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Biological Control Agent in Bell Pepper Infected by Powdery Mildew (*Leveillula taurica*) (Lev.) Arn.: A Biochemical Study

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ABSTRACT

Biological control agents and botanicals are increasingly being used as alternative strategies to chemicals in the control of plant diseases. The effect of some of the biological control agents and botanicals viz., *Trichoderma viride*, *T. harizianum* and *Pseudomonas fluorescens* IIHR+3, emulsified neem (*Azadirachta indica*) oil and NSKE (neem seed kernel extract) as foliar spray in powdery mildew infected bell pepper, grown in a polyhouse were compared with fungicides such as dinocap® and Contaf® in treated and untreated – positive control (healthy) and negative control (diseased) – plants. Estimation of biochemical changes associated with all the above treatments was assessed. Our data show that *T. viride* recorded maximum levels of 1.23, 0.92, 6.75 mg g^{-1} at 35 DAT and 1.0, 0.71, 8.1 mg g^{-1} at 50 DAT in total phenolics, ortho-dihydroxy phenolics and total soluble sugar, respectively. The next best treatment was *T. harizianum*. Similarly, the maximum amount of chlorophyll *a*, *b*, and total chlorophylls was observed in *T. viride*- and *T. harizianum*-treated plants. Overall total phenolics and ortho-dihydroxy phenolics decreased at 50 DAT more than at 35 DAT suggesting that foliar spray of biological control agents, botanicals and fungicides could control powdery mildew disease after one or two sprays which resulted in low levels of pathogen load and ultimately lower levels of defense-related biochemical components. Interestingly, during this study we found increased levels of biochemical constituents at 50 DAT in *T. viride* and *T. harizianum* even though powdery mildew was less than 5%. These fungal biological control agents may have played a vital role in enhancing the defense related biochemical components.

Keywords: botanicals, induced biochemical changes, total phenolics, ortho-dihydroxy phenolics, total soluble sugar, total chlorophylls

INTRODUCTION

Bell pepper is one of the most popular and highly remunerative vegetable crops grown for fresh fruits throughout the world. In India, capsicum is grown in 4,783 ha and production is 42,230 tones (Madavi Reddy 2003) and productivity of 8.83 t/ha (Sidhu 1998). In Karnataka, the area under this crop during 2000 was estimated 4,057 ha with a total production of 25,107 tons (Anon. 2001).

High consumption and market for bell pepper is attributed to increasing demand by urban consumers in India. However, its productivity in India is very low compared to western countries because it suffers from many diseases, pests and disorders that reduce fruit quality and yield. Diseases can be caused by wide range of biological agents, including fungi, bacteria, viruses, insects, nematodes, birds and mammals (Bosland 2003). The most important diseases caused by fungi and Oomycetes are powdery mildew, anthracnose, *Cercospora* leaf spot, charcoal rot, *Choanephora* blight, damping-off, root rot, *Fusarium* stem rot, *Fusarium* wilt, gray leaf spot, gray mold, *Phytophthora* blight, southern blight, white mold and *Verticillium* wilt. Among these fungal diseases powdery mildew, *Leveillula taurica* (Lev.) Arn. an obligate pathogen takes heavy toll in field conditions every year all over the world (Palti 1988) and 10-15 per cent yield loss under greenhouse conditions (Cerkauskas *et al*. 1999).

Disease induces many morphological and biochemical changes (Teiz and Zeiger 1998). Morphological changes are characterized by chlorosis, necrosis, wilting, stunted growth, deformed plant organs etc. Infection by a fungal pathogen induces quantitative variations of secondary metabolites, synthesis of defense related compounds such as phytoalexins, PR-proteins and several oxidative enzymes such as PAL, peroxidase etc., are generally observed (Vidhyasekaran 1988).

Thirteen (systemic and non-systemic) fungicides have been reported to control powdery mildew of bell pepper (Manoj Kumar 2007) Out of three systemic viz., fenarimol (rubigan), carbendazim (bengard), tridemorph (calixin) and 4 non-systemic viz., dinocap (karathane), elemental sulphur (sulfex), mancozeb (dithane M-45) fungicides, fenarimol the most economic treatment for controlling pea powdery mildew (Panja and Chaudhuri 1994). Triazole fungicides such as bromuconazole, hexaconazole, difenconazole, penconazole and triadimefon gave good control against *L. taurica* causing powdery mildew of artichoke (Fiori *et al.* 1996), pea (Nagaraja and Naik 1998). Dhruj *et al.* (2000) reported that seven fungicides viz., propiconazole, penconazole, hexaconazole, triadimefon, tridemorph, dinocap and sulphur used to control powdery mildew (*L. taurica*) of fenugreek significantly reduced the disease as compared to the control. However, hybrids grown under intensive cultivation indiscriminately receive very high doses of fungicides and insecticides resulting in the development of resistance to many diseases, pests, outbreak of secondary pests and accumulation of pesticide residues in the final produce. Environmental and consumer concerns have focused interest on development of biological control agents as an environmentally friendly strategy for the protection of agricultural and horticultural crops against phyllopathogens (Dunne *et al.* 1998). Hence, these became an alternative to chemicals (Ravikumar 1998; Biju 2000) and plant products (Amadioha 1998) have been gaining more importance in modern day agriculture.

Biological control agents or antagonists play an important role in the life of plants by reducing the susceptibility, or increasing the tolerance of plants to pathogens (Mandavia *et al.* 1990; Padgett and Morrison 1990; Jalali *et al.* 1991; Thiagarajan and Ahmed 1994; Biju 2000; Brand *et al.* 2002;

Received: 1 October, 2007. Accepted: 5 December, 2007.

Original Research Paper

Tsror *et al*. 2003). These authors showed that modifications in plant physiology following antagonist infection may explain the decrease in susceptibility to pathogens. These effects may arise through changes in sugar levels, phenol synthesis, amino acid concentrations, pectic and lignin formation, oxidative enzyme activities or ethylene production following antagonist infection. Different studies revealed that simultaneous inoculation with *Glomus mosseae* and *Rhizobium leguminosarum* increased plant tolerance to a variety of pathogens (Mahima *et al.* 1990; Smith 1990; Seikhon *et al.* 1992; Lynd and Ansman 1994). Other bio-control agents such as *T. harzianum*, *T. viride* and *P. fluorescens* were used against powdery mildew of pea (Biju 2000). *Ampelomyces quisqualis* (AQ10) significantly reduced powdery mildew on organic pepper (Tsror *et al*. 2003).

Since a little is known about the intervening biochemical events occurring in bell pepper treated with biological control agents and botanicals, the present study aimed to examine the effect of biological control agents, botanicals and fungicides on total phenolics, ortho-dihydroxy (O.D.) phenolics, total soluble sugars, and chlorophylls *a* and *b* of bell pepper plants sprayed with these agents against powdery mildew infection.

MATERIALS AND METHODS

A polyhouse experiment was conducted at the Indian Institute of Horticultural Research (ICAR), Bangalore, India. Indra, a variety of bell pepper was selected to assess powdery mildew disease in polyhouse using the following treatments and its influence on biochemical components. Seedlings of cv. 'Indra' were raised in Protrays® (60 ×15 cm) containing soilrite in nursery for 25 days and were transplanted to 1.5 m^2 blocks in a polyhouse containing a mixture of red loamy soil, sand and farm yard manure in the ratio of 2:1:1. Samples were collected (third nodal leaf from top) at 25, 35 and 50 DAT (days after transplantation) from all plots for biochemical analysis. After collecting the samples at 25 DAT powdery mildew spore solution was sprayed on all plots. At the onset of powdery mildew disease, different treatments viz., *Trichoderma viride* Pers.:Fr. (2%), *T. harzianum* Rifai (2%) and *Pseudomonas fluorescens* IIHR^{+}3 (2%), emulsified neem (*Azadirachta indica* A. Juss) oil (0.5%), NSKE (Neem Seed Kernel Extract) (10%), dinocap (Karathane®) (1 ml/L) (Bayer Pvt. Ltd, Mumbai, India) and Contaf® (0.05%) (Ralis Pvt. Ltd, Bangalore, India) were given as foliar spray to manage the powdery mildew disease. Isolates of *T. viride*, *T. harzianum* and *P. fluorescens* were collected from IIHR, Bangalore, India. Two sprays of the above treatments were given at fortnight intervals for the management of powdery mildew at 30 and 45 DAT (Manoj Kumar 2007). Two sets of unsprayed controls viz., positive control (healthy) and negative control (diseased) were maintained as a check.

Preparation of powdery mildew spore solution (Manoj Kumar *et al.* 2006)

The lower leaves of bell pepper infected by powdery mildew, *L. taurica* was freshly collected form the unsprayed control plots of experimental field at I.I.H.R, Bangalore, India. Later the lower sporulated surface of leaves was washed with sterile distilled water and conidial spore load was adjusted to 5×10^5 spores/ml. Freshly prepared powdery mildew spore solution was uniformly sprayed to all blocks of bell pepper plants 25 DAT and powdery mildew disease was ensured. The inoculum spray was performed during late evening hours. Control (healthy) blocks were maintained separately without powdery mildew spore spray and free of disease.

Preparation of antagonists

Fungal antagonists viz., *T. harzianum, T. viride* were multiplied on Potato Dextrose Broth at 27 ± 1°C for 7 days. The bacterial antagonist *P. fluorescens* was multiplied on nutrient broth at 35 ± 1°C for three days at 120 rpm in an incubator shaker.

Extraction and estimation of metabolites

Metabolites were extracted by a modified method of Barnett and Naylor (1996). Leaf tissue (0.5 g) was homogenized in 80% ethanol (v/v) and centrifuged for 10 min at 10,000 rpm. The homogenate was refluxed thrice for 15 min on a water bath at 60°C. The supernatants were pooled together and a final volume was made up to 25 ml with ethanol and used for estimation of total soluble sugars (Yemm and Willis 1954), total phenolics (Amorim *et al*. 1977), ortho-dihydroxy phenolics (Johnson and Schaal 1957). The estimation of chlorophylls (*a* and *b*) were done as per the methods of Litchenthaler (1987).

Statistical analysis

The experiment was conducted in a completely randomized design (CRD) with seven treatments and each treatment had five replications (each block one replication). The resultant data was subjected to analysis of variance (ANOVA) followed by mean separation by the Student Newman-Keuls' test (p=0.01 and p=0.05). All analyses were performed using the SAS (1996) package.

RESULTS AND DISCUSSION

The results of the biochemical investigation of bell pepper treated with different disease controlling molecules are presented in **Tables 1** to **4**. It is obvious from **Table 2** that at 25 DAT (before powdery mildew spore inoculation) there was no significant difference among the treatments in the estimated values of total phenolics i.e., 0.9 mg g^{-1} in NSKE,

Table 1 Estimation of chlorophyll content in bell pepper treated with biological control agents, botanicals and fungicides.

Treatments	Chlorophyll *a**		Chlorophyll *b**		Total chlorophyll*	
	35 DAT	50 DAT	35 DAT	50 DAT	35 DAT	50 DAT
T. viride	0.12 a	0.11 a	0.14 a	0.13 a	0.26 a	0.24 a
T. harzianum	0.11 a	0.11 a	0.14 a	0.13 a	0.25 ab	0.24 a
P. fluorescens	0.10 a	0.10 a	0.10 ab	0.09 bcd	0.20 cd	0.19 bc
Neem oil	0.11 a	0.10 a	0.11 ab	0.11 ab	0.21 bcd	0.21 ab
NSKE	0.10 a	0.09 a	0.12 ab	0.11 ab	0.21 bcd	0.20 ab
Dinocap	0.11 a	0.11 a	0.13 ab	0.12 ab	0.24 abc	0.23 ab
Contaf®	0.06 b	0.06 b	0.07 b	0.07 cd	0.13 e	0.13 a
Control-Healthy	0.10 a	0.10 a	0.11 ab	0.11 ab	0.21 bcd	0.21 ab
Control-Diseased	0.06 b	0.06 b	0.06 b	0.06 d	0.12 e	0.15 cd
CV	12.20	11.32	11.8	10.48	8.30	6.573
CD (P=0.01%)	0.031	0.025	0.04	0.027	0.04	0.032
SE ±	0.009	0.006	0.01	0.006	0.01	0.008

*mg g^{-1} fresh weight

Values followed by different letters within a column are significantly different by SNK (p<0.01).

Table 2 Changes in the levels of total phenolics against the different treatments to control powdery mildew infection in bell pepper.

Treatments	25 DAT*	35 DAT*	50 DAT*	PDI at 30 DAT*	PDI at 45 DAT*
T. viride	0.89 b	1.23 a	1.00 a	45 (6.75) c	5.0 (2.34) b
T. harzianum	0.89 b	1.11 ab	0.97 ab	45 (6.75) c	5.0 (2.34) b
P. fluorescens	0.89 b	0.90 b	0.79 c	33 (5.78) b	3.8 (2.06) b
Neem oil	0.89 b	0.99 b	0.88 abc	44 (6.67) c	4.0 (2.11) b
NSKE	0.90 a	0.94 b	0.85 abc	45 (6.75) c	4.2 (2.16) b
Dinocap	0.89 b	0.94 b	0.86 abc	44 (6.67) c	5.0 (2.34) b
Contaf®	0.89 b	0.91 b	0.84 bc	44 (6.67) c	5.0 (2.34) b
Control-Healthy	0.90 a	0.91 b	0.89 abc	0.0 (1.00) a	0.0 (0.71) a
Control-Diseased	0.90 a	0.95 b	0.96 ab	45 (6.75) c	62 (7.91) c
CV	2.26	11.4	9.71	3.34	5.75
CD (P=0.05%)	0.04	0.23	0.15	0.34	0.27
SE ±	0.01	0.07	0.05	0.11	0.09

*mg g^{-1} fresh weight

Means followed by common letters within a column are non significant at 5%.
Figures in parentheses are square root transformed values.

healthy and diseased controls and 0.89 mg g^{-1} in *T. viride* and *T. harzianum*, *P. fluorescens*, neem oil, dinocap and Contaf®. Since at 25 DAT all plants maintained in the polyhouse were free of powdery mildew disease all the plants had almost similar values of total phenolics. After the powdery mildew spore inoculation, when the percent disease incidence (PDI) was taken at 30 DAT, 45% of powdery mildew incidence was observed in *T. viride* and *T. harzianum*, NSKE and control (diseased), 44% PDI in neem oil, dinocap and Contaf® and 33% PDI in *P. fluorescens* was observed in contrast no disease observed in separately maintained control (healthy) plants. At the onset of powdery mildew disease, a first spray of different treatments were given to individual plants in order to control the disease. The biochemical estimations after five days of the first spray i.e. at 35 DAT shows the significant difference among the treatments. High levels of total phenolics i.e., 1.23 and 1.11 mg g^{-1} were observed in *T. viride* and *T. harzianum*, respectively followed by neem oil-treated plants (0.99 mg g^{-1}), NSKE (0.94 mg g^{-1}), dinocap (0.94 mg g^{-1}), Contaf® (0.91 mg g^{-1}) and control (healthy) (0.91 mg g^{-1}) (**Table 2**). Low levels of total phenolics were detected in *P. fluorescens*-treated plants vs. 0.95 mg g^{-1} in the untreated control (diseased). Accumulation of more phenolics against fungal infection (Galzener 1982) was the reason for increased values of total phenolics in all infected plants except control (healthy) plants. However, at 50 DAT the total phenolics decreased and the values ranged from 0.79 to 0.88 mg g^{-1} in all treatments except in *T. viride* and *T. harzianum* (respectively, 1.0 and 0.97 mg g^{-1}) vs. control-healthy (0.89 mg g^{-1}) and control-diseased (0.96 mg g^{-1}). But, the percent disease incidence in all treatments ranged from 3.8 to 5.0 (**Table 2**). A maximum of 62 PDI was recorded in the control (diseased) and no disease symptoms/incidence was observed in control (healthy) plants. Hence, the disease was significantly controlled by treatment sprays which could have resulted in a decrease in phenolics in all treated plants. In contrast, an outstanding observation was made in *T. viride*- and *T. harzianum*-treated plants which showed lowest disease and the highest amount of phenolics. These high levels of phenolics could be induced by fungal biological control agents. Similarly, *Vicia faba* infected with *Botrytis fabae* showed increased concentrations of phenolics compared to that of non-infected plants. In contrast, dual inoculation of *Rhizobium* and VA mycorrhizae further increased phenol concentration suggesting a distinct improvement of the plants (Rabie 1998). Mayama and Shishiyama (1978) suggested that localized accumulation of more phenolic compounds at penetration site of fungus might have controlled the infectivity of the mildew fungus in barley. Parallel reports of increased total phenols in infected plants compared to healthy plants were observed by Galzener (1982) in tomato, Biechn *et al*. (1967) in soybean and Vidyasekeran (1975) in finger millet.

Among the different phenolics, ortho-dihydroxy (OD) phenolics are known to be highly fungi-toxic (Vidyasekeran 1998). In the present investigation, no significant observation was made at 25 DAT. The quantity of OD phenolics were 0.63 mg g^{-1} in *T. viride* and *T. harzianum*, neem oil, dinocap and control (diseased) followed by 0.61 mg g^{-1} in NSKE, Contaf® and control (healthy) (**Table 3**). The OD phenolics content was maximum during the onset of disease in *T. viride* (0.92 mg g^{-1}) and *T. harzianum* (0.87 mg g^{-1}) vs. control (healthy) (0.63 mg g^{-1}) at 35 DAT, while significantly less content was observed in neem oil (0.78 mg g^{-1}), dinocap (0.75 mg g^{-1}) and NSKE (0.74 mg g^{-1}). Minimum OD phenolics were found in *P. fluorescens* (0.67 mg g^{-1}). Subsequent treatments sprays (two) at 15 day intervals had successfully controlled powdery mildew disease (Manoj Kumar 2007) which ultimately decreased the OD phenolics in all treatments except in *T. viride* (0.71 mg g^{-1}) and *T. harzianum* (0.69 mg g^{-1}), unlike the control (healthy) (0.62 mg g^{-1}) at 50 DAT.

Table 3 Changes in the levels of O.D. phenolics against the different treatments to control powdery mildew infection in bell pepper.

Treatments	25 DAT*	35 DAT*	50 DAT*
T. viride	0.63 a	0.92 a	0.71 b
T. harzianum	0.63 a	0.87 a	0.69 bc
P. fluorescens	0.60 a	0.67 d	0.60 d
Neem oil	0.63 a	0.78 b	0.62 cd
NSKE	0.61 a	0.74 bc	0.61 cd
Dinocap	0.63 a	0.75 bc	0.62 cd
Contaf®	0.61 a	0.69 cd	0.61 cd
Control-Healthy	0.61 a	0.63 d	0.62 cd
Control-Diseased	0.63 a	0.79 b	0.81 a
CV	3.92	4.32	7.11
CD (P=0.05%)	0.04	0.06	0.08
SE ±	0.01	0.02	0.03

*mg g^{-1} fresh weight

Means followed by common letters within a column are non significant at 5%.
Figures in parentheses are square root transformed values.

However, few variations in the biochemical components of control (healthy) plants are because of their age and not due to any physiological disease as described by Teiz and Zeiger (1998). Further, the decrease of total phenolics and OD phenolics at 50 DAT compared to 35 DAT suggests that foliar spray of the biological control agents, botanicals and fungicides controlled the powdery mildew disease after one and two sprays, resulting in low levels of pathogen load and ultimately lower level of these defense-related biochemical components.

Many pathogens prefer simple sugars for their growth; hence the quantity of soluble sugars could vary with the infection (Vidhyasekaran and Kandasamy 1972). Similarly, in the present study, the total soluble sugars varied with a narrow range of 5.21-5.2 mg g^{-1} in all the treatments at 25 DAT (**Table 4**). Gradually with the powdery mildew infection, the sugars decreased in neem oil, NSKE, dinocap, Contaf®, and *P. fluorescens* and control (diseased) treatments. In con-

Table 4 Changes in the levels of total soluble sugars against the different treatments to control powdery mildew infection in bell pepper.

Treatments	25 DAT*	35 DAT*	50 DAT*
T. viride	5.20 a	6.75 a	8.05 a
T. harzianum	5.21 a	6.61 a	7.90 a
P. fluorescens	5.20 a	5.02 bc	5.00 f
Neem oil	5.20 a	4.75 c	6.60 bc
NSKE	5.21 a	4.27 d	6.20 cd
Dinocap	5.20 a	4.05 de	5.95 de
Contaf®	5.20 a	3.80 e	6.80 b
Control-Healthy	5.20 a	5.10 b	5.70 e
Control-Diseased	5.21 a	3.30 f	3.35 g
CV	2.39	3.81	4.01
CD (P=0.05%)	0.21	0.34	0.40
SE ±	0.07	0.10	0.17

*mg g^{-1} fresh weight

Means followed by common letters within a column are non significant at 5%. Figures in parentheses are square root transformed values.

trast, there was a significant increase of total soluble sugar contents in *T. viride* and *T. harzianum*.

Overall the resultant data of total phenolics, OD phenolics and total soluble sugars were found to be significant. When the powdery mildew disease incidence was high, all the biochemical components increased compared to healthy controls (as normal disease-free plants). But, these biochemical components in *T. viride* and *T. harzianum* were found to be higher than control (healthy) plants, which would be induced by these fungal antagonists. Subsequent sprays of biological control agents, botanicals and fungicides ultimately decreased the powdery mildew incidence of bell pepper which resulted in a decrease of these biochemical components which is comparatively similar with healthy controls. In other words, healthy plants do not show any changes in these defense-related biochemical molecules but infected plants do.

Irrespective of the treatments, chlorophyll *a* and *b* contents did not show any significant decline, which indicates the active metabolic activity at both 35 and 50 DAT (**Table 1**). The minor reduction of chlorophyll content at 35 and 50 DAT may be due to photoxidation of chlorophyll (Ramachandran *et al*. 1991). Overall, *T. viride* and *T. harzianum* had 0.26 and 0.25 mg g^{-1} of total chlorophyll at 35 DAT and 0.24 mg g^{-1} at 50 DAT respectively; in contrast, 0.21 and 0.12 mg g^{-1} of total chlorophyll at 35 DAT and 0.21 and 0.15 mg g^{-1} at 50 DAT, respectively were observed in control-healthy and control-diseased plants.

ACKNOWLEDGEMENTS

The first author is thankful to Prof. Udayakumar and Dr. Rohini Sreevathsa, UAS, GKVK, Bangalore for providing facilities and support. The authors are also grateful to Dr. Girija Ganeshan, Principal Scientist, IIHR, Bangalore for her co-operation and help during the course of investigation.

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