Proc. Indian Acad. Sci. (Plant Sci.), Vol. 98, No. 6, December 1988, pp. 483–488.

N_2-fixation (N_2-ase activity) in *Azospirillum* strains

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MS received 12 April 1988; revised 16 September 1988

Abstract. The nitrogenase activity (N_2-fixation) of *Azospirillum* isolated from the root region of C_4 *Digitaria, Chloris, Cynodon, Dichanthium, Lasiurus* and *Cenchrus* growing in different tracts of Rajasthan, was studied in spot batch cultures on sodium malate medium both under air and 1% oxygen at regular intervals of 24 h for a span of 7 days. The morphological and cultural characteristics of *Azospirillum* strains belonging to *lipoferum* and *brasilense* group (both nir^+ and nir^-) have also been studied. The strains of *lipoferum* group performed better and exhibited more N_2-ase activity on agar surface under 1% O_2 and associated with the roots compared to the strains of *brasilense* group.

Keywords. Nitrogenase activity; *Azospirillum brasilense*; *Azospirillum lipoferum*; C_4 grasses; *Chloris*; *Cenchrus*; *Cynodon*; *Dichanthium*; *Digitaria*; *Lasiurus*; root nitrogenase activity.

1. Introduction

Döbereiner and Day (1976) observed a considerable N_2-ase activity in the rhizosphere of tropical forage grasses. Day *et al* (1975) reported an associative or semisymbiotic nitrogen fixing bacterium, *Spirillum lipoferum* (*Azospirillum brasilense*) from the roots of these grasses. Berggold and Werner (1987) reported a new nitrogen fixing isolate of *Azospirillum lipoferum* from the rhizosphere of *Sorghum bicolar*. Many workers (Smith and Patriquin 1978; Cohen *et al* 1980; Favilli *et al* 1983) surveyed various cereal crops, grasses and dicotyledonous weeds in different countries and concluded that the N_2-ase activity by associative symbiosis in their roots approached that of tropical forage grasses. In the present paper the existence of this system (grass—*Azospirillum* semi-symbiosis) if any in some C_4 grasses of Rajasthan and the performance of best strains of *Azospirillum* are discussed.

2. Materials and methods

C_4 test grasses *Digitaria adscendens, Chloris virgata, Cynodon dactylon, Dichanthium annulatum, Lasiurus sindicus* and *Cenchrus pennisetiformis* were collected from different places of Rajasthan with roots and soil intact. The endophytic bacterium was isolated on (sodium malate) enrichment medium (Döbereiner and Day 1976) for *Azospirillum*, purified and Koch's postulated employing Gibson's (1968) technique. The root section of 30 days old grass seedling was observed for the colonization and establishment by trichloro tetrazolium chloride reduction test (Patriquin and Döbereiner 1978).

The bacterial strains were identified by morphological and cultural characteristics. The cysts formation was observed by malechite green safranin staining technique. After the preliminary screening for nitrogenase activity of all the bacterial strains in

semisolid malate medium the specific nitrogenase activity both under air and 1% oxygen of the spots of the best strains of this bacterium was studied by gas chromatograph according to Wilcockson and Werner (1976) at regular intervals of 24 h for a span of 7 days. For nitrogenase activity of spot culture, the strains were grown in 50 ml nutrient broth upto one optical density at 600 nm containing $1{\cdot}5 \times 10^8$ cells/ml of Asp L-4 (*Chloris*), $1{\cdot}2 \times 10^8$ cells/ml of Asp L-175 (*Cenchrus*), $1{\cdot}8 \times 10^8$ cells/ml of Asp L-272 (*Dichanthium*), $2{\cdot}1 \times 10^8$ cells/ml of Asp L-288 (*Dichanthium*), $1{\cdot}5 \times 10^8$ cells/ml of Asp B-128 (*Digitaria*), $1{\cdot}5 \times 10^8$ cells/ml of Asp B-132 (*Cynodon*) and 1×10^8 cells/ml of Asp B-466 (*Lasiurus*). Fifty μl suspension of each of above strain was spotted at 4 places in a petri plate containing N free sodium malate medium and incubated at 28°C ± 1. The total bacterial protein was estimated as described by Herbert *et al* (1971). Root associated nitrogenase activity of 30 days old grass seedlings raised in sterilized field soil, inoculated by respective strains of *Azospirillum* was studied according to Döbereiner and Day (1976).

3. Results

On the basis of the morphological and cultural characteristics the strains of the associative (semisymbiotic) bacterium obtained from root crushing of the test grasses were conformed to the genus *Azospirillum* (Tarrand *et al* 1978).

The strains of *Azospirillum* isolated from *C. virgata*, *D. annulatum* and *C. pennisetiformis* belong to group II showing the ability to grow on glucose in place of sodium malate, production of acid on peptone glucose medium, showing pleomorphism in semisolid N free sodium malate medium and biotin requirement for growth conforms to the species identical with *A. lipoferum*. On the other hand, the strains of *Azospirillum* obtained from *D. adscendens*, *C. dactylon* and *L. sindicus* belong to group I with the absence of good growth on glucose in place of sodium malate, absence of acidification on glucose peptone medium, absence of pleomorphism in semisolid N free sodium malate medium and nonrequirement of biotin for growth. These strains conform to *A. brasilense* nir^+ (nitrate reductase positive) or nir^- (nitrate reductase negative) (Tarrand *et al* 1978; Becking 1985).

In the preliminary screening for N_2-ase activity, all the 25 strains of *A. lipoferum* isolated from *Chloris*, 4 out of 22 from *Dichanthium* and 10 out of 37 from *Cenchrus* showed N_2-ase activity both under air and under 1% oxygen while the remaining strains of the same from *Dichanthium* (18) and *Cenchrus* (27) exhibited N_2-ase activity only under 1% oxygen. Similarly, all the 25 strains of *A. brasilense* isolated from *Digitaria*, 21 out of 31 strains of the same from *Cynodon* and 5 out of 23 strains of the organism from *Lasiurus* showed N_2-ase activity both under air and under 1% oxygen while the remaining strains of the same bacterium from *Cynodon* (10) and *Lasiurus* (18) exhibited N_2-ase activity only under 1% oxygen.

The specific N_2-ase activity of the best strain of *Azospirillum* isolated from different test grasses from different places was documented (figure 1).

The maximum N_2-ase activity of Asp B-128 isolated from the root of *D. adscendens* growing in Durgapura Agricultural Farm, Jaipur was $238{\cdot}475 \pm 9{\cdot}1$ nmol $C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$ under air after 24 h and $126{\cdot}970 \pm 5{\cdot}870$ nmol $C_2H_4 \cdot mg\ protein^{-1} \cdot h^{-1}$ under 1% oxygen after 72 h. The activity decreased up to 72 h under air and 120 h under 1% oxygen and stopped thereafter. The pH of the spot changed from 6·8–9·5 in 7 days.

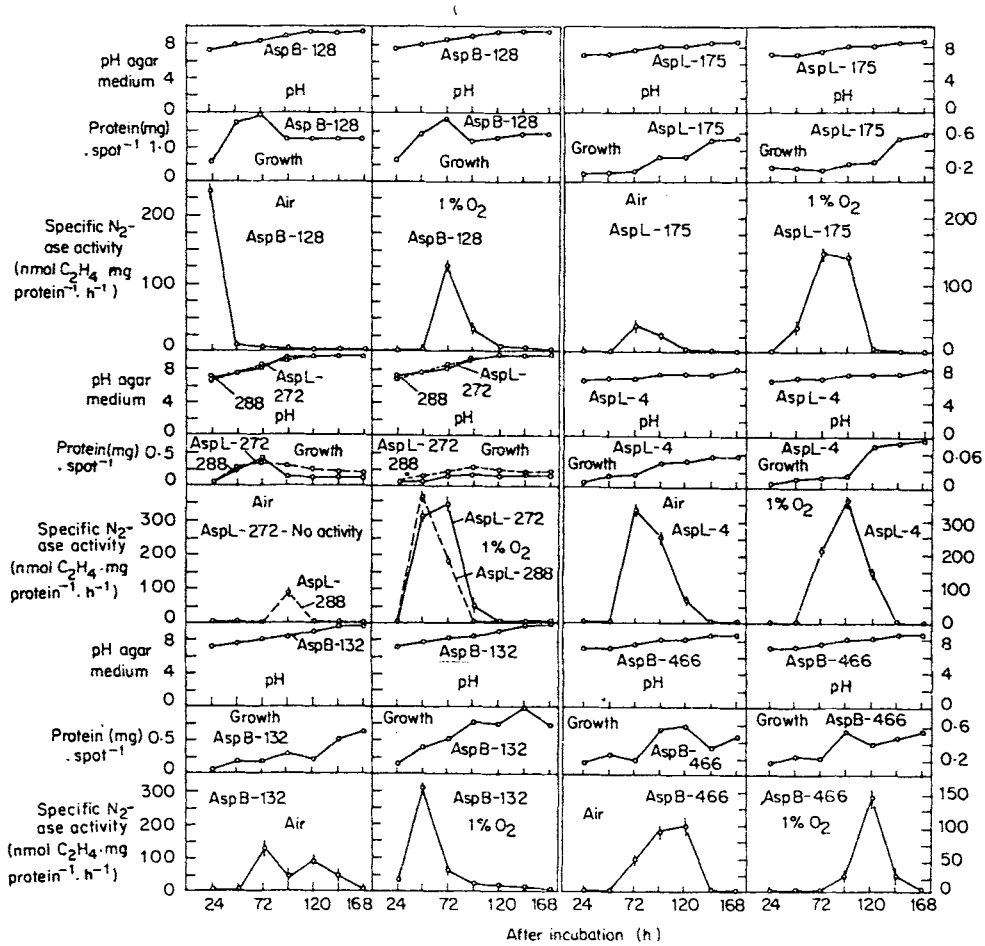

Figure 1. Growth, specific N_2-ase activity (air and 1% O_2) and pH of spot batch culture of *Azospirillum* (sodium malate medium).

The highest N_2-ase activity of strain of *A. brasilense* isolated from *C. dactylon* was of Asp B-132 from Borekheda Agricultural Farm, Kota. This showed 122·242 ± 6·520 nmol C_2H_4·mg protein^{-1}·h^{-1} under air in 72 h and 304·750 ± 3·742 nmol C_2H_4·mg protein^{-1}·h^{-1} under 1% oxygen in 48 h. The activity decreased subsequently and was absent after 144 h both under air and under 1% oxygen. There was a secondary peak of 80·204 ± 1·07 nmol C_2H_4·mg protein^{-1}·h^{-1} after 120 h under air. The pH of the spot changed from 7–9·5 in 7 days.

The strain of *A. brasilense* (Asp B-466) isolated from *L. sindicus* from Desert National Park, Jaisalmer showed the highest N_2-ase activity of 99·89 ± 9·17 nmol C_2H_4·mg protein^{-1}·h^{-1} under air and 140·78 ± 11·85 nmol C_2H_4·mg protein^{-1}·h^{-1} under 1% oxygen in 120 h. The same was absent in 144 h under air and in 168 h under 1% oxygen. The pH at the spot changed from 7–8·5 in 7 days.

Amongst the strains of *A. lipoferum* isolated from *C. virgata*, the maximum N_2-ase activity was 335·658 ± 3·370 nmol C_2H_4·mg protein^{-1}·h^{-1} under air in 72 h and 365·520 ± 2·86 nmol C_2H_4·mg protein^{-1}·h^{-1} under 1% oxygen in 96 h by Asp L-4

obtained from Durgapura Agriculture Farm, Jaipur. It dwindled in 120 h and stopped in 144 h. The pH of the spot changed from 6·8–8 in 7 days.

The strain Asp L-272 isolated from *D. annulatum* from Sikar, did not show N_2-ase activity under air but showed activity of 348·979 ± 12·186 nmol C_2H_4·mg protein^{-1}·h^{-1} in 72 h under 1% oxygen. This activity decreased in 96 h and was absent in 120 h. On the other hand, the N_2-ase activity of Asp L-288 isolated from the same host vegetating in Udaipur showed 78·642 ± 1 nmol C_2H_4·mg protein^{-1}·h^{-1} in 96 h under air and 369·368 ± 1·15 nmol C_2H_4·mg protein^{-1}·h^{-1} in 48 h under 1% oxygen. The latter decreased in 72 h and disappeared in 96 h. The pH of the spots in both the cases changed from 7–9·5 in 7 days.

The maximum N_2-ase activity of 35·97 ± 9·25 nmol C_2H_4·mg protein^{-1}·h^{-1} in 72 h under air and 247·95 ± 7·92 nmol C_2H_4·mg protein^{-1}·h^{-1} in 72 h under 1% oxygen at 7·5 pH of the spot, was shown by Asp L-175 isolated from *C. penniseti-formis* collected from Bird Sanctuary, Bharatpur. The activity commenced after 48 h reached climax in 72 h, dwindled thereafter and was absent after 144 h onwards under 1% oxygen while the same appeared after 72 h under air. The pH at the spots changed from 7–8·5 in 7 days.

The strains of *Azospirillum* belonging to both *lipoferum* and *brasilense* groups were further tested for their efficiency to fix nitrogen (nitrogenase activity) in the roots of their respective host plants. Of the 6 test grasses, the maximum nitrogenase activity associated with the roots was 210·50 nmol C_2H_4·g dry root^{-1}·h^{-1} in *C. virgata* inoculated by strain Asp L-4 belonging to *lipoferum* group. This was followed by the activity in *D. annulatum* inoculated by strain Asp L-288 (185·20 nmol C_2H_4·g dry root^{-1}·h^{-1}) and strain Asp L-272 (150·35 nmol C_2H_4·g dry root^{-1}·h^{-1}), *C. pennisetiformis* with strain Asp L-175 (110 nmol C_2H_4·g dry root^{-1}·h^{-1}), *L. sindicus* with strain Asp B-466 (95·10 nmol C_2H_4·g dry root^{-1}·h^{-1}), *D. adscendens* with Asp B-128 (91 nmol C_2H_4·g dry root^{-1}·h^{-1}) and *C. dactylon* with Asp B-132 (51·50 nmol C_2H_4·g dry root^{-1}·h^{-1}) recording the lowest nitrogenase activity in the roots.

It was observed that all the strains of *lipoferum* group exhibited more root associated nitrogenase activity compared to the strains of *brasilense* group.

4. Discussion

Although *Azospirillum* was microaerophilic and was known to show N_2-ase activity under reduced oxygen concentration. Thirty-nine out of 84 strains of *A. lipoferum* and 51 out of 79 strains of *A. brasilense* (both nir$^+$ and nir$^-$) exhibited significant N_2-ase activity on the agar surface under air. Such a behaviour was also observed by Papen and Werner (1980) in *A. brasilense*. Besides, the test organisms were also a little slimy. Further, in the strains of *A. lipoferum* and *A. brasilense* which showed N_2-ase activity both under air and under 1% oxygen, the activity under 1% oxygen was more than that under air. This suggested that the strains had increased confirmational protection besides respiratory protection (Postgate *et al* 1981).

The peak period of N_2-ase activity could be the same both under air and 1% oxygen as in Asp B-466 and Asp L-175, or postponed under 1% oxygen as in Asp B-128 and Asp L-4, or preponed as in Asp B-132 and Asp L-288 (figure 1). The postponement of peak period of N_2-ase activity under 1% oxygen by 24 h was reported by Jagnow (1983) in *A. lipoferum*. In all the efficient strains, pH of agar

medium seemed to affect specific N_2-ase activity. In a week long lasting batch cultures, pH of the medium increased slowly and steadily with the alkali production and after pH 7·8 the nitrogenase activity was found to decrease and with further rise in pH of about 8 and above the N_2-ase activity completely ceased (figure 1). Similar observation was also made by Okon *et al* (1976) when they found that at pH 7·8 nitrogenase activity was drastically decreased in *Azospirillum*. Papen and Werner (1982) demonstrated higher rates of N_2 fixation in *A. brasilense* ATCC 29145 increasing the phosphate buffer concentration to 20-fold in sodium malate medium. This increased pH of the medium from 6·8–7·6 during 14 day period suggesting that medium with higher buffer concentration could maintain the nitrogenase activity in *Azospirillum* as compared to a medium with low buffer concentration.

The strain Asp B-132 *C. dactylon* showed oscillating N_2-ase activity (first peak of 122·242 nmol C_2H_4·mg protein^{-1}·h^{-1} in 72 h and second peak of 80·204 nmol C_2H_4·mg protein^{-1}·h^{-1} in 120 h) under air in spot batch culture (figure 1). Similar situation was reported by Papen and Werner (1982) in *A. brasilense* under reduced oxygen concentration. They attributed this to encystation of vegetative cells and their subsequent return to the active vegetative state. In Asp B-132 cysts were not found earlier than 72 h of cultivation. Cysts were detected during 72–96 h cultivation time. At this time most of the cells were immotile and the nitrogenase activity was at its minimum. During the maximum of encystation, no significant increase in growth was observed. After 24 h, the nitrogenase activity increased and the cysts decreased rapidly thereafter. Further all the strains of *Azospirillum* belonging to both *lipoferum* and *brasilense* group when inoculated on their respective hosts exhibited nitrogenase activity (N_2-fixation) associated with the roots. The maximum nitrogenase activity was observed in *C. virgata* inoculated with the strain Asp L-4. The lowest root associated nitrogenase activity was recorded in *C. dactylon* inoculated with the strain Asp B-132. Such low rates of nitrogen fixation by *C. dactylon* has also been reported by Döbereiner and Day (1975) and Ahmad (1979). The performance of strains of *lipoferum* group in terms of root associated nitrogen fixation was better as compared to the strains of *brasilense* group.

Acknowledgement

The authors are grateful to the Department of Science and Technology, New Delhi for financial support.

References

Ahmad M H 1979 Nitrogenase activity associated with roots of tropical grasses; *Z. Pflanzenphysiol.* **94** 363–369

Becking J H 1985 Pleomorphism in *Azospirillum*; in *Azospirillum* III: *Genetics, physiology and ecology* (ed.) W Klingmüller (Berlin, Heidelberg, New York: Springer Verlag) p 262

Berggold R and Werner D 1987 Sucrose uptake in an *Azospirillum lipoferum* isolated from *Sorghum* rhizospheres; *Angew. Bot.* **61** 347–358

Cohen E, Okon Y, Kigel J, Nur I and Henis Y 1980 Increase in dry weight and total nitrogen in *Zea mays* and *Setaria italica* associated with nitrogen-fixing *Azospirillum* spp; *Plant Physiol.* **66** 746–749

Day J M, Neeves M C P and Döbereiner J 1975 Nitrogenase activity in the roots of tropical forage grasses; *Soil Biol. Biochem.* 7 102–112

Döbereiner J and Day J M 1975 Nitrogen fixation in the rhizosphere of tropical grasses; in *Nitrogen*

fixation by free-living microorganisms (ed.) W D P Stewart (Cambridge: Cambridge University Press) vol. 6, pp 39–56

Döbereiner J and Day J M 1976 Associative symbioses in tropical grasses; characterization of microorganisms and nitrogen fixing sites; in *Symposium on nitrogen fixation* (eds) W E Newton and C J Nyman (Washington: University Press) vol. 2, pp 518–538

Favilli F, Belloni W, Cresta E and Messini A 1983 *Azospirillum* spp. Ecology of Italian soils; *Proceedings Advances in Nitrogen Fixation* (Holland: Waganingen) pp 339–340

Gibson A H 1968 Nodulation failure in *Trifolium subterraneum* (Woogene llup syn. Marrar); *Aust. J. Agric. Res.* **19** 907–918

Herbert D, Phipps P J and Strange R E 1971 Chemical analysis of microbial cells; *Methods Microbiol.* **35** 209–244

Jagnow G 1983 Nitrogen fixation of *Azospirillum lipoferum*, *Klebsiella* and *Enterobacter* spp. from cereal roots in liquid and semisolid media at different temperatures and oxygen concentrations; *Experientia suppl.* **48** 127–137

Okon Y, Albrecht S L and Burris R H 1976 Factors affecting growth and nitrogen fixation of *Spirillum lipoferum*; *J. Bacteriol.* **127** 1248–1254

Papen H and Werner D 1980 Biphasic nitrogenase activity in *Azospirillum brasilense* in long lasting batch cultures; *Arch. Microbiol.* **128** 209–214

Papen H and Werner D 1982 Organic acid utilization, succinate excretion encystation and oscillating nitrogenase activity in *Azospirillum brasilense* under microaerobic conditions; *Arch. Microbiol.* **132** 57–61

Patriquin D G and Döbereiner J 1978 Light microscopy observations of tetrazolium reducing bacteria in the endorhizosphere of maize and other grasses in Brazil; *Can. J. Microbiol.* **24** 734–742

Postage J R, Eady R R, Dixon R A, Hill S, Kalrn D, Kennedy C, Patridge P, Robson R and Yates M G 1981 Some aspects of the physiology of dinitrogen fixation; in *Biology of inorganic nitrogen and sulfer* (eds) H Bothe and A Trebet (Berlin, Heidelberg, New York: Springer Verlag) pp 103–115

Smith D and Patriquin D G 1978 Survey of Angiosperms in *Nova scotia* for rhizosphere nitrogenase (acetylene-reduction) activity; *Can. J. Bot.* **56** 2218–2223

Tarrand J J, Krieg N R and Döbereiner J 1978 Taxonomic study of *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species *Azospirillum lipoferum* (Beijerinck) Coms. nov. and *Azospirillum brasilense* sp. nov; *Can. J. Microbiol.* **24** 967–980

Wilcockson J and Werner D 1976 Nitrogenase activity by *Klebsiella* and *Rhizobium* on solid substrata exposed to air; *Ber. Dtsch. Bot. Ges.* **89** 587–607