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Antioxidant activity and lipid peroxidation in response to citrus canker bacterial infection

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ABSTRACT: Asiatic citrus canker caused by Xannthomonas citr subsp. citri (Xcc) is endemic in many citrus-growing areas damaging citrus industry. It is expected that citrus plants respond to bacterial infection through generation of more reactive oxygen species, which is an indication of stress. In order to evaluate the stress level caused via Xcc, antioxidant activity, chlorophylls content and lipid peroxidation were measured in lime (Citrus aurantifollia) at 1, 4, and 7 days post inoculation. Two Xcc strains, namely LMG-9322 and NIGEB-88 (10^8 cfu/ml), were used to inoculate one-year old limes and the results were compared with mock inoculated plants with distilled water. Foliar and apoplastic catalase, apoplastic peroxidase and ascorbate peroxidase activities were increased, while foliar ascorbate peroxidase showed a decline in its activity. Treated plants responded indifferently to two bacterial strains. It seems that the different anti-oxidative capacity accompanied by the pathogen-induced response contributes to a better protection of citrus plant against bacterial pathogen stress

***Keywords*:** Antioxidant, Citrus, Lipid Peroxidation, Reactive Oxygen Species, Xanthomonas

Abbreviation: APX-Ascorbate Peroxidase, AWF- Apoplastic Washing Fluid, CAT-Catalase, MDA-Malondialdehyde, PX- Peroxidase, ROS-Reactive Oxygen Species

INTRODUCTION

Bacterial phytopathogens do have adverse effects on plant growth and yield and therefore influencing the economy. Xanthomonas, a Gram-negative bacterial genus, causes serious diseases in about 400 plant species (Robert et al., 2011). One of which is citrus canker caused by Xanthomonas citri subsp. citri. The subspecies appear as a few pathotypes that Type A, also known as Asiatic citrus canker, has shown to be the most widespread and sever pathotype. The pathogenicity of Xcc starts at plant stomata and wounded plant parts, followed by bacterial colonization within apoplastic milieu, causing epidermis break due to cell hyperplasia (Burnings and Gabriel, 2003; Schaad et al., 2006). Oxidative burst on plant side is the common response to bacterial pathogens due to the production of reactive oxygen species (ROS) (Gayoso et al., 2004). Although ROS appears to be useful in signaling cascades and plant reinforcement (Peng and Kuc, 1992; Otte and Orlandi, 1993; Baker and Orlandi, 1995; Foyer et al., 1997), it may damage the plant tissues by affecting the cellular biomolecules (Mandal et al., 2008). Therefore, an enzymatic balance needs to be put in place to control producing and

scavenging of ROS molecules. Multiple antioxidant enzymes such as Superoxide dismutase (SOD, EC 1.15.1.1) react with the superoxide radical to produce H2O2. Hydrogen peroxide is scavenged by catalases (CAT, EC 1.11.1.6) and peroxidases (POX, EC 1.11.1.7). Among peroxidases, ascorbate peroxidases (APX, EC 1.11.1.11) and glutathione peroxidase (GPX, EC 1.11.1.9) which uses ascorbate and glutathione as electron donors, respectively, are well known for their role in H2O2 detoxification in plant (Apel and Hirt, 2004). Change in antioxidant enzyme activity by xanthomonas infection was observed in tomato (Chandrashekar and umesh, 2012), kumquat citrus (kumar et al., 2011) and sweet orange (kumar et al., 2011).

Towards better comprehension of citrus-canker interaction, foliar and apoplastic protein samples from inoculated Citrus auarantifolia were collected in different timepoints and enzymes that are involved in ROS scavenging phenomenon were assayed.

MATERIALS AND METHODS

2.1. Plant material and treatment

Citrus plant (*Citrus aurantifolia*) were provided from southern region of Iran. Tow strain of xanthomonas bacteria NIGEB-88(A*) and LMG-9322(A) were used to inoculation of plant. Distilled water used for inoculation of control plant. Control and Infected plant 1,4and 7days interval after inoculation sampled and kept in -80 freezer for further analyses.

2.2. Extraction of Enzyme

Soluble apoplastic enzyme were extracted using vacuum infiltration by a method that described by (polle et al., 1990). leaves were washed three times with distilled water, placed in dishes containing 200 mL of infiltration solution consisting of either 50 mMMes/KOH buffer (pH 6.0), 40 mM KCl, and 2 mM CaCl2. The infiltration dishes were placed in a vacuum desiccator and the leaves were infiltrated for 15min at70 kPa. They were then blotted and place in falcon tube .AWF was recovered by centrifugation at 1500g for 15 min. for extraction whole leaves enzyme 0.5 g leaves were ground in liquid nitrogen and mortar by the same buffer.

2.3. Determination of enzyme activity

2.3.1. Catalase activity (CAT)

Catalase activity was assayed by measuring disappearing of H2o2 at 240nm (*Aebi, 1984*).Reaction mixture consist 50mM phosphate buffer,10mMH2o2 and 100µ of enzyme extract. The decomposition of H2O2 was determined at 240nm by extinction coefficient 39.4mMcm^{-1}. Enzyme activity was expressed as µgH2o2 min^{-1}mg^{-1}protein.

2.3.2. Peroxidase activity (PX)

Peroxidase activity was assayed in a reaction mixture containing 50mM potassium phosphate buffer (pH 7.0), 10mM H2O2, and 0.05% guaiacol and 100µ enzyme. The activity was determined by the increase in absorption at 470nm due to guaiacol oxidation by extinction coefficient 26.6mMcm^{-1}.Enzyme activity was expressed as µmol guaiacol min^{-1}mg^{-1}protein (chance and maehly, 1955).

2.3.3. Ascorbate peroxidase activity (APX)

APOD activity was assayed in a reaction mixture containing 50mMpotassiumphosphate buffer (pH7.0), 0.1mM EDTA, 1.0mM H2O2, 0.25mM ascorbic acid and enzyme. The activity was determined by the rate of ascorbate oxidation at 290nm by extinction coefficient 2.8mMcm^{-1}.Enzyme activity was expressed µmol ascorbate min^{-1}mg^{-1}protein (Nakano and Asada, 1981).

2.4. Oxidative damage

Lipid peroxidation was measured in terms of MDA content as an oxidative damage. Leaves were first homogenized and then extracted in 0.1% TCA in ratio 1:5 (w/v) and centrifuged at 12,000g for 30 min at 4C. One ml of supernatant was incubated with 4 ml of 20% TCA containing 0.5% thiobarbituric acid for 30 min at 95C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10,000gfor 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-1and pressed as µmol g^{1}FW (Heath and parker,1968).

RESULTS AND DISCUSSION

Result

3.1. Lipid peroxidation

To determine of cell damage caused by oxidative stress related to the plant response to pathogen infection, we estimated the product of membrane lipid peroxidation. Lipid peroxidation measured in terms of malondialdehyde (MDA) content, which result showed an increase in MDA content in all infected treatment compare with non-infected treatment, also we observed an large increase of MDA content after 4day inoculation in both two strain of bacteria infected leaves (fig1).it is necessary to mention that there is no significant effect between to strain of bacteria in all treatment (table1).

3.2. Enzyme activity

3.2.1. Determination of catalase activity

Both whole-leaf and apoplastic extracts had had similar increasing catalase activity (fig.2, fig.3). Also result showed that there is no significant difference between two strains of bacteria compare with control but time of infection had significant effect on foliar and apoplastic enzyme activity.

3.2.2. Determination of peroxidase activity

Result showed that peroxidase activity was affected by bacteria infection. We observed significant difference between non-infected and infected leaves enzyme activity (fig.4, fig.5). In apoplastic extraction, enzyme activity an increase observed after 7 day after inoculation in both two strain bacteria.

3.2.3. Determination of ascorbate peroxidase activity

Inoculation of bacteria affected both foliar and apoplastic enzyme activity (table.1). Generally in leaf extract, enzyme activity showed decrease on the contrary to apoplastic (fig.6, fig.7).Enzyme activity had significant differences in time course infection compare with control in both leaf and apoplastic extract.

Discussion:

Production of ROS including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) at the site of pathogen infection, is the earliest response of plant to pathogen attack (Wojtaszek, 1997). Accumulation of H_2O_2 in infected zone may be toxic and suppress the bacterial population (Peng and Kuc, 1992), nevertheless it leads to oxidative damage of nucleic acid, protein, pigment and lipid (mandal, 2008). We report here elevated level of MDA production associated with increased antioxidant enzyme activity of both leaf and apoplastic extraction. The peroxidation of unsaturated lipids of biological membranes is the most prominent symptom of oxidative stress in animals and plants (mandal et al., 2008). Production of lipid peroxides has been proved to be induced by pathogens (Gobel et al., 2003). In this present study, we observed a high increase in lipid peroxidation measured as MDA content after at 4 day after inoculation (fig.1) but no significant difference between 4 and 7 day after inoculation was observed. It may be possible that the intensive lipid peroxidation at later stage is an indication of compatible nature of the host–pathogen that indicates plant defense system is activated to suppress ROS production.

Antioxidant enzymes protect plants against damage of oxidative stress. Multiple antioxidant enzymes such as Superoxide dismutase react with the superoxide radical to produce H_2O_2. Hydrogen peroxide is scavenged by catalases and peroxidases. Among peroxidases, ascorbate peroxidases and glutathione peroxidase are well known for their role in H_2O_2 detoxification in plant (Apel and Hirt, 2004).Infection of sweet orange leaves by Xanthomnas axopodonis subsp citri showed increase in activity of catalase, peroxidase and ascorbate peroxidase activity(kumar et al.,2011). Also in barley infected by fungi (vanaker et al., 1998), tomato leaves infected by fosarium (mandal et al., 2008), the change in antioxidant enzyme activity was observed. In our study catalase activity increased in whole leaf and apoplastic extraction after inoculation (fig.2, fig.3). Catalase was shown to be

involved in regulation of H2O2 levels in plant tissues (Bolwell and wotjaszek,1997;Lamb and Dixon,1997).It seems plant has high capacity to use catalae for detoxification of H2O2 in the late stage of pathogen attack. Oxidative cross-linking of structural proteins and possibly other polymers, which mediated by peroxidase, makes the cell wall refractory to digestion by microbial protoplasting enzymes (Brisson et al., 1994). In our study peroxidase activity of whole leaf and apoplastic increase in inoculated leaves in compare with control. This increased peroxidase activity might be involved in reduction of peroxides and thus protecting membranes and proteins from oxidation (Navari et al., 1997).

In this present study the ascorbate peroxidase activity was decreased in foliar extraction but on the contrary was increased in apoplastic extraction (fig.6, fig.7). ascorbate peroxidase might be responsible for the fine modulation of ROS for signaling (Grant et al.,2000). Increase of enzyme activity associated with increase MDA concentration indicates tis mechanism prevent cell damage of infected tissues. The apoplastic antioxidant enzyme activities showed an almost universal increase in response to inoculation compared with control. This may suggest that increased apoplastic antioxidant defenses were a feature of the establishment of biotrophy in susceptible host (Vanaker et al., 1998).

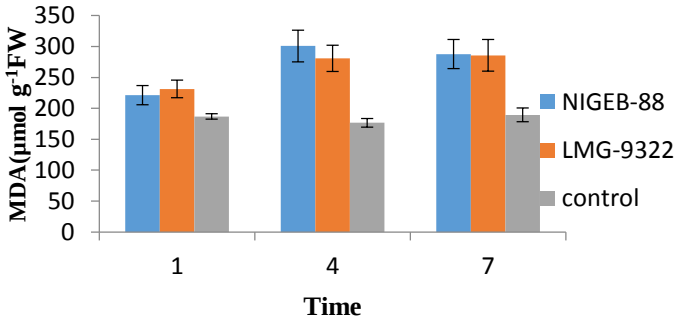

Figure 1. Lipid peroxidation measured in terms of malondialdehyde (MDA) content and expressed as µmol g^{-1}FW) in leaf of lime plants on a time course(day past inoculation) after inoculation of the plants with xanthomonas citri subsp citri and in the control. Data presented in graphs are the means ±SD of three replicates

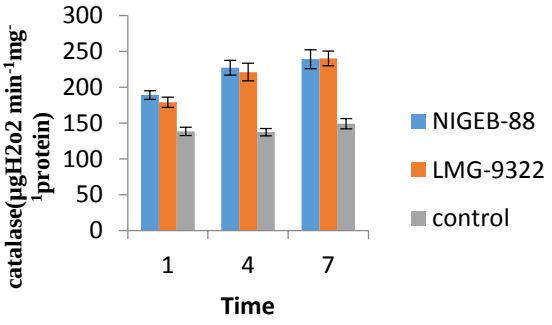

Figure 2. Enzyme activity of ctalase measured and expressed as µgH2o2 $min^{-1}mg^{-1}$protein in leaf of lime plants on a time course(day past inoculation) after inoculation of the plants with xanthomonas citri subsp citri and in the control. Data presented in graphs are the means±SD of three replicates

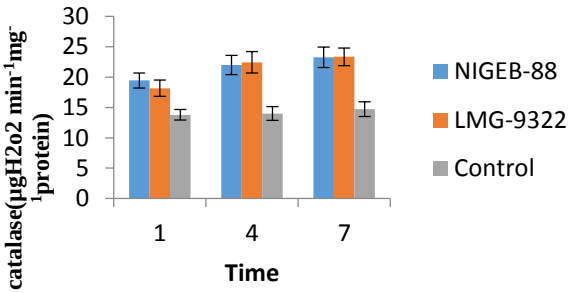

Figure 3. Enzyme activity of catalase measured and expressed as µgH2o2 $min^{-1}mg^{-1}$protein in and expressed as µmol g^{-1}FW) in apoplast of lime plants on a time course(day past inoculation) after inoculation of the plants with xanthomonas citri subsp citri and in the control. Data presented in graphs are the means±SD of three replicates

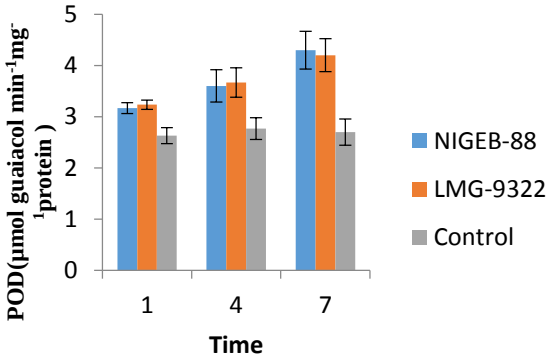

Figure 4. Enzyme activity of peroxidase measured and expressed as µmolguaicol $min^{-1}mg^{-1}$protein in leaf of lime plants on a time course(day past inoculation) after inoculation of the plants with *xanthomonas citri* subsp *citri* and in the control. Data presented in graphs are the means ±SD of three replicates

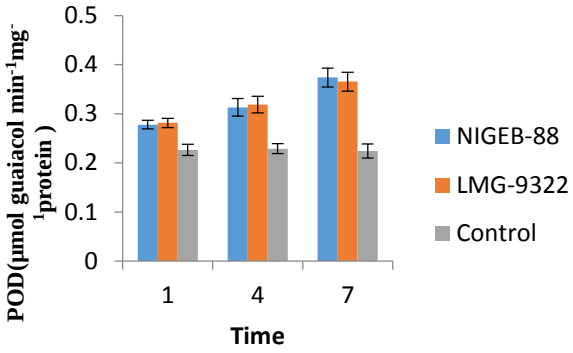

Figure 5. Enzyme activity of peroxidase measured and expressed as µmolguaicol $min^{-1}mg^{-1}$protein in apoplast of lime plants on a time course(day past inoculation) after inoculation of the plants with xanthomonas citri subsp citri and in the control. Data presented in graphs are the means ±SD of three replicates

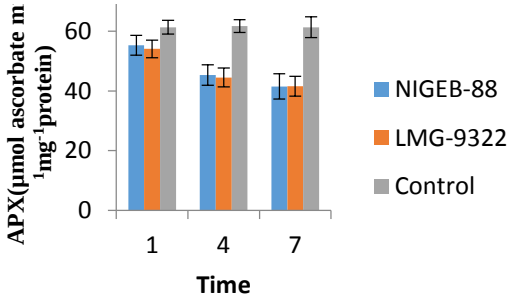

Figure 6. Enzyme activity of ascorbate peroxidase measured and expressed as µmol ascorbate $min^{-1}mg^{-1}$protein in leaf of lime plants on a time course(day past inoculation) after inoculation of the plants with xanthomonas citri subsp citri and in the control. Data presented in graphs are the means ±SD of three replicates

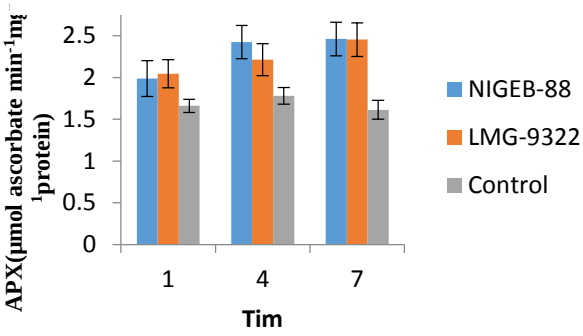

Figure 7. Enzyme activity of ascorbate peroxidase measured and expressed as µmol ascorbate $min^{-1}mg^{-1}$protein in apoplast of lime plants on a time course(day past inoculation) after inoculation of the plants with xanthomonas citri subsp citri and in the control. Data presented in graphs are the means ±SD of three replicates

Table1. Variance analyses of interested trait of lime under pathogen infection

		Mean square						
sov	df	Foliar CAT	Apoplastic CAT	Foliar PX	Apoplastic PX	Foliar APX	Apoplastic APX	MDA
Rep	2	39.8^{*}	6.31^{ns}	0.111^{ns}	0.001^{*}	3.56^{ns}	0.004^{ns}	1331.66
Inoculation	2	11311.5^{**}	86.605^{**}	2.967^{**}	0.064^{**}	623.975^{**}	0.952^{**}	19063.7^{**}
Time	2	6404.4^{**}	64.009^{**}	1.176^{**}	0.005^{**}	185.228^{**}	0.309^{**}	11754.76^{**}
Time*Inoculation	4	1806.5^{**}	15.12^{**}	0.261^{**}	0.001^{**}	48.088^{**}	0.071^{**}	5471.76^{*}
Error	16	8.981	1.15	0.032	0.00025	2.362	0.003	1777.3

*indicate significant level of 0.05.** indicate significant level of 0.01

CONCLUSION

In conclusion the oxidative burst is the earliest defense system of plant that activated against pathogen attack. It seems less efficient of antioxidative, particularly late activation enzymatic system leads to ROS accumulation and then observed high concentration of lipid peroxidation. It indicates that antioxidant system has not enough strong

system to completely protect plant against pathogen. However this system is a weapon against biotrophic pathogen.

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