MYCORRHIZAS UNDER ARCTIC SHRUB EXPANSION: EFFECTS OF TEMPERATURE, NUTRIENT LIMITATION, AND HOST SPECIES ON SYMBIOTIC ROOT PROCESSES

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A Dissertation

Submitted in Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

in Biology

Northern Arizona University

April 2021

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ABSTRACT

MYCORRHIZAS UNDER ARCTIC SHRUB EXPANSION: EFFECTS OF TEMPERATURE, NUTRIENT LIMITATION, AND HOST SPECIES ON SYMBIOTIC ROOT PROCESSES

HALEY DUNLEAVY

In the nutrient limited Arctic tundra, mycorrhizae greatly influence nutrient cycling, providing up to 86% of nitrogen (N) to their host plants. As the Arctic warms, both nutrient availability and mycorrhizal host shrub abundance are increasing in tundra ecosystems. The resulting changes in mycorrhizal community and function are likely to affect not only nutrient, but also carbon (C) dynamics. In this dissertation, I studied how mycorrhizal-associated nutrient cycling may change with future warming, increased nutrient availability, and shifts in host plant abundance by pairing measurements of degradative extracellular enzymes on mycorrhizal roots with 1) mycorrhizal fungal identity, 2) aboveground plant abundance and height, and 3) another symbiotic root process occurring on the same shrub—N fixation. In chapter 2, I tested the effects of long-term experimental warming and fertilization on ectomycorrhizal fungal community and root enzyme activity. Warming tended to increase enzyme activity while fertilization decreased activity. Though these responses were partially explained by changes in EcM fungal community, our findings also suggest changes in enzyme activity were taxon-specific. In chapter 3, I further tested how nutrient limitation affected EcM as well as ericaceous shrub abundance and root enzyme activity, measuring non-linear responses across a long-term experimental soil fertility gradient. I found evidence of the co-expansion of ericaceous shrubs along with deciduous EcM shrubs and support for the potentially increased relative importance of EcM root enzymes compared to those of ericaceous roots in the degradation soil organic matter a future tundra. In chapter 4, I explored drivers of root enzyme activity and N fixation on Siberian alder, a

deciduous EcM shrub found in boreal and Arctic Alaska. Across a latitudinal gradient, root enzyme activity and N fixation were correlated. Furthermore, the direction of this correlation appears to depend on relative availability of soil N and phosphorus. Overall, mycorrhizal root enzyme activity is highly responsive to changes in temperature, nutrient availability, and host species. However, these responses are nuanced and the resulting changes in tundra nutrient cycling will depend on mycorrhizal fungal identity, shrub species, the magnitude of heightened nutrient availability, interactions with other symbiotic partners to shrubs, and, most commonly, the type of enzymes present.

ACKNOWLEDGEMENTS

I thank my advisor, Dr. Michelle Mack, for her invaluable mentorship over the past five years. Michelle, thank you for your guidance, your trust, and your advocacy; thank you for always finding time to discuss not just my research, but my career interests; and thank you for your compassionate and uplifting support during those moments when I was not successful in my endeavors. Your approach and dedication to science and sincere joy for ecology will forever be a source of inspiration to me.

This dissertation would not have been possible without the support of my committee, fellow Mack lab members, and several outside collaborators. Thank you to Matt Bowker, Paul Dijkstra, and Kitty Gehring for your insightful feedback and conversations that helped broaden my ecological perspective. Thank you to all current and past members of the Mack lab—from learning how to run the TICTOC to correcting random effects structures to detailed feedback on countless presentations and analyses, you all helped me grow and learn as an ecologist. Special appreciation to Julia Stuart, Sedona Spann, and Emily Brooke for their extensive support in the field. Thank you to the Arctic Long-Term Ecological Research (LTER), the Bonanza Creek LTER, Toolik Field Station, the Environmental Genetics and Genomics Laboratory, Lela Andrews, Adair Patterson, and the Gehring Lab for providing critical resources, training, lab space, and support for this project.

Lastly, I am so grateful to my family and friends who helped support me in my graduate career. To my parents, Nancy and Dennis, for taking a few deep, calming breaths 10+ years ago and supporting my decision to travel over 3000 miles away from home to attend college in Alaska. I do not know what path my career would have taken had my interest in ecology not been sparked by the coastal rainforests and alpine tundra of southcentral Alaska. To my Nonna,

the late Maryrose Dunleavy, whose loving phone calls served as a weekly high point and source of motivation throughout the early years of my graduate degree. Finally, eternal gratitude and love for my partner Tyler Katzmar for remaining my patient, caring, and steady support throughout the entirety of this degree and beyond.

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DEDICATION

To the Arctic tundra—my love for this place rests somewhere between a perfectly ripened cloudberry and the midnight alpenglow emitted by summer's first sunset.

PREFACE

The chapters presented here are each written in a format for publication in scientific peer-reviewed journals. The first chapter is an overall introduction and establishes the topics and themes investigated in this dissertation. The second chapter, "Long-term experimental warming and fertilization have opposing effects on ectomycorrhizal root enzyme activity and fungal community composition in Arctic tundra," was published in *Soil Biology and Biochemistry* in March 2021. The third chapter, "Non-linear responses of ericaceous and ectomycorrhizal shrub cover and root enzyme activity across a long-term experimental soil fertility gradient in Arctic tundra," is formatted for *Journal of Ecology*. The fourth chapter, "N_2 fixation and ectomycorrhizal root enzyme activity are coupled on Siberian alder across an Arctic-boreal gradient" is formatted for *Ecology*. The final chapter is the overall discussion of results and conclusions. These articles are organized into chapters as per university formatting requirements, which has resulted in some redundancy.

CHAPTER 1: INTRODUCTION

Mycorrhizal fungi, in symbiosis with plant roots, exert a strong influence over nutrient cycling in terrestrial biomes. Distributed along distinct biogeographical regions, mycorrhizal fungi separate across three dominant mycorrhizal types—ericoid mycorrhizas, ectomycorrhizas, and arbuscular mycorrhizas—varying in root colonization structures, nutrient acquisition strategy, and phylogeny. As climate change induces biome-level shifts in plant communities, the dominant mycorrhizal type in each biome is changing. These changes have the potential to alter ecosystem processes such as nutrient cycling (Read & Perez-Moreno, 2003) and soil C storage (Averill *et al.*, 2014; Zak *et al.*, 2019). As a result, positive feedbacks with both vegetation and climate may be developed with shifts in mycorrhizal type. Though the subsequent shifts in mycorrhizal communities following shifts in plant communities are well-documented, far fewer studies investigate the associated changes in mycorrhizal function. This knowledge gap persists despite the consequences these changes may have across ecosystems.

The greening of the Arctic tundra presents a model system in which to explore the responses of mycorrhizal function following biome-level shifts in vegetation. Arctic ecosystems are highly sensitive to recent climatic changes (Serreze & Barry, 2011). As the Arctic warms at twice the rate as the global average (Cohen *et al.*, 2014), shrubs are expanding across tundra ecosystems (Sturm *et al.*, 2001b; Tape *et al.*, 2006; Myers-Smith *et al.*, 2011; Elmendorf *et al.*, 2012). In the North American Arctic, these shifts are thought to be dominated by deciduous ectomycorrhizal (EcM) shrubs such as *Betula* and *Alnus spp*. Yet, in the Scandanavian Arctic, evergreen ericaceous shrubs may also be expanding (Van Wijk *et al.*, 2003; Vowles & Björk, 2018). Because of the mycorrhizal association of expanding shrubs, these vegetations shifts could coincide with changes in the dominant mycorrhizal type from low diversity, low biomass

ericoid mycorrhizae (ErM) with high degradative ability to high diversity, high biomass ectomycorrhizae (EcM) with variable degradative ability, ranging from high to non-existent depending on fungal taxa. A shift in mycorrhizal type or abundance may alter nutrient cycling in a system co-limited by both nitrogen (N) and phosphorus (P) (Shaver *et al.*, 2001).

Arctic shrub expansion is hypothesized to result from warmer temperatures and enhanced shrub nutrient uptake. Warmer Arctic temperatures are predicted to increase nutrient availability, both by stimulating microbial decomposition and nutrient mineralization (Nadelhoffer *et al.*, 1991; Hobbie, 1996; Schimel *et al.*, 2004; Salmon *et al.*, 2016) and by releasing previously unavailable substrates to biological processes via permafrost thaw (Keuper *et al.*, 2012; Salmon *et al.*, 2018). Experimental warming and nutrient addition increase shrub dominance and biomass (Chapin *et al.*, 1995; Bret-Harte *et al.*, 2001; Shaver *et al.*, 2001; Van Wijk *et al.*, 2003; Mack *et al.*, 2004; DeMarco *et al.*, 2014b). These same factors behind shrub expansion are likely driving mycorrhizal community responses. Warming could stimulate mycorrhizal activity while nutrient increases may alter the nature of the relationship between EcM host plant and fungi by alleviating plant nutrient limitations (Högberg *et al.*, 2003, 2010; Brzostek *et al.*, 2014), ultimately decreasing the influence of EcM over nutrient cycling.

The implications of shrub expansion for tundra nutrient cycling have been examined in past studies. Shrub expansion has the potential to alter nutrient dynamics by affecting biochemical and biophysical drivers of nutrient cycling. For instance, the transition to deciduous shrub tundra may coincide with the development of a positive feedback loop known as the snow-shrub hypothesis (Sturm *et al.*, 2001a). Taller, larger patches of shrubs trap more snow, insulating and warming soils during winter. This warmth may promote microbial decomposition and N mineralization, which stimulates growth of deciduous shrubs (Schimel *et al.*, 2004; Sturm

et al., 2005). In addition, despite having poorer leaf litter quality than prevailing graminoid vegetation, deciduous shrub tundra has been associated with faster decomposition rates and N mineralization (Hobbie, 1996; DeMarco *et al.*, 2011, 2014a; Sistla *et al.*, 2013; McLaren *et al.*, 2017). While many have tested the snow-shrub hypothesis, studies involving realistic increases in snow depth have yet to find strong support for the positive influence of snow (DeMarco *et al.*, 2011, 2014a; Christiansen *et al.*, 2018). Instead, the changes in litter quality and quantity that increase rates of nutrient release from soil organic matter appear to be a more important driver in determining how shrub expansion alters tundra nutrient cycling.

Despite the importance of mycorrhizae in tundra nutrient cycling and the large research effort to understand the effects of shrub expansion on nutrients, few studies have focused on identifying how shifts in mycorrhizal function associated with vegetation shifts alter ecosystem nutrient dynamics. To access nutrients locked in soil organic matter, ericoid and ectomycorrhizae exude degradative extracellular enzymes. These enzymes allow for plant nutrient acquisition in an environment with limited nutrient mobilization and can affect soil C cycling (Lindahl & Tunlid, 2015). Mycorrhizal-associated enzyme activity responds to changes in the soil environment (Rineau & Garbaye, 2009; Courty *et al.*, 2010, 2016; Jones *et al.*, 2012; Nicholson & Jones, 2017). Sharp increases in soil nutrient availability reduce activity by shifting mycorrhizal fungal communities towards nitrophilic species with low degradative capacity (Lilleskov *et al.*, 2002). Additionally, mycorrhizal enzyme activity also varies depending on both the host plant and mycorrhizal fungal species involved in the symbiosis. Genetically speaking, ErM often have a higher enzymatic capacity than EcM and at times can act saprophytically (Martino *et al.*, 2018; Perotto *et al.*, 2018). Some evidence suggests preferential acquisition of organic forms of nitrogen (N) by ericaceous plants even in the presence of inorganic N (Read,

1991; Michelsen *et al.*, 1996). Among EcM fungi, species similarly vary in their genetic ability to produce plant-cell wall degrading enzymes (Cairney & Burke, 1994; Read & Perez-Moreno, 2003; Read *et al.*, 2004; Bödeker *et al.*, 2009, 2014; Kohler *et al.*, 2015; Lindahl & Tunlid, 2015; Pellitier & Zak, 2018). Furthermore, certain host plants, especially those that also associate with N-fixing bacteria promote higher enzyme activity than others (Walker *et al.*, 2014; Queralt *et al.*, 2019; Ruess *et al.*, 2019). Changes in the activity of degradative root enzymes, concurrent with the aboveground expansion of shrubs, will alter tundra soil processes and could affect C dynamics in a globally important C-rich ecosystem (Schuur *et al.*, 2015).

Here, I explore three different instances in which mycorrhizas could alter tundra nutrient cycling as their host shrubs expand. In chapter two, I test how EcM-associated root enzyme activity and community responds to long-term experimental warming and nutrient addition, two drivers of shrub expansion. In chapter three, I dive deeper into the effects of nutrient addition on shrub response, testing the functional responses of aboveground dominance and root enzyme activity of two contrasting shrub functional types to a long-term experimental soil fertility gradient. In chapter four, I turn my attention to a shrub that has the potential to play a unique role in the future Arctic: Siberian alder, which hosts a tripartite symbiosis with N-fixing actinobacteria *Frankia* and EcM. I characterize drivers of N fixation and EcM-associated root enzyme activity on alder and test a hypothesis that these two processes are coupled. Together these chapters tackle knowledge gaps in the role of warming, nutrient limitations, and shrub species in determining the future impacts of mycorrhizal function in Arctic tundra. Understanding the implications of these shifts will aid in navigating the ecosystem dynamics of a new, shrubbier Arctic.

CHAPTER 2: LONG-TERM EXPERIMENTAL WARMING AND FERTILIZATION HAVE OPPOSING EFFECTS ON ECTOMYCORRHIZAL ROOT ENZYME ACTIVITY AND FUNGAL COMMUNITY COMPOSITION IN ARCTIC TUNDRA

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Key words: Arctic tundra; *Betula nana*; ectomycorrhizae; fertilization; long-term experiment; warming

Highlights

- Fertilization had a strong effect on EcM, decreasing root and community enzyme activities.
- Warming had no effect on EcM root activity, but it increased EcM root abundance.
- Changes in EcM root community correlated with changes in root enzyme activity.
- We also observed taxon-specific responses that were counter to community changes.

Abstract

As the Arctic rapidly warms, deciduous ectomycorrhizal (EcM) shrubs are expanding across the tundra. While we know how EcM host plants respond to warming and the associated nutrient release predicted for a future Arctic, considerably less is known about how EcM function will respond, despite their important role in plant nutrient acquisition in the nutrient-limited tundra. To explore how EcM-associated nutrient cycling may change, we characterized EcM-associated root enzyme activity and community in a 28-year full factorial warming and fertilization experiment in Arctic tundra. We measured activity at the individual root tip-level ($pmol \cdot min^{-1} \cdot mm^{-2}$ root) and used EcM root tip abundance to scale to community-level activity ($pmol \cdot min^{-1} \cdot cm^{-3}$ soil). We then Sanger sequenced fungi on the same root tips to pair identity with function. Linear mixed effects models and multivariate analysis showed warming and fertilization generally had opposing, and sometimes interacting, effects on EcM root-associated activity. Responses also differed depending on the scale. Fertilization decreased activity on both scales while warming dampened the effect of fertilization. Additionally, warming increased EcM root tip abundance, and therefore community-level enzyme activity. Ectomycorrhizal root tip communities changed with fertilization, but not with warming. Changes in enzyme activity were

moderately correlated with both changes in fungal community and soil inorganic nitrogen concentrations. The contrast in responses of root tip-level activity and community-level activity point to a potential shift in allocation to EcM function: whereas the production of degradative enzymes may become less important for nutrient acquisition, the exploration of the soil environment through increased number of EcM root tips may become more important. Furthermore, the future role of EcM in a warmer Arctic likely depends on the magnitude of nutrient release that comes with warming. While warming may increase the importance of EcM-associated nutrient cycling if nutrient availability remains low, it has the potential to decrease their importance if nutrient availability greatly increases.

1. Introduction

In the nutrient-limited Arctic tundra, mycorrhizal fungi exert a strong influence over nutrient cycling (Read and Perez-Moreno, 2003), providing up to 86% of nitrogen (N) to their host plants (Hobbie and Hobbie, 2006). However, rapid warming in the Arctic and its ensuing effects on plant and soil processes may alter mycorrhizal community composition and function, potentially changing nutrient dynamics in an ecosystem where carbon (C) and nutrient cycling are tightly coupled (Hobbie et al., 2002; Mack et al., 2004; Shaver et al., 1992). Though some studies have measured changes in mycorrhizal fungal community composition with experimental manipulation, few studies have linked community shifts with measured changes in mycorrhizal function or quantified these effects at varying scales, leaving the resulting impacts on tundra ecosystem pools and fluxes understudied.

Although ericoid mycorrhizae (ErM) typify tundra plant associations (Soudzilovskaia et al., 2017), ectomycorrhizae (EcM) may play an important role in the future dynamics of the North American Arctic tundra. Both EcM- and ErM-associated shrubs are expanding into tundra ecosystems across the globe as temperatures warm (Elmendorf et al., 2012; Myers-Smith et al., 2011; Sturm et al., 2001; Vowles et al., 2017; Vuorinen et al., 2017). In the North American low Arctic, tall deciduous EcM shrubs are thought to dominate vegetation shifts (Mekonnen et al., 2018; Tape et al., 2006). To improve our predictions of the impact of deciduous shrub expansion in this region, particularly on ecosystem nutrient cycling, it is necessary to understand how EcM function responds to warming. While EcM readily take up dissolved inorganic nutrients, they also acquire nutrients bound in soil organic matter (SOM) by exuding extracellular enzymes (Hodge, 2017). The amount and type of enzymes produced depend on both the soil environment (Courty et al., 2010; Nicholson and Jones, 2017; Rineau and Garbaye, 2009), especially resource

availability (Courty et al., 2016; Jones et al., 2012), and the genetic ability of each fungal species to transcribe certain enzymes (Kohler et al., 2015). Genes encoding for hydrolytic enzymes, often implemented in N and phosphorus (P) acquisition, are common among fungal lineages. Genes for oxidative enzymes, though, which are implemented in the breakdown of lignin and other phenolic compounds, are only retained in a select number of EcM fungi (Bödeker et al., 2014, 2009; Cairney and Burke, 1994; Kohler et al., 2015; Lindahl and Tunlid, 2015; Pellitier and Zak, 2018; Read et al., 2004; Read and Perez-Moreno, 2003). Changes in the soil environment and EcM fungal community that occur with warming, therefore, have a strong potential to affect EcM enzyme activity, but the direction of this effect is unknown. Because changes in EcM-associated enzyme activity with shrub expansion could not only feedback positively into plant community shifts and the associated stimulation of ecosystem nutrient cycling, but also impact the large stocks of Arctic soil C (Schuur et al., 2015), it is crucial to further study how EcM and their function will respond to warming.

The response of EcM to warming likely depends on the associated increases in soil nutrient availability, which may negatively affect EcM biomass and enzyme activity (Lilleskov et al., 2019). Warmer Arctic temperatures are predicted to increase nutrient availability, both by stimulating microbial decomposition and nutrient mineralization (Hobbie, 1996; Nadelhoffer et al., 1991; Salmon et al., 2016; Schimel et al., 2004) and by releasing previously unavailable substrates to biological processes via permafrost thaw (Keuper et al., 2012; Salmon et al., 2018). Additionally, changes in litter quality and quantity resulting from vegetation shifts have been shown to increase rates of N mineralization (Christiansen et al., 2018; DeMarco et al., 2014a, 2011; McLaren et al., 2017). These nutrient increases may alter the nature of the relationship

between EcM host plant and fungi by alleviating plant nutrient limitations (Brzostek et al., 2014; Högberg et al., 2010, 2003), ultimately decreasing the influence of EcM over nutrient cycling. Across studies on the effects of long-term warming and fertilization on tundra plant communities and ecosystem fluxes and pools, fertilization repeatedly had a stronger effect than warming. This held true for soil C stocks (Mack et al., 2004; Sistla et al., 2013), aboveground plant biomass (Boelman et al., 2003; Chapin et al., 1995; DeMarco et al., 2014b; Van Wijk et al., 2003) and deciduous shrub growth (Bret-Harte et al., 2001; Zamin and Grogan, 2012). Additionally, changes that occurred aboveground were often not indicative of changes belowground (Gough et al., 2012; McLaren et al., 2017; McLaren and Buckeridge, 2019). For example, while aboveground C pools increased with fertilization, belowground C pools generally decreased with added nutrients (Mack et al., 2004).

Several studies have observed shifts in EcM fungal abundance and community composition with warming and fertilization in Arctic tundra, but the magnitude and direction of these responses were inconsistent. For example, some found EcM fungal richness sharply decreased with warming (Geml et al., 2015; Morgado et al., 2015) while others found it increased (Deslippe et al., 2011) despite study sites being located within 2.5km of each other. Deslippe *et al.* (2011) also found N and P fertilization did not alter EcM root colonization while Urcelay *et al.* (2003) found it decreased EcM colonization, again despite similar levels of fertilization and study sites <1 km away. Both EcM mycelial production and fungal biomass in fine roots increased with warming and fertilization (Clemmensen et al., 2006). However, the increase in aboveground host plant biomass was greater than the increase in EcM fungal root biomass, indicating reduced C allocation to EcM relative to allocation aboveground. Additionally, the increase of EcM fungal abundance with warming correlated highly with

aboveground biomass of host plants, indicating warming may have a strong indirect effect on EcM (Clemmensen et al., 2006). Despite these discrepancies in the response of EcM abundance and fungal community structure, ecological market theory presents a strong case for reduced plant allocation to mycorrhizal roots in the presence of increased nutrient availability (Franklin et al., 2014).

Common to all studies was the existence of taxon-specific responses (Deslippe et al., 2011; Geml et al., 2015; Morgado et al., 2015; Semenova et al., 2015). With these responses, several studies then attempted to predict community functional shifts of EcM through linking taxa and exploration types (morphological classifications based on the extent and pattern of extraradical mycelium; Agerer, 2001) to our current understanding of their enzymatic capability. High biomass exploration types are hypothesized to have a high potential for enzymatic acquisition of nutrients to support their high C demand to the plant (Hobbie and Agerer, 2010; Lilleskov et al., 2011). In comparison, low biomass exploration types may be associated with the acquisition of more labile nutrient pools. Warming tended to decrease the abundance of low biomass exploration types, such as *Russula spp.* or in those in the family Helotiaceae, while it increased the abundance of higher biomass types, such as the highly degradative *Cortinarius spp.* (Deslippe et al. 2011, Geml et al. 2015, Morgado et al. 2015). Fertilization increased the presence of *Laccaria bicolor*, a nitrophilic species with lower biomass exploration type (Deslippe et al. 2011). Although these results point to a shift towards higher EcM-associated enzyme activity with warming and lower with increased nutrient availability, changes in enzyme activity concurrent with these community shifts have yet to be directly measured. This is a key component in understanding the future role of EcM in Arctic tundra.

To address this knowledge gap, we characterized both community and functional responses of EcM root tips to long-term warming and fertilization in Arctic tundra, pairing function with fungal taxa on individual root tips. Additionally, we used scaling methods to inform not only how individual root tips were responding, but how this response might affect broader ecosystem processes by calculating an EcM community-level functional activity rate. We hypothesized that while both warming and higher nutrient availability would increase ectomycorrhizal host plant cover, they would have opposing effects on EcM-associated root enzyme activity:

1. By increasing host plant cover, but not alleviating nutrient limitations to growth, warming will increase allocation to EcM root tips, resulting in increased EcM root activity per unit root area and per volume of soil, increased EcM root tip abundance, and a fungal community shift towards taxa with high biomass and a high degradative capacity, such as *Cortinarius spp*.
2. Although fertilization will also increase host plant cover, it will alleviate nutrient limitations to the host plant, thereby reducing the dependence of host plants on their fungal symbionts for nutrient acquisition. This will result in decreased allocation to EcM root tips, resulting in decreased activity per unit root area and per unit volume of soil, decreased EcM root tip abundance, and a fungal community shift towards nitrophilic taxa, such as *Laccaria spp.*

Additionally, we predicted that changes in the soil physical and chemical environment with warming and increased nutrient availability would alter EcM fungal community, which would inescapably affect EcM function.

To test these hypotheses, we measured extracellular enzyme activities on EcM root tips and identified the fungi colonizing a subset of those root tips in a 28-year full factorial warming and fertilization experiment at a moist acidic tussock tundra site in Arctic Alaska. Root enzyme activities provide an estimate of allocation towards the acquisition of organically-sourced nutrients, and therefore a relative measurement of EcM-associated nutrient cycling. We selected five enzymes to represent the range in possible mechanisms of SOM degradation (hydrolytic vs. oxidative), specific nutrients or substrates targeted (C vs. N vs. P), and quality of SOM catalyzed (labile vs. recalcitrant).

2. Materials and Methods

2.1 Study site

We conducted this study near Toolik Lake at the Arctic Long-Term Ecological Research site (68˚38' N, 149˚34'W, 780 m above sea level) on the North Slope of Alaska. The site is located on a north-facing 5˚ slope in moist acidic tussock tundra. Vegetation is dominated by roughly equal amounts of graminoids, dwarf deciduous and evergreen shrubs, and mosses. Of the common plant species, deciduous shrubs *Betula nana* and *Salix spp.*, approximately 20% and 4% of aboveground biomass, respectively, form ectomycorrhizae. Soils consist of a moist organic horizon with a mean thickness of 30 cm and pH of 4.4 over a silty mineral horizon with continuous permafrost. Atmospheric N deposition is minimal, ranging from 8 to 56 mg N m^{-2} yr^{-1} (Hobara et al., 2006; National Atmospheric Deposition Program database). All data is archived in the Arctic Data Center repository under doi:10.18739/A2MK65830.

The warming and fertilization experiment was established in 1989 and has four replicate blocks of full factorial warming and N and P fertilization treatments. Ambient temperature plots

measure to 5 m x 20 m. Greenhouses, made of wooden frames (2.5 x 5 m, 1.5 m in height) covered by 0.15 mm polyethylene sheets, have historically elevated air temperature by 2-3.5˚C and soil temperature by 1.75-2.5˚C at 10 cm depth (Chapin et al., 1995; Deslippe et al., 2011). The polyethylene sheets were removed during the fall, winter, and spring months to avoid unintended consequences such as snowfall or herbivore exclusion. Fertilized plots received 10 g N m^{-2} yr^{-1} as ammonium nitrate (NH_4NO_3) and 5 g P m^{-2} yr^{-1} as triple superphosphate (P_2O_5) every spring following snowmelt until 2012. Since 2012 plots have been fertilized with ammonium chloride and sodium nitrate (NH_4Cl and $NaNO_3$); P fertilization has remained the same. Though the switch in fertilizer type has the potential to affect soil salinity and pH via retention of Na^+ and Cl^-, it has not caused significant change in soil pH (M. Könönen unpublished data). Twenty-eight years of treatments have resulted in an increase of the aboveground biomass of EcM shrub *B. nana*.

2.2 Sample collection

We sampled organic soils 5 cm down from the top of the green moss to 15 cm deep using a 4.2 cm diameter metal corer between 5-15 July 2017, when vegetation is nearing peak biomass. In ambient plots, cores for root tip analysis were taken at nine points across a 4 x 3 grid in a 4 x 8 m area (Fig. S1). We took an additional three cores within each sampling area per plot for soil chemical and physical analyses. At the time of sampling, we also measured active layer thaw depth (distance to frozen soil) at three points within each plot. Cores were stored intact at 4˚C for no more than 72 hours before excising root tips and running enzyme assays. Soil analyses were started the day of core collection.

Vegetation cover was visually estimated in eight permanent 1 m^2 quadrats per plot in July 2019 by the Arctic LTER. Estimates included vascular plant species in addition to broad categories of moss, lichen, litter, and standing dead *Betula nana.* Methods used are described by Gough (2019). Air and soil temperatures were continuously monitored by the Arctic LTER described in DeMarco et al. (2014b) and acquired from Shaver (2019).

2.3 Root tip analysis

2.3.1 Enzyme activity

To characterize EcM enzyme activity and allocation to organic nutrient acquisition, we conducted extracellular enzyme assays on EcM root tips. We attempted to harvest EcM tips exclusively from *B. nana* roots by identifying their red-brown color and irregular angle between secondary and tertiary roots. However, a small portion of our EcM root tip samples may consist of *S. pulchra* roots. The same day of assays, we excised a maximum of 12 healthy EcM root tips from each core, verified by turgidity and a white inner pith under a stereomicroscope, for a maximum of 80 root tips per plot. Total number of root tips sampled per plot varied between 44 and 80 tips due to a lack of healthy EcM in some plots. To capture the heterogeneity of EcM within a plot, we took no more than two root tips from each fine root segment within a core. Excised EcM root tips were stored in 100 µl tap water in 96-well plates at 4˚C for no more than 9 hours before starting enzyme assays.

Following the root tip enzyme assay protocol described in Pritsch *et al.* (2011) and the peroxidase assay in Johnsen and Jacobsen (2008), we measured potential activities of five enzymes—two associated with acquisition of more labile forms of organic N [leucine amino peptidase (EC 3.4.11.1) and N-acetyl glucosaminidase (hereafter chitinase; EC 3.2.1.14)], one

with P acquisition [phosphomonoesteratase (hereafter phosphatase; EC 3.1.3.2)], and two with nutrient acquisition via oxidative breakdown of complex carbon compounds [laccase (hereafter phenol oxidase; EC 1.10.3.2) and peroxidase (EC 1.11.1.7)]—on EcM root tips in a series of fluorometric and colorimetric assays. Details of enzyme assays can be found in Methods S1. Following assays, EcM root tips were scanned in 50 μl tap water in a 96-well plate and projected surface area was measured in ImageJ. Potential enzyme activity was calculated as pmol substrate converted mm^{-2} root tip min^{-1} (hereafter pmol mm^{-2} min^{-1}) using standard curves for hydrolase substrates and the Beer-Lambert law for substrates in phenol oxidase ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}; Pritsch *et al.*, 2011) and peroxidase assays ($\varepsilon_{450} = 5.9 \times 10^4$ M^{-1} cm^{-1}; Josephy *et al.*, 1982). All liquid was removed from wells and root tips were stored for molecular analysis at -20˚C.

To assess the response of EcM root tips on a broader community level, we calculated EcM root tip density and community-level activities of EcM root tip-associated enzymes. We counted total healthy EcM root tips in three randomly selected cores per plot and averaged EcM densities by plot to quantify EcM root tip abundance. We then extrapolated potential enzyme activities on individual root tips to community-level activity (pmol cm^{-3} soil min^{-1}), multiplying the average root tip activity in each core (pmol mm^{-2} min^{-1}) by the average of EcM tip surface area (mm^2 EcM root tip^{-1}) in each core and the plot average of EcM density (EcM root tip cm^{-3} soil). For an additional metric of healthy EcM root tips within a plot, we calculated a healthy fraction by dividing sampled root tips per plot by sampling effort (the attempted 80 tips we intended to collect).

2.3.2 Molecular identification of fungal communities

In addition to root tip enzyme activity, we identified fungal taxa on a subset of assayed EcM root tips via Sanger sequencing. We extracted DNA from a randomly selected subset of 40 root tips per plot using a random number generator and ensuring that each core was roughly equally represented. We then amplified the combined ITS1 and ITS2 regions using the forward primer ITS1-fungal (Gardes and Bruns, 1993) and reverse primer ITS4 (White et al., 1990) in a polymerase chain reaction (PCR). Details of DNA extraction and PCR can be found in Methods S2. PCR product was cleaned with an ethanol wash and cycle sequenced using BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Scientific, USA) and the reverse primer. Cycle sequences were read following a final ethanol wash via capillary electrophoresis in an ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) at the Environmental Genetics and Genomics laboratory at Northern Arizona University. Reads were filtered for quality in Geneious (Biomatters Limited, NZ), where we trimmed primers and regions with an error probability >0.5%. We then identified to species or genus using the NCBI BLAST nucleotide database at the 97% and 95% identity cutoff, respectively. Sequences matching to the same genus were then aligned in Geneious and manually trimmed to be the same length before collapsing fungal identities into operational taxonomic units (OTUs) at 95% similarity. We opted for clustering at 95% after 97% appeared to artificially separate sequences that matched to the same GenBank sequence. Relative abundance of each OTU per plot was calculated by dividing number of sequences of each OTU by total sequences identified per plot. Fungal ITS sequences are archived in GenBank under accession MT278352 - MT278808.

2.4 Soil analysis

We measured soil moisture, bulk C:N, total dissolved organic carbon (TOC), total dissolved nitrogen (TN), and dissolved inorganic N (DIN; NH_4-N and NO_3-N) of organic soil from the 5-15 cm depth increment. The day of collection, coarse material (litter, roots, and rhizomes > 2 mm) was removed from cores and remaining soil was homogenized by hand. After drying a subsample of soil at 60˚C for 48 hours, soil moisture was calculated as the difference of wet weight from dry weight divided by dry weight. The dried sample was ground in a Wiley Mill and bulk C:N content was measured via combustion mass spectrometry on an Elemental Analyzer (Costech Analytical Technologies Inc., USA). Total organic carbon and TN were extracted from 10 g of field moist soil in 50 mL 0.5 M K_2SO_4. Extracts were shaken for 2 hours on a shaker table, placed overnight at 4˚C to settle, and vacuum-filtered through a Whatman GF/A filter. Filtrate was frozen and stored at -20˚C until further analysis. A subsample of filtrate was processed for TOC and TN via combustion on a Total Organic Carbon Analyzer TOC-L (Shimadzu Corporation, Japan) and a separate subsample was processed for DIN on a SmartChem 200 Discrete Chemistry Analyzer (Unity Scientific, USA) via colorimetry using the Ammonium Salicylate method (Unity Scientific Method 210-203D) for NH_4-N detection and the Nitrate method (Unity Scientific Method NO3-001-A) for NO_3 -N detection. All extract concentrations were calculated as g N or g C per g dry soil and were corrected for soil moisture content. Dissolved organic nitrogen (DON) was calculated as TN minus DIN.

2.5 Statistical analysis

We analyzed the responses of root tip enzyme activities and fungal community to warming, fertilization, and soil properties using a combination of multivariate and univariate approaches. All analyses were conducted using R 3.6.1 (R Core Team, 2019). For our

multivariate analysis of enzyme activities, we relativized the average root tip activities of each enzyme within each plot by dividing by the maximum value within each enzyme. We tested for homogeneity of dispersion among treatments using the betadisper function in the *vegan* package (Oksanen et al., 2019) and an ANOVA. After verifying dispersions were homogenous, we then conducted a permutational multivariate analysis of variance (perMANOVA) assessing the correlation of warming, fertilization, their interaction with the Bray-Curtis dissimilarities among enzyme profiles using the adonis function in *vegan*. We generated a nonmetric multidimensional scaling (NMDS) ordination, created with the metaMDS function in *vegan*. We then tested the correlations of NH_4-N, NO_3-N, TOC, TN, %N, %C, C:N, *B. nana* and *Salix pulchra* cover, thaw depth, and moisture with the ordination using the *vegan* function envfit. Points representing plots were scaled by the sum of relativized enzyme activities as an estimate for total EcM root tip enzyme activity of a site. To assess whether variation in EcM root tip fungal communities were related to warming, fertilization, and soil properties, we repeated our multivariate approach used in enzyme analysis, this time using the relative abundances of OTUs in each plot. Both ordinations were fit on 2-dimensions after preliminary ordinations revealed additional dimensions did not lower the stress more than 0.05.

To determine if changes in root tip enzyme activities with warming and fertilization were correlated with changes in fungal communities, we conducted a Mantel test between the Bray-Curtis dissimilarity matrices of root tip-level enzyme activity and fungal OTU relative abundance using the mantel function from *vegan*.

We then tested the responses of EcM-associated enzyme activities, fungal community, and soil variables to warming and fertilization using general linear mixed effects models. For each of the five enzymes measured, we modeled fixed effects of warming and fertilization on

individual root tip enzyme activities and on community enzyme activities using the glmmTMB function from the *glmmTMB* package (Magnusson et al., 2017). In all models, we accounted for spatially nested sampling by including a dependency structure of random effects of core within plot within block. The full model contained the fixed effects of warming, fertilization, and their interaction. We then used backward model selection to choose the best fit model according to AICc values. When data displayed a log-distribution, we created models using log-gamma distributions after adding a non-zero minimum value to data with zero values. We visually examined model residuals to assure for homoscedasticity. After selecting the best fitting model, we ran Tukey HSD pairwise tests using the leastsquares function from the *emmeans* package (Lenth, 2019). We repeated the described model selection process to determine the effects of warming and fertilization on soil properties, plant species cover, EcM root tip density, healthy fraction of root tips (ratio of root tips we were able to collect out of the intended 80), and alpha diversity metrics (OTU richness and Simpson's diversity index obtained using *vegan*). Models of rarified OTU richness were performed to confirm no difference in responses of raw and rarefied richness (Table S1).

To identify enzymatic response of individual fungal taxa to warming and fertilization, we modeled root tip-level activity of each of the five enzymes as a function of the three-way interaction between warming, fertilization, and fungal genus. Due to the limited number of taxa that colonized a sufficient amount of root tips in each treatment, we conducted this analysis only on activities of roots colonized by *Russula, Lactarius*, and *Cortinarius.*

3. Results

3.1 Soil properties and plant cover

Twenty-eight years of warming and fertilization treatments altered environmental conditions and soil properties at 5-15 cm depth (Table 1). In general, DIN concentrations (μg N g^{-1} dry soil) increased with fertilization, but not warming. Dissolved organic N (μg N g^{-1} dry soil) and bulk soil %N, however, did not change with treatments. Warming increased TOC (mg C g^{-1} dry soil), but decreased bulk soil %C in unfertilized plots. Soil C:N ratios decreased in plots that were both warmed and fertilized. Thaw depths at time of sampling were deepest in unfertilized, warmed plots, but were not different among the other plots. Soil moisture levels were unchanged by either treatment.

Warming and fertilization also changed plant community structure. Both fertilization and warming increased cover of deciduous EcM shrub *Betula nana*, forb *Rubus chamaemorus,* and litter while decreasing cover of graminoids, ericaceous shrubs, other forbs such as *Pedicularis spp.* and *Polygonum bistorta*, bryophytes, and lichen (Table 2). *B. nana* cover was highest in warmed and fertilized plots. Due to an ongoing unknown disease outbreak (L. Gough, *pers. comm. Feb. 2020*), there was a high amount of standing dead *B. nana* (26-43%) in ambient fertilized plots.

3.2 Enzyme response to warming and fertilization

In total, we collected and conducted enzyme assays on 998 EcM root tips. Warming and fertilization had an interactive effect on EcM root tip enzyme profiles (perMANOVA, $F = 3.08$, $R^2 = 0.12$, $p = 0.041$). Visually, enzyme profiles were grouped by fertilization treatment in the ordination (Fig. 1a). Unfertilized plots in general were associated with higher activities of leucine aminopeptidase, a hydrolytic N-associated enzyme, and phenol oxidase, an oxidative enzyme associated with breakdown of phenolic compounds. Unfertilized, warmed plots were

associated with higher phenol oxidase and peroxidase activities, both oxidative enzymes associated with breakdown of phenolic compounds. Ammonium concentrations had the strongest correlation with enzyme profiles ($r^2 = 0.65$, $p = 0.002$), where overall enzyme activity decreased with increasing ammonium concentrations. Higher NO_3^--N concentrations ($r^2 = 0.50$, $p = 0.005$) and C:N values ($r^2 = 0.42$, $p = 0.03$) were also moderately positively correlated with fertilized and control sites, respectively. *B. nana* cover, *S. pulchra* cover, thaw depth, moisture, %N, %C , DON and TOC did not significantly correlate with enzyme profiles.

Univariate linear mixed effects model selection determined the full model with interaction was the best fit for two of the three hydrolytic enzymes—leucine aminopeptidase and chitinase, but not phosphatase (Table 3, S2). With respect to these hydrolytic N-associated enzymes, fertilization decreased EcM root tip activities within ambient plots, but had no effect in warmed plots (Fig. 2, Table S2). Warming also decreased EcM root tip activities of leucine aminopeptidase, but only in in unfertilized plots. Warming had no effect on chitinase. Neither warming nor fertilization had an effect on phosphatase activities, a hydrolytic P-associated enzyme. Of the two oxidative enzymes, associated with the breakdown of phenolic compounds, the full model with interaction was selected only for phenol oxidase. Fertilization decreased activities in both ambient and warmed plots. Warming increased activities only in fertilized plots. Neither warming nor fertilization had an effect on peroxidase activities.

EcM root tip density increased with warming (Tukey HSD, $p = 0.05$; Table 4), but did not change with fertilization. Healthy fraction of sampled root tips, however, were 0.14 lower in fertilized plots (Tukey HSD, $p = 0.02$) than in unfertilized plots.

Unlike the responses of individual root activities, the full model with the interaction was selected for community level activities of only two enzymes—leucine aminopeptidase and

phenol oxidase (Tables 5, S2). For leucine aminopeptidase, fertilization decreased activities in ambient, but not warmed plots (Fig. 2). Warming had no effect. For phenol oxidase, fertilization decreased activities in all plots. Warming increased activities only in fertilized plots. For the remaining three enzymes—chitinase, phosphatase, and peroxidase—the reduced, additive model was the best fit. Fertilization decreased while warming increased chitinase, phosphatase, and peroxidase activities.

3.3 EcM fungal community response

We extracted DNA from 672 root tips. After filtering for quality, we obtained a total of 480 sequences. Identification, alignment, and clustering resulted in a total of 44 OTUs, 11 of which were ascomycetes and 33 of which were basidiomycetes (Table S3). Ninety-one percent of identified root tips were colonized by basidiomycetes, the majority of which were within the family Russulaceae (62% of identified root tips). Within this family, *Russula spp.* consisted of eight OTUs found among 237 sequenced root tips, and *Lactarius spp.* of four OTUs among 59 tips. *Cortinarius spp.* (9% of identified root tips) were diverse relative to their abundance—11 OTUs were found among 44 tips.

Warming and fertilization had an interactive effect on Simpson's diversity index, but no effect on OTU richness (Table 4). Fertilization decreased Simpson's diversity index in ambient (Tukey HSD, $p = 0.01$), but not warmed plots. Warming increased Simpson's diversity index in fertilized plots (Tukey HSD, $p = 0.006$), but not unfertilized.

Fungal community composition was affected by fertilization, but not warming. Fertilization weakly correlated with the Bray-Curtis dissimilarities among plots (perMANOVA, $F = 4.1$, $R^2 = 0.22$, $p = 0.002$). Similar to that of enzyme profiles, the community ordination

grouped fungal root communities by fertilization (Fig. 1b). Unfertilized plots were associated with OTU affiliates *Cortinarius croceus 2*, *Cortinarius sp. 1*, *Cortinarius sp. 2*, and *Tomentella sublilacina* while fertilized plots were associated with *Cortinarius delibutus*, *Laccaria laccata*, *Thelephora terrestris*, *Pseudotomentella sp. 1*, *Rhizoscyphus ericae*, *Meliniomyces variabilis*, and *Russula nitida/sphagnicola*. Ammonium concentrations ($r^2 = 0.39$, $p = 0.04$) moderately correlated with the fungal community ordination. Nitrate ($r^2 = 0.3$, $p = 0.09$) had a marginally significant correlation with fungal communities. *B. nana* and *S. pulchra* cover, thaw depth, moisture, C:N, %N, %C, DON, and TOC did not significantly correlate with fungal composition. A Mantel test indicated the dissimilarities among enzyme profiles positively correlated with dissimilarities among community composition (Mantel $r = 0.48$, $p = 0.001$).

Warming and fertilization had a varied effect on the relative abundance of fungal genera (Fig. 3a). Warming alone increased the relative abundance of *Russula*, but decreased it in fertilized plots. Conversely, the relative abundance of *Cortinarius.* decreased with both warming and fertilization. Neither treatment seemed to affect the relative abundance of *Lactarius.* Fertilization favored some fungal taxa. Members of the family Thelephoraceae generally increased with fertilization, and *Laccaria,* completely absent from only warmed plots, were most abundant in fertilized plots. However, fertilization also decreased the abundance of other fungal taxa. *Cenoccocum* decreased in ambient fertilized plots, and *Leccinum* were only present in unfertilized plots. Lastly, ascomycetes, particularly members of the family Helotiales, increased with warming.

The changes in absolute abundances with warming and fertilization generally echoed those in relative abundances (Fig. 3b). *Cortinarius spp.* decreased in absolute abundance with both warming and fertilization. Ascomycetes increased with warming, and members of the

family Thelephoraceae increased with fertilization. However, some differences were notable. In ambient fertilized plots for instance, the density of root tips colonized by *Lactarius* and *Russula* decreased while members of the family Thelephoraceae became the dominant taxa. Additionally, the increase in ascomycetes in unfertilized, warmed plots became more pronounced as roots colonized by *Cenoccocum* increased in density.

3.4 EcM root tip enzyme responses by genus

We tested whether enzyme activities on root tips colonized by the three most prevalent fungal genera colonizing roots—*Russula, Lactarius,* and *Cortinarius*—varied by warming, fertilization, and fungal generic identity. Genus had an interactive effect with warming and fertilization on activities of chitinase, phosphatase, phenol oxidase, and peroxidase, but had only an additive effect on leucine aminopeptidase activities (Table 6). Roots colonized by *Russula* were the most responsive—four of the five enzymes decreased in activity at least one of the treatments (Fig. 4). Roots colonized by *Cortinarius* decreased in activity of only hydrolytic N-associated enzymes. Activities on roots colonized by *Lactarius* responded to warming and fertilization for only one enzyme, leucine aminopeptidase.

The responses of root activities to each treatment varied by enzyme in complex ways. For instance, hydrolytic N-associated enzymes did not respond similarly across genus or treatment. Leucine aminopeptidase activities on roots colonized by all three genera decreased in warmed plots. Chitinase activities on roots colonized by *Cortinarius* also decreased with warming while those on roots colonized by *Russula* were not affected by warming. Instead, activities decreased in fertilized plots. Phosphatase activities were not affected by warming and fertilization across

genera. In contrast to hydrolytic enzymes, oxidative enzymes responded only on roots colonized by *Russula*. Activities of both phenol oxidase and peroxidase decreased in fertilized plots.

4. Discussion

Overall our hypotheses were supported. While warming and fertilization both increased host plant cover, they had opposing effects on EcM root tip density and community-level activity. Warming generally increased EcM community-level activities and abundance while fertilization decreased activities at both the root tip and community levels. Warming and fertilization also altered the EcM fungal community, but only in the manner we hypothesized with fertilization. Warming did not increase the abundance of taxa with high biomass and degradative capacity; instead it increased the abundance of taxa with low biomass, contact exploration types such as *Russula spp.* Fertilization though, increased the abundance of nitrophilic taxa such as *Laccaria spp.* These community shifts explained some, but not all of the changes in enzyme activity. Additionally, responses of EcM root-associated enzyme activity and community shifts were strongly correlated with changes in nutrient availability resulting from the experimental treatments.

The compounding effects of warming and fertilization on EcM and soil responses may indicate changes in limitations to the growth of host plants and fungi (Rastetter and Shaver, 1992). Generally, warming dampened the strong effects of fertilization. We hypothesize that the positive response of growth and activity to warming increased nutrient demand, causing nutrients to remain a limiting factor despite high levels of fertilization. This would maintain host plant reliance on EcM fungal partners for nutrient acquisition. Other studies in Arctic tundra have also observed the dampening effect of warming on responses to N and P fertilization in soil DIN

concentrations (DeMarco et al., 2014b) and ecosystem respiration (Boelman et al., 2003). In a warmer and more nutrient-rich Arctic, these compounding effects and their impacts on nutrient limitation will likely guide future responses of EcM to shrub expansion.

4.1 Enzyme response

As hypothesized, activities of most enzymes sharply decreased with fertilization. This occurred at both the root tip level, where activities of three out of five enzymes decreased, and the community level, where activities of all five enzymes decreased. However, the effect of warming on enzyme activity depended on scale of measurement. We observed a contrasting response between root-level and community-level enzyme activities to warming. Whereas warming either decreased or had no effect on root tip-level enzyme activity, it increased EcM root tip abundance, resulting in increased community-level activity. Clemmensen *et al.* (2006) also found increased fungal abundance in EcM host plant roots after long-term warming. This suggests despite decreased root activity, the overall influence of EcM on ecosystem nutrient cycling may grow as the Arctic warms. These divergences also point to a potential shift in allocation, where enzymatic breakdown of SOM for nutrient acquisition by EcM may become less important as the exploration of the soil environment via increased root tips becomes more important. Importantly, these scale-dependent responses stress the necessity of obtaining functional responses at multiple ecological levels of organization to more fully identify mechanisms of change.

Some enzymes stood out as unique in their responses, or lack thereof, to treatments. Leucine aminopeptidase was the only enzyme where root-tip activities decreased with warming and where genus did not have an interaction with this response. This is an enzyme ubiquitously produced by many types of fungi, associated with the acquisition of more readily available forms

of organic N, and considered to be metabolically inexpensive to produce (Zheng et al., 2020). Thus, we were surprised to find the negative response to warming when activities of more metabolically expensive and less widely produced enzymes such as phenol oxidase did not respond (though showed trends of increasing). However, root tip activities of chitinase, also a hydrolytic N-associated enzyme, showed similar trends, decreasing with warming in unfertilized plots. This suggests that our assay might have been more sensitive for detecting leucine aminopeptidase activities than those of other enzymes. Overall, these trends may indicate that warming induced a shift from the production of hydrolytic to oxidative enzymes. This may have been needed to support the degradation of the observed increases in litter and moss cover, whose litter can be highly recalcitrant (Cornelissen et al., 2007). We also found that two enzymes—phosphatase and peroxidase—were not affected by either warming or fertilization at the scale of root tip-level activity. In addition to being produced by root endophytes like mycorrhizal fungi, plants frequently exude phosphatases and peroxidases for internal and external nutrient cycling (Courty et al., 2011; Gramss and Rudeschko, 1998; Lee, 1988; Marschner, 1996; Passardi et al., 2004). Because the nature of root tip assays makes it impossible to selectively measure activities produced only by fungi, our results could have included enzymes of host plant origin that were not associated with mycorrhizal nutrient acquisition. This would reduce the assays' sensitivity to show potential effects warming or fertilization had on these two enzymes. Alternatively, our observation of no change in root enzyme activity despite sometimes drastic differences in environment aligns with previous findings that indicate functional complementarity among different communities (Jones et al., 2010).

Responses of EcM-associated enzyme activity to warming and fertilization were driven by both changes in the soil chemical environment and in root-tip fungal community composition.

EcM-associated root enzyme activity profiles were correlated both with differences in fungal community composition and with soil DIN and C:N values. However, we also observed significant responses of EcM root tip-associated enzyme activities to warming and fertilization within the most prevalent fungal genera. Similar to other studies where enzyme activity and response highly depends on fungal identity (Jones et al., 2012, 2010), these responses were taxon specific. We found enzyme activities associated with roots colonized by *Russula spp.* to be the most sensitive to both warming and fertilization, decreasing in response to either, while roots colonized by *Lactarius spp.* appeared to be resistant to treatments, maintaining their enzyme activity and relative community abundance. These changes in enzyme activity in *Russula* and *Cortinarius* that are independent of changes in community composition highlight the importance of measuring functional responses in addition to community shifts.

Our results partially support measurements of bulk soil enzyme activity from previous studies in these same sites. While we saw fertilization reduced community-level activities of the three hydrolytic enzymes we measured (leucine aminopeptidase and chitinase, and phosphatase), Koyama et al. (2013) observed reductions only in activities of phosphatase and chitinase, but not leucine aminopeptidase. McLaren and Buckeridge (2019) similarly found fertilization reduced phosphatase activities, but did not affect chitinase activities. Bulk soil enzyme activities were less sensitive to warming than our measurements of EcM-associated community-level activities were; out of the same five enzymes we measured, only phenol oxidase activities was altered, decreasing with warming (Sistla and Schimel, 2013). The greater responsiveness of our measurements of EcM root tip enzymes could result from differences in the stability and sorption of enzymes in the rhizosphere versus in the soil (Burns, 1982), in the amount of intracellular enzymes from lysed free-living microbial cells during bulk soil enzyme assay preparation

(German et al., 2011), or from the biological differences between producers of enzymes on EcM-associated roots and those in bulk soil.

4.2 Fungal community response

Fertilization and soil DIN were stronger drivers of change in EcM root fungal community change than warming, plant community, and other soil physical characteristics. Other studies have also found increased soil inorganic N, whether through fertilization, atmospheric deposition, or naturally occurring gradients, was a strong determinant of EcM fungal communities (Kranabetter et al., 2009; Lilleskov et al., 2002, 2001; Morrison et al., 2016; Sterkenburg et al., 2015; Timling et al., 2012; Toljander et al., 2006), though how these communities changed often depended on the extent and manner of N increase. Naturally occurring increases in soil N positively affected or had no effect on EcM abundance (Kranabetter et al., 2009; Sterkenburg et al., 2015; Toljander et al., 2006). Artificial gradients such as those induced by fertilization or heavily affected by the atmospheric deposition of anthropogenic N, however, appeared to negatively impact richness and drive increases in nitrophilic fungi (Lilleskov et al., 2002, 2001; Morrison et al., 2016), seemingly due to the steeper increases in soil N. Fertilization levels in our experiment fall into the latter category of artificial amendment. While we observed increases of several fungal OTUs with fertilization that have been previously identified as nitrophilic fungi, such as *Laccaria spp.*, or N-tolerant, such as members of the family Thelephoraceae (Lilleskov et al., 2002), it is important to note that the effects of increased nutrient availability in the Arctic tundra could be different depending on how much warming will stimulate nutrient mobilization.

While there was generally an overlap between the EcM fungal community we measured and those of other studies in the immediate area, there was one notable conflict. Deslippe *et al.*(2011) and Geml *et al.* (2015) found increases in *Cortinarius spp.* and decreases in *Russula spp.* with warming at the same site while we observed the opposite. One explanation for this divergence could lie within the spatial and temporal heterogeneity of EcM fungi, where communities can appear vastly different depending on location sampled, even at highly localized scales, and are subject to "hotspot" accumulation of a particular fungal species. To account for this, we sampled root tips from nine cores within each plot while Deslippe *et al.* (2011) sampled three and Geml *et al.* (2015) five. Additionally, those studies occurred after 18 years of warming while ours after 28 years. In boreal forest, EcM fungal community succession was still developing even after 30 years, moving from high biomass EcM fungi such as *Cortinarius spp.* to low biomass fungi such as *Russula spp.* (Clemmensen et al., 2014), and could be the same case in this experiment, pointing to a common, but important, result of long-term experiments: short- and long-term responses often differ, even after decades (Knapp et al., 2012).

This study adds to our understanding of EcM SOM decomposition and points to differences among EcM fungal species. There has been a large effort to genetically quantify the potential of EcM fungi in SOM decomposition by searching genomes for genes specific to enzyme production (Bödeker et al., 2009; Kohler et al., 2015; Martin et al., 2008; Shah et al., 2016). However, to gain a fuller understanding of the influence of different EcM taxa in soil nutrient and carbon dynamics, we need more field-based measurements that link taxa-specific effects to the ecosystem scale (Bödeker et al., 2014). Across the globe, dominant mycorrhizal types mediate C cycling and storage (Averill et al., 2014; Averill and Hawkes, 2016; Clemmensen et al., 2014; R. P. Phillips et al., 2013; Soudzilovskaia et al., 2019), but studies

identifying mechanisms behind these patterns have been limited to EcM-arbuscular mycorrhizal transition zones in the temperate and southern boreal forests. Considering the rapid changes occurring in the Arctic, future research investigating taxa-specific effects of EcM on ecosystem C processes should incorporate the northern edge of the range of EcM.

This was not a complete characterization of EcM fungal communities and function in Arctic tundra. Sequencing individual root tips over bulk soil tends to underestimate diversity (Taylor, 2002) and has a bias for species that produce highly recognizable hyphal sheaths. Additionally, root-associated enzyme activities have been criticized in the past for biases involved with measuring root tip enzymes rather than those of extramatrical hyphae (Talbot et al., 2013; Wright et al., 2005) and the inability to distinguish between fungal-, plant-, and microbe- derived enzymes. While caution should be taken in the extrapolatory power of these results, particularly because this study was constrained to one, albeit ubiquitous, EcM host shrub and one tundra type, this study served as one initial step in understanding the ecosystem-wide consequences of shifts in mycorrhizal function with Arctic shrub expansion. Critical next steps include studies that ask how ErM, the opposite side of this mycorrhizal coin, respond as ericaceous shrubs co-expand with EcM shrubs and how the overall shift in mycorrhizal function ultimately affects C sequestration (Vowles and Björk, 2018).

4.3 Conclusions

We investigated the effects of long-term warming and fertilization on EcM root-associated enzyme activity and fungal community. This was the first study to pair functional responses of EcM roots to long-term experimental warming and fertilization in Arctic tundra with changes in fungal communities. Our results suggest that the future role of EcM in a

warming Arctic tundra depends on the amount of increased nutrient availability. If nutrient availability rises to levels that alleviate plant limitations to growth, EcM may play a lesser role in the enzymatic degradation of SOM. But if the anticipated nutrient release with warming is low, EcM may become more influential players. Additionally, our two levels of EcM root activity—on a per unit root area and a per unit soil volume basis—highlight the importance of scale in predicting future changes.

Acknowledgements

This research was supported by the National Science Foundation award NSF-DEB-1556496 (to MCM). We thank the Arctic LTER (NSF-DEB-1637459), especially Laura Gough and Jim Laundre, and Toolik Field Station for providing access to the long-term experiment and associated vegetation cover and weather data; Catherine Gehring, Adair Patterson, Lela Andrews, and the Environmental Genetics and Genomics Laboratory at Northern Arizona University for guidance and assistance in molecular analyses; and Samantha Miller for aid in soil chemical analyses. Additional thanks to Sedona Spann, Ruby An, Ian Kuplar, and Kai Lynöe for help in the field and lab and to Rebecca Hewitt for feedback on study design and this manuscript.

Tables

Table 2.1. Mean growing season temperature and soil characteristics ± standard error (SE) among experimental treatments. Letters indicate significant differences among treatments.

Characteristic	Control	Fertilized	Warmed	Warmed & Fertilized
Temperature (˚C) Air | Soil at 10 cm	9.5 | 0.83	9.9 | 2.1	11.1 | 2.3	12.4 | 2.5
Soil moisture	3.7 ± 0.3	2.9 ± 0.2	3.3 ± 0.3	3.3 ± 0.3
thaw depth (cm)	20.7 ± 2.0 ^{a}	18.7 ± 2.0^{a}	28.8 ± 2.0 ^{b}	20.1 ± 2.0 ^{a}
%C	40.6 ± 1.6 ^{a}	34.6 ± 1.6 ^{ab}	34.0 ± 1.7 ^{b}	40.7 ± 1.7 ^{a}
%N	1.3 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.9 ± 0.1
C:N	29.9 ± 1.9 ^{a}	23.7 ± 1.9 ^{ab}	24.1 ± 1.9 ^{ab}	20.7 ± 1.9 ^{b}
extractable TOC (mg C g^{-1} soil)	3.1 ± 0.2 ^{ac}	2.0 ± 0.2 ^{a}	6.2 ± 2.2 ^{b}	4.0 ± 1.0 ^{bc}
extractable DON (μg N g^{-1} soil)	236.9 ± 21.1	290.3 ± 77.7	300.4 ± 58.9	368.1 ± 80.8
extractable NH_4^+ (μg N g^{-1} soil)	33.5 ± 6.4 ^{a}	587.2 ± 78.6 ^{b}	51.1 ± 22.3 ^{a}	172.9 ± 36.9 ^{c}
extractable NO_3^- (μg N g^{-1} soil)	2.4 ± 0.5 ^{a}	354.6 ± 112.5 ^{b}	4.3 ± 1.9 ^{a}	63.0 ± 22.8 ^{c}

Table 2.2. Mean percent cover ± SE of aboveground vegetation among experimental treatments. Letters indicate significant differences among treatments.

Species	Control	Fertilized	Warmed	Warmed & Fertilized
Betula nana	10.3 ± 1.8 [a]	20.3 ± 3.8 [b]	26.8 ± 2.9 [bc]	38.3 ± 5.0 [c]
Salix pulchra	2.6 ± 0.6 [a]	0.3 ± 0.2 [b]	3.6 ± 0.9 [a]	4.0 ± 1.6 [ab]
Standing dead *B. nana*	1.7 ± 0.6 [a]	36.1 ± 2.7 [b]	16.1 ± 2.5 [c]	16.6 ± 2.1 [c]
Graminoid	13.8 ± 1.1 [a]	0.1 ± 0.1 [b]	4.6 ± 1.2 [c]	3.1 ± 1.0 [c]
Ericaceous	24.2 ± 2.0 [a]	0.5 ± 0.2 [b]	11.0 ± 1.8 [c]	5.3 ± 1.2 [d]
Moss	15.8 ± 1.5 [a]	0.8 ± 0.4 [b]	4.1 ± 1.0 [c]	1.4 ± 0.5 [bc]
Rubus chamaemorus	9.75 ± 1.6 [a]	23.9 ± 3.1 [b]	25.0 ± 4.0 [b]	26.3 ± 2.8 [b]
Other forbs	1.2 ± 0.1 [a]	0 ± 0.1 [b]	0 ± 0.1 [b]	0 ± 0.1 [b]
Lichen	3.5 ± 0.5 [a]	0.4 ± 0.1 [b]	0.1 ± 0.03 [c]	0.05 ± 0.03 [c]
Litter	6.2 ± 0.6 [a]	19.9 ± 1.8 [b]	11.6 ± 0.9 [c]	9.6 ± 1.0 [c]

Table 2.3. Negative Log-likelihood (-log(L)), corrected Akaike information criteria (AICc), AICc differences from the lowest scoring model (ΔAICc), and Akaike weight (w) from EcM root tip enzyme activity linear mixed effects models. All models contain a random effect of (~1|Block/Plot/Core). Selected model is in **bold**.

Enzyme	Model	-log(L)	AICc	ΔAICc	w
Leucine aminopeptidase	~ 1	-2006	4022	19	0
	~ Warming + Fertilization	-1998	4011	8	0.02
	~ Warming * Fertilization	**-1993**	**4003**	**0**	**0.98**
Chitinase	~ 1	-3795	7601	7	0.02
	~ Warming + Fertilization	-3791	7596	2	0.22
	~ Warming * Fertilization	**-3789**	**7594**	**0**	**0.76**
Phosphatase	**~ 1**	**-4695**	**9401**	**0**	**0.48**
	~ Warming + Fertilization	-4694	9402	1	0.22
	~ Warming * Fertilization	-4693	9402	1	0.30
Phenol oxidase	~ 1	-5278	10568	14	0
	~ Warming + Fertilization	-5271	10556	2	0.22
	~ Warming * Fertilization	**-5269**	**10554**	**0**	**0.78**
Peroxidase	**~ 1**	**-7117**	**14245**	**0**	**0.55**
	~ Warming + Fertilization	-7116	14246	1	0.33
	~ Warming * Fertilization	-7116	14248	3	0.12

Table 2.4. Mean EcM root abundance, healthy fraction of root tips, and alpha diversity metrics among treatments ± SE. Letters indicate significant differences within each enzyme among treatments. * indicates singular effect of fertilization.

	Control	Fertilized	Warmed	Warmed & Fertilized
EcM root tip/ cm^3 soil	0.5 ± 0.1 ^{ab}	0.4 ± 0.2 ^{a}	1.3 ± 0.4 ^{c}	1.0 ± 0.3 ^{bc}
Healthy fraction of root tips	0.80 ± 0.04	0.64 ± 0.04*	0.88 ± 0.04	0.77 ± 0.07
OTU richness	9.3 ± 1.6	6.0 ± 0.6	8.5 ± 1.0	9.3 ± 1.8
Simpson's diversity index	0.80 ± 0.04 ^{a}	0.54 ± 0.08 ^{b}	0.70 ± 0.04 ^{ab}	0.82 ± 0.04 ^{a}

Table 2.5. Negative Log-likelihood (-log(L)), corrected Akaike information criteria (AICc), AICc differences from the lowest scoring model (ΔAICc), and Akaike weight (w) from EcM community-level enzyme activity linear mixed effects models. All models contain a random effect of (~1|Block/Plot). Selected model is in **bold**.

Enzyme	Model	-log(L)	AICc	ΔAICc	w
Leucine aminopeptidase	~ 1	-219	446	9	0.01
	~ Warming + Fertilization	-213	439	2	0.24
	~ Warming * Fertilization	**-211**	**437**	**0**	**0.75**
Chitinase	~ 1	-450	909	7	0.02
	~ Warming + Fertilization	**-444**	**901**	**1**	**0.63**
	~ Warming * Fertilization	-444	902	0	0.35
Phosphatase	~ 1	-563	1133	6	0.04
	~ Warming + Fertilization	**-557**	**1128**	**0**	**0.65**
	~ Warming * Fertilization	-557	1129	1	0.31
Phenol oxidase	~ 1	-707	1422	27	0
	~ Warming + Fertilization	-693	1399	4	0.10
	~ Warming * Fertilization	**-690**	**1395**	**0**	**0.90**
Peroxidase	~ 1	-873	1755	17	0
	~ Warming + Fertilization	**-863**	**1738**	**0**	**0.71**
	~ Warming * Fertilization	-863	1740	2	0.29

Table 2.6. Negative Log-likelihood (-log(L)), corrected Akaike information criteria (AICc), AICc differences from the lowest scoring model (ΔAICc), and Akaike weight (w) from EcM root enzyme activity by taxa linear mixed effects models. All models contain a random effect of (~1|Block/Plot/Core). Selected model is in **bold**.

Enzyme	Model	-log(L)	AICc	ΔAICc	w
Leucine aminopeptidase	~ 1	-646	1302	52	0
	~ Warming * Fertilization + Genus	**-614**	**1249**	**0**	**0.68**
	~ Warming * Fertilization * Genus	-608	1251	2	0.32
Chitinase	~ 1	-1276	2562	39	0
	~ Warming * Fertilization + Genus	-1260	2540	17	0
	~ Warming * Fertilization * Genus	**-1245**	**2523**	**0**	**1**
Phosphatase	~ 1	-1660	3330	7	0.01
	~ Warming * Fertilization + Genus	-1652	3324	1	0.39
	~ Warming * Fertilization * Genus	**-1645**	**3323**	**0**	**0.60**
Phenol oxidase	~ 1	-2087	4185	139	0
	~ Warming * Fertilization + Genus	-2022	4064	18	0
	~ Warming * Fertilization * Genus	**-2006**	**4046**	**0**	**1**
Peroxidase	~ 1	-2510	5029	50	0
	~ Warming * Fertilization + Genus	-2595	5011	32	0
	~ Warming * Fertilization * Genus	**-2473**	**4979**	**0**	**1**

Figures

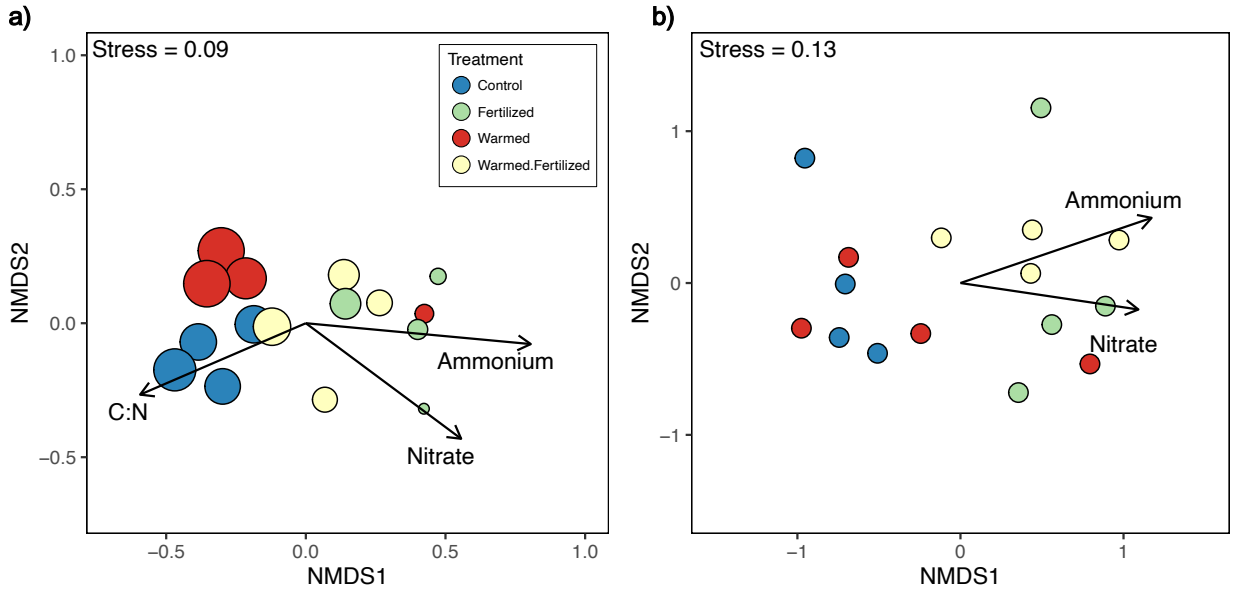

Fig. 1. Nonmetric multidimensional scaling of the Bray-Curtis distances between relativized a) root tip enzyme profiles and b) fungal relative abundances from a 28-year warming and fertilization experiment at Arctic LTER, Toolik Lake, AK. Points are scaled by relativized total enzyme activity (a). Vectors represent the direction and magnitude of significant correlations with environmental variables.

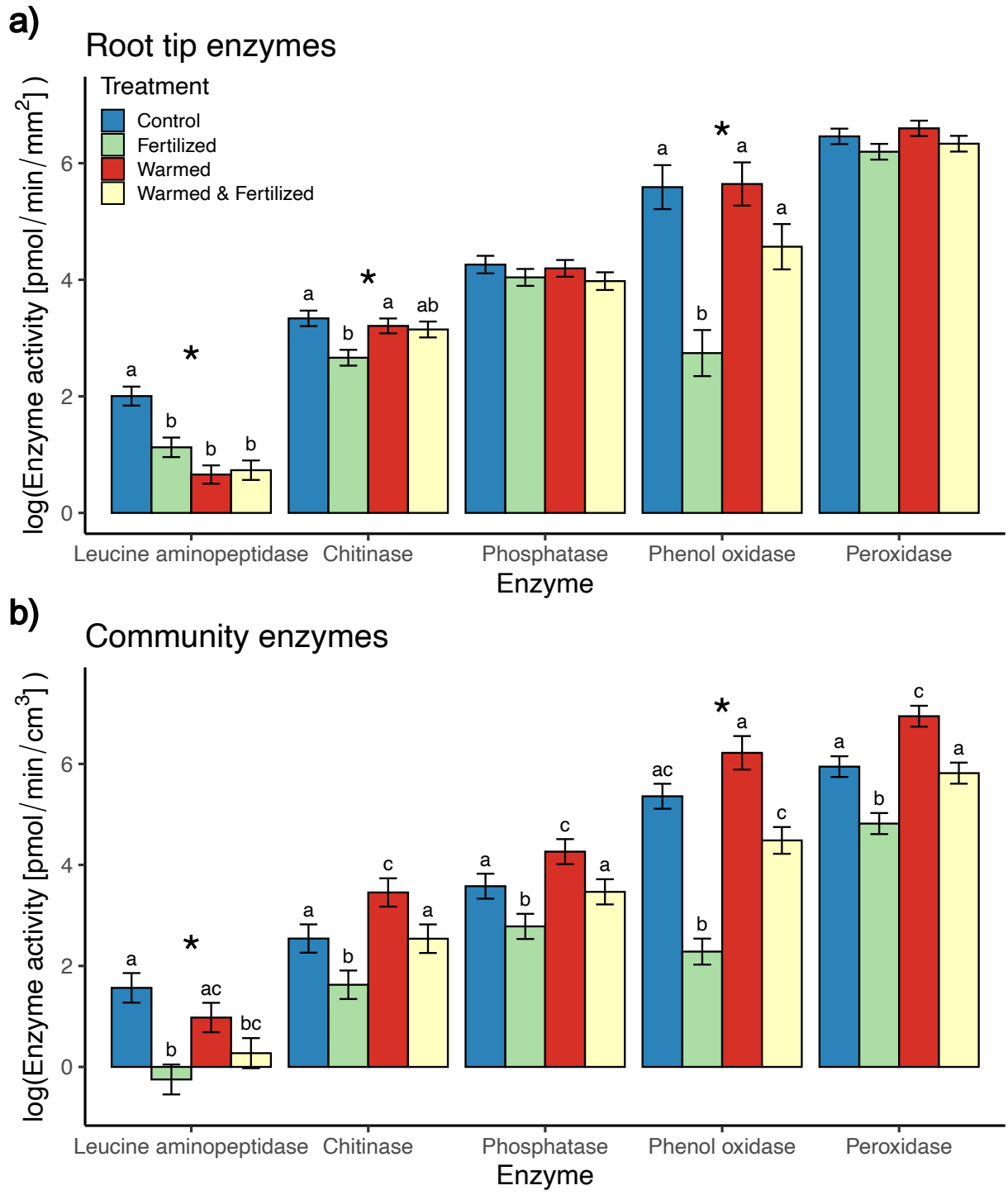

Fig. 2. Mean potential enzyme activity ± SE for five enzymes for a) a root tip-level activities and b) community-level activities among treatments. Activities have been log-transformed. * indicates a significant warming x fertilization interaction. Letters indicate significant differences within each enzyme among treatments.

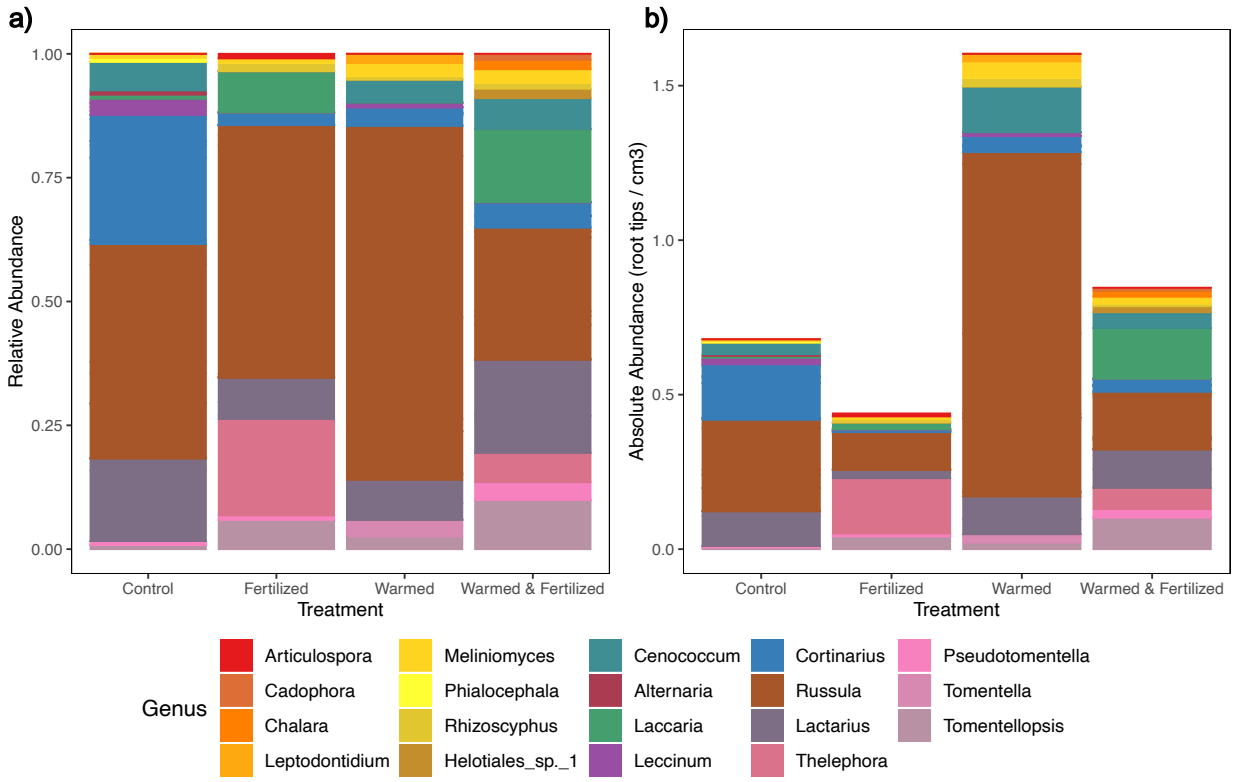

Fig. 3. a) Relative and b) absolute abundances of root-associated fungal genera among treatments.

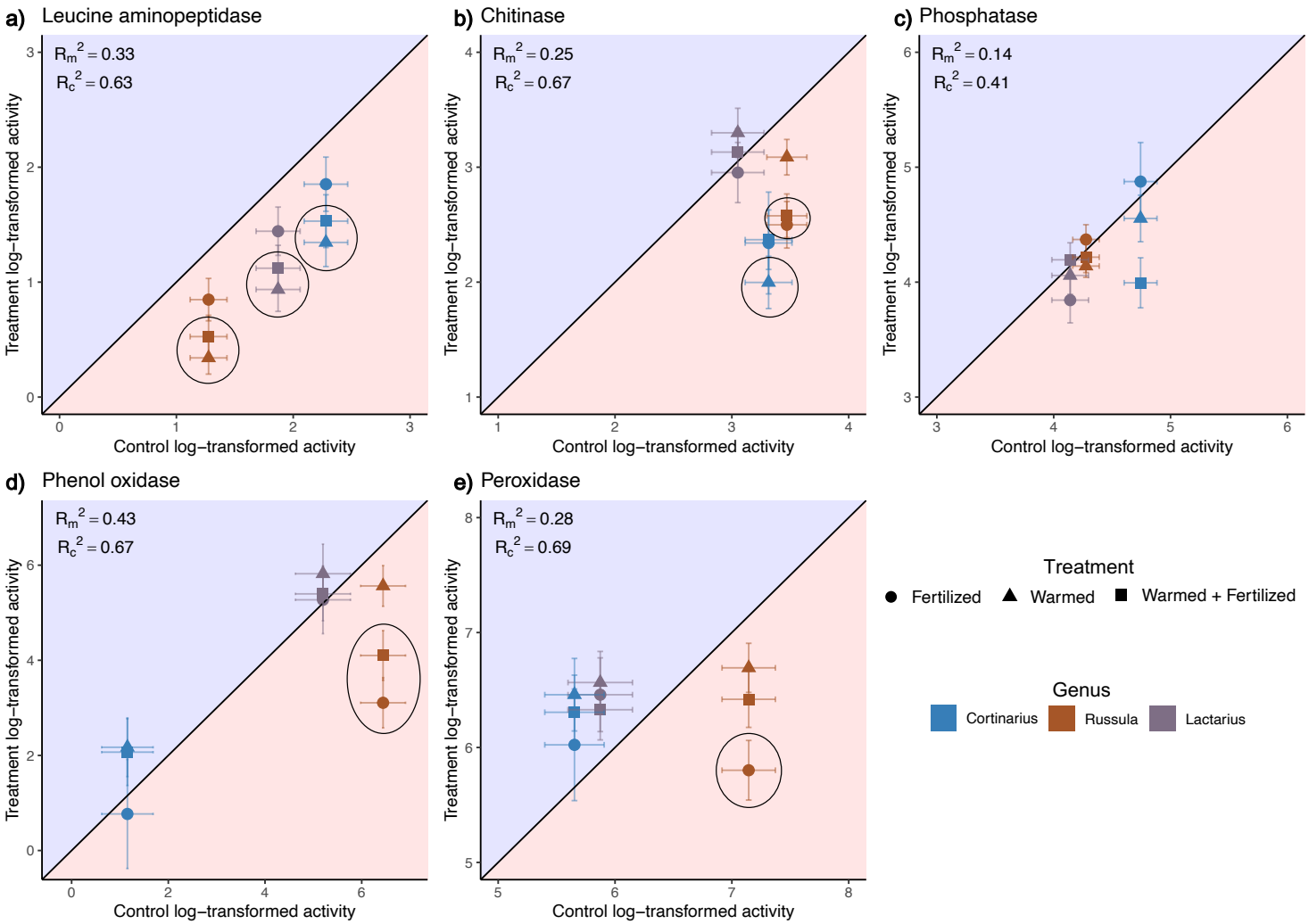

Fig. 4. Responses of log-transformed a) leucine aminopeptidase, b) chitinase, c) phosphatase, d) phenol oxidase, and e) peroxidase activities (pmol mm-2 min-1) of roots colonized by Cortinarius, Russula, and Lactarius spp. to treatments. Plotted line is the 1:1 line. Points above the line represent a positive response to treatment, points below represent a negative response, and points on the line represent no change. Error bars indicate SE. Circled points denote a significant difference in activity from the control. Marginal (R^2_m) and conditional R2 (R^2_c) from linear mixed effects models provided in top left corner of each graph.

CHAPTER 3: NON-LINEAR RESPONSES OF ERICACEOUS AND ECTOMYCORRHIZAL SHRUB COVER AND ROOT ENZYME ACTIVITY ACROSS A LONG-TERM EXPERIMENTAL SOIL FERTILITY GRADIENT IN ARCTIC TUNDRA

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Keywords: Arctic tundra; *Betula nana*; Ericaceae; fertilization; long-term experiment; root enzyme; shrub expansion; soil fertility gradient

Abstract

1. In Arctic tundra, warming is anticipated to stimulate nutrient release and potentially alleviate plant nutrient limitations. Typically simulated by high dose fertilization experiments that saturate plant nutrient demand, the future increases in soil fertility are thought to favor ectomycorrhizal (EcM) rather than ericaceous shrubs, and have often been identified as a key driver of Arctic shrub expansion. However, the projected increases in soil fertility will likely range in the extent to which they alleviate nutrient limitations. The resulting responses of shrubs and their mycorrhizal partners across the gradient of nutrient limitation may be non-linear and could contradict current predictions surrounding tundra vegetation shifts.
2. We compared the functional responses of two dominant tundra shrubs, EcM *Betula nana* and ericaceous *Rhododendron tomentosum*, and their root enzymes across a long-term nitrogen and phosphorus fertilization gradient experiment in Arctic Alaska. We measured shrub cover, height, and root enzyme activities. We then tested their responses via linear mixed effects modeling to two soil fertility indices derived from a multivariate ordination of soil chemical properties.
3. We found that *Betula nana* cover and height linearly increased with increasing soil fertility. In contrast, *Rhodendron tomentosum* cover initially increased, but began decreasing after surpassing intermediate levels of increased soil fertility. Its height did not change. Enzyme activity did not respond to soil fertility on EcM-colonized *B. nana* roots, but sharply declined on *R. tomentosum* roots with increasing fertility.
4. *Synthesis:* Overall, the non-linear responses of shrub cover and root enzyme activity to the soil fertility gradient demonstrate the importance of experiments grounded in

replicated regression design. Our results indicate that under moderate increases in soil fertility, Arctic shrub expansion may not only include deciduous EcM shrubs, but ericaceous shrubs as well. Regardless of plant community shifts aboveground, changes in root enzyme activity belowground point to EcM playing a more influential role in tundra soils; as EcM roots remained steady in their liberation of soil organic nutrients with heightened soil fertility, degradative enzyme activity on ericaceous roots dropped—even at small levels of nutrient increase.

Introduction

Almost a century of research has determined that Arctic tundra plants are nutrient limited (Russell *et al.*, 1940; Bliss, 1971; Haag, 1974; Shaver & Chapin, 1980; Chapin *et al.*, 1995). In testing hypotheses of nutrient limitation, researchers implemented experimental fertilization at high doses that were intended to saturate plant nutrient demand (Chapin et al., 1986). These experiments were designed to answer a binary question: is tundra vegetation nutrient limited or not? Decades of measuring changes in these studies have not only found ample support for the presence of plant nutrient limitation but have also molded our understanding of tundra ecosystem processes.

From these experiments, we have learned that 1) not all plants or tundra ecosystem types equally benefit from added nutrients (Chapin et al., 1995; Shaver et al., 2001; Van Wijk et al., 2003), 2) short-term responses often differ from long-term trends, and 3) nutrient addition interacts with other environmental factors to determine responses of ecosystem pools and fluxes (Bret-Harte et al., 2008; Mack et al., 2004). Yet, as current research attempts to apply the knowledge gained from these studies to predictions of future Arctic vegetation change—specifically woody shrub expansion—the binary experimental design is proving to be insufficient. This design is unable to capture the potentially nuanced, non-linear plant responses that occur across a gradient of nutrient limitation (Cottingham et al., 2005). Given that warming-induced nutrient release will likely be spatially and temporally heterogeneous (Keuper et al., 2012; Salmon et al., 2018, 2016), identifying the functional shape of plant response across a soil fertility gradient is increasingly important as we consider the pace and implications of Arctic vegetation change. In light of this, a new question has emerged: how do tundra shrubs respond when nutrient limitations are alleviated, but not fully relieved?

Most studies of relationships between nutrient availability and Arctic shrub expansion have focused on deciduous ectomycorrhizal (EcM) shrubs rather than evergreen ericaceous shrubs (Myers-Smith et al., 2011; Vowles and Björk, 2018). In high dose fertilization experiments, deciduous EcM shrubs, such as *Betula spp.*, frequently become dominant canopy species and outcompete evergreen ericaceous shrubs (DeMarco et al., 2014b; Shaver et al., 2001; Zamin et al., 2014). This has been hypothesized to result from enhanced nutrient acquisition (Bret-Harte et al., 2008; Clemmensen et al., 2006; Deslippe et al., 2011) and plastic growth strategies (Bret-Harte et al., 2001; Heskel et al., 2013; Prager et al., 2020; Zamin and Grogan, 2012). However, when increases in soil fertility do not fully lift nutrient limitations, ericaceous shrubs may remain competitive with EcM shrubs (Chapin, 1980; Grime, 1977). Evergreen ericaceous shrubs respond positively to warming and subtler increases in fertility (Elmendorf et al., 2012; Vowles and Björk, 2018; Weijers et al., 2018; Zamin et al., 2014). Their growth, therefore, may have a quadratic, or hump-shaped, relationship, with soil fertility, which would imply ericaceous shrubs could co-expand with EcM shrubs at intermediate stages of the nutrient limitation gradient. Co-expansion of these shrubs would broaden the pool of species we consider to be important when considering impacts of expansion. Despite the differing effects these contrasting shrub functional types may have on important ecosystem properties and functions, such as litter input (Christiansen et al., 2018; DeMarco et al., 2014a, 2011; Hobbie, 1996; Hobbie and Gough, 2004), albedo (Chapin et al., 2005; Loranty et al., 2011; Sturm et al., 2005a), or mycorrhizal activity (Read and Perez-Moreno, 2003; Vowles and Björk, 2018), the response of ericaceous shrubs across a soil fertility gradient and the level of fertility at which EcM shrubs dominate remain largely unexplored topics. Experimental soil fertility gradients that capture responses of both these contrasting shrub

functional types are needed to understand the potential for co-expansion and to incorporate non-linear responses into ecosystem models.

Shifts in belowground mycorrhizal function are likely to occur as EcM and ericaceous shrubs change in abundance. Ecto- and ericoid mycorrhizae (ErM) exert a strong influence over nutrient cycling in Arctic tundra through their exudation of degradative enzymes (Hobbie and Hobbie, 2006; Read and Perez-Moreno, 2003). These enzymes target organically-bound nutrients in soil and allow for plant nutrient acquisition in an environment with limited nutrient mobilization. As shrubs travel the gradient from nutrient limited to not, their mycorrhizal-associated enzyme activity may have a quadratic response. Sharp increases in soil fertility can reduce activity by shifting mycorrhizal fungal communities towards nitrophilic species with low degradative capacity (Lilleskov et al., 2002). Additionally, heightened soil fertility could reduce host plant C allocation to their fungal partners, and thus enzyme production, through 1) decreasing dependence on mycorrhizal nutrient acquisition or 2) decreasing host plant success in interspecific competition. Subtle increases in fertility, by contrast, may stimulate activity. Changes in the activity of degradative root enzymes, concurrent with the aboveground expansion of shrubs, will alter tundra soil processes and could affect C dynamics in a globally important C-rich ecosystem (Schuur et al., 2015). Therefore, pairing aboveground shrub responses with those of root enzyme activity across a fertility gradient is a necessary step to understanding the nuanced consequences of shrub expansion.

Direct measurements of shrub-associated mycorrhizal enzyme activity are limited. In Arctic tundra, EcM fungal communities and abundance change with naturally and experimentally increased soil nutrient availability (Clemmensen et al., 2006; Deslippe et al., 2011; Timling et al., 2012; Urcelay et al., 2003), though, sometimes in opposing ways. Our previous work indirectly

points to a quadratic relationship between soil fertility and EcM-associated root enzyme activity: long-term high dose fertilization reduced enzyme activity while warming moderately increased soil fertility and stimulated the activity of some enzymes (Dunleavy & Mack, 2021). The decreases with fertilization occurred even as host shrub cover became monodominant. The enzymatic response of ericaceous roots to increased tundra soil fertility is unknown. Some evidence suggests preferential acquisition of organic forms of nitrogen (N) by ericaceous plants even in the presence of inorganic N (Michelsen et al., 1996; Read, 1991). Genetically, ErM often have a higher enzymatic capacity than EcM and at times can live as independent saprotrophs (Martino et al., 2018; Perotto et al., 2018). Given this, ericaceous root enzymes may be less sensitive to heightened soil fertility than the enzymes of obligately partnered EcM roots.

In this study, we compared the functional responses of two dominant tundra shrubs, EcM *Betula nana* and ericaceous *Rhododendron tomentosum*, and their root enzymes across a long-term nutrient addition gradient experiment in Arctic Alaska. To capture a rough estimate of shrub volume, we measured shrub cover and height. We also assayed potential root activities across a suite of five enzymes varying in targeted substrate and degradative mechanism. We hypothesized cover, height, and root enzyme activities of both shrub species would initially rise as soil fertility increased and nutrient limitations were alleviated (Fig.1). Once nutrient limitations were fully relieved, *B. nana* biomass would continue to linearly increase, yet *R. tomentosum* would decrease as informed by prior high fertilization studies (Bret-Harte et al., 2001; Shaver et al., 2001; Zamin et al., 2014). At this point, both shrubs would decrease in their root activity. In *B. nana,* this decrease would be in response to a weakening of its reliance on mycorrhizal nutrient acquisition; in *R. tomentosum*, it would be in parallel to its aboveground decline. Additionally, we hypothesized the ericaceous *R. tomentosum* root enzymes would be less sensitive to changes in soil fertility than

those of the EcM-associated *B. nana* because of their putative preference for organic N and versatile mycorrhizal-saprobic lifestyle.

Materials and Methods

Study site

We conducted this study near Toolik Lake at the Arctic Long-Term Ecological Research site (68˚38' N, 149˚34'W, 780 m above sea level) on the North Slope of Alaska. The site is located on a gradual east-facing slope in moist acidic tussock tundra. Vegetation is dominated by roughly equal amounts of graminoids, dwarf deciduous and evergreen shrubs, and mosses. Soils consist of a moist organic horizon with a mean thickness of 30 cm over a silty mineral horizon underlain by continuous permafrost.

A nutrient addition gradient experiment was established in 2006. This experiment consists of four replicate blocks that contain six 5 m x 20 m plots. Each plot receives one of six treatments. Treatments include control, 0.5, 1, 2, 5, and 10 g N m^{-2} yr^{-1} fertilization with half the amount of P. Plots were fertilized with ammonium nitrate (NH_4NO_3) and triple superphosphate (P_2O_5) every spring following snowmelt until 2012. Since 2012 plots have been fertilized with ammonium chloride and sodium nitrate (NH_4Cl and $NaNO_3$); P fertilization has remained the same. Previous studies report on plant community, ecosystem C fluxes, and leaf traits (Prager et al., 2020, 2017).

Sample collection

We sampled organic soils from 5 cm below to surface of the green moss down to 15 cm deep using a 4.2 cm diameter metal corer between 2-13 July 2018, when vegetation is nearing

peak biomass. Nine cores were taken from each of the six plots for EcM-colonized *Betula nana* root tip collection across a 3 x 3 grid in a 3 m x 3 m area (Fig. S1). In control plots and fertilizer plots that received 2 g and 10 g N m^{-2} yr^{-1}, we also sampled *Rhododendron tomentosum* rhizomes and roots for ericaceous root tip collection at the same points we cored. These were verified to be *R. tomentosum* by connecting belowground material to aboveground growth. We took an additional three cores within each sampling area per plot for soil chemical analyses. Cores and plants were stored intact at 4˚C for no more than 72 hours before excising root tips. Soil analyses were conducted the day of core collection.

Betula nana and *R. tomentosum* cover was visually estimated in five permanent 1 m^2 quadrats per plot in late July 2020 by the Arctic LTER and Toolik Field Station Spatial and Environmental Data Center, using methods described by Gough (2019). Shrub height was measured as the stem length from the base of each stem to the apical tip, taken from four random shrubs per quadrat per species.

Soil analysis

We measured soil moisture, bulk %C and %N, and total dissolved organic carbon (TOC), total dissolved nitrogen (TN), dissolved inorganic N (DIN; NH_4-N and NO_3 -N), and orthophosphate (PO_4-P) concentrations. The day of collection, coarse material (> 2 mm) was removed from cores and remaining soil was homogenized by hand. A subsample was dried at 60˚C for 48 hours for gravimetric soil moisture and bulk C:N analysis. The dried sample was ground and bulk C:N content was measured via combustion mass spectrometry on an Elemental Analyzer (Costech Analytical Technologies Inc., USA). Total organic carbon and TN were extracted from 10 g of field moist soil in 50 mL 0.5 M K_2SO_4. Extracts were shaken for 2 hours

on a shaker table, placed overnight at 4˚C to settle, and vacuum-filtered through a Whatman GF/A filter. Filtrate was frozen and stored at -20˚C until further analysis. A subsample of filtrate was processed for TOC and TN via combustion on a Total Organic Carbon Analyzer TOC-L (Shimadzu Corporation, Japan) and a separate subsample was processed for DIN on a SmartChem 200 Discrete Chemistry Analyzer (Unity Scientific, USA) via colorimetry using the Ammonium Salicylate method (Unity Scientific Method 210-203D) for NH_4-N detection and the Nitrate method (Unity Scientific Method NO3-001-A) for NO_3 -N detection. Dissolved organic nitrogen (DON) was calculated as TN minus DIN. Orthophosphate was extracted from 2.5 g of air-dry soil in 10 ml of 0.025 N H_2SO_4 and 0.05 N HCl double acid solution. Extracts were shaken for 5 min on a shaker table and vacuum-filtered through a Whatman No. 5 filter. We measured PO_4-P concentrations of filtrate using the Ascorbate method (Murphy and Riley, 1962). Absorbances were read at 880 nm on a Powerwave XS microplate reader (Biotek, USA).

Root enzyme activity

To characterize root enzyme activity and allocation to organic nutrient acquisition, we conducted extracellular enzyme assays on EcM and ericaceous root tips, corresponding to *B. nana* and *R. tomentosum*, respectively. On the same day that we conducted assays, we excised a maximum of 6 healthy root tips from each core and plant, verified by turgidity and a white inner pith under a stereomicroscope, for a maximum of 40 EcM root tips and 40 ericaceous root tips per plot. Total number of root tips sampled per plot ranged between 16 and 40 tips due to a lack of healthy roots in some plots. To capture the heterogeneity among roots within a plot, we took no more than two root tips from each fine root segment within a core or from each plant. Excised

root tips were stored in 100 μl tap water in 96-well plates at 4˚C for no more than 9 hours and on average 5 hours before starting enzyme assays.

Following the root tip enzyme assay protocol described in Pritsch *et al.* (2011) and the peroxidase assay in Johnsen and Jacobsen (2008), we measured potential activities of five enzymes—two associated with labile N acquisition [leucine amino peptidase (EC 3.4.11.1) and N-acetyl glucosaminidase (hereafter chitinase; EC 3.2.1.14)], one with P acquisition [phosphomonoesteratase (hereafter phosphatase; EC 3.1.3.2)], and two with nutrient acquisition via oxidative breakdown of complex carbon compounds [laccase (hereafter phenol oxidase; EC 1.10.3.2) and peroxidase (EC 1.11.1.7)]—on root tips in a series of fluorometric and colorimetric assays. Activities of three hydrolytic enzymes were measured using fluorogenic substrates [4-methylumbelliferone (MUB) N-acetyl glucosaminide for chitinase and 4-MUB-phosphate for phosphatase; 7-amino-4-methylcoumarin labeled leucine for leucine aminopeptidase]; activities of two oxidative enzymes were measured using colorimetric substrates [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), hereafter ABTS, for phenol oxidase and 3,3',5,5'-tetramethylbenzidine, hereafter TMB, for peroxidase].

During assays, we sequentially incubated EcM root tips in concentrated substrate solutions in buffers at the appropriate pH associated with each enzyme. Root tips were incubated in the dark at room temperature on a shaker table. At the end of leucine aminopeptidase, chitinase, and phosphatase assays, we transferred incubated solution to clean, black microplates filled with 150 μl 1 M Tris stop solution and measured fluorescence at 360 ± 20 nm excitation and 460 ± 20 nm emission. At the end of phenol oxidase assays, incubation solutions were transferred to clean, clear plates and absorbance was measured at 420 nm. Incubation solutions from peroxidase assays were transferred to clean, clear plates filled with 30 μl 1 M H_2SO_4 and

absorbance was measured at 450 nm. All fluorescence and absorbance measurements were taken on a Synergy HTX microplate reader (Biotek, USA). Root tips were rinsed between incubations with diluted incubation buffer before beginning the assay for the next enzyme. After completing the final assay, root tips were scanned in 60 μl tap water in a 96-well plate and projected surface area was measured in WinRHIZO software program (Regent Instruments, Inc., Québec, Canada). Potential enzyme activity was calculated as pmol substrate converted mm^{-2} root tip min^{-1} (hereafter pmol mm^{-2} min^{-1}) using standard curves for hydrolase substrates and the Beer-Lambert law for ABTS ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}; Pritsch *et al.*, 2011) and TMB ($\varepsilon_{450} = 5.9 \times 10^4$ M^{-1} cm^{-1}; Josephy *et al.*, 1982).

Statistical analysis

We analyzed the effects of nutrient addition on aboveground and belowground shrub traits using linear mixed effects models. All analyses were performed in R version 4.0.2 (R Core Team, 2019). General linear mixed effects models were fit using the function 'glmmTMB' in the package *glmmTMB* (Magnusson et al., 2017).

To ensure fertilizer treatments produced the intended increases in nutrient availability, we tested the effect of nutrient addition on soil DIN and PO_4-P using log-gamma linear mixed models. Models included fixed effects of amount of fertilizer added, with both 1^{st} and 2^{nd} order polynomial terms, and random effects of plot within block. We fit subsequently reduced models and chose the best fitting model based on AIC values.

To account for the collinearity of relationships among soil chemical variables, we created soil fertility indices from the axes of an NMDS ordination to use in subsequent analyses. We fit a two-dimensional ordination using the Bray-Curtis dissimilarity matrix of relativized DIN, PO_4,

DON, TOC, and bulk soil C:N ratio, %C, and %N using the function metaMDS in the package *vegan* (Oksanen et al., 2019). We then extracted the axis values as soil fertility indices that we used as fixed effects in linear mixed effects models described below. We tested the Pearson's correlation between each NMDS axis and each soil chemical variable to characterize the changes in soil chemistry represented by each index. Axis 1 and axis 2 correlated with inorganic and organic soil chemical variables, respectively, and thus hereafter referred to as the inorganic and organic indices.

We used linear mixed effects models to assess the effect of soil fertility indices and species on shrub cover, shrub height, and root enzyme activity. Shrub cover and height models were fit using a normal distribution; enzyme activity models were fit using a log-gamma distribution. Models were comprised of the interactive fixed effects of species with inorganic and organic indices. Because we hypothesized a quadratic relationship between soil fertility and some of our dependent variables, we included 1st and 2nd order polynomial terms for both indices in our full models. To account for the spatial dependencies in our nested sampling design, we included nested random effects of plot within block for shrub cover models; quadrat within plot within block for shrub height models; and core within plot within block for enzyme activity models. We used backward model selection to find the best fitting model for each of our dependent variables based on AIC_c values. Afterward, residuals for each best fitting model were visually inspected for homoscedasticity. We then calculated the marginal and conditional R^2 for the best fit model via the trigamma method using the function 'r.squaredGLMM' in the package *MuMIn* (Barton and Barton, 2012).

To reconcile zeros within our enzyme activity data with a log-gamma distribution, we added a value of 1×10^{x} that was on the same order of magnitude as the minimum activity each

enzyme. For leucine aminopeptidase, phosphatase, and peroxidase, zero values made up 0.5, 0.1, and 0.9%, respectively, of the total sample size. For phenol oxidase, zero values made up 35% of the sample size. To ensure adding a minimum value did not alter model outcome, we ran preliminary parallel models excluding zeros for leucine aminopeptidase, phosphatase, and peroxidase activities, and a zero-adjusted gamma model for phenol oxidase activity. We did not find that adding a minimum value affected model residuals or coefficient estimates.

Sensitivity of root enzymes

To evaluate whether EcM or ericaceous roots were more sensitive to changes in each soil fertility index, we compared the rates of change for each enzyme with respect to each index. Because many of the best fitting models included a quadratic term, we solved the partial derivatives with respect to each soil fertility index for each enzyme and root type to calculate the rates of change:

$$f(x_i, x_o)_{root} = \log(y) = \beta_{0_{root}} + \beta_{1_{root}} x_i + \beta_{2_{root}} {x_i}^2 + \beta_{3_{root}} x_o + \beta_{4_{root}} {x_o}^2$$

$$\frac{\partial f(x_i, x_o)_{root}}{\partial x_i} = \beta_{1_{root}} + 2\beta_{2_{root}} x_i$$

$$\frac{\partial f(x_i, x_o)_{root}}{\partial x_o} = \beta_{3_{root}} + 2\beta_{4_{root}} x_o$$

Where $f(x_i, x_o)_{root}$ is the model for each enzyme dependent on root type, x_i and x_o refer to the inorganic nutrient and organic indices, respectively, and β_n are the coefficient estimates from our final model.

To calculate which root type was more sensitive to the inorganic and organic indices, we plotted the absolute values of partial derivatives (i.e. magnitude of change) of each root type with

respect to the same index. We considered the root type with the higher magnitude of change to be more sensitive.

Results

Soil fertility

In general, nutrient addition increased nutrient availability in the intended manner. Fertilization was linearly related to DIN concentration and quadratically related to PO_4-P concentration (Table S1). Dissolved inorganic N increased with increasing amount of fertilizer added; concentrations increased gradually until 5 g N m^{-2} $year^{-1}$ after which concentrations increased rapidly ($R_m^2 = 0.36$, $R_c^2 = 0.40$; Fig. 2a). Orthophosphate concentrations increased until a similar inflection point as DIN, after which they decreased ($R_m^2 = 0.31$, $R_c^2 = 0.31$; Fig. 2b). Total dissolved organic C, DON, and bulk soil %C, %N, and C:N ratio did not correlate with fertilization.

To capture the overall change in soil chemical environment with nutrient addition, we created a two-dimensional NMDS ordination (stress = 0.14) and extracted two indices of soil fertility from its two axes (Fig. 3). The first axis, hereafter referred to as the inorganic index, positively correlated with DIN, DON, and PO_4-P (Table 1). The second axis, hereafter the organic index, positively correlated with bulk soil %C, %N, and PO_4-P. This axis also negatively correlated with TOC, DON, and bulk soil C:N ratio.

Aboveground shrub cover

We modeled shrub cover and height as a function of the quadratic fixed effects of the inorganic and organic indices and their interaction with species to test our hypothesized

relationship of cover and height with soil fertility (Table S2). Shrub cover was strongly explained by the inorganic index, though the nature of the relationship depended on species (R_m^2 = 0.39, R_c^2 = 0.49; Fig. 4a). In support of our hypothesis, *B. nana* cover linearly increased with the inorganic index while *R. tomentosum* cover displayed a negative quadratic relationship. *Rhododendron tomentosum* cover increased until intermediate nutrient levels after which it sharply decreased. Both *B. nana* and *R. tomentosum* were quadratically related to the organic index, initially slightly decreasing until intermediate index values, after which cover returned to levels similar to levels that were at low index values (Fig. 4b). Stem heights linearly increased with inorganic index, but were not related to the organic index (R_m^2 = 0.49, R_c^2 = 0.57; Fig. 4c, d). *B. nana* stem heights increased with the inorganic index while those of *R. tomentosum* did not change.

Root enzyme activity

We modeled root activities of each enzyme as a function of the quadratic fixed effects of the inorganic and organic indices and their interaction with species to test for the hypothesized quadratic relationship to soil fertility and for differences among species (Table S3). Contrary to our hypothesis, the relationship between enzyme activities and indices did not form the predicted negative quadratic shape and differed greatly between EcM *B. nana* and ericaceous *R. tomentosum* roots. In four of the five enzymes, we found a significant interaction between soil fertility indices and species (Table 2). Generally, activities on EcM roots did not significantly respond to indices (Table 2). In contrast, we found that activities on ericaceous roots responded non-linearly to fertility indices, as shown by significant 2nd order polynomial model parameters (Table 2). However, they did not form the quadratic shape we predicted (Figs 5-7). Additionally, intercept estimates of ericaceous root activities were significantly lower than those of EcM roots

for all enzymes (Table 2). Activities of most enzymes tended to be related to the inorganic rather than the organic index. The best fitting models for four of the five enzymes contained the fixed effects of the inorganic index while those for only two of the five enzymes contained the fixed effects of the organic index (Table 2). However, the shape of the relationship to each index varied among enzymes, even within their broad classes (Figs 5-7).

Responses of hydrolytic enzymes to either fertility index were mixed. Leucine aminopeptidase activities did not support our quadratic hypothesis and instead linearly decreased with the inorganic index on both ericaceous and EcM roots. Model results showed increased inorganic fertility resulted in decreased activity (Fig. 5a). Chitinase activities on EcM roots were the only activities that responded in the predicted quadratic shape (Fig. 5d). However, the estimated model parameter was not significantly different from zero (Table 2). Chitinase activities on ericaceous roots were quadratically related to the organic index. These activities initially remained stable, but then increased at higher organic index values, showing activities increased when DOC, DON, and bulk soil C:N values strongly decreased. Phosphatase activities on ericaceous roots were related to both indices. These activities were quadratically related to the inorganic index, but again, not in the predicted shape. Activities sharply decreasing initially and then reaching a plateau, showing that even low increases in nutrient availability negatively affected ericaceous phosphatase activity (Fig. 6a). Ericaceous phosphatase activities also linearly decreased with the organic index, showing activities decreased with increasing DOC, DON, and bulk soil C:N (Fig. 6b). Ectomycorrhizal phosphatase activities, though, were not significantly related to either index.

Responses of oxidative enzyme activities, similarly to those of hydrolytic enzymes, did not support our hypothesized quadratic relationship. Oxidative enzymes correlated with the

inorganic index, but not the organic index. Phenol oxidase activities were quadratically related to the inorganic index on ericaceous roots, sharply decreasing initially and then reaching a plateau (Fig. 7a). Ectomycorrhizal phenol oxidase activities, though, did not change with either index. Peroxidase activities on EcM roots linearly increased with increasing inorganic index, suggesting increased nutrient availability steadily increased EcM activities (Fig. 7c). In contrast, ericaceous peroxidase activities were quadratically related to the inorganic index. Initially, these activities slightly decreased with increasing index values but then returned to similar levels of activity at the highest index values as were at lowest values.

Sensitivity of root enzymes to indices

Overall, enzyme activities on ericaceous roots were more sensitive than those on EcM roots. The magnitudes of change for chitinase, phosphatase, and phenol oxidase activity models were generally greater on ericaceous than EcM roots, indicating that activities on ericaceous roots were more sensitive than EcM roots (Fig. 8). Only among peroxidase activities were magnitudes of change greater on EcM roots than ericaceous roots, showing that peroxidase activities on EcM roots were more sensitive than those on ericaceous roots. Regression line slopes for leucine aminopeptidase models were equal between root types, indicating equal sensitivity of both root types. In some hydrolytic enzymes, root sensitivity depended on the index value. Magnitudes of change among chitinase activities were initially greater on EcM roots, but became greater on ericaceous roots towards intermediate index values, showing EcM roots were more sensitive at high TOC, DOC, and bulk soil C:N ratio values, but ericaceous roots became more sensitive as those values decreased. In contrast, magnitudes of change among phosphatase activities with respect to the inorganic index were initially greater on ericaceous roots, but

became greater on EcM roots at high index values, showing ErM roots became less sensitive to increased nutrient index at high inorganic nutrient concentrations.

Discussion

We tested the functional responses of cover, height, and root enzyme activity of two contrasting dominant tundra shrubs to an experimental soil fertility gradient. We hypothesized that responses would 1) differ between shrub species and 2) be non-linear. Our results supported our hypotheses on shrub cover and height, which were heavily informed by prior knowledge. While root enzymes responded differently between shrub species and were generally non-linear, they did not form the quadratic shape that we hypothesized. *Betula nana* cover and height increased linearly throughout the soil fertility gradient while its root enzyme activities remained relatively unchanged. Conversely, *R. tomentosum* cover initially increased, but decreased after surpassing intermediate levels of soil fertility. Its root enzyme activity decreased throughout the gradient. In some enzymes, this decrease occurred with even the slightest levels of enhanced soil fertility. Below, we explore interspecific competition and shifts in mycorrhizal allocation as underlying drivers of the observed shrub responses to increasing soil fertility.

Co-expansion of ericaceous and EcM shrubs

Our results showed that ericaceous shrubs benefited from moderate increases in soil fertility. This suggests that ericaceous shrubs, often overlooked in studies of shrub expansion, are likely co-expanding with EcM shrubs in response to heightened soil fertility. Increasing abundance of ericaceous shrubs like *R. tomentosum* is not a new finding. Support for ericaceous shrub expansion has been reported by both observational and experimental studies in Europe and North America (Elmendorf et al., 2012; Vuorinen et al., 2017; Zamin et al., 2014). The building

evidence for ericaceous shrub expansion demands its further study, especially given the potential ecosystem consequences of this vegetation shift (Vowles & Björk, 2019). Ericaceous shrubs have different litter quality and quantity than EcM shrubs (S. E. Hobbie, 1996), associate with different mycorrhizal fungi, interact differently with herbivores (Vowles et al., 2017), and likely will not affect snow cover in the same manner that taller EcM shrubs do. Additionally, like their EcM counterparts, ericaceous shrubs are active at the thaw front and acquire recently released N from thawing permafrost via their mycorrhizal fungi (Hewitt et al., 2020). These attributes could positively affect ecosystem C storage by either boosting plant growth or moderating decomposition rates in a warmer environment. Incorporating the quadratic response of ericaceous shrubs found in this study into models of vegetation change will improve our ability to predict future co-expansion and its implications for ecosystem processes.

Though *R. tomentosum* benefited from small to moderate increases in nutrient availability, we identified a point at which cover declines—when *B. nana* height begins to exceed the maximum height of *R. tomentosum* (Fig. 4c) . Interspecific competition with *Betula nana* has previously been hypothesized to drive the decrease in ericaceous shrubs at high levels of soil fertility (Bret-Harte et al., 2001). Experimentally increased nutrient availability initiates a plastic growth response in *Betula spp.*, where it grows long shoots and shades competing ericaceous shrub and graminoid species. Throughout our soil fertility gradient, *B. nana* height steadily increased while *R. tomentosum* did not change. Though this is not direct evidence that *B. nana* outcompeted *R. tomentosum* for light in its journey towards dominance across the gradient, it provides further support for this hypothesis. Additionally, these results offer insights for instances when *B. nana* does not initiate shoot elongation. In the Scandinavian Arctic, *B. nana* was relatively unresponsive aboveground to long-term high dose fertilization (Van Wijk et al.,

2003). Instead, plant communities remained similar to unfertilized plots as biomass of all vascular species increased. Varying mechanisms have been proposed to explain the observed regional differences in whether or not *B. nana* grows long shoots, including localized soil characteristics (Jonasson, Michelsen, Schmidt, & Nielsen, 1999) and investment in defensive phenolic compounds (Graglia et al., 2001). Our results in combination with those of past studies suggest that *R. tomentosum* abundance may not shift from increasing to decreasing with higher soil fertility if *B. nana* did not grow long shoots.

Root enzyme responses

Enzyme activities on ericaceous roots were more sensitive to changes in soil fertility than those on EcM roots. In contrast to relatively stable activities on EcM roots, ericaceous root activity declined even as ericaceous shrub cover increased. We offer two possible explanations. On one hand, greater sensitivity could reflect that *R. tomentosum* readily reduces enzyme production—a metabolically expensive process—to account for changes in soil fertility. In tundra and other ecosystems, increased soil fertility can increase plant allocation to aboveground material (Iversen et al., 2015; Poorter et al., 2012). For *R. tomentosum*, this shift may be initially beneficial, but lose its effectiveness once interspecific competition for light with *B. nana* becomes a larger factor. On the other hand, the greater sensitivity of ericaceous roots may indicate that these shrubs respond poorly to inorganic nutrient addition, perhaps physiologically. In this case, the observed drop in root activity—at times instantaneous—serves as an early sign of the detrimental effect of added nutrients. Zamin *et al.* (2014) found negative responses of ericaceous shrubs to high dose fertilization regardless of EcM shrub presence. While the decrease of ericaceous shrubs was speculated to result from trade-offs associated with higher leaf nutrient concentrations, our results

suggest it could relate to belowground processes. Overall, the relative insensitivity of *B. nana* root enzyme activity compared to the sharp sensitivity of *R. tomentosum* could convey a competitive advantage to *B. nana* for nutrient acquisition. Regardless of the drivers behind the greater sensitivity of ericaceous enzyme activity, its implications point to a decreasing influence of ericaceous shrubs in tundra soil nutrient cycling as climate warms and nutrient availability increases.

We were surprised to find activities of most enzymes on EcM roots did not change, even at the highest levels of soil fertility. We hypothesized higher soil fertility levels would reduce EcM-associated root activities based on the ecological market theory involved in mycorrhizal relationships (Franklin, Näsholm, Högberg, & Högberg, 2014). This predicts that when nutrients are readily available, plants will lower their C allocation to mycorrhizae. Furthermore, our prior research in a nearby 28-year experiment found sharp declines in root activity with high dose fertilization. While we expected to find similar results, the difference in the length of these two experiments—more than a decade and a half—could explain the conflicting results. Similar to the lack of EcM response in our study, the initial fifteen years of fertilization in the longer-running experiment did not affect EcM biomass and colonization (Clemmensen et al., 2006; Deslippe et al., 2011). Long-term studies in Arctic tundra demonstrate temporal dependencies in a variety of responses to a changing climate, including plant communities (F Stuart Chapin et al., 1995; G. R. Shaver et al., 2001), soil microbial communities (Koyama, Wallenstein, Simpson, & Moore, 2014; Rinnan, Michelsen, Bååth, & Jonasson, 2007), and ecosystem C and nutrient cycling (Christiansen et al., 2018; Mack et al., 2004). Mycorrhizal root communities and function maybe be no different than these other variables in their temporal response. Although EcM root enzyme activity generally did not respond to increasing soil fertility, the ratio of root activity to aboveground cover and

height decreased. This points to a drop in allocation towards enzymatic acquisition of nutrients and is consistent with other studies that identify a proportional decrease in belowground allocation in EcM shrubs (DeMarco, Mack, Bret-Harte, et al., 2014; Zamin et al., 2014), backing our hypothesized weakening of host shrub reliance on mycorrhizae.

One enzyme—peroxidase—served as an exception to the general insensitivity found in EcM root activities. Ectomycorrhizal-associated peroxidase activities rose with soil fertility and were more sensitive than those on ericaceous roots. We posit that increased peroxidase activities on EcM roots may be in response to increases in woody litter with greater shrub cover. Heightened peroxidase activity might be a mechanism that allows EcM shrubs to maintain dominance by supporting fungal partners that can readily degrade their senesced material. Other studies have found fertilization stimulated the activities of other C-degrading enzymes, similar to peroxidase, on EcM roots (Jones, Phillips, Treu, Ward, & Berch, 2012) and in bulk tundra soils (Koyama, Wallenstein, Simpson, & Moore, 2013). Nutrient-related enzymes in bulk tundra soil, however, either did not respond or decreased with fertilization (Koyama et al., 2013; McLaren & Buckeridge, 2019). Furthermore, high dose fertilization near these sites accelerates decomposition and reduces ecosystem C storage (Mack et al., 2004). Together, these results indicate that faster decomposition rates occurring with experimentally increased soil fertility likely arose from stimulated activity of free-living soil microbes and EcM-associated C-degrading enzymes rather than that of ericaceous root enzymes.

N and P interactions with increased soil fertility

Similar to previous studies, we found inorganic nutrient concentration was an important driver of shrub dynamics in our experiment. Of the two soil fertility indices, the inorganic index,

associated with increased concentrations of DIN and PO_4-P, better explained changes in above- and belowground shrub variables compared to the organic index. However, the question remains of whether N or P contributes more to plant community shifts.

Phosphorus has the potential to be an important element in deciduous shrub expansion. Across several tundra ecosystems, N and P co-limit plant growth (F Stuart Chapin et al., 1995). At the highest levels of fertilization, we found that DIN concentrations steeply rose while PO_4-P concentrations began to decrease. This non-linear relationship suggests a potential shift in the limiting element from N to P at these higher levels of DIN concentration. Recent evidence for co-limitation is variable. In a long-term experiment in Arctic Canada, plants in plots fertilized with only N experienced a weaker positive response than those in plots fertilized with both N and P (Zamin & Grogan, 2012). In contrast, McLaren & Buckeridge (2019) found evidence supporting a primary limitation by N in a factorial N and P addition experiment near our study site. Given that N availability may increase through warming and thawing soils as well as future anthropogenic N deposition in the Arctic, P may become a more important nutrient in vegetation and C dynamics (Jiang et al., 2016; Street, Mielke, & Woodin, 2018; Wieder, Cleveland, Smith, & Todd-Brown, 2015). As permafrost thaw deepens, weathering materials will become more accessible, likely opening a new source of P into tundra ecosystem cycling. The extent to which potential P inputs will alleviate plant P demand, however, is understudied. Further research should be conducted to understand the constraint future P limitation may have on shrub expansion and how increases in plant-accessible P through permafrost thaw may alter these constraints.

Conclusions

This study adds to our understanding of the mechanisms and consequences of Arctic shrub expansion by measuring responses of two contrasting shrub functional types across a long-term experimental soil fertility gradient. Because future nutrient releases in tundra soils are likely to be spatially and temporally heterogeneous, field-based regression analyses over a continuous gradient, such as those conducted in this study, are increasingly important in developing predictive models of shrub expansion. Given that the effects of heightened soil fertility can differ across tundra ecosystem types, further research should compare shrub responses to environmental gradients among various tundra types. The results presented here suggest that as soil fertility increases in Arctic tundra, the resulting vegetation shift heavily depends on the amount of nutrients released. In areas with weaker increases in soil fertility, both deciduous EcM and evergreen ericaceous shrub cover will increase. In areas with stronger increases in fertility, however, deciduous EcM shrubs will dominate while evergreen ericaceous shrubs decrease. Additionally, we predict that as we move to a greener tundra with higher nutrient availability, EcM fungi will play a larger role in liberating soil organic nutrients as their host plant expands and as ericaceous roots decrease in degradative enzyme activity.

Acknowledgements

This study was conducted on the unceded lands of the Inupiaq, Gwich'in, Koyokuk peoples, whose legacy of stewardship continues today in their fight against the impacts of colonialism. This work was funded by U.S. National Science Foundation grant NSF-DEB-1556496 to MCM and an ARCS Foundation Award to HRD. We thank Ruby An, Jim Laundre, and Laura Gough at the Arctic Long-Term Ecological Research site (grant NSF-DEB-1637459); Emily Brooke, Julia Stuart, Samantha Miller, and Moises Ceja for help in the field and lab; and

Toolik Field Station and Environmental Data Center for coordination of a remote field access and additional support.

Author contributions

MCM and HRD conceived the ideas and designed methodology. HRD collected and analyzed the data and led the writing of the manuscript. MCM acquired the funding, supervised the project, and contributed critically to manuscript drafts. Both authors provided final approval for publication.

Data availability

Data can be accessed at the Arctic Data Center online repository under DOI…

Tables

Table 3.1. Pearson's correlation test results of soil chemical variables with NMDS axes 1 and 2. Significance values ($p < 0.1$ = *, $p < 0.05$ = **, $p < 0.001$ = ***)

Axis	Soil variable	r	P
inorganic	NH_4-N	0.84	<0.001 ***
index	NO_3-N	0.89	<0.001 ***
(NMDS1)	PO_4-P	0.70	<0.001 ***
	dissolved organic N	0.35	0.089 *
	bulk soil C:N	0.04	0.829
	bulk soil %C	-0.30	0.152
	bulk soil %N	-0.30	0.156
	total organic C	0.25	0.248
organic	NH_4-N	0.14	0.522
index	NO_3-N	-0.15	0.479
(NMDS2)	PO_4-P	0.55	0.005 **
	dissolved organic N	-0.57	0.004 **
	bulk soil C:N	-0.51	0.011 **
	bulk soil %C	0.56	0.005 **
	bulk soil %N	0.74	<0.001 ***
	total organic C	-0.55	0.005 **

Table 3.2. Parameter estimates, SE, z-values, P values, and marginal and conditional R^2 from the best fitting model for activities of each enzyme on EcM *B. nana* and ericaceous *R. tomentosum* roots. Significance values (P < 0.1 = *, P < 0.05 = **, P <0.001 = ***)

Enzyme	Final model variables	Coefficient	SE	z-value	P	R^2_m	R^2_c	N (roots)
Leucine	(Intercept)	4.0	0.1	36.6	<0.001 ***	0.07	0.34	n = 757 EcM
aminopeptidase	inorganic index	-1.0	0.3	-3.1	0.002 **			n = 430 eri.
	R. tomentosum (intercept)	-0.6	0.1	-8.9	<0.001 ***			
Chitinase	(Intercept)	6.3	0.1	84.8	<0.001 ***	0.51	0.61	n = 760 EcM
	organic index	2.6	1.9	1.4	0.171			n = 430 eric.
	organic index^2	-2.5	1.7	-1.5	0.141			
	R. tomentosum (intercept)	-1.7	0.1	-36.0	<0.001 ***			
	R. tomentosum:organic index	2.1	1.6	1.3	0.192			
	R. tomentosum:organic index^2	5.7	1.9	3.0	0.003 **			
Phosphatase	(Intercept)	7.1	0.1	88.2	<0.001 ***	0.38	0.51	n = 739 EcM
	inorganic index	2.7	1.6	1.7	0.098*			n = 382 eri.
	inorganic index^2	1.3	1.5	0.9	0.373			
	organic index	0.1	0.4	0.3	0.746			
	R. tomentosum (intercept)	-1.3	0.1	-23.4	<0.001 ***			
	R. tomentosum:inorganic index	-11.4	1.6	-7.0	<0.001 ***			
	R. tomentosum:inorganic index^2	6.6	1.8	3.6	<0.001 ***			
	R. tomentosum:organic index	-1.0	0.4	-2.4	0.018 **			
Phenol oxidase	(Intercept)	3.8	0.3	14.3	<0.001 ***	0.11	0.23	n = 760 EcM
	inorganic index	-0.9	10.2	-0.1	0.926			n = 433 eri.
	inorganic index^2	-0.1	9.2	-0.02	0.988			
	R. tomentosum (intercept)	-3.2	0.2	-14.4	<0.001 ***			
	R. tomentosum:inorganic index	-20.6	5.8	-3.6	<0.001 ***			
	R. tomentosum:inorganic index^2	22.6	7.0	3.2	0.001 **			
Peroxidase	(Intercept)	6.1	0.1	63.3	<0.001 ***	0.37	0.60	n = 750 EcM
	inorganic index	6.9	3.8	1.8	0.069 *			n = 425 eri.
	inorganic index^2	2.8	3.4	0.8	0.418			
	R. tomentosum (intercept)	-1.6	0.1	-27.3	<0.001 ***			
	R. tomentosum:inorganic index	-7.9	1.6	-5.0	<0.001 ***			
	R. tomentosum:inorganic index^2	4.3	2.0	2.2	0.027 **			

Figures

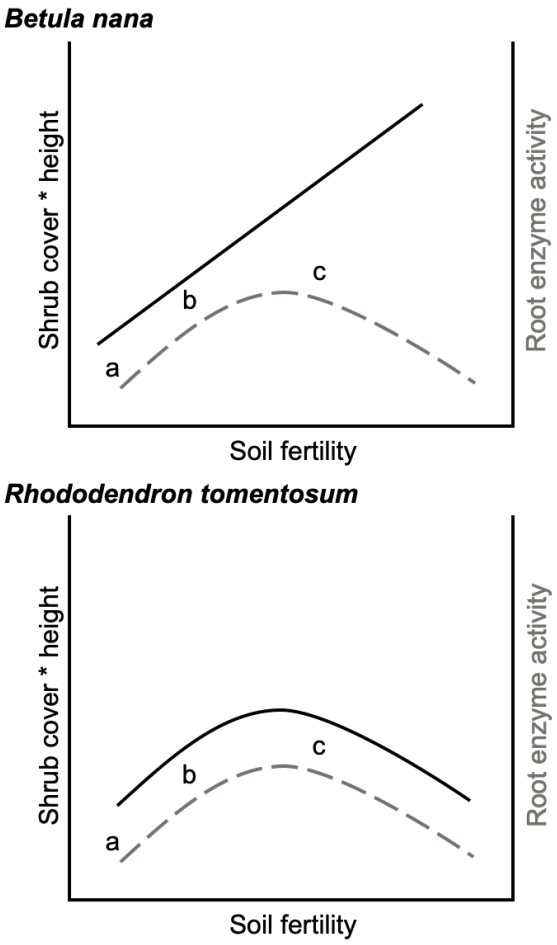

Fig. 1. Hypothesized relationship of *B. nana* and *R. tomentosum* cover, height, and root enzyme activity with soil fertility. Low fertility levels at (a) result in low shrub growth and root activity. As fertility rises at (b), cover and activities increase. Nutrient limitations are relieved at (c): Betula nana continues to increase in aboveground mass, but *R. tomentosum* decreases. Root activities decrease on both species. Note: hypothesized relationships are not drawn to scale.

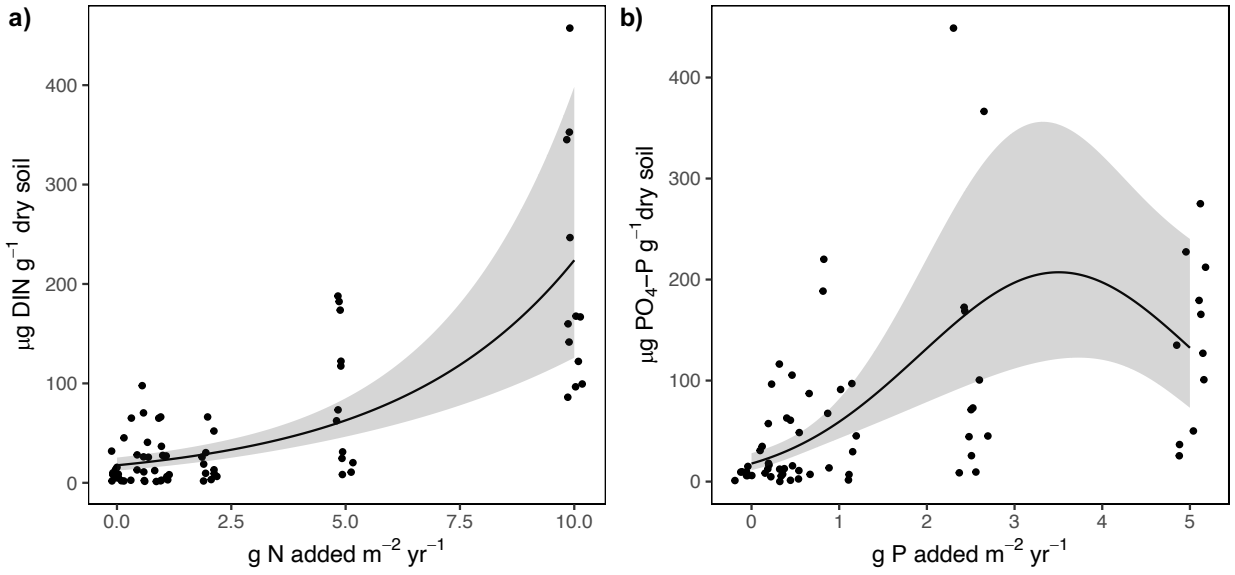

Fig. 2. Responses of soil extractable a) DIN (n = 71 cores) and b) PO_4-P (n = 66 cores) to 12 years of experimental fertilization at Arctic LTER, Toolik Lake, Alaska. Lines and shading represent linear mixed effects model results with 95% confidence limits.

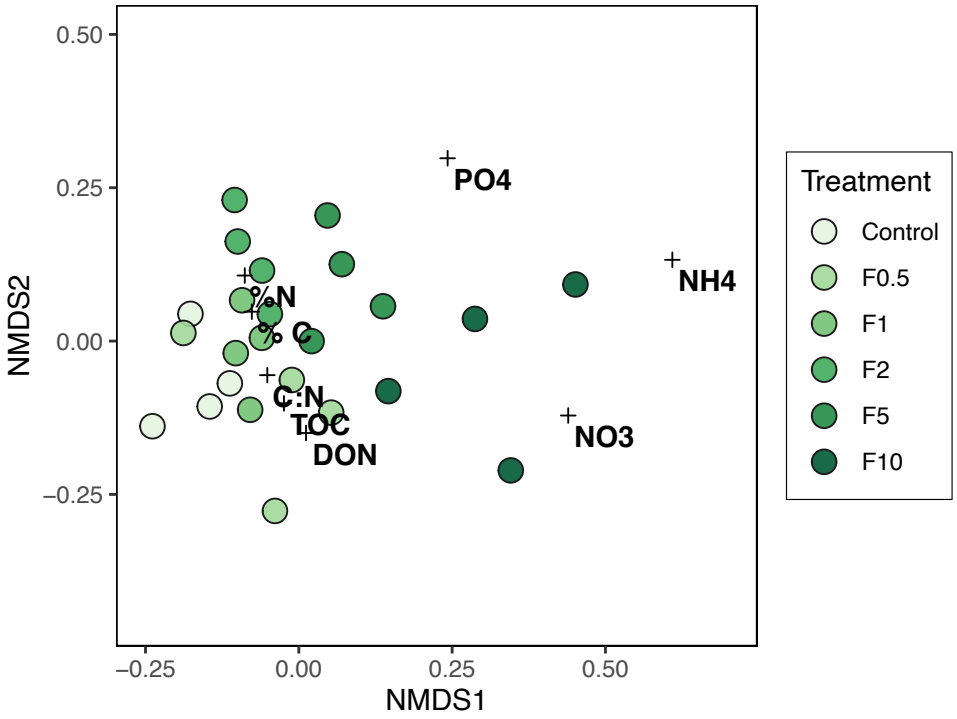

Fig. 3. Nonmetric multidimensional scaling of the Bray-Curtis distances between relativized soil chemical variables among experimental plots. Circles represent plots (n = 24) and are colored by fertilization level. + represent the loadings of each soil chemical variable on the ordination and show the influence of each variable on axes values.

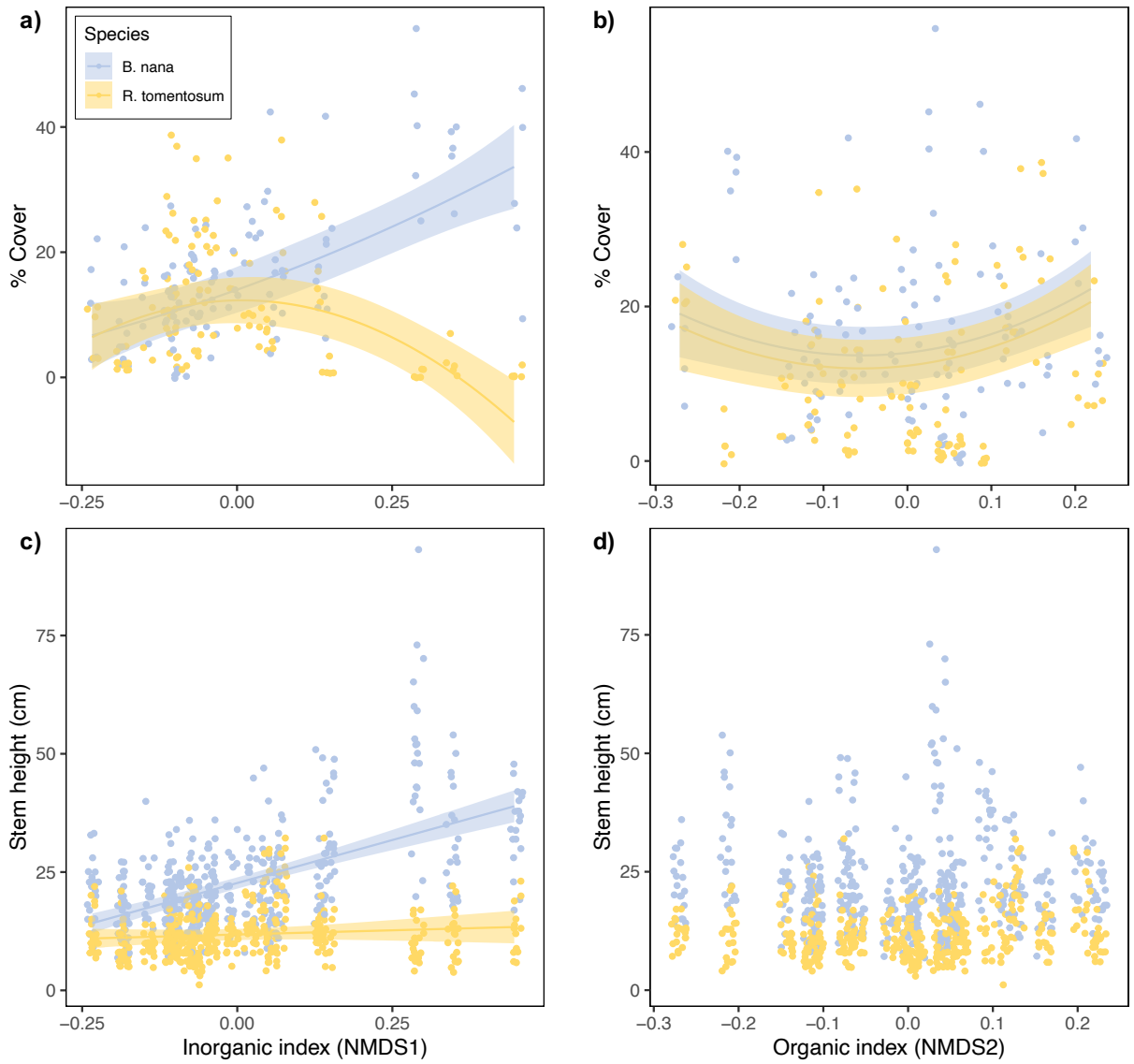

Fig. 4. Responses of *Betula nana* and *Rhododendron tomentosum* cover (a, b) and stem height (c, d) to the inorganic and organic soil fertility indices. Lines and shading represent linear mixed effects model results with 95% confidence limits. Cover: n = 120 *B. nana* and *R. tomentosum* quadrats each. Stem height: n = 458 *B. nana* stems and n = 465 *R. tomentosum* stems.

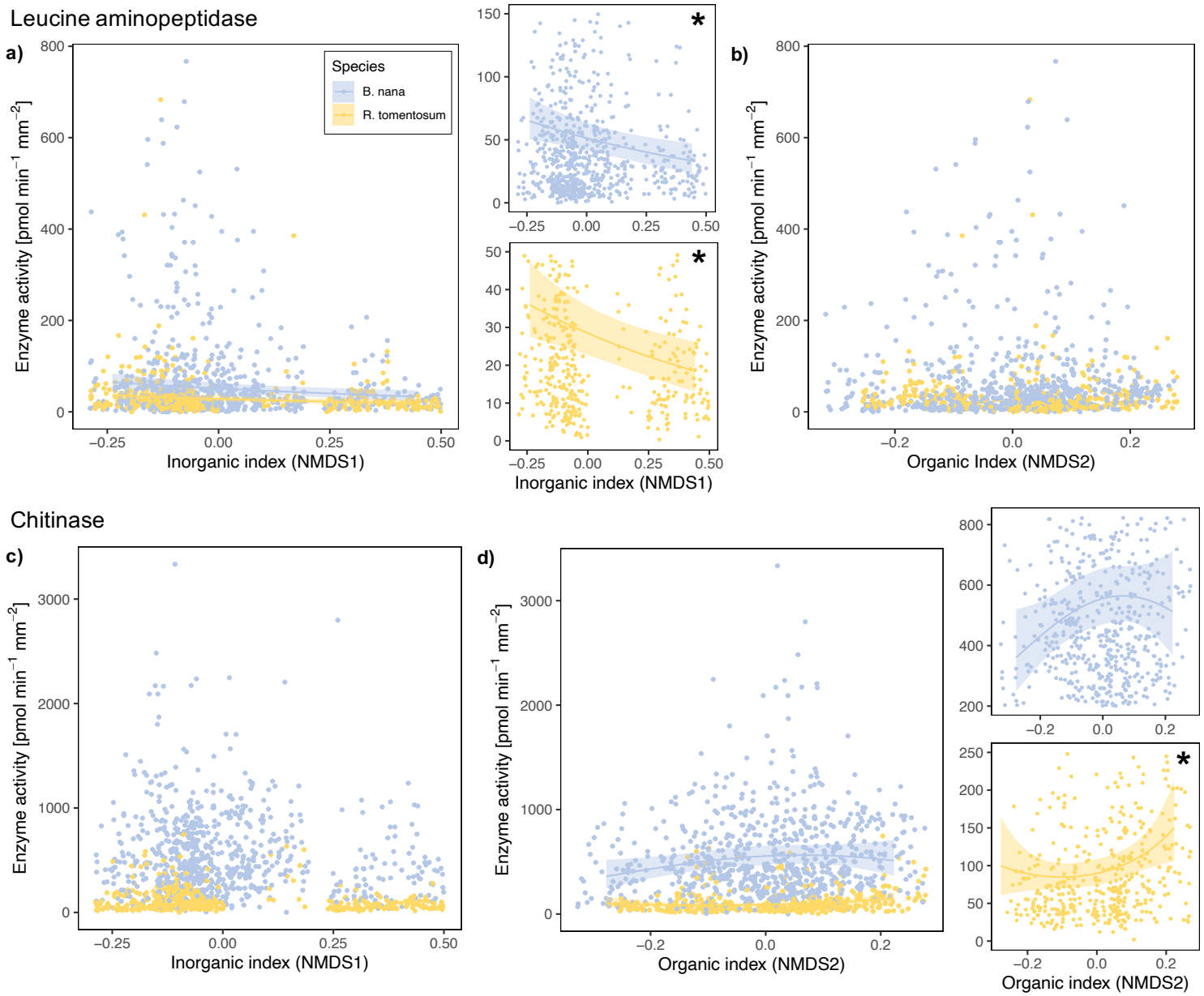
Leucine aminopeptidase
a)
Species
B. nana
R. tomentosum
Enzyme activity [pmol min−1 mm−2]
Inorganic index (NMDS1)
*
*
Inorganic index (NMDS1)
b)
Enzyme activity [pmol min−1 mm−2]
Organic Index (NMDS2)
Chitinase
c)
Enzyme activity [pmol min−1 mm−2]
Inorganic index (NMDS1)
d)
Enzyme activity [pmol min−1 mm−2]
Organic index (NMDS2)
*
Organic index (NMDS2)

Figure 5. Responses of leucine aminopeptidase (a, b) and chitinase (c, d) activities to inorganic and organic soil fertility indices. Because of the large differences in the ranges of enzyme activities between EcM *B. nana* and ericaceous *R. tomentosum* roots, we present model results on the full scale and on a scale proportional to the interquartile range of each root type's activity. Colors represent root type. Lines and shading represent linear mixed effects model results with 95% confidence limits. * represent where parameter estimates are significantly different from zero.

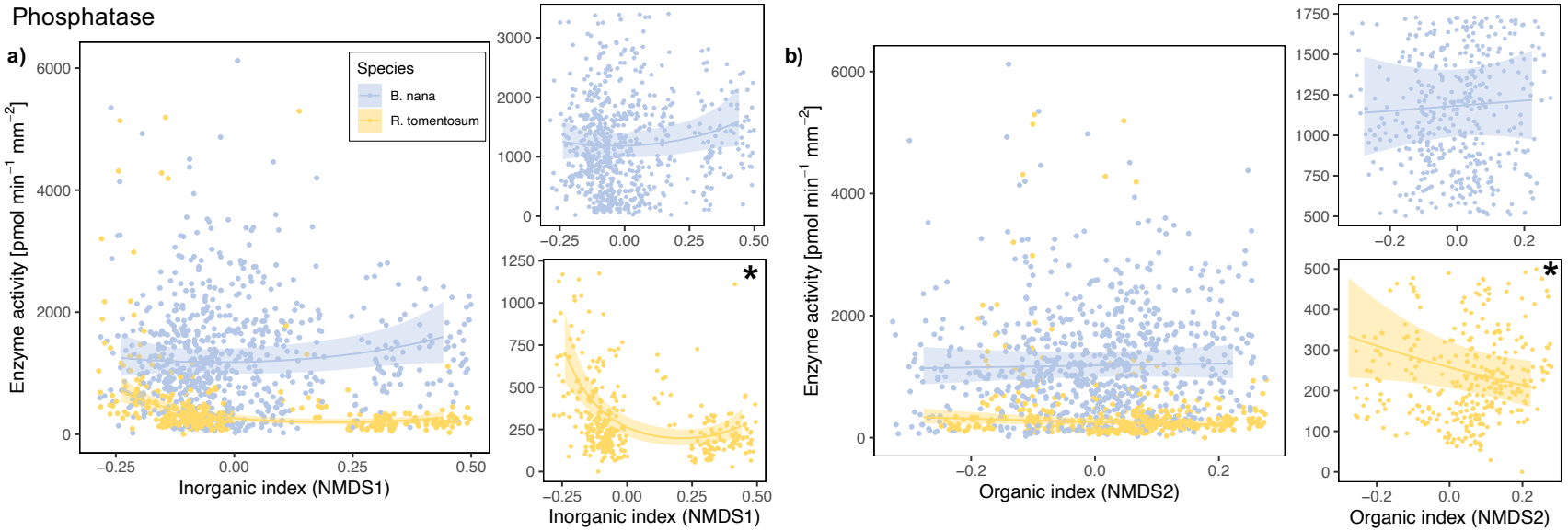

Figure 6. Responses of phosphatase activities to inorganic (a) and organic (b) soil fertility indices. Because of the large differences in the ranges of enzyme activities between EcM *B. nana* and ericaceous *R. tomentosum* roots, we present model results on the full scale and on a scale proportional to the interquartile range of each root type's activity. Colors represent root type. Lines and shading represent linear mixed effects model results with 95% confidence limits. * represent where parameter estimates are significantly different from zero.

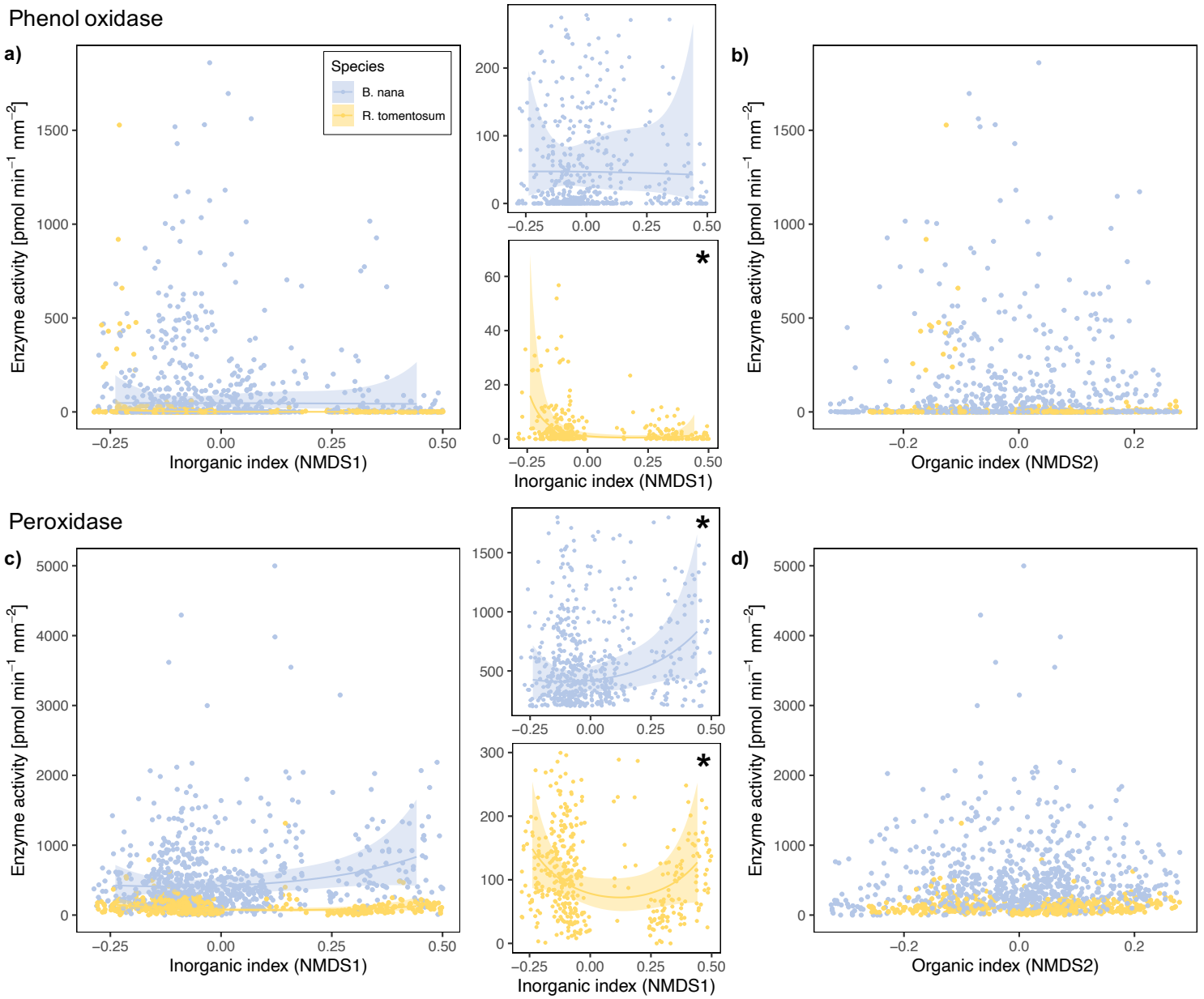
Phenol oxidase
a)
b)
Species
B. nana
R. tomentosum
Enzyme activity [pmol min^{-1} mm^{-2}]
Inorganic index (NMDS1)
Organic index (NMDS2)
*
Peroxidase
c)
d)

Figure 7. Responses of phenol oxidase (a, b) and peroxidase (c, d) activities to inorganic and organic soil fertility indices. Because of the large differences in the ranges of enzyme activities between EcM *B. nana* and ericaceous *R. tomentosum* roots, we present model results on the full scale and on a scale proportional to the interquartile range of each root type's activity. Colors represent root type. Lines and shading represent linear mixed effects model results with 95% confidence limits. * represent where parameter estimates are significantly different from zero.

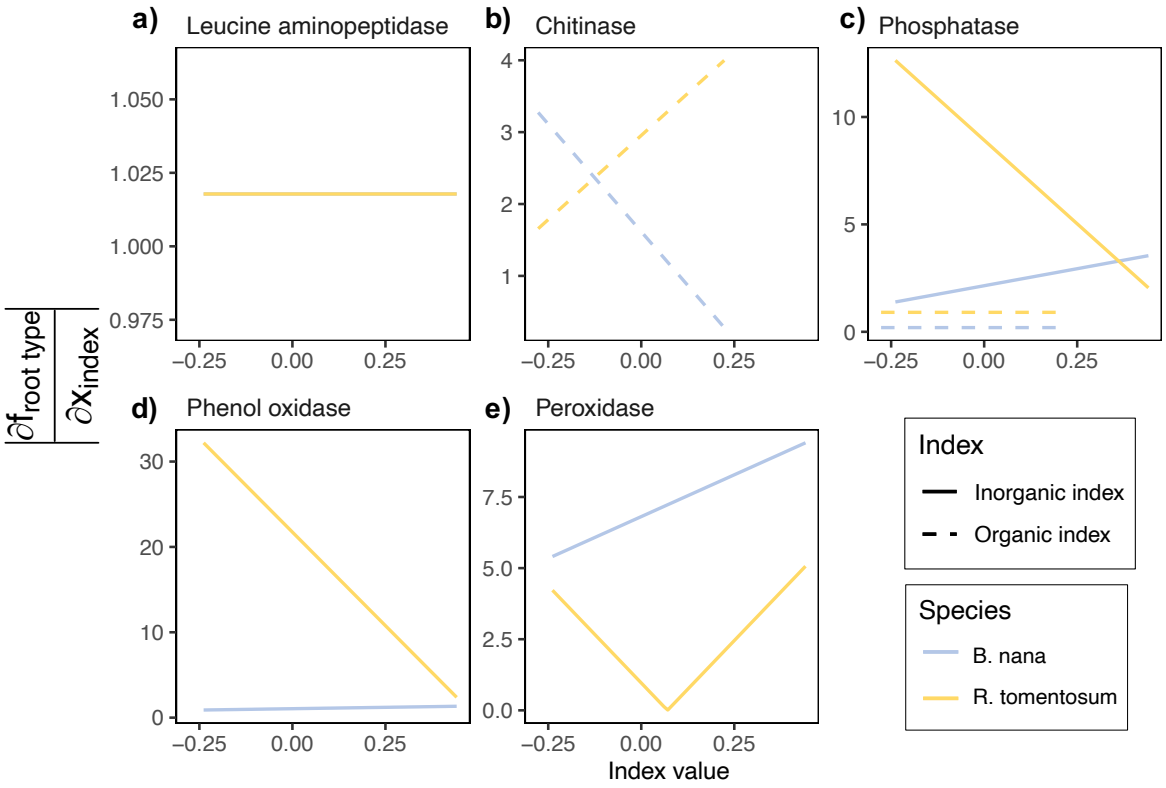

Figure 8. Model estimates of the absolute values of the partial derivatives for activities of leucine aminopeptidase (a), chitinase (b), phosphatase (c), phenol oxidase (d), and peroxidase (e) with respect to the inorganic and organic soil fertility indices. Higher values indicate a higher magnitude of change and thus, higher sensitivity. Colors represent root type. Solid lines represent the magnitudes of change with respect to the inorganic index and dashed lines represent those with respect to the organic index.

CHAPTER 4: N_2 FIXATION AND ECTOMYCORRHIZAL ROOT ENZYME ACTIVITY ARE COUPLED ON SIBERIAN ALDER ACROSS AN ARCTIC-BOREAL GRADIENT

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Key words: actinorhizal*; Alnus viridis fruticosa*; Alaska; Arctic shrub expansion; Arctic tundra; boreal forest; ectomycorrhiza; latitudinal gradient; N_2 fixation; root enzyme

Abstract

Siberian alder is a tall shrub expanding across Alaska. Host to ectomycorrhizal (EcM) fungi and nitrogen (N)-fixing actinobacteria *Frankia*, alder not only facilitates the addition of new N to its ecosystems through fixation, it may also alter phosphorus (P) availability and transform soil organic matter (SOM) via the exudation of EcM degradative enzymes. To understand alder's potential for overhauling nutrient dynamics in northern biomes as it expands, we identified drivers of root enzyme activity and N fixation across a latitudinal gradient in Arctic and boreal Alaska. We paired measurements of enzyme activity and fixation, testing for 1) relationships between each symbiotic root process and environmental and alder properties and 2) how these two processes interacted with one another. We found mean annual temperature was the largest driver of root enzyme activity, correlating with 95% of the variation in enzyme profiles. Warmer, southern sites had high activities dominated by hydrolytic enzymes while colder, Arctic sites had low activities that were dominated by phenol oxidase. In contrast, fixation was best explained by depth of the soil organic layer and foliar d13C rather than by temperature. The relationship between fixation and enzyme activity depended on nutrient limitation (indicated by foliar N:P value). When N was more limiting than P, high fixation rates were associated with lower phosphatase activities relative to phenol oxidase. When P was more limiting, high fixation rates were associated with higher phosphatase activities relative to phenol oxidase. Our results suggest that as alder expands with warming, its potential heightened influence on ecosystems will be mediated by local edaphic conditions. In particular, as warming stimulates nutrient releases, the resulting changes in N limitation relative to P will control whether inputs of fixed N are accompanied by EcM-induced increases in either soil P availability or the transformation of lignin-rich SOM.

Introduction

Symbiotic root processes are increasingly thought to exert strong controls over ecosystem pools and fluxes (Averill et al., 2014; Soudzilovskaia et al., 2019; Terrer et al., 2018). Mycorrhizal fungi can govern nutrient limitation (Franklin et al., 2014), stimulate or stifle decomposition (Fernandez and Kennedy, 2016; Gadgil and Gadgil, 1971; Zak et al., 2019), and stabilize soil organic matter (SOM) through recalcitrant mycelial necromass (Clemmensen et al., 2013; Fernandez et al., 2016). Likewise, nitrogen (N) fixing bacteria add ecologically important amounts of bioavailable N to ecosystems, boosting plant growth and supporting vegetation succession. In warming northern latitudes, woody shrubs, and presumably their microbial partners, are expanding (Myers-Smith et al., 2011; Sturm et al., 2001; Tape et al., 2006). The coinciding changes in symbiotic root processes with vegetation shifts have been studied with respect to their individual effects on carbon (C) and nutrient cycling (Dunleavy and Mack, 2021; Salmon et al., 2019). However, despite the potential for symbionts to be living on the same root—competing for resources or facilitating each other's resource acquisition—it remains uncertain how root symbionts will interact with one another as they and their host plants expand across these nutrient-limited ecosystems.

Siberian alder (*Alnus viridis ssp. fruticosa*; hereafter alder*)* has a uniquely high potential for restructuring nutrient dynamics in the Arctic and boreal biomes compared to other expanding species. Alder is a tall shrub that forms partnerships with both ectomycorrhizal (EcM) fungi and N-fixing actinobacteria, *Frankia*. Alder facilitates the fixation of 6.6 to 11.5 kg N ha^{-1} yr^{-1} in Alaskan boreal forests (Houseman et al., 2020; Mitchell and Ruess, 2009) and 5.3 to 19.5 kg N ha^{-1} yr^{-1} in Arctic tundra (Salmon et al., 2019). However, its effects on ecosystems are not limited to the input of new N. Nitrogen fixation is a process often limited by phosphorus (Houlton et al.,

2008; Ruess et al., 2013; Uliassi and Ruess, 2002). In their effort to support higher fixation rates, alder and other N-fixing plants can alter pools of bioavailable P—either depleting (Mitchell and Ruess, 2009) or mobilizing them (Houlton et al., 2008). One way that alders acquire P is through their ectomycorrhizal (EcM) partners, which exude degradative extracellular enzymes, like phosphatases, to release nutrients locked in SOM (Bödeker et al., 2009; Read et al., 2004). Through their transformation of SOM, alder EcM are not just taking part in the cycling of P (Ruess et al., 2019), but are also contributing to decomposition (Lindahl and Tunlid, 2015; L. A. Phillips et al., 2013). As alder expands, the multi-faceted effects of fixation and EcM-associated enzyme activity on soil processes—N inputs, P depletion or mobilization, and enzymatic transformation of SOM—could compound to restructure vegetation and soil microbial communities and ultimately shift ecosystem C balance. Despite the weight the potential impacts carry in these globally important C-rich northern ecosystems, the driving factors behind how alder's root symbionts interact remains unknown.

How alder's tripartite symbiosis may reshape its ecosystems will likely depend on the interaction between EcM and *Frankia*. It could be hypothesized that these two symbionts compete for C allocation by the alder host. Both require energetically costly investments from their host plant: allocation to mycorrhizas in other plants account for up to 20% of NPP (Hobbie and Hobbie, 2008, 2006), and *Frankia* nodules account for 2-4% of alder biomass and respire up to ~6 g C g^{-1} N fixed (Ruess et al., 2013; Uliassi et al., 2000). Under this framework, EcM root enzyme activity and N fixation may negatively correlate as they compete for host C. As a result, the influence of expanding alder on soil processes would be limited to either N input or degradative enzymes. However, it has been alternatively hypothesized that EcM and *Frankia* act in conjunction to maximize alder's as well as their own nutrient demand. To support *Frankia*'s

high demand for P, alder could harness its EcM to enzymatically access soil P (Horton et al., 2013; Walker et al., 2014). Higher mycorrhizal enzyme activity, especially that of phosphatases, would facilitate further N fixation. In turn, the resultingly higher fixation rates could support an increase in the production of N-rich phosphatases (Houlton et al., 2008), creating a positive feedback between phosphatase production and nitrogen fixation. In this case, EcM and *Frankia* relationship would be characterized as facultative rather than competitive. Together, their effects on soil processes would coalesce to substantially change alder's new environments.

As climate warms, alder is expanding northward and into higher elevations. In boreal and Arctic Alaska, alder grows across a temperature gradient, where EcM and *Frankia* may span the facilitation-competition continuum. Warmer environments likely increase alder productivity and hence C allocation to its symbionts, reducing competition between EcM activity and N fixation. Contrarily, cold environments might reduce NPP and increase competition. Additionally, throughout alder's range there are varying edaphic conditions that potentially modify the effects of temperature. For instance, nutrient limitation could determine whether or not allocation towards symbiotic N and P acquisition is stoichiometrically economical for alder. Phosphorus fertilization nearly doubled N fixation rates by nodules on *Alnus tenuifolia* while N fertilization halved fixation (Ruess et al., 2013). Similarly, N fixation in Arctic tundra increased with decreased P limitation (Salmon et al., 2019). As northern latitudes not only rapidly warm, but also experience related changes in soil conditions—such as nutrient release from thawing permafrost—it is important to understand whether temperature or edaphic conditions have a greater influence on N fixation, root enzyme activity, and their interaction to better predict the future impacts of alder expansion.

In this study, we characterized the effects of temperature and edaphic conditions on alder's symbiotic root processes. We also asked if these two processes were coupled, pairing EcM-associated root enzyme activity and nodule N fixation measurements on alders across a latitudinal gradient in Interior and Arctic Alaska. Within each region of the gradient, we sampled sites that differed in nutrient concentration and alder growth traits. We predicted that temperature would be the most important driver of enzyme activities and fixation rates across the latitudinal gradient, where rates of both processes would increase with warmer temperatures. However, relative soil N and P availability would explain variation in these processes within regions. Furthermore, we hypothesized N fixation and root enzyme activity would be related and that the direction of this relationship would depend on whether N or P is relatively more available to alder growth (Fig. 1).

1. When N is more available than P, fixation would increase with root enzyme activity as higher enzyme activities support greater P acquisition.
2. When P is more available, fixation would decrease with increased root enzyme activity as the symbionts behind these two processes compete for plant C allocation.

Materials and Methods

Study area and sampling

We conducted this study across a latitudinal transect spanning from Interior Alaska to the North Slope. We selected nine sites across four different regions between Delta Junction, AK and Sagwon Bluffs. We chose sites and regions to represent the range of variation in alder allometric and leaf traits and ecosystems in which alder grows (Heslop and Ruess *in prep*). Sites ranged from warm, mesic mixed forests dominated by *Picea spp.* and hardwoods such as *Betula neoalaskana* and *Populus tremuloides* in the south to cold, tussock dominated tundra in the

north. Site coordinates and descriptions can be found in Table 1. Across this latitudinal gradient, alder traits varied. In deciduous and mixed spruce forests, alder lives as a tall understory shrub with thick ramets and high specific leaf area (SLA). Alders are rarer in tundra ecosystems. When present, they dominate the canopy, yet are still shorter than individuals in forests, and have generally lower SLA.

In June through August 2019, we collected leaves, roots, and nodules from six alders at each site. To ensure that roots tips were from alder and not neighboring EcM species, we traced fine roots back to aboveground material. Upon collection, roots were immediately placed into plastic containers on ice in a field cooler and kept moist with 0.5M $CaCl_2$ buffer during transport back to the lab. Nodule N fixation was measured in the field immediately upon excavating nodules. Ten leaves were collected from each alder for foliar analysis. Additionally, we sampled organic and mineral soil within a 1 m radius extending from the basal center of each shrub using a 7 cm diameter core. We sampled three cores per shrub, collecting organic soil from within the top 0-15 cm of the soil profile (at some sites the organic horizon was < 15 cm) and the top 0-5 cm of the mineral horizon. Cores from each horizon were combined for each individual alder and stored on ice for transport back to the lab.

We also characterized alder and site properties. We counted the number and basal diameter of ramets per individual alder (determined by emergence from a central location), and calculated ramet basal diameter of each shrub. Mean annual temperature (MAT) and mean annual precipitation (MAP) for each site were extracted from Terraclimate (Abatzoglou et al., 2018) using the *terra* package in R (Hijmans, 2020).

Soil and foliar chemical analyses

We processed soil cores on the same day of collection to characterize soil chemistry. We homogenized each sample by hand and removed coarse (>2 mm) woody debris, roots, and rocks. Organic soil was split into three subsamples. One subsample was oven dried at 60˚C for 48 hrs to measure gravimetric moisture content. On this same sample, we measured bulk soil %C, %N, $\delta^{15}N$, and $\delta^{13}C$ via combustion mass spectrometry on an ECS4010 Elemental Analyzer (Costech Analytical Technologies Inc., USA) coupled to a Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Fisher Scientific, USA) at Northern Arizona University. A second subsample was analyzed for total dissolved organic C (TOC), total dissolved N (TN), and dissolved inorganic N concentrations (DIN). We extracted dissolved C and N by shaking 10 g of soil in 50 mL of 0.5 M K_2SO_4 for 2 hours. Extracts rested overnight at 4˚C and was then vacuum filtered through a Whatman GF/A filter. Filtrate was stored at -20˚C until measured for TOC, TN, and DIN. We measured TOC and TN of filtrate via combustion on a Total Organic Carbon Analyzer TOC-L (Shimadzu Corporation, Japan) and DIN on a SmartChem 200 Discrete Chemistry Analyzer (Unity Scientific, USA) via colorimetry using the Ammonium Salicylate method (Unity Scientific Method 210-203D) for NH_4-N detection and the Nitrate method (Unity Scientific Method NO3-001-A) for NO_3 -N detection. We computed dissolved organic N as TN minus DIN. A third soil subsample was air dried and analyzed for orthophosphate concentration. We extracted orthophosphate by shaking 2.5 g soil in 10 mL of a double acid solution (0.025N H_2SO_4 and 0.05 N HCl) for 5 min. Extracts were vacuum filtered through a Whatman No. 5 filter. Filtrate was stored at 4˚C for no more than a week. We measured PO_4-P concentrations of filtrate via colorimetry using the ascorbate method (Murphy and Riley, 1962). Absorbances were read at 880nm on a Powerwave XS microplate reader (Biotek, USA). Mineral soil was air dried and measured for pH using a 1:1 soil:water rehydration.

Foliar samples were processed for physical and chemical traits. We measured average SLA for each shrub, drying leaves at 60˚C. Leaves were then ground and combined by individual shrub. Foliar material was run through the same elemental analyzer-mass spectrometer mentioned above for %C, %N, $\delta^{15}N$, and $\delta^{13}C$. For foliar %P, a subsample of ground foliar material was ashed in a muffle furnace at 500˚C for 5 hrs and fully digested in 1 ml of 6 N HCl. We measured %P using the ascorbate method (Murphy and Riley, 1962).

Alder root enzyme activity

Roots were stored overnight at 4˚C. On the day following collection, we selected 20 healthy root tips from each alder with no more than two root tips from the same fine root segment. We determined healthy root tips by inspecting for turgidity and a white inner pith under a dissecting microscope. Isolated tips were placed into wells of a 96-well microplate filled with 100 μL 0.5 M $CaCl_2$. We then sequentially measured potential activities of β-glucosidase (EC 3.2.1.21), N-acetylglucosamindase (EC 3.2.1.14; hereafter chitinase), acid phosphatase (EC 3.1.3.2; hereafter phosphatase), and laccase (EC 1.10.3.2; hereafter phenol oxidase) following the protocol in Pritsch *et al.* (2011). These enzymes represent a range in both degradative mechanisms (hydrolytic vs. oxidative) and targeted nutrients and substrates (N vs. P vs. C). Activities of the three hydrolytic enzymes were measured using 4-methylumbelliferon (MUB) fluorogenic substrates (MUB for β-D-glucopyranoside, MUB N-acetyl glucosaminide for chitinase, and 4-MUB-phosphate for phosphatase). Phenol oxidase activity was measured using the colorimetric substrate 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid).

During assays, we sequentially incubated EcM root tips in concentrated substrate solutions in buffers at the appropriate pH associated with each enzyme. Root tips were incubated in the dark at room temperature on a shaker table. At the end of β-glucosidase, chitinase, and

phosphatase assays, we transferred incubated solution to clean, black microplates filled with 150 µl 1 M Tris stop solution and measured fluorescence at 360 ± 20 nm excitation and 460 ± 20 nm emission. At the end of phenol oxidase assays, incubation solutions were transferred to clean, clear plates and absorbance was measured at 420 nm. All fluorescence and absorbance measurements were taken on a Synergy HTX microplate reader (Biotek, USA). Root tips were rinsed between incubations with diluted incubation buffer before beginning the assay for the next enzyme. After completing the final assay, root tips were scanned in 60 µl tap water in a 96-well plate and projected surface area was measured in WinRhizo. Potential enzyme activity was calculated as pmol substrate converted mm^{-2} root tip min^{-1} (hereafter pmol mm^{-2} min^{-1}) using standard curves for MUB and the Beer-Lambert law for ABTS ($\varepsilon_{420} = 3.6 \times 10^4$ M^{-1} cm^{-1}; Pritsch *et al.*, 2011).

Alder N fixation

Nitrogen fixation was measured following the $^{15}N_2$ incubation method described by Anderson *et al.* (2004). A detailed description of N fixation analysis and measurements can be found in Heslop and Ruess (*in prep*). In short, paired samples of nodules from each shrub were isolated from alder roots—one to be incubated in $^{15}N_2$ enriched air and the other analyzed for natural abundance. Nodules were incubated in the field and then transported to the lab in liquid nitrogen to cease activity, dried, and ground. We measured ^{15}N content on the same mass spectrometer mentioned above. Fixation was calculated as µmol N fixed g^{-1} nodule dry weight hr^{-1} (hereafter µmol N g^{-1} hr^{-1}).

Statistical analysis

All analyses were conducted in R version 4.0.2 (R Core Team, 2019). Data can be accessed at the Bonanza Creek LTER data repository under doi XX.

We implemented both a multivariate and a univariate approach to explore differences and test drivers in the root enzyme activity of each sampled alder. First, we explored differences in the enzymatic fingerprint—or the profile of relativized activities—among alders and tested for correlations with climate, edaphic properties, and alder traits. To account for the large ranges in activities among alders and enzymes, activities were square root transformed and relativized by the maximum activity within each of the four measured enzymes. We created a nonmetric multi-dimensional scaling (NMDS) ordination of the Bray-Curtis distances among alder samples using the relativized activities in the vegan function *metaMDS*. The NMDS was fit on 2 axes after determining that adding more dimensions did not lower stress by more than 0.05. We then tested for correlations between the ordination and climate, alder, and soil variables (MAT and MAP; foliar C:N, N:P, $\delta^{15}N$, $\delta^{13}C$, SLA, basal area, and ramets/alder; soil organic layer thickness, moisture, pH, TOC, DON, DIN, PO_4-P, C:N, and $\delta^{15}N$). Before testing correlations, all predictor variables were z-score standardized.

Following our exploratory NMDS ordination, we tested whether alder and soil properties predicted root enzymatic fingerprints within regions using partial Canonical Correspondence Analysis (pCCA). We created a categorical variable to use as a conditional variable, characterizing sites 1-3 as “south” and sites 4-9 as “north”. This categorization was heavily informed by NMDS axis 1 values, where 95% of the variation was explained by MAT. Our predictor matrix contained covariates that significantly correlated with the NMDS ordination (except for MAT). We reduced our pCCA model to improve fit and remove collinearities using

the vegan function *step.CCA*. Significance of our final model against a null and of its axes and covariates were tested with the permutations-based function *anova.cca*.

To test the effects of climate, edaphic, and alder properties on the root activities of each enzyme (rather than enzymatic fingerprint), we fit generalized linear mixed effects models and used backwards model selection based on AIC values. Because we found in our multivariate analysis that the north vs. south category explained a large amount of variation among our enzyme values, we ran separate models for each region for each enzyme. The full model for each enzyme and region included the significant covariates from the pCCA. To account for the spatial dependencies within our sampling design separate from latitudinal designation, we included the nested random effects of alder individual within site within region. We reduced the full model using the function *step.AIC* and selected the best fitting model among our full, reduced, and null (random effects only) models. Models of β-glucosidase, chitinase, and phosphatase were fit using a log-gamma distribution, and residuals were visually inspected for goodness-of-fit. Phenol oxidase models were fit using zero-inflated log-gamma distribution.

To test whether the same variables drove N fixation, we repeated our GLMM analysis with N fixation rate (per unit nodule biomass) as a response variable. Because we did not observe a strong influence of north vs. south category on fixation, we fit one model for both regions. The full model included covariates from the explanatory NMDS matrix. We selected models based on AIC_c values due to small sample size.

Lastly, we tested whether N fixation and enzyme activity were related and whether their relationship was influenced by N and P limitation using GLMM. Foliar N:P ratios can serve as an indicator of N and P limitation, where values <16 denote N limitation and values >16 denote P limitation. We fit a model predicting N fixation as a function of the interaction between

standardized activities of each enzyme and foliar N:P ratio. We conducted backwards model selection based on AIC_c values. We fit an additional separate model to test the interactive effects of enzymatic fingerprint and foliar N:P ratio on N fixation, using pCCA axes 1 and 2 as covariates.

Results

Site characteristics

Site characteristics varied both within and among regions. Mean annual temperature decreased northward with latitude while MAP was highest in the middle latitudes (Table 1). In general, mineral soil pH varied across regions while other variables differed among site moisture classes and vegetation descriptions regardless of region or latitude (Table 2).

Enzyme activities

We measured enzyme activities on 1015 roots among 54 alders across 9 sites. Visually, root enzymatic fingerprints of individual alders differed between the southern sites 1-3 and northern site 4-9 (Fig. 2). Axis 2 captured differences between alders in southern vs. northern sites (R^2 = 0.95). Axis 2 seemed to explain differences among alders within the same sites (R^2 = 0.03). Alders in the south had higher total activities than northerly sites, driven by higher activities of the hydrolytic enzymes β-glucosidase, chitinase, and phosphatase rather than the oxidative enzyme phenol oxidase. Conversely, northern alders were associated with higher oxidative activity rather than hydrolytic. Despite the large differences between southern and northern sites expressed by axis 1, predictor variables were mostly related with the variation along axis 2. Only MAT and, to a lesser extent, SLA and TOC explained the differences between sites 1-3 and sites 4-9. Higher MAT (r^2 = 0.60, p = 0.001), SLA (r^2 = 0.13, p = 0.02), and soil TOC (r^2 = 0.35, p = 0.001), correlated with alders that had higher hydrolytic enzyme activity over oxidative. Soil

organic layer thickness ($r^2 = 0.38$, $p = 0.001$), moisture ($r^2 = 0.13$, $p = 0.03$), DIN ($r^2 = 0.12$, $p = 0.03$), and C:N ratio ($r^2 = 0.21$, $p = 0.003$); foliar $\delta^{15}N$ ($r^2 = 0.20$, $p = 0.007$) and $\delta^{13}C$ ($r^2 = 0.34$, $p = 0.001$); alder basal area ($r^2 = 0.12$, $p = 0.03$); and MAP ($r^2 = 0.14$, $p = 0.02$) correlated with variation within sites in enzymatic fingerprints along axis 2.

The pCCA accounted for 69% of total inertia, or observed variation, among the root enzymatic fingerprints of alders (model chi-square = 0.03, F = 4.0, p = 0.001). The majority of that variation (46% of total inertia) was captured by our conditional south vs. north variable. After accounting for the differences among southern and northern sites, several climate, alder, and soil properties captured 23% of variation. However, roughly a third of the variation between enzymatic fingerprints remained unexplained by our model (31% of total inertia).

The first two axes of the pCCA explained most of the variation among root enzymatic fingerprints (Fig. 2). Axis 1 and 2 captured 72% ($p = 0.001$) and 23% ($p = 0.097$) of the accumulated eigenvalues within the pCCA. Axis 1 delineated a gradient ranging from enzymatic fingerprints with relatively higher phosphatase activities on the left-hand side to those with higher phenol oxidase activities on the right-hand side. Along this gradient, higher phosphatase activities were associated with higher soil moisture ($p = 0.004$) and foliar $\delta^{15}N$ ($p = 0.04$) while higher phenol oxidase activities were associated with higher foliar C:N value ($p = 0.05$), alder basal area ($p = 0.05$), TOC ($p = 0.03$), DIN ($p = 0.02$), PO_4-P ($p = 0.01$), and soil $\delta^{15}N$ ($p = 0.07$). β-glucosidase and chitinase activities varied along axis 2. Higher β-glucosidase activities were related to higher TOC, soil PO_4-P, and foliar C:N value. High chitinase activities were related to higher soil $\delta^{15}N$ and alder basal area. However, since axis 2 captured an insignificant proportion of the variation, the differences in β-glucosidase and chitinase activities were not likely to be

strong drivers of the overall differences in enzymatic fingerprints within southern and northern sites.

Individual responses of each enzyme to soil properties and alder traits differed among enzyme identity and between southern and northern sites. In southern sites, activities of three out of four enzymes—β-glucosidase, chitinase, and phenol oxidase—were explained by edaphic and alder properties (Table 3). In contrast, two out of the four enzymes—chitinase and phosphatase—were explained by conditions in northern sites. For the rest, the null model was found to be the best fit. Though the effect of edaphic and alder properties varied by each enzyme, some general trends emerged. Higher TOC concentration decreased activities when included in the model while higher PO_4-P, DIN, and foliar C:N value increased activities (Fig. 3). Soil moisture, alder basal area, and foliar $\delta^{15}N$ had mixed effects, increasing activities of some enzymes while decreasing those of other enzymes. In southern sites, soil moisture and foliar $\delta^{15}N$ decreased chitinase and phenol oxidase activities, but increased phosphatase activity in northern sites. All edaphic and alder properties included in our models affected more than one enzyme except for soil $\delta^{15}N$, where higher values increased activities of only chitinase in southern sites. Similarly most properties affected activities in both southern and northern sites with the exception of TOC, DIN, and soil $\delta^{15}N$, which were included in the best fitting models for only southern sites.

N fixation

We measured N fixation rates on 50 alders across nine sites (we attempted to measure fixation on 54 alders, but four out of the six alders in site 1 did not have nodules). Drivers of root enzyme activity did not act as drivers of N fixation rates. Instead, N fixation was explained by soil organic layer thickness and foliar $\delta^{13}C$ (Table 4). Fixation increased with higher foliar $\delta^{13}C$

and decreased with deeper organic layers (Fig. 4). A large proportion of the variance still remained unexplained by the best fitting model.

Fixation was also related to both root enzyme profile and the activities of individual enzymes. The relationship between enzymatic fingerprint and N fixation depended on foliar N:P ratio ($R^2_m = 0.10$, $R^2_c = 0.63$; Table 5a). N fixation increased with pCCA axis 1 at low N:P values, but decreased with axis 1 at high N:P values (Fig. 5). Ecologically, axis 1 represents the gradient of enzymatic fingerprints with relatively higher phosphatase at lower values to those with relatively higher phenol oxidase activities at higher values. When N:P values were low, indicating N was more limiting, alders that were more active in producing phenol oxidase than phosphatase resulted in higher N fixation. When N:P values were high and P was more limiting, alders that were more active in phosphatase resulted in higher fixation. At intermediate N:P values, there was no effect of enzymatic fingerprint on fixation. Random effects of region were included in the final model.

In contrast, activities of individual enzymes did not interact with foliar N:P values to affect N fixation. Instead, N fixation was best explained by an additive model of phosphatase and phenol oxidase root activities ($R^2 = 0.12$; Table 5b). N fixation decreased with higher phosphatase activities and increased with higher phenol oxidase activities (Fig. 5). Additionally, phosphatase activity had a marginally greater effect on N fixation than phenol oxidase. Random effects of site within region did not improve the model and were not included in the final model.

Discussion

Most field research on alder's root symbionts has focused on either N fixation or ectomycorrhizal fungal community and function. Given the high potential for the combined

effects of *Frankia* and EcM to substantially alter ecosystem function as alder expands across the Arctic tundra, we characterized N fixation and EcM-associated root enzyme activity across a latitudinal gradient. To our knowledge, this was the first study to pair measurements of fixation and enzyme activity on alder in the field. Temperature overwhelmingly explained differences in root enzyme activity, strongly correlating ($r^2 = 0.60$) with enzyme profiles along NMDS axis 1, which explained 98% of the variation in enzyme profiles. Higher temperature resulted in higher total root enzyme activity (Fig. 2). We did not detect an effect of temperature on N fixation rates. Instead, fixation was best modeled by soil organic layer thickness and foliar $\delta^{13}C$, which together explained about a fifth of the variation among fixation rates. In testing the relationship of fixation and EcM root enzyme activity and whether it depended on relative N or P limitation, we found alder's symbiotic root processes were coupled despite having different drivers. However, our results offer mixed support for the dependence on N and P limitations.

Different drivers of root enzyme activity and N fixation

Though experimental manipulations are necessary to further test the effect of warming, our findings tentatively suggest that overall EcM-associated root enzyme activity will increase as temperatures warm. However, warming may not equally affect the activity of all enzymes. Alders in warmer sites had higher hydrolytic enzyme activity relative to that of the oxidative enzyme compared to colder sites, which had relatively higher oxidative enzyme activity. Hydrolytic enzymes are generally thought to target more labile forms of SOM while oxidative more recalcitrant forms. This suggests warming will be accompanied by not only higher root enzyme activity, but also a shift in the enzymatic fingerprint from being dominated by oxidative activity to hydrolytic. If so, soils under alder may experience faster cycling of labile SOM

compared to recalcitrant SOM, resulting in shifts towards a larger pool of lignin-rich SOM and decreased saprotrophic soil microbial activity as communities compete for labile SOM with mycorrhizal fungi.

After accounting for the predominant effect of temperature, root enzyme activity was explained by varying edaphic conditions. However, very few patterns emerged surrounding which drivers were important among enzymes and between regions. One of the more intriguing patterns was the importance of PO_4-P, indicated by the high positive coefficient value. In three of the five linear mixed effects models, the most influential predictor of enzyme activity was PO_4-P. In all instances, higher inorganic P concentration was associated with higher enzyme activity. The importance of inorganic P could relate to known patterns in alder's EcM fungal community. Alder partner with a limited number of EcM fungi compared to other host plants, and their EcM fungal communities include species that are highly specific to alder (Põlme et al., 2013; Tedersoo et al., 2009). Many researchers have hypothesized that selective pressure induced by the tripartite symbiosis optimizes for alder EcM communities that specialize in P acquisition to meet high host demand (Horton et al., 2013; Ruess et al., 2019; Walker et al., 2014). The positive correlation between soil PO_4-P and activities of several enzymes, including phosphatase, likely reflects that alder's EcM enzymatic activity increases P availability rather than higher P availability increasing enzyme activity. This may indicate support for the previously hypothesized importance of P acquisition in alder's EcM functional capacity.

We were surprised to find that N fixation did not correlate with mean annual temperature nor with variables indicating relative bioavailability of N to P. Previous studies have found these variables are important drivers of N fixation on alder and other N-fixing plants (Houlton et al., 2008; Ruess et al., 2013; Salmon et al., 2019; Uliassi and Ruess, 2002). The scale at which

temperature affects N fixation might explain why we did not detect any effects of MAT on fixation. Nitrogen fixation varies both with seasonal temperature variation at localized scales, increasing at the warmest parts of the growing season (Uliassi and Ruess, 2002), and at global scales as MAT decreases from the tropics poleward (Houlton et al., 2008). In this study, mean annual temperature may not have been a precise enough measurement to capture any seasonal variation in fixation that occurred. Along a similar line of thought, the regional latitudinal gradient may not have been on a large enough scale to capture the variation previously observed in fixation rates across global temperature gradients. Caution should be taken in applying our lack of an observed relationship between fixation and temperature to future research.

Instead, N fixation in our study increased with thinner soil organic layer. This suggests that the ease of accessing the mineral layer, which provides plants with a source of inorganic P, may be an important driver of fixation. Alder is not evenly expanding across northern latitudes. Instead, its spread is highly driven by disturbances where mineral soil has been exposed at or near the ground surface, such as in frost boils, thermokarsts, water tracks, fire scars, or floodplains (Frost et al., 2013; Lantz et al., 2010, 2009; Tape et al., 2012). These environments feature shallow to non-existent soil organic layers, in which fixation rates are likely high according to our results. Our findings, therefore, point to increased fixation as a possible mechanism for supporting alder growth and success once seedlings establish in disturbed areas.

Not all root symbioses have evolved from a similar extent of interdependence. Although *Frankia* can be found as a free-living soil microbe, it is intimately tied to alder when living in symbiosis in complexly chambered nodules (Hay et al., 2020; Pawlowski, 2008). The relationship between EcM and alder is, in contrast, arguably looser. By definition, EcM fungi do not penetrate the alder's cells but instead form a Hartig net of hyphae between cells through

which to facilitate resource transfer (Smith and Read, 2008). From there, EcM can build extensive mycelial structures that explore up to meters beyond the root tip to obtain their nutrients (Agerer, 2001; Anderson and Cairney, 2007). Some evidence suggests that EcM fungi may withhold N from their host plant (Franklin et al., 2014; Hewitt et al., 2020), even as they continue to obtain plant C (Näsholm et al., 2013). Furthermore, EcM, even among alder's species-limited associations, represent a polyphyletic group ranging in a variety of traits; *Frankia,* conversely, are monophyletic and while species differ in function (Ruess et al., 2013), the group arguably varies much less than EcM. Because of these differences in the nature of alder's two root symbioses, it is not surprising that the drivers behind fixation and enzyme differ.

Evidence for coupled symbiotic root processes

Our results provide tentative evidence that N fixation and EcM-associated root enzyme activity are correlated in alder's tripartite symbiosis. We modeled the relationship between fixation and enzyme activity in two ways: 1) as an additive function of standardized activities of each enzyme and 2) as an interactive function between an enzymatic fingerprint index (pCCA axis 1) and foliar N:P values. The first model showed that higher phosphatase activities decreased fixation rates while higher phenol oxidase activities increased fixation rates. This is opposite of what we expected—that higher phosphatase activity would increase P acquisition and thus, increase fixation. However, the second model may more accurately capture the relationship between fixation and enzyme activity. The use of an enzymatic fingerprint index incorporates the potential interplay between enzymes as they are produced. As discussed above, enzymatic fingerprints on alder were high in either phosphatase or phenol oxidase activity—one or the other. Through the second model, we found that the relationship between fixation and enzyme

activity depended on whether N or P was relatively more available. Our results suggest that as alder expands with warming, its potential heightened influence on ecosystems will be mediated by local edaphic conditions. In particular, as warming stimulates nutrient release, the resulting changes in N availability relative to P will control whether inputs of fixed N are accompanied by EcM-induced increases in either soil P availability or the transformation of lignin-rich SOM.

The nature of the relationship between EcM root enzyme activity and N fixation relationship and its underlying mechanisms remain unresolved. Though we found statistical evidence that root processes are coupled, the models explained a relatively small proportion of the variation in fixation. Furthermore, a modeled relationship does not necessarily imply that these microbial symbionts directly interact with one another. Likely, the relationship between *Frankia* and EcM is moderated by alder's C economy and allocation in response to nutrient availability. Past studies have estimated that phosphatases cost 15 g N g^{-1} P acquired (Treseder and Vitousek, 2001; Wang et al., 2007). This cost for enzymatically accessing P might be beneficial only when it is relatively not available. The interaction we observed between *Frankia* and EcM's relationship and foliar N:P values implies that when N and P are equally available, fixation and enzyme activity are no longer correlated. This brings into question the meaning of tripartite: if their coupling ceases depending on nutrient availability, are *Frankia*, EcM, and alder truly tripartite or is it simply two independent symbiotic relationships?

There are a limited number of studies that have compared N fixation and mycorrhizal colonization or activity in alder. A common finding was that though dual inoculation by both N-fixers and mycorrhizal fungi increased host plant growth, mycorrhizal colonization tended to reduce nodulation and nitrogenase activity, implying a competitive interaction (Chen et al., 2020; Ekblad and Huss-Danell, 1995; Yamanaka et al., 2005). Yet, other research has found the

opposite to be true, where mycorrhizal colonization improved nodulation (Chatarpaul et al., 1989; Yamanaka et al., 2003). These studies occurred on seedlings, where allocation can be quite different compared to mature shrubs, and with highly controlled symbiotic communities grown in greenhouses. In some cases, just one species of mycorrhizal fungi was used in inoculation. In contrast, our field study on mature alder shrubs that were likely colonized by more diverse *Frankia* and EcM communities suggests that as soil nutrient conditions change, alder consequently shifts allocation towards its root symbionts. Furthermore, as indicated by the observed trade-off between relative phosphatase and phenol oxidase activity, these shifts in allocation may not be generalizable as resulting from competitive or facultative interactions between fixation and total mycorrhizal activity, but instead are specific to each enzyme.

Implications as alder expands

As alder expands, its effects on Arctic ecosystems will be extensive (Wallace and Baltzer, 2020). Alder is a tall shrub that captures snow, insulating winter soils (Sturm et al., 2005b), prolonging spring melt (Wilcox et al., 2019), and decreasing winter albedo (Chapin et al., 2005). Its dominance can create islands of high N availability via increased mineralization (Salmon et al., 2019) and increase bulk soil enzyme activity, presumably accelerating decomposition (Heslop, 2020). Here, we add one more to the list of alder's future impacts: the potential for its root symbionts to reshape ecosystem nutrient cycling. This study indicates that warming temperatures will increase EcM root enzyme activity, but potentially have no effect on N fixation. Instead, fixation will be most affected by changes in soil organic layer thickness. Increased disturbances or accelerated decomposition could be a mechanism for future decreases in soil organic layer depth and thus increase fixation. Due to the limitations in using MAT as this

study's temperature measurement however, future research should experimentally explore the effects of temperature and edaphic conditions on these root processes in a field setting, especially N fixation. Most importantly our results show that the effects of alder expansion on ecosystem nutrient cycling will not be limited to that of one symbiotic process. As alder's root symbionts respond to their associated drivers, they are also interacting with one another. The causal pathway for their coupling is unclear, but likely surrounds alder's C economy. Regardless of whether this interaction is occurring proximally or through distal causes, changes in mycorrhizal activity will accompany changes in fixation, and vice versa. Therefore, to predict how alder's root symbionts will restructure nutrient cycling, models cannot consider N fixation alone, but must also incorporate mycorrhizal activity.

Tables

Table 1. Site location and characteristics.

Site	Region	Description	Latitude (˚N)	Longitude (˚ W)	Elevation (m)	MAT (˚C)	MAP (cm)	Organic layer thickness (cm)	soil pH	Moisture class
1	Interior	Tussock tundra	63.772	-145.080	511	-0.2	22.8	> 28*	4.7**	sub-hygric
2	Interior	Mixed forest	64.760	-148.252	318	1.3	20.4	6.9	4.7	sub-mesic
3	Fish Creek	Tussock tundra	66.536	-150.790	250	-1.7	35.8	23.6	5.3	sub-hygric
4	Wiseman	Water track	67.409	-150.062	466	-3.1	28.2	17	4.3	mesic
5	Wiseman	Mixed forest	67.275	-150.160	332	-2.8	31.1	20	4.5	sub-mesic
6	Wiseman	Tussock tundra	67.384	-150.107	371	-3.1	27.4	21.7	6.0	sub-hygric
7	Sagwon Bluffs	Water track	69.413	-148.628	200	-4.6	20.4	5.4	6.8	mesic
8	Sagwon Bluffs	Mesic ridge	69.415	-148.622	204	-4.6	20.4	14.5	6.9	sub-mesic
9	Sagwon Bluffs	Tussock tundra	69.417	-148.609	212	-4.6	20.1	9.7	3.3	sub-hygric

* Soil organic layer had not thawed to the mineral layer at the time of collection and therefore its thickness was not measured.
** pH from organic soil

Table 2. Alder density and site mean values ± SE for alder size and foliar properties.

Site	Alder density (ramets m^{-2})	Alder basal area (cm^2)	# of ramets / alder	SLA ($cm^2 g^{-1}$)	Foliar C:N	Foliar N:P	Foliar $\delta^{13}C$	Foliar $\delta^{15}N$
1	3.1	18 ± 7	13 ± 5	124 ± 5	20.0 ± 0.8	24.3 ± 1.7	-27.7 ± 0.1	-1.55 ± 0.04
2	0.3	123 ± 25	9 ± 2	218 ± 13	17.8 ± 0.7	15.0 ± 0.4	-30.9 ± 0.2	-1.27 ± 0.03
3	1.3	28 ± 7	10 ± 2	168 ± 16	13.9 ± 0.6	21.0 ± 1.0	-29.1 ± 0.2	-1.32 ± 0.04
4	25.4	102 ± 25	15 ± 2	133 ± 5	22.0 ± 0.7	17.3 ± 1.1	-28.0 ± 0.3	-1.48 ± 0.09
5	0.3	31 ± 7	5 ± 1	165 ± 6	15.4 ± 0.5	19.1 ± 1.9	-27.8 ± 0.4	-1.43 ± 0.03
6	0.5	18 ± 6	7 ± 1	109 ± 4	23.0 ± 0.8	21.6 ± 1.3	-26.6 ± 0.2	-1.54 ± 0.02
7	39.6	85 ± 10	10 ± 1	163 ± 6	14.9 ± 0.4	24.7 ± 0.4	-30.0 ± 0.3	-1.87 ± 0.10
8	1.9	97 ± 27	19 ± 3	126 ± 2	17.5 ± 1.1	25.2 ± 1.3	-28.5 ± 0.2	-1.39 ± 0.07
9	0.5	27 ± 14	11 ± 3	114 ± 6	18.7 ± 0.8	23.3 ± 0.9	-29.1 ± 0.1	-1.49 ± 0.07

Table 3. Model performance for root activity of each enzyme, including negative Log-likelihood (-log(L)), corrected Akaike information criteria (AICc), AICc differences from the lowest scoring model (ΔAICc), and Akaike weight (w). Selected model is in **bold**.

Enzyme	Region	Model	df	-log(L)	AIC	ΔAIC	weight	R_c^2	R_m^2	N (roots)
β-glucosidase	North	Full	13	-1286	2597	4	0.07	--	--	707
		Intermediate	11	-1286	2593	0.1	0.46			
		Null	**5**	**-1292**	**2593**	**0**	**0.47**			
	South	Full	13	-1446	2918	9	0.01	0.20	0.36	276
		Intermediate	**8**	**-1446**	**2909**	**0**	**0.93**			
		Null	5	-1452	2914	6	0.06			
Chitinase	North	Full	13	-2848	5721	5	0.08	0.15	0.30	709
		Intermediate	**10**	**-2848**	**5717**	**0**	**0.91**			
		Null	5	-2858	5727	10	0.01			
	South	Full	13	-1919	3863	6	0.06	0.15	0.20	283
		Intermediate	**10**	**-1919**	**3858**	**0**	**0.86**			
		Null	4	-1927	3862	5	0.08			
Phosphatase	North	Full	13	-2664	5353	7	0.03	0.11	0.14	709
		Intermediate	**9**	**-2664**	**5346**	**0**	**0.97**			
		Null	4	-2681	5371	25	0			
	South	Full	13	-2083	4193	10	0.01	--	--	282
		Intermediate	8	-2085	4186	3	0.16			
		Null	**4**	**-2087**	**4182**	**0**	**0.83**			
Phenol oxidase	North	Full	13	-1992	4011	6	0.04	--	--	709
		Intermediate	11	-1993	4008	4	0.14			
		Null	**5**	**-1997**	**4005**	**0**	**0.82**			
	South	Full	16	-560	1152	0	0.64	0.39	0.39	284
		Intermediate	**13**	**-563**	**1153**	**1**	**0.36**			
		Zero-inflated	8	-572	1160	9	0			
		Null	6	-577	1167	16	0			

Table 4. Coefficient values for the best fitting model of N fixation as a function of edaphic conditions (N = 50 alders).

	Final model variables	coefficient	SE	z-value	p-value
N fixation (umol N/g/hr)	Intercept	3.6	0.3	10.7	<0.001
	Organic layer thickness	-1.0	0.4	-2.5	0.01
	Foliar $\delta^{13}C$	0.7	0.4	2.0	0.05

Table 5. Coefficient values for the best fitting model of N fixation as a function of a) pCCA axes and of b) root enzyme activities and their interaction with foliar N:P ratio.

	Final model variables	coefficient	SE	z-value	p-value	N (alder)
a)	Intercept	1.1	0.3	3.3	<0.001	50
	pCCA axis 1	0.03	0.09	0.4	0.7	
	Foliar N:P	0.02	0.10	0.1	0.9	
	pCCA axis 1: Foliar N:P	-0.24	0.08	-3.1	0.002	
b)	Intercept	1.2	0.09	14.3	<0.001	50
	Phosphatase	-0.3	0.09	-3.5	<0.001	
	Phenol oxidase	0.2	0.1	2.2	0.03	

Figures

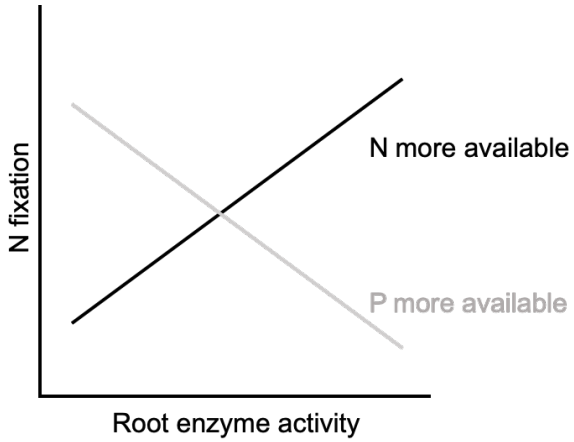

Fig. 1. Conceptual diagram of the hypothesized relationship between N fixation and root enzyme activity. The direction of the relationship will depend on whether N or P is relatively more available than the other.

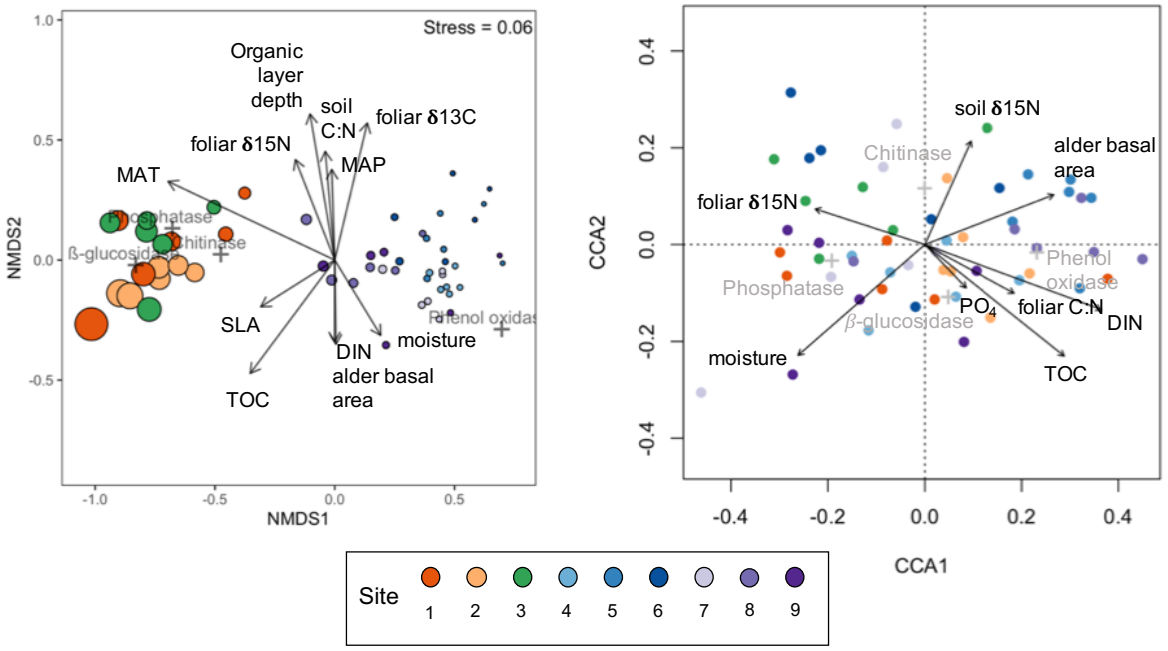

Figure 2. a) NMDS ordination of the Bray-Curtis distances of root enzymatic fingerprints among individual alders. b) Partial CCA of root enzymatic fingerprints with south vs. north conditional variable. Points are colored by site and in (a) scaled relative to total enzyme activity. + designate the scores, or optimal positions, of enzymes in ordination space. Arrows represent the direction and magnitude of significant correlations with climate, alder, and soil variables.

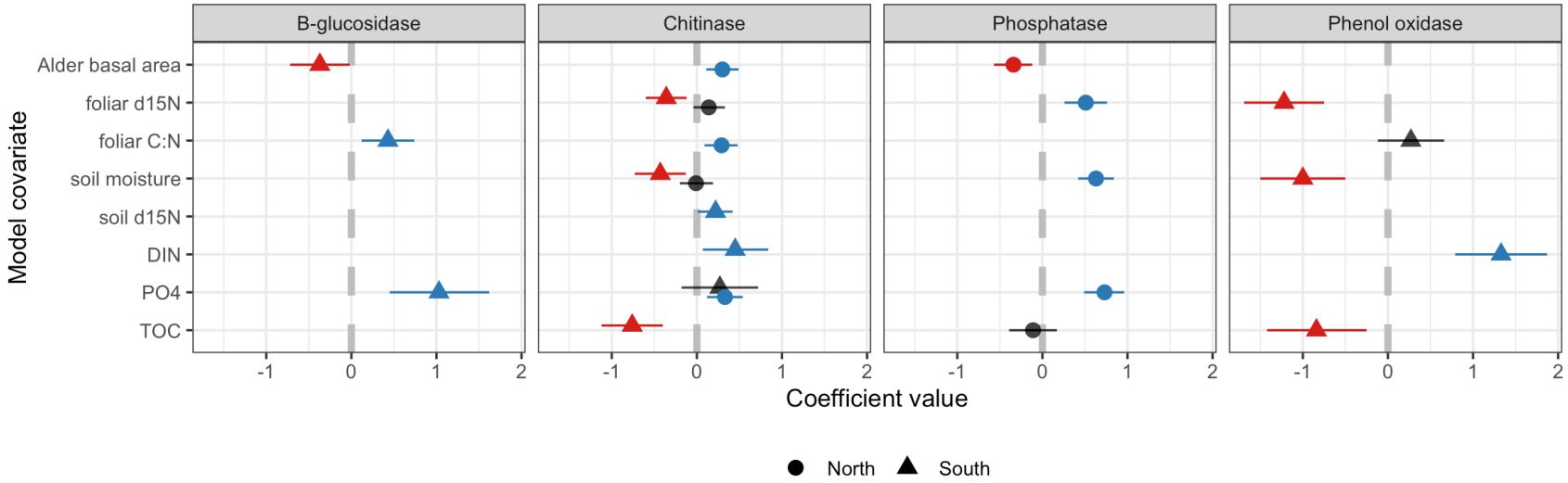

Fig. 3. Coefficients from the best fitting models for each enzyme and region. Point shape corresponds to the region-specific model. Red colored points represent negative effects; blue represent positive effects; and dark grey represent non-significant effects. All coefficients are presented on the log-scale.

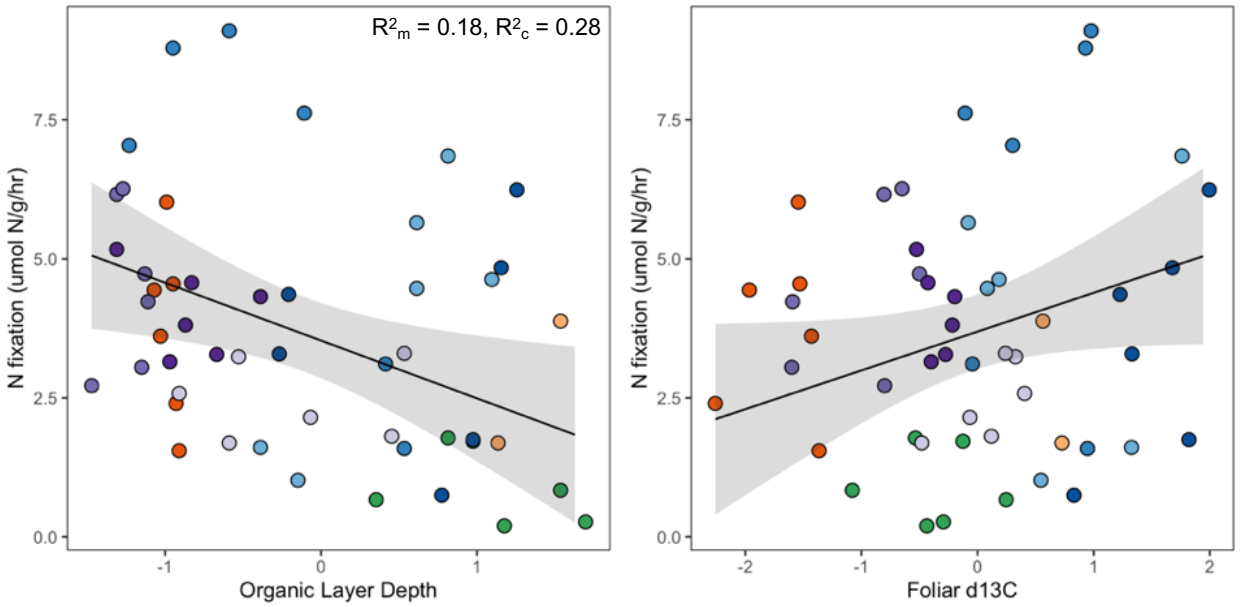

Fig. 4. Modeled relationship of N fixation with a) soil organic layer thickness and b) foliar $\delta^{13}C$. Shading represents the 95% confidence interval. Best fitting model was Fixation ~ Organic layer thickness + Foliar $\delta^{13}C$ + nested random effects of (1|Region/Site).

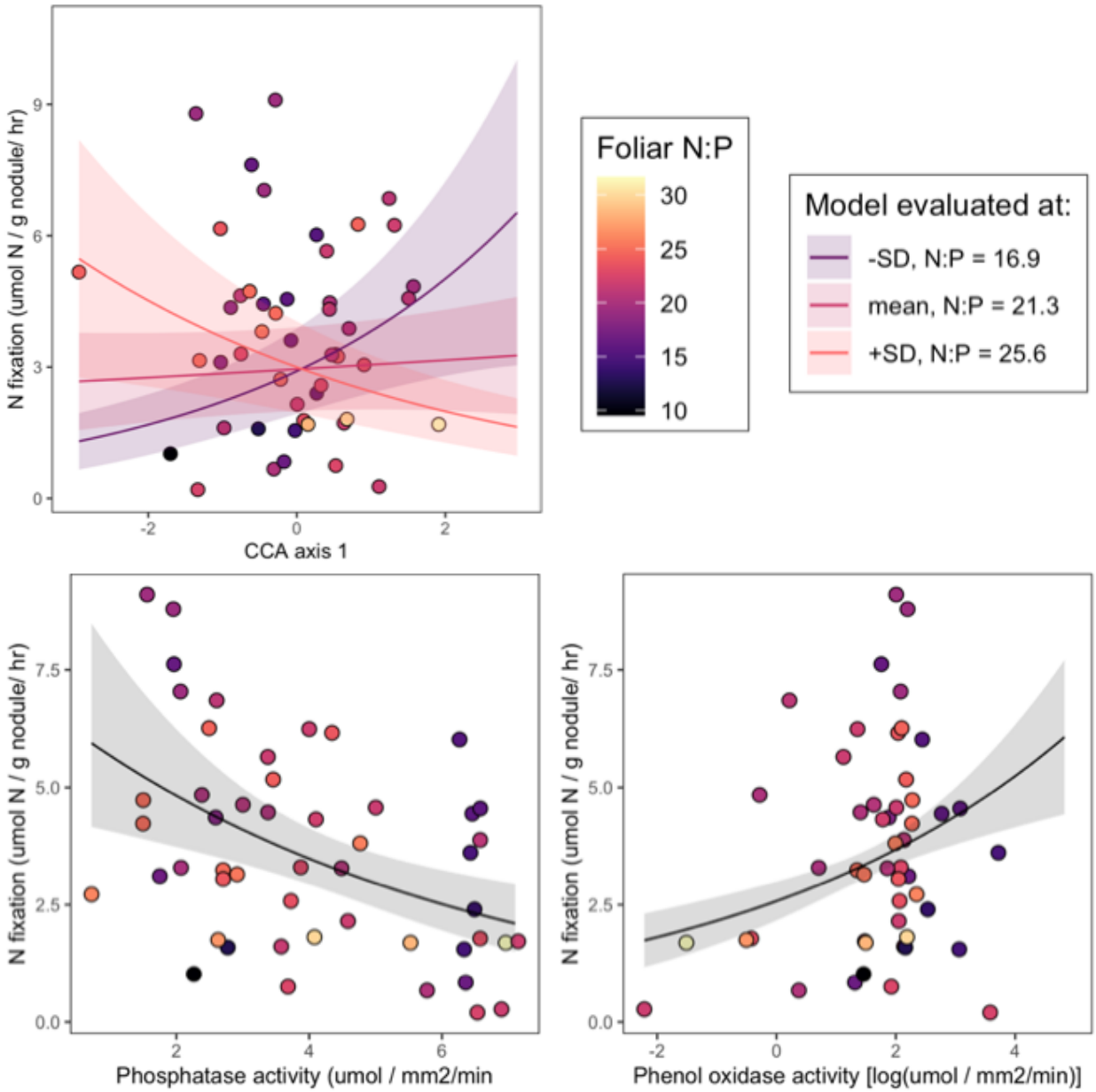

Fig. 5. Modeled relationship between N fixation and a) pCCA axis 1, b) phosphatase activity, and c) phenol oxidase activity. Foliar N:P values interacted with pCCA axis 1 but not phosphatase or phenol oxidase activity.

DISCUSSION OF RESULTS AND CONCLUSIONS

This dissertation explored the ways Arctic mycorrhizal function responded to three consequences of climate change: warming, increased nutrient availability, and host shrub expansion. In chapters 2 and 3, I tested hypotheses on the effects of long-term reductions in nutrient and temperature limitation on mycorrhizal root enzyme activity. Chapter 2 focused on EcM roots and the extent to which fungal communities drive responses to both warming and nutrient addition. I found that warming and fertilization had predominantly opposing and sometimes interactive effects on EcM enzyme activity, where warming dampened the negative effect of fertilization. Although fungal community shifts explained some of the changes in root enzyme activity, there also appeared to be an individual physiological response, where changes in enzyme activity occurred within the most dominant fungal genera. Additionally, responses of EcM function to warming depended on the scale of measurement, reinforcing the necessity of detecting responses at multiple scales to identify mechanisms behind change.

Following the strong effect of nutrient addition on mycorrhizal activity in chapter 2, I expanded on how alleviations in nutrient limitation affect mycorrhizae and their host shrubs in chapter 3, testing responses in two contrasting shrub types across a long-term fertilization gradient. This study provided evidence for the potential co-expansion of deciduous EcM and evergreen ericaceous shrubs and proposed some mechanisms behind co-expansion. My findings suggest that as warming stimulates nutrient mobilization and the expansion of their host shrub, EcM may become more influential in transforming soil organic matter via their relatively unresponsive extracellular enzyme activity compared to the sharply decreasing activity on ericaceous roots. Furthermore, these results greatly contrasted with the observed negative effect of nutrient addition on EcM root enzyme activity in chapter 2. This points to the potential for

temporally dependent responses in EcM even after 15 years of experimental change and supports prior research in that mycorrhizal communities are still undergoing succession in boreal forest 30 years post disturbance (Clemmensen et al., 2014).

Chapter 4 centered on the unique potential of the tripartite symbiosis between *Frankia*, EcM, and Siberian alder to alter nutrient cycling as alder expands in northern latitudes. In this observational study, I explored drivers of alder's two different symbiotic processes—N fixation and EcM root enzyme activity—along a latitudinal gradient from boreal to Arctic Alaska. I found that while variations in mean annual temperature was the strongest driver of root enzyme activity, it did not explain patterns of N fixation. Instead, shallow soil organic layers—a potential indication of root access to mineral P—increased fixation. Additionally, root enzyme activity and fixation were correlated, but that this direction of their correlation depends on relative availability of N and P. As alder both expands into new environments and responds to climate change-related shifts in temperature and edaphic conditions, its impacts on ecosystem nutrient cycling will not be limited to that of one symbiotic process, but instead both.

Combined, these three chapters point to the potential for shifts in mycorrhizal function to substantially alter tundra nutrient cycling. The observed changes to mycorrhizal function could have large effects on overall ecosystem nutrient cycling in an environment where nutrient cycling tightly controls the cycling of its globally important C stocks (Mack et al., 2004). Future research should therefore investigate the connections between mycorrhizal shifts and changes in soil C dynamics to better understand the global consequences, if any, of mycorrhizal response.

Mycorrhizal relationships are not only present in most environments, they are considered important moderators of ecosystem function and stability. As ecosystems around the globe undergo dramatic changes, shifts in mycorrhizas are hypothesized to control how ecosystems

respond. Past studies disentangling the hypothesized role of mycorrhizae in ecosystem function have focused on the southern boreal and temperate forests as well as grasslands. Yet, shifts in mycorrhizas could be more rapid in the climate sensitive Arctic. I hope this dissertation not only illustrates how mycorrhizas may affect Arctic and boreal ecosystems, but also offers some broader perspective on the ecology of symbioses.

APPENDIX

Supplemental Information for Chapter 2

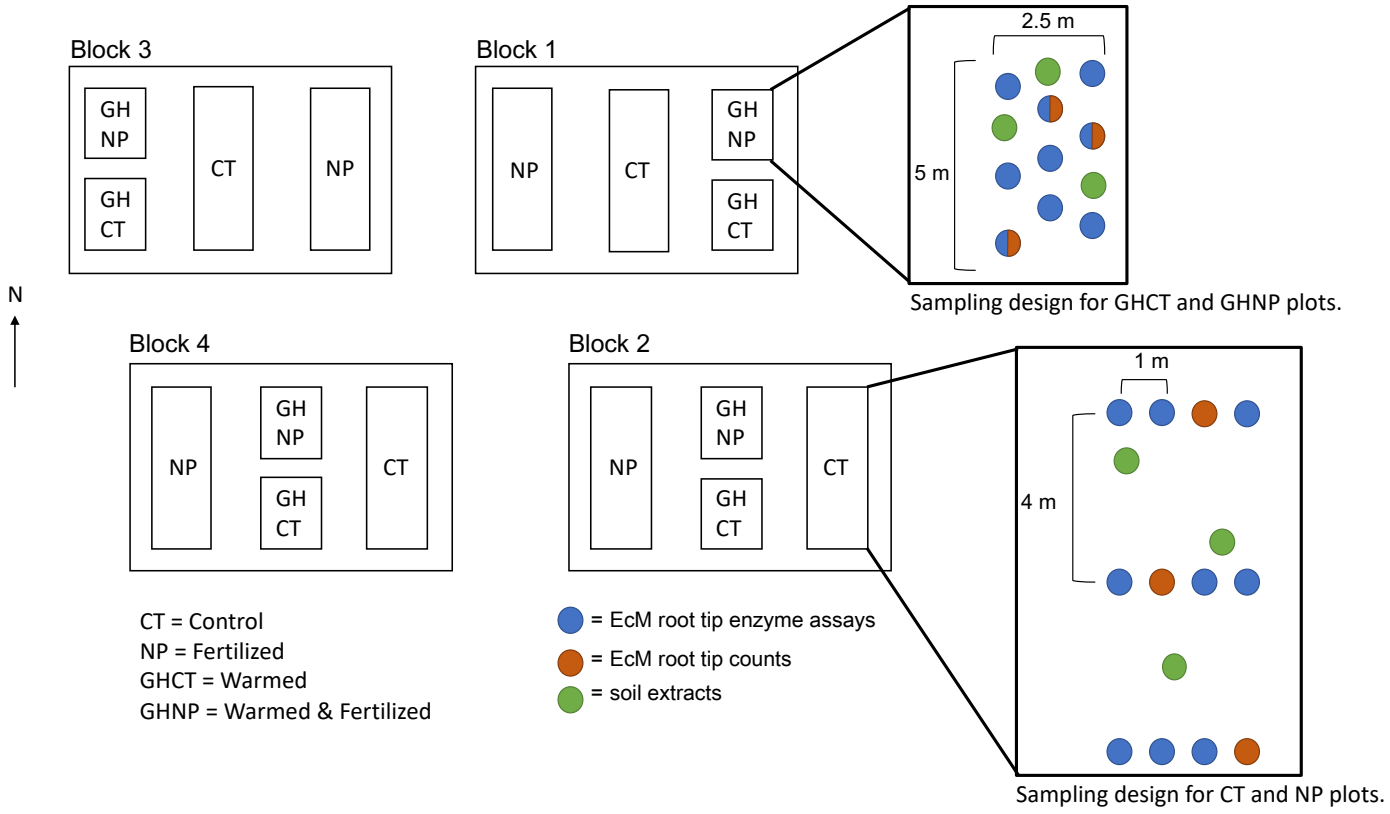

Figure S2.1 Experimental design Map of the full factorial replicated block warming and fertilization experiment. Each of 4 blocks contains 4 plots consisting of one of four treatments (CT, NP, GHCT, GHNP). In ambient plots (CT and NP), three cores were randomly chosen for EcM root tip from each row. In warmed plots (GHCT and GHNP), three cores were randomly chosen to be sampled for both enzyme assays and EcM root tip counts from the entire plot. Each dot represents a core and is colored by what was sampled from each core. Figure is not to scale.

Methods S2.1 Expanded details on root tip extracellular enzyme assays

Root tip enzyme assays

Activities of three hydrolytic enzymes were measured using fluorogenic substrates [4-methylumbelliferone (MUB) N-acetyl glucosaminide for chitinase and 4-MUB-phosphate for phosphatase; 7-amino-4-methylcoumarin labeled leucine for leucine aminopeptidase]; activities of two oxidative enzymes were measured using colorimetric substrates [2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), hereafter ABTS, for phenol oxidase and 3,3',5,5'-tetramethylbenzidine, hereafter TMB, for peroxidase]. During assays, we sequentially incubated EcM root tips in concentrated substrate solutions in buffers at the appropriate pH associated with each enzyme. Root tips were incubated in the dark at room temperature on a shaker table. At the end of leucine aminopeptidase, chitinase, and phosphatase assays, we transferred incubated solution to clean, black microplates filled with 150 µl 1 M Tris stop solution and measured fluorescence at 360 ± 20 nm excitation and 460 ± 20 nm emission. At the end of phenol oxidase assays, incubation solutions were transferred to clean, clear plates and absorbance was measured at 420 nm. Incubation solutions from peroxidase assays were transferred to clean, clear plates filled with 30 µl 1 M H_2SO_4 and absorbance was measured at 450 nm. All fluorescence and absorbance measurements were taken on a Synergy HTX microplate reader (Biotek, USA). Root tips were rinsed between incubations with diluted incubation buffer before beginning the assay for the next enzyme.

Methods S2.2 Expanded details on root tip DNA extraction and ITS amplification

DNA extraction and ITS amplification

Frozen root tips were ground with steel and garnet beads. Cells were lysed and genomic DNA precipitated using Thermo Scientific MagJET Plant gDNA kit (Thermo Scientific, USA).

Lysate was cleared with chloroform:isoamyl OH 24:1. DNA was isolated from lysate in a PEG solution with carboxylated beads and cleaned with an ethanol wash.

Polymerase chain reactions consisted of 1 µl gDNA, 3.2 µl H_2O, 0.4 µl of forward and reverse primer each, 0.1 µl $MgCl_2$, 0.05 µl bovine serum albumin, and 5 µl Maxima HotStart Taq Mastermix. Initial denaturing stage occurred at 95˚C for 2 min, after which we ran 35 cycles of denaturing at 95˚C for 30 sec, annealing at 55˚C for 30 sec, extending at 72˚C for 1 min.

Table S2.1 Comparison of raw and rarefied OTU richness

Table S2.1 a) Mean ± SE raw and rarefied OTU richness

Treatment	OTU Richness	Rarefied OTU Richness (n = 15)
Control	9.3 ± 1.6	6.8 ± 0.8
Fertilized	6.0 ± 0.6	4.7 ± 0.4
Warmed	8.5 ± 1.0	5.8 ± 0.2
Warmed & Fertilized	9.3 ± 1.8	7.4 ± 1.4

Table S2.1 b) Model selection of effects of warming and fertilization on rarefied OTU richness. Not different than results from raw OTU richness.

Model	-log(L)	AICc	ΔAICc	weight
Null	**-31.8**	**71.5**	**0**	**0.90**
Reduced	-31.2	78.3	6.8	0.07
Full	-27.65	76.6	5.1	0.03

Table S2.2 Detailed output from enzyme linear mixed effects models and post-hoc tests

Table S2.2 a) Parameter statistics of the best fitting model for root tip enzyme activities.

Enzyme	Model parameter	z	p-value	R^2_m	R^2_c
Leucine aminopeptidase	Warming	-7.4	>0.001	0.19	0.50
	Fertilization	-4.6	>0.001		
	Warming x Fertilization	3.6	>0.001		
Chitinase	Warming	-0.7	0.485	0.10	0.43
	Fertilization	-3.5	>0.001		
	Warming x Fertilization	2.3	0.023		
Phosphatase	Null model best fit				
Phenol oxidase	Warming	0.1	0.919	0.11	0.29
	Fertilization	-5.2	>0.001		
	Warming x Fertilization	2.3	0.021		
Peroxidase	Null model best fit				

Table S2.2 b) Tukey HSD results for root tip enzyme activity models.

Enzyme	Fertilization effect		Warming effect	
	Control - Fertilized	Warmed - Warmed & Fertilized	Control - Warmed	Fertilized – Warmed & Fertilized
Leucine aminopeptidase	**t = 4.6, p < 0.001 ***	t = -0.4, p = 0.978	**t = 7.4, p < 0.001 ***	t = 2.0, p = 0.18
Chitinase	**t = 3.5, p = 0.002 ***	t = 0.3, p = 0.988	t = 0.7, p = 0.898	**t = -2.52, p = 0.057 ***
Phosphatase				
Phenol oxidase	**t = 5.2, p < 0.001 ***	t = 2.0, p = 0.187	t = -0.1, p = 0.999	**t = -3.3, p = 0.005 ***
Peroxidase				

Table S2.2 c) Parameter statistics of the best fitting model for community-level enzyme activities.

Enzyme	Model parameter	z	p-value	R^2_m	R^2_c
Leucine aminopeptidase	Warming	-1.8	0.080	0.32	0.49
	Fertilization	-5.3	<0.001		
	Warming x Fertilization	2.3	0.021		
Chitinase	Warming	3.1	0.002	0.32	0.58
	Fertilization	-3.1	0.002		
Phosphatase	Warming	2.6	0.010	0.27	0.53
	Fertilization	-3.0	0.003		
Phenol oxidase	Warming	2.0	0.046	0.34	0.35
	Fertilization	-8.7	<0.001		
	Warming x Fertilization	2.4	0.016		
Peroxidase	Warming	4.6	<0.001	0.40	0.50
	Fertilization	-5.2	<0.001		

Table S2.2 d) Tukey HSD results for community-level enzyme activity models.

	Fertilization effect		Warming effect	
Enzyme	Control - Fertilized	Warmed - Warmed & Fertilized	Control - Warmed	Fertilized - Warmed & Fertilized
Leucine aminopeptidase	**t = 5.3, p < 0.001 ***	t = 2.1, p = 0.169	t = 1.8, p = 0.303	t = -1.5, p = 0.434
Chitinase	**t = 3.1, p = 0.013 ***		**t = -3.1, p = 0.013 ***	
Phosphatase	**t = 3.0, p = 0.018 ***		**t = -2.6, p = 0.056 ***	
Phenol oxidase	**t = 8.7, p < 0.001 ***	**t = 4.1, p < 0.001 ***	t = -2.0, p = 0.197	**t = -6.0, p < 0.001 ***
Peroxidase	**t = 5.2, p < 0.001 ***		**t = -4.6, p < 0.001 ***	

Table S3 a) Sequence accession numbers, GenBank matches, and taxonomic affiliate for fungal root tip sequences

Can be found online at
https://www.sciencedirect.com/science/article/pii/S0038071721000237?casa_token=uq0xjtqMTE0AAAAA:pjej9ae8Gq6qzwaw7_ZLRCYSrx_JPLbVPR95acEVVt7A36dJb5g6IR15OF10gmhqiZEisWDSHA

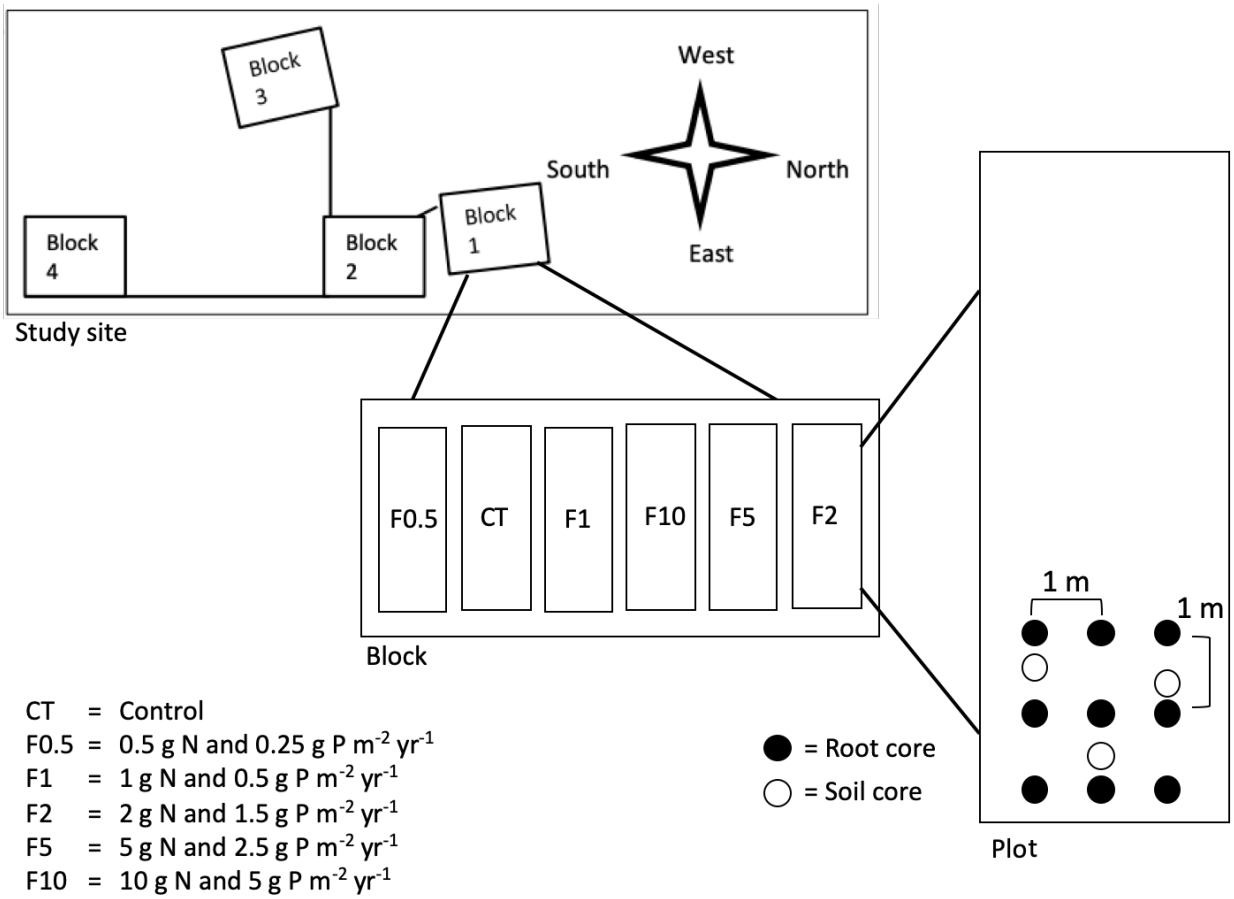

Fig. S1. Experimental and sampling design. Map of the full factorial replicated block fertilization gradient experiment. Each of four blocks contains six plots consisting of one of six treatments (CT, F0.5, F1, F2, F5, F10). Figure is not to scale.

Table S1. Linear mixed effects model results for soil DIN and PO_4-P concentrations as a function of the fixed effects of Fertilizer added^2 + Fertilizer added and the nested random effects of plot within block.

a) Soil DIN and PO_4-P concentrations model performance.

	Model	df	-log(L)	AIC	ΔAIC	weight
DIN	Quadratic	6	-333	678	1.5	0.3
	Linear	**5**	**-333**	**676**	**0**	**0.7**
	Null	4	-347	702	25	0
PO_4-P	**Quadratic**	**6**	**-330**	**672**	**0**	**0.99**
	Linear	5	-335	681	9	0.01
	Null	4	-345	698	26	0

b) Parameter estimates, SE, z-values, and P-values from the best fitting model for soil DIN and PO_4-P concentrations.

	Final model variables	coefficient	SE	z-value	P
DIN	Intercept	2.9	0.2	15.7	<0.001
	Fertilizer added	0.3	0.04	6.8	<0.001
PO_4-P	Intercept	4.0	0.1	31.5	<0.001
	Fertilizer added	5.9	1.1	5.5	<0.001
	Fertilizer added^2	-3.8	1.1	-3.3	<0.001

Table S2. Linear mixed effects model results for shrub cover and height as a function of the fixed effects of inorganic and organic indices and the nested random effects of plot within block (for cover) and quadrat within plot within block (for height).
a) Shrub cover and height model performance.

	Fixed effects structure	df	-log(L)	AIC	ΔAIC	weight
Cover	Inorganic index^2 * Species + Organic index^2 * Species	13	-829	1683	3	0.2
	Inorganic index^2 * Species + Organic index^2	**11**	**-839**	**1681**	**0**	**0.76**
	Inorganic index * Species + Organic index * Species	9	-843	1704	24	0
	Inorganic index^2 * Species	9	-835	1688	6	0.04
	Inorganic index * Species	7	-844	1702	22	0
	Null	4	-892	1792	111	0
Height	Inorganic index * Species + Organic Index * Species	10	-3082	6185	1	0.34
	Inorganic index* Species + Organic Index	9	-3084	6186	2	0.21
	Inorganic index* Species	**8**	**-3084**	**6184**	**0**	**0.45**
	Null	5	-3369	6748	564	0

b) Parameter estimates, SE, z-values, and P-values from the best fitting model for shrub cover and stem height.

	Final model variables	coefficient	SE	z-value	P
Cover	Intercept	16.3	1.6	9.9	<0.001
	Inorganic index	101.6	12.4	8.2	<0.001
	Inorganic index^2	9.7	12.2	0.8	0.4
	Organic index	19.2	10.4	1.8	0.07
	Organic index^2	33.5	9.7	3.5	<0.001
	R. tomentosum intercept	-5.1	1.0	-5.1	<0.001
	R. tomentosum: inorganic index	-147.2	15.2	-9.7	<0.001
	R. tomentosum: inorganic index^2	-61.6	15.3	-4.0	<0.001
Height	Intercept	22.7	0.6	37.3	<0.001
	Inorganic index	36.3	3.6	10.1	<0.001
	R. tomentosum intercept	-10.9	0.4	-25.0	<0.001
	R. tomentosum: inorganic index	-32.8	2.6	-12.4	<0.001

Table S3. Performance of the top 5 and null models for root activity of each enzyme.

Enzyme	Model	df	-log(L)	AIC	ΔAIC	weight
Leucine aminopeptidase	Inorganic index^2 * Species + Organic index^2 * Species	14	-5686	11398	2	0.16
	Inorganic index * Species + Organic index^2 * Species	12	-5687	11396	0	0.46
	Organic index^2 * Species	10	-5692	11402	6	0.02
	Inorganic index * Species	8	-5692	11398	2	0.15
	Inorganic index + Species	**7**	**-5693**	**11397**	**1**	**0.18**
	Null	5	-5736	11481	83	0
Chitinase	Inorganic index^2 * Species + Organic index^2 * Species	14	-7780	15588	5	0.04
	Inorganic index^2 + Organic index^2 * Species	12	-7780	15584	2	0.26
	Organic index^2 * Species	**10**	**-7781**	**15582**	**0**	**0.58**
	Organic index * Species	8	-7786	15588	5	0.03
	Organic index + Species	7	-7788	15589	7	0.02
	Null	5	-8206	16422	834	0
Phosphatase	Inorganic index^2 * Species + Organic index^2 * Species	14	-8508	17043	3	0.12
	Inorganic index^2 * Species + Organic index * Species	**12**	**-8508**	**17040**	**0**	**0.62**
	Inorganic index^2 * Species	10	-8511	17041	2	0.27
	Inorganic index * Species + Organic index * Species	10	-8516	17052	12	0
	Inorganic index * Species	8	-8519	17055	15	0
	Null	5	-8772	17555	515	0
Phenol oxidase	Inorganic index^2 * Species + Organic index^2 * Species	14	-3230	6489	1	0.40
	Inorganic index * Species + Organic index * Species	10	-3239	6498	10	0
	Inorganic index^2 * Species	**10**	**-3234**	**6488**	**0**	**0.57**
	Inorganic index * Species	8	-3239	6494	6	0.02
	Species	6	-3245	6502	15	0
	Null	5	-3333	6675	187	0
Peroxidase	Inorganic index^2 * Species + Organic index^2 * Species	14	-7737	15502	1	0.29
	Inorganic index * Species + Organic index^2 * Species	12	-7741	15506	5	0.04
	Inorganic index * Species + Organic index * Species	10	-7742	15505	4	0.06
	Inorganic index^2 * Species	**10**	**-7740**	**15501**	**0**	**0.46**
	Inorganic index * Species	8	-7744	15503	2	0.14
	Null	5	-8061	16132	631	0

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