Effects of endophyte infection, environmental stress and competition on *Lolium perenne* populations from the Mediterranean region

By

Kristin Kane

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of requirements
for the degree of Doctor of Philosophy, The City University of New York

2012

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

__Gregory. P Cheplick__________
Chair of Examining Committee

______04/19/2012___________________
Date

__ Dr. Laurel Eckhardt__________
Executive Office

______05/01/2012___________________
Date

_________Dr. William L'Amoreaux_____________________
_______________Dr. Lisa Manne______________________
____________Dr. James White_________________________
_________Dr. Faith Belanger____________________________

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

By: Kristin Kane

Effects of endophyte infection, environmental stress and competition on *Lolium perenne* populations from the Mediterranean region

Advisor: Gregory P. Cheplick, PhD

My thesis is a study of the variable outcomes of the interaction between the common forage grass species *Lolium perenne* and its fungal symbiont *Neotyphodium lolii* to explain the existing variation in endophyte infection frequencies. *N. lolii* is a systemic fungal endophyte that colonizes the aerial tissues of its host plant and is vertically transmitted to the next generation via seeds. *Neotyphodium* endophytes in agronomic grass species are considered plant mutualists, because they improve the resistance of the host against various abiotic and biotic stresses. Empirical evidence suggests variable fitness effects of this endophyte on wild populations of *L .perenne* and this association may vary from mutualism to antagonism and may depend upon environmental conditions and the genotypes of these interacting species. I used wild infected grass populations which are part of the native distributional range of *L. perenne*, to understand how the overall ecological outcome of this interaction depends upon host genotype, water availability, inter-genotype competition between endophyte-infected (E+) and non-infected (E-) grasses, and the native habitat of this grass species. I also examined a possible relationship between climate in the Mediterranean and infection frequencies of *N. lolii* in natural *L. perenne* populations.

In some populations under drought, E+ plants had more tillers, greater tiller lengths, total dry mass and green shoot mass than non-infected E- plants, suggesting a positive effect of endophyte infection on host growth. Total tiller length and the number of tillers showed significant population*treatment*infection interactions for 4 of 6 populations which shows endophyte effects specific for particular accessions and cultivars under different water treatments. E+ genotypes outperformed E- genotypes under inter-genotype competition. E- genotypes experienced the effects of competition more severely than E+ genotypes although E+ genotypes didn't significantly outperform E- genotypes in all populations. In addition I found a negative relationship between infection frequency and a precipitation climate variable.

Interpretation of the results leads to the conclusion and support of the hypothesis that the interaction of infection, genotype and the environment is crucial in determining the outcome of the grass-endophyte symbiosis.

Acknowledgements:

I would like to extend special thanks to my advisor, Gregory P. Cheplick for invaluable assistant during the preparation of this dissertation and all experiments held within. Thanks to my committee members : William L'amoreaux, Lisa Manne, James White and Faith Belenger. I would also like to thank Richard Veit for all of his useful comments and help with statistics. Thank you to CSI for its support over the last five years and to the Microscopy and Microanalysis Society for its travel and presidential award. Special thanks to Stephen L. Clement, Plant Germplasm Introduction and Testing Research Unit, United States Department of Agriculture for freely providing the seeds used in the experiments and information pertaining to the accessions and also two anonymous reviewers for providing thoughtful comments on the manuscript.

Table of Contents

List of Tables

List of Figures

Figure 5.3. E+ *Lolium perenne* genotype, endophytes shown in green and propidium iodide-stained chloroplasts (red).

Figure 5.4. E+ *Lolium perenne* genotype. Endophytes (green) visually apparent inside the vascular bundle (red).

Chapter 1

General Introduction

Plant- microbial symbioses are ubiquitous, and the nature of these relationships can range across a continuum from parasitic to mutualistic (Johnson et al., 1997; Saikkonen et al., 1998; Jones and Smith, 2004: Hirsch, 2004; Neuhauser and Fargione, 2004). The degree and nature of these symbioses may be constant through time or they may be sensitive to changes in biotic or abiotic stressors (Thompson, 1988; Bronstein, 1994; Herre et al., 1999). Symbioses that are mutualistic between plants and microbes are usually based on an exchange of benefits between the two organisms. For example, plant hosts commonly supply nutrients, space, and reproduction for the symbiotic microbe, in return for microbial-mediated advantages against abiotic and biotic stress (Rodriguez et al., 2008; Timmusk and Wanger, 1999).

Microbial symbioses that decrease abiotic stress for the plant can have significant positive impacts on their host plants. For example, mycorrhizae and rhizobacteria increase the nutrient acquisition of the host plants they occupy, often conferring significant advantages to the host plant such as increased growth (Hetrick, 1989; Bolan, 1991; van Loon, 2007; Lugtenberg and Kamilova, 2009). Other abiotic stress benefits from plant-microbial mutualisms are associated with tolerance to heat (Redman et al., 2002; Rodriguez et al., 2008; Rodriguez et al., 2004), salt (Rodriguez et al., 2008; Yano- Melo et al., 2003), or drought (Timmusk and Wanger, 1999; Rodriguez et al., 2004; Arshad et al., 2008; Kannadan and Rudgers, 2008). Microbially-mediated abiotic stress tolerance can even allow host plants to survive in environments that they could not without the mutualistic relationship (Redman et al., 2002; Rodriguez et al., 2008; Rodriguez and Redman, 2008).

Plant-microbial mutualisms can also reduce biotic stress to the plant by reducing herbivory (Miller et al., 2002; Miller, 1986; Hartley and Gange, 2009) or increasing disease resistance of the host plant (Graham and Menge, 1982; Volpin et al., 1994) which may alter competitive interactions in the community (Rudgers et al., 2005; 2007). For example, mycorrhizae can alter herbivory of host plants by changing nitrogen availability, which may impact the carbon and nitrogen ratio of the plant in a way that is detrimental to potential herbivores (Gange and West, 1994; Bennett et al., 2006; Hartley and Gange, 2009). Mycorrhizal disease resistance can be the result of mycorrhizae consuming root exudates otherwise used by pathogens (Graham and Menge, 1982) or by a mycorrhizae induced defense response (Volpin et al., 1994).

While most plant-microbial mutualisms are species-specific, they can have significant effects across multiple ecological scales, ultimately influencing plant community and ecosystem structure, and function (van der Heijden, 2010 ; Clay and Holah, 1999; Rudgers et al., 2004;

2005; 2007; Rudgers and Clay, 2008). By reducing abiotic or biotic stress to the plant, plant-microbial interactions can alter inter- and intra-specific competition (Zobel and Moora, 1995; Allen and Allen, 1990; Hart et al., 2003), plant community structure (Hartnett and Wilson, 2002; van der Heijden, 2010) as well as nutrient cycling (Fogel 1980; van der Heijden, 2010). Because of the ecological importance of plant – microbe symbioses, knowing their response to climate change is critical in predicting the response of ecosystems to increasing atmospheric CO2, temperature, and altered precipitation regimes. Previous research has shown that plant-microbial symbioses are sensitive to differences in temperature (Smith and Bowen, 1979; Staddon et al., 2002; Smith and Roncadori, 2006), and there is increasing evidence that plant-microbial symbioses may be responsive to climate change (Staddon et al., 2002; Garcia et al., 2008; Rogers et al., 2009). For example, recent work has illustrated elevated CO_2 may increase ectomycorrhizal colonization of some plant species (Garcia et al., 2008) and increase microbial nitrogen fixation in some legumes (Rogers et al., 2009) - both of which may result in alterations in nutrient availability, capture and use in the ecosystems where they occur. One important type of plant-micobial symbiosis is that of plants and aboveground endophytic fungi.

Wilson (1995) defined endophytes as fungi or bacteria which, for most or all of their life cycles, invade the tissues of living plants and cause unapparent infections entirely within plant tissue and cause no symptoms of plant disease. In literature, the term endophyte is often treated as a synonym with mutualism (Saikkonen et al., 2004). Endophytic fungi are the most common group of fungal symbionts worldwide; every plant species examined thus far has harbored at least one fungal endophyte (Arnold et al. 2000; Faeth and Fagan, 2002). Endophytic fungi are very common in grasses. More than 80 grass genera in all subfamilies of the *Poaceae* family are capable of forming relationships with fungal endophytes (Clay, 1990; White, 1987). Of the common grass-endophyte relationships, the perennial ryegrass-*Neotyphodium lolii* and tall fescue- *Neotyphodium coenophialum* relationships are perhaps the best studied and most well-known.

Perennial ryegrass, *Lolium perenne*, is a widespread, ecologically and economically important cool-season grass that is known to associate with the endophytic fungus *N. lolii* (Latch,Christensen and Samuels). Native to Europe, perennial ryegrass has been widespread in New Zealand pastures since the last quarter of the19th century (Easton and Fletcher, 2007). When an improved strain of perennial ryegrass became available in the 1930's, this was continuously selected and reselected and improved in New Zealand for the next four decades. Most old ryegrass pastures in New Zealand are intensively infected with endophytic fungi (Latch and Christensen 1982; Widdup and Ryan 1992), accounting for the animal toxicosis that became a growing concern (Bush and Buckner, 1973). When *N. lolii* was identified as responsible for ryegrass staggers (Fletcher and Harvey, 1981) in livestock, it was proposed that endophyte- free ryegrass (E-) would solve the problem. However, research quickly revealed that endophyte-free ryegrass was less robust and not resistant to pasture pests such as the Argentine stem weevil (*Listronotus bonariensis)* (Prestidge and Gallagher, 1988; Prestidge et al., 1982). These results prompted further research into the relationship between *Neotyphodium* endophytes and perennial ryegrass.

N. lolii is an obligate, symbiotic fungal endophyte of perennial ryegrass, depending on its plant host for shelter, nutrients and reproduction (Isaac, 1992). *Neotyphodium* is an entirely asexual genus, and evidence suggests it evolved from sexual *Epichloë* species (Schardl andLeuchtmann, 2005; Wilkinson and Schardl, 1997). *N. lolii* does not produce sexual structures but instead grows into the ovaries and developing seeds of its host (White et al.,1993) and by this very efficient mode of reproduction the fungus is vertically transmitted. *Neotyphodium* endophytes are important in ecosystems because they have considerable impact on the fitness of the grass species they infect. These are symbiotic associates of grasses which form asymptomatic systemic infections in the above ground parts of plants. *Neotyphodium* hyphae grow intercellularly in the above-ground portion of perennial ryegrass and are typically concentrated in the leaf sheath and stems (Herd et al.,1997; Keogh et al., 1996; Tan et al., 2001). Since the fitness of the endophytic fungi and their host plant are closely linked, fungal endophyte-grass symbioses are generally assumed to evolve toward mutualism (Clay, 1990; Marks et al., 1991; White et al., 1993; Elberson and West, 1996; Clay and Holah, 1999).

The mutualistic nature of some grass-endophyte symbioses is supported by strong empirical evidence (Clay and Schardl, 2002). Reported benefits of endophyte infection to *L. perenne* include deterrence of herbivores by way of alkaloid production by this fungus (Van Heeswijick and McDonald, 1992; Schardl and Panaccione, 2005; Breen, 1994; Bush et al., 1997). Benefits also include resistance to abiotic stress such as drought (Ravel et al., 1997; Malinowski et al., 2005), flooding (Saikkonen et al., 1998) or low soil nutrients (Ravel et al., 1997; Cheplick et al., 2007; Malinowski et al., 2005). Marks et al., (1991) and Clay et al., (1993) have recorded greater competitive ability of infected hosts under certain environmental conditions. The fungus acquires host sugars and amino acids for nutrition and receives a means of propagation to the next generation by vertical transmission into host seeds (Clay, 1990; White et al., 1993).

However, the benefits of fungal symbionts do not come without associated costs. Since the fungus receives all of its nutrition from the host plant, harboring the fungus causes expenses for the host (Ahlholm et al., 2002; Saikknonen et al., 2004). The net effect of this relationship depends on whether the benefits to the host plant exceed the associated costs. Recorded costs of infection to *L. perenne* include growth under limited soil nutrients (Cheplick et al., 1989; Cheplick 1988), or under low water supply (Cheplick et al., 2000; Cheplick et al., 2004; Hesse, 2004). Reduced competitive ability has also been reported in infected genotypes, relative to uninfected *L. perenne* (Marks et al., 1991; Richmond et al., 2003). The grass-endophyte symbiosis is believed to span a continuum of interactions from mutualistic to antagonistic (Cheplick 2004, Cheplick and Faeth, 2009, Hesse et al. 2004, Lewis 2004, Ravel et al. 1997) and the outcomes of this interaction are influenced by plant genotype, endophyte genotype, the environment and their interactions.

Nearly all studies and experiments involving population, community and ecosystem level effects of endophytes have involved two artificially cultivated grass systems, tall fescue and perennial ryegrass (Cheplick, 1998; Clay and Holah, 1999). Recent investigations of endophyte-grass interactions in natural grass populations indicate that consequences of infection for the fitness of the host plant can be variable, particularly with regard to herbivore resistance (Saikkonen et al., 1999, Schulthess and Faeth 1998, Cheplick and Faeth, 2009). Endophyte-grass interactions, particularly those arising from natural populations, remain evolutionarily complex in nature. The outcome of the endophyte grass interaction can vary widely with plant and endophyte genotype and the environmental conditions in which the interaction occurs so it can be argued that mutualisms may be the exception rather than the rule for endophyte infected populations (Faeth 2002). For example, Cheplick (2007) used infected perennial ryegrass genotypes originating from its native distributional range to test the hypothesis that endophyte infection imparts a growth cost under extreme resource limitation in *L. perenne.* He found a cost of infection that included a reduction in root:shoot ratio and in photosynthetic shoot mass. Also, the effects of infection varied among host genotypes indicating the effects of endophytes depend on specific host-endophyte combinations within populations, which has the potential for genotypic sorting by natural selection for specific host endophyte combinations. In addition, Hesse et al., (2003) used three genotypes arising from natural habitats which differed in water availability to test for the effects of the endophyte *Neotyphodium* on *L. perenne* growth and seed yield under drought. His results suggested that environmental conditions in the original habitat of the plants may influence the symbiotic interaction between plants and fungi.

If the asexual endophyte acts as plant mutualists then natural selection through herbivore resistance, competitive abilities or resistances to stresses such as drought should favor infected plants and endophyte-infected populations should become 100% over time (Clay 1998, Wilkinson and Schardl 1997). While *Neotyphodium* endophytes are well-studied agricultural systems, data on their distribution and abundance in non-agricultural systems at larger scales across landscapes is lacking.

Thesis Outline

This thesis is centered on the *Lolium perenne -Neotyphodium lolii* symbiosis native to Europe, Asia and Africa. To study this symbiosis, I used seeds obtained from wild grasses of *L. perenne* from these regions. I use the terms “wild grasses” or “natural populations” to mean populations with their naturally occurring endophyte from its endemic habitat without any type of manipulation or selective breeding. In this thesis I investigate the interaction continuum hypothesis (Bronstein 1994, Saikkonen et al. 1998, Müller and Krauss 2005) for this grass-endophyte symbiosis. Since I am using natural populations which are subject to spatial and temporal heterogeneity of both the physical and biological environment, I expect variable outcomes of this interaction.

Endophyte- infected (E+) populations of *L. perenne* are believed to survive in areas that are under constant stress and selection due to seasonal drought periods. In the second chapter I conducted a drought stress experiment employing wild genotypes of *L. perenne* from four populations within the native range. My objective was to understand whether endophytes can ameliorate the effects of drought stress for populations from the Mediterranean region.

In natural populations competitors are a major component of the biotic environment and both inter and intraspecific competitive pressures are expected in populations (Bazzaz 1996, Turkington and Mehrhoff, 1990). Competitive ability is a well-known benefit of endophyte infected grasses (Hill et al. 1991), but studies of interspecific competition are limited in this grass species. The range of possible outcomes of interspecific competition is likely to span the continuum from mutualistic to parasitic. I conducted an inter-genotype and intra-genotype competition experiment (Chapter 3) to determine whether E+ genotypes competing with a different E- genotype would outperform an E+ genotype competing with an identical E- genotype. Inter-genotype and intra-genotype competition has never been studied for this endophyte-grass interaction.

To study the environment component of the continuum hypothesis, I investigated temperature and precipitation patterns in countries from the Mediterranean region in which perennial ryegrass is known to exist. I used geographic distribution analyses to model habitat suitability. I then modeled habitat suitability in the year 2050 to project climate change (Chapter

4). Only a few studies have investigated the response of the grass-fungal endophyte symbiosis to atmospheric and climate change factors. No other study but that presented here has actually projected climate change in the areas in which this grass-endophyte system exists naturally. The premise of this model is to understand the climate in the Mediterranean where *L. perenne* survives, and understand the impacts of climate change on its distributional range. A projected increase or decrease in its distributional range because of a change in this grasses' range of growth conditions could predict responses of grass-fungal endophytes to climate change. Potential global climate change is likely to impact biotic interactions in natural communities which may shift the symbiotic continuum and have significant impacts on levels of endophyte-infection frequencies in natural populations. I assessed endophyte infection frequencies in these natural populations and studied the potential relationship between infection frequencies with current temperature and precipitation levels (Chapter 4).

Another aspect of the grass-endophyte symbiosis is the physical relationship between the fungus and the vasculature of the host plant. Previous studies using light and electron microscopy have found that *Neotyphodium* endophytes from natural associates have the potential to colonize vascular bundles (Christensen et al. 2000). To investigate the physical relationship of the fungus *Neotyphodium lolii* to the vasculature of its host plant, I describe in chapter 5 the disruption of the plant cell membrane only in endophyte infected grasses using a novel confocal microscopy technique incorporating the fluorescent dyes wheat germ agglutinin (WGA) and propidium iodine (PI)

Chapter 1: Literature Cited

Ahlholm, J.U., M. Helander, S. Lehtimäki, P. Wäli, and K. Saikkonen. 2002. Vertically transmitted fungal endophytes: Different responses of host-parasite systems to environmental conditions. Oikos 99:173-183.

Allen, E.B., and M.F. Allen. 1990. The mediation of competition by mycorrhizae in successional and patchy environments. In Grace, J.B and D. Tilman (eds) *Perspectives in Plant Competition*. Academic Press, San Diego, CA. pgs. 367-389.

Arnold, A.E., Z. Maynard, G.S. Gilbert, P.D. Coley, and T.A. Kursar. 2000. Are tropical fungal endophytes hyperdiverse? Ecology Letters 3:267-274.

Bennett A.E., J. Alers-Garcia, and J.D. Bever. 2006. Three-way interactions among mutualistic mycorrhizal fungi, plants and plant enemies: Hypothesis and synthesis. American Naturalist 167:141-52.

Bolan, N.S. 1991. A critical review of the role of mycorrhizal fungi in the uptake of phosphorus. Plant and Soil 134: 189-207.

Breen, J.P., 1994. *Acremonium* endophyte interactions with enhanced plant resistance to insects. Annual Review of Entomology 39, 401–423.

Bronstein, J. L., 1994. Our current understanding of mutualism. The Quarterly Review of Biology 69: 31D51.

Bush L., and R.C. Buckner., 1973. Tall fescue toxicity. Pgs. 99-112. Anti-quality components of forages. Ed. Matches, A.G. Crop Science Society of America, Madison, WI.

Bush, L.P., Wilkinson, H.H., Schardl, C.L., 1997. Bioprotective alkaloids of grass–fungal endophyte symbiosis. Plant Physiology 114, 1–7.

Cheplick, G.P., Clay, K., Marks, S., 1989. Interactions between infection by endophytic fungi and nutrient limitation in the grasses *Lolium perenne* and *Festuca arundinacea*. New Phytologist 111, 89–97.

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

Cheplick, G.P., Perera, A., Koulouris, K., 2000. Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. Functional Ecology 14, 657–667.

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

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

Cheplick, G.P., Faeth, S.H., 2009. Ecology and Evolution of the Grass–Endophyte Symbiosis. Oxford University Press, New York.

Clay, K. 1990. Fungal endophytes of grasses. Annual Review of Ecological Systems 21:275-297.

Clay, K., Marks, S., Cheplick, G.P., 1993. Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. Ecology 74,1767–1777.

Clay, K., and J. Holah. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. Science 285:1742-1744.

Clay, K., Schardl, C., 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Naturalist 160, S99–S127.

Easton, H.S., and L.R. Fletcher. 2007. The importatnce of endophyte in agricultural systems-changing plant and animal productivity. Proceedings of the 6th International Symposium on Fungal endophytes of grasses. pgs. 11-18.

Faeth, S.H., and W.F. Fagan. 2002. Fungal endophytes: common host plant symbionts but uncommon mutualists. Integrative and Comparative Biology 42:360-368.

Fletcher L.R., Harvey I.C. (1981) An association of a *Lolium endophyte* with ryegrass staggers *New Zealand Veterinary Journal* 29, 185-186.

Fogel, R. 1980. Mycorrhizae and nutrient cycling in natural forest ecosystems. New Phytologist 86:199-212.

Gange A.C., and H.M. West. 1994. Interactions between arbuscular mycorrhizal fungi and foliar feeding insects in *Plantago lanceolata* L. New Phytol 128:79-87.

Garcia, M., O., T. Ovaspyan, M. Greas, and K. K. Treseder. 2008. Mychorrizal dynamics under elevated CO_2 and nitrogen fertilization in a warm temperate forest. Plant Soil 303: 301-310.

Graham, J.H. and J.A. Menge. 1982. Influence of vesicular-arbuscular mycorrhizae and soil phosphorus on take-all disease of wheat. Phytopathology 72:95-98.

Hart, M.M., R.J. Reader and J.N. Klironomos. 2003. Plant coexistence mediated by arbuscular mycorrihizal fungi. Trends in Ecology and Evolution 18:418-422.

Hartley, S. E., and A. C. Gange. 2009. Impacts of Plant Symbiotic fungi on Insect Herbivores: Mutualism in a Multitrophic Context. Annual Review of Entomology 54***:***323-342.

Hartnett, D.C., and G.W.T. Wilson. 2002. The role of mycorrhizas in plant community structure an dynamics: lessons from grasslands. Plant and Soil 244: 319-331.

Herre, E.A., N. Knowlton, U.G. Mueller, and S.A. Rehner. 1999. The evolution of mutualisms: exploring the paths between conflict and cooperation. Trends in Ecology 14:49-53.

Hesse, U., Schoberlein, W.,Wittenmayer, L., Forster, K., Warnstorff, K., Diepenbrock, W., Merbach, W., 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne L.* genotypes. Grass and Forage Science 58, 407–415.

Hesse, U., Hahn, H., Andreeva, K., Forster, K., Warnstorff, S.W., Diepenbrock, W., 2004. Investigations on the influence of *Neotyphodium* endophytes on plant growth and seed yield of *Lolium perenne* genotypes. Crop Science 44, 1689–1695.

Hirsch A.M. 2004. Plant-microbe symbioses: A continuum from commensalism to parasitism. Symbiosis 37:345-363.

Johnson N.C., J.H. Graham, and F.A. Smith. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytologist 135:575-585.

Jones M.D., and S.E. Smith. 2004. Exploring funcational definitions of mycorrhizas: are mycorrhizas always mutualisms? Canadian Journal of Botany 82:1089-1109.

Kannadan S, and J. A. Rudgers. 2008. Endophyte symbiosis benefits a rare grass under low water availability. Functional Ecology 22: 706-713.

Latch, G.C.M., Christensen, M.J., 1982. Ryegrass endophyte, incidence and control. New Zealand Journal of Agriculture and Research 25, 443–448.

Lugtenberg B., and F. Kamilova. 2009. Plant-Growth-Promoting Rhizobacteria. Annual Review of Microbiology 63:541-556.

Malinowski, D.P., H. Zuo, B.A. Kramp, J.P. Muir, and W.E. Pinchak. 2005. Obligatory summer-dormant cool-season perennial grasses for semiarid environments of the Southern Great Plains. Agronomy Journal 97:147-154.

Marks, S., K. Clay. G.P. Cheplick. 1991. Effects of fungal endophytes on interspecific and intraspecific competition in the grasses *Festuca Arundinacea* and *Lolium Perenne*. Journal of Applied Ecology 28:194-204.

Miller, D.J., S. Mackenzie, M. Foto, G.W. Adams, J.A. Findlay. 2002. Needles of white spruce inoculated with rugulosin producing endophytes contain rugulosin reducing spruce budworm growth rate. Mycological research 106:471-479.

Miller, J. D., 1986. Toxic metabolites of epiphytic and endophytic fungi of conifer needles. In: N. J. Fokkema and J. van den Heuvel (eds), *Microbiology of the Phyllosphere*. Cambridge University Press, Cambridge, England: pgs. 223-231.

Neuhauser, C. and J.E. Fargione. 2004. A mutualism-parasitism continuum model and its application to plant-mycorrhizae interactions. Ecological Modeling 117:337-352.

Ravel, C., Courty, C., Coudret, A., Charmet, G., 1997. Beneficial effects of *Neotyphodium lolii* on the growth and water status in perennial ryegrass cultivated under nitrogen deficiency or drought stress. Agronomie 171, 173–181.

Redman R.S.,K.B. Sheehan,R.G. Stout, R.J Rodriguez,and J.M Henson. 2002 Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. Science 298: 1581–1582.

Rodriguez, R.J., R.S. Redman, and J.M Henson. 2004. The role of fungal symbioses in the adaption of plants to high stress environments. Mitigation and Adaption Strategies for Global Change 9:261-272.

Rodriguez, R.J., J. Henson, E. VanVolkenburgh, M. Hoy, L. Wright, F. Beckwith, Y. Kim, and R.S. Redman. 2008. Stress tolerance in plants via habitat-adapted symbiosis. International Society for Microbial Ecology 2:404-416.

Rogers, A., E. A. Ainsworth, and A. D. B. Leakey. 2009. Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes? Plant Physiology 151:1009-1016

Rudgers, J.A., J.M. Koslow, and K. Clay. 2004. Endophytic fungi alter relationships between diversity and ecosystem properties. Ecology Letters 7:42-51.

Rudgers, J.A., W.B. Mattingly, and J.M. Koslow. 2005. Mutualistic fungus promotes plant invasion into diverse communities. Oecologia 144:463-471.

Rudgers, J.A., J. Holah, S.P. Orr, and K. Clay. 2007. Forest succession suppressed by an introduced plant-fungal symbiosis. Ecology 88:18-25.

Rudgers, J.A., and K. Clay. 2008. An invasive plant-fungal mutualism reduces arthropod diversity. Ecology Letters 11:1-10.

Saikkonen, K., S.H. Faeth, M. Helander, and T.J. Sullivan. 1998. Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Seystematics 29:319-343.

Saikkonen, K., P. Wali, M. Helander, and S.H. Faeth. 2004. Evolution of endophyte –plant symbioses. Trends in Plant Science 9:275-280.

Schulthess, F.M., Faeth, F.M., 1998. Distribution, abundances, and associations of the endophytic community of Arizona fescue (Festuca arizonica). Mycologia 90, 569–578.

Smith G.S. and R.W. Roncadori. 2006. Response of three vesicular – arbuscular mycorrhizal fungi at four soil temperatures and their effects on cotton growth. New Phytologist 104:89-95.

Staddon, P.L., A. Heinemeyer, and A.H. Fitter. 2002. Mycorrhizas and global environmental change: research at different scales. Plant and Soil 244: 253-261.

Thompson, J.N. 1988 Variation in interspecific interactions. Annual Review of Ecology, Evolution, and Systematics. 19: 65–87.

Timmusk S., and E.G.H. Wagner.1999. The plant growth promoting rhizobacterium P*aenibacillus polymyxa* induces changes in *Arabidopsis thalianan* gene expression: A Possible connection between biotic and abiotic stress responses. Molecular Plant-Microbe Interacttions 12:951–959.

van der Heinden, M.G. 2010. Mycorrhizal fungi reduce nutrient loss from model grassland ecosystems. Ecology 91:1163-1171.

van Loon, L.C. 2007. Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant Pathol 119:243-254.

Volpin, H., Y Elkind, Y. Okon, and Y. Kapulnik. 1994. A vesicular arbuscular mycorrhizal fungus (*Glomus intraradix*) induces a defense response in alfalfa roots. Plant Physiology 104:683-689.

White, J.F., Jr. 1987. Widespread distribution of endophytes in the Poaceae. Plant Disease 71:340-342.

White, J.F., Jr., G. Morgan-Jones and A.C. Morrow. 1993. Taxonomy, life cycle, reproduction and detection of *Acremonium* endophytes. Agriculture, Ecosystems and Environment. 44:13-37.

Widdup, K.H., and D.L. Ryan. 1992. Forage potential of wild populations of perennial ryegrass collected from southern New Zealand farms. Proceedings of the New Zealand Grassland Association 54: 161-165.

Wilkinson, H.H., Schardl, C.L, 1997. The evolution of mutualism in grass-endophyte associations. In: Bacon, C.W., Hill, N.S. (Eds.), Neotyphodium/Grass Interactions. Plenum Press, New York, pp. 13–25.

Wilson, D. 1995. Endophyte- the evolution of a term, and clarification of its use and definition. Oikos 73:274-276.

Yano-Melo A.M., O.J. Saggin, and L.C. Maia. 2003. Tolerance of mycoffhized banana (*Musa* sp. Cv. Pacovan) plantlets to saline stress. Agriculture Ecosystems & Environment 95:343-348.

Zobel, M. and M. Moora. 1995. Interspecific competition and arbuscular mycorrhizae: importance for the coexistence of two calcareous grassland species. Folia Geobotanica Phytotaxon 30:223-230.

Chapter 2: Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region*

Abstract:

Perennial ryegrass (*Lolium perenne)* is a cool-season grass species that is often infected by the leaf-inhabiting endophyte *Neotyphodium lolii.* This particular endophyte is asexual and has the potential to impact host survival, growth and reproduction. The objective was to assess the potential costs or benefits of endophyte infection on drought stress tolerance of native perennial ryegrass accessions originally collected from Italy, Morocco, Tunisia, and Turkey. Sixty infected (E+) individuals from each accession were planted in a greenhouse. Half of these individuals were treated with a systemic fungicide to eliminate the endophyte (E-). For two drought periods water was withheld for 10-14 days and then allowed a one week recovery period following each. In some accessions under drought, E+ plants had more tillers, greater tiller lengths, total dry mass and green shoot mass than E- plants, suggesting a positive effect of endophyte infection on host growth. Total tiller length and the number of tillers showed significant population*treatment*infection interactions for 4 of 6 populations. This work is unique in that it documents the effects of endophyte infection for a common forage grass species from wild populations native to its distributional range. The results suggest that endophyte infection can help ameliorate abiotic stress such as drought and there may be a selective advantage for grasses from certain Mediterranean regions.

Keywords: Endophytes; *Lolium perenne* (perennial ryegrass); Mediterranean; Drought Stress

Introduction

On a global scale there is accumulating evidence that climate is changing. In the Mediterranean region, climate models predict increases in temperature and decreases in precipitation (Cubash et al., 1996), which may lead to the increase in duration and severity of summer droughts. The magnitude and rate of climate change will critically affect the extent to which plants in ecological systems can withstand stress and their ability to adapt. Stationary organisms such as plants may be the most limited in their ability to cope with environmental change (Jump & Peňuelas, 2005) as environmental conditions such as temperature, light and water availability play a key role in defining the role and distribution of plants in natural ecosystems. In addition, the response of plants to an altered environment may depend on their ability to form symbiotic relationships with microorganisms.

Fungal endophytes are obligate biotrophs that live in symbiotic relationships with hundreds of grass species worldwide (White, 1987; Clay, 1990). In a few cultivated grass species, fungal endophytes can improve host competitive abilities through increased vegetative growth and reproduction (Cheplick et al., 1989; Marks et al., 1991; Malinowski et al., 1997), resistance to herbivores and seed predators (Cheplick and Clay, 1988; Clay et al., 1993; Breen, 1994; Bush et al., 1997) and enhanced tolerance to abiotic stressors such as drought and flooding (Saikkonen et al., 1998; West, 1994; Read and Camp, 1986; West, 1993). The ability of fungal endophytes to confer stress tolerance to plants may provide a novel strategy for appeasing the impacts of global climate change on agricultural and native plant communities.

Perennial ryegrass, *L. perenne,* is one of the most important pasture, forage, and turfgrasses in the world. It is a cool-season grass that has been introduced into many temperate regions of North America, and is native to Northern Europe, Asia and Africa (Jung et al., 1996).

It can tolerate a wide range of environmental stresses and grazing conditions that has undeniably contributed to its ecological success. It is possible that this success is due to the presence of the leaf-inhabiting fungus *Neotyphodium lolii,* which may be responsible for imparting some of the benefits this grass species exhibits in forage ecosystems and the range of biotic and abiotic stresses it tolerates.

In *L. perenne,* the effects of endophytes on drought and recovery from drought have been ambiguous and like most grass-endophyte symbioses observed in nature, this may span a continuum of interactions from mutualistic to antagonistic (Cheplick et al., 2000; Cheplick, 2008; Wilkinson and Schardl, 1997; Saikkonen et al., 1998; Faeth and Sullivan, 2003; Ahlholm et al., 2002; Hahn et al., 2008). Perennial ryegrass populations in nature exist in a mixture of endophyte infected and uninfected grasses (Bradshaw 1959; Clay 1990; Leuchtmann 1992). Endophyte infection frequencies are the proportion of individuals in a population that contain a fungal endophyte (Cheplick and Faeth, 2009). If endophyte infection is mutualistic it would be expected for populations to have high endophyte infection frequencies. Infection frequencies of agronomic grasses usually increase within a few years to 100% because of increases in general competitive abilities (Clay and Holah, 1999). *Neotyphodium* infection frequencies in natural grass populations are much more variable ranging from 0-100%. This variability in infection frequencies among native grass species suggest that the costs and benefits of endophyte infection differ among environments (Faeth, 2002). Variability could also be explained by imperfect transmission, where the endophyte fails to grow into some seeds or if the endophyte is randomly lost through hyphae inviability in seeds or adult plants (Siegel et al. 1984). Assessing lifetime costs and benefits has been challenging because the frequency of asexual endophytes, such as *Neotyphodium*, in natural populations is quite variable and it not always near or at 100%

infectivity. Within grass species that harbor asexual endophytes, the frequency of infection can vary among populations, individuals, and tillers within individuals (Saikkonen et al., 2000, Schulthess and Faeth, 1998).

While systemic endophytes in agronomic grasses have been well studied, the interactions between host plants and endophytes in natural populations and communities are poorly understood (Faeth, 2002; Saikkonen et al., 2006; Cheplick and Faeth, 2009). Given its importance in pasture and turf communities it is critical to elucidate the role of *N. lolii* on the growth of perennial ryegrass under a variety of environmental conditions. Here I tested the hypothesis that the endophyte *N. lolii* would ameliorate the effects of drought stress for *Lolium perenne* populations from its native range in the Mediterranean region.

2. Material and Methods

2.1 Seed sources for experiments

Seeds of *Lolium perenne* were obtained from the Western Regional Plant Introduction Station, United States Department of Agriculture, Pullman, Washington, USA (National Plant Germplasm System; http://www.ars-grin.gov/npgs). To ensure adequate genetic diversity, seeds of each accession were collected from 50-100 plants with space isolation of 50m to minimize outcrossing between *Lolium* and *Festuca* accessions/species (Clement et al., 2001). As a predominately outcrossing species (Beddows, 1967), *Lolium perenne* is genetically variable within and between cultivars and individuals arising from seeds are expected to represent distinct genotypes (Huff, 1997, Kubik et al., 2001).

The accession from Italy (PI 598931) was collected in Sardinia (41◦5’9”__N, 9◦21’_31”__E), the accession from Turkey (PI 598512) was collected along a mountainside in Antalya at 840m elevation (Nelson et al., 1993). The accession from Morocco (PI 598854) was collected from a moist meadow (33◦29’9”_N, 6◦8’_45”_W) and the Tunisia accession (PI 598909) was obtained in Skalba near a moist floodplain (36◦49’29”_N, 10◦58’_47’_E).

Two cultivars of *L. perenne* known to be infected were also used for comparison: seeds of Repell II and Palmer II, were obtained from Loft’s/Pennington Seed Company, Inc. (Madison, Georgia, USA). Information on the development of these registered cultivars from clones collected from old turf areas in Maryland, New York, and New Jersey, USA can be found in Hurley et al. (1994 a, b).

2.2 Endophyte assessment and elimination of the endophyte

About 100 seeds from each accession and cultivar were sown into pots containing a 1:1 soil mix of topsoil and vermiculite. Three-four weeks later, 60-70 seedlings per accession and cultivar were randomly selected and replanted individually into 9 oz plastic cups. When individuals were large enough, all leaf sheaths were excised and cleared in 70% ethanol for at least one day. Each segment was stained for 10 min with aniline blue-lactic acid (Bacon and White, 1994) and examined with a light microscope at 400 X. The presence of intercellular hyphae indicated endophyte presence.

Sixty E+ seedlings were randomly selected and transplanted into 9 oz plastic cups in a 2:1:1 mixture of topsoil, peat moss and vermiculite. Plants were maintained in a greenhouse and served as a stock culture. Three months later when the genotypes had produced multiple ramets, half the ramets of each genotype were subjected to fungicide treatment to eradicate the endophyte. The fungicide benomyl (methyl 1-[(butylcarbamoyl]-2-benzimidazolcarbamate)-(Sigma-Aldrich Chemical Company, Saint Louis, Missouri, USA) was added to the soil as a 1 g/L aqueous solution. Past research has indicated that this fungicide is an effective way of eliminating *Neotyphodium* endophytes without showing phytotoxic effects (Latch and Christensen, 1982) and has no apparent effect on plant growth (Cheplick et al., 1989; Marks et al., 1990; Marks et al., 1991). Plants were allowed to grow in the greenhouse where ample sunlight was provided, until enough time had elapsed for a new tiller to become available. After two months, the leaf sheath from the new tiller was collected, stained and microscopically analyzed to determine the efficacy of the fungicide. Only newly formed daughter ramets, made by fungicide treated parental ramets were used in the experiment, which began about 3 months after fungicide treatment. The other half of the plants remained endophyte infected (E+). Before the start of the experiment, all genotypes were grown in round plastic pots containing a 2:1:1 mixture of topsoil, peat moss and vermiculite.

2.3 Drought stress experiment

In total, there were 360 plants at the beginning of the experiment. There were 60 plants per accession and cultivar, half of which were E+ and the other half E-. Thirty E+ and thirty E- plants were planted into 9oz plastic cups containing a 2:1:1 mixture of topsoil, peat moss and fine vermiculite. These plants were then separated into three water treatments so that10E- and 10E+ genotypes per accession/cultivar were in each treatment. All plants were maintained in the greenhouse and watered as needed.

All plants were watered before the first drought period so that soil moisture was at 100%. A soil moisture probe (Field Scout TDR 100, Spectrum Technologies Inc, Plainfield, Illinois, USA) was used to determine the percentage of soil saturation. There were two sequential drought periods each lasting 10-14 days and after each, a one -week recovery period. There was a high water treatment that served as a control where plants were watered as needed. When watered, the soil moisture level in this treatment always reached a maximum of 100%. For the drought treatment water was withheld to induce drought conditions and soil moisture was less than 10%. In the medium water treatment, percent soil moisture ranged from 50-80%. When the soil saturation reached a level below 50%, water was given until soil moisture reached 100%.

2.4 Response variables

A few days prior to the first drought, the initial number of tillers (ITTL) was recorded and used as a covariate. Total tiller length (TTL), which is the total summed length of all tillers for each plant, was also measured and recorded. TTL was recorded by measuring with a ruler the length of each tiller from the base to the tip of the leaf. After each recovery period, I recorded the number of tillers and the total tiller length. At the end of the experiment, the shoots were clipped 1 cm above the soil surface and the roots were washed, bagged and dried in a drying oven at 60° C. Clipped shoots, including both green (alive) and brown (dead) tillers, and tiller bases were separately retained and dried to a constant mass. Using an electronic balance, the dry mass of roots, shoots and tiller bases were obtained.

2.5 Data Analysis

Analysis of covariance (ANCOVA) was employed to evaluate the primary factors using a general linear model that included treatment (control, medium water, and drought), population (four accessions and two cultivars), and infection status (E- or E+) and all interactions. The variable

ITTL was treated as the covariate, and was recorded prior to the first drought period. Root: shoot ratio (log10-transformed) was also calculated and analyzed as above.

The GLM procedure of the Statistical Analysis System, Version 8.2 (SAS institute, Cary, North Carolina, USA) was used for all analyses. If the covariate was significant, least square means, adjusted for the covariate, were used for data tables and figures. If the covariate was not significant, then a three-way ANOVA was used for the three factors involved and their interactions. The Tukey post- hoc test was utilized to make comparisons among means.

3. Results

3.1 After the first drought

The number of tillers after the first drought did not depend on the experimental treatment (watered vs. drought). Across all accessions and cultivars, the number of tillers was significantly affected with E+ plants having significantly more tillers than E- plants (table 1). There was also a significant interaction of treatment with infection (table 1, figure 1A) and this demonstrates the effect of endophyte on tiller production varied under the different soil moisture levels. E+ plants had significantly more tillers in the drought treatment compared to E- plants (mean ±1SE 6.13 ± 0.05 E+ vs. 4.62 ± 0.05 E-).

Total tiller length after the first drought was also significantly affected by infection (table 1) with infected plants having greater tiller lengths than uninfected plants. There was also a significant treatment by infection interaction (table 1, figure 1B). The total tiller length of endophyte- infected plants under drought was significantly greater than E- plants in drought (95.55± 6.37 mm E+ vs. 67.21± 6.16 mm E-).

3.2 After the second drought

The number of tillers after the second drought was significantly affected by treatment, population and their interaction (table 1). Drought significantly reduced the number of tillers for all plants exposed to drought stress compared to the control and medium group (mean ±1SE 6.49±.023 Control, 6.27±.023 Medium vs. 1.78±.023 for drought). Although, the effect of infection was not significant, there was a significant three-way interaction (treatment x population x infection) (Table 1, Fig.2.2), which indicates the effects of endophyte infection on tiller numbers depended on the experimental treatment and the accession. For the accessions from Morocco and Tunisa, endophyte-infected plants under drought had significantly more tillers than uninfected plants under drought. In the accession from Italy there were no significant differences between E+ and E- plants or plants in the control vs. drought. Grasses from Turkey grew significantly better in the control and medium water treatments, with differences between E+ and E- grasses. There was no difference between E+ and E- grasses under drought for this accession.

Total tiller length after the second drought was affected by treatment, population, and infection (Table 1). The amount of water available affected total summed lengths of tillers for all plants. Drought significantly reduced tiller length (25.66±3.84mm in drought vs. 113.17±3.84mm for control and 90.47±3.84 mm for medium water treatment). Infected individuals were significantly larger than uninfected individuals (101.92±3.20mm for E+ and

70.95±3.20mm for E-). There was a significant treatment by population interaction (Table 1) and all accessions except Italy exhibited different responses to drought. Grasses from Morocco, Palmer, Repell, Turkey, and Tunisia showed a decrease in tiller length under drought compared to grasses in the control. There was a significant three way interaction (treatment X population X infection) for total tiller length measured after the second drought period (Table 1, Fig. 2.3). E+ grasses from Morocco and Tunisia had greater tiller lengths under drought than E- grasses (Fig. 2.3B, D). There was no significant difference in the control group for E+ and E- individuals. For the accession from Turkey, E+ grasses grew better in the control where water was continuously available and under the medium water treatment where the soil was constantly moist. There was no difference between E+ and E- plants from Turkey under drought. The tiller lengths of E+ grasses were also longer than E- grasses in the cultivars Palmer II and Repell II (Fig. 2.3 E, F).

Across all accessions and cultivars, green shoot mass was 128.45± 6.544 mg for E+ and 107.22 ±6.53 mg for E- plants. The only accession that showed a significant difference between E+ and E- for this was Turkey and E+ (194.79±15.64mg) plants had more photosynthetic tissue than E- (103.30±15.64mg) plants (table 2,figure 2.4A). The effect of endophytes on green shoot mass varied across accessions and water treatments.

Total dry mass at harvest was significantly greater in the control groups (mean ±1 SE=525.5±16.7mg in control vs.359.6±16.7mg in the drought), and there was an interaction of population and infection Table 2, Figure 4.) Figure 4B illustrates this interaction for Turkey. Total dry mass in the accession from Turkey was greater for E+ 628.43±33.52mg plants then for E- 445.51±33.51mg.

Drought significantly reduced shoot mass and root mass compared to the control and medium water treatment (Table 2, 4, Fig.5). E+ grasses from Turkey had greater shoot mass than did uninfected grasses (E+ 431.54 mg vs. E- 302.12 mg). For both of these variables there was an interaction of treatment by populations (table 2, Figure 5A, B). At the end of the experiment, grasses from the accessions Morocco, Turkey and Tunisia under drought had less shoot mass than in the control group (5A). The accession from Turkey also had significantly less root mass under drought than in the control; the cultivar Palmer exhibited the same response (figure 5B).

Discussion

4.1 Benefits of endophyte infection on growth of perennial ryegrass

Fungal endophytes are generally considered to be plant mutualists (Clay, 1990, Clay and Schardl, 2002) providing a variety of advantages to infected hosts. However, few studies in natural systems have directly examined this issue since cultivated plants receive far more attention (Saikkonen et al., 2006). For example, *Neotyphodium coenophialum*, the endophyte which infects tall fescue (*Lolium arundinaceum*), ameliorates the effects of drought stress through altered water potential, enhanced osmotic adjustment and survival and growth of infected compared to noninfected plants (Malinowski and Belesky, 2000, Elmi and West, 1995, West, 1994). However, the reported benefits of the endophyte *Neotyphodium lolii* on the drought stress tolerance of perennial ryegrass have been inconsistent. Some have reported positive effects (Ravel et al., 1997, Lewis et al., 1997, Hesse et al., 2003, Hesse et al., 2004 and Hahn et al., 2008), while Cheplick et al., (2000), Cheplick (2004), and Eerens et al., (1998) found negative effects and Barker et al., (1997) found no measurable effect of endophyte infection. Enhanced drought tolerance is a well-known benefit of endophyte infection in tall fescue and perennial ryegrass (Arechavaleta et al., 1989; Bouton et al., 1993; Lewis et al., 1997; Malinowski et al., 1997) and may be the most thoroughly documented feature of abiotic-stress tolerance in endophyte-infected plants (Malinowski and Belesky, 2000). Future studies using native grasses from areas where severe drought is known to occur will give insight into how endophytes have helped plants adapt to their native environments and if endophytes can help mitigate the effects of climate change.

In this experiment, accessions from native populations of endophyte infected *Lolium perenne* were selected because they are widespread throughout Europe, where stress from summer drought is common. It is suggested that summer drought conditions may impart a selection pressure in favor of infection (Lewis et al., 1997) and since high infection frequencies of this symbiosis have been reported endophyte infection should promote drought tolerance. Future climate projections predict that areas in the Mediterranean will experience longer, more severe drought periods (Cubash et al., 1996). The results from this experiment suggest that infection may play some role in success of perennial rye grass Mediterranean regions under climate change.

All accessions from the native distributional range of *L. perenne* significantly varied in tiller numbers, tiller length, green shoot mass, and total dry mass, including shoot and root mass. These results are not surprising given the substantial genetic variation found with *L. perenne* populations (Cheplick 1998, 2004; Hesse et al., 2003, 2004). Also, there may be a continuum of interactions of endophyte effects on host growth (Cheplick et al., 1989, Ahlholm et al., 2002, Cheplick, 1998, Cheplick et al., 2000, Cheplick, 2004, Hesse et al., 2004, Cheplick, 2007) that depend on the interacting effects of abiotic, biotic and genetic factors which include endophyte and host genotype (Clay, 1988; Saikkonen et al., 1998, Cheplick and Faeth, 2009).

Significant three way interactions were observed for the number of tillers, and total tiller length showing endophyte effects specific for particular accessions and cultivars under different water treatments. These effects were apparent for endophyte-infected grasses from Morocco and Tunisia. Drought significantly reduced the tiller length of grasses from these regions, but endophyte infected hosts from these populations grew better under drought than non-infected hosts. In the accession from Turkey I demonstrated that E+ grasses from this region grew better under watered conditions compared to E- but there was no statistical difference between E+ and E- under drought. The results imply that it is important to recognize the natural habitat in which the populations originate. Endophyte plant associations are adapted to their native range of environmental growth conditions (Hesse, 2003), implying that environmental change can disturb the balance of the interaction, resulting in negative endophyte effects on plant growth. For example, Hesse (2003) found that infected *Lolium perenne* genotypes in Germany from a dry and flood-dry environment grew better under drought than uninfected genotypes, whereas the genotype that lives naturally in a wet environment was negatively affected by endophyte infection when placed under drought stress.

It is also prudent to examine native grasses infected by variable endophyte genotypes if a complete understanding of the ecological genetics of the grass endophyte symbiosis is to be met. This is due to the existing interaction between different endophyte and host genotype combinations and different effects on host growth and reproduction are to be expected for different associations. Studies using isozymes (Leuchtmann and Clay 1990) and DNA markers (van Zijll de Jong et al., 2003) have concluded that the genotypic diversity of *Neotyphodium* endophytes in tall fescue and perennial ryegrass is relatively low. Currently, there is not much known about whether genetic variation in endophytic fungi impacts host grass populations. Other studies demonstrate that endophyte genotype can influence host growth (Hill 1996), having more studies of this kind are greatly needed.

In this study, I demonstrated that the presence of the *N. lolii* endophyte significantly affected morphological growth responses (tiller numbers and tiller length) of the host plant in some populations. Differences between E+ and E- grasses under drought were found for 4 out of 6 populations and the results do support a protective role for the endophyte under water deficit. In conclusion, the results suggest that for some *L. perenne* populations from the Mediterranean region, endophyte infection can help alleviate abiotic stressors such as drought and there may be a selective advantage for grasses from some Mediterranean regions to have mutualistic interactions with endophytes.

Chapter 2: Literature Cited

Arachevaleta, M., Bacon, C.W., Hoveland, C.S., Radcliffe, D. E. 1989. Effect of the tall fescue endophyte on plant response to environmental stress. Agronomy Journal 81, 83-90.

Ahlholm, J. U., Helander, M., Lehtimaki, S., Wali, P., Saikkonen, K., 2002. Vertically transmitted fungal endophytes: different responses of host-parasite systems to environmental condition. Oikos 99:173-183.

Bacon, C.W., and White, J.F., 1994. Stains, media and procedures for analyzing endophytes. In: Bacon, C.W., and White Jr., J.F. (Eds.), Biotechnology of Endophytic Fungi of Grasses. CRC Press, Boca Raton, FL. Pp. 47-56.

Barker, D.J., Hume, D.E., Quigley, P.E., 1997. Negligible physiological responses to water deficit in endophyte-infected and uninfected perennial ryegrass. In: Bacon, C.W., Hill, N.S., (Eds.). Neotyphodium/Grass Interactions. Plenus Press, New York, pp. 137-139.

Beddows, A.R., 1967. Biological flora of the British Isles: *Lolium perenne* L. J. Ecol. 55,567-587.

Bradshaw, A.D., 1959. Population differentiation in *Agrotis tenuis* Sibth II. The incidence and significance of infection by *Epichloe typhina*. New Phytologist, 58, 310-315.

Breen, J.P., 1994. *Acremonium* endophyte interactions with enhanced plant resistance to insects. Annual Review of Entomology 39, 401-423.

Bush, L.P, Wilkinson, H. H., Schardl, C. L., 1997. Bioprotective alkaloids of grass-fungal endophyte symbiosis. Plant Physiology 114,1-7.

Bouton, J.H., Gates, R.N., Belesky, D.P., Owsley, M., 1993. Yield and persistence of tall fescue in the southeastern coastal plain after removal of its endophyte. Agron. J. 85,52–55.

Cheplick, G.P, Clay, K., 1988. Acquired chemical defenses in grasses: the role of fungal endophytes. Oikos 52, 309-318.

Cheplick, G.P., Clay, K., Marks, S., 1989. Interactions between infection by endophytic fungi and nutrient limitation in the grasses *Lolium perenne* and *Festuca arundinacea*. New Phytologist 111, 89-97.

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

Cheplick, G.P., Perera, A., Koulouris, K., 2000**.** Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. Functional Ecology 14, 657-667.

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

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

Cheplick, G.P., 2008. Host genotype overrides fungal endophyte infection in influencing tiller and spike production of *Lolium perenne* (Poaceae) in a common garden experiment. American Journal of Botany 95, 1063-1070.

Cheplick G. P., Faeth S. H. 2009. *Ecology and evolution of the grass-endophyte symbiosis*. Oxford University Press, New York.

Clay, K., 1990. Fungal endophytes of grasses. Annual Review of Ecology and Systematics 21, 275-297.

Clay, K., Marks, S., Cheplick, G.P., 1993. Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. Ecology 74, 1767-1777.

Clay, K., Holah, J., 1999. Fungal endophyte symbiosis and plant diversity in successional fields. Science, 285, 1742-1744.

Clay, K, Schardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Naturalist 160, S99-S127.

Clement, S.L., Elberson, L.R., Youssef, N.N., Davitt, C.M., Doss, R.P., 2001. Incidence and diversity of Neotyphodium fungal endophytes in tall fescue from Morocco, Tunisia, and Sardinia. Crop Science 41, 570-576.

Cubasch, U., von Storch, H., Waszkewitz, J., Zorita, E., 1996. Estimates of climate change in southern Europe derived from dynamical climate model output. Clim. Res., 7, 129-149.

Elmi, A.A., West, C.P., 1995. Endophyte infection effects on stomatal conductance and osmotic adjustment, and drought recovery of tall fescue. New Phytologist 131, 61-67.

Eerens, J.P.J., Lucas, R.J., Easton, S., White, J.G.H., 1998. Influence of the endophyte (Neotyphodium lolii) on morphology, physiology, and alkaloid synthesis of perennial ryegrass during high temperature and water stress. NZ J. Ag. Res. 41, 219-226.

Faeth, S.H., 2002. Are endophytic fungi defensive plant mutualists? Oikos 98, 25-36.

Faeth, S. H., Sullivan, T. J., 2003. Mutualistic asexual endophytes in a native grass are usually parasitic. American Naturalist 161, 310-325.

Hahn, H., McManus, M.T., Warnstorff, K., Monahan, B.J., Young, C.A., Davies, E., Tapper, B.A., Scott, B., 2008. *Neotyphodium* fungal endophytes confer physiological protection to perennial ryegrass (*Lolium perenne* L.) subjected to a water deficit. *Environmental and Experimental Botany* 63, 183-199.

Hesse, U.,, Schoberlein, W., Wittenmayer, L., Forster, K., Warnstorff ,K., Diepenbrock, W., Merbach, W., 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. *Grass and Forage Science* 58, 407-415.

Hesse, U., Hahn, H., Andreeva, K., Forster, K., Warnstorff, S.W., Diepenbrock, W., 2004. Investigations on the influence of *Neotyphodium* endophytes on plant growth and seed yield of *Lolium perenne* genotypes. Crop science 44, 1689- 1695.

Hill, N. S., Pachon, J.G., Bacon, C.W., 1996. *Acremonium coenophialum* mediated short and long-term drought acclimation in tall fescue. Crop science 36, 665-672.

Huff, D.R., 1997. RAPD characterization of heterogeneous perennial ryegrass cultivars. Crop Science 37, 557-564.

Hurley, R.H., Lehman, V.G., Meyer, W.A., Rose-Fricker,C.A., Dickson, W. K., Bara, R.F., Funk, D.C., Funk, C.R., 1994a. Registration of `Palmer II' perennial ryegrass. Crop Sci. 34, 539.

Hurley, R.H., Lehman, V.G., David C. Funk, D.C., C. R. Funk C.R., 1994b. Registration of ‘Repell II’ perennial ryegrass. Crop Science 34, 1409-1410.

Jump, A.S., Peňuelas, J., 2005. Running to stand still: Adaptation and the response to plants to rapid climate change. Ecology Letters, 81010-1020.

Jung, G.A., van Wijk, A.J.P., Hunt, W.F., Watson, C.E., 1996. Ryegrasses. In: Moser LE, Buxton DR, Casler MD, eds. *Cool-season forage grasses*. Madison WI, USA: American Society of Agronomy, Crop Science Society of America, Soil Science Society of America. p. 605-641.

Kubik, C., Sawkins, M., Meyer, W.A., Gaut, B.S., 2001. Genetic diversity in seven perennial ryegrass (*Lolium perenne* L.) cultivars based on SSR markers. Crop Science 41, 1565-1572.

Latch, G.C.M., Christensen, M.J., 1982. Ryegrass endophyte, incidence and control. New Zealand Journal of Agriculture and Research. 25, 443-448.

Leuchtmann, A., Clay, K., 1990. Isozyme variation in the Acremonium/ Epichloe fungal endophyte complex. Phytopathology 80, 1133-1139.

Leuchtmann, A., 1992. Systematics, distribution, and host specificity of grass endophytes. Natural toxins 1, 150-162.

Lewis G.C., Ravel C., Naffaa W., Astier C., Charmet G., 1997. Occurrence of *Acremonium* endophytes in wild populations of *Lolium* spp. in European countries and a relationship between level of infection and climate in France. Annals of Applied Biology 130, 227-238.

Malinowski, D. P., Belesky, D. P., 2006. Ecological Importance of *Neotyphodium* spp. grass endophytes in agroecosystems. Grassland Science 52, 1-14.

Malinowski, D., Leuchtmann A., Schmidt D., Nosberger J., 1997. Symbiosis with *Neotyphodium uncinatum* endophyte may increase the competitive ability of meadow fescue. Agronomy Journal 89, 833-839.

Marks, S., Clay, K., 1990. Effects of CO_2 enrichment, nutrient addition, and fungal endophyte-infection on the growth of two grasses. Oecologia 84, 207-214.

Marks, S., Clay, K., Cheplick, G.P., 1991. Effects of fungal endophytes on interspecific and intraspecific competition in the grasses *Festuca arundinacea* and *Lolium perenne*. Journal of Applied Ecology 28, 194-204.

Nelson, L.R., Marshall, D., Tunali, B., 1993. Exploration of fungal endophyte in *Lolium* and other grass species of central Turkey. In Hume, D.E., Latch, G.C.M., Easton, H.S., (Eds.), Proceedings of the Second International Symposium on Acremonium/Grass Interactions. Palmerston North, New Zealand.

Ravel, C., Courty, C., Coudret, A., Charmet, G., 1997. Beneficial effects of *Neotyphodium lolii* on the growth and water status in perennial ryegrass cultivated under nitrogen deficiency or drought stress. Agronomie 171, 173-181.

Read, J.C., Camp, B.J.,. 1986. The effect of fungal endophyte *Acremonium coenophialum* in tall fescue on animal performance and toxicity, and stand maintenance. Agronomy Journal 78, 848-850.

Saikkonen, K., Faeth, S.H., Helander, M., Sullivan, T.J., 1998. Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Systematics 29, 319-343.

Saikkonen, K., 2000. Kentucky 31, far from home. Science 287, 1887.

Saikkonen, K., Lehtonen, P., Helander, M., Koricheva, J., Faeth, S.H., 2006. Model systems in ecology: dissecting the endophyte-grass literature. Trends in Plant Science 11, 428-433.

Schulthess, F.M., and Faeth. 1998. Distribution, abundances, and associations of the endophytic community of Arizona fescue (*Festuca arizonica*). Mycologia 90, 569-578.

van Zijll de Jong, E., Guthridge, K.M., Spangenberg, G.C., Foster, J.W., 2003. Development and characterization of EST-derived simple sequence repeat (SSR) markers for pasture grass endophytes. Genome 46, 277-290.

West, C.P., Izejor, K. E., Turner, and A. A. Elmi., 1993. Endophyte effects on growth and persistence of tall fescue along a water-supply gradient. Agronomy Journal 85, 264-270.

West, C.P., 1994. Physiology and drought tolerance of endophyte-infected grasses. In *Biotechnology of endophytic fungi of grasses*, eds. C.W. Bacon and J.F. White, Jr., pp. 87-99. Boca Raton , FL:CRC Press.

Wilkinson, H. H., Schardl, C. L. 1997. The evolution of mutualism in grass-endophyte associations. In *Neotyphodium*/grass interactions, eds C.W. Bacon and N.S. Hill, pp 13-25. New York:Plenum Press.

White, J. F., Jr. 1987. Widespread distribution of endophytes in the *Poaceae*. Plant Disease 71,340-342.

Chapter 2: Tables

Table 2.1.

First Drought period Second Drought Period

		No. of Tillers			Total Tiller Length			No. of tillers			Total Tiller Length		
Source	Df	MS	F	P	MS	F	P	MS	F	P	MS	F	P
ittl	1	559.85	130.63	**0.000**	179233.7	152.23	**0.000**	709.79	112.4	**0.000**	105482.0	59.37	**0.000**
Treat	2	2.97	0.69	0.501	1036.2	0.88	0.416	846.11	134.0	**0.000**	247426.4	139.25	**0.000**
Pop	5	23.34	5.45	**0.000**	5541.0	4.71	**0.000**	51.69	8.19	**0.000**	14541.7	8.18	**0.000**
Infect	1	25.99	6.07	**0.014**	5220.4	4.43	**0.036**	8.54	1.35	0.246	10019.8	5.64	**0.018**
Treat*pop	10	5.96	1.39	0.183	1969.6	1.67	0.086	45.43	7.19	**0.000**	12678.1	7.14	**0.000**
Treat*infect	2	21.71	5.06	**0.007**	8960.2	7.61	**0.000**	5.38	0.85	0.427	769.6	0.43	0.649
Pop *infect	5	4.15	0.97	0.436	819.4	0.69	0.627	13.74	2.18	0.056	3451.9	1.94	0.087
Treat*pop*infect	10	4.89	1.14	0.331	1379.7	1.17	0.309	15.14	2.39	**0.009**	3973.4	2.24	**0.015**
Error	323	4.28			1177.4			6.31					

Table 2.2

		GSM			SM			Total Mass			Root Mass		
Source	Df	MS	F	P	MS	F	P	MS	F	P	MS	F	P
ittl	1	125134.5	17.14	**0.000**	264800.8	130.18	**0.000**	442630.3	131.94	**0.000**	80915.04	50.94	**0.000**
Treat	2	720116.2	98.65	**0.000**	34229.5	16.83	**0.000**	91276.4	27.21	**0.000**	15680.22	9.87	**0.000**
Pop	5	110845.8	15.18	**0.000**	28950.3	14.23	**0.000**	54969.2	16.39	**0.000**	29555.63	18.61	**0.000**
Infect	1	37069.3	5.08	**0.025**	12028.4	5.91	**0.015**	26682.7	7.9535	**0.005**	11949.72	7.52	**0.006**
Treat*pop	10	39379.2	5.39	**0.000**	5384.9	2.65	**0.004**	11016.9	3.2839	**0.000**	4969.21	3.13	**0.000**
Treat*infect	2	27879.9	3.82	**0.023**	2912.5	1.43	0.240	907.5	0.2705	0.763	2514.01	1.58	0.207
Pop *infect	5	21145.7	2.89	**0.014**	4344.6	2.14	0.061	7610.7	2.2686	**0.047**	1629.21	1.03	0.402
Treat*pop*infect	10	18386.9	2.52	**0.006**	3064.2	1.51	0.135	5436.9	1.6206	0.099	1675.55	1.05	0.397
Error	323	7299.8			2034.1			3354.8			1588.36		

Figure 1.

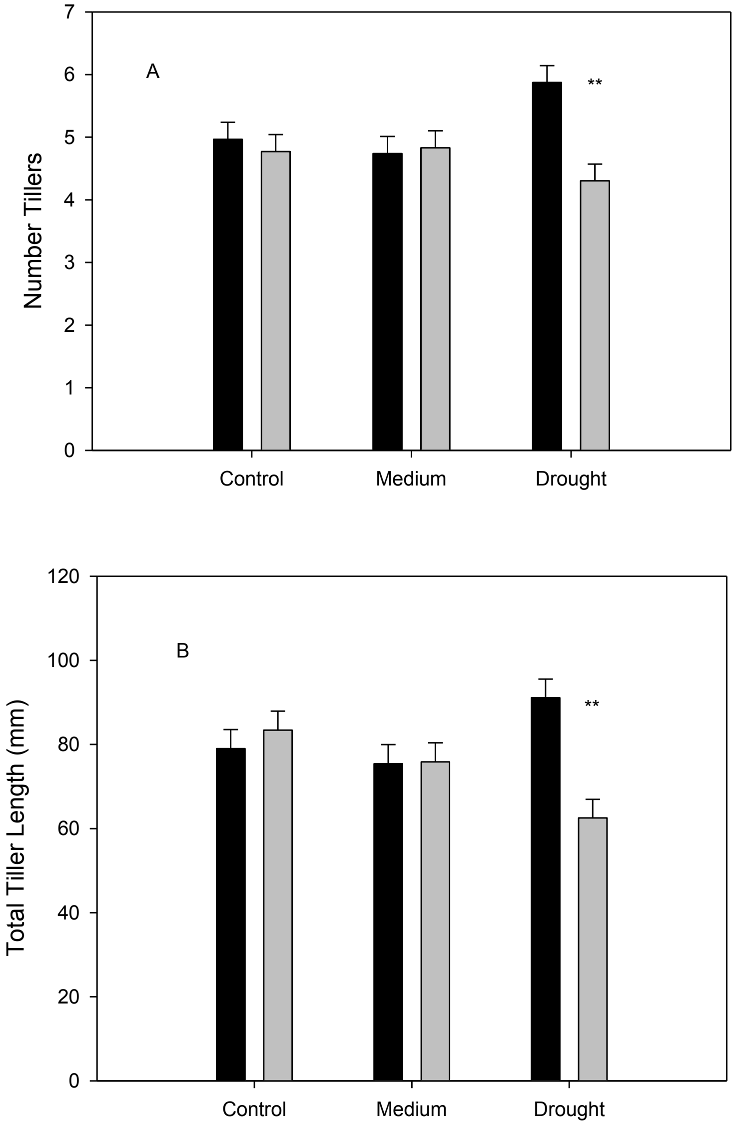

Figure 2.1.

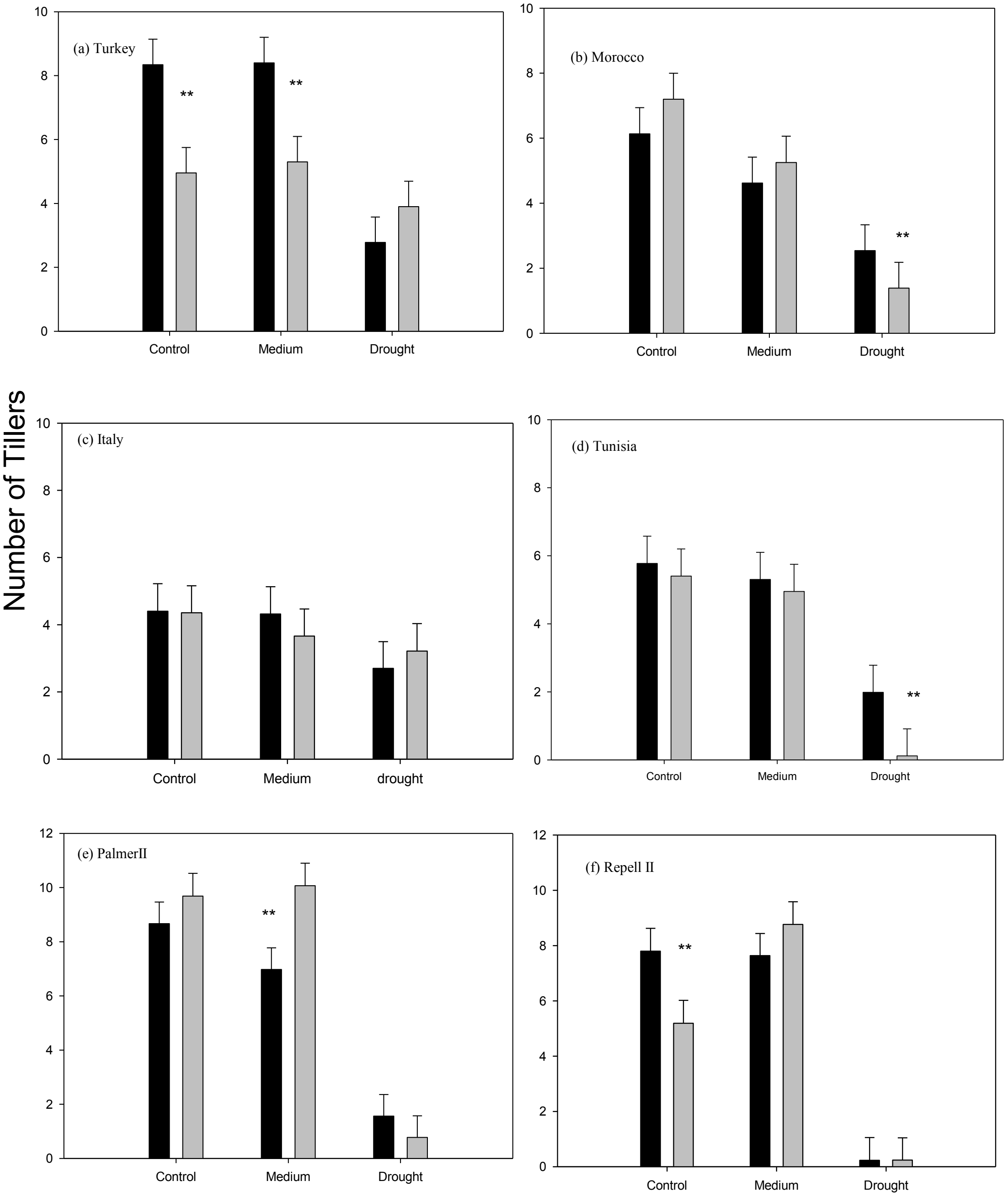
Number of Tillers
(a) Turkey
(b) Morocco
(c) Italy
(d) Tunisia
(e) PalmerII
(f) Repell II
0
2
4
6
8
10
12
**
Control
Medium
Drought
drought

Figure 2.2

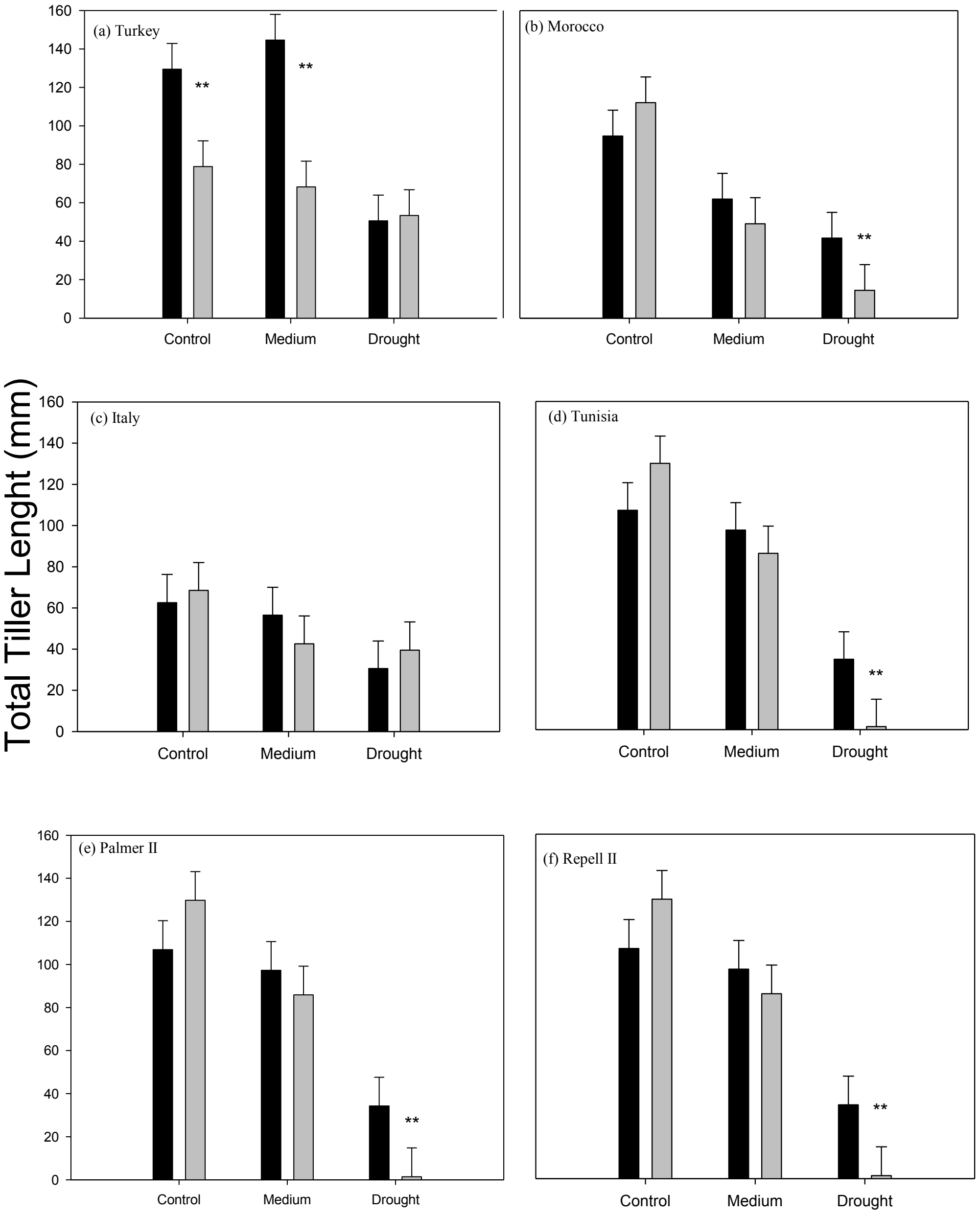
Total Tiller Lenght (mm)
(a) Turkey
(b) Morocco
(c) Italy
(d) Tunisia
(e) Palmer II
(f) Repell II
0
20
40
60
80
100
120
140
160
Control
Medium
Drought
**

Figure 2.3

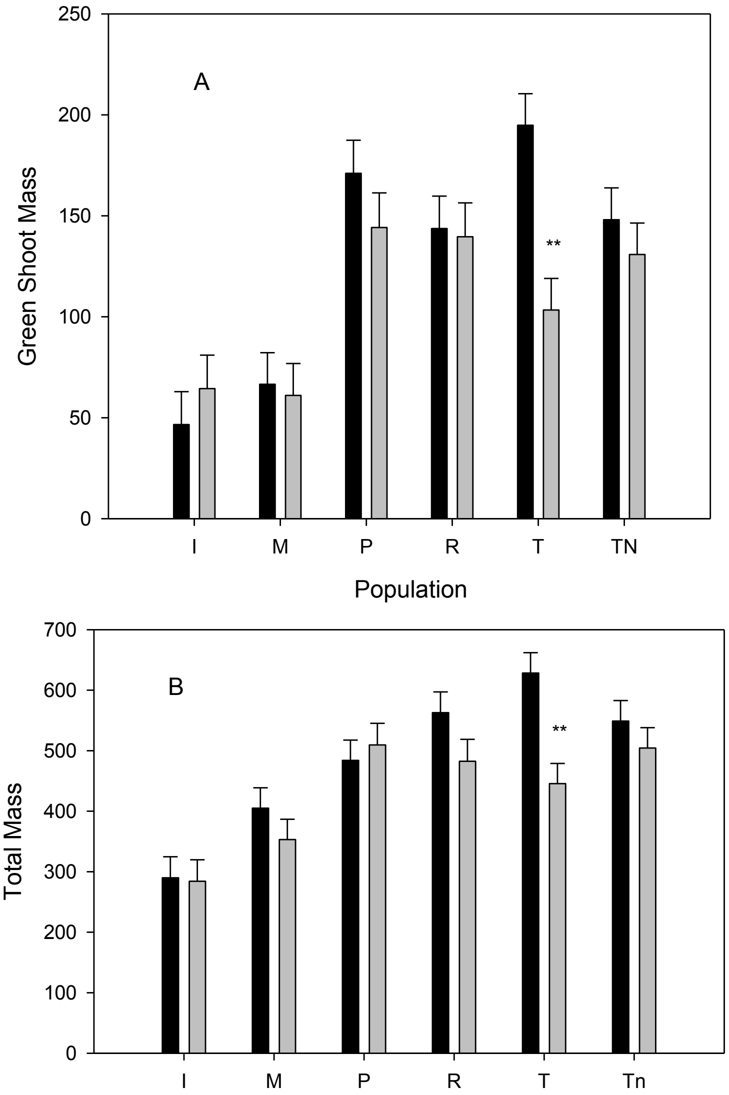
A
Green Shoot Mass
250
200
150
100
50
0
I
M
P
R
T
TN
**
Population
B
Total Mass
700
600
500
400
300
200
100
0
I
M
P
R
T
Tn
**

Figure 2.4

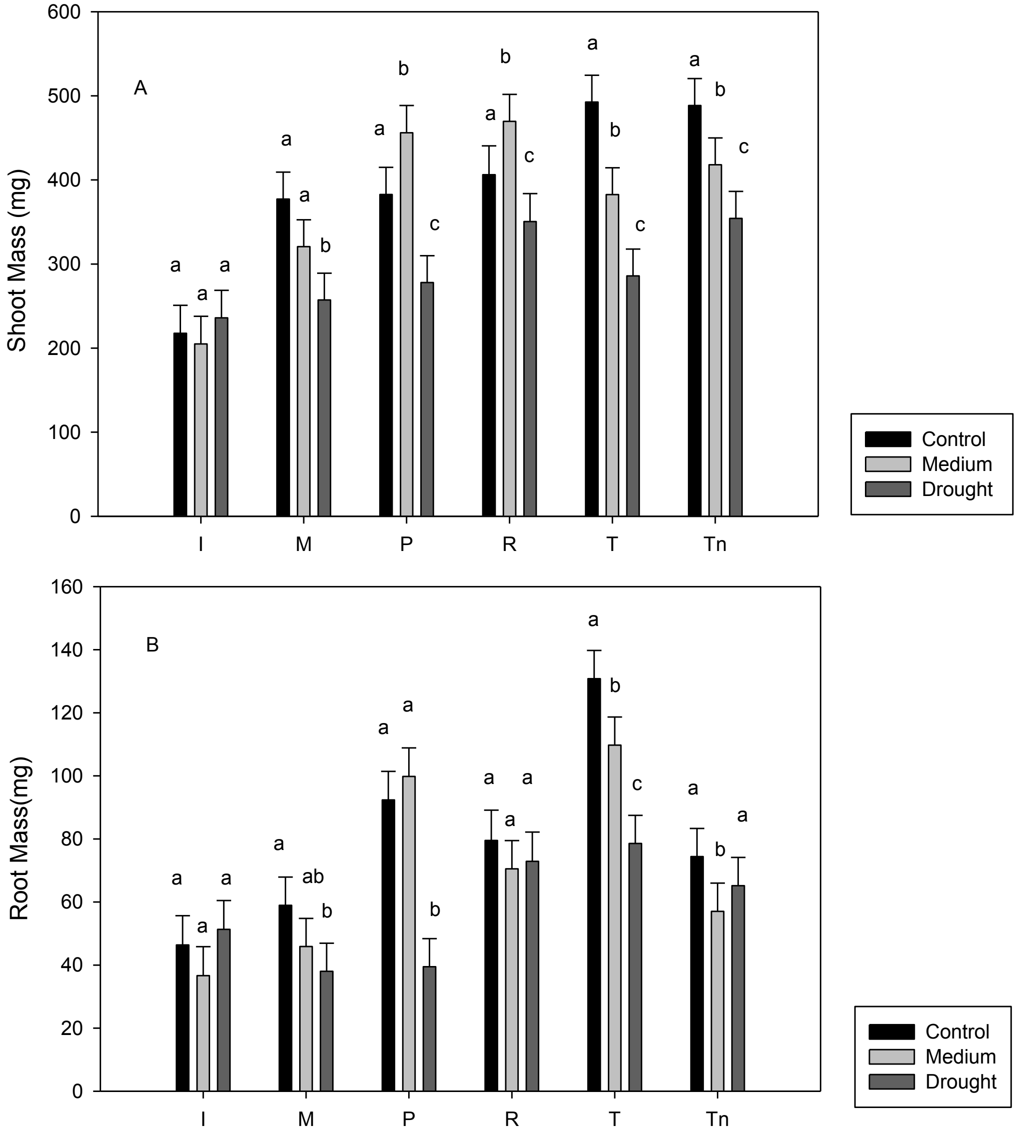
A
Shoot Mass (mg)
0
100
200
300
400
500
600
I
M
P
R
T
Tn
a
a
a
a
a
b
a
b
c
a
b
c
a
b
c
a
b
c
Control
Medium
Drought
B
Root Mass(mg)
0
20
40
60
80
100
120
140
160
I
M
P
R
T
Tn
a
a
a
a
ab
b
a
a
b
a
a
a
a
b
c
a
b
a
Control
Medium
Drought

Figure 2.5

Chapter 3: Inter-and Intra-genotypic competition in endophyte infected *Lolium perenne* populations from the Mediterranean region.

Introduction

Endophytes may alter physiological, developmental or morphological properties of host plants such that competitive abilities are enhanced, especially in stressful environments (Clay 1988, Bacon 1993, Malinowski and Belesky 1999). These properties include modifications in shoot and root growth (Latch et al. 1985, De Battista et al. 1990, Malinowski et al. 1997a, Malinowski et al. 1997b), physiological responses to abiotic stress (West 1994, Malinowski et al. 1997a, Malinowski et al. 1997b), production of anti-herbivore substances (Barker et al. 1983, Clay 1988, Siegel et al. 1990, Schardl and Philips 1997) and allelopathic compounds (Petroski et al. 1990). Asexual, vertically transmitted endophytes in the *Neotyphodium* genus are well known for increasing competitive abilities of several agronomic grasses (Marks et al 1991, Clay et al 1993, Clay and Holah 1999), but little is known about endophyte-host interactions in grasses native to a particular region (Saikkonen et al. 1999).

For example, Faeth et al. (2004) tested whether the asexual *Neotyphodium* endophyte enhances competitive abilities in a native grass, Arizona fescue (*Festuca arizonica*). The results did not support their hypothesis that this particular endophyte conferred a competitive advantage over uninfected plants, but instead E- plants consistently outperformed E+ plants in terms of root and shoot biomass. These results were surprising because previous studies of tall fescue, an agronomic species have shown that infection typically increases competitive abilities under water stress (Bacon and White 1994, Elbersen and West 1996). Furthermore, in other grass species similar results have been found for the native woodland grass species *Brachypodium* *sylvaticum* and *Bromus benekenii* (Brem and Leuchtmann, 2002). Indicated by dry matter yield, infected *Br. benekenii* were stronger competitors than uninfected plants, whereas plants of *B. sylvaticum* were better competitors when uninfected.

Studies of these types using native grasses and native populations of agronomic grasses are greatly needed to achieve a more realistic and holistic view of the plant- endophyte interaction. Clearly the mutualistic nature of this association is not universal, especially among native and wild grass populations. A reason for this may be because tall fescue and perennial ryegrass are introduced Eurasian grasses. Cultivars of these grass species, and probably their endophytes, have limited genetic diversity because of selective breeding and cultivation relative to grasses and endophytes in natural populations. Plant genotypes may respond differently to endophyte infection; for example Cheplick et al. (2000) found that in *Lolium perenne* drought tolerance varied significantly among host plant genotypes.

Since there are no studies directed at determining the competitive abilities of native *L. perenne* grasses infected with the asexual endophyte *Neotyphodium lolii*, I tested the competitive abilities of E+ and E- genotypes of *L. perenne* from their native habitats under intergenotype and intragenotype competition. The questions addressed are 1.) Are endophyte- infected grasses stronger competitors than E- genotypes under intra- and intergenotype competition? 2.) Within *L. perenne* accessions are there genotype-specific responses to competition?

Methods

Experimental design

The *L. perenne* accessions used in this experiment originated from Morocco (M), Tunisia (TN), Portugal (P), and New Zealand (NZ). To obtain infected replicates of genotypes, infected seeds were grown in a 2:1 mixture of soil and vermiculite, and when plants were large enough and produced multiple tillers, those tillers were extracted and placed in separate pots (3.5cm diameter-13.5cm length) and were allowed to grow until large enough for use. Three genotypes for each population were used and were identified by assigning a random number. There were 12 replicates for each genotype (E+ and E-). E- replicates were created and verified in a similar fashion to that which has already been described in chapter 2 (Kane 2011). The experiment included three different treatments: a monoculture treatment which served as a control and two plant genotype mixture treatments. Each treatment was replicated three times for a total of 288 plants.

The monoculture treatment contained only one genotype per pot. For each genotype and accession there were two monoculture treatments. There was an infected monoculture treatment, and an uninfected monoculture treatment for each genotype. The intra- and intergenotype treatments had two genotypes of the same size and were transplanted into small tubular pots (3.5cm diameter-13.5cm length) with the competitor plant about1.5cm from the target plant. To ensure that any one genotype did not have a competitive size advantage over another at the start of the experiment, genotypes were matched for initial fresh weight (iSM) to <10mg. To keep initial size constant across populations all plants were trimmed to the same height of 5cm before the start of the experiment. The intragenotype treatment consisted of 2 plants per pot of the same genotype, one E+ and the other E-. The intergenotype treatment contained one E+ genotype competing with a different genotype (E-), with genotypes from the same population (accession). Pots were randomly arranged in the greenhouse and the soil was a 2:1:1 mixture of topsoil, vermiculite and peat moss.

The effects of competition on shoot mass, total mass (shoot mass and root mass), total tiller length, root mass and root:shoot ratio were analyzed. At harvest total tiller length was measured with a ruler for all plants. The plants were placed in a drying oven for two weeks so that a dry weight could be obtained. All plants were first weighed with shoots and roots attached to achieve total mass, and then the root was separated from the shoot and weighed separately. To conform to rules of normality, all variables were log-transformed before the statistical analysis began.

Statistical analysis

The statistical design was a partially nested, mixed ANCOVA. Sources of variation were treatment (control; intergenotype competition; intragenotype competition), endophyte infection (E+ and E-), population (M, NZ, TN, P), host genotype nested within population (genotype(pop)) and their interactions. All were treated as fixed except for genotype(pop) which was treated as a random factor because the genotypes chosen for each competition experiment were a random subset of all genotypes available for each population. Because each genotype belongs to only one population, genotype is nested within each population. The variable iSM was used as the covariate. All interactions with genotype(pop) made those interactions random. The GLM procedure of the Statistical Analysis System, Version 8.2 (SAS institute, Cary, North Carolina, USA) was used for statistical analyses.

Results

Monoculture Treatments

Averaged over all populations, the mean shoot mass of E- grasses was significantly larger (P=0.0295) than E+ grasses in monoculture (Fig. 3.1B). When analyzing total mass, E- genotypes were larger but this result was not significant (Fig. 3.1A). In Tunisia, and New Zealand, 2 out of 3 E- genotypes had significantly larger tiller lengths than E+ genotypes when growing alone (Fig. 3.8 A and B, Fig. 3.2 B and C respectively).

Some E- genotypes from Morocco and Portugal had longer tiller lengths than E+ genotypes (Fig.3.6 B and C, Fig.3.7 B and C) but this was not significant. Two E+ genotypes had greater shoot mass in the control than E- genotypes for Morocco but this was not significant (Fig. 3.9 B,C). The shoot mass of E- genotype 16 from Tunisia was significantly larger than E- (Fig. 3.3B). In New Zealand and Portugal 2 out of 3 E- genotypes were larger than E+ but only one of these was significant for NZ, and P (Fig. 3.5, and Fig. 3.4).

Intragenotype competition

The response variables shoot mass and total dry mass were both significantly lower for all genotypes when growing under intragenotype competition compared to when growing alone (table 3.1, Fig. 3.1), indicating that competition had occurred. Figure 3.1A shows the treat*infection interaction for total mass. There was no difference between E+ and E- genotypes in response to intragenotype competition. For shoot mass there was a significant three way interaction of treatment*infection*genotype(pop) (table 1). This means that E+ and E- genotypes responded in different ways to the different treatments. For example, figure 3.3 shows this three-way interaction for competing genotypes from Tunisia. Figure 3.3B shows this interaction for genotype 16. Although there is no significant difference between infected and non-infected genotype 16, there was a significant decline in shoot mass for E- 16 in the intragenotype treatment compared to the control. A sharp decrease occurred for E- genotype 15 between the control and intragenotype treatment, but this result was not significant. The shoot mass of non-infected replicates of genotype 17 however, stayed consistently the same under all treatments. There was again no real difference between infected and non-infected replicates of genotype 17 under intragenotype competition. Although, there was a marginal decline in shoot mass for infected replicates of genotype 17 when comparing the control and intragenotype treatment (P=0.0444).

Figure 3.4 shows the three- way interaction for the shoot mass of the three genotypes from Portugal. The only genotype that showed a statistical difference between E+ and E- replicates in the intragenotype treatment was genotype 9. E- replicates of this genotype were significantly larger than E+ replicates (E-=140.73±26.71mg and E+ 50.56±26.71mg). In addition there were significant declines in shoot mass when comparing the controls with the intragenotype treatment for both E+ and E- replicates of genotype 9. For the genotypes within the accession from New Zealand, there was no significant difference between E+ and E- replicates. For genotypes 2 and 3 E- replicates were significantly smaller in the intragenotype treatment than the control (Fig.3.5

B,C). For all three genotypes, the shoot mass of E+ replicates remained consistent between the control and intragenotype competition treatment suggesting that these replicates did not respond to competition. E+ and E- genotypes in Morocco did not significantly differ in shoot mass under intragenotype competition (Fig. 3.9).

Total tiller length was significantly affected by the three- way interaction treatment*infection*genotype (pop) (table 2). Figure 3.6 shows this interaction for grasses from Morocco. In genotype 11 there was no marked difference between E+ and E- replicates under intragenotype competition (Fig. 3.6A). E- and E+ replicates of genotype 2 showed no decrease in tiller length when comparing the control with the intragenotype treatment (Fig. 3.6B). This trend was also present in genotype 14. Although there were declines in tiller length, this was not significant. Another trend was that E- replicates were larger than E+ replicates, although the difference was not significant.

This interaction was also significant for grasses from Portugal. In the intragenotype treatment, infected genotype 2 had greater tiller lengths than its E- counterpart (Fig. 3.7A). When competing with a different genotype there was no difference between the E- and E+ genotypes, but E+ genotype 2 in the intragenotype treatment had greater tiller length than E- in the intergenotype treatment.

In the control, genotype 16 (E-) from Tunisia (mean=64.75±6.96mm) was significantly greater than the infected replicate of genotype 16 (25.33±5.68mm). But under competitive pressure E- genotype 16 was significantly smaller (16.05±6.96mm) than its E+ counterpart (37.35±6.96mm) under intragenotype competition (Fig. 3.8B). E- genotypes from New Zealand were consistently larger across all treatments, but they were not significantly larger than E+ genotypes (Fig. 3.2 A,B,C).

Intergenotype Competition

Total tiller length for E- genotype 2 from Morocco in the intergenotype treatment was significantly larger (mean=24.82±4.41mm) than E+ (mean=11.91±4.02mm) (Fig. 3.6B). The crossing of lines indicates that the growth response for genotype 14 from Morocco under different treatments depends on its infection status(Fig. 3.6C). Also since the crossing of lines is apparent only for the intergenotype competition treatment, the response of this genotype is also dependent upon whether it is competing with a different genotype. Also, for genotype 14, E+ replicates were larger than E- but this result was not significantly different (Fig. 3.6C). For genotypes from the accessions Portugal, New Zealand and Tunisia, there were no significant differences between E- and E+ replicates under intergenotype competition (Fig. 3.7, 3.5 and 3.8 respectively). Two out of three E+ replicates from Portugal had longer but not significantly different tiller lengths than E- replicates(Fig. 3.7). However, E+ and E- genotypes from Tunisia (Fig. 3.8) and New Zealand (Fig. 3.2) appear to respond in a similar fashion to intergenotype competition, showing little to no difference in tiller length.

Infected genotypes 15 from Tunisia (Fig. 3.3A) and 12 from Portugal (Fig. 3.4C) had significantly greater shoot mass than their non-infected counterparts. Two out of three endophyte infected genotypes from Morocco had greater shoot mass than non-infected genotypes but this result was not significant (Fig.3.9). Although two E- genotypes from New Zealand were larger than E+ genotypes there were no significant differences (Fig. 3.5).

For the variables root mass and root:shoot ratio, there were significant population by infection interactions (tables 2 and 3). Figure 3.10 shows this interaction for root mass. New Zealand E- genotypes had greater root mass than E+ genotypes (P=0.0079), whereas E+ genotypes had greater root mass than E- genotypes (P=0.0073) from Tunisia. Infected and non-infected genotypes from Morocco and Portugal had similar root masses. In Portugal (Fig. 3.11), E- genotypes had significantly reduced root:shoot ratio compared to E+ genotypes (P=0.0070).

Discussion

Endophyte-infected genotypes under intergenotype competition performed better than non-infected genotypes which lends support to the hypothesis that infection by the asexual endophyte *Neotyphodium lolii* in perennial ryegrass confers a competitive advantage over uninfected genotypes. E+ genotypes also had significantly larger total mass than E- genotypes in the intergenotype competition treatment as indicated by the treatment*infection interaction (Fig. 3.1). The influence of genotype(pop) on shoot mass, total tiller length, root mass and total mass was much more pronounced than infection, infection by itself was not significantly related to any of the morphological response variables measured in this experiment (tables 1, 2 and 3). These results are similar to the common garden experiment conducted by Cheplick (2008) in which E+ and E- genotypes performed in a similar manner. Interpretation of the results also leads to the conclusion and support of the hypothesis that the interaction of infection, genotype and the environment is crucial in determining the outcome of the grass-endophyte symbiosis (Cheplick and Faeth 2009, Cheplick 2004, Cheplick et al., 2000, Lewis 2004, Ravel et al., 1997)).

The significant three- way interactions for geno(pop) x infection x treatment for the variables shoot mass and total tiller length exhibit the genotype- specific responses of E+ and E- replicates within accessions. Under intragenotype competition, there was no significant trend where one outperformed the other when comparing E+ and E- genotype replicates within accessions. From the data, it appears that E- genotypes experienced the effects of competition more severely that E+ genotypes. For example, the E- replicates of genotype 16 from Tunisia had significantly greater shoot mass in the control than the E+ replicate, but it experienced a significant 75% decline in shoot mass when competing with its E+ replicate (Fig. 3.3B). The same trend is also shown for E- genotypes 2 and 3 from New Zealand (Fig. 3.5). Although there was no difference between E+ and E- replicates in the intragenotype treatment, these two NZ genotypes experienced significant declines in shoot mass when competing with an E+ replicate of the same genotype as compared to the control. E- genotype 2 experienced a 72.6% decline (P=0.0112), and E- genotype 3 from New Zealand experienced a 57.8 % decline (P=0.0082). 2 out of 3 E+ genotypes from Morocco had greater shoot mass under intergenotype competition but this result was not significant (Fig. 3.9), E- genotypes from New Zealand had greater shoot mass, again this was not significant, and E+ genotypes were larger than E- genotypes in Tunisia and Portugal but only 1 out of 3 E+ genotypes for each of these populations were significantly larger than an E- genotype.

There is a strong selective advantage for endophyte-infected grasses in populations that include noninfected plants (Clay 1988). The reason is endophyte infection stimulates production of an array of bioprotective alkaloids (Bush et al. 1997) and non-alkaloid secondary metabolites (Koshino et al. 1989) in the host grass. In this way E+ grasses have direct allelopathic effects on their competitors (Malinowski and Belesky 2000). Loline alkaloids are one such class of endophyte related secondary metabolites with allelopathic properties. Alkaloids from infected grasses may be leached into the soil and inhibit growth of competing grasses (Mathews and Clay 2001). Although loline concentration in roots was not directly measured in this experiment, the production of lolines may be one explanation as to why E+ grasses outperformed E- grasses especially under inter-genotype competition. Siemens et al. 2002, proposed that constitutive alkaloids may have dual functions as herbivore defenses and as allelopathic agents that increase performance during plant competition. The lolines have been suggested to inhibit seed germination or growth in other grass species. Petroski et al. 1990, revealed endophyte infected alkaloids to reduce germination rate of moncot and dicot seed, showing that loline alkaloids may enhance the competitive abilities of E+ grasses by retarding the establishment of competitors in a sward. In another study, the abundance of white clover (*Trifolium repens* L.) declined in endophyte-infected perennial ryegrass pastures as compared with E- perennial ryegrass (Percival and Duder 1983, and Sutherland and Hoglund 1989). Seed extracts of E+ tall fescue inhibited germination of *Trifolium* spp. (Springer 1997).

In this experiment, the hypothesis was that E+ genotypes will outperform E- genotypes under competitive stress. The results show greater overall yield for E+ genotypes in the intergenotype competition treatment and are in line with the resource partitioning hypothesis. According to this hypothesis, genetically variable individuals should experience less competitive reduction in performance as compared to genetically similar or identical individuals (Cheplick and Kane 2004, Cheplick 1992, Maynard Smith 1978). If this true in the present context, then an E+ genotype competing with a different E- genotype will perform better than one competing an identical E- genotype. E+ genotypes outperformed E- genotypes under intergenotype competition as compared to E+ genotypes under intragenotype competition. Also, there were greater competitive reductions under intragenotype competition as opposed to the intergenotype competition treatment. There was also no demonstrable cost to infection as E+ genotypes grew just as well as or slightly better than E- genotypes under all types of competition.

In the present study, effects of endophyte presence on root mass was inconsistent. E- grasses from NZ had significantly more root mass than E+ grasses which does not support a benefit of *N. lolii* on nutrient uptake during competition. E+ grasses from Tunisia had significantly more root mass allocated to nutrient uptake, but there were no differences between grasses from Portugal and Morocco. Increased root allocation commonly occurs in grass species like *L. perenne,* and greater partitioning of dry matter to roots when soil nutrients are low may be one way to improve nutrient uptake (Marriott and Zuazua, 1996).

The results presented show E- genotypes experienced the effects of competition more severely than E+ genotypes although E+ genotypes didn't significantly outperform E- genotypes in all cases. This data further suggests that the outcome of the endophyte- plant interaction in different *L. perenne* accessions which occupy different habitats may not be so predictable (Cheplick and Faeth 2009, Clay 1993, Clay and Schardl 2002, Saikkonen et al., 1998, Hesse et al., 2004, Ahlholm et al., 2002).

Competition experiments with *L. perenne* have revealed a range from positive to negative effects of infection by *N. lolii* under various conditions. Marks et al. (1991) conducted both intraspecific and interspecific greenhouse experiments using *L. perenne* cv. Repell. E+ and E- plants were grown together and also with E+ and E- plants of tall fescue. E- target plants produced more dry mass than E+ plants under both interspecific and intraspecific competition suggesting a cost to endophyte-infection in this cultivar. Richmond et al., 2003 also observed negative endophyte effects under interspecific competition. His investigations were between E+ and E- *L. perenne* cv. Palmer II and crabgrass (*Digitaria sanguinalis)*. A significant detrimental effect of infection on the competitive ability of *L. perenne* was found. In his study, the production of tillers and aboveground dry mass was reduced for E+ plants when growing with crabgrass. Crabgrass showed greater mass and overall seed yield when competing with E+ perennial ryegrass as compared to competing with E- perennial ryegrass.

Conclusions

The intra- and intergenotype competition experiment showed that endophyte infection had marginal effects on the competitive performance of *Lolium perenne* accessions from its native range. This is in line with the view that strictly asexual fungal endophytes sometimes act as plant mutualists and to results from studies using agronomic grass cultivars of the same plant species (Marks et al., 1991, Clay and Holah 1999). The results from this study are more closely related to the predictions made in Saikkonen et al. (1998) that the influence of endophyte infection is variable in natural grass populations. Indeed, this may be the case given the fact that natural grass populations have enormous genotypic variation and variation in endophyte infection frequencies (Lewis et al. 1997, Clement et al. 2001), which in turn allows for the costs and benefits of infection to change among different environments (Thompson 1999, 2005, Faeth 2002, Faeth and Bultman 2002, Ahlholm 2002).

Chapter 3: Literature Cited

Ahlholm, J. U., Helander, M., Lehtimaki, S., Wali, P., Saikkonen, K., 2002. Vertically transmitted fungal endophytes: different responses of host-parasite systems to environmental condition. Oikos 99:173-183.

Bacon, C.W. 1993. Abiotic stress tolerances (moisture, nutrients) and photosynthesis in endophyte-infected tall fescue cultivars. Agriculture, Ecosystems and environment 44:123-141.

Bacon, C.W., and White, J.F., 1994. Stains, media and procedures for analyzing endophytes. In: Bacon, C.W., and White Jr., J.F. (Eds.), Biotechnology of Endophytic Fungi of Grasses. CRC Press, Boca Raton, FL. Pp. 47-56.

Barker, G. M., R. P. Pottinger, and P. J. Addison. 1983. Effect of tall fescue and ryegrass endophytes on Argentine stem weevil. Proceedings of the New Zealand weed control conference 36:216-219.

Brem, D., and Leuchtmann, A. 2002. Intraspecific competition of endophyte infected vs. uninfected plants of two woodland grass species. Oikos 96:281-290.

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

Clay, K., Marks, S., Cheplick, G.P., 1993. Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. Ecology 74,1767–1777.

Clay, K., and J. Holah. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. Science 285:1742-1744.

Clay, K, Schardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Naturalist 160, S99-S127.

Cheplick, G.P., Perera, A., Koulouris, K., 2000**.** Effect of drought on the growth of *Lolium perenne* genotypes with and without fungal endophytes. Functional Ecology 14, 657-667.

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

Cheplick, G.P., 2008. Host genotype overrides fungal endophyte infection in influencing tiller and spike production of *Lolium perenne* (Poaceae) in a common garden experiment. American Journal of Botany 95, 1063-1070.

Cheplick G. P., Faeth S. H. 2009. Ecology and evolution of the grass-endophyte symbiosis. Oxford University Press, New York.

Cheplick G.P., Kane K. 2004. Genetic Relatedness and competition in *Triplasis Purpurea* (POACEAE) Resource partitioning or kin selection. International Journal of Plant Science 165:623-630.

Cheplick, G.P. 1992. Sibling competition in plants. Journal of Ecology 80:567–575.

Clement, S. L. et al. 2001. Incidence and diversity of Neotyphodium fungal endophytes in tall fescue from Morocco, Tunisia, and Sardinia. Crop Sci. 41: 570-576.

de Battista, J.P., Bacon, C.W., Severson, R., Plattner, R.D., and Bouton J.H. 1990. Indole acetic acid production by the fungal endophyte of tall fescue. Agronomy Journal 82:878-880.

Elbersen, H. W., and C. P. West. 1996. Growth and water relations of field-grown tall fescue as influenced by drought and endophyte. Grass and Forage Science 51:333-342.

Faeth S.H. 2002. Are endophytic fungi defensive plant mutualists? Oikos 98:25-36.

Faeth, S.H. and Bultman T.L. 2002. Endophytic fungi and interactions among host plants, herbivores and natural enemies. Pages 89-123, In, T. Tscharntke and B.A. Hawkins, eds. Multitrophic level interactions. Cambridge University Press, Cambridge, UK.

Faeth,S.H., Helander, M.L., Saikkonen, K. 2004. Asexual *Neotyphodium* endophytes in a native grass reduce competitive abilities. Ecology Letters 7:304-313.

Hesse, U.,, Schoberlein, W., Wittenmayer, L., Forster, K., Warnstorff ,K., Diepenbrock, W., Merbach, W., 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne* L. genotypes. Grass and Forage Science 58, 407-415.

Kane, K. Effects of endophyte infection on drought stress tolerance of *Lolium perenne* accessions from the Mediterranean region. 2011. Experimental and Environmental Botany 71, 337:344.

Latch, G.C.M., and Christensen, M.J. 1985. Endophytic fungi affect growth of perennial ryegrass. New Zealand Journal of Agricultural Research 28:165-168. Annals of Applied Biology 144:53-63.

Lewis G.C., Ravel C., Naffaa W., Astier C., Charmet G., 1997. Occurrence of *Acremonium* endophytes in wild populations of *Lolium* spp. in European countries and a relationship between level of infection and climate in France. Annals of Applied Biology 130, 227-238.

Lewis, G.C. 2004. Effects of biotic and abiotic stress on the growth of three genotypes of *Lolium perenne* with and without infection by the fungal endophyte *Neotyphodium lolii*.

Malinowski, D., Leuchtmann, A., Schmidt, D., Nosberger, J., 1997a. Symbiosis with *Neotyphodium uncinatum* endophyte may increase the competitive ability of meadow fescue. Agronomy Journal 89, 833–839.

Malinowski, D., A. Leuchtmann, D. Schmidt, and J. Nösberger. 1997b. Growth and water status in meadow fescue {Festuca pratensis) is differently affected by its two natural endophytes. Agronomy Journal 89:673-678.

Marks, S., K. Clay. G.P. Cheplick. 1991. Effects of fungal endophytes on interspecific and intraspecific competition in the grasses *Festuca Arundinacea* and *Lolium Perenne*. Journal of Applied Ecology 28:194-204.

Maynard Smith, J. 1978b. Optimization theory in evolution. Annu. Rev. Ecol. Syst. 9: 31–56.

Petroski, R. J., D. L. Dornbos, and R. G. Powell. 1990. Germination and growth inhibition of annual ryegrass (*Lolium multiflorum*) and alfalfa (*Medicago sativa*) by loline alkaloids and synthetic N-acetylloline dérivâtes. Journal of Agriculture and Food Science 38:1716-1718.

Ravel, C. et al. 1997. The effect of imperfect transmission on the frequency of mutualistic seed-borne endophytes in natural populations of grasses. Oikos 80: 18-24.

Richmond, M.D., Grewal, P.S., Cardina J. 2003. Competition between *Lolium perenne* and *Digitaria sanguinalis*: ecological consequences for harbouring an endosymbiotic fungus. Journal of Vegetation Science 14:835-840.

Saikkonen, K., S.H. Faeth, M. Helander, and T.J. Sullivan. 1998. Fungal endophytes: a continuum of interactions with host plants. Annual Review of Ecology and Systematics 29:319-343.

Saikkonen, K., M. Helander, S. H. Faeth, F. Schulthess, and D. Wilson. 1999. Endophyte-grass-herbivore interactions: the case of *Neotyphodium* endophytes in Arizona fescue populations. Oecologia 121:411-420.

Schardl, C. L., and T. D. Philips. 1997. Protective grass endophytes. Plant Disease 81:430-438.

Siegel, M. R., G. C. M. Latch, L. P. Bush, F. F. Fannin, D. D. Rowan, B. A. Tapper, C. W. Bacon, and M. C. Johnson. 1990. Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. Journal of Chemical Ecology 16:3301-3315.

Thompson, J.N. 1999. The raw material for coevolution. Oikos 84: 5-16.

West, C.P., 1994. Physiology and drought tolerance of endophyte-infected grasses. In: Bacon, C.W., White Jr., J.F. (Eds.), Biotechnology of Endophytic Fungi of Grasses. CRC Press, Boca Raton, FL, pp. 87–99.

Chapter 3: Tables

Table 1.

---------Shoot mass---------------------------Total Mass-----------------

Source	d.f.	MS	F	P	MS	F	P
Population	3	5752.63	7.62	**0.0482**	17708.13	2.46	0.0644
Treatment	2	37864.40	17.68	**<0.0001**	184698.99	25.66	**<0.0001**
Genotype(pop)	8	6055.27	2.83	**0.0057**	782.37	0.11	**0.0233**
Infection	1	2813.36	1.31	0.2533	16507.84	2.29	0.7420
Pop*infect	3	4207.76	1.97	0.1211	34698.05	4.82	**0.0030**
Treat*pop	6	3266.87	1.53	0.1725	8550.14	1.19	0.3149
Treat*infect	2	11069.69	5.17	**0.0066**	33752.41	4.69	**0.0104**
Treat*pop*infect	6	2435.63	1.14	0.3427	12257.80	1.70	0.1230
Treat*geno(pop)	15	1444.82	0.67	0.8068	4539.81	0.63	0.8467
Infect*geno(pop)	8	3359.14	1.57	0.137	11943.26	1.66	0.1116
Treat*infect*geno(pop)	15	3872.34	1.81	**0.0369**	10036.11	1.39	0.1547
Error	171	2141.29					

Table 2.

		---Total Tiller Length---			-------Root Mass---------		
Source	d.f	MS	F	P	MS	F	P
ISM	1	194.06	2.04	0.1555	114.92	0.04	0.8505
Population	3	5752.63	2.69	**0.0482**	17708.13	2.46	0.0644
Treatment	2	37864.40	17.68	**<0.0001**	184698.99	25.66	**<0.0001**
Genotype(pop)	8	6055.27	2.83	**0.0057**	782.37	0.11	**0.0233**
Infection	1	2813.36	1.31	0.2533	16507.84	2.29	0.7420
Pop*infect	3	4207.76	1.97	0.1211	34698.05	4.82	**0.0030**
Treat*pop	6	3266.87	1.53	0.1725	8550.14	1.19	0.3149
Treat*infect	2	11069.69	5.17	**0.0066**	6148.48	1.92	0.1501
Treat*pop*infect	6	2435.63	1.14	0.3427	12257.80	1.70	0.1230
Treat*geno(pop)	15	1444.82	0.67	0.8068	4539.81	0.63	0.8467
Infect*geno(pop)	8	3359.14	1.57	0.1370	11943.26	1.66	0.1116
Treat*infect*geno(pop)	15	3872.34	1.81	**0.0369**	10036.11	1.39	0.1547
Error	169	2141.29					

Table 3.

Source	DF	MS	F	P
population	3	0.0648	2.80	**0.0419**
Treatment	2	0.1476	6.37	**0.0022**
genotype(pop)	8	0.0169	0.73	0.6640
infection	1	0.0279	1.20	0.2743
pop*infect	3	0.0867	3.74	**0.0124**
treat*pop	6	0.0401	1.73	0.1170
treat*infect	2	0.0125	0.54	0.5843
treat*pop*infect	6	0.0279	1.21	0.3055
treat*geno(pop)	15	0.0268	1.16	0.3091
infect*geno(pop)	8	0.0424	1.83	0.0752
treat*infect*geno(pop)	15	0.0401	1.73	**0.0495**
Error	161	0.0231		

Chapter 3: Figures

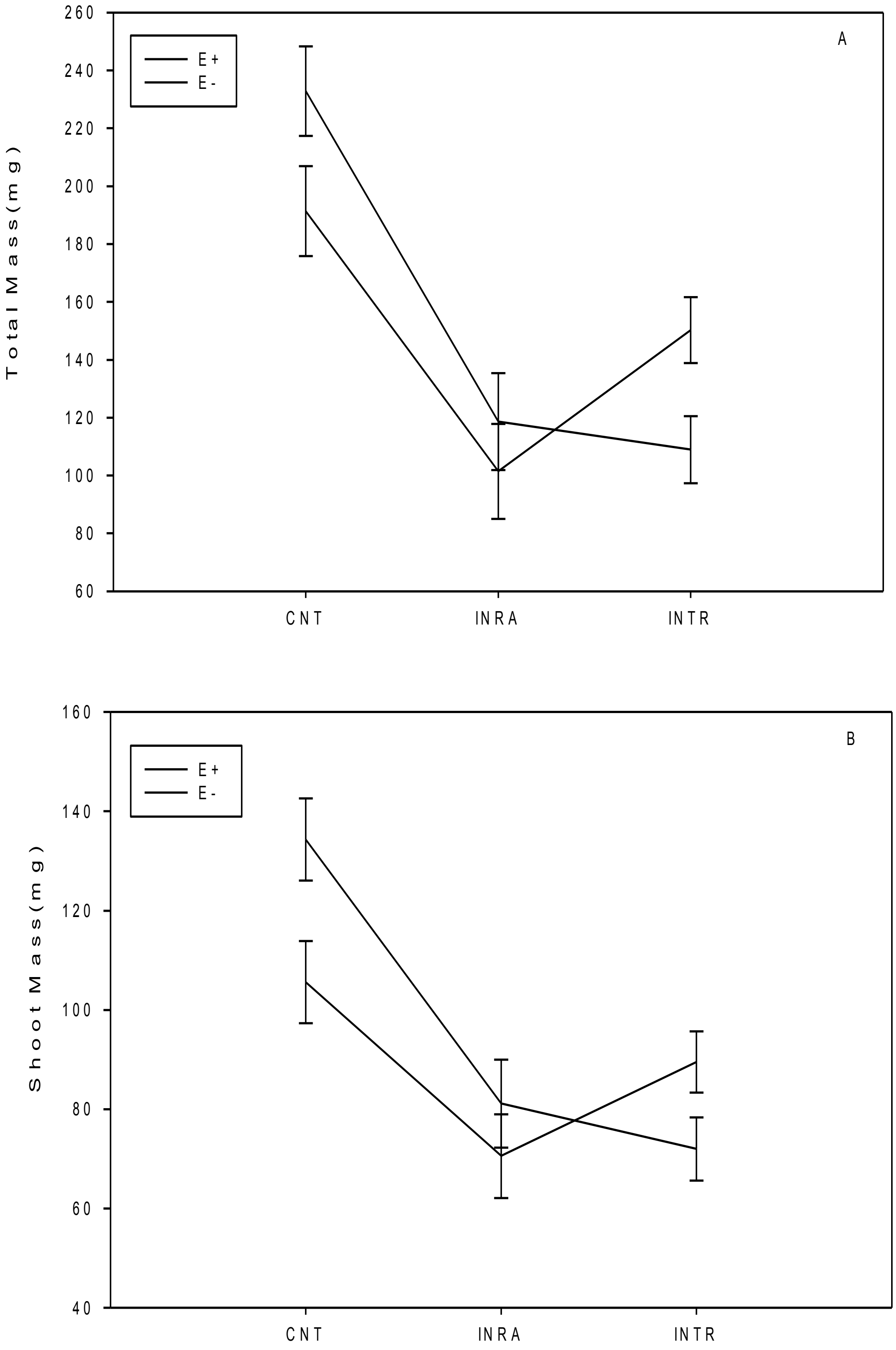

Figure 3.1

New Zealand

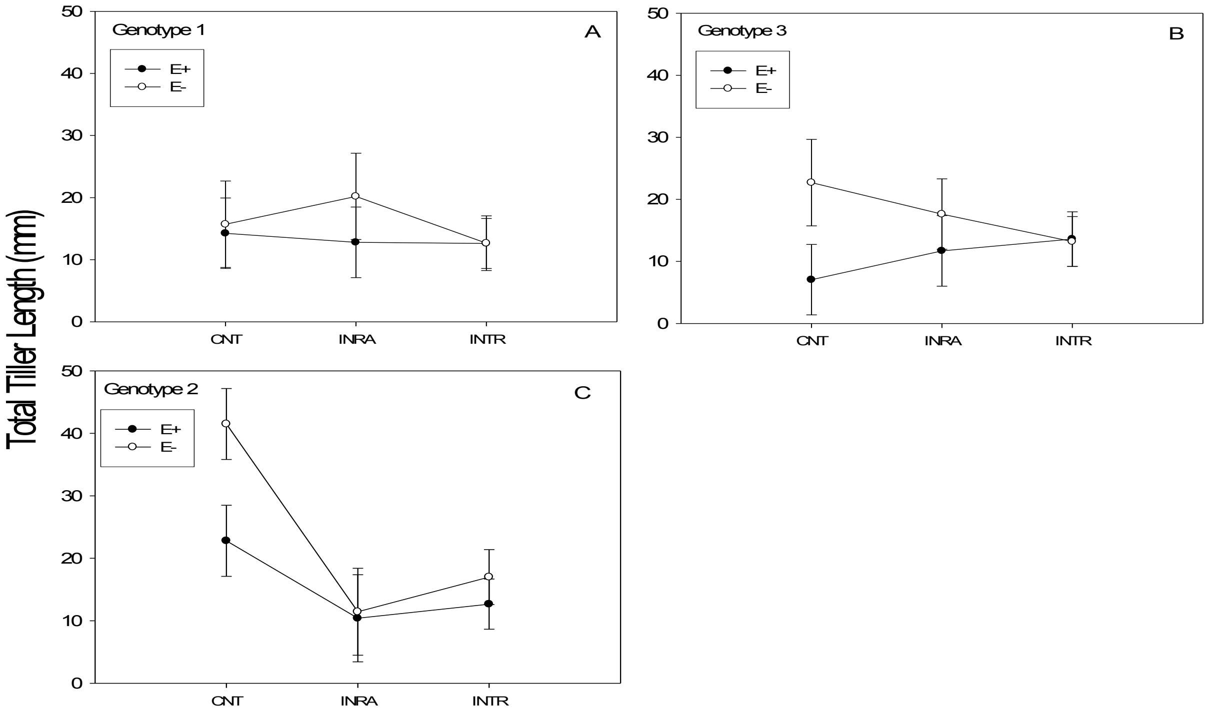

Figure 3.2

Tunisia

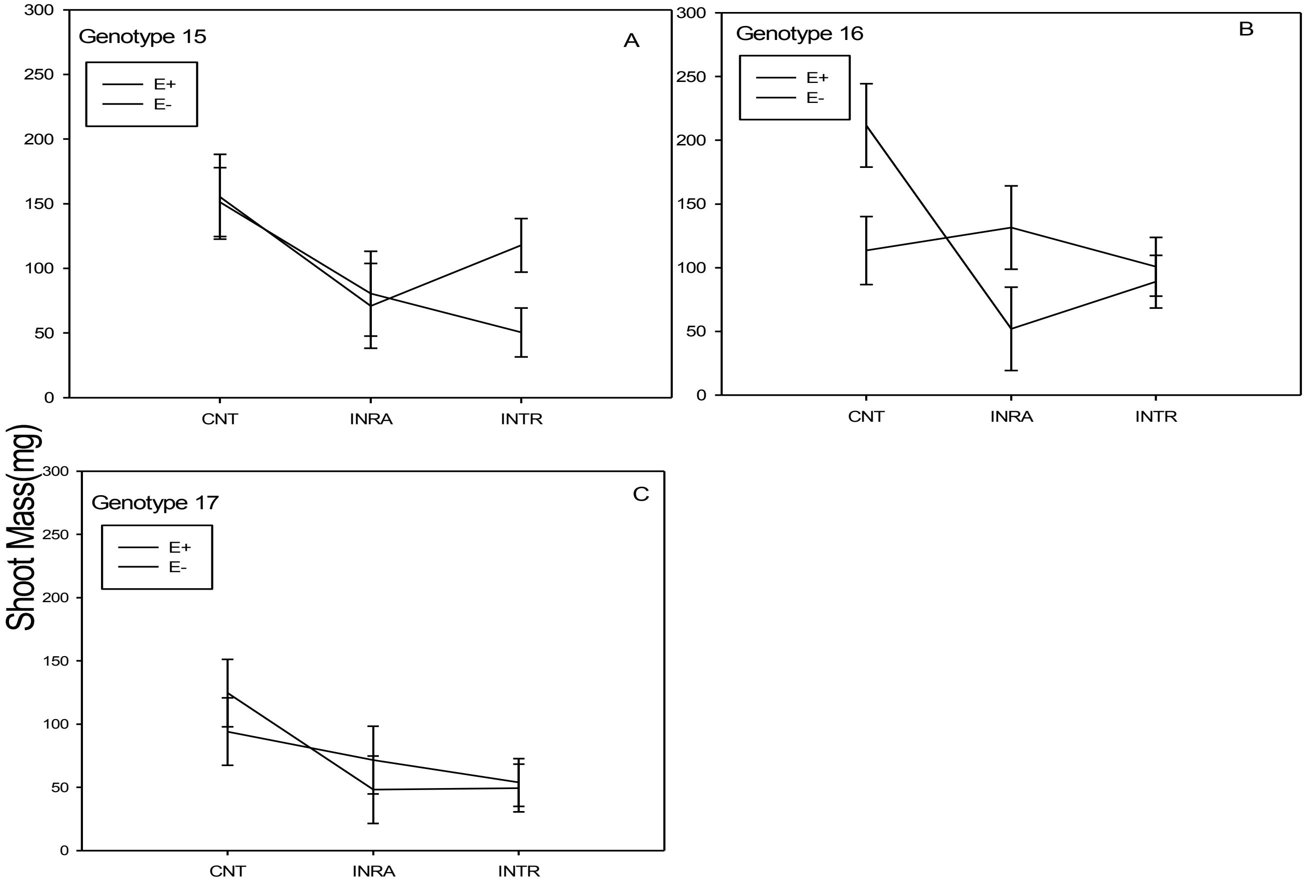

Figure 3.3

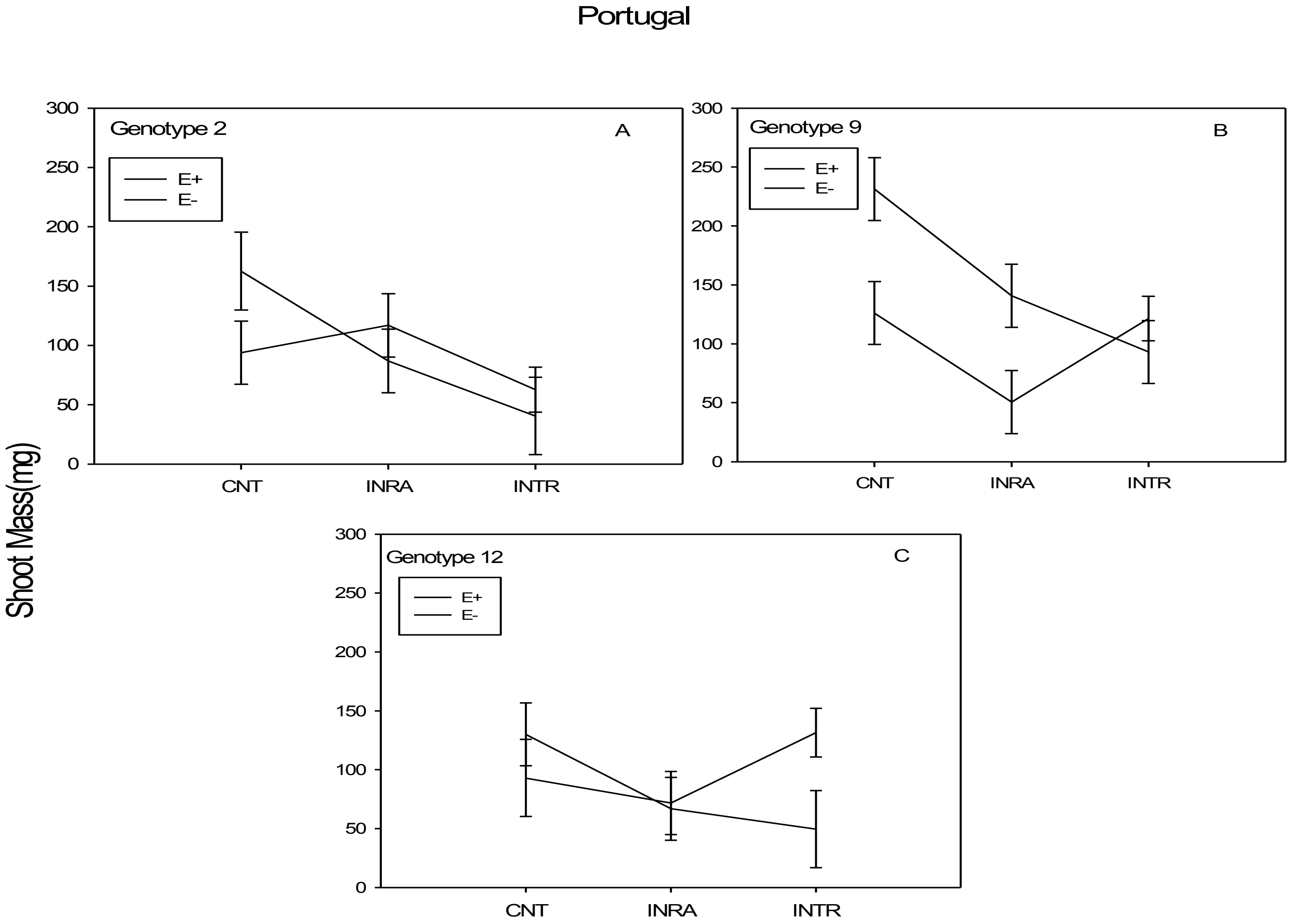
Portugal
Shoot Mass(mg)
Genotype 2
A
E+
E-
300
250
200
150
100
50
0
CNT
INRA
INTR
Genotype 9
B
E+
E-
300
250
200
150
100
50
0
CNT
INRA
INTR
Genotype 12
C
E+
E-
300
250
200
150
100
50
0
CNT
INRA
INTR

Figure 3.4

New Zealand

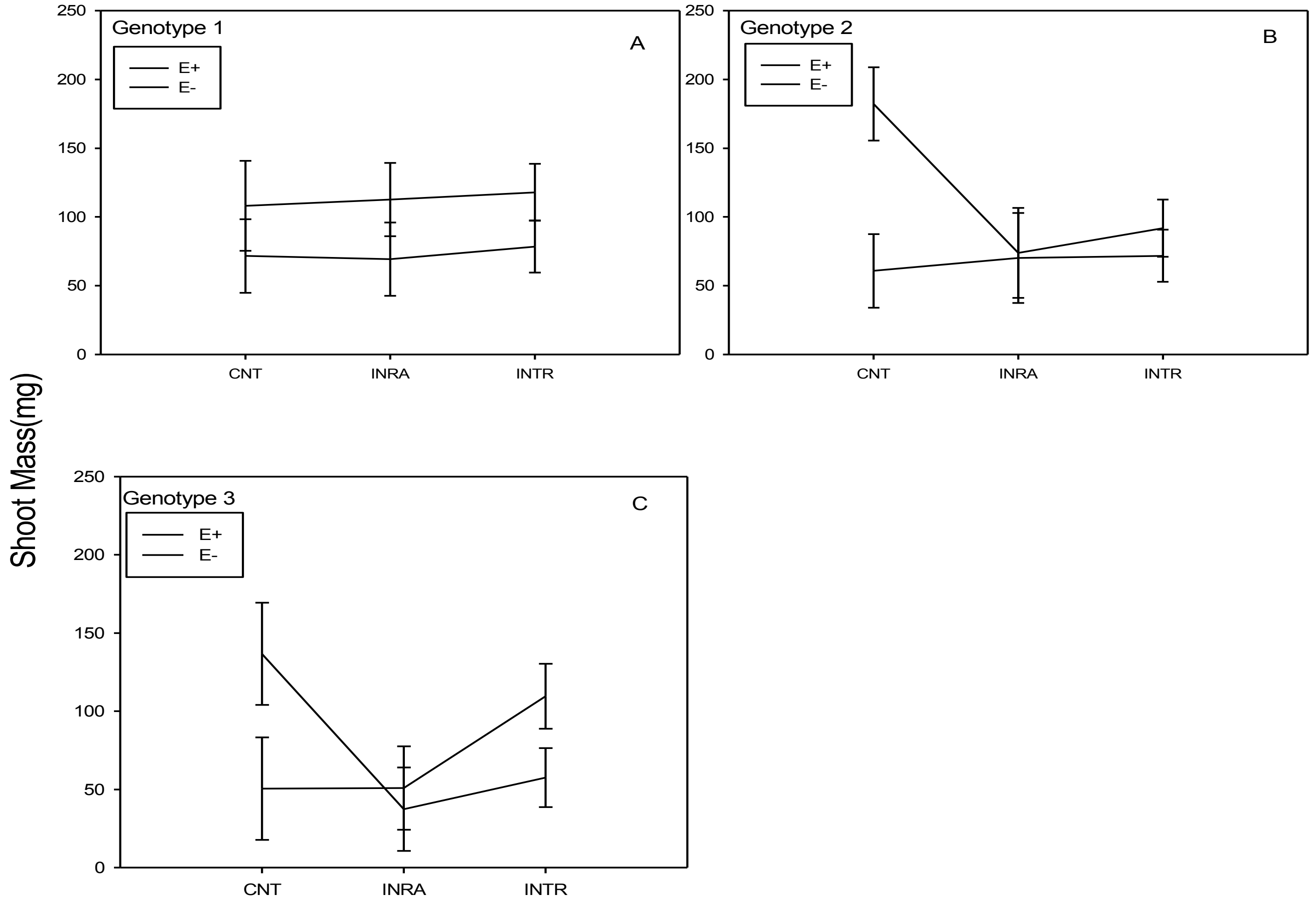

Figure 3.5

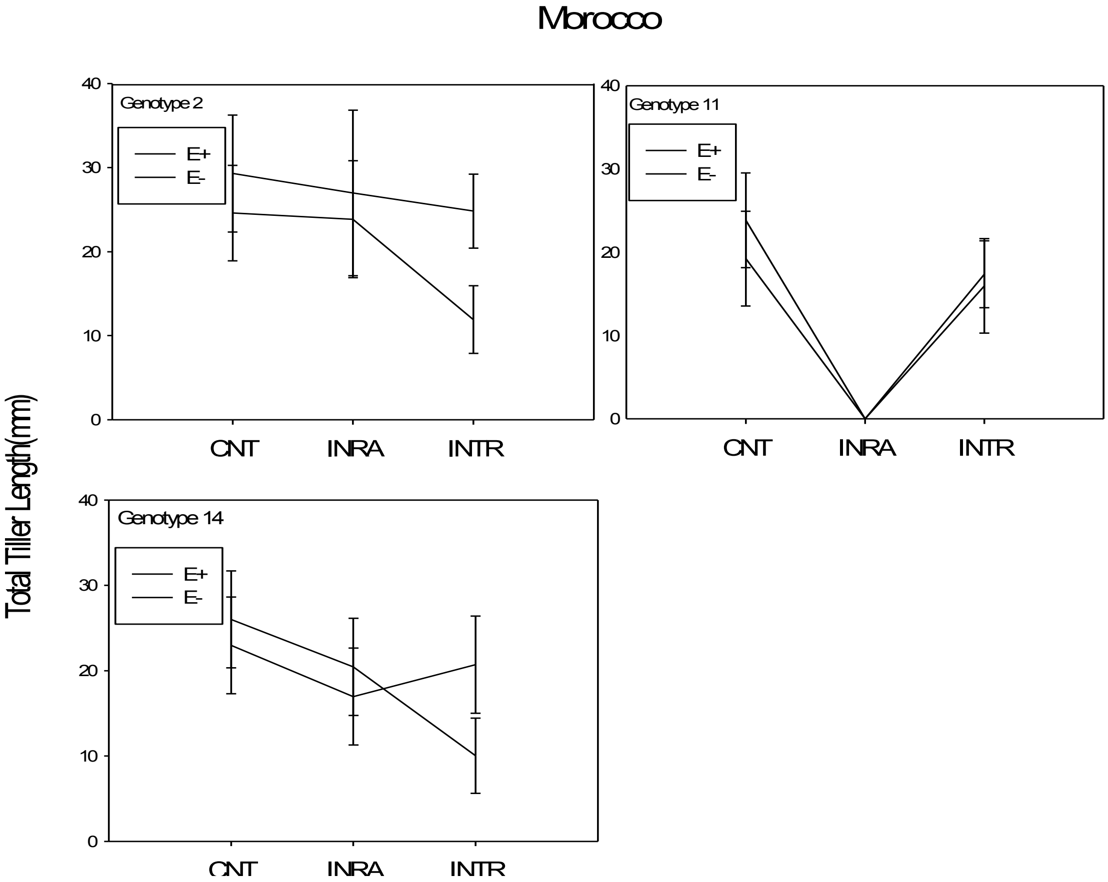
Morocco
Total Tiller Length(mm)
Genotype 2
Genotype 11
Genotype 14
E+
E-
0
10
20
30
40
CNT
INRA
INTR

Figure 3.6

Portugal

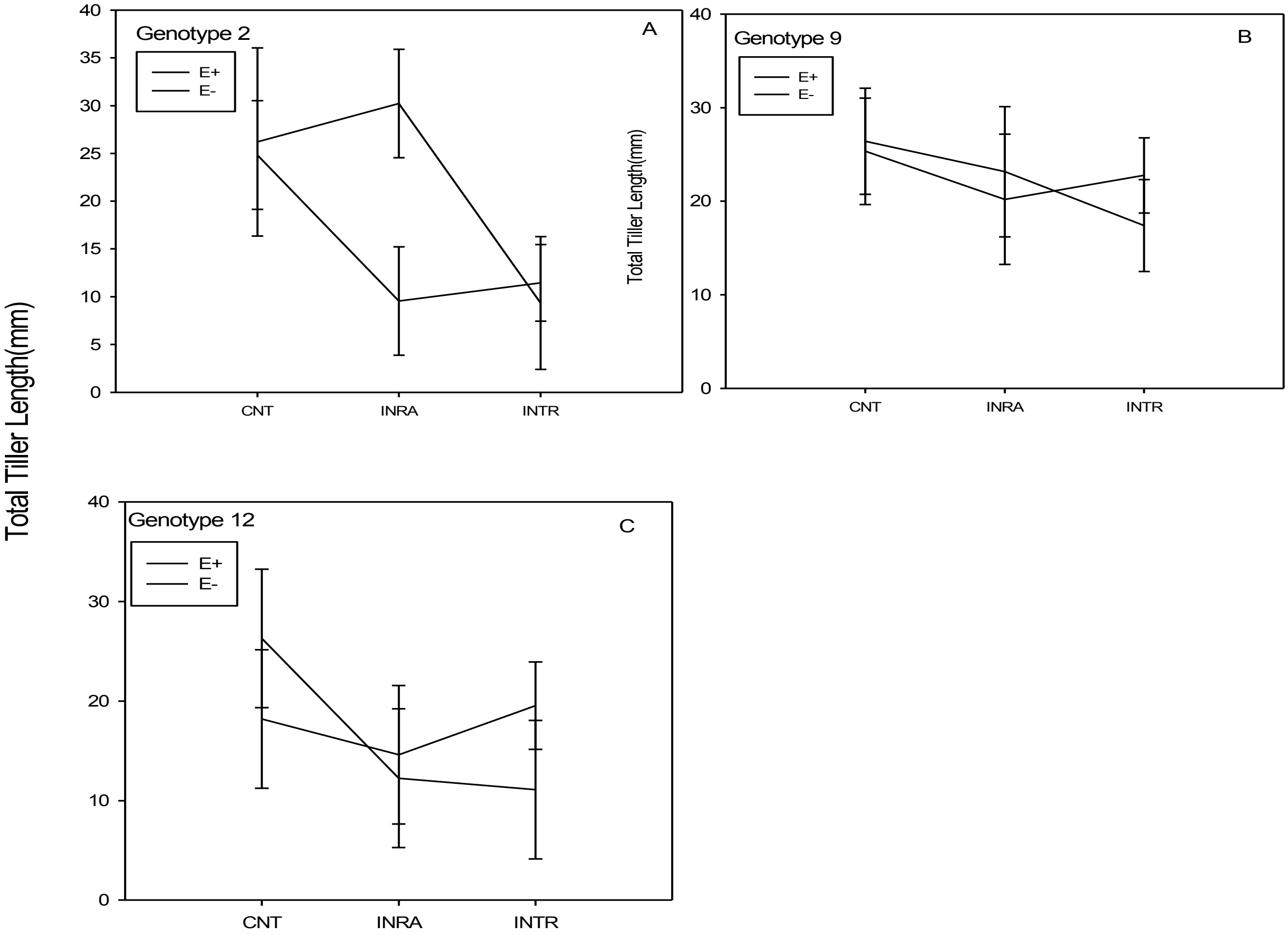

Figure 3.7

Tunisia

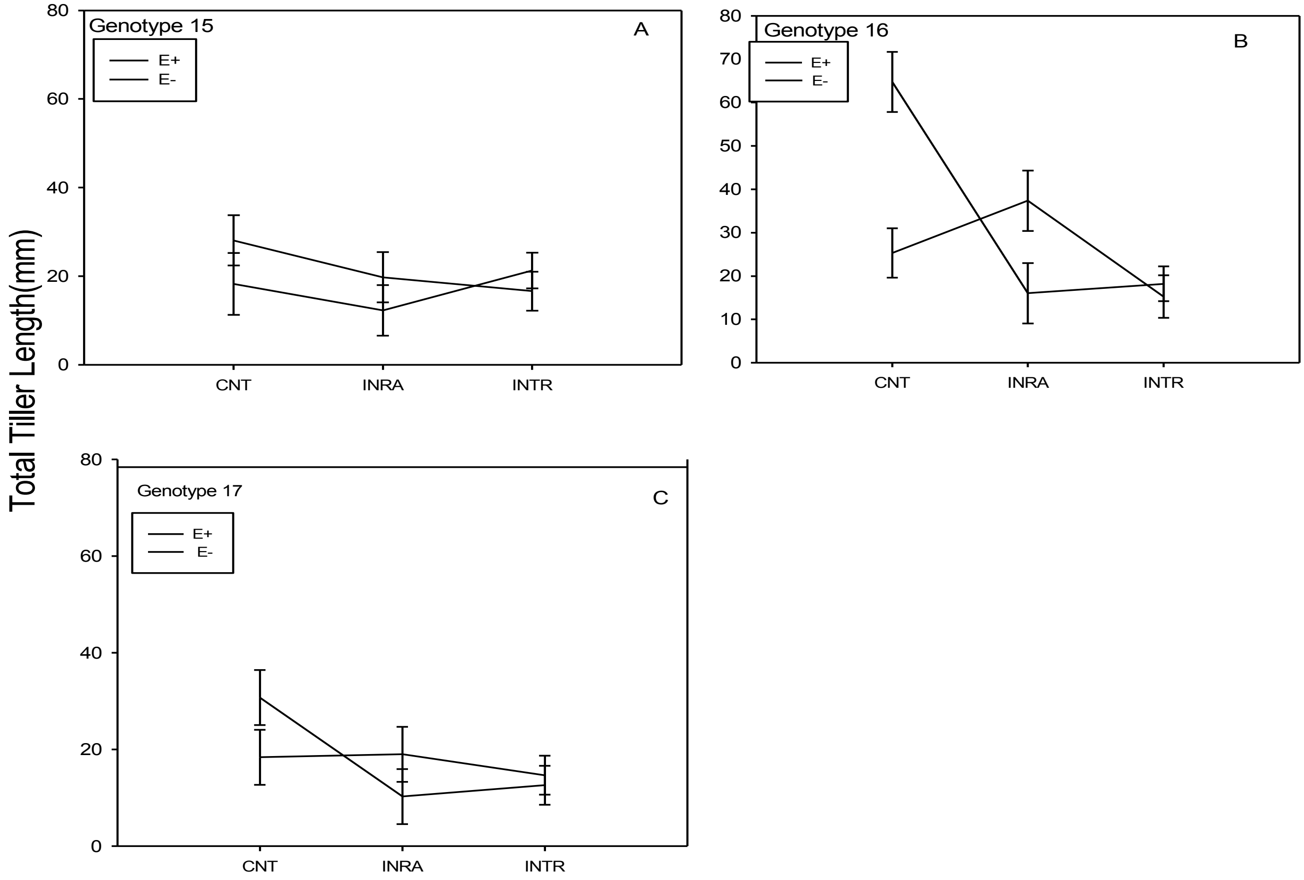

Figure 3.8

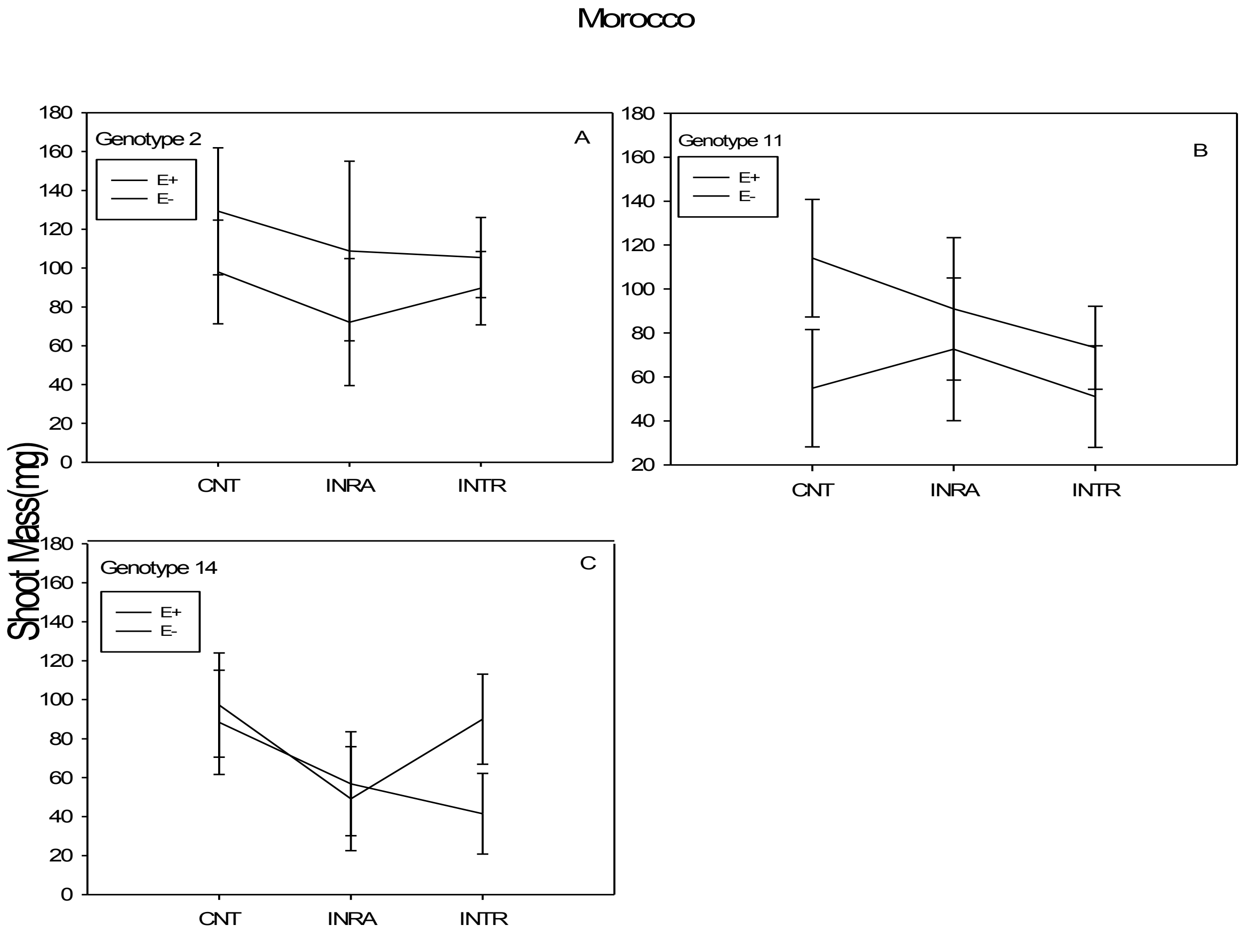
Morocco
Shoot Mass(mg)
Genotype 2
A
E+
E-
0
20
40
60
80
100
120
140
160
180
CNT
INRA
INTR
Genotype 11
B
E+
E-
20
40
60
80
100
120
140
160
180
CNT
INRA
INTR
Genotype 14
C
E+
E-
0
20
40
60
80
100
120
140
160
180
CNT
INRA
INTR

Figure 3.9

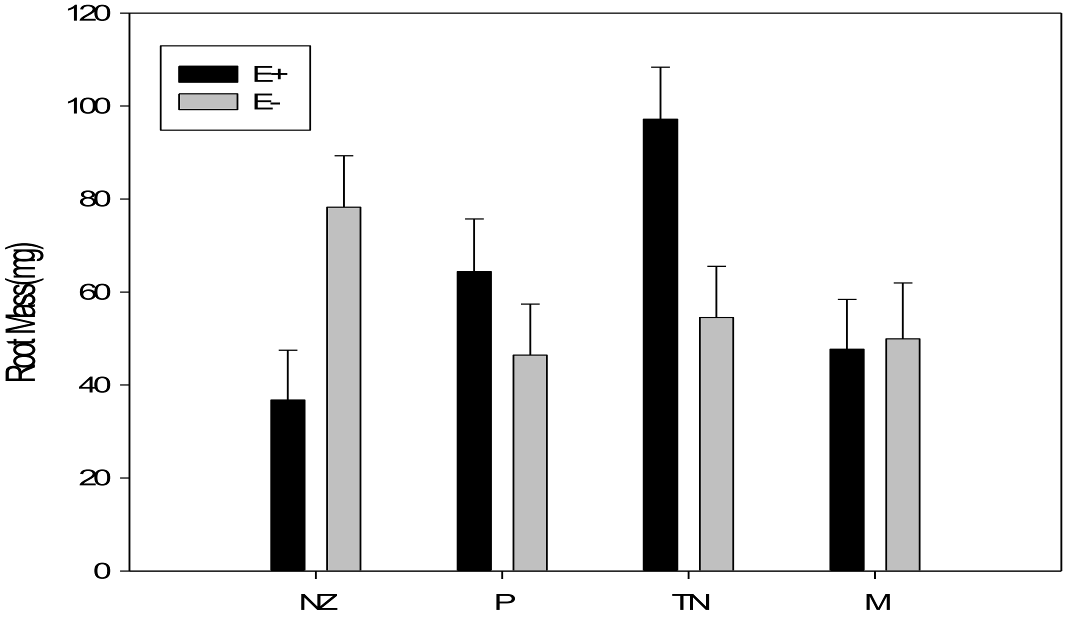

Figure 3.10

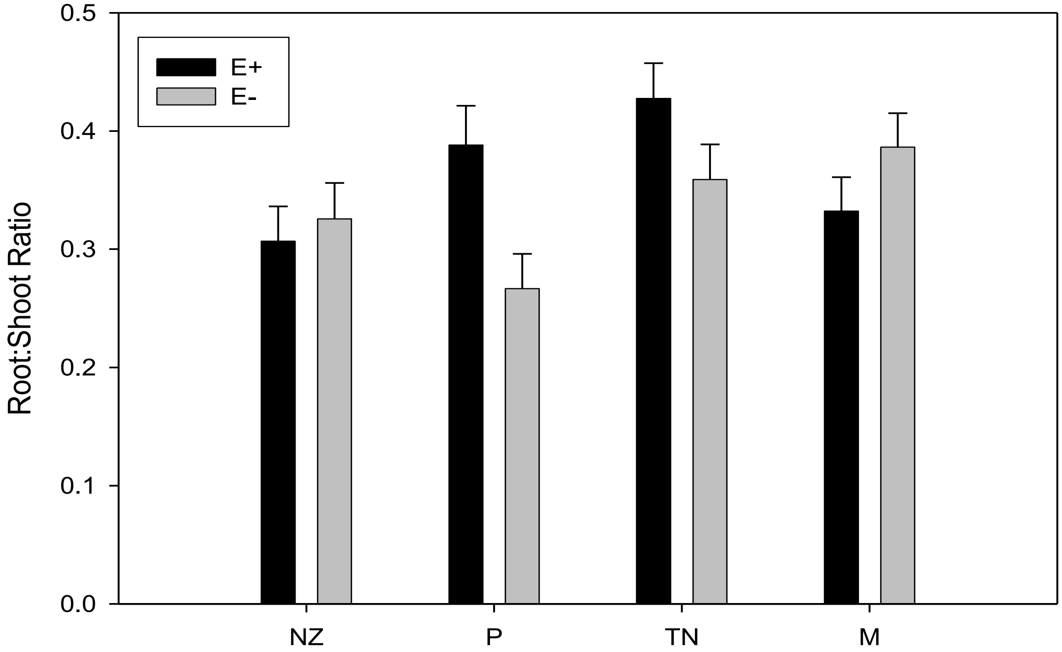

Figure 3.11

Chapter 4

Relationship between Climate factors and *Neotyphodium* infection frequencies and the Impact of climate change in the Mediterranean region on *Lolium perenne* populations

Introduction

In vertically transmitted symbiotic organisms such as *Neotyphodium lolii*, variation exists in their frequency and abundance within and among host populations (Saikkonen et al. 2000, Schulthess and Faeth 1998). Factors that alter symbiont abundance are linked to elements that affect the costs and benefits of the symbiosis. For example, exposure to herbivores can increase the frequency of endosymbiotic fungi that confer herbivore resistance to host grasses, relative to populations protected from herbivory (Clay et al. 2005). In addition to such factors, transmission mode of the endophyte may affect the frequency of the symbiosis. In vertical transmission, endophytes are inherited maternally via seeds, whereas horizontal transmission involves the contagious spread of symbionts. Since in vertical transmission, symbionts depend entirely on hosts for their own reproduction (Fine 1975, Ewald 1987, Gundel et al., 2008) this should select against pathogenic symbionts. In this case, the frequency and abundance of maternally transmitted fungal endophyte populations are ultimately predicted to reach 100% (Clay 1998, Wilkinson and Schardl 1997, Cheplick and Faeth 2009). Rudgers et al. (2009) examined patterns of endophyte frequencies in natural populations for systemic fungal endophytes that varied in their mode of transmission. Their results were consistent with expectations that infections by *Neotyphodium* endophytes are beneficial to their host plants. Across the 118 host species used, they found that *Neotyphodium* frequencies were 40-130% greater than in hosts of mixed and horizontally transmitted *Epichloë* endophytes. Their results showed a strong association between variation in endophyte frequencies and endophyte transmission mode.

Limited studies have proposed that geographic variation in endophyte frequency is due to environmental factors such as climate (Lewis and Clements 1986, White and Baldwin 1992). These studies reported that populations of *L. perenne* were highly symbiotic in New Zealand and the United States, and the prevailing climate conditions were proposed as driving forces. Lewis et al, (1997) and Malinowski and Belesky (2006) found positive correlations between the frequency of infection and hot, dry climatic conditions in Europe. In support of this hypothesis, manipulative experiments confirmed that endophytes in *L. perenne* can enhance plant tolerance to drought (Malinowski and Belesky 2000). Other studies lend support for a latitudinal gradient in Europe with a high frequency of symbiotic plants at low latitudes and a low frequency of symbiotic plants at high latitudes (Townsend et al. 2002, Bazely et al. 2007). This data represents a mixture of studies in natural and agricultural systems. Evidence from agricultural grass systems support the prediction that frequencies of infected grasses should increase over time in plant populations if endophytes increase the fitness of host plants (Clay 1996, Leuchtmann and Clay 1997, Saikkonen et al. 1998). Recent studies on natural grass systems indicate that infection frequencies are more variable in wild grass populations (Lewis et al. 1997, Schulthess and Faeth 1998, Saikkonen et al. 2000). Natural systems have not been subjected to artificial selection and it would be useful to analyze these systems independently.

Fungal endophyte-plant interactions have the ability to impact plant community structure and function (Rudgers et al. 2010, Clay, Holah and Rudgers 2005, Rudgers and Clay 2008), and alter relationships between diversity and ecosystem properties (Rudgers et al. 2004). Since plant- endophyte relationships are complex tri- trophic interactions affected by many factors, it is worthwhile to study the impact of climate change on endophyte-infected populations from the Mediterranean region. Climate models predict that temperatures could rise by over 4°C by 2100 over many inland areas and by more than half of this over the Mediterranean region. Over the same period, annual precipitation is projected to decline by 10 to 40% over much of Africa and southeastern Spain, with smaller, but potentially significant changes elsewhere (IPCC 2007). As a consequence, the frequency and severity of droughts could increase across the region and these changes have the ability to impact species interactions and the structure and function of ecosystems.

Given the predicted changes in climate for the Mediterranean region, I devised a model of species occurrence for *L. perenne*. I used current climate files (representative of the year 2010), to model the current distribution and habitat suitability of *L. perenne,* and then used future climate files (representative of the year 2050), to understand the impact of climate change on this species' distribution. I used the maximum entropy method (Maxent version 3.3.3k, for free download see http://www.cs.princeton.edu/~schapire/maxent/) to make these models. The model for a species is determined from a set of environmental or climate layers for a set of grid cells in a landscape, together with a set of sample locations where the species has been observed. Maxent (Pearson et al. 2006, Phillips et al. 2006) estimates the realized niche of a species based on presence-only occurrence records and environmental or climate layers known to describe important aspects of the habitat that influence the species' presence. The Maxent algorithm for determining habitat suitability assigns a probability to each pixel in the study area. The study area is the space in which the Maxent probability distribution is defined. It computes a species' distribution based on the probability assigned to each pixel and the value of pixels are constrained by environmental conditions at presence localities. A high value at a particular grid cell is predicted to have suitable conditions for that species. The computed model is a probability distribution over all of the grid cells.

As with any modeling approach, the fit or accuracy of the model should be tested to determine its relevance. This is done in two ways: 1) through receiver operating characteristic plots and 2) through defined thresholds. The ROC plot is a plot of sensitivity and 1-specificity. Sensitivity represents how well the data correctly predicts presence, whereas specificity provides a measure of correctly predicted absences (Baldwin 2009). To develop a ROC plot, a certain percentage of the data is selected for training data and the other portion for test data. AUC-area under the receiver operator curve, measures the ability of the model to discriminate between sites where a species is present versus those where it is absent. AUC provides an indication of the usefulness of the model for prioritizing areas in terms of their relative importance as habitat for a particular species. AUC ranges from 0-1 where a score of 1 indicates perfect discrimination. A score of 0.5 implies predictive discrimination (no better than random) and a score of less than 0.5 indicates a model worse than random (Phillips et al. 2006).

The second approach involves selecting thresholds to establish sites that are considered suitable or unsuitable for the species of interest. These thresholds are established by maximizing sensitivity while minimizing specificity (Fielding and Bell 1997). The use of Maxent is an effective method for generating unique probability distributions based on an incomplete state of knowledge, which recently has been applied to species distribution modeling (Elith andGraham 2006 , Pearson et al. 2006).

I also asked whether a relationship exists between infection frequencies and climate in the Mediterranean. This would give an indication of whether climate factors alter the frequency and abundance of endophytes in populations. If there is a relationship, then infection frequency should be particularly important under climate change especially if a warmer, drier climate is expected. Since plant- endophyte relationships are complex tri- trophic interactions affected by many factors, including abiotic and biotic environmental conditions as well as host and endophyte genotypes, they are likely to produce large-scale patterns of abundance that are highly variable and difficult to interpret.

In this chapter I am trying to understand how climate factors (temperature and precipitation) alter the variation of endophyte infection frequencies in natural populations. I screened the proportion of *Neotyphodium* endophyte-infected adult plants grown from seeds sampled from across the Mediterranean region to determine the variation in endophyte frequencies in natural populations and searched for a relationship between these frequencies and temperature and precipitation to better understand the nature of the host-endophyte interaction in native populations.

In this chapter I ask:

1. Is there a significant relationship between infection frequencies and climatic variables in the Mediterranean region?
2. Are the most heavily infected populations situated in regions with high temperatures and low precipitation?
3. As the resulting climate changes over the next several decades will the habitat suitability of *Lolium perenne* populations increase or decrease?

Methods

I used the freely available Maxent software version 3.3.3k (http://www.cs.princeton.edu/~schapire/maxent), which generates and estimate of probability of presence of the species that varies from 0 to 1, where 0 being the lowest and 1 the highest probability. I used 99 occurrence records freely available from the National Plant Germplasm System; (http://www.ars-grin.gov/npgs), and 19 bioclimatic variables available from Worldclim (www.worldclim.org) to model potential habitat distribution of *L. perenne.*

I used 10,000 background pixels to represent the environmental conditions present in the study region. I used Maxent's default settings for the convergence threshold and maximum number of iterations (500). The Maxent models predictions were presented as logistic output.

Testing or validation is required to assess the predictive performance of the model. Ideally an independent data set should be used for testing model performance, however, in many cases this will not be available. The most commonly used approach is to partition the data randomly into "training" and "test" sets, creating independent data for model testing (Fielding and Bell 1997). Training data are used to build the model and test data are used to measure predictive success of the model. I randomly selected 25 occurrence records as test data and the other 74 records were reserved as training data.

The climate database WorldClim (www.worldclim.org) provides monthly precipitation (prec), maximum (tmax), minimum (tmin) and mean temperatures (tmean) climate files. Nineteen current bioclimatic variables (representative of the years 1950-2000) were downloaded directly from the website. These bioclimatic variables are: annual mean temperature, mean diurnal range, isothermality, temperature seasonality, maximum temperature of warmest month, minimum temperature of the coldest month, temperature annual range, mean temperature of wettest quarter, mean temperature of driest quarter, mean temperature of warmest quarter, mean temperature of coldest quarter, annual precipitation, precipitation of wettest month, precipitation of driest month, precipitation seasonality, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter and precipitation of coldest quarter. To understand the impacts of climate change on the suitability of *L. perenne* populations, the bioclimatic variables used as inputs for the Maxent software were derived from the three basic climatic variables (i.e. tmin, tmax, prec). I downloaded future climate files for the year 2050 from the Woldclim website, and converted these files from grid files to an ASCII format that is suitable for Maxent.

To define a relationship between *Neotyphodium* infection levels and the climate in the Mediterranean I randomly selected a subsample of 28 accessions from the sample of 99 accessions available in the NPGS (table 4.1). I planted 100 seeds from each accession in top soil and allowed the seeds to germinate in the greenhouse and produce large enough tillers which could be removed. 30 tillers from each accession were prepared for microscopic detection. There were 870 leaf sheath samples viewed in this manner. A strip of epidermis was peeled from the inner surface of the leaf sheath, near the base and mounted on a slide in aniline blue stain (Latch et al., 1987). The stain was allowed to penetrate the leaf sheath by warming the slide on a slide warmer. The stain was rinsed and I viewed the slides under 400X to examine for presence of the endophyte *Neotyphodium lolii.* Presence of endophytic hyphae was a positive indication of endophyte infection. To achieve an infection frequency, I counted how many individual tillers (for each accession) were endophyte infected, and divided that number by 30 and multiplied by 100 to get a percent.

Using the Climate/Extract function in DIVA-GIS, Version 7.1 (www.diva-gis.org) which assigns environmental and climatic data to point localities, I created climate files for point localities at which perennial ryegrass were recorded.

I then used the climatic data available for each locality in a multiple regression analysis to determine a relationship between climate and infection frequencies found in perennial ryegrass populations in the Mediterranean region (Fig.4.1). Multiple regression and model selection was performed using the backward stepwise regression technique. I used the R function leaps as the model selection tool. I ran a full linear model with infection frequency as the response variable and the 19 bioclimatic variables as the predictor variables. With this technique, initially all variables are included in the model and the contribution of each variable to the model is computed using AIC values. Variables that did not contribute significantly to the model were rejected. Using a correlation matrix with infection frequency as the dependent variable, I selectively removed any climate variables that were correlated with one another.

Results

Infection by *Neotyphodium lolii* was detected in all countries from which *Lolium perenne* populations were sampled (table 4.1). More than half of the accessions (19) had an infection frequency greater than 60% and each accession within a population varied in their percent infection frequency. Morocco and Italy had the highest average percent infection (70.57% ± 27.24 SD and 69.6% ± 12.28 SD respectively). The multiple regression analysis (table 4.2) reveals a negative parameter estimate for the precipitation of coldest quarter, which indicates that infection frequencies are high when the precipitation is low (Fig 4.4).

Figure 4.1 illustrates the collection sites for the 99 accessions of *Lolium perenne* used in the climate model. Figure 4.2 shows that in addition to the 6 countries used, the Maxent model developed for *L. perenne* coincides with the observed distribution of the species. The model predicts suitable habitat in Greece, Turkey, Switzerland, and Northern France, all countries in which perennial ryegrass naturally exists.

The climate change model (Fig 4.3), reveals few new suitable areas expected for this species. Climate change appears to negatively impact habitat suitability in Portugal and France. However, habitats in Morocco, Tunisia, and Italy appear to become more suitable as a result of climate change. Tables 4.3-4.6 show the average predicted change in key climate variables for *L. perenne*. Increases in temperature and declines in precipitation are expected for populations in Italy, Morocco, Tunisia, Italy and France. For E+ populations from France, annual temperature is expected to increase by 20% and annual precipitation is expected to decline by 15.6%. Populations in Morocco are expected to have temperature increases by 18.2% by 2050 and precipitation declines by 26.2%. Tunisia expects the smallest percent increase in annual temperature (11.7%) and has an expected 9% decline in annual precipitation. Annual temperatures in Italy are expected to change by 19.7% and annual precipitation should decline by 7.3%.

Discussion:

Experimental results using cultivated grasses often show a mutualistic relationship between endophytes and the grasses they infect. Increasing evidence from studies using wild grass populations often contradict these results. The persistence of vertically transmitted fungal endophytes in natural grass populations is perplexing, given that infected plants do not consistently exhibit increased fitness relative to non-infected grasses. Although infections are widespread in native grass species, infection levels are relatively low within species and variation in infection frequencies among local populations can be very high (Saikkonen et al. 2000). There are four possible explanations for the observed intermediate infection rates in natural grass populations. 1.) If these asexual endophytes are not at 100% frequency in populations it has been suggested that they have not yet reached equilibrium through natural selection (Clay 1993, 1998). It is predicted that over time these populations should become 100% infected (Clay 1998, Leutchtmann and Clay 1997, Wilkinson and Schardl 1997) because natural selection should favor infected plants if they exhibit herbivore resistance, enhanced competitive abilities and resistances to abiotic stresses such as drought. 2.) Imperfect transmission or the failure of the endophyte to grow into an ovule or seed or hyphal inviability (Siegal et al. 1984, Ravel et al. 1997) could lead to loss of infection. 3.) Since the interactions of asexual endophytes with host grasses span a continuum of interactions, the costs and the benefits of the symbiosis will vary spatially and temporally and infection frequencies will vary accordingly (Saikkonen et al. 1998). 4.) Local persistence of endophyte infection should be possible in the absence of mutualistic effects as a result of metapopulation dynamics (Saikkonen et al. 2002).

In this chapter, I hypothesized that climate factors were related to the infection frequency of *Neotyphodium lolii* in wild populations of *L. perenne*. I expected to see a positive relationship between infection frequency and temperature and a negative relationship between frequency of infection and precipitation. If *Neotyphodium* endophytes are mutualistic then high infection frequencies should occur in highly stressful habitats for plants. In order to fully grasp the range of endophyte-mediated effects on host grasses a better understanding of the natural range of environmental conditions in which the symbiosis exists is greatly needed. Benefits of evolutionary adaptations to a specific environment for E+ to E- grasses may fail completely once host plants are exposed to contrasting conditions (Hesse et al. 2003). If we remove endophyte infected grasses from habitats in which they are adapted and put them in a novel habitat we may be changing the direction and magnitude of the symbiosis. This may be true, especially for perennial ryegrass accessions from their native distributional range where high temperatures and seasonal droughts occur.

Although the P-value was not significant for the model (table 4.2) a significant relationship was found between infection frequency and precipitation of the coldest quarter. However, the results do not indicate a strong linear relationship between infection frequency and precipitation (Fig. 4.4). If there were a relationship than a generalization about the significance of *Neotyphodium lolii* in populations of *L. perenne* could be made. If the populations with the greatest infection frequency occur in habitats with high temperatures or low precipitation we could summarize the possible role of endophytes in these populations. It would provide evidence for the hypothesis that infected plants have a superior fitness to uninfected plants in hot and dry conditions.

From the climate files I generated, I found that infected accessions I used come from areas with variable temperature and precipitation patterns. For example, the annual temperature range from accessions in Italy ranges from 0.36-15.7°C and from Morocco 10.7-15.1°C. In Tunisia precipitation ranges from 275-934mm of rainfall per year and in Italy the yearly precipitation ranges from 636-1124mm. Although these extremes in temperature and precipitation patterns were found, annual precipitation and annual temperature were not significantly related to infection frequencies. The results from the climate models suggest an increase in habitat suitability for *L. perenne* in populations from Morocco, Tunisia, and Italy. This is surprising given the great percent change in temperature and precipitation. One would expect declines in the habitat suitability. However, according the FAO's Ecocrop database (Hijmans et al., 2005), the populations that are expected to have an increase in habitat suitability have temperatures and precipitation that are within the minimum to maximum ranges that are optimal for this species to exist. The minimum average temperature at which this plant grows optimally is at 14ºC and the maximum average temperature is 25ºC. Even with the temperature increases in Morocco, Tunisia and Italy, *L. perenne* is still in its optimal temperature growth range. Habitat suitability in France is predicted to decrease. Table 4.3 shows the climate variables for France and the % change in 2050. Populations in France appear to be the most vulnerable to climate change and this may be due to a 20% increase in temperature. Most notable is the 9.8% change in precipitation in this region where rainfall is frequent.

Although individual accessions varied in their percent infection, the average for each country was between 63-70% (table 4.1). Because of the well documented benefits of *Neotyphodium* endophytes under abiotic stress and drought, I hypothesized that there would be a significant relationship between infection and climate factors. My results do not indicate that high infection frequencies occur in high temperature and low precipitation habitats. This is in contrast to Lewis et al. (1997) who found significant correlations between percentage of infected seeds and climate. My results are more in line with Glade Brosi et al. 2011, who found endophyte infection frequency to be significantly higher under elevate CO2, but did not find support for their hypothesis that infection was significantly higher under altered precipitation, temperature or the treatment combinations.

Chapter 4: Literature cited

Baldwin, R.A. 2009. Use of maximum entropy modeling in wildlife research. Entropy 11:854-866.

Bazely, D. R. et al. 2007. Broad-scale geographic patterns in the distribution of vertically transmitted, asexual endophytes in four naturally-occurring grasses. Ecography 30: 367 – 374.

Brosi, G.B., McCulley, P.L., Bush, L.P., Nelson, J.A., Classen, A.T., Norby, R.J., 2011. Effects of multiple climate change factors on the tall fescue- fungal endophyte symbiosis: infection frequency and tissue chemistry. New Phytologist 189: 797-805.

Cheplick, G.P., Faeth, S.H., 2009. Ecology and Evolution of the Grass–Endophyte Symbiosis. Oxford University Press, New York.

Clay, K. 1996. Fungal endophytes, herbivores, and the structure of grassland communities. In Multitrophic interactions in terrestrial systems (ed. A. C. Gange), pp. 151-169. Oxford, UK: Blackwell Science.

Clay, K. 1998. Fungal endophyte infection and the population dynamics of grasses. In Population biology of grasses, ed. G.P. Cheplick, pp. 255-285. Cambridge: Cambridge University Press.

Clay, K., Holah, J. & Rudgers, J. A. 2005 Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. Proc. Nail Acad. Sei. USA 102, 12 465-12 470.

Ewald, P.W., 1987. Transmission mode and evolution of the parasitism-mutualism continuum. Annals of the New York Academy of Sciences. 503:295-306

Fielding, A.H., and Bell J.F., 1997. A review of methods for the assessment of prediction presence/absence models. Environmental Conservation 24:38-49.

Fine, P.E.M., 1975. Vectors and vertical transmission: an epidemiologic perspective. Annals of the New York Academy of Science 266: 173-194.

Gundel, P. E. et al. 2008a. *Neotyphodium* endophyte infection frequency in annual grass populations: relative importance of mutualism and transmission efficiency. Proc. R. Soc. B 275: 897 – 905.

Hesse, U., Schoberlein, W.,Wittenmayer, L., Forster, K., Warnstorff, K., Diepenbrock, W., Merbach, W., 2003. Effects of *Neotyphodium* endophytes on growth, reproduction and drought-stress tolerance of three *Lolium perenne L.* genotypes. Grass and Forage Science 58, 407–415.

IPCC. 2007: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. In. Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K.B. Averyt, M.Tignor and H.L. Miller (eds.). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. 996p.

Latch, G.C.M., Potter, L.R., and Tyler, B.R. 1987. Incidence of endophytes in seeds from collections of *Lolium* and *Festcua* species. Annals of Applied Biology 111: 59-64.

Leuchtmann, A. & Clay, K. 1997 The population biology of grass endophytes. In The mycota. V. Plant relationships, Part B (ed. G. C. Carroll & P. Tudzynski), pp. 185-204. Berlin: Springer.

Lewis, G. C. and Clements, R. O. 1986. A survey of ryegrass endophyte (*Acremonium loliae*) in the U.K. and its apparent ineffectually on a seedling pest. – J. Agric. Sci. 107: 633 – 638.

Lewis, G. C. et al. 1997. Occurrence of *Acremonium* endophytes in wild populations of *Lolium* spp. in European countries and a relationship between level of infection and climate in France. – Ann. Appl. Biol. 130: 227 – 238.

Malinowski, D. P. and Belesky, D. P. 2006. Ecological importance of *Neotyphodium* spp. grass endophytes in agroecosystems. Grasslands Sci. 52: 1 – 14.

Malinowski, D. P. and Belesky, D. P. 2000. Adaptations of endophyte-infected cool-season grasses to environmental stresses: mechanisms of drought and mineral stress tolerance. Crop Sci. 40: 923 – 940.

Pearson, R.G., Raxworthy, C.J., Nakamura, M., Peterson, A.T. 2007. Predicting species distributions from small numbers of occurrence records: a test case using cryptic geckos in Madagascar. Journal of Biogeography 34: 102-117.

Phillips,S.J., Anderson, R. P., Schapire, R.E. Maximum entropy modeling of species geographic distributions**.** *Ecological Modelling*, 190:231-259, 2006.

Rudgers, J. A., J. M. Koslow, K. Clay 2004. Endophytic fungi alter relationships between diversity and ecosystem properties. Ecology Letters 7: 42-51.

Rudgers, J. A., and K. Clay 2008. An invasive plant-fungal mutualism reduces arthropod diversity. Ecology Letters 11:831-840

Rudgers, J. A., Afkhami, M.E., Rua, M.A.,Davitt, A.J., Hammer, S., and Huguet, V.M. 2009. A fungus among us: broad patterns of endophyte distribution in the grasses. – Ecology 90: 1531 – 1539.
Rudgers, J.A., Fischer, S., and Clay K. (2010) Managing plant symbioses: Fungal endophyte genotype alters plant community composition. Journal of Applied Ecology 47: 468-477

Saikkonen, K., Faeth, S. H., Helander, M. L. & Sullivan, T. J. 1998. Fungal endophytes: a continuum of interactions with host plant. A. Rev. Ecol. Syst. 29, 319-343.

Saikkonen, K., Ahlholm, J., Helander, M., Lehtimaki, S. and Niemelainen, O. 2000. Endophytic fungi in wild and cultivated grasses in Finland. Ecography 23, 360-366.

Schulthess, F. M. and Faeth, S. H. 1998 Distribution, abundance and associations of the endophytic fungal community of Arizona fescue (*Festuca arizonica* Vasey). Mycologza 90, 569-578.

Siegel, M.R., Johnson, M.C., Varney, D.R., Nesmith, W.C., Buckner, R.C., Bush, L.P., Burrus, P.B., II, Jones, T.A., Boling, J.A. (1984) A fungal endophyte in tall fescue: incidence and dissemination. Phytopathology 74: 932-937.

Townsend, C., Harper, J. L.,Begon, M. 2002. Essentials of ecology. Blackwell

Wilkinson, H.H., Schardl, C.L, 1997. The evolution of mutualism in grass-endophyte associations. In: Bacon, C.W., Hill, N.S. (Eds.), Neotyphodium/Grass Interactions. Plenum Press, New York, pp. 13–25.

White, J. F. and Baldwin, N. A. 1992. A preliminary enumeration of grass endophytes in west central England. Sydowia 44: 78 – 84.

Chapter 4 tables:

Table 4.1.

Origin	Accession Number	Infection Frequency %
Tunisia	598909	77
	610924	100
	598911	67
	598913	44
	610925	58
	610950	43
	610927	75
	610926	66
Average		**66.25 ± 18.65 SD**
Morocco	632590	91
	598839	77
	598877	88
	598888	85
	598902	36
	598904	27
	598854	90
Average		**70.57± 27.24 SD**
Italy	598429	50
	598432	70
	598434	86
	610962	63
	598931	60
	610939	80
	598935	73
Average		**69.6±12.28 SD**
France	628693	75
	W69286	85
	598438	68
	598439	73
	610795	57
	577255	34
Average		**65.3± 16.32 SD**

Table 4.2.

Climate Variable	DF	Sum SQ	Mean SQ	F-value	P-Value	
Temperature Seasonality	1	26.1	26.05	0.0648	0.80149	
Min Temperature of Coldest Month	1	11.9	11.93	0.0296	0.86487	
Mean Temperature of Wettest Quarter	1	8.0	8.01	0.0199	0.88909	
Precipitation of Wettest Month	1	469.5	469.5	1.1671	0.29171	
Precipitation of Wettest Quarter	1	438.2	438.18	1.0892	0.30798	
Precipitation of Coldest Quarter	1	2432.5	2432.5	6.0465	**0.02227**	
Error	22	8850.7	402.30			

***= multiple regression different from zero at probability level of 0.05%. Pr>F of the model= 0.2578**

Table 4.3

Origin	Climate variable	Present	2050	%Change
France	Annual Temperature (° C)	10.05	12.06	20
	Maximum Temperature in warmest Month (° C)	23.96	26.66	11.3
	Temperature Seasonality ° C	635.88	643.07	1.1
	Annual Precipitation (mm)	801	722.37	-9.8
	Precipitation of Driest Quarter (mm)	171	144.25	-15.6
	Precipitation of Coldest Quarter	205.25	154.86	-24.55

Table 4.4

Origin	Climate variable	Present	2050	%Change
Morocco	Annual Temperature (° C)	12.84	15.18	18.2
	Maximum Temperature in warmest Month (° C)	32.76	36.01	9.9
	Temperature Seasonality (° C)	680.426	692.34	1.7
	Annual Precipitation (mm)	606.33	447.21	-26.2
	Precipitation of Driest Quarter (mm)	33.43	24.64	-26.3
	Precipitation of Coldest Quarter(mm)	206.5	150.5	-27.12

Table 4.5

Origin	Climate variable	Climate Variable	Present	2050	%Change
Tunisia	Annual Temperature (° C)	Annual Temperature	17.10	19.10	11.7
	Maximum Temperature in warmest Month (° C)	Maximum Temperature in warmest Month	33.41	35.69	6.8
	Temperature Seasonality (° C)	Temperature Seasonality	637.00	636.34	0.35
	Annual Precipitation (mm)	Annual Precipitation	715.66	648.42	-9.0
	Precipitation of Driest Quarter (mm)	Precipitation of Driest Quarter	31.58	25.08	-20.5
	Precipitation of Coldest Quarter (mm)	Precipitation of Coldest Quarter	323.58	281.58	-13

Table 4.6

Origin	Climate variable	Climate Variable	Present	2050	% Change
Italy	Annual Temperature (° C)	Annual Temperature	11.47	13.73	19.7
	Maximum Temperature in warmest Month (° C)	Maximum Temperature in warmest Month	24.68	27.68	12.0
	Temperature Seasonality (° C)	Temperature Seasonality	587.58	591.60	0.68
	Annual Precipitation (mm)	Annual Precipitation	797.65	739.04	-7.3
	Precipitation of Driest Quarter (mm)	Precipitation of Driest Quarter	84.31	68.88	-18.3
	Precipitation of Coldest Quarter (mm)	Precipitation of Coldest Quarter	255.72	220.44	-13.8

Figure 4.1

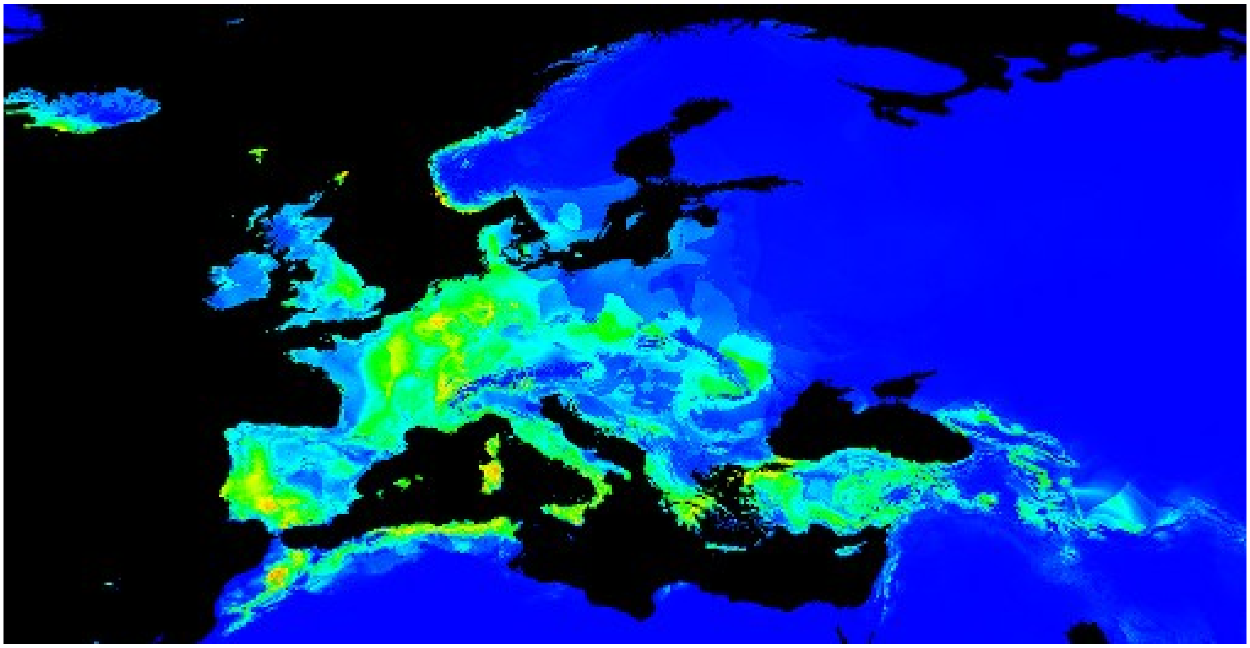
Figure 4.2

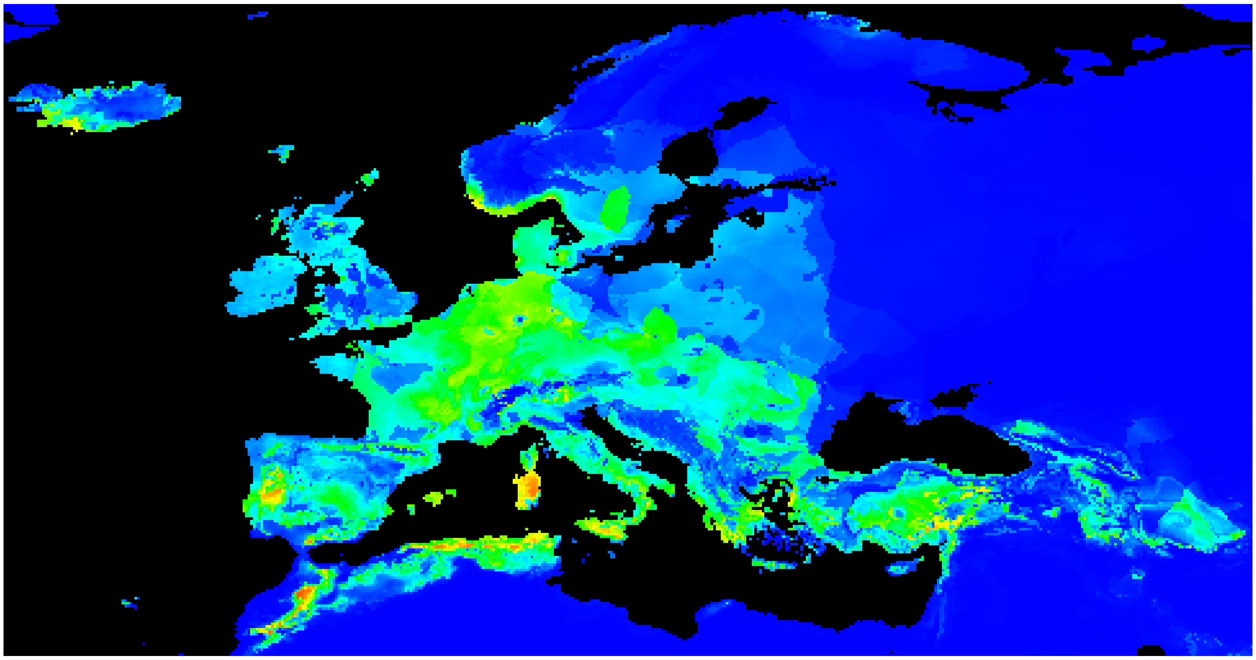

Figure 4.3

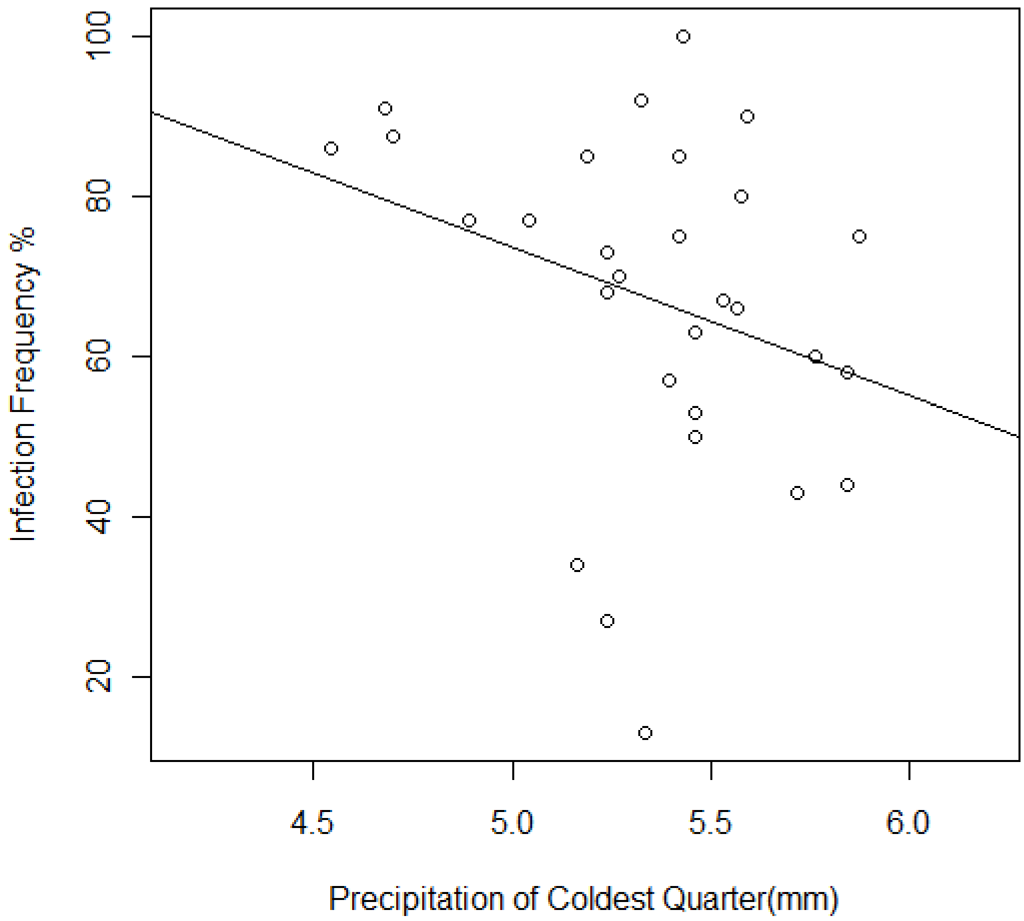
Infection Frequency %
20
40
60
80
100
4.5
5.0
5.5
6.0
Precipitation of Coldest Quarter(mm)

Figure 4.4

Chapter 5

Evidence for membrane disruption by endophytic fungi in perennial ryegrass *(Lolium perenne)*

Introduction

Fungal endophytes in the genus *Neotyphodium* (formerly *Acremonium*; Glenn et al. 1996) are obligate biotrophs that live in symbiotic relationships with hundreds of grass species (White 1987, Clay 1990) some of which are important forage and turf grasses. Endophytes grow as elongate, convoluted hyphae in between host plant cells, apparently obtaining host photosynthates. They also often form close associations with host plant cell walls (Christensen, Bennette and Schmid, 2002). These fungi express different symbiotic lifestyles ranging from mutualism, parasitism and commensalism (Cheplick, Clay and Marks 1989, Ahlholm et al. 2002, Hesse et al. 2004, Cheplick 2007) due to environmental and genetic conditions. In mutualistic associations, endophyte infected grasses can show improved ability to tolerate abiotic stresses such as drought or low soil fertility (West 1994, Malinowski, Belesky and Lewis 2005, Clay and Schardl 2002, Latch 1997, Saikkonen et al. 1998). From its host, the fungus receives nutrients and a means of propagation through the seeds. Fungal produced alkaloids result in resistance to small insects and herbivores (Clay 1988, Breen 1994, Bush, Wilkinson and Schardl 1997), thus benefiting the growth and competitive abilities of the host plant (Clay, Marks and Cheplick1993, Cheplick and Clay 1988, Cheplick et al. 1989, Malinowski et al. 1997). However, fungal derived alkaloids may be detrimental to livestock resulting in ryegrass staggers (Fletcher and Harvey 1981), or fescue toxicosis (Bacon 1977) which may cause severe health problems for the livestock.

Since infection by *Neotyphodium* endophytes is asymptomatic, their presence in plants has primarily been determined by microscopic examination of host leaf sheaths. Many methods have been developed to assess infection frequency and infection intensity. These methods include histochemical (Bacon and White 1994), immunological (Hiatt et al. 1999, Hahn et al.

2003), PCR-based (Doss 1998, Groppe & Boller 1996) and grass tissue culture techniques. Although useful, some of these methods are expensive, time consuming, and do not give an indication of viable endophyte.

In this study, we used a fluorescent based technique that allowed us to study the relationship and distribution of *Neotyphodium lolii* within the vasculature of its host plant *Lolium perenne.* Fungal cell walls containing chitin were fluorescently labeled with Wheat germ agglutinin (WGA), while the vascular bundle was stained with Propidium iodide (PI). The infected (E+) and non-infected (E-) grasses were viewed using confocal microscopy. Our results imply a breakdown of the mesophyll cell membranes, which allowed PI to cross into the mesophyll and subsequently breakdown the membranes of the chloroplasts. As we describe this phenomenon in infected plants only, the data provides evidence of a compromised membrane, suggesting a mechanism by which fungal endophytes may extract nutrients from their hosts.

Materials and Methods

Grass Sources. Seeds representing distinct genotypes of *L. perenne* were obtained from the Western Regional Plant Introduction Station, United States Department of Agriculture, Pullman, WA, USA (National Plant Germplasm System;(http://www.ars-grin.gov/npgs). Some leaf sheaths were imaged for propidium iodide staining alone while others were imaged for dual localization of the plant and fungus. Both (E+) and (E-) genotypes from each population were used. Endophyte status was already determined using the analine blue method described in (Bacon and White 1994) and then viewed under 400X with a light microscope.

Pre-treatment of the plant. Lolium perenne genotypes were grown to maturity in pots containing vermiculite. Propidium iodide (PI) was diluted 1:1000 in water and added to the soil. Propidium iodide is an intercalating agent and fluorescent molecule that can be used to stain cells. It is often used as a counterstain in multicolor fluorescent techniques. The vital dye is impermeable to live cells as it cannot breach the plasma membrane. The original rationale for this approach was that the propidium iodide could be taken up by the plant through its root system and then transported and visualized within the vascular bundle.

Post-treatment of the plant. Following a 16 hour uptake of the propidium iodide, a leaf sheath was excised from each plant and then the samples were rinsed three times in water. To label the fungal wall, we used 10 µg/ml wheat germ agglutinin (WGA) conjugated with Alexa Fluor 488 (Invitrogen/Molecular Probes, Eugene OR). To prepare a stock solution, we dissolved 5.0 mg of lyophilized WGA conjugate in 5.0 ml of phosphate buffer solution (PBS). We stored the stock solution in the freezer at -20ºC. Wheat germ agglutinin is a lectin or a sugar binding protein as it binds to N-acetyl-D-glucosamines which are naturally found in the chitin of fungi. The excised leaf sheath was allowed to incubate in the WGA overnight and after the leaf sheaths were rinsed twice in buffer. The sheath was mounted on a slide and covered by #1.5 cover glass. The cover glass was raised by forming a well around the sheath with parafilm. The cover glass was sealed to the slide with nail polish and the samples imaged with confocal laser scanning microscope (Leica SP2 AOBS) using a 488 nm laser for the WGA and 594 nm for the PI.

Results

By allowing the plants to uptake the PI, it is possible for the PI to be transported throughout the grasses vascular bundle. If the membrane of the mesophyll surrounding the chloroplasts were to remain intact, the PI would not be found to be associated with any double stranded DNA (nuclear, mitochondrial or chloroplast). Only when there is disruption of the mesophyll plasma membrane, does the PI cross into the cell and enter the chloroplasts (fig 3,4). We demonstrated the uptake of PI into the cells of the leaf sheath, but only in those cells infected with the endophyte. Using the PI-specific spectrum for data collection, we were able to demonstrate the presence of PI in the chloroplasts. Our images provide the evidence that the PI was found incorporated in the vascular tissue suggesting it was taken up by the root system. This strongly suggests that the plasma membrane of the plant had been compromised, allowing staining of the chloroplasts. When we examined non-infected plants, we were unable to demonstrate the incorporation of PI into chloroplasts (Figure 1). When staining with fluorescent WGA, we can detect *N. lolii* in arrays perpendicular to the vascular bundle (Figure 2,4) undulating around (Figure 3) or though (Figure 4) a vascular bundle.

Discussion

Currently, our knowledge regarding the ways in which endophytes obtain nutrients from plant cells is limited. Fungi in the family Clavicipitaceae have never been observed to produce haustoria or haustoria- like feeding structures that penetrate plant cells and because of this it was generally believed that endophytes live intercellularly within plant tissues and passively absorb nutrients that leak from plant cells to the apoplast. The current hypothesis regarding nutrient extraction from host cells involves secretion of reactive oxygen species and auxins by endophytes onto host cell walls and membranes inducing nutrient leakage from cells.

ROS production and secretion by endophytes was demonstrated by Tanaka et al. 2006, as part of a mechanism by which clavicipitalean endophytes alter cell membranes to facilitate leakage of nutrients into the apoplastic spaces where they may be absorbed by fungal hyphae. ROS secreted onto the plasma membrane of host cells oxidize and denature the membranes, causing leakage of nutrients from plant cells to hyphae located outside of the cells (White and Torres 2010). In support of this, Torres and White (unpublished) demonstrated greater membrane lipid peroxidation in E+ clones of fine fescue. Also consistent with this hypothesis, Richardson et al. 1992, reported a greater accumulation of glucose and fructose plant sugars in the apoplastic spaces of tall fescue plants infected with the endophyte *Neotyphodium coenophialum* compared to that of non-infected plants.

In nature, fungal secreted hydrolytic enzymes are known to degrade large molecules to smaller molecules that can be used for nutrition (Wessels 1993). There is reported evidence that hydrolytic enzymes possessed by fungal endophytes can be used in nutrient acquisition from the plant apoplast (Lam et al. 1995, Li et al. 2004, Moy et al. 2002, Lindstrom and Belanger, 1994). Li et al., 2004 reported a novel fungal produced endochitinase which was the major secreted protein in culture and also in the apoplastic fluid in the infected host grass, *Poa ampla.* This secreted chitinase may have roles in the nutrition, growth or defense of the endophyte.

We used confocal microscopy as a technique to understand how endophytic hyphae interact with host plant cells. We used *N. lolii* and *L. perenne* as the model for examining this interaction. In this experiment we provide further evidence for the hypothesis that endophytes enhance nutrient leakage by showing plant membrane disruption by fungal endophytes. We show that the intercalating agent and fluorescent molecule propidium iodide crossed the cells plasma membrane only when the plants were endophyte-infected. This provides evidence that the endophyte enhances nutrient leakage possible through the production of ROS.

More studies should be conducted to explore the effects of endophyte produced secondary metabolites to test the hypothesis that they may have a role in nutrient acquisition from host cells. Secondary metabolites such as alkaloids may alter host cell physiology and permit acquisition of nutrients by endophytes from the closely associated host cells through a membrane depolarization mechanism resulting in increased leakage of nutrients from plant cells.

References

Ahlholm J. U., Helander M. Lehtimaki S. Wali P. and Saikkonen K. (2002) Vertically transmitted fungal endophytes: different responses of host-parasite systems to environmental conditions. Oikos 99, 173-183.

Bacon C.W., Porter J.K. Robbins J.D. and Luttrell E.S. (1977) *Epichloë typhina* from toxic tall fescue grasses. Applied and Environmental Microbiology 34,76-81.

Bacon C.W., and White J.F. (1994) Stains, media, and procedures for analyzing endophytes. *In Biotechnology of endophytic fungi of grasses*, eds. C.W. Bacon and J.F. White., pp. 47-56. Boca Raton, Fl:CRC Press.

Breen J.P. (1994) *Acremonium* endophyte interactions with enhanced plant resistance to insects. Ann. Rev. Entomol., 39, 401–423.

Bush L. P., Wilkinson H. H. and Schardl C. L. (1997) Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiology. 114, 1-7.

Cheplick G.P., and Clay K. (1988) Acquired chemical defenses in grasses: the role of fungal endophytes. Oikos 52,309-318.

Cheplick G.P., Clay K. and Marks S. (1989) Interactions between Infection by endophytic Fungi and nutrient limitation in the Grasses *Lolium perenne* and *Festuca arundinacea.* New Phytologist, 111, 89-97.

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

Christensen M. J., Bennette R.J. and J. Schmid. (2002) Growth of *Epichloë/Neotyphodium* and p-endophytes in leaves of *Lolium* and *Festuca* grasses. Mycological Research 106,93-106.

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

Clay K. (1990) Fungal endophytes of grasses. Annual Review of Ecology and Systematics 21,275-297.

Clay K., Marks S, Cheplick G.P. (1993) Effects of insect herbivory and fungal endophyte infection on competitive interactions among grasses. Ecology 74, 1767-1777.

Clay K., Schardl C. (2002) Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. American Naturalist 160, S99-S127.

Doss R.P. (1998) A PCR-Based Technique For Detection Of Neotyphodium Endophytes In Diverse Accessions Of Tall Fescue. Plant Disease 87, 738-740.

Fletcher L.R., Harvey I.C. (1981) An association of a *Lolium endophyte* with ryegrass staggers New Zealand Veterinary Journal 29, 185-186.

Glenn AE., Bacon C.W. Price R. Hanlin R.T. (1996). Molecular phylogeny of *Acremonium* and its taxonomic implications. Mycologia 88,369-383.

Groppe K., and Boller T. (1996) PCR Assay Based On Microsatellite- Containing Locus For Detection And Quantification Of *Epichloe* Endophytes In Grass Tissue. Applied and Environmental Microbiology 63, 1543-1550.

Hahn H., Huth W. Schoberlein W. and Diepenbrock W. (2003) Detection of Endophytic Fungi In *Festuca* Spp. By Means Of Tissue Print Immunoassay. Plant Breeding 122, 217-222.

Hesse U., Hahn H. Andreeva K. Forster K. Warnstorff K. Schoberlein W. and Diepenbrock W. (2004) Investigations on the influence of *Neotyphodium* endophytes on plant growth and seed yield of *Lolium perenne* genotypes. Crop Science 44,1689-1695.

Hiatt E. E., Hill N. S III. Bouton J. H. and Stuedemann J. A. (1999) Tall Fescue Endophyte Detection: Commercial Immunoblot Test Kit Compared with Microscopic Analysis. Crop Science. 39, 796-799.

Lam C.K., Belanger F.C. White J.F. Daie J. (1995) Invertase activity in *Epichloe/Acremonium* fungal endophytes and its possible role in choke disease. Mycological Research 99,867-873.

Latch G.C.M., (1997). An overview of *Neotyphodium*–grass interactions. p. 1–11. *In* C.W. Bacon and N.S. Hill (ed.) *Neotyphodium*/ grass interactions. Plenum Press, New York.

Li H.M., Sullivan R. Moy M. Kobayashi D.Y., Belanger, F.C. (2004) Expression of a novel chitinase by the fungal endophyte in *Poa ampla*. Mycologia 96, 526-536.

Lindstrom J.T., Belanger F.C. (1994) Purification and characterization of an endophytic fungal proteinase that is abundantly expressed in the infected host grass. Plant physiology 106,7-16.

Malinowski D.P., Leuchtmann A. Schmidt D. and Nösberger J. (1997) Symbiosis with Neotyphodium uncinatum endophyte may increase the competitive ability of medow fescue. Agronomy Journal 89, 833-839.

Malinowski D.P., Belesky, D.P. Lewis, G.C. (2005) Abiotic stresses in endophytic grasses. In: Roberts, C.A., West, C.P., Spiers, D.E., (Eds.), *Neotyphodium* in Cool-season Grasses.

Blackwell Publications, Ames, Iowa, pp. 187–199.

Moy M., Li H.M. Sullivan R. White J.F. Belanger F.C. (2002) Endophytic fungal β-1,6-glucanase expression in the infected host grass. Plant physiology 130, 1298-1308.

Saikkonen K., Faeth S.H. Helander M. Sullivan T.J. (1998) Fungal endophytes: a continuum of interactions with host plants. Ann. Rev. Ecol. Syst. 29, 319–343.

Tanaka A., Christensen M.J., Takemoto D., Park P., Scott B. (2006). Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell 18: 1052-1066.

Wessels J.G.H., (1993) Wall growth, protein excretion and morphogenesis in fungi. New phytologist 123,397-413.

West C.P. (1994) Physiology and drought tolerance of endophyte-infected grasses. In *Biotechnology of endophytic fungi of grasses,* eds. C.W. Bacon and J.W. White, Jr., pp. 87-99. Boca Raton, FL:CRC Press.

White J. F. (1987) Widespread distribution of endophytes in the Poaceae. Plant disease 71,340-342.

White J.F., Torres M.S., 2010. Is plant endophyte-mediated defensive mutualism the result of oxidative stress protection? Physiological Plantarum 138, 440-446.

Figures:

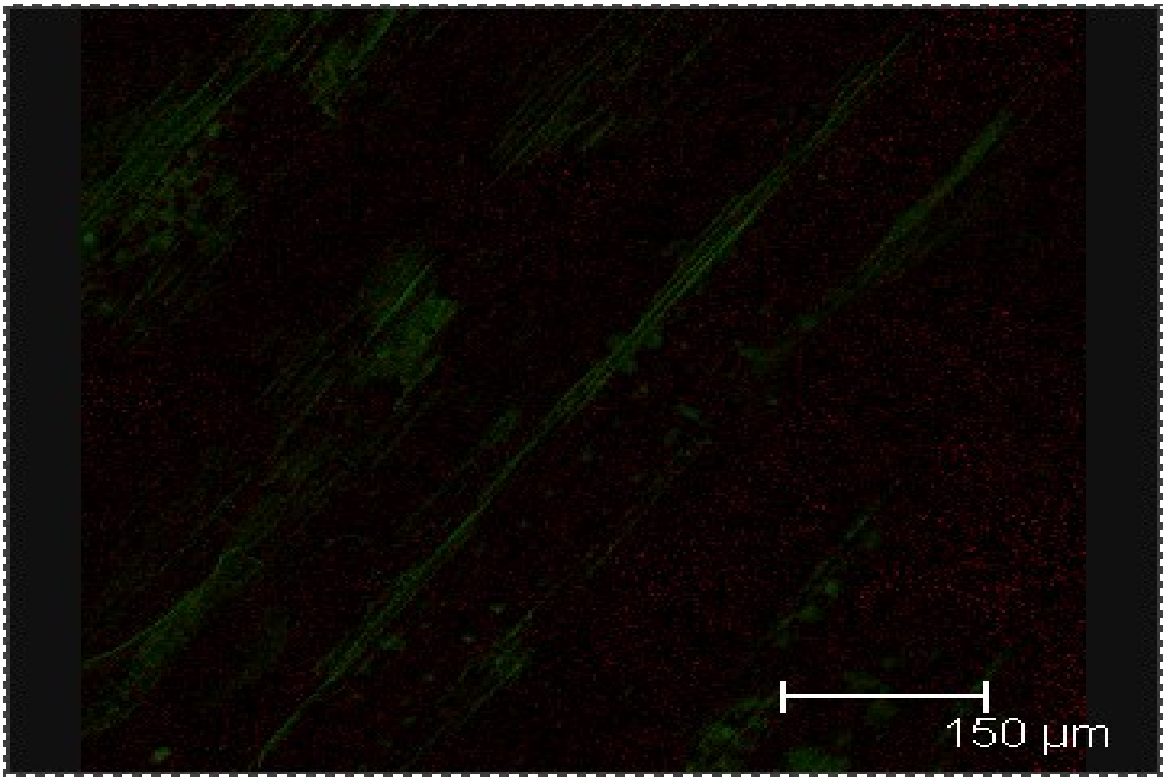

Figure1.

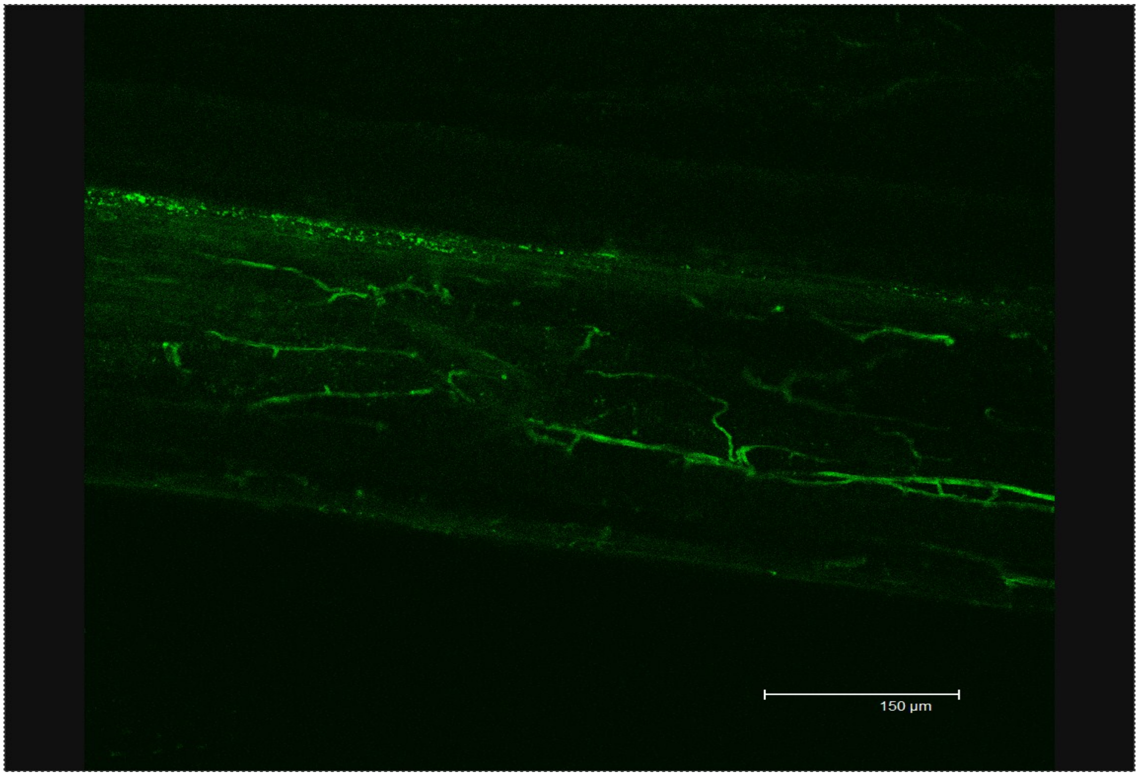

Figure 2.

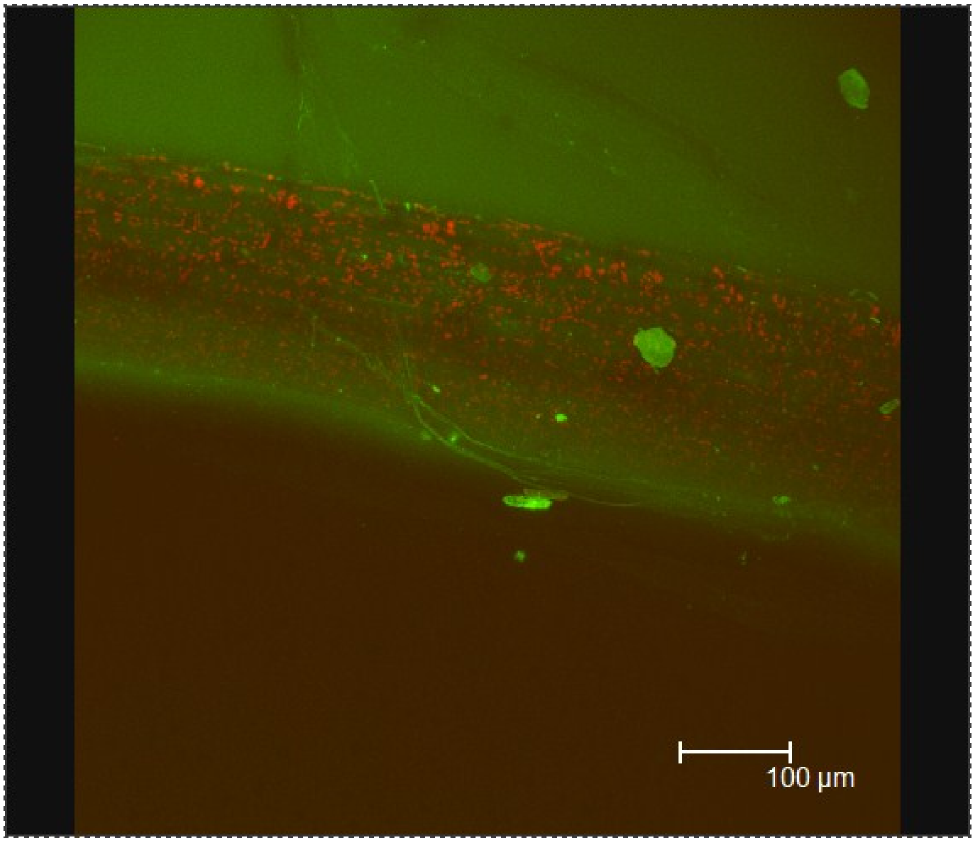

Figure 3

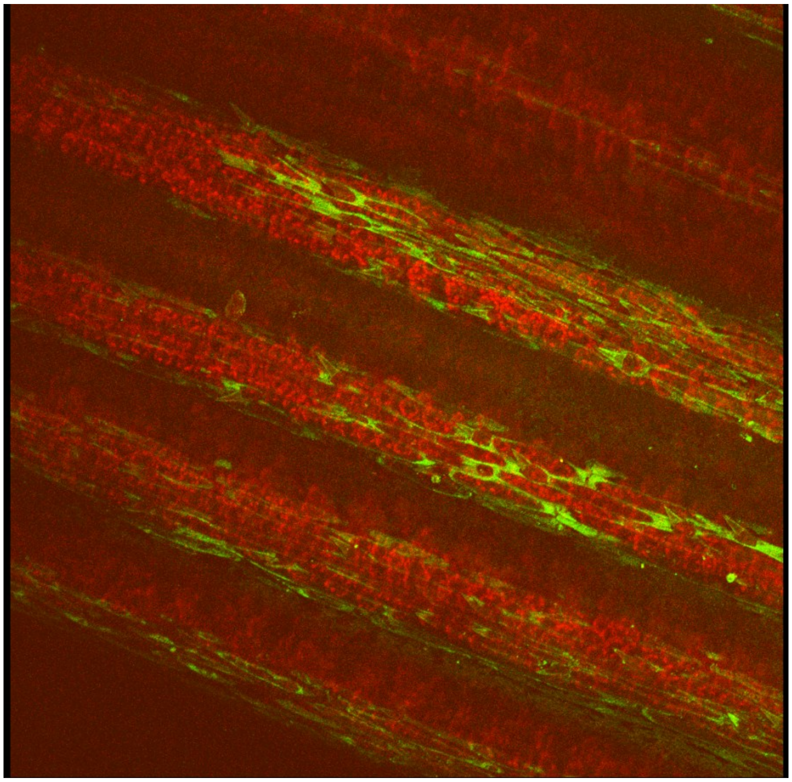

Figure 4.