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Management of Cucumber (*Cucumis sativus* L.) Mildews through Azoxystrobin-Tolerant *Pseudomonas fluorescens*

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ABSTRACT

The compatibility studies of *Pseudomonas fluorescens* (Pf1) with azoxystrobin at different concentrations *viz.,* 100, 150, 200, 250 and 300 ppm revealed that it was compatible with all the concentrations of azoxystrobin tested and the growth of the bacterium was unaffected even at the maximum concentration of 300 ppm. The field experiment revealed a foliar application of Pf1 (2.5 kg ha^{-1}) and azoxystrobin (250 ml ha^{-1}) combined, reduced downy mildew as well as powdery mildew disease severities more than azoxystrobin (250 and 500 ml ha^{-1}) alone. An application of Pf1+azoxystrobin treatment recorded only 2.22 and 1.00 Percent Disease Index (PDI) of downy mildew and 1.85 and 0.50 PDI of powdery mildew during the first and second seasons, respectively. The treatment also recorded a maximum fruit yield of 14.30 and 15.65 tonnes ha^{-1} for the first and second seasons, respectively. Application of Pf1 along with azoxystrobin significantly increased the survival of Pf1 in the phylloplane of cucumber crop. In addition, there was multifold increase in peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, ß-1, 3 glucanase, chitinase and phenolics in plants treated with Pf1+azoxystrobin.

Keywords: Cucumber, *Erysiphe cichoracearum*, Integrated disease management, *Pseudoperonospora cubensis.*

INTRODUCTION

The present agricultural scenario signifies the importance of integrated disease management (IPM) strategies in crop protection. Biological control of foliar diseases has been less successful compared to a control of soilborne diseases in a wide range of agricultural and horticultural crops. Biocontrol agents for the control of foliar diseases are available, but inconsistent performance of the introduced agents on the phylloplane poses a limitation on their extensive adoption (Andrews, 1992). A modification of the delivery systems or supplementation of nutrients and other additives is likely to enhance the performance of biocontrol agents in the phylloplane (Knudsen and Spurr, 1988; Yuen *et al.,* 2001; Guetsky *et al*., 2002). Supplementation with specific compounds may provide a competitive advantage for the establishment of the introduced biocontrol agents leading to an improvement of the biocontrol.

Fungicide tolerance of some selected biocontrol agents has been utilized in their use as key components in IPM. Combining a fungicide tolerant biocontrol agent with fungicides improved the degree of disease control, also reducing the quantity of fungicides required for effective disease management (Frances *et al*., 2002; Buck, 2004). Integrated use of biocontrol agents and chemical fungicides was effective against late leaf spot of groundnut (Kishore *et al*., 2005), *Rhizoctonia* root rot, take-all of spring wheat (Duffy, 2000) as well as post harvest diseases of fruits (Chand-

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Goyal and Spotts, 1996) as compared with the individual components use in disease management.

Management of downy mildew (*Pseudoperonospora cubensis* DC.) and powdery mildew (*Erysiphe cichoracearum* DC.) diseases is highly dependent on chemical fungicides; that being so because adequate levels of host-plant resistances are scarce in many of the cultivated cucumber varieties. Azoxystrobin (Amistar 25 SC), is a potent strobilurin fungicide having novel biochemical mode of action (Hewitt, 1998). Its fungicidal activity results from the inhibition of mitochondrial respiration in fungi, which is achieved by the prevention of electron transfer between cytochrome b and cytochrome c (Becker *et al*., 1981). It is widely used against grapevine, cucumber and tomato foliar diseases. However, the need for repeated application of fungicides is an obstacle for the wide adoption of chemical management by the resource poor farmers due to increased cost of chemicals. Thus, there exists a greater demand for economic and sustainable biocontrol technologies for downy mildew and powdery mildew disease management. *Pseudomonas fluorescens* (Pf1) is one of the most potent biocontrol agent used nowadays in a majority of seed, soil and foliar treatments' due to its efficient antagonistic activity against various plant pathogens (Bharathi *et al*., 2004; Ramamoorthy *et al.,* 2002; Vidhyasekaran *et al*., 1997). Fluorescent pseudomonads control plant pathogens through different modes of action such as competition for nutrients and space (Elad and Chet, 1987), antibiosis (Howie and Suslow, 1991), production of siderophores (Loper, 1988) and lytic enzymes (Frindlender *et al*., 1993). In addition, induction of resistance by fluorescent pseudomonads is an additional mechanism by which these bacteria protect several crop plants against pests and diseases (Chen *et al.,* 1998; Vivekananthan *et al.,* 2004; Viswanathan and Samiyappan, 2006; Saravanakumar *et al.,* 2007). The objective of the study was to test the compatibility or tolerance of Pf1 in conjunction with azoxystrobin at different concentrations under *in vitro* conditions. Pf1 was tested under field conditions against cucumber downy mildew and powdery mildew diseases for its usefulness in controlling these diseases as when combined with a reduced dose of azoxystrobin. The present study is also to suggest the use of biotic inducer strain, Pf1 and azoxystrobin to protect cucumber, and to elucidate the role of PR proteins such as chitinase, β-1,3-glucanase and the enzymes involved in the synthesis of such secondary toxic metabolites as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase in Pf1 and azoxystrobin treated cucumber plants against *P. cubensis*.

MATERIALS AND METHODS

Source of Azoxystrobin, *P. fluorescens* (Pf1) and Growth Conditions

The fungicides azoxystrobin, mancozeb and carbendazim were obtained from M/s Syngenta Pvt. Ltd., India. *P. fluorescens* isolate Pf1 was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, India. Pf1 was stored at –80°C in 44 percent glycerol, cells from stock being first grown in King's B broth (KB). Inoculum was raised by transferring two loopfuls of culture from the stock culture to 100 ml of KB broth in a 250 ml Erlenmeyer flask and incubated at room temperature of 28±2°C (on a shaker running at 150 rpm) for 48 hours. The strain was subcultured once in a month and maintained until the end of the experiment in KB slants.

Compatibility Test with Pf1

Azoxystrobin at different concentrations of 100, 150, 200, 250 and 300 ppm were tested *in vitro* against Pf1 through turbidometric method. One ml of the Pf1 culture was transferred to a 250 ml sidearm flask containing 50 ml of King's B (KB) broth amended with azoxystrobin technical standard at five different concentrations *viz.,* 100, 150, 200, 250 and 300 ppm. An inoculated control was also

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maintained without addition of azoxystrobin technical standard in KB broth. Five replications were maintained for each concentration. The flasks were incubated at 28±1°C in a psychotherm shaker. The optical density values of the culture broth were determined through a spectrophotometer at 610 nm and at regular intervals of 6 hours.

Preparation of Talc-based Formulation of Pf1

A loopful of bacterium was inoculated into the KB broth and incubated in a rotary shaker running at 150 rpm for 48 hours at room temperature (28±2°C). After 48 hours of incubation, the broth containing 9×10^8 cfu ml^{-1} was used for the preparation of talc-based formulation. To 400 ml of bacterial suspension, 1 Kg of purified talc powder (sterilized at 105° C for 12 hours), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose 10 g (adhesive) were mixed under sterile conditions following the method described by Vidhyasekaran and Muthamilan (1995). After over night shade drying, the mix was packed in polypropylene bag and sealed. At the time of application, the population of bacteria in talc formulation was 2.5 to 3×10^8 cfu g^{-1}.

Bio Efficacy of Pf1 and Azoxystrobin against Cucumber Downy Mildew and Powdery Mildew

A field experiment was conducted to study the effect of talc-based formulation of Pf1 combined with azoxystrobin against downy mildew and powdery mildew diseases on hybrid Malini during Feb–May, 2004 in the farmer's holdings at Bolluvampatti, Coimbatore district of Tamil Nadu, India. The experiment was laid out in a randomized block design with four replications with a plot size of 5×4 m (20 m^2). Regular agronomic practices were followed as per the crop production guide of Tamil Nadu Agricultural University. The treatments of the experiment were T_1- Azoxystrobin at 500 ml ha^{-1}, T_2- Pf1 at 2.5 kg ha^{-1}, T_3- Azoxystrobin (250 ml ha^{-1}) with Pf1 (2.5 kg ha^{-1}), T_4– Azoxystrobin at 250 ml ha^{-1}, T_5- Mancozeb at 1 Kg ha^{-1}, T_6- Carbendazim at 500 g ha^{-1} and finally T_7– Control (water spray). The talc-based product of Pf1 was dissolved in water (5 g l^{-1}) and allowed to settle for 1 hour, filtered through muslin cloth and the filtrate was then sprayed on cucumber plants. Pf1 and azoxystrobin along with standard checks (mancozeb and carbendazim) were applied 35 days after sowing at the time of an initial appearance of the diseases, using a high volume ASPEE backpack sprayer with a spray fluid volume of 500 l ha^{-1}. The disease incidence was recorded after a second spray.

Another field experiment was conducted during Aug–Nov, 2004 on a farmer's field at Alandurai, Coimbatore district of Tamil Nadu, India with Malini hybrid of cucumber in the same method as to confirm the results obtained in the field experiment I.

Method of Assessment

The intensity of downy mildew and powdery mildew diseases was assessed with the score chart of 0 to 5 scale (0-No infection, 1-0 to10, 2-10.1 to15, 3-15.1 to 25, 4-25.1 to 50 and 5-More than 50 percent of leaf area being covered with mildew growth) as described by Jamadar and Desai (1997) and Tajider Singh *et al.* (1994). Percent disease index (PDI) was calculated, using the following formula (Mckinney, 1923).

$$\frac{\textit{Sum of numerical ratings}}{\textit{Total number of leaves observed}} \times \frac{100}{\textit{Maximum disease grade in the score chart}}$$

Yield

The fruits were harvested after maturity and the average yield recorded for each treatment.

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Phylloplane Survival of Pf1

The population dynamics of Pf1 either applied singly or in combination with azoxystrobin (0.5 ml l^{-1}) in the cucumber (Malini) phylloplane, was determined using rifampicin resistance as a marker. The bacterium (Pf1) was applied as a foliar spray (10^8 cfu ml^{-1}) at 35 days after sowing in the field. In different treatments, foliar application of Pf1 was combined with 0.5 ml l^{-1} azoxystrobin. In each treatment five randomly selected leaves were excited from different plants at regular intervals of 24 hours. The leaves were suspended in 50 ml of 0.1M phosphate buffer, pH 7, and incubated for 30 minutes at 180 rpm and 30°C. Serial dilutions of the suspensions were plated on King's B medium (containing 100 µg ml^{-1} rifampicin) with three plates for each dilution. The plates were incubated at 30°C and observed for the number of cfu after 48 hours. Phylloplane survival of Pf1 was expressed as log cfu g $leaf^{-1}$. The experiment was conducted during the two crop seasons with five replications in each treatment.

Induction of Defense Related Compounds

The induction of defense reaction in cucumber plants was studied under glasshouse conditions. Cucumber seeds were sown in earthen pots (60×90 cm) filled with sterilized potting soil at two seeds per pot. Thirty days after sowing, one set of cucumber plants were treated with Pf1 (2.5 kg ha^{-1}), azoxystrobin (500 ml ha^{-1}), Pf1 (2.5 kg ha^{-1}) plus azoxystrobin (250 ml ha^{-1}) and azoxystrobin (250 ml ha^{-1}). One day after treatment, the treated plants were challenge inoculated with *P. cubensis* (conidial suspension at 2×10^4 conidia ml^{-1}). Plants neither treated with chemical, Pf1 nor challenged by the pathogen were kept as control. Three replications were maintained in each treatment; each replicate consisted of six pots. The relative humidity was maintained around 80% in the greenhouse. The temperature was adjusted to 26°C (day)/20°C (night). Leaves from sprayed and unsprayed plants were collected at 0 (after 1 hour), 1, 3, 5, 7 and 9 days after pathogen inoculation.

Enzyme Extraction

Leaf tissues collected from plants were immediately homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of sodium phosphate buffer, 0.1M (pH 7.0) at 4°C. The homogenate was centrifuged at 4°C for 20 minutes at 10,000 rpm. Protein extracts prepared from leaves were used for the estimation of peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), *ß*-1,3 glucanase and chitinase.

Spectrophotometric Assay of Defense Enzymes/Compounds

PO

PO activity was assayed spectrophotometrically as in Hartee (1955). The reaction mixture consisted of 1.5 ml of 0.05M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 per cent H_2O_2. The reaction mixture was incubated at room temperature (28±1° C). The change in absorbance at 420 nm was recorded at 30 seconds intervals for 3 minutes and while the boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance of the reaction mixture min^{-1} g^{-1} on fresh weight basis.

PPO

PPO activity was determined as in the procedure given by Mayer *et al.* (1965). The reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reac-

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tion, 200 µl of 0.01M catechol was added and the activity expressed as changes in absorbance at 495 nm $min^{-1}g^{-1}$ fresh weight of tissue.

PAL

The PAL assay was done as per the method described by 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 minutes. The reaction was arrested by adding 2N HCl. Later 1.5 ml of toluene was added, vortexed for 30 seconds, centrifuged (1000 rpm, 5 minutes) and trans-cinnamic acid containing toluene fraction separated. The toluene phase was measured at 290 nm against toluene blank. Standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. The enzyme activity was expressed as n moles of cinnamic acid min^{-1}g fresh $tissue^{-1}$.

β-1, 3-glucanase

The activity of β-1,3-glucanase was colorimetrically assayed (Pan *et al.,* 1991). Crude enzyme extract of 62.5 µl was added to 62.5 µl of 4 per cent laminarin and incubated at 40°C for 10 minutes. The reaction was stopped by adding 375 µl of dinitrosalicylic acid (DNS) and heated for 5 minutes on boiling water bath (DNS prepared by adding 300 ml of 4.5 per cent NaOH to 880 ml containing 8.8 g of DNS and 22.5 g potassium sodium tartarate). The resulting coloured solutions were diluted with distilled water, vortexed and the absorbance 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 fresh $weight^{-1}$.

Chitinase

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

Assay Procedure

The reaction mixture consisted of 10 µl of 0.1M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg). Following incubation for 2 hours at 37°C, the reaction was stopped by centrifugation at 1000 rpm for 3 minutes. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3 per cent (w/v) snail gut enzyme for 1hour. After 1 hour, the reaction mixture was brought to pH 8.9 by the addition of 70 µl 0.1M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 minutes and then rapidly cooled in an ice-water bath. After addition of 2 ml of DMAB, the mixture was incubated for 20 minutes at 37°C and the absorbance measured at 585 nm using *N*-acetylglucosamine (GlcNAc) as standard. The enzyme activity was expressed as nmoles GlcNAc equivalents min^{-1} g fresh $weight^{-1}$.

Phenolics

Phenol content was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One g of leaf tissue was homogenized in 10 ml of 80 per cent methanol with pestle and mortar and agitated for 15 minutes at 70°C. One ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of folin ciocalteau reagent (1N) and the solution kept at 25°C. After 3 minutes, one ml of saturated solution of sodium carbonate and one ml of distilled water

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were added and the reaction mixture incubated for 1 hour at 25°C. The absorption of the developed blue colour was measured using UV-Visible Spectrophotometer (Varian Cary 50, Victoria, Australia) at 725 nm. Total soluble phenols content was calculated (according to a standard curve obtained from folin ciocalteau reagent with phenol solution (C_6H_6O)) and expressed as catechol equivalents g^{-1} tissue weight.

Statistical Analysis

All the experiments were repeated once with similar results. Field experiments were conducted in a randomized block design and the data subjected to analysis of variance (ANOVA) or Dunkan's Multiple Range Test (DMRT) using IRRISTAT version 92-1 programme developed by biometrics unit at International Rice Research Institute, The Philippines. In glasshouse experiments, the data were analyzed as in a completely randomized design and the percentage values of the disease index were arcsine transformed before statistical analysis (Gomez and Gomez, 1984). Data values of bacterial survival in the phylloplane were log-transformed before being subjected to ANOVA analysis.

RESULTS

Compatibility of Azoxystrobin with Pf1

The study on compatibility of azoxystrobin at different concentrations *viz*., 100, 150, 200, 250 and 300 ppm with Pf1 revealed that the bacterial growth was not affected by azoxystrobin even at a high concentration of 300 ppm (Figure 1). Even though no significant difference was observed in OD value of the bacterial growth tested at any of the azoxystrobin concentrations, the turbidity increased with increase in incubation time in all the treatments as that in the inoculated control (with no addition of azoxystrobin).

Bioefficacy

Downy Mildew

In the first season experiment, the results revealed that the foliar application of combined Pf1 (2.5 kg ha^{-1}) and azoxystrobin (250 ml ha^{-1}) significantly reduced the mildew disease as compared to an application of either azoxystrobin (250 and 500 ml ha^{-1}) or *P. fluorescens* (2.5 kg ha^{-1}) alone (Tables 1 and 2). Pf1+azoxystrobin treatment recorded 2.22 per cent incidence of downy

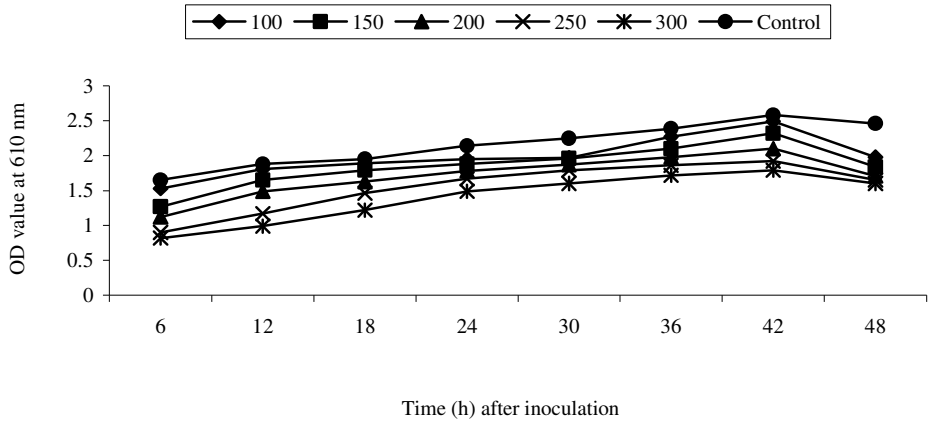

Figure 1. Compatibility of Pf1 with different concentrations of azoxystrobin (100, 150, 200, 250 and 300 ppm).

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mildew as against 4.42, 7.32 and 8.90 PDI of downy mildew incidence in azoxystrobin at 500 ml ha^{-1}, 250 ml ha^{-1} and Pf1 alone treated plots, respectively. The fungicides mancozeb and carbendazim treated plots recorded 9.22 and 20.44 PDI, respectively. The rate of progression of the disease was found to decrease in treated plots. Control plots recorded 5.02 PDI initially, progressing upto 44.55 PDI as observed at the end of the experiment (Table 1).

In the second season also, the same trend of results were observed. Pf1 (2.5 kg ha^{-1}) along with azoxystrobin (250ml ha^{-1}) recorded the least per cent disease index of 1.00 followed by azoxystrobin at 500 ml ha^{-1}, 250 ml ha^{-1} and Pf1 alone treated plots (2.92, 5.45 and 7.90 PDI, respectively) while control plots recorded a PDI of 39.44 (Table 2).

Powdery Mildew

In the first season trial, the results revealed that Pf1+azoxystrobin was effective against powdery mildew the treatment recording a 1.85 per cent of incidence. Azoxystrobin at 500 ml ha^{-1}, 250 ml ha^{-1} and Pf1 at 2.5 kg ha^{-1} sprayed plots recorded 5.22, 9.20 and 9.60 PDI, respectively, while mancozeb and carbendazim recorded 12.46 and 15.12 PDI, respectively. The rate of progression of the disease was found to decrease in treated plots as compared to control plots. The control plots recorded an initial 9.75 PDI which progressed up to 40.85 PDI as observed at the end of the experiment (Table 1).

A similar trend was observed in the second season too. The Pf1+azoxystrobin mix was found to be the most effective among the treatments with a lowest PDI record of 0.50. Azoxystrobin (250 ml ha^{-1}) and Pf1 (2.5 kg ha^{-1}) alone sprayed plots recorded 3.88 and 6.60 PDI, respectively. The recommended dose (500 ml ha^{-1}) of azoxystrobin treated plots recorded a PDI of 1.92. The standard checks *viz*., mancozeb and carbendazim recorded PDI of figures 7.38 and 9.09 respectively while in the control plots a higher PDI figure of 37.22 was recorded (Table 2).

Table 1. Effect of azoxystrobin and *P. fluorescens* on downy mildew and powdery mildew of cucumber (Trial-I).

Treatments	Per cent Disease Index (PDI) [a]					
	Downy mildew			Powdery mildew		
	Before spray	After spray	Disease reduction over control (%)	Before spray	After spray	Disease reduction over control (%)
Azoxystrobin (500 ml ha^{-1})	6.78 (15.10)[a]	4.42 (12.13)[e]	90.08	9.96 (18.40)[a]	5.22 (13.21)[e]	87.22
P. fluorescens (2.5 kg ha^{-1})	7.22 (15.59)[a]	8.90 (17.35)[c]	80.02	8.70 (17.16)[a]	9.60 (18.02)[d]	76.50
Azoxystrobin (250 ml ha^{-1})+ *P. fluorescens* (2.5 kg ha^{-1})	7.99 (16.41)[a]	2.22 (8.57)[f]	95.02	10.11 (18.54)[a]	1.85 (7.82)[f]	95.47
Azoxystrobin (250 ml ha^{-1})	7.50 (15.89)[a]	7.32 (15.69)[d]	83.57	10.01 (18.64)[a]	9.20 (17.65)[d]	76.28
Mancozeb (1 Kg ha^{-1})	7.90 (16.32)[a]	9.22 (17.68)[c]	79.30	10.78 (19.16)[a]	12.46 (20.67)[c]	69.49
Carbendazim (500 g ha^{-1})	7.88 (16.30)[a]	20.44 (26.88)[b]	54.12	8.72 (17.18)[a]	15.12 (22.88)[b]	62.98
Control	5.02 (13.21)[b]	44.55 (49.22)[a]	--	9.75 (18.19)[a]	40.85 (42.78)[a]	--

[a] Mean of four replications.
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.
Values in parentheses are arcsine transformed values.

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Table 2. Effect of azoxystrobin and *P. fluorescens* on downy mildew and powdery mildew of cucumber (Trial - II)**.**

Treatments	Per cent Disease Index (PDI) [a]					
	Downy mildew			Powdery mildew		
	Before spray	After spray	Disease reduction over control (%)	Before spray	After spray	Disease reduction over control (%)
Azoxystrobin (500 ml ha^{-1})	4.64 (12.48)[a]	2.92 (9.83)[e]	92.89	2.96 (9.90)[b]	1.92 (7.90)[e]	94.84
P. fluorescens (2.5 kg ha^{-1})	6.07 (14.26)[a]	7.90 (16.32)[c]	79.97	4.78 (12.63)[a]	6.60 (14.88)[c]	82.27
Azoxystrobin (250 ml ha^{-1})+ *P. fluorescens* (2.5 kg ha^{-1})	6.23 (14.45)[a]	1.00 (5.74)[f]	97.46	5.21 (13.19)[a]	0.50 (4.05)[f]	98.66
Azoxystrobin (250 ml ha^{-1})	6.24 (14.46)[a]	5.45 (13.50)[d]	86.18	3.44 (10.68)[ab]	3.88 (11.36)[d]	89.58
Mancozeb (1 Kg ha^{-1})	6.10 (14.29)[a]	8.52 (16.97)[c]	78.39	3.33 (10.51)[ab]	7.38 (15.76)[c]	80.17
Carbendazim (500 g ha^{-1})	4.82 (12.68)[b]	19.08 (25.90)[b]	51.62	4.58 (12.35)[a]	9.09 (17.55)[b]	75.57
Control	4.50 (12.24)[b]	39.44 (38.90)[a]	--	5.10 (13.05)[a]	37.22 (37.59)[a]	--

[a] Mean of four replications.
In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.
Values in parentheses are arcsine transformed values.

Yield

The data revealed that a higher yield of 14.30 tonnes ha^{-1} was recorded for Pf1 (2.5 kg ha^{-1})+azoxystrobin (250 ml ha^{-1}) treated plots, significantly superior to the other treatments *viz*., azoxystrobin (500 ml ha^{-1}), azoxystrobin (250 ml ha^{-1}) and Pf1 (2.5 kg ha^{-1}) which recorded yields of 13.23, 12.15 and 10.54 tonnes ha^{-1}**,** respectively. Pf1+ azoxystrobin treatment recorded an increased yield by 250.92 per cent over control. Control plots recorded the lowest yield of 4.08 tonnes ha^{-1}. In the second season trial, the same dose of azoxystrobin along with Pf1 recorded a maximum yield of 15.65 tonnes ha^{-1} with 238.01 per cent increase over control. Mancozeb and carbendazim treated plots recorded 9.55 and 8.22 tonnes ha^{-1} while control plots yielding only 4.63 tonnes ha^{-1} (Figure 2).

Survival of Pf1 in the Phylloplane

In the field tests, the population of Pf1 decreased in the cucumber phylloplane from log 7.8 to 3.4 and log 7.9 to 3.8 cfu g^{-1} by 7 days after their application in the first and second season, respectively. The decrease in the phylloplane population of Pf1 was less when the bacterium was applied along with 250 ml l^{-1} of azoxystrobin. However, in both cases, the decline in bacterial population on the phylloplane with lapse of time is observed as significant (Figures 3a and b).

Induction of Defense Related Compounds

Studies on the induction of defense mechanisms revealed that a higher accumulation of PO, PPO, PAL, ß-1,3 glucanase, chitinase and phenolics was observed in Pf1 +azoxystrobin treated cucumber plants challenged with *P. cubensis.* Accumulation of these defense enzymes and phenolics started one day after challenge inoculation. The maximum accumulation was observed at 3-5th day after challenge inoculation. Moreover, the accumulation of defense related enzymes and phenolics was observed to be less pronounced in azoxystrobin and Pf1 alone treated cucumber plants when chal-

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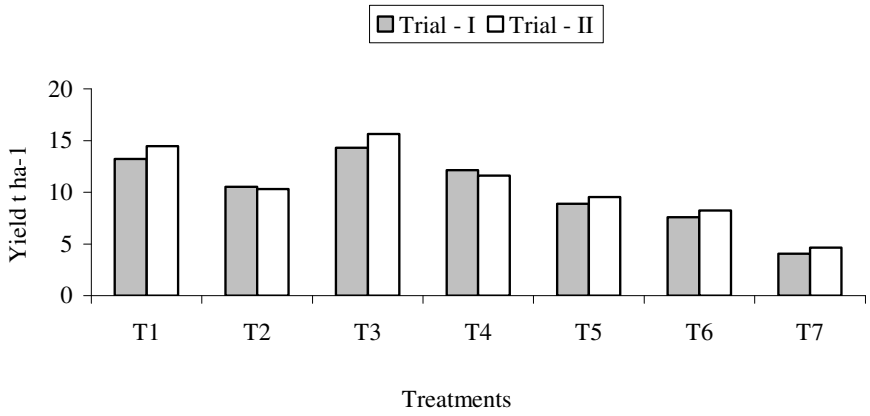

Figure 2. Effect of Pf1 and azoxystrobin on fruit yield of cucumber.
T1= Azoxystrobin 500 ml ha^{-1}; T2= Pf1 at 2.5 kg ha^{-1}; T3= Pf1 (2.5 kg ha^{-1})+Azoxystrobin (250 ml ha^{-1}); T4= Azoxystrobin (250 ml ha^{-1}); T5= Mancozeb 1 Kg ha^{-1}, T6= Carbendazim 500 g ha^{-1} and T7= Control.

lenged with inoculations of *P. cubensis* (Figures 4a-f).

DISCUSSION

Biocontrol agents have been used along with fungicides of no toxic effect on antagonists (Papavizas and Lumsden, 1980). The present investigation was made in connection with the effect of different concentrations of azoxystrobin on the growth of Pf1. Pf1 did not show any deleterious effect when mixed with azoxystrobin even at a concentration higher than 300 ppm. This is due to azoxystrobin being an inhibitor of electron from cytochrome b to c in mitochondria (Hewitt, 1998). Hence, it may well be mixed with azoxystrobin for tank sprays. Kataria *et al.* (2002) reported that lower rates of azoxystrobin in combination with *P. fluorescens* strain 36 resulted in better antagonist interactions against *Rhizoctonia solani* Kuhn. Sendhil Vel *et al*. (2004) found that the growths of Pf1 and *Bacillus subtilis* were not affected by azoxystrobin even at high concentrations. *P. aeruginosa* isolates GSE 18 and isolate GSE 19 were tolerant to chlorothalonil (Kavach) concentrations up to 2000 µg ml^{-1} (Kishore *et al*., 2005).

A combined application of Pf1 (5 g l^{-1}) and azoxystrobin (0.5ml l^{-1}) recorded a maximum disease reduction in cucumber followed by sprayings of azoxystrobin (1 ml l^{-1}), azoxystrobin (0.5 ml l^{-1}), and Pf1 (5 g l^{-1}) individually. *Pseudomonas* spp. are known to have broad spectrum activity (Haas and Keel, 2003) and are frequently identified as effective bio control agents of soil borne as well as foliar fungal diseases. In this context, cucumber seed treatment and soil application with a talc based powder formulation of *P. putida* strain 89B-61 reduced the severity of cucumber diseases and resulted in a simultaneous increase in cucumber yield both in glasshouse and in the field (Wei *et al*., 1996). Similarly, prophylactic foliar application of broad spectrum antifungal *P. aeruginosa* isolates protected perennial ryegrass turf from gray leaf spot infection caused by *Pyricularia grisea* (Viji *et al*., 2003).

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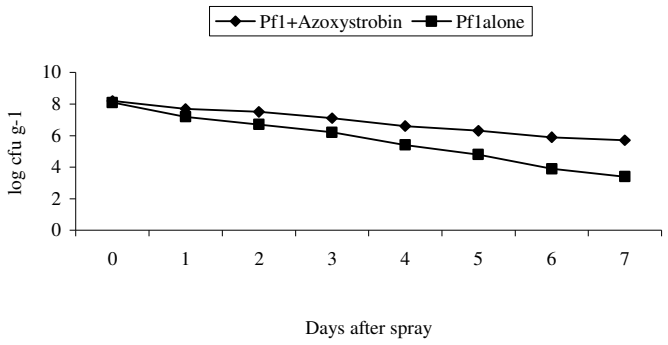
(a)



(b)

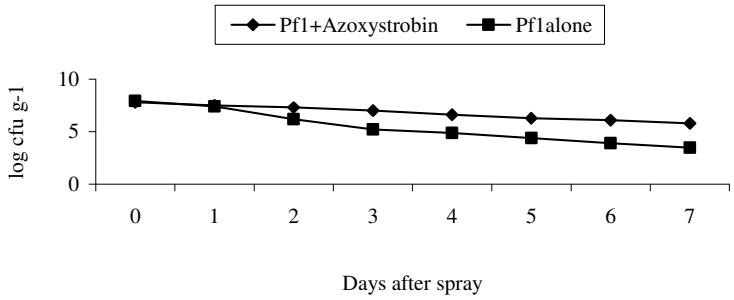


Figure 3. Survival and multiplication of Pf1 with and without 0.5 ml l^{-1} azoxystrobin in cucumber phylloplane in the field. a: Trial I and b: Trial II.

Pf1 was tolerant to the recommended field application rate of azoxystrobin. Fungicide tolerance is not uncommon in *Pseudomonas* spp. In the study reported here, integrated use of Pf1 and azoxystrobin reduced the fungicide requirement to a half of the normal for a control of cucumber downy mildew as well as powdery mildew. Similarly, Kishore *et al.* (2005) found that a combination of *P. aeruginosa* GSE 18 and chlorothalonil (500 µg ml^{-1}) reduced the severity of late leaf spot in groundnut comparable to chlorothalonil (2000 µg ml^{-1}) alone. In other developments, a use of chlorothalonil–tolerant pseudomonads together with a one fourth concentration of the recommended field dose of chlorothalonil doubled pod yield as compared with the untreated control. *P. fluorescens* EPS 288 and *B. subtilis* Rb14-C, in combination with reduced doses of fungicides, were equally as effective as the standard fungicides alone in control of *Penicillium expansum* on pear fruits (Frances *et al.,* 2002) as well as in damping off in tomato plants (Kondoh *et al.,* 2001), respectively. Synergistic action of fungicides and fungicide tolerant biocontrol isolates was reported to be beneficial in management of other phytopathogenic fungi (Conway *et al.,* 1997; Buck, 2004).

Improved control of downy mildew and powdery mildew by a combined application of Pf1 and azoxystrobin corresponded with the enhanced phylloplane survival of the bacterium in the field. Although the microclimate

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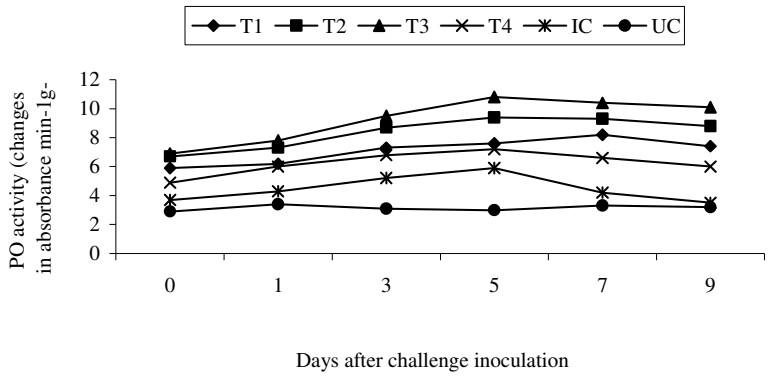
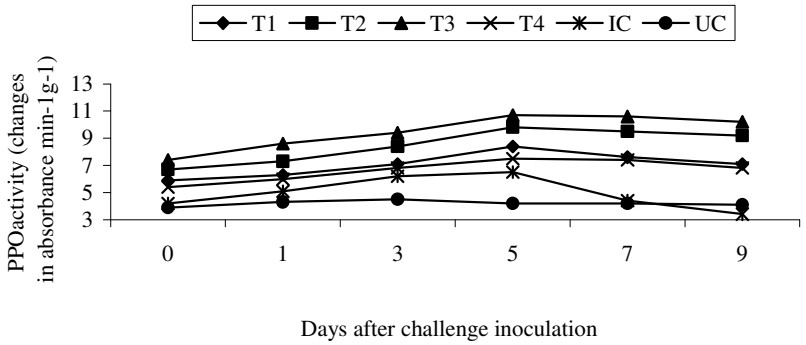
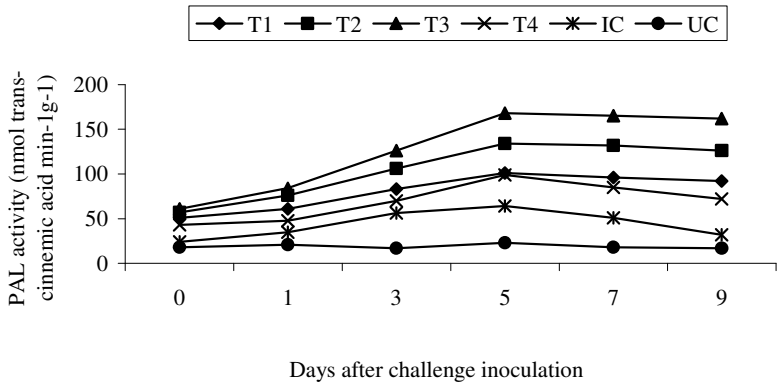
(a)



(b)



(c)



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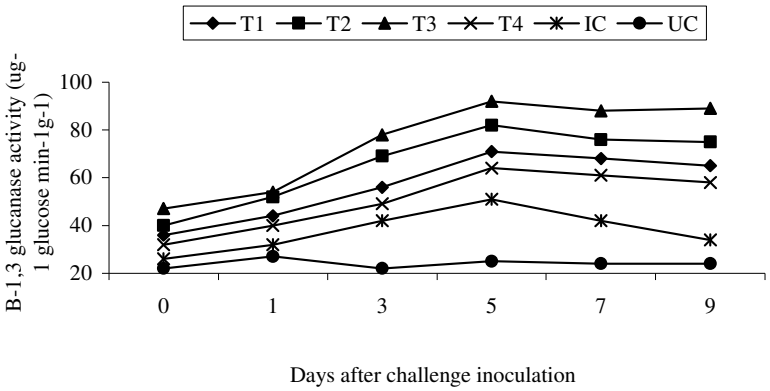
(d)



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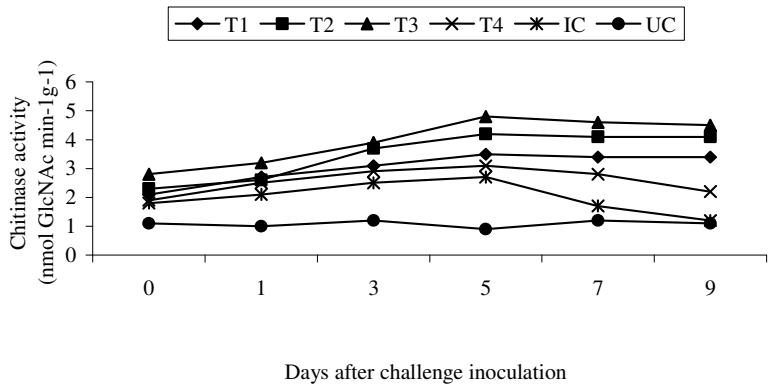

(f)

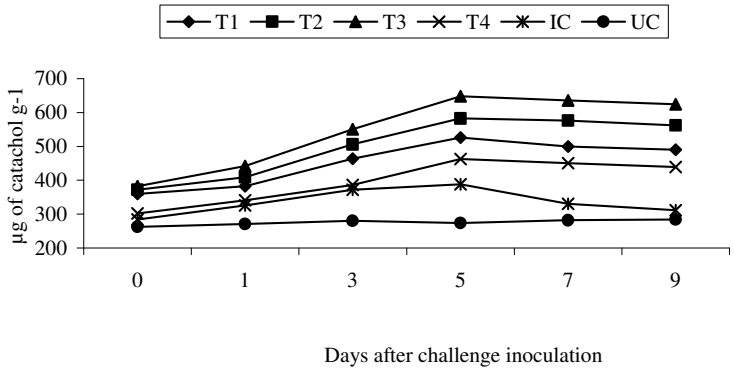

Figure 4. Induction of defense enzymes and chemicals in cucumber plants treated with Pf1 and azoxystrobin against downy mildew pathogen. a: PO; b: PPO; c: PAL; d: β-1,3 glucanase, e: Chitinase and f: Phenolics. T1= Azoxystrobin 500 ml ha^{-1}, T2= Pf1 at 2.5 kg ha^{-1}, T3= Pf1 (2.5 kg ha^{-1}) +Azoxystrobin (250 ml ha^{-1}), T4= Azoxystrobin (250 ml ha^{-1}), IC= Inoculated control, UC= Uninoculated control.

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remained the same in both treatments, the presence of azoxystrobin reduced the microbial competition (that leads to increased nutrient availability) may be the reason for increased survival of Pf1 in the cucumber phylloplane. Similarly, phylloplane survival of the bacteria was enhanced by combined application of *P. aeruginosa* and low doses of chlorothalonil (Kishore *et al.,* 2005). Due to enhanced phylloplane survival, in the present study it was revealed that Pf1 increased the activities of various defense-related enzymes and chemicals in a response to infection by the pathogen. It is well known that all plants are endowed with defense genes that are quiescent in nature and for activation of which appropriate stimuli or signals are needed. It has been reported that application of Pf1 triggers/activates plants' latent defense mechanisms in response to infection by pathogen (Vivekananthan *et al*., 2004). Inducing a plant's own defense mechanism by prior application of a biological agent is a novel strategy in plant disease management. In the present study, it has been observed that application of Pf1 along with azoxystrobin has increased the activities of various defense-related enzymes, leading to the synthesis of defense related chemicals in the plants. PAL plays an important role in the biosynthesis of phenolic phytoalexins (Daayf *et al.,* 1997). Cucumber plants treated with *P. corrugata* had an initially high level of PAL the level being lowered after the plant being challenged with *Pythium aphanidermatum* (Chen *et al.,* 2000). In the present study, increased activity of PAL was recorded in Pf1 +azoxystrobin- treated cucumber plants challenged with the pathogen; it reached its maximum on the third day after being challenged by the inoculation and was maintained at higher levels throughout the experimental period. In plants inoculated with the pathogen alone the activity declined greatly on the fourth day after the challenging inoculation. Invasion of root tissues by the pathogen might have resulted in decreased activity of PAL whereas earlier and increased activity of PAL due to Pf1 treatment might have prevented fungal invasion and thus maintaining the activity at higher levels. PO and PPO catalyze the last step in the biosynthesis of lignin and other oxidative phenols. In the present study, treatment with Pf1+azoxystrobin induced the activities of PO and PPO. A higher PO activity was noticed in cucumber roots treated with *P. corrugata* challenged with *P. aphanidermatum* (Chen *et al.,* 2000). Maurhofer *et al*. (1994) reported that induction of systemic resistance by *P. fluorescens* was correlated with an accumulation of chitinase and β-1,3 glucanase. These enzymes act upon the fungal cell wall, resulting in degradation and loss of the inner contents of cells (Benhamou *et al*., 1996). In our study, chitinase and β-1,3 glucanase have been induced to an enhanced level in cucumber plants through an application of Pf1+azoxystrobin against the downy mildew pathogen. Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In the present study, application of Pf1+azoxystrobin resulted in increased accumulation of phenolic substances in response to infection by the pathogen. M'Piga *et al.* (1997) reported that *P. fluorescens* isolate 63–28 induced the accumulation of phenolics in tomato root tissues. *P. fluorescens* isolate Pf1 also induced the accumulation of phenolic substances and PR-proteins in response to infection by *C. capsici* in pepper (Ramamoorthy and Samiyappan, 2001). Since several defense-related genes encoding proteins are synthesized in ISR by fluorescent pseudomonads, it has been hypothesized that induced resistance by Pf1 is related to multigenic/polygenic (horizontal) resistance in plants which is effective against multiple pathogens/races of pathogens. Tuzun (2001) described that constitutive accumulation of defense-related gene products was an integral part of both multigenic resistance and ISR. Induced resistance by fluorescent pseudomonads covers a broad spectrum activity against several fungal, bacterial and viral diseases (Hoffland *et al.,* 1996 and 1997; Maurhofer *et al.,* 1994; Wei *et al*., 1991 and 1996; Zehnder *et al*., 2001).

In conclusion, foliar application of Pf1 combined with a half of the recommended dose of azoxystrobin is of practical significance, since an application of fungicide alone requires three to four further following sprays for an effective control of downy and powdery mildew

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diseases; also the biochemical nature of resistance due to the application of azoxystrobin and Pf1 (dealt with in the present study) helps towards an understanding of the nature of action going on in the host plant.

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مدیریت سفیدک‌های خیار (*Cucumis sativus* L.) با استفاده از *Pseudomonas fluorescens* متحمل به ازاکسی‌استروبین

ت. آنند، ا. چندراسکاران، س. کوتالام، ت. راجوچاندر و ر. سامی‌یاپان

چکیده

مطالعات سازگاری باکتری *Pseudomonas fluorescens* (Pf) با غلظتهای ۱۰۰، ۱۵۰، ۲۰۰، ۲۵۰ و ۳۰۰ پی‌پی‌ام ازاکسی‌استروبین نشان داد که این باکتری با همه غلظت‌های مورد استفاده این قارچکش سازگار بوده و رشد آن حتی در غلظت ۳۰۰ پی‌پی‌ام نیز تحت تأثیر قرار نگرفت. در آزمایش مزرعه‌ای استفاده همزمان باکتری (۲/۵ کیلوگرم/هکتار) و ازاکسی‌استروبین (۲۵۰ میلی‌لیتر/هکتار) در قسمتهای هوایی گیاه، شدت بیماری‌های سفیدک داخلی و سفیدک سطحی را حتی بیشتر از قارچکش ازاکسی‌استروبین (۲۵۰ و ۵۰۰ میلی‌لیتر/هکتار) کاهش داد. شاخص درصد بیماری (Percent Disease Index, PDI) در فصل زراعی اول و دوم، در تیمار کاربرد توأم قارچ‌کش و باکتری علیه سفیدک پودری بترتیب ۲/۲۲ و ۱/۰۰ و سفیدک داخلی ۱/۸۵ و ۰/۵۰ بود. همچنین بالاترین میزان عملکرد به میزان ۱۴/۰۳ و ۱۵/۶۵ تن/هکتار، به ترتیب در دو فصل زراعی اول و دوم حاصل گردید. استفاده همزمان باکتری و ازاکسی‌استروبین به طور معناداری میزان بقای باکتری *P. flourescens* بر روی سطح برگهای خیار را افزایش داد. علاوه بر این میزان پراکسیداز، فنل‌اکسیداز، فنیل‌آلانین آمونیا لیاز، بتا-۱، ۳ گلوکاناز، کیتیناز و ترکیبات فنلی در گیاهان تیمار شده تا چندین برابر افزایش یافت.

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