Short communication

Diversity of endophytic bacteria from *Piper* spp. with antagonistic property against *Phytophthora capsici* causing foot rot disease in black pepper (*Piper nigrum* L.)

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Abstract

Endophytes, especially bacteria, which inhabit healthy plant tissues without causing any damage to the host, have immense potential as biological control agents against plant diseases. Though the presence of antagonistic endophytic bacteria have been reported from black pepper (*Piper nigrum* L.), no information about antagonistic bacterial endophytes of *Piper colubrinum*, a wild exotic relative of black pepper resistant to the foot rot pathogen, are available. The culturable bacterial endophytic communities associated with *Piper nigrum* and *Piper colubrinum* were isolated and screened for antagonistic potential against *Phytophthora capsici*. Out of the 47 bacterial endophytic isolates obtained, 12 showed *in vitro* antagonism against *P. capsici*. The isolates were identified based on biochemical and molecular characterization. Phylogenetic analysis of 16S rRNA gene sequences showed considerable diversity of antagonistic bacterial endophytes present in *Piper* spp. It was observed that *Bacillus* spp. were predominant among the endophytes that have antagonistic activity against *P. capsici*. An endophytic bacterial isolate belonging to *Rhizobium* sp that showed *in vitro* antagonism against *P. capsici* was also obtained from *P. colubrinum* root tissues.

Keywords: Antagonists, Diversity, Endophytic bacteria, *Phytophthora capsici, Piper colubrinum*, *Piper nigrum*.

Black pepper, known as "The King of Spices" is a major spice crop of Kerala (India), and has its origin in the Western Ghats region. Foot rot incited by *Phytophthora capsici* is one of the major constraints in the production of black pepper. The disease affects all parts of the plant at all the stages of crop. No cultivated variety has been found resistant to this disease and no genetic basis for disease resistance has been identified yet, although most of the available germplasm has been screened against the pathogen.

Endophytes are microorganisms which reside within the plant tissues without causing any harm to the host plants. These organisms can be detected within tissues of healthy plants and their presence causes no symptoms on the host (Schulz and Boyle, 2005). Endophytic bacteria are of increasing interest due to their potential uses in agriculture (Kobayashi and Palumbo, 2000). They have been recognized as good candidates for the biological control of plant pathogens (Hallmann et al., 1997; Kobayashi and Palumbo, 2000; Sturz et al., 2000; Ryan et al., 2008; Senthilkumar et al., 2011). Bacterial endophytes are ecologically highly competitive as they effectively colonize the rhizosphere including the endorhizosphere (Hallmann et al., 1997; Upreti and Thomas, 2015; Govindasamy et al., 2017).

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Under natural field conditions in highly diseased black pepper plantations, disease escape is noticed in some of the vines. Since genetic basis for such tolerence has not been found, some other mechanisms are thought to play a role. Colonization of endophytic bacteria in *Piper* spp. is a natural phenomenon (Philip et al., 2000; Kulkarni et al., 2007). Since there are reports of endophytic colonization in black pepper and other related species, it is hypothesized that endophytes enhance the plant's defence mechanism against *P. capsici*. An understanding of diversity of bacterial endophytes will enable more use of their beneficial characteristics.

Biological control, as an ecofriendly approach, has been considered a potential alternative to chemical control. To initiate a biocontrol programme using new candidate microorganisms from certain specific niches, screening and identification of potential antagonists are necessary pre-requisites. Very little is known about endophytic bacteria in black pepper and related species particularly their potential as biocontrol agents against *P. capsici* induced diseases (Aravind et al., 2009). *P. colubrinum*, an exotic wild relative of black pepper, is found to have resistance to *P. capsici* (Ravindran and Remashree, 1998). This report describes the bacterial endophytic diversity associated with *P. colubrinum* and *P. nigrum* based on biochemical characteriza-tion, molecular characterization and phylogenetic analysis, with special emphasis on antagonism against the foot rot pathogen.

Endophytic bacteria were isolated from the internal tissues of roots, stem and leaves of *P. colubrinum* and *P. nigrum* (var. Karimunda) by trituration of surface disinfected plant tissues (Hallmann et al., 1997). Plant samples (root, stem and leaves of *P. colubrinum* and *P. nigrum*) were collected from the fields of Instructional farm of College of Agriculture, Vellayani. The samples were washed in running tap water and cut into pieces of approximately one centimetre size. Four pre-washes were given with sterile distilled water. Surface sterilization was carried out by soaking the sample pieces in 4% sodium hypochlorite for three minutes and then they were rinsed four times in sterile distilled water (SDW) to clear them of sodium hypochlorite. Sterility checks were carried out to monitor the efficiency of the surface sanitization procedure. For these checks, either 0.1 ml of the last wash was transferred to Nutrient Agar (NA), King's B (KB) or Tryptic Soy Agar (TSA) using spread plate method or, alternatively, 0.1 ml of the final wash was transferred to 9.9 ml of Nutrient Broth, King's B Broth and Tryptic soy broth (TSB), and incubated at room temperature. After 48 h, if no bacterial growth occurred in the sterility check, the recovered bacteria in the isolation processes were considered to be endophytes. For isolation of endophytes, the plant tissue was triturated in one ml phosphate-buffered saline solution (PBS with pH 7.4) with mortar and pestle under aseptic condition. From the macerate, 0.1ml was spread on NA, KB, TSA and agar plates. Also, 0.1 ml of the macerate was mixed with 0.9 ml of sterile water and vortexed to get 10^{-1} dilution and 0.1 ml of the diluted suspension was spread on NA, KB, TSA and agar plates. The agar plates were incubated at 28° C for 2-3 days. Bacterial colonies that appeared frequently and looked morphologically different were selected for further studies.

The plant associated bacterial isolates were screened for their effectiveness in inhibiting the growth of *P. capsici* on Potato Dextrose Agar (PDA) using dual culture plate assay. Mycelial disc of 4 mm diameter obtained from an actively growing *P. capsici* culture on PDA was placed at the centre of a Petri plate containing PDA medium. Two streaks of 1.5 cm length were made on opposite edges of the plate with a fresh growth of the endophytic bacterial isolate after 3 days of mycelial inoculation. Plates were incubated at 28° C for five to six days. The inhibition of mycelial growth based on presence or absence of an inhibition zone in dual culture assay was assessed.

Morphological characters like cell morphology,

colour of the colony, colony appearance (colony configuration, margin and elevation), Gram reaction, cell arrangement and sporulation were examined and recorded following standard protocols (Cappuccino and Sherman, 1992).

Biochemical characterization of selected bacterial isolates was done by performing various biochemical tests and carbohydrate utilization tests by using readymade Himedia kits (Hi-Carbo Part A, B and C, Hi-Bacillus and Hi-Assorted) as per the manufacturer's instructions. Colour change observed on the biochemical amended media of the kit after spot inoculating culture suspensions of selected isolates followed by incubation for 24 h indicated the reaction with respect to different biochemical or carbohydrates as positive or negative. The results of biochemical tests were used to arrive at a tentative genus level identification of isolates as per the Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

Molecular characterization of bacterial isolates was done by analyzing the internal transcribed regions of DNA of 16S rRNA of isolates which were amplified using CAGGCCTAACACATGCAAGTC as forward primer and GGGCGGWGTGTACAAGGC as reverse primer in PCR at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. Sequencing of the PCR product was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufacture's protocol. Blast search details of sequenced DNA in NCBI data base were then used to reveal the identity of the isolates. The acquired sequences were compared with those available in the GenBank, using the BLASTN program to determine their approximate phylogenetic affiliations and 16S rRNA gene sequence similarities (Altschul et al., 1997).

Phylogenetic relationship between the bacterial endophytes with related species in the data base were analysed using the ClustalX (Version 2.1) package and the resultant phylogenetic tree was viewed using TreeviewX software.

A total of 47 morphologically different bacterial endophytes were isolated from the genus *Piper*, 26 from *P. colubrinum* and 21 from *P. nigrum* on NA, KB and TSA media. The bacterial endophytes obtained in the study were given proper abbreviations. PCRE, PCSE, PCLE were for endophytes of *P. colubrinum* from root, stem and leaves respectively. *P. nigrum* endophytes isolated from root, stem and leaves were abbreviated as PNRE, PNSE and PNLE respectively. The isolates were serially numbered with proper abbreviations. Preliminary screening for the antagonistic potential of the bacterial endophytes were done by dual culture plate assay on PDA medium that supported the growth of both the bacterial isolates and the pathogen. Results of the dual culture plate assay indicated that out of the total 47 bacterial isolates obtained, twelve showed *in vitro* antagonism against *P. capsici*. The occurrence of antagonistic bacterial endophytes was more in *P. colubrinum* compared to those of *P. nigrum* (Table 1 and 2). Diverse group of endophytic bacteria in black pepper and their beneficial traits in suppressing *Phytophthora* infection were earlier reported (Aravind et al., 2009; 2012). In the screening procedure used in the present study, primary emphasis was given on occurrence of antibiosis as a mechanism of pathogen inhibition. Results of dual culture plate assay revealed that in

Table 1. Endophytic bacteria isolated from *Piper* spp.

Characteristics of endophytes	Species of *Piper*							
	P. colubrinum				*P. nigrum*			
	Root	Stem	Leaf	Total	Root	Stem	Leaf	Total
Number of endophytes isolated	10	12	4	26	7	10	4	21
Endophytes with antagonistic property against *P. capsici*	3	3	1	7	2	3	-	5

Table 2. Antagonistic potential of endophytes from *P. colubrinum* and *P. nigrum* in dual culture plate assay

Source	Isolates	Inhibition zone (mm)*
P. colubrinum	PCRE1	5.60
	PCRE9	5.40
	PCRE10	2.40
	PCSE5	4.80
	PCSE8	5.80
	PCSE10	5.80
	PCLE3	4.20
P. nigrum	PNRE4	3.80
	PNRE5	3.40
	PNSE3	1.40
	PNSE4	2.60
	PNSE5	2.20

*Inhibition zone was measured from the edge of the bacterial colony to the advancing edge of the fungal growth. Mean of five replications.

P. colubrinum, 7 out of 26 isolates (26.92%) showed inhibition of mycelia growth of *P. capsici*. In *P. nigrum*, 23.81% (5 out of 21 isolates) showed *in vitro* inhibition. It is thus hypothesized that endophytes from *P. colubrinum* have more innate potential in suppressing the infection caused by *P. capsici*. *P. colubrinum* is highly resistant to *P. capsici* infection and has immense potential as a donor plant in breeding programmes for the improvement of the cultivated species, *P. nigrum* (Dicto and Manjula, 2005). Moreover, a partly fertile interspecific hybrid with partial resistance has been developed by hybridizing *P. nigrum* and *P. colubrinum* which contributed to a remarkable breakthrough in the introgression of disease resistance from the wild species *P. colubrinum* to the cultivated *P. nigrum* (Vanaja et al., 2008).

The morphological and cultural characteristics of antagonistic endophytic isolates from *Piper* are presented in Table 3. Biochemical characterization of various isolates was performed by carrying out various biochemical tests and carbohydrate utilization tests (Table 4). Tentative identification of bacteria was performed as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). The bacterial isolates were identified to be belonging to genus *Streptomyces, Bacillus, Rhizobium, Pantoea,* and *Acinetobacter*. The results of biochemical tests hinted at the occurrence of a wide variety of bacterial isolates inhabiting *Piper* spp. possessing biocontrol potential against *P. capsici*. A molecular level identification of the isolates was also carried out by analyzing the internal transcribed regions of DNA of 16S rRNA. Blast search details of amplified DNA in NCBI data base revealed the identity of the isolates (Table 5). Different *Bacillus* spp. were isolated from both *P. colubrinum* and *P. nigrum*. An actinomycete *Streptomyces deccanensis* PCRE1 was isolated from the roots of *P. colubrinum*. Antagonistic activity of endophytic *Streptomyces* sp has been reported by many authors (Cao et al., 2005; Gangwar et al., 2012; Sreeja and Gopal, 2013). Two *Acinetobacter*

Table 3. Morphological and cultural characteristics of antagonistic endophytes

Isolates	Colony morphology	Colour	Appearance	Gram reaction	Cell arrangement	Sporulation
PCRE1	Rough, discrete	White	Discrete	G^+	Filamentous	+
PCRE9	Round	Light yellow	Flat, spreading	G^+	Single rod	+
PCRE10	Round	Light beige	Semi translucent, raised	G^-	Single rod	-
PCSE5	Round	Yellow	Smooth, mucoid	G^-	Single rod	-
PCSE8	Round	Off white	Irregular mucoid	G^+	Single rod	+
PCSE10	Round	Off white	Spreading	G^+	Single rod	+
PCLE3	Irregular	White	Irregular raised	G-	Paired rod	-
PNRE4	Round	Off white	Flat	G^+	Single rod	+
PNRE5	Round	Dull yellow	Raised	G^+	Single rod	+
PNSE3	Round	Cream	Slimy	G^-	Paired rod	-
PNSE4	Round, glassy	Transparent	Mucoid	G^+	Single rod	+
PNSE5	Irregular	Cream	Spreading	G^+	Single rod	+

+ Presence of sporulation

Table 4. Biochemical characterization of endophytic bacterial isolates having antagonism against *P. capsici*

Biochemical test	PCRE1	PCRE9	PCRE10	PCSE5	PCSE8	PCSE10	PCLE3	PNRE4	PNRE5	PNSE3	PNSE4	PNSE5
Citrate utilization	-	-	-	+	-	-	-	-	-	+	+	-
Lysine utilization	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine utilization	-	-	-	-	-	-	-	-	-	+	-	-
Urease	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine Deamination	-	-	-	-	-	-	-	-	-	+	-	-
H_2S production	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	+	+	+	+	-	+	+	-	+	+
Catalase	-	-	-	-	-	-	+	-	-	+	-	-
Arginine Lyase	-	-	-	-	-	-	-	-	-	+	-	-
Malonate utilization	-	-	-	-	-	-	-	-	-	+	+	-
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-	-
ONPG	-	-	-	-	-	-	+	-	-	-	-	-
Glucose	-	-	-	+	+	-	+	+	-	+	+	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	+	+	-	-	+	-	-	+	-	+
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-
Sucrose	-	-	-	+	+	-	+	-	-	-	+	-
Mannitol	-	-	-	+	-	-	-	-	-	-	-	-
Trehalose	-	-	+	+	-	-	+	-	-	-	-	-
Xylose	-	-	+	+	-	-	+	-	-	+	-	-
Maltose	-	-	+	+	-	-	+	-	-	-	-	-
Fructose	-	-	+	+	-	+	+	+	+	-	-	-
Dextrose	-	+	+	+	-	+	+	+	+	+	+	+
Galactose	-	-	+	+	-	-	+	-	-	+	-	-
Raffinose	-	-	+	-	-	-	+	-	-	-	-	-
Melibiose	-	-	+	+	-	-	+	-	-	-	-	-
L-arabinose	-	+	+	+	-	-	+	-	-	+	-	-
Mannose	-	-	+	+	-	-	+	-	-	+	-	+
Inulin	-	-	-	-	-	-	+	+	-	-	-	-
Sodium gluconate	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	-	+	+	-	-	+	-	-	-	-	-	+
Salicin	-	+	+	-	-	-	-	-	-	-	-	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	+	-	-	-	-	-	-	-	-
Sorbitol	-	-	+	-	-	+	-	+	+	-	-	-
Adonitol	-	-	+	-	-	-	-	-	-	-	-	-
Arabitol	-	-	-	+	-	-	-	-	-	-	-	-
Erithritol	-	-	-	-	-	-	-	+	-	-	-	-
Alpha Methyl D-glucoside	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	+	-	-	-	-	-	-	-	-
Cellobiose	-	-	+	-	+	+	-	+	+	+	+	+
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-
Alpha Methyl D-mannoside	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	-	+	+	+	+	+	-	+	+	-	+	+
D- arabinose	-	-	-	+	-	-	-	-	-	+	+	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-
Tentative identification of isolates	*Streptomyces* sp	*Bacillus* sp	*Rhizobium* sp	*Pantoea* sp	*Bacillus* sp	*Bacillus* sp	*Acinetobacter* sp.	*Bacillus* sp	*Bacillus* sp	*Acinetobacter* sp.	*Bacillus* sp	*Bacillus* sp

+ Positive for biochemical reaction / Carbohydrate utilized - Negative for biochemical reaction / Carbohydrate not utilized

Table 5. BLAST search details of the sequences producing most significant alignment of the plant associated bacterial isolates

Isolate	Description	Gene showing maximum homology	Identity (%)
PCRE-1	*Streptomyces deccanensis*	*Streptomyces deccanensis* strain WJA64 16S ribosomal RNA gene, partial sequence	100%
PCRE-9	*Bacillus* sp	*Bacillus* sp JS, complete genome	100%
PCRE-10	*Rhizobium* sp	*Rhizobium* sp A2 16S ribosomal RNA gene, partial sequence	100%
PCSE-5	*Pantoea dispersa*	*Pantoea dispersa* strain Y08 16S ribosomal RNA gene, partial sequence	100%
PCSE-8	*Bacillus velezensis*	*Bacillus velezensis* strain JTYP2 chromosome, complete genome	100%
PCSE-10	*Bacillus velezensis*	*Bacillus velezensis* strain sx01604 chromosome, complete genome	100%
PCLE-3	*Acinetobacter* sp	*Acinetobacter* sp CJ-S-MA3 16S ribosomal RNA gene, partial sequence	100%
PNRE-4	*Bacillus* sp	*Bacillus* sp 275 chromosome, complete genome	99%
PNRE-5	*Bacillus* sp	*Bacillus* sp strain ICA 13 16S ribosomal RNA gene, partial sequence	100%
PNSE-3	*Acinetobacter baumannii*	*Acinetobacter baumannii* strain WKA02 chromosome, complete genome	100%
PNSE-4	*Bacillus* sp	*Bacillus* sp Ant-2a partial 16S rRNA gene	100%
PNSE-5	*Bacillus subtilis*	*Bacillus subtilis* strain VV2, complete genome	100%

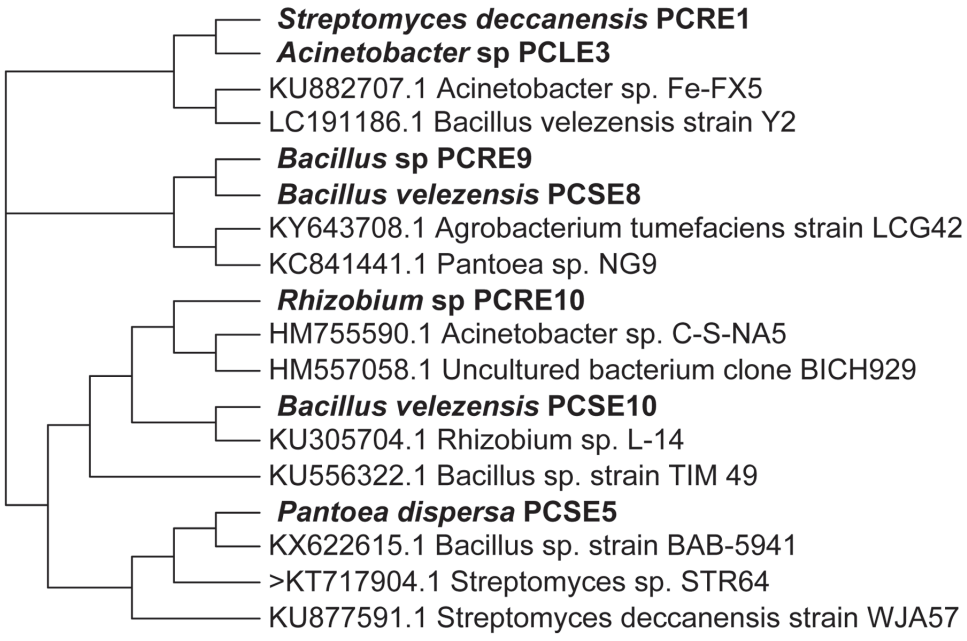

Figure 1. Phylogenetic relationship of endophytes from *Piper colubrinum* with antagonistic potential against *P. capsici* compared with bacterial strains showing sequence similarity. Endophytes of *Piper colubrinum* shown in bold

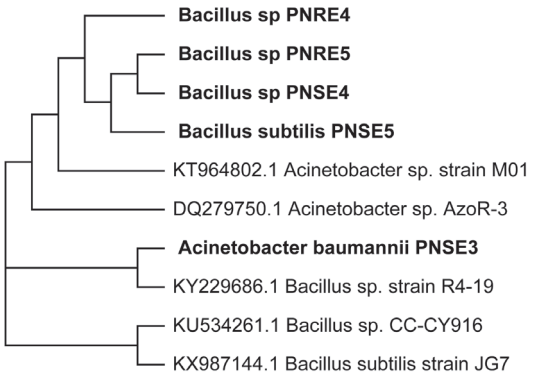

Figure 2. Phylogenetic relationship of endophytes from *P. nigrum* with antagonistic potential against *P. capsici* compared with bacterial strains showing sequence similarity. Endophytes of *P. nigrum* are shown in bold

spp. and one *Pantoea* sp were also isolated. *Bacillus velezensis* was isolated from stem tissues of *P. colubrinum*.

A *Rhizobium* sp was isolated from *P. colubrinum* and this study reports the first evidence of *Rhizobium* sp colonizing *P. colubrinum* as endophyte, though there is a report regarding occurrence of *Rhizobium* sp as an endophyte in another species of *Piper, P. tuberculatum* (Nascimento et al., 2015). *Rhizobium* is normally occurring as a microbe surviving saprophytically in soil in the absence of a legume host. However, the present study confirms that *Rhizobium* can also occupy other endophytic niches, inside *Piper* spp. The presence of endophytic *Rhizobium* sp inside roots of rice plants were reported earlier (Yanni et al., 1997; Chi et al., 2005).

The phylogenetic tree showing the relationship between various antagonistic plant associated bacterial isolates from *Piper* spp. with antagonistic potential against *P. capsici* compared with existing sequences showing similarity is depicted in Figure 1 and Figure 2. The distribution of different isolates in the phylogenetic tree showed great diversity among the species. It also revealed that wide variety of endophytic bacteria with antagonistic potential against *P. capsici* are present in the host tissues. Majority of the endophytic bacteria from *Piper* spp. with antagonistic potential against *P. capsici* belonged to *Bacillus* spp.

It may be concluded from the study that endophytic bacteria from *P. colubrinum* had more antagonistic action against *P. capsici* than those from *P. nigrum*. This work provides the first evidence of *P. colubrinum* associated endophytic bacteria as antagonists against the foot rot pathogen of black pepper.

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