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Isolation and Identification of Mycorrhization Helper Bacteria in Norway spruce, *Picea abies* (L.) Karst.

By

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Key words: Mycorrhization Helper Bacteria (MHB), rhizosphere, fluorescent pseudomonads, *Bacillus*, ectomycorrhiza.

Summary

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We have isolated bacteria from bulk soil, the mycorrhizosphere and the hyphosphere of Norway spruce (*Picea abies* (L.) Karst) seedlings in mycorrhizal association with the fungus *Amphinema byssoides* (Pers.) J. Erikss.. Out of 157 bacterial strains, ten (6.4 %) were fluorescent pseudomonads and 12 (7.6 %) were from the genus *Bacillus*. The selected strain *P. fluorescens* 53a, tested for its effect on growth and mycorrhization of Norway spruce seedlings, grown in vitro in the presence of the ectomycorrhizal fungus *Paxillus involutus* (Batsch. ex Fr.) Fr., showed a positive effect on growth (fresh and dry weight after one and two months of growth) and mycorrhization (number of mycorrhizal seedlings after three months) and a negative effect on the total root length, root surface area and total root volume after 3 months.

Introduction

The rhizosphere is defined as the plant-root surface and the surrounding soil which is influenced by the living root (DANDURAND & KNUDSEN 1996). The operational definition of the rhizosphere, in other studies as well as in ours, is the thin layer of soil adhering to the root system after the loose soil particles have been removed by shaking (DANDURAND & KNUDSEN 1996). In the rhizosphere, fungi

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and bacteria can be found, living on organic compounds released by the root. They can have a positive or a negative effect on plant growth. They also affect the symbiotic establishment of mycorrhizal fungi on plant roots in various ways (GARBAYE 1994). MHBs (Mycorrhization Helper Bacteria) are bacteria associated with mycorrhizal roots and mycorrhizal fungi. They selectively promote the establishment of mycorrhizal symbiosis (GARBAYE 1994). MHBs have a potential use in forest tree seedling production for promoting growth and as protection against pathogenic microorganisms. The two most studied groups of MHBs are fluorescent pseudomonads and the genus *Bacillus*, which are also known to have a PGPR effect (Plant Growth-Promoting Rhizobacteria) (HÖFLICH & al. 1994, PEROTTO & BONFANTE 1997). The mycorrhization helper effect was observed in various symbiotic partnerships (GARBAYE & BOWEN 1989, GARBAYE & al. 1992, GARBAYE 1994, DUNSTAN & al. 1998). The ectomycorrhizal combination of Douglas fir (*Pseudotsuga menzeisii* Mirb. Franco) and *Laccaria laccata* Scop. ex Fr. has been studied most extensively (DUPONNOIS & GARBAYE 1991, DUPONNOIS & GARBAYE 1991a, DUPONNOIS & al. 1993, GARBAYE 1994). For this study, Norway spruce seedlings were chosen. We studied the diversity of bacteria in the bulk soil, the rhizosphere and the hyphosphere of the nursery, the effects of bacteria on the growth of seedlings in vitro, and the effects of bacteria on mycorrhization.

Materials and Methods

Isolation and identification of bacterial strains. We isolated bacteria from bulk soil, the mycorrhizosphere and the hyphosphere (VAN ELSAS & SMALLA 1996) of 4- to 6-year-old Norway spruce seedlings ectomycorrhizal with the fungus *Amphinema byssoides* (AGERER 1987-1998), grown in the nursery of the Slovenian Forestry Institute. Samples of bulk soil (~ 0.1 g) were suspended in 1 ml of 0.9 % NaCl mixed by vortexing for 30 seconds. Mycorrhizal roots without a central cylinder (length up to 0.5 cm), representing the mycorrhizosphere, and rhizomorphs (length up to 1 cm) representing the hyphosphere were macerated, suspended in 1 ml of 0.9 % NaCl and vortexed for 30 seconds. Each sample was diluted (10x, 100x and 1000x) and 0.1 ml was inoculated on three different solid media: tripton soy agar (TSA) (GERHARDT & al. 1994), King B (BACON & HINTON 1996) and 0.2 % brain heart infusion (BHI) supplemented with 1 % peptone, 0.2 % yeast extract, 0.2 % NaCl, and 0.2 % D-glucose (pH 7.2) and incubated at room temperature for two days. Colonies with different morphology were isolated in pure cultures. Each sample (0.1 ml) was also inoculated in two liquid enrichment media for pseudomonads: in 30 ml of tryptophan medium (GERHARDT & al. 1994) and in 3 ml of succinate-salts medium (GERHARDT & al. 1994). The tryptophan medium was incubated with shaking (100 rpm) at room temperature for four days, then 0.1 ml of culture was inoculated in fresh medium and incubated with shaking (100 rpm) at room temperature for two days. The culture was then diluted (10x, 100x and 1000x) and inoculated on plates of solidified tryptophan medium. Pure cultures of colonies with different morphology were isolated. The liquid succinate-salts medium was incubated for four days at 30°C, then 0.1 ml of diluted culture (10x, 100x and 1000x) was inoculated on plates of solidified succinate-salts medium and incubated for two days at 30°C. Morphologically different colonies were isolated in pure cultures.

Pure cultures obtained from primary plates were characterised by colony morphology, Gram staining, oxidase and catalase reaction, and type of metabolism on OF-glucose (oxidation-fermentation test). All Gram-negative, glucose-nonfermenting, fluorescent pigment-producing strains were further identified with the following tests: growth at 4°C, 37°C and 42°C, growth on McConkey agar, growth on 6.5 % NaCl, indole production, production of arginine dihydrolase and lysine decarboxylase, production of lecithinase and lipase, nitrate reduction, production of gas from

nitrate, hemolysis, degradation of gelatin, OF sucrose, acid production from D-mannitol, and motility. Gram-positive, sporulating bacilli, preliminarily classified as the genus *Bacillus*, were further identified with these additional tests: Voges-Proskauer test, acid production from D-glucose, L-arabinose, D-xylose and D-mannitol, degradation of casein and gelatin, production of lecithinase, indole production, hemolysis, growth at 50°C, and motility. Production of endospores was confirmed with heat-shock and differential staining after Schaeffer. All test were carried out as described in MOLINA & PALMER 1984, SNEATH & al. 1986, CLAUS & BALKWILL 1989, GERHARDT & al. 1994, BACON & HINTON 1996, WEYANT & al. 1996.

Identification with classical biochemical tests was compared with commercially available tests for identification (API 20 NE for fluorescent pseudomonads and API 50 CH with medium API 50 CHB for the genus *Bacillus*, BioMérieux, France).

Effect of the selected bacterial strain on the growth of Norway spruce seedlings. The bacterial strain *P. fluorescens* 53a (or *P. putida* as identified with API 20 NE) isolated from the mycorrhizosphere was tested for its effect on Norway spruce seedlings. Spruce seeds were surface sterilised in 30 % H₂O₂ for one hour, washed four times in sterile water and germinated on wet filter paper in Petri dishes (diameter 20 cm). After 24 days, seedlings were planted in pots (diameter 5,5 cm x 9,5 cm) with transparent covers and approx. 80 ml of sterile substrate added: vermiculture-peat mix (1:1-v:v) supplemented with 12 ml of liquid modified MNM medium with 10 g/l D-glucose (MARX 1969), and then grown in growth chambers (LTH, Škofja Loka, Slovenia, lights: Tungsram white, 16 hours light at 24°C and 8 hours dark at 21°C). Seedlings were inoculated with the ectomycorrhizal fungus *Paxillus involutus* (Batsch. ex Fr.) Fr. strain 1444 (generously given by Doz. Dr. Ingrid Kottke, Univ. Tübingen, Botanisches Institute – Spezielle Botanik – Mykologie, Germany) with or without the selected bacterial strain. *Paxillus involutus* was grown on Hagem medium (MOLINA & PALMER 1984) at 20°C in the dark. Fresh and dry weight and the number of root tips after 1, 2 and 3 months were measured and counted and the total root length, root surface area and total root volume after 3 months were analysed with the computer system WinRHIZO™ (version 3.10b, Régent Instruments Inc., Canada). The results of the number of root tips were transformed by arcsin. The mean values of each treatment were compared with analysis of variance and Student's t-test at 0.05 probability level.

Results and Discussion

Isolation and identification of bacterial strains. 157 bacterial strains were isolated: 68 from the mycorrhizosphere, 56 from the hyphosphere and 33 from the bulk soil. Ten (6.4 %) of them were fluorescent pseudomonads and all were identified as *Pseudomonas fluorescens* with classical biochemical tests (Table 1). Three strains identified as *P. fluorescens* were tested with an API 20 NE commercial test. The first was confirmed as *P. fluorescens*, the second as *P. putida*, and the third could be either *P. fluorescens* (89.3 %) or *P. putida* (10.7 %). Twelve (7.6 %) strains showed cell and colony morphology typical for the genus *Bacillus*. Seven of them were identified as *Bacillus subtilis*, four strains as *B. cereus* and one as *Bacillus sp.* (Table 1). Three strains of *Bacillus* were checked with API 50 CH. The strain of *B. cereus* was confirmed as *B. cereus* 1, whereas the strain of *B. subtilis* showed a substrate pattern similar to *B. megaterium* 2 as well as *B. subtilis*. *Bacillus sp.* could be identified as *B. megaterium* 2 or *B. circulans*.

Table 1. Identification of fluorescent pseudomonads and the genus *Bacillus* with classical biochemical and commercial tests.

No. of strains	identification with classical biochemical tests	identification with commercial tests API 20 NE
1	<i>Pseudomonas fluorescens</i>	<i>P. fluorescens</i> (99.8 %)
1	<i>Pseudomonas fluorescens</i>	<i>P. putida</i> (99.5 %)
1	<i>Pseudomonas fluorescens</i>	<i>P. fluorescens</i> (89.3) or <i>P. putida</i> (10.7 %)
7	<i>Pseudomonas fluorescens</i>	/
No. of strains	identification with classical biochemical tests	identification with commercial tests API 50 CH
1	<i>Bacillus cereus</i>	<i>B. cereus</i> 1
1	<i>Bacillus subtilis</i>	<i>B. megaterium</i> 2 or <i>B. subtilis</i>
1	<i>Bacillus sp.</i>	<i>B. megaterium</i> 2 or <i>B. circulans</i>
3	<i>Bacillus cereus</i>	/
6	<i>Bacillus subtilis</i>	/

Symbol: / not done

Cultivation of bacterial isolates have variously been reported to recover only about 0.01 to 10 % of bacteria enumerated by direct counts (DANDURAND & KNUDSEN 1996). Recovery of populations from the rhizosphere depends on the method used (washing, vortexing, sonification, and blending or maceration) (DANDURAND & KNUDSEN 1996). Therefore the number of strains in the tested material might be much higher than 157. We identified 6.4 % out of 157 isolates as fluorescent pseudomonads and 7.6 % as the genus *Bacillus*, which agrees with the previously known data for percentages of different bacteria species isolated from soil and the rhizosphere (GARBAYE & BOWEN 1989, GROOMBRIDGE 1992). Variation in certain characteristics was observed among strains within the species *P. fluorescens* (growth at 4°C and 37°C, growth at 6.5 % NaCl, production of arginine dihydrolase, production of lipase, nitrate reduction, degradation of gelatin) and *B. subtilis* (acid production from L-arabinose, D-xylose and D-mannitol, degradation of gelatin and casein), which indicate that not just a single clone of the identified strains colonized the tested material. Instead, it seems that growth conditions in the nursery selectively promote various strains of the identified bacterial species.

Effect of the bacterial strain *Pseudomonas fluorescens* 53a on the growth of Norway spruce seedlings. Three months after the inoculation of spruce seedlings, we observed structures similar to Hartig net under the microscope on two seedlings inoculated with the fungus, and seven seedlings inoculated with the fungus and the bacterial strain (Table 2). A positive effect of the bacterial strain on fresh weight after one month, and on dry weight after one and two months after inoculation, was found, whereas there was no significant effect on seedling biomass after three months (Table 2). After three months of incubation we observed a significantly smaller number of root tips in the group inoculated with the fungus and bacteria, but there were no differences after one and two months (data not shown). The total root length, root surface area and total root volume were meas-

ured only after three months incubation time and they were all significantly smaller in the group inoculated with the fungus and bacteria (data not shown).

Table 2. Effect of the bacterial isolate *P. fluorescens* 53a on Norway spruce seedlings in vitro.

group	S	S+F	S+F+B
number of all seedlings	30	60	60
average fresh weight (mg)			
after 1 month	44.0	32.6	42.9*
after 2 months	67.0	51.9	60.1
after 3 months	74.4	75.9	68.6
average dry weight (mg)			
after 1 month	13.2	9.6	15.7*
after 2 months	19.3	16.0	20.0*
after 3 months	19.2	18.4	19.0
number of seedlings with the structure similar to Hartig net			
after 3 months	0	2	7

Symbols: S: seedling, F: fungus, B: bacteria,
*:significantly different from the control group (S+F)

We conclude that the bacterial strain *P. fluorescens* 53a has an effect on the mycorrhization and growth of Norway spruce seedlings, observed as an increased number of mycorrhizal seedlings and as an increase in fresh and dry weight. The difference between the test and the control groups decreased after the third month of growth, which might be the consequence of relatively small samples, high variation and standard error.

A c k n o w l e d g e m e n t s

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