Phytopathol. Mediterr. (2007) 46, 259–271

RESEARCH PAPERS

Chemically and biologically mediated systemic resistance in cucumber (*Cucumis sativus* L.) against *Pseudoperonospora cubensis* and *Erysiphe cichoracearum*

Theerthagiri ANAND, Thiruvengadam RAGUCHANDER, Gandhi KARTHIKEYAN, Velappan PRAKASAM and Ramasamy SAMIYAPPAN

Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, Coimbatore 641 003, India

Summary. Azoxystrobin at concentrations of 0.25, 0.5 and 1.0 ml l^{-1}, mancozeb at 2 g l^{-1} and *Pseudomonas fluorescens* at 10 g l^{-1} were evaluated in cucumber for their efficacy in inducing defense enzymes against *Pseudoperonospora cubensis* and *Erysiphe cichoracearum*. The activity of the defense enzymes peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), ß-1,3-glucanase, chitinase, catalase and defense-inducing chemicals (total phenols) increased in the azoxystrobin and *P. fluorescens* treated cucumber plants. Increased expression of specific isoforms of PO and PPO was observed due to induced systemic resistance (ISR).

Key words: Azoxystrobin, defense enzymes, induced systemic resistance (ISR), *Pseudomonas fluorescens*.

Introduction

Cucumber (*Cucumis sativus* L.), a popular fresh market vegetable prepared as salads, is cultivated throughout India. The total area under cucumber cultivation in India is around 0.02 million ha and production is 0.12 million tonnes (Anonymous, 2004). The major constraints to cucumber production in India are downy mildew and powdery mildew caused by *Pseudoperonospora cubensis* DC and *Erysiphe cichoracearum* DC, respectively. Protective spray schedules require frequent application of fungicides as the disease cycle is completed in 3–7 days, and several quick cycles cause widespread infection within a short period. In addition, the continual use of systemic fungicides such as fenarimol, triadimefon and buprimate to control these diseases has led to the development of tolerant strains (Gupta and Shyam, 1996). Frequent sprays of copper containing Bordeaux mixture, copper oxychloride and certain other groups of fungicides are required to check the diseases, and this increases the cost of cultivation, besides posing residue problems.

Induced resistance to fungicides encourages the search for an alternative approach to plant protection (Schoenbeck, 1996). Induced systemic resistance (ISR) by definition refers to protecting plants systemically by enhancing the plant's defensive capacity against a broad spectrum of pathogens. The induction of systemic resistance to

Corresponding author: T. Anand
E-mail: barathiana@yahoo.com

various diseases by chemicals and plant-growth promoting rhizobacteria (PGPR) is a highly beneficial approach in crop protection. ISR differs fundamentally from other mechanisms. First, it is based on defense mechanisms that are activated by the inducing agents. Secondly, once it is expressed, ISR activates multiple potential defense mechanisms that include the increased activity of chitinases, ß-1,3-glucanases and peroxidases (POs) (Maurhofer *et al.*, 1994; Schneider and Ullrich, 1994; Dalisay and Kuc, 1995; Xue *et al.*, 1998). Thirdly, an important aspect of ISR is that a wide spectrum of pathogens can be controlled by a single inducing agent (Dean and Kuc, 1985; Hoffland *et al.,* 1996; Wei *et al.*, 1996). Thus ISR appears to be the result of several mechanisms which together are effective against wide range of fungal, bacterial and viral pathogens. Induced responses and induced resistance have been well documented and have been found in many plant taxa (Karban and Baldwin, 1997).

Systemic resistance mechanisms are induced in crop plants by treatment with chemical inducers, such as isonicotinic acid (INA), benzothiadiazole (BTH), probenazole and salicylic acid (SA) (Kessman *et al*., 1994; Gorlach *et al*., 1996; Pieterse *et al*., 1998; De Meyer *et al*., 1999; Sakamoto *et al*., 1999). In leaves treated with probenazole and inoculated with *Pyricularia oryzae* Cav., the activity of PO, PPO, PAL, tyrosine ammonialyase and catechol-*o*-methyl transferase was higher than in the untreated and/or inoculated leaves. This indicated that the disease controlling mechanism of probenazole was due to a host-mediated defense reaction (Hewitt, 1998). The objective of the present study was to employ azoxystrobin and *Pseudomonas fluorescens* to induce various defense-related genes encoding proteins implicated in strengthening the plant cell walls in response to infection by *P. cubensis* and *E. cichoracearum*.

Materials and methods

Azoxystrobin at concentrations of 0.25, 0.5 and 1.0 ml l^{-1}, *P. fluorescens* at 10 g l^{-1} and mancozeb at 2 g l^{-1} were used to induce a defense reaction in cucumber plants in pot culture. Cucumber seeds (2 seeds per pot) were sown in earthen pots filled with sterilized potting soil. Thirty days after sowing, one set of cucumber plants was treated with either azoxystrobin at the three different concentrations, or *P. fluorescens* or mancozeb. One day after treatment, one part of these treated plants was challenge-inoculated with *P. cubensis* and *E. cichoracearum* (conidial suspension) while the remaining part was not challenged. Plants neither treated with the chemicals or *P. fluorescens,* nor challenged with the pathogens, were kept as control. Three replications were maintained for each treatment, each replicate consisting of six pots. The experiments were conducted using a randomized block design on a greenhouse bench. The humidity in the greenhouse was maintained at around RH 80%. The temperature was adjusted to 26°C (day) and 20°C (night). Leaves from sprayed and unsprayed plants were collected after 0, 1, 2, 3, 4 and 5 days.

Enzyme extraction

Leaf tissues collected from the plants were immediately homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1 M (pH 7.0) at 4°C. The homogenate was centrifuged at 4°C for 20 min at 4000 ×*g* Protein extract prepared from the leaves were used to estimate PO, PPO, PAL, ß-1,3-glucanase and chitinase and catalase.

Spectrophotometric assay

Peroxidase. PO activity was assayed spectrophotometrically (Hartee, 1955). 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. The reaction mixture was incubated at room temperature (28±1°C). Changes in absorbance at 420 nm were recorded at 30 s intervals for 3 min and the boiled enzyme preparation served as a blank. Enzyme activity was expressed as the change in the absorbance of the reaction mixture min^{-1} g^{-1} on a fresh weight basis (Hammerschmidt *et al*., 1982).

Polyphenol oxidase. PPO activity was determined following Mayer *et al*. (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 μl of the enzyme extract. To start the reaction, 200 μl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} g^{-1} fresh weight of tissue.

Phenylalanine ammonia lyase. The PAL assay was conducted following Ross and Sederoff (1992). The assay mixture containing 100 μl of enzyme,

500 μl of 50 mM Tris HCl (pH 8.8) and 600 μl of 1 mM L-phenylalanine was incubated for 60 min. The reaction was arrested by adding 2 N HCl. Later, 1.5 ml of toluene was added, vortexed for 30 s, centrifuged (400 × *g*, 5 min) and the toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. The standard curve was drawn with graded amounts of cinnamic acid in toluene as described above. Enzyme activity was expressed as nmoles of cinnamic acid min^{-1} g^{-1} fresh tissue.

β-1,3-glucanase. Enzyme activity was assayed colorimetrically (Pan *et al*., 1991). Crude enzyme extract (62.5 μl) was added to 62.5 μl of 4% laminarin and incubated at 40°C for 10 min. The reaction was stopped by adding 375 μl of dinitrosalicylic acid (DNS) and heated for 5 min in a boiling water bath (DNS prepared by adding 300 ml of 4.5% NaOH to 880 ml containing 8.8 g of DNS and 22.5 g potassium sodium tartrate). The resulting coloured solutions were diluted with distilled water and vortexed, and the absorbance was read at 500 nm. The crude extract preparation mixed with laminarin at zero time incubation served as blank. The enzyme activity was expressed as μg equivalents of glucose min^{-1} g^{-1} fresh weight.

Chitinase. The colorimetric assay of chitinase was carried out following Boller and Mauch (1988). The reagents used were colloidal chitin, snail gut enzyme, dimethyl amino benzaldehyde (DMAB) and buffer.

Assay procedure

The reaction mixture consisted of 10 μl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg). After incubation for 2 h at 37°C, the reaction was stopped by centrifugation at 400 × *g* for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 μl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 μl of 3% (w:v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 μl 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a 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 the absorbance was measured at 585 nm using *N*-acetylglucosamine (GlcNAc) as standard. Enzyme activity was expressed as nmoles GlcNAc equivalents min^{-1} g^{-1} fresh weight.

Catalase. Catalase activity was estimated following Dekock *et al*. (1960). Five hundred mg of the sample was homogenized in 10 ml of ice cold 0.067 M phosphate buffer (pH 7.0) and centrifuged and the supernatant was used as the enzyme source. The reaction mixture consisted of 3 ml of hydrogen peroxide - phosphate buffer and 0.03 ml of enzyme extract. The reaction mixture was shaken well and the absorbance value was noted immediately and at intervals of 10 or 20 s. The time required for absorbance to decrease from 0.45 to 0.4 was noted and catalase activity was expressed as units g^{-1} tissue.

Total phenols. The content of total phenols present in the leaves was estimated following Bray and Thorpe (1954). Fresh leaves samples (0.5 g each) were blended with 10 ml of 80% ethanol and boiled at 50ºC for 30 min. The extracts were filtered through cheese cloth and then with Whatman No. 41 filter paper and centrifuged. The volume was made up to 10 ml with ethanol. An aliquot of one ml each was placed in a series of boiling tubes and made up to 3 ml with distilled water. To this, one ml of Folin Ciocalteu reagent and two ml of 20% sodium carbonate were added. The tubes were heated for one min in a boiling water bath and cooled in running water. The solution was diluted to 10 ml with distilled water and the intensity of the blue colour was measured at 660 nm in a spectrophotometer against a blank (a blank was maintained with three ml of distilled water instead of the extract, and the colour was developed as described above) for which three replications were maintained. Catechol was used to prepare the standard graph from which the amount of phenol in a given sample was calculated. The content of total phenols was expressed as catechol equivalents in mg 100 g^{-1} fresh weight.

Activity gel electrophoresis

Peroxidase. Activity gel electrophoresis was carried out to study the expression pattern of different isoforms of PO with different treatments. For native anionic polyacrylamide gel electrophoresis, a resolving gel of 8% acrylamide and stacking gel of 4% acrylamide were prepared. After electrophoresis, the gels were incubated in a solution containing 0.15% benzidinein and 6% NH_4Cl for 30 min in the dark. Then drops of 30% H_2O_2 were added with

constant shaking till the bands appeared. After staining, the gel was washed with distilled water and photographed (Sindhu *et al.*, 1984).

Polyphenol oxidase. PPO was extracted following the same procedure reported in the enzyme extraction section. After native electrophoresis, the gel was equilibrated for 30 min in 0.1% *p*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by addition of 10 mM catechol in the same buffer. Then a gentle shaking was given which resulted in the appearance of the dark brown discrete protein bands.

Statistical analysis

The data were statistically analyzed (Gomez and Gomez, 1984) and the treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRI-stat Version 92-a developed by the International Rice Research Institute Biometrics Unit, The Philippines.

Results

Peroxidase

Azoxystrobin sprayed at doses of 0.25, 0.5 and 1.0 ml l^{-1}, mancozeb and *P. fluorescens* all brought about changes in PO enzyme activity. It was inferred that azoxystrobin and *P. fluorescens* increased PO activity up to the 3rd day after challenge-inoculation, when compared with the control. This increase in PO activity was observed very early in *P. fluorescens*-pretreated cucumber plants challenge-inoculated with *P. cubensis* and *E. cichoracearum*. With azoxystrobin the increase was somewhat less. PO activity reached maximum levels on the 3rd day after challenge-inoculation with the downy mildew and powdery mildew pathogens (Fig. 1a, b and c).

PO isoforms

Native gel electrophoretic separation of enzyme extract from cucumber treated with *P. fluorescens* and from cucumber treated with azoxystrobin showed different PO patterns. The bacterized cucumber plants after challenge-inoculation with *P. cubensis* showed induction of different isoforms of PO as compared with plants treated with mancozeb and control plants. Levels of PO isozymes remained low with mancozeb-treated and with control plants, whether inoculated with the mildew agents or not (Fig. 2).

Polyphenol oxidase

Activity of PPO increased in cucumber plants challenge-inoculated with the downy mildew and powdery mildew pathogens. Application of *P. fluorescens* and of azoxystrobin led to an increase in PPO activity up to the 3rd or 4th day as compared

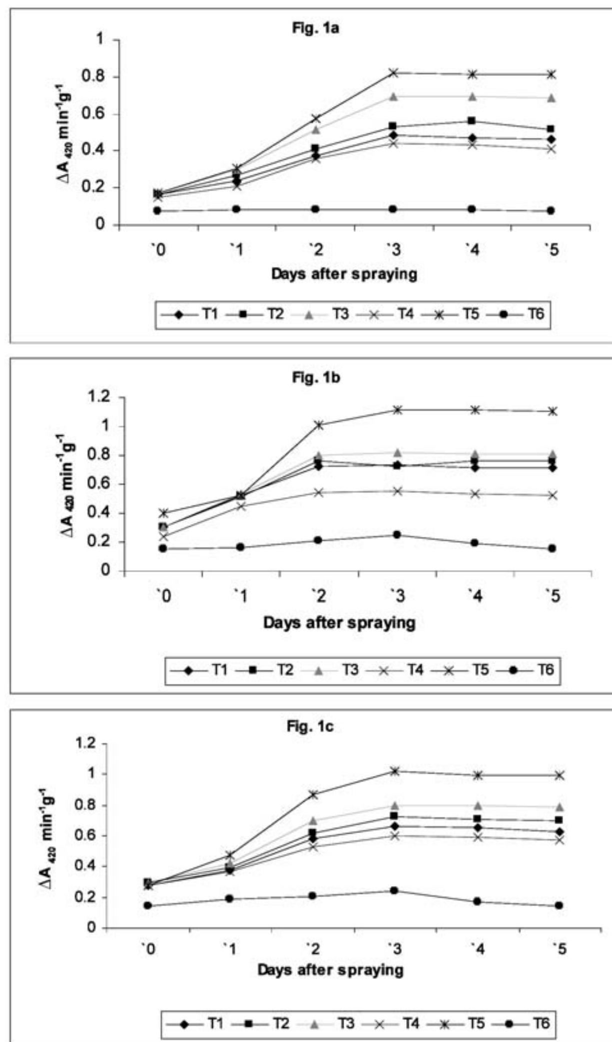


Fig. 1. Changes in PO activity in cucumber plants: 1a, uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens*; 1b, challenge-inoculation of downy mildew pathogen; 1c, challenge-inoculation of powdery mildew pathogen.

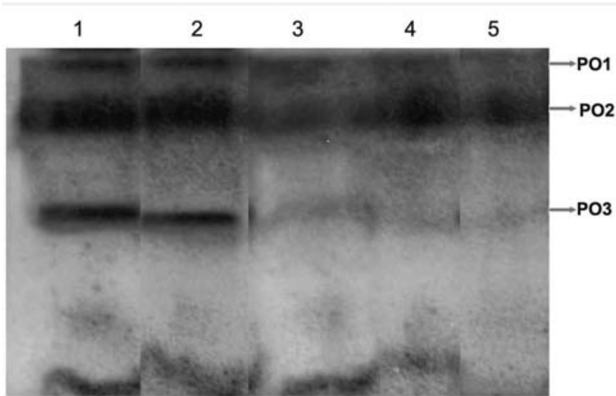


Fig. 2. Native PAGE profile of peroxidase (PO) induced in response to challenge-inoculation of *Pseudoperonospora cubensis* in cucumber.

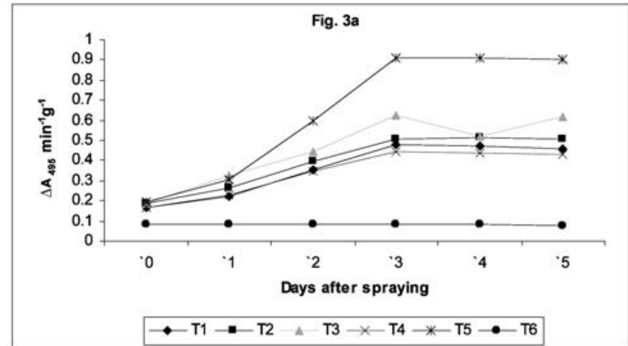
with the control. Initially, induction of PPO in *P. fluorescens* treated plants was on a par with that of azoxystrobin (1 ml l^{-1}) treated plants. Cucumber plants inoculated with the pathogen(s) alone recorded comparatively less PPO activity, and activity was very low on the 5th day after inoculation when compared with the uninoculated control (Fig. 3a, b and c).

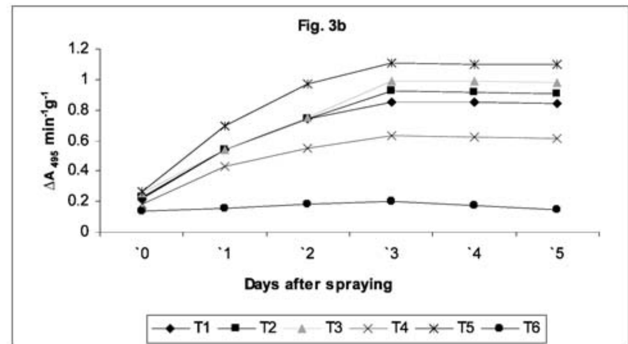
PPO isoforms

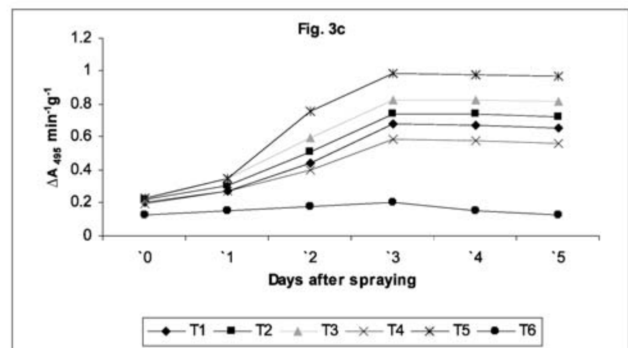
The isoform pattern of PPO in cucumber plants challenge-inoculated with *P. cubensis* was studied. All treatments led to an induction of isoforms after challenge-inoculation with the downy mildew pathogen. The increased intensity of the induced PPO was found in azoxystrobin and in *P. fluorescens* treated plants. In addition, azoxystrobin and *P. fluorescens* treated plants showed a greater intensity of the PPO isoform after challenge inoculation with *P. cubensis* than did the uninoculated plants. Inoculated control plants showed a lower intensity of PPO than did uninoculated (healthy) control plants (Fig. 4).

Phenylalanine ammonia lyase

Cucumber plants treated with azoxystrobin and with *P. fluorescens* were used to test total PAL activity after challenge-inoculation with *P. cubensis* and *E. cichoracearum*. The *Pseudomonas* spray activated PAL in the leaves of the cucumber plants. *P. cubensis* inoculation induced a rapid but transient accumulation of PAL at the site of infestation. Moreover, PAL activity increased with time on







T1 - Azoxystrobin 0.25 ml l^{-1}, T2 - Azoxystrobin 0.5 ml l^{-1}
T3 - Azoxystrobin 1.0 ml l^{-1} T4 - Mancozeb 2 g l^{-1}
T5 - *P. fluorescens* 10 g l^{-1} T6 – Control

Fig. 3. Changes in PPO activity in cucumber plants: 3a, uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens*; 3b, challenge-inoculation of downy mildew pathogen; 3c, challenge-inoculation of powdery mildew pathogen.

the 2nd and 3rd day after pathogen infection. With all treatments, bacterized plants showed significantly higher levels of PAL activity than did control plants. Plants inoculated with the pathogen alone showed a greater activity of PAL for 2-3 days but thereafter PAL activity declined drastically (Fig. 5a, b and c).

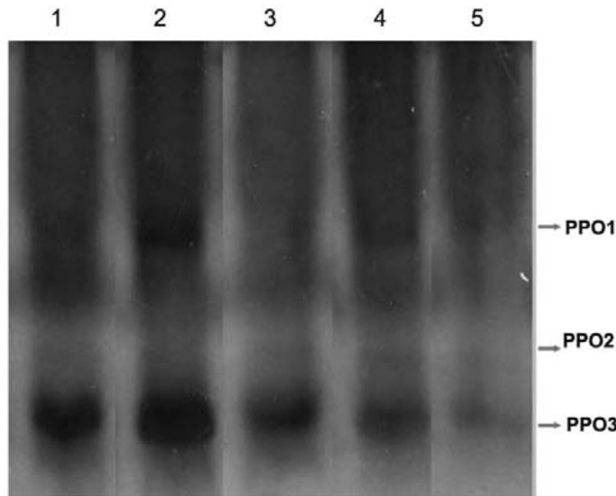
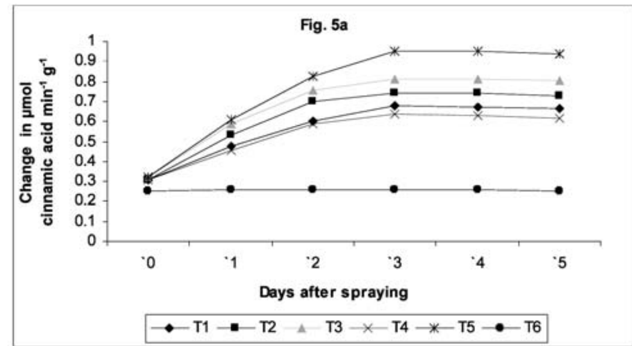
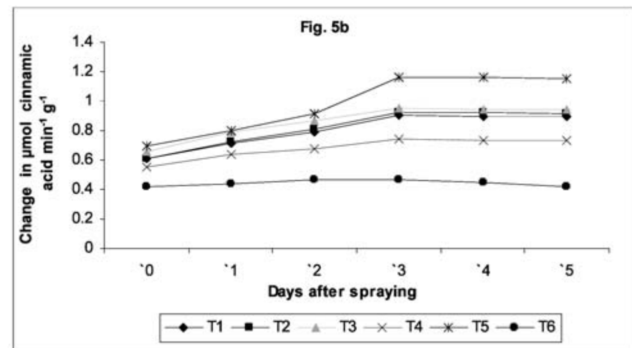
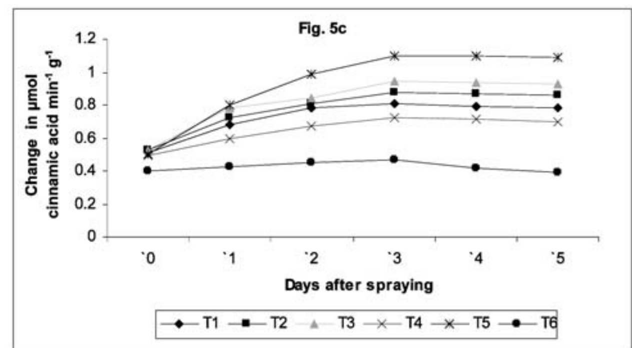


Fig. 4. Native PAGE profile of polyphenol oxidase (PPO) induced in response to challenge-inoculation of *Pseudoperonospora cubensis* in cucumber. Lane 1, Azoxystrobin; lane 2, *Pseudomonas fluorescens*; lane 3, mancozeb; lane 4, control (with inoculation); lane 5, control (no inoculation).







T1 - Azoxystrobin 0.25 ml l^{-1}, T2 - Azoxystrobin 0.5 ml l^{-1}
T3 - Azoxystrobin 1.0 ml l^{-1} T4 - Mancozeb 2 g l^{-1}
T5 - *P. fluorescens* 10 g l^{-1} T6 – Control

Fig. 5. Changes in PAL activity in cucumber plants: 5a, uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens;* 5b, challenge-inoculation of downy mildew pathogen; 5c, challenge-inoculation of powdery mildew pathogen.

ß-1,3-glucanase

The effect of *P. fluorescens* and that of azoxystrobin on glucanase was similar 24 h after inoculation.

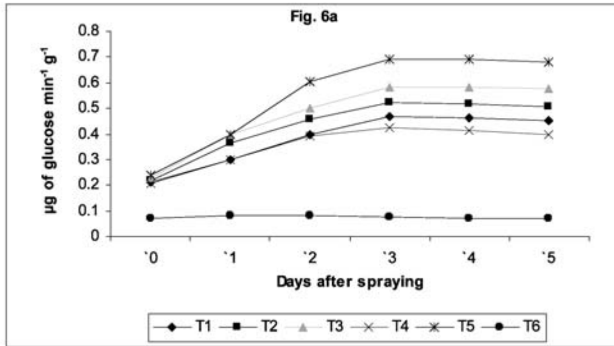
Activity peaked on the 3rd day after challenge-inoculation with *P. cubensis* and *E. cichoracearum*. It was higher in plants treated with *P. fluorescens*, and somewhat lower in plants treated with azoxystrobin (Fig. 6a, b and c).

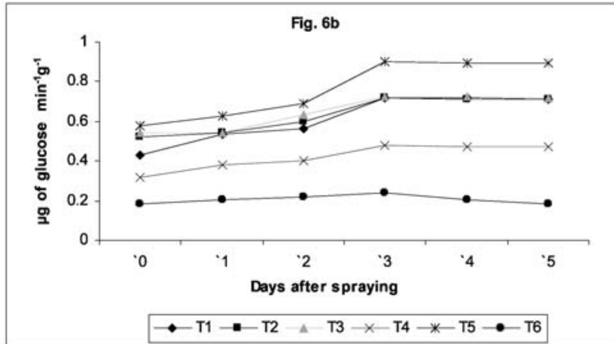
Chitinase

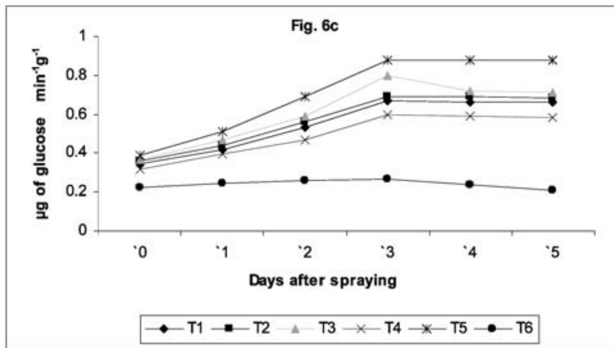
Chitinase activity was higher in plants sprayed with *P. fluorescens* and somewhat lower in plants treated with azoxystrobin (1 ml l^{-1}). Initially, there was no significant difference at the 5% level between the different doses of azoxystrobin, which were initially on a par with *P. fluorescens* (Fig. 7a, b and c).

Catalase

Catalase activity was higher in the bacterized cucumber plants challenge-inoculated with the pathogens; it was somewhat lower in inoculated cucumber treated with azoxystrobin (1 ml l^{-1}). The maximum increase in catalase was observed 3 days after challenge-inoculation. Though enzyme activity decreased 3 days after challenge-inoculation, the level remained higher than the initial level. In plants inoculated with the pathogen alone there was a sharp increase in catalase activity up to 3 days after inoculation, followed by a rapid decrease, and 5 days after inoculation, catalase activity

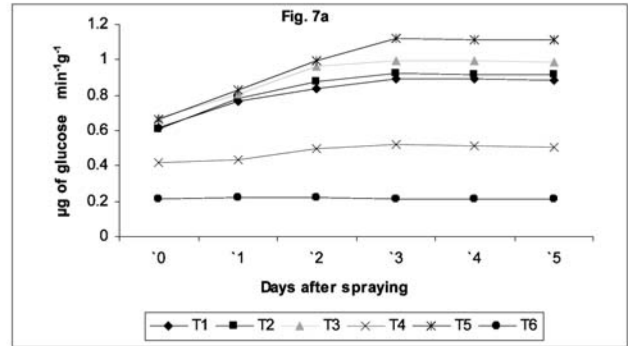


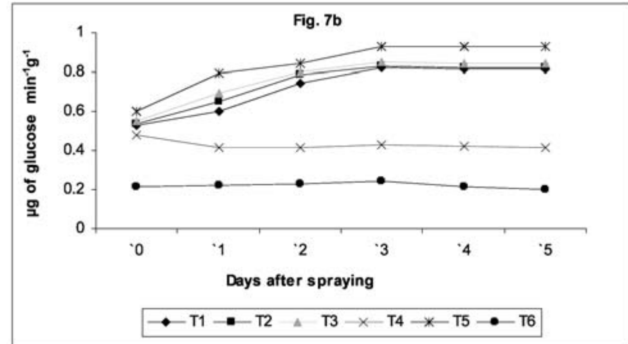




T1 - Azoxystrobin 0.25 ml l^{-1}, T2 - Azoxystrobin 0.5 ml l^{-1}
T3 - Azoxystrobin 1.0 ml l^{-1} T4 - Mancozeb 2 g l^{-1}
T5 - *P. fluorescens* 10 g l^{-1} T6 – Control

Fig. 6. Changes in beta- 1, 3-glucanase activity in cucumber plants: 6a, uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens;* 6b, challenge-inoculation of downy mildew pathogen; 6c, challenge-inoculation of powdery mildew pathogen.





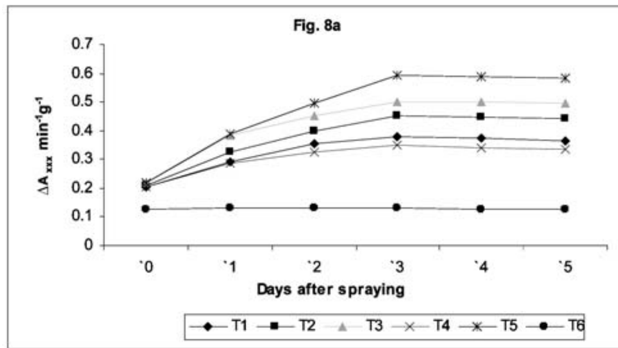
T1 - Azoxystrobin 0.25 ml l^{-1}, T2 - Azoxystrobin 0.5 ml l^{-1}
T3 - Azoxystrobin 1.0 ml l^{-1} T4 - Mancozeb 2 g l^{-1}
T5 - *P. fluorescens* 10 g l^{-1} T6 – Control

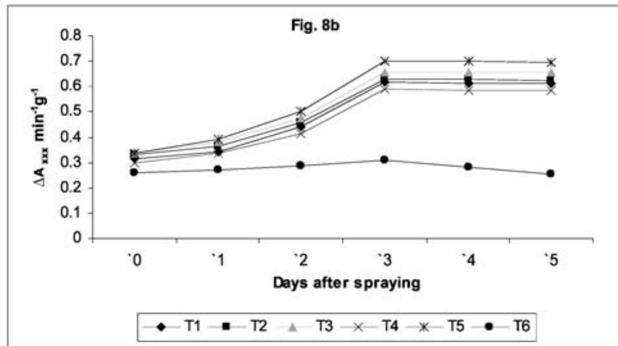
Fig. 7. Changes in chitinase activity in cucumber plants: 7a, uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens*; 7b, challenge-inoculation of powdery mildew pathogen.

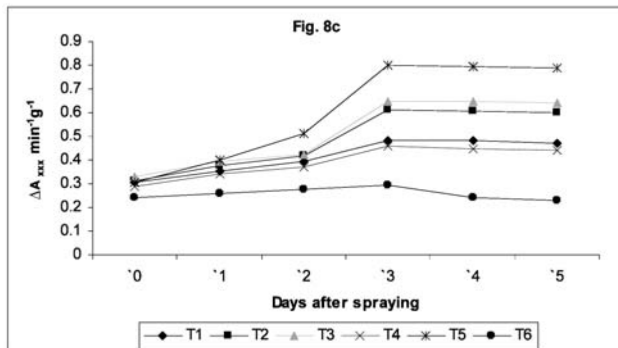
was more or less the same as on day 0. Cucumber plants inoculated with *P. fluorescens* alone showed a marginal increase in catalase activity, and in the uninoculated control plants there was no change in catalase activity throughout the experimental period (Fig. 8a, b and c).

Total phenols

In general, total phenols in cucumber plants increased with all treatments as compared with the uninoculated control. The increase in total phenols was significant in *Pseudomonas*-treated plants challenge-inoculated with *P. cubensis* and *E. cichoracearum*. The accumulation of total phenols started one day after challenge-inoculation and peaked 3 days after challenge-inoculation. Plants inoculated with the pathogens alone also had higher levels of total phenols, and these levels started decreasing after 3 days of inoculation. Cucumber plants treated with *P. fluorescens* alone had a marginal increase in the total phenol level as compared with the uninoculated cucumber plants (Fig. 9a, b and c).

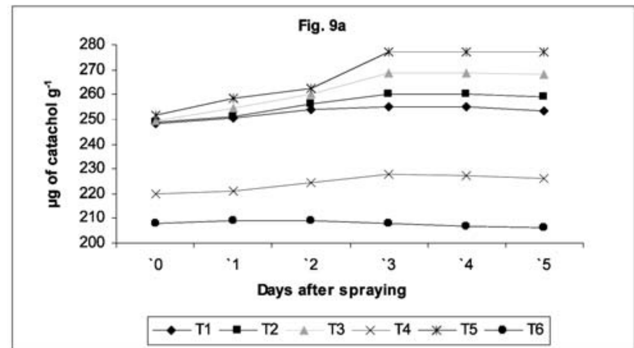


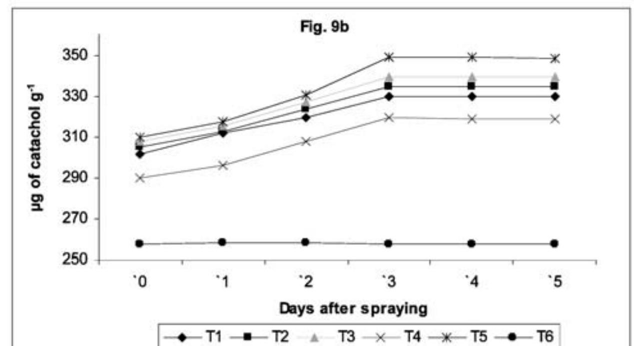


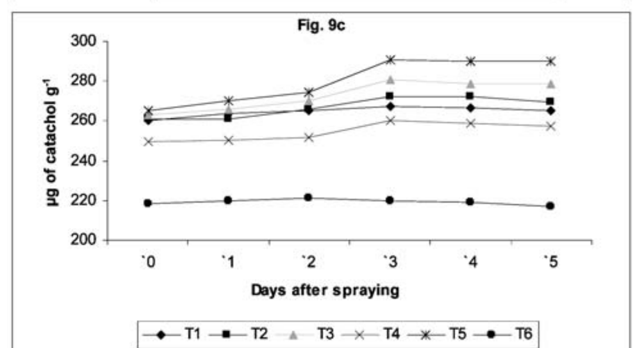


T1 - Azoxystrobin 0.25 ml l^{-1}, T2 - Azoxystrobin 0.5 ml l^{-1}
T3 - Azoxystrobin 1.0 ml l^{-1} T4 - Mancozeb 2 g l^{-1}
T5 - *P. fluorescens* 10 g l^{-1} T6 – Control

Fig. 8. Changes in catalase activity in cucumber plants: 8a uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens*; 8b. challenge-inoculation of downy mildew pathogen; 8c. challenge-inoculation of powdery mildew pathogen.







T1 - Azoxystrobin 0.25 ml l^{-1}, T2 - Azoxystrobin 0.5 ml l^{-1}
T3 - Azoxystrobin 1.0 ml l^{-1} T4 - Mancozeb 2 g l^{-1}
T5 - *P. fluorescens* 10 g l^{-1} T6 – Control

Fig. 9. Changes in total phenols in cucumber plants: 9a, uninoculated and treated with azoxystrobin and *Pseudomonas fluorescens*; 9b, challenge-inoculation of downy mildew pathogen; 9c, challenge-inoculation of powdery mildew pathogen.

Discussion and conclusions

Induced systemic resistance by azoxystrobin

Chemical fungicides often induce systemic resistance to pathogens in plants. The present study revealed that azoxystrobin brought about remarkable changes in PO, PPO, PAL, ß-1,3-glucanase, chitinase, catalase and total phenols. Native gel electrophoresis also showed induction of isoforms in PO and PPO.

These results are consistent with Sendhil Vel

(2003), who found that the activity of PO, PPO, PAL, ß-1,3-glucanase, chitinase and total phenols was higher in azoxystrobin-treated grapevine plants. Hewitt (1988) reported that the systemic fungicide probenazole induced higher levels of PO, PPO, PAL, tyrosine ammonia lyase and catechol-o-methyl transferase in the leaves, which indicated that the disease-controlling effect of azoxystrobin and probenazole was caused by a host-mediated reaction. The accumulation of PAL in tomato leaves treated with Fosetyl-Al® to control *Fusarium* wilt has also been reported (Bompeix *et al*., 1981).

Activity of PO was high in the resistant variety IIHR 517 A of chilli, and in the improved variety Pusa Jawala, as reported by Subhas Chander (1992). A higher peroxidase activity is correlated with disease resistance in many plants (Vidhyasekaran, 1988).

The fungicide probenazole also induced pathogenesis-related (PR) proteins, heat shock proteins and thylakoid protein in rice leaves and was correlated with the efficacy of probenazole against bacterial leaf blight disease (Lalithakumari and Dhakshinamoorthy, 1995). Similarly, triazole compounds such as epoxiconazole and propiconazole have a role in inducing systemic resistance against *Colletotrichum lagenarium* (Pass.) Ellis and Halsted in cucumber plants (Oostendorp *et al*., 1996).

Peroxidase and ß-1,3-glucanase are related to the cross-linking of cell wall components, the polymerization of lignin and suberin monomers and the subsequent resistance to pathogens in several host pathogen interactions (Reuveni *et al*., 1995). Mustard leaves sprayed with BTH (benzothiadiazole) three days prior to inoculation with *Albugo candida* (Lev.) Kunze showed higher levels and enhanced activity of PO at 11 days after inoculation (Kaur and Kolte, 2001). Earlier, several workers found that PO was involved in lignin biosynthesis, and in the production of toxic quinones and phytoalexins at the onset of resistance (Glazener, 1982; Hammerschmidt *et al*., 1982; Daayf *et al*., 1997).

Shahina Kalim *et al*. (2000) reported that there was an increase in the specific activity of PO and PPO, and a reduction in the specific activity of catalase in the roots of plants raised from carbendazim-treated seeds, as compared with untreated seeds. Therefore the greater activity of PO and PPO, along with the higher amount of total phenols, may enhance host resistance.

Induced systemic resistance by *P. fluorescens*

Plants possess various defense-related genes and it is well known that defense genes are sleeping genes, and that 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. The induction of systemic resistance by *P. fluorescens* has earlier been reported by several workers (Zehnder *et al*., 2000). Seed treatment with *P. fluorescens* suppressed foliar pathogens (anthracnose and mildews) by inducing systemic resistance (Wei *et al*., 1991 and 1996). The lipopolysaccharides present in the bacterial cell wall acted as signal molecules and elicited various defense compounds.

The Pf1 isolate of *P. fluorescens* and the secondary metabolite of *Strobilurus tenacellus* (Pers. ex Fr.) Singer (azoxystrobin) were found to protect cucumber against downy mildew and powdery mildew. In the present study, *P. cubensis* and *E. cichoracearum* in cucumber plants that had been sprayed with *Pseudomonas* strongly induced the synthesis and accumulation of PO, PPO, PAL, ß-1,3-glucanase, chitinase, catalase and total phenols. Sendhil Vel (2003) reported similar findings with grapevine plants pretreated with *P. fluorescens* and challenge-inoculated with downy mildew and powdery mildew pathogens. The activity of these defense enzymes and chemicals was more pronounced in a compatible reaction (control plants) of cucumber plants with downy and powdery mildew than in an incompatible interaction (PGPR treated-plants). *P. fluorescens* can act as a strong elicitor of plant defense reactions (M' Piga *et al*., 1997). Recent studies have reported that prior application of *P. fluorescens* strengthens the host-cell wall structures, creating a barrier that restricts the pathogen invading the plant tissue (Benhamou *et al*., 2000; Chen *et al*., 2000). However, only limited information is available on plant-mediated defense reactions induced by *P. fluorescens* to defend plants against pathogen invasion.

The present study clearly showed that the enzymes of phenylpropanoid metabolism and the accumulation of PR-proteins in cucumber induced by *P. fluorescens* isolate Pf1 in response to challenge-inoculation with *P. cubensis* and *E. cichoracearum* prevented further infection by the pathogens. This preventive capacity of *P. fluorescens* may be

attributed to the availability of specific receptors for the inducers in cucumber.

In the present study, increased PO activity was recorded in *P. fluorescens*-treated cucumber plants challenge-inoculated with *P. cubensis* and *E. cichoracearum*. Maximum PO activity occurred 3 days after challenge-inoculation and activity remained high throughout the experimental period. Plants inoculated with the pathogens alone had comparatively less PO activity. PPO activity also increased in *P. fluorescens*-treated cucumber plants. Ramamoorthy and Samiyappan (2001) reported a similar increase in chilli plants inoculated with *Colletotrichum capsici*. PO is a key enzyme in the biosynthesis of lignin and other oxidised phenols (Bruce and West, 1989). PO catalyzes the oxidation of hydroxy cinnamyl alcohols into free radical intermediates, which subsequently are coupled into lignin polymers (Gross, 1980). PO and PPO mediate the oxidation of phenols, and oxidized phenols are also highly toxic to the mildew pathogens (Sequeira, 1983).

Peroxidase and PPO catalyse the oxidation of phenolic compounds through a PPO-PO-H_2O_2 system (Srivastava, 1987). A number of studies have found a correlation between PPO and the resistance response (Velazhahan and Vidhyasekaran, 1994). PO itself was also found to inhibit the spore germination and mycelial growth of certain fungi (Joseph *et al*., 1998).

Increased PAL activity was recorded in *P. fluorescens*-pretreated cucumber plants challenged with *P. cubensis* and *E. cichoracearum* when compared with the untreated control. The induction of defense enzymes like PAL is one of the ways in which the host responds to treatment with biocontrol agents. An increase in the mRNAs encoding for PAL and chalcone synthase was recorded in the early stage of the infection of bean roots with various rhizobacteria (Zdor and Anderson, 1992). PAL activity can be induced by plant pathogen interactions and by fungal elicitor treatment (Ramanathan *et al*., 2000). De Meyer *et al*. (1999) reported that rhizosphere colonization by *P. aeruginosa* (Schroeter) Migula 7 NSK2 activated PAL in bean roots and increased salicylic acid levels in the leaves. Ramamoorthy and Samiyappan (2001) also found that chilli seed treatment with *P. fluorescens* isolate Pf1 increased PAL activity, and that *Pseudomonas*-pretreated chilli leaves challenged with *C. capsici* showed an additional increase in PAL activity. However, Chen *et al*. (2000) reported that high levels of PAL were induced in cucumber roots inoculated with *Pythium aphanidermatum,* but that when the roots were treated with *Pseudomonas corrugata* Roberts and Scarlett they initially had higher levels of PAL, but these levels became lower after the plant was challenged with *P. aphanidermatum*. Sendhil Vel, (2003) made a similar observation in grapevine plants inoculated with *Uncinula necator* and *Plasmopara viticola.* The present study also revealed that plants treated with *P. fluorescens* increased PAL activity, and that *P. fluorescens*-pretreated cucumber plants challenged with *P. cubensis* and *E. cichoracearum* showed an additional increase in PAL activity. PAL is a key enzyme of phenylpropanoid metabolism, which leads to the synthesis of phenols (Massala *et al*., 1980). The biocontrol agent *P. fluorescens* induced a host defense mechanism by increasing PAL activity with a consequent increase in phenol levels.

PR-proteins and host-coded proteins are induced by different types of pathogens and abiotic stress (Van Loon *et al*., 1998). The synthesis and accumulation of PR-proteins is reported to play an important role in plant defense (Maurhofer *et al*., 1994; Van Loon *et al*., 1998; Sendhil Vel, 2003). Maurhofer *et al*. (1994) stated that the induction of systemic resistance by *P. fluorescens* was correlated with the accumulation of chitinase and ß-1,3-glucanase. Ineffective isolates of *P. fluorescens* did not trigger an accumulation of chitinase and ß-1,3-glucanase and did not induce systemic resistance in tobacco against tobacco mosaic virus. Pea seed treated with *P. fluorescens* isolate 63-28 caused higher levels of hydrolytic enzymes such as chitinase and ß-1,3-glucanase at the site of penetration of the fungal hyphae of *F. oxysporum* f. sp. *pisi* (Linford) Snyder and Hansen. These enzymes act upon the fungal cell wall causing the degradation and loss of the inner contents of the cells (Benhamou *et al*., 1996). In our study, chitinase and ß-1,3-glucanase were induced by *P. fluorescens* against *P. cubensis* (only ß-1,3-glucanase) and *E. cichoracearum* in cucumber plants. Chitinase and ß-1,3-glucanase are also implicated in the defense against downy mildew and powdery mildew pathogens in leaves of cucumber.

Catalase activity was also very high in *P. fluorescens*-pretreated cucumber plants challenged with

P. cubensis and *E. cichoracearum*. In plants inoculated with the pathogens alone, catalase activity increased initially but then declined drastically. Gupta *et al*. (1995) also reported that catalase activity was considerably higher at the initial stages of infection by *Alternaria* in all the *Brassica* species, but dropped markedly at later stages.

Studies on the induction of defense mechanisms revealed higher levels of total phenols in *P. fluorescens*-pretreated cucumber plants challenge-inoculated with *P. cubensis* and *E. cichoracearum*. Levels of total phenols began to rise one day after challenge-inoculation. The highest level occurred on the 3rd day after challenge inoculation. Plants inoculated with the pathogens alone also had increased total phenols, but the level declined drastically 3 days after inoculation. Moreover, the levels of total phenols were lower than those in the *P. fluorescens*-treated plants challenged with the pathogens. When *P. fluorescens* treatment alone was given, the total phenols increased the day after treatment and the level remained almost static throughout the experimental period, and always remained higher than that of the untreated control. It is well known that phenolic compounds are fungitoxic. Moreover, they increase the physical and mechanical strength of the host cell wall and thus inhibit fungal invasion. M' Piga *et al*. (1997) reported that seed treatment with *P. fluorescens* raised levels of phenolics in tomato. The hyphae of *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen surrounded by phenolic substances exhibited considerable morphological derangement, including cytoplasmic disorganization and loss of protoplasmic content. Resistance in pea to *Pythium ultimum* Trow. and *F. oxysporum* f. sp. *pisi* due to accumulation of phenolics by prior application of *P. fluorescens* has also been reported (Benhamou *et al*., 1996). Similar observations were made on chilli plants treated with *P. fluorescens* and challenge-inoculated with *C. capsici* (Ramamoorthy and Samiyappan, 2001).

In conclusion, the prior treatment of cucumber plants with azoxystrobin and *P. fluorescens* triggered a plant-defense mechanism protecting the plant against infection from *P. cubensis* and *E. cichoracearum*. Earlier studies revealed that azoxystrobin and *P. fluorescens* are also effective against various other diseases of cucumber. Thus azoxystrobin and *P. fluorescens* give broad-spectrum protection against different pathogens attacking the same crop.

Literature cited

Anonymous, 2004. *The Hindu Survey of Indian Agriculture*. The Hindu publications, New Delhi, India, 184 pp.

Kaur A. and S.J. Kolte, 2001. Protection of mustard plants against the staghead phase of white rust by foliar treatment with benzothiadiazole, an activator of plant defense system. *Indian Journal of Mycology and Plant Pathology* 31(2), 133–138.

Benhamou N., R.R. Belanger and T.C. Paulitz, 1996. Induction of differential host responses by *Pseudomonas fluorescens* in Ri T-DNA-transformed pea roots after challenge with *Fusarium oxysporum* f. sp. *pisi* and *Pythium ultimum*. *Phytopathology* 86, 114–118.

Benhamou N., J.W. Kloepper and S. Tuzun, 2000. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic strain: ultrastructure and cytochemistry of the host response. *Planta* 204, 153–168.

Boller T. and F. Mauch, 1988. Colorimetric assay for chitinase. *Methods in Enzymol*ogy 161, 430–435.

Bompeix G., F. Fettouche and P. Saindrenan, 1981. Mode d'action du phosethyl Al. *Phytiatrie et Phytopharmacie* 30, 257–272.

Bray H.G. and W.V. Thorpe, 1954. Analysis of phenolic compounds of interest in metabolism. *Methods in Biochemical Analysis* 1, 27–52.

Bruce R.J. and C.A. West, 1989. Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiology* 91, 889–897.

Chen C., R.R. Belanger, N. Benhamou and T.C. Paullitz, 2000. Defense enzymes induced in cucumber roots by treatment with plant-growth promoting rhizobacteria (PGPR). *Physiology and Molecular Plant Pathology* 56, 13–23.

Daayf F., A. Schmitt and R.R. Belanger, 1997. Evidence of phytoalexins in cucumber leaves infected with powdery mildew following treatment with leaf extracts of *Reynoutria sachalinesis*. *Plant Physiology* 113, 719–727.

Dalisay R.F. and J.A. Kuc, 1995. Persistence of reduced penetration by *Colletotrichum lagenarium* into cucumber leaves with induced systemic resistance and its relation to enhanced peroxidase and chitinase activity. *Physiology and Molecular Plant Pathology* 47, 329–338.

De Meyer G., K. Capieau, K. Audenaert, A. Buchala, J.P. Metraux and M. Hotfite, 1999. Nanogram amounts of salicyclic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK 2 activate the systemic acquired resistance pathway in bean. *Molecular Plant-Microbe Interaction* 12, 450–458.

Dean R.A. and J. Kuc, 1985. Induced systemic protection in plants. *Trends in Biotechnology 3*, 125–129.

Dekock P.C., K. Commisiong, V.C. Farmer and H.E. Inkson, 1960. Interrelationships of catalase, peroxidase, hematin and chlorophyll. *Plant Physiology* 35, 599–604.

Glazener J.A., 1982. Accumulation of phenolic compounds in cells and formation of lignin-like polymers in cell walls of young tomato fruits after inoculation with *Botrytis cinerea*. *Physiology and Molecular Plant Pathology* 20, 11–25.

Gomez K.A. and A.A. Gomez, 1984. *Statistical Procedures for Agricultural Research*. John Wiley and Sons, New York, NY, USA, 680 pp.

Gorlach J., S. Volrath, G. Knauf- Beiter, O. Hengy, U. Beckhove, K.H. Kogel, M. Oostendorp, T. Staub, E. Ward, H. Kessmann and J. Ryals, 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* 8, 629–643.

Gross G.G., 1980. The biochemistry of lignification. *Advances in Botanical Research* 8, 25–63.

Gupta S.K., P.P. Gupta and C.D. Kaushik, 1995. Changes in leaf peroxidase, polyphenol oxidase, catalase and total phenols due to *Alternaria* leaf blight in *Brassica* species. *Journal of Mycology and Plant Pathology* 25, 175–180.

Gupta S.K. and K.R. Shyam, 1996. Antisporulant activity of some fungicides against *Pseudoperonospora cubensis* on cucumber. *Journal of Mycology and Plant Pathology* 26(3), 293–295.

Hammerschmidt R., E.M. Nuckles and J. Kuc, 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. *Physiology and Plant Patho*logy 20, 73–82.

Hartee E.F., 1955. Haematin compounds. In: *Modern Methods of Plant Analysis* (K. Peach, M. Tracy, ed.), Springer-Verlag, New York, NY, USA, 197–245.

Hewitt H.G., 1988. Systemic acquired resistance. In: *Fungicides in Crop Protection.* CAB International, New York, NY, USA, 140–145.

Hewitt H.G., 1998. Strobilurins. In: *Fungicides in Crop Protection*. CAB International. New York, NY, USA, 128–129.

Hoffland E., J. Hakulinem and J.A. Van Pelt, 1996. Comparison of systemic resistance induced by avirulent and nonpathogenic *Pseudomonas* species. *Phytopathology* 86, 757–762.

Joseph L.M, T.K. Tan and S.M. Wong, 1998. Antifungal effect of hydrogen peroxide and peroxidase on spore germination and mycelial growth of *Pseudocercospora* species. *Canadian Journal of Botany* 76, 2119–2124.

Karban R. and I.T. Baldwin, 1997. *Induced Responses to Herbivory*. University of Chicago Press, Chicago, IL, USA, 56–63.

Kessman H., T. Staub, C. Hofmann, T. Maetzke and J. Herzog, 1994. Induction of systemic acquired disease resistance in plants by chemicals. *Annual Review of Phytopathology* 32, 439–459.

Lalithakumari D. and M. Dhakshinamoorthy, 1995. Control of bacterial leaf blight of paddy by probenazole treatment. *Journal of Mycology and Plant Pathology* 25, 118–119.

M' Piga P., R.R. Belanger, T.C. Paulitz and N. Benhamou, 1997. Increased resistance to *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato plants treated with endophytic bacterium *Pseudomonas fluorescens* strain 63-28. *Physiology and Molecular Plant Pathology* 50, 301–320.

Massala R., M. Legrand and B. Fritig, 1980. Effect of α-aminooxyacetate, a competitive inhibitor of phenylalanine ammonia lyase, on the hypersensitive resistance of tobacco to tobacco mosaic virus. *Physiology and Plant Pathology* 16, 213–226.

Maurhofer M., C. Hase, P. Meuwly, J.P. Metraux and G. Defago, 1994. Induction of systemic resistance of tobacco to tobacco necrosis virus by the root-colonizing *Pseudomonas fluorescens* strain CHAO: influence of the gacA gene and of pyoverdine production. *Phytopathology* 84, 139–146.

Mayer A.M., E. Harel and R.B. Shaul, 1965. Assay of catechol oxidase, a critical comparison of methods. *Phytochemistry* 5, 783–789.

Oostendorp M., H. Van Kessman, F. Lu, A. Geissmann, J. Gorleach, G. Hengy, R. Nordmeyer, R. Reist, W. Rucss and T. Staub, 1996. Influence of plant activator Bion (R) and of triazole fungicides on plant defense mechanisms. *Gesunde Pflanzen* 48, 260–264.

Pan S.Q., X.S. Ye and J. Kuc, 1991. Association of ß-1, 3-glucanase activity and isoform pattern with systemic resistance to blue mold in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. *Physiology and Molecular Plant Pathology* 39, 25–39.

Pieterse C.M., S.C. Ewws, J.A. van Pelt, M. Knoester, K. Laan, H. Gerrits, P.J. Weisbeek and L.C. Van Loon, 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *Plant Cell* 10, 1571–1580.

Ramamoorthy V. and R. Samiyappan, 2001. Induction of defense-related genes in *Pseudomonas fluorescens* treated chilli plants in response to infection by *Colletotrichum capsici*. *Journal of Mycology and Plant Pathology* 31, 146–155.

Ramanathan A., R. Samiyappa, and P. Vidhyasekaran, 2000. Induction of defense mechanisms in green gram leaves and suspension-cultured cells by *Macrophomina phaseolina* and its elicitors. *Journal of Plant Disease and Protection* 107, 245–257.

Reuveni M., V. Agapov and R. Reuveni, 1995. Induced systemic protection to powdery mildew in cucumber plants by phosphate and potassium salts: effect of inoculum concentration and post inoculation treatment. *Canadian Journal of Plant Pathology* 17, 247–251.

Ross W.W. and R.R. Sederoff, 1992. Phenylalanine ammonia lyase from loblolly Pine: purification of the enzyme and isolation of complementary DNA clone. *Plant Physiology* 98, 380–386.

Sakamoto K., Y. Tada, Y. Yokozeki, H. Akari, N. Hayashi, T. Fujimura and N. Ichikawa, 1999. Chemical induction of disease resistance in rice correlated with the expression of a gene encoding a nucleotide binding site and leucine-rich repeats. *Plant and Molecular Biology* 40, 847–855.

Schoenbeck F., 1996. Induced resistance: mechanisms and evaluation. In: *Modern Fungicides and Antifungal Compounds* (H. Lyr, P. Russel, H.D. Sisler, ed.), Intercept Ltd., Andover, UK, 447–450.

Schneider S. and W.R. Ullrich, 1994. Differential induction of resistance and enhanced enzyme activities in cucumber and tobacco caused by treatment with various abiotic and biotic inducers. *Physiology and Molecular Plant Pathology* 45, 291–304.

Sendhil Vel V., 2003. *Evaluation of Azoxystrobin 25 SC against Downy Mildew and Powdery Mildew of Grapevine*. PhD Thesis, Tamil Nadu Agricultural University, Coimbatore, India, 190 pp.

Sequeira L., 1983. Mechanisms of induced resistance in plants. *Annual Review of Microbiology* 37, 51–79.

Shahina Kalim Y., P. Luthra and S.K. Gandhi, 2000. Influence of Bavistin seed treatment on morphophysiological and biochemical parameters of cowpea roots susceptible to *Rhizoctonia* species. *Journal of Mycology and Plant Pathology* 30(3), 375–379.

Sindhu J.S., S. Ravi and J. L. Minocha, 1984. Peroxidase isozyme patterns in primary trisomics of pearl millet. *Theoretical and Applied Genetics* 68, 179–182.

Srivastava S.K., 1987. Peroxidase and polyphenol oxidase in *Brassica juncea* plants infected with *Macrophomina phaseolina* (Tassio) Goid and their implication in disease resistance. *Journal of Phytopathology* 120, 249–254.

Subhas Chander M., 1992. Biochemical properties associated with resistance to powdery mildew in chillies. *Plant Disease Research* 9(1), 103–104.

Van Loon L.C., P.A.H.M. Bakker and C.M.J. Pieterse, 1998. Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology* 36, 453–483.

Velazhahan R. and P. Vidhyasekaran, 1994. Role of phenolic compounds, peroxidase and polyphenol oxidase in resistance of groundnut to rust. *Acta Phytopathologica Entomologica Hungarica* 29, 23–29.

Vidhyasekaran P., 1988. *Current Trends in Physiological Plant Pathology*, Association of Plant Pathologists of India, Lucknow, India, 148 pp.

Wei G., J.W. Kloepper and S. Tuzun, 1991. Induction of systemic resistance of cucumber to *Colletotrichum* by selected strains of plant growth promoting rhizobacteria. *Phytopathology* 81, 1508–1512.

Wei L., J.W. Kloepper and S. Tuzun, 1996. Induced systemic resistance to cucumber diseases and increased plant growth promoting rhizobacteria under field conditions. *Phytopathology* 86, 221–224.

Xue L.,P.M. Charest and S.H. Jabaji-Hare, 1998. Systemic induction of peroxidases, ß-1, 3-glucanases, chitinases and resistance in bean plants by binucleate *Rhizoctonia* species. *Phytopathology* 88, 359–365.

Zdor R.E. and A.J. Anderson, 1992. Influence of root colonizing bacteria on the defense responses in bean. *Plant and Soil* 140, 99–107.

Zehnder G.W., C. Yao, J.F. Murphy, F. Sikora and J.W. Kloepper, 2000. Induction of resistance in tomato against cucumber mosaic cucumovirus by plant growth promoting rhizobacteria. *Biocontrol* 45, 127–137.

Accepted for publication: July 4, 2007