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An improved method for selection of antagonistic bacteria against *Phytophthora capsici* Leonian infections in black pepper (*Piper nigrum* L.)

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Abstract

An improved and rapid method for selection of bacteria primarily based on its inhibitory effect on pathogenesis of *Phytophthora capsici* on black pepper (*Piper nigrum*) was developed. Reductions in mycelial growth of *P. capsici* in dual plating assay and inhibition of lesion on excised black pepper shoots *in planta* assay were calculated. The inhibition ranged from 0.0% to 92.8% among the 19 bacterial isolates tested against *P. capsici* in the *in planta* assay III where the bacterized shoots were challenge inoculated with *P. capsici*. The *in planta* assay III could select only two bacterial strains among the 19 isolates as effective against *P. capsici* whereas, four and six bacterial strains in the *in planta* assays I and II, respectively. The dual plate method, where only two-way interaction between the pathogen and antagonist was studied, could select four bacterial strains as effective ones. The *in planta* selected bacterial strains could protect the black pepper plants from foot rot infection when tested in the nursery. The *in planta* assay based on this three-way interaction among black pepper (host)-*P. capsici* (pathogen)-candidate bacterial strains (antagonist) was found to be an effective method to select antagonistic bacteria against the foot rot pathogen.

Keywords: biological control, black pepper, *in planta* assay, *Phytophthora capsici, Piper nigrum.*

Introduction

Phytophthora capsici Leonian causes foot rot disease in black pepper (*Piper nigrum* L.) and other plantation crops in India and other tropical countries. It affects all parts of black pepper plants namely, roots, stems, shoots, leaves and spikes. In nurseries, where the plants are propagated through runner shoots, the pathogen infects stems, shoots and emerging roots resulting in rotting of plants. Excised runner shoots with single node obtained from the field serve as propagation material for large-scale production of planting materials in nurseries. The pathogen is transmitted, by and large, to the main field through infected rooted cuttings and soil. Controlling the disease at the nursery stage itself assumes significance for production of disease-free rooted cuttings thereby avoiding disease spread and successful establishment of black pepper plantations. Prophylactic application of fungicides such as metalaxyl, copper oxychloride, and phosphonate are generally recommended for the management of the disease. However, the necessity for frequent application of fungicides makes this method

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of disease control unsustainable and unsafe. This has necessitated the search for safer, environment-friendly disease management alternatives such as biological control using microorganisms. However, the success of biological control of plant disease depends largely on the antagonistic potential of the candidate microbes against the target pathogen. Conventionally, the antagonistic microflora is selected by adopting dual plate assay where the inhibitory effect of the prospective biological control agent is tested against the pathogen *in vitro* (Weller & Cook 1986). This kind of selection may not result in a potential biological control agent. Therefore, it has been suggested to use *in planta* assay for selection of antagonistic microorganisms against a given target pathogen (Albouvette *et al.* 1993; Anith *et al.* 2003). In the present paper, an improved, efficient, and rapid bioassay method is reported for selection of candidate microorganisms based on their effect on pathogenesis of *P. capsici* on excised shoot of black pepper.

Materials and methods

Culture conditions and organisms

Nineteen putative endophytic bacterial strains were isolated from black pepper var. Panniyur-5, cv. Karimunda and *in vitro* plantlets by adopting the method of Sturz *et al.* (1999) and stored in frozen glycerol at -80°C (Table 1). The isolates were tentatively identified as fluorescent pseudomonads (3 strains), non-fluorescent pseudomonads (3 strains), *Bacillus* spp. (5 strains), *Arthrobacter* spp. (2 strains), *Micrococcus* spp. (3 strains) and three unidentified strains, based on biochemical tests conducted as recommended in Bergey's Manual. Individual bacterial isolates were streaked on nutrient agar (peptone 5 g l^{-1}, beef extract 2 g l^{-1} and sodium chloride 5 g l^{-1}) amended with 2,3,5 - tri phenyl tetrazolium chloride (30 mg l^{-1}), and incubated at 28°C for 24–48 h. An isolated colony of each bacterium was then transferred to 100 ml nutrient broth amended with tryptophan (100 µg l^{-1}) which was incubated for 24 h at 28°C, with constant agitation (120 rpm). The bacterial isolates were assayed for antagonism by adopting conventional dual plate method as well as by three other *in planta* assay methods as described below.

In vitro *antagonism by bacterial isolates*

The bacterial isolates were evaluated for their ability to inhibit *P. capsici* on agar plates (Weller & Cook 1986). A mycelial plug of actively growing *P. capsici* was placed onto the centre of carrot agar medium and the putative endophytic bacterial strain was streaked 2 cm away on either side of *P. capsici.* The plates were then incubated at 28°C for 5 days or until the leading edge of fungus in the control plate reached the edge of the plate. The radial growth of fungal mycelium was measured and per cent inhibition of growth over control was calculated.

In planta assay I

Runner shoots (about 8 cm length) with at least one node, excised from healthy black pepper (cv. Karimunda) vine, were washed thoroughly with tap water and surface sterilized with sodium hypochlorite 0.1% solution for 10 min. The shoots were then washed five times with sterile distilled water and blot dried on a sterile filter paper for about 30 min. They were bacterized by completely dipping them in bacterial suspension for 1 h and then spread on a sterile blotting paper for drying excess moisture (Anith *et al.* 2003). The bacteria treated shoots were challenge inoculated with mycelial plug of *P. capsici* (Strain IISR 97-166, obtained from National Repository of *Phytophthora,* Indian Institute of Spices Research, Calicut). The fungus was inoculated onto each shoot 2 cm above the nodal region after making a pinprick on the stem surface. The inoculated shoots were kept in a plastic tray, with moist blotting paper and incubated at 25°C for 3 days. The length of dark lesion that developed along the inoculated spot on the excised shoots was measured after 96 h. Shoots dipped in nutrient medium and metalaxyl-mancozeb (1.25 g l^{-1}) served as control.

Table 1. List of bacterial isolates used in the study

Bacterial isolate	Source of isolation	Remarks
Bp-12	Roots of black pepper var. Panniyur-5	Nal^R, Chl^R, HCN^+, Suc^+
Bp-25	Roots of black pepper var. Panniyur-5	Nal^R, Chl^R, Suc^+
Bp-30	Stems of black pepper var. Panniyur-5	$Spec^R$, Amp^R, HCN^+
Bp-35	Stems of black pepper var. Panniyur-5	Str^R, $Spec^R$, Amp^R, HCN^+, Suc^+
Bp-40	Stems of black pepper var. Panniyur-5	Kan^R
Bp-41	Stems of black pepper var. Panniyur-5	Nal^R
Bp-42	Stems of black pepper var. Panniyur-5	Gen^R, Nal^R, Tet^R
Bp-44	Stems of black pepper var. Panniyur-5	$Spec^R$, Amp^R
Bp-47	Roots of black pepper var. Panniyur-5	$Spec^R$, Amp^R
Bp-52	Roots of black pepper cv. Karimunda	$Spec^R$, Amp^R
Bp-55	Roots of black pepper cv. Karimunda	Str^R, Chl^R, Kan^R
Bp-56	Roots of black pepper cv. Karimunda	$Spec^R$, Amp^R
Bp-60	Stem of black pepper cv. Karimunda	$Spec^R$, Amp^R
Tc-5	*In vitro* plantlets of cv. Karimunda	$Spec^R$, Amp^R
Tc-8	*In vitro* plantlets of cv. Karimunda	Kan^R
Tc-9	*In vitro* plantlets of cv. Karimunda	Str^R
Tc-10	*In vitro* plantlets of cv. Karimunda	Nal^R
Tc-16	*In vitro* plantlets of cv. Karimunda	$Spec^R$, Amp^R
Tc-17	*In vitro* plantlets of cv. Karimunda	Str^R

Amp=Ampicillin; Chl=Chloramphenicol; Gen=Gentamycin; HCN^+= Hydrogen cyanide producer; Kan=Kanamycin; Nal=Nalidixic acid; R=Resistance; Spec=Spectinomycin; Str=Streptomycin; Suc^+=Utilization of succinic acid; Tet=Tetracycline.

In planta *assay II*

In this bioassay, the methodology mentioned above was slightly modified and only the bottom portion of the excised shoot (2–3 cm) below the node was dipped in bacterial suspension for 1 h and *P. capsici* was inoculated above the node after pinprick on the surface of cuttings. The shoots after pathogen inoculation were incubated in a moisture chamber at 25°C for 96 h. Dark brown to black lesion that developed on the shoot after 96 h was measured.

In planta *assay III*

This assay is a modification of the above method wherein only the bottom part of the shoot cuttings (2–3 cm) below the node was dipped in bacterial suspension for 1 h and the mycelial plug of *P. capsici* was inoculated on the same bacterized cut end of the shoot without pinprick. The excised bacterized shoot, after inoculation was incubated in moisture chamber at 25°C for 96 h. Dark brown to black lesion that developed after 96 h was measured.

In vivo *evaluation of bacterial isolates*

In order to confirm the results obtained *in vitro*, an *in vivo* trial was conducted wherein the bacterial isolates were evaluated in rooted plants. The roots of black pepper rooted cuttings var. Pournami were dipped in the bacterial suspension (10^{10} cell ml^{-1}) for 30 min and replanted in pot mixture (soil: sand: farmyard manure 2:1:1). Root dipping with metalaxyl-mancozeb (1 g l^{-1}) served as control. The plants were challenged inoculated with zoospores of *P. capsici* after 15 days. The disease incidence was recorded at weekly intervals up to 5 months. The diseased plants were examined on PVPH amended carrot agar (Tsao & Ocana 1969) for confirming the *P. capsici* infection.

Results and discussion

Reductions in radial growth of mycelium in dual plating and lesion length on excised shoots of black pepper in three *in planta*

Fig. 1. Dual plate assay

assays were compared. In dual plate assay, the inhibition ranged from 4.0% to 72.5% among the bacterial isolates tested against *P. capsici* (Fig. 1). A few isolates were on par with the fungicide, metalaxyl-mancozeb, which is recommended for the control of *Phytophthora* infection in black pepper. Six of the bacterial strains evaluated caused more than 50% mycelial growth inhibition. The reduction in lesion length on black pepper shoots ranged from 4.7% to 77.4% among the bacterial isolates in *in planta* assay I. Among the 19 bacterial strains evaluated, 10 of them induced more than 50% inhibition of lesion in both *in planta* assays I and II (Figs. 2 and 3). The reduction in lesion length ranged from 9.5% to 68.3% *in planta* assay II. When assayed by *in planta* assay III, where *P. capsici* was inoculated on the bacterized nodal end of the shoots, the reduction of lesion ranged from 0.0% to 92.8 % among different isolates (Figs. 4 and 6). Unlike the other methods, this method showed wide range of lesion inhibition and hence antagonistic and non-antagonistic isolates could be differentiated. The assay was validated using another 57 putative endophytic bacteria. Only one strain (Bp-35) could reduce over 90.0% lesion indicating its potential for biological control of *P. capsici*. When evaluated *in vivo* the strain Bp-35 could successfully control *P. capsici* infection in black pepper (Fig. 5). This strain was found to inhibit the mycelial growth of *P. capsici* in dual plate assay also. Few isolates though showed mycelial inhibition in dual plate assay, failed to protect the plants from *P. capsici* infection. This clearly indicated the inconsistency and inefficiency of the dual plate assay.

Several procedures have been practiced for selection of antagonistic microbes against plant pathogens in various host-pathogen systems (Broadbent *et al.* 1971; Randhawa & Schaad 1985; Rhodes *et al.* 1987; Kloepper 1991; Han *et al.* 2000). Among them, the "dual plating assay" is widely practiced for rapid screening of antagonists. Though popular, this method lacks consistency, as it depends heavily on the interaction between the pathogen and the test antagonist on the nutrient media. This assay does not involve the host plant during the selection process besides being conducted in a controlled environment. Probably this could be one of the reasons for inconsistencies encountered in biological control of plant diseases in several instances. To circumvent this problem of inconsistency, Anith *et al.* (2003) has recommended a novel *in planta* assay for selection of antagonistic bacteria against *P. capsici* infection in black pepper. In this assay, the pathogen was inoculated on the bacterized shoot cuttings after making an injury by pinprick to enable the infection by *P. capsici*. However, the possible variation and influence of the pinprick on the shoot on the outcome of the bioassay is largely ignored in this assay. Hence in the current study a

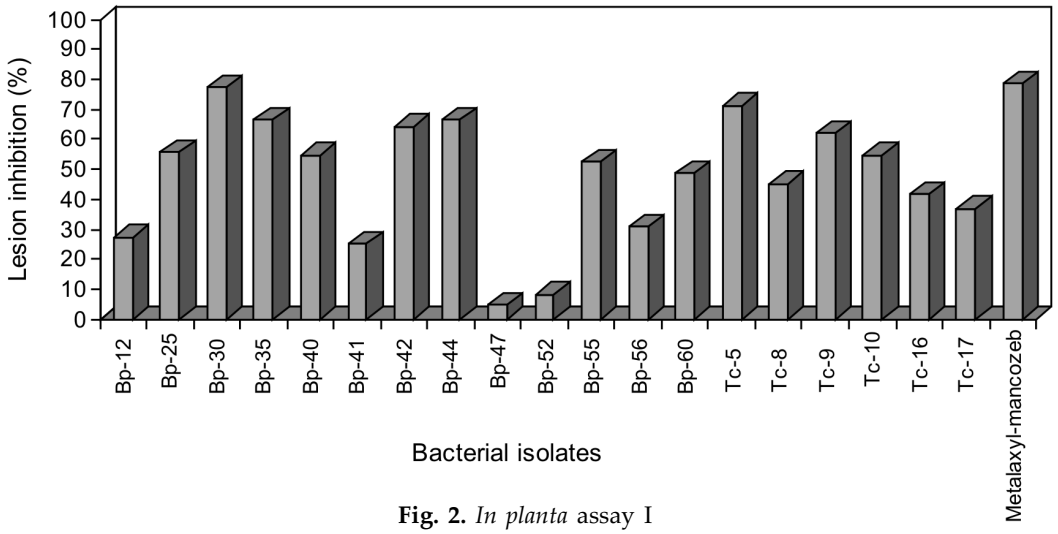

Fig. 2. *In planta* assay I

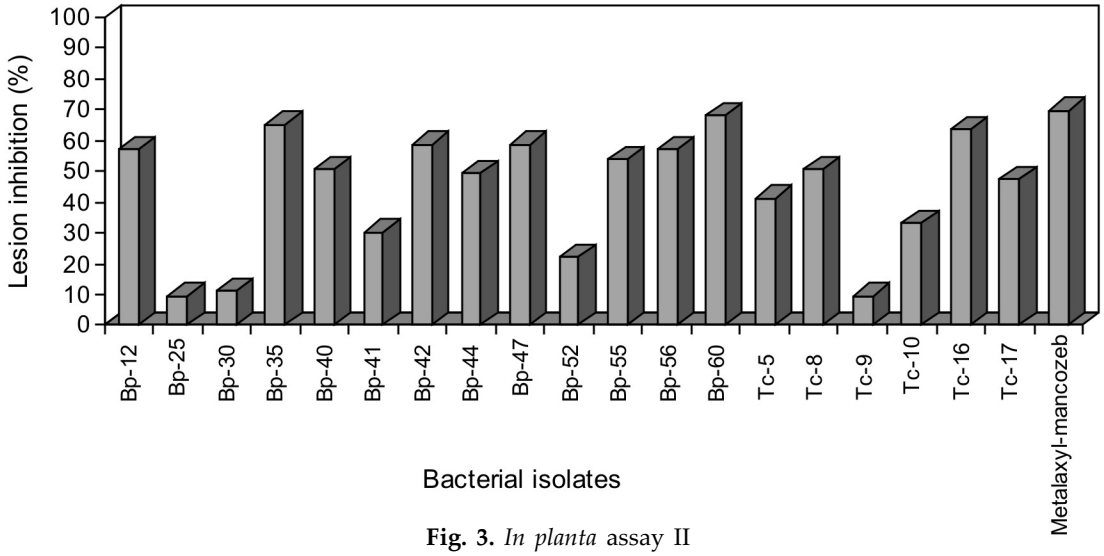

Fig. 3. *In planta* assay II

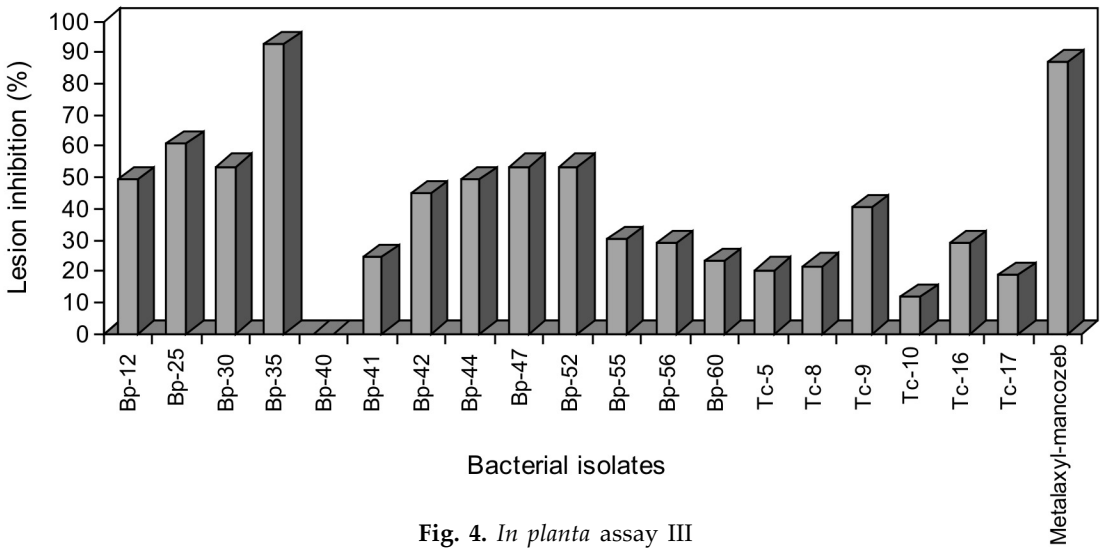

Fig. 4. *In planta* assay III

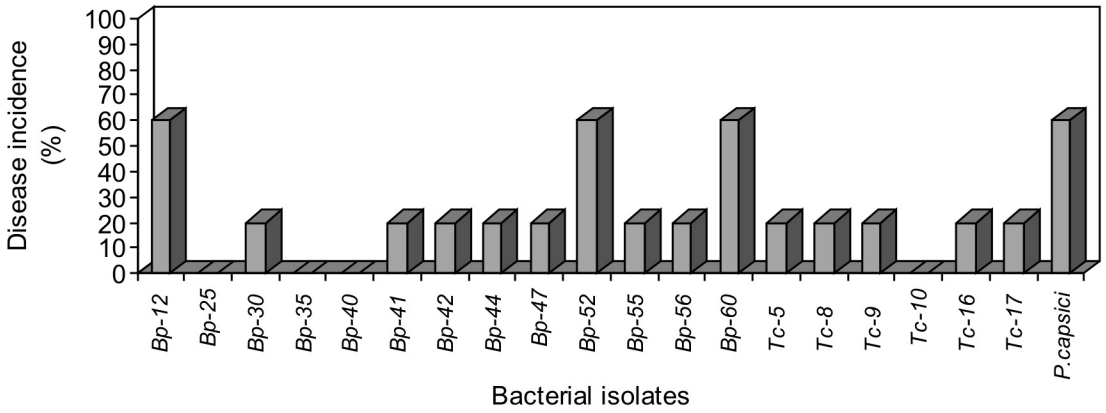

Fig. 5. Suppression of *Phytophthora* foot rot of black pepper by various strains of bacteria in pot culture experiment

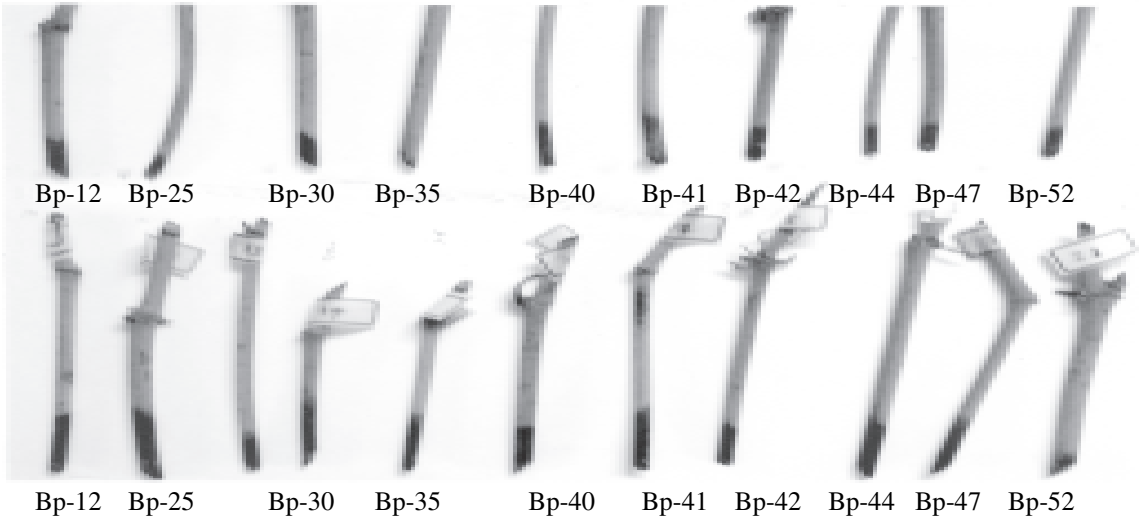

Fig 6. Evaluation of bacterial strains on lesion development on black pepper shoot (*in planta* assay III)

modified method was adopted by challenge inoculating the pathogen at the cut end of the shoot where bacterial inoculation was done before. The modified method does not necessitate any additional injury on the shoot prior to inoculating the pathogen. Though *P. capsici* attacks all parts of black pepper plants, excised runner shoots were selected for screening as they are widely used as planting material in nurseries.

Although many of the bacteria treated cut shoots or "bacterized shoots" succumbed to the disease after 48 to 72 h of pathogen inoculation, few bacterial isolates could effectively prevent the rotting (black to brown lesion) induced by *P. capsici*. This inhibition of rotting on the shoots can be exploited for identifying the right antagonistic bacterial strain that prevent the pathogenesis. This assay depends largely on the effect of bacteria on the pathogenesis of the *P. capsici* rather than on their direct effect on the mycelial growth alone. The number of strains found effective against *P. capsici* infection by the modified *in planta* assay is considerably less (10.2%) as compared to the other assays indicating the high level of selection process of this method. Several isolates of bacteria (Bp-52 and Bp-60), though successfully suppressed the mycelial growth *in vitro*, failed to prevent the lesion formation to desired level on the shoots when tested *in vivo* on the

cut shoots of black pepper. The current results proved that the *in vitro* assay is only next to *in vivo* assay to select any prospective biocontrol agents against *P. capsici*. Therefore an improved *in planta* assay involving the pathogen (*P. capsici*), the host plant (black pepper) and the antagonist (bacterial isolates) is proposed as an effective selection method for antagonistic bacteria against *P. capsici* infection black pepper.

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