The effect of silver and copper nanoparticles on the growth and mycorrhizal colonisation of Scots pine (*Pinus sylvestris* L.) in a container nursery experiment [(§)]

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Recent research points to the possibility of nanoparticles being used as fertilisers, growth stimulators, and promoters of plant resistance or pesticides. In this study, we sought to determine the influence of nanoparticles of silver and copper (AgNPs and CuNPs) on growth parameters and spontaneous mycorrhizal colonisation of roots in 2-year-old container-grown seedlings of Scots pine. Foliar applications of nanoparticles were made through two growing seasons, four times a season, at concentrations of 0, 5, 25 and 50 ppm. Comparisons of the ultrastructures characterising the needles, stems and roots of the treated or untreated pines were conducted with transmission electron microscopy (TEM). The deployed CuNPs stimulated mycorrhizal colonisation at all concentrations, although the growth of seedlings was only promoted at a concentration of 25 ppm. Higher concentrations of AgNPs (25 and 50 ppm) inhibited the formation of mycorrhizae, though the lowest concentration (5 ppm) produced an increase in both mycorrhizal colonisation and the dry mass of roots. The species of ectomycorrhizal fungi found were *Thelephora terrestris*, *Suillus bovinus* and *Sphaerosporella brunnea*. The TEM results comparing treated and control (untreated) needles revealed changes in the chloroplasts from lens-shaped to spherical. Furthermore, an increase in the number of plastoglobules and the presence of large osmophilic globules in the cytoplasm associated solely with the needles of pines receiving 50 ppm nanoparticles were observed. In contrast, ultrastructural changes in stems and roots associated with the applications of NPs were not found. Overall, the results indicated that CuNPs and AgNPs could be used as stimulators of growth in general, and mycorrhizal colonisation in particular, among container-grown Scots pines. However, further work is needed to determine their optimal doses and concentrations.

Keywords: Nanoparticles, Ectomycorrhizae, Toxicity, Growth Stimulation

Introduction

Nanomaterials are engineered structures with at least one dimension between 1 and 100 nm (Nel et al. 2006). In the past decade, they have gained wide application worldwide owing to electronic, optical, me- chanical, magnetic and chemical properties that are unique and can be quite different from those characterising the same materi- als present in bulk (Ruffini Castiglione & Cremoni 2009). Nanotechnology has been applied in plant production (agriculture and forestry) as fertilisers to increase plant growth and disease resistance and as pesti- cides seeking to improve pest and disease management (Servin et al. 2015).

Thus far, most research on the influence of nanoparticles on plants has been con-

ducted in hydroponic culture (Bernhardt et al. 2010), with only a few trials involving plants grown in soil (Josko & Oleszczuk 2013) or in the peat-based substrate used in nursery production (Olchowik et al. 2017). The results of these studies document positive, negative and neutral influences of nanoparticles on plants (Servin et al. 2015, Rizwan et al. 2017). The positive influence is manifested in stimulated germination, increased growth rates, higher plant biomass, a greater number of flowers/fruits/seeds, higher contents of gluten and starch, and higher chlorophyll content with a faster rate of photosynthesis (Linglan et al. 2008, Jaberzadeh et al. 2013). Further, an indirectly favourable influence of nanoparticles on plants reflects growth and/or increased metabolic activity within the population of rhizospheric microorganisms (Raliya & Tarafdar 2013, Tarafdar et al. 2014).

The negative influence is manifested in obstructed seed germination, reduced shoot or root length and dry/fresh mass, as well as a reduced chlorophyll content (Shaw & Hossain 2013, Nair & Chung 2014).

These differences in the results of research carried out so far may reflect that the biological influence of nanoparticles depends on their size, chemical composition, surface structure, solubility, shape, and level of aggregation (Nel et al. 2006). Their influence also seems to depend on the plant species involved, growth conditions, the type of substrate (soil or different nutrient media), temperature, and light intensity and, of course, the concentrations of nanoparticles involved, the manner of the application (foliar or soil) and the dosage (Ruffini Castiglione & Cremoni 2009, Josko & Oleszczuk 2013, Rizwan et al. 2017). The large number of factors involved complicates the comparison of results among studies (Sweet & Singleton 2015).

Scots pine (*Pinus sylvestris* L.) is the most widely distributed conifer species in the northern hemisphere and the most important forest-forming species throughout eastern-central Europe (Krakau et al. 2013). In Polish forests, it covers over 58.2% of the forest area, while accounting for 60.8% of the volume (Central Statistical Office 2017). Pines are thought to be dependent on ectomycorrhizal symbiosis for optimal development under natural conditions. Ectomycorrhizal fungi facilitate both nutrient and water uptake, increase resistance to certain root diseases, and enhance the stress tolerance of trees (Smith & Read 2008).

Currently, little research is being conducted on the influence of nanoparticles on the state and structure of mycorrhizal fungi. The majority of this research concerns arbuscular mycorrhiza, and as in the influence of nanoparticles on plants, the results are ambiguous. Some studies reveal a negative influence on mycorrhizal colonisation of plant roots (Dubchak et al. 2010), while others suggest no influence on the level of mycorrhization at low nanoparticle

concentrations but negative impacts when concentrations are high (Judy et al. 2015, Cao et al. 2017). In contrast, Feng et al. (2013) demonstrated a stimulatory influence of silver nanoparticles (AgNPs) on the formation of an arbuscular mycorrhizae, irrespective of the concentrations applied. Similarly, ambiguous results have been obtained for ectomycorrhizae. Sweet & Singleton (2015) showed that contamination of soil by AgNPs may result in a drastic reduction in the diversity of ectomycorrhizal fungi in the roots of seedlings of *Pinus muricata*. Additionally, Olchowik et al. (2017) documented disparate influences of metal nanoparticles on ectomycorrhizae formation in seedlings of pedunculate oak for both type and concentration. While AgNPs stimulated formation irrespective of concentration, nanoparticles of copper (CuNPs) were found to have a stimulatory effect at low concentration, but an inhibitory impact when the concentration was high.

Notwithstanding the many relevant studies previously conducted, the influence of nanomaterials on plants and the environment remains unclear (Bernhardt et al. 2010). Thus, this paper had the following objectives: (i) to determine the influence of foliar applications of AgNPs and CuNPs at different concentrations on the growth of container-grown 2-year-old Scots pine seedlings; (ii) to compare the ultrastructures of needles, stems and roots of pines treated or untreated with nanoparticles with TEM; and (iii) to determine the extent of root mycorrhization and proportion of different ectomycorrhizal species in relation to the types and concentrations of nanoparticles applied. The basic assumption to be tested was that foliar applications of AgNPs and CuNPs would stimulate both the growth of pines and the formation of mycorrhizae.

Materials and methods

Plant material

The study was conducted in the nursery of the Forest Experimental Station of Warsaw University of Life Sciences, Poland (located at Rogów – 51° 40′ N, 19° 55′ E, 195 m a.s.l). Scots pine seedlings were grown in V-120 plastic container trays (40 pots per tray with a capacity of 120 cm^3) in a peat-perlite substrate, purchased from the container nursery in Nedza, Rudy Raciborskie Forest District, Poland. The substrate (pH=4.5) contained 85% sphagnum peat from Estonia and 15% coarse-grained perlite (No. 3) which improves aeration. Seedlings received extra nutrients *via* a mixture of Osmocote Exact Standard controlled-release fertilisers of different release characteristics: 3-4 M, 5-6 M, and 8-9 M (1:1:1). Fertiliser was added to the substrate at the same time as the pine seeds (of local origin from a managed forest) were sown. On 12 May 2014, two seeds were planted in each pot to provide seedlings. Upon germina-

tion, one plant per pot was selected at random and the other was removed. The containers were then placed in a foil-covered greenhouse (height 2.8 m, width 6 m and length 30 m) and watered regularly as necessary. The temperature in the greenhouse during the experiment was 25-30 °C. At the end of the first growing season (30 September 2014), seedlings were replanted in larger pots. We then used V-360 container trays (15 pots per tray with a capacity of 360 cm^3) and the same peat-perlite substrate as in the first year. In the second year, seedlings were grown outdoors.

Nanoparticles and treatments

The two types of metal nanoparticle chosen were AgNPs and CuNPs. Samples of commercially available solutions of AgNPs and CuNPs were purchased from Nano-Koloid Sp. z o.o, Warsaw, Poland, as a licensee of Nano Technologies Group, Inc. (NY, USA), manufactured under European patent EP2081672 A2. As the producer notes, these nanoparticles are generated in a physical process, consist of approximately 100 atoms and are suspended in demineralised water. The concentration of nanoparticles in the commercially available product is 50 ppm.

Each year (between May and July), there were four spray treatments of the aerial parts of the seedlings using 1000 l ha^{-1} (=100 ml m^{-2}) aqueous solutions of NPs (AgNPs, CuNPs) at 4 concentrations: 0, 5, 25 and 50 ppm. The NPs had been suspended in deionised water prior to application by vigorous shaking for at least 10 min. NPs were applied to foliage on 27 May, 11 and 25 June and 9 July in 2014; and on 17 June and 1, 15 and 29 July 2015. Sprayings were performed in the early morning with a manual compressed air sprayer (model KK-PS5000®, Kisan Kraft, Bangalore, India). The experimental design was comprised of two factors: the type of metal nanoparticles (Ag or Cu) and the 4 concentrations (0, 5, 25 and 50 ppm). The experiment was performed using a randomised complete block design with 4 blocks.

Sampling and biometric parameters

At the end of the second growing season (in October), 40 seedlings (10 plants × 4 blocks) were harvested from each treatment at random. Thus, a total of 320 Scots pine seedlings were sampled. At this time, the features of shoot growth measured were shoot length (cm) and root-collar diameter (mm), as well as total root length (cm) determined by analysis of the root systems using an Epson Perfection 4990 Photo scanner integrated with WinRhizo software (Regent Instruments Inc., Quebec, Canada). Then, dry masses of shoots and roots (g) were determined, following drying at 105 °C for 24 h.

Microscopic investigation

For the purposes of transmission electron microscope (TEM) imaging, ten seedlings

in each treatment were selected at random following the first growing season. The adsorption and assimilation of NPs in the needles, shoots and roots of the tested pine plants were observed by TEM, 8 weeks after the last treatment. Tissue samples were collected from the middle parts of the needles, shoots and roots of the pine seedlings. The plant material obtained was fixed in 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 0.05M cacodylate buffer (pH 7.2), following the methods of Karnovsky (1965), for 4 h at room temperature. The material was then rinsed four times with cacodylate buffer. The samples were contrasted in 1% OsO_4 for 2 h at 4 °C and rinsed four times with the same buffer prior to dehydration in a series of aqueous solutions of ethanol, and subsequently in propylene oxide. Finally, the material underwent gradual saturation with resin (Epon, Fluka) before being polymerized for 24 h at 60 °C. The Epon blocks with plant material were cut for TEM with an ultramicrotome (Leica) into ultrathin sections (~90 nm thick), which were collected on Formvar coated slot-grids and contrasted with 1% lead citrate and 2% uranyl acetate. The material was then examined using a Morgagni 268D (FEI) electron microscope, while photographic documentation was obtained using a Morada (SIS) digital camera and the iTEM (SIS) computer programme.

Assessment of ectomycorrhizae

The roots of harvested seedlings were analysed under a Delta IPOS-808 stereoscopic microscope coupled with a digital camera at 10-40× magnification. The degree of mycorrhization was determined by classifying and counting mycorrhizal and non-mycorrhizal root tips. The proportion of mycorrhizal root tips was calculated as (mycorrhizal root tips) / (mycorrhizal root tips + non-mycorrhizal root tips) × 100 and

presented as degree of mycorrhization. Ectomycorrhizal tips were identified under a dissecting microscope by the absence of root hairs, shape (hypertrophy) and colour of fine roots, as well as the presence of mycelial mantles and emanating fungal structures (hyphae and rhizomorphs). The initial identification of morphotypes was based on the literature (Agerer 2008). Representative mycorrhizal root tips of each morphotype were photographed and deposited in an internal database, together with fungal descriptions and molecular information. To determine mycorrhizal species/taxa, we collected tip material from three to five mycorrhizal root tips per morphotype and transferred them into Eppendorf tubes filled with 70% EtOH for molecular analysis.

The process of identification of mycorrhizal fungi entailed PCR amplification of selected regions of internal transcribed spacer (ITS) rDNA using the ITS1F / ITS4 primer pair, with sequencing of the resulting PCR product. We used direct PCR amplification of fungal DNA from ectomycorrhizal samples bypassing conventional DNA extraction procedures. PCR products were assessed by loading 2 μL onto a 1% agarose gel (0.5× TAE buffer) and visualised under UV light using the GeneRuler™ 1 kb Plus DNA Ladder (Fermentas). Bi-directional dye-terminator cycle sequencing was performed using BigDye Terminator v3.1 Chemistry (Applied Biosystems) and one of each PCR primer. The resulting fragments were analysed on a 3730 DNA Analyser at the Department of Botany and Biodiversity Research of the University of Vienna, Austria. Identification of sequenced fungi was based on the results of BLAST searches against the National Centre for Biotechnology Information (NCBI) public database, with subsequent phylogenetic placement and queries to the UNITE database.

Statistical analysis

We tested the relationships among biometric parameters depending on different concentrations of AgNPs and CuNPs using a one-way analysis of variance (ANOVA) with Tukey's *post-hoc* tests. Analysis of the

Tab. 1 - Biometrical parameters (mean ± SD) of 2-year-old Scots pine seedlings treated with different concentrations of AgNPs and CuNPs. Ag0, Cu0 - 0 ppm; Ag5, Cu5 - 5 ppm; Ag25, Cu25 - 25 ppm; and Ag50, Cu50 - 50 ppm. Different letters indicate significant differences between treatments with different applied concentrations of AgNPs and CuNPs as assessed using Tukey's test (α=0.05).

Treatment (n=40)	Length of shoot (cm)	Root collar diameter (mm)	Total root length (cm)	Dry mass of shoot (g)	Dry mass of root (g)
Ag0	22.2 ± 4.95 [a]	4.72 ± 0.91 [a]	24.8 ± 5.10 [a]	4.67 ± 1.25 [ab]	1.42 ± 0.50 [a]
Ag5	18.5 ± 4.78 [a]	4.82 ± 1.79 [a]	25.6 ± 5.46 [a]	4.07 ± 1.74 [ab]	3.00 ± 0.74 [c]
Ag25	19.9 ± 6.46 [a]	4.49 ± 0.92 [a]	22.9 ± 4.08 [a]	3.52 ± 1.88 [a]	1.60 ± 0.82 [a]
Ag50	19.5 ± 4.48 [a]	5.07 ± 0.84 [a]	23.5 ± 5.10 [a]	4.66 ± 1.85 [b]	2.20 ± 0.74 [b]
Cu0	21.7 ± 3.57 [a]	4.65 ± 0.61 [ab]	21.9 ± 3.86 [a]	4.01 ± 1.59 [a]	1.59 ± 0.57 [a]
Cu5	21.6 ± 5.89 [a]	5.00 ± 0.58 [ab]	25.2 ± 5.23 [ab]	4.53 ± 1.45 [a]	1.70 ± 0.95 [a]
Cu25	26.2 ± 6.73 [b]	5.38 ± 0.86 [b]	25.8 ± 6.08 [b]	5.83 ± 2.21 [b]	2.54 ± 1.02 [b]
Cu50	19.5 ± 5.05 [a]	4.48 ± 0.98 [a]	25.0 ± 5.26 [ab]	4.05 ± 1.81 [a]	2.44 ± 0.84 [b]

Fig. 1 - The ultrastructures of Scots pine needles under TEM. (a) Cross-sections of mesophyll cells in control needles. (b, c) Plants treated with 50 ppm CuNPs; chloroplasts with disturbed ultrastructure and a large number of plastoglobules and osmophilic globules are visible. (d) The chloroplasts from the 50 ppm AgNP-treated plants have been modified from lenticular to round. (cw): cell wall; (cn): cell nucleus; (m): mitochondrion; (ch): chloroplast; (og): osmophilic globule; (white arrow): plastoglobule. Scale bars represent 2 μm.
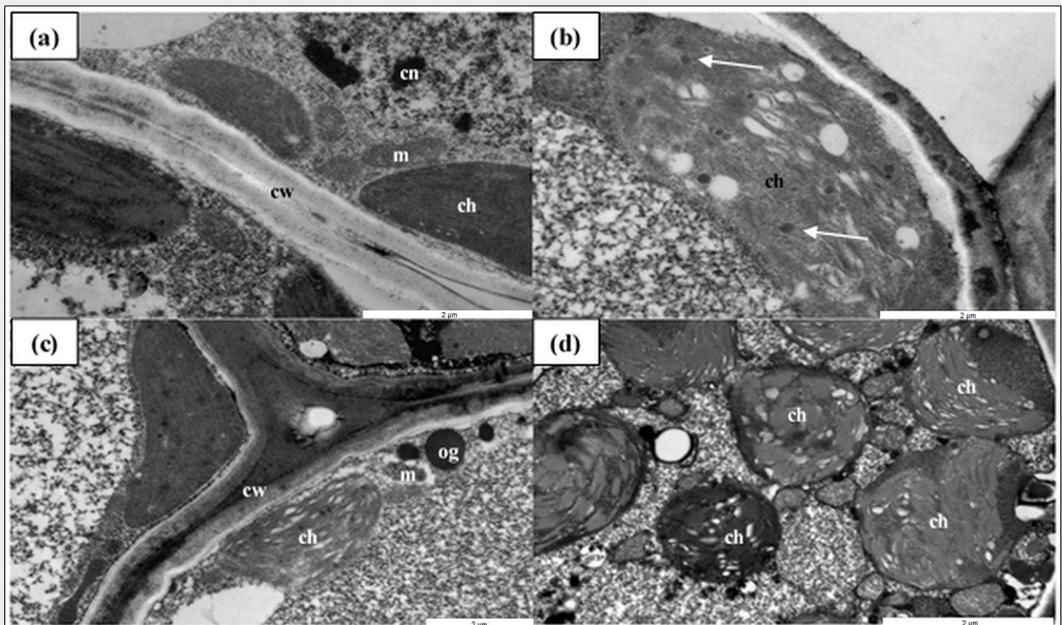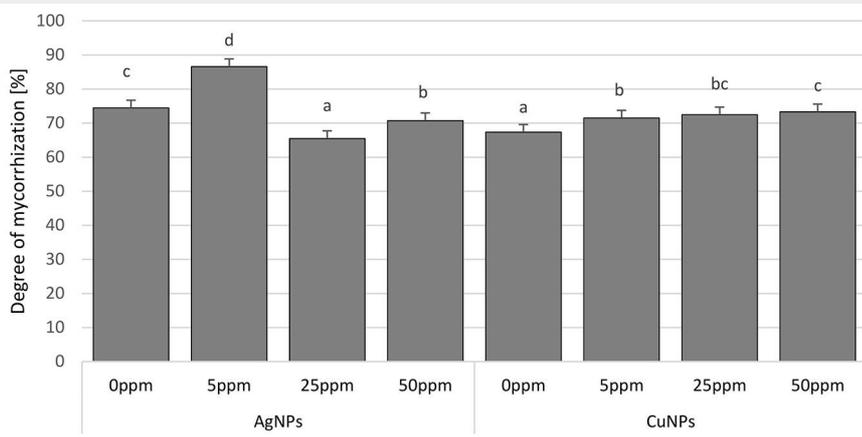
Fig. 2 - Degree of mycorrhization (%) of Scots pine seedlings treated with AgNPs and CuNPs. Concentrations of NPs: 0 ppm, 5 ppm, 25 ppm, and 50 ppm. Different letters indicate significant differences between treatments with different applied concentrations of AgNPs and CuNPs as assessed using Tukey's test (α=0.05).

Fig. 3 - Mycorrhizal morphotypes observed on Scots pine seedlings. (a) *Thelephora terrestris*, (b) *Suillus bovinus*, and (c) *Sphaerosporella brunnea*. White bars = 1 mm.
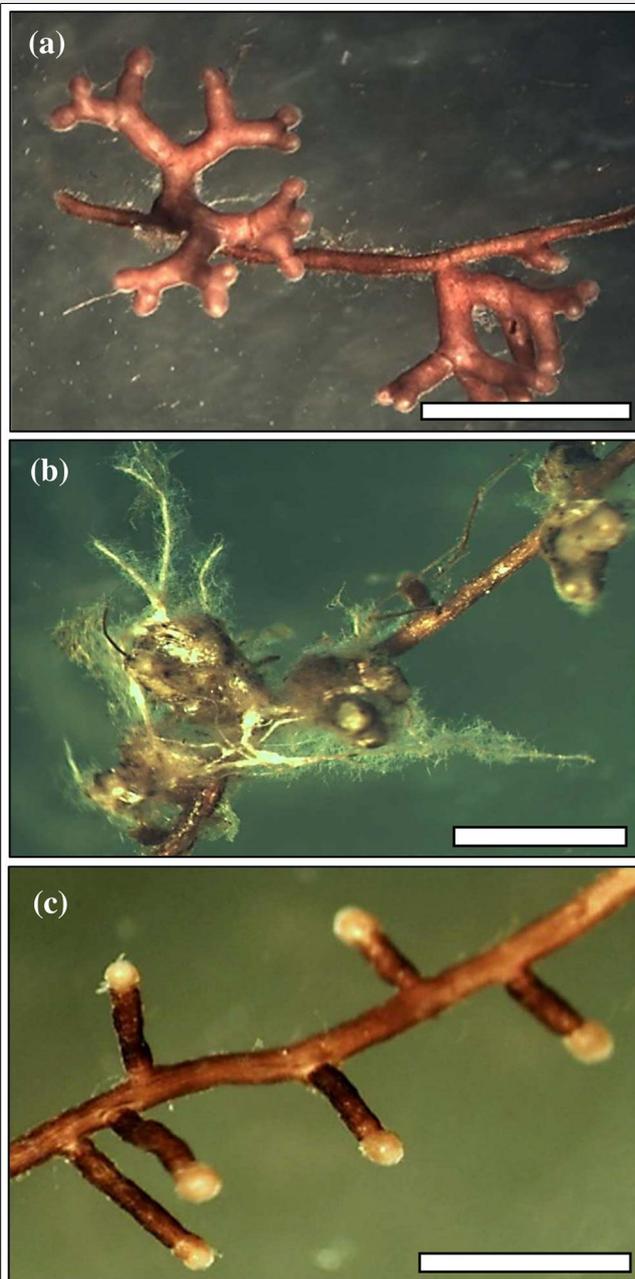
effects of the different concentrations of AgNPs and CuNPs on the degree of mycorrhization and abundance of mycorrhizal fungi was performed using generalised linear models (GLM) for a randomised complete block design with a binomial probability distribution. The Tukey's test was used in pairwise comparisons (as a *post-hoc* test) between different concentrations of AgNPs and CuNPs. The GLM model for the proportion of the defined root types (mycorrhizal and non-mycorrhizal) is given by (eqn. 1):

$$p_{ijk} = \theta + a_i + b_{j(i)} + g_k + e_{ijk} \qquad (1)$$

where p_{ijk} is the numerical proportion of specific root tips (mycorrhizal and non-mycorrhizal) for the *j*-th concentration of nanoparticles nested in *i*-th type of metal nanoparticles in the *k*-th block, θ is the grand mean, a_i the effect of the *i*-th type of metal nanoparticle, $b_{j(i)}$ is the effect of the *j*-th concentration nested in the *i*-th type of metal nanoparticle, g_k is the random effect of the *k*-th block and e_{ijk} is the random experimental error. The statistical analysis was performed using R version 3.3.3 (R Core Team 2012) with the accepted level of significance set at p<0.05.

Results

Both types of NPs did not consistently stimulate growth in the 2-year-old pines. Seedlings treated with AgNPs at concentrations of either 5 or 50 ppm had significantly greater dry masses of roots (3.00 and 2.20 g, respectively). The remaining growth parameters in pine seedlings sprayed with AgNPs did not differ from those of untreated seedlings. For the variant involving CuNPs, at the application concentration of 25 ppm, seedlings had significantly greater values for all biometric parameters analysed except girth of the root collar (Tab. 1).

The ultrastructures of the shoots and roots were similar in nanoparticle-treated and control plants (data not shown). In contrast, plants treated with CuNPs and AgNPs (at only the highest concentration of 50 ppm) exhibited disrupted ultrastructure in their needles, especially in the photosynthetic apparatus. In the control plants (no AgNP and CuNP), chloroplasts filled the cells and contained single plastoglobules, while the NP-treated plants exhibited a large number of plastoglobules, and the shape of chloroplasts of NP-treated plants were modified from lenticular to round. In addition, large, osmophilic globules were present in the cytoplasm of NP-treated needle cells. Application of the CuNPs, compared with the AgNPs, resulted in more limited disturbance to the ultrastructure of mesophyll cells in the pine needles. Other organelles (*e.g.*, mitochondria, cell walls and vacuoles) remained unchanged. These results indicate that Cu- and AgNPs at high concentrations (50 ppm) were able to exert an influence on the ultrastructure

of chloroplasts in pine needles (Fig. 1).

After two years of growth in a peaty substrate in the containers, just over 70% mycorrhization had occurred on average. This process was stimulated by CuNPs at all concentrations and by AgNPs at the lowest applied concentration (5 ppm). Application of AgNPs at the higher concentrations (25 and 50 ppm) was shown to be associated with a more limited role for ectomycorrhizae than in the control (Fig. 2).

The species of ectomycorrhizal fungi found on seedling roots were *Thelephora terrestris*, *Suillus bovinus* and *Sphaerosporella brunnea* (Fig. 3). In comparison with other species, *T. terrestris* had higher colonisation and statistically greater growth when concentrations of both NPs were higher. In contrast, *S. bovinus* was a more abundant coloniser of roots among seedlings not treated with NPs (20.6% and 35.2% with AgNPs and CuNPs, respectively). The participation of this fungus was more limited with successively higher concentrations of the two NPs. The presence of *S. brunnea* was the most limited of the three and was significantly greater where neither AgNPs nor CuNPs were applied (16.1% and 13.6%, respectively – Fig. 4).

Discussion

Two growing seasons of treatment of pine seedlings with AgNPs and CuNPs revealed a significant impact on all of the parameters considered, *i.e.*, growth, the degree of ectomycorrhizal colonisation of roots, species composition of fungi and needle ultrastructure.

Both NPs could stimulate growth, although there was a differential effect, with the use of AgNPs leading, rather unaccountably, to an increase (over the control) in dry masses of roots at doses of both 5 and 50 ppm. In addition, growth stimulation of seedlings treated with CuNP was only observed with the 25 ppm application, although some stimulation was visible for most of the parameters investigated. Copper is a micronutrient essential for proper plant growth. The most abundant copper-containing protein is plastocyanin, which is involved in photosynthetic electron transport in the thylakoid lumen of chloroplasts. Furthermore, copper acts as a prosthetic group of copper/zinc superoxide dismutase, which protects the photosynthetic apparatus from oxidative stress. Despite its physiological importance, excess copper is toxic to plants because of its potential participation in the Fenton reaction (Yamasaki et al. 2008). On the other hand, while organic and peat soils both have copper contents adequate for plant growth, they may be low in forms that can be readily taken up, reflecting the fact that the organic acids (humic, crenic and fulvic) present in peat strongly bind copper in complexes in which the element is rendered plant-unavailable (Schnitzer & Hansen 1970). Thus, the use of CuNPs with pine seedlings grown in a peaty substrate
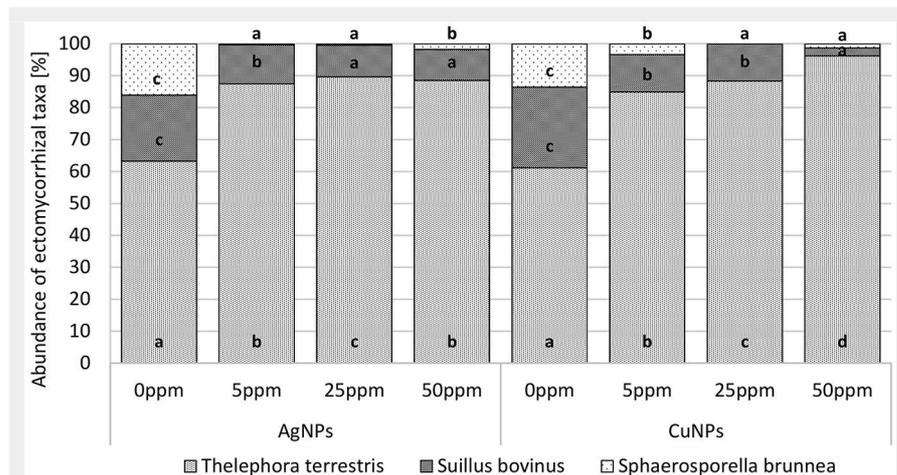
Fig. 4 - Abundance of ectomycorrhizal fungi (%), *Thelephora terrestris, Suillus bovinus* and *Sphaerosporella brunnea*, of Scots pine seedlings treated with AgNPs and CuNPs. Concentrations of NPs: 0 ppm, 5 ppm, 25 ppm, and 50 ppm. Different letters indicate significant differences between treatments with different concentrations of AgNPs and CuNPs for each ectomycorrhizal taxon as assessed using Tukey's test (α=0.05).

and in soil in a nursery may yield different results. Our experiment using 25 ppm CuNPs might have represented a source of copper well above that present in the Osmocote fertiliser, with the dose being markedly higher than in the 5 ppm application and hence, capable of influencing the course of photosynthesis with a stimulatory impact on seedling growth. Moreover, the 50 ppm does appear to have induced certain changes in chloroplasts, even if these changes do not actually reflect toxic effects of the CuNPs. Thus, the benefits of any stimulation due to the extra source of Cu may have been suppressed, resulting in the growth of pine seedlings being comparable with that of the control.

The experiment presented here revealed chloroplasts as cell organelles sensitive to the action of NPs. Deliberate exposure of young pines to the highest concentrations (50 ppm) of NPs was sufficient to produce a change in the shape of chloroplasts from lenticular to spherical, with a marked increase in numbers of plastoglobules, and large osmophilic globules in the cytoplasm, which is likely an indication of the final stage of chloroplast disintegration. The application of CuNPs resulted in a more limited disturbance of ultrastructure in needle mesophyll cells than for application of AgNPs. Indeed, similar results were obtained by Lalau et al. (2014), who found greater numbers of plastoglobules in the aquatic plant *Landoltia punctata* when it was treated with copper (II) oxide nanoparticles. Plastoglobules are subcompartments of thylakoids, containing enzymes that participate in lipid metabolic pathways. It is well documented that under conditions of biotic and abiotic stress, plastoglobules increase in both size and number (Selga & Selga 1996, Piller et al. 2012). Clearly, the results obtained demonstrate that the highest concentrations (50 ppm) of both NPs exert limited, but nevertheless phytotoxic,

impacts.

To date, only a few studies concerning the influence of NPs on plant growth have been conducted on trees. However, in the cases that are known, involving willows treated with TiO₂ nanoparticles, wild pear seedlings receiving nanoparticles of SiO₂ and pedunculate oak seedlings treated with AgNPs and CuNPs (as here), negative influences on growth have not been reported (Zarafshar et al. 2015, Olchowik et al. 2017). Only the work by Sweet & Singleton (2015) revealed a significant inhibitory impact of AgNPs on the growth of bishop pine. However, the fact that the research cited involved different species of trees precludes any comparison with our results. Nevertheless, many factors can affect the impact of NPs on plant growth, with target species of plant being regarded among the most important (Servin et al. 2015).

Scots pine is strongly dependent on ectomycorrhizal symbiosis (Wang & Qiu 2006). In our studies, ectomycorrhizal colonisation was between 65.5% (with AgNPs at 25 ppm) and 86.6% (with AgNPs at 5 ppm). The level of mycorrhizal colonisation depends on several factors, such as applied cultivation system (bare-root *vs.* container, outdoor *vs.* greenhouse), plant species (Menkis et al. 2005) and the location and identity of the nursery in which seedlings are grown (Iwanski et al. 2006). It is typical for bare-root seedlings to achieve almost 100% mycorrhizal colonisation (Leski et al. 2010, Pietras et al. 2013), while the level is often lower in the case of container-grown seedlings (Menkis et al. 2005).

In our case, ectomycorrhizal colonisation of the 2-year-old pines varied in relation to the types and concentrations of NPs applied. Use of CuNPs was observed to stimulate the formation of ectomycorrhizae irrespective of concentration. In contrast, with AgNPs, only the lowest concentration (5 ppm) produced increased colonisation of

roots by ectomycorrhizal fungi, with the higher concentrations actively inhibiting the process. Given that the formation and further functioning of ectomycorrhizae is conditioned by a range of factors linked with both host species, fungal partner and the environment (Smith & Read 2008), it is possible to propose several hypotheses that might account for the results we obtained. For example, an inhibitory influence due to higher concentrations (25 and 50 ppm) of AgNPs might reflect the stronger antifungal properties of silver (Kashyap et al. 2012).

Ectomycorrhizal fungi are very much dependent on products of photosynthesis reaching plant roots (Högberg et al. 2008). Many studies reveal how NPs have a favourable influence on chlorophyll content in plants and on the course of photosynthesis (Yang et al. 2007, Linglan et al. 2008, Jaberzadeh et al. 2013). Thus, the stimulation of the formation of mycorrhizae in our experiment may reflect larger amounts of sugars produced in photosynthesis reaching the seedling roots. This scenario does not have to equate to simultaneous stimulation of growth because seedlings with a higher level of mycorrhization are actually characterised by less-favourable growth parameters (Stenström & Ek 1990).

The formation and functioning of mycorrhizal symbiosis is influenced by not only the host plant and fungal partner but also interactions with mycorrhizal helper bacteria (Smith & Read 2008). The application of nanoparticles may increase the population of soil microorganisms and their activity in the rhizosphere (Raliya & Tarafdar 2013, Tarafdar et al. 2014). Ge et al. (2012) have shown that a large group of bacteria, including *Bacillus, Pseudomonas, Lactococcus* and *Serratia*, have no response to nanoparticles. As the mycorrhizal helper bacteria may be included in this group, the influence on mycorrhizal associations may be a positive one (Dahm 2009).

For the stimulated formation of ectomycorrhizae owing to the use of AgNPs at the lowest concentration, it is also possible to invoke the "ethylene hypothesis". Ethylene is a plant growth hormone serving in a wide range of functions in plants. Its influence on physiological processes is not merely dose-dependent but also reflects the sensitivity of tissues changes in the course of the development of plant organs (Yang 1987). Ethylene is also involved in plant responses to microbial pathogens and herbivorous insects, as well as in interactions between plants and beneficial microbes and insects (Broekgaarden et al. 2015). Ethylene treatment was found to impede fungal colonisation of roots during mutualistic symbiosis between *Laccaria bicolour* and *Populus* (Plett et al. 2014). Additionally, it is also known that silver ions block receptors for ethylene in cells, limiting the hormone action on the plant (McDaniel & Binder 2012). Stimulated formation of mycorrhizae may occur in this way.

The roots of our pine seedlings were found to host ectomycorrhizal fungi of the species *T. terrestris, S. bovinus* and *S. brunnea*. However, *T. terrestris* was dominant in all experimental treatments, with a more limited role played by either *S. bovinus* or *S. brunnea*. Equally, significant differences from the control (receiving 0 ppm of AgNPs and CuNPs) were noted for treatments with the two types of nanoparticle in relation to the roles played by the different species of fungi. A higher proportion of *S. bovinus* and *S. brunnea* was observed where AgNPs and CuNPs applications were absent, while our results clearly demonstrated that the formation of mycorrhizae by these two species was obstructed by applications of both kinds of NP at all tested concentrations. Inhibition of *S. bovinus* and *S. brunnea* gave rise to a nearly 90% increase in the role played by what is normally only a weakly competitive species, *i.e.*, *T. terrestris* (Villeneuve et al. 1991). The major role played by the latter in our experiments may not be surprising as this species is a pioneer mycobiont well adapted to nursery conditions (Colpaert 1999) and often is the dominant ectomycorrhizal fungus in nurseries (Iwanski et al. 2006). On the other hand, *T. terrestris* is regarded as only weakly competitive with other ectomycorrhizal fungi (Villeneuve et al. 1991) and cannot survive, even in the absence of competing species, at sites adversely influenced by the presence of heavy metals (Smith & Read 2008). Silver and copper are typical heavy metals. Similarly, research by Sweet & Singleton (2015) found that *Thelephora* sp. was very vulnerable to the action of AgNPs, whose presence was associated with the lack of the mycorrhizae typically formed by the species in infected soil. Furthermore, the high level of sensitivity of *S. bovinus* to the action of both NPs at all concentrations was surprising, given that *Suillus* fungi are regarded as very resistant to the impacts of industrial air pollutants, including heavy metals, when compared with other species. Attempts have even been made to use *Suillus* species in bioremediation (Dahlberg & Finlay 1999). However, it is possible that the results we obtained reflected the high degree of variability present between one strain of the fungus and another (Aleksandrowicz-Trzcinska & Grzywacz 1997). Perhaps, the strain of *S. bovinus* colonising the roots of pines in our experiment are among those more vulnerable to both of the nanoparticles used (Capuana 2011).

Conclusion

Both types of nanoparticles were found to have a stimulatory effect on both the growth of seedlings in Scots pine and their generation of ectomycorrhizae. The effect of the application of CuNPs was more favourable than that of AgNPs. Both types of nanoparticles produce minor changes in the ultrastructures of pine needles that relate to the chloroplasts (but only at a con-

centration of 50 ppm). Our results indicate the possibility of CuNPs and AgNPs being used as stimulators of the growth and mycorrhizal colonisation of container-grown Scots pine trees. However, further research is needed to determine the optimal doses and concentrations of the NPs.

List of abbreviations

The following abbreviations have been used throughout the paper:
• AgNPs: silver nanoparticles;
• CuNPs: copper nanoparticles;
• Nps; nanoparticles;
• TEM: transmission electron microscope.

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