

## Nitrogen-fixing (acetylene-reducing) bacteria associated with ectomycorrhizae of Douglas-fir

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**Summary** Nitrogenase activity, measured by acetylene reduction, was detected on nursery-grown, surface-sterilized ectomycorrhizae of Douglas-fir, formed with *Laccaria laccata*, *Hebeloma crustuliniforme*, *Rhizopogon vinicolor*, and *Thelephora* sp. Detached mycorrhizae were incubated in nitrogen-free liquid medium under microaerophilic conditions. Nitrogenase activity was attributed to *Clostridium* spp. and *Azospirillum* spp.

### Introduction

Studies with  $^{15}\text{N}_2$  have demonstrated that inoculation with the ectomycorrhizal fungus *Rhizopogon roseolus* (Corda) Th. Fries increased the total nitrogen content of Monterey pine (*Pinus radiata* D. Don) seedlings, presumably through associated nitrogen-fixing bacteria<sup>16</sup>; however, the responsible bacteria were not isolated or identified. Bacteria have been reported<sup>9</sup> throughout the mycorrhizal mantle both within and between symbiont cells of mycorrhizal eucalypts, but their significance was not known. Free-living diazotrophs have been reported in and on the roots of crops and grasses<sup>3,6,8,12,20</sup>.

This study explores the possible association of diazotrophs with ectomycorrhizae of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco).

### Materials and methods

Seventeen-month-old Douglas-fir ectomycorrhizae, formed with *Laccaria laccata* (Scop.:Fr.) Berk. and Br., *Hebeloma crustuliniforme* (Bull. ex. St. Am.) Quél., and *Thelephora* sp. were excavated from inoculated nursery beds of International Paper Co., Western Forest Research Center, Lebanon, Oregon. Ectomycorrhizae on 9-month-old Douglas-fir, formed after inoculation with spore slurries of *Rhizopogon vinicolor* Smith, were obtained from the Georgia-Pacific container nursery at Cottage Grove, Oregon.

The mycorrhizae were thoroughly washed under tap water to remove soil particles. They were immersed in 1% Chloramine-T (J. T. Baker Chemical Co., Phillipsburg, NJ) for 1 h, shaken frequently, then washed in five changes of sterile water<sup>6,13</sup>. (The use of trade or firm names is for reader information and does not imply endorsement by the US Department of Agriculture). The mycorrhizae were then aseptically cut into 0.2–0.5 cm segments. Fifteen to 20 segments were inoculated into 20-ml nitrogen-free liquid medium in 60-ml serum bottles with malate as a carbon source<sup>4</sup>. The bottles were capped and flushed for 5 min with nitrogen gas. Oxygen was injected into the bottles to obtain an atmospheric mixture of  $\text{N}_2\text{—O}_2$  (99:1). The bottles were incubated in the dark for 3–7 days at 30°C.

Bottles showing bacterial growth were assayed for acetylene reduction; 10 percent of the atmosphere in the bottles was replaced with acetylene, and bottles without acetylene were the controls. After 18 h at 30°C, 0.1-ml gaseous samples from each bottle were removed and analyzed for ethylene and acetylene with a Hewlett-Packard 5830A gas chromatograph fitted with a 2-m × 2.1-mm, 80–100 mesh, Poropak R column. Oven temperature was adjusted to 70°C. Injection and flame ionization detector temperatures were adjusted to 100°C. Nitrogen carrier gas flow rate was adjusted to 40 ml/min.

Acetylene-reducing bacteria in bottles of *L. laccata* mycorrhizae were isolated by repeatedly streaking the culture on Döbereiner's agar medium containing 0.002% yeast extract and incubating it under anaerobic conditions by means of GasPak anaerobic systems (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD). Similar procedures were used to isolate the nitrogenase active bacteria on trypticase soy agar from the bottles of *H. crustuliniforme* mycorrhizae.

Acetylene-reducing bacteria from *Thelephora* sp. and *R. vinicolor* mycorrhizae were purified by streaking the culture on Döbereiner's nitrogen-free agar medium containing 0.002% yeast extract.

The isolated pure cultures were tested for acetylene reduction. An aqueous bacterial suspension (0.01 ml), prepared by suspending the isolate in sterile distilled water, was inoculated into serum bottles that contained 20 ml Döbereiner's nitrogen-free liquid medium supplemented with yeast-extract and vitamins<sup>2</sup>. The bottles were capped with sterile serum-stoppers and incubated under nitrogen atmosphere at 30°C for the bacteria isolated from *L. laccata* and *H. crustuliniforme* mycorrhizae, or incubated under microaerophilic conditions (99% N<sub>2</sub> + 1% O<sub>2</sub>) at 30°C for the bacteria isolated from *Thelephora* sp. and *R. vinicolor* mycorrhizae. After 3–4 days, acetylene was injected into the bottles; the aforementioned procedures of acetylene and ethylene determinations followed.

Bacterial cells in the serum bottles were harvested and washed with cold 5% trichloroacetic acid. Cell protein was solubilized in 0.5 N NaOH in a boiling water bath for 10 min<sup>1</sup> and measured by the modified Lowry method<sup>10</sup>.

### Results and discussion

The bacteria isolated from Douglas-fir ectomycorrhizae, formed with *L. laccata* and *H. crustuliniforme*, were anaerobes that produced nitrogenase activity, as measured by acetylene reduction, in the nitrogen-free liquid medium under nitrogen atmosphere (Table 1). The bacteria, isolated from Douglas-fir ectomycorrhizae formed with *Thelephora* sp. and *R. vinicolor*, are aerobes, but they showed nitrogenase activity under microaerophilic conditions (Table 1).

The bacteria from *L. laccata* and *H. crustuliniforme* mycorrhizae were *Clostridium* spp. The isolates differed markedly, however. The isolate from *L. laccata* grew on Döbereiner N-free medium but not on trypticase soy agar. The isolate from *H. crustuliniforme* mycorrhizae grew on both these nutrient media. The bacteria isolated from *Thelephora* and *R. vinicolor* mycorrhizae were *Azospirillum* spp., which formed a distinctive pellicle with nitrogenase activity in Döbereiner's semi-solid medium. The two isolates, however, exhibited different morphologies on nutrient agar.

Table 1. Nitrogen-fixing bacteria isolated from ectomycorrhiza of Douglas-fir and their nitrogenase activity\*

Ectomycorrhizal type	Diazotroph isolated	Nitrogenase activity (nmoles ethylene formed/mg protein per h)
<i>Laccaria laccata</i>	<i>Clostridium</i> sp.	86b
<i>Hebeloma crustuliniforme</i>	<i>Clostridium</i> sp.	66b
<i>Thelephora</i> sp.	<i>Azospirillum</i> sp.	22a
<i>Rhizopogon vinicolor</i>	<i>Azospirillum</i> sp.	16a

\* Data are means of four replicates. Treatment means not sharing a common letter differ significantly by Tukey's test at  $P = 0.05$ .

The successful isolation of nitrogen-fixing bacteria from the surface-sterilized ectomycorrhizae indicates that the organisms are probably present within the ectomycorrhizae. We have no data on the population dynamics of *Azospirillum* and *Clostridium* with Douglas-fir mycorrhizae, but reports by others have shown that the inner root *Azospirillum* populations increase with age of the plants, reaching a maximum during the productive stage of the plants<sup>14</sup>. In addition to providing fixed nitrogen to the plants, *Azospirillum* is noted to benefit the plants by producing phytohormones<sup>4</sup>, and by increasing mineral uptake and drought resistance<sup>18</sup>.

Nitrogen-fixing *Clostridium* spp. are widely distributed in both forest and agricultural soils<sup>5,17</sup>. As much as 33 kg nitrogen per hectare is estimated to be fixed by *Clostridia* in flooded meadow-bogs and rice fields; they also actively fix nitrogen in nonflooded soil in association with aerobic bacteria<sup>11</sup>.

The mutualistic relationship between *Clostridium* and other soil bacteria is well known. Emtsev<sup>7</sup> found that the presence of *Bacillus closteroides* in cultures of *C. pasteurianum* enhanced growth and nitrogen fixation. Rice and Paul<sup>15</sup> observed that decomposition products of other soil bacteria can be utilized by nitrogen-fixing *Clostridia*. We have also detected nitrogenase activities of *Clostridium* spp. with mixtures of non-N<sub>2</sub>-fixers under aerobic conditions. Vose<sup>19</sup> reported greater nitrogenase activity of *Azospirillum* with mixtures of *Bacillus*-like non-N<sub>2</sub>-fixers.

Mycorrhizal fungi can take up phosphorus from inert soil phosphates. The energy requirement for the nitrogenase reaction of N<sub>2</sub>-fixers comes from adenosine triphosphate (ATP). When phosphorus is deficient, the levels of phosphate-containing energy source ATP becomes limiting for nitrogenase activity. Ectomycorrhizal fungi could therefore be important in supplying phosphorus needed for nitrogen fixation by the associated N<sub>2</sub>-fixers.

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