Original Article

Validation of molecular heterogeneity of Fluorescent Pseudomonas spp. and correlation with their potential biocontrol traits against fusarium wilt disease

Nellickal Subramanyan Jayamohan, Savita Veeranagouda Patil, Belur Satyan Kumudini*

Department of Biotechnology, School of Sciences, JAIN (Deemed-to-be University), Bengaluru 560011, India

ARTICLE INFO

Article history:
Received 1 March 2017
Accepted 6 June 2018
Available online 24 October 2018

Keywords:
Fluorescent Pseudomonas
Fusarium wilt
Genomic diversity
Random amplification of polymorphic DNA (RAPD)
Rep-polymerase chain reaction (PCR)
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

ABSTRACT

Fluorescent Pseudomonas (FPs) are major rhizospheric bacteria with a variety of plant growth promotion attributes having potential field applications. The genomic and outer membrane protein (OMP) diversity were valued of 11 shortlisted FPs exhibiting potential biocontrol activity. The taxonomic diversity of isolates was studied using genomic fingerprinting assays—random amplification of polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC), BOX, repetitive extragenic palindromic (REP) and (GTG5), and OMP analysis with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Genomic diversity parameters were calculated: total allelic number, polymorphic information content, expected heterozygosity, dominance, and the Shannon and Simpson indices. All isolates were used to prime tomato seeds and plants were challenge-inoculated with Fusarium oxysporum and the disease incidence was assessed under greenhouse condition. Cluster analyses of RAPD and combined Rep analyses classified isolates into two major clusters. SDS-PAGE analysis of OMP resulted in bands in the range 29–66 kDa. Furthermore, the isolates treated with tomato seeds followed by challenge-inoculation with F. oxysporum under greenhouse conditions showed that the three FPs, M80 (SUB1688209 Seq1 KX570929), M96 (SUB1688209 Seq1 KX570930) and T109 (SUB1688209 Seq1 KX570931) were able to induce systemic resistance. The study examined the possible correlation between strain-specific molecular diversity of FPs and provided an account of their potential biological control activity against F. oxysporum.

Copyright © 2018, Kasetsart University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

The rhizosphere is one of the most diverse ecological niches with innumerable types of microorganisms due to the rich nutrient availability from hosts (Venturi and Keel, 2016). This mutualistic relationship between host and rhizosphere microbes makes it a highly competitive ecosystem in nature. Through this, both the host plant and its concomitant microbiome gain an evolutionary advantage to survive under various adverse conditions by establishing tight interplays (Mapelli et al., 2013). Plant growth-promoting rhizobacteria (PGPR) efficiently colonize the rhizosphere arena and promote plant growth, stimulate immune defenses, influence hormonal balance, protect from pathogens and mobilize nutrients (Haas and Defago, 2005; Venturi and Keel, 2016). The major PGPR candidates belong to the phyla Cyanobacteria, Bacteriodetes, Actinobacteria, Firmicutes and Proteobacteria (Figueiredo et al., 2010).

The bacterial genus Pseudomonas belonging to the phylum Proteobacteria, is ubiquitous, metabolically versatile and an exceptional rhizosphere colonizer (Santoyo et al., 2012). It is well adapted to survival in the rhizosphere hence delivering protection against various phytopathogens, which makes it one of the most studied biocontrol agents (Couillerot et al., 2009; Goswami et al., 2016). Although the listed number of members in the genus Pseudomonas have varied from time to time, the most advanced phylogenetic strategy showed that the genus comprises 144 species (Gomila et al., 2015). This is reportedly the largest Gram-negative bacterial genus with the highest number of species. Enormous phenotypic variability, genomic heterogeneity, frequent description of novel species (P. protegens), subspecies (P. brassicacearum subsp. Neoaurantiaca), and the inclusion of new strains such as Pseudomonas sp. UW4 in the Pseudomonas fluorescens group make...
it a real challenge for categorization into a single phylogenetic group (Garrido-Sanz et al., 2016). In addition, the complete sequence of the P. fluorescens F113 genome shows the existence of manifold strain-specific genes (Redondo-Nieto et al., 2013).

The biocontrol potential of fluorescent Pseudomonas (FP) has a direct correlation with its capability to produce antibiotics and lytic enzymes (Upadhyay and Srivastava, 2011). Extensive molecular level work carried out on FPs revealed that genetic regulatory elements play a major role in their plant protection efficiency, especially through the Gac/Rsm signal transduction pathway (Lapouge et al., 2008). A comparative study of 23 genes from 302 PGPR strains showed evidence of the parallel acquisition of plant-beneficial function contributing genes (Bruto et al., 2014), which hypothesizes that the gene acquisition took place separately in taxonomically different Proteobacteria and involved the acquisition of prehistoric genes. In addition, whole genome sequencing of P. fluorescens PF-5 showed that approximately 5.7% of its genome (approximately 400 kb) is dedicated only to secondary metabolite production (Paulsen et al., 2005). Such metabolites produced by FPs are closely linked with fungal inhibition; consequently, the role of FP genetic elements in plant growth regulation is stronger than previously thought.

Hence, this study was designed with the aim of correlating with the population diversity of the shortlisted FPs. In addition, the study also looked into the ability of FPs to induce resistance against F. oxysporum in tomato under greenhouse conditions.

Materials and methods

Candidate isolates, reference strains and genomic DNA isolation

Eleven FPs were shortlisted from 144 rhizospheric isolates obtained from eight different samples based on plant growth-stimulating traits (Subramanian and Satyan, 2014). The study also used two reference strains of P. fluorescens (PC1 and PC2) and two identified strains of FP from the microbial type culture collection (MTCC), Chandigarh, (Pseudomonas aeruginosa 10311 (PA)) and P. putida 6809 (PP).

Molecular identification of bacterial strains

All the FPs and reference strains were grown on King’s B agar plates. Single fluorescent colonies were inoculated into Luria Bertani broth (HiMedia™, Mumbai, India) and incubated in a shaker incubator for 48 h. The cell pellets were separated using centrifugation and washed twice with Tris-ethylenediaminetetraacetic acid (EDTA) buffer (25 mM Tris and 10 mM EDTA at pH 7.5). The bacterial genomic DNA was extracted from the cell pellet using an Indigene™ DNA isolation kit and stored at –20 °C and served as the template for molecular analysis. This procedure entailed three independent replications to compare the genomic diversity.

Partial 16S rRNA amplification of FPs was carried out using the Pseudomonas-specific primer pair Psmn289-5′-GGTGTACGGACTTATCC-3′ and Psmn1258-5′-TTAGCTCCACCTCGCGGC-3′ (BioServe: Hyderabad, India) as per Widmer et al. (1998). The best candidate isolates were identified by sequencing of partial 16S rRNA using the oligonucleotide primers 397F (5′-CDGGHCTANCAVTGKWAGTS-3′) and 1398R (5′-GMCGKTGKGTACHAGGY-3′).

Genomic diversity analysis of fluorescent Pseudomonas using random amplified polymorphic (DNA) and Rep-polymerase chain reaction primers

Random amplification of polymorphic DNA (RAPD) analyses of FPs were carried out using 40 primers (20 D series and 20 E series). Polymerase chain reaction (PCR) amplification and reaction mixtures were according to Sethia et al. (2015). Diversity in repetitive bacterial genome sequences was analyzed using Rep-PCR, enterobacterial repetitive intergenic consensus (ERIC), BOX A1R and REP (Lupski and Einstock, 1992) and GTG5 primers (Di Conza et al., 2007). Standardized thermocycling conditions for ERIC, BOX A1R, REP and GTG5 were optimized as per the protocol adopted by Jayamohan et al. (2015).

Individual FP colonies were subcultured for 72 h, centrifuged, the cell pellets separated and washed thrice with Tris-EDTA buffer. The OMP of FPs was separated using N-lauroylsarcosine (Himedia™, Mumbai, India) as per Winder et al. (2000), with minor modifications as optimized according to Jayamohan et al. (2015). The OMP samples were subjected to 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis along with the standard protein marker (29–205 kDa range, GeNei™) followed by silver nitrate staining (Gromova and Celis, 2006).

Fusarium wilt disease severity of tomato on fluorescent Pseudomonas priming under green-house conditions

Surface sterilized tomato seeds (Variety: Arka, Lot no. 60) were used for greenhouse study. Bacterization of seeds was carried out as per Subramanian and Satyan (2014) at a concentration of approximately 1 × 108 colony forming units/mL (optical density = 0.3) and were grown under sterilized soil conditions. Twenty-day-old seedlings with uniform physical parameters were selected and transplanted into pots filled with sterile potting soil arranged in a randomized design. Solarized tomato plants treated only with distilled water (UTC) and challenged with F. oxysporum (CC) were maintained as controls in the study. Seedlings were re-treated with the same FPs after 10 d transplantation. The first three compound leaves of the tomato plants were challenge-inoculated using the drop inoculation method, with a spore suspension of F. oxysporum (MTCC1755) of 2 × 105 spores/mL in 0.85% (weight per volume; w/v) sterile saline. Plants were maintained under identical conditions and observed periodically following 20 d of fungal challenge and their disease index was assessed (Bhattacharya et al., 1985).

Data compilation and evaluation

The banding patterns from molecular diversity analysis were detected using ultraviolet fluorescence and SDS-PAGE with silver nitrate staining. A binary matrix data pool was constructed on the basis of presence or absence of each band (coded 1 or 0, respectively) and cluster analysis was performed using combined data and Ward’s algorithm with Euclidean distances. The total allele frequency (TAF), expected heterozygosity (EH), dominance, Shannon H index and Simpson index were calculated. Mean centered principal component analysis (PCA) was carried out using the PAST 3.01 analytical software. The polymorphism information content (PIC) was also calculated using the online AGL-PIC calculator (University of Liverpool, UK).

Greenhouse experiments were carried out using a randomized block design. The results were compared using one-way analysis of variance with Duncan multiple range test in the SPSS 20 software package and significance was tested at p ≤ 0.05. Graphs were generated using the Graph Pad Prism 6.1 software.
Results

The FP isolates were tested for their potential secondary metabolite characteristics, (direct and indirect) regarding plant growth promotion. Qualitative and quantitative characters were systematically graded and were shortlisted based on performance-based ranking using statistical tools. The highest ranking (1) was recorded for the isolate M140 and lowest (10) for R96, M80 and T109 consistent with previous study (Subramanian and Satyan, 2014). Eleven shortlisted fluorescent pseudomonad isolates were further considered for detailed investigation of their intra-species genetic and OMP diversity using molecular fingerprinting analyses and correlation with their biological control ability.

Identification of strains using partial polymerase chain reaction amplification of 16S RNA using a Pseudomonas-specific primer

To confirm the genomic identity of isolates, a partial amplification of 16S ribosomal RNA of all eleven isolates was performed using *Pseudomonas* specific primers. Amplified PCR products were run on agarose gel and generated a distinct 957 bp amplified product against the 1 kb DNA marker (Thermoscientific) and confirmed that all representative isolates belonged to the genus *Pseudomonas*.

Diversity analysis of FPs using RAPD primers

Eleven fluorescent pseudomonad isolates were screened against 40 RAPD primers and nine RAPD primers were shortlisted based on polymorphism and considered for the final study. The nine shortlisted RAPD primers yielded bands in the range 0.2–3.5 kb size and percentage polymorphism in the range 85.7–100. Overall, 64 polymorphic bands were generated with 95.52% polymorphism on RAPD analysis. Evaluation of the RAPD data showed highest values for dominance (0.47) by OP-D16, for Shannon H index (2.85) by OPE16 and for Simpson index (0.94) by OPD16, E16, E17 and E19. Additionally, the average TAF and PIC of RAPD analysis were 429 and 0.11, respectively, with similar EH values (Table 1).

The similarity matrix analysis based on RAPD data displayed the highest similarity coefficient of 42 (between the isolates PA-M139 and T109 consistent with previous study (Subramanian and Satyan, 2014)). The binary data of Rep-PCR analysis was performed on the similarity matrix and 31 was the highest similarity coefficient (between isolates PC1-M140, PC2-M140, PA-PC1 and PA-PC2) as depicted in Table 1. Based on the similarity matrix that showed highest similarity values than 2 kb, with 59 polymorphic bands, 92.18% total polymorphism with TAF of 376 and PIC and EH of 0.26, as depicted in Table 1.

Cluster analyses of combined Rep-PCR data (ERIC, BOX A1R, REP and GTG5) generated two major clusters (Fig. 2). The binary data of Rep-PCR were analyzed based on the similarity matrix and 31 was the highest similarity coefficient (between isolates PC1-M140, PC2-M140, PA-PC1 and PA-PC2) as shown in Table 3. Based on derived eigen values, PCA was carried out to analyze the relationship between bacterial genotypes depending on polymorphism and the output was depicted using a coordinate scale with an X and Y axis (Fig. 3). All FPs were distributed into three different clusters in the line plot, with maximum and minimum eigen values of 4.84 and 0.63, respectively, and percentage of variance of 15.99 and 2.47, respectively.

Outer membrane protein diversity of fluorescent Pseudomonas

OMP profiling of the 11 isolates with four FP control strains together generated 50 distinct bands with silver nitrate staining. The highest number of OMP bands was perceived in the 29–66 kDa range.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>PL</th>
<th>GC%</th>
<th>AT (C)</th>
<th>BS (kb)</th>
<th>NSB</th>
<th>NPB</th>
<th>PoP</th>
<th>TAF</th>
<th>PIC</th>
<th>EH</th>
<th>DOM</th>
<th>SHI</th>
<th>SI</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP-D16</td>
<td>AGGCGGTAAAG</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.25–3.5</td>
<td>7</td>
<td>7</td>
<td>100.00</td>
<td>31</td>
<td>0.14</td>
<td>0.14</td>
<td>0.47</td>
<td>2.79</td>
<td>0.94</td>
</tr>
<tr>
<td>OP-D18</td>
<td>CAAGCCCAAC</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.2–3.5</td>
<td>7</td>
<td>7</td>
<td>100.00</td>
<td>33</td>
<td>0.14</td>
<td>0.14</td>
<td>0.08</td>
<td>2.58</td>
<td>0.92</td>
</tr>
<tr>
<td>OP-E02</td>
<td>GTGGCCGGAA</td>
<td>10-mer</td>
<td>70</td>
<td>35</td>
<td>0.25–3</td>
<td>8</td>
<td>8</td>
<td>100.00</td>
<td>37</td>
<td>0.05</td>
<td>0.05</td>
<td>0.11</td>
<td>2.21</td>
<td>0.89</td>
</tr>
<tr>
<td>OP-E03</td>
<td>CCAGATGCCAC</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.25–3.5</td>
<td>8</td>
<td>8</td>
<td>87.50</td>
<td>41</td>
<td>0.06</td>
<td>0.06</td>
<td>0.09</td>
<td>2.42</td>
<td>0.91</td>
</tr>
<tr>
<td>OP-E09</td>
<td>CTCCACCGGA</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.25–3</td>
<td>7</td>
<td>7</td>
<td>100.00</td>
<td>35</td>
<td>0.04</td>
<td>0.04</td>
<td>0.08</td>
<td>2.66</td>
<td>0.92</td>
</tr>
<tr>
<td>OP-E15</td>
<td>AGCCAAACC</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.25–3</td>
<td>7</td>
<td>6</td>
<td>85.70</td>
<td>43</td>
<td>0.32</td>
<td>0.32</td>
<td>0.10</td>
<td>2.34</td>
<td>0.90</td>
</tr>
<tr>
<td>OP-E16</td>
<td>GTGTAAGCTTG</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.35–3.5</td>
<td>8</td>
<td>8</td>
<td>100.00</td>
<td>43</td>
<td>0.15</td>
<td>0.15</td>
<td>0.06</td>
<td>2.85</td>
<td>0.94</td>
</tr>
<tr>
<td>OP-E17</td>
<td>CATTCTGGCTG</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.35–3.5</td>
<td>7</td>
<td>7</td>
<td>100.00</td>
<td>53</td>
<td>0.33</td>
<td>0.33</td>
<td>0.08</td>
<td>2.71</td>
<td>0.94</td>
</tr>
<tr>
<td>OP-E19</td>
<td>AGGCGGTAGC</td>
<td>10-mer</td>
<td>60</td>
<td>35</td>
<td>0.25–3</td>
<td>8</td>
<td>7</td>
<td>87.50</td>
<td>51</td>
<td>0.21</td>
<td>0.21</td>
<td>0.22</td>
<td>2.79</td>
<td>0.94</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.20–3.5</td>
<td>67</td>
<td>64</td>
<td>95.52</td>
<td>429</td>
<td>0.11</td>
<td>0.11</td>
<td>0.15</td>
<td>2.54</td>
<td>0.91</td>
</tr>
</tbody>
</table>

*All the RAPD, BOX A1R and GTG5 experiments were single primer-centered PCR reactions, whereas ERIC and REP were analyzed by dual primer assay (forward and reverse primers). PL: Primer length; AT: Annealing temperature; BS: Average band size; NSB: Number of scored bands; NPB: Number of polymorphic bands; PoP: Percentage of polymorphism; TAF: Total number of alleles; PIC: Polymorphism information content; EH: Expected heterozygosity; DOM: Dominance; SHI: Shannon H Index; SI: Simpson index.*
range using SDS-PAGE. The binary coding data generated from SDS-PAGE banding were subjected to cluster analysis using Ward’s algorithm and disclosed two major clusters (Fig. 4). The cluster analysis based on the SDS-PAGE banding pattern showed a lower linkage distance compared to genomic diversity analyses. The similarity matrix based on binary coding of reproducible SDS-PAGE bands had a highest similarity coefficient of 2.83 (Table 4). The binary variables of SDS-PAGE were subjected to PCA to study the relationship between distance matrix elements based on their first two principal coordinates. The principal component scatter plot based on SDS-PAGE banding showed that fluorescent pseudomonad isolates were grouped into three different clusters, similar to the genomic variance analysis in the cluster relationship analysis (Fig. 5). The maximum and minimum eigen values recorded were 11.37 and 1.14, respectively, with respective percentages of variance of 36.75 and 1.52. For the two genomic variance

Table 2
Similarity matrix of Random amplification of polymorphic DNA analysis of fluorescent Pseudomonas, where PC1 and PC2 are Pseudomonas fluorescens NBAII and PFDWD, respectively and PA is P. aeruginosa MTCC10311 and PP is P. putida MTCC6809.

<table>
<thead>
<tr>
<th>R69</th>
<th>R78</th>
<th>M80</th>
<th>M96</th>
<th>T109</th>
<th>M 127</th>
<th>M132</th>
<th>M135</th>
<th>M137</th>
<th>M139</th>
<th>M140</th>
<th>PC1</th>
<th>PC2</th>
<th>PA</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>R69</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R78</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M80</td>
<td>24</td>
<td>27</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M96</td>
<td>33</td>
<td>28</td>
<td>23</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T109</td>
<td>25</td>
<td>26</td>
<td>19</td>
<td>28</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M127</td>
<td>30</td>
<td>29</td>
<td>32</td>
<td>29</td>
<td>29</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M132</td>
<td>35</td>
<td>32</td>
<td>33</td>
<td>34</td>
<td>26</td>
<td>31</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M135</td>
<td>36</td>
<td>29</td>
<td>22</td>
<td>17</td>
<td>31</td>
<td>20</td>
<td>35</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M137</td>
<td>30</td>
<td>37</td>
<td>40</td>
<td>33</td>
<td>33</td>
<td>26</td>
<td>33</td>
<td>34</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M139</td>
<td>39</td>
<td>26</td>
<td>37</td>
<td>22</td>
<td>36</td>
<td>25</td>
<td>28</td>
<td>19</td>
<td>35</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M140</td>
<td>25</td>
<td>26</td>
<td>19</td>
<td>24</td>
<td>28</td>
<td>19</td>
<td>30</td>
<td>15</td>
<td>33</td>
<td>26</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>32</td>
<td>31</td>
<td>28</td>
<td>27</td>
<td>25</td>
<td>30</td>
<td>33</td>
<td>26</td>
<td>36</td>
<td>35</td>
<td>33</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>19</td>
<td>22</td>
<td>27</td>
<td>22</td>
<td>26</td>
<td>27</td>
<td>28</td>
<td>25</td>
<td>31</td>
<td>26</td>
<td>20</td>
<td>31</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>25</td>
<td>32</td>
<td>33</td>
<td>32</td>
<td>38</td>
<td>27</td>
<td>38</td>
<td>31</td>
<td>27</td>
<td>42</td>
<td>30</td>
<td>35</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>PP</td>
<td>31</td>
<td>32</td>
<td>39</td>
<td>28</td>
<td>40</td>
<td>29</td>
<td>24</td>
<td>33</td>
<td>33</td>
<td>28</td>
<td>26</td>
<td>35</td>
<td>22</td>
<td>28</td>
</tr>
</tbody>
</table>

Fig. 1. Dendrogram showing the approximate relationship among isolates based on random amplification of polymorphic DNA primer analyses of fluorescent Pseudomonas along with four control strains based on the Ward method with squared Euclidean distances where branch lengths are based on the linkage distance values and PC1 and PC2 are Pseudomonas fluorescens NBAII and PFDWD, respectively, PA is P. aeruginosa MTCC10311 and PP is P. putida MTCC6809.

Fig. 2. Dendrogram obtained with combined data of enterobacterial repetitive intergenic consensus (ERIC), BOX, repetitive extragenic palindromic (REP) and GTG5 primer analyses of fluorescent Pseudomonas along with four control strains using the Ward method, with squared Euclidean distances, where branch lengths are based on the linkage distance values, PC1 and PC2 are Pseudomonas fluorescens NBAII and PFDWD, respectively, PA is P. aeruginosa MTCC10311 and PP is P. putida MTCC6809.
analyses, RAPD generated 431 (43.1 per primer reaction) and Rep PCR generated 381 (95.25 per primer reaction) polymorphic bands, whereas in SDS-PAGE, the 15 samples together generated 127 bands.

Experiments to study the fusarium wilt disease severity on fluorescent Pseudomonas priming under green-house conditions

From the extended greenhouse investigation using FP priming on tomato seeds, it was observed that all 11 isolates were capable of inducing resistance on S. lycopersicum at different levels. FP isolates M80, M96 and T109 were the best in host resistance and fusarium wilt inhibition in tomato plants under greenhouse conditions (Fig. 6). The least disease incidence was displayed by priming with M80 (mean ± SE, 0.75 ± 0.02) followed by M96 (0.93 ± 0.00) and T109 (0.93 ± 0.07), with almost no visible symptoms observed on tomato leaves, indicating a systemic type of resistance induction.

The three best isolates, M80, M96 and T109 were identified as P. putida through 16S rRNA sequencing. Sequences were deposited in the NCBI GenBank with accession numbers M80: SUB1688209 Seq1 KX570929, M96: SUB1688209 Seq1 KX570930 and T109: SUB1688209 Seq1 KX570931.

Discussion

Previous reports have showed that PGPR foster plant growth through multifaceted mechanisms (Saharan and Nehra, 2011). Direct effects encompass enhanced nutrient availability, stimulated root expansion and induced systemic resistance or both; indirect beneficial effects entail competition or antagonism towards phytopathogens (Bruto et al., 2014).

Polymorphism in a population is due to the existence of genetic variants, represented by the allele number at a locus and their distribution frequency. Bacterial genomes are regularly subjected to various rearrangements, by either homologous recombination events or site-specific recombination (Orozco-Mosqueda et al., 2009). Numerous hypotheses propose that genomic rearrangements in bacteria may represent an advantage in adapting to drastic changes in the environment. Bacterial subpopulations with different genomic architecture could be generated which adapt to environmental transformations through this mechanism, thus allowing the survival of a portion of the population enabling them to survive in harsh climates (Santoyo et al., 2012).

Studies by Silby et al. (2009) showed that differences in the distribution and abundance of repetitive elements among closely related strains carry additional importance as the evolution of these
genetic elements are independent of core bacterial genome. This is particularly apparent from the comparative study of two closely related strains *P. fluorescens* SBW25 and Pf0-1. Although belonging to the same genus with a quite high degree of genetic resemblance, the FPs are highly dissimilar in terms of nature, profusion and distribution of interspersed repetitive elements. It has been reported that multivariate methods are very constructive and useful in microbial ecology studies and simultaneously this facilitates a reduced complexity in analysis (Costa et al., 2014).

Apparently in the current study, six RAPD primers showed 100% polymorphism. On further investigation, it was perceived that OP-E17 produced maximum primer efficiency with the maximum number of alleles, PIC and heterozygosity. In a similar way, the REP primer indicated maximum polymorphism (100%) whereas the maximum number of alleles, PIC and heterozygosity were shown by ERIC primers. RAPD analysis facilitated more polymorphic bands, a higher percentage of polymorphism and TAF than the Rep-PCR analysis, whereas the average molecular weight of bands (less than 2–8 kb), PIC and EH were high in the Rep-PCR reactions. In the analyses using RAPD and Rep-PCR, RAPD showed higher diversity. Nevertheless, in the present study, the RAPD primers were able to generate a similar diversity pattern to that of the Rep primers, and GTG5 primer amplification resulted in the least diversity from the four Rep primers, though this was still higher than with the RAPD primers.

Gram-negative bacterial OMP acts as a protective barrier from the exterior environment as a permeable fence, as the narrow porin channels limit penetration of hydrophobic molecules into the cell (Cloete, 2003). It was demonstrated that extracellular metabolites usually contain many active substances which play a major role in plant health (Dobrivoj et al., 2013). Furthermore, outer membrane

Table 4

<table>
<thead>
<tr>
<th></th>
<th>R69</th>
<th>R78</th>
<th>M80</th>
<th>M96</th>
<th>T109</th>
<th>M127</th>
<th>M132</th>
<th>M135</th>
<th>M137</th>
<th>M139</th>
<th>M140</th>
<th>PC1</th>
<th>PC2</th>
<th>PA</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>R69</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R78</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M80</td>
<td>2.24</td>
<td>1.73</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M96</td>
<td>2.45</td>
<td>2.45</td>
<td>2.65</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T109</td>
<td>2.45</td>
<td>2.45</td>
<td>2.24</td>
<td>2.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M127</td>
<td>2.45</td>
<td>2.45</td>
<td>1.73</td>
<td>2.45</td>
<td>2.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M132</td>
<td>2.45</td>
<td>2.45</td>
<td>2.24</td>
<td>2.45</td>
<td>2.83</td>
<td>2.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M135</td>
<td>2.24</td>
<td>2.65</td>
<td>2.45</td>
<td>2.65</td>
<td>2.24</td>
<td>2.24</td>
<td>1.73</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M137</td>
<td>2.45</td>
<td>2.45</td>
<td>2.24</td>
<td>2.45</td>
<td>2.00</td>
<td>1.41</td>
<td>2.00</td>
<td>2.24</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M139</td>
<td>2.24</td>
<td>2.24</td>
<td>2.00</td>
<td>2.65</td>
<td>2.65</td>
<td>1.73</td>
<td>1.00</td>
<td>1.41</td>
<td>1.73</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M140</td>
<td>2.45</td>
<td>2.45</td>
<td>2.24</td>
<td>2.45</td>
<td>2.83</td>
<td>2.00</td>
<td>0.00</td>
<td>1.73</td>
<td>2.00</td>
<td>1.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1</td>
<td>2.45</td>
<td>2.45</td>
<td>2.24</td>
<td>2.45</td>
<td>2.83</td>
<td>2.00</td>
<td>0.00</td>
<td>1.73</td>
<td>2.00</td>
<td>1.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA</td>
<td>2.45</td>
<td>2.45</td>
<td>2.24</td>
<td>2.45</td>
<td>2.83</td>
<td>2.00</td>
<td>0.00</td>
<td>1.73</td>
<td>2.00</td>
<td>1.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td>2.65</td>
<td>2.65</td>
<td>2.45</td>
<td>1.73</td>
<td>2.65</td>
<td>2.65</td>
<td>1.73</td>
<td>2.00</td>
<td>2.65</td>
<td>2.00</td>
<td>1.73</td>
<td>1.73</td>
<td>1.73</td>
<td>1.73</td>
<td>0.00</td>
</tr>
</tbody>
</table>
receptor proteins transport the siderophore-iron complex into the bacterial cell and this plays a major role in providing iron for bacterial metabolic functioning (Meyer, 2000). In the present study, diversity analysis based on membrane protein banding pattern using SDS-PAGE showed a linkage distance of 5.5. The hypothesis behind performing OMP diversity was the highly conserved nature of the channel proteins.

The present study attempted to demonstrate the diverse plant probiotic traits of shortlisted FPs through a combination of genetic and membrane protein variance by using molecular fingerprinting assays. However, similar studies carried out earlier such as Rameshkumar et al. (2012) and Sethia et al. (2015), considered FP isolates from a single rhizosphere. Unlike these reports, the isolates used in the present study were from different rhizospheres and were systematically shortlisted based on multiple plant growth promotion attributes. In addition, OMP was considered as one of the diversity markers. An important study by Charan et al. (2011) depicted the genomic diversity of 15 FP isolates from different rhizosphere samples using RAPD and Rep-PCR profiling; however the present study excluded any potential correlation of diversity with resistance induction. Kumar et al. (2013) classified a larger number of PGPR species, predominantly FPs, from the Western Ghats region based on their secondary metabolite profiling using denaturing gradient gel electrophoresis. Multivariate analysis of 2211 strains of rhizosphere bacteria by Costa et al. (2014) proposed a different classification model based on niche colonization according to soil nutritional status. Conversely, none of the aforementioned diversity studies of FPs are linked to the possible variability with respect to OMP. The first such report was from Jayamohan et al. (2015), where a set of hydroxamate siderophore-producing FPs were classified based on genomic and OMP diversity. The present study utilized genomic and OMP as possible diversity markers and tried to link these with plant disease protection mechanisms, indeed an unconventional approach.

From the outcomes of the present study, it can be concluded that the diversity analysis of functionally divergent FPs using RAPD and Rep primers clearly distinguished the isolates. Cluster analysis based on RAPD and Rep-PCR showed identical root cluster trees. Taxonomical output results based on repetitive sequences reinforced Rep-PCR as a competent and reliable method for exploring genetic variation in the context of evolution. Nevertheless, it is reported that PGPR strains exhibit host specificity in terms of crop and cultivar (Bailey et al., 2014). The present study did not produce any such evidence in terms of genomic or OMP diversity.

Observations on the control set of plants suggest that the defense responses elicited against *F. oxysporum* in test plants were substantially elevated through bacterial seed priming. The extended greenhouse investigation on 11 FPs showed M80, M96 and T109 had the best rhizosphere competence and fusarium wilt inhibition. All the three aforesaid isolates showed similar linkage distances in RAPD analysis and were placed in the same cluster in Rep-PCR. This indicated a substantial link between genomic diversity and host defense signal activation against pathogens and this needs to be analyzed further. Indeed, the use of such a molecular marker-based strategy could be considered to overcome the limitations of probiotic trait-based conventional PGPR shortlisting.

Although, the concept of PGPR is about 40 years old (Kloepper and Schroth, 1978), the molecular characteristics that define PGPR remain vague, especially in Proteobacteria, because of two major reasons. First, PGPR may harbor different microbial habitats and they range from saprophytic free-living soil bacteria to endophytes. Second, several bacteria display alternate ecological niches and at times some may function as PGPR (Bruto et al., 2014). In general, the present study has shown the wide genomic diversity of listed FPs, with potential plant growth-promoting attributes through mitigating fusarium wilt disease symptoms in a model interaction system. However, these results should be looked at as a starting point where molecular diversity markers can be used to identify the potential biological control activity of a particular strain. A major limitation in adopting the strategy proposed by Latour et al. (2003) for shortlisting potential PGPR isolates is the deviation in the expected rate of disease inhibition under field conditions. Studies such as Garcia-Gutierrez et al. (2012) showed a similar response, as lower-ranking isolates outperformed the highest-ranking isolates under greenhouse conditions. The major impact of this work has been in attempting to define an alternative strategy to shortlist potential PGPR strains based on the molecular diversity index supported with greenhouse studies. This methodology is particularly advantageous without using highly sophisticated molecular tools. Moreover, the present work methodology can be easily modified by using extra primers, with sufficient greenhouse data support.

**Conflict of interest**

The authors declare that there are no conflicts of interest in this work.
Acknowledgements
The authors are grateful to JAIN (Deemed-to-be University) and DST-SERB (YSS/2015/1905) for infrastructural and financial support, respectively. The authors are extremely thankful to Prof. Shailaja Hittalman (Emeritus Scientist, Department of Genetics and Plant Breeding, GVKK Bengaluru, Karnataka, India), Dr. R. Vani (Associate Professor, Department of Biotechnology, JAIN, Bengaluru, Karnataka, India) and Dr. S. H. Manohar (Principal Scientist, Biocon Research Limited, Bengaluru, Karnataka, India) for their technical guidance.

References