MATERIALS AND METHODS

3.1 Experimental Site

Three varieties viz. Kufri sindhuri, Kufri chipsona-3 and Kufri lauvkar were assessed for their rhizospheric and non-rhizospheric microbial diversity. These work sites of varieties were generously provided by the Central Potato Research Station, Maharajpura, Gwalior, MP State of India. Gwalior is situated at Latitude 26° 13’ 5.8332” N and longitude 78° 10’ 58.1916” E. The Institute is spread over an area of nearly 400 acres and is under the professional management of the Indian Council for Agricultural Research, New Delhi.

3.2 Selection of plant

The agro climatic conditions of Gwalior are not ideal for potato production because the area being extremely hot in summer and cold in winter. It is a therefore, climatically low development and of low out area for potato production. This may thus effect potato production by both abiotic and biotic factors. The selected plant for the present study was “Potato plant” (Solanum tuberosum L.). Rhizospheric and non-rhizospheric soil (bulk) samples were collected from three different potato varieties viz. Kufri sindhuri, Kufri
Materials and Methods

chipsona-3 and Kufri lauvkar, from 6 to 30 cm depth from the surface with the help of sterilised suitable equipment and packed in sterile plastic bags. These were stored at 4°C. Sampling extended from the seedling up to harvesting of potato plants at fortnightly intervals.

![Kufri Luvkar, Kufri Sindhuri, Kufri Chipsona-3](Images)

**Fig. 3.1:** Different cultivars of potato plant

![Rhizospheric soil, non-rhizospheric soil (bulk)](Images)

**Fig. 3.2:** Collection of soil sample from rhizospheric and non-rhizospheric (bulk) soil
3.3 Soil pH and EC analysis

The soil pH was determined by the method of Jackson (1967) with a slight modification. 10 gm of soil sample was suspended in 90 ml distilled water. The suspension was shaken vigorously and allowed to settle overnight. Systronic pH meter analytic was used to determine pH.

The Electrical conductivity of the soil was determined in the same suspension as used in the pH measurement, with the help of a conductivity meter following modified Jackson (1967) method. The EC is expressed in deci Siemens per meter (dSm\(^{-1}\)) at 25\(^0\)C. Systronic analytic and sysmatic, conducting meter was used for the purpose.

3.4 Preparation of dilutions, inoculations and observations

3.4.1 Dilution Method

In 250 ml capacity of conical flasks, 10 g of soil both from rhizosphere and non- rhizosphere separately were added in the already contained 90 ml of sterile distilled water. The flask was shaken for 10 min on a rotary shaker. One ml of the shaken suspension was added to 9 ml sterile distilled water in a sterile test tube and re-shaken for 2 min. This represented 10\(^1\) dilutions. Similarly, a series up to 10\(^7\) dilutions was prepared under aseptic conditions. These suspensions were spread on the plates of solid Nutrient agar in petri dishes autoclaved at 15 Lbs pressure for 20 minutes. (NA) medium contain (5.0 g
peptone, 1.5 g yeast extract, 1.5 g beef extract, 5.0 g NaCl, 20 g Agar in one liter pH 7.2).

3.4.2 Spread plate method

0.1ml (100 µl) aliquot of this suspension was spread on the plates of Nutrient agar (NA) medium. Plates were incubated for 48hrs at 28±1°C and the different bacterial colonies observed. Isolated single colony was re-streaked on fresh NA medium plate and re incubated.

Fig 3.3: Spread plate method (www.wikipedia.com)

3.4.3 Standard plate count method (CFU)

Standard plate count method was used to enumerate the bacterial cultures. 100µl sample from each dilution was spread over the NA plate and the colonies were formed after incubation then colonies were counted. The number of bacterial colonies in each were referred to as colony forming units (CFU). Colonies exhibiting good variable growth were selected for further streaking on fresh plates. Further purification and multiplication of isolates was done by streaking on fresh plates. The CFU was determined by the relation (Usha rani et al., 2012).
CFU/g = Average no of colonies/inoculation volume plated (ml) x Dilution Factor

3.4.4 Streak plate method

The streak plate method was standardised for obtaining discrete colonies and pure cultures. The inoculating loop or transfer needle was flame sterilised till red hot, cooled and dipped in a diluted suspension of organisms or touched with a single bacterial colony. This was then streaked on the surface of an already solidified agar plate to make a series of parallel, non overlapping streaks. After streaking the loop was again flame sterilised. The petriplates were sealed with a parafilm and incubated at 28-30°C for 24-48 hrs in an inverted position.

Fig. 3.5: Streaking of pure culture of different bacterial colony on NA media
3.5 **Maintenance of isolates**

These Bacterial pure cultures were maintained on the NA medium slants in glass culture tubes. All these isolates were maintained at 4°C. For longer duration 70% culture broth and 30% glycerol in sterilised vials or eppendorf tube were stored at -20 °C.

3.6 **Bacterial identification**

The incubated colonies were observed visually and by magnification to study morphological characteristics for colour, colony shape, colony margins, and their elevation following Goenadi and Sugiarto (2000). Bacterial slides were prepared for Gram staining, so as to differentiate the isolated bacteria into gram positive and gram negative groups (Aneja, 2007).

Bacterial identifications were done using VITEK-2 method at Supratech Micropath Laboratory, Ahmadabad. The reagent cards have 64 wells that can each contain an individual test substrate. Each card has a pre-inserted transfer tube used for inoculation. Cards have bar codes that contain information on product type, lot number, expiration date, and a unique identifier that can be linked to the sample either before or after loading the card onto the system. Four reagent cards are available for the identification of different organism classes as follows:

1. GN - Gram-negative fermenting and non-fermenting bacilli
2. GP - Gram-positive cocci and non-spore-forming bacilli
3. YST - yeasts and yeast-like organisms
4. BCL - Gram-positive spore-forming bacilli

3.7 in vitro Screening of PGPR isolates for their PGPs traits

3.7.1 Phosphate solubilisation activity in different medium

(a) Qualitative assay of phosphate solubilisation

1. Pikovskaya agar medium containing Methyl red

The Phosphate solubilisation potential of selected bacterial isolates were tested by spot inoculation on the Pikovskaya agar medium added with 1% methyl red (Parikh and Jha, 2012) and containing 0.50 g Yeast extract, 0.00001g (arbritary) Ferrous sulphate, 10.0 g Dextrose, 5.0 g tricalcium phosphate, 0.50 g Ammonium sulphate, 0.20 g Potassium chloride, 0.10 g Magnesium sulphate, 0.0001 g Manganese sulphate, 15.0 g Agar in 1000 ml of distilled water maintained at pH 7.2 (Pikovskaya, 1948). Plates after 96 hrs incubation at 28±1°C were observed for the development of clear zone around the specific colony.

2. Pikovskaya agar medium containing Methyl Red orange

The phosphate solubilisation bacteria were characterised by spot inoculation of the single bacterial isolate on the Pikovskaya agar medium (Pikovskaya, 1948) containing tricalcium phosphate pH 6.6 modified with 1% of methyl red orange. Plates after 96 hrs incubation at 28±1°C were observed for the clear zone around the colony.
3. **Pikovskaya agar medium containing Bromophenol blue**

The phosphate solubilisation bacteria were characterised by spot inoculation of the single bacterial isolate on the Pikovskaya agar medium (Pikovskaya, 1948) containing tricalcium phosphate pH 6.0 with 1% of bromophenol blue (Gupta et al., 1994). Plates after 96 hrs incubation at 28±1°C were observed for the clear zone around the colony.

(b) **Quantitative assay of phosphate solubilisation**

NBRIP (National Botanical Research Institute’s phosphate) growth medium as suggested by Nautiyal, (1999) was used in the study for quantitative assay. The NBRIP medium containing 10g Glucose, 5g (Tricalcium phosphate) Ca₃(PO₄)₂, 5g (Magnesium chloride) MgCl₂.6H₂O, 0.25g (Magnesium sulphate) MgSO₄.7H₂O, 0.2g KCl, 0.1g (NH₄)₂SO₄ in one liter as broth. 50ml portions of this broth were dispensed in each 250 ml erlenmeyer flask. These flasks were autoclaved at 120°C and 15 lbs.in⁻² pressure for 15 min. Inoculation was done with 500µl suspension of an already grown each isolates culture and incubated at 28±1°C for 5 days on shaker incubator with 60 rpm. Uninoculated broth served as control. The cultures were centrifuged at 10,000 rpm for 20 min. Presence of yellow colour in the supernatant produced after addition of ammonium molybdate and ammonium vandate (1:1) confirmed phosphate solubilising activity. Colour intensity
Materials and Methods

of the solution was measured with the help of systronics spectrophotometer at 420nm after 25 minutes of reagent addition for quantification of phosphate solubilisation. The standard curve was made with ascending logarithmic concentrations of monobasic KH$_2$PO$_4$ (Kumar et al., 2012; Mehta and Nautiyal, 2001).

![Standard curve of KH$_2$PO$_4$](image)

**Fig. 3.6: The standard calibration curve of KH$_2$PO$_4$ plotted as X-Y slope**

3.7.2 Indole Acetic Acid (IAA) presence

50 ml of nutrient broth in 250 ml erlenmeyer flasks were added with 0.1% DL-tryptophan. Inoculation was done with 500µl of 24 hrs old bacterial isolate. These were then placed for 48 hrs on a Shaker at 28±1°C speed of 60 rpm. The bacterial cultures were centrifuged at 10,000 rpm for 10 min at 4°C on Remi ultracentrifuge. 2ml of supernatant was mixed with 4 ml Salkowski reagent containing 1 ml of 0.5 M FeCl$_3$ and 50 ml of 35% HClO$_4$ (perchloric acid). IAA production was indicated by the development of pink colour. Absorbance as
optical density was measured at 530 nm with the help of systronics spectrophotometer. Standard curve of IAA was obtained by a plot made with dilution from a stock ranging between 10-100 µg/ml of IAA (Hi-media) (Ahmad et al., 2008; Kumar et al., 2012).

![Standard curve of IAA](image)

Fig. 3.7: The standard calibration curve of IAA plotted as X-Y slope

### 3.7.3 Ammonia (NH₃) production

10 ml capacity culture tubes containing peptone water were incubated with bacterial isolates for 72 hrs at 28±1°C. Development of brown to yellow colour by the addition of Nessler’s reagent (0.5 ml) indicated NH₃ production (Cappuccino and Sherman, 1992; Ahmad et al., 2008).

### 3.7.4 Hydrogen cyanide (HCN) production

Solidified agar plates were made by pouring 25ml of agar from a stock of one liter nutrient agar containing 4.4 g glycine. Bacterial isolates were streaked on
these plates by platinum loops. Whatman filter paper 3mm discs soaked in 2% sodium carbonate solution made in 0.5% picric acid were placed over the surface of the media plate. Plates were sealed with parafilm and incubated at 28±1°C for 4 days. Filter paper discs turning brown to dark brown indicated HCN production (Lorck, 1948; Kumar et al., 2012).

3.7.5 Siderophore presence
Siderophore production by isolates was detected by the universal method after using blue agar plates containing the dye chrome azurol S. Fresh bacterial culture were spot inoculated on chrome azurol S (CAS) agar plates {contain 60.5 mg CAS was dissolved in 50 ml distilled water and mixed with 10 ml iron III. solution (1 mM FeCl$_3$6H$_2$O, 10 mM HCl) and this solution was slowly mixed in 72.9 mg HTAB (hexadecyl trimethyl-ammonium bromide) dissolved in 40 ml distilled water} and incubated for 4 days at 28±1°C. Development of yellowish orange halo around the colony was considered as positive for siderophore production (Schwyn and Neilands, 1987; Bholay et al., 2012).

The quantitative estimation of siderophore was done by CAS-shuttle assay, in which the isolates were grown on JNFb’ broth medium (0.5% of malic acid, 0.06% of K$_2$HPO$_4$, 0.18% of KH$_2$PO$_4$, 0.02% of MgSO$_4$.7H$_2$O, 0.01% of NaCl, 0.02% of CaCl$_2$.2H$_2$O, 0.0002% of Na$_2$MO$_4$, 2H$_2$O, 0.45% of KOH, 5ml/L of bromothymol blue {0.5% in 0.2 N KOH} and 2µM FeCl$_3$) (Döbereiner, 1995). These were incubated at 28±1°C for 3 days. Cultures were
centrifuged at 8000 rpm for 5 minutes. 0.5 ml supernatant was mixed with 0.5 ml CAS assay solution. The color obtained was measured as absorbance using systronic spectrophotometer at 630 nm. 0.5ml of uninoculated medium with 0.5 ml CAS solution was used as a blank. The percentage of siderophore units was estimated as the proportion of CAS color using the relation:

\[
\% \text{ Siderophore units} = \left(\frac{Ar - As}{Ar}\right) \times 100
\]

where, \(Ar\) is the absorbance of blank and \(As\) the absorbance of the sample at 630nm (Payne,1994; Bholay et al., 2012).

(a) **Hydroxamate-type**

Hydroxamate-type of siderophore was estimated in the culture supernatant by added pinch of tetrazolium salt then add 1-2 drops 2N NaoH and then 0.1ml sample instant deep red color observed (Atkin et al., 1970).

(b) **Catecholate -type**

This siderophore was estimated in the 1ml culture supernatant then add 1ml of 0.5 N HCl and then added 1ml of Na nitrite and Na molybdate (1:1) solution produced yellow color (Arnow, 1937).

3.7.6 **Antagonistic effects of bacterial isolates against Fusarium and pythium sp.**

Inhibition activity of the selected 40 bacterial isolates against *Fusarium* and *pythium* (pathogen cultures provided by the Plant Pathology lab. of the SOS
Botany, Jiwaji University) were evaluated based on dual culture technique. Potato dextrose agar (PDA) was prepared by using 200g peeled potato; 20g dextrose; 15g agar heated and refluxed in one liter water. The PDA was poured warm into sterile Petri dishes of 10cm dia. 5 mm mycelia plug of the respective fungus was inoculated at the centre of petri dish. Bacteria were spot inoculated against the fungal pathogen and plates were incubated at 28°C for 3 days. Radial growth of the test fungus was measured by percentage growth inhibition using the relation:

\[
\% \text{ inhibition} = \frac{(C - T)}{C} \times 100
\]

where, C is control and T is test (Sakthivel and Gnanamanickam, 1987; Fatima et al., 2009).

### 3.8 Molecular Characterisation of PGPR

On the basis of culture screening 5 PGPR were ultimately used for treatment of pot cultured potato plants. Basis for selection being that these 5 isolates produced significantly good growth results under pot culture for potato varieties. Hence these only were used for molecular characterisation.

#### 3.8.1 Genomic DNA Isolation

Fresh bacterial culture was grown overnight in nutrient broth for further genomic DNA isolation. Isolation was performed using manual isolation protocol (Sambrook and Russell, 2001). Fresh bacterial strain cultures were inoculated overnight in 50ml flask in nutrient broth at 120rpm in incubator
shaker. Further, overnight cultures were centrifuged at 7000 rpm for 10 minutes at 4°C. Subsequently, supernatant were discarded and the pellet was resuspended into 1400 µl of TES buffer (5ml of 1M Tris pH 8.0, 2ml of 0.25M EDTA, 0.3g NaCl, 50ml MilliQ water) respectively, which were incubated on ice for 10 min then added 100 µl (20mg/ml) of lysozyme and incubated at 37°C for 30 minutes in water bath. After thirty minutes, Proteinase K (2mg/ml) was added (190 µl) and mixture was kept at 45°C for 30 minutes, 150 µl 20% SDS was incorporated and incubated at 65°C for 30 minutes in a water bath. Each tube were added with 40µl RNase (10mg/ml) for the removal of RNA contamination with incubation at 37°C for 35 minutes. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1v/v) mixture was added to the homogenate and it was mixed gently by inverting the tubes for 10 minutes. After homogenisation, tubes were centrifuged at 8000 rpm for 40 minutes at 40°C. Upper aqueous phase was collected and mixed with equal volume of chloroform: isoamyl alcohol (24:1v/v) mixture. The homogenate was equalized by inverting the tubes and centrifuged at 8000 rpm for 20 minutes. Aqueous phase was taken into a fresh tube and added with equal amount of chilled isopropanol. Each tube were mixed properly and kept at 0°C for 1 hour. After one hour, tubes were centrifuged at 8000 rpm for 15 minutes and pellet was extracted and treated with 200 µl of 70% chilled ethanol twice with centrifugation at 8000 rpm for 15 minutes after each step. Finally supernatant was discarded and pellet was obtained and kept at 37°C for
drying. 200 µl, 10mM TE buffer (pH 8) was added to the dried pellet. DNA isolated from each of the culture form was utilised further for amplification of 16s rRNA gene.

3.8.2 Gel Electrophoreses

Preparing agarose gel involved melting a 0.8% of agarose in 1X TBE buffer, cooling the solution, and pouring it into the gel casting tray with ethidium bromide. Gels solidify in 15-20 minutes. Solidified gels were transferred to gel tank and electrical lead to the gel tank was attached firmly. 10 µl tracking dye as control, 10 µl (7 µl DNA + 3 µl tracking dye), and 10 µl (7 µl 1 kb ladder + 3 µl tracking dye) were loaded in wells. Electric supply at constant current 90 mA and voltage 75 volt at least for 90 minutes was given. DNA run was continued until the tracking dye had migrated an appropriate distance through the gel. The electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.8.3 Primer for 16s rRNA-PCR analysis

DNA was isolated as reported above from 5 PGPR culture tagged as PR1 and PR3, rhizobial and PB10, PB11 and PB12 as non rhizobial. Universal bacterial 16s rRNA primer was got synthesised from Labreq Bio Scientific, Ahmadabad, India.
Table 3.1: The motif sequence of the reverse and forward primers

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Primer Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward universal bacterial</td>
<td>5’GAGAGTTTGATCCTGGCTCAG3’</td>
<td>Lane, 1991</td>
</tr>
<tr>
<td>primer (27F)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse universal bacterial</td>
<td>5’AGAGTTTGATCCTGGCTCAG3’</td>
<td>Lane, 1991</td>
</tr>
<tr>
<td>primer (1492R)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR amplification was carried out in a final volume of 20 µl using the bacterial DNA as template. The reaction was carried out as given below:

1. PCR cocktail comprised of reaction mixture 20µl taken in a 0.1 ml PCR tube
   a) Emerald Amp master mix from Takara (2x) : 10 µl
   b) synthesised forward primer : 1 µl
   c) synthesised reverse primer : 1 µl
   d) milliQ water : 7 µl
   e) template DNA : 1 µl

2. The reaction mixture was thoroughly mixed by gentle pipetting and the tube was then placed on to the thermal cycler (Bio-Rad, USA).
The thermal amplification programmed was:

<table>
<thead>
<tr>
<th>Temp</th>
<th>Time</th>
<th>No. of cycles</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>45 sec</td>
<td>35</td>
<td>Denaturation</td>
</tr>
<tr>
<td>55°C</td>
<td>60 sec</td>
<td></td>
<td>Annealing</td>
</tr>
<tr>
<td>72°C</td>
<td>60 sec</td>
<td></td>
<td>Elongation</td>
</tr>
<tr>
<td>72°C</td>
<td>10 min</td>
<td></td>
<td>Final extension</td>
</tr>
</tbody>
</table>

PCR product was electrophoretically separated on 2% agarose gel and visualised under uv-transilluminator (Biotech R & D, Laboratory). Further documentation of these gels was carried out using gel doc system (BioRad). The size and quantity of the PCR amplicon was confirmed by 2% agarose gel electrophoresis. Agarose gel was prepared with ethidium bromide approaching final concentration to 0.5 µg/ml. 10 µl of the PCR amplicon was mixed with 2 µl of 6X Gel loading dye and loaded along with a known molecular weight markers of 3000, 1500 and 250 DNA ladder, from lambda hind three digest marker, in well. The electrophoresis was carried out at 80 volts. After the electrophoresis, the amplicons were visualised in a gel documentation system (Bio-Rad, USA) and were photographed.

3.8.4 Elution and purification of Amplified PCR Product

The amplified product was eluted from gel using Agarose Gel Extraction Kit obtained from Genei (Merk) India. Unincorporated PCR product primers and dNTPs from PCR products were removed by using montage PCR clean up Kit
(Millipore) and the eluted product was confirmed by agarose gel electrophoresis. This was further used for sequencing.

3.8.5 16s rRNA gene sequencing and bioinformatic analysis

Purified PCR products were further subjected for the sequencing at Labreq Bio Scientific, Ahmadabad, India, using Big Dye terminator cycle sequencing kit (Applied Biosystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). Obtained nucleotide sequence of 16s rRNA gene were edited by applying Chimera Ver.2 (online Tool) and sequences were subjected to sequence similarity with the global available database search using BLASTn tool at NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1997). The similarity hits with maximum identity and higher percentage of query coverage were selected for identification of isolated PGPR.

Pairwise and Multiple sequence alignment (MSA) of bacterial 16s rRNA gene sequences was to determine by the MEGA6 software (Tamura et al., 2007). The phylogenetic evolutionary dendrogram was inferred using UPGMA tool of MEGA6. The partial sequence of 16s rRNA gene of identified microorganism sequences were subsequently submitted to NCBI database under their accession numbers given ahead.
3.9 Collection of soil sample for Arbuscular Mycorrhizal Fungi

Soil samples were collected from the Central Potato Research Station, Maharajpura, Gwalior, (M.P.), India from different soils where potato cultivars Kufri Sindhuri, Kufri Chipsona-3 Kufri Lauvkar were grown. Samples were taken from a depth down to approximately 12 cm, and then stored at 4°C in a refrigerator till subjected to analysis, specifically for spore isolation. Soil closely clinging to the roots constituted rhizospheric soil and the soil 5-10 cm around roots the non-rhizospheric or bulk soil in this study.

3.9.1 AMF root clearing and staining

Roots from each potato variety were used for observing colonisation in them. The methods as described by Phillips and Hayman (1970) and later modified by Kormanik et al., (1980) was employed for root clearing and staining. Tender roots at the deepest to middle level were selected randomly from the plants of each variety and then cut into 3cm segments. After washing thoroughly with water roots were cleared in 10% KOH (10% KOH was made by dissolving 10 gm KOH pellets in 100 ml distilled water). Each time freshly prepared KOH was used. Clearing was performed over a water bath for 15-20 minutes at 100 °C and time determined according to the tenderness of roots. Cleared roots were rinsed 4-5 times with distilled water and transferred to 1N HCl (1N HCl was prepared by diluting 8.33 ml concentrated HCl in 100 ml of distilled water) for 5 minutes. The solution was decanted. The cleared roots were then placed overnight in 0.05% trypan blue staining solution (0.05 gm
Trypan blue was dissolved in a solution containing 40 ml of Lactic acid and 5 ml of glycerol. The final volume of 100 ml was made by adding 55 ml of distilled water) and subsequently destained 1-2 times in a glass petri plate containing destaining solution of 50% lacto glycerol (lactoglycerol was made by adding 60 ml glycerol to 876 ml of lactic acid. The final volume of 1000 ml was made with double distilled water). The cleared roots were cut into one cm segments and placed on clean glass slides and followed by mounting in 1% (v/v) polyvinyl lactoglycerol (Polyvinyl alcohol solution contained 15 g of polyvinyl alcohol in 100 ml of lactic acid and 10 ml of glycerol. 100 ml of distilled water was finally added to this solution). Ones mounted, the excess mountant was drained out and subsequently covered with another clean slide. 10 pieces of cleared roots were mounted on each glass slide. Ten slides were prepared per sample and observed under a compound light microscope.

3.9.2 AMF colonisation of potato roots

The root colonisation extent was quantified by using frequency distribution of Biermann and Lindermann, (1981). It was assessed as proportion of root length colonised by mycorrhizal fungi. Slides were observed under compound microscope for locating any of the AMF associated structures such as hyphae, vesicles or arbuscules.
Materials and Methods

Percent root colonisation was calculated using following relation:

\[
\text{Percent root colonisation} = \frac{\text{Total number of colonised roots}}{\text{Total number of roots examined}} \times 100
\]

3.9.3 AMF Spore density

Separation of AMF spores from the rhizosphere was achieved by wet sieving and decanting method as is proposed by Gerdemann and Nicolson (1963). 100 gm. rhizosphere soil was suspended in 1000 ml of tap water. The mixture was stirred for half an hour and the heavier soil and sand particles were allowed to settle down to the bottom. The soil water mixture was decanted through sieves arranged in descending mesh in the order of 240µm, 120µm, 60µm, 30µm. Top sieve captured roots and debris. Debris from the remaining sieves was collected separately in beakers. Spores were collected in a petridish of 10 cm diameter and were examined under leica stereo-microscope.

For the quantification of AMF spore density the protocol as proposed by The Energy and Resource Institute, New Delhi (TERI) was employed (Gaur and Adholeya, 1994). The girded petri plate along with the sievate from each sieve was examined under stereo microscope. Spore density was calculated as the total number of AMF spores recorded in all petri plates (Gaur and Adholeya, 1994). Quantification was carried out in 10 cm dia per petri dishes with a gridline of 1 cm square under a stereoscopic microscope at 50x (Lugo and Cabello, 2002). Ten divisions were counted and related to the total number of spores by using the modified method of McKenney and
Lindsey, (1987). The surface sterilisation of spores was done by transferring these to the sterile water in a petri plate, using micropipette for picking, and then transferring these for 2 minutes to another petri plate containing 0.05% cetrimide solution. After a thorough washing 5 to 6 times with sterile distilled water, different types of spores were collected with the help of a micropipette and mounted in a drop of polyvinyl lactoglycerol (PVLG) on a glass slide (Omar et al., 1979; Koske and Tessier, 1983) with or without Melzers reagent (Morton, 1988). Coverslip of one of the slides was gently pressed to break open the spores and observed under a compound microscope.

The spore identification was done using manual of Schenck and Perez, (1990) after comparisons with original spore descriptions and reference isolates from the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi. AMF spores were identified according to their morphology and wall characters (Walker and Treppe 1993; Wu, 1993; Schenck and Perez, 1990; Morton and Benny, 1990).

The sequence to taxonomic characteristics of spores included colour, shape, size and hyphae respectively. Colour range extended from colourless to brown or dark brown and the same was based upon the characterisation provided by Kornerup and Wanscher (1983). Colours of spores were determined when they were in water and observed under a dissecting microscope. Colours of spore wall layers and germinal wall layers were
determined in spores crushed in either water or PVLG. Colours of spore were firm according to Kornerup and Wanscher (1983). The shape and size of spores were characterised based on 50 intact spores which mounted in a drop of water or lactic acid placed on a microscopic slide. The dimensions were determined using a light microscope equipped with an occular micrometer. The thickness of layers of spore wall and germination walls was measured in spores freshly isolated and crushed in PVLG and observed under a light microscope equipped with a micrometer eyepiece. The measurements of mycorrhizae were made using a light microscope equipped with an occular micrometer or a micrometer eyepiece. Spores of mycorrhiza were preserved in drops of PVLG on microscope slides and are stored in plastic boxes.

3.10 AMF-PGPR treatments in pot culture

Uniformly sized, approximately 2 cm dia of potato tubers of the three cultivars, Kufri sindhuri, Kufri lauvkar and Kufri chipsona-3 were generously provided by the Central Potato Research Station, of Indian Council for Agriculture Research (ICAR), Maharajpura, Gwalior, M.P. as seeding material.

PGPR for use in the study were selected from those isolated pure cultures which were produced as monocultures from both rhizo and bulk soils. These isolates were labelled as PR1 (*Bacillus amyloliquifaciens*), PR3 (*Bacillus subtilis*), PB10 (*Lysinibacillus boronitolerans*), PB11 (*Pseudomonas brassicacearum*) and PB12 (*Bacillus subtilis*) for convenience. Only those
PGPRs were used which showed at least four-five PGPR like traits during analysis. These selected PGPR isolates to be used as inoculums for pot experiments with accession numbers ||KR817724||, ||KR817727||, ||KR817726||, ||KT031988||, ||KR817725|| were the ones characterised molecularly and having submitted to the NCBI, New York.

The inoculii of above isolates were prepared in a Nutrient Broth (NB) and each of these were individually transferred further to 500 ml flasks containing NB. Further growth was aerobically done by placing flasks on a rotating shaker with 100 rpm for 24 hrs at 27 °C modified from Aslantas et al., (2007). An optical density measure at 535 nm of the broths containing PGPR was standardised to achieve uniform cell density $10^9$ CFU by dilution. Out of this suspension, 10 ml was applied to the pot.

Inoculum of *Glomus intraradices*, an AMF was procured from The Energy Resources Institute (TERI), New Delhi, and was propagated with maize plants in 4 Kg pots containing sterile mixture of sand: soil (3:1). Pots with maize plants were kept in the green house at 25±2°C temperature for four months, after which aerial part of plants were cut off and the soil taken off by inverting the pots. The soils containing maize roots were thoroughly mixed and used as AMF inoculums of *G. Intraradices*. Number of spores for inoculation were with approximation kept at 100 spores per 10 g soil. 50g inoculum soil
was added to each pot containing 3 kg soil. This method used earlier by Sarikhani and Aliasgharzad, (2012) was standardised with some modifications.

Tubers were washed and then surface sterilised with 0.1% (v/v) sodium hypochlorite for 3 min, and were rinsed three times with distilled water. This was further followed by the dips in 0.01% cetrimide for 5min for bacterial free surface. Approximately a 10 g piece of each potato var. KS, KL and KC-3 was taken from the middle part of tuber containing one sprout (eye) under aseptic conditions, sliced and washed twice with sterile distilled water. Pots were separately prefilled with sterile soil and sand (3:1) for germination of potato (Sarikhani and Aliasgharzad, 2012). Each pot after 1/4th filling with the sterile soil sand mixer was given a 50g AMF inoculum (*Glomus intraradices*) as a layer of nearly 1.0 cm thickness. The seed potato tuber with eye was placed eye upwards above the AMF inoculum. The pot then was filled with soil sand mixture. PGPR suspension was added with the help of a sterile micropipette. Pots with seed tuber without any inoculums of AMF and PGPRs served as control. The quantum of AMF and PGPRs inoculi for each treatment was standardised so that an almost equal quantity of soil inoculum reached each pot. Pots were maintained under green house conditions with regular watering.
The treatment schedule is given in the following table;

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Treatments</th>
<th>Microbial inoculums</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Uninoculated control</td>
<td>Without PGPR and AMF inoculums</td>
</tr>
<tr>
<td>2</td>
<td>AMF (GI)</td>
<td><em>Glomus intraradices</em> (GI)</td>
</tr>
<tr>
<td>3</td>
<td>PR1</td>
<td><em>Bacillus amyloliquifaciens</em></td>
</tr>
<tr>
<td>4</td>
<td>PR3</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>5</td>
<td>PB10</td>
<td><em>Lysinibacillus boronitolerans</em></td>
</tr>
<tr>
<td>6</td>
<td>PB11</td>
<td><em>Pseudomonas brassicacearum</em></td>
</tr>
<tr>
<td>7</td>
<td>PB12</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>8</td>
<td>AMF (GI)+ PR1</td>
<td>GI+B. <em>amyloliquifaciens</em></td>
</tr>
<tr>
<td>9</td>
<td>AMF (GI)+ PR3</td>
<td>GI+B. <em>Subtilis</em></td>
</tr>
<tr>
<td>10</td>
<td>AMF (GI)+ PB10</td>
<td>GI+L. <em>Boronitolerans</em></td>
</tr>
<tr>
<td>11</td>
<td>AMF (GI)+ PB11</td>
<td>GI+ P. <em>Brassicacearum</em></td>
</tr>
<tr>
<td>12</td>
<td>AMF (GI)+ PB12</td>
<td>GI+B. <em>Subtilis</em></td>
</tr>
</tbody>
</table>

Each treatment had four replicates. As the three potato varieties KS, KC-3 and KL viewed similar treatments and replicates each pot was planted with 5 potato tuber slices with eyes. The growth parameters recorded were those of plant heights, fresh weight, dry weight of root, shoot and tubers, root colonisation and spore density after 90 DAP when potato tuber were harvested.
The plant height was measured in centimeter with the meter scale from the base of the plant to the top most leaf of the plant, root length and the total tuber number (yield) per plant was also observed. The plants after uprooting were segregated into root, shoot and tubers. After taking fresh weights of the shoot, root and tuber these were oven dried in a forced draught oven at 95±2.0 °C for 96 hrs and dry weight noted.

All the data were statistically analyzed by analysis of variance with the MSTAT C PROGRAM (Mich. University, East Lasing Mich., USA). Significant differences at P<0.05 were tested using Duncan’s multiple range tests.