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Nitrogen availability regulates proline and ethylene production and alleviates salinity stress in mustard (*Brassica juncea*)

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ABSTRACT

Proline content and ethylene production have been shown to be involved in salt tolerance mechanisms in plants. To assess the role of nitrogen (N) in the protection of photosynthesis under salt stress, the effect of N (0, 5, 10, 20 mM) on proline and ethylene was studied in mustard (*Brassica juncea*). Sufficient N (10 mM) optimized proline production under non-saline conditions through an increase in proline-metabolizing enzymes, leading to osmotic balance and protection of photosynthesis through optimal ethylene production. Excess N (20 mM), in the absence of salt stress, inhibited photosynthesis and caused higher ethylene evolution but lower proline production compared to sufficient N. In contrast, under salt stress with an increased demand for N, excess N optimized ethylene production, which regulates the proline content resulting in recovered photosynthesis. The effect of excess N on photosynthesis under salt stress was further substantiated by the application of the ethylene biosynthesis inhibitor, 1-aminoethoxy vinylglycine (AVG), which inhibited proline production and photosynthesis. Without salt stress, AVG promoted photosynthesis in plants receiving excess N by inhibiting stress ethylene production. The results suggest that a regulatory interaction exists between ethylene, proline and N for salt tolerance. Nitrogen differentially regulates proline production and ethylene formation to alleviate the adverse effect of salinity on photosynthesis in mustard.

Introduction

The availability of a critical amount of nitrogen (N) is important for optimal growth and development of plants. An amount of N greater or smaller than the required may slow or inhibit growth. The increasing area of saline agricultural soils, estimated to be about 800 million ha, has resulted in a substantial decrease of crop yield worldwide (Rengasamy, 2010; Khan et al., 2014). Salt stress causes lipid peroxidation, disturbs water and osmotic balance and nutrient uptake due to the overproduction of reactive oxygen species (ROS) (Munns, 2005; Fatma et al., 2013; Khan et al., 2014) resulting, for example, in damage to the photosynthetic apparatus (Fatma et al., 2014; Nazar et al., 2014).

Salinity affects the metabolisms of carbon (C) and N in plants, which are the key physiological processes in determining plant growth and development (Touchette and Burkholder, 2007). Salinity stress reduces the uptake of nutrients such as N, calcium (Ca) and potassium (K) (Reda et al., 2011; Syeed et al., 2011). The interactive effect of salinity and N plays an important role in determining the photosynthetic potential of plants and salinity tolerance. Exposure of plants to salt stress decreases N assimilation and photosynthesis, while the supplementation of N increases the photosynthetic capacity of leaves through an increase in stromal and thylakoid protein and regulating stomatal movement under salt stress (Syeed et al., 2011; Nazar et al., 2011).

The production of compatible solutes such as proline has been shown to be related to N assimilation and salt tolerance (Iqbal et al., 2014). Proline facilitates water uptake, maintains osmotic balance and protects cells against ROS under salt stress (Ashraf and Foolad, 2007; Filippou et al., 2014; Iqbal et al., 2014). The synthesis of proline in plants occurs mainly from glutamate via pyrroline-5-carboxylate (P5C) and is catalyzed by P5C synthetase (P5CS)

Abbreviations: Φ PSII, quantum yield efficiency of PSII; ACC, 1-aminocyclopropane carboxylic acid; ACS, 1-aminocyclopropane carboxylic acid synthase; AVG, 1-aminoethoxy vinylglycine; DAS, days after sowing; GK, γ-glutamyl kinase; N, nitrogen; NUE, nitrogen use efficiency; P5CS, pyrroline-5-carboxylate synthetase; ROS, reactive oxygen species; Rubisco, ribulose 1,5-bisphosphate carboxylase; TBARS, thiobarbituric acid reactive substances.

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and P5C reductase. Proline degradation occurs in mitochondria via sequential action of proline dehydrogenase or proline oxidase producing P5C from proline, and P5C dehydrogenase, which regenerates glutamate from P5C. Another pathway of proline synthesis is via ornithine, which is transaminated first by ornithine-delta-aminotransferase and then converted to proline.

Phytohormones also regulate N metabolism and proline production and modulate salt tolerance (Iqbal et al., 2014). Ethylene, a gaseous plant hormone, plays an important role in tolerance to abiotic stress (Abeles et al., 1992; Khan and Khan, 2014). Ethylene may be involved in salinity tolerance through its effect on N metabolism and proline production. Application of ethephon (ethylene source) has been shown to increase N assimilation and photosynthesis in *Brassica juncea* grown under different N levels (Khan et al., 2008; Iqbal et al., 2011). Reports suggest that ethylene regulates proline production in *Triticum aestivum* under heat stress (Khan et al., 2013) and cadmium (Cd) stress (Khan et al., 2015). Recently, Nazar et al. (2014) have shown that ethylene protected photosynthesis of *B. juncea* under salt stress by increasing sulfur (S) assimilation and antioxidant metabolism. However, information on how N availability regulates proline and ethylene production and alleviates salt stress is not available. In the present study, we show that N availability differentially influences ethylene production, which regulates proline synthesis and protects photosynthesis of *B. juncea* under salt stress. The involvement of ethylene in the regulation of proline synthesis and protection of photosynthesis under salt stress was substantiated by applying the ethylene biosynthesis inhibitor, 1-aminoethoxy vinylglycine (AVG).

Materials and methods

Plant material and growth conditions

Mustard (*Brassica juncea* L.) cv. Pusa Jai Kisan seeds obtained from Indian Agricultural Research Institute, New Delhi were surface sterilized with 0.1% $HgCl_2$ and were washed repeatedly with deionized water. The seeds were sown in 15-cm-diameter earthen pots filled with acid-washed sand, purified according to Hewitt (1966). The pots were kept in the herbal garden of the Department of Botany, Jamia Hamdard, New Delhi, India under natural day/night conditions with day and night temperature of 24/18 ± 3 °C and relative humidity of 68 ± 6%.

Two plants per pot were maintained and were subjected to control conditions (0 mM NaCl) or treatments (100 mM NaCl, 5 mM N, 10 mM N and 20 mM N). An amount of 300 mL of 100 mM NaCl or different N levels (5, 10, 20 mM), either alone or in combination, in the form of modified full strength Hoagland's nutrient solution was provided per pot every other day and 250 mL of deionized water daily. Potassium nitrate was used for obtaining 5, 10 or 20 mM NO_3^- concentrations and K^+ was maintained in all treatments including control by the addition of KCl. Plants grown in nutrient solution served as control. The nutrient solution was replaced weekly. The control groups of plants were fed with 300 mL nutrient solution every alternate d and 250 mL of deionized water daily.

In the experiment with the ethylene biosynthesis inhibitor, 50 μM 1-aminoethoxy vinylglycine (AVG) was applied to plants as foliar spray at 20 d after sowing (DAS) together with 0.5% surfactant teepol. The plants grown with 20 mM N alone and 100 mM NaCl plus 20 mM N were treated with 50 μM AVG. The control was maintained as above.

The experiment followed a randomized complete block design and the number of replicates for each treatment was four ($n = 4$). Measurements were done at 30 DAS and care was taken to select leaves of the same age for the determinations.

Estimation of leaf Na^+ and Cl^- content

Content of leaf Na^+ and Cl^- was determined by digesting leaf tissues (500 mg) with 19 mL of Tri acid mixture; a mixture of 16 mol L^{-1} concentrated nitric acid (10 mL), 18 mol L^{-1} concentrated sulfuric acid (5 mL) and 11.65 mol L^{-1} concentrated perchloric acid (4 mL) prepared in the ratio of 10:5:4 (v/v). The content of Na^+ was estimated using a flame photometer, whereas the Cl^- content was determined by titration against 0.02 N silver nitrate solution using 5% K_2CrO_4 as indicator.

Determination of H_2O_2 and thiobarbituric acid reactive substances (TBARS) content

Content of H_2O_2 and TBARS was determined adopting the methods of Okuda et al. (1991) and Dhindsa et al. (1981), respectively. The details of the methods have been described earlier (Nazar et al., 2011).

Estimation of proline content and assay of P5CS, γ-glutamyl kinase activity (GK) and proline oxidase activity

Proline content was determined spectrophotometrically using ninhydrin adopting the method of Bates et al. (1973). Fresh leaf tissues (300 mg) were homogenized in 3 mL of 3% sulphosalicylic acid. One milliliters each of acid ninhydrin and glacial acetic acid was added to the homogenate filtrate and the reaction was carried for 1 h in a test tube placed in a water bath at 100 °C. The mixture was extracted with toluene and the absorbance was measured in a spectrophotometer (UV–vis L164, Elico, New Delhi) at 520 nm using L-proline as a standard.

To determine the activity of P5CS (EC 2.7.2.11/1.2.2.41), GK (EC 2.7.2.11) and proline oxidase (EC 1.5.99.8), enzyme extract was prepared by homogenizing 500 mg leaf sample in 0.1 M Tris–HCl buffer, pH 7.5 at 4 °C. The homogenate was centrifuged at 30,000 × *g* for 30 min. The supernatant was used as the crude extract enzyme preparation for P5CS activity and the pellet was collected and used as extract for the assay of GK and proline oxidase. Activity of P5CS and GK was assayed according to the method of Hayzer and Leisinger (1980) with a slight modification. The assay mixture of 1.0 mL contained 100 M L-P5C and 100 mM sodium phosphate buffer at pH 7.5. The decrease in absorbance was measured at 340 nm for P5CS. For determining the activity of GK, the extract was kept in freezer at −20 °C. The frozen sample was suspended in 10 mL of 0.1 M Tris–HCl buffer containing 1 mM 1,4-dithiothreitol (DTT) to rupture the cell and centrifuged at 30,000 × *g* for 30 min. The assay mixture contained 50 mM L-glutamate, 10 mM ATP, 20 mM $MgCl_2$, 100 mM hydroxylamine HCl and 50 mM Tris–HCl, pH 7.0 with 200 μL of desalted extract in a final volume of 500 μL. The reaction was started by the addition of enzyme extract. After 30 min of incubation at 37 °C, the reaction was stopped by the addition of 1.0 mL $FeCl_3.3H_2O$ (2.5%, w/v) and trichloroacetic acid (6%, w/v) in 2.5 M HCl. Protein was precipitated and removed by centrifugation at 12,000 × *g* (4 °C) and absorbance was recorded at 540 nm against a blank identical to the above but lacking ATP. The amount of γ-glutamyl hydroxamate was determined by measuring the absorbance at 540 nm and by comparison with a standard curve of γ-glutamyl hydroxamate. Activity of GK was expressed in U mg^{-1} protein. One Unit (U) of enzyme activity is defined as μg of γ-glutamyl hydroxamate produced min^{-1} mg^{-1} protein.

The method of Huang and Cavalieri (1979) with slight modification was adopted to determine the activity of proline oxidase. The pellet obtained after centrifugation of enzyme extract was mixed with 1 mL Tricine, KOH buffer (pH 7.5) containing 6 M sucrose. This extract was used for the enzyme assay. The assay mixture contained 1.2 mL of 50 mM Tris–HCl buffer (pH 8.5), 1.2 mL of 5 mM