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Actinobacteria may influence white truffle (*Tuber magnatum* Pico) nutrition, ascocarp degradation and interactions with other soil fungi

Aleksandar PAVIĆ[a,1], *Slaviša STANKOVIĆ*[b], *Elmira SALJNIKOV*[e], *Dirk KRÜGER*[c], *François BUSCOT*[c,d], *Mika TARKKA*[c,d], *Žaklina MARJANOVIĆ*[a,*]

[a]*Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1a, 11030 Belgrade, Serbia*
[b]*Chair of Microbiology, Faculty of Biology, University of Belgrade, Studentski Trg 16, 11000 Belgrade, Serbia*
[c]*UFZ – Helmholtz Centre for Environmental Research, Department of Soil Ecology, Theodor-Lieser-Str. 4, D-06120 Halle, Germany*
[d]*University of Leipzig, Institute of Biology, Chair of Soil Ecology, Johannisallee 21-23, D-04103 Leipzig, Germany*
[e]*Institute for Soil Science, Teodora Drajzera 7, 11 000 Belgrade, Serbia*

ARTICLE INFO

Article history:
Received 6 June 2012
Revision received 21 March 2013
Accepted 29 April 2013
Available online 14 September 2013
Corresponding editor:
D. Jean Lodge

Keywords:
Chelating compounds production
Curtobacterium
Ectomycorrhizal fungi-associated bacteria
Phosphate solubilisation
Rhodococcus
Tuber magnatum
Verticillium leptobactrum

ABSTRACT

To test the hypothesis that truffle-associated bacteria may improve truffle nutrition, we isolated bacteria from white truffle ascocarps and tested Actinobacteria for their ability to solubilise phosphate and iron, nutrients that have limited availability in white truffle grounds. Two isolates with sequence similarities to *Curtobacterium flaccumfaciens* and *Rhodococcus* sp. were characterized in detail. Both solubilised $Ca_3(PO_4)_2$ in a way that was dependent on the nitrogen and carbon sources present. Neither strain broke down phytate, but both produced chelating compounds, performed ammonification, and broke down β-glucan. Additionally, *C. flaccumfaciens* decomposed chitin, pectin, lipids and proteins, while *Rhodococcus* sp. exhibited urease activity. Three potentially fungicolous fungi were isolated from diseased white truffle ascocarps and bioassayed against the isolated Actinobacteria. The *Rhodococcus* isolate inhibited *Verticillium leptobactrum*, neither bacterium affected *Clonostachys rosea*, while both isolates promoted growth of *Trichoderma* sp. The results suggest that Actinobacteria might be involved in improving truffle nutrition, ascocarp degradation and establishing relationships with other soil fungi.

* *Corresponding author*. Tel.: +381 112078460; fax: +381 113055289.
E-mail addresses: zaklina@imsi.bg.ac.rs, zaklina@yahoo.com (Ž. Marjanović).
[1] Present address: Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a, 11010 Belgrade, Serbia.

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http://dx.doi.org/10.1016/j.funeco.2013.05.006

Introduction

Ectomycorrhizas (ECM) are symbiotic relationships between long-lived plants (trees and shrubs) and their fungal partners that facilitate survival in climatic conditions characterized by intense seasonal dynamics of temperature and/or precipitation. The longevity of woody plants would be impossible without the ability to adapt to changes in the nutrient and water content status of the soil. It is widely accepted that promiscuous symbioses with diverse communities of ectomycorrhizal fungi enable plants to scavenge water and low-available nutrients from the dynamic soil environment (Smith & Read 2010). Most of the currently available experimental data on ECM originate from ecosystems in which ECM are ubiquitous, with acidic soils and relatively cool climates (Read & Perez-Moreno 2003). However, trees and their ectomycorrhizal fungal partners in Mediterranean and sub-Mediterranean regions usually inhabit alkaline calcareous soils and, therefore, have to adapt to a rather different environment. The most extensively studied ectomycorrhizal fungi native to these environments are truffles.

Truffles (*Tuber* spp.) are ascomycetous fungi that are most abundant in the warmer temperate regions of the northern hemisphere, establish ectomycorrhizas with angiosperms and gymnosperms, and form hypogeous ascocarps. *Tuber magnatum* is both the most prized and most ecologically specialised of the commercially important European truffles. Its main natural habitats are located in small areas of Italy and the Balkan Peninsula (for a detailed list of known habitats, see Marjanović *et al.* 2010), and attempts at planting inoculated trees have not yet proved successful. As such, the species has become the focus of increasing scientific interest. The climate of the sub-Mediterranean regions inhabited by *T. magnatum* is characterized by hot and dry summers, significant rainfall in spring and autumn, and relatively short and mild winters with snow (Marjanović *et al.* 2010). The soils in natural white truffle grounds are usually calcareous, well-aerated and weakly alkaline (Bragato *et al.* 2010). The main fertility problems of such soils in warm regions are low availabilities of soluble phosphates and iron due to the formation of insoluble salts and complexes, as well as nitrogen, which is mainly present in organic forms and can become a growth-limiting factor under dry conditions (Marschner 1995).

While it is clear that the soil conditions under which *T. magnatum* grows differ significantly from those for many previously-described ECM symbioses, there have been no studies on the physiology of its ECM because it has not yet been possible to maintain pure cultures of its mycelium or to achieve axenic mycorrhization in sterile systems. However, data on bacteria that may influence nutrient acquisition by ECM mycelia have become widely available (Frey-Klett *et al.* 2007). Bacteria associated with the mycelium of mycorrhizal fungi may cooperate with the fungi and promote nutrient acquisition (Barea *et al.* 2005).

Previous studies on culturable truffle ascocarp-associated bacteria have focused on *Pseudomonas* and Bacillaceae strains, and provided evidence that these species may be involved in ascocarp degradation and spore release, or mycelium differentiation (Gazzanelli *et al.* 1999; Sbrana *et al.* 2000; Citterio *et al.* 2001). Barbieri *et al.* (2007) published a comprehensive report on the molecular diversity of bacteria inhabiting the ascocarps of *T. magnatum*. Their subsequent report on the diversity and expression of genes involved in nitrogen fixation (*nif*H) isolated from ascocarps was the first attempt at exploring the influence of bacteria on truffle metabolism (Barbieri *et al.* 2010). Despite these authors' efforts, little is known about the metabolic features of actinobacterial strains associated with the ascocarp of *T. magnatum* or their role in nutrient acquisition.

The main environmental challenges that white truffles must overcome in their role as ECM symbionts are the high soil pH values (7–8.5) typically encountered in their natural habitats, which indicate that the soil has significant buffering capacity, and the soil water content that changes substantially among seasons. The uptake of substances that have limited solubility under such conditions (e.g. phosphate) therefore requires acidification or chelation. However, it is very difficult to achieve substantial localised reductions in soil pH under well-buffered soil conditions. Some bacteria are capable of excreting protons or organic acids produced during sugar fermentation that solubilise insoluble phosphates such as $Ca_3(PO_4)_2$ and thus possess phosphate solubilising activity (PSA). While there are no published data on the PSA of any truffle mycelia, it seems reasonable to suppose that the bacteria that are associated with the ascocarps may facilitate mycelial uptake of phosphate or iron in alkaline soils.

Barbieri *et al.* (2007) provided clear evidence that Actinobacteria are relatively abundant in the ascocarps of *T. magnatum*. Building on this finding, we sought to isolate and identify actinobacterial strains from *T. magnatum* ascocarps that are capable of growing on nitrogen-poor media and to determine their metabolic specificities. Potential metabolic specificities consistent with some of the previously-described effects of mycorrhiza helper bacteria (MHB, Frey-Klett *et al.* 2007) were selected on the basis of the physicochemical properties of the soil at the location where the ascocarps were growing, which were determined by analyzing soil samples acquired in that area. We investigated the possible ability of these strains to facilitate mycelial nutrient uptake, the possibility that they might play a role in ascocarp or plant debris decomposition, and their activity against selected filamentous fungi found in the ascocarp.

Material and methods

Isolation and preliminary characterization of bacteria

Two ripe, fresh, undamaged and healthy ascocarps of *T. magnatum* were collected from a natural truffle ground in Western Serbia, transported to the laboratory under cold conditions, and instantly processed. Ascocarps were carefully washed with sterile water, surface-flamed, and cut in half under sterile conditions. The maturation stages of the ascocarps were determined according to Zeppa *et al.* (2002). To collect ascocarp-associated bacteria from the entire cut surface, an aliquot (100 μl) of sterile PBS–Tween 20 solution (0.8 g NaCl, 0.02 g KCl, 0.176 g K_2HPO_4, 0.024 g KH_2PO_4 and 200 μl Tween 20) was applied over the gleba and then