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Improving Chilling Tolerance of Maize Seedlings under Cold Conditions by Spermine Application

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Abstract

Low temperature is an important abiotic stress which reduces crops growth and productivity and causes physiological damages to cellular structures. The aim of this study was to investigate the probability of spermine application to improve chilling tolerance of maize under stress conditions. The treatments were included seed priming with spermine (30, 60 and 90 mg/l solutions) and normal and stress condition. Seed emergence was improved by spermine priming on both conditions and mean emergence time (MET) was also decreased with priming. Shoot and root length was highly reduced under stress conditions, but the treated seeds were improved along with increased spermine concentration. Seedling dry weight was also affected by priming and reduced weight of stressful seedlings was alleviated by spermine priming. Decreased relative water content on seedlings under stress was elevated by the treatments and significantly increased. Electrolyte leakage was also recovered by applied treatments while it was adversely decreased on cold conditions. Antioxidative system was highly responded to spermine application. Superoxide dismutase (SOD) activity increased on both normal and stress conditions, but a little decrease was observed on seedlings treated with 90 ppm level and under chilling conditions. Catalase activity was also amplified by spermine treatments. Priming had a great effect on ascorbate peroxidase (APX) activity on both stressful and normal seedlings and increased it compare with non treated seedlings. It is also important to note that with increasing spermine concentration to 90 ppm, no considerable differences were observed. Thus, 60 ppm concentration could be proposed as the appropriate level of spermine in order to improve chilling tolerance of maize seedlings.

Keywords: antioxidants activity, maize, polyamine, seed, stress

Introduction

Low temperature is known as an important limiting factor which reduces plants productivity around the world. Maize (*Zea mays* L.) is a thermophilic crop and optimum temperature for maize germination is between 25-28°C. Low temperature causes injuries to maize germination and seedling growth, so it will be damaging specially for early spring planting (Parera and Cantliffe, 1994).

Chilling stress could induce different kinds of damages such as reducing growth rate (Sowinski *et al*., 2005; Verheul *et al*., 1996) water uptake disturbance (Aroca *et al*., 2003a) photosynthesis efficiency (Foyer *et al*., 2002; Haldimann, 1997) changes in membrane properties (Pinhero *et al*., 1997) and particularly considerable increase in reactive oxygen species (ROS) production (Foyer *et al*., 2002) as well as enzymatic and non-enzymatic antioxidants (Leipner *et al*., 1999; Skrudlik *et al*., 2000).

A common consequence of most environmental stresses (Hayat *et al*., 2007; Muthuchelian *et al*., 2001; Sairam *et al*., 2005) is an increased production of ROS. Chilling has also similar effects which was studied by some researches (Farooq *et al*., 2008a; 2008b). These ROS, such as superoxide radical (O^-), hydrogen peroxide (H_2O_2) and hydroxyl (HO^-) are exceedingly toxic to cellular and subcellular structures (Schutzendubel and Polle, 2002). However, plants have an efficient antioxidant system includes enzymatic and non-enzymatic antioxidants (superoxide dismutase, peroxidase, catalase, and glutathione reductase, carotenoids, glutathione, ascorbic acid) and also some important metabolites like proline to counter with oxidative stress and protect the plants from oxidative damage (Apel and Hirt, 2004).

Polyamines (PAs) are ubiquitous low-molecular-weight amines involved in of plant growth and development regulation (Martin-Tanguy, 2001). Polyatomic nature of this compounds made them able to interact with proteins, nucleic acids and membrane phospholipids and this leads to activating or stabilizing these molecules. The diamine putrescine (Put), the triamine spermidine (Spd) and the tetraamine spermine (Spm) are the most common polyamines which may be present in the free, soluble conjugated and insoluble bound forms.

There have been several mechanisms proposed for the protective effects of PAs as well as stabilizing DNA structure (Kasinathan and Wingler, 2004) impeding lipid peroxidation and membrane integrity (Ha *et al*., 1998). Kubis (2008) also indicated that PAs may acts as free radicals scavengers. In fact, PAs accumulation could protect plant cells when they exposed to environmental stresses (Nayyar and Chander, 2004; Sanchez *et al*., 2005).

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Roy *et al*. (2005) and Kubis (2008) showed that exogenous application Spd could prevent the electrolyte and amino acid leakage or recovering the plasma membrane damage in rice cultivars in response to salinity, in chilling tolerance and in protection of water stressed cucumber leaves. The protective role of Spm against salt stress has been well established in Arabidopsis (Yamaguchi *et al*., 2006).

Several studies reported priming advantages on germination and stand establishment improving (Basra *et al*., 2005; Farooq *et al*., 2006a; 2006b; 2006c). Priming provides faster and synchronous seedling emergence (McDonald, 1999). Moreover, priming has also been found effective to increase the germination under chilling conditions. Farooq *et al*. (2008a) proposed seed priming as a tool to improve chilling tolerance in late-sown wheat.

In the present study, the effects of seed priming with different concentrations of spermine solutions on maize plants under normal and chilling conditions were investigated to explore the possible biochemical basis of chilling tolerance.

Materials and methods

Plant materials and growth conditions

Seeds of Maize (Single cross 704) were obtained from the Center of Agricultural Researches and Natural Resources, Khorasan Razavi, Iran. Seeds were surface sterilized with 2% $HgCl_2$ for 20 min, washed twice with distilled water and air dried. For seed treatments, seeds were soaked in 30, 60 and 90 ppm solutions of spermine (solved in distilled water) for 24 hours. Untreated seeds (treated by water) were considered as control.

Treated and untreated seeds were sown in 1 liter plastic pots containing water-washed sand, and then placed in a growth chamber with a photosynthetically active photon flux density of 320 mmol m^{-2} s^{-1} and a photoperiod of 16/8 h light/dark. One series of pots was placed at optimal temperature (27°C) as control, while the other was kept at chilling stress (15°C) during the period of study. The number of emerged seedlings was daily recorded. Time required to reach 50% emergence of seedlings (E50) was calculated by following formulae of Coolbear *et al*. (1984) modified by Farooq *et al*. (2005):

$$E50 = t_i + \frac{[(N/2)] - n_i](t_j - t_i)}{n_j - n_i}$$

Where N is the final number of emerged seeds, and n_i and n_j are the cumulative number of seeds emerged counts at times t_i and t_j when $n_i < N/2 < n_j$.

Mean emergence time (MET) was calculated according to the equation of Ellis and Roberts (1981):

$$MET = \frac{\sum D_n}{\sum n}$$

Where n is the number of seeds which emerged on day D, and D is the number of days counted from the beginning of emergence.

After 24 days of emergence, samples were collected for biochemical analysis. Seedlings were carefully removed to evaluate seedling shoot and root length. Seedling fresh weight was determined immediately after harvest while dry weight was taken after drying at 70°C for 2 days.

Membrane permeability

Membrane permeability was determined by measuring the electrolyte leakage using the method of Blum and Ebercon (1981). Six leaf samples were washed with distilled water and soaked in 6 mL of distilled water for 12 h. The conductivity of the solution (C1) was measured with a conductivity meter. Samples were then heated in boiling water for 20 min and then cooled to room temperature. The conductivity of killed tissues (C2) was again measured. Membrane permeability was calculated as the ratio between C1 and C2.

Relative water content

In order to determine relative water content (RWC) fresh leaf discs with 2 cm^2 diameter were weighted floated on deionized water for saturation until 24 hours and saturated leaf weight was recorded. Dry mass was also noted after dehydration at 70°C for 48 h. the following formula was used to calculate RWC (Hayat *et al*., 2007):

$$RWC = \frac{Fresh\ weight - dry\ weight}{Turgor\ weight - dry\ weight}$$

Extraction and determination of enzyme activities

Leaves tissue (100 mg FW) were placed into liquid nitrogen and then homogenized with a pre-chilled mortar and pestle under ice cold-conditions in 4 mL 50 mM potassium phosphate buffer, pH 7.0, with adding 1 mM EDTA. The homogenate was centrifuged at 15000 rpm, at 4°C for 20 min. The supernatant was stored at -20°C and used for determination of enzyme activity.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assessed by the method of Giannopolitis and Ries (1977). The inhibition of photochemical reduction of NBT was measured and the color was developed by adding 2.4 mL of 50 mM potassium phosphate buffer solution (pH 7.8), 0.2 mL of 195 mM methionine, 0.1 mL of 0.3 mM EDTA, 0.2 mL of 1.125 mM NBT and 0.2 mL of 60 μM riboflavin to 50 μL enzyme extract. Reaction mixtures were illuminated for about 15 min at 5000 Lux light intensity. The solution absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme causing half-maximal inhibition of the NBT reduction.

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured based on the method of Nakano and Asada, 1981. The reactive solution contained 50 mM sodium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and 10 µl of enzyme extracts. The decrease in absorbance at 290 nm was recorded. Extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate enzyme activity. One unit of APX was defined as the amount of degrading 1 µmol of ascorbate min^{-1} mg $protein^{-1}$ under the assay conditions.

Catalase (CAT, EC 1.11.1.6) activity was determined following the utilization of H_2O_2 at 240 nm for 1 min (Aebi, 1984). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50 µl of enzyme extract in final volume of 3 ml. The enzyme activity was calculated using the extinction coefficient (39.4 mM^{-1} cm^{-1}) and expressed as units (1 µmol of H_2O_2 decomposed per minute) per mg protein.

Statistical analysis

The experiment was a 2×4 factorial based on a randomized complete block design with four replications and three seedlings on each pot. Analysis of variance performed using SAS 9.1 and general linear models (PROC GLM) procedure (SAS Institute, Cary, NC). The LSD test was used to comparison of means at p= 0.05.

Results and discussion

Growth parameters

Exogenous application of spermine improved seed emergence under both optimal and stress conditions (Fig. 1A). Application of 30 ppm spermine seed priming significantly decreased the E50, but it was increased with increasing spermine concentration. There were no significant differences between the 60 and 90 ppm treatments, but still had the lower E50 compared with control treatment. (Xiong *et al*., 2002).

Chilling impairments mainly consist of alteration of metabolic processes, decrease in enzymatic activities, reduction of photosynthetic capacity and changes in membrane fluidity among others (Dubey, 1997). Plants adopt with the stressful conditions through different kind of mechanism, mostly by acclimation (Lee *et al*., 2002). There is a group of evidences which approved that cold acclimation could be achieved by exogenous application of PAs (Groppa and Benavides, 2008; Xiong *et al*., 2002). These compounds regulate plant growth and development through different physiological processes (Martin-Tanguy, 2001; Paschalidis and Roubelakis-Angelakis, 2005).

Mean emergence time (MET) was also significantly affected by priming such that along with increasing the spermine levels, MET was decreased (Fig .1B)., There was also no difference between the 60 and 90 ppm treatment. The seedlings under chilling condition showed a noticeable decrease in shoot length, as well as root length, but it was improved on account of spermine treatments (Fig. 2A and B). There was a little decrease in both shoot and root length at 90 ppm spermine concentration, which was not significant. It can be concluded that limiting effects of chilling on seedlings emergence and growth could be alleviated by spermine seed priming, but the suitable concentration is 60 ppm and higher level will not be more efficient. Exogenous application of spermine as an important PAs improves germination properties and seedling length, which is related to the role of PAs on germination and primary growth regulation. Xu *et al*. (2010) were also observed the improving of germination percentage, index and mean germination time of chilling exposed *Nicotiana tabacum* primed with putrescine.

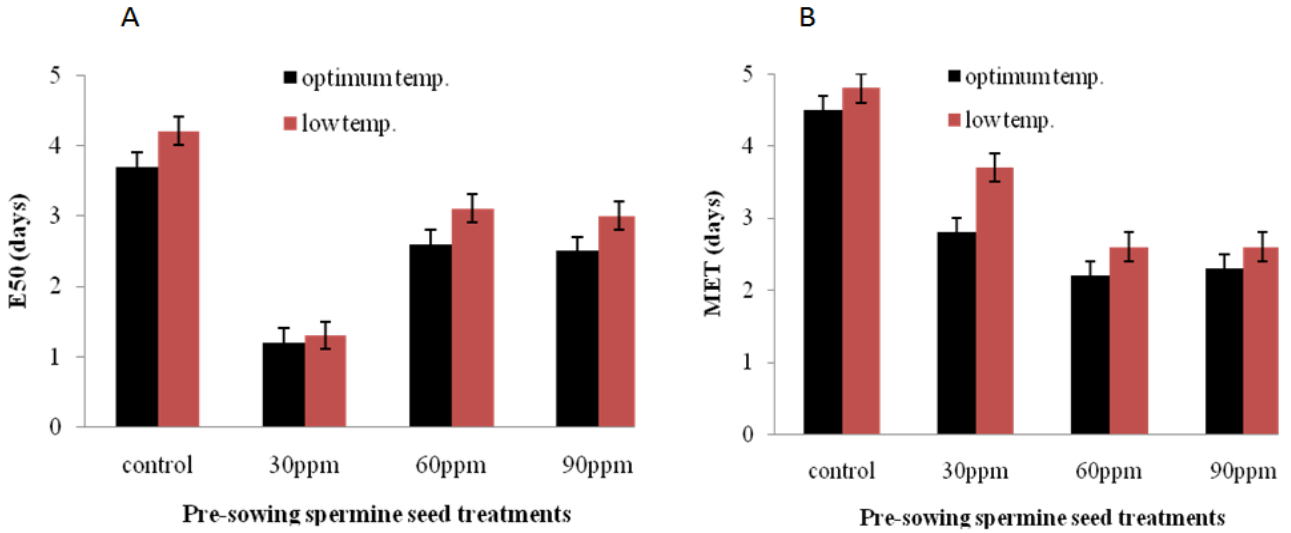

Fig. 1. Effect of spermine application on mean emergence time (MET) (A) and E50 (B) of maize seedlings under optimum and low temperature conditions. Data are the mean value of three replications ± SE which represented by the vertical bar in each graph

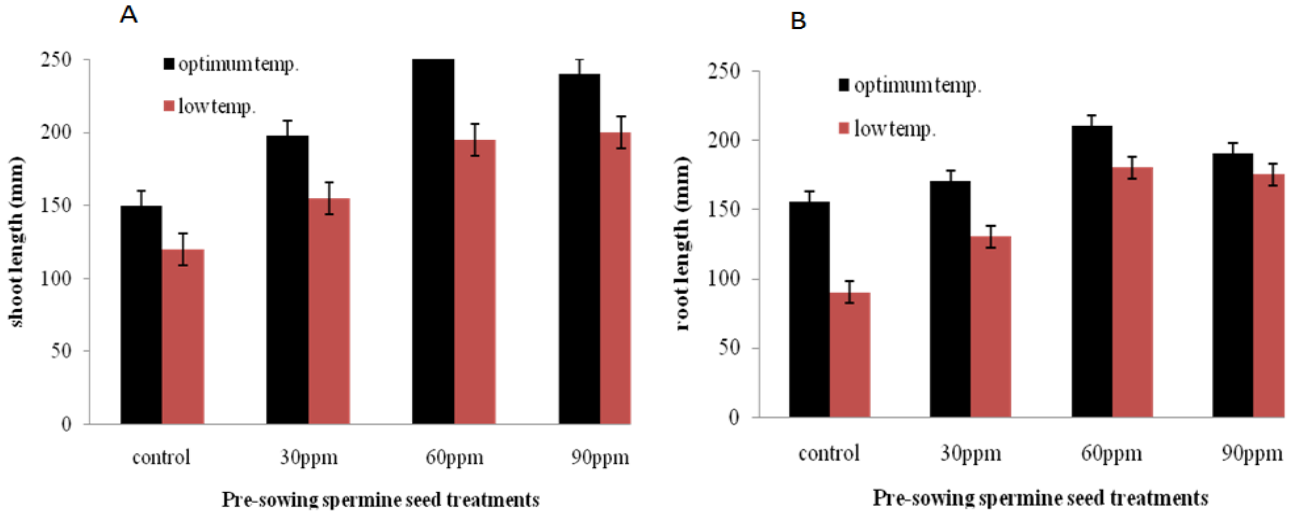

Fig. 2. Effect of spermine application on shoot (A) and root (B) length of maize seedlings under optimum and low temperature conditions. Data are the mean value of three replications ± SE which represented by the vertical bar in each graph

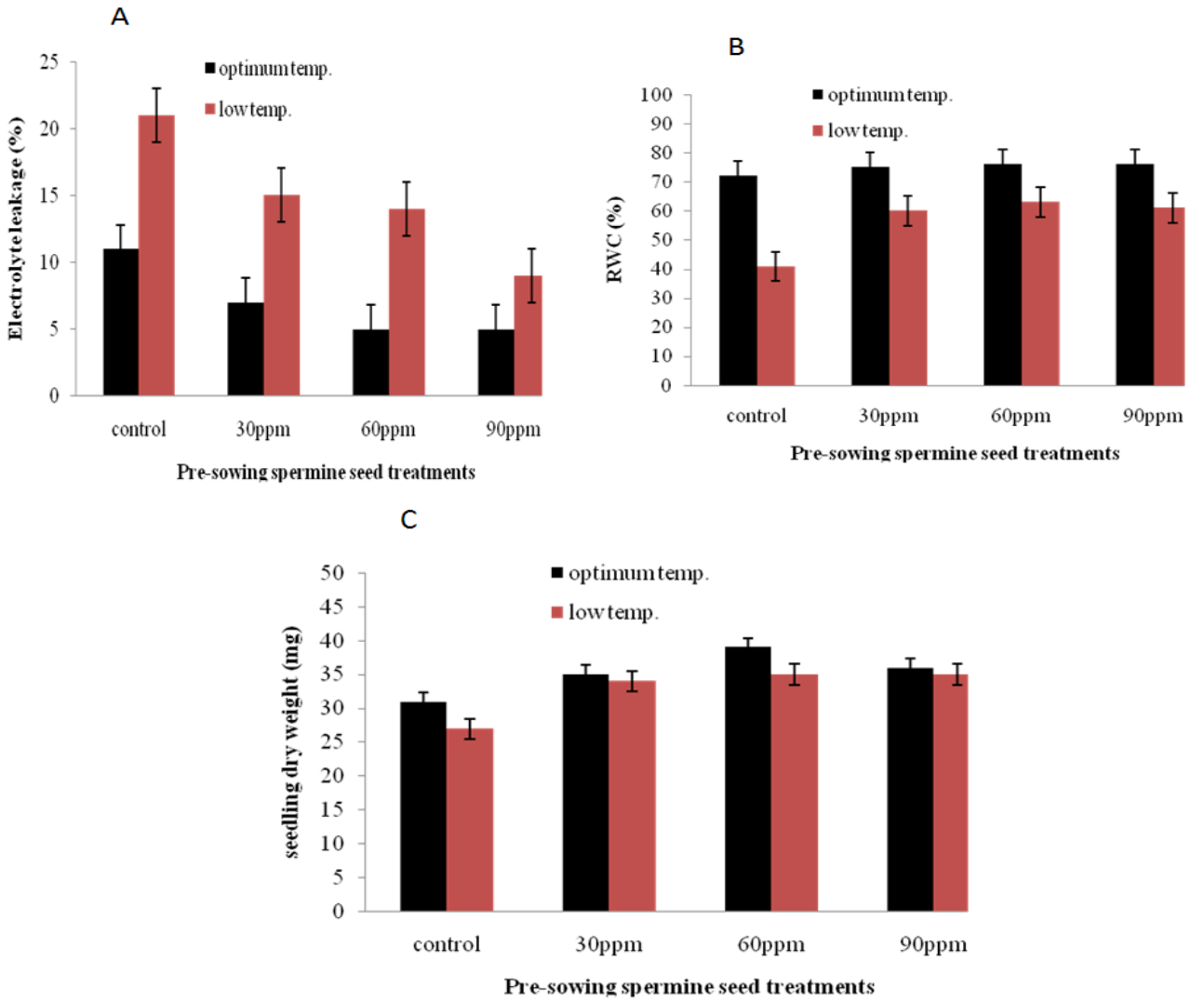

Fig. 3. Effect of spermine application on electrolyte leakage (A), relative water content (RWC) (B) and seedling dry weight (C) of maize seedlings under optimum and low temperature conditions. Data are the mean value of three replications ± SE which represented by the vertical bar in each graph

Relative water content

There was a drastic decrease on chilling exposed seedlings relative water content (RWC). But exogenous spermine application could enhance the RWC content and increased it, significantly (Fig. 3B). Plants grown under normal conditions showed a very slight increase on RWC. The considerable point here is that different spermine concentrations had no effect on RWC on both normal and stress conditions and RWC contents were nearly the same. So, the enhancement effect of spermine may be restricted to the low levels and increasing the applied concentration even may have negative side effects.

Electrolyte leakage and seedling dry weight

A decline on electrolyte leakage on both normal and stressful seedlings due to the spermine treatment was recorded during this experiment (Fig. 3A). Electrolyte leakage was adversely increased when the seedlings was exposed to chilling condition with no priming, but spermine treatment leads to reduce the electrolyte leakage and 90 ppm spermine concentration had the lowest amount. These effects on seedlings under none chilling conditions were less clear. Seedling dry weight was significantly affected by spermine treatments and it was increased on chilling stressed seedlings, as the others on control treatment (Fig. 3C). Like the previous, 90 ppm spermine treatment was not efficient and seedling dry weight was a little decreased on optimum temperature, while it had no change on stressful seedlings.

Membranes are the major targets of environmental stresses (Leshem, 1992). Induced changes on plant cells under chilling stress are mainly related to an increase in membrane permeability, affecting membrane integrity and cell compartmentation under stress conditions (Campos *et al*., 2003). Enhanced electrolyte leakage was considered to be a symptom of stress-induced membrane damage and deterioration (Feng *et al*., 2003). It was reported that Exogenous application of spermidine as an important

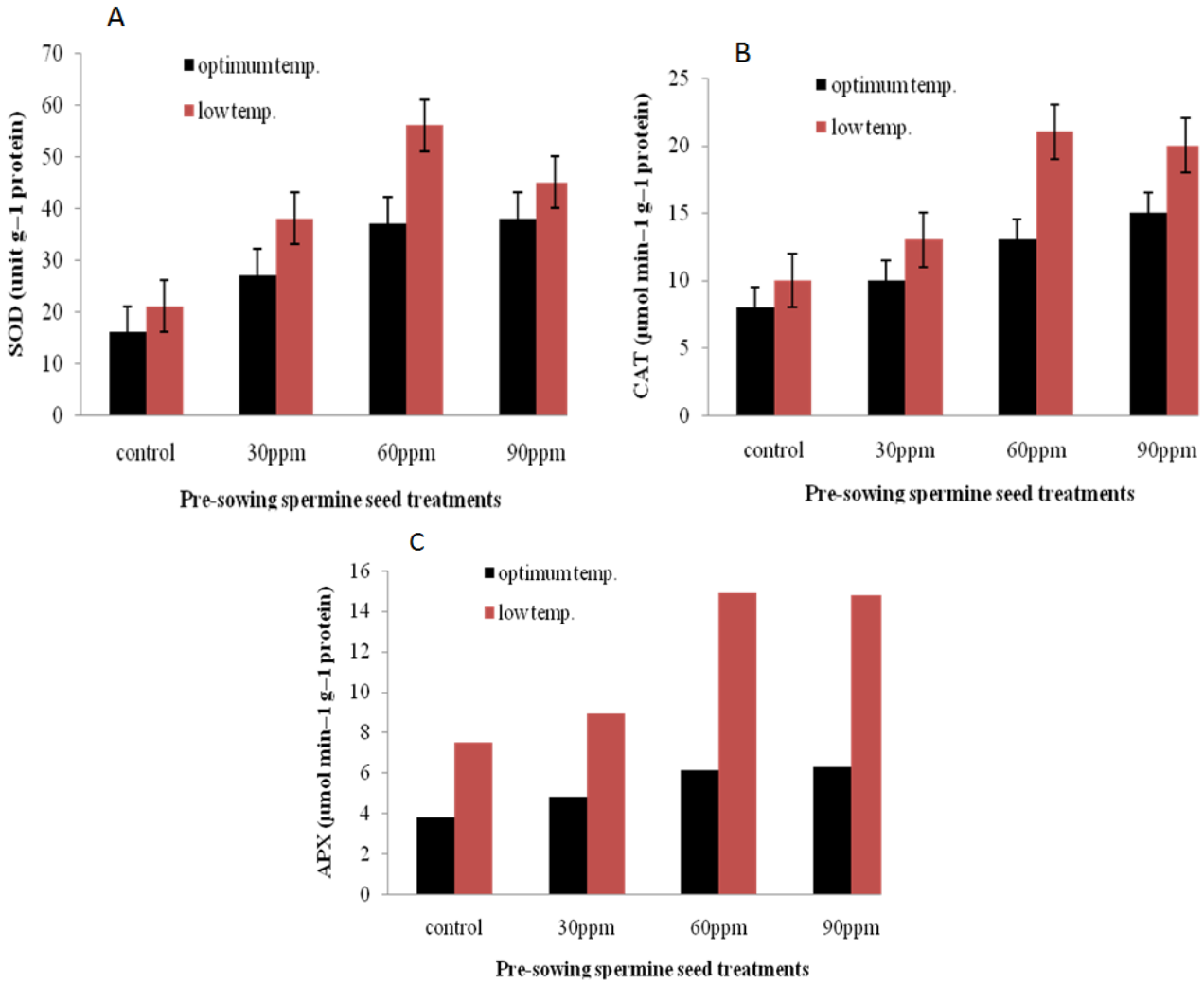

Fig. 4. Response of Antioxidative system of maize seedlings under optimum and low temperature conditions to spermine application including Superoxide dismutase (SOD) (A), Catalaze (B) and Ascorbate Peroxidase (C). Data are the mean value of three replications ± SE which represented by the vertical bar in each graph

polyamine plays vital roles in preventing the electrolyte leakage and recovering the plasma membrane damage in rice cultivars in response to salinity, in chilling tolerance and in protection of water stressed cucumber leaves (Kubis, 2008; Roy *et al*., 2005). Apparently, the effect of spermine on improving the damage of electrolyte leakage caused by chilling stress is achieve to a great extent on high concentrations of spermine. However, the 30 ppm level could significantly reduce the electrolyte leakage of the seedlings under stress and normal conditions.

Antioxidant system activity

Antioxidant system was efficiently responded to the stress conditions and the priming treatment. The activity of superoxide dismutase (SOD) as an important enzymatic antioxidant was highly increased caused by spermine priming treatment on seedlings under both optimal and low temperature (Fig 4A). The highest value of SOD activity was obtained on seedlings primed with 60 ppm spermine and under low temperature conditions. Although the increasing trend of SOD activity was continued with increased level of spermine on non stressful seedlings, a little decrease was observed on 90 ppm level and under chilling conditions seedlings.

Catalase (CAT) activity was also affected by applied spermine levels (Fig 4B). Catalase activity was increased due to Chilling treatments compared with control plants and it was significantly amplified as the spermine concentration increased. There were no differences between 60 and 90 ppm spermine level regards to catalyze activity.

Priming had a great effect on Ascorbate peroxidase (APX) activity on both stressful and normal seedlings (Fig 4C). Specially, 60 ppm spermine level treatment increased APX activity to a great extent comparing with the previous level. But similarly like CAT, 90 ppm level had no advantage about the enzymes activity related to the 60 ppm level treatment, which may suggest the optimum level of spermine application with respect to the results of the other parameter.

PAs are also widely known to play a vital role on activating the defense mechanisms of plants to various environmental stresses (Cuevas *et al*., 2008; Groppa and Benavides, 2008; Katsukabe *et al*., 2004; Xhang *et al*., 2009), especially oxidative stress (Durmus and Kadioglu, 2005; Rider *et al*., 2007).

The activation of several enzymatic (superoxide dismutase, GPX, APX, glutathione reductase and non-enzymatic antioxidants can detoxify produced reactive oxygen species in stressed cells (Blokhina *et al*., 2003). It is well documented that polyamines counteract oxidative damage in plants by acting as direct free radical scavengers or binding to antioxidant enzyme molecules to scavenge free radical (Bors *et al*., 1989). Higher endogenous levels of polyamines, particularly spermine and Spermidine are positively correlated with greater increase in antioxidant enzymes in response to environmental stresses. It was suggested earlier that the PAs may act indirectly by elevating the levels of antioxidants or gene expression of antioxidant enzymes (Shen *et al*., 2000; Tang and Newton, 2005; Verma and Mishra, 2005; Wi *et al*., 2006) resulting in tolerance to numerous abiotic stresses. Result of the enzymatic antioxidants assessment in this experiment is similar to the mentioned achievements.

Conclusions

Based on the results of this experiment, this can be concluded that spermine has a protective effect on plant tissues exposed to cold stress and this role is mainly related to membrane stabilizing and activating antioxidants production processes in cellular structures.

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