Exogenous nitric oxide protects against drought-induced oxidative stress in *Malus* rootstocks

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Abstract: Drought is a major environmental stress that limits the growth and productivity of fruit trees in semiarid and arid regions. We evaluated the potential of exogenous nitric oxide (NO) to improve the drought tolerance of apple rootstocks (*Malus* spp.). Leaves of 2-year-old seedlings of drought-sensitive *Malus hupehensis* (Pamp.) Rehd. and drought-tolerant *Malus sieversii* (Ledeb.) M.Roem. rootstocks were sprayed with NO donor sodium nitroprusside (SNP) at 0–400 µmol L^{-1}, and then the plants were subjected to drought stress. Among all SNP treatments, the 300 µmol L^{-1} SNP treatment mostly alleviated drought-induced ion leakage and the accumulation of malondialdehyde and soluble proteins in *M. sieversii* and *M. hupehensis* leaves. These changes helped to maintain leaf water potential and relative water content of the apple rootstocks under drought stress. The activities of several antioxidant enzymes in leaves increased under drought stress, whereas photochemical efficiency decreased. The adverse effects of drought were exacerbated by treatment with the NO scavenger cPTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt; 400 µmol L^{-1}); however, this effect was offset by NO application. These results suggested that the NO donor SNP effectively protected *Malus* seedlings from drought-induced oxidative damage by enhancing antioxidant enzyme activities and photosynthetic performance.

Key words: Drought tolerance, nitric oxide, *Malus hupehensis*, *Malus sieversii*, antioxidant enzymes

1. Introduction

Agriculture is currently the largest consumer of water resources in the world, especially in vast arid and semiarid areas (Seckler et al., 1997). Understanding the effects of drought on plants is essential for improving management practices and breeding strategies in agriculture (Chaves et al., 2003). In recent decades many studies have focused on plant responses to drought. Such studies have covered subjects ranging from the genetics of enhanced water-use efficiency to the physiological and biochemical processes that reduce the reliance of agriculture on fresh water resources (Chaves et al., 2002, 2003; Ashraf, 2010).

In China, the northwestern region of the Loess Plateau is becoming an important area for apple production. The vast apple-growing area (1.14 × 10^6 ha) in this region accounts for approximately 22% of the world's total apple growing areas. *Malus*, an apple genus native to the temperate zone of the northern hemisphere, is widely used as rootstocks for apple cultivation in the semiarid areas of the Loess Plateau. In an effort to improve apple production, researchers have focused on improving the

positive mechanisms of *Malus* against drought. Various studies have focused on elucidating the biochemical responses of apple cultivars during drought resistance (Bai et al., 2011), on selecting drought-resistant rootstocks (Liu et al., 2010), on promoting drought-related gene expression (Wang et al, 2011), and on determining the effects of exogenous substances, such as abscisic acid, jasmonic acid, and glycine betaine, on drought resistance (Bai et al., 2009). Application of exogenous abscisic acid was shown to increase the drought tolerance of 1-year-old *Malus sieversii* and *Malus hupehensis* seedlings to drought stress (Ma et al., 2008).

Nitric oxide (NO), a small molecule that is ubiquitous in plants, has many physiological roles. As a labile free radical, NO can act as an antioxidant to directly scavenge reactive oxygen species (ROS), thus protecting plants against various environmental stresses (Beligni, 1999, 2002; Arasimowicz et al., 2007; Lei et al., 2007). In plants, NO is involved in regulating tissue growth and development (Delledonne et al., 1998; Xu et al., 2010), protective response to oxidative stress (García-Mata et al.,

2001; Wang et al., 2012), organ maturation and senescence (Guo et al., 2005; Arasimowicz et al., 2007), and stomatal closure (Desikan et al., 2004).

Application of exogenous NO to whole plants or cell cultures has been shown to affect specific physiological and biochemical processes. For example, exogenous NO was shown to mediate NO synthase-like activity in the water stress signaling pathway, induce stomatal closure, and enhance the adaptive response to drought stress (García-Mata et al., 2001; Hao et al., 2008). Exogenous NO was shown to have dose-dependent effects on plant physiological responses, namely a promoting effect at low concentrations and an inhibitory effect at high concentrations (Qiao et al., 2008). Thus, spraying an appropriate amount of exogenous NO could alleviate oxidative damage caused by drought stress. In practice it is difficult to measure the amount of endogenous NO (Xu et al., 2010). Therefore, to confirm the effects of NO in experiments, cPTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) has been used to scavenge endogenous NO (Beligni et al., 1999; García-Mata et al., 2002). Although previous studies have described NO-based strategies for improving the drought tolerance of plants (García-Mata et al., 2001; Zhu et al., 2002), there have been no studies on whether exogenous NO can alleviate drought-induced damage in *Malus* rootstocks.

The aim of the present study was to determine whether the NO donor sodium nitroprusside (SNP) could activate protective responses in *Malus* rootstocks against drought stress by increasing the activity of antioxidant enzymes and decreasing photosynthesis. To this end, we monitored the changes in antioxidant enzyme activities, photosynthetic characteristics, and fluorescence parameters in leaves of seedlings of two *Malus* rootstocks treated with SNP, cPTIO, or a combination of these compounds under drought stress. The results of these experiments provide reference data that can be used to develop management strategies for *Malus* rootstocks for improving their drought tolerance in arid and semiarid regions.

2. Materials and methods

2.1. Plant materials and growth conditions
In autumn 2010, seeds of drought-sensitive *M. hupehensis* (Pamp.) Rehd. and drought-tolerant *M. sieversii* (Ledeb.) M.Roem (Bai et al., 2011; Liu et al., 2011) were respectively collected from Pingyi, Shandong Province (35°07′N, 117°25′E), and the Gongliu, Xinjiang region (42°07′N, 86°37′ E), China.

The field experiments were conducted at the Northwest Agricultural & Forestry University, Yangling (34°20′N, 108°24′E), China. Seeds were stratified on sand at 4 °C for 35–40 days and then planted in plastic pots (12 × 12 cm, one seed per pot) filled with sand. The pots were placed

in a greenhouse under natural light and temperature conditions. At the two-true-leaf stage, the seedlings were transplanted into larger pots (25 × 35 cm, one plant per pot) filled with soil (0–20 cm surface loam soil from an area near the university) and a mixture of perlite, vermiculite, and manure (volume ratio 1:1:1), and then grown as described by Bai et al. (2011).

In March 2012, we selected 48 *M. hupehensis* seedlings and 48 *M. sieversii* seedlings at a similar growth stage and replanted them in larger plastic pots (40 × 35 cm, one plant per pot) filled with a mixture of soil and perlite–vermiculite–manure (1:1, 28.4% field moisture capacity, 1.4 kg mixed soil per pot). The plants were grown for another 4 months under a rain shelter in natural environmental conditions.

We established two soil moisture content treatments using the weighing method of Shao et al. (2007): moist soil (70%–75% of field moisture capacity, adjusted by weighing) and severe drought (40%–45% of field moisture capacity, achieved by withholding water for 7 days). The upper side of the pots was covered with a white plastic bag (45 × 40 cm) to prevent evaporation of soil moisture through the soil surface, and the pots were wrapped in reflective film to avoid excessive heating of the soil (Zhang et al., 2013).

2.2. Experimental treatments
Experiment 1: Aqueous solutions of the NO donor SNP dehydrate (Sigma, St. Louis, MO, USA) were prepared at concentrations of 0, 50, 100, 200, 300, and 400 μmol L⁻¹. The concentration range was chosen by referring to the literature (Beligni et al., 1999, 2000; Xu et al., 2010). Each assay was repeated three times and included a no-drought control (T0). A total of 18 seedlings were sprayed with the SNP solutions on both leaf surfaces until droplets formed at 0900 hours, 12 July 2012. Each concentration of the solution was applied to the leaves five times a day. The leaves were allowed to dry after each application.

The soil moisture content was 72.1% of the field moisture capacity before treatment, and it decreased to 40.7% after 7 days of withholding water. Plants grown with drought-stress conditions and under natural humidity served as the control. All experiments were carried out in a completely randomized block design. Leaves of the same age were sampled at three different positions on the plant (upper, middle, and lower; two leaves at each position), and each replicate was taken from a different plant on day 7. The leaf samples were detached and wrapped in wet absorbent gauze, and were then immediately taken to the laboratory to determine membrane permeability (MP) and malondialdehyde (MDA) and soluble protein (SP) contents. Each assay was repeated four times.

Experiment 2: The optimum concentration of SNP (as determined in experiment 1, 300 μmol L⁻¹ SNP) and

400 μmol L^{-1} carboxy-PTIO potassium salt (cPTIO, a NO scavenger; 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; Sigma) were applied to both leaf surfaces on the remaining 30 seedlings at 0900 hours, 22 July 2012. Each assay included six replicates. In each treatment, the solution was applied to the leaves five times on the same day, as described above. SNP and cPTIO were applied sequentially. The soil moisture content was 71.6% of the field moisture capacity before the treatment, and it decreased to 42.3% after 7 days of withholding water.

The seedlings were divided into five treatment groups: control (T0), no drought or NO; T1, drought; T2, drought + SNP; T3, drought + cPTIO; and T4, drought + SNP + cPTIO. Leaf samples of the same age were collected from different positions of each plant at 0900 hours, 29 July 2012, as described above. Some of the leaves were wrapped in wet absorbent gauze and immediately taken to the laboratory, and the rest were quickly frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. MP, MDA, and SP analysis
We determined MP according to Sairam and Srivastava (2002) with slight modifications. Discs were removed from fresh leaves using a hole punch (l cm diameter), and 20 leaf discs were placed in a glass beaker containing deionized water. The solutions were incubated at 25 °C for 2 h, and then conductivity was measured using a calibrated conductivity meter (HI 8633, Hanna Instruments, Bedfordshire, UK). The solutions were boiled for 15 min and cooled to room temperature, and then conductivity was measured again. The percentage of electrolyte leakage was calculated as follows: EC (%) = (C1/C2) × 100, where EC is conductivity and C1 and C2 are the electrolyte conductivities measured before and after boiling, respectively.

To determine MDA and SP contents, fresh leaves (0.5 g) were homogenized in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1% Triton X-100, and 2% (w/v) PVP with a chilled mortar and a pestle. The homogenates were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatants were used for analyses. The MDA content in leaf samples was determined as described by Hodges et al. (2000). The leaves were extracted with 10% trichloroacetic acid, and the absorbance of leaf extracts was measured at 450, 532, and 600 nm against 0.6% thiobarbituric acid as the blank. The SP content in leaf samples was determined using Coomassie blue according to the method of Cusido (1987) with bovine serum albumin (Sigma) as the standard.

2.4. Measurements of water potential and relative water content
We measured leaf water potential (WP) using a pressure chamber (Model 100, PMS Instrument Co., Corvallis, OR, USA). The leaves were selected from the outside of the crown in the middle of an annual shoot. The measurements

were carried out at 0900 hours, 7 days after spraying with SNP + cPTIO, when the soil moisture content was 42.3% of the field moisture capacity (severe drought). To determine leaf relative water content (RWC), leaves per plant were rapidly weighed, floated on the surface of deionized water, and allowed to fully hydrate for 3 h; then they were reweighed and finally dried to a constant weight at 65 °C. Each assay was repeated three times.

2.5. Measurements of photosynthetic characteristics and fluorescence parameters
Photosynthetic responses of apple leaves were measured in the field using a portable photosynthesis system (CIRAS-2, PP System, UK). These analyses were conducted between 0830 and 1130 hours to avoid photoinhibition resulting from high-light stress at midday. Measurements were made under saturating photosynthetic photon flux density (1800 μmol m^{-2} s^{-1}) from an LED light source and ambient relative humidity. The leaf temperature was controlled at approximately 25 °C (similar to the mean daily growth temperature). Net photosynthetic rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) were measured.

Chlorophyll fluorescence of photosystem II was determined using a chlorophyll fluorescence measurement system (CF-1000; P.K. Morgan Instruments, Andover, MA, USA). The parameters measured were maximal (F_m) and variable (F_v) chlorophyll fluorescence, from which photochemical efficiency (F_v/F_m) was obtained. Dark-acclimation cuvettes, which had a shutter gate to eliminate ambient light when inserting the fiber optic light source, were fixed to the same leaf used for gas exchange measurements, and leaves were acclimated for at least 15 min before measurement. The light source was inserted into the cuvette from the abaxial side of the leaf, and a pulse of 1000 μmol m^{-2} s^{-1} of actinic light (680 nm) was applied for 60 s.

2.6. Extraction and assays of antioxidant enzymes
Enzymes were extracted according to Grace and Logan (1996) with slight modifications. Frozen leaf tissue (0.5 g) was homogenized in 100 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 0.1% Triton X-100, and 2% (w/v) PVP with a chilled mortar and a pestle. The homogenates were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatants were used for antioxidant enzyme activity assays. The specific activity of all enzymes was calculated as units g^{-1} fresh leaf weight.

Superoxide dismutase (SOD) activity was estimated by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) (Dhindsa et al., 1981). One unit of SOD was defined as the amount of enzyme required to inhibit NBT reduction by 50%, as determined by measuring absorbance at 560 nm.

Peroxidase (POD) activity was assayed using guaiacol as the substrate, as described by Ngo and Lenhoff (1980). The change in the absorbance of the assay mixture was measured at 470 nm. One unit of POD activity was defined as the rate of guaiacol oxidized in 3 min. Catalase (CAT) activity was determined by measuring the decrease in the absorbance of H_2O_2 at 240 nm (Deng et al., 2012). One unit of CAT activity was defined as the amount of enzyme catalyzing the decomposition of 1 μmol H_2O_2 per minute.

Ascorbate peroxidase (APX) and dehydroascorbate reductase (GR) activities were assayed using the method of Cheng (2012). The change in the absorbance of the APX assay mixture was measured at 290 nm. One unit of APX activity was defined as the amount of enzyme catalyzing the oxidation of 1 mmol ascorbate per minute. The change in the absorbance of the GR assay mixture was measured at 340 nm. One unit of GR activity was defined as the amount of enzyme that reduced 1 mmol oxidized glutathione per minute.

To determine monodehydroascorbate reductase (MDHAR) activity, the 1-mL reaction mixture contained 50 mM phosphate buffered saline (PBS) (pH 7.8), 1 mM coenzyme (NADH), 2.5 mM ascorbic acid (AsA), 25 units of AsA oxidase, and enzyme extract. The reaction was initiated by adding AsA oxidase and the change in absorbance at 340 nm was measured. One unit of MDHAR activity was defined as the amount of enzyme that oxidized 1 mmol NADH per minute.

To measure dehydroascorbate reductase (DHAR) activity, the 1-mL reaction mixture contained 50 mM PBS (pH 7.8), 20 mM reduced glutathione, 2 mM dihexyl adipate (DHA), and 1 mM EDTA-Na_2. The reaction was initiated by adding DHA and the change in absorbance at 265 nm was measured (Bai et al., 2009). One unit of DHAR activity was defined as the amount of enzyme producing 1 mmol AsA per minute.

2.7. Statistical analysis
Each treatment included four replicates. Data shown in figures are arithmetic mean values ± standard error of replicate measurements. Analysis of variance (ANOVA) was performed for group comparisons using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The significance of difference among mean values was determined at the 95% confidence interval.

3. Results
3.1. Effects of SNP application on leaf MP and MDA contents in Malus rootstocks under drought stress
Application of SNP resulted in larger decreases in MP and MDA contents in the leaves of drought-sensitive *M. hupehensis* (74.94% and 85.01%, respectively) than in the leaves of drought-tolerant *M. sieversii* (17.76% and 41.10%, respectively) under drought stress (Figure 1). Different concentrations of exogenous SNP (50–400 μmol L^{-1}) affected leaf MP and MDA contents of the two *Malus* rootstocks to a different extent. Under drought stress, the lowest levels of MP and MDA in the leaves of *M. sieversii* and *M. hupehensis* were in the 300 μmol L^{-1} SNP treatment (Figure 1), suggesting that this SNP concentration had the strongest inhibitory effect on drought-related physiological responses in these two rootstocks. Thus, 300 μmol L^{-1} SNP was used in the following experiments.

3.2. Effects of NO and cPTIO application on WP and RWC in Malus rootstocks under drought stress
To clarify the physiological role of endogenous NO in the drought resistance of *Malus* rootstocks under drought stress, cPTIO was applied to leaves to scavenge endogenous NO. Exogenous NO and cPTIO had opposite effects on WP and RWC in the two *Malus* species (Figure 2). Under both control (no drought) and drought conditions, the relative
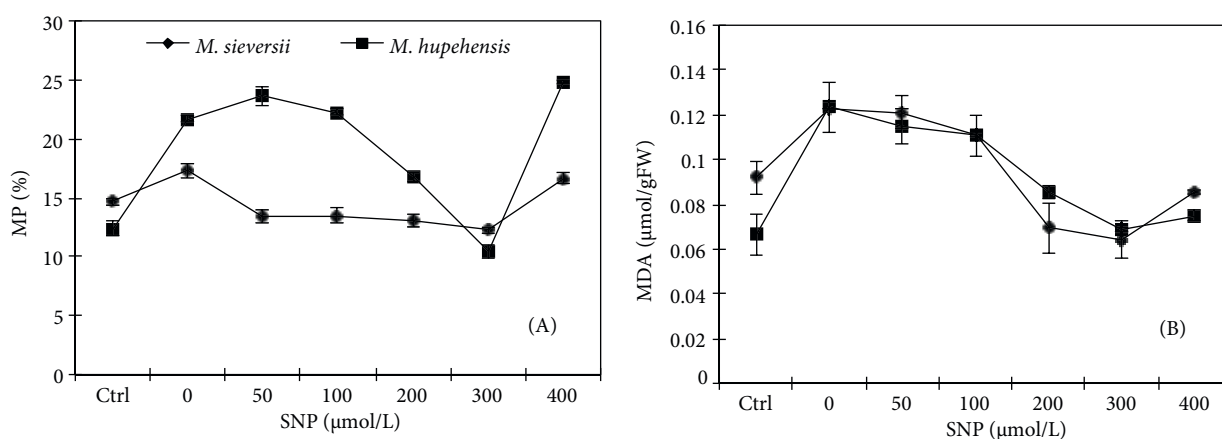

Figure 1. Effect of nitric oxide donor sodium nitroprusside (SNP) on leaf membrane permeability (MP, **A**) and malondialdehyde content (MDA, **B**) in 2-year-old seedlings of *Malus hupehensis* and *Malus sieversii* rootstocks under drought stress (water withheld for 7 days). Values are means ± standard error (n = 3).
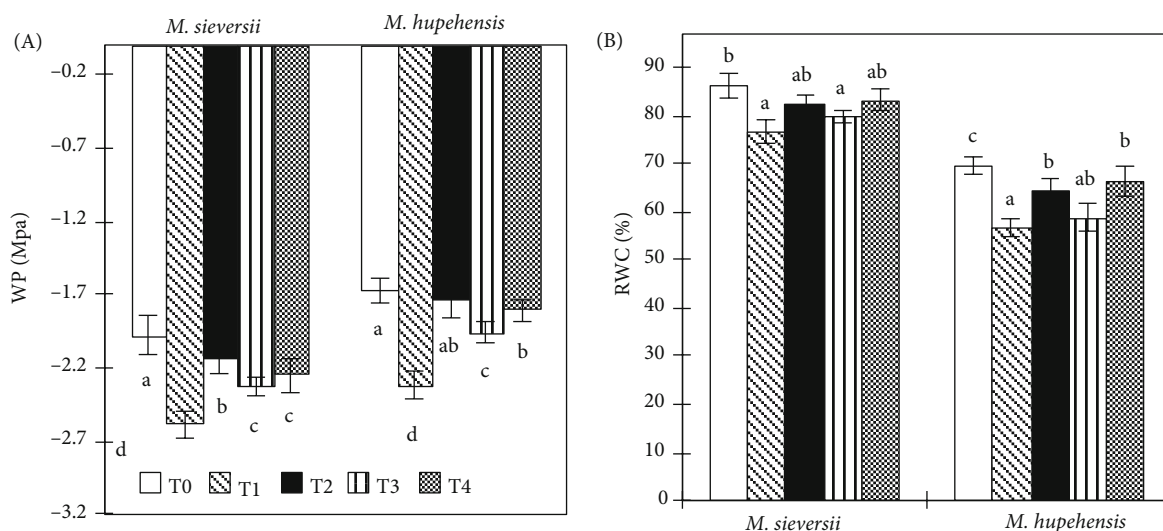
Figure 2. Water potential (WP, **A**) and relative water content (RWC, **B**) in 2-year-old seedlings of *Malus hupehensis* and *Malus sieversii* rootstocks under drought stress, with or without sodium nitroprusside (SNP) and carboxy-PTIO potassium salt (cPTIO). T0: Control, no drought or NO; T1: drought; T2: drought + SNP; T3: drought + cPTIO; T4: drought + SNP + cPTIO. Values are means ± standard error (n = 3); different letters above or below bars indicate significant differences among treatments (P < 0.05).

values of WP and RWC were higher in drought-tolerant *M. sieversii* than in drought-sensitive *M. hupehensis* (T0 and T1). Application of exogenous NO substantially increased WP and RWC in both rootstocks under drought stress (T2) compared to those of untreated drought-stressed rootstocks (T1). For both rootstocks, the plants treated with exogenous NO showed similar WP and RWC under drought stress (T2) to those of plants in the no-drought control.

Drought stress suppressed the growth of both *Malus* rootstocks. After 7 days of withholding water, the degree of wilting increased to 72% in drought-tolerant *M. sieversii* and to 95.1% in drought-sensitive *M. hupehensis*. Exogenous NO application (T2) alleviated leaf dehydration stress (i.e. increased WP and RWC values) to some extent, thus reducing the degree of wilting. However, this alleviation was inhibited by cPTIO (T4).

3.3. Effects of NO and cPTIO application on leaf photosynthetic characteristics and photochemical efficiency in *Malus* rootstocks under drought stress
The values of leaf Pn, Tr, and Gs were significantly lower in plants under drought stress (T1) than in unstressed plants (T0). Drought stress significantly increased leaf Ci concentration in drought-tolerant *M. sieversii* (Figure 3). Under drought stress, leaf Pn, Tr, Gs, and Ci were lower in plants treated with exogenous NO (T2) than in untreated plants (T1). For both *Malus* species, plants treated with cPTIO (T3) under drought stress showed higher leaf Pn, Tr, Gs, and Ci than plants treated with NO (T2). For both *Malus* species, plants treated with both NO and cPTIO (T4)

showed lower leaf Pn, Tr, Gs, and Ci than plants treated only with cPTIO (Figure 3), suggesting that exogenous NO acted as a signal to induce drought tolerance despite the inhibitory effect of cPTIO.

The F_v/F_m value reflects the photochemical efficiency of photosystem II. The F_v/F_m value was significantly lower in drought-stressed plants (T1) than in unstressed plants (8.84% lower in *M. sieversii* and 10.38% lower in *M. hupehensis*). For both rootstocks, exogenous NO increased the F_v/F_m value of drought-stressed plants, with or without cPTIO (T2 and T4), to a level similar to that of control plants (Figure 4).

3.4. Effects of NO and cPTIO on leaf MP, SP, and MDA levels in *Malus* rootstocks under drought stress
As compared to the unstressed plants (T0), plants under drought stress (T1) showed significantly higher leaf MP. Under drought stress, the leaf MP was higher in drought-sensitive *M. hupehensis* than in drought-tolerant *M. sieversii* (Figure 5A). Under drought stress, *M. sieversii* and *M. hupehensis* plants treated with exogenous NO (T2) showed lower MP than that of untreated plants under drought stress (T1). The decreasing effect of exogenous NO on MP under drought stress was largely diminished when cPTIO was applied (T4) (Figure 5A).

Application of exogenous NO also decreased SP and MDA contents in *M. sieversii* and *M. hupehensis* leaves under drought stress (Figures 5B and 5C). The effect of exogenous NO (T2) to decrease leaf SP content was barely affected by cPTIO (T4). In contrast, the effect of exogenous NO to decrease leaf MDA content was significantly
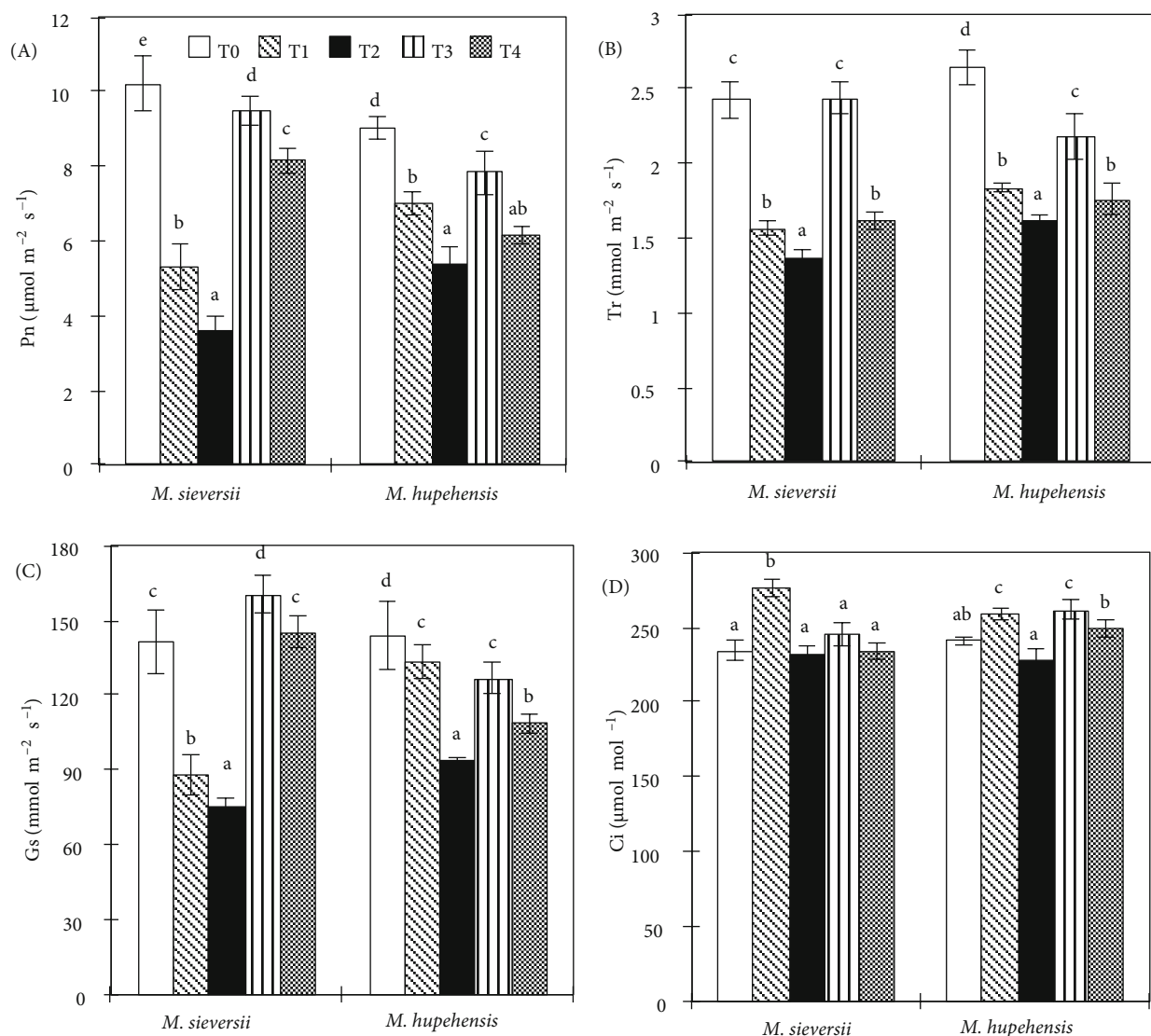
Figure 3. Net photosynthetic rate (Pn, **A**), transpiration rate (Tr, **B**), stomatal conductance (Gs, **C**), and intercellular CO_2 concentration (Ci, **D**) in 2-year-old seedlings of *Malus hupehensis* and *Malus sieversii* rootstocks under drought stress, with or without sodium nitroprusside (SNP) and carboxy-PTIO potassium salt (cPTIO). T0: Control, no drought or NO; T1: drought; T2: drought + SNP; T3: drought + cPTIO; T4: drought + SNP + cPTIO. Values are means ± standard error (n = 3); different letters above bars indicate significant differences among treatments (P < 0.05).

diminished by cPTIO in both *Malus* rootstocks under drought stress (Figure 5B).

3.5. Effects of NO and cPTIO on leaf antioxidant enzyme activities under drought stress
In all treatments, the SOD activity was higher in drought-tolerant *M. sieversii* leaves than in drought-sensitive *M. hupehensis* leaves (Figure 6A). Drought stress (T1) increased leaf SOD activity by 4.9% in *M. sieversii* rootstocks and by 8.5% in *M. hupehensis* rootstocks, as compared with the unstressed control (T0). In both rootstocks, exogenous NO (T2) further increased leaf SOD activity to levels significantly higher than in untreated plants under

drought stress (T1). Application of cPTIO significantly decreased leaf SOD activity in *M. sieversii*, but not in *M. hupehensis*. The effect of cPTIO to decrease SOD activity under drought stress was largely overcome by exogenous NO (Figure 6A). With a few exceptions, the activities of SOD, POD, CAT, APX, DHAR, GR, and MDHAR showed similar responses to the various treatments, albeit on different scales (Figures 6A–6G). This study showed significant effects of different treatments and species on most leaf parameters measured, as determined with ANOVA. However, the effects of the different treatments on MDA, Gs, Ci, and CAT activity were not significant.
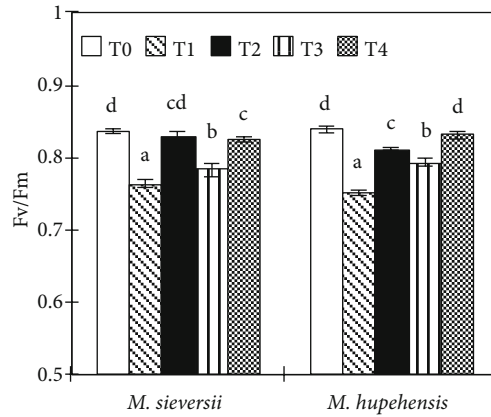
Figure 4. Leaf photochemical efficiency (i.e. variable/maximal chlorophyll fluorescence, F_v/F_m) in 2-year-old seedlings of *Malus hupehensis* and *Malus sieversii* under drought stress, with or without sodium nitroprusside (SNP) and carboxy-PTIO potassium salt (cPTIO). T0: Control, no drought, or NO; T1: drought; T2: drought + SNP; T3: drought + cPTIO; T4: drought + SNP + cPTIO. Values are means ± standard error (n = 3); different letters above bars indicate significant differences among treatments (P < 0.05).
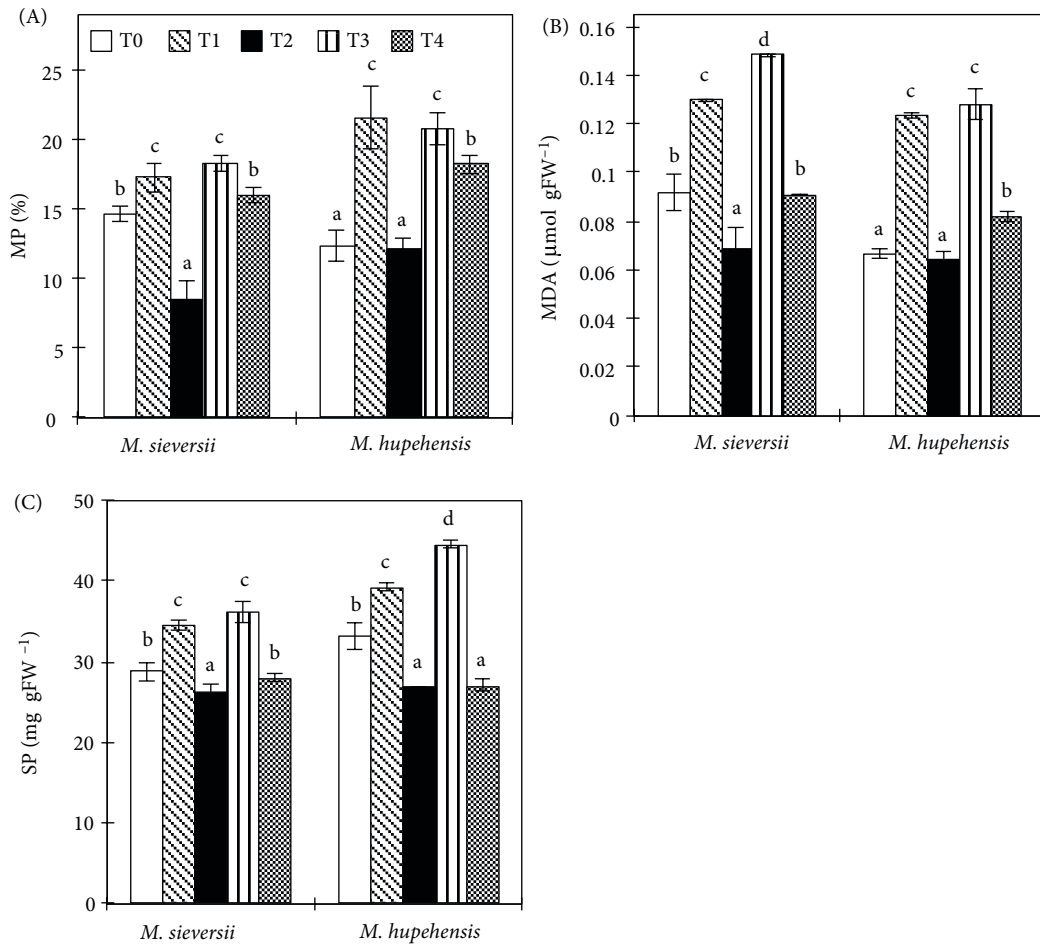
Figure 5. Leaf membrane permeability (MP, **A**), malondialdehyde content (MDA, **B**), and soluble protein content (SP, **C**) in 2-year-old seedlings of *Malus hupehensis* and *Malus sieversii* rootstocks under drought stress, with or without sodium nitroprusside (SNP) and carboxy-PTIO potassium salt (cPTIO). T0: Control, no drought or NO; T1: drought; T2: drought + SNP; T3: drought + cPTIO; T4: drought + SNP + cPTIO. Values are means ± standard error (n = 3); different letters above bars indicate significant differences among treatments (P < 0.05).
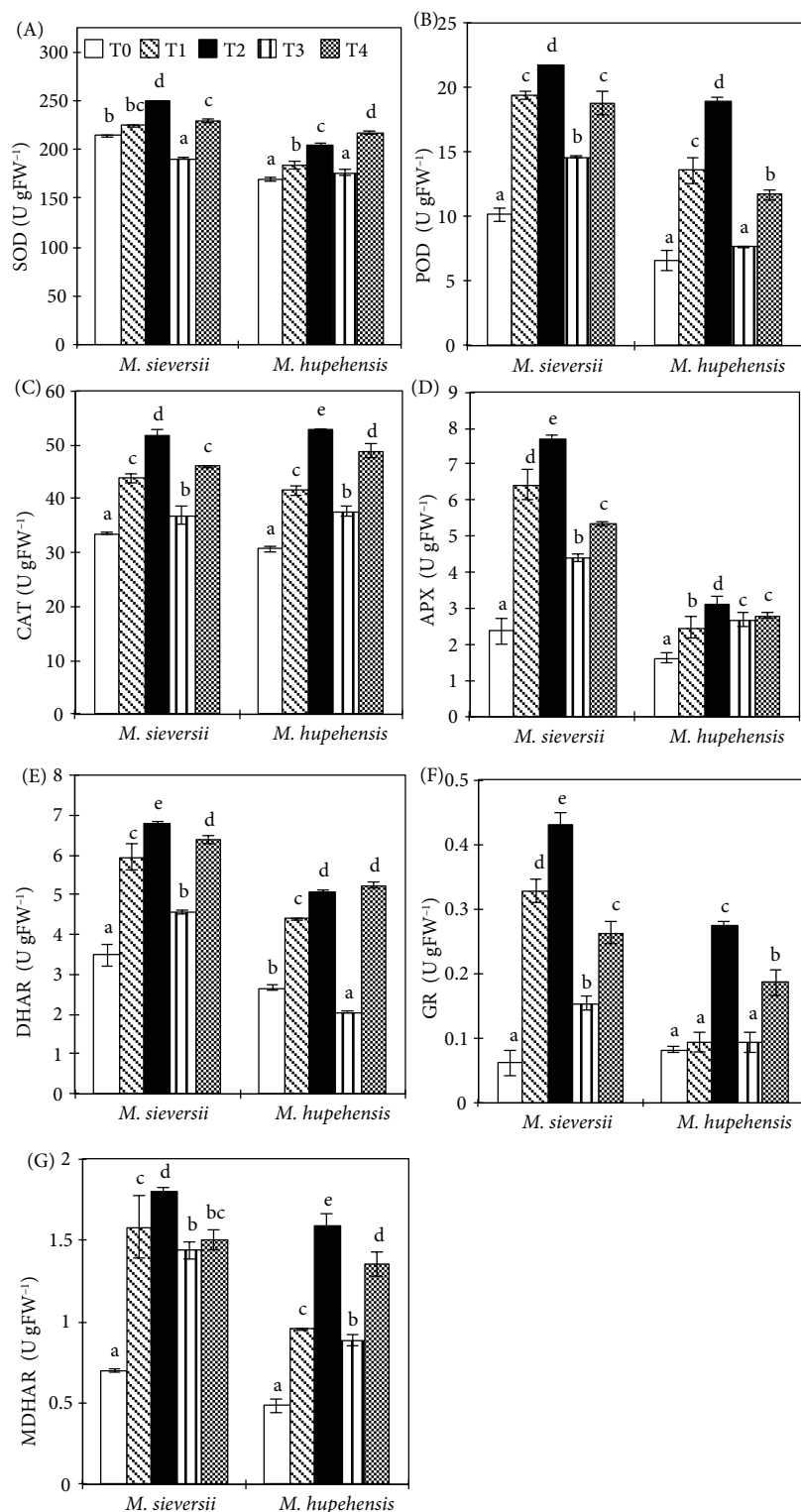
Figure 6. Leaf activities of superoxide dismutase (SOD, **A**), peroxidase (POD, **B**), catalase (CAT, **C**), ascorbate peroxidase (APX, **D**), dehydroascorbate reductase (DHAR, **E**), glutathione reductase (GR, **F**), and monodehydroascorbate reductase (MDHAR, **G**) in 2-year-old seedlings of *Malus hupehensis* and *Malus sieversii* under drought stress, with or without sodium nitroprusside (SNP) and carboxy-PTIO potassium salt (cPTIO). T0: Control, no drought or NO; T1: drought; T2: drought + SNP; T3: drought + cPTIO; T4: drought + SNP + cPTIO. Values are means ± standard error (n = 3); different letters above bars indicate significant differences among treatments (P < 0.05).

4. Discussion

Water shortages and the resulting losses in plant production are major problems in North China, an important agricultural region (Xia et al., 2007; Ashraf, 2010). Plants usually respond to drought stress by suppressing specific types of growth and accumulating ROS. ROS can cause lipid peroxidation and membrane disruption, resulting in oxidative damage (Liu et al., 2000; Jiang et al., 2001). Membrane permeability and the lipid peroxidation product MDA are commonly considered as important indexes of drought stress (Bai et al., 2011). Thus, they have been used to distinguish between drought-tolerant and drought-sensitive plant genotypes in many studies. In the present study, the MP and MDA content increased in two *Malus* rootstocks under drought stress, and there was greater accumulation of MDA and MP in *M. hupehensis* leaves than in *M. sieversii* leaves (Figure 1). These results might be related to the stronger drought tolerance in the latter species than in the former rootstock, consistent with previous findings (Bai et al., 2011; Wang et al., 2012).

Drought stress resulted in decreased WP and RWC in the two *Malus* rootstocks (Figure 2). This could be interpreted as a mechanism to concentrate solutes in the cell sap, thereby lowering the osmotic potential and contributing to osmotic adjustment (Lissner et al., 1999). Nitric oxide is one of the key elements in the complex signaling pathway leading to stomatal closure: it induces reversible protein phosphorylation and Ca^{2+} release from intracellular stores (Lamattina et al., 2003; Ördög et al., 2013). Therefore, under drought stress, plants treated with NO showed WP and RWC values similar to those in the control (no drought). In the present study, application of 300 µmol L^{-1} SNP to leaf surfaces enhanced the drought tolerance of both *Malus* rootstocks by alleviating dehydration stress and decreasing ion leakage, lipid peroxidation, water potential, and the degree of wilting in the leaves (Figures 2 and 5).

As a NO scavenger, cPTIO can reverse the effects of NO donors on plant physiology (Beligni et al., 2002; Piterková et al., 2012). Thus, we used cPTIO to verify the physiological role of endogenous NO in the drought tolerance of the two *Malus* rootstocks. Treatment with cPTIO exacerbated membrane damage and lipid peroxidation to some extent in both *M. sieversii* and *M. hupehensis* leaves, with greater responses in *M. sieversii* than in *M. hupehensis* rootstocks (Figure 6). This result is consistent with the fact that NO reacts with cPTIO to give NO_2, which is very reactive and can cause severe damage (Shao et al., 2007). Plants resist stress-induced ROS production by increasing the amounts and/or activities of various components of their defensive systems. Plant cells are normally protected against the effects of ROS by a complex antioxidant system, which includes enzymatic antioxidants (Cheng et al., 2004; Bai et al., 2009).

Application of exogenous NO to both *Malus* rootstocks significantly decreased drought-related ion leakage, lipid peroxidation, and SP content (Figure 5) and enhanced leaf water attributes (Figure 2) and photochemical efficiency (Figure 4), thus alleviating leaf dehydration stress and scavenging more ROS. The activity of SOD is induced by the substrate. O_2^- is generated by drought stress and then induces a significant increase in SOD activity (Beligni et al., 2002; Zhu, 2002). After exogenous SNP, SOD activity increased significantly, so drought stress was largely released (Figure 6A). Under drought conditions, the activities of POD, CAT, APX and other antioxidant enzymes that can degrade H_2O_2 were decreased, while NO significantly promoted SOD, POD, CAT, and APX, thereby strengthening the capabilities of the defense system to scavenge free radicals (Figure 6). Moreover, the harmful effects of drought oxidative stress on *Malus* seedlings were alleviated, membrane permeability was reduced, and MDA content was decreased (Figure 5). Exogenous NO also changed the photosynthetic characteristics and photochemical efficiency of the two *Malus* rootstocks. These changes could explain the increased drought tolerance of the rootstocks (García and Lamattina, 2001; Ma et al., 2005). However, the application of cPTIO inhibited the beneficial effects of NO, thus decreasing the drought tolerance of *Malus* rootstocks. These results concurrently suggest that NO, produced in the two *Malus* rootstocks under drought stress, might serve as a signal to induce drought tolerance.

The results of this study provide evidence that NO increased the antioxidant capacity of apple rootstocks under drought stress. Although we did not measure the amount of endogenous NO released by SNP treatments in these experiments, there were significant effects at very low SNP concentrations (Delledonne et al., 1998). Our results showed that drought stress adversely affected the leaf water attributes of two *Malus* rootstock species (the drought-sensitive *M. hupehensis* and the drought-tolerant *M. sieversii*) that are widely grown in semiarid areas of the Loess Plateau. Exogenous application of an appropriate amount of the NO generator SNP (300 µmol L^{-1} SNP) to leaf surfaces effectively enhanced the drought tolerance of both *Malus* species by increasing the activities of antioxidant enzymes and enhancing photosynthetic performance under drought conditions. Compared with other exogenous substances, NO (i.e. SNP) is considerably less expensive and is suitable for universal application in plant production. By determining the appropriate amount of NO generator for each species or variety, this leaf-spraying method could be used to overcome the adverse effects of drought stress on apple trees by enhancing their photosynthesis and antioxidant responses. To better understand the role of exogenous NO in ameliorating

oxidative stress in *Malus* rootstocks, in our future research we plan to analyze the transcript levels of genes involved in NO metabolism by quantitative PCR.

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