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Synergistic effect of Nitrogen Fixing Bacteria (NFB) and Phosphate Solubilizing Bacteria (PSB) on *Cicer arietinum.*

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ABSTRACT

In the present research work, agricultural soil samples were taken for the isolation and characterization of phosphate solubilizing bacteria (PSB) and nitrogen fixing bacteria (NFB). Four isolates of PSB and NFB were obtained out of which two bacterial isolates (P3 and N2) were selected and characterized morphologically and biochemically. Strains P3 of PSB was selected because of its high solubilization efficiency (306.78%) on Pikovskaya's Agar medium. The effect of individual as well as combined inoculation of both bacterial strains on *Cicer arietinum* was studied with respect to Plant height, Shoot fresh weight per plant, Number of leaves per plant, Number of branches per plant and Number of nodules per plant. It was found that the co-inoculation of nitrogen fixing bacteria and phosphate solubilizing bacteria significantly increased the vegetative growth as well as nodulation in *C. arietinum* as compared to individual treatments and control.
Keywords : Nitrogen fixing bacteria, phosphate solubilizing bacteria, Pikovskaya Agar Medium, Coinoculation , *Cicer arietinum***.**

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INTRODUCTION

India is primarily an agricultural economy and is the largest producer and consumer of pulses in the World contributing about 25-28 per cent of total global production according to FAOSTAT, 2010. With the increasing population of the country as well as the World, there is a huge pressure on agricultural sector to supply feed to this population. The increasing demand for food supply can be met by increasing the yield and quality of food crop on the limited land resources. During most of the last century, emphasis was on increasing the crop productivity by addition of chemical fertilizers, pesticides, insecticides etc. But eventually, the effects of excessive use of chemicals started showing in the form of aggravation of the land and water pollution which came up as another burning problem. So during the latter half of the past century, efforts were made to come up with more eco-friendly alternatives to the chemicals including biofertilizers.

The improvement of soil fertility is one of the most common strategies to increase agricultural production which can be done by maintaining high levels of available nitrogen (N) and phosphorus (P), the two most limiting nutrients in soil. Nitrogen and phosphorus are two of the major elements which have been found to have an enormous effect on yield and quality of crop plants. The discovery of the role of the nitrogen fixing bacteria, both free livng (eg. *Azotobacter* spp, *Clostridium* spp. etc) as well as symbiotic (eg. *Rhizobium* spp*, Azorhizobium* spp etc) and phosphate solubilizing bacteria (eg *Bacillus* spp*, Pseudomonas* spp*.* etc) paved the way for use of these biofertilizers for crop improvement. Their individual as well as synergistic effect has been explored by a number of research groups [1-6]. In soils where native rhizobial population densities are low, Biological Nitrogen Fixation (BNF) can be enhanced by inoculation of legume seeds with an effective and persistent *Rhizobium* strain or any NFB. However, the process of nitrogen fixation is sensitive to P deficiency, which results in reduced nodule mass and low ureide production. P plays an important role in N cycling as adenosine triphosphate is required in large quantities for legumes to undergo N2 fixation [7-8]. Chickpea (*Cicer arietinum* L.) is one of the major pulse crops in the World and provides high quality protein for the people in South, West and East Asia and North Africa. It is also used as feed for livestock and has a significant role in farming systems [9]. In this regard, *Rhizobium* inoculation of chickpea seed may substitute costly N fertilizers in chickpea production [10]. In the current studies, synergistic effect of Nitrogen Fixing Bacteria (NFB) and Phosphate Solublizing bacteria (PSB) isolated from agricultural soils was studied on the vegetative growth of *Cicer aeritinum.*

METHODOLOGY

Soil samples were collected at a depth of 3-6cm from agricultural land. The samples were air-dried and mixed well to isolate bacterial strains. Pikovskaya agar media of pH 7 was prepared for the isolation of PSBs while Yeast Extract Mannitol Agar (YEMA) medium was used for the isolation of *Rhizobium.*

Appropriately diluted soil sample was spread plated on petriplates containing sterile medium. The plates were incubated for 2 days at 30±2ºC. On the basis of the zone of hydrolysis formed (in mm), four PSB strains was selected, purified by streaking and maintained on agar slants. Phosphate solubilizing efficiency of the individual isolates was calculated as:

Solubilizing Efficiency (%SE) = Z-C/C *100

Where Z is Solubilization Zone (in mm)
and C is Colony Diameter (in mm)

Similarly, four individual colonies of Nitrogen fixing bacteria were selected. Then primary culture was subcultured onto medium containing congo red at ±30ºC for 2 days and kept for further study and preserved at 4^{0} C for future use.

Characterization of Isolates

Morphological Characterization: The unknown cultures were characterized on the basis of colony morphology on agar medium, culture characteristics and staining reactions. The shape, surface, consistency, configuration, margin, elevation and color of the colonies; shape, motility, gram staining and endospore staining of isolates were studied.

Biochemical Characterization: The culture was subjected to various biochemical tests including catalase test, MR, VP, Indole Test, Nitrate Reduction, Citrate utilization, urea hydrolysis, starch hydrolysis and deaminase test.

Synergistic effect of co-inoculation of NFB and PSB on *Cicer arietinum*

On the basis of %SE, P3 isolate of PSB was selected for co-inoculation with randomly selected N2 strain of nitrogen fixing bacteria. Pot experiments were set up in order to study the effect of co-inoculation of NFB and PSB on *C. arietinum.* For combined inoculation, the 2 days old broth culture of both bacterial strains (P3 and N2) was mixed in equal proportions. Seeds were washed properly with tween-20 and soaked into mixed culture for 3-4 hrs. 1-2 seeds were sown in each pot containing sterilized soil. Seeds treated with only P3 and only N2 formed a separate set of trials. Seeds devoid of treatment with any microbial culture served as control. A set of 10 pots was set up for each treatment. The seeds were allowed to germinate into seedlings and plantlets and data for various parameters including plant height (PH), shoot fresh weight per plant (SFWPP), Number of leaves per plant (NLPP), Number of branches per plant (NBPP) and Number of nodules per plant (NNPP) were recorded.

RESULTS AND DISCUSSION

When the bacterial culture inoculated on Pikovskaya agar medium were incubated for 2 days at ±30ºC, colonies of PSB were detected by clear zones of solubilization around the colonies. P3 was found to have highest solubilization efficiency (306.78%) followed by P1 (295.83%, Table 1). Bacterial culture inoculated on YEMA medium were incubated for 2 days at ±30ºC temperature and colonies of NFB were detected by transparent white color and shining.

Table 1: Percent Solublization efficiency of Phosphate Solubilizing Bacterial Isolates

Strain	Zone of Solubilization (mm)*	Colony Diameter (mm)*	Solubilization efficiency (%)
P1	19	4.8	295.83
P2	12	4.6	160.89
P3	24	5.9	306.78
P4	ND	4.4	NC
CD (@5%)	0.63	0.59	-

*Average of three replicates
ND: Not Detected
NC: Not Calculated

Colony Characteristics

The growth, shape, surface, consistency, configuration, margin, elevation and colour of the colonies of all isolates PSB and NFB were observed (Results not shown). Isolate P3 formed cream colored, rapid growing, circular, convex and viscous colonies with a zone of phosphate solubilization around them. Similar colony characteristics on pikovskaya agar medium was studied previously by Ranjan *et al* who found a zone of phosphate solublization around the bacterial culture [11]. Isolate N2 formed rapid growing, cream colored, circular/ irregular, smooth shiny and viscous colonies. Similar results were obtained by Kucuk *et al* for colony characteristics of NFB on YEMA medium [12].

Biochemical Characterization

Table 2 shows the results of various biochemical tests obtained for all the isolates. Isolate P3 was found to be Gram positive forming, non motile, endospore forming rod which gave positive test for citrate utilization and deaminase but was negative for indole production, nitrate reduction, MRVP test, catalase production, urea hydrolysis and starch hydrolysis. Isolate N2 was gram negative, non ensospore forming, non motile rod which was positive for nitrate reduction, VP, catalase production, and starch hydrolysis while the rest of the tests were negative.

Table 2: Morphological and Biochemical characteristics of bacterial isolates.

Characteristic	P1	P2	P3	P4	N1	N2	N3	N4
Shape	Rod	Rod	Rod	Coccus	Spiral	Rod	Rod	Rod
Gram Staining	+	+	+	+	-	-	-	+
Endospore staining	-	+	+	-	-	-	-	-
Motility	-	+	-	-	-	-	-	-
Indole test	-	-	-	+	-	-	-	-
Citrate utilization test	+	+	+	-	+	-	-	-
Nitrate reduction test	+	+	-	+	-	+	-	+
Methyl red test	+	+	-	-	-	-	+	+
Voges-Proskauer Test	-	-	-	-	+	+	-	-
Catalase test	-	-	-	+	+	+	+	+
Deaminase test	-	-	+	+	+	-	-	-
Urea Hydrolysis	-	-	-	-	-	-	+	-
Starch Hydrolysis	+	+	-	+	+	+	-	-

+: Positive Result, -: Negative Result

Synergistic effect on *C. aeritinum*

Effect on Seed Germination

After three days of sowing (DAS), increased seed germination as well as seed growth was observed in P3 and N2 treated seeds as compared to control. Seed germination took 70hrs for control, 48hrs for single isolate treatment (P3 and N2 alone) and about 40hrs for co-inoculation treatment. The seeds were allowed to germinate into seedlings and plantlets and data for various parameters was recorded and documented.

Effect on Vegetative Growth

The number of leaves and plant height were found to be more efficient for single or mixed isolate treated seeds as compared to control after 45 DAS. Both separate and combined application of inoculation and P significantly improved shoot fresh weight plant^{-1} (SFWPP) and number of branches plant^{-1} (NBPP) of chickpea. Very few nodules could be seen after 30 DAS even in the presence of inoculum. This could be due to the relatively long time required for BNF to begin, which usually takes place between 2-5 weeks after planting [13]. In contrast, inoculation improved nodulation significantly at 45 DAS (Table 3). During the beginning of formation, the nodules were very small, pale and white but by 45DAS, the nodules became bigger and pinkish indicating the presence of leghaemoglobin in them.

Table 3: Effect of inoculation of N2 and P3 on *Cicer arietinum*

Parameters	15 DAS				30 DAS				45 DAS				CD (@5%)
PH (cm)*	1.8	2.2	2.2	2.5	12.6	15.7	14.1	19.4	39.0	43.2	41.1	43.5	A: 0.56 B: 0.48 A*B:0.97
SFWPP (g)*	2.1	2.3	2.3	2.5	4.0	5.2	4.9	5.5	6.9	8.3	8.6	9.1	A: 0.25 B: 0.22 A*B: 0.44
NLPP	25	34	30	45	92	99	104	115	174	207	198	234	A: 6.33 B:5.48 A*B: 10.97
NBPP	Nil	0.6	Nil	2	2	3	3	3	7	9	9	11	A: 0.70 B: 0.60 A*B: 1.22
NNPP	Nil	Nil	Nil	2	7	10	11	10	15	43	43	52	A: 1.86 B: 1.60 A*B:3.22

PH: Plant Height; SFWPP: Shoot Fresh Weight Per Plant; NLPP: Number of Leaves Per Plant; NBPP: Number of Branches Per Plant; NNPP: Number of Nodules Per Plant

*Average of three replicates, A: Inoculum Treatments, B: No of Days after sowing

A positive and significant response of legume nodulation to inoculation was also reported by others [14-16] in Kenya, Iran and India respectively. Similar to the effect of inoculation, P application did not improve nodulation of chickpea in the early stage of plant growth, i.e. at 30 DAS. This could be related to the fact that P dissolution takes about 3-6 weeks. Nonetheless, the application of P resulted in a highly significant improvement of nodulation in the later stages (45 DAS). Such positive effects of P on legume nodulation have also been reported by several other studies conducted on chickpea, haricot bean and faba bean in Ethiopia, Tanzania and Pakistan [17-19]. The role of inoculation in enhancing nitrogen fixation and thereby increasing N uptake by plants contributed to improved vegetative growth of chickpea. On the other hand, the poor shoot weight recorded from the control treatment might be due to the low population of native rhizobia of soil. Such positive response of chickpea shoot development to inoculation and P fertilization was also reported elsewhere [16, 20].

CONCLUSION

The current studies showed that the co-inoculation of NFB and PSB have a positive and significant effect on the vegetative growth of chickpea which can result in better crop productivity.

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