Fusarium wilt of tomato: study of host-pathogen-fungicides interaction and their effect on host biochemical balance

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Abstract

In conventional agriculture, chemical fungicides are basic mean to manage plant diseases. At commercial level application of fungicides is cumbersome and some have been proved carcinogenic. Experiments were performed *in vivo* with commercially available fungicides *i.e.* benedict (iprobenfos), treaty (tebuconazole), hiten (fentin hydroxide) and wisdom (fosetyl-aluminium) to investigate polygonal interaction of host-pathogen-fungicides in term of growth, protein and gene expression in *Solanum lycopersicum*. Fungicides phytotoxicity resulted in stunted growth of plant along with chlorosis and necrotic burning on leaves that resulted in reduction of growth and biomass of the infected tomato seedling. Protein profiling by SDS-PAGE exposed some new bands, and peroxidase and chitinase genes expressed appropriately after pathogen infection and fungicides application. These results suggested that fungicides may induce/activate defense related proteins in tomato against invasion of *F. oxysporum* f. sp. *lycopersici*, however deleterious phytotoxicity of fungicides hinder their proper functioning in plant.

Keywords: Tomato, *Fusarium oxysporum* f. sp. *lycopersici*, SDS-PAGE, Gene expression.

Introduction

Tomato (*Solanum lycopersicum* L.) originated in America, belongs to extremely large family Solanaceae, therefore, extensively utilizes as an excellent model for plant-pathogenic interaction (Ercolano *et al*., 2012). Tomato is the most extensively cultivated crop of Pakistan with annual production of 468.14 thousand tones cultivated on 46.23 thousands hectares. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.) Snyd & Hans is considered as one of the most economically important and destructive diseases of tomato worldwide that is responsible for causing massive loss in tomato production (Ignjatov *et al*., 2012). Seedlings infected by the wilt fungus show yellowing of the lower leaves, often only on one side of the plant succeeded by stunted growth, drooping of leaves and plant death at later stages (Anitha and Rabeeth, 2009).

Utilization of pesticides is an uneconomic and eco-toxic way of controlling plant diseases but is the only immediate way to cop up with the diseases at commercial level (Vinale *et al*., 2008; Sharma, 2011; Dias *et al*., 2012). There is some controversy about positive outcome that could be generated by fungicides. Accordingly these chemicals are responsible for altering physiological and metabolic activities in the plant (Garcıa *et al*., 2003), by forming more reactive biomolecules (Dane and Dalgiç, 2005). Many previous investigation revealed that pesticides applicant drastically effect growth of plant by altering nutrient availability to the plants that results in impairment plant photosynthesis, translocation and respiration (Petit *et al*. 2009, 2012, Shoaib *et al*., 2014). When pesticides negatively reacts the general defense mechanism of plant activate through change in activity of defense related enzyme like catalase, peroxidase and superoxide dismutase etc. However, the intensity of the stress induced by pesticide application and the intensity of plant response will thus have a subsequent impact on the growth process if the crops overcome the stress. So far, plants during its early stages and during reproductive stages of growth are more sensitive to negative effect of pesticides (Petit *et al*., 2012).

It is, therefore, imperative to understand mechanism of bilateral interaction of host-pathogen and then correlate it with multilateral interaction of host-pathogen-fungicides. It has been documented that plant normally exhibited ability to increase expression of defense gene against pathogen infection or after fungicide application (Conrath, 2009). Understanding function of plant defense mechanisms or defense related gene against various pathogens and certain stimuli can help in effective disease control methods. In this connection, 17 families of pathogen-related (PR) protein have been described on the basis of sequence of amino acid and biochemical functions (Van Loon *et al*., 2006). Peroxidases (PR9) are regarded as principle enzymes that induce resistance in various plant species against phyto-pathogens through building cell wall barrier that impede pathogen access and spread in plant cells (Taheri and Tarighi, 2011). Other important families

of PR protein are PR-3 and 4 that comprised of chitinases that found to accumulate in plant in contact with pathogen. Their function is hydrolysis of chitin residues (*β*-1,4 linkages between N-acetylglucosamine) in many fungi, while degradation products (oligomer) of the fungal cell wall act as resistance elicitors (Misawa and Kuninaga, 2010). A combination of chitinase and 1,3-glucanase is known to be more effective than each enzyme alone against many fungi (Wubben *et al*., 1996).

Above review of host-pathogen interaction will help to investigate the impact of multilateral interaction of host-pathogen and fungicides on growth, protein, chitinase and peroxidases expression in tomato plant.

Materials and Methods

F. oxysporum f. sp. *lycopersici* was isolated from infected tomato plants collected from tomato fields at Institute of Agricultural Sciences (IAGS), University of the Punjab, Lahore. Pakistan. The diseased portion was cut into 0.5 cm pieces and surface sterilized by 0.1% sodium hypochlorite solution for 2-3 minutes and then rinsed 3-4 times with sterilized distilled water. These pieces were placed on pantachloronitrobenzene media (PCNB) under aseptic conditions and inoculated plates were incubated at 25±2 °C for 7 days. PCNB was prepared by adding 20g agar, 15 g peptone, 1 g PCNB, 1 g KH_2PO_4 and 0.5 g $MgSO_4$ in 1000 mL of distilled water. The fungal colonies appeared on PCNB plates were further purified and fungal pathogen was identified up to morphological level by compound microscope (El-Kazaz *et al*., 2008). Seven days old culture was utilized for preparation of spore suspension of the fungus.

Four fungicides *viz.* benedict (iprobenfos), treaty (tebuconazole), hiten (fentin hydroxide) and wisdom (fosetyl-aluminium) were procured from KANZSO Seeds Envyol Group, Lahore. Pakistan. The solutions of each fungicide were prepared according to their recommended doses. The experiment was conducted in plastic pots (13 cm × 9 cm) in a completely randomized design with three replications. Soil was sandy loam (sand 37: silt 50: clay 12) having 1.11% organic matter, 7.8 pH, 1200 μS cm^{-1} electrical conductivity (EC), 0.05% nitrogen (N), 798 mg kg^{-1} and 11.27 meq L^{-1} potassium (K^+).

Soil was sterilized by sprinkling 10% formalin solution (Miller, 1950). About 300 g of sterilized soil was filled in each pot, inoculated with spore suspension (1×10^9) of *F. oxysporum* f. sp. *lycopersici* and left for 5 days under natural environmental conditions for establishment of inoculum. Tomato variety LA-2662 seedlings with 4–5 leaves were sown in each pot. Recommended dose of each fungicide (50 mL) was applied as soil drenching to each pot. Pots for the positive control were inoculated with fungus only, while the negative control treatments were not included either fungal inoculum or fungicides treatment. Both the positive and he negative control treatments received just 50 mL of distilled water. Experiment was designed for a total of 25 days. The highest and the lowest temperatures during the experimental periods were 26–35 °C and 21–25 °C, respectively and relative humidity was 70%. Bright sunshine duration ranged from 8–10 h day^{-1}. Pots were irrigated occasionally with tap water and were kept weed free by hand weeding. Plants were harvested 25 days after sowing. Growth parameter like root/shoot length, and fresh/dry weights were recorded. For the dry weight measurement, each roots and shoot were placed in paper envelops and oven dried at 80 °C for 24 h and weighed.

Protein profiles characterization was accomplished by utilizing one dimensional sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 300 mg of plant mass was crushed in sterilized pestle and mortar in 500 µL of cold extraction buffer (10 mM Na_2PO_4, 200 mM NaCl, 10% (v/v) glycerol, 0.1 mM PMSF, pH 7). Crushed cells were centrifuged at 10,000 rpm for 1 min at room temperature. The supernatant was transferred to fresh ice-cold tube and centrifuged again at 18,000 rpm for 45 min at 4 ºC to collect the protein. The membrane pellet was suspended in 50 µL of cold extraction buffer. Protein samples were run on 10% SDS- PAGE gels (separating gel: 0.37 mM Tris pH 8.8, 0.1% SDS (w/v), 10% (v/v) acrylamide, 0.05% (w/v) $(NH_4)_2S_2O_8$, 0.05% (v/v) TEMED; stacking gel: 125 mM Tris pH 6.8, 0.125% (w/v) SDS, 5% acrylamide, 0.5% $(NH_4)_2S_2O_8$, 0.5% (v/v) TEMED). Two µL of this protein was mixed with 8 µL 1x running buffer loading dye (60 mM Tris pH 6.8, 25% (v/v) glycerol, 5% (w/v) SDS, 1% (v/v) saturated bromophenol blue. After incubating for 30 min at room temperature, protein were run in 1x SDS running buffer (250 mM Tris pH 8.3, 500 mM glycine, 1 % (w/v) SDS at 200 volts with a protein size marker, until the dye was 1–2 mm from the end of the gel. Gel was stained in a Coomassie Blue stain solution (0.1% (w/v) Coomassie brilliant blue, 45% (v/v) methanol and 10% (v/v) acetic acid) for 20-30 min and then washed with PAGE-destain (10% (v/v) acetic acid, 45% (v/v) ethanol) for several times to visualize protein bands.

Total RNA was extracted from tomato leaves by TRIZOL reagent (Bio Basic) and their qualities were determined by ethidium bromide staining of agarose gels. Afterward, cDNA was synthesized with the gene specific primers i.e. chitinase (*LOC544149*) and peroxidase (*CEVI-1*), Super Script Reverse Transcriptase (In vitrogen) and used for PCR amplification. cDNA was quantified spectro-photometrically by taking the absorbance at 260 nm using water as blank in spectrophotometer BMS (Biotechnology Medical Services, UV-1900). The

quantity of DNA was calculated by applying the following formula;

Amount of DNA (µg mL^{-1}) = 50 × OD at 260 nm × Dilution factor.

Equal quantity of cDNA of each treatment was taken and use in PCR. The reaction mixture in PCR was used in the following composition i.e. 2 µL of cDNA, 2.5 µL primer mixture, 2.5 µL 10 × PCR buffer (including $MgCl_2$), 2 µL of 2.5 mM dNTPs, 0.2 µL of Taq DNA polymerase and 15.8 µL dH_2O to make final volume of 25 µL. The programs for PCR were as follows: pre amplification denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The amplified products were electrophoresed in 1% agarose gel and photographed under UV light.

Data obtained on different growth parameters were subjected to Analysis of Variance (ANOVA) and means were compared by LSD-test using software Statistix 8.1.

Results and Discussion

The polygonal interaction of host-pathogen-fungicides was deliberated *In vivo* in term of growth, SDS-PAGE and gene expression in *L. esculentum*.

Growth assays

Length, fresh and dry weight of 25-days old tomato seedlings was significantly declined by 50%, 70% and 72%, respectively due to infection of *F. oxysporum* f. sp. *lycopersici* (positive control) as compared to negative control (Table 1). It has been documented that when host plant becomes fully infected with wilt pathogen, it probably disturbs normal physiological activity including nutritional imbalance and water potential. Disturbance in plant transpiration rate likely to cause yellowing and wilting of lower leaves that progress to upward as the fungus spread within the host with chlorosis, wrinkling and drying of whole plant few days after infection (Hibar *et al*., 2007; Abd-llah *et al*., 2011; Abdel-Monaim, 2012).

All four fungicides insignificantly affected length of shoot and root, but significantly decreased biomass (fresh and dry) over negative as well as positive control treatments. Application of benedict, hiten and treaty significantly declined fresh and dry weight by 80% over negative control and by 30% over positive control. The difference among three fungicides was non-significant. However, insignificance effect of wisdom was recorded on shoot length, fresh and dry biomass as compared to positive control, while affect was significant over negative control (Table 1). When inoculated tomato seedlings were incorporated with either of fungicides, instead of typical symptoms of Fusarium wilt, stunting, chlorosis, necrotic burning was recorded on leaves. Present results were strongly supported by previous investigation, who reported chlorosis, necrosis and stunting in different plants with the application of high rates of benomyl and tebuconazole (Garciäa *et al*., 2002; Berger *et al*., 2007). The documented results on plant growth and biomass due to fungicides application are in consistency with previous literature as well (Berger *et al*., 2007; Ahemad, 2011). The differences in plant response to fungicides could be attributed to different chemical composition of each fungicide. So far, treaty belongs to triazole group and this group has been reported to interfere with sterol biosynthesis in fungi (Hewitt, 1998). The iprobenfos is active ingredient in benedict and its antifungal activity was positively correlated by inhibiting the synthesis of phospholipids (Roberts and Hutson, 1999). Fentin hydroxide in hitin is known to hinder oxidative phophorylation and ATP synthesis in fungi (FRAC, 2006). Fosetyl aluminium is the organic phosphate compound and active ingredient of wisdom. Thus it is always advisable not to generalize the effects of pesticide groups on crop plants (Shoaib *et al.*, 2014). Meiotic abnormalities induced by most the fungicides appears to be negative effect occurs at cytological level in plants (van Loon *et al*., 2006). Reduction in root biomass/numbers could be due to toxic effects of fungicides on root cells, these cells are supposed to mobilizes a set of detoxifying mechanisms which are largely dependent on ATP in order to maintain a possible normal growth rate (Zablotowizc and Reddy, 2004). Fungicides toxicity may cause ATP demand to raise along with oxygen consumption, the alteration in plant normal growth rate reduce plant biomass.

Re-isolation of *F. oxysporum* f. sp. *lycopersici* from roots of positive control as well as fungicides incorporated treatments confirmed presence of fungus. It seems that fungicides restricted the growth of fungus but not completely inhibited it. There was also possibility of synergistic effect of *F. oxysporum* f. sp. *lycopersici* and fungicides that probably induce a negative influence on overall growth and morphology of the plant. Current findings appear to explain the harmful effects caused by the fungicide on young tomato seedlings in our experiment.

SDS-PAGE

The observed changes included modification in band intensities, appearance of new bands and disappearance of pre-existing bands. In pathogen inoculated treatment some new band were observed with molecular weights ranging between 60-250 KD. A total 7 band of protein were recorded in wisdom, 6 in hiten, 5 in benedict and 4 in treaty with molecular weights ranging between 50–250 KD (Fig. 1). Protein analysis by SDS-PAGE generally gives information of structural genes along their regulatory system that govern the biosynthetic pathway of that protein (Siddiqui *et al*., 2004). The presence or absence of protein bands in pathogen inoculated

treatments might be results of activities of inducers in plant, which may act as key factors for defense mechanism of tomato against *F. oxysporum* f. sp. *lycopersici* (Hassan, 2007). The changes in protein banding pattern in tomato leaves provided with fungicide have been attributed to the some alteration at gene level that possibly lead to mutation in gene or induce cytological aberrations. The absence of some bands might be due to the deletion of their corresponding gene

Transcription analyses

Peroxidase gene was expressed in all treatments and maximum increase in gene expression was observed in treatments treated with wisdom (Fig. 2). Expression of peroxidase in all treatments indicated role of in lignin production by phenyl propanoid pathway (Sareena *et al*., 2006) along with phenolics deposition into plant cell walls during resistance responses (van Loon *et al*., 2006). It can be stated that level of reactive oxygen species in plant-pathogen-fungicides interaction shoot up and probably peroxidase was not sufficient to counter the negative effect of reactive oxygen species (ROS) on overall growth of plant (Taheri and Tarighi, 2011).

Chitinase gene was not expressed in negative control, but expressed in all treatments where pathogen was inoculated with each of four fungicides. However, increased expression was observed after treatment with benedict and hitin (Fig. 2). Chitinase belonging to the PR group of proteins appears to be a potential candidate for disease resistance by degrading fungal cell wall (Misawa and Kuninaga, 2010). Presently, it seems fungicides toxicity hinder the proper functioning of defense related gene. It could be concluded that both peroxidase and chitinase gene are very important in determining the level of basal resistance in plants however it is imperative to quantify the expression of gene through real time PCR.

Conclusion

Young tomato seedlings was senetive to recommended doses of fungicides. Considerable change in normal metabolic activities resulted in reduction in plant biomass of the infected tomato plants after fungicide application. Protein profiling by SDS-PAGE exposed some new bands, and transcription analyses of peroxidase and chitinase genes expressed variably. These results suggested that fungicides may induce/activate defense related proteins in tomato against invasion of *F. oxysporum* f. sp. *lycopersici*, however fungicides deleterious phytotoxicity culminating their successful practical application.

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Table 1: Effect of fungicides on growth parameters of 25-days-old tomato seedling.

Treatments	Shoot length (cm)	Shoot fresh weight (g)	Shoot dry weight (g)	Root length (cm)	Root fresh weight (g)	Root dry weight (g)
Negative Control	10.31 a	1.15 a	0.55 a	7.53 a	1.11 a	0.21 a
Positive Control	6.10 b	0.36 b	0.16 b	3.81 b	0.29 b	0.06 c
Benedict	6.53 bc	0.25 c	0.11 d	3.41 bc	0.17 c	0.04 d
Hiten	6.95 b	0.26 c	0.12 d	3.52 bc	0.22 c	0.04 d
Treety	6.12 bc	0.28 c	0.11 d	3.61 bc	0.19 c	0.04 d
Wisdom	6.19 b	0.31 bc	0.14 c	3.95 b	0.32 b	0.07 b

Values with bold letters in each column show significant difference ($P \leq 0.05$) as determined by LSD-test.

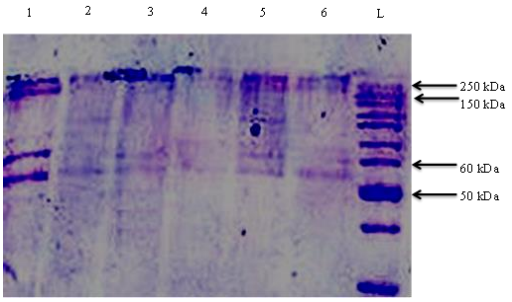

Fig. 1: SDS-PAGE for protein bands of tomato leaves. Lane 1: + Control; Lane 2: - Control; Lane 3: Treaty; Lane 4: Hiten; Lane 5: Wisdom; Lane 6: Benedict.

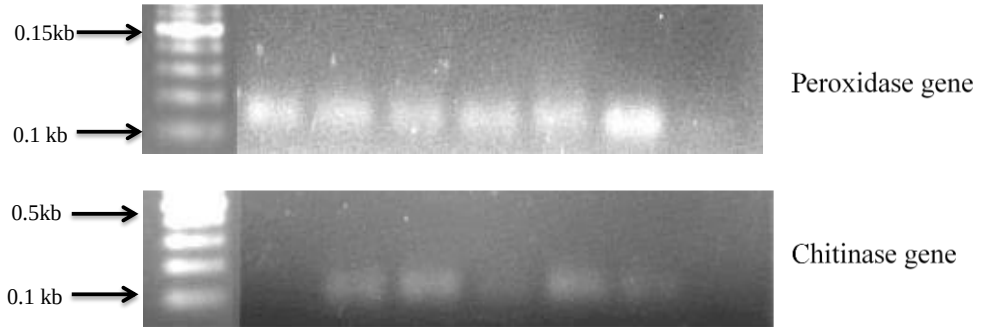

Fig. 2: Expression of peroxidase and chitinase genes in tomato leaves. Lane 1: Wisdom; Lane 2; + Control; Lane 3: Benedict; Lane 4: Treaty; Lane 5 Hiten; Lane 6: - Control

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