Heavy metal localisation in mycorrhizas of *Epipactis atrorubens* (Hoffm.) Besser (Orchidaceae) from zinc mine tailings

A. Jurkiewicz 1, K. Turnau 1,* J. Mesjasz-Przybyłowicz 2, W. Przybyłowicz 2,** and B. Godzik 3

Institute of Botany, Jagiellonian University, Krakow, 2 Materials Research Group, National Accelerator Centre, Faure, and 3 Institute of Botany, Polish Academy of Sciences, Krakow

Received April 10, 2001
Accepted July 5, 2001

Summary. The metal distribution within mycorrhizal and non-mycorrhizal roots of *Epipactis atrorubens* collected from zinc mine tailings and an area rich in heavy metal ores (both located in southern Poland) was investigated. The tailings, consisting of post-flotation material, were characterised by high levels of toxic elements such as Zn, Pb, and Cd, while soil outside the tailings was also strongly enriched in heavy metals. Atomic absorption spectrometry and proton-induced X-ray emission analysis revealed that heavy metals were mostly accumulated within orchid roots. Elemental maps from proton-induced X-ray emission showed that plant root epidermis and fungal coils which had developed within cortical cells of roots collected from the zinc mine tailings were the main places of Zn and Pb accumulation, associated with increased concentrations of Fe, Cd, Ti, Mn, Si, Ca, and S. The mean content of Pb and Zn in the coils was 4 to 5 times higher than in the root epidermis. In mycorrhizal roots from the tailings a statistically significant decrease in Pb and Zn content towards the inside of the root was observed. The mean content of Pb in coils from roots of plants growing outside the tailings was about 1% of the concentration in root coils from the tailings. Coils selected from orchid roots originating from a site outside the tailings contained comparatively high concentrations of Zn, Cd, and Cu, which was probably due to the high content of these elements in the soil. The results presented suggest a biofiltering effect against heavy metals by orchid mycorrhizal fungi.

Keywords: Orchid mycorrhiza; Heavy metal; Detoxification; Industrial mine tailing; Proton-induced X-ray emission; X-ray microanalysis.

Introduction

Natural processes as well as several anthropogenic factors strongly affect ecosystem stability and plant, animal and fungal populations, often leading to the extinction of the more vulnerable species (Smith et al. 1993, Lawton and May 1995). Orchids are generally considered as becoming rare or seriously endangered (Zajac and Zajac 1997), although certain species become more common (Arditti 1992). Recently, abundant populations of *Epipactis atrorubens* (Hoffm.) Besser, *E. helleborine* (L.) Crantz, and *Dactylorhiza majalis* (Rchb.) P. F. Hunt & Summerh. were observed on several mine tailings in southern Poland. These tailings were created as a by-product of the extraction of Zn and Pb from ore-rich, metalliferous dolomite rock material. They are poor plant growth media and contain high levels of heavy metals (Turnau 1998; Turnau et al. 1996, 2001). Heavy metal-rich areas have already been exploited as an important source of metal-resistant fungal strains. Ectomycorrhizal and arbuscular mycorrhizal fungi isolated from such places may be used in restoration practices, since both have been found to alleviate metal toxicity to their symbionts (Leyval et al. 1997, Hildebrandt et al. 1999, Jentschke et al. 2000). Properly selected cultivars and fungal associates could be an efficient tool in site decontamination (Hildebrandt et al. 1999, Ernst 2000). The use of mycorrhizal orchids in restoration practices would be rather impractical; however, research on this kind of symbiosis might improve our knowledge of the mechanisms allowing these plants to survive under stressful conditions. Fungi isolated from polluted areas might be used to inoculate endangered orchid species, which are suffering from anthropogenic impact. The main aim of this study was to find whether the presence of fungal mycelium affects heavy metal distribu-
tion within orchid roots. *Epipactis atrorubens*, occurring naturally on a number of sites in southern Poland (mostly mixed or beech forests) was chosen to compare the localisation of heavy metals within mycorrhizal and nonmycorrhizal roots of orchids collected from zinc mine tailings and from an area located some distance away from the tailings.

The study was started by preliminary investigations carried out with rhodizonate staining (Turnau 1998), which is a simple method used to select plant tissues containing lead (Wierzbicka 1987). Detailed microanalyses were performed with the nuclear microprobe, employing the combination of proton-induced X-ray emission (PIXE) and proton backscattering (BS) (Przybyłowicz et al. 1999). This method allows for quantitative assessment of the distribution of several elements in biological material.

**Material and methods**

*Epipactis atrorubens* specimens originated from three sites: a 40-year-old zinc tailings (silt loam type) located in Chrzanów (southern Poland), planted with 15-year-old pines (*Pinus sylvestris* L.) and birches; a mixed forest developed on coarse sandy soil in Borowiec (southern Poland) about 30 km from the tailings, this site being rich in heavy metals due to the presence of a below-ground zinc and lead ore bed; a nonpolluted site characterised by low heavy metal content, with soil of medium-heavy loam type, located in the Pieniny Mts. (Table 1). Due to legal restrictions only a few specimens from natural stands were collected, depending on the size of the population, while 20 specimens were taken from the tailings.

**Rhodizonate test**

Specimens of *E. atrorubens* were transported from the collection sites within soil samples. The roots were washed in distilled water and selected for the investigation on the basis of the presence of fungal coils. Mycorrhizas were soaked in the staining solution containing 2 g of potassium rhodizonate (Sigma) per liter in tartaric acid-sodium bitartrate buffer (15 g of tartaric acid and 19 g of sodium bitartrate per liter in distilled water; pH 2.8) for 5 h, washed with buffer, and observed with a dissecting microscope. In lead-containing tissues, characteristic purple precipitates of basic lead rhodizonate were formed (Garty and Theiss 1990).

**Atomic absorption spectrometry of soil and plant material**

Bulk soil samples (each consisting of 5 pooled subsamples) were collected from a depth of 0–10 cm. The plant material was dried to constant weight at 85 °C and weighed with an accuracy of 0.01 g. The dried and ground plant material was subsequently mineralised in a 4 : 1 mixture of ultrapure concentrated HNO₃ and HClO₄ (Merck) (Pinta 1977, Grodzifiska 1978), and the soil in HClO₄ (Merck). The dissolved fraction of metal content was measured in 1 M Ca(NO₃)₂ (Weissenhorn et al. 1995). The total concentration of heavy metals was determined by atomic absorption spectrometry (Varian 20BO), P by the molybdate-vanadate method, N by the Kjeldahl method, and organic matter by the Tiurin method.

**Structural observations by scanning electron microscopy**

Structural observations of mycorrhizas were carried out with a JEOL JSM-3400 scanning electron microscope on root samples fixed for 1 h in 3% glutaraldehyde in 0.2 M HEPES buffer, dehydrated in ethanol series, critical-point dried, and after longitudinal sectioning mounted on carbon stubs and coated with carbon and gold.

**Nuclear microprobe analysis**

Roots were cryofixed in isopentane cooled with liquid nitrogen, freeze-dried in an Edwards ETD4 tissue dryer, and cut into ca. 300 μm thick sections. Some fungal coils were removed from the sections and analysed separately for technical reasons. Oxalate crystals were analysed in situ. The material was placed between carbon-coated Formvar films spread on Al frames and coated with carbon. Two-dimensional PIXE maps of elemental distribution were obtained with the nuclear microprobes of the National Accelerator Centre in Faure (South Africa) (Przybyłowicz et al. 1999). A proton beam of 3.0 MeV energy and current of ca. 100 pA was used, focused to an area of 3 by 3 μm, and raster scanned over regions of interest. An external 125 μm Be absorber was interposed between the PXE Si(Li) detector and the specimen to shield the detector from back-scattered protons and to attenuate X-rays from major, light elements. The size of scanned areas was usually 1.5 by 1.5 mm in the case of general maps of the whole sections. Smaller regions of particular interest (such as fungal coils and oxalate crystals) were also analysed, with sizes ranging from 150 by 150 μm to 40 by 40 μm. The GeoPIXE suite of programs (Ryan et al. 1990a, b) was used for the analysis of PIXE spectra and to accumulate on-line elemental maps with Dynamic Analysis (Ryan and Jamieson 1993, Ryan et al. 1995).

**Table 1. Soil chemical characteristics**

<table>
<thead>
<tr>
<th>Stand</th>
<th>Mean concentration ± SD or percentage of soil component:</th>
<th>pH (H₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pb (mg/kg)</td>
<td>Zn (mg/kg)</td>
</tr>
<tr>
<td></td>
<td>total</td>
<td>available</td>
</tr>
<tr>
<td>Pieniny Mts.</td>
<td>52.3 ± 5.8</td>
<td>nd</td>
</tr>
<tr>
<td>Borowiec</td>
<td>102.4 ± 9.6</td>
<td>0.1 ± 0.2</td>
</tr>
<tr>
<td>Zinc waste in</td>
<td>15976 ± 10920</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>Chrzanów</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Not determined*