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Evaluation of indigenous *Azospirillum* isolates for growth promotion in black pepper (*Piper nigrum* L) rooted cuttings

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Abstract

The aim of the present work was to evaluate the inoculation effects of indigenous *Azospirillum* spp. selectively isolated from various black pepper growing locations of Kerala and Karnataka in enhancing the growth and nutrient uptake of black pepper cuttings. *Azospirillum* isolates BPaz4 and BPaz9 recorded 67% more plant height in rooted cuttings than untreated cuttings. The nitrogen, phosphorus and potassium uptake and total dry weight was significantly superior in treated plants. Increase in the uptake of iron, manganese, zinc and copper was found in BPaz9 treatment. Colonization of rhizosphere soil and nonrhizosphere by these isolates was found to be high. The N_2-fixation capacity of the isolates BPaz4 and BPaz9 showed 8.68 and 8.52 mg g^{-1} malate. Thus the application of these inoculants viz. BPaz4 and BPaz9 is suggested for the ecofriendly production of rooted cuttings of black pepper.

Keywords: Azospirillum, Growth promotion, IAA, N_2- fixers

Introduction

In India, soil fertility is diminishing gradually due to soil erosions, loss of nutrients, accumulation of toxic elements, water logging and unbalanced nutrient compensation. Organic manure and biofertilizers are the alternate sources to meet the nutrient requirement of crops. In recent years, biofertilizers have emerged as a promising component of integrated nutrient supply system in agriculture. Among biofertilizers benefiting the crops are *Azosprillum*, *Azotobacter*, P-solubilising microorganisms, blue green algae and mycorrizae (Hedge *et al.* 1999). The promotion of plant growth by *Azospirillum* has been reported in field and nursery plants, resulting in significant changes in several characteristics of plants. The *Azospirillum* inoculation responses in non-leguminous plants are still difficult to estimate (Bashan *et al.*1995).

The indigenous bacterial strains belong to a particular region is also likely to perform better than the exotic strain (Thakuria *et al.* 2001). Indigenous strains have performed better than introduced strains in promoting the growth of crops due to their superior adaptability to the environment as found in A. brasilense in wheat (Kapulnik *et al.* 1983; Abbas *et al.* 2007).

Black pepper is one of the most important export oriented spice crops of India. The production of spices can be increased considerably through integrated nutrient management (Sadanandan 2000).The successful establishment of black pepper in the main field depends up on the quality of rooted cuttings. Beneficial effect of *Azospirillum* inoculation has been reported in black pepper (Govindan & Chandy 1985; Bopaiah & Khader 1989; Kandiannan et al. 2000).However, reports are very scanty on the use of *Azospirillum* native to black pepper.

In the present investigation an attempt has been made to isolate *Azospirillum* from black pepper rhizosphere in order to evaluate them for growth promotion in rooted cuttings of black pepper.

Materials and methods

Isolation

Rhizosphere samples from black pepper representing Kerala and Karnataka were collected and isolated specific group of organisms using selective media. Based on incubation time and morphology of colonies, isolates were selected for evaluation in black pepper cuttings. *Azospirillum* was isolated from soil and root samples based on standard methodology (Tarrand *et al*.1978; Enrique, 1982). Isolated colonies were characterized, sub cultured and maintained at 28±10°C.

In planta evaluation

The experiments were conducted at Indian Institute of Spices Research, experimental farm, Peruvannamuzhi, Kerala. Apparently healthy rooted cuttings of black pepper variety IISR Sreekara was used in the experiment. The strains isolated were compared for their growth promotion efficacy under completely randomized design (CRD) with 30 plants for each strain. Surface disinfected rooted cuttings were root dipped in suspension of *Azospirillum* isolates at 10^8 cfu mL-1 for 15min and transplanted into polythene bags filled with sterilized potting mixture consisting of soil: farm yard manure: sand (1:1:1). The plants were repeat inoculated with *Azospirillum* at monthly interval as soil drench up to three months and the inoculated plants were compared with uninoculated plants. No nutrient and pesticides were applied to the cuttings. Cuttings were irrigated as and when necessary to maintain soil moisture at field capacity level.

Observations

Growth Promotion

Growth parameters such as height of the plant (cm/pot), number of leaves, leaf and internodal length were recorded at monthly interval for four months.

Nutrient uptake

The fresh weight of the shoot as well as the root was recorded and the plants were used for biomass estimation and nutrient analysis. Major, secondary and micronutrients were estimated in roots, shoots and soil by standard protocols. (Bremner 1996; Jackson 1973).

Root/Rhizosphere colonization

Rhizosphere colonization by the isolates was monitored at 30 day interval thrice up to 120 days. Root (endorhizosphere) colonization of the introduced microbes was estimated at the end of the experiment. The roots were washed thoroughly and root pieces from different portions of root (lower, middle and upper) were selected randamonly, surface sterilized and placed in nitrogen free bromothymol (Nfb) medium tubes with five replications (three pieces/tube). The number of tubes with blue colour was considered positive for the presence of *Azosprillum*.

Invitro tests

Putative nitrogen fixing bacteria were screened in the ACC deaminase defined medium, except the N source was eliminated and agar was reduced to 1.75g L-1. The isolates that grew after being sequentially transferred for 10 times to the same medium were considered presumptive positive for N2-fixation. (Day & Dobereiner 1976).Nitrogen fixing ability was determined using Nfb medium. The isolates were grown in semisolid medium for 72 h, homogenized, digested with H_2SO_4 and the aliquots were estimated for total nitrogen. Indole acetic acid production (IAA) was determined in L-tryptophan agar using Salkowski reagent (Sarwar & Kremer 1995).

Short-listing of Promising isolates

The isolates were short-listed based on in vivo and in vitro growth promoting characteristics. The data collected were analyzed statistically using MSTATC.

Results and discussion

Isolation

A total of 11 isolates were obtained based on the colony characteristics and other properties unique to *Azospirillum*.They were isolated from roots and soil samples representing Kerala and Karnataka (Table.1)

Table 1. List of *Azospirillum* isolates used in the study

Designation of isolate	Location	source
BPaz1	Wayanad	Root
BPaz2	Wayanad	Root
BPaz3	Wayanad	Root
BPaz4	Appangala	Root
BPaz5	Gonikoppal	Soil
BPaz6	Appangala	Soil
BPaz7	Idukki	Root
BPaz8	Thrissur	Root
BPaz9	Idukki	Root
BPaz10	Parambikulam	Soil
BPaz11	Parambikulam	Soil

In planta evaluation

The initial nutrient status of potting mixture is as follows.N- 84.0, P- 11.0, K- 180 and Ca - 525, Mg- 182, Fe-30.4, Mn- 5.4, Zn- 1.1 and Cu 0.30 ppm.The PH was in the range of 5.5 to 6.0.The *Azospirillum* isolates significantly enhanced the growth parameters of black pepper cuttings (Table 2). The maximum height was shown by BPaz4 (67%), followed by BPaz9 (60% over control).However, the number of leaves did not differ significantly among treatments. The isolate BPaz9 inoculation produced total biomass of 9.75 g plant-1 followed by BPaz4 (9.74g plant-1) which is 47% over the control. The isolate also showed significant increase in internodal and leaf length.

The total nutrient uptake of host plants was found to be influenced by the inoculants. Among *Azospirillum* isolates, maximum nitrogen uptake was shown by the isolate BPaz4, followed by BPaz9 (33 and 43% each.).The uptake of P, K, Ca and Mg did not differ significantly among the treatments (Table 3). The isolate BPaz3 andBPaz9 showed significant increase in the uptake of Fe, Mn, Zn and Cu over control. The soil nitrogen content was also higher in treatment with isolates BPaz3, BPaz4 and BPaz9.

The population of *Azospirillum* in the rhizosphere was high in inoculated plants (fig1). The Azospirillum isolates BPaz1, BPaz4

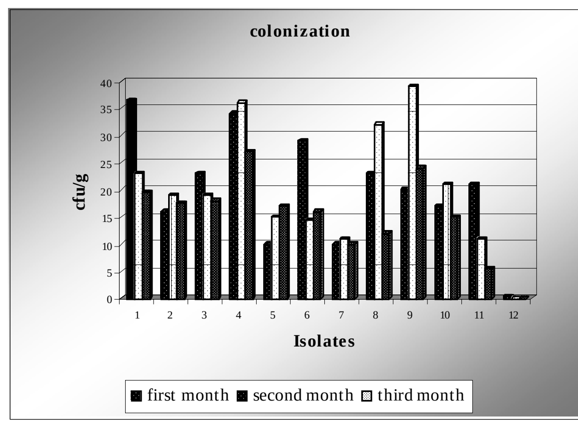

Fig 1. Colonization of *Azospirillum* isolates during different periods of plant growth

and BPaz6 was found to colonize better than than other isolates. The colonization ability increased significantly during the second month except BPaz1, BPaz6 and BPaz11.The isolates BPaz4 and BPaz9 showed highest colonization in the second month (36 and 39X10^4cfu/g). However, the population density decreases gradually in the third month except BPaz5 and BPaz6.It may be observed that endophytic colonization (root) followed the same trend as noticed in the soil. The isolates BPaz4 and BPaz9 also showed better colonization in different portions of root.

The *Azospirillum* isolates BPaz4 and BPaz9 grow in M9 minimal medium even after 10

Table 2. Growth promotion in rooted cuttings inoculated with *Azospirillum* inoculants in soil

Azospirillum Isolates	Plant Height(cm)	No. Leaves	Internodal length(cm)	Leaf Length(cm)	Leaf Breadth (cm)	Total dry wt (gm)
BPaz1	111.0	13.6	6.2	9.8	5.4	4.311
BPaz2	100.3	12.7	4.8	8.6	5.8	5.593
BPaz3	75.00	13.0	5.0	10.3	5.4	7.433
BPaz4	125.4	12.9	5.3	10.0	5.7	9.740
BPaz5	115.0	12.2	5.1	10.1	6.0	7.103
BPaz6	120.2	12.9	5.0	9.4	5.0	8.557
BPaz7	100.0	13.0	4.8	9.5	4.9	6.600
BPaz8	110.0	12.8	5.2	10.0	5.9	8.010
BPaz9	120.0	13.0	6.0	10.2	5.4	9.750
BPaz10	83.00	13.5	5.6	9.0	6.0	5.810
BPaz11	95.00	12.5	5.8	9.5	5.0	6.660
Control	75.00	13.0	4.8	8.5	4.8	6.600
CD(P=0.05)	18.2	2.33	0.42	1.06	0.71	2.07

Table 3. Nutrient uptake as effected by *Azospirillum* inoculants in black pepper rhizosphere

Isolates	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
			g					mg	
BPaz1	0.084	0.007	0.056	0.038	0.014	1.64	0.284	0.056	0.068
BPaz2	0.114	0.007	0.069	0.048	0.013	2.21	0.397	0.045	0.051
BPaz3	0.154	0.014	0.157	0.071	0.026	1.94	1.632	0.338	0.248
BPaz4	0.176	0.010	0.095	0.058	0.022	2.54	0.271	0.119	0.148
BPaz5	0.150	0.009	0.078	0.058	0.020	3.02	0.563	0.117	0.176
BPaz6	0.167	0.010	0.093	0.056	0.025	4.92	0.501	0.153	0.162
BPaz7	0.118	0.010	0.083	0.053	0.018	1.84	0.264	0.115	0.108
BPaz8	0.160	0.009	0.116	0.060	0.024	4.68	0.483	0.105	0.152
BPaz9	0.190	0.011	0.156	0.083	0.027	3.58	0.985	0.292	0.195
BPaz10	0.120	0.009	0.065	0.038	0.015	1.74	0.465	0.093	0.093
BPaz11	0.133	0.008	0.072	0.052	0.017	1.45	0.535	0.106	0.106
Control	0.132	0.010	0.094	0.049	0.021	1.35	0.204	0.099	0.132
CD(P=0.05)	0.081	0.057	0.05	0.05	0.05	1.73	0.655	0.099	0.152

successive subcultures confirms presumptive positive for N2-fixation.The isolates showed wide variations in their nitrogen fixing abilities ranging from 3.20 to 8 mg N g-1of malic acid. The N2-fixation capacity of the isolates BPaz4 and BPaz9 tested invitro showed 8.68 and 8.52 mg N g-1 malate. The isolates also produced indole acetic acid.

The beneficial effects of *Azospirillum* are well known in agriculture, not only due to biological nitrogen fixation in the rhizosphere, but mainly, for the best efficacy in the absorption of water and nutrients, which happens due to a more developed root system, increasing the soil area explored by the roots. In several earlier investigations, exotic strain of *Azospirillum* has been reported (Fages 1994; Bashan & Holguin 1997; Reis *et al.* 2000).In order to develop crop specific strains of *Azospirillum* an investigation was carried out.11 isolates of *Azospirillum* were isolated from black pepper rhizosphere representing major pepper growing regions in South India. These isolates, when evaluated in black pepper rooted cuttings, performed well in terms of growth promotion and nutrient mobilization.

The increased dry matter in BPaz4 and BPaz9 could be attributed to the increased plant height, which is one of the main contributing factors for shoot biomass. The increased height and biomass is the resultant of the increased nutrient uptake by different parts of the plant system as a result of enhanced microbial activity observed in these treatments. This is in agreement with the earlier findings that *Azospirillum* inoculation could improve ion uptake and contributed to the significant elevation of plant growth (Lin *et al.*1983; Bashan & Holguin 1997; Molla *et al.* 2001).

The ability of *Azospirillum* to attain significant populations on the host root system has been shown to be a prerequisite for their beneficial effects on plant growth (Bashan 1986). The isolates BPaz4 and BPaz9 also showed better colonization in different portions of root as well as in the soil. The total population density of the isolates BPaz4 and BPaz9 in the inoculated microcosms were higher than that of uninoculated microcosms. The metabolic differences among the strains of *Azospirillum* and their ability to utilize the carbon sources might have influenced the differential colonization of strains (Wani 1992).

Though the isolates recorded growth in nitrogen free conditions, significant variation was observed among the isolates. The fluctuations in soil redox potential, pH and organic matter govern the nitrogen fixing activity of the *Azospirillum* spp. (Charyulu and Rajaramamohan Rao, 1980).

The present study clearly indicated that the inoculation of plants with the isolates BPaz4 and BPaz9 resulted in better growth of black pepper cuttings. The co-inoculation of these isolates in different combinations, under varying levels of fertilizers and organic amendments may contribute more to the growth and nutrient uptake.

Table 4. *Invitro* growth promoting capacity of isolates

Azospirillum isolates	N2-Fixation mg g-1 malate	N2-Fixation Growth after successive subcultures in ACC medium*	IAA
BPaz1	4.00	++	+++
BPaz2	5.80	+++	-
BPaz3	7.32	+	-
BPaz4	8.68	+++	++
BPaz5	3.22	++	-
BPaz6	3.80	+++	+
BPaz7	4.20	++	-
BPaz8	6.70	++	-
BPaz9	8.52	+++	+
BPaz10	6.12	+++	-
BPaz11	3.20	++	-

* 5-+++ 5-++ 3-+

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