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Nitrogen Fixation by *Azospirillum* in the Rhizoplane and Endorhizosphere of Western Hemlock (*Tsuga heterophylla*) Growing on Decaying Wood

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Abstract

Nitrogen fixing *Azospirillum* was isolated from the rhizoplane and endorhizosphere of mycorrhizal and non- mycorrhizal plants of western hemlock growing on decaying wood. The roots colonized with *Coenococcum geophilum*, *Piloderma* sp. and *Alpova smithi* showed more acetylene reduction activity (ARA) compared to the non-mycorrhizal roots. ARA was more in rhizoplane than in endorhizosphere. Roots colonized by *Coenococcum geophilum* showed more ARA followed by roots colonized by *Piloderma* spp. The least ARA was in roots colonized by *Alpova smithi*. ARA of pure culture of *Azospirillum* isolated from the rhizoplane and endorhizosphere showed a similar trend.

Introduction

Microbial processes are influenced by nutrient inputs and transfer within biosystems. Association of nitrogen fixing bacteria within sporocarps of ectomycorrhizal fungi has been reported (Li and Castellano, 1987; Tilak *et al.*, 1988). Microorganisms growing on decaying plant materials are known to fix atmospheric nitrogen (Aho *et al.*, 1974) and convert them into forms available for plant use. Microorganisms facilitated by decaying materials, such as ectomycorrhizal fungi, serve to export this nitrogen to forest vegetation (Harvey *et al.*, 1976). The present report deals with nitrogen fixation in the rhizoplane and endorhizosphere of western hemlock (*Tsuga heterophylla*) growing on decaying wood.

Material and Method

Twelve to eighteen months old plants of western hemlock growing on decaying wood in the forests of North West Pacific regions of Oregon, USA were selected for the present study. Mycorrhizal and non-mycorrhizal roots were collected and cut into

3 mm length segments. One gram of the root segments was thoroughly rinsed in sterile distilled water several times. For enumerating nitrogen fixing *Azospirillum* in rhizoplane the root segments were transferred, after washing them for 3-4 times in sterile phosphate buffer of pH 7.0, into nitrogen free semi-solid sodium malate medium (Okon *et al.*, 1977) For endorhizosphere population, the segments were treated with 1% chloramine-T for 30 minutes in order to avoid the organisms residing on the root surface. They were washed 4 times in sterile phosphate buffer of pH 7.0 and were plated on semi-solid nitrogen-free sodium malate agar in petriplate or immersed in 10 ml of the medium in screw capped tubes and incubated at 30°C. *Azospirillum* appeared on the second day as sub-surface white colonies originating initially from the cut ends of root segments and later spreading around the entire segment in petriplates. In tubes, the bacteria appeared as white pellicles 1-2 cm below the surface of the semi- solid medium.

In tubes showing *Azospirillum* growth, acetylene was injected to constitute 10% of the total gas volume. Tubes without added acetylene served as controls. After 24 h, 0.1 ml gaseous sample from each tube was removed and analysed for ethylene with a Hewlett Packard 5830 A gas chromatograph fitted with 2 mm x 2.1 mm 80-100 mesh poraback R column with oven temperature at 70°C and flow rate of the N carrier gas adjusted to 40 ml/min. Those tubes which showed the pellicle and also the acetylene reduction activity (ARA) were considered as *Azospirillum* positive.

Acetylene - reducing bacteria from mixed cultures were purified by repeated streaking on N-free sodium malate medium supplemented with 0.002% yeast extract (Barber and Evans, 1976). The characteristics of the isolates were typical of the genus *Azospirillum* (Krieg and Dobereiner, 1984).

To test the nitrogenase activity of isolated bacteria, as measured by ARA, an aqueous suspension of each bacterial isolate was inoculated into screw capped tubes that contained N-free sodium malate medium supplemented with 0.002% yeast extract. The tubes were incubated under microaerophilic conditions (99% N_2 + 1% O_2) at 30° for 3 days. Acetylene was injected into each tube. The formation of ethylene was determined as described earlier.

Bacterial cells in the tubes were harvested and washed with cold 5% TCA. Cell protein was solubilized with 0.5 N NaOH in a boiling water bath for 10min. (Agarwal and Keister, 1983) and measured by modified Lowry method (Markwell *et al.*, 1978). Phosphorus concentration in root was estimated by Vanadomolybdate method (Chapman and Pratt, 1961).

The ectomycorrhizal fungi associated with roots of western hemlock were identified (Dominik, 1969; Zak, 1973) as *Coenococcum geophilum*, *Piloderma* sp. and *Alpova*

smithi. Three different ectomycorrhizal fungi were observed to form the mycorrhiza. The mycorrhiza formed by the 3 fungi were morphologically distinct and hence collected and studied for *Azospirillum* association separately.

Results and Discussion

The nitrogen fixing *Azospirillum* were isolated from the rhizoplane as well as the endorhizosphere of both mycorrhizal and non-mycorrhizal roots. However, the mycorrhizal roots showed more ARA than the non-mycorrhizal roots (Table I). This may be attributed to better establishment of the diazotroph in the presence of mycorrhizal fungi. The environment around the mycorrhizal roots may also be more conducive for the growth of *Azospirillum*. The ARA was more on rhizoplane than in the endorhizosphere of plants which may be attributed to the fact that the root exudates might have played a significant role in increasing the population of the *Azospirillum* on the root surface. Roots colonized by *Coenococcum geophilum* showed more ARA activity followed by roots colonised by *Philoderma* sp. The least ARA activity, among the roots colonized by the 3 fungi, was recorded in *Alpova smithi* colonized roots.

The ARA of the pure cultures of *Azospirillum* isolated from the rhizoplane and endorhizosphere of western hemlock roots colonized by the 3 different ectomycorrhizal fungi, showed a similar trend (Table II).

It was interesting to note that the mycorrhizal roots had more P concentration than the non-mycorrhizal roots (Table I). The energy requirement for nitrogenase activity of diazotrophs comes from phosphates which can be mobilized by ectomycorrhizal fungi. In the present investigation a significant positive correlation between the P concentration of roots and nitrogenase activity (+0.725) was noticed. This suggests

TABLE I

Acetylene reduction activity (ARA) and total P content of the rhizoplane and endorhizosphere of mycorrhizal and non mycorrhizal western hemlock

Treatment	ARA (n moles acetylene reduced /h/mg protein		
	Rhizoplane	Endorhizosphere	P concentration of root (mg/g root)
Non-mycorrhizal root	55	25	2.5
Mycorrhizal root:			
Piloderma sp.	185	155	3.5
Coenococcum geophilum	205	163	4.2
Alpova smithi	150	105	4.0
LSD at P = 0.05	32.5	27.8	0.52

TABLE II

Acetylene reduction activity (ARA) of pure cultures of Azospirillum isolated from rhizoplane and endorhizosphere of mycorrhizal and non-mycorrhizal western hemlock.

Treatment	ARA (n moles acetylene reduced/h/mg protein	
	Rhizoplane	Endorhizosphere
Non-mycorrhizal root	32	50
Mycorrhizal root:		
Philoderma sp.	155	255
Coenococcum geophilum	185	302
Alpova Smithi	105	225
LSD at P = 0.05	42.9	

that mycorrhizal fungi play an important role in supplying P needed for nitrogen fixation by *Azospirillum* in the rhizoplane and endorhizosphere of western hemlock.

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