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Nitric oxide alleviates boron toxicity by reducing oxidative damage and growth inhibition in maize seedlings (*Zea mays* L.)

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Abstract

Nitric Oxide (NO) is an important signal molecule modulating the plants responses to abiotic stresses. In this study the effects of exogenous NO as sodium nitroprusside (SNP) on boron (B)-induced oxidative damage and growth in maize (*Zea mays* L.) were investigated. The addition of B significantly reduced the growth of plants and increased the values of electrolyte leakage, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents. SNP (100 µM) that was applied to seeds before germination significantly increased plant height (respectively, 8 and 5%), fresh weight (respectively, 9 and 6%), and dry weight (respectively, 15 and 12%) of both 11 and 15 day old maize. Furthermore, the measured B-induced oxidative stress increased MDA, electrolyte leakage, H_2O_2 content when compared to a supplementation of NO. SNP application also increased activities of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX). The results suggest that the increased NO, resulting from SNP application, improved the antioxidant capacity of maize plants against B-induced oxidative stress.

Keywords: boron; toxicity; nitric oxide; oxidative stress; antioxidant enzymes; maize.
Abbreviations: B: Boron, NO: Nitric oxide, SNP: Sodium nitroprusside, FW: Fresh weight, DW: Dry weight, PH: Plant height, MDA: Malondialdehyde, H_2O_2: Hydrogen peroxide, SOD: Superoxide dismutase, POX: Peroxidase, CAT: Catalase.

Introduction

Boron(B) is an essential element required for the normal growth of higher plants and in the amount of B the threshold between deficiency and toxicity is narrow (Yau and Ryan, 2008; Herrera-Rodriguez et al., 2010; Siddiqui et al., 2012).The requirement for B varies among plants species and among genotypes of the same species. For example, sensitive plants (that is, avocado, apple and bean) can be safely grown at concentrations of 0.3 mg B L^{-1}, semi-tolerant plants (that is, oat, maize and potato) at 1–2 mg B L^{-1}, and tolerant plants (that is, carrot, alfalfa and sugar beet) at 2–4 mg B L^{-1} (Keren and Bingham, 1985). Soils show important variations in B content. Some soils contain insufficient B as boric acid to support normal plant growth, while containing excess of B, which causes toxicity in some plants (Esim et al., 2012). Worldwide, crop production is limited because B in the soil is either insufficient or at toxic levels (Rerkasem et al., 2003).

B toxicity is an especially serious threat to agriculture in arid and semiarid regions. B is frequently associated with saline soils and inland desert areas (Gupta, 1979). The main sources of elevated B are surface mining, fly ash, and industrial chemicals (Nable et al., 1997). B toxicity causes some physiological and morphological defects such as decreased shoot and root growth (Lovatt and Bates, 1984; Nable et al., 1997), inhibition of photosynthesis, lower stomatal conductance (Lovatt and Bates, 1984), decreased proton extrusion from roots (Roldan et al., 1992), decreased root cell division (Liu et al., 2000; Choi et al., 2007), deposition of lignin and suberin in roots (Ghanati et al., 2002), increased membrane permeability, peroxidation of lipids and changed activities of antioxidant enzymes (Karabal et al., 2003; Ardic et al., 2009a, b; Herrera-Rodriguez et al., 2010;Esim et al., 2012).

Similar to other abiotic stress (that is, salinity, heavy metals, drought, cold, heat), excess B also causes oxidative damage induced by the formation of Reactive Oxygen Species (ROS) such as superoxide ($O^{\cdot-}$), hydroxyl ($OH^{\cdot-}$) radicals and hydrogen peroxide (H_2O_2), which are strong oxidizers of lipids, proteins, and nucleic acids (Cervilla et al., 2007; Ardic et al., 2009a), and destabiliser of cellular homeostasis (Tombuloglu et al., 2012). Cells are able to protect themselves using enzymatic mechanisms against increasing ROS. Enzymatic ROS scavenging mechanisms in plants include superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT) (Apel and Hirt, 2004). Most studies have concluded that SOD decomposes $O^{\cdot-}$ to O_2 and H_2O_2, which are further oxidized to molecular oxygen and H_2O by the enzymes of CAT and POX (Mathews et al., 1984; Karabal et al., 2003; Molassiotis et al., 2006). In spite of the obvious importance of B, the mechanisms of B tolerance and toxicity in plants are poorly understood (Reid et al., 2004; Cervilla et al., 2007; Fitzpatrick and Reid, 2009; Herrera-Rodriguez et al., 2010;Esim et al., 2012). The relationships between nutrient and plant signal molecules are very important for plant growth and development under both normal and stress conditions (Herrera-Rodriguez et al., 2010).

Nitric Oxide (NO) is a signal molecule involved in many physiological functions under normal and stress conditions in plant cells (Arasimowicza and Floryszak-Wieczorek, 2007; Corpas et al., 2008; Neill et al., 2008). NO was shown to participate in a wide spectrum of these physiological functions, including germination, root growth, gravitropic

Table 1. The different treatments conducted in this study.

Plants	Treatment	Interpretation
11-days	CK	Control
	SNP	Seeds were imbibed with 100 µM SNP during 24-h before germination
	B	Seedlings were treated with 2 day of 2 mM boron
	SNP + B	Seedlings were treated with 2 day of 2 mM boron after germination of seeds were imbibed with 100 µM SNP
15-days	CK	Control
	SNP	Seeds were imbibed with 100 µM SNP during 24-h before germination
	B	Seedlings were treated with 6 day of 2 mM boron
	SNP + B	Seedlings were treated with 2 day of 2 mM boron after germination of seeds were imbibed with 100 µM SNP

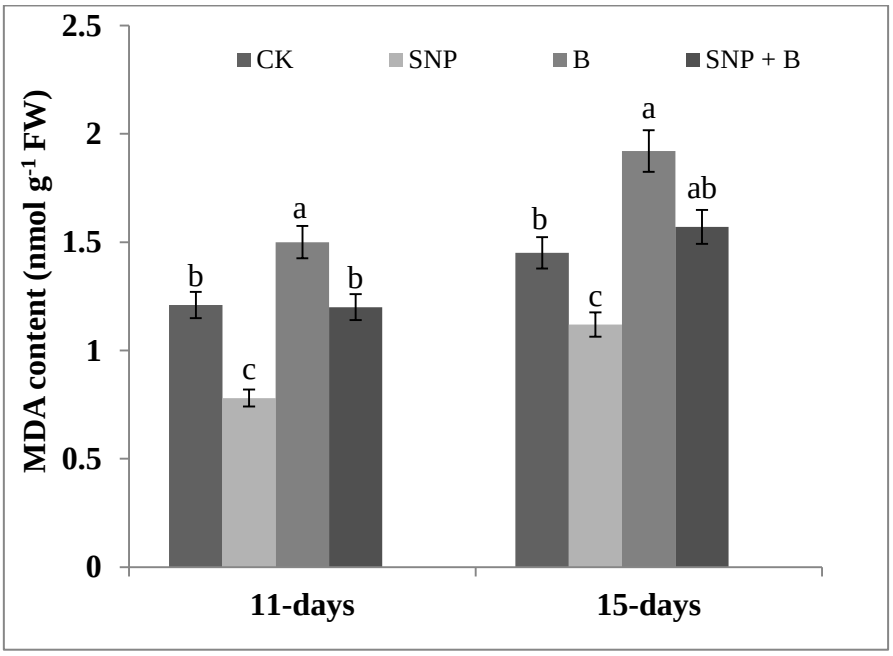

Fig 1. Content of MDA in maize seedling after SNP and B treatments. CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars indicate Standard error of the mean (n = 3).

bending, control of the timing of flowering, stomatal closure, and growth regulation of pollen tubes (Wilson et al., 2008; Besson-Bard et al., 2009). Furthermore, the effect of NO has been observed in the plants as an adaptive response to biotic and abiotic stresses, notably by acting as a signalling molecule (Delledonne, 2005). Tolerance to drought, salt, and heat stress was enhanced in wheat (*Triticum aestivum*) and rice (*Oryza sativa*) seedlings when the plants were treated with the NO donor, sodium nitroprusside (SNP) (Garcia-Mata and Lamattina, 2001; Uchida et al., 2002). It has also been indicated that NO protects plant cells against oxidative stress by reducing ROS accumulation (Wink and Mitchell, 1998; Xu et al., 2010). However, NO improved the tolerance of plants against oxidative stress caused by metals such as copper (Wang et al., 2010), cadmium (Laspina et al., 2005), aluminium (Wang et al., 2011), and arsenic (Singh et al., 2009), although the effect of NO on B toxicity in plants has not been fully understood. This is the first study elucidating the effects of NO on resistance against B-induced oxidative damage and growth inhibition of plants. In a previous study, we have reported that a high level of B causes the oxidative damage and growth inhibition in maize (Esim et al., 2012).

Maize is the largest grown (785 million tons) cereal in the world with doubled 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. However, the B toxicity effect on maize is far from clear. The aim of this study is to evaluate the interactive effect of high level of B and exogenous NO, ascertain the role of NO in the resistance against B toxicity, and make clear the signal cross-talk between B and NO at the physiological level using maize seedlings.

Results

Plant growth characteristics

The present experiment demonstrated that Plant Height (PH), Fresh Weight (FW), and Dry Weight (DW) were reduced in B-treated plants compared to the control plants. Rates on PH, FW, and DW in control plants were recorded to be 39.24, 79.58, 16.11 cm for 11-days and 54.87, 109.54, 22.26 cm for 15-days, respectively. However, rates of PH, FW, and DW when compared to control plants in pre-treatment SNP reached to the maximum after 11 and 15- old-days, while those rates in 2 mMB decreased by 12%, 15%, 11% for 2

Table 2. Effects of SNP and B on the growth performance of maize seedlings (*Zea mays* L.). CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Different letters within columns indicate significant differences ($p < 0.05$), according to Duncan's multiple range test. Error bars indicate standard error of the mean (n = 3).

Plants	Treatments	Plant height (cm)	Plant fresh weight (g)	Plant dry weight (g)
11- days	CK	39.24 ± 1.3 b	79.58± 4.1 b	16.11 ± 2.3 b
	SNP	42.3 ± 1.5 a	86.36± 5.3 a	18.45 ± 2.1 a
	B	34.53 ± 1.9 c	67.21± 3.7 d	14.21 ± 1.8 d
	SNP + B	37.67±1.1 bc	74.53± 3.9 c	15.98 ± 1.4 c
15-days	CK	54.87 ± 2.3 b	109.54± 5.9 b	22.6 ± 2.9 b
	SNP	57.6 ± 2.1 a	116.12± 5.7 a	24.68 ± 2.4 a
	B	45.23± 3.1 d	96.34± 4.6 d	18.73 ± 2.1 c
	SNP + B	50.61 ± 2.4 c	103.45± 5.4 c	21.56 ± 2.3 b

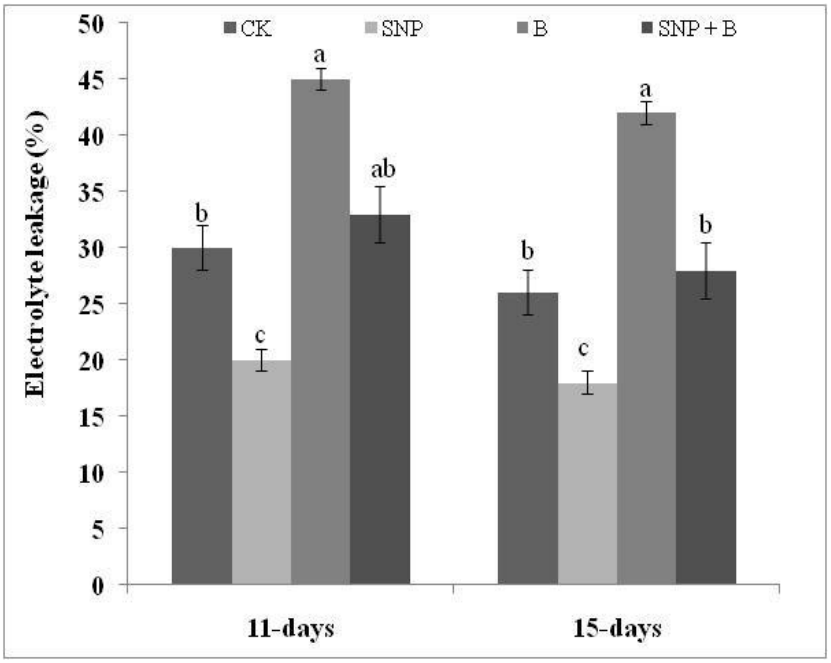

Fig 2. Rate of electrolyte leakage in maize seedling after SNP and B treatments. CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars indicate Standard error of the mean (n = 3).

days, and 17%, 12%, 17% for 6 days, respectively (Table 1).The application of SNP + B significantly ameliorated PH, FW, and DW when compared to the application of B alone. For example, when compared to the 2mM B-treated alone, SNP + B resulted in the increment of 12%, 11%, and 12% in PH, FW, and DW after applying for 2 days, and 11%, 10%, and 15% in those after applying for 6 days, respectively.

Electrolyte leakage, malondialdehyde, and hydrogen peroxide

The level of lipid peroxidation is expressed by the malondialdehyde (MDA) concentration in seedling. The B-treated plants exhibited a higher rate of electrolyte leakage, lipid peroxidation and hydrogen peroxide (H_2O_2) in the maize, indicating significant oxidative stress. At B-treated plants, the levels of electrolyte leakage, lipid peroxidation, and H_2O_2 in plants were higher than the control, SNP and SNP + B-treated plants (Fig. 1-3). Furthermore, SNP treatment lowered the membrane destruction caused by B stresses in plants.The electrolyte leakage was recorded to be 30% and 26% for the control plants, 20% and 18% for SNP-treated plants, 45% and 42% for B-treated plants, and 33 % and 28 % for SNP + B treated plants at the end of 11 and 15 days, respectively (Fig. 1). The MDA contents were 39.3 and 44.3 nmol g^{-1} for the control plants, 0.78 and 1.12 nmol g^{-1} for SNP-treated plants, 1.5 and 1.92 nmol g^{-1} for B- treated plants and 1.2 and 1.57 nmol g^{-1} for SNP+B treated plants at the end of 11 and 15 days, respectively (Fig. 2). The H_2O_2 contents were 39.3 and 44.3 ng g^{-1} for the control plants, 38.1 and 44.6 ng g^{-1} for SNP-treated plants, 45.2 and 50.4 ng g^{-1} for B-treated plants, 40.8 and 47.2 ng g^{-1} for SNP + B treated plants at the end of 11 and 15 days, respectively (Fig. 3).

Antioxidant enzyme activities

The application of SNP and B significantly influenced the activities of antioxidant enzymes (Fig. 4-6). The applicationof SNP and B, alone and in combination significantly relatively increased the activities of SOD, CAT, and POX when compared to the control (Fig. 4-6). The activities of SOD, CAT and POX were significantly higher than any other treatment incombination SNP + B. The SOD activity was recorded to be 63.23 and 59.4 EU g^{-1} for the control plants, 86.4 and 93.6 EU g^{-1} for SNP-treated plants, 78.6 and 87.9 EU g^{-1} for B-treated plants, 89.3 and 104.5 EU g^{-1} for SNP + B treated plants at the end of 11 and 15 days, respectively (Fig. 4). The CAT activity was recorded to be

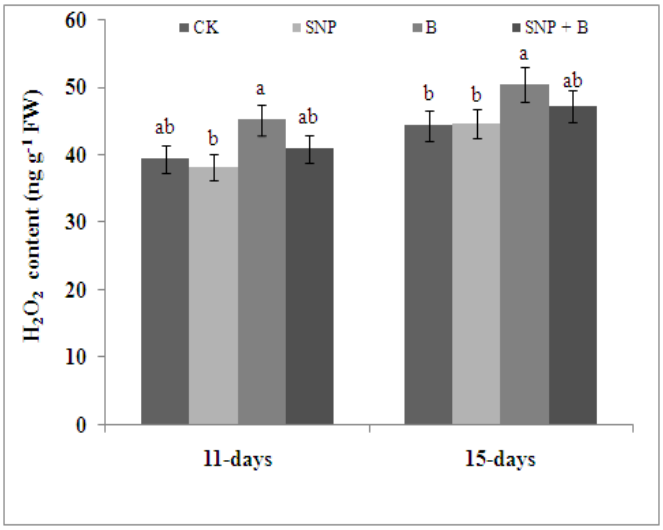

Fig 3. Content of H_2O_2 in maize seedling after SNP and B treatments. CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars indicate Standard error of the mean (n = 3).

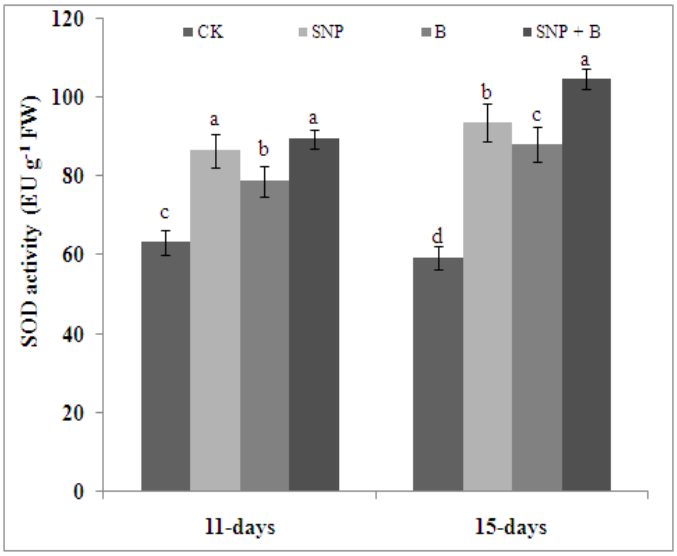

Fig 4. Activity of SOD in maize seedling after SNP and B treatments. CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Bars showing the same letter are not significantly different at $p \leq 0.05$ as determined by Duncan's multiple range test. Error bars indicate Standard error of the mean (n = 3).

51.7 and 61.4 EU g^{-1} for the control plants, 51.4 and 63.6 EU g^{-1} for SNP-treated plants, 57.6 and 97.9 EU g^{-1} for B-treated plants, 68.3 and 116.5 EU g^{-1} for SNP + B treated plantsat the end of 11 and 15 days, respectively (Fig. 5). The POX activity was recorded to be 8600 and 4320 EU g^{-1} for the control plants, 8780 and 4690 EU g^{-1} for SNP-treated plants, 8680 and4528 EU g^{-1} for B-treated plants, 8950 and 5140 EU g^{-1} for SNP + B treated plants at the end of 11 and 15 days, respectively (Fig. 6).

Discussion

Plants have a complex antioxidant system to avoid the harmful effects of Reactive Oxygen Species (ROS). It is well known that excess B causes oxidative stress in plants, as has already been reported by us and other groups (Liu et al., 2000; Cervilla et al., 2007; Ardic et al., 2009a,b; Herrera-Rodriguez et al., 2010; Siddiqui et al., 2012; Esim et al., 2012). However, there is still limited data about the precise mechanisms by which this metal produces oxidative damage. In this work we demonstrated that the toxic effect induced by B on maize seedling was counteracted by NO levels (as SNP), and this effect might be described as NO capability to scavenge ROS, therefore preventing the increase in electrolyte leakage, MDA and H_2O_2 content. This study also showed the effects of NO on growth parameters such as plant height, fresh weight and dry weight decreased by excess B. Excess B leads to a significant growth inhibition that was measured as plant height, fresh weight and dry weight, which were partially restored by NO pre-treatment before germination in maize (Table 1). A similar effect for NO was described by some researchers in plants under salt, heavy metals (such as copper, cadmium, and aluminium), heat stress (Uchida et al., 2002; Wang et al., 2010; Wang et al., 2011). High levels of B (2mM) suppressed plant growth, which might be due to its toxic effects on root cell division, cell wall expansion (Liu et al., 2000; Herrera-Rodriguez et al., 2010; Siddiqui et al., 2012; Esim et al., 2012). Metal toxicity can affect plasma membrane permeability and there is enough evidence to show that the NO donor protects plasma membrane integrity (Hsu and Kao, 2004; Wang et al., 2005; Hu et al., 2007). In plant responses to salt, and heat stresses, NO has also been implicated (Uchida et al., 2002; Zhao et al., 2004). Application of NO donor has also been reported to confer increased resistance of plants to metals such as cadmium, copper, aluminium (Laspina et al., 2005; Wang et al., 2005; Yu et al., 2005). In spite of all the effects of NO, there is no study on the toxic effects of B. In this point of view, NO-mediated amelioration of B toxicity in plants has been for the first time observed in this study. Lipid peroxidation, which was measured through malondialdehyde (MDA) as an indicator of B-induced ROS formation, is one of the first consequences of oxidative damage. B toxicity may have caused cellular dysfunction by increasing MDA, electrolyte leakage and H_2O_2 content (Fig. 1-3). These results corroborate previous findings of increases in MDA, H_2O_2 content and electrolyte leakage in response to B toxicity (Cervilla et al., 2007; Ardic et al., 2009a,b; Herrera-Rodriguez et al., 2010; Esim et al., 2012). Although MDA, H_2O_2, and electrolyte leakage were moderate under B stress, NO almost completely prevented this effect in the seedlings of NO + B treated plants, apparently acting as an efficient ROS scavenger and/or a membrane stabilizer. Some researchers reported that membrane damage might be caused by high H_2O_2 levels, which could trigger the Haber–Weiss reaction, resulting in hydroxyl radical and thus resulted in lipid peroxidation (Mittler, 2002; Aftab et al., 2012).In this study results indicate that exposure of plants to NO donor (SNP) suppressed the B-induced production of ROS and peroxidation of lipids. These results suggest that the NO-mediated antioxidant activity in plant species might be responsible for the promotion of plant growth under stress conditions. The reason for the favourable plant response to NO donor application, which causes the decrease in MDA level, could, presumably, be ascribed to the decrease in B uptake in plants (Han et al., 2009). Therefore, we postulate that the application of NO donor protected the plants from B

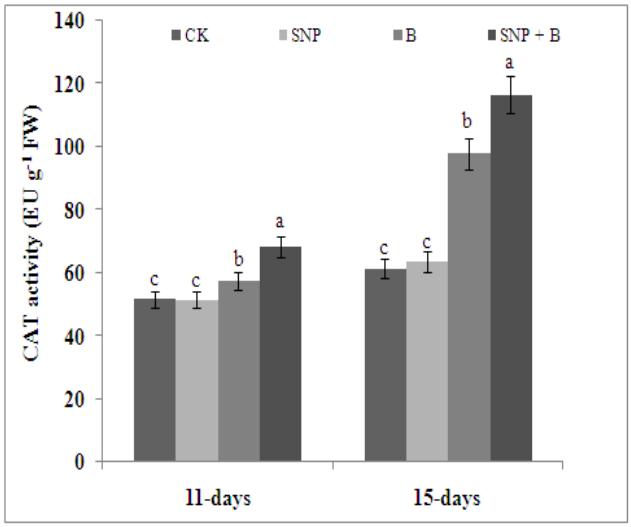

Fig 5. Activity of CAT in maize seedling after SNP and B treatments. CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Different letters indicate significant difference among treatments at the 0.05 significance level based on Duncan's multiple-range test. Error bars indicate Standard error of the mean (n = 3).

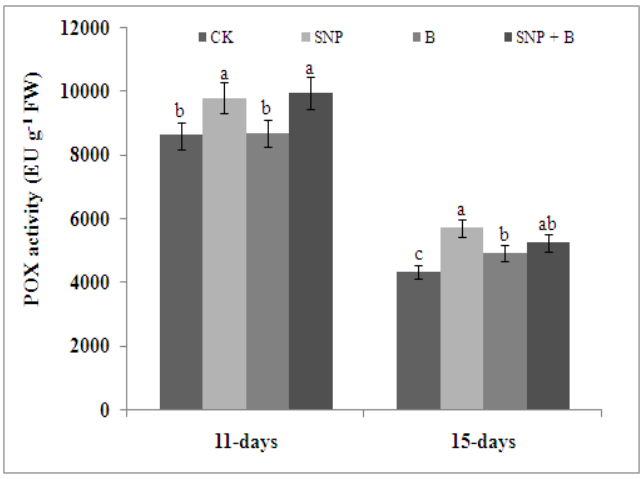

Fig 6. Activity of POX in maize seedling after SNP and B treatments. CK, the control; SNP, seeds were applied with 100 µM SNP; B, seedlings were applied with 2 mM B; SNP + B, seedlings were treated with 2 and/or day of 2 mM B after germination of seeds were imbibed with 100 µM SNP. Different letters indicate significant difference among treatments at the 0.05 significance level based on Duncan's multiple-range test. Error bars indicate Standard error of the mean (n = 3).

toxicity. Under normal conditions, the total amount of ROS formed in the plant is determined by the balance between the multiple ROS producing pathways and the ability of the enzymatic and non-enzymatic mechanisms dealing with them. Under stress, ROS formation is high and this could result in oxidative damage, depending on anti-oxidative enzyme activities (Laspina et al., 2005). Environmental stresses increase the formation of ROS that oxidize the membrane lipids, protein and nucleic acids (Aftab et al., 2012). As revealed in the present study, SOD, CAT, and POX activities increased significantly in B- treated plants with respect to the control plants (Fig. 4-6). Previous studies have demonstrated an increase in activities of anti-oxidative enzyme by high level of B (Cervilla et al., 2007; Han et al., 2009; Herrera-Rodriguez et al., 2010; Esim et al., 2012). Activities of antioxidant enzyme increased in B-treated plants, but they did not protect plants from B-induced oxidative damage.In the present study, although the antioxidant enzyme activities increase by application of B, this increase cannot decrease MDA and H_2O_2 contents which are the indicators of oxidative damage in B-treated plants. However, application of SNP before germination increased the antioxidant enzyme activities both alone and in combination with B (Fig. 4-6). Not only did pre-treatment of SNP increase the activities of anti-oxidative enzymes but only decreased the MDA and H_2O_2 contents augmented by excess B. Enzymes like CAT and POX appear to play an essential protective role in the scavenging process of ROS when coordinated with SOD (Aftab et al., 2012). SOD initiates detoxification of superoxide anion by forming H_2O_2, which is also toxic and is eliminated by conversion to H_2O in the subsequent reactions. In plants, a number of enzymes regulate intracellular H_2O_2 levels, but CAT and POX are considered the most important ones (Noctor and Foyer, 1998; Aftab et al., 2012). Superoxide radicals are toxic by-products of oxidative metabolism and can interact with H_2O_2 to form highly reactive hydroxyl radicals, which are thought to be primarily responsible for O_2 toxicity in the cell. The dismutation of superoxide radicals into H_2O_2 and oxygen, catalysed by SOD, is an important step in protecting the cell from superoxide radicals. In many studies, the alleviation of oxidative damage by NO was ascribed to the induction of activity of various ROS scavenging enzymes (Hsu and Kao, 2004; Hu et al., 2007; Han et al., 2009). In the present study, application of SNP also increased the activity of antioxidant enzymes when applied to stressed or non-stressed plants (Fig 4-6). One possibility is that NO might activate antioxidant systems to scavenge ROS. Alternatively, NO may directly abrogate superoxide anion ($O_2^{\cdot-}$) mediated cytotoxic effects through the conversion of $O_2^{\cdot-}$ into peroxynitrite ($ONOO^-$), thus conferring resistance to plants against B-induced oxidative stress (Neill et al., 2003). Such an effect might indicate the antioxidant property of NO for suppressing the high levels of ROS triggered by B- stress (Han et al., 2009).

Materials and methods

Plant cultures and chemicals treatments

Maize (*Zea mays* L. cultivar Arifiye-2) seeds were obtained from the Agricultural Research Institute in Sakarya (Turkey). Seeds were surface sterilized with 3% sodium hypochlorite solution for 10 minutes. They were washed and after that some were imbibed in distilled water and the others in 100µM sodium nitroprusside (SNP), a donor of Nitric Oxide (NO), for 1 day. Namely, SNP was applied to seeds before germination. After pre-imbibitions, approximately 10–12 seeds were placed to germinate in plastic pots (15cm) containing sand rinsed with distilled water after being washed with 1% hydrochloric acid. After the germination, seedlings were grown until ninth days in a growth chamber at 25±1°C with 16 hours light/8 hours dark photo cycle at a light intensity of 500 µmol m^{-2} s^{-1}. On the ninth day, Hoagland's modified nutrient solution containing 2 mM (200 mg Kg^{-1}) boric acid (H_3BO_3) was applied to the growth environment of the seedlings. Control plants were applied with Hoagland's solution including the normal value of B, and the B treated

and non-treated plants were grown in growth chamber under the same physical parameters for 2 and 6 days. The range of concentration of B (that is 2mM) selected for this study was based on our earlier experiments (Esim et al., 2012). In order to assess their growth characteristics Plant Height (PH), Fresh Weight (FW), and Dry Weight (DW), and physiological aspects [hydrogen peroxide (H_2O_2), malondialdehyde (MDA), electrolyte leakage, and activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD)], plants were sampled on the second and sixth days after application.

Plant growth characteristics

Plant Height (PH) was measured using a meter scale after removal of plants from the pots. After recording the Fresh Weight (FW) with a balance, plants were placed in a 60°C oven for 48 hours and then were weighed for Dry Weight (DW).

Determinations of malondialdehyde, electrolyte leakage and hydrogen peroxide content

Malondialdehyde (MDA) content was determined according to the method of Heath and Packer, (1968). Leaves were weighed and homogenates containing 10% trichloroacetic acid (TCA) and 0.65% 2-thiobarbituric acid (TBA) were heated at 95°C for 60 minutes and then cooled to room temperature and centrifuged at 10.000 x *g* for 10 minutes. The absorbance of the supernatant was read at 532 and 600nm against a reagent blank. MDA content was expressed as nmol g^{-1} FW. Electrolyte leakage was used to assess membrane permeability, as described by Lutts et al. (1995). Samples were washed three times with double distilled water to remove surface contamination. Leaf discs were cut from young leaves and placed in sealed vials containing 10mL of double distilled water and incubated on a rotatory shaker for 24 hours; subsequently, the electrical conductivity of the solution (EC_1) was determined. Samples were then autoclaved at 120°C for 20 minutes and the electrical conductivity was measured again (EC_2) after cooling the solution to room temperature. Electrolyte leakage (%) was calculated as (EC_1/EC_2) x 100 %. Levels of H_2O_2 were measured by monitoring the absorbance at 410 nm of the titanium-peroxide complex according to He at al. (2005). One gram of leaf was extracted using 10 mL cold acetone and centrifuged at 3.000 x *g* for 20 minutes. An aliquot (1 mL) of supernatant was added to 0.1 mL 20% titanium reagent and 0.2 mL of 17 mol ammonia solutions. The solution was centrifuged at 3.000 x *g* at 4°C for 10 minutes and the supernatant was discarded. The pellet was dissolved in 3 mL of 1 mol sulphuric acid. The absorbance of the solution was measured at 410 nm. Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2.

Antioxidant enzyme extraction and assays

Roots (0.5 g) were homogenised in 50 mM sodium phosphate buffer (pH 7.0) containing 1% polyvinylpyrolidine. The homogenate was centrifuged at 20.000 x *g* for 15 minutes at 4ºC, and the supernatant used for the assays to determine the activities of superoxide dismutase (SOD) (EC 1.15.1.1), catalase (CAT) (EC 1.11.1.6) and peroxidase (POX) (EC 1.11.1.7). Activity of SOD was estimated by recording the decrease in optical density of nitro blue tetrazolium (NBT) dye by the enzyme (Dhindsa et al., 1981). The reaction mixture of 3 mL contained 2 mM riboflavin, 13 mM methionine, 75 mM NBT, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50 mM phosphate buffer (pH 7.8), 50 mM sodium carbonate and 0.05 mL enzyme fraction. The reaction was started by adding riboflavin solution and placing the tubes under two 30W fluorescent lamps for 15 minutes. A complete reaction mixture without enzyme, which gave the maximal colour, served as a control. The reaction was stopped by switching off the light and putting the tubes in the dark. A non-irradiated complete reaction mixture served as a blank. The absorbance was recorded at 560 nm, and 1 unit of enzyme activity was taken as the amount of enzyme that reduced the absorbance reading to 50% in comparison with the tubes with no enzyme (Sairam and Srivastava, 2000). Activity of CAT 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 mmol H_2O_2 min^{-1} (Gong et al., 2001). Activity of POX was measured by monitoring the increase in the 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 min^{-1} (Yee et al., 2002).

Statistical analysis

Each experiment was repeated at least three times in two replicates. Analysis of variance (ANOVA) was conducted one-way ANOVA test using SPSS 13.0 for Microsoft windows, and means were compared by Duncan test at the 0.05 level of confidence.

Conclusion

To summarise, the present study detected that MDA, H_2O_2 contents and electrolyte leakage increased when a high level of B was applied. However, exogenous NO significantly reduced the adverse effects of B toxicity by mitigating cellular oxidative damage through improved free radical scavenging by antioxidant enzymes. This effect was mainly attributed to prevention of growth inhibition that allowed the plant to cope better with B toxicity. This experiment therefore indicates that supplemental irrigation with NO is an effective crop management practice for reducing B toxicity.

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