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Genetic analysis of plant endophytic *Pseudomonas putida* BP25 and chemo-profiling of its antimicrobial volatile organic compounds☆

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ABSTRACT

Black pepper associated bacterium BP25 was isolated from root endosphere of apparently healthy cultivar Panniyur-5 that protected black pepper against *Phytophthora capsici* and *Radopholus similis* – the major production constraints. The bacterium was characterized and mechanisms of its antagonistic action against major pathogens are elucidated. The polyphasic phenotypic analysis revealed its identity as *Pseudomonas putida*. Multi locus sequence typing revealed that the bacterium shared gene sequences with several other isolates representing diverse habitats. Tissue localization assays exploiting green fluorescence protein expression clearly indicated that PpBP25 endophytically colonized not only its host plant – black pepper, but also other distantly related plants such as ginger and arabidopsis. PpBP25 colonies could be enumerated from internal tissues of plants four weeks post inoculation indicated its stable establishment and persistence in the plant system. The bacterium inhibited broad range of pathogens such as *Phytophthora capsici, Pythium myriotylum, Giberella moniliformis, Rhizoctonia solani, Athelia rolfsii, Colletotrichum gloeosporioides* and plant parasitic nematode, *Radopholus similis* by its volatile substances. GC/MS based chemical profiling revealed presence of ***Heneicosane; Tetratetracontane; Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl); Tetracosyl heptafluorobutyrate; 1-3-Eicosene, (E)-; 1-Heneicosanol; Octadecyl trifluoroacetate*** and ***1-Pentadecene*** in PpBP25 metabolite. Dynamic head space GC/MS analysis of airborne volatiles indicated the presence of aromatic compounds such as ***1-Undecene; Disulfide dimethyl; Pyrazine, methyl-Pyrazine, 2,5-dimethyl-; Isoamyl alcohol; Pyrazine, methyl-; Dimethyl trisulfide***, *etc.* The work paved way for profiling of broad spectrum antimicrobial VOCs in endophytic PpBP25 for crop protection.

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1. Introduction

It is well established that microbes interacting with below ground (rhizosphere), above ground (phyllosphere) and within the plant (endosphere) in the same plant are different and unique (Turner et al. 2013). This natural interaction between plants and their microbes is vital to plant fitness, modulating growth promotion, disease suppression and the very survival (Bulgarelli et al. 2013; Vorholt 2012). Among the diverse microflora, bacterial endophytes colonize and share an ecological niche similar to that of phytopathogens, which makes them ideal for biocontrol (Berg et al. 2005). Most of the endophytic bacteria colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls and xylem vessels as well as reproductive organs of plants. Indeed, several reports have shown that endophytic microorganisms can have the capacity to control plant pathogens (Aravind et al. 2010), insects (Azevedo et al. 2000) and nematodes (Aravind et al. 2010) besides promoting plant establishment and growth under stress conditions (Azevedo et al. 2000; Bent and Chanway 1998; Chanway 1997; Hallmann et al. 1997; Lodewyckx et al. 2002; Schulz and Boyle 2006). Hundreds of bacterial genera representing 16 phyla have been reported as endophytes which

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include bacteria belonging to *Acidobacteria, Actinobacteria, Aquificae, Bacteroidetes, Cholorobi, Chloroflexi, Cyanobacteria, Deinococcus, Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospira, Planctomycetes, Proteobacteria, Spirochaetes* and *Verrucomicrobiae* (Berg and Hallmann 2006; Mengoni et al. 2009; Manter et al. 2010; Sessitsch et al. 2012). However, the most studied endophytes belong to *Actinobacteria*, *Proteobacteria* and *Firmicutes* and include members of *Azoarcus* (Krause et al. 2006), *Gluconobacter* (Bertalan et al. 2009), *Bacillus* (Deng et al. 2011), *Enterobacter* (Taghavi et al. 2010), *Burkholderia* (Weilharter et al. 2011), *Herbaspirillum* (Pedrosa et al. 2011), *Pseudomonas* (Taghavi et al. 2009), *Serratia* (Taghavi et al. 2009), *Stenotrophomonas* (Ryan et al. 2009) and *Streptomyces*. Among the bacterial genera, *Pseudomonas* species are ubiquitously found in plants and members of this species have a broad metabolic versatility, which allows them to adapt different habitats and including plant endosphere (Johnsen et al. 1996; Palleroni 1992). The endophytic *Pseudomonas* of plant origin has shown promise for broad spectrum of activity against several soil borne pathogens. There are evidences which clearly indicate that the endophytes are a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential (Azevedo et al. 2000; Ryan et al. 2007). Among the various species of *Pseudomonas, P. putida* is a well known plant associated prokaryote with proven effect on plant growth and biological control of several plant pathogens (Wu et al. 2011).

Pseudomonas putida BP25 (PpBP25R) was isolated as an endophyte in root tissues of apparently healthy black pepper cultivar, Panniyur 5, collected from southern Indian state of Kerala. This bacterium exhibited antagonistic activity against foot rot causing oomycete, *Phytophthora capsici* and a nematode pest of black pepper, *Radopholous similis* (Aravind et al. 2009, 2010, 2012). The present study was performed to genetically characterize the endophytic PpBP25R in order to establish its identity at strain level by adopting polyphasic phenotypic and genotypic tools. The bacterium was further analyzed for its endophytic ability in a range of plant species such as black pepper, ginger and arabidopsis by tagging the strain with green fluorescence protein (gfp) gene that enabled us to localize the bacterium in plant internal tissues (Ramos et al. 2000; Molina et al. 2000). Our attempts to elucidate the mode of action of the bacterium on plant pathogens revealed that the bacterium released airborne antimicrobial volatiles against pathogens belong to diverse taxa. Experiments were also conducted to identify major compounds secreted by PpBP25 using gas chromatography–mass spectroscopic (GC/MS) technique.

2. Materials and methods

2.1. Identification and genotyping of Pseudomonas putida BP25R

2.1.1. Bacterial strain, media, growth conditions, development of rifamycin resistant strain

The bacterial strains used were isolated from black pepper root. Unless stated otherwise, bacterial strains were routinely grown on LBA [Luria Bertani Agar ($g\,L^{-1}$) Tryptone 10; yeast extract 5; sodium chloride 10; Agar 18] at 37 °C. The endophytic bacterium was subjected to Biolog based phenotypic finger printing assay, sequence analysis of 16S rDNA as well as multilocus sequence typing (MLST) for establishing intraspecific identity. Spontaneous rifamycin resistant mutant was developed and designated as PpBP25R and used in various experiments.

2.1.2. Identity confirmation using Biolog, other biochemical assays and electron microscopy

The bacterium BP25R was subjected to Biolog based identification assay as per manufacturer's protocol (Biolog Inc, Hayward, USA). Apart from Biolog, ten other tests such as Gram staining, KOH test, Indole test, Methyl red test, Oxidase test, Citrate test, Starch hydrolysis, Protease production, Gelatin liquefaction and Siderophore production tests were also carried out. The bacterial strain was imaged in Transmission electron microscopy (Jeol, Japan) and Scanning electron microscopy (Zeiss, Germany) at a magnification of above 10,000× by adopting standard sample preparation procedures.

2.1.3. Identification using ribosomal DNA sequencing and multilocus sequence typing

2.1.3.1. Isolation of genomic DNA. Genomic DNA was prepared from overnight cultures of PpBP25R grown in 1.0 mL of LB broth. Bacterial cells were washed thrice with 0.9% NaCl and used. Total genomic DNA for PCR templates was isolated by standard CTAB method (Kumar et al. 2004). DNA was quantified on a Biophotometer (Eppendorf, Germany) and 50–100 ng of DNA was used for each PCR reaction.

2.1.3.2. Identification using ribosomal DNA sequencing. For ribosomal DNA sequencing, prokaryotic universal primers 27F-5′ AGAGTTTGATCCTGGCTCAG 3′ and 1492R-5′ GGTTACCTTGTTACGACTT 3′ were used. An amplicon of size 1500 bp was eluted from agarose gel by using SV gel and PCR clean up system as per manufacturer's instructions (Promega Corporation, USA) and sequenced. Sequence data was end trimmed, contig assembled and used for blast search in NCBI database and the identity was confirmed by closest match.

2.1.3.3. Selection of MLST loci. Whole genome sequences of *P. putida* strains KT2440, S16, GB1, ND6, F1, BIRD1, DOT-T1E available in GenBank (NCBI) or www.pseudomonas.com were consulted and used (Winsor et al. 2011). After analyzing several highly conserved housekeeping genes using CLC workbench, twelve of them such as *aroE, dnaG, guaA, gyrB, ligA, pgi, recF, recN, rpoB, rpoD, tpiA* and *trpC* were finally selected for sequencing. The details of the housekeeping genes are present in the supplementary data (Supplementary Table 1).

2.1.3.4. Amplification and DNA sequencing. PCR primers specific for *aroE, dnaG, guaA, gyrB, ligA, pgi, recF, recN, rpoB, rpoD, tpiA* and *trpC* were designed from whole genome sequences of *P. putida* using Primer3plus (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) (Supplementary Table 1). Prior to use, primers were *in silico* validated at http://www.basic.northwestern.edu/biotools/oligocalc.html and used to sequence housekeeping genes in PpBP25R. Amplification of targeted DNA was carried out in 50 μL reaction volumes, each containing final concentrations of the following reagents from Promega PCR kit (Go Taq Buffer – 1×, $MgCl_2$ – 1.5 mM, dNTPs – 200 μM), forward/reverse primers – 10 picomoles each, DMSO – 6%, Taq polymerase – 1 U, DNA – 200 ng. PCR amplification was performed at initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing for 1 min (Supplementary Table 1) and extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min. Amplified PCR products were purified by using SV gel and PCR clean up system as per manufacturer's instruction (Promega Corporation, USA). The cycle sequencing reaction was performed with 20–30 ng of purified amplicon using the ABI PRISM BigDye Terminators v3.1 cycle sequencing kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer's instruction. The purified product was sequenced bi-directionally so as to obtain maximum coverage of the gene. The sequences were end-trimmed, edited, and contig-assembled using DNA baser (http://www.dnabaser.com/download/DNA-Baser-sequence-assembler/). Sequences were further subjected to Basic Local Alignment Search Tool (NCBI

nucleotide BLAST) in order to establish their identity (Altschul et al. 1997). Gene sequences were annotated with the help of EXPasy server (http://web.expasy.org/translate/) and blastP analysis was done to confirm the correctness of sequenced genes (www.ncbi.nlm.nih.gov/BLAST). Multiple alignments and other phylogenetic interpretations were made with the help of CLC bio sequence viewer (Version 8). All sequences were submitted to GenBank and accession number assigned. Sequences for all 12 were concatenated, and served as input for establishing phylogeny. A maximum likelihood tree was constructed with concatenated sequence length of 10,835 bp using RDP v.4.03. Entries from public databases such as NCBI, EMBL and DDBJ were included in the analysis (Supplementary Table 2). The topology was compared with trees generated by the minimum evolution method using the maximum composite likelihood model in MEGA v. 5.01 (Tamura et al. 2011).

2.2. Endophytic colonization of PpBP25R in plants

2.2.1. Transformation of PpBP25R expressing reporter genes

P. putida BP25R strain was transformed and tagged with stable green fluorescence protein (gfp) gene. The gfp gene was delivered and inserted in a neutral site in the genome. For tagging the bacterium, we have used Tn7 based gfp construct pBKminiTn7gfp$_2$Gm$_{10}$ maintained in *E. coli* XL1 Blue (Gentamycin 20 μg mL^{-1}) and a helper plasmid pUXBF$_{13}$Amp$_{100}$ maintained in *E. coli* XL1 Blue (Ampicillin 100 μg mL^{-1}). The gfp gene was delivered into PpBP25R by triparental mating method. The transformants were selected on LBA with Rifamycin (100 μg mL^{-1}) and Gentamycin (75 μg mL^{-1}) at 28°C for 48–72 h. Putative transformants appeared on the selection plates were further subjected to PCR confirmation for the insertion using forward primer specific for insert DNA and the reverse primer specific for bacterial genomic region (Craig et al. 1989). Briefly, primer pairs GlmSF: 5′-AATCTGGCCAAGTCGGTGAC-3′ and Tn7R109: 5′-CAGCATAACTGGACTGATTTCAG-3′ were used in PCR reaction using GoTaq PCR kit of Promega (GoTaq Buffer – 1×, $MgCl_2$ – 1.5 mM, dNTPs – 200 μM, forward/reverse primers – 10 picomoles each, DMSO – 6%, Taq polymerase – 1 U) at an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 7 min. PCR amplicon of 150 bp was visualized in 2% agarose gel electrophoresis. Expression of gfp gene was confirmed in Confocal Laser Scanning Microscopy [CLSM] (DM6000, Leica Microsystems, Germany). Cells of PpBP25R::gfp cells were immobilized on 1% agarose and observed at different magnifications. Wild type PpBP25 served as check. Selected transformants was designated as PpBP25R::gfp and used in the *in planta* experiments.

2.2.2. Plant species, growth conditions and bacterization of plantlets

Transformed PpBP25R (PpBP25R::gfp) was used to track its colonization in diverse plant species such as *Piper nigrum* cv. Sreekara, *Zingiber officinale* cv. Mahima and *Arabidopsis thaliana* ecotype Columbia-0. PpBP25R::gfp was cultured at 37°C on LB amended with rifamycin (50 μg mL^{-1}) and gentamycin (75 μg mL^{-1}). Overnight cultures of PpBP25R::gfp at 1.0 OD unit (absorbance at 600 nm) were used in the plant inoculation assays. Population density on the root surface and plant endosphere was estimated by serial dilution plate technique.

2.2.2.1. Black pepper and ginger. The roots of two-leaf stage black pepper rooted cuttings (cv. IISR-Sreekara) and roots of two-month old ginger plantlets (cv. IISR-Mahima) were treated with bacterial cell suspension (∼×10^8 cfu mL^{-1}) for 30, 60 and 120 min, respectively. The plantlets were planted in pots containing 500 g of potting mixture (sterile coir pith: farmyard manure 3:1) and grown under greenhouse conditions at 28–30 °C with 60–80% humidity. Sampling was done at weekly intervals and endogenous population size of PpBP25R::gfp was estimated using plate count method.

2.2.2.2. Arabidopsis. Seeds of *Arabidopsis thaliana* ecotype Columbia-0 were surface sterilized using 70% ethanol and 1% NaOCl followed by five time washing in sterile distilled water. Thus obtained sterilized seeds were placed on half strength Murashige and Skoog medium (Murashige and Skoog, 1962) and different concentrations of PpBP25R::gfp suspension (10 μL per seed) were spot inoculated on seeds. PpBP25R::gfp cultured overnight in LB amended with rifamycin (50 μg mL^{-1}) and gentamycin (75 μg mL^{-1}). Bacterial suspension was prepared in water and decimal dilutions were prepared so as to obtain 10^{10}, 10^9, 10^8 and 10^7 cells per mL. Five seeds were arranged in line on the surface of the medium with 10 μL of cell suspension and incubated for 48 h at 4 °C. The seeds were transferred to growth chamber and allowed to germinate and grow at 22/20°C (day/night) temperature, 24-h light period and 40% relative humidity. Sampling was done 21 dpi for estimation of endogenous population size of PpBP25R using serial dilution plate count method.

2.2.3. Plate assay to determine endogenous bacterial population in plants

The bacterized black pepper and ginger (30, 60 and 120 min) were aseptically partitioned into roots, shoots and leaves. One gram of tissue was surface sterilized with sodium hypochlorite (0.5%) + tween 20 (0.01%) for 20 min, ethyl alcohol (70%) for 1 min followed by rinsing with sterile distilled water for 2–3 times. Each sample was ground aseptically with 2 mL of Phosphate Buffered Saline [PBS, g L^{-1} NaCl 8; KCl 0.2; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH – 7.4] and serially diluted up to 10^{-3}. One mL of serially diluted samples was pour plated in LBA amended with rifamycin (50 μg mL^{-1}) and gentamycin (75 μg mL^{-1}) and incubated at 28°C for 48 h. Colonies were counted and expressed as CFU per gram of fresh tissue. For *A. thaliana*, the whole plantlet harvested on 21 dpi was ground with PBS, serially diluted upto 10^{-5} and plated for estimation of endogenous population as described above.

2.2.4. Bio-PCR assay to determine endogenous bacterial population in plants

Bio-PCR was adopted to detect bacterial cells in the plant endosphere. Bio-PCR involves culturing an organism prior to PCR amplification, making it a particularly useful method for very low population of bacterium in asymptomatic tissue and ensuring that only viable organisms are examined. The plantlets were surface sterilized with 1% sodium hypochlorite for 1 min, and then washed three times in distilled water (1 min each time). The samples were then ground with a pestle in sterile micro centrifuge tubes containing 1.0 mL of distilled water for approximately 1 min. The homogenates were vortexed for 5 s. Out of this 1 mL, 500 μL was inoculated into two 5 mL LB Broth amended with rifampicin (50 μg mL^{-1}) and gentamycin (75 μg mL^{-1}) and incubated at 37°C for 48 h. Broth culture (2 μL) was used as template for PCR confirmation using primer tpiA_F: 5′-CGAATTCGTCTGCGTTCAG-3′; tpiA_R: 5′-GAGCTGACCAAAGGCTTGAG-3′ that is known to yield 667 bp amplicon.

2.2.5. Confirmation using confocal microscopy (CLSM) and scanning electron microscopy (SEM) for endophytic behavior of BpBP25R in endogenous tissues of Arabidopsis thaliana

Plantlets of three weeks old Col-0 ecotype emerged from seeds inoculated with PpBP25::gfp2 (OD600: 1.0) were washed in sterile

water and thin sections fixed in paraformaldehyde (4%) for 12 h at 4 °C. The tissues were scanned and imaged in CLSM (DM6000, Leica microsystems) at multiple sites on the sections. For SEM, whole roots from mock and bacteria inoculated Columbia-0 seedlings were taken and fixed overnight at 4°C in 2.5% (v/v) glutaraldehyde in 50 mM cacodylate buffer (pH 7.2). Fixed samples were rinsed three times in 0.1 M phosphate buffer for 15 min at 4°C and then dehydrated in a successive acetone series (30%, 50%, 70%, 80%, 90%, 95% and 100%) for 15 min with each concentration. Then samples were processed for critical point drying with liquid CO_2 at 31.5°C and pressure 1100 psi. Finally, samples were cut of (~) 1 cm, mounted on Al stubs, then coated with 35 nm thick films of silver, scanned and imaged in SEM (Zeiss, Germany)

2.3. Bioassay for antagonistic activity of PpBP25R on plant pathogens

In order to determine the effects of the PpBP25R on pathogens, dual-culture confrontation assays were performed to test the effect of bacterial volatiles on growth and development of pathogens. Identity of the pathogen was determined using ITS sequencing by adopting established protocols for fungal identification. PpBP25R was tested against range of pathogens such as *Phytophthora capsici* (ITS-GenBank: KC300238.1), *Pythium myriotylum* (ITS-GenBank: KM434129), *Rhizoctonia solani* (ITS-GenBank: KM434130), *Gibberella moniliformis* (ITS-GenBank: KM434131), *Colletotrichum gloeosporioides* (ITS-GenBank: KM434128), *Athelia rolfsii*, (ITS-GenBank: KM434132) and *Radopholus similis*. Bioassay for antimicrobial VOCs was performed in M9 minimal medium with glucose as a sole carbon source as well as Tryptic Soy Agar (TSA). M9 minimal medium (M9 salt solution 1×; Glucose 0.4%; Thiamine 1 mM; $MgSO_4$ 1 mM; $CaCl_2$ 0.1 mM; Agar 1.8%) was used in the bioassay. M9 Salt solution (10X), prepared by dissolving (g L^{-1}) $Na_2HPO_4{\cdot}12H_2O$ 171.9; KH_2PO_4 30.0; NaCl 5.0; NH_4Cl 10.0 was used in the assay. Mycelia disk from three days old PDA cultures of fungal and oomycetes pathogens were placed in the middle of potato dextrose agar (PDA; HiMedia, India) in Petri dish. Similarly 200 μL of 10^9 cfu mL^{-1} mid-log phase PpBP25R culture was spread on M9 or Tryptic Soy Agar (TSA, HiMedia, India) in another Petri plate. The lids of both the plates were removed and the inoculated plates were positioned face to face and fastened using parafilm followed by petriseal in such a way that the pathogen was directly exposed to air borne bacterial volatiles released during the growth of bacteria. This set up ensured that no volatile escaped during the course of experimentation. The plates were incubated at a common incubation temperature of 28 °C for seven days. The experiment was performed in three replications and was repeated three times. Plates without bacterial inoculum served as mock.

2.4. Solvent extraction (SE) and analysis of volatile compounds

Volatile organic compounds (VOCs) were extracted from 100 mL overnight broth culture of PpBP25R grown at 28°C. This overnight culture was serially extracted with solvents such as Hexane and Dichloromethane according to protocols suggested by Gil-Chavez et al. (2013). The fractions were concentrated using rotary evaporator at 28°C (Rotary Flash Evaporator, Buchi India Ltd). The final concentrate was dissolved in the same solvents, hexane or dichloromethane and subjected to gas chromatography–mass spectroscopic (GC/MS) analysis. GC–MS analysis was performed on a capillary gas chromatograph directly coupled with mass spectrometer having thermal desorption system (Shimadzu QP-2010 TD 20, Japan). AB-Innowax column (60 m length × 0.25 mm id × 0.25 μm film thickness) was used under the following conditions: Column oven temp: 100°C, injection temp: 250°C, pressure 90.4 kPa, total flow: 16.3 mL min^{-1}, column flow: 1.21 mL min^{-1}, the volume of injected sample 1 μL, split ratio 1:10, ion source temperature: 230°C, interface temp: 260°C with scan *m*/*z* starts from 40.00 and end at *m*/*z*: 650.00. The chemical constituents were identified by comparison of mass spectra with the NIST library.

2.5. Dynamic head space sampling of bacterial volatiles

The volatiles released from PpBP25 in broth culture were assessed by dynamic head space sampling method at room temperature. A loopful of PpBP25 colony was inoculated into a 1 L conical flask containing 250 mL of TSB and incubated at 28°C for 24 h. Gentle stream of air was sucked through activated charcoal at 30 mL min^{-1}. It was allowed to pass over the matrix held in a 1 L conical flask. The odor laden air was trapped in glass tube containing Propak Q absorbent (Supelco) 30 mg (50–80 mesh) with glass wool on either side as stoppers. Volatiles from uninoculated TSB served as technical control and compounds from this run were subtracted from the sample run to enumerate the VOC from the bacterial culture. The collection was made for 6 h. The trapped volatiles in the adsorbent were eluted with HPLC grade dichloromethane (400 μL) and condensed to 50 μL by passing gentle stream of ultra pure nitrogen. The compounds were determined in GC/MS (Model: Agilent Technologies GC 7890A, MS 5975C). From the collections, 1 μL of the sample was injected into injection port of GC/MS. All the samples were analyzed in a HP 5MS Phenyl methyl siloxy capillary column. The temperature of column and oven were maintained at 40°C for 1 min and then increased at 20°C min^{-1} to 280°C and held at 300°C for 10 min. The injector and column temperature were 250°C. The total run was for 23 min. The volatile constituents were identified by comparison of mass spectra with the NIST library.

3. Results

3.1. Identification and genotyping of Pseudomonas putida BP25R

Bacterium BP25 is Gram negative and tested positive for oxidase and citrate tests (Table 1). Identity of BP25R was further confirmed as *Pseudomonas putida* Biotype B (similarity coefficient of 94%) by adopting phenotypic fingerprinting method of Biolog (Supplementary Table 3; Supplementary Figure 1). Transmission and scanning electron microscopic image of cells of PpBP25R is depicted in Supplementary Figure 2. Blast analysis of 16S rDNA sequence (GenBank accession: KM376218) further confirmed its identity as *P. putida* as the similarity percentage was well above 99% to that of entries in the GenBank.

Forward and reverse nucleotide sequences of housekeeping genes such as *aroE, dnaG, guaA, gyrB, ligA, pgi, recF, recN, rpoB, rpoD, tpiA, trpC* obtained from PpBP25R were end trimmed, quality assessed and assembled in order to obtain maximum length. The assembled sequences were annotated using EXPasy server and submitted to GenBank with accession numbers (Supplementary Table 1). BlastP analysis was done to confirm the accuracy of annotations. Multiple alignments and other phylogenetic interpretations were performed using CLC bio sequence viewer. Sequences of all those 12 genes were made into a concatenated sequence with a length of 10,835 bp and used to decipher the genetic relationship of the PpBP25R with other *P. putida*. The Bayesian clustering based phylogenetic analysis revealed that PpBP25R grouped with other isolates representing diverse habitats such as soil, fresh water, hospital and industrial strains of *P. putida* (Fig. 1).

3.2. Endophytic colonization of PpBP25R in black pepper and ginger

Over 70 putative transformants of PpBP25R observed on the gentamycin selection plates was subjected to PCR confirmation that

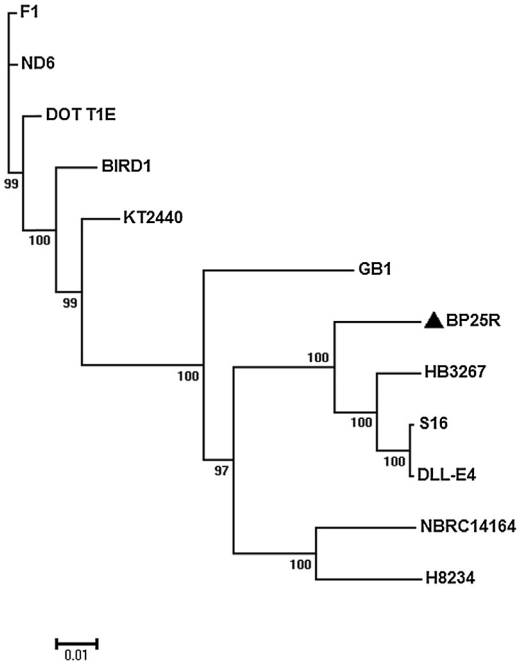

Fig. 1. Maximum likelihood tree of 10,835 bp alignment of concatenated sequences from *Pseudomonas putida* strains constructed using RDP4 v. 4.03. The topology was compared with trees generated by the minimum evolution method using the maximum composite likelihood model in MEGA v. 5.01 (Tamura et al. 2011). Nodal robustness was tested by bootstrap analysis. The passport information of the isolates is given in the Supplementary Table 3.

yielded 150 bp amplicon confirming the integration (Supplementary Figure 3). Transformed PpBP25R was found to express gfp gene (Supplementary Figure 3) and displayed resistance against gentamycin. Coupled with intrinsic rifamycin resistance, the marked isolate was used for enumeration in rhizoplane and endogenous plant tissues. In rooted cuttings of black pepper, PpBP25R displayed a spatiotemporal pattern in endophytic colonization (Fig. 2). The bacterium colonized rhizoplane and root endosphere in high population but not the stem and leaf of black pepper. Results of the colony counts in pepper and ginger plantlets indicated a stable population densities upto 5–6 $\log_{10}$ CFU in rhizoplane and an order less in the root endospheric region. Interestingly the bacterial population could be detected in ginger rhizome and leaf at 1–2 $\log_{10}$ CFU per gram (Fig. 3). However, highly sensitive Bio-PCR using PpBP25R specific primer on extract obtained from bacterized black pepper yielded PpBP25R specific amplicon of 667 bp that further confirmed the endogenous presence of low population level (Figs. 2 and 3). Collectively the data indicated an acropetally reducing endogenous population in root, stem or rhizome and leaf of ginger and black pepper. Duration of bacterization did not affect the bacterial colonization as the endogenous population size for 30 min, 60 min and 90 min of bacterial treatment recorded nearly same (Figs. 2 and 3).

3.3. Endophytic localization of PpBP25R in arabidopsis

Arabidopsis plants recorded nearly identical endogenous population for all concentration of PpBP25R used in bacterization as revealed by plate count as well as bio-PCR results. The endogenous population of PpBP25::gfp ranged from 7.45–7.93 log CFU per gram of tissue on fresh weight basis (Fig. 4). Bio-PCR using PpBP25R specific primer yielded specific amplicon of 667 bp that further confirmed the endogenous presence (Fig. 4). Seed inoculated PpBP25R::gfp could be observed in plant interiors in CLSM and SEM imaging thus confirming its endophytic behavior (Fig. 4).

3.4. Bioassay for antagonistic activity of PpBP25R on plant pathogen

The data on pathogen inhibition clearly revealed that the volatiles released during the growth of PpBP25R in M9 medium were found inhibitory to broad range of pathogens such as *Py. myriotylum, P.capsici, G. moniliformis, Rh. solani, A. rolfsii* and *C. gloeosporioides.* All the targeted pathogens were inhibited to the

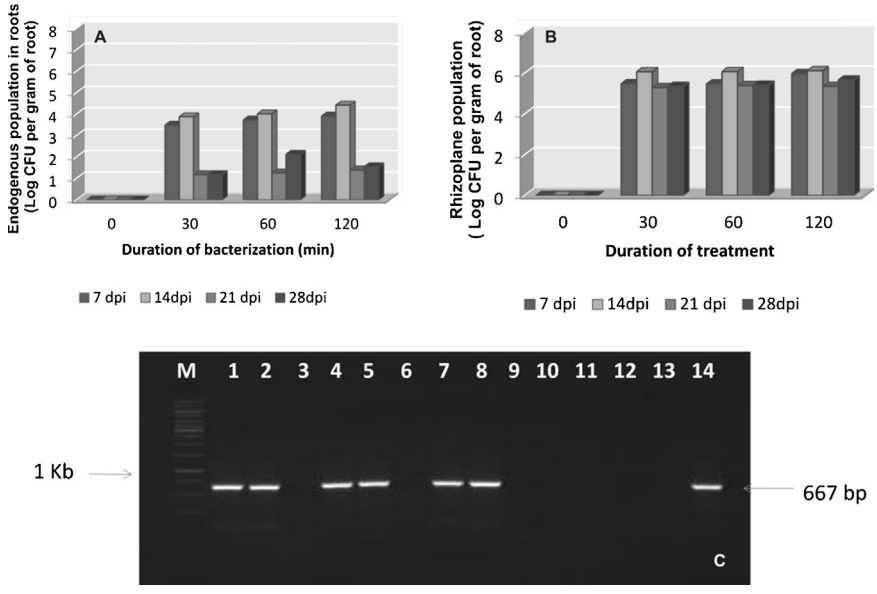

Fig. 2. Assay for endophytic colonization of PpBP25R::gfp in *Piper nigrum*. Endogenous population size of PpBP25R in rhizoplane (A) and root endosphere (B) of black pepper plantlets emerged from bacterized plantlets as detected in dilution plate method; black pepper roots were bacterized for 30, 60 and 120 min in suspension of PpBP25R::gfp. Sampling for plating was done at 7 days interval upto 28 dpi. Experiments were performed twice in triplicate. Water was used in mock treatment. (C) Bio-PCR based detection of PpBP25R in surface disinfected plant parts; Amplicon size of 667 bp confirms presence of bacterium in plant tissues; Lane M: 1 kb DNA ladder used as size marker; Lane 1–3: plantlets emerged from 30 min of bacterization; 1. Root, 2. Stem, 3. Leaf; Lane 4–6: plantlets emerged from 60 min of bacterization; 4. Root, 5. Stem, 6. Leaf; Lane 7–9: Plantlets emerged from 120 min of bacterization; 7. Root, 8. Stem, 9. Leaf; Lane 10–12: mock treatment; 10. Root, 11. Stem 12. Leaf; Lane 13: Negative control-No template DNA; Lane 14: Positive control-PpBP25R genomic DNA.

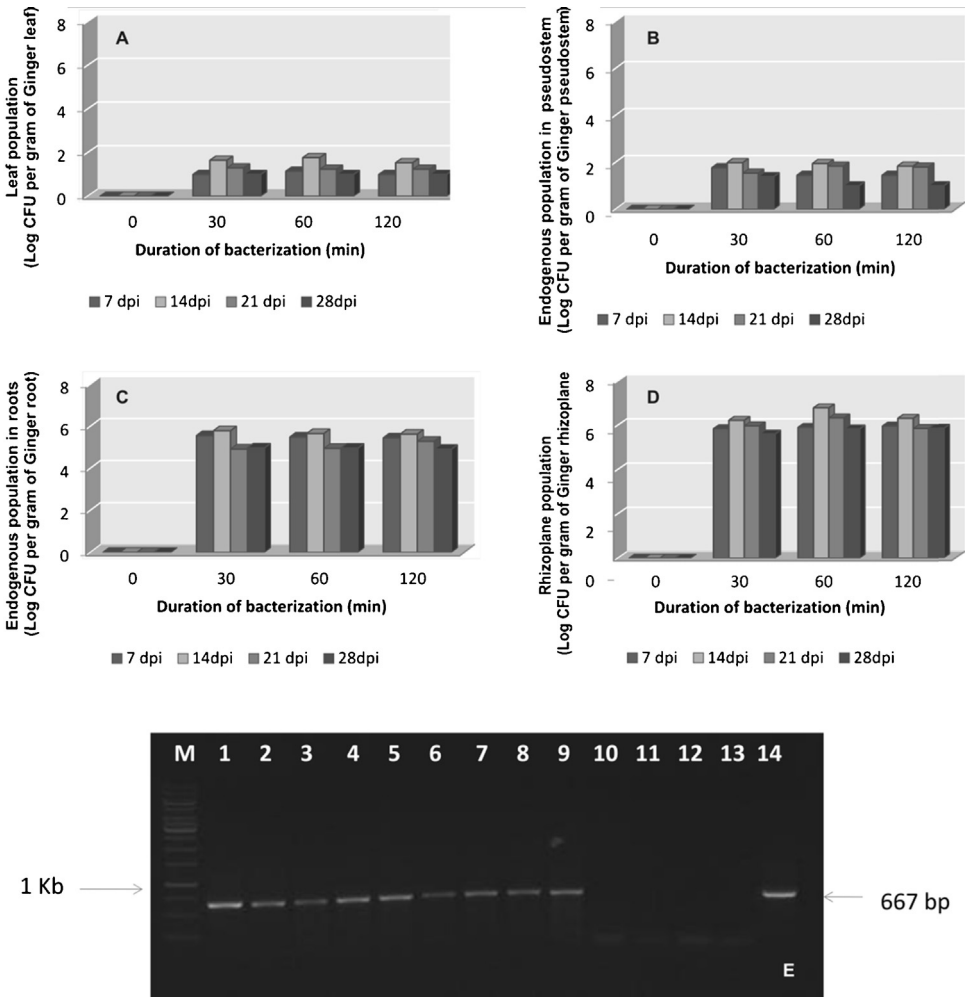

Fig. 3. Assay for endophytic colonization of PpBP25R::gfp in *Zingiber officinale*. Endogenous population size of PpBP25R in various parts of *Zingiber officinale* plantlets emerged from bacterized rooted plantlets as detected in dilution plate method; rooted plantlets were bacterized for 30, 60 and 120 min in bacterial suspension of PpBP25R::gfp. Sampling for plating was done at 7 days interval upto 28 dpi. Experiments were performed twice in triplicate. Water was used in mock treatment. (A) Leaf; (B) pseudostem; (C) root; (D) rhizoplane. (E) Bio-PCR based detection of PpBP25R in surface disinfected plant parts; Amplicon size of 667 bp confirms presence of bacterium in plant tissues; Lane M: 1 kb DNA ladder used as size marker; Lane 1–3: plantlets emerged from 30 min of bacterization; 1. Root, 2. Pseudostem, 3. Leaf; Lane 4–6: Plantlets emerged from 60 min of bacterization; 4. Root, 5. Pseudostem, 6. Leaf; Lane 7–9: plantlets emerged from 120 min of bacterization; 7. Root, 8. Pseudostem, 9. Leaf; Lane 10–12: mock treatment; 10. Root, 11. Pseudostem 12. Leaf; Lane 13: Negative control-No template DNA; Lane 14: positive control-PpBP25R genomic DNA.

level of 85–90% of which *P.capsici* and *G. moniliformis* were highly inhibited at 89–90% followed by others at 85–88% (Fig. 5). The plant parasitic nematode, *R. similis* was also inhibited (92.0%) by VOCs of PpBP25R (Data not shown). Nearly identical observation was made on TSA medium where PpBP25R significantly inhibited the growth of pathogens mentioned above (Supplementary Figure 4).

3.5. Profiling of VOC in solvent extracts of PpBP25

Having observed significant inhibitory activities of volatiles released by PpBP25 on broad range of pathogen belong to fungi, oomycetes and nematode, the volatiles were extracted by adopting standard chemical extraction procedures and subjected to chemical fractionation in GC/MS. GC/MS analysis revealed presence of range of chemicals in the volatiles of PpBP25 (Supplementary Figure 5). A total of 75 compounds could be detected in hexane and dichloromethane extracts. Chemical profiling of hexane and dichloromethane fractions of PpBP25R has yielded 42 and 33 compounds, respectively. Various chemicals identified in the hexane extract represented the compounds that belong to hydrocarbons (combined peak area 97.81%), sulfoxides (combined peak area 1.04%), acids (combined peak area 0.74%), esters (combined peak area 0.04%) and nitrogenous compounds (combined peak area 0.24%). The dichloromethane extract represented chemicals belong to hydrocarbons (combined peak area 16.08%), esters (combined peak area 27.98%), alcohols (combined peak area 7.42%), ketones (combined peak area 45.89%) and aldehydes (combined peak area 0.92%). Diverse groups of chemical detected are furnished in Table 2.

Further scrutiny of the peaks found in hexane fraction revealed the dominance of compounds such as (i) ***Heneicosane*** with the combined peak area of over 61.21% that appeared with multiple retention times between 13.602 and 17.686 min and (ii) ***Tetratetracontane*** with combined peak area of 32.31% with multiple retention times between 17.415 and 23.827 min (Table 3). As for dichloromethane fraction the predominant compounds identified are (i) ***Pyrrolo [1,2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)*** with a combined peak area of 43.25% appeared between 11.980 and 14.744 min; (ii) ***3-EICOSENE, (E)-*** with peak area of 6.81% appeared at 11.536–11.718 min, (iii) ***1-Pentadecene*** with peak area of 4.19% appeared at 9.946 min, (iv) ***1-Heneicosanol*** with a peak area of 7.24% appeared at 14.170 min, (v) ***Octadecyl trifluoroacetate*** with a peak area of 7.36% appeared at 15.324 min, and (vi) ***Tetracosyl heptafluorobutyrate*** with peak area of 11.47%

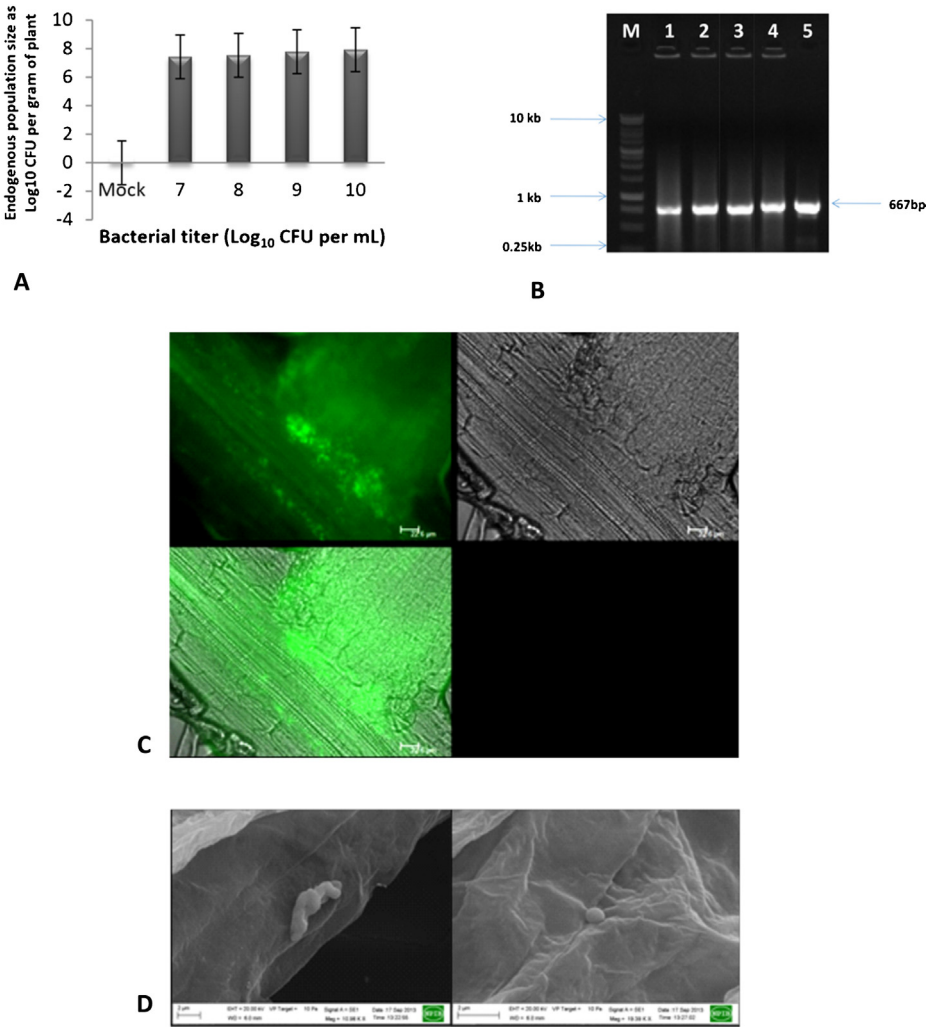

Fig. 4. Assay for endophytic colonization of PpBP25R::gfp in *Arabidopsis thaliana* Col-0. (A) Endogenous population size of PpBP25R in *Arabidopsis* Col-0 seedling emerged from bacterized seeds on 21 dpi as detected in dilution plate method; whole plantlet of *Arabidopsis* was used in the experiment. Experiments were performed twice in triplicate. Error bar represents standard error of mean difference. (B) Bio-PCR based detection of PpBP25R in surface disinfected plantlets; Amplicon size of 667 bp confirms presence of bacterium in plant tissues; Lane M: 1 kb ladder; Lane 1: *Arabidopsis* inoculated with PpBP25R (10^7 cfu mL^{-1}); Lane 2: *Arabidopsis* inoculated with 10^8 cfu mL^{-1}; Lane 3: *Arabidopsis* inoculated with 10^9 cfu mL^{-1}; Lane 4: *Arabidopsis* inoculated with 10^{10} cfu mL^{-1}; Lane 5: positive control – PpBP25R genomic DNA. (C) Endogenous localization of PpBP25R in internal root tissues of *Arabidopsis thaliana* Col-0 using CLSM. Note: Fluorescing cells of PpBP25R in root sections. Bacterized roots were fixed using paraformaldehyde and imaged using gfp filter. (D) Localization of PpBP25R on root surface of *Arabidopsis thaliana* Col-0 using SEM (cluster of cells and single cell).

appeared at 12.875–13.875 min (Table 4). Unlike in solvent extraction method, the dynamic head space sampling of bacterial culture revealed presence of several specific aromatic compound such as ***Pyrazine, 2,5-dimethyl-; Isoamyl alcohol; Pyrazine, methyl; 1-Undecene; Disulphide, dimethyl-; Pyrazine, 2-ethyl-5-methyl-; Octadecyl vinyl ether; Heptamethyl-2-nonene***, *etc.* in the volatile (Table 5; Supplementary Figure 6).

4. Discussion

Pseudomonas putida is a highly versatile and adapted bacterium in diverse habitats such as soil, water, plant rhizosphere and endosphere and occasionally as opportunistic pathogen in immune compromised patients (Andreote et al. 2009; Molina et al. 2011, 2014; Roca et al. 2013). The ecological ubiquitousness is best witnessed in its wide spread usage as bioremediation agent in polluted soils (Iyer et al. 2011). Hundreds of articles have been published about various aspects of *P. putida* physiology, enzymology and genetics by microbiologists and biochemists, in addition to more applied studies by chemists, plant pathologists and environmental microbiologists utilizing *P. putida* and its products for green chemistry applications and bioremediation. The plant associated *P. putida* is one of the well known rhizosphere inhabiting bacteria playing a key role in shaping plant root growth and protection from soil borne pathogens (Kruijt et al. 2009). However, reports on *P. putida* colonizing endosphere region of plants are very scanty with an exception of poplar endophyte *P. putida* W619 and potato endophyte *P. putida* strain P9 (Andreote et al. 2009; Weyens et al. 2013; Khan et al. 2014). It is speculated that the endophytic bacterium would colonize the living tissues of the host plant and participate in a variety of relationships where they support the plant growth by producing a plethora of substances that ultimately confer survival advantage to the plant (Strobel and Daisy 2003). Here, we report unique traits of black pepper associated endophytic *P. putida* BP25 isolated from evergreen Western Ghat forest region of Southern Indian state of Kerala. The bacterium protected rooted cuttings of black pepper against *R. similis* and *P. capsici* (Aravind et al. 2009, 2011). The bacterium was identified as *P. putida* in Biolog based phenotypic finger printing test. Ribosomal DNA sequence comparison further reconfirmed its identity as *P. putida*. In order to establish its strain identity as well as to determine it genetic relationship with other *P. putida*, polyphasic phenotypic and genotypic

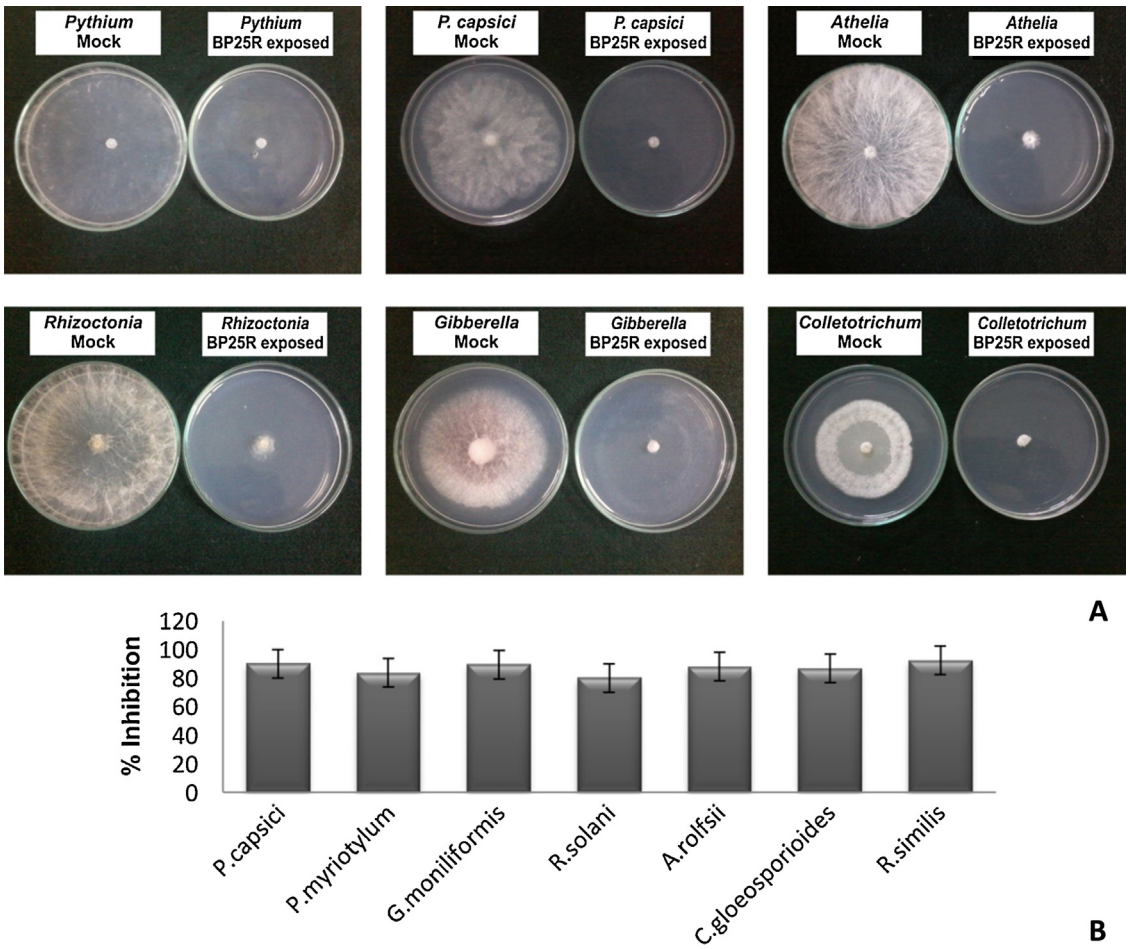

Fig. 5. (A) Effect of volatiles of *Pseudomonas putida* BP25 (grown on M9 Minimal media) on diverse plant pathogen. Water was used in mock treatment. For fungal and oomycete pathogen, mycelial suppression was scored. For nematode, number of dead ones was counted and expressed as inhibition percentage (data not shown). Experiments were performed twice in triplicate. (B) Percent inhibition of mycelial growth of fungal pathogens and nematode (*R. similis*) growth suppression over mock is shown in graph. Error bar represents standard error of mean difference.

tools were used. Multi Locus Sequence Typing using 12 housekeeping genes revealed that endophytic bacterium *P. putida* BP25R is genetically identical to several other strains of *P. putida* representing habitats as diverse as soil, fresh water, plant rhizosphere and even hospital environments (Wang et al. 2007). Versatile functions of *P. putida* are reported by Weyens et al. (2013) who proposed to exploit the poplar endophytic strain for bioremediation in polluted soil.

In order to determine the endophytic colonization of PpBP25R, the bacterium was genetically tagged with pBKminiTn7gfp$_2$Gm$_{10}$ having genes for stable green fluorescence as well as for gentamycin resistance. Coupled with intrinsic rifamycin resistance, we precisely tracked and estimated the bacterial population within the tissues of plants. Our study showed that PpBP25R not only colonized its parent host black pepper but also ginger as well as the model plant arabidopsis. On black pepper and ginger, the bacterium attained a population size of 5–6 log units per gram of root tissue. The bacterium could not attain high population in stem and other aerial tissues of black pepper that corroborated the findings of Aravind et al. (2009) who originally isolated PpBP25 from internal root tissues of black pepper. Interestingly, the bacterium could be tracked in pseudostem and leaf of ginger plantlets emerged from bacterized rooted plantlets. *Arabidopsis* supported the multiplication of PpBP25R to 10–100 order more as compared to black pepper and ginger. High population size of PpBP25 in arabidopsis could be attributed to aseptic *in vitro* assay that was free of other competing microflora in contrast to 'microbe rich' competitive soil environment of black pepper and ginger. The bacterial colonization in tissue was further confirmed in Bio-PCR, CLSM as well as SEM based detection/localization assays. Results of highly sensitive and specific bio-PCR further provided a strong proof for low population size of bacterium in stem tissues of black pepper also. Taken together, our polyphasic endophytic colonization assays have yielded evidences for endophytic colonization of PpBP25 in plants. The versatility of PpBP25R for its colonization in diverse plant species clearly indicated its adaptability in root tissue – a trait important for persistent and sustainable biological control against soil borne plant pathogens (Brader et al. 2014). One of the interesting observations on colonization by PpBP25 in plant is its ability to regulate its endogenous population. The endogenous population size of PpBP25 did not increase with increasing bacterial inoculum or extended duration of bacterization that confirmed internal regulation of endogenous bacterial population size. Interestingly the colonized *Arabidopsis* plantlets survived (data not shown). Regulation of endogenous population is very essential for being a genuine endophyte as uncontrolled increase in endogenous bacterial population could be detrimental for the plant growth as observed in vascular wilt caused by *Ralstonia solanacearum* in plants.

One of the useful biological traits of PpBP25 was its remarkable ability to release volatile organic compounds (VOCs). The volatiles of PpBP25 released upon its growth in chemically defined as well as semi-synthetic medium were found to suppress a wide range

Table 1
Phenotypic characteristics of PpBP25.

Taxonomic/functional traits	Results
General features	
Plant origin	Root tissues of black pepper
Geographical origin	Rainforest of Western Ghats in Kerala, India
Growth temperature range	28–40°C (best at 37°C)
Biolog^{R} analysis (94 biochemical tests)	Closest match to *P. putida* (94% similarity)
16S rDNA analysis	Closest match to *P. putida* (99% similarity)
Swarming motility on soft KB medium	+
Casein hydrolysis	–
Starch hydrolysis	–
Methyl red	–
Citrate, indole	+
Siderophore	+
Antibiotic resistance	Growth occurred at ampicillin (30 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), amoxicillin (30 μg mL^{-1}), trimethoprim (30 μg mL^{-1}), nalidixic acid (30 μg mL^{-1}), cephalothin (30 μg mL^{-1}), cephalexin (30 μg mL^{-1}), oxacillin (30 μg mL^{-1}), novobiocin (30 μg mL^{-1})
Antibiotic sensitivity	No growth occurred at cephotaxime (30 μg mL^{-1}), ciprofloxacin (30 μg mL^{-1}), colistin (25 μg mL^{-1}), doxycycline hydrochloride (30 μg mL^{-1}), polymyxin B (100 units), kanamycin (30 μg mL^{-1}), gentamycin (20 μg mL^{-1}), tetracycline (40 μg mL^{-1}),
Functional traits	
Biological activity	Inhibition of hyphal growth of *Phytophthora capsici*; suppression of lesion expansion of *P. capsici* on stem cutting of black pepper; protects rooted plants of black pepper against *P. capsici*-induced rot; suppression of *Radopholus similis*
Secondary metabolites and enzymes	
HCN	–
Protease	–
Cellulase	–
Catalase	+
Oxidase	+
Plant growth promotion-related features	
Nitrogen, ammonia production	+
Indole acetic acid (IAA)	+

+, Positive for production; –, negative for production.

of pathogens belong to all major fungal taxa. *P. capsici, Py. myriotylum, G. moniliformis, Rh. solani*, *C. gloeosporioides, A. rolfsii* and *R. similis* were found significantly inhibited by VOCs. Bacterial airborne volatile has been a subject of intense investigation during the past decade and its production is widespread among rhizobacteria and depends on culture conditions (Blom et al. 2011; Schulz and Dickschat 2007). Bacterial volatiles have been attributed to number of multifaceted biological traits such as plant growth promotion

Table 2
Significant chemical groups identified in dichloromethane and hexane extract of *Pseudomonas putida* BP25R using GC/MS.

Name	Combined peak area (%)			
	Hexane	Significant chemical identified	Dichloromethane	Significant chemical identified
1. Hydrocarbons	97.81	Tetradecane Tetramethyl hexadecane Cyclobutanediyl benzene Nonadecane Methyl nonadecane Heneicosane Pentacyclo octacosane Nonacosane Tetracontane Methyl heneicosane Cyclohexane Hexatriacontane	16.08	Hexadecene Heptadecane Tetramethyl hexadecane Pentadecene Eicosene Heptyl pentadecene Cyclopropane Tetra contane Tetradecene Pentatriacontane Heptacosane
2. Alcohols	–	–	7.42	Dimethyl octanol Heneicosanol
3. Aldehydes	–	–	0.92	Propenal
4. Acids	0.74	Benzene dicarboxylic acid	–	–
5. Esters	0.04	Ecosyl acetate	27.98	Tetracosyl heptafluoro butyrate Octadecyl trifluoro acetate Octyl phthalate Tricosyl trifluoroacetate Octacosyl trifluoro acetate Hexacosyl heptafluoro butyrate
6. Ketones	–	–	45.89	Octanone Pyrrolopyrazine 1,4,dione Tetrabutyl oxaspiro dione
7. Nitrogenous compounds	0.24	Benzonitrile	–	–
8. Sulfoxides	1.04	Diphenyl cyclopropyl sulfoxide	–	–

Table 3
Chemical profiling of hexane fraction of *Pseudomonas putida* BP25R using GC/MS.

Peak	Retention time (min)	Area (AU)	Area (%)	Name	Similarity index
1	8.134	690,338	0.32	Tetradecane	96
2	10.850	373,309	0.18	Hexadecane, 2,6,10,14-tetramethyl-	95
3	11.475	347,777	0.16	Benzene, 1,1′-(1,2-cyclobutanediyl) bis-, trans-	89
4	11.625	387,023	0.18	Hexadecane, 2,6,10,14-tetramethyl-	92
5	12.167	278,313	0.13	1,2-Benzenedicarboxylic acid, Bis(2-methylpr	86
6	12.309	168,061	0.08	Nonadecane	92
7	12.972	307,411	0.14	Nonadecane	94
8	13.218	275,132	0.13	10-Methylnonadecane	90
9	13.602	314,886	0.15	Heneicosane	95
10	14.205	1,001,579	0.47	Heneicosane	98
11	14.783	4,094,351	1.93	Heneicosane	98
12	15.340	10,759,966	5.06	Heneicosane	98
13	15.417	87,430	0.04	Eicosyl acetate	71
14	15.528	1,384,807	0.65	Pentacyclo[19.3.1.1(3,7).1(9,13).1(15,19)]octacosa-1(25),3,5,	57
15	15.787	520,566	0.24	Benzonitrile, m-phenethyl-	78
16	15.874	21,186,854	9.97	Heneicosane	98
17	16.133	1,286,483	0.61	1,2-Benzenedicarboxylic acid	97
18	16.203	361,442	0.17	Nonacosane	93
19	16.314	1,484,187	0.70	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	87
20	16.412	31,265,907	14.71	Heneicosane	96
21	16.497	712,230	0.34	(2,3-Diphenylcyclopropyl)methyl phenyl sulfoxide, trans-	87
22	16.771	810,959	0.38	Nonacosane	95
23	16.839	207,048	0.10	Tetratriacontane	88
24	17.006	32,011,152	15.06	Heneicosane	97
25	17.415	1,276,608	0.60	Tetratetracontane	95
26	17.493	368,940	0.17	3-Methylhenicosane	90
27	17.686	29,451,233	13.86	Heneicosane	97
28	18.164	1,291,267	0.61	Nonacosane	95
29	18.258	492,021	0.23	Pentacosane	91
30	18.486	24,082,423	11.33	Tetratetracontane	97
31	19.058	1,697,892	0.80	Tetratetracontane	96
32	19.172	491,926	0.23	Tetratetracontane	91
33	19.446	17,390,028	8.18	Tetratetracontane	97
34	20.143	808,980	0.38	Tetratetracontane	93
35	20.283	499,474	0.24	Tetratetracontane	91
36	20.614	11,991,234	5.64	Tetratetracontane	97
37	20.872	242,202	0.11	Cyclohexane, eicosyl-	88
38	21.468	482,227	0.23	Hexatriacontane	92
39	21.644	247,779	0.12	Tetratetracontane	87
40	22.051	6,717,574	3.16	Tetratetracontane	97
41	23.827	3,456,949	1.63	Tetratetracontane	97
42	26.037	1,197,578	0.56	Tetracontane	96
		212,503,546	100.00		

(Ryu et al. 2003; Farag et al. 2006), induced systemic resistance in plants (Ryu et al. 2004; Farag et al. 2006), antimicrobial activities (Fiddaman and Rossall 1994; Kai et al. 2007; Howell et al. 1988; Voisard et al. 1989; Chaurasia et al. 2005; Fernando et al. 2005; Vespermann et al. 2007) and also involved in their interaction with fungi that co-habitat in the ecological niche (Wheatley 2002)

Chemical profiling of VOCs in PpBP25 using GC–MS analysis revealed number of chemicals that includes ***Heneicosane*** and ***Tetratetracontane*** in the hexane fraction. ***Henicosane*** is reported as a pheromone for attracting the female *Aedes aegypti* (Bhutia et al. 2010). However, their natural function and specific role in plant pathogen suppression warrants further studies. Other most dominant chemical found in PpBP25 was ***Pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)*** that was recently identified as a major compound with antimicrobial activity in the metabolites of an endophytic *Mortierella alpina* strain isolated from the Antarctic moss *Schistidium antarctici* (Melo et al. 2013).

Nearly identical observation was made in dynamic head space sample of PpBP25 that revealed presence of ***Pyrazine*** derivatives such as ***methyl pyrazine; 2,5-dimethyl pyrazine; trimethyl pyrazine*** and ***3-ethyl-2,5-dimethyl pyrazine*** as significant chemicals in VOCs. Pyrazines – a group of 1,4 dinitrogen substituted benzenes, are a class of compounds that occur ubiquitously in nature in plants, animals and microorganisms and can be synthesized chemically or biologically (Rajini et al. 2011). A vast majority of ***pyrazine*** derivatives have been found to possess diverse pharmacological properties such as antibacterial, antifungal, antimycobacterial, anti-inflammatory, analgesic, anticancer, antidiabetic and antiviral that triggered increasing attention by researchers (Ferreira and Kaiser 2012).

Other aromatic compounds found in the volatile metabolite of PpBP25 are ***Heptamethyl-1-nonene; Dodecane; Tridecane; Heptamethyl-1-nonene; Tetradecane; Heptamethyl-2-nonene; Pentadec-7-ene, 7-bromomethyl-;*** β***-Naphthol; Ethylhexanol; n-Nonadecanol-1; 2-Undecanethiol, 2-methyl-; Tetradecane, 2,6,10-trimethyl; 1-Hexadecanol, 2-methyl-*** and ***Cyclohexane, 1-methyl-2-pentyl-*** that clearly indicated the volatile diversity in *P. putida*. Similar observation was made by Zechman and Labows (1985) who identified diverse chemicals such as ***1-butanol; isopentanol; toluene; 1-undecene; 2-butanone; 2-heptanone; 2-nonanone*** and ***2-undecanone*** in the VOCs of *P. putida* using an automated headspace concentrator incorporating a gas chromatograph after 24 h incubation in TSA. Tenorio-Salgado et al. (2013) identified several compounds such as ***dimethyldisulfide (DMDS); toluene*** and ***terpenoid*** compounds such as α***-pinene; limonene*** and ***ocimene*** in *Burkholderia tropica*. Several of these volatile compounds, such as ***DMDS; methyl ketone*** and ***toluene*** have been previously detected in the headspace analysis of many strains of *Pseudomonas* spp. and from *B. cepacia* cultured in trypticase soy (Labows et al. 1980). Most of the plants resist invading plant pathogens in parts through

Table 4
Chemical profiling of dichloro methane fraction of *Pseudomonas putida* BP25R using GC/MS.

Peak	Retention time (min)	Area (AU)	Area (%)	Name	Similarity index
1	8.024	6,565,128	1.38	3-Hexadecene, (Z)-	97
2	9.120	1,105,807	0.23	Heptadecane	96
3	9.757	581,762	0.12	Hexadecane, 2,6,10,14-tetramethyl-	89
4	9.946	19,907,518	4.19	1-Pentadecene	98
5	10.475	294,514	0.06	Decane, 2,3,4-trimethyl-	83
6	10.711	407,276	0.09	1-Octanone, 1-(2-octylcyclopropyl)-	73
7	11.163	5,264,443	1.11	4-Nonylphenol	83
8	11.325	4,374,287	0.92	2-Propenal, 3-(1-aziridinyl)-3-(dimethylamino)-	71
9	11.536	31,397,305	6.61	3-Eicosene, (E)-	96
10	11.718	963,989	0.20	3-Eicosene, (E)-	94
11	11.980	73,494,487	15.48	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylprop	84
12	12.473	12,533,853	2.64	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	89
13	12.632	27,272,770	5.74	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylprop	92
14	12.773	82,775,819	17.43	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylprop	95
15	12.875	53,914,342	11.35	Tetracosyl heptafluorobutyrate	88
16	13.375	584,366	0.12	Tetracosyl heptafluorobutyrate	88
17	13.596	1,117,647	0.24	8-Heptylpentadecane	92
18	14.040	875,491	0.18	1-Octanol, 3,7-dimethyl-	85
19	14.176	34,362,803	7.24	1-Heneicosanol	97
20	14.744	21,845,252	4.60	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylprop	79
21	15.142	996,207	0.21	5,8-Tridecadione	72
22	15.324	34,939,113	7.36	Octadecyl trifluoroacetate	95
23	16.135	1,093,336	0.23	Di-n-octyl phthalate	90
24	16.265	664,200	0.14	Cyclopropane, 1-(2-methylbutyl)-1-(1-methylpropyl)-	90
25	16.396	18,932,065	3.99	Tricosyl trifluoroacetate	97
26	17.007	4,930,389	1.04	Tetratetracontane	93
27	17.353	845,338	0.18	1-Tetradecene	82
28	17.670	14,657,948	3.09	Octacosyl trifluoroacetate	97
29	18.489	3,295,601	0.69	Tetratetracontane	97
30	19.428	6,186,304	1.30	Hexacosyl heptafluorobutyrate	97
31	20.618	2,044,441	0.43	Pentatriacontane	94
32	22.025	3,122,597	0.66	Hexacosyl heptafluorobutyrate	97
33	26.003	3,511,324	0.74	Heptacosane, 1-chloro-	69
		474,857,722	100.00		

the secretion of antimicrobial compounds that can be originated from plant associated microorganisms. It is speculated that plant origin isoamyl salicylate is actually a microbe produced chemical that is used by plants as a pheromone to sensitize other plants of pathogens such as tobacco mosaic virus (Frosch et al. 2002).

Although chemical profiling of bacterial VOCs leading to identification of chemicals have been conducted by several workers, reports on their biological activity against microbial pathogens are scanty and confirmed only in few cases. Bacterial volatile chemicals such as ***benzothiazole; cyclohexanol;* n*-decanal; dimethyl trisulfide; 2-ethyl 1-hexanol*** and ***nonanal*** identified from *Pseudomonas* species were found to inhibit sclerotium formation in *Sclerotinia sclerotiorum* (Fernando et al. 2005). Similarly *Pseudomonas* spp. is also capable of producing organic volatiles such as ***cyclohexanol; decanol; 2-ethyl-1-hexanol; nonanal; benzothiazole*** and ***dimethyl trisulfide*** whose *in vitro* antifungal nature has been demonstrated against *Phytophthora vignae* in cowpea (Fernando and Linderman 1994). Recently, antifungal nature of VOCs against soil borne fungus, *A. rolfsii* SY4 from *Pseudomonas fluorescens* is identified as an activity of compound **DMDS** (Zhou et al. 2014). **DMDS** along with ***phenyl ethanone; nonane; phenol; 3,5-dimethoxy-toluene; 2,3-dimethyl-butanedinitrile; 1-ethenyl-4-methoxy-benzene; benzeneacetaldehyde; 2-nonanone; decanal*** and ***2-undecanone*** released from *Bacillus megaterium* were found to display nematicidal activity (Huang et al. 2010).

The foregoing reports along with our data on PpBP25 strongly suggested that *P. putida* released diverse groups of air borne volatile organic compounds that displayed broad spectrum antimicrobial activities against wide range of plant pathogens belong to diverse taxa. The plant endophytic *P. putida* was found genetically close to many environmental isolates of *P. putida* used in bioremediation which broadens its scope beyond biocontrol. Furthermore, the bacterium PpBP25R uniquely colonized diverse plant species – an important trait for an antagonistic bacterium for sustained protection against pathogens. Taken together it can be concluded that the bacterium PpBP25R possessed several useful, unique and beneficial biological activities ideal for commercial exploitation as microbial formulation for crop protection. Furthermore, the work paved way for microbial bioprospecting of *P. putida* for development of novel chemicals for crop protection. It would be very interesting to quantify, identify and assign the role of individual chemical compound released by PpBP25R against pathogens especially within the plant tissue.

Table 5
Head space GC/MS analysis of PpBP25 volatiles.

Name of the compound	BP 25 R (peak area %)
1. Pyrazine, 2,5-dimethyl-	39.526
2. Isoamyl alcohol	20.984
3. Pyrazine, methyl-	10.506
4. 1-Undecene	7.411
5. Disulphide,dimethyl	6.395
6. Pyrazine, 2-ethyl-5-methyl-	3.049
7. Dimethyl trisulfide	1.449
8. Heptamethyl-2-nonene	1.199
9. β-Naphthol	1.164
10. Octadecyl vinyl ether	1.071
11. Tetradecane, 2,6,10-trimethyl	0.937
12. Cyclobutene, 2-propenylidene-	0.794
13. Heptamethyl-1-nonene	0.771
14. 1,8-Nonadien-3-ol	0.411
15. Octadecanal,2-bromo	0.385
16. 2-Ethyl-3,6-dimethyl pyrazine	0.231

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2015.02.001.

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