Molecular characterization of effective PGPRs from rhizosphere of banana against tip over disease caused by *Erwinia carotovora* subsp. *carotovora*

Soumya D Rotti and MR Ravikumar

Abstract

In recent years, there has been a reverse interest in the search of plant growth promoting rhizobacteria (PGPRs) for sustainable crop production. Banana is an economically important tropical fruit crop, which is subjected to infection by fungi, bacteria, virus and nematodes. A total of 134 PGPRs were isolated from rhizosphere of banana. Twelve out of 64 isolates of *Bacillus* spp. and seven out of 70 isolates of *Pseudomonas* spp. were found to effective against the *Erwinia carotovora* subsp. *carotovora in vitro*. Among them most effective isolate of PGPRs were further subjected for molecular characterization. The molecular studies confirmed them to be as *Bacillus pumilis, Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa and Pseudomonas patula*.

Keywords: Plant growth promoting rhizobacteria, Banana, *Erwinia carotovora* subsp. *carotovora, in vitro, Bacillus pumilis, Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa, Pseudomonas patula*.

Introduction

Biological control of plant pathogens by antagonistic microorganisms is a potential non-chemical means (Harman, 1991) [1] and is known to be a cheap, effective and eco-friendly method for the management of crop diseases (Cook and Baker, 1983) [5]. The use of biological control agents as an alternative to fungicides and bactericides is increasing rapidly in the present day agriculture due to the deleterious effects of chemical pesticides. Efforts to control plant diseases with antagonistic bacterial agents have been made successfully (Chen *et al.*, 1995) [3]. *Bacillus* spp. have special characteristics that make them good candidates as biological control agents. Members of the genus *Pseudomonas* have long been known for their potential to reduce the plant disease and they have gained considerable importance as potential antagonistic microorganisms (Pant and Mukhopadhyay, 2001) [9]. Among these, the bacterial antagonists have the twin advantage of faster multiplication and higher rhizosphere competence hence, *Pseudomonas* spp. *And Bacillus* spp. have been successfully used for biological control of several plant pathogens (Ramamoorthy *et al.*, 2001) [10] and biological control using PGPR strains especially from the genus *Pseudomonas* is an effective substitute for chemical pesticides to suppress plant diseases (Compant *et al.*, 2005) [4]. The soil bacteria that aggressively colonize the root zone and promote plant growth are generally termed as Plant Growth Promoting Rhizobacteria (PGPRs). Gechemba *et al.* (2016) [6] reported that the tropical banana rhizosphere harbor’s a wide diversity of antagonistic bacteria that may not only aid in beneficial symbiotic relationships but also stimulate the plant growth by suppressing pathogenic organisms.

Tip over is one of the important disease of banana caused by *Erwinia carotovora* subsp. *carotovora* causing yield losses upto 65.28 percent (Totagi, 2012) [12] and the disease is transferred through tissue cultured materials, infected seedlings, soil and water.

Material and Methods

Isolation of PGPRs from the rhizosphere of banana plant

Rhizospheric soil samples were collected from the neighbouring healthy plants of banana in the field. The collected soil were transferred to sample collection bags, antagonistic bacterium was isolated by following serial dilution and Pour plate method by using Hicrome Bacillus agar, Nutrient agar and King’s B media.

Isolation of *Pseudomonas* spp.

Fluorescent Pseudomonads were isolated from soil using a specific media viz., King’s B (KB)
medium following serial dilution and pour plate technique was done. The plates were incubated at 28 ± 1 °C for 24 h. Colonies were observed under UV light. The fluorescent colonies observed under UV light were picked up, was purified by repeated streaking on same medium and checked for their fluorescence. Further well isolated single colonies were transferred to 20 percent glycerol stock for preservation.

**Isolation of Bacillus spp.**

Different species of Bacillus were isolated from soil using a specific media viz., Hicrome bacillus agar medium following serial dilution and plating technique was done. Then the plates were incubated at 28 ± 1 °C for 48 h. Colonies formed were picked up and purified by repeated streaking on the Nutrient agar medium. Well isolated colonies were transferred to 20 percent glycerol stock for preservation.

**In vitro evaluation for efficacy of isolated PGPRs against Erwinia carotovora subsp. carotovora**

Isolated PGPRs were evaluated for their efficacy against the growth of *Erwinia carotovora* subsp. *carotovora* by well isolation method. A heavy suspension of *Erwinia carotovora* subsp. *carotovora* was multiplied in nutrient broth (20 ml) and was mixed with lukewarm nutrient agar medium in flask. The inoculated flasks were incubated at 28 ± 1 °C for 48 h. The bacterial suspension was then seeded to the lukewarm nutrient agar medium. The seeded medium was poured into the sterilized Petri plates and was allowed to solidify. Then, a well with a diameter of 6 to 8 mm was punched aseptically with a sterile cork borer and a volume (20-100 µL) of the isolated PGPRs cultured in the nutrient broth was introduced into the well. The inoculated plates were incubated at 28 ± 1 °C for 48 h. The observations for the production of inhibition zone around the PGPRs was measured by taking mean diameter of the zone formed and then were analyzed statistically.

**Molecular characterization of effective PGPRs**

The total genomic DNA from pure culture of the different isolates of bacteria was extracted by the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Murray and Thompson, 1980) [8] with some modifications. PCR amplification of rDNA sequences were conducted by using the universal primers (16S rDNA amplification of rDNA sequences were conducted by using Thompson, 1980)

**Results and Discussion**

**Isolation of PGPRs from rhizosphere of banana**

Number of isolates collected from rhizosphere of banana varied from one place to other place. A total of 134 isolates were collected from surveyed area. Among the total isolates, 64 isolates were identified as *Bacillus* spp. and remaining 70 isolates were identified as *Pseudomonas* spp. Similarly, Apastambh *et al.* (2016) [1] isolated 8 strains of fluorescent pseudomonas and 4 strains of *Bacillus* from banana rhizosphere.

**In vitro evaluation of isolated PGPRs against Erwinia carotovora subsp. carotovora**

Among 64 isolates of isolated *Bacillus* spp. 12 isolates were found to be effective compared to other isolates (Table 1 & Fig 1). Among the 70 isolates of isolated *Pseudomonas* spp. 7 isolates were found to be effective compared other isolates (Table 2 & Fig 2). Among 12 effective isolates of *Bacillus* spp. maximum inhibition (16.67 mm) was observed by Belagavi isolate 13 and minimum inhibition (12.00 mm) was observed by Haveri isolate 5. Among 7 effective isolates of *Pseudomonas* spp. maximum inhibition (18.00 mm) was observed by Belagavi isolate 8 and minimum inhibition (12.00 mm) was shown by Dharwad isolate 2. *Pseudomonas* spp. was found to suppress *Erwinia carotovora* subsp. *carotovora* with maximum inhibition (18.00 mm) whereas, *Bacillus* spp. showed the maximum inhibition (16.67 mm). Hence, it indicated that *Pseudomonas* spp. are more efficient than *Bacillus* spp. Similarly Snehalatharani and Khan (2009) [11] reported that the efficacy of three antagonistic microorganisms. *Pseudomonas* *fluorescens*, *Pseudomonas* *aeruginosa* and *Bacillus* *subtilis*. Among antagonistic microorganisms, *Pseudomonas* *aeruginosa* was found to be most effective in *in vitro* conditions followed by *Pseudomonas* *fluorescens*.

**Molecular characterization of the effective PGPRs**

Out of 12 effective isolates of *Bacillus* spp. 4 most effective isolates and of 7 effective isolates of *Pseudomonas* spp. 6 most effective isolates were characterized molecularly. The isolated DNA was amplified at 1500 bp (plate 1). Molecular characterization of effective *Bacillus* spp. were identified as *Bacillus cereus* (Belagavi isolate 13 and Vijayapur isolate 2), *Bacillus subtilis* (Vijayapur isolate 7) and *Bacillus pumilis* (Vijayapur isolate 8). These results were in similar with the results of biochemical characterization. Molecular characterization of effective *Pseudomonas* spp. were identified as *Pseudomonas aeruginosa* (Belagavi isolate 8, Belagavi isolate 9, Haveri isolate 3, Belagavi isolate 4 and Vijayapur isolate 3) and *Pseudomonas putida* (Bagalkote isolate 5). Balayogan and Marimuthu (2014) isolated and molecularly characterized the potential plant growth promoting *Bacillus cereus* GGBSTD1 and *Pseudomonas* spp. GGBSTD3 from Vermisources.

1. Belagavi isolate 13

The Microbe was found to be most *Bacillus cereus* strain LB8, Sequence ID (Accession no.): MH187637. The next closest homologue was found to be *Bacillus cereus* strain SML_M123, Sequence ID: MG937670.

**>16SRDNAF**

CATGCAGTCGACGGAATGGAATATGAAGCTTTGCCTTT
ATGAAAGTTAGCGCGGAGCGGTGAGTAAACGCTGG
GTAACCTGCCATTAAGTCTTGAGGAAAACCCGGAGGA
CCGGGCTTATACCGGTACATTGAAATTGGATCT
GTTCGAAATTGTAAGGCGCGTGGCTGTGATCTTAT
GGATTCGACCCCGCTCGCAATTAAGCTGAGGTTAG
AACGCGTCAACCAAGGCAAGATGCTGATGCCCGT
AGAAGGTGTAGCCCACTCGGCAGTGACGACCG
CCAGAAGTCCCTAAGGAGGCGACAGTGAGGATCTT
CCGCAATGAGCGCAGAAGATGCTAGCGAGACGC
GGAGTAGTAAAGGCTTGGGGCTGTAAACACTCT
GTGGTAAAGGAAGAAGAAATGGTGTGTTAGAATGCTTG
CACCTTGAGCCTACCTAACCAGAAGACCCAGCCTAA
CTACGTGAGCCACAGCCCGGTGAACTAGTGATGG
AGCGTTATCGCGGATTAGTTGGCGTAAAGGCAGCG
AGGTTGTTCCTAAATGCTGTGATGAACCCACGCG
TCAACCGGTAGAGGTCAAATCTGGAATGAGGAC
AGTGCAGAAGAAAGAAGGTAAATCCGTCGTAGCG
GTGAAATCCGTAGAGATATGGAGGAACACGAGG

“2388”
2. Vijayapur isolate 7
The Microbe was found to be most Bacillus subtilis strain GF14, Sequence ID (Accession no.): MG976623. The next closest homologue was found to be Bacillus subtilis strain GF3, Sequence ID: MG976621.

>16SRDNA
TGGATCGAGCGACGGAGGGAGATTTGCTGCTCTCTATATGGTGAGGCGGAGAGGCTGGAGGCTATCCTGGACGTGCTTGGGTCTCGTCGCTACGGTGACGTTTGCAACCATGACTGCTTAGAGTGACCCGACTAGAGCCGTCAGGACATGGGATGCGGATCCGACCTGAAAGGGTGATCCTGCACACTGGGACTGACCACACTCCTAACTCCTACGGGAGGGGGGAATTGGGAATCTTGGCAATTGGGACTGATCCTGACGGACAACGCCGCATGAGTGATGAAGGTTTTCGGATCACTTTTCTGTAGAATAACCCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATAAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATA

3. Vijayapur isolate 2
The Microbe was found to be most Bacillus cereus strain AMB_17, Sequence ID (Accession no.): JX971533. The next closest homologue was found to be Bacillus cereus strain H25, Sequence ID: MH045979.

>16SRDNA
TCAACACGCTATACTGAAGGTTTTAGTGTACGGGTGACCAACGTGGGTAACCTGCCATAAGACTGGGATACCTCCGGGAAACCGGGCCTAATACCGCATACGTCCTGAGGGAGAAAGTGGGGGATCTTCCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGAAGTCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTTGGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAAGTTAATACCTTGCTGTTTTGACGTTACCAACAGAAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCTAGGTGGTTCAGCAAGTTGGATGTTAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAACACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAGGAAACACCAGTGGCGAAGGCGACCACCTGGACTGACTCTGACACTGAGGTGCGAAAGCGTGGGGAGCAAAACAGGATTAGATACCTCTGTTAGTCCTATATGGTGACCCGACTAGAGCCGTCAGGACATGGGATGCGGATCCGACCTGAAAGGGTGATCCTGCACACTGGGACTGACCACACTCCTAACTCCTACGGGAGGGGGGAATTGGGAATCTTGGCAATTGGGACTGATCCTGACGGACAACGCCGCATGAGTGATGAAGGTTTTCGGATCACTTTTCTGTAGAATAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATAAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATA

4. Vijayapur isolate 8
The Microbe was found to be most Bacillus pumilis strain SCSGAB0102, Sequence ID (Accession no.): JX315311. The next closest homologue was found to be Bacillus pumilis strain AIMST 1.A.sub3L, Sequence ID: JF819677.

>16SRDNA
TGGATCGAGCGACGGAGGGAGATTTGCTGCTCTCTATATGGTGAGGCGGAGAGGCTGGAGGCTATCCTGGACGTGCTTGGGTCTCGTCGCTACGGTGACGTTTGCAACCATGACTGCTTAGAGTGACCCGACTAGAGCCGTCAGGACATGGGATGCGGATCCGACCTGAAAGGGTGATCCTGCACACTGGGACTGACCACACTCCTAACTCCTACGGGAGGGGGGAATTGGGAATCTTGGCAATTGGGACTGATCCTGACGGACAACGCCGCATGAGTGATGAAGGTTTTCGGATCACTTTTCTGTAGAATAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATAAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATA

5. Belagavi isolate 8
The Microbe was found to be most Pseudomonas aeruginosa strain CCUG 70744, Sequence ID (Accession no.): CP023255. The next closest homologue was found to be Pseudomonas aeruginosa strain AR_0446, Sequence ID: CP029660.

>16SRDNA
AGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAAACCGCGGTAGTCAAACCTGGACTGCAAAACAGCAGTGAATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTTACACCTCCGCACTACTGACATTGCGATCATTGCGACTTCTCGTGGTCTGACACTGAGGTGCGAAAGCGTGGGGAGCAAAACAGGATTAGATACCTCTGTTAGTCCTATATGGTGACCCGACTAGAGCCGTCAGGACATGGGATGCGGATCCGACCTGAAAGGGTGATCCTGCACACTGGGACTGACCACACTCCTAACTCCTACGGGAGGGGGGAATTGGGAATCTTGGCAATTGGGACTGATCCTGACGGACAACGCCGCATGAGTGATGAAGGTTTTCGGATCACTTTTCTGTAGAATAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATAAACCCGCTGAGAACTGCTTGCACCTTGACTGCACCTAACCCAGAAAGCCACCATATACCTCTGCTGACTGGAGGGTAGTTTGGCAATGTTGAGGATA

6. Belagavi isolate 9
The Microbe was found to be most Pseudomonas aeruginosa strain Dut-lxmg702, Sequence ID (Accession no.): MF100795. The next closest homologue was found to be
7. Haveri isolate 3
The Microbe was found to be most *Pseudomonas aeruginosa* strain CNSG21, Sequence ID: KY962356. The next closest homologue was found to be *Pseudomonas aeruginosa* strain RA5, Sequence ID: MH160762.

8. Belagavi isolate 4
The Microbe was found to be most *Pseudomonas aeruginosa* strain DM11, Sequence ID (Accession no.): KT297444. The next closest homologue was found to be *Pseudomonas putida* strain CG29, Sequence ID: KF782801.

9. Bagalkote isolate 5
The Microbe was found to be most *Pseudomonas putida* strain Sp16, Sequence ID (Accession no.): KF768787. The next closest homologue was found to be *Pseudomonas putida* strain CP027166.

10. Vijayapur isolate 3
The Microbe was found to be most *Pseudomonas aeruginosa* strain AR_0357, Sequence ID (Accession no.): CP027166. The next closest homologue was found to be *Pseudomonas aeruginosa* strain AR_0446, Sequence ID: CP029660.
ATTAGCTAGTTGGTGGAATGCAAGGGCTACCAAGGCC
ACGATCCCATGTGATTGAGAGTATCAGTCACT
ACTGGAACACTGAGACCGTCAGCTACCTACCCCGG
GGACAGTGTGGGAATAATGGCAACTGGGCAAAGGC
CTGATCCAGCCATGGCCTGTGTTGAAGAGGGTCT
GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCA
GTAAGTTAATACCTTGGCTGGTACGGGAAACCAAGC
GTAAGTTAATACCTTGGCTGGTACGGGAAACCAAGC
GTAAGTTAATACCTTGGCTGGTACGGGAAACCAAGC
GTAAGTTAATACCTTGGCTGGTACGGGAAACCAAGC
Table 1: *In vitro* evaluation of *Bacillus* spp. against the growth of *Erwinia carotovora* subsp. *carotovora*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Isolates</th>
<th>Mean diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Vijayapur isolate 2</td>
<td>15.33 (4.04) *</td>
</tr>
<tr>
<td>T2</td>
<td>Vijayapur isolate 4</td>
<td>14.33 (3.92)</td>
</tr>
<tr>
<td>T3</td>
<td>Vijayapur isolate 5</td>
<td>14.00 (3.87)</td>
</tr>
<tr>
<td>T4</td>
<td>Vijayapur isolate 7</td>
<td>16.00 (4.12)</td>
</tr>
<tr>
<td>T5</td>
<td>Vijayapur isolate 8</td>
<td>14.67 (3.96)</td>
</tr>
<tr>
<td>T6</td>
<td>Haveri isolate 5</td>
<td>12.00 (3.61)</td>
</tr>
<tr>
<td>T7</td>
<td>Dharwad isolate 1</td>
<td>14.00 (3.87)</td>
</tr>
<tr>
<td>T8</td>
<td>Belagavi isolate 12</td>
<td>15.00 (4.00)</td>
</tr>
<tr>
<td>T9</td>
<td>Belagavi isolate 13</td>
<td>16.67 (4.20)</td>
</tr>
<tr>
<td>T10</td>
<td>Bagalkote isolate 15</td>
<td>13.00 (3.74)</td>
</tr>
<tr>
<td>T11</td>
<td>Belagavi isolate 16</td>
<td>14.00 (3.87)</td>
</tr>
<tr>
<td>T12</td>
<td>Belagavi isolate 17</td>
<td>13.67 (3.83)</td>
</tr>
<tr>
<td>S. Em. ±</td>
<td></td>
<td>0.059</td>
</tr>
<tr>
<td></td>
<td>C. D. at 1%</td>
<td>0.235</td>
</tr>
</tbody>
</table>

* - √ x+1 transformed values

Table 2: *In vitro* evaluation of *Pseudomonas* spp. against the growth of *Erwinia carotovora* subsp. *carotovora*

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Isolates</th>
<th>Mean diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Vijayapur isolate 3</td>
<td>12.17 (3.63) *</td>
</tr>
<tr>
<td>T2</td>
<td>Dharwad isolate 2</td>
<td>12.00 (3.61)</td>
</tr>
<tr>
<td>T3</td>
<td>Haveri isolate 3</td>
<td>15.00 (4.00)</td>
</tr>
<tr>
<td>T4</td>
<td>Belagavi isolate 4</td>
<td>14.00 (3.87)</td>
</tr>
<tr>
<td>T5</td>
<td>Belagavi isolate 8</td>
<td>18.00 (4.36)</td>
</tr>
<tr>
<td>T6</td>
<td>Belagavi isolate 9</td>
<td>16.00 (4.12)</td>
</tr>
<tr>
<td>T7</td>
<td>Bagalkote isolate 5</td>
<td>13.00 (3.74)</td>
</tr>
<tr>
<td>S. Em. ±</td>
<td></td>
<td>0.030</td>
</tr>
<tr>
<td></td>
<td>C. D. at 1%</td>
<td>0.128</td>
</tr>
</tbody>
</table>

* - √ x+1 transformed values

---

**PGPR isolates**

Fig 1: *In vitro* evaluation of *Bacillus* spp. against the growth of *Erwinia carotovora* subsp. *carotovora*
Fig 2: In vitro evaluation of Pseudomonas spp. against the growth of Erwinia carotovora subsp. carotovora

Plate 1: PCR amplification of effective PGPRs

References


