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# Formulation of a compatible microbial consortia of nitrogen fixing, phosphate solubilising and potash mobilizing bacteria for optimizing nutrient supplementation to tuberose

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**Abstract**
The present investigation was conducted with the aim to isolate and characterize *Azotobacter*, phosphate solubilising bacteria (PSB) and potash mobilizing bacteria (KMB) from rhizosphere soil of tuberose and formulate consortium of *Azotobacter*, PSB and KMB. Three isolates each of *Azotobacter*, PSB and KMB were obtained from soil samples collected from three different locations of Pune region. Based on morphological and biochemical characterization, the highly efficient nitrogen fixing isolate (A I), PSB isolate (P III) and KMB isolate (K II) were identified as *Azotobacter chroococcum*, *Bacillus subtilis* and *Frateuria aurantia* respectively and used for the formulation of microbial consortium. Among the different culture media formulated, M III media having Glucose (10 g/L), Ammonium sulphate (0.4 g/L) and Yeast extract (1 g/L) recorded maximum growth and c.f.u. count of *Azotobacter*, PSB and KMB. These three beneficial organisms were found compatible with each other when grown on M III culture medium.

**Keywords:** *Azotobacter chroococcum*, *Bacillus subtilis*, *Frateuria aurantia*, consortium

## Introduction
Soil microorganisms play a crucial role in promoting effective soil conditions essential for stimulating plant growth by solubilization, mobilization and recycling of nutrients in soil. Among a variety of beneficial microorganisms, bacteria like *Azotobacter* play an important role in the nitrogen cycle in the nature, binding atmospheric nitrogen, which is inaccessible to plants, and releasing it in the form of ammonium ions into the soil. Phosphorus solubilising bacteria (PSB) are beneficial bacteria capable of solubilising inorganic phosphorus from insoluble compounds (Chen *et al*., 2006) [6]. Potash mobilizing bacteria (KMB) such as *Frateuria aurantia*, *Bacillus mucilagenosus*, and *Bacillus edaphicus* are examples of microorganisms that are used as potassium biofertilizer and this stands a novel solution to convert insoluble form of soil potassium into soluble (plant available) form (Kammar *et al*., 2016) [13]. Different types of culture media are required for growth of different microorganisms *In-vitro*. The cultural conditions exhibit a wide variation for growth activities of the microorganisms. Selection of a suitable media compatible for simultaneous synthesis of bioactive compounds of several microorganisms is a challenging task which pre occupies many possible variations. Jensen's media, Pikovskaya media and Alexandrov's media are suitable for individual growth of *Azotobacter*, PSB and KMB respectively. Hence, the present study was aimed towards formulation of such a culture medium which is suitable for growth of all these three beneficial microorganisms in a consortium.

## Material and methods
### Rhizospheric soil samples of tuberose
Fresh rhizospheric soil samples were collected from NARP, Ganeshkhind, Pune; Hi-tech Floriculture Project, College of Agriculture, Pune and Tamhanevasti, Chikhli, Pune and used for isolation of *Azotobacter*, PSB and KMB isolates.

**Isolation of *Azotobacter* from rhizospheric soil of tuberose**

The *Azotobacter sp*. were isolated on Jensen's agar medium by serial dilution pour plate technique given by Subba Rao (1986) [19] and three bacterial isolates were selected as nitrogen fixers and christened as AI, AII and AIII (Table 1).

**Isolation of phosphate solubilizing bacteria (PSB) from rhizospheric soil of tuberose**

The isolation of phosphate solubilizing bacteria on Pikovskaya's medium was carried out by serial dilution of soil and agar plating method (Aneja, 2003) [1]. Three isolates selected as phosphate solubilisers (P I, P II and P III) were purified and maintained for further study (Table 2).

**Isolation of potash mobilizing bacteria (KMB) from Rhizospheric soil of tuberose**

The isolation of potash mobilizing bacteria on Alexandrov's agar medium was carried out by serial dilution of soil and agar plating method (Aneja, 2003) [1]. Colonies exhibiting zone of clearance indicating potassium solubilization were selected. Three bacterial isolates were selected as potash mobilizers and named as K I, K II and K III (Table 3).

**Characterization of *Azotobacter* isolates**

The growth and colony characters of individual isolates (A I, A II, A III) *viz.*, shape, surface colour, pigmentation, gram staining were studied by raising the individual *Azotobacter* isolate on Jensen's agar media. The nitrogen fixing ability of *Azotobacter* isolates was estimated using Microkjeldhal method (Reis *et al.*, 1994) [15]. The nitrogen fixing ability of the MPKV (Mahatma Phule Krishi Vidyapeeth) isolate (*Azotobacter chroococcum*) was used as control. The highly efficient nitrogen fixing *Azotobacter* isolate was further subjected to biochemical tests including catalase, oxidase, gelatin hydrolysis, indole test, methyl red test, Voges-Proskaeur test, growth on carbon sources and growth at various temperatures for confirmation (Cappuccino and Sherman, 2014) [4].

**Characterization of PSB isolates**

The formation of clear zone of P-solubilisation around the colonies grown on Pikovskaya's medium were selected, purified, subcultured and maintained on the slants of Pikovskaya's agar for further use. The ability of the bacterial isolates to solubilise insoluble inorganic phosphate was tested by spotting 10 µl overnight cultures on Pikovskaya's agar plates and incubating at 28-30°C for 2-3 days. The phosphate solubilizing ability of the MPKV isolate (*Bacillus subtilis*) was used as control. The bacterial isolates positive for P solubilisation on Pikovskaya's agar medium were subjected to quantification of Pi released from TCP in broth medium (Jackson, 1973). The highly efficient phosphate solubilizing bacterial isolate was tested for different biochemical characters. Catalase test, oxidase test, methyl red test, Voges-Proskauer (VP) test, gelatin hydrolysis test, starch hydrolysis and $H_2S$ production test were performed (Cappuccino and Sherman, 2014) [4].

**Characterization of KMB isolates**

All the selected isolates (K I, K II and K III) were examined for their colony morphology, cell shape, Gram reaction. The isolates showing zone of solubilisation on Alexandrov's agar medium were further examined for their ability to release 'K' from broth media. The amount of 'K' released from muscovite mica in the broth by the isolates was studied at 7, 15 and 20 days after incubation (DAI) in lab condition as described by Parmar *et al*., (2016) [14]. The amount of 'K' released from muscovite mica in the broth by the MPKV isolate (*Frateuria aurantia*) was used as control. The biochemical characterization of the highly efficient potash mobilizing isolate was carried out as per the procedures outlined by Cappuccino and Sherman, (2014) [4]. Sugar utilization, methyl red test, Voges-Proskauer (VP) test, urea hydrolysis, nitrate reduction test, gelatin hydrolysis test, catalase test, oxidase test, starch hydrolysis, casein hydrolysis and $H_2S$ production test were performed.

**Results and discussion**

**Colony and morphological characterization of *Azotobacter*, PSB and KMB isolates**

All the *Azotobacter* isolates (A I, A II and A III) formed circular colonies with entire margins and convex elevation, exhibited light brown pigmentation and reported unevenness in colour, with dull to clear white colour. All the isolates were gram negative in reaction with rod shaped bacterial cells. Similar findings were reported by Upadhyay *et al.*, (2015) [20].

Three bacterial isolates exhibiting halozone, thus showing the capability of P solubilization, were selected as phosphate solubilizers. The zone of phosphate solubilization varied from 3 mm to 7 mm among the different PSB isolates. The maximum zone of 7 mm was produced by P III isolate (Table 2). The phosphate solubilizing isolates (P I, P II and P III) formed circular colonies with entire margins, convex elevation on Pikovskaya's agar medium. All the isolates were gram positive in reaction with rod shaped bacterial cells. The results are in agreement with the findings of Wasule *et al.*, (2019) [22].

The potash mobilizing isolates (K I, K II and K III) exhibited round shaped colonies and possessed entire margins with convex elevations. All the isolates exhibited white pigmentation, were gram negative in reaction with rod shaped bacterial cells. The diameter of zone of solubilization formed by the isolates ranged from 2 mm to 5 mm at 72 hours after incubation (HAI). The isolate K II recorded maximum solubilization zone of 5 mm (Table 3). The results are in agreement with the findings of Kammar *et al.*, (2016) [13] and Parmar *et al.*, (2016) [14].

**Biochemical characterization of nitrogen fixing, phosphate solubilising and potash mobilizing bacterial isolates**

**Nitrogen fixing ability of isolates**

The bacterial isolates along with MPKV isolate (*Azotobacter chroococcum)* were subjected for estimation of nitrogen fixation by Microkjeldhal method. The A I isolate fixed highest amount of nitrogen (18.65 mg of nitrogen/g of sucrose used) than the other isolates (Table 1). The results of the present investigation are in agreement with results of Upadhyay *et al.*, (2015) [20] and Sanoria and Sundara Rao (1975) [18].

**Phosphate solubilizing ability of the PSB isolates**

All the three PSB isolates along with MPKV strain (*Bacillus subtilis*) were tested for their ability to solubilize inorganic phosphate both qualitatively and quantitatively and their results are presented in Table 2.

**Quantitative estimation of Pi released from TCP**

The amount of Pi released from tri-calcium phosphate by the isolates in Pikovskaya's broth was estimated at 10 days after inoculation. The amount of Pi released from TCP by the

isolates at 10 DAI ranged from 29.80 to 34.17 *per cent*. The isolate P III recorded significantly highest P-solubilization (34.17%) than the other isolates (Table 2).

**Decrease in pH of medium during phosphate solubilization**

The decrease in pH of TCP broth from initially adjusted pH of 7.0 was also noted at 10 days after inoculation. The significant reduction in pH of the medium i.e. pH 3.01 was recorded by P II isolate followed by P I and P III isolates which reduced the pH of the medium to 3.27 and 3.48 respectively (Table 2). The correlation of decrease in pH of the medium with the amount of Pi released was also worked out. At 10 days after incubation, they had significant positive correlation (r = + 0.219). The results comply with the findings of Wasule *et al.*, (2019) [22] and Whitelaw (2000) [23].

**Quantitative estimation of 'K' solubilization**

All the isolates exhibited zone of solubilization and were examined for their ability to solubilize muscovite mica on Alexandrov media supplemented with mica at one *per cent*. The amount of K released from muscovite mica in the broth by the isolates were studied at 7, 15 and 20 days after incubation (DAI) in lab condition and found in the range of 29.38 µg/mL to 41.48 µg/mL at 20 DAI. The results indicated that the amount of 'K' increased as the days of incubation increases and the highest amount of 'K' was present at 20 DAI. The highest solubilization after 20 DAI was observed in isolate K II (41.48 µg/mL) (Table 3). The results are in close conformity with the findings of Parmar *et al.*, (2016) [14].

On the basis of nitrogen fixing, phosphate solubilising and potash mobilizing ability, highly efficient nitrogen fixing *Azotobacter* isolate (A I), phosphate solubilising isolate (P III) and potash mobilizing isolate (K II) were tested for different biochemical tests.

**Biochemical characterization and identification of nitrogen fixing, phosphate solubilizing and potash mobilizing bacterial isolate**

The highly efficient nitrogen fixing *Azotobacter* isolate (A I) was tested for different biochemical characters *viz*., gram staining, gelatin liquefaction, catalase test, oxidase test, methyl red test, Voges-Proskaeur test, growth on carbon sources and growth at various temperatures (Table 4). The cells of bacterial isolate (A I) selected for consortium formulation were rod shaped and gram negative in reaction. The isolate A I reported positive for catalase and oxidase test while negative for indole production test, gelatin hydrolysis, methyl red test and Voges-Proskaeur test (Table 4). Glucose, sucrose, and mannitol were used as a sole carbon source for growth by bacterial isolate. Moreover, the growth of bacterial isolate was positive at various concentrations (5, 10, 20 and 30%) of sugar. The growth of bacterial isolate was positive (++) at 20ºC, 30ºC, and 40ºC temperature with highest (+++) growth observed at 30ºC, but was negative (-) at 10ºC and 50ºC. These results are in conformity with findings of Islam *et al.*, (2008) [11]. According to Bergey's manual of Systematic Bacteriology (Holt and Krieg, 1984) [10], the isolate A I was identified as *Azotobacter chroococcum* and used for formulation of microbial consortium. The results of the present investigation are in agreement with the results of Upadhyay *et al.*, (2015) [20]; Hala and Ali (2019) [9] and Gomare *et al.*, (2013) [7].

The highly efficient phosphate soubilising bacterial isolate P III was tested for different biochemical characters *viz*., gram staining, gelatin liquefaction, catalase test, oxidase test, starch hydrolysis, methyl red test, Voges-Proskaeur test and $H_2S$ production test. The cells of bacterial isolate (P III) selected for consortium formulation were rod shaped and gram positive in reaction. The isolate P III reported positive for catalase activity, gelatin liquefaction, starch hydrolysis and Voges-Proskaeur test while the isolate reported negative for $H_2S$ production test, oxidase activity and methyl red test (Table 4). The highly efficient phosphate solubilizing bacterial isolate P III showing highest solubilization of phosphate in TCP medium (P III- 34.17%) was identified by morphological examination, cultural studies and biochemical characterization as *Bacillus subtilis* according to the method given by Buchanon and Gibbons (1974) [3] and used for formulation of microbial consortium. The results of the present findings are in agreement with the findings of Anjhana and Sasikala, (2017) [2] and Vijayalakshmi and Murali (2015) [21].

The highly efficient potash mobilizing bacterial isolate K II was tested for different biochemical characters *viz*., sugar utilization, gram staining, gelatin liquefaction, catalase test, oxidase test, casein hydrolysis, methyl red test, Voges-Proskaeur test, urea hydrolysis, nitrate reduction, starch hydrolysis test and $H_2S$ production test (Table 4). The isolate K II was gram negative in reaction, reported positive for catalase activity, Voges-Proskaeur test and $H_2S$ production test while the isolate reported negative for oxidase test, starch hydrolysis, casein hydrolysis, urea hydrolysis, nitrate reduction, methyl red test and gelatin liquefaction (Table 4). The highly efficient bacterial isolate K II showing highest solubilization of potassium mineral (mica) in liquid medium (K II- 41.48 µg/mL) was identified as *Frateuria aurantia* by morphological examination, cultural studies and biochemical characterization and used for formulation of microbial consortium. The results of the present findings are in agreement with the findings of Chandra and Greep, (2006) [5].

**Growth and microbial count of *Azotobacter*, PSB and KMB on different culture media**

Efficient strains of *Azotobacter*, PSB and KMB were inoculated in broth of each culture media *viz.*, M I, M II, M III and M IV (Table 7). The data presented in Table 5. Revealed that the maximum growth of *Azotobacter*, PSB and KMB were found in M III culture medium containing Glucose (10 g/L), Ammonium sulphate (0.4 g/L) and Yeast extract (1 g/L). All these three beneficial microorganisms were found to be compatible with each other on culture media M III (Plate 1). However, among all the culture media, maximum c.f.u. count of *Azotobacter* (8.3 $X10^5$), PSB (7.7 $X10^5$) and KMB (8.0 $X10^5$) were recorded in M III culture medium (Table 6). The c.f.u. count of all the efficient isolates of *Azotobacter*, PSB and KMB was further recorded on their respective growth medium i.e. Jensen's agar medium, Pikovskaya's agar medium and Alexandrov's agar medium respectively (Table 6). Growth of *Azotobacter*, PSB and KMB was maximum in medium M III since it contained all the essential nutrients in sufficient amounts required by the bacteria for growth i.e. it contained $MgSO_4$, $CaCO_3$ and $K_2HPO_4$ etc. It has been reported that $MgSO_4$ prolongs the viability of microorganisms suspended in potassium phosphate buffer (Gunter, 1954) [8]. Also, Rojas *et al.*, (2011) [16] reported optimum growth of *Azotobacter* when supplemented with medium containing sucrose and yeast extract 13.06 g/L and 3.70 g/L respectively. Sagervanshi *et al.*, (2014) [17] studied the effect of different nitrogen sources like Ammonium sulphate, Casein, Sodium

nitrate and Urea and found the best optimized source was Ammonium sulphate for the maximum "P" solubilization. Furthermore, Chandra and Greep, (2006) [5] reported luxurious growth of *Frateuria aurantia* when supplemented with medium containing Mannitol 15g/L, Yeast extract 3g/L, Peptone 2g/L and trace elements 1 mL/L.

The microbial consortium formulated was used to study the inoculation effect on growth and yield of tuberose *cv. Phule Rajani.* Inoculations of consortium of *Azotobacter*, PSB and KMB with or without chemical fertilizers resulted in increased growth parameters and yield of tuberose against control.

**Table 1:** Nitrogen fixing ability of isolates by Microkjeldhal method

| S. No. | Isolate | Nitrogen fixing ability (mg of Nitrogen/g of sucrose) |
|---|---|---|
| 1. | A I | 18.65 |
| 2. | A II | 11.84 |
| 3. | A III | 7.32 |
| 4. | MPKV isolate (*Azotobacter chroococcum*) | 16.25 |

**Table 2:** Zone of 'P' solubilization on Pikovskaya's agar and *per cent* Pi released from TCP broth by the PSB isolates

| S. No. | Isolate | Zone of solubilization TCP (mm) | % Pi released from TCP after 10 days | Decrease in pH of medium (from initial pH 7.0) after 10 days |
|---|---|---|---|---|
| 1. | P I | 3.0 | 29.80 | 3.27 |
| 2. | P II | 4.0 | 31.48 | 3.01 |
| 3. | P III | 7.0 | 34.17 | 3.48 |
| 4. | MPKV isolate (*Bacillus subtilis*) | 6.0 | 28.35 | 3.50 |
| | Correlation (r) of Pi released with decrease in pH | | | 0.219 |

**Table 3:** Solubilization of muscovite mica by the KSB isolates

| S. No. | Isolate | Zone of solubilization (mm) | 7 DAI (µg/mL) | 15 DAI (µg/mL) | 20 DAI (µg/mL) |
|---|---|---|---|---|---|
| 1. | K I | 4.0 | 18.65 | 24.64 | 29.38 |
| 2. | K II | 5.0 | 24.84 | 36.61 | 41.48 |
| 3. | K III | 2.0 | 15.32 | 27.52 | 31.32 |
| 4. | MPKV isolate (*Frateuria aurantia*) | 5.0 | 23.25 | 35.10 | 39.25 |

**Table 4:** Biochemical characterization of nitrogen fixing, phosphate solubilizing and potash mobilizing isolate

| S. No. | Biochemical characteristics | Isolate A I | Isolate PIII | Isolate K II |
|---|---|---|---|---|
| 1. | Gram staining | - | + | - |
| 2. | Cell shape | Rod shape | Rod shape | Rod shape |
| 3. | Gelatin hydrolysis | - | + | - |
| 4. | Catalase test | + | + | + |
| 5. | Oxidase test | + | - | - |
| 6. | Casein hydrolysis test | | | - |
| 7. | Indole production test | - | | |
| 8. | Methyl red test | - | - | - |
| 9. | Voges-Proskaeur test | - | + | + |
| 10. | $H_2S$ production test | | - | + |
| 11. | Nitrate reduction test | | | - |
| 12. | Urea hydrolysis | | | - |
| 13. | Starch hydrolysis | | + | - |
| 14. | Sugar utilization | | | |
| | a) Maltose | + | | + |
| | b) Sucrose | + | | + |
| | d) Mannitol | + | | + |
| 15. | Growth at various concentration of sugar | | | |
| | a) 5% | + | | + |
| | b) 10% | + | | + |
| | c) 20% | + | | + |
| | d) 30% | + | | + |
| 16. | Growth at various temperatures | | | |
| | a) 10ºC | - | | |
| | b) 20ºC | ++ | | |
| | c) 30ºC | +++ | | |
| | d) 40ºC | ++ | | |
| | e) 50ºC | - | | |

**Table 5:** Growth of *Azotobacter*, PSB and KMB in different culture medium

| Culture medium | *Azotobacter* (c.f.u./g) | PSB (c.f.u./g) | KMB (c.f.u./g) |
|---|---|---|---|
| M I | + | + | + |
| M II | ++ | - | ++ |
| M III | +++ | +++ | +++ |
| M IV | ++ | + | - |
| Jensen's agar medium | +++ | - | - |
| Pikovskaya's agar medium | - | +++ | - |
| Alexandrov's agar medium | - | - | +++ |

**Table 6:** C.f.u. count of *Azotobacter*, PSB and KMB in different culture medium

| S. No. | Culture medium | *Azotobacter* ($X10^5$ c.f.u./g) | PSB ($X10^5$ c.f.u./g) | KMB ($X10^5$ c.f.u./g) |
|---|---|---|---|---|
| 1. | M I | 1.7 | 1.3 | 1.3 |
| 2. | M II | 1.0 | - | 3.3 |
| 3. | M III | 8.3 | 7.7 | 8.0 |
| 4. | M IV | 3.3 | 2.1 | - |
| 5. | Jensen's agar medium | 6.7 | - | - |
| 6. | Pikovskaya's agar medium | - | 6.3 | - |
| 7. | Alexandrov's agar medium | - | - | 6.7 |

**Table 7:** Composition of culture media for consortium of *Azotobacter*, PSB and KMB

| S. No. | Chemicals | Composition of culture media (g) | | | |
|---|---|---|---|---|---|
| | | M I | M II | M III | M IV |
| 1. | Glucose | 35 | 20 | 10 | 9 |
| 2. | Iron chloride | 0.005 | 0.1 | 0.1 | 0.1 |
| 3. | Tri calcium phosphate | 5 | 5 | 4.5 | 4 |
| 4. | Ammonium sulphate | 0.5 | 0.5 | 0.4 | 0.3 |
| 5. | Yeast extract | 0.5 | 1 | 1 | 1 |
| 6. | Magnesium sulphate | 0.1 | 0.6 | 0.5 | 0.3 |
| 7. | Potassium Chloride | 0.2 | 0.2 | 0.2 | 0.2 |
| 8. | Manganese sulphate | 1 | 0.1 | 0.1 | 0.1 |
| 9. | Ferrous sulphate | 0.2 | 0.1 | 0.1 | 0.1 |
| 10. | Calcium carbonate | 2.1 | 0.1 | 0.1 | 0.1 |
| 11. | Potassium alumino silicates | 2 | 2 | 2 | 2 |
| 12. | Dipotassium hydrogen orthophosphate | 1 | 0.5 | 0.4 | 0.3 |
| 13. | Sodium Chloride | 0.5 | 0.1 | 0.1 | 0.1 |
| 14. | pH | 6.9 | 7.1 | 6.9 | 7.0 |
| 15. | Distilled water | 1000 ml | 1000 ml | 1000 ml | 1000 ml |

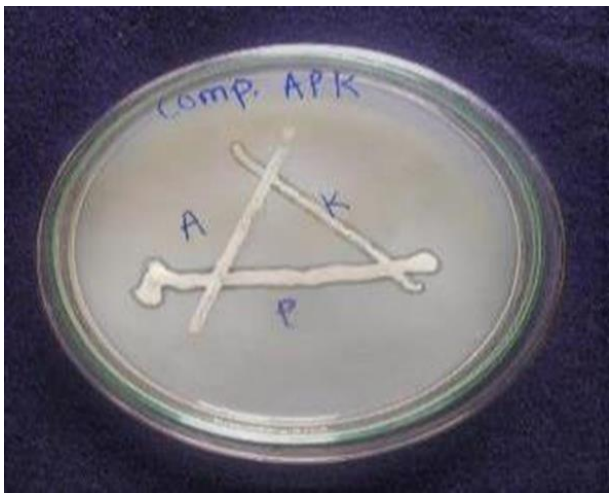


**Plate 1:** Compatibility of *Azotobacter*, PSB and KMB on M III culture medium

## Inoculum preparation

Inoculums of *Azotobacter chroococcum*, *Bacillus subtilis* and *Frateuria aurantia* were prepared in selective medium M III. The media was inoculated in 500 mL conical flask containing 150 mL medium and incubated at 28±2ºC under shaking at 100-150 rpm for three days to give an optical density of 0.5 recorded at 535 nm. Lignite powder used as carrier was sterilized at 121ºC and 1.04 kg/cm$^2$ pressure for one hour and inoculated with broth cultures of *Azotobacter chroococcum*, *Bacillus subtilis* and *Frateuria aurantia* (500 mL per 100 g of lignite powder). Lignite powder based inoculum was incubated at 28±2ºC for three days by adding 10% sugar solution to increase the population of respective microbe.

## Conclusion

Considering the production of *Azotobacter*, PSB and KMB biofertilizers separately which increases the cost of production and is also tedious to farmers for application of individual biofertilizer to the crop. Therefore, one mixed formulation of these biofertilizer strains suitable to crop will boost adoption and spread of biofertilizers technology. A culture medium which proves effective with respect to population stability of individual strain and effectiveness of consortia on growth and yield of a particular crop can be formulated. The findings show avenues for developing mixed consortia for other symbiotic and nonsymbiotic nitrogen fixing bacteria with phosphate solubilizing and potash mobilizing bacteria.

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