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RESEARCH ARTICLE

Salicylic acid and nitric oxide alleviate osmotic stress in wheat (*Triticum aestivum* L.) seedlings

Seyed Mehdi Naser Alavi^{a*}, Mohammad Javad Arvin^b and Khosrow Manoochehri Kalantari^a

^aDepartment of Biology, Shahid Bahonar University of Kerman, Kerman, Iran; ^bDepartment of Horticulture, Shahid Bahonar University of Kerman, Kerman, Iran

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Salicylic acid (SA) and nitric oxide (NO) are reported to alleviate the damaging effects of stress in plants rather similarly when applied at appropriate low concentrations. An experiment was therefore conducted to study the impact of SA, sodium nitroprusside (SNP; as NO donor), and methylene blue (MB; as a guanylate cyclase inhibitor) on wheat seedling performance under osmotic stress. Osmotic stress significantly reduced shoot fresh weight (SFW), chlorophyll contents (Chla, Chlb, total Chl), and membrane stability index (MSI) and also increased malondialdehyde (MDA) level, lipoxygenase (LOX) activity, and hydrogen peroxide production. Moreover, enzymatic antioxidant activities including superoxide dismutase, guaiacol peroxidase, and glutathione reductase activity were enhanced under osmotic stress. On the contrary, SA or SNP pretreatment reduced the damaging effects of osmotic stress by further enhancing the antioxidant activities that led to increased SFW, Chl, and MSI and reduced MDA level and LOX activity. However, pretreatment of plants with MB reversed or reduced the protective effects of SA and SNP suggesting that the protective effects were likely attributed to NO signaling. Therefore, NO may act as downstream of SA signaling in reduction of induced oxidative damage in wheat seedlings.

Keywords: nitric oxide; osmotic stress; oxidative stress; salicylic acid; wheat

Introduction

Water deficit or drought stress induces several physiological, biochemical, and molecular responses in plants (Misra et al. 2011) which are characterized by reduction of water content, diminished leaf water potential and turgor loss, closure of stomata, and decrease in cell enlargement and growth (Jaleel et al. 2008). Severe water stress may result in the arrest of photosynthesis, disturbance of metabolism followed by reduction of growth, dry matter, and harvestable yield in a number of plant species (Jaleel et al. 2008).

One of the inevitable consequences of drought stress is enhanced reactive oxygen species (ROS) production in different cellular compartments, namely in the chloroplasts, the peroxisomes, and the mitochondria (Cruz & Maria 2008) that activates expression of genes for antioxidant enzymes (Misra et al. 2011).

Some molecules, such as calcium, hydrogen peroxide (H₂O₂), abscisic acid, jasmonic acid, ethylene, salicylic acid, and nitric oxide (NO) have been suggested to be signal transducers or messengers. These substances have obtained particular attention because of inducing a protective effect on plants under abiotic stresses (Simaei et al. 2011).

Salicylic acid (SA, 2-hydroxybenzoic acid) is considered as a hormone-like endogenous regulator, which influences a range of diverse processes in plants, including seed germination, ion uptake and transport, membrane permeability, and photosynthesis (Simaei et al. 2011). SA is an important signal involved in the activation of plant defense responses against abiotic

and biotic stresses and plays a crucial role for the regulation of physiological and biochemical processes (Saruhan et al. 2012). Effects of SA on plants are concentration dependent, treatment duration, plant species, age at treatment, and plant organ examined used for pretreatment (Shi et al. 2009).

NO is a colorless gas and a bioactive molecule involved in many biological events, which belongs to the free radical-type molecules. It has an unpaired electron in its π orbital, making NO as a special molecule which may act as pro-oxidant as well as an antioxidant in plants (Corpas et al. 2011).

NO possesses antioxidant properties and as an antioxidant agent is able to scavenge ROS, also, as a signaling molecule leading to alterations of anti-oxidative gene expression and thus protects plant cells from oxidative damage under abiotic stress (Arasimowicz et al. 2007). There is evidence indicating that NO can act in plants similarly as in animals, through the cyclic guanosine monophosphate (cGMP)-dependent or cGMP-independent pathway, by increasing cytosolic Ca²⁺ concentration, and modulating the activity of protein kinases (Arasimowicz et al. 2007). NO can be generated non-enzymatically by chemical breakdown of NO donor molecules, such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine, S-nitrosoglutathione, and 3-morpholinodimethylamine (Simaei et al. 2011).

Soluble guanylate cyclase is the main intracellular receptor of NO. This enzyme catalyzes biosynthesis of second messenger, guanosine 3',5'-monophosphate (cGMP), a potent regulator of cell metabolism that

*Corresponding author. Email: malavi@uk.ac.ir

significantly influences various cell functions (Murad 1994). Most effects of NO are realized via stimulation of soluble guanylate cyclase and accumulation of cGMP (Pyatakova & Severina 2012).

Methylene blue (MB, methylthionine chloride) is a heterocyclic aromatic chemical compound (Wiklund et al. 2007), cationic thiazine dye, and deep blue in the oxidized state while it is colorless in its reduced form (leucomethylene blue). MB and leucomethylene blue exist as a redox couple in equilibrium and together form a reversible oxidation–reduction system or electron donor–acceptor couple (Wiklund et al. 2007). MB blocks accumulation of cGMP by inhibiting the enzyme guanylate cyclase (Mayer et al. 1993a). It has been reported that MB blocks the activity of NO-dependent guanylate cyclase via the oxidation of the active haemo center (Mayer et al. 1993a) or by inactivation of its haemo-deficient apoenzyme (Mayer et al. 1993b), and it has been used as NO signaling inhibitor by many researcher (Chow et al. 2013; Luna-Vázquez et al. 2013).

However, there is no report on the combined effect of MB with SA under stress condition and therefore the objectives of the present experiment were to investigate the impact of SA or NO on wheat seedling tolerance to osmotic stress and to test if the SA is exerting its effect via NO signaling.

Materials and methods

Plant materials and plant treatment

The uniform seeds of wheat (*Triticum aestivum* L. cv Roshan) were surface sterilized with 5% NaOCl for 10 min, rinsed with distilled water, imbibed for 24 h in room temperature, and then transferred to the gauze on the tap of plastic aerated pots containing ½ Hoagland nutrient medium (pH 6.2). The plants were grown in a self-regulating culture room and the light/dark period was 16/8 h, temperature 25/20 °C, and air humidity 60%.

The 21-day-old seedlings (5-leaf stage) were exposed to six treatments. The treatments consisted of 100 µM of the following: SA, SNP, MB, SA + MB, NO + MB, and a control. After 48 h of initial treatment, three pots of each treatment were exposed to polyethylene glycol (PEG-6000) of 15.4% (w/v) strengths to achieve water (osmotic) stress level of −0.4 MPa. After 72 h of root osmotic stress the shoot samples were collected, washed for 2 min by distilled water, immediately preserved in liquid N₂, and stored at −80 °C prior to assay for various biochemical estimations.

Determination of physiological and biochemical parameters

Photosynthetic pigments concentration

The amount of photosynthetic pigments (Chla and Chlb) was determined according to the method of Lichtenthaler (1987). Fresh leaves (0.25 g) were homogenized in acetone 80%, centrifuged at 3000 × g, and absorbance was recorded at wavelengths of 646.8 and 663.2 nm for

chlorophyll assay. Chla, Chlb, and total Chl were calculated using the following formulas:

$$\text{Chla} = 12.25A_{663.2} - 2.79A_{646.8}$$

$$\text{Chlb} = 21.21A_{647.8} - 5.10A_{663.2}$$

$$\text{Total Chl} = \text{Chla} + \text{Chlb}$$

Membrane stability index

Membrane stability index (MSI) of leaves was determined by recording the electrical conductivity of leachates in de-ionized water at 40 and 100 °C according to the method of Sairam et al. (1997).

H₂O₂ determination

H₂O₂ content was determined using the method given by Velikova et al. (2000). Shoot samples were extracted with 5 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 15 min. Then 0.5 ml of supernatant was mixed with 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 ml of 1 M potassium iodide and the absorbance was determined at 390 nm. The amount of H₂O₂ was calculated using the extinction coefficient 0.28 mM cm^{−1} and expressed as µmol/g FW.

Malondialdehyde content

Malondialdehyde (MDA) assay was estimated by the thiobarbituric acid (TBA) test according to the modified method of Heath and Packer (1968) and expressed as the amount of MDA as the products of lipid peroxidation nmol/g fr wt. One gram of fresh tissue was ground in 10 ml of 0.5% TBA in 20% (w/v) TCA. The mixture was incubated at 95 °C in a water bath for 30 min and quickly cooled in an icebath. Then, the homogenate was centrifuged at 10,000 × g for 15 min. The absorbance of the supernatant was read at 532 nm. After subtracting the non-specific absorbance at 600 nm, the MDA concentration was calculated using an extinction coefficient of 155 mM cm^{−1}.

Enzyme extraction and activity determination

Leaves (500 mg) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% soluble polyvinyl pyrrolidone, 1 mM ethylenediamine tetraacetic acid (EDTA), and 1 mM phenylmethanesulfonyl fluoride with the addition of 10 mM ascorbic acid for example in the case of the ascorbate peroxidase (APX) assay. The homogenate was centrifuged at 20,000 × g for 20 min and the supernatant used for determination of enzyme activity. All procedures were performed at a temperature of −4 °C.

Lipoxygenase (EC 1.13.11.12)

Lipoxygenase (LOX) activity was estimated according to the method of Doderer et al. (1992). For measurement of LOX activity, the substrate solution was prepared by adding 35 μ l linoleic acid to 5 ml distilled water containing 50 μ l Tween-20. The solution was kept at pH 9.0 by adding 0.2 M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 6.5 by adding 0.2 M HCl, 0.1 M phosphate buffer (pH 6.5) was added to make a total volume of 100 ml. LOX activity was determined spectrophotometrically by adding 50 μ l of enzyme to 2.95 ml substrate. Solution absorbance was recorded at 234 nm, and the activity was expressed as a change in absorbance/min/mg protein in the leaves.

Superoxide dismutase (EC 1.15.1.1)

Superoxide dismutase (SOD) activity was determined by the ability of SOD to inhibit reduction of nitrobluete-trazolium (NBT) to formazane (Giannopolitis & Ries 1977). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM Na-EDTA, 75 μ M riboflavin, 13 mM methionine, and 0.05 ml the enzyme extract. The unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photoreduction by 50%. SOD activity values are given in units (U)/mg protein.

Catalase (E.C 1.11.1.6)

Catalase (CAT) activity was measured according to the modified method of Dhindsa et al. (1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 15 mM H₂O₂, and 100 μ l enzyme extract. The decomposition of H₂O₂ was followed by measuring the decrease in absorbance at 240 nm. The activity was calculated using an extinction coefficient of 40 mM cm⁻¹. The U of CAT activity was defined as the amount of enzyme that decomposed 1 mM H₂O₂/min/mg protein in 100 μ l enzyme extract are given in U/mg of protein in g FW.

Guaiacol peroxidase (EC 1.11.1.7)

Guaiacol peroxidase (GPX) activity was determined in a reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.3% H₂O₂, 1% guaiacol, and 20 μ l enzyme extract, a method derived from Plewa et al. (1991). The increase of absorbance, due to tetraguaiacol formation, was recorded at 470 nm and enzyme activity was determined using an extinction coefficient of 25.5 mM cm⁻¹.

Glutathione reductase (EC 1.6.4.2)

The oxidized glutathione-dependent (GSSG-dependent) oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) was followed at 340 nm in a 1 ml

reaction mixture containing 100 mM sodium phosphate buffer (pH 7.8), 0.5 mM GSSG, 0.1 mM NADPH, and 0.05 ml extract. Glutathione reductase (GR) activity was calculated using an extinction coefficient of 6.2 mM cm⁻¹ and expressed as U/mg of protein. One unit of GR activity is defined as the amount of enzyme that oxidizes 1 nM of NADPH in 1 min (Foyer & Halliwell 1976).

Statistical analysis

In the present research, two factors, osmotic stress (Control, PEG) and chemicals (Control, MB, SA, SA + MB, SNP, SNP + MB), were factorially arranged in completely randomized design. The experiments were repeated thrice with three replicates each. Statistical analyses were performed by analysis of variance using the Statistical Analysis System software. Treatment means were separated by least significant difference (LSD) test ($p \leq 0.05$).

Results***Effects of osmotic stress (PEG) on physiological and biochemical parameters***

Osmotic stress (PEG) significantly led to strong oxidative stress and reduced shoot fresh weight (SFW), chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophyll (total Chl), and MSI by 37%, 21%, 39%, 25%, and 41%, respectively, relative to the control (Tables 1 and 2). It also raised the MDA content, LOX activity, and H₂O₂ level by 3.65, 10.4 and 5.76 folds, respectively (Table 2).

Osmotic stress also enhanced the activity of SOD, GPX, GR, and CAT by 1.58, 2.89, 1.65, and 2.84 folds, respectively, compared with the control (Table 3).

Effect of SA or SNP on physiological and biochemical parameters

Under non-stress conditions, SA or SNP increased SFW and chlorophylls only and had little effects on other parameters recorded (Tables 1–3). However, under osmotic stress, SA or SNP were significantly effective on all parameters recorded and their response was very similar.

Under osmotic stress and compared with corresponding control, SA or SNP increased SFW (35% and 29%), Chl_a (17% and 14%), Chl_b (44% and 31%), total Chl (20% and 17%), and MSI (46% and 46%), respectively (Tables 1 and 2).

Moreover, pretreatment by SA or SNP further increased the activity of antioxidant systems. Relative to controls, SA or SNP increased the activity of SOD (33% and 34%), GPX (18% and 20%), and GR (40% and 45%), respectively (Table 3). The increased activity of antioxidants by SA and SNP significantly reduced the content of MDA (49% and 51%), activity of LOX (59% and 58%), and the level of H₂O₂ (32% and 30%), respectively (Table 2).

Table 1. The effects of exogenous SA, SNP, and MB on SFW, Chla, Chlb, and total Chl content in wheat seedlings under osmotic stress.

Treatment	SFW (mg/plant)		Chla (mg/g FW)		Chlb (mg/g FW)		Total Chl (mg/g FW)	
	Control	PEG	Control	PEG	Control	PEG	Control	PEG
Control	4.68 ^b ± 0.06	2.94 ^d ± 0.08	18.41 ^b ± 0.02	14.47 ^d ± 0.15	5.06 ^b ± 0.13	3.07 ^d ± 0.36	23.47 ^b ± 0.15	17.53 ^d ± 0.33
MB	4.62 ^b ± 0.12	2.81 ^d ± 0.15	17.99 ^b ± 0.12	14.92 ^d ± 0.17	5.15 ^b ± 0.23	3.08 ^d ± 0.26	23.14 ^b ± 0.32	18.00 ^d ± 0.09
SA	5.08 ^a ± 0.17	3.96 ^c ± 0.12	19.65 ^a ± 0.29	16.87 ^c ± 0.42	6.56 ^a ± 0.17	4.17 ^c ± 0.42	26.16 ^a ± 0.41	21.04 ^c ± 0.16
SNP	5.07 ^a ± 0.11	3.80 ^c ± 0.09	19.60 ^a ± 0.25	16.54 ^c ± 0.13	6.28 ^a ± 0.05	4.02 ^c ± 0.23	25.92 ^a ± 0.26	20.58 ^c ± 0.10
SA + MB	4.58 ^b ± 0.07	2.90 ^d ± 0.14	17.86 ^b ± 0.20	15.02 ^d ± 0.51	5.35 ^b ± 0.35	3.09 ^d ± 0.17	23.22 ^b ± 0.17	18.11 ^d ± 0.63
SNP + MB	4.58 ^b ± 0.15	2.95 ^d ± 0.05	17.74 ^b ± 0.18	14.74 ^d ± 0.34	5.24 ^b ± 0.28	3.12 ^d ± 0.25	22.99 ^b ± 0.37	17.86 ^d ± 0.09

Note: Values are means ± SE ($n = 9$). For each trait, different letters indicate significant differences among treatments at $p \leq 0.05$ according to LSD's test.

Table 2. The effects of exogenous SA, SNP, and MB on MSI, MDA content, LOX activity, and H₂O₂ content in wheat seedlings under osmotic stress.

Treatment	MSI (%)		MDA (nmol/g FW)		LOX activity (U/mg protein)		H ₂ O ₂ (μmol/g FW)	
	Control	PEG	Control	PEG	Control	PEG	Control	PEG
Control	88.08 ^a ± 1.32	52.05 ^{de} ± 2.01	2.05 ^{de} ± 0.10	7.49 ^a ± 0.30	0.045 ^d ± 0.007	0.468 ^{ab} ± 0.019	0.73 ^d ± 0.05	4.21 ^a ± 0.18
MB	84.62 ^a ± 2.37	50.71 ^e ± 4.86	2.53 ^{de} ± 0.16	7.81 ^a ± 0.24	0.053 ^d ± 0.004	0.474 ^a ± 0.005	0.75 ^d ± 0.06	4.29 ^a ± 0.15
SA	90.51 ^a ± 0.49	76.01 ^b ± 2.44	1.87 ^e ± 0.06	3.83 ^c ± 0.28	0.047 ^d ± 0.005	0.192 ^c ± 0.028	0.65 ^d ± 0.03	2.85 ^c ± 0.32
SNP	90.24 ^a ± 0.52	76.19 ^b ± 2.86	1.82 ^e ± 0.02	3.90 ^c ± 0.32	0.044 ^d ± 0.002	0.195 ^c ± 0.041	0.70 ^d ± 0.02	2.93 ^c ± 0.13
SA + MB	86.11 ^a ± 0.68	57.43 ^{cd} ± 1.62	2.42 ^{de} ± 0.20	6.00 ^b ± 0.21	0.050 ^d ± 0.008	0.424 ^b ± 0.005	0.77 ^d ± 0.03	3.48 ^b ± 0.30
SNP + MB	85.57 ^a ± 1.29	60.62 ^c ± 2.63	2.22 ^{de} ± 0.27	5.98 ^b ± 0.43	0.048 ^d ± 0.011	0.454 ^{ab} ± 0.007	0.73 ^d ± 0.05	3.47 ^b ± 0.14

Note: Values are means ± SE ($n = 9$). For each trait, different letters indicate significant differences among treatments at $p \leq 0.05$ according to LSD's test.

However, in all cases, no significant changes were observed between control and MB alone or in combination with SA or SNP (Tables 1–3) and MB reversed or reduced the protective effects of SNP or SA under osmotic stress and to lesser extents under non-osmotic stress.

Discussion

In present study, osmotic stress negatively affected SFW and chlorophyll contents. Osmotic stress induces water deficit and consequently closes stomata, a process which decreases CO₂ availability and photosynthesis, leading to excess ROS formation in chloroplasts (Parida & Das 2005). Osmotic stress also increased the level of MDA and LOX activity which are considered as the biomarkers of stress. Under osmotic stress, plants produce ROS such as H₂O₂, hydroxyl radicals (OH•), and superoxide anion (O₂^{•-}), which are harmful to plant growth due to their detrimental effects on the subcellular components

and metabolism of the plant, leading to the oxidative destruction of cells (Gunes et al. 2007; Maksup et al. 2014). Active oxygen species cause deterioration of membrane lipids, leading to increased MDA, and leakage of solutes from membranes (Gunes et al. 2007). Also, LOX activity as an oxidative enzyme contributes to lipid peroxidation increased in drought conditions. Under non-stress conditions, ROS are efficiently eliminated by non-enzymatic and enzymatic antioxidants. But, under drought conditions the production of ROS exceeds the capacity of the anti-oxidative systems to remove the oxidative stress (Sofa et al. 2005). The activated antioxidant systems are beneficial for plant performance and have a vital role in alleviating osmotic stress damage in plants, by removing excess ROS and inhibiting lipid peroxidation (Misra et al. 2011). We observed that SOD activity was enhanced under osmotic stress. Although SOD functions as the first line of defense against ROS, its end product is the toxic H₂O₂. Therefore, an efficient H₂O₂ scavenging system is

Table 3. The effects of exogenous SA, SNP, and MB on SOD, GPX, GR, and CAT activities in wheat seedlings under osmotic stress.

Treatment	SOD activity (U/mg protein)		GPX activity (U/mg protein)		GR activity (U/mg protein)		CAT activity (U/mg protein)	
	Control	PEG	Control	PEG	Control	PEG	Control	PEG
Control	18.84 ^{de} ± 0.71	29.75 ^b ± 1.61	58.38 ^e ± 7.81	168.61 ^b ± 4.60	1.06 ^d ± 0.09	1.75 ^b ± 0.12	3.70 ^e ± 0.18	10.52 ^a ± 0.55
MB	17.67 ^c ± 2.11	28.37 ^{bc} ± 0.77	57.54 ^e ± 1.88	167.46 ^b ± 0.71	0.92 ^d ± 0.03	1.50 ^{bc} ± 0.05	3.64 ^e ± 0.08	9.99 ^a ± 0.74
SA	22.38 ^{de} ± 1.30	39.49 ^a ± 1.26	54.11 ^c ± 5.38	199.28 ^a ± 3.99	1.07 ^d ± 0.10	2.45 ^a ± 0.10	3.57 ^e ± 0.23	7.87 ^b ± 0.34
SNP	23.25 ^{cd} ± 1.57	39.83 ^a ± 0.89	56.73 ^c ± 3.84	203.18 ^a ± 3.38	1.15 ^d ± 0.08	2.54 ^a ± 0.18	3.12 ^e ± 0.47	7.77 ^b ± 0.76
SA + MB	20.63 ^{de} ± 1.01	30.44 ^b ± 2.27	54.43 ^c ± 2.14	161.35 ^b ± 3.34	0.97 ^d ± 0.06	1.68 ^c ± 0.06	3.65 ^e ± 0.18	9.69 ^a ± 0.55
SNP + MB	20.92 ^{de} ± 1.49	29.52 ^b ± 1.25	54.28 ^c ± 1.44	165.32 ^b ± 1.13	1.07 ^d ± 0.03	1.46 ^{bc} ± 0.16	3.13 ^e ± 0.51	9.92 ^a ± 0.46

Note: Values are means ± SE ($n = 9$). For each trait, different letters indicate significant differences among treatments at $p \leq 0.05$ according to LSD's test.

required to enable rapid removal of H_2O_2 in the plant cells. A number of enzymes regulate H_2O_2 intracellular levels, but CAT, APX, and GR are considered the most important (Parida & Das 2005).

SA and NO increased SFW and chlorophylls content. SA or NO usually enhances growth via protecting reaction of the photosynthetic pigments (El-Tayeb 2005; Simaei et al. 2011).

Bai et al. (2011) found that NO affected a series of protein accumulation involving in material and energy metabolism, including ATPase and LOX.

Signaling compounds such as SA or NO have dual functions as potent oxidant or effective antioxidant mostly depends on their concentrations and on the status of the environments (Shi et al. 2009). When applied at appropriate low concentrations, they are able to reduce the severity of stresses in many species (Horvath et al. 2007), by inducing transient oxidative stress in plants, which acts as a hardening process, increasing antioxidant capacity of plants (Horvath et al. 2007). It appears that they induce redox signal (H_2O_2 as a secondary messenger) and leading to increase in antioxidant activity is linked to inhibition of CAT or plasma membrane linked NADPH oxidase (Horvath et al. 2007). However, to induce antioxidant activity, low concentration of H_2O_2 is required. Improved performance of plant species treated with SA or NO has been reported under stress conditions (Khodary 2004; El-Tayeb 2005). The decrease in membranes damage which is associated with an improved performance of plants in response to SA or NO may be related to the induction of antioxidant responses that protect the plant from oxidative damage, a similar mechanism to be responsible for SA-induced multiple stress tolerance in bean and tomato plants (Senaratna et al. 2000). SA- or NO-induced increases in SOD, CAT, GPX, and GR activity have also been reported (El-Tayeb 2005; Simaei et al. 2011; Kong et al. 2014).

Similar to NO, SA alleviates the damaging effect of long-term drought stress, decreased water loss, and increased relative water content during drought stress (Kadioglu et al. 2011). Decreases in the stomatal conductance were lower in SA-treated plants than control (Saruhan et al. 2012). The NO regulation of stomatal closure may be via modulating intracellular Ca^{2+} in guard cells. Fan and Liu (2012) reported that NO played a critical role in mediating stomatal movement and the role of cGMP synthesis and signaling and Ca^{2+} increase via cADPR signaling of plant NO response. Liu et al. (2003) suggested that both cGMP and cADPR might mediate the signal transduction of SA and NO-induced stomatal closure. Therefore, it seems the stomatal closure induced by SA or NO shares similar mechanism (s) and involves NO release and drastic changes in Ca^{2+} levels. Arfan (2009) reported that, SA-induced SOD activity accompanied with an increase in shoot Ca^{2+} supported the view that if sufficient Ca^{2+} is present, it acts as a second messenger and causes a transient increase in H_2O_2 , which in turn induces antioxidant enzymes leading to decrease in ROS on

long-term basis. It has also been reported that NO treatment further improved protein accumulations and enzyme activities including APX, Mn-SOD, GR, and growth via G proteins (Bai et al. 2011).

Moreover, Karpets et al. (2011) reported that SNP induced heat resistance of wheat coleoptiles depended on calcium and ROS, whose production is probably boosted by activation of NADPH oxidase and MB reduced the elevation of wheat resistance induced by NO donor.

However, in all cases, MB reversed or reduced the protective effects of SA and SNP on parameters recorded under osmotic stress. As it has been frequently shown that the stomatal closure of leaf by NO is by cGMP and MB is an inhibitor of the guanylate cyclase and cGMP signaling, it may be concluded that, the similar effects of SA and SNP on plant performance before and after MB application may imply that SA is exerting its effects via NO through cGMP signaling.

Conclusion

In conclusion the results of present study revealed that effects of osmotic stress was partially overcome by the application SA and SNP thereby increasing antioxidant enzyme activation and SA probably operated through guanylate cyclase and cGMP pathway.

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