OXIDATIVE ENZYME RESPONSES OF SIX CITRUS ROOTSTOCKS INFECTED WITH *PHOMA TRACHEIPHILA* (PETRI) KANTSCHAVELI AND GIKASHVILI

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SUMMARY

Citrus trees are often exposed to severe infectious diseases. Mal secco caused by *Phoma tracheiphila* (Petri) Kantschaveli and Gikashvili is one of the most destructive fungal diseases of lemons (*Citrus limon* Burm. F.). In the present study, antioxidant enzyme activity in different mal secco-resistant and susceptible citrus rootstocks including Cleopatra mandarin (*C. reshni* Tan.), sour orange (*C. aurantium* L.), rough lemon (*C. jambhiri* Lush.), Volkameriana (*C. volkameriana* Tan. and Pasq.), Carrizo citrange (*Poncirus trifoliata* L. Raf. X *C. sinensis* L. Osbeck) and trifoliate orange (*P. trifoliata*) was investigated. Possible differences in constitutive levels of these antioxidant enzymes and correlations between enzyme levels and mal secco caused by *P. tracheiphila* were examined. Among the rootstocks, Cleopatra mandarin was found to be resistant to mal secco, whereas rough lemon, sour orange and trifoliate orange were highly susceptible. Total peroxidase (TPX; EC: 1.11.1.7) activity increased in all infected rootstocks. Ascorbate peroxidase (APX; EC: 1.11.1.11) activity increased in most of the rootstocks and no correlation was found between catalase (CAT; EC: 1.11.1.6) activity and mal secco resistance. This study indicates that overall TPX activity is upregulated and APX activity is up- and down-regulated depending on the type of rootstock in response to *P. tracheiphila* infection.

INTRODUCTION

Free radicals generated by a partial reduction of O_2 cause a serious hazard to tissues and vital organs, especially membrane lipids, connective tissues, and nucleic acids of cells. Thus, they have to be neutralized by the antioxidative defence mechanism of plants. This mechanism comprises a variety of antioxidant molecules and enzymes encoded by genes. The capacity and activity of the antioxidative defence system are important in both limiting the oxidative damage and destroying active oxygen species that are produced in excess of those normally required for metabolism (Arora *et al.*, 2002).

Many enzymatic and non-enzymatic antioxidant defences exist, including superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) enzymes and glutathione (GSH), beta-carotene (vitamin A), ascorbic acid (vitamin C) and alpha-tocopherol (vitamin E) chemical compounds (Mates and Sanchez-Jiménez, 1999). The balance between SOD and APX or CAT activities in cells is crucial

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for determining the steady-state level of superoxide radicals and hydrogen peroxide. Different affinities of APX and CAT for hydrogen peroxide suggest that they belong to two different classes of H_2O_2-scavenging enzymes: APX possibly being responsible for fine modulation of reactive oxygen intermediates (ROIs) for signalling and CAT being responsible for the removal of excess ROIs during stress (Mittler, 2002).

The effects of fungal, bacterial and virus infections on the activity of antioxidative enzymes in plants have been previously reported. The response of antioxidative enzymes against plum pox virus (PPV) was examined in two apricot (*Prunus armeniaca* L.) cultivars, which behaved differently against PPV infection (Hernandez *et al.*, 2001). Kumquat [*Fortunella margarita* (Lour.) Swingle] 'Nagami' leaves were exposed to bacteria of *Xanthomonas axonopodis* pv. *citri* and a higher CAT activity was observed in inoculated leaves (Kumar *et al.*, 2011). Wu *et al.* (2006a) reported increased antioxidative enzymes (SOD, CAT and APX) in *Citrus tangerine* roots infected with an arbuscular mycorrhizal (AM) fungus under water stress. Similarly, higher levels of SOD, CAT and peroxidase activities were also reported in micropropagated mycorrhizal *P. trifoliata* plants than those in non-mycorrhizal plants (Wu *et al.*, 2006b). Infection of the strawberry leaves with *Mycosphaerella fragariae* fungi resulted in an increase in SOD activities of resistant and susceptible cultivars (Ehsani-Moghaddam *et al.*, 2006). In another study, Reverberi *et al.* (2008) reported that the synchronous presence of hydrolytic enzymes, toxic compounds, oxidative stress inducers and membrane transporters in the fungus, and the differential ability to modulate the lipoperoxidative pathway in the host, can play a significant role in *Phoma tracheiphila* infection of *C. limon*. Recently, Zhang *et al.* (2010) reported that male poplar trees showed higher antioxidant activities and less H_2O_2 accumulation than did females after being infected by *Melampsora laricipopulina* Kleb., responsible for rust disease.

In the Mediterranean Basin, mal secco caused by *P. tracheiphila* is one of the most destructive fungal diseases of lemons. The disease occurs in Italy, Spain, Greece, Tunisia, Algeria, Cyprus, Turkey and Israel, and reduces the quantity and quality of lemon production in the areas where the pathogen is present. Complete control of mal secco with chemicals and other control measures such as sanitary applications is not possible. Furthermore, the mechanism of tolerance to this disease is unknown. Development of cultivars tolerant or resistant to this disease is essential (Gulsen *et al.*, 2007; Tuzcu *et al.*, 1989; Uzun *et al.*, 2009b). Variations in resistance of citrus rootstocks against mal secco were reported. For instance, while Cleopatra mandarin (*C. reshni* Hort. ex Tan.) was intermediate resistant, Volkameriana (*C. volkameriana* Tan. and Pasq.), rough lemon (*C. jambhiri* Lush.), sour orange (*C. aurantium* L.) and trifoliate orange (*P. trifoliata* (L.) Raf.) were susceptible (Tuzcu *et al.*, 1989).

To date, no information on effects of mal secco infection on activated antioxidative enzymes in citrus rootstocks is available. In the present work, antioxidant enzyme activity in different mal secco-resistant and susceptible citrus rootstocks was examined to evaluate the possible differences in constitutive levels of these antioxidant enzymes and correlations between enzymatic levels and mal secco susceptibility/resistance in citrus rootstocks.

Table 1. Disease ratings of *Citrus* rootstocks to mal secco in this study.

Rootstocks	Damage ranking	Resistance
Cleopatra mandarin (*Citrus reshni* Tan.)	0.60	Resistant
Carrizo citrange (*Poncirus trifoliata* L. Raf. X *C. sinensis* L. Osbeck)	1.80	Intermediate resistant
Volkameriana (*C. volkameriana* Tan. and Pasq.)	3.40	Susceptible
Sour orange (*C. aurantium* L.)	4.20	Highly susceptible
Rough lemon (*C. jambhiri* Lush.)	4.40	Highly susceptible
Trifoliate orange (*P. trifoliata* Raf.)	4.40	Highly susceptible

MATERIALS AND METHODS

Plant material

Six citrus rootstocks including Cleopatra mandarin, volkameriana, rough lemon, sour orange, trifoliate orange and Carrizo citrange were used as plant material in this study (Table 1). They vary in resistance to mal secco caused by *P. tracheiphila* (Tuzcu *et al.*, 1989). Seeds were obtained from 25-year-old plants from the citrus collection plot of the Alata Horticultural Research Station (AHRS), Mersin, Turkey. Seeds were treated with benomyl to eliminate pest development and germinated in boxes containing river sand in a semi-controlled greenhouse. When plants reached to 15 cm, 12 plants were transferred to 4-L pots containing a mixture of sand, organic matter and soil (1:1:1) for further plant growth. All plants were managed according to the same standard local commercial practices.

Inoculation and evaluation of mal secco

Phoma tracheiphila isolation, propagation, inoculation and further processes were performed according to Solel and Spiegel-Roy (1978) and Gulsen *et al.* (2007). The pathogen was isolated from 1-year-old tissue of the tree with severe mal secco symptoms located on the orchard of the AHRS, where the disease is epidemic. Five replications of 10-month-old seedlings for each rootstock were inoculated by inserting pathogen plaque under cambium tissue 20 cm above the soil level in February. Then, cambium tissue was covered with wet cotton and wrapped to maintain moisture for pathogen development. Plants were kept in temperature range between 20 and 24 °C. Five seedlings of each 10-month-old rootstock were maintained as control treatment in another greenhouse. Aluminium foil and cotton were removed after 15 days. Mal secco symptoms were evaluated after three months of inoculation using a scale: 0 = no symptoms; 1 = shoot tip is died; 2 = side branches and twigs are died; 3 = whole crown down to infection site is died; 4 = whole crown down to bud union is died; 5 = whole tree including rootstock is died (Tuzcu *et al.*, 1989). Plant resistance was classified in accordance with Tuzcu *et al.* (1989) as follows:

0.00–0.99: resistant; 1.00–1.99: intermediate resistant; 2.00–3.99: susceptible; 4.00–5.00: highly susceptible.

Enzyme extraction and assays

Leaf samples of six citrus rootstocks were collected one month after inoculations when mal secco symptoms were visible on susceptible rootstocks. At the same

time, leaf samples of non-infected control plants were also collected. Soluble proteins were extracted from 20 mg of plant tissue, using a standard sap extraction method described by Gulsen *et al.* (2010) with little modifications. Plant tissues were placed between two rollers of a sap extraction apparatus (Ravenel Specialities Co., Seneca, SC). One and a half ml solution of a 20-mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer at pH 7.2, containing a protease inhibitor cocktail [0.3 g L^{-1} g of tissue of 4-(2-aminoethyl) benzenesulfonyl fluoride, bestatin, pepstatin A, E-64, leupeptin, 1,10-phenanthroline (Sigma, St. Louis, MO)] and 1% polyvinylpyrrolidone (PVP) was dropped on the top of the roller. Homogenate was collected from the bottom of the roller and centrifuged at 15,000 rpm for 15 min at 4 °C. Supernatant was collected and placed at 4 °C (<4 h) for further analysis.

Ascorbate peroxidase (APX) activity was estimated according to Nakano and Asada (1981) with some modifications. The reaction mixture contained a 50-mM potassium-phosphate buffer (pH 7.6), 0.25 mM L(-) ascorbic acid, 12 mM H_2O_2 and 1 μg mL^{-1} protein extract. Volume of the reaction mixture was adjusted to 200 μL and the reaction was evaluated every 30 s for 2 min. Activity of APX was calculated from oxidized ascorbate concentration by using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. One enzyme unit was defined as mmol mL^{-1} oxidized ascorbate per min.

Total peroxidase (TPX) activity was measured by monitoring increase in absorbance at 470 nm for 2 min as described by Gulsen *et al.* (2010). The reaction mixture included 2 μL of 30% hydrogen peroxide to wells of a 96-well microplate containing 60 μL of 18 mM guaiacol, 20 μL of 200 mM HEPES (pH 7.0), 117 μL of distilled water and 1 μL of enzyme extract. Specific activity of peroxidase was determined using molar absorptivity of guaiacol at 470 nm (26.6 × 10^3 M^{-1} cm^{-1}).

Catalase activity was assayed according to Cakmak and Marschner (1992). The reaction mixture included a 25-mm phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme. Decomposition of H_2O_2 was followed at 240 nm (39.4 mM cm^{-1}).

STATISTICAL ANALYSIS

Data were analysed by using JMP software (Version 5.0.1; SAS Institute, 2002). Statistical significance was judged at the $p < 0.05$ level and least significant difference (Student's multiple range test) procedure was used to separate means.

RESULTS

Responses of rootstocks to mal secco

Citrus rootstocks showed differences with regard to resistance against mal secco. Damage ranking of rootstocks varied between 0.60 and 4.40 (Table 1). While Cleopatra mandarin was the most resistant rootstock, Carrizo citrange was classified as intermediate resistant rootstock (1.80). On the other hand, Volkameriana was susceptible whereas the rest, rough lemon, sour orange and trifoliate orange, were highly susceptible.
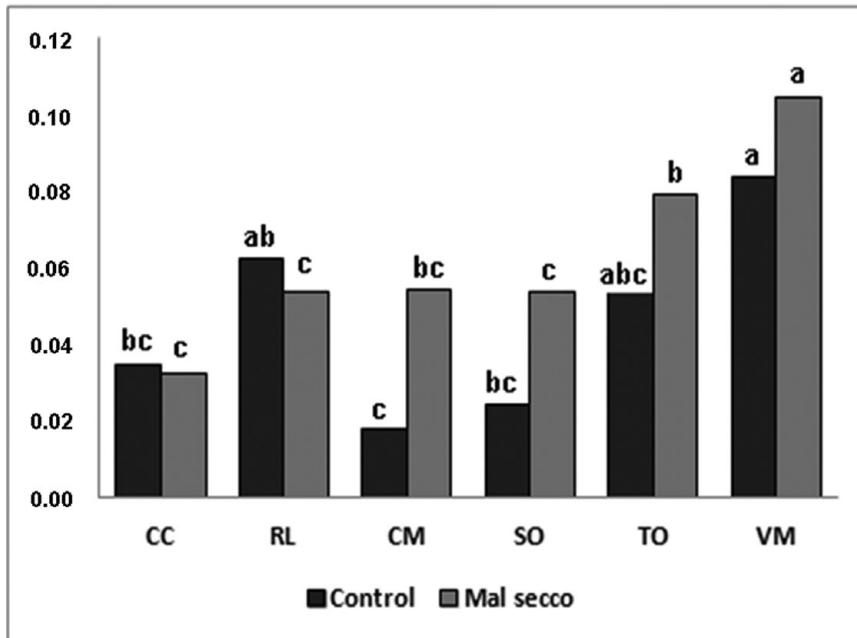
Figure 1. Ascorbate peroxidase activity of rootstock leaves in response to mal secco. Enzyme activity was expressed as (mg protein)$^{-1}$. Each point is the average value of three independent measurements. CC: Carrizo citrange; RL: rough lemon; CM: Cleopatra mandarin; SO: sour orange; TO: trifoliate orange; VM: Volkameriana. Columns with different letters are significantly different ($p < 0.05$) according to Student's multiple range test.

Antioxidative enzyme activity

Several antioxidative enzyme activities were determined in leaves of control and *P. tracheiphila* inoculated plants in order to assess responses of the six citrus rootstocks to mal secco. For APX activity, there were differences between the control and infected plants (Figure 1). In control plants, the highest activity was observed in Volkameriana, whereas Cleopatra mandarin had the lowest activity. Infected plants increased the APX activity except for Carrizo citrange and rough lemon compared to the control plants. Inoculated plants of Cleopatra mandarin, resistant rootstock, showed a significant increase of about 2.5-fold compared to control plants. Similarly, APX activity of sour orange also increased 2.5-fold. The inoculation did not affect APX activity of Carrizo citrange, while this activity slightly decreased in highly susceptible rough lemon.

TPX activity of control plants except for Cleopatra mandarin showed little difference. The highest activity was obtained from highly susceptible trifoliate orange, whereas the lowest activity was determined in mal secco-resistant Cleopatra mandarin (Figure 2). For all rootstocks, TPX activity increased in inoculated plants compared to the control plants. Mal secco-resistant Cleopatra mandarin showed a significant increase of over 3.5-fold in infected plants compared to the control plants. The rest varied between 1.31- (Carrizo citrange) and 1.48-fold (trifoliate orange).

There were significant differences in CAT activities of the control plants. Rough lemon showed the highest activity and Cleopatra mandarin showed the lowest activity
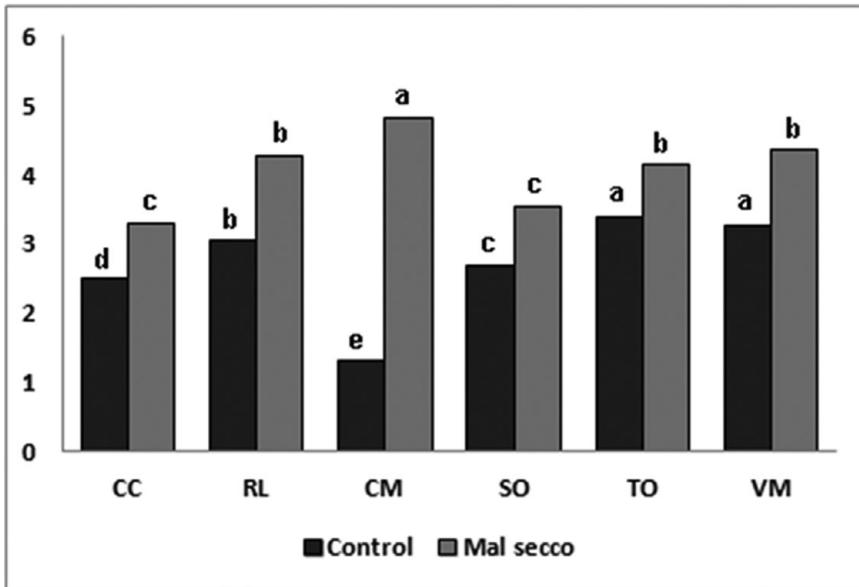
Figure 2. Total peroxidase activity of rootstock leaves infected with *P. tracheiphila*. Enzyme activity was expressed as (mg protein)$^{-1}$. Each point is the average value of three independent measurements. CC: Carrizo citrange; RL: rough lemon; CM: Cleopatra mandarin; SO: sour orange; TO: trifoliate orange; VM: Volkameriana. Columns with different letters are significantly different ($p < 0.05$) according to Student's multiple range test.

(Figure 3). Inoculation with *P. tracheiphila* produced different responses in CAT activity for rootstocks. Enzyme activity of Carrizo citrange, sour orange and Volkameriana increased with inoculation. In contrast, CAT decreased nearly threefold in diseased rough lemon plants compared to control plants. Also, trifoliate orange showed decrease in enzyme activity with inoculation. CAT activity of mal secco-resistant rootstock, Cleopatra mandarin, did not change in response to inoculation.

DISCUSSION

Mal secco is a serious disease of *Citrus*, especially of lemons. Development of resistant or tolerant cultivars is an essential way to prevent mal secco in *Citrus* (Gulsen *et al.*, 2007; Tuzcu *et al.*, 1989; Uzun *et al.*, 2009b). The disease does not show constant epidemics. Different ecological factors and cultural measures affect the response of hosts and desirable results have not been obtained from the breeding programmes (Tuzcu *et al.*, 1989). While Cleopatra mandarin was found to be resistant, Carrizo citrange was classified as intermediate resistant in the present study. Similarly, Tuzcu *et al.* (1989) reported Cleopatra mandarin as intermediate resistant rootstock. On the other hand, Volkameriana was susceptible, whereas the rest of three rootstocks, rough lemon, sour orange and trifoliate orange, were highly susceptible. These four rootstocks were found as susceptible in a previous study (Tuzcu *et al.*, 1989). Current results as regards responses of rootstocks to mal secco were mostly consistent with the previous study.
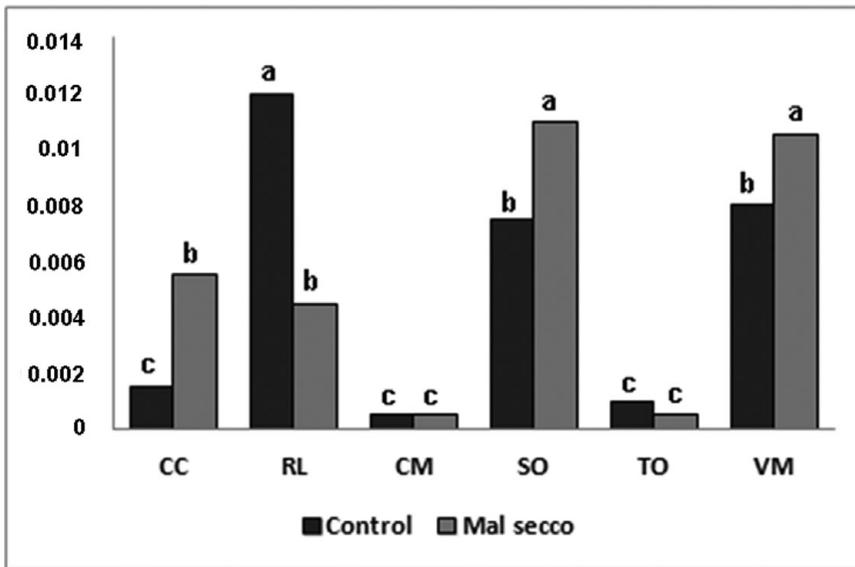
Figure 3. Catalase activity of rootstocks leaves infected with *P. tracheiphila*. Enzyme activity was expressed as (mg protein)$^{-1}$. Each point is the average value of three independent measurements. CC: Carrizo citrange; RL: rough lemon; CM: Cleopatra mandarin; SO: sour orange; TO: trifoliate orange; VM: Volkameriana. Columns with different letters are significantly different ($p < 0.05$) according to Student's multiple range test.

Note that one of the most important events in the early phase of the incompatible plant–pathogen interaction was the rapid and transient production of activated oxygen species (AOS), such as superoxide radical (O_2 $^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen (O_2), called the oxidative burst (Baker and Orlandi, 1995; Hernandez *et al.*, 2004). In order to cope with this stress, plants evolved some mechanisms called antioxidative systems to detoxify these oxidative chemicals. These include enzymes such as SOD, CAT and peroxidases (Hernandez *et al.*, 2004). Changes in response to *P. tracheiphila* were investigated in this study. Change of enzyme activities of rootstocks as regards this infection differed. APX activity increased in mal secco-resistant Cleopatra mandarin, susceptible Volkameriana, and highly susceptible sour orange and trifoliate orange but with varying levels. APX did not change in intermediate resistant Carrizo citrange, while decreased in highly susceptible rough lemon. APX induction might cause reduction in the H_2O_2 level, and thus it may have a limited signal transduction effect, by effectively removing H_2O_2, as it was formed (Hernandez *et al.*, 2001). APX level was found to be higher in mycorrhizal fungus-inoculated *C. tangerine* roots than non-inoculated roots (Wu *et al.*, 2006a). Similar results were also obtained from shoots of *O. europaea* ssp. *sylvestris* that was inoculated with mycorrhizal fungi (Alguacil *et al.*, 2003). APX activity showed a gradual increase compared to control after the beginning of fungi (*Mycosphaerella fragariae*) inoculation in strawberry genotypes tested and peaked on the third day, after which activities returned to initial levels (Ding *et al.*, 2011). Higher increase of APX activity was found in the leaves of resistant genotypes when compared to susceptible ones. Increased

APX activity was due to the increased H_2O_2 production and the resistant genotypes obviously had greater capacity to metabolize H_2O_2 to H_2O to alleviate the injury to strawberry leaves. It was notified that an increase in APX activity could not stop the development of symptoms caused by infection, but may have helped to reduce severity of the disease and possibly to allow the plants to recover (Hernandez *et al.*, 2004). This may apply in this study. In another study, APX activity decreased in orange fruit flavedo and albedo infected with *Penicillium digitatum* compared to control (Ballester *et al.*, 2006). Similarly, activity of APX markedly decreased in tomato leaves following the inoculation of *Botrytis cinerea* fungi (Kuzniak and Sklodowska, 2005).

It was previously suggested that increased peroxidase activity in resistant plants might allow the plant to detoxify peroxides and therefore sustain less tissue damage than susceptible plants (Heng-Moss *et al.*, 2004; Hildebrand *et al.*, 1986). Increased TPX activity was observed in all resistant and susceptible rootstocks in response to mal secco, and indicated the highest correlation with disease rating of infected plants. Similarly, total peroxidases also increased in apple fruits inoculated with *Penicillium expansum* (Torres *et al.*, 2003). Accordingly, tomato peroxidase activity increased with inoculation of *Fusarium oxysporum* f. sp. lycopersici (Mandal *et al.*, 2008).

In the present study, there was no positive correlation between CAT activity and mal secco resistance. It did not change in resistant Cleopatra mandarin, whereas it increased in highly susceptible sour orange and decreased in highly susceptible rough lemon. Similar results were also reported for other plants. Increased catalase activity was not associated with increased levels of resistance to insects (Heng-Moss *et al.*, 2004; Hildebrand *et al.*, 1986). CAT activity was higher in the leaves of mycorrhizal *P. trifoliata* than those in non-mychorrizal plants (Wu *et al.*, 2006b). In *O. europaea* plants, the inoculation treatments of mycorrhizal fungus increased the CAT level compared to non-inoculated plants (Alguacil *et al.*, 2003). CAT activity in fungus (*Botrytis cinerea)* inoculated tomato leaves was induced at the initial phase of the tomato–*B. cinerea* interaction, later CAT activity decreased (Kuzniak and Sklodowska, 2005). The researchers reported that the role of CAT in the biotic stress process might be more complex than in abiotic stress. An increase of foliar CAT activity was also observed in both susceptible and resistant oats (*Avena sativa* L.) in responses to attack by the biotrophic fungal pathogen *Blumeria graminis*, which causes powdery mildew (Vanacker *et al.*, 1998). On the other hand, it was reported that plants overproducing CAT have a decreased resistance to pathogen infection (Mittler, 2002; Polidoros *et al.*, 2001). Abedi and Pakniyat (2010) argued that reduction of CAT activity was probably due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under stress conditions. They also reported that downregulation of CAT activity was due to degradation caused by induced peroxisomal proteases or due to photo-inactivation of the enzyme.

In the present study, morphological and antioxidant enzyme responses of six citrus rootstocks inoculated with *P. tracheiphila* were evaluated. This was the first report to evaluate antioxidative enzyme responses of citrus rootstocks infected with *P. tracheiphila*. Resistances of rootstocks to mal secco varied and the results were generally consistent with previous reports. As regards antioxidant enzymes, high-level increases of APX

and TPX in resistant Cleopatra mandarin may be associated with the resistance to mal secco. This study brought valuable insights into defence mechanisms of six citrus rootstocks with varying genetic compositions (Gulsen *et al.*, 2001; Uzun *et al.*, 2009a). Further studies including other antioxidant enzymes could allow a better understanding of the relation between disease resistance and antioxidant enzyme activity.

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