REACTIVE OXYGEN SPECIES SIGNALING IN EGGPLANT IN RESPONSE TO *RALSTONIA SOLANACEARUM* INFECTION

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SUMMARY

This study is aimed at investigating reactive oxygen species formation, lipid peroxidation, induction of antioxidant enzymes and cell wall strengthening as initial responses of eggplant to *Ralstonia solanacearum* infection. The oxidative burst triggered during the course of interaction may be an initial defense response of the host targeted against the invading bacterial pathogen. The concentration of H_2O_2 in inoculated roots was 53 µmol g^{-1} at 48 h post inoculation (hpi), which was about three times higher than in the control. The increase in $O_2\cdot^-$ generation resulting from inoculation with *R. solanacearum* was evident prominently from 24 hpi, but was highest at 48 hpi. At 24 hpi, the increase in OH· generation resulting from *R. solanacearum* inoculation was at 3.9 A/g/h. Lipid peroxidation was higher at later stage of eggplant-*R. solanacearum* interaction. The activities of the antioxidative enzymes, *viz*. superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) increased in response to pathogen inoculation. SOD activity was more than three times higher than in non-inoculated plants at 48 hpi. CAT activity showed a decrease after 30 hpi and finally it reached the level in control plants. The increase in activities of GPX and APX was significant in inoculated roots. The highest phenolic and lignin contents were determined at 54 hpi in the inoculated plants. However, the antioxidative system in the plant was rather weak and the rate of lipid peroxidation was higher. We conclude that the biochemical events favored *R. solanacearum* rather than the eggplant, making this host-pathogen interaction a compatible combination.

Key words: Antioxidative enzymes, lipid peroxidation, oxidative burst, *Ralstonia solanacearum*, *Solanum melongena*.

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INTRODUCTION

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide anion ($O_2\cdot^-$), hydroxyl radical (OH·) and singlet oxygen ($^1O_2$) and they are ubiquitous signaling molecules generated during the course of regular cellular metabolism (Blomster *et al*., 2011). ROS are produced, *inter alia*, after incomplete reduction of oxygen (H_2O_2, $O_2\cdot^-$ and OH·) (Gechev *et al*., 2006). These ROS become rapidly metabolized with the help of constitutive antioxidative enzymes and nonenzymatic antioxidant scavenging systems such as antioxidant vitamins, proteins and thiols during normal conditions (Kovtun *et al*., 2000). However, ROS production takes upswing under the influence of environmental stresses such as pathogen attack, cold, ozone, drought, salt stress, UV irradiation, etc. Under the stressed condition, activation of additional defense mechanisms is required to counteract this excessive accumulation of ROS. If these ROS are not scavenged efficiently, they pose danger to lipids in cellular membranes, proteins and other cellular components leading to dysfunction and ultimately to the appearance of necrotic lesions (Foyer and Noctor, 2005). A vast network of antioxidants is constantly on the alert for rising ROS concentrations and provides effective scavenging for it (Apel and Hirt, 2004; Gechev *et al*., 2006). A host of enzymatic systems take part in ROS scavenging during the pathogen invasion in plants (Bolwell *et al*., 2002). Most important ROS scavenging enzymes e.g., 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) are put in place rapidly in plant cells to prevent cellular damage by ROS. These enzymes are believed to maintain ROS homeostasis in different compartments of the plant cell (Mittler *et al*., 2011). They could restrict the ROS-dependent damage or finely tune ROS-dependent signal transduction in plant system.

During plant-microbe interactions, ROS have been suggested to participate in plant defense signaling (Levine *et al*., 1994). ROS can directly cause cell wall reinforcement through oxidative cross-linking of glycoproteins or lipid peroxidation and membrane damage (Montillet *et al*., 2005). ROS have taken a centre stage in signal transduction network of stress-inducible genes in higher plants and it is emerging that a balanced amount of ROS is crucial for many different metabolic processes in plants (Kotchoni *et*

al., 2006). Additional regulatory functions for ROS in defense occur in conjunction with other plant signaling molecules, particularly with salicylic acid (SA) and nitric oxide (NO). Evidence indicates that the superoxide-generating NADPH oxidases are the main sources of extracellular ROS generated during infection of plants with pathogen (Nuhse *et al*., 2007).

Eggplant or brinjal (*Solanum melongena*) is an important vegetable crop and eggplant-based diet is recommended as a choice for the management of type 2 diabetes. Bacterial wilt caused by *Ralstonia solanacearum* is one of the threatening diseases and a major constraint to production of this important vegetable. *R. solanacearum* is present worldwide and has a wide host range, including several hundreds of susceptible species in at least 50 different plant families, which makes the pathogen most destructive and most difficult to control (Kelman, 1954). Accumulation of ROS in plants in response to pathogen infections is emphasized with the aim of elucidating the mechanisms of disease resistance acquisition in higher plants. We attempted to delve into ROS generation, antioxidative mechanisms and cell wall strengthening as initial defense response of eggplant (line CHBL-54) to *R. solanacearum* infection.

MATERIALS AND METHODS

Chemicals. Analytical grade chemicals were used in sample preparation. All authentic standards used in the study were procured from Sigma-Aldrich (Bangalore, India).

Establishment of hydroponic culture of eggplants. Eggplants (line CHBL-54) were grown in hydroponic culture according to Mandal *et al.* (2009a) with modifications. Briefly, eggplant seeds were sown in nursery bed soil and grown to the 4-week stage in open ambient climate. Plants were removed from the soil and placed into hydroponic vessels (250 ml conical flasks) containing 300 ml Hoagland nutrient medium. The vessels were then covered with brown paper on the exterior to decrease light penetration to prevent algal growth in the liquid medium. Hydroponically-grown eggplants were maintained under a 14-h photoperiod regime throughout the experiment.

The pathogen. A virulent strain of *R. solanacearum* (phylotype I biovar 3) was isolated on *Pseudomonas solanacearum* medium (HiMedia, India) from a wilted eggplant found in the experimental plot at the Central Horticultural Experiment Station, Bhubaneswar (India). The biovar of the strain was determined based on its ability to utilize disaccharides and hexose alcohols. Phylogenetically meaningful classification scheme based on DNA sequence analysis was developed for *R. solanacearum* (Champoiseau *et al*., 2009), by which the species complex is divided into four phylotypes. Broadly, these phylotypes reflect the ancestral relationships and geographical origins of the strains (Fegan and Prior, 2005). According to this DNA sequence-based scheme, the strain isolated from eggplant and used in the ROS signaling study in eggplant may fall in phylotype I (i.e., the strains of Asian origin) (Mandal *et al*., 2011).

Pathogen inoculation of eggplants for the study of oxidative burst. Bacterial inoculum was prepared from agar plates by flooding with sterile distilled water, and adjusted at an optical density of 0.05 at 600 nm with a BioMate™ 3 spectrophotometer (Thermo Spectronic, USA), corresponding to *ca.* 7.0×10^6 CFU/ml. After establishment in hydroponic culture, the plants were inoculated by adding 2.5 ml of bacterial inoculum (2.5 ml sterile distilled water in the control) in the hydroponic medium (*ca.* 300 ml). Tap and fibrous roots were harvested on a time course to perform analyses.

Assay of hydrogen peroxide (H_2O_2) concentration during eggplant-*Ralstonia* interaction. Eggplant roots (both inoculated and non-inoculated) were homogenized in 0.1% trichloroacetic acid (TCA) at 1:10 (w/v) ratio and centrifuged at 12,000*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 390 nm (Veliková, 2000). 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 µmol/g FW.

Assay of superoxide anion ($O_2\cdot^-$) concentration during eggplant-*Ralstonia* interaction. Measurement of reduction of nitro-blue tetrazolium (NBT) was used for determination of $O_2\cdot^-$ (Doke, 1983). Five root discs (0.5 cm diameter) were immersed in 3 ml of 0.01 M potassium phosphate buffer pH 7.8 containing 0.05% NBT and 10 mM NaN_3 for 1 h. After removing the leaf discs, the mixture was heated at 85°C for 15 min and cooled. The NBT-reducing activity of the discs was expressed as increased absorbance at 580 nm/h/g FW.

Assay of hydroxyl radical (OH·) concentration during eggplant-*Ralstonia* interaction. Hydroxyl (OH·) radicals were measured as described by Tiedemann (1997). 2-Deoxyribose (DOR) was used as a scavenger and molecular probe for OH· radicals. DOR is slightly sensitive to degradation by OH· radicals, resulting in the accumulation of thiobarbituric acid-reactive degradation products. Ten root discs (0.5 cm diameter) were immersed in 1 ml 1 mM DOR and incubated at room temperature (22°C) in the dark for 45 min. Then, 0.5 ml of the sugar solution (10%) was added to a preheated mixture of 0.5 ml thiobarbituric acid (TBA) 1% w/v in 0.05 M NaOH and 0.5 ml TCA 2.8% w/v, and immediately boiled for 10 min. Finally, samples were

cooled on ice for 10 min. Absorbance was measured at 540 nm. The results are given as absorbance units/h/g FW.

Determination of lipid peroxidation during eggplant-*Ralstonia* interaction. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content, a thiobarbituric acid reactive substance as per Heath and Packer (1968). Eggplant roots were extracted in 0.1% TCA in ratio 1:5 (w/v) and centrifuged at 12,000*g* for 30 min at 4°C. One ml 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*g* for 15 min. The absorbance of the reaction product was measured at 532 nm. MDA concentration was determined using the extinction coefficient of 155/mM/cm and expressed as µmol/g FW.

Study of antioxidative enzyme activities in relation to oxidative burst. Eggplant 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*g*. After concentration through AmiconR™ Ultra-4 CFU membrane (Millipore, USA), the supernatant was used as enzyme extract for determination of superoxide dismutase, catalase, guaiacol peroxidase and ascorbate peroxidase activities. Protein concentration was measured according to Bradford method (Bradford, 1976)

SOD activity assay during eggplant-Ralstonia *interaction.* Superoxide dismutase activity was assayed according ro Beauchamp and Fridovich (1971) by measuring its ability to inhibit the photochemical reduction of nitroblue 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 watt fluorescent lamps for 15 min at 25°C, transferred to dark for 15 min, then its absorbance was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit reduction of NBT by 50%.

CAT activity assay during eggplant-Ralstonia *interaction.* Catalase activity was assayed by measuring the rate of disappearance of H_2O_2 at 240 nm as per the method of Cakmak and Marschner (1992). 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 protein.

GPX activity assay during eggplant-Ralstonia *interaction.* Guaiacol peroxidase activity was assayed by measuring the 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 enzyme extract (0.2 ml) in a final volume of 3 ml (Chance and Maehly, 1955). The linear portion of the activity curve was used to express the enzyme activity (expressed as nkat/mg protein). One unit of enzyme activity represented the amount of enzyme catalyzing the oxidation of 1 µmol guaiacol in 1 min.

APX activity assay during eggplant-Ralstonia *interaction.* Ascorbate peroxidase activity was measured by following the oxidation of ascorbic acid at 290 nm as described by Nakano and Asada (2001). The reaction mixture (2 ml) consisted of 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.25 mM ascorbic acid, 1.0 mM H_2O_2 and 0.2 ml enzyme extract. Decrease in absorbance was noticed 60 sec after addition of the enzyme extract at 290 nm. One unit was defined as a change in absorbance of 0.1 under the conditions of the assay. The enzyme activity was expressed as nkat/mg protein.

Estimation of total phenolics in *Ralstonia*-inoculated eggplant roots. The total phenolic content was determined as described by Mandal *et al.* (2009b) using Folin-Ciocalteau reagent. Briefly, the reaction mixture contained 100 µl of methanolic extract of eggplant root tissues, 200 µl deionised water with 500 µl of Folin-Ciocalteau reagent. After 5 min, 800 µl of 20% sodium carbonate (Na_2CO_3) was added and after 1 h incubation, the absorbance was measured at 254 nm. Standard curve was prepared with *p*-hydroxybenzoic acid in 50% (v/v) methanol.

Determination of lignin in *Ralstonia*-inoculated eggplant roots. Lignin was extracted according to the method of Bruce and West (1989) with slight modifications. Briefly, root segments (elicited and control) were homogenized in 80% methanol. The homogenate was filtered through Whatman no. 4 filter paper and rinsed with methanol, then the residue was dried at 60°C for 24 h. The dried alcohol insoluble residue (AIR) was used for lignin determination. To 50 mg of AIR in glass screw-cap vials 5 ml HCI (2 N) and 0.5 ml thioglycolic acid (TGA) were added and the mixture was placed in boiling water for 4 h. The mixture was then centrifuged at 20,000*g* for 15 min and the pellet washed with 5 ml deionised water. It was then suspended in 5 ml of 0.5 N NaOH, shaken at 25°C for 2 h and centrifuged at 20,000*g* for 15 min. Concentrated HC1 (1 ml) was added to the supernatant and the lignin-thioglycolic acid was allowed to precipitate at 4°C for 4 h. After centrifugation at 10.000*g* for 10 min, the orange-brown pellet was dissolved in 10 ml of 0.5 N NaOH, again centrifuged and the absorbance of TGA-derivatives in the supernatant measured at 280 nm. Results were expressed as the increase in A_{280} nm/g of AIR fresh weight.

Disease assessment. Assessment of disease severity was done according to Ishikawa *et al.* (2005) with

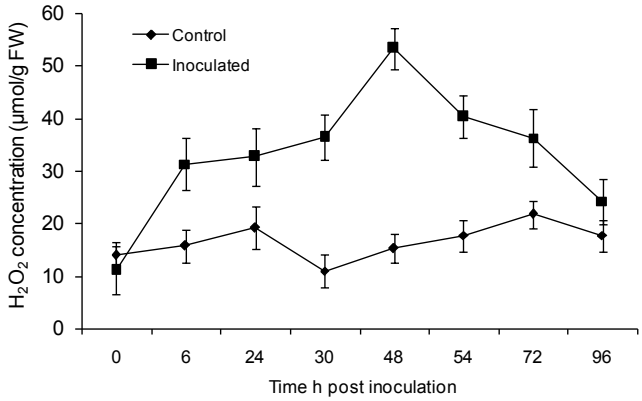

Fig. 1. H_2O_2 generation (expressed as µmol/g FW) in eggplant roots on a time course after inoculation of the plants with *Ralstonia solanacearum*, and in the control. Data presented are the means ± SD of three replicates.

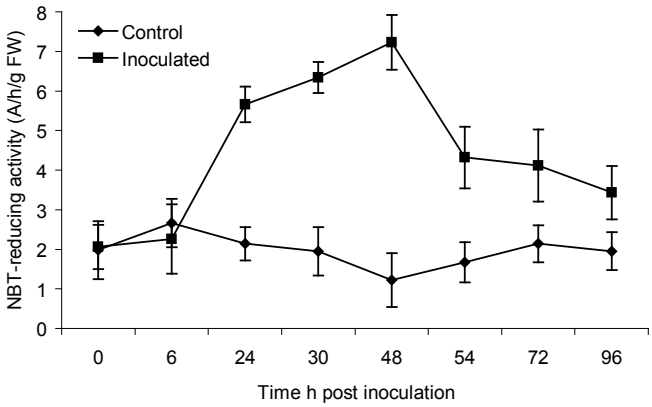

Fig. 2. Nitro-blue tetrazolium (NBT)-reducing activity for determination of superoxide anion ($O_2^{\cdot -}$) (expressed as A/g/h FW) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia solanacearum*. Data presented are the means ± SD of three replicates.

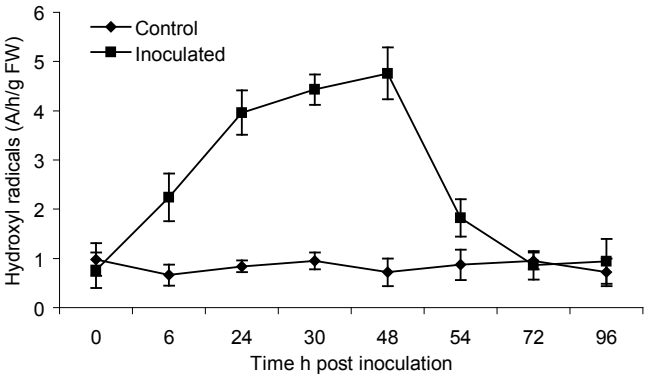

Fig. 3. Hydroxyl radical (OH·) concentration (expressed as A/g/h FW) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia solanacearum*. Data presented are the means ± SD of three replicates.

modifications. Two weeks after challenge of the eggplants with *R. solanacearum*, the disease index (on a 1-5 scale) on each plant was recorded based on vascular browning and the mean value was calculated as disease severity. For vascular browning evaluation, the basal stems were cut and the discoloration was rated on a scale where 1 = no vascular browning; 2 = 1-25% vascular browning; 3 = 26-50% vascular browning; 4 = 51-75% vascular browning; 5 = more than 75% vascular browning. Disease severity in respect to wilting was recorded with the same scale.

Statistical analysis. Growing, inoculation and sampling of eggplants were done in three independent experiments. Statistical analysis was done using Student's *t*-test, with level of significance P = 0.05. SD was calculated and its range is shown in the figures.

RESULTS

Hydrogen peroxide generation during eggplant-*Ralstonia* interaction. H_2O_2 is most studied among ROS in relation to oxidative burst during plat-pathogen interaction. The level of H_2O_2 generation was higher in eggplants inoculated with *R. solanacearum* beginning 6 h post inoculation (hpi) as compared to the non-inoculated plants (Fig. 1). H_2O_2 generation continued throughout and a characteristic peak was detected at 48 hpi. The concentration of H_2O_2 in inoculated roots was 53 µmol/g FW at this point of time, which was about 3 times higher than control plants. After this increase, there was a sharp decrease in the level of H_2O_2 up to 96 hpi.

Superoxide anions generation during eggplant-*Ralstonia* interaction. Superoxide anion generation in the tissues of infected plants is detected after few hours of pathogen invasion. The increase in $O_2^{\cdot -}$ generation resulting from inoculation with *R. solanacearum* was evident prominently from 24 hpi, but was highest at 48 hpi. At this point, the value for $O_2^{\cdot -}$ was 7.2 A/g/h FW in root tissues of the inoculated plants and the same was only 1.2 A/g/h FW in the roots of non-inoculated plants (Fig. 2). After 48 hpi, the increase showed a decline and this trend continued till end of the time course study.

Hydroxyl radicals generation during eggplant-*Ralstonia* interaction. Hydroxyl radical generation is a rapid phenomenon in the tissues of pathogen-infected plants and is detected after just few hours from pathogen attack. Increase of OH· was detected from 6 hpi of eggplants with *R. solanacearum*. This sharp increase continued and a peak was observed at 48 hpi. At 24 hpi, the increase in OH· generation resulting from *R. solanacearum* inoculation was at a value of 3.9 A/g/h FW and it was 4.7 A/g/h FW at 48 hpi (Fig. 3). Hereafter, a sharp decrease was noticed in OH· generation in the inoculated roots and it reached the level of control plants at 72 hpi.

Determination of lipid peroxidation during eggplant-*Ralstonia* interaction. The extent of cell damage caused by the production of reactive radicals and oxidative stress related to plant response to pathogen infection can be estimated by determining the products of membrane lipids peroxidation. Lipid peroxidation is measured in terms of malondialdehyde (MDA) content, a thiobarbituric acid reactive substance. It was observed that the increase in lipid peroxidation was very slow in the initial hours after *R. solanacearum* inoculation. However, the increase was unabated and the concentration of MDA was 5.3 µmol/g FW in *Ralstonia*-inoculated eggplant roots at 48 hpi. This increasing trend continued and the concentration of MDA was 8.2 µmol/g FW in inoculated roots at 96 hpi (Fig. 4).

Dynamics of antioxidative enzymes during eggplant-*Ralstonia* interaction. Various enzyme systems participate in ROS metabolism during plant-pathogen interactions. Superoxide dismutase, the first enzyme in ROS metabolism, catalyzes dismutation of O_2^- and OH· to H_2O_2. Catalase and ascorbate peroxidase belong to important H_2O_2-scavenging enzymes that remove H_2O_2 through a mechanism known as the 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.

SOD activity during eggplant-Ralstonia *interaction.* The stimulating effect of inoculation of eggplant roots with *R. solanacearum* on SOD activity was studied. SOD activity started increasing in a few hours of challenge inoculation with *Ralstonia* (Fig. 5). The increasing trend continued peaking at 48 hpi (21.8 nkat/mg protein). This level of activity was more than 3 times higher than in non-inoculated plants. A slow decrease was observed after this point, but the activity of SOD in inoculated plants never returned to the level of controls.

CAT activity during eggplant-Ralstonia *interaction.* An increase in CAT activity was observed from 6 hpi onwards A stiff increase in CAT activity was found to be the hallmark of this plant-pathogen interaction upto 24 hpi (Fig. 6). However, the peak of activity was attained at 30 hpi in *Ralstonia*-inoculated roots with a value of 4 nkat/mg protein, which was more than 4 times higher than the control. Immediately after this point, there was a gradual decrease in CAT activity in inoculated roots. Finally its activity reached the level of control at 96 hpi.

GPX activity during eggplant-Ralstonia *interaction.* Correlating with H_2O_2 accumulation, the activity of GPX also increased in inoculated plants. GPX activity was detected in higher level at 6 hpi (Fig. 7). The highest increase in GPX activity was observed at 72 hpi (104 nkat/mg protein),

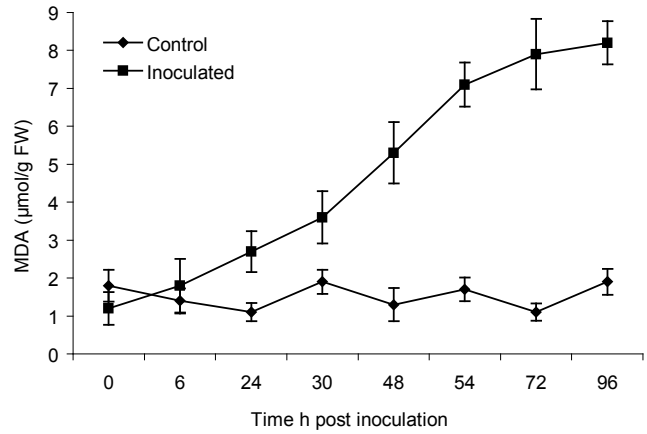

Fig. 4. Lipid peroxidation (measured in terms of malondialdehyde (MDA) content and expressed as µmol/g FW) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia solanacearum* and in the control. Data presented in graphs are the means ± SD of three replicates.

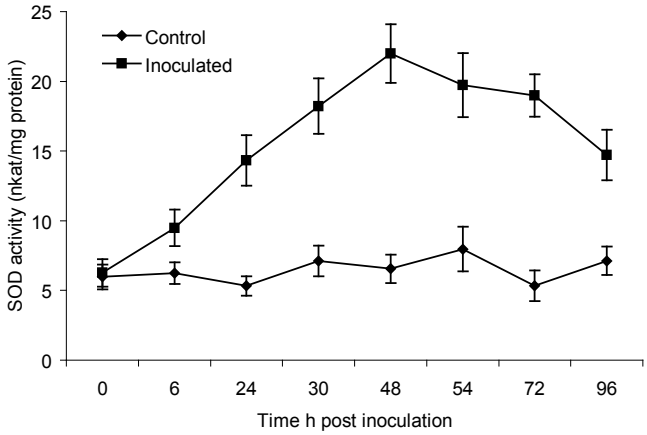

Fig. 5. Superoxide dismutase activity (expressed as nkat/mg protein) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia* and in the control. Data presented in graphs are the means ± SD of three replicates.

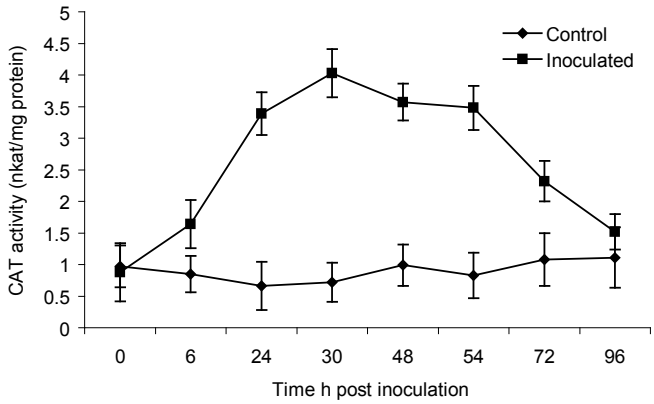

Fig. 6. Catalase activity (expressed as nkat/mg protein) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia solanacearum* and in the control. Data presented in graphs are the means ± SD of three replicates.

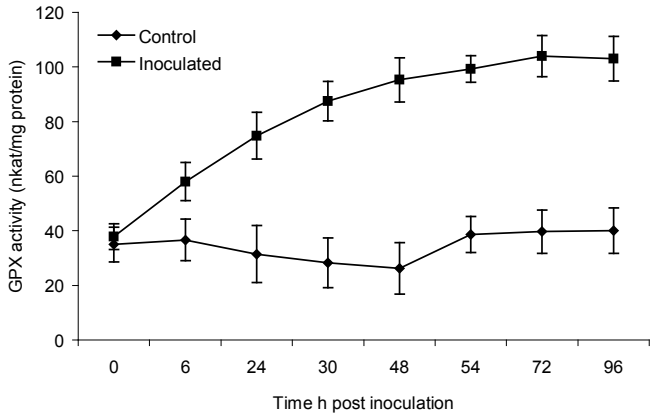

Fig. 7. Guaiacol peroxidase activity (expressed as nkat/mg protein) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia solanacearum* and in the control. Data presented in graphs are the means ± SD of three replicates.

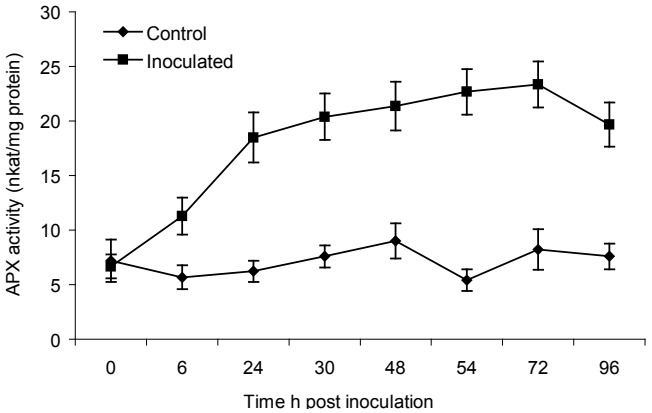

Fig. 8. Ascorbate peroxidase activity (expressed as nkat/mg protein) in roots of eggplants on a time course after inoculation of the plants with *Ralstonia solanacearum* and in the control. Data presented in graphs are the means ± SD of three replicates.

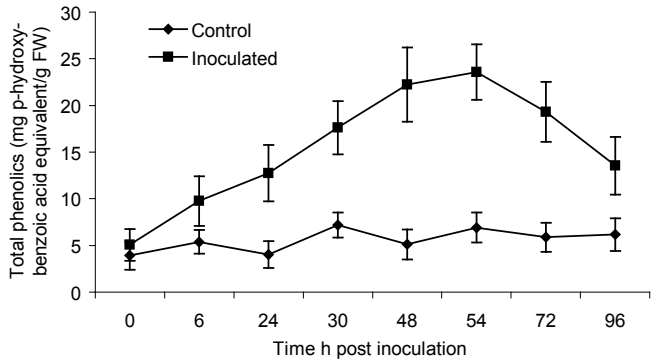

Fig. 9. Total phenolics content (expressed as mg *p*-hydroxybenzoic acid equivalent/g FW) in roots of eggplant on a time course after inoculation of the plants with *Ralstonia solanacearum* as compared with the control. Data presented in graphs are the means ± SD of three replicates.

which was about 2.5 times higher in-inoculated roots than in the control. Thereafter, a very slow decrease in activity of GPX was observed.

APX activity during eggplant-Ralstonia *interaction.* APX in eggplant roots responded to pathogen attack with a sharp increase in activity immediately after the interaction eggplant-*Ralstonia* began, with a peak at 24 hpi (18.4 nkat/mg protein). Afterwards, the increase of APX activity was marginal and this trend continued till 72 hpi (Fig. 8), when a real decrease in enzyme activity was observed.

Determination of total phenolics during eggplant-*Ralstonia* interaction. Total phenolic content in eggplant roots was found to increase from the beginning in inoculated as compared with control plants (Fig. 9). At 24 hpi, the phenolic acids content was about 3 times that of the control and was highest at 54 hpi in inoculated plants (23.59 mg *p*-hydroxybenzoic acid equivalent/g FW) compared to the control (6.92 mg). This peak was followed by a decrease so that at 96 hpi, the content of total phenolics was 13.54 mg in inoculated root tissues.

Determination of lignin deposition during eggplant-*Ralstonia* interaction. There was no increase in lignin content in inoculated eggplant roots up to 6 hpi when lignin deposition in the cell walls started to increase. Its content was about 3 TGA-derivatives at 280 nm/g of AIR FW at 24 hpi in inoculated roots, whereas it was about 1 TGA-derivatives at 280 nm/g of AIR FW in control roots (Fig. 10). The highest lignin deposition was detected at 54 hpi in inoculated roots (5.24 TGA-derivatives at 280 nm/g of AIR FW) and continued to increase though at a lower rate. In any case, the lignin content remained about 4-fold higher in inoculated than in control plants.

Assessment of disease severity on the eggplants challenged with *Ralstonia*. Inoculation of eggplants (line CH-BL-54) with *R. solanacearum* through the hydroponic medium resulted in infection. Disease severity was assessed by scoring (on an arbitrary scale of 1-5) vascular browning and whole plant wilting two weeks after inoculation (Fig. 11). A value of 4.38 was scored as regards to vascular browning in the stems of inoculated plants, while the value was only 0.93 in control plants. A 3.93 severity value was determined in inoculated plants in respect to whole plant wilting, whereas there was no wilting in the controls.

DISCUSSION

ROS (H_2O_2, $O_2^{\cdot -}$, $OH^{\cdot}$ and $^1O_2$) are essential signaling molecules in plant development and in response to biotic and abiotic stresses (Mittler *et al.*, 2011). ROS are known to play dual role depending on their accumulation levels. High intracellular concentration of ROS can cause extensive cell injury or death. During periods of biotic or abiotic stress, ROS levels can rise excessively, leading to an oxidative stress state (Apel and Hirt, 2004). We report here higher levels of ROS production in conjunction with

increased activities of antioxidant enzymes in root tissues of eggplants inoculated with *R. solanacearum*.

A wide range of environmental stimuli lead to a transient rise in cellular H_2O_2 levels. In these cases, H_2O_2 can be viewed as a signal that relays the initial stimuli to downstream effectors (Petrov and Van Breusegem, 2012). In the present study, eggplant roots inoculated with *R. solanacearum* experienced transient increment in the H_2O_2 level. Tomato leaves infected by *R. solanacearum* contained hydrogen peroxide, and concentrations of this ROS increased as pathogen populations increased. These results reveal that *R. solanacearum* is exposed to ROS during pathogenesis and that it has evolved a redundant and efficient oxidative stress response to adapt to the host environment and cause disease (Flores-Cruz and Allen, 2009). *Erwinia carotovora* pv. *betavasculorum* was found to induce H_2O_2 production in sugar beet (Bargabus *et al*., 2003). Our study showed increase in concentration of $O_2\cdot^-$ up to 48 hpi. Increasing evidence points to superoxide-generating NADPH oxidases as the main sources of extracellular ROS produced during pathogen infection or elicitation (Nuhse *et al*., 2007). $O_2\cdot^-$ is produced in the apoplast by respiratory burst oxidase homologues (RBOHs). The first role to be characterized for RBOHs was in ROS production during pathogen defense (Torres *et al*., 2006). $O_2\cdot^-$ production was observed in the mitochondrial fraction isolated from *Pseudomonas cichorii*-infected lettuce leaves much more intense than in water-treated leaves (Kiba *et al*., 2009). Higher levels of $O_2\cdot^-$ and HO· were detected in soybean lines after infection with *Sclerotinia sclerotiorum* (Malenčić *et al*., 2010).

Recent research shows that some pathogens may induce ROS production to their own advantage. It has been shown that the necrotrophic pathogen *Botrytis cinerea* induces ROS formation in plants, resulting in hypersensitive cell death that facilitates fungal colonization (Schouten *et al*., 2002; Govrin *et al*., 2006). There are also reports of ROS being produced, together with increased levels of ROS detoxification enzymes, during compatible interactions involving viruses (Clarke *et al*., 2002). ROS is produced as part of a complex network of signals that respond to pathogen attack and mediate multiple responses, sometimes with opposite effects, in different contexts or in response to different pathogens (Torres *et al*., 2006). In spite of all these developments, there is emerging complexities in ROS production and its implication in cell signaling during plant-pathogen interaction (Vellosillo *et al*., 2010).

The peroxidation of unsaturated lipids of biological membranes is the most prominent symptom of oxidative stress in animals and plants. The production of lipid peroxides were proven to be induced by pathogens (Göbel *et al*., 2003). In our study, a very high increase in lipid peroxidation measured as production of MDA was observed during the course of the study. Higher rate of lipid peroxidation indicates that the pathogen may develop a

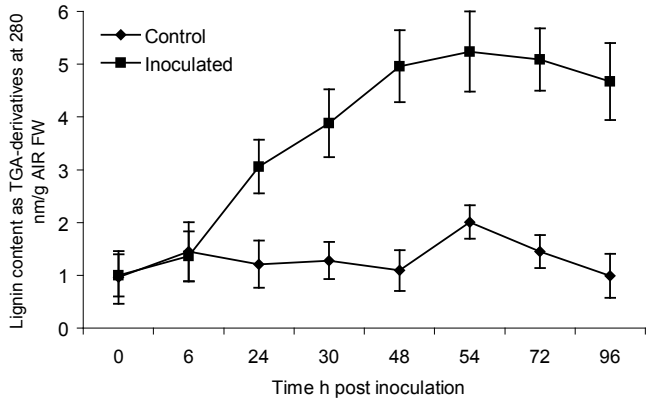

Fig. 10. Deposition of lignin (expressed as thioglycolic acid derivatives at 280 nm/g alcohol insoluble residue (AIR) FW) in the cell wall on a time course after inoculation of the eggplants with *Ralstonia solanacearum* as compared with the control. Data presented in graphs are the means ± SD of three replicates.

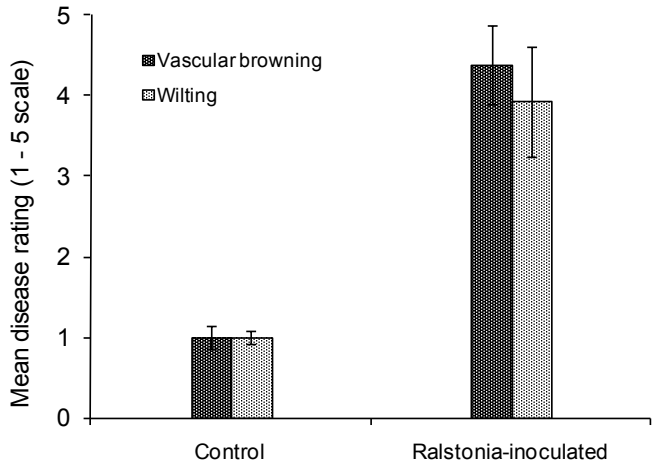

Fig. 11. Mean percentage vascular browning and wilting after inoculation of the eggplants with *Ralstonia solanacearum* as compared with the control. Data on vascular browning of stem and wilting of plants were recorded 3 weeks after challenge-inoculation of the eggplants with *Ralstonia solanacearum*. Columns represent the mean disease rating on a 1-5 scale as described in Materials and Methods section.

compatible nature of the host-parasite interaction, leading to development of disease in the brinjal plants.

The major ROS-scavenging enzymes of plants are SOD, GPX, APX and CAT; and these antioxidative enzymes protect the plants from the damage of oxidative stress. Infection of the strawberry leaves with *Mycosphaerella fragariae* resulted in increase in SOD activities, reached the highest level at the 2nd day post inoculation and then slowly declined afterward (Ehsani-Moghaddam *et al*., 2006). Significant increase in SOD activity in brinjal roots inoculated with *R. solanacearum* was found in consistency with published literature. The effective concentration of free radicals in plant tissues is a result of a dynamic equilibrium between the rates of their production and

scavenging. SOD and CAT were shown to be involved in regulation of H_2O_2 levels in plant tissues (Bolwell and Wojtaszek, 1997). In tobacco, the overexpression of CAT led to more disease sensitive plants (Polidoros *et al*., 2001). We observed a higher CAT activity at 30 hpi in inoculated brinjal tissues than in the control, then followed by decline. This lower level of activity of this enzyme might have rendered it less efficient in antioxidative activities. pH-dependent peroxidases in the cell wall can be a source of apoplastic H_2O_2 in the presence of a reductant released from responding cells (Wojtaszek, 1997). The expression of these enzymes was induced following recognition of bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Chittoor *et al*., 1997). In our study, GPX activity started increasing after 6 hpi and was higher through out the time course study. To protect cells under stress conditions and maintain the level of ROS, peroxidases regulate H_2O_2 levels (Blokhina *et al*., 2003). APX might be responsible for the fine modulation of ROS for signaling (Grant and Loake, 2000). Activity of APX increased significantly in brinjal roots inoculated with the bacterial pathogen.

The soluble phenolics appear to play an important role in apoplastic redox regulation in plant tissues (Baker *et al*., 2013). Phenol content has been reported to increase in plant tissues in response to pathogen infection. In this study, concentration of free phenolics increased in the tissues in consistent with increase of H_2O_2 level and antioxidant enzyme activities. Level of free phenols was reported to get elevated dramatically in a susceptible genotype of tomato in response to *Oidium neolycopersici* (Tománková *et al*., 2006). H_2O_2 has been shown to be involved in cell wall reinforcement by increasing protein cross-linking and incorporation of phenolics in the cell wall (lignification) which is an effective defense mechanism to both biotrophics and necrotrophics (Kang, 2008).

Disease progress was monitored after the inoculation of the brinjal plants with *R. solanacearum*. Development of symptoms (vascular browning and wilting) on the inoculated plants indicate that the interaction between brinjal and *R. solanacearum* was a compatible one. The oxidative burst triggered during the interaction between brinjal (line CHBL-54) and *R. solanacearum* may be an initial defense response of the host targeted against the invading bacterial pathogen. The antioxidative system was rather weak and rate of lipid peroxidation was higher in the present investigation. We conclude that the biochemical events favoured *R. solanacearum* rather than brinjal line CHBL-54, making this host-pathogen interaction a compatible combination. Our findings were in good correlation with recent reports. *Fusarium oxysporum-Arabidopsis* interaction induced ROS production which contributed to pathogen survival and disease development (Berrocal-Lobo and Molina, 2008). *R. solanacearum* Dps gene was demonstrated to contribute quantitatively to host plant colonization and bacterial wilt virulence. It was further demonstrated that *R. solanacearum* must overcome oxidative stress during the bacterial wilt disease cycle (Colburn-Clifford *et al*., 2010). Another study reported that the virulence of the *oxyR* mutant strain was significantly reduced in both tomato and tobacco host plants, demonstrating that *Ralstonia solanacearum* is exposed to inhibitory concentrations of ROS *in planta* and that OxyR mediated responses to ROS during plant pathogenesis are important for *R. solanacearum* host adaptation and virulence (Flores-Cruz and Allen, 2011).

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REFERENCES

Apel K., Hirt H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology* **55:** 373-399.

Baker C.J., Kovalskaya N.Y., Mock N.M., Deahl K.L., Whitaker B.D., Roberts D.R., Hammonda R.W., Aver'yanov A.A., 2013. Real-time monitoring of the extracellular redox potential of cell suspensions during plant/bacterial interactions. *Physiological and Molecular Plant Pathology* **82**: 20-27.

Bargabus R.L., Zidack N.K., Sherwood J.E., Jacobsen B.J., 2003. Oxidative burst elicited by *Bacillus mycoides* isolate Bac J, a biological control agent, occurs independently of hypersensitive cell death in sugar beet. *Molecular Plant-Microbe Interaction* **16**: 1145-1153.

Beauchamp C., Fridovich I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry* **44**: 276-287.

Berrocal-Lobo M., Molina A., 2008. *Arabidopsis* defense response against *Fusarium oxysporum*. *Trends in Plant Science* **13**: 145-150.

Blokhina O.B., Virolainen E., Fagerstedt K.V., 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany* **91**: 179-194.

Blomster T., Salojärvi J., Sipari N., Brosché M., Ahlfors R., Keinänen M., Overmyer K., Kangasjärvi J., 2011. Apoplastic reactive oxygen species transiently decrease auxin signaling and cause stress-induced morphogenic response in Arabidopsis. *Plant Physiology* **157**: 1866-1883.

Bolwell G.P., Wojtaszek P., 1997. Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. *Physiological and Molecular Plant Pathology* **55**: 347-366.

Bolwell G.P., Bindschedler L.V., Blee K.A., Butt V.S., Davis D.R., Gardner L.R., 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. *Journal of Experimental Botany* **53**: 1367-1376.

Bradford M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-54.

Bruce R.J., West C.A., 1989. Elicitation of casbene synthetase activity in castor bean: The role of pectic fragments of the plant cell wall in elicitation by a fungal endopolygalacturonase. *Plant Physiology* **69**: 1181-1188.

Cakmak I., Marschner H., 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves. *Plant Physiology* **98**: 1222-1227.

Champoiseau P.G., Jones J.B., Allen C., 2009. *Ralstonia solanacearum* race 3 biovar 2 causes tropical losses and temperate anxieties. Online. *Plant Health Progress* doi:10.1094/PHP-2009-0313-01-RV.

Chance B., Maehly M., 1955. In: Colowick S.P., Kaplan N.P. (eds). *Methods in Enzymology*, p 764. Academic Press, New York, NY, USA.

Chittoor J.M., Leach J.E., White F.F., 1997. Differential induction of a peroxidase gene family during infection of rice by *Xanthomonas oryzae* pv. *oryzae*. *Molecular Plant-Microbe Interaction* **10**: 861-871.

Clarke S.F., Guy P.L., Burritt D.J., Jameson P.E., 2002. Changes in the activities of antioxidant enzymes in response to virus infection and hormone treatment. *Physiologia Plantarum* **114**: 157-164.

Colburn-Clifford J.M., Scherf J.M., Allen A., 2010. *Ralstonia solanacearum* Dps contributes to oxidative stress tolerance and to colonization of and virulence on tomato plants. *Applied and Environment and Microbiology* **76**: 7392-7399.

Doke N., 1983. Generation of superoxide anion by potato tuber protoplasts during the hypersensitive response to hyphal wall components of *Phytopthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity. *Physiological Plant Pathology* **23**: 359-367.

Ehsani-Moghaddam B., Charles M.T., Carisse O., Khanizadeh S., 2006. Superoxide dismutase responses of strawberry cultivars to infection by *Mycosphaerella fragariae*. *Journal of Plant Physiology* **163**: 147-153.

Fegan M., Prior P., 2005. In: Allen C., Prior P., Hayward A.C. (eds). Bacterial wilt: The disease and the *Ralstonia solanacearum* species complex, pp. 449-462. APS Press, St. Paul, MN, USA.

Flores-Cruz F., Allen C., 2009. *Ralstonia solanacearum* encounters an oxidative environment during tomato infection. *Molecular Plant-Microbe Interaction* **22**: 773-782.

Flores-Cruz F., Allen C., 2011. Necessity of OxyR for the hydrogen peroxide stress response and full virulence in *Ralstonia solanacearum*. *Applied Environment and Microbiology* **77**: 6426-6432.

Foyer C.H., Noctor G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* **17**: 1866-1875.

Gechev T.S., Van Breusegem F., Stone J.M., Denev I., Laloi C., 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays* **28**: 1091-1101.

Göbel C., Feussner I., Rosahl S., 2003. Lipid peroxidation during the hypersensitive response in potato in the absence of β-lipoxygenases. *Journal of Biological Chemistry* **278**: 52834-52840.

Govrin E.M., Rachmilevitch S., Tiwari B.S., Solomon M., Levine A., 2006. An elicitor from *Botrytis cinerea* induces the hypersensitive response in *Arabidopsis thaliana* and other plants and promotes the gray mold disease. *Phytopathology* **96**: 299-307.

Grant J.J., Loake G.J., 2000. Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiology* **124**: 21-30.

Heath R.L., Packer L., 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* **125**: 189-198.

Ishikawa R., Shirouzu K., Nakashita H., Lee H.Y., Motoyama T., Yamaguchi I., Teraoka T., Arie T., 2005. Foliar spray of validamycin A or validoxylamine A controls tomato *Fusarium* wilt. *Phytopathology* **95**: 1209-1216.

Kang N.J., 2008. Inhibition of powdery mildew development and activation of antioxidant enzymes by induction of oxidative stress with foliar application of a mixture of riboflavin and methionine in cucumber. *Scientia Horticulturae* **118**: 181-188.

Kelman A., 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance in tetrazolium medium. *Phytopathology* **44**: 693-695.

Kiba A., Lee K.Y., Ohnishi K., Park P., Nakayashiki H., Tosa Y., Mayama S., Hikichi Y., 2009. Induction of reactive oxygen generation and functional changes in mitochondria and their involvement in the development of bacterial rot in lettuce caused by *Pseudomonas cichorii*. *Physiological and Molecular Plant Pathology* **74**: 45-54.

Kotchoni O.S., Kuhns C., Ditzer A., Kirch H.-H., Bartels D., 2006. Over-expression of different aldehyde dehydrogenase genes in *Arabidopsis thaliana* confers tolerance to abiotic stress and protects plants against lipid peroxidation and oxidative stress. *Plant Cell and Environment* **29**: 1033-1048.

Kovtun Y., Chiu W.-L., Tena G., Sheen J., 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proceedings of National Academy of Science USA* **97**:2940-2945.

Levine A., Tenhaken R., Dixon R., Lamb C., 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. *Cell* **79**: 583-593.

Malencic D., Kiprovski B., Popovic M., Prvulovic D., Miladinovic J., Djordjevic V., 2010. Changes in antioxidant systems in soybean as affected by *Sclerotinia sclerotiorum* (Lib.) de Bary. *Plant Physiology and Biochemistry* **48**: 903-908.

Mandal S., Mallick N., Mitra A., 2009a. Salicylic acid-induced resistance to *Fusarium oxysporum* f. sp. *lycopersici* in tomato. *Plant Physiology and Biochemistry* **47**: 642-649.

Mandal S., Mitra A., Mallick N., 2009b. Time course study on accumulation of cell wall-bound phenolics and activities of defense enzymes in tomato roots in relation to *Fusarium* wilt. *World Journal of Microbiology and Biotechnology* **25**: 795-802.

Mandal S., Das R.K., Mishra S., 2011. Differential occurrence of oxidative burst and antioxidative mechanism in compatible and incompatible interactions of tomato and *Ralstonia solanacearum*. *Plant Physiology and Biochemistry* **49**: 117-123.

Mittler R., Vanderauwera S., Suzuki N., Miller G., Tognetti V.B., Vandepoele K., Gollery M., Shulaev V., Van Breusegem F., 2011. ROS signaling: the new wave? *Trends in Plant Sciences* **16**: 300-309.

Montillet J.-L., Chamnongpol S., Rusterucci C., Dat J., Van de Cotte B., Agnel J.-P., Battisti C., Inze D., Van Breusegem F., Triantaphylides C., 2005. Fatty acid hydroperoxides and H_2O_2 in the execution of hypersensitive cell death in tobacco leaves. *Plant Physiology* **138**: 1516-1526.

Nakano Y., Asada K., 2001. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell and Physiology* **22**: 867-880.

Nuhse T.S., Bottrill A.R., Jones A.M., Peck S.C., 2007. Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. *The Plant Journal* **51**: 931-940.

Petrov V.D., Van Breusegem F., 2012. Hydrogen peroxide - a central hub for information flow in plant cells. *AoB PLANTS*, pls014; doi:10.1093/aobpla/pls014.

Polidoros A.N., Mylona P.V., Scandalios J.P., 2001. Transgenic tobacco plants expressing the maize Cat2 gene have altered catalase levels that affect plant-pathogen interactions and resistance to oxidative stress. *Transgenic Research* **10**: 555-569.

Schouten A., Tenberge K.B., Vermeer J., Stewart J., Wagemakers L., Williamson B., van Kan J.A.L., 2002. Functional analysis of an extracellular catalase of *Botrytis cinerea*. *Molecular Plant Pathology* **3**: 227-238.

Tiedemann A., 1997. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. *Physiological and Molecular Plant Pathology* **50**: 151-166.

Tománková K., Luhová L., Petřivalský M., Peč P., Lebeda A., 2006. Biochemical aspects of reactive oxygen species formation in the interaction between *Lycopersicon* spp. and *Oidium neolycopersici*. *Physiological and Molecular Plant Pathology* **68**: 22-32.

Torres M.A., Jones J.D.G., Dangl J.L., 2006. Reactive oxygen species signaling in response to pathogens. *Plant Physiology* **141**: 373-378.

Veliková V., 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Science* **151**: 59-66.

Vellosillo T., Vicente J., Kulasekaran S., Hamberg M., Castresana C., 2010. Emerging complexity in reactive oxygen species production and signaling during the response of plants to pathogens. *Plant Physiology* **154**: 444-448.

Wojtaszek P., 1997. Oxidative burst: an early response to pathogen infection. *Biochemistry Journal* **322**: 681-692.

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