DROUGHT TOLERANCE OF PERENNIAL RYEGRASS (*LOLIUM PERENNE* L.) AND THE ROLE OF *EPICHLOË* ENDOPHYTE

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Abstract

Perennial ryegrass is the most important grass species in New Zealand. Due to climate change, drought will become more severe and frequent in New Zealand, which makes it increasingly important to improve drought tolerance of perennial ryegrass. There are many ryegrass cultivars in the seed market; however, very limited information is available about drought tolerance of these cultivars. Therefore, the first aim of this thesis was to compare drought tolerance of several market-leading perennial or long-rotation ryegrass cultivars in order to provide cultivar information for pastoral industry. *Epichloë festucae* var. *lolii* fungal endophyte naturally colonises perennial ryegrass. Reported effects of endophyte on drought tolerance of the host perennial ryegrass are multifarious. Therefore, the second aim of this thesis was to investigate effects of endophyte on drought tolerance of perennial ryegrass comprehensively.

Two main experiments were conducted in this PhD project. In the first experiment, endophyte-free (E–) and endophyte-infected (E+) cloned plants of seven perennial or long-rotation ryegrass cultivars (Grasslands Commando, Ceres One50, Banquet II, Alto, Bealey, Trojan and Avalon), an un-released elite perennial ryegrass line (URL) and one Mediterranean tall fescue cultivar (Grasslands Flecha) were subjected to a cycle of drought and rehydration from December 2012 to May 2013 while other clones of the same plants were irrigated. In the second experiment, two perennial ryegrass cultivars One50 and Commando infected with and without the AR37 endophyte were subjected to a glasshouse experiment. Eight genotypes of each cultivar with and without endophyte infection were either under irrigation or withheld irrigation for two weeks and then rehydrated for one month. A series of plant morphological and physiological responses were measured in each experiment.

In the rainout shelter experiment, it was found that Flecha tall fescue was more tolerant to drought than ryegrass cultivars, but this was attributed to its small plant size induced by the partial summer dormancy. Introducing germplasm from Mediterranean areas would be an option to improve drought tolerance of perennial ryegrass in New Zealand. Among evaluated ryegrass cultivars, Banquet II was relatively more drought tolerant than other cultivars, which was also mainly due to its small plant size. In the glasshouse experiment, it was found that Spanish

germplasm based One50 was more drought tolerant than ‘Mangere’ ecotype based Commando, suggesting that Spanish germplasm has conferred enhanced drought tolerance to perennial ryegrass in New Zealand.

Under both irrigated and non-irrigated conditions, endophyte infection reduced the herbage yield, decreased the relative water content, osmotic potential and stomatal conductance (as indicated by carbon isotope discrimination) and increased the proline concentration of the host compared to E– plants. Also, a majority of these effects were more pronounced in the URL (infected with AR37) and One50 (infected with AR1). It was concluded that E+ plants are at a disadvantage compared to E− plants when insect pressure is artificially controlled, no matter whether the water availability is high or low.

KEY WORDS: *Epichloë coenophiala*, *Epichloë festucae* var. *lolii*, *Festuca arundinacea*, gas exchange, nitrogen uptake, pasture production, plant water relations, water deficit.

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Glossary of Abbreviations

Abbreviation	Full name/meaning	Unit
$\Delta^{13}C$	Carbon isotope discrimination	‰
ABA	Abscisic acid	
ABB	Africa black beetle	
AMF	Arbuscular mycorrhizal fungi	
APX	Ascorbate peroxidase	
ART	Aligned rank transformation	
ASW	Argentine stem weevil	
ATP	Adenosine triphosphate	
CAT	Catalase	
CF	Chlorophyll fluorescence	
DM	Dry matter	g/plant
E−	Endophyte-free	
E+	Endophyte-infected	
EC	Electric conductivity	
EL	Electrolyte leakage	%
FC	Field capacity	
FW	Fresh weight	g
GAPDH	Glyceraldehyde-3-P-dehydrogenase	
GLM	General linear model	
GPX	Glutathione peroxidase	
G_s	Stomatal conductance	mol H_2O m^{-2} s^{-1}
I−	Non-irrigation	
I+	Irrigation	
LER	Leaf elongation rate	mm/tiller/day
LSR	Leaf senescence rate	mm/tiller/day
LWP	Leaf water potential	bars
MDA	Malondialdehye	nmol/g leaf DM
NADP	Nicotinamide adenine dinucleotide phosphate	
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen	
NIWA	National Institute of Water and Atmospheric Research	
NRL	New root length	cm
OA	Osmotic adjustment	bars
OM	Organic matter	g/plant
OP	Osmotic potential	bars
P_n	Net photosynthesis rate	µmol CO_2 m^{-2} s^{-1}
POD	Peroxidase	
PWP	Permanent wilting point	
RC	Ring colonization	

RD	Reproductive development	
RLDM	Regrowth leaf dry matter	g/plant
ROS	Reactive oxygen species	
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase	
RWC	Relative water content	%
RSR	Root: shoot ratio	
SBP	Sedoheptulose-1,7-bisphophatase	
SOD	Superoxide dismutase	
SWC	Soil water content	%
N%	Total nitrogen concentration	%
TP	Turgor pressure	bars
T_r	Transpiration rate	mmol H_2O m^{-2} s^{-1}
TSR	Tiller survival rate	
TTN	Total tiller number	
TW	Turgid weight	g
WSC	Water soluble carbohydrates	
WUE	Water use efficiency	
$\delta^{13}C$	Carbon isotope composition	‰
$\delta^{15}N$	Nitrogen isotope composition	‰

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Chapter 1 Introduction

1.1 Background

New Zealand has about 29.8 million sheep, 3.7 million beef cattle and 6.7 million dairy cows (Statistics New Zealand, 2014). Dairy farming in New Zealand accounts for about 3% of world milk production (but over 30% of internationally traded dairy produce) and contributed 29% of the total value that New Zealand earned from its merchandise exports (Livestock Improvement Corporation Ltd. and DairyNZ Ltd., 2015). The competitive advantage of New Zealand in the international market mainly arises from the low-cost pastoral farming systems. Perennial ryegrass (*Lolium perenne* L.), with desirable characteristics of easy establishment and management, high palatability and digestibility and good persistence, has been the most commonly sown grass species in New Zealand farming systems, especially in the North Island where 74% of the New Zealand's dairy herds are located (Livestock Improvement Corporation Ltd. and DairyNZ Ltd., 2015). Reduced herbage production of perennial ryegrass because of summer drought has caused feed shortage for livestock and increased capital input due to supply of supplements (Macdonald et al., 2011). Moreover, the National Institute of Water and Atmospheric Research (NIWA) has predicted that, drought will be more frequent and severe by the middle of this century (NIWA, 2013), which makes improving drought tolerance of perennial ryegrass increasingly important.

Since the 1980s, there has been awareness that perennial ryegrass in New Zealand pastures usually has an endophyte symbiont (Fletcher & Harvey, 1981). The endophyte-infected perennial ryegrass pastures have been anecdotally claimed to have better performance than endophyte-free pastures in summer drought and it has been well documented that endophyte produced alkaloids protect their host from a range of insects (Easton, 1999; Thom et al., 2014; Thom et al., 2013). However, it is not clear whether endophyte infection improves the drought tolerance itself of perennial ryegrass or merely because of the insect deterrence. Therefore, there is a need to clarify this question.

1.2 Objectives

In the seed market, perennial ryegrass cultivars are commonly available with selected endophyte strains and these novel plant-endophyte associations have a great agricultural value. However, very little research information exists on their comparative drought tolerance or on the role of selected endophyte strains in drought tolerance of the host. Thus, the objectives of this thesis were: 1) to assess and compare drought tolerance of a selection of market-available perennial (or with behaviour like perennial) ryegrass cultivars; and 2) to investigate the effects of selected endophyte strains on drought tolerance of the host cultivar.

1.3 Thesis structure

Nine chapters are included in this thesis. Following this introduction, Chapter 2 provides a literature review describing the biological background and breeding history of perennial ryegrass in New Zealand, drought in New Zealand and common plant drought responses, the taxonomy, life cycle, discovery history and metabolism of endophyte, and a review of published studies on effects of endophyte on drought tolerance of forage grasses.

A rainout shelter experiment was designed to assess and compare drought tolerance of eight perennial ryegrass cultivars (or breeding lines) and one tall fescue cultivar with and without endophyte infection. Materials and methods for this experiment are reported in Chapter 3; results for yield and related morphological traits are given in Chapter 4. In Chapter 5, data of herbage yield was further explored to test whether endophyte affects drought tolerance of some specific genotypes within each cultivar. Leaf water relations and other physiological traits are presented in Chapter 6. The nitrogen uptake and nitrogen concentration of shoots are demonstrated in Chapter 7. Chapter 8 provides results from a glasshouse experiment, which included two cultivars infected with and without the same endophyte AR37 to further understand the mechanisms of drought tolerance of perennial ryegrass and the effect of endophyte on drought tolerance of the host. Chapter 9 summarises the main findings of this PhD project and discusses the implications of the results for plant breeding.

Chapter 2 Literature review

2.1 Perennial ryegrass

2.1.1 Biology of perennial ryegrass

Perennial ryegrass is one of the 13 currently recognised species in the genus *Lolium* (Poeae tribe, Pooideae subfamily, Poaceae family). All species in the genus *Lolium* are naturally diploid (2n = 2x = 14). While the genus has some self-fertilizing species, e.g. *L.remotum* and *L.temulentum*, perennial ryegrass is an outbreeding species (largely self-incompatible) (Cooper, 1951; McCraw & Spoor, 1983); and therefore, exhibits a great genetic variability within and among cultivars (Casler, 1995). It has been suggested that perennial ryegrass arose as a hybrid between Italian ryegrass (*L.multiflorum*) and meadow fescue (*Festuca pratensis*) (Yamada et al., 2005).

Perennial ryegrass was originally found in Europe, temperate Asia, and North Africa, but it has now been distributed by human activity to many other parts of the world, including North and South America, New Zealand and Australia. There are over 6 million hectares of perennial ryegrass based pastures in Australia and 7 million hectares in New Zealand (Foot, 1997). Now perennial ryegrass is also widely distributed throughout the temperate regions of the world as a forage and turf grass (Lee et al., 2012). One reason for the wide adoption of perennial ryegrass is because this species is able to adapt to many soil and climate types and can be easily established (it germinates in 7–10 days) and easily managed. Perennial ryegrass is also highly regarded as a source of both forage and hay, because of its high palatability and digestibility; furthermore, it can tolerate trampling and recover rapidly from heavy grazing, which makes it an attractive pasture species choice in a wide range of farm and amenity uses.

Hannaway et al. (1997) stated that perennial ryegrass is intolerant of high temperatures, i.e. when day time temperatures exceed 31°C and night time temperatures exceed 25°C; perennial ryegrass is tolerant of a wide pH range (5.1–8.4), but grows best with a pH range 5.5–7.5. Vernalisation followed by a long photoperiod (> 16h) are two factors effectively inducing flowering of perennial ryegrass (Heide, 1994).

2.1.2 Ryegrass in New Zealand

Perennial ryegrass seeds were first brought by British immigrants in early 19th century to New Zealand. During the late 19th century general trade with Britain for pasture establishment materials continued and the imports did not decrease substantially until 1912 by which time most seeds used in New Zealand were locally produced (Stewart, 2006). The European germplasm (most from Ireland, Ayrshire and Devon) was winter-dormant, while the winter in New Zealand is milder than that in Europe and capable of supporting growth; therefore, the early goal of plant breeding in perennial ryegrass was to improve the winter growth (Stewart, 2006). Another problem of the European germplasm was that it was susceptible to crown or stem rust. Increasing the resistance to crown and stem rust has been a constant breeding objective in New Zealand (Easton et al., 1989; Lancashire & Latch, 1970).

In the 1920s, E.B. Levy and W.M. Davies examined seed lines at the Plant Research Station (later Grasslands Division of the Department of Scientific and Industrial research (DSIR Grasslands), now AgResearch) in Palmerston North, and found that the purity and quality of perennial ryegrass seeds that have been sold in New Zealand were quite variable. From this work, superior perennial ryegrass populations were identified, most sourced from Hawkes Bay and Poverty Bay. In 1929/30, the government seed certification scheme was introduced by the Government Seed Testing Station in Palmerston North to ensure the faithful multiplication of the elite lines (Scott, 1980). After several generations of recurrent selection, the first 'New Zealand perennial ryegrass pedigree strain' was developed from the Hawkes Bay superior ecotype in 1936 (was renamed Grasslands Ruanui in 1964), with increased leaf production, persistence and resistance to crown rust as well as improved winter and spring herbage yield (Wratt & Smith, 1983).

Italian ryegrass was noted to have high winter growth, therefore, by hybridising elite plants of previously selected perennial ryegrass and Italian ryegrass breeding lines, a hybrid cultivar H1 (renamed Grasslands Manawa in 1964) was developed and released in 1943 (Corkill, 1949). However, this is a short-rotation cultivar. In the 1950s and 1960s, further backcrossing of Grasslands Manawa to perennial ryegrass lead to the release of a 'long-rotation hybrid' cultivar, Grasslands Ariki, which functioned as a perennial (Barclay, 1963).

In the late 1960s, a Mangere ecotype from the farm of Mr Trevor Ellett in South Auckland was noticed by him. This germplasm was found to be distinct from Grasslands Ruanui and Grasslands Ariki. Points of difference included: more erect larger leaves and tillers, higher winter production, greater resistance to summer drought, a more rapid response to autumn rains (Bahmani et al., 2002; Corkill, 1980), and greater crown rust (*Puccinia coronata*) resistance, but more susceptibility to stem rust (*Puccinia graminis*) (Stewart, 2006). The discovery of the Mangere ecotype was a milestone in New Zealand perennial ryegrass breeding, since this ecotype formed the basis of many cultivars well adapted to the North Island in following decades. The cultivar Grasslands Nui was the first perennial ryegrass cultivar derived from this Mangere ecotype, and was released in 1975 (Armstrong, 1977). Concurrently with the development of Grasslands Nui at DSIR Grasslands, the Yates Corporation (a New Zealand family company well known at that time for providing plant materials for home gardeners) also developed a cultivar from this ecotype, which was certified in 1980 and marketed as Grassland Ellett (Wratt & Smith, 1983). An experiment was carried out to compare Grasslands Nui, Ellet and Ruanui sown with clover at different levels of irrigation under sheep grazing in Canterbury, results showed that Grasslands Nui was a more persistent and higher yielding cultivar than either Grasslands Ruanui or Grasslands Ariki, although the sheep live weight gain was similar (Hayman, 1980).

The artificial doubling of the chromosome number using colchicine was first achieved in *Lolium* species in the 1930s in the USA (Myers, 1939) and was first explored in New Zealand in the late 1950s. In 1968, the first tetraploid cultivar Grasslands Tama, an annual form of ryegrass, was released. Later, more tetraploid ryegrass cultivars (both perennial and hybrid ryegrass) were developed including Grasslands Greenstone, Nevis, Quartet, Ceres Horizon, Grasslands Sterling, Bealey and Banquet. Tetraploid cultivars, in general, have characteristics of increased tiller size and palatability, meanwhile a reduction in tiller number and dry matter yield (Ahloowalia, 1967). It was suggested that tetraploid cultivars have the potential to improve the animal intake and production for farms with high soil fertility and good farm management (Lee et al., 2012).

In the 1980s, based on recognition that the climate in North West Spain was similar to that in North Island in New Zealand, germplasm from mild oceanic regions of

North West Spain was introduced to New Zealand and provided valuable improvements in a number of traits including winter activity, late flowering, low vernalisation response and excellent resistance to crown and stem rust (Stewart, 2006). After Plant Variety Rights Legislation was enacted in 1987, private seed companies such as NZ Agriseeds Ltd., PGG Wrightson Seeds Ltd. and Cropmark Ltd. were also involved in forage grasses breeding, which accelerated the release of new perennial ryegrass cultivars. In the 1990s, Grasslands Impact was bred from the Spanish germplasm and Grasslands Nui. Later more cultivars including Tolosa, Arrow and the tetraploid Banquet were derived from Grasslands Impact. The development of other cultivars such as Trojan and Ceres One50 also incorporated Spanish germplasm. National trials evaluating perennial ryegrass cultivars were started in 1991 organized by New Zealand Plant Breeding and Research Association (NZPBRA), an association of seed companies. New cultivars released after 1991 were compared to old cultivars released before 1991 in a network of trials. Results showed that new cultivars yielded 6% average more herbage annually and 9% in summer than old cultivars, which was mainly due to the introduction of Spanish germplasm (Easton et al., 2001).

Currently, there are at least 27 perennial or long-rotation ryegrass cultivars available in New Zealand pasture seeds market (Table 2.1) (collected from websites of several seeds companies, updated on 29/09/2015). Wang et al. (2014) constructed a neighbour-joining tree for 27 ryegrass cultivars or breeding lines, including 19 perennial ryegrass cultivars or breeding lines (Figure 2.1). Breeding line LP534 was released commercially as Trojan in New Zealand and Impact II in Australia. It was demonstrated that the structure of the neighbour-joining tree of the perennial ryegrass group was complex and revealed close affinities, such as those between Bealey (Bealey is the autotetraploid derivate of Tolosa), Tolosa and PG150; Banquet (Banquet is the autotetraploid derivate of Impact), Impact and Alto; Grasslands Nui and Commando; Aberdart and Expo; LP534 (Trojan) and Arrow. These relationships can be assumed to indicate flow of germplasm between cultivars in the various cultivar breeding programmes.

The genetic variation of perennial ryegrass in New Zealand is very limited compared with that in Europe and Middle East (Stewart, 2006). Therefore, New Zealand public and private breeders organised a collection expedition in 2000 to the south east coast

of the Black Sea in Turkey, one of the oceanic climate zones in the eastern Mediterranean (Stewart, 2006). The collected seeds are under exploration by breeders now. Easton et al. (2011) suggested that New Zealand might need to import more genetic resources from other regions in order to cope with climate change.

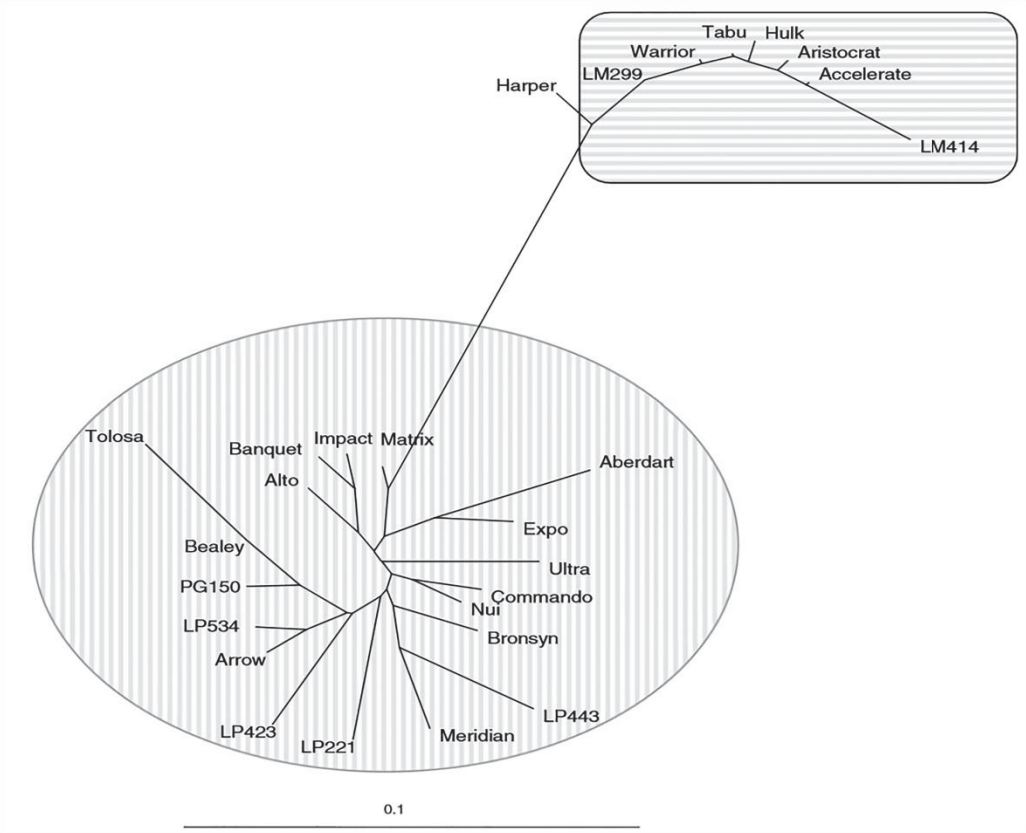

Figure 2.1 Neighbour-joining tree of 27 cultivars from perennial ryegrass, Italian ryegrass and their hybrid (Harper), the scale bar indicates length of branches in Nei's genetic distance units. Cultivars of perennial ryegrass are enclosed in an oval with vertical line shading while cultivars of Italian ryegrass are enclosed in a rectangle with horizontal line shading (reproduced with permission from Wang et al. (2014)).

Table 2.1 Commercial available perennial or long-rotation ryegrass cultivars in New Zealand pasture seeds market that collected from the websites of four main seed marketers.

Cultivar	Marketer	Ploidy	Heading date[1]	Endophyte strains
Ceres Kingston	Agricom	diploid	-3	WE
Grasslands Commando[2]	Agricom	diploid	+1	AR37
Grasslands Hillary	Agricom	diploid	\	AR1
Grasslands Supreme^{PLUS} (75% perennial)	Agricom	tetraploid	+15	AR1/WE
Alto	Agriseeds	diploid	+14	AR37/AR1/WE
Arrow	Agriseeds	diploid	+7	AR1/WE
Bealey	Agriseeds	tetraploid	+25	NEA2
Bronsyn	Agriseeds	diploid	\	AR1
Rohan	Agriseeds	diploid	+18	NEA2/WE
Trojan	Agriseeds	diploid	+16	NEA2
Cropmark Matrix	Cropmark	diploid	+23	WE
Cropmark Ultra	Cropmark	diploid	+20	AR1/WE
Banquet II	PGG Wrightson	tetraploid	+18	Endo5
Base	PGG Wrightson	tetraploid	+22	AR37/AR1
Excess	PGG Wrightson	diploid	+7	AR37/AR1
Expo	PGG Wrightson	diploid	+21	AR37/AR1
Extreme	PGG Wrightson	diploid	0	AR37/AR1
Grasslands Kamo	PGG Wrightson	diploid	0	AR37
Grasslands Pacific	PGG Wrightson	diploid	+1	WE
Quartet II	PGG Wrightson	diploid	\	\
Rely	PGG Wrightson	diploid	0	AR37/AR1
Ceres One50	PGG Wrightson &Agricom	diploid	+20	AR37/AR1/WE
Grasslands Halo	PGG Wrightson& Agricom	tetraploid	+25	AR37/AR1
Grasslands Ohau (75% perennial)	PGG Wrightson &Agricom	tetraploid	+8	AR37/AR1/WE
Grasslands Prospect	PGG Wrightson &Agricom	diploid	+12	AR37/AR1
Grasslands Request	PGG Wrightson &Agricom	diploid	0	AR37/AR1
Grasslands Samson	PGG Wrightson &Agricom	diploid	+3	AR37/AR1/WE

Note: WE = wild type endophyte.

[1]The heading date is labelled relative to day 0, which is the date that traditional ryegrass variety Grasslands Nui flower.

[2]Commando is no longer available on the website of the marketers at the time of writing.

2.2 Drought and drought responses

2.2.1 Definition of drought

Crop growth and development is usually influenced by environmental stress, which consequently decrease the productivity of the plant. From all environmental stress types, drought stress is considered the most devastating to plant productivity (Lambers et al., 2008).

Drought should not be confused with aridity, with the former being a type of temporary weather event and the latter being more permanent climatic condition. Wilhite and Glantz (1985) defined four basic categories of drought: meteorological, agricultural, hydrological and socioeconomic. This research is primarily interested in agricultural drought which is generally defined in a similar way to meteorological drought (e.g. precipitation shortage, prolonged departure from normal, high evapotranspiration due to high temperature and/or low humidity and/or high wind speed and so forth). The concept of agricultural drought, however, also considers agricultural impacts (Wilhite & Glantz, 1985). The water demand of plants not only depends on meteorological conditions, but also the specific biological characteristics and growth stage of the plant as well as the soil properties (Wilhite & Glantz, 1985). It was suggested that agricultural drought should be expressed in terms of soil moisture requirement of a particular crop at a particular time (Hisdal & Tallaksen, 2000; Wilhite & Glantz, 1985); therefore, drought in this research is defined as: insufficient rainfall during the summer period resulting in growth reduction of perennial ryegrass.

There are no precise classifications for drought severity, since severity is a relative term. The plant impact of a given soil moisture deficit, regardless of how it is measured, depends on a number of factors, including soil types, plant species and environmental factors. In the literature, drought severity is usually described based on soil water content as a percentage of field capacity (Boutraa et al., 2010); plant water dehydration level (Kaiser, 1987); days after withdrawing irrigation (Huang et al., 1998a); and the portion of given water of the pot weight loss (Hayatu & Mukhtar, 2010).

2.2.2 Drought in New Zealand

The rainfall in most areas of New Zealand is 600 to 1600 mm, spread throughout the year but with a dry period in summer in most regions and years where evaporation exceeds precipitation leading to soil moisture deficit, especially the North Island and the east part of the South Island. In 2013, a severe drought occurred in the North Island of New Zealand, where the water deficit for pastures was reported as 362 mm; the previous highest record of the water deficit was 361 mm over the period of 1945–1946 (NIWA, 2013). Summer (November–March) rainfall data from 2004 to 2013 of five pastoral regions in New Zealand was collected from the CLIFLO data base (Table 2.2). The summer rainfall needed for pastures to reach yield potential for each region was estimated using the simulation model LINGRA (LINtul-GRAss) calibrated for perennial ryegrass, following the methodology of Matthew et al. (2012). Values obtained were 575 mm, 667 mm, 612 mm, 661 mm and 563 mm for Waikato, Taranaki, Manawatu, Canterbury and Southland respectively (Matthew et al., 2012). Averaged over these 10 years, Canterbury region experienced the greatest water deficit (447 mm), then Manawatu (251 mm) and Waikato (187 mm). Southland (71 mm) and Taranaki (2 mm) regions almost reached the ideal rainfall (Table 2.2). However, in some years, Taranaki and Southland also experienced dry summers. Over the study period, the greatest summer moisture deficits experienced by Taranaki and Southland were 203 and 222 mm, respectively.

It was reported that the economic loss to New Zealand due to drought was more than $500 million in 2007 and more than $1 billion in 2008 (NIWA, 2013). NIWA models suggest that by 2050, most North Island regions, as well as eastern regions of the South Island will experience 5–10% more days per year in drought.

Table 2.2 Summer (November–March) rainfall (mm) of five main pastoral regions of New Zealand from 2004 to 2013. The ideal rainfall is the rainfall for pastures to reach yield potential, which was estimated by using the simulation model LINGRA (LINtul-GRAss) calibrated for perennial ryegrass.

	Waikato	Taranaki	Manawatu	Canterbury	Southland
2004	576	1110	580	138	472
2005	346	634	363	265	639
2006	422	580	299	205	638
2007	367	616	340	262	489
2008	190	456	261	248	341
2009	410	613	387	230	463
2010	331	464	392	138	515
2011	515	796	334	220	502
2012	519	947	438	251	441
2013	208	433	221	178	422
Average	388	665	361	214	492
Ideal	575	667	612	661	563
Deficit	187	2	251	447	71

2.2.3 Plant responses to drought stress

Generally, responses of plants to drought have been described as a sequence of three successive stages of soil dehydration (Figure 2.2) (Serraj & Sinclair, 2002; Sinclair & Ludlow, 1986).

Stage I: at the beginning of drought, soil has water storage thus water is still freely available to the plant. At this stage, both stomatal conductance and water vapour loss are not limited by soil water availability. The transpiration rate during this stage is therefore determined by environmental conditions like air temperature, air humidity and wind speed.

Stage II: with more depletion of soil water by plant use, the soil water storage is reduced. At this stage, the rate of plant water uptake cannot match the potential transpiration rate; therefore, the stomatal conductance, which controls the

transpiration rate to be at a rate similar to that of water uptake, declines in order to maintain the water balance in the plant. At end of this stage, plant growth no longer occurs.

Stage III: the soil available water cannot meet the plant transpiration even though the stomatal conductance is at a minimum. At this stage, the plant is dehydrated and, eventually, desiccates and dies if there is no additional irrigation. The ability of a plant to conserve water content in this stage until water becomes available again is critical for its survival.

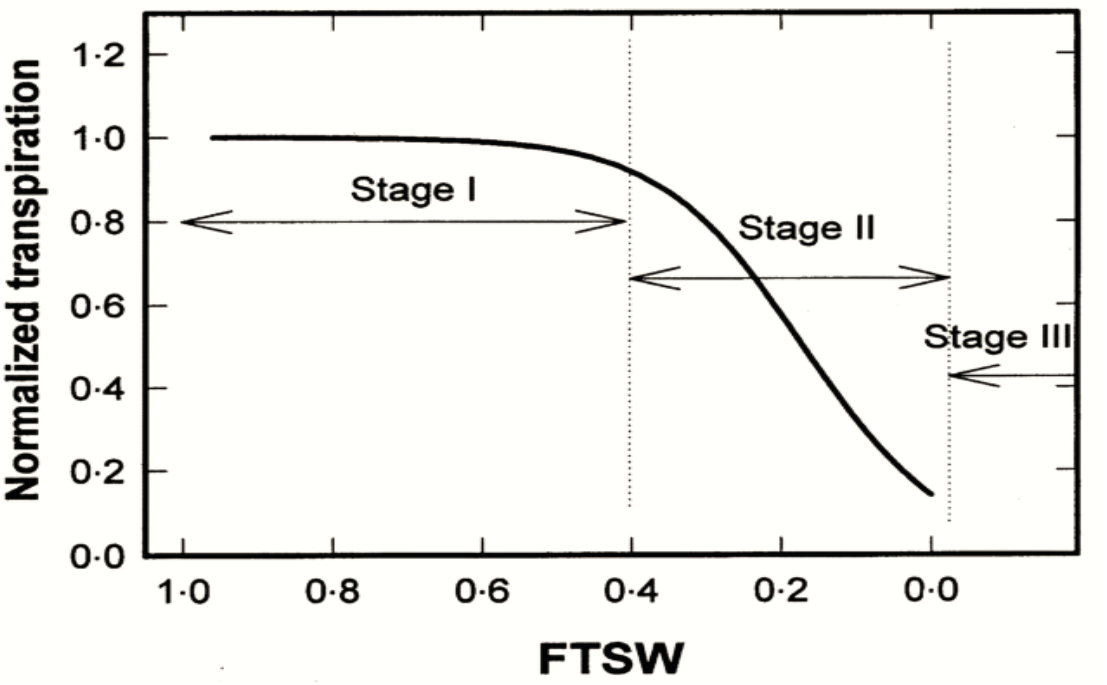

Figure 2.2 Typical plot of normalised leaf transpiration against the fraction of transportable soil water (FTSW). Data was obtained from Sinclair & Ludlow (1986), diagram was created by Serraj & Sinclair (2002).

2.2.3.1 Leaf morphology

Leaf area generally decreases under drought conditions. For forage grass species, the reduced leaf area is a result of slower leaf expansion, leaf appearance, tiller appearance, and greater leaf senescence (Barker & Caradus, 2001). Leafe et al. (1977) reported that the canopy photosynthesis, on a ground area basis, was markedly reduced by drought stress, but the individual leaf photosynthesis (canopy photosynthesis/ leaf area index) was not affected by this, indicating that the reduced

dry matter yield was determined more by reduced leaf area than by reduced photosynthesis rate of individual leaves. However, in another field experiment, the reduced canopy photosynthesis of perennial ryegrass was attributed to both reduced leaf area and reduced individual leaf photosynthesis (mainly due to decreased stomatal conductance) (Jones et al., 1980a). Since reduced leaf area resulted in reduced canopy photosynthesis and eventually lower productivity, despite enabling plants to decrease water loss, it should not be neither considered as an adaptation to drought, nor be considered as a drought tolerance trait (Turner, 1986). Leaf rolling, which is caused by loss of turgor pressure of bulliform cells (bubble-shaped epidermal cells) (Begg, 1980), is considered a drought adaptation trait, as it allows plants to reduce the heat load and transpiration water loss (Frank et al., 1996; Turner, 1986).

In addition to leaf area reduction and leaf rolling, researchers also found drought-stressed perennial ryegrass had thicker leaves, smaller epidermal cells, smaller but more frequent stomata, and more pronounced leaf ridging than well-watered plants (Jones et al., 1980b; Leafe et al., 1977).

2.2.3.2 Root morphology

Roots, the plant organ with a critical role in water uptake, are commonly investigated in drought experiments (Comas et al., 2013; Thomas, 1997). An increased rooting depth under drought will improve water uptake from the deep soil, providing water is stored there (Jordan, 1983). A systematic analysis of plant traits to increase grain yield of maize and sorghum on limited water supplies demonstrated that the increased rooting depth resulted in a crop yield increment (Sinclair & Muchow, 2001). Such crop yield increment is inevitably associated with plant transpiration; when plants take up more water from the soil under drought, the transpiration is maintained, which results in an increased yield (Serraj & Sinclair, 2002).

Roots are generally less vulnerable to drought stress than shoots. It has been reported that root cells and lateral root growth of perennial ryegrass only significantly decreased under severe drought stress. The most dramatic reduction of the percentage of roots with live cortex and root hairs was observed to occur at –30 to –40 bars soil matric potential, and the death of root tips in both main roots and first-

order laterals occurred when soil matric potential was below –100 bars (Jupp & Newman, 1987).

The root:shoot ratio is usually higher under drought stress compared to that in well-watered conditions. Some researchers have demonstrated that this is the result of a greater proportion of assimilates diverted into root growth (Otoole & Bland, 1987). Blum (2005) claimed that this is mainly due to reduced shoot growth rather than more root dry matter. Roots of two tall fescue cultivars (Kenturcky-31 and MIC18) were investigated under increasing soil moisture deficit during withholding of water. Compared to well-watered counterparts, the total root dry weight was reduced by 17%, 12%, 16% and 19% for MIC18 and by 12%, 8%, 8% and 15% for Kentucky-31 at 7, 14, 21 and 28 days after withholding water. Root:shoot ratio of both cultivars under drought was higher than that of control plants, and this was first observed at 7 days dry down for MIC18 and at 14 days dry down for Kentucky-31(Huang et al., 1998b). The decreased total root dry matter and increased root:shoot ratio indicated that the shoot growth was constricted more than that of the root growth under water deficit. Possible reasons for this include, differential sensitivities of the shoot and root to endogenous abscisic acid (ABA), and greater osmotic adjustment or turgor maintenance in the root than that in the shoot (Sharp & Davies, 1989).

2.2.3.3 Stomatal control

Stomata are formed by pairs of specialized epidermal guard cells and they are generally closely surrounded by subsidiary cells. Due to the impermeable waxy cuticle of the epidermis, stomata are the major gateways of gas exchange between the plant and the surrounding atmosphere. Stomatal opening is necessary for carbon dioxide (CO_2) uptake, but transpiration is an inevitable consequence. Under unfavourable conditions, stomatal closure reduces water loss but also sacrifices CO_2 intake; thus stomatal closure is problematic for a plant. As summarised in the review by Araujo et al. (2011), the stomata aperture is regulated by a range of environmental factors such as, light intensity, air humidity, atmospheric CO_2 concentration, temperature and soil water availability. Roots are considered to be the first organ to sense soil water deficit, with the response being an increased level of ABA in the root. ABA is then transferred via the xylem and perceived by the stomatal guard cells

where it triggers changes in ion fluxes; as a result, water moves out of the guard cells, thus leading to stomatal closure Schroeder et al. (2001).

As drought occurs, air humidity is usually also dramatically reduced and in some geographic areas such as the Mediterranean region, high air temperature occurs together with drought. At Stage I of the soil dehydration process, soil water is freely available for plants. Stomatal aperture at this stage is mainly regulated by air humidity and temperature. In studies of perennial ryegrass, it has been found that stomata tended to close as air humidity decreased (or leaf to air vapour pressure deficit increased) (Woledge et al., 1989). This applies as well to other plant species (Aliniaeifard et al., 2014; Hall et al., 1975; Morison & Gifford, 1983). An experiment was conducted with different plant species grown in day time temperatures ranging from 15°C to 36°C to examine the effect of temperature on stomatal aperture. It was found that the stomatal aperture increased with increasing air temperature, with the exception of two cool climate species, the widest aperture occurred at 27°C to 30°C; aperture decreased slightly when temperatures were higher than 30°C (Hofstra & Hesketh, 1969). In another experiment with bean leaf segments incubated in darkness floating for 30 minutes on water at temperatures ranging from 20 to 50°C, the stomatal aperture increased with the temperature and the stomatal opening was fully reversible (Feller, 2006).

Stomatal closure not only restricts CO_2 intake, but also has other negative effects, such as increased canopy temperature due to reduced transpirational cooling (Kimball & Bernacchi, 2006), reduced uptake and transportation of nutrients (Renkema et al., 2012; Yingjajaval, 2013) and increased photorespiration (Wingler et al., 1999). However, the benefits of water retention by stomatal closure outweigh the negative effects when water supply is limited (McCree & Richardson, 1987). Decreased stomatal conductance under drought has been commonly considered as an adaptive response in dehydration postponement (Blum, 1996; Turner, 1986).

The ability of stomata to control water loss is varied among forage grass species under drought. For example, the leaf stomatal conductance of orchard-grass (cocksfoot, *Dactylis glomerata* L.) was 33% greater than perennial ryegrass under well-watered conditions, while it was 25% lower than perennial ryegrass under drought (Thomas, 1986). Both European and Mediterranean tall fescue varieties have

been observed to display higher stomatal conductance than perennial ryegrass in both well-watered and drought conditions (He et al., 2013; Jiang & Huang, 2001b). The sensitivity of stomatal aperture to drought is also influenced by the plant growth stage. In a study of perennial ryegrass Thomas and Evans (1990) found that stomatal closure occurred more slowly in flowering plants than in plants in vegetative growth.

2.2.3.4 Photosynthesis

Photosynthesis is the most fundamental but complex physiological process in all green plants. This process consists of two main sets of reactions: light reactions and dark reactions. Light reactions occur in the grana of the chloroplasts, involving two photosystems (PS I and PS II). An electron transport chain is created in the thylakoid membranes, leading to the ultimate reduction of nicotinamide adenine dinucleotide phosphate ($NADP^+$) to nicotinamide adenine dinucleotide phosphate hydrogen (NADPH), as well as a proton gradient, which drives adenosine triphosphate (ATP) synthesis. The dark reactions occur in the stroma of the chloroplast, where CO_2 is fixed into carbohydrates by utilising NADPH and ATP as a reducing agent and an energy source, respectively.

During mild and moderate droughts, stomatal closure is the dominant factor limiting the photosynthesis due to a reduced internal CO_2 concentration, which is rapidly reversible. However, under severe drought, the reduced photosynthesis involves a larger component of non-stomatal limitations as the photosynthesis cannot be completely recovered by increasing internal CO_2 concentration (Flexas & Medrano, 2002; Signarbieux & Feller, 2011). The non-stomatal limitations include: a reduced amount of ATP as a result of loss of ATP synthase activity (Tezara et al., 1999); a reduced total chlorophyll content, especially chlorophyll b (Moran et al., 1994; Zuilyfodil et al., 1990); and decreased levels of photosynthetic enzymes including sedoheptulose-1,7-bisphophatase (SBP), transketolase (TK), NADP-glyceraldehyde-3-P-dehydrogenase(NADP-GAPDH) and Ribulose-1,5-bisphosphate carboxylase/-oxygenase (RuBisCO) activase (Bayramov et al., 2010).

Chlorophyll fluorescence (CF) is the light that is re-emitted after being absorbed by chlorophyll molecules. Light energy that is absorbed by plant leaves will be dissipated through three pathways: capture by the electron transport chain (photochemical quenching), CF, and heat dissipation (non-photochemical

quenching). These three processes dissipate the entire incident light; for a fixed light intensity, if the rate of one process increases, the light flux for the other two processes will decrease. Therefore, the fluorescence yield is highest when the photochemical absorption and heat dissipation are at the lowest point. The reaction centre of PS II is open (all primary acceptors oxidised and capable of accepting an electron for photo-reduction) in dark adapted leaves; therefore, the photochemical quenching is maximised and the CF is minimised in this condition (F_0). With a very strong, short pulse of light applied to the dark adapted leaf, the reaction centre of PS II is closed (the primary electron acceptors of PS II are reduced and the electron cannot be transferred downstream) and thus, the photochemical quenching is quickly minimised. Non-photochemical quenching (or dissipation as heat) will not be affected because the flash is short, and the fluorescence will be maximised (F_m). The maximal quantum yield is estimated from the ratio $(F_m - F_0)/F_m$. The entity $(F_m - F_0)$ is often referred to as F_v (variable fluorescence). F_v/F_m is the most commonly used parameter for measuring the maximum photochemical efficiency of PS II (Jiang & Huang, 2001b; Maxwell & Johnson, 2000; Signarbieux & Feller, 2011). When plants are under environmental stresses, such as extreme light, temperature or water stress, a decrease of F_v/F_m is frequently observed, thus F_v/F_m is often used to monitor stress (Baker, 2008). For example, the F_v/F_m value of tall fescue plants declined significantly when RWC dropped below 60% after withholding water for 12 days (Huang et al., 1998a). The F_v/F_m value is approximately in the range of 0.79−0.84 in many plant species (Maxwell & Johnson, 2000).

2.2.3.5 Plant water relations

Water potential is defined as the potential energy per unit mass of water with reference to pure water at zero potential (atmospheric pressure and 20°C) (Taiz & Zeiger, 2010). Water moves from regions with high water potential to regions with low water potential spontaneously. In the soil-plant-atmosphere continuum, water moves from soil into the plant, then moves from the plant into the atmosphere in response to a water potential gradient.

Soil water potential mainly depends on the matric potential, except saline soil for which osmotic potential is another important component. Soil water potential at field capacity (FC) is usually close to 0 bars (−0.1 to −0.3 bars) except salty soils, while

the soil water potential at the permanent wilting point (PWP) varies with the plant species. For examples, the PWP for potato is approximately −10 bars, and for wheat is approximately −30 bars (Campbell, 2015). Water is absorbed by roots due to the water potential gradient between roots and the neighbouring soil, and then transported into xylem, with the driving force of transpiration. It is eventually distributed from xylem to other cells due to the water potential gradients between xylem and neighbouring cells.

In plants, the main components of water potential are osmotic potential (OP) and turgor pressure (TP). OP describes the effect of dissolved solutes on water potential; the greater amount of solutes dissolved per volume of water, the more negative the OP. Commonly (without drought stress), OP of most crop plants is in a range of −15 bars to −20 bars (Kramer, 1983). The TP in living cells is considered to be positive as a result of pressure from cell walls, and it is often estimated as the difference between leaf water potential (LWP) and OP: TP = LWP − OP. LWP describes leaf water energy status which varies throughout the day, with the least negative value occurring predawn and the most negative value occurring around midday (Jones et al., 1980a) (Figure 2.3). Thus these are two critical times during the day to measure LWP (Ritchie & Hinckley, 1975; Williams et al., 2012). Compared to LWP, OP is relatively stable throughout the day (Figure 2.3).

Relative water content (RWC) is a measure of plant tissue water status which is often used to evaluate the dehydration level of a plant. Plant tissue physiological injury and death occurs at a critical RWC value of about 50%, but can vary among species and tissue types (Taiz & Zeiger, 2010). For example, leaf RWC of 25% is critical for Kentucky bluegrass survival of drought stress (Chai et al., 2010; Wang & Huang, 2004).

As a soil water deficit develops, daily plant water uptake eventually falls below transpiration, thus plant RWC and LWP decrease. For example, the LWP of two tall fescue cultivars became significantly more negative, moving from approximately −5 bars to −20 bars after 10 days withholding water, while the RWC significantly declined from approximately 90% to 50% after 12 days withholding water for a drought tolerant tall fescue cultivar (Kentucky-31). The LWP and RWC dropped

more slowly than the drought sensitive cultivar (MIC18) (Huang et al., 1998a).

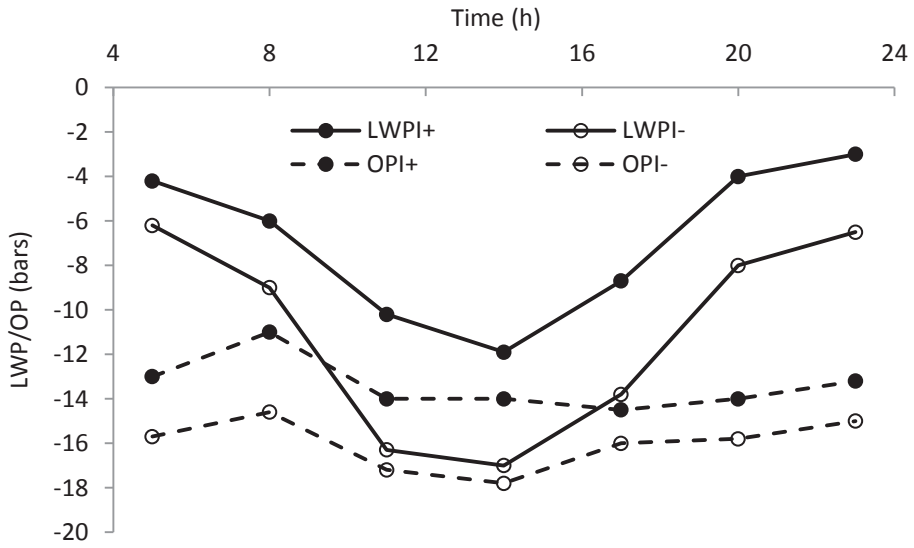

Figure 2.3 Diurnal measurements of leaf water potential (LWP) and osmotic potential (OP) for irrigated (I+) and non-irrigated (I−) perennial ryegrass field swards (graph is reproduced based on data from Jones et al. (1980a)).

2.2.3.6 Osmotic adjustment

Plant dehydration directly causes more negative OP but a further accumulation of solutes in the cytoplasm and vacuoles of plant cells also further reduces the OP, with the latter called osmotic adjustment (OA). OA plays a role in maintaining cell turgidity under drought (Begg, 1980), thus OA has been commonly considered an important adaptive response to drought (Blum, 1996; Turner, 1986; Zlatev & Lidon, 2012). Experiments on wheat showed that there was a greater depth of water extraction in high OA lines than in that of low OA lines (Morgan, 1995; Morgan & Condon, 1986).

Osmotic compounds include carbohydrates (e.g. sucrose, trehalose, glucose, fructose etc.) and cyclitols (e.g. D-pinitol, mannitol); amino acids (e.g. proline, aspartic acid and glutamic acid); methylated quaternary ammonium compounds (e.g. glycine betaine and alanine betaine); and hydrophilic proteins (e.g. late embryogenesis abundant) (Chaves et al., 2003; Farooq et al., 2009). These osmotic compounds not only assist in maintaining the cell turgor pressure, but also in the retention of cellular membrane stability and metabolic machinery under plant dehydration. For example, glycine betaine has been shown to play a role in protecting functional proteins,

enzymes (e.g. RuBisCO), and lipids, as well as maintaining electron flow through the thylakoid membranes (Xing & Rajashekar, 1999). Proline not only acts as a scavenger for reactive oxygen species (Hamilton & Heckathorn, 2001), but also a molecular chaperone in stabilising the structure of protein and enzyme (Samuel et al., 2000), as well as having other possible functions like buffering cytosolic pH (Verbruggen & Hermans, 2008).

To estimate OA, the component of reduced OP attributable to water loss, should be adjusted for. To date, there are four methods to estimate OA:

Method 1(Morgan, 1992): The RWC and OP are obtained from consecutive measurements during a drought stress cycle. OP_0 is estimated from the tissue OP ascribed to the mere loss of water at each given RWC according to: $OP_0 = OP_w (RWC_w/RWC_d)$. The OP_w and RWC_w refer to the OP and RWC of well-watered plants and, the RWC_d refers to the RWC of drought-stressed plants. The measured OP from drought-stressed plants (OP_d) and OP_0 are then plotted against RWC_d. OA is calculated from the two regressions as the difference between OP_d and OP_0 at any given RWC. This method is considered the best estimate of OA but it is precision work requiring large labour and consuming quantities of plant material.

Method 2 (Wilson et al., 1979): The OA is estimated from the difference in OP between well-watered and drought-stressed plants, but both OPs are calculated at a well-watered state (OP_{100}). $OP_{100} = OP\ [(RWC - B)/(100 - B)]$, where B refers to a correction for tissue apoplastic water. Different plant species have different values of B, for rice, it is 18% and constant within cultivar (Turner et al., 1986).

Method 3 (Begg, 1980; Blum, 1989): The OA is estimated from the difference of the OP between well-watered plants and rehydrated drought-stressed plants. Stressed plants are irrigated in the evening and sampled the next morning for the OP determination.

Method 4 (Morgan, 1995): This method is based on the regression of the RWC_d on OP_0 used in Method 1 above; a higher RWC at any given OP indicates higher OA in those plants.

Babu et al (1999) compared these four methods in 12 rice cultivars and found that the mean OA over 12 cultivars was 8.9 bars, 5.1 bars and 7.2 bars for Method 1, 2 and 3

respectively. Simple correlation coefficients of Method 2, 3 and 4 with Method 1 were 0.54, 0.76 and 0.87 respectively. The coefficient variation (calculated from the OA of 12 cultivars) as an indicator of error was 47% in Method 1, 31% in Method 2, 21% in Method 3, and 24% in Method 4. Method 2 and 3 required less labour and plant materials than Method 1 and 4, with the conclusion that Method 3 can be considered as a replacement for Method 1, which is fast, economic and accurate.

2.2.3.7 Reactive oxygen species and antioxidants

In general, reactive oxygen species (ROS) generation begins in the aerobic metabolism of chloroplasts and mitochondria, superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), singlet oxygen ($^1O_2$) and alkoxy radicals (RO) are major ROS. The antioxidant defence system consists of non-enzymic antioxidants, for example ascorbate, glutathione, tocopherol, flavonoids, alkaloids, carotenoids and free amino acids (Gomes et al., 2010; Hussain et al., 2008; Rodriguez & Redman, 2005); also enzymatic antioxidants, for example, superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). SOD is the key enzyme in this defence system. Under non-stress conditions, ROS and antioxidant levels are in a balance in plants. However, stresses such as drought, extreme temperatures, and presence of heavy metals increase ROS production and break this balance, potentially resulting in the oxidative damage of organic molecules such as proteins, lipids, carbohydrates and DNA (Demidchik, 2012; Hamilton & Bauerle, 2012). Oxidative damage compromises the cell membrane and cellular functions, thus increasing the probability of cell death (Hamilton & Bauerle, 2012).

Enzymatic antioxidant activities fluctuate as drought develops. For examples, in one experiment in the initial drying phase of Kentucky bluegrass and tall fescue, both SOD and POD activity increased; however, in the prolonged drying phase, SOD activity decreased to levels below those of well-watered control plants while POD decreased to the level of the control group. The transient increase in SOD and POD during initial periods of drying might protect plants from oxidative injury. However, the decline in SOD and POD activity in the prolonged drying phase indicated that the scavenging function of the antioxidant enzymes, especially SOD, was impaired (Fu & Huang, 2001). In an experiment where maize seedlings were subjected to different

stress levels by irrigation with a polyethylene glycol solution at 0, −5, −10 and −20 bars OP for three weeks. SOD, POD and CAT activities were enhanced progressively by each incremental reduction in OP in a drought tolerant genotype; while for a drought sensitive genotype, the SOD activity increased as above with increased drought stress but the POD and CAT activities were not increased in plants at −20 bars stress, compared to plants at − 10 bars (Moussa & Abdel-Aziz, 2008).

Lipid peroxidation is a very damaging intracellular transformation known to occur in a wide spectrum of living organisms. Malondialdehyde (MDA), a product of peroxidation of unsaturated fatty acid, has been considered a good indicator of lipid peroxidation under a variety of environmental stresses (Davey et al., 2005; Smirnoff, 1993). When two wheat genotypes with different levels of drought tolerance were subjected to drought stress, the MDA concentration did not rise, compared to well-watered plants, in plants of the drought tolerant genotype subjected to drought stress. By contrast, MDA concentration increased from 69 to 89 μmol/g in the drought sensitive genotype (Hameed et al., 2013). In another experiment, the MDA concentration was doubled in both tall fescue and Kentucky bluegrass at 18 days after withholding water (Jiang & Huang, 2001a).

2.2.4 Drought tolerance

Drought tolerance with high (less negative) plant water potential (dehydration postponement) and drought tolerance with low (more negative) plant water potential (dehydration tolerance) as well as drought escape, are three plant strategies for drought resistance that have been widely recognised (Turner, 1986).

Drought escape is the ability of a plant to complete its life cycle before the onset of severe soil water deficit, which is achieved by rapid phenological development and developmental plasticity (Turner, 1986). Drought escapers are usually annual plants. Perennial, cool-season grasses are not true drought escape plants because their life cycle is not completed after seeds are produced (Frank et al., 1996).

To maintain a high water status, plants either increase water uptake or reduce water loss; therefore, plants avoid being stressed and plant tissues are not exposed to dehydration. Increased rooting depth or root density enable plants to maintain water uptake from the soil; stomatal closure and leaf rolling reduce plant water loss.

Dehydration tolerance is the ability of a plant to conserve the plant functions in a dehydrated state. OA plays a role in maintaining cell turgor and some of the accumulated solutes have functions in stabilising the cell membrane (Barker & Caradus, 2001; Blum, 1996; Turner, 1986). Extreme dehydration is called desiccation and most plants are unable to survive desiccation. However, a small group of vascular angiosperms termed resurrection plants have evolved desiccation tolerance. These include *Anastatica hierochuntica* and *Boea hygrometrica*. The late embryogenesis abundant proteins, which are very hydrophilic, are associated with desiccation tolerance, as reviewed by Bartels (2005).

When evaluating drought tolerance, plant responses such as plant growth and yield, photosynthesis rate, CF (F_v/F_m), RWC, LWP, MDA concentration and electrolyte leakage to drought are usually used to compare drought tolerance of plants, while plant responses such as leaf rolling, root characteristics, stomatal conductance, OA, antioxidant activities, water soluble carbohydrate concentration, proline concentration and ABA to drought are measured to explain drought tolerance mechanisms. For examples, the forage species *Medicago truncatula* (M) was concluded to be more tolerant to drought than the species *Sulla carnosa* (S), mainly because the shoot dry matter reduction appears to be less in M (50%) than in S (70%), M had higher RWC (73%) than S (63%) under drought conditions although drought induced RWC reduction was similar between M and S, and the greater ability of M to protect membrane integrity than S as indicated by the fact that drought induced a significant increase in MDA concentration and electrolyte leakage in S but not in M (Rouached et al., 2013); in another study, water deficit increased the MDA concentration of both drought tolerant (cv Manhattan-5) and drought sensitive (cv Silver Dollar) perennial ryegrass cultivars, however, Manhattan-5 exhibited lower level of MDA than Silver Dollar under drought conditions, this was attributed to its higher activity of SOD, CAT, APX and POD than that of Silver Dollar (Zhang et al., 2015).

2.3 Endophyte

Endophytes are endosymbionts, often bacteria or fungi, living within a plant for their part or entire life cycle without causing symptoms to the host. Endophytes are ubiquitous in all plant species that have been studied so far (Stone et al., 2000), and also occur in lichens (Grube et al., 2009) and algae (Flewelling et al., 2013).

2.3.1 Diversity and taxonomy of the *Epichloë* endophyte

Epichloë endophytes, one of the most widely studied fungal endophytes, are known to date in cool-season grasses (subfamily Pooideae). Among the 15 tribes in the subfamily, most *Epichloë* endophytes have been found in the tribe *Poeae*, but occurrence in other tribes has also been reported, including the *Aveneae* (Gentile et al., 2005), *Brachypodieae* (Meijer & Leuchtmann, 1999), *Brachyelytreae* , *Bromeae* (Gentile et al., 2005), *Meliceae* (Gentile et al., 2005; Moon et al., 2007), *Stipeae* (Moon et al., 2007) and *Triticeae* (Card et al., 2014) (Table 2.3).

The genus *Epichloë* belongs to the fungal family Clavicipitaceae. This fungi family is found throughout the tropical and temperate regions of the world and has been observed to infect a variety of hosts including grasses, sedges, other ascomycetes and insects. The family Clavicipitaceae consists of three subfamilies: Oomycetoideae, Cordycipitoideae and Clavicipitoideae (Diehl, 1950). The subfamily Clavicipitoideae is further divided into three tribes: Clavicipiteae, Balansieae and Ustilaginoideae, and most of the pathogens belong the tribe Balansieae (Diehl, 1950). The tribe Balansieae consists of five teleomorphic genera: *Atkinsonella, Balansia*, *Balansiopsis*, *Epichloë*, and *Myriogenospora* (Diehl, 1950) (Figure 2.4). Among all five genera, *Epichloë* is the only genus in which all the known species have developed an endophytic habit.

The anamorphs of both *Epichloë* typhina and the tall fescue endophyte (*Acremonium coenophialum* Morgan-Jones & W.Gams), and other genera including *Cephalosporium, Gliomastix* and monophialidic species of *Paecilomyces* were all merged into one genus *Acremonium* (belonging to the family Hypocreaceae) (Morgan-Jones & Gams, 1982). However, *Acremonium* was a very heterogeneous genus containing distantly related fungi. In order to alleviate the heterogeneity within *Acremonium*, Glenn et al (1996) proposed a new genus *Neotyphodium* which

included the anamorphs of *Epichloë* and other closely related asexual grass endophytes. *Neotyphodium coenophialum*, *Neotyphodium lolii*, *Neotyphodium uncinatum* were three best known fungal endophyte species that affect tall fescue, perennial ryegrass and meadow fescue, respectively.

However, in 2014, Leuchtmann et al. (2014) proposed a nomenclatural realignment of *Neotyphodium* species with the genus *Epichloë* in order to follow the principles of the International Code of Nomenclature for algae, fungi and plants, i.e. each species should have a single name, covering all growth and spore stages; also more importantly to align with a broader understanding of the phylogenetic relationships and common features of these grass endophytes. As a result, all previously described *Neotyphodium* species were placed within the genus *Epichloë*, with the exceptions of *Neotyphodium chilense* and *Neotyphodium starrii*. So far, there are 43 unique taxa in *Epichloë*, including distinct species, subspecies, and varieties (Leuchtmann et al., 2014). Following usage of the term "endophyte" refers to *Epichloë* endophyte.

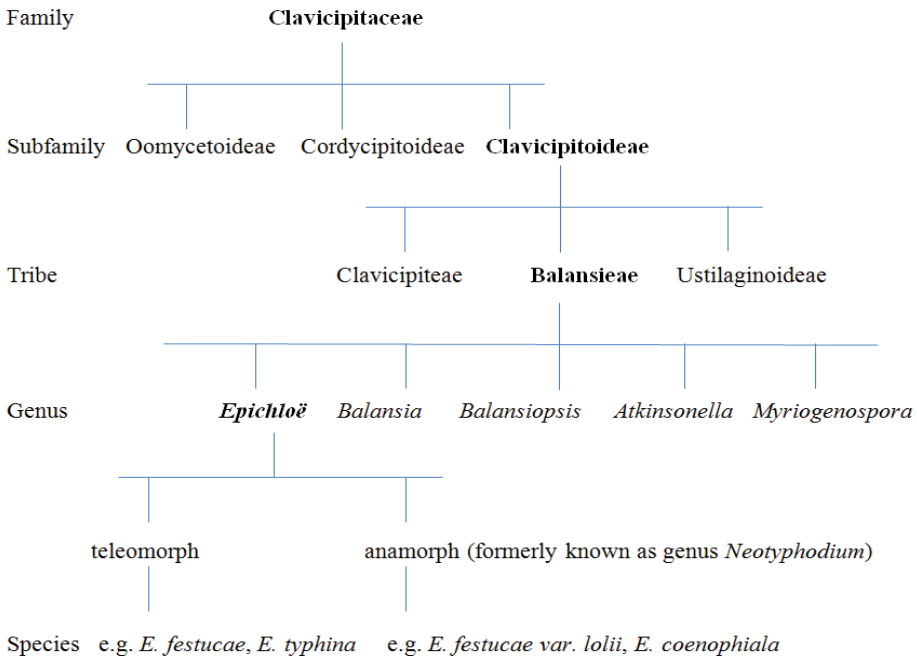

Figure 2.4 Taxonomic position of the endophyte genus *Epichloë* within the family Clavicipitaceae endophyte.

Table 2.3 Endophytes of the genus *Epichloë* and their host grasses.

Epichloë spp.	Host grass spp.	Host tribe
E.amarillans	*Agrostis hyemalis* *Elymus virginicus* *Sphenopholis obtusata*	Poeae Triticeae
E. aotearoae	*Echinopogon ovantus*	Poeae
E. australiensis	*Echinopogon ovantus*	Poeae
E. baconii	*Agrostis stolonifera* *Agrostis tenuis* *Calamagrostis villosa*	Poeae
E. brachyelytri	*Brachyelytrum erectum*	Brachyelytreae
E. bromicola	*Bromus benekenii* *Bromus erectus* *Bromus ramosus* *Elymus repens* *Hordelymus europaeus* *Hordeum brevisubulatum* *Leymus chinensis* *Roegneria kamoji*	Bromeae Triticeae
E. cabralii	*Phleum alpinum*	Poeae
E. canadensis	*Elymus canadensis*	Triticeae
E. chisosa	*Achnatherum eminens*	Stipeae
E. coenophiala	*Festuca arundinaceus*	Poeae
E. danica	*Hordelymus europaeus*	Triticeae
E. disjuncta	*Hordelymus europaeus*	Triticeae
E. elymi	*Elymus villosus* *Bromus kalmii*	Triticeae Bromeae
E. festucae	*Festucar ubra* *Koeleria pyramidata* *Lolium giganteum*	Poeae
E. festucae var. lolii	*Lolium perenne*	Poeae
E. festucae var. lolii × typhina	*Lolium perenne*	
E. funkii	*Achnatherum robustum*	Stipeae
E. gansuensis	*Achnatherum inebrians* *Achnatherum pekinense*	Stipeae
E. gansuensis var. inebrians	*Achnatherum inebrians*	Stipeae
E. glyceriae	*Glyceria striata*	Meliceae
E. guerinii	*Melica ciliata*	Stipeae
E. hordelymi	*Hordelymus europaeus*	Triticeae
E. liyangensis	*Poa pratensis*	Poeae
E. melicicola	*Melica decumbens*	Stipeae
E. mollis	*Holcus mollis*	Poeae
E. occultans	*Lolium rigidum* *Lolium multiflorum*	Poeae
E. pampeana	*Bromus auleticus*	Bromeae

E. schardlii	*Cinna arundinacea*	Poeae
E. sibirica	*Achnatherum sibiricum*	Stipeae
E. siegelii	*Lolium pratense*	Poeae
E. sinica	*Roegneria sp.*	Triticeae
E. sinofestucae	*Festuca parvigluma*	Poeae
E. stromatolonga	*Calamagrostis epigeios*	Poeae
E. sylvatica	*Brachypodium sylvaticum* *Hordelymus europaeus*	Brachypodieae
E. sylvalica subsp. *pollinensis*	*Hordelymus europaeus*	Triticeae
E. tembladera	*Bromus auleticus* *Bromus setifolius* *Poa huecu* *Festuca arizonica* *Melica stuckertii* *Phleum alpinum*	Bromeae Meliceae Poeae
E. typhina	*Anthoxanthum odoratum* *Brachypodium pinnatum* *Brachypodium phoenicoides* *Dactylis glomerata* *Lolium perenne* *Phleum pratense* *Poa trivialis* *Puccinellia distans*	Brachypodieae Poeae Stipeae
E. typhina subsp. *clarkii*	*Holcus lanatus*	Poeae
E. typhina subsp. *poae*	*Poa pratensis* *Poa sylvestris* *Poa secunda* *Poa nemoralis*	Poeae
E. typhina subsp. *Poae var. aonikenkana*	*Bromus setifolius*	Bromeae
E. typhina subsp. *Poae var. canariensis*	*Lolium edwardii*	Poeae
E. typhina subsp. *Poae var. huerfana*	*Festuca arizonica*	Poeae
E. uncinata	*Festuca pratensis*	Poeae

2.3.2 Life cycles of *Epichloë* endophytes

The genus *Epichloë* includes both sexual and asexual species (previously *Neotyphodium* endophytes). The asexual species are derived either from individual sexual species or more commonly, from hybrids with at least two ancestral sexual species (Moon et al., 2004; Tsai et al., 1994).

Asexual *Epichloë* endophyte species, such as *E. festucae var. lolii*, *E. coenophiala* and *E.uncinata* disseminate within the host grass (vertical transmission) (Figure 2.5)

Endophyte hyphae live in the intercellular air space of plant tissues (Figure 2.6) and, their growth is synchronised with host growth. This is predominantly by tip growth within the leaf primordia (Tan et al., 2001), but further elongation is achieved by intercalary extension in elongating grass leaves (Christensen et al., 2008). When the flowering stem starts to elongate, the hyphae grow with the inflorescence and colonise many flower tissues including the lodicules, stamens and stigmas, but not pollen grains. They also infect the maternal tissues of the ovule (Philipson & Christey, 1986). At the seed maturity, the hyphae are found between the seed coat and the proteinaceous aleurone layers and between the cells of the embryo. The hyphae remain dormant until the seed starts to germinate. Fungal colonisation then proceeds via systemic invasion through the apical meristem.

For the sexual *Epichloë* species, such as *E. festucae*, *E. typhina*, *E.amarillans*, before inflorescences emerge from the boot, a yellow-orange fungal stroma is formed on the immature inflorescences embedded in and penetrating the flag leaf sheath, which arrests the inflorescence development. This condition is known as 'choke disease'. The spermatia (mitotic spores) are then transferred by flies (*Botanophila sp*., previously known as *Phorbia sp*.) between chokes which results in the fungal fertilisation. A few weeks later, filamentous ascospores (meiotic spores) are forcibly ejected from the mature stroma, dispersed by wind and land on the florets of other plants. Fungal growth down the stigma to the ovule follows, and eventually infected seeds are formed (Figure 2.5) (Leuchtmann, 2003; Schardl & Phillips, 1997). This process is called horizontal transmission. The rest of the sexual endophyte life cycle is similar to the asexual one.

The life cycle of the same *Epichloë* species may also vary between different plant species, for example, *E. festucae* is associated with numerous fescues (*Festuca* species). It always cause 'choke diseases' in *Festuca* species, while choke diseases have never been observed when isolated *E. festucae* is artificially introduced into perennial ryegrass (Schardl & Phillips, 1997).

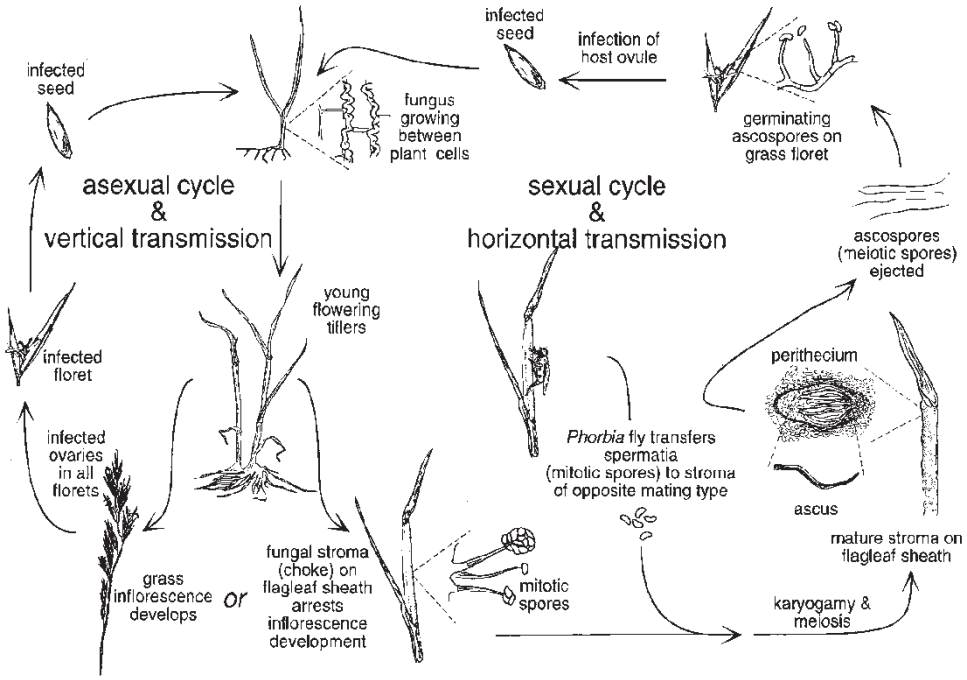

Figure 2.5 Life cycles of asexual and sexual *Epichloë* endophytes (Schardl & Phillips, 1997).

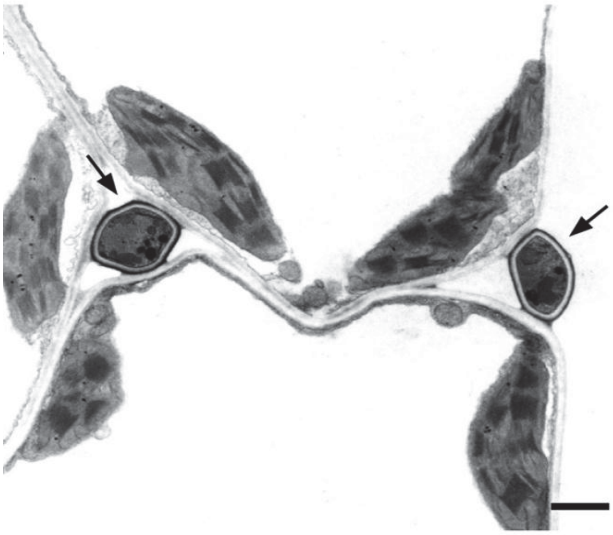

Figure 2.6 Two *Epichloë festuca var. lolii* endophyte hyphae in the intercellular spaces of a perennial ryegrass leaf blade. The hyphae are not round and appear firmly attached to host mesophyll cells (scale bar = 1 μm) (Christensen et al., 2008).

2.3.3 Discovery and development of *Epichloë* endophyte in New Zealand

By importing perennial ryegrass seeds from Europe in the early 19th century, the endophyte was also introduced to New Zealand. Endophyte presence in perennial ryegrass and tall fescue in New Zealand was first described by Neill (1941). It has been long observed that animals have health problems after grazing on tall fescue or perennial ryegrass pastures, such as tall fescue lameness in cattle (Cunningham, 1949). The hypothesis of animal disorders associated with endophytes was first tested by Cunningham (1958), but the experiment failed to prove this hypothesis. However, in the late 1970s, Bacon et al. (1977) proved that fungal endophyte in tall fescue was responsible for the fescue toxicosis in cattle. In the early 1980s, Fletcher and Harvey (1981) and Prestidge et al. (1982) proved that endophyte infection was associated with ryegrass staggers and ryegrass resistance to Argentine stem weevil (ASW), respectively. Effects of endophyte on herbivores were further confirmed by other studies (Bacon, 1995; Barker et al., 1984; Gallagher et al., 1981; Lyons et al., 1986; Mortimer & Menna, 1983; Prestidge et al., 1985; Riedell et al., 1991; Rowan & Gaynor, 1986; Rowan et al., 1986). So far, four main classes of alkaloids have been identified: ergot alkaloids, principally ergovaline; indole diterpene alkaloids, principally epoxyjanthitrems and lolitrem B; pyrrolopyrazine alkaloids, principally peramine; and aminopyrrolidine, principally loline (Figure 2.7). Effects of each main alkaloid on grazing livestock and pasture invertebrates are summarised in Table 2.4.

Table 2.4 Classification of the main alkaloids and their effects on grazing livestock and pasture invertebrates.

Alkaloid classification	Mycotoxin	Effects to grazing livestock	Effects on pasture invertebrates
Ergot alkaloids	Ergovaline	-fescue-foot syndromes of cattle - heat stress of cattle and sheep	-deters adult ASW -deters ABB
Indole diterpene	Lolitrem B	- ryegrass staggers	- affects ASW larvae growth and development
	Epoxyjanthitrems	- ryegrass staggers	- against a broad spectrum insect pest
Pyrrolopyrazine	Peramine	-No observed toxic effect	- deters adult ASW and larvae
Aminopyrrolidine	Loline	-No observed toxic effect	- against a broad spectrum insect pest

Note: ASW = Argentine stem weevil (*Listronous bonariensis* (Kuschel) (Coleóptera: Curculionidae)); ABB = African black beetle (*Heteronychus arator*, (F.) (Coleóptera: Scarabaeidae)).

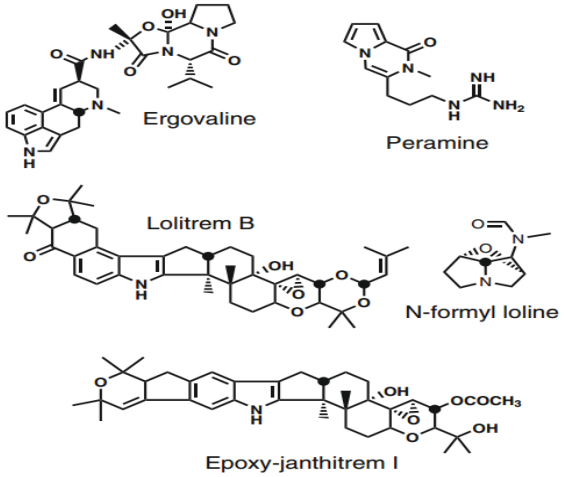

Figure 2.7 The chemical structure of five alkaloids produced by *Epichloë* endophyte

The common toxic endophyte (*E. festucae var. lolii*) that associated with perennial ryegrass produces peramine, lolitrem, epoxyjanthitrems and ergovaline; and the common toxic endophyte (*E. coenophiala*) that associated with tall fescue produce peramine, ergovaline and loline. To overcome the animal disorders caused by fungal endophyte in forage grasses, eliminating the endophyte from the grass was the initial solution; however, this resulted in reduced grass production and persistence (Tapper & Latch, 1999). This dilemma remained until it was realised that different endophyte strains have different alkaloid profiles. However, initially a survey of perennial ryegrass plants from around New Zealand failed to find endophyte-infected plants with low levels of lolitrem B. As a result, attention turned to the international seed collections in the Margot Forde Germplasm Centre at Palmerston North New Zealand (Latch & Tapper, 1988). In this collection, a few endophyte strains that produce peramine but not lolitrem B were identified from several hundred seed accessions.

Techniques for isolating endophyte strains from plants and re-inoculating into other plants were developed in 1985 (Latch & Christensen, 1985) and made it possible to achieve the ideal combinations of endophyte with elite grass germplasm. In the early 1990s, the first selected endophyte strain, 'Endosafe' (produces peramine and ergovaline but not lolitrem B) was commercially released in two ryegrass cultivars. Endosafe protects its host against ASW attack and does not cause ryegrass staggers (Fletcher et al., 1991). However, as awareness developed of the potential for ergovaline intoxication with symptoms such as heat stress and lameness (Fletcher, 2010), industry concerns were raised and Endosafe was withdrawn from sale within a few years of release. In 2001, the second selected endophyte strain AR1 (produces peramine but not ergovaline and lolitrem B) was released after extensive tests. This endophyte strain is harmless to animals (Thom et al., 2013) and protects the host against ASW and pasture mealy bug. Thus, it was quickly accepted by farmers. AR1 is now licenced into 31 ryegrass cultivars through 10 companies, and exported off shore into Australia and Chile, and is being evaluated in USA, Europe and Argentina (Johnson et al., 2013). However, it was then found that AR1 does not provide effective protection against African black beetle (ABB) and root aphid (Popay & Baltus, 2001). In 2005, another two selected endophyte strains NEA2 (a mixture of strains) (produces low levels of lolitrem B, peramine and ergovaline) and Endo 5

(produces peramine and low levels of ergovaline) were released. These were developed in commercial programs by New Zealand Agriseeds and PGGWrightson Seeds, to protect against insect attack but low enough to have minimal impact on grazing animals (Fletcher, 2010). In 2007, the latest AgResearch selected endophyte strain AR37 (produces expoxyjathitrems) was released. This endophyte protects against a wide range of insects (Pennell et al., 2005; Thom et al., 2014) and does not cause animal disorders (Thom et al., 2013), but sometimes induce ryegrass staggers when fed to sheep (Roberts et al., 2005). AR37 now has been included in 11 ryegrass cultivars (Johnson et al., 2013).

In a parallel discovery programme in tall fescue, the endophyte strain AR542 (does not produce any ergovaline but produces loline and peramine) was developed combination with improved tall fescue cultivars, and is sold as MaxQ® and MaxP®. This product was commercially released in 2000 in the USA and then in New Zealand and Australia in 2003, respectively (Johnson et al., 2013). Both have broad spectrum insect resistance and do not cause fescue toxicosis in grazing animals (Bouton et al., 2002).

In 2010, endophyte strains AR601 (branded Avanex®) in tall fescue and AR94/95 (branded Avanex®) in perennial ryegrass was commercially released in use in the airport or sport fields to deter birds (Pennell & Rolston, 2012; Pennell et al., 2010). It was estimated that selected endophyte strains have contributed approximately $200 million per annum to the New Zealand economy (Johnson et al., 2013).

On a worldwide basis, other grass-endophyte associations are also of research interest due to their impacts on grazing animals; such as *E. funkkii* in sleepy grass (*Achnatherum robustum*) in North America (Faeth et al., 2006; Petroski et al., 1992; Shymanovich et al., 2015), which causes horses go to sleep for two to three days after consumption of relatively small quantities of the grass (Vasey, 1887). The ergot alkaloid lysergic acid amide has been identified to be sleep-inducing agent in sleepy grass (Petroski et al., 1992). *E. gansuense* and *E. gansuense* var. *inebrians* in drunken horse grass (*Achnatherum inebrians*) in China (Chen et al., 2015; Li et al., 2007; Xiaopeng et al., 1992; Zhang et al., 2009), cause staggers for horses and even death within 24 hours if severely affected (Bruehl et al., 1994). Lysergic acid amide and ergonovine have been identified as the toxic compounds (Miles et al., 1996). *E.*

tembladera in huecú toxicosis (*Poa huecu*) in Argentina causes staggers and is frequently lethal to animals (Cabral et al., 1999; Gentile et al., 2005). Glycoproteins have been found to be the toxic compound (Pomilio et al., 1989).

2.3.4 Metabolic aspects of grass-endophyte associations

2.3.4.1 Nutrient acquisition

Endophyte hyphae are found in the leaf blades, sheaths, seeds, crowns and rarely in roots, with the highest concentration occurring in sheaths and seeds (Siegel et al., 1984). Endophyte colonises the intercellular spaces of the plant and firmly attaches to the plant cell walls; therefore, the prevalent hypothesis is that any nutrients required by the endophyte are obtained from leaked apoplastic fluid in the intercellular space of the plant (Christensen et al., 2002; Hinton & Bacon, 1985). However, there is no direct evidence to prove this hypothesis. Rasmussen et al. (2008) demonstrated that leaf samples of endophyte-infected (E+) perennial ryegrass had a significantly lower neutral detergent fibre content than endophyte-free (E–) plants, which is consistent with a lower level of cell wall hemicellulose caused by carbohydrate hydrolysis. In another study, Rasmussen et al. (2012) showed that a fungal monosaccharide transporter (mstN) preferentially catalyses the uptake of mannose, a monosaccharide mainly found in polymeric cell wall carbohydrates, and a higher expression of putative endophytic cell wall hydrolases (α-mannosidase, cellulase and β-1,6-glucanase) in planta than in culture. All of this evidence suggests that endophyte very likely hydrolyses plant polymeric cell wall carbohydrates as a supplementary carbon source.

2.3.4.2 Alkaloids distribution and synthesis

Since toxicity syndromes of livestock were linked to endophyte presence in grasses, a large volume of research has been conducted to understand the alkaloid distribution. Each class of the alkaloids has its own characteristic intra-plant distribution in the host. Ergovaline is concentrated in the stem and basal leaf sheath of intermediate age; lolitrem B accumulates over time in older tissue and is present at low levels in young tissue; peramine is fairly evenly distributed in plant tissues and does not accumulate in older tissues (Spiering et al., 2002; Spiering et al., 2005). One of the explanations for these differences in distribution could be that lolitrem B

remains within the endophyte hyphae and ergovaline is associated with fungal growth in particular tissue, while peramine is translocated from the endophyte into plant intercellular spaces where it is either metabolized or mobilized (Koulman et al., 2007).

As noted earlier, different endophyte strains might vary in alkaloid productions. In addition to endophyte strains, host genotypes (Faeth et al., 2002) and environmental factors such as soil moisture (Thom et al., 2014), air temperature (Salminen et al., 2005), soil nitrogen supply (Hunt et al., 2005; Rasmussen et al., 2007) and CO_2 concentration (Hunt et al., 2005) also influence alkaloid production in the host grasses.

Gene clusters for the biosynthesis of some alkaloids have been identified. The ergot alkaloid synthesis (EAS) gene clusters, compromising 11 genes, have been identified as encoding for the biosynthesis of ergot alkaloids (Fleetwood et al., 2007; Panaccione et al., 2001; Schardl et al., 2013; Wang et al., 2004). The lolitrem (LTM) gene clusters, comprising 11 genes, have been characterised and shown to be required for lolitrem B biosynthesis (Saikia et al., 2012; Young et al., 2005; Young et al., 2006; Young et al., 2009). Peramine synthesis is encoded by a single multifunctional non-ribosomal peptide synthetase gene, *per*A (Tanaka et al., 2005); and the 11 loline alkaloid biosynthesis (LOL) genes are responsible for the loline biosynthesis (Schardl et al., 2007; Schardl et al., 2013).

2.3.4.3 Other metabolites

The presence of endophyte and its metabolic activities contribute substantially to the metabolite profile of the grass-endophyte association. Mannitol, a fungal-produced carbohydrate, was identified in E+ plants but not in E– plants of tall fescue (Richardson et al., 1992) and perennial ryegrass (Rasmussen et al., 2008), and the concentration is significantly correlated to the endophyte biomass (Rasmussen et al., 2008). Beatriz et al. (2013) found that E+ red fescue (*Festuca rubra*) plants had significantly higher concentration of nitrogen, phosphorus, and zinc in shoots than E– plants. Phenolic compounds are the largest group of secondary metabolites. Malinowski et al. (1998) found that E+ tall fescue plants had higher total phenolic concentration than their E– clones in both roots and shoots, especially under

phosphorous limiting conditions. Rasmussen et al (2008) compared the metabolic profiles of E+ and E– perennial ryegrass plants. There were 66 major metabolic variables in leaf blades quantified, and for 41 of these 66 metabolic variables, occurrence differed significantly between E+ and E– plants. To be more specific, nitrogenous compounds, such as free amino acid, nitrate, total proteins, and total nitrogen were reduced in E+ plants compared with E– plants. For amino acids, L-asparagine and L-proline were reduced the most. Conversely, carbon compounds were increased significantly in E+ plants compared with E– plants, including the total water soluble carbohydrates, organic acids (especially quinate and shikimate, the precursors of aromatic acids), and phenolics.

2.3.5 Methods of endophyte detection and elimination

There are two commonly used methods to check the endophyte status in grasses: microscopy examination and immuno-detection. With the former, endophyte hyphae can be directly observed by examining the stained (e.g. 0.05% aniline blue) epidermal plant layer from the adaxial surface of the leaf sheath under a light microscope (Clark et al., 1983; Latch & Christensen, 1982). This method is very labour intensive and time consuming. The latter is based on serological detection of fungal antigens, by cutting a mature tiller from the tiller base and pressing against a nitrocellulose membrane, then exposing the nitrocellulose membrane to a series of antibody solutions. Endophyte presence can be identified by reading the colour of the tiller imprint (red blots represent endophyte infection and pink blots represent endophyte free) (Simpson et al., 2012). This method can screen a large number of plants in a short period of time. Other detection methods include: (i) microscopy examination of seeds after treatment of seeds with dilute nitric acid or sodium hydroxide to soften the seed, followed by staining with aniline blue (Clark et al., 1983); (ii) isolating endophyte from plant tissue or seeds in culture (Bacon et al., 1977; Clark et al., 1983; Latch & Christensen, 1982); (iii) the polymerase chain reaction assay, which can not only detect but also quantify endophyte in the plant tissue (Groppe & Boller, 1997; Rasmussen et al., 2007).

Endophyte in growing grasses and seeds can be eliminated by treating with fungicides, such as benomyl, dichlorobutrazol, triadimefon, etaconazole, propiconazole, prochloraz (Latch & Christensen, 1982). However, some of these

fungicides, such as etaconazole, propiconazole and prochloraz, adversely affect seedling growth (Latch & Christensen, 1982). Endophyte in seeds can be removed by exposing seeds to a high temperature of 47°C and relatively humidity of 45% for about 21 days (Siegel et al., 1984). However, the seed germination rate is also decreased due to the heat stress to the seeds.

2.4 Effects of endophyte on drought tolerance of the host

Besides effects of endophyte presence on grazing animals and insect herbivores, much research effort has also been directed towards other aspects of the endophyte interactions with its host, including effects of endophyte on the host tolerance to fungal pathogens (Li et al., 2007; Wäli et al., 2006; Zabalgogeazcoa, 2008), drought stress (Malinowski & Belesky, 2000; West et al., 1993), salinity (Song et al., 2015), heavy metals (Zhang et al., 2010) and nutrient shortage (Li et al., 2012). In this section, effects of endophyte on drought tolerance of the host are reviewed.

In the literature, several studies have demonstrated that endophyte infection improves drought tolerance of tall fescue. For examples, Arachevaleta et al. (1989) demonstrated that E+ plants (one plant genotype was included in the experiment) were more productive under mild drought stress (–0.5 bars) and improved plant survival rate following exposure to a water deficit of –5 bars than E– plants. However, the drought severity was low in this experiment. Richardson et al (1992) found that E+ plants (one plant genotype was included in the experiment) had greater concentrations of fructose and glucose in blades and higher glucose levels in sheaths than E– clones under drought conditions. Elmi and West (1995) reported that E+ plants (two plant genotypes were included in the experiment) had greater OA and higher post-drought tiller survival rate and leaf elongation rate compared to the E– clones. Similarly, Nagabhyru et al. (2013) reported that E+ plants (two plant genotypes were included in this experiment) accumulated free sugars, sugar alcohols, and amino acids earlier than E– plants, which was correlated with the greater plant survival rate of E+ plants than E– plants. Swarthout et al. (2009) used plants that were germinated from E+ and E– seeds, they found that E+ plants (10 plants) had a

significantly higher photosynthesis rate, stomatal conductance and instantaneous water use efficiency (WUE) than E– plants at the end of drought period where plants had been exposed to severe stress (30% FC). Interestingly, all those studies used the tall fescue cultivar Kentucky-31 as the plant material, presumably because Kentucky-31has been the best-known tall fescue cultivar in the USA, renowned for its excellent agronomic attributes under difficult growth conditions, such as drought and poor soils (Young et al., 2014).

Enhancement of host drought tolerance by endophyte infection has also have been reported for other grass species, such as grove bluegrass (*Poa alsodes* A.Gray), a perennial C3 grass native to moist woodland habitats from north-eastern to central USA (Kannadan & Rudgers, 2008); arizona fescue (*Festuca arizonica* Vasey), a dominant understory grass in the Ponderosa pine forests of southwest USA (Morse et al., 2002); and in meadow fescue (Malinowski et al., 1997).

However, it is not always the case that endophyte infection enhances drought tolerance, especially for perennial ryegrasses. By searching the key words of perennial ryegrass or ryegrass, drought or water deficit and endophyte, 11 articles and 1 PhD thesis was found to have investigated effects of endophyte on drought tolerance of perennial ryegrass.

Four of the above studies concluded that endophyte infection improved drought tolerance of perennial ryegrass. Kane (2011) stated that for 4 out of 6 perennial ryegrass populations, endophyte infection helped alleviate drought stress as indicated by increased total tiller number and total tiller length compared to the E− plants under drought conditions. Hahn et al. (2008) reported that E+ plants had higher RWC and less negative OP than E− plants under drought conditions. Amalric et al. (1999) showed that E+ plants had similar leaf dry matter, but greater tiller numbers and less negative LWP under drought conditions, and higher stomatal conductance and net photosynthesis rate than E− plants under both well-watered and drought conditions. (Ravel et al., 1997) reported that E+ plants had 10% greater tiller number

and 13% more negative OP than that of E− plants at the end of a drought stress period (there was no well-watered control in this experiment).

A further four studies concluded that endophyte infection had no effect on drought tolerance of perennial ryegrass. Briggs et al. (2013) found no difference in SOD activity between E+ and E− plants under drought conditions. Marks and Clay (2007) showed that E+ plants had higher root biomass, total biomass and root:shoot ratio than E− plants, but only under well-watered conditions not under drought conditions. Cheplick et al. (2000) found that endophyte infection had no effect on number of live tillers, live leaf area or total plant biomass after a second drought and recovery period. Barker et al. (1997) did not find any endophyte main effect or interaction between water treatment and endophyte on the LWP, OA, RWC, TP and stomatal conductance in four experiments with perennial ryegrass in New Zealand.

One study concluded that endophyte infection is detrimental for the perennial ryegrass host under drought conditions. Cheplick (2004) tested effects of endophyte infection on recovery from three sequential drought periods in 10 genotypes of a perennial ryegrass cultivar, and found that E+ plants had fewer tillers, reduced leaf area and total plant mass, compared to E− plants for both well-watered and drought-stressed plants and concluded that "the symbiotic relationship between perennial ryegrass and its endophyte primarily benefits the fungus, not the host, under many environmental conditions".

For the other three studies, authors concluded that effects of endophyte on drought tolerance depend on original habitats (from a dry or wet areas) (Hesse et al., 2003, 2005) and inherent drought tolerance of the host (Zhou, 2014).

2.5 Summary

Perennial ryegrass is one of the most important forage grass species in temperate areas. However, its growth and yield production has been largely restricted by summer drought in New Zealand. Drought in New Zealand is predicted to be more frequent and severe in the near future; therefore, improving persistence and production of perennial ryegrass under summer drought is becoming increasingly important. It is of great value to identify drought tolerance of market available cultivars. Selected endophyte strains have been released with modern ryegrass cultivars for decades. Despite the fact that effects of endophyte on drought tolerance of perennial ryegrass are multifarious, a majority of the published research has been conducted with the wild type endophyte and with a limited selection of plant cultivars included. Also, different morphological and physiological traits were measured in different studies to draw conclusions. Experiments which test effects of selected endophyte on drought tolerance of the associated perennial ryegrass cultivars by measuring a comprehensive range of plant responses to drought would contribute greatly to the current knowledge.

Chapter 3 Introduction to the rainout shelter experiment

3.1 Introduction and structure

3.1.1 Introduction

Plant domestication and modern breeding practices have been very successful in optimising plant performance to suit the needs of farmers and consumers (Moose & Mumm, 2008). This strong selective pressure exerted by humans on plant genetic diversity has a history of about ten thousand years for crops like wheat, and a few centuries for European forage grasses. However, selection pressure has resulted in genetic bottlenecks and therefore reduced the capability of the improved cultivars to adapt to the changing environment of many crops (Tanksley & McCouch, 1997). Perennial ryegrass is one of the most widely distributed grass species throughout the temperate regions of the world. Perennial ryegrass in Australasia was imported from Europe, and in New Zealand two superior ecotypes referred to as 'Hawkes Bay' and 'Mangere' ecotypes and Spanish germplasm have contributed strongly to the genetic structure of the cultivars currently sold to farmers (Stewart, 2006). However, germplasm from various other sources such as the European high sugar ryegrasses has also been used e.g. cultivar Aberdart (Stewart, 2006), and in Australia the possible use of Mediterranean germplasm has been explored among other initiatives (Silsbury, 1961).

The rainfall requirement for unrestricted perennial ryegrass growth depends on site factors such as temperature, evaporation potential, and soil water holding capacity. A recent New Zealand assessment of perennial ryegrass water requirements, using models developed at Wageningen, indicated that a potential perennial ryegrass yield of 15.8 t DM/ha/year in cooler conditions of Southland would require 1208 mm annual rainfall while a potential yield of 19.9 t DM/ha/year in warmer conditions of Waikato would require 1332 mm annual rainfall (Matthew et al., 2012). Against this, average annual rainfall (2001–2010) was 1134, and 1121 mm for Southland and Waikato, respectively, indicating a moisture deficit of over 200 mm per year in Waikato. Moreover, perennial ryegrass is commonly grown in drier regions of South Australia, Victoria, New South Wales and New Zealand regions with annual rainfall of 600–1000 mm. This implicit water deficit and associated production limitation is

compounded by the occurrence of drier years and a trend towards warming temperatures and decreasing rainfall in some regions (Smith, 2012). To develop perennial ryegrass genetics that will ensure productivity from grazed pasture under water limitation, there is a need to identify genetic variation in plant performance of existing perennial ryegrass cultivars under summer drought.

Members of the fungal family Clavicipitaceae are found throughout the tropical and temperate regions of the world with many forming associations with various fungal, plant and invertebrate hosts. These relationships span the continuum from pathogenic, as with the entomopathogenic genus *Metarrhizium*, to mutualistic as with endophytic asexual species of *Epichloë* that colonise certain members of the grass family Poaceae (Leuchtmann et al., 2014). *Epichloë festucae* var. *lolii* naturally colonises perennial ryegrass (Leuchtmann et al., 2014). The wild type endophyte (common toxic endophyte) produces toxic metabolites which protect hosts from insects while also causing health issues for the grazing animals (Johnson et al., 2013; Thom et al., 2012). Selected endophytes, usually identified by screening a range of collected wild type strains for absence or reduced levels of toxic alkaloids harmful to mammals while retaining those alkaloids responsible for insect deterrence (Thom et al., 2014; Thom et al., 2013), are now widely used in the Australasian pastoral industries with the intention of enhancing ryegrass and tall fescue persistence.

Current research focuses on the use of selected symbiotic endophytes as a means to improve the tolerance of grasses, including cereals such as wheat (Hubbard et al., 2014; Simpson et al., 2014), to biotic and abiotic stressors. Published results on the effects of endophyte status on drought tolerance of perennial ryegrass are quite inconsistent, with some showing enhanced drought tolerance in endophyte infected plants, e.g. compared to endophyte-free plants, endophyte-infected plants showed more live tiller number (Amalric et al., 1999; Kane, 2011; Ravel et al., 1997), higher leaf water status (Amalric et al., 1999; Hahn et al., 2008) and more osmotic adjustment (Ravel et al., 1997) under drought conditions. In response to endophyte infection, perennial ryegrass has been found to show increased root dry matter (Hesse et al., 2003; Latch et al., 1985) or modified root distribution (Crush et al., 2004), however, no direct relationship between these root characteristics and drought tolerance was documented for endophyte-infected plants. Some other research

showed no effect of endophyte on drought tolerance of perennial ryegrass (Barker et al., 1997; Briggs et al., 2013; Cheplick et al., 2000; Marks & Clay, 2007), and sometimes even detrimental (Cheplick, 2004). However, a majority of this published research was done with the wild type endophyte. So far, there is limited information about the role of selected endophyte strains in drought tolerance of their associated perennial ryegrass cultivars.

Therefore, based on the information needs identified above, an experiment was set up at Palmerston North, New Zealand in 2012, aiming to i) evaluate variability in adaptation to moisture deficit in modern perennial or long-rotation ryegrass cultivars; and ii) identify whether presence of a commercial endophyte symbiont in the respective cultivar would improve plant performance during and after moisture deficit stress. Associated with these aims, research hypotheses were: (1) evaluated ryegrass cultivars would differ in agronomic drought tolerance, as assessed by herbage mass reduction under water deficit, compared to adequate watering, (Chapter 4); and (2) since perennial ryegrass is an out-crossing plant species, genotypes within each cultivar would show different drought tolerance levels (Chapter 5); (3) herbage mass responses to drought would likely be accompanied by differences in leaf water relations, and study of these responses might enhance understanding of defence against water deficit in ryegrass (Chapter 6); (4) leaf water relations might be modified by the presence of fungal endophyte, which in turn might vary depending on the host cultivar and endophyte strain (Chapter 6); (5) nutrient uptake is usually limited under drought conditions, although this response is not a criterion for drought tolerance evaluation, the author was interested to test whether cultivars show different nitrogen uptake abilities in response to drought; also, whether endophyte presence influences nitrogen uptake of the host under different water regimes (Chapter 7).

3.1.2 Structure

This chapter introduces the plant material and methods of the rainout shelter experiment. Results of this experiment are presented in Chapters 4 to 7. The morphological data, including shoot dry matter, score of tiller survival rate and reproductive development and total length of new roots are given in Chapter 4. Chapter 5 explores genotype variations within each cultivar and interactions between plant genotype and irrigation treatment or endophyte status for herbage yield. The physiological data, including plant water relations, chlorophyll florescence, proline concentration and carbon isotope discrimination are described in Chapter 6. Data for nitrogen uptake and nitrogen concentration of shoots are reported in Chapter 7.

3.2 Methods and materials

3.2.1 Plant materials

Seeds of seven perennial or long-rotation ryegrass cultivars with varying genetic backgrounds and a significant market-place profile at the time the experiment was set up, one un-released perennial ryegrass breeding line (URL), and one tall fescue cultivar (Grasslands Flecha (Flecha)) infected with their specific endophyte strain(s) were used (Table 3.1). Seeds were sown in October 2011 (mid-spring) in pots in a glasshouse. Tall fescue is closely related to perennial ryegrass. A tall fescue cultivar was included with the aim of comparing the behaviour of the endophyte in tall fescue (*Epichloë coenophiala*), with that in perennial ryegrass in water deficit.

After three months, four endophyte-infected (E+) vigorously growing individual plants (genotypes) of each cultivar were selected, and each plant was split into 16 clonal ramets with each ramet consisting of three tillers of similar size. To generate endophyte-free (E–) clonal copies, half of the ramets from each plant were fully immersed in Benomyl fungicide solution (2g/L) within test tubes for approximately 3 hours; then planted in sand (or vermiculite) within a plastic cup and fungicide solution added until the root zone was covered. Each plastic cup was weighed every day and the weight kept constant by addition of water so that concentration of fungicide did not change. After approximately 3–4 weeks, each plant was planted into potting mix. The other half of the ramets were subjected to the same procedure but using distilled water, and thus remained endophyte infected. As a result,

genetically identical clones of each cultivar with and without endophyte were developed. All plants were kept in the glasshouse under natural light with regular irrigation to allow tiller number increase. Endophyte status of three randomly selected newly emerged tillers of each plant was checked using immunoblotting (Simpson et al., 2012), and confirmed by microscopy examinations (Latch & Christensen, 1982). Fungicide treatment was repeated approximately every six weeks until no endophyte was detected in the E– clones. When all plants had the required endophyte status, they were kept in the greenhouse for another two months and then transferred to a concrete block outside for one month in order to eliminate side effects (if any), caused by the fungicide, and also to allow vernalisation. It has been reported that about six weeks of cold temperatures below 10°C are required to fully vernalise perennial ryegrass (Evans, 1964). Ideally, plants should be placed in a cold room for vernalisation, but the cold temperatures in winter is often considered to be effective in practice, the average air temperature in August and September was about 10°C and the average daily minimum air temperature was 6−7°C (Figure 3.4a).

Table 3.1 The list of cultivars and their associated endophytes in the rainout shelter experiment.

Cultivar	Endophyte strain	Ploidy of the cultivar
URL	AR37	Diploid
Grasslands Commando (Commando)	AR37	Diploid
Banquet II	Endo5	Tetraploid
Ceres One50 (One50)	AR1	Diploid
Alto	AR37	Diploid
Bealey	NEA2	Tetraploid
Trojan	NEA2	Diploid
Avalon	AR1	Diploid
Grasslands Flecha (Flecha)	AR542	Unknown

3.2.2 Experiment design

The experiment was located in a field at AgResearch Grasslands (40.3798°S, 175.6067°E) where a rainout shelter was set up to close automatically when rainfall occurred and also, every night between 8 pm to 6 am to avoid dew forming on plant leaves. Soil at the site is a Manawatu mottled silt loam, with good to imperfect drainage, no significant rooting barrier within 1 metre, and with dry bulk density of topsoil and subsoil being 1.09 g/cm³ and 1.30 g/cm³, respectively. The soil volumetric water content at full FC is 44% (v/v) (data from the National Soil Database and personal communication with Dr. Alan Palmer). Initial soil nutrient analysis was carried out and based on the soil test results, soil nutrient status was adjusted to meet the recommendations of Roberts and Morton (2009).

On 18th September 2012, a total of 432 plants (9 cultivars × 4 plant genotypes × 2 endophyte status × 2 irrigation treatments × 3 replications) were transplanted in a split-plot design with the main factor being irrigation treatment (I+, irrigation and I–, non-irrigation) in a randomised complete block design (Figure 3.1 and 3.2). In each main plot, plants were randomly arranged in a row-column design at a spacing of 45 cm, i.e. one cultivar only appeared once in each column and twice in each row (E+ and E– clones pairwise). A PVC ring (height 1.5 cm × diameter 5.0 cm) cut from a pipe was fully inserted into the soil surrounding each plant to avoid plant horizontal expansion (Hatier et al., 2014). Trenches (width 80 cm, depth 50 cm) were dug and coated with polythene plastic to prevent underground water flow between plots. Each main plot was surrounded by a row of edge plants to reduce marginal effects (Figure 3.1 and 3.2). A systemic insecticide, Confidor (active ingredient 0.125 g/L imidacloprid), was applied at a rate of 2.5 ml (8 ml–12 ml Confidor/L) per plant during the course of the experiment to control insects and avoid the biological benefits generated by E+ plants arising from insect deterrent effects.

A drip irrigation system was installed in all plots and from 18th September to 19th December 2012, all plants were irrigated at 5 am every two days. From 20th December 2012 (early summer), irrigation was withdrawn for the I– plots until 15th March 2013 (early autumn), a total of 85 days. Following the non-irrigation period, all plants were rehydrated for two months.

At periodic intervals approximately one month apart from December to May, a total of six 'harvests' were conducted in which as many measurements as possible were completed in as short a time as possible, usually about two weeks. The main activity dates for the six harvest periods were, 10th December, 13th January, 4th February, 4th March, 2nd April and 14th May (Figure 3.3), respectively. Hereafter, these harvest dates refer to Dec, Jan, Feb, Mar, Apr, and May. Measurements conducted in each harvest are listed in Table 3.2.

Figure 3.1 The rainout shelter field at AgResearch Grasslands in Palmerston North. The rainout shelter is open in a sunny day in this picture.

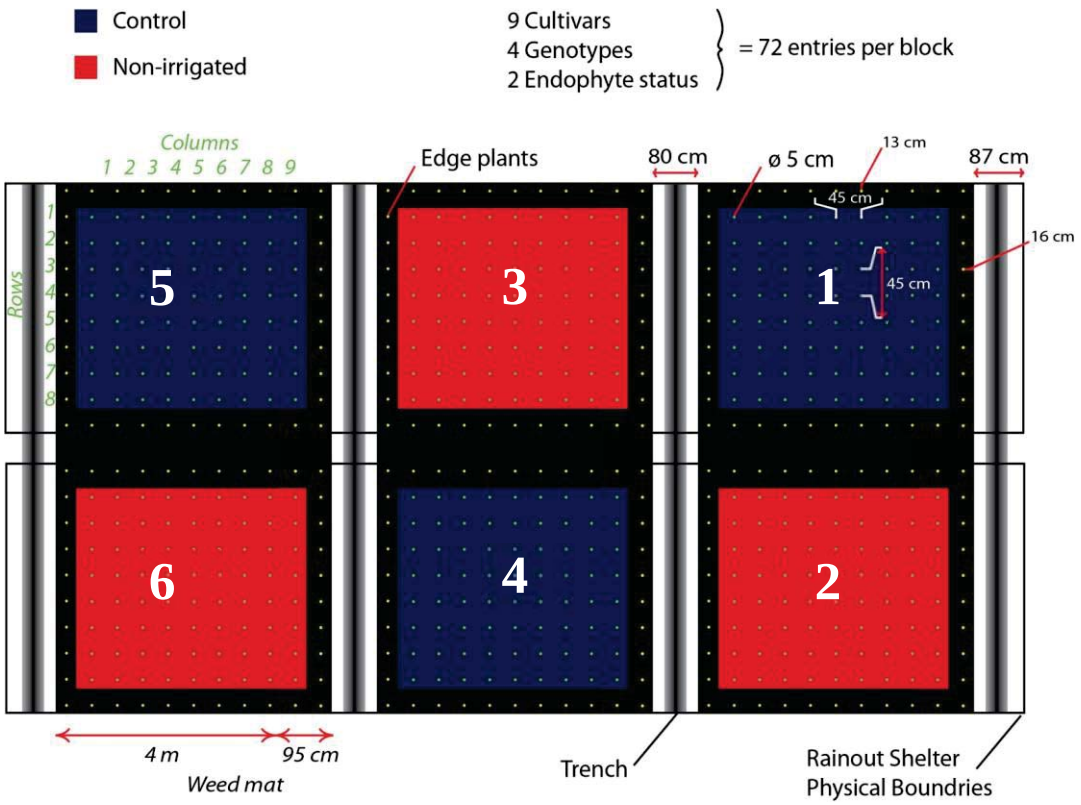

Figure 3.2 Diagram of the experimental design. Plots 1, 4 and 5 were irrigated control, and plots 2, 3 and 6 were non-irrigated treatment. In each plot, 72 plants were arranged in a row-column design at a spacing of 45 cm.

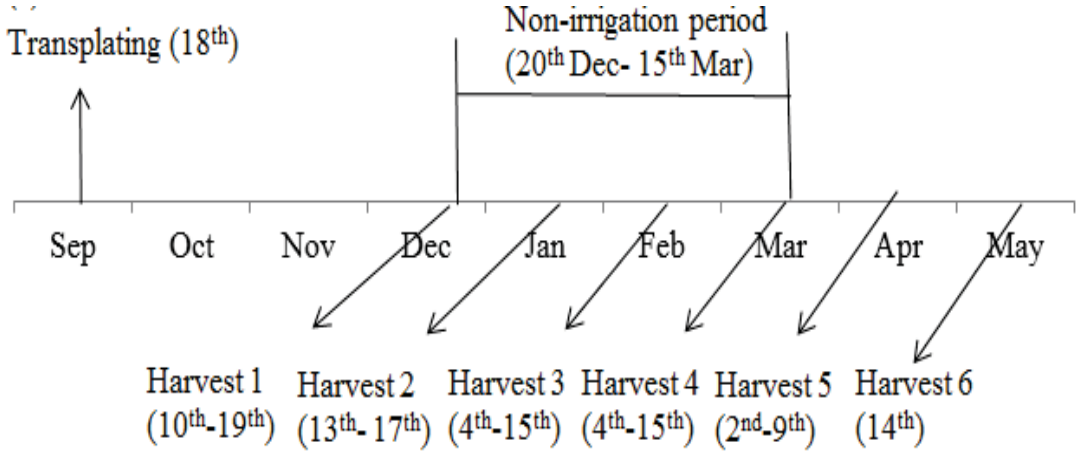

Figure 3.3 Timeline for the experiment. Plants were transplanted in 18th September 2012 to the field and six harvests were conducted monthly from December 2012 to May 2013.

Table 3.2 Measurements conducted in each harvest from December 2012 to May 2013.

Harvest 1 (Dec)	SWC, Shoot DM, RWC, LWP, OP, CF, RC, RD, TTN, TSR, $\delta^{13}C$, $\delta^{15}N$, proline
Harvest 2 (Jan)	SWC, Shoot DM, RWC, CF, NRL
Harvest 3 (Feb)	SWC, Shoot DM, RWC, LWP, OP, CF, RD, TSR
Harvest4 (Mar)	SWC, Shoot DM, RWC, LWP,OP, RD, TSR, $\delta^{13}C$, $\delta^{15}N$, NRL, proline
Harvest 5 (Apr)	SWC, Shoot DM, RWC, LWP, OP, proline
Harvest 6 (May)	Shoot DM

Note: SWC, soil water content; RWC, relative water content; LWP, leaf water potential; OP, osmotic potential; DM, dry matter; CF, chlorophyll fluorescence; RC, ring colonisation score; RD, reproductive development score; TTN, total tiller number; TSR, tiller survival rate score; NRL, new root length; $\delta^{13}C$, carbon isotope composition; $\delta^{15}N$, nitrogen isotope composition; proline, proline concentration.

3.3 Climate

Weather data for the experimental period was collected from the Palmerston North Ews NIWA weather station located adjacent to the experimental site (Agent Number 21963, Network Number EO536D, 40.38195°S, 175.60915°E). During the non-irrigation period from January to March, the average air and soil temperatures were 17.42°C ± 0.16 and 21.50°C ± 0.08, respectively, these values being on average 25% and 32% higher than those of the other months during the experiment. The average daily maximum and minimum temperatures fell within a range from 25°C to 5°C during the experiment, but the average daily minimum temperature from December to March was higher than 10°C (Figure3.4a). The relative humidity was typically near 70% from January to March (Figure3.4b) and this was approximately 10% lower than values for the other three months (December, April and May).

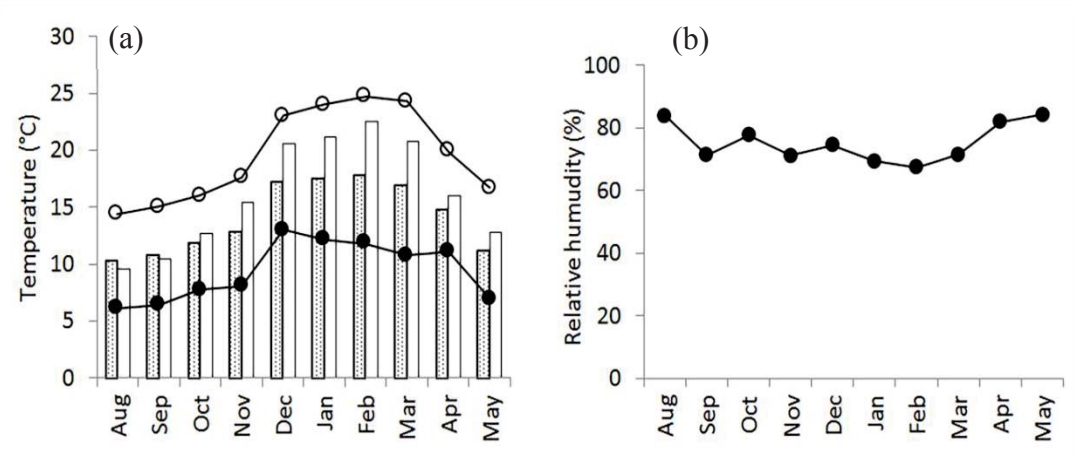

Figure 3.4 (a) Changes in daily average air (open bars) and 20 cm depth soil temperatures (dot filled bars) as well as average daily maximum (empty circles) and average daily minimum (filled circles) air temperatures; (b) Average daily relative humidity in the experimental period. Data were collected from the AgResearch Palmerston North electronic weather station located adjacent to the experimental site (National Institute of Water and Atmospheric Research, Agent number 21963).

3.4 Measurements

3.4.1 Soil water content

Volumetric SWC was measured periodically by averaging two readings adjacent to each plant using a 20 cm CS620-Hydrosense digital Time-domain reflectometer (Campbell Scientific Inc.). The 20 cm rod was chosen because for perennial ryegrass, root growth occurs mainly within this horizon (Jacques, 1943; Reid & Crush, 2013).

3.4.2 Plant growth

Ring colonisation score

In order to evaluate plant establishment rate and vigour, a visual estimate of the proportion of the ring that was occupied by each plant was used. Dead plants were scored 0; plants that filled less than 10% of the ring were scored 1; plants that filled about 50% of the ring were scored 2; plants that 100% filled the ring were scored 3.

Total tiller number and tiller survival rate score

Total tiller number was manually counted for each plant. Five score levels were applied to describe plant TSR. Dead plants without any live tillers scored 0; plants with 15% live tillers scored 1; plants with 50% live tillers scored 2; plants with 75% live tillers scored 3; plants with 100% live tillers scored 4.

Reproductive development score

Four score levels were developed to describe the RD of plants. Plants that were completely vegetative scored 0; plants with visible reproductive tillers but no visible head scored 1; plants at heading stage scored 2; plants with mature or flowering heads scored 3.

3.4.3 Shoot dry matter

Shoot DM production of each plant was determined by periodically cutting 5 cm above ground level, oven-drying herbage at 80°C for 48 hours, and weighing.

3.4.4 New root length and nitrogen fertiliser application

Before initiation of drought treatment, in early December, a core (diameter 5 cm) was inserted 20 cm away from the plant and at a 45 degree angle to the ground surface, so that with 28 cm length of the core tool penetrating the soil, the end of the core hole was directly beneath each plant. The original soil was taken out, and 20 ml (0.98 g/L) ^{15}N labelled $(NH_4)_2\ SO_4$ (equal to 4.38 mg ^{15}N /plant) was applied at the bottom of the hole created with the core tool. In this way the ^{15}N label was deposited at 20 cm depth under each plant (Figure 3.5). The ^{15}N labelled $(NH_4)_2\ SO_4$ was intended as a tag of nitrogen uptake during the water deficit treatment. In addition it was intended to re-sample from the same core hole a month after placing ^{15}N, to gauge new root development activity. For this purpose core holes were refilled with fine sand to differentiate the core hole from surrounding soil and a 15 cm long plastic sleeve of internal diameter 1mm larger than the external diameter of the core (machined from PVC water pipe) was driven into the hole just close to the ground surface in order to provide a guide to keep the core in the same hole when recovering samples before fresh sand was refilled in the hole.

In January, sand in the core (in the soil depth of 10 cm−20 cm) with new roots was taken out and stored at 5°C room for later root length determination, and then fresh sand was refilled again after a second dose of 20 ml (0.98 g/L) ^{15}N labelled $(NH_4)_2SO_4$ was applied in the same way to all plants. In March, sand with new roots was collected again from each plant for two replications, only, because of time constraints.

Roots were washed out of the sand and then counted manually as described by Tennant (1975), using in a 1 cm × 1 cm grid. The total root length of new grown roots in this certain volume of soil was calculated as: NRL (cm) = 11/14 × number of intercepts × grid unit square size. This NRL was taken to estimate the new root production activity of the plant.

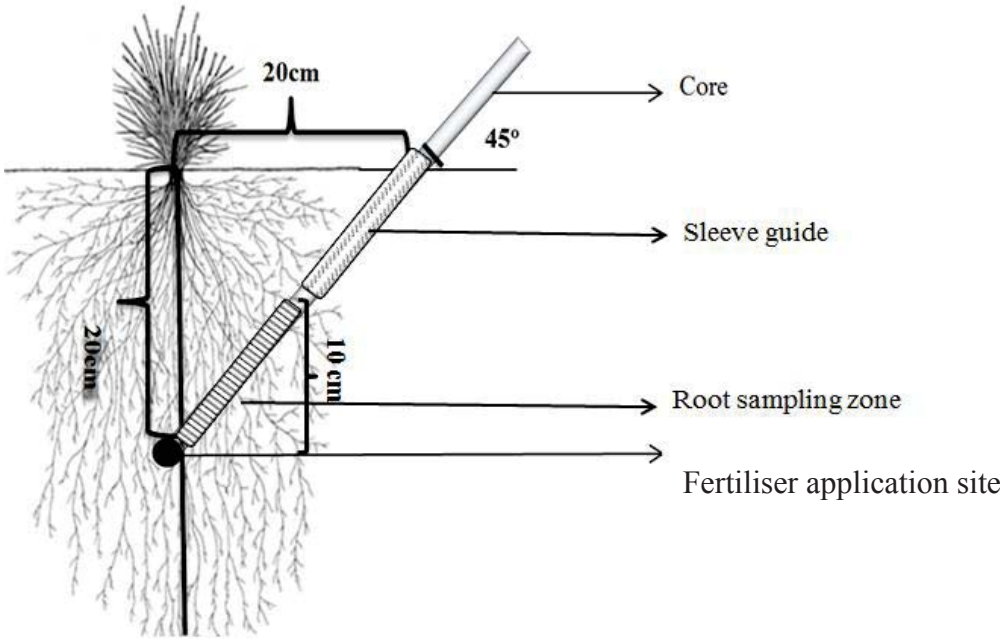

Figure 3.5 Diagram of ^{15}N application method

3.4.5 Plant water relations

Measurements of plant water relations, chlorophyll fluorescence and proline concentration were carried out on the first fully expanded leaves of randomly selected tillers of each plant, for consistency of leaf age when comparing data. Carbon isotope composition and nitrogen isotope composition was measured from a subsample of the shoot DM.

The RWC was determined according to the following equation:

$$RWC\ (\%) = [(FW - DW)/ (TW - DW)] \times 100\%, \qquad \text{Equation 3.1}$$

where FW is the leaf fresh weight, DW is the dry weight of leaves after drying 80°C for 48 hours, and TW is the turgid weight of leaves after soaking in water overnight in darkness at room temperature. The predawn LWP was measured between 5 a.m. and 8 a.m. by using a Scholander pressure chamber (Soil Moisture Equipment Crop., Santa Barbara. CA) (Scholander et al., 1965). After taking leaf samples to determine LWP, the lower 2 cm of the same leaves were cut immediately as samples for the OP determination. The OP was measured using a number of Wescor C-52 sample chambers (Wescor, Logan, UT, USA) by the dew-point method (Turner, 1981), in conjunction with the Wescor HR-33T microvolt meter. In order to convert the μV values to bars, a standard curve was prepared for each of the chambers by measuring a series of NaCl solutions with different molalities (0. 0.2, 0.4, 0.6, 0.8, 1.0 m) according to the Wescor C-52 manual. The standard curve determined for each chamber can be found in Appendix 1. TP was not calculated because the LWP and OP measured in the field generally are not very precise compared to that in a more controlled environment, which would generate even larger errors for TP when TP was calculated from the difference between LWP and OP.

3.4.6 Chlorophyll fluorescence

To obtain a larger surface area for measurement, three to four leaves were fastened together side by side (with adaxial leaf surface upward and without leaf overlap) using a plastic tape. A 'Dark Leaf Clip' DLC-8 was placed on each plant at least one hour before data collection to allow leaves doing dark adaption. The CF from the adaxial surface of stuck leaves was measured using a photosynthesis yield analyser Mini-PAM (WALZ, Germany). The maximum quantum efficiency of PS II (Y) was automatically calculated according to the equation:

$$Y = (F_m - F_o)/F_m = F_v/F_m, \qquad \text{Equation 3.2}$$

where F_m and F_o is the maximum and minimum fluorescence, respectively, and F_v refers to the variable fluorescence (Maxwell & Johnson, 2000; Signarbieux & Feller, 2011).

3.4.7 Proline concentration

Leaf samples for quantifying the proline concentration were freeze dried for three days and ground to powder. Approximately 30 to 50 mg tissue powder of each plant was subjected to the analysis. Proline concentration was determined using the colorimetric method described by Magne and Larher (1992). Briefly, Leaf samples were homogenised in liquid nitrogen, and then 1.2 mL of 3% (w/v) sulphosalicylic acid was added and mixed with the ground leaf powder. The homogenate was then centrifuged at 3,000 r/min for 10 minutes at room temperature. A 200 µL aliquot of the resulting supernatant was transferred to a fresh 1.5 mL centrifuge tube and combined with 400µL water and 800µL of 1% ninhydrin reagent and incubated for 1h at 98 °C in a water bath. Centrifuge tubes were then placed into ice to stop the ninhydrin reaction. All the liquid in each centrifuge tubes was transferred individually into a test tube with 800µL toluene to extract the proline-ninhydrin complex. Test tubes were vortexed for 15 seconds and allowed to stand for 5 minutes for phase separation to occur. A 600 µL portion of the upper toluene phase containing the chromophore was transferred to a 1mL quartz cuvette for spectrophotometric analysis at 518 nm using a Bausch & Lomb Spectronic 20 Spectrophotometer.

3.4.8 Carbon isotope discrimination and nitrogen uptake

The isotope analysis was carried out on a fully automated Europa Scientific 20/20 isotope analyser located at the Waikato Stable Isotope Unit, the University of Waikato. The carbon isotope composition ($\delta^{13}C$) of plant samples was determined as:

$$\delta^{13}C\ (‰) = (R_p/R_s - 1) \times 1000, \qquad \text{Equation 3.3}$$

where R_p and R_s refers to the ratio of $^{13}C/^{12}C$ of a plant sample and an accepted international standard limestone Pee Dee belemnite, respectively. Carbon isotope discrimination ($\Delta^{13}C$) of a plant to atmospheric air was then calculated as:

$$\Delta^{13}C\ (‰) = (\delta_a - \delta_p)/ (1+ \delta_p), \qquad \text{Equation 3.4}$$

where δ_a is the $\delta^{13}C$ of the atmospheric air, which is approximately −8‰ (Farquhar et al., 1989; Seibt et al., 2008) and δ_p is the $\delta^{13}C$ of the plant sample.

The nitrogen isotope composition ($\delta^{15}N$) of a plant was determined as:

$$\delta^{15}N\ (‰) = (R_p/R_s - 1) \times 1000\ ‰, \quad \text{Equation 3.5}$$

where R_p and R_s refer to the ratio of the $^{15}N/^{14}N$ of the plant sample and the atmospheric nitrogen, respectively, the R_s has a value of 0.0036765 (Mariotti, 1983).

The ^{15}N atom percentage (Atm) was then converted from the $\delta^{15}N$ and calculated as:

$$Atm\ (\%) = [R_s \times (\delta^{15}N/1000 + 1)]/[1 + R_s \times (\delta^{15}N/1000 + 1)] \times 100\%, \quad \text{Equation 3.6}$$

The total ^{15}N capture by plants was calculated as:

^{15}N capture (mg) = shoot DM (g/plant) × 1000 × total nitrogen concentration (%) × Atm (%), Equation 3.7

The ^{15}N concentration was calculated as:

^{15}N concentration (mg ^{15}N/ g DM) = N% × Atm (%) × 1000 Equation 3.8

3.5 Data analyses

Plants in I+ plots and I– plots should have similar plant size and vigour before irrigation treatment initiation as these factors would influence plant drought tolerance (Hatier et al., 2014). Therefore, data of all measurements were filtered by RC scores in December and only data for plants with RC score = 3 (i.e. the ring was fully occupied by the plant) were included in the statistical analysis (409 of 432 plants). The ANOVA assumption of normal error distribution was checked in a set of preliminary analyses. Where necessary, some data were log10 transformed before further analysis. A general linear model procedure (Proc GLM) was employed using SAS software (version 9.4, SAS institute, Cary, NC, USA). A TEST option was included in the model to request the GLM test the irrigation effect using the "irrigation × block" interaction as the denominator. If an interaction between cultivar and irrigation or between cultivar and endophyte was detected in the GLM analysis, then irrigation or endophyte effects for each cultivar were evaluated using a SLICE function, i.e. *Cultivar × irrigation (or endophyte) / slice = cultivar pdiff*, which requests a P value to detect the effect of irrigation or endophyte on each cultivar (SAS code is provided in Appendix 2). Least square means (LS-means) rather than

arithmetic means are presented because LS-means are preferred in an unbalanced design (SAS Institute, 1999) (There were missing values in this experiment because of the omission of data for plants with RC score ≠ 3). For TSR scores, a non-parametric factorial analysis (NPFA) was applied. Data was 'aligned rank transformed' (ART) to generate normally distributed ranks by using the ARTool package in R, then subjected to ANOVA testing (Wobbrock et al., 2011). However, the results were very close to the GLM analysis in SAS with non-transformed scores, therefore, the GLM analysis was presented for consistency with the other analyses (The NPFA results are provided in Appendix 3).

Chapter 4 Morphological traits of ryegrass and Mediterranean tall fescue plants with and without *Epichloë* endophyte under two water regimes

4.1 Abstract

Many perennial ryegrass cultivars are available in the market, but little research information exists on their comparative drought tolerance or on the role of selected endophyte strains in drought tolerance of the host. To provide such data, cloned plants of seven perennial or long-rotation ryegrass cultivars (Grasslands Commando, Ceres One50, Banquet II, Alto, Bealey, Trojan and Avalon), an un-released elite perennial ryegrass line (URL) and one Mediterranean tall fescue cultivar (Grasslands Flecha), in all cases both *Epichloë* endophyte-free (E–) and endophyte-infected (E+) plants were subjected to a cycle of drought and rehydration from December 2012 to May 2013 while other clones of the same plants were irrigated. Here data for shoot dry matter (shoot DM), tiller survival rate (TSR), reproductive development and new root length (NRL) assessed approximately monthly during the experiment. In the second month of drought, among all evaluated cultivars, only Banquet II and Flecha did not show a significant shoot DM reduction under water deficit. In the third month of drought, shoot DM of all cultivars was significantly reduced with the percentage reduction ranging from 43% to 85% compared with irrigated plants. The TSR of Banquet II, Avalon and Flecha was not significantly reduced by water deficit. New root formation did not differ between irrigated and non-irrigated plants, although cultivar differences were observed. During rehydration, growth of previously non-irrigated plants typically exceeded growth of irrigated clones across all cultivars. In this experiment, Banquet II was more drought tolerant compared to other ryegrass cultivars evaluated and Flecha was also drought tolerant because of their high yield stability under drought, however, these two cultivars had low yield potential under irrigated conditions, which was not ideal in commercial farm systems. Irrespective of irrigation treatment, the shoot DM of E+ plants of the URL (infected with AR37) and of One50 (infected with AR1) was reduced by almost 50% compared to their E– counterparts in each harvest from December to May, suggesting that some novel cultivar-endophyte associations are at a disadvantage compared to E– plants when the plant environment is without an insect pressure component.

4.2 Introduction

Herbage yield is a trait of high interest to farmers and breeders, and is the principal factor influencing pasture productivity and profitability (Williams et al., 2007). In agronomy, drought tolerance have been often evaluated based on yield stability under water deficit compared to well watering (Ebrahimiyan et al., 2012; Menezes et al., 2014; Rad & Abbasian, 2011; Saraswati et al., 2004). The hypothesis of this chapter was that evaluated ryegrass cultivars would differ in agronomic drought tolerance, as assessed by herbage mass reduction under water deficit, compared to adequate watering; and endophyte presence would affect agronomic drought tolerance, and this endophyte effect would have interactions with host cultivar.

An introduction to the experiment, and information for methods and materials were provided above in Chapter 3. In this chapter, morphological data including shoot dry matter (shoot DM), scores for tiller survival rate (TSR), the reproductive development (RD) scores, and new root length (NRL) data are presented. The data presentation format adopted is to first present ANOVA results to show which effects were statistically significant for the various measurements, and then to present relevant graphs and tables to elucidate the significant effects. A majority of the data presentation is constructed to present I+ and I– or E+ and E– effects for the eight cultivars and the URL listed in Table 3.1.

4.3 Results

The SWCs fell in both I+ and I– plots from December to January, then the SWCs of I– plots separated from those of I+ plots statistically, from January to March. The SWCs of both I+ and I– plots were increased to about 40% in April after re-watering (Figure 4.1). In December, when all plots were under irrigation, plants in I+ plots had similar shoot DM and tiller number to those of plants in I– plots (Table 4.1 and 4.2). In January, none of the cultivars was significantly affected by water deficit (Table 4.1 and Figure 4.2). In February, compared to the I+ plants, water deficit significantly reduced the shoot DM of the URL (63% reduction), Commando (51% reduction), One50 (58% reduction), Alto (61% reduction), Bealey (55% reduction), Trojan (46% reduction) and Avalon (45% reduction) but not significant for Banquet II (42% reduction) and tall fescue cultivar Flecha (15% reduction) (Table 4.1 and

Figure 4.2). In March, water deficit significantly reduced the shoot DM of all cultivars, the percentage reduction ranged from 43% (Flecha) to 85% (the URL) (Table 4.1 and Figure 4.2). The TSR scores of cultivars Banquet II, Avalon and Flecha were not significantly decreased by water deficit in both February and March (Table 4.2 and Figure 4.4), and the TSR scores of these three cultivars were less than 3 even when grown under I+ conditions (Figure 4.4). The NRL was not affected by water deficit (Table 4.3). During rehydration, plants that had experienced water deficit produced similar shoot DM to that of constantly irrigated plants in April and surpassed the constantly irrigated plants in May (Figure 4.2). There was a significantly positive correlation ($r = 0.7361$, $p < 0.0001$, $N = 181$) between shoot DM in April and TSR score of I– plants in March (Figure 4.8a).

The E+ plants of the URL and One50 consistently had lower shoot DM than their E– clones from December to May (Table 4.1 and Figure 4.3), with the E+ plants' shoot DM averaging only about 50% of that of the E– plants over this period. Endophyte infection also tended to reduce the shoot DM of Alto and Trojan in a majority of the harvests, but was only statistically significant occasionally (Figure 4.3). E+ plants of all ryegrass cultivars on average had significantly lower TSR scores than E– plants, but significant for the URL, One50 and Alto in March (Table 4.2 and Figure 4.5). The NRL of E+ plants of the URL were consistently less than that of E– plants in January and March (Figure 4.7). In contrast to ryegrass cultivars, E+ plants of tall fescue Flecha had slightly greater shoot DM (Figure 4.3) and a numerically higher TSR score than their E– clones (Figure 4.5).

The majority of reproductive growth observed occurred in December. Numerically, ryegrass cultivars One50, Bealey and Avalon had a higher RD score than other cultivars in December, with some aftermath heading in February and March not seen in other cultivars. Flecha had a higher RD score than the ryegrass cultivars in all three months (Figure 4.6). Since the majority of plants were in a vegetative growth state (RD score = 0) in March, no correlation between shoot DM in April and plant RD score of I– plants in March was detected (Figure 4.8b).

4.3.1 SWC

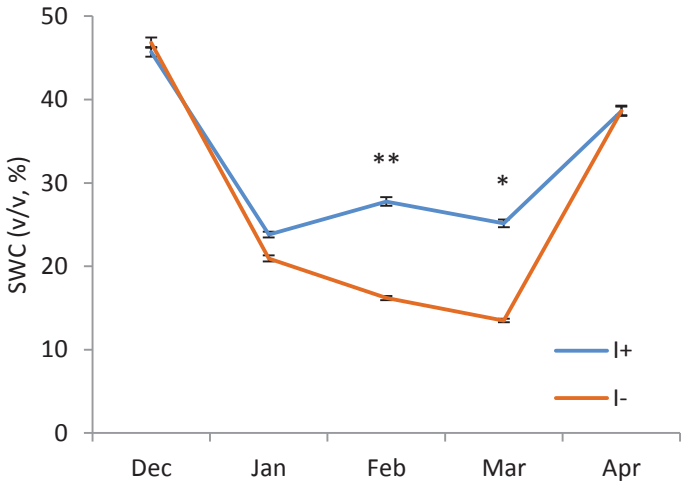

Figure 4.1 Average soil water content (SWC) at 20 cm soil depth of irrigated (I+) and non-irrigated (I−) plots from December to April. Vertical bars indicate standard errors; single asterisk and double asterisks denotes significant difference between I+ and I− plots at $P < 0.05$ and $P < 0.01$, respectively.

4.3.2 Shoot DM

Table 4.1 Results from GLM ANOVA for shoot dry matter from December to May.

Source	*df*	Dec		*df*	Jan		*df*	Feb	
		F	P		F	P		F	P
I	1	7.07	0.117	1	5.73	0.139	1	163.86	**0.006**
C	8	6.33	**<.0001**	8	7.09	**<.0001**	8	4.95	**<.0001**
C × I	8	0.69	0.705	8	0.70	0.694	8	2.21	**0.026**
E	1	12.16	**0.001**	1	21.05	**<.0001**	1	16.41	**<.0001**
E × I	1	0.02	0.894	1	0.56	0.456	1	0.00	0.948
C × E	8	2.68	**0.007**	8	3.21	**0.002**	8	4.04	**0.0001**
C × E × I	8	0.55	0.820	8	0.58	0.795	8	0.45	0.891
Error	365			358			354		
Source	*df*	Mar		*df*	Apr		*df*	May	
		F	P		F	P		F	P
I	1	66.56	**0.015**	1	0.00	0.974	1	16.04	**0.057**
C	8	4.59	**<.0001**	8	9.77	**<.0001**	8	11.32	**<.0001**
C × I	8	4.43	**<.0001**	8	1.17	0.314	8	0.97	0.461
E	1	13.73	**0.0002**	1	13.87	**0.0002**	1	6.33	**0.012**
E × I	1	0.84	0.361	1	0.6	0.440	1	2.56	0.111
C × E	8	2.35	**0.018**	8	3.02	**0.003**	8	3.47	**0.001**
C × E × I	8	0.59	0.788	8	0.97	0.457	8	1.53	0.146
Error	325			347			341		

Note: I, irrigation treatment; C, plant cultivar; E, endophyte status.

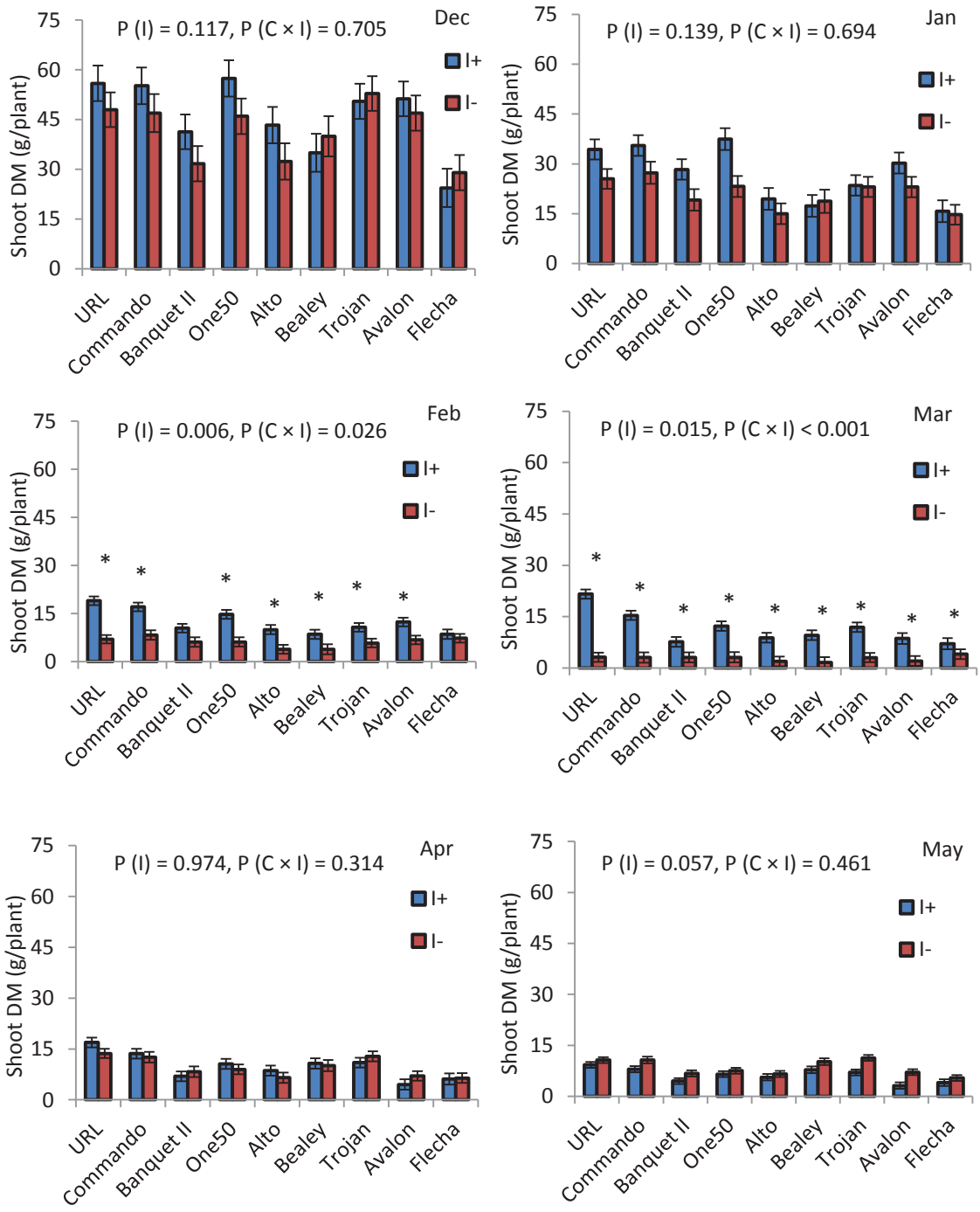

Figure 4.2 Shoot dry matter (shoot DM) of irrigated (I+) and non-irrigated (I–) plants of each cultivar from December to May. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

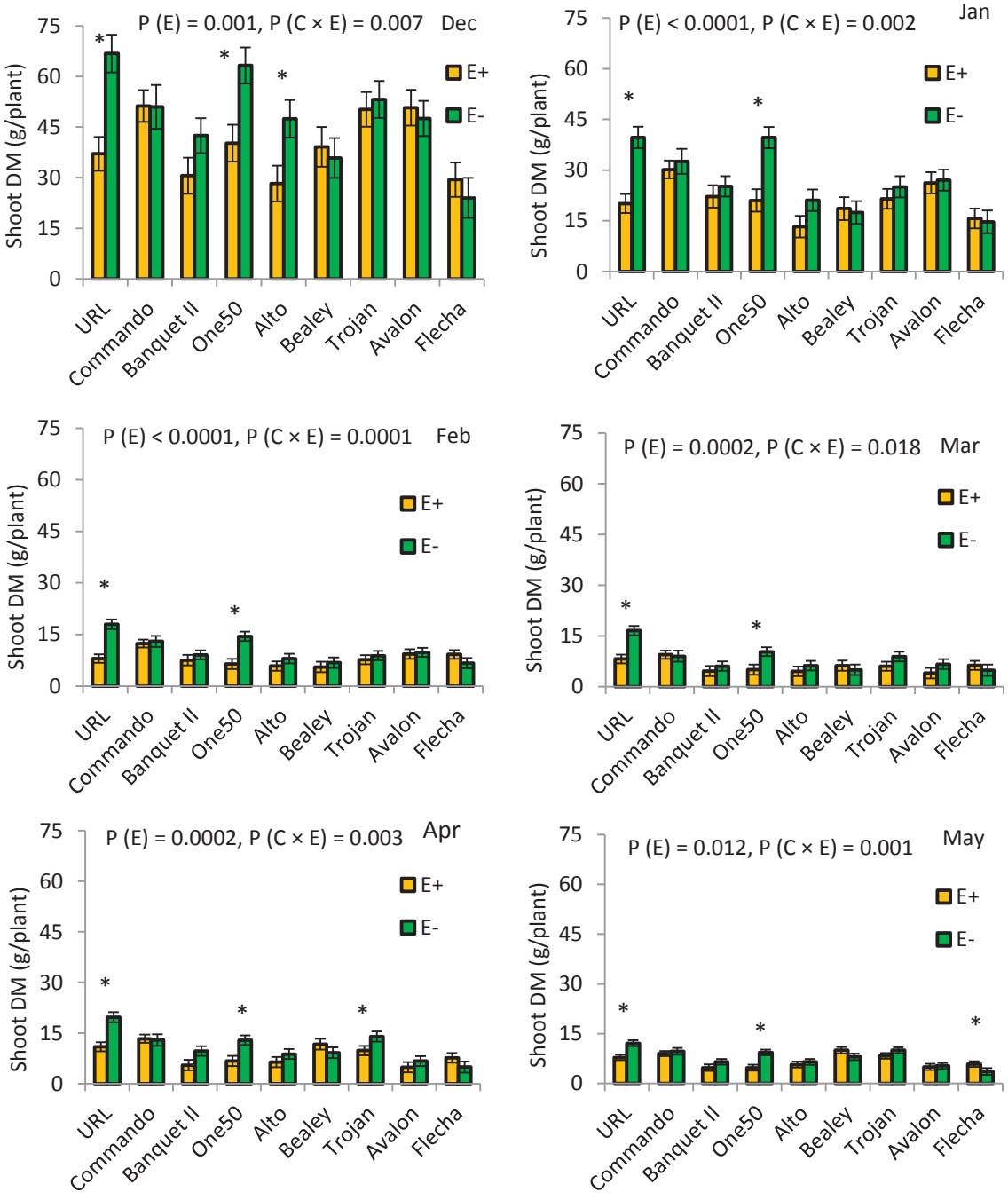

Figure 4.3 Shoot dry matter (shoot DM) of endophyte-infected (E+) and endophyte-free (E–) plants of each cultivar from December to May. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

4.3.3 TSR score

Table 4.2 Results of GLM ANOVA for total tiller number in December and tiller survival rate score in February and March.

Source	*df*	Dec		*df*	Feb		*df*	Mar	
		F	P		F	P		F	P
I	1	0.01	0.937	1	384.02	**0.003**	1	39.15	**0.025**
C	8	3.16	**0.002**	8	4.51	**<.0001**	8	4.51	**<.0001**
C × I	8	0.39	0.927	8	2.75	**0.006**	8	5.18	**<.0001**
E	1	0.91	0.341	1	10.33	**0.001**	1	10.29	**0.002**
E × I	1	0.27	0.602	1	1.36	0.244	1	1.28	0.259
C × E	8	1.53	0.147	8	1.18	0.308	8	2.01	**0.045**
C × E × I	8	0.55	0.816	8	0.91	0.506	8	0.38	0.933
Error	242			368			369		

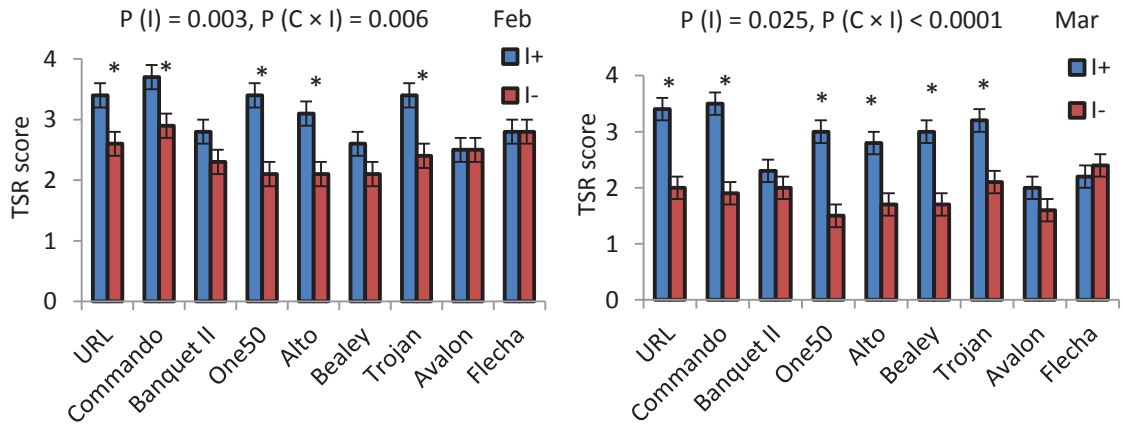

Figure 4.4 Score of tiller survival rate (TSR) of irrigated (I+) and non-irrigated (I–) plants of each cultivar in February and March. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

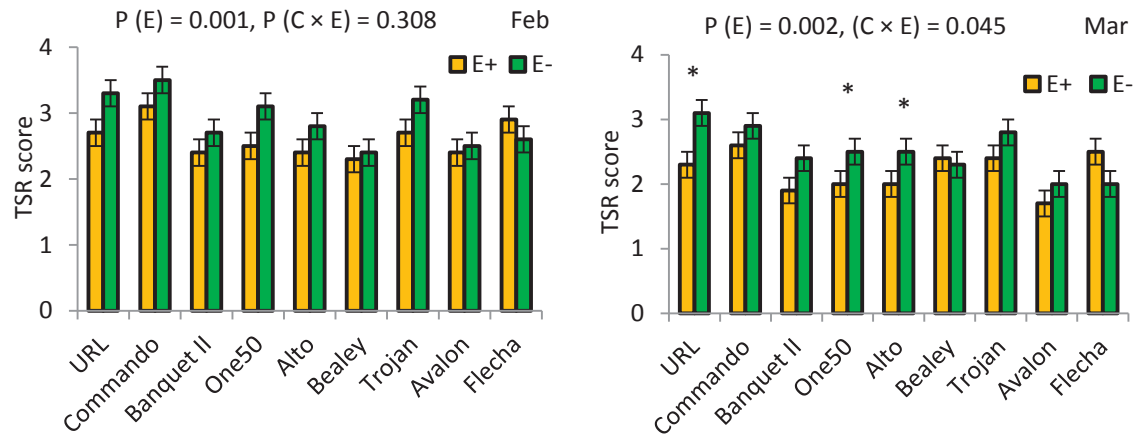

Figure 4.5 Score of tiller survival rate (TSR) of endophyte-infected (E+) and endophyte-free (E−) plants of each cultivar in February and March. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

4.3.4 RD score

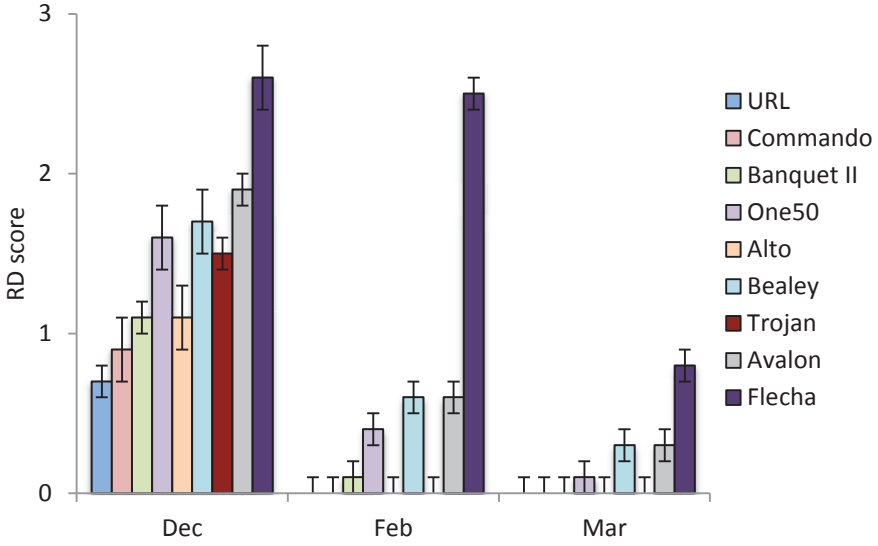

Figure 4.6 Reproductive development (RD) score in December, February and March. Vertical bars indicate standard errors.

4.3.5 NRL

Table 4.3 Results of GLM ANOVA for new root length in January and March.

Source	*df*	Jan		*df*	Mar	
		F	P		F	P
I	1	0.58	0.524	1	5.4	0.257
C	8	8.09	**<.0001**	8	4.51	**<.0001**
C × I	8	0.90	0.517	8	1.05	0.398
E	1	0.72	0.395	1	0.32	0.572
E × I	1	1.56	0.213	1	2.69	0.103
C × E	8	1.84	0.068	8	1.53	0.147
C × E × I	8	1.21	0.290	8	1.79	0.080
Error *df*	351			220		

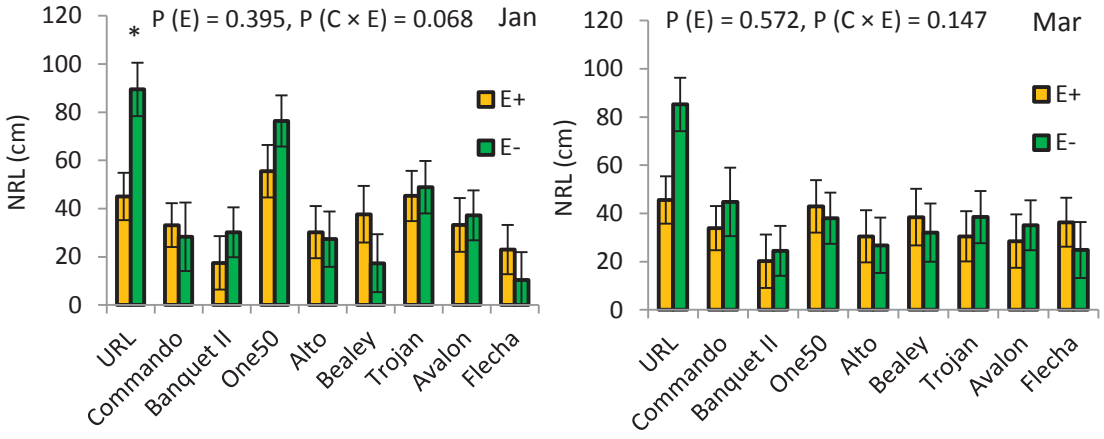

Figure 4.7 New root length (NRL) of endophyte-infected (E+) and endophyte-free (E−) plants of each cultivar in January and March. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

4.3.6 Correlations

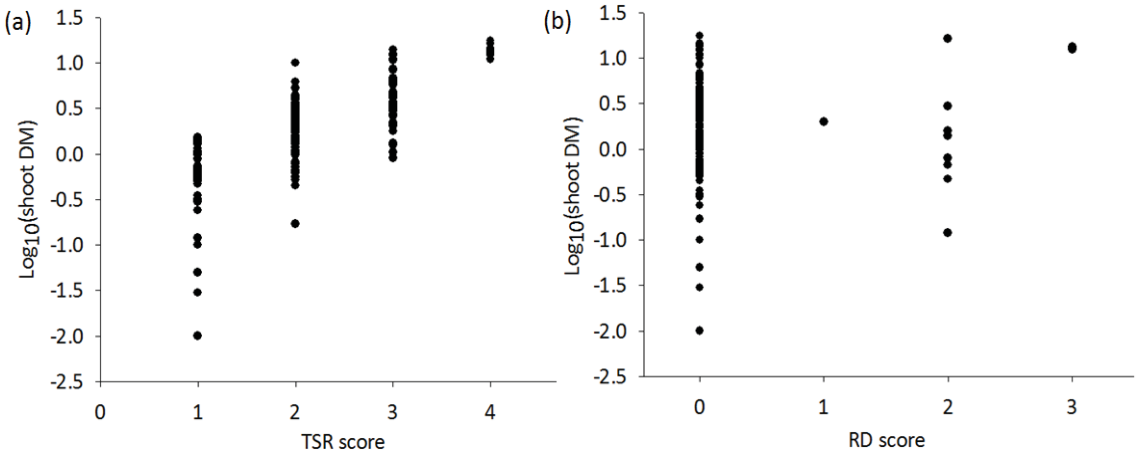

Figure 4.8 Correlation plots of (a) tiller survival rate (TSR) score (r = 0.7361, P < 0.0001, N = 181) or (b) reproductive development (RD) score (r = 0.0635, P = 0.3958, N = 181) of non-irrigated plants in March and their post-drought shoot dry matter (shoot DM) in April.

4.4 Discussion

Perennial ryegrass is currently one of the most important grass species in temperate regions but its productivity is restricted by occasional drought; hence, improving its tolerance to drought is necessary to secure profitability for farmers. Herbage yield is a trait of high interest to farmers and breeders, and is the principal factor influencing pasture productivity and profitability (Williams et al., 2007). The yield stability has been commonly used as an index to evaluate drought tolerance in many crops (Ebrahimiyan et al., 2012; Menezes et al., 2014; Rad & Abbasian, 2011; Saraswati et al., 2004). Thus, in this chapter, drought tolerance is mainly evaluated by comparing herbage yield stability.

It is understood that local climate factors such as evaporation, air humidity and air temperature also contribute to the drought stress (Rickard & Radcliffe, 1976; Wilhite & Glantz, 1985); therefore, this drought treatment was applied from January to March which corresponded to the naturally occurring summer dry period in New Zealand. Perennial ryegrass is intolerant of high temperatures, defined as day time

temperatures exceeding 31°C and night time temperatures exceed 25°C (Hannaway et al., 1997). During the course of this experiment, the average daily maximum air temperatures were no more than 25°C (Figure3.4a), which indicated that heat stress would not be a major concern during the summer dry period at the experiment site. Therefore, the reduction of pasture yield can be attributed mainly to moisture deficit.

During the period of gradually declining SWC from January to March in the I– plots (Figure 4.1), no cultivars in January, seven cultivars in February, and all cultivars in March showed significantly reduced shoot DM associated with water deficit (Figure 4.2). From December to January, all ryegrass cultivars were able to maintain herbage yield in slight drought stress, where soil water was freely available to plants; therefore, plant water uptake would have met the transpiration demand. However, the diminishing soil water status as the experiment progressed elicited the classic drought response of reduced plant size, which has been shown elsewhere to arise from reduced leaf elongation and accelerated leaf senescence (Assuero et al., 2002; Laidlaw, 2009), and results in reduction of water demand.

The URL, Commando and One50 were three high-yielding cultivars under I+ conditions, however, the water deficit linked yield reduction for these cultivars was also the most pronounced in both February and March (Figure 4.2). Banquet II and Flecha were not significantly reduced in shoot DM by water deficit in February, but in both cases, shoot DM and TSR were comparatively lower than for the majority of cultivars even when irrigated (Figure 4.2 and 4.4). The smaller plant size of Banquet II and Flecha would have slowed water use under the water deficit conditions. Banquet II is a hybrid (*Lolium perenne* × *Lolium bouchianum*) tetraploid cultivar, and tetraploid cultivars usually have lower yield than diploid cultivars (Ahloowalia, 1967). Flecha is a Mediterranean cultivar and exhibits a partial degree of endogenous summer dormancy, with markedly reduced growth associated with partial foliage senescence (Norton et al., 2006). Summer dormancy has been considered as an important trait that contributes to plant survival during the long hot-dry summer in Mediterranean area (Volaire & Norton, 2006). For Bealey and Avalon in February,

and for Banquet II, Avalon and Flecha in March, the shoot DM was significantly reduced by the water deficit while the TSR was not, suggesting that the shoot DM reduction for these cultivars probably mainly caused by decreased growth rate rather than increased senescence rate.

During rehydration, plants that had been un-irrigated in summer produced similar herbage yield in the first month of rehydration to that of I+ cloned plants, and even surpassed the yield of I+ plants in the second month of rehydration (Figure 4.2). An explanation that has been advanced for this effect is that nutrient uptake by plants is restricted in conditions of limited water supply and reduced growth, and as a result the nutrients remain available in the soil, so increasing growth when water becomes available again (Renkema et al., 2012; Yingjajaval, 2013). Another reason for the increased herbage yield of I– plants during rehydration is that non-structural carbohydrates accumulated during water deficit are remobilised to accelerate plant regrowth (Volaire et al., 1998; Yang et al., 2013).

It has been suggested that plant RD and TSR during drought are two factors affecting plant herbage production during rehydration. Thomas and Evans (1990) found that previously drought stressed flowering plants regrew more slowly than watered controls after drought, whereas previously stressed vegetative plants regrew more quickly than the controls. However, as noted above, in the current experiment, since the majority of plants were completely vegetative in March (Figure 4.6), no correlation between shoot DM production during rehydration and the RD score during drought was detected (Figure 4.8b). A high correlation ($r = 0.93$, $P < 0.01$) has been observed between plant regrowth after drought and wilting level for perennial ryegrass (wilting score was on a scale of 0 to 9, with 0 indicating fully wilted and 9 indicating no wilting damage) at the end of drought (Jonaviciene et al., 2014). Similar results showing a close correlation between TSR during drought and plant regrowth after drought were reported by Volaire et al. (1998). Similarly, in the current experiment, the shoot DM after drought had a strong correlation with TSR

during drought (Figure 4.8a), plants with higher TSR during drought produced greater shoot DM when water was available again.

There was no interaction between endophyte status and irrigation treatment detected for the shoot DM and TSR (Table 4.1 and 4.2), suggesting that E+ and E– plants had similar responses to drought. However, the main endophyte effect and interaction between cultivar and endophyte status were detected for shoot DM and TSR (Table 4.1 and 4.2). Endophyte infection significantly reduced the shoot DM (about 50% reductions) and TSR of the URL and One50 (Figure 4.3 and 4.5). These effects were unexpected and are not easy to account for. The possibility that plants might have been mixed at planting was considered and so stored leaf samples were profiled for alkaloids after the conclusion of the experiment to check endophyte status, and the shoot DM reduction was confirmed to be linked to E+ plants. The results suggested that endophyte presence has a metabolic cost of the host and this is dependent on host cultivar and endophyte strain. Also, since the URL and One50 were infected with AR37 and AR1, respectively, herbage yield of the URL and One50 infected with other selected endophyte strains would need to be tested in a future experiment. The older tillers/leaves may be more vulnerable than the young tillers/leaves to endophytic metabolic cost, as endophyte hyphal concentration has been found to be much higher in the older tillers/leaves (Christensen & Voisey, 2007). Also, older tillers/leaves translocate photo-assimilates (Carvalho et al., 2006) and nitrogen (Mohammad et al., 2010) to young tillers/leaves. Total nitrogen concentration of leaves (including leaf lamina and pseudostem) of E+ plants was significantly lower than that of E– plants, especially for older leaves, which was 3.98% for E+ plants and 4.40% E– plants across two ryegrass cultivars and one tall fescue cultivar in a smaller linked experiment (unpublished data), which indicates evidence to support this hypothesis. Ryan et al. (2015) showed that the endophyte concentrations of novel perennial ryegrass-endophyte associations were strongly dependent on plant genotype and fungal strain. It is possible that the concentrations of endophyte hyphae in the URL and One50 were higher than that of other cultivars. This was not checked

and could be a point for future investigation. Consistent with the reduced shoot DM, E+ plants of the URL also displayed a reduction in NRL compared to E− plants, indicating that endophyte infection reduced the new root formation of the URL as well as shoot biomass. Endophyte infection related plant biomass reduction has been reported in some other grass species, for example, arizona fescue (*Festuca arizonica*) (Faeth et al., 2004).

For the tall fescue cultivar Flecha, numerically, the E+ plants had a slightly higher shoot DM (Figure 4.3, statistically significant in May) and TSR score compared to E– counterparts (Figure 4.5). Previously, Malinowski et al. (2005) also showed that E+ (AR542) plants of cultivar Flecha had 10–50% greater tiller density than E– plants over three years in a semiarid zone in the USA. However, benefits of endophyte infection to the host cultivar Flecha were not observed in all experiments, for example, West et al. (2007) reported that endophyte infection (AR542) had no effect on TSR of cultivar Flecha, both in irrigation and drought conditions. These different results suggested that effects of AR542 endophyte on plant growth of Flecha were probably influenced by other environmental factors.

4.5 Conclusions

Among the eight ryegrass cultivars and one tall fescue cultivar evaluated, all cultivars were able to maintain growth in the first month after withholding irrigation, two cultivars (Banquet II and Flecha) could do so in the second month after withholding irrigation, and no cultivar could do so in the third month, in this experiment. However, these two cultivars that were able to continue growth in the second month after withholding irrigation both had low yield potential under optimum conditions, which was not ideal in commercial farm systems. The URL, Commando and One50 are three cultivars with high yield potential but also vulnerability to water deficit. Drought-exposed plants commonly showed compensatory growth during rehydration but all cultivars had similar recovery ability. Novel cultivar-endophyte associations have a great agricultural value, however, these selected endophyte strains did not improve the yield stability of their associated cultivars in response to drought. Instead, independently of the water

availability, some endophyte strains caused significant herbage yield reduction in their host cultivars when insect pressure was artificially controlled, which indicates that endophyte infection has a metabolic cost that could potentially place E+ plants at a disadvantage compared to E− plants in certain conditions.

Chapter 5 Exploring effects of genotype within each cultivar and the genotype interactions with endophyte and irrigation for plant yield

5.1 Abstract

The objective of this chapter is to examine whether genotypes within each cultivar differed in drought tolerance, and whether effects of endophyte status on drought tolerance of the host vary within cultivars. In agronomy, plant productivity is the most important trait in evaluating drought tolerance, thus the data for shoot dry matter (shoot DM) in February, March and May was selected for further exploration in this chapter. No plant genotype × irrigation treatment interaction effect for the shoot DM was found in any of the cultivars, indicating that all the evaluated genotypes within each cultivar showed a similar degree of drought tolerance. Nor was any plant genotype × irrigation treatment × endophyte status interaction detected for the shoot DM, indicating that endophyte had no effect on any particular plant genotype within the respective cultivars in response to drought. A plant genotype × endophyte status interaction effect was detected only twice in the data set, suggesting that such effects should not be a major barrier in plant improvement work to achieve cultivar-endophyte combinations with stable behaviour.

5.2 Introduction

Due to its self-incompatible and out crossing pollination pattern, perennial ryegrass shows genetic variability not only between cultivars but also within cultivars (Casler, 1995). Cultivars of outbreeding forage grass species such as perennial ryegrass and tall fescue are typically ‘synthetic’ cultivars. Seeds of a synthetic cultivar are derived from multiplication over several generations of seeds from a polycross of a set of parent plants that have been selected and preserved. Thus the seeds of the synthetic cultivar are not genetically identical but a population made up of various re-combinations of the parent plant gene pool, and seed lines produced at different times share the same gene pool, since they share the same parents in the first generation. Perennial ryegrass is generally susceptible to drought stress, but individual genotypes within a particular cultivar probably have different drought

tolerance levels. This is the question to be explored in this chapter, by comparing the four genotypes of each cultivar included in the experiment.

Reports on the effect of endophyte on the drought tolerance of the host have been inconsistent. One of the reasons could be that the endophyte effect depends on the host genotype. Previously, three-factor interactions (plant genotype × irrigation treatment × endophyte status) on root:shoot ratio and plant biomass allocation to tiller bases have been reported in perennial ryegrass (Cheplick, 2004; Cheplick et al., 2000), but not on tiller number, leaf area or total biomass. Also, some researchers have shown that endophyte infection improved drought tolerance of the host genotypes (or ecotypes) that have adapted to drought conditions or are drought tolerant (Hesse et al., 2003, 2005; Zhou, 2014). The present data also allow examination of endophyte × plant genotype × irrigation treatment interactions to test this question.

Hence, the research questions in this study were: 1) Do genotypes within each cultivar show different drought tolerance levels? 2) Does the endophyte effect on drought tolerance of the host vary among genotypes within cultivars? The associated hypotheses were: since perennial ryegrass is an out-crossing plant species, genotypes within each cultivar would show different drought tolerance levels; and endophyte effect on drought tolerance would have interactions with host genotypes within each cultivar.

In agronomy, plant yield is the most important trait in evaluating drought tolerance (Farshadfar & Sutka, 2002; Golabadi et al., 2006; Talebi et al., 2009), also, plant yield is a primary interest of farmers who sow these cultivars, thus the data of shoot DM in February and March (the second and third month after withholding irrigation) and May (the second month after rehydration) were chosen as the trait to be considered when examining the data for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status. Similar analysis of other traits would also be interesting but time and space do not allow for that in this thesis.

5.3 Data analysis

Data was sorted by cultivar (SAS command: Proc Sort By), and then data for each cultivar was analysed using the GLM model described in Section 3.5.

5.4 Results

Only effects of genotype and genotype related interactions are included in this chapter, as effects of irrigation and endophyte have been discussed previously in Chapter 4.

For the URL, One50, Alto, Bealey and Trojan, no genotype effects on shoot DM and no interactions between plant genotype and endophyte status and/or irrigation treatment were detected (Table 5.1 5.4, 5.5 5.6 and 5.7). An effect of plant genotype on the shoot DM was observed 5 times, for Commando in March and May, Avalon in May and Flecha in March and May (Table 5.2, 5.8 and 5.9). An interaction between plant genotype and irrigation treatment for the shoot DM was found in Avalon in February and May (Table 5.8). One genotype of Avalon behaved differently from other 3 genotypes, this genotype under I+ condition had lower shoot DM than I− counterparts and also lower shoot DM compared to other 3 genotypes under I+ conditions (Figure 5.1). An interaction between plant genotype and endophyte status was seen for the shoot DM of cultivars Commando and Banquet II in February (Table 5.2 and 5.3). For both Commando and Banquet II, two genotypes out of four genotypes had similar shoot DM between E+ and E− plants, the other two genotypes with E+ plants had either higher or lower shoot DM than E− counterparts (Figure 5.2).

Table 5.1 Results of GLM ANOVA for shoot dry matter of the URL in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	837.46	0.0010	75.63	0.0130	0.63	0.5100
G	0.94	0.43442	1.84	0.1672	2.93	0.0517
G × I	0.86	0.4764	0.78	0.5159	0.54	0.6594
E	23.23	<.0001	15.68	0.0006	12.71	0.0014
E × I	0.55	0.4651	1.46	0.2380	0.43	0.5163
G × E	0.22	0.8821	0.24	0.8641	0.37	0.7747
G × E × I	0.79	0.5110	0.19	0.8997	0.34	0.7967
Error *df*	27		24		27	

Note: I, irrigation treatment; G, plant genotype; E, endophyte status.

Table 5.2 Results of GLM ANOVA for shoot dry matter of Commando in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	10.27	0.0852	18.13	0.0510	3.22	0.2144
G	1.96	0.1442	3.12	**0.0450**	5.62	**0.0044**
G × I	2.86	0.0561	1.11	0.3630	0.40	0.7515
E	1.24	0.2752	0.09	0.7613	0.53	0.4732
E × I	0.69	0.4154	0.98	0.3325	0.31	0.5850
G × E	3.02	**0.0480**	1.53	0.2334	0.69	0.5695
G × E × I	0.35	0.7881	0.15	0.9294	0.38	0.7673
Error *df*	26		24		25	

Table 5.3 Results of GLM ANOVA for shoot dry matter of Banquet II in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	0.51	0.5484	1.71	0.3215	2.43	0.2595
G	1.10	0.3695	0.81	0.5030	0.62	0.6117
G × I	0.71	0.5557	0.23	0.8778	0.57	0.6428
E	0.06	0.8104	0.41	0.5300	0.52	0.4767
E × I	0.29	0.5965	0.59	0.4520	7.05	0.0145
G × E	4.12	**0.0181**	1.87	0.1653	0.81	0.5038
G × E × I	0.06	0.9821	0.16	0.9220	0.12	0.9448
Error *df*	23		21		22	

Table 5.4 Results of GLM ANOVA for shoot dry matter of One50 in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	204.48	0.0050	61.54	0.0159	0.09	0.7871
G	0.31	0.8164	0.06	0.9804	1.22	0.3250
G × I	0.78	0.5194	0.57	0.6386	1.29	0.3023
E	10.73	0.0044	4.69	0.0420	13.95	0.0010
E × I	0.21	0.6544	0.78	0.3870	0.68	0.4180
G × E	0.78	0.5201	0.97	0.4250	1.97	0.1448
G × E × I	0.44	0.7267	0.65	0.5904	0.59	0.6281
Error *df*	22		21		24	

Table 5.5 Results of GLM ANOVA for shoot dry matter of Alto in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	101.04	0.0098	97.61	0.0100	0.06	0.8350
G	2.21	0.1142	2.9	0.0601	0.35	0.7870
G × I	1.28	0.3052	1.73	0.1933	0.41	0.7454
E	2.4	0.1349	2.06	0.1666	0.29	0.5940
E × I	0.41	0.5270	0.05	0.8185	0.58	0.4549
G × E	0.74	0.5378	0.25	0.8610	1.64	0.2113
G × E × I	0.89	0.4698	1.37	0.2794	1.88	0.1637
Error *df*	23		20		21	

Table 5.6 Results of GLM ANOVA for shoot dry matter of Bealey in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	180.82	0.0055	50.01	0.0194	6.00	0.1340
G	2.16	0.1305	0.52	0.6773	1.54	0.2427
G × I	0.19	0.9003	0.67	0.5846	2.12	0.1381
E	0.06	0.8039	0.58	0.4586	1.40	0.2539
E × I	0.75	0.3992	0.14	0.7141	1.12	0.3049
G × E	0.58	0.6350	0.24	0.8682	1.09	0.3813
G × E × I	1.12	0.3702	0.52	0.6774	1.56	0.2368
Error *df*	17		17		16	

Table 5.7 Results of GLM ANOVA for shoot dry matter of Trojan in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	9.99	0.0872	8.98	0.0956	6.15	0.1313
G	0.03	0.9939	0.34	0.7993	1.33	0.2879
G × I	1.42	0.2607	0.59	0.6293	1.14	0.3529
E	1.38	0.2514	3.66	0.0682	2.73	0.1118
E × I	0.17	0.6798	0.23	0.6330	0.30	0.5915
G × E	0.85	0.4802	1.37	0.2783	1.10	0.3683
G× E × I	1.11	0.3624	2.06	0.1339	0.49	0.6896
Error *df*	26		23		24	

Table 5.8 Results of GLM ANOVA for shoot dry matter of Avalon in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	6.74	0.1219	11.02	0.0800	3.27	0.2125
G	2.18	0.1155	0.70	0.5660	4.79	**0.0102**
G × I	5.24	**0.0060**	0.27	0.7639	3.40	**0.0358**
E	0.10	0.7601	3.58	0.0748	0.03	0.8577
E × I	0.00	0.9528	2.49	0.1320	1.90	0.1815
G × E	0.58	0.6306	0.44	0.7299	0.21	0.8863
G× E × I	1.55	0.2254	2.78	0.0887	1.51	0.2429
Error *df*	25		18		22	

Table 5.9 Results of GLM ANOVA for shoot dry matter of Flecha in February, March and May to test for plant genotype and interaction effects between genotypes and irrigation treatment or endophyte status.

Sources	Feb		Mar		May	
	F	P	F	P	F	P
I	0.00	0.9627	2.25	0.2725	0.16	0.7295
G	0.99	0.4151	4.43	**0.0153**	5.92	**0.0040**
G × I	1.50	0.2393	1.28	0.3070	1.22	0.3267
E	3.52	0.0725	0.91	0.3521	0.95	0.3396
E × I	0.67	0.4199	0.43	0.5203	0.62	0.4403
G × E	2.76	0.0634	0.51	0.6831	1.68	0.1996
G× E × I	1.31	0.2946	0.07	0.9311	0.69	0.5112
Error *df*	25		20		22	

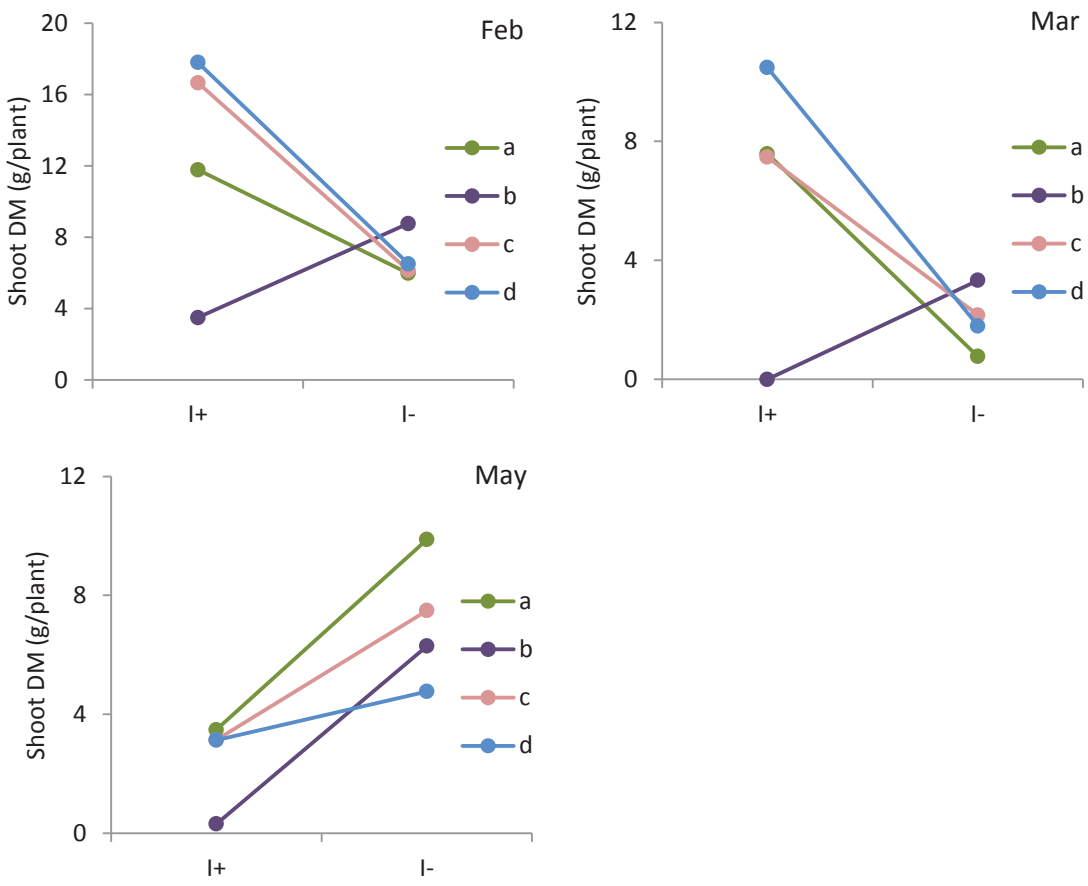

Figure 5.1 Shoot DM of the 4 genotypes within cultivar Avalon under irrigated (I+) and non-irrigated (I−) conditions in February, March and May, to examine the nature of the plant genotype × irrigation treatment statistical interaction from Table 5.8.

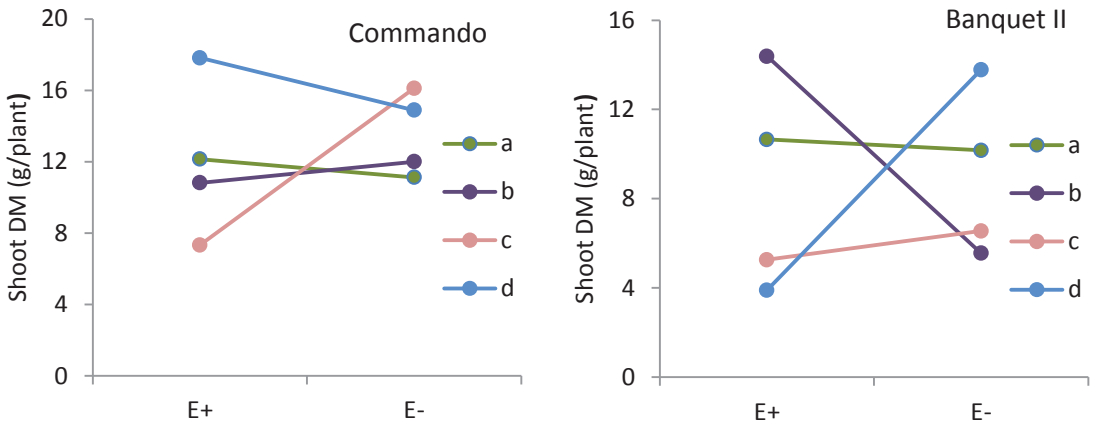

Figure 5.2 Shoot DM of the 4 genotypes within cultivar Commando and Banquet II with (E+) and without (E−) endophyte in February, to examine the nature of the plant genotype × endophyte status statistical interaction from Table 5.2 and 5.3, respectively.

5.5 Discussion

This rainout shelter experiment was not designed to investigate the effect of plant genotype; however, it was of interest to know whether these four genotypes within each cultivar differed from each other in drought tolerance and whether endophyte affected this drought tolerance of each host genotype similarly or differently in some cases, or whether genotypes within cultivars had comparatively uniform behaviour.

5.5.1 Plant genotype and interaction effects with irrigation treatment

From nine cultivars across three harvests (in total 27 cases), there were only five cases involving four genotypes within cultivars where variations in plant yield were detected. For the remaining 22 cases, the means of the four genotypes did not separate statistically, indicating the four genotypes within each cultivar were generally quite uniform.

There were only two cases of an interaction between plant genotypes and irrigation treatment detected (Table 5.8). Plants of one genotype of Avalon had very low plant yield under I+ conditions in all three harvests; lower even than the yield of their I− counterparts in February and March (Figure 5.1). As mentioned in Section 3.5, only

data for plants for which 100% of the ring was occupied by the plant in December was included in the statistical analysis. This suggests that this genotype of Avalon established and grew well from September to December but for some reasons nearly died in February. The data of this genotype was eliminated from cultivar Avalon and the analysis was done again. In this re-analysis, there was no significant interaction between plant genotypes and irrigation treatment but only a significant effect of genotype on the shoot DM in May (data not presented).

Overall, there was no interaction between plant genotypes and irrigation treatment for plant yield, indicating the lack of diversity in drought tolerance of genotypes within cultivars in this experiment. Thus selection for elite genotypes will likely require screening many individuals or outcrossing with external germplasm.

5.5.2 Interaction effects between plant genotypes and endophyte status

There was a plant genotype × endophyte status interaction effect on the shoot DM of Commando and Banquet II in February (Table 5.2 and 5.3), indicating that for these two cultivars, effects of endophyte on shoot DM was dependent on host genotypes. Also, the effect of genotype × endophyte status interaction was only detected in February but not in March and May, suggesting that the plant genotype × endophyte status interaction of these two cultivars probably also related to other environmental factors, such as temperature, air humidity, as the temperature/air humidity in February was higher/lower than that in other months (Figure 3.4). It has been noted that endophyte infection significantly reduced the shoot DM of the URL and One50 in every harvest (Figure 4.3); here it can be further noted that there was no plant genotype × endophyte status interaction effect on the shoot DM of these two cultivars, indicating that reduced herbage yield of the URL and One50 when infected by endophytes was a general effect common to the four tested genotypes, and not a result of one or two genotypes having a typical behaviour.

In Chapter 4, it was concluded that endophyte has no effect on plant yield stability in response to drought. Here it is demonstrated that the endophyte infection also does not generally influence the yield stability of particular genotypes within cultivars as indicated by the lack of a significant three-factor (plant genotype, endophyte status and irrigation treatment) interaction effect on the shoot DM. Cheplick (2004; 2000)

evaluated effects of drought on the growth of E+ and E– clones of a number of perennial ryegrass genotypes within cultivar Yorktown III. In those studies he found plant genotype × endophyte status × water treatment interaction for root:shoot ratio but not for shoot DM and tiller number. Hesse et al. (2005) collected three genotypes (or ecotypes) of perennial ryegrass from three different sites in the centre of Germany, a dry site, a wet site and either a wet or dry site. It was found that for the genotype collected from dry sites, the E+ and E– clones showed similar plant growth under drought, whereas E+ plants had significantly higher shoot, root and total dry weights and root:shoot ratio than E– plants during drought recovery. For the genotype collected from either the wet or dry site, the E+ and E– plants reacted similarly under drought and recovery. For the genotype collected from the wet site, the E+ plants were more sensitive to drought stress than E– plants. In another study, Zhou (2014) selected one drought tolerant E+ (DTE+) genotype and one drought sensitive E+ (DSE+) genotype of the perennial ryegrass cultivar Nine-o-One under a severe drought stress (25% FC), then the E+ and E– clones of both genotypes were subjected to drought stress with well-watered counterparts as control. In this test, E+ and E− plants of both DT and DS genotypes had similar RWC, CF (F_v/F_m) and total tiller number under well-watered conditions, while DTE+ had significantly higher RWC, CF (F_v/F_m) and total tiller number than DTE– under drought conditions, but there was no significant difference between DSE+ and DSE– plants under drought conditions. It is possible that the plant growth under drought stress or during recovery can only be enhanced by endophyte infection if the host-endophyte associations have adapted to a drought environment.

5.6 Conclusion

Based on the data of shoot DM, the most important trait in evaluating drought tolerance, it is concluded that genotypes within cultivars generally behaved similarly in their expression of drought tolerance and endophyte status did not affect the drought tolerance of individual host genotypes differently. The infrequent occurrence of interactions between plant genotype and endophyte status suggested that this effect should not be a major barrier to achieving cultivar-endophyte combinations with stable behaviour in plant improvement work.

Chapter 6 Physiological traits of ryegrass and Mediterranean tall fescue plants with and without *Epichloë* endophyte under two water regimes

6.1 Abstract

Physiological responses are often considered to provide specific insight about differences in drought tolerance strategies and different physiological traits have different sensitivities to drought stress. In this experiment, the objective was to compare physiological responses including plant water relations, chlorophyll fluorescence, proline concentration and carbon isotope discrimination of ryegrass cultivars and a Mediterranean tall fescue cultivar in a simulated summer drought and recovery, and to investigate the impacts of associated commercial endophytes on the physiological responses of their host cultivars. Plants responding to drought stress exhibited significantly decreased stomatal conductance (as indicated by carbon isotope discrimination ($\Delta^{13}C$)), decreased leaf relative water content (RWC) and osmotic potential (OP), and increased proline concentration, but leaf water potential (LWP) was less sensitive to drought stress. Flecha tall fescue was more drought tolerant than ryegrass cultivars, in the sense that RWC and CF of Flecha was not significantly influenced by water deficit, but this could be mainly attributed to summer dormancy rather than their having developed physiological adaptation mechanisms as the osmotic potential (OP) and proline concentrations also did not respond to drought significantly in Flecha. The measured physiological traits of endophyte-infected (E+) and endophyte-free (E−) plants responded to drought in similar magnitudes, suggesting that endophyte presence did not influence drought tolerance of the host. However, in most harvests, endophyte presence decreased the RWC, OP, $\Delta^{13}C$ and increased the proline concentration of the host. Effects of endophyte on these physiological traits were more pronounced in the URL and One50, suggesting that these physiological traits likely relate to the endophyte-linked herbage yield reduction in the URL and One50.

6.2 Introduction

Physiological responses are often considered to provide specific insight about differences in drought tolerance strategies and different physiological traits have different sensitivities to drought stress. In this chapter, the tested hypothesis was that evaluated cultivars would show different levels of physiological responses to water deficit; and physiological responses to water deficit would be modified by the presence of fungal endophyte, and this in turn might vary depending on the host cultivar and endophyte strain.

As for Chapter 4, an introduction to the experiment, and information methods and materials were provided above in Chapter 3. In this chapter, data for physiological traits including relative water content (RWC), leaf water potential (LWP), osmotic potential (OP), chlorophyll fluorescence (CF), proline concentration and carbon isotope discrimination ($\Delta^{13}C$) are presented. Following the data presentation format of Chapter 4, ANOVA results are presented to show which effects were statistically significant for the various measurements, followed by relevant graphs and tables to elucidate the significant effects.

6.3 Results

6.3.1 Effects of drought

Plants were transplanted in the field in September and grew for three months before the first harvest in December. All plants were well-watered to this point and the volumetric SWC of both irrigated (I+) and non-irrigated (I−) plots was about 45% (full FC) in December (Figure 4.1). Irrigation for the I− plots was withheld from January to March while plants in I+ plots were irrigated regularly during this period of time. From January to March, the SWC of I− plots gradually diverged from that of I+ plots, with significant differences occurring in February and March (Figure 4.1). A significant difference between I+ and I− plants was initially observed in OP in February (Table 6.1). A majority of the physiological traits were measured in March. I+ plants had statistically decreased RWC and $\Delta^{13}C$, increased proline concentration and a decrease trend in LWP compared to I− plants (Table 6.1 and Figure 6.1). A cultivar × irrigation treatment interaction was detected for RWC, OP and proline concentration in March (Table 6.1 and 6.2). RWC for all ryegrass cultivars was

significantly decreased by water deficit but not for Flecha tall fescue, and the extent of RWC reduction in Banquet II was numerically smaller than other ryegrass cultivars (Figure 6.2). I− plants of the URL, Trojan and Avalon had significantly more negative OP than their I+ counterparts (Figure 6.6). I− plants of the URL, Commando, Alto and Trojan significantly increased proline concentration compared to their I+ counterparts (Figure 6.10). After rehydration in April, the volumetric SWC in both I+ and I− plots was 39% (89% FC) (Figure 4.1). Rewatering restored the RWC, LWP, OP and proline concentration to a level similar to that of consistently irrigated plants (Table 6.1 and Figure 6.1).

6.3.2 Effects of endophyte

In general, cultivar × endophyte status and endophyte status × irrigation treatment interactions were uncommon and a main effect of endophyte was more commonly indicated in the statistical analyses. This main effect of endophyte was frequently detected in the RWC, OP, proline concentration and $\Delta^{13}C$ data (Table 6.1 and 6.2). Where this occurred, E+ plants had a lower RWC, more negative OP, lower $\Delta^{13}C$ and higher proline concentration than E− plant, and this pattern was seen in most harvests (Figure 6.1). Averaging across I+ and I− plants in all harvests, the RWC, OP, proline concentration and $\Delta^{13}C$ values for E− plants were, respectively, 90%, −14.9 bars, 3.0 mg/g DM, and 18.79‰. Corresponding values for E+ plants were 89%, −15.6 bars, 3.6 mg/g DM, 18.34‰, respectively (Figure 6.1).

A cultivar × endophyte status interaction was found for RWC, OP, CF, proline concentration, and $\Delta^{13}C$ in some harvests (Table 6.1 and 6.2). Among these detected cultivar × endophyte status interactions, significant differences between E+ and E− plants were most frequently detected for the URL and One50 (Figure 6.3, 6.7, 6.9, 6.11 and 6.13).

Endophyte status × irrigation treatment interactions were only observed for RWC in February ($P = 0.052$) and for OP in March ($P = 0.030$) (Table 6.1). More specifically, in February there was no difference in RWC between E+ and E− plants under I+ conditions, while E+ plants had lower RWC than E− plants under I− conditions. For OP in March, E+ plants had more negative OP than E− plants under I+ conditions, while less negative than E− plants under I− conditions (Figure 6.1).

6.3.3 Correlation between shoot DM and physiological traits

The shoot DM was positively correlated with RWC, LWP, OP, CF and $\Delta^{13}C$ and negatively correlated with proline concentration under drought conditions in February and March (Table 6.3).

Table 6.1 Results of GLM ANOVA analysis for relative water content (RWC), leaf water potential (LWP) and osmotic potential (OP).

	Sources	RWC		LWP		OP	
		F	P	F	P	F	P
Dec	I	3.90	0.187	0.28	0.647	0.72	0.484
	C	8.00	**<.0001**	3.44	**0.001**	3.82	**0.0003**
	C × I	0.72	0.677	0.92	0.497	0.91	0.512
	E	4.59	**0.033**	0.15	0.695	3.89	**0.049**
	E × I	0.02	0.876	0.71	0.399	0.33	0.568
	C × E	0.50	0.855	1.14	0.334	2.26	**0.023**
	C × E × I	0.85	0.563	0.58	0.796	0.60	0.778
	Error *df*	347		369		342	
Jan	I	5.70	0.140	–	–	–	–
	C	2.05	**0.040**	–	–	–	–
	C × I	1.08	0.378	–	–	–	–
	E	2.58	0.109	–	–	–	–
	E × I	0.93	0.336	–	–	–	–
	C × E	1.31	0.238	–	–	–	–
	C × E × I	0.44	0.896	–	–	–	–
	Error *df*	351		–		–	
Feb	I	7.02	0.118	5.25	0.148	41.68	**0.023**
	C	1.14	0.339	4.85	**<.0001**	7.67	**<0.0001**
	C × I	0.63	0.755	0.84	0.567	0.77	0.632
	E	3.14	**0.077**	0.00	0.975	13.18	**0.0003**
	E × I	3.82	**0.052**	1.19	0.277	0.24	0.6214
	C × E	0.93	0.493	0.96	0.466	1.18	0.309
	C × E × I	0.69	0.699	0.75	0.648	0.99	0.441
	Error *df*	295		352		343	
Mar	I	20.69	**0.045**	9.82	0.088	5.84	0.137
	C	1.90	0.060	1.67	0.104	3.58	**0.001**
	C × I	2.12	**0.034**	0.92	0.501	2.21	**0.026**
	E	6.30	**0.013**	0.08	0.779	0.47	0.495
	E × I	0.24	0.625	0.89	0.346	4.73	**0.030**
	C × E	1.10	0.363	0.90	0.520	0.98	0.454
	C × E × I	1.11	0.358	1.42	0.186	1.37	0.208
	Error *df*	298		325		318	
Apr	I	0.59	0.522	1.18	0.390	3.82	0.190
	C	1.52	0.147	2.65	**0.008**	1.78	0.081
	C × I	0.65	0.736	0.29	0.969	2.46	**0.013**
	E	1.25	0.264	1.00	0.319	0.24	0.625
	E × I	3.06	0.081	0.00	0.948	0.63	0.427
	C × E	1.99	**0.047**	0.62	0.761	1.20	0.296
	C × E × I	1.03	0.414	1.38	0.206	1.07	0.381
	Error *df*	331		344		341	

Note: I, irrigation treatment; C, cultivar; E, endophyte status.

Table 6.2 Results of GLM ANOVA analysis for chlorophyll fluorescence (CF), proline concentration (Proline) and carbon isotope discrimination ($\Delta^{13}C$).

	Sources	CF		Proline		$\Delta^{13}C$	
		F	P	F	P	F	P
Dec	I	1.33	0.367	0.01	0.936	1.37	0.363
	C	0.90	0.517	12.03	**<.0001**	40.98	**<.0001**
	C × I	1.24	0.273	1.84	0.068	1.03	0.409
	E	0.36	0.548	12.70	**0.0001**	32.06	**<.0001**
	E × I	0.10	0.747	0.45	0.501	0.02	0.876
	C × E	0.60	0.780	1.99	**0.047**	2.21	**0.026**
	C × E × I	0.72	0.676	0.58	0.796	0.47	0.875
	Error *df*	343		359		358	
Jan	I	0.02	0.902	–	–	–	–
	C	2.99	**0.003**	–	–	–	–
	C × I	0.82	0.583	–	–	–	–
	E	0.23	0.630	–	–	–	–
	E × I	2.30	0.131	–	–	–	–
	C × E	1.99	**0.047**	–	–	–	–
	C × E × I	1.09	0.370	–	–	–	–
	Error *df*	360		–		–	
Feb	I	9.32	0.092	–	–	–	–
	C	0.92	0.499	–	–	–	–
	C × I	0.80	0.599	–	–	–	–
	E	3.13	0.078	–	–	–	–
	E × I	2.03	0.156	–	–	–	–
	C × E	1.53	0.145	–	–	–	–
	C × E × I	0.82	0.582	–	–	–	–
	Error *df*	346		–		–	
Mar	I	–	–	18.06	**0.051**	37.06	**0.026**
	C	–	–	5.07	**<.0001**	35.52	**<.0001**
	C × I	–	–	1.90	0.060	1.30	0.241
	E	–	–	5.92	**0.016**	10.32	**0.001**
	E × I	–	–	2.97	0.086	2.38	0.124
	C × E	–	–	1.08	0.379	0.50	0.859
	C × E × I	–	–	0.48	0.871	0.61	0.768
	Error *df*	–		304		321	
Apr	I	–	–	2.87	0.231	–	–
	C	–	–	5.48	**<.0001**	–	–
	C × I	–	–	1.15	0.332	–	–
	E	–	–	6.75	**0.010**	–	–
	E × I	–	–	0.40	0.529	–	–
	C × E	–	–	0.78	0.620	–	–
	C × E × I	–	–	1.84	0.069	–	–
	Error *df*	–		321		–	

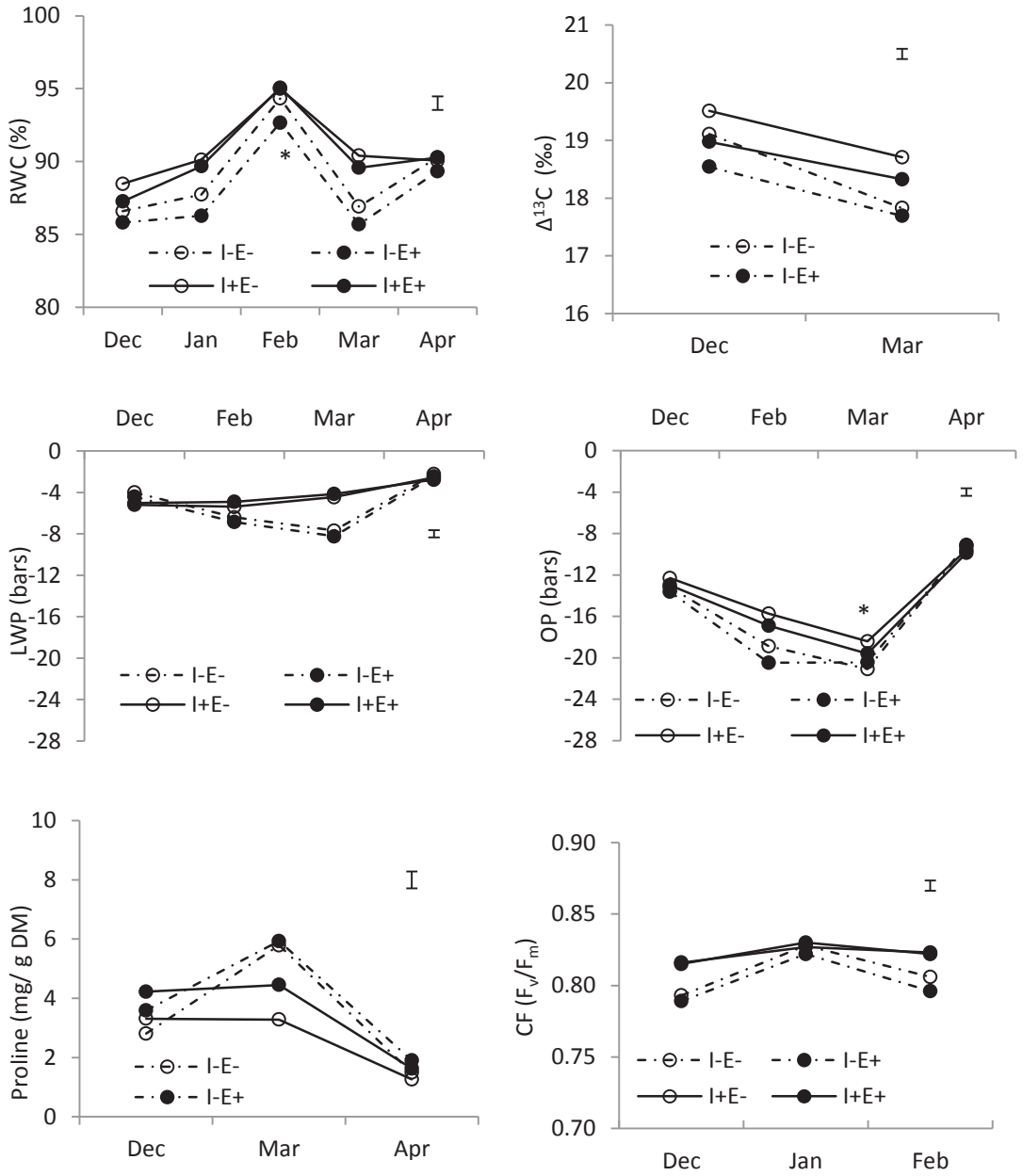

Figure 6.1 Relative water content (RWC), leaf water potential (LWP), osmotic potential (OP), carbon isotope discrimination ($\Delta^{13}C$), free proline concentration (Proline) and chlorophyll florescence (CF) of endophyte-infected (E+) and endophyte-free (E–) plants under irrigated (I+) and non-irrigated (I–) conditions from December to April. Vertical bar refers to mean standard error of all the means. An asterisk denotes significant difference of RWC between E+ plants and E– plants under I– condition and significant difference of OP between E+ plants and E– plants under I+ condition at $P < 0.05$.

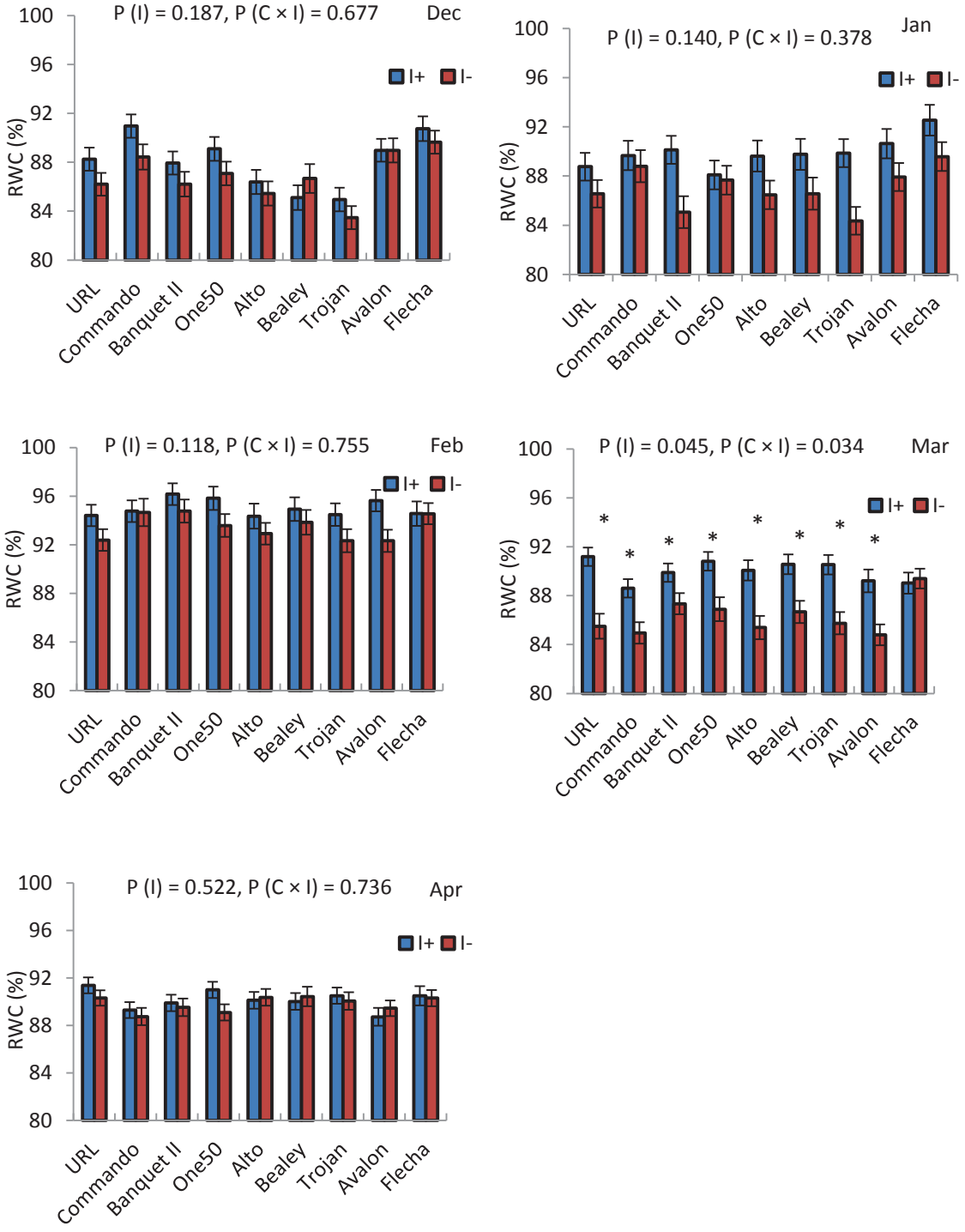

Figure 6.2 Relative water content (RWC) of irrigated (I+) and non-irrigated (I–) plants of each cultivar from December to April. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

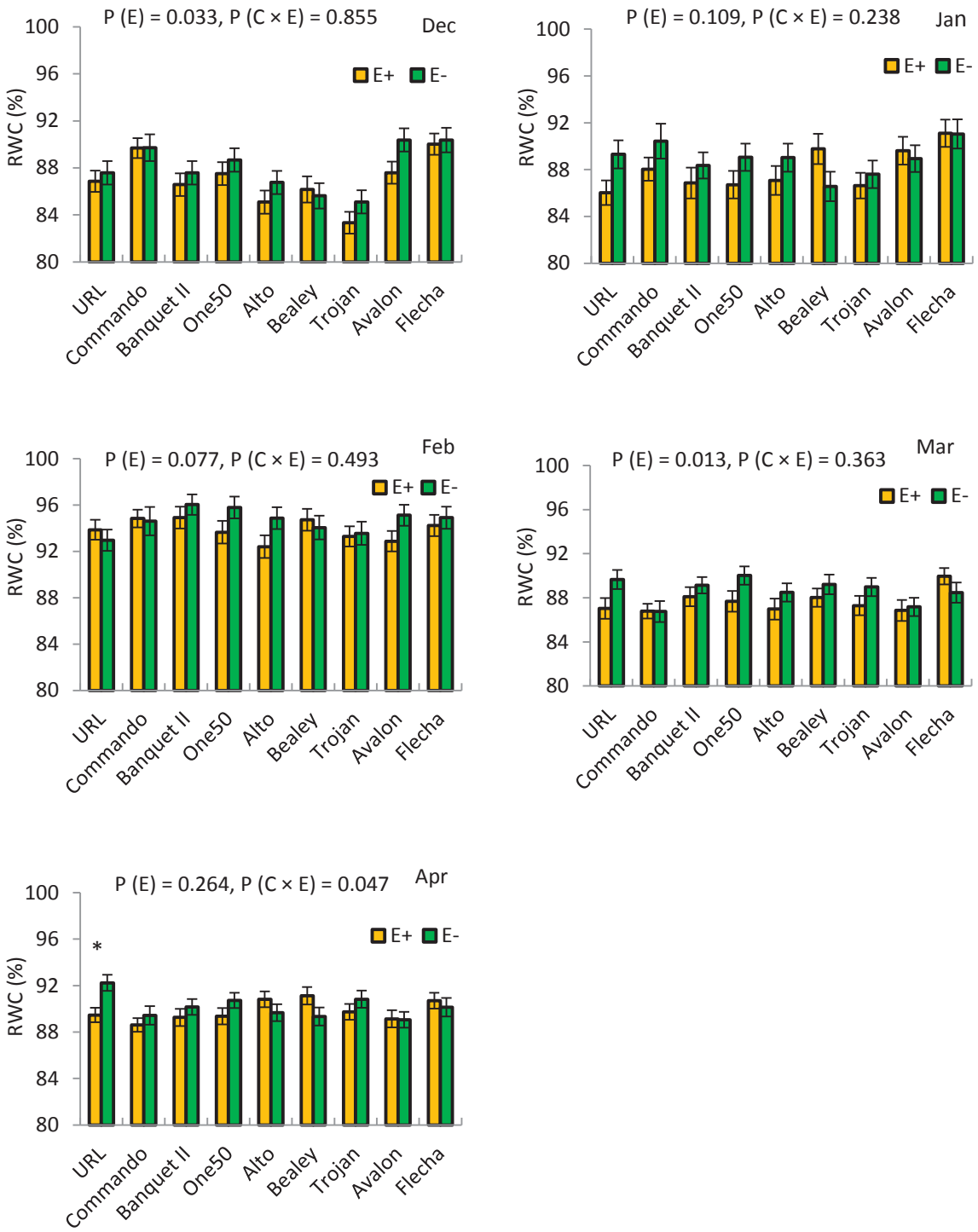

Figure 6.3 Relative water content (RWC) of endophyte-infected (E+) and endophyte-free (E−) plants of each cultivar from December to April. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

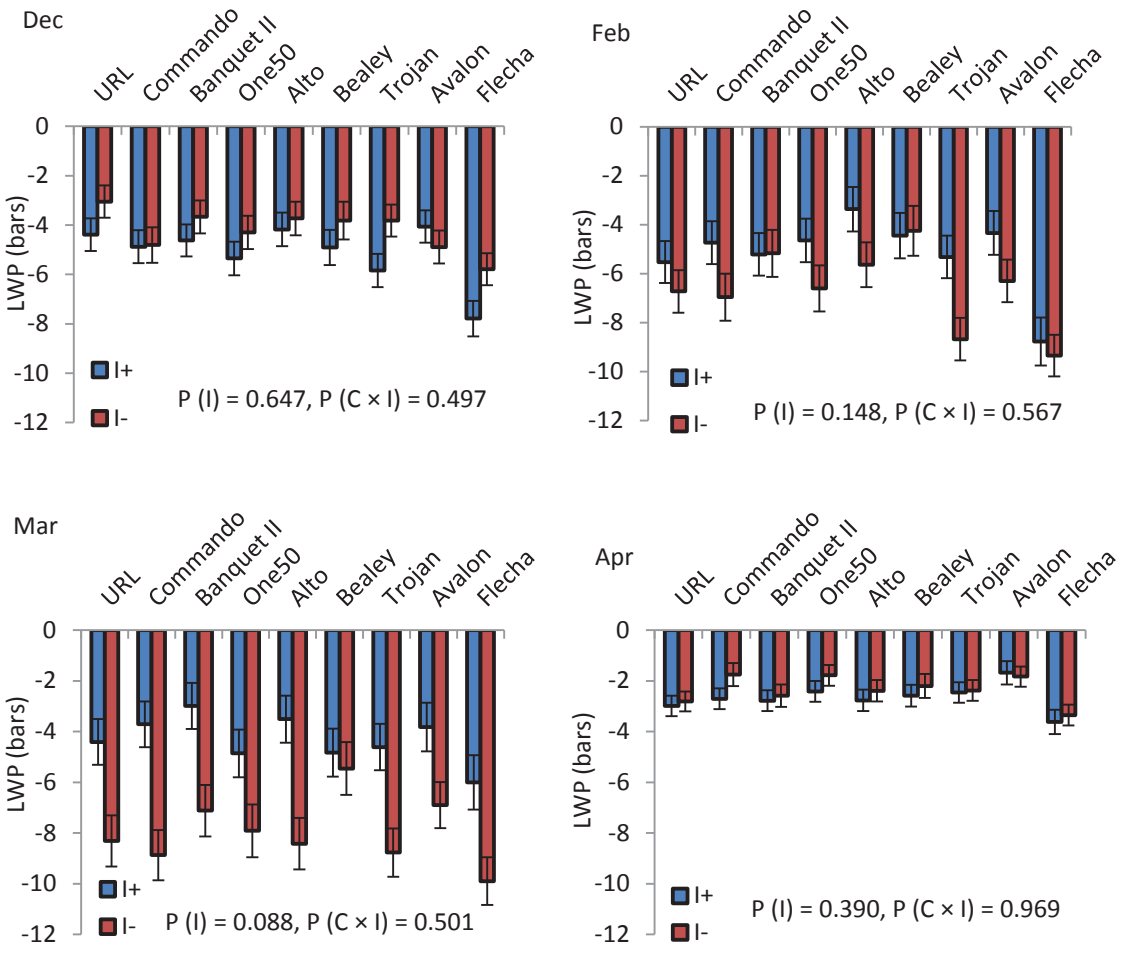

Figure 6.4 Leaf water potential (LWP) of irrigated (I+) and non-irrigated (I–) plants of each cultivar from December to April. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

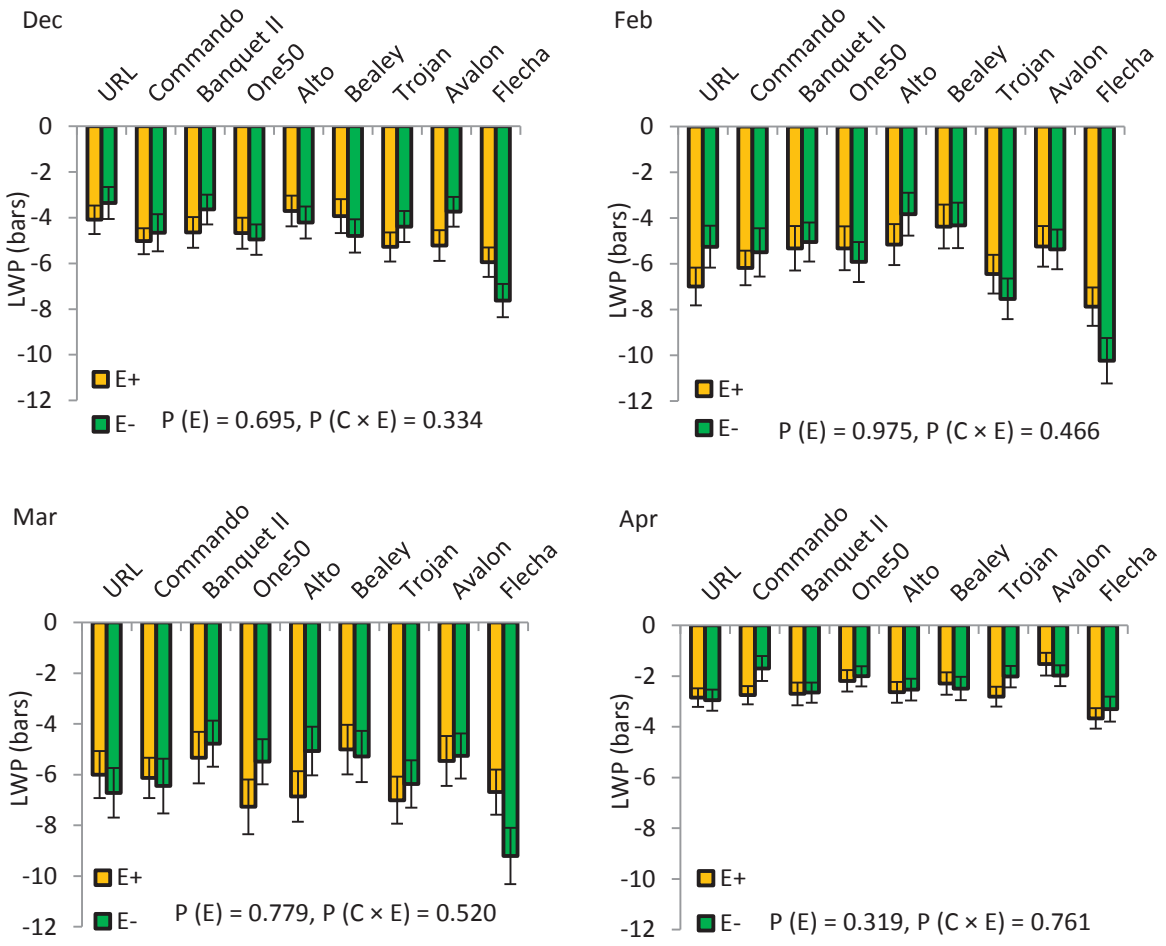

Figure 6.5 Leaf water potential (LWP) of endophyte-infected (E+) and endophyte-free (E–) plants of each cultivar from December to April. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

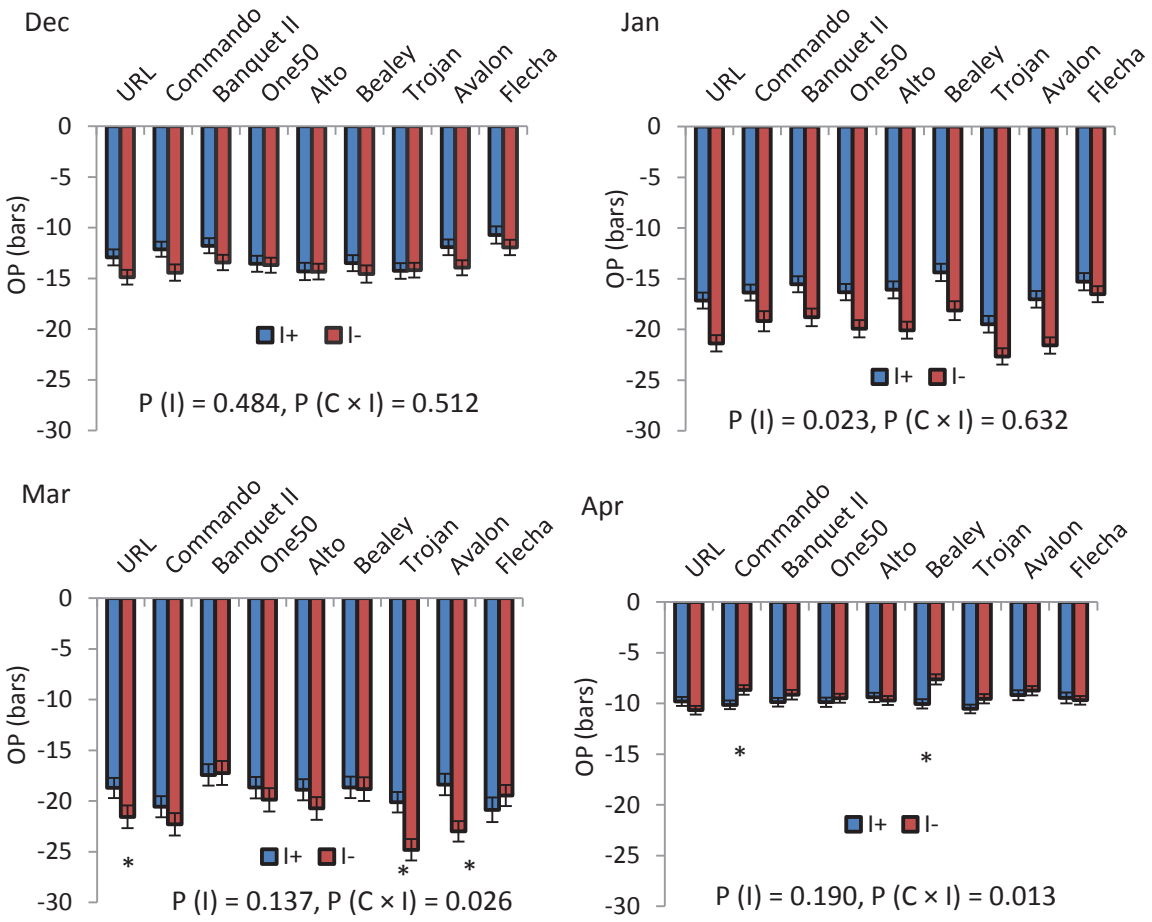

Figure 6.6 Osmotic potential (OP) of irrigated (I+) and non-irrigated (I–) plants of each cultivar from December to April. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

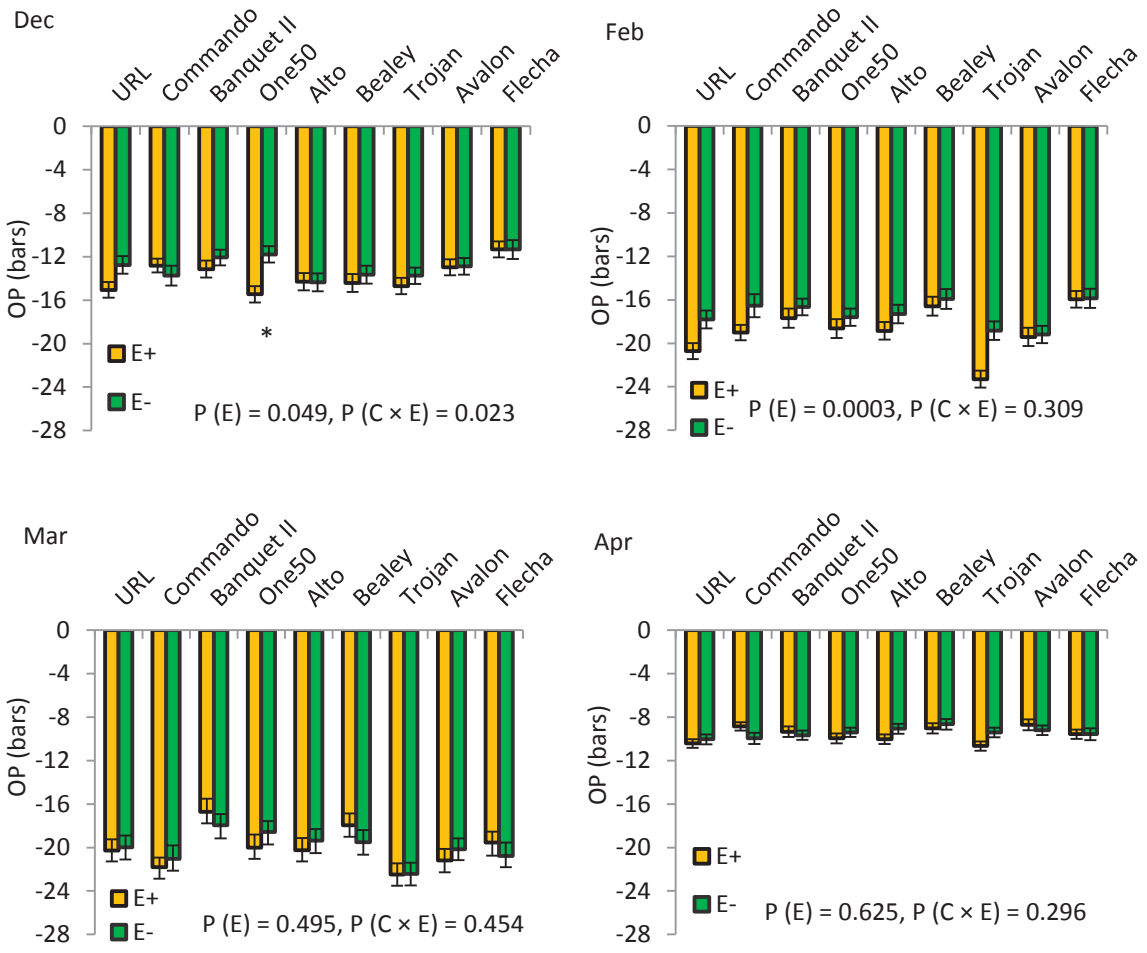

Figure 6.7 Osmotic potential (OP) of endophyte-infected (E+) and endophyte-free (E–) plants of each cultivar from December to April. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

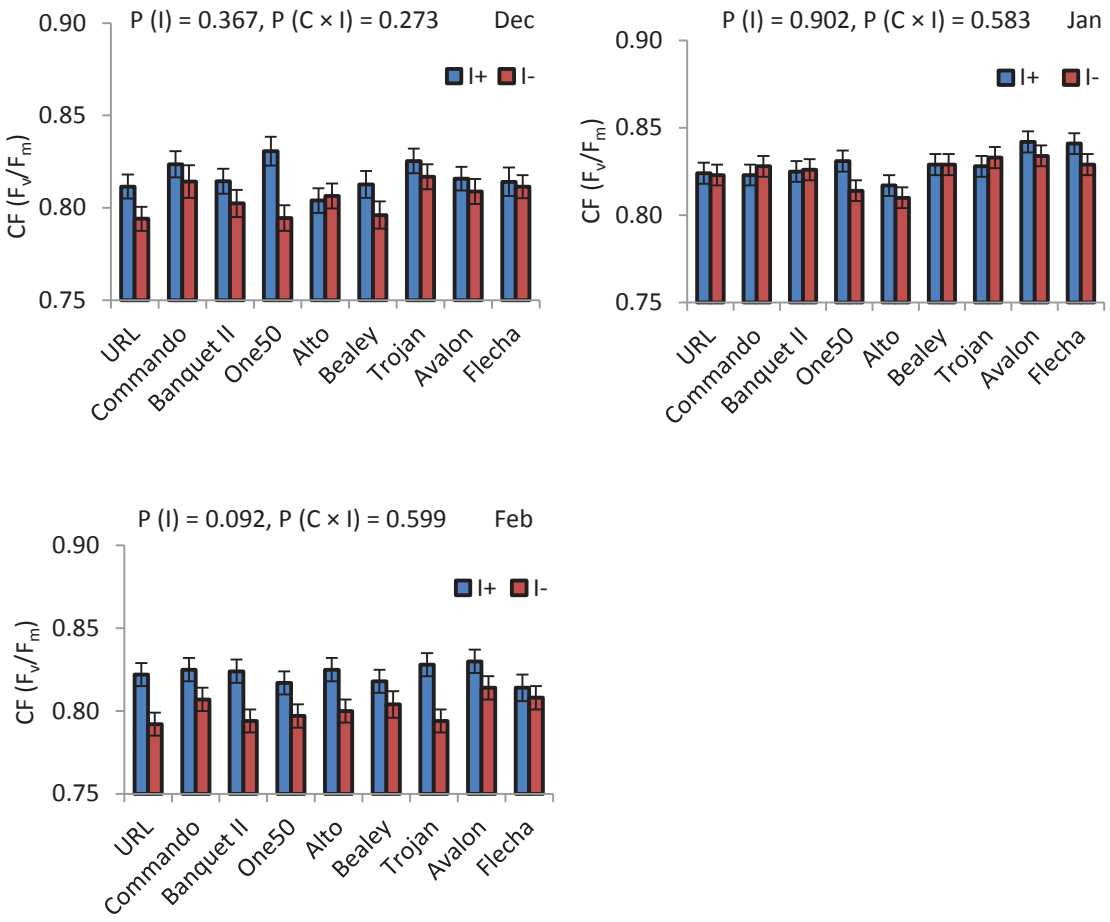

Figure 6.8 Chlorophyll fluorescence (CF) of irrigated (I+) and non-irrigated (I–) plants of each cultivar from December to February. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

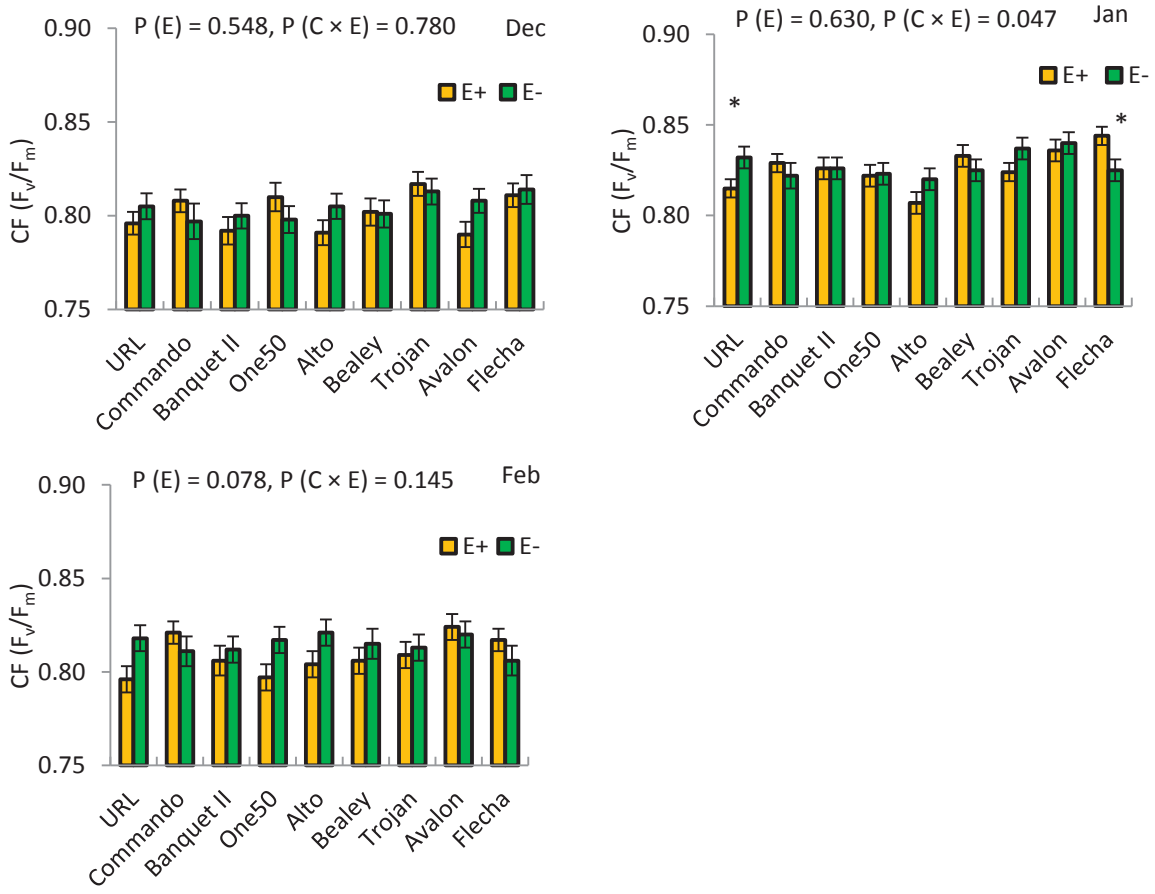

Figure 6.9 Chlorophyll fluorescence (CF) of endophyte-infected (E+) and endophyte-free (E–) plants of each cultivar from December to February. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

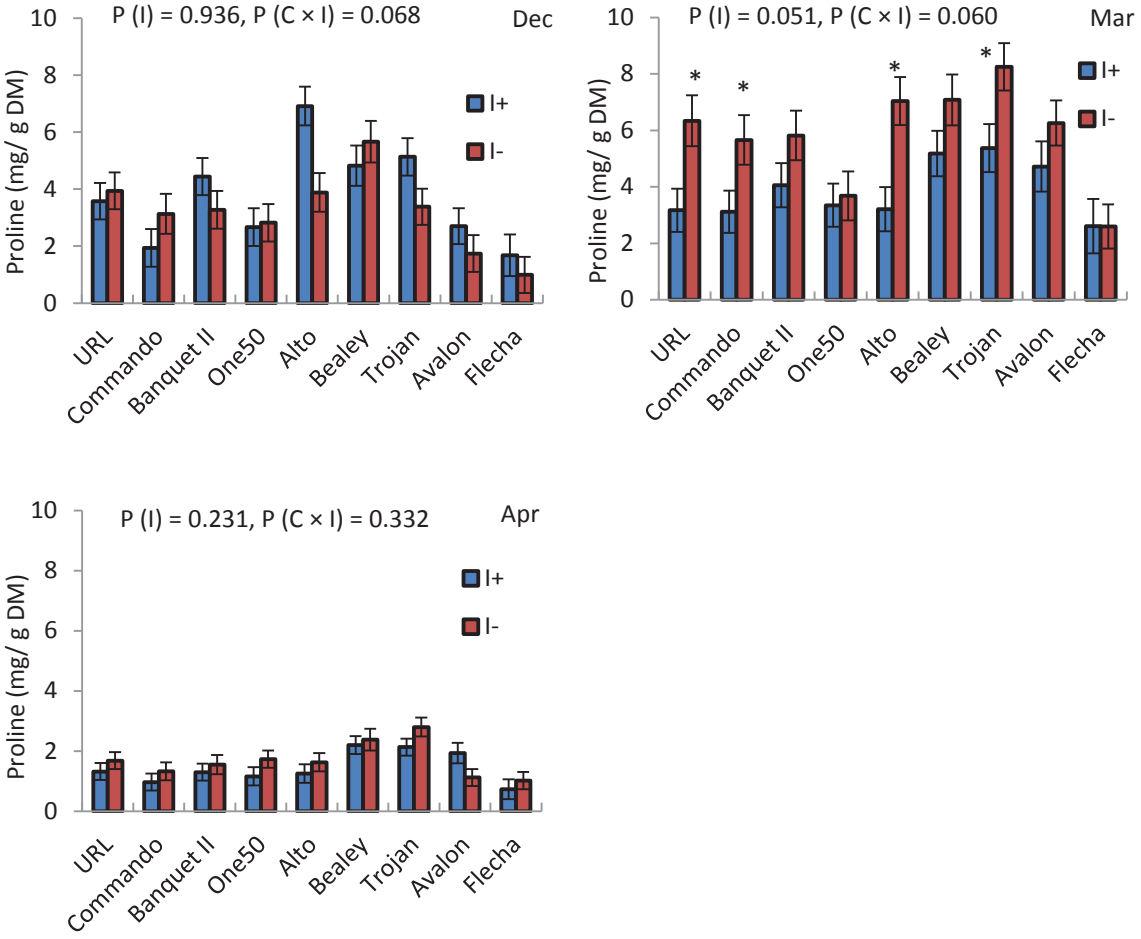

Figure 6.10 Proline concentration of irrigated (I+) and non-irrigated (I–) plants of each cultivar in December, March and April. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

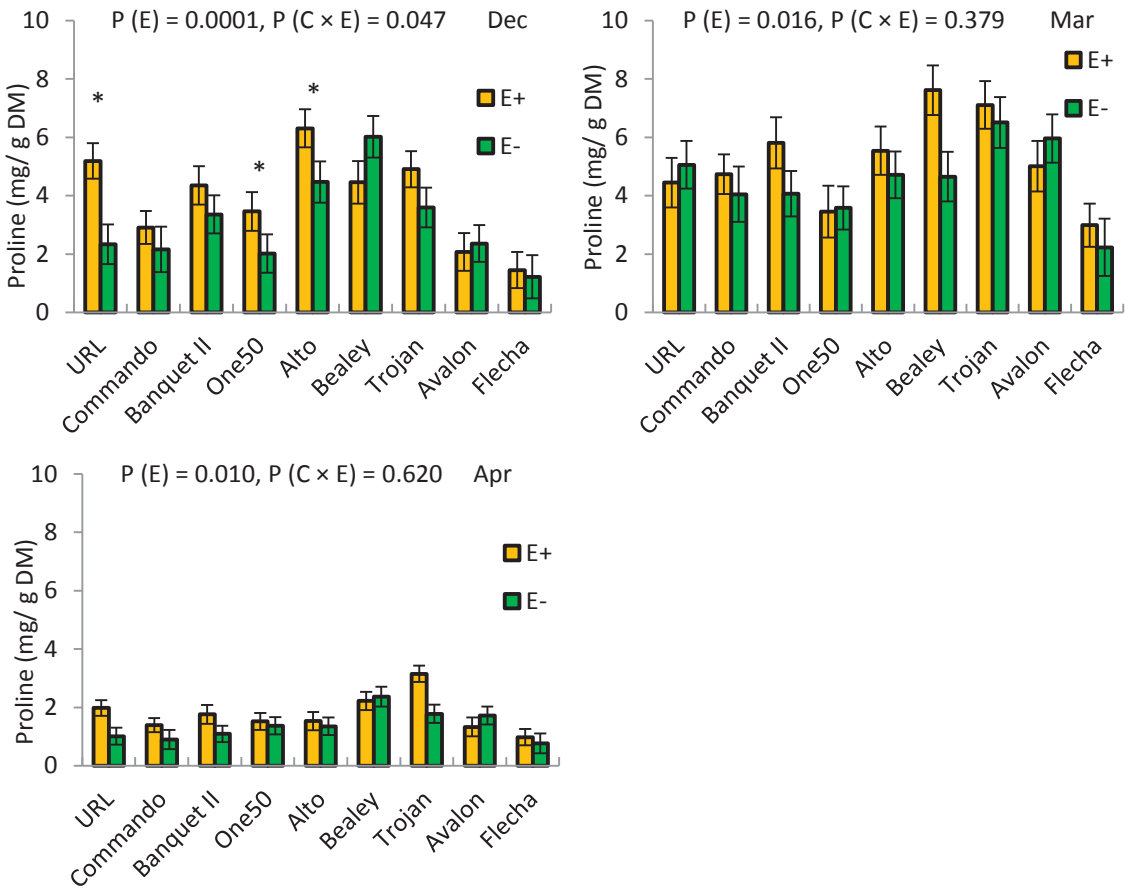

Figure 6.11 Proline concentration of endophyte infected (E+) and endophyte-free (E–) plants of each cultivar in December, March and April. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

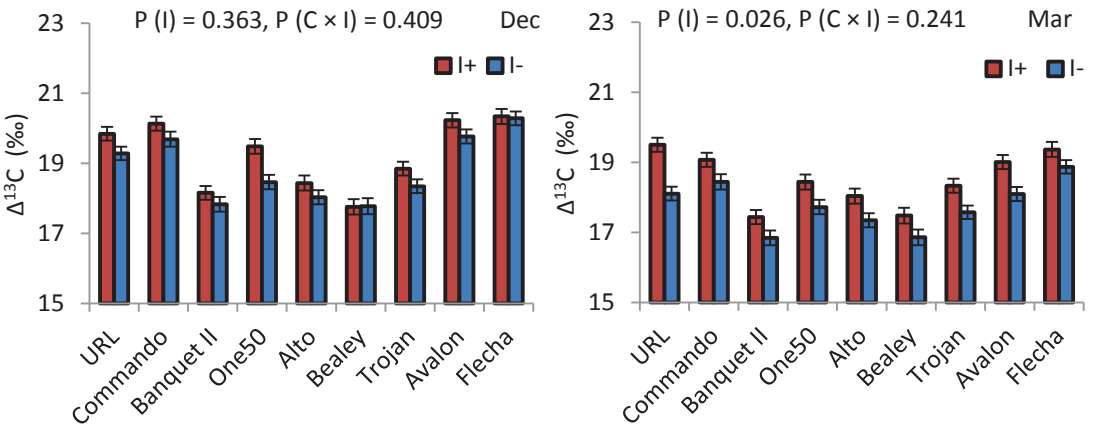

Figure 6.12 Carbon isotope discrimination ($\Delta^{13}C$) of irrigated (I+) and non-irrigated (I–) plants of each cultivar in December and March. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

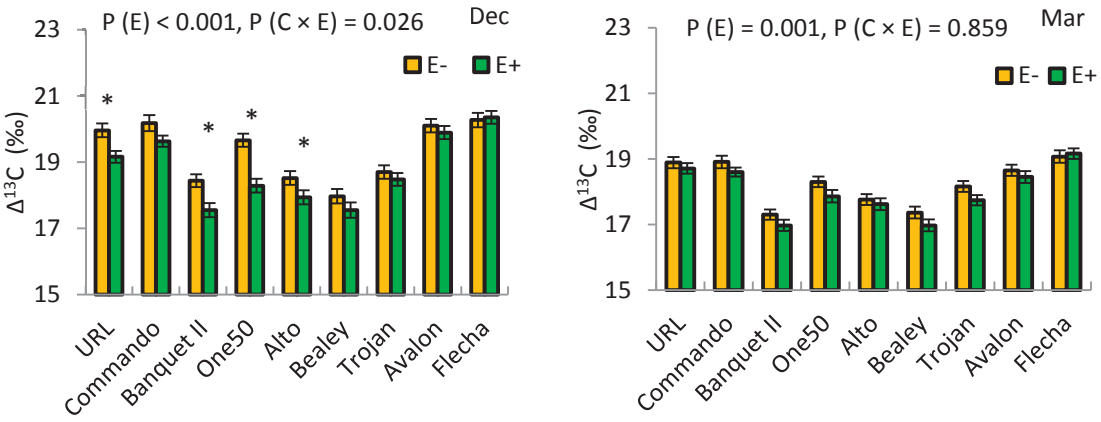

Figure 6.13 Carbon isotope discrimination ($\Delta^{13}C$) of endophyte-infected (E+) and endophyte-free (E–) plants of each cultivar in December and March.Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

Table 6.3 Correlations between shoot dry matter (shoot DM) and physiological traits including relative water content (RWC), leaf water potential (LWP), osmotic potential (OP) and chlorophyll florescence (CF), proline concentration (Proline) and carbon isotope composition ($\Delta^{13}C$) of non-irrigated plants in February and March.

February		RWC	LWP	OP	CF	
Shoot DM	r	0.3631	0.2441	0.1439	0.2514	
	P	**<0.0001**	**0.0006**	**0.0574**	**0.0005**	
	N	166	192	175	188	
March		RWC	LWP	OP	Proline	$\Delta^{13}C$
Shoot DM	r	0.4615	0.1662	0.2049	−0.2610	0.3691
	P	**<0.0001**	**0.0303**	**0.0075**	**0.0009**	**<0.0001**
	N	149	170	169	160	175

6.4 Discussion

6.4.1 Drought treatment

It has been claimed that drought treatments imposed in experimental systems should be similar to the stresses that occur naturally (da Silveira Pinheiro, 2003). Also, in an agricultural system, defoliation of the vegetative organs of the grasses by livestock is unavoidable. Thus, in order to have a simulated drought environment, irrigation was withheld from December to March, which is a typical time and duration for drought occurrence in New Zealand. Also, artificial removal of herbage was carried every month to simulate animal grazing. Removing the vegetative organs that generate transpiration would potentially slow down the water consumption rate.

Drought stress to plants in temperate environments usually results from a combination of soil water deficit and high air temperature. In this experiment the monthly average daily maximum air temperature during the experimental period was not over 30°C, meaning that the plant growth should not have been limited by the high temperature. However, the high temperature and low humidity from January to March would have increased the evapotranspiration, which resulted in decreased SWC even in I+ plots from January to March compared to that in December and April (Figure 4.1).

6.4.2 Plant physiological responses to drought

Before withholding irrigation in I− plots in December, plants in I+ and I− plots were under the same water regime and thus showed similar plant physiological conditions. From January to March, for plants in the I+ plots, there were two water sources, irrigation and soil storage water, while for plants in the I− plots, storage water was the only water source. During this period of time, the volumetric SWC in the I+ plots in January, February and March was maintained at about 60% FC while the SWC in the I− plots was dropped from full FC in December to 48% FC in January, 37% FC in February and 31% FC in March, suggesting that I+ plants were in a mild state of stress but receiving sufficient water from irrigation and did not further access soil storage water from January to March; while I− plants had to gradually consume the storage water.

The significant shoot DM reduction due to water deficit occurred in February (Figure 4.2), while the significant effect of water deficit on the leaf RWC occurred in March, suggesting that plant growth slowed down earlier than leaf dehydration. Evidence has been accumulating that shows stomatal closure is the initial response to drought for most plant species and stomata close progressively as drought progresses (Chaves, 1991; Yan et al., 2016). Since stomatal closure reduces the transpiration water loss, it is also commonly considered as a drought adaptation trait (Blum, 1996; Turner, 1986). In this experiment, the stomatal conductance was not directly measured due to the logistical difficulty of using a photosynthesis measurement system in the field with a large number of plants, but the $\Delta^{13}C$ data is able to provide some indication of changes in stomatal conductance. The $\Delta^{13}C$ of C3 plants is expressed as:

$$\Delta^{13}C = a + (b - a)\,(C_i/C_a), \qquad \text{Equation 6.1}$$

where a is the fraction occurring due to diffusion in air; b is the net fraction caused by carboxylation mainly the discrimination by RuBisCO; C_i/C_a is the ratio of

intercellular and atmospheric CO_2 concentration; *a* and *b* has a theoretical value of 4.4‰ and 27‰, respectively (Farquhar et al., 1982). Thus stomatal closure causes a decrease of C_i/C_a in response to drought will result in a decrease of $\Delta^{13}C$. Here, among all the physiological traits measured in March, $\Delta^{13}C$ was the only trait influenced by irrigation treatment independently of cultivar (Table 6.2). The I– plants displayed significantly lower $\Delta^{13}C$ compared to I+ plants (Figure 6.12), suggesting that stomatal closure is a common strategy of dehydration postponement. A significant correlation between shoot DM and $\Delta^{13}C$ ($r = 0.3691$, $P < 0.0001$) (Table 6.3) was detected in March, suggesting that decreased C_i/C_a largely explained the herbage yield reduction under drought.

Stomatal conductance is also a bridge that connects $\Delta^{13}C$ and water use efficiency (WUE). The WUE is expressed as:

$$WUE = P_n/G_w = G_c\,(C_a - C_i)/G_w = C_a\,(1 - C_i/C_a)/1.6, \qquad \text{Equation 6.2}$$

where P_n, G_c, G_w are the net photosynthesis rate, and stomatal conductance to CO_2 and water vapour, respectively, the factor 1.6 refers to the relative diffusivities of CO_2 and water vapour in air (Condon et al., 2002; Polley et al., 1993). Stomatal conductance decreases in response to drought stress, which will result in a decrease of C_i/C_a, and thus causes a decrease in $\Delta^{13}C$ and an increase in WUE. This explains why a negative correlation between $\Delta^{13}C$ and WUE is frequently observed in many plant species (Ebdon et al., 1998; Johnson & Bassett, 1991). Since the realised value of $\Delta^{13}C$ is highly heritable, $\Delta^{13}C$ is also recommended as a tool to screen for improved WUE in plant breeding programs (Condon et al., 2002; Farquhar & Richards, 1984). Here, a decreased $\Delta^{13}C$ of I– plants also indicated that I– plants had an increased WUE compared to I+ plants. However, under severe drought stress, if the photosynthesis is not only limited by the stomatal conductance but also limited by non-stomatal factors such as impairment in RuBP regeneration capacity, ATP synthesis and RuBisCO activity (Flexas & Medrano, 2002; Signarbieux & Feller,

2011), the WUE might be decreased, rather than enhanced. For example, such effects have been shown in wheat (Boutraa et al., 2010) and cowpea (Hayatu & Mukhtar, 2010), although the WUEs in these studies were expressed in agronomic term, the agronomic WUE should be consistent with the physiological WUE described here.

CF (F_v/F_m), the photochemical efficiency of PS II, has been commonly measured to monitor stresses. In this experiment, the CF was not significantly affected by water deficit in February (Table 6.2). However, a positive correlation between the shoot DM and CF in February was detected ($r = 0.2514$, $P = 0.0005$) (Table 6.3), suggesting that the slightly decreased CF caused by water deficit in February was also a factor contributing to the herbage yield reduction.

RWC is often measured to estimate the dehydration level of plant tissues. In this experiment, as the drought progressed from January to March, the leaf RWC was only significantly reduced by water deficit in March (Table 6.1 and Figure 6.1). However, the dehydration level was not severe as indicated by an average RWC value of 86% across all cultivars under drought (Figure 6.1). Kaiser (1987) considered the leaf RWC above 70% as mild to moderate drought stress, between 30% and 70% as severe drought stress and below 30% as extreme severe drought stress as a general standard. Even though the plant leaf dehydration was mild to moderate in this experiment, according to Kaiser (1987)'s classification, the herbage yield was very sensitive to dehydration, as indicated by significant correlation between shoot DM and RWC in February ($r = 0.3631$, $P < 0.0001$) and March ($r = 0.4615$, $P < 0.0001$) (Table 6.3).

LWP, is the plant water energy status. Comparing to RWC, LWP was less sensitive to drought. As drought developed from January to March, plants were able to maintain a comparatively high (less negative) LWP, with a value of −6 bars to −8 bars of I− plants in February and March, respectively (Figure 6.1). More negative OP can be induced by two components, reduced plant water content and solute

accumulation, the latter is called osmotic adjustment (OA). It has been commonly agreed that OA plays a role in maintaining cell turgidity (Levitt, 1980; Turner, 1986). At the end of the second month of withholding irrigation (in February), I− plants were not significantly dehydrated as indicated by similar RWC to I+ plants (Figure 6.1), thus the decrease in OP of I− plants from January to February was attributed to the accumulated solutes. At the end of the third month of withholding irrigation (in March), the OP of I− plants would be expected to be much more negative than that of I+ plants as I− plants were not only dehydrated but expected to accumulate more solutes. However, the OP for I+ and I− plants was very similar (Table 6.1 and Figure 6.1), which was mainly because the OP values of I+ plants also became more negative in March compared to those values in December and February. The fall in OP of I+ plants between February and March measurements could be because I+ plants had become drought stressed in that time. However, the patterns of SWC, RWC and LWP in I+ plants from December to March do not support this. Therefore this observation could mean that ryegrass plants tend to naturally develop a more negative OP in mid-summer in response high temperatures or some other related climatic stimulus. It has been reported that OA is not only an adaptation strategy under drought conditions but also under extreme temperatures (Siddiqui et al., 2015).

Numerous publications have reported proline accumulation in responses to environmental stresses such as extreme temperatures, salinity, heavy metals and drought, as reviewed by (Hayat et al., 2012). It has been understood that the transcriptional up-regulation of proline synthesis from glutamate and down-regulation of proline catabolism both contribute to proline accumulation under stress (Verslues & Sharma, 2010). Proline concentration is very sensitive to drought stress and the more severe the drought the higher the proline concentration (Hahn et al., 2008; Lum et al., 2014; Quan et al., 2016). However, proline not only acts as osmolyte, but more importantly, as an ROS scavenger, as a molecular chaperone in stabilising the structure of proteins, has a role in buffering cytosolic pH and to

balance the cell redox status, and as a stress signal (Hayat et al., 2012; Verbruggen & Hermans, 2008). In March, all ryegrass cultivars dehydrated as indicated by RWC (Figure 6.2), while the URL, Commando, Alto and Trojan exhibited significantly increased proline concentration (Figure 6.10), suggesting that these four cultivars developed a better protection system than other dehydrated cultivars. One 50 and Flecha showed no separation in proline concentration between I+ and I− plants and other cultivars showed trends that might have been biologically real though non-significant (Figure 6.10).

6.4.3 Mediterranean tall fescue behaved differently from ryegrass cultivars in summer drought

Tall fescue is taxonomically and genetically closely related to perennial ryegrass, but tall fescue is usually considered more drought tolerant than perennial ryegrass (Turner et al., 2012). In New Zealand, tall fescue has been recommended as an alternative where summer drought limits the growth of ryegrass. Tall fescue also has a different *Epichloë* endophyte species from perennial ryegrass. Thus, in this experiment, it was of interest to know the physiological differences between tall fescue and ryegrass, with their respective commercial endophytes, in response to water deficit. A 'European' rather than a 'Mediterranean' tall fescue cultivar which has summer dormancy would have been included in this experiment but at the time of endophyte elimination from the E+ parent plants at the state of the experiment, no suitable E+ plants of a European tall fescue were available.

The most noticeable differences were found in data for OP and CF in February and, RWC and proline concentration in March between ryegrass cultivars and Flecha tall fescue. A majority of ryegrass cultivars (or sometimes all ryegrass cultivars) showed more negative OP, decreased CF, decreased RWC and increased proline concentration in response to water deficit, while Flecha tall fescue plants maintained similar physiological status to their I+ counterparts (Figure 6.2, 6.6, 6.8 and 6.10). There are two possible ways for a plant to remain hydrated as Flecha did: increased

water uptake; or, reduced water demand. Flecha is a partially summer dormant cultivar (Norton et al., 2006), which had slower growth rate compared to ryegrass. It thus would have consumed the stored soil water more slowly than the ryegrass cultivars.

6.4.4 Differences between E+ and E− plants

Endophyte is a heterotrophic organism which is completely dependent on the host grass for nutrients. The total amount of endophyte DNA represents only between 0.5% and 2% of the association (Young et al., 2005), however, endophyte infection causes dramatic changes in the expression of over one third of the host genes and triggers reprogramming of the host metabolism (Dupont et al., 2015) and contributes substantially to the metabolism of the association (Rasmussen et al., 2009). A general down-regulation of primary metabolism (e.g. genes involved in transcription and nucleotide metabolism) due to endophyte presence has been reported for perennial ryegrass (Dupont et al., 2015) and red fescue (Ambrose & Belanger, 2012). Here, it was found that endophyte presence decreased leaf RWC, OP, and stomatal conductance and increased proline concentration of the host.

Endophyte metabolism requires water and nutrients from the host. Here, it was shown that endophyte presence decreased the leaf RWC of the host (Table 6.1 and Figure 6.1), but only about 1%. It is possible water lost from plant tissue was consumed by endophyte metabolism. The more negative OP of E+ plants compared to E− plants could be due to lower RWC but also very likely due to greater solute accumulation. Dupont et al. (2015) showed that E+ plants had more accumulated solutes than E− plants including arabitol, threitol and mannitol; Hunt et al. (2005) showed that E+ plants of perennial ryegrass (cultivar Samson) had higher concentrations of both high-molecular-weight and low-molecular-weight carbohydrates than E− plants. Proline is also often considered to be one of the osmotic compounds (Verbruggen & Hermans, 2008), however the proline concentration was only increased 0.6 mg/ g DM due to endophyte infection in this

experiment (Table 6.2 and Figure 6.1), which would have contributed only −0.02 bars to the OP (this calculation was based on the Morse equation, OP = iCRT, where i is the ratio of the number of particles in the solution to the number of molecule dissolved, R is the ideal gas constant (8.32 J mol^{-1} K^{-1}), T is the absolute temperature in degrees Kelvin). Compared to the −0.7 bars of OP linked to endophyte presence, the contribution of proline was negligible. The endophyte effect on OP was found in December and February but not in March and April, suggesting that endophyte presence probably influences the solute accumulation in a seasonal pattern, possibly triggered by some environmental factor such as ambient temperature.

Endophyte produces endogenous reactive oxygen species (ROS) that play an important role in regulating the growth of endophyte hyphae (Scott et al., 2007; Tanaka et al., 2006). It is well-known that ROS can cause oxidative damage of organic molecules such as proteins, lipids, carbohydrates and DNA (Demidchik, 2012; Hamilton & Bauerle, 2012). Also, ROS are key players in plant stress signalling (Baxter et al., 2014). Proline is an amino acid related to alkaloid metabolism. It is a product of ergot alkaloids breakdown (Stoll & Hoffmann, 1965) and peramine synthesis (Siegel et al., 1990). In this experiment, several endophytes (AR1, AR37, Endo5, NEA2 and AR542) were included with their respective host cultivars and each endophyte has different alkaloid profiles, but the proline concentration was generally higher in E+ plants than E− plants, suggesting that endophyte effect on proline concentration must be related to some other mechanism. Fabro et al. (2004) and Ben Rejeb et al. (2014) demonstrated that ROS signalling induces proline accumulation under biotic and abiotic stress. Thus, it is possible that the endogenous endophyte ROS induced proline accumulation of the host, if there was transfer of these ROS into the host symplast. Proline accumulation linked to endophyte presence has previously been reported in perennial ryegrass by Ma et al. (2015) and also in drunken horse grass (*Achnatherum inebrians*) (Liu et al., 2015).

There was also a possible endophyte-linked reduction in CF (F_v/F_m) in February (P = 0.078) (Table 6.2 and Figure 6.1), which could be biologically real. The CF measures the maximum quantum efficiency of PS II, which depends on the activity of photosynthetic reaction centres (a complex of several proteins, pigments and other co-factors, Section 2.2.3.4). The endophyte produced ROS could have slightly damaged the activity of photosynthetic reaction centres, thus resulted in a decreased CF of E+ plants compared to E− plants. However, reduced CF in E+ plants might equally have arisen indirectly from one of the other physiological changes associated with endophyte presence.

Endophyte presence modified the stomatal conductance of the host as indicated by decreased $\Delta^{13}C$ values of E+ plants compared to E− plants (Table 6.2 and Figure 6.1). Dupont et al. (2015) demonstrated that the expression of genes encoding key enzymes involved in biosynthesis and signalling by the hormone abscisic acid (ABA) was up-regulated in E+ perennial ryegrass, which suggested that ABA levels may be elevated in E+ plants. It is well-known that ABA induces stomatal closure (Li et al., 2000). By directly measuring the stomatal conductance, Dupont et al. (2015) also confirmed that E+ plants had reduced stomatal conductance compared to E− plants.

In some harvests, effects of endophyte on these physiological traits were more pronounced for the URL and One50, and the shoot DM of the URL (infected with AR37) and One50 (infected with AR1) was also significantly reduced by endophyte presence in every harvest (Chapter 4), suggesting that these physiological traits likely are related to the endophyte-linked herbage yield reduction in the URL and One50.

Since no major irrigation treatment × endophyte status interaction was detected for physiological responses in this study, it is concluded that E+ and E− plants had similar tolerance to drought stress. However, if comparing E+ and E− plants merely under drought conditions (in absence of the insect pressure), it would appear that E+ plants are slightly more stressed than E− plants as indicated by greater dehydration,

lower CF (although not statistically significant) (Figure 6.1) and significant shoot DM reduction (Figure 4.3).

6.5 Conclusions

This experiment is possibly unique for any forage grass in terms of the wide range of plant physiological traits measured on clonally replicated plants of a variety of cultivars infected with and without their commercially associated endophyte strain, before, during and after drought. Plants respond to summer drought with a series of physiological changes including stomatal closure, leaf dehydration, osmotic adjustment and proline accumulation. Stomatal closure is a common dehydration postponement strategy in all cultivars. However, significant proline accumulation was only developed in the URL, Commando, Alto and Trojan while all ryegrass cultivars were significantly dehydrated. Flecha tall fescue was more drought tolerant than ryegrass cultivars, in the sense that RWC and CF of Flecha were not significantly influenced by water deficit, but the better physiological status of Flecha plants could be attributed to their summer dormancy rather than their having developed OA or proline accumulation. Endophyte presence decreased the RWC, OP, stomatal conductance and increased proline concentration under both I+ and I− conditions and effects of endophyte on these physiological traits were more pronounced in URL and One50, suggesting that these physiological traits likely are related to the endophyte-linked herbage yield reduction in the URL and One50.

Chapter 7 Nitrogen uptake of ryegrass and Mediterranean tall fescue plants with and without *Epichloë* endophyte under two water regimes

7.1 Abstract

The objective of this measurement within the previously described experiment was to use ^{15}N labelled fertiliser as an indicator to investigate the nitrogen uptake of different cultivars in response to drought stress, as well as to detect effect of endophyte on nitrogen uptake of the host. As described in Chapter 3, 20 ml (0.98 g/L) ^{15}N labelled $(NH_4)_2SO_4$ (equal to 4.38 mg ^{15}N /plant) fertiliser was applied to each plant at 20 cm depth in the soil in early December and January, respectively. ^{15}N capture (mg ^{15}N/plant), ^{15}N concentration (mg ^{15}N/g shoot DM) and total nitrogen concentration (N%) in shoots of each plant were measured in March. Nitrogen uptake of all cultivars was significantly decreased by water deficit, and this was mainly due to the shoot DM reduction as the ^{15}N concentration was not affected by drought. N% was not affected by water deficit, except that two cultivars had increased N% compared to their irrigated counterparts. Endophyte presence increased the ^{15}N concentration of shoots while the ^{15}N capture was decreased due to the lower shoot DM of E+ plants compared to E− plants. N% was not affected by endophyte presence. It is concluded that nitrogen uptake is more sensitive than nitrogen concentration to drought and, endophyte presence decreases the nitrogen uptake of shoots as a result of the metabolic cost of hosting the endophyte.

7.2 Introduction

Plants absorb nitrogen from the soil mainly in the form of nitrate and ammonium and may also absorb amino acids under particular soil conditions, and nitrogen is transported from roots to shoots via the xylem in the form of nitrate, dissolved ammonia and amino acids (Masclaux-Daubresse et al., 2010). Many factors influence the amount of nitrogen uptake of plants, including shoot and root system size (Pang et al., 2015), soil nitrogen level (Presterl et al., 2002) and environmental stresses (Alam, 1999). The nitrogen uptake rate of plants under drought is generally decreased due to the decreased diffusion rate of nitrogen from the soil matrix to the

root (Nye & Tinker, 1977); which can be attributed to the reduced transpiration rate under drought (Turner et al., 2001); changes in root morphology such as decreased root hair length (Bibikova & Gilroy, 2002); reduced hydraulic conductance of roots (Cruz et al., 1992); and diminished activity of soil organisms involved in mineralization is diminished (Borken & Matzner, 2009).

Endophytes, as they are heterotrophic organisms, obtain nutrients solely from their host plants. It has been shown that endophyte presence modifies the metabolic profiles of the host grasses, including the total carbon, total nitrogen, some nitrogenous compounds and other metabolic compounds (Rasmussen et al., 2008; Rasmussen et al., 2009). Therefore, it is likely that endophyte presence has an effect on the processes of using of nitrogen of host plants.

The hypotheses of this experiment were: 1) cultivars would have different nitrogen uptake in response to water deficit; and 2) endophyte presence would affect nitrogen uptake and nitrogen concentration of the host.

As for Chapter 4 and Chapter 6, an introduction to the experiment, and information on methods and materials were provided above in Chapter 3. In this chapter, data for the amount of ^{15}N captured by plants from the applied ^{15}N labelled fertiliser (calculation based on Equation 3.7), ^{15}N concentration (calculation based on Equation 3.8) and total nitrogen concentration (N%) of shoots are presented.

7.3 Results

In March, ^{15}N capture of all cultivars was significantly decreased by water deficit, while the ^{15}N concentration was not affected by water deficit (Table 7.1 and Figure 7.1). An interaction between cultivar and irrigation treatment was detected for the ^{15}N capture (Table 7.1); reduction of ^{15}N capture percentage caused by water deficit ranged from about 80% (the URL, Commando and Bealey) to about 40% (Banquet II and Flecha) (Figure 7.1). N% of only two cultivars was increased by water deficit (Table 7.1 and Figure 7.2). E+ plants had higher ^{15}N concentration but lower ^{15}N capture compared to E− plants (Table 7.1 and Figure 7.3).

Table 7.1 Results of GLM ANOVA analysis for ^{15}N capture, ^{15}N concentration and total nitrogen concentration (N%) in shoots of plants.

Sources	^{15}N capture		^{15}N concentration		N%	
	F	P	F	P	F	P
I	23.86	**0.0395**	0.06	0.8348	0.97	0.4290
C	3.59	**0.0005**	5.49	**<.0001**	6.67	**<.0001**
C × I	3.66	**0.0004**	1.06	0.3882	3.34	**0.0011**
E	4.77	**0.0298**	9.25	**0.0026**	0.54	0.4628
E × I	0.03	0.8710	1.38	0.2414	0.32	0.5701
C × E	1.23	0.2803	0.51	0.8522	0.71	0.6796
C × E × I	0.79	0.6126	0.28	0.9708	0.58	0.7940
Error *df*	259		259		332	

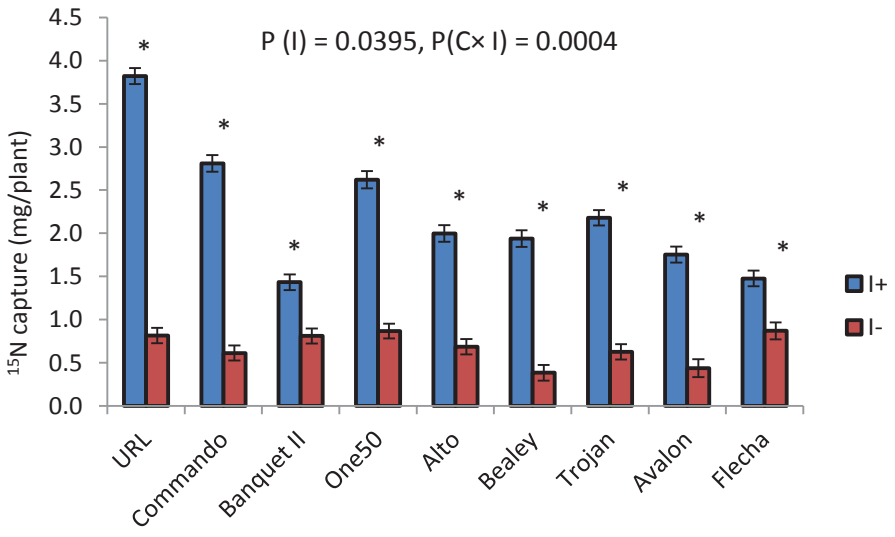

Figure 7.1 ^{15}N capture in shoots of irrigated (I+) and non-irrigated (I–) plants of each cultivar in March. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

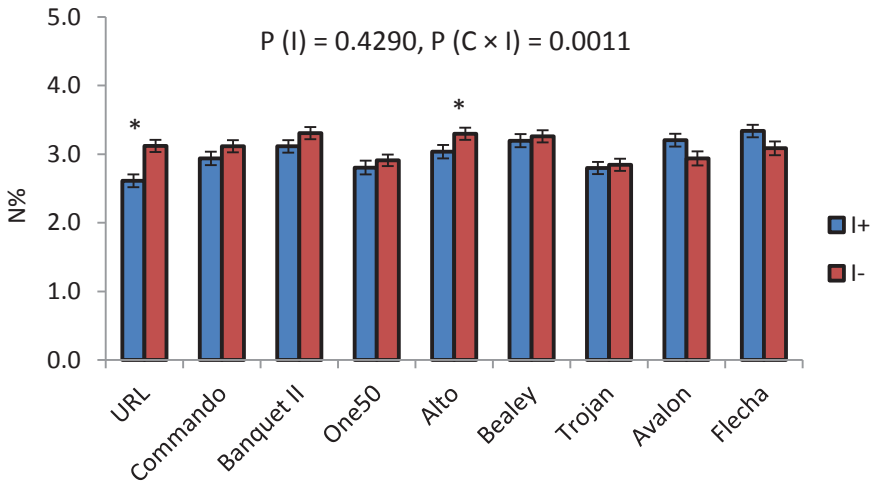

Figure 7.2 Total nitrogen concentration (N%) in shoots of irrigated (I+) and non-irrigated (I–) plants of each cultivar in March. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

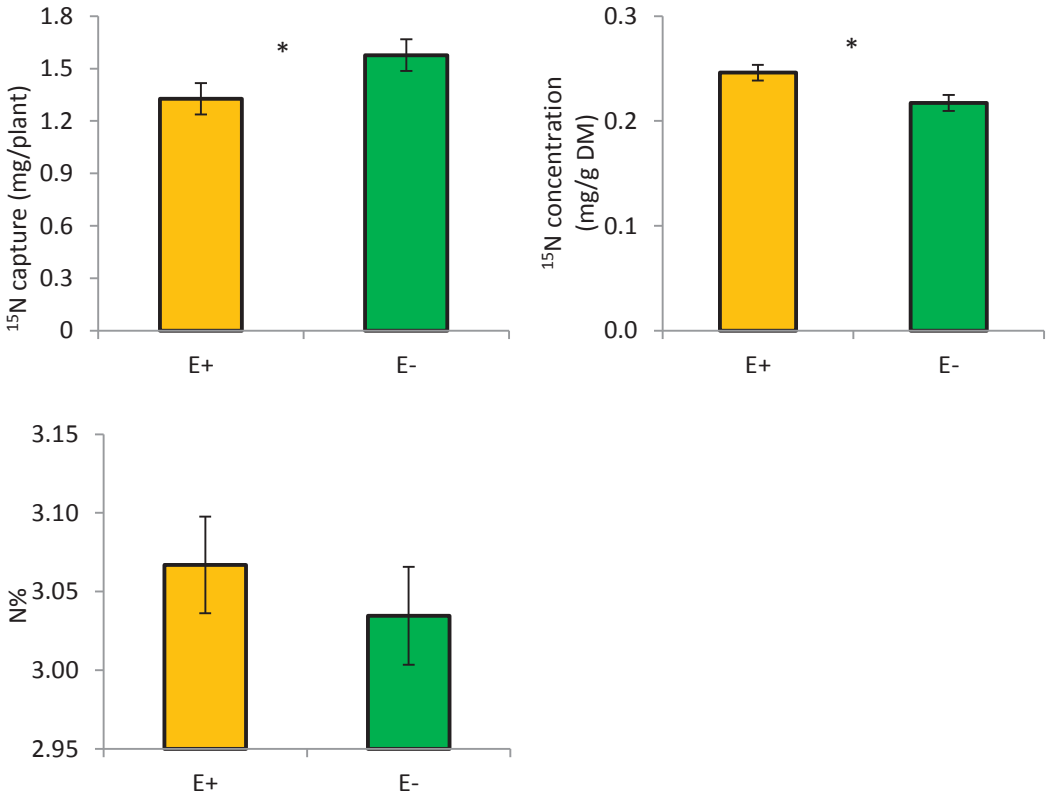

Figure 7.3 ^{15}N capture, ^{15}N concentration and total nitrogen concentration (N%) in shoots of endophyte-infected (E+) and endophyte-free (E−) plants. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

7.4 Discussion

In this experiment, since the ^{15}N labelled fertiliser was applied to each plant, the ^{15}N capture of shoots was used to estimate nitrogen uptake in shoots, that is, nitrogen amount that had been transferred to shoots during the period from February to March (the shoot DM in March was used to calculate the ^{15}N capture from February to March).

In the third month after withholding irrigation (in March), nitrogen uptake was decreased by water deficit (Table 7.1 and Figure 7.1). As mentioned in Section 7.2, a series of soil or plant characteristics contribute the nitrogen uptake reduction caused by water deficit. In this experiment, the stomatal conductance was decreased (as indicated by carbon isotope composition, Figure 6.1) and this provides evidence that the transpiration was also decreased (as would be expected because of reduced soil water availability in drought conditions), which is a major factor in nitrogen uptake reduction. However, the reduced nitrogen uptake appears not to be linked to the level of root formation activity, since the new root formation was not affected by water deficit (Table 4.3).

Effects of drought on plant nitrogen and phosphorus concentrations have been inconsistent in past studies. He and Dijkstra (2014) conducted a meta-analysis to examine drought effects on plant nitrogen and phosphorus concentration and concluded that drought stress generally decreases nitrogen and phosphorus concentration, however, nitrogen and phosphorus concentrations are usually unaffected in long term drought (>90 days) where plants may have adapted to the new conditions and established a new equilibrium between plant growth and plant nitrogen and phosphates uptake. In this experiment, plant N% was not affected by water deficit, except for the URL and Alto, which exhibited increased N% under I− conditions compared to I+ counterparts (Figure 7.2).

It has been reported for chewing fescue (*Festuca rubra* L. ssp. commutata Gaud) that endophyte infection increased nitrogen uptake (in both shoots and roots) of the host, but this was associated with the increased plant biomass (Richardson et al., 1999). Here, it was found that endophyte presence decreased the ^{15}N capture of the host compared to E− plants (Figure 7.1), which could arise mainly from the lower average shoot DM of E+ plants compared to E− plants (Figure 4.3), as the ^{15}N concentration

was increased by endophyte presence. The ^{15}N concentration was calculated from N% and nitrogen isotope composition (Equation 3.8), and the N% was not significantly affected by endophyte (Table 7.1 and Figure 7.3), thus the major reason why E+ showed an increased ^{15}N concentration compared to E− plants was the increased ^{15}N nitrogen isotope composition. This means endophyte presence decreased the discrimination against ^{15}N during one or more processes of nitrogen uptake, assimilation and transport. A number of studies have shown that mycorrhizal fungi affect plant $\delta^{15}N$ values because mycorrhizal fungi transfer isotopically depleted nitrogen to their host plants. As a result, mycorrhizal fungi are enriched while associated plants are depleted in ^{15}N (Hobbie et al., 1999; Hobbie et al., 2000). A possible explanation for the results obtained here is that endophyte presence influenced host $\delta^{15}N$ values indirectly through an influence on the activity of host mycorrhizal fungi.

Effects of endophyte on nitrogen concentration are multifarious in past studies. For examples, (Beatriz et al., 2013) reported that endophyte presence increased the nitrogen concentration in shoots but not in roots in red fescue (*Festuca rubra*); (Rogers et al., 2011) reported that effects of endophyte status on nitrogen concentration of the whole plant were dependent on plant genotypes and maturity in tall fescue; and (Ren et al., 2014) reported that nitrogen concentration in leaves was reduced by endophyte presence under drought conditions but not under well-watered conditions in a perennial rhizomatous grass (*Leymus chinensis*). Here, it was found that the N% in shoots was similar between E+ and E− plants (Table 7.1).

7.5 Conclusion

Nitrogen capture was greatly decreased by water deficit, while the nitrogen concentration was relatively unaffected under drought conditions, compared to nitrogen capture, but effects of drought on N% depend on plant cultivars. In this experiment, N% was only determined once during the drought. It would be of interest to monitor the N% and shoot biomass of plants at different stages of drought in order to have a better understanding of drought effects on N%. Although endophyte presence decreased the total nitrogen uptake of shoots, the N% was similar to that of E− plants in this experiment. However, different results for endophyte effects on N% in different experiments suggest that other factors are also involved in determining N% of the grass-endophyte symbionts.

Chapter 8 Drought responses of two perennial ryegrass cultivars with and without AR37 endophyte

8.1 Abstract

In order to further understand ryegrass responses to water deficit and the role of endophyte in drought tolerance of the host, plants of two perennial ryegrass cultivars, Ceres One50 and Grasslands Commando with and without AR37 endophyte were studied in a glasshouse experiment. Six replicates of eight genotypes of each cultivar infected with endophyte and without endophyte were kept under contrasting irrigated or unirrigated water regimes for two weeks and then rehydrated for one month. During the period of non-irrigation, a series of plant growth parameters, plant water relations, gas exchange parameters and some stress indicators were measured. Leaf dry matter yield in the rehydration period was also determined. Plants responded to water deficit with decreased leaf elongation rate, relative water content, leaf water potential, osmotic potential, photosynthesis rate, stomatal conductance and transpiration rate and increased leaf senescence rate, root: shoot ratio, proline concentration and electrolyte leakage. One50 had less LER reduction and greater proline accumulation than Commando, suggesting that One50 was more tolerant to drought than Commando, which may be attributable to the incorporation of Spanish germplasm in the breeding process of One50. A positive correlation between the extent of osmotic adjustment and leaf dry matter regrowth indicated that osmotic adjustment promoted the plant regrowth during recovery. However, high osmotic adjustment was related to more restricted shoot growth under drought, thus breeding for high OA plants create a dilemma. 'High OA' plants probably exhibit lower shoot dry matter production during drought but better post-drought recovery. Among all the measured morphological and physiological traits, endophyte infection significantly affected only the root:shoot ratio and this effect was independent of cultivar and irrigation. It is concluded that AR37 endophyte had no effect on drought tolerance of the host One50 and Commando plants in this experiment.

8.2 Introduction

The discovery of the Mangere ecotype in the late 1960s was a milestone in New Zealand perennial ryegrass breeding, since this ecotype formed the basis of many cultivars well adapted to the North Island in following decades. The cultivar Grasslands Nui was the first perennial ryegrass cultivar derived from this Mangere ecotype (Armstrong, 1977). Grasslands Commando that was collected from New Zealand old pastures (Stewart, 2006) has a close affinity to Grasslands Nui (Wang et al., 2014). In the 1980s, based on recognition that the climate in North West Spain was similar to that in North Island in New Zealand, germplasm from mild oceanic regions of North West Spain was introduced to New Zealand and provided valuable improvements in a number of traits including winter activity, late flowering, low vernalisation response and excellent resistance to crown and stem rust (Stewart, 2006). In the 1990s, Grasslands Impact was bred from the Spanish germplasm and Grasslands Nui. Later more cultivars including Tolosa, Arrow and the tetraploid Banquet were derived from Grasslands Impact. The development of other cultivars such as Trojan and Ceres One50 also incorporated Spanish germplasm. National trials evaluating perennial ryegrass cultivars showed that new cultivars (released after 1991) yielded on average 6% more herbage annually and 9% more in summer than old cultivars (released before 1991), which was mainly due to the introduction of Spanish germplasm (Easton et al., 2001). However, very limited scientific information is available about whether the introduction of Spanish germplasm has improved drought tolerance of New Zealand perennial ryegrass population.

Effects of endophyte on drought tolerance of the host could be associated with the intrinsic drought tolerance level of the host plant. Hesse et al. (2005) found that for the genotype collected from dry sites, E+ plants had significantly higher shoot, root and total dry weights and root:shoot ratio than E– plants during drought recovery. Also, Zhou (2014) showed that E+ plants had significantly higher RWC, CF (F_v/F_m) and total tiller number than E– under drought conditions for drought tolerant genotype of perennial ryegrass, but this result was not observed for drought sensitive genotype.

In this experiment, by measuring a series of plant morphological and physiological responses, the aim was to further understand how perennial ryegrass plants respond

to drought stress and explore whether endophyte plays a role in drought tolerance of the perennial ryegrass host. Associated with these aims, research hypotheses were: (1) One50 which was bred from Spanish germplasm would show improved drought tolerance compared to Commando; (2) endophyte presence would modify some of the plant responses to drought, but this would depend on the host cultivar, probably only affect the drought tolerant cultivar not the drought sensitive cultivar. In the previous experiment, the herbage yield of One50 was reduced by AR1 endophyte, thus it is also of interest to test whether another endophyte strain (AR37) would have similar impact on the herbage yield of One50.

8.3 Methods and Materials

8.3.1 Plant material

Seeds of cultivar Ceres One50 (One50) and Grasslands Commando (Commando) infected with AR37 endophyte (E+) or free of endophyte infection (E–) were obtained from PGG Wrightson Seeds Ltd. Seeds were placed in an incubator under conditions of 30°C/20°C (light 16 hour/ dark 8 hour) and 40% humidity from 18th November 2013. One week later, seedlings with similar size were transplanted to plastic pots containing potting mix and slow release fertiliser. Seedlings were kept in a glasshouse under natural light and well irrigated for one and half months. Endophyte status was checked using immunoblotting (Simpson et al., 2012).

8.3.2 Experiment design

On 17th January 2014, ten individual plants with the required endophyte status were selected to represent each cultivar-endophyte association, and each plant was split into six clones. Each clone, consisting of four adult tillers, was transplanted into a PVC pipe (height 50 cm, diameter 10 cm). From the bottom to top, the pipe was filled with 15 cm of sand (dry bulk density 1.42 g/ml), 10 cm of a mixture (50%, 50%) of air-dried Manawatu silt loam (dry bulk density 1.25 g/ml) and sand, and 20 cm of an air-dried Egmont loam soil (a typic orthic allophanic soil in the New Zealand soil classification system) A horizon (dry bulk density 0.74g/ml). Two layers of fine wind netting mesh were taped on the end to hold soil in the pipe. After plants were transplanted into pipes, a PVC ring (height 15 mm, diameter 35 mm) cut from a pipe was fully inserted into the soil surrounding each plant (as in the field

experiment) to avoid plant horizontal expansion and to achieve similar plant size before drought treatment was initiated. Every pot was weighed after the air dried soil was filled in and weighed again after fully watered (24 hours later) to estimate the amount of water that can be held in the pot (931 ml on average).

Plants in their pipes were arranged in groups in a split-plot experimental design, with irrigation treatment (I+, irrigation and I–, non-irrigation) as the main-plot factor. Ten individual plants (ten genotypes) of each cultivar-endophyte association were randomly arranged in each group. Groups of pipes were considered equivalent to plots in a field experiment, to allow for possible temperature or light intensity gradients within the glasshouse. Plants were fully irrigated in the first day after being transplanted into the pipes, and 150 ml water was supplied daily to each plant over the following two weeks. Thereafter, 300 ml water was supplied to each plant daily. On 17th March 2014, plants that had not fully colonized the PVC ring were discarded, leaving eight genotypes of each cultivar-endophyte association in the experiment. Leaf water potential and gas exchange parameters of a certain number of plants were determined one week before drought treatment began, in order to confirm there was no difference between plants in I+ and I– plots at this stage. From 3rd April 2014, irrigation was withheld for two weeks for plants in the I– plots and followed with one month rehydration, while plants in I+ plots were consistently well irrigated. All plants were in vegetative growth in this experiment as they were not vernalised.

During the months of the experimental period from January to May inclusive, the monthly means for daily maximum temperature in the glasshouse were 28.6°C ± 0.5, 29.6°C ± 0.6, 26.4°C ± 0.5, 23.8°C ± 0.6, 21.1°C ± 0.2, respectively, and the average minimum daily temperature were 16.6°C ± 0.5, 17.6°C ± 0.3, 15.0°C ± 0.4, 14.6°C ± 0.4, 11.2°C ± 0.6, respectively.

8.3.3 Measurements

Measurements including leaf elongation rate, leaf senescence rate, plant water relations, gas exchange parameters, electrolyte leakage, plant sample collection for proline and malondiadehye (MDA) concentration analysis were conducted during the drought period. On 18th April 2014, half of the plants (four genotypes of each

cultivar-endophyte association from each plot, in total 96 plants) were clipped to 7.5 cm above ground level to determine leaf dry matter, and then plants were transferred to 4°C walk-in refrigerator room for storage while awaiting further sampling for stubble dry matter, root organic matter, and gravimetric soil water content (sample processing was completed within 2 weeks). The remaining half of the plants were also trimmed and rehydrated for one month, after which the regrowth leaf dry matter was determined at the end of the rehydration period.

Plant water relations, gas exchange parameters, electrolyte leakage, proline concentration and MDA concentration were all determined in the first fully expanded leaves of a/several randomly selected adult tillers of each plant.

8.3.3.1 Soil water content

Soil water content (SWC) determination at root harvest. Approximately 300 g soil samples were recorded before and after oven-dried at 105°C for 24 hours. The SWC (%, w/w) was calculated as:

SWC (%, w/w) = (FW – DW)/DW× 100%,

where FW and DM refers to fresh weight and dry weight of the soil samples, respectively.

8.3.3.2 Plant growth parameters

Three adult tillers of each plant were randomly selected and labelled with plastic tapes with different colours. Initial leaf length (from leaf ligule to tip, or to the boundary of the chlorotic portion if senescence had begun) of each leaf on each labelled tiller was recorded on 7^{th}–8^{th} April (L_1) and again 15^{th} –16^{th} April (L_2). The leaf elongation rate (LER) and leaf senescence rate (LSR) of each plant were calculated as:

LER (mm/tiller/day) = $\sum (L_2 - L_1)$/3 tillers /8 days, when $L_2 > L_1$;

LSR (mm/tiller/day) = $\sum (L_1 - L_2)$ /3 tillers/ 8 days, when $L_1 > L_2$.

Leaf dry matter (leaf DM) and the stubble dry matter (Stubble DM) (mainly pseudostem) were harvested on 18^{th} April 2014 and oven-dried at 80°C for 48 hours. The sum of the leaf DM and stubble DM was considered as the total shoot DM.

For the 96 plants in the storage room as mentioned above, all visible roots were picked out of the soil, washed and oven-dried. The root organic matter (root OM) was determined by combusting samples in a porcelain mortar in a muffle furnace at 650°C for 2 hours (Schuurman & Goedewaagen, 1965). Root OM was calculated as the difference between weight (mortar, oven-dried root and residual soil) before and after combustion. The root OM was recorded instead of the root DM was because soil particles could not be washed off completely from the roots, which would contribute errors to the determination of root DM. The root:shoot ratio was calculated as: root:shoot ratio (RSR) = root OM/ shoot DM.

8.3.3.3 Plant water relations

The relative water content (RWC), leaf water potential (LWP) and osmotic potential (OP) was measured from 12th April to 14th April, one replication per day. Methods of measuring RWC, LWP and OP were as described in Section 3.4.

On 18th April, the 96 un-harvested plants were fully irrigated in the evening and the OP of these plants was determined the next morning (before dawn). Osmotic adjustment (OA) was calculated as the difference OP between rehydrated I– plants (OP_1) and the consistently I+ counterparts (OP_0): $OA = OP_1 – OP_0$ (Begg, 1980; Blum, 1989).

8.3.3.4 Gas exchange parameters

It was possible to measure the gas exchange parameters of a maximum of 12 plants in a day between 9 am and 11 am, after which time gas exchange might have been curtailed by falling LWP and stomatal closure. Therefore, only three genotypes of cultivar One50 with and without endophyte in each plot were measured from 12th to 14th April, one replication per day.

The rate of photosynthesis (P_n, μmol CO_2 m^{-2} leaf s^{-1}), transpiration rate (T_r, mmol H_2O m^{-2} leaf s^{-1}) and stomatal conductance (G_s, mol H_2O m^{-2} leaf s^{-1}) were measured for each entry, using a portable photosynthesis system (Li6400, LiCor Inc., USA) fitted with a standard 20 mm × 30 mm leaf chamber, leaf thermocouple and a blue-red LED light source. The photosynthetically active radiation was 1500 μmol m^{-2} s^{-1}, ambient CO_2 concentration was 390 μmol CO_2 mol^{-1} air, temperature of the leaf

chamber was 20°C, and the relative humidity in the chamber was controlled within a range from 50–75%.

Figure 8.1 Measuring gas exchange parameters using a portable photosynthesis Li6400 system before drought treatment commenced.

8.3.3.5 Proline and MDA concentration

On 17th April 2014, leaf samples for free proline and MDA analysis were cut and placed in a plastic seal bag and immersed in liquid nitrogen immediately. Samples were then stored in a –80 °C freezer. Leaf samples were freeze-dried then ground to powder by using a Retsch MM200 mixer mill.

About 40 mg of leaf powder was weighed in a centrifuge tube and a free proline concentration analysis conducted using the proline assay kit for plant tissue (Product No. A107, Nanjing Jiancheng Bioengineering Institute Ltd.). Absorbance was read at 518 nm using a Bausch & Lomb Spectronic 20 spectrophotometer.

A further sample of about 40 mg leaf powder was analysed for MDA using the MDA assay kit from the same company (Product No. A003-3, Nanjing Jiancheng Bioengineering Institute Ltd.). Absorbance was read at 532 nm and 600 nm (Biochrom Libra S60PC Double Beam Spectrophotometer, Cambridge, England). The absorbance for the nonspecific turbidity at 600 nm was subtracted from the reading at 532 nm.

8.3.3.6 Electrolyte leakage

The electrolyte leakage (EL) was determined from 12th to 14th April 2014. Leaf samples were collected and placed in a plastic seal bag on ice, then immediately transferred to the lab after sample collection was complete. Leaf samples were washed with running RO water followed with deionized water to remove any electrolytes adhering to the leaf surface, and then cut into 1 cm long segments and put in a test tube containing 15 ml of deionized water. Tubes were shaken for 30 minutes at room temperature to allow electrolyte diffusion from the leaf tissue. The initial conductance (EC_1) was determined by using an electric conductivity meter. Tubes were put in the autoclave at 120°C for 30 minutes to completely release electrolytes, then brought to room temperature, shaken for 24 hours and conductance was measured again (EC_2). The percentage of electrolyte leakage were calculated as: EL (%) = $EC_1/EC_2 \times 100\%$ (Bayat et al., 2009; Blum & Ebercon, 1981).

8.3.4 Data analysis

A general linear model procedure (Proc GLM) using SAS software (version 9.4, SAS institute, Cary, NC, USA) was employed for all analyses. The residuals of GLM for RWC, TP and proline were not normally distributed even after data transformation, so a non-parametric factorial analysis was applied. The data were Aligned Rank Transformed (ART) to generate normal distributed ranks by using ARTool package in R, then subjected to ANOVA test (Wobbrock et al., 2011).

8.4 Results

Before the drought treatment commenced, the average LWPs of plants in I+ plots and I– plots were – 2.0 bars and – 1.6 bars, respectively, indicating that all plants were well hydrated. Gas exchange parameters did not show significant differences between I+ and I– plants. The average values for P_n, G_s and T_r were 16.54 ± 0.45 μmol CO_2 m^{-2} leaf s^{-1}, 0.42 ± 0.02 mol H_2O m^{-2} leaf s^{-1}, and 4.61 ± 0.13 mmol H_2O m^{-2} leaf s^{-1}, respectively.

At the end of drought treatment, the SWC of I– pots was significantly lower than that of I+ pots in all three soil layers (Figure 8.2). The LER of I– plants was reduced more than 50% compared to I+ plants, meanwhile, the LSR of I– plants was 3 times

higher than that of I + plants (Table 8.1 and Figure 8.3). The leaf DM, stubble DM, total shoot DM, root OM and RSR were not significantly influenced by water deficit (Table 8.1 and Figure 8.3). The LWP, OP and RWC were reduced from values of −2 bars, −10 bars and 90% for I+ plants to about −7 bars, −15 bars and 70% for I– plants, respectively (Figure 8.5). The TP of I− plants remained similar to that of I+ plants (Table 8.2 and Figure 8.5). The LER was positively correlated with the RWC of plants under water deficit ($r = 0.6195$, $P < 0.0001$, $N = 96$) (Figure 8.10a) and the LSR was negatively correlated with the RWC of plants under water deficit ($r = −0.5635$, $P < 0.0001$, $N = 96$) (Figure 8.10b). As noted above, gas exchange parameters were only measured on cultivar One50. The G_s, P_n, and T_r were markedly reduced by water deficit (Table 8.3 and Figure 8.6). Proline concentration increased dramatically in response to water deficit, and was on average about 10 times higher than that of I+ plants (Table 8.4 and Figure 8.7). The EL was increased about 50%, and the MDA was not significantly increased compared to the I+ plants (Table 8.4 and Figure 8.7). After rehydration for one month, the I+ and I− plants did not show a difference in the RLDM (Table 8.5). The RLDM was negatively correlated with the LER ($r= −0.5624$, $P < 0.0001$, $N = 48$) and positively correlated with the level of the OA ($r = −0.4680$, $P = 0.0023$, $N = 40$) (Figure 8.10c and d).

An interaction between cultivar and irrigation was detected for the LER and proline concentration (Table 8.1 and 8.4). Both One50 and Commando responded to drought with decreased LER and increased proline concentration (Figure 8.2 and 8.6). However, Commando had higher LER than One50 under I+ conditions but similar LER to One50 under I− conditions (Figure 8.2). The proline concentration was similar between One50 and Commando under I+ condition, while One50 had higher proline concentration than Commando under I− conditions (Figure 8.6). However, this effect on proline concentration was also interfered by endophyte status, as indicated by a significant three-factor interaction between cultivar, irrigation and endophyte (Table 8.4). E+ and E− plants of One50 and Commando had similar proline concentration under I+ condition, while under I− conditions, E+ and E− plants of One50 showed a difference in proline concentration but this was not so for Commando (Figure 8.8).

Among all the measured parameters, endophyte status only had a significant effect on the RSR (Table 8.1), where E+ plants had higher RSR than E− plants (Figure 8.4). An interaction between cultivar and endophyte for RLDM was detected (Table 8.5). Endophyte infection increased the RLDM of Commando but not of One50 (Figure 8.9).

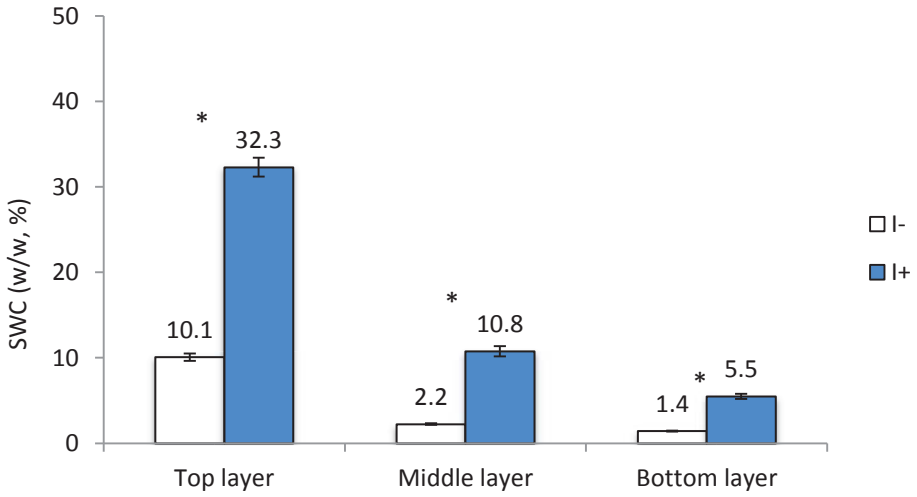

Figure 8.2 Gravimetric soil water content (SWC) of three soil layers in 96 harvested plants at the end of drought treatment. Vertical bars indicate standard errors; an asterisk denotes significant difference between irrigated (I+) plants and non-irrigated (I–) plants at $P < 0.05$.

Table 8.1 GLM analysis for leaf elongation rate (LER), leaf senescence rate (LSR), leaf dry matter (leaf), stubble dry matter (stubble), shoot dry matter (shoot), root organic matter (root) and root: shoot ratio (RSR). Only P values are presented in the GLM analysis tables, a complete analysis output can be found in Appendix 4.

Source	LER	LSR	Leaf	Stubble	Shoot	Root	RSR
I	**0.0262**	0.0754	0.1521	0.1944	0.3018	0.1154	0.0761
C	**0.0017**	**0.0033**	**0.0024**	0.1576	**0.0084**	**0.0070**	0.1754
C × I	**0.0328**	0.1819	0.2665	0.5365	0.3132	0.3270	0.8036
E	0.4714	0.7280	0.9971	0.9218	0.9639	0.2073	**0.0270**
E × I	0.6100	0.6668	0.9937	0.5898	0.8181	0.7461	0.8019
C × E	0.1561	0.6133	0.4483	0.8897	0.5720	0.7757	0.2678
C × E × I	0.1985	0.6225	0.4122	0.4324	0.3749	0.8794	0.2968

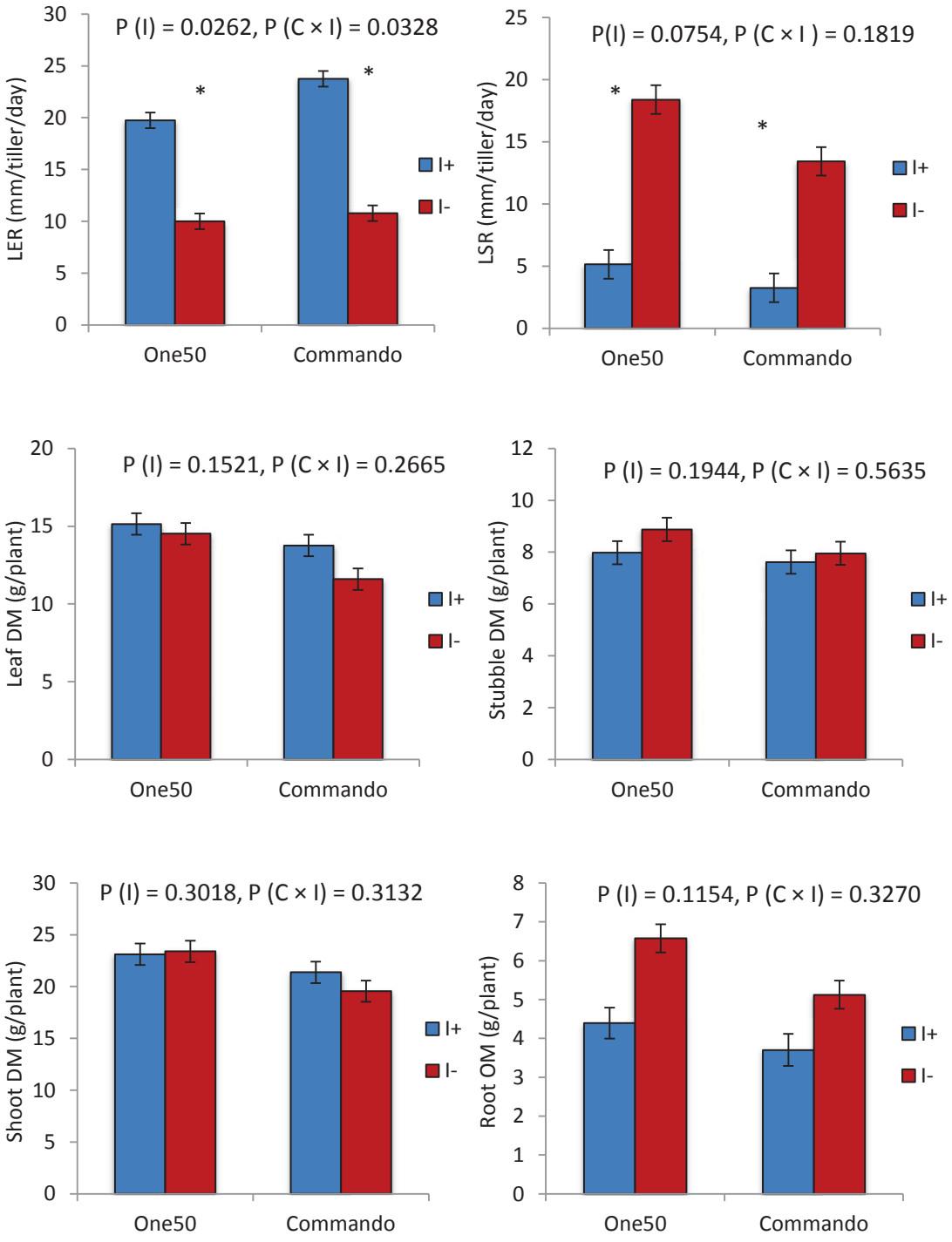

Figure 8.3 Plant growth parameters including leaf elongation rate (LER), leaf senescence rate (LSR), leaf dry matter (leaf DM), stubble dry matter (stubble DM), shoot dry matter (shoot DM) and root organic matter (root OM) of cultivar One50 and Commando plants under irrigated (I+) and non-irrigated (I–) conditions. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

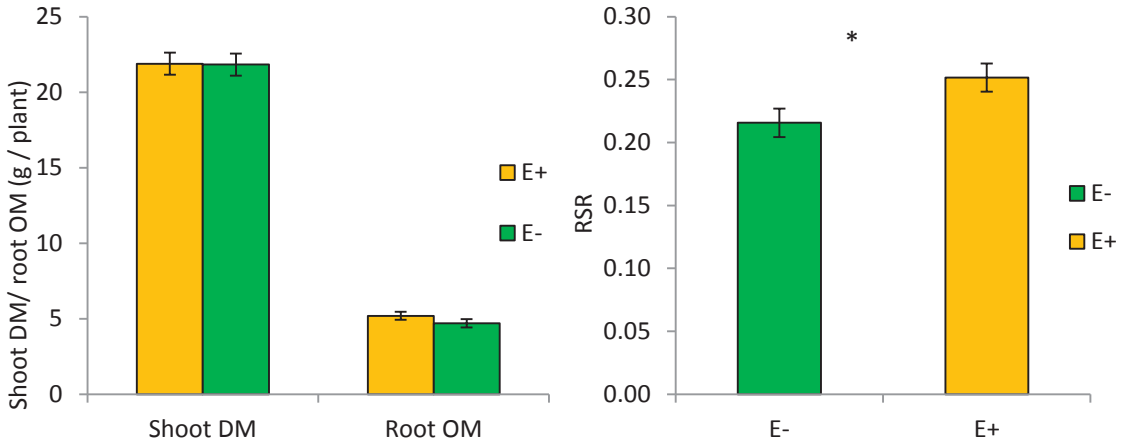

Figure 8.4 Shoot dry matter (shoot DM), root organic matter (root OM) and root:shoot ratio (RSR) of endophyte-infected (E+) and endophyte-free (E–) plants. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

Table 8.2 GLM analysis for the plant water relations including relative water content (RWC), leaf water potential (LWP), osmotic potential (OP) and turgor pressure (TP).

Source	RWC	LWP	OP	TP
I	**<0.0001**	**0.001**	**0.0040**	0.6453
C	**0.0171**	0.8477	0.2023	0.2758
C × I	0.1699	0.2602	0.5766	0.2415
E	0.0764	0.7163	0.8841	0.5117
E × I	0.7290	0.9201	0.5998	0.2354
C × E	0.9366	0.4639	0.9509	0.9779
C × E × I	0.7687	0.9236	0.9372	0.8895

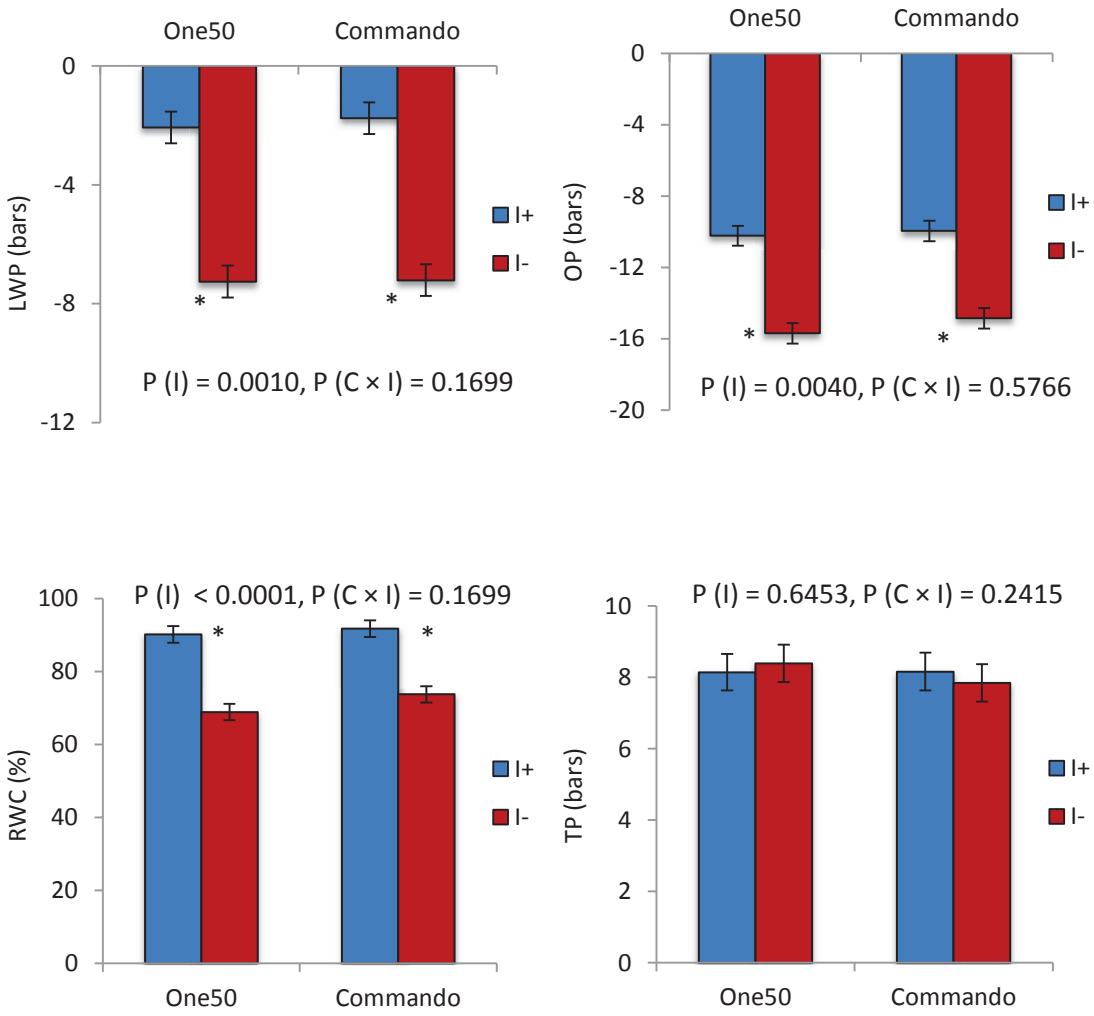

Figure 8.5 Plant water relations including leaf water potential (LWP), osmotic potential (OP), relative water content (RWC) and turgor pressure (TP) of One50 and Commando plants under irrigated (I+) and non-irrigated (I–) conditions. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

Table 8.3 GLM analysis for the gas exchange parameters including net photosynthesis rate (P_n), stomatal conductance (G_s) and transpiration rate (T_r).

Source	P_n	G_s	T_r
I	**0.0067**	**0.0437**	**0.0100**
E	0.3195	0.1313	0.2832
E × I	0.8357	0.1660	0.6613

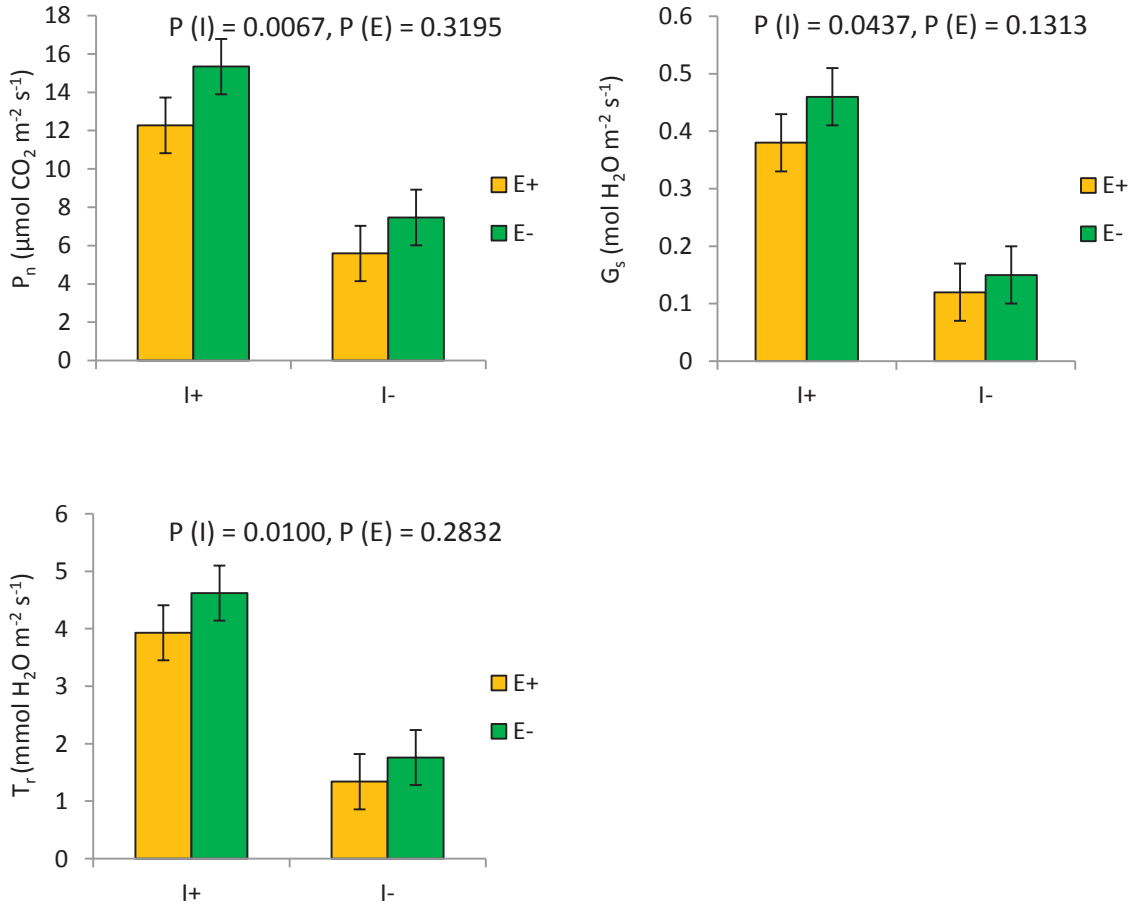

Figure 8.6 Gas exchange parameters including net photosynthesis rate (P_n), stomatal conductance (G_s) and transpiration rate (T_r) of endophyte-infected (E+) and endophyte-free (E–) plants under irrigated (I+) and non-irrigated (I–) conditions. Vertical bars indicate standard errors.

Table 8.4 GLM analysis for electrolyte leakage (EL), proline concentration (Proline) and malondialdehyde concentration (MDA).

Source	EL	Proline	MDA
I	**0.0004**	**<0.0001**	0.2547
C	**0.0312**	**0.0004**	0.1217
C × I	0.2307	**0.0002**	0.9786
E	0.0912	**0.0364**	0.2893
E × I	0.8730	**0.0182**	0.4655
C × E	0.4393	**0.0211**	0.2370
C × E × I	0.6713	**0.0489**	0.2825

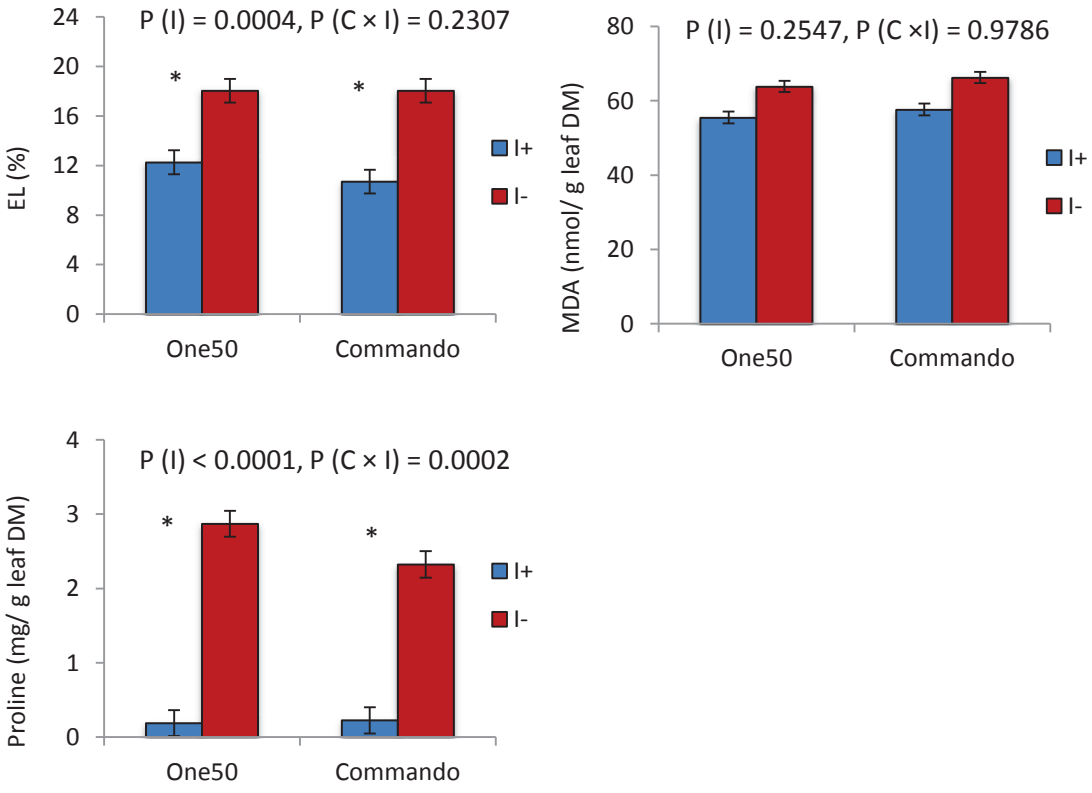

Figure 8.7 Electrolyte leakage (EL), malondialdehyde (MDA) and proline concentration (Proline) of One50 and Commando plants under irrigated (I+) and non-irrigated (I–) conditions. Vertical bars indicate standard errors; an asterisk denotes significant difference between I+ and I– plants at $P < 0.05$.

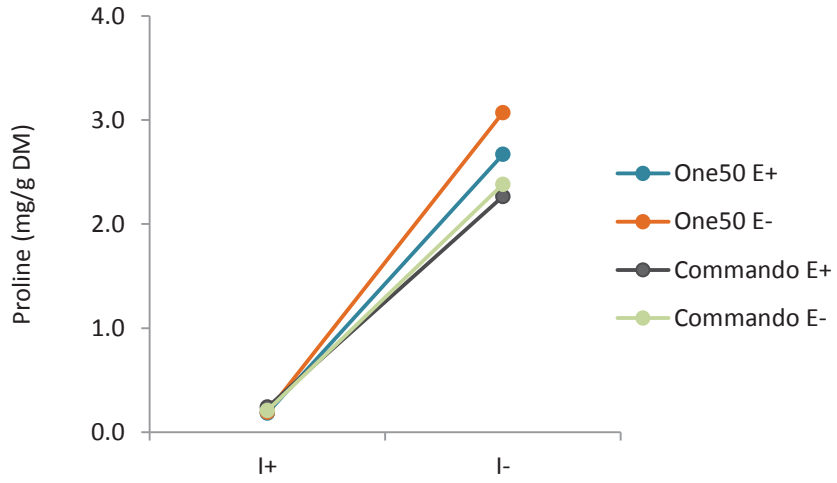

Figure 8.8 Proline concentration of One50 and Commando with endophyte (E+) and without endophyte (E−) under irrigated (I+) and non-irrigated (I−) conditions. (Line graphs are presented here to explain the three-factor-interaction between cultivar, irrigation treatment and endophyte status).

Table 8.5 GLM analysis for the regrowth leaf dry matter (RLDM) and osmotic adjustment (OA).

Source	RLDM	OA
I	0.3283	\
C	0.1335	0.5414
C × I	0.6957	\
E	0.5669	0.6887
E × I	0.5999	\
C × E	**0.0196**	0.2398
C × E × I	0.3796	\

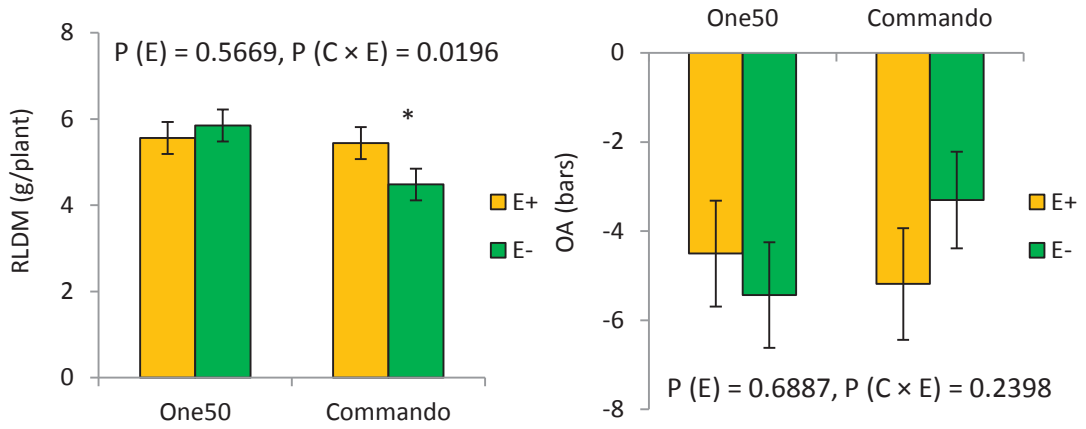

Figure 8.9 The regrowth leaf dry matter (RLDM) and osmotic adjustment (OA) of endophyte-infected (E+) and endophyte-free (E−) plants of cultivar One50 and Commando. Vertical bars indicate standard errors; an asterisk denotes significant difference between E+ and E– plants at $P < 0.05$.

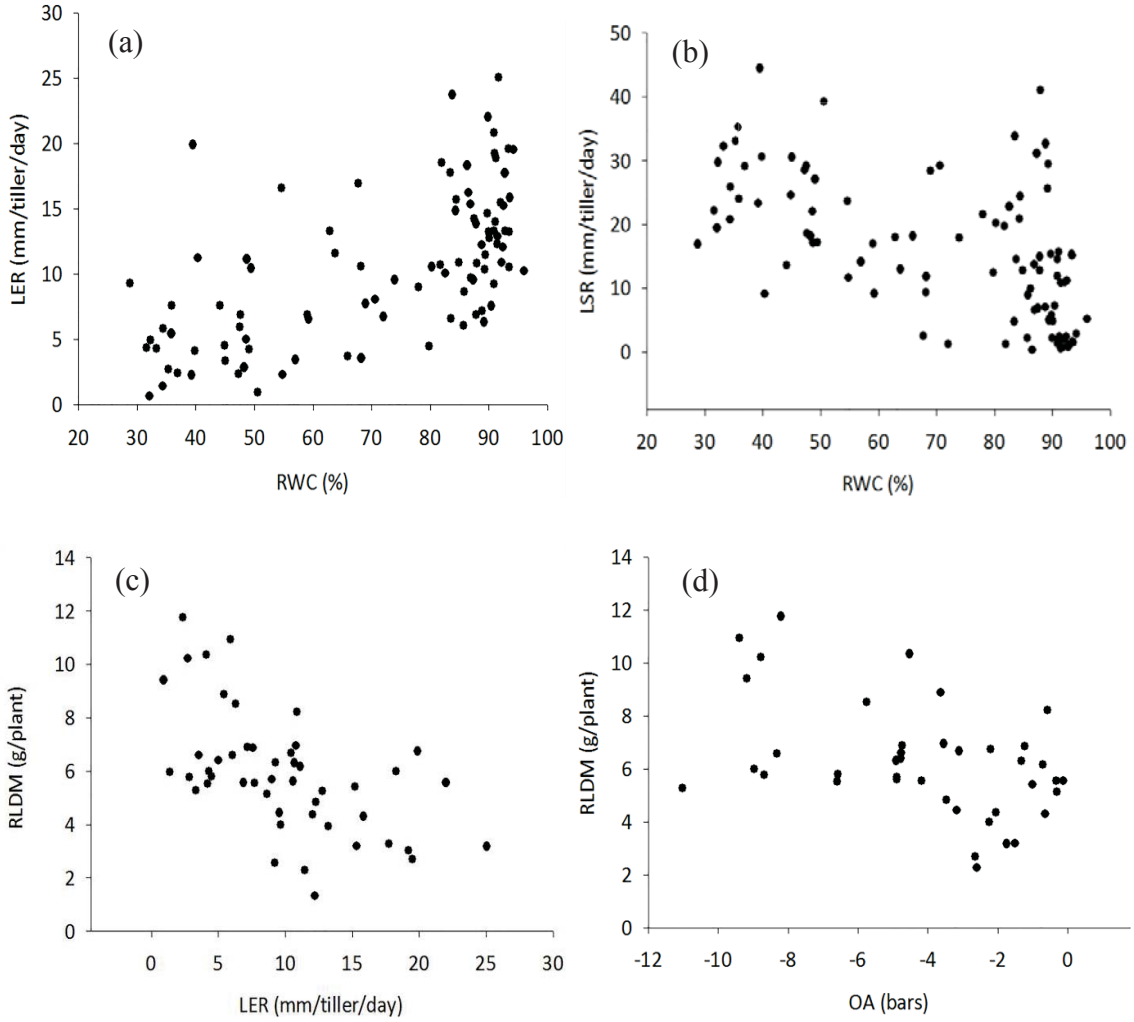

Figure 8.10 Correlation in non-irrigated plants between (a) the relative water content (RWC) and leaf elongation rate (LER) ($r = 0.6195$, $P < 0.0001$, $N = 96$); (b) RWC and leaf senescence rate (LSR) ($r = -0.5635$, $P < 0.0001$, $N = 96$); (c) regrowth leaf dry matter (RLDM) and LER ($r = -0.5624$, $P < 0.0001$, $N = 48$); and (d) RLDM and osmotic adjustment (OA) ($r = -0.4680$, $P = 0.0023$, $N = 40$).

Table 8.6 Correlations between the root:shoot ratio (RSR), shoot dry matter (shoot DM), root organic matter (root OM) and relative water content (RWC) under non-irrigated conditions.

		Shoot DM	Root OM	RWC
RSR	r	−0.2228	0.7663	0.1389
	P	0.1615	**<.0001**	0.3864
	N	41	41	41
Shoot DM	r		0.4140	−0.0882
	P		**0.0071**	0.5512
	N		41	48
Root OM	r			0.0280
	P			0.8622
	N			41

8.5 Discussion

8.5.1 Plant responses to drought

Based on the SWC of I− pots and the amount of water that can be held in the pot (see details in Section 8.3.2), it was estimated that plants in I− pots consumed 768 ml water in average from each pot during the drought period (soil evaporation from the pot is negligible as pots were fully covered by plants, Figure 8.1). Since plant size was large when drought treatment was initiated, the daily water consumption was 200–250 ml (estimated during the experiment in a sunny day), thus plants in the I− pots likely consumed a majority of the soil available water in the first three days after withholding irrigation.

Plant growth was restricted by water deficit as indicated by decreased LER and increased LSR (Table 8.1 and Figure 8.3). The LSR was even higher than the LER of

I− plants (Figure 8.3), which would eventually result in herbage yield reduction. However, since the drought period was relatively short (only two weeks), the herbage accumulation of I+ plants and I− plants was not significantly different (Table 8.1 and Figure 8.3).

Stomatal closure is usually the earliest plant response to water deficit (Schroeder et al., 2001), which enables plants to reduce the transpiration water loss, but with a concomitant reduction of photosynthesis due to restricted CO2 uptake, as indicated by decreased Gs, Pn and Tr of I− plants compared to I+ plants (Table 8.3 and Figure 8.6). When water supply cannot meet the water demand of plants, plants will get dehydrated. Here, the RWC of I− plants decreased from 90% of I+ plants to 70% (Figure 8.5). The more severe plant dehydration, the more the LER was limited and the greater LSR, as indicated by negative correlation between LER and RWC and positive correlation between LSR and RWC (Figure 8.10a and b). Reactive oxygen species are usually over produced and cause oxidative damage of organic molecules when plants are under drought stress, resulting in damage to cell membranes, as indicated by increased EL this experiment (Table 8.4 and Figure 8.7). The non-significant increase of MDA in I– plants compared to I+ plants suggests that lipid oxidation is less sensitive to drought stress. It may be that lipid oxidation occurs only under more severe or more prolonged drought stress.

Among plant responses, OA development is generally considered to be one of the plant adaptation traits to drought stress (Blum, 1996; Turner, 1986; Zlatev & Lidon, 2012), as OA has the effect to maintain cell turgor and at the same time, certain types of accumulated solutes help to protect cellular proteins, enzymes, and cellular membrane (Chaves et al., 2003; Farooq et al., 2009). Here, the TP was maintained even though plants were dehydrated (Table 8.2 and Figure 8.5), which could be attributed to the function of OA. However, if LWP and OP were measured in the midday, the TP between I+ and I− plants would probably be different, as the water deficit induced TP decrease is more pronounced in the midday than in the early

morning (Jones et al., 1980a). Diurnal measurements of LWP and OP for I+ and I− perennial ryegrass field swards can be found in Figure 2.3.The positive correlation between OA and RLDM (Figure 8.10d) suggests that the accumulated solutes benefited plant regrowth during recovery from drought. The RLDM was also negatively correlated with LER during drought (Figure 8.10c), which indicates that the more plant growth was restricted during drought, the more solutes accumulated in plant cells. It has been claimed that the accumulation of solutes during water deficit is because the cell expansion and elongation rates fall more rapidly than the photosynthesis rate, thus resulting in the supply of photosynthate exceeding its utilisation (Munns & Weir, 1981; Van Volkenburgh & Boyer, 1985; Wardlaw, 1969). The relationship between OA and crop yield was reviewed by Serraj and Sinclair (2002), who pointed out that most published papers indicated no effect or a negative influence of OA on crop yield during drought stress. In the current experiment, it was seen that the higher OA level benefited plant growth in rehydration but with a sacrifice of yield during the drought stress.

Plant species that inhabit dry sites generally have higher RSR than those of wet habitat, and the RSR is usually increased under drought stress as reviewed by Wu and Cosgrove (2000). Blum (2005) claimed that the increased RSR mainly arises from reduced shoot growth rather than increased root dry matter; therefore, the RSR should not be considered a good indicator of drought tolerance nor a selection criteria for drought tolerant plants. However, in this experiment, it was shown that the increased RSR was mainly due to increased root OM rather than decreased shoot DM, as indicated by significant correlation between RSR and root OM and non-significant correlation between RSR and shoot DM under drought conditions (Table 8.6). This experiment also demonstrated that the increased root OM and RSR under drought had no effect on delaying dehydration, as indicated by the non-significant correlations between RSR or root OM and RWC (Table 8.6), which agrees with Blum's statement that root biomass and RSR should not be considered as selection

criteria for drought tolerance. However, it should be noted that drought in this experiment was only of two weeks duration. Sinclair and Muchow (2001) assessed traits for crop yield under drought conditions and found that an increase in rooting depth consistently increased crop yield. Therefore, instead of root total biomass and RSR, rooting depth could be one of the selection criteria for enhancing drought tolerance of perennial ryegrass.

8.5.2 Drought tolerance of One50 and Commando

One50 and Commando were bred from different germplasm sources. As mentioned in the Section 2.1.2, One50 incorporates Spanish germplasm while Commando was derived from the 'Mangere' ecotype. Stewart (2006) stated that the Spanish germplasm provided valuable improvements in a number of traits including winter activity, late flowering, low vernalisation response and excellent resistance to crown and stem rust to New Zealand perennial ryegrass. In this experiment, an interaction between cultivar and irrigation was detected for LER and proline concentration. One50 had a smaller reduction in LER (Figure 8.3) and a larger increase in proline concentration (Figure 8.7) in response to drought than Commando, suggesting that One50 was more drought tolerant than Commando, and that the larger accumulation of proline may partially contribute to the lesser growth restriction of One50 (more information about proline function under drought conditions can be found in Section 6.4.2). In addition to the improved traits that have been summarised by Stewart (2006), Spanish germplasm has also provided improvement in drought tolerance of perennial ryegrass in New Zealand.

8.5.3 Role of endophyte

In the previous experiment, AR1 endophyte presence decreased the herbage yield of cultivar One50 and AR37 endophyte presence had no effect on herbage yield of cultivar Commando. In this experiment, both cultivars were infected with endophyte AR37, and AR37 did not influence the herbage yield and herbage yield components

(LER, LSR, leaf DM and stubble DM) of either One50 or Commando. The different effects of AR1 and AR37 on herbage yield of cultivar One50 suggest that different endophyte strains might have different levels of metabolic cost of the host. Further work is needed to clarify this point.

Among all the measured morphological and physiological parameters, main effect of endophyte status was only detected for RSR. The RSR was significantly increased by endophyte infection, which was mainly attributable to the increased root OM (Table 8.1 and Figure 8.4). Since this endophyte main effect was not associated with any interaction with irrigation treatments, it appeared that endophyte related increase in the RSR was independent from water availability. Hesse et al. (2003) also reported that endophyte presence increased root dry matter and RSR of three perennial ryegrass genotypes that had been collected from three different sites in Germany with different edaphic characteristics (wet, dry and neither wet or dry).

Proline concentration differed between plant cultivars, with endophyte status and with water availability, as well as there being interactions detected. Similar results were found in tall fescue (Bayat et al., 2009). As mentioned in Section 6.4.2, proline is not only a drought indicator but also plays a role in cell protection, thus results of proline concentration have to be examined together with other plant characteristics when comparing drought tolerance. In this study, E+ and E− plants of One50 and Commando had similar proline concentration under I+ conditions, while under I− conditions, E+ and E− plants of only One50 showed a difference in proline concentration (Figure 8.8). However, this pattern was not found for other plant characteristics, thus the three-factor interaction detected for proline is not considered to be evidence of different drought tolerance of E+ and E− plants.

8.6 Conclusions

One50 was more tolerant to drought than Commando, which can probably be attributed to the introduction of Spanish germplasm to One50. OA has often been considered as a drought adaptation traits and selection criteria, however, here it was demonstrated that in perennial ryegrass, OA promotes plant regrowth during plant rehydration but with a sacrifice of plant growth during drought stress. Thus breeding for high OA plants raises a dilemma. 'High OA' plants are likely to exhibit lower shoot dry matter production during drought but better post-drought recovery. Among all the measured morphological and physiological traits, AR37 endophyte infection only significantly increased the RSR and this effect was independent of cultivar and irrigation. It is concluded that AR37 endophyte had no effect on drought tolerance of the host One50 and Commando plants in this experiment.

Chapter 9 General discussion

9.1 Introduction

The two main objectives of this research were to evaluate the drought tolerance of some selected market-leading cultivars and to ascertain impacts of endophyte infection on drought tolerance of the host perennial ryegrass cultivars. The results from experiments which were discussed in each chapter in this thesis provided further understanding of drought tolerance of perennial ryegrass and the changes associated with endophyte-infection (E+) compared to endophyte-free (E−) plants. Data sets for ryegrass comparing cultivar leaf water relations of clonally identical plants with and without endophyte in the field are rare, and the present data set may be almost unique. In this chapter, the major findings, implications of the results and future possible follow-up research will be discussed.

9.2 Summary of plant responses to drought

Plants respond to drought stress with a series of complex mechanisms from genetic expression, biochemical metabolism, and physiological processes to plant morphological changes. There are several comprehensive reviews of plant responses to drought (Chaves et al., 2003; Farooq et al., 2009). In this research, we mainly investigated the morphological and physiological responses of perennial ryegrass to drought.

Yield reduction is the most dramatic and obvious response to drought. For forage grasses, the yield reduction is normally attributable to a lower rate of leaf expansion, leaf appearance, tiller appearance, and a greater rate of leaf senescence and tiller death (Barker & Caradus, 2001). In this research, it was observed that the tiller survival rate (visually scored) was significantly decreased under drought conditions compared to the irrigated plants in the rainout shelter experiment, while the leaf elongation rate was decreased and leaf senescence rate was increased in the glasshouse experiment. These morphological responses are not ideal from an agronomic point of view since this pattern of response necessarily involves yield loss. However, the reduced total leaf area, as a result of these responses, reduces the transpiration water loss and plant water demand, which favours the water status of

the surviving tissues and enables those tissues to maintain their active metabolic activities. In the rainout shelter experiment, the major herbage yield reduction occurred in the second month after withholding irrigation while the surviving leaves were remaining well hydrated at this stage as indicated by the relative water content (RWC).

Soil water deficit is primarily perceived by roots. In this project, roots were able to maintain growth under water deficit, as indicated by similar new root formation and root organic matter between irrigated (I+) and non-irrigated (I−) plants in the rainout shelter experiment and glasshouse experiment, respectively. Root:shoot ratio (RSR) depends on the dynamics of shoot and root growth. In the glasshouse experiment, it was demonstrated that the RSR was increased under drought as a result of slightly increased root biomass compared to I+ plants. However, in another study, the increased RSR of drought-stressed plants was attributed to the larger decrease of shoot biomass than root biomass in response to drought (Huang et al., 1998b). These different results are probably due to different drought severities. No matter whether roots maintain or reduce growth under drought conditions, in both cases roots become a proportionately greater allocation priority under drought.

When roots sense drought, a chemical message, abscisic acid, is produced by roots and travels to shoots via the xylem and controls the stomatal aperture (Schroeder et al., 2001). In the glasshouse experiment, the stomatal conductance (G_s) was decreased in response to drought, and as a consequence of stomatal closure, both transpiration and photosynthesis rate were reduced, and transpiration rate was inhibited more than photosynthesis rate, which would lead to increased water use efficiency (WUE), though lower productivity. It is known that G_s, WUE, and carbon isotope discrimination ($\Delta^{13}C$), crop yield are related to each other. For example, it has been found that: $\Delta^{13}C$ is negatively correlated to WUE in many crop species such as wheat (Farquhar & Richards, 1984) and cool-season forage grasses (Johnson & Bassett, 1991); $\Delta^{13}C$ is positively correlated to crop yield of bean (Zacharisen et al., 1999); and G_s is positively correlated to yield of cotton (Ulloa et al., 2000). However, their relationships under different soil water conditions are not consistent (Jensen et al., 2002), and the reasons for this are not fully understood. To date, there is a lack of research investigating the relationships among these traits under different

water regimes in perennial ryegrass. In the rainout shelter experiment, only the $\Delta^{13}C$ and herbage yield was measured, a positive correlation between $\Delta^{13}C$ and herbage yield at both I+ and I− conditions was detected (Appendix 5).

The regrowth of perennial grass species after drought is very important for pasture re-establishment. In the rainout shelter experiment, compensatory growth following drought was observed, in the form of higher shoot DM in I− than I+ plants in May (Figure 4.2). As discussed in the Section 4.4, the compensatory growth was likely attributed to the remaining nutrients in the soil and/or OA. The total nitrogen capture was significantly decreased by water deficit (Figure 7.1). In the glasshouse experiment, it was shown that the degree of osmotic adjustment (OA) during drought was positively correlated with the plant regrowth during recovery from drought. It is important to recognise that OA is achieved by the accumulation of a multitude of solutes. Proline has been considered one of the osmolytes (Verbruggen & Hermans, 2008), however, not a major one. The role of proline in prevention of protein denaturation and cell membrane integrity may be more important than its role in OA (Hamilton & Heckathorn, 2001; Samuel et al., 2000). In the glasshouse experiment, proline accumulation contributed only 5% to the OA (calculated according to the Morse equation). In many plant species, water soluble carbohydrates are the major accumulated solutes contributing to the OA under drought (DaCosta & Huang, 2006; De Diego et al., 2013; Volaire & Lelievre, 1997). Fructans, synthesised and stored in the vacuole (Wagner et al., 1983), are the major carbohydrate storage compounds for forage grasses. It has been reported that fructan accumulation is increased when grasses are under drought conditions (Amiard et al., 2003; Thomas & James, 1999). It was planned to measure leaf carbohydrates in the glasshouse experiment but problems with equipment resulted in samples being lost. However, it was likely that the accumulated fructans act as an energy reserve for promoting plant regrowth during rehydration. It has been shown that total fructans decrease to a level similar to that of control plants during rehydration of cocksfoot (*Dactylis glomerata*) and perennial ryegrass (Amiard et al., 2003; Volaire & Lelievre, 1997). It has been reported that high OA breeding lines produced more seed/grain yield than low OA breeding lines under drought conditions in sunflower (*Helianthus annuus* L.) (Chimenti et al., 2002), castor (*Ricinus communis* L.) hybrids (Babita et al., 2010), sorghum (*Sorghum bicolor* L.) (Ludlow et al., 1990), barley (*Hordeum vulgare* L.)

(Gonzalez et al., 2008) and wheat (*Triticum aestivum* L.) (Fischer et al., 2005). However, based on results from this project, it is deduced that selecting high OA in grass species that are harvested for herbage biomass might result in low herbage production during drought but improved herbage yield after drought, which is in agreement with statements of Munns (1988), who suggested that OA might be an adaptation for surviving stress rather than for growing during stress.

Malondialdehye (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids (the major component of cell membranes), thus MDA accumulation and electrolyte leakage (EL) are commonly measured to assess the stress-induced injury of cell membrane (Agarie et al., 1995; Bajji et al., 2002; Bandurska & Jozwiak, 2010; Chai et al., 2010; Labudda, 2013). Both EL and MDA accumulation have been linked to the accumulation of reactive oxygen species (ROS) (Demidchik et al., 2014). In the glasshouse experiment, the cell membrane of I− plants was injured at least to some extent as indicated by the significantly increased EL.

9.3 Drought tolerance of evaluated cultivars

Flecha tall fescue was identified as the most drought tolerant cultivar as indicated by its having the least yield reduction and highest retention of RWC in I− plants compared to I+ counterparts. As mentioned in Section 4.4, Flecha is a Mediterranean cultivar which exhibits partial endogenous summer dormancy (leaf growth is constrained, associated with moderate levels of foliage senescence) (Norton et al., 2006; Volaire et al., 2009). The small plant size of Flecha as a result of summer dormancy would likely slow down water consumption from the soil, and therefore, can be considered as a dehydration postponement strategy. In reality, summer dormancy is the most important strategy for plants to survive summer drought in Mediterranean regions, especially in severe summer drought (Volaire et al., 2009). Since the New Zealand climate is heading towards a Mediterranean climate with a tendency toward warmer and drier summers (Richards et al., 2010), it may be worth evaluating Mediterranean perennial ryegrass cultivars for future use in New Zealand, or incorporating Mediterranean sources into New Zealand-bred perennial ryegrass cultivars. Some initial work explores the potential for introgression of the drought resistance characteristics of Medea by studying a structured population of hybrids

and parents of Medea and Grasslands Samson cultivars has been done recently (Hussain, 2013). Some F1 and F2 families with promising trait combinations were found, but this work was not continued. In the glasshouse experiment, it was found that the Spanish germplasm based One50 was more drought tolerant than 'Mangere' ecotype based Commando as indicated by less LER reduction and higher proline accumulation of One50 compared to Commando responding to drought, suggesting that the Spanish germplasm has contributed to the drought tolerance of perennial ryegrass in New Zealand. As New Zealand perennial ryegrass cultivars are mainly developed from three germplasm sources, 'Hawkes Bay' ecotype, 'Mangere' ecotype and collections from North West Spain (Stewart, 2006), there is now rather limited genetic variations within perennial ryegrass varieties available in New Zealand. Easton et al. (2011) also suggested that New Zealand needs to import more genetic resources from other regions in order to cope with the climate change, and by crossing and backcrossing with locally adapted material, valuable gene combinations might be achieved.

Another option for improving ryegrass drought tolerance is to hybridise perennial ryegrass with drought tolerant grass species. In general, the *Festuca* species are believed to be more drought and cold tolerant than the *Lolium* species (Bandurska & Jozwiak, 2010; Humphreys, 2003). Cultivar Matrix and Ultra has been developed by Cropmark Ltd by hybridising perennial ryegrass with meadow fescue (*Festuca pratensis*). However, to date, it is not clear whether these two cultivars are more drought tolerant than other perennial ryegrass cultivars, although one experiment has been done to compare drought tolerance of Matrix, Medea, Samson and One50 (Hussain, 2013), in which experiment, interaction between cultivar and irrigation was only detected for seed-head count to tiller count ratio. Unfortunately it was not possible to include one of these cultivars in the current experiment because of workload constraints, but in the National Forage Variety Trial data, the summer yield of both Matrix and Ultra are mid-ranked rather than high ranked among the cultivars tested (New Zealand Plant Breeding and Research Association, 2015).

9.4 Effect of endophyte on drought tolerance of perennial ryegrass

In this research, no major interactions between irrigation and endophyte were detected for plant responses. Under both I+ and I− conditions, endophyte infection

influenced plant growth and physiological traits, including reduced herbage yield, decreased leaf water status, increased proline concentration and decreased stomatal conductance, especially for AR37 endophyte infected URL and AR1 endophyte infected One50. The possible explanations for these endophyte effects have been discussed in the Chapter 4 and Chapter 6. More information of effects of endophyte infection on the host metabolism of perennial ryegrass are given by Dupont et al. (2015) and Rasmussen et al. (2009). Since endophyte linked yield reduction and plant tissue dehydration (although only 1%) not only exist under I+ conditions but also exist under I− conditions, it would appear some E+ plants, especially AR37 infected URL and AR1 infected One50, are dehydrated more than the E− counterparts under drought conditions when insect predation is removed by chemical means. Kannadan and Rudgers (2008) particularly compared E+ plants and E− plants of grove bluegrass under either high water treatment conditions (with 50 ml water twice daily) or low water treatment conditions (with 50 ml water once daily) in a three-month period. They found E+ plants and E− plants had similar plant performance under high water treatment conditions, however, under low water treatment conditions, E+ plants had 14% more shoot biomass, 24% more root biomass and 29% less leaf senescence than E− plants, but lower leaf RWC, and there was no difference between E+ and E− plants in root morphology, leaf area or WUE (as measured by carbon isotope composition), and concluded that endophyte may ameliorate the negative effects of drought stress for grove bluegrass. Cheplick (2004) exposed E+ and E− plants of perennial ryegrass to three sequential droughts (water withheld for 11-14 days) with well-watered clones as control, and plant growth characteristics were determined 1 week, 4 weeks and 7 weeks after drought. In both control and drought treatment, E+ plants had fewer tillers, smaller leaf area and total mass than E− plants, although interactions between plant genotypes, endophyte status and water treatment were detected for root:shoot ratio. That author concluded "the symbiotic relationship between perennial ryegrass and its endophyte primarily benefits the fungus, not the host, under many environmental conditions". Considering the fact that endophyte presence decreased the herbage yield and RWC of the host under both I+ and I− conditions in this experiment, it is concluded that E+ plants are at disadvantage compared to E− plants when insect predation is removed by chemical means. This presumably reflects the metabolic cost to the plant of hosting the endophyte. However, in practice, there would seldom be a time when this

cost of having endophyte in the plants would be a problem, because of the biological benefits generated by E+ plants arising from insect deterrent effects.

9.5 Future research

In the glasshouse experiment, the plant traits were only determined once during the two-week drought. Ideally, the irrigation could have been gradually reduced rather than withheld abruptly, so that drought onset would be more gradual and plant traits could have been measured at successive stages in a dry-down cycle e.g. every week. Also, at least one of the enzyme-antioxidants could have been measured in order to have a more in depth understanding of the mechanisms of drought tolerance of perennial ryegrass.

Since no robust information is available for drought tolerance of Matrix and Ultra compared to other perennial ryegrass cultivars, it would be interesting to conduct a future experiment to evaluate whether introgression of *Festuca* genes has conferred improved drought tolerance to those cultivars.

AR37 and AR1 endophyte significantly decreased the herbage yield of the URL and of One50. It would be necessary to confirm these results and also to test whether other endophyte strains have the similar effect on these two cultivars. Ryan et al. (2015) showed that endophyte concentrations of novel perennial ryegrass-endophyte associations were strongly dependent on plant genotype and fungal strain. It is possible that the concentration of endophyte hyphae in the URL and One50 was higher than that of other cultivars. Thus this is another point that needs to be confirmed in a future investigation.

Effects of endophyte status on N% of the host were inconsistent in different studies, which indicate there must be a complex interaction between a range of factors such as soil nitrogen availability and plant age. Therefore, an experiment investigating effects of endophyte status on N% of the host might be conducted in order to have a better understanding of how the ryegrass-endophyte symbiosis impacts on nitrogen relations of the host.

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Appendices

Appendix 1 Water potentials of NaCl solutions at temperatures between 0−40°C and the calibration equations for each C-52 chamber

TABLE I

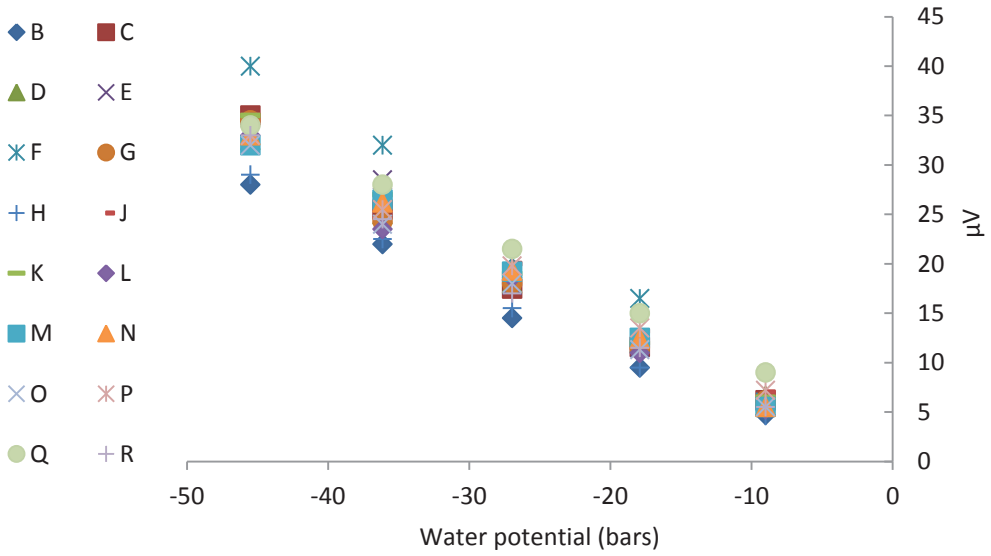
WATER POTENTIALS OF NaCl SOLUTIONS AT TEMPERATURES BETWEEN 0-40°C

(Lang, 1967)

Temperature / Molality	0°C	5°C	10°C	15°C	20°C	25°C	30°C	35°C	40°C
0.05	-2.14	-2.18	-2.22	-2.26	-2.30	-2.34	-2.38	-2.42	-2.45
0.1	-4.23	-4.31	-4.39	-4.47	-4.54	-4.62	-4.70	-4.77	-4.85
0.2	-8.36	-8.52	-8.68	-8.84	-9.00	-9.15	-9.30	-9.46	-9.61
0.3	-12.47	-12.72	-12.97	-13.21	-13.44	-13.68	-13.91	-14.15	-14.37
0.4	-16.58	-16.93	-17.27	-17.59	-17.91	-18.23	-18.55	-18.86	-19.17
0.5	-20.70	-21.15	-21.58	-22.00	-22.41	-22.81	-23.22	-23.62	-24.02
0.6	-24.84	-25.39	-25.93	-26.44	-26.94	-27.44	-27.94	-28.43	-28.91
0.7	-29.01	-29.67	-30.30	-30.91	-31.51	-32.10	-32.70	-33.28	-33.85
0.8	-33.20	-33.98	-34.72	-35.43	-36.12	-36.82	-37.51	-38.18	-38.85
0.9	-37.43	-38.32	-39.17	-39.98	-40.79	-41.58	-42.27	-43.14	-43.90
1.0	-41.69	-42.70	-43.66	-44.59	-45.50	-46.40	-47.29	-48.15	-49.01
1.1	-45.99	-47.13	-48.20	-49.24	-50.26	-51.27	-52.26	-53.22	-54.18
1.2	-50.32	-51.60	-52.78	-53.94	-55.07	-56.20	-57.30	-58.35	-59.41
1.3	-54.70	-56.11	-57.42	-58.69	-59.94	-61.19	-62.39	-63.54	-64.71
1.4	-59.12	-60.68	-62.10	-63.50	-64.87	-66.23	-67.54	-68.80	-70.06
1.5	-63.59	-65.29	-66.84	-68.37	-69.86	-71.34	-72.76	-74.11	-75.48
1.6	-68.11	-69.96	-71.63	-73.30	-74.91	-76.52	-78.05	-79.50	-81.07
1.7	-72.60	-74.60	-76.40	-78.20	-80.00	-81.70	-83.30	-84.90	-86.50
1.8	-77.30	-79.40	-81.30	-83.30	-85.20	-87.00	-88.80	-90.40	-92.10
1.9	-81.90	-84.30	-86.30	-88.40	-90.40	-92.40	-94.30	-96.00	-97.80
2.0	-86.70	-89.20	-91.30	-93.60	-95.70	-97.80	-99.80	-101.60	-103.50

WATER POTENTIAL (BARS)

This table from C-52 instruction/ service manual shows the relationship between different NaCl molality and water potential at different temperatures. In our experiment, the temperature was around 20−22°C, therefore the corresponding water potential at 20°C was regarded as providing the X values in the following graph which shows the standard curve for each chamber. The calibration equation for each chamber is displayed below.



Chamber No.	Calibration Equation
B	y = −1.5331x − 2.9633
C	y = −1.2582x − 2.9872
D	y = −1.402x − 0.7077
E	y = −1.249x − 3.0392
F	y = −1.0768x − 2.3283
G	y = −1.295x − 2.6177
H	y = −1.4708x − 3.2382
J	y = −1.3193x − 0.3915
K	y = −1.2609x − 2.0026
L	y = −1.3313x −2.7582
M	y = −1.3585x − 1.0923
N	y = −1.3257x − 1.5349
O	y = −1.3846x − 1.9497
P	y = −1.4321x + 1.2612
Q	y = −1.4474x + 4.0249
R	y = −1.3313x − 2.7313

Appendix 2 Example of SAS code for GLM analysis

Normality test

```
proc glm data=DM;
class rep water endophyte cultivar ;
model DM1= rep rep*water water|cultivar|endophyte / ss3;
test h=water e=rep*water;
output out=resid r=resid1;
run;
proc univariate data=resid normal plots;
var resid1;
run;
```

GLM analysis code

```
proc glm data=DM;
class rep water endophyte cultivar ;
model logDM1= rep rep*water water|cultivar|endophyte / ss3 ;
test h=water e=rep*water;
lsmeans water*cultivar / slice = cultivar pdiff stderr;
lsmeans cultivar*endophyte/ slice = cultivar pdiff stderr;
lsmeans water*endophyte/ slice = water pdiff stderr;
run;
```

Appendix 3 Non-parametric factorial analysis for tiller survival rate in February and March in the rainout shelter experiment.

February					
Resources	SS	df	Df.res	F	P
Cultivar (C)	430415	8	372	3.8959	0.0001973 ***
Endophyte (E)	66807	1	372	4.4907	0.0347415 *
Irrigation (I)	681899	1	372	51.4997	3.91E–12 ***
C × E	182191	8	372	1.5626	0.1344678
C × I	236908	8	372	2.0579	0.0390938 *
E × I	25245	1	372	1.6850	0.1950681
C× E × I	114169	8	372	0.9729	0.4568130
March					
Resources	SS	df	Df.res	F	P
Cultivar (C)	499764	8	373	4.5113	3.002E–05 ***
Endophyte (E)	120280	1	373	8.1185	0.004625 **
Irrigation (I)	1298583	1	373	111.2506	< 2.2E–16 ***
C × E	230384	8	373	1.9811	0.047743 *
C × I	537961	8	373	4.9176	8.555E–06 ***
E × I	53492	1	373	3.5542	0.060174 .
C× E × I	60370	8	373	0.5047	0.852777

Appendix 4 GLM and ART ANOVA analysis output in the glasshouse experiment

- LER

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	257.686379	128.84319	4.76	0.0097
Rep × Irrigation	2	338.126079	169.06304	6.24	0.0024
Irrigation (I)	1	6192.222588	6192.222588	228.54	<.0001
Cultivar (C)	1	274.730776	274.730776	10.14	0.0017
C × I	1	125.437167	125.437167	4.63	0.0328
Endophyte (E)	1	14.11043	14.11043	0.52	0.4714
I ×E	1	7.072513	7.072513	0.26	0.61
C × E	1	54.965901	54.965901	2.03	0.1561
I × C × E	1	45.134105	45.134105	1.67	0.1985
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	6192.222588	6192.222588	36.63	0.0262

- LSR

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	378.2754	189.1377	2.98	0.0532
Rep × Irrigation	2	1117.563	558.7815	8.81	0.0002
Irrigation (I)	1	6581.84	6581.84	103.75	<.0001
Cultivar (C)	1	564.338	564.338	8.9	0.0033
C × I	1	113.9446	113.9446	1.8	0.1819
Endophyte (E)	1	7.700013	7.700013	0.12	0.728
I ×E	1	11.79588	11.79588	0.19	0.6668
C × E	1	16.25759	16.25759	0.26	0.6133
I × C × E	1	15.43034	15.43034	0.24	0.6225
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	6581.84	6581.84	11.78	0.0754

- Leaf DM

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	128.8623	64.43115	5.68	0.0049
Rep × Irrigation	2	18.17636	9.088179	0.8	0.4523
Irrigation (I)	1	46.5095	46.5095	4.1	0.0461
Cultivar (C)	1	110.897	110.897	9.77	0.0024
C × I	1	14.19882	14.19882	1.25	0.2665
Endophyte (E)	1	0.00015	0.00015	0	0.9971
I ×E	1	0.000704	0.000704	0	0.9937
C × E	1	6.583538	6.583538	0.58	0.4483
I × C × E	1	7.706667	7.706667	0.68	0.4122
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	46.5095	46.5095	5.12	0.1521

- Stubble DM

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.304502	0.152251	0.03	0.9693
Rep × Irrigation	2	4.999444	2.499722	0.51	0.6008
Irrigation (I)	1	9.244209	9.244209	1.9	0.1722
Cultivar (C)	1	9.913776	9.913776	2.03	0.1576
C × I	1	1.878801	1.878801	0.39	0.5365
Endophyte (E)	1	0.047259	0.047259	0.01	0.9218
I ×E	1	1.428376	1.428376	0.29	0.5898
C × E	1	0.094376	0.094376	0.02	0.8897
I × C × E	1	3.035259	3.035259	0.62	0.4324
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	9.244209	9.244209	3.7	0.1944

- Shoot DM

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	128.9422313	64.4711156	2.51	0.087
Rep × Irrigation	2	15.0198521	7.509926	0.29	0.7469
Irrigation (I)	1	14.283551	14.283551	0.56	0.4576
Cultivar (C)	1	187.125426	187.125426	7.3	0.0084
C × I	1	26.407526	26.407526	1.03	0.3132
Endophyte (E)	1	0.0527344	0.0527344	0	0.9639
I ×E	1	1.365651	1.365651	0.05	0.8181
C × E	1	8.254401	8.254401	0.32	0.572
I × C × E	1	20.414926	20.414926	0.8	0.3749
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	14.28355104	14.28355104	1.9	0.3018

- Root OM

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	19.09403	9.547015	3.14	0.049
Rep × Irrigation	2	18.35313	9.176564	3.02	0.0549
Irrigation (I)	1	66.01327	66.01327	21.75	<.0001
Cultivar (C)	1	23.40767	23.40767	7.71	0.007
C × I	1	2.956775	2.956775	0.97	0.327
Endophyte (E)	1	4.916294	4.916294	1.62	0.2073
I ×E	1	0.320697	0.320697	0.11	0.7461
C × E	1	0.24839	0.24839	0.08	0.7757
I × C × E	1	0.070434	0.070434	0.02	0.8794
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	66.01327	66.01327	7.19	0.1154

- Root OM/shoot DM

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.018145	0.009073	1.76	0.1786
Rep × Irrigation	2	0.030991	0.015495	3.01	0.0554
Irrigation (I)	1	0.180731	0.180731	35.15	<.0001
Cultivar (C)	1	0.009631	0.009631	1.87	0.1754
C × I	1	0.00032	0.00032	0.06	0.8036
Endophyte (E)	1	0.026227	0.026227	5.1	0.027
I ×E	1	0.000326	0.000326	0.06	0.8019
C × E	1	0.006412	0.006412	1.25	0.2678
I × C × E	1	0.00568	0.00568	1.1	0.2968
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	0.180731	0.180731	11.66	0.0761

- art(RWC)

Sources	SS	df	DF.res	F value	P
Cultivar (C)	17317	1	183	5.7958	0.01706 *
Endophyte	9616	1	183	3.1764	0.07637 .
Irrigation (I)	179163	1	183	82.3294	< 2E–16 ***
C × E	20	1	183	0.0063	0.93662
C × I	5800	1	183	1.8987	0.16990
E × I	370	1	183	0.1204	0.72904
C × E × I	267	1	183	0.0868	0.76868

- LWP

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.04297	0.021485	0.26	0.7699
Rep × Irrigation	2	0.022125	0.011063	0.13	0.8739
Irrigation (I)	1	11.4055	11.4055	139.04	<.0001
Cultivar (C)	1	0.003034	0.003034	0.04	0.8477
C × I	1	0.104642	0.104642	1.28	0.2602
Endophyte (E)	1	0.010869	0.010869	0.13	0.7163
I ×E	1	0.000827	0.000827	0.01	0.9201
C × E	1	0.044191	0.044191	0.54	0.4639
I × C × E	1	0.000756	0.000756	0.01	0.9236
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	11.4055	11.4055	1031	0.001

- OP

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.016852	0.008426	0.69	0.5012
Rep × Irrigation	2	0.009609	0.004805	0.4	0.674
Irrigation (I)	1	1.192	1.192	98.11	<.0001
Cultivar (C)	1	0.0199	0.0199	1.64	0.2023
C × I	1	0.003802	0.003802	0.31	0.5766
Endophyte (E)	1	0.000259	0.000259	0.02	0.8841
I ×E	1	0.003356	0.003356	0.28	0.5998
C × E	1	4.62E–05	4.62E–05	0	0.9509
I × C × E	1	7.55E–05	7.55E–05	0.01	0.9372
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	1.192	1.192	248.09	0.004

- art(TP)

Sources	SS	df	df.res	F value	P
Cultivar (C)	3550.8	1	178	1.1952	0.2758
Endophyte	1285.9	1	178	0.4322	0.5117
Irrigation (I)	634.1	1	178	0.2126	0.6453
C × E	2.3	1	178	0.0008	0.9779
C × I	4102.0	1	178	1.3809	0.2415
E × I	4185.8	1	178	1.4175	0.2354
C × E × I	57.9	1	178	0.0194	0.8895

- EL

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.02277828	0.01138914	4.18	0.0167
Rep × Irrigation	2	0.00013049	0.00006524	0.02	0.9763
Irrigation (I)	1	0.16135428	0.16135428	59.28	<.0001
Cultivar (C)	1	0.01282945	0.01282945	4.71	0.0312
C × I	1	0.00393726	0.00393726	1.45	0.2307
Endophyte (E)	1	0.00784774	0.00784774	2.88	0.0912
I ×E	1	0.00006974	0.00006974	0.03	0.873
C × E	1	0.00163549	0.00163549	0.6	0.4393
I × C × E	1	0.00049162	0.00049162	0.18	0.6713
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	0.16135428	0.16135428	2473.06	0.0004

- art(proline)

Sources	SS	df	df.res	F value	P
Cultivar (C)	36912	1	183	13.0324	0.0003951***
Endophyte	13323	1	183	4.4422	0.0364227 *
Irrigation (I)	266404	1	183	160.7132	< 2.2E–16 ***
C × E	16439	1	183	5.4083	0.0211372 *
C × I	39102	1	183	13.8858	0.0002587 ***
E × I	16873	1	183	5.6770	0.0182138 *
C × E × I	12078	1	183	3.9303	0.0489191 *

- MDA

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.261358	0.130679	23.63	<.0001
Rep × Irrigation	2	0.11977	0.059885	10.83	<.0001
Irrigation (I)	1	0.149667	0.149667	27.06	<.0001
Cultivar (C)	1	0.013376	0.013376	2.42	0.1217
C × I	1	0.000004	0.000004	0	0.9786
Endophyte (E)	1	0.00625	0.00625	1.13	0.2893
I ×E	1	0.002959	0.002959	0.54	0.4655
C × E	1	0.007788	0.007788	1.41	0.237
I × C × E	1	0.006427	0.006427	1.16	0.2825
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	0.149667	0.149667	2.5	0.2547

- Pn

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.121404	0.060702	0.52	0.6008
Rep × Irrigation	2	0.053036	0.026518	0.23	0.7986
Irrigation (I)	1	3.899807	3.899807	33.34	<.0001
Endophyte (E)	1	0.12013	0.12013	1.03	0.3195
I × E	1	0.005125	0.005125	0.04	0.8357
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	3.899807	3.899807	147.06	0.0067

- Gs

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	357.9884	178.9942	0.12	0.8842
Rep × Irrigation	2	331.9102	165.9551	0.11	0.8921
Irrigation (I)	1	3550.571	3550.571	2.45	0.1286
Endophyte (E)	1	3499.906	3499.906	2.42	0.1313
I × E	1	2929.335	2929.335	2.02	0.166
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	3550.571	3550.571	21.39	0.0437

- Tr

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.163812	0.081906	0.8	0.4581
Rep × Irrigation	2	0.063288	0.031644	0.31	0.7358
Irrigation (I)	1	3.130289	3.130289	30.68	<.0001
Endophyte (E)	1	0.122137	0.122137	1.2	0.2832
I × E	1	0.020003	0.020003	0.2	0.6613
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	3.130289	3.130289	98.92	0.01

- RLDM

Source	df	Type III SS	Mean Square	F Value	Pr > F
Rep	2	0.23459558	0.11729779	4.97	0.0091
Rep × Irrigation	2	0.17387702	0.08693851	3.68	0.0293
Irrigation (I)	1	0.14292291	0.14292291	6.05	0.0159
Cultivar (C)	1	0.05417827	0.05417827	2.3	0.1335
C × I	1	0.00363716	0.00363716	0.15	0.6957
Endophyte (E)	1	0.0078035	0.0078035	0.33	0.5669
I ×E	1	0.0065459	0.0065459	0.28	0.5999
C × E	1	0.13367292	0.13367292	5.66	0.0196
I × C × E	1	0.01841842	0.01841842	0.78	0.3796
Tests of Hypotheses Using the Type III MS for Rep × Irrigation as an Error Term					
Source	df	Type III SS	Mean Square	F Value	Pr > F
Irrigation	1	0.14292291	0.14292291	1.64	0.3283

- OA

Source	df	Type III SS	Mean Square	F Value	Pr > F
Cultivar (C)	1	5.350315	5.350315	0.38	0.5414
Endophyte (E)	1	2.295795	2.295795	0.16	0.6887
C × E	1	20.09831	20.09831	1.43	0.2398

Appendix 5 Correlation between carbon isotope discrimination ($\Delta^{13}C$) and shoot dry matter (shoot DM) of irrigated (I+) ($r = 0.5345$, $P < 0.0001$, $N = 183$) and non-irrigated (I−) plants ($r = 0.2940$, $P < 0.0001$, $N = 175$) in March in the rainout shelter experiment.