Copper-Adapted *Suillus luteus*, a Symbiotic Solution for Pines Colonizing Cu Mine Spoils

K. Adriaensen, T. Vrålstad, J.-P. Noben, J. Vangronsveld, and J. V. Colpaert

Hasselt University, Centre of Environmental Sciences, Environmental Biology Group, Agoralaan, Gebouw D, 3590 Diepenbeek, Belgium; Department of Biology, University of Oslo, P.O. Box 1066 Blindern, 0316 Oslo, Norway; and Hasselt University, Biomedical Research Institute, Agoralaan, Gebouw A, 3590 Diepenbeek, Belgium

Received 7 February 2005/Accepted 23 June 2005

Natural populations thriving in heavy-metal-contaminated ecosystems are often subjected to selective pressures for increased resistance to toxic metals. In the present study we describe a population of the ectomycorrhizal fungus *Suillus luteus* that colonized a toxic Cu mine spoil in Norway. We hypothesized that this population had developed adaptive Cu tolerance and was able to protect pine trees against Cu toxicity. We also tested for the existence of cotolerance to Cu and Zn in *S. luteus*. Isolates from Cu-polluted, Zn-polluted, and nonpolluted sites were grown in vitro on Cu- or Zn-supplemented medium. The Cu mine isolates exhibited high Cu tolerance, whereas the Zn-tolerant isolates were shown to be Cu sensitive, and vice versa. This indicates the evolution of metal-specific tolerance mechanisms is strongly triggered by the pollution in the local environment. Cotolerance does not occur in the *S. luteus* isolates studied. In a dose-response experiment, the Cu sensitivity of nonmycorrhizal *Pinus sylvestris* seedlings was compared to the sensitivity of mycorrhizal seedlings colonized either by a Cu-sensitive or Cu-tolerant *S. luteus* isolate. In nonmycorrhizal plants and plants colonized by the Cu-sensitive isolate, root growth and nutrient uptake were strongly inhibited under Cu stress conditions. In contrast, plants colonized by the Cu-tolerant isolate were hardly affected. The Cu-adapted *S. luteus* isolate provided excellent insurance against Cu toxicity in pine seedlings exposed to elevated Cu levels. Such a metal-adapted *S. luteus*-Pinus combination might be suitable for large-scale land reclamation at phytotoxic metalliferous and industrial sites.

Severe anthropogenic enrichment with heavy metals can cause a rapid change in the biotic communities living in and on a soil. Very few plant species are able to survive on or colonize highly polluted soils through fast selection of metal-tolerant ecotypes (2, 6). For most plants a rapid unprecedented change in the amount of available metals in a soil is likely to exceed their adaptive potential; this is especially true for tree species, which have long reproductive cycles (23). Since plants are sessile organisms with limited mechanisms for stress avoidance, they need flexible means for acclimation to changing environmental conditions. It has been suggested that trees can resist extreme environments through their great phenotypic plasticity and through their association with well-adapted ectomycorrhizal (ECM) fungi (30). Plant adaptation to selective pressures is often considered to be regulated by the plant genome, but it is likely that mutualistic microorganisms also can alleviate heavy metal toxicity in plants (1, 12).

Under natural conditions the majority of woody plants in temperate and boreal forests are associated with ECM fungi, and it seems that this mutualistic symbiosis persists on strongly metal-contaminated sites, which are slowly colonized by mycorrhizal tree species, such as birches, pines, and willows (17, 29). Although soil fungi (at least saprotrophs) seem to be more resistant to heavy metal contamination than soil bacteria (21), extreme conditions impose selection pressure that may trigger additional evolutionary adaptation in the fungal populations toward even greater resistance. Such genetic adaptation to toxic Zn concentrations has been described previously for ECM fungal populations of suillloid fungi thriving in pioneer pine forests that colonize sites severely contaminated by Zn smelters in northeast Belgium (7). In a dose-response experiment with pines as a host (1), we previously showed that a Zn-sensitive *Suillus bovinus* isolate developed poorly and failed to acquire sufficient nutrients when the mycorrhizal root system was exposed to Zn concentrations representative of the soluble Zn fraction present in the pore water of soils close to the Zn smelters. In contrast, a Zn-adapted fungal genotype hardly suffered from severe Zn stress. As a consequence, the better performance of this fungus significantly improved the nutrient status of its host.

In 2001, we discovered a *Suillus luteus* population which was associated with small pine trees that colonized mine waste heaps from a small abandoned Cu mine in central Norway. We hypothesized that this *S. luteus* population had developed adaptive Cu tolerance. In a first experiment, we compared the in vitro Cu tolerance of *S. luteus* isolates collected from the Cu-contaminated site, from a Zn-contaminated site, and from nonpolluted sites. The objective was to identify differential Cu sensitivity in the populations and to test whether there is any cotolerance for Cu and Zn in *S. luteus*. In a second experiment, the Cu sensitivity of nonmycorrhizal (NM) *Pinus sylvestris* seedlings was compared to the Cu sensitivity of mycorrhizal seedlings colonized by either a Cu-sensitive *S. luteus* isolate or a Cu-tolerant *S. luteus* isolate. This experiment was performed in order to explore (i) whether mycorrhizal pine seedlings performed better than NM seedlings when they were exposed to...
toxic Cu concentrations and (ii) whether a Cu-tolerant isolate offered better host plant protection than a Cu-sensitive isolate.

MATERIALS AND METHODS

Fungal material. In September 2001 fungal cultures were prepared from seven *S. lutes* (L.: Fr.) Roussel sporocarps collected in pioneer Scots pine forests in Norway at Hedmark, Follidal, Geitryggen on a Cu mine spoil. In September 2002 additional cultures were prepared from seven sporocarps collected from uncontaminated sites in Norway, including four sporocarps from Hedmark, Follidal, Vassaga, one sporocarp from Telemark, Kragere, Kureldala, and two sporocarps from Oslo, Østmarka. For the in vitro metal screening tests, we included 14 *S. lutes* isolates collected in Belgium. One half of these isolates were Zn tolerant and were collected in pioneer forests at severely Zn-contaminated sites in Lommel, Maatheide, and Lommel, Sahara; the other half were Zn sensitive and originated from an uncontaminated site in Paal (7, 9). To ensure that genetically different individuals were obtained, the minimum distance between sporocarps collected for isolation was 10 m. Genetic fingerprints confirmed that this distance is sufficient to avoid sampling of the same individuals of *S. lutes* in pioneer conditions (19).

Soil characteristics. Soil samples (~1 kg) from the mine spoil and from the Belgian collection sites were transferred to a greenhouse, and pore water samples were obtained with Rhizon soil moisture probes (Eijkelkamp Equipment, Giesbeek, The Netherlands) as described by Knight et al. (15). Pore water was analyzed to determine the pH and the Cu and Zn concentrations. The total metal in a 0.5-g soil sample was extracted in aque regia using microwave digestion (Milestone 1200 MEGA).

Fungal metal tolerance analysis. The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu tolerance was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%). The fungi were isolated, grown, and screened for Cu and Zn sensitivity in vitro as previously described (7). Cu sensitivity was measured using a growth inhibition test (90%).
The in vitro EC50s of the Belgian isolates and the isolates from the uncontaminated sites in Norway were reached at 0.3 mM Cu, whereas the average EC50 for the Cu mine spoil isolates was more than 4.8 mM Cu. On Zn-supplemented media, only the *S. luteus* population from the Zn-contaminated area in Belgium showed a high degree of Zn tolerance (Fig. 1, lower panel). An average EC50 of 19.5 mM Zn was determined for these isolates (7). The average EC50s of Zn were 2.8 mM for the Norwegian isolates from the Cu mine spoil and 2 mM for the Norwegian isolates from the uncontaminated sites.

Two *S. luteus* isolates were selected for the plant experiment. The EC50 of Cu for the Cu-sensitive *S. luteus* isolate (UH-Slu-Lsk10) was 0.25 mM, whereas the in vitro EC50 of Cu for the Cu-tolerant isolate (UH-Slu-Fg1.6) from the Folldal copper mine spoil was 4.6 mM. The EC50 of Zn was 2.2 mM for isolate UH-Slu-Fg1.6 but was more than 15 mM for the UH-Slu-Lsk10 isolate. Hence, the latter isolate originating from Lommel was highly Zn tolerant.

**Plant growth.** Copper toxicity gradually resulted in a severe reddish discoloration of needles in NM plants. This symptom was less pronounced in pines colonized by UH-Slu-Lsk10 and was not observed in pines colonized by UH-Slu-Fg1.6 (Fig. 2). In the absence of an elevated Cu concentration, there were no significant differences in shoot biomass between the inoculation treatments (Table 2), whereas the root biomass of NM plants was significantly higher than that of mycorrhizal plants. With increasing Cu concentrations, a significant decrease in aboveground biomass was observed in NM plants and in plants colonized by UH-Slu-Lsk10. Root growth was affected by Cu toxicity more than shoot growth (Table 2); in all inoculation treatments the root biomass was significantly reduced by 30 /H9262 M Cu. The toxic effect of Cu on root growth was greater in NM plants. This was also illustrated by the significant increase in the shoot/root ratio in NM plants with 30 and 60 /H9262 M Cu, whereas there was no such effect on the shoot/root ratio of mycorrhizal plants. Compared to the control Cu treatment, the total biomass of the NM plants was reduced by 76% with 60 /H9262 M Cu, the biomass of pines colonized by UH-Slu-Lsk10 was reduced by 41%, and the biomass of seedlings colonized by UH-Slu-Fg1.6 was reduced by only 23%.

**Fungal growth in symbiosis.** At the start of the Cu treatments, 5 weeks after inoculation, at least 50% of the short roots were mycorrhizal in the inoculated plants. Noninoculated

---

**TABLE 1.** Zn and Cu concentrations in soil samples collected at the study sites

<table>
<thead>
<tr>
<th>Site</th>
<th>Total Pore water</th>
<th>Cu concn (mg kg⁻¹)</th>
<th>Zn concn (mg kg⁻¹)</th>
<th>Cu concn (μM)</th>
<th>Zn concn (μM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folldal</td>
<td></td>
<td>1,692 ± 446&lt;sup&gt;b&lt;/sup&gt;</td>
<td>273 ± 52</td>
<td>226 ± 198</td>
<td>18 ± 16</td>
<td>3.5 ± 1.0</td>
</tr>
<tr>
<td>Lommel</td>
<td></td>
<td>54 ± 11</td>
<td>1,529 ± 297</td>
<td>&lt;1</td>
<td>120 ± 44</td>
<td>4.7 ± 1.0</td>
</tr>
<tr>
<td>Paal</td>
<td></td>
<td>11 ± 4</td>
<td>26 ± 4</td>
<td>&lt;1</td>
<td>1.8 ± 0.4</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Total contents (aqua regia extraction) and concentrations in soil pore water, as well as pH values, were determined.

<sup>b</sup> The values are means ± standard errors of the means (n = 3).
plants remained nonmycorrhizal throughout the experiment. Excess Cu caused minor changes in the active fungal biomass in roots colonized by UH-Slu-Fg1.6 (Table 2). However, the UH-Slu-Lsk10 isolate clearly suffered more from the Cu stress. The toxic effect of Cu was most obvious in the amount of active extraradical mycelium produced by this sensitive isolate. At 30 μM Cu there was a significant decrease, and at the highest Cu concentration the UH-Slu-Lsk10 isolate almost failed to form active extraradical mycelium. In contrast, the development of extraradical hyphae by UH-Slu-Fg1.6 was not affected by the Cu treatments (Table 2).

**Nutrient uptake capacities.** After 4 and 10 weeks of Cu treatment, P<sub>i</sub> and NH<sub>4</sub><sup>+</sup> uptake rates were calculated from the depletion of P<sub>i</sub> and NH<sub>4</sub><sup>+</sup> in the nutrient solution circulated through the plant containers for 5 h. P<sub>i</sub> uptake rates are shown in Fig. 3. In the absence of an elevated Cu concentration, there was a significant increase in P<sub>i</sub> uptake potential between weeks 4 and 10 as a result of the expanding (mycorrhizal) root system. The Cu treatments had a striking differential impact on the nutrient uptake of the plants. The uptake of P<sub>i</sub> was maintained much better in seedlings associated with the UH-Slu-Fg1.6 isolate (Fig. 3A). In contrast, relatively low Cu concentrations (15 μM) disturbed P<sub>i</sub> uptake in roots colonized by the UH-Slu-Lsk10 isolate (Fig. 3B). In any case, ECM seedlings took up P<sub>i</sub> more efficiently than NM seedlings (Fig. 3C). There was no significant difference between the sensitive plant-fungus association and NM plants only at the highest Cu concentration tested (60 μM). P<sub>i</sub> uptake in NM plants and plants inoculated with UH-Slu-Lsk10 decreased to almost zero by the end of the experiment, illustrating the ultimate failure of the nutrient uptake system (Fig. 3B and 3C). Similar data were obtained for NH<sub>4</sub><sup>+</sup> uptake (data not shown). Taking into account root biomass, specific uptake rates for P<sub>i</sub> and NH<sub>4</sub><sup>+</sup> could be calculated when plants were harvested after 10 weeks of Cu treatment (Fig. 4). The data obtained largely confirmed the different nutrient uptake capacities and thus Cu sensitivities of the *S. luteus* isolates.

**Cu accumulation in the plants.** Exposure to elevated Cu concentrations significantly increased the Cu accumulation in the aboveground parts of the plants (Fig. 5). Pine seedlings colonized with UH-Slu-Fg1.6 seemed to control Cu influx better than NM plants and plants colonized with UH-Slu-Lsk10, resulting in transfer of less Cu to the pine needles. For all treatments, the Cu concentrations in roots were considerably higher than Cu concentrations measured in shoots. In plants exposed to 60 μM Cu, the percentages of Cu translocated to the shoots were 0.8, 0.7, and 1.2% of the total Cu content for the mycorrhizal plants with the tolerant and sensitive isolates and the NM plants, respectively.

![FIG. 3. P<sub>i</sub> uptake rates at different Cu levels for pine seedlings that either were inoculated with a Cu-tolerant isolate (A) or a Cu-sensitive isolate (B) of *S. luteus* or were nonmycorrhizal (C). The P<sub>i</sub> uptake measurements were obtained 4 weeks (dotted lines) and 10 weeks (solid lines) after Cu addition was initiated. The error bars indicate standard errors of the means (n = 5).](image-url)
plants. The error bars indicate standard errors of the means (n/Cu-sensitive S. luteus with a Cu-tolerant elevated Cu concentrations for 10 weeks. Open bars, plants inoculated that may trigger adaptive Cu tolerance. Relatively few plants these extreme conditions also impose great selection pressure of soil microorganisms and plants colonize such habitats, but colonize the mine spoil (29). Especially Cu-resistant ecotypes low pH and high Cu toxicity, trees, shrubs, and grasses sparsely was probably due to oxidation of metal sulfides. Despite the high acidity and Cu concentration in the pore water of the Folldal mine spoil confirmed that this substrate is highly acidic and strongly contaminated with Cu (Table 1). The high acidity was probably due to oxidation of metal sulfides. Despite the low pH and high Cu toxicity, trees, shrubs, and grasses sparsely colonize the mine spoil (29). Especially Cu-resistant ecotypes of soil microorganisms and plants colonize such habitats, but these extreme conditions also impose great selection pressure that may trigger adaptive Cu tolerance. Relatively few plants seem to be able to evolve Cu-tolerant ecotypes (16, 22, 31). In tree species such genetic modifications are even less likely because of the long generation times (23, 30). Nevertheless, trees do colonize severely metal-contaminated sites. In the present study we demonstrated that the S. luteus population of the Folldal mine spoil has evolved adaptive Cu tolerance (Fig. 1, upper panel) that results in highly significant protection of pine seedlings against Cu toxicity. It is therefore likely that pine trees colonizing the Cu mine spoil are strongly assisted by their ECM associations with Cu-tolerant fungi inhabiting this toxic soil. S. luteus is a pioneer ECM bolete that specifically colonizes roots of Pinus spp. Zn-tolerant populations of this fungus have been discovered previously in Zn-contaminated soils (7).

In our in vitro screening experiment, all seven S. luteus isolates collected from the mine spoil exhibited high Cu tolerance (Fig. 1, upper panel), whereas Cu tolerance was not detected in populations from nonpolluted and Zn-polluted soils. However, the possibility that Cu tolerance was present at a low frequency in nonexposed populations could not be excluded. In previous reports, considerable inter- and intraspecific variation in response to excess Cu was observed in ECM fungi, but adaptive Cu tolerance was never discovered (5, 9, 14). It should be clear that the EC50s obtained in the in vitro study cannot be directly extrapolated to the natural soil environment. We assume that the rich nutrient composition of most in vitro media used to cultivate and test microorganisms, including ECM fungi, is a major factor that determines the upward shift of the toxicity range. The isolates grow much faster in vitro than in symbiosis (7).

The tests on Zn-supplemented media showed that the Cu mine isolates do not possess increased Zn tolerance (Fig. 1, lower panel). Similarly, Zn-tolerant isolates originating from Zn-contaminated sites do not exhibit any Cu tolerance (Fig. 1, upper panel), indicating that there was evolution of metalspecific tolerance mechanisms and that strong cotolerance was absent. The absence of cotolerance for Cu and Zn is supported by studies on the genetic basis of metal tolerance in bacteria and higher plants, which have shown that Cu tolerance and Zn tolerance are under the control of different genes (18, 22). The in vitro metal screening test allowed us to select two S. luteus isolates with contrasting Cu tolerance characteristics, and these isolates were subsequently used to inoculate pine seedlings. The copper sensitivity of myco- and phytobionts was determined in a dose-response experiment by analyzing several responses, including growth of the symbiotic partners, Cu accumulation, and the nutrient uptake potential of the seedlings. NM pines are very sensitive to elevated Cu concentrations, which result in fast inhibition of root growth (Table 2) and reduced nutrient uptake (Fig. 3C and 4), as well as a greater influx of Cu (Fig. 5). The average shoot concentration of Cu was 55 μg g (dry weight)−1 at the highest Cu concentration applied, a value that greatly exceeds the critical level for Cu toxicity in pine needles, which is estimated to be 20 μg g (dry weight)−1 (4). Nevertheless, most Cu was retained in the nonmycorrhizal root system. The strong belowground Cu retention and the very poor growth of nonmycorrhizal roots led us to believe that the root cells that are in direct contact with the Cu solution are the first target of excess Cu. Severe membrane damage in these cells results in an immediate disturbance in nutrient uptake. At the highest Cu concentration, the Cu/P
ratio in NM plants (1.4) was considerably higher than the ratios in mycorrhizal plants (0.4 for the UH-Slu-Fg1.6 isolate and 0.7 ratio in NM plants (1.4) was considerably higher than the ratios in other studies. A study performed by ADRIAENSEN ET AL. APPL. ENVIRON. MICROBIOL. showed that different metals in excellent insurance against Cu toxicity in pine seedlings exposed to Cu stress (28). In both studies, the ameliorating effect of the ECM fungi on host fitness could be ascribed to better plant nutrition and reduced uptake of Cu. Ecromycorrhizal fungi largely control the pathway of nutrients, whether in excess or at trace levels, from the soil to the plant. The fungal sheath may act as a barrier, either through its ability to transform soluble heavy metal forms into insoluble forms (17) or by preventing apoplastic radial transport of water and ions to the root surface and thus reducing metal influx (3, 10). A biofiltering pattern has been observed in S. luteus-P. sylvestris mycorrhizas by the use of elemental imaging techniques (25).

However, there is considerable variation in the extent of the fungal protective effect among the ECM fungi. In the present study, seedlings colonized by UH-Slu-Lsk10 were affected in nutrient uptake capacity more quickly than plants colonized by UH-Slu-Fg1.6 (Fig. 3 and 4), and this effect was apparent even before there was clear growth inhibition of UH-Slu-Lsk10. At the highest Cu concentrations development of the external mycelium in particular was very poor and was in sharp contrast to the good growth of UH-Slu-Fg1.6 (Table 2).

We concluded that the better growth and nutrient status of the plants associated with the tolerant fungal isolate strongly suggest that metal-adapted isolates of ECM fungi provide excellent insurance against Cu toxicity in pine seedlings exposed to elevated Cu concentrations. The existence of tolerance to different metals in S. luteus provides opportunities to breed isolates with combined metal tolerances. Such a heavy-metal-adapted Suillus-Pinus combination might be most suited for large-scale land reclamation at metalliferous and industrial sites that have little plant cover due to their phytotoxicity. Phytostabilization of vast areas of metal-contaminated soils or wastes that resulted from previous mining and smelting activities is urgently needed to reduce further dispersion of heavy metals. Phytostabilization, combined with in situ immobilization of heavy metals, is a low-cost option that can deal with this problem (27). Reforestation would be especially attractive for industrial and mining sites since forests reduce erosion, restrict the dispersal of metals, and are sinks for atmospheric carbon dioxide.

ACKNOWLEDGMENTS

We thank S. Poorters, C. Put, A. Wijgaerts, and F. Van Bellegheem for experimental and technical assistance. This research was supported by FWO-Vlaanderen (grant G0001.01) by the EU (MYCOREM grant QLK-1999-00897), by a postdoctoral grant to T.V. from the Norwegian Research Council (grant NFR-145324/V40), and by a grant from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) to K.A.

REFERENCES


