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Genotyping and identification of broad spectrum antimicrobial volatiles in black pepper root endophytic biocontrol agent, *Bacillus megaterium* BP17

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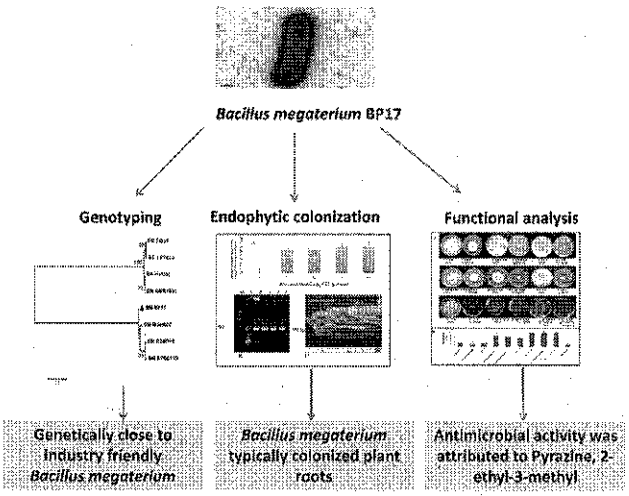
HIGHLIGHTS

- *Bacillus megaterium* BP17 is genetically divergent from clinical *Bacillus* strains.
- *Bacillus megaterium* BP17 is an endophytic colonist in diverse plant species.
- *Bacillus megaterium* BP17 showed volatiles mediated antimicrobial activity on plant pathogens.
- Antimicrobial activity of endophytic antagonist was attributed to Pyrazine group of chemicals.
- Pyrazine, 2-ethyl-3-methyl is identified as an antimicrobial compound against plant pathogens.

GRAPHICAL ABSTRACT



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ABSTRACT

Black pepper root endophytic *Bacillus* BP17 was identified as *Bacillus megaterium* (BmBP17) by a panel of phenotypic and genotypic methods. BmBP17 was found genetically close to industrially significant *B. megaterium* WSH002 and divergent from clinical *Bacillus* strains. Tracking with genetically tagged BmBP17 revealed its endophytism in diverse plant species such as *Piper nigrum*, *Zingiber officinale* and *Arabidopsis thaliana*. BmBP17 released antimicrobial volatiles against several pathogens viz., *Phytophthora capsici*, *Pythium myriotylum*, *Athelia rolfsii*, *Gibberella moniliformis*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani*, *Magnaporthe oryzae*, *Ralstonia solanacearum*, and *Xanthomonas axonopodis* pv. *punicae*. Chemical compounds belong to hydrocarbons, heterocyclics, esters and sulfoxides were dominantly present in solvent extracts of BmBP17 in GC/MS profile. Dynamic head space GC/MS

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Endophytic bacteria
Ginger
Volatile organic compounds

analysis revealed broad spectrum antimicrobials such as Pyrazine, 2-ethyl-3-methyl-; Pyrazine, 2,5-dimethyl-; Pyrazine, ethyl-; and Pyrazine, methyl- in the volatiles of BmBP17. Pyrazine, 2-ethyl-3-methyl was found most inhibitory followed by Pyrazine, 2-ethyl-; Pyrazine, 2, 5-dimethyl and Pyrazine, 2-methyl which can be exploited for crop protection.

1. Introduction

Plant species on earth are inhabited and colonized by microorganisms that include several rhizospheric, phyllospheric and endophytic microorganisms (Hallmann et al., 1997; Lodewyckx et al., 2002; Schulz and Boyle, 2006). Among these endophytic microorganisms, bacteria are unique as they are not only primary colonizers of plants but also influence their growth and health (Ryan et al., 2007). Endophytic bacteria of plant origin have shown promise because of their broad spectrum of activity against several plant pathogens. They are often touted as a rich and reliable source of bioactive and chemically novel compounds (Ryan et al., 2007).

Bacillus megaterium is a spore-forming bacterium found in diverse habitats such as soil, seawater, sediments, rice paddies, dried food, honey, milk as well as in plant tissues as an endophyte (Vary, 1994; Aravind et al., 2009; Salgaonkar et al., 2013). Akin to its adaptive behaviour, the economic use of *B. megaterium* is highly versatile. *B. megaterium* is known to produce vitamin B_{12}, antiviral agent-oxetanocin and penicillin amidase besides its use in AIDS diagnostics and as a host to express foreign proteins without degradation (Vary, 1994; Morita et al., 1999). In agriculture, *B. megaterium* is known for its plant growth promotion and biocontrol ability against plant pathogens. Growth promotion is largely due to air borne volatiles such as acetoin and 2-pentylfuran (Lopez-Bucio et al., 2007; Zou et al., 2010). Huang et al. (2010) reported that *B. megaterium* released nematicidal compounds such as phenyl ethanone; nonane; phenol; 3,5-dimethoxy-toluene; 2,3-dimethyl-butanedinitrile; 1-ethenyl-4-methoxy-benzene; benzene acetaldehyde; 2-nonanone; decanal; 2-undecanone; and dimethyl disulphide. Li et al. (2012) reported nematicidal volatiles from *B. megaterium* against *Panagrellus redivivus* and *Meloidogyne incognita*. Apart from direct inhibitory activities on pathogens, bacterial volatiles are known to trigger induced systemic resistance in plants (Ryu et al., 2004). In order to develop an ecofriendly technology for production of pesticide-free black pepper, plant endophytic bacteria were explored as biological control agent (Aravind et al., 2009). *B. megaterium* BP17 (BmBP17) was isolated as a root endophyte in apparently healthy black pepper cultivar Panniyur 5 (Aravind et al., 2010, 2012). BmBP17 exhibited disease suppressive activity *in planta* against the foot rot causing oomycete pathogen, *Phytophthora capsici* and a nematode pest *Radopholus similis* (Aravind et al., 2010, 2012). The objective of the present study was to characterize BmBP17 by adopting polyphasic approaches which included genotypic and phenotypic methods for determining intraspecific taxonomic position. The bacterium was further analyzed for its endophytic ability in plants by tagging the strain with genetic markers conferring resistance to antibiotics. Experiments were also conducted to elucidate the mode of antagonistic action on different plant pathogens and to identify major volatile compounds secreted by BmBP17 using head space chromatography–mass spectroscopic analysis. The microbial volatile organic compounds (MVoCs) identified in BmBP17 were evaluated against a range of plant pathogens representing oomycetes, fungi, bacteria and nematodes.

2. Materials and methods

2.1. Bacterial strain, growth conditions and development of a rifamycin resistant strain

B. megaterium BP17 was isolated from black pepper root. Unless stated otherwise, bacteria were routinely grown on LBA [Luria Bertani agar ($g\,l^{-1}$) Tryptone 10; Yeast extract 5; Sodium chloride 10; agar 18] at 37 °C. A spontaneous rifamycin resistant mutant was developed by growing the bacterium overnight and plating on LBA plates amended with rifamycin (50 $\mu g\,ml^{-1}$). Colonies obtained were again streaked on rifamycin amended plates (Enne et al., 2004). The mutants obtained were designated as BmBP17R.

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

The bacterium was subjected to Biolog based identification assays as per manufacturer's protocol (Biolog Inc, Hayward, USA). Apart from Biolog based phenotypic finger printing, the following biochemical tests were also carried out.

KOH test: Two drops of a 3% solution of potassium hydroxide (KOH, HiMedia, India) were placed on a glass slide. A 2-mm loopful of bacterial growth, obtained from a 48 h culture, was stirred in a circular motion in the KOH solution. The loop was occasionally raised 1–2 cm from the surface of the slide. Gram-positive bacteria suspended in the KOH solution generally displayed no reaction (absence of stringing) whereas string formation was observed in case of Gram-negative bacteria (Suslow et al., 1982; Powers, 1995).

Indole test: The bacterium was inoculated in peptone (HiMedia, India) water, which contains amino acid tryptophan (HiMedia, India) and incubated overnight at 37 °C. Following incubation a few drops of Kovac's reagent (HiMedia, India) were added. Formation of a red or pink colored ring at the top was scored as positive (Vashist et al., 2013).

Methyl red test: The bacterium was inoculated into glucose phosphate broth (HiMedia, India) and incubated at 37 °C for 48 h. After 48 h, pH of the medium was tested by adding 5 drops of Methyl red reagent (HiMedia, India). Development of red color was recorded as positive (Vashist et al., 2013).

Oxidase test: 1–2 drops of 1% oxidase reagent (HiMedia, India) was placed on a 6 cm square piece of Whatman filter paper. A small colony of bacterium was transferred using a loop onto soaked filter paper and observed for purple color development. (Vashist et al., 2013).

Citrate test: The bacterium was streaked on Simmons Citrate Agar (HiMedia, India) and incubated at 37 °C for 48 h. After 48 h, change in color from green to blue was observed (Vashist et al., 2013).

Starch hydrolysis: The bacterium was streaked on starch agar (HiMedia, India) plate and incubated at 37 °C for 48 h. After incubation, iodine solution was flooded with a dropper for 30 s on the starch agar plate. A clear zone of hydrolysis was observed around the bacterial growth (Kaur et al., 2012).

Protease production: The bacterium was streaked on milk agar (HiMedia, India) plate and incubated at 37 °C for 48 h. After 48 h, formation of a clear zone around the colonies resulting from casein

hydrolysis was taken as evidence of proteolytic activity (Bizuye et al., 2014).

Gelatin liquefaction: Bacterial colony from a pure culture was stabbed into the nutrient gelatin (HiMedia, India) deep tubes that contain 12% gelatin. The gelatin media was incubated for at least 48 h and then placed a refrigerator for approximately 30 min. The medium was observed for liquefaction that indicated production of gelatinase (Vashist et al., 2013).

Siderophore production test: Bacterial culture was spot inoculated onto chrome azurol sulfonate assay agar (blue agar) (HiMedia, India) and incubated at 37 °C for 24–48 h. The results were interpreted based on the color change due to transfer of ferric ion from its intense blue complex to siderophore. The sizes of yellow orange haloes around the growth indicated siderophore activity (Gokarn and Pal, 2010).

The bacterial strain was imaged in transmission electron microscopy (Jeol, Japan) at a magnification of 10,000×. Bacterial colony was fixed at 4 °C overnight in 1.5% glutaraldehyde (HiMedia, India) solution prepared in 0.1 M cacodylic acid (HiMedia, India) buffer (pH 7.3). Dehydration of sample was done in 50% ethanol for 5 min, 70% ethanol for 10 min, 80% ethanol for 10 min, 90% ethanol for 15 min, and 99.9% ethanol twice for 20 min at room temperature. Sample was then passed through a "transition solvent" propylene oxide, infiltrated and embedded in a liquid resin such as epoxy and LR white resin. After embedding, the resin block was thin sectioned into sections of 50–70 nm thickness and collected on metal mesh 'grids', stained with electron dense stains and viewed in TEM (Burghardt and Droleskey, 2006).

2.3. Gene sequence based species identification

2.3.1. Isolation of genomic DNA

Genomic DNA was prepared from overnight cultures of BmBP17R grown in 1.0 ml of LB broth. Bacterial cells were washed thrice with 0.9% NaCl and used to isolate total genomic DNA for PCR templates by 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.

2.3.2. Identification using ribosomal DNA and cobalamine biosynthesis gene sequences

For ribosomal DNA sequencing, prokaryotic universal primers were used (Lane, 1991; Stackebrandt and Liesack, 1993) (Table S1). Amplification of targeted DNA was carried out in 50 μl reaction volumes, containing final concentrations of GoTaq Buffer-1X; $MgCl_2$ – 1.5 mmol l^{-1}; dNTPs – 200 μmol l^{-1}; Forward/Reverse primers – 10 pmol each; DMSO – 6%; Taq polymerase – 1 Unit; DNA 200 ng (Promega Corporation, Madison, USA). PCR amplification was performed at initial denaturation at 95 °C/5 min; 35 cycles of denaturation at 95 °C/1 min; annealing at 58 °C/1 min and extension at 72 °C/1 min followed by final extension at 72 °C/10 min. An amplicon of size 1500 bp was eluted from agarose gel by using SV gel and PCR clean up system (Promega Corporation, USA) and sequenced. Sequence data was end trimmed, good quality sequence reads were contig assembled, blast searched in NCBI database and their identity was confirmed by the closest match. Identity of bacterium was further confirmed using cobalamine biosynthesis genes *cbiD* and *cbiG*, specific to *B. megaterium*. PCR primers were designed using Primer3plus (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) (Table S1). Primers were *in silico* validated at http://www.basic.northwestern.edu/biotools/oligocalc.html and used.

2.3.3. Selection of loci, their amplification and sequencing

Whole genome sequences of *B. megaterium* strains DSM319, QM B1551 and WSH002 available in GenBank (NCBI) were referred for designing PCR primers for housekeeping genes such as *dnaB* and *gyrA*. The details of the housekeeping genes are present in the Supplementary data (Table S1). PCR primers were designed and validated as mentioned above. Amplification of targeted DNA was carried out in 50 μl reaction volumes containing final concentrations of GoTaq Buffer-1X; $MgCl_2$ – 1.5 mmol l^{-1}; dNTPs – 200 μmol l^{-1}; Forward/Reverse Primers (Table S1) – 10 pmol each; DMSO – 6%; Taq polymerase – 1 Unit; DNA 200 ng (Promega Corporation, Madison, USA). PCR amplification was performed at initial denaturation at 95 °C/5 min; 35 cycles of denaturation at 95 °C/1 min; annealing for 1 min (Annealing temperature for each gene is given in Table S1) and extension at 72 °C/1 min followed by final extension at 72 °C/10 min. Amplified PCR products were purified by using SV gel and PCR clean up system (Promega Corporation, USA). The cycle sequencing reaction was performed with 20–30 ng of purified amplicon using ABI PRISM Big Dye Terminators v1.1 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 sequence. 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. Gene sequences were annotated with the help of EXPasy server (http://web.expasy.org/translate/) and blastP analysis was done to confirm the accuracy (www.ncbi.nlm.nih.gov/BLAST). Multiple alignments were made with the help of CLC bio sequence viewer (Version 6). All sequences were submitted to GenBank and accession numbers were assigned (Table S3). Sequences for two loci *dnaB* and *gyrA* were concatenated to a total length of 1626 bp and a phylogenetic tree was constructed in MEGA 6 (Molecular Evolutionary Genetic Analysis) (Tamura et al., 2013). The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura–Nei model (Tamura and Nei, 1993).

2.4. Endophytic colonization of BmBP17 in plants

2.4.1. Introduction of erythromycin resistance marker by genetic transformation

For precise quantification of endophytic population of BmBP17, dual antibiotic resistance of BmBP17RE was used. Briefly, erythromycin resistance marker gene was introduced in spontaneous rifamycin resistant BmBP17R, using plasmid pMUTIN-GFP+ having gene for erythromycin resistance as well as inducible gfp-gene (Biedendieck et al., 2006). BmBP17R was transformed by protoplast transformation protocol with modifications (Biedendieck et al., 2006) (Supplementary protocol 1). Transformants were PCR confirmed using pMUTIN-GFP gene specific primers (Table S1) that were designed from gfp gene sequences for amplification of 359 bp. PCR was performed in GoTaq PCR kit of Promega (GoTaq Buffer-1X, $MgCl_2$ – 1.5 mmol l^{-1}, dNTPs – 200 μmol l^{-1}, Forward/Reverse primers – 10 pmol each, DMSO – 6%, Taq polymerase – 1 Unit) at an initial denaturation at 95 °C/5 min, 35 cycles of denaturation at 95 °C/1 min, annealing at 56 °C/1 min and extension at 72 °C/1 min followed by final extension at 72 °C/7 min. PCR amplicon was visualized in agarose gel electrophoresis. Erythromycin resistant transformant was designated as BmBP17RE and used in colonization assays.

2.4.2. Black pepper and ginger

Roots of two-leaf stage black pepper rooted cuttings (cv. IISR-Sreekara) and two-month old ginger plantlets (cv. IISR-Mahima) were treated by placing in bacterial cell suspension (~ × 10^8 cfu ml^{-1}) for 30, 60 and 120 min. 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 BmBP17RE was estimated using plate count method (Sheoran et al., 2015).

2.4.3. Arabidopsis

Seeds of *Arabidopsis thaliana* ecotype Columbia-0 (ABRC, Ohio State University) were surface sterilized using 70% ethanol and 1% NaOCl followed by washing five times in sterile distilled water. These sterilized seeds were placed on half strength Murashige and Skoog medium (MS, HiMedia, India) (Murashige and Skoog, 1962) and decimal dilutions of BmBP17RE cell suspension (10 µl per seed) were spot inoculated on seeds. Bacterial suspensions were prepared in water and dilutions were prepared so as to obtain 10^{10}, 10^{9}, 10^{8} and 10^{7} cells ml^{-1} and 10 µl of suspension were used for seed inoculation (Sheoran et al., 2015). Five seeds were arranged in line on the surface of the medium and incubated for 48 h at 4 °C. Seeds transferred on a growth chamber were allowed to germinate and grow at 22/20 °C (day/night) temperature. Sampling was done on 21 dpi and endogenous population size of BmBP17RE was estimated using plate count method (Hallmann et al., 2000).

2.5. Estimation of epiphytic and endophytic bacterial population in plants

2.5.1. Plate assay

Upon harvest, inoculated plants were aseptically partitioned into roots, shoots and leaves. For determination of population of BmBP17RE on rhizoplane, 1 g of roots were agitated in bottles containing 100 ml of Phosphate Buffered Saline (PBS, HiMedia, India) [PBS – g l^{-1}, NaCl 8; KCl 0.2; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH-7.4] at 200 rpm for 30 min. Each sample was serially diluted up to 10^{-6} and plated on LBA amended with rifampicin (50 µg ml^{-1}) and erythromycin (1 µg ml^{-1}). Colonies were counted after incubation at 28 °C for 48 h. To estimate endogenous population, 1 g of tissue was surface sterilized with sodium hypochlorite (0.5%) + tween 20 (0.01%) for 20 min, ethyl alcohol (70%) for one min followed by rinsing with sterile distilled water for two to three times. Each sample was ground aseptically with 2 ml of PBS 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 erythromycin (1 µg ml^{-1}), and incubated at 28 °C for 48 h. Colonies were counted and expressed as cfu g^{-1} of fresh tissue. For *A. thaliana*, the whole plantlet harvested on 21 dpi was ground with PBS, serially diluted up to 10^{-5} and plated for estimation of endogenous population as described above (Hallmann et al., 2000).

2.5.2. Bio-PCR assay

For detection of endogenous BmBP17RE in *Arabidopsis* plants bio-PCR was performed. 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 ml of distilled water for approximately 1 min. The homogenates were vortexed for 5 s and 500 µl inoculated into LB broth amended with rifampicin (50 µg ml^{-1}) and erythromycin (1 µg ml^{-1}), and the broth was incubated at 37 °C for 48 h. Two µl of this broth culture was used as a template for PCR confirmation using BmBP17 specific primers (Table S1).

2.5.3. Scanning electron microscopy (SEM)

For localization of bacterium on surface of plant roots, whole roots from *A. thaliana* Columbia-0 seedlings were subjected to SEM (Zeiss, Germany) as per the protocol of Fischer et al. (2012).

2.6. Bioassay for antagonistic activity of BmBP17 on plant pathogens

In order to determine the effects of the BmBP17 on plant pathogens, dual-culture confrontation assays that test the effect of bacterial volatiles on pathogen growth and development were performed (Fernando et al., 2005). BmBP17 was tested against *P. capsici, Pythium myriotylum, Athelia rolfsii, Gibberella moniliformis, Colletotrichum gloeosporioides, Rhizoctonia solani, Magnaporthe oryzae, Ralstonia solanacearum, Xanthomonas axonopodis* pv. *punicae* and *R. similis*. For fungal/oomycetes pathogens a mycelial disk from three-day-old potato dextrose agar (PDA; HiMedia, India) culture was placed in the middle of a PDA plate, for bacteria 10 µl of 10^{9} cfu ml^{-1} was spot inoculated in the middle of LBA plate and for *R. similis*, approximately 25 nematodes were inoculated on water agar plate. Similarly 200 µl of 10^{9} cfu ml^{-1} mid-log phase BmBP17 culture was spread on Tryptic Soy Agar (TSA, HiMedia, India) in another Petri plate. 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 bacterial volatiles released during the growth of BmBP17. This set up ensured that no volatiles escaped during the course of experimentation. The plates were incubated at 28 °C for 7 days. The experiment was performed in three replications and was repeated three times. The same bioassay was also performed in M9 minimal medium with glucose as the sole carbon source (Kuzma et al., 1995). Plates without bacterial inoculum were the blank treatment.

2.7. Identification of bacterial volatiles

2.7.1. Solvent extraction (SE) of BmBP17

Compounds were extracted from overnight broth culture (100 ml) of BmBP17 grown at 28 °C. The culture was serially extracted with hexane and dichloromethane as suggested by Gil-Chavez et al. (2013). The fractions were condensed using a flash rotary vacuum evaporator at 28 °C (Buchi, Switzerland). Dried fraction dissolved in hexane or dichloromethane was injected to GC/MS having a thermal desorption system (Shimadzu QP-2010 TD 20, Japan). An 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: 250.00 °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: 10, ion source temperature: 230.00 °C, interface temp: 260 °C with scan m/z starts from 40.00 and end at m/z: 650.00.

2.7.2. Dynamic head space sampling of bacterial volatiles

The volatiles released from BmBP17 in broth culture were assessed by dynamic head space sampling method at room temperature. Volatiles from the culture were trapped over a 6 h time period in 30 mg Propak Q absorbent (50–80 mesh, Supelco) contained in a glass tube. Volatiles from uninoculated TSB served as a technical control and compounds from this run were subtracted from the sample run to enumerate the MVoCs from the bacterial culture. 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 7890 A, MS 5975 C). All 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 (National Institute of Standards and Technology, Gaithersburg, MD, USA).

2.8. Assay of antimicrobial activity of MVoCs identified in BmBP17

The GC–MS identified volatile chemicals such as Pyrazine, 2,5-dimethyl-; Pyrazine, ethyl-; Pyrazine, methyl-; and Pyrazine, 2-ethyl-3-methyl which were tested on pathogens representing oomycetes – *P. capsici*; fungus – *M. oryzae*; bacterium – *R. solanacearum* and nematode – *R. similis*. Chemically synthesized molecules procured from commercial sources were used in the assay (Sigma–Aldrich, USA) (Fernando et al., 2005). Each compound was tested at a range of concentrations i.e. 21, 42, 84, 168, 336, 504 and 672 μg ml^{-1} by soaking sterile filter paper disks with chemical placed on TSA (HiMedia, India). The pathogens were placed or streaked onto PDA or LBA (HiMedia, India) plates and for *R. similis*, approximately 25 nematodes were exposed to chemicals on a water agar (HiMedia, India) 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 volatile compounds and no volatiles escaped. The plates were incubated at 28 °C. After 3 days, (a) diameter of the fungal colony was measured and per cent inhibition over control was calculated; (b) growth of the bacterial colony was monitored and recorded; (c) the number of live nematodes were counted and per cent inhibition over control was calculated. The experiment was performed in three replications and was repeated three times. To calculate per cent inhibition in case of *R. solanacearum* a broth experiment was also performed. Two days old *R. solanacearum* cell suspension was inoculated in 1 ml CPG broth [Casamino acid 1; Peptone 10; glucose 5 g l^{-1}] in microfuge tube (2 ml) with various concentrations (21, 42, 84, 168, 336, 504, 672 μg ml^{-1}) of MVoCs. CPG broth inoculated with bacteria, but devoid of MVoC served as mock. Tubes were sealed with parafilm and incubated at 28 °C with constant shaking of 150 rpm. After 72 h, turbidity was measured at 600 nm in a spectrophotometer (Biophotometer plus, Eppendorf, Germany) and per cent inhibition over control was calculated.

2.9. Statistical analysis

Analysis of variance (ANOVA) of data was done using the online statistical software OPSTSAT (hau.ernet.in/about/opstat.php) and appropriate normalized data sets were used for statistical analysis. Whereas angular transformation was adopted for data on per cent inhibition, logarithmic transformation was adopted for colony count data. Means were compared using Critical Difference (CD) at $P = 0.05$.

3. Results

3.1. Identification and genotyping of BmBP17

The bacterium BmBP17 is Gram positive and positive for starch hydrolysis, protease production, siderophore production and gelatin liquefaction. Identity of BmBP17 was further confirmed by Biolog phenotypic fingerprinting which showed a similarity coefficient of 99.9% with *B. megaterium* (Table S2). Various other phenotypic features of BmBP17 are furnished in Table 1. Transmission electron microscopic image of BMBP17 cell is presented in Fig. S1. 16S rDNA sequence and sequences of cobalamine biosynthetic genes further confirmed its identity as *B. megaterium*. Phylogenetic analysis with 1626 bp concatenated sequence clearly revealed that BmBP17 was genetically similar to *B. megaterium* WSH002 and is divergent from clinical strains such as *Bacillus cereus* and *Bacillus anthracis* (Fig. S2). Sequences of the *cbiD, cbiG, dnaB* and *gyrA* obtained from BmBP17 were curated and submitted to GenBank with accession numbers (Table S3).

Table 1
Phenotypic characteristics of *Bacillus megaterium* BmBP17.

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	28–55 °C (best at 37 °C)
Biolog^{R} analysis (95 biochemical tests)	Closest match to *B. megaterium* (99.9% similarity)
16S rDNA analysis	Closest match to *B. megaterium* (99% similarity)
Swarming motility on soft KB medium	+
Casein hydrolysis	+
Starch hydrolysis	+
Methyl red	–
Citrate, indole	–
Antibiotic resistance	–
Antibiotic sensitivity	No growth occurred at cephotaxime (30 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), ciprofloxacin (30 μg ml^{-1}), colistin (25 μg ml^{-1}), doxycycline hydrochloride (30 μg ml^{-1}), nalidixic acid (30 μg ml^{-1}), novobiocin (μg ml^{-1}), oxacillin (μg ml^{-1}), piperacillin (100 μg ml^{-1}), polymyxin B (100 units), cephalothin (μg ml^{-1}), kanamycin (μg ml^{-1}), gentamycin (20 μg ml^{-1}), tetracycline (40 μg ml^{-1}), ampicillin (30 μg ml^{-1}), trimethoprim (30 μg ml^{-1}), amoxicillin (30 μg ml^{-1}), cephalexin (30 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 *R. similis*
Secondary metabolites and enzymes	
HCN	+
Protease	+
Cellulase	+
Catalase	+
Oxidase	–
Vitamin B12	+ (Genes participating in cobalamine biosynthesis such as cbiD and cbiG could be found)
Plant growth promotion-related features	
Nitrogen, ammonia production	+
Indole acetic acid (IAA)	+
Siderophore production	+

+ Positive for production; – negative for production.

3.2. Epiphytic and endophytic colonization of BmBP17 in plants

Transformation of BmBP17RE was PCR confirmed in pMUTIN-GFP specific PCR assay (Fig. S3). Transformed BmBP17RE displayed resistance against erythromycin and this antibiotic resistance was used as a marker for selective isolation and enumeration of BmBP17RE in black pepper and ginger roots as well as whole *A. thaliana* plantlets. In rooted cuttings of black pepper, BmBP17RE displayed a spatial pattern for endophytic colonization. The population densities were higher in rhizoplane (5 $\log_{10}$ cfu g^{-1}) than in root interior (1–3 $\log_{10}$ cfu g^{-1}) (Table 2). The bacterium was not detected in stem and other above ground plant tissue. Similar observations were made for ginger where the bacterium attained population size of 3–4 $\log_{10}$ cfu g^{-1} in rhizoplane and 2.5–2.8 $\log_{10}$ cfu g^{-1} in root interior (Table 3). No bacterial colonies could be isolated from stem and leaf tissues. The endogenous population of BmBP17 was found stabilized in both black pepper and ginger after 4 weeks at 1–2 $\log_{10}$ cfu g^{-1}. The bacterium could endophytically colonize the plantlets of *Arabidopsis* where

Table 2
Endogenous and rhizoplane (epiphytic) population of *Bacillus megaterium* BP17 in black pepper.

Duration of treatment		Number of colonies per gram of tissue (log 10 cfu g^{-1})				Mean	Mean (duration of treatment)
		7 dpi	14 dpi	21 dpi	28 dpi		
30 min	Rhizoplane	5.409	5.902	5.108	5.351	5.44	3.96[b]
	Root	3.008	3.451	1.15	2.259	2.47	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
60 min	Rhizoplane	5.451	5.882	5.295	5.461	5.52	4.07[a]
	Root	3.122	3.616	1.301	1.409	2.61	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
120 min	Rhizoplane	5.476	5.795	5.278	5.464	5.50	4.10[a]
	Root	3.056	3.529	1.87	2.36	2.70	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
Control	Rhizoplane	0	0	0	0	0	0[c]
	Root	0	0	0	0	0	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
Mean (days)		3.19[b]	3.52[a]	2.50[d]	2.91[c]		
CD ($P = 0.05\%$)	0.125						
CV (%)	2.036						

Table 3
Endogenous and rhizoplane (epiphytic) population of *Bacillus megaterium* BP17 in ginger.

Duration of treatment		Number of colonies per gram of tissue (log 10 cfu g^{-1})				Mean	Mean (duration of treatment)
		7 dpi	14 dpi	21 dpi	28 dpi		
30 min	Rhizoplane	3.922	4.079	4.088	4.178	4.06	1.67[a]
	Root	2.562	2.833	2.561	2.488	2.61	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
60 min	Rhizoplane	4.096	4.154	4.016	4.252	4.13	1.72[a]
	Root	2.835	2.883	2.789	2.495	2.75	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
120 min	Rhizoplane	4.112	4.203	4.13	4.19	4.15	1.69[a]
	Root	2.865	2.88	2.5	2.596	2.62	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
Control	Rhizoplane	0	0	0	0	0	0[b]
	Root	0	0	0	0	0	
	Stem	0	0	0	0	0	
	Leaf	0	0	0	0	0	
Mean (days)		1.27[ab]	1.31[a]	1.23[b]	1.26[ab]		
CD ($P = 0.05\%$)	0.65						
CV (%)	1.997						

colonization pattern was typically density dependent. Plantlets treated with high bacterial titer (10 OD units) recorded more bacterial population of 6 log units per gram of tissue than the plantlets treated with low bacterial titer (Fig. 1A). Bio-PCR using BmBP17RE specific primers on plant extract obtained from surface disinfected plant yielded BmBP17RE specific amplicon of 862 bp (Fig. 1B). The bacterium could be observed on the *Arabidopsis* root surface in SEM imaging (Fig. 1C).

3.3. Bioassay for antagonistic activity of BmBP17 on plant pathogens

The data on pathogen inhibition revealed that the volatiles released during the growth of BmBP17 on TSA medium inhibited a broad range of pathogens. *M. oryzae*, *R. solanacearum* and *X. axonopodis* pv. *punicae* were completely inhibited whereas *A. rolfsii*, *C. gloeosporioides* and *R. solani* were inhibited at 63.2%, 56.8% and 47.1%, respectively. Others were inhibited in the range of 19–32% (Fig. 2 and Table 4). Identical observation was made on M9 minimal medium (Data not shown).

3.4. Profiling of MVoCs in solvent extracts of BmBP17

Having observed significant antimicrobial activities of volatiles on broad range of pathogens, the metabolites were extracted by adopting standard chemical extraction procedures and subjected to chemical fractionation in GC/MS. A total of 100 compounds were detected in hexane (63 compounds) and dichloromethane (37 compounds) extracts (Figs. S4 and S5). Chemicals detected in hexane extract included hydrocarbons (combined peak area 70.55%), sulfoxides (combined peak area 8.84%), alcohols (combined peak area 3.61%), acids (combined peak area 2.44%), esters (combined peak area 1.37%) and ketones (combined peak area 0.58%). Heterocyclics (combined peak area 62.68%), hydrocarbons (combined peak area 17.63%), esters (combined peak area 12.94%), sulfoxides

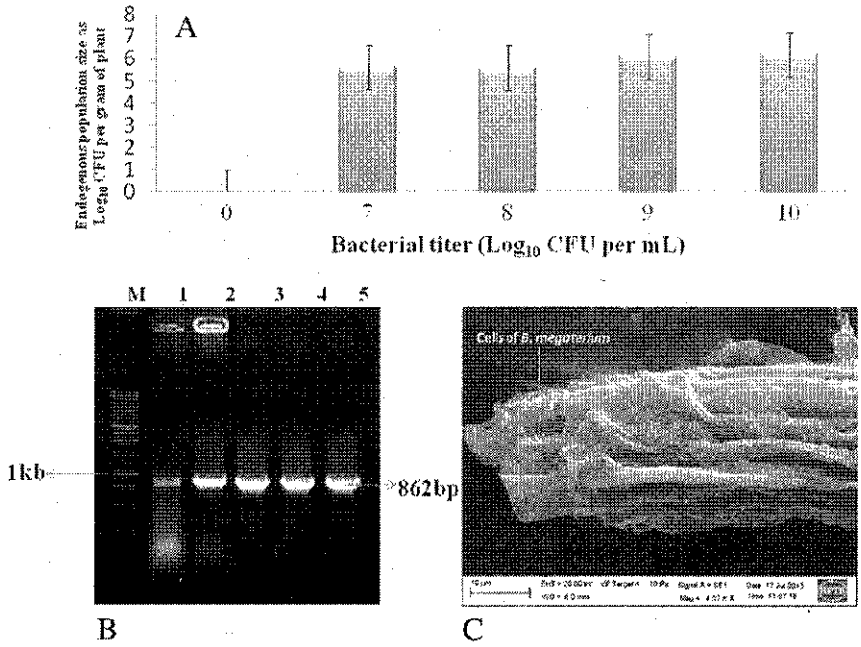

Fig. 1. (A) Endogenous population size of *Bacillus megaterium* BP17RE in *Arabidopsis* Col-0 seedling on 21 dpi; whole plantlet of *Arabidopsis* was used in the experiment. Experiments were performed twice in triplicates. Error bars indicate mean values with SEM. (B) Bio-PCR confirmation of *B. megaterium* BP17RE in *Arabidopsis*; Lane M: 1 kb ladder; Lane 1: *Arabidopsis* inoculated with 10^7 cfu ml^{-1} of bacterium; Lane 2: *Arabidopsis* inoculated with 10^8 cfu ml^{-1} of bacterium; Lane 3: *Arabidopsis* inoculated with 10^9 cfu ml^{-1} of bacterium; Lane 4: *Arabidopsis* inoculated with 10^{10} cfu ml^{-1} of bacterium; Lane 5: Positive control-DNA template from BmBP17RE. (C) Localization of *B. megaterium* BP17RE on root surface of *Arabidopsis thaliana* Col-0 using SEM.

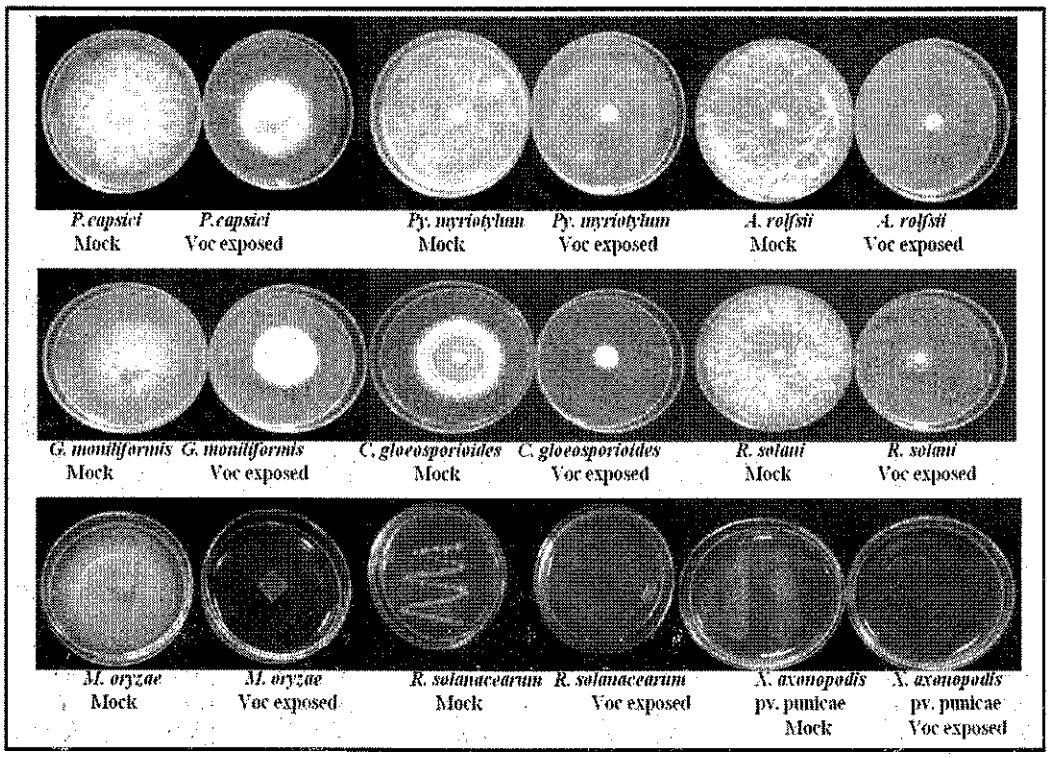

Fig. 2. Effect of volatiles of *Bacillus megaterium* BP17 on diverse plant pathogens.

(combined peak area 8.84%), alcohols (combined peak area 5.58%), ketones (combined peak area 0.58%) and acids (combined peak area 0.26%) were found in dichloromethane extract. Table 5 summarizes the patterns of chemical abundance. Further scrutiny of the peaks observed in hexane fraction revealed dominance of compounds such as Heneicosane with the combined peak area of over 27.83% that appeared with multiple retention times between 11.871 and 17.682 min, tricosane with the total peak area of 12.2% that appeared between 14.623 and 16.408 min and tetratetracontane with combined peak area of 10.95% with multiple retention

Table 4
Effect of volatiles of *Bacillus megaterium* BP17 on growth of diverse plant pathogens *in vitro*.

Pathogen	*Inhibition (%) over control
Phytophthora capsici	27.80 (32.66)
Pythium myriotylum	24.50 (28.47)
Athelia rolfsii	79.70 (63.23)
Gibberella moniliformis	27.00 (31.29)
Colletotrichum gloeosporioides	70.10 (56.82)
Rhizoctonia solani	58.80 (47.18)
Magnaporthe oryzae	100.00 (90.00)
Ralstonia solanacearum	100.00 (90.00)
Xanthomonas axonopodis pv *punicae*	100.00 (90.00)
Radopholus similis	16.15 (19.14)
C.D.	0.771
SE(m)	0.238
SE(d)	0.336
C.V.	0.688

Data in the parentheses are angular transformed values.
* $\frac{\text{Pathogen growth in mock} - \text{Pathogen growth in MVoC exposed plate}}{\text{Pathogen growth in mock}} \times 100$.

Table 6
Head space GC/MS analysis of volatiles produced by *Bacillus megaterium* BmBP17.

Name of the compound		BP17 R (Peak area %)
1.	Pyrazine, 2,5-dimethyl-	16.932
2.	Isoamyl alcohol	12.382
3.	Pyrazine, ethyl-	9.206
4.	Pyrazine, methyl-	8.069
5.	Sulfurous acid, butyl cyclohexylmethyl ester	5.076
6.	Pyrazine, 2-ethyl-3-methyl-	4.927
7.	Heptamethyl-1-nonene	4.394
8.	Dodecane	4.192
9.	Tridecane	3.500
10.	Heptamethyl-1-nonene	2.701
11.	Tetradecane	2.641
12.	Heptamethyl-2-nonene	2.545
13.	Pentadec-7-ene, 7-bromomethyl-	1.953
14.	β-Naphthol	1.922
15.	Ethylhexanol	1.237
16.	n-Nonadecanol-1	1.220
17.	2-Undecanethiol, 2-methyl-	1.140
18.	Tetradecane, 2,6,10-trimethyl	1.093
19.	1-Hexadecanol, 2-methyl-	0.999
20.	Cyclohexane, 1-methyl-2-pentyl-	0.816

times between 18.482 and 23.818 min (Table S4). As for dichloromethane fraction predominant compounds identified were Pyrrolo [1,2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) with a combined peak area of 46.33% appeared between 11.996 and 14.922 min, Pyrrolo [1,2-a] pyrazine-1, 4-dione, hexahydro-3-(phenyl methyl) with peak area of 13.41% appeared at 15.551 min, 1-Nonadecene with peak area of 6.47% appeared at 12.918 min, 1-Heneicosanol with a peak area of 4.76% appeared at 14.170 min, Octadecyl trifluoroacetate with a peak area of 5.99% appeared at 15.315 min and 1-Heptadecene with peak area of 4.41% appeared at 11.532 min (Table S5). Unlike solvent extraction method, the dynamic head space sampling of bacterial culture revealed presence of several specific aromatic compounds such as Pyrazine, 2,5-dimethyl; Isoamyl alcohol; Pyrazine, ethyl; Pyrazine, methyl; Sulfurous acid, butyl cyclohexylmethyl ester; Pyrazine, 2-ethyl-3-methyl; Heptamethyl-1-nonene; Dodecane and tridecane in the volatiles (Table 6; Fig. S6).

3.5. Assay of antimicrobial activity of MVoCs identified in BmBP17

In general all pyrazine derivatives were inhibitory to *P. capsici*, *M. oryzae* and *R. solanacearum* where the growth inhibition was found to increase with increasing concentration of compounds. Among them pyrazine, 2-ethyl-3-methyl was significantly more inhibitory at 168 μg ml^{-1} than Pyrazine, 2-ethyl; Pyrazine, 2,5-dimethyl and Pyrazine, 2-methyl (Table 7–9). Complete inhibition was observed with Pyrazine, 2-ethyl-3-methyl at 504 μg ml^{-1} and 672 μg ml^{-1} for *P. capsici* (Fig. S7, Table 7) and *R. solanacearum* (Fig. S8, Table 8), respectively and at 168 μg ml^{-1} for *M. oryzae* (Fig. S9, Table 9). The compounds were less inhibitory to *R. similis* as compared to other pathogens (Data not shown).

4. Discussion

Endophytic microorganisms are known to colonize the living tissues of the host plants and participate in a variety of mutualistic relationships where they help the plants by producing a plethora of substances that ultimately confer survival advantage to the plants (Strobel and Daisy, 2003). Deployment of biocontrol technology based on "safe microorganisms" without any clinical affiliations is emphasized in recent times, as species that are not known to be pathogens are far more likely to be commercially viable (Kumar et al., 2013). The identity of root endophytic bacterium BP17 was confirmed as *B. megaterium* (BmBP17) in Biolog-based identification system as well as 16S rRNA and cobalamine biosynthetic gene sequences. In order to establish the strain identity and to determine its genetic relationship with other clinical strains of *Bacillus*, genetic characterization based on housekeeping genes such as *dnaB* and *gyrA* was adopted. *B. megaterium* BP17 is genetically close to free living *B. megaterium* WSH002 and not related to clinically significant *Bacillus* spp. (Eppinger et al., 2011; Liming et al., 2011). Genes for cobalamine biosynthesis were found in endophyte BmBP17 which broaden its scope for industrial use for vitamin B12 production.

Table 5
Significant chemicals identified in dichloromethane and hexane extracts of *Bacillus megaterium* BP17R using GC/MS.

Name	Combined peak area (%)			
	Hexane	Significant chemical identified	Dichloro methane	Significant chemical identified
1. Hydrocarbons	70.55	Several	17.63	Several
2. Sulfoxides	8.84	Diphenyl sulfoxides	0.00	–
3. Alcohols	3.63	Dronabinol; Arcidinol	5.58	Heneicosanol; tetra methyl hexadecanol; trimethyl butyl octanol
4. Acids	2.44	1,2 benzene dicarboxylic acid; benzene dicarboxylic acid; tetrahydroabietic acid	0.26	Tricycle decane carboxylic acid
5. Esters	1.37	Nonadecyl acetate; dodecyl ester; hexadecanoic acid, methyl ester	12.94	Octadecyl trifluoroacetate; benzene carboxylic acid ditridecyl ester; heneicosyl heptafluorobutyrate; tetratriacontyl heptafluorobutyrate; dotriacontyl heptafluorobutyrate
6. Ketones	0.58	Eicosanone 1,3 diphenyl heptanone	0.58	–
7. Heterocyclics	0.00	–	62.68	Pyrrollo pyrazine derv. oxaspiro derv.

Table 7
Anti-oomycetes activities of MVoCs identified in *Bacillus megaterium* BP17 on *Phytophthora capsici*.

Concentration (μg ml^{-1})	*Mycelial inhibition (%) over control			
	2,5-Dimethyl pyrazine	2-Ethyl-3-methyl pyrazine	2-Ethyl pyrazine	2-Methyl pyrazine
21	26.32 (30.89)	15.63 (27.62)	6.25 (18.38)	12.50 (20.07)
42	36.84 (36.84)	40.63 (40.26)	15.63(23.34)	12.50 (21.16)
84	50.00 (44.67)	65.63 (53.32)	37.50 (37.04)	25.00 (30.71)
168	60.53 (50.77)	100.00 (90.00)	43.75 (41.17)	37.50 (37.59)
336	84.21 (67.31)	100.00 (90.00)	71.88 (58.09)	62.50 (53.34)
504	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	78.13 (62.24)
672	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)	100 (90.00)
C.D.	2.02	0.98	2.01	1.86
SE(m)	0.67	0.32	0.66	0.62
SE(d)	0.95	0.46	0.94	0.87
C.V.	2.26	0.93	2.57	2.71

Data in the parentheses are angular transformed values.
* $\frac{\text{Mycelial growth in mock} - \text{Mycelia growth in MVoC exposed } \textit{Phytopthora capsici}}{\text{Mycelial growth in mock}} \times 100$.

Table 8
Anti-bacterial activities of MVoCs identified in *Bacillus megaterium* BP17 on *Ralstonia solanacearum*.

Concentration (μg ml^{-1})	*Growth inhibition (%) over control			
	2,5-Dimethyl pyrazine	2-Ethyl-3-methyl pyrazine	2-Ethyl pyrazine	2-Methyl pyrazine
21	6.41 (14.64)	21.59 (27.66)	11.21 (18.95)	10.80 (19.16)
42	7.45 (15.80)	29.81 (33.08)	14.53 (22.07)	13.91 (21.65)
84	16.78 (23.51)	48.15 (43.92)	19.77 (26.17)	15.64 (23.12)
168	22.59 (28.24)	56.48 (48.70)	23.97 (29.23)	20.93 (27.19)
336	30.83 (33.69)	72.89 (58.61)	31.83 (34.31)	22.93 (28.54)
504	69.75 (56.74)	91.65 (73.28)	40.89 (39.71)	25.82 (30.51)
672	79.87 (63.54)	95.90 (78.29)	55.12 (47.92)	31.54 (34.14)
C.D.	7.214	2.13	6.708	4.331
SE(m)	2.386	0.704	2.218	1.432
SE(d)	3.374	0.996	3.137	2.026
C.V.	13.995	2.684	14.075	10.767

Data in the parentheses are angular transformed values.
* $\frac{\text{Turbidity } (OD_{600nm}) \text{ in mock} - \text{Turbidity } (OD_{600nm}) \text{ in MVoC exposed } \textit{Ralstonia solanacearum}}{\text{Turbidity } (OD_{600nm}) \text{ in mock}} \times 100$.

Table 9
Anti-fungal activities of MVoCs identified in *Bacillus megaterium* BP17 on *Magnaporthe oryzae*.

Concentration (μg ml^{-1})	*Mycelial inhibition (%) over control			
	2,5-Dimethyl pyrazine	2-Ethyl-3-methyl pyrazine	2-Ethyl pyrazine	2-Methyl pyrazine
21	32.75 (34.24)	44.44 (41.91)	3.19 (10.30)	3.19 (11.89)
42	36.84 (36.90)	54.39 (46.58)	7.35 (18.17)	5.43 (17.56)
84	46.49 (41.58)	58.19 (51.49)	15.97 (22.92)	18.21 (25.92)
168	58.19 (50.53)	100.00 (90.00)	26.52 (30.18)	26.52 (31.06)
336	63.16 (52.75)	100.00 (90.00)	60.70 (52.65)	42.49 (46.04)
504	67.84 (54.96)	100.00 (90.00)	67.09 (59.18)	64.86 (53.64)
672	73.68 (58.93)	100.00 (90.00)	100.00 (90.00)	100.00 (90.00)
C.D.	2.46	2.14	2.45	1.90
SE(m)	0.81	0.71	0.81	0.63
SE(d)	1.15	1.00	1.15	0.89
C.V.	3.42	1.96	3.97	3.16

Data in the parentheses are angular transformed values.
* $\frac{\text{Mycelial growth in mock} - \text{Mycelia growth in MVoC exposed } \textit{Magnaporthe oryzae}}{\text{Mycelial growth in mock}} \times 100$.

BmBP17 was an endophytic colonist of black pepper, ginger roots and model plant, *Arabidopsis* when introduced to these plants. The endophytic assays clearly indicated that BmBP17 specifically colonized roots of black pepper and ginger, but not the stem or other aerial plant parts. The unique root endophytic colonizing ability of BmBP17 might be traced to its root origin as Aravind et al. (2009) originally isolated BmBP17 from endophytic root tissues of black pepper. BmBP17 attained endophytic population size of 1–3 log cfu g^{-1} of root tissue which suggested a regulated endogenous multiplication. BmBP17 could sustain its population in the interiors of plantlets even 4 weeks after release. *Arabidopsis* plantlets supported higher population levels than black pepper and ginger roots. The versatility of BmBP17 for colonizing diverse plant species clearly revealed its adaptability in root tissue, a trait essential for sustained biological control against soil borne root infecting pathogens. The ability of BmBP17 to endophytically colonize diverse plant species can be exploited in sustainable biocontrol systems, especially in producing disease free planting

material of black pepper and ginger. Aravind et al. (2012) reported *B. megaterium* based pre-planting bacterization technology for production of healthy black pepper rooted cuttings. Other reports of plant endophytic *B. megaterium* are from cotton, ginger and ginseng (McInroy and Kloepper, 1995; Vendan et al., 2010; Chen et al., 2014).

Endophytic BmBP17 suppressed a wide range of plant pathogens belonging to diverse taxa. Among fungi, *A. rolfsii, G. moniliformis, C. gloeosporioides, R. solani* and *M. oryzae* were significantly inhibited. Bacterial pathogens *R. solanacearum* and *X. axonopodis* pv. *punicae* were also completely inhibited by BmBP17. The data on pathogen suppression by BmBP17 clearly suggests that the root associated bacterium is not only a broad spectrum endophyte but also a broad spectrum antagonist as well. The strong antagonistic activity of BmBP17 could be attributed to its organic volatile compounds as non-volatile metabolites did not show any antimicrobial activity (Data not shown). *Bacillus* species are known to release diverse MVoCs belonging to ketones, alcohols, aldehydes, pyrazines, acids, esters, pyridines and benzene with ketones and alcohols as the most dominant ones (Li et al., 2015). MVoCs have long been considered important signals involved in plant growth promotion; pathogen suppression and induced systemic resistance against biotic stresses (Ryu et al., 2003; Ping and Boland, 2004).

Chemical profiling of solvent extracts of BmBP17 confirmed presence of compounds such as Heneicosane; tricosane; tetratetracontane; Pyrrolo [1,2-a] pyrazine-1, 4-dione; hexahydro-3-(2-methylpropyl); Pyrrolo [1,2-a] pyrazine-1, 4-dione, hexahydro-3-(phenylmethyl); 1-Nonadecene; 1-Heneicosanol; Octadecyl trifluoroacetate and 1-Heptadecene. Results of our study corroborates other reports on MVoC profiles of *B. megaterium* which is known to release wide variety of volatile compounds such as acetoin; Phenylethyne; acetic acid; Propanoic acid, 2-methyl; Butanoic acid, 2-methyl; Thymol; Tetradecanoic acid; Pentadecanoic acid; hexadecanoic acid; 9-hexadecanoic acid and octadecanoic acid (Lopez-Bucio et al., 2007).

Dynamic head space analysis of MVoCs of BmBP17 revealed presence of diverse pyrazine derivatives as major chemical compounds. Among them 2,5-dimethyl pyrazine was a major compound followed by ethyl pyrazine-; methyl pyrazine and 2-ethyl-3-methyl pyrazine. Pyrazine derivatives were reported from several other species of *Bacillus* such as *Bacillus subtilis, B. megaterium, Bacillus amyloliquefaciens* and *B. cereus* (Owens et al., 1997; Larroche et al., 1999; Adams and Kimpe, 2006). Particularly alkyl-pyrazine is reported as major flavour compound in the MVoCs of *B. megaterium* and *B. subtilis* (Owens et al., 1997; Larroche et al., 1999). Microorganisms are known to produce pyrazines during primary or secondary metabolism (Besson et al., 1997). Pyrazine, 2-ethyl-3-methyl-; Pyrazine, 2, 5-dimethyl-; Pyrazine, ethyl-; and Pyrazine, methyl identified in BmBP17 could suppress the pathogens when evaluated *in vitro*. *M. oryzae P. capsici*, and *R. solanacearum* were found completely inhibited by Pyrazine, 2-ethyl-3-methyl and partially by other pyrazine derivatives such as Pyrazine, 2,5-dimethyl-; Pyrazine, ethyl-; and Pyrazine, methyl. Antifungal activity of methyl pyrazine against *C. gloeosporioides* was reported (Zheng et al., 2013). Our data on chemical profile of BmBP17 along with other published data strongly suggest that *B. megaterium* produces diverse group of MVoCs that displayed broad spectrum antimicrobial activities against diverse plant pathogens including economically significant bacterial pathogens such as *R. solanacearum* and *X. axonopodis* pv. *punicae* causing bacterial wilt in solanaceous vegetables and leaf blight in pomegranate, respectively. The natural functions of pyrazine in microbes are still unclear.

Apart from pyrazines, other significant compounds found in MVoCs of BmBP17 were dodecane; tridecane and tetradecane which are found in other species of *Bacillus* such as *Bacillus simplex, B. subtilis, Bacillus weihenstephanensis* and *B. amyloliquefaciens* (Gu et al., 2007; Farag et al., 2013). While dodecane is reported as nematicidal chemical against *P. redivivus* and *Bursaphelenchus xylophilus*, tetradecane showed antifungal activity against *Fusarium oxysporum* (Gu et al., 2007; Yuan et al., 2012). Other interesting function of dodecane is its plant growth promoting ability (Farag et al., 2006). However, role of several other related MVoCs such as decane; undecane; undecane-2-one; tridecane; tridecane-2-one and tridecan-2-ol released from *B. subtilis* is not known (Farag et al., 2013).

Taken together it can be concluded that the plant endophytic BmBP17 is a potential biocontrol agent genetically similar to industrially important *B. megaterium* which broadens its scope beyond agriculture. Furthermore, BmBP17 is unique for its broad spectrum endophytic ability and wide antagonistic activity against several plant pathogens mediated by MVoCs. The broad spectrum endophytic and antagonistic capabilities are ideal for commercial exploitation not only as microbial formulation but also as a valuable source for untapped antimicrobial compounds such as Pyrazine, 2-ethyl-3-methyl-; Pyrazine, 2,5-dimethyl-; Pyrazine, ethyl and Pyrazine, methyl which can be exploited as new chemical agents for crop protection in future.

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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.biocontrol.2015.09.005.

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