Application of fungistatics in soil reduces N uptake by an arctic ericoid shrub (*Vaccinium vitis-idaea*)

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Abstract: In arctic tundra soil N is highly limiting, N mineralization is slow and organic N greatly exceeds inorganic N. We studied the effects of fungistatics (azoxystrobin [Quadris®] or propiconazole [Tilt®]) on the fungi isolated from ericaceous plant roots in vitro. In addition to testing the phytotoxicity of the two fungistatics we also tested their effects on growth and nitrogen uptake of an ericaceous plant (*Vaccinium uliginosum*) in a closed Petri plate system without root-associated fungi. Finally, to evaluate the fungistatic effects in an in vivo experiment we applied fungistatics and nitrogen isotopes to intact tundra soil cores from Toolik Lake, Alaska, and examined the ammonium-N and glycine-N use by *Vaccinium vitis-idaea* with and without fungistatics. The experiments on fungal pure cultures showed that Tilt® was more effective in reducing fungal colony growth in vitro than Quadris®, which was highly variable among the fungal strains. Laboratory experiments aiming to test the fungistatic effects on plant performance in vitro showed that neither Quadris® nor Tilt® affected *V. uliginosum* growth or N uptake. In this experiment *V. uliginosum* assimilated more than an order of magnitude more

ammonium-N than glycine-N. The intact tundra core experiment provided contrasting results. After 10 wk of fungistatic application in the growth chamber *V. vitis-idaea* leaf %N was 10% lower and the amount of leaf ^{15}N acquired was reduced from labeled ammonium (33%) and glycine (40%) during the 4 d isotope treatment. In contrast to the in vitro experiment leaf ^{15}N assimilation from glycine was three times higher than from $^{15}NH_4$ in the treatments that received no-fungistatics. We conclude that the function of the fungal communities is essential to the acquisition of N from organic sources and speculate that N acquisition from inorganic sources is mainly inhibited by competition with complex soil microbial communities.

Key words: amino acid, nitrogen cycle, plant nutrition, tundra

INTRODUCTION

Nitrogen is the most limiting resource in most terrestrial ecosystems (Vitousek et al. 1997, Gough et al. 2000). In arctic tundra, where water saturation and low temperatures limit mineralization, decomposition is slower than new carbon inputs from plants (Nadelhoffer et al. 1992). As a result most N is in organic matter and nutrients remain limiting (Shaver and Chapin 1980) and N available in the soil amino acid pools far exceeds N available in the inorganic N pools (Kielland 1994, 1995).

A variety of plants have been shown to use organic N (Schimel and Chapin 1996; Näsholm et al. 1998, 2000; McKane et al. 2002; Miller and Bowman 2002; Grelet et al. 2009a). However whether organic N use occurs significantly without the fungal symbioses in situ remains controversial (Jones et al. 2005). Ericaceous plants that are an important codominant component in arctic tundra ecosystems preferentially may rely on organic N sources even more so than other plants (Michelsen et al. 1996, Michelsen et al.

1998, Jonasson and Shaver 1999), although even this issue is debated (Kielland 1994). In heath tundra Michelsen et al. (1998) showed that ericoid mycorrhizal (ErM) plants were the most ^{15}N depleted among non-mycorrhizal, arbuscular mycorrhizal and ectomycorrhizal plants; the study hypothesized that this pattern was due to fractionation during fungal uptake and/or translocation of organic N and reduced reliance on inorganic N sources that are enriched by the activity of saprobic microbes. The ericaceous plant dependence on organic N indeed might be essential in bypassing competition for inorganic N with saprobic microbes in the arctic (Kielland 1994, Michelsen et al. 1996, Jonasson and Shaver 1999, Schimel and Bennett 2004).

Read (1996) postulated that plant co-existence might be aided by root-associated fungal communities. In particular ErM are important because they participate in N uptake from organic sources (Bajwa and Read 1985, Xiao and Berch 1999, Whittaker and Cairney 2001). Although these diverse fungal associations (Bougoure et al. 2007) have been proposed to be key to the success of ericaceous plants in low-nutrient environments (Read 1991, 1996; Cairney and Meharg 2003) investigation of nutrient provisioning has been hampered by the production of toxins and release of nutrients during the sterilization of natural substrates (Smith and Read 2008).

To explore the reliance of ericaceous tundra plants on fungi for uptake of organic N we reduced soil and root-associated fungal activity by fungistatic applications and determined plant N uptake with ^{15}N-labeled glycine or ammonium. We employed two broad spectrum fungistatics, azoxystrobin (Quadris®, Syngenta) and propiconazole (Tilt®, Syngenta), that have been marketed as effective against ascomycetes and basidiomycetes. Although fungistatics reduce fungal activities across all nutritional

modes, including soilborne saprobes, our goal was to broadly target fungi including ascomycetes that most frequently form ErM (Read 1996, 1991) as well as basidiomycetes that also might play a prominent role (Berch et al. 2002, Allen et al. 2003). Fungistatic use in mycorrhizal studies (mainly benomyl) had shown strong inhibition of arbuscular mycorrhizal (AM) colonization (Newsham et al. 1994, Merryweather and Fitter 1996, Hartnett and Wilson 1999), but results on ascomycetous inhibition are variable because some fungi (e.g. *Alternaria* spp.) are resistant to benomyl (Schild et al. 1988, Summerbell 1988). In other experiments short-term application of propiconazole inhibited the activity of AM external hyphae, while internal plant colonization was not affected (Kjøller and Rosendahl 2000). We are not aware of any previous studies to examine the effects of fungistatics on ericaceous plant N uptake. Our objectives were to evaluate the effect of fungal inhibition on plant N uptake from a free amino acid and to determine whether this inhibition had different effects on plant N uptake from an organic vs. an inorganic source.

Because of the limited precedence for studies aiming to use fungistatics or fungicides to control ErM communities or ErM colonization we conducted a series of supporting, preliminary in vitro experiments. The goal of these additional experiments was to evaluate the effects of fungistatic application on fungi commonly isolated from ericaceous roots in the arctic tundra. In addition because we were concerned about potential non-target effects of the fungistatics on the host plants we selected a commonly occurring ericaceous plant (*Vaccinium uliginosum*) and empirically tested the fungistatic effects on its growth and N acquisition from both organic and inorganic sources.

MATERIALS AND METHODS

Site characteristics.—Soil cores were collected and ericaceous root-associated fungi isolated near Toolik Lake, Alaska, (68°38′N, 149°38′W, 720 m) at the Arctic Tundra Long Term Ecological Research (LTER)

site in the northern foothills of the Brooks Range. The soils consist of a 10–50 cm organic (peat) layer over a silty mineral soil and permafrost. Tundra vegetation at the site is representative of tundra across the Alaskan North Slope (Shaver and Chapin 1991), northern Canada and eastern Siberia (Bliss and Matveyeva 1992). The target plant species for this study, *Vaccinium uliginosum* and *V. vitis-idaea*, are codominant in erect-shrub tundra communities that form a major component of the circumpolar arctic vegetation (CAVM Team 2003).

Fungal sensitivity to fungistatics in vitro.—To understand the resident fungal sensitivity to azoxystrobin (active ingredient of Quadris®, 22.9%) and propiconazole (active ingredient of Tilt®, 41.8%) a total of seven isolates representing the most commonly isolated fungi from ericaceous roots from our research site at the Toolik Lake LTER were tested. The choice of fungi to test and their identification were based on a large isolate library that has been analyzed with molecular techniques and verified by resynthesis (Walker et al. unpubl). All isolates remained sterile and did not produce conidia or conidiophores. As a result they initially were grouped based on macromorphology, the groupings further refined by an RFLP analysis of the internal transcribed spacer (ITS) region of the ribosomal RNA gene and representatives assigned to putative taxa based on the closest BLAST (Altschul et al. 1997) affinities. Of the seven isolates three were assigned to *Phialocephala fortinii*, two to *Cryptosporiopsis ericae*, one to *Lachnum pygmaeum* and one to *Lachnum* cf. *virgineum*. The ITS sequences for the isolates are deposited respectively at GenBank under accession Nos. EF026057, EF026058, EF026064, EF026067, EF026068, EF026050 and EF026054.

Fungal isolates were grown on PDA, and 6 mm plugs of mycelia from actively growing margins of 3 wk old cultures were transferred onto plates of PDA with 10^0 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} or 0 g/L of either filter-sterilized Quadris® or Tilt® and incubated in the dark at room temperature. The dilution series were replicated three times to allow the use of estimated fungal sensitivities as independent observations in the further analyses. Note that all calculations were adjusted to represent actual concentrations of the active ingredients. In other words the Quadris® volume used for the dilution series was nearly twice that used for Tilt®.

The vegetative expansion of the mycelial colony was observed every other day and the colony diameter recorded after 10 d incubation. Longer incubations risked expanding to the edge of the medium. To provide a single metric to characterize the fungal sensitivities to the fungistatics a concentration that

effectively reduced growth by 50% (EC_{50}) relative to the unamended control (0.0 g/L) was determined for each isolate and each fungistatic by regressing the colony diameter data against the $\log_{10}$ of the fungistatic concentration and by interpolation (in some cases the EC_{50} was extrapolated because fungal sensitivity was far lower than the range used in the experiments) of the 50% intercept (Pasche et al. 2004). The EC_{50} responses to main treatment effects (fungistatic, fungal isolate) and their interaction were assessed by ANOVA in JMP (7.01, SAS Institute, Gary, North Carolina). To satisfy the assumptions of normality and equal variances EC_{50} were $\log_{10}$ transformed before analyses. The pairwise comparisons among isolates were performed with Tukey's HSD at $\alpha = 0.05$.

Plant sensitivity to fungistatics in vitro.—To understand the plant sensitivity to the two fungistatics (Quadris® and Tilt®) a total of 81 *Vaccinium uliginosum* plants were grown in vitro in the laboratory (for description of the system see Xiao and Berch 1999). We were unable to obtain *V. vitis-idaea* seed commercially and substituted the target species for the field experiments with a congeneric and commonly co-occurring taxon. The seeds were surface sterilized in 30% H_2O_2 10 min, germinated on sterile filter paper, and nine replicate plants were randomly assigned to each of the nine treatments in a fully randomized factorial design with two factors (fungistatic, ^{15}N-source) with three levels each (water control, Quadris®, Tilt® for fungistatic; water control, ^{15}N-ammonium, ^{15}N-glycine for ^{15}N-source). Each seedling was transferred on a Petri dish containing 20 mL one-tenth strength Murashige and Skoog (MS) basal salt media (Sigma Aldrich Co., St Louis, Missouri) and a 2.5 cm fiberglass filter disk for a patch onto which the fungistatic and N treatments were applied. The plants were incubated 3 mo under artificial light (12 h/12 h day/night; PAR 250 μmol $m^{-2}s^{-1}$). During incubation stochastic mortality across the treatments reduced the replication to 5–9 per treatment and the total sample size to 59.

Two weeks before harvest the filter disk patches were amended with 200 μL of two fungistatics, Tilt ® and Quadris®, at concentrations providing 1 mg l^{-1} propiconazole or 1000 mg l^{-1} azoxystrobin, or with 200 μL sterile water (control). The treatment concentrations for this experiment were determined based on the EC_{50} or maximum values in the previous experiment focusing on the effects on root-isolated fungi. Four days before harvesting 200 μL of 500 μM of 99% enriched ^{15}N treatments (ammonium chloride [$^{15}NH_4Cl$–F.W. 54.49 g mol^{-1}] or glycine [$^{15}NH_2CH_2COOH$–F.W. 76.06 g mol^{-1}]) were added to the filter disk patches for a total of 1.5 μg ^{15}N. Isotope controls received 200 μL sterile water at the time of isotope

application. The whole plants were harvested, roots were rinsed briefly in deionized water and in dilute CaCl (0.05g/L) and plants dried at 50 C for 48 h for the root, shoot and total dry mass. Due to concern that superficial isotope contamination might occur in the root tissues, we chose to analyze only the shoots. Shoots were ground in a Wig-L-Bug (Reflex Analytical Corp., Ridgewood, New Jersey) and analyzed for $\delta^{15}N$, atom $\%^{15}N$ ($[^{15}N/\{^{14}N+^{15}N\}]*100$) and %N with a ThermoFinnigan Delta Plus mass spectrometer with a CE 1110 elemental analyzer for solid sample combustion and a Conflo II interface by the Stable Isotope Mass Spectrometry Laboratory, Division of Biology, Kansas State University. The ^{15}N abundance was used to calculate the amount and proportion of the N taken up and transferred to plant shoots. The biomass, root to shoot ratio and N responses to treatments (fungistatic, ^{15}N-source) and their interaction were assessed by two-way ANOVA in JMP (7.01, SAS Institute, Gary, North Carolina). To satisfy the assumptions of normality and equal variances, the response variables were $\log_{10}$ transformed before analyses. The pairwise comparisons among the treatments, where necessary, were performed with Tukey's HSD at $\alpha = 0.05$.

N assimilation in intact tundra samples.—Randomly located sections of tundra containing *V. vitis-idaea*, 8 cm × 8 cm × 6.5 cm deep, were cut intact from tundra 29 Aug 2003 and placed in plastic nursery pots. These samples were packed in coolers, transported to the laboratory at Kansas State University and allowed to stabilize 6 wk in the growth chamber. During experiment the plants were maintained in a Conviron (Controlled Environments Inc., Pembina, North Dakota) growth chamber at 12 C with 18 h photoperiod. The average daily temperature (lights on) was 14 C, and the minimum nightly temperature (lights off) was 12 C. The pots were kept in nursery flats (F1020, Hummert International, Earth City, Missouri), with transparent plastic lids (Propagation Dome for F1020 flat, Hummert International, Earth City, Missouri) and were watered evenly and regularly with DI water (~15 mL/d) until just before ^{15}N application. Light intensity was approximately 600 $\mu mol/m^2s$ beneath the lids.

The pots were distributed randomly into six plastic trays with mesh bottoms for drainage, and two trays were randomly assigned to each fungistatic treatment. The trays in the growth chamber and the pots within the trays (fungistatic treatments) were rearranged weekly to eliminate any stochastic effects of the experimental unit's position in the growth chamber. Fungistatics were applied every 2 wk for 8 wk (a total of five applications); N isotopes were applied starting 1 wk after the final fungistatic application. All 108

samples were assigned randomly to factorial combinations of three fungistatic treatments (no-fungistatic, Quadris®, Tilt®) and three N sources (no-N, universally labeled $^{15}N^{13}C$-glycine, $^{15}NH_4$) for 12 replicates for each of the nine treatment combinations. N treatments were applied to three randomly chosen pots within each treatment combination and injected with the N isotopes on four consecutive days. To account for the effects of the timing the four consecutive days were considered temporal blocks in the data analyses. Plants were harvested 4 d after N treatment application. Given the four temporal blocks and 4 d treatment period, the fungistatics therefore were present in the soil for a total of 9.5–10 wk at the time the plants were harvested.

The three fungistatic treatments were Quadris®, Tilt® and no-fungistatic. The initial fungistatic treatments were applied at 13.78 μL/pot Quadris® (active ingredient concentration 22.9% azoxystrobin) and 15.10 μL/pot Tilt® (active ingredient concentration 41.8% propiconazole) diluted to 25 mL/pot with DI water before application. Because no information was available on soil applications for Tilt® we used double the rate of the active ingredient but approximately an equal rate across fungistatics. Fungistatic applications were made with 30 mL plastic syringes, distributing the aliquot evenly over the soil surface. To avoid excessive accumulation of the fungistatics the four subsequent biweekly reapplications were made with half the initial concentrations. The pots were rearranged finally so that each flat contained a random assortment of equal number from each fungistatic treatment to enable the application of ^{15}N sources to pots in separate flats.

The two ^{15}N sources were $^{15}NH_4$, and $^{15}N^{13}C$-glycine (labeled at all positions); no-N (D.I. H_2O) was control. Application amounts were based on 0.05 g $^{15}N/m^2$, or 0.32 mg $^{15}N/64$ cm^2 pot. Due to weighing inaccuracy for the small amounts of isotope used in the stock solution, the actual applications were 0.332 mg^{15}N/pot for NH_4 and 0.320 mg ^{15}N/pot for glycine. The two N sources and the no-N source were injected in 10 mL/pot aliquots with a repeating syringe (Minipet, Bel-Art, Pequannock, New Jersey) with a needle designed to distribute the isotope evenly throughout the upper portion of the root zone. Each needle had four alternating rows of ports with 1cm between ports in a row and ports beginning 6.5 cm above the bottom of the pot, approximately 0.5 cm below the soil surface and ending 1.5 cm from the bottom of the pot. Ten 1 mL injections per pot were administered with templates that were suspended

above the pot with openings evenly distributed on a 7 × 7 cm grid. A funnel placed below the template surrounded the needle to eliminate foliar contamination.

To control for soil water content the pots first were brought to field capacity then not watered 3 d before injections to avoid leakage of the isotope solution through the drain holes. The lids were replaced after the 10 mL injection, and all plants not injected on a given day received approximately 10 mL DI water mist daily until harvested. The pots were weighed before injection, dried (55 C) and reweighed post harvest (without plants) to estimate soil water content at time of injection. The water content of the pots was significantly higher in the Tilt® fungistatic treatment (135 +/− 10 mL) than in the Quadris® fungistatic treatment (106 +/−10 mL; $P < 0.02$) at the time of isotope injection. Increased dilution in the Tilt® treatment might have exaggerated the observation of reduced ^{15}N uptake due to the inhibition of ErM by this fungistatic if dilution in the soil matrix was related to uptake. There was no difference however in water content among N treatments at the time of injection.

All *V. vitis-idaea* leaves were wiped free of debris and harvested (both new and older growth, stems and petioles removed). All *V. vitis-idaea* roots were manually cleaned free of soil, rinsed briefly in DI water, then rinsed briefly in dilute CaCl (0.05 g/L). Each sample was dried at 55 C, weighed, then ground in a Wig-L-Bug (Reflex Analytical Corp., Ridgewood, New Jersey) and analyzed for $\delta^{15}N$, atom $\%^{15}N$ ($[^{15}N/\{^{14}N+^{15}N\}]*100$) and %N with a ThermoFinnigan Delta Plus mass spectrometer with a CE 1110 elemental analyzer for solid sample combustion and a Conflo II interface by the Stable Isotope Mass Spectrometry Laboratory, Division of Biology, Kansas State University. The standard error for $\delta^{15}N$ was 0.2 based on internal references. The proportional ^{15}N uptake (μg ^{15}N/g tissue or ppm) was calculated based on the atom percent enrichment of the sample vs. controls (no-fungistatic, no-isotopes) and the total percent N in the sample. We did not determine total ^{15}N uptake by including biomass in this calculation because the *V. vitis-idaea* biomass in each pot was highly variable and the total belowground biomass of the target plants was impossible to separate. Because ^{13}C also was present in the label the samples were analyzed for $\delta^{13}C$, although we did not expect ^{13}C label to be present in the shoots after the 4 d treatment.

Roots from a random subsample of 10 pots per fungistatic treatment were used to estimate (i) ericoid mycorrhizal diversity and (ii) root colonization parameters. We attempted to estimate the diversity of ericoid mycobionts by surface sterilizing (30% H_2O_2 1–2 min, no rinse) and plating (water agar media)

five 1 cm segments of fine root from each subsampled pot. Isolates originating from these roots were sorted morphologically and by RFLP typing of the ITS region and the 5.8S rRNA gene flanked by the 18S and 25S subunits of the nuclear rRNA gene (see above). Because of the low recovery rate of ErM fungi from the roots with the culture-based method we were unable to compare ErM diversity among the fungistatic treatments.

Root colonization parameters were estimated by means of a standard magnified intersection method (McGonigle et al. 1990) adapted for ErM fungi. The proportion of root length colonized by hyphae and intracellular coils (fungal nutrient exchange structures formed within root cells) or with hyphae on the root surface was estimated separately by observing the presence or absence of the ErM structures at the intersection of the microscope eyepiece crosshairs for 100 locations along a 10 cm transect of the root at 200× magnification.

Statistical analyses of harvested $\delta^{15}N$, proportional ^{15}N uptake, %N and water content at the time of injection were performed with a fully randomized fully factorial two-way ANOVA (factors: fungistatics and ^{15}N sources) in JMP (7.01, SAS Institute, Gary, North Carolina). The temporal block was not significant and was omitted from final data analyses. Because our initial analyses indicated that plant biomass was variable from the beginning of the experiment fungistatic effects on plant biomass were not estimable and therefore omitted. However *V. vitis-idaea* biomass was not considered to be a confounding factor because the biomass was not significantly different among treatments at the time of harvest. The pairwise comparisons among the treatments, where necessary, were performed with Tukey's HSD at $\alpha = 0.05$. Root colonization parameters from a subsample of 10 pots per fungistatic treatment were similarly analyzed by two-way ANOVA followed by Tukey's HSD at $\alpha = 0.05$.

RESULTS

Fungal and plant sensitivity to fungistatics.—The two fungistatics differed in their effects on the vegetative mycelial expansion in the fungal culture sensitivity tests (ANOVA, $F_{1,41} = 70.85$, $P < 0.0001$; FIG. 1). Tilt® had consistently lower EC_{50} values indicating a greater sensitivity among fungi to Tilt® than to Quadris®. Furthermore growth of many strains was not affected by Quadris® within the range of concentrations (< 1 g L^{-1});

extrapolated EC_{50} estimates exceeded 1000 g L^{-1} indicating virtually complete resistance to Quadris®. The fungal strains also differed in their sensitivity to fungistatics (ANOVA, $F_{6,41} = 5.89$, $P = 0.0005$) and sensitivities of the individual isolates differed among the fungistatics as indicated by the significant interaction term (ANOVA, $F_{6,41} = 2.71$, $P = 0.0337$).

Our closed Petri dish studies indicated no fungistatic effects on *V. uliginosum* growth or N acquisition; the observed differences were driven mainly by the form in which ^{15}N was supplied to the plant. *Vaccinium uliginosum* growth (shoot, root, or total biomass and root to shoot ratio) or N content were not affected by the fungistatic, ^{15}N treatments, or their interaction (ANOVA, TABLE I; FIG. 2a–c). In contrast shoot N concentration, ^{15}N recovery and N derived from the applied isotopes varied among ^{15}N treatments but not among fungistatic treatments (ANOVA, TABLE I; FIG. 2d–f). There were no significant interactions among the fungistatic and ^{15}N treatments, suggesting consistent and invariable responses to the isotope treatments across fungistatic treatments. Total N concentration (%N), when the isotope was applied as ^{15}N-ammonium, did not differ from either of the two other isotope treatments (^{15}N-glycine and water control) (FIG. 2d). However when the isotope was applied as ^{15}N-glycine the average %N was slightly higher than in the water control. In contrast the N content (total mg N) in the *V. uliginosum* shoots did not differ across treatments (FIG. 2c), suggesting that the N uptake over plant incubation was not strongly affected by ^{15}N applications and that the observed differences might have been correlated with biomass parameters across the experiment.

The ^{15}N acquisition by *V. uliginosum* varied across N treatments as indicated by ^{15}N recovered from the plant shoots (ANOVA, TABLE I). These differences are attributable to far greater ^{15}N acquisition from ^{15}N-ammonium than from ^{15}N-glycine (FIG. 2e) in the fungus-free Petri dish system. It is notable that the ^{15}N acquired from ammonium is more than an order of magnitude greater (24 times) than that acquired from glycine. Although the ^{15}N acquisition from glycine was limited and did not differ from the water control in a two-way ANOVA, separate *t*-tests indicated that some N acquisition from glycine did occur ($t_{16} = 3.0147$, one-tailed $P = 0.0041$ for the hypothesis that ^{15}N [μg] acquired is greater than zero).

Fungistatic effects on N acquisition in intact tundra cores.—Our goal was to improve our understanding of the soil fungal community function by contrasting a highly artificial Petri dish system without any soilborne organisms with one that would include a complex microbial community that we aimed to manipulate with fungistatic applications. We first tested whether the fungistatic treatment affected ^{15}N natural abundance during the 10 wk application. We observed no differences in foliar ^{15}N natural abundance among fungistatic and no-fungistatic treatments (ANOVA, TABLE II; FIG. 3a), indicating that the fungistatics themselves did not result in any discernable N fractionation in the intact tundra cores. Similarly we were unable to see any evidence for $^{13}C^{15}N$-glycine use as an intact monomer; the $\delta^{13}C$ natural abundance in the $^{13}C^{15}N$-glycine applications did not significantly differ from those in the no-^{15}N controls (ANOVA, TABLE II). Leaf N concentration was slightly (~ 10%) but significantly lower in both Quadris® and Tilt® treatments compared to no-fungistatic treatments but was unaffected by the ^{15}N source (FIG. 3b), suggesting an overall inhibition of N acquisition by the fungistatics. In contrast

to the Petri dish experiment the plants took up three times more glycine than ammonium when compared only among no-fungistatic samples (FIG. 3d). Although fungistatics seemed to reduce plant ^{15}N uptake from both sources compared to the no-fungistatic controls, only Tilt® was significant (ANOVA, TABLE II; FIG. 3e). We observed no significant interaction between fungistatic and N-source treatments (TABLE II), although ^{15}N-ammonium uptake seemed to be lowered by Tilt® but not by Quadris® (FIG. 3e).

Root colonization.—In comparison to a larger fungal diversity dataset from *V. vitis-idaea* plants collected nearby at Toolik Lake (Walker et al. unpubl) the dominant fungus previously cultured from roots was present in all fungistatic treatments (*Phialocephala fortinii*, ITS sequence deposited at GenBank under accession No. DQ004263). Because low recovery rates are typical with culture-based isolation methods (Xiao and Berch 1999, Berch et al. 2002) and relatively few isolates were recovered we were unable to analyze changes in the fungal species composition or abundance among fungistatic treatments.

The fungistatics did not reduce *V. vitis-idaea* root colonization, as reported for AM fungi (Kjøller and Rosendahl 2000). Neither of the two fungistatics reduced extramatrical hyphae (hyphae on the root surface) compared to no-fungistatic treatment (ANOVA, TABLE III; FIG. 4a). In contrast both fungistatic treatments increased intracellular coils (hyphal coils within cortical root cells) and internal hyphae (hyphae within the root but not forming intracellular coils) compared to no-fungistatic treatment (FIG. 4b–c). In addition colonization by intracellular coils was lower in the ^{15}N-ammonium treatment compared to ^{15}N-glycine or no-N treatments (FIG. 4b). The significant but small differences in the fungal root colonization might or might not be

biologically meaningful. Regardless we did not estimate the key factor, hyphal activity, which has been shown to be reduced by propiconazole in AM fungi (Kjøller and Rosendahl 2000).

DISCUSSION

Vaccinium *N uptake*.—Our Petri dish experiment indicated that *V. uliginosum* growth and N uptake are not directly inhibited by the application of fungistatics, even if fungistatic concentrations were high and the application period extended to nearly 20% of the duration of the intact tundra core experiment. However the plate experiment suggested that, in absence of the rhizosphere- and soil-associated microbial communities, *V. uliginosum* had only a limited ability to acquire and assimilate N from an organic monomer, in our case a simple amino acid glycine. In contrast an inorganic N source, ammonium, was assimilated readily in this artificial system during the 4 d trace period. This is in contrast with the findings of Grelet et al. (see FIG. 3, 2009b), whose analyses show that ^{15}N acquired from glutamate did not differ significantly from that acquired from ammonium in non-mycorrhizal *Vaccinium* plants. In our in vitro experiment we estimated that $0.14 \times 10^{-2} \pm 0.09 \times 10^{-2}$ % of N in the shoots was from the ammonium application. In comparison N derived from glycine during the same 4 d chase was only $0.58 \times 10^{-4} \pm 0.42 \times 10^{-4}$%, or more than 20 times less than that derived from ammonium. Although the inorganic N source was far more readily used we were able to confirm that some N, albeit small amounts, was acquired from the organic source: *t*-tests indicated that ^{15}N acquired from glycine was greater than zero. Taking these results and those of Grelet et al. (2009b) together it seems that the autonomous plant use of organic N is highly dependent on the form of N available.

The intact tundra core experiment provides interesting and important contrasts. While glycine was poorly used in the Petri dish experiment, three times more ^{15}N accumulated in the plant leaves from organic than from inorganic N sources during the 4 d chase in the intact tundra core experiment. Taken together these data from the separate and independent experiments suggest that *Vaccinium* plants are able to readily use both organic and inorganic N sources but their ability to assimilate even simple organic monomers might be limited in absence of active microbial communities. Furthermore the intense competition for inorganic N in a complex soil environment with diverse microbial communities likely resulted in the higher use of the organic ^{15}N-glycine in the intact tundra core experiment compared to the inorganic source. We speculate that in the intact tundra experiment a variety of soil microbes (bacteria and fungi) likely immobilized much of the inorganic ^{15}N, limiting its availability to our target plant. Microbial immobilization of inorganic N also was proposed by McFarland et al. (2002) to explain similar results from a boreal forest ecosystem. Because the resident amino acid pools in arctic tundra soils are much greater than inorganic N pools (Kielland 1994, 1995) competition in a soil environment might be less intense for organic N. Fungi associated with ericaceous hosts reportedly are able to depolymerize complex N sources (e.g. Leake and Read 1989, Kerley and Read 1995) and readily use simple organic N sources (Bajwa and Read 1986, Midgley et al. 2004). Although fungi associated with ericaceous plants clearly are capable of NH_4 assimilation (Bajwa and Read 1986, Grelet et al. 2009a) our data suggest that these fungi might be more important for organic N acquisition from arctic tundra soil (see also Grelet et al. 2009b).

Nitrogen with its numerous organic forms can be considered a complex niche dimension in organic soils (Kielland 1994). Accordingly plant diversity in the arctic tundra is likely tied to organic N acquisition (McKane et al. 2002). Our results support the idea that ericaceous plants depend on fungal communities for N acquisition, especially for N acquisition from organic sources. Our low average natural abundance (−6.0 $\delta^{15}N$) from controls (no-fungistatic, no-N) combined with our observations of reduced N use when fungal activities were inhibited further support the notion that the ericaceous plant reliance on organic N explains depleted ^{15}N natural abundances (Michelsen et al. 1998) and that the fungal community is essential in accessing those sources from the soil (Read 1996). The importance of the fungal community function is emphasized further by *V. vitis-idaea* leaf N concentrations, which were reduced cumulatively during the ~ 10 wk fungistatic treatment.

Effects of fungistatics.—Although the ericaceous host growth and N uptake were insensitive to the fungistatic treatments in the Petri dish experiment, the responses to fungistatics differed among the seven commonly isolated fungal strains in our pure culture tests. Taken together from the results of the three experiments we propose that fungistatic applications had only a small effect on host performance but modified the activity of the fungal community in the intact core experiment and therefore led to altered (in our case reduced) N acquisition from ^{15}N treatments.

It is essential to note that our applications of fungistatics in the Petri dish experiment were designed to assess the non-target effects on an ericaceous plant, whereas the intact tundra core experiment aimed at general modification of the fungal activity in the soil and roots. Although our observed reduction of N uptake in the tundra core

experiment is an outcome of inhibition of *V. vitis-idaea*-associated mutualists as well as inhibition of soil-inhabiting saprobes that compete for available soil N, it is tempting to speculate on the importance of the fungal contribution on plant N uptake. If we assume that fungistatics completely inhibited the activity of those fungi that are mutualistic with *V. vitis-idaea* and do not account for the reduced competition from the saprobic fungi in soil or other changes to competitive soil microbes, one could argue that the still substantial organic N uptake in the fungistatic treatments (FIG. 3) is attributed to direct plant uptake. If so autonomous plant uptake exceeded by 40% the fungal provision of the plant-acquired organic N. However in our fungal sensitivity tests the EC_{50} for Tilt® was in the 1 ppt range for most isolates. Accordingly our treatment at ~ 0.05 ppt would be expected to have only a moderate or weak effect and unlikely to have eliminated the symbiont-facilitated N acquisition. Furthermore the effects on the general soilborne community remain uncertain. However it seems likely that saprobic fungi would function as competitors for N when supplied directly as a monomer and therefore fungistatic control of saprobic fungi would be unlikely to cause the reduced uptake of ^{15}N from the glycine monomer that we observed.

We saw no negative effect of the fungistatics on the abundance of ErM structures in the plant roots in the intact tundra core experiment. When considering the mechanisms by which the two fungistatics inhibit fungi this is not completely unexpected. Propiconazole is known as a sterol biosynthesis inhibitor (Loeffler and Hayes 1992) and azoxystrobin inhibits mitochondrial respiration (Steinfeld et al. 2001). Because the two fungistatics primarily affect fungal physiological activity and may be less concentrated in the root systems compared to in the soil solution the fungistatic treatments might not

affect ericaceous root colonization but instead reduce the hyphal extension to the soil matrix or just reduce the physiological activity of hyphae. Similar to our lack of reduced fungal colonization of *V. vitis-idaea* roots, Kjøller and Rosendahl (2000) did not see reduced arbuscular mycorrhizal colonization after applications with propiconazole.

Vaccinium vitis-idaea *^{13}C and ^{15}N*.—Comparisons of molar ratios of ^{13}C and ^{15}N assimilated from universally labeled amino acids over appropriately short treatment periods have evidenced uptake of intact amino acids by arctic (Lipson and Monson 1998) and boreal (Näsholm et al. 1998, McFarland et al. 2002) plants. For the related ericaceous species *Vaccinium myrtillus*, Näsholm et al. (1998) estimated that 91% of the glycine absorbed was taken up intact based on samples taken 6 h after isotope application. Furthermore Sokolovski et al. (2002) demonstrated that amino acid uptake kinetics of *Calluna vulgaris* roots were enhanced by mycorrhization. Extrapolation from these experiments allows conclusions that ericoid mycorrhizal fungi may directly acquire amino acid monomers without enzymatic cleaving of the amino groups and that the acquired amino acids may be rapidly metabolized once assimilated by the plant.

Because we aimed to investigate N assimilation and not uptake stoichiometry we chose a longer, 4 d trace. This choice might have compromised our ability to interpret ^{13}C data but aided detecting the effects of the fungistatics. During the trace ^{13}C supplied in as $^{13}C^{15}N$-glycine possibly was respired by the plant, associated fungi, or both. Notwithstanding, our inability to detect elevated ^{13}C in the *V. vitis-idaea* foliage does not negate the ^{15}N acquisition by ericoid fungi. Whether ^{15}N was acquired as an intact glycine monomer, some ^{15}N, albeit to a significantly lower degree than from ammonium, was acquired from ^{15}N-glycine in our Petri dish experiment. This suggests that slow

acquisition of amino acid N is possible even in the absence of soil and rhizosphere-associated microbial communities. However the importance of these communities is illustrated by the fungistatic reduction of the ^{15}N assimilated into the *V. vitis-idaea* foliage in the intact tundra core experiment. Although elevated ^{13}C was not detected in our plants it is unlikely that the organic N was assimilated by the plants after first being mineralized by the non-mycorrhizal soil microbial pool because the uptake of inorganic N was relatively low in the intact tundra core experiment.

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LEGENDS

FIG. 1. Fungal sensitivities (EC_{50}) of ErM fungal isolates of dominant taxa from the study area to the fungistatics (Quadris®, Tilt®) used for the isotope experiment in vitro on PDA. Groups of bars with different letters are significantly different at $\alpha = 0.05$. Lowercase letters = comparisons among Quadris® treatments; uppercase letters = comparisons among Tilt® treatments.

FIG. 2. *Vaccinium uliginosum* responses to fungistatic (water control, Quadris® and Tilt®) and nitrogen (water control, ^{15}N-ammonium and ^{15}N-glycine) treatments in a Petri dish experiment. a. Biomass, shoot mass above and root mass below the x-axis. b. Root to shoot ratio. c. Shoot N content. d. Shoot N concentration. e. ^{15}N-recovery. f. N derived from ^{15}N treatments. Corresponding ANOVA tables are provided (TABLE I). Note that there were no significant interaction terms. Significant pairwise differences (Tukey's HSD at $\alpha = 0.05$) among the main effects are indicated by letters after the labels or legends.

FIG. 3. *Vaccinium vitis-idaea* responses to fungistatic (water control, Quadris® and Tilt®) and nitrogen (water control, ^{15}N-ammonium and $^{13}C^{15}$N-glycine) treatments in an intact tundra core experiment. a. ^{15}N natural abundance among the fungicidal treatments that received no-^{15}N treatment. b. leaf N concentration. c. δ^{15}N among the treatments that received ^{15}N. d. Foliar ^{15}N-recovery in excess of natural abundance (μg g^{-1} leaf dry weight) among the treatments that received no-fungistatic. e. Foliar ^{15}N-recovery in excess of natural abundance (μg g^{-1} leaf dry weight). Corresponding ANOVA tables are provided (TABLE II). Note that there were no significant interaction terms. Significant pairwise differences (Tukey's HSD at $\alpha = 0.05$) among the main effects are indicated by letters after the x-axis labels or legends.

FIG. 4. *Vaccinium vitis-idaea* root colonization responses to fungistatic (water control, Quadris® and Tilt®) and nitrogen (water control, ^{15}N-ammonium and $^{13}C^{15}$N-glycine) treatments in an intact tundra core experiment. a. Extramatrical hyphae. b. Intracellular coils. c. Intraradical hyphae. Corresponding ANOVA tables are provided (TABLE III). Note that there were no significant interaction terms. Significant

pairwise differences (Tukey's HSD at $\alpha = 0.05$) among the main effects are indicated by letters after the x-axis labels or legends.

FOOTNOTES

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TABLE I. ANOVA tables for the *Vaccinium uliginosum* responses to the stable isotope tracer (^{15}N ammonium, ^{15}N glycine, or water control) and the fungistatic (Quadris®, Tilt®, or water control) treatments in a closed Petri dish system. All response variables were Log_{10} transformed before ANOVA to satisfy assumptions of normality and equal variances. The responses directly estimating ^{15}N incorporation were analyzed after the control in which the ^{15}N tracer was replaced with sterile water was omitted.

Response	Fungistatic	Nitrogen	F * N interaction
Shoot mass (mg)	$F_{2,58} = 0.20^{ns}$	$F_{2,58} = 0.97^{ns}$	$F_{4,58} = 0.80^{ns}$
Root mass (mg)	$F_{2,58} = 0.50^{ns}$	$F_{2,58} = 0.78^{ns}$	$F_{4,58} = 1.02^{ns}$
Total mass (mg)	$F_{2,58} = 0.23^{ns}$	$F_{2,58} = 0.82^{ns}$	$F_{4,58} = 0.54^{ns}$
Root:Shoot F	$_{2,58} = 0.03^{ns}$	$F_{2,58} = 1.26^{ns}$	$F_{4,58} = 0.98^{ns}$
Shoot N (%)	$F_{2,58} = 0.09^{ns}$	$\mathbf{F_{2,58} = 4.52^{*}}$	$F_{4,58} = 2.17^{ns}$
Shoot N (mg)	$F_{2,58} = 0.23^{ns}$	$F_{2,58} = 1.61^{ns}$	$F_{4,58} = 0.95^{ns}$
NdfT (%)[a]	$F_{2,35} = 1.83^{ns}$	$\mathbf{F_{1,35} = 4.72^{*}}$	$F_{2,35} = 1.68^{ns}$
^{15}N Recovery (μg)	$F_{2,35} = 2.06^{ns}$	$\mathbf{F_{1,35} = 15.09^{***}}$	$F_{2,35} = 1.94^{ns}$

[a] NdfT = proportion of N derived from tracer.

TABLE II. ANOVA tables for the *Vaccinium vitis-idaea* responses to the stable isotope tracer (^{15}N ammonium, ^{15}N glycine, or water control) and the fungistatic (Quadris®, Tilt®, or water control) treatments in an intact tundra core experiment. All response variables were Log_{10} transformed before ANOVA to satisfy assumptions of normality and equal variances. Note that some of the treatment combinations were omitted for the analyses estimating the ^{15}N incorporation (see footnotes for clarification).

Response	Fungistatic	Nitrogen	F * N interaction
Leaf			
Natural $\delta^{15}N$ (‰)[a]	$F_{2,34} = 0.09^{ns}$ NA		NA
Natural $\delta^{13}C$ (‰)[b]	$F_{2,66} = 2.73^{ns}$	$F_{1,66} = 0.09^{ns}$	$F_{2,66} = 0.64^{ns}$
N (%)	$\mathbf{F_{2,99} = 4.83^{**}}$	$F_{2,99} = 2.34^{ns}$	$F_{4,99} = 0.59^{ns}$
^{15}N Recovery (µg)[c] NA		$\mathbf{F_{2,33} = 28.35^{***}}$	NA
^{15}N Recovery (µg)	$\mathbf{F_{2,99} = 3.18^{*}}$	$\mathbf{F_{2,99} = 71.02^{***}}$	$F_{4,99} = 1.66^{ns}$

[a] Analyses included only the controls that received no ^{15}N tracer.
[b] Analyses omitted ^{15}N-ammonium treatments.
[c] Analyses included only the controls that received no fungistatic.

TABLE III. ANOVA tables for the *Vaccinium vitis-idaea* root colonization responses to the stable isotope tracer (^{15}N ammonium, ^{15}N glycine, or water control) and the fungistatic (Quadris®, Tilt®, or water control) treatments in an intact tundra core experiment.

Response	Fungistatic	Nitrogen	F * N interaction
Root			
Extramatrical hyphae	$\mathbf{F_{2,21} = 3.45^{*}}$	$F_{2,21} = 1.55^{ns}$	$F_{4,21} = 0.09^{ns}$
Intraradical hyphae	$\mathbf{F_{2,21} = 4.45^{*}}$	$F_{2,21} = 1.28^{ns}$	$F_{4,21} = 0.23^{ns}$
Intracellular coils	$\mathbf{F_{2,21} = 11.45^{***}}$	$\mathbf{F_{2,21} = 7.81^{**}}$	$F_{4,21} = 0.98^{ns}$

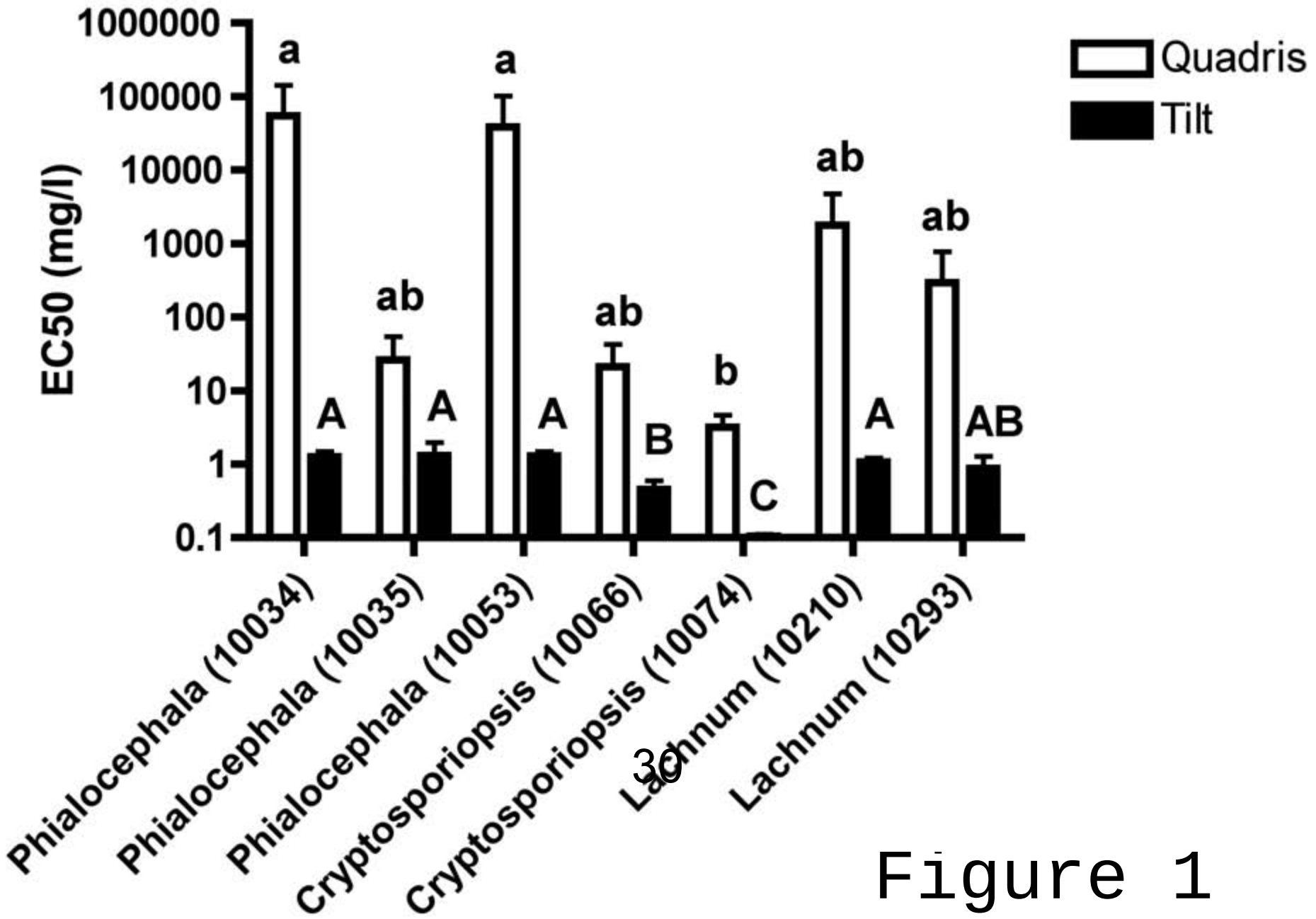

1000000
100000
10000
1000
100
10
1
0.1
EC50 (mg/l)
Quadris
Tilt
a
A
ab
A
a
A
ab
B
b
C
ab
A
ab
AB
Phialocephala (10034)
Phialocephala (10035)
Phialocephala (10053)
Cryptosporiopsis (10066)
Cryptosporiopsis (10074)
Lachnum (10210)
Lachnum (10293)

Figure 1

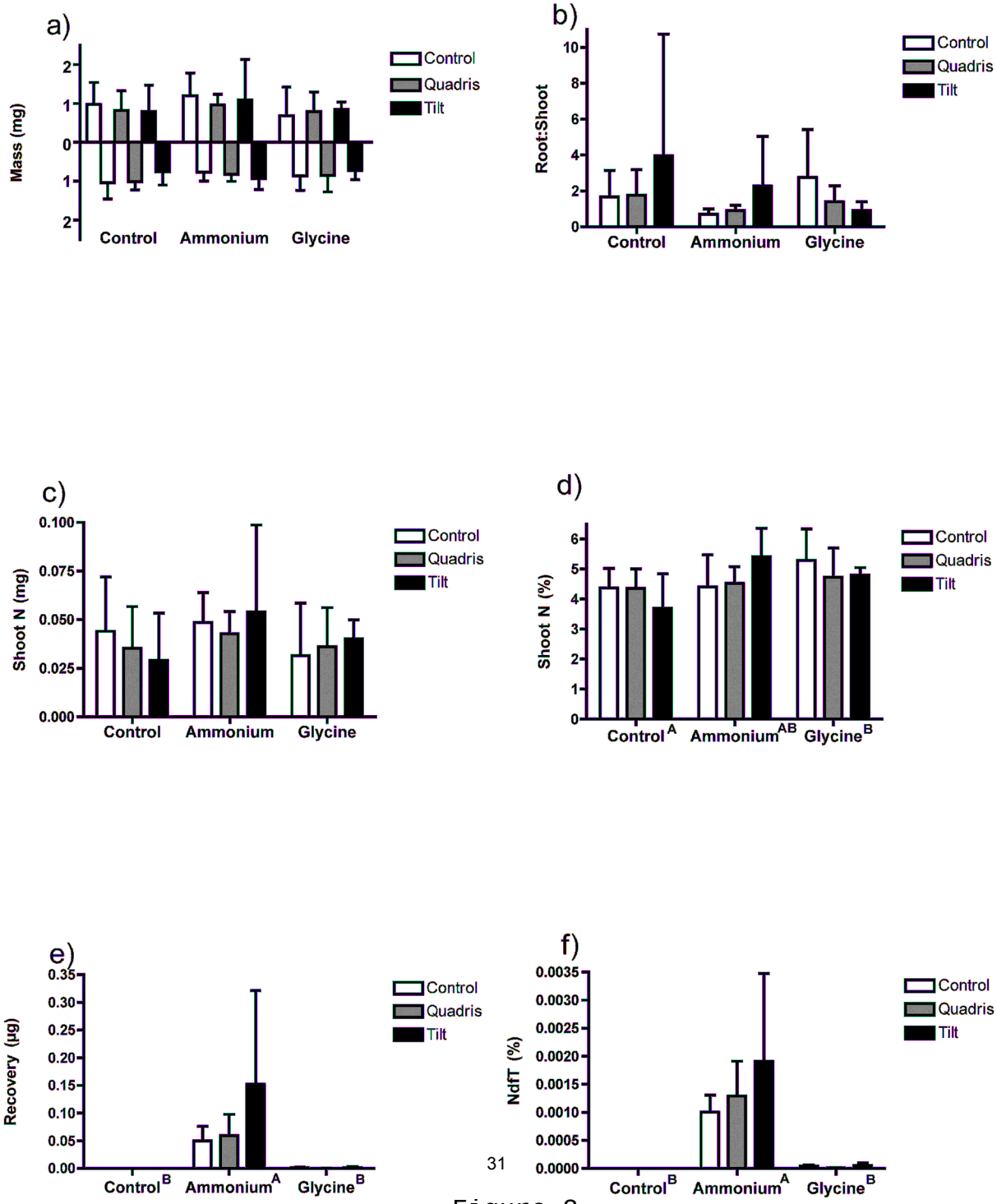
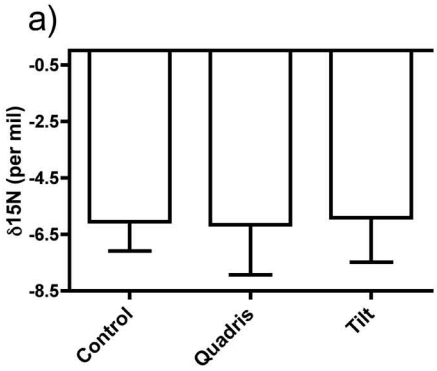
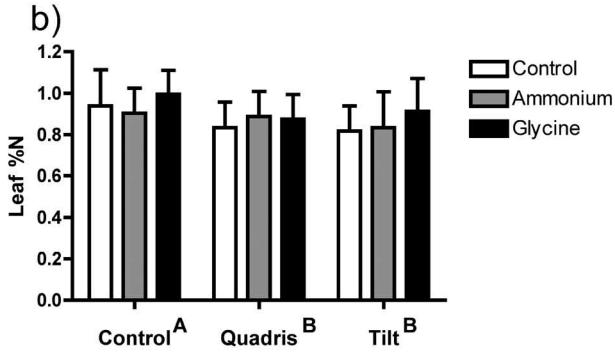
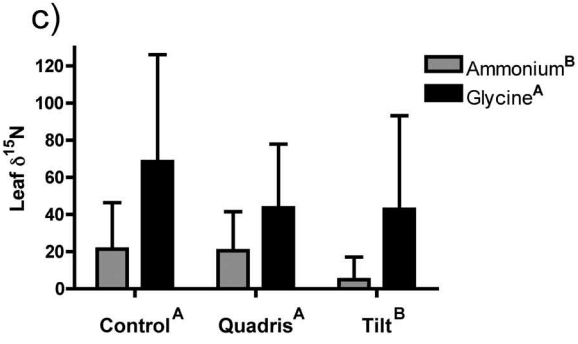
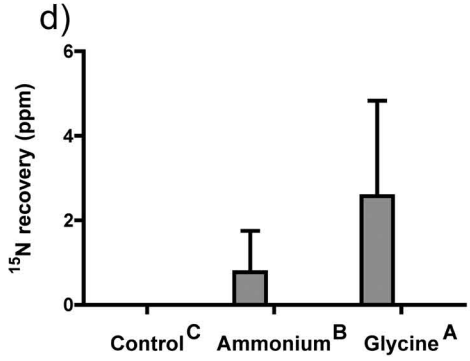

a)
Mass (mg)
2
1
0
1
2
Control
Ammonium
Glycine
Control
Quadris
Tilt
b)
Root:Shoot
10
8
6
4
2
0
Control
Ammonium
Glycine
Control
Quadris
Tilt
c)
Shoot N (mg)
0.100
0.075
0.050
0.025
0.000
Control
Ammonium
Glycine
Control
Quadris
Tilt
d)
Shoot N (%)
6
5
4
3
2
1
0
Control A
Ammonium AB
Glycine B
Control
Quadris
Tilt
e)
Recovery (µg)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Control B
Ammonium A
Glycine B
Control
Quadris
Tilt
f)
NdfT (%)
0.0035
0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Control B
Ammonium A
Glycine B
Control
Quadris
Tilt

Figure 2


a)
δ15N (per mil)
-0.5
-2.5
-4.5
-6.5
-8.5
Control
Quadris
Tilt


b)
Leaf %N
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Ammonium
Glycine
Control A
Quadris B
Tilt B


c)
Leaf δ15N
120
100
80
60
40
20
0
Ammonium B
Glycine A
Control A
Quadris A
Tilt B


d)
15N recovery (ppm)
6
4
2
0
Control C
Ammonium B
Glycine A

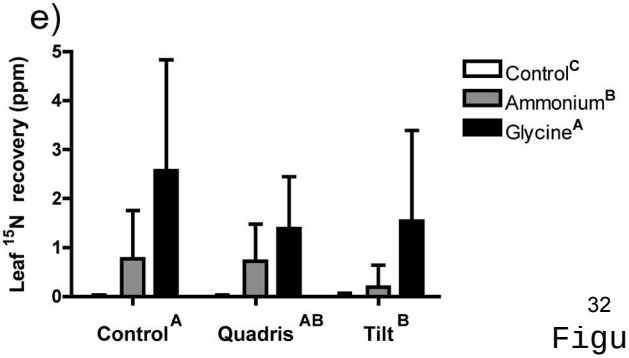

e)
Leaf 15N recovery (ppm)
5
4
3
2
1
0
Control C
Ammonium B
Glycine A
Control A
Quadris AB
Tilt B

Figure 3

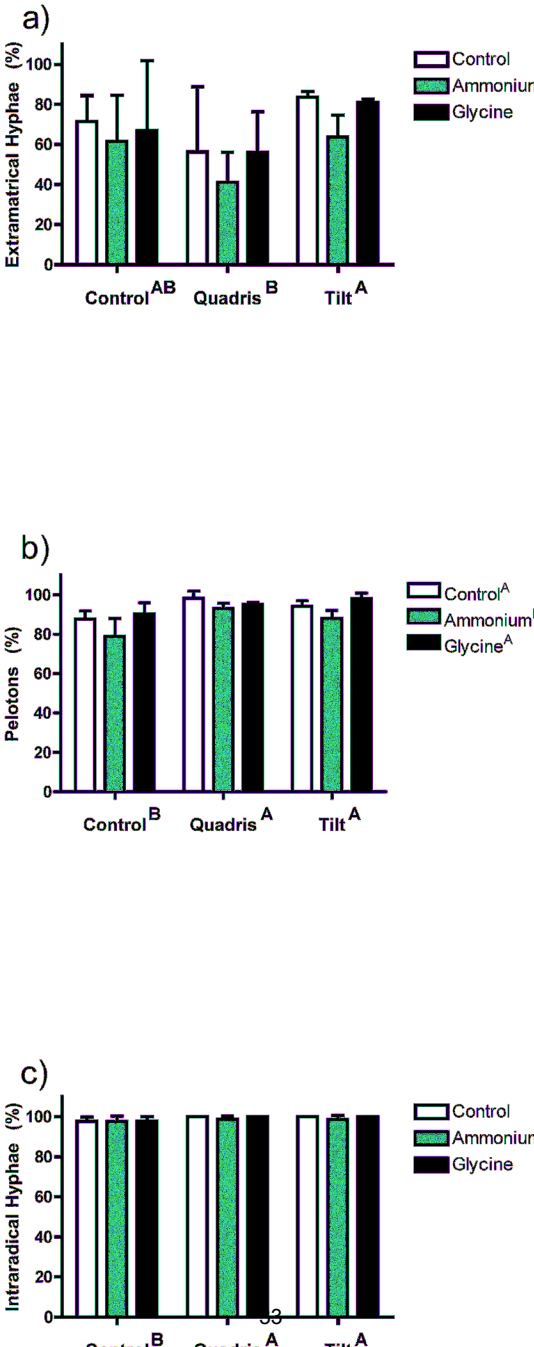

a)
Extramatrical Hyphae (%)
0
20
40
60
80
100
Control
Ammonium
Glycine
Control AB
Quadris B
Tilt A
b)
Pelotons (%)
0
20
40
60
80
100
Control A
Ammonium B
Glycine A
Control B
Quadris A
Tilt A
c)
Intraradical Hyphae (%)
0
20
40
60
80
100
Control
Ammonium
Glycine
Control B
Quadris A
Tilt A

Figure 4