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Synergistic effect of plant growth promoting bacterium *Pseudomonas fluorescens* and phosphate solubilizing fungus *Aspergillus awamori* for growth enhancement of chickpea

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Many rhizosphere microorganisms solubilize the fixed phosphorus present in soil and make it available to the plants and also contribute towards better growth and yield of plants through other direct and indirect plant growth promoting activities. This communication deals with synergistic effect and compatibility of two rhizosphere microorganisms, *Pseudomonas fluorescens* BAM-4 and *Aspergillus awamori* S-19 *in vitro* and *in planta* in chickpea. BAM-4 and S-19 solubilized 354.41 and 361.12 mg kg^{-1} of P *in vitro*, respectively. BAM-4 also showed indole acetic acid (IAA) production and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity. Under pot culture conditions, the overall plant growth in all treatments assayed increased significantly over the untreated soil. The length and fresh and dry weight of plants were significantly higher in co-inoculation treatment by BAM-4 and S-19 as compared to single inoculation of either microorganism, showing the positive synergistic effect. The number of pods and weight of pods per plant were maximum in soil + BAM-4 + S-19 treatment with and/or without TCP. Maximum total chlorophyll content was 4.28 mg g^{-1} fresh weight in dual inoculation treatment with TCP. The results indicated the potential usefulness of co-inoculation by rhizosphere bacteria and fungi in stimulation of plant growth and yield in chickpea for sustainable environment and agriculture.

Keywords: Biofertilizers, Bioinoculants, Bioremediation, *Cicer arietinum*, PGPR, Phosphate solubilizing fungi (PSF), Rhizosphere bacteria and fungi, Seed bacterization

Excessive applications of synthetic fertilizers in agriculture may contaminate the soil and waterways. The high solubility of chemical fertilizers also exacerbates their tendency to degrade ecosystems, particularly through eutrophication. Therefore, there is a need to look for efficient substitutes and biofertilizers can be a viable alternative. The soil of rhizosphere is populated by a diverse array of microorganisms. Due to their inhabitation in rhizosphere soil, these multiple microbial communities interact and colonize with plant roots, and produce plant beneficial substances[1]. Most of the plants survive by releasing root exudates of precise chemical composition to activate their friendly soil fungi and bacteria, which will, in turn, make certain elements available to the plant at that time. Certain microorganisms such as phosphate solubilizing bacteria and fungi associated with the plant rhizosphere are known to convert insoluble inorganic phosphorus (P) into a soluble form that could be utilized by the plants.

Plant growth promoting rhizobacteria (PGPR) can improve plant growth and soil fertility by different mechanisms such as increased mobilization of insoluble nutrients and subsequent enhancement of nutrient uptake by the plants, production of plant hormones, and biocontrol of phytopathogenic organisms. *Pseudomonas* is a widespread bacterial genus in agricultural soils and has many traits that make it well-matched as PGPR[2]. Many plant–associated strains of *Pseudomonas* promote the plant growth by suppressing pathogenic microorganisms, synthesizing growth–stimulating hormones and promoting increased plant disease resistance[3-6]. Similarly, the beneficial effect of phosphate solubilizing fungi (PSF) on various crops has been demonstrated with many species of *Aspergillus*[7]. *A. awamori* is one of the commonly used bioinoculants cited in the literature for agricultural crops such as cowpea, chickpea, fenugreek, mulberry and others[8-13].

A substantial amount of literature is available on interactions of microbes with vesicular-arbuscular

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mycorrhiza[14-15]. However, researchers on the synergistic effect of a PGPR co-inoculated with a PSF on plant growth enhancement are scarce. Therefore, for agronomic utility, assays of seed bacterization by mixed cultures of PGPR and PSF are necessary to take advantage of their beneficial properties for plant yield enhancement. Chickpea (*Cicer arietinum* L.) is the world's third most important food legume and a good source of protein in many countries. South and Southeast Asia contribute about 81% to the global chickpea production, with India as the principal chickpea producing country (84% share in the region). The chickpea area slightly increased from 9.1 to 9.5 million ha during the last three decades.

The objective of the present study was to determine the effect of co-inoculation of a PGPR strain, *Pseudomonas fluorescens* BAM-4 and PSF strain *Aspergillus awamori* S-19 *in vitro* and *in vivo* on chickpea crop in semi-arid climatic conditions for their potential use in agriculture field as bioinoculants.

Materials and Methods

Bioinoculants and fermentation media

Pseudomonas fluorescens BAM-4 and *Aspergillus awamori* S-19 were selected as bioinoculants for the study. These were previously isolated from the rhizosphere of agricultural crops grown in semi-arid soil of Banasthali (India) and identified by authors' research group and partial sequences submitted at National Center for Biotechnology Information (NCBI). The accession numbers obtained from NCBI are FJ887893 and HQ891667 for *P. fluorescens* BAM-4 and *A. awamori* S-19, respectively.

The growth media used were nutrient broth and potato dextrose broth for bacterial and fungal cultures. They were maintained on agar slants at 4°C, subcultured every month, and their stability was periodically checked. Whereas, the production medium used for phosphate solubilization consisted of Pikovskaya (PVK) broth with tricalcium phosphate (TCP), 0.2%.

Plant material and seed disinfection

Seeds of chickpea (*Cicer arietinum* L. var. RSG-9631) were obtained from Krishi Vigyan Kendra (KVK) of Banasthali University. The seeds were surface disinfected by immersing in 70% ethanol and then 3% sodium hypochlorite for three minutes each, followed by repeated rinsing with sterile distilled water, dried and used for sowing. All the steps were carried out under aseptic conditions in laminar flow (LF) clean bench.

Phosphate solubilization in PVK broth

Phosphate solubilization was checked using TCP as insoluble phosphate. Erlenmeyer flasks (250 mL) with 100 mL of modified PVK medium containing TCP (0.2%)[16,17] were prepared in triplicate, inoculated with 1 mL of bacterial/ fungal culture adjusted to 10^8 cfu mL^{-1}/5×10^5 cfu mL^{-1} and incubated at 30°C and 130 rpm on a rotary incubator shaker for 9 days. Autoclaved, uninoculated medium served as control. The estimation of soluble P in the culture supernatant was done every 48 h by the molybdenum-blue method[18]. In each experiment, the pH was recorded using glass electrode pH meter.

Growth promoting activities of *Pseudomonas* strain

Production of indole acetic acid (IAA) by *Pseudomonas fluorescens* strain was estimated according to the method of Gordon and Weber[19], whereas ACC (1-aminocyclopropane-1-carboxylate) deaminase assay was done by using the method of Penrose and Glick[20]. To study the production of ammonia, the test strain was grown in peptone water broth at 37±2°C for 4 days. The accumulation of ammonia was detected by addition of Nessler's reagent at the rate of 1 mL per tube. Yellow colour indicated the production of ammonia. The siderophore production was determined by performing the chrome azurol sulfonate (CAS) agar diffusion assay. Culture filtrate was tested for identification of hydroxamate and catechol types of siderophores following the standard method of Schwyn and Neiland[21].

The bacterial strain was also tested for its sensitivity towards various antibiotics at a concentration of 30 µg $disc^{-1}$ by disk diffusion assay. Plates having a zone of inhibition around the antibiotic discs showed that the strain was sensitive to the respective antibiotic. The diameter of inhibition zone (in cm) was noted.

In vitro effect on seed germination and vigour index

To see the *in vitro* effect on seedling germination and growth, surface sterilized chickpea seeds were immersed in fungal and bacterial culture broths for 30 min and placed on solid agar plates at the rate of 10 seeds per plate. The experiment was done in triplicate, and untreated seeds were maintained as a control. All the plates were incubated at 28±2°C, and 37±2°C for *Aspergillus* and *Pseudomonas* strains, respectively and observations were made at 2 and 7 days for length of radical and plumule. The vigour index was calculated by following formula[22]:

Vigour index = (Mean root length + Mean shoot length) × Seed germination (%).

Soil-plant experiment

For seed pelleting, the inoculum was prepared by inoculating a single colony of bacterial isolate BAM-4 and fungal isolate S-19 in 50 mL of nutrient and potato dextrose broth, respectively and incubated at 30±2°C in an orbital shaker. The concentration was adjusted approximately at 10^7 cells or spores mL^{-1}. Sterilized jaggery slurry was prepared in distilled water and mixed with both bacterial and fungal inocula as an adhesive agent. The sterilized chickpea seeds were dipped in single and mixed cultures of bacteria and fungi.

The experiment was conducted in earthen pots using unsterile field soil (sandy loam, pH 8.3, electric conductivity 0.28 dSm^{-1}, organic matter 0.78%, K 280 kg ha^{-1}). The soil contained 4.98 kg ha^{-1} available-P which falls under the low category. The soil was thoroughly mixed and sieved (mesh, 2 mm) to remove large particulate matter, then 1 g insoluble TCP per kg soil mixture was prepared, and pots were filled with 6 kg of this soil-phosphate mixture. Single super phosphate (SSP) and a commercially available phosphatic biofertilizer named Biogold (National Organic Fertilizer, India) were also taken to compare the results. There were a total of 11 treatments and three pots for each treatment in the experiment: (1) soil; (2) soil + TCP; (3) soil + SSP; (4) soil + SSP + TCP; (5) soil + Biogold; (6) soil + BAM-4; (7) soil + BAM-4 + TCP; (8) soil +S-19; (9) soil +S-19 + TCP; (10) soil +BAM-4 + S-19 and (11) soil +BAM-4 + S-19 + TCP.

At the time of sowing, the soil from 20 mm depth was removed from earthen pots and nine seeds, rolled in a mixture of jaggery slurry and inoculum, were placed at equal distance. The seeds were then covered with a uniformly spread 20-mm thick soil layer. After an hour, the pots were sprinkled with water. Water was added periodically to maintain soil moisture during the experiment. After 1 week of germination, plants were thinned to five in number per pot.

Plants were harvested after 90 days of sowing and their shoot and root lengths and weights and pod number and weight were recorded. For measuring shoot and root dry weight, the samples were dried in an oven for 48 h at 60°C. Besides, chlorophyll-a, -b and total chlorophyll contents were also estimated by the method of Arnon[23].

Statistical analysis

Data obtained by *in vitro* experiments are expressed as the mean ± SE of 3 independent replicates, whereas *in vivo* experiments were subjected to Analysis of Variance (ANOVA) followed by LSD and post hoc multiple comparison tests were done using SPSS (version 16.0). The values of $P < 0.05$ were considered as statistically significant.

Results

***In vitro* phosphate solubilization**

Phosphate solubilization and the change in pH by *P. fluorescens* BAM-4 and *A. awamori* S-19 in PVK broth were recorded up to 9 days (Table 1) and the maximum soluble phosphorus *i.e.* 354.4 and 361.1 mg kg^{-1}, respectively was found on the 3rd day of incubation. In the case of *P. fluorescens*, there was a steep increase of soluble P on the 1st day, which further increased up to 3rd day of incubation but thereafter a sharp decline was observed. After that a slight increase was recorded, whereas *A. awamori* showed almost constant amount of soluble P ranging from 330 to 361 mg kg^{-1} from first day to the end of the experiment. Co-inoculation of BAM-4

Table 1 — Solubilization of insoluble TCP and variations in pH in PVK broth by bioinoculation

Bacterial and fungal strains	Days after inoculation											
	0		1		3		5		7		9	
	SP (mg kg^{-1})	pH	SP (mg kg^{-1})	pH	SP (mg kg^{-1})	pH	SP (mg kg^{-1})	pH	SP (mg kg^{-1})	pH	SP (mg kg^{-1})	pH
Control	91.2± 5.3	6.2± 0.05	90.7± 4.7	6.1± 0.1	90.1± 6.2	6.3± 0.05	92.2± 5.2	6.3± 0.11	88.2± 4.1	6.4± 0.11	91.1± 4.5	6.4± 0.05
Pseudomonas fluorescens BAM-4	98.4± 5.0	6.2± 0.05	328.5± 1.5	**3.8± 0.05**	**354.4± 3.3**	4.5± 0.35	93.0± 5.1	6.5± 0.20	114.8± 0.9	6.1± 0.23	127.2± 3.7	6.0± 0.15
Aspergillus awamori S-19	93.5± 4.9	6.2± 0.05	330.5± 16.9	4.0± 0.05	**361.1± 3.7**	4.0± 0.37	341.9± 0.9	**3.7± 0.17**	348.4± 3.2	4.4± 0.4	330.5± 2.6	4.6± 0.47
P. fluorescens BAM-4 + *A. awamori* S-19	100.8± 0.9	6.2± 0.05	340.1± 4.9	4.0± 0.05	**370.3± 1.5**	4.0± 0.37	250.2± 0.9	**3.7± 0.17**	224.6± 5.1	4.0± 0.05	246.0± 4.5	6.0± 0.15

[Values are the mean of replicates ± SD, SP- soluble phosphorus. Lowest pH and maximum SP values are shown in bold]

Table 2 — Indole acetic acid (IAA) production (μg mL^{-1}) by *P. fluorescens* BAM-4 in the presence of different concentrations of tryptophan

IAA	Tryptophan (mg mL^{-1})						
	0	0.5	1.0	1.5	2.0	2.5	3.0
Day 1	9.2 ± 0.7	15.4 ± 0.5	17.0 ± 0.3	21.4 ± 0.3	23.4 ± 0.4	25.4 ± 0.4	28.2 ± 0.3
Day 2	17.0 ± 0.3	17.8 ± 0.2	19.3 ± 0.4	22.1 ± 0.2	24.6 ± 0.3	28.2 ± 0.3	**31.4 ± 0.5**
Day 3	15.4 ± 0.5	17.0 ± 0.3	19.1 ± 0.2	21.6 ± 0.5	23.6 ± 0.4	25.1 ± 0.2	30.4 ± 0.5

[Values are the mean of replicates ± SD, Highest value shown in bold]

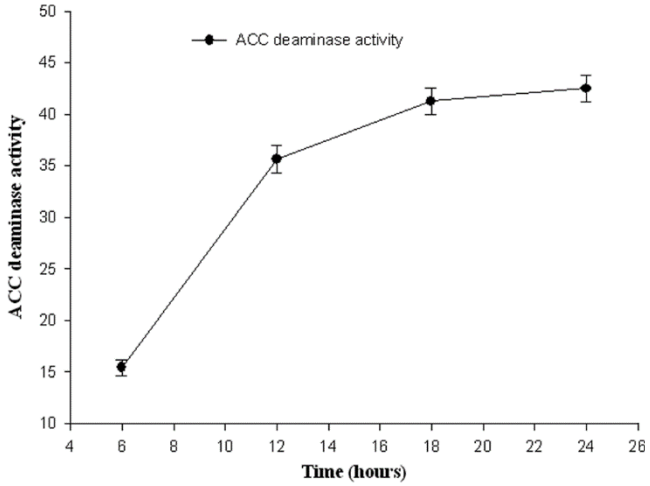

Figure 1 — ACC (1-Aminocyclopropane-1-Carboxylate) deaminase activity of *P. fluorescens* BAM-4 expressed as μM α ketobutyrate mg^{-1} protein h^{-1}, Bars indicate standard error.

and S-19 showed the best P solubilization as compared to single inoculations on 3rd day *i.e.* 370.3 mg kg^{-1} to decrease slightly thereafter and then it remained almost constant. The corresponding pH drop was also observed with phosphate solubilization in both the cultures, when taken either separately or in combination, reaching the lowest pH values of 3.8 for BAM-4 and 3.7 for both S-19 strain and co-inoculation treatment.

PGPR activities of *Pseudomonas fluorescens*

P. fluorescens BAM-4 exhibited healthy plant growth promoting traits. It showed an orange halo zone around the colonies after 48 h of incubation on CAS agar plate which indicated the production of siderophore which was of hydroxamate type. The strain was also found to be positive for ammonia production. The plant growth-promoting characteristics like IAA production and ACC deaminase activity were determined quantitatively. It produced IAA with a maximum of 31.35 μg mL^{-1} on the 2nd day of incubation in the presence of 3 mg mL^{-1} of tryptophan, and then it declined slightly on the 3rd day (Table 2). ACC deaminase activity of the isolate increased from 15.22 to 42.14 μM α ketobutyrate mg^{-1} protein h^{-1} between 6 and 24 h of incubation (Fig. 1).

Table 3 — Intrinsic antibiotic resistance profile of *P. fluorescens* BAM-4

Antibiotics	Diameter of inhibition zone (mm)
Cephalxin	18
Chloramphenicol	30
Ciprofloxacin	32
Erythromycin	30
Gentamicin	32
Kanamycin	32
Nalidixic acid	16
Neomycin	28
Norfloxacin	32
Streptomycin	30
Tetracyclin	30

The strain also showed strong antibiotic resistance profile against 11 antibiotics out of 15 checked during the study (Table 3).

Effect of bio-inoculation on chickpea in soil-plant system

Both the strains selected for the study showed significant *in vitro* enhancement in length of radical and plumule and vigour index over control as depicted in Table 4. Table 5 shows the results of length and weight of root and shoot by single and dual inoculations. Seed bacterization of chickpea with both strains, singly or in combination, significantly enhanced the development of root and shoot after 90 days of sowing as compared to un-inoculated control. In almost all the treatments the addition of TCP proved to be good for enhancement of growth parameters. The length and fresh and dry weights were significantly higher in dual inoculation with or without TCP as compared to single inoculation of two strains. It can be inferred from the results that *Pseudomonas* strain acted synergistically with *Aspergillus* strain and effectively promoted the growth of chickpea. SSP addition also showed substantially higher length and weight than control and was more or less equivalent to single inoculations in some parameters.

Similarly, the crop yield significantly increased when BAM-4 and S-19 strains were inoculated together with or without TCP in comparison to control

Table 4 — *In vitro* effect of bioinoculation on radical and plumule length in chickpea

	Plumule length (cm)		Radical length (cm)		Vigour Index
	After 2 days	After 7 days	After 2 days	After 7 days	
Control	1.25±0.43	5±0.71	1.5±0.5	3.25±0.43	570.6
P. fluorescens	3.3±0.19* (164)	10.88±0.08* (117.6)	6.35±0.11* (323.3)	6.15±0.11* (89.2)	2118.7* (271.1)
A. awamori	3.1±0.17* (148)	7.75±0.43* (55)	6.1±0.1* (306.7)	11.9±0.1* (283.9) (90.2)	2073.5* (263.4)

[Values are the mean replicates ± SE; * Significantly different than respective uninoculated control according to LSD (P <0.05). Values in bracket indicate percent increase over control]

Table 5 — Effect of co-inoculation of *P. fluorescens* and *A. awamori* on length and weight of root and shoot of chickpea plants in pot trial.

Treatments	Shoot length (cm)	Shoot weight (g)		Root length (cm)	Root weight (g)	
		Fresh	Dry		Fresh	Dry
Soil (UC, uninoculated control)	22.5±1.80g	0.47±0.09e	0.39±0.08d	6.80±0.10c	0.038±0.010c	0.021±0.003e
UC + TCP	24.53±0.61fg (9.0)	0.65±0.09d (38.3)	0.39±0.03d (0.0)	7.90±1.37bc (16.2)	0.056±0.008bc (47.4)	0.024±0.001de (14.3)
UC + SSP	26.16±1.75ef (16.3)	0.72±0.11cd (53.1)	0.49±0.02bc (25.6)	8.43±1.53bc (23.9)	0.059±0.006bc (55.3)	0.023±0.002de (9.5)
UC + SSP + TCP	28.2±2.8e (25.3)	0.86±0.13abc (82.9)	0.59±0.06a (51.3)	8.23±0.83bc (21.0)	0.055±0.006bc (44.7)	0.025±0.002de (19.0)
Soil + Biofertilizer	33.53±1.15cd (48.9)	0.72±0.04cd (53.1)	0.52±0.07abc (33.3)	7.83±0.30bc (15.1)	0.057±0.003bc (50.0)	0.028±0.003d (33.3)
Soil + BAM-4	32.5±1.64d (44.4)	0.76±0.11cd (61.7)	0.56±0.05ab (43.6)	8.53±0.35bc (25.4)	0.060±0.007bc (57.9)	0.037±0.001c (76.2)
Soil + BAM-4 + TCP	34.6±0.87bcd (53.8)	0.78±0.13bcd (65.9)	0.58±0.05a (48.7)	8.73±0.56b (28.4)	0.076±0.039b (100.0)	0.038±0.003c (80.9)
Soil + S-19	34.4±2.19bcd (52.9)	0.96±0.15ab (104.3)	0.46±0.09cd (17.9)	9.13±0.55ab (34.3)	0.07±0.018b (84.2)	0.037±0.001c (76.2)
Soil + S-19 + TCP	36.8±1.18bc (63.6)	0.88±0.06abc (87.2)	0.56±0.03ab (43.6)	9.43±0.41ab (38.7)	0.065±0.025bc (71.1)	0.046±0.003ab (119.0)
Soil + BAM-4 + S-19	37.23±2.41b (65.4)	0.96±0.02ab (104.3)	0.59±0.02a (51.3)	10.7±1.75a (57.4)	**0.113±0.005a (197.4)**	**0.049±0.004a (133.3)**
Soil + BAM-4 + S-19 +TCP	**42.06±2.10a (86.9)**	**0.99±0.05a (110.6)**	**0.60±0.01a (53.8)**	**10.83±0.40a (59.3)**	0.108±0.008a (184.2)	0.042±0.003bc (100)
Std. Error	1.162	0.061	0.031	0.598	0.010	0.002
LSD	3.21	0.168	0.085	1.65	0.027	0.005

[Values are the mean of replicates ± SD. Data were subjected to analysis of variance (ANOVA) using LSD and post-hoc multiple range test at 5% level of significance ($P \leq 0.05$). Values in the parentheses indicate percentage increase over control. Data in bold show highest values. TCP: tricalcium phosphate; SSP: single super phosphate; BAM-4: *Pseudomonas fluorescens*; S-19: *Aspergillus awamori*]

and other treatments as illustrated in Table 6. Number of pods per plant was maximum (*i.e.* 3.66) in co-inoculation of BAM-4 and S-19 treatment (with or without TCP), whereas weight of pods per plant was highest (*i.e.* 0.86 g) in Soil + BAM-4 + S-19 + TCP, followed by same treatment in the absence of TCP without showing any significant difference. As regarding the maximum total chlorophyll content, it was 4.28 mg g^{-1} fresh wt. in dual inoculation with TCP and 4.22 mg g^{-1} fresh wt. without TCP indicating increased photosynthetic rates. The increase was significant compared to the control and S-19 single inoculation. Maximum chlorophyll-a and -b were 2.47 and 1.78 mg g^{-1} fresh wt. in BAM-4 + S-19 + TCP and BAM-4 + S-19 treated soil, respectively (Table 6). For chlorophyll-a, all the treatments showed significant increase over the control but gave non-significant differences among the treatments.

Discussion

A few bacteria and fungi are known for solubilizing the fixed soil phosphorus and make it available to the plants. However, before carrying out *in situ* experiments, the compatibility between the two associate members needs to be checked *in vitro*. In our study, both the selected strains were found as potential

Table 6 — Effect of co-inoculation of *P. fluorescens* and *A. awamori* on number of pods per plant, weight of pods per plant and chlorophyll content of chickpea plant in pot trial

Treatment	Number of pods per plant	Weight of pods per plant (g)	Chlorophyll content (mg g^{-1} fresh wt.)		
			Chlorophyll a	Chlorophyll b	Total
Soil (UC, uninoculated control)	1.333±0.577d	0.216±0.060d	1.962±0.39b	1.246±0.045b	2.887±0.083c
UC + TCP	1.666±0.577cd (24.9)	0.228±0.051d (5.6)	2.292±0.05a (16.8)	1.278±0.246b (2.6)	2.933±0.816c (1.6)
UC + SSP	2.333±0.577bcd (75.0)	0.270±0.08cd (25)	2.299±0.080a (17.2)	1.374±0.545ab (10.3)	3.566±0.414abc (23.5)
UC + SSP + TCP	2.000±0.00bcd (50.0)	0.441±0.113bc (104.2)	2.387±0.103a (21.7)	1.389±0.119ab (11.5)	3.942±0.109ab (36.5)
Soil + Biofertilizer	2.333±0.577bcd (75.0)	0.386±0.097cd (78.7)	2.386±0.051a (21.6)	1.572±0.161ab (26.2)	3.827±0.090ab (32.6)
Soil + BAM-4	2.333±0.577bcd (75.0)	0.433±0.110bc (100.5)	2.374±0.038a (20.9)	1.562±0.195ab (25.4)	3.559±0.340abc (23.3)
Soil + BAM-4 + TCP	2.666±0.577abc (100)	0.570±0.066b (163.9)	2.411±0.032a (22.9)	1.737±0.044a (39.4)	3.946±0.218ab (36.7)
Soil + S-19	2.333±0.577bcd (75.0)	0.585±0.088b (170.8)	2.336±0.086a (19.1)	1.610±0.260ab (29.2)	3.348±0.058bc (15.9)
Soil + S-19 + TCP	2.666±0.577abc (100)	0.582±0.086b (169.4)	2.447±0.186a (25.4)	1.746±0.062a (40.1)	4.051±0.863ab (40.3)
Soil + BAM-4 + S-19	**3.666±0.577a (175.0)**	0.814±0.167a (276.9)	2.457±0.059a (25.2)	1.751±0.014a (40.5)	4.224±0.249a (46.3)
Soil + BAM-4 + S-19 +TCP	**3.666±0.577a (175.0)**	**0.862±0.045a (299.1)**	**2.474±0.050a (26.1)**	**1.788±0.178a (43.5)**	**4.284±0.066a (48.4)**
Std. Error	0.395	0.061	0.111	0.143	0.262
LSD	1.091	0.168	0.306	0.395	0.724

[Values are the mean of replicates ± SD; Data were subjected to analysis of variance (ANOVA) using LSD and post-hoc multiple range test at 5% level of significance ($P \leq 0.05$). Values in the parentheses indicate percentage increase over control. Data in bold shows highest values. TCP: tricalcium phosphate; SSP: single super phosphate; BAM-4: *Pseudomonas fluorescens*; S-19: *Aspergillus awamori*]

solubilizers of inorganic phosphate for growth enhancement of chickpea, which is similar to the findings on groundnut by Bhatia *et al*.[24] and on mung beans by Jain *et al*.[13] and Minaxi and Saxena[5], who reported the strains of Pseudomonads and Aspergilli as one of the best phosphate solubilizers. Both of these strains were found compatible for P solubilization when co-inoculated *in vitro* and the soluble P in a medium was either more or at par with the single inoculations. The decline in pH of the medium was observed throughout the experiment which supported the mechanism of P solubilization by the production of organic acid(s) by these microbes[25]. In soil, these organic acids can either dissolve the phosphorous directly by lowering the pH of the soil, which can help in ion exchange of PO_4^{-2} by acid ions, or they can chelate heavy metal ions and release associated phosphorous with them[26,27]. Earlier, *A. awamori* S-19 has been validated for production of malic, fumaric, succinic, citric and oxalic acids; malic acid being the major organic acid[28].

Inoculation of vegetable and cereal plants with PGPR strains has been found to show a wide range of effects on plant growth that varied with different strains and different hosts[29]. BAM-4 strain showed many plant growth promoting traits. It produced IAA which concurs with the earlier reported observations suggesting induction of IAA production in the presence of tryptophan[30]. IAA biosynthesis and secretion have been correlated with stimulation of root proliferation by rhizosphere bacteria, which enhanced uptake of nutrients by the associated plants. A variety of bacteria belonging to different genera such as *Pseudomonas*, *Agrobacterium*, *Arthrobacter*, *Klebsiella*, *Mycobacterium* and *Streptomyces* have been reported to produce IAA and aid in plant growth[31-34]. As for ACC deaminase activity, our result corroborates with Saravana Kumar and Samiyappan[35] who reported 342 nM α-ketobutyrate mg^{-1}, protein h^{-1} by *P. fluorescens.* The role of ACC deaminase in decreasing ethylene levels by the enzymatic hydrolysis of ACC into α-ketobutyrate and ammonia has been documented as one of the major mechanisms of PGPR in promoting root and plant growth[20,36].

Bacterization of chickpea seeds with BAM-4 and S-19 showed enhanced vegetative growth in terms of root

and shoot length and weight. These two strains also showed positive effect on radical and plumule length in *in vitro* experiments. In green gram (*Vigna radiata*), the inoculation with different phosphate solubilizing bacteria (PSB) such as *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia* and *Xanthomonas* resulted in higher nodule number, nodule dry weight, shoot dry matter and total dry matter. The majority of PSB were able to improve growth parameter of green gram was significantly compared to rock phosphate control and single superphosphate control[37]. Similarly, seed inoculation of cowpea (*Vigna unguiculata*) by *Gluconacetobacter* sp. and *Burkholderia* sp. helped in improved nodulation, root and shoot biomass, straw and grain yield and phosphorous and nitrogen uptake of crops. Out of these, best results were shown by *Burkholderia* sp.[38]. Enhancement of crop yield by inoculation with PGPR strains has also been observed in pot and field experiments by Ozturk *et al*.[39] and Cakmakci *et al*.[40] in wheat and other plants. In another study, BAM-4 was found to have high potential as biocontrol agent[5]. It was observed that *Pseudomonas* BAM-4 caused hyphal deformities such as fragmentation, lysis, and swelling of mycelia of plant pathogen *Macrophomina phaseolina* which may be due to the production of antifungal secondary metabolites, siderophores and chitinase enzyme.

Dual inoculations showed increase in total chlorophyll and both chlorophyll-a and -b contents which may positively affect the photosynthesis. Bacon and White[41] were of the view that interactions between host and microbes lead to physiological changes and translocation of sugars resulting in changes in photosynthetic rates of leaves and their metabolic activities. The significant increase in plant growth and chlorophyll content was observed by Singh and Siddiqui[42] when biocontrol agents such as *A. awamori*, *B. subtilis* and *P. fluorescens* were inoculated separately and in consortium in diseased tomato plants. Jha *et al*.[43] showed equal or more leaf area in seed bacterized plants than SSP treated ones. They also found more photosynthetic yield in bacterial treated moong bean plants, which is directly related to leaf area[43].

Interaction of PSB with other microorganisms has been reviewed by Khan *et al*.[44] There are several studies on co-inoculation of PSB and *Rhizobium*[45,46], PSB and non-symbiotic bacteria[47,48] and PSB and vesicular arbuscular mycorrhiza[49-51]. However, the synergistic effect of P-solubilizing bacterial and fungal species has been seldom discussed. Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea was demonstrated by Zaidi *et al*.[1] and Mittal *et al*.[10]. In India, a microbial preparation, Indian Agricultural Research Institute (IARI) microphos culture, a carrier based inoculant of *Aspergillus awamori, Bacillus polymyxa* and *Pseudomonas striata* is used as commercial biofertilizer with positive effect on growth and yield of different plants[52]. Dubey and Billore[53] showed an increase in yield of legumes after inoculation with rock phosphate and P-solubilizing bacteria, *Bacillus megaterium* and *Pseudomonas striata* and P-solubilizing fungus, *A. awamori* and suggested the use of low-grade rock phosphate for both neutral and alkaline soils with P-solubilizing inoculants. The two microbial strains selected for the present study showed synergistic effect and the growth and yield of chickpea plants was significantly higher than when either is used separately.

It can be concluded that *Pseudomonas fluorescens* BAM-4 and *Aspergillus awamori* S-19 are good phosphorus solubilizers for leguminous crops when used separately as well as in combination. They are compatible with each other and show positive synergism towards growth and yield of chickpea plants. Thus, these rhizosphere microorganims can be exploited as co-inoculants for better crop productivity and have potential to be developed as biofertilizer after confirmational field studies, which are in the offing.

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