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Induction of induced systemic resistance in capsicum against *Colletotrichum truncatum* through fungal biocontrol agents

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Abstract
To induce the systemic resistance in capsicum against *Colletotrichum truncatum* an experiment was conducted with two biocontrol agents *viz., Trichoderma harzianum* and *Trichoderma asperellum* in pot culture. The induced resistance was monitored by increase in activities of five defense related enzymes *viz.,* peroxidase (PO), polyphenol oxidase (PPO) phenylalanine ammonia lyase (PAL), catalase (CAT), and superoxide dismutase (SOD) and the accumulation of phenols and β -1,3-glucanase were also noticed in capsicum upon challenge inoculation with *C. truncatum* the causal agent for anthracnose or fruit rot in capsicum. The activities of defense enzymes reached a peak at eight days after inoculation (DAI) with the pathogen. Native PAGE analysis revealed the expression of an additional isoforms of PO and PPO were observed in biocontrol agents treated seedlings due to induced systemic resistance (ISR) introduction.

Keywords: Bioagents, induced resistance, defense related enzymes, phenols, peroxidise, polyphenol oxidase, chitinase, β-1,3-glucanase

Introduction
The capsicum (*Capsicum annuum* L. *var. grossum* Sendt; 2n = 24) is one of the most popular and highly remunerative annual herbaceous vegetable crops which is commonly known as Sweet pepper, Bell pepper, Cherry pepper, Green pepper and Shimla mirch. It is different from chilli (*Capsicum annuum* L. var. *longum*) in size and shape of the fruits, capsaicin content and belongs to family Solanaceae. In India, it is being cultivated in an area of 0.885 million ha with annual production of 0.9 million MT (National Horticultural Board, 2019-20). It is extensively cultivated as summer crop in Jammu and Kashmir, Gujarat, Himachal Pradesh, Uttarakhand, Arunachal Pradesh and West Bengal as an autumn crop in Uttar Pradesh, Madhya Pradesh, Jharkhand, Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh and Bihar (Chadha, 2005). In Andhra Pradesh, it occupied an area of 2,136 ha with a production of 32, 092 MT (National Horticultural Board, 2019-20). *Colletotrichum* is an important pathogenic genus worldwide. These fungi cause disease symptoms that are generally known as anthracnose in a wide range of vegetables, fruits and other crops. In capsicum, anthracnose is a destructive disease caused by a complex of *Colletotrichum* species that causes extensive yield losses at both the pre- and post-harvest stages during warm and rainy seasons. Induced resistance may provide an alternative approach to plant protection especially for problems not satisfactory controlled by various fungicides (Schoenbeck, 1996) [20]. Induced resistance is defined as an enhancement of the plant defensive capacity against broad spectrum pathogens that is acquired after appropriate stimulation. The resulting elevated resistance due to an inducing agent upon infection by a pathogen is called ISR or SAR (Hammerschmidt and Kuc, 1995) [23]. Plant has endogenous defense mechanisms that can be induced in response to attack by insects and pathogens (Bostock *et al.,* 2001) [1]. Defense reaction occurs due to the accumulation of PR-proteins, phytoalexins, chalcone synthase, PAL, PO, PPO and phenolics. The objective of the present.

Experimental material and chemicals
Induction of systemic resistance in capsicum by biocontrol agents
The effective biocontrol agents *viz., Trichoderma harzianum* (Th1), *Trichoderma viride* (Tv1) selected based on *in vitro* and pot culture studies were formulated using talc as a carrier. Capsicum seedlings of variety Arka Mohini were treated separately with the formulated biocontrol agents and planted in pots containing rooting medium.

Instead of soil, rooting medium (coir pith: vermicompost @ of 5:3 v/v) was used for raising capsicum plants. Experiments were conducted in completely randomized design with three replications in each treatment. The biocontrol agents were sprayed in 30 days old plants and challenge inoculated with pathogen after two days. The treatments also included seedling treatment followed by foliar spray of biocontrol agents at 30 DAP without challenge inoculation. Leaf samples were collected at 0, 2, 4, 8 and 10 days after challenge inoculation with pathogen to assay the changes in activities of defense related enzymes *viz*., phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), b-1-3-glucanase, catalase (CAT), superoxide dismutase (SOD) and phenol. The plants inoculated only with pathogen and also healthy plants were maintained for comparison.

Phenylalanine ammonia lyase (PAL) Assay

One g of capsicum leaf was homogenized in 2 ml of ice-cold 0.1 M sodium borate buffer, pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4 ºC. The supernatant was used to assay the enzyme activity. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson *et al.*, 1984) [4]. A sample extract of 0.4 ml was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 1 ml of 12 mM L- phenylalanine and incubated for 1 h at 30 ºC. The reaction initiated by L- phenylalanine was stopped with 0.5 ml of 2 N HCl. A blank was maintained by adding L-phenylalanine after the addition of 2 N HCl. The absorbance was read at 290 nm and the results were expressed as nmol trans-cinnamic acid/min/g of fresh tissue.

Peroxidase (PO) Assay

The activity of PO was determined as detailed by Hammerschmidt *et al.* (1982) [6]. One g of leaf sample was homogenized in 1 ml of 0.1 M phosphate buffer pH 7.0 in a pre-cooled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min at 4 ºC. The supernatant was used to assay activities of PO and PPO. 1.5 ml of 0.05 M pyrogallol and 0.1 ml of enzyme extract were taken and added to the cuvette. To initiate the reaction 0.5 ml of 1% H_2O_2 was added. The change in absorbance was recorded at 420 nm at 30 sec interval for three min from zero second of incubation at room temperature. The results were expressed as a change in absorbance/min/g of fresh tissue.

Polyphenol oxidase (PPO) Assay

The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer pH 6.5 with 0.1 ml of enzyme extracts. To this 0.2 ml of 0.01 M catechol was added to initiate the reaction. The change in absorbance was recorded at 490 nm and the results were expressed as a change in absorbance/min/g of fresh tissue (Mayer *et al.*, 1965) [12].

Catalase (CAT) Assay

The catalase activity was estimated following the procedure described by Dekock *et al.* (1960). Five hundred mg of the sample was homogenized in 10 ml of ice cold 0.067 M sodium phosphate buffer (pH 7.0) and centrifuged and the supernatant was used as 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 sec. The activity was expressed as µ mol of H_2O_2 consumed/min/g fresh tissue.

Superoxide dismutase (SOD) Assay

The enzyme extract was prepared by homogenizing 1g leaf tissue in two ml of 0.2 M citrate phosphate buffer (pH 6.5) at 4 ºC. The homogenate was centrifuged at 12,000 rpm at 4 ºC for 30 min. The supernatant served as enzyme source and SOD activity (EC 1.15.1.1) was determined as its ability to inhibit the photochemical reduction of Nitro Blue Tetrazolium (Giannospolitis and Ries, 1977). The assay mixture (3 ml) contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin. 0.1 mM EDTA and 100 µl of the enzyme extract and the riboflavin was added at the end. Tubes were shaken and placed under a 40-W fluorescent lamp at 25 ºC. The reaction was initiated and terminated by turning the light on and off respectively. The absorbance at 560 nm was measured against identical non-illuminated in parallel to the sample tubes for blank. Each extract was subtracted from the blank and mathematical difference was then divided by blank and multiplied by 100 to obtain the percentage inhibition of NBT photo-reduction. The SOD activity was expressed in SOD units g^{-1} fresh tissue (50% NBT inhibition = 1 unit) (Belid El-Moshaty *et al.*, 1993).

Total Phenolic Content

Leaf samples were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 ºC. To 1 ml of the extract, 5 ml of distilled water and 250 ml of Folin-ciocalteau reagent (1 N) were added and incubated at 25 ºC for 3 min. After that 1 ml of 20% sodium carbonate was added and mixed well. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was read at 750 nm and catechol was used as the standard. The total phenol content was expressed in µg of catechol/g of fresh tissue (Zieslin and Ben Zaken 1993) [19].

β-1, 3-glucanase Assay

Crude enzyme extract of 62.5 ml was added to 62.5 ml of laminarin and then incubated at 40 ºC for 10 min the reaction was stopped by adding 375 ml of dinitrosalicylic acid and heated for 5 min on boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with laminarin with zero time incubation served as blank. The activity was expressed as µg equivalent of glucose/ min/g of protein (Maurhofer *et al.*, 1994) [21].

Native-polyacrylamide gel electrophoresis (PAGE) analysis for isozyme induction PO and PPO

Native-PAGE analysis was used to find out the expression of PO and PPO isoforms. Samples was homogenized with 1 ml of 0.1 M sodium phosphate buffer pH 7.0 and centrifuged at 10,000 rpm for 20 min at 4 ºC. The protein content of the sample was determined by Bradford (1976) [2] method. Samples (50 µg protein) were loaded onto 8% polyacrylamide gel. After electrophoresis, the gel was stained in 0.2 M acetate buffer at pH 4.2 containing 0.05% benzidine for 30 min in dark. Then drops of H_2O_2 (0.03%) was added slowly with constant shaking to visualize the PO isoforms. After staining the gel was washed with distilled water (Nadlony and Sequerira, 1980) [13]. For PPO, the gel was immersed in P-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer pH 7.0 for 30 min. Later 10 mM catechol was added and kept in a shaker with gentle shaking and observed for dark brown protein bands (Jayaraman *et al.*, 1987) [7].

Statistical analysis

All the analyses were repeated once with similar results. The data were statistically analyzed by using the IRRISTAT package developed by International Rice Research Institute, Biometrics Unit, Philippines. The treatments means were compared by DMRT.

Results and Discussion:

Induction of systemic resistance in capsicum by biocontrol agents:

The induced resistance against anthracnose disease was measured in capsicum cv. Arka Mohini through biochemical analysis of leaf samples collected from *Colletotrichum truncatum* inoculated and biocontrol agents treated plants (Plate 4.43). The results revealed the increased activities of the enzymes *viz.*, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, superoxide dismutase β-1,3-glucanase, catalase and phenols in the biocontrol agents treated capsicum plants against disease. In isozyme analysis, additional PO and PPO isoforms with greater intensity were induced with biocontrol agents that were absent in control.

Phenylalanine ammonia lyase (PAL)

Significant difference in phenylalanine ammonia lyase activity was observed in all treatments (Table 4.116 and Figure 4.16). Gradual increase in phenylalanine ammonia lyase activity was observed in all the treatments up to 6 DAI and thereafter the activity was decreased in all the treatments. The treatment T_6 (TH1+TV1+Pathogen) recorded the maximum activity of phenylalanine ammonia lyase (6.42 n mol transcinnamic acid/min/g of fresh tissue) at 6 DAI which was on par with the treatment T_3 (TH1+TV1) (6.12 n mol transcinnamic acid/min/g of fresh tissue). Comparatively less activity of phenylalanine ammonia lyase was observed in the treatment with pathogen inoculated control (2.23 n mol transcinnamic acid/min/g of fresh tissue) at 6 DAI.

Other researchers had observed increased activity in these enzymes in the host tissues in response to pathogenic infections (Abo-Elyousr *et al.*, 2010 and Ojha *et al.*, 2012). Chen *et al.* (2000) reported that the increased activity of PAL can also be contributed for enhancing the resistance in tomato plants against fungal pathogen, *F. oxysporum* f.sp. *Lycopersici* and induction of PAL by Fluorescent pseudomonas in cucumber against *P. aphanidermatum.*

Peroxidase (PO)

At 6 DAI the enhanced activity of PO was observed in capsicum cv. Arka Mohini (Table 4.114 and Figure 4.14). The treatment T_6 (TH1+TV1+Pathogen) recorded the maximum activity of peroxidase (1.054 changes in A420/min/g of fresh tissue) at 6 DAI which was on par with the treatment T_3 (TH1+TV1) (1.013 changes in A420/min/g of fresh tissue).

These findings are, in agreement with those of several workers. Bio formulation of *T. virens* sprayed on leaves and flowers increased the induction of peroxidase activity in cucumber (Wei *et al.*, 1996) [25]. Bradford. (1976) [2] reported that increased PO activity has been correlated with resistance in many species including barley, cucurbits, cotton, tobacco, wheat and rice and these enzymes are involved in the polymerization of proteins and lignin or suber in precursor into plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration of cell walls or movement through vessels.

Polyphenol oxidase (PPO)

In the present study, the trend of increasing PPO activity was similar to that of PO in all the treatments (Table 4.115 and Figure 4.15). The enzyme activity was maximum (2.782 changes in A490/min/g of fresh tissue) at 6 DAI when the plants were pre-treated with TH1+TV1 challenged with the pathogen.

Radja commare (2002) reported that *P. fluorescence* induced PPO isoenzymes in rice against *R. solani.* Chen *et al.* (2000) reported that various rhizobacteria and *P. aphanidermatum* induced PPO activity in cucumber root tissues.

Superoxide dismutase (SOD)

The superoxide dismutase activity was significantly increased up to 6 days after inoculation in all the treatments and thereafter it was observed as declining (Table 4.120 and Figure 4.20). Among the treatments, the treatment T_6 with TV1+TH1 and challenge inoculation with pathogen recorded the maximum superoxide dismutase activity (19.24 unit/min/g of fresh tissue). Similar observations were recorded by Chakrabarthy *et al.* (2002) in grey mildew susceptible cotton lines.

Catalase (CAT)

Catalase activity was measured in capsicum by leaf samples collected from pathogen inoculated and biocontrol agents pretreated plants. In general, there was an increasing trend of catalase activity up to 6 days after inocualtion and subsequently gradually decreased in all the treatments (Table 4.118 and Figure 4.18). The enhanced activity of catalase was observed in the treatment T_6 with TV1+TH1 and challenge inoculation with pathogen (3.78 μmol H_2O_2 min^{-1} g^{-1} of fresh tissue). Similar observations were recorded by Chakrabarthy *et al.* (2002) in grey mildew susceptible cotton lines.

β-1,3-glucanase

It was measured in capsicum plant by leaf samples collected from pathogen inoculated and biocontrol agents pretreated plants. In general, there was an increasing trend of β-1,3-glucanase activity up to 6 days after inoculation and subsequently gradually decreased in all the treatments (Table 4.119 and Figure 4.19). The enhanced activity of β-1,3-glucanase was observed in the treatment T_6 with TV1+TH1 and challenge inoculation with pathogen (201.96 µg of glucose/min/g of fresh tissue).

Several literatures have documented the use of biocontrol agents in combination was more effective for management of plant diseases and pathogens compared to individual agents. It significantly increased the induction of PR proteins and enzymatic activity of phenols, PAL, PO, PPO SOD and CAT in groundnut plants upon challenged with *Sclerotium rolfsii* (Young *et al.*, 2008).

Total phenols

Phenolics are fungi toxic in nature and increase the physical and mechanical strength of the host cell wall. Plant phenolics and their oxidation products such as quinines are highly toxic to invading fungi thereby offering resistance against a wide range of pathogens (Cahill and Mccomb, 1992) [3]. In our study at rendonphenol accumulation was noticed in all plants treated with the bio-control agents had a profound effect on the accumulation of phenols in plants upon challenge inoculation with pathogen. Its accumulation increased from third day and attained peak on 6 DAI (Table 4.117 and Figure 4.17). Maximum (595.20 µg of catechol/min/g fresh tissue) accumulation of phenols was noticed in treatment T_6 with TH1+TV1 challenged with the pathogen at 6 DAI when compared to plants inoculated with the pathogen alone. Rathod and Vakharia (2011) reported similar results in chickpea cultivars under diseased environment of *Fusarium* sp.

Native PAGE analysis for Isozyme induction PO and PPO

Native PAGE analysis showed four isoforms (PO1 to PO4) of peroxidase was observed in all treatments except healthy control. However, the intensity of the isoform was more in plants treated with biocontrol agents than those challenged with the pathogen (Plate 1). The treatments challenged with the *C. truncatum* revealed the presence of four isoforms (PPO1 to PPO4) with more pronounced expression which was absent in healthy and inoculated control (Plate 2).

Discussion

Induction of systemic resistance by PGPR against various diseases was considered as the most desirable approach in crop protection. There are major differences in ISR when compared to other mechanisms. First, the action of ISR is based on the defense machanim that is activated by inducing agents. Second, ISR expresses multiple potential defense mechanism that include increased activity of chitinase, -1-3 glucanase and peroxidase (Maurhofer *et al.*, 1994; *Xue et al.*¸1998) [21, 22] and accumulation of antimicrobial low molecular substances- phytoalexins and formation of protective biopolymers *viz.*, lignin, callose and hydroxyproline rich glycoprotein (Hammerschmidt and Kuc, 1982) [23]. Third, an important aspect of ISR is the wide spectrum of pathogens that can be controlled by single inducting agent (Hoffland *et al.*, 1996; Wei *et al.*, 1996) [24, 25]. In the present investigation revealed enhanced activities of defense related enzymes PO, PPO, CAT, SOD and PAL and accumulation of phenol, β -1,3-glucanase in capsicum plants treated with biocontrol agents and challenged with *C. capsici.* In isozyme analysis, additional PO and PPO isoforms with greater intensity were induced with biocontrol agents that were absent in control.

Phenyl propanoid metabolism starts with the conversion of L-Phenylalanine into transcinnamic acid by PAL and supplies the precursors for flavanoid pigments, lignin and phytoalexins (Massala *et al.*, 1980; Hahlbrock and Scheel, 1989) [11, 5]. Increase in PAL activity subsequently increases the phenolic contents leading to disease resistance (Klessig and Malamy, 1994) [8]. Peroxidase (PO) is a component of an early response in plants to pathogen infection and plays a major role in the biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West, 1989). The products of the enzyme in the presence of hydrogen donor and hydrogen peroxide have antimicrobial activity (VanLoon and Callow, 1983) [16]. PO is one of the key enzyme involved in phenyl propanoid pathway and it is associated with disease resistance in plants (Hammerschmidt *et al.*, 1982) [6]. Bradley *et al.* (1992) reported that increased PO activity has been correlated with resistance in many species including barley, cucurbits, cotton, tobacco, wheat and rice and these enzymes are involved in the polymerization of proteins and lignin or suberin precursor into plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration of cell walls or movement through vessels. Polyphenol oxidase (PPO) is enzymes which use molecular oxygen to catalyze the oxidation of monophenolic and orthophenolic compounds. In the present study, the trend of increasing PPO activity was similar to that of PO in all the treatments. Phenolics are fungitoxic in nature and increase the physical and mechanical strength of the host cell wall. Plant phenolics and their oxidation products such as quinones are highly toxic to invading fungi thereby offering resistance against a wide range of pathogens (Cahill and Mccomb, 1992) [3]. In our study a trend on phenol accumulation was noticed in all plants treated with the biocontrol agents had a profound effect on the accumulation of phenols in plants upon challenge inoculation with *C. capsici*. Its accumulation increased from third day and attained peak on 6 DAI. Some phenolics may act as a signal molecules or antioxidants and thus induce resistance (Malamy *et al.*, 1990) [9].

Accumulation of pathogenesis-related (PR) proteins is known to be associated with systemic acquired resistance (SAR) in plants. Studies have shown that PR-proteins are also induced in plants upon treatment with *P. fluorescens*. PR proteins like chitinase and β-1, 3-glucanase have the potential to hydrolyze chitin and β-1, 3-glucan respectively, which are major components of fungal cell walls. Moreover the chitinase and glucanase release elicitors from the walls of fungi which, in turn, stimulate various defense responses in plants. Scavengers of active oxygen species like catalase (which catalyzes the decomposition of H2O2) and superoxide dismutase (which scavenges O_2) suppress the oxidative burst and inhibit tissue necrotization.

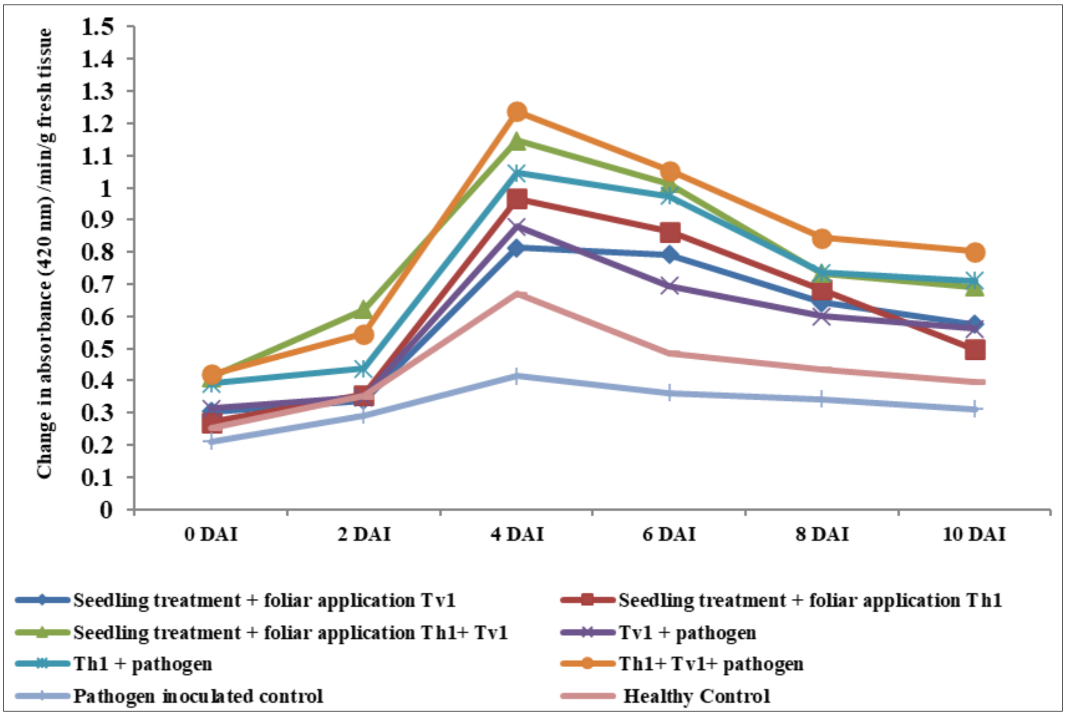

Fig 1: Induction of peroxidase in capsicum var. Arka Mohini treated with biocontrol agents

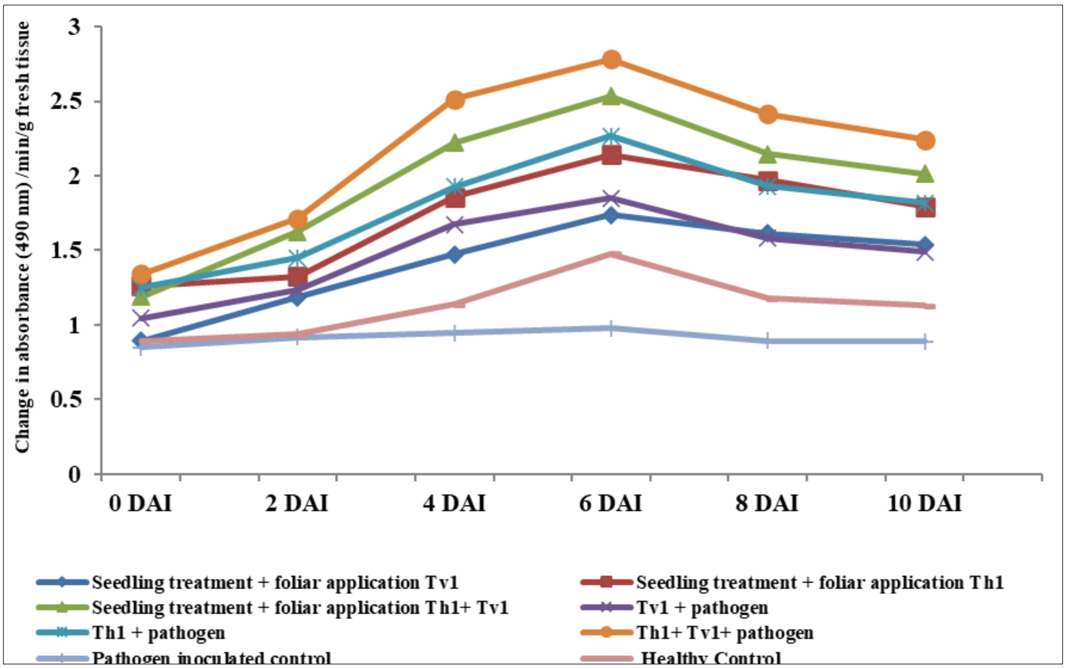

Fig 2: Induction of polyphenol oxidase in capsicum var. Arka Mohini treated with biocontrol agents

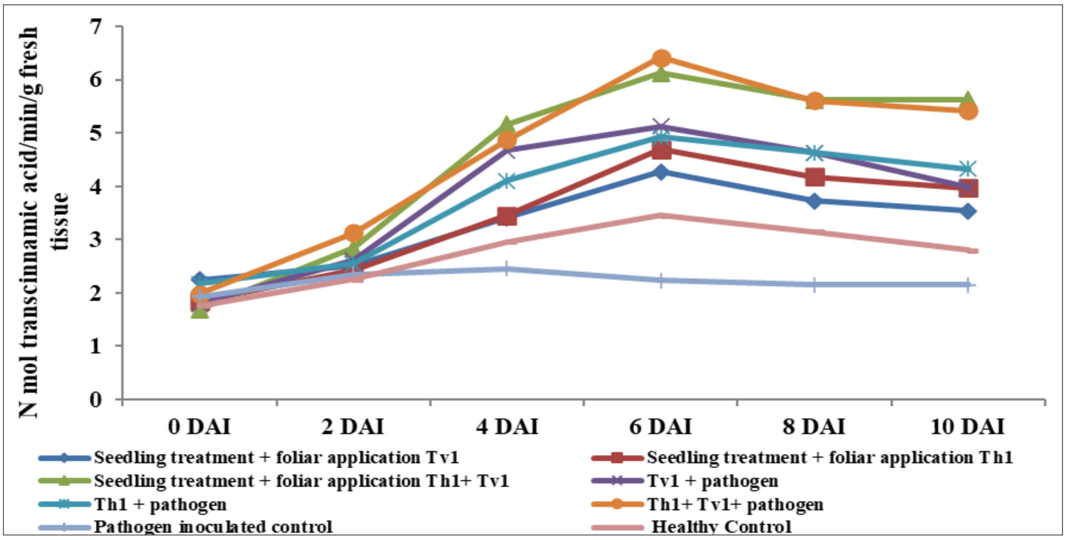

Fig 3: Induction of phenylalanine ammonia lyase in capsicum var. Arka Mohini treated with biocontrol agents

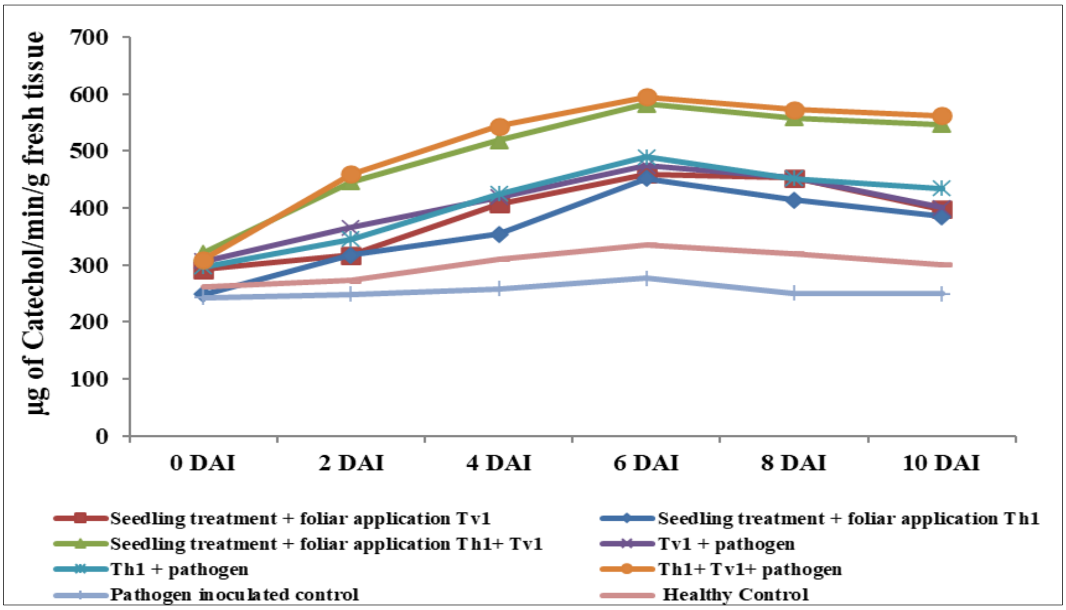

Fig 4: Accumulation of phenols in capsicum var. Arka Mohini treated with biocontrol agents

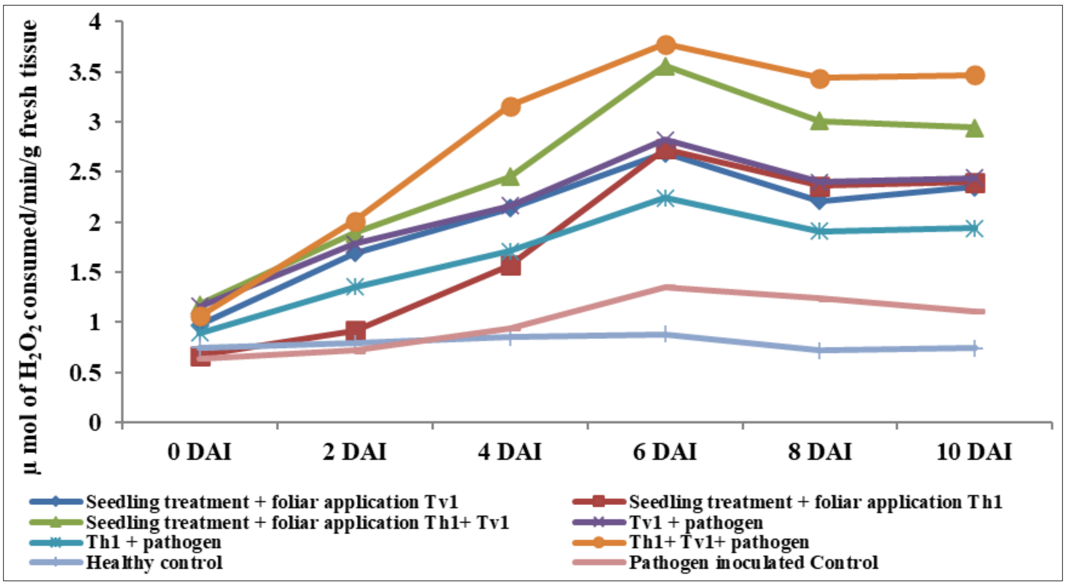

Fig 5: Induction of catalase in capsicum var. Arka Mohini treated with biocontrol agents

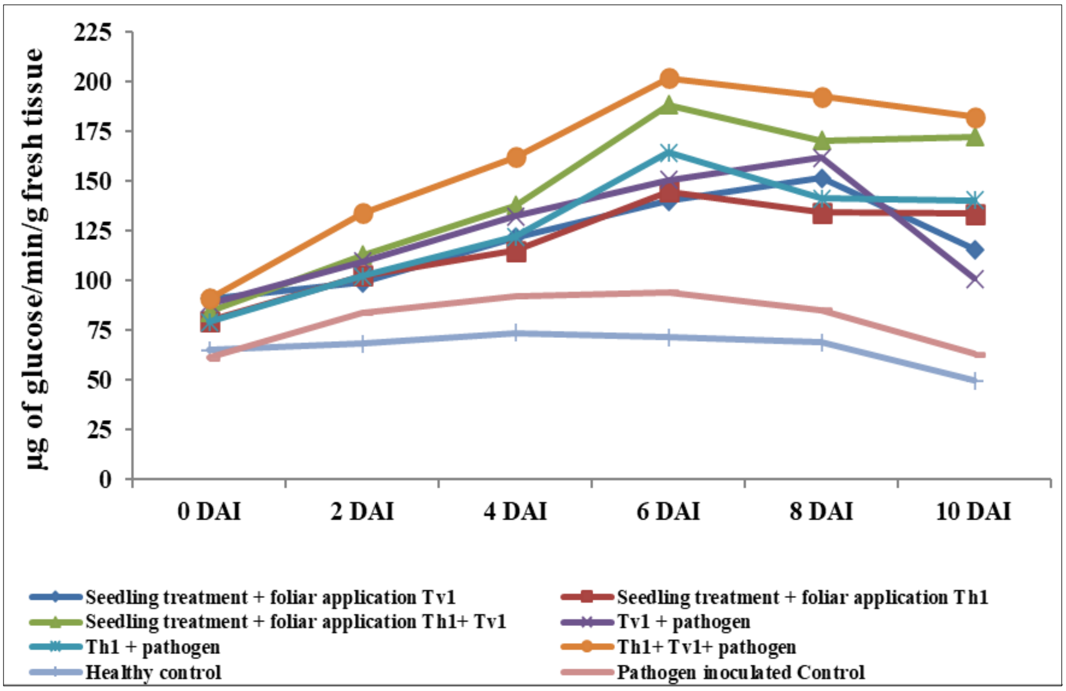

Fig 6: Accumulation of β -1, 3-glucanase in capsicum var. Arka Mohini treated with biocontrol agents

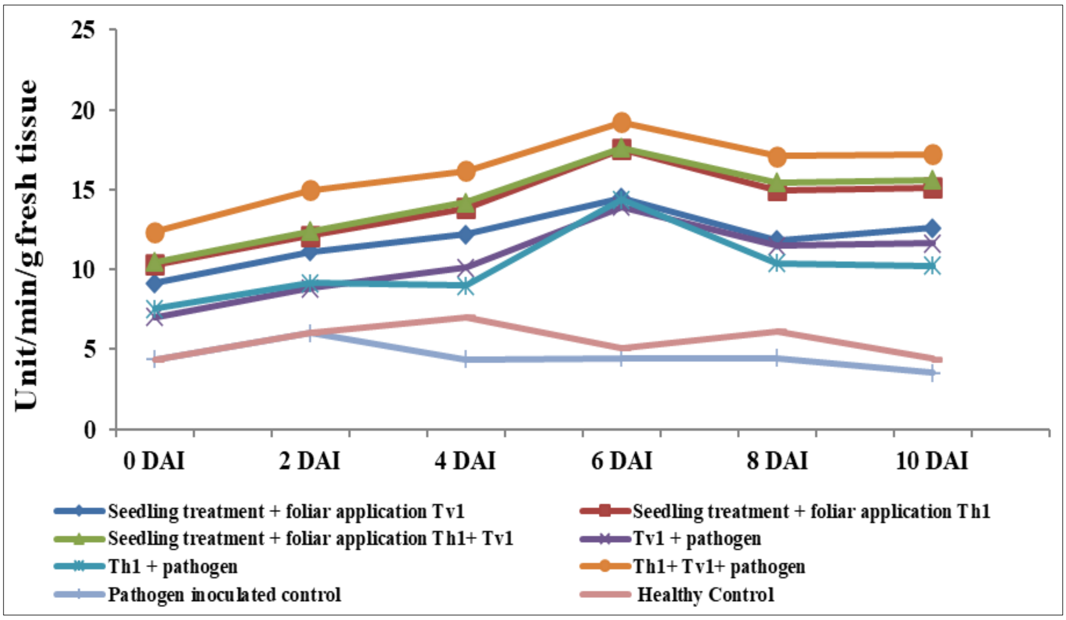

Fig 7: Induction of superoxide dismutase in capsicum var. Arka Mohini treated with biocontrol agents

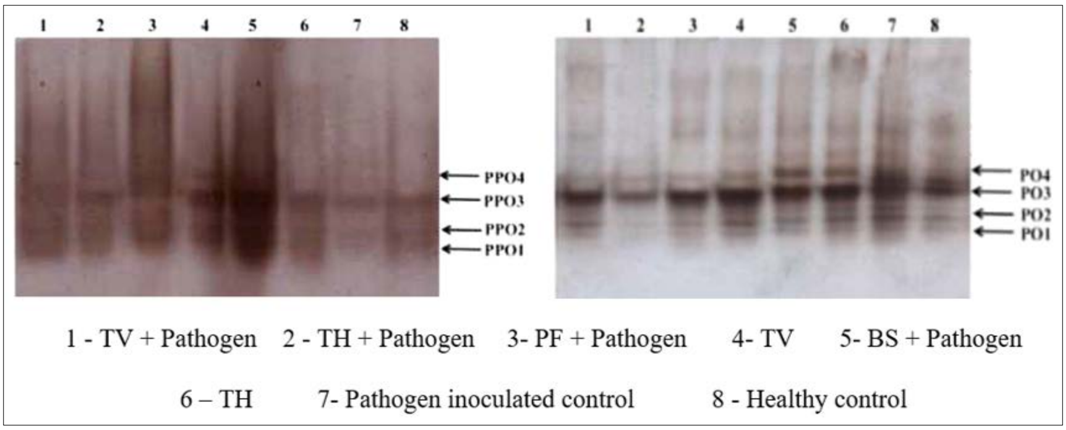

Plate 1: Differential expression of peroxidise and polyphenol oxidase isoforms in capsicum treated with biocontrol agents challenged with *Colletotrichum capsici*

Conclusion

In conclusion, prior treatment of capsicum seedlings with biocontrol agents triggered the plant defense mechanism in response to infection by *C. capsici.* Hence, it is speculated that among the various direct antagonistic tools, ISR is also the one indirect tool by which the tested biocontrol agents afforded resistance to capsicum against the pathogen.

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