Novel *in planta* assay for selection of antagonistic bacteria against *Phytophthora capsici* on black pepper (*Piper nigrum*. L)

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Introduction

Foot rot of black pepper caused by *Phytophthora capsici* is estimated to cause an annual loss of 4.5-7.5 million dollars on global sales. Stem cuttings with single node obtained from the runner shoots of black pepper serve as the planting material for large-scale production of plantlets in state and privately owned nurseries. The pathogen is transmitted to the main field through the infected rooted cuttings and rooting medium besides premature rooting of cuttings in the nursery itself. Controlling the disease at the nursery level assumes significance for production of disease free vigorous rooted cuttings for successful establishment of pepper plantations. Prophylactic application of fungicides such as metalaxyl, copper based fungicides, phosphonates are recommended for the management of the disease. However, continuous application of fungicides resulted in emergence of resistant isolates of pathogen to fungicides particularly against metalaxyl makes this method of disease control unsustainable (Ham *et al*., 1991). The need for agriculture to become more sustainable and less dependable on chemical pesticides has necessitated the search for safer, environment friendly disease management alternatives. One of the alternatives to chemical control of plant disease is biological control using introduced microorganisms, which holds great promise in the era of organic agriculture.

Success of biological control of plant pathogen depends largely on the potential of the candidate antagonist against the target pathogen and its ability to cause disease in plants. Conventionally the antagonistic microflora were found by adopting ‘dual plating’ where only the inhibitory effect of the candidate microbe was tested on the pathogen (Dennis and Webster., 1971). This kind of screening assay is not always effective in finding genuine biocontrol agent. Therefore it has been suggested to conduct '*in planta*' assay for screening antagonistic microorganisms against target pathogens (Alabouvette *et al*., 1993, Anith *et al*.., 2003). In the present investigation we report an efficient, reliable and rapid bioassay for investigating the effect of candidate microorganism on pathogenesis of pathogen *P. capsici* on black pepper stem cuttings. Three way interaction among black pepper – *P. capsici* - bacterial strains was studied where the pathogenic potential of *P. capsici* was assayed on bacterized stem cuttings.

Material and methods

Bacterial strains were isolated from black pepper plants (Sturz *et al*. 1999), and stored as frozen glycerol stocks. List of strains and their colony characters are furnished in (Table 1). Individual bacterial isolates were streaked on nutrient agar plate amended with 2,3,5 tri phenyl tetrazolium chloride, and was incubated at 28°C for 48 h. An isolated colony of each bacterium was then transferred to 100ml nutrient broth amended with tryptophan (100µg /ml). It was then 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.

In vitro* antagonism by bacterial isolates on *P. capsici

Bacterial isolates were tested for their ability to inhibit *P. capsici* on agar plates (Dennis and Webster., 1971). A mycelial plug of actively growing *P.capsici* was placed on to the centre of carrot agar medium and endophytic bacterial strain was streaked 2 cm away on either side of mycelial plug. Plates were then incubated at 28°C for about five 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 percent inhibition of growth was estimated

***In planta* assay I using single node cuttings** (Anith *et al*., 2003)

Stem cuttings of about 8 cm length with at least one node were made from runner shoots of healthy black pepper (c.v *Karimunda*) plant. The cuttings were washed thoroughly with tap water and surface sterilized with 0.1% sodium hypochlorite solution for 10 min. The cuttings were then washed five times with sterile distilled water and blot dried on a sterile filter paper. Stem cuttings were bacterized by completely dipping them in bacterial suspension for 1 h and then spread on a sterile filter paper for drying. The stem cutting after bacterization was challenge inoculated with *P. capsici* (mycelial plug) after pinprick. The inoculated cuttings were kept in a tray, with moist filter paper to maintain humidity and incubated at 25°C. Three replications were maintained for each bacterial isolate. Length of black lesion developed around the inoculated region of the stem cuttings was recorded after 96 h. Stem cuttings dipped in nutrient medium and metalaxyl (1.25g/l) served as check. Stem cuttings without pinprick served as absolute control.

***In planta* assay II**

In this bioassay, the above methodology was slightly modified where only the lower portion of the cuttings (2 - 3 cm) below the node was dipped in bacterial suspension for 1h and *P. capsici* was inoculated above the node after pinprick on cuttings. The stem cuttings, after inoculation was incubated in a moisture chamber. Dark brown lesion to black lesion developed after 96 hours was measured and percent lesion inhibition was calculated.

***In planta* assay III**

This assay is modified from the above method where lower part of the cuttings (2 – 3 cm) below the node was dipped in bacterial suspension for 1h and the mycelial plug of *P.capsici* was inoculated on the bacterized cut end of the stem without pinprick. The stem cuttings, after inoculation was incubated in a moisture chamber. Dark brown lesion to black lesion developed after 96 hours was measured and percent lesion inhibition was calculated. This assay allowed us to study induction of defense reaction by the candidate microorganism.

Results

Reduction in mycelial growth for dual plating and lesion developed on black pepper stem cuttings in three *in planta* assay were measured. The percent reduction in mycelial growth and lesion length as compared to untreated control was calculated. The data was subjected to statistical analysis and was tabulated (Table 2 and Table 3).

In dual plate assay, the percent inhibition ranged from 4 -72.5% among the bacterial isolates tested against *P.capsici*. It is interesting to note that few isolates are on par with the fungicide, metalaxyl that is generally recommended for the control of *Phytophthora* infection in crop plants including black pepper. Six of the bacteria evaluated caused growth inhibition more than 50%.

The lesion inhibition or reduction in lesion length on black pepper stem cutting was in the range of 4.7 – 77.4% among the bacterial isolates in *in planta* assay I. Among the nineteen bacteria evaluated, ten of them had caused more than 50% inhibition of lesion in both *in planta* assay II and I. Similarly the reduction in lesion length was in the range of 9.5% - 68.3% in *in planta* assay II.

When assayed by *in planta* assay III screening method, where the *P.capsici* was inoculated on the bacterized nodal end of the stem cutting, the reduction of lesion was in the range of 0 – 92.8 % among different isolates. Unlike the other methods, this method has shown wide range of lesion

inhibition where highly inhibitory isolates and non – effective isolates have been found. Five of the bacteria caused lesion length more than 50 %.

Discussion

Biological control of soil borne pathogen is a promising alternative to chemical control, which can be practical in organic production of black pepper. One of the prerequisite for the success of biological control of plant diseases using introduced microorganism is availability of very efficient strains with high degree of antagonistic activity against the pathogen.

Several procedures have been suggested for selection of antagonistic microbes against plant pathogen in various host - pathogen systems (Broadbent *et al*., 1971; Han *et al*., 2000; Kloepper *et a*l., 1991; Randhawa and Schaad 1985; Rhodes *et al*., 1987). Among them the dual plating assay for quick screening of efficient strain is widely practiced. Though used widely this method lacks consistency, as it depends only on interaction between the pathogen and biological control agent. More over the pathogenic potential of the pathogen on the host is not studied in this method. To circumvent this problem of in consistency Anith et al., (2003) has recommended an *in planta* assay for selection of antagonistic bacteria against *P. capsici*. In this assay pathogen was inoculated after pinprick, fresh phenolics exuded from the injure portion can influence the ability of the microbe.

In the third *in planta* assay *P. capsici* was inoculated on the cut end of the stem below the node without pinprick. This closely resembled condition prevailing in pepper nurseries. Though *P. capsici* attacks all parts of pepper plants stem cuttings were selected as the host plant for screening, as they are the planting material of the crop. Number of strains selected in *in planta* assay III is less compared to the other assays, phenolics exuded from the injured portion might have influenced the disease prevalence. *In planta* assay III that closely mimics the nursery condition would be ideal for the effective screening of *P. capsici* against pepper cuttings.

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Sl.No	Isolates	Source	Varieties	Characteristics and remarks
1.	Bp-12	Black pepper root	Panniyur-5	Nal^R, Ch^Rl, HCN^+, Suc^+
2.	Bp-25	Black pepper root	Panniyur-5	Nal^R, Chl^R, Suc^+
3.	Bp-30	Black pepper stem	Panniyur-5	Spc^R, Amp^R, HCN^+
4.	Bp-35	Black pepper stem	Panniyur-5	Str^R, Spc^R, Amp^R, HCN^+, Suc^+
5.	Bp-40	Black pepper stem	Panniyur-5	Kan^R,
6.	Bp-41	Black pepper stem	Panniyur-5	Nal^R
7.	Bp-42	Black pepper stem	Panniyur-5	Gen^R, Nal^R, Tet^R
8.	Bp-44	Black pepper stem	Panniyur-5	Spc^R, Amp^R
9.	Bp-47	Black pepper root	Panniyur-5	Spc^R, Amp^R
10.	Bp-52	Black pepper root	Sreekara	Spc^R, Amp^R
11.	Bp-55	Black pepper root	Sreekara	Str^R, Chl^R, Kan^R
12.	Bp-56	Black pepper root	Sreekara	Spc^R, Amp^R
13.	Bp-60	Black pepper stem	Sreekara	Spc^R, Amp^R
14.	Tc-5	Tissue culture plant	Sreekara	Spc^R, Amp^R
15.	Tc-8	Tissue culture plant	Sreekara	Kan^R
16.	Tc-9	Tissue culture plant	Sreekara	Str^R
17.	Tc-10	Tissue culture plant	Sreekara	Nal^R
18.	Tc-16	Tissue culture plant	Sreekara	Spc^R, Amp^R
19.	Tc-17	Tissue culture plant	Sreekara	Str^R

Table 1. Details of strains used in the study.

- Nal : Nalidixic acid, Str : Streptomycin, Kan : Kanamycin, Amp : Ampicilin, Chl : chloromphenicol, Spc : Spectinomycin, Gen : Gentamycin, Tet : Tetracyline, ^{R} : Resistance, HCN^+: Hydrogen cyanide production, Suc^+: Utilization of succinic acid

Sl. No	Bacteria	Dual plating	*In planta* assay I	*In planta* assay II	*In planta* assay III
1	Bp-12	55.3 (48) G	27.4 (31.5) HI	57.1(49.2) BCD	49.3 (44.6) C
2	Bp-25	21.9 (27.9) J	55.9 (48.5) BCDEFG	9.5(15.8) F	60.9 (51.5) C
3	Bp-30	59.7 (50.6) F	77.4 (61.6) BC	11.1(14.1) F	53.6 (47.1) C
4	Bp-35	72.5 (58.4) C	66.7 (54.8) BCDE	65.1 (53.8) B	92.8 (75) B
5	Bp-40	29.8 (33.1) I	54.7(47.8) BCDEFG	50.8 (45.5) BCD	0 (3.6) H
6	Bp-41	15.5 (23.2) K	25 (26.3) I	30.2 (33.1) DE	24.6 (29.7) EF
7	Bp-42	15.5 (23.2) K	64.3 (53.4) BCDE	58.7 (50.2) BCD	44.9 (42) CD
8	Bp-44	29.8 (33.1) I	66.7 (54.8) BCDE	49.2 (44.5) BCD	49.3 (44.6) C
9	Bp-47	34.5 (36) H	4.7 (11.1) J	58.7 (50.1) BCD	53.6 (47.1) C
10	Bp-52	66.7 (54.8) D	8.3 (12.4) J	22.2 (24.7) EF	53.6 (47.1) C
11	Bp-55	4 (11.5) O	52.4 (46.4) CDEFGH	53.9 (47.3) BCD	30.4 (32.8) DEF
12	Bp-56	6.4 (15) N	30.9 (33.6) GHI	57.1 (49.1) BCD	29 (32.6) DEF
13	Bp-60	63.5 (52.9) E	48.8 (44.3) DEFGH	68.3 (55.7) B	23.2 (28.7) EFG
14	Tc-5	10.5 (18.9) M	71.4 (57.7) BCD	41.3 (39.2) BCDE	20.3 (26.7) FG
15	Tc-8	62.4 (52.2) E	45.2 (42.2) DEFGH	50.8 (45.5) BCD	21.7 (27.3) FG
16	Tc-9	16.3 (23.8) K	61.9 (52.2) BCDEF	9.5 (16) F	40.6 (39.5) CDE
17	Tc-10	28.3 (32.1) I	54.7 (47.7) BCDEFG	33.3 (35.1) CDE	11.6 (17.6) G
18	Tc-16	9.6 (18.1) M	41.6 (39.9) EFGHI	63.5 (52.9) BC	29 (32.3) DEF
19	Tc-17	12 (20.2) L	36.9 (37.3) FGHI	47.6 (43.6) BCD	18.8 (25.5) FG
20	Fungicides	76.3 (60.9) B	78.6 (62.4) B	69.8 (56.7) B	86.9 (69.5) B
21	Absolute	100 (90) A	100 (90) A	100 (90) A	100 (90) A
	Cv%	2.8 (2)	22.2 (17.9)	27.3 (21.8)	22.2 (15.8)
	CD	1.24	13.29	15.43	10.49

Table 2. Percent inhibition by *P.capsici* on blackpepper stem cutting by bacterial isolates in different methods

- Data with same letter designation are not different according to DMRT at p= 0.05.
- Data in the parenthesis are Arc Sin transformed data.

Sl. No	Bacteria	Dual plating	*In planta* assay I	*In planta* assay II	*In planta* assay III
1	Bp-12	55.3 (48)	27.4 (31.5)	57.1(49.2)	49.3 (44.6)
2	Bp-25	21.9 (27.9)	55.9 (48.5)	9.5(15.8)	60.9 (51.5)
3	Bp-30	59.7 (50.6)	77.4 (61.6)	11.1(14.1)	53.6 (47.1)
4	Bp-35	72.5 (58.4)	66.7 (54.8)	65.1 (53.8)	92.8 (75)
5	Bp-40	29.8 (33.1)	54.7(47.8)	50.8 (45.5)	0 (3.6)
6	Bp-41	15.5 (23.2)	25 (26.3)	30.2 (33.1)	24.6 (29.7)
7	Bp-42	15.5 (23.2)	64.3 (53.4)	58.7 (50.2)	44.9 (42)
8	Bp-44	29.8 (33.1)	66.7 (54.8)	49.2 (44.5)	49.3 (44.6)
9	Bp-47	34.5 (36)	4.7 (11.1)	58.7 (50.1)	53.6 (47.1)
10	Bp-52	66.7 (54.8)	8.3 (12.4)	22.2 (24.7)	53.6 (47.1)
11	Bp-55	4 (11.5)	52.4 (46.4)	53.9 (47.3)	30.4 (32.8)
12	Bp-56	6.4 (15)	30.9 (33.6)	57.1 (49.1)	29 (32.6)
13	Bp-60	63.5 (52.9)	48.8 (44.3)	68.3 (55.7)	23.2 (28.7)
14	Tc-5	10.5 (18.9)	71.4 (57.7)	41.3 (39.2)	20.3 (26.7)
15	Tc-8	62.4 (52.2)	45.2 (42.2)	50.8 (45.5)	21.7 (27.3)
16	Tc-9	16.3 (23.8)	61.9 (52.2)	9.5 (16)	40.6 (39.5)
17	Tc-10	28.3 (32.1)	54.7 (47.7)	33.3 (35.1)	11.6 (17.6)
18	Tc-16	9.6 (18.1)	41.6 (39.9)	63.5 (52.9)	29 (32.3)
19	Tc-17	12 (20.2)	36.9 (37.3)	47.6 (43.6)	18.8 (25.5)
20	Fungicides	76.3 (60.9)	78.6 (62.4)	69.8 (56.7)	86.9 (69.5)
21	Absolute	100 (90)	100 (90)	100 (90)	100 (90)

Table 3. Percent inhibition by *P.capsici* on blackpepper stem cutting by bacterial isolates in different methods (combined analysis).

- Data in the parenthesis are Arc Sin transformed data.
- CD at p= 0.05 is 11.3

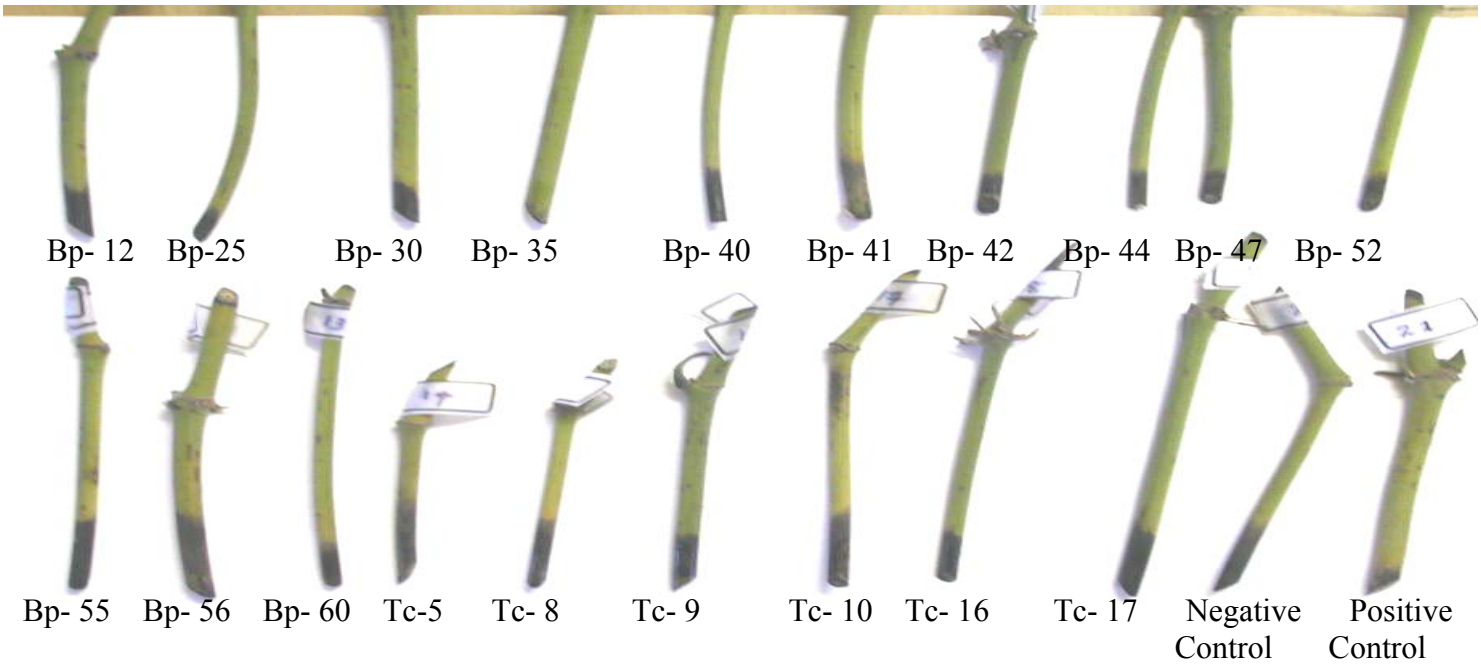

Figure 1. ***In planta assay*** **III**