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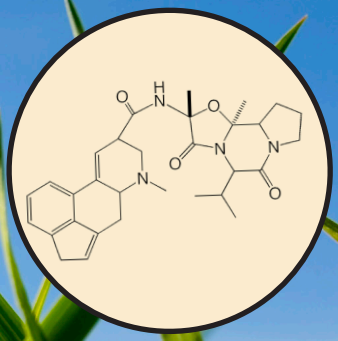

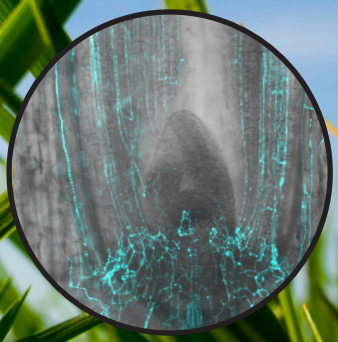

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4.2 Influence of the *Neotyphodium* - Tall fescue symbiosis on belowground processes

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ABSTRACT

Much of the work to date on the relationships between cool season grasses and *Neotyphodium* fungal endophytes has focused on the physiological, biochemical, and genetic ramifications of the host-fungus relationship and the subsequent influence these effects have on ruminant nutrition, plant adaptation to environmental stresses, and aboveground ecological processes. Relatively little attention has been paid to effects on belowground parameters. In this paper, we review the research evaluating the impact of one endophyte-grass association, the *Neotyphodium* – tall fescue symbiosis, on underground ecological and biogeochemical processes. We also present some preliminary data showing that the quantity and nature of tall fescue root exudates are influenced by the plant cultivar and fungal genotype. This body of work clearly indicates that effects of the *Neotyphodium*-tall fescue symbiosis extend to belowground processes; however, additional research is needed to understand the mechanisms driving many of the observed root and soil endophyte effects.

KEYWORDS: fungal endophyte, *Neotyphodium*, rhizodeposition, rhizosphere, soil microbes, tall fescue

INTRODUCTION

Research on the *Neotyphodium coenophialum* - tall fescue (*Lolium arundinaceum*) symbiosis has historically focused on the toxic alkaloids present in the aboveground portions of endophyte-infected (E+) tall fescue and the effects these compounds have on aboveground fauna (Bacon 1995), perhaps in part because *Neotyphodium* and its associated alkaloids are rarely, if ever, observed in belowground portions of infected plants. As a consequence of this aboveground focus, much less is known about the influence of these fungal endophytes on belowground plant or soil processes. Yet research indicates that endophyte presence can alter a multitude of belowground parameters, such as root exudate production, soil fauna, microbial activity and community structure, organic matter decomposition, and resulting nutrient pools (Table 1).

There are several possible mechanisms by which aboveground endophytes may alter belowground parameters. Toxic alkaloids (or other allelochemicals) produced by the endophyte and contained in aboveground plant material may leach into the soil environment and have deleterious effects on soil microbial and faunal communities. Support for this mechanism comes from a study by Antunes et al. (2008) where aqueous extracts from shoots of E+ tall fescue material were shown to reduce mycorrhizal spore germination 10% more than extracts of endophyte-free (E-) controls. In addition, Siegrist et al. (2010) found that measurable quantities of ergot and loline alkaloids persisted in decaying E+ plant litter for ~50 days in the field, and Franzluebbers and Hill (2005) reported detectable quantities of ergot alkaloids in surface soils supporting E+ tall fescue stands in Georgia. It is also possible that endophyte presence, by altering aboveground plant production, community composition, micro-environmental conditions, and/or levels of aboveground herbivory, may indirectly alter belowground parameters (Lemons et al., 2005; Omacini et al., 2005). And, finally, endophyte presence may directly alter allocation to roots, which could influence root biomass and/or the quantity and nature of root exudates – all of which could impact belowground food webs and nutrient cycling. These possible mechanisms are not mutually exclusive and may be operating in concert to generate the various observed effects.

For the purpose of this paper, we will focus on the potential direct effects of endophyte presence on tall fescue root exudation. Roots can release anywhere from 10 to 250 mg C/g root produced (Newman 1985), and these rhizodeposits can greatly influence the chemical, physical and biological properties of the surrounding soil structure (Pirha et al., 1999; Hamilton and Frank, 2001; Innes et al., 2004; Batten et al., 2006; Broekling et al., 2008). The composition and quantity of rhizodeposits are dictated by plant species and by soil biotic and abiotic characteristics (Prithiviraj et al., 2007). Previous work by Malinowski and colleagues has demonstrated that endophyte infection increases tall fescue root exudation of phenolic-like compounds, which may help the plant obtain phosphorus, especially under low phosphorus conditions, and protect the plant from metal toxicity

Table 1. Evidence of *Neotyphodium* – tall fescue symbiotic effects on belowground communities and processes.

Belowground Parameter	Endophyte Effect	Supporting Work
Tall fescue root exudation & nutrient uptake	+	Malinowski and Belesky, 1999b; Van Hecke et al., 2005
	+/0	Malinowski and Belesky, 1999a; Malinowski et al., 1998a, b, 2004
	+/-	Malinowski et al., 2000; Rahman and Saiga, 2005
Root symbioses (mycorrhizae)	-	Antunes et al., 2008; Chu-Chou et al., 1992; Guo et al., 1992; Mack and Rudgers, 2008
	0	Chen et al., 2007
Root pathogens (nematodes)	-	Bacetty et al., 2009a, b; Elmi et. al., 2000; Kimmons et al., 1990; Timper et al., 2005; West et al, 1988
	0	Kimmons et al, 1990
Soil microbial community	1	Rudgers and Orr, 2009 iqbal et al., 2012
	+/-	Franzluebbers et al., 1999; Jenkins et al., 2006
	0	Van Hecke et al., 2005
	-	Buyer et al., 2010 Buyer et al., 2011
Soil microbial activity & decomposition rates	+	Van Hecke et al., 2005
	0	Franzluebbers 2006; Lemons et al., 2005 iqbal et al., 2012
	-	Franzluebbers and Hill 2005; Buyer et al., 2011
Soil nutrient pools	+	Franzluebbers et al., 1999; Franzluebbers and Stuedemann 2002; iqbal et al., 2012
	+/0	Franzluebbers and Hill 2005; Franzluebbers 2006; Handayani et at., 2011
Soil fauna: Earthworms	+	Humphries et al., 2001
	0	Davidson and Potter, 1995
Other invertebrates	+/0/-	Rudgers and Clay, 2007
	0	Davidson and Potter, 1995

(+) increase, (-) decrease, (0) no change, (1) a change occurred but unclear whether increase or decrease

(e.g., aluminum, iron, and copper) (Malinowski et al., 1998a, b; Malinowski and Belesky, 1999a, b; Malinowski et al., 2004). Van Hecke et al. (2005) found E+ tall fescue root exudates had higher total carbon and carbohydrate content than E- plants; however, contrary to expectations, these exudates appeared to stimulate microbial activity and caused no apparent change in the microbial community structure. Bacetty et al. (2009a) reported that methanol extracts of E+ tall fescue roots affected the chemotaxis activities of nematodes, but this effect was not consistently observed across the experiment. In many of these experiments, endophyte effects on root exudates and nutrient uptake have been shown to be environment and genotype (for both plant and fungus) dependent (Rahman and Saiga, 2005; Malinowski and Belesky, 2006).

To date, very little is known about the specific compounds in root exudates that are altered by endophyte infection. Bertin et al. (2007) identified *m*-tyrosine in the root exudates of fine leaf fescues

(*Festuca rubra)*, demonstrated its broad phyto-toxicity, and hypothesized that this root exudate likely contributes to the improved persistence observed in these fescue cultivars. Unfortunately, this study did not report the endophyte status of the plant material utilized, although it is known that this species is capable of forming symbioses with fungal endophytes similar to *N. coenophialum*. Bacetty et al. (2009b) also identified several polyphenols in whole root extracts from tall fescue (chlorogenic acid, 3,5-dicaffeoylquinic acids, caffeic acid, and two unidentifiable compounds); however, the presence and quantity of these compounds did not depend on endophyte status of the plant, despite the fact that E+ root extracts from the same experiment were shown to be nematistatic. Given the widespread and sometimes contradictory effects of endophye symbiosis on belowground processes (Table 1), it seems clear that a better understanding of the mechanisms involved, including changes in the quantity and composition of root exudates, and the spatio-temporal conditionality of these responses is needed.

Neotyphodium - Tall Fescue Root Exudate Study

As a first step in evaluating the influence of the endophyte -tall fescue symbiosis on root exudation, we conducted preliminary studies in which wild-type endophyte infected (E+), endophyte free (E-) Kentucky 31 (KY31) tall fescue and a novel endophyte infected (AR 542; NE), experimental tall fescue cultivar derived from KY31 (KY9301) were each grown in pure culture conditions to determine the basal composition and quantities of root exudates. Approximately 100 surface sterilized tall fescue seed of each endophyte status (E+, E- and NE) were placed in sterilized Growtek™ culture vessels (Krackeler Scientific, Inc, Albany, NY) containing 100 ml of liquid minimal nutrient media (MNM) which were placed on a rotary shaker (Barnstead International, Dubuque, Iowa) set to 90 rpm under cool white fluorescent light (45 mmol m^{-2} s^{-1}; 16hrs light/8hrs dark) and grown for 14 days at 25 ± 5°C. Each treatment was reproduced in triplicate and placed in a randomized block arrangement. Culture vessels containing only MNM with no plants were used as a control for each treatment. Verification of endophyte infection status was performed at the termination of the experiment (14 days post germination) using the Phytoscreen™ greenhouse grow-out tiller endophyte detection kit (Agrinostics Ltd. Co., Watkinsville, GA).
At the termination of the experiment, portions of the un-fractionated ("whole") growth solutions containing exudates were retained and used to analyze for total phenolics. The remaining solution was then filtered using a 0.45mm nylon syringe filter, concentrated via lyophilization, weighed and the dry powder stored with desiccant at -20°C for later use in bioassays and analytical analysis as described below.

As a relative estimate of differences in root exudate composition among the cultivar-endophyte combinations, total phenolic content was assessed in the whole fraction

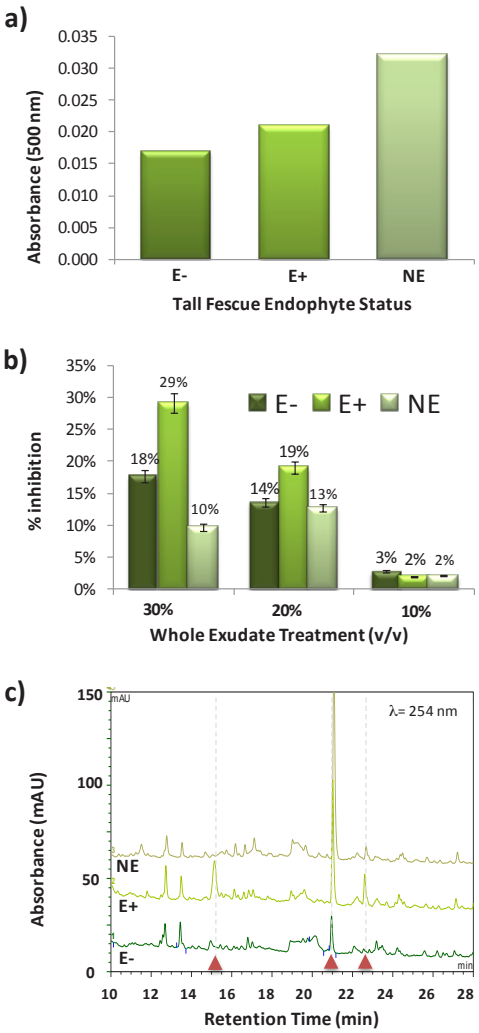

Figure 1: Characteristics of whole root exudates collected from the pure-culture growth media of endophyte-free (E-), wild-type endophyte-infected (E+), and novel endophyte-infected (NE) – tall fescue combinations. (a) Colorimetric assay of phenolic content; (b) bioactivity assay (% inhibition of S. meliloti); and (c) chromatograms showing the presence of unique peaks (i.e. chemical components) and variable peak intensities (Δ).

collected on day 14 from each of the replicates using a modification of the spectrophotometric method established by Arnow (Arnow 1937a, b), where greater absorbance readings indicate the presence of larger quantities of phenolic compounds. We found that compared to E-, the E+ and NE infected tall fescue combinations produced 19 and 47% greater amounts of total phenolic compounds, respectively (Figure 1a). This result lends credence to the hypothesis that the endophyte alters root exudate composition (Malinowski et al., 1998a; Bacetty et al., 2009b).

To evaluate if the altered exudate composition had an influence on microbial growth, the bioactivity of the exudates was assessed, via the broth micro-dilution antimicrobial susceptibility test outlined by the National Committee for Clinical Laboratory Standards (NCCLS) (Jones et.al., 1985), using the reconstituted whole root exudates and bacterial cultures of *Sinorhizobium meliloti* as the indicator organism (Figure 1b). *Sinorhizobium meliloti* was chosen because it represents a key symbiotic soil microorganism forming associations with many plant species often grown in close association with tall fescue (e.g., clover), in which case, monitoring its response to tall fescue root exudates could possibly indicate indirect effects on plant species interactions. Cultures were grown to an optical density (O.D.) of 0.02 (λ=600nm) and dispensed into 96-well, sterile, flat-bottomed microtiter plates (Nalge Nunc International, Roskilde, Denmark) containing increasing concentrations (10-30% (v/v)) of whole exudate fractions to a final volume of 200 ml. To account for possible dilution and plant nutrient media interferences, control wells contained 200 ml of bacteria alone with freeze-dried and reconstituted nutrient media from the control (i.e. no-plants) Growtech™ vessels. Plates were covered and the absorbance (λ=600 nm) in each well measured at the beginning and after 24 hours of incubation using a Victro^2 microtiter plate reader (Perkin-Elmer Shelton, CT). Each assay was performed twice with 3 replicates. Net bacterial growth after 24hrs was calculated by subtracting the 0 hr reading from the 24 hr reading, and then used to calculate the % inhibition using the following formula ([(control – exudate treated)/ control] x100). Using this methodology, preliminary bioassays indicated that whole root exudates produced by the NE-tall fescue cultivar combination were less inhibitory than the exudates produced by the E- and E+ KY31 pairs (Figure 1b), despite NE producing more total phenolic compounds (Figure 1a), and overall, E+ KY31 whole root exudates were more inhibitory to this microbe than the E- or NE. While exudate inhibition of other bacterial and fungal species has yet to be assessed, these results support the hypothesis that the composition of root exudates and their effectiveness at mediating microbial activity is influenced by the endophyte status of the plant.

To start identifying the components of the root exudates responsible for the observed differences in bioactivity, we chromatographically separated compounds within the whole root exudate fraction of the various endophyte-tall fescue cultivar combinations. The whole root exudate samples were first partitioned into polar and non-polar fractions using a methanol- hexane fractionation method. The non-polar fraction (hexane extractable) was separated and stored at -20^{o}C until future analysis. The polar fraction (methanol extractable) was concentrated using dry filtered air and the powder remaining after drying reconstituted in methanol and chromatographed by methanol:water gradient elution on a 25 cm x 4.6 mm reverse phase, C_{18} column (Acclaim 120; Dionex Sunnyvale, Ca) using a Dionex Ultimate 3000 chromatography system with a photodiode array variable UV-Vis detector (PDA-3000). Preliminary chromatograms of the whole root exudates from the E-, E+, and NE tall fescue plants used in the total phenolic (Figure 1a) and bacterial bioassays (Figure 1b) are shown in Figure 1c. We observed a unique peak at ~15 min in the E+ chromatogram and a shared unique peak in the E+ and NE chromatograms at ~22.5 min, with that of the E+ being more intense then NE. This additional evidence strongly suggests that the presence of the shoot-specific endophyte is indeed altering the composition of root exudates, that this alteration influences microbial activity, and that this behavior may be attributable to a specific chemical component (15 min peak), mixture of components (15, 21 and 22.5 min peaks) or the exudation of larger amounts of certain components (21 min peak) in the root exudates. Future work will chromatographically isolate portions of these exudates and further characterize their bioactivity and chemical nature.

CONCLUSIONS

The fungal endophyte, *Neotyphodium coenophialum*, despite primarily occurring in aboveground plant material, is clearly capable of altering belowground plant and soil processes. Endophyte presence appears to directly alter the quantity and quality of tall fescue root exudates which may in turn affect soil microbial and faunal communities and ultimately rates of nutrient cycling and storage. These effects are likely sensitive to tall fescue and fungal endophyte genotypes and their interaction with surrounding environmental conditions. Additional work exploring the mechanisms driving the observed belowground endophyte effects, the influence of tall fescue cultivar and fungal genotypes on exudate composition and quantity, and genotype by environment interactions is currently under way.

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