Isolation and characterization of rhizobacteria against *Fusarium* wilt diseases of Cucurbitaceae

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Abstract

The bacteria present around the rhizosphere, suppress diseases and promote the plant growth are known as plant growth promoting rhizobacteria (PGPR). The PGPR metabolites directly or indirectly influence the plant growth. Cucurbitaceae is one of the largest groups of summer vegetables includes, 118 genera and 825 species most of them possessing medicinal value. *Fusarium* spp. cause major infection in curcurbits. In present investigation 128 bacterial isolates were isolated from rhizosphere soil samples collected in and around Mysore district, Karnataka, India. All the isolates were identified based on the morphological structure, IAA, siderophore, HCN, phosphate solubilisation, hydrolytic enzymes present and by molecular characterization. Out of the 128 isolates only nine isolates were confirmed as PGPR. The PGPR strains showing more than two traits showed good antifungal activity against *F. oxysporum* f. sp. *cucumerinum*. These data provide evidence that the PGPR isolates have potentiality to protect the cucumber plant from *Fusarium* wilt disease.

Keywords : PGPR, *Fusarium oxysporum*, Wilt and Cucurbitaceae

Introduction

Plant growth promoting rhizobacteria (PGPR) are root-colonizing rhizobacteria which help in either growth promotion or and biological control of plant diseases (Compant *et al.*, 2010). In developing countries to control or minimize the use of chemicals in agriculture and to increase the nutrient availability to plants, PGPR are being used from past three decades. PGPR associated with the plant - rhizosphere influence the soil microfauna by stimulating plant growth, crop yield and disease control (Glick, 2012). Root exudates release organic acids, sugars, amino acids, nucleic acid and vitamins but these are affected by plant species, root region, plant age, pH, temperature and surrounding microbes. There are many direct and indirect mechanisms to enhance the growth of plants which include production of hormones like indole acetic acid (IAA) (Patten and Glick, 2002) and gibberellic acid (GA) (Liu *et al.*, 2013), production of cell wall degrading enzymes like chitinase (Shanmugaiah, 2008), β-1,3-glucanase (Kumar *et al.*, 2010), production of hydrogen cyanide (HCN) (Jayaprakashvel *et al.*, 2010), iron-chelating siderophores (Yu *et al.*, 2011), 1-amino-cyclopropane-1-carboxylate (Penrose and Glick, 2003), mineralization or solubilisation of soil phosphorous (Hariprasad and Niranjana, 2009) and antagonism (Idris *et al.*, 2007). All these multiple plant growth promoting traits in the PGPR increase the crop yield (Vessey, 2003).

The rhizosphere has complex and diverse group of microbes, which secrete diverse chemical compounds
for cell to cell communication and alter their activity. Fusarium spp., are the major soil-borne pathogens distributed worldwide. The fungi cause wilt and rot diseases in 80 plant species, including cucurbits and cause up to 100% yield loss (Santos et al., 2002). Pathogens survive in soil in the form of chlamydospores for many years, mycelia enter the epidermal tissues invading through roots, extend to the vascular bundles and form spores in plants (Chen et al., 2013). Soil-borne fungi can be controlled by using chemical fungicides (Fravel et al., 2005), but residues of fumigants or fungicides in the soil increase human health risk and environmental pollution. Chemical fertilizers used increase the resistance of the soil-borne pathogens, besides soil solarisation, crop rotation and grafting were also used to control the root diseases (Zhao et al., 2011).

In sustainable agriculture soil borne pathogens can be controlled by PGPR as they colonize host root and create competition for space as well as nutrition. The PGPR are ecofriendly, stimulate the plant growth factors and reduce the incidence of crop diseases. PGPR- induced systemic resistance has been demonstrated in many plant species against fungi, bacteria and viruses (Glick, 2012). In modern agriculture PGPR are used as biofertilizer as well as biocontrol against certain seed and soil-borne plant pathogens. Some PGPR successfully tested in greenhouse studies include Fusarium wilt of tomato (Hariprasad et al., 2009), cucumber (Koike et al., 2001), muskmelon (Bora et al., 2004), sorghum (Idris et al., 2007) and bitter gourd (Yang and Sung, 2011). In the present study the PGPR isolated from cucurbitaceae were subjected to biochemical tests, molecular characterization and screened for potential ability to control Fusarium wilt caused by F. oxysporum f. sp. cucumerinum in cucurbits.

Materials and methods
Isolation of rhizobacteria
Rhizosphere soil samples were collected from cucurbitaceae growing fields from Mysore (12°12’ 36″ N, 76° 29’ 24″ E) and Mandya (12° 31’ 12″ N, 76° 54′ 0″E) regions of Karnataka during May to August, 2010 and stored at 4°C until use. The soil samples were mixed with 10 ml of sterile 0.9% NaCl solution and vortexed for 10 min; suitable dilutions were spread plated onto nutrient agar (NA) and incubated at 37±2°C for 24 - 48 h. Each bacterial colony differing in its colony morphology was pure cultured onto NA slants and maintained at 4°C. The PGPR strains were confirmed by rapid screening bioassay (Silva et al., 2003).

Sarratia spp. MIC1, Pseudomonas aeruginosa MIC2, Bacillus cereus MIC3, B. subtilis MIC4, B. cereus MIC5, B. amyloliquefaciens MIC6, B. cereus MIC7, B. licheniformis MIC9 and B. subtilis MIC10 were isolated from the collected soil samples. PGPR were Standard PGPR strains were collected from the Microbial type culture collection (MTCC) Chandigarh, India, which included Pseudomonas aeruginosa MTCC 2581, Bacillus coagulans MTCC3543 and Bacillus subtilis MTCC2763, used as positive controls. The fungal culture Fusarium oxysporum f. sp. cucumerinum was isolated from cucurbitaceae growing field soil and confirmed by the pathogenicity test after inoculating to healthy seed in pot experiments. Rhizobacterial samples were characterised on the basis of biochemical tests as described by Cuppcciono and Sherman (2008).

The PGPR traits were confirmed by standard tests
HCN Production
Glycine (4.4 g/l) and FeCl₃,6H₂O (0.3 mM) were amended with 10% tryptcase soya agar slants. Alkaline picrate solution saturated with Whatman no.1 filter paper strips were placed on the sides of the slants.
and incubated for 2-3 days at 37±2°C. The relative quantification of HCN was done spectrophotometrically at 625 nm as described by Nagarajkumar et al. (2004).

**Indole acetic acid production**

The rhizobacterial isolates on NA medium amended with tryptophan were subjected to qualitative screening for IAA using Salkowski’s reagent [2% of 0.5 M FeCl₃ in 35% HClO₄]. Development of pink colour confirmed IAA production (Ahmad et al., 2008).

**Phosphate solubilisation**

The isolates were inoculated on Pikovskaya’s agar media and the plates were incubated for four days. The Opaque zone around the rhizobacterial colonies confirmed phosphate solubilization (Hariprasad and Niranjana, 2009).

**Siderophore**

Siderophore production was performed as described by Yu et al. (2011). Briefly, chrome azurol S, a blue indicator dye was incorporated into CAS agar medium and inoculated with isolated rhizobacteria. The isolates exhibiting an orange halo after 3 - 4 days of incubation at 37±2°C were considered positive for the production of siderophores.

**Amylase**

The rhizobacterial isolates were streaked onto starch agar medium and incubated for 2 - 3 days at 37°C. Plates were flooded with Lugol’s iodine solution. Appearance of clear zone of hydrolysis around the bacterial growth indicates hydrolysis of starch (Cuupuccino and Sherman, 2008).

**Hydrolytic activity of enzymes of PGPR isolates**

Cellulase production was determined by the medium supplemented with 0.1% carboxymethyl cellulose (CMC) incubated at 37°C for 24 h (Pastor et al., 2012). The rhizobacterial isolates were inoculated onto the pectin agar plates, incubated for 4 days at 37°C. After staining with 0.1% aqueous ruthenium red, those agar plates which were pink with dark pink zones around colonies are an indication of pectinase activity (Cattelan et al., 1999). Casein hydrolysis was assayed by spot inoculating rhizobacterial isolates on 10% casein agar media and incubating for 2-4 days at 37°C. Hydrolysis activity was detected by the translucent area around the colony (Suresh et al., 2010). Production of chitinase was determined on 0.5% colloidal chitin agar plates (Shanmugaiah et al., 2008), β-1, 3-glucanase activity was detected using the laminarin incorporated medium as described by Kumar et al. (2010).

**Inherent antibiotics resistance assay**

Resistance against 20 different antibiotics with different concentrations was determined as described by Hariprasad et al. (2009). Briefly the discs amended with standard antibiotics were placed on previously swabbed rhizobacterial cultures on the nutrient agar medium plates and incubated for 2 - 3 days at 37±2°C.

**In-vitro antagonism assay**

The antagonistic nature of all rhizobacterial isolates were tested against phytopathogen Fusarium oxysporum f. sp. cucumerinum isolated from the cucurbitaceous field by employing a dual culture technique (Idris et al., 2007). Bacterial isolates were seeded at the edges of a 90 mm Petri plate containing PDA and incubated for 36 h at 28 ± 2°C. A 9 mm diameter plug of fungus was placed on the centre of the circle. Plates were incubated at 28 ± 20°C for 7 days. The radii of the fungal colony towards and away from the bacterial colony were measured. The per cent growth inhibition was calculated using the formula,

\[
\text{Per cent inhibition} = \left( \frac{R - r}{R} \right) \times 100
\]

Where, \( r \) is the radius of the fungal colony
opposite to the bacterial colony and R is the maximum radius of the fungal colony away from the bacterial colony.

**PCR amplification and Phylogenetic analysis**

Bacterial isolates were grown on NA medium for 24-48 h and genomic DNA was isolated using DNA isolation Kit (Bangalore Genei, India). All genomic DNA amplified using set of universal primers 27 F (5’-AGAGTTTGATCCTGCTCAG-3’) and 1492 R (5’-TACGGCTACCTTGTTACGACTT-3’) (Luo et al., 2011). PCR amplifications were performed in thermal cycler (ABI Thermocycler). All amplified PCR products were purified and sequenced (Eurofins Genomics India Pvt. Ltd, Bangalore, India). The resulting 16s rRNA sequences were analysed with the reference sequence available at NCBI database. Multiple sequence alignment was achieved using Clustal W (Thompson et al., 1994). The evolutionary distances calculated by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Phylogenetic dendrogram was constructed by Neighbour-Joining (NJ) method and tree topologies were evaluated by performing bootstrap analysis of 1000 replicates using MEGA 5 (Tamura et al., 2011).

**Seed bacterization**

All PGPR strains were grown in nutrient broth medium for 48 h and centrifuged at 8000 rpm for 5 min to get the pellet and washed with sterile distilled water. The final optical density was set to $10^8$ cfu/ml. PGPR suspensions with an adhesive CMC (0.1g/10 ml) were used to treat the surface sterilized cucurbit seeds for 6 h on a rotary shaker at 150 rpm. Seeds soaked in distilled water amended with CMC served as control (Ramamoorthy et al., 2002). The overnight, drained seeds were subjected to seed germination test of the paper towel method (ISTA, 2005), and seedling vigor index was calculated by Abdul-Baki and Anderson, (1973) method. Each rhizobacterial isolate was maintained in triplicate and all experiments were carried out in triplicate.

**Fatty acid methyl esterase (FAME)**

PGPR cultures were grown on the nutrient agar media. The selected samples were dissolved with nonpolar solvents like hexane and BCI3-methanol and heated at 600°C for 10 min. Organic layer was separated and sodium sulphate was added as well as used for the FAME analysis (Koubek et al., 2012).

**Results**

**Isolation and characterization of rhizobacterial isolates**

The eight selected PGPR isolates (Table 1) screened from cucurbitaceae rhizosphere soils were designated as MIC1, MIC2, MIC3, MIC4, MIC5, MIC6, MIC7 MIC9 and MIC10. The isolates were characterized by morphological, physiological, biochemical as well as molecular techniques. Among them, three were Gram negative, non spore formers and seven were Gram positive, spore formers with white colonies (Table - 2). All PGPR isolates were rods, catalase positive, grew well in 2% salt and at 40°C. MIC2 and MIC6 showed oxidase activity, whereas none of the isolates were positive for H$_2$S production. Except MIC9, the rest of the rhizobacteria failed to produce gas in lactose, glucose and sucrose peptone broth. Fermentation was observed with acid production among MIC1 to MIC7 in glucose, MIC2 as well as MIC9 in Sucrose and MIC7 in Lactose. Gelatinase enzyme produced by MIC9, MIC5 and MIC1 at room temperature confirmed liquefaction of gelatin. MIC7, MIC9 and MIC1 degraded lipid forming an opaque zone around the bacteria.

**PGPR traits of isolates**

MIC1 and MIC6 showed phosphate solubilisation with opaque zone around the colonies on Pikovskaya’s
Table - 1. PGPR isolates from different cucurbitaceae crops and place of soil collection

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Isolates</th>
<th>PGPR isolated from field</th>
<th>Crops in the field</th>
<th>Place of soil collection</th>
<th>Accession number *</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MIC1</td>
<td>Sarratia spp.</td>
<td>Cucumis melo (Muskmelon)</td>
<td>Hunsur</td>
<td>KC428743</td>
<td>89%</td>
</tr>
<tr>
<td>2.</td>
<td>MIC2</td>
<td>Pseudomonas aeruginosa</td>
<td>Luffa cylindrica (Sponge Gourd)</td>
<td>H.D.kote</td>
<td>KC567886</td>
<td>100%</td>
</tr>
<tr>
<td>3.</td>
<td>MIC3</td>
<td>Bacillus cereus</td>
<td>Luffa acutangula (Ridge gourd)</td>
<td>Hunsur</td>
<td>KC567887</td>
<td>100%</td>
</tr>
<tr>
<td>4.</td>
<td>MIC4</td>
<td>Bacillus subtilis</td>
<td>Cucumis melo (Muskmelon)</td>
<td>Pandavapura</td>
<td>KC567893</td>
<td>100%</td>
</tr>
<tr>
<td>5.</td>
<td>MIC5</td>
<td>Bacillus cereus</td>
<td>Cucumis sativus (Cucumber)</td>
<td>Pandavapura</td>
<td>KC567891</td>
<td>100%</td>
</tr>
<tr>
<td>6.</td>
<td>MIC6</td>
<td>Bacillus amyloliquefaciens</td>
<td>Cucumis sativus (Cucumber)</td>
<td>Pandavapura</td>
<td>KC567892</td>
<td>100%</td>
</tr>
<tr>
<td>7.</td>
<td>MIC7</td>
<td>Bacillus cereus</td>
<td>Momordica charantia (Bitter gourd)</td>
<td>Mysore</td>
<td>KC567890</td>
<td>100%</td>
</tr>
<tr>
<td>8.</td>
<td>MIC9</td>
<td>Bacillus licheniformis</td>
<td>Luffa acutangula (Ridge gourd)</td>
<td>Pandavapura</td>
<td>KC567889</td>
<td>99%</td>
</tr>
</tbody>
</table>

* All the cultures isolated and used in the study deposited in NCBI database.

Table - 2. Morphological, physiological and biochemical characteristics of bacterial isolates from cucurbitaceae rhizosphere.

<table>
<thead>
<tr>
<th>Biochemical characteristics</th>
<th>PGPR Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram’s test</td>
<td>-ve</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod</td>
</tr>
<tr>
<td>Endospore</td>
<td>Rod</td>
</tr>
<tr>
<td>Growth at 40°C</td>
<td>Rod</td>
</tr>
<tr>
<td>KOH test</td>
<td>Rod</td>
</tr>
<tr>
<td>Urease Test</td>
<td>Rod</td>
</tr>
<tr>
<td>Catalase Test</td>
<td>Rod</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>Rod</td>
</tr>
<tr>
<td>Oxidase Test</td>
<td>Rod</td>
</tr>
<tr>
<td>Lipase Medium</td>
<td>Rod</td>
</tr>
<tr>
<td>H₂S Production</td>
<td>Rod</td>
</tr>
<tr>
<td>Indole Production</td>
<td>Rod</td>
</tr>
<tr>
<td>Bacillus cereus MIC3</td>
<td>Rod</td>
</tr>
<tr>
<td>Bacillus cereus MIC5</td>
<td>Rod</td>
</tr>
<tr>
<td>Bacillus cereus MIC7</td>
<td>Rod</td>
</tr>
<tr>
<td>Bacillus licheniformis MIC9</td>
<td>Rod</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa MTCC2581</td>
<td>Rod</td>
</tr>
<tr>
<td>Bacillus subtilis MTCC2763</td>
<td>Rod</td>
</tr>
<tr>
<td>Bacillus coagulans MTCC3543</td>
<td>Rod</td>
</tr>
</tbody>
</table>
agar plate. All PGPR isolates produced minimum amount of IAA using tryptophan as a substrate and MIC6 gave high concentration of IAA (212 µg/ml) compared to others. MIC1, MIC2, MIC5 and MIC9 isolates produced minimum HCN, compared to maximum amount produced by MIC2 and MIC5 against *F. oxysporum* f. sp. cucumerinum. Siderophores high-affinity chelating agent for ferric iron produced by MIC1 and *Pseudomonas aeruginosa* MTCC2581 gave orange spots around the colonies on CAS agar. Among the rhizobacteria except MIC5 and MIC6, all other isolates showed resistance to Penicillium G, Erythromycin, clindamycin, linezolid, co-trimoxazole, vancomycin, ticarcillin, Imipenam and Piperacillin (Table - 3).

### Hydrolytic enzymes

Production of hydrolytic enzymes by 8 isolates of PGPR stains are summarized in Table 4. The isolates hydrolyzed starch, CMC, pectin, casein, chitin and laminarian under the test conditions. Cellulase and pectinase have been the most frequently detected in the genera *Pseudomonas* and *Bacillus*. However, production of chitinase was observed in *Serratia* spp. MIC1 and *B. cereus* MIC5. Similarly β-1, 3 glucanase was detected in *Pseudomonas aeruginosa* MIC2 and *B. amylo liquefaciens* MIC6. The PGPR isolates screened produced more than one extracellular enzyme as for example three isolates namely, *P. aeruginosa* MIC2, *B. cereus* MIC3 and *B. subtilis* MIC4 exhibited amylase, cellulase, protease and pectinase activity. Therefore, the production of hydrolytic enzymes by PGPR strains indicates their importance in plant protection. The increase in root and shoot germination of cucurbit was observed in treatments of *Pseudomonas aeruginosa* MIC2, *Bacillus subtilis* MIC4, *B. cereus* MIC5, *B. amylo liquefaciens* MIC6 and *P. aeruginosa* MTCC 2581 as compared to the control (Fig.-1).

### Antagonistic activity

Inhibition of mycelial growth of *F. oxysporum* f. sp. cucumerinum by MIC2, MIC3 was 52% and
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40.37%, respectively whereas *F. oxysporum* f. sp. cucumerinum 2 was inhibited maximum (59.66%) by MTCC 2851 followed by 35% suppression by MIC2 (Table - 3). No inhibition of mycelia was observed by isolates MIC4, MIC6, MIC7, MIC9, MTCC2763 and MTCC3543 whereas control plates showed complete growth of phytopathogens.

**Phylogenetic analysis**

The 16s rRNA partial sequence was compared with the reference sequence available at the NCBI

| Table - 4. Hydrolytic enzyme activity of PGPR isolated from cucurbitaceae rhizosphere |
|----------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Isolates                              | Amylase | Cel lulase | Protease | Pectinase | Chitinase | β-1,3-glucanase |
| Sarratia spp. MIC1                    | -       | -           | -          | -          | ++          | -               |
| Pseudomonas aeruginosa MIC2           | +       | +           | +          | +          | -           | -               |
| Bacillus cereus MIC3                  | +       | +           | +          | +          | -           | -               |
| Bacillus subtilis MIC4                | +       | +           | +          | +          | -           | -               |
| Bacillus cereus MIC5                  | +       | +           | -          | -          | ++          | -               |
| Bacillus amyloliquifaciens MIC6       | -       | -           | -          | +          | -           | -               |
| Bacillus cereus MIC7                  | -       | -           | -          | +          | E, LZ       | -               |
| Bacillus licheniformis MIC9           | -       | -           | +          | +          | -           | -               |
| Pseudomonas aeruginosa MTCC2581       | +       | +           | +          | +          | P, E, CD, LZ, VA | -               |
| Bacillus subtilis MTCC2763            | +       | -           | +          | +          | E, TCC, IPM, PI, COT | -               |
| Bacillus coagulans MTCC3543           | -       | -           | -          | +          | -           | -               |

+ Positive reaction, - Negative reaction. All the tests were repeated for thrice for each bacterial isolates.
data-base using BLAST search. Phylogenetic analysis of closely related bacteria were constructed with MEGA 5 software using the neighbour-Joining (NJ) method. The trees formed six major clusters, of the nine bacterial isolates compared with other similar isolates in the NCBI database. Cluster I formed Bacillus cereus MIC7 (KC567890), Bacillus cereus MIC5 (KC567891), Bacillus amyloliquefaciens MIC6 (KC567892), Bacillus subtilis MIC4 (KC567893), Bacillus subtilis MIC10 (KC567888). Cluster III formed Bacillus licheniformis MIC9 (KC567889), cluster IV formed Serratia sp. MIC1 (KC428743), Pseudomonas aeruginosa MIC2 (KC567886), Cluster V formed Bacillus cereus MIC3 (KC567887) (Table 1 and Fig.-2).

**Fatty acid methyl esterase (FAME)**

Among the nine PGPR strains isolated only two were used for the FAME analysis. The analysis was based on their molecular and biochemical tests. The chromatograms of strains were compared with the standard once. In FAME analysis, both the strains were confirmed as *Pseudomonas aeruginosa* MIC2 and *Bacillus cereus* MIC5 (Fig.-3).

**Discussion**

Solubilisation of calcium phosphate on Pikovskaya’s agar medium forming clear zone around the bacterial colonies by *Serratia* spp. MIC1 and *Bacillus amyloliquefaciens* MIC6 is the indication that the isolates increase the availability of phosphate in soil. Jones and Oburger, (2011) confirmed the formation the of translucent zone around the bacterial colonies by release of organic acids, low pH around rhizosphere dissolves mineral phosphate thereby enhancing the available phosphate content in soil. A wilt disease caused by *Fusarium oxysporum* was controlled by the use of PSRB thereby reducing infection and enhancing the total yield (Hariprasad and Niranjana, 2009). L-tryptophan stimulates auxin production by bacteria and we observed varied amounts of IAA being produced by different PGPR strains, but *Bacillus cereus* MIC5 gave high concentration of IAA compared to others. To utilize L-tryptophan microbes produce a row of enzyme tryptophan transaminase and decarboxylase in the L-tryptophan dependent pathway in the plant growth promoting (Idris et al., 2007). Earlier reports state that increased concentration of L-tryptophan stimulates more auxin production by the PGPR exuding number of lateral roots and root hairs (Ahmad et al., 2008).

*Serratia* spp. MIC1, *Pseudomonas aeruginosa* MIC2, *Bacillus cereus* MIC5 and *B. licheniformis* MIC9 isolates produced HCN volatile antibiotics against pathogens in maximum amount. HCN production by microbes has shown a beneficial property for plants by controlling phytopathogens by volatile antibiotics (Ramette et al., 2003). Jayaprakashvel et al. (2010) reported that out of 24 strains only selected strains of PGPR produced more cyanogenic activity as well as fungistatic quality against *Rhizoctonia solani*. The *Pseudomonas fluorescens* PnMDU2 and PnMDU3 produced the maximum amount of HCN to inhibit the mycelial growth of *Rhizoctonia solani* by in-vitro.
The isolates Sarratia spp. MIC1 and Bacillus amyloliquefaciens MIC6 produce a hydroxamate type of siderophore at neutral pH, a high-affinity chelating agent for ferric ion. This gives orange spots around the colonies on CAS agar. Different types of siderophores form different colors at neutral pH by the ferric ion. Hydroxamate type produces orange and catechol type purple color (Chaiharn et al., 2009). Siderophore production in plant growth promotion is by two mechanisms—direct supply of iron to plants and indirectly depriving fungal pathogens of iron (Ahmad et al., 2008). Siderophore produced in various media shows strong biological control against different phytopathogens (Hu and Xu, 2011).

Bellis and Ercolani (2001) showed the rootlet elongation in cucumbers grown under gnotobiotic conditions.

**Fig. 2.** Phylogenetic analyses of the 16S rRNA partial sequences are compared with closely related bacteria obtained from the NCBI GeneBank database by MEGA 5. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown to the branches. (Nagarajkumar et al., 2004). The isolates Sarratia spp. MIC1 and Bacillus amyloliquefaciens MIC6 produce a hydroxamate type of siderophore at neutral pH, a high-affinity chelating agent for ferric ion.

**Fig. 3.** FAME chromatogram of selected PGPR siderophore production in plant growth promotion is by two mechanisms—direct supply of iron to plants and indirectly depriving fungal pathogens of iron (Ahmad et al., 2008). Siderophore produced in various media shows strong biological control against different phytopathogens (Hu and Xu, 2011).

Bellis and Ercolani (2001) showed the rootlet elongation in cucumbers grown under gnotobiotic conditions.
conditions by siderophore production by *Pseudomonas* spp. Some of the major cell wall degrading enzymes like cellulases, proteases, chitinase and β-1,3-glucanase contribute to the antagonism against the phytopathogens to control the plant diseases by enzymatic degradation (Shanmugaiah et al., 2008). Among these Chitinase and β-1,3-glucanase are the major fungal cell wall degrading enzymes produced by microbes to degrade long chain carbon molecules chitin and glucan. In our study *Pseudomonas aeruginosa* MIC2, *B. cereus* MIC5, *P. aeruginosa* MTCC2581 degrade fungal cell wall and most of the tested strains were able to produce both chitinase and β-1,3-glucanase, these were shown as better antagonism against *F. oxysporum* f. sp. cucumerinum. Kumar *et al.* (2010) confirmed the production of hydrolytic enzymes by *Sinorhizobium fredii* and *Pseudomonas* spp. along with their activity against the wilt of *Fusarium in Cajanus cajan*. Out of 78 rhizobacterial isolates, 23 showed 66% inhibition of mycelial growth against the root rot of *Sorghum* and among them KBE5-7, KBE5-1, KBE2-5, NAE5-5 resulted in 100% disease control without any root symptoms compared to the control seedlings (Hari-prasad and Niranjana, 2009). *E. meliloti* and *R. leguminosarum* inhibited the growth of *F. oxysporum* to 70% in dual culture and it was revealed by SEM examination the formation of clear halo cell vacuole, squeezing, empty cell formation, swelling and lysis of mycelium (Kumar *et al.*, 2011).

To know the antagonism effect with *Pseudomonas aeruginosa* MIC2, *Bacillus cereus* MIC5 and *P. aeruginosa* MTCC2581 isolates on the *F. oxysporum* f. sp. *cucumerinum* by dual culture assay. The *Bacillus* strain BC121 isolated from sorghum rhizosphere soil and antagonised *C. lunata* (Basha and Ulaganathan, 2002). Sang *et al.* (2008) confirmed the control of Phythoptora capsici by and ISE14. In *Fusarium* spp. mycelial growth and appearance changed from white to reddish brown or red due to the inhibition by antibiosis and other antifungal metabolites such as siderophores, hydrogen ions and gaseous products including ethylene, hydrogen cyanide and ammonia (Saravanan *et al.*, 2004).

Molecular typing of bacterial isolates based on 16S rRNA sequences are good indicators of phylogenetic relationships among the intra- and interspecies levels (Solanki *et al.*, 2012). Kumar *et al.* (2011) sequenced 18 rhizobacterial isolates among them 15 isolates belong to group of *Bacillus* with *Pseudomonas* sp, *Pantoea agglomerans* and *Staphylococcus intermedius*. Phylogenetic analyses of the strains based on the basis of the NJ method clustered in a 4 major clusters. In our study 16s rRNA partial sequence of nine bacterial isolates was compared with reference sequence available at NCBI database. Phylogenetic analyses of closely related bacteria were constructed with MEGA 5 software using NJ method. The nine bacterial isolates forms six major clusters in the tree with seven *Bacillus*, one *Serratia* and one *Pseudomonas* isolate. Among nine PGPR strains only selected two PGPR strains were analysed by FAME. In FAME analysis, fatty acids are extracted from the PGPR samples, methylated and analysed by gas chromatography. Cellular fatty acids can be influenced by temperature and nutrition (Kirk *et al.*, 2004). FAME provides information about microbes based on their groupings of fatty acids profiles. Fatty acid populations were used for representing the microbial community (Kelly, 1999).

In conclusion, the rhizobial strains showing important PGP traits proved to be very efficient in managing *F. oxysporum* f. sp. *cucumerinum*. The selected PGPR promoted shoot and root growth with possessed better root colonization property. *P. aeruginosa* MIC2, *B. subtilis* MIC4, *B. cereus* MIC5 and *B. amyloliquefaciens* MIC6 showed improved growth in the green house compared to the control.
P. aeruginosa MIC2 and B. cereus MIC3 have good antagonist property and could be used as biocontrol agents against F. oxysporum f. sp. cucumerinum in cucurbitaceae.

References


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