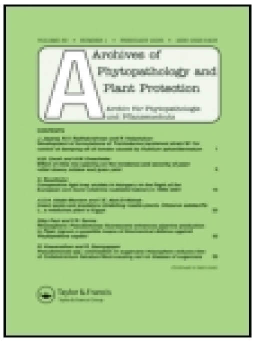

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Archives of Phytopathology and Plant Protection

ISSN: 0323-5408 (Print) 1477-2906 (Online) Journal homepage: http://www.tandfonline.com/loi/gapp20

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Archives of Phytopathology and Plant Protection
Vol. 45, No. 9, May 2012, 1115–1126

Pre-plant bacterisation: a strategy for delivery of beneficial endophytic bacteria and production of disease-free plantlets of black pepper (*Piper nigrum* L.)

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(*Received 2 February 2011; final version received 27 May 2011*)

Naturally occurring endophytic bacteria from black pepper vines were found to exhibit strong antagonistic activities against *Phytophthora capsici* and *Radopholus similis*. In order to deliver these bacterial strains, as well as to produce disease-free plantlets of black pepper, a pre-plant stem and root bacterisation was standardised. Stem bacterisation with endophytic *Pseudomonas* spp. was found to suppress *P. capsici* infection (over 90% reduction in lesion length) on cut shoots. Pre-plant root bacterisation with *Pseudomonas aeruginosa, Pseudomonas putida* and *Bacillus megaterium* yielded over 60% of plantlets free from *P. capsici* infection on roots. *Curtobacterium luteum* and *B. megaterium* recorded over 70% reduction of nematode population in soil with concomitant production of over 65% of nematode-free plantlets. Besides protecting the plants from the pathogens, the bacteria were also found to enhance the growth of rooted cuttings. The biocontrol potential of the above endophytic bacteria and their exploitation for disease management in the black pepper nursery are discussed.

Keywords: black pepper; *Piper nigrum*; endophytic bacteria; *Phytophthora capsici*; *Radopholus similis*

Introduction

One of the challenges in the production of black pepper plantlets is their freedom from biotic stress that often limits the successful establishment of the transplants in the field besides reducing the marketable plantlets. Conventional method of black pepper propagation in soil system is vulnerable to *Phytophthora* rot caused by *Phytophthora capsici* and nematode infestation by *Radopholus similis* (Anandaraj and Sarma 1995; Anandaraj 2000). The pathogens are often carried to the black pepper plantation through the latently infected rooted cuttings as well as in the rooting medium which results in transplantation failures. One of the strategies to prevent the spread of pathogens across different pepper growing regions is to produce and distribute disease-free vigorous plantlets. Traditionally, the nursery diseases and nematode infestation are managed by application of chemical pesticides like metalaxyl or copper-based fungicides or phorate. Public concern over harmful effects of chemical pesticides on the environment has necessitated development of environment friendly disease management alternatives such as resistant varieties,

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ISSN 0323-5408 print/ISSN 1477-2906 online

http://dx.doi.org/10.1080/03235408.2012.657894
http://www.tandfonline.com

biological control, coupled with sanitary and phytosanitary (SPS) procedures. Success of these ecofriendly options, particularly the biocontrol, depends on a multitude of variables stemmed from varietal, soil, agronomic and geographical factors (Gnanamanickam et al. 1992). Endophytic microorganisms with biocontrol activities can be ideal under such circumstances for consistent and reliable control of diseases in a perennial crops like black pepper. Evidence has shown that bacteria naturally occurs in healthy plant organs where they establish themselves without causing visible host damage (Jacobs et al. 1985; Misaghi and Donndelinger 1990), promote plant growth and reduce disease symptoms (Chen et al. 1995). Several endophytic bacteria have showed promise in control of fungal plant pathogens (Pleben et al. 1995; Sturz and Matheson 1996; Sharma and Nowak 1998; Vilich et al. 1998), insects (Petrini et al. 1989; Azevedo et al. 2000), and nematodes (Hallmann et al. 1998) in a variety of host plants. Aravind et al. (2009) reported diverse group of endophytic bacterial species in black pepper and their usefulness for suppressing *Phytophthora* infection. One of the issues pertaining to the use of biocontrol agent for crop protection is their delivery on the crop. The success of the biocontrol system against soil-borne plant diseases depends largely on the activity of the biocontrol agents in the rhizosphere region of the crop plants, which in turn is influenced by the method of delivery of the biocontrol agents. Several methods of delivery of biocontrol agents are reported which includes seed treatment (seed biopriming), bacterisation of plant propagation material, soil application (Bressan and Borges 2004) and even foliar application. Endophytic bacteria can be directly delivered into the succulent plant system prior to the planting in the soil. Being a vegetatively propagated plant species, black pepper shoots are amenable for bacterisation by endophytic bacteria. Such a pre-emptive bacterisation using endophytic bacteria to suppress the *Phytophthora* and nematode infection/infestation can be one of the methods to produce disease-free plantlets of black pepper. This article deals with a pre-plant stem and root bacterisation of black pepper using black pepper associated endophytic bacteria for the production of disease-free plantlets in the nursery.

Materials and methods

Endophytic bacterial strains

Endophytic bacterial strains isolated from black pepper used in this trial and their beneficial traits are listed in Table 1. The bacterial strains were streaked on nutrient agar (g l^{-1} 5 peptone, 3 beef extract, 3 yeast extract, 5 NaCl and pH 7.0) and incubated at 28°C for 24 h. Nutrient broth was used for mass culturing of the bacterium and diluted 10-fold in sterile distilled water prior to stem and root bacterisation.

Pre-plant stem bacterisation

Stem cuttings (cv. Panniyur-1) of about 16.0 cm length, with at least two nodal primordia, were washed thoroughly with tap water and blot dried. Stem cuttings were treated for 30 min in bacterial suspension and control plants were treated with 10-fold diluted nutrient broth. Bacterised stem cuttings were planted in poly bags (500 g per bag) containing river sand and maintained in nursery at 28°C and 95% relative humidity.

Table 1. Biocontrol and growth promotion attributes of four endophytic bacterial species isolated from black pepper (*Piper nigrum* L.).

	Secondary metabolite[a]		Hydrolytic enzyme[a]					Growth promoting character[a]			
Endophytic bacterial isolate	HCN production	Biosurfactant production*	Chitinase	Protease	Lipase	Cellulase	Pectinase	Phosphate solubilisation	Nitrogen fixation	Indole Acetic Acid (IAA)	Ammonia production
B. megaterium (MTCC – 5412)	–	–	–	++	–	+++	–	–	+	+++	+
P. putida	–	+	–	–	+	–	–	–	+++	+	++
P. aeruginosa (MTCC – 5410)	+++	+++	–	++	++	–	–	+	+++	–	+++
C. luteum	–	–	+	–	–	–	–	–	–	–	++

[a]: – indicates no *in vitro* production, + indicates low *in vitro* production, ++ indicates moderate *in vitro* production,+++ indicates high *in vitro* production; *Based on Drop Collapse and SW medium based assay (Siegmund and Wagner 1992).

Establishment of the bacterised stem cuttings was evaluated in a greenhouse. The treatments included four endophytic bacteria and a control. Each bag contained four stem cuttings. There were 50 bags per treatment. The treated stem cuttings were maintained in a temperature controlled growth chamber (25°C, 95% relative humidity, 16 h of day light) to allow the cuttings to root. The booster application of bacterial cell suspension ($\sim 10^9$ cfu per ml) of each culture was made in the soil at 15 days interval up to two months.

After three months, the stem cuttings were removed from the bags and observations were taken based on germination percentage, number of leaves and disease incidence. Number of roots showing rot or lesion was taken as a measure by visual scoring using a 0–5 scale, where 0: no root rotting, 1: 0 to 10% roots with rot/lesion, 2: 10 to 20% roots with rot/lesion, 3: 20 to 50% roots with rot/lesion, 4: 50 to 80% roots with rot/lesion and 5: 80 to 100% roots with rot/lesion.

Pre-plant root bacterisation

Rooted cuttings (IISR Sreekara) of black pepper were bacterised for 30 min while control plants were treated with sterile distilled water. Bacterised rooted cuttings were planted in potting mixture containing soil, sand and farmyard manure at 2:1:1 ratio in poly bags (2 kg soil per bag) in a greenhouse.

Disease suppressive effect of the endophytic bacteria and the production of disease free plantlets were evaluated in a greenhouse in a factorial CRD design with six treatments imposed on plants challenged with pathogens. The treated rooted cuttings were maintained in a temperature controlled growth chamber (25°C, 70% relative humidity, 16 h of day light). The booster application of the bacterial cell suspension ($\sim 10^9$ cfu per ml) of each culture was made by drenching the soil in each poly bag at 15 days interval up to two months.

Preparation of pathogen inoculum

Phytophthora capsici

P. capsici (IISR 99–166) was cultured on carrot agar for four days at room temperature (28°C) in the dark. Mycelial discs (1 cm diameter) were cut from the plate and placed in another plate with mycelium on the top and sterile distilled water was added to partially immerse the discs. The plates were incubated under continuous fluorescent light for four days. The mycelial discs were observed under an inverted microscope for the presence of mature sporangia. The plates were, then, given a cold shock for 10 min and observed under the microscope for release of zoospores. The zoospores were counted using a hemocytometer and the zoospore suspension of *P. capsici* (10^6 zoospores ml^{-1}) was made.

Radopholus similis

The nematodes were extracted from infected black pepper roots. The collected nematodes were surface sterilised in sodium hypochlorite (1%), washed in sterile water and subsequently treated with 0.01% streptomycin sulphate and washed again with sterile water. The nematodes were inoculated on carrot discs placed on water agar (2%) and were maintained at 28 ± 1°C.

Pathogen inoculation

The bacterised plants were challenged with the pathogens individually or in combination of *P. capsici* (10^4 zoospores g^{-1}) and *R. similis* (100 nematodes bag^{-1}). After three months, the number of diseased plants was recorded and the percentage decrease over control was calculated, normalised by angular transformation and statistically analysed using MstatC.

Observations

The root rot and root lesion indices were observed after three months by destructive sampling. Number of roots showing lesions/rotting was taken as measures by visual scoring using a 0–5 scale as described above. The growth parameters such as plant height, number of leaves and root biomass were also recorded.

Statistical analyses

Data obtained in the greenhouse experiments were analysed using ANOVA and two-factorial completely randomised design (CRD). Least significant differences were calculated at the 5% probability level of significance for comparison of different treatments.

Results

Effect of endophytic bacteria on growth of black pepper plantlets

Stem cuttings

Pre-bacterised stem cuttings recorded significantly less rot index with concomitant increase in the number of sprouts. The stem bacterisation significantly decreased the lesion size on the stem cuttings (Figure 1).

The sprouting behaviour of black pepper stem cuttings was superior with *P. aeruginosa, B. megaterium* and *C. luteum* treatments than the untreated stem

Figure 1. Pre-plant stem treatment using endophytic bacteria: a strategy against *P. capsici* infection on stem cuttings. Stem cuttings were treated in bacterial suspension (*P. aeruginosa*) ($\sim 10^9$ cfu ml^{-1}) for 30 min (shoot bacterisation). A mycelial plug of *P. capsici* was inoculated on the bacterised cut end of the shoot and incubated in a moisture chamber 28 ± 1°C and lesions on the stem cuttings were recorded.

cuttings. Number of leaves was more in plants treated with *B. megaterium* or *P. aeruginosa* than with *C. luteum* and *P. putida* (Table 2). Reduction in root rot lesion was recorded in bacterial treatment which was on par, but not in the untreated one.

Rooted cuttings

Pre-plant root bacterisation of black pepper plantlets had positive effect on growth performances such as height of plantlets, number of leaves, root biomass and total biomass. Root bacterisation with *P. putida* significantly increased all the growth promoting parameters (Figure 2).

Effect of endophytic bacteria on P. capsici *and* R. similis *infection on rooted cuttings*

P. capsici

The root rot and root lesions indices observed on *P. capsici* challenged and endophyte treated are presented in Figure 3. The root rot and root lesions were recorded as less in chemical treatments which were on par with *P. aeruginosa, P. putida* and *B. megaterium*. These three endophytic bacteria showed more than 60% black pepper rooted plantlets free from infection. Among the endophytic bacteria, *P. aeruginosa* recorded least rot and lesion indices which had reflected on production of plantlets free of *Phytophthora* infection in pathogen challenged trials (Figure 3). In absence of *P. capsici*, the endophytic bacterial treatment showed improved growth performances of rooted cutting as observed on plant height, number of leaves, roots biomass and total biomass of the rooted cuttings (Data not shown).

R. similis

Bacterised plants when challenged with *R. similis*, least root rot and root lesions were observed for *C. luteum* and *B. megaterium* which were on par with the nematicide, phorate. In *R. similis* sick soil, *C. luteum* and *B. megaterium* performed equally well for suppression of nematodes and its infestation on roots (Figure 4). Pre-plant root treatment with endophytic bacteria resulted in more number of healthy plants than the untreated check. It is interesting to observe that the nematode suppression by the endophytic bacteria on the root resulted in enhancing growth parameters such as

Table 2. Effect of pre-plant stem bacterisation on sprouting and growth of black pepper plantlets.

Treatments	Sprouting (%)	Number of leaves	Rot index	Lesion inhibition (%)*
B. megaterium	63.0 (52.7) a	2.8 a	1.0 b	39.03 (38.61) c
P. putida	53.3 (46.9) ab	1.8 b	1.0 b	67.63 (55.36) b
P. aeruginosa	70.3 (57.3) a	2.3 ab	0.5 b	90.43 (73.93) a
C. luteum	61.0 (51.6) a	2.0 b	1.5 b	40.93 (39.71) c
Control	32.8 (34.9) b	0.5 c	3.0 a	–

Values in the indices are Arc Sine transformed values. Means followed by the same letter designation are not significantly different according to the Duncan's Multiple Range Test at the $p = 0.05$. *Lesions on the stem cuttings were recorded and the percentage decrease over control (lesion inhibition [%]) was calculated, normalised by angular transformation and statistically analyzed using MstatC.

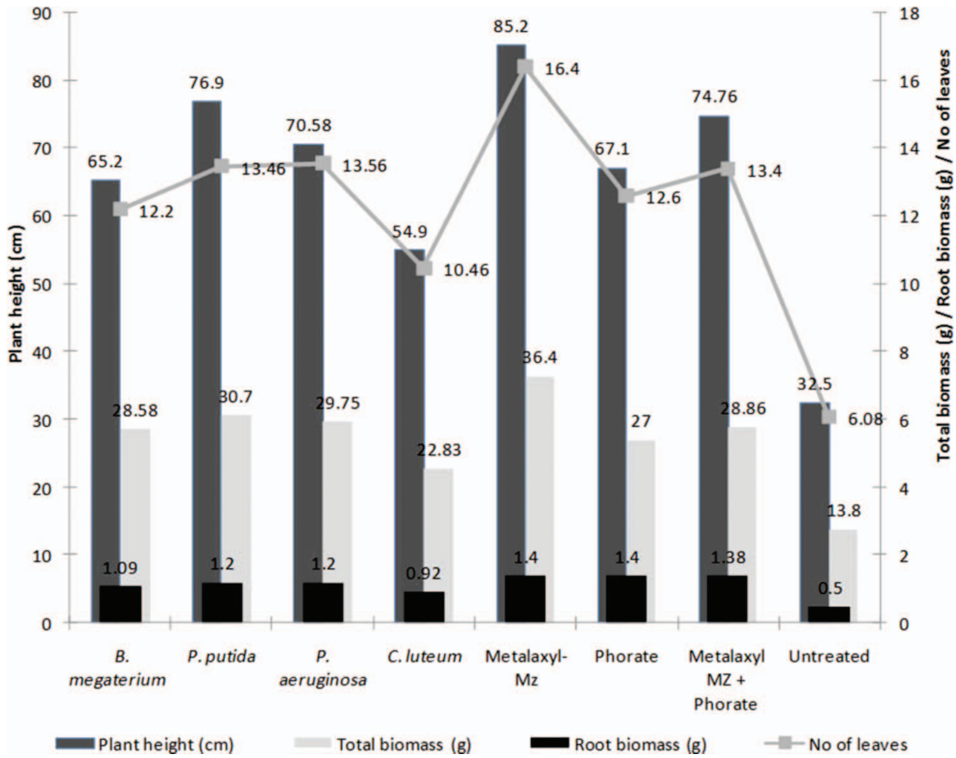

Figure 2. Growth attributes of endophytic bacteria treated plantlets of black pepper (Pooled mean of four independent trials). $LSD_{(p=0.05)}$: Plant height 34.6; Total biomass 12.7; Root biomass 0.7; Number of leaves 6.3.

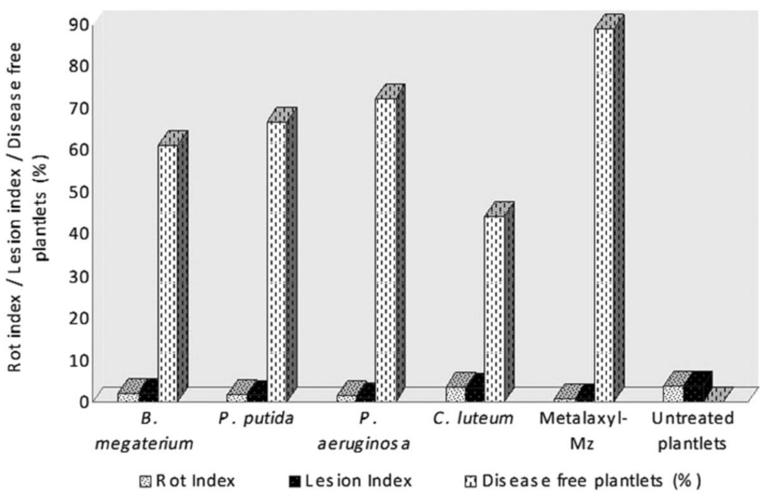

Figure 3. Rot and lesion indices of endophytic bacteria treated plantlets *vis-à-vis* production of *Phytophthora*-free plantlets in soil inoculated with *P. capsici*. Values presented are the mean of nine replicates. $LSD_{(p=0.05)}$: Rotting index – 1.84; Lesion index – 1.86; Disease-free plantlets (%) – 18.7.

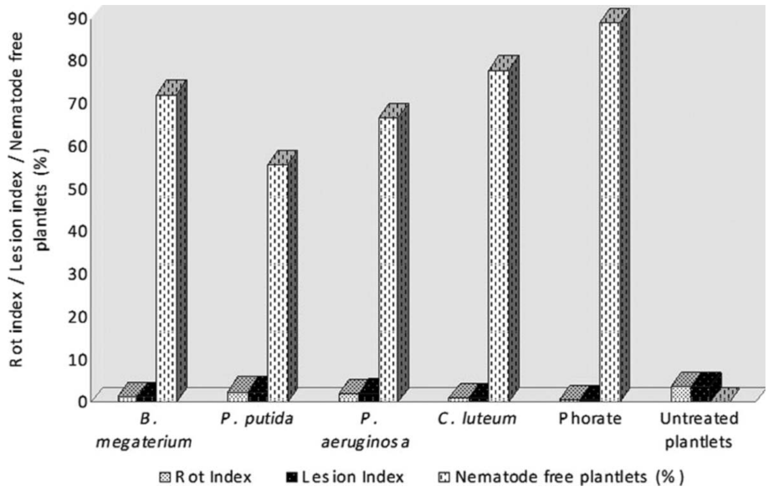

Figure 4. Rot and lesion indices of endophytic bacteria treated plantlets *vis-à-vis* production of nematode-free plantlets in soil inoculated with *R. similis*. Values presented are the mean of nine replicates. $LSD_{(p=0.05)}$: Rotting index – 1.82; Lesion index – 1.85; nematode-free plantlets (%) – 17.31.

plant height, number of leaves, root biomass and total biomass on rooted cuttings (Data not shown).

Interestingly, total biomass and plant height of the black pepper rooted cuttings were superior in *Bacillus* and *Curtobacterium* treated plants than *Pseudomonas* treated ones when the plants were subjected to nematode (*R. similis*) inoculations. This is in contrast to the result obtained when the plant was subjected to *Phytophthora* inoculation where *C. luteum* performed poorly.

Combined inoculation of **P. capsici** *and* **R. similis**

When the rooted cuttings were inoculated with *P. capsici* and *R. similis* together, three of the endophytic bacteria except *C. luteum* significantly reduced the root rot as well as the root lesions which was on par with chemicals used in the trial. In *P. capsici* and *R. similis* sick soil, *P. aeruginosa* and *B. megaterium* performed equally well with more than 65% plantlets free from *R. similis* and *Phytophthora* infection (Figure 5). As a result, protection offered by the endophytic bacteria against the pathogens had a positive effect on the growth parameters such as plant height, number of leaves, root biomass and total biomass on rooted cuttings.

Discussion

High quality of planting material is a critical input for success of any horticulture venture and it is especially true for a high-value perennial spice crop such as black pepper. To ensure the quality of the planting materials, an effective production and protection system is of paramount importance. Propagating material used in black pepper nurseries includes rooted cuttings and nodal cuttings which are known reservoirs of pathogen inoculum and often difficult to visualise. It is, therefore, pre-requisite to select the ''Pathogen Free'' starting material followed by their protection

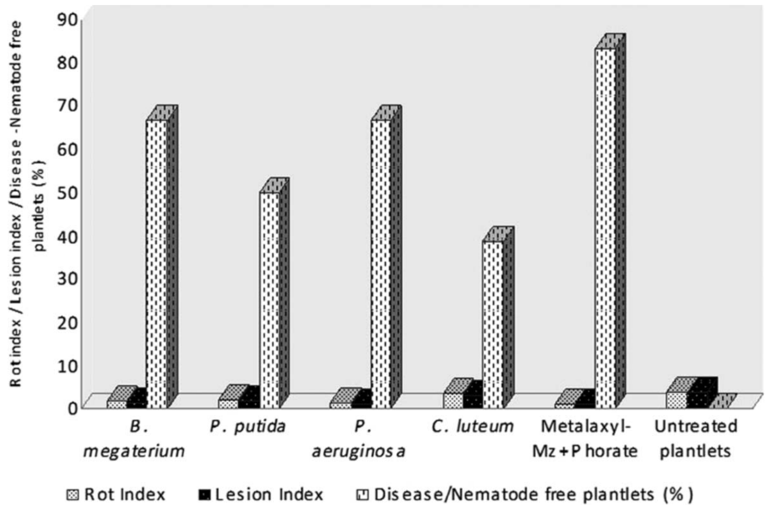

Figure 5. Rot and lesion indices of endophytic bacteria treated plantlets *vis-à-vis* production of disease/nematode-free plantlets in soil inoculated with *R. similis* and *P. capsici*. Values presented are the mean of nine replicates. $LSD_{(p=0.05)}$: Rotting index – 1.55; Lesion index – 1.46; Disease-/nematode-free plantlets (%) – 20.02.

by effective crop protection strategies (Parthasarathy et al. 2007). This ''clean start'' can be achieved by selecting the propagating material from disease-free areas, inspecting and indexing them carefully before they enter a disease-free nursery. Planting materials can be treated with chemicals that kill pathogens on their surfaces. Alternatively, planting materials can be treated with biologicals that prevent the infection besides inducing growth promotion. Though effective for disease management, the indiscriminate application of pesticides poses a serious threat to public health and the environment, besides responsible for emergence of resistant types of pathogens. Therefore, integrated pest management (IPM) strategies involving biological control with introduced microorganisms has been identified as an inevitable component of organic agriculture (Cook 1993). Attempts have been made on the biological control of foot-rot of black pepper using bacterial antagonists (Jubina and Girija 1998). Anith et al. (2002) have identified bacterial antagonists which can colonise and protect the planting material against *P. capsici*-induced wilt of black pepper in the nursery. Several rhizo and endophytic bacterial communities have been identified in black pepper that can be exploited for production of plantlets of black pepper in a nursery (Tran et al. 2008; Aravind et al. 2009, 2010).

We have shown that the naturally occurring plant associated endophytic bacterial species such as *P. aeruginosa*, *P. putida*, *B. megaterium* and *C. luteum* can be delivered into the black pepper plantlets through stem or rooted cuttings, the two major starting material for large-scale production of black pepper planting material. Such a delivery mechanism for endophytic bacteria during early stage of its development would ensure production of disease-free planting material. Such a strategy resulted in production of over 60% plantlets free from *Phytophthora* or *Radopholus*. Besides producing disease-free plantlets, these bacteria enhanced the growth attributes such as the number of leaves, root biomass and total biomass of the plantlets. Biocontrol potential of black pepper associated endophytic bacterial communities on *P. capsici* and *R. similis* has been discovered in the current investigation. The disease suppressive activities of *P. aeruginosa* MTCC5410 against *P. capsici* infection in the cut shoots of black pepper was earlier demonstrated by Dinu et al. (2007) and subsequently by several others (Rajina 2007; Priyanka 2008; Aravind et al. 2009). Among the endophytes, *P. aeruginosa* protected black pepper stem cuttings in a density dependent manner with the concentration of bacterium of $10^{5–6}$ per gram of stem tissues (Rajina 2007). Interestingly, species of *Pseudomonas* were found superior when compared to the other two bacterial genera for growth promotion especially when the plants were stressed due to both pathogens together (*P. capsici* and *R. similis*). Ability of *Pseudomonas* to protect the plants from biotic stress especially from fungal and bacterial pathogens is well known (Beckers and Conrath 2007). Endophytes offer a wide range of benefits to plants such as promoting growth (Barka et al. 2002; Kang et al. 2007) and reducing disease severity (Coombs et al. 2004; Senthilkumar et al. 2007). Endophytic nature of *Pseudomonas* in plant tissue, which improves the plant growth and also acts as biocontrol agents has been published in various reports (Shin et al. 2007; Prieto and Mercado-Blanco 2008).

Unlike *Phytophthora* management, the nematode management poses challenges as there is a paucity of nematicides in the market. The use of phorate for nematode suppression is under scanner due to its extreme toxicity to the environment. Being a migratory endoparasite, locating an efficient biocontrol agent against *R. similis* is still a big challenge (Chabrier and Queneherve 2003). Mendoza and Sikora (2009) reported the endophytic bacteria *Bacillus firmus* produce bioactive compounds against *R. similis* in banana. We have further shown that plant associated endophytic bacteria such as *C. luteum* and *B. megaterium* can be exploited for suppression of *R. similis* and its infestation in black pepper plantlets.

The plants can be bioprimed during the initial stages of its establishment for protection against pathogens (Beckers and Conrath 2007; El-Mougy and Abdel-Kader 2008). Priming in beneficial plant–microbe associations has most extensively been studied in the interaction of plants with ISR-inducing plant growth promoting rhizobacteria (PGPR) (Conrath et al. 2002, 2006; Paré et al. 2005). Such a biopriming would reduce the need for excessive use of chemicals for producing planting material of black pepper for large-scale distribution. The prophylactic effect of these bacteria on two important nursery pathogens has been demonstrated. *P. aeruginosa* and *B. megaterium* were superior when compared to the other two bacteria for disease suppression when the plants were dually challenged with both *P. capsici* and *R. similis*. Pseudomonads are aggressive colonisers of the root and shoot which have broad spectrum of antagonistic activity against root-infecting fungi and root-knot nematodes (Sharma and Nowak 1998; Siddiqui et al. 2000, 2001). Based on our greenhouse trails we found that these four endophytic bacteria can be used in black pepper nurseries for production of disease-free planting materials.

One of the interesting observations in our study is a sound delivery mechanism for the endophytic bacteria in black pepper and their ability to improve the growth performance of black pepper rooted cuttings. This pre-emptive stem and root bacterisation yielded significantly more healthy plants even under pathogen challenged conditions. The most significant recommendation that emerged from these trials is pre-plant stem or root bacterisation as delivery strategy for the endophytic bacteria with concomitant production of disease-free planting material of black pepper.

Acknowledgements

The authors are grateful to Department of Biotechnology (DBT), New Delhi for funding the project. Facilities obtained from the Head of the Division of Crop Protection, Bioinformatics Center and the Director of the Indian Institute of Spices Research, Calicut are gratefully acknowledged.

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