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Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd

Erik Krznaric [a], Nathalie Verbruggen [c], Jan H.L. Wevers [a], Robert Carleer [b], Jaco Vangronsveld [a], Jan V. Colpaert [a,*]

[a] Environmental Biology Group, Centre for Environmental Sciences, Hasselt University, Agoralaan, Gebouw D, 3590 Diepenbeek, Belgium
[b] Laboratory of Applied Chemistry, Centre for Environmental Sciences, Hasselt University, Agoralaan, Gebouw D, 3590 Diepenbeek, Belgium
[c] Laboratoire de Physiologie et de Génétique Moléculaire des Plantes, Université Libre de Bruxelles, Campus Plaine, CP242, Bd du Triomphe, 1050 Brussels, Belgium

The evolutionary adaptation for higher Cd tolerance in Suillus luteus, an ectomycorrhizal fungus, is of major importance for the amelioration of Cd toxicity in pine trees exposed to high Cd concentrations.

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Soil metal pollution can trigger evolutionary adaptation in soil-borne organisms. An *in vitro* screening test showed cadmium adaptation in populations of *Suillus luteus* (L.: Fr.) Roussel, an ectomycorrhizal fungus of pine trees. Cadmium stress was subsequently investigated in Scots pine (*Pinus sylvestris* L.) seedlings inoculated with a Cd-tolerant *S. luteus*, isolated from a heavy metal contaminated site, and compared to plants inoculated with a Cd-sensitive isolate from a non-polluted area. A dose–response experiment with mycorrhizal pines showed better plant protection by a Cd-adapted fungus: more fungal biomass and a higher nutrient uptake at high Cd exposure. In addition, less Cd was transferred to aboveground plant parts. Because of the key role of the ectomycorrhizal symbiosis for tree fitness, the evolution of Cd tolerance in an ectomycorrhizal partner such as *S. luteus* can be of major importance for the establishment of pine forests on Cd-contaminated soils.

1. Introduction

In many industrial areas heavy metals have accumulated in soils through anthropogenic activities. In most terrestrial ecosystems Cd has no known function as a nutrient; moreover, it is toxic at very low concentrations (Schützendübel and Polle, 2002). Cd accumulation in plants affects gene expression (Kovalchuk et al., 2005), inhibits DNA repair (Banerjee and Flores-Rozas, 2005), causes reductions in photosynthesis, diminishes water and nutrient uptake (Di Toppi and Gabbrielli, 1999), and results in visible symptoms of injury such as chlorosis, growth inhibition, browning of root tips, and finally death (Kahle, 1993). In needles of spruce seedlings (*Picea abies*) chlorophyll and water contents decrease in response to Cd, as well as CO_2 fixation (Schlegel et al., 1987).

The ecological success of many plant species depends to a large extent on fungal symbionts that improve nutrient acquisition and protect plants against various biotic and abiotic stresses (Selosse et al., 2004; Rodriguez and Redman, 2008). On metal contaminated soils, symbiotic micro-organisms may improve plant fitness through an improved nutrition and (or) through a reduced toxicity of the metals. In previous experiments it was demonstrated that tree species inoculated with ectomycorrhizal fungi performed better than non-mycorrhizal seedlings when exposed to toxic metal concentrations (Jentschke and Goldbold, 2000). However, the extent of this ameliorating effect of the mycorrhizal symbiosis depends on the fungal species and genotype. Several authors have suggested that particular ectomycorrhizal fungi can directly reduce toxicity of Cd through compartmentation, chelation and intra- and extracellular complexation mechanisms (Blaudez et al., 2000; Bellion et al., 2006, 2007). These mechanisms undoubtedly can reduce the toxic effects of Cd for the fungus. Nevertheless such mechanisms are probably present within many fungal species and it remains unclear to what extent they also avoid transfer of Cd to a host plant. Accumulation of Cd in vacuoles could greatly increase the transfer of Cd to the host plant because motile tubular vacuoles are an important vector in the transport chain of mineral nutrients from the site of uptake at hyphal tips to the exchange region in the mycorrhizal root (Ashford and Allaway, 2002).

An increase in the concentration of bio-available heavy metals in soil may result in severe toxicity which will ultimately trigger selection pressure for higher tolerance in exposed biota. Such adaptive metal tolerances have evolved in particular plants

* Corresponding author. Tel.: +32 (0)11 268304; fax: +32 (0)11 268301.
E-mail address: jan.colpaert@uhasselt.be (J.V. Colpaert).

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(Bradshaw and McNeilly, 1981; Clemens, 2006), soil bacteria (Mergeay et al., 2003) and mycorrhizal fungi (Colpaert et al., 2000). Zinc-tolerant ectomycorrhizal fungi have been found around zinc smelters, whereas copper-tolerant ones thrived on Cu mine spoils (Adriaensen et al., 2005). Naturally selected Cd tolerance was suggested for a *Suillus luteus* population (Colpaert et al., 2000); nevertheless adaptive Cd tolerance is a rare phenomenon in plants and their symbiotic partners (Roosens et al., 2003; Wu et al., 2007). In the present work the occurrence of adaptive Cd tolerance in *S. luteus* was further explored and the Cd accumulation pattern in Cd-tolerant and sensitive isolates was identified. The significance of the adaptive Cd tolerance for a host plant was studied in a dose–response experiment with Scots pine seedlings that were inoculated either with a Cd-tolerant or a non-tolerant *S. luteus* isolate. Mycorrhizal plants were exposed to five different Cd concentrations, from 0 to 20 μM Cd. We hypothesized that seedlings inoculated with a Cd-adapted fungus would perform better than with a non-adapted fungus at high external Cd. We therefore measured fungal growth, nutrient acquisition and Cd accumulation in the seedlings.

2. Materials and methods

2.1. Fungal material and study site

In Northern Limburg, acidic sandy soils were contaminated through atmospheric deposition of heavy metals from four non-ferro smelters (Colpaert et al., 2004). *Suillus luteus* (L.: Fr.) Roussel is an important ectomycorrhizal fungus that grows in pioneer pine forests growing on the sandy soils. Forty-eight *S. luteus* isolates belonging to six populations were screened for Cd tolerance *in vitro*. Half of the isolates were collected from Lommel-Maatheide (Lm), Lommel-Sahara (Ls) or Neerpelt (N), all sites severely contaminated by a mixture of metals: Zn, Cd and Pb. The other half of isolates were from control sites with low levels of pollution: Maasmechelen (Mm), Paal (P) or Meeuwen-Gruitrode (MG). The three populations from polluted soil were approximately 0.5 (Lm), 1.2 (N) or 1.4 km (Ls) separated from a metal smelter; the control sites were at a distance of 12.4 (Mm), 16.6 (P) or 21.4 km (MG). More details on the collection sites and *Suillus* populations are provided in Colpaert et al. (2004). We previously showed that the Lm, N and Ls isolates were Zn-tolerant whereas the strains from the control sites were all Zn-sensitive. Fungal cultures were established from basidiocarps collected in 2000, 2001 or 2002. Cadmium tolerance was tested on solid modified Fries medium (Colpaert et al., 2004) supplemented with 0, 9, 18, 45, 90 or 180 μM Cd, through addition of $3CdSO_4 \cdot 8H_2O$ to the medium. The pH of the final media was adjusted to 4.5, the Zn concentration to 150 μM. For each isolate-treatment combination, there were three replicates. Mycelia were incubated at 23 °C in darkness and were harvested after 10 days of exposure. Mycelia were frozen at −80 °C, and subsequently freeze-dried before weighing. The Cd tolerance index was calculated for each isolate as the percentage of biomass retained on the Cd-enriched media as compared to growth on the control Fries medium. The EC_{50} concentration (Cd concentration which inhibits growth by 50%) was determined for each isolate.

Five Cd-tolerant and five sensitive isolates were analysed for Cd content. Freeze-dried mycelia were wet digested with concentrated acid (15.7 M HNO_3 /12.1 M HCl) and the concentration of Cd was determined with ICP-OES (inductively coupled plasma optical emission spectroscopy). The analyses were performed on triplicate samples and certified reference material was included: spinach leaves (Standard reference material® 1570a, National Institute of Standards & Technology, Gaithersburg, USA).

Cadmium pollution at the collection sites was assessed by analyzing Cd in soil pore water. At each study site, 4–6 soil samples (to a depth of 20 cm) of about 2 kg each were collected beneath *Suillus* basidiocarps. Soils were transferred to large pots and incubated in a greenhouse under moist conditions. Pore water was sucked 4 weeks later with Rhizon soil moisture samplers (Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands) according to the procedure described in Knight et al. (1998). The pH of the pore water was measured and Cd was subsequently analysed with AAS.

2.2. Inoculation of the host

For the *in symbiosis* experiments, fungi were inoculated on 6-week-old pine seedlings. Surface sterilized seeds were sown in perlite, moistened with a balanced nutrient solution (mass ratios: 100 N/9 P/54 K/6 Ca/6 Mg/9 S + micronutrients) for *Pinus sylvestris* (Ingestad and Kähr, 1985; Colpaert and Verstuyft, 1999). Uniform seedlings (fresh weight) were inoculated using a sandwich technique. The root system of pine seedlings were brought into close contact with fresh, actively growing mycelia grown on an agar medium covered with cellophane (Van Tichelen and Colpaert, 2000). In this way the root system was inoculated quickly and evenly. The pine seedlings were inoculated with a Cd-tolerant isolate, UH-Slu-Lsc4, or a non-tolerant isolate UH-Slu-P13. After inoculation, pines were transplanted in 150 ml containers filled with perlite. They were grown in a semi-hydroponic system in which nutrient supply is tuned to nutrient uptake by plants. Nutrients were added every other day to obtain a relative growth rate of approximately 3% day^{-1}. The pH of the solution was adjusted to 5. Plants were grown in a growth chamber with 250 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic active radiation, relative air humidity of 70%, and with a day/night rhythm of 18/6 h and a temperature of 22/15 °C. After approximately 4 weeks roots and perlite were well colonized with mycelia and Cd treatments were started.

2.3. Cd treatment

For the Cd treatments, nutrient solution was enriched with different concentrations of Cd, added as $3CdSO_4 \cdot 8H_2O$. Five levels were applied: 0, 1, 5, 10 and 20 μM Cd. This Cd range is environmentally relevant (Ernst et al., 2008) and the higher part of the range is known to cause stress and toxicity in tree seedlings (Schlegel et al., 1987; Balsberg Påhlsson, 1989). In addition to the regular application of nutrient solution, plant containers were flushed every two weeks with a large volume (200 ml) of treatment solution to maintain the appropriate Cd level. There were five replicas for each metal-inoculation treatment. The treatments lasted for 8 weeks; halfway through this period phosphate uptake capacity of each plant was measured in a non-destructive way. At the start of the treatments five plants of each plant–fungus combination were harvested in order to determine the initial weight of the plants and to allow calculation of the relative growth rate of both plants and fungi during the Cd exposure period.

2.4. Nutrient uptake capacity under Cd stress

Nutrient uptake of all individual plants was measured 4 and 8 weeks after the start of the Cd treatment. The depletion of ammonium and phosphate was determined in a nutrient solution (100 ml) that circulated during 5 h through the plant containers. The measurements were non-destructive as the integrity of the fungus–root system was maintained (Colpaert et al., 1999) and they reflected the proper functioning of the mycorrhizal symbiosis. Reductions in nutrient uptake capacity of ectomycorrhizal root systems are an early diagnostic sign of metal stress (Van Tichelen et al., 1999; Adriaensen et al., 2004). The concentrations of ammonium and phosphate were determined colorimetrically with a flow injection analyser (FIA). Uptake rates were calculated from individual depletion curves between 20 and 30 μM external phosphate and between 200 and 300 μM external ammonium.

2.5. Harvest and analyses

After 8 weeks of Cd treatment, plants were harvested. Subsamples of root and perlite were frozen in liquid N_2 in order to determine fungal biomass. The ergosterol concentration was used for estimation of active fungal biomass. Ergosterol was extracted from freeze-dried roots and perlite. Quantification was performed with HPLC (Nylund and Wallander, 1992). Plant material (shoot, stem, roots) was dried at 70 °C, weighed and subsequently pulverized to powder. Relative growth rates (RGR) of plants and fungi were calculated (Hoffmann and Poorter, 2002). Subsamples of pulverized plant material were destroyed in concentrated acid (HNO_3/HCl). The concentration of Cd was determined with ICP-OES.

2.6. Statistical analysis

Results are controlled for significance with one-way or multi-way analysis of variance (ANOVA) when the influence of one or more predictor variables on the response variable is assessed, after checking the necessary assumptions for those tests (homogeneity of variance, normal distribution of residuals). When the null hypothesis is rejected ($\alpha = 0.05$), the differences between the groups are tested with post-hoc Tukey–Kramer tests.

3. Results

3.1. Cd pollution and Cd tolerance in S. luteus

Heavy metal pollution of the soils that harboured the *S. luteus* isolates is shown in Table 1. The Cd concentration in the pore water from the control sites was lower than 0.1 μM and the pH was 4.2 on average. Pore waters in the polluted area reached values up to 10 μM Cd, the pH averaged on 4.7. In the pore water from the most polluted soil, Lommel-Maatheide (Lm), Cd ranged from 1.0 to 10 μM; in Neerpelt (N) and Lommel-Sahara (Ls) it varied from 0.3 to 1.8 μM Cd.