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

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Abstract

Fungal endophytes of the genus *Epichloë* often form stable, symbiotic, and mutualistic relationships with grasses of the Pooideae, including perennial ryegrass (*Lolium perenne* L.). The endophyte provides various benefits to its grass host, through the production of secondary metabolites, which are exploited in New Zealands pastoral agriculture systems. The endophyte can give its host grass an ecological advantage in certain challenging environments, such as during seedling establishment, where young plants are especially vulnerable to insect predation, such as feeding by adult Argentine stem weevil (ASW, *Listronotus bonariensis*).

This thesis focuses on understanding the alkaloid concentrations that occur in endophyte-infected perennial ryegrass seedlings during the early establishment phase. A glasshouse experiment was conducted in which fungal alkaloid concentrations (peramine, lolitrem B, ergovaline, and epoxy-janthitrems) were measured in perennial ryegrass seedlings infected with *Epichloë festucae* var. *lolii* strains AR1, AR37, NEA2, and NZ_{CT} for 69 days after sowing. From the data it is inferred that an initial translocation of alkaloids stored in seed during maturation into the developing shoot of the germinating seedling occurs, followed by a period of alkaloid dilution due to seedling expansion, and finally production of newly metabolised alkaloids in the plant. Alkaloid concentration were found to peak in 8–10 day old seedlings, giving the seedling a "kick start" in protection of the emerging seedling from adult ASW feeding during the first 11 days after sowing.

The influence of adult ASW feeding on alkaloid concentrations in endophyte-infected perennial ryegrass seedlings was also tested. The study demonstrated that adult ASW feeding can influence alkaloid production, although peramine, the main alkaloid responsible for adult

ASW deterrence was not significantly affected. Findings from this thesis improve understanding of the role of fungal alkaloids in endophyte-infected perennial ryegrass seedlings during establishment, and help explain results from earlier studies describing seedling susceptibility to adult ASW.

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Chapter 1 Introduction & literature review

New Zealand`s temperate climate enables livestock farming through pasture based grazing systems. Livestock usually graze throughout the year, with summer and autumn-stockpiled pasture fed in winter. Supplementary feeds derived from surplus pasture such as silage or hay, some temporary grazing of animals off farm, and some use of forage crops, is used to overcome seasonal feed deficits or unfavourable climate conditions. Pasture based systems offer a sustainable, efficient, low-price feed, making New Zealand one of the most competitive countries for food and fibre production. Therefore, New Zealand's economy heavily relies on productive pastures, making pasture husbandry an essential tool to enhance and maintain farm production.

The most commonly sown pasture type in New Zealand is a mixture of perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). Key factors such as pasture persistence, dry matter production, and botanical composition have a major impact on the earning potential from pastures. These factors can be influenced by environmental pressures, such as heat, moisture, disease, insect pressure, and by human factors such as grazing management, which can significantly affect pasture persistence and production (Tozer*, et al.*, 2011). However, one of the important factors for pasture persistence in New Zealand is the presence of an asexual fungal endophyte from the genus *Epichloë* (Popay and Hume, 2011). By producing secondary metabolites, particularly alkaloids, *Epichloë* fungi have developed a strategy to protect themselves and their host from predators such as herbivorous insects or mammals. One of these insect herbivores is Argentine stem weevil (ASW, *Listronotus bonariensis* Kuschel), a serious pest of New Zealand's improved pastures. However, some of these alkaloids are toxic to livestock (Gallagher*, et al.*, 1981), whereas others confer a

significant benefit by controlling pests and enhancing the productivity and survival of endophyte-infected pasture (Ball, *et al.*, 1995; Mortimer and Di Menna, 1983). The type of alkaloid produced by the fungus is a property of the strain itself. Endophyte-free grasses, which do not contain fungal alkaloids, are highly susceptible to some herbivorous insects resulting in high pasture losses (Thom, *et al.*, 2014), making endophyte infection and alkaloid production an essential part of New Zealand's farming environment.

In the 1980s, study of the role of endophytes in pasture systems led to the exploitation of endophytes that provide protection against insect pests, while minimizing or eliminating negative effects on grazing livestock. This biological defence has been commercially utilized by research providers such as AgResearch, and by seed companies which produce and market endophyte-infected seeds, contributing an estimated NZ$ 200 million dollars annually to New Zealand's economy (Johnson, *et al.*, 2013).

1.1 Perennial ryegrass pastures

New Zealand's most agriculturally important plant species were originally imported during the late 18th century and 19th century from Europe. One of these species is perennial ryegrass, a cool season grass member of the grass family Poaceae. Perennial ryegrass is a winter active grass with an abundant number of tillers. The leaves are hairless, smooth, narrow dark-green, which are shiny on the underside. Perennial ryegrass has a short ligule and small auricles in comparison to other grass species (Stewart, *et al.*, 2014). The base of the tiller is purple to bright red (Figure 1.1). The inflorescences of perennial ryegrass are awnless spikes and are comprised of individual spikelets which are sessile and arranged alternately along the spike (Figure 1.1).

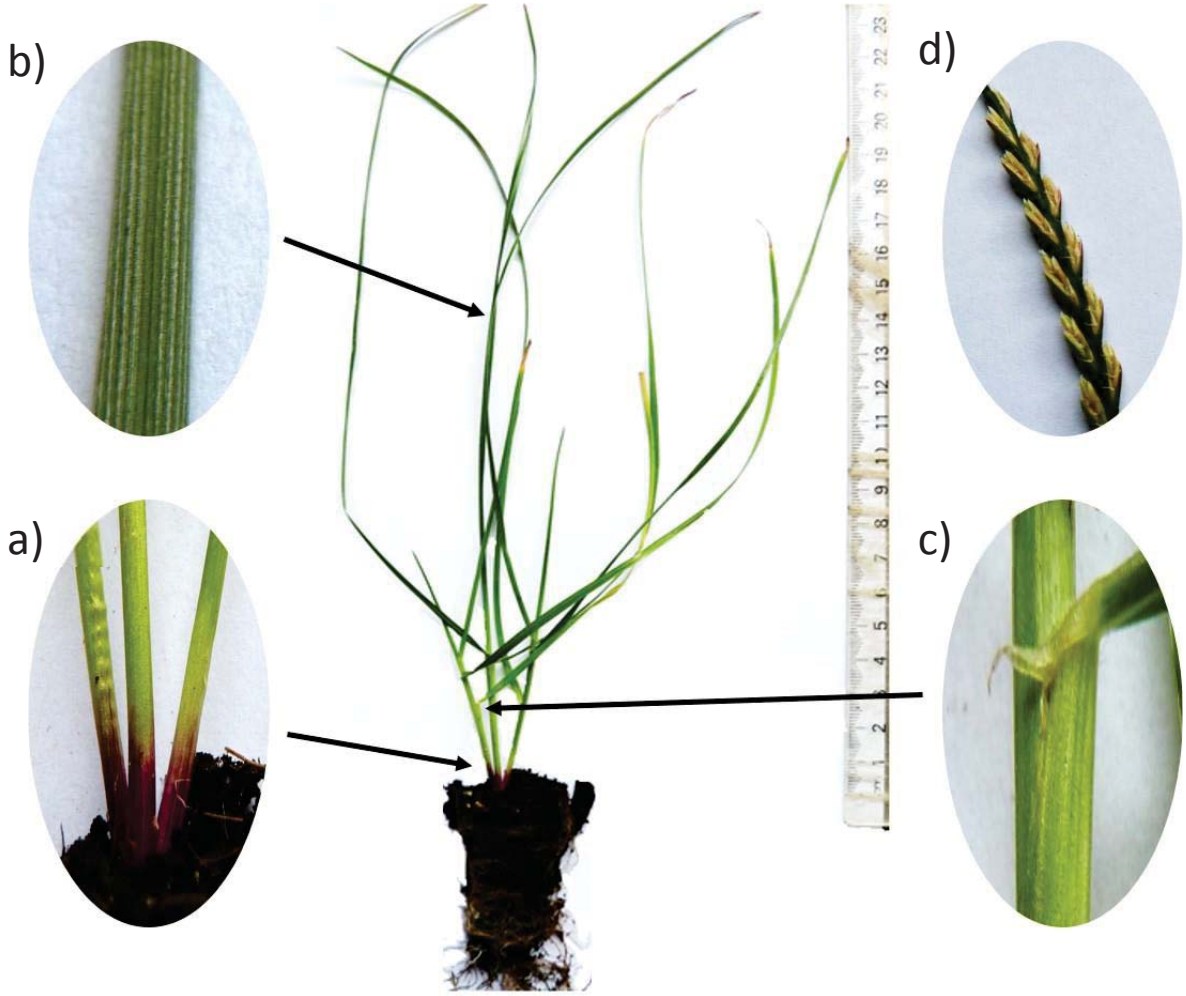


Figure 1.1: Growth characteristics of perennial ryegrass (*Lolium perenne* L.); a) The base of the tiller is purple to bright red, b) The leaves are hairless, smooth, narrow dark-green, which are shiny on the underside, c) short ligule and small auricles, d) inflorescences are awnless spikes.

So-called bush burn pastures in New Zealand essentially sought to recreate the community structure of a European meadow (Levy and Madden, 1933). Gradually practice evolved towards use of perennial ryegrass as a sole grass species with a clover as companion species for symbiotic nitrogen fixation and forage herbs such as chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.). For agricultural purposes, perennial ryegrass has gained

acceptance due to its high productivity on fertile soils, high nutritive value, palatability, grazing/treading tolerance, and fast establishment after sowing (Young, *et al.*, 2013). Perennial ryegrass can grow in a wide range of environmental conditions throughout New Zealand and is widely utilised (Stewart, *et al.*, 2014). However, it can perform poorly in extreme high or low temperatures or drought, and it is intolerant of low soil nutrient status. Perennial ryegrass is winter active and can yield up to 25t DM/hectare/ year (Stewart, *et al.*, 2014). Dry matter production is mainly influenced by temperature, available soil moisture, and soil fertility, as well as grazing management. Optimum temperature for perennial ryegrass is 18°C (Mitchell, 1956). Currently around 30 perennial ryegrass cultivars are commercially available (Stewart, *et al.*, 2014).

1.2 *Epichloë* endophytes

As mentioned, perennial ryegrass is commonly infected with an asexual fungal endophyte from the genus *Epichloë*, which significantly affects the performance of livestock and pasture persistence. Perennial ryegrass endophytes were unknowingly introduced to New Zealand inside imported seeds and can nowadays be found throughout New Zealand.

1.2.1 Taxonomy

Fungal endophytes of the genus *Epichloë* are grouped in the tribe Balansieae of the Clavicipitaceae family. *Epichloë* endophytes can infect a number of grasses of the subfamily Pooideae including perennial ryegrass, tall fescue (*Festuca arundinacea* syn. *Lolium arundinaceum* Schreb.), fine fescue (*Festuca* subgenus *festuca* L.), and meadow fescue (*Festuca pratensis* Huds.) (Christensen, *et al.*, 2002). The *Epichloë* genus is split into sexual (teleomorph) and asexual (anamorph) endophytes, with the latter being formerly described as *Neotyphodium* (and prior to that *Acremonium*). Nomenclature changes were made to improve the understanding of sexual and asexual *Epichloë* endophytes based on their similarities (Leuchtmann, *et al.*, 2014). Asexual *Epichloë* endophytes are host specific, meaning that each endophyte strain is adapted to live in a specific host (Schardl and Clay, 1997). The endophyte *Epichloë festucae* var. *lolii* Latch, Christensen, & Samuels specifically colonises perennial ryegrass, while *E. coenophiala* Morgan-Jones & W.Gams colonises tall fescue. However, of particular importance for this thesis is the asexual endophyte *E. festucae* var. *lolii* (formerly known as *Neotyphodium lolii*) which infects perennial ryegrass.

1.2.2 Biology of asexual *Epichloë* endophytes

Epichloë endophytes exclusively complete their entire life cycle within their host grass, never having a free-living state or phase. These fungi are transmitted vertically via hyphal colonization of the host's seed. Asexual *Epichloë* are obligate biotrophs and typically do not produce ascospores. The fungus within the seed is found between the scutellum of the embryo and the starchy endosperm, and within the remnants of the nucellus tissue, which lies between the seed coat (pericarp) and the aleurone cells (Card*, et al.*, 2011). The fungus penetrates through the rachilla at the base of the ovary in the ovular nucellus (Majewska-Sawka and Nakashima, 2004). Young embryos do not contain fungal endophyte hyphae, but as they mature the hyphae penetrate through the scutellum. The hyphae, 1–2µm wide, grow within the intercellular spaces without breaching the host cell walls, and are distributed unequally within the plant. The greatest concentration of endophyte hyphae in the vegetative plant is found in the base of the leaf sheath and the seed, where the mycelium is abundantly branched (Bacon and Siegel, 1988). The endophyte hyphae throughout the tiller are aligned parallel to the longitudinal leaf axis, and are seldom branched (Christensen*, et al.*, 2008). Hyphae are in close contact with the plant cells and appear to be firmly attached to the walls of mesophyll cells. Endophyte hyphae undergo intercalary elongation as the leaf cells expand, preventing fragmentation of hyphae as the plant grows (Christensen*, et al.*, 2008). No endophyte hyphae are found in the roots or pollen but hyphae are found in the radicle apex (di Menna*, et al.*, 2012; White and Morgan-Jones, 1987).

The growth rate of the fungus and host are completely synchronized, meaning that when the plant stops growing the hyphae between the plant cells stop growing as well, although the fungus remains metabolically active (Tan*, et al.*, 2001). During tillering the endophyte grows into the generative flower stem (EC 39–59 (Gustavsson, 2011; Majewska-Sawka and Nakashima, 2004). Once the endophyte has colonized the new seed it stays dormant until seed

germination. The life cycle of the endophyte starts again when the seed germinates (Figure 1.2).

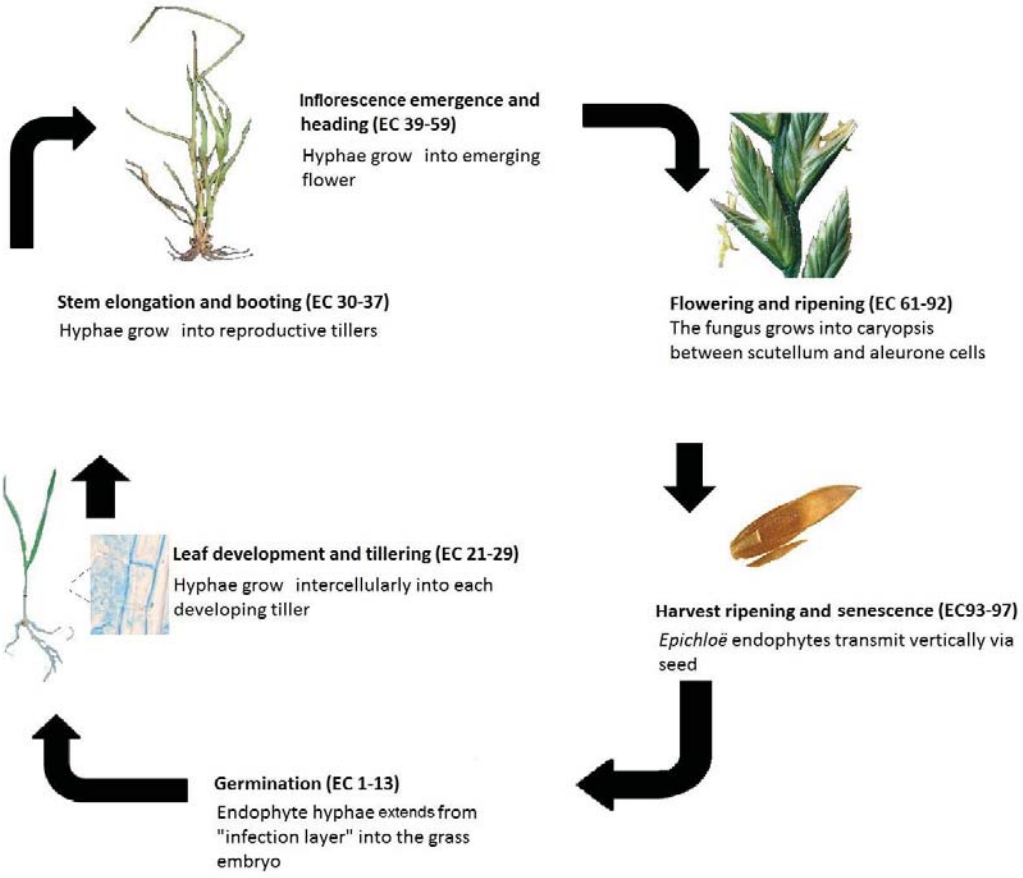


Figure 1.2: Life cycle of asexual *Epichloë* endophytes (Figure modified from Dapprich (1996)).

Asexual *Epichloë* endophytes are restricted to vertical transmission and completely rely on the host for reproduction and survival. However, vertical transmission of the fungus can be inadequate, with the result that not all seeds are infected (Wheatley, *et al.*, 2007). This occurs mainly if seed heads are harvested prematurely and endophyte fails to grow into all plant ovaries (Gundel, *et al.*, 2009).

1.2.3 Symbioses

The association of asexual *Epichloë* endophytes with host grasses is described as a mutualistic relationship (Bacon and Siegel, 1988). Both partners benefit; the plant supplies the endophyte with a habitat, long-term protection, nutrients, and a chance to reproduce. In turn, the endophyte enhances the plant's competitive ability to cope with biotic stress factors such as insects (Popay*, et al.*, 2012), pathogens (Pańka*, et al.*, 2013), mammalian herbivores (Fletcher and Sutherland, 2009), and nematodes (Bacetty*, et al.*, 2009) by producing specific alkaloids. Furthermore, endophyte infection can improve the ability of its host plant to cope with abiotic stress such as drought (Ravel*, et al.*, 1997). These benefits make endophyte-infected grasses more competitive than endophyte-free grasses.

1.3 Secondary metabolites

Grasses have relatively poorly developed defence mechanisms compared to other plant species and appear to optimise for herbivore tolerance, such as grazing tolerance, rather than chemical defence. However, endophytes produce a number of fungal bioprotective secondary metabolites, commonly known as alkaloids. These alkaloids are organic, nitrogen containing compounds that are not directly involved in cell function, development or reproduction of the host organism. Alkaloid concentrations within the plant are influenced by environmental factors, including soil moisture, nitrogen availability (Arechavaleta, *et al.*, 1992), and temperature (Huizing, *et al.*, 1991). Other factors such as host-endophyte interaction can also greatly alter the alkaloid concentration within the plant (Spiering, *et al.*, 2005), as well as host performance (Easton, *et al.*, 2002; Faeth, *et al.*, 2002). *Epichloë* endophytes are currently known to produce four main classes of alkaloids and these are expressed in various combinations and levels in different endophyte strains: indole diterpenes (lolitrems and epoxy-janthitrems), ergot alkaloids (clavines, lysergic acid, and derivative alkaloids), pyrrolopyrazine (peramine), and pyrrolizidines (lolines). However, lolines are not produced by *E. festucae* var. *lolii* and therefore play a minor role in this thesis. Some of the chemical structures are outlined in Figure 1.3.

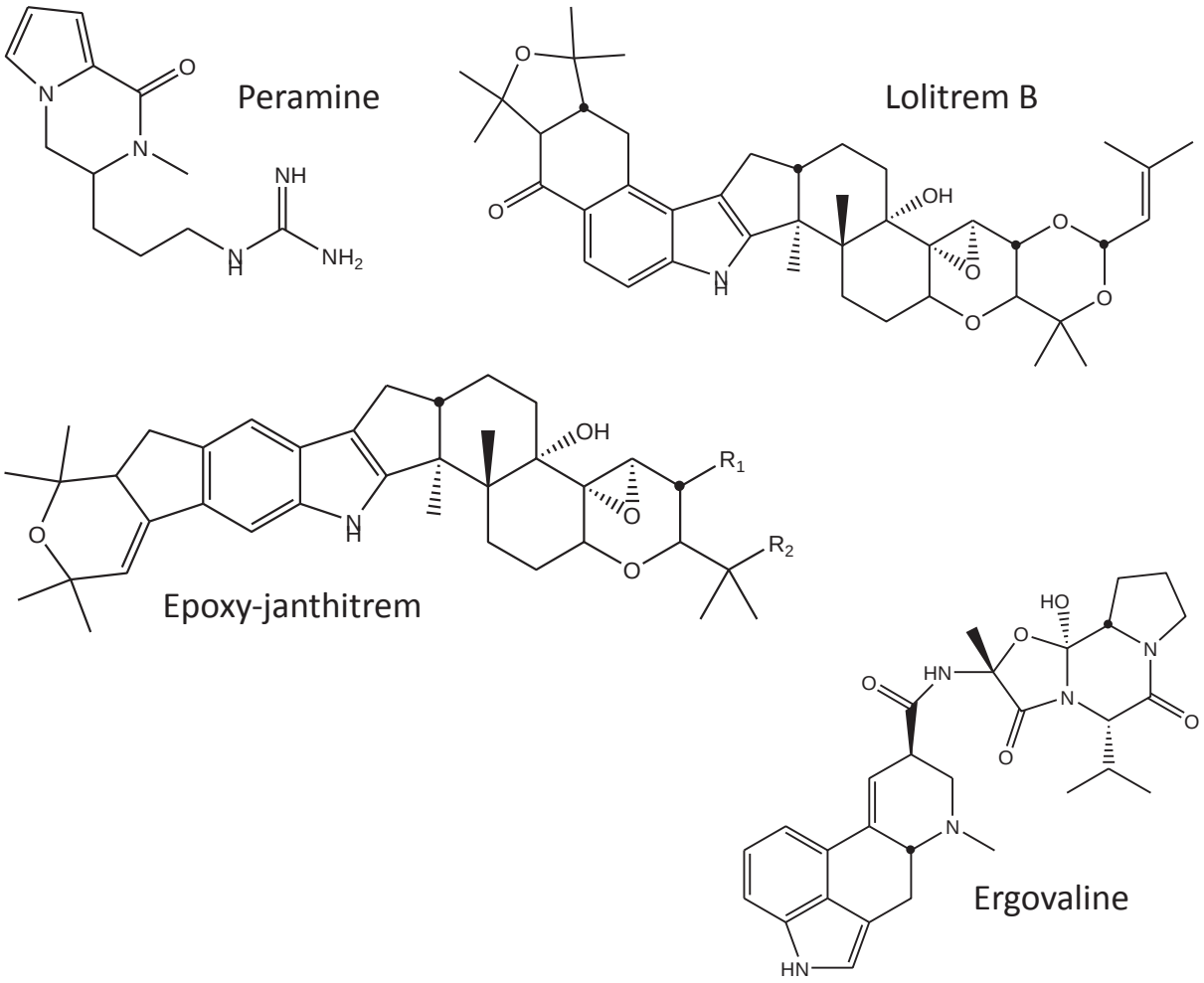


Figure 1.3: Chemical structures of alkaloids produced by asexual *Epichloë* endophytes. Epoxy-janthitrem structure shows the basic structure of the five main epoxy-janthitrem compounds. Modified from (Johnson, *et al.*, 2013).

1.3.1 Peramine

Peramine is a pyrrolopyrazine alkaloid made up of a lipophilic ring system and hydrophilic guanidinium group (Tanaka, *et al.*, 2005). Peramine is the only alkaloid in the pyrrolopyrazine group and can be produced by *E. festucae* var. *lolii* in ryegrass and *E. coenophiala* in tall fescue (Mortimer and Di Menna, 1983). This alkaloid is continuously produced by many endophyte strains and is a highly active feeding deterrent to insects such as ASW (Rowan and Latch, 1994). Peramine is translocated freely in the plant and can be found throughout the leaves and stems (Popay, *et al.*, 1990), but with a reduced presence in senescent leaf sheaths, the crown,

and roots (Davies, *et al.*, 1993). The highest peramine concentration can be found in seed, leaf sheaths, and leaf blades of vegetative tillers (Ball, *et al.*, 1997b). Peramine is hydrophilic and is mobilized by the host plant into plant fluids (Koulman, *et al.*, 2007). Concentrations in endophyte-infected ryegrass can reach between 10–50 µg/g dry weight (Ball, *et al.*, 1997b), and are dependent on the host genotype and endophyte strain (Latch and Tapper, 1988). There is a negligible peramine concentration in the roots of perennial ryegrass (Fannin, *et al.*, 1990). Peramine has no known health impacts on grazing mammals (Fannin, *et al.*, 1990).

1.3.2 Lolitrem B

Lolitrem B belongs to the indole diterpene class of alkaloids and is the most abundant of the lolitrem compounds (Miles, *et al.*, 1992) and is produced by some *E. festucae* var. *lolii* strains (Gallagher, *et al.*, 1985). Lolitrem B is the main causative agent of the mammalian disorder ryegrass staggers (Gallagher, *et al.*, 1981). The highest concentration of lolitrems can be found in the basal leaf sheaths and older leaves, where the abundance of fungal mycelium is the greatest (Keogh, *et al.*, 1996). Furthermore, lolitrem B concentration in the base of the plant can be three times higher than in other parts of the plant (Ball, *et al.*, 1997c). High concentrations can also be found in the outer leaf sheaths (Ball, *et al.*, 1997c), while concentrations in young plant tissue tend to be lower (Spiering, *et al.*, 2005). Concentration is higher in the pseudostem compared to leaf tissues (di Menna, *et al.*, 1992). Lolitrem B concentrations in endophyte-infected ryegrass pasture can average 0.2−2 µg/g dry weight (Gallagher, *et al.*, 1985), with peaks of up to 10 µg/g dry weight (Latch and Tapper, 1988). Lolitrem B concentrations of 2 µg/g in pasture or seed are considered sufficient to cause ryegrass staggers in sheep and cattle (Prestidge, 1989). Lolitrems can also be found in the roots

where endophyte is largely absent (Ball, *et al.*, 1997c). Lolitrem B contributes to plant resistance by reducing development of ASW larvae (Prestidge and Gallagher, 1985).

1.3.3 Ergovaline

Ergovaline is the main alkaloid of the ergopeptine group containing an ergoline ring. Its concentration is higher in the basal leaf sheaths, seed heads, and pseudostems than in other plant parts (Davies, *et al.*, 1993). Ergovaline can be produced by *E. festucae* var. *lolii* and *E. coenophiala* (Mortimer and Di Menna, 1983; Siegel, *et al.*, 1990). Ergot alkaloid concentrations in tall fescue and ryegrass seeds or herbage can reach 1.0–14 µg/g dry matter but are dependent on temperature, drought and nitrogen fertilization (Lyons, *et al.*, 1986). Ergovaline concentration is lower in perennial ryegrass compared to infected tall fescue (Lane, *et al.*, 1997). The main characteristic of the ergot alkaloids is their activity against vertebrates. Ergovaline is a vaso-constrictor leading to elevated body temperature and respiration rate of affected animals (heat stress). Furthermore, livestock grazing endophyte-infected tall fescue can suffer from fescue toxicosis (Lyons, *et al.*, 1986) and fescue foot (a condition where blood flow to the feet and tail is so restricted that tissue death occurs and gangrene may set in) (Bluett, *et al.*, 2005b). Ergovaline concentrations of 0.05 µg/g dry matter are considered sufficient to cause fescue toxicosis (Cornell, *et al.*, 1990). The threshold for fescue foot disease in combination with cooler temperatures is 0.4–0.75 µg/g dry matter for cattle and 0.5–0.8 µg/g dry matter for sheep (Tor-Agbidye, *et al.*, 2001). Ergovaline has been recorded to have similar concentrations in root tissue compared with above-ground plant pseudostem (Popay A., personal communication). Ergovaline concentration is not affected by ensiling pasture (Turner, *et al.*, 1993). Ergot alkaloids contribute to plant resistance to insects such as African black

beetle (*Heteronychus arator* Fabricius) (Belesky and Hill, 1997), and to nematodes (e.g. *Pratylenchus scribneri)* (Bacetty*, et al.*, 2009).

1.3.4 Epoxy-janthitrems

Epoxy-janthitrems are indole-diterpenoids and are similarly structured to lolitrem B (Rasmussen*, et al.*, 2009). To date, epoxy-janthitrems have only been found in an endophyte strain called AR37 in perennial ryegrass. Epoxy-janthitrems are a group of five compounds: epoxy-janthitriol, epoxy- janthitrem I, epoxy- janthitrem II, epoxy-janthitrem III, and epoxy-janthitrem IV (Tapper and Lane, 2004). Epoxy-janthitrems are less toxic to grazing mammals than the lolitrems. Their concentration in root tissue is lower relative to the above-ground plant material (Popay, A. personal communication). Epoxy-janthitrems have a low tremorgenic potential but can cause ryegrass staggers in sheep and horses grazing AR37 pastures (Fletcher and Sutherland, 2009). A relatively high concentration of epoxy-janthitrems can be found in AR37-infected perennial ryegrass during the summer and autumn months with peaks above 35 µg/g in perennial ryegrass (Finch*, et al.*, 2012). However, concentrations can reach up to 50 µg/g (Mace, W.: personal communication). Due to the variability in ryegrass staggers occurrence it is currently unknown at what concentration epoxy-janthitrems induce staggers (Finch, S.: personal communication).

1.4 Agricultural use of asexual *Epichloë* endophytes

As noted, New Zealand`s temperate climate enables livestock farming through a pasture based grazing system. In the early 1900s farmers noticed a disease which came to be named ryegrass staggers after livestock had been grazing on perennial ryegrass pasture (not to be confused with hypomagnesaemia). Around 80 years later the link between pasture infected with a toxic endophyte strain and ryegrass staggers was made (Fletcher and Harvey, 1981). Ryegrass staggers is a neurological disorder affecting sheep, cattle, horses, deer, and alpaca grazing on toxic endophyte-positive perennial ryegrass pastures (Prestidge, 1993). Ryegrass staggers has also been observed in a white rhinoceros (*Ceratotherium simum* Burchell) at Auckland Zoo after consuming hay infected with a toxic endophyte (Bluett, *et al.*, 2004). Affected animals suffer from severe muscular spasms resulting in poor coordination and a hypersensitivity to external stimulations. Ryegrass staggers can also reduce live weight gains (Fletcher and Barrell, 1984) and milk production in dairy cows (Bluett, *et al.*, 2005a). The disease itself does not kill, although in some cases death from falls or drowning in watercourses or creeks can occur (Mortimer and Di Menna, 1985).

Ryegrass staggers is caused by lolitrem B, which is produced by the endophyte (NZ_{CT}) commonly found in New Zealand (also called standard or wild type endophyte) (Johnson, *et al.*, 2013). This endophyte can be found in the majority of pastures sown prior 2001. Grasses infected with the NZ_{CT} endophyte strain have also been found to cause heat stress in grazing cattle, due to high levels of ergovaline (Fletcher, 1993). Affected animals can suffer from an elevated body temperature, restricted blood flow, reduced weight gain, reduced milk production, decreased fertility, high rectal temperatures, increased respiration rate, and the inability to dissipate body heat (Paterson, *et al.*, 1995; Strickland, *et al.*, 1996).

As soon as it was recognized that endophyte-infected pasture grasses were responsible for animal toxicoses, it was suggested that removal of the endophyte from the sward would alleviate animal disease symptoms and improve animal health and productivity (Bacon, *et al.*, 1981). However, it was subsequently found that endophyte-negative plants (in comparison with ryegrass infected with NZ_{CT}), were susceptible to predation such as from ASW (Prestidge, *et al.*, 1982), pasture mealy bug (*Balanococcus poae* Marksell) (Pennell, *et al.*, 2005), and adult African black beetle (Popay and Baltus, 2001). The insect resistance was attributed to alkaloids such as peramine, ergovaline, and lolitrem B produced by the endophyte (Ball, *et al.*, 1995).

Knowledge of the effects of endophyte-produced alkaloids has led to the selection of endophyte strains which retain insect resistance, but do not produce the mammalian toxins lolitrem B and ergovaline (or produce ergovaline at lower levels than NZ_{CT}, providing some protection against insects but which do not pose a major threat to livestock health). Grass hosts containing such endophytes have been obtained globally. Endophytes with a desirable alkaloid profile are isolated from their original host and used to produce synthetic host-endophyte combinations using an inoculation technique (Latch and Christensen, 1985). In this way suitable endophyte strains can be established in high-yielding pasture to create elite-forage germplasm (Easton and Fletcher, 2007). The first commercial products containing plant/endophyte associations that retain insect resistant properties, but with reduced impacts on animal performance, have been available since the early 1990s (Johnson, *et al.*, 2013). Although NZ_{CT} endophyte is still commonly found in older perennial ryegrass pastures, it has been superseded by other endophyte strains with equal or better insect protection, but fewer animal health issues in younger pastures.

Major commercially selected endophyte strains available in New Zealand include amongst others: AR1, AR37, and NEA2. Endophyte strain AR1, originally from Italy, was commercialised in 2001 by AgResearch Ltd., and is available in a broad range of perennial and hybrid ryegrass cultivars. This endophyte does not produce lolitrem B or ergovaline known for their mammalian toxicity, and therefore does not cause ryegrass staggers or heat stress (Fletcher, 1999). AR1 protects its perennial ryegrass host against ASW (Popay, *et al.*, 1999) as well as reducing pasture mealybug populations (Pennell, *et al.*, 2005), which led to a rapid uptake in the New Zealand pasture market (Milne, 2007). However, studies have shown that despite the optimal alkaloid profile against ASW, the protection from AR1 was inferior to the protection from the NZ_{CT} against adult African black beetle (Popay and Baltus, 2001). African black beetle can have a significant impact on pasture in the northern part of New Zealand. AR1-infected perennial ryegrass pasture is not recommended in regions with a high abundance of African black beetle such as the upper and coastal North Island in New Zealand. Furthermore, AR1 offers no protection against root aphids (Pennell, *et al.*, 2005), and in some cases increases plant susceptibility (Popay, *et al.*, 2004).

Another endophyte strain was released by AgResearch Ltd. in 2007 named AR37, which was discovered in France. Nowadays, AR37 is available in various perennial ryegrass cultivars. AR37 does not produce the alkaloids peramine, lolitrem B, or ergovaline. AR37 differs from other commercial endophyte strains in that it only produces alkaloids called epoxy-janthitrems (Tapper and Lane, 2004). Despite the lack of peramine, AR37 gives protection against ASW larvae (Popay and Wyatt, 1995), African black beetle (Ball, *et al.*, 1994), pasture mealybug (Pennell, *et al.*, 2005), and root aphid (Popay, *et al.*, 2004), as well as porina (*Wiseana* spp. Walker) (Jensen and Popay, 2004). In some trials, perennial ryegrass infected with AR37 has exhibited increased dry matter production of up to 36%, compared to the same ryegrass cultivar

infected with other endophyte strains (Hume, *et al.*, 2007). Pastures infected with endophyte strain AR37 are nowadays widely distributed throughout New Zealand and the financial benefit derived from this strain is estimated by the entity that markets it to have added NZ$42 million dollars revenue to New Zealand`s farming sector (Caradus, *et al.*, 2013).

NEA2 is another commercially available endophyte strain that has been on the market since 2002 (Agriseeds Ltd.). NEA2 is used in association with the tetraploid cultivar Bealey and the diploid cultivar Trojan. NEA2 seed mixes are known to contain at least two different endophyte genotypes, listed separately in the NZ Plant Variety Rights system as NEA6 and NEA2 (New Zealand Agriseeds Ltd., 2013). NEA2 endophytes provide resistance to African black beetle, pasture mealy bug, ASW, and partially to root aphid (Johnson, *et al.*, 2013). NEA2 endophytes can cause ryegrass staggers in ewe lambs and hoggets (Logana, *et al.*, 2015). Some staggers were also observed in elks (*Cervus canadensis* Erxleben) after grazing NEA2-infected pastures (New Zealand Agriseeds Ltd., 2013).

Evidence for endophytes improving ryegrass persistence, induced by the deterrent effect on herbivorous insects is substantial (Popay and Hume, 2011). In order to gain maximum benefit, it is essential to choose the right endophyte strain considering the area, environmental conditions, and insect pest pressure. However, currently no endophyte strain is known to deter all major pasture insects, without effecting livestock production. Furthermore, farmers as well as seed producers face the challenge of reducing contamination of the selected endophyte with the NZ_{CT} strains (Dombrowski, *et al.*, 2006). In New Zealand, traded endophyte-infected seed mixtures can have a maximum of 5% contamination with NZ_{CT} and a minimum of 70% viable endophyte infection rate. However, no official seed certification scheme for grass

variety/endophyte combinations exists (Rolston and Agee, 2007). A summary of insect control of commercial endophytes in perennial ryegrass is given in Table 1.1.

Table 1.1: Summary of most common pasture insect pests in New Zealand and the ability of specific endophyte strains to control them (Table adapted from Stewart, *et al.* (2014). Pictures: AgResearch, Te Ara, Peter Bailey).

Insect species		AR1	NEA2	AR37	NZ_{CT}	Endophyte-free
Argentine stem weevil		++++	+++	++++ [1]	++++	-
Pasture mealy bug		++++	(++++)	++++	++++	-
African black beetle		+	+++	+++	+++	-
Root aphid		-[2]	++	++++	++	-
Porina		-	Not tested	+++	+	-

-	No control
+	Low level of control: Endophyte may provide a measurable effect, but is unlikely to give control in the field
++	Moderate level of control: Endophyte may provide some protection in the field, with a low to moderate reduction of pest population
+++	Good level of control: Endophyte significantly reduces insect damage under low to moderate insect pressure. Damage might occur during high insect pressure
++++	Very good control: Endophyte significantly reduces insect damage and pest population even under high pest pressure
( )	Provisional rating: Testing is ongoing, further data is required to support rating
1	AR37 only deters the more damaging ASW larvae not adult
2	AR1 plants are more harmed than plants without endophyte

1.5 Pasture renewal and seedling establishment

Pasture renewal is a major component in farm management practises to increase pasture and farm production (Glassey, *et al.*, 2010). It offers short and long term benefits through improved plant genetics and plant breeding. Pasture renewal includes the replacement of an existing low value pasture by one with a preferred plant species or a mixture that increases pasture productivity and long-term pasture persistence. In many farming situations pasture production deteriorates over time, where heavy weed infestation, poor yield, or removal of NZ_{CT} are the biggest drivers behind pasture renewal.

Perennial ryegrass is the most vulnerable at its seedling stage, and insect predation at an early age can have a significant long-term effect on pasture persistence and plant community composition (Thom, *et al.*, 2011). In farming situations, seeds are usually treated with an insecticidal, fungicidal, and occasionally nutrient, or growth-hormone based seed coat (seed treatment) to protect the vulnerable seed/seedling from insect pest and diseases during germination and early establishment.

Newly-sown untreated endophyte-free ryegrass pastures can be seriously affected by adult ASW (Stewart, 1985), greatly affecting pasture establishment (Pottinger, 1961). Detailed information about the impact of adult ASW and the role of alkaloids in developing seedlings is discussed in Chapter 4.

1.6 Alkaloids in seed and seedlings

Alkaloids are responsible for endophyte-induced insect resistance in mature plants (Bush, *et al.*, 1997). However, the endophyte and its alkaloids also play an important role in endophyte-infected seeds and developing seedlings. Endophyte infection significantly reduces seed predation by insects (Popay, *et al.*, 2000) and birds (Madej and Clay, 1991), who concentrate their feeding on seeds lacking the fungus. Resistance to seed predation is due to high alkaloid concentrations in seed, for example concentrations of peramine (Ball, *et al.*, 1997b) and lolitrem B (Ball, *et al.*, 1997c) can be greater in seeds than in mature plants. High alkaloid concentrations in seeds may have arisen as a defence against seed-and/or seedling-eating herbivores (Knoch, *et al.*, 1993), and are a function of a large quantity of endophyte mycelium in the seed (Ball, *et al.*, 1994).

While the endophyte survives indefinitely in the vegetative plant (Bacon and Siegel, 1988), the survival of the endophyte within the seed is not indefinite. In general, endophyte viability in seed decreases faster than the seed viability itself (Hume, *et al.*, 2011). The survival of the fungus is dependent on the length of storage time, seed moisture content, as well as storage temperature and relative humidity (Siegel, *et al.*, 1985). Seeds containing a non-viable endophyte still maintain high alkaloid concentrations (Ball, *et al.*, 1993) protecting young seedlings from insect attack for a short period, estimated to be approximately 5 days after emergence (Stewart, 1985). After the initial resistance, seedlings growing from seed infected with a non-viable endophyte will grow into ASW-susceptible mature plants. The loss of an initial resistance to ASW adult feeding was reported, even before the discovery of the effects of endophyte (Trought, 1976).

Perennial ryegrass seedlings infected with viable endophyte also possess a limited level of resistance to adult ASW. Seedlings infected with peramine-producing endophyte strains possess an initial resistance against adult ASW, after which seedlings were found to be increasingly susceptible to feeding until about 8 weeks after planting (Ruppert*, et al.*, 2016).

While the pattern of growth synchronization between the fungus and mature host and alkaloid production in normal vegetative growth are reasonably well understood (Christensen*, et al.*, 2008; Tan*, et al.*, 2001), it is less well known how the endophyte colonises young seedlings. Furthermore, little research has been done investigating the alkaloid concentrations in endophyte-infected seedlings that could explain the earlier documented insect susceptibility in endophyte-infected seedlings. A delay in alkaloid production possibly occurs at a stage when seed-borne endophyte alkaloids have been diluted by seedling expansion and the seedling endophyte is not yet producing its own alkaloids. This would result in a period of seedling vulnerability to adult ASW, when concentrations of alkaloids such as peramine fall below the estimated adult and larvae ASW field threshold of proposed 15–20 μg/g DM (Popay and Wyatt, 1995), and new alkaloid production has not commenced.

1.7 Objectives and thesis aims

This thesis will focus on

i) determining how alkaloid concentrations change in developing endophyte-infected perennial ryegrass seedlings for up to 69 days after planting

ii) investigating if adult ASW feeding on developing endophyte-infected seedlings influences the alkaloid production

The first part of this thesis describes work aimed at measuring alkaloid levels during the early establishment phase of perennial ryegrass seedlings infected with different endophyte strains. The research involved regular sampling of ryegrass seedlings containing commercial endophyte strains AR1, AR37, and NEA2, and compared the alkaloid concentration during establishment with that of NZ_{CT}-endophyte infected seedlings. The expressed alkaloid concentration of peramine, ergovaline, lolitrem B, and epoxy-janthitrems was measured *in planta*. Such information was expected to be valuable because endophyte alkaloid concentration is indicative of the bioprotection provided by these alkaloids against potential insect herbivores. A working hypothesis was that stored fungal alkaloids in the seed are diluted by seedling expansion during germination, and that the alkaloid concentration increases only after the endophyte becomes metabolically active. The research was expected to define the time courses for these processes.

The second part of this thesis explored the possibility that adult ASW feeding on developing seedlings influences the alkaloid production of endophyte-infected seedlings compared to those grown without insect predation. Information obtained from this study provides information about the ability of endophyte-infected, untreated seedlings to protect its host grass during

establishment. The hypothesis is that endophyte-infected seedlings attacked by adult ASW during establishment have an increased alkaloid production compared to seedlings grown without insect pressure.

Both questions are of relevance to pasture establishment practices in New Zealand and may provide specific information useful for minimising seedling damage from insect attack, thereby maximising pasture establishment and subsequent yield.

1.8 Thesis structure

This general introduction and review of the literature on asexual *Epichloë* endophytes, their alkaloids and their importance in New Zealand agricultural systems (Chapter 1), is followed by Chapter 2 describing the general methods used in both experiments. Chapter 3 investigates how alkaloid concentrations change in endophyte-infected perennial ryegrass seedlings during the early establishment phase. Chapter 4 explores if insect pressure in the form of adult ASW feeding influences the alkaloid production in endophyte-infected seedlings. Chapter 5 and Chapter 6 cover a general discussion as well as conclusions from this thesis.

Chapter 2 General Materials and Methods

This chapter describes the materials and methods which are in common to both experiments in this thesis. Additional detailed methodology for each experiment is found in 3.2 and 4.2.

2.1 Cultivars

2.1.1 One50

One50 is a diploid late heading perennial ryegrass developed by PGG Wrightson Seeds Ltd.. The cultivar was obtained from crossings between Spanish and New Zealand germplasm (Agricom, 2012), and is closely genetically related to cultivar "Tolosa" bred by NZ Agriseeds Ltd. (Wang, *et al.*, 2014). One50 is reported to be selected for Australian conditions with improved tolerance to crown rust (*Puccinia coronata* Corda) and stem rust (*P. graminis* Pers.), and high dry matter production over a long period (Agricom, 2012). One50 is commonly used for rotational grazing, as it is late flowering (20 days later than benchmark cultivar Nui), and remains vegetative up until late-spring and early-summer. One50 is recommended by the breeder to be sown in the field at 18–22 kg/ha along with white clover, red clover, and plantain.

2.1.2 Trojan

The diploid Trojan ryegrass is classified as *Lolium boucheanum* due to the low incidence of tip awns on some seed. Functionally, Trojan performance is similar to perennial ryegrass (Stewart, *et al.*, 2014). The cultivar Trojan has been bred through re-selection from cultivar Tolosa. Trojan is recommended by breeders to be sown in the field at 18–22 kg/ha along with white clover, red clover and plantain.

2.2 Endophyte strains

Various endophyte strains within perennial ryegrass are currently on the market. The endophytes chosen for this study are successfully established in the pasture market in New Zealand and Australia (Table 2.1). Endophyte strains AR1 and AR37 were inoculated into the elite forage cultivar One50. Endophyte strain NEA2 was not inoculated into its host but was present as a natural symbiont in maternal parents at the foundation of the breeding programme and inherited through the various stages in the breeding of Trojan. NEA2 seed mixes are known to contain at least two different endophyte. Endophyte strain NZ_{CT} is commonly found in New Zealand pastures, although it is nowadays largely replaced by more animal friendly endophyte strains. The endophyte strain-host combinations used in this research with their alkaloid profiles are outlined in Table 2.1.

Table 2.1: The occurrence of peramine, lolitrem B, epoxy-janthitrems and ergovaline in commercial ryegrass endophyte associations used in this experiment. Constitution and comparison of alkaloid concentration are estimated on a mid-summer harvest. Peramine: high (+30µg/g), low/medium (2.5–15µg/g). Lolitrem B: medium (0.1–1.0µg/g), high (1.0–5.0 µg/g). Epoxy-janthitrems: medium (20–50µg/g). Ergovaline: medium (0.2–1.0µg/g), high (1.0–2.5µg/g) (Wade Mace, personal communication).

Alkaloid classification	**Alkaloid compound**	**endophyte strain-host combination**			
		AR1 *One50*	AR37 *One50*	NEA2 *Trojan*	NZ_{CT} *One50*
Pyrrolopyrazine	Peramine	high	-	low/medium	high
Indole diterpenes	Lolitrem B	-	-	medium	high
	Epoxy-janthitrems	-	medium	-	-
Ergot alkaloid	Ergovaline	-	-	medium	high

- Not produced by this endophyte

2.3 Tissue print immuno blot method of endophyte detection

The viable *E. festucae* var. *lolii* infection frequency of the perennial ryegrass plants was confirmed by a tissue print immunoblot method similar to the method described by Simpson, *et al.* (2012). A positive and a negative control tiller were blotted onto the membrane for comparison using plants of known endophyte status. Blotted membrane was stored in a refrigerator at 4˚C until it was processed. Blot sheets were developed within three weeks. Plants with ambiguous immunoblot results were checked by microscopy of leaf sheath material (Card, *et al.*, 2011).

2.4 Alkaloid extractions

Harvesting techniques and sample preparation for each experiment are described in detail in chapters 3.2.1 and 4.2.2. Generally, fresh plant material was shock frozen with liquid nitrogen, freeze dried, and then ground using a modified blade-style coffee grinder and stored at -20°C. Approximately 50 mg (±5 mg) of lyophilized, ground seedling sample was weighed into a weighed 2 mL screw cap vial along with two 3 mm stainless steel beads. Samples were ground further using a bead ruptor (FastPrep FP120, Savant Instruments Inc., Farmingdale, NY, USA) (10 s at 5 m/s) to increase the efficiency of alkaloid extraction. The exact sample weight was recorded enabling calculation of the alkaloid concentration in ppm (µg/g).

Ergovaline was extracted from seedling material using a similar method to Moore*, et al.* (2015). 1 mL of prepared extraction solvent was added to each sample (50% methanol, 0.54 µg mL^{-1} ergotamine tartrate (Sigma Chemical Co., St. Louis, MO, USA) as an internal standard[1]). All suspensions were mixed in the dark using an overhead shaker at 30 rotations/min for 1 h at ambient temperature. Samples were then centrifuged (5000 g, 5 min) and the aqueous layer was transferred via a 0.45 µm syringe filter (PVDF) into 2 mL HPLC vials for analysis. Ergovaline was analysed by HPLC using a Gemini-NZ C18 150 x 2.0 mm (3 µm) column (Phenomenex, Torrance, CA, USA). Targeted analytes were detected with a Shimadzu RF-10Axl fluorescence detector (excitation at 310 nm, emission detected at 410 nm). A linear gradient profile (eluent A = 10 mM ammonium carbonate with 20% acetonitrile (v/v), eluent B = acetonitrile; T_0 = 8% B, T_9 = 25% B, $T_{7.5}$ = 60% B, T_8 = 83.3% B, T_9 = 83.3% B followed

[1] An internal standard is a chemical compound that is very similar, but not identical to the alkaloid of interest in the sample. An internal standard is correcting for the loss of alkaloids during sample preparation and is important to calculate the concentrations from a calibration curve.

by equilibration to primary conditions over the following 9 min) was used to separate targeted analytes at a flow rate of 200 µL/min. The limit of quantification was 0.1 µg/g DM (0.1 ppm).

Peramine was extracted from seedling material using a method similar to that of Moore*, et al.* (2015). Samples were extracted as described previously using an extraction solvent (50% methanol with 1.70 ng mL^{-1} homoperamine nitrate (AgResearch Grasslands) as an internal standard). All suspensions were mixed in the dark using an overhead shaker at 30 rotations/min for 1 h at ambient temperature. Samples were centrifuged and the aqueous layer transferred into 2 mL HPLC vials as described previously. Targeted analytes were separated through a Synergi Polar-RP 100 x 2.00 mm (2.5 µm) column (Phenomenex, Torrance, CA, USA) using a linear gradient profile (eluent A = aqueous 0.1% formic acid, eluent B = acetonitrile; T_0 = 5% B, T_9 = 40% B, T_{11} = 90% B, T_{12} = 90% B, followed by equilibration to initial conditions over the next 8 min). Quantitation was achieved using mass spectroscopy (MSQ, Thermo Scientific) with the parameters described in Rasmussen*, et al.* (2012). The limit of quantification was 0.1 µg/g DM (1 ppm) with a 5 µL injection volume.

Epoxy-janthitrems produced by the endophyte strain AR37 were extracted using a modified method from Moate*, et al.* (2012). To each lyophilized, ground seedling sample (50 mg) 1 mL of extraction solvent, made out of 80% acetone and 0.512 µg mL^{-1} BzNI as an internal standard, was added and spun on an orbital shaker for 1 h in dark (30 rotations/min). Samples were centrifuged and the aqueous layer transferred into HPLC vials as described previously. Chromatographic separation was obtained using a reverse phase 4.6 x 250 mm (5 µm) ODS C18 column (Phenomenex, Torrance, CA, USA) through an isocratic flow of water-acetonitrile suspension (1:19, 1 mL/min). The injection volume was 5 µL. Compounds were quantified by HPLC-fluorescence (Dionex ultimate 3000) (excitation at 333 nm, emission detection at 385 nm). The limit for quantification was 0.1 µg/g DM (0.1 ppm).

Lolitrem B was extracted from seedling material using the method of Moore*, et al.* (2015). Each sample of lyophilized, ground material was extracted for 1 h in the dark with 1 mL of 2:1 dichloromethane/methanol. Samples were centrifuged and the aqueous layer transferred as described previously. Targeted analytes were separated using an isocratic flow (1 mL/min, 80% dichloromethane/ 20% acetonitrile) using a Luna Silica 250 x 2.0 mm (5 µm) column (Phenomenex, Torrance, CA, USA). The lolitrem B peak was detected with a Shimadzu RF-10Axl fluorescence detector (emission detection at 410 nm, excitation at 260 nm). The limit of quantification was 0.1 µg/g (0.1 ppm).

Identification of ergovaline, lolitrem B, peramine, and epoxy- janthitrems was confirmed through comparison of the acquired spectra with known spectra for authentic standards routinely analysed at AgResearch, Grasslands New Zealand. Quantification was achieved through comparison with calibrated reference samples.

2.5 Data processing

Data processing of raw chromatographic output was achieved using a range of proprietary data processing software. Peramine data was processed using Thermo LCuan version 2.7.0 SP1.28 software (Thermo Scientific, MA, USA). Ergovaline and lolitrem B data was processed using LabSolutions software (3.20.216, Shimadzu Corporation, Japan). Epoxy-janthitrem data was processed using Thermo Scientific Dionex Chromeleon 7 version 7.2.0.3765 software (Thermo Scientific, MA, USA).

Each alkaloid produced a chromatographic peak that was integrated and the area used to determine concentration of the alkaloids. Calibration curves had previously been prepared, and were used in conjunction with the peak areas of the standards (internal or external) and target alkaloids to determine the concentration (Figure 2.1).

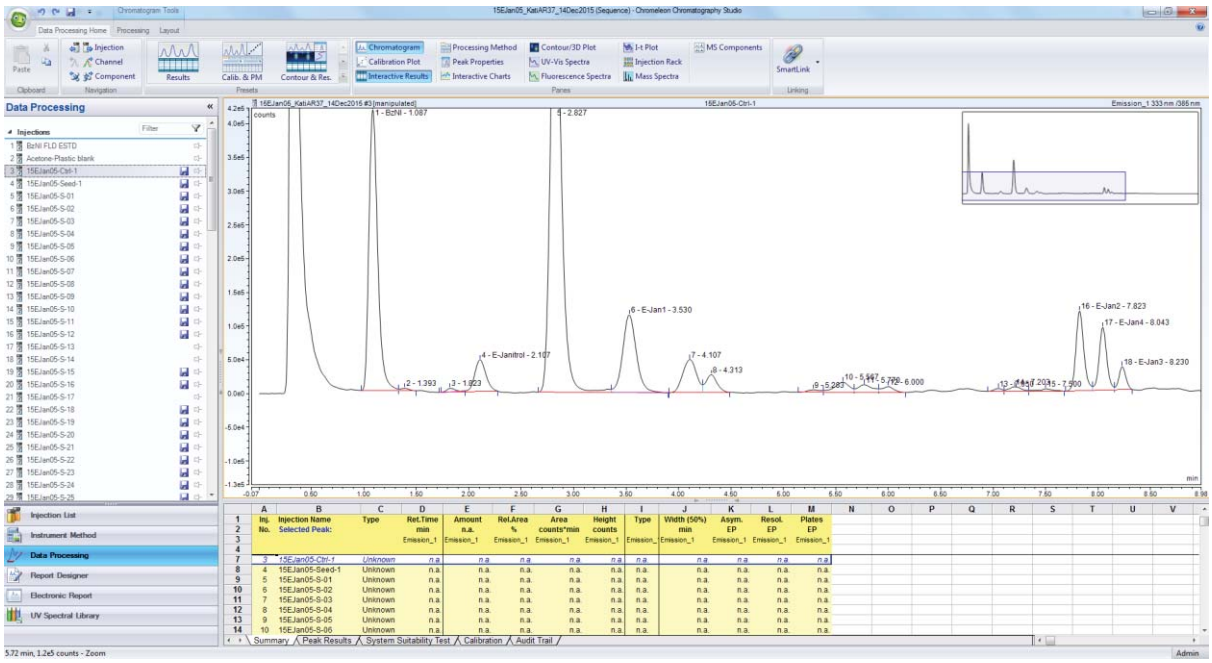


Figure 2.1: Data processing of epoxy-janthitrem peaks using Chromeleon 7 software.

Chapter 3 Alkaloid concentrations in perennial ryegrass seedlings infected with *E. festucae* var. *lolii*

3.1 Introduction

Mature endophyte-infected perennial ryegrass can produce a wide range of alkaloids. The combination of alkaloid compounds produced is strain dependent. However, alkaloid concentrations can be affected by many factors including host genotype, nutrient availability to the host, and environmental conditions (Rasmussen, *et al.*, 2009). Information on alkaloid identity and concentrations in plants are important for determining the ability of different *Epichloë* strains to deter herbivorous insects. Choice feeding experiments with artificial diets give valuable information regarding what alkaloid compound, and at what concentration, a specific insect species is affected (Prestidge and Gallagher, 1988). For example adult African black beetles are deterred from feeding on artificial diets containing 5 µg/g ergovaline, but feed freely on artificial diets containing lolitrem B or peramine with the same concentrations (Ball, *et al.*, 1997a).

Little research is available regarding what role alkaloids play in developing endophyte-infected perennial ryegrass seedlings. Before the discovery of the effects of endophyte on ASW, seedling resistance to this insect was often found to be limited to the early stages of seedling emergence (Goldson and Penman, 1979; Kain, *et al.*, 1982), lasting about five days after seedling emergence when adult ASW were present (Stewart, 1985). As the seedling ages it can be seriously damaged by adult ASW for up to 46 days, even though the seedling is infected with a peramine-producing endophyte (Ruppert, *et al.*, 2016). A previous study has shown that

alkaloid concentrations of endophyte-infected ryegrass seedlings decreased as seedlings aged (7 to 35 days after emergence) (Qawasmeh, 2012). Further studies were needed to determine alkaloid levels beyond 35 days post emergence. This will clarify the time course of changes in alkaloid concentrations during seedling development to capture information on when the endophyte becomes metabolically active and starts producing new alkaloids. Therefore, this study was designed to determine alkaloid concentrations in endophyte-infected perennial ryegrass seedlings up to 69 days after planting in order to predict possible impact on adult ASW feeding on developing seedlings.

3.2 Materials and Methods

3.2.1 Experimental design

The experiment was conducted over 10 weeks (69 days) during the winter months from 30th July to 7th September 2015 in a glasshouse located on the AgResearch Grasslands Campus, Palmerston North, New Zealand. Perennial ryegrass seedlings, infected with various endophyte strains, (Table 2.1) were regularly harvested and tested for their alkaloid concentrations. The experiment was set up in three parts (Table 3.1) to meet the growth requirements of seedlings. The first alkaloid measurements were taken from seeds, followed by seedlings germinated in petri dishes and then plants grown from seeds sown in propagation trays filled with standard seed raising mix, enabling analyses of alkaloid concentrations over the early establishment phase.

Table 3.1: Alkaloid measurements in developing perennial ryegrass seedlings infected with *E. festucae* var. *lolii* were set up in three parts. The first alkaloid measurement was conducted on seeds followed by seedlings grown in petri dishes and plants grown from seed sown in propagation trays filled with standard seed raising mix.

Seedling age in days when harvested (days post planting)	**Experiment type**
0	Seeds
6	Petri dish
8	Petri dish
10	Petri dish
13	Grow-out
20	Grow-out
27	Grow-out
34	Grow-out
41	Grow-out
48	Grow-out
55	Grow-out
62	Grow-out
69	Grow-out

3.2.2 Seed experiment

To determine the initial alkaloid concentration in endophyte-positive, developing perennial ryegrass seeds, 18 seeds per endophyte treatment were placed into 2 mL screw cap vials and shock frozen in liquid nitrogen. Samples were lyophilized for 72 h (Labconco, Free-Zone Plus, Model 7752030, Kansas, USA) and a total dry-weight for each treatment was recorded.

3.2.3 Petri dish experiment

To determine alkaloid concentration in germinating seedlings, a petri dish experiment was set up to obtain sufficient mass for chemical analyses. Fifty seeds of each endophyte treatment were germinated in sterile plastic petri dishes lined with moist filter paper (90mm). Seeds were regularly watered using tap water and germinated under glasshouse conditions. At each harvest the numbers of germinated seedlings were recorded to allow a calculation of alkaloid expression on a per seedling basis. The developing shoots were severed from the caryopsis using a scalpel 6, 8, and 10 days after planting (Table 3.1). Shoots were placed into 2 mL screw cap vials and shock frozen in liquid nitrogen (Figure 3.1). Samples were lyophilized for 72 h (Labconco, Free-Zone Plus, Model 7752030, Kansas, USA) and a total dry-weight for each treatment was recorded.

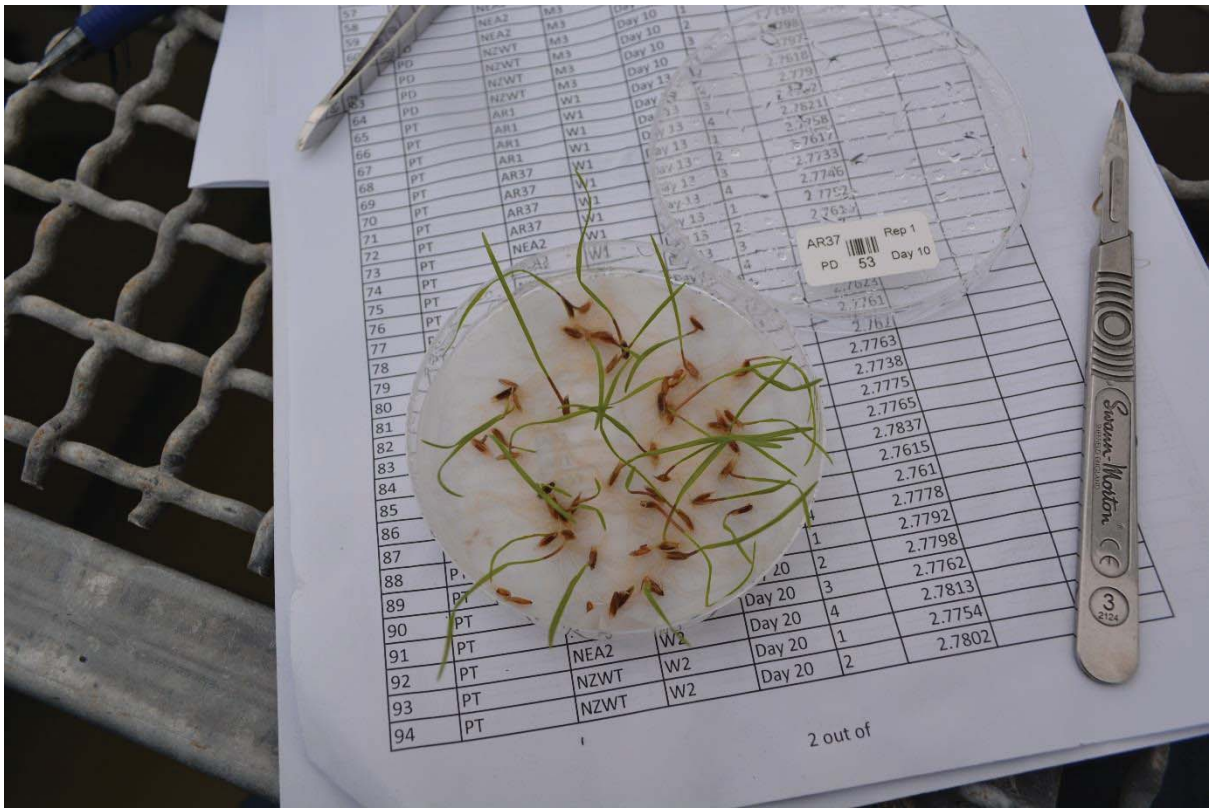


Figure 3.1: Harvesting 10-day old perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strain AR37, which had been germinated in petri dishes, for alkaloid analysis.

3.2.4 Grow-out experiment

The grow-out experiment was set up in 12 x 12 plastic propagation trays (40 x 40 x 5 cm). One seed per plug was placed in standard seed raising mix (75% screened fine bark, 12.5% coir fibre, 12.5% pumice; NPK 16-3.9-10) and germinated under glasshouse conditions for 13 days. Seedlings were harvested weekly from day 13 until day 69 post planting (Table 3.1). Seedlings within a treatment replicate were trimmed at ground level with a scalpel and transferred into either 2 mL, 5 mL, or 50 mL screw cap vials, depending on the plant size, and shock frozen in liquid nitrogen. Each sample consisted of bulked material from 18 planted seeds. At each harvesting time point the number of emerged seedlings was recorded to allow a calculation of alkaloid expression on a per seedling basis. Samples were lyophilized for 72 h (Labconco, Free-

Zone Plus, Model 7752030, Kansas, USA), and a total dry-weight for each treatment block was recorded.

Figure 3.2: Harvesting 13-day old perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strains AR1, AR37, NEA2, and NZ_{CT} from the grow-out experiment for alkaloid analysis into 2 mL screw cap vials.

All plants were maintained in the glasshouse under natural light conditions. Throughout the experiment, air temperature and relative humidity were recorded at 10 minute intervals using a Digitech QP-6013 data logger. Trays were placed on a capillary bed so that seedlings received water as required.

The frequency of viable endophyte infection for each endophyte treatment was determined prior to the experiment using a random set of plants from the same seed lot. To get confirmation of viable endophyte frequency, 96 seeds per endophyte strain were grown in standard potting mixture (17% peat, 43% screened fine bark, 10% coir fibre, 20% pumice, 10% sterilised river sand) to at least 3–4 tiller stage before detection of endophyte was undertaken. Endophyte infection status was confirmed by tissue print immunoblot method (see 2.3.). Endophyte infection rate was used to scale the results to determine the alkaloid production on a per seedling basis.

3.2.5 Endophyte alkaloid analysis

Alkaloids from all experiments were analysed according to the methods described in 2.4.

3.2.6 Statistical data analysis

A 4 x 4 Latin-square design was used with the four endophyte treatments randomised in each row and column, so that each treatment occurred 4 times per harvesting day. Two trays, each with four rows and two columns, were combined to form the 4 x 4 Latin square layout, as shown in Table 3.2. Each harvesting day had a different randomised layout.

Table 3.2: Example of experimental design with four endophyte treatments (strains) and four replications per harvesting date for day 13. Each endophyte treatment consisted of 18 seedlings which were bulked up to one sample.

Week 1	**Day 13**			
	Tray 1		**Tray 2**	
	Column Labels			
Row Labels	**1**	**2**	**3**	**4**
1	NZ_{CT}	AR1	NEA2	AR37
2	NEA2	AR37	NZ_{CT}	AR1
3	AR1	NZ_{CT}	AR37	NEA2
4	AR37	NEA2	AR1	NZ_{CT}

Separate statistical analyses were performed for the response variables alkaloid concentration (µg/g) and total amount of alkaloid per seedling (µg/seedling), for the four alkaloids (peramine, lolitrem B, ergovaline, and total epoxy-janthitrems). Separate analyses were also performed for alkaloid concentrations of un-germinated seeds, seeds germinated in petri dishes, and seeds grown in soil. All alkaloid concentrations in un-germinated seeds were assessed using One-way ANOVA on the factor endophyte treatment. An example showing analysis of peramine

concentration in un-germinated seed and the relevant GenStat output is given in Appendix 1. Alkaloid concentration (µg/g) and total amount of alkaloid (µg/seedling) from material collected from seeds grown in petri dishes as well as seeds grown in soil were assessed using a two-way ANOVA on the factors endophyte treatment and harvest day.

Data for the total amount of alkaloid (µg/seedling) from seeds germinated in petri dishes were analysed in conjunction with data from seedlings grown in soil. ANOVA for these data examined the significance of endophyte treatments over the duration of the experiment from 6–69 day old seedlings. The endophyte infection levels and the number of germinated seeds were taken into account for all alkaloid concentrations and total amount of alkaloid calculations.

Where necessary to meet the statistical assumption of homogeneity of variance between endophyte treatment and seedling ages, alkaloid concentrations were square root or log transformed prior to analysis. Fisher's Least Significant Difference (LSD) test was used to assess significance between alkaloid concentration or weight means at α=0.05. ANOVA residuals were checked for normality using graphs and Shapiro-Wilk test and for homogeneity using Bartlett`s test. All analyses were carried out using GenStat 16 statistical software package (VSN International, 2016).

3.3 Results

3.3.1 Endophyte infection frequencies

Viable endophyte infection frequencies, tested by the tissue print immuno blot method, as well as seedling emergence percentages for each endophyte treatment are indicated in Table 3.3.

Table 3.3: Seedling emergence (%) and viable endophyte infection frequency (%) for selected host-endophyte combinations. Endophyte infection was tested using tissue print immuno blot method.

Host cultivar	Endophyte strain	Seedling emergence		Viable endophyte infection	
		%	±SEM	%	±SEM
One50	AR1	93.8	0.027	87.5	0.035
Trojan	NEA2	92.7	0.029	62.5	0.050
One50	NZ_{CT}	89.6	0.033	82.2	0.040
One50	AR37	93.8	0.027	83.3	0.040

3.3.2 Fungal alkaloid occurrence in endophyte-infected seedlings

Alkaloid measurements are presented in two formats; i) Total amount of alkaloid in µg/seedling (Table 3.4) and ii) mean alkaloid concentration in µg/g (Table 3.5). From the alkaloid concentration it was possible to calculate the total amount of alkaloid (µg/seedling).

$$= \left(\frac{Alkaloid\ concentration\ \frac{\mu g}{g} * dry\ matter\ shoot\ weight\ g}{viable\ endophyte\ infection\ \% * number\ of\ harveste\ seedling}\right)/100$$

This was used to identify the timing of when the endophyte starts to produce alkaloids within the emerging seedling, and the rate of increase over time. The increase of total amount of alkaloid is presented with the mean dry weight increase of the plants. In comparison, the mean alkaloid concentration shows the changes in alkaloid concentrations in the developing seedling. Changes in alkaloid concentrations and total amount were measured on the expected alkaloid profile of each endophyte strain.

Table 3.4: Total amount of alkaloid per seed (µg/seed) and seedling (µg/seedling) of selected alkaloids from perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strains AR1, NEA2, NZ_{CT}, and AR37 during the early establishment phase. Significance of difference in total amount of alkaloid was tested for each alkaloid compound separately and can be compared between endophyte strains (column) and seedling age (row) (values followed by the same letter within each alkaloid compound are not significantly different at $p<0.05$). Standard error of the mean (± SEM) is given for each treatment underneath total amount of alkaloid. Analyses of alkaloid concentration in seeds (0), 6–10 day old plants, and 13–69 day old plants were performed separately.

Selected alkaloid	Endophyte strain	Seedling age in days												
		0	6	8	10	13	20	27	34	41	48	55	62	69
Peramine	AR1	0.090 ^{a}	0.003 ^{v}	0.009 ^{t}	0.013 ^{rst}	0.029 ^{no}	0.049 ^{klm}	0.068 ^{jk}	0.119 ^{i}	0.278 ^{h}	0.437 ^{g}	0.694 ^{ef}	1.368 ^{c}	2.143 ^{b}
		±0.040	<0.000	±0.001	±0.002	±0.004	±0.007	±0.009	±0.016	±0.038	±0.060	±0.095	±0.188	±0.294
	NEA2	0.077 ^{b}	0.005 ^{u}	0.014 ^{qrs}	0.019 ^{pq}	0.043 ^{mn}	0.045 ^{lm}	0.064 ^{jkl}	0.087 ^{ij}	0.476 ^{fg}	0.482 ^{fg}	0.527 ^{fg}	1.210 ^{cd}	1.323 ^{c}
		±0.040	±0.001	±0.002	±0.003	±0.006	±0.006	±0.009	±0.012	±0.065	±0.066	±0.072	±0.166	±0.182
	NZ_{CT}	0.071 ^{b}	0.004 ^{uv}	0.010 st	0.015 ^{qr}	0.028 ^{op}	0.059 ^{klm}	0.124 ^{i}	0.237 ^{h}	0.619 ^{efg}	0.829 ^{de}	1.697 ^{bc}	2.145 ^{b}	3.374 ^{a}
		±0.040	±0.001	±0.001	±0.002	±0.004	±0.008	±0.017	±0.033	±0.085	±0.114	±0.233	±0.294	±0.463
Lolitrem B	NEA2	0.09 ^{a}	<0.000 ^{o}	<0.000 ^{no}	<0.000 ^{mno}	<0.000 ^{klm}	0.001 ^{jkl}	0.001 ^{hi}	0.004 ^{g}	0.017 ^{ef}	0.012 ^{f}	0.016 ^{ef}	0.067 ^{cd}	0.135 ^{c}
		±0.003	<0.000	<0.000	<0.000	<0.000	<0.000	<0.000	±0.001	±0.005	±0.003	±0.005	±0.020	±0.040
	NZ_{CT}	0.034 ^{a}	<0.000 ^{lmn}	0.001 ^{kl}	0.001 ^{ijk}	0.001 ^{hij}	0.003 ^{gh}	0.012 ^{f}	0.036 ^{de}	0.092 ^{c}	0.113 ^{c}	0.696 ^{b}	0.834 ^{ab}	1.781 ^{a}
		±0.013	<0.000	<0.000	<0.000	<0.000	±0.001	±0.003	±0.011	±0.027	±0.033	±0.206	±0.247	±0.526
Ergovaline	NEA2	0.112 ^{a}	0.001 ^{l}	0.002 ^{jkl}	0.003 ^{ghi}	0.002 ^{ijkl}	0.002 ^{hijkl}	0.006 ^{f}	0.011 ^{e}	0.054 ^{c}	0.119 ^{b}	0.143 ^{b}	0.341 ^{a}	0.388 ^{a}
		±0.006	<0.000	<0.000	±0.001	<0.000	<0.000	±0.001	±0.002	±0.010	±0.021	±0.026	±0.061	±0.070
	NZ_{CT}	0.045 ^{b}	0.002 ^{kl}	0.003 ^{ghij}	0.004 ^{gh}	0.003 ^{ghijk}	0.002 ^{jkl}	0.004 ^{fg}	0.006 ^{f}	0.021 ^{d}	0.024 ^{d}	0.066 ^{c}	0.129 ^{b}	0.137 ^{b}
		±0.004	<0.000	±0.001	±0.001	±<0.000	±<0.000	±0.001	±0.001	±0.004	±0.004	±0.012	±0.023	±0.025
Epoxy-janthitrems	AR37	0.157	0.002 ^{j}	0.004 ^{i}	0.007 ^{h}	0.009 ^{h}	0.021 ^{g}	0.084 ^{f}	0.201 ^{e}	0.511 ^{d}	0.743 ^{d}	1.527 ^{c}	2.380 ^{b}	4.191 ^{a}
		±0.002	±0.003	±0.001	±0.001	±0.001	±0.003	±0.012	±0.028	±0.071	±0.104	±0.213	±0.332	±0.584

Table 3.5: Mean alkaloid concentration (µg/g) of selected alkaloids from seedlings infected with *E. festucae* var. *lolii* strains AR1, NEA2, NZ_{CT}, and AR37 during the early establishment phase. Significance of difference in total amount of alkaloid was tested for each alkaloid compound separately and can be compared between endophyte strains (column) and seedling age (row) (values followed by the same letter within each alkaloid compound are not significantly different at $p<0.05$). Standard error of the mean (± SEM) is given for each treatment underneath mean alkaloid concentration. Analyses of alkaloid concentration in seeds (0), 6–10 day old plants, and 13–69 day old plants were performed separately

Selected alkaloid	Endophyte strain	Seedling age in days												
		0	6	8	10	13	20	27	34	41	48	55	62	69
Peramine	AR1	38.2 $^{\alpha}$ ±2.4	21.2 $^{\gamma}$ ±4.5	34.7 $^{\gamma}$ ±4.5	32.3 $^{\gamma}$ ±4.5	28.1 ^{a} ±3.4	13.7 ^{cd} ±1.7	6.1 ^{ijk} ±0.7	4.2 ^{lm} ±0.5	5.0 ^{jkl} ±0.6	5.0 ^{jkl} ±0.6	4.2 ^{lm} ±0.5	6.0 ^{ijk} ±0.7	6.4 ^{hij} ±0.8
	NEA2	31.4 $^{\alpha}$ ±2.4	24.8 $^{\gamma}$ ±4.5	38.7 $^{\gamma}$ ±4.5	39.9 $^{\gamma}$ ±4.5	31.1 ^{a} ±3.8	11.5 ^{de} ±1.4	4.4 ^{klm} ±0.5	3.8 ^{lm} ±0.5	7.6 ^{ghi} ±0.9	4.8 ^{jkl} ±0.6	3.2 ^{m} ±0.4	4.5 ^{kl} ±0.6	3.9 ^{lm} ±0.5
	NZ_{CT}	36.0 $^{\alpha}$ ±2.4	22.2 $^{\gamma}$ ±4.5	25.8 $^{\gamma}$ ±4.5	43.2 $^{\gamma}$ ±4.5	24.3 ^{ab} ±3.0	17.3 ^{bc} ±2.1	10.3 ^{defg} ±1.3	10.1 ^{defg} ±1.2	11.3 ^{de} ±1.4	9.1 ^{efgh} ±1.1	10.7 ^{defg} ±1.3	7.9 ^{fghi} ±1.0	10.7 ^{def} ±1.3
Lolitrem B	NEA2	3.6 $^{\beta}$ ±1.3	0.5 $^{\varepsilon}$ ±0.2	0.4 $^{\varepsilon}$ ±0.2	0.4 $^{\varepsilon}$ ±0.2	0.3 ^{ef} ±0.1	0.1 ^{fg} ±0.0	0.1 ^{g} ±0.0	0.2 ^{fg} ±0.1	0.3 ^{ef} ±0.1	0.1 ^{fg} ±0.0	0.1 ^{g} ±0.0	0.3 ^{ef} ±0.1	0.4 ^{de} ±0.1
	NZ_{CT}	17.1 $^{\alpha}$ ±6.3	1.4 $^{\delta}$ ±0.2	1.5 $^{\delta}$ ±0.2	2.4 $^{\gamma}$ ±0.2	1.1 ^{c} ±0.3	0.8 ^{cd} ±0.3	1.0 ^{cd} ±0.3	1.5 ^{bc} ±0.5	1.7 ^{bc} ±0.5	1.2 ^{c} ±0.4	4.4 ^{a} ±1.4	3.1 ^{ab} ±1.0	5.6 ^{a} ±1.7
Ergovaline	NEA2	30.9 $^{\alpha}$ ±2.1	5.4 $^{\gamma}$ ±1.6	3.7 $^{\gamma}$ ±1.6	7 $^{\gamma}$ ±1.6	1.5 ^{ab} ±0.3	0.5 ^{cdef} ±0.1	0.4 ^{efg} ±0.1	0.5 ^{defg} ±0.1	0.9 ^{bc} ±0.2	1.2 ^{b} ±0.3	0.9 ^{bcd} ±0.2	1.3 ^{b} ±0.3	1.2 ^{b} ±0.2
	NZ_{CT}	21.5 $^{\beta}$ ±2.1	9.7 $^{\gamma}$ ±1.6	7.3 $^{\gamma}$ ±1.6	11.4 $^{\gamma}$ ±1.6	2.3 ^{a} ±0.5	0.6 ^{cde} ±0.1	0.3 ^{fg} ±0.1	0.3 ^{g} ±0.1	0.4 ^{efg} ±0.1	0.3 ^{g} ±0.1	0.4 ^{efg} ±0.1	0.5 ^{cdef} ±0.1	0.4 ^{efg} ±0.1
Epoxy-janthitrems	AR37	63.5 ±1.4	12.7 $^{\gamma}$ ±2.2	13.6 $^{\gamma}$ ±2.2	16.5 $^{\gamma}$ ±2.2	6.8 ^{cd} ±0.8	5.0 ^{d} ±0.6	6.5 ^{cd} ±0.8	7.3 ^{bc} ±0.9	8.7 ^{bc} ±1.0	7.2 ^{bc} ±0.8	10.1 ^{ab} ±1.2	9.0 ^{bc} ±1.1	13.0 ^{a} ±1.5

3.3.2.1 Peramine

Seedlings infected with AR1, NEA2, and NZ_{CT} showed a significant increase in the total amount of peramine from day 6 to day (Table 3.4). The total amount of peramine per seedling continued to increase in all three endophyte strains throughout the 69-day assessment period, with the total amount of peramine in seedlings infected with NZ_{CT} increasing more rapidly than AR1 and NEA2 (Figure 3.3).

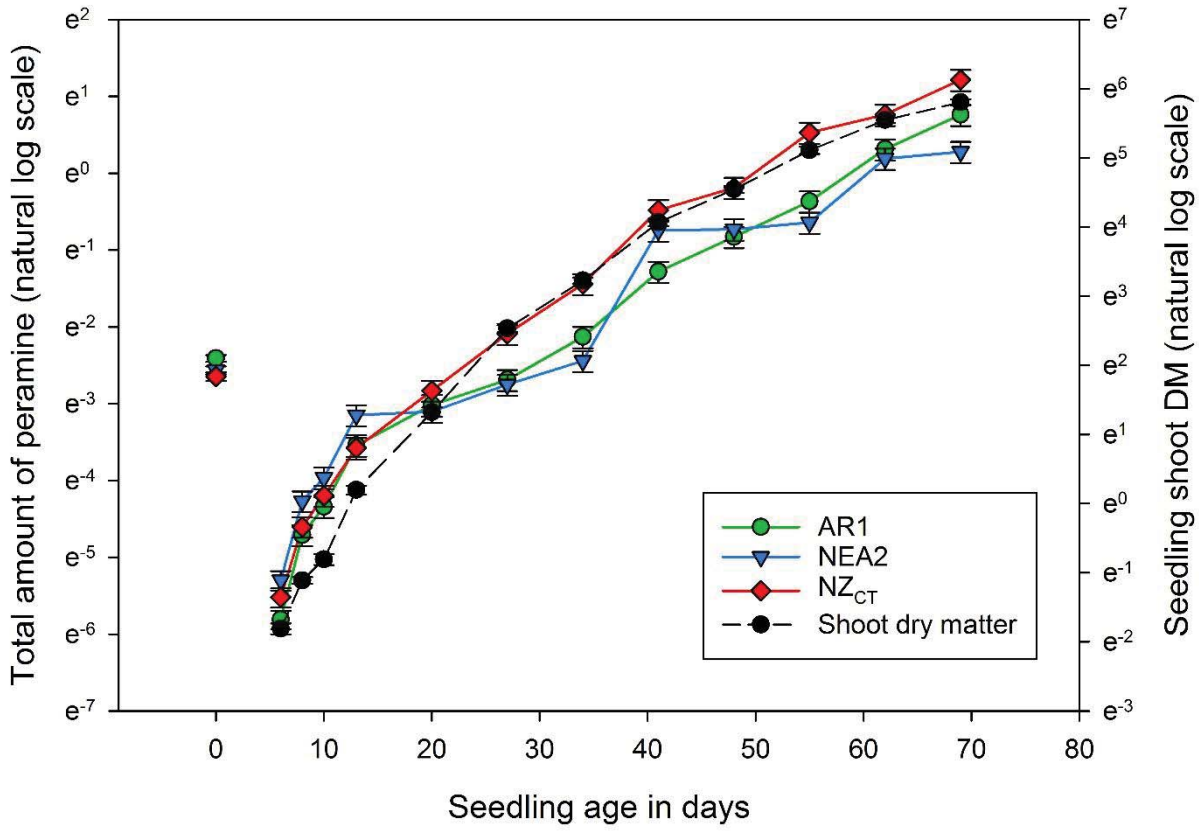


Figure 3.3: Natural logarithmic scale of the total amount of peramine (µg/seedling) and mean shoot dry matter (mg/seedling) of seedlings infected with *E. festucae* var. *lolii* strains AR1, NEA2, and NZ_{CT} during the early establishment phase. Error bar represent standard error of the mean (±SEM). Shoot dry matter are the means of the harvested seedlings across all endophyte treatments.

Although not significant, peramine concentration was highest in seeds (Day 0) containing AR1, in comparison to seeds infected with NEA2 or NZ_{CT} endophytes (p=0.189) (Figure 3.4). A transient peak in peramine concentrations was seen in 8–13 day old seedlings and concentrations then steadily decreased until seedlings were about 27 days, after which time concentrations were approximately constant (Figure 3.4). From day 20, seedlings infected with NZ_{CT} contained significantly higher peramine concentrations than seedling infected with AR1 or NEA2 ($p<0.001$) (Table 3.5).

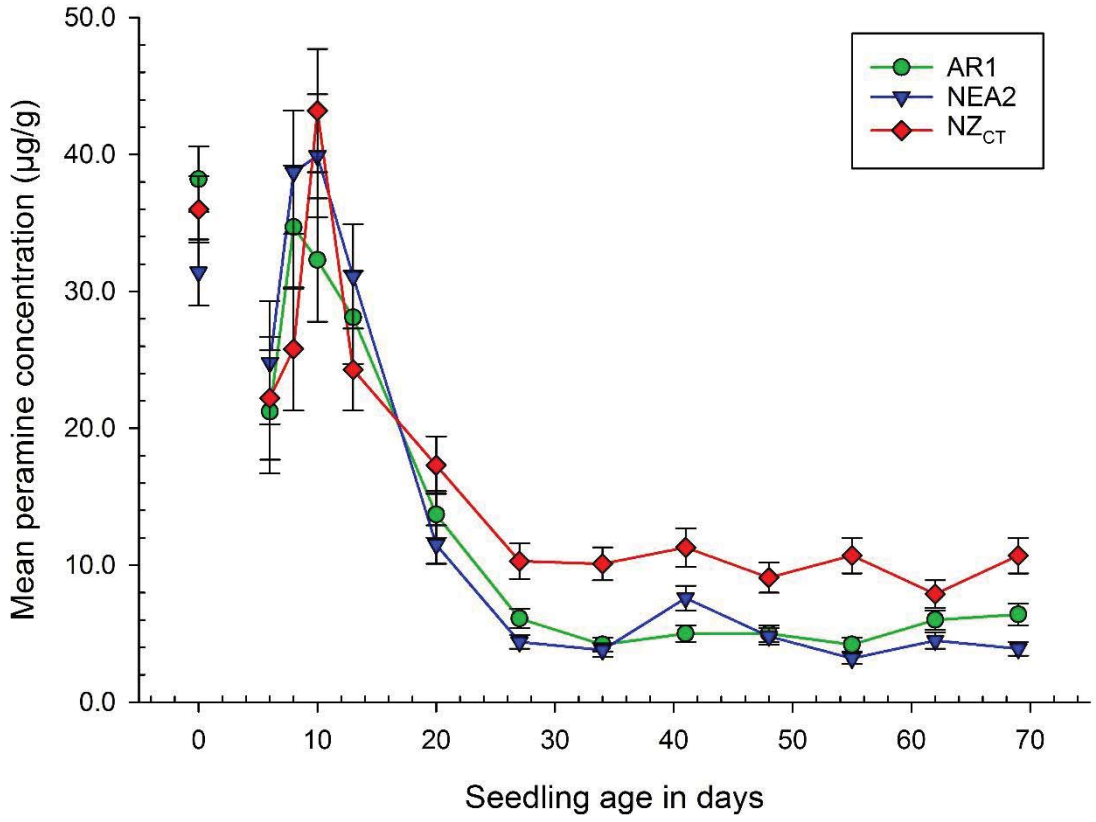


Figure 3.4: Mean peramine concentration of seeds and seedlings (µg/g) infected with *E. festucae* var. *lolii* strains AR1, NEA2, and NZ_{CT} during the early establishment phase. Error bar represent standard error of the mean (±SEM). Each data point is the mean for 18 seeds for day 0 and 13–69 day old seedlings, and 50 seeds for 6–10 day old seedlings analysed as one sample.

3.3.2.2 Lolitrem B

Seedlings infected with the NZ_{CT} endophyte showed a small but significant increase in the total amount of lolitrem B from 20 days after planting. Even though the total amount of lolitrem B then increased rapidly in 55-day old seedlings infected with NZ_{CT} (Table 3.4), total amount of lolitrem B increase matched plant growth (Figure 3.5). By contrast the total amount of lolitrem B in NEA2-infected seedlings increased significantly 27 days post planting, but only accumulated minimally over the assessment period of 69 days (Figure 3.5).

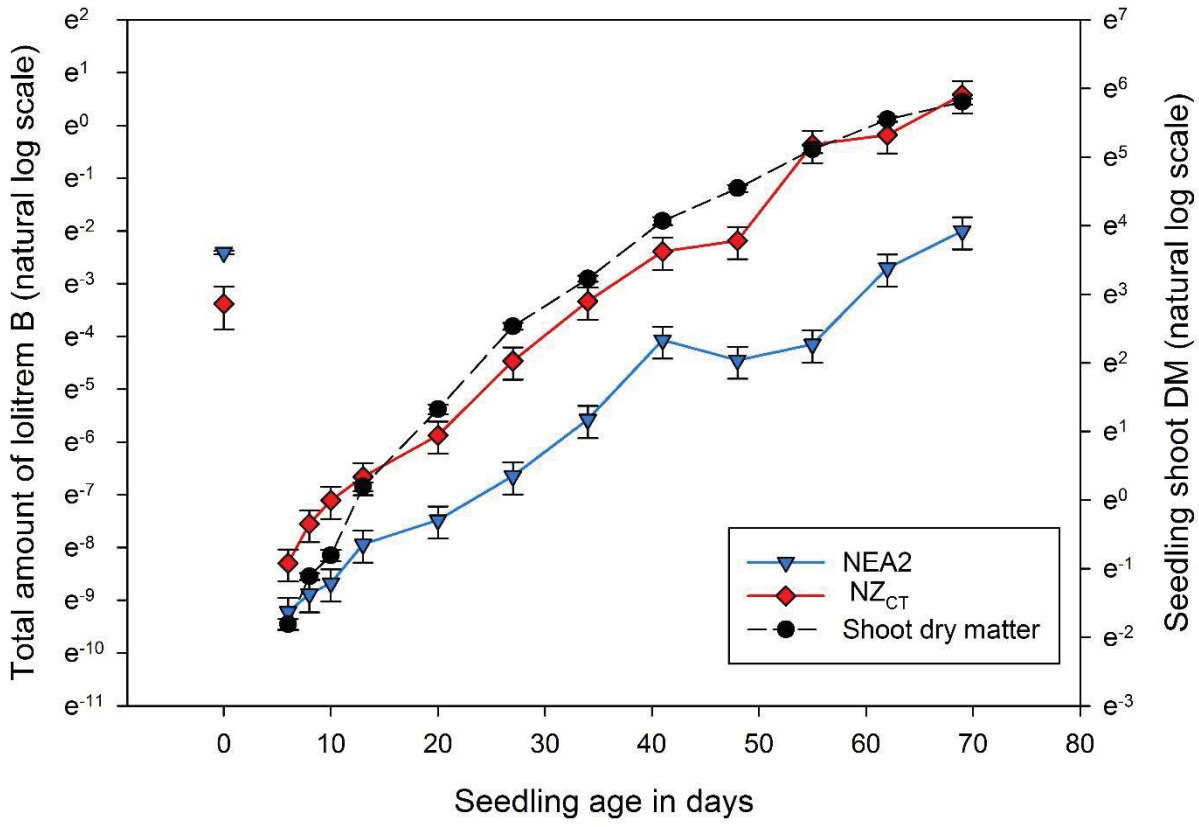


Figure 3.5: Natural logarithmic scale of the total amount of lolitrem B (µg/seedling) and mean shoot dry matter (mg/seedling) of seedlings infected with *E. festucae* var. *lolii* strains NZ_{CT}, and NEA2 during the early establishment phase. Error bar represent standard error of the mean (±SEM). Shoot dry matter are the means of the harvested seedlings across all endophyte treatments.

In this experiment seeds infected with NZ_{CT} stored significantly higher lolitrem B concentrations (µg/g), compared to seeds infected with NEA2 ($p=0.024$). Similarly, seedlings infected with NZ_{CT} endophyte contained significantly higher lolitrem B, than seedlings infected with NEA2 ($p<0.001$). A significant transient peak in lolitrem B concentration was seen in 6–10 day old seedlings infected with NZ_{CT}, followed by a decrease in concentration as the seedlings further developed (Figure 3.6). Lolitrem B concentration in these seedlings increased sharply in 55-day old seedlings, compared with the previous 48-day harvest, and remained high for harvests at day 62 and day 69, with a peak in lolitrem B concentration of 5.6 µg/g in 69-day old seedlings (Table 3.5). In contrast, seedlings infected with NEA2 showed only trace levels and little variation in lolitrem B concentrations in 62–69 day old seedlings (Figure 3.6).

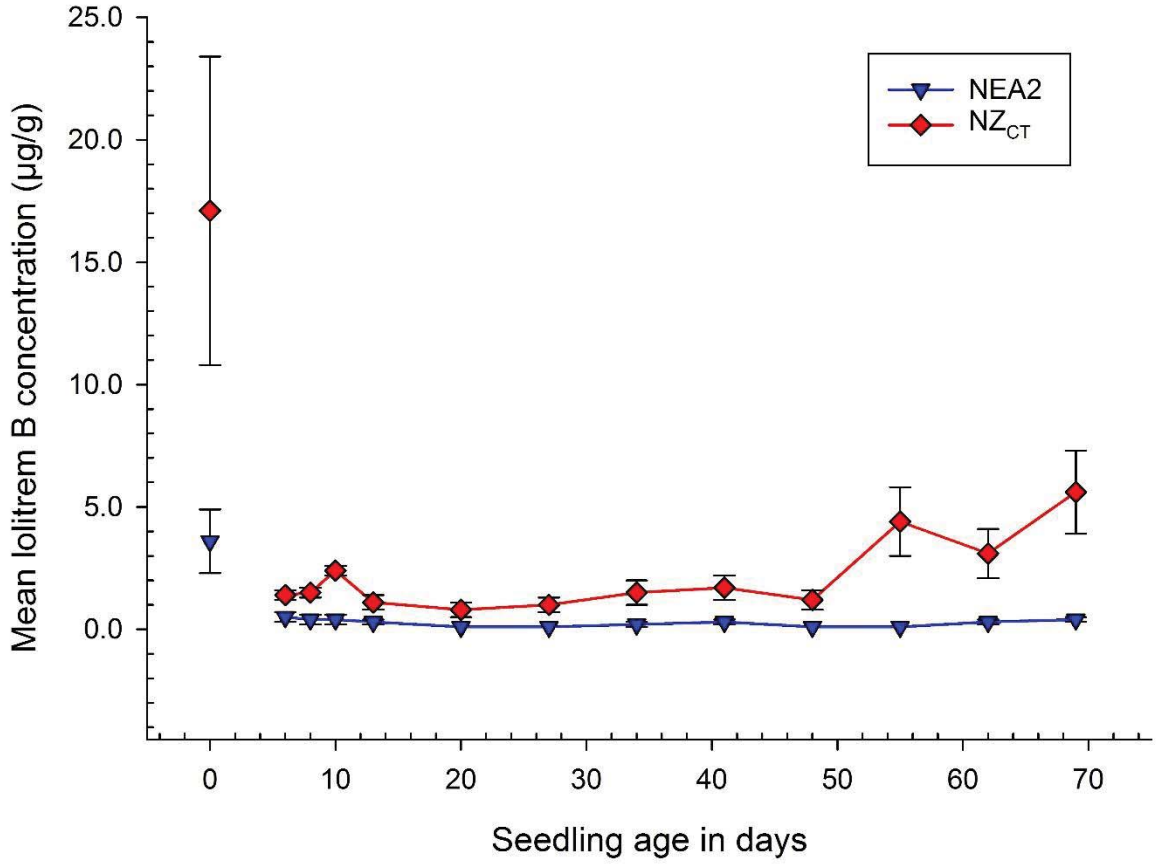


Figure 3.6: Mean lolitrem B concentrations of seeds and seedlings (µg/g) infected with *E. festucae* var. *lolii* strains NEA2 and NZ_{CT} during the early establishment phase. Error bar represent standard error of the mean (±SEM). Each data point is the mean for 18 seeds for day 0 and 13–69 day old seedlings, and 50 seeds for 6–10 day old seedlings analysed as one sample.

3.3.2.3 Ergovaline

Seedlings infected with NEA2 and NZ_{CT} displayed a small but significant increase in the total amount of ergovaline in 10 and 8-day old seedlings respectively (Table 3.4). As seedlings developed, the total amount of ergovaline increased more rapidly in seedlings containing NEA2, compared to seedlings infected with NZ_{CT} (Figure 3.7).

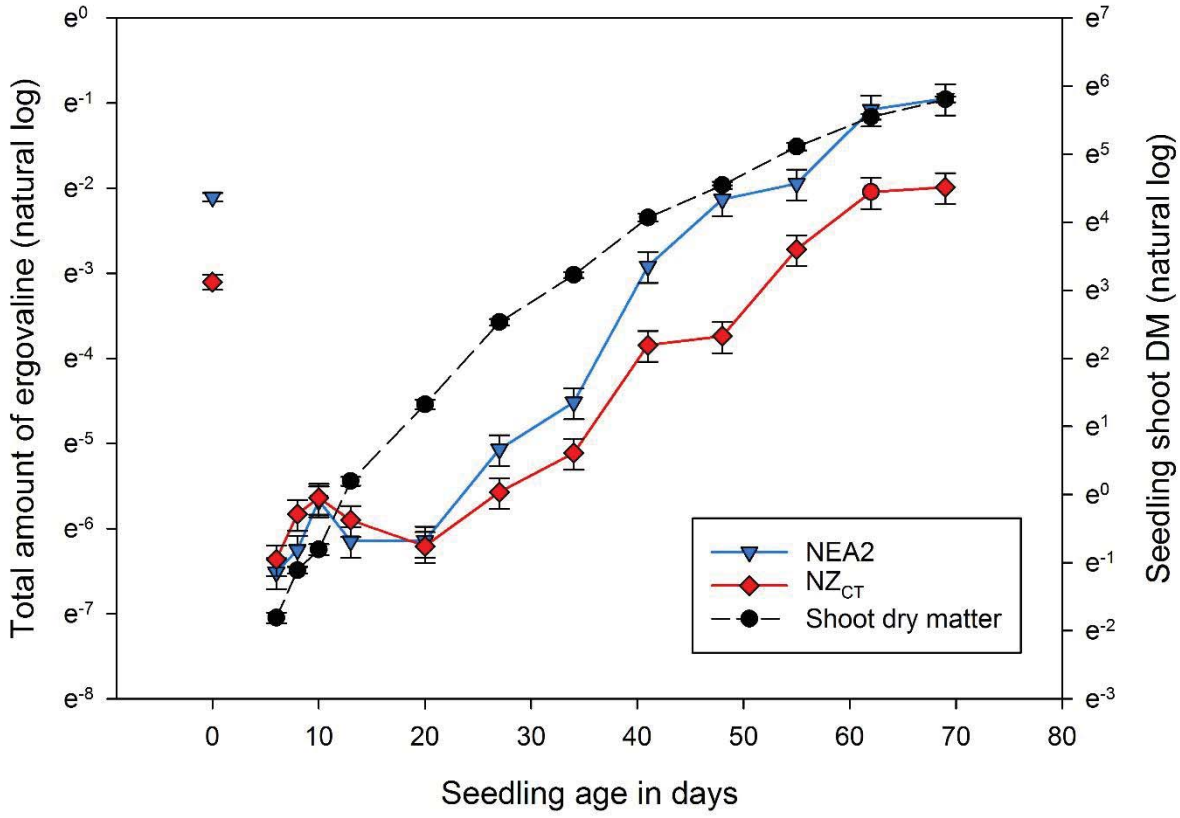


Figure 3.7: Natural logarithmic scale of the total amount of ergovaline (µg/seedling) and mean shoot dry matter (mg/seedling) of seedlings infected with *E. festucae* var. *lolii* strains NZ_{CT} and NEA2 during the early establishment phase. Error bar represent standard error of the mean (±SEM). Shoot dry matter are the means of the harvested seedlings across all endophyte treatments.

Seeds infected with NEA2 contained significantly higher ergovaline concentrations than seeds containing NZ_{CT} (p=0.018). Ergovaline concentrations decreased from 6–8 days of age, and then increased at 10 days of age before falling sharply to below 1 µg/g by day 20 and remaining at that low level for the remainder of the observation period. After 10 days post planting ergovaline concentrations decreased and levelled out after 20 days, with a mean ergovaline concentration at 69 days for seedlings infected with NEA2 and NZ_{CT} of 1.2 µg/g and 0.4 µg/g respectively (Table 3.5; Figure 3.8).

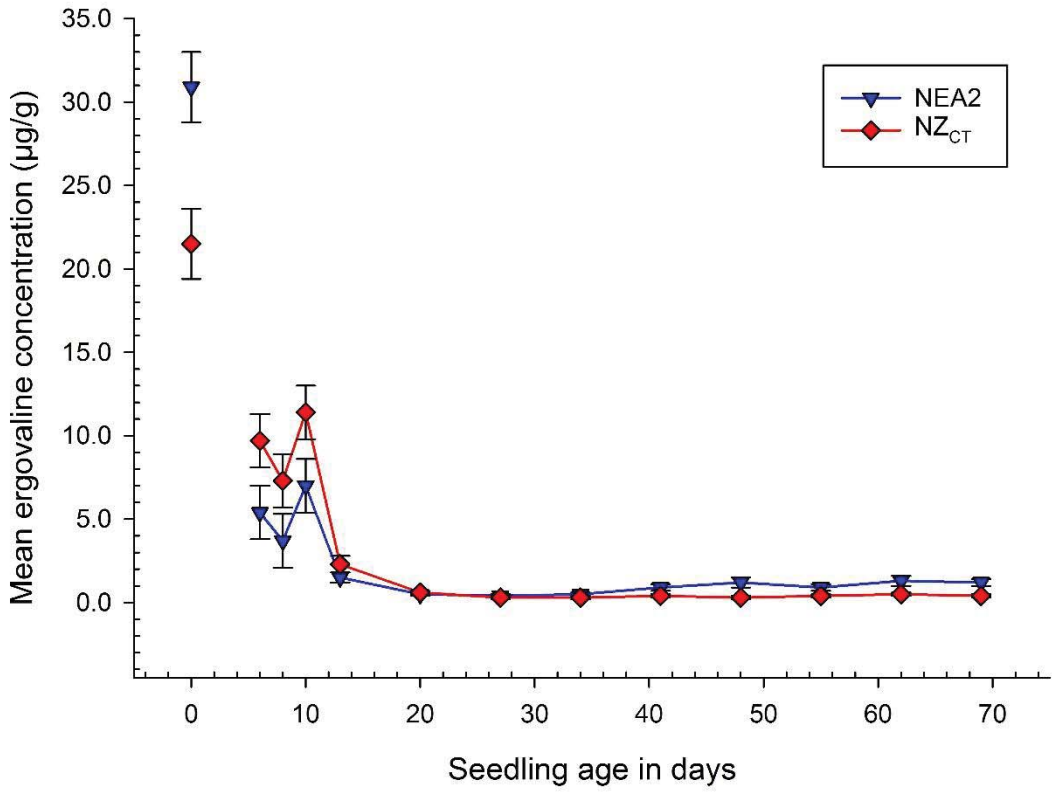


Figure 3.8: Mean ergovaline concentration of seeds and seedlings (µg/g) infected with *E. festucae* var. *lolii* strains NEA2 and NZ_{CT} during the early establishment phase. Error bar represent standard error of the mean (±SEM). Each data point is the mean for 18 seeds for day 0 and 13–69 day old seedlings, and 50 seeds for 6–10 day old seedlings analysed as one sample.

3.3.2.4 Epoxy-janthitrems

Seedlings infected with AR37 displayed a gradual increase in the total amount of epoxy-janthitrems (the sum of all known epoxy-janthitrems; epoxy-janthitriol, epoxy-janthitrem I, epoxy-janthitrem II, epoxy-janthitrem III, and epoxy-janthitrem IV) from 8 days of age until the end of the assessment period (Figure 3.9).

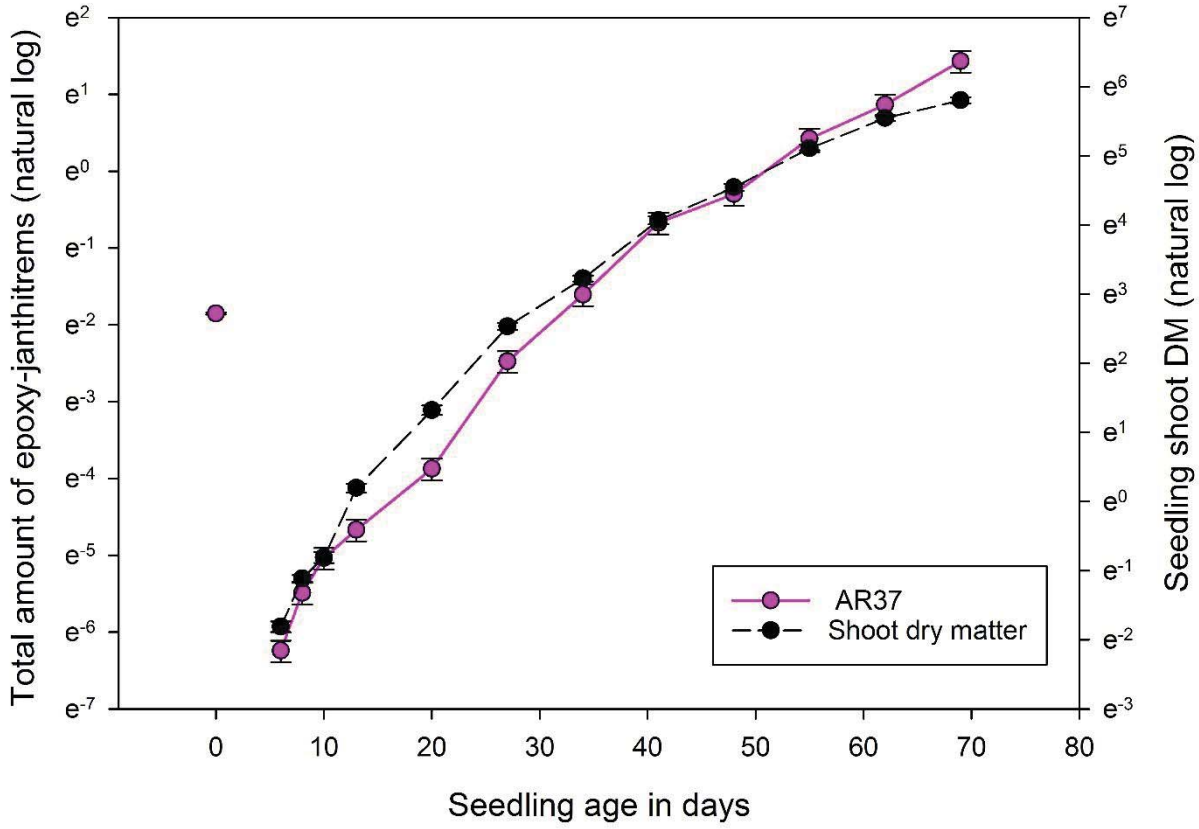


Figure 3.9: Natural logarithmic scale of the total amount of epoxy-janthitrems (µg/seedling) and mean shoot dry matter (mg/seedling) of seedlings infected with *E. festucae* var. *lolii* strain AR37 during the early establishment phase. Error bar represent standard error of the mean (±SEM). Shoot dry matter are the means of the harvested seedlings across all endophyte treatments.

Epoxy-janthitrem concentrations in seeds were significantly higher compared to developing 69 day old seedlings ($p<0.001$) (Figure 3.10). Although not significant, epoxy-janthitrem concentrations increased from 6 day old until 10 day old seedlings (Table 3.5). After that time epoxy-janthitrem concentrations dropped to 5 µg/g in 20-day old seedlings followed by a steady increase with mean concentration of 13 µg/g at the end of the 69-day assessment period (Table 3.5).

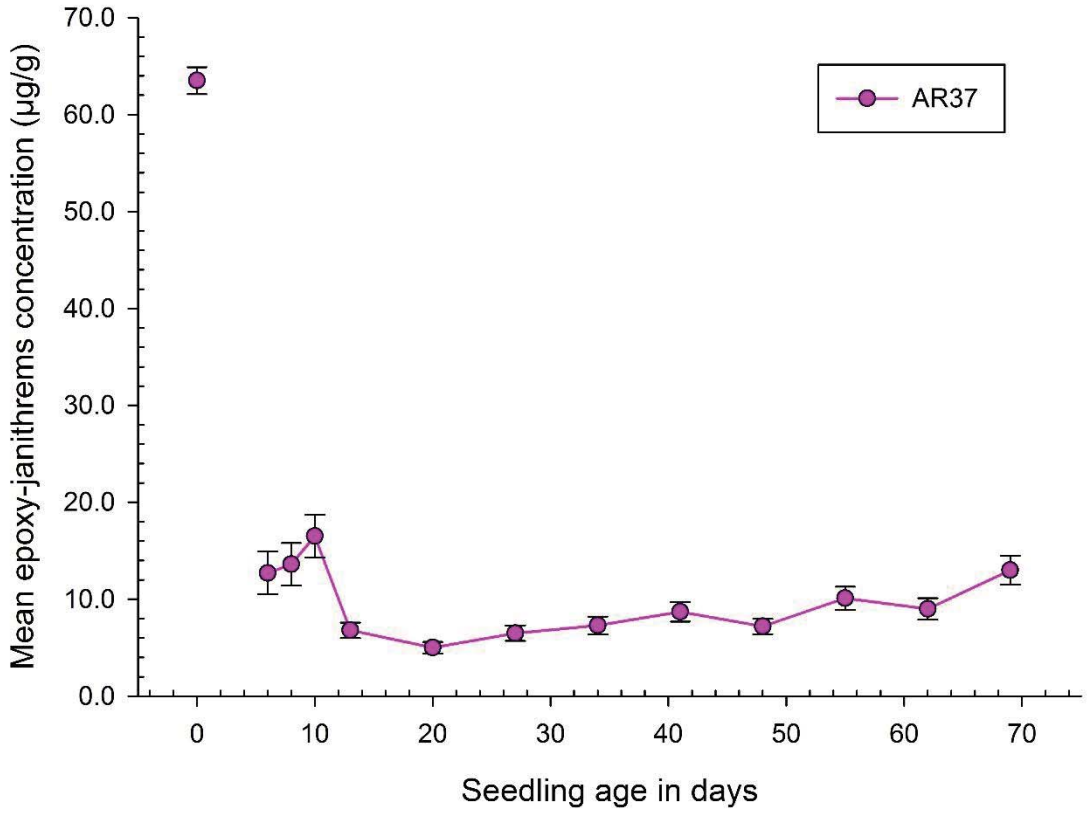


Figure 3.10: Total epoxy-janthitrem concentrations of seeds and seedlings (µg/g) infected with *E. festucae* var. *lolii* strain AR37 during the early establishment phase. Error bar represent standard error of the mean (±SEM). Each data point is the mean for 18 seeds for day 0 and 13–69 day old seedlings, and 50 seeds for 6–10 day old seedlings analysed as one sample.

3.3.3 Glasshouse temperatures

The mean glasshouse temperatures and mean relative humidity for each experimental week are recorded in Figure 3.11. The highest temperature recorded during the experiment (41.6°C, for 10 minutes) occurred during week 10, while the lowest temperature (6.4°C, for 20 minutes) occurred during the week 8.

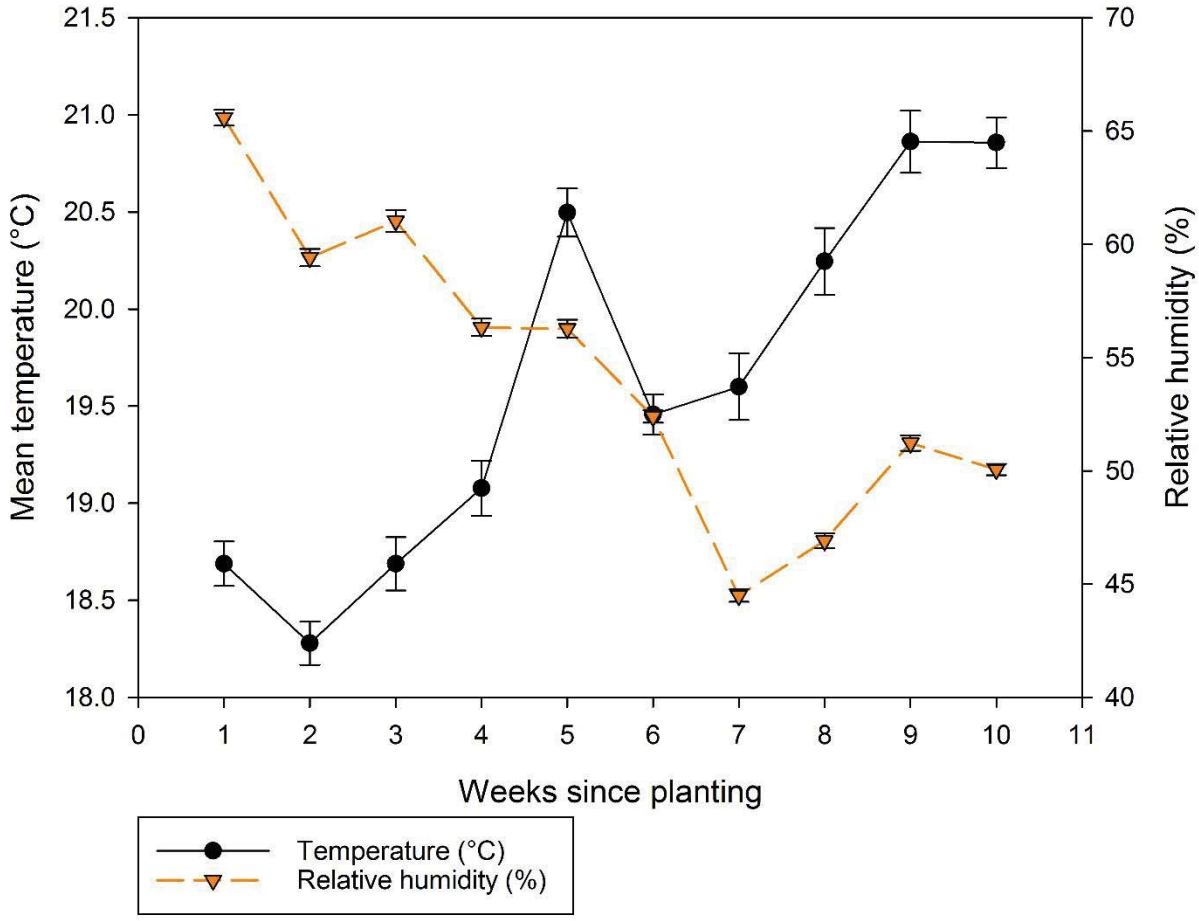


Figure 3.11: Mean glasshouse temperature (°C) and mean relative humidity (%) recordings from 29th June to 7th September 2015. Error bar represent standard error of the mean (±SEM).

3.4 Discussion

Endophyte-infected seeds can contain extremely high alkaloid concentrations which has been further confirmed in this study. As an example, seeds infected with AR37 had more than 15-fold higher epoxy-janthitrem concentrations than 69-day old seedlings, with 63.5 µg/g and 4.2 µg/g respectively (Figure 3.10). High alkaloid concentrations in seed have also been found by Ball, *et al.* (1997c) and Bush, *et al.* (1982), who reported that endophyte-infected seeds have substantially higher alkaloid concentrations than other parts of the grass. Elevated alkaloid concentration in seed is thought to have arisen as a defence mechanism against seed predation from herbivorous insects and mammals (Knoch, *et al.*, 1993). The concentrations of stored alkaloids in seeds are believed to be dependent on the environmental conditions in which the plant sets seed, as these can greatly influence alkaloid concentration (Malinowski and Belesky, 2000; Rasmussen, *et al.*, 2006). Therefore, no generalisation of alkaloid concentrations between endophyte strains used in this study can be made, based on the unknown environmental conditions prior to seed harvest.

Mean alkaloid concentrations were found in this study to be lower in the first emerging shoot in 6-day old seedlings compared to those found in the seed, with a subsequent peak in concentration in 10–13-day old seedlings (Table 3.5). As the plant continued to develop, alkaloid concentrations gradually declined as the seedling age increased from 10–20 days (Table 3.5). These results corroborate previous studies showing a decrease in alkaloid concentration with seedling age (Ball, *et al.*, 1993).

Peramine concentrations have been found to be highest 7 days after seedling emergence and subsequently decline (Qawasmeh, 2012). Similar results have been reported by Dymock, *et al.* (1989), who reported a reduction in peramine concentration in endophyte-infected annual

ryegrass *L. temulentum* L. and *L. persicum* Boiss. & Hohen seedlings aged 20-58 days old. To understand the occurrence of a peak peramine concentration followed by an *in planta*, it is useful to draw together the interacting factors of peramine concentration, total amount of peramine and shoot dry matter in one graph on a logarithmic scale (Figure 3.12).

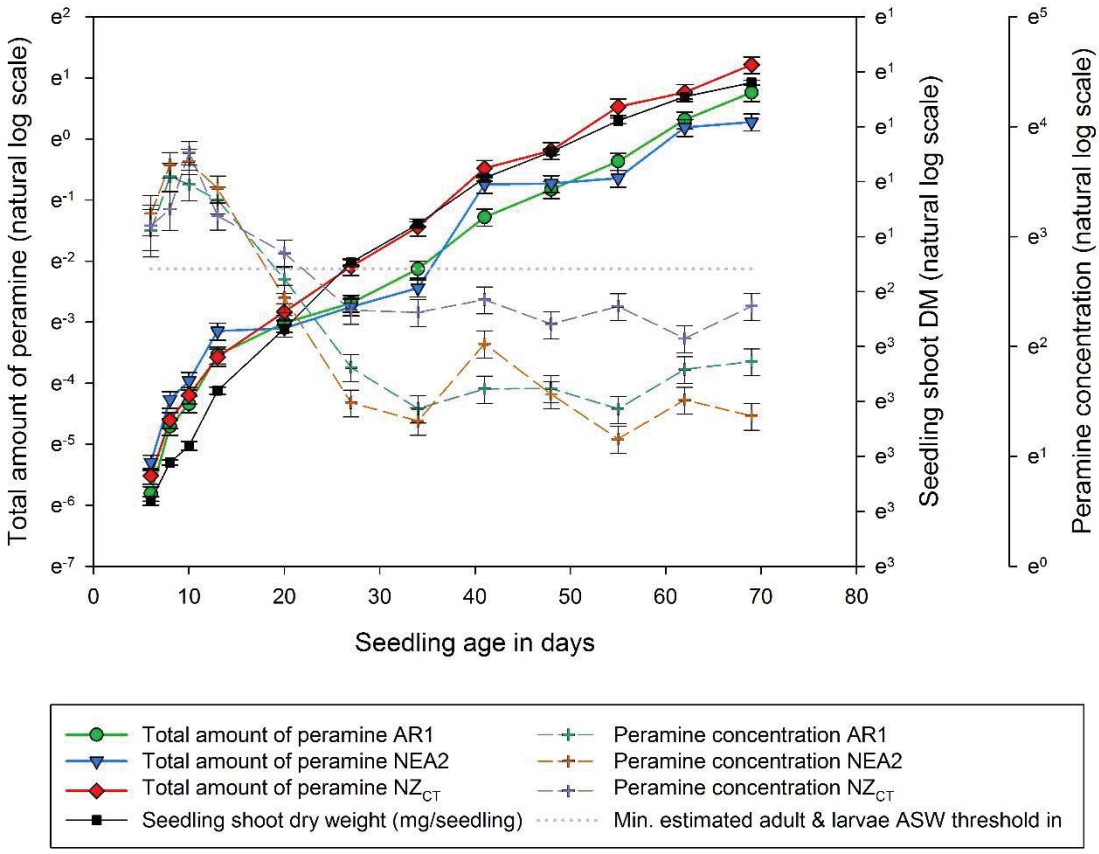


Figure 3.12: Natural logarithmic scale of total amount of peramine (µg/seedling), peramine concentration (µg/g), and mean shoot dry matter (mg/seedling) of seedlings infected with *E. festucae* var. *lolii* strains AR1, NEA2, and NZ_{CT} during the early establishment phase. Dry matter values are the means of all seedlings across all endophyte treatments. Minimum adult and larvae ASW peramine threshold in the field is estimated to be 15 µg/g (Popay and Wyatt, 1995). Error bar represent standard error of the mean (±SEM).

Shoot dry matters measured in this thesis were observed to increase very little in 8–10 day old seedling. Such a growth slowdown was also observed by Anslow (1962), who reported a constant total seedling weight in 7–11 day old plants with a transfer of weight from the caryopsis to the emerging shoot. In this experiment, even though plant growth slowed down in 8–10 day old seedlings, the total amount of peramine continued to increase, accounting for the peramine concentration peak in 8–10 day old seedlings (Figure 3.12). The rate of increase in the total amount of peramine during this time is greater than those in older seedlings, indicating that either the endophyte is producing peramine at a much greater rate in very young seedlings, or that the emerging seedling is getting additional peramine from the caryopsis. Peramine is hydrophilic allowing an easy translocation from the seed into the developing shoot (Ball, *et al.*, 1993) and is relatively evenly distributed throughout the plant (di Menna, *et al.*, 1992). From the current study is would appear that seed-stored peramine is translocated out of the seed once the seedling germinates, with measurable amounts in 6-day old seedlings with peak concentration occurring 10–13 days post planting. Peramine continued to be translocated into the developing shoot as the seedling developed, while some remained in the seed residue, or small quantities are transferred into the root system (Ball, *et al.*, 1997b; Ball, *et al.*, 1997c). It is possible that in addition to the seed-stored alkaloid translocation phase the endophyte produces peramine in low levels, as Ball, *et al.* (1993) observed that seedlings infected with a viable endophyte had higher peramine concentrations 24 days after planting, than seedlings infected with a non-viable endophyte, although this was not statistically different. As the seedling matures from 10–27 day old plants, peramine concentrations were found to decrease, likely due to a dilution effect where plant growth increase is not synchronised with alkaloid seed translocation and/or early alkaloid synthesis (Figure 3.12). In approximately 27-day old seedlings peramine concentration was observed to equilibrate with mean concentrations above

10 µg/g for NZ_{CT} and below 10 µg/g for seedlings infected with NEA2 and AR1 (Figure 3.4). In 27–69 day old plants, the rate of peramine production matches the increase in plant growth, indicating that peramine production and plant growth are completely synchronised, explaining the constant peramine concentration in the plant (Figure 3.12). However, these concentrations were lower than those observed in mature plants infected with the same endophyte strains (Popay et al. in press), and below the estimated adult and larvae ASW field threshold of 15–20 µg/g (Popay and Wyatt, 1995). Accordingly the low peramine levels observed here in 27–69 day old seedlings infected with AR1, NEA2, and NZ_{CT}, might not be deterring the feeding of adult and larvae ASW. This provides an understanding of the results of earlier research showing an increase in seedling susceptibility after an initial period of resistance to adult ASW in glasshouse grown seedlings up to 43 days old, even though they contained a peramine producing endophyte strain (Ruppert, *et al.*, 2016).

In a previous study Ball, *et al.* (1993) reported a significant increase in peramine concentration between sampling of 48 and 120 day old seedlings, after trimming plants at 70 days old. It is unknown if this trimming event led to an inducement of alkaloid production observed in the Ball, *et al.* (1993) study. However, alkaloid concentrations can be influenced by plant clipping (Salminen, *et al.*, 2003) as well as plant age (Fuchs, *et al.*, 2013), which might have caused an increase in alkaloid production (Ball, *et al.*, 1993). In this current study alkaloid concentrations were monitored out to 69 days with no trimming to influence the alkaloid production, and a steady state peramine concentration was observed.

The total amount of lolitrem B significantly increased from 20–27 days in seedlings infected with the NZ_{CT} endophyte strain (Table 3.4), indicating that new lolitrem B was being produced in very small quantities by the endophyte or small quantities were being translocated into the

developing shoot (Figure 3.6). Lolitrem B is hydrophobic and it is not as evenly distributed in the plant in comparison to other alkaloid compounds (Spiering, *et al.*, 2005). Furthermore, Ball, *et al.* (1993) showed that lolitrem B is not as easily translocated from the seed into other parts of the plant in comparison to peramine. Even though it is possible that lolitrem B production might be activated as early as 12 days post planting (Ball, *et al.*, 1993), new lolitrem B production appears to be very slow in young seedlings, based on the small increase in the total amount of lolitrem B up to 27 days after sowing (Table 3.4). In about 27-day old seedlings plant growth and lolitrem B production in NZ_{CT} infected seedlings are highly synchronised, based on the logarithmic increase of both lines (Figure 3.5).

In this research a slight increase in lolitrem B concentration was observed in 10-day old seedlings infected with NZ_{CT} (Figure 3.6), likely due to the previously described seedling growth slow down. Such a concentration peak was also observed by Ball, *et al.* (1993), although in 24-day old seedlings. Possible reasons for an earlier expression of this peak in this experiment might be due to temperature difference, which may have caused the seedling to go into its steady growth phase sooner than in (Ball, *et al.*, 1993). Ball, *et al.* (1993) measured alkaloids in plants grown in a controlled environment at 20°C in a 16 hour light and 8 hours dark regimes. In contrast, plants from this study were grown in natural light conditions in a glasshouse environment with fluctuating temperatures peaking as high as 41.6°C (Figure 3.11).

As the seedling matured, lolitrem B concentrations in seedlings infected with NZ_{CT} rapidly increased approximately 55 days post planting (Figure 3.6). These finding are consistent with those of Ball, *et al.* (1993), who reported a steep increase in lolitrem B concentration in 48-day old seedlings. A general increase in lolitrem B concentration with seedling age has also been

reported by Gallagher, *et al.* (1987). Furthermore, Spiering, *et al.* (2005) reported a lolitrem B concentration increase of 1.5 to 2 fold between 40 and 60 days of age.

A similar increase, as seen with lolitrem B and peramine concentrations, occurred for ergovaline in 8–10 day old seedlings (Figure 3.8). Ergovaline distributes heterogeneously in the plant with a similar low mobility as lolitrem B (Spiering, *et al.*, 2005). However, it can be translocated in the plant as demonstrated by detection in the guttation fluid in endophyte-infected tall fescue (Koulman, *et al.*, 2007). It appears that ergovaline is being translocated from the seed into the emerging shoot, likely not to the same degree as peramine. Similar to peramine, seedlings infected with NEA2 and NZ_{CT} displayed an increase in the total amount of ergovaline in 10 and 8-day old seedlings, respectively (Table 3.4). However, concentrations levelled out much sooner than for peramine, and in a similar way to those of lolitrem B (Figure 3.8).

The pattern for epoxy-janthitrem concentrations display a time course similar to that for lolitrem. It appears that the growth rate of the seedling and total amount of epoxy-janthitrems in the seedling are in synchrony in 6 day old seedling onwards (Figure 3.9), which would indicate that there is very little in the way of translocation of epoxy-janthitrems from the seed into the seedling. Lolitrem B and epoxy-janthitrems are both indole diterpenoid, hydrophobic (lipophilic) compounds with similar biosynthetic pathways (Saikia, *et al.*, 2012), hence do not easily translocate through the aqueous conditions of the plant cells and apoplastic medium. Epoxy-janthitrem concentrations reached a minima in 20-day old seedlings and steadily increased thereafter, reaching a mean concentration of 13.0 μg/g at the end of the assessment period (69 days) (Table 3.5). The increase in epoxy-janthitrem concentrations starting in 20-day old seedlings contrast with those of Qawasmeh (2012), who reported epoxy-janthitrem

concentrations decline and became undetectable 27 days after seedling emergence in AR37-infected perennial ryegrass seedlings. Factors that are likely to have contributed to such a difference in epoxy-janthitrem concentrations may be that plants in Qawasmeh (2012) experiment were kept in a shade house exposed to 12 h light period with average temperatures during day and night of 14°C and 4°C, respectively. Low temperatures are known to decrease epoxy-janthitrem concentrations in *planta* (Hennessy*, et al.*, 2016) and might have contributed to low epoxy-janthitrem concentrations in Qawasmeh (2012) research.

It appears there are two phases of alkaloid appearance in endophyte-infected seedlings, in which alkaloids appear rapidly in approximately 6–13 day old seedlings and a slower appearance phase in approximately 13–69 day old plants (Figure 3.12). For example, plant growth and peramine appearance display a constant exponential increase in the shoot from 6–10 day old seedlings. During the very early alkaloid measurements in 6–13 day old seedlings, alkaloid levels might be boosted by translocation of the stored alkaloids in the caryopsis, as well as log linear de novo alkaloid synthesis. The combined effect of alkaloid translocation from the seed and early synthesis of new alkaloids might cause the total amount of alkaloid weight to increase in 6–13 day old seedlings. In 13-day old seedlings around 42% of the seed stored peramine present in the emerging shoot, possibly giving the seedling an alkaloid "kick start" from the caryopsis translocation. Such an increase in the total amount of alkaloids in the above ground seedling was found to be dependent on the alkaloid compound; ASW deterrent peramine increased earlier than lolitrem B, in about 13-day old seedlings (Table 3.4). Lolitrem B is known for its toxicity against mammals, which increased in 27-day old seedlings. If so this would constitute an 'evolutionary pressure' accounting for the pattern of alkaloid production during seedling establishment that was observed in this experiment.

In the second phase, alkaloid appearance seems to slow down in 13–69 day old seedlings, with an exponential constant rate, possibly expressing a shoot endogenous alkaloid production phase. It is unknown if alkaloid translocation from the seed continued after the exponential peramine increase in 6–10 day old seedlings. Ball, *et al.* (1993) measured decreasing peramine concentrations in seed residues up to 36 days after sowing, indicating that peramine might be metabolised with the seed symplast/apoplast or translocated into the seedling. However, when the total amount of alkaloid in seedlings rises above the original total amount of alkaloid in the seed, then this is the time the endophyte must have become metabolically active and has started to produce new metabolised alkaloids. For example, seedlings infected with AR1 and NEA2 showed higher amounts of peramine (µg/seedling) than in the seed in 34–41 day old seedlings (Table 3.4), indicating that any increase after this date must come from the endophyte producing peramine. Although the time frame of when the total amount of alkaloids in seedlings rises above the total amount of alkaloid in the seed gives a valuable indication of when the endophyte must have started to metabolise new alkaloids, the research question of when exactly the endophyte becomes metabolically active could not be answered in this study. This is due to the experimental set up, which focussed on measuring alkaloid concentrations and total amount of alkaloids in only above ground seedling material. It is believed that some alkaloids remained in the seed residue, or were transferred into the root system. A future hypothesis for further investigation is that endogenous *Epichloë* shoot mycelium production of peramine starts with seedling germination with a translocation increase from the caryopsis into the seed up to day 13. In order to determine the start and the end of the alkaloid translocation phase and the shoot endogenous alkaloid production phase, further research should be aimed at measuring alkaloid concentrations and the total amount of alkaloids in all parts of developing seedlings (root, shoot, and seed residue) during seedling establishment. Furthermore, the same

experiment should be compared with seedlings infected with a non-viable endophyte, as they still maintain high alkaloid concentrations (Ball*, et al.*, 1993). Regular harvesting of seedlings as well as seed residue containing non-viable endophyte will give more detailed information of how long it takes until transfer of stored alkaloids to the developing seedling is completed.

Chapter 4 Influence of adult ASW feeding on alkaloid production in *E. festucae* var. *lolii* infected perennial ryegrass seedlings during establishment

4.1 Introduction

Since the mid-1980s, comprehensive evidence has demonstrated that grasses utilize endophyte toxins as a chemical defence against herbivores (Johnson, *et al.*, 2013; Popay and Hume, 2011). Endophytes improve the insect resistance of grasses to a wide variety of insects from different orders, such as sod webworms (Lepidoptera: *Crambus* spp.) (Funk, *et al.*, 1983), black field cricket (Orthoptera: *Teleogryllus commodus*) (Quigley, *et al.*, 1993), and most importantly ASW (Prestidge, *et al.*, 1994). ASW have been a serious pest of New Zealand's improved pasture and turf for more than 100 years, greatly affecting plant growth, reproduction and yield production of pastures. They can affect many pasture grasses, including perennial ryegrass, tall fescue, and poaceous crops like maize (Kelsey, 1958). The larval stage of ASW is particularly damaging to pastures. Larvae bore through the epidermis of the grass leaf sheath into the tiller destroying the apical meristem tissue, resulting in tiller death. Adult weevils normally cause minor damage to mature plants, but can have a significant impact on developing seedlings. Newly sown seeds and emerging seedlings in agricultural systems are often exposed to adult ASW feeding. Severe feeding at the base of a seedling in the region of the meristem can cause plant death or severe stunting of the plant (Ruppert, *et al.*, 2016), which can have a significant impact on pasture establishment and persistence. In 1991 it was estimated that ASW causes NZ$46–202 M/year financial loss to New Zealand's agriculture sector (Prestidge et al. 1991).

However, the impact of ASW on New Zealand's pastoral industry has been lessened through endophyte-induced plant resistance as well as through the introduction of a parasitoid wasp, *Microctonus hyperodae* Loan. ASW adults strongly prefer endophyte-free plants for feeding and oviposition when the endophyte-infected plants produce peramine (Popay and Hume, 2011), as peramine is the main alkaloid compound responsible for ASW deterrence. Adult ASW were deterred from feeding on artificial diets containing peramine levels as low as 0.1 µg/g (Rowan*, et al.*, 1990). However, ASW larvae need much higher levels of peramine to be deterred, with the threshold estimated to be 10 µg/g (Rowan, et al., 1990).

The concentration of peramine in NZ_{CT}-infected ryegrass is usually greater than the levels at which peramine shows feeding deterrent activity against ASW in laboratory assays (Rowan, 1993; Tapper, et al., 1989). A minimum peramine concentration of 15–20 µg/g *in planta* has been estimated to provide effective control of adult and larvae ASW in the field (Popay*, et al.*, 2003; Popay and Wyatt, 1995).

Other alkaloids produced by the endophyte play a minor role in ASW resistance in plants. Although adult ASW are highly sensitive to ergovaline (Popay*, et al.*, 1990), ergovaline is found only in low concentrations in leaf blades where adult ASW usually feed (Lane*, et al.*, 2013), whereas lolitrem B has antibiotic activity toward ASW larvae, but does not deter adult ASW (Prestidge and Gallagher, 1985). Accordingly, ergovaline and lolitrem B are not essential for sustaining a high level of ASW deterrence in endophyte-infected ryegrass pastures (Popay and Wyatt, 1995).

Alkaloid concentrations can be influenced by various biotic and abiotic factors including temperature (Breen, 1992), host and endophyte genotype (Spiering*, et al.*, 2005), as well as cultural practices like grass clipping (Salminen*, et al.*, 2003). The induced synthesis of

alkaloids by the endophyte is described as "exogenous resistance", determined by the endophyte rather than the host grass (Sullivan, *et al.*, 2007). This induced response allows the endophyte to produce increased amounts of chemical defence compounds only when they are needed.

In a previous chapter it was found that 27–69 day old endophyte-infected seedlings maintain peramine and ergovaline concentrations at low levels, around 10 µg/g and 0.6 µg/g, respectively, although the seedling increases dry weight production (Figure 3.4; Figure 3.8). These low levels of peramine and ergovaline might leave the seedling susceptible to insect predation in the field. In the previous experiment described in Chapter 3 seedlings were grown in a glasshouse environment with optimal growing conditions without insect pressure. Accordingly, endophytes may have no need for increased alkaloid protection if it is not stressed. Therefore, the host plant might have more energy resources available, which can be allocated towards plant growth rather than chemical defence. It would be of interest to know if insect pressure at this development stage might trigger an increase in alkaloid production as a defence response. To date, no research has been undertaken to determine if adult ASW feeding affects the alkaloid concentrations in developing seedlings resulting in the seedlings being better able to withstand high insect pressure. This study focuses on the question of whether adult ASW feeding induces an increase in alkaloid production in developing endophyte-infected seedlings.

4.2 Materials and Methods

4.2.1 Insect material

Adult ASW were captured in late-summer on the 8th & 9th of February 2016 on a Ruakura Research farm, using a leaf blower converted to provide vacuum, to suck the insects into a removable net recessed into the inlet pipe. Sampled paddocks contained tetraploid short rotation (hybrid) ryegrass "Delish AR1", and were regularly grazed by sheep and cattle. Collections were made by dragging the vacuum device along a 200m transect across each paddock. Weevils were removed from the litter and separated from other non-targeted insects and held in 33 x 21 x 12 cm containers with a moistened filter paper floor at room temperature. Weevils were fed weekly with fresh endophyte-negative, diploid- perennial ryegrass (One50) until needed. Dead and parasitized weevils or parasitoids were removed weekly.

4.2.2 Experimental design

The experiment was conducted from February 12th to March 24th 2016 in a glasshouse located at AgResearch Grasslands campus, Palmerston North. Four bioassays were set up, where adult ASW were placed onto developing seedlings at different plant ages and compared to plants without insect pressure (Table 4.1). Each bioassay consisted of four ASW cages and four control trays without adult ASW exposure, for comparison. Bioassay 3 and 4 consisted of two cages and two controls, due to a technical problem during the experiment, resulting in the split of bioassay 3 into two separate bioassays. In each bioassay adult ASW were able to choose between seedlings that were infected with one of four different endophyte strains (Figure 4.2).

Table 4.1: Experimental set up for adult ASW bioassays. Adult ASW were able to feed on developing seedlings for five consecutive days before plants were harvested for alkaloid analysis.

Bioassay	**Seedling age (in days after planting) when exposed to ASW**	**Seedling age (in days after planting) when harvested for alkaloid analysis**
1	36-41	41
2	43-48	46
3	50-55	55
4	57-62	62

One seed per plug was germinated in 12x12 plastic propagation trays (40x40x5cm) filled with standard seed raising mix (75% screened fine bark, 12.5% coir fibre, 12.5% pumice, NPK 16-3.9-10) and grown under glasshouse conditions for 35 days without ASW exposure. For each ASW bioassay cage, 100 adult ASW were left to feed for 5 consecutive days before plants were harvested for alkaloid analysis. The weevils were contained in a cubical cage constructed from an aluminium frame and fabric mesh (mesh size 1.0 x 0.5 mm) (Figure 4.1).

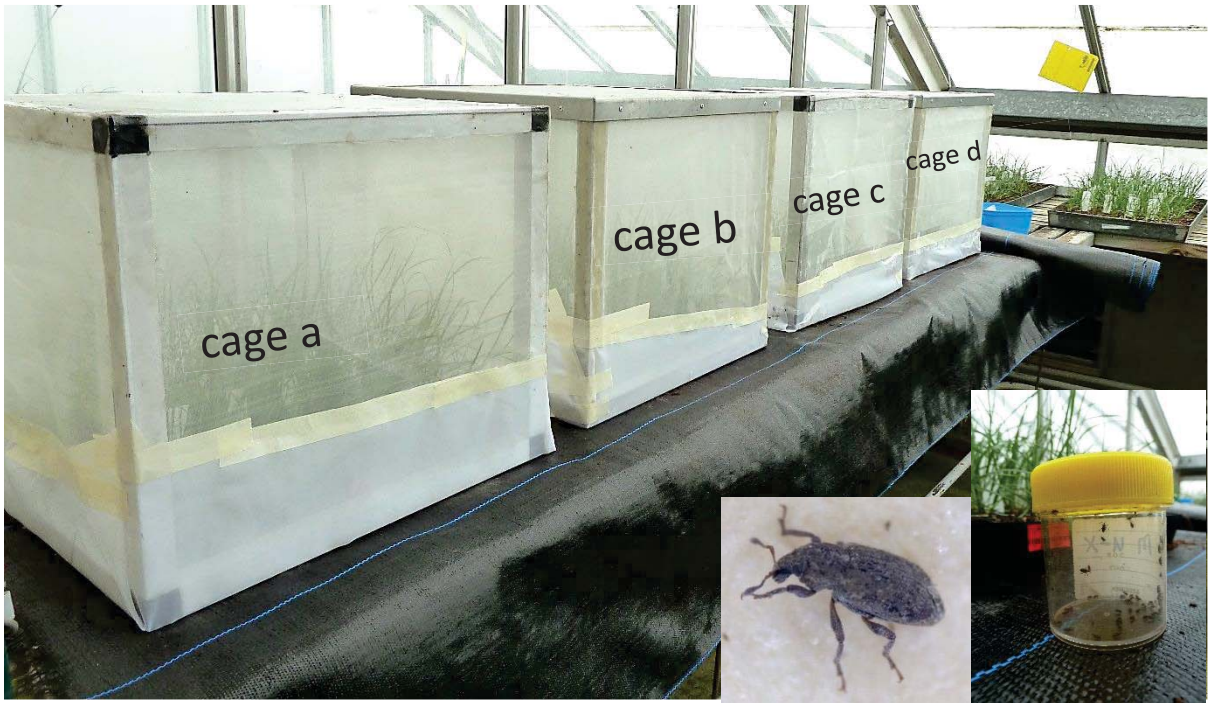


Figure 4.1: A bioassay in progress. One bioassay consisted of four cages. In each cage adult ASW were able to feed for 5 days on seedlings infected with *E. festucae* var. *lolii* strains AR1, AR37, NEA2, and NZ_{CT}. Developing seedlings were then harvested and analysed for alkaloid concentrations.

Covers were removed for seedling harvests, along with the corresponding control bioassays. Plants within a treatment replicate were trimmed at ground level with a scalpel and transferred into 50 mL screw cap vials and shock frozen in liquid nitrogen. Samples were lyophilized (Labconco, Free-Zone Plus, Model 7752030, Kansas, USA) for 72 h, and a total dry-weight for each treatment block was recorded. Alkaloids were extracted as described in 2.4. All plants were maintained in the glasshouse under natural light conditions. Throughout the experiment, air temperatures and relative humidity were recorded at 10 min intervals using a Digitech QP-6013 Data logger. Trays were placed on a capillary bed so that seedlings received water as required.

4.2.3 Statistical data analysis

A 4 x 4 Latin Square design was used with the four endophyte treatments randomly allocated within in each row and column of the block layout, so that each treatment occurred four times in each of the four bioassays (Figure 4.2). One bioassay consisted of four cages, where each cage was randomised differently. The same cage layouts were used for each bioassay. Four seedling samples from one endophyte treatment were bulked up to one sample (Figure 4.2). Seed infected with NZ_{CT} were planted around the Latin square design, to minimise weevil movement on the propagation tray. Grey shadowed data were excluded from data analysis.

Column / **Row**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1												
2		NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	
3		NZ_{CT}	AR1	AR1	NZ_{CT}	NZ_{CT}	AR37	AR37	NEA2	NEA2	NZ_{CT}	
4		NZ_{CT}	AR1	AR1	NZ_{CT}	NZ_{CT}	AR37	AR37	NEA2	NEA2	NZ_{CT}	
5		NZ_{CT}	AR37	AR37	NEA2	NEA2	AR1	AR1	NZ_{CT}	NZ_{CT}	NZ_{CT}	
6		NZ_{CT}	AR37	AR37	NEA2	NEA2	AR1	AR1	NZ_{CT}	NZ_{CT}	NZ_{CT}	
7		NZ_{CT}	NZ_{CT}	NZ_{CT}	AR1	AR1	NEA2	NEA2	AR37	AR37	NZ_{CT}	
8		NZ_{CT}	NZ_{CT}	NZ_{CT}	AR1	AR1	NEA2	NEA2	AR37	AR37	NZ_{CT}	
9		NZ_{CT}	NEA2	NEA2	AR37	AR37	NZ_{CT}	NZ_{CT}	AR1	AR1	NZ_{CT}	
10		NZ_{CT}	NEA2	NEA2	AR37	AR37	NZ_{CT}	NZ_{CT}	AR1	AR1	NZ_{CT}	
11		NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	NZ_{CT}	
12												

Figure 4.2: Latin square design with four endophyte strains AR1, AR37, NEA2, NZ_{CT} and four replications per bioassay. Grey shadowed data were excluded from data analysis. The same cage layouts were used for each bioassay.

The impact of adult ASW feeding on alkaloid concentration and shoot dry weight were analysed using one-way ANOVA, with the response variables being seedling age and insect pressure. An example showing analysis of peramine concentration from control plants and seedlings attacked by ASW and the relevant GenStat output is given in Appendix 2. Further exploratory analysis including a two-way ANOVA, with the response variables being seedling age, insect pressure, and endophyte strain. Where necessary, to meet the statistical assumption of homogeneity of variance between endophyte treatment and seedling age, alkaloid concentrations were square root or log transformed prior to analysis. Fisher's Least Significant Difference (LSD) test was used to assess whether differences between alkaloid concentration and adult ASW feeding were significant. ANOVA residuals were checked for normality (Shapiro-Wilk test) and homogeneity (Bartlett`s test).

4.3 Results

4.3.1 Influence of adult ASW feeding on alkaloid concentration

The effect of adult ASW on alkaloid concentrations was influenced by the seedling age (Appendix 3), which is discussed in detail in this chapter for each alkaloid compound. Although not always significant, a consistent pattern was observed where younger plants produced higher alkaloid levels after adult ASW feeding than control plants. However, as the seedlings matured, alkaloid concentrations were lower in seedlings browsed by ASW than in control plants. The effect of ASW attack on alkaloid concentration for each endophyte strain is given in Appendix 4.

4.3.1.1 Peramine

Adult ASW feeding had a marginally significant impact on peramine concentrations in seedlings (p=0.053). No significant interaction was found between insect pressure, seedling age and the different endophyte strains (p=0.136) (Appendix 4). Peramine concentrations in 41 and 48-day old seedlings appeared to be higher in plants exposed to ASW than in control plants, yet these differences were not significant (Figure 4.3). In contrast to this, 55 and 62-day old seedlings appeared to have lower peramine concentrations after adult ASW feeding than control plants (Figure 4.3).

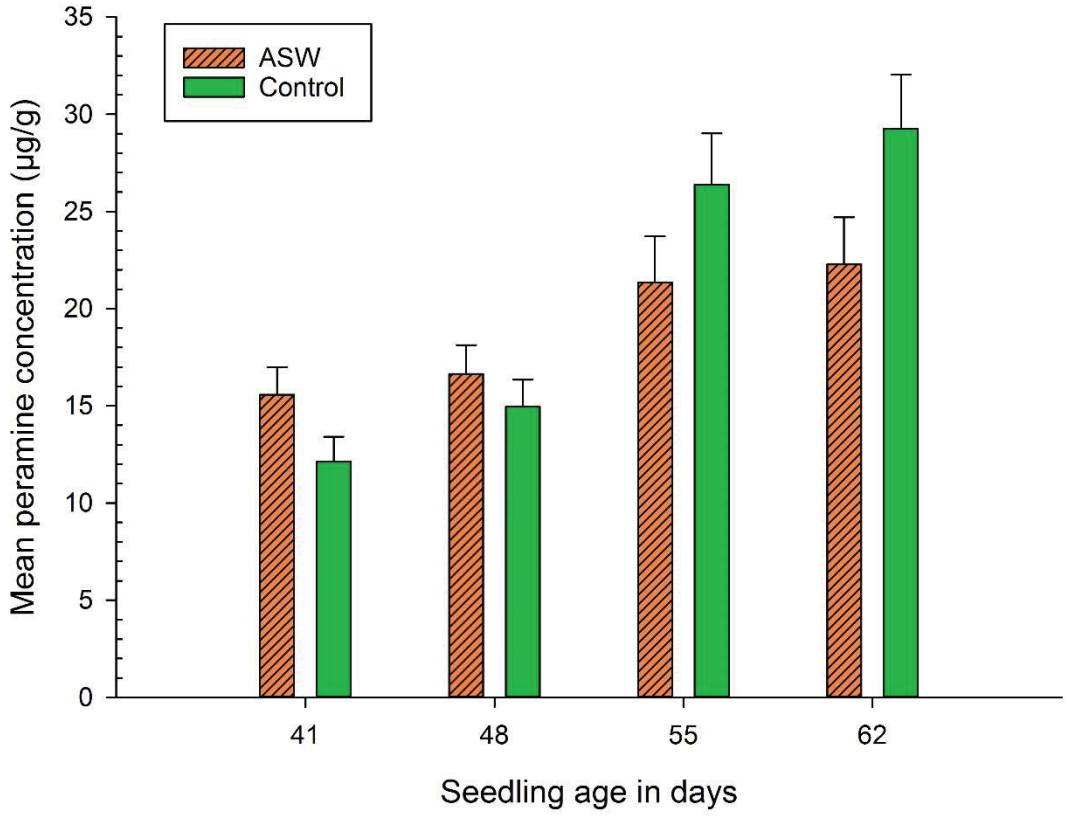


Figure 4.3: The effect of adult ASW feeding on mean peramine concentrations (µg/g) in developing perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strains AR1, NEA2, and $NZ_{CT.}$ Adult ASW had a marginal significant impact on peramine concentration overall (p=0.053). Error bar represent standard error of the mean (+SEM).

4.3.1.2 Lolitrem B

An induced alkaloid response was most significant for lolitrem B, where a significant interaction was found between two parameters: seedling age and presence of ASW (Appendix 3). Adult ASW feeding had significant impact on lolitrem B concentrations, where 62-day old seedlings produced significantly lower lolitrem B levels when exposed to adult ASW ($p=0.036$) (Figure 4.4). Furthermore, a significant difference was found in the interaction between three parameters: seedling age, presence of ASW, and endophyte strain (Appendix 4). Forty-one and 48-day old seedlings infected with NZ_{CT} produced significantly higher lolitrem B levels after adult ASW browsing than control plants ($p<0.001$) (Appendix 4). Seedlings infected with NZ_{CT} produced significantly higher lolitrem B concentrations than seedlings infected with NEA2 ($p<0.001$) (Appendix 4).

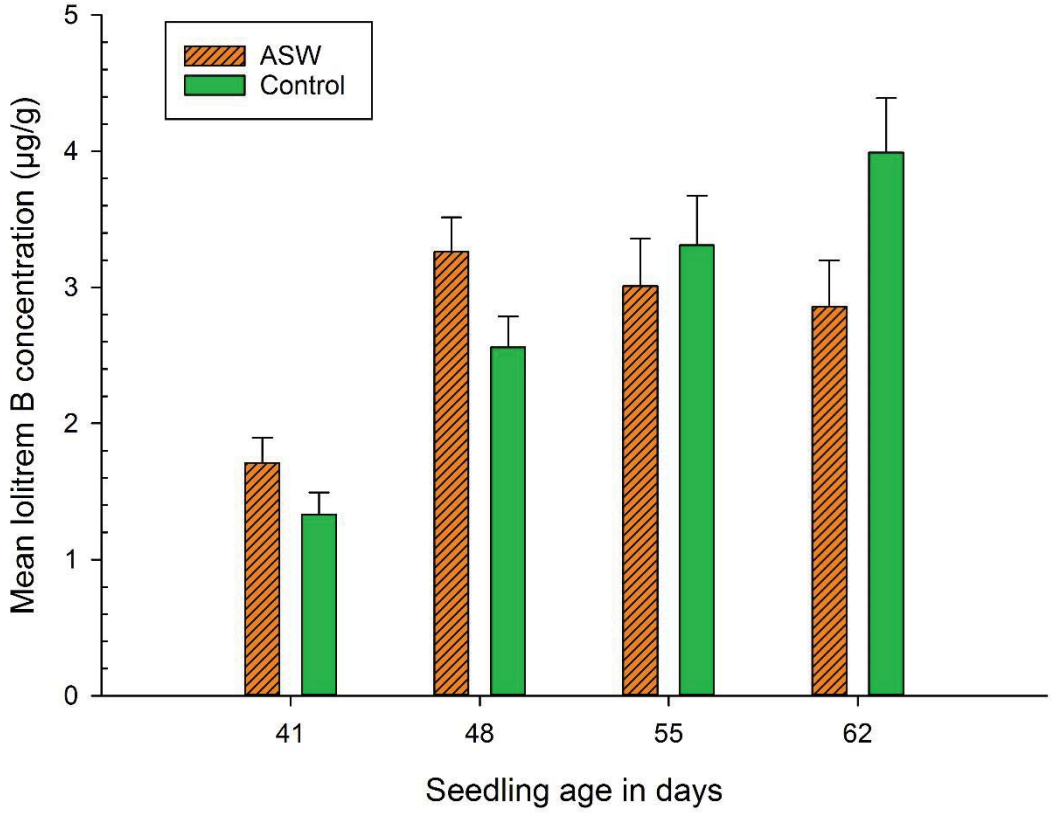


Figure 4.4: The effect of adult ASW feeding on mean lolitrem B concentrations (µg/g) in developing perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strains NEA2 and NZ_{CT}. In 62-day old plants lolitrem B concentrations were significantly lower in plants exposed to ASW feeding (p=0.036). Error bar represent standard error of the mean (+SEM).

4.3.1.3 Ergovaline

Adult ASW feeding had a significant impact on ergovaline concentrations, whereby 41-day old seedlings produced significantly higher ergovaline levels when exposed to adult ASW, than control plants (p=0.014) (Figure 4.5).

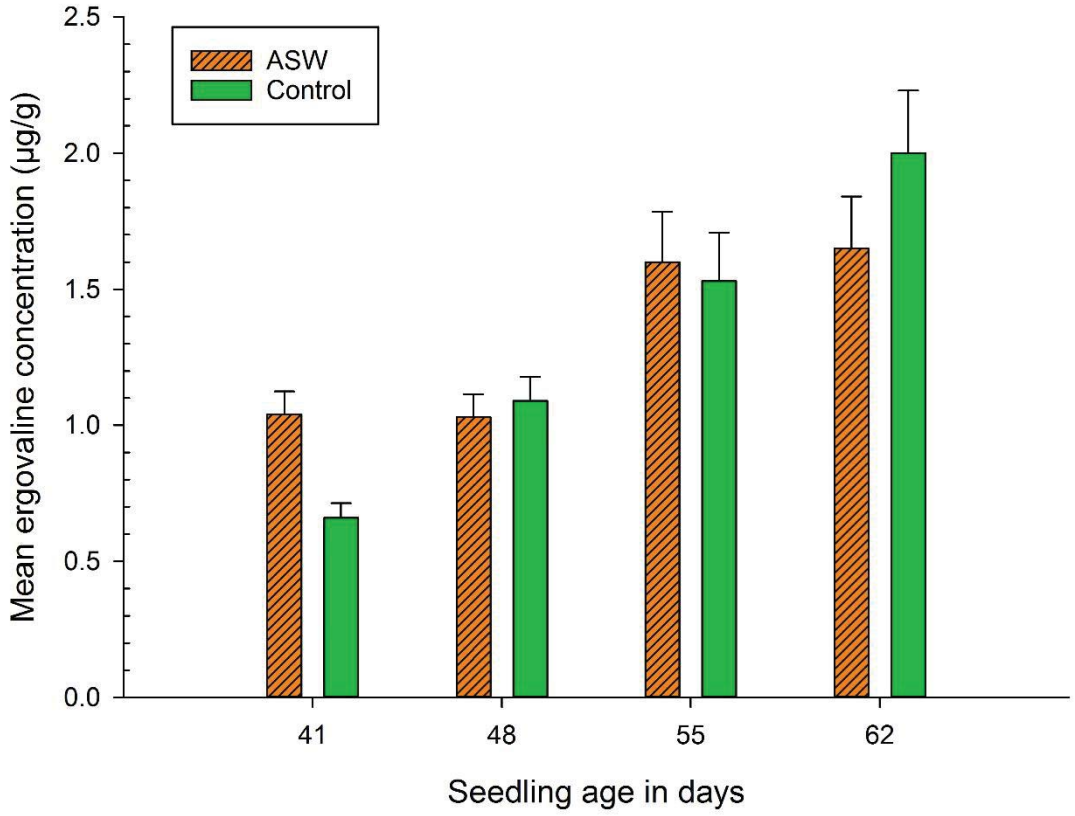


Figure 4.5: The effect of adult ASW feeding on mean ergovaline concentrations (µg/g) in developing perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strains NEA2 and NZ_{CT}. Adult ASW feeding significantly increased ergovaline concentration in 41-day old plants. In contrast 41-day old seedlings produced significantly lower ergovaline levels when exposed to adult ASW than control plants (p=0.014). Error bar represent standard error of the mean (+SEM).

4.3.1.4 Epoxy-janthitrems

Adult ASW feeding had no significant impact on epoxy-janthitrem concentrations (p=0.437) (Appendix 3). Although not significant epoxy-janthitrems concentrations appear to be higher in 41 and 48 day old seedlings exposed to ASW than in control plants (Figure 4.6). In contrast to this, 55 and 62-day old seedlings appear to have lower epoxy-janthitrem concentrations after adult ASW browsing than control plants (Figure 4.6).

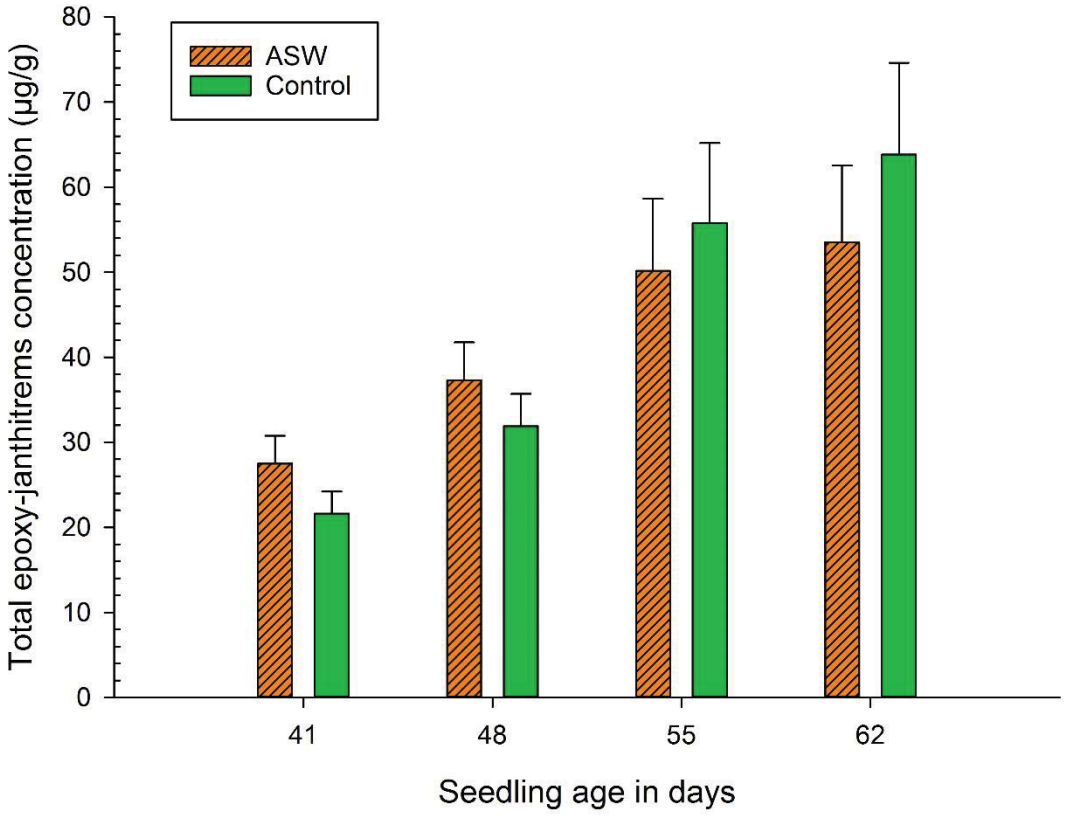


Figure 4.6: The effect of adult ASW feeding on mean epoxy-janthitrem concentrations (µg/g) in developing perennial ryegrass seedlings infected with *E. festucae* var. *lolii* strain AR37. Adult ASW had no significant impact on epoxy-janthitrem concentrations overall (p=0.437). Error bar represent standard error of the mean (+SEM).

4.3.2 Shoot dry matter

ASW feeding had a significant impact on shoot dry matter, where 41-day old plants produced significantly higher dry matter after ASW feeding than control plants ($p<0.05$). As the plant aged to 55 and 62 day old, control plants produced significantly more dry matter than plants browsed by ASW ($p<0.05$). There was no significant interaction between ASW feeding, seedling age, and endophyte strains ($p=0.970$) (Appendix 5).

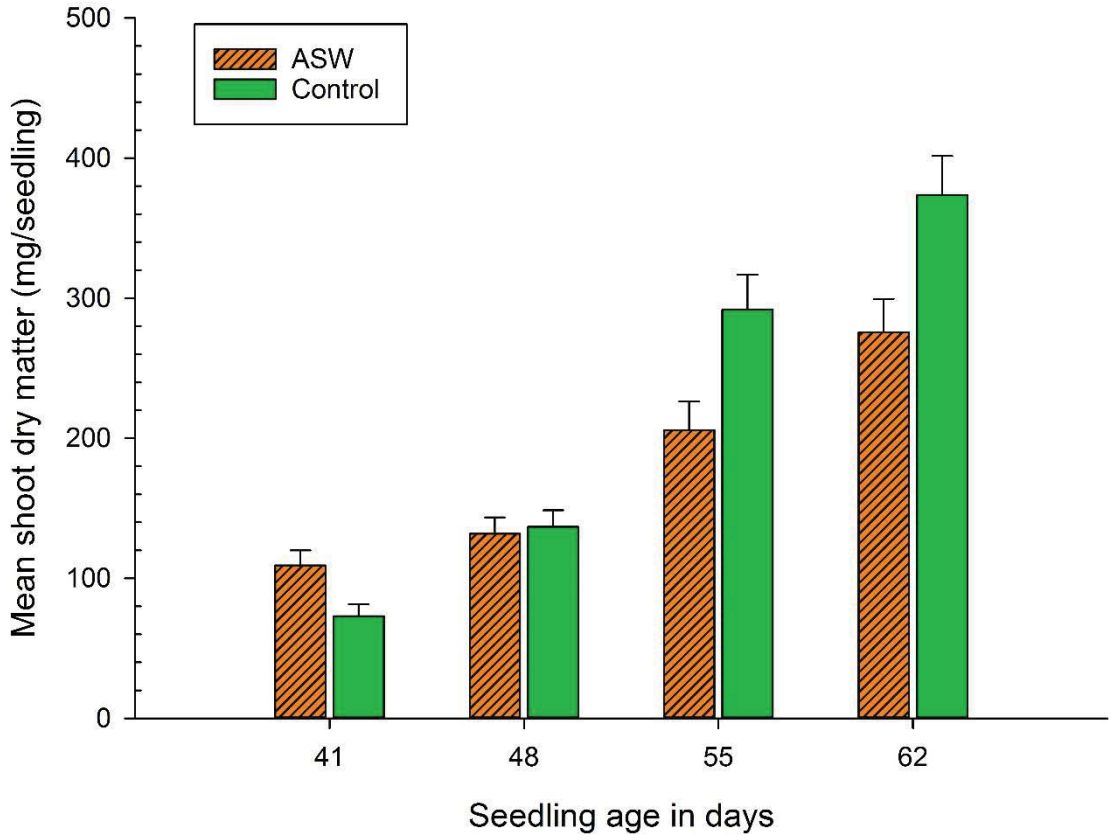


Figure 4.7: The effect of adult ASW feeding on dry matter production (mg/seedling) of *E. festucae* var. *lolii* infected perennial ryegrass seedlings. Error bar represent standard error of the mean (+SEM).

4.3.3 Glasshouse temperature

The mean glasshouse temperature, as well as mean relative humidity are recorded in Figure 4.8. The highest temperature recorded during the experiment was during bioassay 1, with 38.5°C, while the lowest temperature of 11.8°C occurred during the fourth bioassay.

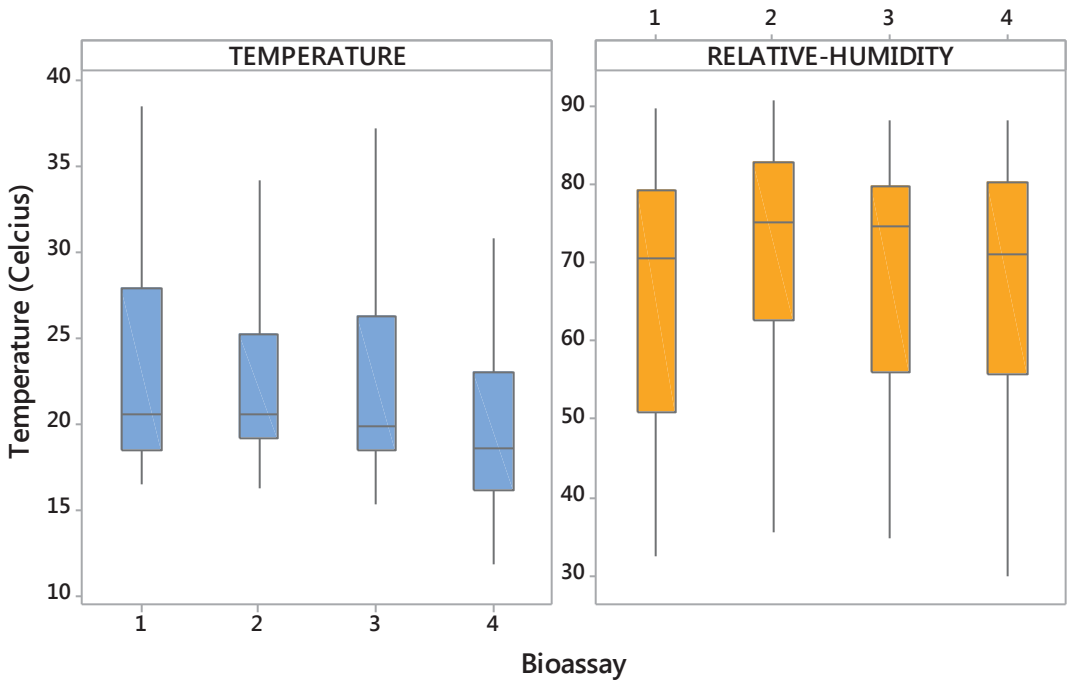


Figure 4.8: Boxplot of temperature (°C) and relative humidity (%) recordings for each adult ASW bioassay (Bioassay 1=36–41 day old seedlings; bioassay 2= 43–48 day old seedlings; bioassay 3= 50–55 day old seedlings; bioassay 4= 57–62 day old seedlings).

4.4 Discussion

A general pattern for all alkaloid compounds was observed where young seedlings (41–48 days old) appear to produce higher alkaloid levels after adult ASW feeding than control plants, although not always significant. This induced alkaloid response was most significant for lolitrem B, where a significant interaction was found between two parameters: seedling age and presence of ASW (Appendix 3), as well as the interaction between three parameters: seedling age, presence of ASW, and endophyte (Appendix 4). Lolitrem B concentration in 41–48 day old seedlings infected with NEA2 were significantly higher after ASW browsing, compared to seedlings grown without insect pressure ($p<0.001$; Appendix 4). A similar result for induced alkaloid response was observed for ergovaline, where 41-day old seedlings showed significantly higher ergovaline concentrations after ASW feeding, compared to control plants ($p=0.014$) (Figure 4.5). However as the seedlings matured (55–62 day old seedlings), a reverse pattern was observed, where control plants produce higher alkaloid concentrations than seedlings browsed by insects. In 62-day old seedlings lolitrem B concentration was significantly higher in control plants with an average of 3.99 µg/g compared to seedlings attacked by adult ASW with an average of 2.86 µg/g (Appendix 3).

Additionally, a similar pattern as seen with alkaloid concentrations was observed for dry matter production, where younger seedlings aged 41-days old that were exposed to ASW adult feeding produced significantly higher shoot dry matter than control plants (Figure 4.7). On the other hand, seedlings aged 55–62 days old produced significantly lower shoot dry matter after adult ASW browsing, than control plants (Figure 4.7). This pattern was similar across all tested endophyte strains (Appendix 5). A reason for this might be that young seedlings protect themselves though increased alkaloid concentrations, as well as with increased growth rates as

they perceive ASW feeding damage and compensate for that. It is unknown why alkaloid concentrations and dry matter production appear to be higher in control plants as they mature. Bultman, *et al.* (2004) found that the induced alkaloid response and resistance of endophyte-infected tall fescue to aphids (*Rhopalosiphum padi* Linn.) after plant damage (clipping), resulted in reduced plant growth rates. It is believed that alkaloid production and plant growth are inextricably linked (Christensen and Voisey, 2007), where reduced plant growth may also result in reduced alkaloid concentration in the plant.

Perennial ryegrass needs regular cutting or grazing to maintain growth (Davies, *et al.*, 1981). Plants from this experiment were not trimmed, which might have slowed the production of dry matter. Furthermore, because no additional fertilisers were applied during the experiment, plants were likely deprived of nutrients as they matured in the restricted soil volume. These reasons might have slowed down plant growth which subsequently resulted in reduced alkaloid concentrations in 55–62 day old plants, particularly in ASW exposed plants.

Induced resistance has been reported by Bultman and Ganey (1995), who observed that endophyte-infected perennial ryegrass plants showed a greater resistance to fall army worm (*Spodoptera frugiperda* Smith) after plants were artificially damaged. Although Bultman and Ganey (1995) did not measure alkaloid concentrations, it might be that plants reacted to insect feeding with increased alkaloid concentrations. Sullivan, *et al.* (2007) demonstrated that loline concentrations increased in endophyte-infected tall fescue after fall army worm feeding. The increased chemical defence after herbivory was correlated with the upregulation of the LolC gene cluster, which is responsible for loline synthesis (Sullivan, *et al.*, 2007). Even though the ryegrass endophyte *E. festucae* var. *lolii* does not produce lolines, parallel changes in

expression of genes involved in production of other alkaloids may be occurring after ASW feeding activity.

The reason that the induced alkaloid response on younger plants and reduction of alkaloid concentration in older plants after adult ASW browsing are not always significant, might be due to the experimental design. It is possible that we did not apply sufficiently strong ASW feeding pressure to developing plants (1 adult ASW/plant) and/or that exposure to feeding (5 days) was too short. Adult ASW feeding has only a minor influence on mature perennial ryegrass plants in comparison to fall armyworm, which was used by Sullivan*, et al.* (2007). Characteristic adult ASW damage appears as small rectangular "windows" in the leaf due to leaf tissues being scraped off, leaving only the cuticle and occasionally broken veins. It might be that the more damage herbivores are causing, the bigger the chemical response of the plant. Further research should be aimed at having longer exposure periods and higher adult or larval ASW pressure to determine if these differences between plants exposed to insect browsing and control plants would increase.

Our hypothesis that adult ASW feeding induced an increased chemical response has been proven, although this was only statistically significant for lolitrem B and ergovaline in 41 and 48-day old seedlings. Nevertheless all alkaloids exhibited a very similar pattern. This pattern seems to be reversed as seedlings age, where alkaloid concentrations appeared to be higher in control plants compared to seedlings browsed by ASW. Further research should be aimed in repeating this experiment and verifying that these reverse patterns are actually caused by ASW feeding. It might be that reduced alkaloid concentration and dry matter production in older plants are a by-product of experimental conditions as described earlier.

Chapter 5 General Discussion

Grasses have co-evolved with *Epichloë* endophytes, which provide the grass with an effective defence system through alkaloid production against invertebrate herbivores. This defence mechanism was explored in this thesis during seedling establishment of endophyte-infected perennial ryegrass.

Seedlings are particularly vulnerable to insect predation due to their small size, where tissue loss caused by herbivorous feeding, such as by adult ASW, can have a great impact on seedlings causing severe stunting and seedling death (Ruppert, *et al.*, 2016). During pasture renewal practises, seedling protection from pests is generally provided through the use of insecticide seed treatments, such as clothianidin. However, the presence of *Epichloë* fungal endophytes in the seed can play a major role in enhancing ryegrass seedling survival (Ruppert, *et al.*, 2016). Endophyte-infected seedlings exhibit a strong anti-herbivore defence mechanisms, which already starts in the seeds. Endophyte-infected seeds contain extremely high alkaloid concentrations, and were found to reduce seed predation by insects (Popay, *et al.*, 2000) and birds (Madej and Clay, 1991).

The results of this thesis show an extremely high alkaloid concentration in seed compared to other parts of the plant. As seedlings germinated, alkaloid concentrations were found to peak in 8–10 day old emerging seedlings, such as with peramine, which had concentrations of up to 43.2 µg/g in NZ_{CT}-infected seedlings (Table 3.5). Such a burst of alkaloid concentration is believed to be caused by the combined effect of translocation from seed stored alkaloids out of the caryopsis, and early synthesis of alkaloids in the emerged seedling (see Chapter 3.4). Furthermore, the peak in alkaloid concentration observed for all tested alkaloid compounds in

10–13 day old seedlings was also likely influenced by the seedling growth rate, where the plant is not growing as fast and yet the alkaloids are still translocated from the seed into the shoot. The success of the early translocation phase is dependent on the alkaloid compound, where hydrophilic compounds such as peramine are more easily translocated out of the caryopsis into the seedling than hydrophobic compounds such as lolitrem B or epoxy-janthitrems.

Nonetheless, the increase in alkaloid concentration in 8–10 day old seedlings appears to be of benefit to seedling survival in a high insect pressure environment. In 10-day old seedlings it is estimated that the amount of peramine in the shoot corresponds to approximately 20% of that which was contained in the seed (Table 3.4). As the production of peramine by the endophyte in the shoot at this stage is expected to be very low, the majority of this peramine is likely a result of translocation from the seed into the shoot, possibly giving the seedling a "kick start" in alkaloid levels/concentrations and protection of the emerging seedling from adult ASW feeding during the first 11 days after sowing. During these first 11 days after sowing adult ASW were able to distinguish between endophyte-infected, peramine-containing seedlings and endophyte-free seedlings and concentrated their feeding on those seedlings lacking endophyte or infected with a non-peramine producing strain (Ruppert*, et al.*, 2016). However, after the initial protection period, seedlings became increasingly susceptible to adult ASW predation and seedling survival was significantly reduced, even though they contained a peramine producing endophyte strain. This thesis provides data consistent with the conclusion of Ruppert*, et al.* (2016) that the loss of adult ASW resistance is linked with a reduction in chemical defence as seedlings age (Figure 5.1). In NZ_{CT} infected seedling for example, peramine concentrations decreased from 43.2 µg/g in 10 to 27 day old seedlings to 10.3 µg (Figure 3.4).

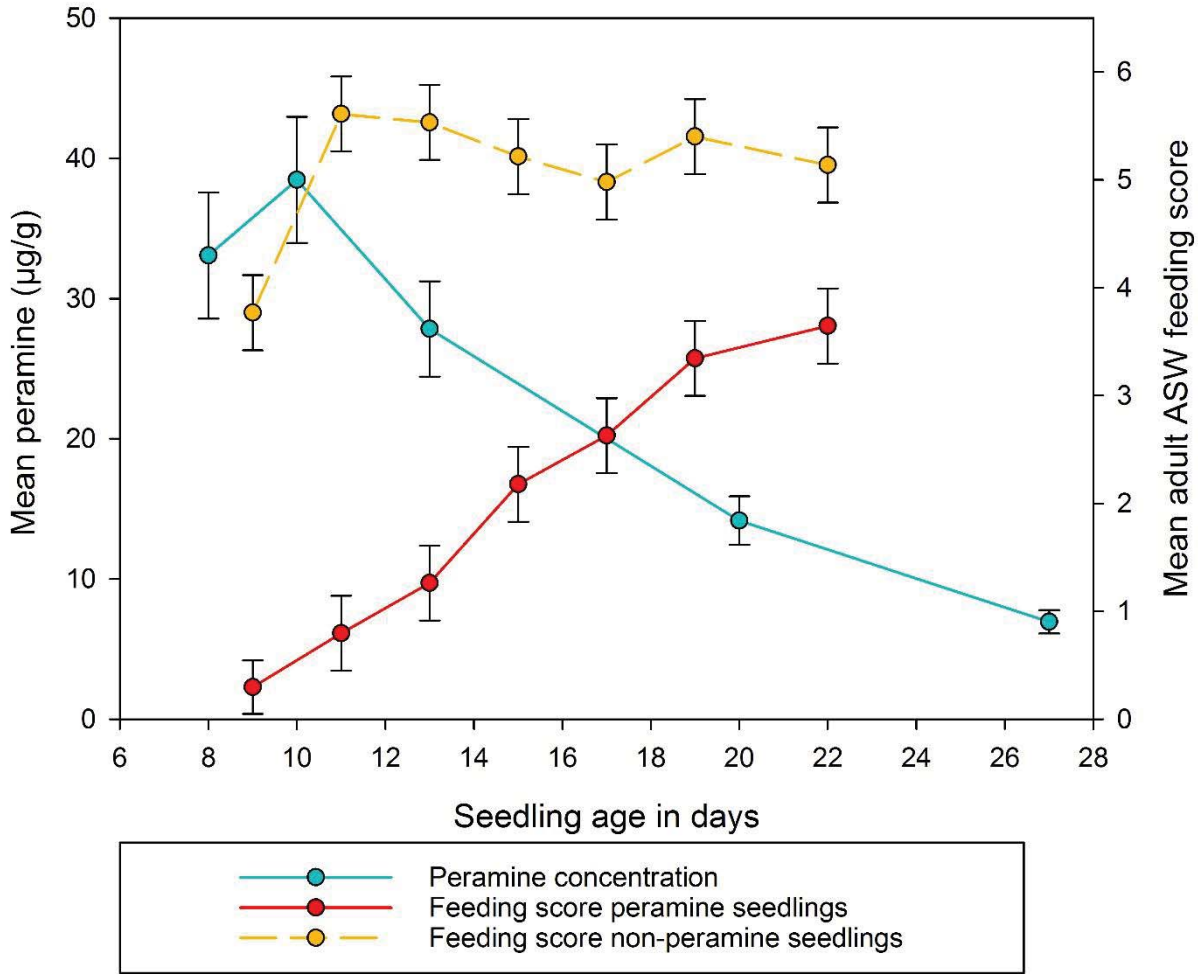


Figure 5.1: The relationship between peramine concentration (mean of AR1, NEA2, and NZ_{CT}) and adult ASW feeding in *E. festucae* var. *lolii* infected perennial ryegrass seedling during the early establishment phase. Adult ASW feeding scores were reproduced from Ruppert, *et al.* (2016) (on a scale of 0–6 whereby: 0= no damage, 1= little feeding; test nibbles can be seen, 3= feeding on base of tiller, 4= feeding on base and tip of tiller, 5= seedling completely chewed off and detached from seed and 6= seedling died after extensive feeding. Feeding score of peramine containing seedlings is the mean of AR1, NEA2, and NZ_{CT}. Feeding score of non-peramine containing seedlings is the mean of AR37 and endophyte-free seedlings. Error bar represent standard error of the mean (+SEM).

A similar scenario can be postulated from the work of Ball, *et al.* (1993) who showed that seedlings infected with a non-viable endophyte exhibited high alkaloid concentrations in the first 12 days after sowing with peramine levels above 30 µg/g. During this time the plant is

likely protected from insect feeding by the inherited endophyte alkaloids, but then grows into ASW susceptible mature plants. Before the endophyte discovery Kain and Atkinson (1977) reported similar results where seedling resistance was often found to be limited to the early stages of seedling emergence. Because the endophyte has died within the seed, no new metabolised alkaloids are produced, resulting in ASW susceptible mature plants. However, plants from this experiment were infected with a viable endophyte and after seed stored alkaloids were diluted as the seedling grew, the endophyte started to produce new metabolised alkaloids. For example seedlings infected with NZ_{CT} rapidly increased their lolitrem B concentrations 48 days after planting (Figure 3.6), indicating a production of new metabolised alkaloids must have commenced. Based on the alkaloid concentration pattern of endophyte-infected seedlings, results from this thesis are likely confirming our hypothesis of an initial translocation of seed stored alkaloids, followed by a period of dilution by seedling expansion, and finally production of new metabolised alkaloids in the plant.

However, not all alkaloid compounds showed such an increase in alkaloid concentration once the endophyte became metabolically active. In contrast to lolitrem B, peramine concentrations in 27–69 day old plants were found to level out without a significant increase during that period (Figure 3.4). The original hypothesis of Ruppert*, et al.* (2016) was a temporary loss of alkaloid protection between caryopsis alkaloid being diluted and new shoot endophyte alkaloid production commencing. Once the endophyte has become metabolically active peramine concentrations were thought to increase, protecting the young plant from ASW feeding. However, even though the endophyte is metabolically active and has started to produce new peramine, concentrations in this experiment in 27–69 day old plants remained low with mean concentration of 5.3 µg/g for AR1, 4.6 µg/g for NEA2, and 10.0 µg/g for NZ_{CT} (Table 3.5). During this time seedlings were probably vulnerable to adult ASW feeding, as peramine

concentrations were below the adult and larvae ASW field threshold of 15–20 µg/g (Popay and Wyatt, 1995). However, in a previous study, plants with the same cultivar, infected with the same endophyte strains, and grown under similar glasshouse conditions, showed little adult ASW feeding damage in 46–64 day old plants (Ruppert, *et al.*, 2016). (Ruppert, *et al.*, 2016). After seedlings reached the four tiller stage, adult ASW feeding caused very minor damage to plants, as plants grew faster than the adult weevils could feed.

When given a choice adult ASW were deterred from peramine containing artificial diets as low as 1.0 µg/g (Popay, *et al.*, 1990) and preferred feeding on endophyte-free plants, or those which were infected with a non-peramine producing endophyte strain. The peramine levels recorded during this study in 27–69 day old plants were probably sufficient to deter adult ASW feeding during seedling establishment but effect on oviposition at those concentrations are unknown. Low peramine levels recorded in 27–69 day old AR1 and NEA2-infected plants were below the estimated threshold of 10 µg/g against ASW larvae (Rowan, et al., 1990). ASW larvae are able to migrate from neighbouring plants to other tillers if food supply decreases, where they confine most of their feeding to the meristem region (Barker, *et al.*, 1984). Accordingly, plants form this study had likely high enough peramine levels to deter adult ASW feeding and possibly oviposition, but were probably susceptible to migrating ASW larvae from neighbouring plants. Although peramine concentrations in young AR1 and NEA2-infected seedlings are very similar, peramine concentration between these two strains differ greatly as they mature, where mature plants of Trojan NEA2 produced significantly lower levels of peramine than another diploid perennial cultivar, Samson, infected with AR1 or NZ_{CT}, and this results in higher levels of larval damage (Popay et al. in press).

In New Zealand, pasture renewal usually takes place during spring or autumn, with the majority taking place during autumn. Pasture renewal in autumn was shown to significantly reduce adult ASW damage in swards (Goldson and Penman, 1979), as adult weevils undergo a reproductive diapause in which they feed little (Goldson, 1981). Accordingly, seedlings infected with a peramine producing endophyte strain sown in autumn might increase seedling survival through the initial peramine translocation from the seed into the emerging shoot, early synthesis of peramine, as well as the reduced feeding caused by the reproductive diapause.

Furthermore, we provided evidence that adult ASW can influence alkaloid concentration in developing, endophyte-infected perennial ryegrass plants. Although not always significant, a general pattern for all alkaloid compounds was observed, where young seedlings (41–48 days old) showed an induced alkaloid response after adult ASW browsing. . This induced alkaloid response was most apparent in lolitrem B, an alkaloid of no importance to adult ASW feeding. Adult ASW browsing increased lolitrem B concentration up to 35% and 42% in NZ_{CT}-infected 41 and 48-day old seedlings, respectively (Appendix 4). It is unknown why lolitrem B was most affected by adult ASW feeding, as lolitrem B does not deter ASW from feeding (Prestidge and Gallagher, 1985). However, an induced lolitrem B response may be beneficial to the plant, as ASW larvae reared on artificial diets containing a lolitrem B concentration of 5 µg/g exhibited slower development rates and higher mortality (Prestidge and Gallagher, 1985). Peramine, the main alkaloid compound affecting adult ASW, was only marginally affected by adult ASW browsing, with 41 and 48-day old plants infected with NZ_{CT} appeared to produce higher peramine concentrations after adult ASW browsing, than control plants (Figure 4.3). Alkaloid concentrations, particularly with peramine, varied greatly among replicates with peramine concentration in 41-day old seedlings ranging from 1.00–41.3 µg/g. Therefore, it is unknown if the observed induced alkaloid response in seedlings is due to biological variability

in alkaloid concentration or a true response caused by the adult ASW. Furthermore, great variability in alkaloid concentrations were observed between the two experiments in this thesis, even though the exact same seed material was used. While we measured relatively low peramine concentrations in the first experiment in 27–69 day old plants, peramine concentrations from similar aged control plants in the second experiment showed much greater levels, even though they were not exposed to insect browsing. Peramine concentrations in the second experiment in NZ_{CT}- infected 62-day old plants were more than 6-fold higher than those infected with the same endophyte and same age from the first experiment. Such big variation in alkaloid concentrations between the two experiments may be due to different environmental conditions. Mean temperatures in the first experiment increased from 18.2°C to 20.9°C (Figure 3.11), while mean temperatures in the second experiment decreased from 23.2°C to 19.6°C (Figure 4.8). Environmental conditions such as temperature and season can greatly influence alkaloid concentration (Eerens*, et al.*, 1998), making it difficult to compare alkaloid concentrations between experiments. Accordingly, due to such variation within the second experiment as well as in comparison to the first experiment, the original hypothesis of an induced alkaloid response after adult ASW browsing needs further testing. However, the data does indicate that there is an induced alkaloid response after insect feeding.

It is noteworthy that as seedlings aged to 55–62 day old plants, it appeared that adult ASW browsing caused a reduction in alkaloid and dry weight production (not always significant). Adult ASW browsing reduced lolitrem B concentration by up to 38% in 62 day old NZ_{CT}-infected seedlings (Appendix 4). It is unknown why adult ASW browsing appears to suppress alkaloid concentration in older seedlings. This unexpected result may be a by-product of the experiment condition possibly resulted in nutrient deprived older plants (see 4.5). The reduced plant growth in older plants subsequently resulted in reduced alkaloid concentration. Further

research should be aimed at repeating this experiment in order to confirm these reverse patterns, as well as investigating the reasons of such alkaloid and dry matter production loss (possibly by increasing the nutrient availability of the plants).

Generally an induced alkaloid response, as found with younger plants, allows the plant to allocate resources only when they are needed and may enable the plant to overcome biotic stress, while the plant is still very vulnerable to insect predation. However, adult ASW feeding in this experiment on 41–62 day old plants had little impact on plants (data not shown). Nonetheless, during this time peramine concentrations were lowest in 41-day old NEA2 infected plants with 5.04 µg/g (Appendix 4), which is probably still likely to give protection to adult ASW feeding, but not against ASW larvae (Rowan*, et al.*, 1990). Even though adult ASW browsing appeared to increase peramine concentrations in 41-day old plants (Appendix 4; not statistically significant), such an increase was not sufficient to increase peramine concentration above the ASW larvae threshold of 10 µg/g (Rowan*, et al.*, 1990). Accordingly, the induced alkaloid response found in this experiment did not increase alkaloid levels to the extent that they might have benefited the plant in allowing it to be better able to withstand the more damaging ASW larvae feeding pressure. However, peramine concentrations were likely high enough to deter adult ASW feeding.

Chapter 6 Conclusion

Adult ASW can have a major impact on seedling survival in non-insecticide treated sown seed mixtures. Endophyte strains such as AR1, NEA2, and AR37 can provide significant benefit by increasing pest resistance during seedling establishment via the presence of the deterrent alkaloids.

Endophyte alkaloids, particularly peramine, provide the young seedling with an initial resistance to adult ASW, likely due to translocation of seed stored alkaloids into the emerging seedling as well as early synthesis of new alkaloids. However, alkaloid concentrations were then found to decrease as the seedling matured, explaining the previously reported increase in seedling susceptibility to adult ASW feeding in older seedlings as being due to a reduction in chemical defence. Although the decrease in chemical defence allows adult ASW to feed on plants, the impact on the plant lessens as it matures. However, ASW larvae might be expected to cause damage during this time, when alkaloid concentrations fall below the deterrence threshold. Eventually the endophyte starts producing new metabolised alkaloids leading to a stabilising or increasing concentration of alkaloids in the maturing seedlings.

It was also demonstrated that adult ASW browsing can influence alkaloid concentration, where ASW triggers an increase in alkaloid production. A general pattern for all alkaloid compounds was observed, where young seedlings appeared to produce higher alkaloid levels after adult ASW feeding, than control plants, although this was not always significant. However, as the seedling matures alkaloid concentrations appear to be lower in seedlings exposed to insect feeding than in control plants, although this pattern requires further testing.

Further research should be aimed at measuring alkaloid concentrations and the total amount of alkaloids in all parts of developing seedlings (root, shoot, and seed residue) during seedling establishment. This may confirm when the seedling ceases to use the seed as a resource for chemical defence and stops further translocation from the seed residue into the developing shoot.

These results can be used to inform farmers and improve their understanding of seedling establishment during pasture renewal. Given the vulnerability of seedlings to insect damage from ASW and other pests, farmers are encouraged to use insecticide-treated seeds regardless of which endophyte strain they use, to ensure ryegrass is protected during the important establishment phase.

Appendices

Appendix 1: GenStat code and output for peramine concentrations in seed statistical analysis.

```
135  "General Analysis of Variance"
 136  BLOCK "No Blocking"
 137  TREATMENTS Endophyte
 138  COVARIATE "No Covariate"
 139  ANOVA [PRINT=aovtable,information,means; FACT=32; CONTRASTS=7;
PCONTRASTS=7; FPROB=yes;\
 140   PSE=lsd,means; LSDLEVEL=5] PeramineConcentrationFinal
```

Analysis of variance

Variate: PeramineConcentrationFinal

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Endophyte	2	94.69	47.34	2.02	0.189
Residual	9	211.09	23.45		
Total	11	305.78			

Tables of means

Variate: PeramineConcentrationFinal

Grand mean 35.2

Endophyte	AR1	NEA2	NZ_{CT}
	38.2	31.4	36.0

Standard errors of means

Table	Endophyte
rep.	4
d.f.	9
e.s.e.	2.42

Least significant differences of means (5% level)

Table	Endophyte
rep.	4
d.f.	9
l.s.d. 7.75	

Variances and degrees of freedom

Endophyte	Var_	d_f
AR1	9.63	3
NEA2	17.34	3
NZ_{CT}	43.39	3

Bartlett's test for homogeneity of variances

Chi-square 1.51 on 2 degrees of freedom: probability 0.471

Appendix 2: GenStat code and output from statistical analysis from the influence of adult ASW feeding on peramine concentrations in seedlings infected with *E.festucae* var. *lolii* strain AR1, NEA2, and NZ_{CT}. Peramine concentrations were square-root transformed.

```
2048  "General Analysis of Variance"
 2049  BLOCK DayCage
 2050  TREATMENTS
InsectPressure*HarvestDays+Endophyte*InsectPressure*HarvestDays
 2051  COVARIATE "No Covariate"
 2052  ANOVA [PRINT=aovtable,information,means; FACT=32; CONTRASTS=7;
PCONTRASTS=7; FPROB=yes;\
 2053   PSE=lsd,means; LSDLEVEL=5] sqrt_PerConc
```

Analysis of variance

Variate: sqrt_PerConc

Source of variation	d.f.	(m.v.)	s.s.	m.s.	v.r.	F pr.
DayCage stratum						
InsectPressure	1		0.0433	0.0433	0.03	0.871
HarvestDays	3		85.4995	28.4998	18.01	<.001
InsectPressure.HarvestDays	3		15.0394	5.0131	3.17	0.053
Residual	16		25.3230	1.5827	3.54	
DayCage.*Units* stratum						
Endophyte	2		315.1199	157.5599	352.90	<.001
InsectPressure.Endophyte	2		1.3958	0.6979	1.56	0.212
HarvestDays.Endophyte	6		7.9968	1.3328	2.99	0.008
InsectPressure.HarvestDays.Endophyte	6		4.3962	0.7327	1.64	0.136
Residual	245	(3)	109.3863	0.4465		
Total	284	(3)	560.0636			

Tables of means

Variate: sqrt_PerConc

Grand mean 4.220

InsectPressure	ASW	control
	4.232	4.208

HarvestDays	41	48	55	62
	3.714	3.974	4.878	5.066
rep.	96	96	48	48

InsectPressure	HarvestDays	41	48	55	62
ASW		3.945	4.081	4.621	4.722
	rep.	48	48	24	24
control		3.483	3.867	5.136	5.410
	rep.	48	48	24	24

Endophyte	AR1	NEA2	NZ_{CT}
	4.455	2.838	5.367

InsectPressure	Endophyte	AR1	NEA2	NZ_{CT}
ASW		4.408	2.948	5.341
control		4.503	2.728	5.393

HarvestDays	Endophyte	AR1	NEA2	NZ_{CT}
41		3.927	2.581	4.635
	rep.	32	32	32
48		4.243	2.592	5.087
	rep.	32	32	32
55		5.095	3.205	6.335
	rep.	16	16	16
62		5.297	3.476	6.424
	rep.	16	16	16

InsectPressure	HarvestDays	Endophyte	AR1	NEA2	NZ_{CT}
ASW	41		4.088	2.916	4.833
		rep.	16	16	16
	48		4.375	2.656	5.212
		rep.	16	16	16
	55		4.735	2.966	6.162
		rep.	8	8	8
	62		4.790	3.578	5.798
		rep.	8	8	8
control	41		3.767	2.246	4.438
		rep.	16	16	16
	48		4.111	2.528	4.963
		rep.	16	16	16
	55		5.456	3.444	6.508
		rep.	8	8	8
	62		5.805	3.375	7.050
		rep.	8	8	8

Standard errors of means

Table	InsectPressure	HarvestDays	InsectPressure HarvestDays	Endophyte	
rep.	144	unequal	unequal	96	
d.f.	16	16	16	245	
e.s.e.	0.1048	0.1816	0.2568	0.0682	min.rep
		0.1284	0.1816		max.rep

Table	InsectPressure Endophyte	HarvestDays Endophyte	InsectPressure HarvestDays Endophyte	
rep.	48	unequal	unequal	
e.s.e.	0.1311	0.2271	0.3212	min.rep
d.f.	38.35	38.35	38.35	
e.s.e.		0.1606	0.2271	max.rep
d.f.		38.35	38.35	

Except when comparing means with the same level(s) of

InsectPressure	0.0964			
d.f.	245			
HarvestDays		0.1670		min.rep
d.f.		245		
		0.1181		max.rep
d.f.		245		
InsectPressure.HarvestDays				
			0.2362	min.rep
d.f.			245	
			0.1670	max.rep
d.f.			245	

Least significant differences of means (5% level)

Table	InsectPressure	HarvestDays	InsectPressure HarvestDays	Endophyte	
rep.	144	unequal	unequal	96	
d.f.	16	16	16	245	
l.s.d.		0.5444	0.7699		min.rep
	0.3143	0.4715	0.6667	0.1900	max-min
		0.3849	0.5444		max.rep

Table	InsectPressure Endophyte	HarvestDays Endophyte	InsectPressure HarvestDays Endophyte	
rep.	48	unequal	unequal	
l.s.d.		0.6500	0.9192	min.rep
d.f.		38.35	38.35	
l.s.d.	0.3753	0.5629	0.7961	max-min
d.f.	38.35	38.35	38.35	
l.s.d.		0.4596	0.6500	max.rep
d.f.		38.35	38.35	
Except when comparing means with the same level(s) of				
InsectPressure	0.2687			
d.f.	245			
HarvestDays		0.4653		min.rep
d.f.		245		
		0.4030		max-min
d.f.		245		
		0.3290		max.rep
d.f.		245		
InsectPressure.HarvestDays				
			0.6581	min.rep
d.f.			245	
			0.5699	max-min
d.f.			245	
			0.4653	max.rep
d.f.			245	

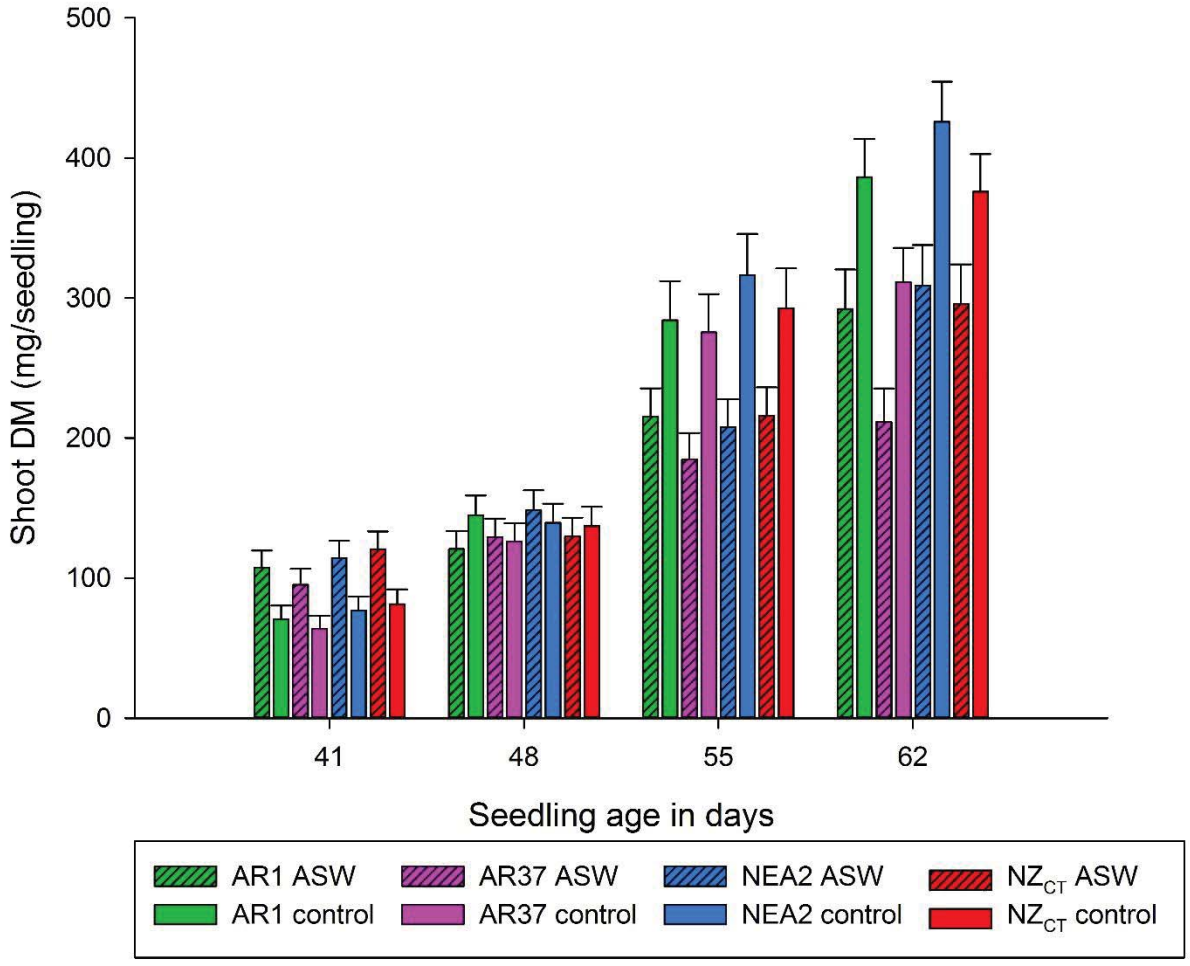
Appendix 3: Mean alkaloid concentrations (µg/g) from *E. festucae* var. *lolii* infected perennial ryegrass seedlings after five days exposure to adult ASW feeding at different seedling ages (bioassays). Significance of difference in alkaloid concentrations were tested for each alkaloid compound separately in the interaction between the occurrences of adult ASW feeding (ASW/Control) across the four bioassays. Values followed by the same letter within each alkaloid compound are not significantly different at given p-value. Standard error of the mean (± SEM) is given for each treatment underneath mean alkaloid concentration.

	Bioassay 1		Bioassay 2		Bioassay 3		Bioassay 4		p-value
Seedling age in days	41		48		55		62		
Selected alkaloid									
	ASW	Control	ASW	Control	ASW	Control	ASW	Control	
Peramine	15.56 [a] ±1.43	12.13 [a] ±1.27	16.65 [a] ±1.48	14.95 [a] ±1.40	21.35 [a] ±2.37	26.38 [a] ±2.64	22.3 [a] ±2.43	29.27 [a] ±2.78	p=0.053
Lolitrem B	1.71 [c] ±0.18	1.33 [c] ±0.16	3.26 [ab] ±0.26	2.56 [b] ±0.23	3.01 [ab] ±0.35	3.31 [ab] ±0.36	2.86 [b] ±0.34	3.99 [a] ±0.40	p=0.036
Ergovaline	1.04 [b] ±0.09	0.66 [c] ±0.05	1.03 [b] ±0.08	1.09 [b] ±0.09	1.6 [a] ±0.18	1.53 [a] ±0.18	1.65 [a] ±0.19	2.00 [a] ±0.23	p=0.014
Epoxy-janthitrems	27.49 [a] ±3.28	21.61 [a] ±2.58	37.3 [a] ±4.45	31.88 [a] ±3.81	50.15 [a] ±8.48	55.76 [a] ±9.42	53.52 [a] ±9.04	63.82 [a] ±10.78	p=0.437

Appendix 4: Mean alkaloid concentrations (µg/g) from seedlings infected with *E. festucae* var. *lolii* strains AR1, NEA2, NZ_{CT}, and AR37 after 5-day exposure to adult ASW feeding at different seedling ages (bioassays). Significance of difference between alkaloid concentrations were tested for each alkaloid compound between the occurrence of adult ASW feeding, seedling age and endophyte strain. Values followed by the same letter within the alkaloid compound are not significantly different at given p-value. Standard error of the mean (± SEM) is given for each treatment underneath mean alkaloid concentration.

		Bioassay 1		Bioassay 2		Bioassay 3		Bioassay 4		p-value
Seedling age in days		41		48		55		62		
Selected alkaloid										
Peramine		ASW	Control	ASW	Control	ASW	Control	ASW	Control	
	AR1	16.71 [a] ±1.86	14.19 [a] ±1.71	19.14 [a] ±1.99	16.9 [a] ±1.87	22.42 [a] ±3.04	29.77 [a] ±3.50	22.94 [a] ±3.08	33.7 [a] ±3.73	
	NEA2	8.50 [a] ±1.32	5.04 [a] ±1.02	7.05 [a] ±1.21	6.39 [a] ±1.15	8.80 [a] ±1.91	11.86 [a] ±2.21	12.80 [a] ±2.30	11.39 [a] ±2.17	p=0.136
	NZ_{CT}	23.36 [a] ±2.20	19.70 [a] ±2.02	27.16 [a] ±2.37	24.63 [a] ±2.25	37.97 [a] ±3.96	42.35 [a] ±4.18	33.62 [a] ±3.72	49.70 [a] ±4.53	
Lolitrem B	NEA2	0.35 [g] ±0.16	0.32 [g] ±0.15	0.78 [f] ±0.21	0.84 [f] ±0.22	0.69 [fg] ±0.28	0.54 [fg] ±0.26	0.72 [fg] ±0.29	0.60 [fg] ±0.27	
	NZ_{CT}	4.12 [d] ±0.67	3.03 [e] ±0.50	7.45 [b] ±1.34	5.21 [c] ±0.86	6.98 [b] ±1.74	8.41 [ab] ±2.25	6.43 [bc] ±1.56	10.37 [a] ±3.10	p<0.001
Ergovaline	NEA2	1.68 [a] ±0.27	0.75 [a] ±0.12	1.50 [a] ±0.24	1.49 [a] ±0.24	2.42 [a] ±0.55	2.01 [a] ±0.46	2.42 [a] ±0.55	3.81 [a] ±0.87	
	NZ_{CT}	0.65 [a] ±0.10	0.59 [a] ±0.09	0.70 [a] ±0.11	0.80 [a] ±0.13	1.05 [a] ±0.38	1.17 [a] ±0.27	1.13 [a] ±0.16	1.05 [a] ±0.24	p=0.333
Epoxy-janthitrems	AR37	27.49 [a] ±3.28	21.61 [a] ±2.58	37.3 [a] ±4.45	31.88 [a] ±3.81	50.15 [a] ±8.48	55.76 [a] ±9.42	53.52 [a] ±9.04	63.82 [a] ±10.78	p=0.437

Appendix 5: The effect of adult ASW feeding on the mean shoot dry matter (mg/seedling) of seedlings infected with *E. festucae* var. *lolii* strains AR1, AR37, NEA2, and NZ_{CT}. Error bar represent standard error of the mean (+SEM).



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