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RESEARCH ARTICLE

PRODUCTION OF HYDROGEN PEROXIDE AND PHYSIOLOGICAL CHANGES IN TOMATO AFTER INFECTION OF *Alternaria alternata*

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ABSTRACT

Plant defense mechanisms against necrotrophic pathogens such as *Alternaria alternata* are considered to be complex and different from those that are effective against biotrophs. Tomato is an economically important vegetable crop affected severely by *A. alternata* that causes leaf spot, stem canker and fruit rot diseases. In the present work physiological parameters were found to be decreased in tomato plants after infection by the pathogen. The infection also leads to production of reactive oxygen species which are involved in antimicrobial activities for restricting pathogen's ingression in plants. Hydrogen peroxide production was observed by 3, 3'-diaminobenzidine staining, which showed reddish-brown color precipitation in leaves. Estimation of hydrogen peroxide showed highest level of production of H_2O_2 after 48 hours after infection and then declined after 48 hour up to 96 hours. Following H_2O_2 production activities of different antioxidant enzymes namely ascorbate peroxidase, catalase and glutathione s-transferase level were checked which were found to be altered due to ROS production.

INTRODUCTION

Several microorganisms affect plants in wide range environmental conditions. Among them fungi have greater role to play. *Alternaria alternata* (Fr.) Keissler has several pathogenic variants, most of them producing a unique host specific toxins (HST) and causing disease in different host plants (Nishimura, 1983; Hatta, 2002; Ito, 2004; Kohmoto, 1991; Otani, 1996). Necrotrophs kill their host cells by secreting toxic compounds or lytic enzymes and, in addition, produce an array of pathogenicity factors that can subdue host defenses. HSTs produced by *Alternaria* pathotypes are chemically diverse, ranging from low-molecular-weight compounds to cyclic peptides. *A. alternata* causes major loss to tomato production by producing leaf spot, stem canker and fruit rot diseases on tomato plants. Tomato is one of the most important vegetable crop, which has been identified as a food of great interest and is known as a powerhouse of nutrition. It contains a multitude of vitamins and minerals that act to support health. Its availability is round the year. When pathogen attacks plants, the first response is the production of reactive oxygen species (ROS) which is also known as oxidative burst (including O_2, H_2O_2) that is caused by activation of a membrane bound NADPH oxidase. Different roles of ROS in plants during infection are known as direct antimicrobial agents, defense genes activator (Doke, 1996; Lamb, 1997; Low, 1996), agents for cross linking proteins to restrict pathogen infections (Brisson, 1994) and producers of the hypersensitive response (HR), cell death, salicylic acid production, and systemic acquired resistance (SAR) (Doke, 1996; Lamb, 1997; Low, 1996). H_2O_2 is emerging as a key signaling molecule generated by plants in response to both biotic and abiotic stresses such as drought, UV-radiation, ozone, high and low temperatures, and pathogen attack (Prasad *et al*., 1994; Bartosz, 1997; Foyer *et al*., 1997; Dat *et al*., 2000). In green tissues, H_2O_2 is produced at high flux rate in chloroplast (Asada, 1999) and in mitochondria (Moller, 2001) via electron transport. In response to ROS, plants develop an efficient antioxidative protection system against them. Antioxidants play a crucial role in plant defense mechanism against reactive oxygen species (Hassan *et al*., 2013). Several enzymes are present for removal of dangerous ROS within the plant cells. Halliwell-Asada pathway is comprised of such enzymes (Noctor and Foyer, 1998). Enzymes ascorbate peroxidase (APX), catalase (CAT), glutathione S-transferase (GST) are the main enzymes in this pathway, which results in detoxification of H_2O_2 using reducing power derived from NADPH. APX scavenge ROS and detoxify lipid peroxidation products (Baker and Orlandi, 1995). GST is involved in detoxification and solubilization of toxic lipid oxidation by products for their removal from the cell and it is very rapidly induced during oxidative stress response (Edwards *et al*., 2000). Catalase is also an important ROS detoxification enzyme which directly converts H_2O_2 into water (Chamnongpol *et* al., 1996; Willekens *et al*., 1997). Pathogen infection also alters other different parameters as chlorophyll content, which plays a major role in plant growth and development, to know the effect of *A. alternata* on growing tomato plants and what are the primary reactions occurring in plants due to infection of pathogen, the present study has been done. The present work shows the production of hydrogen peroxide in tomato plants after infection of *A. alternata* and its estimation at a different time interval. Physiological changes have also been observed during infection.

MATERIALS AND METHODS

Isolation of the Pathogen

Alternaria was isolated from infected tomato plants. Plant parts were cut with razor blade and immersed in 1% sodium hypochlorite followed by 70% ethanol for 1 mm and finally rinsing with sterile

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distilled water. They were transferred to Potato Dextrose Agar (PDA) medium and incubated (25˚-27˚C) for 4-5 days. After proper growth of culture in Petri-plates 1mm diameter agar plug is cut and transferred to Potato Dextrose Broth (PDB) for mycelia production. *Alternaria alternata* was identified by morphological features and the spores examined by light microscope which was confirmed with the help of standard manual (Simmons, 2007).

Pathogenecity Test

Tomato (*Lycopersicon esculentum* cv. Punjab chhuhara) plants were grown in greenhouse conditions under 14 hour light and 10 hour dark cycle at 27°C and 22° C, respectively. Autoclaved soil was used and the plants were allowed to grow and attain a height of 15-20 cm before testing for sensitivity to the pathogens and for other experiments. Pathogenicity test was carried out by inoculating 15-20 cm long plants with spore suspension of *Alternaria alternata* containing 4×10^5 spores /ml followed by 48 hours of incubation in moist chamber after that the plants were kept back to the green house bench. Again isolation of the pathogen was done from this manually infected tomato plants. Spores and culture morphology were compared with the isolates from naturally infected plants.

Determination of Chlorophyll Content

Chlorophyll content was determined as described by Arnon (1949). Samples (1gm of fresh leaves) were ground in 20 ml of 80% acetone and the supernatant was transferred to 100 ml volumetric flask after centrifugation. It was repeated until the residue became colorless (Gomathi and Rakkiyapan, 2011). The absorbance of extracts was measured at 663 and 647 nm using the UV spectrophotometer. The amount of chlorophyll (chlorophyll a, chlorophyll b and total chlorophyll) was thus recorded.

Estimation of Hydrogen Peroxide Accumulation

The in vivo detection of H_2O_2 during tomato-*Alternaria* interaction was done by 3, 3-diaminobenzidine (DAB) staining according to Thordal-Christensen *et al*. (1997). Three hours before each sampling time point, *A. alternata* infected tomato leaves were cut from 8-day old plants. The cut ends of the leaves were immersed in a solution containing 1 mg mL^{-1} DAB dissolved in water and the pH was maintained to 3.8 by adding HCl to solubilize the DAB. The leaves were then incubated in the growth chamber for an additional 8-h period to allow proper DAB uptake and reaction with H_2O_2 and peroxidase. At specific time points after inoculation, the DAB reactions were examined on three replicate leaves of the control and treated plants. To allow resolution of fungal structures and host cell responses, the leaves were fixed and cleared. H_2O_2 was visualized as a reddish-brown coloration in DAB-treated leaves while viewing under Dewinter microscope. The NADPH-dependent formation of H_2O_2 was quantified as described by Thurman *et al*. (1972) with some Modifications. Pathogen treated leaves of tomato were ground adding 5% trichloroacetic acid (TCA). After removal of precipitated protein by centrifugation, 0.4 ml of 10 mM ferrous ammonium sulfate followed by 0.2 ml of 2.5 M potassium thiocyanate were added to a 1.6 ml aliquot of the supernatant. The absorption of the red ferrithiocyanate complex formed in the presence of peroxides was measured at 480 nm with the help of Hitachi U-2900 spectrophotometer. Hydrogen peroxide standards were prepared from 500 µM stock solution and observed in a range of 1-100 µM. Standard graph was plotted taking absorbance of each known concentration of the samples at 480nm at Y-axis and 1-100 µM H_2O_2 concentration at X-axis.

Protein Isolation and Estimation

Protein was isolated by Tris-Buffer method in which 1 gram of tissue was homogenized in 0.1M Tris-Buffer (pH- 6.8), crushed material was collected in fresh centrifuge tube and harvested at 12000 rpm at 4˚C for 20 minutes. The supernatant was collected in another fresh tube and again centrifuged at 14000 rpm at 4˚C for 20 minutes. The supernatant was stored at -20˚C. Protein was estimated according Bradford method (Bradford, 1976). The protein content was calculated with reference to standard graph obtained by Bradford method.

Assay of Antioxidant Enzymes

For determination of catalase, 0.5 gm of plant tissue was homogenized in phosphate buffer (0.067 M) and centrifuged at 15000g for 15 min. The supernatant was used for extraction of the enzymes. Assay of CAT was performed by taking H_2O_2-phosphate buffer (2 mM) in cuvette, adding enzyme extract and after mixing properly, the time required for a decrease in absorbance by 0.05 units was recorded at 240 nm with the spectrophotometer (Luck *et al*., 1974; Ramkumar *et al*., 2012). APX activity was determined by adding enzyme extract in reaction mixture that contained 50 mM potassium phosphate (pH 7), 0.1 mM H_2O_2, 0.5 mM ascorbic acid and 0.1 mM EDTA (Li *et al*., 2008). The reaction was started by addition of hydrogen peroxide and oxidation rate of ascorbic acid was estimated by monitoring the decrease in absorbance at 290nm with an absorption coefficient of 2.8 $mM^{-1}cm^{-1}$(Nakano and Asada, 1981). For extraction of GST (Hamda *et al*., 2012), 0.5 g of plant tissues was homogenized in phosphate buffer (0.1M, pH 6.5) and the homogenates were centrifuged at 5000 rpm for 10 minutes and the supernatants were used for the assay. The activity of the enzyme was determined by observing the change in absorbance at 340 nm. The reaction mixture contained 0.1 ml of GSH, 0.1ml of 1-chloro, 2, 4-dinitrobenzene (CDNB) and phosphate buffer in a total volume of 2.9 ml. The reaction was initiated by the addition of 0.1ml of the enzyme extract. The readings were recorded after every 15 seconds at 340nm against distilled water as blank for a minimum of three minutes in the spectrophotometer (Habig *et al*., 1974). The assay mixture without the extract served as control to monitor non specific binding of the substrates. GST activity was calculated using the extinction co-efficient of the product formed (9.6 $mM^{-1}cm^{-1}$) that was expressed as nmoles of CDNB conjugated per minute.

Statistical analysis

Each experiment was repeated two times with three replicates each and the data presented are mean values of independent experiments.

RESULTS AND DISCUSSION

Isolation of the Pathogen and Pathogenecity Tests

Isolates of *Alternar*ia growing on PDA plates were identified by colony characters having grayish black colonies and cottony texture. The conidiophores were observed under microscope as brown straight, bearing light brown conidia with short beak at the tip. The conidia were produced in chains and showed 3-8 transverse and longitudinal septa. Upon infection with the isolated *A. alternata*, the tomato plants when raised in green house showed symptoms of necrotic lesions and typical black/brown concentric rings after 7-10 days. When the reisolated pathogen showed similar morphology, colony characteristics and pathogenecity as the previous isolates from natural conditions. The pathogenicity test confirmed the isolated pathogen as *Alternaria alternata* f. sp. *lycopersici* which was also compared with already isolated pathogen in the laboratory (Prasad and Upadhyay, 2010).

Effect of Pathogen on Chlorophyll content of Tomato

Physiological parameters in terms of chlorophyll content were checked to see the effect of pathogen infection. Plant biomass was harvested at 24, 48, 72 and 96 hours after treatment (HAT). Fresh tissue samples were collected at pre treatment and at each post treatment for chlorophyll analysis. Chlorophyll content level was found to decrease in *A. alternata* treated tomato plants (Fig. 1). The

percent inhibition due to pathogen infection on plants was found as 8.15% for chlorophyll a, 9.6% for chlorophyll b and 25.1% for total chlorophyll content as compared with the control.

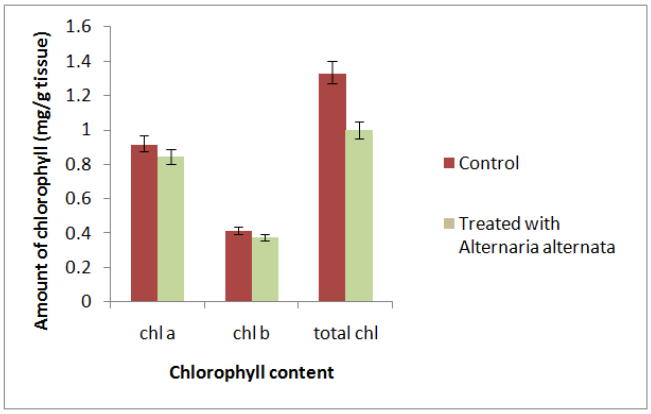

Fig. 1. Effect of the pathogen treatment on chlorophyll content of tomato plants

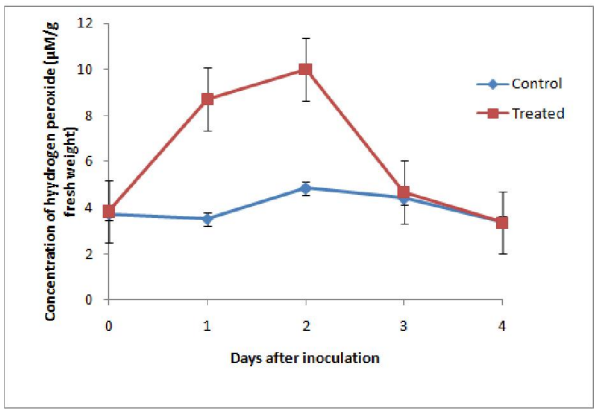

Fig.3. Hydrogen peroxide level in untreated control plants and *Alternaria alternata* treated tomato plants

autofluorescent compounds in attacked epidermal cells. Similar phenomenon was seen in our experiment, the rate of H_2O_2 production was started after pathogen infection. The amount which was

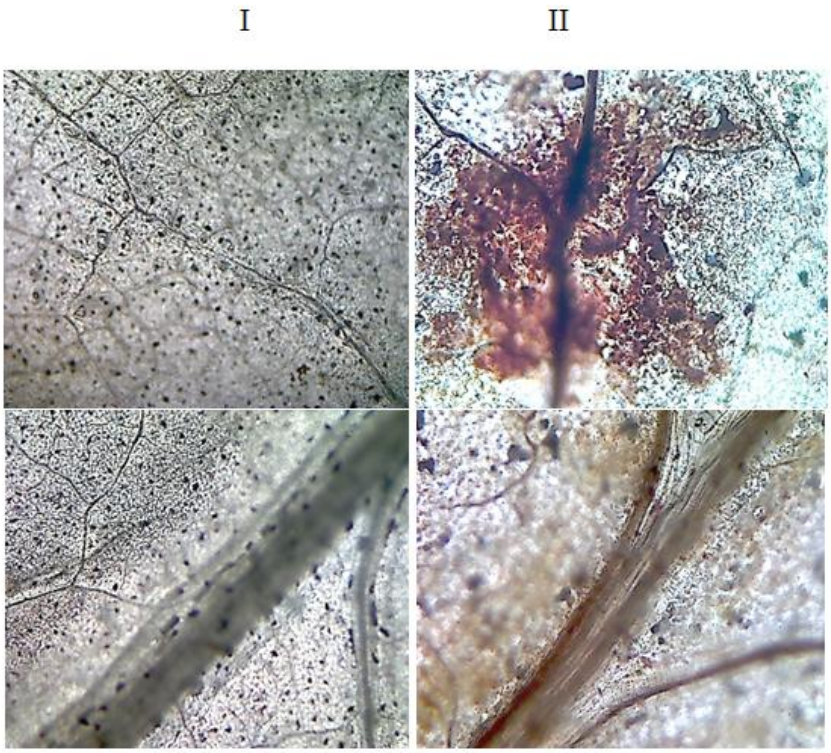

Fig.3. DAB staining pictures at 4X and 10X resolution (A) Control Plant leaves (B) *Alternaria alternata* treated leaves.

H_2O_2 Accumulation by DAB and its Estimation

The presence of H_2O_2 was detected in dead cells using DAB as a substrate. H_2O_2 revealed as reddish brown precipitate of polymerized DAB which was localized in the wall of epidermis after infection with pathogen. DAB polymerizes locally as soon as it comes in contact with H_2O_2 in the presence of peroxidase and produce a reddish-brown polymer (Fig. 2). DAB was taken up by living plant tissue and can be used to show H_2O_2 production when peroxidase activity is present. These precipitates were not observed in untreated (control) plant leaves when observed under microscope. Pathogen treated plants produced an oxidative burst releasing H_2O_2. Rapid production of oxidants is an early characteristic feature of the HR response (Kiraly *et al.*, 2008). H_2O_2 was convinced to be one of the effective oxidants involved in defense response to tomato diseases. Vanacker *et al*. (2000) showed that early in the HR response, cells other than the attacked cell respond by transient H_2O_2 production. This response precedes H_2O_2 accumulation and subsequent accumulation of significant after 24 hours and highest concentration of 10 µM/g fresh weight was seen after 48 hours after treatment followed by a gradual decrease by 4.2 and 3.8 µM/g fresh weight after 72 and 96 hours (Fig. 3). Comparatively, in untreated control plants there was no significant increase in concentration of H_2O_2. So it was confirmed from the data that the pathogen infection was responsible for production of ROS, an oxidative burst resulting a gradual rise followed by a fall in concentration of H_2O_2 depending on days post infection (dpi).

Protein Estimation and Antioxidant Enzyme Activity

Protein was isolated and quantified according to Bradford method using Bovine Serum Albumin (BSA) as a standard. ROS production in tomato plants due to pathogen infection altered the antioxidant enzyme activities (such as APX, CAT and GST activity) after a certain time interval. CAT, APX and GST are important antioxidant enzymes in plants. The APX activities in tomato plant treated with

A. alternata increased significantly that came to a maximum level with 0.14 µM/min/mg tissue at 48 hour after pathogen inoculation as compared to the control. APX activity decreased to 0.04 µM/min/mg at 96 hour, and then remained similar after 96 hour after inoculation (Fig. 4). The activity of catalase, in tomato treated with *A. alternata* increased suddenly and reached maximum after 24 hours with 8.5 nm/min/mg protein thereafter it gradually declined when checked after every 24 hours interval (Fig. 5). This trend showed that the enzyme was active at 24 hours after treatment with the pathogen as the activity

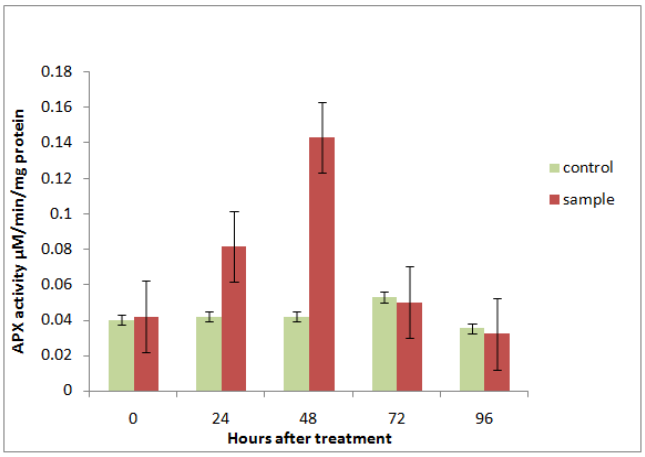

Fig.4. Ascorbate peroxidase activity in tomato plant after infection with *Alternaria alternata* at different time interval

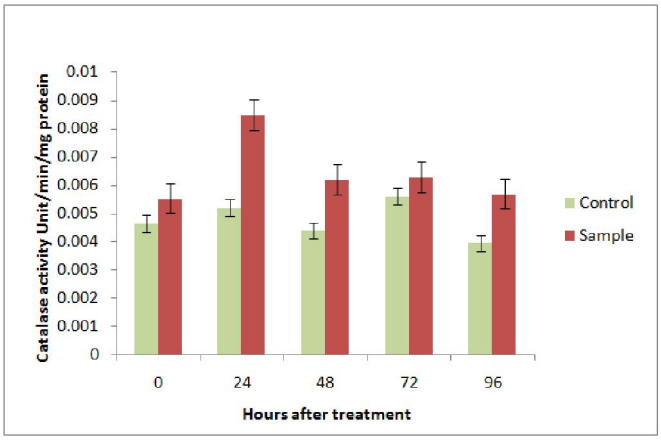

Fig.5. Catalase activity in tomato plant after infection of *Alternaria alternata* at different time interval

was in higher proportion at which it converts H_2O_2 into water. Our study indicates that APX and CAT enzymes showed same phenomenon. They first increased up to a certain time and then decreased gradually after a regular interval of time. In contrast to these enzymes, GST showed a different phenomenon where it gradual decreased after pathogen infection in tomato plants showing that it was started declining after 24 hours and further decreases up to 96 hours was recorded that reached to a minimum level of 4nm/min/mg protein (Fig. 6) role of GST was known. It is located in cytosol, detoxifies lipid hydroperoxides generated by active oxygen species (Berhane *et al*., 1994), and induced GST induction in response to low doses of H_2O_2 providing cellular protectants to block oxidant-mediated programmed cell death in cells surrounding the oxidative burst (Levine *et al*., 1994; Tenhaken *et al*., 1995).

Conclusion

We conclude that *A. alternata* is responsible for cell death by reactive oxygen species production in tomato plants resulting in a rise in the level of hydrogen peroxide and by altering the level of different

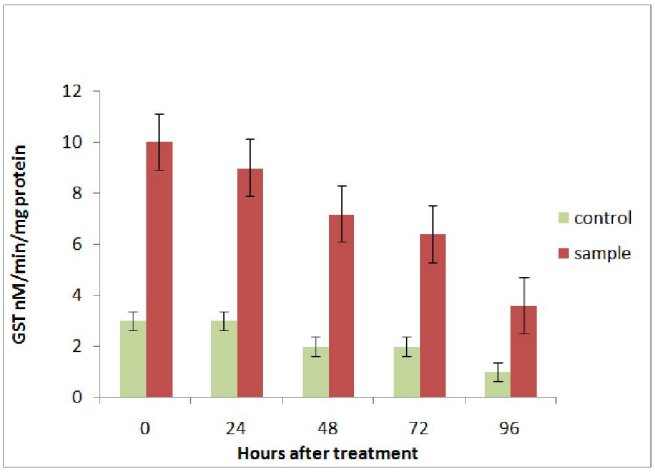

Fig.6. Glutathione S-transferase activity in tomato plant after infection of *Alternaria alternata* at different time interval

antioxidant enzymes (as APX, CAT and GST) related to reactive oxygen species production. *A. alternata* also minimizes chlorophyll content of tomato plants as compared to untreated control plants.

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REFERENCES

Arnon, D.I. 1949. Copper enzymes in isolated chloroplasts, polyphenoxidase in beta vulgaris. Plant Physiol. 24: 1-15.

Asada, K. 1999. The water-water cycle in chloroplast: Scavenging of active oxygens and dissipation of excess photons. Ann. Rev. Plant Physiol. Plant Mol. Biol. 50: 601–639.

Baker, C.J. and Orlandi, E.W. 1995. Active oxygen in plant pathogenesis. Ann. Rev. Phytopathol. 33: 299-321.

Bartosz, G. 1997. Oxidative stress in plants. Acta Physiologia. Plantarum. 19: 47–64.

Berhane, K., Widersten, M., Engström, A., Kozarich, J.W. and Mannervik, B. 1994. Detoxification of base propenals and other ß-unsaturated aldehyde products of radical reactions and lipid peroxidation by human glutathione transferases. Proc. Natl. Acad. Sci. 91: 1480-1484.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 7(72): 248-54.

Brisson, L.F., Tenhaken, R. and Lamb, C. 1994. Function of Oxidative Cross-Linking of Cell Wall Structural Proteins in Plant Disease Resistance. Plant Cell. 6**:** 1703–1712.

Chamnongpol, S., Willekins, H., Langebartels, C., Monatagu, M.V., Inze, D. and Camp, W.V. 1996. Transgenic tobacco with a reduced catalase activity develops necrotic lesions and induces pathogenesis-related expression under high light. The Plant J. 10(3): 491-503.

Dat, J., Vandenbeele, S., Vranova, E., Van Montagu, M., Inze, D. and Van Breusegem, F. 2000. Dual action of the active oxygen species during plant stress responses. Cellular Mol. Life Sci. 57: 779–795.

Doke, N., Miura, Y., Sanchez, L.M., Park, H.J., Noritake, T., Yoshioka, H. and Kawakita, K. 1996. The oxidative burst protects plants against pathogen attack: mechanism and role as an emergency signal for plant bio-defence-a review**.** Gene. 179: 45–51.

Edwards, R., Dixon, D. and Walbot, B. 2000. Plant glutathione Stransferases: enzymes with multiple functions in sickness and in health. Trends in Plant Sci. 5: 193–198.

Foyer, C.H., Lopez-Delgado, H., Dat, J.F. and Scott, I.M. 1997. Hydrogen peroxide and glutathione-associated mechanisms of acclimatory stress tolerance and signalling.Physiologia Plantarum. 100**:** 241–254.

Gomathi, R. and Rakkiyapan, P. 2011. Comparative lipid peroxidation, leaf membrane thermostability, and antioxidant system in four sugarcane genotypes differing in salt tolerance. Int. J. Plant Physiol. Biochem. 3(4): 67-74.

Habig, W.H., Pabst, M.J and Jakoby, W.B. 1974. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249: 7130-7139.

Hamda, A., Al-Dhaheri. and Al-Deeb, M.A. 2012. Mortality and GST Enzyme Response of Saw-toothed grain beetles, *Oryzaephilus surinamensis* (Coleoptera-Silvanidae) Exposed to low insecticide concentrations. J. Entomol. 9(6): 396-402.

Hassan, N.M., Abu-Doubara, M.I., Waly, M.A., Nemat Alla, M.M. 2013. Efficacy of a pyrimidine derivative to control spot disease on Solanum melongena caused by *Alternaria alternata*. J. Adv. Res. 4: 393–401.

Hatta, R., Ito, K., Hosaki, Y. 2002. A conditionally dispensable chromosome controls host-specific pathogenicity in the fungal plant pathogen *Alternaria alternata*. Genet. 161: 59–70.

Ito, K., Tanaka, T., Hatta, R., Yamamoto, M., Akimitsu, K and Tsuge, T. 2004. Dissection of the host range of the fungal plant pathogen *Alternaria alternata* by modification of secondary metabolism. Mol. Microbiol. 52(2): 399–411.

Kiraly, L., Hafez, Y.M, Fodor, J. and Kiraly, Z. 2008. Suppression of tobacco mosaic virus-induced hypersensitive-type necrotization in tobacco at high temperature is associated with down regulation of NADPH oxidase and superoxide and stimulation of dehydroascorbate reductase. J. Gen. Virol. 89: 799-808.

Kohmoto, K., Akimitsu, K. and Otani, H. 1991. Correlation of resistance and susceptibility of citrus to *Alternaria alternata* with sensitivity to host-specific toxins. Phytopathol.81:719–722.

Lamb, C. and Dixon, R.A. 1997. The Oxidative Burst in Plant Disease Resistance. Annu. Rev. Plant Physiol. Mol. Biol. 48: 251–275**.**

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. 1994. H_2O_2 from the oxidative burst

Li, Shi-Mo., Hua, Gou-Gen., Liu, Hong-Xia., Guo, Jian-Hua. 2008. Analysis of defence enzymes induced by antagonistic bacterium *Bacillus subtilis* strain AR12 towards *Ralstonia solanacearum* in tomato. Annals of Microbiol. 58 (4): 573-578.

Low, P.S. and Merida, J.R. 1996. The oxidative burst in plant defense: Function and signal transduction. Physiologia Plantarum. 96: 533-542.

Luck, H. 1974. Estimation of catalase. In: Methods in Enzymatic Analysis. (Eds). Bergmeyer Academic Press. 885.

Moller, I.M. 2001. Plant Mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive species. Ann. Rev. Plant Physiol. Plant Mol. Biol. 52: 561–91.

Nakano, Y. and Asada, K. 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: 867-880.

Nishimura, S. and Kohmoto, K. 1983. Host-specific toxins and chemical structures from *Alternaria* species. Ann. Rev. Phytopathol. 21: 87–116**.**

Noctor, G., Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Bio. 49: 249-279. Orchestrates the plant hypersensitive disease resistance response. Cell. 79: 583-593.

Otani, H., Kohmoto, K. and Kodama, M. 1996. Alternaria toxins and their effects on host plants. Canadian Journal of Botany.73: 453–458. oxidative burst in hypersensitive disease resistance. Proc. Natl. Acad. Sci. 92: 4158-4163.

Prasad, T.K., Anderson, M.D., Martin, B.A. and Stewart, C.R. 1994. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. The Plant Cell. 6: 65–74.

Prasad, V. and Upadhyay, R.S. 2010. *Alternaria Alternata* f.sp. *Lycopersici* and its Toxin Trigger Production of H_2O_2 and Ethylene in Tomato. J. Plant Pathol. 92 (1): 103-108.

Ramkumar, L., Ramanathan, T., and Johnprabagaran, J. 2012. Evaluation of nutrients, trace metals and antioxidant activity in *Volvariella volvacea* (Bull. Ex. Fr.) Sing. Emir. J. Food Agric. 24 (2): 113-119.

Simmons, E.G. 2007. Alternaria An Identification Manual, CBS Biodiversity Series No. 6, CBS Fungal Biodiversity Centre, Utrecht, the Netherlands. 775.

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C. 1995. Function of the

Thordal-Christensen, H., Zhang, Z., Wei, Y, Collinge, D.B. 1997. Subcellular localization of H_2O_2 in plants, H_2O_2 accumulation in papillae and hypersensitive response during barley-powdery mildew interaction. Plant J. 11:1187–1194.

Thurman, R.G., Ley, H.G., Scholz, R. 1972. Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. Eur. J. Biochem. 25: 420-430.

Vanacker, H., Carver, T.L.W. and Foyer, C.H. 2000. Early H_2O_2 Accumulation in Mesophyll Cells Leads to Induction of Glutathione during the Hyper-Sensitive Response in the Barley-Powdery Mildew Interaction. Plant Physiol. 123: 1289–1300.

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Montagu, M. V., Inze, D. and Camp, W.V. 1997. The EMBO J. 16: 4806-4816.

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