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SALINITY STRESS

The Effects of Foliar Application of Ascorbic Acid (Vitamin C) on Antioxidant Enzymes Activities, Lipid Peroxidation and Proline Accumulation of Canola (*Brassica napus* L.) under Conditions of Salt Stress

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Abstract

The effects of salt stress on protein (PROT) content, lipid peroxidation, proline accumulation, chlorophyll (Chl) content, and superoxide dismutase (SOD; EC 1.15.1.1), catalase (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) activity were studied in the leaves and roots of canola (*Brassica napus* L. cv. Okapi). Four weeks after sowing (at the V_4 stage), plants were exposed to salt stress by the application of NaCl solution (200 mM) for 6 days daily, After 6 days followed by foliar application of ascorbic acid (AsA) solution (25 mM). The activity of all the antioxidant enzymes assayed (except SOD in the roots) was increased significantly in the plants under conditions of salt stress. The application of AsA decreased enzyme activity in the leaves, but it had no effect on enzyme activity in the roots. The total PROT content of the leaves and roots decreased under the conditions of high salinity. AsA treatment of plants under salt stress increased the total PROT content significantly in both leaves and roots. Measurement of the malondialdehyde content of leaves and roots showed that lipid peroxidation was increased by interaction with damaging reactive oxygen species during salt stress, and that application of AsA reduced lipid peroxidation only in the leaves. The Chl content was also affected by salt stress. There was significant difference between the controls and salt-stress treatments in Chl content. The results of the present study indicate that usage of AsA reduces the harmful effects of salinity and increases resistance to salinity in canola plant.

Introduction

High salinity is a common abiotic stress factor that seriously affects crop production in some parts of the world, particularly in arid and semi-arid regions. Irrigation with poor quality water is one of the main factors that lead to salt accumulation and the resulting decrease in agricultural productivity. Saline soils and saline irrigation waters present potential hazards to canola production. Brassica oilseed species now hold the third position among the oilseed crops and are an important source of vegetable oil (Ashraf and McNeilly 2004). The crop has considerable potential to grow in salt-affected areas. Under conditions of salt stress and other types of environmental stress, reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, are generated (Zhu 2000). In chloroplasts, ROS can be generated by direct transfer of excitation energy from chlorophyll (Chl) to produce single oxygen atoms, or by univalent oxygen reduction of photosystem I, in the Mehler reaction (Asada 1999). These free radicals can damage essential membrane lipids as well as proteins (PROT) and nucleic acids (Noctor and Foyer 1998). Different plant species have developed various defense systems for the scavenging of ROS. One of these systems is the antioxidant system, which involves antioxidant substances such as tocopherols and ascorbic acid (AsA). A complex antioxidative defense system, composed

of both non-enzymic and enzymic constituents, is present in all plant cells (Foyer et al. 1994). In response to the increased production of oxygen radicals the capacity of the antioxidant defense system is increased (Gressel and Galun 1994). However, under conditions of environmental stress, production of ROS can increase and endogenous protective activity may then become inadequate. Various associations between water stress and endogenous levels of water-soluble antioxidants have been described (Tsugane et al. 1999). Antioxidant enzymes ascorbate peroxidase (POX), glutathione reductase and non-specific POX also increased significantly under water stress (Sairam and Saxena 2000).

Ascorbic acid is a small, water-soluble antioxidant molecule that acts as a primary substrate in the cyclical pathway for detoxification and neutralization of superoxide radicals and singlet oxygen (Noctor and Foyer 1998). Ascorbate has been shown to play multiple roles in plant growth, such as in cell division, cell wall expansion and other developmental processes (Pignocchi and Foyer 2003). Ascorbate functions in coordination with glutathione and several enzymatic antioxidants to counteract the O_2^- radicals that are produced by the Mehler reaction and photorespiration (Noctor and Foyer 1998). In addition, ascorbate is believed to detoxify $^1O_2$ and OH^- (Asada 1999). It has also been reported that application of exogenous ascorbate can increase resistance to salt stress and reduce oxidative stress (Shalata and Neumann 2001).

The primary free radical scavenger in plant cells is superoxide dismutase (SOD). This enzyme converts O_2^- to H_2O_2, which is eliminated by ascorbate POX in association with dehydroascorbate reductase; this process regenerates AsA (Asada 1994). POX plays an important role in eliminating H_2O_2 by utilizing ascorbate as its specific electron donor to reduce H_2O_2 to water, with the concomitant generation of monodehydroascorbate (MDHA). MDHA is converted to AsA by MDHA reductase, or is disproportionated non-enzymatically to AsA and dehydroascorbate. Hydrogen peroxide is also scavenged by catalase (CAT) (Comba et al. 1998). Changes in the activities of various antioxidant enzymes under salt stress have been reported (Hernandez et al. 2000). Malondialdehyde (MDA), a product of the decomposition of polyunsaturated fatty acids in biomembranes, shows increased accumulation under salt stress (Gossett et al. 1994a,b). Measurement of cell membrane stability has been widely used to differentiate stress-tolerant and -susceptible cultivars of some crops (Blum and Ebercon 1981), and in some cases higher membrane stability can be correlated with abiotic stress tolerance (Premachandra et al. 1992).

In this study, the effects of salinity and AsA on the antioxidant defense system of canola were investigated. The activity of enzymes involved in the antioxidative defense system, SOD, CAT and POX were assayed in crude extracts of the roots and leaves of canola plants. Because the roots are the first part of the plant to sense saline stress in the soil and are the first to show adaptation reactions, the activity of SOD, CAT and POX was also studied in canola roots. The primary objective of the present investigation was to examine the effect of salt stress and AsA on the activities of different antioxidant enzymes and other biochemical exchanges in leaves and roots of canola. The work was aimed also whether a foliar supply of AsA to plant might be a strategy for increasing the salt tolerance.

Materials and methods

Plant material and growth conditions

Canola seeds (*Brassica napus* L. cv. Okapi) were used in this study. Seeds of canola were surface sterilized for 5 min in sodium hypochlorite solution and then in 96 % ethanol for 30 s. After sterilization, the seeds were grown in plastic pots (six plant in each pot) containing clay–loam soil in a culture room under conditions of 25/20 °C day/night temperature, 55–60 % air humidity, and with a 12 h photoperiod under a photon flux density of 250 μmol photons m^{-2} s^{-1}. Irrigation was performed daily using water with electrical conductivity of 7.5 ms.

Salt stress and ascorbic acid treatments

Four weeks after sowing (at the V_4 stage), salt stress treatment was initiated. Plants were exposed to salt stress by irrigation with the NaCl solution (200 mM, electrical conductivity = 13.5 ms) for 6 days daily. Treatments were include; without salt stress and without AsA (S0V0), without salt stress with AsA (S0V1), salt stress and without AsA (S1V0) and salt stress and AsA (S1V1). After 6 days followed by foliar application of AsA solution (25 mM). tween 20 (1 drop per litre) was added to the AsA solution to increase adhesiveness between the leaves and the applied solution.

Sampling

Three days after the foliar application of AsA, the roots and fourth leaves of each plant were removed. The samples were washed and then frozen in liquid N_2 and then stored at −80 °C pending biochemical analysis.

Preparation of extracts

Leaf and root samples (0.2 g) were homogenized in a mortar and pestle with 3 ml ice-cold extraction buffer

(25 mM sodium phosphate, pH 7.8). The homogenate was centrifuged at 18 000 g for 30 min at 4 °C, and then the supernatant was filtered through paper. The supernatant fraction was used as a crude extract for the assay of enzyme activity and PROT content. All operations were carried out at 4 °C.

Assay of antioxidant enzymes

Catalase activity was estimated by the method of Cakmak and Horst (1991). The reaction mixture contained 100 μl crude enzyme extract, 500 μl 10 mM H_2O_2 and 1400 μl 25 mM sodium phosphate buffer. The decrease in the absorbance at 240 nm was recorded for 1 min by spectrophotometer, model Cintra 6 GBC (GBC Scientific Equipment, Dandenong, Victoria, Australia). CAT activity of the extract was expressed as CAT units per milligram of PROT.

Superoxide dismutase activity was determined according to the method of Giannopolitis and Ries (1997). The reaction mixture contained 100 μl 1 μM riboflavin, 100 μl 12 mM L-methionine, 100 μl 0.1 mM EDTA (pH 7.8), 100 μl 50 mM Na_2Co_3 (pH 10.2), and 100 μl 75 μM nitroblue tetrazolium (NBT) in 2300 μl 25 mM sodium phosphate buffer (pH 6.8), with 200 μl crude enzyme extract in a final volume of 3 ml. SOD activity was assayed by measuring the ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the mixture were illuminated with a fluorescent lamp (120 W); identical tubes that were not illuminated served as blanks. After illumination for 15 min, the absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme activity that was able to inhibit by 50 % the photoreduction of NBT to blue formazan. The SOD activity of the extract was expressed as SOD units per milligram of PROT.

Peroxidase activity was determined by the oxidation of guaiacol in the presence of H_2O_2. The increase in absorbance was recorded at 470 nm (Ghanati et al. 2002). The reaction mixture contained 100 μl crude enzyme, 500 μl H_2O_2 5 mM, 500 μl guaiacol 28 mM and 1900 μl potassium phosphate buffer 60 mM (pH 6.1). POX activity of the extract was expressed as POX units per milligram PROT.

Protein assay

Total PROT content was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976), using 1 ml Bradford solution and 100 μl crude extract. The PROT concentration was calculated from a BSA standard curve.

Lipid peroxidation assay

The level of membrane damage was determined by measuring MDA as the end product of peroxidation of membrane lipids (De Vos et al. 1991). In brief, samples were homogenized in an aqueous solution of trichloroacetic acid (10 % w/v), and aliquots of the filtrates were heated in 0.25 % thiobarbituric acid. The amount of MDA was determined from the absorbance at 532 nm, followed by correction for the non-specific absorbance at 600 nm. The content of MDA was determined using the extinction coefficient of MDA (ε = 155 μM^{-1} cm^{-1}).

Proline assay

Proline (PRO) content was determined according to the method of Bates et al. (1973), which was modified as follows. Samples of leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml sulphosalicylic acid (3 % w/v), and then the homogenate was centrifuged at 18,000 ***g*** for 15 min. Two millilitres of the supernatant were then put into a test tube into which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. Tubes were incubated in a water bath for 1 h at 100 °C, and then allowed to cool to room temperature. Four millilitres of toluene were added and mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min to allow the separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube, and its absorbance was measured at 520 nm in a spectrophotometer. The content of PRO was calculated from a PRO standard curve and was expressed as mmol g^{-1} per fresh weight.

Chlorophyll assay

Chlorophyll was extracted in 80 % acetone from the leaf samples, according to the method of Arnon (1949). Extracts were filtrated and the content of total Chl, Chl *a* and Chl *b* were determined by spectrophotometry at 645 and 663 nm. The content of Chl was expressed as mg g^{-1} FW.

Statistical analysis

All data were analysed using SAS software (SAS Institute Inc. 1997). Each treatment was analysed in three replications. When ANOVA showed significant treatment effects, Duncan's multiple range test was applied to compare the means at $P < 0.05$ (Steel and Torrie 1980).

Results

The results showed that salt stress affected all parameters measured, except for Chl *b* and SOD activity in the leaves and MDA content of the roots. The effect of foliar application of AsA was significant for all variables except CAT and POX activities in the roots.

Antioxidant enzymes

Plants under salt stress showed a significant increase in CAT activity in the leaves compared with control plants (Fig. 1). Application of AsA was not associated with a significant difference in CAT activity in non-stressed plants; however, application of AsA decreased CAT activity in stressed plants. There was no significant difference in SOD activity in the leaves in of all treatments (Fig. 2).

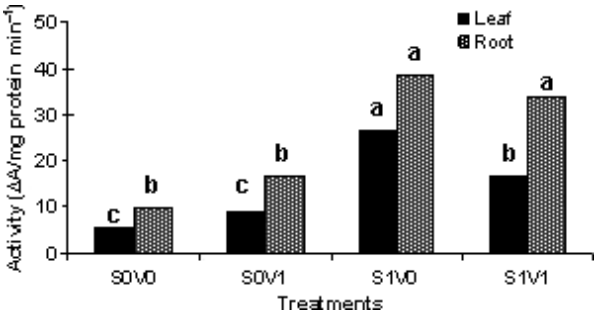

Fig. 1 The effect of NaCl salinity and ascorbic acid on catalase activity in the leaves and roots of canola plants (S = salt stress, V = ascorbic acid treatment, 0 = untreated plants and 1 = treated plants). All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

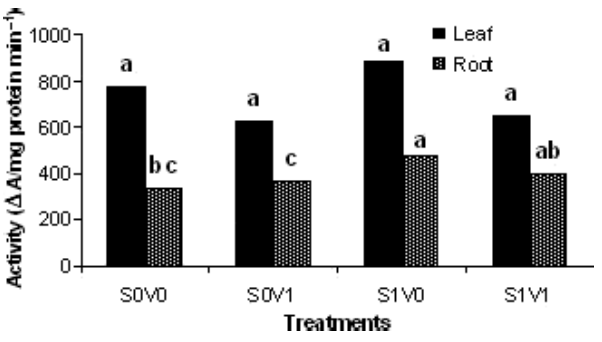

Fig. 2 The effect of NaCl salinity and ascorbic acid on superoxide dismutase activity in the leaves and roots of canola plants (S = salt stress, V = ascorbic acid treatment, 0 = untreated plants and 1 = treated plants). All the values followed by the same letter in each column are not statistically different at the $P < 0.05$ probability level.

POX activity in the leaves was increased by saline treatment. Foliar application of AsA to stressed plants decreased the POX activity in the leaves but did not result in a significant change in POX activity in controls (Fig. 3).

The activity of all antioxidant enzymes in the roots was increased by application of salt stress. Application of AsA to stressed and non-stressed plants had no effect on the activity of antioxidant enzymes in the roots (Fig. 4).

Protein content

Leaf PROT content was affected by salt stress and there was a significant decrease in PROT content in stressed plants relative to control plants (Fig. 5). Foliar application of AsA increased the content of soluble PROT in the leaves of plants exposed to high salinity but did not affect significantly the PROT content of the leaves of control plants. Root soluble PROT decreased under saline stress, but with the application of AsA, it was significantly increased. The root PROT content was not affected by foliar application of AsA in non-stressed plants.

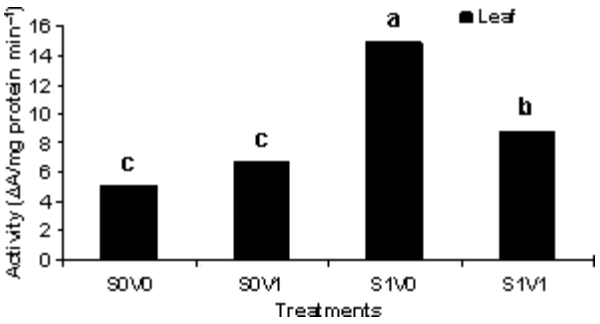

Fig. 3 The effect of NaCl salinity and ascorbic acid on peroxidase activity in the leaves and roots of canola plants.

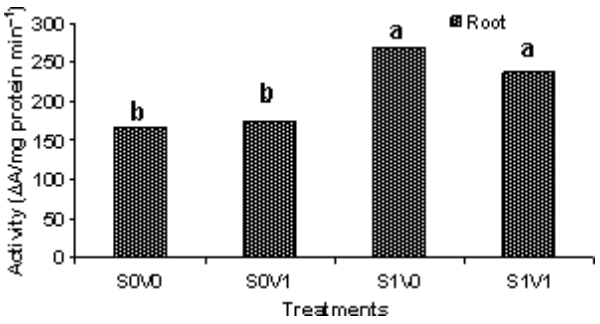

Fig. 4 The effect of NaCl salinity and ascorbic acid on peroxidase activity in the leaves and roots of canola plants.

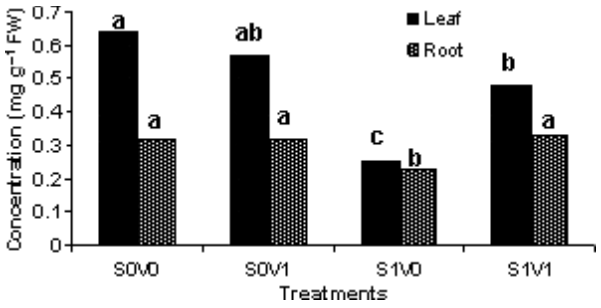

Fig. 5 The effects of NaCl and ascorbic acid on protein content in leaves and roots of canola plants.

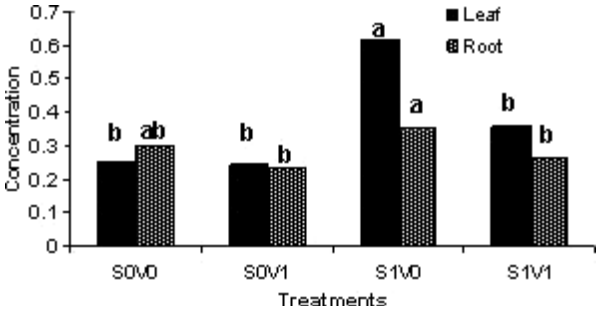

Fig. 6 The effects of NaCl and ascorbic acid on malondialdehyde content in leaves and roots of canola plants.

Lipid peroxidation

The level of lipid peroxidation of both leaves and roots, measured as MDA content, is shown in Fig. 6. The level of MDA was altered in the leaves and roots of plants by salinity. In stressed plants, the MDA content was increased by salt stress treatment. As shown in Fig. 6, the MDA content of the leaves and roots of saline-treated plants was markedly raised. It was observed that AsA decreased the MDA content in the leaves of plants exposed to salinity. Application of AsA to non-stressed plants resulted in no significant difference in MDA compared with control plants.

Proline accumulation

The PRO content in the leaves of plants treated was significantly raised and decreased significantly with application of AsA. However, AsA application did not alter the PRO content in non-stressed plants (Fig. 7).

Chlorophyll

As show in Fig. 8, the plants exposed to salt stress showed a statistically significant decrease in total, Chl *a* and *b* content compared with control plants.

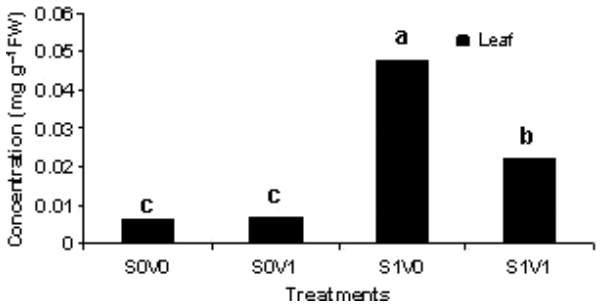

Fig. 7 The effects of NaCl and ascorbic acid effects on proline content in leaves of canola plants.

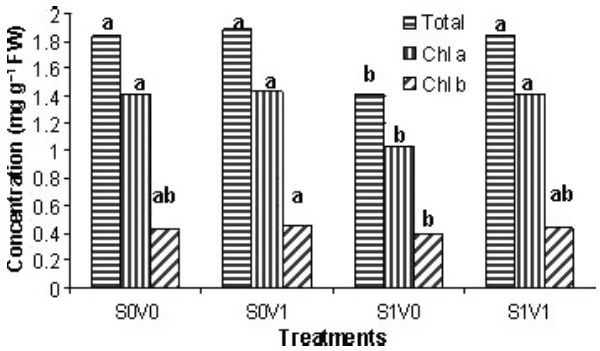

Fig. 8 The effects of NaCl and ascorbic acid effects on total, chlorophyll *a* and *b* content in leaves of canola plants.

Discussion

Abiotic stress, such as salt stress, causes molecular damage to plant cells either directly or indirectly through the formation of ROS. In the present study, the plants exposed to saline showed a significant increase in CAT and POX activity in the leaves, and in CAT, SOD and POX in the roots. The enzymes assayed are scavengers of free radical species. SOD converts one form of ROS (O_2^-) to another equally toxic one (H_2O_2). Hydrogen peroxide is converted to oxygen and water by CAT and POX, which use ascorbate as the hydrogen donor (Hegedus et al. 2001). Our results suggest that salt stress directly or indirectly leads to production of oxygen radicals, which results in increased lipid peroxidation and oxidative stress in the plant. Salt stress may also lead to stomatal closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation. This exposes the chloroplasts to excessive excitation energy, which in turn could increase the generation of free radicals and induce oxidative stress (Gossett et al. 1994a,b).

Similar increases in the activities of these enzymes have been reported in cotton cultivars subjected to salt stress (Rajguru et al. 1999). The increase in SOD activity was

reported in tolerance basmati rice variety (Singh et al. 2007). In our study, AsA decreased the activity of these enzymes maybe by elimination of free radicals. AsA has been found to be loaded in the phloem of source leaves and is then transported to other tissues (Tedone et al. 2004). When AsA was applied to the leaves of plants in our study, there was an obvious decrease in CAT and POX activities in the leaves.

A role of AsA in the ascorbate–glutathione cycle in mitochondria and peroxisomes has been described (Jiménez et al. 1997). It also plays a protective role against ROS that are formed during biotic and abiotic stress (Noctor and Foyer 1998). Ascorbate is oxidized by oxygen free radicals and dehydroascorbate is generated (Noctor and Foyer 1998). This leads to a decline in antioxidant activities, which is followed by an increase in oxidative damage.

One of the best known toxic effects of ROS is damage to cellular membranes, lipids and PROTs. Plasma membranes are oxidized by ROS to generate MDA. In this study, salt stress induced a progressive accumulation of MDA in the roots and leaves of plants. Zhang and Kirkham (1996) reported similar inhibitory effects of exogenous AsA on lipid peroxidation in sunflower seedlings exposed to osmotically induced water stress. We observed a significant reduction in the PROT content and an increase in the MDA content in salt-stressed plants. The reduction in PROT content of the stressed plants was probably due to adverse effects of ROS, which may be mediated by degradation of PROTs (Davies 1987). Exogenous AsA partially inhibits these increases because AsA is a scavenger of ROS (Noctor and Foyer 1998). Although the inhibitory effect of AsA on lipid peroxidation has been demonstrated, the actual mechanism is not yet clear.

Proline is a highly water-soluble amino acid. The accumulation of PRO is a common metabolic response of plants to adversity, and PRO is an indicator of adaptation to adversity and is involved in the succession resistant capability of plants (Bian et al. 1988). PRO protects membranes and PROTs against the adverse effect of high concentrations of ions and may also function as a PROT-compatible hydrotrope and as a hydroxyl radical scavenger (Kavi Kishor et al. 1995). In the current study, salt stress increased the leaf PRO content, and application of AsA scavenged ROS and prevented biosynthesis of extra praline. The concentration of this metabolite usually increases in response to salt stress (Nandwal et al. 2000b).

In plants, oxidative stress and signs of senescence include loss of Chl and PROT and decline in membrane permeability, all of which lead to a progressive reduction in photosynthetic capacity (Thompson et al. 1987). Salt stress leads to an increase in free radicals in chloroplasts and destruction of Chl molecules by ROS, which results in reduction of photosynthesis and growth. Singlet oxygen atoms and O_2^- radicals predominantly attack double-bond-containing compounds (unsaturated fatty acids and Chl), thus damaging the chloroplast membrane system and photosynthetic reaction centres (Zhang et al. 2003). This in turn may result in the release of Chl from the thylakoid membranes. In such a situation, the Chl needs to be degraded quickly to avoid cellular damage by its photodynamic action (Takamiya et al. 2000). Failure of Chl degradation can increase the amount of ROS produced to an extent where the detoxification capacity of the antioxidant systems may be overridden. Inhibition of Chl biosynthesis has been reported in plants under metal and salt stress (Sinha et al. 2003). AsA is a detoxifier and neutralizer of superoxide radicals and other singlet oxygen species; by prevention of the activity of free radicals it can enhance the Chl content.

Conclusions

In conclusion, this study has shown that foliar application of AsA can increase the survival capacity of canola plants under conditions of salt stress. The increase in resistance to salt stress is associated with the antioxidant activity of AsA, a partial inhibition of salt-induced increases in lipid peroxidation by ROS, and a decrease in antioxidant activity. According to these results it can be suggested that usage of AsA can reduce the harmful effects of ROS and improves plant resistance.

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