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Induction of plant defence compounds by *Pseudomonas chlororaphis* PA23 and *Bacillus subtilis* BSCBE4 in controlling damping-off of hot pepper caused by *Pythium aphanidermatum*

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Abstract
Bacillus subtilis strain BSCBE4 and *Pseudomonas chlororaphis* strain PA23 (=*P. aureofaciens*) were effective biocontrol agents against *Pythium aphanidermatum*, the causal agent of damping-off of hot pepper (*Capsicum annum* L.) in greenhouse vegetable production systems. Application of strains BSCBE4 and PA23 at the rate of 20 g kg^{-1} of seed significantly increased the growth of hot pepper seedlings. The efficacies of various carriers in sustaining the population of these strains in storage were assessed. Both the antagonists survived up to 180 days of storage in peat and talc-based formulations. The two bacterial strains induced development of plant defence-related enzymes including phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, phenol content, suppressed incidence of damping-off and increased growth of hot pepper seedlings.

Keywords: *Damping-off*, Pythium aphanidermatum, *hot pepper, induced resistance*, Pseudomonas, Bacillus

Introduction

Hot pepper (*Capsicum annuum* L.) is an important spice crop of the world. Several fungal, bacterial and viral diseases affect it. Among the fungal diseases, damping-off caused by species of *Pythium* is very common in nurseries. *Pythium* species are essentially soil-borne and, consequently, affect the seedlings of vegetables in all nurseries. Pythium is one of the most important root and seedling pathogens in greenhouses, on both vegetables and horticultural crops (Paulitz & Belanger 2001). In British Columbia, *Pythium* spp., were responsible for root disease and crown rot of green house cucumbers (Paulitz et al. 1992). Fungicides offer a degree of protection against pathogens, but their adverse effect on beneficial soil microorganisms and the

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environment cannot be ignored. Therefore, biocontrol agents appear to hold promise in disease management. Since biological control is a key component of integrated disease management, it is important to search for plant growth promoting rhizobacteria (PGPR) active against specific pathogens and evaluate the antagonists for wider application.

Many microorganisms from the rhizosphere can positively influence plant growth and plant health, and are referred to as PGPR. These microbes induce resistance in different plant species against the infection of fungal, bacterial and viral pathogens (Liu et al. 1995; Maurhofer et al. 1998). Strains of *Pseudomonas* have been shown to induce systemic resistance in cucumber to the root pathogen, *P. aphanidermatum.* Chen et al. (2000) reported that *Pseudomonas corrugata* strain 13 and *P. aureofaciens* strain 63-28 systemically suppressed *P. aphanidermatum* in cucumber roots. However, little is known about the ability of these PGPR strains to stimulate higher levels of defence enzymes. Many greenhouse studies (Thomashow & Weller 1988; Kaiser et al. 1989) and field experiments have been conducted (Trapero-Casas et al. 1990) to show the efficacy of PGPR in disease management. Commercial application of PGPR for control of soil-borne diseases depends upon the development of commercial formulations in which bacteria can survive for a considerable length of time, on the development of a suitable method of application to control pathogen establishment and disease development, and assessment of their efficacy under field conditions. The present study reports on the selection of PGPR strains based on disease suppression, growth enhancement, formulation development and the role of defense enzymes phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO), induced by PGPR in the management of damping-off caused by *Pythium* spp.

Materials and methods

Isolation of pathogen

Hot pepper seedlings affected by Pythium damping-off disease were collected from greenhouse nurseries of Tamil Nadu Agricultural University, Coimbatore, India. The pathogen was isolated by the tissue segment method (Rangaswami 1958) on potato dextrose agar (PDA) medium. Seedlings infected by damping-off disease were collected from greenhouse nurseries and washed with sterile water. Infected roots (1 cm length) were excised with a sterile scalpel or blade. Infected tissues were surface sterilized with 0.1% (w/v) mercuric chloride for 60 s. Sterilized root pieces were washed twice with sterile water for 60 s, plated on to sterile Petri plates with 15 mL of PDA, and incubated at room temperature for 5 days. White cottony mycelial growth from the infected root pieces was transferred to plates with 1.5% (w/v) water agar. The growing hyphal tips were transfered to PDA plates. The pathogen was identified as *P. aphanidermatum* (Edson) Fitz, based on the characteristics described by Middleton (1943). Koch's postulates were demonstrated for the pathogen isolates.

Isolation of rhizobacteria. Rhizosphere colonizing *Bacillus subtilis* and *Pseudomonas fluorescens* were isolated from vegetable crops (tomato, brinjal [egg plant] and hot pepper) grown in different geographic areas and soil types of Tamil Nadu Province, India. Root segments (1 g) from different crops were suspended separately in 10 mL of sterile distilled water and vortexed for 5 min. One mL of the suspension was poured and uniformly spread on plates with nutrient agar (NA; 1% (w/v) peptone, 0.5% (w/v)

beef extract, 0.5% (w/v) NaCl, and 1.5% (w/v) agar) and King's B agar (KBA) medium to isolate fluorescent Pseudomonads (King et al. 1954). Four isolates of *P. fluorescens* (PFK1, PFK2, PFK3, and PFK4) were isolated using King's medium B (King et al. 1954). The isolates were characterized based on standard biochemical tests including gelatin liquefaction, nitrate reduction, arginine dihydrolase, and levan formation, growth at 4 and 41°C and utilization of different carbon sources (Hildebrand et al. 1992). *B. subtilis* was isolated by suspending 1 g of fresh roots in 10 mL of sterile water and vortexing the sample as described above. The suspension was held at 75°C for 20 min to kill all vegetative microbial cells. The suspension was then plated on to NA plates and incubated at 37°C for 24 h. Seven *B. subtilis* (BSK1, BSK2, BSK3, BSK4, BSCBE4, BSK5, and BSK6) isolates were recovered. The bacterial colonies were characterized based on their shape, Gram staining, presence of spores, growth under aerobic and anaerobic conditions (Norris et al. 1981; Sneath 1986). *Pseudomonas chlororaphis* strain PA23 was obtained from University of Manitoba, Canada (Savchuk 2002). *Pseudomonas chlororaphis* strain PA23 has shown excellent disease control of phyllosphere (Savchuk & Fernando 2004) and rhizosphere (Mathiyazhagan et al. 2004) plant pathogens.

Screening of antagonistic bacterial isolates

Twelve bacterial strains were screened for antifungal activity against *P. aphanidermatum*. These were *B. subtilis* (BSK1, BSK2, BSK3, BSK4, BSCBE4, BSK5, and BSK6), *P. fluorescens* (PFK1, PFK2, PFK3, PFK4) and *P. chlororaphis* strain PA23. The bacterial isolates were streaked with sterile cotton tips 10 mm from the edge of the plate containing PDA 24 h prior to inoculation of the pathogen. A 5-mm mycelial disc of 5-day-old *P. aphanidermatum* culture was placed at the opposite side of the Petri dish perpendicular to the bacterial streak. Plates were incubated at 28 ± 2°C, and growth of the fungal mycelia towards the bacterial colony and inhibition zone was measured after 72 h incubation.

Shelf life of P. chlororaphis *strain PA23 and* B. subtilis *strain BSCBE4 in different formulations*

P. chlororaphis strain PA23 and *B. subtilis* strain BSCBE4 were most effective. These strains were further used for formulation development. Strains PA23 and BSCBE4 were grown in King's B broth (KMB) and nutrient broth, respectively, for 48 h in shake culture at 150 rpm at room temperature (28 ± 2°C). Shelf life of bacteria was tested in six different carriers: peat, talc, farmyard manure, vermiculite, lignite and gypsum. Carboxy methylcellulose (10 g) was added to 1 kg of the carrier as a sticker and mixed well. The carriers were autoclaved for 45 min at 137.3 kPa pressure. Bacterial suspension (500 mL) containing 11×10^9 colony forming units (cfu) mL^{-1} of broth was added to 1 kg of carrier and mixed well under aseptic conditions. The formulations were air-dried to 20% (w/v) moisture content, packed in three separate polythene bags (three replicates) and incubated at 28 ± 2°C. Samples were taken from each bag containing different carrier material at monthly intervals for up to 6 months. The bacterial population of *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 were assessed on KBA and NA, respectively, using the serial dilution method. Each carrier was replicated three times and populations were estimated at monthly intervals. The experiment was set up as a completely randomized design. Duncan's

multiple range test (DMRT) was used for analysis using the package IRRISTAT version 97 developed by International Rice Research Institute Biometrics Unit, Philippines.

Greenhouse studies

The efficacy of *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 strains to control damping-off of hot pepper in the greenhouse was tested. Seeds of hot pepper were sown in pots containing field soil into which *P. aphanidermatum* at 1:19 ratio (v/v) (sand-maize inoculum/soil) was incorporated. Fifty seeds were sown/pot (15 cm diameter, 30 cm height) and 10 pots/treatment were maintained. The seeds were treated with peat–soil-based formulations of *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 at the rate of 5, 10, 15, and 20 g kg^{-1} of seed. One gram of both the formulated inoculants consisted of 10^9 cfu of bacteria. The fungicide, metalaxyl (4 g kg^{-1}) was included as a standard seed treatment for comparison. For comparing the efficacy of various treatments, both healthy (without pathogen inoculation) and pathogen-inoculated controls were included in the study. All treatments were watered each day. Disease incidence was recorded 25 days after sowing, and shoots and root length were recorded. There were three replicates/treatment and pots were arranged in a completely randomized design. The relative humidity in the greenhouse was maintained at around 80%, and the temperature at 26°C (day) and 20°C (night). The experiment was repeated once.

Induced systemic resistance (ISR)

P. chlororaphis strain PA23 and *B. subtilis* strain BSCBE4 were used for studying ISR. Treatment details were as follows.

Treatment 1: seed treatment with peat-based bacterial strain *P. chlororaphis* (PA23) at the rate of 20 g kg^{-1} of seed;

Treatment 2: seeds treated with bacterial strain PA23 and inoculated with *P. aphanidermatum* 15 days after sowing (50 g sand-maize medium containing 10^3 cfu g^{-1} medium in each pot);

Treatment 3: seed treatment with peat-based bacterial strain *B. subtilis* (BSCBE4) at the rate of 20 g kg^{-1} of seed;

Treatment 4: seeds treated with bacterial strain BSCBE4 and inoculated with *P. aphanidermatum* 15 days after seeding;

Treatment 5: plants inoculated with the pathogen 15 days after seeding; and

Treatment 6: untreated control plants.

Seeds were planted at the rate of 50 seeds/pot (30 cm diameter $\times$ 40 cm height) filled with sterilized potting medium. The potting medium (laterite sandy loam soil:sand:decomposed cow dung manure at 1:1:1, w/w/w) was autoclaved for 1 h on two consecutive days. There were five pots/replicate. There were three replicates/

treatment and five plants were scored/replicate. The pots were arranged in a completely randomized design.

Seedlings with roots were carefully removed from the pots 0, 4, 8, 12, and 16 days after inoculation with the pathogen, and washed several times with sterile distilled water before they were used for extraction of enzymes. Washed seedlings (1 g) from three different replicates were homogenized separately with liquid nitrogen using a pre-chilled mortar and pestle. The homogenized tissues were stored at -70°C.

Assay for detection of phenylalanine ammonia lyase (PAL)

Root and shoots (1 g) of hot pepper seedlings either exposed or not exposed to *P. aphanidermatum* in the growing medium were homogenized in 1 mL of cold 25 mM borate HCl buffer (pH 8.8) containing 0.4 mL of 5 mM mercaptoethanol L^{-1}. The homogenate was centrifuged at 15,000 $\times g$ for 15 min and the supernatant was used as the enzyme source. The assay mixture consisted of enzyme extract (0.2 mL), water (1.3 mL) and borate buffer (0.5 mL), (pH 8.8). The reaction was initiated by the addition of 1 mL of 0.1 M L-phenylalanine,and the reaction mixture was incubated for 1 h at 32°C. The reaction was stopped by the addition of 0.5 mL of 2 N HCl. A blank was run in which phenylalanine was added after adding 2 N HCl. PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm. The enzyme activity was expressed as μmol of cinnamic acid^{-1} min^{-1} mg^{-1} of protein (Dickerson et al. 1984).

Assay for detection of peroxidase activity (PO)

Hot pepper seedlings (1 g) either exposed or not exposed to *P. aphanidermatum* in the growing medium were ground in 1 mL of 0.1 M phosphate buffer (pH 7.0) in a pre-cooled mortar. The homogenate was centrifuged at 15,000 $\times g$ at 4°C for 15 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract and 0.5 mL of 1% H_2O_2, and the reaction mixture was incubated at room temperature ($28 \pm 2^{\circ}$C). The changes in absorbance were recorded at 420 nm at 30-s intervals for 3 min, and the boiled enzyme preparation served as a blank. The enzyme activity was expressed as change in absorbance of the reaction mixture min^{-1} mg^{-1} of protein (Hammerschmidt et al. 1982).

Assay for detection of polyphenol oxidase (PPO)

Hot pepper seedlings (1 g) either exposed or not exposed to *P. aphanidermatum* in the growing medium were ground in 1 mL of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 15,000 $\times g$ for 15 min at 4°C and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 mL of the enzyme extract. The reaction was started with the addition of 0.2 mL of 0.01 M catechol, and the activity was expressed as changes in absorbance at 495 nm at 30-s intervals for 3 min. The enzyme activity was expressed as changes in absorbance min^{-1} mg^{-1} of protein (Mayer et al. 1965).

Estimation of phenol

Hot pepper seedlings (1 g) either exposed or not exposed to *P. aphanidermatum* in the growing medium were ground in a mortar in 10 mL of 80% (v/v) methanol, and the homogenate was centrifuged at $10{,}000 \times g$ for 10 min. The supernatant was evaporated to dryness and the residue was dissolved in 5 mL of distilled water. From this, 0.2 mL was diluted to 3 mL with distilled water, and 0.25 mL of folin-ciocalteau reagent added. One mL of 20% (w/v) sodium carbonate was added after 3 min and mixed thoroughly. The tubes were placed in boiling water for 1 min and cooled. The absorbance was measured at 650 nm against a reagent blank. The phenol activity was expressed in μg catechol g^{-1} of plant tissue (Zieslin & Ben-Zaken 1993).

Results

Screening of antagonistic bacterial isolates

P. chlororaphis strain PA23 and *B. subtilis* strain BSCBE4 showed the largest inhibitory effect of all isolates on mycelial growth of *P. aphanidermatum* on PDA plates, with 37 and 42% reduction from the control plates, respectively (Table I).

Shelf life of B. subtilis *strain BSCBE4 and* P. chlororaphis *strain PA23*

B. subtilis strain BSCBE4 and *P. chlororaphis* strain PA23 survived in all formulations at room temperature ($28 \pm 2^{\circ}$C) for the time period tested (6 months). Peat, talc, farmyard manure (FYM), vermiculite, lignite and gypsum supported the survival of *B. subtilis* strain BSCBE4 up to 150 days of storage (Table II). Peat-, talc- and vermiculite-based formulations supported survival for 180 days of storage, but in other formulations the population density of BSCBE4 declined slowly after 150 days of storage. The population density of BSCBE4 after 180 days of incubation was 2.0–5.3×10^{7} cfu g^{-1} in peat, talc and vermiculite formulations. Although population

Table I. Screening of bacterial isolates against *P. aphanidermatum*.

Bacterial isolates	Mycelial growth of pathogen after 72 h of incubation (mm)[1]	% Reduction of mycelial growth over control	Inhibition zone (mm)
BSK1	77a–d[2]	14.4	2.3e
BSK2	65c–f	27.7	11.0b
BSK3	90a	0.0	0.0 f
BSK4	88ab	2.2	2.0e
BSCBE 4	52f	42.2	36.0a
BSK5	84abc	6.6	2.0e
BSK6	70b–f	22.2	7.0cd
PFK1	73ade	18.8	6.0d
PFK2	76a–d	15.5	3.0e
PFK3	64def	28.8	10.2bc
PFK4	68c–f	24.4	9.0bcd
PA23	56ef	37.7	32.0a
Control	90a	–	–

[1]Plate bioassay on PDA was conducted in Petri dishes of 9 cm diameter at $28 \pm 2^{\circ}$C. [2]Values are the mean of three replicates. Means followed by a common letter within a column are not significantly different ($P = 0.05$) according to DMRT.

Table II. Shelf life of *B. subtilis* strain BSCBE4 in six different formulations.

Formulation	Population after storage at 28±2°C ($\times 10^7$ cfu g^{-1})						
	0 days	30 days	60 days	90 days	120 days	150 days	180 days
Peat	92.0a[1]	80.0a	68.0e	48.0a	32.0a	14.0a	5.3a
Talc	86.5a	76.0a	60.0d	36.5a	28.0a	9.0b	2.0ab
Farmyard manure	90.0a	72.0a	58.0cd	28.0a	10.0b	4.0c	1.0b
Vermiculite	94.0a	66.0a	56.0bc	30.0a	22.0a	10.0ab	2.0ab
Lignite	88.0a	80.0a	52.0ab	14.0a	8.0b	1.1e	1.0b
Gypsum	90.0a	70.0a	48.0a	10.5a	6.0b	2.0d	1.0b

[1]Values are the mean of three replicates. Means followed by a common letter within a column are not significantly different ($P=0.05$) according to DMRT.

decline was observed with all carriers, the population was maintained at a minimum of $1.0–5.3 \times 10^7$ cfu g^{-1} after 6 months storage in different carriers (Table II). Similarly, storage of *P. chlororaphis* strain PA23 maintained a population density of $8.0–11.5 \times 10^7$ cfu g^{-1} in peat and talc formulations after 150 days of storage, but the population declined rapidly after 120 days in vermiculite, lignite, FYM and gypsum carriers (Table III). Although population decline was observed with all carriers, the population was maintained at a minimum of $0.1–1.0 \times 10^7$ cfu g^{-1} after 6 months storage.

Efficacy of B. subtilis *strain BSCBE4 and* P. chlororaphis *strain PA23 against damping-off in hot pepper*

An application rate of 20 g kg^{-1} of peat-based formulation of *B. subtilis* strain BSCBE4 and *P. chlororaphis* PA23 was effective, and was comparable to metalaxyl in reducing the incidence of damping-off. Seed treatment of hot pepper with 20 g kg^{-1} of *B. subtilis* BSCBE4 and *P. chlororaphis* strain PA23 resulted in 68 and 67% reduction of damping-off, respectively, over the control in Trial I, and 65 and 57% reduction, respectively, of damping-off over the control in Trial II. The incidence of damping-off in the inoculated control was 90 and 76% in Trial I and Trial II, respectively. Seed treatment of hot pepper at 20 g kg^{-1} with *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 increased shoot length, but not root length in both trials compared to inoculated and non-inoculated controls (Table IV).

Table III. Shelf life of *P. chlororaphis* strain PA23 in six different formulations.

Formulations	Population after storage at 28±2°C ($\times 10^7$ cfu g^{-1})						
	0 day	30 days	60 days	90 days	120 days	150 days	180 days
Peat	58.0a[1]	50.0a	42.5a	30.0a	22.0a	11.5a	1.0a
Talc	54.0a	48.0a	36.0a	21.0ab	16.0a	8.0b	0.1b
Farmyard manure	49.0a	40.0a	31.0a	12.0bc	7.5a	0.1c	0.1b
Vermiculite	52.0a	42.0a	28.0a	9.0c	1.0b	0.1c	0.1b
Lignite	50.0a	41.0a	26.5a	9.0c	1.0b	0.1c	0.1b
Gypsum	51.0a	39.5a	11.0a	2.5d	0.1c	0.1c	0.1b

[1]Values are the mean of three replicates. Means followed by a common letter within a column are not significantly different ($P=0.05$) according to DMRT).

Table IV. Efficacy of *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 against hot pepper damping-off in the greenhouse.

Seed treatment with bacterial antagonists[1]	Trial I				Trial II			
	Disease incidence (%)	% Reduction over control	Shoot Length (cm)	Root length (cm)	Disease incidence (%)	% Reduction over control	Shoot Length (cm)	Root length (cm)
BSCBE4 (ST-5 g kg^{-1})	62.6b[2]	30.1	12.2e	4.8a–d	44.4bc	41.1	9.6ef	4.1def
BSCBE4 (ST-10 g kg^{-1})	42.3bc	52.7	14.2cd	5.3a–d	41.1bc	45.5	13.3cde	5.0b–f
BSCBE4 (ST-15 g kg^{-1})	33.6c	62.5	15.8abc	6.5abc	40.0bc	47.0	15.6b	5.5a–e
BSCBE4 (ST-20 g kg^{-1})	28.4c	68.3	16.6ab	6.8ab	26.6c	64.7	17.5ab	5.8abc
PA23 (ST-5 g kg^{-1})	59.5b	33.5	13.4d	5.1a–d	43.3bc	42.6	15.6bc	4.8c–f
PA23 (ST-10 g kg^{-1})	46.6bc	47.9	14.8bcd	6.1abc	42.2bc	44.1	14.6bd	5.6a–d
PA23 (ST-15 g kg^{-1})	36.6c	59.0	16.2ab	6.6abc	40.1c	46.9	17.5ab	6.5ab
PA23 (ST-20 g kg^{-1})	30.0c	66.5	16.8a	7.3a	32.2c	57.3	20.6a	6.6a
Ridomil (ST-4 g kg^{-1})	26.4c	70.5	11.0e	4.1cd	25.5c	66.1	11.0de	4.0ef
Control (pathogen-inoculated)	89.6a	–	7.6f	4.3bcd	75.5a	–	6.3f	3.8f
Control (pathogen not inoculated)	–	–	11.6e	4.8a–d	–	–	13.3cde	5.0b–f

[1]BSCBE4, *B. subtilis*; PA23, *P. chlororaphis*; ST, seed treatment. [2]Values are the mean of three replicates. Means followed by a common letter within a column are not significantly different ($P=0.05$) according to DMRT. Percentage data were arcsine transformed prior to analysis.

Induction of defence-related enzymes and phenolic compounds

Plants synthesized higher levels of PAL when hot pepper seeds were treated with either *B. subtilis* strain BSCBE4 or *P. chlororaphis* strain PA23. PAL activity in treatments with *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 was increased 3-fold by the fourth day and 6-fold by the 12th day after inoculation of pretreated seedlings with *P. aphanidermatum*. The activity slowly declined thereafter (Figure 1A). A 2-fold increase in PAL activity was observed in seedlings pretreated with BSCBE4 and PA23 compared to the non-inoculated control. However, in the pathogen-inoculated control, activity of PAL increased up to 4 days and declined thereafter, but the activity was several-fold lower than the plants pretreated with BSCBE4 and PA23 (Figure 1A).

Three- to four-fold increases in the activity of peroxidase and polyphenol oxidase were observed with BSCBE4 and PA23 pretreated hot pepper seedlings inoculated with *P. aphanidermatum* (Figure 1B, C). The activity of PO and PPO increased up to 12 days after soil inoculation with the pathogen and the activity declined thereafter, but the activity of PO and PPO was three to four times greater than the control. The activity reached the maximum level 12 days after pathogen inoculation. The activity was greater with *B. subtilis* strain BSCBE4 than with *P. chlororaphis* strain PA23 (Figure 1B, C).

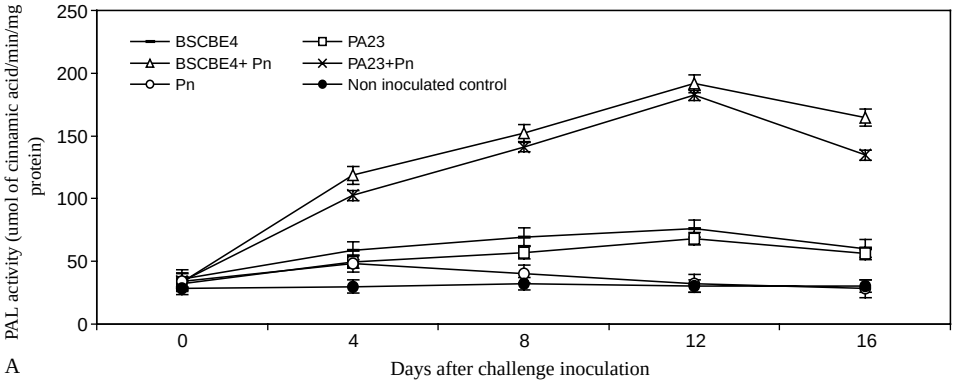
The phenolic content was increased 2-fold in the seedlings pretreated with BSCBE4 and PA23 (Figure 2). The phenolic content slowly increased from 0 to 12 days after pathogen inoculation and started to decline after the 12th day. In plants inoculated with the pathogen alone, the phenol content declined to the initial level, 16 days after inoculation (Figure 2).

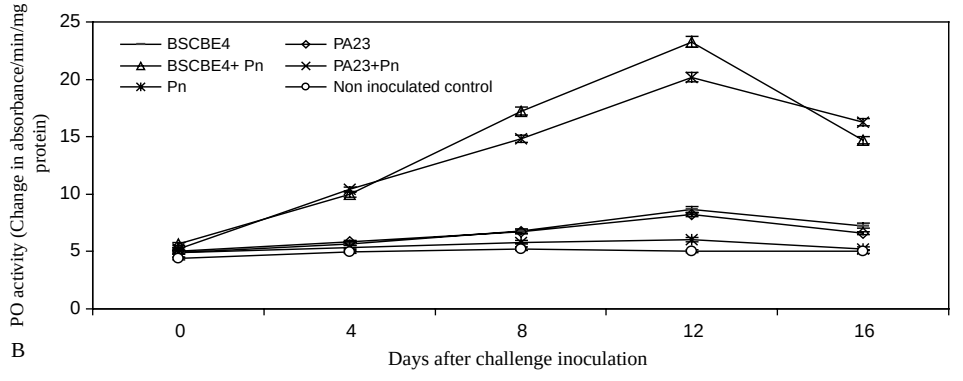
Discussion

B. subtilis strain BSCBE4 and *P. chlororaphis* strain PA23 were both effective against *P. aphanidermatum* and controlled damping-off disease in greenhouse-grown hot pepper. Both bacteria survived well in the formulations, especially in the peat-based formulation for 6 months, while maintaining high population densities. Both were capable of increasing defence-related enzymes (PAL, PO, and PPO) and phenol in hot pepper plants, and inducing systemic resistance of the host to the pathogen.

Seed treatments with cell suspensions of PGPR strains have been found to be effective in controlling several diseases (Kaiser et al. 1989; Trapero-Casas et al. 1990). However, for commercial exploitation, cell suspension treatment is impractical due to difficulty in handling, transport and storage. The present study on the shelf life of different formulations of *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 showed that they survived well in peat- and talc-based formulations for >5 months. Populations of PGPR did not decline in a talc mixture with 20% (w/v) xanthan gum after storage for 2 months at 4°C (Kloepper & Schroth 1981). Bapat and Shah (2000) reported that a formulation of *Bacillus brevis* with vermiculite as a carrier had a shelf life of at least 6 months.

A powder formulation with a longer shelf life would be beneficial. Different carrier formulations of fluorescent pseudomonads have been developed. The talc-based powder formulation containing the antagonistic bacterium was effective against chickpea wilt, pigeon pea wilt, rice blast and rice sheath blight (Vidhyasekaran & Muthamilan 1999). In the present investigation, application of peat-based





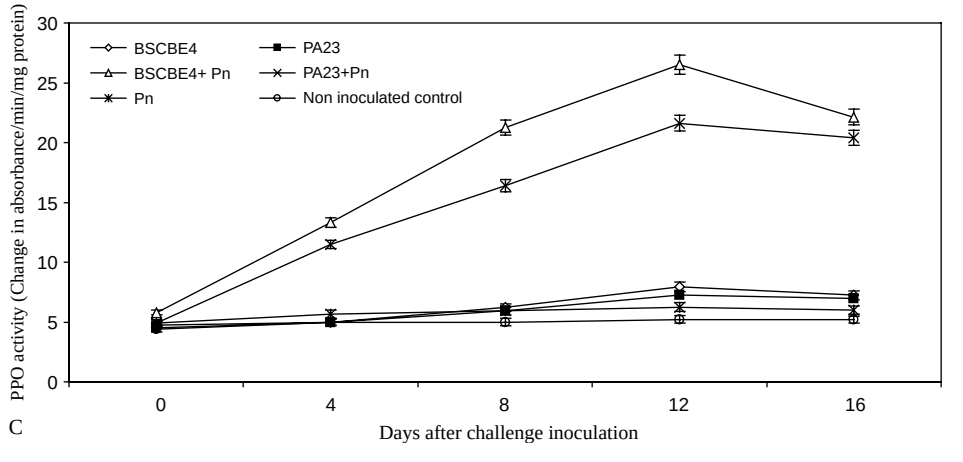


Figure 1. (A) Influence of seed treatment with *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 on the activity of phenylalanine ammonia lyase (PAL) in hot pepper with or without *P. aphanidermatum*. Bars represent the standard deviation. (B) Influence of seed treatment with *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 on the activity of peroxidase (PO) in hot pepper with or without *P. aphanidermatum*. Bars represent the standard deviation. (C) Influence of seed treatment with *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 on the activity of polyphenol oxidase (PPO) in hot pepper with or without *P. aphanidermatum*. Bars represent the standard deviation.

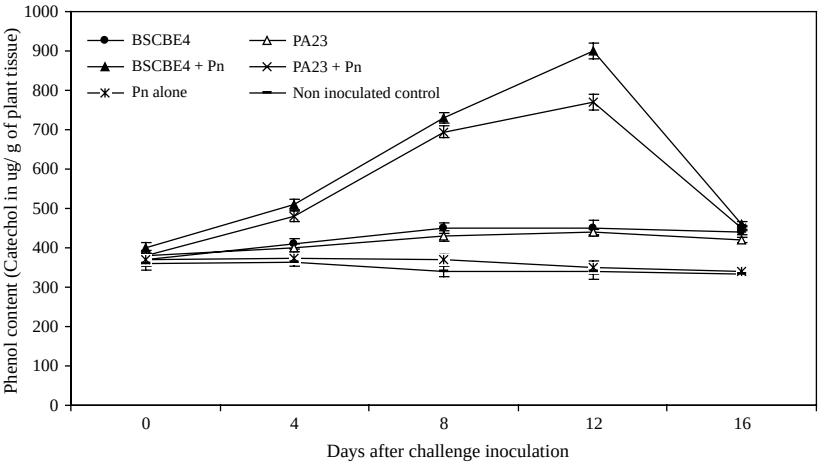


Figure 2. Influence of seed treatment with *P. chlororaphis* strain PA23 and *B. subtilis* strain BSCBE4 on the activity of phenolics content in hot pepper with or without *P. aphanidermatum* (Pn). Bars represent the standard deviation. (BSCBE4, *B. subtilis* strain BSCBE4; PA23, *P. chlororaphis* strain PA23; Pn, pathogen-inoculated control; BSCBE4+Pn, *B. subtilis* BSCBE4 challenged with *P. aphanidermatum*; PA23+Pn, *P. chlororaphis* strain PA23 challenged with *P. aphanidermatum*.)

formulations of BSCBE4 and PA23 as a seed treatment at the rate of 20 g kg^{-1} of seed increased shoot and root length of seedlings, and reduced disease incidence. It was comparable with the metalaxyl seed treatment. The increase in plant growth might be associated with secretion of auxins, gibberellins and cytokinins (Dubeikovsky et al. 1993), and suppression of deleterious microorganisms in the rhizosphere (Gamliel & Katan 1993).

In the present study, seed treatment with bacterial isolates increased the activities of various defence enzymes when the plants were inoculated with the pathogen. PAL plays an important role in the biosynthesis of phenolics and phytoalexins (Daayf et al. 1997). The increase in PAL activity indicates the activation of the phenyl propanoid pathway. The product of PAL is *trans*-cinnamic acid, which is an immediate precursor for the biosynthesis of salicylic acid, a signal molecule in systemic acquired resistance (SAR) (Klessig & Malamy 1994). De Meyer et al. (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean roots and increased salicylic acid levels in leaves. Seed bacterization with *B. subtilis* AF1 resulted in a distinct increase in the activity of PAL in pigeon pea within 24 h (Podile & Laxmi 1998). In the present study, increased activity of PAL was recorded in PA23-treated hot pepper seedlings challenged with *P. aphanidermatum,* and reached a maximum at 12 days. Further, the PAL activity declined after this. In plants inoculated with pathogen alone, the PAL activity declined rapidly. Similarly, the activity of PAL, PO and PPO declined rapidly in tomato after 4 days of inoculation with *Fusarium oxysporum* f.sp. *lycopersici* compared to bacterized plants challenged with the pathogen (Ramamoorthy et al. 2002b). PO and PPO catalyze the last step in the biosynthesis of lignin and other oxidized phenols. The higher PO activity was noticed in cucumber roots treated with *P. corrugata* challenged with *P. aphanidermatum* (Chen et al. 2000). Enhanced PO activity is very often associated with resistance and lignin production (Reuveni et al. 1992). In the present study, seed treatment of *B. subtilis* strain BSCBE4 showed a higher activity 12 days after pathogen inoculation. Increases in

PO and PPO activity at a later stage may contribute to cross linking of hydroxyproline rich glycoproteins (HRGPs), lignifications that will act as barriers against pathogen entry. PO-generated hydrogen peroxide may function as an anti-fungal agent in disease resistance. Hydrogen peroxide inhibits pathogens directly or it may generate other free radicals that are antimicrobial in nature (Chen et al. 2000).

Phenolic compounds are fungitoxic. In the present study, seed treatment and soil application with BSCBE4 and PA23 resulted in increased accumulation of phenolic substances in response to infection by the pathogen. Similarly, the increased accumulation of phenolics was observed between 3 and 5 days in tomato roots bacterized with *P. fluorescens* isolate Pf1 challenged with *F. oxysporum* f.sp. *lycopersici*. In addition, it also increased the activity of PAL, PO, PPO, chitinase and β-1,3-glucanase. Bacterized tomato plants also induced the expression of PR protein chitinase and prevented the establishment of *F. oxysporum*. f. sp. *lycopersici* (Ramamoorthy et al. 2002b). *P. fluorescens* isolate 63-28 induced the accumulation of phenolics in tomato root tissues. The hyphae of *F. oxysporum*. f. sp. *radicis lycopersici* surrounded by phenolic substances exhibited cytoplasmic disorganization and loss of protoplasmic content (M'Piga et al. 1997). Benhamou et al. (2000) reported that an endophytic bacterium *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots following infection by *P. ultimum*. Similarly, Ramamoorthy et al. (2002a) reported that pretreatment of tomato and hot pepper plants with *P. fluorescens* isolate Pf1 challenged with *P. aphanidermatum* induced earlier and higher accumulation of PAL, PO, PPO, and phenolics compared to non-inoculated control.

In conclusion, peat-based formulation developed from *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 reduced the incidence of damping-off of hot pepper. Prior treatment of hot pepper with these PGPR strains triggered the plant-mediated defence mechanism in response to infection by *P. aphanidermatum.* Since the treatments with the two bacterial strains were comparable to metalaxyl in reducing damping-off, it could be used as a component in integrated disease management programmes.

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