Research Article

# Isolation and Characterization of Phosphorus and Potassium Solubilising Microbes from Rhizosphere of Orchard Field and Its Effect on Seedling Growth of Broccoli (*Brassica Oleracea* Var. Italica L.)

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**Abstract**

A nursery trial was performed to study the effect of biofertilizers on broccoli (*Brassica oleracea* var. italica L.) plant seedling which was further transplanted under citrus orchard. The present study interprets the effect of biofertilizers on growth of Broccoli seedlings. Bacterial biofertilizers phosphomicrobes PSB1 and potassium solubilising microbes KSB2 were isolated from the soil of citrus orchard. The strains were screened by employing qualitative plating techniques. The isolates showing maximum zones were screened for further analysis. The microbial inoculants coated seeds were then sown in fields. After 30 days of sowing, the plant growth parameters like morphological parameters were analyzed in *Brassica oleracea* var. italica L. plants. The morphological parameters like number of leaves, root length, shoot length increased in the treatment having potassium solubilizing bacteria ($T_2$) in broccoli than other treatments and control plants. On the basis of the observations, present work suggests that broccoli seedlings could be performed well by seed treatments with solubilising microbes.

**Keywords:** Biofertilizers, Microbes, Morphological, Broccoli, Seedlings

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## Introduction

Broccoli (*Brassica oleracea* L. var. Italica) is an European vegetable and a considerably new introduction to Indian farmers [1, 2]. Biofertilizers can be used for broccoli cultivation as they play a very vital role in improving soil fertility [3-6]**.** There are several reports of *Trichoderma* and *Pseudomonas* mediated growth promotion and development of seedlings of several vegetable crops, namely cabbage, tomato, and capsicum [7-12]. Apart from improving nutritional status and growth of various vegetable crops also used as biocontrol agent against a wide range of soil borne fungal pathogens of crucifers [13]. The benefits of growth promoting microbes in vegetable crop production represent great opportunities for current agricultural practices [14].

A principal problem of organic farming is the low nutrient status of most organic fertilizers. This problem is further compounded by the difficulties in assessing the value of organic fertilizers through direct analysis of total quantities of plant nutrients [15]. Therefore, field experiments are needed to determine the nutrient availability and efficiency of most organic fertilizers. Thus, biofertilizer with Plant Growth Promoting *Rhizobacteria* (PGPR) can be used to increase soil productivity and plant growth in sustainable agriculture through increasing mineral and organic fertilizer use efficiency [16].

Phosphorus is one of the less-abundant (0.1% of total) elements in the lithosphere [17] and often regarded as a limiting nutrient in agricultural soils. Upon application as inorganic phosphorus rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils or with Ca in calcareous soils becomes unavailable to plants [18, 19]**.** Similar to these findings, IAA production by PSB strains such as *Achromobacter xylosoxidans* and *Klebsiella* have also been reported [20-22]**.** *Pseudomonas fluorescens* remarkably increased plant height, shoot and root dry weight, and phosphorous and nitrogen uptake of field crops [23].

The present investigation clearly demonstrated that application of *T. Viride* alone or in combination with other growth promoting microbes (*Bacillus*, *P. fluorescens and Aspergillus*) proved to be a promising factor for improved growth performance of broccoli seedling to an acceptable level in the experimental field of Department of Horticulture, SHUATS, Allahabad, India.

## Materials and Methods

### *Sample collection*

Soil samples were collected from the rhizosphere of citrus orchard. The rhizosphere was dug out with intact root system. The samples were placed in plastic bags and stored at 4ºC.

### *Isolation of Bacillus*

One gram of soil sample was aseptically collected from the institute campus and serially diluted till $10^{-6}$ dilution. 0.1 ml of aliquot was plated on Luria bertani agar media using spread plate technique. The plates were incubated at $30^0$c for 4-7 days. Following incubation, the isolated colonies were pure cultured and Gram stained. Biochemical characterization of the isolated colonies was carried out using standard protocols [24]. Identification was carried out using Bergey's manual ($7^{th}$ edn.).

### *Isolation of Pseudomonas*

Ten grams of rhizosphere soil were taken into a 250 ml of conical flask and 90 ml of sterile distilled water was added to it. The flask was shaken for 10 min on a rotary shaker. One milliliter of suspension was added to 10 ml vial and shaken for 2 min. Serial dilution technique was performed upto $10^{-6}$ dilution. An aliquot (0.1 ml) of this suspension was spread on the plates of King's B agar medium. Plates were incubated for 3 days at 28ºC to observe the colonies of bacteria. Bacterial colonies were streaked to other King's B agar plates and the plates were incubated at 28ºC for 3 days. Typical bacterial colonies were observed over the streak. Well isolated single colony was picked up and re-streaked to fresh King's B agar plate and incubated similarly.

### *Identification of isolates*

The bacterial strain was studied for cultural, morphological and biochemical characteristics based on Bergey's Manual of Systematic Bacteriology [25].

### *Cultural characteristics*

All the isolates were streaked on King's B and Luria bertani agar agar plates. After 3 days of incubation, different characteristics of colonies such as growth, form and colour.

### *Morphological characteristics*

The suspected organisms were subjected to Gram's staining [26]. The bacteria which retained the primary stain called gram +ve while those that lost the crystal violet and counter stained by safranin were referred as gram –ve.

### *Biochemical characterization*

*Methyl Red test*

Tubes containing the sterilized MR–VP broth were inoculated with isolated bacterial strains and one tube as uninoculated comparative control. Then all the inoculated and uninoculated tubes were incubated at 37º C for 48 h. After 48 h of incubation 5 drops of methyl red indicator was added to each tube. When methyl red was added, it remained red which was indicative of positive test while turning of methyl red to yellow indicated the negative test.

*Hydrogen sulfide production test*

The inoculum was stab inoculated onto the SIM agar medium and incubated at 37º C for 48 h. Blackening of the culture medium indicated a positive test while no change in colour of the medium indicated a negative test.

*Catalase test*

The hydrogen peroxide solution (2%) was added to the culture on a slide. The release of free oxygen bubbles indicated a positive test.

*Casein Hydrolysis test*

The casein agar plate was streaked with the isolated organism in sterile condition. After that the plates were incubated at 37ºC for 24-48h in an inverted position [27]. A clear zone surrounding the bacterial growth indicated the positive reaction for extracellular caseinase secretion while absence of clear zone indicated the negative reaction.

*Gelatin Hydrolysis test*

Gelatin agar medium was melted and cooled to 45-50º C to pour into four sterile petri dishes. All the inoculated and uninoculated (control) plates were incubated at 37º C for 4 -7 days. After incubation, plates were placed in refrigerator at 4ºC for 24h. The incubated agar plates were flooded with mercuric chloride solution and the plates were allowed to stand for 5 to 10 minutes. Then plates were examined for clearing zone around the line of growth [28].

*Indole test*

The isolated organism was inoculated into tryptone broth the inoculated and uninoculated (control) tubes were incubated at 37ºC for 48h. After incubation, Kovac's reagent was added to inoculated and control tubes. Development of cherry red colour at the top layer in the form of ring indicated the positive test while its absence indicated the negative test.

*Ammonia production test*

Fresh culture were inoculated into 10mL peptone water and incubated for 48-72h at 37ºC. Nessler's reagent (0.5mL) were added to each tube and development of yellow to brown colour was considered as positive test while no change in colour showed negative test [29].

*Phosphate solubilization test*

The bacterial isolates were inoculated into plates with sterilized Pikovskaya medium containing tricalcium phosphate and incubated at 30ºC for 72h. Formation of clear zone around the colony indicated the phosphate solubilizaton by the bacteria [30].

*Growth under different temperature condition*

Cultures (24 h old) of the isolated bacterial strains were streaked on nutrient Agar plates and incubated for 24-48 h at 4º C, 12º C, 28º C and 37º C. The change in growth and colour were Observed.

*Plan of experiment*

The experiment was carried out at experimental field, Department of Horticulture, Sam Higginbottom University of Agriculture, Technology and Sciences, Allahabad during 2013-14 and 2014-15. The treatments were tested such as four Biofertilizers (*Bacillus1, Bacillus2, Pseudomonas*1 and *Pseudomonas*2), on growth, and quality parameters of *Broccoli* seedlings. The experiment was laid out in Randomized Block Design with four treatments and three replications. The culture solution was prepared by mixing 500 g of *Bacillus1, Bacillus2, Pseudomonas* 1 and *Pseudomonas* 2, separately in 5 litre of 10 percent jaggory solution [15]. Healthy seedlings are taken and roots were dipped in the slurry of *Azotobacter1, Azotobacter2, Pseudomonas* 1 and *Pseudomonas* 2 separately for 30 minutes than transplanted (Second week of November). The cultural operations *viz*. Irrigation, Weed control, intercultural operations, insect pest control etc. were done in the plots uniformly throughout the course of investigation. The data were recorded on various parameters and analysed by Tukey method (31) in order to test the significance of results. The various treatment combinations of bacterial cultures were: $T_0$-CONTROL+100 % RDF, $T_1$-PSB+100 % RDF, $T_2$-KSB+100 % RDF and $T_3$- KSB+ PSB+100 % RDF.

*Seed treatment and sowing*

Seeds were inoculated following the method of Weller and Cook [32]. The seeds of broccoli were surface sterilized using tap water and then with 70% ethanol, disinfected with 0.05% $Hgcl_2$ for 6 min again rinse with water. The strains

selected for the treatments were grown on medium for 48 hours. Growth was scraped and thoroughly mixed with one percent sterile jaggery suspension. Now seeds are treated with PSB and KSB broth cultures and sown in nursery.

## Results

### *Isolation of Bacillus and Pseudomonas strains from citrus orchard rhizosphere*

To isolate the strains soil samples were collected from citrus orchard field and inoculated on King's B and luria bertani agar plates. They were designated as B1, B2, P1 and P2 which were subjected to Cultural, Morphological and Biochemical characterization.

### *Cultural characteristics*

The isolates B1 and B2 were having smooth, irregular and milky white colour colonies (**Figure 1**). P1 and P2 isolate was regular, smooth and dirty white colour colony (**Figure 3)**. All the isolates were odourless (**Table 1**).

### *Morphological characteristics*

The morphological characteristics of B1, B2, P1 and P2 were widely varied (**Table 2**). All the isolates produced rod shaped and were gram negative in reaction (**Figure 2 & Figure 4**).

**Table 1** Cultural characteristics of the Isolates

| Isolate | Elevation | Margin | Colour | Odour |
|---|---|---|---|---|
| B1 | Raised | Smooth | Milky white | Odourless |
| B2 | Raised | Smooth | Milky white | Odourless |
| P1 | Raised | Smooth | Dirty white | Odourless |
| P2 | Raised | Smooth | Dirty white | Odourless |

**Table 2** Morphological characteristics of the Isolates

| Isolate | Shape | Arrangement | Gram reaction |
|---|---|---|---|
| A1 | Rod | Staphylo | +ve |
| A2 | Rod | Staphylo | +ve |
| P1 | Rod | Staphylo | -ve |
| P2 | Rod | Staphylo | -ve |

### *Biochemical characteristics*

*Indole test*

Isolates B1 and B2 were found to be good producer of IAA. On the contrary, P1and P2 were found to be negative for test (**Table 3).**

*Methyl- red tests*

In MR test, on addition of methyl red indicator the isolate B1, B2 showed positive test. While the isolate P1 and P2 showed negative test.

*Voges-Proskaur test*

The test was performed to determine the capability of microorganisms to produce non acidic end products such as Ethanol and Acetoin (acetyl methyl carbinol) from the organic acid. Organism was inoculated in VP broth. After addition of reagent (12 drops of freshly prepared VP-reagent 1 (napthol solution), 2-3 drops of VP-reagent 2 (40% KOH) B1, B2 showed positive result, whereas P1 and P2 showed negative result.

*Starch hydrolysis test*

The test was performed to determine the capability of microorganism to produce amylase. The starch agar plate was streaked with the organism and after that the plate was incubated at 28±2°C for 48 hours in an inverted position. After incubation, the surface of the plate was flooded with Iodine solution with dropper for 30 seconds. The plate was examined for the color change of the medium. A clear zone surrounding the microbial colonies indicated a positive Starch hydrolysis while negative reaction was shown as dark blue coloration of the medium. Isolates B1, B2 showed positive result, whereas P1 and P2 showed negative result.

*Citrate utilization test*

This test was performed to differentiate among enteric bacteria on the basis of their ability to utilize Citrate as the sole carbon source. Simmon's citrate medium (App. 1.25) was prepared and inoculated with the organism to be tested and incubated for 24 hours at 28±2°C. An uninoculated tube was used as control. Growth in the medium with a change in color from green to blue indicated positive citrate utilization by the isolate. Isolates B1, B2 showed positive result, whereas P1 and P2 showed negative result.

*Hydrogen sulphide production test*

The isolates B1, B2, showed positive test. P1, and P2 indicated the negative test.

*Catalase test*

All the isolates B1, B2, P1, and P2 showed the catalase positive test.

*Casein hydrolysis*

The B1 isolate followed by B2, were capable of producing a clear zone surrounding the bacterial growth i.e. positive reaction for extracellular caseinase secretion whereas P1 and P2 isolates showed negative test.

*Hydrolysis of gelatin*

A protein (production of gelatinase): A clear zone was observed around the bacterial growth in presence of mercuric chloride solution in the inoculated petri plates demonstrating the proteolytic hydrolysis of gelatin i.e. positive reaction was shown by all the isolates.

**Table 3** Biochemical characteristics of B1and P1 isolates

| **Test** | **Isolates** | |
|---|---|---|
| | B1 | P1 |
| Indole Tests | + | - |
| Methyl Red Test | + | - |
| Vogus Proskauer Test | + | - |
| Citrate Utilization Test | + | + |
| Catalase Test | + | + |
| Oxidase Test | + | + |
| Motility Test | + | + |
| Casein Hydrolysis Test | + | - |
| Starch Hydrolysis Test | + | - |
| Gelatin Hydrolysis Test | + | + |
| $H_2s$ Production Test | + | - |
| Phosphate Solubilising.Test | + | ++ |
| Carbohydrate Test | + | + |
| *Identification* | *Bacillus* | *Pseudomonas* |

*$NH_3$ production test*

The isolates B1, B2, P1 and P2 were capable of producing ammonia.

*Phosphate solubilization test*

The isolates B1, B2, P1and P2 were capable of solubilizing the phosphorus.

*Growth under different temperature conditions*

The growth of isolates on agar plates varied in temperature (**Table 4**).

**Table 4** The growth of all isolates (B1, B2, P1 and P2) in different temperature range

| ISOLATES | TEMPERATURE | | | |
|---|---|---|---|---|
| | $4^0$C | $12^0$C | $30^0$C | $40^0$C |
| B1 | - | - | ++ | +++ |
| P1 | - | - | ++ | +++ |

**Table 5** Effect of different isolates on growth of broccoli seedlings

| Treatments | Seedling Height | | Number Of Leaves | | Root Height | |
|---|---|---|---|---|---|---|
| | 1st year | 2nd year | 1st year | 2nd year | 1st year | 2nd year |
| $T_0$ | 5.50±0.30$^c$ | 5.20±0.10$^c$ | 5.33±0.58$^a$ | 6.00±0.00$^a$ | 1.97±0.01$^d$ | 2.06±0.01$^c$ |
| $T_1$ | 8.30±0.26$^b$ | 8.27±0.25$^b$ | 4.33±0.58$^a$ | 4.67±0.58$^a$ | 2.63±0.01$^a$ | 2.64±0.01$^a$ |
| $T_2$ | 9.37±0.40$^a$ | 8.87±0.05$^a$ | 5.00±1.00$^a$ | 5.67±0.58$^a$ | 2.57±0.01$^b$ | 2.64±0.02$^a$ |
| $T_3$ | 8.23±0.45$^b$ | 8.23±0.25$^b$ | 4.67±1.15$^a$ | 5.00±1.00$^a$ | 2.46±0.01$^c$ | 2.55±0.01$^b$ |
| | Significant | Significant | Significant | Significant | Significant | Significant |
| F-tab | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 | 2.31 |
| F-value | 62.07 | 234.37 | 0.74 | 2.67 | 1592.13 | 1273.09 |
| Sed | 0.84 | 0.43 | 2.0 | 1.49 | 0.026 | 0.026 |
| CD at 5% | 1.94 | 0.99 | 4.62 | 3.44 | 0.06 | 0.06 |

*Different letters in each column denote significant difference (p>0.005,n=3) according to a tukey's HSD test. Each letter shows relative degree of significance (a=most significant whereas "c" is least significant).

## Field Experiment

Among the isolates P1 showed maximum efficiency as it showed maximum phosphorus solubilising zone whereas B1 showed maximum efficiency as it showed maximum potassium solubilising zone. Therefore, these two isolates were taken for further analysis. The various treatment combinations of bacterial cultures were: $T_0$-CONTROL+100%RDF, $T_1$-PSB+100%RDF, $T_2$-KSB+100%RDF and $T_3$- KSB+ PSB+100%RDF

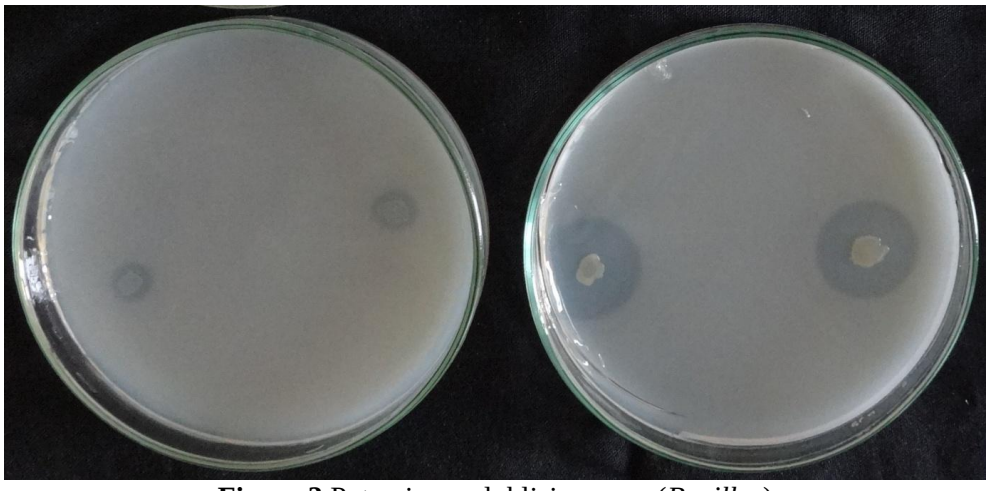

**Figure 3** Potassium solublising zone (*Bacillus*)

The highest root length (2.63cm, 2.64cm) was recorded in $T_2$ among the four treatments in both the years of experiment. The isolates significantly affected the shoot length of broccoli. Results revealed that shoot length increased in culture treated plants over untreated plants as control (**Figure 5**). The highest shoot length (9.37cm,

8.87cm) was recorded in $T_2$ among the four treatments. The highest Number of leaves (5.33, 6.00) was recorded in $T_2$ among the four treatments in both the years of experiment (**Table 5**)

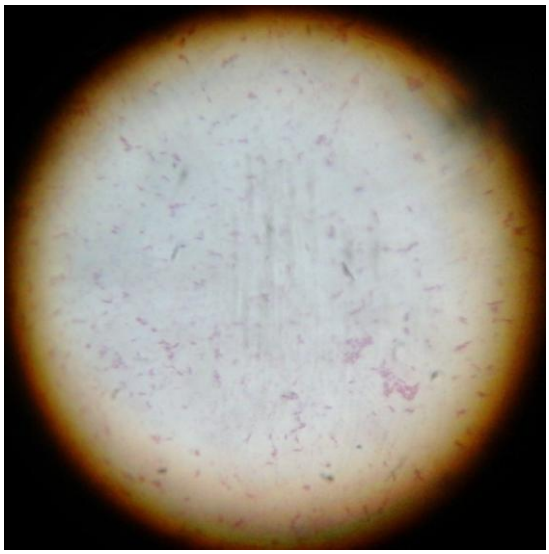

**Figure 4** Microscopic view of *bacillus* (Gram +ve rods)

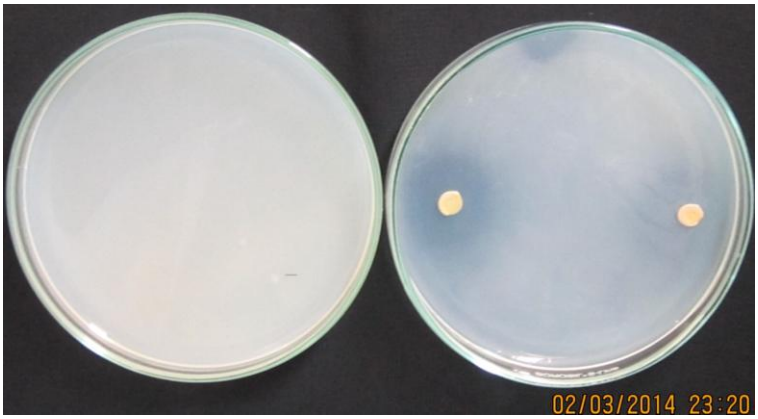


**Figure 5** Phosphorous solublising zone (*Pseudomonas*)

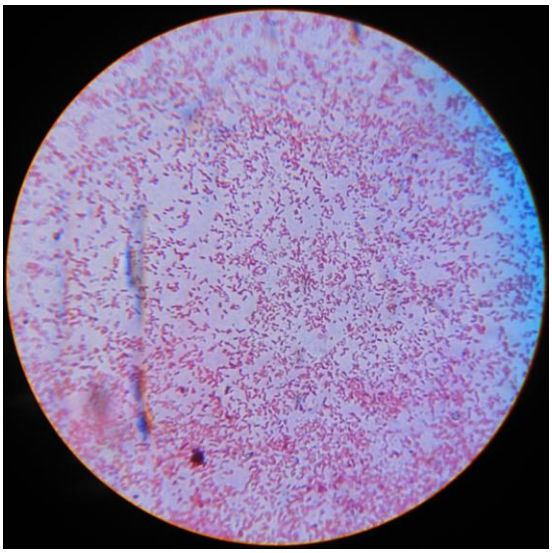

**Figure 6** Microscopic view of pseudomonas (gram -ve rods)

**Figure 7** Seedling growth of Broccoli

**Figure 8** The Experimenter and A field view of broccoli 30 DAP

## Discussion

Phosphorus is one of the major nutrients, second to nitrogen in requirement for plants. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants [33]. In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria was commonly found in the rhizosphere [34]. Many investigators reported that increasing phosphorus levels improved plant growth of broccoli [35, 36]. In the present investigation effect of the PGPR isolates remarkably affected the germination of broccoli seeds (**Figure 5)**. The highest seed germination was recorded when seeds were pre-treated with P2 isolate. A large body of evidence suggests that PGPR enhance the growth and seed emergence. This is the first study to demonstrate that PGPR root inoculation with manure can increase yield, growth, and phosphorus nitrogen efficiency (PNE) of broccoli plants (**Figure 6**). Two years of trials under field conditions showed that inoculations with Bacillus cereus, *Brevibacillus reuszeri*, and *Rhizobium rubi* significantly increased yield, yield parameters, and PNE uptake of broccoli. The effects of a combined treatment of PGPR (mixture of Bacillus sp., B. *subtillis*, B *erythropolis*, B. *pumilus*, and P. *rubiacearum*) plus 50% chemical fertilizer (½ CF + biofertilizer) on the growth of lettuce were compared by [37]. In many studies it has been reported that *Trichoderma* strains colonise the plant roots, establishing

chemical communication and systemically altering the expression of numerous plant genes that alter plant physiology and may result in the improvement of abiotic stress resistance, nitrogen fertiliser uptake, and resistance to pathogens and photosynthetic efficiency [38, 39]. The enhanced vegetative growth of broccoli in *Trichoderma* treated plants could be due to the root colonising ability of the fungus that resulted in better nutrient absorption through increased root biomass improved root and shoot growth in response to all inoculants compared with the control indicates the beneficial role of these *rhizobacteria* [40]. The improving effect of seed inoculation with *rhizobacteria* on shoot dry weight and yield of plant were reported earlier by [41-45]. Such an improvement might be attributed to the $N_2$-fixing and phosphate-solubilizing capacities of bacteria, as well as the ability of these microorganisms to produce growth-promoting substances such as Indole acetic acid (IAA) [46].

The present study demonstrates that biofertilizers applications in addition to manure can be used to increase broccoli seedlings growth without using mineral fertilizer. Most of phosphorus in soil is present in the form of insoluble phosphates and cannot be utilized by the plants [32]. In comparison to non-rhizospheric soil, a considerably higher concentration of phosphate-solubilizing bacteria was commonly found in the rhizosphere [47]. The isolates P1, P2, P3 and P4 were able to solubilise phosphate in the rhizosphere soil. On quantitative estimation of phosphorus among all the isolates P1 showed most efficient of all cultures. The experimental findings showed that seedling root treatment with bio-inoculants offers great potential as organic amendment for broccoli cultivation. Efficient plant nutrition management should ensure both enhanced and sustainable agricultural production and safeguard the environment.

The use of chemical fertilizers and manures cannot be eliminated at this time without drastically decreasing food production. At the same time, the harmful environmental side effects of fertilizer use such as leaching, runoff, emission, and eutrophication of aquatic ecosystems worldwide cannot go un-debated. Hence, there is an urgent need for integrated nutrient management (INM) that targets agricultural inputs and lowers the adverse environmental impacts of agricultural fertilizers and practices. An important nutritional problem of developing countries is micronutrient malnutrition, also called hidden hunger. Data may suggest that inoculations with biofertilizers have some potential to increase use efficiency of organic fertilizer in both organic and conventional farming.

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