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Synergistic Action of Exogenous Salicylic Acid and Arbuscular Mycorrhizal Fungus Colonization in *Avena nuda* Seedlings in Response to NO_2 Exposure

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Abstract Colonization of arbuscular mycorrhizal fungi *Glomus mosseae* or exogenous salicylic acid (SA) treatment can increase *Avena nuda* plant tolerance to elevated NO_2 exposure. The combination of the two factors, namely application of SA to the mycorrhizal plants, further promoted NO_2 tolerance, as indicated by an alleviated plant biomass decrease compared to the respective treatment. The analysis of antioxidant capacity, redox status and photon energy utilization showed that the increased NO_2 tolerance in the treated plants may be associated, at least in part, with scavenging reactive oxygen species, maintaining CO_2 assimilated rate and reducing conditions in cells.

Keywords *Avena nuda* · Arbuscular mycorrhizal symbiosis · Salicylic acid · Nitrogen dioxide

Atmospheric NO_2 exposure at an ambient level increases plant size (Takahashi et al. 2005), however, in some regions of the world, ambient NO_2 has caused reduction and deterioration in crop and vegetable yield and quality (Maggs and Ashmore 2004; Muzika et al. 2004). Accumulation of NO^{2-} resulting from atmospheric NO_2 in leaves leads to the inhibition of photosynthesis, thereby generation of reactive oxygen species (ROS), which might contribute to visible injury caused by NO_2 (Shimazaki et al. 1992). To avoid ROS-caused oxidative damage, plants evolved a complex of antioxidant defense system which is involved in the detoxification of ROS (Mittler 2002). Arbuscular mycorrhizal (AM) symbiosis is an almost ubiquitous rhizospheric interaction (Smith and Read 1997). The typical benefit of arbuscular mycorrhizal fungi (AMF) to the host is mineral uptake, especially phosphate. Nevertheless, non-nutritional effects of AMF on host plants have attracted increasing attention. For example, there are numerous reports of fungal symbionts conferring tolerance to various stresses to host plants (for a review, see Rodriguez and Redman 2008 and references here). In a previous study, we observed that the *Glomus mosseae*/*Avena nuda* symbiosis increases SO_2 tolerance to host plants, even though the SO_2 exposure attenuates the AMF growth in the plant roots (Huang et al. 2008). Although the ameliorative effect of AM symbiosis on the host response to detrimental circumstances has been attributed to a wide variety of mechanisms, the antioxidant defense role of AM symbiosis is generally considered (Huang et al. 2008 and references here).

In addition to its well-established role in plant response to pathogen attack (for a review, see Halim et al. 2006), salicylic acid (SA) has also been intensively investigated in plant adaptation to various abiotic stresses (for a review, see Yuan and Lin 2008). However, to our knowledge, little information is available for SA involving in plant response to NO_2 stress. Although the involvement of SA in enhancing plant resistance to pathogens (fungi) has been well demonstrated, the role of SA in plant-fungus symbiosis is not yet known (for a review, see Zhao and Qi 2008). In this study, we found that application of SA to the *Avena nuda* plants colonized by AMF *Glomus mosseae* synergistically increased the NO_2 tolerance. To our knowledge, this is the first finding of this sort.

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Materials and Methods

The experiment was conducted three times using four treatments: controls, SA treatment, AMF *G. mosseae* [Thaxter and Gerd. (Gerd. and Trappe)] inoculation, and SA treatment + AMF inoculation. Plant growth, AMF inoculation and the colonization determination were carried out as described by a previous report (Huang et al. 2008). For SA application, 4-week-old plants including control (no AMF colonization) and AM symbiosis (at least 20% of root colonization) were sprayed with SA solution (pH 7.0) at a final concentration of 1 mM 24 h prior to NO_2 exposure. For NO_2 exposure, plants were transferred to a glass chamber (0.8 m × 0.8 m × 0.8 m) and NO_2 gas was supplied directly from cylinders, into a dilution reservoir into which charcoal filtered air was drawn. Mean concentration of the chamber NO_2 was monitored using an NO_2 analyzer (Model ML Series). Plants were fumigated during the light period for 6 h per day for 10 day. For the control, filtered air alone was supplied. Except where mentioned in the text, the mean NO_2 concentration of 1 μL L^{-1} was used in this experiment. All the analyses in this experiment were performed at the immediate end of NO_2 exposure.

For biomass determination, the above-ground parts were harvested and oven-dried at 85°C for 2 days and dry mass recorded. The relative biomass increment was expressed as $(W_1 - W_0)/W_0 \times 100\%$, where W_0 is the mean dry weight of plants at the beginning of NO_2 exposure and W_1 is the mean dry weight at the end of NO_2 exposure. Photosynthesis rate and intercellular CO_2 concentration (*Ci*) were measured using a portable photosynthesis system (LI-6200) at ambient climatic conditions. During the measurement the PAR over waveband 400–700 nm was about 500 μM m^{-2} s^{-1} at 25°C. For maximum quantum efficiency of PSII (F_v/F_m) determination, plants were kept for 15 min in darkness to determine minimum fluorescence F_0 with a weak beam from a light-emitting diode, following by a saturating pulse (2,000 μM m^{-2} s^{-1}) to determine maximum fluorescence F_m, and F_v/F_m was calculated following the formula $(F_m–F_0)/F_m$. Total chlorophyll content was determined following the method described by Zhang and Qu (2003).

Hydrogen peroxide (H_2O_2) level was determined according to a method from Mukherjee and Choudhuri (1983). The reduced glutathione (GSH) content was determined as described by Griffith and Meister (1979). Oxidised glutathione (GSSG) content was calculated from the difference between total glutathione from DTT-treated samples and GSH from non-DTT-treated samples. SOD (EC 1.15.1.1) activity was assayed using the photochemical nitroblue tetrazolium (NBT) method as described previously (Beyer and Fridovich 1987). CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm for 3 min as described by Aebi (1983), in which the initial concentration of H_2O_2 is 0.04% (v/v) in PBS, at pH 7.0. POD (EC 1.11.1.7) activity was estimated according to Hemeda and Klein (1990). In all the enzyme preparations, protein concentration was estimated by the method of Bradford (1976) using bovine albumin as standard. The level of malondialdehyde (MDA) was determined following Shalata and Tal (1998). For electrolyte leakage measurement, 20 leaf discs of 8 mm in diameter were placed in glass vials, rinsed three times with 20 mL of deionized water to remove electrolytes released during leaf disc excision. Vials were then filled with 20 mL of deionized water and incubated in the dark for 6 h at room temperature. Electrolytic conductivity (EC1) of the solution was measured with a conductivity meter at the end of incubation. The solution was heated to boiling, and then cooled to room temperature and electrolytic conductivity (EC2) measured again. The percent electrolyte leakage of the leaf discs was calculated as 100× EC1/EC2.

Each result shown in figures was the mean of at least three replicated treatments. Data were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute, Cary NC) and expressed as means ± SD.

Results and Discussion

As shown in Fig. 1, NO_2 exposure at 0.25 μL L^{-1} promoted plant biomass production, while the concentrations of 1 μL L^{-1} or higher inhibited plant growth compared with control. The response in chlorophyll content to NO_2 exposure was similar to that in biomass production (Fig. 1). Based on these data, in all further experiments, 1 μL L^{-1} of NO_2 was used.

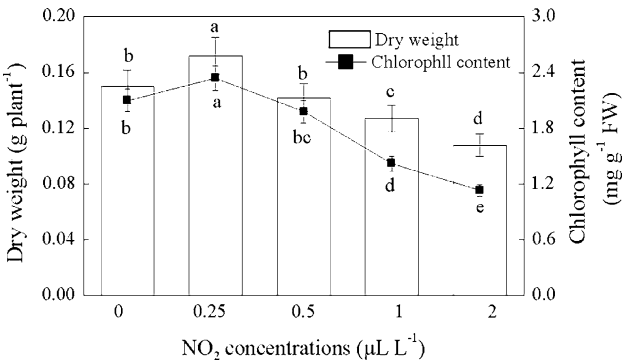

Fig. 1 Effect of NO_2 on plant growth and total chlorophyll content in *Avena nuda*. Plants were exposed to the different concentrations of NO_2 as indicated in the figure for 6 h per day for 10 d. The data shown here from five replicated experiments and represent the mean ± SD. The different letters (*a*, *b*, etc.) in the same series indicate significant difference at $p \leq 0.05$

NO_2 exposure decreased biomass production in all analyzed plants, whereas the decrease was significantly alleviated in the plants treated with SA and AMF colonization, singly or in combination, compared with control, with the combined treatment being most efficient (Fig. 2a). Changes of the photosynthetic rate were well correlated to the result of relative biomass assay (Fig. 2b). To investigate whether the NO_2-caused growth inhibition was induced by disturbances in photosynthetic processes, the ratio of variable to maximal fluorescence (F_v/F_m), as an indicator of damage to the PSII reaction centres has been widely used in estimation of the maximum quantum yield of PSII photochemistry, was measured. NO_2 exposure obviously decreased the ratio of F_v/F_m in control, and in SA-treated or AM symbiosis plants as well compared to their NO_2-unexposed parallels, whereas the ratio in combination-treated plants had no major difference between NO_2-exposed and unexposed samples (Fig. 2c). The result of intercellular CO_2 concentration (*Ci*) analysis was consistent with the altered pattern of F_v/F_m ratio (Fig. 2d). This indicated that the impaired utilization of photon energy may contribute to the NO_2-caused growth inhibition.

Earlier reports proposed that the accumulation of NO_2^- resulting from atmospheric NO_2 might inhibit the photosynthetic fixation and, consequently, accelerated the one-electron reduction of O_2 to generate ROS on the reducing site of PS as a result of the absence of a physiological acceptor of $NADP^+$ (Shimazaki et al. 1992; Foyer 2002), thus we investigated H_2O_2 accumulation and oxidised injury, as well as the plant antioxidant responses to NO_2. The assay of MDA, a product of lipid peroxidation, and electrolyte leakage enabling cell membrane injury to be assessed, showed that NO_2 exposure resulted in oxidised damage to all the tested plants. However, the SA application and AM symbiosis, singly or in combination, markedly mitigated NO_2-caused oxidised injury, with the combined treatment being most effective (Fig. 2e, f). The determination of H_2O_2 levels in these plants (Fig. 3f) suggested that the H_2O_2 accumulation was responsible for the oxidised injury.

Although NO_2 exposure decreased SOD activity in all the plants, it was the highest in plants with the combined treatment compared to others (Fig. 3a). Similarly, the CAT and POD activities also were the highest in the

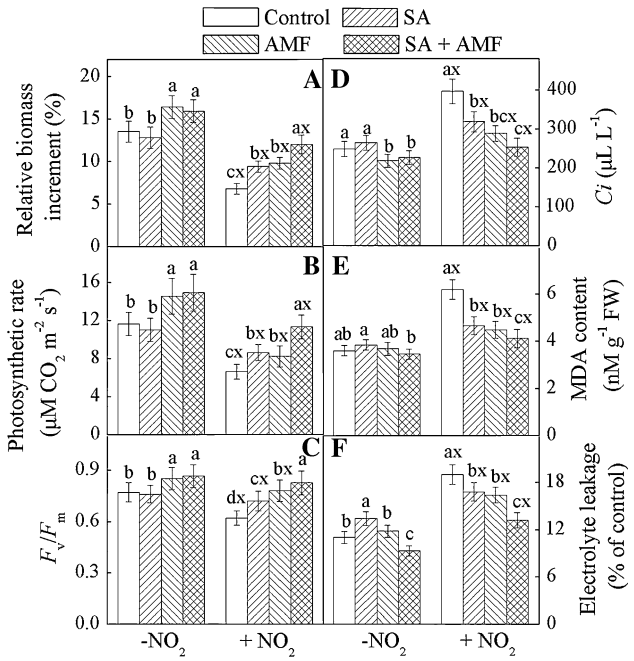

Fig. 2 Effect of Salicylic acid treatment and AM symbiosis, singly or in combination, on relative biomass increment (**a**), photosynthetic rate (**b**), maximum photochemical efficiency (F_v/F_m; **c**), intercellular CO_2 concentration (*Ci*; **d**), malondiadehyde (MDA) content (**e**) and electrolyte leakage (**f**) in *Avena nuda* plants grown in filtered air ($-NO_2$) and 1 μL L^{-1} of NO_2 ($+NO_2$). The data shown here from three replicated experiments and represent the mean ± SD. The different letters (*a*, *b*, etc.) in the same group (by an absence or presence of NO_2) indicate significant difference at $p \leq 0.05$, and *x* indicates significant difference at $p \leq 0.05$ between NO_2-exposed plant and its unexposed control

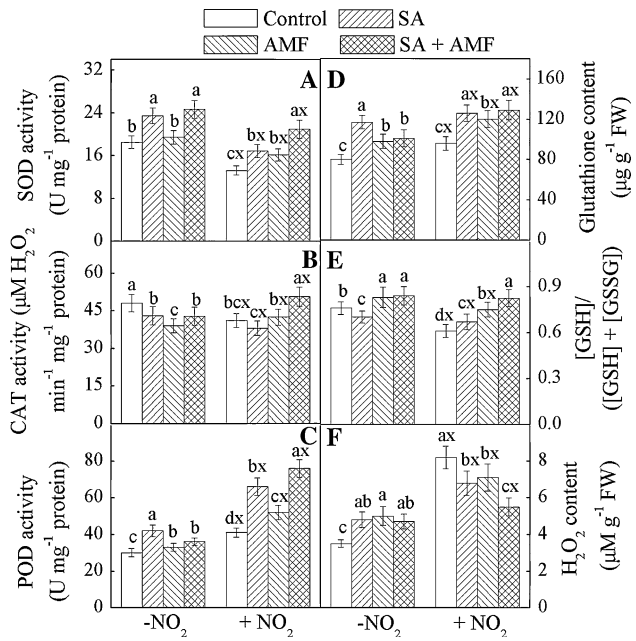

Fig. 3 Effect of Salicylic acid treatment and AM symbiosis, singly or in combination, on superoxide dismutase (SOD) activity (**a**), catalase (*CAT*) activity (**b**), peroxidase (*POD*) activity (**c**), reduced glutathione (*GSH*) content (**d**), ratio between reduced glutathione (*GSH*) and oxidised glutathione (*GSSG*; **e**) and hydrogen peroxide (H_2O_2) content (**f**) in *Avena nuda* plants grown in filtered air ($-NO_2$) and 1 μL L^{-1} of NO_2 ($+NO_2$). The data shown here from three replicated experiments and represent the mean ± SD. The different letters (*a*, *b*, etc.) in the same group (by an absence or presence of NO_2) indicate significant difference at $p \leq 0.05$, and *x* indicates significant difference at $p \leq 0.05$ between NO_2-exposed plant and its unexposed control

combination-treated plants (Fig. 3b, c). In addition, NO_2 exposure increased POD activity in all tested plants (Fig. 3c) and CAT activity in AM symbiosis and combination-treated plants (Fig. 3b), when compared, respectively, with their NO_2-unexposed parallels. The NO_2 exposure increased GSH content in all plants relative to their unexposed parallels, and the SA application and AM symbiosis, singly or in combination, also elevated the GSH levels independent of the NO_2 exposure, in comparison with the untreated control (Fig. 3d). Glutathione exists in both a reduced (GSH) and oxidised form (glutathione disulphide; GSSG), and its influence on cellular redox status depends on both the GSH/GSSG ratio and the concentration of GSH (Schaffer and Buettner 2001). NO_2 exposure decreased the ratio in control, SA-treated or AMF-colonized plants, whereas the ratio remained unchanged in the combination-treated plants (Fig. 3e). With the lowest H_2O_2 level (Fig. 3f) and the weakest lipid peroxidation (Fig. 2e) in combination-treated plants among all the plants under NO_2 exposure, these data indicated that the effective control of ROS by the promoted antioxidant capacity and the maintenance of reducing conditions in cells may contribute the plant tolerance to NO_2 stress.

Based on the result obtained in this study, it was proposed that the improved tolerance to NO_2 in plants with SA treatment and AM symbiosis singly or in combination compared to control, as indicated by an obvious alleviation of NO_2-caused biomass production decrease and photosynthetic rate impairment, was associated with the elevated antioxidant capacity and reducing conditions in cells, resulting in decreased H_2O_2 levels and mitigated lipid peroxidation. In this case, a synergistic action of exogenous SA treatment and AM symbiosis was observed. Growing evidence has shown that AM symbiosis effectively enhances the host antioxidant level, leading to increased tolerance to various abiotic stresses (Huang et al. 2008 and references here). Also, there has been considerable evidence to show that exogenous SA can activate antioxidant enzymes in plants in response to different abiotic stresses such as paraquat (Ananieva et al. 2004), heavy metals (Popova et al. 2009), and salinity (Xu et al. 2008). In addition, increasing evidence indicates that SA-improved plant resistance to biotic and abiotic stresses is associated with the maintenance of reducing conditions or redox homeostasis in cells (Mou et al. 2003; Mateo et al. 2006). To our knowledge, however, there is no report yet to indicate the existence of a synergistic action of exogenous SA treatment and AM symbiosis in plants in response to adverse stresses.

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