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Research article

Involvement of ethylene in gibberellic acid-induced sulfur assimilation, photosynthetic responses, and alleviation of cadmium stress in mustard

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ABSTRACT

The role of gibberellic acid (GA) or sulfur (S) in stimulation of photosynthesis is known. However, information on the involvement of ethylene in GA-induced photosynthetic responses and cadmium (Cd) tolerance is lacking. This work shows that ethylene is involved in S-assimilation, photosynthetic responses and alleviation of Cd stress by GA in mustard (*Brassica juncea* L.). Plants grown with 200 mg Cd kg^{-1} soil were less responsive to ethylene despite high ethylene evolution and showed photosynthetic inhibition. Plants receiving 10 μM GA spraying plus 100 mg S kg^{-1} soil supplementation exhibited increased S-assimilation and photosynthetic responses under Cd stress. Application of GA plus S decreased oxidative stress of plants grown with Cd and limited stress ethylene formation to the range suitable for promoting sulfur use efficiency (SUE), glutathione (GSH) production and photosynthesis. The role of ethylene in GA-induced S-assimilation and reversal of photosynthetic inhibition by Cd was substantiated by inhibiting ethylene biosynthesis with the use of aminoethoxyvinylglycine (AVG). The suppression of S-assimilation and photosynthetic responses by inhibiting ethylene in GA plus S treated plants under Cd stress indicated the involvement of ethylene in GA-induced S-assimilation and Cd stress alleviation. The outcome of the study is important to unravel the interaction between GA and ethylene and their role in Cd tolerance in plants.

1. Introduction

Cadmium (Cd) is a highly toxic element that poses a major threat to the agricultural system (Anjum et al., 2008a; Asgher et al., 2015). The accumulation of Cd in plants stimulates excessive production of reactive oxygen species (ROS) responsible for the damage of photosynthetic apparatus (Masood et al., 2012). Cadmium also inhibits photosynthesis and growth by interacting with mineral nutrient elements (Astolfi et al., 2014). The management of mineral nutrients has shown to counteract the adverse effects of Cd. Sulfur (S) nutrition regulates photosynthesis under both optimal and stressful environments (Khan et al., 2013) as it is an integral part of many metabolites of plants, such as allyl cysteine (Cys), cofactors, Cys, glucosinolates, glutathione (GSH), iron-S clusters, methionine (Met), sulfolipids and vitamins (Abdallah et al., 2010; Khan et al., 2013). It has been shown that adequate S availability is necessary for the protection of photosynthetic pigments, photosystem (PS) II efficiency, activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and growth responses in plants grown under Cd stress (Astolfi et al., 2004; Masood et al., 2012). Recently, Khan et al. (2016) reviewed the role of S in increasing efficiency of an antioxidant system and improvement in the capacity of plants to survive under Cd stress. The enzymes of S-assimilation, ATP-sulfurylase (ATP-S) and serine acetyl transferase (SAT) play important roles in enhancing Cd tolerance in *Triticum aestivum* (Khan et al., 2007). Anjum et al. (2008b) have shown that elemental S supplementation (40 mg S kg^{-1} soil) increased content of leaf AsA and GSH and reduced Cd toxicity in *Brassica campestris*.

Phytohormones are important in the regulation of S-assimilation, antioxidant metabolism (Khan and Khan, 2014) and physiological and biochemical responses of plants under optimal and limited environmental conditions. Gibberellic acid (GA) augments photosynthetic and growth potential and source–sink relation of plants (Iqbal et al., 2011). Earlier, Khan et al. (1998) have shown that GA spraying improves photosynthetic functions and nutrient

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uptake in *Brassica juncea*. Reports are available on the mitigating effects of GA in Cd stressed plants (Rubio et al., 1994; Meng et al., 2009; Masood and Khan, 2013). The plant hormone ethylene has gained much attention in recent years in regulation of photosynthetic capacity (Khan, 2004; Iqbal et al., 2012) and adaptation to abiotic stress (Khan et al., 2014). It has been shown earlier that ethylene was involved in S-mediated alleviation of photosynthetic inhibition by Cd in *B. juncea* (Masood et al., 2012). Also, Fu et al. (2014) have shown regulatory interaction between S-assimilation and ethylene and the tolerance of Col-0 accession to arsenic.

Independent research on GA and ethylene has revealed their potential in regulation of S-assimilation, photosynthetic responses and alleviation of Cd stress. One of the studies has shown that GA spraying improved photosynthesis by improving S-use efficiency (SUE) in mustard plants grown with 100 mg S kg^{-1} soil (Khan et al., 2005). In another study, it was found that 100 mg S kg^{-1} soil alleviates Cd stress through regulation of optimal ethylene formation in mustard (Masood et al., 2012). However, the role of ethylene in GA-induced S-assimilation, photosynthetic responses and alleviation of Cd stress has not been worked out in detail. There is possibility of interaction between ethylene and GA in S-assimilation, photosynthetic responses and Cd tolerance. The reported research was, therefore, carried out to study the involvement of ethylene in GA-induced S-assimilation and photosynthetic responses under Cd stress.

2. Materials and methods

2.1. Plant material and growth conditions

Mustard (*B. juncea* L. Czern & Coss.) cv. Varuna seeds were surface sterilized with 0.01% $HgCl_2$ solution and were repeatedly washed with double distilled water before sowing in 23-cm diameter earthen pots filled with 5 kg of reconstituted soil (sand:clay:peat; 70:20:10 by dry weight). The pots were kept in a naturally illuminated net house of the Department of Botany, Aligarh Muslim University, Aligarh, India with average day/night temperatures of 22/14 ± 3 °C and relative humidity of 62 ± 5%. Plants were grown individually with 200 mg Cd kg^{-1} soil or/and100 mg S kg^{-1} soil or in combination. These plants were treated with 10 μM GA_3 at 20 days after sowing (DAS) to study the potential of GA spraying and S supplementation in the alleviation of Cd stress. A control group of plants was also maintained. Cadmium was applied as $CdCl_2$ at the time of sowing, while elemental S was used for S treatment and applied 15 days before seed sowing. The concentration of S and GA_3 was selected based on earlier findings that S at 100 mg S kg^{-1} soil to Cd-treated plants maximally increased Cd tolerance (Masood et al., 2012), and that application of 10 μM GA_3 to mustard plants increased S availability (Khan et al., 2005). Another experiment was conducted to study the role of ethylene in GA-induced Cd tolerance of S grown plants. In this experiment, ethylene biosynthesis inhibitor, aminoethoxyvinylglycine (AVG) was applied to Cd-treated plants or to plants receiving GA_3 spraying plus S and grown under Cd stress. In addition to control, plants treated with Cd and plants treated with AVG alone were also maintained. Cadmium and S were given at 200 mg Cd kg^{-1} soil and 100 mg S kg^{-1} soil, respectively, while GA_3 was sprayed at 10 μM and AVG at 50 μL L^{-1} concentration. The treatment of GA_3 or AVG was applied on the foliage of plants at 20 DAS. The volume of the spray was 25 mL per pot. At 30 DAS, the measurements on S-assimilation, ethylene evolution, net photosynthesis and plant dry mass were recorded. The experiments followed a completely randomized block design and the number of replicates for each treatment was four (n = 4).

2.2. Determination of Cd content

Leaf and root samples were dried at 80 °C for 48 h, ground to fine powder and digested with concentrated $HNO_3/HClO_4$ (3:1, v:v). Cadmium concentration was determined by atomic absorption spectrophotometer (GBC, 932 plus; GBC Scientific Instruments, Braeside, Australia).

2.3. Determination of H_2O_2 content

The method adopted for the determination of H_2O_2 content and details have been described earlier (Asgher et al., 2014).

2.4. Determination of ATP-sulfurylase activity and content of sulfur, cysteine and methionine

Activity of ATP-S was assayed *in vitro* in leaves by measuring molybdate-dependent formation of pyrophosphate. The content of S was determined in leaf samples digested in a mixture of concentrated HNO_3 and 60% $HClO_4$ (85:1, v/v). Total sulfur in plant samples was estimated according to the turbidimetric method. A 5 mL aliquot was used for turbidity development in 25 mL volumetric flask. Turbidity in 5 mL aliquot was developed by adding 2.5 mL gum acacia (0.25%) solution, 1.0 g $BaCl_2$ sieved through 40–60 mm mesh and the volume was made to 25 mL with de-ionized water. The contents of 25 mL volumetric flask were thoroughly shaken till $BaCl_2$ completely dissolved. The values were recorded at 415 nm within 10 min after the turbidity development. A blank was run simultaneously after each set of determination. For determination of Cys content, fresh leaves (0.5 g) were homogenized in 5% (w:v) ice-cold perchloric acid. The final volume of 4 mL g^{-1} of plant tissue was used. The suspension was centrifuged at 2800×*g* for 1 h at 5 °C and the supernatant was filtered. One mL of the filtrate was treated with acid ninhydrin reagent and the color intensity was read at 580 nm. The amount of Cys was calculated with reference to a calibration curve.

The details of the method determination of ATP-S activity, content of S and Cys have been described earlier (Asgher et al., 2014).

Methionine content was determined by the method of Horn et al. (1947). Fresh leaves (0.5 g) were reflexed with 20.0 mL 6.0 N HCl for 20–24 h followed by evaporation on water bath with the addition of 1.0 g activated charcoal. The filtrate was collected to which 4.0 mL de-ionized water and 2.0 mL of 5 N NaOH were added followed by the addition of 0.1 mL sodium nitroprusside and 2.0 mL glycine solution (3%). Finally, 4.0 mL phosphoric acid was added and the color intensity was read at 450 nm.

2.5. Assay of antioxidant enzymes

Fresh leaf tissue (200 mg) was homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone (PVP) in potassium-phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 15,000×*g* for 20 min at 4 °C. The supernatant obtained after centrifugation was used for the assay of superoxide dismutase (SOD; EC 1.15.1.1), GSH peroxidase (GPX; EC 1.11.1.9) and GSH reductase (GR; EC 1.6.4.2) enzymes. For the assay of ascorbate peroxidase (APX; EC 1.11.1.11), extraction buffer was supplemented with 2 mM ascorbate.

Activity of SOD was determined by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT). Activity of APX was determined by recording the decrease in absorbance of ascorbate at 290 nm. The assay mixture contained phosphate buffer (50 mM, pH 7.0), 0.1 mM EDTA, 0.5 mM ascorbate, 0.1 mM H_2O_2,