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Interactions between the root exudates of pearl millet and *Azospirillum brasilense*

B VENKATESWARLU and A V RAO

Division of Soil-Water-Plant Relationship, Central Arid Zone Research Institute, Jodhpur 342003, India

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Abstract. Root exudates of different pearl millet varieties showed quantitative differences in organic carbon, reducing sugars, total and amino nitrogen. The growth and nitrogenase activity of *Azospirillum* were stimulated by the addition of root exudates in the culture medium. Root exudates were also found to support the growth of *Azospirillum* in the rhizosphere. Inoculation with *Azospirillum* significantly enhanced the root exudation in axenically grown pearl millet plants accompanied by an increase in the permeability of roots. N_2-ase activity of the inoculated plants differed among the varieties and was related to the amount of organic carbon released in the exudates. Addition of extraneous carbon source significantly increased the nitrogenase activity of the roots as the carbon compounds provided by the root exudates appear to be inadequate for the optimum expression of nitrogenase activity. The implications of these results in the pearl millet-*Azospirillum* association were discussed.

Keywords. Pearl millet; root exudates; *Azospirillum* inoculation; nitrogenase activity.

1. Introduction

The associative symbiotic bacterium *Azospirillum* has been receiving increasing attention in view of its nitrogen fixing association with the roots of a number of cereal plants (Dobereiner and Day 1975; Subba Rao 1980; Kapulnik *et al* 1981). This organism has been reported to increase the plant growth and yields through a number of processes like nitrogen fixation (Von Bulow and Dobereiner 1975; Kapulnik *et al* 1981), phytohormone synthesis (Tien *et al* 1979; Venkateswarlu and Rao 1983) and increasing the nutrient uptake (Barea *et al* 1983; Lin *et al* 1983). However the various physiological interactions between the host plant and the bacterium leading to the formation of the symbiosis are not yet fully understood. For example, not much information is available on the interactions between root exudates and the microsymbiont although the activity of the bacterium in the root zone is greatly influenced by these compounds as a source of energy and nutrients (Boddey and Dobereiner 1982). The present investigation therefore was conducted to study the interactions between root exudates of pearl millet and *Azospirillum*, such as the biochemical characterization of the root exudates, effect of root exudates on growth and nitrogenase activity of the bacterium, and the influence of inoculation on the exudation pattern and permeability of the roots.

2. Materials and methods

2.1 *Collection and purification of root exudates*

Four varieties of pearl millet (BJ-104, MP-15, MHB-110 and MHB-118) were grown axenically in glass tubes of 200 x 25 mm size containing acid washed and sterilized quartz sand. Surface sterilized seeds were pre-germinated on plain agar plates and 4 seedlings each were transferred into the tubes which were then kept in an artificially illuminated growth room (14 hr photo period with 30°C day and 25°C night temperatures, light intensity, 12,000 lux). The plants were maintained with Hoagland's half strength mineral nutrient solution containing 50 ppm nitrogen as ammonium nitrate. The moisture level in the tubes was maintained in such a way as to provide adequate water to the plants without causing any water-logging condition around the roots. Plants grew well and a normal root system developed.

Root exudates were collected by removing 12 day old plants from the tubes and rinsing the roots and sand from each tube separately in distilled water. The exudates from all plants of one variety were pooled and centrifuged to remove the suspended matter. The supernatant was concentrated by vacuum evaporation to 1/3 of the original volume and desalted by passing through ion exchange columns (Hussain and McKeen 1963; Rai and Strobel 1966). The crude exudates were passed through Dowex-50 (H^+ form) and Dowex-1 (formate form) columns to separate it into cationic, anionic and neutral fractions. The individual fractions were evaporated to dryness at 60°C and dissolved in distilled water. For quantitative and thin-layer chromatography (TLC) analysis the different fractions were used directly i.e. cationic for amino acids, anionic for organic acids and neutral for sugars. Alternately all the fractions were pooled together, concentrated and adjusted to a final volume of 1·0 ml representing 10 seedlings. This exudate solution was used to study the effects on the bacterial growth etc.

To study the effect of inoculation with *A. brasilense* on the pattern of exudation in pearl millet (var. BJ-104) 1·0 ml cell suspension of 4 day old *A. brasilense* strain isolated from the roots of pearl millet was added to each tube when the plants were 3 days old. The control tubes received the same amount of autoclaved cells. Root exudates at 8, 12 and 15 days after planting were collected from each set and purified as above for biochemical analysis.

2.2 *Biochemical analysis of the exudates*

The organic carbon and total nitrogen contents from the crude exudates were estimated by Walkley and Black rapid titration and micro kjeldahl methods respectively (Jackson 1958). The reducing sugars and amino nitrogen were estimated from the respective fractions by Nelson's arsenomolybdate method (Nelson 1944) and ninhydrin method of Moore and Stein (1948) respectively.

2.3 *Effect of root exudates on A. brasilense*

For growth studies a malate liquid medium containing only 50% of the malic acid (2·5 g/l), but supplemented with 50 ppm ammonium sulphate was used. Filter sterilized root exudates were added in 3 replicate tubes containing 4 ml of the above medium so

as to get different concentrations (5, 10, 15 and 20 %). A double strength medium was prepared initially and appropriate volumes of exudates and sterile distilled water added in each tube to keep the final volume constant. Inoculation was done with 0·1 ml pure washed cell suspension (OD 0·4) of 4 day old *A. brasilense* grown in nutrient broth. The tubes were incubated at 30°C for 72 hr and the optical density was recorded at 520 nm in a Systronics Spectrophotometer. For studying the nitrogenase activity the organism was grown in 7 ml test tubes containing 3 ml of semi-solid malate medium with 50 % of the malic acid (2·5 g/l). The nitrogenase activity was assayed after 48 hr of incubation by acetylene-reduction method as described earlier (Venkateswarlu and Rao 1983).

2.4 *Permeability changes in the pearl millet roots*

Plants (var. BJ-104) were grown in test tubes containing pure sand as described earlier. One set of tubes were inoculated on the third day with 1·0 ml cell suspension, while the control tubes received the same amount of autoclaved cell suspension. After 15 days the plants were removed by emptying the tubes and the root system was slowly and carefully separated from the sand and gently washed with distilled water. Three replicate samples of 0·5 g fresh roots each from inoculated and control sets were weighed and wrapped in a cheese cloth. These were placed in a conical flask containing 40 ml distilled water. The contents were shaken on a rotary shaker for 5 min and kept at room temperature. The conductivity of the bathing solution was measured in a conductivity bridge at every 1 hr interval.

Alternately root material from control plants was impregnated with pure cell suspension of *A. brasilense* under a brief vacuum to adsorb the bacterial cells on to the roots, and then incubated in a sterile petri dish for 15 min. The roots were rinsed with distilled water and the conductivity of the bathing solution was measured as above.

2.5 *Nitrogenase activity in pearl millet-Azospirillum association*

Pearl millet plants (BJ-104, MP-15, MHB-110 and MHB-118) were grown in 200 × 25 mm tubes containing equal volumes of sterilized quartz sand and vermiculite as the growth medium and watered with Hoagland's N-free half strength nutrient solution. When the seedlings were 3 day old 1·0 ml pure cell suspension (OD = 0·38) of 4 day old *A. brasilense* and 4 ml of nutrient solution containing 1 % sucrose were added to one set of tubes while the tubes in the second set received the inoculum and 4 ml of nutrient solution. The nitrogenase activity (C_2H_2-reduction) of intact plants was estimated after 3 weeks without pre-incubation. Acetylene was injected into the tubes directly through an air tight rubber cap and ethylene produced after 24 hr was estimated by gas chromatography (Venkateswarlu and Rao 1983). Appropriate control tubes were included in the assay; uninoculated tubes having plants did not show any acetylene reduction but those inoculated with *A. brasilense* without plants did show some activity which was accounted in the calculations.

2.6 *Counts of Azospirillum in the root zone*

Pearl millet (var. BJ-104) plants were grown in 200 × 25 mm glass tubes containing sand + vermiculite mixture (1 : 1) and maintained with Hoagland's half strength N-free

nutrient solution. Equal number of tubes without plants were also maintained which received the same quantity of nutrient solution each time. On the third day 1·0 ml pure washed cell suspension of 4 day old *A. brasilense* (10^8 cells/ml) was added to all the tubes. Immediately after addition and every 3 days thereafter the numbers of *Azospirillum* in the tubes were estimated by dilution plate count (by transferring whole contents of the tube into the diluent flask) using malate agar medium supplemented with 500 ppm $(NH_4)_2 SO_4$.

The data were statistically analysed for analysis of variance. In case of the root exudate data (table 2) as the number of observations were few, the differences between intervals were tested by Kruskal-Wallis one way analysis of variance (H) test.

3. Results

There were marked quantitative differences in the biochemical composition of the root exudates of 4 varieties (figure 1). The exudates of BJ-104 contained the highest amounts of organic carbon and reducing sugars followed by MHB-110, MP-15 and MHB-118.

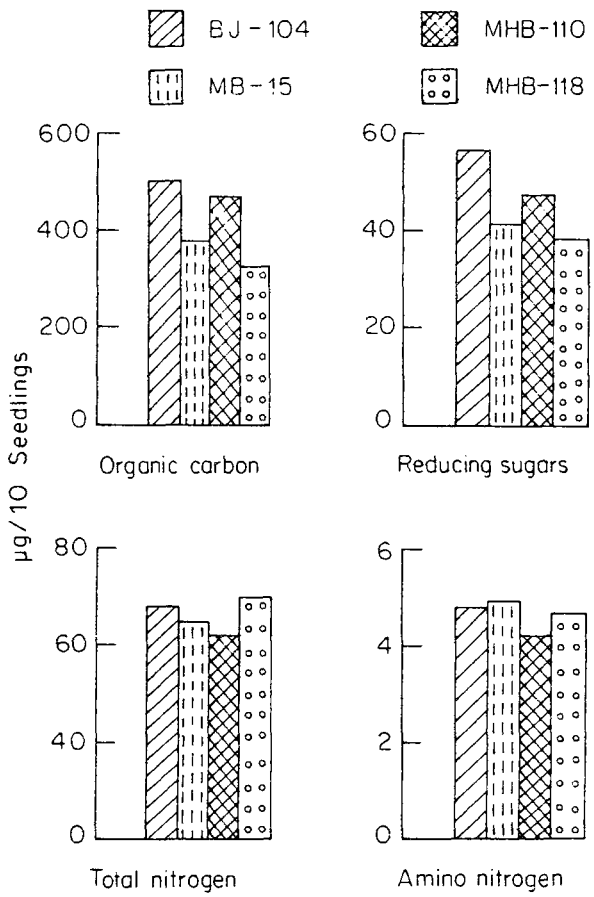

Figure 1. Biochemical composition of the root exudates of pearl millet varieties.

However the differences in total and amino-nitrogen were not significant. There was no relationship between the seed size or the plant dry weight with the quantity of the root exudates. Organic carbon in the exudates varied from 1·6–2·1 % of the plant dry weight in different varieties and C : N ratio from 4·7 to 7·5. Reducing sugars in the exudates were much higher than amino nitrogen. Qualitative analysis of the exudates on TLC showed the presence of amino acids like glutamic acid, tryptophane, cysteine and asparagine besides two unidentified spots. Sucrose, glucose, fructose and xylose were among the sugars identified. Three organic acids viz. citrate, malate and succinate were also detected but in extremely small quantities.

Root exudates when incorporated in the culture medium had a stimulatory effect on the growth of *A. brasilense* which varied with the variety and the maximum was observed with BJ-104 (table 1). There was a gradual increase in the growth with increasing concentration. The exudates from BJ-104 which contained highest amounts of organic carbon and reducing sugars also showed maximum stimulatory effect.

The nitrogenase activity followed a similar trend as that of growth (table 1). With different varieties the increase in the activity with 20 % exudates varied from 12–25 % over control. The pellicle formation was much quicker and dense in the semi-solid medium at higher concentrations.

The exudation of organic carbon, reducing sugars, total and amino nitrogen increased gradually with increasing age of the plant under axenic conditions (table 2). Further inoculation significantly enhanced the exudation of all these compounds and the effect became more pronounced at the later stages. The increase varied from 16·7–19·9 % in case of organic carbon and 15·2–17·3 % in case of reducing sugars from 8th–12th day. The data on organic carbon was consistent with a t value significant at 5 % for inoculation while the Kruskal-Wallis H for intervals was not significant. Although there was an increase in the other 3 components it was not statistically significant whereas the Kruskal-Wallis H for periods was significant at 5 % level. Increased root exudation upon inoculation has been suggested to be due to several reasons including changes in the permeability of the root cells and altering the metabolism of the roots. In the present study inoculated roots showed higher cell

Table 1. Effect of root exudates of pearl millet on the growth and nitrogenase activity of *A. brasilense*.

Conc. of exudates (%)	Growth (optical density at 520 nm)					Nitrogenase activity (n mol C_2H_4 $tube^{-1}$ h^{-1})				
	BJ-104	MP-15	MHB-110	MHB-118	Mean	BJ-104	MP-15	MHB-110	MHB-118	Mean
Control			0·202					214		
5	0·218	0·209	0·215	0·210	0·213	229	222	226	219	224
10	0·236	0·221	0.231	0.225	0.228	238	226	240	228	233
15	0·259	0·235	0·250	0·242	0·246	246	231	245	239	240
20	0·281	0·262	0.273	0.269	0·271	268	241	261	256	256
Mean	0·248	0·231	0.242	0.236	0.239	245	230	243	235	238
SEM ± for varieties			0·016					2·4		
Concentrations			0·016					2·4		
Interaction			0·033					4·7		

Table 2. Changes in root exudation pattern in pearl millet as influenced by *A. brasilense* inoculation.

Treatment	Age of the plants (days)											
	8	12	15	Mean	SD	*t*-value	8	12	15	Mean	SD	*t*-value
	Organic carbon[a]						Total nitrogen[a]					
Control	412	466	492	456·7	36·6	3·31*	59	67	75	67·00	9·91	2·18
Inoculated	481	548	590	539·7	49·40		68	79	88	78·3	9·99	
Mean	446·5	507·0	541·0	498·2			63.5	73·0	81·5	72·7		
Kruskal-Wallis H for periods	5·54						6·34*					
	Reducing sugars[a]						Amino nitrogen[a]					
Control	41	52	66	53	12·07	1·56	3·9	4·8	5·6	4·77	0·97	1·66
Inoculated	58	56	75	63	10·08		4·6	5·9	6·8	5·77	1·12	
Mean	49·5	54·0	70·5	58·0			4·25	5·35	6·20	5·27		
Kruskal-Wallis H for periods	7·65*						6·81*					

[a] μg/10 seedlings.
* significant at 5% level.

Table 3. Permeability changes in pearl millet roots as influenced by inoculation.

Incubation time	Conductivity (μmhos/cm/g fresh root)	
	Uninoculated	Inoculated
1	310 ± 8·5	334 ± 8·5
2	381 ± 7·1	428 ± 28·3
3	418 ± 8·5	456 ± 11·3

Mean ± Standard deviation.

permeability than those treated with autoclaved cells (table 3). The permeability was higher by 12·3% in the inoculated roots incubated for 2 hr. However when the roots were directly impregnated with cell suspension the change in the permeability was much higher (44·6% over the control after 2 hr of incubation).

Nitrogenase activity of the intact plants inoculated with *A. brasilense* differed significantly among varieties (table 4). The activity varied from 12·5 nmol C_2H_4/hr/plant in MHB-118 to 21 nmol in BJ-104. However, the amount of carbon available in the exudates appear to be inadequate for the optimum activity of the microsymbiont. When extraneous carbon source as sucrose is provided in the root zone there was a spurt in the nitrogenase activity of all the varieties (table 4), accompanied by a marked increase in the numbers of *Azospirillum* in the root zone. The increase in the activity due to carbon supplementation varied from 125–272% in different varieties.

Periodical counts of *Azospirillum* in the root zone of the inoculated plants grown axenically showed that plant roots exert a favourable influence on the microorganism in the root zone. The numbers in the rhizosphere increased rapidly from $11·2 \times 10^7$–$17·5 \times 10^7$ cells/tube from day 0–10, while in the non rhizosphere it declined to

Table 4. Nitrogenase activity of inoculated pearl millet varieties and the influence of carbon supplementation.

Variety	Nitrogenase activity (nmol C_2H_4/Plant/hr)	
	Without sucrose	With sucrose
BJ-104	21·0 ± 4·2	49·0 ± 4.5 (133·0)
MP-15	14·5 ± 3·1	32·7 ± 6·2 (125·0)
MHB-110	17·5 ± 2·8	45·0 ± 3·9 (157·0)
MHB-118	12·5 ± 3·5	46·5 ± 5·5 (272·0)

Mean ± Standard deviation.
Figures in parentheses indicate % increase over control.

$10{\cdot}2 \times 10^7$ during the same period. However from day 10 onwards the numbers declined under both the conditions, but more so in the non rhizosphere.

4. Discussion

In the present study a close relationship between carbon compounds exuded by the roots and the *in vitro* growth of *A. brasilense* in the exudate media as well as the nitrogenase activity of the intact plants was observed. Rovira (1965), Lee and Gaskins (1982) and Beck and Gilmour (1983) have emphasized the role of root exudates in the nutrition and colonization of rhizosphere microflora. The quantitative differences observed in different varieties of pearl millet and its relationship with the nitrogenase activity indicates that differences in root exudates may be one of the major factors responsible for the genetic variation in the root associated nitrogenase activity. Von Bulow and Dobereiner (1975) in maize, Vlassak and Reynders (1978) in wheat and Pohlman and McColl (1982) in barley have emphasized the role of root exudates in the varietal variation of nitrogenase activity. The stimulation of *in vitro* growth and nitrogenase activity by the exudates further indicates the favourable effects on the microsymbiont. In general, the *in vitro* growth of the organism and the nitrogenase activity of the intact plants were proportional to the organic carbon and reducing sugars released in the exudates emphasizing the role of energy supply to the organisms. With Sorghum-*Azospirillum* association Lee and Gaskins (1982) using [^{14}C] have found that photosynthetically fixed carbon moves quickly into the roots and the growth of bacteria in the rhizosphere is proportional to the release of carbon compounds in the root zone.

Inoculation under axenic conditions has significantly increased the exudation in pearl millet (table 2). Increase in the root exudation in the presence of microorganisms has been reported by Rovira (1965), which was attributed to the possible changes in the permeability of the roots induced by the microbial metabolites. Lee and Gaskins (1982) in Sorghum and Beck and Gilmour (1983) in wheat have also found increased exudation in the presence of N_2-fixing bacteria. In the present study the cell permeability of inoculated roots has increased markedly which might have contributed to the enhanced exudation. The permeability change was more marked when the roots were impregnated with the pure cells. Although this treatment can not be compared with the other two it suggests a definite physiological interaction between the bacteria

and the root cells. *Azospirillum* has been reported to produce pectinolytic enzymes in the culture media (Garcia *et al* 1978) and it is possible that these cell wall hydrolyzing enzymes may play an important role in increasing the cell permeability.

The population in the root zone increased markedly and remained much higher than in the non-rhizosphere indicating the possible *in vivo* effects of root exudates. However it showed a declining trend after 10 days which might be due to the inadequate supply of the root exudates to the rapidly multiplying bacteria and a possible competition for O_2 between the bacteria and respiring roots in the closed system (Martin and Glatzle 1982).

The nitrogenase activity of the 4 varieties was related to the organic carbon exuded by the roots indicating the role of the exudates as a carbon source to bacteria. However, when external carbon is provided there was a marked increase in the activity and the population in the rhizosphere indicating that the amount of exudates released by the roots may not be adequate for the optimum expression of this association. Hess and Kiefer (1981) and Lethbridge and Davidson (1983) have also reported the presence of considerable latent activity in various grass-diazotrophic associations which was expressed only upon providing extraneous carbon source. However most of these experiments including the present study were conducted under axenic conditions where plants grow with some constraints, whereas the actual quantum of exudates in the soil under natural conditions might be much larger. Further insoluble suspended materials in the root zone have been demonstrated to be equally important in the rhizosphere (Barber and Martin 1976) while most of the studies so far have concentrated on the affects of soluble compounds on the rhizosphere microflora.

Although it is difficult to conclusively evaluate the role of root exudates as a source of energy supply from the present study mainly because of the limitations imposed by investigating small seedlings, the results demonstrate the crucial role of these compounds in the formative stages of the associative symbiosis.

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