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Defense response of *Trichoderma* sp. on tomato seedlings through biochemical analysis (*in-vitro*)

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Abstract
Tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is most important and destructive disease of tomato in Uttar Pradesh, which causes considerable losses in yield of tomato. Therefore, present studies were undertaken to test the efficacy of two fungicides, three bioagents and soil amendments *in vitro* Azad T-6 varieties of tomato in green house against Fusarium wilt of tomato Among the highest chlorophyll content was also observed in Seedlings treatment with vermicompost 3.110 (0.855 chl. A and 2.250 chl. B) followed by poultry manure as 2.596 (0.759 chl. The maximum protein content was continuously higher (all three week) in the Seedlings treatment *Trichoderma viride* (T-05), followed by Neem cake in soil amendment. The maximum PPO activity was found in Soil apply with poultry manure followed by the vermicompost and FYM plants on the pathogen challenge respectively.

Keywords: Tomato wilt, *Trichoderma viride* and FYM plants

Introduction
The word of tomato (*Lycopersicon esculentum* Mill.) krust is derived from Latin words Aztec xitomate or xitomate and its origin is Tropical America (Thompson and Kelly, 1957) [6]. It was introduced into Europe by Spanish explorer in early sixteen century. Subsequently, it was perhaps introduced in India by Portuese though there is no definite record of when and how it came to India. Tomato wilt caused by *Fusarium oxysporum* f.sp. *lycopersici* is most important and destructive fungal disease causing substantial quantitative and qualitative losses. Considering the seriousness of the disease, the present studies were undertaken *in vitro* to test the efficacy of seedlings treatment with *Trichoderma* sp. and fungicides along with different soil amendments on total chlorophyll content, Protein and Polyphenoloxidase of tomato against Fusarium wilt of tomato in green house.

Materials and Methods
Total chlorophyll content in leaves
One gram leaf from each treatments is measured and cut into fine pieces and then grinded with mortar and pestle. Thereafter, 20 ml of 80% acetone and 0.5 g of (MgCo3) powder was added and further grinded gently following the method of Kamble *et al.* (2015) [3]. The mixture was then incubated at 4ºC for 3 hours. The mixture was centrifuged at 2500 rpm for 5 min and the supernatant was transferred to a 100 ml volumetric flask and the volume was made up to 100ml with the addition of 80% acetone and the solution was used for chlorophyll estimation. The absorbance of the solutions was measured at 645 nm and 663 nm in labtronic Spectrophotometer LT-39 taking the 80% acetone solution as blank (Sadasivam & Manickam 1996) [5]. The reading was taken in a triplicate sample and average was considered for calculation of chlorophyll content. The chlorophyll a, b and a + b (total chlorophyll) contents were calculated out by applying the following (Arnon 1949) [1] formulae:-

Calculations
Use Arnon's equation (Arnon, 1949) [1] to convert absorbance measurements to mg Chl g- leaf tissue

Chl a (mg g-1) = [(12.7 × A663) - (2.6 × A645)] × ml acetone / mg leaf tissue
Chl b (mg g-1) = [(22.9 × A645) - (4.68 × A663)] × ml acetone / mg leaf tissue
Total Chl = Chl a + Chl b.

Total protein extraction and quantification

(i) Protein extraction

Total leaf protein was extracted using method developed by Goggin *et al.* (2011) [2]. 0.5g leaves of treated tomato plants were frozen by liquid nitrogen, grinding to a fine powder using mortar and pestle then transferred to a fresh centrifuge tube. Two ml of extraction buffer (Tris-HCl 1M, pH 8, EDTA, 0.25), SDS, 10%, glycerol, 50%) was added and mixed well. The content of the tubes were centrifuge at 12000 rpm for 20 min at 4°C. After centrifugation process supernatant was discarded. Mixed the pellets with 1ml of sample buffer (80% Acetone, 0.07% β- mercaptoethanol and 2mM EDTA) and centrifuged at 12000 rpm for 5 minutes. The process was repeated until all chlorophyll removed. Mixed clear pellet with milli Q water and stored at -20 °C. Protein concentration of all the samples was determined using Lowry assay (*Lowry et al*. 1951) [4]. Estimation of Polyphenoloxidase assay (PPO).

Polyphenol oxidase activity was assayed with 4-methylcatechol as a substrate, according to the method described by Zauberman *et al*. (1991). The reaction mixture contained 0.5 mL of diluted enzyme extract, 2 mL of phosphate buffer (pH 7.0, 0.1 mol·L^{-1}) and 0.5 mL of 100 mmol·L^{-1} 4-methylcatechol. The increase in absorbance at 410 nm at 25 °C was recorded every 30 seconds for 2 min. Enzyme activity was expressed as changes in absorbance in mg^{-1} protein min^{-1}.

Calculation

$$\text{Activity U/ mL} = \frac{\text{Abs at 3 min-Abs at 0min X total reaction vol.}}{\text{Time interval X 0.2}}$$

Where

Reaction vol =1.9

Time interval = 3min

0.2 mL is volume of enzyme taken in reaction mixture U/ mL can be used to back calculate activity units per gram of sample and further per mg of protein in 1g sample.

Results

Chlorophyll content

From the above result and its corresponding Table indicated that the highest chlorophyll content was also observed in Seedlings treatment with vermicompost 3.110 (0.855 chl. A and 2.250 chl. B) followed by poultry manure as 2.596 (0.759 chl. A and 1.837 chl. B), T-02 as 2.546 (0.752 chl. A and 1.794 chl. B), FYM as 2.185 (0.629 chl. A and 1.556 chl. B), carbendazim@0.2% as 1.644 (0.485 chl. A and 1.159 chl. B), neem cake as 1.606 (0.463 chl. A and 1.143 chl. B), Tebuconozole @0.3% as 1.586 (0.465 chl. A and 1.121 chl. B), T-05 as 1.317 (0.392 chl. A and 0.925 chl. B), T-10 as 1.245 (0.375 chl. A and 0.876 chl. B). The least chlorophyll content was recorded in control 0.979 (0.667 chl. A and 0.312 chl. B).

Table 1: Effect of seedlings treatment with *Trichoderma* sp. and fungicides along with different soil amendments on total chlorophyll content, Protein and Polyphenoloxidase in tomato

Treatment	Treatment detail	Chlorophyll content			Protein concentration	Poly phenoloxidase (PPO)
		Base value 645 nm	Base value 663 nm	Total		
		Chl-A	Chl-B			Actual value
T_1	Vermicompost	0.855	2.250	3.110	0.436	0.022
T_2	FYM	0.629	1.556	2.185	0.518	0.013
T_3	Poultry Manure	0.759	1.837	2.596	0.632	0.057
T_4	Neem cake	0.463	1.143	1.606	0.839	0.004
T_5	*T. harzianum* (T-2)	0.752	1.794	2.546	0.496	0.011
T_6	*T. viride* (T-5)	0.392	0.925	1.317	1.072	0.008
T_7	*T. koningiopsis* (T-10)	0.375	0.870	1.245	0.335	0.008
T_8	Carbendazim	0.485	1.159	1.644	0.207	0.002
T_9	Tebuconozole	0.465	1.121	1.586	0.202	0.004
T_{10}	Control	0.667	0.312	0.979	0.125	0.005

Protein content

The variation in total protein content in three weeks after inoculation of *Fusarium oxysporium* f.sp. *lycopersici* Table gives stronger evidence to prove the active metabolic activities in plant system. it shows that in second week after of inoculation, the highest amount of protein was recorded in all treatments as compared to first and third week. The difference of protein content in first and third week was not-significant. Maximum protein content was continuously higher (all three week) in the Seedlings treatment *Trichoderma viride* (T-05), followed by Neem cake in soil treatment and poultry manure. In case of fungicidal seed treatment, total protein content was also increased in 2 weeks but only carbendazim@0.2@ was effective. Minimum protein content was recorded in control.

Polyphenol oxidase activity

PPO activity in all the treatments showed an increasing trend until 12 to 14 days (second week) after inoculation of the pathogen and then gradually decreased Maximum PPO activity was found in Soil apply with poultry manure followed by the vermicompost and FYM plants on the pathogen challenge. An elevated level of PPO activity was also recorded in the treated and pathogen challenged plants. PPO was observed only up to the inoculation of pathogen and thereafter, a drastic reduction in enzyme activities was recorded.

Discussion

The total Chlorophyll, Protein and Polyphenoloxidase content was recorded in above pot experiment at after germination as described in the highest chlorophyll content was also observed in Seedlings treatment with vermicompost 3.110 (0.855 chl. A and 2.250 chl. B) followed by poultry manure as 2.596 (0.759 chl. A and 1.837 chl. B). The least chlorophyll content was recorded in control 0.979 (0.667 chl. A and 0.312 chl. B). The Maximum protein content was continuously higher (all three week) in the Seedlings treatment *Trichoderma viride* (T-05), followed by Neem cake in soil apply and poultry manure. In case of fungicidal seed treatment, total protein content was also increased in 2 weeks but only carbendazim@0.2@ was effective. The maximum polyphenol oxidase (PPO) activity was found in Soil apply with poultry manure followed by the vermicompost and FYM

plants on the pathogen challenge. (Segneanu *et al.* 2013) estimated protein concentration by Lowry method using bovine serum albumin (BSA) as the standard. This chromogenic procedure is inexpensive, easy to perform, very sensitive and highly reproducible, but the major disadvantage is because its accuracy depends on the pH of the solution.

Conclusion

Plants have evolved multiple defense mechanisms against microbial pathogens and various types of environmental stress. Besides anti-microbial secondary metabolite, some of which are performed and some of which are induced by infection. The most significant information that generated in this experiment, effect of seedlings treatment with *Trichoderma* sp. and fungicides along with different soil amendments on total chlorophyll content, Protein and Polyphenoloxidase in tomato.

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