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Isolation of bacteria from ectomycorrhizae of *Tuber aestivum* Vittad.

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Fifteen different cultivation media were used to isolate bacteria with the idea to obtain taxa specifically associated with ectomycorrhizae of *Tuber aestivum*. Ectomycorrhizae were collected at the sampling points previously analyzed for bacterial molecular diversity. We isolated 183 bacterial strains and identified them on the basis of the partial sequence of 16S rDNA. Out of these isolates, only 4 corresponded to operational taxonomic units significantly associated with *T. aestivum* ectomycorrhizae in previous molecular study. Preliminary study of the effect of 12 selected isolates on growth of *T. aestivum* mycelium showed no stimulation and one isolate induced the damage of hyphae. Different isolation strategy has to be developed to increase the probability of cultivation of potentially important components of *T. aestivum* mycorrhizosphere.

Key words: culture, summer truffle, Pseudonocardineae, *Streptomyces*, Rhizobiales

INTRODUCTION

For centuries, truffles have been highly appreciated as a special culinary ingredient, being highly priced on the markets. Although some of the truffle species have successfully been produced in plantations, attempts to culture other species have repeatedly failed and they can only be collected from their primary habitats. Life strategy of different truffle species is understood insufficiently and mainly the interactions between truffles and mycorrhizae- and mycelium-associated microflora merits attention.

Truffles are ectomycorrhizal Ascomycota deriving significant portion, if not all, of their carbon nutrition from a host tree via specialized communication organs, ectomycorrhizae. The soil space directly affected by ectomycorrhizae is called mycorrhizosphere (Linderman 1988) and is characterized by high biological activity (Chalot, Brun 1998). Presence of some bacteria may enhance the formation of ectomycorrhizal structures on the roots and was shown to change gene expression of the ectomycorrhizal fungal hyphae (Deveau et al. 2007). Some of these microbes

referred to as „mycorrhization helper bacteria“ (Garbaye 1994) have received a special attention because they can stimulate mycorrhizal colonization of the host roots by the truffles with economic consequences

Of the few available studies directed to microbial associates of truffles, some have focused on bacteria (Citterio et al. 2001; Bedini et al. 1999; Sbrana et al. 2002) and yeasts (Zacchi et al. 2003) associated with the ascocarp or ectomycorrhizae. The information on the microflora of the soil inhabited by truffles is also infrequent and has been published for yeasts by Zacchi et al. (2003) and, for saprotrophic fungi by Luppi-Mosca (1973, in Napoli et al. 2008) and for bacteria by Sbrana et al. (2002). The most striking result of these studies is a strong association of yeasts *Cryptococcus* spp. with ectomycorrhizae of the truffle ground reported by Zacchi et al. (2003). Some novel information is gradually becoming available using cultivation-independent molecular methods such as high-throughput PCR and DNA sequencing. These approaches allowed insights into mycorrhizal community of the productive spots of *T. magnatum* – showing that the mycorrhizae of this fungus are generally rare and most roots of the host trees are occupied by other mycorrhizal fungi (Murat et al 2005). Molecular analysis of bacterial and fungal communitites associated with the productive spots of *T. magnatum* failed to identify any specific fungi associated with this truffle, whereas they suggested a bacterium *Moraxella osloensis* (a gamma-Proteobacterium) to be preferentially associated with the productive spots of this truffle (Mello et al. 2010). Our previous study (Gryndler et al. 2012) revealed a specific association of some bacteria, including four genera of actinobacterial suborder Pseudonocardineae, with ectomycorrhizae of *Tuber aestivum* Vittad.

In this study, we aimed at isolation of bacteria from the communities associating to the *T. aestivum* ectomycorrhizae. *T. aestivum* (incl. forma *uncinatum*) has been recently rediscovered as a valuable alimentary product in many European countries and is currently considered to be the most common European truffle with gradually increasing commercial value. Knowledge of the interactions of this truffle species with accompanying soil microflora might be of practical importance, for example in formulation of complex truffle inocula used in artificial host seedlings inoculations, containing beneficial (e.g., mycorrhization-helper) bacteria.

MATERIALS AND METHODS

Bacteria were isolated from the *T. aestivum* ectomycorrhizae (samples 34, 36 and 39 mentioned in Gryndler et al., 2012, collected at the locality dominated by *Carpinus betulus*) using dilution plate technique. Mycorrhizae were thrice shaken in 50 ml of sterile water. Fresh 100 mg aliquots were immediately homogenized in 5 ml sterile water using mortar and pestle and then suspended in 50 ml water. Resulting suspension was then diluted by sterile water 1:10 through 1: 10 000 and 25 µl aliquots were spread on the surface of the solid medium A and incubated for 1-4 weeks at 25ºC. Medium A contained malt extract (Fluka 70167) 5 g, potato extract (Fluka 07915) 5g, yeast extract (Oxoid L21) 2 g, $CaCO_3$ 1 g, anhydrous $CaCl_2$ 73 mg, KH_2PO_4 100 mg, KNO_3 19.3 mg, $Ca(NO_3)_2.4H_2O$ 292 mg, $MgSO_4.7H_2O$ 196 mg, Na_2SO_4 70 mg, K_2SO_4 38 mg, NH_4NO_3 2.5 mg and agar 14 g in one liter, pH (before autoclaving) 7.0.

The diluted suspension from the sample 36 was incubated also on other 14 different media sharing the following composition of mineral salts: $(NH_4)_2SO_4$ 4 g, K_2HPO_4 2 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$, agar 10 g per liter, pH 7.0. The organic components of the different media were: yeast extract (0.25%), or casamino acids (0.03%) with yeast extract (0.03%) and glucose (0.03%), or humic acid (0.1%) with vitamins (thiamin-HCl, riboflavin, nicotinic acid, pyridoxin-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid, and biotin, each at concentration of 0.5 mg per l), or yeast extract (0.25%) with cellulose powder (4%), or starch (2%), or glycerol (0.05%) with arginine (0.1%), or oak root powder with yeast extract (0.1 %), or oak root extract (0.02%) with yeast extract (0.1%), or colloidal chitin (0.5%), or colloidal chitin (0.5%) with yeast extract (0.1%), or gelatin (1%), or gelatin (1%) with yeast extract (0.1%), or poly-L-lactate (0.1%) with oxgall (0.01%), or poly-L-lactate (0.1%) with oxgall (0.01%) and yeast extract (0.1%).

Ten replicate plates were established per medium. Growing bacteria were subcultured on medium B with the same composition as the above medium A, except that $CaCO_3$ was omitted and the concentration of anhydrous $CaCl_2$ was increased to 1.27 g per liter. Bacterial colonies of different morphological properties were chosen for subcultivation in order to obtain as many as possible different bacterial taxa.

Bacterial isolates were then inoculated to 10 ml of the liquid medium B (without agar) and bacterial biomass was pelleted by centrifugation. DNA was extracted from the pellet using Nucleo-Spin Soil DNA kit (Macherey-Nagel GmBH & Co., Germany) and a fragment of 16S rDNA was amplified in PCR with forward primer eub530F (5´-gtg cca gcm gcn gcg g-3´) and reverse primer eub1100aR (5´-ggg ttn cgn tcg ttg cg-3´). The primers were modified from Dowd et al. (2008). Cycling conditions were 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 1 min, 62 ºC for 50 s, and 72 ºC for 30 s, and concluded by incubation at followed by 72 ºC for 10 min.

PCR products were then sequenced and the identity of isolates was estimated using comparison of their partial 16S rDNA sequence with GenBank database.

Selected isolates were tested for their possible effects on the growth of culture of *T. aestivum,* strain Tae5 (maintained in the Laboratory of Fungal Biology, Institute of Microbiology ASCR, Prague, Czech Republic). The truffle mycelium was first pre-cultured on the medium PEX (Pebeyre S.A., Cahors, France) for 6 weeks and the bacterium was then applied as one 4-cm line per dish, whose middle was 5 mm apart from the edge of the mycelial colony. The growth of *Tuber* mycelium in the proximity of the bacterial colony was observed after 7 days of co-cultivation.

RESULTS

In total, 183 bacterial isolates were obtained from ectomycorrhizae of *T. aestivum*. Based on the partial sequence of the 16S rDNA, they belong to 6 bacterial orders: Actinomycetales (139 isolates), Burkholderiales (4 isolates), Enterobacteriales (1 isolate), Pseudomonadales (2 isolates), Rhizobiales (33 isolates) and Xanthomonadales (4 isolates).

Among the Actinomycetales, distinct groups of isolates were obtained, corresponding to 9 genera: *Streptomyces* (127 isolates), *Kocuria* (1 isolate), *Microbacterium* (1 isolate), *Micromonospora* (3 isolates), *Nocardia* (1 isolate), *Nocardiopsis*

(1 isolate), *Nonomuraea* (2 isolates), *Rhodococcus* (2 isolates) and *Rothia* (1 isolate). No culture of a member of the Pseudonocardineae suborder was isolated.

The isolates belonging to the order Rhizobiales involved the members of the genera *Phyllobacterium* (23 isolates), *Rhizobium* (6 isolates), *Bosea* (1 isolate), *Ensifer* (1 isolate), *Mesorhizobium* (1 isolate) and *Microvirga* (1 isolate).

The order Pseudomonadales was represented by the genera *Pseudomonas* (possibly *P. putida,* the only organism willing to grow on the medium containing oak root extract) and *Moraxella*.

Two members of the order Burkholderiales were further isolated: *Acidovorax* sp. (3 isolates) and *Xylophilus* sp. (1 isolate).

Escherichia (possibly *E.coli*, 1 isolate) was the only representative of the order Enterobacteriales and the order Xanthomonadales was represented by the genera *Lysobacter* (possibly *L. antibioticus*, 3 isolates) and *Dyella* (1 isolate).

The identification of the isolates is provisional and may be refined in future, if a particular isolate will prove to have beneficial effects on mycorrhizal colonization of truffle-inoculated tree seedlings.

The interactions with culture of *T. aestivum* were tested for *Moraxella* sp., *Lysobacter* sp., *Phyllobacterium* sp., *Rhizobium* cf. *giardinii, Rhizobium* cf. *leguminosarum, Mesorhizobium* sp., *Xylophilus* sp.,3 isolates *of Acidovorax* sp., *Microvirga* sp. and *Nocardia* sp. Some bacterial isolates (*Rhizobium* cf. *leguminosarum*, *Rhizobium* cf. *giardinii*, *Phyllobacterium* sp., *Microvirga* sp.) grew vigorously on the PEX medium, whereas growth of others (*Moraxella* sp., *Lysobacter* sp., *Nocardia* sp.) was slower. After 7 days of co-cultivation, the only interaction of truffle mycelium with a bacterial isolate was cell vacuolization and dying in proximity of *Rhizobium* cf. *leguminosarum* (Fig. 1). No other case of adverse or stimulatory effects was noted.

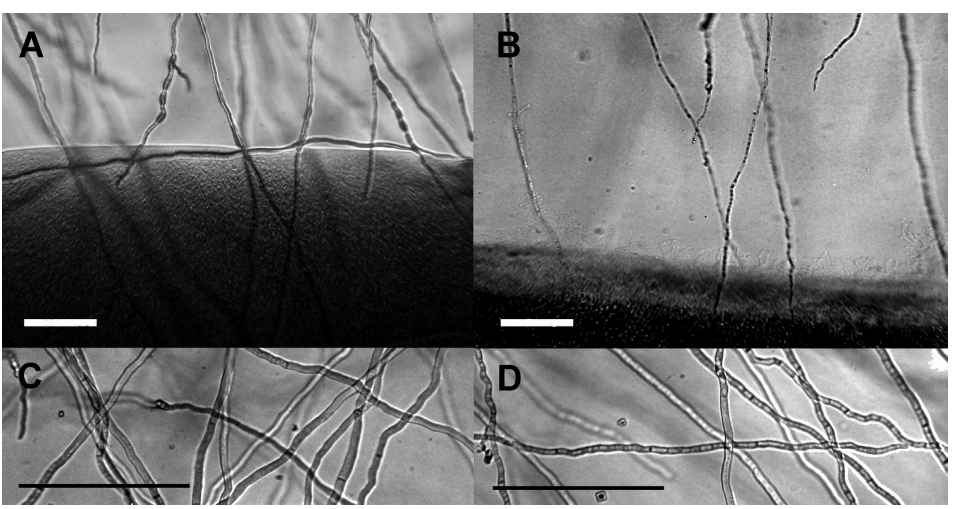

Fig. 1. Interaction of in vitro-cultivated mycelium of *T. aestivum* with *Phyllobacterium* sp. (A, C) or *Rhizobium* cf. *leguminosarum* (B, D). During the 7-day co-cultivation, the bacteria (dark mass in the figure) became to close contact with hyphae (A, B). Whereas hyphae in the proximity of *Phylobacterium* sp. colony had normal morphology (C), the proximity of *Rhizobium* cf. *leguminosarum* caused extensive vacuolization. Scale bar = 100 μm.

DISCUSSION

In spite of extensive effort during the isolation of the bacteria from ectomycorrhizae of *T. aestivum*, we were able to cultivate the members of 6 bacterial orders. This is very low number in comparison with the results of molecular analysis of the same material (Gryndler et al. 2012), which detected operational taxonomic units that can be grouped into a total of 79 bacterial orders. This discrepancy confirms the hypothesis that cultivation-based studies reveal just negligible portion of the microbial diversity of mycorrhizosphere.

Our recently reported effort is the continuation of the past works (Gryndler et al. 2012) led by the intention to cultivate mainly the bacterial taxa that are specifically associated with ectomycorrhizae of *T. aestivum*. This effort was, however, only partially successful and the isolates of *Lysobacter* sp. and *Ensifer* sp. remain the only bacteria that were significantly positively associated with *T. aestivum* ectomycorrhizae. In particular, we repeatedly failed to isolate the members of the actinobacterial genera *Actinosynnema*, *Allokutzneria*, *Kibdelosporangium* and *Lentzea*, the members of the suborder Pseudonocardineae which proved to positively correlate with the presence of *T. aestivum* in ectomycorrrhizae. In spite of the fact that Pseudonocardineae members are generally considered rare organisms in the nature (Jarerat et al. 2002), our molecular data predict that Pseudonocardineae members represent significant portion of biomass of the root-associated microbial community. However, they either do not produce sufficient amounts of colony forming units necessary for isolation or cannot be cultivated using our methodology. At the same time, their colony forming units may be hidden on dilution plates by high numbers of massively sporulating members of Actinomycetales.

There is no information on interactions of the members of this suborder with fungi or plants available in the literature. Some members of this group possess the ability to degrade poly-L-lactate (a kind of plastic, also used as a cultivation medium component in our work), which was originally considered as unique among the actinobacteria (Jarerat et al. 2002). Even though some other actinobacterial degraders of this material were described later (Sukkum et al. 2009), the suborder Pseudonocardineae remains important pool of organisms possessing this activity. This may indicate that the members of this suborder may possess exotic metabolic capabilities, perhaps connected with very specific nutritional demands which were not met during our isolation approach.

Further work and probably different isolation strategy will be needed to obtain the cultures of the bacteria specifically associated with ectomycorrhizae of *T. aestivum*. We hope, this work will be worth of effort as these bacteria may have potentially interesting effects on truffle mycelium and mycorrhiza functioning.

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