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N_2 Fixing Bacterial Isolates of Tea and their Plant Growth Promotional Activities

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ABSTRACT

An investigation was carried out to characterize N_2 fixing bacteria isolated from tea plants grown in tea growing regions of Karnataka, India and analyse their potential to enhance growth and nutrient uptake of tea plants under nursery conditions. Twenty *Azospirillum* and 18 *Beijerinckia* isolates were used in the study. The amount of N_2 fixed by *Azospirillum* isolates ranged from 7.23 to 22.16 mg g^{-1} of malate, whereas that of *Beijerinckia* isolates ranged from 2.86 to 5.54 mg g^{-1} of glucose. Sixteen out of 20 *Azospirillum* isolates produced both Indole Acetic Acid and Gibberellic Acid which were in the range of 334 to 874 and 33 to 230 μg L^{-1} of broth, respectively. Five isolates of *Beijerinckia* were found to produce both Indole Acetic Acid and Gibberellic Acid in the range of 53 to 169 μg and 44 to 90 μg L^{-1} broth, respectively.

Results of nursery evaluation of efficient *Azospirillum* and *Beijerinckia* isolates indicated that nine out of 12 N_2 fixers enhanced growth, dry matter accumulation and nutrient uptake of tea plants significantly over an uninoculated control.

Keywords: Tea plants, *Azospirillum*, *Beijerinckia*, growth promotion, nutrient uptake

INTRODUCTION

Biological nitrogen fixation offers an economically attractive and ecologically sound route for augmenting nitrogen supplies. Of the various rhizosphere associated N_2 fixing bacteria, *Azospirillum* species are extensively studied and shown to have a significant potential for commercial applications for non-leguminous crops (Bashan and Holquin, 1997). They enhance soil fertility by increasing the amount of available N and synthesize several different plant hormones that can act to enhance various stages of plant growth (Bashan and Holquin, 1997). Although the growth promotional effects of *Azospirillum* and *Beijerinckia* have been reported in many crop plants (Alagawadi and Gaur, 1992; Polyanskaya *et al.*, 2002 and Rao and Charyulu, 2006), their impact on plantation crops like tea is yet to be fully explored. Biofertilizer preparations containing such efficient organisms can be an

ecofriendly and cost effective supplement to chemical fertilizers. In this context, present work was undertaken to characterize *Azospirillum* and *Beijerinckia* from tea plants and to analyse their potential to enhance growth and nutrient uptake of tea plants under nursery conditions.

MATERIALS AND METHODS

Twenty *Azospirillum* and 18 *Beijerinckia* isolated from endorhizosphere and phyllosphere of tea plants, respectively in an earlier study (Tennakoon, 2007) were used in the present study. Isolates were screened for their ability to fix atmospheric N_2, phosphate solubilizing ability and production of plant growth promoting substances by standard procedures, as detailed below.

In vitro nitrogen fixation

A loopful of 48 h old culture of *Azospirillum* and *Beijerinckia* were inoculated to 5 ml of semisolid N free malate medium and N free broth medium, respectively and incubated for 48 h. One ml of this culture was inoculated to 50 ml of respective media in 250 ml flasks and three replicates were maintained for each isolate. Uninoculated flasks served as controls. The flasks were incubated at 28 ± 2 ^{0}C for 15 days and 10 ml of this culture was used for estimation of nitrogen by Microkjeldhal method of Jackson (1973) and Bremner (1960).

Phosphate solubilizing ability

Isolates were tested for their ability to solubilize insoluble inorganic phosphate on Sperber's Agar plates by spotting overnight grown cultures and incubating the plates at 30^{0}C for 24 to 48 h. The isolates showing clear zone of solubilization around the colony were taken as positive ones for P-solubilization. The diameter of the zone of solubilization was measured and expressed in centimeters.

Production of plant growth promoting substances

All the isolates of *Azospirillum* and *Beijerinckia* were initially subjected to qualitative analysis for the production of Indole Acetic Acid (Bric *et al.*, 1991) and Gibberellic Acid (Brown and Burlingham, 1968). The overnight positive cultures were inoculated to 50 ml of sterilized Czapeck's solution (Mahadevan and Sridhar, 1982) and incubated at 37 ^{0}C for seven days in dark. The cultures were then centrifuged at 6000 rpm for 20 min. The supernatant was used for the estimation of Indole Acetic Acid (IAA) by the method of Gordon and Paleg (1957) and Gibberalic Acid (GA) by the method of Paleg (1965).

Nursery evaluation of *Azospirillum* and *Beijerinckia*

Isolates were selected based on their efficiency of N_2 fixation and production of IAA and GA *in vitro*. The performance of the selected isolates of *Azospirillum* and *Beijerinckia* in

enhancing the growth and nutrient uptake of tea plants was assessed under nursery conditions.

Three months old nursery tea plants of clone TRI 2025, grown under conventional nursery conditions (one plant in 25 cm x 5 cm polythene sleeve) obtained from Kelagur Estate, Chickmagalur District of Karnataka State, India were used for the study.

The experiment comprised of 13 treatments, consisting of ten efficient isolates of *Azospirillum* (AZOSP 4-1, AZOSP 4-2, AZOSP 11-1, AZOSP 11-2, AZOSP 27, AZOSP 27-1, AZOSP 27-2, AZOSP 27-3, AZOSP 31, AZOSP 34) and two *Beijerinckia* (BEIJ 2 and BEIJ 5) along with one uninoculated control. Plants in triplicate were inoculated with 48 h old culture at 5 ml per plant. The experiment was conducted in a completely randomized design (CRD). The plants were kept under high shade conditions throughout the experimental period. The plants were watered regularly and plant protection measures were taken as and when necessary to protect them against any insect-pest damages. The plants were allowed to grow for 90 days. Observations on plant height, number of leaves per plant and leaf area per plant were recorded on the day of inoculation as well as after 90 days of inoculation (DAI). All other growth parameters and nutrient content were measured at 90 DAI.

Plant height was measured from the base of stem to base of fully opened top leaf and expressed in centimeters. Number of leaves on each plant were counted and recorded. The leaf area was measured by a geometric method. The length and maximum width of each leaf in a plant were measured and used to calculate the leaf area using the formula: a = (l $\leftrightarrow$ b)$\leftrightarrow$ 0.625, where a = leaf area (cm^2), l = length (cm) and b = maximum width (cm) of leaf (Krishnapillai, 1975). The area of all the leaves on each plant were summed up and expressed as total leaf area per plant.

After 90 days of inoculation, plants were carefully uprooted and root system was washed free of soil. Root length was recorded from base of stem to tip of the longest root and expressed in centimeters.

The root and shoot portions were separated from plants and air dried. They were oven dried at 62 ^{0}C to a constant weight. The shoot and root dry weights were recorded separately and expressed in gram per plant. The oven dried plant samples were ground to a fine powder and used for estimation of N and P contents. Total N content in the plant samples was estimated by the Microkjeldahl method and P content by Vanadamolybdate phosphoric yellow colour method (Jackson, 1973) and expressed in percentages.

The total plant uptake of N and P were calculated by multiplying the percent nutrient (N/P) content and respective dry biomass of tea plants.

The data obtained were analysed by Fisher's method of analysis of variance (Panse and Sukhatme, 1985). The level of significance used in 'F' test was P = 0.01. Critical differences were calculated wherever 'F' test was significant.

RESULTS AND DISCUSSION

All the isolates of *Azospirillum* and *Beijerinckia* isolated from tea plants were examined for their N_2 fixing ability. The amount of N_2 fixed by *Beijerinckia* isolates ranged from 2.86 to 5.54 mg g^{-1} of glucose (Table 2), whereas that of *Azospirillum* isolates ranged from 7.23 to 22.16 mg (Table 1), indicating higher N_2 fixing potential. These results are in conformity with the earlier observations made by Veena (1999) and Naikar (2003). Among the *Azospirillum* isolates, AZOSP 27 showed the highest amount of N_2 fixation (22.16 mg g^{-1} of malate added) followed by AZOSP 11-2 (20.3 mg g^{-1} of malate added), which were significantly superior to all the other isolates, but were on par among themselves (Table 1). Among the *Beijerinckia* isolates, BEIJ 2 showed the highest amount of N_2 fixation (5.54 mg g^{-1} of glucose added) followed by BEIJ 5 and BEIJ 14 (4.79 mg and 4.73 mg g^{-1} of glucose added, respectively) and were significantly superior to the rest of the isolates, but were on par among themselves (Table 2).

Three *Azospirillum* isolates *viz.*, AZOSP 4-1, AZOSP 27 and AZOSP 27-1, showed P-solubilizing zones on Sperber's agar (Table 1) indicating their ability to solubilize insoluble phosphate, but none of the *Beijerinckia* isolates showed P-solubilizing ability.

Sixteen out of 20 *Azospirillum* isolates were found to produce both IAA and GA in the range of 334 to 874 and 33 to 230 μg L^{-1} of broth, respectively (Table 1). The isolate, AZOSP 11-2 produced the highest amount of IAA (874 μg L^{-1} of broth), which was however on par with AZOSP 27 and AZOSP 4-1 (852 μg and 790 μg L^{-1} of broth, respectively), but was significantly superior to rest of the isolates. AZOSP 27 produced the highest amount of GA (230 μg L^{-1} of broth), which was significantly superior to the other isolates. Similar observations on N_2 fixation, P-solubilization and production of IAA and GA by *Azospirillum* and *Beijerinckia* have been made earlier (Iswaran and Marhawa, 1982; Tamilvendan and Purushothaman, 1996; Veena, 1999; Naikar, 2003).

Inoculation effects of N_2 fixers on growth and nutrient uptake of tea plants

The data on plant growth parameters of tea plants as influenced by inoculation with *Azospirillum* and *Beijerinckia* isolates are presented on Table 3. Data on shoot, root and total dry weight, plant N and P contents and total uptake are presented in Table 4.

At the initiation of the experiment, growth parameters of plants receiving most inoculation treatments did not differ significantly from uninoculated control. After 90 days of inoculation (DAI), all the inoculas except AZOSP 34, BEIJ 2 and BEIJ 5, had enhanced plant growth, dry matter content and nutrient uptake significantly over the control. Among the isolates, AZOSP 27 and AZOSP 11-2 were significantly superior to the rest of the isolates (Table 3).

Table 1: Beneficial characters of *Azospirillum* isolates

Sl. No.	Isolate	Nitrogen fixation (mg/g of malate)	Mineral phosphate solubilizing ability*		Production of IAA		Production of GA	
			Sperber's agar	Pikovskaya's agar	Qualitative	Quantitative (µg/L)	Qualitative	Quantitative (µg/L)
1	AZOSP 4-1	17.27	0.4*	NZ	+	790	+	122
2	AZOSP 4-2	16.10	NZ	NZ	+	670	+	125
3	AZOSP 6	15.63	NZ	NZ	+	518	+	42
4	AZOSP 9	10.03	NZ	NZ	+	387	+	49
5	AZOSP 10	9.56	NZ	NZ	+	334	+	44
6	AZOSP 11-1	15.86	NZ	NZ	+	693	+	53
7	AZOSP 11-2	20.30	NZ	NZ	+	874	+	185
8	AZOSP 13	9.33	NZ	NZ	+	490	+	68
9	AZOSP 14	9.56	NZ	NZ	-	-	-	-
10	AZOSP 20	9.33	NZ	NZ	+	564	+	49
11	AZOSP 24	9.56	NZ	NZ	-	-	-	-
12	AZOSP 25	7.70	NZ	NZ	+	391	+	65
13	AZOSP 26	9.80	NZ	NZ	-	-	-	-
14	AZOSP 27	22.16	0.8	NZ	+	852	+	230
15	AZOSP 27-1	17.50	0.5	NZ	+	519	+	86
16	AZOSP 27-2	15.63	NZ	NZ	+	595	+	88
17	AZOSP 27-3	15.86	NZ	NZ	+	702	+	83
18	AZOSP 31	11.66	NZ	NZ	+	358	+	40
19	AZOSP 31-1	7.23	NZ	NZ	+	337	+	33
20	AZOSP 34	10.26	NZ	NZ	-	-	-	-
	SEm±	0.62	-	-	-	21.66	-	5.59
	CD at 1%	2.39	-	-	-	83.91	-	21.68

NZ : No zone formation

+ : Positive to test

-: Negative to test

* : Diameter of zone of solubilization (cm)

Table 2: Beneficial characters of *Beijerinckia* isolates

Sl. No.	Isolate	Nitrogen fixation (mg/g of glucose)	Production of IAA		Production of GA	
			Qualitative	Quantitative (µg/L)	Qualitative	Quantitative (µg/L)
1.	BEIJ 1	3.39	-	-	-	-
2.	BEIJ 2	5.54	+	142	+	74
3.	BEIJ 3	3.33	-	-	-	-
4.	BEIJ 4	3.09	-	-	-	-
5.	BEIJ 5	4.79	+	169	+	90
6.	BEIJ 6	3.33	-	-	-	-
7.	BEIJ 7	2.92	-	-	-	-
8.	BEIJ 8	3.33	+	53	+	44
9.	BEIJ 9	3.09	-	-	-	-
10.	BEIJ 10	2.86	-	-	-	-
11.	BEIJ 11	3.09	-	-	-	-
12.	BEIJ 12	3.39	-	-	-	-
13.	BEIJ 13	3.56	-	-	-	-
14.	BEIJ 14	4.73	+	100	+	53
15.	BEIJ 15	3.80	+	67	+	65
16.	BEIJ 16	3.44	-	-	-	-
17.	BEIJ 17	3.15	-	-	-	-
18.	BEIJ 18	2.86	-	-	-	-
	SEm±	0.23	-	9.24	-	6.73
	CD at 1%	0.89	-	41.44	-	30.19

+ : Positive to test

-: Negative to test

The increase in shoot and root growth, dry matter accumulation and N and P content in tea plants inoculated with N_2 fixers could be attributed to efficient N_2 fixation (Berkum and Bahlool, 1980) and production of growth promoting substances (Sattar and Gaur, 1987). It was evident in the present study that the two isolates, AZOSP 27 and AZOSP 11-2, which fixed maximum amounts of N_2 and produced the highest amount of IAA and GA, showed an increase in all measured growth parameters, nutrient uptake and dry matter accumuation of tea plants significantly over the rest of the isolates. Besides that, AZOSP 27 also possessed the ability to solubilize insoluble inorganic phosphate which also might have contributed to growth improvement and nutrient uptake in inoculated plants.

Table 3. Effect of inoculation of selected *Azospirillum* and *Beijerinckia* isolates on growth of tea plants

Sl. No.	Treatment / isolate	Plant growth parameters*									
		At initiation of experiment			After 90 days of inoculation						
		Plant height (cm)	No. of leaves per plant	Total leaf area per plant (cm^2)	Plant height (cm)	% increase over initial height	No. of leaves per plant	% increase in No. of leaves over initial stage	Total leaf area per plant (cm^2)	% increase in total leaf area over initial stage	Root length (cm)
1.	AZOSP 4-1	12.5	3.00	59.0	17.8	42	6.33	111	183.7	211	22.9
2.	AZOSP 4-2	11.3	2.67	50.7	15.5	37	4.67	75	105.0	107	22.8
3.	AZOSP 11-1	13.7	3.33	52.7	19.2	41	6.00	80	174.0	230	14.2
4.	AZOSP 11-2	12.7	3.33	56.7	22.0	73	7.00	110	200.0	253	27.1
5.	AZOSP 27	14.0	2.67	55.7	27.5	96	6.00	125	194.3	249	21.0
6.	AZOSP 27-1	12.5	2.67	56.0	15.3	22	4.33	62	106.3	90	18.2
7.	AZOSP 27-2	13.0	2.67	55.7	18.0	39	5.00	87	141.0	153	19.3
8.	AZOSP 27-3	12.3	2.67	55.0	14.8	20	4.33	62	89.0	62	20.2
9.	AZOSP 31	12.8	2.67	58.7	14.5	13	4.33	62	107.3	83	17.0
10.	AZOSP 34	11.5	2.67	59.0	13.5	17	3.00	12	90.7	54	12.2
11.	BEIJ 2	12.3	2.67	57.0	13.8	12	4.67	75	103.0	81	18.2
12.	BEIJ 5	12.1	2.67	52.7	13.4	11	3.00	12	70.0	33	14.0
13.	Control (UIC)	12.1	2.67	49.3	13.1	8	3.00	12	59.3	20	9.4
	SEm±	0.49	0.32	3.13	0.33	-	0.26		4.95	-	0.92
	CD at 1%	1.94	NS	NS	1.33	-	1.02		19.47	-	3.62

* Average of 3 plants UIC – Uninoculated control NS - Not Significant

Table 4. Effect of inoculation of selected *Azospirillum* and *Beijerinckia* isolates on dry matter content and nutrient uptake of tea plants at 90 DAI

Sl. No.	Treatment / Isolate	Dry matter content *			Plant nutrient content and uptake *			
		Shoot (g/plant)	Root (g/plant)	Total (g/plant)	N content (%)	P content (%)	Total N uptake (mg/plant)	Total P uptake (mg/plant)
1.	AZOSP 4-1	1.86	0.62	2.48	2.36	0.15	58.40	3.69
2.	AZOSP 4-2	1.20	0.72	1.92	2.27	0.11	43.60	2.09
3.	AZOSP 11-1	2.00	0.64	2.64	2.27	0.12	59.90	3.20
4.	AZOSP 11-2	2.17	1.18	3.35	2.40	0.13	80.40	4.35
5.	AZOSP 27	2.23	0.78	3.01	2.46	0.18	74.03	5.38
6.	AZOSP 27-1	1.28	0.51	1.79	2.25	0.13	40.20	2.35
7.	AZOSP 27-2	1.66	0.76	2.42	2.38	0.13	57.60	3.07
8.	AZOSP 27-3	1.01	0.86	1.87	2.25	0.12	42.00	2.19
9.	AZOSP 31	1.13	0.76	1.89	2.24	0.11	42.10	2.07
10.	AZOSP 34	1.05	0.60	1.65	2.18	0.11	36.04	1.85
11.	BEIJ 2	1.30	0.48	1.78	2.11	0.11	37.30	1.96
12.	BEIJ 5	1.07	0.38	1.85	2.19	0.11	39.50	1.72
13.	UIC	0.80	0.32	1.12	1.83	0.11	20.60	1.25
	SEm±	0.04	0.02	0.11	0.01	0.01	2.54	0.20
	CD at 1%	0.16	0.08	0.43	0.04	0.03	9.98	0.81

* Average of 3 plants UIC – Uninoculated control

The favourable influence of *Azospirillum* and *Beijerinckia* on plant growth and plant biomass through N_2 fixation are well known (Berkum and Bohlool, 1980). Nitrogen promotes vegetative growth and encourages the formation of foliage thus promoting the production of more dry matter (Van der Werf *et al.*, 1993). *Azospirillum* and *Beijerinckia* inoculation is also known to improve root development, mineral uptake and plant water relationships (Prasad and Govindarajan, 2001). Plant growth promoting substances produced by these bacteria are known to change the root morphology and increase their biomass enabling the plant roots to contact more soil volume for nutrient uptake (Malik *et al.*, 1997). Thus, such bacteria can complement the beneficial effects of N_2 fixation, phosphate solubilization and geocycles that release nutrients into the rhizosphere. This was clearly evident in the present study that the plants inoculated with N_2 fixers showed significantly improved growth and nutrient accumulation over uninoculated control. In accordance with shoot and root growth, dry matter content of tea plants was also increased. These observations are confirming the earlier findings of Baby *et al.* (2002) in tea and Alagawadi and Gaur (1992) and Rao and Charyulu (2006) in other crop plants.

CONCLUSION

The investigation has clearly brought out the potential of *Azospirillum* in tea plants in enhancing growth and nutrient uptake of clonal teas through multiple beneficial functions. Performance of these efficient isolates either singly or in the form of consortia in combination with other inputs to improve growth of tea plants under field conditions should be investigated in future studies.

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