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Effect of inoculation of *Azospirillum lipoferum* on nitrogen fixation in rhizosphere soil, their association with root, yield and nitrogen uptake by mustard (*Brassica juncea*)

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Summary A microplot field experiment was conducted in the presence or absence of P and N application to evaluate the influence of the seed inoculation of mustard (cv. Baruna T_{59}) with *Azospirillum lipoferum* on N_2-fixation in rhizosphere, association of the bacteria with the roots and grain yield and N uptake. Inoculation significantly increased the N content in rhizosphere soil particularly at early stage (40 days) of plant growth, which was accompanied by the increased association of the bacteria (*A. lipoferum*) in rhizosphere soil, root surface washing and surface-sterilized macerated root. A significant increase in grain yield and N uptake was also observed due to inoculation. Application of P particularly at the 20 kg. ha^{-1} level further enhanced the beneficial effect of *Azospirillum lipoferum* inoculation, while N addition markedly reduced such an effect.

Introduction

The high potential rates of N_2-fixation by the unique association of Azospirillum with roots of some crop plant and forage grasses have long been appreciated[3,4,6,8]. Since then attempts are being made to exploit this association for augmenting the nitrogen nutrition and hence yield of some cereal crops like maize[2], wheat[9,12], rice[10,11], sorghum and pearl millet[11,14]. But association and activities of this N_2-fixing bacteria in root region of oil seed crop like mustard has received less attention.

The present investigation was, therefore, undertaken to study the seed inoculation of mustard with *Azospirillum lipoferum* on the N_2 fixation in rhizosphere soil, association of the bacteria with the roots and the yield and N uptake of the crop under field conditions.

Materials and methods

Inoculum

Azospirillum lipoferum, isolated from a laterite soil and having N_2-fixing capacity of 12.72 mg.g^{-1} malic acid, was used as inoculum. The bacter was grown on potato infusion agar[7] plates at 30°C for 72 hours and the cultures were thereafter scraped out and suspended in a dilute N-free medium containing 1 g malic acid l^{-1} of the media for use as a seed inoculant.

Field experiment

A field experiment was conducted in an alluvial soil (pH 6.8, org. C 0.58%, total N 0.06%, av. P 3.6 ppm) with mustard (cv. Baruna T_{59}) following a randomised block design with 2 × 2 m plot size. The treatments used were (1) control, (2) two levels of P, *viz* 10 and 20 kg.ha^{-1} as single superphosphate and (3) two levels of N, *viz* 30 and 50 kg.ha^{-1} as urea, all applied as basal, each divided into two series, *viz* (a) inoculated and (b) uninoculated with four replications of each treatment combinations.

Seed inoculation

Seeds were soaked in culture suspension for 6 hours, air dried for 30 minutes and thereafter used for sowing in the plots under inoculated treatments, whereas for the uninoculated plots the seeds were similarly treated in blank media.

N content in rhizosphere soil

Soil along with intact root was collected around the plants from several points in each plot at 40 days after sowing and at harvesting. Soil crumbs adhering to the root surface were carefully collected, composite sample for each plot was prepared and analysed for total N by Kjeldahl method as described by Jackson[5].

N_2-fixing capacity

One gm of the rhizosphere soil and 1 ml of root washing (10^{-1} dilution) were inoculated separately in sterile N-free malate broth[7], kept under incubation at 30°C for 1 week and thereafter the whole contents were analysed for total N.

Number of associative bacteria

The most probable number (m.p.n.) of associative bacteria in the rhizosphere soil and root washing was determined by serial dilution technique as described by Alexander[1], using semi-solid malate agar and growing the organism at 30°C for 72 hours and thereafter examining them microscopically. The associative organism present within the roots were also determined. The intact roots after washing were surface-sterilised by dipping them for 2 minutes in 1% (V/V) chloramine-T solution, followed by washing for five times alternatively with equal volume of sterilised distilled water and 2 m*M* phosphate buffer. The roots were then homogenised in sterilised water using a pestle and mortar and the m.p.n. of the associative bacteria in the homogenate was determined following the technique as described before.

Species abundance

The relative abundance of the two species of the associative bacteria (*A. brasilense* and *A. lipoferum*) in the isolates from each of the above three sources *viz* rhizosphere soil, root washing, and surface sterilised root was determined after purification by repeated subculturing the isolates in solidified malate agar medium amended with 1 g l^{-1} NH_4Cl, followed by streaking the subcultures on potato infusion agar and identifying the growth as per Tarrand *et al.*[13].

Grain yield

The crop was harvested at maturity and the grain yield from each of the plots was taken, and also analysed for N-content.

Results and discussion

N-content in rhizosphere soil

Results in Table 1 show that the N content of rhizosphere soil both at 40 days after sowing and at maturity of the crop recorded significant increase due to inoculation. The magnitude of increase being 7.5

Table 1. Influence of the inoculation of *Azospirillum lipoferum* on N content in rhizosphere soil of mustard (N in per cent)

	Uninoculated			Inoculated		
Levels of P or N $kg.ha^{-1}$	R.soil at 40 days of plant growth	R. soil at harvest	Mean	R. soil at 40 days of plant growth	R. soil at harvest	Mean
Control (N_0P_0)	0.067 ± 0.006	0.060 ± 0.003	0.063	0.072 ± 0.005	0.070 ± 0.003	0.071
P_{10}	0.072 ± 0.003	0.069 ± 0.002	0.070	0.076 ± 0.002	0.070 ± 0.001	0.073
P_{20}	0.071 ± 0.004	0.070 ± 0.002	0.070	0.079 ± 0.001	0.070 ± 0.004	0.074
N_{30}	–	0.072 ± 0.002	–	–	0.070 ± 0.005	
C.D. at 5% level: 40 days of plant growth			0.0048			
At harvest			0.0043			

± Standard deviation of the mean of 4 replicates.

and 16.7 per cent respectively over the uninoculated control. Application of P at the rate of $20\,kg.ha^{-1}$ also brought about a significant increase in the N-content of the rhizosphere soil of the inoculated plots over the uninoculated ones, whereas no such effect of inoculation was observed in presence of applied N at either of the levels of harvesting stage.

N_2-fixing capacity

Results (Table 2) show that the N_2-fixing capacity of the rhizosphere soil as well as the root washing was increased due to Azospirillum inoculation excepting the rhizosphere soil of the treatments which received 10 or 20 kg P ha^{-1}. The magnitude of increase being about 48 and 30 per cent respectively, over their uninoculated control counterparts. At $10\,kg.ha^{-1}$ level of P application, inoculation did not show any such effect in respect of rhizosphere soil but a higher level of P application, inoculation caused an increase in N_2-fixing capacity of the root washing to the extent of about 32 per cent over the corresponding uninoculated treatment. At 30 kg and 50 kg of N application per hectare inoculation brought about considerable increase in the N_2-fixing capacity of rhizosphere soil but comparatively much less in the root washing, the increase in the former case being 16 and 24 per cent as compared to 3 and 37 per cent respectively in the latter over the uninoculated ones. Azospirillum possesses a unique tendency to associate themselves with the roots of crop plants. The increased N_2-fixing capacity of the rhizosphere soil as well as of the root washing

Table 2. Nitrogen fixation in enrichment culture of rhizosphere soil and root washing of mustard inoculated with *Azospirillum lipoferum* (N_2 fixation in mg . g^{-1} malic acid used)

Levels of P or N kg . ha^{-1}	Inoculation	R. soil	Root washing
Control	Uninoculated	3.04 ± 0.81	0.84 ± 0.34
(N_0P_0)	Inoculated	4.50 ± 1.83	1.08 ± 0.27
P_{10}	Uninoculated	6.57 ± 0.28	1.28 ± 0.19
	Inoculated	6.40 ± 0.49	1.40 ± 0.49
P_{20}	Uninoculated	7.20 ± 0.37	1.36 ± 0.26
	Inoculated	6.72 ± 0.16	1.68 ± 0.48
N_{30}	Uninoculated	2.68 ± 1.10	1.20 ± 0.17
	Inoculated	3.12 ± 0.53	1.24 ± 0.13
N_{50}	Uninoculated	1.34 ± 0.36	0.76 ± 0.08
	Inoculated	1.67 ± 0.23	1.04 ± 0.44

± Standard deviation of the mean of 4 replicates.

Table 3. Most probable number of associative bacteria in rhizosphere soil, root washing and surface sterilized macerated root of mustard inoculated with *Azospirillum lipoferum*

Levels of P or *N* kg . ha^{-1}	Inoculation	R. soil (X 10^6 cells/g)	Root washing (X 10^5 cells/g)	Surface sterilized macerated root (X 10^3 cells/g)
Control	Uninoculated	46.20 ± 5.35	16.00 ± 4.20	28.00 ± 2.94
(N_0P_0)	Inoculated	73.80 ± 8.66	69.30 ± 4.92	50.30 ± 4.92
P_{10}	Uninoculated	78.30 ± 9.39	47.00 ± 18.38	45.00 ± 11.57
	Inoculated	88.30 ± 8.73	136.60 ± 23.57	110.00 ± 14.72
P_{20}	Uninoculated	70.30 ± 22.10	52.30 ± 8.50	56.00 ± 10.20
	Inoculated	78.50 ± 17.56	163.30 ± 41.90	81.30 ± 6.55
N_{30}	Uninoculated	97.00 ± 17.00	30.00 ± 4.24	29.30 ± 6.24
	Inoculated	130.00 ± 28.28	42.00 ± 4.72	31.00 ± 4.97
N_{50}	Uninoculated	137.00 ± 23.57	30.00 ± 5.51	39.00 ± 4.55
	Inoculated	143.33 ± 20.55	34.00 ± 11.47	32.00 ± 5.66

± Standard deviation of mean of 4 replicates

might be attributed to the increased population of the organism on the root surface and in the root environment due to inoculation.

Population of the organism

Results (Table 3) show that there has been tremendous increase in the population of the associative bacteria in the rhizosphere soil, on the root surface and also inside the roots due to inoculation, the magnitude of increase being about 60, 330 and 80 per cent respectively over the corresponding uninoculated counterparts, suggesting that there had been an association of the inoculated organism with the roots of plants. Application of P appears to have further encouraged this association. Although application of P alone without inoculation increased the population of the organism, the increase in the population due to inoculation in presence of P application was much higher particularly on the root surface. The increase was more at higher level of P (P_{20}) application. Application of P particularly at the lower level (P_{10}) also caused a marked increase in the population inside the root in presence of inoculation over that in the uninoculated treatment. At the higher level of P (P_{20}) the increase due to inoculation was, however, comparatively less than that of the lower level of P (P_{10}). At this level of P application greater number of the organism remained on the root surface, which may be due to their P nutritional requirements. Application of N, on the other hand, both in presence as well as in absence of inoculation greatly increased the population in the rhizosphere soil, but the increase in the population on the root surface and also inside the root was very small and inoculation practically failed to bring about any increase in the population. This suggests that exogenous supply of combined N discouraged the root bacterial association.

Relative distribution of species

Results (Table 4) show that the isolates collected from rhizosphere soil, root surface and inside the root tissue all were dominated by *Azospirillum brasilense* in uninoculated series. Not a single isolate from the rhizosphere soil resembled the species *Azospirillum lipoferum*, although the same from root surface and inside the root tissue contained a few of the latter species (11 and 16 per cent respectively). Inoculation of the seeds with the culture *Azospirillum lipoferum*, however, improved the relative abundance of this species in all the three environments, the percentage of isolates showing the group characteristics of this species being 33, 25 and 50 per cent respectively. This marked increase in the relative proportion of *Azospirillum lipoferum* inside the root tissue provides evidence of root infection by the organism and establishment of root-bacteria association. The infection, however, appeared to be lower than what was expected. Boddey and Dobereiner[2] reported that *Azospirillum lipoferum* preferentially infect plants having C_4 photosynthetic pathway. Mustard being C_3 plant, the roots probably got comparatively less infected.

Grain yield

The data in Table 5 show that inoculation of seeds with *Azospirillum lipoferum* brought about a significant increase in grain yield over that with uninoculated control (N_0P_0), the magnitude of increase being more than 100 per cent. Application of P or N alone at any of the

Table 4. Distribution of isolates resembling characteristic groups of *Azospirillum* spp. in uninoculated and inoculated mustard grown in the field

Source of isolates	Uninoculated			Inoculated		
		Per cent of isolates identified as			Per cent of isolates identified as	
	No. of isolates	*A. brasilense*	*A. lipoferum*	No. of isolates	*A. brasilense*	*A. lipoferum*
R. soil	18	100	0	18	67	33
Unwashed root surface	27	89	11	16	75	25
Surface sterilized macerated root	18	84	16	16	50	50

Table 5. Influence of *Azospirillum lipoferum* inoculation on the yield of mustard (cv. Baruna T_{59})

Levels of P or N kg . ha^{-1}	Grain yield (g/4 sq. m. plot)	
	Uninoculated	Inoculated
Control (N_0P_0)	81.20 ± 6.69	165.50 ± 20.02
P_{10}	164.50 ± 27.44	192.00 ± 19.80
P_{20}	198.50 ± 20.07	259.70 ± 32.26
N_{30}	290.00 ± 14.70	272.00 ± 6.53
N_{50}	342.00 ± 39.65	377.00 ± 47.36
C.D. at 5% level	43.09	

± Standard deviation of the mean of 4 replicates.

Table 6. Influence of *Azospirillum lipoferum* inoculation on the N content in and uptake by grain of mustard (cv. Baruna T_{59})

Levels of P or N kg . ha^{-1}	N content (%)		N uptake (g/4 sq. m. plot)	
	Uninoculated	Inoculated	Uninoculated	Inoculated
Control (N_0P_0)	3.45 ± 0.12	3.62 ± 0.19	2.80 ± 0.23	5.99 ± 0.78
P_{10}	3.90 ± 0.08	3.81 ± 0.16	6.40 ± 1.00	7.31 ± 0.78
P_{20}	3.95 ± 0.10	3.92 ± 0.14	7.85 ± 0.89	10.21 ± 1.56
N_{30}	3.89 ± 0.12	3.80 ± 0.07	11.28 ± 0.61	10.34 ± 0.39
N_{50}	4.06 ± 0.15	4.21 ± 0.36	13.90 ± 1.60	15.86 ± 2.39
C.D. at 5% level	0.24		2.08	

± Standard deviation of the mean of 4 replicates.

doses without inoculation increased the yield significantly. Inoculation in presence of applied N did not show any beneficial effect over the uninoculated counterparts, whereas the same in the presence of 20 kg P/ha brought about a significant increase in yield, the magnitude of increase being about 30 per cent over that in the uninoculated control. At the lower level of P application (10 kg P ha^{-1}) inoculation although brought about some increase, but the same was not statistically significant. Rai and Gour[9] also observed a significant increase in the grain yield as well as N uptake of wheat due to inoculation with *A. lipoferum*. Beneficial effect of inoculation with *A. brasilense* on rice, pearl millet, barley, sorghum and jower was also reported[10,11].

N-content and N-uptake

Neither inoculation alone nor the same in the presence of applied N or P at any of the two levels used could bring any significant change in the N content in the grain. But the total uptake of N was significantly increased by inoculation alone and in the presence of applied P at the level of 20 kg . ha^{-1} but not in the presence of N at any of the two levels, although N alone could increase the uptake 4 to 5 times of that in the control (Table 6).

The results, therefore, clearly show that inoculation of mustard seeds with *Azospirillum lipoferum* alone or in the presence of 20 kg P . ha^{-1} proved to be beneficial so far the grain yield and N uptake were concerned. This was due to better association of the inoculated organism with the roots of plant.

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