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Responses of photosynthesis-related parameters and chloroplast ultrastructure to atrazine in alfalfa (*Medicago sativa* L.) inoculated with arbuscular mycorrhizal fungi

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ABSTRACT

Atrazine is an ingredient in photosynthesis-inhibiting herbicides and has been widely used to combat weeds in farmland. However, most atrazine that is applied fails to degrade in the soil and subsequently affects non-target plants. In this study, we investigated the influence of arbuscular mycorrhizal fungi (AMF), *Funneliformis mosseae* on the photosynthesis-related parameters, chlorophyll content, and chloroplast ultrastructure in alfalfa plants, some of which had been exposed to atrazine. Our results showed that the percentage of AMF hyphal colonization reached 91.23% 35 days after the alfalfa was planted, which suggests a symbiotic relationship between *F. mosseae* and alfalfa roots. *F. mosseae* alleviated the inhibition of net photosynthesis and stomatal function significantly in alfalfa exposed to atrazine for 24 h. A chlorophyll fluorescence analysis revealed that *F. mosseae* prevented a major reduction in the performance of photosystem II (PSII) photochemistry in the presence of atrazine, such as the relative decrease of F_v/F_m between the non-mycorrhizal and *F. mosseae* mycorrhizal treatments was 4.4% and 5.8% after 24 and 48 h of atrazine exposure time. However, *F. mosseae* has no significant alleviation on a sharp reduction in the chlorophyll a, chlorophyll b and carotenoid content in alfalfa exposed to atrazine. For the chloroplast ultrastructure in alfalfa exposed to atrazine, the number of both plastoglobules and partial granal stacks was greater in the presence of *F. mosseae*. In general, our results indicate that the *F. mosseae* inoculation was beneficial to sustain photosynthesis-related performance, such as net photosynthesis, stomatal conductance, the maximum quantum yield (F_v/F_m) and effective quantum yield (Φ_{PSII}) of PSII photochemistry in alfalfa after exposure to atrazine, because the mycorrhizal alfalfa had a greater number of plastoglobules and granal stacks in the chloroplast, thereby enhancing its resistance to the oxidative damage induced by atrazine.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is one of the most widely used herbicides, administered both singly and in combination with other herbicides, to combat grassy and broadleaf weeds (Zhang et al., 2014a). A photosynthesis-inhibiting herbicide, atrazine specifically interrupts photosystem II (PSII) by blocking electron acceptor proteins and inhibiting the electron transport chain in the chloroplast. Atrazine then greatly reduces the production of ATP and nicotinamide adenine dinucleotide phosphate (NADPH), as well as the productivity of the CO_2 fixation process (Hess, 2000; Qian et al., 2014). At the same time, atrazine induces the rapid accumulation of reactive oxygen species (ROS), which leads to membrane injury because of lipid peroxidation, degradation of proteins, inactivation of enzymes, bleaching of pigments and disruption of DNA strands (Wang et al., 2015; Alberto et al., 2016). Because atrazine is often applied in large amounts and usually persists for several decades, atrazine is regularly detected in soils (Jablonowski et al., 2011) and in aquatic ecosystems throughout a watershed (Guo et al., 2016; Qu et al., 2017). In this way, atrazine often affects non-target plants. For example, Zhang et al. (2014b) reported a significant decrease in the elongation of rice shoots and roots, as well as in total chlorophyll, after atrazine exposure. It has also been suggested that atrazine dramatically inhibits the periphyton effective quantum yield of PSII photochemistry (Φ_{PSII}) and the

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maximum quantum yield (F_v/F_m) (Laviale et al., 2011).

Arbuscular mycorrhizal fungi (AMF) form mutualistic symbioses with most terrestrial plant species worldwide (Fan and Song, 2014). AMF can enhance resistance to many abiotic stressors, including heavy metals (Meier et al., 2012; Yang et al., 2015) and organic contaminants (Lu and Lu, 2015; Dong et al., 2016) by alleviating the toxic effects on plants. However, despite the numerous studies on the toxic effect of atrazine on plants, the way in which AMF influences plant responses to atrazine has been overlooked.

Alfalfa (*Medicago sativa* L.) was selected as the host plant in the present study and serves two distinct purposes. First, alfalfa is sensitive to toxic inhibition during the photosynthetic process (Frank et al., 1993), so it can be affected by both atrazine and AMF, which enables us to examine the contributions of these interacting factors. Second, because alfalfa can store a high amount of protein, it is commonly used for animal forage or in crop rotation practices and is often grown in fields that have been treated with atrazine, making this a relevant model organism for farmers. Thus, we are justified in investigating the influence of AMF on the following: (1) the photosynthesis-related parameters and the chlorophyll content (2) the change in chloroplast ultrastructure in alfalfa leaves following atrazine addition; and (3) whether the association of alfalfa with AMF can be applied in fields with atrazine residue.

2. Material and methods

2.1. Experimental design

The AMF inoculum *Funneliformis mosseae* (Nicol. and Gerd.) Walker & Schüβler comb. nov., also known as *Glomus mosseae* (Schüβler and Walker, 2010), was preserved in the Ecology Laboratory of Heilongjiang University. The *F. mosseae* inoculum consisted of a mixture of growth media, mycelium, spores (approximately 25 spores per gram) and root fragments, and was propagated for three months on sorghum.

In alfalfa pot experiment, seeds were surface-sterilized by 10% H_2O_2 and germinated as described in Song et al. (2016). The growth medium used was a mixture of peat soil, sand and vermiculite 5:2:3 (*V*: *V*: *V*) and was autoclaved at 121 °C, 0.1 MPa for 1.5 h to inactivate indigenous AMF. For the *F. mosseae* mycorrhizal treatments (FM treatments, 10 pots), approximately 3.5 kg of the medium was placed in a pot (300 × 100 × 150 mm) and was thoroughly mixed with 30 g of the inoculum. The same amount of growth medium and the inactive inoculum was mixed for use in the non-mycorrhizal treatments (NM treatments, 10 pots). Potted seedlings were placed in an outdoor nursery to receive natural daylight in June 2017, when the temperature averaged 26/17 °C (day/night).

Mycorrhizal colonization was assessed by compound microscope after acetic acid ink staining (Song et al., 2016) each week after planting. The colonization percentage of AMF vesicles and intercellular hyphae was estimated according to the magnified line-intersect method (Mcgonigle et al., 1990). When the colonization percentage reached 90%, 10 mg kg^{-1} atrazine (10 mg active ingredient kg^{-1} growth media) was applied to both the FM and the NM treatments. In total, there were four different treatments: FM treatments without atrazine (5 pots); NM treatments without atrazine (5 pots); FM treatments with atrazine (5 pots); and NM treatments with atrazine (5 pots). After atrazine exposure, leaf photosynthesis-related parameters and the chlorophyll content were determined, and the chloroplast ultrastructure was checked at 24 h and 48 h.

2.2. Photosynthesis-related parameters

Net photosynthesis, stomatal conductance and chlorophyll fluorescence parameters were determined for the third fully expanded young leaves, with five replicates per treatment, using LI-6400 (LiCor, Lincoln, NE, USA). The measurements were carried out at 25 °C with an ambient CO_2 concentration of approximately 350 μmol mol^{-1}, a relative humidity of 75% and with a 1200 μmol m^{-2} s^{-1} photosynthetic photon flux density, which was provided by an LED light.

These chlorophyll fluorescence parameters were adopted from the following equations defined by Murchie and Lawson (2013) and containing F_v/F_m and Φ_{PSII} (Rascher et al., 2000).

$$F_v/F_m = (F_m - F_0)/F_m \quad (1)$$

$$\Phi_{PSII} = (F'_m - F_t)/F'_m \quad (2)$$

where F_m and F_0 are the maximal and minimal fluorescence values of dark-adapted leaves after 30 min, respectively, F_v is the variable fluorescence, F'_m is the maximal fluorescence of the light-adapted leaves, and F_t is the steady state fluorescence.

2.3. Chlorophyll content

Chlorophyll was extracted from 1 g of fresh leaves by suspending them in 5 mL of 80% acetone (v/v). The absorbance of the extracts at 470, 646 and 663 nm was recorded by a spectrophotometer (UV-2800, Unico, Shanghai, China). The chlorophyll a (Chl *a*), chlorophyll b (Chl *b*) and carotenoid content was calculated according to the equations described by Lichtenthaler and Wellburn (1983).

2.4. Transmission electron microscopy (TEM)

Leaf segments (1–2 mm) were cut and immersed in 2.5% glutaradehyde (0.1 M sodium phosphate buffer, pH 6.8) and then fixed for 24 h at 4 °C to prevent air bubbles from entering. The samples were then washed three times in a 0.1 M sodium phosphate buffer, postfixed with 1% osmium tetroxide and washed in a 0.1 M sodium phosphate buffer again. Subsequently, the samples were dehydrated with alcohol, impregnated and embedded in resin. The ultrathin sections were picked up on copper grids, stained with a uranyl acetate solution for 2 min and observed under the TEM (Hitachi H-7650, Tokyo, Japan).

2.5. Statistics

The statistical analysis was performed using the SPSS 19.0 statistical program (SPSS Inc., Chicago, IL, USA). Data were analyzed with a two-way ANOVA of *F. mosseae*, atrazine and their interactions. Duncan's multiple range test was used to determine the significant difference between groups, with $p < 0.05$ as the significance threshold.

3. Results

3.1. Colonization

In FM treatments, the percentage of roots with vesicles and hyphae increased over time and reached 91.23% 35 days after the alfalfa was planted (Fig. 1). Inside the mycorrhizal roots, typical mycorrhizal structures, such as vesicles, arbuscular and intercellular hyphae, were all detected by compound microscope (Fig. 2). This observation suggests a symbiotic relationship between *F. mosseae* and alfalfa roots. As expected, no mycorrhizal colonization was observed in the NM treatments.

3.2. Photosynthesis-related parameters

Net photosynthesis and stomatal conductance of alfalfa were strongly inhibited by atrazine ($p < 0.001$, Table 1), regardless of whether the plant had been inoculated with *F. mosseae*. Their inhibitions by atrazine increased, as expected, with exposure time (Fig. 3). After 48 h of exposure time, net photosynthesis was almost zero (Fig. 3A). Furthermore, the presence of *F. mosseae* significantly increased the net photosynthesis and stomatal conductance of alfalfa