CHANGES IN EXPRESSION OF PATHOGENESIS-RELATED GENE 1, PATHOGENESIS-RELATED GENE 2, PHENYLALANINE AMMONIA-LYASE AND CATALASE IN TOMATO IN RESPONSE TO *PECTOBACTERIUM CAROTOVORUM* SUBSP. *CAROTOVORUM*

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SUMMARY

Pectobacterium carotovorum subsp. *carotovorum* (*Pcc*) is one of the most destructive pathogens causing stem rot disease on tomato. In this study, expression profiling of pathogenesis-related gene 1 (*PR-1*), pathogenesis-related gene 2 (*PR-2*), phenylalanine ammonia-lyase (*PAL*) and catalase (*CAT*) in a partially resistant (Sun) and a susceptible (Early orbano) tomato cultivar in response to *Pcc* were compared. Our results showed more expression of *PR-2*, *PAL* and *CAT* in the partially resistant cultivar than the susceptible cultivar. On the other hand, no significant difference was found in expression of *PR-1* between the both cultivars. These findings suggest the involvement of *PR-2*, *PAL* and *CAT* in defense responses of tomato against *Pcc* that might be considered in plant breeding programs.

Keywords: catalase, pathogenesis-related gene 1, pathogenesis-related gene 2, *Pectobacterium carotovorum* subsp. *carotovorum*, Phenylalanine ammonia-lyase.

INTRODUCTION

During pathogen infection of plants, expression of some defense genes is changed to overcome pathogen attack. The role of pathogenesis-related (PR) proteins in plant defense mechanism is demonstrated (Ebrahim *et al*., 2011). Most PR proteins are acid-soluble, low molecular weight and protease-resistant. PR proteins have been divided into 17 families so far, based on their sequences and functions (Ebrahim *et al*., 2011). PR-1 protein class was identified in the early 1980s (Antoniw *et al*., 1980). It has been found in some plant species such as barley, wheat, maize, tobacco, rice, pepper and tomato (Liu and Xue, 2006). Although *PR-1* is expressed abundantly upon pathogen attack (Mitsuhara *et al*., 2008; Asai *et al*., 2014), the exact mode of action of this protein is yet to be understood. PR-2 as a β-1,3-glucanase enzyme was diagnosed firstly in tomato (Antoniw *et al*., 1980) and afterward discovered in a variety of plants like peanut, chickpea and common bean. Induction of *PR-2* in response to various pathogens is proved (Elvira *et al*., 2008). Phenylalanine ammonia-lyase (PAL) is an important enzyme in the regulation point between primary and secondary metabolism that catalyses the non-oxidative deamination of phenylalanine to *trans*-cinnamate (Dixon and Paiva, 1995; Huang *et al*., 2010). *PAL* gene expression responds to pathogen invasion and numerous abiotic stresses (Dixon and Paiva, 1995; MacDonald and D'Cunha, 2007). Furthermore, rapid and strong accumulation of reactive oxygen species (ROS) is a crucial plant defense mechanism against pathogen invasion (Bolwell *et al*., 2002). Although early research about ROS focused on its direct toxicity, recent studies confirmed the role of ROS as a signaling molecule. ROS involves in hypersensitive response (HR) (Lamb and Dixon, 1997), systemic acquired resistance (SAR) (Alvarez *et al*., 1998), phytoalexin production (Daudi *et al*., 2012) and callose deposition (O'Brien *et al*., 2012). Scavenging enzymes like catalase (CAT) play a key role in balancing of ROS to employ it as a signaling molecule (Mittler *et al*., 2004). Expression of *CAT* in plants upon pathogen attack is shown (Kwak *et al*., 2009; Cheng *et al*., 2012).

Tomato is a major vegetable crop in the world that suffers from numerous pathogens. Stem rot caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) is one of the most important diseases of tomato worldwide (Pérombelon and Salmond, 1995). In recent years, the promoting concern about harmful environmentally effects of pesticides and also appearance of pesticides-resistant pathogen strains has led to search for new more effective and safer techniques. Use of resistant cultivars is one of the popular methods to reach this goal. Additionally, identification of the genes that play role in resistance is a key approach to might be considered in plant breeding programs. There are no reports about the effect of *Pcc* on expression of *PR-1*, *PR-2*, *PAL* and *CAT* in tomato. Hence, the aim of this study was to investigate expression of *PR-1*, *PR-2*, *PAL* and *CAT* in tomato in response to *Pcc*. Moreover, Expression of

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these genes in cultivars Sun (as a partially resistant cultivar) and Early orbano (as a susceptible cultivar) were compared.

MATERIALS AND METHODS

Plant materials and pathogen inoculation. The tomato (*Lycopersicon esculentum*) cultivars Early orbano and Sun were used in this study. Tomato seeds were surface sterilized in 1.0% sodium hypochlorite for 10 min and rinsed four times in sterile distilled water. The seeds were sown in quartz sand in 15-cm plastic pots. The inoculum of *Pcc* 1675 (purchased from Persian Type Culture Collection) was provided in sterile distilled water at a concentration of about 10^8 CFU/ml and each stem of six-week-old plants was infiltrated with 50 µl of the bacterial suspension. Sterile distilled water was employed as a negative control. The plants were incubated in a growth chamber at 28°C with a 16 h photoperiod at 70% relative humidity. The length of rotten lesions (cm) on stems was recorded at 5 and 10 days post pathogen inoculation (dpi).

RNA extraction and cDNA synthesis. Stems were harvested at 24, 48, 72 and 96 h post pathogen inoculation (hpi) with three repetitions. Total RNA was isolated by a RNA extraction kit (DENAzist, Iran) according to the manufacturer's protocol. After treatment of RNA with DNase I (Fermentas, Lithuania) to eliminate genomic DNA contamination, its quality and quantity was evaluated by ethidium bromide staining of agarose gel and spectrophotometrically. First-strand cDNA synthesis was performed using a cDNA synthesis kit (Fermentas, Lithuania), according to manufacturer's guidelines.

Real-time RT-PCR analysis. Real-time RT-PCR reactions were performed with 30 ng of cDNA, 600 nM of each primers, 10 µl (2×) of the SYBR green Master Mix (Ampliqon, Denmark) and RNase free water in a final volume of 20 µl. Primers used in this study had the following sequences: 5'GGATCGGACAACGTCCTTAC and 5'GCAACATCAAAAGGGAAATAAT (Molinari *et al*., 2014) for *PR-1*, 5'AAGTATATAGCTGTTGGTAATGAA and 5'ATTCTCATCAAACATGGCGAA (Molinari *et al*., 2014) for *PR-2*, 5'ACGGGTTGCCATCTAATCTG and 5'AGCTCTTTTCCTGGCTGAAA (Aimé *et al*., 2013) for *PAL*, 5'TGGAAGCCAACTTGTGGTGT and 5'ACTGGGATCAACGGCAAGAG (Zhang *et al*., 2015) for *CAT*, 5'AACCTCCATTCAGGAGATGTTT and 5'TCTGCTGTAGCATCCTGGTATT (Aimé *et al*., 2013) for *β-tubulin*. Real-time RT-PCR experiments were carried out in a Bioneer (South Korea) with a program consist of initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C and extension for 1 min at 72°C; with finalextension for 5 min at 72°C. Each Real-time RT-PCR was repeated three times and the results were averaged.

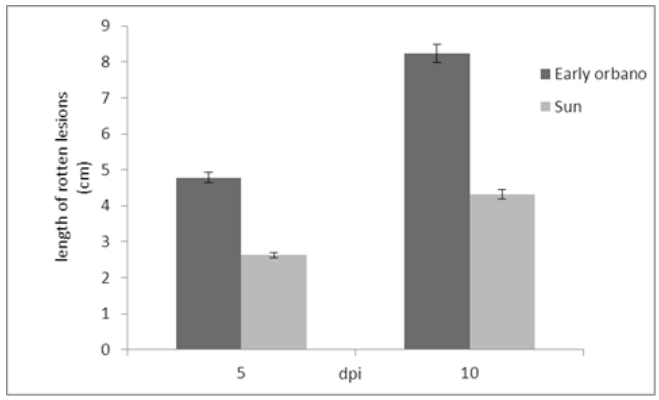

Fig. 1. Length of rotten lesions (cm) on stem in cultivars Sun (partially resistant) and Early orbano (susceptible) inoculated with *Pcc* at 5 and 10 dpi. The length of rotten lesions on cultivar Early orbano was significantly higher compared to cultivar Sun.

Statistical analysis. The CT values of target and reference genes were employed for analysis of data. Relative gene expression was measured using the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001). Treatments were evaluated statistically using SAS 9.1 (SAS Institute, Cary, NC, USA) and a probability of $P < 0.05$ was considered significant.

RESULTS

Disease evaluation. The length of rotten lesions on stems of tomato plants cultivar Early orbano inoculated with *Pcc* was significantly higher than cultivar Sun at 5 and 10 dpi (Fig. 1).

Expression of *PR-1*. *PR-1* expression in cultivar Sun inoculated with *Pcc* increased 3.6-, 3.2-, 2.5- and 2.2- fold at 24, 48, 72 and 96 hpi, respectively compared to controls. Moreover, *PR-1* expression in cultivar Early orbano inoculated with *Pcc* enhanced 3.5-, 3.0-, 2-6- and 2.2- fold at 24, 48, 72 and 96 hpi, respectively compared to controls. No significant difference was found in expression of *PR-1* between cultivars Sun and Early orbano at the all time intervals (Fig. 2).

Expression of *PR-2*. Expression of *PR-2* in cultivar Sun inoculated with *Pcc* was significantly higher than cultivar Early orbano at the all time points. *PR-2* expression in cultivar Sun inoculated with *Pcc* enhanced 4.1-, 3.3-, 2.8- and 2.1- fold at 24, 48, 72 and 96 hpi, respectively compared to controls. On the other hand, *PR-2* expression in cultivar Early orbano inoculated with *Pcc* increased 2.6-, 2.2-, 1.9- and 1.5- fold at 24, 48, 72 and 96 hpi, respectively compared to controls (Fig. 3).

Expression of *PAL*. Expression of *PAL* in cultivar Sun inoculated with *Pcc* was significantly higher than cultivar

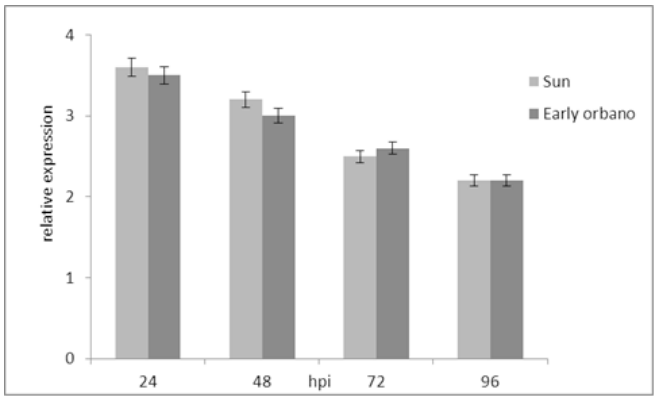

Fig. 2. RT-qPCR relative expression levels of *PR-1* in cultivars Sun (partially resistant) and Early orbano (susceptible) inoculated with *Pcc.* No significant difference was found in expression of *PR-1* between these cultivars at the all time points.

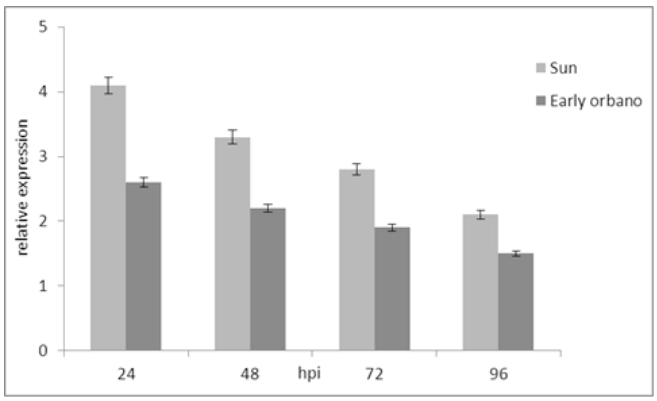

Fig. 3. RT-qPCR relative expression levels of *PR-2* in cultivars Sun (partially resistant) and Early orbano (susceptible) inoculated with *Pcc. PR-2* expression was elevated in cultivar Sun compared to cultivar Early orbano at the all time points.

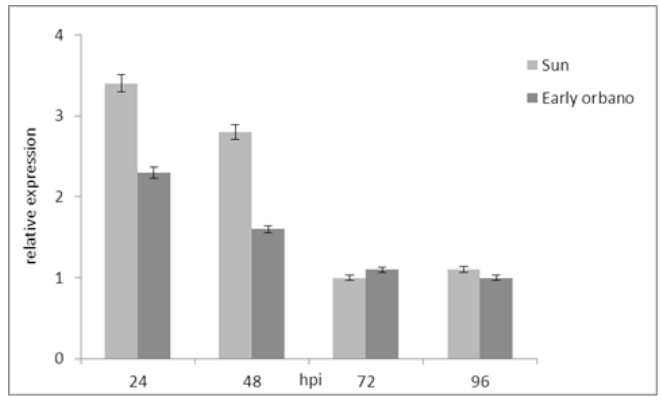

Fig. 4. RT-qPCR relative expression levels of *PAL* in cultivars Sun (partially resistant) and Early orbano (susceptible) inoculated with *Pcc. PAL* expression in cultivar Sun was significantly higher than cultivar Early orbano at 24 and 48 hpi. No significant expression of *PAL* was occurred in the both cultivars in response to *Pcc* at 72 and 96 hpi.

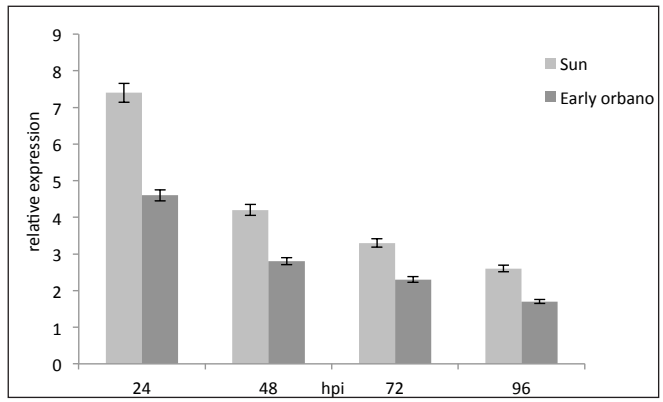

Fig. 5. RT-qPCR relative expression levels of *CAT* in cultivars Sun (partially resistant) and Early orbano (susceptible) inoculated with *Pcc.* In comparison with cultivar Early orbano, higher expression of *CAT* was found in cultivar Sun at the all time points.

Early orbano at 24 and 48 hpi. *PAL* expression in cultivar Sun inoculated with *Pcc* increased 3.4- and 2.8- fold at 24 and 48 hpi, respectively compared to controls. Additionally, *PAL* expression in cultivar Early orbano inoculated with *Pcc* enhanced 2.3- and 1.6- fold at 24 and 48 hpi, respectively compared to control. On the other hand, no significant difference was observed in *PAL* expression between plants inoculated with *Pcc* and controls at 72 and 96 hpi in the both cultivars (Fig. 4).

Expression of *CAT*. Expression of *CAT* in cultivar Sun inoculated with *Pcc* increased 7.4-, 4.2-, 3.3- and 2.6- fold at 24, 48, 72 and 96 hpi, respectively compared to controls. In addition, *CAT* expression in cultivar Early orbano inoculated with *Pcc* increased 4.6-, 2.8-, 2.3- and 1.7- fold at 24, 48, 72 and 96 hpi, respectively compared to controls. Significant difference was found in expression of *CAT* between cultivars Sun and Early orbano at the all time points (Fig. 5).

DISCUSSION

Plants exhibit numerous responses against pathogen attack, many of which involve the expression of defense genes. Activation of these genes leads to physical and physiological changes in plant which are not favorable for pathogen. The involvement of *PR-1*, *PR-2*, *PAL* and *CAT* genes in basal resistance of tomato against *Pcc* was investigated in this study. Moreover, in order to better understanding about resistance mechanisms to *Pcc*, expression profiling of the genes in partially resistant and susceptible cultivars were compared in time course experiments. Our results indicated expression of *PR-1* and *PR-2* in tomato in response to *Pcc.* Mitsuhara *et al.* (2008) displayed up-regulation of *PR-1* in rice upon *Magneporthe grisea* infection. Overexpression of *WRKY48* (as a negative regulator of *PR-1*) in *Arabidopsis* leads to enhancing susceptibility against *Pseudomonas syringae* that is associated with reduced expression of *PR-1* (Xing *et al.*, 2008). Elvira *et al.*

(2008) proved accumulation of some PR proteins such as PR-1 and PR-2 in *Capsicum chinense* plants inoculated with pepper mild mottle virus. Induction of *PR-1* in *Arabidopsis thaliana* in response to *Hyaloperonospora arabidopsidis* is demonstrated (Asai *et al*., 2014). A resistance-derived from the harpin of *Pseudomonas syringae* pv. *syringae* in transgenic tobacco plants against *Fusarium oxysporum* f. sp. *nicotianae* is accompanied by expression of some defense genes like *PR-1* and *PR-2* (Dey *et al*., 2014). Additionally, more expression of *PR-1* in a resistant cultivar of wheat against *Fusarium graminearum* compared to a susceptible cultivar is confirmed (Soltanloo *et al*., 2010). Expression of *PR-1* in the resistant pepper cultivar infected with *Phytophthora capsici* is higher than in the susceptible cultivar (Silvar *et al*., 2008). Although expression of *PR-1* in both tomato cultivars infected with *Pcc* was demonstrated in this study, there was no significant difference in *PR-1* expression between cultivars with different susceptibility. Hence, different resistance in these cultivars is not related to expression of *PR-1* and maybe some other defense genes play role in plant defense mechanism. Salicylic acid-responsive genes have generally been supposed to activate defense mechanisms against biotrophic and hemibiotrophic pathogens while jasmonic acid/ethylene-responsive genes play important role against necrotrophic pathogens. Although this remains somewhat true, the signaling network involved in defense responses appears more complex. It is noteworthy that induction of *PR-1* and *PR-2* as salicylic acid-responsive genes against *Pcc* as a necrotrophic pathogen was approved in the present study. In *Arabidopsis*, induced resistance against *Pcc* can be obtained either by salicylic acid-mediated and jasmonic acid/ethylene-mediated defenses (Kariola *et al*., 2003). This apparent controversy might be due to the overlapping defenses pathways at the different stages of the infection. For instance, salicylic acid-mediated defenses appear to be more efficient during the latent phase of *Arabidopsis* infection by *Pcc* (Kariola *et al*., 2005). On the other hand, jasmonic acid/ethylene-dependent defenses have a more important role during the necrotrophic phase of *Pcc* (Brader *et al*., 2001).

The role of PAL in biosynthesis of salicylic acid and as an important signal in plant systemic resistance is confirmed (Nugroho *et al*., 2002; Chaman *et al*., 2003). *PAL* induction in tomato during infection by *Pcc* was revealed in this study. Campos *et al.* (2003) indicated PAL activity in bean plants inoculated with *Colletotrichum lindemuthianum*. *PAL* mutants of *Arabidopsis thaliana* are more susceptible to *Pseudomonas syringae* than wild-type plants (Huang *et al*., 2010). *PAL*-silenced pepper plants exhibits increased susceptibility to *Xanthomonas campestris* pv. *vesicatoria* infection (Kim and Hwang, 2014). According to our data, *PAL* expression in the partially resistant cultivar to *Pcc* was higher compared to the susceptible cultivar. Higher levels of PAL are found in chickpea resistant cultivar than the susceptible cultivar after treatment with *Fusarium oxysporum* f. sp. *ciceri* (Raju *et al*., 2008). PAL activity in the resistant banana cultivar inoculated with *Mycosphaerella fijiensis* is higher compared to the susceptible cultivar (Torres *et al*., 2012). In our study, expression of *PAL* occurred only at 24 and 48 hpi that suggests *PAL* expression at early stages of infection may be enough to motivate synthesis of secondary metabolites to prevent pathogen invasion.

ROS production is one of the earliest responses of plants against pathogens (Bolwell *et al*., 2002). On the other hand, to prevent the harmful effects of ROS and to maintain the equilibrium of its levels, plants produce some enzymes to scavenge the ROS proficiently (Mittler *et al*., 2004). *CAT* expression in tomato upon *Pcc* infection is displayed in this study. Induction of scavenging enzymes including *CAT* in pepper in response to *Xanthomonas campestris* pv. *vesicatoria* is shown (Kwak *et al*., 2009). *CAT* is expressed in broad bean upon *Puccinia striiformis* attack (Cheng *et al*., 2012). In addition, our results showed an increment in expression of *CAT* in the partially resistant cultivar to *Pcc* when compared to the susceptible cultivar. In sugarcane, CAT activity in resistant cultivar to *Sporisorium scitamineum* is higher than the susceptible cultivar, suggesting that catalase activity may play an important role in resistance to the pathogen (Su *et al*., 2014). Expression of *CAT* is detected in both susceptible and resistant cultivars of cabbage to *Xanthomonas campestris* pv. *campestris*, while the expression in the resistant cultivar is relatively higher than the susceptible cultivar (Kaunain Roohie and Umesha, 2015). In summary, expression of *PR-1*, *PR-2*, *PAL* and *CAT* in tomato in response to *Pcc* was shown in this study. Moreover, we demonstrated more expression of *PR-2*, *PAL* and *CAT* in a partially resistant cultivar than a susceptible cultivar after inoculation with *Pcc*. These findings suggest the partially resistance to *Pcc* in cultivar Sun may is correlated with higher expression of some defense genes such as *PR-2*, *PAL* and *CAT*. Identification of resistant tomato germplasm and the mechanisms involved in resistance to *Pcc* would aid in plant breeding programs.

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Received May 1, 2016
Accepted July 24, 2016