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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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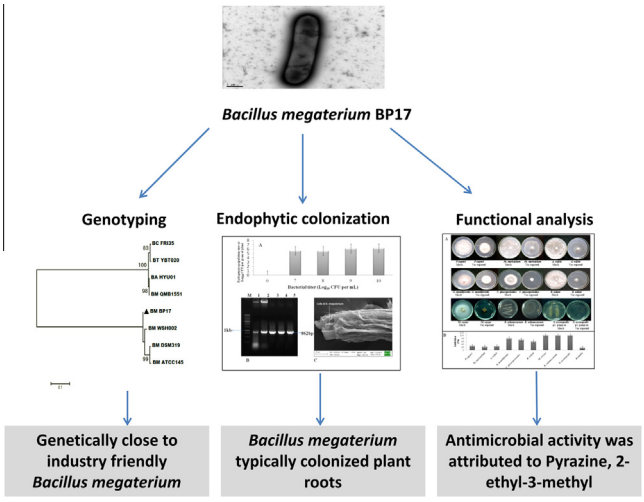
HIGHLIGHTS

- *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