Effects of Cadmium on Radial Growth and Dry Mass Production of Ectomycorrhizal Fungi

Kim, Chang-Gi*, Sally Anne Power[1] and John Nigel Berridge Bell[1]
Institute of Basic Science Research, Kangwon National University, Chuncheon, 200-701, Korea
[1]Department of Environmental Science and Technology, Imperial College London, Silwood Park Campus, Ascot, Berkshire, SL5 7PY, UK

ABSTRACT : The sensitivity to Cd of three ectomycorrhizal fungi, *Paxillus involutus*, *Suillus bovinus* and *Rhizopogon subcaerulescens*, was assessed and compared in terms of radial growth and dry mass production, using both agar and liquid culture. The radial growth of *S. bovinus* and *R. subcaerulescens* was significantly reduced at the lowest concentration (0.1 mg Cd/L). The 50% effective concentration (EC_{50}) values calculated from radial growth rates of the ectomycorrhizal fungi showed that the sensitivity of the fungi to Cd was greatest in *S. bovinus* and lowest in *R. subcaerulescens*. Cadmium addition also significantly decreased dry mass production of the ectomycorrhizal fungi. The sensitivity of the fungi to Cd in terms of dry mass production, was greatest in *S. bovinus* and lowest in *P. involutus*. Higher growth rates of *P. involutus* and melanisation of *R. subcaerulescens* appeared to contribute to reduced Cd toxicity.

Key words : Cadmium toxicity, Dry mass production, Ectomycorrhizal fungi, Effective concentration, Radial growth

INTRODUCTION

Ectomycorrhizal symbiosis has been found to improve plant growth by increasing water (Duddridge *et al*. 1980) and nutrient uptake, such as N, P, K and Zn (Smith and Read 1997) via increases in the absorbing area as a result of hyphal infection. While air pollution may indirectly affect the vitality of ectomycorrhizal fungi, as a result of reduced carbohydrate translocation from host plants to the fungi (Termorshuizen and Schaffers 1991), soil pollutants, such as heavy metals, can also have a direct effect on the fungal partners themselves. Reductions in the number of species and the sporophore production of ectomycorrhizal fungi caused by heavy metals have been found in coniferous forests situated close to a Cu-Pb smelter in Sweden (Rühling and Söderström 1990), and a Zn smelter in Poland (Kowalski *et al*. 1990).

Cadmium has been shown to be one of the most toxic heavy metals for ectomycorrhizal fungi (Paulus and Bresinsky 1989). Studies of ectomycorrhizal fungi in *in vitro* experiments have shown considerable, but differential, sensitivity to Cd (McCreight and Schroeder 1982, Blaudez *et al*. 2000, Colpaert *et al*. 2000). Less sensitive ectomycorrhizal fungi may confer a certain level of Cd resistance to host plants in Cd-contaminated soil (Jongbloed and Borst-Pauwells 1990).

We have previously reported the effects of Cd and mycorrhizal colonisation on growth and Cd accumulation in *Pinus sylvestris* seedlings (Kim *et al*. 2004). It was hypothesised that the least Cd-sensitive ectomycorrhizal fungi in *in vitro* experiments would confer Cd resistance in *P. sylvestris* seedlings. The objective of the present study was to assess and compare the sensitivity of three ectomycorrhizal fungi, *Paxillus involutus*, *Suillus bovinus* and *Rhizopogon subcaerulescens*, to Cd without their host plants, in terms of radial growth and dry mass production, using agar and liquid culture, respectively. These ectomycorrhizal fungi were chosen because they have been observed to form ectomycorrhizas with *P. sylvestris* and because of their relative ease of cultivation and fast growth rates (Kim *et al*. 2003). *Paxillus* has a broad host range (Villeneuve *et al*. 1989), whereas *Suillus* and *Rhizopogon* appears to be ectomycorrhizal exclusively with the members of the Pinaceae (Finlay 1989, Molina and Trappe 1994). Although the effects of Cd toxicity have been reported for *P. involutus* and *S. bovinus* (Darlington and Rauser 1988, Hormilla *et al*. 1996, Blaudez *et al*. 2000), no such studies have yet been carried out for *R. subcaerulescens*.

MATERIALS AND METHODS

Fungal Culture

* Corresponding author; Phone: 82-33-250-8529, Fax: 82-33-251-3990, e-mail: changgikim@empal.com

Cultures of *Paxillus involutus* (Batsch. ex Fr.) Fr. (isolate no. 87017), *Suillus bovinus* (Fr.) O. Kuntze (isolate no. 096) and *Rhizopogon subcaerulescens* Smith (isolate no. 379) were obtained from Professor D.J. Read at the University of Sheffield in April 1999. The stock cultures were grown in an incubator at 20℃, in the dark, on modified Melin-Norkrans' (MMN) agar medium (Marx 1969).

Radial Growth

As a pilot study, radial growth of the ectomycorrhizal fungi was investigated at concentrations of 0, 1, 2, 5, 10 or 50 mg Cd/L. However, since the radial growth was greatly inhibited at levels above 1 mg Cd/L for all fungi, a second experiment was carried out to determine the response of the fungi to levels below 1 mg Cd/L.

Cadmium chloride was incorporated into the MMN agar in order to give final concentrations of 0, 0.1, 0.5 or 1.0 mg/L. Mycelial agar plugs (7 mm diameter) were removed from the edge of actively growing fungal colonies; these were 2~4 weeks old, depending on the growth rates of the fungal species. An agar plug was placed on the centre of the fresh solidified MMN agar medium. The inoculated plates were kept in a 20℃ incubator, in the dark. Six replicates were used for each treatment. Due to the high radial growth rate of the *P. involutus* colony, colony diameters were measured every two days until the control colonies of each species reached the edges of the plates. The mean colony diameter was calculated from the widest diameter and its perpendicular diameter. The radial growth rates of the fungi were determined from a regression analysis of diameter against time. Effective concentrations inhibiting growth by 50% (EC_{50}) were calculated from curve-fitted growth rate data.

Dry Mass Production

Prior to inoculation in liquid MMN medium, 7 mm diameter agar plugs from the fungal colonies on MMN agar were removed and incubated on 15% (w/v) water agar. The plugs were maintained until the mycelium projected around the upper periphery of the plugs (Palmer and Hacskaylo 1970). Cadmium chloride was added into the MMN basal liquid medium in order to give final concentrations of 0, 1, 2, 5, 10 or 50 mg/L. One plug of inoculum incubated on the water agar was floated on the surface of 25 ml of treated medium and then incubated at 20℃. Six plates were prepared for each treatment.

Three replicates of each treatment were harvested at days 14 and 21. The mycelium from a single replicate was rinsed with distilled water and then collected on a Whatman no. 1 filter paper under suction. The collected mycelial mat was dried in an oven at 80℃ for 48 h and weighed. The final dry weight of mycelium was obtained by subtracting from that of the filter paper-mycelial weight, the predetermined oven-dried weight of filter paper and initial dry weight of fungal inoculum which was determined as the mean of the dry weight of 15 plugs. The EC_{50} was calculated for each harvest date.

Statistics

Data were analysed using the STATISTICA (version 5.5, STATSOFT, USA) statistical package. The effect of Cd on the growth of each fungus was evaluated using one-way analysis of variance. Statistically significant differences between means were identified by Tukey's honestly significant difference (HSD) test ($p < 0.05$).

RESULTS

Effect of Cadmium on Radial Growth of Ectomycorrhizal Fungi

Although a smaller amount of Cd was added than in the pilot experiment, the overall effect of Cd on the radial growth of *P. involutus* was still highly significant ($F = 310.1$, $p < 0.001$) (Fig. 1A, Table 1). The growth rates were not affected by the 0.1 mg/L treatment compared to the controls, whereas they were reduced at 0.5 and 1.0 mg/L. The growth rates of *S. bovinus* were significantly reduced by Cd addition ($F = 299.2$, $p < 0.001$) (Fig. 1B, Table 1). The growth rates at levels above 0.5 mg/L were 91 % lower than those in the controls. A difference in the radial growth of *S. bovinus* between 0 and 1 mg/L was seen on day 12 and continued for the rest of the incubation period. The growth rates of *R. subcaerulescens* were also reduced by Cd addition ($F = 57.7$, $p < 0.001$) (Fig. 1C, Table 1). The overall growth rates of *P. involutus* were higher than those of *S. bovinus* or *R. subcaerulescens*. The EC_{50} value for *R. subcaerulescens* ($EC_{50} > 1.0$) was greater than that of *P. involutus* ($EC_{50} = 0.96$) or *S. bovinus* ($EC_{50} = 0.10$).

The mycelial colour of all fungi changed during the incubation. The brown colour of the *P. involutus* mycelium intensified, although

Table 1. Mean growth rates (mm/day) of ectomycorrhizal fungi exposed to Cd on MMN agar. Data are means ± standard errors (n=6). Values within columns with the same letters are not significantly different (Tukey's HSD test, $p < 0.05$)

Cd added (mg/L)	Mean growth rates (mm/day)		
	P. involutus	*S. bovinus*	*R. subcaerulescens*
0.0	5.23±0.10 a	2.30±0.10 a	1.30±0.05 a
0.1	5.06±0.07 a	1.11±0.06 b	1.15±0.09 b
0.5	3.87±0.11 b	0.20±0.03 c	0.94±0.03 c
1.0	2.50±0.14 c	0.12±0.02 c	0.74±0.04 d

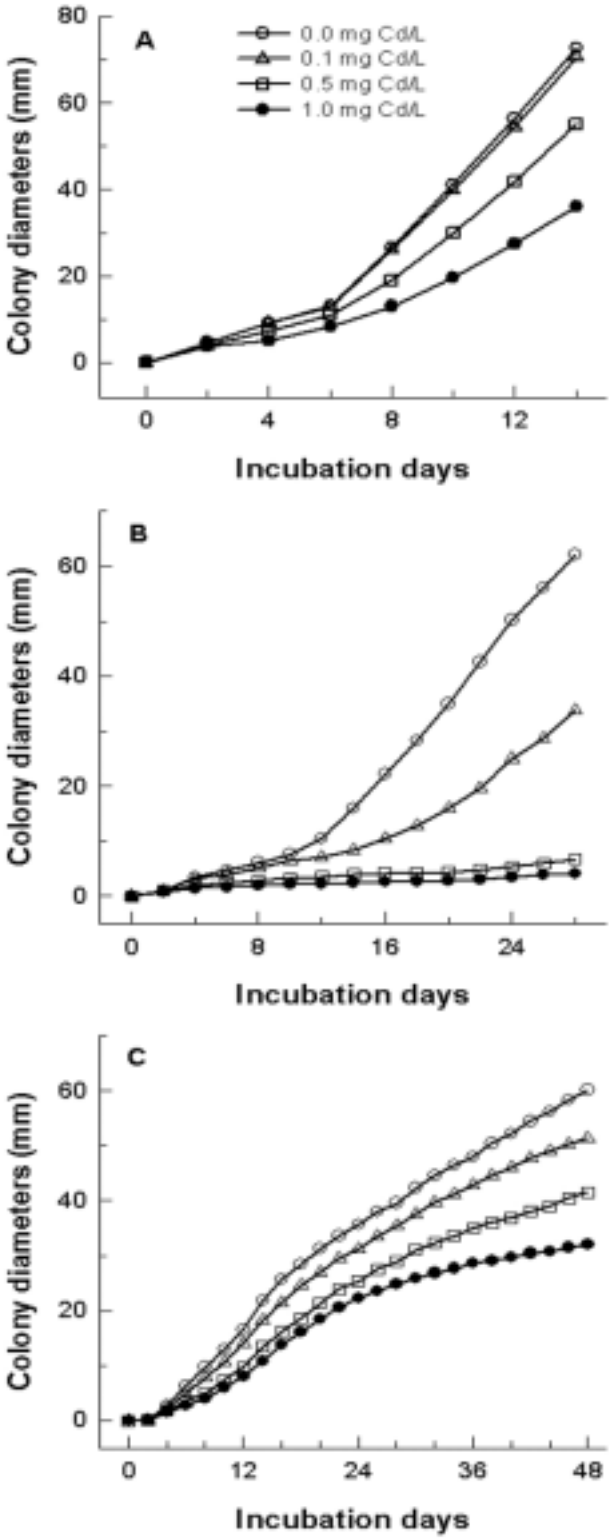

Fig. 1. Radial growth (mm) of (A) *P. involutus*, (B) *S. bovinus* and (C) *R. subcaerulescens* exposed to Cd on MMN agar.

this was not conspicuous compared to changes of the colour of *S. bovinus* or *R. subcaerulescens*. Both the agar itself and the white mycelium of *S. bovinus* also developed a brown coloration in the presence of Cd. Development of brown pigmentation in the centre of the control colony was also observed in *R. subcaerulescens*, with the extent of pigmentation directly related to Cd concentrations.

Effect of Cadmium on Dry Mass Production by Ectomycorrhizal Fungi

Dry mass production of the three fungi was significantly reduced with increasing concentrations of Cd (Table 2). The least sensitive fungus appeared to be *P. involutus*, which produced a greater amount of mycelium than for either *S. bovinus* or *R. subcaerulescens*. On day 14, the EC_{50} for *P. involutus* (EC_{50} = 5.5) was greater than *S. bovinus* (EC_{50} = 1.4) or *R. subcaerulescens* (EC_{50} = 1.6). The reduction in mycelial dry weight caused by Cd addition was similar for both *S. bovinus* and *R. subcaerulescens* on day 14. The difference in mycelial biomass between Cd and control treatments did not differ between days 14 and 21 for *S. bovinus*. However, growth of *R. subcaerulescens* continued between days 14 and 21 in the Cd-treated medium. Therefore, the EC_{50} for *R. subcaerulescens* (EC_{50} = 3.5) was higher on day 21 than on day 14. On day 21, the EC_{50} for *P. involutus* (EC_{50} = 5.7) was still greater than *S. bovinus* (EC_{50} = 1.3) or *R. subcaerulescens*.

DISCUSSION

Cadmium caused a considerable reduction in growth of ectomycorrhizal fungi in the present study. The significant reduction in growth at low Cd concentrations indicates that this metal could contribute to the decline of mycorrhizal fungi in heavy metal-contaminated forest ecosystems.

The sensitivity to Cd in terms of radial growth and dry mass production was highest in *S. bovinus*. The high sensitivity of *S. bovinus* to Cd has been demonstrated in both agar and liquid medium elsewhere. Blaudez *et al*. (2000) observed a 96~99% reduction in dry weight of three isolates of *S. bovinus* at 1 mg Cd/L. Hormilla *et al*. (1996) also reported that the growth of *S. bovinus* was inhibited by 84% and 81% at 3 mg Cd/L in a 35-day incubation in agar and liquid medium, respectively.

Although *P. involutus* was shown to be less sensitive to Cd than *S. bovinus* in the present study, Colpaert and Van Assche (1992) reported that the radial growth rates of *P. involutus* on Fries agar medium were lower than those of *S. bovinus*. Blaudez *et al*. (2000) also reported that *P. involutus* was less tolerant than *S. bovinus* in terms of dry mass production in MMN agar. Out of 19 *P. involutus* isolates tested, growth of 14 isolates was completely inhibited at levels of up to 1 mg Cd/L. Although the present study showed only 26% inhibition in dry mass production of *P. involutus* at 1 mg

Table 2. Mycelial dry weight (mg) of ectomycorrhizal fungi exposed to Cd in liquid MMN medium. Data are means ± standard errors (n = 3). Values for each species and day within columns with the same letters are not significantly different (Tukey's HSD test, $p < 0.05$)

Cd added (mg/L)	Dry weight (mg)					
	P. involutus		*S. bovinus*		*R. subcaerulescens*	
	Day 14	Day 21	Day 14	Day 21	Day 14	Day 21
0	19.9 ± 1.4 a	23.0 ± 0.2 a	13.1 ± 0.1 a	15.5 ± 0.4 a	19.7 ± 0.4 a	19.8 ± 0.5 a
1	15.0 ± 1.2 b	17.1 ± 0.2 bc	7.5 ± 0.0 b	8.1 ± 2.3 b	11.2 ± 0.2 b	12.3 ± 0.4 b
2	13.5 ± 1.0 b	19.7 ± 0.7 b	5.6 ± 0.5 bc	7.1 ± 0.2 bc	9.0 ± 0.7 bc	12.5 ± 0.3 b
5	14.2 ± 0.5 b	15.3 ± 1.3 c	3.4 ± 0.1 d	3.9 ± 0.2 bc	6.5 ± 1.3 cd	9.3 ± 1.2 b
10	4.0 ± 0.5 c	4.2 ± 0.7 d	3.7 ± 0.6 cd	5.8 ± 0.7 bc	4.2 ± 0.4 de	4.5 ± 1.0 c
50	3.9 ± 0.4 c	4.6 ± 0.1 d	2.6 ± 0.8 d	2.3 ± 0.9 c	3.2 ± 0.1 e	2.4 ± 0.6 c
ANOVA						
F-ratio	1381.5	51.6	371.0	73.9	1030.5	83.0
p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

Cd/L, it may not be appropriate to compare both results, since colony dry weight was not determined in liquid culture but in agar culture in the study by Blaudez *et al*. (2000).

The present study showed significantly different degrees of sensitivity between media. The EC_{50} values obtained from radial growth assessments were greater for *R. subcaerulescens* than *P. involutus*. However, EC_{50} values obtained following measurement of dry mass production were greater for *P. involutus* than *R. subcaerulescens*. In contrast, radial growth of *S. bovinus* was more sensitive to Cd than dry mass production. Darlington and Rauser (1988) also reported different levels of sensitivity to Cd in *P. involutus*. Based on radial growth on MMN agar, the growth rate of *P. involutus* was reduced by 85% at 0.1 mg Cd/L compared to the control. However, dry mass production was reduced by 45% at the same concentration in their study. They observed that the hyphae became more dense and that growth was achieved by elongation of submerged hyphae. Growth of the latter can not be accurately recorded in measurements of radial growth on the agar surface. Fungi which have thin mycelia growing very close to the agar surface can also outperform fungi which have denser, thick aerial colonies (Aggangan *et al*. 1998). Dry weight measurement of mycelium in liquid culture may, therefore, provide a more accurate assessment on Cd toxicity than measurements on agar culture.

However, it should be noted that mycelial growth of ectomycorrhizal fungi in liquid culture is likely to be different from growth on solid substrates (Whipps 1987) and can not reflect mycelial growth in soil in a natural environment (Hartley *et al*. 1997). More importantly, the mycelia of symbiotic ectomycorrhizal fungi grow not only on solid surfaces but also in the intercellular spaces within the host plant roots, with a totally different pattern of hyphal growth (Kottke and Oberwinkler 1987). Therefore, there are fundamental limits to *in vitro* tests of pure cultures of ectomycorrhizal fungi on either agar or in liquid culture.

Although the growth of the three fungi tested in the present study was highly inhibited by Cd, the magnitude of the sensitivity differed between species, probably due to differences in Cd detoxification mechanisms. Higher growth rates of *P. involutus* in the present study appeared to contribute to reduced Cd toxicity. Faster growing fungi which produce more extramatrical hyphae may be less sensitive to Cd due to the presence of more binding sites. However, an increase in metal uptake by fungi is far greater than an increase in binding of metals on cell walls (Brown and Hall 1990). Therefore, other mechanisms may be required for detoxification in *P. involutus*. Having observed significant transport of Cd into the vacuoles of *P. involutus*, Blaudez *et al*. (2000) suggested that vacuolar compartmentation, as well as binding of Cd on cell walls, may be two essential detoxification mechanisms in *P. involutus*.

Since the effects of Cd toxicity on radial growth and dry mass production have yet not been reported for *R. subcaerulescens*, Cd distribution in the mycelium needs to be analysed for additional information. Although only visual assessment was possible, the notable melanisation observed in the present study may be an important Cd tolerance mechanism for *R. subcaerulescens*, which was shown to be less sensitive to Cd than *S. bovinus*. Gruhn and Miller

(1991) suggested that melanin may be able to bind Cu and block its entry into the cell. Although a quantitative assessment of melanisation was not possible within the current study, melanin production within *R. subcaerulescens* hyphae may also play a similar role, binding and blocking Cd.

Colpaert and Van Assche (1987) suggested that binding of heavy metals onto the extensive extramatrical hyphae of some species of Suillus may be effective at providing metal tolerance to a host plant. However, *S. bovinus* isolates in the present study appeared to be particularly sensitive to Cd. Vodnik *et al*. (1998) reported an increase in the pigmentation of *S. bovinus* caused by Pb. Although melanisation of *S. bovinus* was also observed in the present study, it did not appear to be effective in reducing Cd toxicity in this species.

From the results of the present study, a decline of *S. bovinus* in Cd-contaminated forests and a higher senitivity to Cd of *S. bovinus*-infected plants than *P. involutus*- or *R. subcaerulescens*-infected plants would be expected. However, the test of the fungal performance in symbiosis with their host plants should be followed, because fungal physiology *in vitro* may be different to that in symbiosis (Marschner *et al*. 1998).

ACKNOWLEDGEMENTS

We thank Professor D.J. Read F.R.S. and Ms. Irene Johnson at the University of Sheffield, for providing cultures of ectomycorrhizal fungi and for advice on culturing and associated research techniques. This research was partially supported by a UK Overseas Research Students Award.

LITERATURE CITED

Aggangan, N.S., B. Dell and N. Malajczuk. 1998. Effects of chromium and nickel on growth of the ectomycorrhizal fungus *Pisolithus* and formation of ectomycorrhizas on *Eucalyptus urophylla* S.T. Blake. Geoderma 84: 15-27.

Blaudez, D., C. Jacob, K. Turnau, J.V. Colpaert, U. Ahonen-Jonnarth, R. Finlay, B. Botton and M. Chalot. 2000. Differential responses of ectomycorrhizal fungi to heavy metals *in vitro*. Mycol. Res. 104: 1366-1371.

Brown, M.T. and I.R. Hall. 1990. Metal tolerance in fungi. *In* A.J. Shaw (ed.), Heavy Metal Tolerance in Plants. CRC Press, Boca Raton. pp. 95-104.

Colpaert, J.V. and J.A. Van Assche. 1987. Heavy metal tolerance in some ectomycorrhizal fungi. Funct. Ecol. 1: 415-421.

Colpaert, J.V. and J.A. Van Assche. 1992. The effects of cadmium and the cadmium-zinc interaction on the axenic growth of ectomycorrhizal fungi. Plant Soil 145: 237-243.

Colpaert, J.V., P. Vandenkoornhuyse, K. Adriaensen and J. Vangronsveld. 2000. Genetic variation and heavy metal tolerance in the ectomycorrhizal basidiomycete *Suillus luteus*. New Phytol. 147: 367-379.

Darlington, A.B. and W.E. Rauser. 1988. Cadmium alters the growth of the ectomycorrhizal fungus *Paxillus involutus*: a new growth model accounts for changes in branching. Can. J. Bot. 66: 225-229.

Duddridge, J.A., A. Malibari and D.J. Read. 1980. Structure and function of mycorrhizal rhizomorphs with special reference to their role in water transport. Nature 287: 834-836.

Finlay, D. 1989. Functional aspects of phosphorus uptake and carbon translocation in incompatible ectomycorrhizal associations between *Pinus sylvestris*, *Suillus grevillei* and *Boletinus cavipes*. New Phytol. 103: 185-192.

Gruhn, C.M. and O.K. Miller, Jr. 1991. Effect of copper on tyrosinase activity and polyamine content of some ectomycorrhizal fungi. Mycol. Res. 95: 268-272.

Hartley, J., J.W.G. Cairney and A.A. Meharg. 1997. Do ectomycorrhizal fungi exhibit adaptive tolerance to potentially toxic metals in the environment? Plant Soil 189: 303-319.

Hormilla, S., M.K. Dunabeitia, P. Cabrerizo, J.I. Pena and J.M. Becerril. 1996. Response of six ectomycorrhizal fungi on pure culture to some environmental stresses. *In* C. Azcón-Aguilar and J.M. Barea (eds.), Mycorrhizas in Integrated Systems from Genes to Plant Development: Proceedings of the Fourth European Symposium on Mycorrhizas. European Commission Report, EU16728EN, pp. 448-451.

Jongbloed, R.H. and G.W.F.H. Borst-Pauwels. 1990. Differential response of some ectomycorrhizal fungi to cadmium *in vitro*. Acta Bot. Neerl. 39: 241-246.

Kim, C.-G., S.A. Power and J.N.B. Bell. 2003. Effects of cadmium on growth and glucose utilisation of ectomycorrhizal fungi *in vitro*. Mycorrhiza 13: 223-226.

Kim, C.-G., S.A. Power and J.N.B. Bell. 2004. Response of *Pinus sylvestris* seedlings to cadmium and mycorrhizal colonisation. Water, Air, Soil Pollut. 155: 189-203.

Kottke, I. and F. Oberwinkler. 1987. The cellular structure of the Hartig net: coenocytic and transfer cell-like organization. Nord. J. Bot. 7: 85-95.

Kowalski, S., W. Wojewoda, C. Bartnik and A. Rupik. 1990. Mycorrhizal species composition and infection patterns in forest plantations exposed to different levels of industrial pollution. Agr. Ecosyst. Environ. 28: 249-255.

Marschner, P., G. Jentschke and D.L. Godbold. 1998. Cation exchange capacity and lead sorption in ectomycorrhizal fungi.

Plant Soil 205: 93-98.

Marx, D.H. 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism of mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59: 153-163.

McCreight, J.D. and D.B. Schroeder. 1982. Inhibition of growth of nine ectomycorrhizal fungi by cadmium, lead, and nickel *in vitro*. Environ. Exp. Bot. 22: 1-7.

Molina, R. and J.M. Trappe. 1994. Biology of the ectomycorrhizal genus, *Rhizopogon*. I. Host associations, host-specificity and pure culture syntheses. New Phytol. 126: 653-675.

Palmer, J.G. and E. Hacskaylo. 1970. Ectomycorrhizal fungi in pure culture. I. Growth on single carbon sources. Physiol. Plant. 23: 1187-1197.

Paulus, W. and A. Bresinsky. 1989. Soil fungi and other microorganisms. *In* E.-D. Schulze, O.L. Lange and R. Oren (eds.), Forest Decline and Air Pollution: A Study of Spruce (*Picea abies*) on Acid Soils. Springer-Verlag, Berlin. pp. 110-120.

Rühling, Å. and B. Söderström. 1990. Changes in fruitbody production of mycorrhizal and litter decomposing macromycetes in heavy metal polluted coniferous forests in north Sweden. Water, Air, Soil Pollut. 49: 375-387.

Smith, S.E. and D.J. Read. 1997. Mycorrhizal Symbiosis, 2nd ed. Academic Press, San Diego.

Termorshuizen, A. and A. Schaffers. 1991. The decline of carpophores of ectomycorrhizal fungi in stands of *Pinus sylvestris* L. in The Netherlands: possible causes. Nova Hedwigia 53: 267-289.

Villeneuve, N., M.M. Grandtner and J.A. Fortin. 1989. Frequency and diversity of ectomycorrhizal and saprophytic macrofungi in the Laurentide Mountains of Quebec. Can. J. Bot. 67: 2616-2629.

Vodnik, D., A.R. Byrne and N. Gogala. 1998. The uptake and transport of lead in some ectomycorrhizal fungi in culture. Mycol. Res. 102: 953-958.

Whipps, J.M. 1987. Method for estimation of chitin content of mycelium of ectomycorrhizal fungi grown on solid substrates. Trans. Brit. Mycol. Soc. 89: 199-203.

(*Received September 3, 2004; Accepted October 15, 2004*)