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Effects of Various Nitrogen Loads on the Nitrate Reductase Activity in Roots and Mycorrhizas of Norway Spruce Seedlings

By

I. BRUNNER[1)], S. BRODBECK[1)] & M. GENENGER[1)]

K e y w o r d s : Nitrogen loads, ammonium, nitrate, nitrate reductase activity, *Picea abies*, mycorrhizas, *Hebeloma crustuliniforme*, *Laccaria bicolor*.

S u m m a r y

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Nitrate reductase activity (NRA, *in vivo*-assay) was measured from mycorrhizal and non-mycorrhizal roots and from excised mycorrhizas of Norway spruce (*Picea abies*) seedlings. Seedlings were grown in a greenhouse and were either non-mycorrhizal controls or inoculated with the mycorrhizal fungi *Hebeloma crustuliniforme* or *Laccaria bicolor*. Nitrogen was applied as nitrate or ammonium in loads of 50, 100 or 800 kg N/hay. Comparing the loads of 100 kg N/hay nitrate versus ammonium, NRA in the roots was significantly enhanced with nitrate. Increasing nitrate loads led to a 3-4 fold enhance of the NRA in non-mycorrhizal and in mycorrhizal roots. The inoculation with the mycorrhizal fungi, however, reduced the NRA significantly at all nitrate treatments, and the reductions were higher in roots associated with *H. crustuliniforme* than in roots associated with *L. bicolor*. In excised mycorrhizas, loads of 100 kg N/hay nitrate versus ammonium enhanced NRA significantly. Increasing nitrate loads enhanced the NRA in excised mycorrhizas 2-6-fold. *L. bicolor*- compared to *H. crustuliniforme*-mycorrhizas had higher NRA at all nitrate loads except at 800 kg N/hay. While *L. bicolor*-mycorrhizas had the highest NRA at a nitrate load of 50 kg N/hay, *H. crustuliniforme*-mycorrhizas showed it at 800 kg N/hay. According to our results, the inoculation with mycorrhizal fungi led to a reduction of the NRA in roots, indicating an efficient uptake of nitrate by the extraradical mycelia, supporting the hypothesis that nutrient acquisition is improved by mycorrhizal fungi.

[1)] Swiss Fed. Inst. Forest, Snow & Landscape Res. (WSL), CH-8903 Birmensdorf, Switzerland.

Introduction

Nitrate reductase in plants is part of the assimilatory nitrate reduction and can be induced within a few hours by the addition of nitrate (see OAKS 1991). In a certain range, the activity of the nitrate reductase reflects the rate of nitrate supply, and, therefore, can be used to monitor temporal changes (HÖGBERG & al. 1998). General nitrogen availability for forest trees has increased due to anthropogenic nitrogen depositions (GEBAUER & SCHULZE 1997), and nitrate leaching from forest soils is one indication of nitrate saturation of forest ecosystems (GEBAUER & SCHULZE 1997). Since most of the tree roots live in symbiosis with mycorrhizal fungi (READ 1999), extramatricular mycelia and mycorrhizas are essential organs of the uptake, metabolism, storage, transport, and transfer of nitrogen. However, the impact of mycorrhizal colonization upon nitrogen source utilization and metabolism is only poorly investigated (see also WALLENDA & KOTTKE 1998, BOTTON & CHALOT 1999). The objectives of the present study were I) to measure NRA in relation to various loads and sources of nitrogen, II) to compare NRA of mycorrhizal roots with that of mycorrhizas, and III) to investigate the effects of the root colonization by various mycorrhizal fungi on the NRA.

Materials and Methods

Experimental setup

Fungal mycelia of *Hebeloma crustuliniforme* (Bull.:St. Amans) Quél. (WSL #6.2) and of *Laccaria bicolor* (R. Mre.) Orton (S-238, WSL #73.1) grown in autoclaved 3 L polycarbonate flasks (Le Lion flask) containing 2 L vermiculite and 450 ml MMN nutrient solution were used as inoculum and mixed (1:1) with a autoclaved vermiculite:peat moss mixture (9:1) in Rootrainers (PETERSON & CHAKRAVARTY 1991). Control treatments contained no fungal inoculum. Seeds of Norway spruce (*Picea abies* (L.) Karst.) were surface sterilized for 40 min in 30 % H_2O_2, and 4 seeds per compartment of the Rootrainers were dispersed and covered with 1 cm of substrate. Rootrainers were put into a greenhouse with temperatures set between 10 and 25°C and air humidity of at least 70 %. As soon as seeds germinated, the seedlings were thinned out to one plant per compartment. Five mL per seedling of a 1:5 diluted modified Melin-Norkrans (MMN) nutrient solution (MARX & BRYAN 1975; without glucose and malt) were applied 4 times. Demineralized water was added twice a week or as needed to prevent drying out. Nitrogen loads were applied either as ammonium (NH_4Cl) or as nitrate (KNO_3) with 10 mL per seedling every second week (in total 9 times), leading to final loads of 50, 100, or 800 kg N/hay. In the treatment of 0 kg N/hay the same amount of deionized water was applied. After 5 months, plants were harvested and 20 seedlings per treatment were cleaned in demineralized water, divided into shoots and roots, and samples of mycorrhizas were excised from mycorrhizal roots with the aid of forceps. All materials were frozen in liquid nitrogen and stored at -80 °C.

Determination of nitrate reductase activity (NRA)

NRA was measured from mycorrhizal and non-mycorrhizal roots and from excised mycorrhizas applying a modified in vivo-assay after YANDOW & KLEIN 1986. Using liquid nitrogen, samples were broken into small pieces in 2 mL tubes with a Retsch Mill (MM2000). Approximately 100 mg fresh weight of the root material (or 50 mg of mycorrhizas) were incubated in 1.5 mL K-phosphate-buffer (0.1 mM, pH 7.5), containing 12 mM KNO_3, 1 % isopropanol, and 30 µg/mL ampicillin. Incubation was carried out at 25°C under vacuum for 3 h in the dark. After the incubation, the sample-buffer was cleared by centrifugation (20 min, 20,000 g, 4°C) and activated charcoal

(about 10 mg/sample) was added. Charcoal was then removed by centrifugation (20 min, 20,000 g). 0.5 mL of the supernatant were used for nitrite detection by adding 0.25 mL sulphanilamide (0.1 % in 2N HCl) and 0.25 mL N-naphtyl ethylendiamine (0.02 %). In blanks, N-naphtyl ethylendiamine was replaced by Millipore water. Colour reaction was measured after 30 min at 546 nm. The remainings of the samples were dried at 105°C for 24 h and weighted. For statistical analyses, activities were conducted to a two-factorial analysis of variance (ANOVA). Least significant differences were calculated at $P \leq 0.05$ using Fisher's PLSD test. All tests were undertaken using StatView 4.5 on a PowerMacintosh.

Results and Discussion

Comparing the application of 100 kg N/hay nitrate with 100 kg N/hay ammonium, NRA in the roots was significantly enhanced with nitrate by a factor of 1.4-4.2 (Fig. 1). The inoculation with the mycorrhizal fungi, however, resulted in a significant reduction of the NRA compared to the non-mycorrhizal control roots (Fig. 1). With increasing nitrate loads a 3-4-fold enhancement of the NRA in non-mycorrhizal and in mycorrhizal roots was measured (Fig. 2). PEUKE & TISCHNER 1991 who used a hydroponic experimental setup with non-mycorrhizal *Picea abies* roots made the similar observation that ammonium inhibited and nitrate induced NRA, and that elevated nitrate loads increased NRA. The inoculation with the mycorrhizal fungi, however, reduced the NRA significantly at all nitrate loads, and the reductions were higher in roots associated with *Hebeloma crustuliniforme* than in roots associated with *Laccaria bicolor* (Fig. 2). Working with *Pinus,* VEZINA & al. 1989 and SARJALA 1991 obtained similar results. They reported a 50-85 % reduction of NRA in roots associated with mycorrhizal fungi compared to non-mycorrhizal control roots.

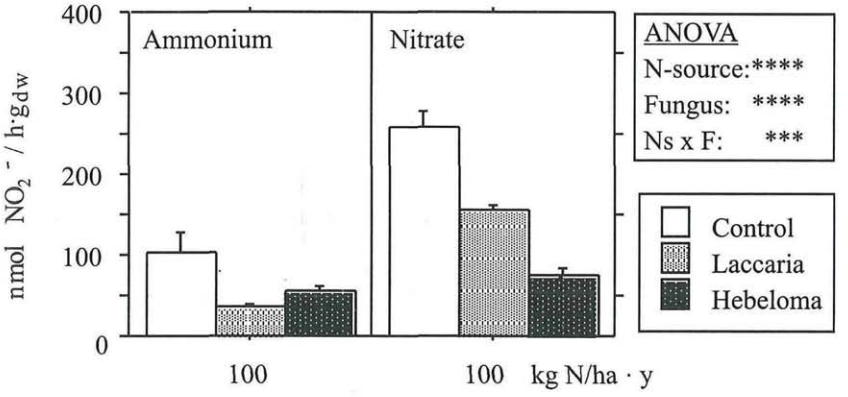

Fig. 1. NRA of non-mycorrhizal control roots and of mycorrhizal roots associated with *Hebeloma crustuliniforme* or *Laccaria bicolor* grown at loads of 100 kg N/hay of ammonium or nitrate. Bars represent ±1 SE of the mean (n=6-8). Probability level for ANOVA: ns=not significant, *= significant at $P \leq 0.05$, **= significant at $P \leq 0.01$, ***= significant at $P \leq 0.001$, ****= significant at $P \leq 0.0001$.

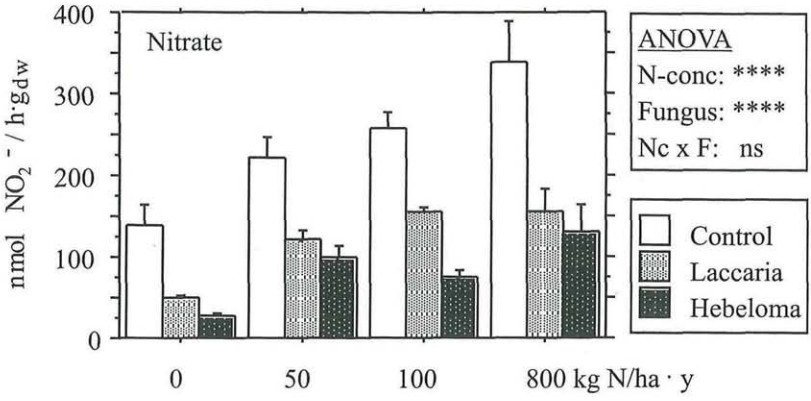

Fig. 2. NRA of non-mycorrhizal control roots and of mycorrhizal roots associated with *Hebeloma crustuliniforme* or *Laccaria bicolor* grown at loads of 0, 50, 100, or 800 kg N/hay of nitrate. Bars represent ±1 SE of the mean (n=3-8). Probability level for ANOVA: ns=not significant, *= significant at P≤0.05, **= significant at P≤0.01, ***= significant at P≤0.001, ****= significant at P≤0.0001.

In excised mycorrhizas, loads of 100 kg N/hay nitrate versus ammonium enhanced NRA significantly (Fig. 3). In *L. bicolor*-mycorrhizas, the treatment with nitrate resulted in a 4 fold higher NRA compared to ammonium treated mycorrhizas. In *H. crustuliniforme*-mycorrhizas, this difference was only 1.2-fold (Fig. 3). Increasing nitrate loads enhanced the NRA in excised mycorrhizas 2-6-fold (Fig. 4). However, *L. bicolor*- compared to *H. crustuliniforme*-mycorrhizas had higher NRA at all nitrate loads except at 800 kg N/hay. While *L. bicolor*-mycorrhizas had the highest NRA at a nitrate load of 50 kg N/hay, *H. crustuliniforme*-mycorrhizas showed it at 800 kg N/hay (Fig. 4). According to FINLAY & al. 1992 both fungal species, *H. crustuliniforme* and *L. bicolor*, grew on media with nitrate as sole nitrogen source, indicating that both fungi are capable to reduce nitrate. However, *H. crustuliniforme* grew as well on nitrate as on ammonium whereas *L. bicolor* showed only intermediate growth on nitrate.

The reduced NRA in mycorrhizal compared to non-mycorrhizal roots indicates an efficient uptake of nitrate by the extramatrical mycelium, supporting the hypothesis that nutrient acquisition is improved by mycorrhizal fungi (BOTTON & CHALOT 1999, READ 1999). Comparing NRA of mycorrhizal roots versus mycorrhizas, a similar pattern of the fungal species has been exhibited. The present data suggest that nitrate at loads of 50 and 100 kg N/hay is possibly utilized less efficiently by the extraradical mycelium in *L. bicolor*-inoculated plants compared to *H. crustuliniforme*-inoculated plants. This result is confirmed by the observations of FINLAY & al. 1992 that *H. crustuliniforme* can utilize nitrate better in comparison to *L. bicolor*. The reduced NRA in mycorrhizal compared to non-mycorrhizal roots in the presence of ammonium, on the other hand, also indicates that fungal inoculation possibly suppresses the expression of the genes encoding for NR (compare also VEZINA & al. 1989). In the *Medicago truncatula/Glomus versiforme* mycorrhizal symbiosis it has been demonstrated, that plant genes in-

volved in phosphate uptake are down-regulated following fungal colonization (LIU & al. 1998).

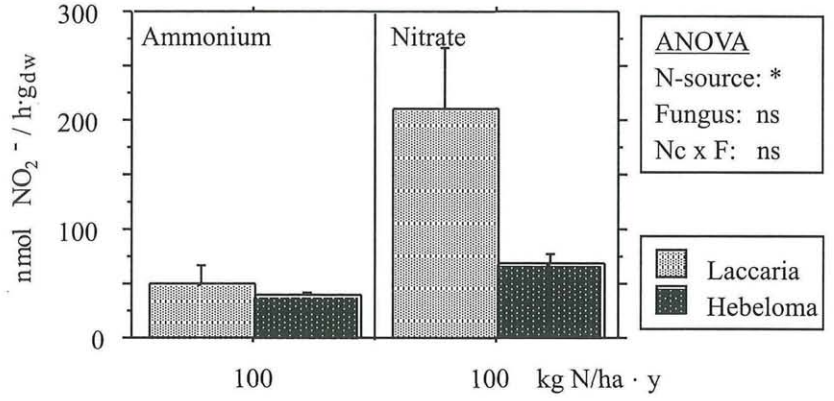

Fig. 3. NRA of mycorrhizas of *Hebeloma crustuliniforme* or *Laccaria bicolor* grown at loads of 100 kg N/hay of ammonium or nitrate. Bars represent ±1 SE of the mean (n=2). Probability level for ANOVA: ns=not significant, *= significant at P≤0.05, **= significant at P≤0.01, ***= significant at P≤0.001, ****= significant at P≤0.0001.

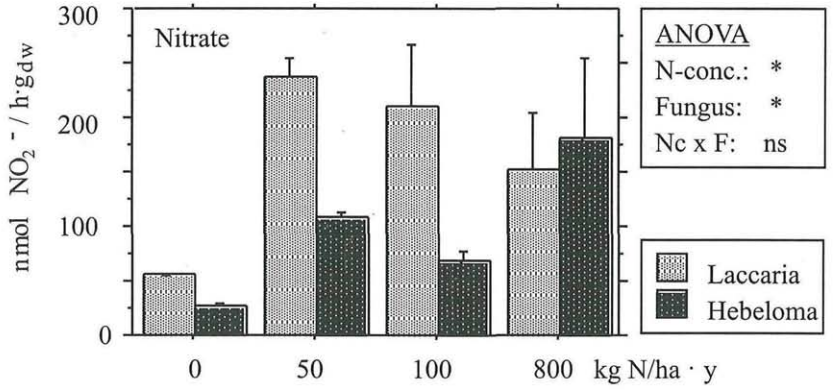

Fig. 4. NRA of mycorrhizas of *Hebeloma crustuliniforme* or *Laccaria bicolor* grown at loads of 0, 50, 100, or 800 kg N/hay of nitrate. Bars represent ±1 SE of the mean (n=2). Probability level for ANOVA: ns=not significant, *= significant at P≤0.05, **= significant at P≤0.01, ***= significant at P≤0.001, ****= significant at P≤0.0001.

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