strain or without endophyte [NIL]) (raw means ± standard deviation [mm]).

	N-AR1	N-AR37	N-CT	N-NIL	S-AR1	S-AR37	S-CT	S-NIL
Aphids of all ages (pooled)[1]	**0.30 ±0.11^{ab}**	**0.27 ±0.13 ^{ab}**	**0.27 ±0.13 ^{a}**	**0.31 ±0.14 ^{ab}**	**0.36 ±0.13 ^{b}**	**0.27 ±0.11 ^{ab}**	**0.33 ±0.13 ^{ab}**	**0.35 ±0.18 ^{ab}**
Immature aphids								
Number of records	11	10	17	24	36	5	37	39
Diameter of roots used	0.33 ±0.12	0.27 ±0.13	0.24 ±0.10	0.32 ±0.15	0.36 ±0.15	0.23 ±0.09	0.31 ±0.13	0.33 ±0.17
Number of records (by PA)	0/1/1/5/2/2/0/0	5/0/0/0/2/1/2/0	1/0/1/6/5/4/0/0	8/6/0/7/2/0/1/0	1/15/14/5/1/0/0/0	4/0/0/0/0/1/0/0	12/3/8/0/3/9/2/0	10/12/4/7/2/4/0/0
Diameter by p. age II	-	0.38 ±0.06	0.09	0.34 ±0.13	0.60	0.22 ±0.11	0.34 ±0.16	0.43 ±0.12^{ab}
III	0.53	-	-	0.35 ±0.23	0.33 ±0.12	-	0.36 ±0.09	0.42 ±0.18^{a}
IV	0.17	-	0.34	-	0.36 ±0.16	-	0.27 ±0.12	0.23 ±0.15^{abc}
V	0.37 ±0.09	-	0.27 ±0.11	0.31 ±0.08	0.43 ±0.17	-	-	0.19 ±0.06^{bc}
VI	0.22 ±0.01	0.23 ±0.04	0.19 ±0.09	0.20 ±0.02	0.21	-	0.24 ±0.11	0.30 ±0.20^{abc}
VII	0.28 ±0.13	0.18	0.25 ±0.08	-	-	0.25	0.33 ±0.12	0.16 ±0.05^{c}
VIII	-	0.09 ±0.00	-	0.21	-	-	0.19 ±0.09	-
Mature aphids								
Number of records	5	0	18	17	19	3	11	14
Diameter of roots used	0.23 ±0.06	-	0.29 ±0.15	0.29 ±0.13	0.36 ±0.10	0.34 ±0.12	0.38 ±0.14	0.42 ±0.18
Number of records (by PA)	0/0/2/3/0/0/0		9/0/0/1/5/3/0/0	3/5/1/1/0/1/4/2	0/1/9/9/0/0/0/0	2/1/0/0/0/0/0/0	1/4/4/0/0/2/0/0	0/2/0/9/1/2/0/0
Diameter by p. age II	-		0.33 ±0.17	0.42 ±0.04	-	0.41 ±0.05	0.27	-
III	-		-	0.35 ±0.17	0.49	0.21	0.41 ±0.15	0.26 ±0.02^{a}
IV	-		-	0.15	0.31 ±0.08	-	0.41 ±0.15	-
V	0.29 ±0.03		0.41	0.31	0.38 ±0.11	-	-	0.53 ±0.11^{a}
VI	0.19 ±0.00		0.20 ±0.13	-	-	-	-	0.16 ^{a}
VII	-		0.32 ±0.10	0.31	-	-	0.30 ±0.13	0.22 ±0.08^{a}
VIII	-		-	0.20 ±0.03	-	-	-	-
IX	-		-	0.20 ±0.01	-	-	-	-
Mature vs immature, *p*-value	***(0.145)***		***(0.273)***	***(0.704)***	***(0.951)***	***(0.250)***	***(0.073)***	***(0.105)***

The measurements were performed on pictures taken at microscope magnifications of 50×; Means with different capital letters were significantly different from other means within the same line at an $\alpha = 0.05$ level. PA: plant age period, see Table A12.2.1.1.

[1] Indicative only, significant effects in general means are likely due to the confounding effects of plant age.

Table A12.2.4.2. Root diameter of feeding sites used by aphids, by plant genotype (N, S)-endophyte status (in symbiosis with an AR1, AR37, common-toxic CT endophyte strain or without endophyte [NIL]) combination (raw means ± standard deviation [mm]).

	N-AR1	N-AR37	N-CT	N-NIL	S-AR1	S-AR37	S-CT	S-NIL
Feeding sites								
Immature aphids								
Number of sites	10	8	15	19	29	5	32	34
Average root diameter at site	0.33 ±0.13	0.29 ±0.13	0.25 ±0.10[a]	0.31 ±0.13	0.36 ±0.15	0.23 ±0.09	0.29 ±0.13	0.34 ±0.17
Mature aphids								
Number of sites	3	0	7	7	11	1	7	7
Average root diameter at site	0.26 ±0.06		0.36 ±0.11[b]	0.31 ±0.14	0.32 ±0.11	0.21	0.33 ±0.15	0.47 ±0.12
Main axis diameter								
Number of sites (U/1st BO/2nd BO)	0 / 2 / 2	0 / 1 / 1	0 / 7 / 5	0 / 7 / 6	0 / 15 / 10	0 / 1 / 1	1 / 5 / 10	2 / 14 / 6
Diameter of roots: Unbranched	-	-	-	-	-	-	0.41	0.47 ±0.13
1st BO	0.47 ±0.08	0.34	0.38 ±0.07	0.37 ±0.13	0.36 ±0.14	0.38	0.35 ±0.06	0.37 ±0.14
2nd BO	0.34 ±0.12	0.36	0.33 ±0.09	0.40 ±0.11	0.34 ±0.08	0.25	0.31 ±0.18	0.43 ±0.15
Diameter by position (pooled BO)								
Number of sites (M / L1 / L2)	5/ 8 / 0	2 / 1 / 0	13 / 4 / 1	13 / 8 / 1	28 / 5 / 2	2 / 2 / 1	19 / 4 / 0	25 / 2 / 0
Diameter at: Main axis (M)	0.42 ±0.10[b]	0.35 ±0.01	0.33 ±0.11[b]	0.39 ±0.12[b]	0.36 ±0.13[b]	0.31 ±0.09	0.32 ±0.14	0.42 ±0.15
Lateral 1 (L1)	0.24 ±0.07[a]	0.43	0.20 ±0.07[a]	0.23 ±0.10[a]	0.20 ±0.06[a]	0.19 ±0.03	0.24 ±0.04	0.38 ±0.33
Lateral 2 (L2)	-	-	0.12 ([ab])	0.15 ([ab])	0.26 ±0.17[ab]	0.14	-	-
Diameter by root colour (all positions)								
Number of sites (DB / PS / W)	8 / 3 / 2	6 / 1 / 1	11 / 5 / 5	9 / 13 / 4	19 / 16 / 4	2 / 0 / 4	11 / 19 / 9	21 / 16 / 3
Diameter: Dark brown roots (DB)	0.31 ±0.11	0.25 ±0.12	0.27 ±0.11	0.35 ±0.12	0.36 ±0.14	0.23 ±(0.03)	0.31 ±0.14	0.44 ±0.15 [b]
Pale brown roots (PS)	0.36 ±0.17	0.34	0.34 ±0.16	0.30 ±0.14	0.35 ±0.14		0.30 ±0.15	0.28 ±0.14 [a]
White roots (W)	0.22 ±0.01	0.45	0.20 ±0.11	0.24 ±0.10	0.26 ±0.18	0.22 ±0.11	0.28 ±0.10	0.15 ±0.05 [a]

The measurements were performed on pictures taken at microscope magnifications of 50×; BO: branching order; Means with different letters were significantly different from other means within the same column section (colour stripe) at $\alpha = 0.05$ level

A12.3. Wax

Table A12.3.1. Properties of the colonies collected during the colony wax observations.

Plant geno-type	Endo-phyte status	N	Plant age at AphP	Age of aphid population at harvest	Number of aphids		Old/ Total ratio [%]	% feeding		Root diameter [mm]	Wax	
					Number	[Range]		Old	Young		Root length [mm]	Area [mm^2]
N	AR1	3	21.00±0.00[a]	27.33±2.08	2.00 ±1.73	[0 – 3]	33 ± 0	100 ± 0	100 ± 0	0.53±0.35	3.45± 3.30 [a]	8.90 ± 4.29
	AR37	3	26.00±0.00[a]	20.00±0.00	5.33 ±3.21	[3 – 9]	23 ±11	100 ± 0	57 ±28	0.63±0.12	4.99± 5.46 [a]	18.46 ±15.14
	CT	6	26.00±2.86[a]	23.17±2.71	2.83 ±2.79	[1 – 8]	54 ±40	60 ±55	38 ±48	0.38±0.20	9.32±10.40 [a]	12.01 ± 8.68
	NIL	6	23.50±2.74[a]	25.00±0.00	6.83 ±6.46	[0 – 18]	16 ±20	75 ±50	92 ±10	0.73±0.43	7.03± 8.20 [a]	44.21 ±23.63
S	AR1	5	22.00±1.34[a]	28.20±1.64	4.80 ±4.21	[0 – 10]	45 ±40	100 ± 0	74 ±31	0.51±0.36	6.03± 5.25 [a]	22.10 ±12.55
	AR37	3	26.00±0.58[a]	22.33±2.89	3.00 ±1.73	[1 – 4]	58 ±38	67 ±58	75 ±35	0.35±0.18	4.65± 4.65 [a]	21.18 ±21.75
	CT	3	26.00±2.89[a]	25.67±2.89	2.33 ±0.58	[2 – 3]	56 ±10	100 ± 0	33 ±58	0.67	7.34± 7.34 [a]	17.15 ± 1.16
	NIL	9	22.00±0.93[a]	26.22±3.27	4.22 ±3.90	[1 – 13]	38 ±32	100 ± 0	47 ±44	0.49±0.30	3.54± 3.80 [a]	17.05 ±11.02
Overall		**38**	**23.39±2.46**	**25.05±3.18**	**4.16 ±3.89**	**[0 – 18]**	**40 ±31**	**88 ±34**	**61 ±40**	**0.54±0.30**	**5.76± 3.10**	**21.25 ±17.01**
		df										
Tests (*p*-value)												
PG (MWU)		**1**	0.140	0.218	0.894		0.215	0.196	0.313	0.303	0.044	0.868
E (KW)		**3**	< 0.001	0.605	0.523		0.257	0.529	0.325	0.967	0.054	0.381
PG×E (KW)		**7**	0.011	0.524	0.742		0.331	0.348	0.330	0.621	0.031	0.109

AphP: aphid placement; df: degree of freedom; E: endophyte status; MWU: Mann-Whitney-U test; KW: Kruskal-Wallis test; N: number of colonies collected; PG: plant genotype; PG×E: plant genotype-endophyte interactions. Means with a same letter were not significantly different at a post-hoc dunn test with Benjamini-Hochberg *p*-adjustment.

Table A12.3.2. Spearman's rank correlation coefficient (Spearman's ρ) for the relationship between number of exuviae, aphids of both age categories and root or colony properties.

Parameter	df	Exuviae		Young aphids		Old aphids		All aphids	
		ρ	(p_ρ)	ρ	(p_ρ)	ρ	(p_ρ)	ρ	(p_ρ)
Colony parameters									
Exuviae	36	-	-	0.24	*(0.144)*	0.06	*(0.724)*	0.28	*(0.084)*
Number of old aphids	36	0.06	*(0.724)*	0.28	*(0.092)*				
Number of young aphids	36	0.24	*(0.144)*			0.28	*(0.092)*		
Wax area	35	0.48	*(0.002)*	0.53	*(< 0.001)*	-0.02	*(0.904)*	0.53	*(< 0.001)*
Wax length	26	0.26	*(0.183)*	0.29	*(0.128)*	-0.11	*(0.571)*	0.26	*(0.174)*
Root parameters									
Root diameter	25	0.55	*(0.003)*	0.56	*(0.002)*	0.20	*(0.315)*	0.60	*(< 0.001)*

df: degree of freedom.

Table A12.3.3. Spearman's rank correlation coefficient (Spearman's ρ) for the relationship between proportion of feeding aphids of both age categories and root or colony properties.

Parameter	df	Proportion of young aphids feeding		Proportion of old aphids feeding		Proportion of all aphids feeding	
		ρ	(p_ρ)	ρ	(p_ρ)	ρ	(p_ρ)
Colony parameters							
Exuviae	28	0.03	*(0.888)*	0.11	*(0.552)*	0.03	*(0.874)*
Number of old aphids	28	0.19	*(0.315)*	0.18	*(0.321)*	0.29	*(0.096)*
Number of young aphids	28	0.38	*(0.041)*	0.35	*(0.051)*	0.30	*(0.078)*
Age of colonies	28	0.15	*(0.433)*	0.40	*(0.023)*	0.36	*(0.018)*
Wax area	28	0.18	*(0.337)*	0.22	*(0.235)*	0.14	*(0.438)*
Wax length	21	-0.16	*(0.455)*	0.14	*(0.525)*	-0.15	*(0.476)*
Root parameters							
Root diameter	20	0.55	*(0.009)*	0.02	*(0.912)*	0.33	*(0.113)*

df: degree of freedom.

```
> # WAX-COVERED ROOT LENGTH
> summary(WaxLenB11.lme<-lme(WaxLenghtRoot1~Group2,random=~1|SampleID,data=WaxD,method="REML",na
action=na.omit))
Linear mixed-effects model fit by REML
 Data: WaxD
       AIC      BIC    logLik
  119.3852 129.3426 -49.69262

Random effects:
 Formula: ~1 | SampleID
        (Intercept) Residual
StdDev:    1.129175 2.163411

Fixed effects: WaxLenghtRoot1 ~ Group2
               Value Std.Error DF   t-value p-value
(Intercept)  3.696333  1.037171 11  3.563861  0.0044
Group2BNNIL  4.560610  1.632400  9  2.793806  0.0209
Group2NAR1  -0.395309  1.749529  9 -0.225952  0.8263
Group2NAR37  1.764077  1.977593  9  0.892032  0.3956
Group2NCT    6.705031  1.749529  9  3.832478  0.0040
Group2SAR1   1.301679  1.823546  9  0.713818  0.4934
Group2SAR37  0.949437  2.013310  9  0.471580  0.6484
Group2SCT    3.641347  2.651623  9  1.373252  0.2029
 Correlation:
             (Intr) G2BNNI G2NAR1 G2NAR3 Gr2NCT G2SAR1 G2SAR3
Group2BNNIL -0.635
Group2NAR1  -0.593  0.377
Group2NAR37 -0.524  0.333  0.311
Group2NCT   -0.593  0.377  0.351  0.311
Group2SAR1  -0.569  0.361  0.337  0.298  0.337
Group2SAR37 -0.515  0.327  0.305  0.270  0.305  0.293
Group2SCT   -0.391  0.249  0.232  0.205  0.232  0.222  0.202

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-1.3540615 -0.5830894 -0.1966120  0.6249104  1.3995572

Number of Observations: 28
Number of Groups: 17
> WaxLenB11B.lme<-lme(WaxLenghtRoot1~1,random=~1|SampleID,data=WaxD,method="REML",na.action=na.om
it)
> anova(WaxLenB11.lmeM<-update(WaxLenB11.lme,method="ML"),WaxLenB11B.lmeM<-update(WaxLenB11B.lme,
method="ML"))
                                                    Model df      AIC
WaxLenB11.lmeM <- update(WaxLenB11.lme, method = "ML")      1 10 137.7725
WaxLenB11B.lmeM <- update(WaxLenB11B.lme, method = "ML")    2  3 145.9667
                                                       BIC    logLik
WaxLenB11.lmeM <- update(WaxLenB11.lme, method = "ML")  151.0945 -58.88623
WaxLenB11B.lmeM <- update(WaxLenB11B.lme, method = "ML") 149.9633 -69.98334
                                                         Test  L.Ratio
WaxLenB11.lmeM <- update(WaxLenB11.lme, method = "ML")
WaxLenB11B.lmeM <- update(WaxLenB11B.lme, method = "ML") 1 vs 2 22.19422
                                                         p-value
WaxLenB11.lmeM <- update(WaxLenB11.lme, method = "ML")
WaxLenB11B.lmeM <- update(WaxLenB11B.lme, method = "ML")  0.0024
> residplot(WaxLenB11.lme)
Error in data.frame(x = tmp$x, y = tmp$y, lab = rownames(mf)) :
  arguments imply differing number of rows: 28, 38
> shapiro.test(WaxLenB11.lme$res[,1])

        Shapiro-Wilk normality test

data:  WaxLenB11.lme$res[, 1]
W = 0.97062, p-value = 0.5972

> shapiro.test(WaxLenB11.lme$res[,2])
```

```
        Shapiro-Wilk normality test

data:  WaxLenB11.lme$res[, 2]
W = 0.95438, p-value = 0.2548

> r.squaredGLMM(WaxLenB11.lme)
      R2m       R2c
0.4907137 0.5997509
> predictmeans(WaxLenB11.lme,"Group2",pairwise=T)
$`Predicted Means`
Group2
  ASNIL   BNNIL    NAR1   NAR37     NCT    SAR1   SAR37     SCT
 3.6963  8.2569  3.3010  5.4604 10.4014  4.9980  4.6458  7.3377

$`Standard Error of Means`
Group2
  ASNIL   BNNIL    NAR1   NAR37     NCT    SAR1   SAR37     SCT
1.03717 1.26056 1.40895 1.68379 1.40895 1.49987 1.72560 2.44037

$`Standard Error of Differences`
 Max.SED  Min.SED Aveg.SED
2.988825 1.632400 2.238779

$LSD
 Max.LSD  Min.LSD Aveg.LSD
 6.76119  3.69275  5.06447
attr(,"Significant level")
[1] 0.05
attr(,"Degree of freedom")
Group2
     9

$`Pairwise LSDs`
         ASNIL    BNNIL    NAR1    NAR37       NCT     SAR1    SAR37      SCT
ASNIL 0.00000 -4.56061 0.39531 -1.76408 -6.70503 -1.30168 -0.94944 -3.64135
BNNIL 3.69275  0.00000 4.95592  2.79653 -2.14442  3.25893  3.61117  0.91926
NAR1  3.95771  4.27669 0.00000 -2.15939 -7.10034 -1.69699 -1.34475 -4.03666
NAR37 4.47363  4.75815 4.96660  0.00000 -4.94095  0.46240  0.81464 -1.87727
NCT   3.95771  4.27669 4.50746  4.96660  0.00000  5.40335  5.75559  3.06368
SAR1  4.12515  4.43210 4.65517  5.10103  4.65517  0.00000  0.35224 -2.33967
SAR37 4.55442  4.83419 5.03950  5.45403  5.03950  5.17203  0.00000 -2.69191
SCT   5.99839  6.21348 6.37451  6.70703  6.37451  6.47980  6.76119  0.00000
attr(,"Significant level")
[1] 0.05
attr(,"Degree of freedom")
Group2
     9

ASNIL   A
BNNIL    BC
NAR1    A
NAR37   ABC
NCT      B
SAR1    A C
SAR37   A C
SCT     ABC
```

A12.4. Population experiment

Table A12.4.1. Aphid population structures and loads at the end of the Population experiment 60 to 140 days after initial plant trim, i.e. 38 to 111 days after aphid placement, on two distinct perennial ryegrass plant genotypes (N, S) living in symbiosis with one of three strains of the endophyte *E. festucae* var. *lolii* (AR1, AR37 or common-toxic CT) or without endophyte (NIL).

Plant genotype-endophyte	N	Duration of aphid occupation [days]	Living adults/ plant	Living older instars/ plant	Living 1st instars/ plant	Dead aphids/plant (% of all aphids)	FA/GSA [aphids/cm²]	FA/Shoot BM [aphids/g]	FA/Green shoot BM [aphids/g]	FA/Root BM [aphids/g]	Population structure [%FA]	
											OIm	Ad
N-AR1	14	60 ± 23	20 ± 14	103 ± 71	199 ± 217	47 ± 93 (9%)	59 ± 80	1025 ± 661	2467 ± 1962	2201 ± 1170	25%	75%
N-AR37	5	48 ± 9	3 ± 3	23 ± 17	13 ± 14	3 ± 2 (6%)	4 ± 4	158 ± 116	271 ± 258	473 ± 401	12%	88%
N-CT	13	51 ± 19	11 ± 14	93 ± 77	140 ± 214	19 ± 48 (7%)	66 ± 179	804 ± 621	1668 ± 1615	1646 ± 1205	16%	84%
N-NIL	13	62 ± 25	12 ± 10	71 ± 50	168 ± 221	16 ± 28 (7%)	58 ± 115	852 ± 502	2272 ± 1705	2235 ± 1810	19%	81%
S-AR1	14	67 ± 19	14 ± 12	59 ± 56	136 ± 194	20 ± 18 (16%)	30 ± 20	808 ± 528	2645 ± 1467	1885 ± 1401	25%	75%
S-AR37	2	62 ± 25	5 ± 5	38 ± 54	11 ± 15	1 ± 1 (25%)	19 ± 25	371 ± 492	909 ± 1164	733 ± 964	55%	45%
S-CT	7	52 ± 18	6 ± 6	26 ± 23	26 ± 33	11 ± 26 (5%)	11 ± 13	251 ± 222	682 ± 785	514 ± 470	33%	67%
S-NIL	14	68 ± 25	15 ± 16	62 ± 52	161 ± 198	21 ± 43 (9%)	57 ± 99	662 ± 507	3123 ± 3092	1188 ± 932	20%	80%
Mean ± SD		**60 ± 22**	**13 ± 13**	**69 ± 61**	**137 ± 193**	**22 ± 49 (10%)**	**46 ± 100**	**739 ± 567**	**2121 ± 2026**	**1606 ± 1334**	**22%**	**78%**
Range		**[39-112]**	**[0-52]**	**[0-210]**	**[0-742]**	**[0-334]**	**[0 -662]**	**[7-2728]**	**[9-9050]**	**[33-6114]**		

Means ±SD; Only the plants colonised by aphids were included in the calculation of the means; Ad: adults; BM: dry biomass weight; GSA: Green shoot area (estimated from the final pictures just before harvest); FA: feeding aphids, i.e. dead and living adults and older instars, as opposed to first instar (having not fed, straight abdomen). N: number of Petri Dishes (plants) colonised; OIm: older immatures

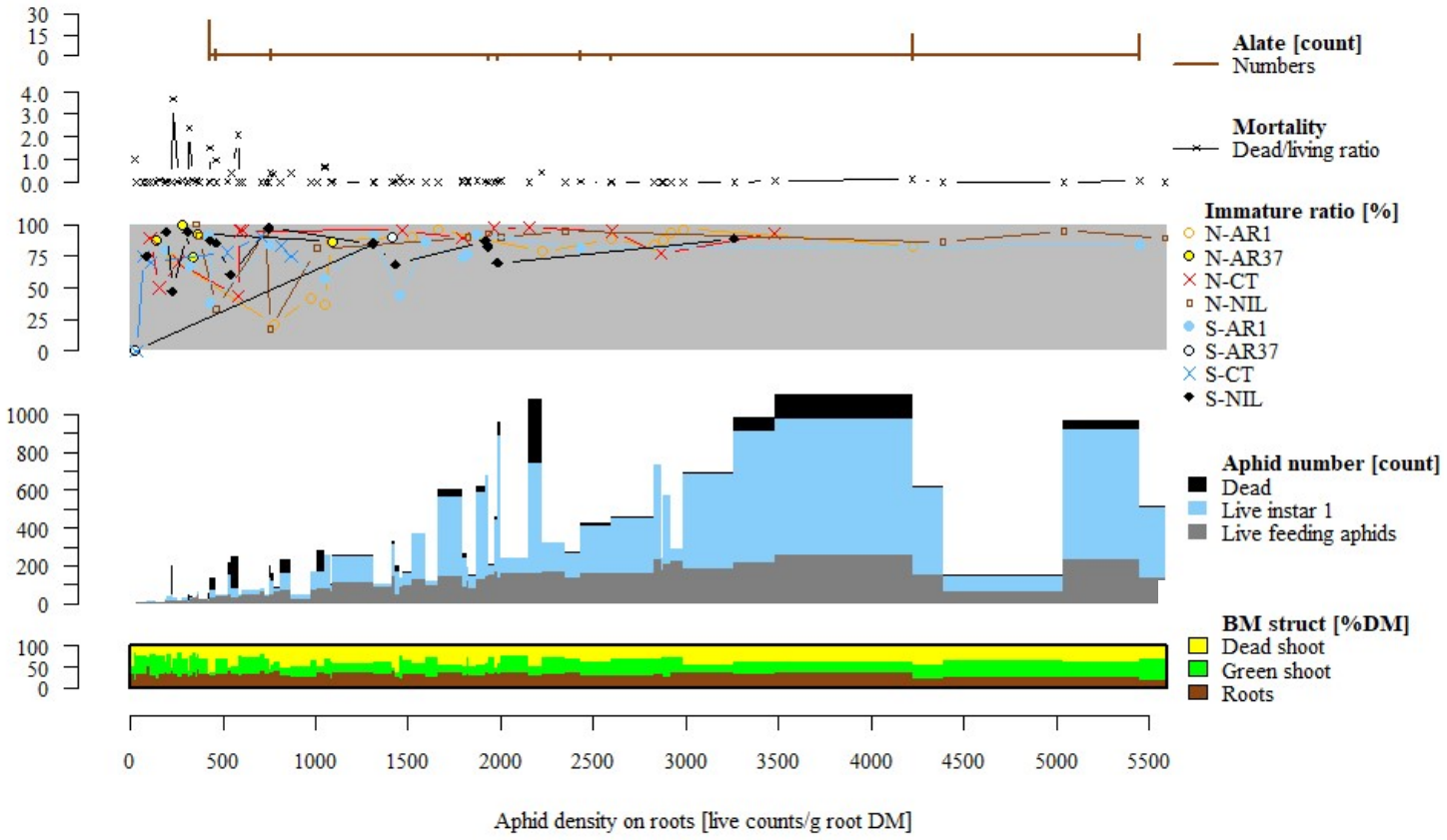


Figure A12.4.1. Colony development during the Population experiment, by aphid loads on roots [number of live older immature or adult aphids per g dry root biomass 38 to 111 days after aphid placement]. Both aphid treatments were pooled. The data are given by Petri dish (raw data) pooling also the plant genotype-endophyte groups for all variables except the immature ratio. Column width for Aphid number and BM struct: interval between measurement x and the next measurement. BM struct: biomass structure; DM: dry matter

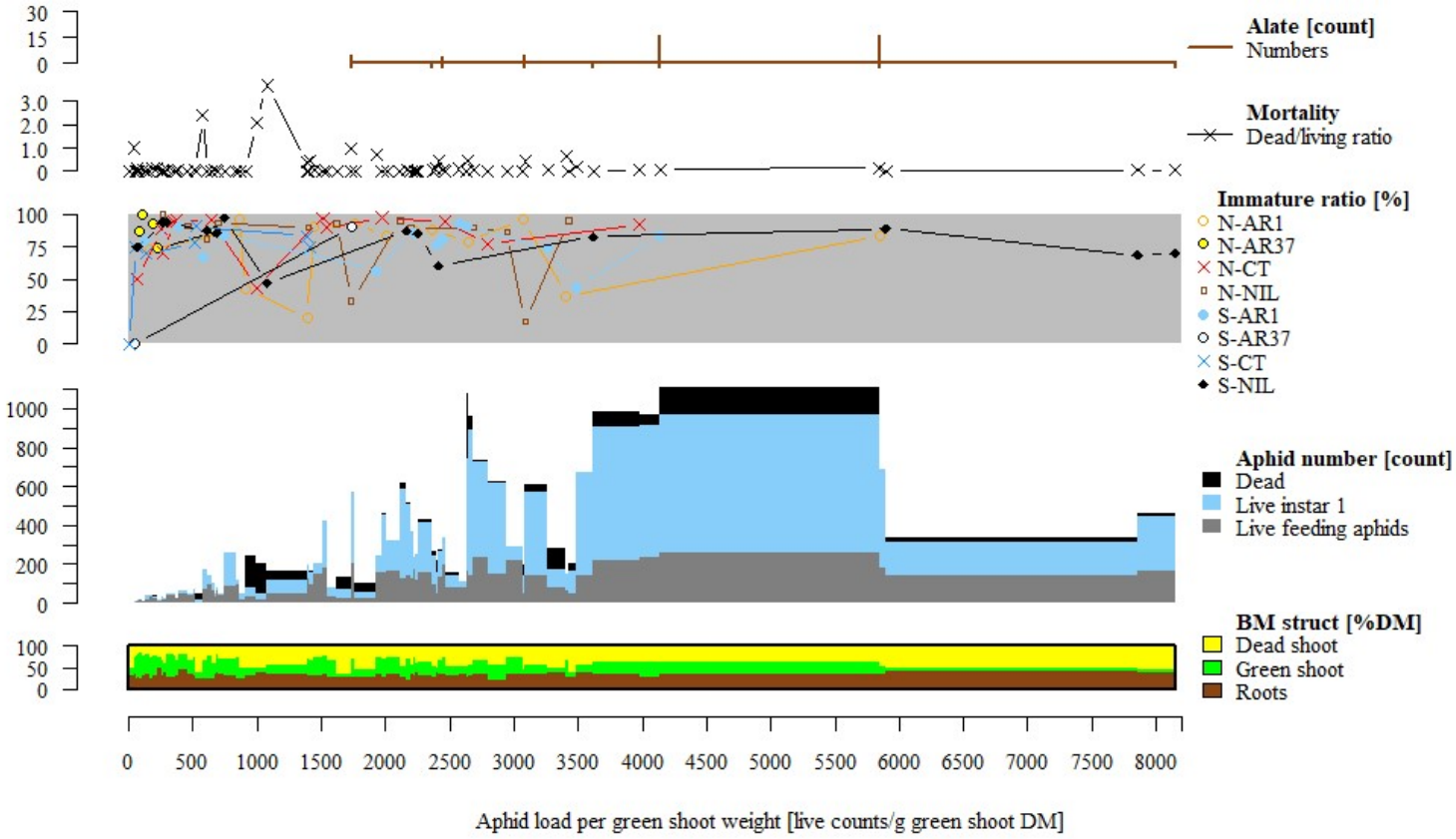


Figure A12.4.2. Colony development during the Population experiment, row data by aphid loads per green shoot weight [number of live feeding aphids/g green shoot dry matter 38 to 111 days after aphid placement]. Both aphid treatments were pooled. The data are presented by Petri dish (raw data) pooling also the plant genotype-endophyte groups for all variables except the immature ratio. Column width for Aphid number and BM struct: interval between measurement x and the next measurement. BM struct: biomass structure; DM: dry matter

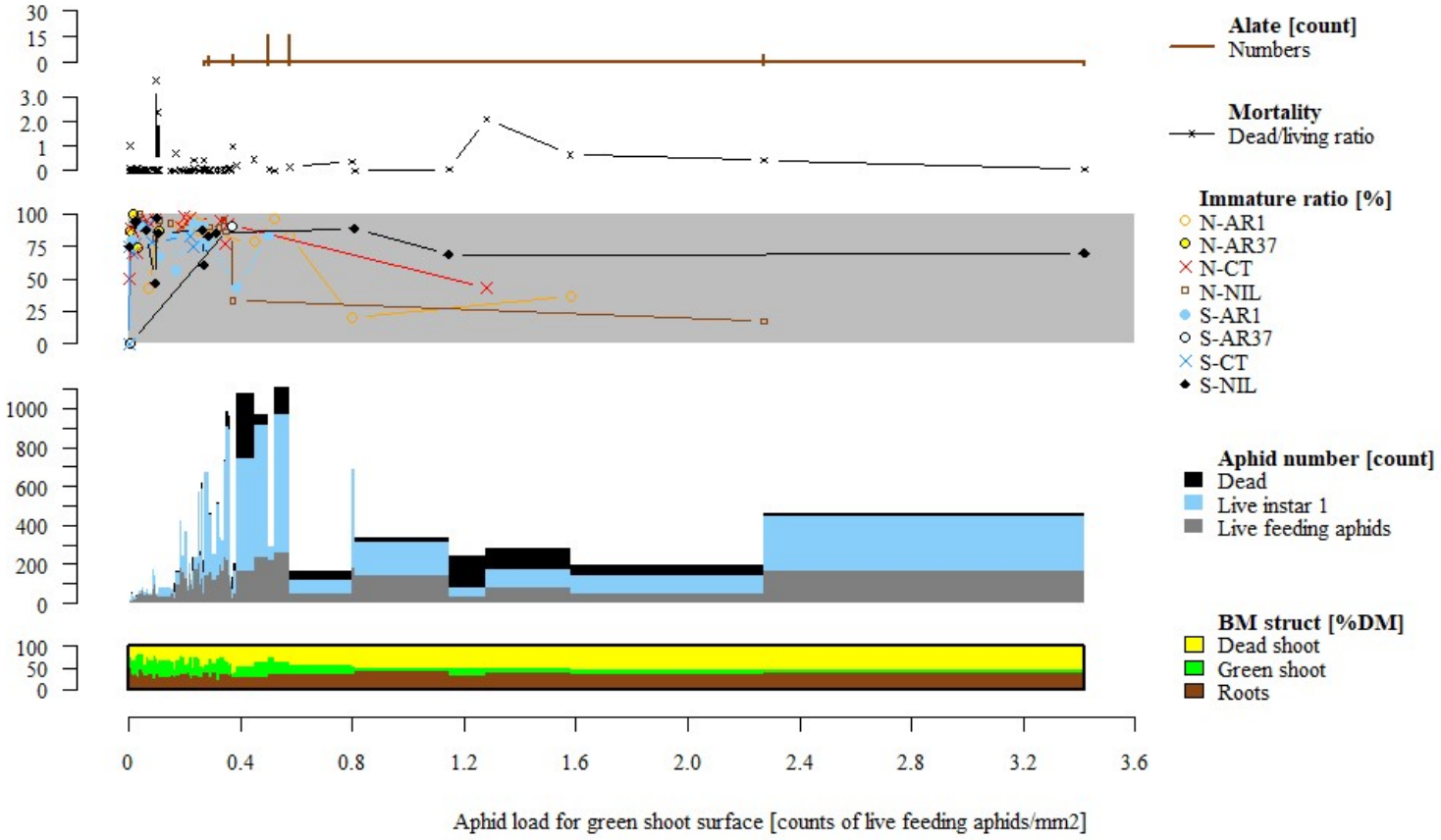


Figure A12.4.3. Colony development during the Population experiment, raw data by aphid loads on green shoot area [Feeding aphids by mm^2 green shoot at the final harvest, 38 to 111 days after aphid placement]. Both aphid treatments were pooled. The data are presented by Petri dish (raw data) pooling also the plant genotype-endophyte groups for all variables except the immature ratio. Column width: interval between measurement x and the next measurement. BM: biomass; DM: dry matter.