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PGPR MEDIATED MANAGEMENT OF STEM BLIGHT OF *PHYLLANTHUS AMARUS* (SCHUM AND THONN) CAUSED BY *CORYNESPORA CASSIICOLA* (BERK AND CURT) WEI

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Bacillus subtilis (BSCBE4), *Pseudomonas chlororaphis* (PA23), endophytic *P. fluorescens* (ENPF1) inhibited the mycelial growth of stem blight pathogen *Corynespora casiicola* (Berk and Curt)Wei under *in vitro*. All these bacterial isolates produced both hydroxamate and carboxylate type of siderophores. But the siderophore production was maximum with the isolate ENPF1. Delivering of talc based formulation of BSCBE4 through seedling dip and foliar application effectively reduced stem blight disease incidence and increased the dry matter production under pot culture and field conditions. Application of BSCBE4, PA23 and ENPF1 increased the defense related enzymes such as peroxidase, polyphenol oxidase, chitinase and β-1,3 glucanase in *P. amarus* up to ten days after challenge inoculation with *C. cassicola*. Native gel electrophoretic analysis revealed that challenge inoculation of pathogen with BSCBE4 and PA23 induced both peroxidase and polyphnol oxidase isoforms.

Keywords: *Phyllanthus amarus*; *Corynespora casiicola*; Peroxidase; Polyphenol oxidase; Chitinase; β-1; 3 glucanase; *Bacillus subtilis*; *Pseudomonas chlororaphis*; Induced systemic resistance

INTRODUCTION

Phyllanthus has been used in ayurvedic medicine for over 2000 years and has a wide number of traditional uses. The World Health Organization has compiled more than 20 000 medicinal plants used in different parts of the world. Among the medicinal plants more than one hundred botanicals have larger potential for commercial exploitation and could be marketed in the world drug markets. *P. amarus*, commonly known as Keelanelli (Tamil), Bhuiaonla (Hindi) belonging to the family Euphorbiaceae occupies a prime position among the commercially cultivated medicinal plants. It

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has a long history of folk use in drug industry for the treatment of dropsy, urinogenital problem, dysentery, diabetes, skin ulcer, dyspepsia, fever, asthma, bronchial infections, tumour and Hepatitis B virus. Though *Phyllanthus* is bestowed with several medicinal principles to cure several human disorders, the crop is susceptible to a number of devastating diseases that affects the quality and quantity of the medicinal principles. With the rise in demand for plant based drugs in global market, commercial cultivation of *Phyllanthus* has gained momentum during the last decade. This has led to the occurrence of stem blight, which caused complete failure of the crop. Since *Phyllanthus* is an export oriented medicinal herb, the current research on the etiology and management of stem blight disease will be a boon to the farming community.

The increased reflection on environmental concern over pesticide use has been instrumental in a large upsurge of biological disease control. Development of fungicide resistance among the pathogens, ground water and foodstuff pollution and the development of oncogenic risks have further encouraged the exploitation of potential antagonistic microflora in disease management. Among the various antagonists used for the management of plant diseases, PGPR play a vital role. Fluorescent pseudomonads have revolutionized the field of biological control of soil-borne plant pathogens. Fluorescent Pseudomonads have been implicated in the control of several wilt diseases caused by *Fusarium* spp (Chen *et al*., 1995) root rot of important crops like wheat, cucumber and tulip (Roberts *et al*., 1997). Red rot in sugarcane caused by *Colletotrichum falcatum* was suppressed by certain strain of Fluorescent pseudomonads (Viswanathan and Samiyappan, 1999). Jayalakshmi *et al*. (1998) reported that managing the fruit rot and die back diseases of chilli by using *B. subtilis*. Ryder *et al*. (1999) reported that *B. cereus* isolate A 47 and *B. subtilis* B 908 consistently reduced the severity of take all of wheat grown in sodic and acid soils. The amount of disease control was similar to that with the biocontrol isolate *P. corrugate* 2140. Gasoni *et al*. (1998) reported that radish seed treatment with *B. cereus* and *P. fluorescens* in peat/vermiculite/clay formulation effectively controlled *Rhizoctonia* damping off in green house studies.

Endophytic bacteria isolated from rape and tomato plants inhibited mycelial growth and reduced the incidence and severity of wilt caused by *V. dahliae* and *F. oxysporium* f.sp. *lycopersici* (Nejad and Johnson, 2000). Endophytic bacteria isolated from rice seed, had strong antifungal activity against *R. solani, P. myriotylum, Gaumannomyces graminis* and *Heterobasidium annosum* (Mukhopadhyay *et al*., 1996). Endophytic strains of *P. fluorescens* 89B.27 was observed to induce resistance in cucumber to *Pseudomonas syringae* pv *lachrymans* as well as to the fungal pathogen *Fusarium oxysporum* f.sp. *cucumerinum* (Liu *et al*., 1995)

MATERIALS AND METHODS

Isolation of pathogen

Phyllanthus amarus seedlings affected by stem blight disease were collected from the field. The pathogen was isolated by tissue segment method (Rangaswami, 1958) and pathogenicity was proved by live plant method and cut stem method.

Isolation of bacterial antagonists

(i) Endophytes

P. amarus seedlings of 30 days old were uprooted and brought to laboratory. Stem and root sections were made using a sterile scalpel. The root and stem samples of 2–3 cm long were excised from 10 plants. Root sections were taken just below the soil line and stem section were taken 5 cm above the soil line. Stem sample were first weighed and surface sterilized with hydrogen peroxide (20%) for 10 min. and rinsed four times with 0.02 M potassium phosphate buffer (pH 7.0). Root samples were surface disinfected with sodium hypochlorite (1.05%) and washed in four changes of 0.02 M phosphate buffer solution. Measured quantity of 0.1 ml aliquot from the final buffer wash was removed and transferred in 9.9 ml tryptic soya broth to serve as sterile check. Samples were discarded, if growth was detected in the sterile check within 48 h. Selected samples were triturated in 9.9 ml of buffer in sterile pestle and mortar. The triturate was serially diluted in potassium phosphate buffer solution and plated on Tryptic Soya Agar (TSA). Representatives of colony morphology were transferred to fresh TSA plated as pure cultures (McInroy and Kloepper, 1995). Identification of plant pathogenic bacteria by the American Phytopathological Society (Schaad, 1992).

(ii) Rhizobacteria

Bacillus subtilis (BSCBE4) was collected from department of plant pathology, Tamil Nadu Agricultural University, Coimbatore, and *Pseudomonas chlororaphis* (PA 23) was obtained from University of Manitoba, Canada.

***In vitro* screening of endophytes and bacterial antagonist**

In vitro antagonism of *P. chlororaphis* (PA23), *B. subtilis* (BSCBE4), five endophytes of *B. subtilis* (ENBS2, ENBS3, ENBS4, ENBS7 and ENBS 9) and five endophytic strains of *P. fluorescens* (ENPF1, ENPF6, ENPF5, ENPF8 and ENPF10) were screened against stem blight pathogen (*C. cassiicola*) of *P. amarus* under *in vitro*.

Testing of endophytes and other bacterial antagonist for siderophores production

Productions of siderophores by bacterial antagonist were assayed by plate assay. The tertiary complex Chrome azural S (CAS)/Fe^{3+}/hexadecyl trimethyl ammonium bromide served as an indicator. The 48 h old culture of the endophytes were streaked on to the succinate medium (Succinic acid-4.0 g, K_2HPO_4-3.0 g, $(NH4)_2SO_4$. $7H_2O$-0.2 g, Distilled water-1 litre, pH-7.0) amended with indicator dye. The resultant dark blue liquid was observed for the formation of bright zone with yellowish fluorescent colour in the dark coloured medium. It was the indication of production of siderophore. The result was scored either positive or negative to this test (Schwyn and Neilands, 1987).

(i) Detection and nature of siderophore

The isolates of *P. fluorescens* and *B. subtilis* were inoculated in 10 ml of King's B broth (Kings *et al*., 1954) and nutrient broth respectively. It was incubated in a rotary shaker

at 120 rpm for 48 h. The bacteria multiplied in the broths were used as the sample for the determination of the nature of siderophore.

(ii) Hydroxamate nature

It was examined by tetrazolium salt test. Instant appearance of a deep red colour by addition of siderophore sample to tetrazolium salt under alkaline conditions indicated the presence of hydroxamate (Snow, 1984)

(iii) Carboxylate nature

It was detected by Vogel's chemical test where the disappearance of pink colour on addition of Phenolphthalein to siderophore sample under alkaline condition indicated carboxylate nature (Vogel, 1987)

Preparation of talc based formulation of bio control agents

A loop full of *P. fluorescens* and *B. subtilis* were inoculated into the King's B broth and Nutrient broth and incubated in a rotary shaker at 150 rpm for 72 h at room temperature ($28 \pm 2^{\circ}C$). After 72 h of incubation the broth containing 9×10^8 cfu/ml was used for the preparation of talc based formulation. To 400 ml of bacterial suspension, 1 kg of the talc powder (sodium ammonium silicate), calcium carbonate 15 g (to adjust the pH to neutral) and carboxy methyl cellulose (CMC) 10 g (as adhesive) were mixed under sterile condition following the method described by Vidhyasekaran and Muthamilan (1995). The product was shade dried to reduce the moisture content to 20% and then packed in polypropylene bags and sealed. At the time of application the population of bacterium in talc formulation was checked to 2.5 to 3×10^8 cfu/g.

Screening of biocontrol agents and fungicides against stem blight disease under glasshouse conditions

To study the biocontrol potential of the antagonistic bacteria (*P. chlororaphis* isolate (PA23), *B. subtilis* isolate (BSCBE4), endophytic *P. fluorescens* (ENPF1) and *T. viride* (Tv-MNT7) an experiment was conducted under pot culture condition. The talc based formulations of the antagonistic bacteria and fungi were delivered as seedling dip, foliar application and combination of seedling dip and foliar application. Foliar spray was given at weekly intervals. The pathogen mass multiplied on stem bits of *P. amarus* was suspended in sterile distilled water and kept in mechanical shaker at 250 rpm to dislodge the conidia. The spore suspension having spore load of 10^4 conidia/ml was sprayed to plants which caused more that 75% infection under glass house condition. The observations on the per cent disease incidence of stem blight were recorded. In addition growth parameters like shoot length, root length and biomatter production was recorded after 90 days of sowing.

Enzyme extraction

The collected samples were homogenized with phosphate buffer (0.1M) (1 g of leaf sample with 1 ml of sodium phosphate buffer pH 7.0). These homogenized samples

were centrifuged at 10 000 *g* for 15 min. The supernatant solution was used as source for analyzing chitinase and β-1, 3 glucanase.

(i) Chitinase

Leaves (1 g) were homogenized in 5 ml of 0.1mol/l sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10 000 × *g* at 4°C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared by taking 2 g of crab-shell chitin (Sigma, USA). It was slowly added into 35 ml of cold concentrated hydrochloric acid with vigorous stirring and placed at 4°C for 24 h. The mixture was filtered through glass wool into ethanol (200 ml) at − 20°C with rapid stirring. The resultant chitin suspension was centrifuged at 10 000 *g* for 20 min and the chitin pellets were washed repeatedly with distilled water until the pH became neutral (Roberts and Selitrennikoff, 1988). The commercial lyophilized snail gut enzyme was desalted as described by Boller and Mauch (1988) for the colorimetric assay of chitinase 10 μl of 1 mol/litre sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml eppendorff tube. After 2 h at 37°C, the reaction was stopped by centrifugation at 1000 × *g* for 3 min. An aliquot of the supernatant (0.3 ml) was pippetted into a glass reagent tube containing 30 μl of 1mol/l potassium phosphate buffer (pH 7.0) and incubated with 20 μl of 3% (w/v) desalted snail gut enzyme for 1 h. After 1 h the reaction mixture was brought pH 8.9 by addition of 70 μl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in the boiling water bath for 3 min and then rapidly cooled in an ice water bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 min at 37°C and immediately thereafter the absorbance was measured at 585 ηm. *N*-acetyl glucosamine (GlcNAc) was used as a standard. The enzyme activity was expressed as ηmol GlcNAc equivalents per min per mg of protein. (Boller and Mauch, 1988).

(ii) β-1, 3 - glucanase

The enzyme activity was colorimetrically assayed. Crude enzyme extract of 62.5 μl was added to 62.5 μl of laminarin (4%) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 μl of dinitro salicylic acid and heated for 5 min. on boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 ηm. The crude extract preparation with laminarin with zero time incubation served as blank. The enzyme activity was expressed as μg equivalent of glucose per min per mg of protein (Pan *et al*., 1991).

Native gel electrophoresis

Since, combined application of bacterial biocontrol agents as seed soaking and foliar spray recorded the maximum PO and PPO activity on the 4th day after challenge inoculation through the colorimetric method, it was subjected for the analysis of PO and PPO isoforms. The isoform profiles of PO and PPO were examined by discontinuous native polyacrylamide gel electrophoresis (native-PAGE) (Laemmli, 1970). Plant samples were collected on the 4th day after

challenged inoculation with pathogen, when the activity of PO and PPO was maximum.

The protein extract was prepared by homogenizing 1 g of plant sample in 1 ml of 0.1 M sodium phosphate buffer (pH 7.0) and centrifuged at 16 000 *g* for 20 min at 4°C. The protein content of the sample was determined (Bradford, 1976). Sample (50 μg protein) were loaded onto 8% polyacrylamide gels (Sigma, USA). After electrophoresis, PO isoforms were visualized by soaking the gels in staining 0.05% benzidine (Sigma, USA) and 0.03% H_2O_2 in acetate buffer (20 mM, pH 4.2) (Nadlony and Sequira, 1980). For assessing PPO isoforms profile, the gels were equilibrated for 30 min in 0.1% p-phenylenediamine followed by addition of 10 mM catechol in the same buffer (Jayaraman *et al.*, 1987).

Testing the efficacy of biocontrol agents and fungicides against stem blight of *P. amarus* under field condition

A field trial with three replications of the different treatment (plot size 4 m × 3 m) was laid out under completely randomized block design. The observations on per cent incidence of stem blight were recorded on 90 days after sowing. The total biomass production was estimated during harvest (90 DAS).

Statistical analysis

The statistical analysis of the data were made using package IRRISTAT version 92 of International Rice Research Institute Biometrics unit, Philippines.

RESULTS

Symptomatology and pathogenicity

The disease was initially characterized by the appearance of water soaked dark brown coloured elongated spots on the basal portion of the stem. Later the spots turned necrotic and subsequently the spots spread over the entire stem. As the disease severity increased, the spots coalesced and resulted in blighting of the stem. The foliage of the blighted plants turned yellow followed by premature defoliation. This disease occurred right from seedling stage (25 days-old) to maturity of the crop. The pathogen responsible for the disease was isolated from the infected portion and identified as *Corynespora cassiicola* (Indian Type Culture Collection (ITCC), I.D.No. 5572.03).

In vitro antagonism of bacterial antagonists

In vitro antagonism of twelve bacterial antagonists (PA23, BSCBE4, ENBS2, ENBS3, ENBS4, ENBS7, ENBS9, ENPF1, ENPF6, ENPF5, ENPF8, and ENPF10) was used against *C. cassiicola*. The bacterial antagonist *B. subtilis* (BSCBE4) isolated from rhizosphere region was found to be effective in inhibiting the radial growth of *C. cassiicola*. The inhibition zone produced by BSCBE4 was 22.0 mm. It was followed by the endophyte *P. fluorescens* (ENPF1) and *P. chlororaphis* isolate (PA23). PA23 and ENPF1 were not found to differ significantly in inhibiting the radial growth of *C. cassiicola* (Table I).

TABLE I *In vitro* antagonism of bacterial and fungal antagonists against *C. cassiicola*

Bacterial antagonists	In vitro *antagonism*	
	Radial growth (mm) of C. cassiicola	*Inhibition Zone (mm)*
PA23	65.0[c]	15.00
BSCBE4	58.0[d]	22.00
ENPF 1	66.0[c]	14.00
ENPF 6	80.0[b]	–
ENPF 5	78.0[b]	–
ENPF 8	82.0[b]	–
ENPF 10	78.0[b]	–
ENBS 2	82.0[b]	–
ENBS 3	85.0[b]	–
ENBS 4	84.0[b]	–
ENBS 7	86.0[b]	–
ENBS 9	85.0[b]	–
TVMNT7	66.4[c]	
Control	90.0[a]	–

Values are mean of three replications. In a column, means followed by a common letter are not significantly different at the 5 % levels by DMRT. ENPF – Endophytic *P. fluorescens,* ENBS – Endophytic *B. subtilis,* PA23 – *P. chlororaphis*, BSCBE4 – *B. subtilis,* TVMNT7 – *T. viride* isolate.

Effect of biocontrol agents and fungicides against stem blight under glasshouse condition

The biocontrol agents and fungicides were evaluated for the management of stem blight under glass house condition. Standardization of delivery system of bacterial and fungal antagonists indicated that delivering bacterial and fungal antagonists through seedling dip and foliar spray were highly effective in inhibiting stem blight incidence rather than individual application of antagonists either through seedling dip (or) foliar spray. Among the bacterial antagonists tested, delivering of BSCBE4 (*B. subtilis*) as seedling and foliar spray recorded the lowest stem blight incidence of 15.60% with increased root and shoot length. It was followed by the application of ENPF1 and PA23 delivered through seedling dip and foliar spray.

In general, the growth parameters like root length and shoot length were also found to be maximum in the treatment that received the combined application of seedling dip and foliar spray. Efficacy of fungicides against stem blight indicated that spraying of Ridomil at 0.2% levels recorded 16.30% stem blight incidence and was comparable with PA23 delivered through both seedling dip and foliar spray (Table II).

Siderophore production

The endophytic bacteria isolated were tested for the production of siderophore under *in vitro*. All the isolates produced yellowish fluorescent pigments which was the indication of siderophore production. Among the different endophytes, surface area production of siderophore was higher with isolates ENPF1 (10.00 cm^2) (Table III).

All the bacterial isolates produced hydroxamate type of siderophores. But the comparison of isolates for the intensity of production of hydroxamate type siderophores indicated that *B. subtilis* (BSCBE4), *P. chlororaphis* (PA23), ENPF1, ENPF6, ENPF5, and ENPF8 produced higher intensity of hydroxamate types. Studies on carboxylate type of siderophore indicated that all the isolates produced carboxylate

TABLE II Effect of biocontrol agent and fungicide against stem blight under glasshouse conditions

Treatment	*Per cent disease incidence (90 DAS)*	*Shoot length (cm)*	*Root length (cm)*	*Dry weight (g/plants)*
BSCBE4 (SD)	18.23^{bc} (25.25)	42.6^{c}	14.5^{bc}	3.42^{a}
BSCBE4(FS)	16.21^{bc} (23.71)	44.5^{b}	16.2^{bc}	3.64^{a}
BSCBE4 (SD + F S)	15.43^{c} (23.11)	47.5^{a}	18.2^{a}	3.85^{a}
ENPF1(SD)	16.22^{bc} (23.72)	32.4^{hi}	12.3^{ef}	3.12^{bc}
ENPF1(FS)	16.60^{bc} (24.01)	34.5^{fg}	13.6^{cde}	3.22^{bc}
ENPF1 (SD + FS)	15.60^{c} (23.23)	36.6^{e}	14.4^{bc}	3.36^{b}
PA23 (SD)	18.30^{bc} (25.30)	30.5^{ik}	12.5^{def}	2.85^{de}
PA23 (FS)	16.46^{bc} (23.93)	33.4^{gh}	14.3^{cd}	2.93^{cd}
PA23 (SD + FS)	16.20^{bc} (23.70)	40.3^{d}	16.2^{b}	3.46^{bc}
TvMNT 7 (SD)	20.00^{bc} (26.54)	32.5^{hi}	12.4^{cf}	2.85^{cd}
TvMNT 7(FS)	18.60^{bc} (25.52)	33.6^{gh}	13.8^{cde}	2.98^{bc}
TvMNT 7 (SD + FS)	19.63^{b} (26.27)	35.4^{ef}	14.6^{bc}	3.38^{ef}
Ridomil (FS)	16.30^{bc} (23.78)	28.3^{l}	11.3^{fg}	2.46^{ef}
Benomyl (FS)	18.34^{bc} (25.33)	29.5^{kl}	10.2^{g}	2.24^{f}
Mancozeb (FS)	17.18^{bc} (24.46)	28.5^{l}	10.8^{fg}	2.64^{de}
Control	82.34^{a} (65.18)	22.5^{m}	10.2^{g}	2.13^{f}

Values are mean of three replications. SD-Seedling dip, FS-Foliar spray. Values in parenthesis are arcsine transformed values. In a column, means followed by a common letter are not significantly different at the 5 % levels by DMRT. ENPF-Endophytic *P. fluorescens,* ENBS-Endophytic *B. subtilis,* PA23-*P. chlororaphis,* BSCBE4-*B. subtilis*.

TABLE III Siderophore production by bacterial antagonists

Bacterial endophytes	*Siderophore production*	*Surface area of siderophore production after 24 h (cm^2)*	*Hydroxamate type*	*Carboxylate type*
ENPF1	+ ve	10.00^{a}	+ +	+
ENPF6	+ ve	8.00^{bc}	+ +	–
ENPF5	+ ve	2.50^{ef}	+ +	+
ENPF8	+ ve	9.20^{ab}	+ +	+
ENPF10	+ ve	4.20^{d}	+	+
ENBS2	+ ve	4.20^{de}	+	+
ENBS3	+ ve)	8.40^{bc}	+	+
ENBS4	+ ve	7.20^{c}	+	+
ENBS7	+ ve	2.20^{f}	+	–
ENBS9	+ ve	1.60^{g}	+	+
PA23	+ ve	8.20^{bc}	+ +	+
BSCBE4	+ ve	4.20^{d}	+ +	+

Values are mean of three replications. In a column, means followed by a common letter are not significantly different at the 5% levels by DMRT. ENPF- Endophytic *P. fluorescens,* ENBS-Endophytic *B. subtilis,* PA23-*P. chlororaphis*, BSCBE4-*B. subtilis*. (+ +) Higher intensity, (+) Low intensity, (−) Negative.

type siderophore, except ENPF6 and ENBS7 in addition to hydroxamate type (Table III).

Peroxidase

Studies on the induction of peroxidase isoforms by bacterial antagonists explained that PO2 was expressed in pathogen inoculated and healthy control. The bacterial

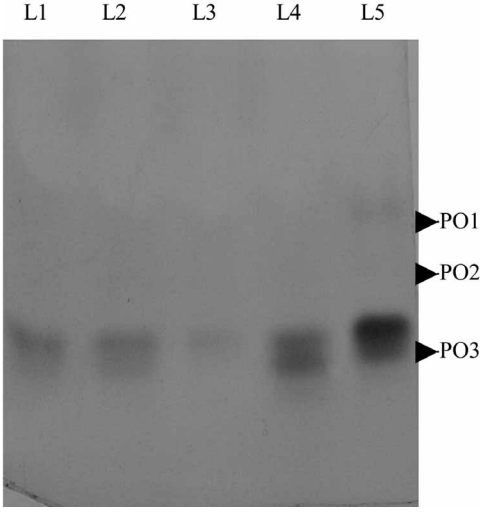
antagonists *B. subtilis* (BSCBE4) induced 3 isoforms PO1, PO2 and PO3 while only PO2 and PO3 was expressed in *P. amarus* treated with *P. chlororaphis* (PA23) challenged against pathogen (Figure 1).

Polyphenol oxidase

Studies on the expression of various isoforms of PPO through native gel electrophoresis indicated that 4 different isoforms (PPO1-PPO4) were induced in all the treatments including pathogen inoculated and healthy control. But the intensity of expression of the isoforms was very less in inoculated control and healthy control. Comparison between biocontrol agents indicated that the intensity of expression of PPO4 was very high with PA23. But the expression of PPO1, 2 and 3 was more in BSCBE4 treated plants than all other treatments. Treatment of *P. amarus* seedlings with ENPF1 challenged against pathogen induced 4 isoforms of PPO (Figure 2).

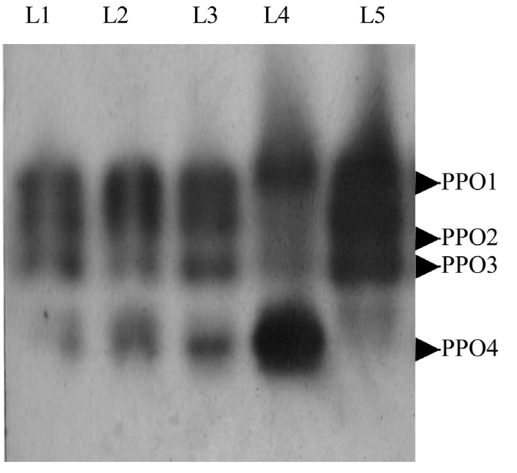
Chitinase and β-1, 3 Glucanase

Studies on the induction of chitinase and β-1, 3 glucanase by PGPR isolates revealed that the expression of chitinase and β-1, 3 glucanase increased irrespective of the PGPR treatments. The activity of chitinase and β-1, 3 glucanase increased up to 595 nmol of GluNAc min^{-1} mg^{-1} of protein and to 585 μ mol equivalent of glucose h^{-1} mg^{-1} of protein respectively on 4 days after challenge inoculation with *C. cassiicola* in *P. amarus* plants pretreated with BSCBE4. Subsequently the activity of both defense enzymes declined slowly up to 10 days after challenge inoculation. In addition, comparison of the activity of chitinase and β-1, 3 glucanase in *P. amarus* plant inoculated with pathogen alone reflected that the activity was lower than the plants pretreated with BSCBE4 (Figures 3 and 4).



Lane-1-Inoculated control, Lane 2- Healthy control, Lane 3- ENPF1 challenged against pathogen, Lane 4- PA23 challenged against pathogen, Lane 5- BSCBE4 challengd against pathogen.

FIGURE 1 Induction of PO isoforms by bacterial antagonists.



Lane-1-Inoculated control, Lane 2- Healthy control, Lane 3- ENPF1 challenged against pathogen, Lane 4- PA23 challenged against pathogen, Lane 5- BSCBE4 challengd against pathogen.

FIGURE 2 Induction of PPO isoforms by bacterial antagonists.

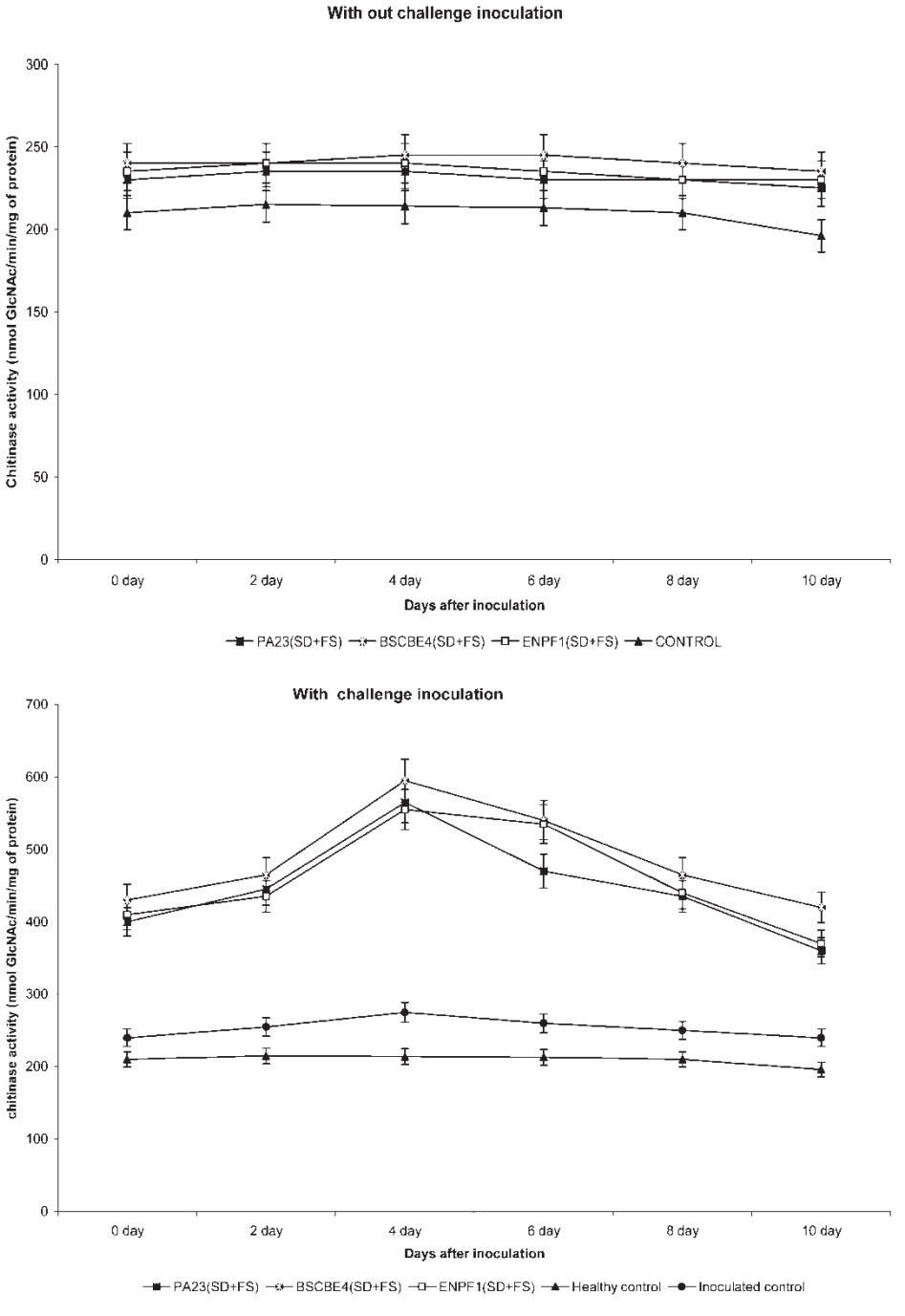
Testing the efficacy of biocontrol agents and fungicides against stem blight of *P. amarus* under field condition

Assessment of biocontrol agents and fungicides under field condition against stem blight indicated that delivery of bacterial and fungal antagonists through seedling dip and foliar spray reduced the incidence of stem blight rather than the individual application of antagonists either through seedling dip (or) foliar spray. Application of BSCBE4 as seedling dip and foliar spray recorded the lowest stem blight incidence (17.45%) and it was followed by the application of PA23, TvMNT7 and ENPF1 and delivered through seedling dip and foliar spray.

The growth parameters like root length and shoot length were maximum with the combined application of seedling dip and foliar spray. Spraying of Ridomil at fortnightly intervals recorded 17.31% incidence of stem blight. It was on par with the application of BSCBE4 delivered as seedling dip and foliar spray table (Table IV).

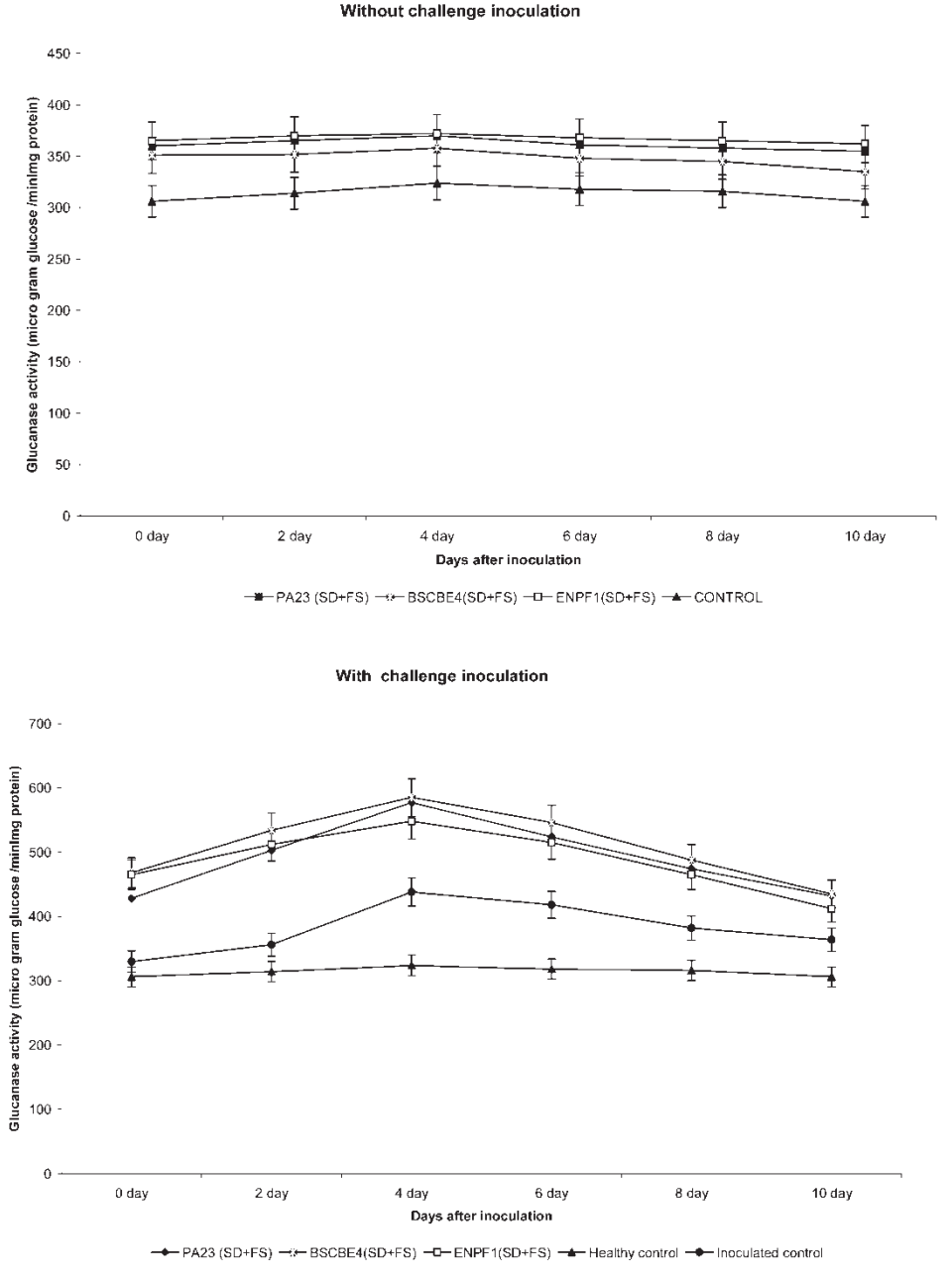
DISCUSSION

In the present study stem blight infected plants showed the presence of water soaked spots on basal portion of the stem followed by the appearance of necrotic spots on entire stem. As the spots enlarged, the stem got blightened followed by premature defoliation of the leaf. Pathogen associated with the disease was identified as *C. cassiicola* (Berk and Curt) Wei. Though this disease is reported for the first time in *P. amarus*, the same pathogen causes target spot on leaf, stem, roots and flower of a wide range of hosts grown in tropical and subtropical countries. The blighting symptom on cucumber caused by this pathogen was reported by Blazquez (1967). It also infects crops like cotton (Johnes, 1961) cowpea (Olive, 1949), egg plant (Onesirosan, *et al.*, 1974), sesame (Stone and Jones, 1960) and tomato (Blazquez, 1972).



SD-Seeding dip, FS- Foliar spray ENPF1- Endophytic *P. fluorescens*, PA23- *P. chloraphis*, BSCBE4 - *B. subtilis*

FIGURE 3 Induction of chitinase activity in *Phyllanthus amarus* treated with PGPR strains against stem blight disease.



SD-Seeding dip, FS- Foliar spray, ENPF1- Endophytic *P. fluorescens*, ENBS-Endopyytic *B. subtilis*, PA23- *P. chloraphis*, BSCBE4 - *B. subtilis*

FIGURE 4 Induction of β-1, 3 glucanase activity in *Phyllanthus amarus* treated with PGPR strains against stem blight disease.

TABLE IV Efficacy of biocontrol agents and fungicides against stem blight of *Phyllanthus amarus* under field conditions

Treatment	*Per cent disease incidence (90DAS)*	*Shoot length (cm)*	*Root length (cm)*	*Dry weight (g/plant)*
BSCBE4 (SD + F S)	17.45^{e} (24.52)	41.50^{a}	16.38^{a}	3.64^{a}
ENPF1 (SD + FS)	20.54^{cd} (26.93)	39.40^{b}	15.80^{a}	3.48^{a}
PA23 (SD + FS)	18.84^{e} (25.70)	38.40^{b}	14.58^{b}	3.36^{b}
TvMNT 7 (SD + FS)	20.38^{cd} (26.81)	38.40^{b}	13.70^{c}	3.37^{b}
Ridomil (FS)	17.31^{e} (24.56)	29.90^{c}	12.50^{d}	2.04^{d}
Benomyl (FS)	20.41^{cd} (26.84)	28.70^{c}	12.80^{d}	2.86^{c}
Mancozeb (FS)	22.21^{bc} (28.43)	29.40^{c}	12.00^{d}	2. 34^{c}
Control	82.00^{a} (62.17)	25.40^{d}	10.40^{e}	2.01^{e}

Values are mean of three replications. Values in parenthesis are arcsine transformed. In a column, means followed by a common letter are not significantly different at the 5% levels by DMRT. SD-Seedling dip, FS-Foliar spray, DAS-days after sowing. ENPF-Endophytic *P. fluorescens,* PA23-*P. chlororaphis,* TvMNT7-*Trichoderma viride*, BSCBE4-*B. subtilis*

Studies on the *in vitro* antagonism of bacterial antagonists indicated that *B. subtilis* BSCBE4 and endophytic *P. fluorescens* resulted in the suppression of the mycelial growth of stem blight pathogen (*C. cassicola*). Several strains of *Pseudomonas* spp. and *Bacillus* spp. have been shown to produce wide array of antibiotics which include lacton, 2,4 diacetylphloroglucinol, HCN, oligomycen, oomycen A, phenazine, pyroluteorin, pyrolnitrin, pyocyanin, iturin, surfactin and several other uncharacterized molecules (Kim *et al*., 1989; Keel and Defago, 1997; Whipps, 1997; Nielson *et al*., 1998).

Plant growth promoting rhizobacteria and bacterial endophytes play a vital role in the management of various fungal diseases. But one of the major hurdles experienced with biocontrol agents is the lack of appropriate delivery system. In the present study combined application of BSCBE4 and ENPF1 through seedling dip and foliar spray gave maximum control of stem blight both *in vitro* and *in vivo*. Similarly seed treatment and soil application of *P. fluorescens* reduced root rot of black gram caused by *Macrophomina phaseolina* (Jayashree *et al*., 2000; Shanmugam *et al*., 2001) panama wilt of banana (Raguchander *et al*., 1997). Seed and foliar application of *P. fluorescens* reduced sheath blight of rice (Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al*., 2001.) The efficacy of biocontrol agents to reduce stem blight under glass house condition might be due to the production of siderophore, antibiotics, lytic enzymes and the induction of defense related enzymes like PO, PPO, PAL, chitinase, β-1,3 glucanase and phenol. The increase in biomatter production by ENPF1 and BSCBE4 may be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake and by producing siderophore or antibiotics to protect plant from deleterious rhizosphere organisms. Van Peer and Schippers (1988) documented an increase in the root length and shoot length of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strain.

Production of siderophores like pseudobactin and pyoverdine which chelate the available iron in the soil results in the death of pathogen due to lack of iron for pathogen survival. (Kloepper *et al*., 1980a,b). Since iron being a compound of cells, its deficiency can cause growth inhibition, decrease in nucleic acid synthesis, inhibition of sporulation and change in cell morphology (Chincholkar *et al*., 2000). In addition also

regulates metabolic processes such as TCA cycle, electron transport chain, oxidative phosphorylation and photosynthesis (Chincholkar *et al*., 2000). The deficit of available iron to pathogens might have resulted in death of *C. cassiicola*. Similar suppression of fungal pathogens by siderophores has been reported by Leong (1986). In this study, the bacterial antagonists *B. subtilis* (BSCBE4), ENPF1 and PA23 produced hydroxamate and carboxylate type of siderophores leading to iron deficit condition. Similarly Kloepper *et al*. (1988) reported the production of fluorescent siderophores by *P. fluorescens* which contributed to its antagonistic action against *P. ultimum*.

Besides direct antagonistic activity by the production of various bacterial metabolites, induction of systemic resistance by fluorescent pseudomonas against diseases has been established as a new mechanism by which the plants defend themselves from pathogen attack (Van Peer *et al*., 1991, Van Loon *et al*., 1998). Prior application of fluorescent pseudomonas as seed treatment induces various defense mechanisms in the plants (Chen *et al*., 2000).

Plant has endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Bostock *et al*., 2001; Heil, 2001). It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. Induced resistance by inducing agents in several crops is associated with enhancement of lignification and also increased activities of enzymes involved in phenyl propanoid pathway and PR protein synthesis (Boller and Mauch, 1988; Hammerschmidt and Kuc, 1995). Recent studies imply that prior application of fluorescent pseudomonas strengthens the host cell wall structures resulting in restriction of pathogen invasion in plant tissue (Benhamou *et al*., 2000; Chen *et al*., 2000).

Peroxidase represents another component of an early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West, 1989). The products of this enzyme in the presence of hydrogen donor and hydrogen peroxide have antimicrobial activity and even antiviral activity (Van Loon and Callow, 1983). Increased activity of cell wall bound peroxidases has been elicited in different plants such as cucumber (Chen *et al*., 2000), rice (Reimers *et al*., 1992) tomato (Mohan *et al*., 1993) and tobacco (Ahl goy *et al*., 1992) due to pathogen infection. In bean rhizosphere, colonization of various bacteria induced the peroxidase activity (Zdor and Anderson, 1992).

Radjacommare (2000) reported that *P. fluorescens* Pf1 induced the activities of PPO in rice against *R. solani*. Similarly Meena *et al*. (2000) reported that the *P. fluorescens* induced the activities of PPO in response to infection by *C. personata* in groundnut. Tea plants treated with *P. fluorescens* at weekly intervals increased activity of PPO under field condition. Similarly significant increase in the activity of PPO was observed in greengram plants treated with *P. fluorescens* along with chitin (Saravanakumar, 2002). In the present study, PPO activity increased from 2nd day after challenge inoculation, but maximum activity was recorded on 4th day after challenge inoculation with pathogen in all the PGPR treated *Phyllanthus* plants. In addition the various isoforms of PO were induced by BSCBE4 after challenge inoculation by pathogen. The intensity of expression of PPO was comparatively more in BSCBE4 treated plants challenged with *C. cassiicola*. The induction of PO and PPO might have resulted in cell wall thickening and would have suppressed the penetration of *C. cassiicola* into *P. amarus* as seen from the cross section of infected stems.

In general fungal cell contain chitin and glucan as their cell wall constituents. The main mode of antagonistic activity of microbes is production of lytic enzymes (chitinase and β-1, 3 glucanase) which act on cell walls of organisms which have chitin and glucan as their cell wall component (Sing *et al*., 1999) and also through induced systemic resistance (ISR) in plant system. Maurhofer *et al*. (1994) reported that PR proteins, viz., PR-1a, 1b, 1c, endochitinase and β-1,3 glucanase were induced in the intercellular fluid of tobacco leaves of plants grown in the presence of *P. fluorescens* strain CHAO.

Earlier Viswanathan and Samiyappan (1999) reported that ISR by fluorescent pseudomonas which was associated with induction of chitinase, as a promising technology for the management of red rot of sugarcane. In the present study, the activity of chitinase and β-1, 3 glucanase was higher in PGPR treated plants challenge inoculated with *C. cassiicola* indicating that it might have resulted in the lysis of invading pathogen.

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