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The Effects of Abscisic Acid and $CaCl_2$ on the Activities of Antioxidant Enzymes under Cold Stress in Maize Seedlings in the Dark

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Abstract: The aim of this study was to determine the effects of Abscisic acid, $CaCl_2$ and acclimation (15°C) pre-treatments on the activities of antioxidant enzyme system under cold stress (5°C) in maize seedlings in the dark. When corn seedlings were imposed to cold stress for 7 day at 5°C in the dark, antioxidant enzyme system was induced and protected the seedlings. At first, malondialdehyde (MDA) levels as a product of lipid peroxidation were measured. ABA at 0.1 ρM and $CaCl_2$ at 0.75 mM decreased MDA levels in roots and shoots significantly, indicating that antioxidant enzymes are activated and cold injury is reduced. Measuring the relative activities of SOD, APX, CAT, GR and GPX revealed that, ABA and $CaCl_2$ promoted their activities in roots and shoots significantly. The enhancing effects of ABA on the activities of all enzymes under cold stress were higher than $CaCl_2$ and 15°C acclimation temperature. Calcium treatments were second in this respect. The role of ABA and Ca^{2+} was discussed in this study.

Key words: Oxidative stress, antioxidative enzymes, SOD, APX, CAT, GR, GPX, maize

INTRODUCTION

Germination and early seedling growth are important in stand establishment of maize (*Zea mays* L.). Because maize is a chilling-sensitive crop, environmental stresses such as low temperature during the early stages of development can be detrimental to subsequent crop establishment and productivity[1,2]. Therefore, it is important to understand the molecular, biochemical and physiological means to devise and protect maize seedlings from such environmental adversities[3,4].

A number of tolerance mechanisms have been proposed based on the physiological and biochemical changes associated with chilling injury[5-10]. Chilling injury causes changes in membrane properties[11], solute leakage[12], reduced transport across the plasmalemma[13], malfunction of mitochondrial respiration[14] and induction of peroxide and indoleacetic acid oxidase levels[15]. Recently, chilling sensitivity has also been shown to be correlated with the extend of fatty acid unsaturation of the phosphatidyl glycerol of chloroplast membranes in genetically engineered tobacco plants[16].

There is increasing evidence that chilling causes elevated levels of active oxygen species[15,17,18], which likely contribute significantly to chilling damage. The active oxygen species -H_2O_2 (hydrogen peroxide), $^1O_2^{\bullet-}$ (super oxide), $OH^{\bullet}$ (hydroxyl radical) and $^1O_2$ (singlet oxygen)- are present in all plants to various degrees as a result of normal aerobic metabolism. Allowed to accumulate, these active oxygen species can cause damage to cellular components, severely disrupting metabolic functions[19]. Under normal conditions, plants possess scavenging systems that keep active oxygen species below damaging levels[20]. Superoxide dismutase (SOD) scavenges $O_2^{\bullet-}$ in the cytosol, chloroplasts and mitochondria. Catalase (CAT) is the primary H_2O_2 scavenger in the peroxisomes and the mitochondria, at least in maize[21,22]. H_2O_2 generated in the chloroplasts is scavenged by an ascorbate-glutathion cycle[23]. In this system, ascorbate peroxidase (APX) utilizes H_2O_2 to oxidize ascorbic acid to monodehydroascorbate radical, which disproportionates to dehydroascorbate (DHA) nonenzymatically. Monodehydroascorbate reductase regenerates ascorbate at the expense of NAD(P)H and dehydroascorbate reductase (DHAR) regenerates ascorbate utilizing GSH to form GSSG. GSH is regenerated at the expense of NADPH by the action of glutathione reductase(GR), the rate limiting step of the cycle[24]. Although this cycle is known to be responsible primarily for H_2O_2 scavenging in chloroplasts, its importance in the cytosol and in nonphotosythetic tissues is also becoming apparent[25]. When a plant is stressed, the production of active oxygen can exceed the capacity of the scavenging systems, resulting in oxidative damage. Thus, the ability of a plant to improve its active-oxygen-scavenging capacity may be a key element in stress tolerance[26].

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In the series of experiments reported in this study, maize seedlings were treated with aqueous solutions of $CaCl_2$, ABA and 15°C to see if their chilling tolerance would be significantly affected and to determine the enhancing effects of these treatments on the activities of antioxidative enzyme system.

MATERIALS AND METHODS

Corn seeds (*Zea mays* L. single cross 704) were obtained from Urmia Agricultural Organization (2004). The germination percentage of the seeds was 96% after 72 h. For each experiment, a necessary amount of seeds were sterilized in 1% sodium hypochlorite solution for 10 min. Then they were washed in tap water and finally rinsed with distilled water. The seeds were placed on Petri dishes and incubated at 25°C for four days. At first, the similar seedlings were selected and treated for 24 h with acclimation temperature (15°C), $CaCl_2$ and ABA before cold stress (for 7 day at 5°C in the dark) and MDA levels[27] as a product of lipid peroxidation were measured. ABA at 0.1 ρM and $CaCl_2$ at 0.75 mM decreased MDA levels in roots and shoots significantly, indicating that antioxidant enzymes are activated and cold injury is reduced. Four days old seedlings in four groups and three replicates were treated with 25°C, 15°C, $CaCl_2$ (0.75 mM), ABA (0.1 ρM) for 24 h before cold stress. Then they were transferred to growth chamber at 5°C for one week at darkness condition. Finally the necessary samples of shoots and roots were taken for each experiment.

Measurement of malondialdehyde content: Malondialdehyde (MDA) contents were measured using a thiobarbituric acid reaction[27].

Preparation of enzyme extract: The 0.5 g FW was homogenized at 4°C in 1 mL of extraction buffer (0.05 *M* Tris-HCl buffer, pH 7.5, 3 mM $MgCl_2$, 1 mM EDTA and 1.5% *w/v* PVPP) with mortar and pestle. The extraction buffer used for the APX assay contained 0.2 mM ascorbate. The homogenate was then centrifuged at 25000 g for 20 min and the supernatant was used as the crude extract for the assays of antioxidant enzyme activity[28].

Enzyme assay: Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of NBT using the method of Dhindsa *et al.*[29].

Catalase (CAT) activity was assayed by measuring the rate of disappearance of hydrogen peroxide using the method of Maehly and Chance[30].

Ascorbate peroxidase (APX) activity was determined according to the method of Chen and Asada[31] with minor modification.

Guaiacol peroxidase (GPX) activity was determined according to Upadhyaya *et al.*[32].

Glutathione reductase (GR) activity was assayed by measuring the decrease in absorbance at 334 nm due to the oxidation of NADPH[33].

Statistical analysis: All experiments were repeated at least twice with similar results. Data were subjected to an analysis of variance and means and standard errors calculated.

RESULTS

When corn seedlings are exposed to chilling temperatures, Active Oxygen Species (AOS) accumulate in different cell compartments. One of the indications of AOS injuries to cell membranes is the production of MDA. MDA is the end product of lipid peroxidation which increases in chilled corn seedlings (shoots and roots). This investigation studied the effects of temperature (15°C) and varying concentrations of ABA and $CaCl_2$ as a pre-treatments for 24 h to see if they can reduce chilling injury to corn seedlings, that were imposed to 5°C for 7 days later (data not shown). We found that $CaCl_2$ at 0.75 mM and ABA at 0.1 ρM are showing promising effects. In this experiment, we pre-treated the four days old corn seedlings with 25°C, 15°C, $CaCl_2$ (0.75 mM), ABA (0.1 ρM) for 24 h and then we exposed all of the plants to 5°C for 7 days. Table 1 shows that, the control plants that were pre-treated at 25°C, accumulated the highest level of MDA in roots and shoots. Pre-treating the seedlings at 15°C for acclimation, decreased MDA levels slightly that was not very significant. But, pre-treating the seedlings with $CaCl_2$ and ABA significantly decreased the MDA levels in comparison to untreated controls. ABA was the most effective.

The hypothesis that, if chilling can induce oxidative stress and the activities of antioxidant enzymes (SOD, APX, CAT, GR and GPX) changes or not was tested. The design of experiment was the same as mentioned above. Results suggested that, chilling has imposed an oxidative stress on corn seedlings grown at 5°C for one week in the dark. Table 2 reveals an increase of 29.86% for stem and 25% for root in SOD activity when seedlings were pre-treated with 0.1 ρM ABA for 24 h. On the other hand, pre-treating the seedlings with 0.75 mM $CaCl_2$ increased the activity of SOD about 18.75% for stem and 14.71% for root. But, the 15°C cold acclimation treatment did not significantly increase the activity of SOD. This finding suggests a role for $CaCl_2$ and ABA under chilling imposed oxidative stress conditions.

Table 1: The effects of treatments of 25, 15°C, $CaCl_2$ and ABA on the malondialdehyde contents. The data are the means of three replications ±SE

Treatments	Stem (μmol $g^{-1}fw^{-1}$)	Root (μmol $g^{-1}fw^{-1}$)
Control (25°C)	15±0.30	11±0.46
Acclimation (15°C)	13.8±0.45	10.6±0.35
$CaCl_2$ (0.75 mM)	12.7±0.32	10.1±0.21
ABA (0.1 ρM)	11.5±0.31	7.2±0.42

Table 2: The effects of treatments of 25°C, 15°C, $CaCl_2$ and ABA on the relative activity of SOD. Means ±SE of different treatments on the relative activity of SOD

Treatments	Stem	Root
Control (25°C)	100±5.51	100±3.70
Acclimation (15°C)	108.33±4.81	107.35±4.73
$CaCl_2$ (0.75 mM)	118.75±4.34	114.71±3.40
ABA (0.1 ρM)	129.86±3.87	125±3.89

Table 3: The effects of treatments of 25, 15°C, $CaCl_2$ and ABA on the relative activity of APX. Means ±SE of different treatments on the relative activity of APX

Treatments	Stem	Root
Control (25°C)	100±2.94	100±3.06
Acclimation (15°C)	108.33±2.62	106.67±4.06
$CaCl_2$ (0.75 mM)	116.11±2.42	118±4.29
ABA (0.1 ρM)	125±2.62	126.67±3.15

Table 4: The effects of treatments of 25, 15°C, $CaCl_2$ and ABA on the relative activity of CAT. Means ±SE of different treatments on the relative activity of CAT

Treatments	Stem	Root
Control (25°C)	100±4.67	100±3.29
Acclimation (15°C)	129.41±5.09	133.33±5.43
$CaCl_2$ (0.75 mM)	164.71±3.45	1160±3.15
ABA (0.1 ρM)	229.41±4.49	233.33±4.06

Table 5: The effects of treatments of 25, 15°C, $CaCl_2$ and ABA on the relative activity of GR. Means ±SE of different treatments on the relative activity of GR

Treatments	Stem	Root
Control (25°C)	100±6.50	100±5.82
Acclimation (15°C)	125±6.17	120±4.94
$CaCl_2$ (0.75 mM)	143.75±2.60	140±4.70
ABA (0.1 ρM)	162.5±5.05	156.92±4.07

Table 6: The effects of treatments of 25, 15°C, $CaCl_2$ and ABA on the relative activity of GPX. Means ±SE of different treatments on the relative activity of GPX

Treatments	Stem	Root
Control (25°C)	100±7.26	100±7.77
Acclimation (15°C)	133.33±6.01	138.46±5.09
$CaCl_2$ (0.75 mM)	156.67±5.36	163.46±7.28
ABA (0.1 ρM)	186.67±6.74	186.54±9.09

Measuring the activity of APX under chilling condition revealed that APX activity shows the same trend as SOD activity. Pre-treating the seedlings with 0.1 ρM ABA increased APX activity about 25% for stem and 26.67% for roots and 0.75 mM $CaCl_2$ about 16.11% for stem and 18% for roots (Table 3).

Studying CAT activity showed a dramatic increase of about 133.33% in comparison to untreated controls, which was 100% (Table 4). Here also the ABA pre-treated seedlings showed the highest CAT activity, while the $CaCl_2$ treated seedlings were in the second place. Meanwhile, the 15°C acclimation treated plants, showed also a significant increase in CAT activity with respect to untreated controls.

GR and GPX activities showed the same trends as SOD, APX and CAT (Tables 5 and 6). Here also, ABA pre-treated seedling showed the highest activities in comparison to control plants.

DISCUSSION

Chilling can lead to increased concentrations of toxic oxygen compounds in susceptible tissues[17,34]. Some of very active oxygen species are $O_2^{\bullet -}$ (super oxide), H_2O_2, $OH^{\bullet}$ and $^1O_2$ (singlet oxygen). H_2O_2 levels are elevated in various tissues of chilled seedlings[18]. Elevation of H_2O_2 is an indication of a state of oxidative stress under which lipid peroxidation and other deleterious effects on membranes[10] and inactivation of various membrane linked metabolic enzymes occur[35]. Elevated H_2O_2 levels can also be resulted from an increased concentration of $OH^{\bullet}$, which is highly reactive and can contribute significantly to cellular damage[19].

Mitochondria are indeed a target for chilling damage in maize seedlings in the dark. Mitochondrial respiration is significantly reduced at low temperatures, which indicates that the immediate response of the tissue to chilling is a depression of respiratory activity. The decline in O_2 uptake rates is apparently a result of damage to the more sensitive cytochrome pathway. The increase in alternative pathway during low-temperature stress was thought to be the source of the electrons for the production of O_2 free radicals during such a stress[3]. Chilling stress decreases the cytochrome oxidase (COX) activity, its protein level and cyanide sensitivity of O_2 uptake. These results are consistent with a possible leakage of electrons from impaired electron transport chain to reduce molecular O_2 to superoxide and H_2O_2. Low temperature also increases electron flow via the alternative pathway. It was speculated that a broader role for the alternative pathway could be one way of protection against extremes[36-38] and therefore, considered as a reserve for the failure of the more sensitive Cyt pathway during chilling. Chilling sensitivity has also been attributed to critical changes in membrane fluidity[14,39]. Chilling, in general, inhibits not only the F_1-ATPase activity but also its protein level, similar to COX. It is likely that efficient oxidative phosphorylation may be important for full recovery from chilling stress. Lyons *et al.*[39] proposed that mitochondria of chilling-sensitive plants do not possess dynamic properties at low temperature and that phosphorylation is

therefore disrupted. The changes in mitochondrial behavior might also be a result of sharply reduced energy generation, since oxidation rates of mitochondria form chilling-sensitive tissues decrease as chilling temperatures are incurred.

In present study, the activity of CAT increased about 133.33% in comparison to unpre-treated controls (Table 4). This suggested that, chilling has imposed an oxidative stress and the level of H_2O_2 has increased highly and CAT has become very active to scavenge the H_2O_2 and prevent its damage. An other result of this study is the role of acclimation (15°C), $CaCl_2$ and ABA in activing the catalase and other antioxidative enzymes. There are reports that, catalase isozymes are rapidly induced by chilling[40,41]. So they play a direct role in stress protection.

The SOD activity also increased about 29.86% in comparison with unpre-treated controls (Table 2). This suggested that chilling has imposed an oxidative stress and $O_2^{\bullet-}$ level has been elevated. Here also, ABA had a promotive effect on SOD activity. The result of present study showed that, following the increased activities of CAT and SOD, the activities of other enzymes of Halliwell-Asada cycle are also increased in order to scavenge any toxic oxygen species from the cells.

Another finding of present study is the differential effect of cold acclimation at 15°C for 24 h on the activities of antioxidative enzymes. While, cold acclimation did not have a significant effect on SOD and APX activities, but it had a meaningful promoting affect on CAT, GR and GPX activities. It is speculating that, under chilling stress, SOD is not becoming very active and probably the elevated levels of $O_2^{\bullet-}$ injuries the membranes and denatures the protein at darkness. But it is possible that elevated levels of H_2O_2 are produced in mitochondria and peroxisomes in the dark, which causes the highly increased activities of CAT, GR and GPX.

An interesting finding of present study is the promoting effects of $CaCl_2$ and ABA in activating of all enzymes of Halliwell-Asada cycle. There are many reports that Ca ions are taken up by plant cells under reduction in temperature. Increased influx of radiolabled Ca^{2+} into maize roots has been observed[42]. The cold-induced influx of Ca^{2+} was found to be due to the opening of calcium channels rather than a general cold-induced increased membrane permeability, because influx of $^{45}Ca^{2+}$ into cold-shocked maize roots was inhibited by lanthanium which is a calcium channel blocker[42,43]. Other workers have reported that in addition to the influx of external calcium, there is also a release of calcium from the vacuole, via IP_3 sensitive channels on tonoplast[44]. So, it is believed that Ca^{2+} is involved in cold stress signaling pathway, gene expression and cold tolerance.

On the other hand Ca^{2+} signaling is involved in oxidative stress[45]. Since, a rapid transient elevation of Ca^{2+} has been observed after treatment with 10 mM H_2O_2. This elevation was inhibited by lanthanium.

The results of present study indicate that, cold shock induces an oxidative stress and Ca^{2+} plays an important role in signal transduction pathway and activating the enzymes of Halliwell-Asada cycle.

There are reports that phytohormones are released in response to various stresses. The ABA involvement also has been reported in cold stress response[46,47]. ABA plays an important role in signaling of low temperature stress and it has been demonstrated that different members of SOD gene family increase at transcriptional level after ABA treatment in maize[48]. Meanwhile, ABA has been shown to increase both GR and APX activities also[49]. At last it appears that, ABA and Ca^{2+} have enhancing effect on the activities of all enzymes of antioxidative system. Probably they are surving in signal transduction pathway and gene expression under cold stress.

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