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Biochemical characterization of oxidative burst during interaction between *Solanum lycopersicum* and *Fusarium oxysporum* f. sp. *lycopersici*

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ABSTRACT

The oxidative burst or rapid and transient production of large amount of reactive oxygen species (ROS) belongs to the fastest and earliest active defense responses to microbial infection known in plants. The aim of this study was to investigate the intensity and timing of the ROS formation, lipid peroxidation and expression of antioxidant enzymes as initial responses of tomato (*Solanum lycopersicum* L.) against the invading necrotrophic pathogen *Fusarium oxysporum* f. sp. *lycopersici*. The concentration of hydrogen peroxide (H_2O_2) was 2.6 times higher at 24 h post-inoculation (hpi) and lipid peroxidation was 4.4 times higher at 72 hpi in the extracts of inoculated roots than in the control. An increase in total phenolic content was also detected in inoculated roots. The activities of the antioxidative enzymes, viz., superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11), increased in response to pathogen inoculation. SOD activity at 48 hpi in inoculated roots was 2.9 times that in the control. CAT activity showed a decrease after 24 hpi and the increase in activities of GPX and APX was insignificant after 24 hpi in the inoculated roots. The oxidative burst generated in the interaction between tomato and *F. oxysporum* f. sp. *lycopersici* may be an early first line of defense by the host mounted against the invading necrotrophic pathogen. However, seemingly less efficient antioxidative system (particularly the decrease of CAT activity after 24 hpi) leading to sustained accumulation of ROS and the observed higher rate of lipid peroxidation indicate that the biochemical events are largely in favour of the pathogen, thus making this host–pathogen interaction a compatible combination. It is discussed that the oxidative burst served as a weapon for the necrotrophic pathogen because the antioxidative system was not strong enough to impede the pathogen ingress in the host.

1. Introduction

Plant cells produce reactive oxygen species during interactions with potential pathogens. The oxidative burst belongs to the fastest active defense responses known in plants. In almost all host–fungus interactions, one of the first events detected in attacked host cells is the rapid and transient generation of activated oxygen radicals, including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). The association of ROS formation and increased activity of enzymes participating in their metabolism with the induction of defense responses has been demonstrated in many plant–pathogen interactions [1–3]. H_2O_2 is generally thought of as the most versatile of the ROS, with a number of different possible functions in a plant's defense strategy. Besides having direct antimicrobial effects, such as inhibiting germination of spores of many fungal pathogens [4], H_2O_2 has been shown to be involved in the oxidative cross-linking of cell wall glycoproteins [5]. H_2O_2 has also been attributed a possible role in orchestrating the hypersensitive cell death response [6]. The ROS may also act as second messengers for the activation of genes encoding protective proteins [7].

However, the enhanced ROS production causes oxidative damage, leads to lipid peroxidation and damages macromolecules such as pigments, proteins, nucleic acids and lipids [8]. Under conditions of normal healthy growth, plants possess a number of enzymatic and non-enzymatic mechanisms of detoxification to efficiently scavenge for either the ROS themselves or their secondary reaction products [9]. Various enzyme systems participate in ROS metabolism during the pathogen attack in plants [10]. Major ROS-scavenging enzymes such as superoxide dismutase (SOD, EC

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; EDTA, ethylene diamine tetraacetic acid; FW, fresh weight; *Fol*, *Fusarium oxysporum* f. sp. *lycopersici*; GPX, guaiacol peroxidase; hpi, hours post-inoculation; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NBT, nitro blue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

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1.15.1.1), catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPX, EC 1.11.1.7) and ascorbate peroxidase (APX, EC 1.11.1.11) are produced to avoid cellular disintegration by ROS [11]. Superoxide dismutase, the first enzyme in ROS metabolism, catalyzes dismutation of O_2^- and $HO_2^{\bullet}$ to H_2O_2. Catalase and ascorbate peroxidase belong to important H_2O_2-scavenging enzymes, removing H_2O_2 through a mechanism known as Halliwell–Asada–Foyer pathway. Guaiacol peroxidase is included in different physiological processes like cross-linking of the cell wall proteins, pectins by diferulic bridges and the oxidation of cinnamyl alcohols prior to their polymerization during lignin and suberin formation. Peroxidases are an important component of an early plant-response system to pathogen attack [12,13].

Phenolic compounds can act as free radical scavengers and protect cells from their oxidative toxicity [14]. On the other hand, simple phenols may cause disorders in plant cell membrane functioning, and cause membrane damage accompanied by lipid peroxidation [15]. Lipid peroxides decompose to produce malondialdehyde, volatile hydrocarbons such as ethane, pentane and are precursors for the synthesis of jasmonic acid [16].

It is widely believed that plants have coevolved with pathogens to develop complex mechanisms, both constitutive and inducible, to cope with the infection [17]. ROS production has been well established in several plant tissues infected by biotrophs and associated with the expression of a hypersensitive response or systematic acquired resistance [18]. In contrast, very little is known about oxidative metabolism in plant resistance to necrotrophic pathogens such as the fungi that invade the plant vascular system [19,20]. Recently, it was reported that *Fusarium* infection resulted in increased activities of antioxidant enzymes together with increased levels of ROS and lipid peroxidation in cucumber roots [21].

To our knowledge, there has been no report published on the biochemical aspects of interaction between *Solanum lycopersicum* and *Fusarium oxysporum* f. sp. *lycopersici*. In the backdrop of this scenario, the aim of the present study was to investigate the intensity and timing of the ROS formation, lipid peroxidation and expression of antioxidant enzymes as initial responses of *S. lycopersicum* L. cv. Arka Saurabh against the invading necrotrophic pathogen *F. oxysporum* f. sp. *lycopersici*, the causal organism of wilt disease which is one of the most prevalent and damaging diseases of tomato world wide.

2. Materials and methods

2.1. Chemicals

Analytical grade chemicals were used in sample preparation. Deionized water for all procedures was obtained from a Barnstead/Thermolyne (Dubuque, USA) Diamond-Nanopure™ water purification system. All authentic standards were procured from Sigma–Aldrich Chemical Co. Ltd (New Delhi, India).

2.2. Plant material and pathogen inoculation

Tomato plants (*S. lycopersicum* L. cv. Arka Saurabh) were grown in hydroponics culture according to Spletzer and Enyedi [22] with modifications.

The causal agent of *Fusarium* wilt on tomato is *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyd. & Hans. *F. oxysporum* f. sp. *lycopersici* strain 1322F (*Fol*), a virulent strain on tomato (i.e., compatible host–pathogen interaction), was obtained from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi. The pathogen was grown on potato dextrose agar medium in light at 26 °C. The spore suspension used for inoculation was prepared from a 2-week-old culture and was applied at a concentration of 5×10^6 spores per 1 ml [23]. Tomato plants were inoculated by the pathogen with addition of the spore suspension in the hydroponics media. As a control, non-inoculated tomato plants were used. The roots were harvested on a time course to perform analyses.

2.3. Assay of H_2O_2 generation during tomato–Fusarium interaction

Tomato roots (both inoculated and non-inoculated) were homogenized in 0.1% trichloroacetic acid (TCA) in ratio 1:10 (w/v) and centrifuged at $12{,}000 \times g$ for 15 min at 4 °C. Supernatant (0.5 ml) was mixed in 1 ml of 1 M potassium iodide (KI) solution and incubated for 5 min before measuring of the oxidation product at A_{390} [24]. The amount of H_2O_2 formed was computed from the standard curve made earlier with known concentrations of H_2O_2 and expressed as $\mu mol\, g^{-1}$ FW.

2.4. Determination of lipid peroxidation

Lipid peroxidation was measured in terms of MDA content, a thiobarbituric acid reactive substance as per Heath and Packer [25]. Tomato roots were first homogenized and then extracted in 0.1% TCA in ratio 1:5 (w/v) and centrifuged at $12{,}000 \times g$ for 30 min at 4 °C. One millilitre of supernatant was incubated with 4 ml of 20% TCA containing 0.5% thiobarbituric acid for 30 min at 95 °C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at $10{,}000 \times g$ for 15 min. The absorbance of the reaction product was measured at 532 nm. The concentration of the MDA was determined using the extinction coefficient of $155\ mM^{-1}\ cm^{-1}$ and expressed as $\mu mol\, g^{-1}$ FW.

2.5. Study of the antioxidative enzyme activities in relation to oxidative burst

Tomato roots were homogenized in 10 ml of chilled 0.1 M phosphate buffer (pH 7.0). The homogenate was centrifuged at 4 °C for 30 min at $15{,}000 \times g$. After concentration through AmiconR™ Ultra-4 CFU membrane (Millipore, Bedford, USA), the supernatant portion was used as enzyme extract for determination of superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase activities. Protein concentration was measured according to Bradford [26].

2.5.1. Superoxide dismutase (SOD) activity

SOD was assayed following the method of Beauchamp and Fridovich [27] by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. The reaction mixture (4 ml) contained 63 μM NBT, 13 mM L-methionine, 0.1 mM EDTA, 13 μM riboflavin, 0.05 M sodium carbonate and 0.5 ml enzyme extract (0.5 ml distilled water in case of control). It was kept under two 15-W fluorescent lamps for 15 min at 25 °C, followed by transferring to dark for 15 min and then the absorbance was read at 560 nm. One unit of the SOD activity was defined as the amount of enzyme required to inhibit reduction of NBT by 50%.

2.5.2. Catalase (CAT) activity

CAT activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm as per the method of Cakmak and Marschner [28]. The reaction mixture (2 ml) consisted of 25 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 0.2 ml enzyme extract. One unit was defined as a change in absorbance of 0.1 under the conditions of the assay. Enzyme activity was expressed as $nkat\, mg^{-1}$ protein.

2.5.3. Guaiacol peroxidase (GPX) activity

GPX activity was assayed by measuring increase in absorbance at 470 nm due to oxidation of guaiacol to tetraguaiacol. The reaction mixture consisted of 20 mM guaiacol (0.5 ml), 0.1 mM acetate buffer (pH 5.0) (2.1 ml), 40 mM H_2O_2 (0.2 ml) and the