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Using fungal biocontrol agents to establish systemic resistance in capsicum chillies against *Colletotrichum truncatum*

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Abstract

Two biocontrol agents, *Trichoderma harzianum* and *Trichoderma asperellum*, were used in a pot culture experiment to generate systemic resistance in capsicum against *Colletotrichum truncatum*. Increases in the activities of five defense-related enzymes—peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), catalase (CAT), and superoxide dismutase (SOD)—were used to track the induced resistance. Additionally, phenols and β -1,3-glucanase accumulation were observed in capsicum following challenge inoculation with *C. truncatum*, which causes anthracnose or fruit rot. Eight days after inoculation (DAI) with the infection, defence enzyme activity peaked. Due to the introduction of induced systemic resistance (ISR), native PAGE analysis showed that extra PO and PPO isoforms were expressed in seedlings treated with biocontrol agents. The plant defence system was activated in response to *C. capsici* infection in capsicum seedlings that had previously been treated with biocontrol agents. Therefore, it is hypothesised that ISR is the only indirect technique among the many direct antagonistic tools that the evaluated biocontrol agents used to provide capsicum resistance against the virus.

Keywords: Bioagents, defense-related enzymes, phenols, peroxidase, polyphenol oxidase, chitinase, and β -1,3-glucanase.

Introduction

One of the most well-liked and lucrative annual herbaceous vegetable crops is the capsicum (*Capsicum annuum* L. var. *grossum* Sendt; $2n = 24$), sometimes referred to as sweet pepper, bell pepper, cherry pepper, green pepper, and Shimla mirch. It is a member of the Solanaceae family and differs from the chilli (*Capsicum annuum* L. var. *longum*) in terms of fruit size, shape, and capsaicin concentration. It is grown on 0.885 million hectares of land in India, producing 0.9 million MT year (National Horticultural Board 2019-20). It is widely grown as an autumn crop in Uttar Pradesh, Madhya Pradesh, Jharkhand, Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh, and Bihar, and as a summer crop in Jammu and Kashmir, Gujarat, Himachal Pradesh, Uttarakhand, Arunachal Pradesh, and West Bengal (Chadha 2005). It covered 2,136 hectares and produced 32,092 MT in Andhra Pradesh (National Horticultural Board 2019-20). Globally, *Colletotrichum* is a significant pathogenic genus. In a variety of fruits, vegetables, and other crops, these fungus produce symptoms that are often referred to as anthracnose. Anthracnose is a devastating disease that affects capsicum during warm and wet seasons. It is caused by a complex of *Colletotrichum* species and results in significant yield losses both before and after harvest. For issues that different fungicides are unable to adequately manage, induced resistance may provide a different strategy for plant protection (Schoenbeck 1996; George & Sujatha 2019). An improvement in a plant's

ability to defend itself against a wide range of diseases that is obtained after suitable stimulation is known as induced resistance. ISR or SAR refers to the ensuing increased resistance brought on by an inducing substance after infection by a pathogen (Hammerschmidt & Kuc 1995). According to Bostock et al. (2001), plants possess endogenous defence systems that may be triggered in reaction to insect and pathogen attacks. The buildup of PR-proteins, phytoalexins, chalcone synthase, PAL, PO, PPO, and phenolics causes a defence response. The current study's goal is to determine how biocontrol agent treatments induce different defense-related genes that encode proteins involved in strengthening plant cell walls in response to *C. truncatum* infection.

Materials and Methods

Induction of systemic resistance in capsicum by biocontrol agents

Talc was used as a carrier in the formulation of the effective biocontrol agents, *Trichoderma harzianum* (Th1) and *Trichoderma viride* (Tv1), which were chosen based on in vitro and pot culture tests. Arka Mohini variety capsicum seedlings were planted in pots with rooting media after being individually treated with the developed biocontrol agents. For growing capsicum plants, rooting media (coir pith: vermicompost @ 5:3 v/v) was used in place of soil. Three replications of each treatment were used in the fully randomised design of the experiments. After two days, the 30-day-old plants were challenged with a pathogen after the biocontrol agents were applied. Additionally, seedling treatment and foliar spraying of biocontrol agents at 30 DAPS without challenge inoculation were included of the treatments. In order to measure changes in the activities of defense-related enzymes, such as phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), β -1-3-glucanase, catalase (CAT), superoxide dismutase (SOD), and phenol, leaf samples were taken at 0, 2, 4, 8, and 10 days following challenge inoculation with pathogen. For comparison, both healthy and pathogen-only infected plants were kept.

Phenylalanine ammonia lyase (PAL) Assay

After homogenising one gram of capsicum leaf in two millilitres of ice-cold 0.1 M sodium borate buffer (pH 7.0), it was centrifuged at 10,000 rpm for twenty minutes at 4 oC. The enzyme activity was measured using the supernatant. The rate at which L-phenylalanine was converted to trans-cinnamic acid at 290 nm was used to calculate PAL activity (Dickerson et al., 1984). A 0.4 ml sample extract was incubated for one hour at 30 oC with 0.5 ml of 0.1 M borate buffer, pH 8.8, and 1 ml of 12 mM L-phenylalanine. 0.5 cc of 2 N HCl was used to halt the reaction that L-phenylalanine had started. After adding 2 N HCl, L-phenylalanine was added to maintain a blank. The data were presented as nmol trans-cinnamic acid/min/g of fresh tissue after the absorbance was measured at 290 nm.

Peroxidase (PO) Assay

According to Hammerschmidt et al. (1982), the activity of PO was determined. A pre-cooled pestle and mortar were used to homogenise one gram of the leaf sample in one millilitre of 0.1 M phosphate buffer pH 7.0. The homogenate was centrifuged for 20 minutes at 4 oC at 10,000 rpm. PO and PPO activities were assayed using the supernatant. 0.1 ml of enzyme extract and 1.5 ml of 0.05 M pyrogallol were added to the cuvette. 0.5 ml of 1% H₂O₂ was added to start the reaction. After three minutes of room temperature incubation, the absorbance change was measured at 420 nm at 30-second intervals. A change in absorbance/min/g of fresh tissue was used to represent the findings.

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).

Catalase (CAT) Assay

The method outlined by Dekock et al. (1960) was used to estimate the catalase activity. After

homogenising 500 mg of the material in 10 ml of ice-cold 0.067 M sodium phosphate buffer (pH 7.0) and centrifuging the mixture, the supernatant was used as a source of enzymes. Three millilitres of hydrogen peroxide-phosphate buffer and 0.03 millilitres of enzyme extract made up the reaction mixture. The absorbance value was recorded right away and every ten or twenty seconds after the reaction mixture was well mixed. The activity was measured in μ mol of H₂O₂ consumed/min/g of fresh tissue.

Superoxide dismutase (SOD) Assay

Assay for superoxide dismutase (SOD) One gram of leaf tissue was homogenised in two millilitres of 0.2 M citrate phosphate buffer (pH 6.5) at four degrees Celsius to create the enzyme extract. The homogenate was centrifuged for 30 minutes at 12,000 rpm and 4 °C. The capacity of SOD activity (EC 1.15.1.1) to prevent the photochemical reduction of Nitro Blue Tetrazolium was measured using the supernatant as an enzyme source (Giannopolitis & Ries 1977). 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 75 μ M NBT, and 2 μ M riboflavin were all included in the test mixture (3 ml). Finally, 100 μ l of the enzyme extract, 0.1 mM EDTA, and riboflavin were added. After shaking the tubes, they were exposed to a 40-W fluorescent light at 25 °C. The light was turned on and off to start and stop the reaction, respectively. For the blank, the absorbance at 560 nm was measured in parallel to identical, non-illuminated sample tubes. The percentage inhibition of NBT photo-reduction was calculated by subtracting each extract from the blank, dividing the mathematical difference by the blank, and then multiplying the result by 100. According to Belid El-Moshaty et al. (1993), the SOD activity was measured in SOD units g⁻¹ fresh tissue (50% NBT inhibition = 1 unit).

Total Phenolic Content

Samples of leaves were homogenised in 10 millilitres of 80% methanol and shaken at 70 degrees Celsius for 15 minutes. After adding 5 ml of distilled water and 250 μ l of Folin-Ciocalteu reagent (1 N) to 1 ml of the extract, the mixture was incubated for three minutes at 25 °C. One millilitre of 20% sodium carbonate was then added and well mixed. The tubes were then cooled after being submerged in hot water for one minute. The standard was catechol, and the absorbance was measured at 750 nm. μ g of catechol/g of fresh tissue was used to represent the total phenol concentration (Zieslin & Ben Zaken 1993).

β -1, 3-glucanase Assay

After adding 62.5 ml of crude enzyme extract to 62.5 ml of laminarin, the mixture was incubated at 40 °C for 10 minutes. The reaction was halted by adding 375 μ l of dinitrosalicylic acid, which was then heated for 5 minutes in a boiling water bath. After diluting the resultant solution with 4.5 millilitres of distilled water, the absorbance at 500 nm was measured. The blank was a crude extract preparation with laminarin and no incubation period. According to Maurhofer et al. (1994), the activity was measured in μ g equivalent of glucose/min/g of protein.

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

PO and PPO isoform expression was determined by native-PAGE analysis. The samples were centrifuged at 10,000 rpm for 20 minutes at 4 °C after being homogenised with 1 cc of 0.1 M sodium phosphate buffer pH 7.0. Bradford's (1976) technique was used to determine the sample's protein content. 50 μ g protein samples were put onto an 8% polyacrylamide gel. Following electrophoresis, the gel was stained for 30 minutes in the dark using 0.2 M acetate solution at pH 4.2 that included 0.05% benzidine. To see the PO isoforms, droplets of H₂O₂ (0.03%) were then gradually added while being continuously shaken. Distilled water was used to wash the gel after staining (Nadlony & Sequerira 1980). For PPO, the gel was submerged in 0.1% P-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) for 30 minutes. After that, 10 mM catechol was added, the shaker was gently shaken, and dark brown protein bands were checked (Jayaraman et al. 1987).

Statistical analysis:

Every analysis was conducted once again, with comparable results. The IRRISTAT tool, created by the International Rice Research Institute's Biometrics Unit in the Philippines, was used to statistically analyse the data. DMRT was used to compare the treatment means.

Results and Discussion

Biocontrol compounds inducing systemic resistance in capsicum Through chemical analysis of leaf samples taken from plants treated with biocontrol agents and infected with *Colletotrichum truncatum*, the induced resistance to hracnose disease was determined in capsicum cv. Arka Mohini. The enzymes peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, superoxide dismutase, β -1,3-glucanase, catalase, and enols were shown to be more active in the biocontrol compounds that protected the capsicum plants against disease. In isozyme analysis, biocontrol agents that were not present produced more PO and PPO isoforms with higher intensities.

Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase activity varied significantly across all treatments (Figure 1). All treatments showed a gradual rise in phenylalanine ammonia lyase activity up to 6 DAI, after which all treatments showed a drop in activity. At 6 DAI, the treatment T6 (TH1+TV1+Pathogen) had the highest phenylalanine ammonia lyase activity (6.42 n mol transcinamic acid/min/g of fresh tissue), which was comparable to the treatment T3 (TH1+TV1) (6.12 n mol transcinamic acid/min/g of fresh tissue). At 6 DAI, phenylalanine ammonia lyase activity was significantly lower in the pathogen-inoculated control treatment (2.23 n mol transcinamic acid/min/g of fresh tissue). In response to pathogenic infections, several researchers have noted elevated activity in these enzymes in the host tissues (Abo-Elyousr et al. 2010; Ojha et al. 2012). According to Chen et al. (2000), fluorescent pseudomonas may induce PAL in cucumber plants and boost tomato plants' resistance to the fungal disease *F. oxysporum* f.sp. *Lycopersici*.

P. aphanidermatum.

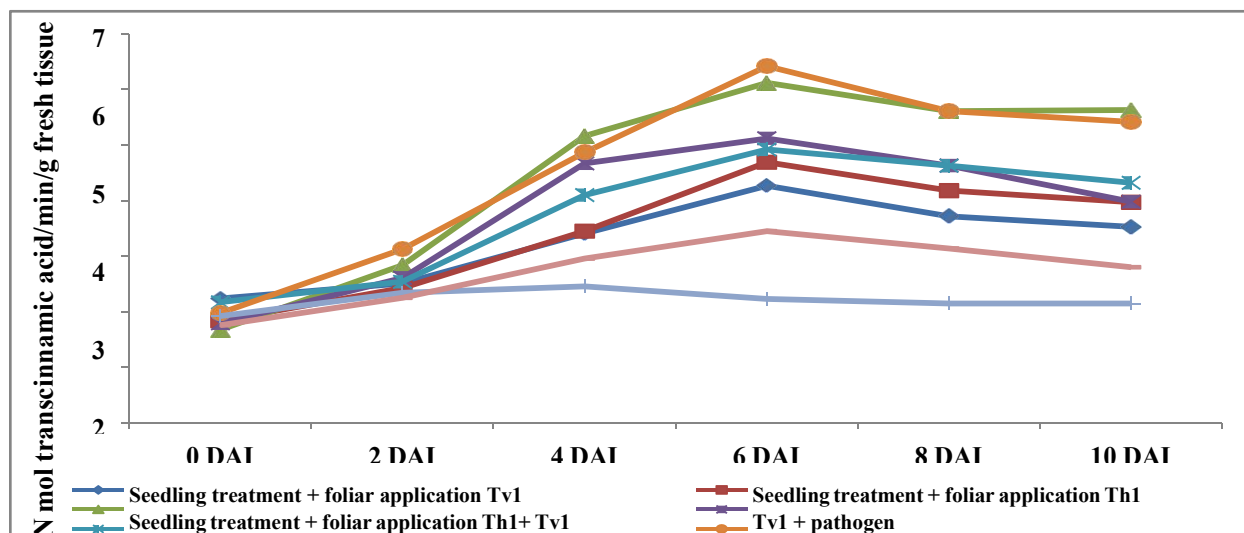


Figure 1. Induction of phenylalanine ammonia lyase in capsicum var. Arka Mohini treated with biocontrol agents

Peroxidase (PO)

In capsicum cv. Arka Mohini, increased PO activity was seen at 6 DAI (Figure 2). At 6 DAI, the treatment T6 (TH1+TV1+Pathogen) had the highest peroxidase activity (1.054 changes in A420/min/g of fresh tissue), which was comparable to the treatment T3 (TH1+TV1) (1.013 changes in A420/min/g of fresh tissue). These results are consistent with the findings of many workers. Cucumber peroxidase activity was boosted when *T. virens* bioformulation was sprayed on leaves and blossoms (Wei et al. 1996). Increased PO activity has been linked to resistance in a number of species, including barley, cucurbits, cotton, tobacco, wheat, and rice, according to Bradford (1976). These enzymes are involved in the polymerisation of proteins and lignin or suberin precursor into plant cell walls, creating a physical barrier that may prevent pathogen penetration of cell walls or movement through vessels.

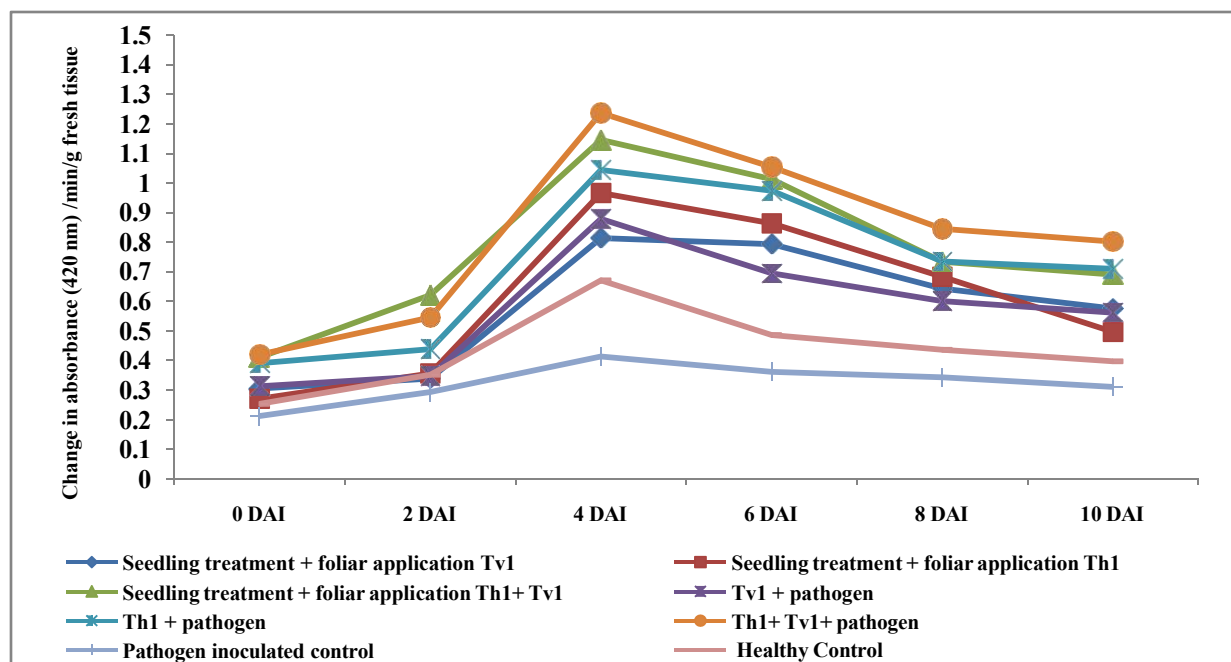


Figure 2. Induction of peroxidase in capsicum var. Arka Mohini treated with biocontrol agents
Polyphenol oxidase (PPO)

The pattern of rising PPO activity in the current research was comparable to that of PO across all treatments (Figure 3). When the plants were pre-treated with TH1+TV1 challenged with the pathogen, the enzyme activity reached its peak (2.782 changes in A490/min/g of fresh tissue) at 6 DAI. Chen et al. (2000) showed that several rhizobacteria and *P. aphanidermatum* produced PPO activity in cucumber root tissues, whereas Radja Commare (2002) revealed that *P. fluorescence* promoted PPO isoenzymes in rice against *R. solani*.

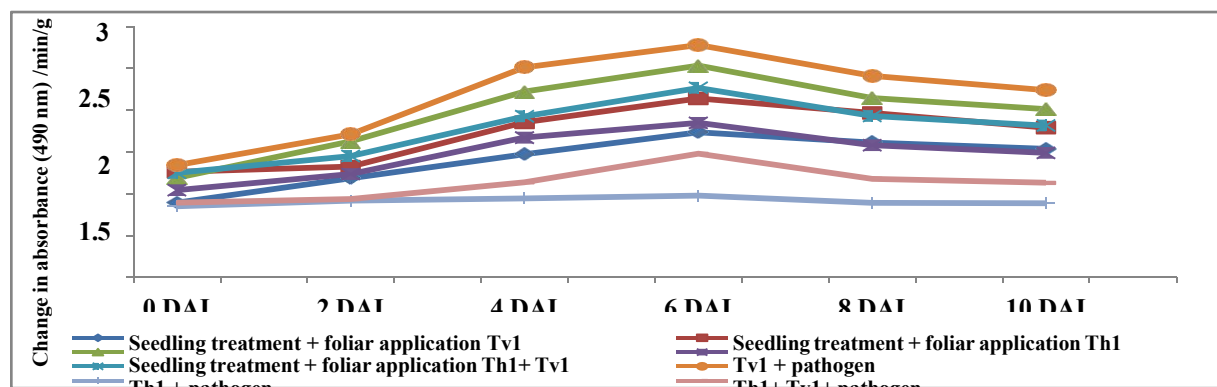


Figure 3. Induction of polyphenol oxidase in capsicum var. Arka Mohini treated with biocontrol agents

Superoxide dismutase (SOD)

All treatments showed a considerable increase in superoxide dismutase activity up to six days after inoculation, after which it was shown to be diminishing (Figure 4). Superoxide dismutase activity was highest in treatment T6 with TV1+TH1 and challenge inoculation with pathogen (19.24 unit/min/g of fresh tissue). Chakrabarthy et al. (2002) reported similar findings in cotton lines vulnerable to grey mildew.

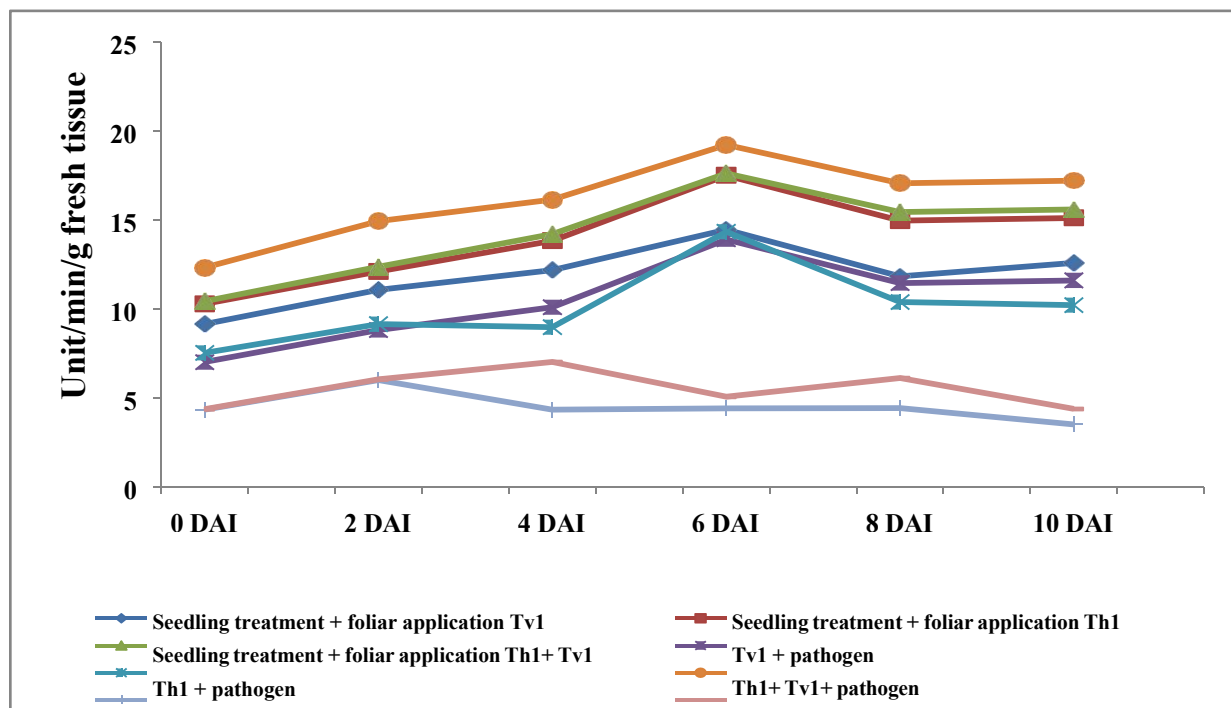


Figure 4. Induction of superoxide dismutase in capsicum var. Arka Mohini treated with biocontrol agents

Catalase (CAT)

Leaf samples from plants treated with biocontrol agents and infected with pathogens were used to assess catalase activity in capsicum. Catalase activity generally increased up to six days after inoculation before progressively declining in all treatments (Figure 5). The treatment T6 with TV1+TH1 and challenge inoculation with pathogen ($3.78 \mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1}$ of fresh tissue) showed increased catalase activity. Chakrabarthy et al. (2002) reported similar findings in cotton lines vulnerable to grey mildew.

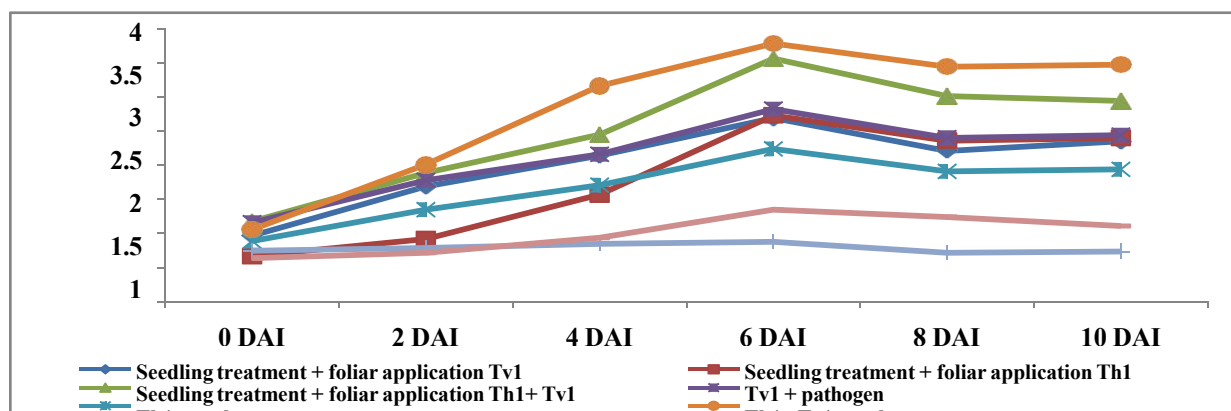
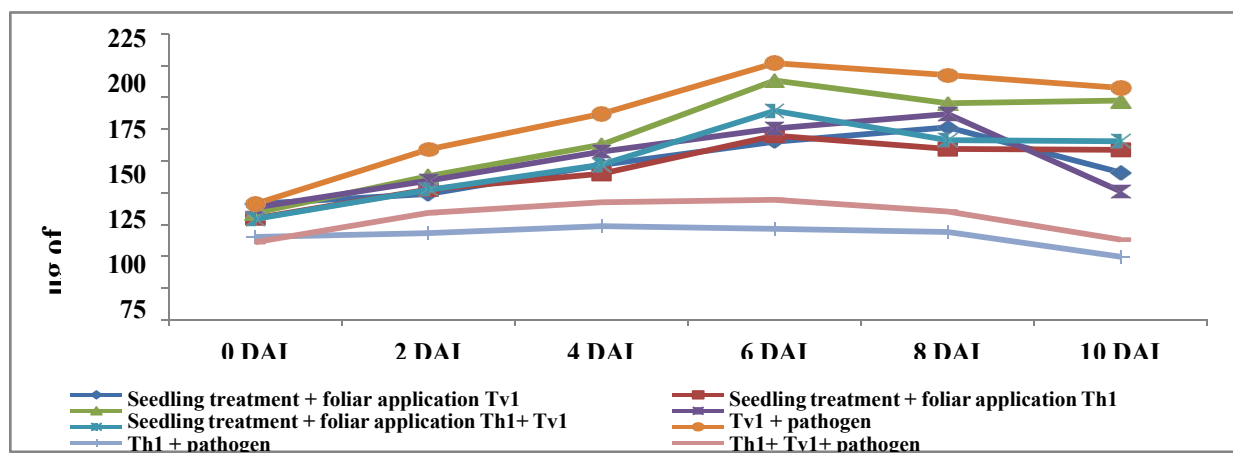


Figure 5. Induction of catalase in capsicum var. Arka Mohini treated with biocontrol agents **β -1,3-glucanase**

Leaf samples from plants treated with biocontrol agents and infected with pathogens were used to measure it in capsicum plants. β -1,3-glucanase activity often increased up to six days after inoculation before progressively declining in all treatments (Figure 6). Treatment T6 with TV1+TH1 and challenge inoculation with pathogen showed increased β -1,3-glucanase activity (201.96 μ g of glucose/min/g of fresh tissue). Numerous studies have shown that managing plant diseases and pathogens using a mix of biocontrol agents is more successful than using individual agents. When groundnut plants were challenged with *Sclerotium rolfsii*, it greatly enhanced the induction of PR proteins and the enzymatic activity of phenols, PAL, PO, PPO SOD, and CAT (Young et al. 2008).

**Figure 6.** Accumulation of β -1, 3-glucanase in capsicum var. Arka Mohini treated with biocontrol agents**Total phenols**

Phenolics are poisonous substances found in fungus that strengthen the host cell wall both mechanically and physically. Because plant phenolics and their oxidation products, such quinines, are very poisonous to invasive fungus, they provide defence against a variety of diseases (Cahill & Mccomb 1992). In our investigation, phenol accumulation was seen in every plant treated with the bio-control agents, which had a significant impact on the accumulation of phenols in plants after pathogen challenge inoculation. Its buildup began to rise on the third day and peaked on the sixth DAI (Figure 7). When compared to plants infected with the pathogen alone, treatment T6 with TH1+TV1 challenged with the pathogen at 6 DAI showed the highest accumulation of phenols (595.20 μ g of catechol/min/g fresh tissue). Similar outcomes in chickpea cultivars with *Fusarium* sp. illness were reported by Rathod & Vakharia (2011).

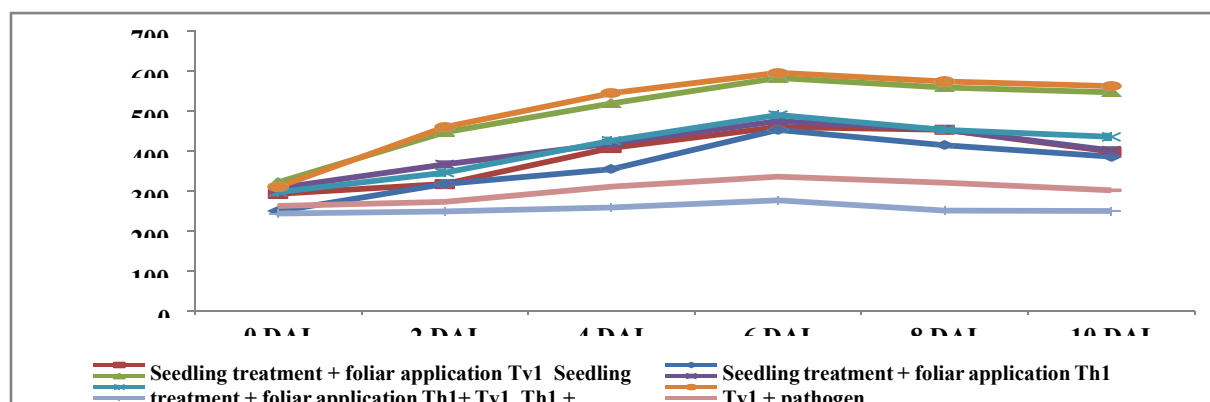
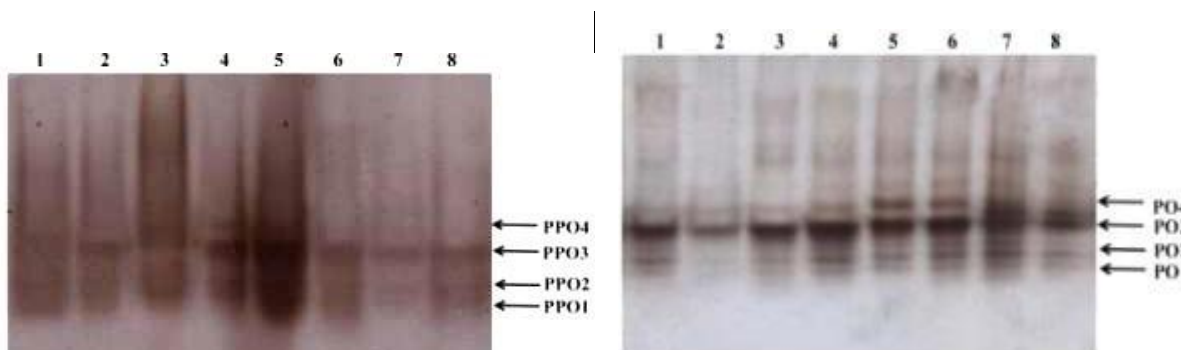


Figure 7. Accumulation of phenols in capsicum var. Arka Mohini treated with biocontrol agents
Native PAGE analysis for Isozyme induction PO and PPO

Four peroxidase isoforms (PO1 to PO4) were found in all treatments with the exception of the healthy control, according to native PAGE analysis. However, compared to plants challenged with the pathogen, the intensity of the isoform was higher in plants treated with biocontrol drugs. Four isoforms (PPO1 to PPO4) with more prominent expression were found in the treatments challenged with *C. truncatum*; they were not present in the healthy and infected control (Plate 1).



1 - TV + Pathogen 2 - TH + Pathogen 3- PF + Pathogen 4- TV 5- BS + Pathogen
 6 – TH 7- Pathogen inoculated control 8 - Healthy control

Plate.1 Differential expression of peroxidase and polyphenoloxidase isoforms in capsicum treated with biocontrol agents challenged with *Colletotrichum capsici*

The best strategy for crop protection was thought to be the induction of systemic resistance by PGPR against a variety of pathogens. ISR differs significantly from other processes. First, the defence mechanism that is triggered by inducing agents is the foundation of ISR's activity. Second, ISR exhibits a variety of potential defence mechanisms, such as elevated activity of chitinase, α -1,3 glucanase, and peroxidase (Maurhofer et al. 1994; Xue et al. 1998), accumulation of antimicrobial low molecular substances, phytoalexins, and formation of protective biopolymers, such as lignin, callose, and hydroxyproline rich glycoprotein (Hammerschmidt & Kuc 1982). Third, the broad range of pathogens that may be managed by a single inducing agent is a significant feature of ISR (Hoffland et al. 1996; Wei et al. 1996). The current study found that capsicum plants treated with biocontrol chemicals and challenged with *C. capsici* exhibited increased activity of defense-related enzymes PO, PPO, CAT, SOD, and PAL as well as buildup of phenol and β -1,3-glucanase. Biocontrol agents that were not present in the control group produced more PO and PPO isoforms with higher intensity in isozyme analysis. Phenyl propanoid metabolism provides the building blocks for flavanoid colours, lignin, and phytoalexins. It begins with PAL converting L-phenylalanine into transcinnamic acid (Massala et al. 1980; Hahlbrock & Scheel 1989). Disease resistance results from a rise in PAL activity, which also raises phenolic contents (Klessig & Malamy 1994). A key player in the manufacture of lignin, which restricts the spread of pathogens, peroxidase (PO) is a part of plants' early response to pathogen infection (Bruce and West, 1989). When hydrogen donor and hydrogen peroxide are present, the enzyme's products exhibit antibacterial action (VanLoon & Callow 1983). Plant disease resistance is linked to PO, one of the important enzymes in the phenyl propanoid pathway (Hammerschmidt et al. 1982). Increased PO activity has been linked to resistance in many species, such as barley, cucurbits, cotton, tobacco, wheat, and rice, according to Bradley et al. (1992). These enzymes are involved in the polymerisation of proteins and lignin or suberin precursor into plant cell walls, creating a physical barrier that may prevent pathogen penetration of cell walls or movement through vessels. Enzymes called polyphenol oxidase (PPO) catalyse the oxidation of monophenolic and orthophenolic substances using molecular oxygen. The pattern of rising PPO activity in the current research was comparable to that of PO across all regimens. Phenolics strengthen the host cell wall's mechanical and physical properties and are fungitoxic. Because they are very poisonous to invasive fungus, plant phenolics and their oxidation products, such as quinones, provide resistance against a variety of diseases (Cahill & McComb 1992). In our investigation, a pattern of phenol accumulation was seen in every plant treated with the biocontrol agents, which had a significant impact on the plants' phenol accumulation after challenge inoculation with *C. capsici*. It began to accumulate on the third day and peaked on the sixth DAI. Certain phenolics may cause resistance by acting as antioxidants or signal molecules (Malamy et al. 1990; Muthusamy et al. 2017). Plant systemic acquired resistance (SAR) has been linked to the accumulation of pathogenesis-related (PR) proteins. Research has shown that *P. fluorescens* treatment also induces PR-proteins in plants. The primary components of fungal cell walls, chitin and β -1, 3-glucan, may be hydrolysed by PR proteins such as chitinase and β -1, 3-glucanase, respectively. Additionally, elicitors are released from fungal walls by chitinase and glucanase, which in turn trigger a variety of defence mechanisms in plants. The oxidative burst is suppressed and tissue necrosis is prevented by scavengers of active oxygen species such as catalase, which catalyses the breakdown of H_2O_2 , and superoxidedismutase, which scavenges O_2 .

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