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Probing the Involvement of Hydrogen Peroxide Elicitation and Endogenous Ethylene Evolution in Tomato after Fusarium Toxin Treatment

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Abstract *: Fusarium wilt of tomato plant, caused by Fusarium oxysporum f.sp. lycopersici is a most prevalent and damaging disease. During disease development and in liquid culture, pathogen secretes toxins, out of which Fusaric acid (FA) is believed to play a important role in pathogenesis. FA, a non-host specific phytotoxin induces generation of reactive oxygen species (ROS) and causes oxidative stress. Among the generated ROS, H_2O_2 plays a central role in the plant defence responses and serves as a signal molecule in plant-pathogen interactions. Besides H_2O_2, Ethylene is also a signal molecule and an endogenous regulators for plant growth, development, senescence and stressed conditions such as pathogen invasions etc. In the present study, leaves of tomato plant were infiltrated with various concentrations of FA. H_2O_2 and Ethylene production were highly stimulated by 250 ug/ml FA concentration. Control plant leaves were infiltrated with sterile distilled water. Leaves were harvested 0 h, 4 h, 8 h, 12 h, 16 h, 20 h, 24 h, 36 h, 48 h and 72 h after FA treatment for estimation of H_2O_2 and Ethylene. FA treatment triggered H_2O_2 production and Ethylene evolution from tomato leaves within 4 h of treatment. FA treatment produced significantly high levels of H_2O_2 in the leaves, as compared with the control and optimum H_2O_2 production was observed after 4 h. With FA treatment Ethylene evolution was initially slower but increased gradually with time and peaked at 12 h and declined thereafter. Infiltration with L-ascorbic acid and aminoethoxy vinyl glycine (AVG) blocked H_2O_2 generation and Ethylene evolution respectively.*

Keywords*: Tomato; Fusarium; Fusaric acid; Hydrogen Peroxide; Oxidative burst; Ethylene*

1. Introduction

Many plant pathogenic microorganisms produce phytotoxins which are non-enzymatic toxic metabolites that damage plants at low concentrations. Host-selective phytotoxins, produced only by a few pathogenic microorganisms, are toxic exclusively to host plants, whereas the non-host specific phytotoxins cause symptoms on many plants. Fusaric acid (FA) is a non-host specific phytotoxin of *Fusarium* species and suspected of being involved in pathogenecity. FA has been shown to stimulate the rapid development of some disease symptoms, such as interveinal necrosis and foliar dessication [1]. It has been detected in plants after *Fusarium* attack and was present in much higher concentration in plant tissues infected with a virulent strain than in those infected with an avirulent one [2].

At the subcellular level, FA induced many biochemical effects. It was found to modify the proton electrochemical gradient across the plasma membrane, to increase electrolyte leakage and to decrease the amount of cellular ATP as well as causing inhibition of some metal containing enzymes (eg. Cytochrome oxidase) which resulted in respiratory impairment [3,4]. Plants have developed complex protection systems to cope with pathogen attack. It has been reported that reactive oxygen species (ROS) and the enzymatic systems that govern their metabolism, for example superoxide dismutase (SOD), catalase (CAT) and peroxidases may play a role in pathogenesis [5]. ROS, which are rapidly produced in plant cells after pathogen attack, are potentially involved in many defence processes including the hypersensitive response (HR), phytoalexin synthesis and oxidative cross-linking of plant cell wall proteins. Many non-host selective microbial phytotoxins are known to induce a generation of ROS and in many cases, disease symptom development can be explained by this mechanism [6]. Therefore, the possibility that ROS might also be involved in the response to FA has been of interest. Several lines of evidences suggest that, from among the generated ROS, H_2O_2 plays a central role in these plant defence responses [7]. H_2O_2 is one such ROS which acts both as toxic by product of cellular metabolism and as an important signalling molecule, depending on its concentration [8]. H_2O_2 serves as a signal molecule under various abiotic stresses [9], in acclimation to photooxidative stress [10] and in plant-pathogen interactions [11].

Ethylene is an endogenous regulators for plant growth, development and senescence [12]. Ethylene production from plants under normal

conditions is very low, but increases when living tissues are subjected to a variety of stress conditions [13] such as wounding, flooding, heavy metal toxicity, pathogen invasion etc. Stress-induced ethylene in turn leads to defence responses such as accelerated senescence, abscission of infected organs, or induction of specific defence proteins [14]. The present investigation demonstrates the phytotoxic action of FA towards Tomato (*Lycopersicon esculentum* Mill.). Thus, present experiments provide a useful model system for the research on oxidative damage and plant cell death resulting from FA.

2. Materials and Methods

2.1 Plant material and growth conditions

Tomato (*Lycopersicon esculentum* Mill.) belongs to Solanaceae family and is an important model plant. The seeds of tomato (*Lycopersicon esculentum,* cv. Sel-7) were obtained from Indian Institute of Vegetable Research, Varanasi and plants were grown in soil in green house with 14 h light and 10 h dark regime, at 27±0.5^{o}C. The plants were grown in thermocol pots of 6 cm diameter and 15 cm height containing autoclaved soil. The plants were allowed to grow and when they attained 6-8 leaves (3-4 weeks old), they were treated with FA.

2.2 FA treatment

FA was extracted and purified from the culture filtrate of *Fusarium oxysporum* f. sp. *lycopersici* (causal agent of Fusarium wilt of tomato) [15] grown at 25^{o}C in 500 ml flasks containing 100 ml potato dextrose broth (PDB) and then analysed by TLC [16] and HPLC [15]. The purified and characterized FA was stored at 4^{o}C until further use. Crystalline FA standard (5-butylpicolinic acid) was supplied by MP Biomedicals, CA, USA.

FA treatment of tomato leaves was performed by infiltration of leaf tissue with the help of a 1 ml syringe fitted with 25 guage needle in the midrib just above the petiole on the lower side of the leaf. FA solution was infiltrated to each leaflet of fully expanded tomato leaves. Control plants were infiltrated with sterile distilled water. The second and third fully expanded leaves from the plant base were used for the experiments. Oxidative burst, antioxidant enzyme activities, lipid peroxidation and ethylene evolution in the leaves of tomato plants were measured at 0, 4, 8, 12, 16, 20, 24, 36, 48 and 72 h after FA treatment.

2.3 H_2O_2 assay

Leaves treated with FA were cut with razor blade 1 cm above the base of the petiole and immediately placed in a beaker containing 1 mg/ml 3,3'-diaminobenzidine hydrochloric acid (DAB-HCl), adjusted to pH 5.6 with NaOH and were incubated in a humid growth chamber for 8 hours in the dark. The leaves were kept in the vertical position with 5 mm of the basal part dipped into the DAB solution. After DAB uptake, the leaves were cleared in 96% boiling ethanol, and examined with light microscope. H_2O_2 is visualized as a reddish-brown coloration.

The H_2O_2 produced was determined according to [17]. The treated and control plant leaf tissue were homogenized in 5% cold trichloro acetic acid (TCA) and the homogenate was centrifuged at 17000g for 10 min at 0^{o}C. The reaction mixture contained 1.6 ml of supernatant, 0.4 ml of 50% TCA, 0.4 ml of ferrous ammonium sulphate and 0.2 ml of potassium thiocynate. The absorbance was recorded at 480 nm after 15 min of incubation. The amount of H_2O_2 was estimated by a calibration curve prepared with known concentrations of H_2O_2 (Merck, India).

2.4 Ethylene evolution assay

For ethylene production, the treated and control leaf tissues of tomato plants were excised and placed in test tubes for 30 min to allow the escape of wound ethylene. After this the tubes were sealed for 2 h. Gaseous samples were then analysed for ethylene content on gas chromatograph (Model CP-3800 GC, Varian, Inc. CA, USA) equipped with an flame ionization detector (FID) and a column (CP-Pora PLOT Q, 25 m × 0.32 mm) packed with fused silica. For measuring ethylene content 1 ml gas sample was injected into the GC column using a Hamilton gastight syringe (Model: 701 RN). The column, injector and detector temperatures were set at 110^{o}C, 130^{o}C and 130^{o}C respectively. Nitrogen gas at a flow rate of 30 ml/min was used as the carrier. The ethylene content was quantified by comparison of peak areas with standard curve constructed from known amounts of ethylene gas (SSG, Alltech Asso. Inc. USA).

3. Results and Discussion

3.1 FA induced H_2O_2 generation

The accumulation of H_2O_2 was visualized by DAB staining where reddish-brown coloration was observed (Figure 1). The leaves treated with FA showed enhanced H_2O_2 release. The amount of H_2O_2 started to increase from 12 h after FA application that reached to maximum at 48 h and decreased thereafter. The control leaves had a relatively basal level of ROS. Infiltration with L-ascorbic acid blocked H_2O_2 generation (Figure 2).

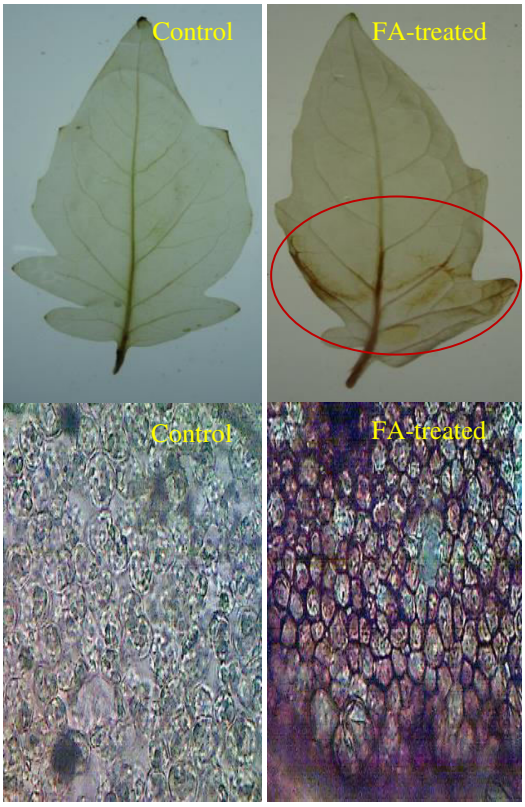

Figure 1. Localization of H_2O_2 in the leaves of tomato by DAB staining method at 24 h of FA treatment.

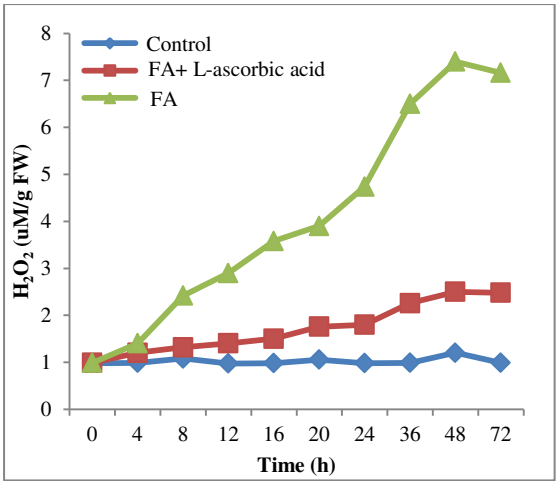

Figure 2. H_2O_2 production in the leaves of tomato plant treated with FA.

3.2 Ethylene evolution

A significant increase in ethylene production was observed following the treatment of FA in comparison to untreated tomato leaves. FA triggered ethylene evolution from tomato leaves within 4 h of treatment.

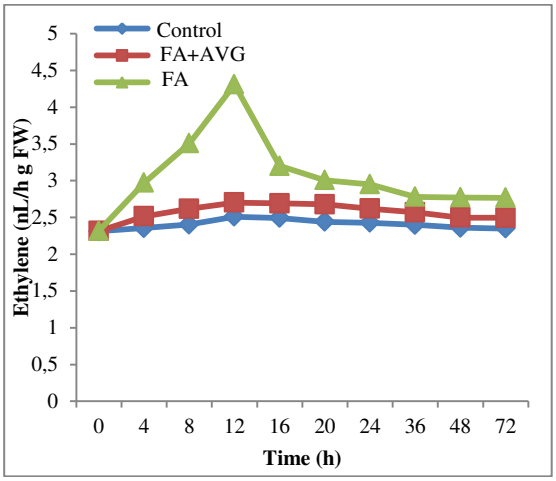

Figure 3. Ethylene production in the leaves of tomato plant treated with FA.

With FA treatment ethylene evolution was initially slower but increased gradually with time, reached at peak at 12 h and declined afterwards. Infiltration with aminoethoxy vinyl glycine (AVG) blocked Ethylene evolution (Figure 3).

4. Conclusion

The present investigation demonstrates that FA causes oxidative stress as evidenced by increased H_2O_2 production and ethylene evolution. FA-induced production of these two signalling molecules (H_2O_2 and Ethylene) may be served as important components to understand the mechanism behind the Fusarium toxin (Fusaric acid) induced oxidative damage and cell death in tomato plants.

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