AN ABSTRACT OF THE THESIS OF

<u>Julaluck Intasin</u> for the degree of <u>Master of Science</u> in <u>Microbiology</u> presented on <u>July 19, 2024</u>.

Title: Determining *Epichloë* Infection Status across Commercial Perennial Ryegrass Cultivars and Evaluating *Epichloë*-mediated Resistance against *Noctua pronuba* in Cool-Season Turfgrass

Abstract approved:

Navneet Kaur

Hannah M. Rivedal

Epichloë spp. are fungal endophytes that infect cool-season grasses, imparting benefits such as insect pest resistance and tolerance to abiotic stresses. These benefits are primarily attributed to the production of alkaloids by *Epichloë*, which can deter or be toxic to insect pests. The implications of grass-*Epichloë* associations on grass seed and turfgrass performance are significant for Oregon's grass seed industry, as they could provide an alternative control method for insect pests of concern. Nonetheless, the status and persistence of *Epichloë* endophytes in turf and grass seed stands have not always been prioritized or given higher market value for Oregon produced seed.

This research aimed to assess the prevalence and identify alkaloid pattern of *Epichloë* in Oregon's turfgrass production and investigate if this symbiotic association can be utilized for the management of winter cutworms (*Noctua pronuba*) in tall fescue [*Festuca arundinacea* Schreb. = *Lolium arundinaceum* (Schreb.) Darbysh. = *Schedonorus arundinaceus* (Schreb.) Dumort] and perennial ryegrass (*Lolium*

perenne L.). In 2021, 15 sets of tiller samples from 51 perennial ryegrass cultivars evaluated in the 2016 NTEP trials at the Oregon State University were collected. Utilizing polymerase chain reactions (PCR) targeting *Epichloë* alkaloid loci, infection rates, genetic variation in alkaloid gene loci, and the relationship between Epichloë incidence and turfgrass quality were determined. The findings revealed highly variable *Epichloë* infections among perennial ryegrass cultivars, with comparable number of cultivars within each range of infection intervals (e.g., 0-20%, 20-40%, etc.). Two distinct profiles of potential chemotypes in *Epichloë* were detected, with most cultivars capable of producing peramine, ergot alkaloid, and indole-diterpene. Moreover, this analysis showed an increase in *Epichloë* incidence in 28 cultivars over the five-year period of the NTEP trial, while some exhibited a decline (17 cultivars) and the others stayed consistent. Surprisingly, traffic application did not significantly impact average quality ratings in 2021; instead, variability in *Epichloë* infection levels emerged as a principal factor affecting quality estimations. Interestingly, these data suggest that achieving a 20% infection rate in perennial ryegrass may suffice to enhance average quality ratings, with no further improvements noted at higher infection rates.

In a subsequent study, responses of *N. pronuba*, a significant lepidopteran pest in Oregon's grass seed production, to tall fescue and perennial ryegrass with varying *Epichloë* infection levels was evaluated. Two no-choice greenhouse assays (trail 1 and 2) were conducted to assess insect survival, weight gain, grass biomass, and feeding damage at multiple time points during the 14-day trial duration. Grass tiller samples were analyzed to confirm *Epichloë* status and alkaloid gene presence postfeeding. Unfortunately, a significant difference both among expected *Epichloë* incidence and within cultivars was not detected in trial 1, regardless of the grass host species. Additionally, observed *Epichloë* infection was not an important factor in estimating observed variability in the dependent variables in trial 1.

Trial 2 results indicated that the expected *Epichloë* infection levels in tall fescue did not significantly affect insect mortality or weight gain but were associated with differences in mean feeding damage at 3 and 12 days. However, the impact of expected *Epichloë* infection status on feeding damage appeared to be overridden by cultivar-specific traits. In perennial ryegrass, low *Epichloë* infection unexpectedly correlated with higher insect mortality, although this trend was not consistent across all parameters considered. Similar to tall fescue, variations in grass performance metrics in perennial ryegrass were predominantly attributable to cultivar-specific characteristics rather than *Epichloë* levels.

Overall, these findings contribute valuable insights into the practical application of grass-*Epichloë* symbioses for turf management and potential *N. pronuba* and other cutworm control, thereby enhancing agronomic and environmental sustainability in grass seed production. In the future, the use of advanced analytical tools such as HPLC and MS will be essential for alkaloid quantification in grass tillers and insects to further the understanding of *Epichloë*-mediated insect resistance.

©Copyright by Julaluck Intasin July 19, 2024 All Rights Reserved

Determining *Epichloë* Infection Status across Commercial Perennial Ryegrass Cultivars and Evaluating *Epichloë* -mediated Resistance against *Noctua pronuba* in Cool-Season Turfgrass

by Julaluck Intasin

A THESIS

submitted to

Oregon State University

in partial fulfillment of the requirements for the degree of

Master of Science

Presented July 19, 2024 Commencement June 2025 Master of Science thesis of Julaluck Intasin presented on July 19, 2024

APPROVED:

Co-Major Professor, representing Microbiology

Co-Major Professor, representing Microbiology

Head of the Department of Microbiology

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Julaluck Intasin, Author

ACKNOWLEDGEMENTS

My deepest appreciation to:

Dr. Navneet Kaur and Dr. Hannah Rivedal for being hardworking scientists and understanding mentors. Thank you for providing the womanhood we all need in academia. Thank you for seeing my values and capability. I appreciate your recognition of my values and capabilities, and for never restricting my imagination but instead creating space for me to evolve into the scientist I aspire to be. Your appreciation of not only my scientific identity but my entire being means a great deal to me.

Navneet lab members for being patient with my frustration and flexible when our schedule was tight. Thank you for your dedication in making all the trials happened. To me, it was beyond mission impossible.

Hannah lab members for allowing me to use the lab space whenever needed, answering all my questions patiently, and going out of your way to assist with my project. Todd Temple, thank you for tolerating my intentional unprofessionalism and for your unwavering support and availability.

The Oregon State University Turf Team for allowing me to participate in the study and for sharing valuable information regarding the NTEP trial. Special thanks to Dr. Alec Kowalewski for providing resources and opportunities to engage with the turf community.

My committee- Dr. Nicole Anderson and Dr. Kimberly Halsey for being my sounding boards and supporting my professional development.

My friends and family for always supporting me and believing in me when I didn't believe in myself. Thank you for showing me we are only a phone call away regardless of time zone you are in. The soil department and my soil cohort for adopting me and teaching me the "dirt"-ey science. Thank you for your emotional support and friendship.

TABLE OF CONTENTS

| - | |
|--|---------|
| General Introduction | 1 |
| Chapter 2 <i>Epichloë</i> infection and alkaloid pattern of perennial ryegrass cultivars in Oregon | ı 15 |
| Chapter 3 Endophyte-mediated resistance against <i>Noctua pronuba</i> in cool-season turfgrass | 38 |
| General Conclusions | 73 |

Page

LIST OF FIGURES

| Figure Page |
|---|
| 2.1 Average quality ratings of perennial ryegrass cultivars evaluated in the 2016 NTEP program under both traffic and non-traffic conditions from 2017 to 2021. The red boxes represent the no-traffic designation, while the green boxes represent the traffic designation. N, no traffic; T, traffic |
| 2.2 Average quality ratings of perennial ryegrass cultivars evaluated in the 2016 NTEP program, categorized by varying frequencies of tiller <i>Epichloë</i> infection, under both traffic and non-traffic conditions in 2021. The red dots represent means of the <i>Epichloë</i> infection range groups. The red boxes represent the no-traffic designation, while the green boxes represent the traffic designation. N, no traffic; T, traffic. * denotes $P < 0.05$, Dunnett's post hoc test |
| 3.1 Mean ± SE feeding damage at 3 days in trial 1 (A) and trial 2 (B) of N. pronuba feeding on tall fescue grown from fungicide treated (F) or no fungicide treated (NF) seed |
| 3.2 Mean \pm SE insect mortality (A) and insect weight gain (B) of <i>N. pronuba</i> feeding on tall fescue cultivars with either high or low expected <i>Epichloë</i> level in trial 1 no-choice feeding experiment |
| 3.3 Mean ± SE insect mortality (A, C) and insect weight gain (B, D) of <i>N. pronuba</i> feeding on the perennial ryegrass cultivars with either high or low expected <i>Epichloë</i> level (A, B) and different perennial ryegrass cultivars (C, D) in trial 1 no-choice feeding experiment |
| 3.4 Mean ± SE feeding damage at 3 days (A) and 12 days (B) of <i>N. pronuba</i> feeding on tall fescue cultivars with either high or low expected <i>Epichloë</i> level in trial 2 no-choice feeding experiment |
| 3.5 Mean ± SE insect mortality (A, C) and insect weight gain (B, D) of N. pronuba feeding on the tall fescue cultivars with either high or low expected Epichloë level (A, B) and different tall fescue cultivars (C, D) in trial 2 no-choice feeding experiment |
| 3.6 Mean ± SE insect mortality (A) and insect weight gain (B) of <i>N. pronuba</i> feeding on perennial ryegrass cultivars with either high or low expected <i>Epichloë</i> level in trial 2 no-choice feeding experiment |
| 3.7 Linear relationships between observed <i>Epichloë</i> level and feeding damage at 3 days of <i>N. pronuba</i> feeding on the perennial ryegrass cultivars |

LIST OF TABLES

| Tabl | <u>Table</u> Pag | |
|------|--|--|
| 2.1 | List of 51 evaluated perennial ryegrass cultivars with seed and tiller <i>Epichloë</i> spp. infection frequencies and predicted alkaloid profiles in Oregon State University's NTEP trials | |
| 2.2 | Primers used in multiplex PCR to determine <i>Epichloë</i> presence and alkaloid potential | |
| 3.1 | Expected and observed <i>Epichloë</i> levels in tiller samples of both tall fescue and perennial ryegrass cultivars from both trials | |
| 3.2 | <i>P</i> -values for mean comparisons between fungicide treatments, expected <i>Epichloë</i> infection levels, and grass cultivars for tall fescue and perennial ryegrass in trial 1 | |
| 3.3 | LM output for insect mortality, insect weight gain, grass biomass, percentage of feeding damage at 3, 12, 15 days in trial 1 | |
| 3.4 | <i>P</i> -values for mean comparisons between fungicide treatments, expected <i>Epichloë</i> infection levels, and grass cultivars for tall fescue and perennial ryegrass in trial 2 | |

CHAPTER 1

General Introduction

Julaluck Intasin

Cool-Season Grass Seed Production Systems in Oregon

Oregon is renowned globally as a primary producer of cool-season grass seed for forage and turf, with a total harvestable acreage of annual ryegrass, perennial ryegrass, and tall fescue estimated to be 302,000 acres (USDA NASS, 2024). Most of the production is located in the Willamette Valley – the "grass seed capital of the world." Grass seed is one of Oregon's leading agricultural commodities with a farmgate value of \$639.2 million USD (Oregon Department of Agriculture 2023). Oregon's grass seed industry has grown since the 1940s, followed by the establishment of the Plant Variety Protection (PVP) Act in 1970. The PVP Act catalyzed a significant increase in breeding initiatives targeting cool-season turf grass cultivars, resulting in a rise in demand for branded turf cultivars (Young and Silberstein 2012). Moreover, the mild climate of the Willamette Valley, characterized by moderate rainfall during winters and dry conditions throughout summers, creates an optimal setting for growing and harvesting grass seed crops. Due to Oregon's dry summers, farmers can cut the crops, dry them naturally in the field, and store them without artificial drying (Taylor and Bartlett 1993). Grass seed crops are susceptible to a variety of seasonal diseases, insects, and weeds (Rinehold and Jenkins 1994). Over the decades, regulations on field burning and other straw management strategies resulted in heavy reliance on chemical interventions for pest management (Shaner 2014).

Tall fescue [*Festuca arundinacea* Schreb. = *Lolium arundinaceum* (Schreb.) Darbysh. = *Schedonorus arundinaceus* (Schreb.) Dumort] and perennial ryegrass (*Lolium perenne* L.) dominate Oregon's grass seed production acreage. Both tall fescue and perennial ryegrass are parts of the subfamily Pooideae (family Poaceae) that are often associated with fungal endophytes of the genus *Epichloë* (family Clavicipitaceae) (Schardl et al. 2004, Leuchtmann et al. 2014) known to provide several plant protection benefits including herbivore feeding and disease resistance (Lyons et al. 1986, Omacini et al. 2001, Meister et al. 2006, Panaccione et al. 2006, Baldauf et al. 2011).

Plant-fungi Relationship

The term "endophyte" refers widely to any microbes – including bacteria and fungi, that colonize living plants intercellularly without immediately causing harm and usually without showing any overt external symptoms of infection. The term "endophyte" does not explicitly refer to their mutualistic relationship with the plant host (Wennström 1994). Some microbial endophytes are commensal for some time and become pathogenic to the plant host under specific conditions (Fesel and Zuccaro 2016). The relationship between plants and associated microbial endophytes is believed to have evolved 60 million years ago when the bacteria rhizobia acquired a capacity to colonize plants via symbiotic horizontal gene transfer and has been crucial for land ecosystems ever since (Sprent 2008). Endophytes are recognized to have an impact on a number of essential functions of the host plant. They can stimulate plant growth, trigger a defense reaction against pathogen invasion, and mitigate abiotic stressors; while the plant host provides nutrients, habitats, and sometimes a mode of fungal transmission via seed (Tetlow and Farrar 1993, Tanaka et al. 2005, Lehtonen et al. 2006, Meister et al. 2006, Song et al. 2015, Trivedi et al. 2020).

Epichloë spp., hereafter "*Epichloë*", are fungal endophytes that form a symbiotic relationship with several cool-season grass hosts. Plant hosts do not appear to have symptoms or exhibit defense responses even though the fungal *Epichloë* infection involves extensive production of hyphae throughout plant tissues (Clay and Schardl 2002). *Epichloë* species can be

transmitted vertically from a mother plant to daughter seeds when hyphae grow into developing seeds of the grass host (Zhang et al. 2017). Several reports have shown the economic impact of grass-*Epichloë* associations in turf and forage systems. *Epichloë* endophytes were found to be associated with increased yield in pasture, enhanced germination rate, heightened biotic and abiotic stress resistance, and increased nutrient content in cool-season grasses (Hoveland 1993, Lehtonen et al. 2006, Saikkonen et al. 2013; Song et al. 2015, Li et al. 2020). Moreover, grass hosts associated with *Epichloë* were found to repel both insect and mammal herbivores through alkaloid production (Meister et al. 2006, Baldauf et al. 2011, Realini et al. 2024).

Epichloë endophytes provide anti-herbivory benefits through mycotoxin production (Lyons et al. 1986, Omacini et al. 2001, Meister et al. 2006, Panaccione et al. 2006, Baldauf et al. 2011). The four major classes of alkaloids produced by *Epichloë* include pyrrolopyrazines or peramine, 1-aminopyrrolizidines or lolines, ergovaline or ergot alkaloids, and indole-diterpenes or lolitrem B (Siegel et al. 1990, Schardl et al. 2013, Realini et al. 2024). Ergot alkaloids and indole-diterpenes, mainly produced by common endophyte strains in cool-season turfgrasses, can lead to considerable problems with animal health (Lyons et al. 1986, Fisher et al. 2004, Panaccione et al. 2006, Finch et al. 2012). The toxicity of ergot alkaloids produced by the Epichloë in mammal grazing grasses was first noticed in 1963, before Epichloë had been identified as the toxin-producing agent in 1977 (Jacobson et al. 1963, Bacon et al. 1977). On the other hand, the indole-diterpenes were found to be a major cause of 'ryegrass staggers,' a neuromuscular impairment in sheep and horses (Gallagher et al. 1981, Miyazaki et al. 2001). Several studies reported that peramine has a deterrent effect on insects (Rowan 1993, Schardl et al. 2012), as well as lolines, which are known for insecticidal and deterrent effects on multiple insect pests (Bacetty et al. 2009, Baldauf et al. 2011, Schardl et al. 2012).

Plant host genotypes and *Epichloë* genetics influence *Epichloë* infection rates and alkaloid levels (Jensen and Popay 2004, Richmond and Bigelow 2009, Young et al. 2014, Vikuk et al. 2019). Variation in *Epichloë* infection levels and alkaloid expression occurs at both the grass cultivar and cultivar × *Epichloë* strain levels within a species (Young et al. 2014), resulting in a diverse array of *Epichloë*-mediated defenses against insect herbivores (Richmond and Bigelow 2009, Baldauf et al. 2011). Indeed, more research is required to elucidate the influences of grass host species, grass host cultivar, and *Epichloë* genetics on the alkaloid type and level expressed in a grass-*Epichloë* relationship.

Epichloë Detection Methods

Several techniques can be used to detect the presence of *Epichloë* endophytes, their alkaloid biosynthesis genes, and assessments of the *Epichloë* chemotype including isozyme analysis (Leuchtmann and Clay 1990), immunoblot assays (Holder et al. 1994), mass spectrometry (MS) (Ranieri and Ciolino, 2008), and high throughput polymerase chain reactions (PCR) (Takach and Young 2014). With isozyme analysis, one can determine the class of alkaloid genes found in the *Epichloë*'s genome; however, this technique is limited to pure cultures of *Epichloë* which can be challenging to acquire (Leuchtmann and Clay 1990, Young et al. 2014). Moreover, a grass host population may consist of up to 52 isolates per host, making isozyme phenotype identification a time-consuming method for a grass cultivar's alkaloid profile determination. Immunoblot assays are efficient in terms of time and the number of plant samples that can be run at once; however, several false negatives were observed when immunoblot tests were used to assess the *Epichloë* infection in seedlings with low fungal biomass (Holder et al. 1994, Cagnano et al. 2019). Mass spectrometry (MS) has been used widely to determine the concentrations of alkaloids in *Epichloë*-infected plant samples. While MS is accurate at quantifying a grass cultivar's alkaloids, the preparation scheme is usually labor-intensive (Ranieri and Ciolino2008). High throughput PCR, on the other hand, is more practical in detecting the presence of alkaloid genes in an *Epichloë*'s genome using grass tiller samples as the DNA extraction material is accurate and the protocol is more user-friendly, compared to other approaches (Takach and Young 2014).

Benefits of Grass-Epichloë Symbiosis on the Plant Host

Some *Epichloë*-mediated resistances to stress factors include a reduced infection rate of plant pathogens (Gwinn and Gavin 1992, Bonos et al. 2005, Clarke et al. 2006), drought tolerance, and hypoxia tolerance (Marks and Clay 1996, Belesky and West 2009, Song et al. 2015). These biotic and abiotic tolerance benefits conferred by the grass-Epichloë association are of interest to breeders and grass producers in light of a changing climate and increasingly limited chemical pest control options. The grass-*Epichloë* association is most known for insect pest resistance which is an advantageous trait in turf, pasture, and seed crop production systems (Funk et al. 1993, Eerens et al 1997, Young et al. 2013, Pennell et al. 2017, Lugtenberg et al. 2016, Gundel et al. 2020). According to Meister et al. (2006), two aphid species: Rhopalosiphum padi and Metopolophium dirhodum showed decreased densities when feeding on grasses infected with E. lolii producing ergot alkaloids and indole-diterpenes. Additionally, the alkaloids were found to reduce *R. padi* life span and fecundity. Furthermore, *Epichloë* may show a tritrophic level effect on insect herbivores' susceptibility to entomopathogens (Grewal et al. 1995, Bixby and Potter 2010). A study by Grewal et al. (1995) suggested that Epichloë enhanced nematode establishment in the root-feeding Japanese beetle Popillia japonica Newman larvae, leading to

an increase in nematode-induced mortality of the larvae feeding on *Epichloë*-infected tall fescue cultivars, compared to *Epichloë*-free grasses.

Lepidopteran pest-*Epichloë* interactions have also been evaluated. Previous studies examined how *Epichloë*-infected grasses impacted black cutworms, *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae), and fall armyworms, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) in turf (Bultman et al. 2009, Richmond and Bigelow 2009, Baldauf et al. 2011). Literature showed that in controlled conditions, *A. ipsilon* larvae had reduced establishment, survival, and biomass when feeding on tall fescue cultivars infected with *Epichloë* (Baldauf et al. 2011). Similarly, experiments with *S. frugiperda* exhibited a preference for leaf blades from *Epichloë*-free tall fescue over those with *Epichloë* (Bultman et al. 2009). Little is known about the effects of a grass-*Epichloë* association on cutworm pests in Oregon's grass seed production systems.

Winter Cutworm

The yellow underwing moth or winter cutworm, *Noctua pronuba* (Lepidoptera: Noctuidae), is an economically important insect pest species of grass seed crops in Oregon (Green et al. 2016). In the Pacific Northwest, *N. pronuba* appears to be a bivoltine species – having two generations per year, with adult moth flights occurring from April through June and from mid-August to October (Crabo et al. 2015, Landolt et al. 2015). *N. pronuba* larvae are highly gregarious and mobile insect pests with a wide host range, including turfgrass, grass seed, alfalfa, and ornamental crops. Since their first detection in the Pacific Northwest region of United States in 2001, periodic outbreaks have occurred throughout Oregon's grass seed production fields, golf courses, sport fields, and lawns (Kaur, personal communication, May 21, 2024). Unfortunately, effective cultural or biological practices against *N. pronuba* have yet to be discovered.

Bioassays to Measure Endophyte-mediated Insect Resistance

Two common laboratory and greenhouse assays used to determine the effects of endophyte infection level and alkaloid contents against insect pests are choice- and no-choice assays. A choice experiment is mainly adopted to study an insect's preference for plant feeding either between endophyte and endophyte-free plant material or between plants infected with different endophyte strains. Nonetheless, with a possible lack of feeding due to the deterrent effect of endophytes, this type of test cannot directly be used to assess the effect of both endophyte and alkaloid on insect performance metrics such as insect survival, establishment, biomass consumption, and fecundity. Additionally, the choice assay may obscure the effects of endophytes on insects if insects avoid feeding on the plant material provided. On the other hand, a no-choice assay allows an insect to feed on only one plant type, so that the assay can be used to investigate the impact of the plant type on insect behavior – either infected with the fungal endophytes or not on insect fitness through the measurable performance metrics mention above. However, the no-choice assay may exaggerate the impacts of endophytes on insect performance if the insects are forced to feed on plant materials that they could have avoided in a non-confined setting.

Conclusion

Pest resistance properties inferred by *Epichloë* could alleviate challenges in turf management in places where the use of pesticides is not desirable, such as sports fields, playgrounds, parks, and roadsides (Funk et al. 1993). Additionally, with 98% of the seed

produced in Oregon being shipped nationally and exported internationally, seed crops with improved insect resistance could be marketed to fetch Oregon seed growers a higher price premium, consequently improving the economics of Oregon's grass seed industry. The following research aims to better understand the current status of *Epichloë* endophytes occurring in United States turfgrass, as well as to evaluate the feasibility of *N. pronuba* control utilizing *Epichloë* endophytes. In Chapter 2, an inventory of the National Turfgrass Evaluation Program's (NTEP) 2021 perennial ryegrass trial was conducted to determine *Epichloë* infection status and mycotoxin profiles for 51 commercially available cultivars. In Chapter 3, a repeated no-choice study evaluated tall fescue and perennial ryegrass cultivars with low and high *Epichloë* infection rates for susceptibility to *N. pronuba* feeding. Together, this work lends more research to the value and practicality of using *Epichloë* endophytes to confer insect resistance and stress tolerance capabilities in the United States grass seed production and other turfgrass systems.

Reference

- Bacetty AA, Snook ME, Glenn AE et al. 2009. Toxicity of endophyte-infected tall fescue alkaloids and grass metabolites on *Pratylenchus scribneri*. Phytopathology 99(12):1336-1345. <u>https://doi.org/10.1094/phyto-99-12-1336</u>.
- Bacon C, Porter J, Robbins J et al. 1977. *Epichloe typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34(5):576-581.
- Baldauf MW, Mace WJ, Richmond DS. 2011. Endophyte-mediated resistance to black cutworm as a function of plant cultivar and endophyte strain in tall fescue. Environ. Entomol. 40(3):639-647. <u>https://doi.org/10.1603/EN09227</u>.
- Belesky DP, West CP. 2009. Abiotic stresses and endophyte effects. In: Fribourg HA, Hannaway DB, West CP. Tall fescue for the twenty-first century. https://doi.org/10.2134/agronmonogr53.c4.
- Bixby AJ, Potter DA. 2010. Influence of endophyte (*Neotyphodium lolii*) infection of perennial ryegrass on susceptibility of the black cutworm (Lepidoptera: Noctuidae) to a baculovirus. Biol. Control 54(2):141-146. https://doi.org/10.1016/j.biocontrol.2010.04.003.

- Bonos SA, Wilson MM, Meyer WA et al. 2005. Suppression of red thread in fine fescues through endophyte-mediated resistance. Appl. Turfgrass Sci. 2(1):1-7. https://doi.org/https://doi.org/10.1094/ATS-2005-0725-01-RS.
- Bultman TL, Rodstrom JL, Radabaugh KR et al. 2009. Influence of genetic variation in the fungal endophyte of a grass on an herbivore and its parasitoid. Entomol. Exp. Appl. 130(2):173-180. <u>https://doi.org/https://doi.org/10.1111/j.1570-7458.2008.00802.x</u>.
- Cagnano G, Roulund N, Jensen CS et al. 2019. Large scale screening of *Epichloë* endophytes infecting *Schedonorus pratensis* and other forage grasses reveals a relation between microsatellite-based haplotypes and loline alkaloid levels. Front. Plant Sci. 10:765. <u>https://doi.org/10.3389/fpls.2019.00765</u>.
- Clarke BB, White Jr JF, Hurley RH et al. 2006. Endophyte-mediated suppression of dollar spot disease in fine fescues. Plant Dis. 90(8):994-998. <u>https://doi.org/10.1094/pd-90-0994</u>.
- Clay K, Schardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat. 160(S4):S99-S127. <u>https://doi.org/10.1086/342161</u>.
- Crabo L, Zack R, Peterson M. 2015. Pacific northwest moths; [accessed 2024 May 21]. http://www.pnwmoths.biol.wwu.edu/.
- Eerens J, Easton H, Lucas R et al. 1997. Influence of the ryegrass endophyte on pasture production and composition in a cool-moist environment. Springer. 157-159.
- Fesel PH, Zuccaro A. 2016. Dissecting endophytic lifestyle along the parasitism/mutualism continuum in *Arabidopsis*. Curr. Opin. Microbiol. 32:103-112. <u>https://doi.org/10.1016/j.mib.2016.05.008</u>.
- Finch SC, Fletcher LR, Babu JV. 2012. The evaluation of endophyte toxin residues in sheep fat. N. Z. Vet. J. 60(1):56-60. <u>https://doi.org/10.1080/00480169.2011.634746</u>.
- Fisher MJ, Bohnert D, Ackerman C et al. 2004. Evaluation of perennial ryegrass straw as a forage source for ruminants. J. Anim. Sci. 82(7):2175-2184. <u>http://dx.doi.org/10.2527/2004.8272175x</u>.
- Funk CR, White RH, Breen JP. 1993. Importance of *Acremonium* endophytes in turf-grass breeding and management. Agric. Ecosyst. Environ. 44(1):215-232. <u>https://doi.org/https://doi.org/10.1016/0167-8809(93)90048-T</u>.
- Gallagher R, White E, Mortimer P. 1981. Ryegrass staggers: isolation of potent neurotoxins lolitrem A and lolitrem B from staggers-producing pastures. N. Z. Vet. J. 29(10):189-190. https://doi.org/10.1080/00480169.1981.34843.
- Green J, Dreves AJ, McDonald BW et al. 2016. Winter cutworm: a new pest threat in Oregon. Oregon State University, Extension Service Corvallis, OR, USA. https://extension.oregonstate.edu/sites/default/files/documents/em9139.pdf.
- Grewal S, Grewal PS, Gaugler R. 1995. Endophytes of fescue grasses enhance susceptibility of *Popillia japonica* larvae to an entomopathogenic nematode. Entomol. Exp. Appl. 74(3):219-224. <u>https://doi.org/10.1111/j.1570-7458.1995.tb01894.x</u>.

- Gundel PE, Biganzoli F, Freitas PP et al. 2020. Plant damage, seed production and persistence of the fungal endophyte *Epichloë* occultans in *Lolium multiflorum* plants under an herbivore lepidopteran attack and ozone pollution. Ecol. Austral. 30(2):321-330. https://doi.org/10.25260/EA.20.30.2.0.1034.
- Gwinn K, Gavin A. 1992. Relationship between endophyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. Plant Dis. 76(9):911-914. https://doi.org/10.1094/PD-76-0911.
- Holder T, West C, Turner K et al. 1994. Incidence and viability of *Acremonium* endophytes in tall fescue and meadow fescue plant introductions. Crop Sci. 34(1):252-254. https://doi.org/10.2135/cropsci1994.0011183X003400010046x.
- Hoveland CS. 1993. Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plant. Agric. Ecosyst. Environ. 44(1-4):3-12. https://doi.org/10.1016/0167-8809(93)90036-0.
- Jacobson EH. 1963. Sojourn research: A definition of the field. J. Soc. Issues 19(3):123-129. https://doi.org/https://doi.org/10.1111/j.1540-4560.1963.tb00451.x.
- Jensen J, Popay A. 2004. Perennial ryegrass infected with AR37 endophyte reduces survival of porina larvae. N. Z. Plant Prot. 57:323-328. <u>https://doi.org/10.30843/nzpp.2004.57.6930</u>.
- Landolt PJ, Zack RS, Roberts D. 2015. Seasonal response of *Noctua pronuba* Linnaeus, 1758 (Lepidoptera: Noctuidae) to traps in Washington State. Pan-Pac. Entomol. 91(1):20-28. https://doi.org/10.3956/2014-91.1.020.
- Lehtonen PT, Helander M, Siddiqui SA et al. 2006. Endophytic fungus decreases plant virus infections in meadow ryegrass (*Lolium pratense*). Biol. Lett. 2(4):620-623. https://doi.org/10.1098%2Frsbl.2006.0499.
- Leuchtmann A, Clay K. 1990. Isozyme variation in the *Acremonium/Epichloë* fungal endophyte complex. Phytopathol. 80(10):1133-1139.
- Leuchtmann A, Bacon CW, Schardl CL et al. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia 106(2):202-215. <u>https://doi.org/10.3852/13-251</u>.
- Li XZ, Simpson WR, Song ML et al. 2020. Effects of seed moisture content and *Epichloe* endophyte on germination and physiology of *Achnatherum inebrians*. S. Afr. J. Bot. 134:407-414. <u>https://doi.org/https://doi.org/10.1016/j.sajb.2020.03.022</u>.
- Lugtenberg BJ, Caradus JR, Johnson LJ. 2016. Fungal endophytes for sustainable crop production. FEMS Microbiol. Ecol. 92(12):1-17. <u>https://doi.org/10.1093/femsec/fiw194</u>.
- Lyons PC, Plattner RD, Bacon CW. 1986. Occurrence of peptide and clavine ergot alkaloids in tall fescue grass. Sci. 232(4749):487-489. <u>https://doi.org/10.1126/science.3008328</u>.
- Marks S, Clay K. 1996. Physiological responses of *Festuca arundinacea* to fungal endophyte infection. New Phytol. 133(4):727-733. <u>https://doi.org/https://doi.org/10.1111/j.1469-8137.1996.tb01941.x</u>.

- Meister B, Krauss J, Härri SA et al. 2006. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. Basic Appl. Ecol. 7(3):244-252. https://doi.org/10.1016/j.baae.2005.06.002.
- Miyazaki S, Fukumura M, Yoshioka M et al. 2001. Detection of endophyte toxins in the imported perennial ryegrass straw. J. Vet. Med. Sci. 63(9):1013-1015. https://doi.org/10.1292/jvms.63.1013.
- Oregon Department of Agriculture. 2023. Oregon releases updated top 20 agricultural commodities. <u>https://content.govdelivery.com/accounts/ORODA/bulletins/355f2ac</u>.
- Omacini M, Chaneton EJ, Ghersa CM et al. 2001. Symbiotic fungal endophytes control insect host–parasite interaction webs. Nature 409(6816):78-81. https://doi.org/10.1038/35051070.
- Panaccione DG, Cipoletti JR, Sedlock AB et al. 2006. Effects of ergot alkaloids on food preference and satiety in rabbits, as assessed with gene-knockout endophytes in perennial ryegrass (*Lolium perenne*). J. Agric. Food Chem. 54(13):4582-4587. <u>https://doi.org/10.1021/jf060626u</u>.
- Pennell C, Rolston M, Van Koten C et al. 2017. Reducing bird numbers at New Zealand airports using a unique endophyte product. N. Z. Plant Prot. 70:224-234. <u>https://doi.org/10.30843/nzpp.2017.70.55</u>.
- Ranieri TL, Ciolino LA. 2008. Rapid selective screening and determination of ephedrine alkaloids using GC-MS footnote mark. Phytochemical Analysis: Int. J. Plant Chem. Biochem. Tech. 19(2):127-135.
- Realini FM, Escobedo VM, Ueno AC et al. 2024. Anti-herbivory defences delivered by *Epichloë* fungal endophytes: a quantitative review of alkaloid concentration variation among hosts and plant parts. Ann. Bot. 133(4):509-520. <u>https://doi.org/10.1093/aob/mcae014</u>.
- Richmond D, Bigelow C. 2009. Variation in endophyte–plant associations influence black cutworm (Lepidoptera: Noctuidae) performance and susceptibility to the parasitic nematode *Steinernema carpocapsae*. Environ. Entomol. 38(4):996-1004. https://doi.org/10.1603/022.038.0406.
- Rinehold J, Jenkins JJ. 1994. Oregon pesticide use estimates for seed and specialty crops, 1992. EM 8568. Oregon State University Extension Service: Corvallis, OR.
- Rowan DD. 1993. Lolitrems, peramine and paxilline: mycotoxins of the ryegrass/endophyte interaction. Agric. Ecosyst. Environ. 44(1-4):103-122. <u>https://doi.org/10.1016/0167-8809(93)90041-M</u>.
- Saikkonen K, Gundel PE, Helander M. 2013. Chemical ecology mediated by fungal endophytes in grasses. J. Chem. Ecol. 39:962-968. <u>https://doi.org/10.1007/s10886-013-0310-3</u>.
- Schardl CL, Leuchtmann A, Spiering MJ. 2004. Symbioses of grasses with seedborne fungal endophytes. Annu. Rev. Plant Biol. 55:315-340. <u>https://doi.org/10.1146/annurev.arplant.55.031903.141735</u>.

- Schardl CL, Young CA, Faulkner JR et al. 2012. Chemotypic diversity of *Epichloae*, fungal symbionts of grasses. Fungal Ecol. 5(3):331-344. <u>https://doi.org/https://doi.org/10.1016/j.funeco.2011.04.005</u>.
- Schardl CL, Florea S, Pan J et al. 2013. The *Epichloae*: alkaloid diversity and roles in symbiosis with grasses. Curr. Opin. Plant Biol. 16(4):480-488. https://doi.org/https://doi.org/10.1016/j.pbi.2013.06.012.
- Shaner DL, Beckie HJ. 2014. The future for weed control and technology. Pest. Manag. Sci. 70: 1329-1339. <u>https://doi.org/10.1002/ps.3706</u>.
- Siegel M, Latch G, Bush L et al. 1990. Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. J. Chem. Ecol. 16:3301-3315. https://doi.org/10.1007/BF00982100.
- Song M, Li X, Saikkonen K et al. 2015. An asexual *Epichloë* endophyte enhances waterlogging tolerance of *Hordeum brevisubulatum*. Fungal Ecol. 13:44-52. https://doi.org/https://doi.org/10.1016/j.funeco.2014.07.004.
- Sprent JI. 2008. 60Ma of legume nodulation. What's new? What's changing? J. Exp. Bot. 59(5):1081-1084. <u>https://doi.org/10.1093/jxb/erm286</u>.
- Takach JE, Young CA. 2014. Alkaloid genotype diversity of tall fescue endophytes. Crop Sci. 54(2):667-678. <u>https://doi.org/10.2135/cropsci2013.06.0423</u>.
- Tanaka A, Tapper BA, Popay A et al. 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol. Microbiol. 57(4):1036-1050. https://doi.org/https://doi.org/10.1111/j.1365-2958.2005.04747.x.
- Taylor GH, Bartlett A. 1993. The climate of Oregon. Climate zone 2, Willamette Valley.
- Tetlow IJ, Farrar JF. 1993. Apoplastic sugar concentration and pH in barley leaves infected with brown rust. J. Exp. Bot. 44(5):929-936. <u>https://doi.org/10.1093/jxb/44.5.929</u>.
- Trivedi P, Leach JE, Tringe SG et al. 2020. Plant–microbiome interactions: from community assembly to plant health. Nat. Rev. Microbiol. 18(11):607-621. https://doi.org/10.1038/s41579-020-0412-1.
- USDA NASS. 2024. Oregon grass seed available for harvest in 2024 are estimated at 302,000 acres. https://www.nass.usda.gov/Statistics_by_State/Washington/Publications/Current_News_ Release/2024/ORGRASS_2024.pdf.
- Vikuk V, Young CA, Lee ST et al. 2019. Infection rates and alkaloid patterns of different grass species with systemic *Epichloë* endophytes. Appl. Environ. Microbiol. 85(17):e00465-00419. https://doi.org/10.1128%2FAEM.00465-19.
- Wennström A. 1994. Endophyte: the misuse of an old term. Oikos 71(3):535-536. https://doi.org/10.2307/3545842.

- Young C, Hume D, McCulley R. 2013. FORAGES AND PASTURES SYMPOSIUM: Fungal endophytes of tall fescue and perennial ryegrass: Pasture friend or foe? J. Anim. Sci. 91:<u>https://doi.org/10.2527/jas.2012-5951</u>.
- Young CA, Charlton ND, Takach JE et al. 2014. Characterization of *Epichloë coenophiala* within the US: are all tall fescue endophytes created equal? Front. Chem. 2:95. https://doi.org/10.3389%2Ffchem.2014.00095.
- Young W, III, Silberstein T. 2012. The Oregon grass seed industry'. In: C. A. Young GEARLMJRSCLS ed.: Ardmore Samuel Roberts Noble Foundation. 49-52.
- Zhang W, Card SD, Mace WJ et al. 2017. Defining the pathways of symbiotic *Epichloë* colonization in grass embryos with confocal microscopy. Mycologia 109(1):153-161. https://doi.org/10.1080/00275514.2016.1277469.

CHAPTER 2

Epichloë Infection and Alkaloid Pattern of Perennial Ryegrass Cultivars in Oregon

Julaluck Intasin

To be submitted to Crop, Forage & Turfgrass Management 5585 Guilford Road Madison WI 53711-5801 USA

Abstract

Perennial ryegrass (Lolium perenne L.) is a valuable turfgrass species grown for seed in Oregon. The quality of experimental and commercial turfgrass cultivars has been assessed under the National Turfgrass Evaluation Program (NTEP) since 1981 (Morris and Shearman 2000, NTEP 2024). In spite of potential agronomic advantages conferred by the grass-*Epichloë* symbiosis, *Epichloë* incidence in turf cultivars has been exclusively assessed in seed samples, while the persistence of *Epichloë* post-establishment – a crucial piece of information for understanding Epichloë impact on turfgrass ecosystems - is underexplored. In this study, infection rates and alkaloid potential of *Epichloë* endophytes in perennial ryegrass cultivars evaluated in Oregon State University's 2016 NTEP trials using molecular tests targeting alkaloid synthesis genes were determined. Integration of endophyte components into the NTEP trial also allowed for the observation of correlations between Epichloë incidence levels and turfgrass quality when subject to traffic pressure applied within the NTEP trials. Infections of Epichloë endophytes among the perennial ryegrass cultivars five years after stand establishment were highly variable. While four profiles of potential chemotypes of *Epichloë* were identified, most examined cultivars were predicted to be able to produce peramine, ergot alkaloid, and indolediterpene. Over the course of the five-year study, an increase in *Epichloë* incidence from seed samples was observed in 28 cultivars. Conversely, 17 cultivars showed a decline in Epichloë infection. Statistical analysis revealed that, contrary to expectations, traffic application did not affect the turf quality rating in 2021. Instead, the range of Epichloë infection was identified as the primary factor influencing the estimation of the quality rating for that year. Interestingly, these results suggest that a perennial ryegrass turf system may only require 20% of plants to be Epichloë-infected to result in an increase in turf quality rating, with no additional improvement

observed in those with higher infection rates. These findings provide valuable insights for turfgrass users and breeders seeking cultivars with robust *Epichloë* associations.

Introduction

Oregon is recognized worldwide for its role as a leading producer of ryegrass seed used for both forage and turf purposes. Perennial and annual ryegrass account for 165,000 acres of the estimated total harvestable area in Oregon (USDA NASS, 2024). Several cool season grasses, including perennial ryegrass, form a symbiotic relationship with *Epichloë* spp. endophytes, hereafter "*Epichloë*". The grass hosts infected with *Epichloë* show no visible symptoms or defense responses, despite the extensive presence of fungal hyphae throughout their tissues (Clay and Schardl 2002). Literature indicates that grasses associated with *Epichloë* may be benefitted by higher yield, germination rates, resistance to both biotic and abiotic stresses, and nutrient content (Hoveland 1993, Lehtonen et al. 2006, Saikkonen et al. 2013, Song et al. 2015).

Furthermore, these grasses deter insect and mammal herbivore feeding due to the presence of *Epichloë*-producing alkaloids (Meister et al. 2006, Baldauf et al. 2011). One of the toxins produced is ergot alkaloid. The toxicity of ergot alkaloid produced by *Epichloë* in grasses used for mammal grazing was first noticed in 1963 before *Epichloë* had been identified as the toxin-producing agent in 1977 (Jacobson et al. 1963, Bacon et al. 1977). These toxins are a concern in grasses produced for forage, due to large grazing mammals, like sheep and cows, being affected by the toxin. Unlike forage grasses, turfgrasses benefit from the presence of ergot alkaloid-producing *Epichloë* because the alkaloids can deter insect pests, birds, and mammals (Stewart et al. 2022), and there is not typically feeding of turfgrass by livestock. Furthermore, the pest resistance properties inferred by *Epichloë* could alleviate management challenges in

turfgrass in places where the use of pesticides is not desirable, such as sports fields, playgrounds, parks, and roadsides (Funk et al. 1993).

Despite the wide range of agronomic advantages that the grass-*Epichloë* relationship offers for turfgrass performance and resilience, a high incidence of *Epichloë* in turfgrass seeds does not currently influence the value of a cultivar in the United States turf seed market. Consequently, breeding for a high *Epichloë* infection has not been a clear priority in the United States compared to breeding for disease resistance and other stress tolerances. Two primary reasons why high *Epichloë*-infected seeds are not currently marketable in the United States include cost and labor-intensive quality control practices, and limited consumer awareness regarding the grass-*Epichloë* mutualism's benefits on turf management (Stewart et al. 2022).

The process of seed production, harvest, storage, and distribution can decrease *Epichloë* viability in grass seeds (Stewart et al. 2022). To minimize the loss of viable *Epichloë* during seed production and distribution, seeds must be stored under 4°C and 30% relative humidity throughout the supply chain to maintain a high infection rate. These stringent environmental requirements pose significant challenges and costs for storage and commercial delivery (Rolston and Agee 2007, Stewart et al. 2022). For grass seed producers to justify investing in guaranteed high *Epichloë* infection levels in turfgrass seeds sold in the United States, turf consumers and managers must be willing to pay a premium for cultivars with such guarantees and additional production costs. This necessitates educating consumers about the advantages of turfgrass cultivars with high *Epichloë* incidence over those with low or no infection. Currently, most research on the importance of *Epichloë* on grass host performance focuses on forage grass cultivars and desired forage quality. Moreover, an *Epichloë* endophyte diversity study in the

18

United States has only been done in forage type tall fescue species (Young et al. 2014). Little is known about the variation of *Epichloë* infection rate and *Epichloë* chemotypes present in Oregon's commercial turfgrass cultivars, and any potential correlation between *Epichloë* incidence, the alkaloid profiles, and turf quality.

The National Turfgrass Evaluation Program (NTEP) is a non-profit organization that has facilitated the assessment of experimental and commercial turfgrass cultivars in cooperation with university-based turfgrass research institutions since 1981 (Morris and Shearman 2000, NTEP 2024). The information regarding turf quality and characteristics provided by NTEP is valuable for various users including the turfgrass seed industry, homeowners, golf course managers, and researchers. Turfgrass quality is visually ranked based on characteristics of color, total coverage, texture, tolerances to drought, pests, and traffic. The scale for turfgrass quality ranges from 1 to 9, with 1 being the poorest possible quality and 9 being the best possible quality (Morris and Shearman 2000). The NTEP program provides an excellent opportunity for education about cultivars that perform best in a specific region or environment, and allow consumers to see variable turf performance in the field. Despite NTEP's acknowledgement of Epichloë benefits for turfgrasses, *Epichloë* persistence in turfgrass after establishment is not one of the primary traits being measured as part of the evaluation program. *Epichloë* presence determination has been exclusively conducted on seed samples (NTEP 2024). This evaluation lacks information about Epichloë viability and its foliar infection (Da Costa et al. 1998, Mohr et al. 2002, NTEP 2024). By incorporating Epichloë persistence in turfgrass stand evaluations after establishment as an assessment into NTEP trials, turfgrass researchers could construct a correlation between observed turfgrass quality characteristics and *Epichloë* incidence levels or chemotype.

The objectives of this study were to determine *Epichloë* incidence levels and chemotypes within perennial ryegrass cultivars that were commercially available and evaluated under Oregon State University's NTEP trials from 2016 to 2021. To evaluate *Epichloë* status and its genetic capability in alkaloid production, molecular assays targeting markers of alkaloid biosynthesis genes were used. Moreover, a relationship between *Epichloë* incidence levels and turfgrass quality was determined. This study could provide a rationale for turf end users and breeders to identify strong grass-*Epichloë* relationship and associated plant protection benefits among experimental and commercial turfgrass cultivars.

Materials and Methods

As part of the 2016 NTEP Perennial Ryegrass Trial program, turf plots were established and maintained by the Oregon State University Extension Turfgrass Program at the Oregon State University Lewis Brown Research Farm, Corvallis, OR, (44°32'59.4"N 123°12'54.5"W). Performance and suitability of 114 perennial ryegrass cultivars were evaluated for quality over five years either under traffic pressure or no traffic pressure. Seeds of the 114 perennial ryegrass cultivars supplied by NTEP were planted in rectangular plots measuring approximately 1.5 by 1.2 meters on September 30th, 2016, at a rate of approximately 16.7 grams of seed per square meter. Each cultivar was replicated three times in a randomized complete block design. No herbicides were applied at planting. Routine maintenance of plots consisted of a total annual application of 22.7 grams of nitrogen per square meter, regular mowing at a height of 5 centimeters with a Toro 3500-D mower, and irrigation daily from approximately May 1st through September 30th at 100% of ET calculated with an onsite Campbell Scientific Weather Station. No fungicides or insecticides were applied. For traffic tolerance evaluation, plots were subjected to traffic and no-traffic conditions. Simulated traffic was applied to plots in 2017, 2018 and 2019 using a John Deere 800 Aerifier modified with artificial feet. In 2020 and 2021, traffic was applied via walking due to technical issues with the John Deere Aerifier. Traffic was applied to half plots across an entire row of plots (i.e. to split blocks) on the west side of plots for replicate 1 and 3, and the east side of plots for replicate 2.

The trial ran for five years ending in June 2021. During the evaluation period, plots were visually rated monthly for overall quality (1.0 - 9.0 scale) (Morris and Shearman 2000). As mentioned, the total perennial ryegrass cultivar entry number was 114; however, only 51 cultivars were commercially available by 2021. Consequently, 51 perennial ryegrass cultivars, including seven standard varieties (Table 2.1) were evaluated for *Epichloë* incidence at the end of the experiment.

Plant sample collection and PCR

In August of 2021, tiller samples were collected from each of the 51 plots of interest (Table 2.1) to determine *Epichloë* status of each perennial ryegrass cultivar. From each plot, five random points were selected for sampling. At each sample point, one to two tillers were collected and placed in an extraction bag (Agdia Inc., Elkhart, IN) for nucleic acid extraction (three replicates, n = 15 tiller-set samples). Samples were stored at -20°C until they could be processed for extraction and PCR.

Total nucleic acids were extracted from frozen plant material using a Qiagen DNeasy plant mini kit, following manufacturer protocol with some modification. The modification involved the use of drill press attached with an Agdia tissue homogenizer (Agdia, Inc.) to grind samples with initial extraction buffer in extraction bags. From that point, the original manufacturer protocol was used. RNase A was added to the ground sample, followed by an incubation in 65°C hot water bath for 10 min. Buffer P3 was added to the reaction before the mixture was placed on ice for five minutes, followed by five minutes of centrifugation at max. The supernatant was mixed with buffer AW1, transferred to a spin column, and followed by one minute of centrifugation at 8000 rpm. The supernatant was discarded before two aliquots of AW2 buffer were added to the spin column. The first and second aliquots of AW2 were followed by 1 min centrifugation at 8000 rpm and 2 min at max, respectively. The total DNA was eluted with 100 ul of buffer AE.

The *Epichloë* infection rate and chemotype were determined using a high throughput multiplex PCR method described by Takach et al. (2012). The protocol amplifies an internal control, the translation elongation factor 1-alpha gene region (*tefA*, abbreviated "TEF") and the following alkaloid biosynthesis-related genes: peramine (*perA*, abbreviated "PER"), loline (*lolC*, abbreviated "LOL"), ergot alkaloid (*dmaW*, abbreviated "EAS"), and indole-diterpene (*idtG*, abbreviated "IDT") from total DNA. All genes have been widely used for *Epichloë* detection and chemotypic analysis of *Epichloë* worldwide (Schardl et al. 2013). The primers used for the multiplex PCR reaction are listed in Table 2.2. The PCR reactions were conducted in a total volume of 25 μ l. Each reaction contained 5× Green GoTaq reaction buffer (Promega Corp., Madison, WI), 10 μ M of each deoxynucleoside triphosphate (dNTP), 1.0-U GoTaq DNA polymerase (Promega Corp.), and each target-specific primer (10 μ M). Amplification conditions included 2 min. of initial denaturation at 94°C, 30 cycles of 15s at 94°C, 30s at 56°C, and one min at 72°C, followed by seven minutes at 72°C (Takach et al. 2012). PCR products were visualized via gel electrophoresis with a 1.5% agarose gel in 1X Tris-borate-EDTA (TBE) buffer

followed by ethidium bromide staining and UV transillumination. The expected fragment sizes for each amplicon are presented in Table 2.2. Each chemotype was deemed present if the appropriately sized band was detected in at least one of 15 samples and if the *tefA* gene was present.

Statistical analysis

Statistical analysis was performed using R version 4.2.2 (R Core Team 2024). To further examine the correlation between quality rating in 2021, traffic pressure, and *Epichloë* infection level, monthly quality ratings in 2021 for each cultivar were averaged. This averaged value was defined as the "quality rating in 2021" and was used in subsequent statistical analyses. A Dunnett's post hoc test with Bonferroni adjustment was applied following a significant Kruskal-Wallis test (P < 0.05) to compare the quality ratings in 2021 among different groups of *Epichloë* infection ranges. Moreover, a linear mixed-effects model was fitted using the *lme* function from the nlme package (Pinheiro 2011). The model included the *Epichloë* infection range and the traffic designation as fixed effects and replication as a random effect. Results were considered different with a *P* value < 0.05.

Results

Epichloë were detected in 47 out of the 51 cultivars evaluated in the NTEP trial. Of the 765 tillers examined, 56% were infected with *Epichloë*. The four cultivars with no detection of *Epichloë* included 'Allstar III,' 'Brightstar SLT,' 'Linn,' and 'Savant.' The 51 perennial ryegrass cultivars were grouped according to their *Epichloë* infection level as follows: 0% (4 cultivars), 1 -20% (6 cultivars), >20 -40% (6 cultivars), >40 -60% (10 cultivars), >60 -80% (10 cultivars), and >80 -100% (15 cultivars) (Table 2.1). Of the 47 *Epichloë*-infected cultivars, 44 contained

the genes involved in synthesis pathways of the following alkaloids: peramine, ergot alkaloid, and indole-diterpenes (Table 2.1). Only few were characterized by their ability to produce either peramine and ergot alkaloid (one cultivar) or only ergot alkaloid (one cultivar). In addition, one cultivar ('Karma') was identified as infected, but none of the alkaloid markers were amplified (Table 2.1).

A comparison of *Epichloë* infection levels reported in the seed (Bonos, personal communication, June 21, 2024) and tiller samples (this study) revealed that 28 of 51 cultivars had an increase in *Epichloë* incidence from seed to tiller after five years of establishment, while six cultivars were found to be at comparable levels (less than 5% change), and 17 cultivars had a decline in the *Epichloë* infection level from seed to tiller (Table 2.1).

A reductive effect of traffic pressure on the average quality rating of the perennial ryegrass cultivars examined was observed in 2017, when turf quality was rated on average above 7 on the quality scale for plots not under traffic and below 6 for plots with traffic pressure (Figure 2.1). In 2018, 2019, and 2020, the difference in mean quality rating between traffic and no traffic plots was much less pronounced. There was no traffic pressure effect in 2021 (Figure 2.1). Therefore, the traffic condition was not considered when comparing the quality rating in 2021 across different *Epichloë* infection level groups (Dunnett's post hoc test). Perennial ryegrass cultivars with 0 - 20% *Epichloë* infection exhibited a lower mean quality rating in 2021, compared to cultivars with greater infection of *Epichloë* (Figure 2.2). However, the mean quality rating of perennial ryegrass cultivars with 20 - 40%, 40 - 60%, 60 - 80%, and 80 - 100% *Epichloë* infection were not different from each other in pairwise comparisons (Figure 2.2).

Moreover, the linear mixed-effects model fitting indicated an effect of *Epichloë* infection status on mean turfgrass quality in 2021 (F(4, 298) = 9.39, P < 0.05), while the traffic designation did not have a significant effect on the mean quality rating in 2021 (F(1, 298) = 0.57, P = 0.45). In other words, the *Epichloë* infection level, and not the traffic regime, was an important factor used to explain a variability in mean quality rating in 2021. The finding indicates that in the NTEP study, *Epichloë* status could be a greater contributor to turfgrass quality than the applied traffic treatment.

Discussion

In this study, the *Epichloë* infection levels of 51 perennial ryegrass cultivars evaluated as a part of the 2016 NTEP Perennial Ryegrass Trial program at Oregon State University were reported. *Epichloë* was detected in 47 cultivars with different infection levels (Table 2.1). On the population level, 56% of tiller samples examined were infected with *Epichloë*, which was higher than those reported by other similar surveys in forage-type perennial ryegrass. For example, in Germany, perennial ryegrass cultivars had Epichloë infections ranging from 15 to 28% (König et al. 2018, Vikuk et al. 2019). However, the average infection rate of the 51 cultivars in this study was lower than those reported in New Zealand and Australia (70%) (Easton and Tapper 2005). After five years of stand establishment, most perennial ryegrass cultivars in this study were infected with some levels of *Epichloë*, with only four cultivars being *Epichloë*-free. The *Epichloë* infection frequency was extremely variable among the 51 cultivars ranging from 0 to 100% (Table 2.1). The variation in Epichloë infection level among grass cultivars grown in Oregon was expected due to the lack of regular testing to ensure *Epichloë* viability in grass seeds (Rolston and Agee 2007). Moreover, 28 out of the 51 perennial cultivars examined in this study had an increase in Epichloë incidence in tillers collected in 2021 from seeds sown in 2017 (Table 2.1). The increase in proportion of *Epichloë*-infected plants over time corresponds with previous reports showing that two perennial ryegrass cultivars exhibited an increase in *Epichloë* infection rate from 3 to 67% and 83% in three years (Francis and Baird 1989). Similarly, a study of Tasmanian pastures reported an increase in *Epichloë* infection rate in perennial ryegrass from 80 to 100% in four years (Cunningham et al. 1993). The observed phenomena were hypothesized to be caused by the selective advantage of *Epichloë* presence, especially under intense pressures such as herbivory and drought stress (Clay et al. 2005, Young et al. 2013). With few traffic applications per year (3 times/year from 2017 – 2019 and 4 times/year from 2020 - 2021), the traffic pressure applied as a part of the NTEP may not be intense enough to drive a rapid fixation in the other 23 cultivars without infection increases, which could be a cultivar-specific or genetic compatibility-driven phenomenon (Saikkonen et al. 2010).

Moreover, by using *Epichloë*-specific primers, four predicted alkaloid profiles were reported as followed: PER, EAS, IDT; PER, EAS; EAS; and none. Most of the infected cultivars exhibited a genetic potential for the following alkaloids: PER, EAS, IDT. The absence of LOL in the population was supported by a report from Germany determining only two predicted alkaloid profiles in perennial ryegrass samples (n = 164) including "PER, IDT" and "PER, EAS, IDT". However, the "PER, IDT" genotype (n = 161) was more prevalent in the German perennial ryegrass population than "PER, EAS, IDT" (n = 3) (Vikuk et al. 2019), opposite of the results in this study. Some perennial ryegrass population studies focusing on alkaloid production indicated that ergovaline, lolitrem B, and peramine could be measured in Australian perennial ryegrass pastural populations (Reed et al. 2000) and New Zealand perennial ryegrass cultivars (Hewitt et al. 2020), suggesting a similarity in the alkaloid production capacity of *Epichloë* infecting perennial ryegrass in Oregon, Australia, and New Zealand.
In addition, this study found that perennial ryegrass cultivars with Epichloë infection above 20% appeared to perform better than those with low *Epichloë* infection (below 20%) (Figure 2.2), suggesting that perennial ryegrass could benefit in turf performance quality when the *Epichloë* incidence in a population reached greater than 20%. However, the mean turf quality rating in 2021 did not increase with the *Epichloë* infections above the 20 - 40%. Little research has been conducted to investigate an association between *Epichloë* infection levels and observed *Epichloë*-mediated benefits in turf systems (Richmond et al. 2000, Stewart et al. 2022). Nevertheless, the observation in this study agrees with Richmond et al. (2000) showing that perennial ryegrass turf infected with *Epichloë* in as little as 35% of stands exhibited a reduction in an insect herbivore population, and the benefit did not increase with the Epichloë infection rate. However, the lack of a strong correlation between *Epichloë* infection and mean quality rating could be explained by a bias toward moderate values in the NTEP's visual assessment system. Krans and Morris (2007) interviewed scientists from 12 universities hosting NTEP trials regarding their ranking protocols and found that most scientists usually use 6 to represent minimally acceptable quality, and some of them avoided using the 9 score. The bias against extreme values could result in minimal variation in quality rating across treatments. The lack of variance homogeneity (tested by Levene's test, data not shown) in the quality rating data in this study also supported this speculation. Moreover, the ranking system might not be sensitive enough to accurately evaluate several components contributing to turfgrass quality (Jayasinghe et al. 2019, Kazemi et al. 2020). An incorporation of objective methodologies such as digital image processing into the NTEP is recommended (Kazemi et al. 2020).

In conclusion, this study demonstrated for the first time that *Epichloë* infection levels among perennial ryegrass cultivars commercially available in Oregon were highly variable, ranging from 0 to 100%. This variability can be attributed to inherent genetic diversity among the grass hosts, as well as the absence of seed certification practices regarding *Epichloë* infection of seeds. Moreover, four predicted alkaloid profiles of the examined perennial ryegrass cultivars were identified, which were slightly different from those determined in German populations (Vikuk et al. 2019). Over the five years of the study, 28 cultivars showed an increase in Epichloë incidence. The decline in Epichloë infection of some cultivars could reflect a low level of stress from minimal traffic applications. Moreover, linear regression analysis revealed the significance of *Epichloë* infection range, and no traffic application, as a factor for estimating turf quality ratings in 2021. This study suggests that a perennial ryegrass turf system only requires 20% of infected plants to exhibit an increase in overall quality, and an additional effect was not observed in those with higher infection rates. However, in future NTEP and turf evaluation studies, it is recommended to incorporate objective methodologies to enhance sensitivity and reliability in assessing turf quality to confirm the 20% Epichloë infection threshold observed in this study. It is necessary to regularly monitor Epichloë infection rates for turfgrass seeds throughout the supply chain and turfgrass stand after establishment to accurately determine Epichloë -mediated stress tolerance in a turf system.

| Cultivar | Source | Epichloë spp. infection | | % Epichloë | Predicted | |
|---------------------|-------------------|-------------------------|---------------------|------------|----------------------|--|
| | | frequency (%) | | spp. | alkaloid | |
| | | Seed ^a | Tiller ^b | infection | profile ^c | |
| | | | | change | | |
| Allstar III | Public | 63 | 0 | -63 | - | |
| Brightstar | Public | 54 | 0 | -54 | - | |
| SLT | | | | | | |
| Linn | Public | 58 | 0 | -58 | - | |
| Derby | Public | 19 | 13 | -6 | PER, EAS, | |
| Xtreme | | | | | IDT | |
| Karma | Public | 83 | 20 | -63 | - | |
| SR 4650 | Public | 28 | 47 | 19 | PER, EAS, | |
| | | | | | IDT | |
| Grand Slam | Public | 62 | 67 | 5 | PER, EAS, | |
| GLD | | | | | IDT | |
| Savant | Columbia Seeds | 24 | 0 | -24 | | |
| Saguaro | Columbia Seeds | 41 | 7 | -34 | PER, EAS, | |
| | | | | | IDT | |
| Mensa | Ledeboer Seed | 4 | 7 | 3 | EAS | |
| | LLC | | | | | |
| Overdrive 5G | Burlingham Seeds, | 22 | 13 | -9 | PER, EAS, | |
| | LLC. | | | | IDT | |
| Evolve | SiteOne Landscape | 4 | 20 | 16 | PER, EAS | |
| | Supply | | | | | |
| Apple 3GL | Mountain View | 70 | 27 | -43 | PER, EAS, | |
| (PPG-PR | Seeds | | | | IDT | |
| 339) | | | | | | |
| Fireball | Bailey Seed & | 43 | 33 | -10 | PER, EAS, | |
| (BWH) | Grain LLC | | | | IDT | |
| Paragon 2 | Turf Merchants, | 16 | 33 | 17 | PER, EAS, | |
| GLR (FP2) | Inc | | | | IDT | |
| Signet | Smith Seed | 0 | 40 | 40 | PER, EAS, | |
| | Services | | | | IDT | |
| ASP0117 (A- | Allied Seed LLC | 27 | 40 | 13 | PER, EAS, | |
| PR15) | | | | | IDT | |
| Man O'War | Lebanon Seaboard | 81 | 40 | -41 | PER, EAS, | |
| | Corp. | <u>.</u> | | | IDT | |
| Homerun LS | Mountain View | 60 | 47 | -13 | PER, EAS, | |
| (PPG-PR | Seeds | | | | IDT | |
| 419) | | | | | | |

Table 2.1 List of 51 evaluated perennial ryegrass cultivars with seed and tiller *Epichloë* spp. infection frequencies and predicted alkaloid profiles in Oregon State University's NTEP trials.

| Cultivar | Breeder | Epichloë spp. infection | | % Epichloë | Predicted | |
|---------------------------------|----------------------------|-------------------------|---------------------|---------------------|----------------------|--|
| | | frequency (%) | | spp. | alkaloid | |
| | | Seed ^a | Tiller ^b | infection change | profile ^c | |
| Superstar GL (PPG-PR 420) | Mountain View Seeds | 31 | 47 | 16 | PER, EAS, IDT | |
| Stellar 4GL (PPG-PR 424) | Mountain View Seeds | 84 | 47 | -37 | PER, EAS, IDT | |
| Intense | Landmark Turf & NS | 82 | 53 | -29 | PER, EAS, IDT | |
| Belize 2 (GO- 142) | Grassland Oregon | 12 | 53 | 41 | PER, EAS, IDT | |
| Paradox GLR (PPG- PR 331) | Turf Merchants, Inc | 41 | 53 | 12 | PER, EAS, IDT | |
| Silver Sport (PST-2CRP) | Rose Agri-Seed, Inc. | 42 | 53 | 11 | PER, EAS, IDT | |
| Gray Hawk (PST-2FIND) | Pure-Seed Testing, Inc. | 43 | 53 | 10 | PER, EAS, IDT | |
| Pharaoh | Lebanon Seaboard Corp. | 67 | 53 | -14 | PER, EAS, IDT | |
| Xcelerator | Landmark Turf & NS | 76 | 67 | -9 | PER, EAS, IDT | |
| ASP0218 (A- 6D) | Allied Seed LLC | 50 | 67 | 17 | PER, EAS, IDT | |
| Fastball 3GL (PPG-PR 329) | Mountain View Seeds | 67 | 67 | 0 | PER, EAS, IDT | |
| UMPQUA | Vista Seed Partners LLC | 26 | 67 | 41 | PER, EAS, IDT | |
| Shield (02BS4) | Smith Seed Services | 88 | 73 | -15 | PER, EAS, IDT | |
| Hatrick (BSP-17) | Bailey Seed & Grain LLC | 44 | 80 | 36 | PER, EAS, IDT | |
| Spie GLS (UF3) | Landmark Turf & NS | 59 | 80 | 21 | PER, EAS, IDT | |
| Cayman (GO-143) | Grassland Oregon | 66 | 80 | 14 | PER, EAS, IDT | |
| Seabiscuit | Lebanon Seaboard Corp. | 80 | 80 | 0 | PER, EAS, IDT | |

Table 2.1 (Continued).

| Cultivar | Breeder | Epichloë spp. infection | | % Epichloë | Predicted | |
|----------------------------|-----------------------|-------------------------|--|------------|----------------------|--|
| | | frequency (%) | | spp. | alkaloid | |
| | | Seed ^a | Tiller ^b | infection | profile ^c | |
| Tee-Me-Up | Bailev Seed & | 87 | 87 | 0 | PER, EAS, | |
| (BSP-25) | Grain LLC | | | - | IDT Í | |
| AllStar Fore | DLF Pickseed | 77 | 87 | 10 | PER, EAS, | |
| (DLFPS- | USA, Inc | | | | IDT | |
| 236/3541) | , | | | | | |
| SR 4700 | DLF Pickseed | 82 | 87 | 5 | PER, EAS, | |
| (DLFPS- | USA, Inc | | | | IDT | |
| 236/3543) | | | | | | |
| Nexus GT | Smith Seed | 37 | 87 | 50 | PER, EAS, | |
| (SNX) | Services | | | | IDT | |
| Green | AMPAC Seed Co. | 68 | 87 | 19 | PER, EAS, | |
| Supreme+ | | | | | IDT | |
| (AMP-R1) | | | | | | |
| Alloy (RRT) | The Scotts Miracle- | 44 | 87 | 43 | PER, EAS, | |
| | Gro Co | | | | IDT | |
| Slider LS | Mountain View | 0 | 87 | 87 | PER, EAS, | |
| (PPG-PR | Seeds | | | | IDT | |
| 241) | | - - | ~ | • | | |
| Furlong | Lebanon Seaboard | 85 | 87 | 2 | PER, EAS, | |
| (LTP-FCB) | Corp. | 20 | 07 | 40 | IDT DED EAG | |
| Ivy (PS1- | The Scotts | 38 | 8 / | 49 | PER, EAS, | |
| 2GTD) Course Welf | Company | 77 | 07 | 10 | | |
| Gray Woll | Kose Agri-Seed, | // | 8/ | 10 | PEK, EAS, | |
| (PSI-2GAL) Figsts Cinco | Inc. DI E Dialwood | 70 | ٥ 7 | 0 | IDI DED EAS | |
| TIESTA CIIICO | USA Inc | 19 | 0/ | 0 | FER, EAS, | |
| (DLF1 5- 236/3554) | USA, IIIC | | | | IDT | |
| 250/5554) ASP0116FXT | Allied Seed LLC | 45 | 93 | 48 | PER EAS | |
| ASI UTIULAT | Amed Seed LLC | ч <i>3</i> |)5 | 10 | IDT | |
| ASP0118GL | Allied Seed LLC | 15 | 93 | 78 | PER EAS | |
| (A-4G) | | 10 | <i>y</i> y | 10 | IDT | |
| Pepper II | Lewis Seed | 65 | 93 | 28 | PER, EAS. | |
| (RAD-PR | Company | | | - | IDT | |
| 103) | -12 | | | | _ | |
| Slugger 3GL | Mountain View | 63 | 100 | 37 | PER, EAS, | |
| (PPG-PR | Seeds | | | | IDT | |
| 343) | | | | | | |

Table 2.1 (Continued).

^a Information provided by Rutgers University, New Brunswick, New Jersey.

^b *Epichloë* infection frequency determined from tiller samples collected at the end of the 2016 NTEP Perennial Ryegrass Trial program at Oregon State University.

^c Predicted alkaloid profile represent the class of genes involved in an alkaloid production and do not always represent a functionally active gene or imply a completion of alkaloid locus in the genome. *PER*, peramine; *EAS*, ergot alkaloid; *IDT*, indole-diterpenes.

| Locus | Primer name | Primer sequence | gDNA size (bp) |
|-------|---------------|---------------------------|----------------|
| TefA | tef1-exon1d-1 | GGGTAAGGACGAAAAGACTCA | 860 |
| | tef1-exon6u-1 | CGGCAGCGATAATCAGGATAG | |
| PER | per T2-F | TCTTCAGGCATCGCAGGAAC | 600 |
| | per T2-R | TCGGCCACCTCCAGCCTGATG | |
| LOL | lolC-3a | GGTCTAGTATTACGTTGCCAGGG | 442 |
| | lolC-5b | TCTAAACTTGACGCAGTTCGGC | |
| EAS | dmaW-F4 | GTGTACTTTACTGTGTTCGGCATG | 282 |
| | dmaW-6R | GTGGAGATACACACTTAAATATGGC | |
| IDT | idtG-F | GAGCTTGAGAAGCTTACGAATCC | 113 |
| | idtG-R | GGGCAATGGAGCGATTCTCTC | |

Table 2.2 Primers used in multiplex PCR to determine *Epichloë* presence and alkaloid potential.



Figure 2.1 Average quality ratings of perennial ryegrass cultivars evaluated in the 2016 NTEP program under both traffic and non-traffic conditions from 2017 to 2021. The red boxes represent the no-traffic designation, while the blue boxes represent the traffic designation. N, no traffic; T, traffic.



Figure 2.2 Average quality ratings of perennial ryegrass cultivars evaluated in the 2016 NTEP program, categorized by varying frequencies of tiller *Epichloë* infection, under both traffic and non-traffic conditions in 2021. The red dots represent means of the *Epichloë* infection range groups. The red boxes represent the no-traffic designation, while the blue boxes represent the traffic designation. N, no traffic; T, traffic. * denotes P < 0.05, Dunnett's post hoc test.

References

- Bacon C, Porter J, Robbins J et al. 1977. *Epichloe typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34(5):576-581.
- Baldauf MW, Mace WJ, Richmond DS. 2011. Endophyte-mediated resistance to black cutworm as a function of plant cultivar and endophyte strain in tall fescue. Environ. Entomol. 40(3):639-647. <u>https://doi.org/10.1603/EN09227</u>.
- Clay K, Schardl C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. Am. Nat. 160(S4):S99-S127. <u>https://doi.org/10.1086/342161</u>.
- Clay K, Holah J, Rudgers JA. 2005. Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. roc. Proc. Natl. Acad. Sci. U.S.A 102(35):12465-12470. https://doi.org/10.1073/pnas.0503059102.
- Cunningham P, Foot J, Reed K. 1993. Perennial ryegrass (*Lolium perenne*) endophyte (*Acremonium lolii*) relationships: the Australian experience. Agric. Ecosyst. Environ. 44(1-4):157-168. <u>https://doi.org/10.1016/0167-8809(93)90044-P</u>.
- Da Costa M, Bhandari B, Carson J et al. 1998. Incidence of endophytic fungi in seed of cultivars and selections in the 1988 national fine fescue test. Proc. New Jersey Turfgrass Expo. Atlantic City, New Jersey, USA. December:8-10.
- Easton S, Tapper B. 2005. *Neotyphodium* research and application in New Zealand. *Neotyphodium* in Cool-Season Grasses:35-42. https://doi.org/10.1002/9780470384916.ch1c.
- Francis SM, Baird DB. 1989. Increase in the proportion of endophyte-infected perennial ryegrass plants in overdrilled pastures. N. Z. J. Agric. Res. 32(3):437-440. https://doi.org/10.1080/00288233.1989.10421764.
- Funk CR, White RH, Breen JP. 1993. Importance of *Acremonium* endophytes in turf-grass breeding and management. Agric. Ecosyst. Environ. 44(1):215-232. <u>https://doi.org/https://doi.org/10.1016/0167-8809(93)90048-T</u>.
- Hewitt KG, Mace WJ, McKenzie CM et al. 2020. Fungal alkaloid occurrence in endophyteinfected perennial ryegrass during seedling establishment. J. Chem. Ecol. 46:410-421. https://doi.org/10.1007/s10886-020-01162-w.
- Hoveland CS. 1993. Importance and economic significance of the *Acremonium* endophytes to performance of animals and grass plant. Agric. Ecosyst. Environ. 44(1-4):3-12. https://doi.org/10.1016/0167-8809(93)90036-O.
- Jacobson EH. 1963. Sojourn research: A definition of the field. J. Soc. Issues 19(3):123-129. https://doi.org/https://doi.org/10.1111/j.1540-4560.1963.tb00451.x.
- Jayasinghe C, Badenhorst P, Wang J et al. 2019. An object-based image analysis approach to assess persistence of perennial ryegrass (*Lolium perenne* L.) in pasture breeding. Agron. 9(9):501. <u>https://doi.org/10.3390/agronomy9090501</u>.

- Kazemi F, Golzarian MR, Nematollahi F. 2020. Quality assessment of turfgrasses using NTEP method compared to an image-based scoring system. J. Ornament. Plants 10(3):167-178.
- König J, Fuchs B, Krischke M et al. 2018. Hide and seek–infection rates and alkaloid concentrations of *Epichloë festucae* var. *lolii* in *Lolium perenne* along a land-use gradient in Germany. Grass Forage Sci. 73(2):510-516. <u>https://doi.org/10.1111/gfs.12330</u>.
- Krans JV, Morris K. 2007. Determining a profile of protocols and standards used in the visual field assessment of turfgrasses: A survey of national turfgrass evaluation programsponsored university scientists. Appl. Turfgrass Sci. 4(1):1-6. https://doi.org/https://doi.org/10.1094/ATS-2007-1130-01-TT.
- Lehtonen PT, Helander M, Siddiqui SA et al. 2006. Endophytic fungus decreases plant virus infections in meadow ryegrass (*Lolium pratense*). Biol. Lett. 2(4):620-623. https://doi.org/10.1098%2Frsb1.2006.0499.
- Meister B, Krauss J, Härri SA et al. 2006. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. Basic Appl. Ecol. 7(3):244-252. https://doi.org/10.1016/j.baae.2005.06.002.
- Mohr M, Meyer W, Mansue C. 2002. Incidence of *Neotyphodium* endophyte in seed lots of cultivars and selection of the 2001 tall fescue test. Proc. New Jersey Turfgrass Expo. Atlantic City, New Jersey, USA. December 33:3-8.
- Morris K, Shearman R. 2000. The National Turfgrass Evaluation Program: assessing new and improved turfgrasses. Diversity 16(1/2):19-22.
- National Turfgrass Evaluation Program (NTEP). 2024. https://www.ntep.org/
- Pinheiro J. 2011. nlme: Linear and nonlinear mixed effects models. R package version 3:1.
- R Core Team. 2024. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.
- Reed K, Leonforte A, Cunningham P et al. 2000. Incidence of ryegrass endophyte (*Neotyphodium lolii*) and diversity of associated alkaloid concentrations among naturalised populations of perennial ryegrass (*Lolium perenne* L.). Aust. J. Agric. Res. 51(5):569-578. <u>http://dx.doi.org/10.1071/AR99182</u>.
- Richmond DS, Niemczyk HD, Shetlar DJ. 2000. Overseeding endophytic perennial ryegrass into stands of Kentucky bluegrass to manage bluegrass billbug (Coleoptera: Curculionidae). J. Econ. Entomol. 93(6):1662-1668. <u>https://doi.org/10.1603/0022-0493-93.6.1662</u>.
- Rolston M, Agee C. 2007. Delivering quality seed to specification-the USA and NZ novel endophyte experience. NZGA Res. Pract. Ser. 13:229-231. http://dx.doi.org/10.33584/rps.13.2006.3065.
- Saikkonen K, Wäli PR, Helander M. 2010. Genetic compatibility determines endophyte-grass combinations. PLoS One 5(6):e11395. <u>https://doi.org/10.1371/journal.pone.0011395</u>.

- Saikkonen K, Gundel PE, Helander M. 2013. Chemical ecology mediated by fungal endophytes in grasses. J. Chem. Ecol. 39:962-968. <u>https://doi.org/10.1007/s10886-013-0310-3</u>.
- Schardl CL, Young CA, Pan J et al. 2013. Currencies of mutualisms: sources of alkaloid genes in vertically transmitted *Epichloae*. Toxins 5(6):1064-1088. https://doi.org/10.3390/toxins5061064.
- Song M, Li X, Saikkonen K et al. 2015. An asexual *Epichloë* endophyte enhances waterlogging tolerance of *Hordeum brevisubulatum*. Fungal Ecol. 13:44-52. https://doi.org/https://doi.org/10.1016/j.funeco.2014.07.004.
- Stewart AV, Barcellos G, Brilman L. 2022. Use of endophytic fungi in turfgrasses: Difficulties in delivery to the market. Int. Turfgrass Soc. Res. J. 14(1):1070-1073. <u>https://doi.org/10.1002/its2.131</u>.
- Takach JE, Mittal S, Swoboda GA et al. 2012. Genotypic and chemotypic diversity of *Neotyphodium* endophytes in tall fescue from Greece. Appl. Environ. Microbiol. 78(16):5501-5510. <u>https://doi.org/10.1128/aem.01084-12</u>.
- USDA NASS. 2024. Oregon grass seed available for harvest in 2024 are estimated at 302,000 acres. https://www.nass.usda.gov/Statistics_by_State/Washington/Publications/Current_News_ Release/2024/ORGRASS_2024.pdf.
- Vikuk V, Young CA, Lee ST et al. 2019. Infection rates and alkaloid patterns of different grass species with systemic *Epichloë* endophytes. Appl. Environ. Microbiol. 85(17):e00465-00419. <u>https://doi.org/10.1128%2FAEM.00465-19</u>.
- Young C, Hume D, McCulley R. 2013. FORAGES AND PASTURES SYMPOSIUM: Fungal endophytes of tall fescue and perennial ryegrass: Pasture friend or foe? J. Anim. Sci. 91:<u>https://doi.org/10.2527/jas.2012-5951</u>.
- Young CA, Charlton ND, Takach JE et al. 2014. Characterization of *Epichloë coenophiala* within the US: are all tall fescue endophytes created equal? Front. Chem. 2:95. https://doi.org/10.3389%2Ffchem.2014.00095.

CHAPTER 3

Endophyte-mediated resistance against Noctua pronuba in cool-season turfgrass

Julaluck Intasin

To be submitted to Environmental Entomology

170 Jennifer Rd., Suite 230

Annapolis, MD 21401-7995 USA

Abstract

Winter cutworms (*Noctua pronuba*) are highly gregarious and mobile insect pests with a wide host range, including turfgrass and grass seed crops in Oregon. Chemical controls have limited effect due to N. pronuba larvae's winter-feeding habit. Fungal endophytes (Epichloë spp.) grow symbiotically within cool-season grasses and provide several benefits, including resistance to insect herbivory through the production of alkaloids. This research evaluates the *Epichloë*-mediated resistance response against *N. pronuba* in eight commercial cultivars of tall fescue and perennial ryegrass grown for seed in Oregon. In the summer and fall of 2023, two nochoice greenhouse experiments were conducted to measure the impact of expected Epichloë infection on insect survival, insect weight gain, grass biomass, and feeding damage at three different time points. It is important to note that mean comparisons between the high and low *Epichloë* infection levels were based on *Epichloë* infection status either predetermined in a previous study or provided by breeders. Individual grass tillers were collected at the end of the 14-day no-choice experiments to validate the Epichloë infection status and presence of alkaloidproducing genes after feeding. A significant difference in the examined parameters was not detected in trial 1 when comparing among expected Epichloë incidence and within cultivars, regardless of the grass host species. In trial 2, expected *Epichloë* infection status in tall fescue did notaffect insect mortality and weight gain but did influence feeding damage at 3 and 12 days. However, the observed effects of expected Epichloë infection on feeding damage (one-way ANOVA) could be more influenced by cultivar differences. In trial 2 perennial ryegrass, a low Epichloë infection level unexpectedly correlated with higher insect mortality, yet the observed trend was not supported by the other considered parameters. Similar to the tall fescue experiment, grass host performance variations in perennial ryegrass were primarily due to

cultivar-specific traits rather than *Epichloë* levels. These findings help inform the practical use of the grass-*Epichloë* symbioses for controlling *N. pronuba* and other cutworms.

Introduction

Grass seed is one of Oregon's leading agricultural commodities with a farmgate value of \$639.2 million (Oregon Department of Agriculture 2023). The Willamette Valley of Oregon is known as the "grass seed capital of the world" because most grass seeds in the national and international markets are from Oregon (Young and Silberstein 2012). Tall fescue and perennial ryegrass are ranked first and third, respectively, among grass species produced for seed in Oregon, encompassing a significant acreage of the grass seed crops harvested - 187,000 acres in total (USDA NASS, 2024). Both tall fescue and perennial ryegrass are cool-season grass species that are often associated with *Epichloë* spp. – hereafter "*Epichloë*" (Schardl et al. 2004, Leuchtmann et al. 2014).

Epichloë species can be transmitted vertically from mother plant to daughter seeds when hyphae grow into developing seeds of the grass host (Zhang et al. 2017). The grass-*Epichloë* symbiosis provides the host plant with advantages including abiotic stress tolerances, disease suppression, and anti-herbivory properties (Clarke et al. 2006, Meister et al. 2006, Song et al. 2015, Realini et al. 2024). *Epichloë* -mediated insect herbivory resistance is predominantly attributable to the fungi's ability to produce a wide range of alkaloids or secondary metabolites: ergot alkaloids, peramine, lolines, and indole-diterpenoids (Siegel et al. 1990, Realini et al. 2024). Specifically, peramines and lolines are found to be deterrent and toxic to insect herbivores (Bacetty et al. 2009, Schardl et al. 2012), while ergot alkaloids and indole-diterpene are responsible for fescue toxicosis and ryegrass staggers in grazing mammals (Bacon et al. 1977, Fletcher and Harvey 1981, Caradus et al. 2022).

The magnitude and effectiveness of alkaloid-dependent anti-herbivory traits vary across host-symbiont associations (Jensen and Popay 2004, Bastias et al. 2017). The classes and concentrations of alkaloids produced in a grass-*Epichloë* symbiosis can be dictated by environmental conditions such as temperature, season, and soil fertility (Breen 1992, Ball et al. 1995, Belesky et al. 1988, Bultman and Bell 2003, Fuchs et al. 2017). For instance, Fuch et al. (2017) found that the ergot alkaloid, indole-diterpene and peramine concentrations in perennial ryegrass was seasonally dependent across three years of observation, with the highest concentrations in summer and lowest concentrations in winter. Moreover, the authors reported that the concentration of peramine reached the toxicity threshold for invertebrates in August and October in the first year and from May to September of the second year of their study. The observation suggests that the level of *Epichloë*-mediated insect resistance could vary with grass stand age or climatic conditions.

Genotypes of the plant host and *Epichloë* strain often play a significant role in determining the *Epichloë* infection incidence and alkaloid titers and therefore resistance to insect herbivory (Jensen and Popay 2004, Richmond and Bigelow 2009, Young et al. 2014, Vikuk et al. 2019). Moreover, within a grass species, the variation in *Epichloë* infection level and alkaloid expression exist in the grass cultivar and cultivar \times *Epichloë* strain combination levels (Young et al. 2014), resulting in a unique range of *Epichloë*-mediated defense against insect herbivores (Richmond and Bigelow 2009, Baldauf et al. 2011). For example, Richmond and Bigelow (2009) found that all four tall fescue cultivars tested in their study exhibited varying levels of *Epichloë* infection and ergot alkaloid concentration. Moreover, in the same experiment, the researchers observed that black cutworm (*Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae)) survival was different between tall fescue cultivars in a greenhouse assay, although there were no correlations between the larvae survival, *Epichloë* infection rate, and ergot alkaloid concentration. Variations in *A. ipsilon* survival and biomass were observed among ten different tall fescue × *Epichloë coenophiala* strain combinations. *N*-acetyl norloline was identified as the best alkaloid predictor for *A. ipsilon* survival, with increasing levels of *N*-acetyl norloline observed with decreased *A. ipsilon* survival at 5 and 10days of active feeding on infected leaf clippings (Baldauf et al. 2011).

Earlier choice or no-choice assays in the United States investigating *Epichloë*-mediated herbivory resistance against lepidopteran pests in turf systems were limited to *A. ipsilon*, and fall armyworms, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). *A. ipsilon* larvae settling, larval survival, and larval biomass were found to be negatively affected when feeding on *Epichloë* infected-grass cultivars in no-choice assays. (Baldauf et al. 2011). A similar observation was made in a *S. frugiperda* study in which the *S. frugiperda* preferred leaf blades from *Epichloë*-free tall fescue over grasses infected with *Epichloë*, in choice experiments (Bultman et al. 2009). A recent study by Kaur et al. (2024) demonstrated that high *Epichloë* infection in tall fescue cultivars grown for seed in Oregon could provide protection against sod webworm (*Chryoteuchia topiaria* Zeller) feeding. These findings indicate that the symbiotic relationship between grasses and *Epichloë* endophytes holds promise for managing other lepidopteran pests in Oregon's grass seed crops.

The winter cutworm, *Noctua pronuba*, has become a serious agricultural pest in Oregon's grass seed production, golf courses, sport fields, and lawns (Green et al. 2016). The larvae actively feed during fall and winter months inflicting chewing damage near the crown, followed by pupation in early spring (Difonzo and Russell 2010, Green et al. 2016). The main challenge

with controlling *N. pronuba* using pesticides is that the larvae feed in the late fall and winter months when field conditions are too wet for equipment operation. Moreover, the temperature during the winter months, when the insects are active, is usually lower than the recommended range for full efficacy of the insecticide. Thus, chemical applications are difficult to appropriately time (Anderson unpublished data).

Unfortunately, effective cultural or biological practices against N. pronuba have yet to be determined. With most of the seed produced in Oregon being exported nationally and internationally (Young and Silberstein 2012), Epichloë-mediated anti-herbivory properties could result in a substantial reduction in pesticide use for turf, pasture, and cover crop end-users across the globe. Additionally, seed crops with improved insect resistance could be marketed to fetch Oregon seed growers a higher premium, consequently improving the economics of Oregon's grass seed industry. Therefore, the objective of this study was to evaluate Epichloë-mediated resistance against N. pronuba in tall fescue and perennial ryegrass cultivars with differential *Epichloë* infection levels by conducting a series of no-choice experiments under greenhouse conditions. The resistance response was also studied in tall fescue and perennial ryegrass cultivars after the eradication of *Epichloë* upon seed treatment using a systemic fungicide. The hypothesis of this study was that grass cultivars, regardless of grass species, with high levels of *Epichloë* infection would exhibit a greater resistance response against N. pronuba, compared to grass cultivars with low Epichloë infection. An additional hypothesis was that with no regard to a grass cultivar's initial *Epichloë* incidence level, the *Epichloë*-infected grasses group would show greater resistance against N. pronuba than the Epichloë-eradicated grasses group. The information gained from this project could be used to determine an N. pronuba management strategy in both turfgrass and grass seed production systems of Oregon.

Materials and Methods

Two no-choice bioassays were conducted under greenhouse conditions during summer and fall of 2023 at the West Greenhouses, Oregon State University, Corvallis, OR. Treatments were arranged in a completely randomized design to compare two grass species (tall fescue, perennial ryegrass), two levels of *Epichloë* infection (high, low), and two fungicide treatments (no fungicide, fungicide), along with simultaneous *N. pronuba* feeding (*N. pronuba* present, *N. pronuba* absent control), across two trials. The first trial was conducted in summer 2023 with six replications and the second trial was conducted in fall 2023 with five replications. In these trials, *N. pronuba* mortality, larval weight, aboveground grass biomass, aboveground grass feeding damage at three different time points, and individual plant *Epichloë* infection were quantified.

Plants

Cultivars of tall fescue and perennial ryegrass with either a high *Epichloë* infection rate (above 85%) or a low *Epichloë* infection rate (below 20%) were identified. Two cultivars of each grass species-*Epichloë* level combination (e.g. tall fescue-high *Epichloë*, tall fescue-low *Epichloë*, perennial ryegrass-high *Epichloë*, perennial ryegrass-low *Epichloë*) were used in the experiment (Table 3.1).

Seeds of the eight cultivars of both grass species were obtained from five different seed storage and distribution facilities in Oregon during Spring 2023, as detailed in Table 3.1. The seeds were stored at 4°C when not in use. These seeds were sown in a 40-cell tray insert (150 cm³ per cell) containing RESiLIENCE® potting mix (comprising 35-45% Canadian sphagnum peat moss, processed softwood bark, perlite, coir, and dolomite) (Sun Gro Horticulture, Agawam, MA). Five seeds per cultivar were sown in each cell at a depth of 1 cm. One week after

germination, seedlings were manually thinned to one per cell. The grasses were maintained under a 16:8h L:D photoperiod with supplemental lighting from 1000 W sodium vapor bulbs and watered as needed. Day and night temperatures were set at 25° and 20°C, respectively. Another set of the same seeds was treated with a systemic fungicide (Banner Maxx II) containing propiconazole as the active ingredient (Syngenta, Wilmington, DE) to create fungicide-treated counterparts. 4000 mg seeds were soaked in the fungicide solution (0.005g a.i./10 ml DI water) for 25 min and air-dried on paper towels overnight. The appropriate fungicide rate for controlling *Epichloë* in seedlings was previously determined. Fungicide-treated seeds were planted and maintained in the greenhouse as described above.

Insects Colony Maintenance

Noctua pronuba eggs were collected from commercial grass seed fields in Oregon, USA ($45^{\circ}11'14.4"N 123^{\circ}14'59.7"W$ and $44^{\circ}33'12.3"N 123^{\circ}18'02.8"W$) from August to September 2023. Eggs were checked for parasitism before the introduction into a laboratory for rearing. Healthy eggs and collections without any prior exposure to insecticides were used in the laboratory colony. The insect colonies were maintained on a general-purpose lepidopteran diet (Frontier Scientific Inc., Newark, DE) using a method slightly modified (Hervet et al. 2016) under laboratory conditions ($20 \pm 2 \ ^{\circ}C$; 9:15 (L:D) h photoperiod; 70% humidity). One liter of diet was prepared as per the protocol modified from the recipe provided by Frontier Scientific. To a 2L beaker, 875 ml of DI water was boiled on a hotplate with a stirring bar, after which 105 g of dry mix were slowly added to the beaker. A rubber spatula was used to vigorously mix the slurry to prevent burning on the bottom of the beaker. Once the diet was fully mixed, 19 g of agar was added to the mixture, followed by vigorous mixing for five minutes. The diet was then

transferred into 30 ml-cups, filling them up to ¼ of their volume. Insects (eggs and neonates) were transferred to fresh diet cups every 3 days until they were ready for testing (2nd-3rd instars).

Insect Performance in No-choice Assays Trial 1 and 2

Eight to nine weeks after the seeds germinated, five plant plugs of each cultivar-fungicide treatment combination were transferred into an approximately 20-liter plastic storage container filled with potting mix (1:1 ratio of G&B Simples Seed Starter Mix comprising peat moss, perlite, pumice, washed sand; Kellogg Garden Products, Carson, CA) and autoclaved kiln-dried sand (Marion Ag Services, Inc., St. Paul, OR)). The container bases and lids were drilled with 6 (8 mm) and 2 holes (50 mm), respectively, to enhance ventilation. Mesh netting (1000 microns) was glued over the holes on the lid and placed between the base and potting mix to prevent larval escape.

Five 2^{nd} - 3^{rd} instar *N. pronuba*, with a total weight ranging from 0.14 - 0.55g for trial 1 and 0.33 - 1.13g for trial 2, were added to each plastic container containing five transferred test plants. Every three days after the insect release, the numbers of observed live and dead larvae were recorded, and the aboveground grass area was photographed using a camera attached to a frame with 26 cm height from the container's top edge to ensure depth of field consistency. The *N. pronuba* larvae were allowed to feed on the grasses for 14 days before they were removed from the arena. Missing larvae were considered dead, and all live larvae were weighed. Each plant was cut at ground level and weighed for aboveground biomass data before three representative tillers were taken and stored in an Agdia mesh sample bag at -20°C for *Epichloë* detection using the previously described multiplex PCR method (Takach et al. 2012).

Feeding Damage Determination using ImageJ

The photographed aboveground grass area was analyzed by computing the total vegetation area using the built-in particle analysis plugin in ImageJ software (Schneider et al. 2012). Feeding damage at 3, 12, and 15 days were determined by subtracting a total vegetation area on day 0 from a total vegetation area on day 3, day 12, and day 15, respectively. Then, the differences were divided by the total vegetation area on day 0. The numbers were multiplied by 100% to obtain feeding damage percentages. A positive percentage number represents an increase in total vegetation area from day zero, while a negative percentage number represents a decrease in total vegetation area from day zero.

Molecular Epichloë Determination Methods

Total nucleic acids were extracted from frozen plant material using the protocol modified from Dellaporta et al. (1983). The modification involved the use of drill fitted with an Agdia tissue homogenizer (Agdia, Inc., Elkhart, IN) to grind samples in Agdia's mesh sample bags. From that point, the original protocol was used. To each ground sample, 10% sodium dodecyl sulfate was added, followed by an incubation in 65°C hot water bath for 10 min. Acidified 5M potassium acetate was added to the sample before the mixture was placed on ice for 5 min, followed by 10 min centrifugation at max speed. The supernatant was mixed with cold isopropanol, followed by incubation on ice and centrifugation as mentioned above. The supernatant was discarded before the pellet was mixed with cold 70% ethanol. The reaction was centrifuged for 2 minutes at max speed before the supernatant and residual alcohol were discarded. The pellets were re-suspended with 300 µL DI water.

The *Epichloë* infection status, infection rate, and chemotype were determined using a high throughput multiplex PCR method described by Takach et al. (2012). The translation

elongation factor 1-alpha gene region (tefA, abbreviated "TEF") is an internal positive control for fungal infection, and the following alkaloid biosynthesis-related genes: peramine (perA, abbreviated "PER"), loline (lolC, abbreviated "LOL"), ergot alkaloid (dmaW, abbreviated "EAS"), and indole-diterpene (*idtG*, abbreviated "IDT") were amplified from total DNA. All genes have been widely used for *Epichloë* detection and chemotypic analysis of *Epichloë* worldwide (Schardl et al. 2013). The primers used for the multiplex PCR reaction are listed in Table 2.2. The PCR reactions with a total volume of 25 μ l each contained 5× Green GoTag reaction buffer, 10 µM of each deoxynucleoside triphosphate (dNTP), 1.0-U GoTaq DNA polymerase (Promega Corp., Madison, WI), and each target-specific primer (10 μ M). Amplification conditions were two min of initial denaturation at 94°C, 30 cycles of 15s at 94°C, 30s at 56°C, and one min at 72°C, followed by seven min at 72°C (Takach et al. 2012). PCR products were visualized via gel electrophoresis with a 1.5% agarose gel in 1X Tris-borate-EDTA (TBE) buffer following ethidium bromide staining and UV transillumination. The expected fragment sizes for each amplicon was presented in Table 2.2. Each chemotype was characterized using banding patterns.

Statistical Analysis

Statistical analysis was performed using R version 4.2.2 (R Core Team, 2024). Each trial was evaluated separately. The Shapiro-Wilk test was performed to assess the normality of residuals. To assess homogeneity of variances, a scatterplot of residuals against fitted values was generated to visually inspect the pattern of residuals across the range of fitted values. Outliers were identified in the dataset using the Z-score rule (\pm 3). These outliers were subsequently removed from the analysis due to their potential to skew results. Insect mortality, insect weight

gain, grass biomass, feeding damage at 3, 12, and 15 days were compared by expected *Epichloë* level (high or low) and cultivar using one-way ANOVA when the assumptions were satisfied. Otherwise, a Dunnett's post hoc test with Bonferroni adjustment was applied following a significant Kruskal-Wallis test (P < 0.05). Moreover, a linear regression model was fitted using the *lm* function in R (R Core Team, 2024). The model included observed *Epichloë* infection and cultivar as fixed effects. When the assumptions were met, the effects of the observed *Epichloë* infection (factor 1) and cultivar (factor 2) on the six different parameters examined in this study were evaluated using two-way ANOVA. Otherwise, multiple comparisons using Tukey's adjustment were performed using the *lsmeans* function from the car package (Fox and Weisberg 2018) to determine if cultivars differ significantly from each other. Results were considered different with a *P*-value < 0.05.

Results

The observed results of multiplex PCR and *Epichloë* levels in tiller samples collected at the end of the experiments did not match the expected *Epichloë* levels that were either reported by breeders or previously determined in Chapter 2 (Table 3.1). Grass cultivars that were considered either high or low in *Epichloë* infection were expected to have an *Epichloë* infection frequency above 85% and below 20%, respectively. None of the grass cultivars expected to have high *Epichloë* levels, regardless of grass species, demonstrated an *Epichloë* infection exceeding 85%. Moreover, none of the examined tall fescue cultivars expected to have low *Epichloë* levels met the 20% standard. Only perennial ryegrass 'Mensa' *Epichloë* levels were consistently low in both trials; while perennial ryegrass 'Sienna' was infected with a higher level of *Epichloë* than expected in trial 1 and as expected in trial 2 (Table 3.1).

Moreover, regardless of grass species and expected *Epichloë* level, the observed *Epichloë* levels in tiller samples were not consistent across the two trials. For example, the tall fescue cultivar 'Sidewinder' observed *Epichloë* level was 53.33% in trial 1, but dropped to 20% in trial 2 (Table 3.1). Similarly, the perennial ryegrass 'Furlong' observed *Epichloë* level was 53.33% in trial 1 and 28% in trial 2 (Table 3.1).

Insect and Grass Performances in No-choice Feeding Assays

Trial 1

Tall Fescue

In trial 1, there were no significant differences in mean insect mortality, mean insect weight gain, and mean grass biomass, and mean feeding damage at the three time points between tall fescue grasses grown from seeds treated with the fungicide and those not treated with the fungicide (p > 0.05) (Table 3.2). Similarly, mean feeding damage feeding damage at 3, 12, and 15 days were not different between the two fungicide treatments (p > 0.05) (Table 3.2). However, there was an effect of the fungicide treatment on mean feeding damage at 3 days, with a higher feeding damage on grass cultivars not treated with fungicides than on the cultivars treated with fungicides ($\bar{Y}_{No \ fungicide} = 97.42\%$, $\bar{Y}_{Fungicide} = 29.15\%$, p = 0.045) (Table 3.2 and Figure 3.1A).

Mean insect mortality (Figure 3.2A), mean insect weight gain (Figure 3.2B), mean grass biomass, and mean feeding damage at 3, 12, and 15 days were not different between the tall fescue cultivars with high *Epichloë* incidences and those with low *Epichloë* incidences (p > 0.05) (Table 3.2). Furthermore, mean insect mortality, mean insect weight gain, mean grass biomass, and mean feeding damage at 3, 12, and 15 days were not different among the four tall fescue cultivars (p > 0.05) (Table 3.2). Analysis of the effect of observed *Epichloë* infection and tall fescue cultivar on insect mortality, insect weight gain, and feeding damage at the three time points revealed no effect of both observed *Epichloë* infection and cultivar (P > 0.05) (Table 3.3).

Perennial Ryegrass

There were no differences in mean insect mortality, insect weight gain, grass biomass, and feeding damage at 3, 12, and 15 days between perennial ryegrass cultivars treated with fungicide and those not treated with fungicide (p > 0.05) (Table 3.2).

Mean insect mortality (Figure 3.3A), mean insect weight gain (Figure 3.3B), mean grass biomass, and mean feeding damage at 3, 12, and 15 days were not different between the perennial ryegrasses with high *Epichloë* incidences and those with low *Epichloë* incidences when the seeds were not subject to the fungicide treatment (p > 0.05) (Table 3.2). Similarly, mean insect mortality (Figure 3.3C), mean insect weight gain (Figure 3.3D), mean grass biomass, and mean feeding damage at 3, 12, and 15 days were not significantly different among the four perennial ryegrass cultivars (p > .05) (Table 3.2). Moreover, an analysis of the effect of observed *Epichloë* infection and perennial ryegrass cultivar on insect mortality, insect weight gain, and feeding damage at the three time points revealed no effect of either factor (P > 0.05) (Table 3.3).

Trial 2

Tall Fescue

There were no differences in mean insect mortality, mean insect weight gain, and mean grass biomass between tall fescue grasses grown from seeds treated with fungicide and those not treated with fungicide (p > 0.05) (Table 3.4). Similarly, mean feeding damage at 12 days and 15

days were not different between the two fungicide treatments (p > 0.05) (Table 3.4). However, there was an effect of the fungicide treatment on mean feeding damage at 3 days ($\bar{Y}_{No \text{ fungicide}} = 47.15\%$, $\bar{Y}_{Fungicide} = -13.46\%$, p = 0.031) (Table 3.4 and Figure 3.1B).

In the no-fungicide treatment group, mean feeding damage at 3 days for tall fescue cultivars with high *Epichloë* incidence was found to be higher than that of tall fescue cultivars with low *Epichloë* incidences ($\overline{Y}_{\text{High Epichloë}} = -5.83\%$, $\overline{Y}_{\text{Low Epichloë}} = 100.12\%$, p = 0.034) (Table 3.4 and Figure 3.4A). Moreover, mean feeding damage at 12 days for tall fescue cultivars with high *Epichloë* incidence was found to be higher than that for tall fescue cultivars with low *Epichloë* incidence ($\overline{Y}_{\text{High Epichloë}} = -47.34\%$, $\overline{Y}_{\text{Low Epichloë}} = 58.30\%$, p < 0.001) (Table 3.4 and Figure 3.4B). However, insect mortality (Figure 3.5A), insect weight gain (Figure 3.5B), grass biomass, and feeding damage at 15 days were not different between tall fescue cultivars with the two different *Epichloë* infection levels (p > 0.05) (Table 3.4).

Furthermore, within the no-fungicide treatment group, mean insect mortality ($\overline{Y}_{\text{Sidewinder}} = 100\%$, $\overline{Y}_{\text{Blacktail}} = 60\%$, $\overline{Y}_{\text{Bronson}} = 100\%$, $\overline{Y}_{\text{Goliath}} = 56\%$, p = 0.003) (Table 3.4 and Figure 3.5C), mean insect weight gain ($\overline{Y}_{\text{Sidewinder}} = -0.156 \text{ g}$, $\overline{Y}_{\text{Blacktail}} = 0.188 \text{ g}$, $\overline{Y}_{\text{Bronson}} = -0.154 \text{ g}$, $\overline{Y}_{\text{Goliath}} = 0.132 \text{ g}$, p < 0.001) (Table 3.4 and Figure 3.5D), mean feeding damage at 3 days ($\overline{Y}_{\text{Sidewinder}} = -42.40\%$, $\overline{Y}_{\text{Blacktail}} = 30.75\%$, $\overline{Y}_{\text{Bronson}} = 42.13\%$, $\overline{Y}_{\text{Goliath}} = 158.11\%$, p < 0.001), and mean feeding damage at 12 days ($\overline{Y}_{\text{Sidewinder}} = -48.27\%$, $\overline{Y}_{\text{Blacktail}} = -46.41\%$, $\overline{Y}_{\text{Bronson}} = 61.72\%$, $\overline{Y}_{\text{Goliath}} = 54.89\%$, p = 0.007) (Table 3.4) were different among tall fescue cultivars. However, mean grass biomass and feeding damage at 15 days were not different among the tall fescue cultivars (p > 0.05) (Table 3.4).

Analysis of the effect of observed *Epichloë* infection and cultivar on insect mortality, insect weight gain, and feeding damage at 3 days revealed a strong effect of cultivar (P < 0.001) (Table 3.3), whereas the effect of observed *Epichloë* infection was not significant. Interestingly, the linear regression model indicated a subtle to no effect of cultivar on feeding damage at 12 days (P = 0.061) and 15 days (P = 0.258) (Table 3.3). There was no observed effect of *Epichloë* infection on feeding damage at 15 days was not detected (Table 3.3).

Perennial Ryegrass

There were no differences in mean insect mortality, mean insect weight gain, and mean grass biomass between perennial ryegrass cultivars treated with fungicide and those not treated with fungicide (p > 0.05) (Table 3.4). Similarly, mean feeding damage at 15 days was not different between the two fungicide treatments (p > 0.05) (Table 3.4). However, there were effects of the fungicide treatment on mean feeding damage at 3 days ($\bar{Y}_{No fungicide} = 37.04\%$, $\bar{Y}_{Fungicide} = -28.47\%$, p < 0.001) and 12 days ($\bar{Y}_{No fungicide} = 8.60\%$, $\bar{Y}_{Fungicide} = -62.92\%$, p < 0.001) (Table 3.4).

In the no-fungicide treatment group, mean insect mortality was lower in perennial ryegrass cultivars with high *Epichloë* incidence, compared to those with low *Epichloë* incidence $(\bar{Y}_{\text{High } Epichloë} = 62\%, \bar{Y}_{\text{Low } Epichloë} = 82\%, p = 0.028)$ (Table 3.4 and Figure 3.6A). Nonetheless, significant differences between the high and low *Epichloë* levels within the perennial ryegrass group were not observed in terms of mean insect weight gain (Table 3.4 and Figure 3.6B), mean grass biomass, and mean feeding damage at 3, 12, and 15 days (p > 0.05) (Table 3.4).

Furthermore, within the no-fungicide treatment group, mean grass biomass ($\bar{Y}_{Furlong} = 0.89 \text{ g}, \bar{Y}_{Tee-Me-Up} = 1.28 \text{ g}, \bar{Y}_{Mensa} = 0.52 \text{ g}, \bar{Y}_{Sienna} = 2.07 \text{ g}, p = 0.008$), mean feeding damage at 3

days ($\bar{Y}_{\text{Furlong}} = 16.24\%$, $\bar{Y}_{\text{Tee-Me-Up}} = 136.28\%$, $\bar{Y}_{\text{Mensa}} = -19.98\%$, $\bar{Y}_{\text{Sienna}} = 69.79\%$, p = 0.002) and 12 days ($\bar{Y}_{\text{Furlong}} = -41.38\%$, $\bar{Y}_{\text{Tee-Me-Up}} = 69.38\%$, $\bar{Y}_{\text{Mensa}} = -66.72\%$, $\bar{Y}_{\text{Sienna}} = 73.14\%$, p = 0.005) (Table 3.4) were different among perennial ryegrass cultivars; however, mean insect mortality, mean insect weight gain, and mean feeding damage at 15 days were not different among the cultivars (p > 0.05) (Table 3.4).

Analysis of the effect of observed *Epichloë* infection and cultivar on the insect mortality, insect weight gain, and feeding damage at 15 days revealed no effect of observed *Epichloë* infection and cultivar. Additionally, linear regression indicated a strong effect of cultivar on grass biomass (P = 0.025) and feeding damage at 3 days (P = 0.004), whereas the effects of observed *Epichloë* infection were not significant (Table 3.3). Interestingly, the analysis showed that the effect of observed *Epichloë* infection on feeding damage at 12 days was statistically significant (P = 0.017) (Table 3.3 and Figure 3.7); however, the model's adjusted R-squared value was considerably low (0.38).

Discussion

The effects of *Epichloë*-mediated insect resistance on *N. pronuba* and grass performance were highly variable across two trials in both tall fescue and perennial ryegrass. The results from trial 1 did not show an association between *Epichloë* infection and grass cultivar across six different parameters (insect mortality, insect weight gain, grass biomass, feeding damage at 3, 12, and 15 days) in both grass species (Table 3.2, 3.3). This could be driven by an effect of seasonal variability or response to environmental conditions such as amount of sunlight and temperature on the grass-*Epichloë* association and alkaloid production (Belesky et al. 1988, Ball et al. 1995, Marks and Clay, 2007, Repussard et al. 2014). Marks and Clay (2007) showed that

the grass-*Epichloë* spp. association was the most beneficial to plant growth when the grass host was under full sun, but the symbiosis became commensal or adverse when the light resource was limited. The trial 1 was from September – October 2023 when the amount of sunlight was relatively higher than October – November 2023 when trial 2 was run. The high amount of sunlight the grasses were exposed to during the first trial could enhance the vegetative growth of all plants, regardless of grass species, *Epichloë* infection frequency, and cultivar, resulting in the observed comparable mean grass biomass and mean feeding damage at all three time points (Table 3.2). Furthermore, the mean insect mortality and mean insect weigh gain observed in trial 1 were not statistically different when compared among cultivars and expected *Epichloë* incidences (Table 3.2). This could be a result of relatively low concentrations of ergovaline – the alkaloid found to be toxic to some insect herbivores (Schardl et al. 2006) – in *Epichloë*-infected grasses during the summer when the trial 1 took place (Belesky et al. 1988, Repussard et al. 2014).

In Trial 2, the expected status of *Epichloë* infection in tall fescue (Table 3.4) did not affect the mean insect performance metrics, specifically mortality and weight gain. However, significant differences in mean feeding damage at 3 and 12 days were apparent when comparing expected levels of *Epichloë* infection, potentially influenced by cultivar effects for two primary reasons. First, the observed *Epichloë* infection levels, as determined by PCR analysis, exhibited no detectable variation among tall fescue cultivars (Table 3.1). Second, the linear regression analyses did not identify *Epichloë* infection level as an important factor for capturing the variation in mean feeding damage at 3 and 12 days (Table 3.3). These findings in tall fescue suggest that cultivar or plant genotype likely contributes to the observed variability in both insect and grass performance metrics.

Similarly, variations in mean grass biomass and feeding damage at 3 and 12 days were observed among perennial ryegrass cultivars, but not in comparisons involving Epichloë infection levels (Table 3.4). The only effect of *Epichloë* infection in perennial ryegrass was observed in terms of insect mortality, where mean insect mortality was higher in grasses with low Epichloë infection levels. The linear regression model assessing the effects of Epichloë infection level and cultivar on mean feeding damage on day 3 revealed an effect of cultivar on the feeding damage estimation, but not the *Epichloë* infection level (Table 3.3). Nonetheless, both Epichloë infection level and cultivar were not important factors for explaining variability in other metrics (Table 3.3). This suggests that *N. pronuba* survived better when feeding on perennial ryegrass with high Epichloë infection levels. However, it is important to note that the observed levels of Epichloë infection in perennial ryegrass cultivars expected to be high did not meet the 85% criterion - the observed values were 28% and 4% for 'Furlong' and 'Tee-Me-Up', respectively (Table 3.1). Moreover, this observed phenomenon was not supported by other metrics. Similar to the tall fescue case, variations in perennial ryegrass performance could be explained by cultivar-specific differences rather than *Epichloë* levels.

Previous studies have shown that plant genotype or inherent genetic differences between grass cultivars could be a more important determinant of insect resistance than *Epichloë* infection levels (Richmond et al. 2000, Popay and Gerard 2007, Richmond and Bigelow 2009). Specifically, variations in *Epichloë* infection seem to have a minor to no effect on the performance of caterpillar feeding on *Epichloë*-infected grasses (Williamson and Potier 1997, Richmond and Bigelow 2009, Baldauf et al. 2011, Miller et al. 2017). For instance, Richmond and Bigelow (2009) showed that *A. ipsilon* survival did not depend on *Epichloë* infection level but cultivar of grass host in a tall fescue study. A recent study by Miller et al. (2017) investigated the effects of two tall fescue cultivars harboring two different *Epichloë* strains (wild-type and AR601) on several invertebrates and found that the effects of *Epichloë* infection on survival and growth of *A. ipsilon* and *S. frugiperda* were not apparent, with similar and, in some cases, better larval growth and survival on the tall fescue cultivar infected with AR601.

The enhanced performance of caterpillars reared on Epichloë-infected grasses have been reported in the literature. Interestingly, Ball et al. (2006) found that S. frugiperda larval performance was enhanced on *Epichloë*-infected tall fescue. Similarly, Bultman and Bell (2003) showed that S. frugiperda developed faster and grew larger on Epichloë-infected tall fescue. These observations could be a result of ergot alkaloid's negative multitrophic impact on lepidopteran natural enemies, such as entomopathogenic viruses and nematodes (Kunkel et al. 2004, Richmond and Bigelow 2009, Bixby and Potter, 2010). For example, in a study by Bixby and Potter (2010), the endophytic alkaloids in grasses have a deterrent effect on A. ipsilon, resulting in a reduced consumption of grasses that were pre-sprayed with the entomopathogenic virus (Agrotis ipsilon nucleopolyhedrovirus; family Baculoviridae; AgipMNPV) and consequently, a decrease in virus-induced larvae mortality. Similarly, Richmond and Bigelow (2009) found that in a no-choice greenhouse experiment, A. ipsilon larvae feeding on perennial ryegrass or tall fescue infected with *Epichloë* were less susceptible to an entomopathogenic nematode (Steinernema carpocapsae Weiser) – a generalist parasite with a wide lepidopteran host range, than those feeding on uninfected counterparts. Kunkel et al. (2004) showed that, in a laboratory setting, the Epichloë-producing alkaloids could be toxic to a nematode's mutualistic bacterium, Xenorhabdus nematophila, which is responsible for a lethal bacterial septicemia in A. ipsilon. Accordingly, consumption of Epichloë-harboring grass by A. ipsilon larvae could be considered an acquisition of resistance against the entomopathogenic virus and nematode,

thereby decreasing *A. ipsilon* mortality (Kunkel et al. 2004, Richmond and Bigelow 2009, Bixby and Potter, 2010).

The lack of apparent effects of *Epichloë* infection could be because the no-choice assays performed in this study might not be able to capture the insect deterrence nature of peramine-producing *Epichloë*. Ball et al. (2006) discussed that *S. frugiperda* feeding on *Epichloë*-infected tall fescue could be influenced by the insect's hunger when confined to a chamber in no-choice assays. Moreover, the presence of the markers involved in alkaloid production pathways within the genome of a grass-*Epichloë* association does not necessarily indicate gene activity or the presence of other genes required to complete the alkaloid production (Berry et al. 2015, Vikuk et al. 2019). Without analytical techniques such as High-Performance Liquid Chromatography and MS, one cannot assume the production and storage of specific alkaloids in grass tillers (Young et al. 2014).

Half of the three-tiller samples (n = 480) were expected to have no *Epichloë* infection as they were grown from seeds that were treated with a systemic fungicide with propiconazole as an active ingredient; however, the fungicide did not completely eradicate the *Epichloë* in seeds – the treatment was 77.5% effective in trial 1 and 90% effective in trial 2 (data not shown). The observation was not in agreement with a previous fungicide treatment study by Latch and Christensen (1982) that indicated a complete eradication of *Epichloë* in perennial ryegrass seedlings grown from seeds treated with propiconazole or prochloraz at 0.5g.a.i /kg seed. However, an investigation by Leyronas et al. (2006) revealed that many fungicides including prochloraz failed to completely reduce *Epichloë* load in tall fescue and perennial ryegrass seeds. Unfortunately, the study did not include propiconazole. The reduced fungicide effectiveness in controlling *Epichloë* in grass seeds could be because, over time, fungicide resistance in *Epichloë* populations have been selected as systemic fungicides have been heavily used to control other fungal pathogens in seed crops (Chynoweth et al. 2012). These fungicides could have broad-spectrum effects and indirectly impact fitness of *Epichloë*. However, without the fungicide treatment, the *Epichloë* infection levels in most cultivars were still unexpectedly low in both trials (Table 3.1). A similar phenomenon was observed in a study by Richmond et al. (2000) in which the observed *Epichloë* infections in tiller samples of two perennial ryegrass cultivars marketed as *Epichloë*-enhanced cultivars were lower than expected (58% and 31%). This could be a result of unfavorable transportation and storage conditions to *Epichloë* viability in seeds, leading to a decreased rate of *Epichloë* colonization in seedlings (Rolston and Agee 2007).

In conclusion, this study demonstrated that *Epichloë* infection status in tall fescue did not affect *N. pronuba* mortality or weight gain, but did impact feeding damage on certain days. Variability in tall fescue cultivar insect damage metrics were more attributable to cultivar effects than *Epichloë* infection levels. In perennial ryegrass, *Epichloë* infection levels had an effect on insect mortality, with higher mortality observed in grasses with expected low *Epichloë* infection. However, similar to tall fescue cases, variability in perennial ryegrass insect damage metrics were primarily due to cultivar-specific differences rather than *Epichloë* levels. Previous studies and literature indicated that cultivar often played a more important role in determining insect resistance than *Epichloë* infection levels, which aligned with the findings of this study. Moreover, alkaloid gene analysis revealed that peramine was the most common alkaloid gene present in the infected plant population, but this did not correlate with observed insect performance metrics in the trials, suggesting that no-choice assays may not capture the full insect determence potential of peramine-producing *Epichloë*. An extended period of bioassays could be

essential to observe sub-lethal effects of alkaloids on *N. pronuba*. The unexpectedly low *Epichloë* infection levels in many cultivars across both trials emphasize the inconsistency and unreliability of *Epichloë* infection status in turfgrass seeds throughout the Oregon grass seed supply chain. These findings underscore the complex interactions between grass cultivars, *Epichloë* infection levels, and environmental conditions in determining insect resistance and grass performance metrics. Analytical tools such as HPLC and MS will be essential to characterize and quantify alkaloids in grass tillers and within insect bodies, enabling a comprehensive understanding of alkaloid production by grass-*Epichloë* associations and alkaloid uptake by insects. This deeper understanding is crucial for comprehending how *Epichloë*-mediated insect resistance functions against *N. pronuba*.

Table 3.1 Expected and observed Epichloë levels in tiller samples of both tall fescue and perennial ryegrass cultivars from both trials.

| Cultivar | Seed supplier | Expected <i>Epichloë</i> level | Observed level (%) | d <i>Epichloë</i>) ^c | |
|---------------------------|---------------------------|-----------------------------------|-----------------------|-------------------------------------|--|
| | | | Trial 1 | Trial 2 | |
| Tall fescue | | | | | |
| Sidewinder | Sidewinder AMPAC Seed Co. | | 53.33 | 20 | |
| Blacktail | il Columbia Seeds, LLC | | 20 | 28 | |
| Bronson | AMPAC Seed Co. | | 43.33 | 24 | |
| Goliath | AMPAC Seed Co. | Low ^a | 40 | 28 | |
| Perennial ryegrass | | | | | |
| Furlong | ng Lebanon Seaboard Corp. | | 53.33 | 28 | |
| Tee-Me-Up | Bailey Seed & Grain, LLC | High ^b | 10 | 4 | |
| Mensa Ledeboer Seed, LLC | | Low ^b | 10 | 12 | |
| Sienna Ledeboer Seed, LLC | | Low ^a | 40 | 0 | |

^a Information from breeders/sources

^b Determined from tiller samples collected from Chapter 2 PCR testing ^c Determined from tiller samples collected at the end of the present experiments using PCR testing

| Table 3.2 P-values for mean comparisons between fungicie | le treatments, expected <i>Epichloë</i> infection levels, and grass |
|--|---|
| cultivars for tall fescue and perennial ryegrass in trial 1. | |

| Comparisons | Insect | Insect weight | Grass | Feeding damage | Feeding damage | Feeding damage |
|-----------------------|---------------|---------------|-------------|----------------|----------------|----------------|
| | mortality (%) | gain (g) | biomass (g) | at 3 days (%) | at 12 days (%) | at 15 days (%) |
| Tall fescue | | | | | | |
| Fungicide treatment | >0.05 | >0.05 | >0.05 | 0.045* | >0.05 | >0.05 |
| <i>Epichloë</i> level | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| Cultivars | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| Perennial ryegrass | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| Fungicide treatment | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| <i>Epichloë</i> level | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| Cultivars | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |

* denote significant difference in means (P<0.05



Figure 3.1 Mean \pm SE feeding damage at 3 days in trial 1 (A) and trial 2 (B) of *N. pronuba* feeding on tall fescue grown from fungicide treated (F) or no fungicide treated (NF) seed.



Figure 3.2 Mean \pm SE insect mortality (A) and insect weight gain (B) of *N. pronuba* feeding on tall fescue cultivars with either high or low expected *Epichloë* level in trial 1 no-choice feeding experiment.
Table 3.3 LM output for insect mortality, insect weight gain, grass biomass, percentage of feeding damage at 3, 12, 15 days in trial 1.

| Trial | Factor | Insect mortality (%) | Insect weight gain (g) | Grass biomass (g) | Feeding damage at 3 days (%) | Feeding damage at 12 days (%) | Feeding damage at 15 days (%) |
|-------|--------------------|-------------------------|------------------------------|----------------------|------------------------------------|--|--|
| 1 | Tall fescue | | | | | | |
| | Epichloë infection | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| | Cultivar | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| | Perennial ryegrass | | | | | | |
| | Epichloë infection | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| | Cultivar | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| 2 | Tall fescue | | | | | | |
| | Epichloë infection | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| | Cultivar | 0.003637** | 0.01** | >0.05 | 0.0046** | >0.05 | >0.05 |
| | Perennial ryegrass | | | | | | |
| | Epichloë infection | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| | Cultivar | >0.05 | >0.05 | >0.05 | 0.0036** | >0.05 | >0.05 |

*, **, and *** denote significant difference in means (P < 0.05, P < 0.01, and P < 0.001, respectively).



Figure 3.3 Mean ± SE insect mortality (A, C) and insect weight gain (B, D) of *N. pronuba* feeding on the perennial ryegrass cultivars with either high or low expected *Epichloë* level (A, B) and different perennial ryegrass cultivars (C, D) in trial 1 no-choice feeding experiment.

| Comparison | Insect mortality (%) | Insect weight gain (g) | Grass biomass (g) | Feeding damage at 3 days (%) | Feeding damage at 12 days (%) | Feeding damage at 15 days (%) |
|-----------------------|-------------------------|---------------------------|----------------------|---------------------------------|----------------------------------|----------------------------------|
| Tall fescue | | | | | | |
| Fungicide treatment | >0.05 | >0.05 | >0.05 | 0.03093 * | >0.05 | >0.05 |
| <i>Epichloë</i> level | >0.05 | >0.05 | >0.05 | 0.03428 * | 0.0003199 *** | >0.05 |
| Cultivars | 0.01 * | 0.01 * | >0.05 | 0.0007512 *** | 0.006706 ** | >0.05 |
| Perennial ryegrass | | | | | | |
| Fungicide treatment | >0.05 | >0.05 | >0.05 | 0.00007051 *** | 0.0005856 *** | >0.05 |
| <i>Epichloë</i> level | 0.02819 * | >0.05 | >0.05 | >0.05 | >0.05 | >0.05 |
| Cultivars | >0.05 | >0.05 | 0.008289 ** | 0.002374 ** | 0.005169 ** | >0.05 |

Table 3.4 *P*-values for mean comparisons between fungicide treatments, expected *Epichloë* infection levels, and grass cultivars for tall fescue and perennial ryegrass in trial 2.

*, **, and *** denote significant difference in means (P < 0.05, P < 0.01, and P < 0.001, respectively)



Figure 3.4 Mean ± SE feeding damage at 3 days (A) and 12 days (B) of *N. pronuba* feeding on tall fescue cultivars with either high or low expected *Epichloë* level in trial 2 no-choice feeding experiment.



Figure 3.5 Mean \pm SE insect mortality (A, C) and insect weight gain (B, D) of *N. pronuba* feeding on the tall fescue cultivars with either high or low expected *Epichloë* level (A, B) and different tall fescue cultivars (C, D) in trial 2 no-choice feeding experiment.



Figure 3.6 Mean \pm SE insect mortality (A) and insect weight gain (B) of *N. pronuba* feeding on perennial ryegrass cultivars with either high or low expected *Epichloë* level in trial 2 no-choice feeding experiment.



Figure 3.7 Linear relationships between observed *Epichloë* level and feeding damage at 3 days of *N. pronuba* feeding on the perennial ryegrass cultivars.

References

- Bacetty AA, Snook ME, Glenn AE et al. 2009. Toxicity of endophyte-infected tall fescue alkaloids and grass metabolites on *Pratylenchus scribneri*. Phytopathology 99(12):1336-1345. <u>https://doi.org/10.1094/phyto-99-12-1336</u>.
- Bacon C, Porter J, Robbins J et al. 1977. *Epichloe typhina* from toxic tall fescue grasses. Appl. Environ. Microbiol. 34(5):576-581.
- Baldauf MW, Mace WJ, Richmond DS. 2011. Endophyte-mediated resistance to black cutworm as a function of plant cultivar and endophyte strain in tall fescue. Environ. Entomol. 40(3):639-647. <u>https://doi.org/10.1603/EN09227</u>.
- Ball OJ-P, Coudron TA, Tapper BA et al. 2006. Importance of host plant species, *Neotyphodium* endophyte isolate, and alkaloids on feeding by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae. J. Econ. Entomol. 99(4):1462-1473. <u>https://doi.org/10.1603/0022-0493-99.4.1462</u>.
- Ball OJ, Prestidge RA, Sprosen JM. 1995. Interrelationships between Acremonium lolii, peramine, and lolitrem b in perennial ryegrass. Appl. Environ. Microbiol. 61(4):1527-1533. <u>https://doi.org/10.1128/aem.61.4.1527-1533.1995</u>.
- Bastias DA, Martínez-Ghersa MA, Ballaré CL et al. 2017. *Epichloë* fungal endophytes and plant defenses: not just alkaloids. Trends Plant Sci. 22(11):939-948. https://doi.org/10.1016/j.tplants.2017.08.005.
- Belesky D, Stuedemann J, Plattner R et al. 1988. Ergopeptine alkaloids in grazed tall fescue. Agron. J. 80(2):209-212. <u>https://doi.org/10.2134/agronj1988.00021962008000020014x</u>.
- Berry D, Takach JE, Schardl CL et al. 2015. Disparate independent genetic events disrupt the secondary metabolism gene perA in certain symbiotic *Epichloë* species. Appl. Environ. Microbiol. 81(8):2797-2807. <u>https://doi.org/10.1128/AEM.03721-14</u>.
- Bixby AJ, Potter DA. 2010. Influence of endophyte (*Neotyphodium lolii*) infection of perennial ryegrass on susceptibility of the black cutworm (Lepidoptera: Noctuidae) to a baculovirus. Biol. Control 54(2):141-146. https://doi.org/10.1016/j.biocontrol.2010.04.003.
- Breen JP. 1992. Temperature and seasonal effects on expression of *Acremonium* endophyteenhanced resistance to *Schizaphis graminum* (Homoptera: Aphididae). Environ. Entomol. 21(1):68-74. <u>https://doi.org/10.1093/ee/21.1.68</u>.
- Bultman TL, Bell GD. 2003. Interaction between fungal endophytes and environmental stressors influences plant resistance to insects. Oikos 103(1):182-190. https://doi.org/10.1034/j.1600-0706.2003.11574.x.
- Bultman TL, Rodstrom JL, Radabaugh KR et al. 2009. Influence of genetic variation in the fungal endophyte of a grass on an herbivore and its parasitoid. Entomol. Exp. Appl. 130(2):173-180. <u>https://doi.org/https://doi.org/10.1111/j.1570-7458.2008.00802.x</u>.

- Caradus JR, Card SD, Finch SC et al. 2022. Ergot alkaloids in New Zealand pastures and their impact. N. Z. J. Agric. Res. 65(1):1-41. <u>https://doi.org/10.1080/00288233.2020.1785514</u>.
- Chynoweth R, Rolston M, Kelly M et al. 2012. Control of blind seed disease (*Gloeotinia temulenta*) in perennial ryegrass (*Lolium perenne*) seed crops and implications for endophyte transmission. Agron. N. Z. 42(1):141-148.
- Clarke BB, White Jr JF, Hurley RH et al. 2006. Endophyte-mediated suppression of dollar spot disease in fine fescues. Plant Dis. 90(8):994-998. https://doi.org/10.1094/pd-90-0994.
- Dellaporta SL, Wood J, Hicks JB. 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1:19-21.
- Difonzo C, Russell H. 2010. *Noctua pronuba* (Lepidoptera: Noctuidae): An outbreak in emails. J. Integr. Pest Manag. 1(1):B1-B6. <u>https://doi.org/10.1603/ipm10005</u>.
- Fletcher L, Harvey I. 1981. An association of a *Lolium* endophyte with ryegrass staggers. N. Z. Vet. J. 29(10):185-186. https://doi.org/10.1080/00480169.1981.34839.
- Fox J, Weisberg S. 2019. An R companion to applied regression, third edition. Thousand Oaks, California: Sage publications. https://socialsciences.mcmaster.ca/jfox/Books/Companion/.
- Fuchs B, Krischke M, Mueller MJ et al. 2017. Herbivore-specific induction of defence metabolites in a grass–endophyte association. Funct. Ecol. 31(2):318-324. https://doi.org/https://doi.org/10.1111/1365-2435.12755.
- Green J, Dreves AJ, McDonald BW et al. 2016. Winter cutworm: a new pest threat in Oregon. Oregon State University, Extension Service Corvallis, OR, USA. <u>https://extension.oregonstate.edu/sites/default/files/documents/em9139.pdf</u>.
- Hervet VAD, Laird RA, Floate KD. 2016. A review of the mcmorran diet for rearing lepidoptera species with addition of a further 39 species. J. Insect Sci. 16(1). <u>https://doi.org/10.1093/jisesa/iev151</u>.
- Jensen J, Popay A. 2004. Perennial ryegrass infected with AR37 endophyte reduces survival of porina larvae. N. Z. Plant Prot. 57:323-328. <u>https://doi.org/10.30843/nzpp.2004.57.6930</u>.
- Kaur N, Rivedal H, Intasin J et al. 2024. Response of sod webworm *Chrysoteuchia topiaria* Zeller (Lepidoptera: Crambidae) to endophyte infection and mycotoxin profiles of coolseason turfgrass species grown for seed in Oregon. Crop Forage Turfgrass Manage. <u>https://doi.org/https://doi.org/10.1002/cft2.20291</u>.
- Kunkel BA, Grewal PS, Quigley MF. 2004. A mechanism of acquired resistance against an entomopathogenic nematode by *Agrotis ipsilon* feeding on perennial ryegrass harboring a fungal endophyte. Biol. Control. 29:100-108. <u>https://doi.org/10.1016/s1049-9644(03)00119-1</u>.
- Latch G, Christensen M. 1982. Ryegrass endophyte, incidence, and control. N. Z. J. Agric. Res. 25:443-448. <u>https://doi.org/10.1080/00288233.1982.10417910</u>.

- Leuchtmann A, Bacon CW, Schardl CL et al. 2014. Nomenclatural realignment of *Neotyphodium* species with genus *Epichloë*. Mycologia 106(2):202-215. <u>https://doi.org/10.3852/13-251</u>.
- Leyronas C, Mériaux B, Raynal G. 2006. Chemical control of *Neotyphodium* spp. endophytes in perennial ryegrass and tall fescue seeds. Crop Sci. 46(1):98-104. https://doi.org/10.2135/cropsci2005.0135.
- Marks S, Clay K. 2007. Low resource availability differentially affects the growth of host grasses infected by fungal endophytes. Int J Plant Sci. 168(9):1269-1277. https://doi.org/10.1086/521834.
- Meister B, Krauss J, Härri SA et al. 2006. Fungal endosymbionts affect aphid population size by reduction of adult life span and fecundity. Basic Appl. Ecol. 7(3):244-252. https://doi.org/10.1016/j.baae.2005.06.002.
- Miller DM, Redmond CT, Flythe MD et al. 2017. Evaluation of 'Jackal'AR601 (Avanex) and Kentucky-31 endophytic tall fescues for suppressing types of invertebrates that contribute to bird strike hazard at airports. Crop Forage Turfgrass Manage. 3(1):1-11. https://doi.org/10.2134/cftm2017.03.0023.
- Oregon Department of Agriculture. 2023. Oregon releases updated top 20 agricultural commodities. <u>https://content.govdelivery.com/accounts/ORODA/bulletins/355f2ac</u>.
- Popay A, Gerard P. 2007. Cultivar and endophyte effects on a root aphid *Aploneura lentisci* in perennial ryegrass. N. Z. Plant Prot. 60:223-227. https://doi.org/10.30843/nzpp.2007.60.4624.
- R Core Team. 2024. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.
- Realini FM, Escobedo VM, Ueno AC et al. 2024. Anti-herbivory defences delivered by *Epichloë* fungal endophytes: a quantitative review of alkaloid concentration variation among hosts and plant parts. Ann. Bot. 133(4):509-520. <u>https://doi.org/10.1093/aob/mcae014</u>.
- Repussard C, Zbib N, Tardieu D et al. 2014. Endophyte infection of tall fescue and the impact of climatic factors on ergovaline concentrations in field crops cultivated in southern France. J. Agric. Food Chem. 62(39):9609-9614. <u>https://doi.org/10.1021/jf503015m</u>.
- Richmond D, Bigelow C. 2009. Variation in endophyte–plant associations influence black cutworm (Lepidoptera: Noctuidae) performance and susceptibility to the parasitic nematode *Steinernema carpocapsae*. Environ. Entomol. 38(4):996-1004. https://doi.org/10.1603/022.038.0406.
- Richmond DS, Niemczyk HD, Shetlar DJ. 2000. Overseeding endophytic perennial ryegrass into stands of Kentucky bluegrass to manage bluegrass billbug (Coleoptera: Curculionidae). J. Econ. Entomol. 93(6):1662-1668. https://doi.org/10.1603/0022-0493-93.6.1662.

- Rolston M, Agee C. 2007. Delivering quality seed to specification-the USA and NZ novel endophyte experience. NZGA Res. Pract. Ser. 13:229-231. http://dx.doi.org/10.33584/rps.13.2006.3065.
- Schardl CL, Leuchtmann A, Spiering MJ. 2004. Symbioses of grasses with seedborne fungal endophytes. Annu. Rev. Plant Biol. 55:315-340. https://doi.org/10.1146/annurev.arplant.55.031903.141735.
- Schardl CL, Panaccione DG, Tudzynski P. 2006. Chapter 2 Ergot Alkaloids Biology and Molecular Biology'. In: Cordell GA ed.: Academic Press. 45-86.
- Schardl CL, Young CA, Faulkner JR et al. 2012. Chemotypic diversity of *Epichloae*, fungal symbionts of grasses. Fungal Ecol. 5(3):331-344. https://doi.org/https://doi.org/10.1016/j.funeco.2011.04.005.
- Schardl CL, Young CA, Pan J et al. 2013. Currencies of mutualisms: sources of alkaloid genes in vertically transmitted *Epichloae*. Toxins 5(6):1064-1088. <u>https://doi.org/10.3390/toxins5061064</u>.
- Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 9(7):671-675. https://doi.org/10.1038/nmeth.2089.
- Siegel M, Latch G, Bush L et al. 1990. Fungal endophyte-infected grasses: alkaloid accumulation and aphid response. J. Chem. Ecol. 16:3301-3315. https://doi.org/10.1007/BF00982100.
- Song M, Li X, Saikkonen K et al. 2015. An asexual *Epichloë* endophyte enhances waterlogging tolerance of *Hordeum brevisubulatum*. Fungal Ecol. 13:44-52. https://doi.org/https://doi.org/10.1016/j.funeco.2014.07.004.
- Takach JE, Mittal S, Swoboda GA et al. 2012. Genotypic and chemotypic diversity of *Neotyphodium* endophytes in tall fescue from Greece. Appl. Environ. Microbiol. 78(16):5501-5510. <u>https://doi.org/10.1128/aem.01084-12</u>.
- USDA NASS. 2024. Oregon Grass Seed Forecast. https://www.nass.usda.gov/Statistics_by_State/Washington/Publications/Current_News_ Release/2024/ORGRASS_2024.pdf.
- Vikuk V, Young CA, Lee ST et al. 2019. Infection rates and alkaloid patterns of different grass species with systemic *Epichloë* endophytes. Appl. Environ. Microbiol. 85(17):e00465-00419. https://doi.org/10.1128%2FAEM.00465-19.
- Williamson CR, Potier DA. 1997. Turfgrass species and endophyte effects on survival, development, and feeding preference of black cutworms (Lepidoptera: Noctuidae). J. Econ. Entomol. 90(5):1290-1299. <u>https://doi.org/10.1093/jee/90.5.1290</u>.
- Young CA, Charlton ND, Takach JE et al. 2014. Characterization of *Epichloë coenophiala* within the US: are all tall fescue endophytes created equal? Front. Chem. 2:95. https://doi.org/10.3389%2Ffchem.2014.00095.

- Young W, III, Silberstein T. 2012. The Oregon grass seed industry'. In: C. A. Young GEARLMJRSCLS ed.: Ardmore Samuel Roberts Noble Foundation. 49-52.
- Zhang W, Card SD, Mace WJ et al. 2017. Defining the pathways of symbiotic *Epichloë* colonization in grass embryos with confocal microscopy. Mycologia 109(1):153-161. https://doi.org/10.1080/00275514.2016.1277469.

CHAPTER 4

General Conclusions

Julaluck Intasin

Epichloë are fungal endophytes infecting cool-season grasses and provide several benefits to the grass hosts through insect pest resistance and abiotic stress tolerances. Insect pest resistance is associated with Epichloë's ability to produce alkaloids which can be toxic or deterrent to some insects. The benefits of grass-Epichloë associations on turf performance could improve the economics of Oregon's grass seed industry. However, the Epichloë status and persistence in turf stands are not always the highest priority in grass cultivar breeding programs or a considering component for turfgrass quality assessed in NTEP trials. This research investigated the prevalence of *Epichloë* in Oregon turfgrass production and explores it's potential for N. pronuba control. The first objective was to determine Epichloë infection rate, Epichloë genetic variation in terms of alkaloid gene loci, and a correlation between Epichloë incidence level and turfgrass quality in commercially available cultivars from the 2016 NTEP trial. This study provides a comprehensive analysis of Epichloë infection levels in perennial ryegrass cultivars commercially available in Oregon, highlighting their significant variability ranging from 0 to 100%. This variability is attributed to inherent genetic diversity among grass hosts and a lack of seed certification practices ensuring *Epichloë* viability in grass seeds. The study identified four predicted alkaloid profiles in perennial ryegrass cultivars, with minor variation in the profile frequency. Over five years, fluctuations in Epichloë incidence were noted among cultivars. Notably, this study suggests that maintaining at least 20% infected plants could enhance turf quality, though further validation with objective methodologies is recommended.

The second objective aimed to assess the susceptibility of tall fescue and perennial ryegrass cultivars with varying *Epichloë* levels to *N. pronuba* feeding in a repeated no-choice experiment. In tall fescue, pre-trial *Epichloë* status and post-trial infection rate did not affect mean insect mortality, mean insect weight gain, mean grass biomass, and mean feeding damage

at the three time points in this study for both trials. Similarly, in perennial ryegrass, variability in some insect damage metrics were predominantly cultivar-specific rather than driven by *Epichloë* levels. These results align with existing literature highlighting the dominant role of plant genotype in determining insect resistance. Additionally, the lack of consistency in *Epichloë* infection level in this study underscores the reliability of *Epichloë* infection status in turfgrass seeds across Oregon's supply chain.

These findings contribute to understanding the utility of *Epichloë* endophytes in enhancing insect resistance and stress tolerance in both seed and turfgrass production systems in the US. Moving forward, employing advanced analytical tools such as HPLC and MS or developing quantitative molecular methods (quantitative PCR) will be crucial to accurately characterize and quantify alkaloids within grass tillers and insect bodies. This approach is essential for better understanding how *Epichloë*-mediated insect resistance functions against lepidopteran pests, such as *N. pronuba*. Regular monitoring of *Epichloë* infection rates throughout the seed supply chain and turfgrass establishment is recommended to accurately determine *Epichloë* presence in a turfgrass system.