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Exogenous calcium induces tolerance to atrazine stress in Pennisetum seedlings and promotes photosynthetic activity, antioxidant enzymes and *psbA* gene transcripts

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ABSTRACT

Calcium (Ca) has been reported to lessen oxidative damages in plants by upregulating the activities of antioxidant enzymes. However, atrazine mediated reactive oxygen species (ROS) reduction by Ca is limited. This study therefore investigated the effect of exogenously applied Ca on ROS, antioxidants activity and gene transcripts, the D1 protein (psbA gene), and chlorophyll contents in Pennisetum seedlings pre-treated with atrazine. Atrazine toxicity increased ROS production and enzyme activities (ascorbate peroxidase APX, peroxidase POD, Superoxide dismutase SOD, glutathione-S-transferase GST); but decreased antioxidants (APX, POD, and Cu/Zn SOD) and psbA gene transcripts. Atrazine also decreased the chlorophyll contents, but increased chlorophyll (a/b) ratio. Contrarily, Ca application to atrazine pre-treated seedlings lowered the harmful effects of atrazine by reducing ROS levels, but enhancing the accumulation of total chlorophyll contents. Ca-protected seedlings in the presence of atrazine manifested reduced APX and POD activity, whereas SOD and GST activity was further increased with Ca application. Antioxidant gene transcripts that were down-regulated by atrazine toxicity were up-regulated with the application of Ca. Calcium application also resulted in up-regulation of the D1 protein. In conclusion, ability of calcium to reverse atrazine-induced oxidative damage and calcium regulatory role on GST in Pennisetum was presented.

1. Introduction

Atrazine (2-chloro-4-ethylamine-6-isopropylamino-S-triazine) is a common selective triazine herbicide used to control broadleaf and grassy weeds at low cost in diverse crop plantations such as maize, sorghum and sugarcane (Kiely et al., 2004; Moore and Kröger, 2010; Jiang et al., in press). Atrazine may be absorbed by the plant roots and transported to the above ground part (Akbulut and Yigit, 2010). The presence of atrazine in the plant system can inhibit plant development by inducing the production of reactive oxygen species (ROS), denaturing proteins, lipids, and nucleic acids (Zhao et al., 2005). Exposure to atrazine has been reported to induce the production of ROS in *Arabidopsis thaliana* seedlings (Sulmon et al., 2007).

Atrazine affects plant photosynthesis by blocking the electron acceptor protein photosystem II (PSII), thereby inhibiting electron transfer in the photosynthesis process (Qian et al., 2014). The inhibition of PSII electron transport prevents the conversion of chlorophyll-absorbed light energy into electro-chemical energy and results in the production of triplet chlorophyll and singlet oxygens (Perez-Jones et al., 2009). The rapid and prolonged accumulation of these highly reactive radicals otherwise referred to as the ROS, beyond the quenching capacity of cellular scavenging antioxidants in the vicinity of PSII, cause severe oxidative damage of proteins, lipids and pigments (Zhu et al., 2009). The ROS can also induce substantial damage to a number of macromolecules such as DNA and RNA (Zouari et al., 2016a). Majorly, physiological processes such as photosynthesis are severely affected, reducing the photosynthetic rate by induction of stomatal or nonstomatal limitations (Song et al., 2014).

Exogenous applications of various treatments have been utilized to alleviate ROS-induced damages (oxidative damages). Sulmon et al. (2007) showed that exogenous treatment by soluble sugars, especially sucrose, successfully relieved *Arabidopsis*

Abbreviations: ROS, reactive oxygen species; PSII, Photosystem II; CAT, catalase; POD, peroxidase; SOD, Cu/ZnSOD, cupper-zinc superoxide dismutase; APX, ascorbate peroxidase; Ca, calcium ion; Chl, chlorophyll; CLSM, laser scanning fluorescence microscopy; DHE, dihydroethidium; H_2O_2, hydrogen peroxide; $O_2^{\bullet -}$, superoxide anion radical

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thaliana or mustard weeds of atrazine induced oxidative damages. Likewise, proline was used to alleviate cadmium induced stress on young date palms (Zouari et al., 2016b, 2016c). Other similar treatments that have been used to alleviate oxidative damages in plant studies include the exogenous application of glycine-betaine to cotton seedlings (Bharwana et al., 2014) and ornamental shrubs (Cirillo et al., 2016), and application of nitric oxide to lettuce leaves (Silveira et al., 2015).

Application of mineral nutrients, such as calcium (Ca) has been shown to play significant role in controlling membrane structure and function (Burstrom, 1968), stress tolerance, induction of antioxidant enzyme activities and reduction of lipid peroxidation (Siddiqui et al., 2011; Xu et al., 2013). Calcium binding to the phospholipid stabilizes lipid bilayers and thus provides structural integrity to cellular membranes (Hanson, 1984). In a series of leaf abscission and tissue senescence, Poovaiah and Leopold (1976) reported that Ca inhibited or slowed these processes. Calcium ability to retard the loss of chlorophyll, protein, and free space in maize and rumex leaf disks has also been shown (Poovaiah and Leopold, 1973a); suggesting that the ion plays a regulatory role in maintaining and controlling membrane structure and function (Poovaiah and Leopold, 1973b). Furthermore, Khan et al. (2010) reported the ability of Ca in inducing antioxidative defense and osmoprotection in linseeds (*Linum usitatissimum*) subjected to salt (NaCl) stress. Siddiqui et al. (2011) also reported Ca ability to induce tolerance to nickel toxicity in *Triticum aestivum*. As noted by Yang et al. (2014), Ca-involved signal transduction pathway helped plants adapt to stress by evoking gene expression and activating a series of biochemical responses. It has also been reported that Ca improvement of photosynthesis is related to enhancing the activity of antioxidant enzymes to alleviate ROS accumulation (Yang et al., 2014).

Pennisetum (*Pennisetum* sp.) is a C_4 plant with high growth rate and biomass production (Hu et al., 2005), and most commonly used as a forage plant for feeding livestock (Hu et al., 2007). Besides, the ability of Pennisetum, alone or in combination with soil isolated microorganisms, to degrade s-triazine herbicides at the rhizosphere level has been reported. Pennisetum was used to degrade two triazine herbicides (atrazine and simazine) in cement blocks of a long-term (approx. 15 yrs.) contaminated soil (Singh et al., 2004). After 80 days exposure, atrazine and simazine concentrations decreased to 55% and 48%, respectively, of the original level in the rhizosphere soil. Similarly, Zhang and colleagues have reported the ability of Pennisetum in combination with *Arthrobacter* sp. DNS10 to degrade atrazine in a contaminated medium (Zhang et al., 2013). They reported plant alone or plant-microbe joint interaction degraded 66.71% or 98.10%, respectively, of the atrazine after a 30-day exposure time. Moreover, the tolerance of Pennisetum plants to high levels of atrazine was recently reported by Jiang et al. (in press). In their report, they noted that exposure of Pennisetum seedlings to high atrazine concentrations (100 mg kg^{-1} and 200 mg kg^{-1}) induced a down regulation of the antioxidant enzyme activities; whereas, malondialdehyde (MDA) content, an indicator of lipid and membrane peroxidation, in the leaves and roots of the plants did not increase significantly under the atrazine treatments, compared to the control plants.

The present investigation was thus performed to determine the (1) effect of exogenously applied calcium on atrazine detoxification in Pennisetum (*Pennisetum americanum* (L.) K. Schum) seedlings by assessing the effect of treatments on the response of antioxidant enzymes activity and gene transcripts; and alleviation of atrazine-induced oxidative stress, through the reduction of ROS in Pennisetum seedlings; and (2) effect of Ca on chlorophyll contents and photosystem II-related protein (psbA) in atrazine-stressed Pennisetum seedling. In order to fulfill these objectives, a greenhouse experiment was carried out to assess the effects of exogenously applied calcium on Pennisetum grown under atrazine toxicity. Untreated and treated plants were compared in terms of the above mentioned physiological and molecular parameters.

2. Material and methods

2.1. Herbicide, soil and plant seed

The atrazine (> 97% purity) used in this study was obtained from Shandong Pesticide Research Institute, China. The Pennisetum seeds (*Pennisetum americanum* (L.) K. Schum) were purchased from Jiang Su Xinglong Seedling Company, China. The test soil was taken at a depth of 0–30 cm from the experimental field located in Acheng District, Harbin, Heilongjiang Province, China (45°32′ 8.498″N–127°1′0.795″E). The soil samples were dried at room temperature, crushed and sieved through a 2 mm mesh to separate small rocks and litters. The chemical properties of the test soil are given as: pH 7.92; organic matter 2.78%, ammonium nitrogen 16.60 mg kg^{-1}; nitrate nitrogen 4.78 mg kg^{-1}; available potassium 86.00 mg kg^{-1}. The textural content of the soil is clay, silt and sand of 14.46%, 56.17% and 29.37%, respectively.

2.2. Plant materials and growth conditions

The Pennisetum seeds were washed thoroughly with distilled water and soaked in deionized water for 3 h, afterwards were fully rinsed with deionized water. The rinsed seeds were pre-germinated in the dark (27 ± 1 °C) on moist Whatman No. 1 filter papers (Sigma-Aldrich) in petri dishes (90 mm diameter). Seedlings of good vigour were selected and transplanted into germination pots (40 cm length × 25 cm wide × 7.5 cm deep) (about 50 seedlings per pot) containing a mixture of soil and vermiculite (2.0 kg per pot; soil and vermiculite of ratio 1:3). Before transplanting of seedlings, the growth medium was pre-spiked with atrazine (100 mg kg^{-1} growth medium).

The pots were maintained in the greenhouse under ambient environmental conditions with natural sunlight and temperature (28 ± 1 °C/20 ± 1 °C in day/night), and relative air humidity of 50–60%. The plants were irrigated using Hoagland solution (Hoagland and Arnon, 1938), comprising 2.5 mM KNO_3, 0.5 mM NH_4NO_3, 0.5 mM $NH_4H_2PO_4$, 0.5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 4.5 μM $MnCl_2 \cdot 4H_2O$, 23 μM H_3BO_3, 0.4 μM $ZnSO_4 \cdot 7H_2O$, 0.15 μM $CuSO_4 \cdot 5H_2O$, 0.05 μM H_2MoO_4, and 4.5 μM EDTA-Fe. The amount of water used for irrigation during the experimental period was determined by gravimetric method (Reynolds, 1970), thus: the moist soil sample was oven dried (105 °C) to constant weight. The water weight between the wet and oven dry samples was used to determine the soil moisture content. After 10 d, in order to determine the effect of Ca, a 30 mL $Ca(NO_3)_2$ (20 mM) was sprayed uniformly on foliage using a spray bottle once daily, and were further grown for 15 d. At the end, four treatment groups were realized: (1) control group with no atrazine or Ca treatment, *CK*; (2) Ca treated plants with no atrazine, *Ca*; (3) atrazine (100 mg kg^{-1}) treated plants with no Ca amendment, *ATZ*; and (4) Ca amended atrazine-treated plants, *ATZ+Ca*. The experiment was performed with three replicates per treatment.

2.3. ROS detection by confocal laser scanning fluorescence microscopy (CLSM)

Superoxide anion radicals in Pennisetum leaves were detected using the fluorescent probe dihydroethidium (DHE) (Sigma-Aldrich). Newly emerging Pennisetum leaves were cut into segments of about 20 mm^2 from the apex and were incubated overnight at 25 °C, in the dark, with 10 μM DHE (Sandalio et al., 2008). Then the