MATERIALS AND METHODS

Microbial cultures

Strains of nitrogen fixing and phosphate mobilizing microorganisms were obtained from the culture collection of Division of Microbiology, Indian Agricultural Research Institute, New Delhi, and the Institute of Microbial Technology, Chandigarh. One phosphate mobilizing microorganism (*Penicillium variable*) was isolated from a soil sample obtained from the experimental beds of the Institute of Agriculture, Aligarh Muslim University, Aligarh. The names of each group of microorganisms included in the study are given below:

Nitrogen-fixing microorganisms

(a) Symbiotic nitrogen fixers (Plate 1)
   i. *Rhizobium* sp. (F-75)
   ii. *Bradyrhizobium* sp. (Vigna)

(b) Free-living nitrogen fixer (Plate 1)
   *Azotobacter chroococcum*

Phosphate-mobilizing microorganisms

(a) Phosphate solubilizers
   i. *Pseudomonas striata*
   ii. *Penicillium variable*

(b) Non-solubilizer
   *Glomus fasciculatum* (VAM fungus)

Media

Different strains of *Rhizobium* sp., *Bradyrhizobium* sp.,
Plate 1: Streaked plates showing bacterial colonies

A and B: *Rhizobium* sp. and *Bradyrhizobium* sp. on yeast extract mannitol medium supplemented with Bromthymol blue.

C: *Azotobacter chroococcum* on Jensen’s medium.
Azotobacter sp. and phosphate solubilizers were normally grown in yeast extract mannitol medium (YEM), Jensen's medium and Pikovskaya's medium respectively, except for in vitro interaction studies where the modified media were used. For the maintenance of VAM fungus, *Glomus fasciculatum*, open pot culture method of Gilmore (1968) was used. The constituents of various media (g/1) are given below:

**Yeast Extract Mannitol Agar** (Vincent, 1970)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO$_4$$\cdot$7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

10 ml of Congo red stock solution (0.25g/100 ml) was added to the medium used for plating to count rhizobia.

**Pikovskaya's medium (g/l)** (Pikovskaya, 1948)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$</td>
<td>5.0</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>0.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO$_4$$\cdot$7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
</tbody>
</table>
KCl 0.2
MnSO₄ Traces
FeSO₄ Traces
Agar 20.0
pH 7.0

**Jensen’s Medium**

Sucrose 20.0
K₂HPO₄ 1.0
MgSO₄·7H₂O 0.5
NaCl 0.5
FeSO₄ 0.1
Na₂MoO₄ 0.005
CaCl₂ 0.2
Agar 20
pH 7.5-8.0

**Culturing of the VAM fungus**

Inoculum of the VAM fungus, *Glomus fasciculatum* was obtained from the Indian Agricultural Research Institute, New Delhi and maintained on rhodes grass (*Chloris gayana* Kunth.), following open pot culture method of Gilmore (1968). Seedlings of the grass were raised in clay pots, each containing 3.5 kg sterile sandy clay loam soil. Hoagland solution was applied soon after sowing and then regularly at 15 days interval. The grass was grown for 4 months.
Inoculation was done by placing 125 g of the inoculum containing roots of the rhodes grass infected with the VAM fungus, *Glomus fasciculatum* and its spores. Inoculum was covered with a soil layer of 2 cm, over which seeds were sown. Again seeds were covered with a layer of soil.

Numbers of *G. fasciculatum* spores in the inoculum were determined by the wet-sieving and decanting technique (Gerdemann and Nicolson, 1963). The percentage mycorrhizal infection of the roots was determined by the root slide technique after clearing the roots with 10% KOH and staining with trypan blue (Phillips and Hayman, 1970).

**Isolation and screening of phosphate mobilizing microorganism**

For isolation of phosphate mobilizers, soil samples were collected from the rhizosphere of potato (*Solanum tuberosum* L.), onion (*Allium cepa* L.), lettuce (*Lactuca sativa* L.), okra (*Abelmoschus esculentus* Moench.), tomato (*Lycopersicum esculentum* L.), chilli pepper (*Capsicum annum* L.), brown mustard (*Brassica campestris* L.) and maize (*Zea mays* L.) growing in the fields at the Institute of Agriculture, Aligarh Muslim University, Aligarh. Physical, chemical and microbiological analyses of the soil carried out in this experiment are given in Appendix 1 and 2.
(a) Preparation of the soil samples

The soil samples were air-dried and ground to pass through a 2 mm sieve. Sufficient amount of this soil was kept in clean and sterilized bottles for isolation and microbiological, chemical and physical analyses.

(b) Method of isolation

Enrichment culture technique (in liquid medium) was used for the isolation of phosphate solubilizing microorganisms. One gram of rhizosphere soil from each sample was added to 250 ml conical flask containing 100 ml modified Pikovskaya broth and incubated at 28°C±2°C temperature. Three successive transfers were made at 7 day intervals to enrich the culture. From the final flask, a loop-full of the culture was streaked on plates containing solidified Pikovskaya’s medium. The colonies showing clear zones of tricalcium phosphate solubilization around them developed within 48 h were transferred to agar slants of Pikovskaya’s medium and allowed to grow at 28°C±2°C for three days. The cultures were then repeatedly plated using the same agar medium till pure culture of the isolated strains were obtained and finally 8 bacterial and 34 fungal cultures were maintained on Pikovskaya’s medium.

**Modified Pikovskaya’s medium (g/l) for isolation**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$ or Rock phosphate</td>
<td>20.0</td>
</tr>
<tr>
<td>(NH$_4$)$_2$ SO$_4$</td>
<td>0.5</td>
</tr>
</tbody>
</table>
NaCl 0.2
MgSO₄·7H₂O 0.1
Yeast extract 0.5
MnSO₄ Traces
FeSO₄ Traces
Distilled water 1 lit.
pH 7.0

Modified Pikovskaya broth used for isolation was sterilized at 15 lb/sq inch pressure for 15 minutes.

Screening of the isolated cultures

All the cultures of the isolated phosphate solubilizers were screened on the basis of maximum solubilization of tricalcium phosphate or rock phosphate in liquid culture medium. (Table 8). The most efficient strain of rock phosphate solubilizing fungus was identified as Penicillium variable and selected for further studies.

Interaction of phosphate mobilizing microorganisms with nitrogen fixing microorganisms in liquid culture medium

Interactions of nitrogen fixing and phosphate solubilizing bacteria were studied in vitro using modified medium devoid of soluble phosphate source. Their individual or interactive effects on the population count, change in pH and phosphate solubilizing and nitrogen fixing activities were determined.

Modified medium for interaction studies

Modified medium was prepared by choosing the required nutrients from both Pikovskaya and yeast extract mannitol medium.
as given below (g/l):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.0</td>
</tr>
<tr>
<td>Ca$_3$(PO$_4$)$_2$</td>
<td>5.0</td>
</tr>
<tr>
<td>(NH$_4$)$_2$ SO$_4$</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
<tr>
<td>MgSO$_4$$\cdot$7H$_2$O</td>
<td>0.2</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>MnSO$_4$</td>
<td>Traces</td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>Traces</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 lit.</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

(a) Interaction of phosphate solubilizing bacteria *Pseudomonas striata* with symbiotic nitrogen fixing *Rhizobium* sp. or *Bradyrhizobium* sp.

Strains of *Bradyrhizobium* sp. and *Rhizobium* sp. specific for greengram (*Bradyrhizobium* sp. (vigna) and chickpea (*Rhizobium* sp. (F-75) were grown either singly or in combination with *P. striata* in modified medium devoid of soluble form of phosphorus to study their growth, pH change and phosphate solubilizing activity.

For this study 50ml of the modified medium was dispensed in 250 ml flasks which were sterilized at 15 psi for 15 min at 121°C. A set of flask containing 50 ml of yeast extract mannitol broth, without insoluble tricalcium phosphate, was also prepared and
sterilized simultaneously. The flasks were then inoculated with 0.1 ml of 48 h old culture suspension of either *Rhizobium* sp. or *P. striata* or both as per the treatments given below

$T_1$ *Rhizobium* sp. or *Bradyrhizobium* sp. in YEM broth (without tricalcium phosphate)

$T_2$ *Rhizobium* sp. or *Bradyrhizobium* sp. in the modified medium.

$T_3$ *P. striata* in the modified medium

$T_4$ *Rhizobium* sp. or *Bradyrhizobium* sp. and *P. striata* in the modified medium

Three sets of experiments were conducted using each strain of *Rhizobium* sp. or *Bradyrhizobium* sp. with similar treatments as mentioned above. A set of uninoculated control of yeast extract mannitol broth and modified medium was also maintained and used for phosphate estimation at different stages of incubation. Nine replicates were used for each treatment. All the flasks were incubated at $28^\circ C \pm 2^\circ C$.

**Observations**

The following observations were made:

1. **Population counts**

   Population counts of *Rhizobium* sp. or *Bradyrhizobium* sp. and *P. striata* were made by dilution plating at an interval of 24 h for six days. For *Rhizobium* sp. or *Bradyrhizobium* sp., yeast
extract mannitol agar medium supplemented with congo red and for *P. striata*, Pikovskaya’s agar medium was used.

2. **Change in pH**

On 8, 15, 22 day of incubation, the change in pH of the medium was noted using a digital pH meter (Elico, Hyderabad).

3. **Soluble phosphates and nitrogen estimation**

Three replicates of each treatment were used for phosphate and nitrogen estimations on 8, 15 and 22 day of incubation. The amount of phosphate solubilized was estimated by the procedure given by King (1932) and later modified by Jackson (1967).

For estimation of the soluble phosphate at the end of each incubation period, the volume of the medium in each flask was made upto 50 ml in volumetric flask with double distilled water and the culture suspension was centrifuged at 15000 rpm for 20 min in high speed centrifuge (Remi centrifuge, India). One ml of supernatant was taken in a 50 ml volumetric flask to which 10 ml of chloromolybdic acid was added and mixed thoroughly. Double distilled water was added to make the volume approximately 40 ml and 5 drops of chlorostannous acid were added to it. Immediately the volume was made upto 50 ml with double distilled water. After 15 min. the colour (blue) was read in a spectrophotometer at 610 nm using a reagent blank. Simultaneously, a standard curve was also plotted using various concentrations of standard 2 ppm KH$_2$PO$_4$ solution. The amount of phosphorus
solubilized was calculated from the standard curve.

**Chloromolybdic acid reagent**

The reagent was prepared by dissolving 15 g ammonium molybdate in 300 ml warm double distilled water to which 342 ml of concentrated HCl was added and finally the volume was made upto one liter with double distilled water.

**Chlorostannous acid reagent**

This reagent was prepared by dissolving 2.5 g of SnCl₂⋅2H₂O in 10 ml concentrated HCl and the volume was made upto 100 ml with distilled water.

Nitrogen content in the culture was estimated by modified Kjeldahl method given by Iswaran and Marwaha (1980). 50 ml of the culture medium was taken in the Kjeldahl flask, moistened with 5 ml water, containing 15 ml N/100 H₂SO₄ and shaken thoroughly. This was followed by the addition of N KMnO₄ in small amount till pink colour appeared. The catalyst mixture (3g K₂SO₄, 0.3g FeSO₄⋅5H₂O and 0.15g CuSO₄⋅5H₂O) was then added and sample was digested for 30 min on low flame till the mixture was yellowish green in colour. The ammonia in the digest was determined by usual micro-Kjeldahl method.

**(b) Interaction of phosphate solubilizing P.striata with free living nitrogen fixer A. chroococcum**

An efficient strain of N₂-fixing A.chroococcum and P.striata were grown singly or in combination in modified Jensen’s medium.
devoid of soluble phosphorus source to study their interaction in in vitro. The interactive effects were measured by determining their growth, effect on pH and phosphate solubilizing and nitrogen fixing activities under in vitro conditions.

**Modified Jensen's medium (g/l)**

This medium was prepared by choosing the required nutrients from both Jensen’s medium and Pikovskaya’s medium.

- Glucose: 15.0
- Tricalcium phosphate: 5.0
- NaCl: 0.5
- KCl: 0.2
- MgSO$_4$$\cdot$7H$_2$O: 0.5
- Yeast extract: 0.5
- (NH$_4$)$_2$SO$_4$: 0.5
- MnSO$_4$: Traces
- Na$_2$MoO$_4$: 0.00
- CaCl$_2$: 0.2
- FeSO$_4$: 0.1
- Distilled water: 1 lit.
- pH: 7.0

In modified Jensen’s medium, soluble phosphate sources were replaced by insoluble tricalcium phosphate and sucrose was replaced by glucose. For this study 50 ml of modified medium was dispensed in 250 ml flasks which were sterilized at 15 psi for 15
minutes at 121°C. A set of flasks containing 50 ml of Jensen's medium (broth), without insoluble tricalcium phosphate, was also prepared and sterilized simultaneously. The flasks were then inoculated with 0.1 ml of 48 h old culture suspension of either *A. chroococcum* or *P. striata* or both as per the treatments given below:

\[ T_1 \quad A.\textit{chroococcum} \text{ in Jensen's broth} \]

\[ T_2 \quad A.\textit{chroococcum} \text{ in modified medium} \]

\[ T_3 \quad P.\textit{striata} \text{ in modified medium} \]

\[ T_4 \quad A.\textit{chroococcum} \text{ and } P.\textit{striata} \text{ in modified medium.} \]

A set of uninoculated control of Jensen's broth and modified medium was also maintained and used for phosphate estimation at different stages of incubation. Nine replicates were used for each treatment. All the flasks were incubated at 28±2°C.

**Observations**

The following observations were made at different stages of incubation.

1. **Population counts**

Population counts of *A. chroococcum* and *P. striata* were made by dilution plating on 0, 2, 5, 10, 15, 20, 25 & 30 days of incubation. For *A. chroococcum* Jensen’s agar medium and for *P. striata*, Pikovskaya’s agar medium was used.
2. Change in pH

On 0, 2, 5, 10, 15, 20 days of incubation, the change in pH of the medium was noted using a digital pH meter (Elico, Hyderabad).

3. Soluble phosphates and nitrogen estimation

Three replicates of each treatment were used for phosphate and nitrogen estimations on 10, 20 and 30 days of incubation. The amount of phosphate solubilized was estimated by the procedure given by King (1932) and modified by Jackson (1967) and nitrogen was estimated by modified Kjeldahl method given by Iswaran and Marwaha (1980) as mentioned earlier.

Interaction of phosphate mobilizing microorganisms with nitrogen fixing microorganisms in soil and their effect on different crops

Preparation of soil

The sandy clay loam soil from the Institute of Agriculture, A.M.U., Aligarh, was used throughout the study. After sieving, the soil was thoroughly mixed with Mussoorie rock phosphate (obtained from Pyrite phosphates and Chemicals Ltd., New Delhi) @ 45 kg P₂O₅/ha, urea @20/ kg N/ha and muriate of potash @ 40 kg K₂O/ha and filled in clay pots (25 cm diameter). The pots along with 3 kg of soil were sterilized three times in an autoclave at 15 psi for 3 h on alternate days. Physical, chemical and microbiological analyses of the soil were done and data are given in Appendix 1 and 2.
Interaction of phosphate mobilizing microorganisms with symbiotic nitrogen fixers and their effect on legume crops

The experiment was conducted to study the associative effect of *Rhizobium* sp., *P. striata* or *P. variable* and *G. fasciculatum* on greengram (*Vigna radiata* (L.) Wilczek var. T44) and chickpea, (*Cicer arietinum* (L.) var. T3).

**Treatments**

- **T₁**  Control
- **T₂**  *Rhizobium* sp. or *Bradyrhizobium* sp.
- **T₃**  *Pseudomonas striata*
- **T₄**  *Penicillium variable*
- **T₅**  *Rhizobium* sp. or *Bradyrhizobium* sp. + *P. striata*
- **T₆**  *Rhizobium* sp. or *Bradyrhizobium* sp.+*Penicillium variable*
- **T₇**  *Rhizobium* sp. or *Bradyrhizobium* sp. + *P. striata* + *G. fasciculatum*
- **T₈**  *Rhizobium* sp. or *Bradyrhizobium* sp. + *P. variable*+ *G. fasciculatum*

Same treatments were given to both the legume crops changing the strain of *Rhizobium* sp. with *Bradyrhizobium* sp. specific for the crop. Eight replicates were maintained for each treatment.
Seed treatment and sowing

Seeds were surface sterilized with 0.1% mercuric chloride and inoculated with *Rhizobium* sp. or *Bradyrhizobium* sp. by soaking in 6 day old broth culture as per treatment for one hour and sown after drying at room temperature. For combined inoculation, the required cultures were mixed in equal proportions and then seeds were soaked in it. Fungal culture was filtered, the mycelial mat was suspended in sterile distilled water and shaken vigorously before use. Mycorrhizal fungus was added into the soil before sowing as mentioned earlier. Ten seeds each of chickpea and greengram were sown in each pot during October to January and June to August respectively. After germination, thinning was done to retain 4 plants per pot. The pots were watered regularly to maintain the optimum moisture level. Eight replicates were maintained for each treatment.

Observations

Four replicates of each treatment were used for taking observations at 45 and 90 day in chickpea and 35 and 60 day in greengram. Plants were depotted and soil was removed by washing gently under running tap water and the number of nodules and percent root infection were recorded. The plants were dried at 60°C in hot air oven for 72 h. Dry weight of plants, nodules and grains were determined. For chemical analysis of plants and grains, samples were digested. Total nitrogen was estimated by modified Kjeldahl method (Iswaran and Marwaha, 1980) and
phosphorus by the method given by Jackson (1958). Population count of rhizobia and phosphate solubilizing microorganisms and available phosphorus and available nitrogen of the rhizosphere soil were also estimated.

**Interaction of phosphate mobilizing microorganisms with free living nitrogen fixing microorganisms and their effect on wheat crop**

The associative effect of *A.chroococcum*, *P.striata* or *P.variable* and mycorrhizal fungus *G.fasciculatum* on wheat (*Triticum aestivum* L. var. PBW154) was also studied. Wheat was grown from November to April. For this experiment, nitrogen was added @ 90 kg/ha as urea, rock phosphate @ 60 kg P₂O₅/ha and muriate of potash @ 40 kg K₂O/ha to the soil. Treatments and observations were almost same as in the earlier experiment except, instead of *Rhizobium* sp. or *Bradyrhizobium* sp., *A.chroococcum* was inoculated and observations were made at 40 and 80 days.

**Statistical analysis of the data**

Data obtained from the laboratory and pot experiments were analysed statistically by the method of analysis of variance (Fisher, 1958). Standard error and critical difference at 5% level of significance were calculated.