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Nitrogen Fixation by *Azospirillum* in Some Tropical Plants

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The nitrogen-fixing bacterium, *Azospirillum* has been found associated with the root environments of plants such as maize, finger millets, kudiraivali, guinea grass, nut grass and hariyali grass. The rhizosphere and inner rhizoplane of these plants harboured a denser population of *Azospirillum* than in the rhizoplane. *Azospirillum* isolates from these plants have exhibited a high potential for nitrogen fixation. Pre-incubation of the excised roots with different energy sources greatly enhanced the nitrogenase activity. There was no relationship between the population of *Azospirillum* and nitrogenase activity in the root system.

Key Words: Nitrogen Fixation, Associative Symbiont, *Azospirillum*, **Nitrogenase,** *Rhizosphere rhizoplane*

Introduction

The associate symbiont, *Spirillum lipoferum* (syn. *Azospirillum lipoferum* Beij.) is receiving greater attention in recent years as in many tropical grasses and grain crops *S. lipoferum* is found to be the major nitrogen-fixing bacterium (Day et al. 1975, Newton et al. 1977). In Brazil, Neyra and Döbereiner (1977) made extensive studies on this bacterium and suggested that the root environments of most tropical plants like maize, sorghum and *Digitaria* serve as conducive 'microbiological niches' for the activities of this bacterium. The impressive data collected by Okon et al. (1976) revealed that of the total number of bacteria colonising the rhizosphere of maize ca. 60-90% was constituted by *Azospirillum* alone. Recent findings of Subba Rao (1979) emphasize the role of *Azospirillum* in the nitrogen economy of cereal cropsin India.

It is of interest to observe that most of the plant species examined thus far for the activities of *Azospirillum* and found efficient in nitrogen fixation fall under C_4 type (Okon et al. 1976, Barber et al. 1979). By virtue of their unique photosynthetic pathway, the C_4 plants are known to exude through the root system appreciable amounts of energy source which in turn help the colonization of *Azospirillum* (Jagnow 1979). In the present study, the quantitative

occurrence of this bacterium and nitrogen fixation in the root environment of a few C_4 plants have been investigated.

Materials and Methods

Maize (*Zea-mays* var. Ganga) finger millet (*Eleusine coracana* var. Co. 7) Kuthiraivali (*Echinochola colona* var. *frumentace*) guinea grass (*Panicum maximum*) nut grass (*Cyperus rotundus*) and hariyali grass (*Cynodon dactylon*) grown in the University farm were chosen for the study. From plants at flowering stage of growth the rhizosphere soil samples were collected (Pramer & Schmidt 1966). Samples of rhizoplane, the soil-free root surface were also obtained by gently washing the roots with sterile distilled water. For enumerating *Azospirillum* occurring within the root tissue, a weighed quantity of freshly collected root was washed thoroughly in changes of sterile distilled water, macerated in a surface-sterilized porcelain pestle and motar and the slurry after serial dilution in sterile dist water was used. The MPN (Most Probable Number) technique was used for enumeration (Okon et al. 1977, Hegazi et al. 1979). The formation of subsurface characteristic white pellicles in malate semi-solid medium, a change to dark blue colour of the medium, positivity in acetylene reduction test (more than 5 n moles of C_2H_4/hr), the appearance of pink irregular dense colonies when streaked on special potato agar medium and the occurrence of typically curved cells on microscopical examination served as indices in the quantification of *Azospirillum* (Döbereiner et al. 1976).

For studying the influence of pre-incubation of roots with energy sources, excised fresh root bits (510-mm) of the plants were incubated under reduced oxygen pressure (PO_2=ca. 0.2 atm) for 8 hr in (i) 50 nm of sodium malate (ii) 50 nm of sodium succinate (iii) 20 nm of sodium bicarbonate (iv) 50 nm of glucose, and (v) distilled water. Root bits without pre-incubation served as control. The nitrogenase activity was determined in a Perkin Elmer Gas Liquid Chromatograph Model F. 33 following the procedure detailed elsewhere (Purushothaman et al. 1979).

In order to compare the *in vivo* nitrogenase activity of the roots with the activity of purified cultures of *Azospirillum*, the isolates were grown in semi-solid malate medium in 15 ml serum tubes and after 96 hr of growth the cultures were assayed for nitrogenase activity in GLC.

Results and Discussion

The results presented in table 1 reveal that *Azospirillum* is very widely distributed in the root environments of the plants. The rhizosphere and inner rhizoplane registered more *Azospirillum* counts than in the rhizoplane. That all the six plant species examined harboured this organism suggests the widespread occurrence in tropical soils. Nearly 60% of the soil samples in Africa, USA and Brazil have been reported to contain this organism (Döbereiner et al. 1976). The effect of pre-incubation of root tissue with different substrates (table 2) indicated that sodium malate, sodium succinate, glucose and sodium bicarbonate have stimulated the nitrogenase activity. In a similar study, Döbereiner (1977) also observed that pre-incubation of fresh excised roots of sorghum with malate increased the nitrogenase activity by 100%. Since *Azospirillum* prefers malic acid or succinic acid as energy sources and also that during the pre-incubation period there might be increase in the population of *Azospirillum*, there was an increased

Table 1 *Distribution of* Azospirillum *in the root environments of certain C_4 plant species*

Plant species	Rhizosphere (10^3/g soil)	Rhizoplane (10^2/g of fresh root)	Inner rhizoplane (10^3/g of fresh root)
Zea mays Linn.	3.0 ± 0	12.0 ± 1.0	89.0 ± 5.0
Eleusine coracana Linn.	10.5 ± 1.5	16.0 ± 6.0	94.0 ± 18.0
Cyperus rotundus Linn.	2.0 ± 0	11.5 ± 3.5	146.5 ± 18.5
Cynodon dactylon Pers.	7.0 ± 3.0	20.0 ± 6.0	64.0 ± 22.0
Echinochloa colona var. *frumentacea* Link.	18.5 ± 2.5	25.0 ± 7.0	133.0 ± 13.0
Panicum maximum Gaertn	22.0 ± 4.0	40.0 ± 2.0	177.0 ± 39.0

Table 2 *Effect of the pre-incubation of excised root tissue on the nitrogenase activity* (Data represent mean of two determinations)*

Treatment	*Zea mays*	*Eleusine coracana*	*Cyperus rotundus*	*Cynodon dactylon*	*Echinochloa colona* var. *frumentacea*	*Panicum maximum*
Excised roots + 50 mM sodium malate	470.0±90.0	376.0±144.0	255.5±69.5	281.5±36.5	435.0±89.0	216.0±30.0
Excised roots + 50 mM sodium succinate	368.0±50.0	186.0± 10.0	349.0±63.0	394.0±18.0	252.0±34.0	289.0±29.0
Excised roots + 20 mM sodium bicarbonate	162.0±58.0	81.0± 9.0	121.0±25.0	49.5±22.5	187.5±22.5	121.0±23.0
Excised roots + 50 mM glucose	95.0±25.0	130.0± 18.0	138.5±26.5	160.0±48.0	190.0± 6.0	94.0±18.0
Excised roots+dist. water	24.0±10.0	51.0± 33.0	50.0± 2.0	59.0± 5.0	59.0± 3.0	64.0±12.0
Excised roots (control) no pre-incubation	16.2± 3.8	8.0± 2.0	12.8± 0.25	38.0± 6.0	31.0±13.0	15.0± 3.0

*Nitrogenase activity expressed as n moles of C_2H_4 formed g/hr at 28°C

nitrogenase activity. Interestingly there was, however, no correlation between the population of *Azospirillum* and nitrogenase activity. *Zea mays* though harboured less population of *Azospirillum* recorded the highest nitrogenase activity suggesting perhaps the state of metabolic activity of the organism rather than the population that matters in N_2 fixation. All isolates of *Azospirillum* from the six plant species exhibited high nitrogenase activity (table 3), however, the isolates from *Cyperus rotundus* and *Zea mays* recorded higher nitrogenase activity.

Table 3 *Nitrogenase activity of certain isolates of* Azospirillum *in vitro*

Isolate No.	Plant species	n moles of* C_2H_4 formed/ hr/tube
Zm.1	*Zea mays*	240.0
EC.5	*Eleusine coracana*	165.0
Cyp.3	*Cyperus rotundus*	260.0
Echi.4	*Echinochloa colona* var. *frumentacea*	98.0
pm.6	*Panicum maximum*	185.0

*Mean of two estimations
Assay conditions: PO_2=0.02atm; Temp. 28°C, Age of culture=96 hr in 5m

In higher plants as much as 0.5 to 5.0% of the photosynthate flows down to the root system as root exudation (Barber & Lynch 1977 and Newman 1978). Malate, the preferred carbon source contained in the root exudates of C_4 plants stimulates N_2 fixation. Our earlier experiments have indicated that without pre-incubation, soil or plant roots recorded only very low nitrogenase activity. However, these data reveal the potential of substantial nitrogen fixation under the root system of tropical plants. Recently, Baltensperger et al. (1979) recorded increased growth of Bermuda grass (*Cynodon doctylon*) due to *Azospirillum* inoculation.

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