Effect of *Sphaerosporella brunnea* mycorrhizas on mycorrhization of *Quercus ilex* x *Tuber melanosporum*

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Abstract It is generally accepted that *Sphaerosporella brunnea* is a significant ectomycorrhizal contaminant in nurseries producing plants mycorrhized with various species of *Tuber,* and subsequently in truffiéres after outplanting. At the University of Alcalá, Spain, 397 small plants of *Quercus ilex* which were mycorrhized with *Tuber melanosporum* were inadvertently contaminated with *S. brunnea,* and this contamination was then monitored for 2 years. Sixty percent of the plants were contaminated and had *S. brunnea* ascomata on the surface of the container on one or several occasions. However, a Spearman test provided no evidence that *S. brunnea* mycorrhizas affected *T melanosporum* mycorrhization whereas other contaminating ectomycorrhizal fungi significantly did. Therefore, it appears that *S. brunnea* is not detrimental to plants which are well mycorrhized with *T melanosporum.*

Keywords black truffle; mycorrhized contamination; Mediterranean mushroom; mycorrhizal fungi; *Tuber melanosporum*

INTRODUCTION

Sphaerosporella brunnea (Alb. and Schwein.) Svrcek and Kubicka (Otideaceae, Pezizales) is a mycorrhizal discomycete that can prevent or displace *Tuber* mycorrhizal infections during the nursery production of truffle-infected plants throughout Europe, North America, Oceania, and Asia. It does this through a massive production of spores that germinate rapidly and which quickly infects host plants. For these reasons, contamination with *S. brunnea* has caused significant economic losses in commercial truffle-infected plant production where it has sometimes been necessary to destroy entire batches of mycorrhized plants. Moreover, *S. brunnea* is considered to be a mycorrhiza which contaminates truffle woods and nurseries producing plants mycorrhized with *Tuber* (Danielson 1984; Egger & Paden 1986; Meotto & Carraturo 1988; Chevalier & Poitou 1990; Meotto et al. 1992; Donnini 1994; Bencivenga et al. 1995a; Donnini & Bencivenga 1995; Román & De Miguel 1999; Yu et al. 2001; De Miguel & Sáez 2005).

Danielson (1984) reported that the origin of *S. brunnea* contamination in agroforestry nurseries was unknown. Meotto & Carraturo (1988) directly broadcasted *S. brunnea* spores over the plant containers in an agroforestry nursery and were able to mycorrhize plants with *S. brunnea* successfully. These authors and Meotto et al. (1992) indi cate that

S. brunnea mycorrhizas have a negligible initial presence in plants mycorrhized with *Tuber magnatum* Pico, but over time *S. brunnea* becomes highly competitive with *T. magnatum* in environments with high and constant humidity. Conversely, Bencivenga et al. (1995a) and Chevalier & Poitou (1990) indicated that the spread of *S. brunnea* decreased over time in comparison with *Tuber melanosporum* Vittad. mycorrhizas and other contaminating fungi, which may even recolonise many root tips previously mycorrhized by *S. brunnea.*

The concern over the effects of *S. brunnea* on agroforestry nurseries has given rise to studies of its metabolism and the molecular characterisation of its mycorrhizas, to verify and elucidate the competitive processes between the mycorrhizas of *Tuber* spp. and other fungi with *S. brunnea* (Bertini et al. 1998; Mello et al. 1998; Amicucci et al. 2000; Zambonelli et al. 2000). Palazón et al. (2005) began experiments on fungicide treatments *in vitro* and *in situ* for the purpose of controlling *S. brunnea* contamination in nurseries. Nevertheless, most of the studies on *S. brunnea* have failed to supply long-term quantitative information or statistical demonstration of the detrimental effects of *S. brunnea* on commercial mycorrhized plants in agroforestry nurseries.

In 1996, Di Massimo et al. (1996) reported an accidental contamination with *S. brunnea* in an agroforestry nursery used for mycorrhiza research at the University of Alcalá. As a result of this event, we initiated preliminary studies of this contamination (García-Montero et al. 1997; García-Montero & Manjón 1999). The aim of this work was to study the development of *S. brunnea* contamination over 2 years in the nursery and to carry out a quantitative statistical analysis to determine whether *S. brunnea* and other pollutant mycorrhizas interfere with the development of *T melanosporum* mycorrhizas.

MATERIALS AND METHODS

Cultivation procedure

Various *T. melanosporum* mycorrhization tests were conducted on 397 plants of *Quercus ilex* L. subsp. *bailóla* (Desf.) Samp., which had been inoculated with a low-concentration *T. melanosporum* spore suspensión, under various treatments in semi-sterile conditions. The spore suspensions were obtained from *T. melanosporum* ascomata stored at 4°C, using the methodology proposed by Bencivenga (1982) but modified according to Manjón & García-Montero (1996). The suspensions were prepared using a low concentration of spores (half a gram of ascomata per plant), to obtain poorly mycorrhized plants for experimental purposes with no application in truffle cultivation.

Acorns and spore suspensions were raised in plástic forestry containers 40 cm deep and 1 litre in volume. The substrate used for seeding was a limestone soil from a natural truffle-producing área mixed with vermiculite and perlite, which was sterilised in an autoclave at 120°C. The plants were kept under controlled environmental conditions at the Juan Carlos I Royal Botanical Garden at the University of Alcalá (Madrid, Spain).

The plants were housed in a greenhouse with an average daily temperature of 20-25°C; relative humidity of between 60% and 70%; watered by micro-sprinkler between one and three times a day for 1-3 min depending on the time of year; and under natural light conditions. These parameters were controlled automatically by computer. The parameters were also randomly checked by means of mínimum and máximum temperature thermometers and hand-held hygrometers. The plants were not fertilised.

Contamination monitoring

In the first year of growth the spontaneous appearance of *S. brunnea* ascomata was observed on the surface of numerous containers. The plants were then monitored over a 2-year period, under the growing conditions described. The ascomata of *S. brunnea* were carefully counted in a total of five controls in all, carried out every 6 months and ending in June 1998. Throughout the study the locations of the contaminated plants remained unchanged.

Many of the contaminated plants showed repeat fruiting of *S. brunnea* ascomata. Therefore in the five regular controls carried out to analyse the spread of the contamination, the plants which fruited for the first time were distinguished from those which showed repeat fruiting of *S. brunnea* ascomata.

Taxonomy studies

The taxonomic study and description of *S. brunnea* were described in García-Montero et al. (1997). From a systematic point of view, the identification of *S. brunnea* does not present any difficulties owing to its taxonomic characteristics.

Study of mycorrhizas

At the end of the second year of monitoring the contamination, a sample of 100 plants was harvested (25% of the population), and the roots were analysed. The mycorrhizas of 50 randomly-selected plants

which had never shown any *S. brunnea* ascomata were analysed, together with the mycorrhizas of 50 plants which had shown the greatest abundance of *S. brunnea* ascomata.

Mycorrhizas were identified using a stereoscopic microscope (Leica WildMZ8) and a microscope (Leica LeitzDMRB) following the descriptions and indications recommended by Agerer (1987-91), Bencivenga et al. (1995b), Granetti (1995), and Verlhac et al. (1990). The degree of mycorrhization was expressed in percentages of mycorrhized root tips, using Bencivenga et al.'s procedure (1987).

All mycorrhizas present in roots were checked and recorded, but only mycorrhizas of *T. melanosporum* and *S. brunnea* were identified. *T. melanosporum* mycorrhizas were identified from the surface ornamentation of the mantle and the shape of the cystidia. The mycorrhized categories are mutually exclusive, i.e., a tip mycorrhized with *T. melanosporum* cannot also be mycorrhized with *S. brunnea.* The microscopic features of *S. brunnea* mycorrhizas are very different and so easily distinguishable from those of *T. melanosporum* (Di Massimo et al. 1996).

Quantitative analysis

The statistical treatment was performed with Statistica v.6 (StatSoft, Inc., Tulsa, OK, United States 1999). Normality was checked using the Shapiro Wilks Test. Mycorrhization percentages obtained in the roots of the plants analysed did not follow a normal distribution, so non-parametric distribution was used, and the Spearman test applied.

RESULTS

Over a period of 2 years, of the 397 *Q. ilex* subsp. *ballota* plants lightly mycorrhized with *T. melanosporum,* 60% of the plants were contaminated and had *S. brunnea* ascomata on the surface of the container on one or several occasions. The number of *S. brunnea* ascomata which fruited on the containers varied between one and 14. The fruiting of these ascomata took place throughout all months of the year and did not follow any pattern. In the first year, *S. brunnea* formed ascomata for the first time in 52% of the plants and in the second year the figure was 8% (Table 1).

At the end of the second year of monitoring, 100 plants were harvested and their roots were studied. Table 2 summarises the analysis of the roots of 50 *Q. ilex* subsp. *ballota* seedlings which showed the greatest abundance of *S. brunnea* ascomata. Mycorrhization percentages were low, with an average of 11 % of the tips mycorrhized by *S. brunnea.* Moreover, it was observed that in 12 plants, *S. brunnea* ascomata had formed when only 1% or 2% of the root tips had been colonised. *S. brunnea* mycorrizas only occurred in plants with *S. brunnea* ascomata (see Tables 2 and 3). It can also be seen that *S. brunnea* mycorrhizas are persistent over time. Thus 15 plants which had ascomata in June 1996 retained their mycorrhizas for another 2 years, until harvesting in June 1998.

Finally the Spearman test was used to determine whether there had been any interaction between the mycorrhizas of the different species of fungi (Table 4). The test indicated that the mycorrhization of *T. melanosporum* was not affected by *S. brunnea* to any significant degree *(P* = 0.2751).

The colonisation of *T. melanosporum* was affected by the mycorrhization percentages of the remaining species of unidentified contaminating fungi, both in the 50 plants with ascomata of *S. brunnea* and in all 100 plants analysed *(P* < 0.0001) (Table 4). The Spearman test also showed that the mycorrhization of *S. brunnea* was also significantly affected by the presence of mycorrhizas of other species of fungi *(P* = 0.0337).

Microscopic study of the roots' morphological characteristics permitted clear identification of *S. brunnea* mycorrhizas. Diagnostic characterisation is summarised in the following Ítems. Macroscopic appearance: mycorrhizas which are a yellow-ochre colour when young and dark brown, almost black when oíd, always lighter in colour at the apex, with

Table 1 Monitoring of contamination over a 2-year period: 6-month intervals with numbers of plants showing *Sphaerosporella brunnea* ascomata on container surface.

Periodic monitoring of the presence of ascomata	1996: 06	1996: 12	1997: 06	1997: 12	1998: 06
Plants showing ascomata for the first time (no.)	50	113	43	25	8
Contaminated plants (with ascomata)	50	163	206	231	239
Contaminated plants (%)	13	41	52	58	60

an elongated and sinuous shape, either without branches or with monopodial pinnate branches; they are 1-3 mm long and 150-260 *pan.* in diameter at the main axis. The measurements of the branches are smaller; these are straight or slightly curved. Cystidium: from the surface of the mantle there emerges a mycelium composed of translucent, septate and branched hyphae with a diameter of 4—5 *piva.* and a distance between septa of 50-120 *piva.,* with thickening at the points where they insert into the mantle and characteristic narrowing in all its septa, which is more pronounced in juvenilemycorrhizas. Mantle: opaque appearance, pseudoparenchymatic construction, with cells (or pseudocells) on the mantle surface with clearly visible walls, polygonal in shape with rounded corners or long and sinuous, with a length at the longest axis of 19-57 *piva.* and 18-34 *piva.* at the shortest axis.

DISCUSSION

The results indicate that the presence of mycorrhizas of contaminating fungi did indeed have a negative significant effect on the mycorrhization of *T. melanosporum.* This confirms the importance of the heat sterilisation of substrate (or using other procedures) to avoid the proliferation of mycorrhizas of contaminating fungi in mycorrhized-plant-producing nurseries.

Heat sterilisation of the substrate decreases the proliferation of unwanted mycorrhizas which, in conjunction with the low-intensity mycorrhization by *T. melanosporum* in the first year of mycorrhized plant production, provides a favourable environmental condition for *S. brunnea* development. Meotto & Carraturo (1988) indicate that *S. brunnea* fruits rapidly as soon as it establishes its first mycorrhizas, which encourages its pioneering behaviour under suitable circumstances in nurseries.

Table 2 Summary of the incidence of mycorrhizas of 50 plants with the highest abundance of ascomata.

Mycorrhizas by fungi species	Mean%	SD	Range
Total mycorrhized root tips (%)	36	16	70 (5, 75)
Root tips mycorrhized with *Tuber melanosporum* (%)	8	14	40 (0, 40)
Root tips mycorrhized with *Sphaerosporella brunnea* (%)	11	10	39(1,40)
Root tips mycorrhized with other fungi species (%)	17	16	50 (0, 50)

Table 3 Summary of the incidence of mycorrhizas of 50 randomly-selected plants which did not have *Sphaerosporella brunnea* ascomata.

Mycorrhizas by fungi species	Mean%	SD	Range
Total mycorrhized root tips (%)	23	20	70 (0,70)
Root tips mycorrhized with *Tuber melanosporum* (%)	14	19	70 (0,70)
Root tips mycorrhized with *S. brunnea* (%)	0	0	0
Root tips mycorrhized with other fungi species (%)	8	12	60 (0,60)

Table 4 Estimates of Spearman Rank Order Correlations between mycorrhizas of different species *(S.b., Sphaerosporella brunnea; T.m., Tuber melanosporum).*

Sample	Association between different species		*t (N-2)*	*P* level
50 plants with *S.b.* ascomata	*S.b.* and *T m.*	0.15737	1.1041	0.2751
50 plants with *S.b.* ascomata	*S.b.* and other fungi	-0.30091	-2.1861	0.0337
50 plants with *S.b.* ascomata	*T.m.* and other fungi	-0.69867	-6.7657	< 0.0001
100 plants: 50 with and 50 without *S.b.* ascomata	*T.m.* and other fungi	-0.54794	-6.48445	< 0.0001

Sphaerosporella brunnea contaminated the majority of the lightly-mycorrhized *Q. ilex* plants in our study. This fungus became established extremely rapidly and fruited numerous ascomata in all the plants which had its mycorrhizas, even when only 1% of the root tips were mycorrhized. Moreover, this contamination persisted over time. Di Massimo (1997) has observed saprophytic behaviour of *S. brunnea* in some Italian agroforestry nurseries. This facultative capacity would explain why *S. brunnea* is so difficult to eradicate from the nurseries it contaminates.

However, our results showed that contaminated *Q. ilex* presented a low percentage of *S. brunnea* mycorrhizas, compared to other observadons in the natural environment and in other nurseries (Etayo et al. 1999; Román & De Miguel 1999, 2005). Therefore the appearance of numerous *S. brunnea* ascomata in a plant's container does not mean that this fungus has massively mycorrhized its roots. This conclusión concurs with the observations of Turnau (1995) in deteriorated natural áreas, where *S. brunnea* forms numerous ascomata, although it may not be a dominant mycorrhiza in the host plants studied. Finally, the results showed that the colonisation of *T. melanosporum* mycorrhizas was not significantly affected by *S. brunnea,* although the humidity conditions were favourable. Therefore, our results diverge from those of Meotto & Carraturo (1988) and Meotto et al. (1992).

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