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Anuradha Rai, Pradeep K Rai, Jay S Singh, Surendra Singh

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Multifarious native plant growth promoting fluorescent pseudomonads associated with rhizosphere of *Aloe barbadensis* miller

Anuradha Rai[1], Pradeep Kumar Rai[1], Jay Shankar Singh[2], Surendra Singh[1*]

[1]*Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi (UP) 221005, India*

[2]*Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow-226025, Uttar Pradesh, India*

[*]Corresponding author: E-mail: surendrasingh.bhu@gmail.com

ABSTRACT

Medicinal plants provide an enormous bioresource of potential use in modern medicine and agriculture. Phosphorous deficiency is a major constraint to plant production. Sustainable agriculture could be promoted by harnessing the plant beneficial bacteria particularly the fluorescent pseudomonads associated with the rhizosphere of plants, to mobilize soil inorganic phosphate and also to increase its bioavailability to the plants. Total five hundred seven fluorescent *Pseudomonas* isolates were obtained from four different *Aloe barbadensis* (Miller) growing locations of Varanasi. These *Pseudomonas* strains were further evaluated *in vitro* for their ability to solubilize phosphate and to produce indole acetic acid (IAA), hydrogen cyanide (HCN), siderophore and aminocyclopropane (ACC) deaminase. Total 119 fluorescent *Pseudomonas* isolates from the rhizospheric soil (RS) and 25 isolates from the endorhizosperic (ER) region solubilized phosphate.Whereas 53 (36.8%) *Pseudomonas* isolates produced IAA and siderophore, 36(25%) and 31 (21.5%) isolates, however, produced HCN and ACC deaminase. Out of 119 phosphate solubilizing bacteria (PSB) from RS region, 51 (42.9%) isolates and 9

(36%) isolates out of 25 PSBs from ER region lacked plant growth promoting traits (PGPTs). Among the phosphate solubilizing fluorescent pseudomonads showing PGPT, 59 isolates have multiple traits and showed more than two types of PGPT. A positive correlation exists between siderophore and ACC deaminase producers. Clustering by principal component analysis (PCA) showed that RS was the most important factor influencing the ecological distribution and physiological characterization of PGPT- possessing PSB. Geographical Information System (GIS) and Kriging Interpolation method was used to map and establish spatial variation of soil properties of the study site.

Keywords: Aloe barbadensis; PGPR activity; Mapping; 1-Aminocyclopropane-1-carboxylate (ACC)-deaminase; GIS

Introduction

An intensive farming practice with greater yield and quality requires high levels of nutrients like phosphate and nitrogen, supplied in the form of chemical fertilizers. However, repeated and excessive use of chemical fertilizers deteriorates the soil quality. Currently world is shifting towards environmental friendly, sustainable and organic agricultural practices (*Esitken et al., 2005*). Use of plant growth promoting microorganisms (PGPMs) as bio-inoculants instead of chemical fertilizers is increasing tremendously to increase the plant yields, nutrient availability and soil productivity (*O'Connell, 1992*). Plant growth promoting rhizobacteria (PGPR) enhance the plant growth and yield either directly or indirectly, without conferring pathogenicity (*Hariprasad et al., 2009*). Indirect plant growth promotion includes the prevention of the deleterious effects of phytopathogenic organisms. This can be achieved by the production of

siderophores, hydrogen cyanide (HCN), antibiotics and fungal cell wall degrading enzymes, e.g., chitinase, ß-1, 3-glucanase etc. Direct plant growth promotion includes production of phytohormones and volatile compounds, nitrogen-fixation and mineral nutrient solubilization that affect the plant signaling pathways.

Phosphate, the second most important plant growth limiting mineral nutrient next to nitrogen, is present in the form of insoluble phosphates and cannot be utilized by the plants (*Pradhan and Sukla, 2006*). Of the total phosphate exists in a soluble form, only 0.1% is available for plant uptake (*Zhou et al., 1992*) due to its fixation into an unavailable form. Phosphate solubilizing microorganisms (PSMs) play an important role in supplying phosphate to plants through various mechanisms of solubilization and mineralization. Among the different organic acids, gluconic acid production seems to be the most common mechanism of phosphate solubilization used by PSMs. Microbial solubilization of phosphate in soil was correlated with the ability of microbes to produce selected organic acids or extracellular polysaccharides (*Kim et al., 1998; Halvorson et al., 1990*), which are involved in plant growth promotion and biological control against phytopathogens.

Among PSB, fluorescent pseudomonads aggressively colonize to the plant roots, and due to their plant growth promotion and biocontrol ability, they are considered as most important group of bacteria. Fluorescent pseudomonads are Gram-negative, motile, rod-shaped, aerobic γ-proteobacteria (*Galli et al., 1992*). They are metabolically and functionally diverse group of PGPR that can promote plant growth by producing phytohormones, solubilizing phosphate, sequestering iron by siderophore (*Salisbury, 1994; Ayyadurai et al., 2007; Ravindra Naik et al., 2008; Budzikiewicz, 1993*) and by suppressing phytopathogenic microorganisms by producing antibiotic (*Thomashow, et al., 1990; Ayyadurai et al., 2007; Ravindra Naik et al., 2008*).

Whereas several reports are available on *Pseudomonas* as PGPR and biocontrol agents in cereals and fodder crops (*Mittal et al., 2008; Dey et al., 2004; Gulati et al., 2009*), very few reports are, however, available in case of medicinal plants.

Aloe barbadensis is an important drought-resistant, succulent, medicinal plant belonging to the family Liliaceae and has wide applications in pharmaceutical, food and cosmetic industries. It is a perennial and semitropical plant cultivated commercially in many parts of India; and is one of the 250 species of *Aloe* (*Das and Chattopadhay, 2004*). The gel present in the leaves of *A.barbadensis* contains a diverse array of compounds mainly aloin A, aloesin, isoaloeresin D, aloeresin E, carbohydrates, proteins, amino acids, vitamins and minerals (*Roy et al., 2012; Saeed et al., 2004; Patidar et al., 2012*). It has been widely used as antioxidant (*Miladi and Damak, 2008*), antidiabetic (*Jones, 2007*), anticancer (*Naveena et al., 2011*), antimicrobial (*Bashir et al., 2011*), immunomodulatory (*Atul et al., 2011*) and several other pharmaceutical activities. Due to its unique and structurally divergent secondary metabolites *A. barbadensis* hosts a specific and diverse rhizospheric and endophytic phosphate solubilizing PGPR. In this study an attempt has been made to isolate phosphate-solubilizing PGPR associated with the rhisosphere and endorhizosphere of *A. barbadensis* plants and also to evaluate their plant growth promotion ability such as production of indole acetic acid (IAA), HCN, siderophore and aminocyclopropane (ACC) deaminase. The major factors influencing the ecological and physiological characters of PSB possessing PGPT are also discussed.

Material and Methods

Study site and sampling

The soil samples were collected from the rhizosphere region of the planted *A. barbadensis* growing at four different locations *viz.*, Kaazisarai, Manduadih, Banaras Hindu

University (BHU) campus and Tengara of Varanasi, India which is located at a latitude of 25°19'14·86 N and longitude of 82°58'12·30 E (Fig. 1). Ten plants from each location of different sampling sites were randomly selected. Sampling was done in the month of June 2013. Samples were collected in plastic bags, immediately brought to the laboratory and stored at 4°C for further processing. Soil samples were air dried and sieved (2 mm) prior to its physico-chemical analysis.

Mapping and geospatial analysis

Global Positioning System (GPS) and Geographical Information System (GIS) were used for mapping and studying the spatial variation of physico-chemical properties of soil of the different sampling locations. Mapping and geospatial analysis of all the soil parameters were done by using ArcGIS 10.1 software. Kriging interpolation method was used for mapping and predicting the property of unsampled location. It also allows to compare the performances for interpolating soil analysis. In kriging, spherical, exponential and Gaussian models were fitted using the variogram. Interpolation is used to convert data from point observations to continuous fields so that the spatial patterns sampled by these measurements can be compared with spatial patterns of other spatial entities (*Christos et al., 2009*). Once the variogram is known, the value of an attribute at any point in a mapping unit can be predicted from the available data points using kriging (*Omran et al., 2012*).

Soil Characterization

Soil characteristics such as pH and electrical conductivity (EC) were determined by using pH and EC meter, respectively according to Sparks (1996). Organic carbon (OC) was determined

following the chromic acid digestion method (*Walkley and Black, 1934*). The diethylene triamine penta-acetic acid (DTPA) extractable micronutrients (Fe, Cu, Zn and Mn) in the soil samples were determined by the method of Lindsay and Norwell (1978). Available nitrogen (N), phosphorus (P), potassium (K) and sulphur (S) were determined by the methods of Subbiah and Asija (1956), Olsen et al. (1954), Hanway and Heidal (1952) and Chesin and Yein (1952), respectively.

Isolation of RS and ER fluorescent pseudomonads

Fluorescent pseudomonads were isolated from the rhizospheric soil. Soil samples (10g) tightly adhered to the roots of *A. barbadensis* plants were added to 90 ml sterile distilled water and the content was agitated for 20 min at 160 rpm. The soil suspension thus obtained was serially diluted in 0.15 M NaCl, spread on King's B (KB) agar medium (King et al., 1954) and the plates were incubated at 28°C for 2 days. Endorhizobacteria residing inside the roots of *A. barbadensis* were isolated according to the method described by Sturz et al. (1998). Roots were rinsed with tap water to remove soil and then treated with commercial bleach (5.25% available chlorine) for 3 min. The treated roots were transferred to 3% hydrogen peroxide (H_2O_2) solution for 3 min and finally rinsed three times with sterile distilled water. The outer surface of sterile roots were trimmed, the pieces were further macerated in Ringers solution (215 mg of NaCl, 7.5 mg of KCl, 12 mg of $CaCl_2$ (dihydrate), 50 mg of $Na_2S_2O_3.5H_2O$ in 100 ml of distilled water, pH - 6.6) and was serially diluted upto 10^{-3} dilution. From this dilution, 0.1 mL suspension was plated onto KB medium and the plates were incubated at 28°C for 3-4 days. Single bacterial colonies were selected and streaked onto a new KB plates. Colonies showing fluorescence under

UV light were selected and transferred onto fresh KB plates. Purified colonies were preserved in 50% glycerol at -80°C.

Gram's reaction

Gram's reaction was performed by the KOH method (*Ryu, 1940*).Visible amount of overnight grown cells were taken from agar plate and smeared onto glass slide containing 3% aqueous KOH solution. The strains producing viscous gel that string out along with the loop was identified as gram negative.

Phosphate solubilization assay

Phosphate solubilization ability of pseudomonads isolates was assayed according to Mehta and Nautiyal (2001). Pseudomonads strains were streaked onto NBRIP medium containing per liter: glucose, 10 g; $Ca_3(PO4)_2$, 5 g; $MgCl_2$.6H2O, 5 g; $MgSO_4.7H_2O$, 0.25 g; KCl, 0.2 g, $(NH_4)_2SO_4$, 0.1 g, and bromophenol blue (BPB), 0.025 g. The plates were incubated for 3 days at 28°C. Appearance of clear halo zone around the colonies was indicated the phosphate solubilization.

IAA production

IAA production was determined following the standard method of Brick et al. (1991). Overnight grown Pseudomonas cultures were inoculated on LB medium containing per liter: 10g tryptone, 5g yeast extract, 5g NaCl amended with 5mM L-tryptophan, 0.06 sodium dodecyl sulphate (SDS) and 1% glycerol and the plates were incubated at 28°C for 48 h. Cultures were pelleted by centrifugation at 4000 rpm for 15 minutes, supernatants (2ml) were mixed with 100

µl of o-phosphoric acid and 4 mL of Salkowski's reagent (50 ml 35% perchloric acid; 1 mL 0.5M $FeCl_3$) and kept at room temperature for 30 min. Development of pink color indicated IAA production.

Siderophore assay production

Siderophore production assay was performed on blue agar chrome azurol S (CAS) medium containing CAS and hexadecyltrimethylammonium bromide (HDTMA) as indicators (*Schwyn and Neilands, 1987*). Pseudomonas isolates were grown in KB broth at 28°C for 48 h. All the isolates (10 µl) were inoculated onto the center of CAS medium and incubated at 28°C for 48 h. Development of yellowish orange halos around the colonies indicated the siderophore production.

HCN production

HCN production was assayed according to Bakker and Schippers (1987). The pseudomonads isolates were grown in screw-cap test tubes containing 5 ml of King's B broth supplemented with 4.4 g/L of glycine, at 28°C on a rotary shaker. Whatman No. 1 filter paper was cut into uniform strips of 9 cm long and 0.5 cm wide, saturated with alkaline picrate solution (0.5% picric acid and 2.0% Na_2CO_3) and placed inside the screw cap tubes in a hanging position. After incubating the tubes at 28°C for 48 h, a change in the filter paper colour from yellow to orange-brown was indicative of HCN production.

ACC deaminase activity

ACC deaminase activity was determined as described by Ramamoorthy et al. (2001) on Dworkin and Foster (DF) minimal salts medium, which contains (per litre): 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2 g glucose, 2 g gluconic acid and 2 mg citric acid with trace element solution (1 mg $FeSO_4.7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4.H_2O$, 124.6 µg $ZnSO_4.7H_2O$, 78.22 µg $CuSO_4.5H_2O$ and 10 µg MoO_3). Filter sterilized ACC solution (3 mM) was spread over the agar plates inoculated with pseudomonads strains and allowed to dry for 10 min. The growth of pseudomonads isolates was observed after 2 days of incubation at 28°C. Growth of the pseudomonads isolates on the DF minimal salt medium indicated ACC deaminase production.

Statistics

All the statistical analysis was conducted by using SPSS 20.0 (Analytical Software). Principal component analysis (PCA) and Analysis of Variance (ANOVA) were carried out to know the influence of RS and ER region on PGPT-possessing PSB distribution and to clarify the most determining factor in grouping.

Results

Physico-chemical characteristics of soil

High resolution soil quality information getting through manual field survey is time consuming, expensive and labour intensive. Keeping this in mind we have carried out mapping and geospatial variations analysis of soil samples by using ArcGIS 10.1 software through Kriging interpolated method. Mapping was used to know the physic-chemical characteristics of soil in relation to soil fertility status of *A. barbadensis* growing areas of Varanasi. Ten composite rhizospheric soil samples from each site of four locations namely Kaazisarai, Manduadih,

Tengara and BHU campus were analyzed and the data presented in Table 1. Tha data on mapping and geospatial variations analysis of soil pH, EC, available N, P, K S and micronutrients of all the *A. barbadensis* growing locations are presented in Fig. 2. Soils of all the *A. barbadensis* growing locations were alkaline in nature. The pH and EC of roots of different locations ranged from 7.3 to 8.6 and 0.032 to 0.610 dSm^{-1}, respectively. The soil organic carbon varied from 0.27 to 0.58 %. Soil of Kaazisarai had maximum organic carbon (0.58%) while minimum organic carbon (0.26%) was recorded for the soil of BHU campus. The available N was maximum (194.08 kg h^{-1}) in the soil of Manduadih while minimum (72.14 kg h^{-1}) in the soil of Tengara. The available P was maximum (44.27 kg h^{-1}) in the soil of Kaazisarai and minimum (14.40 kg h^{-1}) in the soil of Manduadih. The K value in soil ranged from 69.60 to 367.36 kg ha^{-1} it was recorded minimum and maximum in the soils of BHU campus and Kaazisarai, respectively. Maximum (23.05 mg kg^{-1}) and minimum (8.02 mg kg^{-1}) available S, was recorded for the soils of Kaazisarai and Manduadih, respectively. Among the DTPA extractable micronutrients, Fe (15.34 mg kg^{-1}) and Mn (11.22 mg kg^{-1}) contents were maximally recorded in the soil of Manduadih and minimum (3.12 and 2.49 mg kg^{-1}, respectively) in the soil of BHU campus. However, the amounts of other two micronutrients Cu (2.18 mg kg^{-1}) and Zn (1.76 mg kg^{-1}) were higher in the soil of Kaazisarai and lower (1.01 and 0.54 mg kg^{-1}, respectively) in the soil of Manduadih.

Phenotypic characterization of fluorescent pseudomonads

Total 407 isolates of fluorescent *Pseudomonas* were isolated from the rhizospheric and endorhizospheric regions of healthy *A. barbadensis* (Miller) plants from four different locations in Varanasi (Kaazisarai, Manduadih, Tengara and BHU campus). All the isolates were rod

shaped, Gram negative and fluoresced under UV light (254 nm), however the intensity of fluorescence varied among the isolates. Some of the isolates showed green pigmented colonies while others showed light green and orange colonies. The shape of the colonies was round and irregular.

Screening for phosphate solubilizing phenotype

Total 144 isolates produced zone of solubilization on the NBRI medium indicating their ability to solubilize phosphate. One hundred nineteen phosphate solubilizing isolates from the rhizosphere (RS) and 25 from endorhizosperic (ER) region exhibited their ability to solubilize phosphate. Thirty phosphate solubilizing isolates were isolated from the RS of Kaazisarai, and 7 from the ER of the Kaazisarai. Thirty two, 30 and 27 phosphate solubilizing isolates were obtained from RS of Manduadih, Tengra and BHU campus, respectively. However, 5, 7 and 6 phosphate solubilizing isolates were obtained from the ER of the Manduadih, Tengra and BHU campus. Phosphate solubilizing isolates associated with RS and ER of *A. barbadensis* from each location with their PGPTs are given in Table 2.

Comparison of PGPTs of the isolates

Fluorescent pseudomonad isolates were screened for their PGPTs such as production of IAA, HCN, siderophore and ACC deaminase. Whereas 53 (36.8%) isolates produced IAA and siderophores, 36(25%) and 31 (21.5%) isolates, however, produced HCN and ACC deaminase, respectively. The ratio of phosphate solubilizing isolates lacking PGP attributes was relatively higher in the RS of BHU campus (55.5%) and ER of Kaazisarai (57.1%). In contrast, the ratio of phosphate solubilizing isolates lacking PGP attributes was; however, lower in RS of Tengara

(26.6) and ER of Manduadih (20%). Out of 119 phosphate solubilizing isolates from the RS 51 (42.9%) isolates and 9 (36%) isolates out of 25 phosphate solubilizing isolates from the ER samples lacked PGPTs.

Among the four sites, the percentage of phosphate solubilizing isolates having the ability to produce IAA was highest in RS (9.3%) and ER (20%) of Manduadih. Siderophore producing phosphate solubilizing isolates were maximum in RS (10%) of Tengara and ER (14.2%) of Kaazisarai and Tengara. Phosphate solubilizing isolates having the ability to produce HCN were present only in RS of Kaazisarai (3.3%) and ER of Tengara (14.2%); while absent in other samples. Phosphate solubilizing isolates exhibiting ACC deaminase activity were maximum in RS (6.2%) of Manduadih but absent in all the ER samples. The number of phosphate solubilizing isolates producing IAA (13.6%), siderophores (9.0%), and HCN (4.5%) were highest in ER whereas phosphate solubilizing isolates exhibiting ACC deaminase activity were maximally present in RS (3.3%).

Phosphate solubilizing isolates having the ability to produce IAA and siderophores were highest in RS (10%) and ER (14.2%) of Tengara. However, phosphate solubilizing isolates having the ability to produce maximum IAA and HCN were present in RS (9.3%) of Manduadih and ER (20.0%) of BHU campus. Whereas phosphate solubilizing isolates exhibiting IAA and ACC deaminase activity were maximally present in RS (6.6%) of Tengara. Those having the ability to produce siderophore and HCN were, however, maximum in RS (7.4%) and ER (16.6%) of BHU campus. Phosphate solubilizing isolates having the ability to produce siderophores and ACC deaminase were maximally present in Tengara (10.0%) but absent in all the samples of ER. Whereas phosphate solubilizing isolates having the ability to produce ACC deaminase and IAA were maximum in ER (16.6%) of BHU campus, however, these were absent in all the samples of

RS. Overall, the ratio of phosphate solubilizing isolates having the ability to produce IAA and siderophores (7.5%), and IAA and HCN (5.0%) was relatively higher in ER. Similarily phosphate solubilizing isolates having the ability to produce siderophores and HCN were relatively higher in ER (8.0%). Phosphate solubilizing isolates having the ability to produce ACC deaminase and IAA were present in only ER samples. Phosphate solubilizing isolates having the ability to produce IAA and ACC deaminase (3.3%), and siderophore and ACC deaminase (4.2%) were abundantly present in RS.

Ratio of phosphate solubilizing isolates displaying combination of triple PGPTs i.e. IAA production, siderophore synthesis and ACC deaminase activity was highest in ER (17.4%) and RS (6.2%) of Tengara and Manduadih, respectively. Phosphate solubilizing isolates having the ability to produce IAA and HCN and to synthesize siderophore were maximally present in ER of Manduadih (20.0%) and RS of Kaazisarai and Tengara (6.6%). Phosphate solubilizing isolates displaying triple activities of IAA, HCN and ACC deaminase were maximally present in RS of BHU campus (3.7%) but these were absent in all the samples of ER. Phosphate solubilizing isolates having the ability to synthesize siderophore and to produce HCN and ACC deaminase were found only in the ER of Tengara (14.2%) and RS of Manduadih (3.1%). On the basis of total ratio of phosphate solubilizing isolates, those having the ability to produce IAA, HCN and siderophores were relatively higher in ER (4.0%). However, phosphate solubilizing isolates displaying IAA, HCN and ACC deaminase activity were highest in RS (2.5%).

Phosphate solubilizing isolates having the ability to produce IAA and HCN and to synthesize ACC deaminase and siderophores isolated from the four *A. barbadensis* growing locations were shown in Fig. 3. Phosphate solubilizing isolates exhibiting four PGP activities i.e IAA, siderophore, HCN and ACC deaminase were maximally found in RS (6.6%) of Tengara

and ER (20.0%) of Manduadih. Overall the ratio of total phosphate solubilizing isolates, having four PGP activities was relatively higher in RS (5.0%) than the other lacations. However the ratio of phosphate solubilizing isolates having the ability to produce IAA was maximum in Manduadih (123.7%) followed by Tengara (71.8%), Kaazisarai (61.8%) and BHU campus (55.5%). Phosphate solubilizing isolates having the ability to produce IAA were maximally present in ER (80.0%) of Manduadih and in RS (43%) of both Manduadih and Tengara. Phosphate solubilizing isolates having the ability to synthesize siderophores were maximum in Tengara (110.4%) followed by Manduadih (74.3%), Kaazisarai (55.2%) and BHU campus (49.9%). On the basis of their ability to produce HCN, phosphate solubilizing isolates can be arranged as Manduadih (85.0%) >Tengara (55.1%) > BHU campus (38.8%) > Kaazisarai (37.5%). Phosphate solubilizing isolates having the ability to produce HCN were maximally recorded in the RS (26.6%) of Tengara. Phosphate solubilizing isolates, on the basis of their ability to produce ACC deaminase appeared in the order of Tengara (61.8%) > Manduadih (48.2%) > BHU campus (31.4%) > Kaazisarai (24.2%). However, the ability to synthesize ACC deaminase was recorded highest in the phosphate solubilizing isolates of RS (33.3%) Tengara and lowest (14.2%) in ER of Kaazisarai.

Correlation analysis

No significant correlations existed between the physico-chemical properties of the soil samples (pH, EC, organic carbon, available N, P, K and DTPA extractable micronutrients Fe, Cu, Zn and Mn) and PSB having the ability to produce IAA, siderophores, HCN and ACC deaminase (data not shown).

The data on the correlation analysis of the PSB possessing PGPT are shown in Table 3. A positive correlation (0.98, $p<0.05$) existed between IAA producers from the RS samples and the total IAA producers. A significant positive correlation (0.99) was found between IAA and HCN producers from the RS samples. A significant positive correlation (0.99, $p< 0.05$) was also recorded between the siderophores producers from the RS samples and the total PSB possessing the ability to produce siderophores without considering RS and ER samples. A positive correlation (0.95 $p< 0.05$) was also recorded between total PSB having the ability to produce siderophores and ACC deaminase from the root samples and between HCN producers from the root samples and total PSB having the ability to produce HCN.

Cluster analysis

To find out and further to ascertain whether any relationship exists between PGPT of the PSB from RS and ER samples, the data of PSB having the ability to produce IAA, siderophore, HCN and ACC deaminase in the RS or ER of each samples were used to perform cluster analysis through PCA (Fig. 4). The summary of PCA of gathered data based on abundance of PGPT of PSB (Table 4), shows that out of eight principal components, two components, PC1 and PC2 had eigen value more than one and were retained for further analysis. PC1 and PC2 had eigen value 4.69 and 2.53, respectively. The first component (PC1) explained 58.62% variability while the second one (PC2) explained 31.59% of total variation. In PC1, ER from Kaazisarai (KER) had highest coefficient value (0.979) followed by Tengara (TER) having coefficient value of 0.876 in PC2. On transposition of biplot, the two components showed 90.21% variability. Whereas TER fell in the PC1 over +, - coordinate, BRS, TRS and KER, however, fell over +, + coordinate in PC2. KRS and MRS come over +, + coordinate close to PC2. MER and BER fell on PC2 over

343 closer to +, - coordinates. PCA data (PC1 and PC2 values of each condition) were used to carry
344 out ANOVA to clarify the most important factor in grouping. Patterns formed by PC1 ($p<0.01$)
345 was significantly affected by the sampling positions (RS or ER) although PGPT types were
346 affected slightly by the patterns formed by the PC1 and PC2. However, the distribution of PSB
347 possessing PGPT was affected by the RS and ER, since the RS was the portion of a plant's root
348 where bacteria are closely associated with the host plants.
349

350 **Discussion**

351 In the present scenario the conventional agriculture is shifting towards a more
352 sustainable one. Application of soil beneficial microbes increases the soil fertility through P–
353 solubilization by releasing organic acids, chelation and phytohormone production (*Omar, 1998;*
354 *Narula et al., 2000; Whitelaw, 2000*). PGPR have been employed in agriculture and horticulture
355 and have been considered very important due to their potential of ecological amelioration. The
356 present work contributes to the knowledge of beneficial microbial community associated with
357 rhizosphere and endorhizosphere of *A. barbadensis* and frequencies of fluorescent *Pseudomonas*
358 and PGPR traits associated with this group. Plant growth promoting PSB have been isolated
359 from food and fooder crops (*Yanes et al., 2012; Patrick et al., 2009*), however, very little is
360 known about the PGP fluorescent *Pseudomonas* from the medicinal plants. *A. barbadensis* is an
361 important medicinal plant and has wide applications in pharmaceutical, food and cosmetic
362 industries. This is the first study of isolation of PGP fluorescent *Pseudomonas* from the ER and
363 RS of *A. barbadensis* and exploration of environmental and physico-chemical properties of soil
364 interacting with PSB associated with *A. barbadensis*. Fluorescent pseudomonads often
365 predominate among plant rhizosphere associated bacteria (*Glick et al., 1995; Sunish et al., 2005*).

366 Fluorescent *Pseudomonas* has been taken as a keystone species in this study because it is a major
367 component of rhizospheres and exhibits multifunctional PGPTs such as solubilization of
368 inorganic phosphate and production of phytohormones and antimicrobial metabolites (*Morrissey*
369 *et al., 2002*).

370 In this study, soil physico-chemical characteristics indicated that each sampling site
371 differed in its soil types (Fig. 2). Interpolation of the mapping of soil samples indicated a high
372 degree of variation among all the study sites. However, no significant differences were found in
373 the frequency of PGP fluorescent *Pseudomonas* per plants from the RS and ER of four *A.*
374 *barbadensis* growing locations. It clearly indicates that different soil regime did not affect the
375 number of culturable fluorescent *Pseudomonas* (*Patrick et al., 2009*).

376 PSB lacking the PGP attributes were slightly higher (42.9%) in RS as compared to ER
377 (36.0%). Endophytic microorganisms have been studied from several host plants (*Cocking,*
378 *2003*). However, according to our knowledge naturally occurring root endophytic fluorescent
379 *Pseudomonas* of *A. barbadensis* have not yet been studied. The population density and diversity
380 of endophytes with PGPTs are highly variable attributes and depend mainly on the bacterial
381 species, host genotypes, developmental stage and environmental conditions (*Ahn et al., 2007;*
382 *Marschner et al., 2004; Mendes et al., 2007; Rosenblueth and Martinez-Romero, 2006*).

383 The PSB isolates from the RS and ER exhibited two, three and four PGPR traits
384 simultaneously, suggesting that the application of PSB with multifunctional traits is more
385 beneficial for plant growth promotion. PSB with multifunctional PGP traits have also been
386 reported from the rhizosphere of apple (*Mehta et al., 2013*). The percent ratio of PSB exhibiting
387 binary activities of IAA production and siderophore synthesis was 7.5%, while the percent ratio
388 of PSB producing IAA and ACC deaminase was 3.3%. However, the ratio of rhizobacteria

389 isolated from *Carex leiorhyncha* showing siderophore(s) synthesis and IAA production was
390 reported to be 0.9% while the PGPR ratio showing IAA production and ACC deaminase activity
391 was 2.6% (*Cattelan et al., 1999*). Among 144 PSB isolated from the RS and ER, the proportion
392 of the IAA producing PSB was 36.8%. However, the proportion of PSB associated with RS and
393 ER of apple having the ability to produce IAA was 24.2% (*Mehta et al., 2013*). Similarly 44%
394 rhizobacteria isolated from *Brassica campestris* sp. *pekinensis* have the ability to produce IAA
395 (*Poonguzhali et al., 2006*). Thus, *Pseudomonas* isolates from the RS and ER have significant
396 ability to produce IAA and HCN. IAA stimulates the root development, resulting in better
397 absorption of water and nutrients from the soil and stimulates the release of plant metabolites
398 (*Lambrecht et al., 2000*).

399 *Pseudomonas* isolates from RS and ER exhibited three and four PGPTs. Percentages of
400 PSB having the ability to produce IAA, ACC deaminase and siderophore were 2.5 and 4% in the
401 RS and ER of *A. barbadensis*, respectively. Percentage of PSB exhibiting four PGPTs was 5%.
402 However, percentage of rhizobacteria having three PGPT was reported to be 4.2% in the RS of
403 *C. leiorhyncha* (*Koo et al., 2010*). Siderophores play an important role in the plant growth
404 because of their ability to supply iron (*Ramos-Solano et al., 2010*). ACC deaminase decreases
405 ethylene biosynthesis by sequestering ACC, an ethylene precursor (*Genrich et al., 1998*) and
406 producing positive effect on root elongation (*Esashi, 1991*).

407 In the present study the diverse population of PSB with multiple PGPTs (production of
408 IAA, HCN, siderophore and ACC deaminase), differed significantly amongst the sites as well as
409 sampling source (rhizosphere and endosphere). It is evident that PGPTs were not correlated with
410 the source of isolates (i.e. rhizosphere, endorhizosphere, nature of the sampled plant or sampling
411 site). This variation could be attributed to several factors like physico-chemical characteristics of

412 soil, developmental stages and agronomic practices among others that affect the populations of
413 fluorescent pseudomonads (*Costa et al., 2006; Picard and Bosco, 2008*). Presence of PGPR with
414 multifunctional traits from the RS and ER of *A. barbadensis* suggested that this medicinal plant
415 is a potential niche for P- solubilizing fluorescent pseudomonads possessing PGP attributes.

416 Occurrence of a significant positive correlation (r =0.99) between IAA and HCN
417 producers and HCN and ACC deaminase producers suggested that the release of HCN by
418 rhizospheric microbes has been considered a possible line of defense against soil-borne plant
419 pathogens (*Blumer and Hass 2000; Hoflich, 1994*). HCN and IAA production by the PGP P-
420 solubilizing fluorescent pseudomonads may play an important role in the plant growth promotion
421 and development of resistance in *A. barbadensis* against soil-borne phytopathogens. It was
422 speculated that the production of phytohormone by P-solubilizing microorganisms may
423 contribute to their stimulatory effect on plant growth (*Azcon et al., 1978; Sattar and Gaur,*
424 *1987*). However, no significant correlation was found between PGPR traits and physico-
425 chemical properties of the soil. Similar results have also been reported where no correlation
426 existed between soil physico-chemical properties and PGPTs (*Koo et al., 2010*).

427 Spatial variation and mapping of the soil physico-chemical properties reflect condition of
428 the soil nutrient content and help to analyze variation perfectly. Spatial mapping provides a
429 visual representation of the ecological factors shaping microbial community. It provides the
430 microlevel soil analysis information at enough and accurate scale. This information will be
431 helpful in management of the societal demands, guiding policy decisions and soil sustainablity of
432 the study area (*Griffiths et al., 2015*). Besides plant species, plant health and developmental
433 stage, the composition and diversity of rhizospheric microbial communities are governed by the
434 soil type, nutrients, season, pedoclimate, climate and several other biotic and abiotic factors

435 (*Singh and Mukerji, 2006; Berg and Smalla, 2009*).GIS interpolation mapping showed
436 significant variation in soil type and nutrient in the rhizospheric region of *A. barbadensis* which
437 possibly may be due to the plant root exudates. It generates a selective environment for the
438 proliferation of specific kind of PGP fluorescent pseudomonads in the rhizospheric region as
439 well as within the root (endorhizosphere). The informations generated through mapping may
440 help to apply the experimental data according to the soil condition for farming *A.barbadensis*
441 and can also be used as a tool in guiding farmers to produce same at commercial level. GIS
442 mapping may also facilitate in preliminary information prediction regarding microbial
443 population, as well as production and application of native bioiniculants according to soil
444 physico-chemical properties without taking much of time, resource and labour. The information
445 on soil physico-chemical properties are necessary for management of the commercial cultivation
446 of medicinal plants.
447
448 **Conclusions**
449 A significant variation exists in the rhizospheric and endorhizospheric PSB population
450 and rhizosphere is the most important factor of distribution. Absence of significant correlation
451 between soil properties and PGPTs of PSB, indicates that fluorescent pseudomonads possessing
452 multifunctional plant growth promoting abilities are profoundly present in the rhizosphere and
453 endorhizosphere of *A. barbadensis*. The presence of diverse population of fluorescent
454 pseudomonads in the rhizosphere and endorhizosphere of *A. barbadensis* suggests that the
455 rhizosphere and endorhizosphere of *A. barbadensis* are unique niche supporting the existence of
456 fluorescent pseudomonads possessing different multifunctional PGPTs. Information obtained on
457 the biodiversity of P- solubilizing fluorescent pseudomonads having multifunctional properties

will be helpful in designing the strategies for using these strains as inoculants for commercial cultivation of medicinal plants. The data on spatial variation of soil nutrients can be used as a fundamental map for guiding the researchers and farmers in applying bioinoculants and in preparing bioformulation according to their need. This can also help to formulate a long-term experience-oriented strategy for applying bio-fertilizer/bioinoculants to raise the economic efficiency for commercial production. It not only help to apply fertilizers properly, but also has very important theoretical and practical value in region-based management of fertilizer use and to guide people scientifically to produce medicinal plants at commercial level.

Conflict of interest

There are no conflicts of interest among authors.

Acknowledgments

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656

Figure 1(on next page)

FIGURES

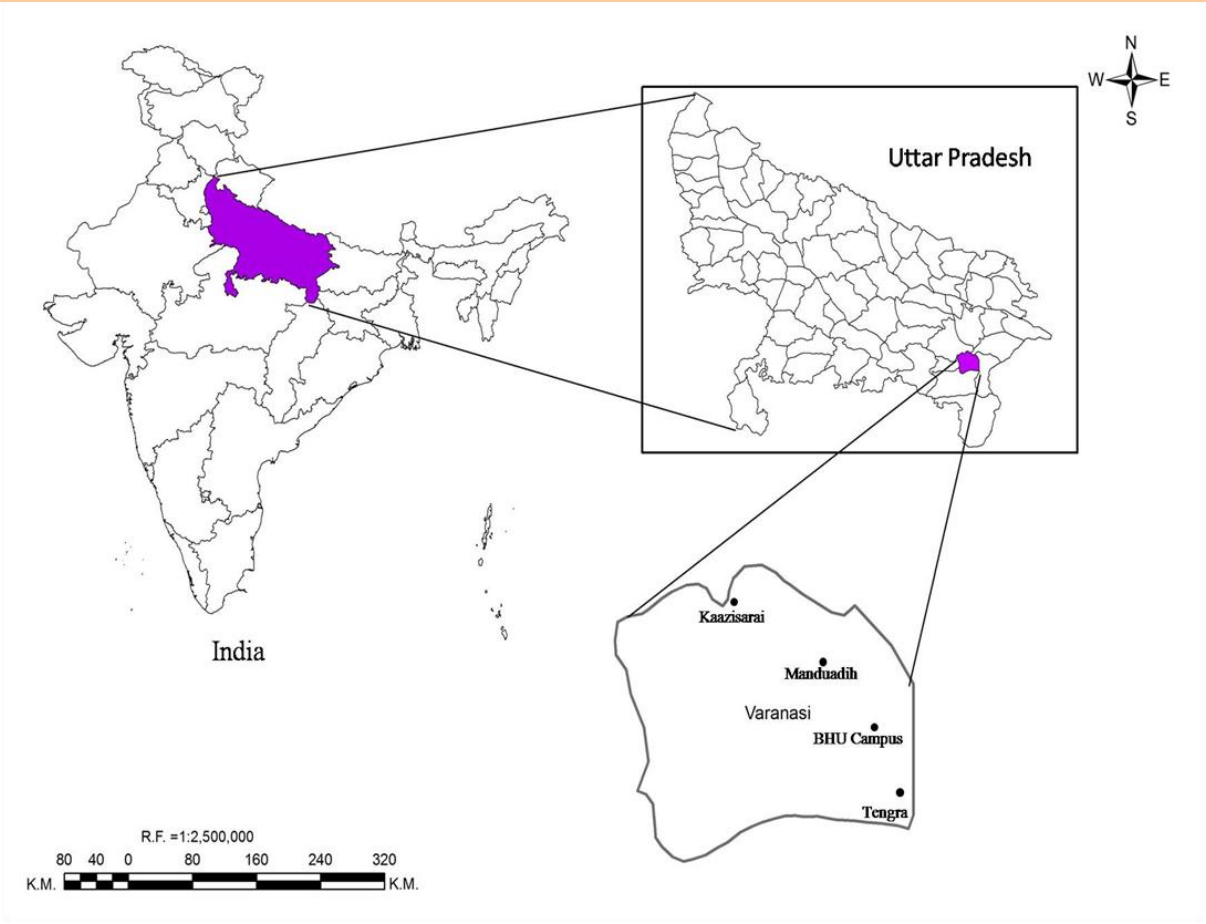

Fig. 1. Location map of the study area

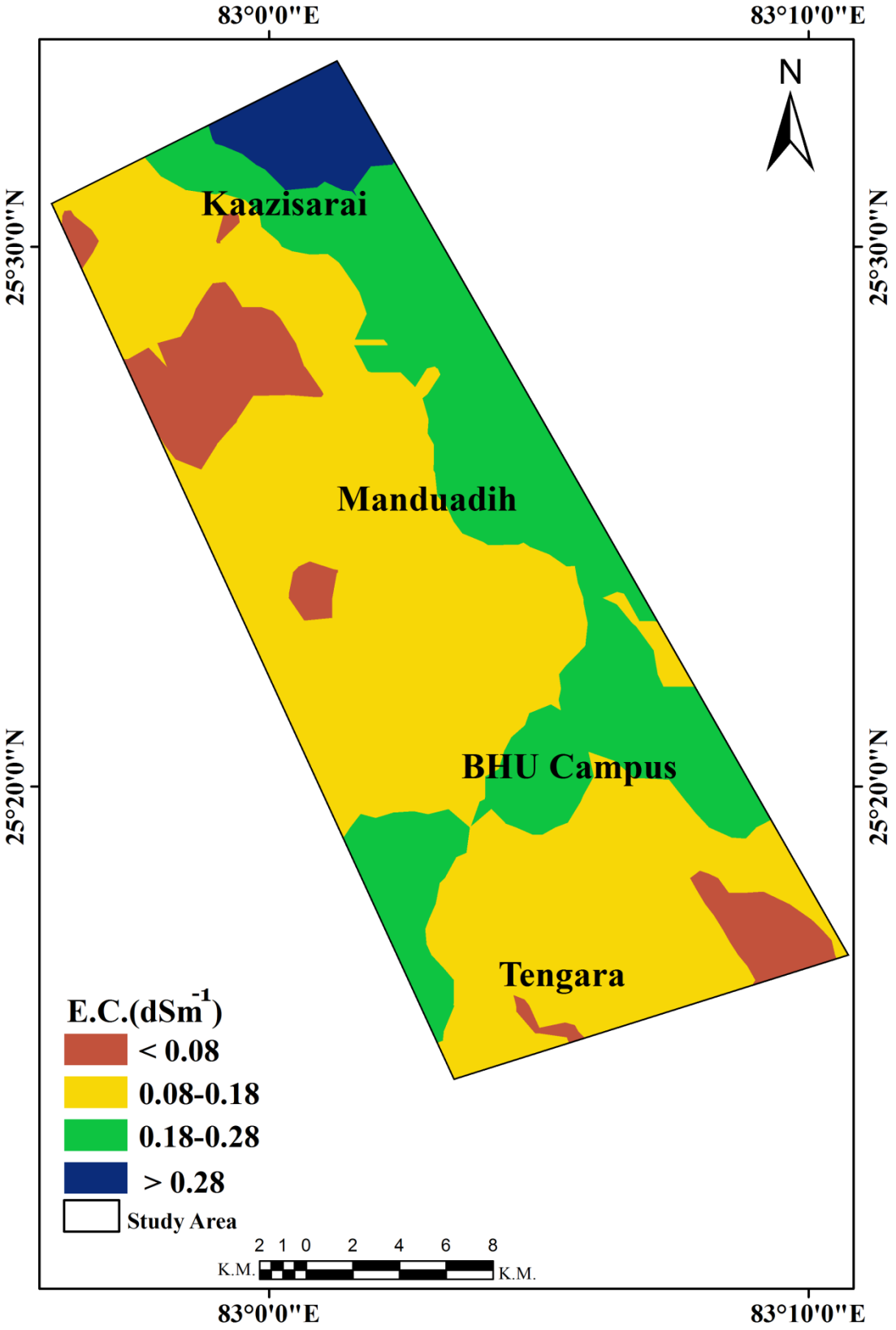

Fig. 2. (A) Geospatial variation of soil of *A. barbadensis* growing locations: EC

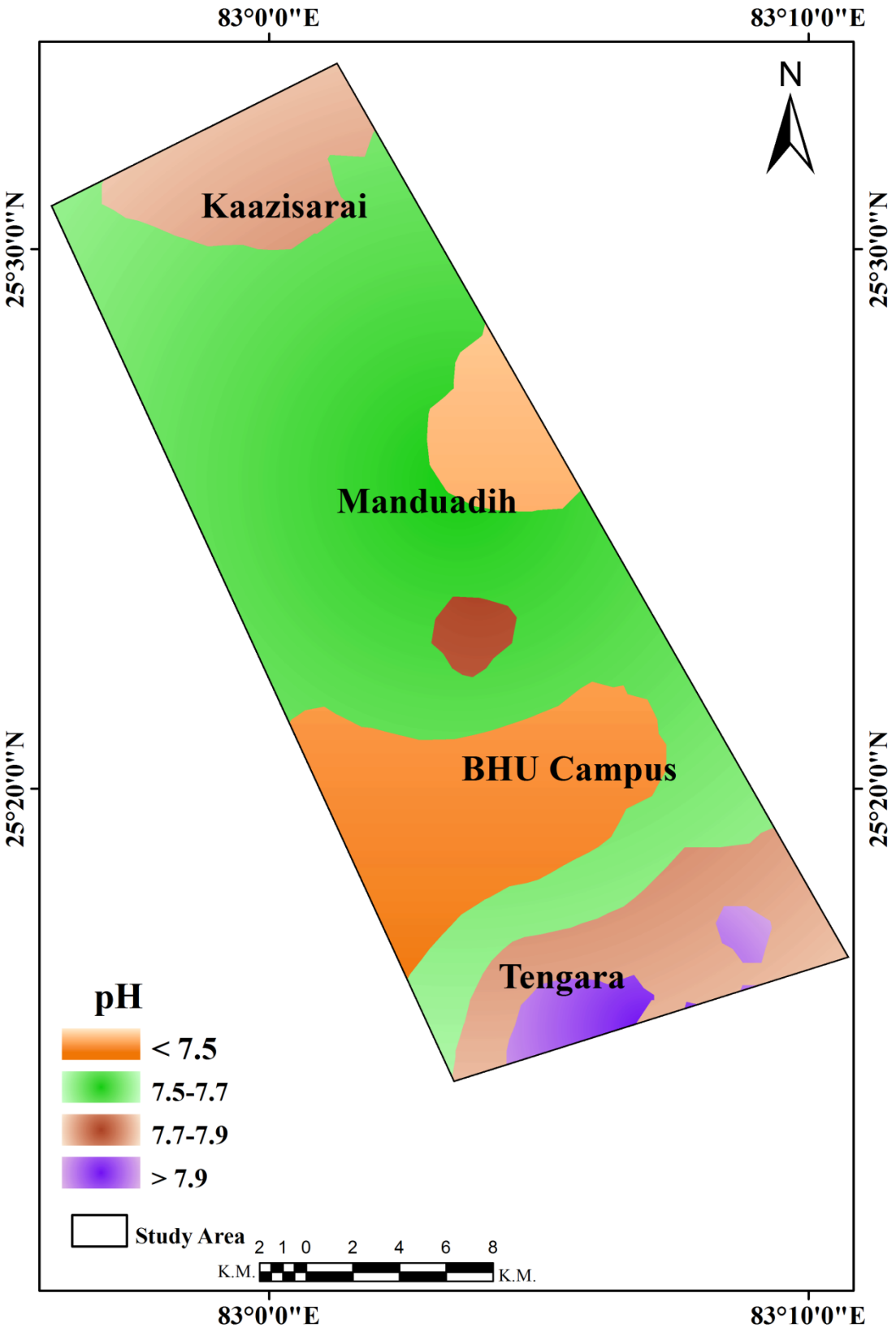

Fig. 2. (B) pH

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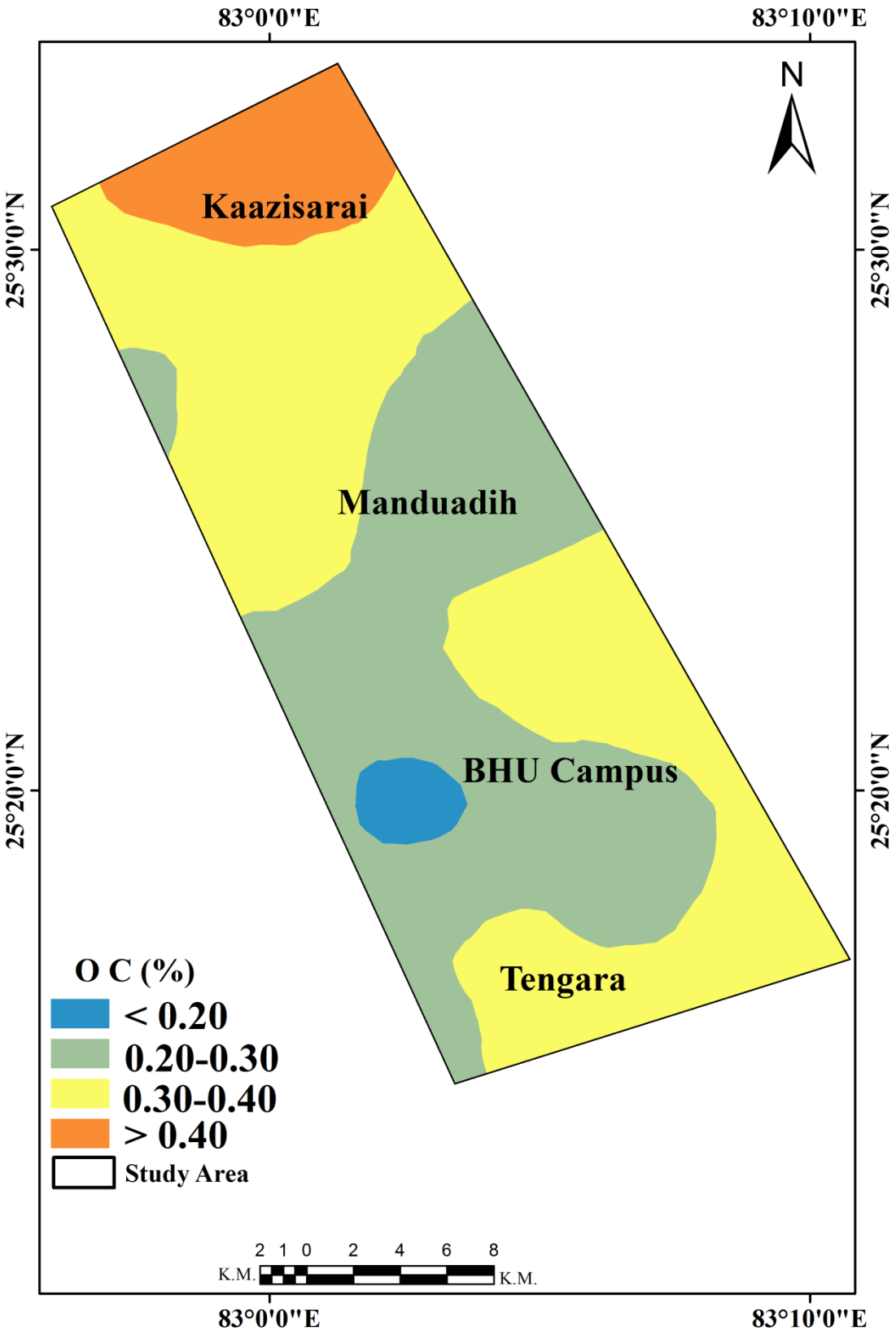

Fig. 2. (C) Organic carbon

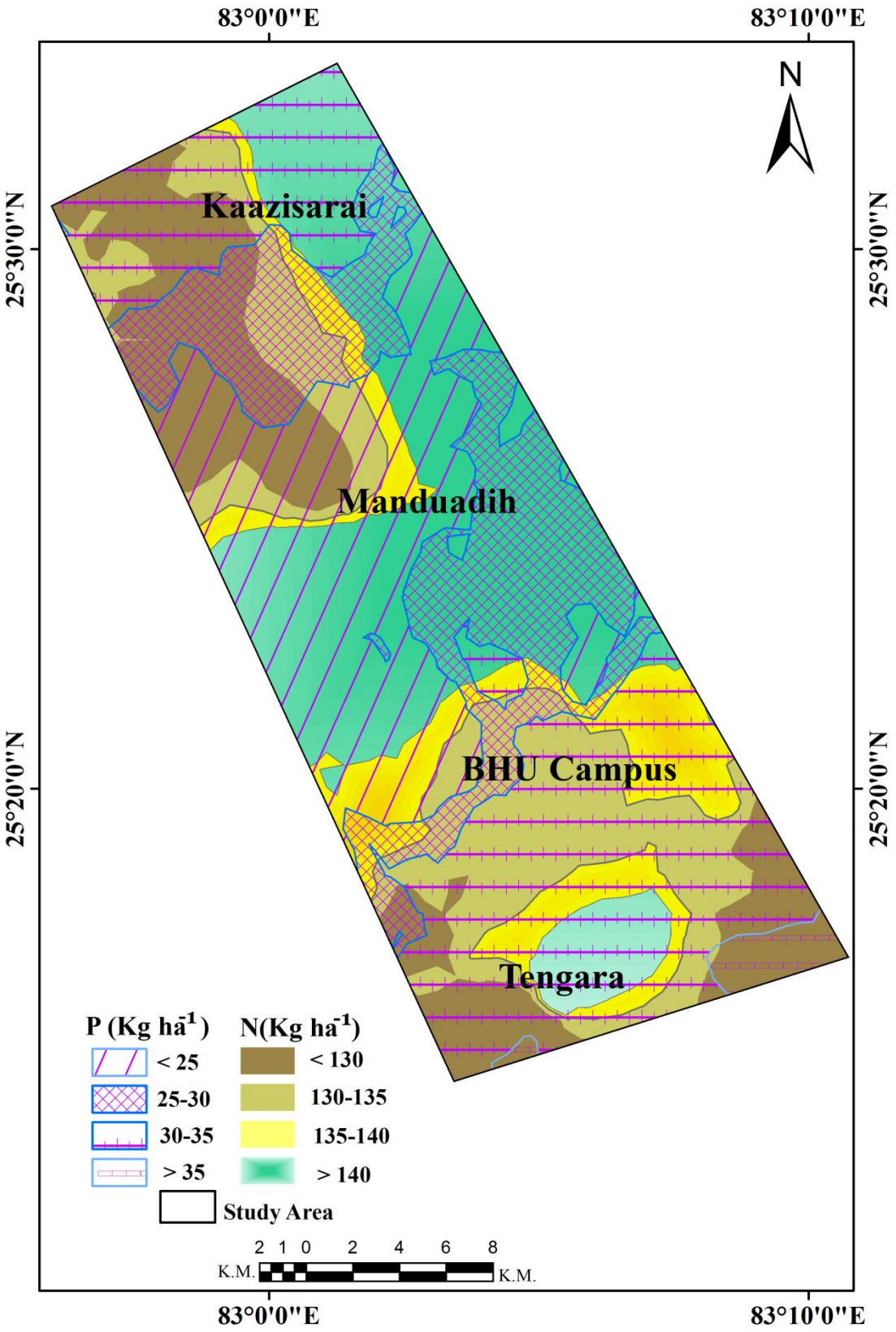

Fig. 2. (D) Available N, P

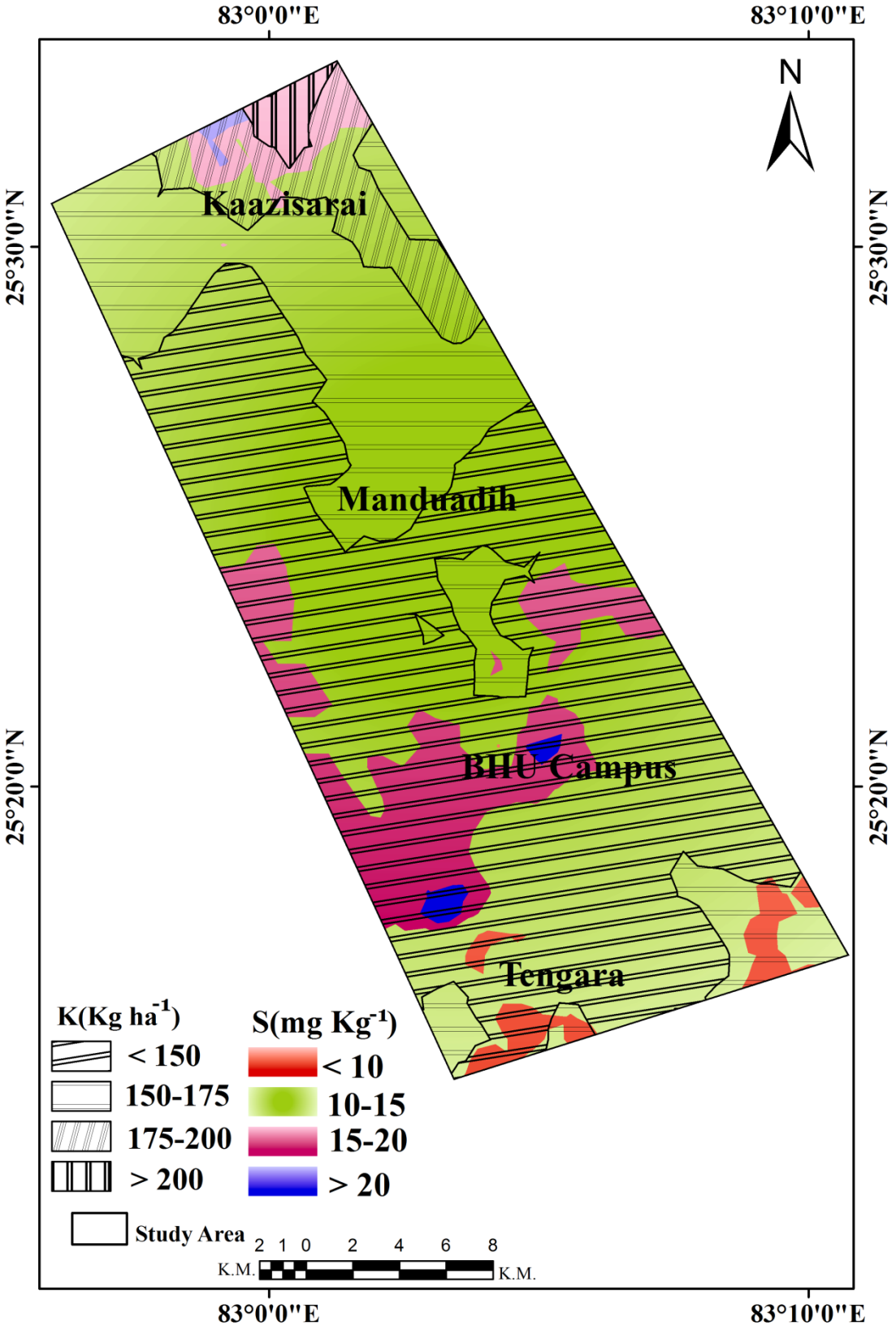

Fig. 2. (E) Available K, S

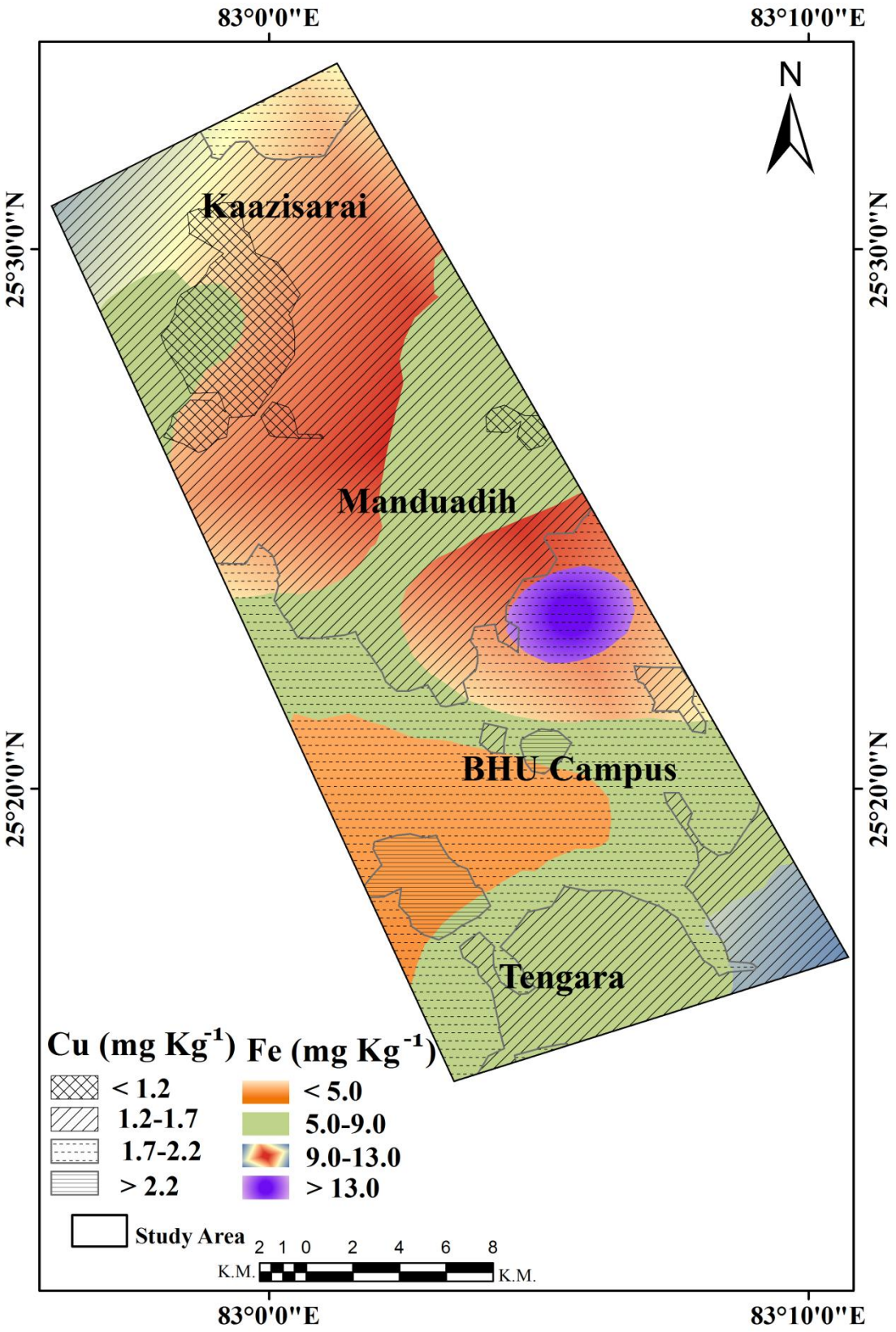

Fig. 2. (F) Micronutrients Fe, Cu

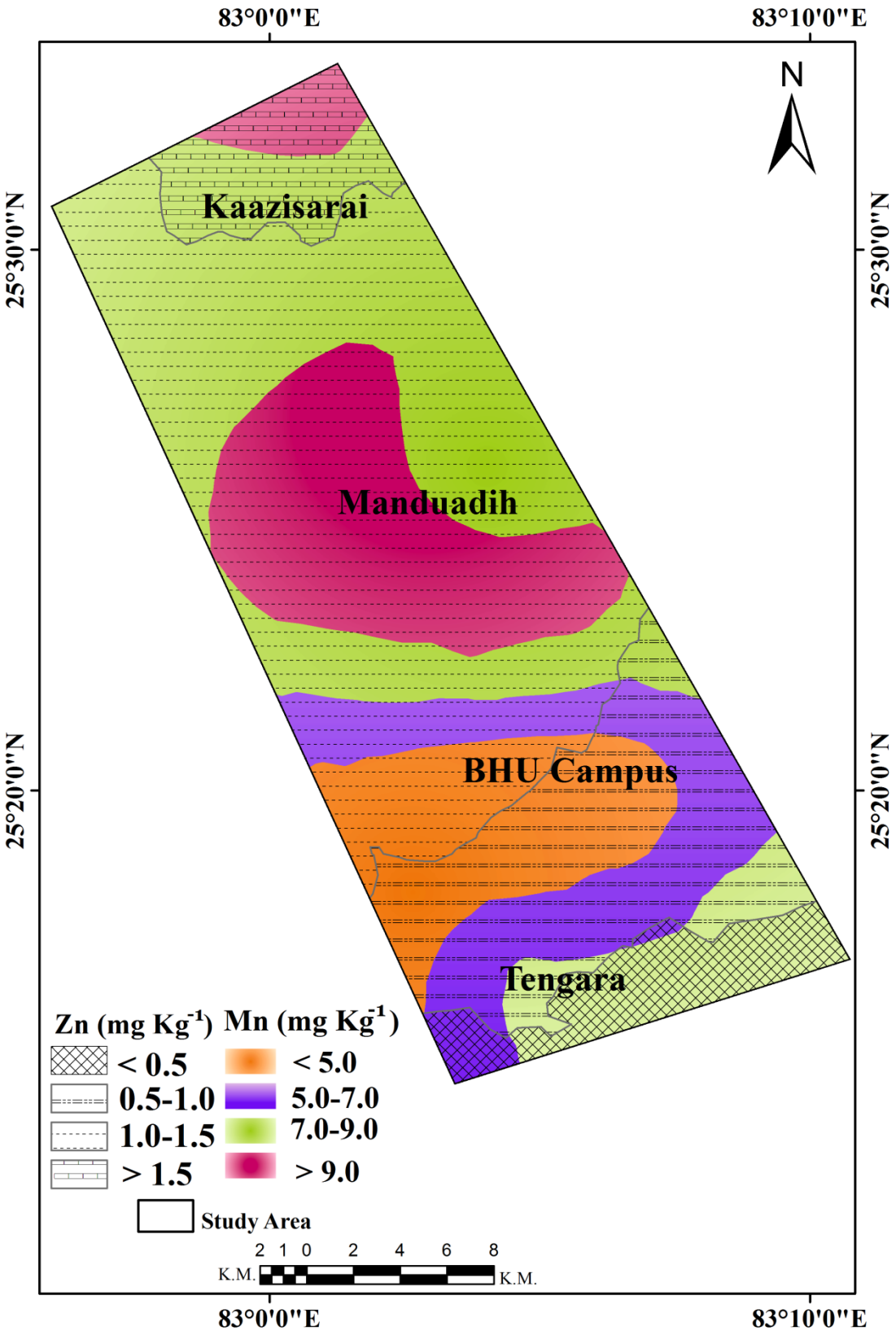

Fig. 2. (G) Micronutrient Zn, Mn

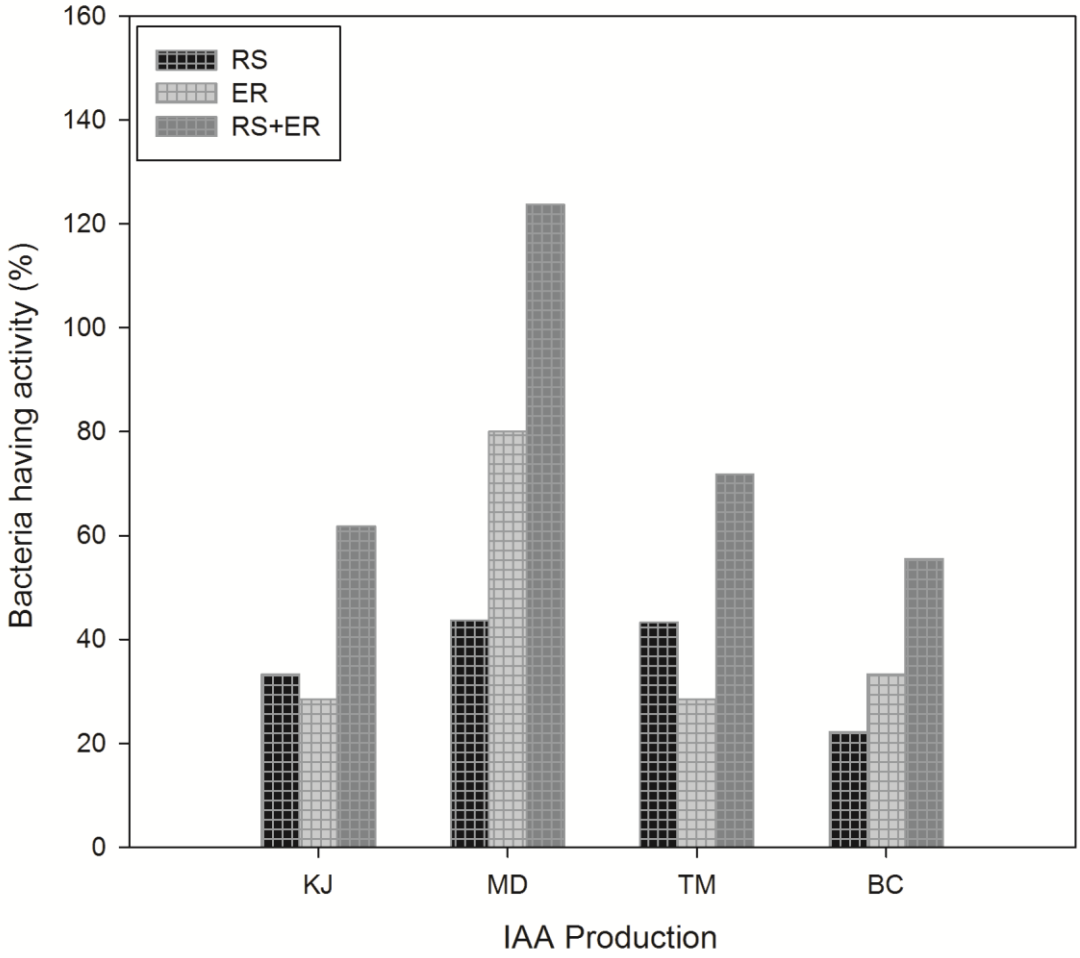

Fig. 3. (A) Percentages of rhizospheric (RS) and endorhizospheric (ER) phosphate-solubilizing fluorescent pseudomonads of four locations with multifarious PGPTs: IAA production

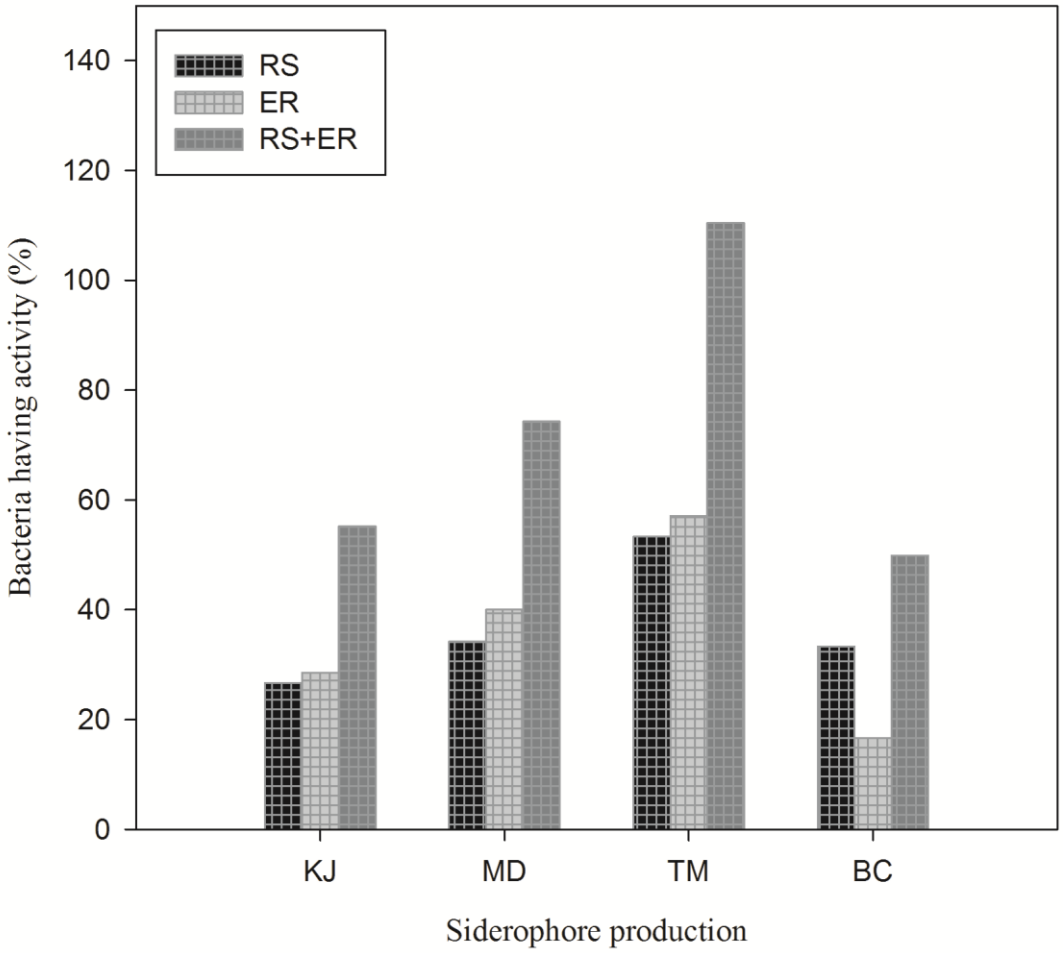

Fig. 3. (B) siderophore synthesis

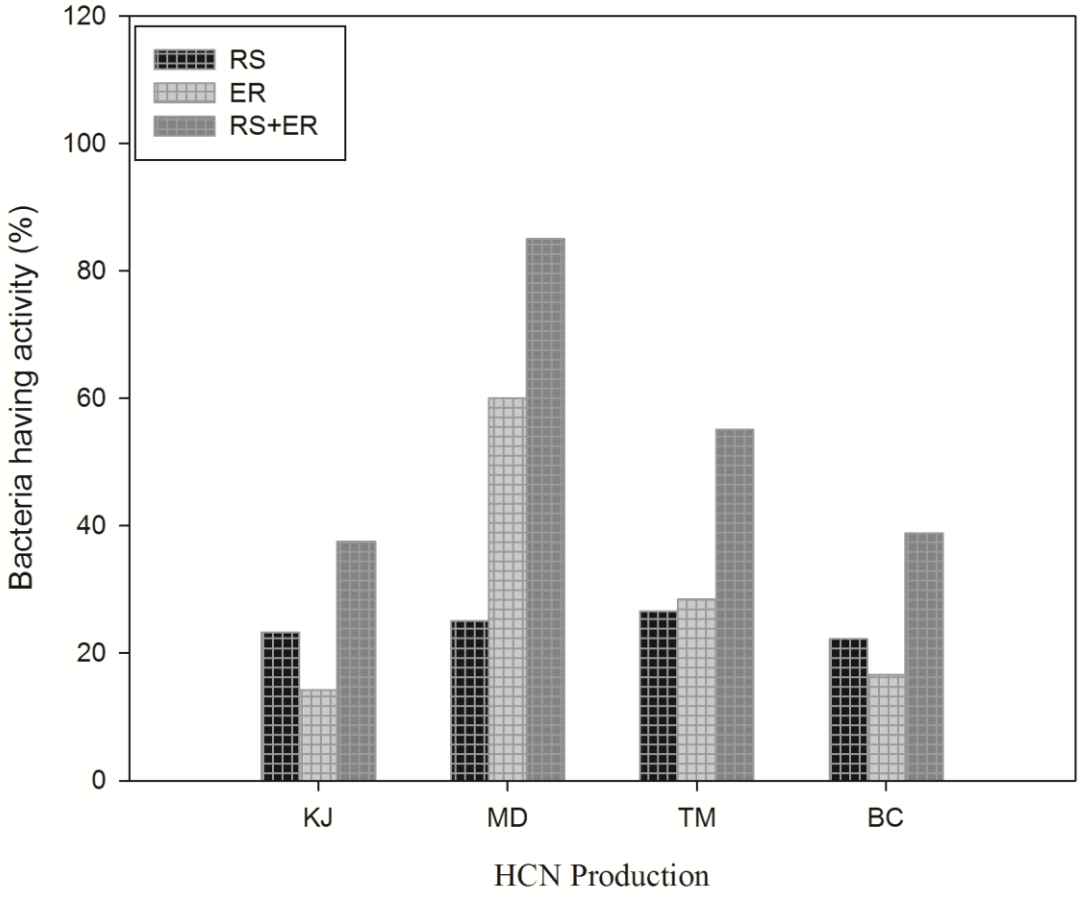

Fig. 3. (C) HCN production

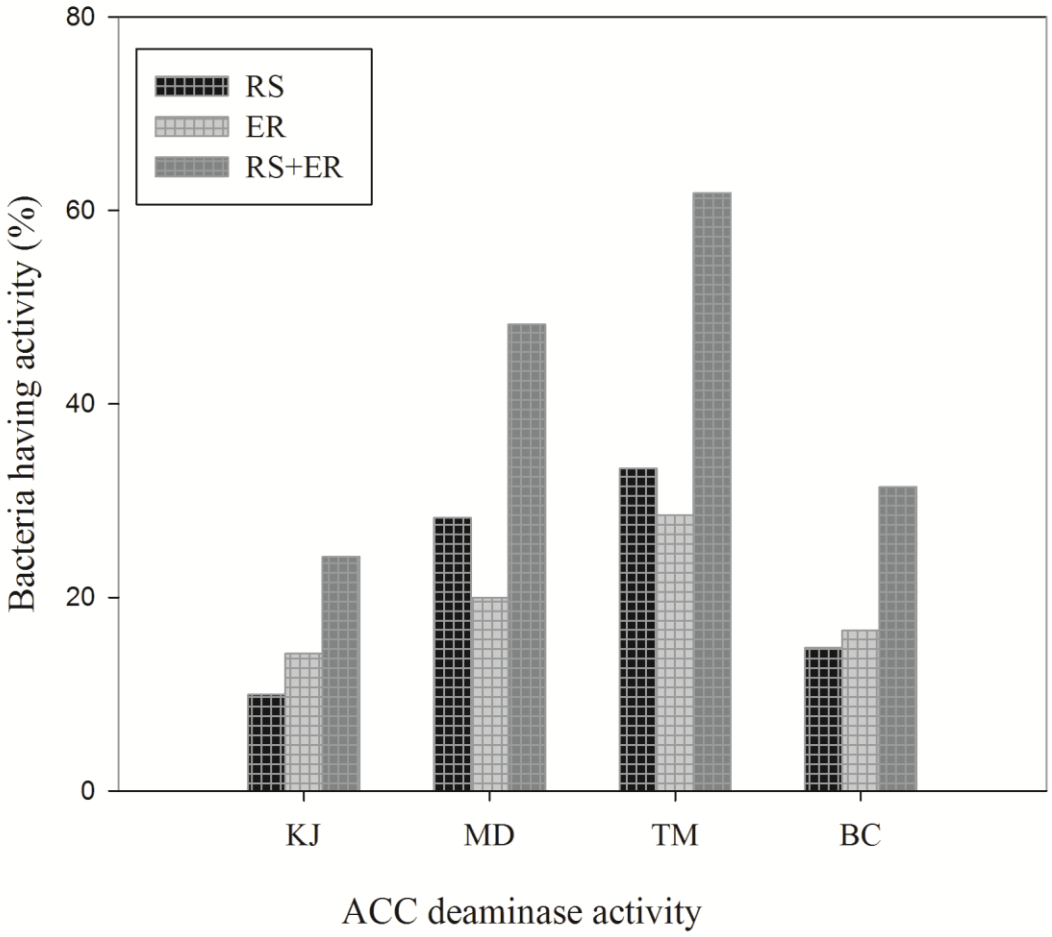

Fig. 3. (D) ACC deaminase activity

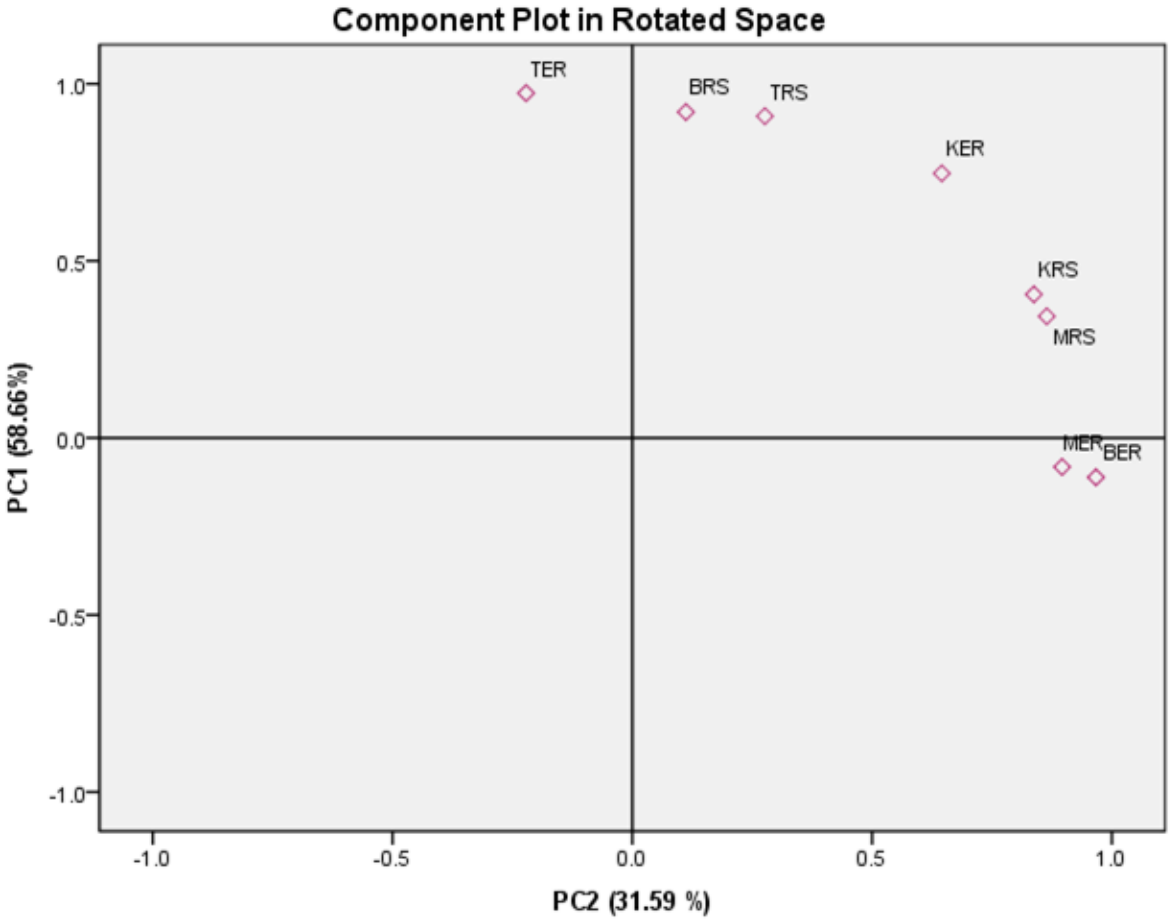

Fig. 4. Principal component analysis of the percentages of phosphate solubilizing rhizobacteria possessing PGPTs considering *A. barbadensis* growing locations, sampling position and PGPTs types

Table 1(on next page)

TABLES

Table 1

Physico-chemical characteristics of the soil collected from different *A. barbadensis* growing locations

Sampling sites		pH	EC	OC	N	P	K	S	Fe	Cu	Mn	Zn
			(dSm^{-1})	(%)	(Kg h^{-1})			(mg kg^{-1})				
Manduadih	Max	8.4	0.327	0.48	194.08	44.01	216.48	21.32	15.34	2.12	11.22	1.43
	Min	7.4	0.043	0.27	104.37	14.40	84.90	8.02	5.11	1.01	5.52	0.54
Tengara	Max	8.6	0.118	0.48	175.62	41.23	245.68	16.66	9.17	1.92	8.56	0.89
	Min	8.1	0.032	0.33	72.14	25.44	120.96	10.89	7.47	1.22	6.58	0.68
Kaazisarai	Max	8.3	0.610	0.58	175.62	44.27	367.36	23.05	11.78	2.18	9.22	1.76
	Min	8.0	0.032	0.33	108.32	14.53	136.08	12.54	8.05	1.03	6.59	0.76
BHU Campus	Max	7.5	0.341	0.45	138.98	28.20	115.60	22.50	6.75	4.24	3.89	1.33
	Min	7.3	0.062	0.26	128.71	15.30	69.60	17.40	3.12	2.02	2.49	0.91

Table 2

Sampling sites	No. of PSB without PGP Activity	No. of PSB with single PGP Traits				No. of PSB with binary PGP Traits						No. of PSB with triple PGP traits				No. of PSB with four PGP traits
		I	S	H	A	I+S	I+H	I+A	S+H	S+A	A+I	I+S+A	I+S+H	I+A+H	S+A+H	I+S+A+H
Kaazisarai																
RS	16/30 (53.3)	2/30 (6.6)	2/30 (6.6)	1/30 (3.3)	0/30 (0.0)	2/30 (6.6)	1/30 (3.3)	1/30 (3.3)	1/30 (3.3)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	2/30 (6.6)	1/30 (3.3)	0/30 (0.0)	1/30 (3.3)
ER	4/7 (57.1)	1/7 (14.2)	1/7 (14.2)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	1/7 (14.2)	0/7 (0.0)	1/7 (14.2)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)
Manduadih																
RS	12/32 (37.5)	3/32 (9.3)	1/32 (3.1)	0/32 (0.0)	2/32 (6.2)	2/32 (6.2)	3/32 (9.3)	1/32 (3.1)	1/32 (3.1)	1/32 (3.1)	0/32 (0.0)	2/32 (6.2)	1/32 (3.1)	0/32 (0.0)	1/32 (3.1)	2/32 (6.2)
ER	1/5 (20.0)	1/5 (20.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	1/5 (20.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	0/5 (0.0)	1/5 (20.0)	0/5 (0.0)	0/5 (0.0)	1/5 (20.0)
Tengara																
RS	8/30 (26.6)	1/30 (3.3)	3/30 (10.0)	0/30 (0.0)	1/30 (3.3)	3/30 (10.0)	1/30 (3.3)	2/30 (6.6)	2/30 (6.6)	3/30 (10.0)	0/30 (0.0)	1/30 (3.3)	2/30 (6.6)	1/30 (3.3)	0/30 (0.0)	2/30 (6.6)
ER	2/7 (0.0)	0/7 (0.0)	1/7 (14.2)	1/7 (14.2)	0/7 (0.0)	1/7 (14.2)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	0/7 (0.0)	1/7 (14.2)	0/7 (0.0)	0/7 (0.0)	1/7 (14.2)	0/7 (0.0)
BHU Campus																
RS	15/27 (55.5)	0/27 (0.0)	2/27 (7.4)	0/27 (0.0)	1/27 (3.7)	2/27 (7.4)	1/27 (3.7)	0/27 (0.0)	2/27 (7.4)	1/27 (3.7)	0/27 (0.0)	0/27 (0.0)	1/27 (3.7)	1/27 (3.7)	0/27 (0.0)	1/27 (3.7)
ER	3/6 (50.0)	1/6 (16.6)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	1/6 (16.6)	0/6 (0.0)	1/6 (16.6)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)	0/6 (0.0)
SUM (%)																
RS	51/119 (42.9)	6/119 (5.0)	8/119 (6.7)	1/119 (0.84)	4/119 (3.3)	9/119 (7.5)	6/119 (5.0)	4/119 (3.3)	6/119 (5.0)	5/119 (4.2)	0/119 (0.0)	3/119 (2.5)	6/119 (5.0)	3/119 (2.5)	1/119 (0.84)	6/119 (5.0)
ER	9/25 (36.0)	3/25 (12.0)	2/25 (8.0)	1/25 (4.0)	0/25 (0.0)	1/25 (4.0)	1/25 (4.0)	0/25 (0.0)	2/25 (8.0)	0/25 (0.0)	2/25 (8.0)	1/25 (4.0)	1/25 (4.0)	0/25 (0,0)	1/25 (4.0)	1/25 (4.0)

Characterization of phosphate-solubilizing fluorescent pseudomonads isolates form RS and ER of *A. barbadensis* for their multifarious PGPTs

I indole acetic acid, **S** siderophore, **A** ACC deaminase activity, **H** HCN production, **RS** rhizosphere soil, **ER** root endosphere
Figure in parentheses denote the percent of P-solubilizing fluorescent pseudomonads with plant growth promoting (PGP) traits

Table 3

Correlation matrices showing relationship amongst various PGPTs of the phosphate-solubilizing fluorescent pseudomonad isolates from *A. barbadensis*

	IAA			**SID**			**HCN**			**ACCd**		
	RS	*ER*	*RS+ER*	*RS*	*ER*	*RS+ER*	*RS*	*ER*	*RE+ER*	*RS*	*ER*	*RS+ER*
IAA												
RS	1											
ER	0.60	1										
RS+ER	0.98*	0.74	1									
SID												
RS	0.60	0.00	0.50	1								
ER	0.68	-0.13	0.54	0.89	1							
RS+ER	0.63	-0.04	0.53	0.99*	0.94	1						
HCN												
RS	0.99*	0.52	0.96*	0.68	0.76	0.72	1					
ER	0.85	0.87	0.92	0.49	0.35	0.46	0.82	1				
RE+ER	0.92	0.73	0.98*	0.62	0.58	0.62	0.95*	0.95*	1			
ACCd												
RS	0.81	0.47	0.79	0.88	0.72	0.86	0.84	0.84	0.88	1		
ER	0.42	-0.33	0.27	0.94	0.93	0.95*	0.52	0.17	0.37	0.66	1	
RS+ER	0.79	0.39	0.75	0.92	0.77	0.90	0.83	0.79	0.85	0.93	0.73	1

IAA indole acetic acid, **Sid** siderophore, **HCN** hydrogen cyanide production, **ACCd** aminocyclopropane deaminase activity, **RS** rhizosphere, **ER** endorhizospheric

* Correlation is significant at 0.05 level of significance

Table 4

Loading of coefficients of percentages of phosphate-solubilizing fluorescent pseudomonad isolates possessing PGPTs for the first two principal components

Component matrix[a]		
Variables	Principal Components	
	PC1	PC2
KER	0.979	0.131
KRS	0.896	-0.249
MRS	0.872	-0.321
TRS	0.810	0.495
BRS	0.694	0.615
TER	0.481	0.876
BER	0.649	-0.725
MER	0.615	-0.656
Eigen value	4.69	2.53
% of Variance	58.62	31.59
Cumulative percentage of total variance	58.62	90.22

Extraction method: Principal Component Analysis.

a. 2 components extracted.

K Kaazisarai, **M** Manduadih, **T** Tengara, **B** BHU Campus, R**S** rhizosphere soil, **ER** root endorhizosphere