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Effect of PSB on Growth and Development of Chilli and Maize Plants

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Abstract: The objectives of this research was isolation and characterization of PSB from elephant dung sample and evaluate their potential for P solubilization from insoluble P compounds for the effective plant growth promotion. In the present study, four different types of PSB were isolated and screened for their P solublization. It was observed that out of four isolates screened, the isolate PSB4 was the most potential isolate and it was used for further experimental analysis. The selected isolate was mass cultured and inoculated in maize and chilli plants to test their efficiency on the growth and biomass production. The strain was identified to be members of *Pseudomonas* sp, by 16S rDNA sequence analysis and biochemical tests. At the end of incubation time, it appeared that, phosphate solubilization resulted from a combined effect of pH decrease of the media and organic acids production. The available phosphorus is not only by the action of PSB but also by the production of biologically active substance like IAA. After 30 days of inoculation, growth and development was monitored through pot experiment. The total soluble sugars in the leaves of all maize and chilli plant showed an increase in PSB inoculated seedlings than un-inoculated seedlings. The starch content was maximum in seedling treated with PSB when compared to control crops. The result of PSB inoculation in the protein content showed a greater increase in PSB plants than in control seedlings. *In vitro* characterization of the strain PSB4 was studied by the siderophore production and antifungal activity. All the morphological and biochemical measurement showed a great response in PSB treated plants thus confirming the efficiency of the selected isolate PSB4 as a phosphate solubilizer.

Key words: PSB · *Pseudomonas* sp · 16S rDNA · maize · Chilli

INTRODUCTION

Converting soil insoluble phosphates (both organic and inorganic) to a form available for plants is a necessary goal to achieve sustainable agricultural production. Extensive use of chemicals as fertilizers to improve plant health and productivity and for control of pathogens has disturbed the ecological balance of soil and has led to the depletion of nutrients. Hence there is a need to search for alternative strategies to improve soil health without causing damage to environment as well as soil. Biological control is an alternative or a supplemental way of decreasing the use of chemicals in agriculture. New species of bacteria may provide potentially new biological control agents with novel mechanisms of disease suppression active in a range of environments [1]. Phosphorus is a component of ATP, DNA and RNA. It plays a major role in respiration, photosynthesis, cell division, energy storage and several other processes in the living plant. It promotes early root formation, plant growth and it improves the quality of fruits, vegetables and grains and is vital to seed formation. It increases nitrogen fixing capacity of plant, water utilizing efficiency and contribute to disease resistance in plants. Phosphorus is an essential element for plant development and growth making up about 0.2% of plant dry weight [2].

The plant growth promoting rhizobacteria such as *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are used as biofertilizers or biocontrol agents for rural improvement. Several reports have reported that the ability of different

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bacteria releasing phosphate from inorganic phosphate such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite and rock phosphate through solubilization and mineralization [3, 4]. Solubilization can be accomplished by a range of mechanisms, which include excretion of metabolites such as organic acids, proton extrusion or production of chelating agents [5].

Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic and succinic acid have also been identified among phosphate solubilizers. These organic acids solubilize insoluble forms of phosphate to a soluble form, such as orthophosphate, thus increasing the potential availability of phosphate for plants. Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers [6].

The reductions of pathogenic microorganisms mostly due to the synthesis of antifungal compounds, antibiotics, cyanide or siderophores are produced by bacteria. Siderophores are low-molecular weight, iron-chelating ligands synthesized by microorganisms which compete effectively against other organisms for the available iron [7]. Fluorescent *Pseudomonads* are used as biocontrol agent because they can produce large amounts of secondary metabolites to protect plans from phytopathogen and stimulate plant growth. A microorganism that colonizes roots is ideal for use as a biocontrol agent against soil-borne pathogens. The high concentration of L-tryptophan would enhance the production of IAA by PGPR, resulting in the inhibition of root elongation [8].

Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* have increased root and shoot elongation in canola, lettuce and tomato as well as crop yields in potato, radishes, rice, sugar beet, tomato, lettuce, apple, citrus, beans, ornamental plants and wheat. Wheat yield increased up to 30% with *Azotobacter* inoculation and up to 43% with *Bacillus* inoculants and a 10–20% yield increase in the same crop was reported in field trials using a combination of *Bacillus megaterium* and *Azotobacter chroococcum*. Inoculations with PSBs. have increased shoot length and root length of plants in both green houses [9].

Currently, PSB have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield. Several studies have shown that *Pseudomonas fluorescens* as a soil bioinoculant improves the growth of plants by various mechanisms like production of antifungal compounds, siderophore production, plant hormone production and phosphate solubilization [10]. Hence the present work was undertaken to investigate the effect of PSB on growth and development of chilli and maize plants.

MATERIALS AND METHODS

In the present investigation, the sample was collected in Asian elephant dung (*Elephas maximus indicus*) from Senbagathoppu hills in Srivilliputtur, Tamilnadu, India by using sterile container and it used for further experimental analysis.

Isolation and Screening of Phosphate Solubilizing Bacteria: Pikovskaya's medium was prepared and sterilized. The isolated colonies were streaked on the plate and incubated at 37°C for 48 hours. The zone around the bacterial growth indicates the phosphate solubilization. The isolated colonies on these plates were maintained on nutrient agar slants at 4°C for further experimental analysis. Halo surrounding the colonies were measured and the solubilizing efficiency (SE) was calculated by the following formula [11]. The isolated bacterial colonies were further characterized for their morphological and biochemical characters. Identification was carried by the standard procedures described in Bergy's manual of determinative bacteriology.

Identification of the Selected PSB by 16S rDNA Sequencing: The strain was identified by the analysis of their 16S rRNA gene sequences and sequence homologies were determined using BLAST. The identified gene sequences were submitted to GenBank/NCBI under the accession number **JF906501.**

Quantitative Analysis of Phosphate Solubilization in Culture Broth: The phosphate solubilization potential of selected strains was tested *In vitro* for available phosphorous in the PVK medium amended with tricalcium phosphate as a substrate. Quantitative analysis of phosphate solubilization was carried out using Erlenmeyer flasks containing 50 ml of PVK medium. Before inoculation, the bacterium was cultured on nutrient broth at 30°C for 24 hours. After the preparation of PVK medium, pH was adjusted to 7 and sterilized. Then it was inoculated with the isolated efficient PSB and incubated at 30°C for 5 days on orbital shaking incubator at 180rpm. After incubation period, the culture was harvested by centrifugation at 6,000 rpm for 15 minutes. At the same time, sterile un-inoculated medium served as control.

Remaining phosphate in the culture supernatant was estimated the 500 µl culture supernatant was mixed with 500µl of 10% (w/v) trichloroacetic acid in a test tube to which 4 ml of color reagent was added (1:1:1:2 ratio of 3 M H_2SO_4/2.5% (w/v) ammonium molybdate/10% (w/v) ascorbic acid and distilled water) and incubated at room temperature (26 ± 2°C) for 15 minutes. The absorbance of the developing blue color was measured at 820 nm using spectrophotometer. The amount of soluble phosphorus was detected from the standard curve of KH_2PO_4. All phosphate determinations were made in triplicate.

Determination of Plant Hormones

Estimation of IAA: The Indole Acetic Acid was quantitatively analyzed by the method of Gotdan and Webber [12]. The strain PSB 4 was grown in LC medium in the presence of tryptophan (100mg/l) and incubated at 30ºC. The IAA production by bacterial strains was measured every 24 hours of incubation. A 2 ml culture was removed and centrifuged at 10,000 rpm for 15 minutes in a cooling centrifuge, 1 ml of supernatant was transferred to fresh tube to which 100 ìl of 10 mM orthophosphoric acid and 2 ml of reagent consisting of 1 ml of 0.5% $FeCl_3$ in 50 ml of 35% $HClO_4$ were added sequentially. The absorbance of the developed pink color was read at 530 nm after 25 min in a UV-VIS Spectrophotometer (SHIMADZU UV-1700, Japan). IAA concentration in the culture was determined by using a calibration curve of pure IAA as a standard.

pH Estimation: The Pikovskaya broth was prepared and the pH of the medium was adjusted to 7 and sterilized. Then the isolated phosphate solubilizing bacteria was inoculated under aseptic condition into the sterile Pikovskaya broth and incubated at 30°C for 3 days. The pH of the culture filtrate was measured once in every 24 hours. The pH of the un-inoculated media served as control.

***In vitro* Characterization of Biocontrol Features:** To investigate the biocontrol mechanism, the efficient isolated PSB was tested for the production of siderophore and antifungal activity.

Assay for Siderophore Production: The isolated PSB was inoculated into the nutrient broth and incubated for 24 hours. After centrifugation 0.5 ml supernatant was added with 2% of 0.5 ml ferric chloride. The positive result was observed by the colour change of the supernatant from yellow to orange or brown colour.

Evaluation of Antifungal Activity of Isolates (Cultural Filtrate Assay): The fungal pathogen were inoculated in the PDA broth and incubated at 37°C for 3 days. After incubation the fungal culture was centrifuged at 10,000 rpm for 20 min used for further assay. The PDA agar plates were prepared and the fungal supernatant was swabbed on agar plates. After that 50µl of bacterial culture supernatant was added into the well and then plates were incubated at 37°C. After incubation the zone of inhibition was measured [13].

Effect of PSB on Plant Growth and Development

Pot experiment -Preparation of Sand Soil Mixture: River soil, garden soil and red soil were sieved through 30mesh sieve separately to remove the coarse particles. These soils were mixed in the preparation of 2: 1: 1 (River soil, Garden soil and Red soil) to give a favorable medium for the growth of root system.

Sterilization: The sand soil mixture was moistened with water and sterilized in the autoclave. The sterilization was carried out at 121°C for 2 hours to destroy the various bacterial and other pathogenic organisms and their spores. After this process, the sand soil mixture was aerated overnight and transformed into a container to prevent dust contamination from air. Chilli and maize seeds were washed with sterile water for seven times. Seeds were then treated with 24 hours old bacterial culture isolate for 30 minutes. Without treated seeds with any isolate designated as control. An amount of 0.3 kg sand was placed into a plastic pot. Ten seeds were sown at 4 to 5 cm depth of sand in each plastic pot. After 30 to 45 days the growth promoters like plant height and root length as well as shoot and root dry biomass was recorded.

Analysis of Growth Characters on Plant Height (cm)

Evaluation of Phosphate Solubilizing Isolates on Plant Growth: In the present investigation, 24 hours old bacterial culture isolate was mixed with sterile distilled water in 1:1 ratio prior to application in soil. Plant height, fresh weight and dry weight were recorded 15 days after sowing.

Morphometric Analysis: After the treatment period before plucking, the plants were watered and three plants each from control and treated were randomly uprooted without any damage to the seedlings and it was thoroughly washed well with tap water in order to remove soil and debris particle.. The height of the shoot was

measured and expressed in cm scale on 15days. In uprooted plants (treated and control) the root length was measured with the help of meter scale.

Fresh Weight of Roots and Shoots (g): The plants were removed gently from the soil without disturbing the root system and then the roots were washed with tap water to remove the soil particles. The roots and shoots were weighed separately using electrical balance.

Dry Weight of Shoot and Root (g): The plants were uprooted gently without disturbing the root system and then roots system were washed with tap water to remove the soil particles. The fresh shoot and root from each treatment and control were cut into pieces and kept in an oven at 80°C for 24 hours and then shoot dry weight was recorded. The dried sample was weighed and the dry matter yield was recorded.

Moisture Content: Fresh weight of plant material (leaves, stem and root) was taken and then it was kept in over for 72 hours at 80°C. The moisture content is determined as follows.

$$\text{Moisture (\%)} = \frac{\text{Plant fresh weight} - \text{Plant Dry weight}}{\text{Plant fresh weight}} \times 100$$

Biochemical Analysis

Determination of Chlorophyll: 100 mg leaf sample was homogenized with 10 ml of 80% pre-chilled acetone. The extract was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected. Repeat this procedure until the residue was colorless. The volume of supernatant was made up to 100 ml with 80% acetone and the absorbance was read at 645 nm and 663 nm. 80% acetone was served as blank. Total chlorophyll content was calculated by using the formula,

$$\text{Total chlorophyll} = 20.2\,(A645) + 8.02\,(A\,663) \times V/1000 \times W$$

where, A - OD at specific nm, V- Final volume of plant extract in 80% acetone, W - Fresh weight of leaf tissue used).

Determination of Carotenoids: One gram of leaves were ground with 10 ml of acetone and filtered through the Whatmann No1 filter paper. Repeat the procedure until the residue was colorless. Then the extract was transferred into a separating funnel containing equal volume of petroleum ether and mixed gently. The ether layer was evaporated at 35°C. The residue was dissolved in ethanol and 60% of aqueous KOH was added at the rate of 1ml for every 10 ml of ether extract. It was boiled for 5-10 minutes. To this equal volume of water and ether was added and it was evaporated. Then the residue was dissolved in ethanol. The absorbance was read at 450 nm.

Determination of Proline: Free proline from plant tissues may be selectively extracted in aqueous sulphosalicylic acid and its concentration was measured using ninhydrin method. 100 mg of leaf sample was taken and ground with 10 ml of 3% sulphosalicylic acid and filtered with Whatmann No1 filter paper. Two ml of the extract along with two ml acid ninhydrin and two ml of glacial acetic acid was taken, mixed well and kept in boiling water bath (100°C) for one hour. It was cooled in an ice for five minutes and added with four ml of toluene. The tubes were agitated vigorously for 20-30 seconds. The upper pink chromophore layer was separated and the absorbance was read at 520 nm. The acid ninhydrin reagent was used as blank. The amount of proline was determined using proline as the standard.

Determination of Total Soluble Sugars and Starch: The amount of total soluble sugar and starch present in the leaf extract was determined by Anthrone method. The protein content was determined by the procedure described by Lowry *et al*. [14].

RESULTS AND DISCUSSION

In the present investigation, four isolates of PSB were isolated from the Asian elephant (*Elephas maximus indicus*) dung sample from Senbagathoppu hills in Srivilliputtur, Tamilnadu, India. Isolated organisms were further screened for phosphate solubilization by measuring the zone around the bacterial growth in pikovskaya agar. Among the four phosphate solubilizing bacterial strains, only one bacterium was finally selected for further experimental analysis. Based on the morphological, physiological and biochemical characteristics the suspected bacterial strain was identified as *Pseudomonas aeruginosa* by the following standard keys of Bergey's Manual of Determinative Bacteriology (Table-1). 16S rRNA gene sequences of phosphate solubilizing bacterial isolate revealed that it is more closely related to bacterial geneoic sequences of *Pseudomonas aeruginosa.* Since the isolates had a close similarity, the dendrogram was constructed based on their phylogenetic relationship revealed that all the isolates were distinctly placed under separate clusters. Similarly,

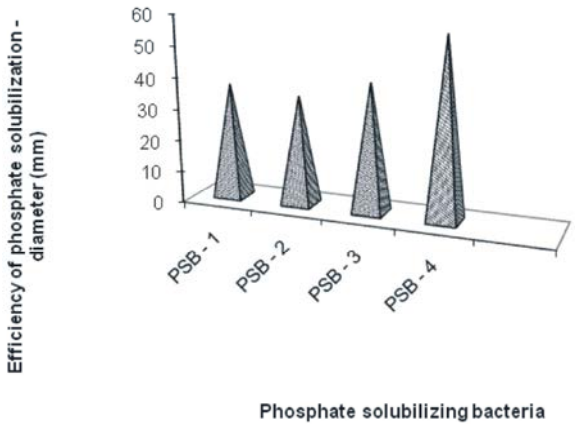

Fig. 1: Screening of PSB based on the efficiency of TCP solubilization on Pikovskaya agar plate

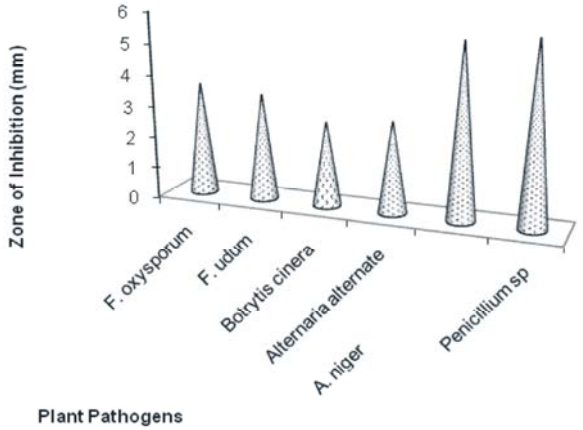

Fig. 2: Evaluation of anti-fungal activity of PSB 4 (culture filtrate assay)

Table 1: Biochemical characterization of the isolated PSB4

Characters	Observation
Indole production	-
Methyl red	-
Voges-Proskauer	-
Citrate utilization	+
Catalase	+
Oxidase	+
Nitrate reduction	+
Gelatin Hydrolysis	+
Urea Hydrolysis	-
Triple sugar iron agar	+
Casein hydrolysis	+
Starch hydrolysis	+
Lipid hydrolysis	+

Table 2: Estimation of IAA production

Incubation time	IAA (mg/ml)
24 hrs	0.073
48 hrs	0.190
72 hrs	0.145

Ahmadzadeh, *et al*. (2006) collected sediment samples from different stations of the Thondi coast, Palk Strait. The isolation of phosphate solubilizing bacteria (PSB) namely such as *Pseudomonas*, *Bacillus*, *Vibrio*, *Micrococcus*, *Flavobacterium*, *Corynebacterium*, *Alcaligenes* and *Enterobacter*. Among the isolated samples, *Pseudomonas* and *Bacillus* were found to solubilize more phosphates than others.

Estimation of Phosphate Solubilization: In the present study, among four isolates, the strain PSB 4 identified as *Pseudomonas aeroginosa,* released soluble phosphates from the culture medium after three days of incubation (Fig. 1). In the same way, Phosphate solubilizing bacteria (PSB 4) secreted organic acids and phosphates to convert the insoluble phosphates into soluble monobasic ($H_2PO_4^-$) and dibasic (HPO_2^-) ions [15].

Estimation of IAA Production: In this study, the ability of *Pseudomonas aeroginosa,* produced 0.190mg/ml indole acetic acid in medium supplemented with tryptophan after 48 hours incubation (Table 2). In accordance with the earlier report of Patten and Glick [16] stated that the *Bacillus* sp have produced more IAA in medium added with tryptophan.

Siderophore Production: In the present study, the selected strain *Pseudomonas aeroginosa* had high affinity for up taking irons based on their colour changes and also concluded that the strain was effective biocontrol agent. Similarly Whipps [17] stated that the biocontrol mechanisms belonging to PGPR groups, including *Bacillus* sp. under iron limiting condition. Several studies have demonstrated that production of siderophore by PGPR was most effective in controlling the plant root pathogens [18].

Evaluation of Antifungal Activity of Isolate: The genus *Pseudomonas* are aggressive colonizers of the rhizosphere of various crop plants and have a good antagonistic activity against plant pathogens, such as antibiosis (the production of inhibitory compounds). In the present investigation, the Isolate PSB4 was tested against common pathogens. The Isolated PSB showed inhibitory action over the above said fungal pathogens (Fig.2). Strains of *Pseudomonas* sp. produced phenazine-1-carboxamide (PCN) and these metabolites

Table 4a: Estimation of chlorophyll in maize

Plant	Mg Chlorophyll a/g	Mg Chlorophyll a/g	Mg Total Chlorophyll/g
Test Plant	19.80	13.09	32.89
Control Plant	8.18	7.43	15.61

Table 4b. Estimation of chlorophyll in chilli

PLANT	Mg Chlorophyll a/g	Mg Chlorophyll b/g	Mg Total Chlorophyll/g
Test plant	15.69	11.80	27.49
Control plant	12.77	6.02	18.79

have been shown to be involved directly in the suppression of all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici* [19].

Estimation of Chlorophyll and Carotenoids Content: The present study, the *Pseudomonas aeruginosa* had improve the chlorophyll content values are calculated as 32.89 mg/g over the control to 15.61 mg/g in maize (Table 4) and 27.49 mg/g over control in 18.79 mg/g in chilli (Table 4). The *Pseudomonas aeruginosa* increased carotenoids content to 0.047 mg/g% over the control 0.033mg/g in maize and 0.038mg/g% over control in 0.030 mg/g% in chilli (Table 5). Maximum amount of chlorophyll pigment may show an efficient rate of photosynthesis. Koide [20] reported that using Mycorrhiza increases leaf chlorophyll content and can positively affect rate of photosynthesis The maximum N and P uptake were observed in bacteria containing treatments. This suggests that there is a direct and positive synergic effect between fungus and bacteria on soil phosphorous availability [21].

pH Estimation: The pH of the culture filtrate was measured once in every 24 hours. The pH of the un-inoculated media served as control. The solubilization effect is generally due to production of organic acids by these organisms. Among the selected isolates, PSB 4 showed better phosphate solubilization in the plates after incubation (Table 6). After 96 hours incubation, the solubilization of TCP in the liquid medium by candidate bacterium was accompanied by a considerable fall in pH i.e. 3 and 6.0 from an original pH of 6–7.0. The soluble-P concentration in the medium ranged between 31.5 and 519.7 mg/l with variations among different isolates [4]. Our findings in accordance with the work of earlier researchers who confirmed the P solubilizing activity of selected strains was related to the release of organic acids and subsequent pH reduction in the medium [4].

Biochemical Analysis: In maize plant, the amount of total soluble sugars present in the test plant was 110 mg(%) and control was 80 mg(%). In chilli plant, it was found to be 180 mg(%) in test and 110 mg(%) in control (Table 7). The above said result was correlated to Sudhalakshmi *et al.* [22]. The infected plants showed 178.26 mg/ml over the control 135.86mg. The concentration of protein found in treated maize plant was 178.26 mg/ml and in control maize plant it was 135.86 mg/ml. At the same time treated chilli plant contained 1270.65 mg/ml and in control it was 701.30 mg/ml (Table 8). The above held report was in accordance with the previous result of Sudhalakshmi *et al*. [22]. The plants grown in soil inoculated with the fungal inoculants showed the maximum protein content over the control. Simultaneously, the amount of starch present in maize was 245 (mg%) over the control plant (135.86 mg%). Whereas, the amount of starch present in chilli was 145 (mg%) over the control plant (Table 9).

Table 5: Estimation of carotenoid (mg/g) in maize and chilli

Plant	Maize (mg/g)	Chilli (mg/g)
Test	0.069	0.173
Control	0.051	0.159

Table 6: pH estimation in culture supernatant

Incubation time	Isolated PSB4	Control
24 hrs	6	7
48 hrs	5	7
72 hrs	4	7
96 hrs	3	7

Table 7: Estimation of total soluble sugars in maize and chilli

Plant	Maize (mg (%))	Chilli (mg (%))
Test	110	180
Control	80	110

Table 8: Estimation of protein in maize and chilli

Plant	Maize (mg/ml)	Chilli (mg/ml)
Test plant	178.26	1270.65
Control plant	135.86	791.30

Table 9: Estimation of starch (mg (%)) in maize and chilli

Plant	Maize (mg (%))	Chilli (mg (%))
Test	245	145
Control	110	50

Table 10: Effect of PSB 4 on maize growth (30 days after sowing)

		Dry weight g/Plant		Fresh weight g/Plant	
Treatment	Plant height	Shoot	Root	Shoot	Root
Control	14.3	1.01	0.54	1.34	0.66
PSB	38.0	1.35	0.61	1.49	0.71

Table 11: Effect of PSB 4 on chilli growth (30 days after sowing)

	Dry weight g/Plant		Fresh weight g/Plant	
Treatment	Shoot	Root	Shoot	Root
Control	1.01	0.54	1.34	0.66
PSB	1.35	0.61	1.49	0.71

Plate. 1: Control Test

Control plant Treated plant

Plate 2: Pot experiment (Maize)

Control plant Treated plant

Plate 3: Pot experiment (Chilli)

Estimation of Plant Growth

Evaluation of Isolated PSB 4 on Maize and Chilli Plant Growth: In our study, both maize and chilli plants were inoculated with PSB 4 to induce plant growth.

Plate 4: Effect of PSB 4 on plant growth and development

As phosphate solubiliser, it showed better growth in shoot as well as root. It was found that, there is a significant enhancement in both root and shoot dry and fresh weight in maize and chilli plants. When evaluating the results, bacterial inoculum used for the present study showed significant differences between the treated plants and untreated control plants (Plate 2-5).

After 30 and 60 days the plants were removed and washed with tap water carefully and then washed with distilled water without causing a damage to the root system and then the following morphological growth characters like plant height (cm), shoot length (cm), root length (cm), fresh weight of root (g), fresh weight of shoot (g), dry weight of shoot and root (g) and moisture content were analyzed. It was represented in the Table 10-11. In the present study, *Pseudomonas aeruginosa* showed positive effect on maize and chilli based on their activity of shoot length root length fresh weight and dry weight. Similarly, Sachin [23] *Azotobacter chroococcum* had a positive effect on the growth parameters of bamboo and maize under *In vitro* condition as well as pot experiment. The bacterial inoculums caused significant increased on growth parameters such as seed germination, root and shoot length, dry weight of root and shoot of bamboo and with talc formulation of *Bacillus* sp. Koide [20] also endorsed our results that the optimistic result of Mycorrhiza fungus on crop yield due to the increase of nutrients uptake (predominantly phosphorus and zinc).

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