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ARTICLE

Mycorrhizal colonization by *Tuber aestivum* has a negative effect on the vitality of oak and hazel seedlings

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ABSTRACT Ectomycorrhizal fungi have a great impact on the ecosystem in boreal and temperate regions, and it has commercial, silvicultural and crop importance as well. The summer truffle (*Tuber aestivum*), a common mycorrhizal partner of several trees, is a valuable ectomycorrhizal fungus since its fruit bodies (ascomata) are a popular and expensive product on the global markets. To understand the physiology and ecology of a natural forest or a plantation, the participants and relationships between them should be examined. Hence, the maximal quantum efficiency of photosystem II centers, that is vitality of half a year old oak (*Quercus robur*) and hazel (*Corylus avellana*) seedlings inoculated with summer truffle was measured. The relation between the vitality of the plants and the rate of colonization of the fungus was examined applying single and multiple linear regressions. In the case of the oak seedlings contamination of *Scleroderma* spp. morphotype colonization was observed. Negative relationship between rate of colonization and the vitality was detected in the case of hazel seedling and non-contaminated oak seedlings. Multiple linear regression analysis revealed that there is no effect of truffle and contaminant fungi together, but alone the truffle has a negative impact. Consequently, the *Scleroderma* ectomycorrhiza seemed to have a balancing effect on the negative impact of summer truffle.
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One of the most common interactions between plants and fungi are the mycorrhizal associations. In boreal and temperate forest regions, the ectomycorrhizal (ECM) fungi are the dominant mycorrhizal partners of trees (Smith and Read 2008). Basically, this association is widely accepted as a mutualistic relationship, in which the fungus, due to its very efficient nutrient uptake, provides water and nutrients for the plant partner and receives assimilates from the host (Peterson et al. 2004; Kirk et al. 2008; Smith and Read 2008). Nevertheless, ECM associations are not always truly mutualistic. At different stages in the life cycle of ECM fungi and depending on the environmental conditions, the same fungus can have saprotrophic, mutualistic, endophytic or necrotrophic stages (Nylund et al. 1982; Downes et al. 1992; Sen et al. 1999; Koide et al. 2011; Vaario et al. 2012; see Figure 2. in Hall et al. 2003 and Figure 1. in Brundrett 2004). As the impact of ECM fungi on the ecosystem is evident (Smith and Read 2008), the study of the physiological effects of ECM fungus species on different woody plant species has an emerging importance.

Several studies showed the positive effect of ECM on the vitality of land plants (Bougher et al. 1990; Muhsin and Zwiazek 2002; Corrêa et al. 2006; Danielsen and Polle 2014), where the vitality was indicated on a dry weight (e.g. Bougher et al. 1990), total chlorophyll content (e.g. Vodnik and Gogala 1994; Kraj and Grad 2013) or photosynthetic activity basis (e.g. Corrêa et al. 2006). The photosynthetic apparatus, especially the photosystem II (PSII) is very sensitive to environmental changes. Among others, both of nutrient deficiencies (Lippemeier et al. 2001), salinity stress (Chen et al. 2004), drought stress (Colom and Vazzana 2003) and pathogen attacks (Berger et al. 2007) decrease the maximum quantum efficiency of PSII. The direct reasons for the measureable decrease are the disturbances of the PSII acceptor side (Šetlík et al. 1990) and the inhibition of the Calvin-cycle (Takahashi and Murata 2005). Thus, photosynthetic activity is strongly related with the vitality of plants (Tsimilli-Michael and Strasser 2008; Baker 2008) and it is widely used in stress detection in plant physiology.

Summer truffle (*Tuber aestivum*) was shown to be associated with numerous temperate European and North-American tree species, such as *Quercus* spp., *Fagus sylvatica*, *Corylus avellana* and *Pinus* spp., *Castanea sativa* and *Carya illioinensis* (Chevalier and Frochot 1997; Wedén et al. 2009; Benucci et al. 2012). Host trees have commercial, silvicultural and crop importance, and the summer truffle as well. The summer truffle, in contrast to the Périgord black truffle (*Tuber melanosporum*), has a wide distribution and tolerates a broad range of climatic condition thus it is one of the most popular truffles (Hall et al. 2007; Benucci et al. 2011). In spite of the economic importance of this fungus, its biology

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Table. 1. The four datasets of the values of fluorescence induction measurements. Co dataset contains all measurements of *Corylus avellana* seedlings. The measurements of *Quercus robur* seedlings sorted into three dataset. QT dataset contains seedlings of *Quercus* with only *Tuber aestivum* mycorrhiza. QTC dataset contains seedlings of *Quercus* with *Tuber aestivum* and also contaminant fungi. QA dataset contains all oak seedlings, which is the sum of QT and QTC datasets.

	Hazel	Oak		
dataset abbreviation	Co	QT	QTC	QA
Tuber aestivum mycorrhiza	x	x	x	x
contaminant mycorrhiza			x	x
population size	27	33	48	81

is barely studied compared to other commercially valuable fungal taxa (e.g. *T. melanosporum*, *Agaricus bisporus*). Summer truffle was shown to increase the vitality of older sessile oak (*Quercus petraea*) plants on a chlorophyll *a* fluorescence induction based technique, the development of this relationship has been hardly known yet (Solti et al. 2011). Thus, we studied the effect of *T. aestivum* mycorrhizal colonization on the vitality of young oak and hazel seedlings.

Materials and Methods

Plant material

Certificated seedlings (Bach et al. 2010) were produced and nursed by Pannon Szarvasgomba Ltd. Co. Pedunculate oak (*Quercus robur*) and common hazel (*Corylus avellana*) seeds were germinated and grown in plastic pots containing sterile peat-perlit mixture. One month old seedlings were planted to other plastic pots filled with the same medium but compounded summer truffle (*Tuber aestivum*) propagules (spores and mycelium fragments) into it. Plants were kept in greenhouse under controlled humidity conditions. Measurements were performed five month after inoculation. The company provided us nine hazel seedlings (in average height of 32±6.8 cm and stem diameter of 5±0.7 mm) and thirty-one oak seedlings (average height of 61±6.3 cm and stem diameter of 7.8±0.8 mm) for the measurements. Among the oak seedlings, contamination by other mycorrhizal fungi was found, but in every cases *T. aestivum* mycorrhiza was present as well.

Chlorophyll a fluorescence induction

Depend on the number of leaves, two or three (in one case only one) healthy, well developed leaves per seedlings were chosen and measured. Fluorescence induction measurements were carried out with intact leaves using a PAM 101-102-103 Chlorophyll *a* Fluorometer (Walz, Effeltrich, Germany). Leaves were dark-adapted for 15 min. The F_o level of fluorescence was determined by switching on the measuring light (modulation frequency of 1.6 kHz and photosynthetic photon flux density (PPFD) less than 1 μmol m^{-2} s^{-1}) after 3 s illumination with far-red light in order to eliminate reduced electron carriers (Belkhodja et al. 1998). The maximum fluorescence yield, F_m, was measured by applying a 0.7 s pulse of white light (PPFD of 3500 μmol m^{-2} s^{-1}, light source: KL 1500 electronic, Schott, Mainz, Germany). The maximal efficiency of PSII centres were determined as $F_v/F_m = (F_m - F_o)/F_m$.

Measurement of fungus colonisation

After the chlorophyll *a* fluorescence induction measurements, roots were gently washed, and the root tips were visually examined under a stereomicroscope. The percentage of the colonization was estimated by a visual investigation on the whole root system of the individual seedlings. In the case of hazel seedlings the estimation was less precise than that of oak seedlings because of the more dense root branching system.

Statistical analysis

For statistical analysis we used separately the independent chlorophyll *a* fluorescence induction values and analyzed four dataset (Table 1): (1) hazel seedlings (*Coryllus*; Co dataset with 27 data); (2) all oak seedlings, (*Quercus* All; QA dataset with 81 data); (3) a subset of QA dataset where only *Tuber aestivum* mycorrhizae were presented (*Quercus Tuber*; QT dataset with 33 data); (4) a subset of QA dataset which contains only those trees that has contaminant fungi together with *Tuber aestivum* mycorrhiza (*Quercus Tuber* and *Contaminant*; QTC dataset with 48 data). We also checked the relationship between the height of the trees and vitality / rate of colonization. Because the water status of the trees strongly affects the stem diameter (Kanalas et al. 2009) it was found not accurate enough to involve into the analyses.

Relation between the vitality and the rate of colonization were analyzed applying single linear regression using ordinary least squares (OLS) method. In the case of QTC and QA datasets, multiple linear regressions were applied, where two explanatory variables were presented: percentage of *Tuber aestivum* colonization and percentage of contaminant fungi colonization. Significance level of $p = 0.05$ was used in all cases. The normality of variables / residuals and the variance homogeneity of residuals were checked on quantile-quantile plot and scale-location plot respectively. The influential points, detected according Cook's distance, were deleted and also the regression outliers were deleted at 99% confidence interval using externally studentized residuals. The analyses were performed with the statistical software R version 3.0.2. (R Core Team 2014).

Results

On hazel seedlings, the average *Tuber aestivum* colonization percentage was 12±7.5%, while that of on oak seedlings was

the double (24±13%). In the case of QTC dataset (Table 1.), the average percentage of contamination was 11±9.8%. Most of the contaminants were *Scleroderma* spp. morphotypes, but on three seedlings, *Tuber brumale* mycorrhiza and on four seedlings, *Tomentella* spp. morphotypes were also occurred. In both hazel and oak, the PSII maximum quantum efficiency was between 0.78 and 0.83. There was no correlation between the height of the trees and the vitality or the rate of the colonization.

All datasets were normally distributed and no tendencies were found in the variances of residuals. None of the datasets (Table 1.) contains influential points, while in the case of QA dataset four, and in the case of Co dataset one regression outliers were deleted. According the multiple linear regression of QA dataset, no significant common effect of *Tuber aestivum* and contaminant fungi was found, while *T. aestivum* explanatory variable had significant negative effect (p=0.027). The two subset of QA dataset also confirm this result. In the case of QT, *T. aestivum* has significant negative effect (p=0.034) (Fig. 1), while in the case of QTC, no significant common or single effect of *T. aestivum* and contaminant fungi were detected. In the case of hazel seedlings (Co dataset) a significant negative effect of mycorrhization was also found (p=0.005) (Fig. 2).

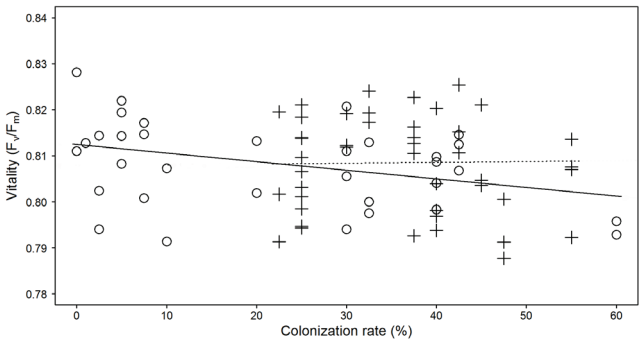

Figure 1. Relationship between the vitality of *Quercus robur* seedlings and rate of colonization of summer truffle. Crosses represent the QTC dataset and open circles represent the QT dataset (see Table 1.). Blank line is fitted to QT dataset, and dotted line is fitted to QTC dataset. In the case of QT dataset the summer truffle has a significant negative effect (p=0.034) on vitality of seedling. In the case of QTC dataset no common or single effects of summer truffle and contaminant mycorrhizal fungi were shown.

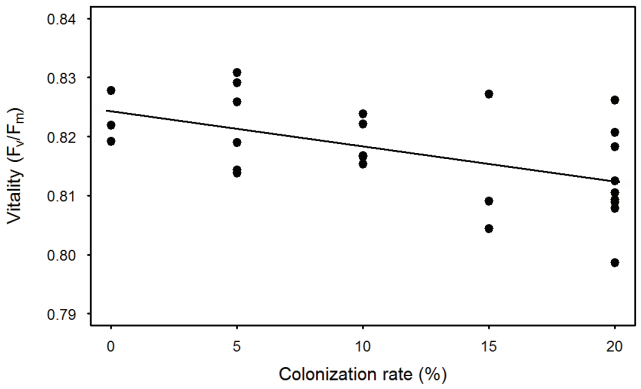

Figure 2. Relationship between the vitality of hazel seedlings and rate of colonization of summer truffle. Mycorrhizal colonization of summer truffle has a significant effect (p=0.005) on the vitality of seedlings.

Discussion

The *Tuber aestivum* mycorrhizal colonization had a significant negative effect on the vitality of seedlings, while the presence of other mycorrhizal fungi seems to fade this effect of summer truffle. Similar results were also shown under the colonization phase of mycorrhiza by other authors. Kraj and Grad (2013) found that mycorrhizal colonization had a negative effect on pigment content of *Pinus sylvestris*. Nevertheless, in a later stage, the pigment accumulation of inoculated plants was enhanced compared to non-inoculated plants. They also showed differences between the effects of different mycorrhizal fungi in respect to the beginning of their beneficial stadium. Colpaert et al. (1992) showed decrease of plant biomass and lower nitrogen content of mycorrhizal plants contrast to control. There is also an example for positive effect of colonized roots on plant dry weight, but no positive relation between the growth rate and the rate of colonization (Lu et al. 1998).

Previous studies have already showed the effect of ECM on green plants. However only just a few article deal with the fluorescence induction parameters of the photosystems. Corrêa et al. (2006) applied chlorophyll *a* fluorescence induction based methods to monitor the effect of *Pisolithus tinctorius* on *Pinus pinaster* vitality through the colonization process. They generally found that mycorrhizal colonization has negative effect on the vitality of plants, but the strength of this effect depends on the availability of the nitrogen source and the age of the plant at the time of inoculation. Solti et al. (2011) found a positive correlation (under 10% of colonization) or no correlation (above 10% of colonization) between the maximal quantum efficiency of PSII centers and summer truffle rate of colonization on *Quercus petraea*. They could observe this positive or no effect because they measured older seedlings.

In the present study, a balancing effect of *Scleroderma* mycorrhization was observed. This could be explained by the differentiation between the physiology of *Scleroderma* sp. and *Tuber aestivum*. *Scleroderma* spp. mycelium is a fast growing one in contrast to *T. aestivum*. The mycorrhiza of *Scleroderma* spp. is a long distance exploration type in contrast to the short distance exploration type that of *T. aestivum* (Agerer 2001, www.deemy.de). Additionally, mycorrhiza of the two species has evolved in two different ways. According the genome of *Tuber melanosporum* (Martin et al. 2010)

and *Laccaria bicolor* (Martin et al. 2008), ascomycetes and basidiomycetes have different 'symbiosis toolbox'. Ragnelli et al. (2013) showed programmed cell death in plant roots caused by ECM of *Tuber* spp., which could ascribe to their degrading enzymes. On one hand, the faster grow of mycelia of *Scleroderma* sp. can cause a faster colonization of the host plant, thus if *Scleroderma* sp. colonization has a negative effect this process could have elapsed and the mycorrhizal relationship may turns to beneficial. Because of the slower mycelia growth of summer truffle it may develop mycorrhiza only when *Scleroderma* sp. is already in its beneficial stage, so the negative effect of summer truffle could be faded by *Scleroderma* spp. On the other hand, exploration types can utilize different nitrogen sources (Hobbie and Agerer 2009), therefore different effects of the colonization rates and nitrogen metabolism may also affect the vitality of the host plant. The common occurrence of *T. aestivum* and *Scleroderma* spp. on Hungarian natural sites have been observed by truffle collectors, that is in dried years *Scleroderma* spp. produces fruit bodies, while in wet years *T. aestivum* has a bigger amount of yield. Furthermore, in the case of *T. melanosporum*, *Scleroderma verrucosum* seems to have neutral effect on truffle orchards (Sourzat 2011). Despite the negative effect of *T. aestivum,* the values of maximal quantum efficiency of PSII centres ranged between 0.78 and 0.83 which can be qualified as a non-stressed condition (Baker 2008).

In conclusion, colonization of *Tuber aestivum* ECM has a little, however measureable negative effect on young host trees. The negative effect might depend on the nutrition demand and acquisition of fungi, and the physiological condition of the plant.

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