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Research Paper

# Plant growth promoting capability and genetic diversity of bacteria isolated from mud volcano and lime cave of Andaman and Nicobar Islands

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## Abstract

Twenty four bacterial strains from four different regions of mud volcano and lime cave were isolated to estimate their diversity, plant growth promoting and biocontrol activities to use them as inoculant strains in the fields. An excellent antagonistic effect against four plant pathogens and plant growth promoting properties such as IAA production, HCN production, phosphate solubilization, siderophore production, starch hydrolysis and hydrolytic enzymes syntheses were identified in OM5 (*Pantoea agglomerans*) and EM9 (*Exiguobacterium* sp.) of 24 studied isolates. Seeds (Chili and tomato) inoculation with plant growth promoting strains resulted in increased percentage of seedling emergence, root length and plant weight. Results indicated that co-inoculation gave a more pronounced effects on seedling emergence, secondary root numbers, primary root length and stem length, while inoculation by alone isolate showed a lower effect. Our results suggest that the mixed inocula of OM5 and EM9 strains as biofertilizers could significantly increase the production of food crops in Andaman archipelago by means of sustainable and organic agricultural system.

**Key words:** plant growth promotion, phytopathogens, mud volcano, lime cave, amplified ribosomal DNA restriction analysis (ADRDA).

## Introduction

Sustainable agriculture has evolved from three perspectives: system of production to achieve self-sufficiency in food, concept of stewardship and means of sustaining rural communities. The indiscriminate use of chemical pesticides not only causes pollution but also leads to uncalled losses of microbial diversity in the natural environment. In view of this, usage of bio based fertilizers and pesticides are one of the promising ways to enhance crop productivity and to manage the plant diseases. In this view, use of plant growth promoting bacteria has an important role in developing promising method for crop management (Sturz *et al.*, 2000). The PGPR mechanisms to promote plant growth are of diverse nature such as phosphorus solubilization, production of plant hormones (Bent *et al.*, 2001; Reyes *et al.*, 2002) and excretion of diverse compounds like antibiotics or proteolytic enzymes. Some plant-beneficial microorganisms are known to antagonize plant pathogens through competition for nutrients; parasitism by means of hydrolytic enzyme production; inhibition of the pathogens by anti-microbial compounds; induction of systemic resistance in host plants (Whipps, 2001; Compant *et al.*, 2005).

Mud volcanoes are featured where mixed fluid-rich fine-grained sediments, associated with fragments of rocks or consolidated mud, are expelled at the earth's surface or on the seafloor (Staffini *et al.*, 1993). It was discovered that the immediate surroundings of these volcanoes are constituted of micro-organisms, of which 99% are still unidentified today. One of 1,100 mud volcanoes is located in the Island of Baratang, part of the Great Andaman archipelago, where there was a significant eruption event in 2003.

In Andaman and Nicobar Islands, out of total geographical area of 8,249 sq. km land, only 9% land is used for vegetable cultivation. The wilt caused by bacterial pathogen *Ralstonia solanacereaum*, fungal pathogen *Fusarium* sp., damping off caused by *Pythium* sp., root rot by *Rhizoctonia* sp. and *Macrophomina* sp., collar rot caused by *Sclerotium rolfsii* and anthracnose caused by *Colletotrichum* sp., were reported to cause severe damage

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to the vegetable crops in Andaman and Nicobar Islands. As a new inch in the search of agriculturally important microorganisms from various sources, here the microorganisms have isolated from Mud Volcano, one of the natural gift of the great Andaman archipelago. Though studies proving that the microorganisms isolated from the terrestrial mud volcano shows potentiality like hydrocarbon utilization and production of methane gas (Alain, 2006) the current study was carried out to find out their potential microbial source as plant growth promoters and effective biocontrol agent.

## Materials and Methods

### Sampling regime and physic-chemical analysis

Volcano samples were collected from the Island of Baratang, part of the great Andaman archipelago, Indian Ocean. The samples has been collected from the different points of mud volcano (12º07'46.5" latitude; 092º47'31.2" longitude) *viz*., point of the eruption (PM), end of the volcano (EM), dried part of the mud volcano (DM), outside of the mud volcano (OM) and the soil sample from Lime Cave (LC) (12º05'37.6" latitude; 092º44'38.5") were collected to obtain a representative composite sample. In subsamples pH, electrical conductivity, available nitrogen, potassium and phosphorous contents as described by Jacob Parnell (1996) and the results were shown in Table 1. For isolation of bacteria, 1 g of each soil sample was suspended into 9 mL of sterile saline solution. Ten-fold of serial dilution was performed then 0.1 mL of aliquot was used for pour plating on nutrient agar plates (Anon, 1957). The plates were maintained at 28 ± 2 °C for 48 to 72 h, total colonies were enumerated and the colonies showing visible morphological differences were isolated. Counts were done after 24 h of incubation at 25 °C and results were expressed as colony forming units (cfu) $g^{-1}$ soil (on a dry-weight basis). A total of 52 bacterial isolates were isolated from five soil samples of mud volcano and lime cave soils were purified and maintained on 20% glycerol stock and nutrient agar slant for the further experiments and analysis.

### Morphological and biochemical characterization

Each isolate was subjected to morphology, motility and 3% KOH solubilization test (Suslow *et al.*, 1982). Biochemical tests were also performed. Nitrate reduction ability of the organisms was determined as described by García de Salamone *et al.* (1996). Oxidase activity was measured using the reactive discs from Himedia, India. Based on morphological and biochemical characterization 24 of 52 isolates were selected for PGPR and diversity analysis.

### Plant growth promoting properties

#### Indole acetic acid production

Inocula were transferred to tryptone broth was used to quantify auxin production. Sterilized tryptone broth was inoculated with the test culture and incubated at 30 °C for 48 and 72 h with occasional shaking. The tubes were centrifuged at 15,500 *g* for 2 min and auxin production was measured as indole-3-acetic acid (IAA) equivalents, as follows: Equal volume of culture supernatant was added with Salkowski reagent (12 g $L^{-1}$ $FeCl_3$ + 7.9 $MH_2SO_4$). The mixture was allowed to stand for 30 min for color development. Color intensity was measured spectrophotometrically at 550 nm using a standard curve (Sarwar and Kremer, 1992).

#### Mineral phosphate solubilization

Phosphate solubilization ability of isolates was assessed qualitatively using Pikovaskaya's agar medium (Pikovaskaya, 1948) containing tricalcium phosphate (5 g/L). Each bacterial culture was spot inoculated in the centre of a plate and incubated at 28 ± 2 °C for 48 h. Clear/halo zone indicates the positive cultures. The halo zone was calculated by subtracting bacterial colony diameter from the total halo zone diameter (Freitas *et al.*, 1997). Each test was replicated three times

#### Siderophore production assay

Qualitative assay was done based on strains competition for iron on medium supplemented with ferric complex of chrome azurol S (CAS) as described by Schwyn and Neilands (1987). The nutrient agar medium was supplemented with CAS 60.5 g in 50 mL, Iron III solution (1 mM $Fecl_3.H_20$ and 10 mM Hcl in 10 mL) and HDTMA (72.9 mg in 40 mL). The isolates were spot inoculated on the plates and incubated for 48 h at 30 °C. Development of yellow-orange halo zone around the culture was considered as positive for siderophore production.

#### Screening for cyanide production

HCN production by the isolates was examined as described by Bakker and Schipper (1957). The isolates were

**Table 1** - Abiotic characteristics of the soils.

| Sample | pH | EC | Available nitrogen (kg/ha) | Available phosphorous (kg/ha) | Available potassium (kg/ha) |
|---|---|---|---|---|---|
| DM | 7.5 | 2.3 | 8.4 | 21 | 95 |
| EM | 8.6 | 5.6 | 5.6 | 16 | 150 |
| LC | 8.5 | 0.4 | 25.2 | 40 | 145 |
| OM | 7.4 | 0.2 | 14.0 | 15 | 145 |
| PM | 8.9 | 4.9 | 5.6 | 20 | 155 |

streaked on plates containing King's B medium amended with glycine (4.4 g/L) and the lid of each Petri plate placed Whatman no.1 filter paper soaked in 0.5% picric acid in 2% sodium carbonate. The plates were then sealed with parafilm and incubated at 28 ± 2 °C for 4 days. Change of color from yellow to orange and then to dark brown in the filter paper indicated as positive reaction and the absence as negative reaction.

### Antagonistic activity

The phytopathogenic fungi *Rhizoctonia solani* (ITCC 6376), *Pythium aphanidermatum* (ITCC 5488) and *Macrophomina* sp. (ITCC 5519) were collected from the Indian Type Culture Collections Centre, IARI, New Delhi and *Sclerotium* (*Athelia*) *rolfsii* (MTCC 288) collected from Microbial Type Culture Collection, IMTECH, Chandigarh.

The antagonistic ability of bacterial isolates was determined by dual culture technique as described by Dennis and Webster (1971). Six millimeter agar discs containing grown mycelia of any of the four phytopathogenic fungi were at approximate distance apart on a PDA dish. Four bacterial isolates were streaked between the agar discs. Inhibition of fungal mycelium around the bacterial colony was scored for 2-3 days by measuring the radial growth of the pathogen. PDA plate inoculated only with pathogen was taken as control. All strains were tested in two replicates on different plates and the tests were carried out twice for each isolate.

Percentage of inhibition was calculated by the following formula

$$I = \frac{C - T}{C} * 100,$$

where, $I$ is percent inhibition of growth, $C$ is mycelial growth in control plate, $T$ is mycelial growth in test plate.

## Production of hydrolytic enzymes

### Screening for cellulase production

The fresh culture was spot inoculated on the pre poured Sodium carboxy methyl cellulose (CMC) medium. The plate was incubated for 2-3 days at 28 ± 2 °C. Plates were then flooded with Gram's iodine to visualize halo zone around the bacterial colony, which indicates positive result in utilizing of the cellulose substrate.

### Screening for protease production

Proteolytic activities of the cultures were screened qualitatively in a medium containing skim milk. Fresh culture was streaked onto the medium and incubated at room temperature for 24-48 h. Clear zone formation around the colony indicates the proteolytic activity of the bacterium as positive and absence as negative.

### Intrinsic antibiotic resistance (IAR) test

Bacterial isolates were screened for their sensitivity or resistance to antibiotics. IAR has also been used in the genotypic identification of bacterial species. Plate of Muller Hinton Agar was swabbed with fresh culture; fourteen different antibiotic discs were pressed gently on the surface of the plate at sufficient distance and incubated at 28 ± 2 °C for 3 days. The presence of inhibition zones around the discs of the different antibiotics were noted (Bauer *et al.*, 1966). Each test was replicated three times.

### Salt tolerance

A plates containing beef extract, peptone plus sodium chloride at different concentration (7-12%) was inoculated with 20 µL aliquot of an overnight test culture. The plates were then incubated at room temperature for 24-48 h. Growth on the culture media plates is recorded as positive and absence of growth is recorded as a negative.

### Genotypic characterization of bacterial isolates

The selected bacterial strains exhibiting pronounced in vitro PGP qualities were characterized for their diversity analysis. Genomic DNA was extracted based on the method described by Chen and Kuo (1993). DNA was electrophoresed on 1% agarose, ethidium bromide gel.

One hundred nanograms of DNA were used as template in PCR procedures. Selected primers pA and pH (5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3' respectively) were used for amplification as described by Edwards *et al.* (1989). The mixture was subjected to initial denaturation of 92 °C for 2 min and 10 s followed by 35 cycles of 92 °C for 1 min, 48 °C for 30 s and 72 °C for 2 min and 10 s and a final extension step of 72 °C for 6 min and 10 s using GeneAmp® PCR system 9700 (Applied Biosystems, USA). The PCR products were resolved using a 1.5% agarose gel photographed under UV transillumination. PCR products of 1.5 kb were sent to commercial gene se-

**Table 2** - Summary of the 16S rDNA sequences from some bacterial strains with plant growth promoting properties.

| Strain Code | 16S rRNA gene identification | GenBank accession No. |
|---|---|---|
| DM 5 | *Pseudomonas* sp. | HQ400616 |
| EM 5 | *Enterobacter aerogenes* | HQ400617 |
| EM 9 | *Exiguobacterium* sp. | HQ400618 |
| OM 2 | *Enterobacter aerogenes* | HQ400619 |
| OM 3 | *Enterobacter hormaechei* | HQ400620 |
| OM 5 | *Pantoea agglomerans* | HQ400621 |
| OM 6 | *Enterobacter aerogenes* | HQ400622 |
| OM 8 | *Enterobacter cloacae* | HQ400623 |
| PM 9 | *Enterobacter cloacae* | HQ400624 |
| LC 1 | *Enterobacter aerogenes* | HQ400625 |

quencing laboratory of Synergic scientific services, Chennai, India for sequencing. Ten partial 16S rDNA nucleotide sequences determined in this report have been deposited in the GenBank. (Table 2)

Amplicons obtained using the primers pA and pH have been used for the restriction analysis using *Hpa*II, and *Msp*I resstrictases. The restriction mixture contained 10 µL of PCR product, 1.5 U of each of the restrictases and manufacturer's recommended buffer (2.5 µL of 10x) up to 25 µL volume. The reaction mixture was incubated at 37 °C for 2 h and the reaction was inactivated by heating at 70 °C for 10 min. Restriction products were separated in 3% agarose gel in TAE buffer. Medium size 100 bp DNA (GeNeI) ladder were used to estimate the size of the digested fragments.

Strains were genotypically characterized by BOX-PCR, as described by Rademaker and De Bruijn (1997) using BOX A1R (5'- CTA CGG CAA GGC GAC GCT GAC G-3') as primer. Reaction was performed in 50 µL reaction mixture containing: 5 µL buffer (10x), 1 µL of BOX A1R, 1.5 µL of each dNTP, 1 µL of Taq DNA polymerase, 36.5 µL of sterile bidistilled water and 4 µL of template DNA. PCR-amplifications were performed in a GeneAmp® PCR system 9700 (Applied Biosystems, USA) and the temperature profile was as follows: initial denaturation at 95 °C for 6 min, 35 cycles at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 65 °C for 8 min followed by final extension at 65 °C for 16 min. Amplified products were separated by horizontal electrophoresis on 1.5% agarose gels.

### Field experiments

Tomato and chili seeds were treated with bacterial suspensions of OM5 and EM9 strains ($10^8$ cfu/mL) as well as their combination (OM5+EM9 in equal parts) for 30 min and were shade dried at 28 °C for 1 h, the control seeds were coated with sterilized tap water. The treated seeds were sown in pots (15-20 cm) sterilized with 0.7% sodium hypochlorite solution, filled with red sand, soil and farmyard manure (2:1:1) which was sterilized at 121 °C for 30 min. Observations were recorded on germination percentage in the beginning, after one month of sowing, 10 seedlings from each replication were assessed for their root length, shoot length, wet weight and dry weight. Each treatment consisted of four replicates of 100 seeds each (25 seeds/pot) and the experiment was repeated three times.

### Data analysis and dendrogram

For the analysis of ARDRA and BOX PCR molecular weight and Rf values of each band was determined by using 'Molecular Analyst software' (Version 1.5). The character state '1' was given for a band, which could be clearly and reproducibility detected in the gel and '0' was assigned if it was not possible to determine. The similarity coefficient and cluster analysis was performed (Janda, 1991) by the unweighted-pair-group method with arithmetic average (UPGMA) of NTSYS-pc 2.02e (Applied Biosciences, Inc., New York, USA).

## Results

### Morphological and Biochemical characteristics

The characterized mud volcano and lime cave isolates (a total of 52 bacterial isolates) showed diverse morphological and cultural characteristics as indicated from variations in colony size and shape. On the basis of their Gram reaction, 10 (19.2%) of the isolates were found to be Gram positive and about 42 (80.8%) were Gram negative bacteria (Data not shown).

Upon subjecting to various biochemical tests, it was observed that 34 isolates (65.4%) were able to degrade tryptophan to indole and pyruvic acid while other isolates showed negative reaction to indole test. Nearly 48.1% (25 isolates) of 52 isolates showed positive result for methyl red test, 27 isolates were shown to produce variable results and negative result for methyl red test. Twenty five isolates showed positive result to Voges Prauskauer test and nearly 51.9% isolates (27 isolates) showed variable and negative result to this test. Twenty three isolates utilized citrate and all the other isolates showed negative result for citrate utilization test. Only 30.8% isolates (16 isolates) were able to reduce nitrate to nitrite, while the rest of the isolates are non-reducers of nitrate. Starch hydrolysis results showed that 26 isolates (50%) were able to hydrolysis starch as a source of carbon and remaining isolates did not hydrolyze the starch. (Data not shown)

### Plant growth promoting traits

Based on morphological and biochemical variations 24 distinct isolates were screened for their PGPR potential. Of the 24 isolates 15 were able to produce IAA. The outer part of the mud volcano sample had the more number of IAA producers in relation to other sites. Only 15 out of the 24 isolates were able to solubilize phosphate. The mud volcano sample collected from the outer part showed the highest number of phosphate solubilizer strains in relation to the other sites. Only 7 isolates of 24 were able to produce siderophores. The outer part of the mud volcano sample presented the highest number of isolates that produce siderophores. Of the 24 isolates 5 were able to produce HCN. Dried part of the mud volcano sample had the highest number of hydrogen cyanide production than other sites. Out of 24, 10 isolate showed protease and cellulose production (Table 3).

Isolates OM5, DM5, DM6, EM9, LC6 showed positive result for more than four properties tested. There was no significant contribution of the area from where the samples collected, as demonstrated in Table 3.

**Table 3** - Bacterial isolates representing the plant growth promoting properties from mud volcano & lime cave.

| Isolate | IAA | Phosphatase | HCN | Sediropore | Protease | Cellulase |
|---|---|---|---|---|---|---|
| PM 9 | + | - | - | - | - | - |
| OM 2 | + | + | - | - | - | - |
| OM 3 | + | + | - | - | - | - |
| OM 4 | + | - | - | - | - | - |
| OM 5 | - | + | + | + | + | - |
| OM 6 | + | - | - | - | - | - |
| OM 7 | + | + | - | - | - | - |
| OM 8 | - | - | - | + | + | - |
| OM 10 | - | - | - | - | - | + |
| OM 11 | - | - | - | + | + | + |
| DM 5 | + | + | + | + | + | + |
| DM 6 | - | + | + | + | + | + |
| DM 7 | + | + | - | - | - | + |
| DM 8 | + | - | - | - | - | + |
| DM 10 | + | - | - | - | - | - |
| EM 4 | - | - | - | - | - | - |
| EM 5 | + | - | - | - | - | + |
| EM 6 | + | - | - | - | + | + |
| EM9 | + | + | + | + | + | - |
| EM 11 | - | - | - | - | + | + |
| LC 1 | + | + | - | - | - | - |
| LC 4 | - | + | - | - | - | - |
| LC 5 | + | - | - | - | + | + |
| LC6 | + | + | + | + | + | - |

**Table 4** - Bacterial isolates representing the antagonistic activity against four plant pathogens.

| Isolate name | *Macrophomina* sp. | *Pythium aphanidermatum* | *Sclerotium rolfsii* | *Rhizoctonia solani* |
|---|---|---|---|---|
| PM 1 | 5 | 29.2 | 11.7 | 24.2 |
| OM 2 | 29.2 | 29.2 | 37.5 | 20.2 |
| OM 3 | 26.7 | 32.7 | 33.3 | 35 |
| OM 4 | 28.3 | 24.2 | 37.5 | 33.3 |
| OM 5 | 55.8 | 48.3 | 41.7 | 55.8 |
| OM 6 | 36.7 | 41.7 | 40 | 25.8 |
| OM 7 | 30 | 41.7 | 38.3 | 23.3 |
| OM 8 | 57.5 | 46.7 | 20.8 | 36.7 |
| OM 10 | 21.7 | 25 | 30 | 8.3 |
| OM 11 | 51.7 | 44.2 | 5.8 | 35.8 |
| DM 5 | 50 | 61.7 | 35 | 65 |
| DM 6 | 29.2 | 29.2 | 22.5 | 38.3 |
| DM 7 | 38.3 | 34.2 | 23.3 | 38.3 |
| DM 8 | 40.8 | 33.3 | 30.8 | 33.3 |
| DM 10 | 30.6 | 33.3 | 43.3 | 32.5 |
| EM 4 | 16.7 | 0 | 3.3 | 1.7 |
| EM 5 | 5 | 1.7 | 3.3 | 1.7 |
| EM 6 | 5 | 1.7 | 1.7 | 6.7 |
| EM 9 | 60 | 65.38 | 40.2 | 60 |
| EM 11 | 3.3 | 1.7 | 3.3 | 8.3 |
| LC 1 | 28.3 | 26.7 | 22.5 | 35 |
| LC 4 | 11.7 | 25.8 | 35 | 38.3 |
| LC 5 | 3.3 | 3.3 | 3.3 | 3.3 |
| LC 6 | 52.5 | 67.2 | 40 | 57.2 |

## Intrinsic antibiotic resistance and salt tolerance

When susceptibility to antibiotics was investigated, isolates LC5, EM10 and EM3 showed sensitive to all antibiotics tested, none of the isolates showed resistant to all antibiotics. All OM isolates showed resistant to Ampicillin, Vancomycin and Amoxicillin. Whereas, DM isolates were resistant to Amoxycillin and Vancomycin and PM isolates showed resistant to Vancomycin. Isolates OM5 and EM4 tolerated high salt concentration of 12% (Data not shown).

## Biocontrol activity of the strains

Isolates OM5, EM9 and LC6 showed antagonistic effects against mycelial growth of root rot fungus *S. rolfsii* (Inhibition zone greater that 40%). Isolates LC6 (67.2%) and EM9 (65.38%) are the only two bacterial isolate which inhibited *P. aphanidermatum* significantly over the control. Mycelial growth of *R. solani* was inhibited by strains DM5 (65%), EM9 (60%) and LC6 (57.2%). The antagonistic effect appeared after 3 days of incubation. Among 24 isolates EM9, OM8, OM5, OM11 and DM5 showed statistically significant highest mycelial inhibition of charcoal rot causing fungus *Macrophomina* sp. (Inhibition zone greater that 50%) (Table 4).

## Molecular identification based on 16S rDNA sequence

Undigested PCR products amplified with pA and pH primers produced a single band of about 1500 bp. ARDRA analysis revealed large variations among the isolates. Restriction pattern obtained after digestion of the amplified fragment with *Hpa*II and *Msp*I revealed 12 and 15 patterns respectively (Fig. 1a, b, c & d).

Similarity analysis was then performed based on composite ARDRA profile, showed low similarity index around 58% (Figure 2). Amplified patterns were observed in the band profiles obtained during genetic analysis. Consequently, we suppose that the PGPR and antagonistic effects would not be associated to a single genotype of wide distribution, but to a pronounced bacterial genetic diversity. Four groups were formed in the composite dendrogram analysis. Group I consists of isolates from dried and end point mud volcano, group II formed with four isolates each from end point and dried mud volcano and two iso-

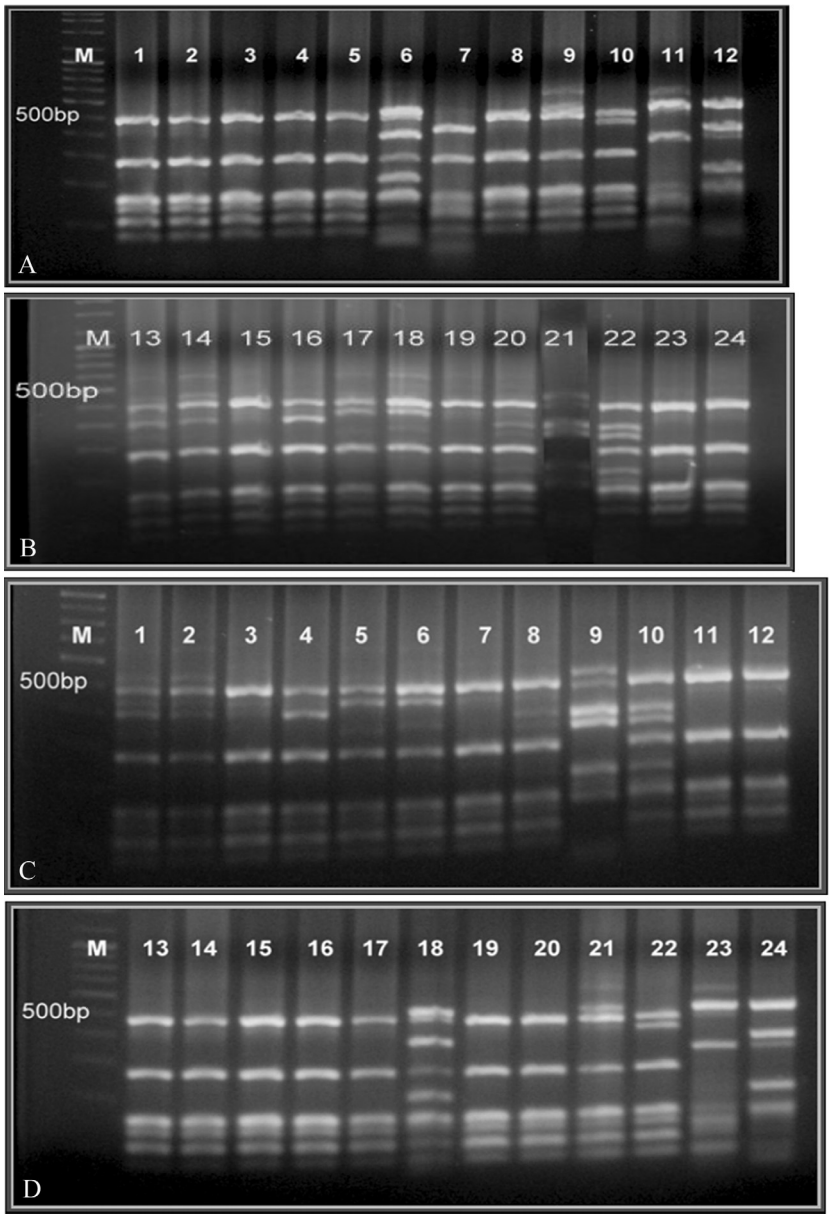


**Figure 1** - Restriction Digestion pattern using enzyme *Msp*I. Lane 1-24 (DM5-8, 10, EM4-6, 9, 11, OM2-8, 10, 11, PM9, LC1, 4-6). c&d Restriction Digestion pattern using enzyme *Hpa*II. Lane 1-24 (DM5-8, 10, EM4-6, 9, 11, OM2-8, 10, 11, PM9, LC1, 4-6).

lates from outside mud volcano. Group III comprised of predominantly outside mud volcano isolates followed by 2 isolates from lime cave and one isolate from point mud volcano. Group IV formed with two isolates of lime cave only. There is no grouping of correlation was observed among the isolates based on their respective antagonism and PGP properties.

## Genomic fingerprinting by BOX-PCR

BOX sequences are regulatory sequences which can increase and decrease the specific gene expression (Lupski and Weinstock, 1992). Amplicons obtained by BOX-PCR were analysed on 3% agarose gel (Figure 3a,b).

Strains were grouped into four clusters with low similarity index (Figure 4). All the strains showed wide variations in fingerprinting pattern due to their high degree of genetic variability and distributed into different clusters.

## Field experiment

Green house experiment analysis showed that there was an average increase of 14.2 and 27.4% in seedling emergence of chili and tomato respectively. Seed bacterization of chili and tomato have increased the secondary root numbers up to 56.8 and 46.5% respectively, significant increase in primary root length of both the plants were also noticed. Stem length of chili and tomato were also increased up to 10.5 and 38.1%. No significant difference

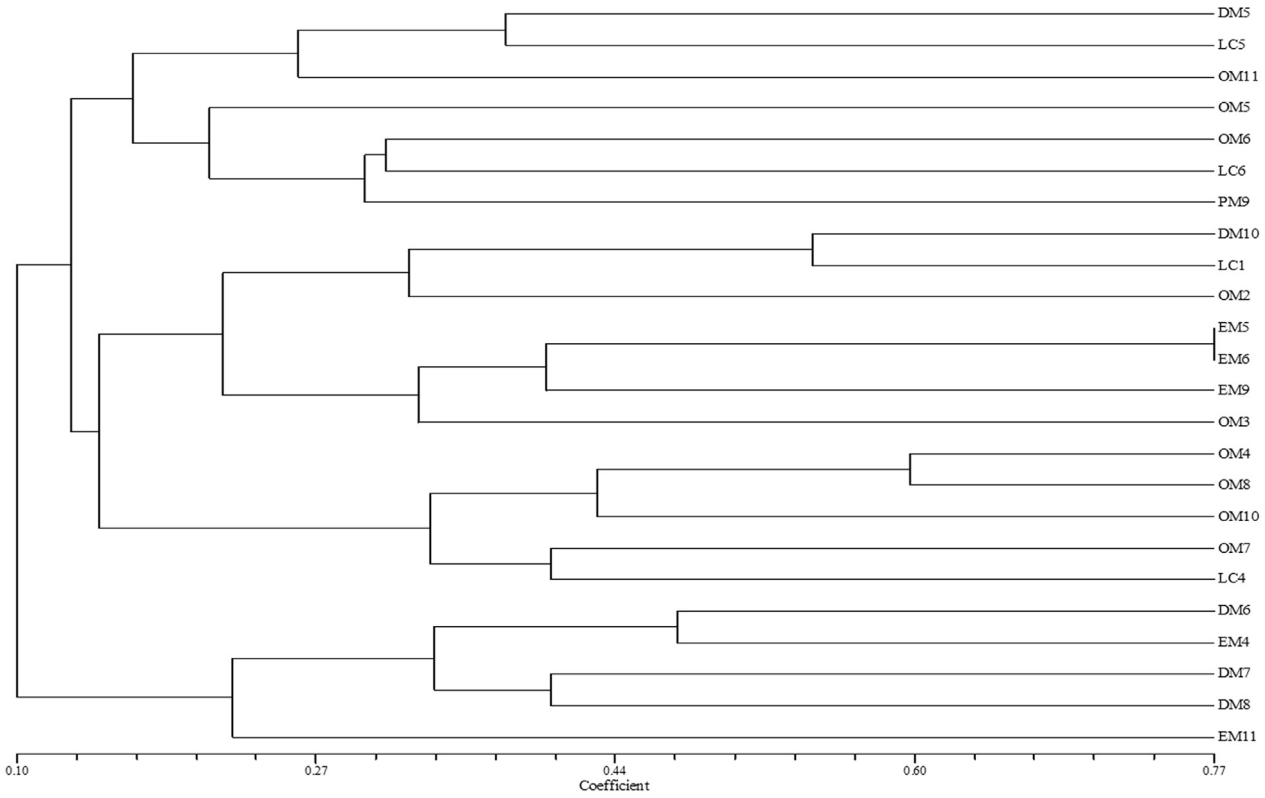


**Figure 2** - Dendrogram showing the similarity co-efficient of Mud volcano & Lime cave isolates based on ARDRA analysis using *Hpa*II and *Msp*I Restriction Enzymes.

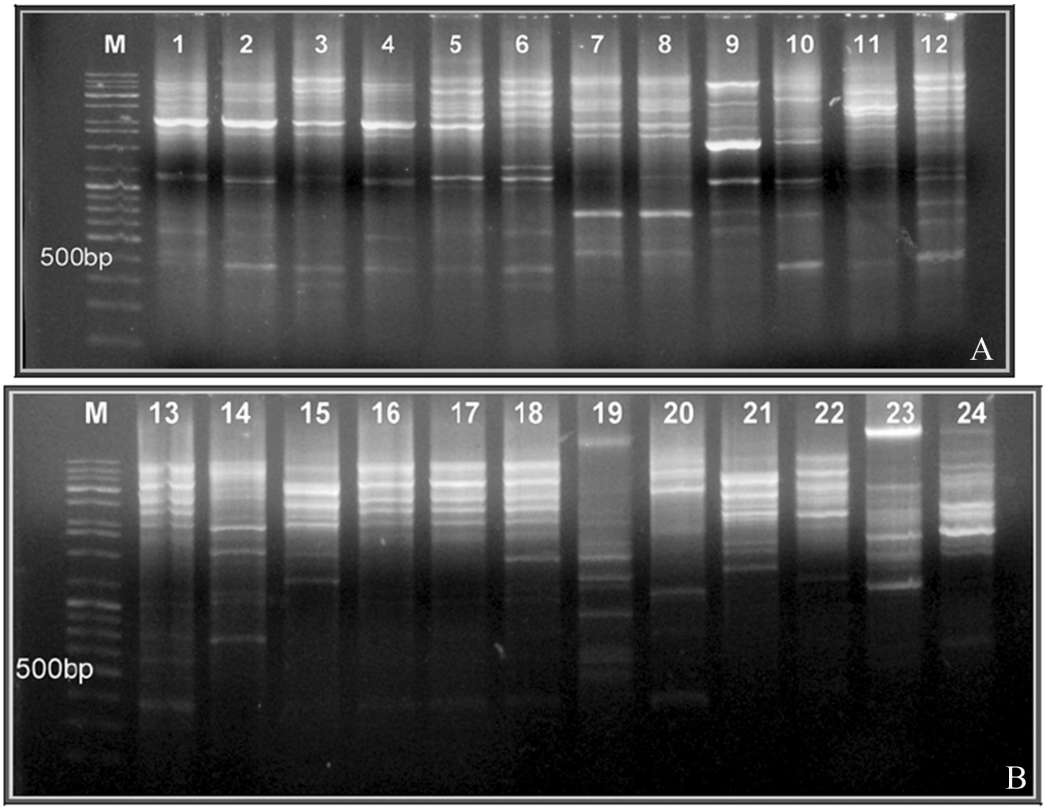


**Figure 3** - Amplified patterns obtained by BOX-PCR Lane 1-24 (DM5-8, 10, EM4-6, 9, 11, OM2-8, 10, 11, PM9, LC1, 4-6).

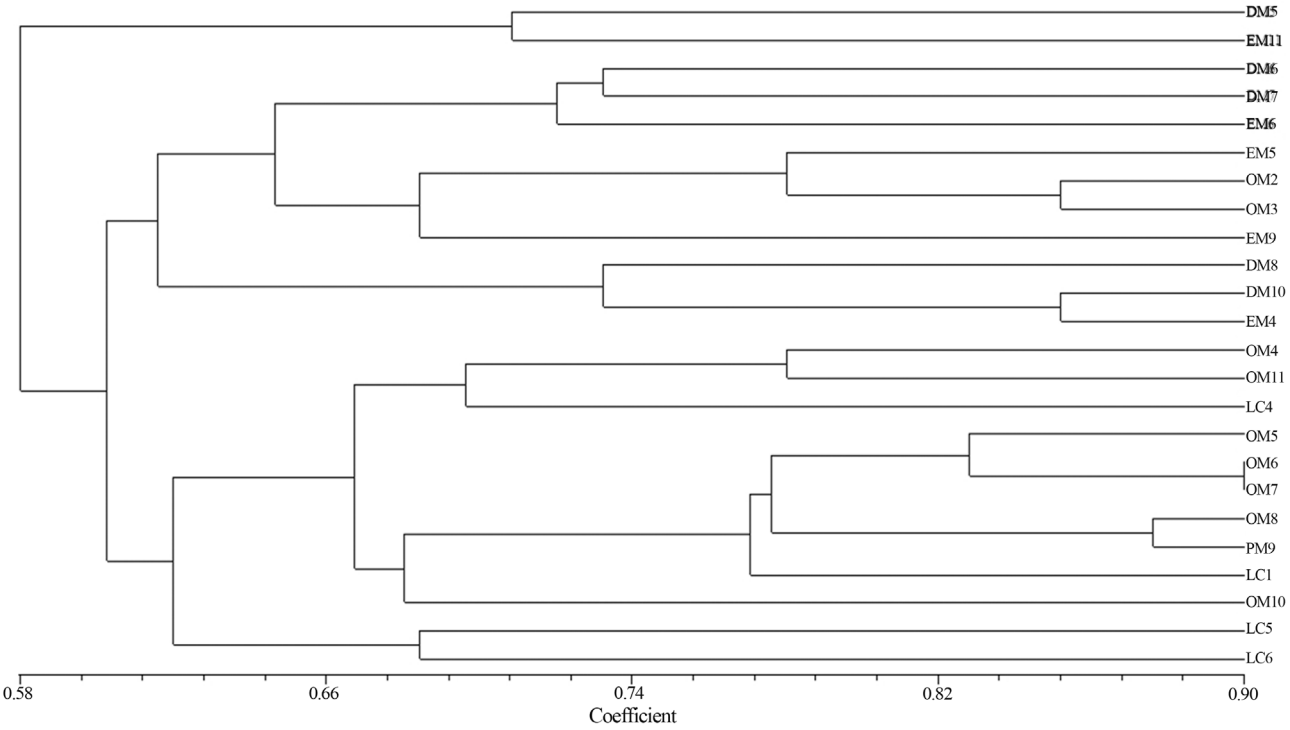


**Figure 4** - Dendrogram showing the similarity co-efficient of Mud volcano and Lime cave isolates based on BOX-PCR analysis.

were noticed in fresh weight and dry weight of tomato plant but it was interesting to note that fresh and dry weight of chili plant was increased up to 91.2 and 135% respectively. Among all the different treatments, consortium of OM5 and EM9 showed best results in all parameters except fresh and dry weight of tomato (Table 5 and Figure 5).

## Discussion

Present study is first to report the plant growth beneficial microorganisms in the active mud volcano and lime cave soil. As reported by several authors (Gilbert *et al.*, 1993) that Gram negative ones are the most abundant rhizobacteria, around 80% of our isolates were Gram negative. Twenty four isolates were screened based on morphological and biochemical features. These isolates were screened for their potential PGPR characteristic. These twenty four isolates do not represent the bacterial diversity of the soil.

Plant auxin IAA produced by the bacteria enhances plant cell elongation or cell division thus stimulates better root growth (Glick *et al.*, 1998). It was interesting to note that about 66% of our isolates are IAA producers. Under field conditions precipitated phosphates should be solubilized to readily available ones for plant growth, which can be done by plant growth promoting bacteria (Verma, 2001). Siderophore produced by bacteria binds to the free iron in the rhizosphere, thus favors the biocontrol agent than those of the pathogen (Siddiqui, 2005). Of the 24 isolates 7 were able to produce siderophores. Siderophores produced by the isolates should be studied for their affinity towards different iron complexes. Isolate DM5 showed positive response to all properties tested. Isolate EM9 showed positive response to all the above properties tested except cellulase production, isolate OM5 showed positive response to all the above properties tested except IAA and cellulase production. The above tested properties have been reported among the mechanisms by which the microorganisms promote plat growth (Niranjan *et al.*, 2005), supporting the further evaluation of our isolates for their potentiality. One of the mechanisms of fungal inhibition is degrading their cell wall by lytic enzymes, hence the isolates were also screened for their protease and cellulase production. Ten isolates of 24 showed positive result for both the enzymes production. Growth and yield of several crop plants has been adversely affected by salinity, interaction of plant growth promoting bacteria helps the survival in adverse conditions. All 24 isolates showed positive result of which OM5 and EM4 showed highest tolerant rate.

Wide variety of bacterial species can be used to protect plants against fungal and bacterial diseases (Ren et *al*., 2006). Our isolates were tested against phytopathogenic fungi *Rhizoctonia solani, Pythium aphanidermatum, Macrophomina* sp. and *Sclerotium* (*Athelia*) *rolfsii* to assess whether they can act as effective biocontrol agent. Isolates OM5 and EM9 showed comparatively effective antagonism against all pathogens tested and can be considered for good candidature as biocontrol agent. Observations indicated that bacteria use to compete with pathogens

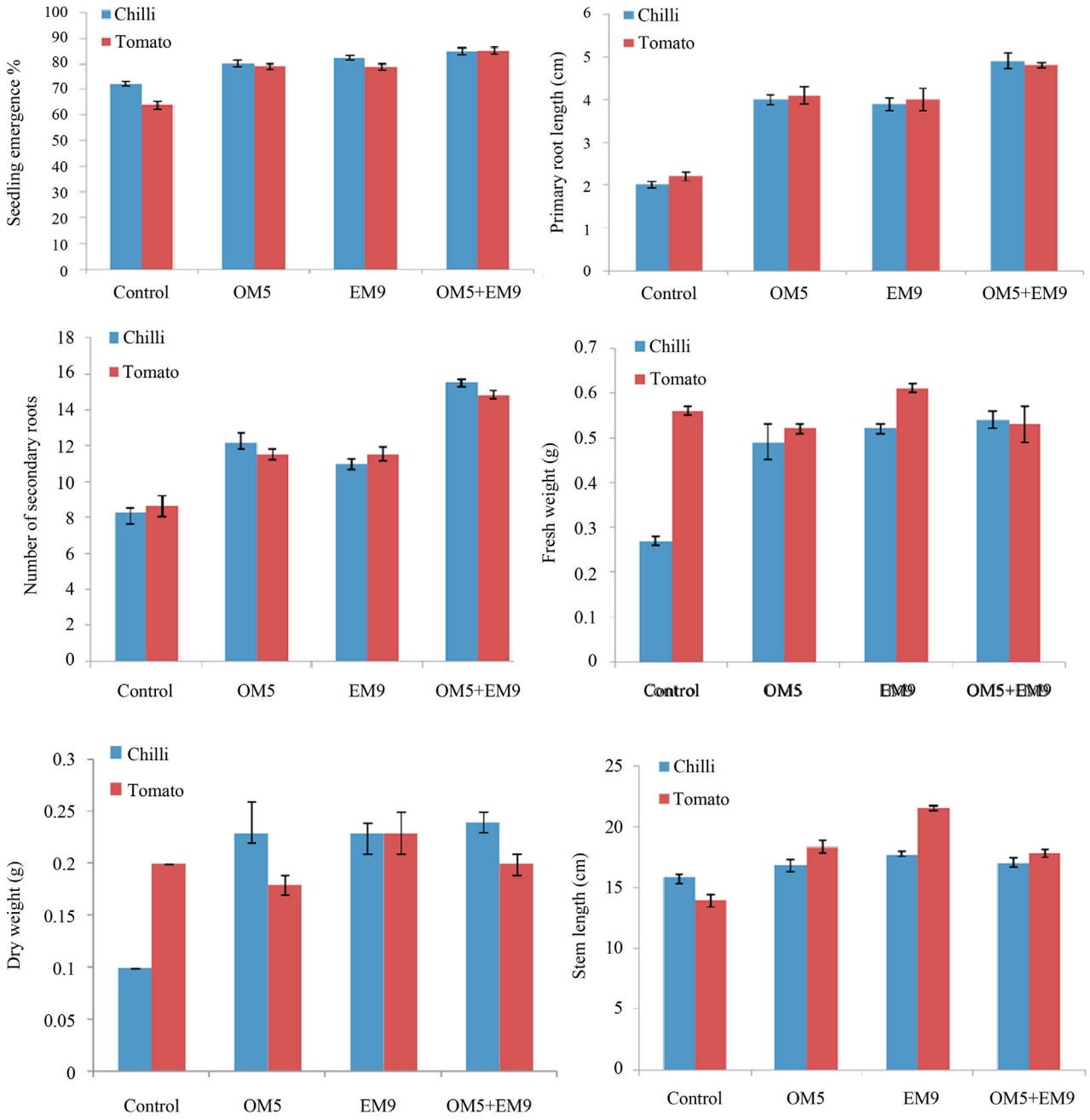


**Figure 5** - Effect of seed bacterization of PGPR OM5 and EM9 (a) Seedling emergence, (b) Primary root length, (c) Number of secondary roots, (d) Fresh weight, (e) Dry weight and (f) Stem length. Seeds without any treatment (control), seeds co-inoculated with OM5 at cell concentration of $10^8$ cell/mL (OM5), seeds co-inoculated with EM9 (EM9) and seeds co-inoculated with consortium of OM5 and EM9 (OM5+EM9).

for the nutrients available in the culture media and also some diffusible compound secreted by the bacterial strains should be responsible for fungal inhibiton. Eventhough *Pantoea agglomerans* is considered as bacterial pathogen for rice, studies proved the ability of these strains to reduce incidence of fire blight in apple, pears and flowers (Elmer *et al.*, 2005). It was also proved as most promising biocontrol agents for variety of bacterial and fungal diseases in plants (Rezzonico *et al.*, 2009).

A similarity analysis was then performed based on composite ARDRA profile from the use of the two restriction endonucleases (*Hpa*II and *Msp*I) showed low similarity index around 58%. In the genomic finger prints of potential isolates showed no particular correlation between the origin of the isolates and the ARDRA groups could be established. In BOX-PCR four groups were formed. There was a broad range of variations in their fingerprinting pattern due to their high degree of genetic variability and distributed into different clusters. On observation of these

**Table 5** - Effect of seed treatments with suspensions of PGPR strains on growth of chili and tomato seedlings.

| Treatment | Seedling emergence (%) | | Primary root length (cm) | | Secondary root numbers | | Fresh weight (g) | | Dry weight (g) | | Stem length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Chili | Tomato | Chili | Tomato | Chili | Tomato | Chili | Tomato | Chili | Tomato | Chili | Tomato |
| Control | $72 \pm 1.0^{a}$ | $63.7 \pm 3.2^{a}$ | $2 \pm .08^{a}$ | $2.2 \pm .10^{a}$ | $8.2 \pm .33^{a}$ | $8.6 \pm .58^{a}$ | $0.27 \pm 0.01^{a}$ | $0.56 \pm 0.01$a | $0.10 \pm 0.00^{a}$ | $0.20 \pm 0.00^{a}$ | $15.8 \pm 0.25^{a}$ | $13.9 \pm 0.53^{a}$ |
| OM5 | $80 \pm 1.1^{b}$ | $79 \pm 1.1^{b}$ | $4.0 \pm .12^{b}$ | $4.1 \pm .21^{b}$ | $12.1 \pm .60^{b}$ | $11.5 \pm .29^{b}$ | $0.49 \pm 0.04^{b}$ | $0.52 \pm 0.01$a | $0.23 \pm 0.03^{b}$ | $0.18 \pm 0.01^{a}$ | $16.8 \pm 0.47^{a}$ | $18.3 \pm 0.48^{b}$ |
| EM9 | $82 \pm 1.0^{b}$ | $78.7 \pm 1.2^{b}$ | $3.9 \pm .15^{b}$ | $4 \pm .26^{b}$ | $11 \pm .22^{b}$ | $11.5 \pm .38^{b}$ | $0.52 \pm 0.01^{b}$ | $0.61 \pm 0.01$a | $0.23 \pm 0.01^{b}$ | $0.23 \pm 0.02^{a}$ | $17.7 \pm 0.31^{b}$ | $21.5 \pm 0.16^{b}$ |
| OM5+EM9 | $84.7 \pm 1.4^{b}$ | $85 \pm 1.5^{b}$ | $4.9 \pm .19^{b}$ | $4.8 \pm .07^{b}$ | $15.5 \pm .14^{b}$ | $14.8 \pm .25^{b}$ | $0.54 \pm 0.02^{b}$ | $0.53 \pm 0.04$a | $0.24 \pm 0.01^{b}$ | $0.20 \pm 0.01^{a}$ | $17 \pm 0.44^{b}$ | $17.8 \pm 0.34^{b}$ |

Mean ± S.E (Standard error) followed by the same letter do not differ significantly according to Dunnett's test at p = 0.05.

results, current study identified a high degree of genetic variability among different species as well as same species.

## Conclusion

In last few decades there is increased need to explore varied ecological niches to find out beneficial microorganisms. Increasing awareness about the chemically synthesized fertilizers based agriculture practices, this study identified two isolates selected and genetically characterized (*Pantoea agglomerans* [OM5], *Exiguobacterium* sp. [EM9]) showed excellent antagonistic property as well as other plant growth promoting properties. Field trail experiments showed increase in the plant growth of tomato and chili by seed bacterization. Hence these isolates can be of good candidate for potential agricultural use as multipurpose organism, effective biocontrol agent and plant growth promoters.

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