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Nitrogen-fixing *Bacillus* sp. associated with Douglas-fir tuberculate ectomycorrhizae

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Abstract

Nitrogenase activities, measured by acetylene reduction, were detected under microaerophilic field conditions in Douglas-fir tuberculate ectomycorrhizae. Tuberculate ectomycorrhizae consist of densely packed clusters of ectomycorrhizal rootlets enclosed in a supplementary fungal peridium-like layer. Nitrogenase activity was primarily in the external layer and was greatly enhanced with added sucrose. The bacterium isolated, a nitrogen-fixing, spore-forming *Bacillus* sp., is an aerobe but requires anaerobic conditions for nitrogenase activity. Respiration in the tuberculate complex by the fungus, roots, and associated mycorrhizosphere microbes probably contributes to maintaining a microaerophilic niche where nitrogen fixation can take place. Water extracts of peridium or mycorrhizal root tips enhanced nitrogenase activity of this associative *Bacillus* sp., thereby indicating a close nutritional relationship between this bacterium and the tuberculate mycorrhizae. Thiamine more significantly enhanced bacterial nitrogenase activity than biotin; no activity was detected with *p*-aminobenzoic acid. Even though the levels of nitrogenase activities in the tubercles in situ were low, as measured by the present methods, they may indicate a significant contribution to the nitrogen dynamics of these nitrogen-limited Douglas-fir forests over a long-term period.

Introduction

Tuberculate ectomycorrhizae (TM) develop as nodule-like clusters of ectomycorrhizal root tips and occur on various tree species (Dell et al., 1990; Dominik, 1963; Masui, 1926; Melin, 1923; Randall and Grand, 1986; Trappe, 1965). On Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco), extreme development of TM may yield up to 2000 root tips packed in one single cluster (Trappe, 1965). The ectomycorrhizal fungi induce a single rootlet to proliferate and form a mass of mycorrhizal root tips tightly enclosed within a peridium-like sheath, effectively sealing off direct contact between soil particles and mycorrhizal roots. Rhizomorphs often radiate out from the peridium into soil.

Zak (1971) identified the fungal symbiont of the Douglas-fir TM as *Rhizopogon vinicolor* A.H. Smith and successfully reconstituted small tubercles in pure culture syntheses. The ectomycorrhizal tubercles commonly occur in decayed stumps, logs, and other woody debris partially buried in the soil but are also found in the litter of Douglas-fir stands. Zak (1971) suggested that the tubercles, with their supplementary fungal sheaths, may confer added benefits to their hosts during water stress or serve as a

barrier against entrance by pathogen or aphid attack, but their ecological significance and function are still not completely understood.

Presence and activity of N_2-fixing bacteria have been demonstrated in various decayed woody substrates (Harvey et al., 1989; Jurgensen et al., 1989; Larsen et al., 1978; Silvester et al., 1982). Because microbial colonization of the ectomycorrhizal tubercles probably is extensive, we investigated the possibility that tuberculate structure might support associative bacteria with nitrogen fixation ability.

Materials and methods

In situ nitrogenase activity

Douglas-fir tubercles studied (Figs. 1, 2 and 3) were in decayed coarse woody debris at 1200 m elevation along the Woods Creek Road on Mary's Peak in the Oregon Coast Range about 15 km southwest of Corvallis. Intact tubercles attached to the parent root were each placed in a 30 cm^3 plastic tube in situ (Fig. 3). The tubercles with portions of the root were sealed from the rest of the root system by using a serum stopper and a caulking agent (Fig. 3). The entire system was incubated with 10% acetylene under microaerophilic conditions (99% N_2, 1% O_2) at field temperature (10°C) for 1 day. The same system without acetylene addition served as control. A 0.1-cm^3 gaseous sample from each tube was removed and assayed for ethylene and acetylene with a Hewlett-Packard 5830 A gas chromatograph fitted with a 2 m × 2.1 mm, 80–100 mesh, Porapak R column with oven temperature at 70°C. Injection temperature and flame-ionization detector temperature were each adjusted to 100°C, and flow rate of the nitrogen carrier gas was adjusted to 40 cm^3 per min. The experiment was carried out with one or two clusters of ectomycorrhizal tubercles located at five different areas within the stand.

In-vitro tests

To demonstrate the presence of N_2-fixing bacteria, sheaths of the tubercles (see Fig. 4) were removed after their surfaces were thoroughly washed in sterile distilled water. The sheath (peridium) and densely packed mycorrhizal tips (see Figs. 4, 5) were each moistened with 0.01% sucrose in the tubes and subsequently incubated separately, in 10% acetylene under microaerophilic conditions for 1 day at 10°C. The same experiment was conducted with soils from around the tubercles. A 0.1-cm^3 gaseous sample was then removed for ethylene and acetylene determinations.

Isolation and characterization of N_2-fixing bacteria

The N_2-fixing bacteria were isolated by streaking the cultures on a nitrogen-deficient Burk's agar medium (Zuberer, 1987) with 0.002% yeast extract and incubating the plates under anaerobic conditions by means of GasPak anaerobic systems *(BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD). The isolated N_2-fixing bacteria were tested for nitrogenase activity and identified by the phenotypic methods described in the Virginia Polytechnic Institute (VPI) Anaerobe Laboratory Manual (Holdeman et al., 1977) and by Moore et al. (1980). Cellular fatty acid profile analysis was performed as described by Moore et al. (1987).

Water extracts of peridium, mycorrhizal tips, or sporocarps of *R. vinicolor* were prepared by homogenizing 2 g of tissue in 100 cm^3 of distilled water with an Omini-mixer at the highest speed. The homogenate was centrifuged for 30 min at 25,000 × *g* and the supernatant was collected and sterilized by filtration through a 0.2-μm filter. A 0.1-cm^3 aliquot of the supernatant was added to the Burke's medium, representing 0.002% tissue extract in the medium, to test for nitrogenase activity. Biotin (0.12 mg L^{-1}), *p*-aminobenzoic acid (0.08 mg L^{-1}), pyridoxine hydrochloride (0.08 mg L^{-1}), and thiamine hydrochloride (0.4 mg L^{-1}) also were each added to the medium to test for the bacterial nitrogenase. Five replicates for each treatment with completely randomized design were tested for this study.

Because the ratio of the largest to smallest mean values of the response variables is larger

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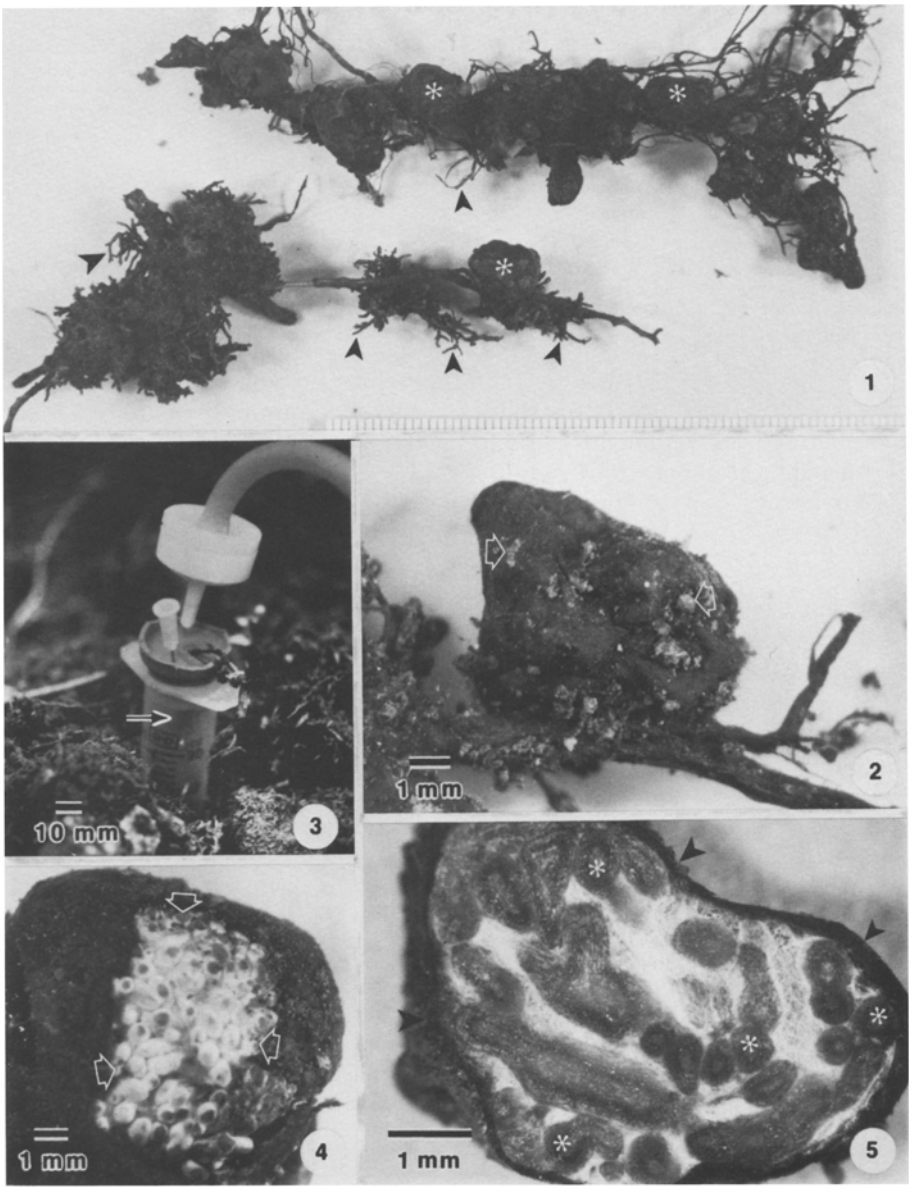

Fig. 1. Photograph showing tuberculate clusters (*) among nontuberculate (arrowheads) Douglas-fir roots. Scale in millimeters.

Fig. 2. Dissecting microscope micrograph showing a tuberculate cluster with a few soil particles (arrows) still attached to the peridium.

Fig. 3. Close-up view, in the field, of a modified syringe being flushed with nitrogen gas. A tuberculate cluster, still attached to the tree, is enclosed (arrow).

Fig. 4. Dissecting microscope micrograph of a tuberculate cluster on which a portion of the peridium was removed revealing numerous mycorrhizal roots (arrows).

Fig. 5. Dissecting microscope micrograph showing a hand-sectioned cluster of ectomycorrhizal roots (tubercle). Note the peridium layer (arrowheads) enclosing the numerous mycorrhizal roots (*).

Table 1. Nitrogenase activity (nmoles C_2H_2 reduced per mg tissue in 24-hr period) of Douglas-fir tuberculate ectomycorrhizae and adjacent soil

Treatment	nmoles acetylene reduced[a]
Tubercles in situ	3.91×10^{-2} a
Peridium of tubercles	3.68×10^{-2} a
Mycorrhizosphere soil with sucrose	20.02×10^{-2} b
Peridium of tubercles with sucrose	8.48 c
Mycorrhizosphere soil in situ	0
Mycorrhizal tips with or without sucrose	0
Surface-sterilized peridium of tubercles with or without sucrose	0

[a] Average of 5 replicates under microaerophilic conditions. Means followed by the same letter are not significantly different (alpha = 0.05) by the Bonferroni test.

than ten, the data were log-transformed to stabilize variance and analyzed for variance by using the Bonferroni procedure at alpha = 0.05 (Sabin and Stafford, 1990).

Results and discussion

Douglas-fir tubercles exhibited in situ nitrogenase activity. The activity was due to N_2-fixing bacteria on the surfaces of the peridium-like sheath of the tubercles. No nitrogenase activity was detected within the densely packed mycorrhizal tips, even when they were treated with sucrose (Table 1). The peridium treated with sucrose solution had significantly higher nitrogenase activity than that without sucrose treatment. No nitrogenase activity was detected in mycorrhizosphere soils unless the soils were treated with sucrose, in which the nitrogenase activity was significantly higher than that of sucrose-treated peridium (Table 1).

Based on the physiological and biochemical characteristics, the N_2-fixing bacterium isolated from the tubercle surface was an aerobic, spore-forming *Bacillus* species that exhibited nitrogenase activity in nitrogen-deficient medium under anaerobic conditions (Table 2). The bacterial surface colonies on blood agar plates incubated either aerobically or anaerobically at 30°C for 48 hr were small (0.5-mm diameter), circular to slightly irregular, white, shiny, and smooth with a mosaic internal structure and were surrounded by zones of beta hemolysis. Subcultures in pre-reduced peptone-yeast extract (PY) broth medium produced moderate to good turbidity without sediment. Cells were straight to slightly curved Gram-positive rods 0.6 to 1.0 μm wide by 3.0 to 8.0 μm long; cells appeared Gram-negative in older cultures. Spores usually were subterminal, but an occasional terminal spore was seen.

The optimum temperature for growth was 25°C to 30°C; growth was less at 37°C and poor at 45°C. Esculin was hydrolyzed and nitrate was reduced to nitrite. Catalase was produced when cells were grown aerobically. Acids produced by peptone-yeast extract-glucose broth cultures (in milliequivalents per 100-cm^3 of culture) were formic (0.63), acetic (0.43), and lactic (0.18). The major fatty acid methylesters of cells grown in peptone-yeast extract-1% glucose-0.02% Tween-80 broth were 15:0 anteiso (42%), 16:0 (13%), 18:1 cis 9 (5%), 14:0 iso (5%), 14:0 (4.5%), 15:0 iso (4.5%), and 16:0 iso (4%).

The pH was not lowered in PY broth to which

Table 2. Nitrogenase activity (nmoles acetylene reduced per mg bacterial protein per h) of *Bacillus* sp., isolated from the peridium surface of Douglas-fir tuberculate ectomycorrhizae, as influenced by various treatments

Treatment	nmoles acetylene reduced[a]
Nitrogen-deficient medium (NDM)	0
NDM + *p*-aminobenzoic acid	0
NDM + 0.002% yeast extract	3.87 a
NDM + pyridoxine	7.91 b
NDM + biotin	18.31 c
NDM + water sporocarp extract of *Rhizopogon vinicolor*	19.97 cd
NDM + water extract of mycorrhizal tip	29.80 cde
NDM + water extract of peridium	50.05 e
NDM + thiamine	163.16 f

[a] Average of 5 replicates under anaerobic conditions. Means followed by the same letter are not significantly different (alpha = 0.05) by the Bonferroni test.

0.5 to 1.0% of the following sugars individually were added: amygdalin, arabinose, cellobiose, erythritol, esculin, fructose, glucose, glycogen, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, pectin, raffinose, rhamnose, ribose, salicin, sorbitol, soluble starch, sucrose, trehalose, or xylose. Neither gelatin, milk, casein, or meat was digested. No lecithinase or lipase was detected on egg yolk agar. Indole was not produced.

The bacteria required yeast extract for growth and nitrogenase activity. Water extract of tubercle sheath-like peridium, mycorrhizal tips without peridium, or sporocarps of *R. vinicolor* promoted significantly higher nitrogenase activity than did yeast extract (Table 2). Thiamine produced the highest nitrogenase activity among the vitamins; *p*-aminobenzoic acid did not support the growth and nitrogenase activity of this bacterium (Table 2). Li and Castellano (1984) reported that sporocarp extract was effective in enhancing growth and nitrogenase activity of N_2-fixing bacteria isolated from mycorrhizal fungi.

Douglas-fir tuberculate mycorrhizae predominantly occur in decaying coarse woody debris, where active fixation of dinitrogen has been demonstrated in various stages of decay (Harvey et al., 1989; Jurgensen et al., 1989; Larsen et al., 1978; Silvester et al., 1982). Stimulation by sucrose of N_2 fixation under anaerobic conditions suggests the existence of an important linkage between nutrient exchange of mycorrhizae with bacteria during decomposition of large woody debris. Assuming an average rate of acetylene reduction in situ for TM of 3.91×10^{-11} moles per mg TM per day, for one year at that level of fixation for a 100 g TM, 13×10^{-3} g of N would be fixed. These nitrogen fixation rates are small compared to those of actinorhizae and the Rhizobium-legume symbiosis (Stowers, 1987), but the nitrogen input on a long-term basis may add significantly to the nitrogen budget of mature stands of Douglas fir where no actinorhizal plants are associated.

This tripartite association in Douglas-fir tuberculate mycorrhizae suggests another close interrelationship between root symbionts and associated microorganisms. Ectomycorrhizal fungi probably enhance associative bacterial performance of ecological functions by providing them with energy. In return, associative bacteria with nitrogenase abilities may fix nitrogen and provide nutrients to both fungus associate and rootlets. A key question is the level of microaerophilic conditions maintained in the tubercles. The bacterium cannot fix nitrogen under aerobic conditions, but high respiration rates of the clustered mycorrhizal rootlets and other associated organisms may lower the oxygen tension, thereby allowing nitrogen fixation.

Other tuberculate mycorrhizae, such as in pine which may form loosely clustered mycorrhizal tips without peridium, need to be investigated to determine of a peridium is prerequisite for associative N_2-fixing bacteria. Li and Hung (1987) and Amaranthus et al. (1990) reported the occurrence if nitrogen-fixing *Azospirillum* and Clostridium spp. in peridium-lacking Douglas-fir ectomycorrhizae; Pachlewski et al. (1992) also reported the colonization of ectendomycorrhizae of *Pinus sylvestris* by nitrogen-fixing *B. polymyxa* in Poland.

As more is known about the microbial ecology of the associated flora (nitrogen-fixers and other groups) within the tubercular structure, we may be able to establish the critical processes and specific roles performed by these in maintaining ecosystem productivity. Angiosperm TM and gymnosperm TM also need to be compared for associative microflora as they are likely to represent different microniches.

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