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Short communication

Mycorrhizal fungi symbiosis as a strategy against oxidative stress in soybean plants

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ABSTRACT

Oxidative stress responses generated by paraquat (PQ), an herbicide that triggers an oxidative stress reaction in leaves, were studied in non-arbuscular mycorrhizal (non-AM) and in arbuscular mycorrhizal (AM) soybean plants inoculated with *Glomus mosseae* (*Gm*) or *Glomus intraradices* (*Gi*). Some oxidative stress symptoms were evident in non-AM after 6 d of PQ application on leaves. Oxidative damage, measured as malondialdehyde content (MDA), was significantly higher, and although no changes were evident in total catalase (CAT, EC 1.11.1.6) and total superoxide dismutase (SOD, EC 1.15.1.1) activity, total ascorbate peroxidase (APX, EC 1.11.1.11) activity was significantly reduced. These effects were correlated with a significant decrease in growth parameters. By contrast, in both AM plants, foliar MDA content was reduced or unaltered and, interestingly, after PQ stress, its level was unchanged and significantly lower than in PQ non-AM plants. Unlike PQ stress in non-AM plants, total APX activity was unaltered or induced by AM plants, while total SOD activity was unchanged and no consistent effects were detected in total CAT activity. All these events coincided with no changes or a significant increase in growth parameters. Since oxidative stress is a common phenomenon triggered by several environmental stresses, these results highlight the importance of mycorrhizal fungi in oxidative stress regulation as a general strategy to protect plants from abiotic and biotic stress.

Introduction

Arbuscular mycorrhizal fungi (AMF) confer plants with an improved ability to tolerate biotic and abiotic stresses (Smith and Read, 2008). A number of different mechanisms have been proposed to explain how AMF enhance stress tolerance (Pedersen and Sylvia, 1996; Smith and Read, 2008). Several of these mechanisms include oxidative stress regulation (Alguacil et al., 2003; Ruiz-Lozano, 2003; Ghorbanli et al., 2004; Wu et al., 2006a,b). Oxidative stress is characterized by the generation of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$). ROS can cause rapid and deleterious oxidation of most cell components (Apel and Hirt, 2004). Therefore, plants have evolved a dynamic antioxidant defense network. Superoxide dismutases (SOD), ascorbate peroxidases (APX), catalases (CAT), and ascorbic acid and glutathione are considered the major ROS scavenging mechanisms in plants (Niyogi, 1999). Although ROS are generally produced during normal cell metabolism and their regulation is a frequent cellular event, oxidative damage is associated with plant stress (Apel and Hirt, 2004; Fazeli et al., 2007). Interestingly, several investigations, mainly developed in roots, have addressed mycorrhiza-induced reduction of oxidative stress. Ruiz-Lozano et al. (1996) reported increased SOD activity in roots of mycorrhizal lettuce plants under drought stress, and this response was confirmed at the transcriptional level by subsequent molecular analyses (Ruiz-Lozano et al., 2001a). Also, AMF considerably increased glutathione reductase activity in roots and nodules of soybean plants subjected to drought stress (Porcel et al., 2003). Schützendübel and Polle (2002) found increased SOD activity and reduced glutathione content in arbuscular mycorrhizal pine roots under heavy metal stress. More recently, Beltrano and Ronco (2008) observed that root colonization by *G. claroideum* in wheat recovered cell membrane permeability in leaves after rewatering stress. On the other hand, under drought conditions, Porcel and Ruiz-Lozano (2004) and Wu et al. (2006b) found reduced lipid peroxide content in both AM roots and leaves of soybean and citrus seedlings, respectively. With respect to biotic stress, Pozo et al. (2002) observed that AMF effectively reduced *Phytophthora*

Abbreviations: AM, arbuscular mycorrhizal; AMF, arbuscular mycorrhizal fungi; APX, ascorbate peroxidases; CAT, catalases; *Gm*, *Glomus mosseae*; *Gi*, *Glomus intraradices*; H_2O_2, hydrogen peroxide; $OH^{\bullet}$, hydroxyl radicals; non-AM, non-arbuscular mycorrhizal; PQ, paraquat; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide; SOD, superoxide dismutases.

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infection symptoms in tomato plants and also induced new SOD isoforms.

While the relationship between oxidative stress and AMF protection has been studied under different conditions of biotic and abiotic stress, an evaluation of AMF behavior under an experimental system that exclusively triggers an oxidative stress reaction in leaves is still lacking.

In the present work, we sprayed non-arbuscular mycorrhizal (non-AM) and arbuscular mycorrhizal (AM) soybean plants with paraquat (PQ). PQ application was selected as a model to study oxidative stress because it is a potent herbicide that exacerbates $O_2^{\bullet -}$ radical production and increases lipid peroxidation of membranes (Bowler et al., 1994). We compared the effects of two AMF, *Glomus mosseae* (*Gm*) and *Glomus intraradices* (*Gi*), on growth parameters of soybean plants subjected to oxidative stress induced by PQ. To determine whether oxidative stress regulation by AMF was involved, the level of oxidative damage was evaluated and some key antioxidant enzymes, such as total CAT, APX and SOD were examined.

Material and methods

Plant material and experimental conditions

Mycorrhizal inoculum was bulked in an open-pot culture of *Trifolium repens* and consisted of spores, mycelia, and colonized root fragments. The AM species were *Glomus mosseae* (Nicolson and Gerdemann) Gerdemann and Trappe and *Glomus intraradices* (Schenck and Smith). Inoculum (20 g) was added to the corresponding pots at sowing.

Soybean (*Glycine max* cv. Don Mario 4800) seeds were sterilized in a 1% NaClO solution for 30 s and placed on cotton and paper towel at 25 °C to germinate. Three-d-old seedlings were transferred to plastic pots containing 1000 g of sterilized substrate: sand, Perlite, and Vermiculite (1:1:1:1, v/v/v/v). The soil had a pH of 6.46; 7.9% organic matter; 0.66% total N; 84.1 $\mu g\, g^{-1}$ P; cationic exchange capacity mequivalent/100 K 2.1; 4.6% organic C. Plants were grown in a glasshouse for 28 d at 20–25 °C and watered with distilled water twice a week. Three inoculation treatments with 20 replicates each were performed: AM plants inoculated with *G. mosseae* (*Gm*), AM plants inoculated with *G. intraradices* (*Gi*), and non-AM plants used as control. When the plants of the three treatments reached the V3 stage (third leaf totally expanded), half were totally sprayed with water + Tween 0.25% (control PQ) and the other half were sprayed totally with PQ (*Gi* PQ and *Gm* PQ). To choose experimental conditions, sampling time and different PQ concentrations (0, 5, 10, 25, 50, and 100 μM PQ + 0.25% Tween solutions) were previously assessed in non-AM plants, and MDA was measured. After 3 d of PQ treatment, no change in MDA content was detected (data not shown). However, on d 6, MDA was significantly increased. Because necrosis and chlorosis were induced with 10–100 μM PQ solutions (data not shown), and no leaf damage was evident at 5 μM PQ, the latter PQ concentration and 6 d after PQ treatment were the experimental conditions selected.

Measurements of oxidative damage and antioxidant defenses

At 6 d after PQ treatment, the third and fourth leaves were collected and frozen in liquid air to analyze oxidative damage and antioxidant defenses. Oxidative damage was evaluated as MDA content, as described by Heath and Packer (1968) and Hodges et al. (1999). Antioxidant defenses were evaluated from 100 mg of leaf samples homogenized with 2 ml of 50 mM potassium phosphate pH 7.0, 1 mM EDTA, 50 mM NaCl, 1% PVPP. When total APX activity was evaluated, 0.1 M ascorbic acid was added to the homogenate. The homogenate was centrifuged at 16,000 × *g* at 4 °C for 20 min and antioxidant enzyme activities were measured in the supernatant. Total CAT and APX activities were assayed according to Aebi (1984) and Nakano and Asada (1981), respectively. Total SOD activity was measured at 560 nm, according to Beauchamp and Fridovich (1973), based on the ability of SOD to inhibit the reduction of nitroblue tetrazolium by superoxide radicals generated photochemically. One unit of SOD was defined as the amount of enzyme required to inhibit the reduction rate of nitroblue tetrazolium by 50% at 25 °C. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as standard.

Measurement of growth parameters and evaluation of AM colonization

At the end of the experiment (28 d of sowing), PQ toxicity was evaluated by measuring the following growth parameters: length and fresh mass (FM) of shoot and root, and dry mass (DM). Water content of leaflets was measured as leaf water content percentage. To determine AM colonization, roots were stained as described in Phillips and Hayman (1970) and the percentage of mycorrhizal root infection was calculated according to the grid-line intersection method (Giovannetti and Mosse, 1980). Briefly, we randomly disperse cleared and stained roots of every plant of all mycorrhized treatments in a dish with grid lines. After that, we assessed mycorrhizal colonization under a dissecting microscope. We followed all horizontal and vertical lines, counting intersects with roots and mycorrhizae separately, and analyzed at least 10 roots/treatment, twice. AM colonization was evaluated when soybean plants reached the V3 stage (third leaf totally expanded) and at the end of the experiment. Means of 10 replicates were calculated from two separate experiments and were subjected to analysis of variance (ANOVA), followed by LSD Fisher's multiple-range test.

Results and discussion

Under natural conditions, roots of many plant species are associated with mycorrhizal symbionts, which have the ability to enhance resistance to biotic and abiotic stresses (Smith and Read, 2008). Among the different mechanisms involved in AMF-induced stress resistance, oxidative stress and antioxidant defenses have received special attention (Schützendübel and Polle, 2002; Ruiz-Lozano, 2003; Porcel and Ruiz-Lozano, 2004; Ghorbanli et al., 2004). However, to the best of our knowledge, this is the first report that shows how an experimental system that generates oxidative stress at the foliar level in plants induces different behavior in AM and non-AM plants.

Tolerance to PQ stress in AM and non-AM plants was evaluated at the end of the experiment, 28 d after sowing. In general, PQ in non-AM plants induced a decrease in growth parameters, which was significant in total length, particularly root length and total FM (Table 1), and tended to decrease in shoot length and leaf DM. This is consistent with the fact that PQ is a contact herbicide, which acts rapidly by causing the plant to bleach and wilt (Ismail et al., 2001) and also by reducing photosynthesis, water, and soluble protein content in pea plants (Iturbe-Ormaetxe et al., 1998). By contrast, in both AM plants, growth parameters were unaltered compared with control non-AM plants and interestingly, this effect was maintained in both AM plants after PQ stress. Moreover, both PQ AM plants showed a significant increase in total length (root and shoot length) and FM compared with PQ non-AM plants. Our results agree with many studies that have shown AM capacity to increase plant growth and yield (Smith and Read, 2008) and evidence the ability of AMF to protect soybean plants from oxidative PQ-induced stress

Table 1
Growth parameters of mycorrhizal and non mycorrhizal soybean plants in control conditions and paraquat treatment.

Treatment	Shoot length (cm plant^{-1})	Root length (cm plant^{-1})	Total length (cm plant^{-1})	Total FM (g plant^{-1})	Leaf DM (g leaf^{-1})	Leaf water content (%)	AM colonization (%)
Control							
Non-AM	21.9 ab	25.4 bc	47.3 b	4.67 b	0.13 b	85.2 a	–
Gm	28.4 c	23.9 ab	52.3 b	4.83 b	0.08 a	91.0 b	18.30a
Gi	26.2 bc	25.9 bc	52.5bc	4.57bc	0.09 a	90.5 b	26.80a
PQ							
Non-AM	19.6 a	22.2 a	41.8 a	4.53 a	0.11 ab	87.9 b	–
Gm	27.6 c	26.9 c	54.5 c	5.47 c	0.09 a	90.0 b	19.70a
Gi	24.6bc	24.1 b	48.7 b	4.61 b	0.11 ab	88.3 b	26.20a

Shoot, root and total plant length, total fresh mass (FM), leaf dry mass (DM) and leaf water content in AM and non-AM soybean plants. AM colonization was expressed as percentage of whole roots, more details in "material and methods". Non-AM: no mycorrhizal plants; *Gm*: *G. mosseae* plants; *Gi*: *G. intraradices* plants; 5 μM PQ: paraquat treatment. The mean values were calculated from the data obtained in two separated experiments. The columns marked with equal letters represents the means which do not differ significantly at α: 0.05. LSD Fisher.

generated. Both leaf water content and leaf DM were unaltered in PQ AM plants and PQ non-AM plants, suggesting that tolerance to PQ stress in AM plants was not related to AM capacity to improve water relations (Auge, 2001) or photosynthetic metabolism regulation (Smith and Read, 2008). On the other hand, PQ stress did not affect AM colonization, and this was correlated with root growth stimulation in AM plants compared with PQ non-AM plants, suggesting that tolerance to PQ stress in AM plants could be associated with an enhanced capacity to increase nutrient uptake by plants (Smith and Read, 2008).

Tolerance to PQ stress has been associated with oxidative stress development. It is well known that, under light conditions, PQ preferentially accepts electrons from photosystem I and donates them to oxygen, generating the $O_2^{\bullet -}$ within the chloroplast (Halliwell, 1982). In the absence of sufficient antioxidant defenses, the presence of both $O_2^{\bullet -}$ and H_2O_2 would ensure the formation of $OH^{\bullet}$, which can then initiate a destructive process of the thylakoid membrane, with peroxidation of membrane lipids and disruption of chloroplast structure (Farrington et al., 1973; Babbs et al., 1989). After exposure to PQ stress, non-AM plants showed a significant increase in MDA content (Fig. 1). When antioxidant defenses were evaluated, total SOD activity was unaltered (data not shown) and no PQ effect on total CAT activity was evident. These results agree with the lack of up-regulation of the plant antioxidant system by PQ (Ekmekci and Terzioglu, 2005). Accordingly, total APX activity was significantly decreased in PQ non-AM plants. This result is in agreement with Mano et al. (2001), who observed that one of the primary targets of PQ was chl APX isoenzymes. Since APX activity catalyzes H_2O_2 detoxification (Asada, 1992), its decay could result in a lack of H_2O_2 regulation (Asada, 1992) or a decrease in ascorbate content (Iturbe-Ormaetxe et al., 1998). All these effects could be involved in oxidative stress development observed in control PQ non-AM plants.

In contrast to non-AM plants, in AM plants, foliar MDA content decreased significantly in *Gm* or remained unaltered in *Gi* (Fig. 1). Interestingly, MDA content was unchanged in AM plants after PQ stress and remained significantly lower than in PQ non-AM plants (Fig. 1). Oxidative damage decrease, evaluated as MDA content, has been observed under different stress conditions in AM plants. For example, Ruiz-Lozano et al. (2001b), Porcel et al. (2003), Porcel and Ruiz-Lozano (2004), and Wu et al. (2006a,b) observed that MDA content was reduced in roots and shoots of AM plants subjected to drought stress, and it was correlated with an enhanced drought tolerance of AM plants. Similarly, in a previous work conducted under biotic stress conditions, we found that, after the inoculation of the soilborne pathogens, *Macrophomina* and *Fusarium*, a low MDA level was maintained in leaves of AM soybean plants (data not shown). Thus, both previous and present results indicate that reduced oxidative damage to lipids in AM plants seems to be a consistent effect of AM symbiosis under several stress conditions. When antioxidant defenses were evaluated in both control AM plants, total CAT activity increased significantly in *Gm* or decreased in *Gi* plants, showing an inconsistent effect on oxidative stress alleviation. However, and surprisingly, total APX activity was significantly diminished in both AM plants (Fig. 1). Porcel and Ruiz-Lozano (2004) showed a similar and significant decrease in total APX activity in roots and shoots in well-irrigated *G. intraradices* soybean plants. Under normal conditions, lower APX activity in both AM plants was related to a signaling phenomenon of defense. Supporting this idea, oxidative damage evaluated as foliar MDA content was lower in AM plants than in non-AM plants. Indeed, H_2O_2 accumulation has been mentioned as a signal both in biotic and abiotic

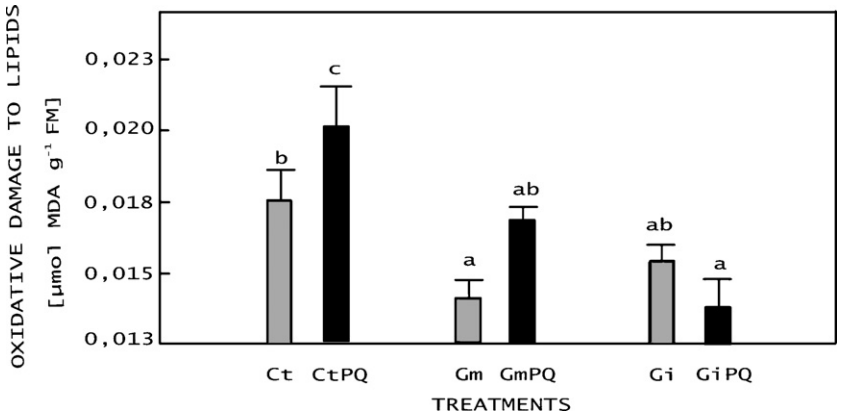

Fig. 1. Effect of paraquat (PQ) stress on oxidative damage to lipids (μmol MDA g^{-1} FM) in leaves of non-AM (control), *Glomus mosseae* (*Gm*) and *Glomus intraradices* (*Gi*) soybean plants. The mean values were calculated from 10 measurements obtained from two separate experiments. In each column, different letters indicate significant differences at α: 0.05 according to LSD test.

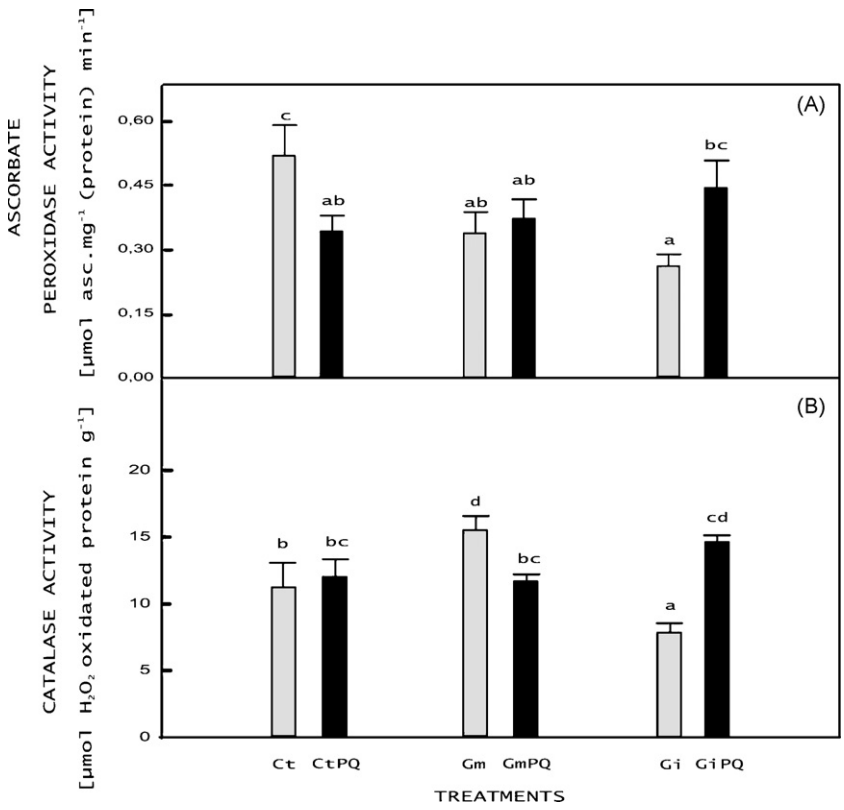

Fig. 2. Effect of paraquat (PQ) stress on ascorbate peroxidase (A) and catalase (B) activities in leaves of non-AM (control), *Glomus mosseae* (*Gm*) and *Glomus intraradices* (*Gi*) soybean plants. The mean values were calculated from 10 measurements obtained from two separate experiments. In each column, different letters indicate significant differences at α: 0.05 according to LSD test.

stresses (Mittler, 2002; Apel and Hirt, 2004) and its relation with APX activity decrease has also been documented (Mittler, 2002). The response of control AM soybean plants to APX activity requires further study. A different situation was observed when AM plants were exposed to PQ stress; both total APX and CAT activity were induced significantly in *Gi* PQ plants, while in *Gm*, PQ total APX activity was unaltered and total CAT activity was reduced to control levels (Fig. 2A and B). Because PQ treatment developed photooxidative damage in leaves, the ability of AM soybean plants to increase or maintain total APX activity could be a strategy to keep oxidative stress at low levels, while total CAT activity did not show consistent effects. Our results agree with findings of Alguacil et al. (2003), Porcel et al. (2003) and Wu et al. (2006a,b), who reported increased antioxidant enzyme activities under other abiotic stresses, suggesting that antioxidant defenses could be involved, at least in part, in the beneficial effects of mycorrhizal colonization.

Overall, after PQ treatment, growth parameters and oxidative stress characters were different in AM soybean plants than in non-AM soybean plants. The typical PQ-induced oxidative stress in AM plants was significantly relieved: oxidative damage in AM plants was under control and antioxidant defense, particularly total APX activity, seems to be included. Since plants that are resistant to one stress are often more resistant to other stresses (Bowler and Fluhr, 2000), the use of mycorrhizal fungi to improve oxidative stress tolerance could be a promising strategy to protect plants from biotic and abiotic stress.

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