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Inoculation effect of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth of black gram (*Phaseolus mungo* Roxb; Eng)

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ABSTRACT

A total of 17 bacterial isolates including Azotobacter chroococcum (N=5), Azotobacter beijerinckii (N=4), Pseudomonas aeruginosa (N=4), and Bacillus sp (N=4) were isolated and tested for siderophore, HCN, ammonia and indole acetic acid production in vitro. The bacterial cultures were positive for siderophore, HCN and ammonia. Among the isolates, The amount of IAA produced by Azotobacter chroococcum and Azotobacter beijerinckii were 23.6 and 17.6 µg ml^{-1} respectiviely, whereas the phosphate solubilizers Pseudomonas aeruginosa and Bacillus sp showed maximum IAA production of 26.5 and 19.8 µg ml^{-1} of IAA in Luria Bertani broth. The single inoculation effects of N_2-fixing and PS bacteria on the growth, chlorophyll content, P and N content of black gram plants in green house experiments varied considerably between the treatments. The N contents in roots and shoots differed considerably among the treatments.

Key words: Nitrogen-fixing bacteria, Phosphate-solubilizing bacteria, Black gram, Plant growth.

INTRODUCTION

Nitrogen and phosphorous are known to be essential nutrients for plant growth and development. The global nitrogen cycle pollutes ground water and increases risk of chemical spills. The production of chemical fertilizers is a highly energy-intensive process using large amounts of fossil energy. High-input farming practices achieving high yields have created environmental problems and degradation in natural resources. Large quantities of chemical fertilizers are used to replenish soil N and P, resulting in high costs and severe environmental contamination. Consequently, there has recently been a growing level of interest in sustainable agricultural practices to alleviate detrimental effects of intensive farming currently practiced. Increasing and extending the role of biofertilizers would reduce the need for chemical fertilizers and decrease

adverse environmental effects. Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers.

Rhizophere associated N2 fixing and P-solubilizing bacteria have increasingly been used in non-legume crop species such as sugar beet, sugar cane, rice, maize and wheat [1,2,3]. Trials with Bacillus sp. indicated yield increases in rice [4], cereals [5,6] and maize [7]. Asymbiotic N-fixing bacteria were reported to replace 60% of N requirements of sugar cane amounting to 200 kg N/ha [8]. Bacillus sp. used as biofertilizers may have direct effects on plant growth through the synthesis of plant growth hormones [9], N fixation and synthesis of the enzymes modulating the level of plant growth promoting rhizobacteria [10].

Some of the above bacteria may also solubilize inorganic phosphate, making soil phosphorous otherwise remaining fixed available to the plants [11] due to excretion of organic acids [12] and through carbon and nitrogen sources, salt, pH, temperature [13]. Phosphate solubilizing bacteria stimulates plant growth through P nutrition, increasing the uptake of N, P, K and Fe. Phosphorous biofertilizers could help increase the availability of accumulated phosphates for plant growth by the increasing the efficiency of biological nitrogen fixation and the availability of Fe, Zn through production of plant growth promoting substances [14]. Therefore, a study was conducted in order to investigate the effect of single inoculations with N_2-fixing and P-solubilizing bacterial species on black gram growth under green house experimental analysis.

MATERIALS AND METHODS

Sediment samples were collected from non-seagrass environment of Thondi coast (latitude of 9º44'N and longitude of 79º19' E), Palk Strait, Southeast coast of India, by sediment sampler (Peterson grab). The sampler was sterilized with alcohol before sampling. The central portion of the sediment sample was taken out with the help of sterile spatula. This sample was then transferred to a sterile petriplates and transported immediately to the laboratory. *Azotobacter* were isolated using the plate technique on Burk's agar medium (HiMedia, India) and the plates were incubated in an inverted position for 72h at 28^0C. For the enumeration of PS bacteria by using Pikovskaya's agar medium [15]. The plates were incubated at 28 ± 2^0C for 3 days, colonies forming a clear halozone around them indicating phosphate solubilizing bacteria. The well developed and morphologically different single colonies were studied for their morphological and biochemical characteristics following standard techniques and their identification confirmed. [16,17].

Bioassay of IAA

The quantitative analysis of indole-3-acetic acid was performed by the method suggested by Gordan and Weber [18] and later modified by Brick *et al.*, [19]. Selected bacterial isolates were grown in Luria Bertani broth (g/l: tryptophan 10; yeast extract 5; NaCl 10 and pH 7.5). A 100 ml of LB broth amended with tryptophan (10, 20, 60, 80 and 100 µg ml^{-1}) was inoculated with 1 ml of culture (No.x10^8 cells ml^{-1}) and incubated for 24 h at 28±1°C on a rotary shaker for 125 rpm. After 24 h, 5 ml of each culture was centrifuged (10000 rpm) for 15 min and 2 ml of Salkowsky reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) was added to 2 ml of supernatant and incubated at 28°C in the dark for 1 h. the IAA concentration was determined using a spectrophotometer (λ

540 nm) against a standard curve. The experiments were conducted three times at different time intervals.

In vitro production assay of siderophore, HCN and ammonia

The analysis of siderophore production by the bacterial isolates was performed following the chrome azurol S (CAS) method of Alexander and Zuberer [20]. For each bacterial isolate, 100 µl of inoculum was dropped in the centre of Petri dishes (8.5 cm diameter) containing 30 ml CAS agar. The dishes were incubated at 28°C for four days and were observed daily. The discoloration of the medium (blue to orange) indicated siderophore production by the bacterial isolates. HCN production by the bacterial isolates was detected by the method of Bakker and Schipper [21]. For HCN production, the bacterial isolates were grown on an HCN induction medium (30 g tryptic soy broth, 4.4 g glycine, 15 g agar l^{-1}) at 28°C for four days. For each bacterial isolate, 100 µl of inoculum (No.x10^8 cells ml^{-1}) was dropped in the centre of the plates. Then a disk of Whatman filter paper dipped in 0.5% picric acid and 2% Na2CO3 was placed in the lid of the Petri dish, which was then sealed with parafilm. After four days of incubation at 28°C, the orange-brown discoloration of the paper indicated HCN production. In the case of ammonia production, bacterial isolates were grown in peptone water (g/l: peptone 10 g, NaCl 5 g, pH 7) and incubated at 30°C for four days. One ml of Nessler reagent was put into each tube and the presence of yellow colour indicating ammonia production was recorded [22].

Plant growth experiment

The bacterial strains *Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Pseudomonas aeruginosa* and *Bacillus* sp, which were positive for IAA, siderophore, HCN and ammonia, were selected and used alone to assess their effect on black gram productivity. Healthy viable black gram (*Phaseolus mungo* Roxb; Eng) seeds were procured from The Seed Center, Tamil Nadu Agriculture University, Coimbatore, India. For the experiments each pot was seeded with 50 healthy seeds. The black gram belongs to the family *Leguminosae* (*Papilonaceae*). Seeds of black gram were surface sterilized [23] and soaked for two hours in three-day-old cultures using 10% gum arabic as adhesive to deliver approximately 10^5 cells ml^{-1} of *Azotobacter* and 10^8 cells ml^{-1} of *Bacillus* and *Pseudomonas aeruginosa*. Thirty seeds were sown in each pot and the pots were watered well. Uninoculated pots were kept as control. The effects of *Azotobacter* spp. on the growth of seedlings were monitored carefully at intervals of 5 days. Only twenty germinated seeds were allowed to grow in each pot, and the effect of the inoculum (*Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Pseudomonas aeruginosa*, and *Bacillus* sp) were observed on the 2^{nd} to 15^{th} day at two days interval. The garden soil was processed and filled in the pots, after making the soil free of stones, fibrous materials etc, which may interfere with the growth of plants. The pots with soil were sterilized at 121 °C for 4 h. The treatments were: T1 control (uninoculation); T2 *Azotobacter chroococcum*; T3 *Azotobacter beijerinckii*; T4 *Pseudomonas aeruginosa*; T5 *Bacillus* sp. The experiments were conducted at each replicated three times. Plants harvest at 15^{th} day and were oven dried before the weights of roots and shoots and total plant biomass were determined. Total chlorophyll contents in foliage were determined at 15 days [24]. Total nitrogen contents in roots, shoots and straw was measured at 15 days of culture as suggested by Iswaran and Marwah [25]. Total P contents in plants were estimated at 15 days by the method of Jackson [26]. The chlorophyll content in the foliage was estimated [27].

RESULTS AND DISCUSSION

In the present study, strains of *Azotobacter chroococcum* (N=5), *Azotobacter beijerinckii* (N=4), *Pseudomonas aeruginosa* (N=4), and *Bacillus* sp (N=4) were isolated from Thondi non-seagrass sediment. Among all the strains were found to be positive for HCN, siderophore, ammonia and IAA Production (Table 1). The amount of IAA produced by *Azotobacter chroococcum* and *Azotobacter beijerinckii* were 23.6 and 17.6 µg ml^{-1} respectiviely, whereas the phosphate solubilizers *Pseudomonas aeruginosa* and *Bacillus* sp showed maximum IAA production of 26.5 and 19.8 µg ml^{-1} respectively. In LB broth supplemented with different range of tryptophan, where as 100 mg ml^{-1} of L-tryptophan supplement showed maximum production of IAA; by 76.5, 98.6 and 120% over Bacillus sp, Azotobacter chroococcum and Pseudomonas aeruginosa, respectively. In general, the IAA production increased with increasing physiological precursor of L-tryptophan concentration for all tested cultures. All the strains were positive production of alkaloids, aminoacids by chromatographic method. Bacterial cultures expressing positive results of IAA, HCN, siderophore and ammonia production, and to assess their effect on black gram under green house conditions. Among the strains, *Azotobacter chroococcum, Azotobacter beijerinckii, Pseudomonas aeruginosa* and *Bacillus* sp have 846, 680, 708 and 505 µg ml^{-1} of protein values at OD 660 nm of 0.61, 0.47, 0.52 and 0.38, respectively (Table 2). The effect of symbiotic of N_2-fixing and PS bacteria, used single inoculation on black gram crops was variable. The tested bacterial cultures produced considerable amounts of plant growth-promoting substances, which not only promoted the growth of the black gram plants but may also have played a significant role in the suppression of pathogens, i.e. biocontrol agent also increased plant growth under green house conditions. It is well–accepted fact that P-solubilizing bacteria not only solubilize insoluble P in the soil but also release considerable amounts PGPS, e.g. auxins [28]. The availability of balanced nutrients (e.g. N by N_2 fixer, Fe by siderophare producers and P by PB) and the release of PGPS, as observed under *in vitro* conditions, it might have resulted in the enhancement of the black gram and consequently an increase in the seed yield. Further, the increased availability of P increases the rhizobial activity in the rhizophere, resulting in better growth [29,30] and nitrogen fixation. The beneficial effect of *Azotobacter* strain were not only due to its ability to release growth–promoting substances, but could also be attributed to the release of other metabolites (e.g. antibiotics) into the surrounding environment, thus inhibiting root pathogens [31]. The bacterial inoculations increased the shoot growth of black gram treated with *Azotobacter chroococcum, Azotobacter beijerinckii, Pseudomonas aeruginosa,* and *Bacillus* sp by enhancing shoot length by 49.3, 49.0, 48.9, 46.8 cms, respectively. The primary and main root length of 22.5, 22.1, 22.0, 21.8 cms were enhancing by *Azotobacter chroococcum, Azotobacter beijerinckii, Pseudomonas aeruginosa* and *Bacillus* sp respectively. Moreover, the root and shoot length was monitored 2 days interval of 14 days of culture period. However all the parameters were induced maximum with the inoculation of *Azotobacter chroococcum* and *Pseudomonas aeruginosa* than the other strains (*Azotobacter beijerinckii* and *Bacillus* sp) and compared with control treatment (Tables 3 and 4). Single inoculation effects of nitrogen-fixing and phosphate-solubilizing bacteria on quantitative study of P content in total plants and N content in roots, shoots and straw showed remarkable variations between the treatments (Table 5). Further also studied the dry mass of root, shoot and total plants of black gram. Likewise, the level of total chlorophyll was increased maximum by 0.93 and 0.91, 0.82 and 0.8 mg g^{-1} with the inoculation of *Pseudomonas aeruginosa, Azotobacter chroococcum, Azotobacter beijerinckii,* and *Bacillus* sp, respectively (Tables 6). Kalaigandhi [32] was reported

that the 12 different *Azotobacter* species isolated from Thondi seagrass ecosystem and also used as a biofertilizer for black gram plant growth. Ravikumar *et al.*, [33] have reported that, halophilic *Azotobacter* species isolated from Pichavaram mangrove forest perform better by the inoculation into Rhizophora seedlings. Bashan *et al.*, [34] have reported that, the increase in the concentration of IAA directly promoted the chlorophyll content in *Anabaena* sp. The present findings thus suggested that microbial cultures should be established and proliferated under green house conditions as a suitable bioinoculant for raising the productivity of the plants. The present findings indicate that a single inoculation of N_2-fixers and Ps bacteria was more effective than control. Further investigation made on the joint application of N_2-fixers and Ps bacterial treatments could be an effective bioresource for developing black gram productivity, also could help to reduce external inputs under field conditions.

Table-1 *In vitro* production of IAA, siderophore, HCN and ammonia by the isolates

Strains	Production of IAA (µg ml^{-1}) at different conc. of L-tryptophan (µg ml^{-1})					Siderophore	HCN	Ammonia
	10	20	60	80	100			
Azotobacter chroococcum	6.5	9.3	12.5	12.9	23.6	+	+	+
Azotobacter beijerinkii	5.8	8.0	12.7	14.1	17.2	+	+	+
Pseudomonas aeruginosa	7.0	14.5	21.2	24.7	26.5	+	+	+
Bacillus sp	7.1	10.2	15.4	16.8	19.8	+	+	+

Values are average of three replicates; +, positive.

Table-2 Protein concentrations of *isolated* strains

Strains	OD 660nm	Conc. of protein (µg ml^{-1})
Azotobacter chroococcum	0.61	846
Azotobacter beijerinkii	0.47	680
Pseudomonas aeruginosa	0.52	708
Bacillus sp	0.38	505

Values are average of three replicates

Table-3 Shoot length of black gram treated with N_2- fixers and phosphate solubilizers

Days	Control	*Azotobacter chroococcum*	*Azotobacter beijerinkii*	*Pseudomonas aeruginosa*	*Bacillus* sp
2	3.2	3.6	3.5	3.5	3.4
4	7.3	8.2	8.1	8.1	7.9
6	13.8	14.6	14.3	14.5	14.2
8	21.2	26.8	26.4	26.2	26.3
10	29.0	31.6	31.2	31.4	30.8
12	36.5	38.7	38.5	38.5	38.1
14	43.6	49.3	49.0	48.9	46.8

Values are average of three replicates

Table-4 Root length of black gram treated with N_2- fixers and phosphate solubilizers

Days	Control	*Azotobacter chroococcum*	*Azotobacter beijerinkii*	*Pseudomonas aeruginosa*	*Bacillus* sp
2	2.1	2.5	2.3	2.3	2.4
4	3.9	4.8	4.6	4.7	4.8
6	6.3	7.1	7.2	7.0	6.9
8	9.0	9.6	9.8	9.0	8.6
10	12.2	13.1	13.4	12.8	12.3
12	16.8	18.3	18.0	18.1	17.8
14	19.6	22.5	22.1	22.0	21.8

Values are average of three replicates

Table-5 Inoculation effects of nitrogen-fixing and phosphate-solubilizing bacteria on P content in plants and N content in roots, shoots and straw of black gram plants

Treatment	P content (mg/g)	N content (mg/g)		
		Root	Shoot	Straw
Control	0.4	3.57	4.8	4.27
Azotobacter chroococcum	0.57	5.73	6.53	7.07
Azotobacter beijerinkii	0.53	3.7	6.93	6.53
Pseudomonas aeruginosa	0.6	3.73	5.0	5.83
Bacillus sp	0.49	3.57	4.8	4.27

Values are average of three replicates

Table-6 Inoculation effects of nitrogen-fixing and phosphate-solubilizing bacteria on mean dry mass of root, shoot, total plants and chlorophyll content of black gram

Treatment	Mean dry mass (g/plant)		Total dry mass (g/plant)	Total chlorophyll (mg/g of tissue)
	Root	Shoot		
Control	0.10	0.40	0.50	0.73
Azotobacter chroococcum	0.21	0.52	0.73	0.91
Azotobacter beijerinkii	0.17	0.45	0.62	0.82
Pseudomonas aeruginosa	0.20	0.5	0.70	0.93
Bacillus sp	0.13	0.47	0.60	0.80

Values are average of three replicates

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