

Changes of antioxidant enzymes of mung bean [*Vigna radiata* (L.) R. Wilczek] in response to host and non-host bacterial pathogens

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Abstract: The natural resistance against the majority of potential pathogens that exist in most plant species is known as non-host resistance. Several reports suggest the role of antioxidant enzymes in non-host resistance. We assayed the expression or activity of four scavenging enzymes during non-host pathogen-plant interaction (*Xanthomonas hortorum* pv. *pelargonii*/mung bean) and host pathogen-plant interaction (*Xanthomonas axonopodis* pv. *phaseoli*/mung bean). The expression of superoxide dismutase (SOD) and ascorbate peroxidase (APX) and the enzyme activity of catalase (CAT) and peroxidase (POX) were investigated. The activities of CAT and POX were higher during non-host pathogen invasion *vs.* host pathogen attack. The expression of SOD and APX were also different between compatible and incompatible interactions. The expression of SOD and APX were higher in the incompatible compared to the compatible interaction. Additionally, induction of the antioxidant enzymes in response to non-host pathogen was earlier than induction in response to host pathogen. Such information is important for plant breeders, and useful when looking for alternative control strategies as well.

Key words: ascorbate peroxidase, catalase, host pathogen, mung bean, non-host pathogen, peroxidase, superoxide dismutase

Introduction

Plants have developed strategies to defend themselves against pathogen attacks. One of the earliest plant defense responses is the production of reactive oxygen species (ROS) after pathogen recognition (Bolwell and Daudi 2009). Reactive oxygen species includes the superoxide anion radicals and the hydroxyl radicals produced as by-products of oxidation/reduction reactions as a consequence of aerobic metabolism (Halliwell 2006). The interaction between pathogen and plant, leads to production of ROS at early time points of the interaction. This oxidative burst seems to be effective in controlling pathogen infection in incompatible interactions (Baker and Orlandi 1995). Plants have a variety of mechanisms for ROS detoxification. Superoxide dismutase (SOD) catalyses the dismutation of O_2^- to H_2O_2 , catalase (CAT) scavenges H_2O_2 to oxygen and water, and ascorbate peroxidase (APX) reduces H_2O_2 to water by utilising ascorbate as specific electron donor. Peroxidases (POX) oxidise phenolic nature (De Gara *et al.* 2003). Superoxide dismutases are classified into three groups: those which consist of iron SOD (Fe-SOD), manganese SOD (Mn-SOD), and copper-zinc SOD (Cu/Zn-SOD), based on the metal co-factor used by them (Smith and Doolittle 1992).

Non-host resistance is a resistance exhibited by an entire plant species to all genetic variants of a non-adapted pathogen species. It is a durable and strong resistance

against a variety of pathogens (Fan and Doerner 2012). Genetic and mechanistic components of non-host resistance are yet to be fully understood. Yet, such an understanding is necessary to engineer crops for durable disease resistance.

Mung bean [*Vigna radiata* (L.) R. Wilczek] is a summer-rainy season pulse crop that is exposed to some pathogens. Bacterial blight of mung bean caused by *Xanthomonas axonopodis* pv. *phaseoli* is an important disease of mung bean. The disease causes losses worldwide. To our knowledge, no physiological studies have been done on the expression of antioxidant defense genes under pathogen invasion. This study aims to compare the induction of SOD, APX, CAT, and POX between compatible and incompatible mung bean-pathogen interactions to validate the role of these antioxidant enzymes in plant defense against pathogens. An investigation was done on the SOD and APX expression using relative real-time PCR. Additionally, CAT and POX expression was surveyed using enzyme extraction.

Materials and Methods

Plant materials and pathogen inoculation

Mung bean (*Vigna radiata* cv. vc6173) seeds were surface-sterilised by 1.0% sodium hypochlorite (20% household

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bleach) for 5 min, then sown in quartz sand in 15 cm plastic pots in a growth chamber. Plants were grown with a 16 h photoperiod at 28°C. *Xanthomonas hortorum* pv. *pelargonii* PTCC 1474 (purchased from the Persian Type Culture Collection) and *X. axonopodis* pv. *phaseoli* k1 (Osdaghi 2014) were used as non-host and host pathogens, respectively. The inoculums of the host or non-host pathogens at a concentration of about 1×10^7 cfu · ml⁻¹ water were sprayed on the leaves of plants which were 25 days old. Sterile water was used as a negative control. Leaf samples were harvested from the control and from the treated plants at 12, 24, 48, 72, and 96 hours after inoculation.

RNA extraction and cDNA synthesis

Total RNA was extracted using a RNA isolation kit (DENAZist, Iran) according to the recommendations of the manufacturer. The RNA pellet was washed with ice-cold 75% ethanol, air-dried and dissolved in 40 µl of diethylpyrocarbonate (DEPC) water. Isolated RNA was treated with DNase I (Fermentas, Lithuania) to remove genomic DNA contamination. RNA quality and quantity was assessed by agarose gel electrophoresis and spectrophotometry. The cDNA synthesis was carried out on total RNA using the cDNA synthesis kit (Fermentas, Lithuania), according to the manufacturer's instructions.

Real-time PCR (RT-PCR) assay

RT-PCR experiments were performed using RealQ PCR 2x master mix (Ampliqon, Denmark). Each reaction was made in triplicate. Primers APX-F (5'CTCAGAGGYT-MATCGCTGAGAAG3') and APX-R (5'CAGCAAACCC-AAGCTCRGARAGC3'), Cu/Zn-SOD-F (5'ATGGTKAAG-GCTGTGGCAGTTC3') and Cu/Zn-SOD-R (5'ACCTTT-CCCAAGATCATCAGGAGGATC3') (Sairam *et al.* 2011) were used for APX and Cu/Zn-SOD, respectively. Also, β-tubulin with corresponding primers Tubulin-F (5'CTT-GACTGCATCTGCTATGTTTCAG3') and Tubulin-R (5'CC-AGCTAATGCTCGGCATACTG3') (Sairam *et al.* 2011) was considered as the house-keeping gene. Total reaction volume was 25 µl and included 10 µl (2X) SYBR green master mix, 100 ng of cDNA, 0.5 µl of 10 µM of each forward and reverse primers, and volume adjusted with water. Thermal cycling conditions consisted of 95°C for 5 min, 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. The real-time PCR was performed in a Bioneer (China). The Cycle Threshold (CT) values of target and house-keeping genes were used for analysis of data. Relative gene expression was calculated using the comparative $\Delta\Delta C_T$ method according to Livak and Schmittgen (2001).

POX and CAT extraction

To maintain enzyme activities, all operations were performed at 4°C. Frozen leaves were crushed to a fine powder with 5% (w/w) polyvinylpyrrolidone (PVPP) in a mortar with liquid nitrogen. Soluble proteins were extracted by suspending 500 mg of the powder in 2 ml extraction buffer containing 50 mM potassium phosphate buffer,

(pH 7.8), 0.1 mM EDTA, 1 mM phenylmethanesulfonyl (PMSF), and 10 mM dithiothreitol (DTT). The homogenate was centrifuged at $13,000 \times g$ for 30 min. Enzyme extracts were stored at -80°C until use.

POX activity assay

The method described by Lin and Kao (1999) was used to determine POX activity. The reaction mixture consisted of 976 µl potassium phosphate buffer (50 mM, pH 7), 10 µl guaiacol (900 mM), 20 µl H₂O₂ (500 mM), and 3 µl enzyme extract. The reaction was started by adding H₂O₂ and the oxidation of guaiacol was determined by measuring the increase in absorbance at 470 nm. Enzyme activity, expressed as µmol · min⁻¹ · mg⁻¹ protein of oxidised guaiacol, was calculated using the extinction coefficient of 26.6 mM⁻¹ · cm⁻¹ for guaiacol.

CAT activity assay

CAT activity was determined by following the decline of absorbency (decomposition of H₂O₂) at 240 nm (Aebi 1984). The reaction mixture consisted of 20 µl H₂O₂ (500 mM) and 10 µl enzyme extract in 970 µl phosphate buffer (50 mM, pH 7). CAT activity, expressed as µmol · min⁻¹ · mg⁻¹ protein, was calculated using the extinction coefficient of 36 M⁻¹ · cm⁻¹ for H₂O₂.

Statistical analysis

The experiments were repeated three times for each sample in two different biological replicas. Statistical analysis was performed using SAS 9.1 (SAS Institute, Cary, NC, USA) and a probability of $p < 0.05$ was considered significant.

Results

Cu/Zn-SOD expression

Cu/Zn-SOD expression in inoculated mung beans with non-host pathogen was higher than in inoculated mung beans with host pathogen. Expression of the gene in inoculated plants with *X. hortorum* pv. *pelargonii* increased 2.1-, 4.4-, 3.6-, and 2.8-fold at 24, 48, 72, and 96 hours, respectively, after inoculation compared to the control. On the other hand, Cu/Zn-SOD expression in inoculated plants with *X. axonopodis* pv. *phaseoli* increased 1.3-, 1.8-, 2.2-, and 1.2-fold at 24, 48, 72, and 96 hours, respectively, after inoculation compared to the control. A significant difference was observed in Cu/Zn-SOD expression between compatible and incompatible interactions at the all time intervals. The highest expression in compatible and incompatible interaction was observed at 74 and 48 hours after inoculation, respectively (Fig. 1).

Ascorbate peroxidase expression

In inoculated plants with non-host pathogen, the APX expression increased 2.2-, 4.6-, 3.5-, and 2.3-fold at 24, 48, 72, and 96 hours, respectively, after inoculation compared to the control. Furthermore, APX expression in in-

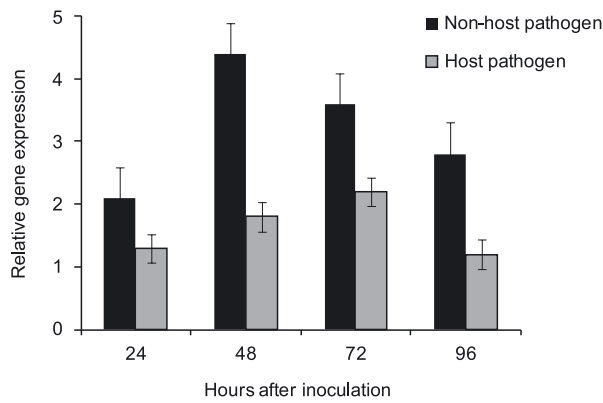


Fig. 1. Expression analysis of Cu/Zn-superoxide dismutase (Cu/Zn-SOD) during infection of mung bean by *X. hortorum* pv. *pelargonii* (non-host pathogen) and *X. axonopodis* pv. *phaseoli* (host pathogen). Quantification of gene expression was carried out using the comparative Ct method (Livak and Schmittgen 2001). Relative expression of the gene is reported as the number of fold increases of the transcript level in infection experiments relative to each corresponding control sample

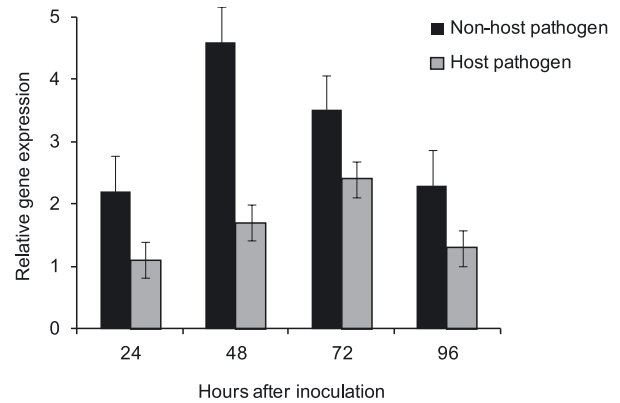


Fig. 2. Ascorbate peroxidase (APX) expression of mung bean in response to non-host pathogen (*X. hortorum* pv. *pelargonii*) and host pathogen (*X. axonopodis* pv. *phaseoli*). Relative gene expression was calculated using the comparative Ct method (Livak and Schmittgen 2001). Relative expression of the gene is reported as the number of fold increases of the transcript level in infection experiments relative to each corresponding control sample

oculated plants with host pathogen increased 1.1-, 1.7-, 2.4, and 1.3- fold at 24, 48, 72, and 96 hours, respectively, after inoculation compared to the control. In inoculated mung beans with *X. hortorum* pv. *pelargonii*, the APX expression was higher than in inoculated mung beans with *X. axonopodis* pv. *phaseoli* and a significant difference was observed between them at the all time points. In a compatible interaction, maximum expression was observed at 48 hours after inoculation, whereas highest expression in incompatible interaction was recorded at 72 hours after inoculation (Fig. 2).

POX activity

In incompatible interaction, POX activity was significantly enhanced compared to the control, at all the time intervals. In inoculated plants with non-host pathogen,

POX activity increased about 1.6-, 2.6-, 2.1-, and 2.1-fold at 24, 48, 72, and 96 hours, respectively, after inoculation compared to the control. Additionally, POX activity in incompatible interaction was significantly higher than in compatible interaction at the all the sampling times. In compatible interaction, POX activity decreased almost 22, 50, 23, and 33% at 24, 48, 72 and 96 hours, respectively, after inoculation compared to incompatible interaction (Table 1).

CAT activity

In inoculated plants with *X. hortorum* pv. *pelargonii*, CAT activity was significantly increased compared to the control, at the all the sampling times. In incompatible interaction, POX activity showed an increment about 3.3-, 4.1-, 3.7-, and 2.3 fold at 24, 48, 72, and 96 hours, respectively,

Table 1. Peroxidase activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) during compatible and incompatible plant-pathogen interactions at 24, 48, 72, and 96 hours after inoculation

Hours after inoculation	The control	Compatible interaction	Incompatible interaction
24	31.1	42.1	52.3
48	31.6	44.1	88.4
72	32.6	54.7	70.5
96	30.4	43.2	64.4

Table 2. Catalase activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) in the control and inoculated plants with host and non-host pathogens at 24, 48, 72, and 96 hours after inoculation

Days after inoculation	The control	Compatible interaction	Incompatible interaction
24	17.5	24.5	57.1
48	18.1	31.3	75.3
72	17.7	37.1	64.2
96	18.2	28.4	42.3

after inoculation compared to the control. Furthermore, CAT activity in inoculated plants with host pathogen was lower than in inoculated mung beans with non-host pathogen. In compatible interaction, CAT activity displayed a decrease almost 58, 59, 43, and 34% at 24, 48, 72, and 96 hours, respectively, after inoculation compared to incompatible interaction (Table 2).

Discussion

One of the earliest responses of the plants to an attempted infection is the production of ROS such as singlet oxygen (O_2), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^\cdot). Reactive oxygen species (ROS) has been implicated in hypersensitive response (HR) (Lamb and Dixon 1997), phytoalexin production (Daudi *et al.* 2012), callose deposition (O'Brien *et al.* 2012), and systemic acquired resistance (SAR) (Alvarez *et al.* 1998). Additionally, ROS molecules have major roles in cell signaling, some physiological processes, and second messengers for the activation of genes encoding protective proteins (Mendoza 2011). It has been showed that an accumulation of ROS plays a central role in a number of non-host interactions like barley/*Blumeria graminis* f. sp. *tritici* (Hückelhoven *et al.* 2001), cowpea/*Erysiphe cichoracearum* (Mellersh *et al.* 2002); and pepper/*Blumeria graminis* f. sp. *tritici* pathosystems (Hao *et al.* 2011). To avoid the detrimental effects of ROS and to maintain the equilibrium of ROS levels, plants possess many enzymes to scavenge the ROS efficiently. Major ROS scavenging enzymes are SOD, APX, CAT, and POX (Bolwell *et al.* 2002). In this work, we have investigated expression of some antioxidant enzymes during interaction of *X. axonopodis* pv. *phaseoli*/mung bean (compatible interaction) and *X. hortorum* pv. *pelargonii*/mung bean (incompatible interaction). Although several studies have been previously performed on a variety of plant-pathogen interactions (Shigeoka *et al.* 2002; Kwak *et al.* 2009), up till now no examination has being carried out about expression of scavenging enzymes in mung bean in response to pathogens. Our results showed that pathogen attack leads to substantial changes in the antioxidant status of mung bean, while there were clear differences between compatible and incompatible interactions. Our data showed increment in expression of antioxidant enzymes in incompatible interaction versus compatible interaction that was in agreement with previous studies. Subramanian *et al.* (2005) showed promotion in the levels of SOD in incompatible interaction between *Brassica carinata* – *Lepidosphaeria maculans* compared to compatible interaction. Cheng *et al.* (2012) displayed more expression of SOD and CAT in inoculated broad beans with a non-host pathogen compared to the control. Moreover, differential induction of SOD in downy mildew-resistant and susceptible genotypes of pearl millet has been demonstrated (Babitha *et al.* 2002). Ádám *et al.* (1995) previously elucidated the activity of SOD during a host-incompatible interaction of bean/*Pseudomonas syringae* pv. *phaseolicola*. More activity of SOD, CAT, and POX in pepper in response to *Xanthomonas campestris* pv. *campestris* (as a non-host pathogen) has been approved (Kwak *et al.* 2009). Inoculation of wheats by *Fusarium graminearum* leads to a significant increase in POX

activity in comparison with the non-inoculated control plants (Mohammadi and Kazemi 2002). Induction of POX in *Cucumis sativus* and *Gynura aurantiaca* upon infection by prunus necrotic ringspot virus has been approved (Bellés *et al.* 2006). Agrawal *et al.* (2003) displayed more expression of APX1 and APX2 in rice in response to *Magnaporthe grisea*. Moreover, APX activity increased along with the activities of other enzymes, such as CAT and SOD (Shigeoka *et al.* 2002). Also, we demonstrated that expression of scavenging enzymes in the incompatible interaction is faster than in the compatible interaction. Therefore, if these responses are important for resistance, they may have occurred too late in the susceptible interaction to afford protection. Non-host resistance comprises multi-layered defense responses and is difficult to overcome by the majority of potential pathogens. Hence, it can provide durable and broad-spectrum resistance. Successful pathogens have evolved counter-defense strategies to overcome non-host resistance (Senthil-Kumar and Mysore 2013). Understanding the signaling and defense mechanisms involved in the execution of non-host resistance might help to improve resistance against pathogens.

Conclusions

We approved induction of SOD, APX, POX, and CAT in mung bean inoculated with *X. axonopodis* pv. *phaseoli* as a host pathogen and *X. hortorum* pv. *pelargonii* as a non-host pathogen compared to non-inoculated plants. In addition, expression of the enzymes was significantly stronger in incompatible interaction than compatible interaction. It seems antioxidant enzymes plays a significant role in plant defense against pathogens.

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