Short communication

Some Characteristics of Bacteria Isolated from the Ectomycorrhiza of Chinese Pine Inoculated with *Cenococcum graniforme*

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Ectomycorrhizal association between fungi and tree species are one of the most important symbiotic relationships in a forest ecosystem. Trees accept inorganic nutrients effectively through symbiotic fungi in forest soils with poor nutrient status and enhance their photosynthetic activity and alter the water regime thus ensuring normal growth even under stressed conditions¹⁴). The function of mycorrhiza cannot be interpreted solely from the viewpoint of the interaction between host plants and symbiotic fungi because there are many other microorganisms in the soil surrounding the mycorrhiza which may affect this symbiotic relationship directly and indirectly. By analogy with a well documented rhizosphere effect, the concept of a mycorrhizosphere has been presented⁴). Some soil bacteria beneficial to the mycorrhiza formation are known as 'helper bacteria'⁵), while some soil microorganisms are reported to inhibit infection of mycorrhizal fungi into the roots^{1,13}). Thus, the microbial community of mycorrhizosphere and its function in the soil seems to be rather complex. Chinese pine can form mycorrhizae with many species of ectomycorrhizal fungi, but the mycorrhizal formation of this tree

with *Cenococcum graniforme* has not yet been reported. Furthermore, the microorganisms associated with the mycorrhiza formed by *C. graniforme* have not been clarified. In this experiment, based on the success in forming ectomycorrhizae between Chinese pine seedlings and *C. graniforme*, we compared the bacterial flora in mycorrhizae and nonmycorrhizal roots as part of a preliminary experiment analysing the function of ectomycorrhiza.

Forest soil was collected from the surface of a Japanese red pine forest at the campus of Shizuoka University, Ohya, Shizuoka Prefecture (N 0.20%, P 0.072%, K 0.67%, pH 4.45, originating from Quaternary sediment and containing 30% clay). A mixture of the soil, vermiculite and washed quartz sand at a ratio of 2:1:1 by volume was sterilized (121°C, 1 h) and put into a transparent square plastic box of 100×140 mm size and 15 mm thickness (Eiken Kizai Co., Tokyo). The box was used vertically with the narrower side up, and a hole of ca. 1 cm diameter was made at the topside to make the plant grow through this hole.

Surface sterilized (0.1% KMnO₄, 2 h) seeds of Chinese pine (*Pinus tabulaeformis* Carr.) were germinated on sterile water agar, then transferred to the sur-

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face of the sterile soil indicated above and cultured in a greenhouse under natural light and temperature ($18^{\circ}\text{C}\sim 30^{\circ}\text{C}$). The culture was watered twice a week and fertilized every 3 months with Hocking's nutrient solution⁶. Four weeks after the transfer, the seedlings were inoculated with the ectomycorrhizal fungus *Cenococcum graniforme* (Sow.) Ferd. and Winge. The fungus had been previously grown on Melin-Norkrans (MMN) agar media⁹ for 6 weeks. An agar plug of 1 cm diameter of the fungal mycelia was introduced into the box to contact with the roots of the plant. Fifty ml of the half-strength MMN liquid media was added into the culture just after the inoculation. MMN agar plugs of the same size but without the fungus were supplied to the roots of the seedlings as a control. After 12 weeks of fungal inoculation, when the roots of the inoculated plants bore apparent mycorrhizal short roots, 20 ml of the soil suspension (50 g of the forest soil described as above in 1,000 ml sterilized water, dry basis) were added to the plant culture to inoculate soil microorganisms. The plants were cultured for a further 9 months.

Bacteria were isolated from both the mycorrhiza of the inoculated plant and the short roots of the control plant. Mycorrhizae and short roots excised from the plants were washed with sterilized water and crushed using a sterile pestle. The number of bacteria in the suspensions was measured using the dilution-plate method with a yeast extract-glucose (YG) medium having a composition: glucose 1 g, yeast extract 1 g, K_2HPO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, agar 15 g, distilled water 1,000 ml, pH 7.0. Fifty colonies from each of the treatments were picked up, purified through successive transfers to a new medium and cultured on it. The number of nitrogen-fixing bacteria associated with the mycorrhiza and non-mycorrhizal roots was determined according to the MPN (Most Probable Number) method using Rennie's semisolid agar medium¹¹. The presence of nitrogen-fixing bacteria was certified by introducing acetylene gas into the MPN tubes and detection of ethylene formation by gas chromatograph after 12 h incubation.

Each isolate was classified according to the primary bacteriological identification method². Gram staining, observation of morphology and motility, oxidase

and catalase formation, fermentation and oxidation test were used as the key characters. Cell fatty acid pattern was used for further grouping. Young bacterial cells cultured on YG agar medium were directly saponified with alkali and cell fatty acids methylated with HCl-methanol and were analyzed by a gas chromatograph (Shimadzu GC 17A) equipped with FID and a capillary column of 30 m length and 0.32 mm diameter (DB-17, J & W Scientific Inc., CA, USA). Some strains belonging to the most abundant group were supplied for identification by MIS (Microbial Identification system, Microbial ID. Inc. Newark, DE, USA). The cell fatty acid methyl esters were analyzed by a gas chromatograph (Hewlett Packard Model 5890) and the fatty acid pattern was compared with the data base of the MIS.

The antibiotic activity of some of the mycorrhiza isolates were examined against several plant-pathogenic fungi. A piece of sterile filter paper was dipped in the supernatant of the YG liquid culture of the isolate, dried and put on a malt agar plate. The plate was inoculated with each of the strains of *Fusarium*, *Rhizoctonia* and *Pythium* (shown in Table 1) and cultured for 2 days. The inhibition of fungal growth was examined. An agar plug of the *C. graniforme* culture was also supplied for examining the presence of antibiotic activity for these pathogenic fungi.

A marked difference was observed between the numbers of bacteria from mycorrhiza and non-mycorrhizal roots (Table 2). The numbers of both total bacteria (cfu) and nitrogen-fixing bacteria of mycorrhiza were about an order lower than those of non-mycorrhizal roots.

The kinds of bacteria from the mycorrhiza and non-mycorrhizal roots were also different from each other (Table 3). From the cell fatty acid pattern, the composition of bacterial flora from the mycorrhiza were shown to be heterogeneous. The largest group of fatty acid pattern H occupied only 20% of the total isolates. All other characteristics except fatty acid pattern were similar. All the isolates were non-motile gram-positive rods being oxidase-positive, catalase-positive and with no acid formation from glucose in either the aerobic or anaerobic cultures (OF test). Among the gram-positive bacteria only 5 isolates of

Table 1. Antifungal activity of *C. graniforme* and the mycorrhizosphere isolate M17 against some pathogenic fungi.

Fungi*	Growth Supression** by	
	<i>C. graniforme</i>	M17
<i>Fusarium oxysporum</i> f.sp. raphani SS-1	++	++
<i>F. oxysporum</i> C1	—	—
T193	+	—
6385	±	±
9761	—	++
9966	±	++
<i>F. solani</i> f.sp. radicicola SS-2	++	+
<i>F. roseum</i> f.sp.cerealis SS-3	±	+
<i>F. moniliforme</i> SS-4	++	+
<i>Rhizoctonia solani</i> Chin-1-1S	—	—
R64	—	—
<i>Pythium irregulare</i>	++	++

* *Fusarium* spp. are the pathogens of crops, and *Rhizoctonia* and *Pythium* spp. are pathogenic for both crops and woody plants.

** The extent of growth suppression was tentatively evaluated by observing the size of the inhibition zone on the agar plate.

Table 2. Number of rhizosphere bacteria (CFU*) and rhizosphere nitrogen-fixing bacteria of Chinese Pine seedlings inoculated with ectomycorrhizal fungi *Cenococcum graniforme*.

	Total bacteria (10 ⁷ per g fresh weight)	Nitrogen-fixing Bacteria (10 ⁴ per g fresh weight)
Mycorrhizal	2.92 (±0.25)	5.98 (2.33~15.4)
Non-mycorrhizal	36.4 (±3.5)	19.8 (7.7 ~50.9)

Numbers in parentheses are the limit of confidence in 5% level.

* Colony-forming unit.

Table 3. Grouping and Characteristics of Bacteria isolated from the Mycorrhizal and Non-mycorrhizal roots of Chinese Pine

Origin	Fatty Acid Pattern*	Number of Isolates	Characteristics					OF-test
			Shape	Gram	Motility	Oxidase	Catalase	
Mycorrhiza	H	10	Rod	+	—	+	+	no acid
	I	7	Rod	+	—	+	+	no acid
	J	4	Rod	+	—	+	+	no acid
	Others**	29	Rod	+	—	+	+	no acid
	Total	50						
Nonmycorrhizal Roots	A	33	Rod	—	+	+	—	oxidative
	B	9	Rod	—	+	+	+	oxidative
	C	4	Rod	—	+ or —	+	+	oxidative
	Others**	4						
	Total	50						

* Difference in the fatty acid pattern was obtained from the distribution of the peaks of the gas chromatogram of each strain.

** Number of the isolates with the same fatty acid pattern was below 2.

miscellaneous fatty acid groups (designated as "others" in Table 3) were spore-forming and were regarded as *Bacillus* spp. The result of the MIS analysis indicated that the bacteria of fatty acid type H from mycorrhiza was *Lactobacillus fermentum*, but the probability of coincidence with the database was only 0.011. Other bacteria could not be identified because of the absence of the corresponding fatty acid patterns in the database.

The microflora from non-mycorrhizal roots was less complex than that from mycorrhiza. About two third of the isolates were regarded as a homogenous group. These were gram-negative motile rods being oxidase-positive, catalase-negative and OF-test oxidative. The second largest group was of gram-negative, oxidase-positive and catalase-positive rods with the OF-test oxidative. The result of MIS analysis of the main groups showed that the most dominant bacteria from non-mycorrhizal roots (fatty acid pattern A) was *Xenorhabdus nematophilus* (probability 0.128) or *Morganella morganii* (probability 0.100). *X. nematophilus* is isolated from the nematode and is not a soil bacterium⁷⁾. The low probabilities for both species also suggest the uncertainty of their taxonomic position. The next main group of bacteria of fatty acid pattern B was shown to correspond to *Burkholderia* (*Pseudomonas*) *gladioli*.

Some mycorrhizal fungi including *C. graniforme* are reported to produce antibiotic substances and protect host plants against pathogenic microorganisms^{3,8,10)}. The kind and nature of rhizosphere microorganisms may also be related to the antibiosis of mycorrhiza. The antibiotic effect of the mycorrhizal fungus and coexistent bacteria on some pathogenic fungi was therefore examined. Not only the ectomycorrhizal fungus *C. graniforme* itself, but also several kinds of bacteria from mycorrhiza suppressed some pathogenic fungi. The results of *C. graniforme* and a typical suppressive bacterium M17 is shown in Table 1. Some of the pathogenic fungi against crops and woody plants were suppressed by both *C. graniforme* and mycorrhizosphere bacteria. Suppressive patterns of *C. graniforme* and M17 were similar, but the pattern against *F. oxysporum* 9761 and 9966, and also against *F. roseum*, was different be-

tween the ectomycorrhizal fungus and coexistent bacteria. Stack and Sinclair¹²⁾ reported that free-living microflora which are stimulated by the ectomycorrhizal fungus *Laccaria*, protected Douglas fir roots from attack by the pathogenic fungus *Fusarium oxysporum*. Garbye⁴⁾ suggested that growth stimulation by ectomycorrhizal infection is partly due to the antibiosis of both the mycorrhizal fungus and coexistent bacteria. The situation in our experiment is similar to the suggestions previously reported by them, and both mycorrhizal fungi and bacteria may protect tree roots against attack by pathogenic microorganisms.

Moreover, the possibility exists that the antibiosis is involved in the selection of mycorrhizosphere bacterial flora, and the antibiosis of mycorrhizosphere microorganisms may elucidate why the bacteria colonizing on or in the non-mycorrhizal roots differ both qualitatively and quantitatively as shown in Table 2 and 3.

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