

# Phthalic Acid Induces Oxidative Stress and Alters the Activity of Some Antioxidant Enzymes in Roots of *Malus prunifolia*

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**Abstract** Apple replant is a widespread agricultural problem documented in all of the major fruit-growing regions of the world. In order to better understand the phytotoxic mechanisms induced by allelochemicals involved with this problem, *Malus prunifolia* plants were grown hydroponically to the six-leaf-stage in the presence of phthalic acid (0 or 1 mM) for 5, 10, or 15 days. Apple plants were evaluated for: shoot and root length, fresh and dry weight, malondialdehyde (MDA) content, hydrogen peroxide ( $H_2O_2$ ) content, superoxide radical ( $O_2^{\cdot-}$ ) generation rate, and antioxidant enzyme activities. Shoot and root lengths and fresh and dry weights of *M. prunifolia* decreased in plants exposed to phthalic acid. MDA and  $H_2O_2$  content increased in phthalic acid-treated plants as did the generation rate of  $O_2^{\cdot-}$  in *M. prunifolia* roots. The activities of superoxide dismutase (EC 1.15.1.1), peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6), ascorbate peroxidase (EC 1.11.1.11), glutathione reductase (EC 1.6.4.2), dehydroascorbate reductase (EC 1.8.5.1), and monodehydroascorbate reductase (EC 1.6.5.4) increased in phthalic acid-stressed roots compared with control roots. These results suggest that activation of the antioxidant system by phthalic acid led to the formation of reactive oxygen species that resulted in cellular damage and the decrease of *M. prunifolia* growth.

**Keywords** Antioxidant enzymes · Growth inhibition · *Malus prunifolia* · Membrane damage · Oxidative stress · Phthalic acid

## Introduction

Allelopathy is widely reported in agroecosystem and silviculture, and is implicated in problems such as exotic plant invasion, replant problems, and soil sickness (Lee et al. 2006; Hao et al. 2007; Fernandez et al. 2008). Allelopathic plants exert detrimental effects via the release of plant compounds (allelochemicals) through leaching, root exudation, volatilization, and/or decomposition of plant materials (Weir et al. 2004) and can interfere with the metabolism of other plants. If the effect of such compounds is harmful to plant growth and development, it becomes a biotic or allelochemical stress (Romero-Romero et al. 2005).

Under normal growth conditions, a dynamic equilibrium exists between the production and detoxification of free radicals in cell organelles. Biotic and abiotic stresses may cause the formation of reactive oxygen species (ROS) such as superoxide radical ( $O_2^{\cdot-}$ ), hydroxyl radical ( $OH^{\cdot}$ ), and/or hydrogen peroxide ( $H_2O_2$ ) that are commonly generated and accumulated in cells (Cho and Seo 2005). Enhanced ROS production can affect membrane permeability, damage DNA and proteins, induce lipid peroxidation, and ultimately lead to programmed cellular death (Ding et al. 2007). Plants have evolved mechanisms that protect cell and subcellular systems from the effects of ROS by using antioxidant systems such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase

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(MDHAR). Tolerance to allelochemicals by various crop plants has been associated with an increase in the activity of antioxidant enzymes (Yu and Matsui 1997).

The apple replant problem is widespread. Studies have shown that allelochemicals in root exudates or decomposition of residues may play a role in this agricultural problem (Borner 1959; Zhang et al. 2007; Bai et al. 2009). However, the mechanisms involved have not been investigated extensively. Reports suggest that allelochemicals cause increased membrane leakage, and enhance  $H_2O_2$  and MDA levels in the target plant tissues. For instance, cinnamic acid enhanced the generation of free radicals, increased lipid peroxidation, and oxidative membrane damage in cucumber and figleaf gourd plants (Ding et al. 2007). Similarly, 2-benzoxazolinone inhibited lettuce growth, caused membrane damage, and increased MDA and  $H_2O_2$  production (Sanchez-Moreiras and Reigosa 2005). Most studies in this regard focused on vegetables and agronomic crops (Baziramakenga et al. 1995; Yu et al. 2003; Ye et al. 2006). However, it is not known whether the inhibitory effects were mediated through production of ROS in apple roots under allelochemical stress. Phthalic acid is a potent allelochemical and inhibits the growth of a number of plants such as *Lactuca sativa* L. (Lee et al. 2006), *Zea mays* L. (Chai and Feng 2007), and *Malus prunifolia* (Bai et al. 2009). Accordingly, its effect on plant growth, ROS generation rate, and ROS-antioxidant enzyme activity were investigated in *M. prunifolia* seedlings.

## Methods and Materials

**Plant Materials and Phthalic Acid Treatment** Seeds of apple rootstock, *M. prunifolia*, were obtained from Fuping County (34°75' N, 109°15' E), Shaanxi Province. Seed sterilization was done according to Zhang et al. (2007) and involved surface-sterilization in 0.3% (v/v)  $H_2O_2$  for 20 min, followed by several rinses with sterile  $H_2O$ . The sterilized seeds were stratified at 4°C for 85 days. Sprouted seeds were sown in plastic pots (9 cm in diameter, 12 cm high; three seeds per pot) filled with sterilized sand. All pots were placed in a greenhouse at the College of Horticulture, Northwest A&F University, Yangling (34°20' N, 108°24' E). Plants were grown without supplementary illumination with night and day temperatures at 20 to 25°C and relative humidity at 65–80%. Seedlings were watered once a week with Hoagland nutrient solution (Hoagland 1920), pH 6.0±0.2. When the seedlings reached the six-leaf-stage, batches of 45 uniform seedlings were transferred into a hydroponic system (plastic container; 45×37×22.5 cm) filled with 5 l half Hoagland nutrient solution at pH 6.0±0.2 and electrical conductivity at 1.2 ms/cm, respectively. The containers were placed in a controlled

growth room with a L/D regime of 12/12 h, 25/20°C, and a photon flux density of 140–160  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Seedlings were allowed to acclimate to the hydroponic conditions for 5 days. Phthalic acid (purchased from Yifang S&T Ltd. Tianjin, China), dissolved in ethanol, was added to the nutrient solution to concentrations of 0 or 1 mM. In the preliminary experiment, thinner *M. prunifolia* leaves and brownish root apices in parallel with a large amount of mucilage was secreted from roots 15 days after application of 1 mM phthalic acid. This indicates that phthalic acid at this concentration is lethal to *M. prunifolia* seedlings. The final concentration of ethanol in control and treatment solutions was 0.1% (v/v). The nutrient solutions in the containers were aerated continuously by air pumps. The solution in the plastic container was kept at the same level by adding half Hoagland nutrient solution at 24 h intervals. Each treatment was replicated three times in a completely randomized design. Root samples were taken from both control and phthalic acid-treated plants on days 5, 10, and 15 after treatment, and the tissue was frozen in liquid nitrogen and stored at -70°C until analysis. At the end of the experiment (15 days after treatment), root and shoot length and fresh and dry weight of seedlings were measured. The seedlings were dried in a forced-air oven at 60°C until constant mass was reached.

**Measurement of MDA,  $H_2O_2$ , and  $O_2^{\cdot-}$  Generation** Lipid peroxidation was followed by measuring MDA accumulation using the method of Baziramakenga et al. (1995) with some modifications. Root samples (0.2 g) were homogenized in 0.1% of trichloroacetic acid in phosphate buffer (5 ml; pH 7.8) and centrifuged at 12,000×g for 15 min. Supernatant (1 ml) was added to 0.5% thiobarbituric acid in 20% trichloroacetic acid (4 ml). The mixture was placed in a water bath at 100°C for 10 min and then quickly cooled in an ice-bath for 15 min. Samples were centrifuged at 12,000×g for 5 min, and then the absorbance of the supernatant was measured at 450, 532, and 600 nm.

$H_2O_2$  in the supernatant was measured according to Patterson et al. (1984). Root samples (0.5 g) were homogenized in 5-ml pre-cooled acetone and centrifuged for 10 min at 1,500×g. Titanium chloride (0.1%, w/v) and concentrated ammonia (0.2 ml) were added into the supernatant (1 ml), the mixture was allowed to react (10 min at 25°C) and the reaction mixture was centrifuged at 1,500×g for 10 min. Absorbance at 410 nm was measured, and the  $H_2O_2$  concentration was calculated according to a standard curve.

The rate of  $O_2^{\cdot-}$  generation was measured as described by Elstner and Heupel (1976) with some modifications. Root tissue (1 g) was homogenized in 65 mM potassium phosphate buffer (3 ml; pH 7.8). The homogenate was centrifuged at (10,000×g for 15 min). The supernatant

(0.5 ml) was added to 65 mM potassium phosphate buffer (0.5 ml; pH 7.8) containing 10 mM hydroxylammonium-chloride (0.1 ml) and incubated (25°C for 20 min). Sulphanilic acid (58 mM; 1 ml) and  $\alpha$ -naphthyl amine (7 mM; 1 ml) were added to the mixture, and it was allowed to incubate (25°C for 20 min). The final solution was mixed with an equal volume of chloroform and the absorbance of the pink phase was measured at 530 nm.

**Extraction and Assay of Enzyme Activities** Antioxidant enzymes (SOD, POD, and CAT) were extracted according to the method of Yu et al. (2003) with some modifications. Root samples (0.5 g) were homogenized in phosphate buffer (8 ml; 0.1 M; pH 7.5) containing 2% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged (12,000 $\times$ g for 20 min) and the supernatant was used for enzyme analysis. All assays were carried out at 2–4°C.

Superoxide dismutase activity was measured according to Beauchamp and Fridovich (1971) with minor modification. The assay medium (3 ml) contained phosphate buffer (50 mM; pH 7.8), EDTA-Na (0.1 mM), L-methionine (12 mM), riboflavin (2  $\mu$ M), and nitrotetrazolium blue chloride (75  $\mu$ M). Riboflavin was added last. The tubes were shaken and placed at a photosynthetic photon flux of 50  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> for 15 min. The reaction was initiated and terminated by turning the light on and off, respectively. The A<sub>560</sub> was measured on a spectrophotometer and tubes containing the assay mixture, but without the root extract (control), were illuminated to determine maximum A<sub>560</sub>.

Peroxidase activity was measured according to Sofo et al. (2004) with some modification. The reaction solution (3 ml) contained phosphate buffer (2.9 ml, 50 mM; pH 7.0), guaiacol (50  $\mu$ l; 10 mM), H<sub>2</sub>O<sub>2</sub> (10  $\mu$ l; 40 mM), and crude enzyme extract (40  $\mu$ l). The increase in A<sub>470nm</sub> due to the oxidation of guaiacol was measured at 20°C.

Catalase activity was assayed by monitoring the decrease in A<sub>240nm</sub> (Aebi 1984). The reaction mixture contained phosphate buffer (50 mM; pH 7.0) and H<sub>2</sub>O<sub>2</sub> (30% w/v) and was started by adding the reaction solution to crude extract (10  $\mu$ l).

AsA-related enzymes were extracted according to Nakano and Asada (1981). Generally, each 0.5 g of roots material was homogenized in KH<sub>2</sub>PO<sub>4</sub>-KOH (6 ml, 50 mM; pH 7.5) containing ethylenediaminetetraacetic acid (0.1 mM), Triton X-100 (0.3% v/v), and insoluble polyvinylpyrrolidone (4% w/v). The homogenate was centrifuged (16,000 $\times$ g for 15 min at 2°C) and the supernatant was used for APX, GR, MDHAR, and DHAR analyses.

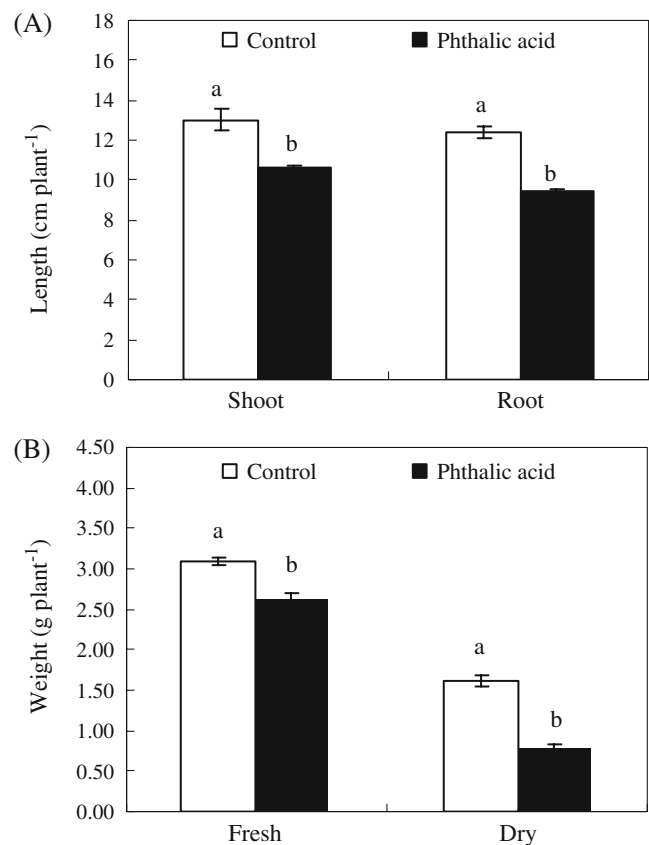
Ascorbate peroxidase was measured by monitoring the decrease in A<sub>290 nm</sub> (Nakano and Asada 1981). The assay mixture (1 ml) contained Hepes-KOH (50 mM; pH 7.6), ethylenediaminetetraacetic acid (0.1 mM), H<sub>2</sub>O<sub>2</sub> (0.2 mM, AsA 0.5 mM), and enzyme extract. The reaction was initiated by adding H<sub>2</sub>O<sub>2</sub>.

Glutathione reductase activity was monitored at A<sub>340 nm</sub> in a 1 ml reaction mixture containing Tris-HCl (100 mM; pH 8.0), ethylenediaminetetraacetic acid (1 mM), oxidized glutathione (GSSG; 1 mM), and NADPH (0.2 mM). The reaction was initiated by adding NADPH (Grace and Logan 1996).

Monodehydroascorbate reductase activity was assayed at 340 nm in a 1 ml reaction mixture containing Hepes-KOH (50 mM; pH 7.6), NADH (0.1 mM), AsA (0.25 mM), and AsA oxidase (EC 1.10.3.3; 0.25 U). The reaction was initiated by adding AsA oxidase (Miyake and Asada 1992).

Dehydroascorbate reductase activity was measured at 265 nm in a 1 ml assay solution containing Hepes-KOH (100 mM; pH 7.0), ethylenediaminetetraacetic acid (1 mM), GSH (2.5 mM), and DHA (0.2 mM). The reaction was initiated by adding DHA (Dalton et al. 1986).

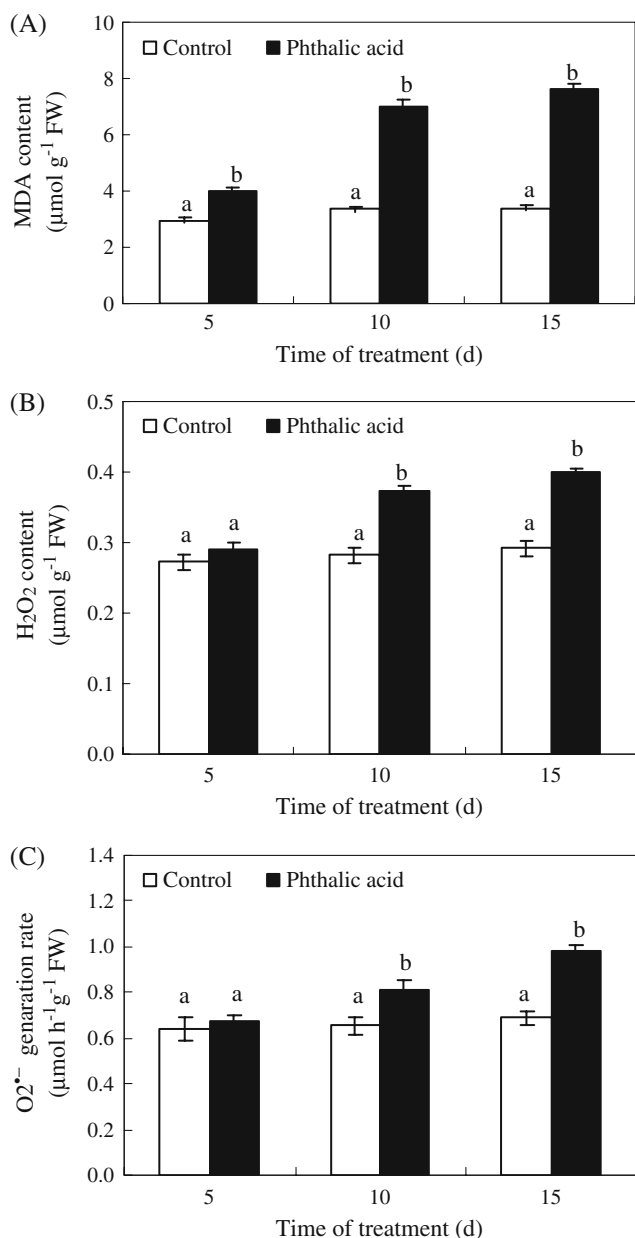
**Statistical Analysis** All data were subjected to analysis of variance, followed by Tukey's Studentized Range Test (SAS Statistical package, version 8.2). Results are presented as the means  $\pm$  standard deviation (SD).



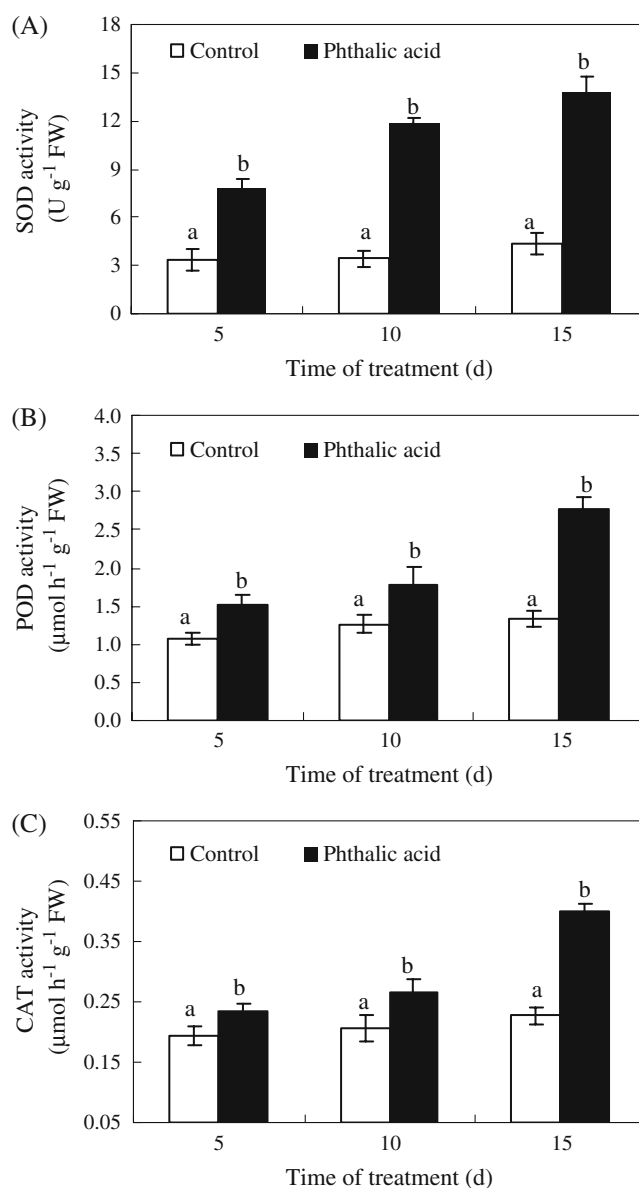
**Fig. 1** Shoot and root length (a) and fresh and dry weight (b) of *Malus prunifolia* plants grown at 0 (control) or 1 mM phthalic acid. Samples were taken 15 days after treatment. Values are means of three replicates  $\pm$  standard error (SE). Significant difference ( $P < 0.05$  level) was tested between the control and 1 mM phthalic acid treatment for each dependent variable and indicated by different letters above the bars

## Results

**Effect on Plant Growth** The toxic effect of 1 mM phthalic acid appeared after 15 days following treatment. The length of shoot and root of *M. prunifolia* plants had reduced shoot and root length compared to lower than controls (Fig. 1a). Fresh and dry weights were 3.09 and 1.61 g plant<sup>-1</sup> in control plants compared to 2.63 and 0.79 g plant<sup>-1</sup> for plants treated with 1 mM phthalic acid (Fig. 1b).



**Fig. 2** MDA (a), H<sub>2</sub>O<sub>2</sub> (b) content, and O<sub>2</sub><sup>-</sup> generation rate (c) in *Malus prunifolia* plants grown at 0 (control) or 1 mM phthalic acid. Values are means of three replicates $\pm$ standard error (SE). Significant difference ( $P<0.05$  level) was tested between the control and 1 mM phthalic acid treatment at each sampling date separately and indicated by different letters above the bars



**Fig. 3** SOD (a), POD (b), and CAT (c) activities of *Malus prunifolia* plants grown at 0 (control) or 1 mM phthalic acid. The values are means of three replicates $\pm$ standard error (SE). Significant difference ( $P<0.05$  level) was tested between the control and 1 mM phthalic acid treatment at each sampling date separately and indicated by different letters above the bars

**Effect on MDA, H<sub>2</sub>O<sub>2</sub>, and O<sub>2</sub><sup>-</sup> Generation** MDA concentration in *M. prunifolia* roots increased significantly after 5, 10, and 15 days of treatment (Fig. 2a). The maximum increase of 125.4% was observed after 15 days. Exposure to phthalic acid also resulted in an increase in the H<sub>2</sub>O<sub>2</sub> content and O<sub>2</sub><sup>-</sup> generation rate, respectively (Fig. 2b, c). The maximum increases of 37.2% and 29.9%, respectively, were observed after 15 days.

**Effect on Enzyme Activity** In response to phthalic acid, activities of SOD, POD, and CAT, as well as enzymes in

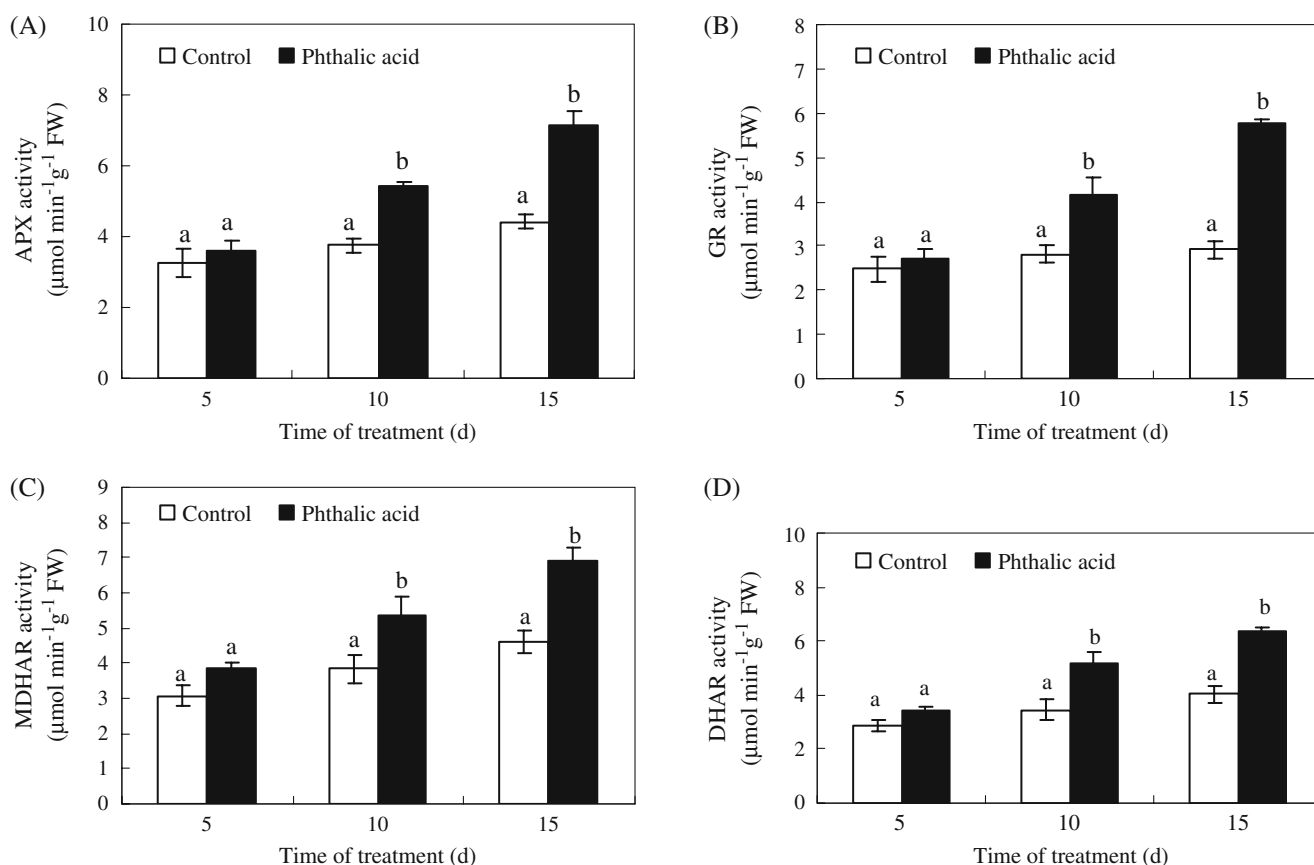
ascorbate-glutathione cycle, e.g., APX, GR, MDHAR, and DHAR, all showed similar trends in the activity of root enzymes in response to phthalic acid. In general, enzyme activities increased progressively during the treatment period (days 5–15) compared to enzymes in the roots of plants not exposed to phthalic acid (Figs. 3 and 4).

## Discussion

Phthalic acid treatment significantly decreases the growth of *M. prunifolia* plants (Fig. 1). This finding is in agreement with studies on *L. sativa* L. (Lee et al. 2006) and *Z. mays* L. (Chai and Feng 2007), and consistent with the inhibitory effects of other allelochemicals on plant growth reported previously (Lin and Kao 2000; Asao et al. 2004; Batish et al. 2008).

The formation of free radicals in cells results in damage to cell membranes due to lipid peroxidation. Thus, the level of MDA, produced during lipid peroxidation, is a good indicator of oxidative damage that could be occurring

within cells (Masia 2003). MDA accumulation due to lipid peroxidation has been reported in response to a variety of abiotic and biotic stresses (Dhindsa et al. 1981; Apel and Hirt 2004). In the present study, enhanced MDA (Fig. 2a) suggests that phthalic acid probably induces oxidative stress, and, as a result, disrupts cellular membrane structure, and causes a loss of cellular integrity. Similar results with other allelochemicals have been found (Wu et al. 2002; Batish et al. 2006; Ye et al. 2006). Many studies show that increased MDA content is associated with increased  $O_2^{\cdot-}$  and  $H_2O_2$  production following biotic and abiotic stresses (Forman et al. 2002; Lara-Nunez et al. 2006). Here, phthalic acid induced  $O_2^{\cdot-}$  and  $H_2O_2$  production in *M. prunifolia* roots (Fig. 2b, c), which suggests that phthalic acid could trigger ROS generation and induce oxidative stress in *M. prunifolia* roots. This observation is consistent with that obtained with mung bean treated with 2-benzoxazolinone (Batish et al. 2006). Increases in SOD activity (Fig. 3a) also indicate that excessive generation of  $O_2^{\cdot-}$  has been triggered by phthalic acid treatment, and, consequently, that SOD activity was up-regulated to mitigate the oxidative damage. Similar responses have been



**Fig. 4** APX (a), GR (b), MDHAR (c), and DHAR (d) activities of *Malus prunifolia* plants grown with 0 (control) or 1 mM phthalic acid. The values are means of three replicates  $\pm$  standard error (SE).

Significant difference ( $P < 0.05$  level) was tested between the control and 1 mM phthalic acid treatment at each sampling date separately and indicated by different letters above the bars

observed in plants treated with other allelochemicals (Yu et al. 2003; Batish et al. 2006; Wu et al. 2002; Lin et al. 2007).

Accumulation of H<sub>2</sub>O<sub>2</sub> in *M. prunifolia* roots in response to phthalic acid treatment enhances lipid peroxidation and causes a severe oxidative stress resulting in disruption of metabolic activities in the cell. Enhanced H<sub>2</sub>O<sub>2</sub> levels are removed by the antioxidant enzymes such as CAT, APX, and POD (Blokhina et al. 2003), and glutathione-ascorbate cycle (Nakano and Asada 1981). In the present study, increases in the activities of these antioxidant enzymes paralleled the accumulation of MDA and H<sub>2</sub>O<sub>2</sub> in *M. prunifolia* root after exposure to phthalic acid (Figs. 2–4). Our observations are consistent with those of Cruz-Ortega et al. (2002), who reported that allelochemical stress causes increases in the level of free radicals and the activity of antioxidant enzymes and suggests that increased induction of these enzymes is necessary to prevent lipid peroxidation (i.e., to counter the higher MDA and H<sub>2</sub>O<sub>2</sub> in roots). Although there were increased levels of antioxidants in roots after exposure to phthalic acid, oxidative stress still occurred. Our data are consistent with those of Batish et al. (2006), who showed that exposure of mung bean to 2-benzoxazolinone increased APX, GR, and CAT activity. An increased POD activity in response to allelochemicals also has been demonstrated in cucumber root (Yu et al. 2003). Oracz et al. (2007) observed an increase in the cell membrane permeability, MDA level, H<sub>2</sub>O<sub>2</sub> concentration, and SOD and CAT activity in mustard treated by sunflower extract. Furthermore, the activity and expression of most antioxidant enzymes is stimulated by ROS accumulation (Apel and Hirt 2004).

In summary, phthalic acid induces oxidative stress in *M. prunifolia* roots through the generation of ROS and decreases plant growth, despite the concomitant increase in antioxidant enzymes. This might be one of the mechanisms responsible for the apple replant problem. The increase in antioxidant enzymes could reflect a defensive response to the cellular damage provoked by phthalic acid treatment. However, this increase was not strong enough to eliminate all the deleterious effects provoked by phthalic acid, only alleviated the impact of stress.

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