Proc. Indian natn. Sci. Acad., **B62**, No. 1, pp 25-30 (1996)

Herbaspirillum Associated with Forage Grasses

B N INDIRA and D J BAGYARAJ

Department of Agricultural Microbiology, G.K.V.K. Campus, University of Agricultural Sciences, Bangalore 560 065

(Received on 14 February 1995; Accepted on 25 September 1995)

Occurrence of *Herbaspirillum* in the rhizosphere and endorhizosphere of seventeen grasses was examined. Enumeration of *Herbaspirillum* showed that the population varied from 10^2 to 10^4 in the rhizosphere and endorhizosphere of the 17 forage grasses. Identity of *Herbaspirillum* was confirmed based on morphological and physiological characters. Colony morphology on different media, Gram reaction, shape, size and motility were used as morphological characters. Physiological characters like positive catalase activity carbon-source utilization, salinity-tolerance, *p*H requirement, nitrate-dissimilation were studied. *Herbaspirillum* isolates were further confirmed and differentiated from *Azospirillum* based on their negative reaction in acidified peptone medium and biotin medium. Acetylene-reduction activity was also measured. A total of 48 *Herbaspirillum* isolates were obtained from endorhizosphere and rhizosphere of 17 forage grasses.

Key Words: ***Herbaspirillum*, *Azospirillum*, Nitrogen fixers, Forage grasses**

Introduction

Nitrogen fixation associated with roots of grasses has been recognised as an important factor in the maintenance of soil fertility (Dart 1986, Dobereiner 1985). Such studies received an impetus with the report of the occurrence of *Azospirillum* in the rhizosphere of various grasses and its close association with the roots (Dobereiner et al. 1976). The case of recognition of *Azospirillum* by its characteristic subsurface pellicle in the semisolid selective medium has led many workers to conclude its occurrence in the rhizosphere and root surfaces (Hegazi et al. 1979, Rao & Venkateswarlu 1985). However close investigation of such pellicles revealed presence of microorganisms other than *Azospirillum* (Bilal et al. 1990, Seldin et al. 1984. Haahtela et al. 1983). *Herbaspirillum seropedicae* was found to be associated with *Azospirillum* spp. (Baldani et al. 1986) isolated from cereals for the first time in Brazil. *Herbaspirillum* resembles *Azospirillum* in forming characteristic subsurface pellicle and shape, and can be isolated using N-free malate (NFB) medium. This led to the assumption that *Herbaspirillum* is another species of *Azospirillum*. RNA/RNA hybridization studies disproved this assumption and a new genus *Herbaspirillum* was established (Gills et al. 1989).

This paper describes the occurrence, isolation and characterization of *Herbaspirillum* from the rhizosphere and endorhizosphere of seventeen forage grasses.

Materials and Methods

Rhizosphere and endorhizosphere samples of the following forage grasses: Co-1 (*Pennisetum purpureum* × *Pennisetum typhoides*), blue panic grass (*Panicum antid-*

otale), green panic grass (*Panicum maximum* var *trichoglume*), green panic mutant (*Panicum maximum*), rhodes katambora (*Chloris gayana*), rhodes mutant (*Chloris gayana*), para grass (*Bracharia mutica*), bahia grass (*Paspalum notatum*), khus grass (*Vetiveria zizaniodes*), dallis grass (*Paspalum dilactum*), sudan grass (*Sorghum sudanense*), majjige grass (*Setaria* sp.), marvel grass (*Dicanthium annulatum*), Arundinella (*Chrysopogan fulvus*), nandisetaria (*Setaria sphacelata* var *sericea*), guinea riversdale (*Panicum maximum*), signal Kennedy (*Bracharia eminli*) were collected from the Crop Museum of the University of Agricultural Sciences, Bangalore.

Enumeration of *Herbaspirillum* sp. in rhizosphere and endorhizosphere of forage grasses was done following the most probable number (MPN) technique (Okon et al. 1979, Hegazi et al. 1979) similar to *Azospirillum*. One g of rhizosphere sample and 1 g of fresh surface sterilized (30% chloramine-T for 15 min) crushed root suspension were diluted serially 10 folds in sterile water and inoculated to semisolid N-free malate (NFb) medium in tubes and plated in solid agar NFb plates following spread plate technique. After incubation of plates for 3-4 days. *Herbaspirillum* appeared as small moist colonies with green or greenish centre. Dilutions which showed the presence of *Herbaspirillum* in plates were checked for pellicle formation in MPN tubes. Presence of pellicles in MPN tubes and appearence of small moist colonies with greenish to green centre were taken as positive for the presence of *Herbaspirillum* sp. *Herbaspirillum* population was determined from MPN tables and expressed as per g dry soil per g dry roots.

Isolation of *Herbaspirillum* was done following the enrichment culture technique (Baldani et al. 1986, Dobereiner 1992). Rhizosphere soil was serially diluted (10 folds) and inoculated to semi-solid N-free malate medium in tubes and incubated for 3-4 days at 30°C. Fresh-root bits were surface sterilized (3% chloramine-T for 15 min), crushed, and the suspension was transferred to semisolid N-free malate medium in test tubes and incubated for 3-4 days. After 3-4 days of incubation at 30°C in semisolid NFb medium characteristic subsurface pellicle was observed. Single colonies were picked from NFb agar plates which were streaked with inoculum picked from pellicle. Colonies were developed small and moist with greenish to green centre. Final purification was done by streaking on potato infusion agar (BMS) as described by Dobereiner (1992) on which they produced small moist colonies with brownish to brown centre, characteristic of *Herbaspirillum*.

Isolates were streaked on YEMA (Yeast extract mannitol agar) supplemented with malate and congo red to differentiate from *Azospirillum*. Further the isolates were identified based on morphological and physiological tests. Microscopic observations of isolates for Gram reaction, shape, size and motility were done. Physiological tests like catalse activity, carbon source utilization, *p*H requirement and salinity tolerance were conducted as described by Baldani et al. (1986). Biotin requirement, acid production in acidified peptone medium and glucose were determined according to Tarrand et al. (1978) to differentiate *Herbaspirillum* from *Azospirillum*. Nitrate dissimilation (Neyra et al. 1977) and acetylene reduction activity of the isolates were also tested (Hardy et al. 1968).

Results

Herbaspirillum was detected in all the 17 forage grasses and their population varied from 0.01 to 10.30×10^3/g in rhizosphere soil and root (table 1). Population of *Herbaspirillum* was more in the endorhizo-

Table 1 *Population of* ***Herbaspirillum*** *sp. occurring in the root zone of forage grasses*

Forage grasses	*Endorhizosphere/ g root* ($\times 10^3$)	*Rhizosphere/ g soil* ($\times 10^3$)
Co-1	5.00	0.10
Blue panic grass	2.00	0.10
Green panic grass	4.00	0.30
Green panic mutant	0.33	0.03
Rhodes mutant	0.45	0.03
Rhodes katambora	10.30	0.24
Signal kennedy	0.10	0.10
Para grass	0 47	0.22
Bahia grass	0.72	0.08
Khus grass	2.40	0.11
Dallis grass	0.59	4.50
Sudan grass	2.70	0.01
Marvel grass	0.89	3.40
Majjige grass	4.20	0.07
Nandi setaria	7.10	0.08
Arundinella	0.41	2.30
Guinea riversdale	0.11	0.03

sphere compared to rhizosphere. The lowest population of *Herbaspirillum* in endorhizosphere 0.41×10^3/g roots) and rhizosphere 0.01×10^3/g soil) was observed in arundinella grass and sudan grass, respectively. Rhodes katambora roots and dallis grass rhizosphere harboured the highest numbers of *Herbaspirillum* (10.3×10^3/g roots and 4.5×10^3/g soil, respectively).

Herbaspirillum isolates were identified, based on their colony morphology on NFb, BMS and congo red media and then further tests were done to confirm the identity of the isolates using Z-67 (ATCC-35892) as reference culture obtained from Dr. Dobereiner, EMBRAPA, Brazil. On NFb medium supplemented with 20 mg/ litre yeast-extract, isolates appeared as moist small colonies of beige colour with greenish to green centre. On BMS agar plates, *Herbaspirillum* isolates appeared after 3-4 days of incubation as small moist colonies with brown to brownish centre and with foul smell emitted from such plates. *Herbaspirillum* isolates did not turn scarlet as *Azospirillum* in N-free YEMA supplemented with malate and congo red which helped in confirming the identity of isolates and differentiating from *Azospirillum.*

Further these 48 isolates tentatively identified as *Herbaspirillum* on microscopic examination revealed that they are Gram negative, vibroid in shape, varying size from 0.1 to 0.8 μm wide and 2.0 to 3.0 μm long and showed typical spiral movement. They did not show poly beta hydroxy butyrate (PHB) inclusions in the cytoplasm. Microscopical observation and colony morphology of the isolates were similar to reference culture Z-67.

All the 48 isolates showed the following physiological activities: Positive catalase activity, aerotaxis (movement towards surface and formation of pellicle), salinity tolerance (1% NaCl in medium) and wider *p*H tolerance (5.4 to 7.8). These isolates did not require plant growth factors and could not dissimilate nitrate completely to N_2. *Herbaspirillum* isolates could neither utilize sucrose and glucose as sole carbon source, nor could they produce acid from glucose or acidified peptone media. Acetylene reduction activity under microaerobic conditions, when measured varied from 0 to 117.4 n moles of acetylene reduced/ hr by different isolates.

Based on morphological and physiological characters and on comparison with reference culture Z-67, identity of the isolates was confirmed as *Herbaspirillum* sp.

Discussion

Occurrence of *Herbaspirillum* was reported from Brazil in cereals, but their popu-

Table 2—*Some morphological and physiological characters of* ***Herbaspirillum*** *sp. and* ***Azospirillum*** *sp.*

	Herbaspirillum sp.	*Azospirillum* spp.
Growth under air	+	+
Microaerobic N_2 fixation	+	+
Aerotaxis	+	+
Gram reaction	−	−
Shape	Spirillum-like	Sprillum-like
Size	0.6-0.8 μm × 2.0-3.0 μm	0.8-1.0 μm × 2.0-4.0μm
Motility	Spiral	Spiral
Presence of PBH granules	−	+
Optimum *p*H	5.4-8.0	6.0-7.0
Salinity tolerance	+(>1.0%)	−
Biotin requirement	−	+/−
Glucose as sole carbon source	−	+
Acid production in acidified peptone medium	−	+

lation was not enumerated (Baldani et al. 1986). This paper reports from the first time in India successful isolation and enumeration of *Herbaspirillum*. The population of *Herbaspirillum* in the rhizosphere of forage grasses varied from 0.01 to 4.50×10^3/g soil and in their endorhizosphere it varied from 0.10 to 10.30×10^3/g root. Morphological characters like Gram reaction, shape, size, absence of PHB granules and motility on comparison with the reference culture Z-67 were identical. Physiological characters: catalase activity, salinity tolerance, wider *p*H tolerance, and response to tests like: Biotin requirement, acid production in acidified peptone medium and from glucoe and nitrate dissimilation were also similar to that of the reference culture Z-67. All these characters helped in confirming the identity of *Herbaspirillum* isolates (table 2).

Acknowledgement

The authors are thankful to Mr Channakeshava, Sr Agronomist, University of Agricultural Sciences, Bangalore for sparing the grass samples used in this study.

References

Baldani J I, Baldani V L D, Selvin L and Dobereiner J 1986 Characterization of *Herbaspirillum seropedicae* gen.nov., sp.nov. a root associated nitrogen fixing bacterium: *Int. J. Syst. Bacteriol.* **36** 86-93

Bilal R, Rasul G, Quershi J A and Malik K A 1990 Characterization of *Azospirillum* and related diazotrophs associated with roots of plants growing in saline soils; *World J. Microbiol. Biotechnol.* **6** 53-56

Dart P J 1986 Nitrogen fixation association with non-legumes in agriculture; *Plant Soil* **90** 303-334

Dobereiner J 1988 Isolation and identification of root associated diazotrophs; *Plant Soil* **110** 207-212

—— 1992 The genera *Azospirillum* and *Herbaspirillum*; in *The Prokaryotes* Vol III pp. 2236-2253 eds A Balwos, H G Truper, M Dworkin, W Harder and K H Schleifer (London: Springer Verlag)

——, Mariel K E and Nerry M 1976 Ecological distribution of *Spirillum lipoferum* Beijerinck: *Can. J. Microbiol.* **22** 1464-1473

Gills M, Kersters K, Hoste B, Janssens D, Kroppenstedt R M, Stephen M P, Teixeira K R S, Dobereiner J and Delay J 1989 *Acetobactor diazotrophicus* sp.nov. a nitrogen-fixing acetic acid bacterium associated with sugarcane: *Int. J. Syst. Bacteriol.* **39** 361-364

Haahtela K, Helander I, Nurmiaho-Iassila E. L and Sundman V 1983 Morphological and physiological characteristics and lipopolysaccharide composition of N_2-fixing (C_2H_2-reducing) root associated *Pseudomonas* sp.; *Can. J. Microbiol.* **29** 874-880

Hardy R W F, Hols'en R D, Jackson E K and Burns R C 1968 The acelytene-ethylene assay for N_2-fixation: Laboratory and field evaluations; *Plant Physiol.* **43** 1185-1207

Hegazi N A, Monib M and Vlassak K 1979 Effect of inoculation with nitrogen fixing spirilla and *Azotobacter* on nitrogenase activity as roots of maize grown under subtropical conditions; *Appl. Environ. Microbiol.* **38** 621-625

Neyra C A, Dobereiner J, Lalande R and Knowles R 1977 Denitrification by nitrogen fixing *Spirillum lipoferum*; *Can. J. Microbiol.* **26** 482-485

Okon Y, Albrecht S L and Burris R H 1979 Methods for growing *Spirillum lipoferum* and for counting it in pure culture and association with plants; *Appl. Environ. Microbiol.* **33** 85-88

Rao A V and Venkateswarlu B 1985 Salt tolerance of *Azospirillum brasilense*; *Acta. Microbiol. Hung.* **31** 221-224

Seldin L, Elsas J D, Van and Penido E G C 1984 *Bacillus azotofixans* sp. nov., a nitrogen fixing species from Brazilian soils and grass roots; *Int. J. Syst. Bacteriol.* **34** 451-456

Tarrand J J, Krieg M R and Dobereiner J 1978 A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov., and two isolates *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov.; *Can. J. Microbiol.* **24** 967-980