AZOSPIRILLUM ISOLATED FROM WITHIN SPOROCARPS OF THE MYCORRHIZAL FUNGI *HEBELOMA CRUSTULINIFORME*, *LACCARIA LACCATA* AND *RHIZOPOGON VINICOLOR*

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Nitrogenase activity was detected when tissue fragments from within sporocarps of *Laccaria laccata* and *Rhizopogon vinicolor* were inoculated into nitrogen-free liquid medium and incubated under micro-aerophilic conditions (99% N_2+1% O_2) at 30 °C. Nitrogenase activity was also detected when tissue homogenate from within sporocarps of *Hebeloma crustuliniforme* was incubated in nitrogen-free semi-solid agar medium. *Azospirillum* spp. were isolated from the nitrogenase-active cultures. *Azospirillum* isolated from *R. vinicolor* had higher nitrogenase activity than the isolate from either *H. crustuliniforme* or *L. laccata*.

Isolation of bacteria from fungal sporocarps has often been reported. Swartz (1929) isolated bacteria from within sporocarps of Lycoperdaceae with either broken or unbroken peridia, although the role of the bacteria is unknown. Larsen *et al.* (1978) demonstrated nitrogenase activity, measured by acetylene reduction, in sporocarps of decay fungi growing on dead tree boles. The responsible organisms were not isolated, but bacteria in sporocarps were detected with scanning electron microscopy. Spano *et al.* (1982) reported isolation of nitrogen-fixing bacteria from within sporocarps of *Fomitopsis pinicola* (Fr.) Karst. growing on decaying wood. The isolates were not tested for nitrogenase activity, however.

We have successfully isolated and identified acetylene-reducing bacteria from within sporocarps of three ectomycorrhizal fungi, *Suillus ponderosa* Smith & Thiers, *Hymenogaster parksii* Zeller & Dodge, and *Tuber melanosporum* Vitt. (Li & Castellano, 1985). In a further study with simple modified procedures, we examined sporocarps of *Hebeloma crustuliniforme* (Bull.) Quél., *Laccaria laccata* (Scop.: Fr.) Berk. & Br., and *Rhizopogon vinicolor* Smith for nitrogen-fixing bacteria, as measured by their acetylene reduction. These three fungi are common ectomycorrhizal associates of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) in the Pacific Northwest.

In 1984 fresh sporocarps of *H. crustuliniforme* and *L. laccata* were collected in the fall, and *R. vinicolor* in the spring, under Douglas fir in the Coast Range of Oregon. For each sporocarp three to five pieces of interior glebal (*R. vinicolor*) or stipe (*L. laccata*) tissue were aseptically removed and placed in 20 ml of Döbereiner's nitrogen-free liquid medium (Döbereiner & Day, 1976) in a 60 ml serum bottle. After 3 d at 30 °C under micro-aerophilic conditions (99% N_2, 1% O_2), the media became turbid. For *H. crustuliniforme*, fragments of tissue were removed from the stipe interior and homogenized in Döbereiner's nitrogen-free liquid medium with a sterilized mortar and pestle. Tissue debris was removed by filtering the homogenate through sterilized cheese cloth; one drop of filtrate was inoculated on to 12 ml of Döbereiner's nitrogen-free semi-solid agar in a 32 ml test tube. After 3 d at 30°, each tube contained a thick pellicle formed below the agar surface.

Acetylene was then injected into each bottle or tube to 10% of the total gas volume. Bottles and tubes without added acetylene served as controls. After 18 h a 0·1 ml gaseous sample from each bottle and tube was removed and analysed for ethylene and acetylene with a Hewlett-Packard 5830A gas chromatograph fitted with a 2 m × 2·1 mm, 80–100 mesh, Porapack R column with oven temperature at 70°*. Injection temperature and flame-ionization detector temperature were each adjusted to 100°, and flow rate of the nitrogen carrier gas was adjusted to 40 ml per min.

Acetylene-reducing bacteria from the mixed cultures were purified by repeated streaking on Döbereiner's nitrogen-free agar medium supplemented with 0·002% yeast extract. To test the nitrogenase activity of isolated bacteria, as measured by acetylene reduction, an aqueous suspension of each bacterial isolate was inoculated into serum bottles that contained Döbereiner's nitrogen-free liquid medium supplemented with yeast extract and vitamins (Barber & Evans, 1976). The bottles were capped with a sterilized serum-stopper and incubated under micro-aerophilic conditions at 30° for 3 d. Acetylene was injected into each bottle. The formation of ethylene was determined as previously described.

* Use of trade names does not imply endorsement or approval by the USDA Forest Service.

Table 1. *Nitrogenase activities of* Azospirillum *isolated from within sporocarps of three ectomycorrhizal fungi**

	Acetylene-reduction activity (nmoles ethylene formed per mg protein per h)
Laccaria laccata	20*a*
Hebeloma crustuliniforme	56*b*
Rhizopogon vinicolor	207*c*

* Data are means of four replicates. Treatment means not sharing a common letter differ significantly by Tukey's test at $P = 0{\cdot}05$.

Bacterial cells in the bottles were harvested and washed with cold 5% trichloracetic acid. Cell protein was solubilized with 0·5 N-NaOH in a boiling water bath for 10 min (Agarwal & Keister, 1983) and measured by the modified Lowry method (Markwell *et al.*, 1978).

Bacteria isolated from within sporocarps of *H. crustuliniforme*, *L. laccata* and *R. vinicolor* were aerobic chemoheterotrophs and grew well on nutrient and trypticase soy agar. They reduced acetylene on nitrogen-free medium under microaerophilic conditions (Table 1), and thus, presumably, are able to fix gaseous nitrogen. All bacteria observed in water under light microscopy showed a characteristic spiral movement. Cells are straight to curved, plump, slightly pointed rods with phase-dense granules. All form a distinctive pellicle with acetylene-reduction activity in nitrogen-free semi-solid agar. These characteristics are typical of the genus *Azospirillum* (Kreig & Döbereiner, 1984; Bashan & Levanony, 1985). The morphology of the isolate colonies showed marked variation on agar media. The *Azospirillum* isolated from *R. vinicolor* had significantly more nitrogenase activity than that isolated from either *H. crustuliniforme* or *L. laccata*.

Energy for the nitrogenase reaction comes from cellular metabolic cycles in the form of adenosine triphosphate (ATP). Where phosphorus is deficient, a phosphate-containing energy source (ATP) may be limiting. Mycorrhizal fungi are known to take up phosphorus from inert soil phosphates and may supply the phosphorus required for the ATP cycle of the dinitrogen-fixing bacteria. *Hebeloma crustuliniforme*, *L. laccata* and *R. vinicolor* produce acid phosphatases that catalyse the hydrolysis of complex phosphorus compounds into a more readily absorbable form (Ho & Zak, 1979). Enhancement of bacterial growth and nitrogenase activity by sporocarp extract has been reported (Li & Castellano, 1985).

Whether the nitrogen fixed by bacteria in fungal sporocarps contributes to nitrogen requirements of the sporulating fungus is not known, but many fungi do use large amounts of nitrogen in producing sporocarps and spores. Fixation of atmospheric nitrogen by associated bacteria could partially satisfy such demands (Cowling & Merrill, 1966).

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IN VITRO SYNTHESIS OF MYCORRHIZA IN ROOT ORGAN CULTURES OF A TROPICAL DIPTEROCARP SPECIES

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A technique for observing mycorrhizal synthesis in root organ cultures of *Shorea roxburghii* under axenic conditions is described.

The dominant Dipterocarpaceae of South-East Asian rainforests are extensively logged for their valuable timber. Natural regeneration is poor and reforestation programmes are hampered by lack of suitable propagules, since dipterocarps fruit irregularly (Cockburn, 1975). Large areas of dipterocarp forests in S. E. Asia are therefore becoming depleted (Ashton, 1981; Jacobs, 1981). The possibility of using micropropagation techniques is now being explored (Smits & Struycken, 1983; Scott, in prep.). Ashton (1981) suggested that mycorrhizal research might shed light upon some of the problems encountered in vegetative propagation by cuttings and by tissue culture, and hence facilitate the re-introduction of dipterocarps in deforested areas. Singh (1966) was the first to report ectomycorrhizas in several genera of Dipterocarpaceae, and Becker (1983) looked at ten types of ectomycorrhiza in a lowland Malaysian rainforest. Little is known about the specificity of the association and the sequence of infection and development.

To date, all in vitro studies on mycorrhizal synthesis have involved temperate species of host and fungus (Fortin, 1966; Molina, 1979; Mason, 1980; Duddridge & Read, 1984; Grellier, Letouze & Strullu, 1984; Yang & Willcox, 1984; Richter & Bruhn, 1986). The need for mycorrhizal research in tropical forestry is crucial if current forestation practices are to be improved. In view of this, a preliminary study of root growth and mycorrhiza formation in axenic conditions was carried out using root organ cultures of a dipterocarp species and a fungal isolate from a tropical Basidiomycete.

Although *Shorea roxburghii* G. Don is native to the Malayan peninsula, especially N. W. Malaysia, it is found in the Singapore Botanic Gardens and was the only dipterocarp to produce a reasonable number of viable fruits during the period of study. Fresh fruits of *S. roxburghii* were washed in detergent solution (2 ml 'Teepol'/l) for 2–3 min and rinsed in tap water for 2–3 h. They were then surface sterilized by soaking in 0·2% mercuric chloride, with two drops of Tween 20, for 40 min, before rinsing five times in sterile distilled water. Using aseptic techniques, the cotyledons were dissected out, cut into pieces and cultured on a modified Murashige & Skoog (1962) MS agar medium with macro-elements at half-strength ($\frac{1}{2}$ MS), 2% (w/v) sucrose, 0·8% (w/v) agar and the plant-growth regulators naphthalene acetic acid (NAA, 0·5 mg l^{-1}) and 6-benzylamino purine (BA, 0·05 mg l^{-1}). This combination stimulated cotyledons to produce large numbers of adventitious roots (Fig. 1); entire roots were excised at the point of emergence from the cotyledon and the roots then cultured on basal $\frac{1}{2}$ MS medium (pH 5·6) to allow further growth.

Various sporophores were collected from the Bukit Timah Nature Reserve, a primary lowland forest in Singapore, where many dipterocarp species occur including *Shorea macroptera* Dyer, *S. curtisii* Dyer ex King, *Hopea mengarawan* Mig and *Dipterocarpus penangianus* Foxw. Spores and sporophores were used as inocula to initiate pure cultures on various nutrient media. A pure mycelial culture was established from the angularly shaped spores of a *Rhodophyllus* sp. which produced a pink spore print. Studies were therefore initiated with this isolate. Horak (1980) has described the various