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Relationships between some endogenous signal compounds and the antioxidant system in response to chilling stress in maize (*Zea mays* L.) seedlings

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Abstract: This study investigated the correlation between endogenous levels of some signal compounds [nitric oxide (NO), salicylic acid (SA), abscisic acid (ABA)] and the antioxidant system in response to chilling stress in maize (*Zea mays* L.). Seedlings grown for 12 or 19 days under normal conditions (24/20 °C) were exposed to chilling stress (10/7 °C) for 2 days. The levels of NO, SA, ABA, hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), and malondialdehyde (MDA) were determined in leaves of seedlings harvested on day 14 and day 21. In the same seedlings, the activities of superoxide dismutase (SOD), peroxidase (POX), catalase (CAT), and nitrate reductase (NR) were analyzed. Chilling treatment enhanced the levels of NO and ABA in the seedlings harvested on both day 14 and day 21 as compared with the control group. However, SA content was elevated by chilling treatment in 14-day-old seedlings, while it did not change significantly in 21-day-old seedlings. As compared with control plants, chilling treatment increased not only antioxidant enzymes activities but also contents of H_2O_2, $O_2^{\cdot-}$, and MDA. NR regarding ABA and NO biosynthesis was increased by chilling in maize. The results showed that chilling stress caused oxidative damage, and changes in signal molecules (NO, SA, and ABA) and NR activity in maize.

Key words: Chilling stress, nitric oxide, salicylic acid, abscisic acid, oxidative damage, antioxidant enzyme, HPLC

1. Introduction

Chilling temperature is among the major factors that limit the production of chilling sensitive cereals and reduce their yield (Janda et al., 1999; Zhang et al., 2011). Chilling temperatures prompt deformation of cell components and dehydration of intracellular spaces (Levitt, 1980). Thus chilling sensitive cereals may undergo metabolic decomposition by exhibiting structural injuries (Janda et al., 1999). During growth and development at low temperatures including chilling, plants can increase the levels of reactive oxygen species (ROS). In fact, ROS are produced as a habitual by-product of cellular metabolism in living organisms (Ahmad et al., 2008). However, biotic and abiotic stresses bring about the excessive production of ROS, causing oxidative damage and even cell death at their increased levels although low levels of ROS are acknowledged as second messengers in response of tolerance to abiotic stresses (Ahmad et al., 2008). Equilibrium between ROS production and their scavenging is a decisive manifestation about whether ROS play a role as signaling molecules or cause oxidative damage in cells (Scandalios, 1993). Oxidative damage arising from excess ROS has also been associated with appearance of chilling stress in plant cells. The toxicity of ROS is due to reactions with numerous cell components, causing a cascade of oxidative reactions and inactivation of enzymes, lipid peroxidation, protein degradation, and even DNA damage (Scandalios, 1993). Plants protect themselves against the detrimental effects of ROS by a complex antioxidant system involving lipid-soluble antioxidants (tocopherol and carotenoids), water-soluble reductants (glutathione and ascorbate), and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Apel and Hirt, 2004). During chilling stress, the activities of antioxidant enzymes are decreased, which leads to increased superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and other ROS (Kang et al., 2003; Liu et al., 2010; Esim and Atici, 2014). Maize (*Zea mays*) seedlings are injured by temperatures below 12 °C, which often occur in spring shortly after sowing and germination. Therefore, cold or chilling is one of the main limiting factors of wider spread and growth of maize (Farooq et al., 2009). Injury to maize plants caused by chilling stress is associated with high oxidative damage at the cellular and plant level (Farooq

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et al., 2009). Several messenger molecules such as abscisic acid (ABA) (Xiong et al., 2003), nitric oxide (NO) (Neill et al., 2003), and salicylic acid (SA) (Ahmad et al., 2008) are involved in the perception and transduction of chilling temperature signals to mediate the stress dependent changes in physiological processes.

ABA, SA, and NO as plant hormones are important signaling molecules in plants and are proposed to have similar/dissimilar effects on tolerance to abiotic stresses including chilling (Neill et al., 2003; Xiong et al., 2003; Ahmad et al., 2008). Endogenous ABA content increased under environmental stress such as chilling (Janowiak et al., 2002; Liu et al., 2011), heat (Teplova et al., 2000), and drought (Wang and Huang, 2003), which adjusts the relatively physiological and biochemical reactions. A role of ABA in the chilling tolerance of maize seedlings and *Chorispora bungeana* suspension culture has also been demonstrated (Janowiak et al., 2002; Liu et al., 2011). In addition, application of exogenous ABA increased chilling tolerance in maize, wheat, *Brassica napus*, chickpea, *Stylosanthes guianensis*, and *Chorispora bungeana* (Zhou et al., 2005; Kumar et al., 2008; Liu et al., 2011). In a previous study, Liu et al. (2011) demonstrated that an inhibitor of endogenous ABA decreased the activities of antioxidant enzymes in *Chorispora bungeana* under chilling stress. NO acts as a signaling molecule with multiple biological functions in plants (Neill et al., 2003). Exogenous NO has also been found to be involved in responses to chilling stresses by activating antioxidant enzymes and by reducing ROS accumulation (Xu et al., 2010; Esim and Atici, 2014; Esim et al., 2014). SA is another vital signal molecule for chilling tolerance in several plant species including maize. It plays an important role in a complex signaling pathway induced by many stresses. Exogenous SA increased tolerance to chilling in maize and barley plants by activating antioxidant enzymes such as SOD, POX, and CAT (Janda et al., 1999; Mutlu et al., 2013).

In plants, nitrate reductase (NR) like NO synthase can intervene in NO production from nitrite in an NAD(P)H dependent manner (Rockel et al., 2002), as NR catalyzes the reduction of nitrate by causing the generation of nitrite during nitrate assimilation. The involvement of NR-caused NO production in physiological processes in plants has been substantiated by using *Arabidopsis* mutants defective for NR activity (Desikan et al., 2002; Bright et al., 2006). Therefore, Zhao et al. (2009) showed that NR-dependent NO production is involved in cold tolerance in *Arabidopsis*. NR activity is also affected by chilling and exogenous SA treatment (Aydın and Nalbantoğlu, 2011).

Maize is the largest grown (785 million tons) cereal in the world with double the grain yield per unit area compared to wheat and barley. In Turkey, maize is produced on approximately 550 thousand hectares with annual production of 3.5 million tons. Maize growing areas in Europe, the United States, Russia, central Asian states, and the Indian subcontinent are confronted by the problem of chilling stress; nevertheless, the severity, duration, nature, and timing vary with regions (Farooq et al., 2009).

In light of the above assessments, we hypothesize that evaluating the relationships between different signal molecules that have roles in stress response and the antioxidant system during chilling stress in plants will be vitally important to advance our knowledge about the roles of endogenous signal molecules during chilling stress. Thus, this study investigated the correlation between endogenous levels of some signal compounds (NO, SA, and ABA), levels of antioxidant system parameters (activities of SOD, POX and CAT) together with ROS (H_2O_2, $O_2^{\cdot-}$) and MDA contents, and NR enzyme activity in response to chilling stress in maize (*Zea mays* L.) seedlings.

2. Materials and methods

2.1. Plant material and chilling treatment

Maize (*Zea mays* cv. Arifiye) seeds were used for the experiment. Before sowing, the seeds were surface sterilized for 10 min with 10:1 water/bleach (commercial NaOCl) solution. Then they were washed six times with distilled water. Later the seeds were planted in sand in 15-cm pots. They were maintained in a growth chamber under controlled environmental conditions (i.e. 25/22 °C day/night temperature, 50% relative humidity, and a photon flux density of 400 µmol/ms photosynthetic active radiation with a 16-h photoperiod) for 12 and 19 days separately. The plants in each group (i.e. 12- and 19-day groups) were transferred to chilling conditions (i.e. 10/7 °C day/night) for an additional 2 days. Control plants were allowed to grow under normal conditions for the same period. The plants' leaves in each group were harvested on day 14 and day 21 to evaluate the endogenous levels of NO, ABA, SA, H_2O_2, $O_2^{\cdot-}$, and MDA, and the activities of CAT, SOD, POX, and NR. The harvested samples were quickly frozen in liquid nitrogen (N_2) and stored at –80 °C until use.

2.2. Extraction, purification, and determination of ABA and SA

The extraction and purification of ABA and SA were conducted according to the method described by Zhang et al. (2008) with a few modifications. The frozen samples (1 g) were powdered in liquid N_2, and 10 mL of cold methanol containing 1 mM butylatedhydroxytoluene as an antioxidant was added to the fine powder and stored at 4 °C for 24 h in the dark. The powder was filtered through Whatman No. 1 filter paper. The filtrates were pooled after the residue was re-extracted once more in the same way. The filtrates were filtered through 0.45-µm

poly-tetrafluoroethylene (PTFE, Sartorius) filters. After evaporating off the methanol at 35 °C under reduced pressure with a rotary evaporator, the extract was re-dissolved in 0.1 M KH_2PO_4 buffer (pH 8.0) and centrifuged at 10,000 rpm for 30 min at 4 °C. About 10 mL of the supernatant was transferred into a 25-cm^3 flask containing 1 g of polyvinylpolypyrrolidone (PVPP), and then mixed well and filtered through Whatman No. 1 filter paper. The filtrates were loaded into Sep-Pak C18 cartridges (Waters, Hichrom Ltd., UK) after being activated with 2 mL of 100% methanol, followed by 2 mL of distilled water. Cartridge-absorbing hormones were loaded with 20 mL of ddH_2O and then they were eluted with 3 mL of cold 80% ethanol. After solvent evaporation, the dry residue was dissolved in 0.5 mL of 20% acetonitrile. The samples were filtered through a 0.45 Millipore filter and were injected into HPLC to detect ABA and SA.

2.3. Determination of endogenous NO content

NO content was determined using the method described by Hu et al. (2003) with slight modifications. Leaves (0.5 g) were ground using a mortar and pestle in 3 mL of 50 mM cool acetate buffer (pH 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected. The pellet was washed with 1 mL of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortexing and filtration, the filtrate was leached and collected. The mixture of 1 mL of filtrate and 1 mL of the Greiss reagent was incubated at room temperature for 30 min. Absorbance was determined at 540 nm. NO content was calculated by comparison to a standard curve of $NaNO_2$.

2.4. Determination of $O_2^{\cdot-}$ generation and H_2O_2 and MDA contents

The content of H_2O_2 was determined by monitoring the absorbance of titanium-peroxide complex at 415 nm according to the method described by He et al. (2005) with some modifications. Leaves (1 g) were ground with liquid nitrogen and the fine powdered materials were mixed with 6 mL of cooled acetone. The homogenate was centrifuged at 5000 rpm for 10 min at 4 °C. The extracted solution (1 mL) was mixed with 0.1 mL of 5% $Ti(SO_4)_2$ and 0.2 mL of concentrated NH_4OH solution. The titanium peroxide complex precipitated and this sediment was dissolved in 4 mL of 2 M H_2SO_4 after centrifugation at 3000 rpm for 10 min. The absorbance was read at 415 nm and the H_2O_2 content was calculated by comparing with a standard drawn with known H_2O_2 concentrations.

Superoxide anion ($O_2^{\cdot-}$) content was measured as described by Elstner and Heupel (1976). A 0.5-g aliquot of leaves was ground and extracted in 2 mL of 65 mM phosphate buffer (pH 7.8). The homogenate was centrifuged at 5000 rpm for 10 min at 4 °C. A 1-mL aliquot of the supernatant was mixed with 0.9 mL of 65 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine hydrochloride, and then the mixture was incubated at 25 °C for 20 min. Then 1 mL of the mixture, 1 mL of 17 mM p-aminobenzenesulfonic acid anhydrous, and 1 mL of 17 mM 1-naphthylamine were mixed and the mixture was incubated at 25 °C for 20 min. The absorbance was monitored at 530 nm after 3 mL of n-butyl alcohol was added to the mixture.

The level of lipid peroxidation was measured using Heath and Packer's (1968) method with slight modifications (Ananieva et al., 2002). Seedling material weighing 0.5 g was homogenized in 3 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 15,000 rpm for 30 min at 4 °C. One milliliter of reagent (0.5% thiobarbituric acid (TBA) in 20% TCA, w/v) was added to a 0.5-mL aliquot of the supernatant. For a negative control, 0.5 mL of 0.1% TCA and 1 mL of reagent were added. The test tubes were heated at 95 °C for 30 min and then were quickly cooled in an ice bath. After cooling and centrifugation to give a clear supernatant, the absorbance of the supernatant at 532 nm was read and the value for the nonspecific absorption at 600 nm was subtracted. The level of malondialdehyde (MDA) was estimated by using an extinction coefficient of 155 mmol L^{-1} FW.

2.5. Determination of antioxidant enzyme activities

Fresh leaves (0.5 g) were homogenized with a mortar and pestle in ice-cold 0.2 M phosphate buffer (pH 7) including 0.1 mM ethylene dinitrilotetra acetic acid (EDTA). The homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was used to determine SOD, CAT, and POX activities. SOD activity was estimated by recording the decrease in absorbance of nitro-blue tetrazolium (NBT) dye by the enzyme (Dhindsa et al., 1981). The reaction mixture contained 2 µM riboflavin, 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate, and 0.1 mL of the extraction. POX activity was measured by monitoring the increase in absorbance at 470 nm in 50 mM phosphate buffer (pH 5.5) containing 1 mM guaiacol and 0.5 mM H_2O_2. One unit of POX activity was defined as the amount of enzyme that caused an increase in absorbance of 0.01 per min (Yee et al., 2002). CAT activity was measured by monitoring the decrease in absorbance at 240 nm in 50 mM phosphate buffer (pH 7.5) containing 20 mM H_2O_2. One unit of CAT activity was defined as the amount of enzyme that used 1 µmol H_2O_2 per min (Gong et al., 2001).

2.6. Determination of nitrate reductase activity

The frozen maize leaves were ground with liquid nitrogen in a chilled mortar. Then extraction buffer (100 mM Tris-HCl pH 7.5, 10 mM cysteine, 1 mM EDTA, and 5 mM FAD) was added to spinach powder (4 mL g^{-1} fresh leaf weight). The homogenate was centrifuged at 15,000 rpm for 25 min

and the resulting supernatant was used for determination of NR activity. The whole extraction procedure was carried out at 4 °C. Nitrate reductase activity was estimated by measuring nitrite formed in an assay system containing 100 mM phosphate buffer (pH 7.5), 1 mM EDTA, 10 mM KNO3, 0.3 mM NADH, and the enzyme preparation (0.2 mL), in a final volume of 1 mL of 1 M barium acetate and 1.9 mL of 96% (v/v) ethanol. After vigorous shaking in a mixer, the mixture was left to stand for 5 min at 4 °C and centrifuged at 2300 rpm for 5 min. Then nitrite was measured on an aliquot from the clear supernatant solution by addition of 1 mL of sulfanilamide (1%, w/v, in 2 M HCl) followed by 1 mL of 0.02 (w/v) N-(1-naphthyl) ethylene-diamine. After 15 min, the absorbance of the sample was read at 540 nm (Barro et al., 1994). The determined values are the means of 4 independent experiments. NR activity (EU g^{-1} leaf) was calculated as NO_2 **µ**g g^{-1} fresh weight of the leaf (Aydın and Nalbantoğlu, 2011).

2.7. Statistical analysis

All experiments were performed three times. Data were analyzed by analysis of variance (ANOVA) and means were compared by Duncan's multiple range test at $P \leq 0.05$.

3. Results and discussion

We investigated interactions between the endogenous levels of signal compounds (NO, SA, and ABA) important for chilling stress response and the contents of antioxidant system parameters (the activities of antioxidant enzymes and ROS level) in response to chilling stress in maize (*Zea mays* L.) seedlings exposed to chilling at different times. Our findings indicated that chilling stress elevated the contents of $O_2^{\cdot-}$, H_2O_2, and MDA in both 14-day-old and 21-day-old maize seedlings exposed to chilling for 2 days (Table 1). To sum up, chilling treatment significantly ($P<0.05$) increased superoxide anion ($O_2^{\cdot-}$) generation by 16% in 14-day-old and by 20% in 21-day-old maize seedlings (Table 1). The highest $O_2^{\cdot-}$ generation rate was 6.63 ng g^{-1} in 14-day-old maize leaves under chilling stress. H_2O_2 contents significantly ($P < 0.05$) increased by 35% in 14-day-old seedlings and by 86% in 21-day-old seedlings. The highest H_2O_2 content was 101 ng g^{-1} in 21-day-old maize leaves under chilling stress. In addition, MDA, an indicator of lipid peroxidation level, increased ($P < 0.05$) by 70% and 36% in 14-day-old and 21-day-old maize, respectively. The highest MDA content was 2.83 nmol g^{-1} in 21-day-old maize leaves under chilling stress. It can be concluded that chilling stress stimulates the level of ROS in both early and late seedlings of maize exposed to chilling. These results were in good agreement with those of previous studies (Kang et al., 2003; Liu et al., 2010; Mutlu et al., 2013; Esim and Atici, 2014; Esim et al., 2014), which showed that chilling or cold stress could cause oxidative damage by increasing ROS production in plants.

Scavenging of the ROS by the antioxidant defense system against oxidative injury is crucial to cell survival under chilling stress as a major mechanism of resistance (Cao et al., 2009). Antioxidant enzymes such as SOD, POX, and CAT, as a significant component of the antioxidant defense system, can play primary roles in detoxifying of ROS. In this way, SOD detoxifies $O_2^{\cdot-}$ into H_2O_2 in different cellular compartments and then CAT and POX detoxify H_2O_2 to water (Noctor and Foyer, 1998). It was shown that the antioxidant enzyme activities were generally stimulated by chilling conditions in plants (Kang et al., 2003; Wang et al., 2009; Liu et al., 2010; Mutlu et al., 2013; Esim and Atici, 2014; Esim et al., 2014). In the present study, activities of SOD, POX, and CAT were provoked by chilling treatment as compared with the control plants (Table 2). In 14-day-old and 21-day-old chilling treated seedlings, for instance, CAT activity was 36% and 27%, and SOD activity was 12% and 44% higher than that of the control plants, respectively. Chilling treatment significantly ($P < 0.05$) increased POX activity, by 30%, in 21-day-old seedlings but did not in 14-day-old seedlings. When the data are compared with the ROS contents and MDA levels, it can be suggested that chilling stress (despite being applied at different times) increases the ROS content and MDA. In response to this, the plant also induces the activities of SOD, CAT, and

Table 1. The effect of chilling (10/7 °C) on oxidative stress parameters in maize seedlings.

Plants		H_2O_2 concentration (ng g^{-1} FW)	$O_2^{\cdot-}$ concentration (ng g^{-1} FW)	MDA content (nmol g^{-1} FW)
14-Day	Control	46.4 ± 1.78 b	5.71 ± 0.062 b	1.5 ± 1.09 b
	Chilling	62.8 ± 1.67 a	6.63 ± 0.043 a	2.55 ± 0.98 a
21-Day	Control	54.2 ± 1.99 b	4.78 ± 0.086 b	2.08 ± 0.77 b
	Chilling	101 ± 2.54 a	5.74 ± 0.094 a	2.83 ± 1.03 a

Means within each plant group followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test. ± represents standard error (SE) of the mean (n = 3)

Table 2. The effect of chilling (10/7 °C) on antioxidant enzymes activities of maize seedlings.

Plants		SOD (U g^{-1} FW)	POX (U g^{-1} FW)	CAT (U g^{-1} FW)
14-Day	Control	100 ± 0.13 b	31,200 ± 1530 b	243 ± 3.8 b
	Chilling	112 ± 0.56 a	23,450 ± 1380 a	330 ± 6.5 a
21-Day	Control	268 ± 0.65 b	57,650 ± 1290 b	172 ± 5.2 b
	Chilling	385 ± 0.3 a	74,800 ± 1300 a	218 ± 3.3 a

Means within each plant group followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test. ± represents standard error (SE) of the mean (n = 3)

POX that scavenge the ROS, resulting in protection of cell membranes from increased MDA to accommodate chilling stress. Similar results can be found in the literature. Some previous researchers reported that chilling stress increased ROS and MDA contents in plants while the activity of antioxidant enzymes was enhanced under chilling stress (Janda et al., 1999; Ananieva et al., 2002; Kang et al., 2003; Wang et al., 2009; Mutlu et al., 2013; Esim and Atici, 2014; Esim et al., 2014). However, pretreatment with exogenous NO (Esim and Atici, 2014; Esim et al., 2014), ABA (Aroca et al., 2003), and SA (Janda et al., 1999; Ananieva et al., 2002; Kang et al., 2003; Wang et al., 2009; Mutlu et al., 2013) increased the activities of SOD, CAT, and POX in chilling or cold-stressed plants, accompanied by reductions in levels of ROS and MDA. In all plants exposed to stress, it is well known that plant growth regulators are involved in stress response (Wendehenne et al., 2004; Tossi et al., 2009; Liu et al., 2010; Cantrel et al., 2011). The searches for signal molecules mediating stress tolerance are an important step in our better understanding of how plants acclimate to adverse environments.

We also evaluated the involvement of plant growth regulators (NO, SA, ABA) together with the antioxidant system in maize under chilling conditions since production of the regulators was an early response of plants to abiotic stresses (Wendehenne et al., 2004; Tossi et al., 2009). Considerable findings were determined for endogenous contents of the plant regulators in chilling-treated/untreated maize seedlings (Table 3). The endogenous levels of NO and ABA showed a remarkable increase during chilling treatment, compared to the control group. The levels of signal molecules were increased by 10% and 46% for NO and by 47% and 30% for ABA in 14- and 21-day-old seedlings after chilling treatment, respectively (Table 3). Chilling exposure has been reported to induce an increase in endogenous NO (Zhao et al., 2009; Liu et al., 2010; Cantrel et al., 2011) and ABA (Aroca et al., 2003; Liu et al., 2010) production in plants. Our data are in good agreement with those of previous studies. ABA content was also higher in chilled maize cultivars (Javier et al., 1997). Although chilling treatment caused an increase in SA content in early young seedlings (14-day-old), it did not affect that of late seedlings (21-day-old). There are also other studies that support our results. For instance, exogenous treatments of NO (Liu et al., 2010; Esim and Atici, 2014; Esim et al., 2014), ABA (Janowiak et al., 2002; Aroca et al., 2003; Kumar et al., 2008), and SA (Janda et al., 1999; Wang et al., 2009; Mutlu et al., 2013) could counteract oxidative damage by stimulating the antioxidant system under chilling conditions. We can conclude that there is a correlation between the triggering of the antioxidant system and the endogenous level of signal molecules (NO, SA, and ABA) in maize under chilling stress, and the plant growth regulators may be involved in the response to chilling

Table 3. The effect of chilling (10/7 °C) on endogenous ABA, NO, and SA in maize seedlings.

Plants		ABA (ng g^{-1} FW)	NO (ng g^{-1} FW)	SA (ng g^{-1} FW)
14-Day	Control	234.05 ± 3.9 b	1.72 ± 0.4 b	85.27 ± 2.3 b
	Chilling	345.65 ± 4.2 a	1.89 ± 0.6 a	102.15 ± 3.1 a
21-Day	Control	165.51 ± 3.1 b	1.82 ± 0.5 b	87.44 ± 0.8 b
	Chilling	215.16 ± 2.8 a	2.67 ± 1.1 a	90.5 ± 0.3 b

Means within each plant group followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test. ± represents standard error (SE) of the mean (n = 3)

stress to improve the antioxidant response mechanism. NO is involved in plant hormone signaling pathways (Neill et al., 2003). ABA induces significant NO production in guard cells. Nitrate reductase (NR) is required for ABA-induced NO generation (Desikan et al., 2002). Therefore, endogenous NO and ABA contents depend on NR activity in plants under stress. In the present study, it was seen that activities of NR of 14-day-old and 21-day-old maize were increased by chilling treatments compared with those of plants grown under control conditions (25/22 °C). As shown in the Figure, chilling treatment significantly increased NR activity by 36% and 32% in 14-day-old and 21-day-old maize, respectively. In previous studies, it was reported that NR activity was increased by cold in black alder leaves and roots, *Pinus sylvestris* needles, winter wheat, and spinach leaves (Pietilainen et al., 1991; Yaneva et al., 2002; Aydın and Nalbantoğlu, 2011). A few studies show evidence related to NR activity under cold stress. A study by Yaneva et al. (2002) demonstrated that short-term low temperature causes a rapid activation of NR in winter wheat leaves resulting from NR protein dephosphorylation by protein phosphatase. The results strongly suggest that structural modification(s) for cold adaptation affects thermodynamic properties of each of the functional domains within the NR holoenzyme (Aydın and Nalbantoğlu, 2011). There are also other studies that have investigated the effect of SA in the short term on NR activity in maize leaves and roots, black gram, mustard, and spinach (Aydın and Nalbantoğlu, 2011).

The correlation coefficients were calculated for the antioxidant enzymes (SOD, POX, CAT) with signal molecules (ABA, NO, SA) (Table 4). Antioxidant enzymes had a statistically significant positive correlation with signal molecules. SOD and CAT had a significant positive correlation with signal molecules both 14- and 21-day-old maize, but had no relation with SA in 21-day-old maize. Similarly, POX had a statistically significant positive correlation with signal molecules in 21-day-old maize, but had an inverse correlation with the signal molecules in 14-day-old maize (Table 4). These results suggest that antioxidant enzymes and signal molecules have a significant correlation in maize exposed to chilling stress.

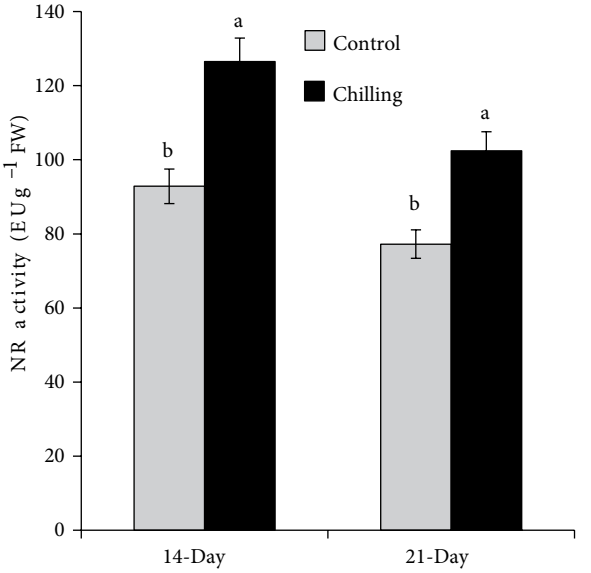

Figure. The effect of chilling (10/7 °C) on nitrate reductase activity in maize seedlings.

In conclusion, the results demonstrated that chilling stress induces a marked increase in endogenous NO, ABA, and SA levels in maize leaves. Chilling treatment also

Table 4. Correlation analysis between antioxidant enzymes and signal molecules.

Antioxidant enzymes	Signal molecules	14-day (r)		21-day (r)	
		Control	Chilling	Control	Chilling
SOD	ABA	0.92	0.75	0.87	0.89
	NO	0.93	0.69	0.94	0.91
	SA	0.84	0.61	0.75	0
POX	ABA	0.92	−0.56	0.81	0.92
	NO	0.90	−0.39	0.89	0.91
	SA	0.84	−0.51	0.76	0
CAT	ABA	0.92	0.78	0.79	0.86
	NO	0.89	0.81	0.83	0.79
	SA	0.84	0.84	0.85	0

r: correlation coefficient

increases the activities of antioxidant enzymes, which then leads to enhanced ROS production and lipid peroxidation. The results indicate that antioxidant enzyme activities are essential for removing ROS and reducing lipid peroxidation, thus conferring tolerance of the seedling to chilling stress. These findings highlight the involvement of endogenous NO, ABA, and SA in cold tolerance in maize during stress, and may provide a new strategy to enhance chilling tolerance in maize by controlling endogenous or exogenous application of signal molecules generation.

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