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Enhancing chilling stress tolerance of pepper seedlings by exogenous application of 5-aminolevulinic acid

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ABSTRACT

In this study, the possibility of enhancing chilling stress tolerance of pepper (*Capsicum annuum* L.) during early growth stages by exogenous application of 5-aminolevulinic acid (ALA) was investigated. To improve chilling tolerance during seedling stage, ALA was applied in various concentrations (0, 1, 10, 25 and 50 ppm) through three different methods (seed soaking, foliar spray, or soil drench). After ALA applications, the plants were subjected to chilling stress at 3 °C for 2 days. Although all ALA application methods improved chilling stress tolerance in pepper seedlings, seed soaking and foliar spray provided better protection against chilling stress compared to soil drench. Exogenous application of ALA provided significant protection against chilling stress compared to non-ALA-treated seedlings, significantly enhancing plant mass and chlorophyll, sucrose, and proline contents. ALA pre-treatment also increased relative water content, stomatal conductance and superoxide dismutase (SOD) enzyme activity and reduced membrane permeability. Of the ALA concentrations, the highest chilling tolerance was obtained with 25 ppm ALA pre-treatment. Results indicate that ALA which is considered as an endogenous plant growth regulator could be used effectively to protect pepper seedlings from damaging effects of chilling stress without any adverse effect on seedling growth.

1. Introduction

The optimum growth temperature for pepper (*Capsicum annuum* L.) is between 21 and 27 °C, with growth reduced below 12 and above 30 °C (Wien, 1997). Exposure of plants from tropical or subtropical origin to chilling temperatures may stunt plant growth, induce wilting, cause necrotic lesions on leaves, and increase susceptibility to diseases and pathogens (Hällgreen and Öquist, 1990; Korkmaz and Dufault, 2001). Plant growth may deteriorate as a consequence of impaired photosynthesis and respiration and disrupted water relations, membrane integrity, and hormonal balance (Allen and Ort, 2001; Korkmaz et al., 2007).

Pepper is grown extensively in western and southern Turkey, and although climatic conditions in these areas are ideal for growing pepper, increasing yield by multiple harvests requires field planting in early March before the last killing frost. For example, in the Kahramanmaras province (one of the major commercial pepper production areas in Turkey), the mean air temperatures range from 10 to 15 °C and the mean min temperatures fluctuate between 3 and 7 °C on typical planting dates (Korkmaz, 2009). Once the seedlings have been transplanted in the field, they may be exposed to temperatures cycling between chilling and optimum for weeks before temperatures finally stabilize. In some cases, late frosts occur which kill the newly transplanted seedlings in the field or if not severe enough, stagnate early plant growth and field establishment.

It is known that 5-aminolevulinic acid (ALA) is a key precursor in the biosynthesis of all porphyrins compounds such as chlorophyll, heme, and phytochrome (Wang et al., 2005). Exogenous applications of ALA have been found to regulate plant growth and development and to enhance chlorophyll biosynthesis and photosynthesis thus increasing crop yield (Hotta et al., 1997). Treating rice, barley, potato and garlic plants at early growth stages with suitable concentrations of ALA promoted plant growth and photosynthetic rates resulting in significant yield enhancements (Tanaka et al., 1992). ALA applied at low concentrations is also known to enhance plant's tolerance to cold (Hotta et al., 1998; Wang et al., 2004) and salinity stresses (Watanabe et al., 2000; Nishihara et al., 2003; Zhang et al., 2006) and exhibit herbicidal effects if used at concentrations over 5 mM (Kumar et al., 1999), suggesting that ALA has a great application potential in agricultural production as a new non-toxic endogenous substance (Wang et al., 2003). In our ongoing research, we previously established that pre-sowing seed treatment with 50 ppm ALA enhanced germination and emergence performance of pepper seeds under chilling stress conditions

Abbreviations: ALA, 5-aminolevulinic acid; EC, electrical conductivity; NBT, nitro blue tetrazolium; RWC, relative water content; SOD, superoxide dismutase.

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(Korkmaz and Korkmaz, 2009). However, to our knowledge, no specific information is available regarding the effects of ALA on chilling stress tolerance of pepper seedlings. Moreover, the mechanisms of ALA in promoting stress tolerance in plants also need to be fully elucidated. Therefore, this study provides the first evidence that ALA protected pepper seedlings against chilling stress. It also contributes significantly to our understanding of the role of ALA in promoting chilling stress tolerance. Our specific objectives were (1) to compare three ALA application methods and (2) to determine the optimum ALA concentration that would provide the best protection against chilling stress.

2. Materials and methods

2.1. Plant material, ALA treatments and chilling stress imposition

Seeds of 'Sena' red pepper, all from the same seed lot, were obtained from Agricultural Research Institute, Kahramanmaras, Turkey. Seeds were disinfested in 1% (active ingredient) sodium hypochlorite solution for 10 min to eliminate possible seed-borne microorganisms, rinsed for 1 min under running water then were dried for 30 min at room temperature.

A single layer of pepper seeds was placed in covered transparent polystyrene boxes (10 cm × 10 cm × 4 cm) on double layers of filter paper wetted with 15 ml of 0, 1, 10, 25 or 50 ppm ALA (Sigma Aldrich, St. Louis, MO, USA) solution. The boxes were kept at 20 °C in the dark for 24 h. After ALA application, seeds were rinsed for 1 min under running water and left to dry on paper towels for 24 h under room conditions (20–22 °C and 50–60% relative humidity).

The seeds were planted at a depth of 1.0 cm into 5.5 cm-deep flat cells (75 cm^3) filled with growth medium consisting of peat and perlite in the ratio of 3:1. The flats were watered regularly with tap water and kept in a growth chamber at 25 ± 1 °C (day/night) under cool fluorescent lamps (100 $\mu mol\ m^{-2}\ s^{-1}$) for 16 h day^{-1}.

A second batch of seeds was also imbibed in distilled water under the same conditions prior to sowing to obtain seedlings for foliar spray and soil drench applications of ALA, and these seedlings were grown under the same conditions. When the seedlings had fully developed 4 true leaves (30–34 days after planting), half of the seedlings were sprayed with 0, 1, 10, 25 or 50 ppm ALA solutions until both sides of the leaves were completely wet while the other half was soil drenched with 25 ml (enough to cause run off) of 0, 1, 10, 25 or 50 ppm ALA solution. For foliar application, a few drops of surfactant (Tween 20) were added to ALA solutions to increase adherence and paper towels were laid on the growth medium to prevent ALA solution from entering the growth medium. Three days after foliar spray and soil drench application of ALA, all plants (seed soaked, foliar sprayed, and soil drenched) were subjected to chilling stress at 3 ± 0.5 °C for 48 h under the same light regime as mentioned above. All plants were watered 2 h prior to and after the chilling stress and were assessed 72 h after the end of chilling stress to determine the extent of chilling injury. The treatments were replicated four times with 12 plants in each replication and all treatments were arranged in a randomized complete block design. For comparison purposes, plants not exposed to chilling stress and grown in the growth chamber at 25 °C were used as the control.

2.2. Determination of visual damage

All plants were visually examined to determine the extent of chilling injury and classified using the following scale: none: no visible symptoms, slight: small necrotic areas on shoots but without growth restrictions (<5% of leaf area necrotic), moderate: well-defined necrotic areas on shoots (5–25% of leaf area necrotic), severe: extensive necrotic areas and severe growth reductions (26–50% of leaf area necrotic but plant still alive), and killed: entire plant necrotic and collapsed. By assigning values of 1, 2, 3, 4, and 5, respectively to each group, the average injury for each treatment was calculated (Korkmaz et al., 2007).

2.3. Chlorophyll content determination

Chlorophyll content was determined by taking fresh leaf samples (0.5 g) from randomly selected three plants per each replicate. The samples were homogenized with 5 ml of acetone (80% v/v) using pestle and mortar and filtered through a filter paper (Whatman No. 2). The absorbance was measured with a UV/visible spectrophotometer (Spectramax Plus 384, Molecular Devices, CA, USA) at 663 and 645 nm and chlorophyll contents were calculated using the equations proposed by Lichtenthaler (1987) given below:

$$\text{Chl } a\,(\text{mg/g FW}) = 11.75 \times A_{663} - 2.35 \times A_{645}$$

$$\text{Chl } b\,(\text{mg/g FW}) = 18.61 \times A_{645} - 3.96 \times A_{663}$$

2.4. Stomatal conductivity

The youngest fully expanded leaves of randomly selected three plants per replicate were chosen for gas exchange measurements, and stomatal conductivity was measured using a portable porometer (Model: AP4, Delta-T Devices, Cambridge, UK). Light intensity during the measurements was maintained at 100 $\mu mol\ m^{-2}\ s^{-1}$ which was the same light intensity that the plants were exposed to in the growth chamber.

2.5. Electrolyte leakage

In order to assess membrane permeability, electrolyte leakage was determined according to Korkmaz et al. (2007). Leaf discs (1 cm in diameter) from randomly chosen two plants per replicate were taken from the middle portion of fully developed youngest leaf and washed with distilled water to remove surface contamination. The discs were placed in individual stoppered vials containing 20 ml of distilled water. After incubating the samples at room temperature on a shaker (150 rpm) for 24 h, the electrical conductivity (EC) of the bathing solution (EC_1) was determined. The same samples were then placed in an autoclave at 121 °C for 20 min and a second reading (EC_2) was determined after cooling the solution to room temperature. The electrolyte leakage was calculated as EC_1/EC_2 and expressed as percent.

2.6. Relative water content

Leaf discs (1 cm in diameter) from randomly chosen two plants per replicate were taken from the middle portion of fully developed third leaf (to exclude the age effect). Discs were weighed (fresh wt, FW) and then immediately floated on distilled water in a petri dish for 5 h in the dark. Turgid weights (TW) of leaf discs were obtained after drying excess surface water with paper towels. Dry weights (DW) of discs were measured after drying at 75 °C for 48 h. Relative water content (RWC) was calculated using the following formula:

$$\text{RWC} = \left[\frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}}\right] \times 100$$

2.7. Proline content determination

Proline content was determined according to the method described by Bates et al. (1973). Fresh leaf material (0.5 g) was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and filtered