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Characterization of Nitrogen-fixing *Azospirillum* isolated from within Sporocarps of Ectomycorrhizal fungi associated with Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco]

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Abstsact Three strains of nitrogen-fixing *Azospirillum* were isolated from within sporocarps of three ectomycorrhizal fungi associated with Douglas-fir : *Hebeloma erustuliniforme*, *Laccaria laccata*, and *Rhizopogon vinicolor*. Each strain possessed characteristics typical of *Azospirillum* and all closely resembled *A. brasilense*. They exhibited maximum nitrogen-fixing ability (acetylene-reducing activity) at pH's ranging from 6.5 to 8.5 and at temperature between 30 and 35°C when grown in N-free, semisolid sodium malate medium. Presence of combined nitrogen (0.1% NH_4Cl) suppressed acetylene reduction by all three strains.

Key words. *Azospirillum*, sporocarps, ectomycorrhizal fungi, Douglas-fir,

Introduction

Association of *Azospirillum* spp. with roots of various crops and grasses has been well documented (1–5). Li and Castellano (6) isolated three strains of acetylene-reducing *Azospirillum* spp. from within sporocarps of three common ectomycorrhizal fungi—*Hebeloma crustuliniforme* (Bull. ex St. Am.) Quel., *Laccaria laccata* (Scop. : Fr.) Berk. & Broome, and *Rhizopogon vinicolor* Smith—associated with Douglas-fir (*Pseudotsuga menziesii* (Mirb. Franco) in forests of the Oregon Coast Range. The present study deals with the morphological and physiological characteristics of the three isolates, obtained from within the sporocarps of the above said ectomycorrhizal fungi.

Materials and Methods

Each isolate was morphologically and physiologically characterized by the methods outlined by Tarrand *et al.* (7). Standard strains of *A. brasilense* (SL 33) and *A. lipoferum* (1 CM 1001) and *A. amazonense* (AMS 91) were obtained from the International Crops Research Institute for Semi-Arid Tropics, Hyderabad, India, for comparison. Effects of various factors, such as pH, temperature, and combined nitrogen, on the nitrogenase activity (acetylene-reducing activity) (ARA) of the three isolates were determined on semisolid sodium malate medium (8). Where combined nitrogen was used, it was incorporated as NH_4Cl, 0.1% by weight. The bacteria were grown in screw-capped 50-ml serum bottles at 30°C (unless otherwise stated) under stationary conditions for 72 h and were assayed for ARA. Ten per cent of the atmosphere in the bottles was replaced with acetylene. After 20 h incubation at 30°C, 0.1-ml gaseous samples from each bottle were removed; production of

Table 1. Characteristics of three strains of *Azospirillum* (isolated respectively from within sporocarps of three ectomycorrhizal fungi) in comparison with known isolates of *A. brasilense* and *A. lipoferum*

Characteristics	*Azospirillum* strains isolated from			*Azospirillum brasilense*	
	Laccaria laccata	*Hebeloma crustuliniforme*	*Rhizopogon vinicolor*	(strain SL 33)	*A. lipoferum* (ICM 1001)
Cell morphology in semi-solid malate medium for 72 h at 35°C	Medium sized, motile rods with round ends. PHB granules conspicuous	Dark colored, medium sized plump rods Highly motile, PHB granules conspicuous	Medium-sized thin rods, with round ends, motile cells, PHB granules conspicuous	Dark colored medium-sized plump rods Highly motile. PHB granules conspicuous	Medium sized plump rods with smooth to pointed ends. Motile, PHB granules conspicuous
Requirement of biotin	Not required	Not required	Not required	Not required	Required
Growth in N-free semisolid medium containing glucose and α-ketoglutarate as sole carbon sources	Poor	Poor	Poor	Poor	Good
Acidification of peptone-based glucose medium after 72 h	Negative	Negative	Negative	Negative	Positive
Production of gas under anaerobic conditions	Negative	Negative	Negative	Negative	Positive
Urease activity	Good	Good	Good	Good	Good
Hydrolysis of gelatin	Negative	Negative	Negative	Negative	Negative
Reduction of NO_3 to NO_2	Positive	Positive	Positive	Positive	Positive
Production of indole	Negative	Negative	Negative	Negative	Negative
Sole source of carbon with[1] $(NH_4)_2SO_4$ as N source :					
α–ketoglutarate	—	—	—	—	++
Glucose	+	+	+	+	+
Mannitol, sorbitol, and ribose	+	+	+	+	+
Glucose, arabinose	+	+	+	+	+

[1]Growth : — no growth; + negligible growth; ++ excellent growth.

ethylene was estimated by use of a Nucon gas chromatograph fitted with a Porapak R column. Oven temperature was adjusted to 100°C. Bacterial cell protein was determined by the method of Lowry *et al.* (9). Acetylene-reducing activity was expressed as n moles C_2H_4 produced mg^{-1} of protein h^{-1}. Acetylene-lacking controls were also analyzed in each case.

All treatments were replicated four times in a completely randomized design. Data were subjected to analysis of variance with differences between means tested by Tuke's test.

Results and Discussion

The three strains isoloted from sporocarps of the three ectomycorrhizal fungi showed characteristic spirillar movement, grew well on nutrient and trypticase soy agar, and reduced C_2H_2 on N-free malate medium under microaerophilic conditions. These characteristics are typical of *Azospirillum* (10). Each isolate showed close similarity to *A. brasilense* by virtue of characteristics (Table 1) such as absence of growth in N-free, semisolid media containing alpha-keto glutarate as sole source of carbon, no requirement of biotin for growth, no acid formation in peptone-based glucose medium, and no gas production when used as a carbon source (7). The isolates did not show any resemblences with *A. amazonense* since they did not form white colonies on potato agar (BMS agar) (10) and their inability to use sucrose as a sole carbon source for nitrogen fixation (11, 12).

All three strains showed maximum ARA at pH 6.5 and above. Maximum ARA occurred between 30 and 35°C (Table 2). Maximum nitrogen-fixing ability of different

Table 2. Nitrogenase activity of three *Azospirillum* strains from within sporocarps of three ectomycorrhizal fungi

Fungal source of isolates	Nitrogenase activity[1] (n moles C_2H_4.mg protein^{-1}.h^{-1})							
	pH				Temperature			
	5 5	6.5	7.5	8.5	25°C	30°C	35°C	40°C
Laccaria laccata	42b	85c	92c	88c	30b	60d	65d	20a
Hebeloma crustuliniforme	30a	92c	95c	95c	28b	75e	85e	16a
Rhizopogon vinicolor	28a	45b	52b	50b	16a	26b	42c	10a

[1]Data are means of four replicates. Treatment means for each row of pH range and temperature not sharing a common letter differ significantly at P=0.05. Grown at different pH's and temperatures in semi-solid sodium malate medium at 30°C.

strains of *Azospirillum* isolated from the roots of rice, maize, sorghum and grasses at temperatures between 30 and 35°C was reported by Subba Rao *et al.* (13). Addition of 0.1% NH_4Cl inhibited the ARA in all the strains tested (Table 3). *Azospirillum* may grow best microaerophilically even not fixin nitrogen (14).

Mycorrhizal fungi are particularly effective in assimilating nutrients (particularly phosphorus) from soil (15). Phosphorus-deficient conditions limit production of the

Table 3. Influence of combined nitrogen on nitrogenase activity of three *Azospirillum* strains from within sporocarps of three ectomycorrhizal fungi

Fungal sources of isolates	Nitrogenase activity[1] (n moles C_2H_4 mg protein^{-1}.h^{-1})	
	No nitrogen added	0.1% NH_4Cl added
Laccaria laccata	85 b (++)	0 (++)
Hebeloma crustuliniforme	105 a (+++)	0 (+++)
Rhizopogon vinicolor	65 c (++)	0 (++)

[1]Data are means of four replicates. Treatment means for each *Azospirillum* strain not sharing a common letter within the column differ significantly at P=0.05. Figures in parentheses indicate growth of the organisms : +++ very good; ++ good. At 30°C (pH 7.5).

phosphate-rich energy source, adenosine triphosphate (ATP). Adenosine triphosphate is needed as energy for nitrogen fixation by diazotroph. Ectomycorrhizal fungi could therefore help meet phosphorus needs of the diazotrophs associated within the sporocarp, diazotroph growth and nitrogenase activity are enhanced by sporocarps extracts of different ectomycorrhizal fungi (16). The interior tissues of fungal sporocarps seem an appropriate habitat for *A. brasilense* because it appears to grow best under microaerophilic conditions (14).

To our knowledge, *A. brasilense* has not been previously detected in soils or organisms from coniferous forests. This species is likely more widely distributed than in just the fields and pastures where it has been previously isolated.

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