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ORIGINAL PAPER

Zinc-tolerant *Suillus bovinus* improves growth of Zn-exposed *Pinus sylvestris* seedlings

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Abstract Scots pine (*Pinus sylvestris* L.) seedlings inoculated or not (NM) by a Zn-sensitive or a Zn-tolerant isolate of the ectomycorrhizal fungus *Suillus bovinus* (L. Fr.) Roussel were exposed to 0.1 or 150 μM Zn^{2+} for 9 months. We hypothesized that inoculation with a Zn-tolerant *S. bovinus* isolate should result in added Zn resistance of the host plant. Plant and fungal growth as well as nutrient profiles and photosynthetic pigments in pine needles were quantified. In NM plants and in plants colonized by the Zn-sensitive isolate, plant growth, N, P, Mg and Fe assimilation were strongly inhibited under Zn stress and concurred with significantly reduced chlorophyll concentrations. In contrast, plants colonized by the Zn-tolerant isolate grew much better and remained physiologically healthier when exposed to elevated Zn. These results provide further evidence for the important role metal-adapted mycorrhizal fungi play as an effective biological barrier against metal toxicity in trees.

Keywords Ectomycorrhiza · Heavy metal adaptation · Scots pine · *Suillus bovinus* · Zn pollution

Introduction

Phytoremediation—using plants and trees to remove or neutralize contaminants—holds great promise for unobtrusive and cost-effective treatment of soils contaminated with heavy metals (van der Lelie et al. 2001). Establishment of vegetation markedly decreases metal leaching to groundwater and prevents dispersal of polluted dusts through wind and rain erosion from severely contaminated sites. However, for the revegetation of degraded soils and the reclamation of industrial sites, stress tolerant plants are required. The availability and uptake of heavy metals into plants depends on complex rhizospheric reactions involving not only exchange processes between soil and plants but also interactions mediated by microorganisms. In this respect, mycorrhizal fungi appear to play a central modulating role (Schützendübel and Polle 2002). The diversity of potential mycobionts is, however, large and it is obvious that not all fungal species are equally efficient in sustaining a host plant's fitness under heavy metal stress. Also within single species, fungal ecotypes may have evolved which exhibit genetic adaptations that favor their own survival and that of their host.

Only a limited number of studies have considered such intraspecific differences in metal tolerance between isolates of ectomycorrhizal fungi from metalliferous and non-metalliferous sites (Meharg and Cairney 2000). Recently, adaptive Zn and Cu tolerance was described in basidiomycete species from the Suilloid clade (Colpaert et al. 2004; Adriaensen et al. 2005), a monophyletic group which mostly forms ectomycorrhiza almost exclusively with Pinaceae (Bruns et al. 2002). The interpopulation differences in the response of the *Suilloid* fungi to Zn^{2+} or Cu^{2+} were clearly related to the specific metal pollution of their habitat of origin. The correlation between Zn resistance and Zn pollution in the three *Suilloid* species studied makes a strong case for a causal relationship, and there exists evidence that Zn exclusion mechanisms are involved in this naturally selected adaptive Zn resistance (Colpaert et al. 2005).

In a former experiment with Zn-exposed ectomycorrhizal Scots pine seedlings, we reported that a Zn-tolerant

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genotype of *S. bovinus*, collected from a Zn-polluted site, could easily sustain the pines' acquisition of nutrients, in contrast to a non-adapted isolate from an uncontaminated area (Adriaensen et al. 2004). Despite the differential response in nutrient uptake, a differential effect on plant growth could not be demonstrated. This was probably due to the relatively short period of the Zn treatment (6 weeks) and the relatively slow physiological adaptation of the pines growth rates to changing environmental conditions, a well-known phenomenon in pine seedlings (Ingestad and Kähr 1985). To evaluate the long-term effects of excess Zn, we set up a new experiment in which mycorrhizal and non-mycorrhizal (NM) pine seedlings were exposed to 150 μM Zn for 9 months. We hypothesized that a long-term exposure to elevated Zn is necessary to demonstrate the superiority of the Zn-tolerant *Suillus* isolates in terms of sustaining host plant growth.

Materials and methods

Two isolates of *Suillus bovinus* were used in the experiment. One isolate, UH-Sbo-Ls1, originated from a pine forest growing on an old industrial site polluted with non-ferrous metals emitted by the abandoned Zn smelter of Maatheide in Lommel (B). The other isolate, UH-Sbo-Mg2, was collected in a forest in Meeuwen-Gruitrode (B), on an uncontaminated sandy soil, 22 km from the nearest zinc smelter. The in vitro EC_{50} value for biomass production on solid Fries medium was 1.8 mM Zn for the UH-Sbo-Mg2 isolate. The Zn-tolerant UH-Sbo-Ls1 from the Lommel population had an in vitro EC_{50} value of 9.0 mM Zn. A sandwich technique was used to inoculate 6-week-old Scots pine seedlings with vigorously growing mycelia of either *S. bovinus* isolate, a non-inoculated control group was included as well. After inoculation, plants were transferred to 70-ml syringes filled with pure perlite. The plants were watered weekly with a balanced Ingestad nutrient solution (Ingestad and Kähr 1985). The final basic solution contained 70 μM K_2SO_4, 96 μM KNO_3, 63 μM KH_2PO_4, 58 μM $K_2HPO_4{\cdot}4H_2O$, 733 μM NH_4NO_3, 36 μM $Ca(NO_3)_2{\cdot}4H_2O$, 62 μM $Mg(NO_3)_2{\cdot}6H_2O$, 5 μM H_3BO_3, 2 μM $Mn(NO_3)_2{\cdot}4H_2O$, 3 μM $FeCl_3{\cdot}2H_2O$, 0.1 μM $Zn(NO_3)_2{\cdot}4H_2O$, 0.1 μM $CuCl_2{\cdot}2H_2O$, and 0.02 μM $Na_2MoO_4{\cdot}2H_2O$. Phosphorus was the growth-limiting element. The pH was adjusted to 4.5. Plants were grown in a growth chamber with 300 μmol m^{-2} s^{-1} photosynthetic active radiation and with a day/night regime of 18/6 h and a temperature of 22/15°C.

After 4 weeks, when mycelia had well established, the Zn treatments were started. Plants were divided at random in two Zn treatments to create a factorial set-up with the factors Zn treatment (0.1, 150 μM Zn^{2+}) and inoculation. Each combined treatment had three replicates. Extra Zn was added to the nutrient solution as sulphate. The 150-μM Zn treatment was imposed because this Zn concentration results in a strong differential response in nutrient acquisition among the inoculation treatments studied (Adriaensen et al. 2004).

After 7 weeks, plants were transferred to 4-l microcosms (20×40×5 cm) filled with perlite, moistened with the respective nutrient solution of both Zn treatments. The microcosms had transparent walls so that root and fungal growth could be observed. Plants were placed in the growth chamber initially at the same temperature and day/night regime as before, but 1 week later the temperature was gradually decreased unto 5/3°C over a 5-week period and the day/night regime was changed to 12/12 h. This cold period was introduced because primary shoot growth had ceased and terminal buds were formed. After 5 weeks of 'winter' conditions, the growth regime was gradually restored to the initial regime. Conifers such as Scots pine need to go through a cold period to break bud dormancy (Savitch et al. 2002). After this cold period, shoot apical growth was initiated.

Finally, after 9 months of Zn treatment, the plants were harvested. Shoots were oven-dried (70°C, 120 h) and milled with a ball mill to a fine powder for analyses of elements. The powdered plant material was wet digested in Pyrex tubes in a heating block, two cycles with the addition of 1 ml HNO_3 (65%), followed by one cycle in 1 ml HCl (37%) at 120°C for about 5 h. Samples were eventually dissolved in HCl and diluted to a final volume of 5 ml (2% HCl). Analyses were performed in duplicate and certified reference material was included as an external standard for element analyses: Virginia tobacco leaves (CTA-VTL-2, Institute of Nuclear Chemistry and Technology, Warszawa, Poland) and spinach leaves (Standard reference material® 1570a, National Institute of Standards and Technology, Gaithersburg, USA). P was analyzed colorimetrically with a flow injection analyzer (Lachat; QuickChem®Method 10-115-01-1-A), N was analyzed as N_2 with an CHNS-O analyzer (Flash EA Series 1112, Thermo Electron) and Zn, Fe, and Mg content were measured by atomic absorption spectroscopy. Chlorophyll content in needles was determined by the method described by Lichtenthaler (1987).

Roots and substrate samples (containing external mycelium) were frozen in liquid N_2 and subsequently lyophilized to determine their ergosterol concentration. Ergosterol, a fungal membrane compound, was used as a biochemical marker of active fungal biomass. Ergosterol was extracted and analyzed by HPLC as described in Nylund and Wallander (1992). Ergosterol data were converted to fungal biomass with a conversion factor of 7.1 and 7.5 mg ergosterol g^{-1} dry weight mycelium for UH-Sbo-Ls1 and UH-Sbo-Mg2, respectively. These conversion factors were

calculated from ergosterol levels determined in freeze-dried fungal mats of in vitro grown *S. bovinus*. Data were analyzed by two-way analysis of variance (ANOVA) after checking their normal distribution. Means were calculated, and when the F ratio was significant, least significant differences were evaluated by the Tukey test with significance level 0.05.

Results

After 7 weeks of Zn treatment, when plants were transferred to the microcosms, there were no obvious symptoms of growth depression in any of the treatments, confirming previous observations (Adriaensen et al. 2004). However, such a differential growth became very obvious when the Zn treatment was extended to 9 months (Fig. 1). In particular, after the cold period, plant growth was very limited in Zn-exposed plants that were non-mycorrhizal or inoculated with UH-Sbo-Mg2. The same was true for fungal growth (Fig. 1). At 150 μM Zn, shoot biomass of NM plants and plants inoculated with UH-Sbo-Mg2 was reduced by 75 and 58%, respectively, whereas shoot biomass in the tolerant association was not significantly reduced ($F_{2,12}$=19.3, $P_{\text{interaction}}$=0.0002). The results also showed that root growth was slightly more sensitive to Zn stress than shoot growth, the former being significantly inhibited in all inoculation treatments ($F_{2,12}$=42.5, $P_{\text{interaction}}$<0.0001). Root weight of NM plants collapsed to only 15% of the biomass produced in controls without zinc. Root biomass was reduced to 34 and 76% of the normal weight in the UH-Sbo-Mg2 and UH-Sbo-Ls1 association, respectively (Fig. 1). Growth of UH-Sbo-Mg2 was also reduced by 70% by excess Zn, whereas active fungal biomass of UH-Sbo-Ls1 tended to increase (plus 10%) under Zn stress (Fig. 1).

After this 9-month period of Zn treatment, we also found significantly higher levels of N and P in needles from the seedlings inoculated with UH-Sbo-Ls1 (Table 1). The exposure to elevated Zn reduced the Fe and Mg concentrations in the needles of the pines (Table 1). This effect was most pronounced in NM plants and in those inoculated with UH-Sbo-Mg2 (significant interaction effects with both elements). Fe in needles is higher in mycorrhizal pines than in NM plants, suggesting an increased Fe uptake mediated by these *S. bovinus* isolates. The improved Fe nutrition, however, cannot eliminate the Zn toxicity in plants colonized with UH-Sbo-Mg2. Previously, we also found no correlation between Zn resistance and mycelial Fe accumulation in *S. bovinus* (Colpaert et al. 2005). Severe yellowing of needles was observed in all Zn-exposed NM plants and in plants inoculated with UH-Sbo-Mg2; chlorophyll concentrations in these plants became very low (Table 1), whereas total carotenoid concentrations were not affected by the Zn treatments.

As expected, exposure to elevated Zn also increased the transfer of Zn to the needles of plants (Table 1). There was no difference found in Zn accumulation pattern between the three inoculation treatments. Metal concentrations in leaf tissues should be interpreted with caution (Jentschke and Godbold 2000). The transfer and accumulation of excess metals in leaves are complex processes, strongly controlled by the transpiration stream. In a former experiment with Zn-exposed pine seedlings, it was shown that the transpiration stream slowed down most dramatically in plants inoculated with the Zn-sensitive fungus. The reduced transpiration concurred with a declining transfer of Zn to the shoots once plants were exposed to external concen-

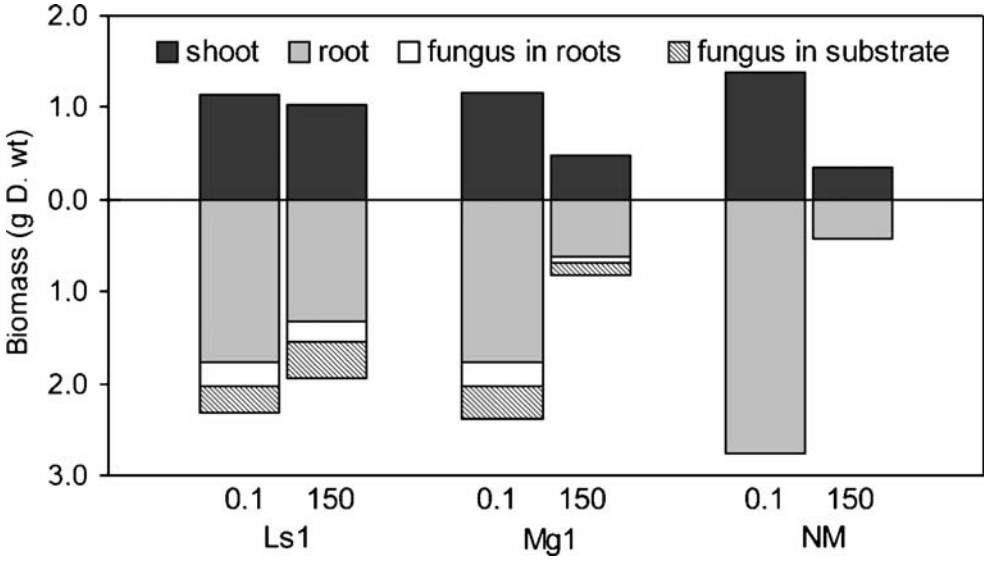

Fig. 1 Plant and fungal biomass of Scots pine seedlings, non-inoculated (NM) or inoculated with the Zn-tolerant UH-Sbo-Ls1 isolate (Ls1) or with the Zn-sensitive UH-Sbo-Mg1 isolate (Mg1). Plants were grown in a control nutrient solution containing 0.1 μM Zn or with a solution enriched to 150 μM Zn. Plants were harvested after 9 months of Zn treatment

Table 1 Nitrogen, phosphorus, zinc, iron, magnesium, and chlorophyll (Chl) concentrations in needles of Scots pines, non-inoculated or colonized by either a Zn-tolerant or a Zn-sensitive *Suillus bovinus* isolate and exposed to elevated Zn for 9 months

Inoculation	Zn treatment						
		N	P	Zn	Fe	Mg	Chl a+b
Zn-tolerant	Control	13.7±1.2	1.13±0.11	59±18	242±35	904±102	762±56
UH-Sbo-Ls1	150 μM	15.4±0.3	1.27±0.05	400±57	223±23	758±29	687±44
Zn-sensitive	Control	14.5±1.6	1.17±0.09	83±10	160±6	851±46	727±119
UH-Sbo-Mg2	150 μM	7.4±0.3	0.70±0.07	508±60	62±13	575±48	218±10
Nonmycorrhizal	Control	11.4±0.6	0.93±0.07	92±2	31±2	999±133	617±58
	150 μM	5.1±1.0	0.59±0.07	716±252	10±1	365±44	201±6
Source of variation	*df*						
Inoculation (Inoc)	2	***	***	ns	***	ns	***
Zn treatment (Zn)	1	***	**	***	***	***	***
Inoc×Zn	2	**	**	ns	*	*	**
df error		12	12	12	12	12	12

Results of the two-way ANOVA: *P* values (significance levels) of the single factor and interaction effects are shown. Data are expressed in mg g^{-1} dry weight for N and P and μg g^{-1} dry weight for Zn, Fe, and Mg and μg g^{-1} fresh weight for Chl. Values are means±SEM (*n*=3).

ns not significant

*$P<0.05$

**$P<0.01$

***$P<0.001$

trations above 150 μM Zn (Adriaensen et al. 2004). In the present experiment, Zn accumulation in the sensitive association has probably slowed down earlier compared to the tolerant association, resulting in similar, final Zn concentrations in the needles (Table 1). In addition, the Zn concentrations measured are total Zn concentrations, and little is known about the Zn speciation and subcellular localization in foliage. Pines with a better nutrient status probably have a higher capability of tightly controlling Zn homeostasis in their cells.

Discussion

Several studies have dealt with a possible alleviation of metal toxicity by mycorrhization, but only few presented direct evidence for such effects in ectomycorrhizal trees (Meharg and Cairney 2000). It is possible that at least in some of these experiments plants were harvested too early to detect differential growth responses; in the majority of the experiments seedlings were exposed for less than 4 months to toxic metal concentrations. The inevitable induction of dormancy in the shoot apex of many perennial plants may mask late effects on growth. To obtain a second boost of shoot growth, the introduction of a hibernation period is the most obvious strategy. The second growth period in our experiment was most successful in revealing the differential growth among the different treatments.

The beneficial effect of ectomycorrhizal fungi is mostly attributed to a general enhanced fitness as a result of the colonization (Dixon and Buschena 1988; Meharg and Cairney 2000). The present results support this hypothesis but in the meantime show that intraspecific differences in Zn tolerance among fungi are at least equally important for sustaining plant protection.

There are several ways in which ectomycorrhizal fungi may ameliorate metal tolerance to their host plant (Godbold et al. 1998). However, regardless of the mechanism, it is obvious that the fungus must be able to develop into the polluted substrate and must remain metabolically active over a reasonable length of time to maintain plant fitness. In the present experiment, the UH-Sbo-Mg2 isolate showed very little growth in the Zn-contaminated substrate and the same was observed for the pine roots. Nevertheless, plants inoculated with this isolate were slightly better off than NM plants, but the mycorrhizal protection of the host plant was not very spectacular, at least not at this particular Zn concentration. On the other hand, the Zn-tolerant UH-Sbo-Ls1 isolate colonized the Zn-contaminated substrate for 100%, while pine roots followed mycelial expansion.

The overall picture shows that NM plants and plants inoculated with UH-Sbo-Mg2 clearly had lower nutrient concentrations under Zn stress than their counterparts inoculated with the Zn-tolerant UH-Sbo-Ls1 isolate. The damage caused by the Zn treatment could be well deduced from the yellowing of the pine needles in NM plants and in those inoculated with the Zn-sensitive fungus. Serious reductions in chlorophyll concentrations lead to reduced C fixation and thus reduced growth. Such a simultaneous reduction in chlorophyll content and growth was also

observed in mycorrhizal and NM birches exposed to Ni stress (Jones and Hutchinson 1988). The data do not allow us to pinpoint the exact cause of the reduced chlorophyll concentration in foliage of the Zn-stressed plants. However, it is likely that the reduction is largely caused by the Zn-induced impairment of nutrient transfer to needles, in particular N, Fe, and Mg, three elements playing a pivotal role in chlorophyll synthesis and functioning.

The present results further support the hypothesis that a Zn-adapted *S. bovinus* strain performs much better under Zn stress than an isolate from an unpolluted site. After two growth periods, pine seedlings are much larger when their root system is colonized by the Zn-tolerant mycobiont. Thus, whether the mechanism of tolerance involved the binding of metal, either internally or externally, or an altered uptake of nutrients, the tolerant genotype was likely to be more effective in protecting its host from metal toxicity. Hardly any biomass reduction of the tolerant plant-fungus association was observed at 150 μM Zn compared to the control. According to Meharg (2003), there are only few examples where the presence of mycorrhizal fungi clearly alleviates metal toxicity of the host: Lux and Cumming (2001) showed that Tulip-poplar (*Liriodendron tulipifera*) colonized by arbuscular mycorrhizal fungi was more Al insensitive than in its non-mycorrhizal state.

Similarly, in ericoid mycorrhiza, colonization considerably increased the growth and survival of *Calluna vulgaris* at excess Zn concentrations (Bradley et al. 1982). Bradley et al. (1982) also confirmed that mycorrhizal plants from a mine population grew better than colonized plants from a non-mine population. Hence, both metal-resistant hosts and mycorrhizal fungi have sometimes co-adapted to the extreme ecological niches provided by metal-contaminated sites (Meharg and Cairney 2000). Nevertheless, there is little evidence that tree species have adapted genetically to grow on metal-contaminated soils (Wilkinson and Dickinson 1995). So tree adaptation to contaminated soils largely depends on the metal adaptive potential of their associated mycorrhizal fungi.

In the present experiment, it was shown that the Zn-adapted *S. bovinus* provided a sustainable "insurance" against Zn toxicity for pine seedlings growing on Zn-polluted soil. The use of such metal-tolerant *Suillus* species may be a promising strategy to develop tools for reclamation of metal-contaminated and disturbed soils. Suilloid species are the principal ectomycorrhiza-forming fungi on pines establishing on sites where mycelial networks of ectomycorrhizal fungi are absent or have been destroyed (Visser 1995; Horton et al. 1998; Ashkannejhad and Horton 2006). Their basidiospores, dispersed by wind and animals, successfully yield ectomycorrhizas on pine seedlings, establishing on disturbed or pristine soils. The group has specific ecological adaptations for establishment of pines in harsh or early successional habitats (Ashkannejhad and Horton 2006), conditions typically encountered on severely metal-contaminated sites.

In situ experiments have been set-up to demonstrate the advantage of inoculation of pines with Zn-tolerant *Suillus* isolates in a Zn-contaminated soil. As Suilloids are able to spontaneously colonize pine seedlings under nursery conditions (Menkis et al. 2005), pre-inoculation of nursery seedlings with metal-tolerant *Suillus* species could be a realistic and desirable strategy in the production process of pine seedlings aimed for the reforestation of metal-contaminated sites. Nevertheless, it is evident that additional work will be necessary to identify the application range of the metal-tolerant fungi in terms of soil types, competitiveness and behavior in soils with a mixed pollution, for example, mixtures of different toxic elements or in combination with harmful organic compounds.

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