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SHORT COMMUNICATIONS

Do silver nanoparticles stimulate the formation of ectomycorrhizae in seedlings of pedunculate oak (*Quercus robur* L.)?

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Abstract
Metal nanoparticles are gaining ever-wider application in agriculture and forestry, as alternatives to chemical agents used as fertilisers, growth stimulators and pesticides, establishing a need for eco-toxicological risk assessment of these agents. We tested the effects of foliar-applied silver nanoparticles (AgNPs) on chlorophyll a fluorescence and on abundance and species composition of ectomycorrhizal (ECM) colonisation. The application of AgNPs at concentrations of 5, 25 and 50 ppm was found to stimulate the formation of mycorrhizae in seedlings of pedunculate oak, with the highest effect at intermediate concentrations (25 ppm). There were non-linear effects on the relative abundance of ECM fungal species. The proportion of dominant *T. terrestris* was highest in the control group, whereas the shares of ECM formed by the two other species, *S. brunnea* and *P. involutus*, were higher in the treatments with intermediate and maximal concentrations of AgNPs, respectively. Maximum quantum yield of photosystem II (Fv/fm) assessed by chlorophyll *a* fluorescence measurements revealed slight debilitation of oak seedlings irrespective of the application of AgNPs and their concentrations. This result offered an indirect indication that photosynthesis capacity had no influence on the level of mycorrhization. We hypothesise that foliar AgNPs treatments at concentrations below thresholds of acute toxicity and in the absence of significant effects on chlorophyll a fluorescence may still exert significant influence on biotic interactions including mycorrhizal symbioses by impacting plant hormonal balance, particularly ethylene, and regulatory pathways involved in host control of ECM colonisation.

Keywords Chlorophyll fluorescence · *Paxillus involutus* · *Thelephora terrestris* · *Sphaerosporella brunnea*

1 Introduction

Nanoparticles (NPs) are atomic or molecular aggregates with two dimensions between 1 and 100 nm (Klaine et al. 2008). It is the combination of such small dimensions and the proportionately large external surface area that is known to confer physico-chemical and bioactive properties on nanoparticles that are markedly different from those characterising bulk material of the same kind (Nel et al. 2006). NPs have a wide range of potential applications in agriculture and forestry, inter alia as alternatives to the chemicals used as fertilisers, growth stimulators and plant protection agents (Abd-Elsalam 2012; Mukhopadhyay 2014). Furthermore, NPs are increasingly used in various industries and consumer products, resulting in the release of synthetic NPs into the environment and causing increasing eco-toxicological concern (Sweet and Singleton 2015).

Research to date points to a differentiated influence of nanoparticles on higher plants, in that both positive and negative effects have been noted (Rizwan et al. 2017). Such differences relate *i.a.* to species of plant, growth conditions (e.g. in soil or different nutrient media), type and concentration of nanoparticles, means of application (foliar or soil) and dose applied (Ruffini Castiglione and Cremoni 2009; Ma et al. 2010; Arruda et al. 2015; Rizwan et al. 2017), This all ensures

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considerable difficulties with any comparing of obtained results (Sweet and Singleton 2015). However, many authors point to a positive influence of nanoparticles on seed-germination, plant growth, chlorophyll content and the course assumed by photosynthesis (Zheng et al. 2008; Sharma et al. 2012; Syu et al. 2014; Razzaq et al. 2016).

Mycorrhizal symbiosis is of key interest to foresters, because the fungi responsible influence the productivity of trees and diversity of stands, playing a key role in the cycling of carbon, nitrogen and phosphorus (van der Heijden et al. 2015). Mycorrhizal development and functioning is shown to be under the marked influence of environmental factors, host plant, the physiology of the symbiotic fungi, and interactions with other soil microorganisms (Smith and Read 2008). Equally, the deployment of nanoparticles (as growth stimulators or plant protection agents) in the nursery production of seedling trees for forestry is linked with certain dangers that influences on both host plant and mycorrhizal partner will prove unfavourable.

Currently, there remains little research on the influence nanoparticles exert on the state and structure of mycorrhizal fungi. Most of the work that has been done concerns arbuscular mycorrhiza, with results resembling those concerning the influence on plants, in that there is ambiguity. While some studies point to a negative influence of nanoparticles on the mycorrhizal colonisation of plant roots (Dubchak et al. 2010), others suggest that, at low concentration, there is no discernible impact on levels of mycorrhizal colonization, even if high concentrations do indeed seem to curb this (Judy et al. 2015; Cao et al. 2017). For their part, Feng et al. (2013) reported a stimulatory influence of AgNPs on the formation of arbuscular mycorrhizae irrespective of concentrations investigated.

Contradictory results have been supplied by different authors researching effects of AgNPs on ECM symbiosis. Sweet and Singleton (2015) showed how soil contamination with high doses of AgNPs contributed to a drastic reduction in the diversity of ectomycorrhizal fungi on the roots of seedling bishop pines (*Pinus muricata*); while Olchowik et al. (2017) found different influences of foliar-applied metal nanoparticles on the formation of ectomycorrhizae of pedunculate oak seedlings, in relation to both type of particle and concentration. While the influence of AgNPs was stimulatory at all tested concentrations, that of copper nanoparticles was shown to be positive at low concentrations, inhibitory at higher ones.

Among the methods by which the impacts on plants of both biotic and abiotic stress factors are researched, it is chlorophyll *a* fluorescence measurement in vivo that has gained wide application (Maxwell and Johnson 2000). Interactions between AgNPs, photosynthesis and chlorophyll a fluorescence may involve diverse mechanisms. Chlorophyll fluorescence quenching by AgNPs was demonstrated in vitro (Falco et al. 2015). In vivo the disruption of energy transport is the main mechanism inhibiting maximum and effective quantum yield of PS II, inhibiting the photo-protective capacity of PS II and resulting in the induction of reactive oxygen species in the chloroplast, in inhibition of Rubisco and in decreased CO2 assimilation (Jiang et al. 2017). AgNP impact on on PS II is a key mechanism of AgNP toxicity to plants, therefore chlorophyll a fluorescence measurements appear to be particularly suited the assess this impact. However, most studies on AgNPs, photosynthesis and chlorophyll a fluorescence used algae or aquatic plants (e.g. Jiang et al. 2017). The more complex and resistant structures of land plants foliage constitute barriers to protect the chloroplasts from environmental stressors, reducing the impact of AgNPs on photosynthesis, too. Furthermore, plants challenged with AgNPs can increase chlorophyll content, potentially overcompensating inhibitory effects on photosynthesis, as demonstrated in *Brassica juncea* (Sharma et al. 2012).

The objectives of the work detailed here were: (1) to assess plant condition on the basis of chlorophyll *a* fluorescence measurements following AgNPs application, (2) to compare the abundance of mycorrhizal fungi on seedlings of pedunculate oak in relation to applications of AgNPs at different concentrations.

2 Materials and methods

2.1 Study design and plant material

The study was conducted at the nursery of the Forest Experimental Station of Warsaw University of Life Sciences (at Rogów, 51°40'N, 19°55'E, 195 m a.s.l). Seedlings of pedunculate oak were grown in V-360 plastic container trays (15 pots per tray with a capacity of 360 cm^3) in a peat–perlite substrate purchased from the container nursery at Nędza, Rudy Raciborskie Forest District, Poland. The substrate (pH = 4.5) comprised sphagnum peat from Estonia - 85%, plus coarse-grained perlite (No. 3) - 15%, the latter being thought to improve the aeration of the substrate. Seedlings were fertilised with a mixture of Osmocote Exact Standard controlled-release fertiliser with differential release characteristics: 3-4 M, 5-6 M, 8-9 M (1:1:1). The seeds of pedunculate oak were of local origin, from managed forest. On May 12th 2015, two oak acorns respectively were seeded to each pot, in order to obtain seedlings in each. After germination, one plant per pot was selected at random, while the others were removed. Colonization by ECM fungi occurred spontaneously. There were four spray treatments of the seedlings' aerial parts using 1000 l/ha (=100 ml/m^2) aqueous solutions of AgNPs at concentrations of 0, 5, 25 and 50 ppm. Previously, the NPs had been suspended in deionised water by way of vigorous shaking for at least 10 min. Nanoparticles were applied to foliage four times, i.e. on 11 and 25 June, 9 July and 6 August 2014. The first treatment was applied one month after sowing, by which time the seedlings had formed two leaves.

Sprayings were done in the early morning, with a manual compressed air sprayer (Kisan Kraft, model KK-PS5000).

The experiment was performed on a randomised complete block design. At the end of the growing season (October), 40 seedlings (10 plants × 4 blocs) were harvested at random from each treatment, 160 seedlings sampled in all.

2.2 Nanoparticles

Samples of commercially available solutions of AgNPs were purchased from Nano-Koloid (Nano Koloid Sp. z o. o), as a licensee of Nano Technologies Group, Inc. (USA), manufactured in accordance with European patent EP2081672 A2. As the producer notes, these are generated by way of a physical process, consist of around 100 atoms each, and are suspended in demineralised water. The concentration of nanoparticles in the commercially-available product is 50 ppm.

2.3 Chlorophyll *a* fluorescence measurements

The fluorescence measurements for chlorophyll *a* were made using a Hansatech FMS-2 fluorimeter (Hansatech Ltd., Kings Lynn, UK), on 1, 10, 17, 24 and 31 July; 7, 14, 21 and 28 August; and 15 and 28 September 2015. They were performed on 10 randomly-selected, fully-developed leaves from 10 oaks in the case of each treatment variant. With the aid of clips, leaves were first adapted to darkness for a period of 20 min before being connected up to an optical fibre. The fluorescence parameters considered in relation to chlorophyll *a* were: Fo – i.e. initial (zero) fluorescence following adaptation to the darkness, Fm – maximum fluorescence following adaptation to the darkness, and Fv/fm – as the maximum efficiency of photosystem II (PSII), where Fv – is the fluorescence variable Fm-Fo.

2.4 Assessment of ectomycorrhizae

The roots of harvested seedlings were analysed under a stereoscopic microscope (Delta IPOS-808), as 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. The abundance of individual ectomycorrhizal fungal taxa was calculated as the number of ectomycorrhizal root tips of each morphotypes divided by the total number of roots tips and multiply by 100. The frequency of ectomycorrhizal taxa was expressed as the percentage of colonized plants. 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 1987–2008). 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, 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.

Identification of mycorrhizal fungi included PCR amplification of selected regions of internal transcribed spacer (ITS) rDNA using the primer pair ITS1F / ITS4 (Gardes and Bruns 1993; White et al. 1990) and sequencing of the resulting PCR product. We used direct PCR amplification of fungal DNA from ECM samples, bypassing conventional DNA extraction procedures (Iotti and Zambonelli 2006). PCR products were assessed by loading 2 μL on to a 1% agarose gel (0.5 x TAE buffer) and visualised under UV light, using the GeneRulerTM 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 the PCR primers each. The resulting fragments were analysed on a 3730 DNA Analyser at the Department of Botany and Biodiversity Research of the University of Vienna. Identification of sequenced fungi was based on the results of BLAST (Altschul et al. 1997) searches against the National Centre for Biotechnology Information (NCBI) public database, with subsequent phylogenetic placement and queries to the UNITE database (Kõljalg et al. 2013).

2.5 Statistical analysis

Analysis of the effects of the different concentrations of AgNPs on the abundance of mycorrhizal fungi was performed using Generalised Linear Models (GLM) for a randomised complete block design with a binomial probability distribution. The Tukey test was used in pairwise comparisons (as a post hoc test) between different concentrations of AgNPs. We tested the significance of changes in fluorescence over time, with different concentrations of AgNPs applied, using a growth-curve model. The statistical analysis was performed using R version 3.3.3, with the accepted level of significance set at $p < 0.05$.

3 Results

Mean values (for all measurement dates) for the maximal efficiency of photosystem II (given by Fv/fm) were found to range between 0.79 where no AgNPs were applied, and 0.76 – in the case of the treatment variant with 50 ppm AgNPs applied (Fig. 1). They were not found to vary significantly in relation to either application of AgNPs and their concentrations, or time of measurement ($p = 0.1693$, $p = 0.1104$ respectively).

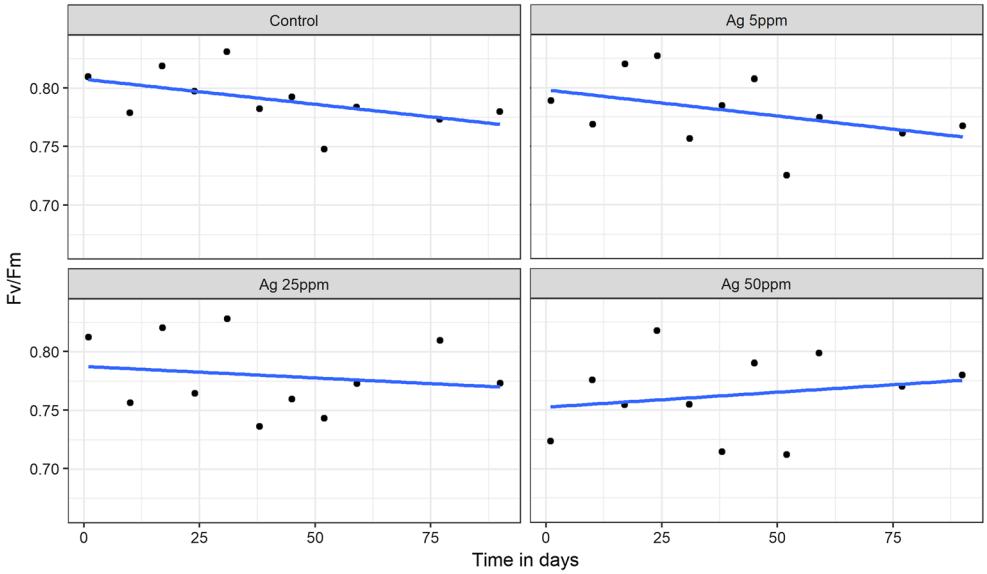

Fig. 1 Chlorophyll *a* fluorescence, i.e. maximal efficiency of photosystem II (Fv/fm) in oak seedlings treated with different concentrations of AgNPs. Points denote mean values established by reference to 10 measurements made on each of 11 different dates (where date 0 = July 1)

The mean proportion of short roots with vital mycorrhizae present in seedlings of pedunculate oak in all of the experimental treatments was 47.8%. The use of AgNPs at all different concentrations was associated with a higher level of mycorrhizal colonisation than was present in the control (42.5%, df = 3, $p < 0.00001$). The highest level reported was the 54.2% associated with the application of AgNPs at a concentration of 25 ppm (Fig. 2).

The 3 fungal species identified were one representative of the Ascomycota (.i.e. *Sphaerosporella brunnea*, KC008076.1, identity 98%), and two of the Basidiomycota (i.e. *Thelephora terrestris*, FJ809998.1, identity 99% and *Paxillus involutus* EU078725.1, identity 98%). *T. terrestris* was the most abundant ECM species in all treatments (32.4% on average), reaching highest levels under control conditions (35.1%) and the lowest levels (27.9%) in the 5 ppm AgNP treatment. There were quite disparate shares of *S. brunnea* on the roots of the seedlings studied - only a third or half as many in the treatment receiving no application of AgNPs (at 4.5%), as compared with the treatments actually receiving the nanoparticles. The largest share was the 16.1% noted where AgNPs had been applied at a concentration of 25 ppm. The proportion of *P. involutus* ECM was highest in the treatment with the highest dose – 50 ppm of AgNPs (6.6%), and lowest in the control treatment (3.1%). 5.3% were recorded in the 5 ppm treatment, 3.4% in the 25% treatment (Fig. 2). The most frequently observed EMC fungus was *T. terrestris* (Table 1).

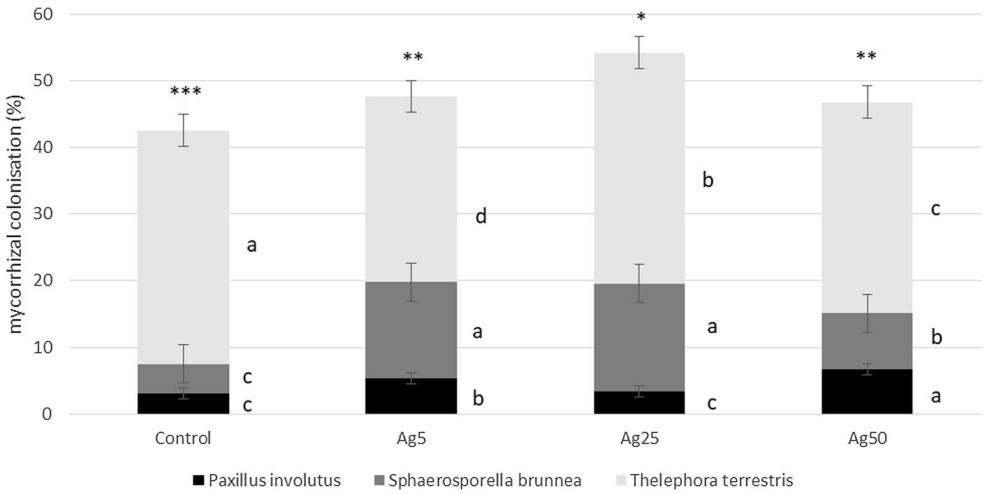

Fig. 2 Degree of mycorrhization and abundance of ECM fungi: *Thelephora terrestris, Sphaerosporella brunnea* and *Paxillus involutus* [%] of oak roots treated with concentrations of AgNPs equal to 0–0 ppm; 5–5 ppm; 25–25 ppm; 50–50 ppm. Numbers of asterisks indicate significant differences between concentrations of AgNPs for degree of mycorrhization assessed with Tukey's test ($\alpha = 0.05$), different letters indicate significant differences between concentrations of AgNPs for each ECM taxa assessed with Tukey's test ($\alpha = 0.05$). While error bars represent standard error

4 Discussion

Fv/fm values between 0.78 and 0.85 are considered indicative of healthy, nonstressed deciduous trees, including *Q. robur* seedlings (Percival 2005). The mean Fv/fm values (all measurement dates averaged) observed, ranging between 0.76 and 0.79, were distributed around the lower margin of the range of reference values for optimal growth conditions.

The absence of significant effects of foliar AgNP treatments on chlorophyll a fluorescence was unexpected, since this parameter is considered to be sensitive to various stressors, and since ultrastructural alterations of chloroplasts had been observed in a previous study under similar experimental conditions (Olchowik et al. 2017). On the other hand, this result is compatible with the lack of effects on the seedlings´ growth parameters (Olchowik et al. 2017). While chlorophyll a fluorescence is not strictly proportional to the intensity of photosynthesis (expressed via the assimilation of CO_2 or emission of O_2; Maxwell and Johnson 2000) it seems unlikely that increased levels of mycorrhizal colonisation linked to AgNPs treatments (see below) were due to increased photosynthesis, which might have increased the amount of sugar reaching the roots and ECM fungi (Högberg et al. 2008).

The mean proportion of short roots with ectomycorrhizae present in seedlings in all of the experimental treatments was 47.8%. The level of mycorrhizal colonisation is typically found to depend on several factors, such as cultivation system (bare-root vs. container, outdoor vs. greenhouse), plant species (Menkis et al. 2005) and the place or nursery in which the seedlings have been grown (Iwański et al. 2006). It is typical for seedlings grown outdoor, with bare-root systems, to be characterised by almost 100% mycorrhizal colonisation (Leski et al. 2010; Pietras et al. 2013), while the level of colonisation of seedlings grown up in peaty substrates in greenhouse is often lower than this (Menkis et al. 2005).

The use of foliar-applied AgNPs at all different concentrations was associated with a higher level of mycorrhizal colonisation than was present in the control. Similar stimulation of the formation of mycorrhizae was observed in our previous research, in which AgNPs were applied as an alternative to fungicides, with a view to powdery mildew of oak being combated (Olchowik et al. 2017). However, results different from ours were obtained by Sweet and Singleton (2015), in their work with the Bishop pine (*Pinus muricata*) exposed to soils treated with AgNPs. Intensified application of AgNPs was found to reduce, not only the level of mycorrhization, but also the biodiversity of ectomycorrhizal fungi at the level of the genus (from 5 to 1).

The 3 fungal species were identified: *T. terrestris*, *S. brunnea* and *P. involutus.* All of these are typical, commonly-occurring ECM partners of forest tree seedlings, be they produced in bare root or in containers nurseries (Menkis et al. 2005; Rudawska et al. 2006; Sánchez et al. 2014).

T. terrestris was the most abundant and frequent ECM species in all treatments (Table 1, Fig. 2). *T. terrestris* is a pioneer mycobiont, well adapted to nursery conditions (Iwański et al. 2006) or plantations, especially at sites that have been treeless, and thus symbiont-free, for many years (Last et al. 1987). Populations of *T. terrestris* which are not adapted to trace metal stress were found to be very sensitive to high concentrations of AgNPs, like other species of ECM fungi (Sweet and Singleton 2015). Furthermore, *T. terrestris* is considered only poorly competitive with other ectomycorrhizal fungi (Villeneuve et al. 1991). Maximal abundance was recorded in the control treatment, but the moderate decrease in AgNP treatments was not linearly related to AgNPs dose. Variation in the abundance of *T. terrestris* at different levels of AgNP treatments might be the result of competitive interactions with the other ECM fungal species present.

The share of *S. brunnea* was two-three times higher as a result of the application of nanoparticles. The pyrophilous ascomycete *S. brunnea* is a pioneer and opportunist ectomycorrhizal species, and the most common fungal competitor in nurseries producing plants mycorrhized with *Tuber* species, where it can reproduce abundantly, via ascomata and via conidiospores (Sánchez et al. 2014). In natural forest environments fruiting of *S. brunnea* is rarely observed and typically restricted to rather recent fireplaces, a more or less alcaline and chemically reactive substrate quite different from forest soils, which is colonised by highly specialised, stress-resistant pioneer species. *S. brunnea* was also reported to occur in zinc mine spoils, in the presence of high concentration

Table 1 Frequency (percent of colonized plants±standard errors) of fungal taxa on roots of *Q. robur* seedlings treated with different concentrations of AgNPs. GenBank accession numbers of sequences (accession no), best match in GenBank, percent of identity between the best match and representative sequence of the taxa (identity). For each fungal EMC taxa, one sequence was deposited

ECM taxa	Reference sequence	Accession no	Identity (%)	Treatment ($n = 40$)			
				Control	Ag5 ppm	Ag25 ppm	Ag50 ppm
Thelephora terrestris	*Thelephora terrestris* (FJ809998.1)	MK660101	99	90.0 ± 1.24	82.5 ± 1.17	80.0 ± 1.05	87.5 ± 1.12
Paxillus involutus	*Paxillus involutus* (EU078725.1)	MK660102	98	22.5 ± 0.87	17.5 ± 0.68	25.5 ± 0.48	40.0 ± 0.81
Sphaerosporella brunnea	*Sphaerosporella brunnea* (KC008076.1)	MK660100	98	32.5 ± 0.81	52.5 ± 0.95	37.5 ± 0.75	32.5 ± 0.74

of diverse heavy metals (Zn, Pb, Cd; Mleczko 2004). It is likely that *S. brunnea* is constitutively resistant to a wide range of stressors, possibly including AgNPs.

The proportion of *P. involutus* ECM was highest in the treatment with the highest dose of AgNPs, and lowest in the control treatment. Again, there was no strictly linear dose-response relationship, but it is clear that ECM formation by *P. involutus* was not impaired by the AgNP treatments applied. *P. involutus* is particularly resistant to metal stress (Denny and Wilkins 1987; Hintikka 1988; Bellion et al. 2007). Furthermore, *P. involutus* is a characteristic representative of ECM Boletales in forming complex rhizomorphs, which qualify this species as a long-distance explorer, in contrast to *T. terrestris* (medium distance explorer) and to *S. brunnea* (contact type) (Agerer 2001). The observation that ECM formation by *P. involution* was highest in the 50 ppm AgNP treatment may be another indication that photosynthesis was not substantially inhibited by the treatments, since this species needs to maintain a complex and abundant rhizomorph system, which is probably costly in terms of carbohydrate requirements.

The shares accounted for by each of the three ECM species reported may also reflect competition between them, or else differentiated sensitivity to the action of AgNPs. Some studies (in in vitro cultures) do point to such a differentiated direct influence of NPs on (pathogenic and wood decay) fungi (Aleksandrowicz-Trzcińska et al. 2018). Our previous in vitro experiment for example found *Amanita cirtina* displaying a complete lack of sensitivity to the action of the nanoparticle, while *Hebeloma crustuliniforme* proved to be highly sensitive. AgNPs stimulate mycelial growth in *Suillus luteus* (unpublished data). On the one hand, these results indicate high selectivity of NP activity in relation to different species of EMC fungi, but on the other there can be no doubt that the reactions of fungi may be completely different under natural conditions.

Ag + and AgNPs are strong antibacterial and antifungal agents, but the potential to adapt to toxic concentrations of trace metals is widespread among populations of ECM fungi (Urban 2011). Former silver mining sites contaminated with diverse heavy metals were reported to be colonised by ECM fungi of the genera *Cortinarius, Hebeloma, Inocybe, Laccaria, Tomentella, Tricholoma* and *Tuber,* with Thelephoraceae (*Tomentella*) being dominant (Hrynkiewicz et al. 2008). Saprotrophic and ECM macrofungi were found to accumulate Ag, in the case of *Amanita* sect. Vaginata and sect. *Lepidella hyperaccumulation* was observed (Borovicka et al. 2007, 2010). In *Amanita strobiliformis* intracellular Ag was demonstrated to be sequestered by multiple metallothioneins, i.e. cysteine-rich peptides involved in heavy metal tolerance of many eukaryotes (Osobová et al. 2011). Fungal species-specific accumulation of Ag and other heavy metals in ectomycorrhizae was suggested to protect host trees (*Picea abies*) from toxicity (Cejpková et al. 2016). These adaptations to Ag + exposition by diverse ECM fungi are in contrast to the nearly complete inhibition of ECM colonisation of *Pinus muricata* roots in experimental AgNP treatments (Sweet and Singleton 2015). The application of synthetic AgNPs to non-adapted ECM communities versus long-term exposure with naturally occurring silver species might explain the discrepancy of results.

The ECM fungi occurring spontaneously in the tree nursery are more or less stress-tolerant pioneer species not preadapted to silver stress. Shoots and leaves were the primary target of AgNP treatments, limited collateral exposure of soil and roots cannot be fully excluded, but was certainly orders of magnitude lower than exposure of foliage. ECM colonisation may have been influenced by (1) effects on plant condition (productivity, patterns of organic carbon allocation, stress responses, plant hormon status), (2) effects of silver (AgNPs od Ag+) internally translocated to the roots or (3) leaching of AgNPs into the containers. Given the available evidence, we hypothesise that the probable impact of AgNPs on plant hormon balance explains best the aggregate effect on ECM development (see below). Shifts between the abundance of different ECM fungal species may be conditioned by the interaction of plant condition, direct AgNP effects on ECM fungal fitness and competition between ECM species, along with random effects. The complexity of these interactions may explain the non-linearity of the observed effects, particularly at the level of ECM fungal species.

Changes in root structure due to AgNPs include disrupted epidermal cells, highly vacuolated cortical cells (Yin et al. 2011: in *Lolium multiflorum*), shortened or completely reduced root hairs (Yin et al. 2011: in *Lolium multiflorum*; Vannini et al. 2013: in *Eruca sativa*), an increased production of lateral roots primordia (Vannini et al. 2014; in *Triticum*) and a reduction of the length of primary tap roots and of the development of lateral and fine roots at higher AgNP concentrations (Sweet and Singleton 2015; in *Pinus muricata*). Complete reduction of root hairs and increased branching are commonly observed in ECM symbiosis. This commonality led us to the hypothesis that AgNPs might induce physiological and structural changes in plant hosts which increase their susceptibility for ECM symbiosis. The development and branching of plant root systems is controlled by the crosstalk of plant hormones via signalling pathways (Smith and De Smet 2012). AgNPs share various effects on plant physiology with silver ions (Ag+; e.g. applied as AgNO3), while others appear to be specific to AgNPs (Yin et al. 2011; Kaveh et al. 2013; Vannini et al. 2013). Effects in polyamine-, ethylene- and calcium- mediated pathways are considered to be mediated by Ag + (Kumar et al. 2009). In addition to ethylene-regulated pathways, AgNPs were reported to interact with genes involved in auxin- and abscissic acid mediated signalling pathways (Kaveh et al. 2013; Syu et al. 2014). Many genes upregulated by AgNPs and Ag + were linked to oxidative stress and metal-induced stress, while downregulated genes were rather associated with response to pathogens and

hormonal stimuli (Kaveh et al. 2013). The inhibitory effect of silver on ethylene action (Beyer Jr. 1976) was demonstrated to be predominantly mediated by ethylene receptor 1 (ETR1) in *Arabidopsis thaliana* (McDaniel and Binder 2012). Ethylene modulates numerous plant physiological processes, including root growth, root branching, root hair formation and interactions with mutualistic or parasitic symbionts (Kaveh et al. 2013; Syu et al. 2014). Ethylene-mediated defense is part of complex regulatory networks, involving inter alia jasmonic acid and salicylic acid. In general, ethylene is considered a negative regulator of mutualistic root symbioses, despite certain exceptions, e.g. *Piriformospora indica* (Khatabi and Schäfer 2012; Broekgaarden et al. 2015). Inhibition of ethylene-induced effects with ≥10 μM silver thiosulfat had a neutral effect on ECM formation by *Pisolithus tinctorius* and a negative effect on *Laccaria laccata* (Rupp et al. 1989). It is important to note that silver thiosulfat was added to the liquid growth medium and may have directly impaired the growth of *L. laccata*. Plett et al. (2014) demonstrated that ethylene and jasmonic acid treatments reduced the development of the Hartig net in *Laccaria bicolor/Populus* ECM symbiosis, this effect was correlated to increased expression of certain transcription factors, including ERF1.

5 Conclusions

Stimulation of ECM formation by foliar-applied AgNPs, combined with neutral effects on seedlings growth parameters (Olchowik et al. 2017) and chlorophyll a fluorescence is a counterintuitive result, resistant to overly simplistic explanations based on photosynthesis efficiency and resulting organic carbon supply. Alternatively, interactions of AgNPs with plant hormones and developmental programs involved in host control of ECM symbiosis might cause variation in ECM colonisation at foliar AgNP exposition levels below acute toxicity. The design of this study on AgNPs, chlorophyll a fluorescence and ECM formation was originally motivated by the potential perspective of NP applications in forest nursery management and by the need to assess potential risks of AgNPs for biodiversity and forestry sustainability. In order to test our hypotheses linking AgNPs, plant hormone balance and ECM symbiotic relationships (Plett et al. 2014) transcriptomic and/or proteomic studies are required. To this end it may be advantageous to shift to a model host tree of ECM symbiosis, such as *Populus*.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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