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Ethylene production is associated with alleviation of cadmium-induced oxidative stress by sulfur in mustard types differing in ethylene sensitivity

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ABSTRACT

We studied the response of ethylene-sensitive (Pusa Jai Kisan) and ethylene-insensitive (SS2) mustard (*Brassica juncea*) cultivars to 0, 0.5, 1.0 and 2.0 mM SO_4^{2-}, and the effect of 1.0 mM SO_4^{2-} was studied in the amelioration of 50 μM cadmium (Cd). The Cd-induced oxidative stress and Cd accumulation were greater in SS2 than Pusa Jai Kisan, but sulfur (S) application alleviated Cd-induced oxidative stress more prominently in Pusa Jai Kisan by increasing S-metabolism and synthesis of reduced glutathione (GSH) and ethylene production; and promoted photosynthesis and plant dry mass under Cd stress. The ethylene-sensitive cultivar responded more to S treatment under Cd stress and showed increased activity of antioxidant system resulting in increased photosynthesis and growth. Cadmium treatment resulted in rapid increase in ethylene formation which adversely influenced photosynthesis and plant dry mass. However, S and ethephon application to Cd-treated plants lowered ethylene formation to optimal range responsible for maximal GSH synthesis and protection against Cd-induced oxidative stress. The similarity of the effectiveness of 1.0 mM SO_4^{2-} with 200 $\mu L\,L^{-1}$ ethylene source as ethephon in alleviation of 50 μM Cd further verifies that differential alleviation of Cd toxicity in the two cultivars by S was dependent on ethylene production. The results suggest that ethylene production determines Cd stress alleviation by S via regulatory interaction with antioxidant metabolism. Thus, ethylene production and sensitivity bear a prominent role in alleviation of Cd stress by S and can be used as a criterion for developing Cd tolerant genotypes.

1. Introduction

Cadmium (Cd) toxicity is one of the most widespread abiotic stresses responsible for limiting plant metabolism and productivity worldwide (Nazar et al., 2012). The uptake and accumulation of Cd in plants can produce excess reactive oxygen species (ROS), having potential to displace essential elements such as zinc (Zn), calcium (Ca), and iron (Fe) from proteins and can inhibit electron transport chain in the chloroplast and mitochondria (Gallego et al., 2012). Cadmium negatively affects growth as a direct consequence of the inhibition of photosynthesis in plants (Mobin and Khan, 2007). It also can cause cell death due to the oxidative damage to membranes, proteins, and nucleic acids resulting in significant reductions of growth and productivity (Nazar et al., 2012). Recently, Dias et al. (2013) have reported that increasing Cd concentration reduces maximum efficiency of pigment system II (PSII) and net CO_2 assimilation rate together with the decrease in ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity. They have also shown that Cd^{2+} ions interfere with Rubisco activation, lower activity and damage its structure by substituting for magnesium (Mg^{2+}) ions. In order to cope with the excess ROS induced by Cd stress in cells, plants develop several strategies including induction of enzymatic and non-enzymatic components of antioxidant defense system.

Sulfur (S) is one of the essential mineral nutrient elements required for growth and development of plants. It regulates plant metabolism under optimal and stressful environments (Iqbal et al., 2013; Khan et al., 2013; Nazar et al., 2014). Study of Guo et al. (2009) have shown that the enzymes of S-assimilatory pathway were induced by Cd stress and plants showed tolerance to Cd stress. Sulfur assimilation pathway is linked to the synthesis of ethylene via cysteine (Cys) and methionine. In Cys synthesis, two enzymes of S-assimiltion, ATP-sulfurylase (ATP-S; EC. 2.7.7.4) and

Abbreviations: ACS, 1-aminocyclopropane carboxylic acid synthase; APX, ascorbate peroxidase; ATP-S, adenosine triphosphate sulfurylase; Cd, cadmium; Cys, cysteine; DAS, days after sowing; GSH, reduced glutathione; GR, glutathione reductase; ROS, reactive oxygen species; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase; SAT, serine acetyl transferase; S, sulfur; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

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serine acetyltransferase (SAT; EC 2.3.1.30) play essential roles. ATP-S catalyzes the activation of sulfate and SAT is responsible for the entry step from serine metabolism to Cys biosynthesis (Fatma et al., 2013).

Ethylene has diverse roles in plants affecting photosynthesis and growth (Iqbal et al., 2013). The response of plants to ethylene depends on its concentration and sensitivity of plants to ethylene (Iqbal et al., 2012). We have shown earlier that the ethylene-sensitive and ethylene-insensitive mustard types can provide a better option to study ethylene-mediated responses for augmenting photosynthesis and growth under optimal environments (Iqbal et al., 2012). The reported study was undertaken based on the information available on the cross talk between S assimilation and ethylene signaling in plants (Iqbal et al., 2013). It was postulated that ethylene-sensitive and ethylene-insensitive cultivars respond differently to S availability for ethylene production and maximal photosynthesis and growth, and ethylene-sensitive cultivar will alleviate Cd-induced oxidative stress more effectively.

2. Materials and methods

2.1. Plant material and growth conditions

Mustard (*Brassica juncea* L. Czern & Coss.) cultivars, ethylene-sensitive (Pusa Jai Kisan) and ethylene-insensitive (SS2) were selected for the study based on our earlier findings of Iqbal et al. (2012). In the study of Iqbal et al. (2012) we screened mustard cultivars for ethylene sensitivity and found that Pusa Jai Kisan and SS2 were ethylene-sensitive and ethylene-insensitive cultivars, respectively. In the present study, we examined the effect of 0, 0.5, 1.0 or 2.0 mM SO_4^{2-} on S-assimilation, reduced glutathione (GSH), ethylene production, photosynthesis and plant dry mass in Pusa Jai Kisan and SS2 cultivars. In another experiment, the potential of 1.0 mM SO_4^{2-} (selected from the first experiment where it increased photosynthesis and plant dry mass maximally) was studied in the alleviation of 50 μM Cd in these two cultivars and studied oxidative stress, antioxidant metabolism, S assimilation, activity of 1-amino cyclopropane carboxylic acid (ACC) synthase (ACS), ethylene production, photosynthesis and plant dry mass. Further, the effectiveness of 1.0 mM SO_4^{2-} was compared with 200 μL L^{-1} ethephon (ethylene source) in alleviation of 50 μM Cd to verify the postulation that differential alleviation of Cd toxicity in the two cultivars by S was dependent on ethylene production.

The seeds were surface sterilized with 0.01% $HgCl_2$ followed by repeated washings with distilled water and then rinsed with de-ionized water. The seeds were sown in 23-cm diameter pots filled with acid-washed sand. Two plants per pot were maintained after seed germination and were fed with 250 mL of modified full strength Hoagland nutrient solution on alternate days. Sulfur in different experiments was given along with the nutrient solution at 15 days after sowing (DAS). $MgSO_4$ was used for obtaining S concentrations and a uniform Mg^{2+} concentration in the treatments was maintained by the addition of $MgCl_2$. Cadmium was applied as $CdCl_2$ in the nutrient solution. The pots were kept in a greenhouse of the Botany Department, Aligarh Muslim University, Aligarh, India under natural day/night conditions with an average day and night temperatures of 22/14 ± 3 °C, photosynthetically active radiation (PAR; 680 μmol m^{-2} s^{-1}) and relative humidity of 62 ± 5%. Treatments in the experiments were arranged in a factorial randomized block design. The number of replicates for each treatment was four (n=4). The measurements in the experiments were done at 30 DAS.

2.2. Activity of ATP-S and SAT

Activity of ATP-S was measured using molybdate-dependent formation of pyrophosphate as described by Lappartient and Touraine (1996). Fresh leaf tissues (1.0 g) were ground at 4 °C in a buffer consisting of 10 mM Na_2 EDTA, 20 mM Tris–HCl (pH 8.0), 2 mM dithiothreitol (DTT), and 0.01 g mL^{-1} polyvinyl pyrrolidone (PVP), using 1:4 (w/v) tissue to buffer ratio. The homogenate was centrifuged at 20,000g for 10 min at 4 °C. The supernatant was used for in vitro ATP-S assay. The reaction was initiated by adding 0.1 mL of extract to 0.5 mL of the reaction mixture, which contained 7 mM $MgCl_2$, 5 mM Na_2MoO_4, 2 mM Na_2ATP, and 0.032 units mL^{-1} of sulfate-free inorganic pyrophosphate in 80 mM Tris–HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture but without Na_2MoO_4. Incubations were carried out at 37 °C for 15 min, after which phosphate was determined using UV–vis spectrophotometer.

Activity of SAT in the leaf extract was determined by the method of Kredich and Tomkins (1966). Fresh leaf tissues (0.5 g) were ground with a chilled mortar and pestle in 2 mL of ice cold extraction buffer Tris–HCl 100 mM (pH 8.0), 100 mM KCl, 20 mM $MgCl_2$, 1% Tween 80, and 10 mM DTT. The samples were centrifuged at 12, 000g for 10 min at 4 °C. The supernatant obtained was used for SAT assay. The enzyme reaction mixture contained 0.1 mM acetyl CoA, 50 mM Tris–HCl (pH 7.6), 1 mM DTNB, 1 mM EDTA, and 1 mM L-serine in 1 mL. Subsequent to reaction initiation by addition of enzyme at 25 °C, the rate was estimated by monitoring the increase in absorbance at 412 nm and calculated using an extinction coefficient of 13, 600. A blank containing all materials except L-Serine was run simultaneously and subtracted from the reaction rate obtained with L-serine.

2.3. Determination of content of Cys, GSH and S

The content of Cys in leaves was determined spectrophotometrically adopting the method of Giatonde (1967). Fresh leaves (500 mg) were homogenized in 5% (w/v) ice-cold perchloric acid. The suspension was centrifuged at 2800g for 1 h at 5 °C and the supernatant was filtered. Following this, 1 mL of filtrate was treated with acid ninhydrin reagent and the absorption was read at 580 nm. The amount of Cys was calculated using the calibration curve obtained for standard Cys.

GSH was determined following the method of Anderson (1985). Fresh leaves (500 mg) were homogenized in 2.0 mL of 5% sulphosalicylic acid under cold conditions. The homogenate was centrifuged at 10,000g for 10 min. To 0.5 mL of supernatant, 0.6 mL of phosphate buffer (100 mM, pH 7.0) and 40 μL of 5'5'-dithiobis-2-nitrobenzoic acid (DTNB) were added and after 2 min the absorbance was read at 412 nm.

S content was determined in leaf samples digested in a mixture of concentrated HNO_3 and 60% $HClO_4$ (85:1 v/v) using the turbidimetric method (Chesnin and Yien, 1950).

2.4. Measurement of ACS activity and ethylene production

Activity of ACS (EC; 4.4.1.14) was measured by adopting the methods of Avni et al. (1994) and Woeste et al. (1999). Leaf tissues (5.0 g) were ground in 100 mM HEPES buffer (pH 8.0) containing 4 mM DTT, 2.5 mM pyridoxal phosphate and 25% PVP. The homogenized preparation was centrifuged at 12,000g for 15 min. One mL of the supernatant was placed in a 30 mL tube and 0.1 mL of 5 mM S-adenosyl methionine (AdoMet) was added and incubated for 2 h at 22 °C. The ACC formed was determined by its conversion to ethylene by the addition of 0.1 mL of 20 mM $HgCl_2$ followed by the addition of 0.1 mL of 1:1 mixture of saturated NaOH/NaCl and placed on ice for 10 min. In the control set, AdoMet was not added.

Ethylene was measured by cutting 0.5 g of leaf material into small pieces that were placed into 30 mL tubes containing moist paper to minimize evaporation from the tissue and were stoppered with secure rubber caps and placed in light for 2 h under the same condition used for plant growth. An earlier experiment showed that 2 h incubation time was adequate for ethylene detection without the interference of wound-induced ethylene, which began after 2 h of leaf incubation. A 1 mL gas sample from the tubes was withdrawn with a hypodermic syringe and assayed on a gas chromatograph (Nucon 5700, New Delhi, India) equipped with a 1.8 m Porapack N (80–100 mesh) column, a flame ionization detector and data station. Nitrogen was used as the carrier gas. The flow rates of nitrogen, hydrogen and oxygen were 30, 30 and 300 mL min^{-1}, respectively. The detector was set at 150 °C. Ethylene was identified based on the retention time and quantified by comparison with peaks from standard ethylene concentration.

2.5. Determination of lipid peroxidation and H_2O_2 content

Lipid peroxidation in leaves was determined by estimating the content of thiobarbituric acid reactive substances (TBARS) as described by Dhindsa et al. (1981). Fresh leaf tissues (500 mg) were ground in 0.25% 2-thiobarbituric acid (TBA) in 10% trichloroacetic acid (TCA) using mortar and pestle. After heating at 95 °C for 30 min, the mixture was rapidly cooled on ice bath and centrifuged at 10,000g for 10 min. To 1 mL aliquot of the supernatant, 4 mL 20% TCA containing 0.5% TBA were added. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The content of TBARS was calculated using the extinction coefficient (155 mM^{-1} cm^{-1}).

The content of H_2O_2 was determined following the method of Okuda et al. (1991). Fresh leaf tissues (500 mg) were ground in ice-cold 200 mM perchloric acid. After centrifugation at 1200g for 10 min, perchloric acid of the supernatant was neutralized with 4 M KOH. The insoluble potassium perchlorate was eliminated by centrifugation at 500g for 3 min. In a final volume of 1.5 mL, 1 mL of the eluate, 400 μL of 12.5 mM 3-(dimethylamino) benzoic acid (DMAB) in 0.375 M phosphate buffer (pH 6.5), 80 μL of 3-methyl-2-benzothiazoline hydrazone (MBTH) and 20 μL of peroxidase (0.25 unit) were added. The reaction was started by the addition of peroxidase at 25 °C and the increase in absorbance was recorded at 590 nm.

2.6. Antioxidative enzymes assay

Fresh leaf tissues (200 mg) were homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) PVP in potassium-phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was