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Soil receptivity for ectomycorrhizal fungi: *Tuber aestivum* is specifically stimulated by calcium carbonate and certain organic compounds, but not mycorrhizospheric bacteria

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ABSTRACT

Mycelium of ectomycorrhizal fungi colonizes soil that is extremely heterogeneous in terms of spatial arrangement as well as chemical and biological properties. Here we addressed which of the soil components would have the greatest influence on hyphal development of an ectomycorrhizal fungus, the summer truffle (*Tuber aestivum*). We tested a range of inorganic and organic compounds and bacterial strains isolated from truffle mycorrhizosphere, added to truffle grounds in small compartments accessible exclusively to the hyphae and not to host plant roots. Our results showed stimulation of truffle hyphal growth by high dosis of lime powder, whereas leaf litter had no effect. Further, we recorded significant stimulation of the hyphal growth by several organic compounds (gallic acid, cellulose and calcium formate), whereas no significant stimulation was observed by any of the inorganic compounds or bacterial cultures mixed with an inert carrier. None of the amendments, however, sustained the truffle growth rate recorded in unsterile field soil. These results indicate that the development of hyphae of *T. aestivum* in soil may well require complex and micro-heterogeneous environment, where specific organic compounds and calcium carbonate play particularly important roles.

1. Introduction

The true truffles (including *Tuber aestivum* Vittad.) are ectomycorrhizal fungi deriving most of their carbon and energy from the host tree. In spite of their predominantly symbiotic life style, mycelia of true truffles have been observed not only as associated with true ectomycorrhizae but, at least in case of *T. aestivum*, they were also detected in microbial films on the surface of roots beyond mycorrhizas, inhabiting decaying suberinized cell layers of old host roots (Gryndler et al., 2013b). Furthermore, the truffle hyphae were also abundant in roots of many herbaceous plant species not known previously as ectomycorrhizal host plants, which were present at the locality where *T. aestivum* naturally occurred (Gryndler et al., 2014). Occurrence of several *Tuber* spp., including *T. aestivum*, often leads to formation of areas devoid of understory vegetation called brûlé (Streiblová et al., 2012). This phenomenon is based mainly on mycelium-produced metabolites which suppress the herbaceous vegetation cover through their negative effects on the non-host plant roots in this particular ecosystem (Lanza et al., 2004). Brûlé is thus considered a marker of the truffle soil mycelial colony.

Beyond plant roots, true truffles significantly interact with the soil environment, which, in turn, may be important for the development of their mycelia and also for their fructification (Bencivenga and Granetti, 1989; Garcia-Montero et al., 2009a, 2009b; Valverde-Asenjo et al., 2009). The complexity of these biological and ecological factors and their unknown interactions are likely the reasons for the variability in sporocarp yields in truffle plantations and in natural truffle grounds (Kues and Martin, 2011). Particularly important role in this regard is thought to be played by soil organic matter, an indispensable component of the soil environment, which represents a key factor affecting soil microbial communities (Lejon et al., 2007).

The effects of organic compounds on the mycelia of ectomycorrhizal fungi in general and true truffles in particular can be very important because these fungi have considerable potential to decompose organic matter (Barry et al., 1993), though the expression and ecological consequences of this potential in the fields is still not fully understood (for a recent overview see Lindahl and Tunlid, 2015). Although this may not be the case for all ectomycorrhizal fungi, there is a possibility

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that at least the truffles could partially behave as saprotrophs. This notion is indirectly supported by at least three pieces of evidence: First, some of the truffle species can be cultured *in vitro* in absence of host plants. Second, the soil space occupied by truffle (*T. magnatum*) mycelium is only poorly linked to ectomycorrhizae, with the latter being only scarcely or not at all detected in the truffle mycelium spots (Iotti et al., 2014). And third, physical association of truffle hyphae with organic particles in the soil may indicate a kind of utilization of such substrates or at least some of its degradation products, released by accompanying, typically saprotrophic microorganisms. Such microbes (e.g., mycorrhiza helper-bacteria) have also been shown to stimulate the growth of mycelium of different species of ectomycorrhizal fungi, providing another line of evidence for an alternative life style of these predominantly symbiotic organisms (Dominguez et al., 2012).

Further, mineral nutrients such as phosphorus and nitrogen play very important roles in physiology of ectomycorrhizal symbiosis. These nutrients may be present as soluble inorganic or organic compounds in the soil solution or can be liberated from decomposing organic matter (Le Tacon et al., 2015). Then, mycelium of *T. aestivum* should explore the soil microsites enriched with organic or mineral nutrients to gain access to these compounds. In particular, available calcium seems to play a crucial role in the receptivity of the soil for true truffles (Garcia-Montero et al., 2009a; Garcia-Montero et al., 2009b) and thus merits particular attention from both scientific and practical application perspectives.

Ectomycorrhizal fungi, interconnecting the root and soil environments, are deeply embedded in complex interaction networks including both abiotic and biotic soil components and should respond to experimental manipulations of the availability of these components. However, there has been little progress so far in understanding the possible effects of various soil amendments on the growth of truffle mycelium, in spite of the fact that molecular methods (e.g., Gryndler et al., 2015, 2013b) now allow relatively simple experiments in this regard. There are several basic unresolved questions directly related to the *Tuber* life strategy and its local ecophysiological function that can be straightforwardly answered via the analysis of mycelial development in traps containing various bait materials. Therefore, within the present field study we aimed at answering the following questions:

- Is the truffle mycelium development affected by organic matter such as tree litter, which is the most abundant source of soil organic matter at truffle grounds?
- Is the content of main mineral nutrients (including calcium) important in this regard?
- Is the presence of truffle-associated soil microorganisms, and/or their extracellular products, important for the development of truffle mycelium?

2. Materials and methods

2.1. Trap fabrication

Two types of traps have been used in this study. The Type 1 traps contained autoclaved and microbially recolonized soil from the *T. aestivum* natural locality and were fabricated from 50-ml conical polypropylene centrifugation tubes (internal diameter 28 mm) and root-exclusion mesh at their openings exactly according to Gryndler et al. (2015). The details of the preparation of the soil for traps are given in Supplementary information.

The volume of the soil in Type 1 traps was divided into 5 mm thick compartments by using round discs of coarse (1 mm) nylon mesh. This allowed easy separation of the compartments upon harvest. The compartments were named A (the closemost compartment to the free soil/tube opening), B, C, D and E (the most distant from the free soil) and contained 4.9 g soil each. The compartment A was separated from the free soil by 42-μm nylon mesh (Silk & Progress, Brněnec, Czech Republic) to prevent entrance of roots and animals.

The Type 2 traps were fabricated from 1.5 ml Eppendorf tubes with removed lids and covered by 42-μm nylon mesh at the opening. The internal space of these traps was not divided into compartments and was filled with various materials as indicated below.

2.2. Materials tested in type 1 traps

Effects of calcium carbonate and pulverized (particle size < 100 μm) hornbeam litter on *T. aestivum* mycelium were tested using the Type 1 traps. The litter (collected in November 2012 on forest floor at "Michelský les" (Prague) was mixed with soil at a concentration of 1% (w:w). Anhydrous calcium carbonate ($CaCO_3$) was finely pulverized (particle size < 25 μm) and used at five different concentrations to reach 10, 30, 100, 1 000 and 10 000 mg of added calcium (Ca) per kg soil. Soil without any additive was used as a control in a separate series of the traps. The soil samples containing or not various amounts of $CaCO_3$ were used to measure water extractable- and exchangeable Ca levels as indicated in Supplementary information.

2.3. Materials tested in type 2 traps

Three different sorts of materials were tested for their effects on the development of *T. aestivum* mycelium using the Type 2 traps: unsterile soils, chemical compounds (inorganic and organic), and bacterial cultures (see Table S1 in the Supplementary information for the full list).

Seven different soil samples were tested in the Type 2 traps. The soils were sampled from the uppermost (0–10 cm) layer of three arable sites (samples 1–3) and four different samples (4–7) from the locality where *T. aestivum* was previously found, but not directly from the colony of this fungus (brûlé area) – see Fig. S1 and Table S2 in the Supplementary information to Gryndler et al. (2014). The sampling of soil from the confirmed truffle locality provided a kind of a reference soil that could with a high degree of certainty be colonized by *T. aestivum* mycelium.

All the tested soils were sieved moist through a 2 mm sieve to homogenize and to reach the same maximum particle size as the Perlite used in the other Type 2 traps. Then, the individual soils were filled in traps (four true replicates per soil treatment). The soils 4, 5, 6 and 7 (*T. aestivum* locality) were pooled and thoroughly mixed to produce one composite soil sample for chemical analyses. The soil samples were subjected to measurement of water extractable and exchangeable Ca levels as described above. The total carbonate content in the composite soil sample from the summer truffle locality was 16% (volumetric method using 10% HCl, expressed as $CaCO_3$).

The soil types, pH, water extractable Ca^{2+} and exchangeable Ca^{2+} levels are given in Table 1, further details are provided in Table S2 (Supplementary information). The absence of truffle mycelium was tested as indicated in the Supplementary information.

Before the use, the soils were tested for their ability to support the

Table 1
The pH and water extractable and exchangeable calcium levels (± SE, 3 technical replicates) in the studied soils.

Soil samples	Soil type	pH (water)	Water extractable Ca	Exchangeable Ca
			mmol kg^{-1}	
1	orthic luvisol	6.6	0.95 ± 0.06	93 ± 7
2	dystric cambisol	7.1	2.17 ± 0.05	57 ± 3
3	orthic luvisol	6.7	0.72 ± 0.08	89 ± 6
4–7 (mixed)	rendzic leptosol	7.6	2.76 ± 0.02	153 ± 2