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Native multi-trait rhizobacteria promote growth and suppress foot rot in black pepper

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Abstract

In this study, 74 PGPR isolates from different varieties of black pepper were characterized for morphological, biochemical and nutrient mobilization traits as well as inhibition of *Phytophthora capsici* (causing foot rot) under *in vitro* conditions. Based on the multiple traits, two PGPR [(*Micrococcus luteus* Schroeter (1872) Cohn 1872 (BRB3) and *Enterobacter aerogenes* Hormaeche and Edwards 1960 (AL) (BRB13)] were shortlisted for further growth promotion studies. For biocontrol studies, three PGPR [(*Burkholderia cepacia* (Palleroni & Holmes 1981) Yabuuchi *et al.* (1993) (BRB21), *Pseudomonas aeruginosa* Schröter (1872) Migula 1900 (BRB28) and *Serratia marcescens* Bizio 1823 (AL) (BRB49)] which showed >70% inhibition of *P. capsici* were shortlisted. The results from green house study on growth promotion indicated that application of *M. luteus* (BRB3) + 75% recommended dose (RD) of N + 100% RD of PK produced taller plants, longer roots, greater fresh biomass, more number of leaves and nodes in black pepper. This suggested a 25% reduction in chemical N fertilizer in the presence of *M. luteus* (BRB3). In the green house study on biocontrol, lowest foot rot (32.77%) and taller plants (332.0 cm) were observed with *B. cepacia* (BRB 21), which was on par with chemical treatment. This is the first report on the potential of PGPR like *M. luteus* for growth promotion and *B. cepacia* for management of *P. capsici* in black pepper.

Keywords: *Phytophthora capsici, Piper nigrum*, plant growth promoting rhizobacteria

Introduction

Black pepper (*Piper nigrum* L.) is an important commodity traded globally and the annual demand for this most important spice is between 200,000-225,000 Mg accounting for 20% of the world spice trade. Presently, a successful black pepper crop requires large amounts of chemical inputs that have an adverse effect on soil quality as well as the produce. Hence, efforts are on to devise methods that help in reducing chemical input use. In this context, plant growth promoting rhizobacteria (PGPR) have been found to be successful and have, therefore, been widely employed in many crops.

PGPR are free living bacteria that aggressively

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colonize and multiply on the roots (Adesemoye *et al*. 2009). The direct effects are through biofertilization, stimulation of root growth and rhizoremediation, while indirect effects are through reduction in disease incidence. Common PGPR include the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* (Lugtenberg & Kamilova 2009).

Several studies have shown that PGPR not only benefit crops (Kurabachew & Wydra 2013) but also improve the use efficiency of fertilizers and manures thus allowing reduced application rates (Adesemoye *et al*. 2009; Dinesh *et al.* 2013). While the beneficial effects of PGPR are widely reported, very little information exists on PGPR for black pepper. Our primary objective was to study the effects of new promising PGPR on black pepper growth promotion and biocontrol of *Phytophthora capsici* causing foot rot.

Materials and methods

Soil sampling

For isolation of PGPR, soils were collected from the rhizosphere of five different varieties of healthy black pepper vines grown in different geographical regions of Kerala state (India) and Karnataka state (India). The soils strongly adhering to the roots and within the space explored by the roots were considered as the rhizosphere soil (Garcia *et al*. 2005). Soil samples were collected from six randomly (between June to July) selected black pepper vines under each variety and immediately transferred to an ice box for transport. In the laboratory, the living plant material and coarse roots were removed prior to estimation of moisture content in the samples.

Isolation of PGPR

The samples were serially diluted upto 10^{-10}, pour plated on nutrient agar (NA) and incubated at 28°C for 2-3 days. The individual bacterial colonies, expressed as number of colony forming units (CFU) per gram of soil were then selected and subcultured on NA. The isolates thus obtained were cryopreserved at –80°C in 40% glycerol for further studies.

Morphological and biochemical characterization

All the isolates were studied for their morphological characters *viz*., cell form and size, gram staining, spore formation, motility, colony pigmentation and production of UV-fluorescent pigments. The biochemical traits studied were tests for methyl red, Voges Proskauer (VP), citrate, presence of oxidase and catalase, succinic acid, carbohydrate utilization patterns, NH_3 production, and growth at varying temperatures (28°C, 37°C, 41°C, 50°C and 60°C) and salt concentrations (1%, 2%, 5%, 7% and 10% NaCl) as described by Holt *et al*. (1994) and Tindall *et al*. (2007). Other traits like indole acetic acid (IAA) was determined by the method of Sawar & Kremer (1995), production of cell-wall-degrading enzymes (á-amylase, cellulase, pectinase and protease) and NH_3 by the methods described by Cappuccino & Sherman (1992), HCN by the method of Kloepper *et al.* (1991), P solubilization by the method of Gaur (1990), K solubilization as described by Hu *et al.* (2006) and Zn solubilization by the method of Venkatakrishnan *et al.* (2003).

In vitro *screening of rhizobacteria for biocontrol*

The rhizobacterial isolates were screened using dual plate culture (Berg *et al.* 2005) against *Phytophthora capsici*, causing foot rot. Briefly, a mycelial plug of actively growing *P. capsici* (isolated from black pepper) was placed in the centre of the carrot agar medium and the PGPR was streaked 2 cm away on either side of mycelial plug. Plates were then incubated at 28°C for 5 days or until the leading edge of the pathogen in the control plate reached the edge of the plate. The radial growth of mycelium was measured and inhibition was estimated using the formula, I= [C-T/C] × 100, where, I is the percent inhibition and C and T are the radial growth of the pathogen in control and treatment, respectively. Rhizobacterial isolates that showed > 70% inhibition of *P. capsici* were short-listed for green house studies.

Identification of bacterial isolates

The preliminary identification of the isolates was done using the Bergey's Manual of

Determinative Bacteriology (Holt *et al.* 1994) and identity of the short-listed isolates was confirmed using 16S rDNA sequence analysis and Biolog. The genomic DNA from the short listed PGPR were extracted using standard protocol (Sambrook & David 2000), which was sequenced and subjected to BLAST analysis and compared with registered sequences in the GenBank database using NCBI Blast server (http://www.ncbi.nlm.nih.gov). The short-listed strains were identified at species level using Biolog Microstation System (Version 5.1.1, RDG Laboratories, Hayward, California, USA) using GEN III software.

Green house evaluation on growth promotion

The recommended dose (RD) of NPK for black pepper is 140-55-270 kg ha^{-1}, respectively. The inorganic sources of NPK *viz*., urea, rock phosphate and muriate of potash, respectively were applied in two splits [45th and 90th day after planting (DAP)]. This fertilization regime was considered as 100% RD and each nutrient was reduced to 75% and was applied alone or in combination with the shortlisted PGPR [(*Micrococcus luteus* (BRB3) and *Enterobacter aerogenes* (BRB13)]. Earthen pots of 20 kg capacity were filled with 15 kg potting mixture (soil: sand: FYM, 2:1:1). The experiment was conducted in 2012 (June-October) in Randomized Block Design (RBD) with 15 treatments and six replications. The treatment details are as follows:

T1- Control; T2- BRB3 (*Micrococcus luteus*); T3- BRB13 (*Enterobacter aerogenes*); T4- 75% N + 100% PK; T5- 75% N + 100% PK + BRB3; T6- 75% N + 100% PK+ BRB13; T7- 100% N + 75% P + 100% K; T8- 100% N + 75% P + 100% K+ BRB3; T9- 100% N + 75% P + 100% K + BRB13; T10- 100% NP + 75% K; T11- 100% NP + 75% K + BRB3; T12- 100% NP + 75% K + BRB13; T13- 100% NPK; T14- 100% NPK + BRB3; T15- 100% NPK + BRB13

Uniform healthy black pepper were planted in the pots and the bacterial suspensions (10^8 CFU mL^{-1}) were drenched at the rate of 250 mL pot^{-1} at the time of planting and booster doses (10^8 CFU mL^{-1}; 250 mL pot^{-1}) were applied thrice at 30 days intervals up to 90 DAP. The plants were uprooted at 150 DAP for measuring various growth parameters.

Green house evaluation for biocontrol

A second green house experiment on biocontrol of *P. capsici* was carried out simulatenously using the shortlisted PGPR *viz*., *B. cepacia* (BRB21), *P. aeruginosa* (BRB28) and *S. marcescens* (BRB4) during 2012 (June- October). The treatments also included application of metalaxyl- mancozeb @ 1.25g L^{-1}. Also included were a pathogen only treatment (inoculated with *P. capsici*) and an absolute control. The experimental design was RBD with six replications

For challenge inoculation, *Phytophthora capsici* 06-04 strain (collected from Phytophthora Repository of IISR, Kozhikode, Kerala, India) was subcultured onto carrot agar plates and incubated for 72h. After incubation, 5mm discs were cut from the culture plate by using cork borer and kept in sterile distilled water for sporulation in a chamber under light. Ten sporulated discs were inoculated into each pot on 90 DAP. No such inoculation was done in the absolute control. For recording foot rot, the plants were destructively sampled 10 days after appearance of disease symptoms. Rot index was done on a 0-4 scale, where 0=no roots affected; 1=25% of roots affected; 2=50% of roots affected; 3=75% of roots affected and 4=>75% affected. The biocontrol efficiency (BE) was calculated using the formula:

BE = [(Disease incidence (DI) in pathogen inoculated treatment) – (DI in PGPR treatment) /(DI of pathogen inoculated treatment)] × 100%

Both the green house experiments were repeated twice. Since the trends were identical we report here only the results of the second experiment.

Statistics

The significance of treatment effects was determined by one-way ANOVA. Where the F values were significant, post hoc comparisons of means were made using the Least Significance Test (LSD) at $P < 0.05$.

Results and discussion

Isolation of PGPR and identification

A total of 74 PGPR were obtained from different varieties of black pepper (Table 1). The dominant genera (Fig. 1) were *Pseudomonas* spp (25 nos), *Bacillus* sp (11 nos) and *Arthrobacter* spp (7 nos). However, 6 isolates (BRB 27, 36, 37, 45, 62 and 68) could not be identified. These findings are comparable to earlier studies that have reported that the most representative genera are *Pseudomonas*, *Clostridium*, *Arthrobacter*, *Achromobacter*, *Micrococcus*, *Flavobacterium*, *Azospirillum*, *Azotobacter* and *Bacillus*, with the latter being the most common group of bacteria isolated from soil (Felici *et al.* 2008).

Table 1. PGPR isolated from different varieties of black pepper

Locations[a]	Name of Variety	Number of PGPR isolated	Genus
Site 1	IISR- Girimunda	7	*Pseudomonas* sp., *Bacillus* sp. & *Klebsiella* sp.
Site 1	IISR- Malabar Excel	6	*Pseudomonas* sp., *Bacillus* sp., *Enterobacter* sp. & *Micrococcus* sp.
Site 2	Panchami	6	*Pseudomonas* sp., *Bacillus* sp., *Arthrobacter* sp., *Serratia* sp. & *Micrococcus* sp.
Site 1	IISR- Shakthi	10	*Pseudomonas* sp., *Bacillus* sp., *Klebsiella* sp., *Arthrobacter* sp., *Enterobacter* sp., *Serratia* sp. & *Micrococcus* sp.
Site 2	Sreekara	11	*Pseudomonas* sp., *Bacillus* sp. *Klebsiella* sp., *Arthrobacter* sp., *Enterobacter* sp., *Serratia* sp. & *Micrococcus* sp.
Site 1	IISR- Thevam	5	*Pseudomonas* sp., *Bacillus* sp. *Klebsiella* sp., *Enterobacter* sp. & *Curtobacterium* sp.
Site 3	Panniyur- 1	18	*Pseudomonas* sp., *Bacillus* sp. *Burkholderia* sp., *Arthrobacter* sp., *Curtobacterium* sp., *Enterobacter* sp., *Serratia* sp. & *Micrococcus* sp.
Site 3	Panniyur- 2	5	*Pseudomonas* sp., *Bacillus* sp.& *Curtobacterium* sp.
	Total	68[b]	

[a]Site 1: Kozhikode, Kerala State, India; Site 2: Wayanad, Kerala State, India; Site 3: Coorg, Karnataka State, India; [b]6 isolates could not be identified

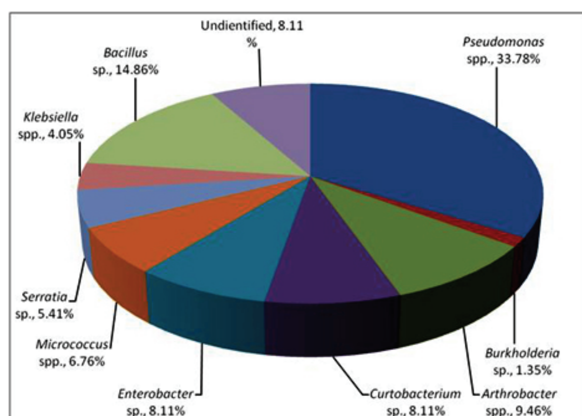

Fig. 1. Major genera of PGPR isolated from black pepper rhizosphere

Growth promotion traits

All the isolates were characterized for various traits including IAA and NH_3 production, hydrolytic enzyme production and nutrient solubilization. The results suggested that among the 74, two PGPR *viz*., *Micrococcus luteus*-BRB3 and *Enterobacter aerogenes*-BRB13 possessed maximum PGP traits. These were shortlisted for further growth promotion studies. The first isolate *M. luteus*-BRB13 was positive for catalase, oxidase, IAA/ NH_3 production, P, Zn and K solubilization, was found to tolerate up to 7% salt concentration and could survive temperatures from 28-55°C. The second shortlisted isolate *E. aerogenes*-BRB13 was

positive for methyl red, VP, citrate, catalase, casein hydrolysis, IAA/ NH_3 production, P, Zn and K solubilization, was found to tolerate up to 10% salt concentration and could survive temperatures ranging from 4-45°C. Such multiple modes of action have been reported to be the prime reasons for the plant growth promotion and disease suppressing ability and similar to our study, a single PGPR has often been found to reveal multiple modes of action (Rodríguez-Díaz *et al.* 2008).

Antifungal activity

All the isolates were screened against *P. capsici*, causing wilt in many crops. However, we provide here the data of only those isolates that showed maximum (>70%) inhibition of *P. capsici* (Fig. 2). While control did not exhibit any inhibition, only three of the 74 isolates

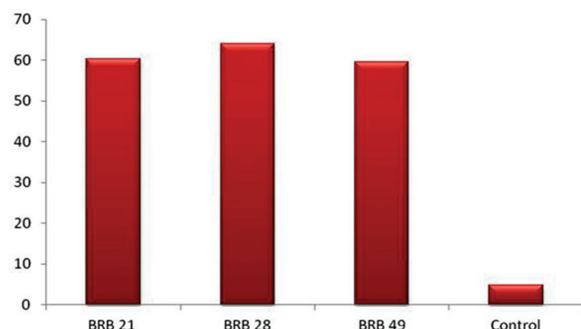

Fig. 2. PGPR with > 70% inhibition of *P. capsici in vitro* (Values shown are based on arc sine transformation of percent inhibition levels; BRB 21-*Burkholderia cepacia*; BRB 28-*Pseudomonas aeruginosa*; BRB 49-*Serratia marcescens*)

(*Burkholderia cepacia*-BRB21, *Pseudomonas aeruginosa*-BRB28 and *Serratia marcescens*-BRB49) exhibited 75.6%, 80.7% and 74.4% inhibition, respectively. These three isolates were, therefore, shortlisted for biocontrol studies in the green house. Such significant antifungal effects *in vitro* has been attributed to the production of a variety of antimicrobial compounds that cause cytolysis, inhibit mycelial growth and protein biosynthesis (Quan *et al.* 2010).

Green house study on growth promotion

The tallest plants (Fig. 3) were observed in the treatment T5 with 75% N + 100% PK + *M. luteus*-BRB3 (368.7 cm) followed by the treatment T14 with 100% NPK + *E. aerogenes*-BRB13 (354.7 cm). These treatments also registered the greatest root length (55.37 and 63.43 cm, respectively). Biomass (dry), number of leaves and number of nodes were also greatest in the treatment T5 (156.7 g plant^{-1}; 54, 56, respectively). The treatments with only PGPR registered longer roots than some of the chemical treatments especially 100% NPK. Only fresh root weight showed marked variations among the treatments and was greatest in the treatment with 100% NPK+ *M. luteus*-BRB3 (14 g plant^{-1}).

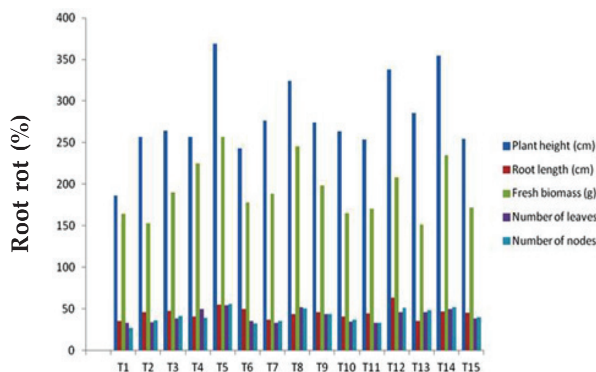

Fig. 3. Effects of shortlisted PGPR (BRB3-*Micrococcus luteus* and BRB13-*Enterobacter aerogenes*) applied in combination with different rates of NPK fertilizers on growth parameters of black pepper (T1- Control; T2- BRB3 alone; T3- BRB13 alone; T4- 75% N + 100% P + 100% K; T5- 75% N + 100% P + 100% K+ BRB 3; T6- 75% N + 100% P + 100% K+ BRB 13; T7- 100% N + 75% P + 100% K; T8- 100% N + 75% P + 100% K+ BRB3; T9- 100% N + 75% P + 100% K + BRB13; T10- 100% N + 100% P + 75% K; T11- 100% N + 100% P + 75% K + BRB3; T12- 100% N + 100% P + 75% K + BRB13; T13- 100% N + 100% P + 100% K; T14- 100% N + 100% P + 100% K + BRB3; T15- 100% N + 100% P + 100% K + BRB13)

In fact, in the treatment with 75% N + 100% PK, plant height was lower by 30%, root length by 26%, biomass by 12%, root weight by 30%, number of leaves by 8.0% and number of nodes by 30% compared to the same treatment applied along with PGPR i.e., 75% N + 100% PK + *M. luteus*-BRB3. This suggested that *M. luteus*-BRB3 did indeed promote growth of black pepper, albeit at varying degrees. PGPR have been reported to promote plant growth through direct mechanisms that include N fixation, nutrient solubilization, production of growth

regulators, increasing nutrient and water uptake (Compant *et al.* 2005). Besides, the observed effect on plant growth can be the result of a tandem or a cascade of mechanisms in which one mechanism stimulates another, yielding enhanced plant growth (Bashan & de-Bashan 2010).

Similar to our results on black pepper, several reports on growth promotion by PGPR in other crops have been made (Almaghrabi *et al.* 2013; Egamberdieva 2011). Also, *Micrococcus* sp. with multiple PGPR attributes beneficial to cowpea (Dastager *et al.* 2010) and maize (Raza & Faisal 2013) have been reported. The superior performance of the treatment 75% N + 100% PK + *M. luteus* (BRB3) indicated the possibility of a 25% reduction in chemical N fertilizer application rate. This could possibly be due to greater acquisition of nutrients due to microbial modification of the absorptive properties of the roots such as increased root length and surface area and number of root hairs (Sessitsch *et al.* 2013). Consistent with our results, there are studies that have reported reduction in chemical fertilizer use when applied along with PGPR (Shaharoona *et al.* 2008; Adsemoye *et al.* 2009).

Green house study on biocontrol

As expected, lowest foot rot (0.81%) was recorded in the absolute control with no challenge inoculation with *P. capsici* (Fig. 4). Conversely, the greatest disease incidence (80.0%) was observed in *P. capsici* inoculated control. Among the treatments, lowest foot rot (32%) and the tallest plants (mean 332 cm) were recorded with *B. cepacia* (BRB21), which was, however, on par with metalaxyl-mancozeb (43% and 313.7, respectively). The performance of *S. marcescens* (BRB49) was poor (72.50%) and was almost identical to pathogen inoculated control (*P. capsici*). The BE was significantly lower (9.0%) with *S. marcescens*-BRB49, while it was markedly greater (59.0) with *B. cepacia* (BRB21). The inconsistency in the performance of *P. aeruginosa* and *S. marcescens* from laboratory to green house conditions can be attributed to their inability to compete with existing microorganisms in the soil, while *B. cepacia* was more resilient and competitive.

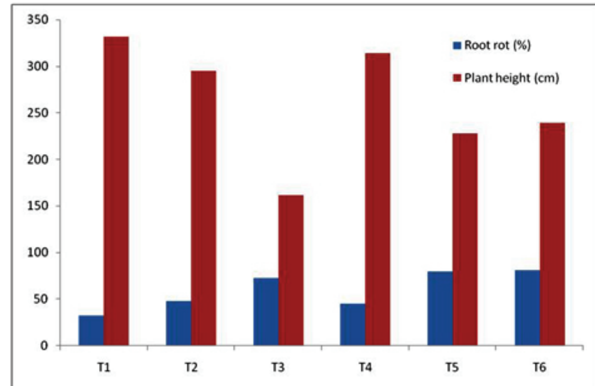

Fig. 4. Effects of shortlisted PGPR (BRB21- *Burkholderia cepacia*, BRB28-*Pseudomonas aeruginosa* and BRB49-*Serratia marcescens*) on foot rot and height of black pepper challenge inoculated with *P. capsici* (T1- BRB21; T2- BRB28; T3-BRB49; T4-Metalaxyl-Mancozeb; T5-Pathogen inoculated control; T6-Absolute control)

Our findings suggested that for management of *P. capsici* in black pepper, *B. cepacia*-BRB21 can be a good alternative to chemical measures. Inhibition of plant pathogens by PGPR can be attributed to production of secondary metabolites (such as antibiotics, HCN, NH_3) and cell wall degrading enzymes that inhibit phytopathogens (Martínez-Viveros *et al.* 2010). Other indirect mechanisms reported are competitive exclusion of pathogens, removal of phytotoxic substances produced by deleterious bacteria and plant roots under stress (Bashan & de-Bashan 2010) and stimulating systemic disease resistance. Besides, the strong antifungal potency of the genera *Burkholderia* has been attributed to secretion of compounds like pyrrolnitrin (Schmidt *et al.* 2009), burkholdines, occidiofungins, xylocandins, and cepacidines etc (Lin *et al.* 2012) and *B. cepacia* has been used as a biocontrol agent against several important soil-borne plant pathogens including *R. solani*, *Sclerotium rolfsii*, *Pythium* spp., *Fusarium* spp. and *P. capsici* and is considered a viable alternative to a variety of chemical pesticides (Quan *et al.* 2006).

Overall, our study involved isolation of PGPR from different sites under black pepper, their characterization and screening for growth promotion and biocontrol traits. The promising rhizobacteria with multiple PGP

traits were shortlisted for further green house studies involving growth promotion and biocontrol of *P. capsici*. The green house studies revealed that application of *M. luteus*-BRB3 + 75% recommended dose (RD) of N + 100% RD of PK led to marked enhancement in black pepper growth. With regard to *P. capsici* inhibition, lowest incidence of foot rot and taller plants were observed with *B. cepacia*-BRB21, which was, comparable to application of metalaxyl-mancozeb. It is, therefore, apparent from this study that the identified PGPR have great promise as a viable alternative to chemical inputs. Earlier, three endophytic bacteria identified as *P. aeruginosa*-IISR BP35, *P. putida*-IISR BP25 and *B. megaterium*-IISR BP17 were found promising for suppression of *P. capsici* (Aravind *et al*. 2009). However, Kumar *et al*. (2013) reported that the endophytic *Pseudomonas aeruginosa* strain BP35 was as virulent as clinical *P. aeruginosa* strains and have, therefore, advised caution while using it for black pepper. Nevertheless, this is the first report on the immense potential of *M. luteus* for growth promotion and *B. cepacia* for control of *P. capsici* in black pepper. Our future strategy would be to test these PGPR using a combination of two or more strains by integrating them into appropriate nutrient and disease management schedules.

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