# Ectomycorrhizal protection of *Pinus sylvestris* against copper toxicity

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## Summary

• Change in the copper (Cu) sensitivity of *Pinus sylvestris* is presented, in response to the ectomycorrhizal fungi, *Suillus bovinus* and *Thelephora terrestris*, common mycobionts on metal contaminated sites.

• Seedlings grown under phosphorus (P) limitation were exposed to a range of Cu concentrations. Plant and fungal development, P nutrition, sorption of Cu on roots and external mycelia as well as transfer of Cu to shoots were assessed.

• Root growth and P nutrition were severely inhibited in nonmycorrhizal pines at elevated Cu compared with mycorrhizal plants. Excess Cu had little effect on the development of mycorrhizal roots and mycelia. *Thelephora terrestris* was less sensitive to Cu stress than *S. bovinus*. The extraradical mycelium of *S. bovinus* retained large amounts of Cu. However, binding of Cu in fungal tissue was not a prerequisite for low Cu sensitivity since *T. terrestris* absorbed considerably less Cu that *S. bovinus*.

• Both ectomycorrhizal fungi protect *P. sylvestris* against Cu toxicity; a benefit that was not due to a metal dilution effect. The mechanisms of mycorrhizal amelioration of Cu toxicity are probably diverse and species-dependent.

**Key words:** copper toxicity, ectomycorrhiza, heavy metals, *Pinus sylvestris* (Scots pine), *Suillus bovinus*, *Thelephora terrestris*.



## Introduction

A common characteristic of Cu distribution in soils is its accumulation in surface horizons, which reflects the bioaccumulation of the metal but also its deposition from anthropogenic sources (Tiller & Merry, 1981). Sources of soil copper contamination are diverse, including application of fertilisers, fungicide sprays, dumping of agricultural or municipal wastes, and emissions by industrial activities such as smelting and mining. High concentrations of Cu in soil have an adverse effect on vegetation (Lepp *et al.*, 1997). Nevertheless, some tree species can establish and survive on Cu-polluted soils. Under natural conditions the majority of woody plants in temperate and boreal forests are associated with ectomycorrhizal (ECM) fungi and it seems that this mutualistic symbiosis persists on strongly metal (Cu)-contaminated sites which become slowly colonized by birches, pines and willows (Leyval *et al.*, 1997; Vrålstad *et al.*, 2000).

Copper, when present at elevated concentrations, is considered to be highly toxic for both plants and fungi (Fernandes & Henriques, 1991; Gadd, 1993). However, Cu sensitivity of ECM plants is poorly documented and there is no clear-cut evidence for a protective role of ECM fungi ( Jones & Hutchinson, 1986; Dixon & Buschena, 1988). There are no studies in which ECM fungi have been demonstrated to alleviate growth depressions of tree seedlings due to toxic effects of Cu (Van Tichelen *et al.*, 1999; Jentschke & Godbold, 2000). Only for the ericoid association between *Calluna vulgaris* and *Hymenoscyphus ericae*, has direct evidence for increased Cu resistance been found (Bradley *et al.*, 1981).

The *in vitro* Cu sensitivity of a number of ECM fungi has been studied in several experiments. Considerable interspecific variation response to Cu was observed, but adaptive Cu tolerance has not been found (Blaudez *et al.*, 2000; Colpaert *et al.*, 2000). Relevant differences in metal sensitivity between ECM fungi and their hosts can, however, be best assessed in symbiosis experiments. The functioning of the mycorrhizal

association as well as the development of the symbiotic partners should be studied together in a dose – response experiment.

In the present study, the Cu sensitivity of *Pinus sylvestris* seedlings and two ECM symbionts (*Suillus bovinus* and *Thelephora terrestris*) with high constitutive Cu resistance *in vitro* was compared. These fungi are common mycobionts under pines on metal contaminated sites (Colpaert & Van Assche, 1987). Phosphorus uptake, sorption of Cu on roots and extraradical mycelia, and Cu transfer to above-ground plant parts were assessed.

## Materials and Methods

### Plant and fungal material

The *Thelephora terrestris* (Ehrh.) Fr. culture was obtained from basidiospores from a sporocarp collected in a pine stand on metal-polluted soil in Lommel (B) (Vangronsveld *et al.*, 1996). The *Suillus bovinus* (Fr.) isolate was collected from a non-polluted area in Paal (B). In a preliminary *in vitro* test on Fries agar medium (Fries, 1981), we compared Cu sensitivity of four *S. bovinus* genotypes from different locations, but no significant differences in Cu sensitivity were found among the isolates. The $EC_{50}$ value – the Cu concentration which inhibits growth by 50% – was estimated approx. 150 mmol $m^{-3}$ for the *S. bovinus* isolate and it was above 790 mmol $m^{-3}$ for the *T. terrestris* isolate.

Scots pine seeds (*P. sylvestris* L., provenance Groenendaal, Belgium, 50°45′ N, 4°25′ E) were surface-sterilized for 10 min in $H_2O_2$ (30%). They were sown in perlite/vermiculite (2 : 1, v/v) and watered weekly with a balanced Ingestad nutrient solution. The nutrient solution contained (in mmol $m^{-3}$): $K_2SO_4$ 70, $KNO_3$ 96, $KH_2PO_4$ 38, $K_2HPO_4 4H_2O$ 35, $NH_4NO_3$ 733, $Ca(NO_3)_2 4H_2O$ 32, $Mg(NO_3)_2 6H_2O$ 62, $H_3BO_3$ 5, $Mn(NO_3)_2 4H_2O$ 4, $FeCl_3 2H_2O$ 4, $Zn(NO_3)_2 4H_2O$ 0.3, $CuCl_2 2H_2O$ 0.3, $Na_2MoO_4 2H_2O$ 0.04. Phosphorus was the growth limiting element and the pH was 4.5. The experiment was carried out in a growth chamber with 300 μmol $m^{-2}$ $s^{-1}$ PAR, at least 70% relative air humidity and with a day/night rhythm of 18/6 h and a temperature of 22/15°C. Thirty-six d after sowing, 72 uniform seedlings were selected for the experiment. Perlite and vermiculite were gently removed from the roots and fresh weight of each plant was measured. A sandwich technique was used to inoculate 48 seedlings (Van Tichelen & Colpaert, 2000). Twenty-four NM seedlings followed the same procedure in the absence of fungal inoculum. At the time of inoculation, six additional seedlings were harvested to determine their P content. After inoculation (3 d), plants were transferred to 70-$cm^3$ transparent syringes (Braun Omnifix®, B. Braun Melsungen AG, Melsungen, Germany) filled with 4.0 g d. wt acid-washed, sieved perlite (2–4 mm particles) (Colpaert *et al.*, 1999). The perlite in each container was covered with small quartz stones and black plastic foil to prevent growth of algae. The syringes hung in holes in a PVC lid put over large dark plastic boxes (60 × 40 cm). Below the syringes, a glass tube (100 ml) was present to collect percolating water. From the moment of transplantation, a constant relative nutrient addition rate of 4% $d^{-1}$ was initiated for each individual plant. The average P content of the seedlings which were harvested at the inoculation time was used as a basis for the calculation of the daily nutrient additions necessary to obtain the 4% $d^{-1}$ nutrient addition rate. Applying this procedure, plants have to adjust their relative growth rate to the relative addition rate (Colpaert & Verstuyft, 1999).

### Copper treatments

After inoculation and transfer to the syringes (3 wk), Cu treatments were started. The plants were divided at random into four Cu treatments in order to create a factorial set-up with the factors Cu treatment (0.3, 16, 32, 47 mmol $m^{-3}$ Cu) and mycorrhizal inoculation (NM, mycorrhizal with *S. bovinus* or *T. terrestris*). In the first subset, Cu concentration was maintained at a control concentration of 0.3 mmol $m^{-3}$, in the other treatment sets the Cu concentrations in the Ingestad nutrient solution was raised with $CuSO_4 5H_2O$ to 16, 32 or 47 mmol $m^{-3}$. The Cu conditions were established by flushing the plant containers with nutrient solution containing the respective Cu concentrations. The Cu concentration in the drained solution was monitored. The plant containers were flushed at a flow rate of ±4.0 $cm^3$ $min^{-1}$, until Cu in the eluent solution reached the concentration of the respective treatments. All containers were flushed with the same volume of nutrient solution. The following days, plants received the small quantity of nutrient solution, necessary to maintain the 4% relative growth rate. The flushing (titration) of the plant containers was repeated every 2 wk, in order to maintain the external Cu concentrations at the intended levels.

### Harvest and analyses

Plants were harvested at two occasions: half of the plants was exposed to elevated Cu for 22 d, the other half was treated for 36 d. On both harvest days, three plants from each treatment group were harvested. Shoots were cut off and plant containers were subsequently flushed with 100 $cm^3$ distilled $H_2O$. Thereafter the syringes were centrifuged for 30 s at 135 g in order to remove the nutrient solution retained in the perlite. Root systems were pulled out of the containers and were separated from the perlite. Fresh weights of shoots and roots were measured. Two subsamples of fine roots (0.2 g) and wet perlite (5 g) were immediately frozen in liquid nitrogen for ergosterol analysis. Shoots, remaining roots and perlite were oven-dried for at least 3 d at 75°C to determine dry mass, fresh : dry mass conversion factors and chitin contents.

Analysis of P and Cu in the plant tissues was performed on ashed samples (4 h at 500°C). Ashes were dissolved in

500 mol $m^{-3}$ HCl. Dried perlite was pulverized with a ball mill at 200 Hz for 2 min. Copper was determined in milled perlite, in order to estimate the Cu accumulation on the extraradical mycelia. This mycelium could not be separated from the perlite. Two subsamples of 0.5 g milled perlite were submerged overnight in a mixture of 1 $cm^3$ $HNO_3$ (65%) and 1 $cm^3$ HCl (35%) in a Pyrex tube. Subsequently, the samples were heated for 30 min in a boiling water bath. After cooling, 5 $cm^3$ of distilled $H_2O$ was added to the samples. Once perlite had settled down, Cu was determined in the supernatant. The Cu concentration in perlite from the NM plants was used to determine background adsorption of Cu on perlite (not colonized by extraradical mycelium). Copper sorbed on extraradical mycelia of the fungi was calculated by correcting for Cu adsorbed on perlite.

Chitin content in the milled perlite was analysed in order to determine total fungal biomass in the perlite (active and dead extraradical mycelium). Two subsamples of 0.3 g milled perlite were hydrolysed for 3 h in 1 $cm^3$ 6000 mol $m^{-3}$ HCl at 120°C. The hydrolysate was evaporated to dryness and redissolved in 1 $cm^3$ distilled $H_2O$. Glucosamine was determined according to Rondle & Morgan (1955). The chitin content of *in vitro* cultivated mycelium was 45 and 32 μg $mg^{-1}$ d. wt mycelium for *S. bovinus* and *T. terrestris*, respectively. These conversion factors were determined in mycelia grown *in vitro* on the above-mentioned double strength Ingestad medium supplemented with 5.0 kg $m^{-3}$ glucose, 100 mg $m^{-3}$ thiamine, 25 mg $m^{-3}$ biotin and 8.0 kg $m^{-3}$ agar.

The amount of active fungal biomass in fine roots and perlite was assessed by measuring the concentration of ergosterol as described by Nylund & Wallander (1992). Ergosterol data were converted to fungal biomass with conversion factors of 6.9 and 3.0 mg $g^{-1}$ d. wt fungus, for *S. bovinus* and *T. terrestris*, respectively. These conversion factors were calculated from ergosterol determined in the same fungal mats used to calculate the chitin conversion factors. Perlite samples were sonicated for 2 min and were then refluxed for 30 min in 10 $cm^3$ KOH (1000 mol $m^{-3}$) in MeOH (100%), root samples were refluxed in 5 $cm^3$ of this solution. After cooling and addition of 0.5 $cm^3$ $H_2O$, the samples were extracted with 10 $cm^3$ *n*-hexane (5 $cm^3$ for root samples). The hexane fraction was pipetted off and evaporated to dryness in a speedvac (30 min). The extract was redissolved in 1 ml *n*-hexane and again evaporated to dryness (10 min). Finally, the extract was dissolved in 0.5 $cm^3$ HPLC-grade methanol and stored at 4°C until quantification. Ergosterol was quantified by HPLC (Waters™ 626 pump linked to a Waters™ 916 Photodiode Array Detector, Millipore Corporation, Milford, MA, USA) using a reverse-phase C-18 column, methanol (99%) as the mobile phase, an injection volume of 100 $mm^3$ and a flow rate of 2 $cm^3$ $min^{-1}$. Peaks were detected at 282 nm.

Fresh mycorrhizas and NM root tips from the 32 mmol $m^{-3}$ Cu treatment were used for a Cu desorption test. In order to remove Cu from the free space, root tips (±20 mg f. wt) were desorbed in 1.5 $cm^3$ of a 5-mol $m^{-3}$ $Pb(NO_3)_2$ solution for 60 min at 4°C, as proposed by Harrison *et al.* (1979). Immediately thereafter, roots were blotted on paper tissue and transferred to a HCl solution of pH 1. After 30 min in HCl, roots were dried overnight and subsequently ashed for Cu analysis as described earlier. Cu was analysed in the desorption solutions and in the desorbed roots.

### Statistical analysis

Data were subjected to a three-way ANOVA using the general linear models procedure in the statistical software package SAS® (SAS Institute Inc., NC, USA) with inoculation treatment, Cu treatment and harvest time as variable factors. A log transformation was applied for the shoot, root and perlite Cu concentration data in order to meet the assumptions of the ANOVA. The Tukey multiple comparisons procedure was used to determine significant effects of pairwise comparisons.

## Results

### Plant growth

At harvest, all plants looked healthy, except for NM seedlings exposed to 32 or 47 mmol $m^{-3}$ Cu concentration. At 47 mmol $m^{-3}$ Cu, viable (white) root tips were not present on NM plants, and the small root system was blackened, wrinkled and easily fragmented; needles of these plants turned reddish and at the last harvest needles started wilting. In the same Cu treatment, ECM plants had green needles and normal root systems with numerous mycorrhizal root tips. Macroscopic differences were not observed among *T. terrestris* mycorrhizas from different Cu treatments. A larger proportion of *S. bovinus* mycorrhizas tended to be yellow at the highest Cu concentration. The pH in the perlite nutrient solution was around 3.5 and did not differ significantly between the different treatments.

In the absence of elevated Cu, there were no significant differences in dry mass of shoots and roots between ECM and NM pine seedlings. In all treatments there was a significant increase in shoot dry mass between both harvests (Table 1, Fig. 1). In contrast, an inoculation × harvest × Cu interaction effect was found on root biomass (Table 1, Fig. 1). Root growth was far more sensitive to Cu toxicity when plants were not inoculated and this became most pronounced at the second harvest. There was no significant increase in root biomass between harvests for NM plants treated with 16, 32 or 47 mmol $m^{-3}$ Cu and for *S. bovinus* associated plants treated with 47 mmol $m^{-3}$ Cu (Fig. 1). At both harvest dates, there was no difference in total root biomass between NM plants from the 0.3 and 16 mmol $m^{-3}$ Cu treatment, but NM roots were significantly smaller when plants were exposed to an external Cu concentration of 32 ($P < 0.01$) or 47 ($P < 0.0001$) mmol $m^{-3}$. In contrast, no adverse effect of Cu

**Table 1** Results of the three-way ANOVA's: *P*-values (significance levels) of the single and interaction effects are shown

| | | Biomass | | | | P concentration | | Cu concentration | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Source of variation | df | Shoot | Root | Mycelium in roots[a] | Extraradical mycelium[a] | Shoot | Root | Shoot | Root | Extraradical mycelium[b] |
| Inoculation (In) | 2 | 0.4577 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | |
| Harvest (H) | 1 | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0138 | 0.8680 | 0.0002 | 0.0081 | 0.0191 |
| Copper (Cu) | 3 | 0.0765 | 0.0001 | 0.2819 | 0.0501 | 0.0633 | 0.0276 | 0.0001 | 0.0001 | 0.0001 |
| In × H | 2 | 0.6126 | 0.0001 | 0.9365 | 0.2687 | 0.1833 | 0.3805 | 0.0477 | 0.1762 | |
| In × Cu | 6 | 0.4470 | 0·0001 | 0.8653 | 0.0040 | 0.0022 | 0.0062 | 0.0016 | 0.0001 | |
| H × Cu | 3 | 0.2488 | 0·2948 | 0.0744 | 0.2130 | 0.2557 | 0.2625 | 0.3020 | 0.0291 | 0.2354 |
| In × H × Cu | 6 | 0.9493 | 0·0002 | 0.7978 | 0.9454 | 0.0034 | 0.0382 | 0.2559 | 0.0127 | |
| df error | | 48 | 48 | 48 | 48 | 48 | 47 | 48 | 48 | 12 |

[a]active fungal biomass calculated from ergosterol data. [b]Cu concentration in extraradical mycelium of *Suillus bovinus* (results of two-way ANOVA).

treatments was found on root growth of plants colonized by *T. terrestris*. Root biomass was reduced in plants colonized by *S. bovinus* exposed for 36 d to 47 mmol $m^{-3}$ Cu ($P < 0.01$).

## Fungal growth

At the start of the Cu additions, 3 week after inoculation, at least 50% of short roots were mycorrhizal in the inoculated plants. Uninoculated plants remained nonmycorrhizal throughout the experiment. At harvest, all short roots on inoculated seedlings were mycorrhizal and active fungal biomass in roots and perlite was high, irrespective of the Cu treatments (Table 2). No ergosterol was detected in NM plants. For all Cu treatments, the fungal biomass in roots and of extraradical mycelium was significantly higher at the second harvest than at the first one, whether determined with the ergosterol (Tables 1, 2) or chitin assay (not shown). At 0.3, 16 and 32 mmol $m^{-3}$ Cu, active fungal biomass in perlite of *S. bovinus* was higher than that of *T. terrestris* ($P < 0.01$). Cu had no effect on the total amount of active fungal biomass in roots. However, the biomass of active extraradical *S. bovinus* mycelium was significantly reduced ($P < 0.01$) to 40 and 65% of the control value after 22 and 36 d of exposure to 47 mmol $m^{-3}$ Cu (Table 2). The elevated Cu concentrations did not reduce the active biomass of extraradical *T. terrestris* mycelium.

The average chitin : ergosterol ratios in the perlite (extraradical mycelia) were 10.8 ± 1.0 and 42.6 ± 4.8 when colonized by *S. bovinus* or *T. terrestris*, respectively. These ratios were significantly different between species ($P < 0.0001$), but there was no difference between the different Cu treatments or harvests. The proportion of active to total mycelium remained constant.

## P nutrition

In the control and 16 mmol $m^{-3}$ Cu treatment, ECM plants had lower shoot P concentrations than their NM counterparts, especially at the second harvest (Tables 1, 3). The opposite was true for P in roots, in particular when colonized by *T. terrestris*. At the second harvest, Cu significantly reduced the P concentration in shoots of NM plants. Such a Cu effect was not observed in ECM plants (Table 3).

## Copper distribution in plants

Cu concentrations in plants from the control Cu treatment were similar between inoculation treatments with average Cu concentrations of 7 μg $g^{-1}$ shoot d. wt and 11 μg $g^{-1}$ root d. wt (Table 4). Shoot Cu concentrations rose in all plants once they were exposed to elevated Cu (Tables 1, 4). This increase was most pronounced in NM plants, grown at 16 and 32 mmol $m^{-3}$ Cu. At the same Cu treatment, shoot Cu concentrations in plants inoculated with *S. bovinus* remained at a much lower level. In roots, Cu concentrations increased once plants were exposed to elevated Cu and they became considerably higher than the concentrations measured in shoots. Under the highest Cu treatment, the small root systems of NM plants accumulated extremely high amounts of Cu, with concentrations reaching on average 3.6 mg $g^{-1}$ d. wt (Table 4).

Cu concentrations in perlite of all plants rose with increasing Cu additions, but the Cu concentration in perlite colonized by *S. bovinus* was consistently higher than the concentration measured in the perlite of NM and *T. terrestris* colonized plants (Table 4). At the experimental pH of 3.5, the *T. terrestris* extraradical mycelium contained very little Cu, since background Cu levels were hardly exceeded in the *T. terrestris* perlite (Fig. 2). However in the case of *S. bovinus*, the Cu concentration in the perlite was up to 5 times higher than the background level (Table 4). Cu concentration in perlite invaded by *S. bovinus* was positively correlated with the biomass of extraradical mycelium (Fig. 2). The Cu concentration

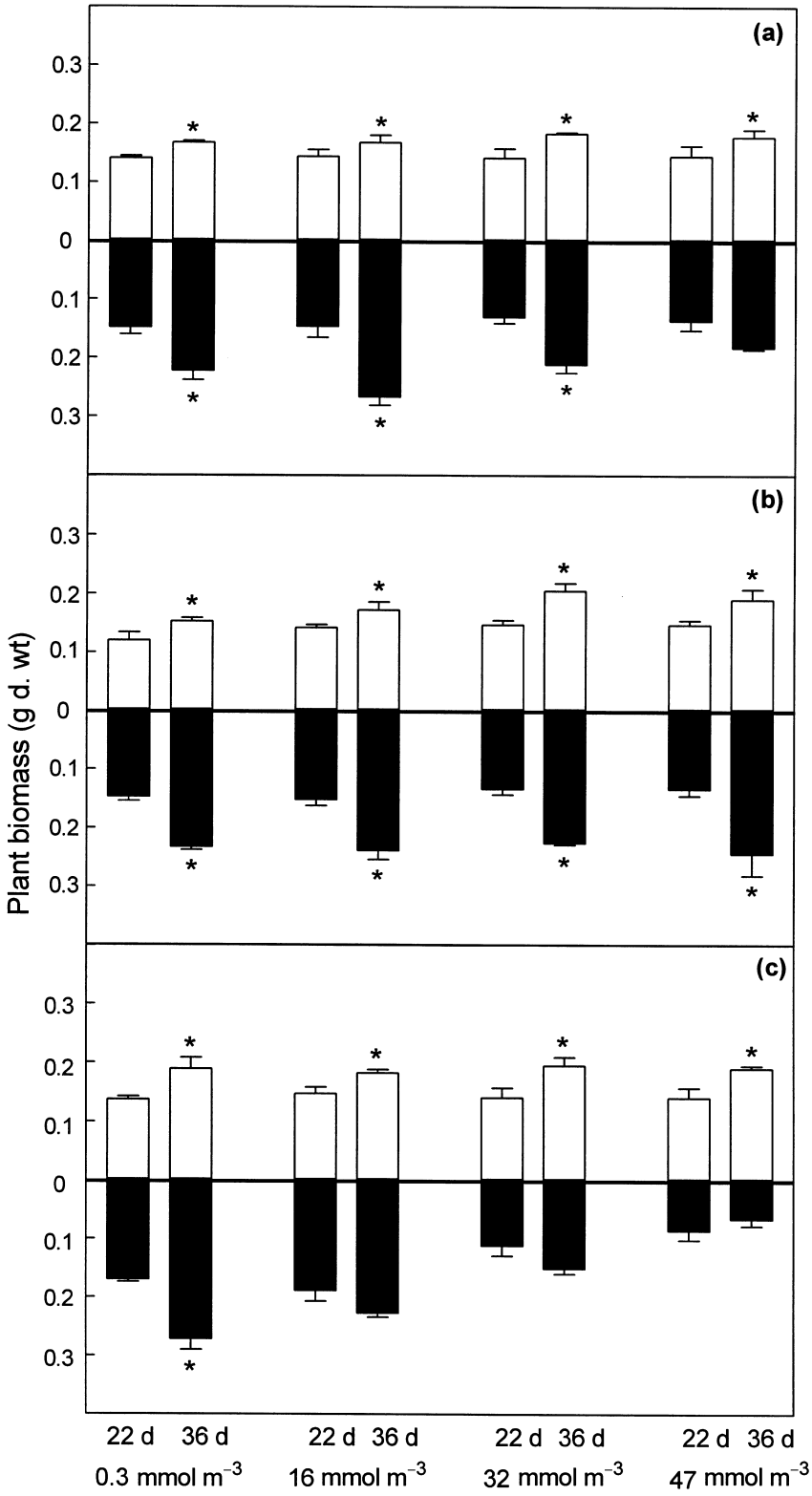


**Fig. 1** Shoot (open column) and root (closed column) biomass of *Pinus sylvestris* seedlings inoculated with (a) *Suillus bovinus* (b) *Thelephora terrestris* or (c) nonmycorrhizal. Plants were exposed for 22 or 36 d to 4 different Cu concentrations: 0.3, 16, 32 or 47 mmol $m^{-3}$ Cu. Bars represent standard errors ($n$ = 3). Paired columns marked with an asterisk indicate a significant increase in biomass between both harvest days ($P < 0.01$, Tukey comparisons).

**Table 2** Active fungal biomass (mg $g^{-1}$ d. wt) in fine roots and in perlite of the mycorrhizal *Pinus sylvestris* seedlings given different Cu and mycobiont treatments

| Mycobiont | Cu treatment (mmol $m^{-3}$ Cu) | Harvest 1 (22d) | | Harvest 2 (36d) | |
|---|---|---|---|---|---|
| | | Root | Perlite | Root | Perlite |
| *Suillus bovinus* | 0.3 | 216 | 1.8 | 351 | 3.1 |
| | 16 | 185 | 2.1 | 402 | 3.5 |
| | 32 | 150 | 2.7 | 330 | 3.4 |
| | 47 | 154 | 0.7 | 361 | 2.0 |
| *Thelephora terrestris* | 0.3 | 202 | 0.7 | 310 | 2.1 |
| | 16 | 203 | 0.9 | 313 | 2.1 |
| | 32 | 329 | 1.2 | 402 | 1.6 |
| | 47 | 205 | 1.4 | 308 | 2.0 |

Data were calculated from the ergosterol measurements using a conversion factor of 6.9 and 3.0 μg ergosterol $mg^{-1}$ d. wt for *Suillus bovinus* and *Thelephora terrestris*, respectively, $n = 3$. No fungal biomass was found in NM plants.

**Table 3** Shoot and root P concentrations (μg P $mg^{-1}$ d. wt) in *Pinus sylvestris* seedlings given different Cu and mycobiont treatments

| Mycobiont | Cu treatment (mmol $m^{-3}$ Cu) | Harvest 1 (22 d) | | Harvest 2 (36 d) | |
|---|---|---|---|---|---|
| | | Shoot | Root | Shoot | Root |
| *Suillus bovinus* | 0.3 | 1.12 | 1.72 | 1.05 | 2.04 |
| | 16 | 1.04 | 1.79 | 1.04 | 1.42 |
| | 32 | 1.01 | 1.54 | 1.12 | 1.56 |
| | 47 | 0.91 | 1.50 | 1.17 | 1.74 |
| *Thelephora terrestris* | 0.3 | 1.07 | 2.43 | 1.08 | 2.39 |
| | 16 | 1.12 | 2.43 | 1.04 | 2.36 |
| | 32 | 1.00 | 1.92 | 1.13 | 2.12 |
| | 47 | 1.11 | 2.05 | 1.28 | 2.19 |
| Nonmycorrhizal | 0.3 | 1.32 | 1.29 | 1.59 | 1.36 |
| | 16 | 1.26 | 1.43 | 1.52 | 1.53 |
| | 32 | 1.17 | 1.69 | 1.25 | 1.37 |
| | 47 | 1.37 | 1.49 | 1.10 | 1.36 |

Data represent mean ($n = 3$).

in the *S. bovinus* mycelium increased with increasing Cu levels and between harvests (Table 1). Mean copper concentrations (± standard error of the mean) in the extraradical *S. bovinus* mycelium were $221 \pm 39$, $3547 \pm 194$, $8227 \pm 296$ and $7807 \pm 1088$ μg $g^{-1}$ total fungal d. wt (chitin based) for the 0.3, 16, 32 and 47 mmol $m^{-3}$ Cu treatments of the second harvest, respectively.

The inoculation treatment had a marked effect on the partitioning of Cu between shoot, root and extraradical mycelium (Fig. 3). Despite its relatively small biomass, the *S. bovinus* extraradical mycelium contained a very high amount of Cu. NM plants transported more Cu to above-ground plant parts. At 16 and 32 mmol $m^{-3}$ Cu, total amount of Cu present in all plant and fungal structures was highest in the *S. bovinus* treatments; at 47 mmol $m^{-3}$ Cu, most Cu was found in NM plants. The pine – *T. terrestris* association accumulated least Cu in all elevated Cu treatments. In all treatments, most Cu was found in belowground biomass (Fig. 3). At 16 or 32 mmol $m^{-3}$ Cu, roots and extraradical mycelium of the *S. bovinus*-inoculated plants had accumulated up to two times more Cu than NM roots or roots associated with *T. terrestris*. Total transfer of Cu to shoots was nevertheless lowest in pine seedlings colonized by *S. bovinus*. A pronounced accumulation of Cu in shoots was observed in NM plants exposed to 32 mmol $m^{-3}$ Cu for 36 d (Fig. 3, Table 4). At 47 mmol $m^{-3}$ Cu, the dead root systems of NM plants accumulated a large amount of Cu, but transfer to shoots was relatively low.

The desorption test of the root tips from the 32 mmol $m^{-3}$ Cu treatment showed that $Pb^{2+}$ is an effective desorbing agent to remove Cu from NM roots and *Thelephora* mycorrhizas (Fig. 4). Most Cu present in *Suillus* mycorrhizas could not be desorbed with Pb or an excess of protons. The largest pool of Cu was found after ashing the desorbed *Suillus* mycorrhizas.

**Table 4** Copper concentrations (μg Cu $g^{-1}$ d. wt) in the different plant parts and in the perlite (+ extraradical mycelium) of *Pinus sylvestris* seedlings given different Cu and mycobiont conditions

| Mycobiont | Cu treatment (mmol $m^{-3}$ Cu) | Harvest 1 (22d) | | | Harvest 2 (36d) | | |
|---|---|---|---|---|---|---|---|
| | | Shoot | Root | Perlite | Shoot | Root | Perlite |
| *Suillus bovinus* | 0.3 | 5.4 | 11.4 | 0.8 | 6.3 | 15.7 | 1.9 |
| | 16 | 10.5 | 66 | 13.6 | 9.9 | 62 | 20.5 |
| | 32 | 18.3 | 176 | 26.6 | 13.3 | 256 | 45.2 |
| | 47 | 19.5 | 556 | 18.6 | 23.4 | 433 | 33.4 |
| *Thelephora terrestris* | 0.3 | 5.9 | 7.4 | 0.5 | 7.3 | 11.9 | 0.9 |
| | 16 | 10.0 | 121 | 4.4 | 16.4 | 103 | 5.3 |
| | 32 | 14.6 | 128 | 6.2 | 24.5 | 284 | 9.3 |
| | 47 | 18.4 | 324 | 9.6 | 23.4 | 443 | 9.5 |
| Nonmycorrhizal | 0.3 | 6.8 | 14.2 | 0.4 | 9.0 | 6.2 | 0.8 |
| | 16 | 22.8 | 70 | 4.6 | 25.9 | 79 | 5.3 |
| | 32 | 30.1 | 275 | 7.3 | 59.7 | 375 | 8.9 |
| | 47 | 39.6 | 2001 | 14.2 | 33.8 | 3630 | 13.4 |

Data represent mean ($n$ = 3).

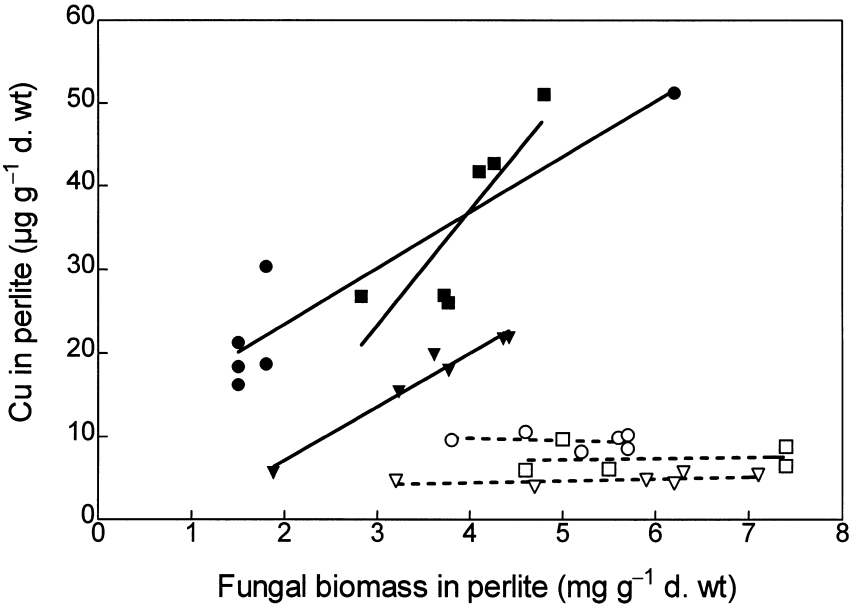


**Fig. 2** Relationship between the total biomass (based on chitin determinations) of the extraradical mycelia and Cu present in the perlite + extraradical mycelium of mycorrhizal *Pinus sylvestris* plants treated with: 16 (open triangles/closed triangles), 32 (open squares/closed squares) or 47 (open circles/closed circles) mmol $m^{-3}$ Cu. Open symbols represent data of perlite colonized by *Thelephora terrestris*, closed symbols represent data of perlite colonized by *Suillus bovinus*. Data points are values of individual plants at both harvests. The regression equations are (open triangles) $y = 0.2x + 3.6$ ($R^2 = 0.26$); (open squares) $y = 0.1x + 6.6$ ($R^2 = 0.01$); (open circles) $y = -0.3x + 10.8$ ($R^2 = 0.04$); (closed triangles) $y = 6.4x - 5.7$ ($R^2 = 0.96$); (closed squares) $y = 13.7x - 17.9$ ($R^2 = 0.72$); (closed circles) $y = 6.7x + 10.1$ ($R^2 = 0.89$).

## Discussion

Metal sensitivity or tolerance in trees is generally assessed in dilute nutrient solutions in hydroponics (Balsberg Påhlsson, 1989), whereas fungi are usually screened *in vitro* on nutrient rich culture media (Hartley *et al.*, 1997). Comparisons of metal toxicity levels for both groups of organisms in absolute terms are consequently difficult and probably not very relevant. Nevertheless, in a metal-contaminated soil, ECM mycelia and roots are exposed to the same soil solution. In unpolluted soils, the water extractable Cu concentration is considerably < 1 μmol $g^{-1}$ soil, but in the vicinity of copper processing factories, this soil copper fraction can increase > 100 μmol $g^{-1}$ (Lepp *et al.*, 1997). An important aspect of the present experiment is that Cu sensitivity of both symbiotic partners was assessed in the same culture system. Cu toxicity was determined in a dose – response experiment using different parameters: growth of both symbiotic partners, P and Cu status of the seedlings. Biomass of the fungi, in the roots as well as in the substrate, had to be quantified indirectly. Therefore, two methods were used for the quantification (Ekblad *et al.*, 1998). Both the chitin and ergosterol assay gave similar results, which indicates that the proportion of active and inactive hyphae was hardly affected by the Cu treatments. The chitin : ergosterol ratio of our ECM fungi fell within the range observed in other ECM mycelia (Martin *et al.*, 1990).

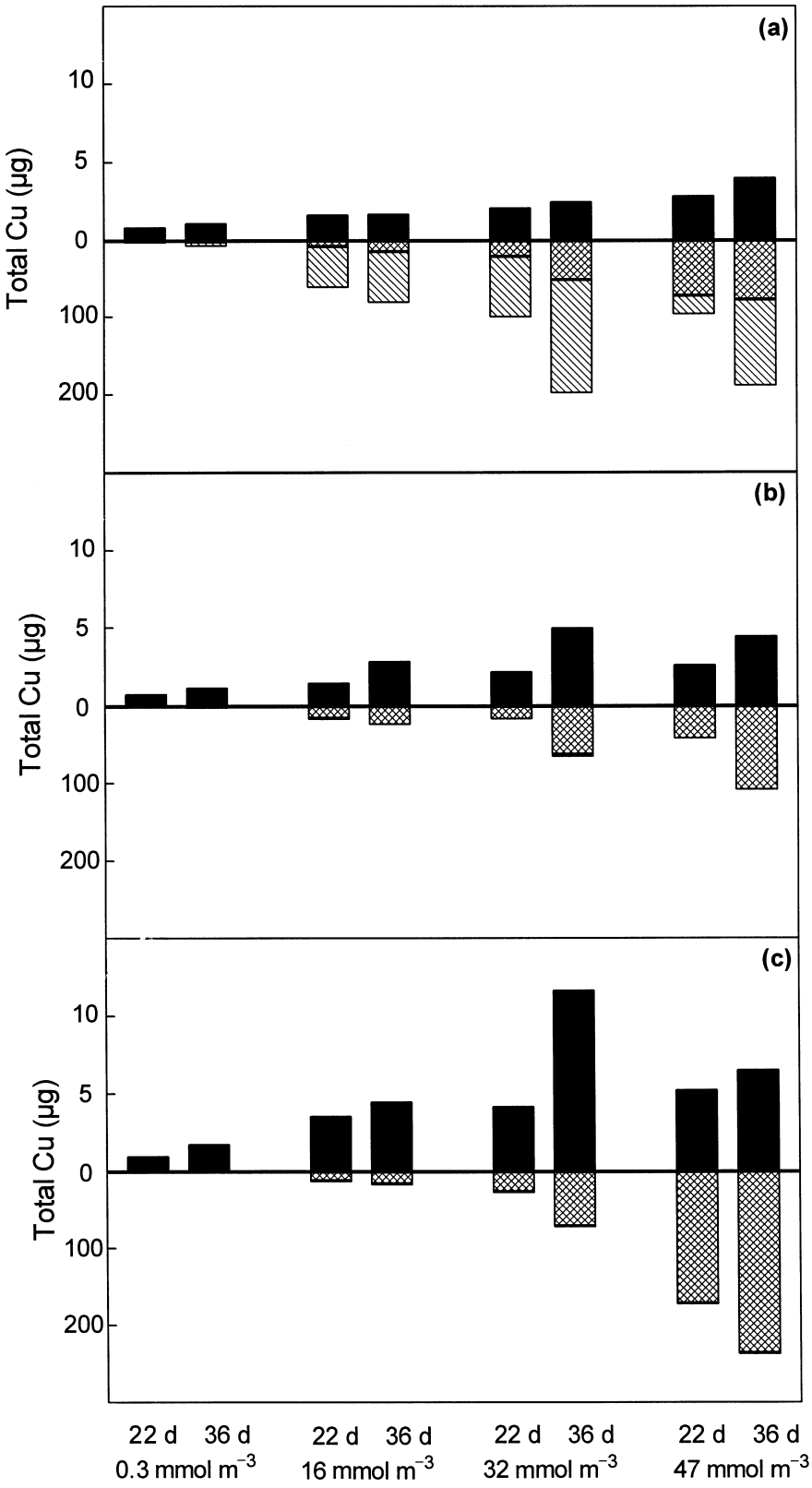


**Fig. 3** Partitioning of Cu between the different plant and fungal structures of *Pinus sylvestris* seedlings mycorrhizal with (a) *Suillus bovinus* (b) *Thelephora terrestris*, or (c) left nonmycorrhizal. Plants were exposed for 22 or 36 d to 4 different Cu concentrations: 0.3, 16, 32 or 47 mmol $m^{-3}$ Cu. Closed columns, shoots; crossed columns, roots; hatched columns, extraradical mycelium ($n$ = 3). Note difference in scale of *Y*-axis above and below *X*-axis.

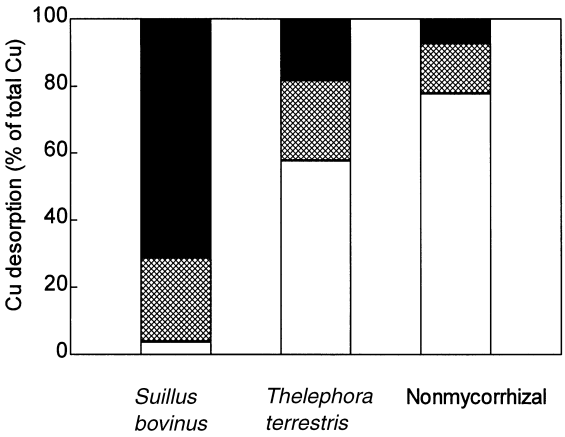


**Fig. 4** Desorbable and nondesorbable Cu fractions of mycorrhizas and NM root tips from the 32 mmol $m^{-3}$ Cu treatment. Open part of column, Cu desorbed with 5 mol $m^{-3}$ $Pb^{2+}$; crossed part, Cu desorbed with HCl (pH = 1); black part of column, Cu-non-desorbed by these solutions.

Recently, Tarhanen *et al.* (1999) successfully used the ergosterol assay to assess the growth of the fungal partner in the lichen *Bryoria fuscescens*, exposed to elevated Cu and Ni. In the present experiment, the fungal biomass in roots was not significantly affected by the Cu treatments. Reductions in biomass of the extraradical *S. bovinus* mycelium indicated that this part of the thallus was more sensitive to Cu stress than the less exposed mantle mycelium on the roots (Tables 1, 2).

A beneficial effect of ECM fungi on host plants exposed to toxic levels of heavy metals has been observed in several experiments (Jentschke & Godbold, 2000). However, there remains a lot of controversy whether ECM fungi can confer added metal resistance to their host trees. In many cases alleviation of metal toxicity was largely based on growth promotion through a better nutrition, and hence was merely a matter of dilution of metals in the host plant tissues (Jentschke *et al.*, 1999). Meharg & Cairney (2000) argue that ECM fungi merely fulfil their normal ecological function in polluted habitats and that most ECM fungi are as sensitive or even more sensitive than their hosts to metal contamination. The reduced transfer of metals to shoots of metal treated ECM plants, a phenomenon observed in many laboratory experiments (Jones & Hutchinson, 1986; Colpaert & Van Assche, 1992; Marschner *et al.*, 1996; Jentschke *et al.*, 1999) would only be active at moderate metal toxicity as long as the fungi survive. Meharg & Cairney (2000) further postulate that this reduced metal transfer aboveground in ECM hosts did not result in decreased metal toxicity. In many experiments, ECM plants did not outperform their NM counterparts at toxic metal levels when growth and nutrient status were considered.

The present experiment, however, shows that our *T. terrestris* and *S. bovinus* isolates are less sensitive to Cu than NM pine roots and it also demonstrated that both fungi could protect root growth and could decrease metal transfer to the host while maintaining P nutrition of the host. The presence of the fungi directly influenced Cu sensitivity of the host plants. A dilution effect in plant biomass was not present because the strictly controlled nutrient addition in the semihydroponics resulted in similar growth rates in ECM and NM plants (Colpaert & Verstuyft, 1999). In these circumstances mycorrhizal colonization may increase nutrient influx potential but not total nutrient assimilation in the host plant (Colpaert *et al.*, 1999).

The most striking result of the experiment was seen in the comparison of the Cu sensitivity of NM pine roots and the fungal symbionts. Despite the well known fungitoxic nature of Cu, the ECM fungi clearly were less sensitive than the NM roots. Both the ergosterol and chitin assay confirmed that fungal growth was still substantial in the 32 and 47 mmol $m^{-3}$ Cu treatment whereas the same Cu concentrations severely hampered or completely inhibited root growth of NM pine. Utriainen *et al.* (1997) found $EC_{50}$ values for root elongation in NM birch clones, originating from metal (Zn, Cu, Ni)-polluted soil, to lie between 8 and 30 mmol $m^{-3}$ Cu in hydroponics. Growth of our *T. terrestris* isolate was not affected at 47 mmol $m^{-3}$ Cu. In the *in vitro* test, no growth inhibition was observed for this isolate at 790 mmol $m^{-3}$ Cu, a concentration which is toxic to most ECM fungi *in vitro* (Hartley *et al.*, 1997). Jones & Muehlchen (1994) report that growth of their *T. terrestris* isolate was hardly affected in liquid cultures at 3200 mmol $m^{-3}$ Cu. These observations indicate that *T. terrestris* might possess a similar or even higher constitutive Cu resistance than *Hymenoscyphus ericae*. This ericoid mycobiont became inhibited *in vitro* at 1600 mmol $m^{-3}$ Cu (Bradley *et al.*, 1982), but it also conferred increased Cu resistance to its sensitive host plants (e.g. *Calluna vulgaris*). The apparently high Cu resistance of *T. terrestris* may explain the survival of this species on Cu polluted soils and it may also contribute to the success of this mycobiont in tree nurseries in which the application of Cu containing fungicides (e.g. copper oxychloride) is still common practice (Manninen *et al.*, 1998).

Our study shows that at least some ECM fungi exhibit a higher constitutive Cu tolerance than the NM roots of their host plant. This finding supports the theory of Wilkinson & Dickinson (1995) who suggested that metal-resistant mycorrhizal fungi may allow trees to colonize extreme environments such as metalliferous soils. So far, there is little evidence that the tree species themselves have adapted genetically to metal contaminants. Both *S. bovinus* and *T. terrestris* clearly protected root growth of their host plant and reduced transfer of Cu to aboveground plant parts. Such a protective effect against elevated Cu has not previously been observed in studies with other fungal symbionts (Jones & Hutchinson, 1986).

Cu toxicity is considered to result in oxidative stress. This process is marked by an increase in plasmalemma permeability caused mainly by non-selective conductance increases and

electrogenic pump inhibition. This process further leads to an imbalance and malfunctioning of transport processes localized in the plasmalemma (Demidchik *et al.*, 1997). We assume that at the higher Cu concentrations, membrane damage was high in NM roots. First, this resulted in a sharp increase in Cu influx and translocation to shoots (32 mmol $m^{-3}$), but eventually it led to a complete inhibition of root growth and Cu accumulation in roots (47 mmol $m^{-3}$). P and Cu were no longer transported to the shoots, probably due to a strongly reduced transpiration stream. Jentschke & Godbold (2000) warned that metal transfer to shoots is a complex process that is affected by transpiration, which in turn might be influenced by metal toxicity and fungal symbionts. The reduced transfer of Cu in needles of NM plants at 47 mmol $m^{-3}$ Cu (Fig. 4) is therefore misleading as it is probably due to a severely reduced transpiration stream. Despite the strong toxic effect of Cu on root development, shoot growth continued for some time in the NM plants although it was accompanied by a discoloration of the needles. Decreasing P concentrations in foliage (Table 3) explains the production of reddish foliage at the highest Cu concentration (Bergmann, 1992). It is also well known that root growth is more sensitive to Cu toxicity than shoot growth so that increases in shoot : root ratio are frequently found for plant seedlings exposed to elevated Cu (Jones & Hutchinson, 1986; Fernandes & Henriques, 1991; Bergmann, 1992).

After 36 d exposure to 16 and 32 mmol $m^{-3}$ Cu, total amounts of Cu transferred to shoots were significantly lower with *S. bovinus* than with *T. terrestris*. The strong retention of Cu in the extraradical mycelia of *S. bovinus* might contribute to the low transfer of Cu to the shoots of its host. NM plants had shoot Cu concentrations above 20 μg $g^{-1}$ d. wt, a critical level for Cu toxicity in pine needles (Balsberg Påhlsson, 1989). Metal sorption on fungal mycelia has often been postulated as a mechanism that restricts metal translocation to the host tissues (Jentschke & Godbold, 2000). Copper binding or sequestering in *H. ericae* was also proposed as a key phenomenon for the amelioration of Cu toxicity in the ericoid mycorrhizal symbiosis (Bradley *et al.*, 1981). The Cu concentration in the extraradical mycelium of *S. bovinus* amounted to 0.13 mmol $g^{-1}$ d. wt. ECM hyphae can adsorb large amounts of cations because of their very high CEC values (Browning & Hutchinson, 1991). Pb adsorption after exposure to 48 mmol $m^{-3}$ $Pb^{2+}$ was as high as 0.2–1.3 mmol $g^{-1}$ d. wt (Marschner *et al.*, 1998). The specific metal adsorbing capacity of mycelia is, however, highly species dependent and is largely affected by pH and other cations (Gadd, 1993). It was surprising that Cu could not be desorbed from the *S. bovinus* mycorrhizas suggesting that Cu is tightly sequestered in poorly soluble compounds or that it became largely enclosed in a hydrophobic fungal apoplast during mycorrhizal development. The production of oxalic acid by many fungi provides a means of immobilizing soluble metal ions as insoluble oxalates, decreasing availability and conferring resistance (Gadd, 1999; Ahonen-Jonnarth *et al.*, 2000). Copper oxalate is very insoluble and has been observed on hyphae growing on Cu treated wood. A reduced availability of Cu can also be obtained when ageing hyphae become more hydrophobic. A considerable portion of the *S. bovinus* mycelia has hydrophobic features, in contrast to the highly hydrophilic *T. terrestris* mycelia (Unestam & Sun, 1995). It is likely that $Cu^{2+}$ adsorbs on the newly formed, least hydrophobic, fungal cell walls of the *S. bovinus* mycorrhiza; during development of the mycorrhiza these cell walls become more hydrophobic and Cu becomes enclosed in the mantle. Hydrophobic cell walls may form a barrier between the fungal plasma membranes and the toxic soil solution. A similar immobilization mechanism is not possible with hydrophilic ECM fungi and explains why relatively more Cu ions can be desorbed from *Thelephora* mycorrhizas.

The excellent growth of *T. terrestris* in this experiment indicates that a high metal immobilization capacity is not an absolute prerequisite for a high constitutive Cu resistance of a mycorrhizal fungus at elevated Cu concentrations. The extraradical mycelia of *T. terrestris* contained far less Cu than those of *S. bovinus*. In addition, adsorbed copper could easily be desorbed by $Pb^{2+}$ and protons. The high Cu resistance of *Thelephora* might be realized by an exclusion mechanism operating in the membranes (reduced uptake, a Cu efflux mechanism). Copper exclusion from cells has been demonstrated for several eukaryotic microorganisms (Meharg & Cairney, 2000).

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