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ORIGINAL PAPER

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# Influence of restoration on arbuscular mycorrhiza of *Biscutella laevigata* L. (Brassicaceae) and *Plantago lanceolata* L. (Plantaginaceae) from calamine spoil mounds

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**Abstract** The arbuscular mycorrhizal status of two plant species, *Biscutella laevigata* L. and *Plantago lanceolata* L., was investigated on calamine spoil mounds in Bolesław (southern Poland). Although *B. laevigata* is a member of the Brassicaceae, a family generally accepted as non-mycorrhizal, this species formed AM symbioses on both heavy metal-contaminated and non-contaminated sites. Besides vesicles and coils, arbuscules were also observed, especially in roots collected prior to seed maturity. Relative mycorrhizal root length and relative arbuscular richness were usually much higher in *P. lanceolata* than in *B. laevigata* but not absolute arbuscule richness. Roots of *P. lanceolata* showed higher colonisation than *B. laevigata*. Although roots were collected from plants in close proximity, no correlation in mycorrhizal parameters was found between the two species.

**Keywords** Brassicaceae · Plantaginaceae · Arbuscular mycorrhiza · Restoration · Heavy metal toxicity

## Introduction

Strip mining to recover metallic ores destroys vegetation, alters microbial communities and results in bare areas with a compact, nutrient-poor substratum and reduced water infiltration (Grunwald et al. 1988). On a calamine mound located in Bolesław near Olkusz (southern Poland), non-mycorrhizal and mycorrhizal plants have formed a community typical of metal-rich soils (Dobrzańska 1955; Szafer and Zarzycki 1972; Pawłowska et al. 1996). Plants from this site are characterised by a high concentration of heavy metals in their tissues, dwarfism, xeromorphism, plagiotrophism, stronger development of underground than aboveground parts and prolonged flowering time (Dobrzańska 1955). The introduction of trees on part of the spoil (restored area) has speeded up the succession of vegetation and resulted in an increase in population size of some less-interesting plant species; plants of particular floristic value have decreased seriously. No differences in plant appearance were apparent between the parts of the zinc waste with and without trees. *Biscutella laevigata*, a member of the Brassicaceae and of particular floristic value, is considered to be a relict of a glacial flora and in Poland grows only in the region of calamine wastes and in the Tatra mountains. Its rarity was the main reason to include part of the calamine mounds in an area under protection (considered as ecological land), excluded from restoration practices (decree XXIII/196/97 of the Bolesław Commune Council).

The biology and ecology of this species has been the subject of intensive study in the area of Polish calamines (Skalińska 1949–1950; Dobrzańska 1955; Godzik 1991, 1993; Wierzbicka and Obidzińska 1998; Wierzbicka 1999). *B. laevigata* was also included in an investigation of plant communities developing on calcareous rocks of the Tatra mountains and listed as a mycorrhizal species of the thamniscophagic (containing arbuscules) type (Dominik et al. 1954). In another investigation on the mycorrhizal status of plants colonising the calamine mounds (Pawłowska et al. 1996), *B. laevigata* was found to be colonised by mycelium forming vesicles; however, arbuscules as a criterion of a functional mycorrhiza were not observed. Detailed studies of the mycorrhizal status of *B. laevigata* and the possible influence of restoration on mycorrhizal colonisation were the main objectives of the present investigation. *Plantago lanceolata* was selected for purposes of comparison and also as a plant whose mycorrhiza could be used potentially to monitor

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**Table 1** Mean concentration of elements (mg $kg^{-1}$ ± SD) and pH in calamine waste substrata (*A*, *B*) and reference areas (*Rb*, *Rp*) (*A* non-restored part; *B* restored part, *E* exchangeable content determined in 1 M $NH_4NO_3$, *nd* not determined, *Rb* reference site for *Biscutella laevigata*, *Rp* reference site for *Plantago lanceolata*, *T* total content)

| Site | Cd | | Pb | | Zn | | P | N | C | pH |
|---|---|---|---|---|---|---|---|---|---|---|
| | T | E | T | E | T | E | | | | |
| A | 183±33 | 6.51±2.64 | 3050±740 | <0.1 | 48500±13200 | 17.7±3.7 | 640±210 | 2310±1020 | 15240±3560 | 7.55 |
| B | 196±54 | 5.65±2.97 | 3000±1040 | <0.1 | 53230±20030 | 19.1±7.0 | 700±190 | 5770±3380 | 29430±15230 | 7.42 |
| Rb | 1.46±0.47 | <0.01 | 520±470 | <0.1 | 160±40 | 0.83±0.34 | 450± 80 | 3500±160 | 30790±8600 | 7.39 |
| Rp | 1.56±0.33 | <0.01 | 50±10 | <0.1 | 110±20 | 0.32±0.15 | nd | 2580±460 | 10110±320 | 7.33 |

changes occurring during the succession. This investigation was part of a broad research project on element uptake by plants of the calamine wastes in Bolesław.

## Materials and methods

### Site characterisation

The investigations were carried out on approximately 100-year-old mine wastes located on the western border of the Cracow-Częstochowa Jurassic formation (50°17′52′′, 19°28′35′′). Triassic oolitic limestones and metalliferous dolomites (containing 8–13% total Ca) are the main components of a non-differentiated profile resembling initial soils, classified as silt loam and composed of 77% stones (>1 mm). The mechanical composition of the <1 mm fraction was as follows: 1–0.1 mm 30%; 0.1–0.05 mm 7%; 0.05–0.02 mm 44%; 0.02–0.006 mm 9%; 0.006–0.002 6%; <0.002 4%. Soil characteristics of the two parts of the waste did not differ significantly (data not shown). The non-restored area is inhabited by plants such as *Armeria maritima* (Mill.) Willd. subsp. *halleri* (Wallr.) Á. Löve & D. Löve, the only species tied to soils rich in heavy metals (Szafer 1959), *Biscutella laevigata* L., *Silene vulgaris* (Moench) Garcke, *Reseda lutea* L., *Gypsophila fastigiata* L., *Erysimum odoratum* Ehrh. and *Cerastium arvense* L. (Grodzińska et al. 2001). Scattered, dwarfed *Pinus sylvestris* L. and *Betula pendula* Roth are also present. The restored part, which was scarified and planted with *Pinus sylvestris* and *Betula pendula* seedlings 30 years ago, is of sward type dominated by grasses and accompanied by the perennial herbs *Plantago lanceolata* L. and *Biscutella laevigata* L. scattered mostly on the edges. Table 1 summarises some of the properties of the wastes. The area is under pressure from industrial pollutants produced by a metallurgical plant in Bukowno and the Upper Silesian Industrial Centre (Godzik 1993). The Jaworzynka Valley in the Western Tatra Mountains, which is triassic dolomitic limestone (shallow rendzina) and where *B. laevigata* grows close to a tourist trail 1,100 m above sea level (19°53′40′′, 49°15′30′′), was selected as a reference area. The reference area for *P. lanceolata* was localised in the calcareous area of Skałki Twardowskiego (near Cracow) and is a close match in Ca content to the calcareous wastes.

### Material sampling and chemical analysis

Soil samples and plant material were collected mainly during the flowering period and early seed formation of *B. laevigata*, on 25 May 2000 at the calamine wastes site and 10 July 2000 in the Tatra Mountains. *P. lanceolata* specimens from the calamine wastes were collected at the same time and from the same places as *B. laevigata*. The two species were at a similar vegetation stage. Ten samples of each species, each composed of 3–5 specimens, were collected from approximately 2-$m^2$ plots from the non-restored, the restored mounds and the reference areas. Additional samples of *B. laevigata* were taken from the zinc waste on 20 April, 5 June, 4 July, 25 July, 5 October and 21 November 2000, when only 3–5 plants were collected to avoid drastic depletion of the population. A total of more than 100 specimens of *B. laevigata* from the zinc wastes and almost 40 specimens from the reference area were collected and analysed. At each collection site, bulk soil samples (each consisting of 3–5 pooled subsamples) were collected from the 0- to 10-cm-deep layer (Table 1). Below this layer, the substratum was composed of solid rock. The finest roots of *B. laevigata* and a sample of roots of *P. lanceolata* were collected for mycorrhizal investigation. The remaining plant material was washed with redistilled water, divided into underground and aboveground parts and dried to constant weight at 85°C. The dried and milled plant material was subsequently mineralised in a 4:1 mixture of ultrapure concentrated $HNO_3$ and $HClO_4$ (Merck) (Pinta 1977; Grodzińska 1978) and the soil in $HClO_4$. The exchangeable metal fraction was extracted in 1 M $NH_4NO_3$ (Carter 1993). Heavy metals were determined by atomic adsorption spectrometry (Varian 20BQ), P using the molybdate-vanadate method, N by the Kjeldahl method and organic matter by the Tiurin method (Nowosielski 1968).

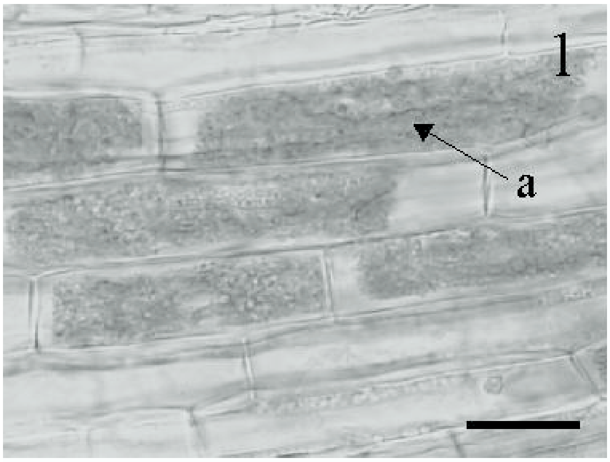
### Mycorrhizal studies

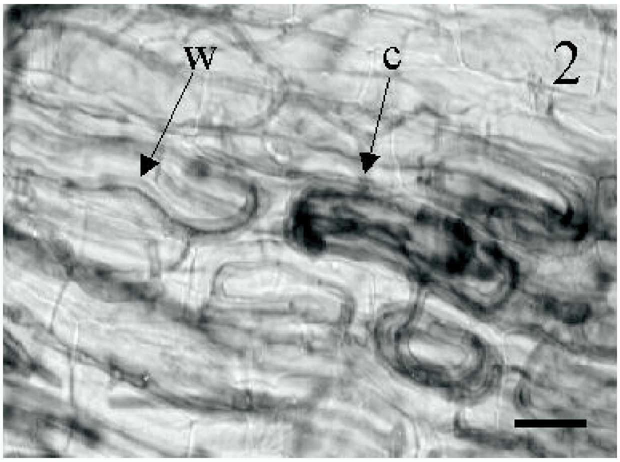
Mycorrhizal research was carried out on roots of *B. laevigata* and *P. lanceolata* collected on five occasions during the vegetation period. Only fine roots attached to the main roots were used to avoid the possibility of collecting roots from another species. *P. lanceolata* specimens for analysis of mycorrhizal parameters were collected at corresponding sampling dates. For the estimation of mycorrhizal colonisation, the roots were prepared according to the modified method of Phillips and Hayman (1970). After careful washing with tap water, the roots were softened in 10% KOH for 24 h, washed in water, bleached in a 10:1 mixture of 30% $H_2O_2$ and 25% $NH_4OH$ for a few minutes, washed again in water, acidified in 5% lactic acid in water for 1–24 h, stained with 0.01% aniline blue in 5% lactic acid for 24 h at room temperature and eventually stored in 5% lactic acid. Mycorrhizal frequency (F%), relative mycorrhizal root length (M%), intensity of colonisation within individual mycorrhizal roots (m%), relative arbuscular richness (A%) and arbuscule richness in root fragments where the arbuscules were present (a%) were assessed (Trouvelot et al. 1986). The roots were also observed with a JEOL JSM-5400 scanning electron microscope. In the case of *B. laevigata*, the samples were stained with aniline blue as described above, lyophilised, sectioned with a razor blade, mounted on carbon stubs and coated with carbon and gold.

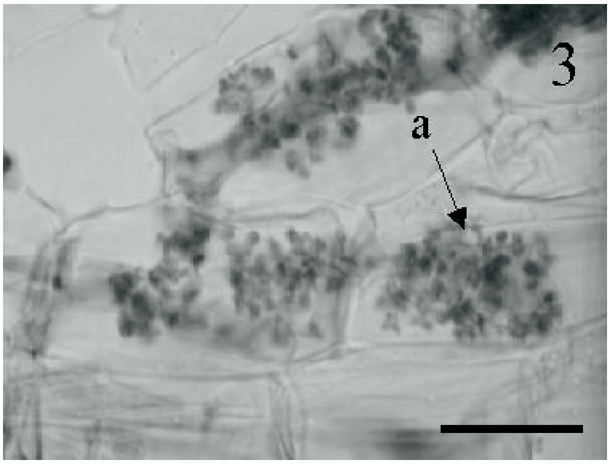
Soil samples from the restored and the non-restored mine waste were also used for estimation of the most probable number of AM propagules according to Adelman and Morton (1986). For this, five bulk soil samples, each consisting of five subsamples taken from 0–10 cm depth and collected at five random points chosen from an area of approximately 1-$m^2$, were collected from both parts of the zinc waste in Bolesław in September 2000. The soil was transported from the waste in plastic bags, sieved through a 3-mm sieve and stored at 4°C for up to 1 week before use.

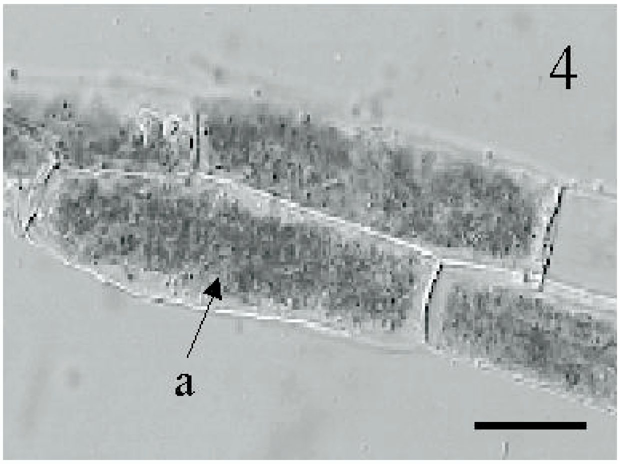
### Statistics

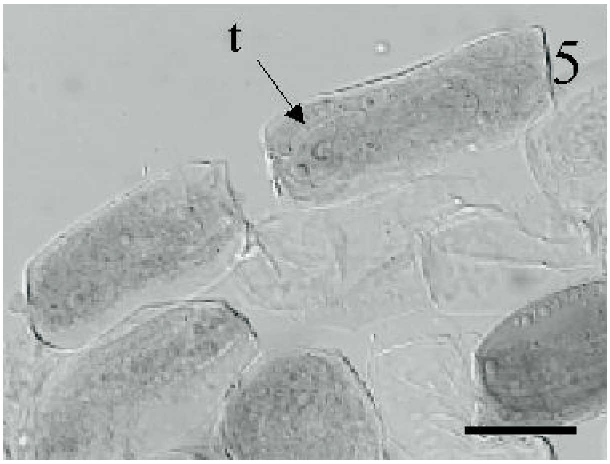
Data for mycorrhizal parameters and for heavy metal content were analysed with the non-parametric Kruskal-Wallis and Mann-Whitney

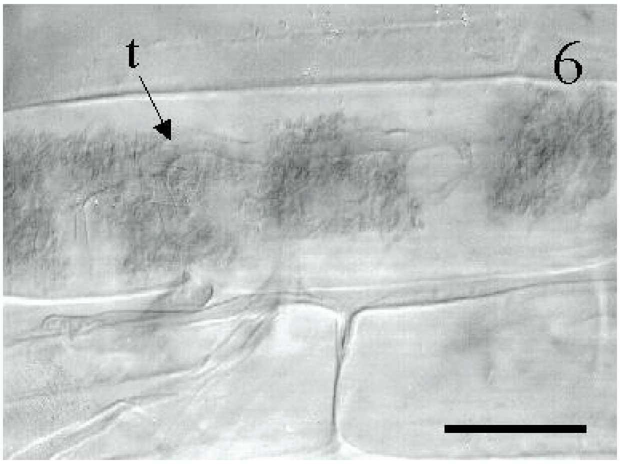












**Figs. 1–6** AM fungal morphotypes observed in *Biscutella laevigata* and *Plantago lanceolata* roots collected from mine wastes in Bolesław; (*a* arbuscule, *c* coil, *t* main trunk, *w* thickened hyphal wall); *bars* 20 µm

**Fig. 1** Morphotype 1, the most common in both plant species, shown here in *Plantago lanceolata* from the restored part of the zinc waste. This is characterised by poorly stained arbuscules (*a*) tightly filling cortical cells and hyphal coils (*c*) with distinctly thick walls (*w*)

**Fig. 2** As Fig. 1

**Fig. 3** Morphotype 2 with strongly stained arbuscules (*a*), characterised by clumped branches, shown here in *Biscutella laevigata* from the restored part of the zinc waste

**Fig. 4** Morphotype 3 with strongly stained arbuscules (*a*) occupying the cortical cells, shown in *Biscutella laevigata* from the Tatra Mountains

**Fig. 5** Morphotype 4 with a clearly visible main trunk (*t*), often relatively long in comparison to trunks of other morphotypes, shown here in *Biscutella laevigata* from the restored part of the zinc waste

**Fig. 6** Morphotype 4 with a clearly visible main trunk (*t*), often relatively long in comparison to trunks of other morphotypes, shown here in *Plantago lanceolata* from the non-restored part of the zinc waste

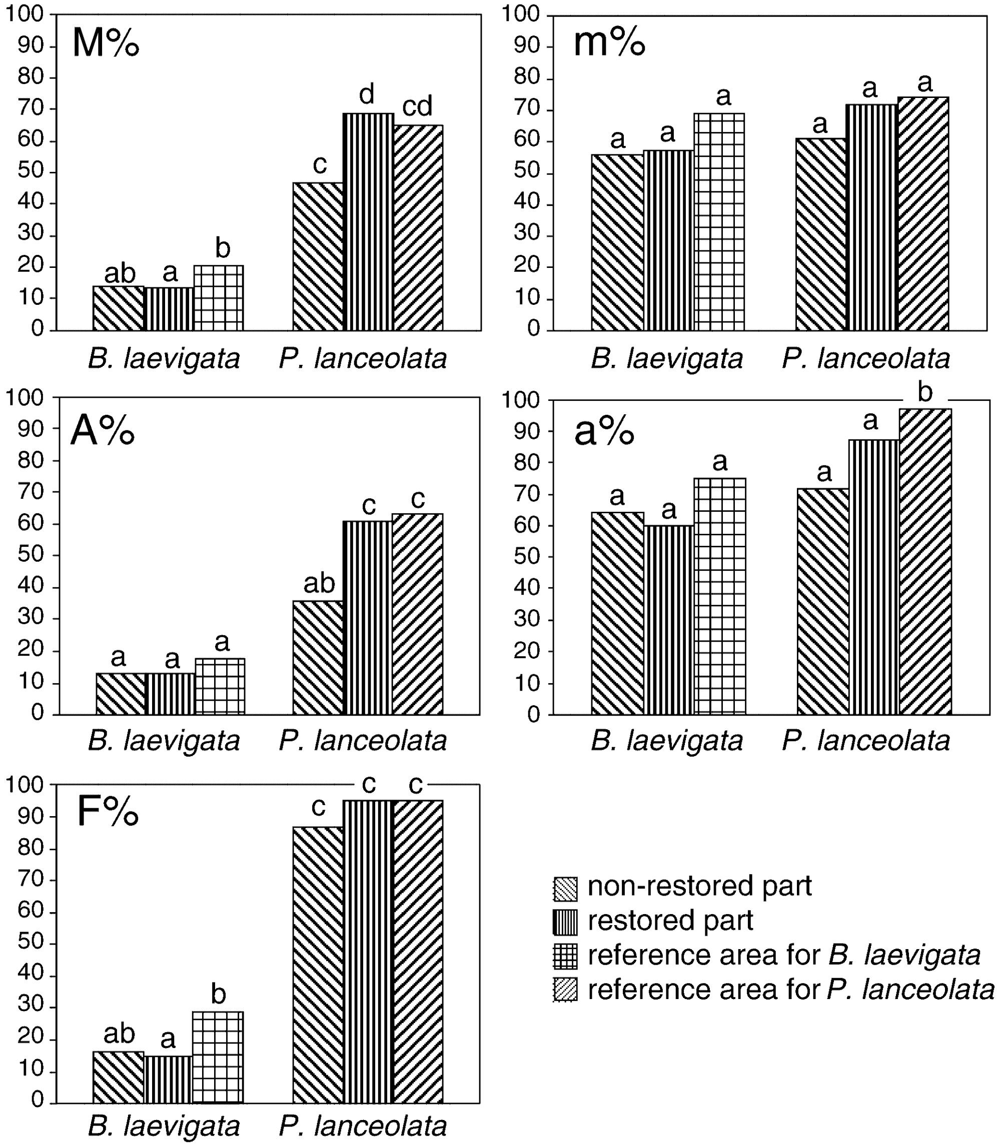


**Fig. 7** Relative mycorrhizal root length (*M%*), intensity of colonisation within individual mycorrhizal roots (*m%*), relative arbuscular richness (*A%*), arbuscule richness in root fragments where the arbuscules were present (*a%*) and mycorrhizal frequency (*F%*) in *Biscutella laevigata* and *Plantago lanceolata* from different collection sites. Different letters above bars indicate statistically significant differences ($P<0.05$)

tests using Statistica (version 5.0) software ($P<0.05$); correlation analysis was performed with Statgraphics (version 5.0).

## Results

### Mycorrhizal status of *Biscutella laevigata*

The finest roots of *B. laevigata* collected at the time of flowering from the calamine wastes and from the Tatra Mountains were colonised by AM fungal hyphae forming vesicles and well-developed arbuscules. The morphological diversity (differences in cell wall thickness of the intraradical mycelium, arbuscule structure and intensity of staining) of the different fungal structures observed within the roots suggested that the roots were colonised by at least four different fungal species (Figs. 1, 2, 3, 4, 5, 6). In samples collected in June, July and October, intraradical colonisation by mycelium forming coils and vesicles was found occasionally at a level of approximately 0–5%, but no arbuscules were observed. Mycorrhiza was present in all samples collected from the Tatra Mountains. In 60% of the samples, mycorrhizal colonisation was observed in up to 15% of total root length, while the rest were mycorrhizal in 30–45% of root length. There were no statistically significant differences in arbuscule richness between samples collected from different stands (Fig. 7). Generally, roots with developed arbuscules had the highest class of arbuscule richness (a%). The same was true of samples collected from the wastes. Although there were no significant differences in mycorrhizal colonisation levels due to high variation of data, a number of samples from the non-restored waste showed apparent higher colonisation levels than most samples from the restored part. Root samples collected from calamine wastes after the period of seed formation (beginning of June) were usually devoid of arbuscules; however, coils were often found within the finest roots (Fig. 8).

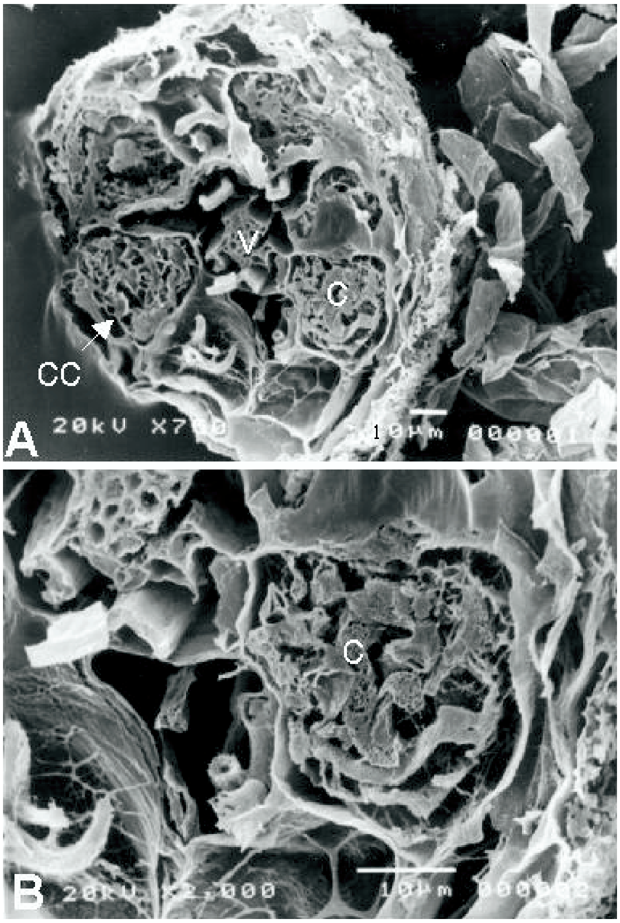


**Fig. 8** Scanning electron micrographs of coils (*c*) visible in cortical cells (*cc*) of *Biscutella laevigata* roots collected after seed formation (*v* vascular tissue)

## Mycorrhizal status of *Plantago lanceolata* and comparison with the mycorrhiza in *Biscutella laevigata*

The frequency of mycorrhizal colonisation (F%) and the relative mycorrhizal colonisation (M%) of *P. lanceolata* were significantly higher than in *B. laevigata* (Fig. 7). There was no correlation in the level of mycorrhiza development between the two analysed species. All samples of *P. lanceolata*, including the samples collected from the reference area, were mycorrhizal. Mycorrhizal morphotypes similar to those found in *B. laevigata* were also observed in *P. lanceolata* from the wastes (Figs. 1, 2, 3, 4, 5, 6). The colonisation of the total root system ranged from 3 to 90%; low colonisation levels were rare. As far as an influence of restoration on mycorrhiza is concerned, the greatest differences (statistically significant) for *P. lanceolata* were observed in the case of M% and A% (Fig. 7). When *P. lanceolata* samples from the non-restored calamine wastes were compared to those from the reference area, significant differences were found mainly in arbuscule development (A%, a%) ($P<0.005$). In the case of *B. laevigata*, significant differences were found only for M% and F% between the reference area and the restored zinc waste.

## Mycorrhiza of *Plantago lanceolata* and *Biscutella laevigata* in relation to the heavy metal contents of plants and soil

Table 2 presents data on metal contents of *B. laevigata* and *P. lanceolata.* Both plants accumulated more Cd in roots than in shoots. *P. lanceolata* accumulated more Pb and Zn in roots, except for Pb in plants from the non-restored site. *B. laevigata* from this site accumulated more Pb in roots. The Cd and Pb contents of *P. lanceolata* roots were lower in samples collected from the restored than from the non-restored wastes (*P* values 0.0379 and 0.0469, respectively).

No correlation was found between any of the parameters characterising mycorrhiza development in either plant species and the total or exchangeable heavy metal contents of soil. There was also no correlation between the total and exchangeable metal contents of the soil and the metal contents of plant tissues. In the case of *P. lanceolata*, there was a negative correlation between the M%, m%, A%, and a% parameters and the Pb content of roots (correlation coefficients –0.5307, –0.5348, –0.6592 and –0.6467, respectively; *P* values 0.0284, 0.0270, 0.004 and 0.0040, respectively).

## AM propagules in non-restored and restored calamine wastes

The most probable number assay showed a high variation in the density of fungal propagules in the substratum of

**Table 2** Element contents (mean ± SD, mg $kg^{-1}$) in *Biscutella laevigata* and *Plantago lanceolata*. Different letters following the values indicate statistically significant differences ($P<0.05$) (*A* aboveground part of the plant, *B* underground part of the plant)

| Site | | Cd | | Pb | | Zn | |
|---|---|---|---|---|---|---|---|
| | | *B. laevigata* | *P. lanceolata* | *B. laevigata* | *P. lanceolata* | *B. laevigata* | *P. lanceolata* |
| Non-restored wastes | A | 4.3±2.5 a | 5.3±2.4 a | 62.0±25.3 a | 44.7±16.6 a | 413.0±183.7 a | 532.8±140.0 a |
| | B | 14.3±5.6 b | 68.6±24.8 b | 97.4±39.9 b | 202.0±134.7 b | 409.7±125.2 a | 2536.5±889.3 b |
| Restored wastes | A | 4.9±4.7 a | 6.2±1.7 a | 101.4±44.3 a | 84.9±53.7 a | 412.4±88.0 a | 683.3±137.1 a |
| | B | 16.2±8.8 b | 44.5±12.1 b | 85.2±34.9 a | 101.1±35.2 a | 317.2±63.1 a | 1901.0±314.1 b |
| Reference area | A | 0.2±0.1 a | 0.8±0.3 a | 2.3±1.9 a | <0.1 a | 32.1±12.3 a | 53.1±13.8 a |
| | B | 0.3±0.2 b | 1.4±0.2 a | 2.7±1.4 a | <0.1 a | 47.4±13.0 a | 75.4±14.1 a |

the waste. In the non-restored part, the number of propagules was in the range of 50 to 280 propagules per 100 g of substratum with a mean of 140 propagules. In the restored part, the range was 3.4–560 with a mean of 220 propagules per 100 g.

## Discussion

The demonstration of mycorrhizal symbiosis in *Biscutella laevigata* was the most surprising outcome of this research. This plant species belongs to the Brassicaceae, a family generally accepted to be non-mycorrhizal (Gerdemann 1968; Trappe 1987). According to DeMars and Boerner (1996), members of this group of plants may be colonised by AM fungi but arbuscules, which are the most important criterion of a functional mycorrhiza, do not develop under glasshouse conditions. No arbuscules were found in *B. laevigata* during the first survey of mycorrhiza in plants colonising calamine wastes in Bolesław (Pawłowska et al. 1996). However, in the present paper we clearly show the presence of arbuscules during the flowering period in plants colonising the zinc wastes and growing in the Tatra Mountains. Only intraradical mycelium forming coils and vesicles was observed after seed formation. High percentages of AM infection usually correlate with the most active growth of the host, as shown by Nicolson and Johnston (1979), Van Duin et al. (1990), and Ietswaart (1992). This may apply to the flowering and seed formation stage of *B. laevigata*. However, investigation of this hypothesis requires the comparison of mycorrhizal and non-mycorrhizal plants. Although the plant grows well under experimental conditions, we were not able to obtain the flowering stage (unpublished data). Additionally, the effect of AM should not be considered only in terms of the effect of P increase on yield. In many cases, impact on the viability of offspring, seed production or improved health status seem more relevant (Khan 1972; Koide and Lu 1992). However, the response of plants to mycorrhizal colonisation can be nil or antagonistic (Francis and Read 1995). There is also no correlation between the degree of mycorrhizal colonisation and the mycorrhizal effect (Sanders and Fitter 1992).

Heavy metal accumulation by *B. laevigata* has been studied in specimens originating from other sites. Most pronounced was the hyperaccumulation of Pb (>1,000 mg kg$^{-1}$) and Zn (>4,500 mg kg$^{-1}$) in plant material collected from the Austrian Alps (Wenzel and Jockwer 1999). Neither in the present study nor in the research carried out previously on the same calamine wastes by Godzik (1991, 1993) were such high concentrations of Pb, Zn and Cd found in Polish plants. Differences in the exchangeable metal content in Polish and Austrian soils may explain the dissimilarities in metal uptake. Compared with *Plantago lanceolata*, mycorrhizal colonisation was found in a relatively low percentage of *B. laevigata* roots. Sampling in the wrong period or destruction of a high percentage of fine roots during sampling may explain the lack of arbuscules in *B. laevigata* in other studies (Pawłowska et al 1996). In the present investigation, the colonisation percentage may have been underestimated due to greater losses of the finest roots.

Mycorrhizal parameters of the two plant species growing in close proximity were not correlated. This may be due to their root systems being of a different kind or to differences in mycorrhizal dependency. *P. lanceolata* developed roots in the uppermost layer of the mound, whereas *B. laevigata* tended to form a long tap root penetrating crevices in the hard, stony substratum. There was no correlation between mycorrhizal parameters and the total or exchangeable heavy metal contents of the soil.

There was a negative (as yet unexplained) correlation between mycorrhizal parameters and Pb content of *P. lanceolata* roots, despite the fact that this element has low mobility in soil (less than Cd and Zn) and that the investigated site was not more polluted with Pb than with the other two elements. It has been suggested that AM fungi associated with metal-tolerant plants contribute to the accumulation of heavy metals in roots in a non-toxic form (Tonin et al. 2001). However, the forms of different elements transferred from the fungus to the plant and the effects at the interface between the symbionts are unknown. Transfer of Pb may destroy the plant plasmalemma, leading to decreased permeability of water (Cumming and Taylor 1990). It is known that mycorrhizal colonisation is highly regulated by the host (Koide and Schreiner 1992). Thus, inhibition of colonisation might be an host plant response to Pb transfer from the fungus.

Both plant species analysed in the present study belong to the so-called pseudo-metallophytes that grow on contaminated as well as non-contaminated soils (Baker 1987). The tolerance to heavy metals of most members of this group is achieved by a metal exclusion strategy involving avoidance of uptake and restriction of metal transport to the shoots (De Vos et al. 1991). In addition, *B. laevigata* is known for its ability to accumulate heavy metals primarily in roots. In autumn, metals are eventually transferred to the shoots (Rascio 1977). In addition to root exudates, mycorrhizal fungi may influence the bioavailability of heavy metals to plants. The mechanisms of this phenomenon are poorly understood, especially in the case of AM fungi. Different fungal species may accumulate different levels of heavy metals and perhaps influence their uptake by the symbiotic plant in different ways. The accumulation of heavy metals within intraradical hyphae of AM fungi has been studied in plants naturally occurring on industrial wastes and in plants cultivated under laboratory conditions (Joner and Leyval 1997; Turnau 1998; Hildebrandt et al. 1999; Tonin et al. 2001). In the present research, the localisation of heavy metals within mycorrhizas was not analysed. Nevertheless, considering the differences in mycorrhizal colonisation found, the role of AM fungi in the survival of the two plant species may vary. Metal bioavailability to the fungus/root system may also be influenced by differences in the root systems of the two species and contrasting physico-chemical soil conditions around mycorrhizal roots. Variation in root development might also explain the lack of correlation between mycorrhizal parameters of plants growing in close proximity. However, similar mycorrhizal morphotypes were observed in the two plant species.

Restoration of the zinc waste had a positive effect on *P. lanceolata* and the grasses' population size, visible as a denser vegetation cover on the restored part. Mycorrhizal inoculum potential, evaluated in the top layer of the substratum, was higher in the restored than the non-restored area, leading to improved development of mycorrhizal associations. Due to the presence of trees, the organic matter content of the top soil was also higher, creating more appropriate conditions for the growth of plants developing their root systems within this layer. Decrease in metal toxicity may result from significant effects of the organic fraction on metal binding (Zimdahl and Skogerboe 1977; Rieuwerts et al. 1998). *B. laevigata*, which develops roots in the deeper layer poor in organic matter, seems to be simply outcompeted by the more prosperous herbaceous plants on the restored areas.

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