

THE RELATIONSHIP BETWEEN NITRIC OXIDE AND PLANT HORMONES IN SNP ADMINISTRATED SUNFLOWER PLANTS UNDER SALT STRESS CONDITION

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Abstract. Nitric oxide (NO) and sodium nitro prusside (SNP) are striking molecules and play important roles in animals and plants. SNP serves as nitric oxide donor in both group. NO can act free radical and impaires important biomolecules functions beside this it has beneficial effect recovery from salinity, drought etc. NO and SNP are beneficial and protectant molecules in cope with stressfull conditions. In plants these molecules are very important, and regulate many physiological events. In the present study, endogen abscisic acid (ABA), indole acetic acid (IAA), gibberellic acid (GA₃) and NO levels were investigated in NaCl, SNP and plant growth regulators treated sunflower plant (*Helianthus annuus* L.) leaves and roots. The content of NO was higher in GA₃ + SNP treated plant leaves at 72 h. The highest IAA level was determined in IAA + SNP treated plant roots at 72 h. In NaCl + ABA treated plant leaves ABA was higher at 72 and GA₃ levels were equal or less than the control group. Our results showed that coadministration of SNP and plant growth regulators cope with salinity stress via elevated internal hormone and NO level versus salinity.

Key words: nitric oxide, sodium nitroprusside, salinity, sunflower

INTRODUCTION

Salinity is the most effective stress factor that significant amount of interest has become over it in recent years. Decreasing in rainfall and high moisture formation in arid and semi-arid areas lead to difficulty in reaching food and water for the plants.

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Thus, salt stress, absence of water and also drought stress occur [Mahajan and Tuteja 2005, Al-Karaki 2006, Porcel et al. 2012]. High salinity induces negative effects on plants due to ion toxicity, nutrient deficiency, replacement of metabolic processes, membrane disorders, declining in cell division and expansion, genotoxicity [Zhu 2007]. Similarly, protein synthesis, photosynthesis and lipid metabolism are negatively affected when NaCl stress starts in plant [Munns 2002, Parida and Das 2005]. Osmotic effect of NaCl stress can be observed immediately after the application of NaCl and cell growth and division were inhibited and stomata were closed [Munns 2002, Flowers 2004]. Long-term salinity causes the plant senescence in mature leaves and causes a decrease in photosynthetic area [Cramer and Nowak 1992]. Plant cells have offensive or protective mechanisms against negative effect of salinity. Taking control of stomata allows osmotic adaptation. Moreover, plants produce secondary metabolites and phytohormones. Thus, plants cope with drought stress and salt stress conditions [Yordanov et al. 2000, Valladares and Percy 2002, Martinez-Ferri et al. 2004, Radhakrishnan and Lee 2013].

Plant growth regulators play an important role in plant growth and development. Some of endogenous plant hormones act as a key molecule in the regulation of signal transduction and gene expression when faced with abiotic stress [Xiong et al. 2002]. Plant growth and development are significantly affected by the cross talk between plant growth regulators [Davies 1995, Miransari and Smith 2014]. Abscisic acid (ABA) plays a role in the regulation of water stress and stress response of the plant for the situations like heavy metal stress. ABA is an important plant hormone that plays a role in response to drought stress and regulates stomatal activity, dormancy and plant activity under the abiotic and biotic stress conditions. The amount of ABA is increased with salinity stress. ABA also plays an important role for plant adaptations at cold temperatures. Cold stress induces the synthesis of ABA and the exogenous application of ABA improves the cold tolerance of plants [Xue-Xuan et al. 2010]. ABA synthesis is one of the fastest responses of plants against abiotic stress and is triggered by ABA-inducible gene expression [Yamaguchi-Shinozaki and Shinozaki 2006]. ABA-mediated signal transduction regulates NaCl stress genes [Moore 1989, Davies and Jones 1991, Weyers and Paterson 2001, Szepesi et al. 2009, Popko et al. 2010]. Another plant hormone indole-3-acetic acid (IAA), also called auxin, plays a major role cell cycle, growth and development, vascular and pollen formation in plants. Auxin has regulatory effects against the stress. Gibberellic acid (GA) considerably regulates plant growth and development. GA reduces the effects of NaCl stress. One of the plant hormones, gibberellins are necessary for seed germination. Gibberellins can influence the production of proteins during pathogenic, oxidative and heavy metal stresses [Marrs 1996, Miransari and Smith 2014]. Nitric oxide (NO) is a molecule that has an effective in plant growth and development in different stages. NO increases NaCl tolerance in plants. Furthermore, NO has important roles in plants such as seed germination, plant growth and development, senescence and stomatal movements. NO has different effects at low and high concentrations in plants. Exogenous NO donor, sodium nitro prusside (SNP) reduces loss of water under arid conditions in wheat leaves and seeds. NO helps to stomatal movements via ABA stimulation. Similarly, the externally applied NO reduces negative effects of biotic

and abiotic stress factors in plants [Durner and Klessig 1999, Bright et al. 2006, Zhang et al. 2006, Neill et al. 2008, Hancock et al. 2011]. NO may affect on the biosynthesis, catabolism, transduction and transport of phytohormones such as auxins, gibberellins and abscisic acid [Freschi 2013]. Root organogenesis, gravitropic responses, root nodule formation, embryogenic cell formation and activation of cell division are related with the synergistic effects of IAA and NO. Exogenous IAA application causes an increase in the amount of nitric oxide. Furthermore, nitric oxide regulates auxin metabolism, transport and signaling. Previous studies have demonstrated that auxin (indole-3-acetic acid) and nitric oxide are plant growth regulators that affect some plant physiological responses. NO and ABA are important stress-related molecules. They work together in signal transduction that is caused by environmental changes such as lacking of water and UV-B radiation. In this way, they provide the induction of adaptive responses such as stomatal closure and antioxidant defense [Neill et al. 2008, Tossi et al. 2009, Hancock et al. 2011]. During the induction of stress responses, NO acts as a downstream molecule in the ABA signaling pathway since the breakdown in NO production or its removal from tissues decreases or even eliminates ABA responses. The inhibition of ABA production does not affect the induction of these responses with exogenous NO treatment [Bethke et al. 2006, Lozano-Juste and Leon 2010 a, b]. The interaction between NO and GA has been described in plants under limited physiological stages. In fact, there are positive and negative interactions between nitric oxide and GA [Freschi 2013]. NO has been described in upstream of GA [Freschi 2013, Bethke et al. 2007] by regulating both GA biosynthesis and perception/transduction [Freschi 2013, Leon and Lozano-Juste 2011]. In addition, mutual antagonism which controls the level of GA and NO has also recently been proposed by Leon and Lozano-Juste [2011]. In the present study, we investigated the effect of exogenously administrated sodium nitroprussid on nitric oxide and plant growth regulators interactions. Nitric oxide and abscisic acid, gibberellic acid and indole-3-acetic acid levels were evaluated to provide information on the differences under salt stress.

MATERIALS AND METHODS

The research was carried out in climate chamber with 16 h light/8 h dark period in 60% humidity. Experiments were performed in hydroponic culture conditions with Hoagland culture solution. Sunflower (*Helianthus annuus* L. cv. TARSAN-1018) was used in this study. The cultivars of sunflower used in the study were obtained from Trakya Agricultural Research Institute. Firstly, maximum salt tolerance of plant was determined, and then seeds were sterilized using sodium hypochloride solution (1% v/v) followed by washing with dH₂O. After sterilization, seeds were incubated in dark for 24 hours. After germinations, seeds were grown using a Hoagland culture solution during 15 days in a 5 liter continuously aerated pots. Nine experimental groups (Control, NaCl, NaCl + SNP, GA₃ + SNP, IAA + SNP, ABA + SNP, NaCl + GA₃, NaCl + IAA and NaCl + ABA) made for NO levels analyses and five experimental groups (Control,

NaCl, NaCl + SNP, Plant Growth Regulator + SNP and NaCl + Plant Growth Regulator) made for plant growth regulators analyses. Each group has three aerated pots and 30 seeds germinated in each pot. Sodium nitro prusside (100 μ M), 10^{-5} M gibberellic acid, 10^{-5} M indole acetic acid and 10^{-5} M abscisic acid treatment were performed independently. Then, the salt stress (300 mM NaCl) was applied for 72 h. Leaves and roots were drawn randomly at 0 (control), 24 and 72 h. The leaves and roots were frozen with liquid nitrogen and stored in the freezer until analyses.

Nitric oxide and plant growth regulators analysis. Leaf and root tissues were cut and weighed and 1 g samples were used for analyses. Tissues were homogenized in PBS at pH 7.4 and centrifuged at 10.000 g for 20 min. Supernatant was used for determining nitric oxide and plant growth regulators content by using manufacturer assay protocol. Absorbance values were determined by microplate reader (Molecular Devices Corp., VersaMax®).

Nitric oxide analysis. NO level of supernatant was measured by using NO assay kit (Cayman Chem. catalog no. 780001) and absorbance values were determined by microplate reader at 550 nm.

IAA, GA and ABA analysis. IAA, GA and ABA contents of supernatants were measured by using assay protocol (Cloud-Clone Corp, catalog no CEA737Ge for IAA, CEA759Ge for GA₃ and CEB218Ge for ABA) and absorbance values were determined by microplate reader at 450 nm.

Statistical analysis. The experimental assays used to obtain all results were repeated three times, under the same conditions, and yielded essentially the same results. A comparative analysis of variance was performed between the control and experimental groups. Statistical analyses of data, was performed using SPSS 15.0 software program. All measurements were subjected to analysis of variance (ANOVA) to discriminate significant differences (defined as $P \leq 0.05$). Data were shown as the mean \pm standard deviation (SD). Results were compared with the control group.

RESULTS

Nitric oxide levels. In the present study we showed that changes in the amount of nitric oxide depending on the application groups in sunflower leaves and roots and all results were higher than the control group. The highest amount of NO was determined in the ABA + SNP treatment at 24 h and 72 h in leaves (14.34 ± 0.31 μ mol/g FW and 11.13 ± 0.28 μ mol/g FW respectively) and similarly in roots the highest amount of NO was determined in GA₃ + SNP and NaCl + SNP treatment (24.27 ± 0.95 μ mol/min g FW and 22.79 ± 0.84 μ mol/g FW respectively). It was determined that root tissues include high nitric oxide level than leaves. According to statistical analysis, the differences between amount of NO levels were determined in the application groups (figs 1 a, b).

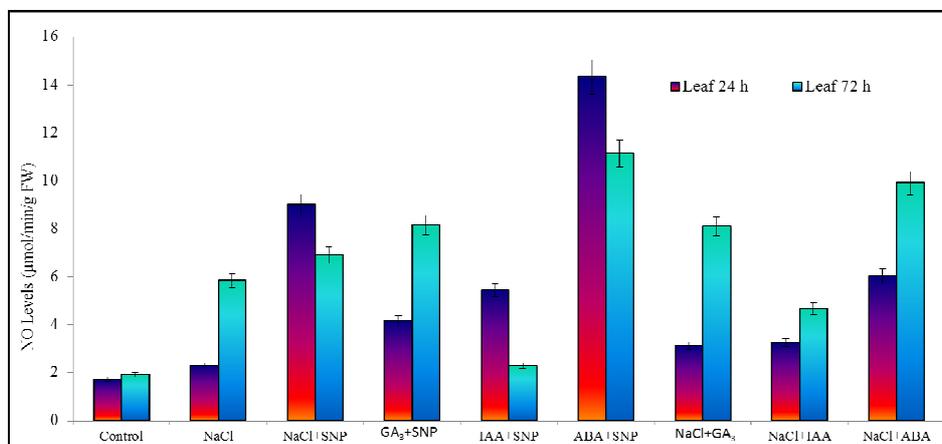


Fig. 1 a. NO levels (µmol/g FW) of *Helianthus annuus* L. cv. TARSAN-1018 leaves

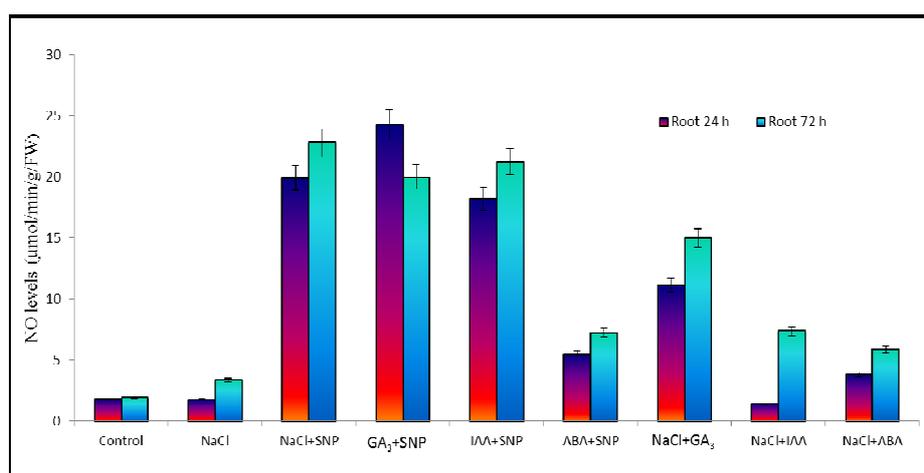


Fig. 1 b. NO levels (µmol/g FW) of *Helianthus annuus* L. cv. TARSAN-1018 roots

IAA Levels. We determined high IAA levels in root tissues than leaves. Figure 2 shows that NaCl, NaCl + SNP and NaCl + IAA applications caused a decrease in IAA levels of leaves and root for 24 h and 72 h compared to control group. The highest IAA levels were observed in the IAA + SNP treatment for 72 h in leaves and root tissues (198.24 ± 7.14 ng/g FW and 302 ± 8.63 ng/g FW, respectively). On the other hand, NaCl application caused significant decrease in IAA levels for both root and leaf tissues of *Helianthus annuus* L. cv. TARSAN-1018 (fig. 2). The SNP treatment has the effect of mitigating consequences. Adding SNP into the salt application caused an in-

crease in the amount of IAA. The synergistic effect of IAA + SNP had positive interaction. The results showed that IAA + SNP-treated leaves and roots had a higher IAA content, while the other treatments had low IAA levels compared to the control leaves and roots.

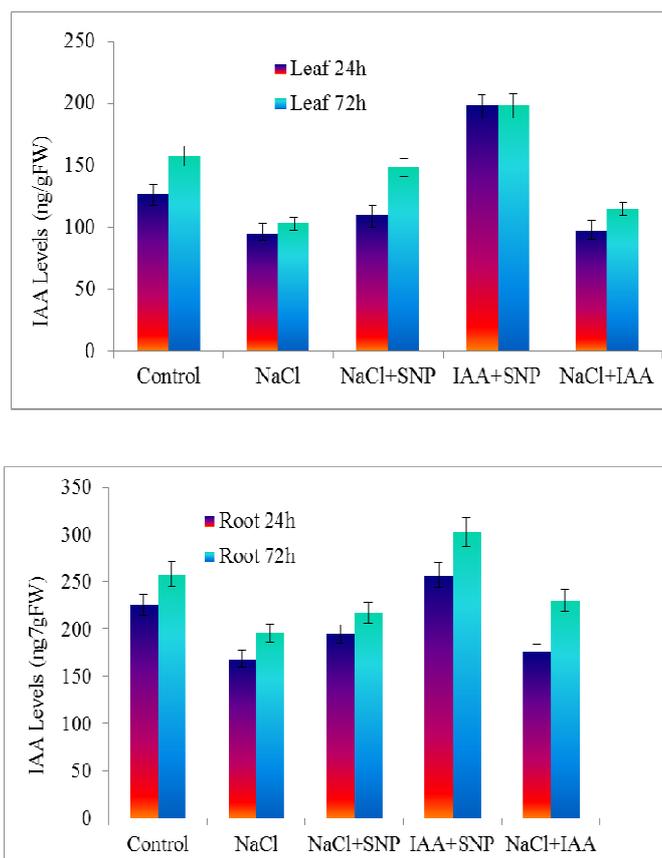


Fig. 2. IAA levels (ng/g FW) of *Helianthus annuus* L. cv. TARSAN-1018 leaves and roots

ABA Levels. Figure 3 shows the time-course of ABA levels as affected by treatment groups. ABA levels of leaves and roots increased by time. The combination of ABA with either the NaCl or the SNP stimulated ABA production. Especially ABA levels of leaves were significantly increased in NaCl+ABA treated in root tissue at 72 h. ABA level increased by 5.95 fold and 2.77 fold in leaves and root tissue respectively at 72 h.

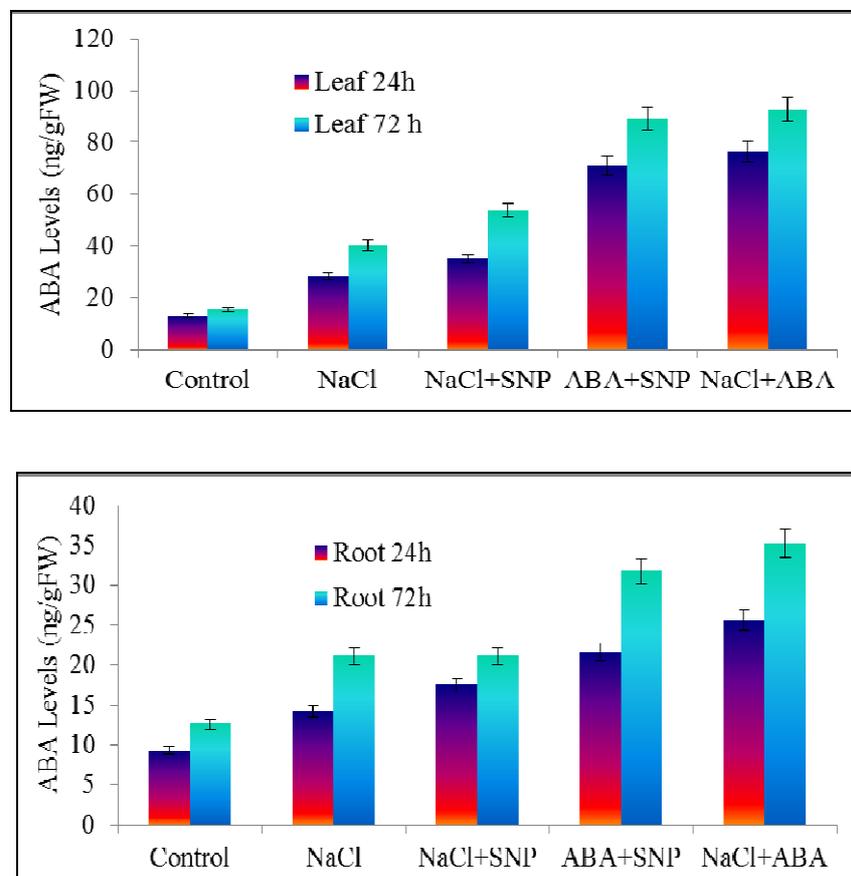


Fig. 3. ABA levels (ng/g FW) of *Helianthus annuus* L. cv. TARSAN-1018 leaves and roots

GA₃ Levels. Results showed that all of the treatment groups have led to a lower GA₃ levels than the control in leaves and roots of *Helianthus annuus* L. cv. TARSAN-1018. On the other hand, combination of GA₃ and SNP caused increase level of GA₃ than other groups (except control group) and the NaCl treatment has reduced GA₃ levels. The combination of GA₃ with SNP stimulated GA₃ production and at 24 h and 72 h compare to plant roots treated with NaCl and NaCl+SNP (fig. 4).

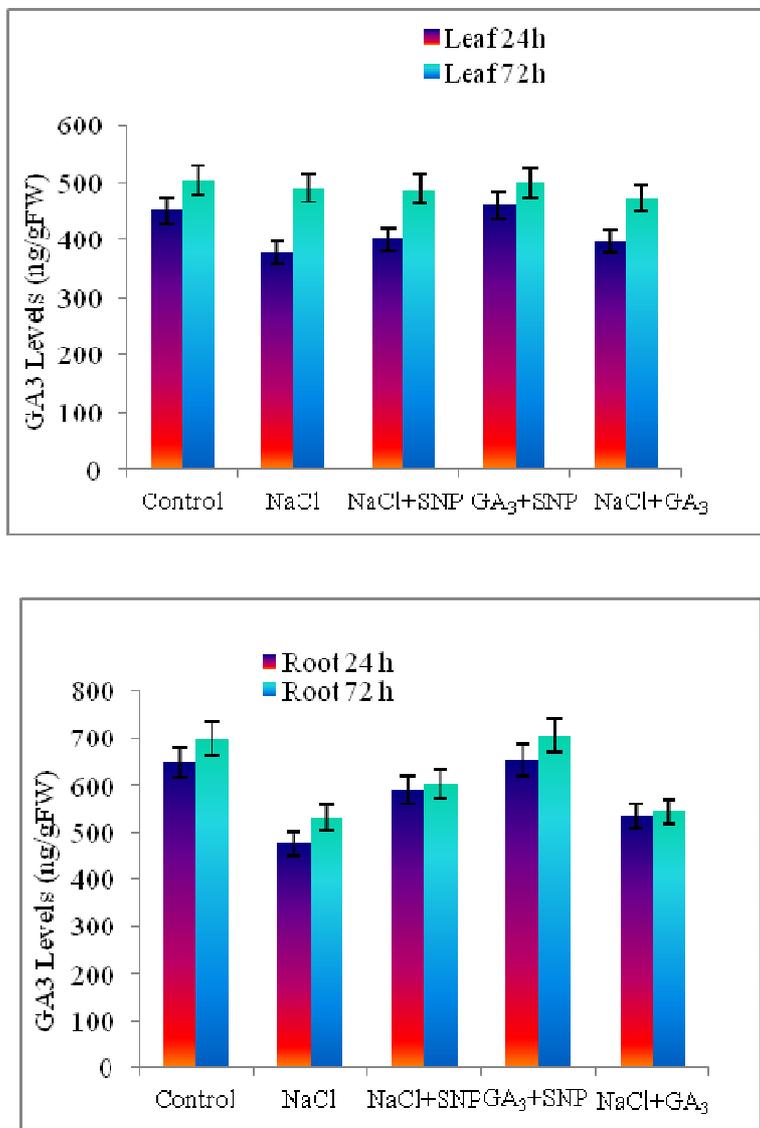


Fig. 4. GA₃ levels (ng/g FW) of *Helianthus annuus* L. cv. TARSAN-1018 leaves and roots

DISCUSSION

There are numerous studies explaining the relationship between nitric oxide and stress conditions on plant growth regulators [Arasimowicz et al. 2007, Hancocok et al. 2011, He et al. 2012, Freschi 2013]. In this study, exogenous SNP (100 μ M) application

and combination of SNP with NaCl and plant hormones changed NO level in *Helianthus annuus* L. cv. TARSAN-1018 leaves and roots at 24 and 72 h (figs 1 a, b).

Combination of SNP and all three hormone applications caused an increase in NO level. On the other hand, combination of NaCl and ABA, IAA and GA₃ provided high NO level at 24 and 72 h, except for NaCl + IAA at 24 h for sunflower root tissues. The present study revealed that extending NaCl stress caused increasing NO level both in root and leaf tissues of sunflower at 24 h and 72 h (figs 1 a, b). Garcia-Mata and Lamattina [2001] reported that Nitric oxide is very important signal molecule and it has defense responses against plant abiotic and biotic stresses including salinity. Plant response to such stressors as drought, high or low temperature, salinity, heavy metals and oxidative stress is regulated by NO [Garcia-Mata and Lamattina 2001, Arasimowicz et al. 2007]. Earlier studies reported that nitric oxide acts as a signaling molecule under osmotic and drought stress conditions in the root tissues of *Petroselinum crispum* L., *Pisum sativum* L. and *Triticum aestivum* L. [Kolbert et al. 2005]. It is reported that externally applied NO donor SNP reduced water loss ion current and transpiration rate and caused the closure of the stomata in leaves and seeds of wheat under dry conditions [Garcia-Mata and Lamattina 2001]. According to the literature, exposing to light stress increased the level of NO in meadow grass plant [Xu et al. 2010]. These results in the present study are similar to the earlier studies. The combination of NaCl + SNP caused significant increase level of NO in sunflower root and leaf tissues. Especially, the root tissues have high NO levels than leaf. These results suggest that the signal transduction is triggered by root. NO levels showed a significant increase with the addition of the SNP. NaCl + SNP application has provided 12-fold increase in NO level at 24 h in root tissue. As clearly shown in Figure 1, the combination of hormones and SNP provided increases NO level. An increase in NO content in ABA + SNP application has been observed and this is consistent with the findings of Sripinyowanich et al. [2013]. However, this increase was not as high as on the NaCl + SNP application. The highest NO level of leaf tissues was provided by ABA + SNP application at 24 h and 72 h and caused approximately 8.5 fold increase of NO level at 24 h. It was also observed that the NO level increased in the application of GA₃ + SNP and IAA+SNP. ABA and NO are stress-related molecules and play critical role together on signaling cascades triggered by some environmental stresses such as water limitation and UV-B radiation [Desikan et al. 2004, Tossi et al. 2009, Hancock et al. 2011]. During the regulation of these plant stress responses, NO mainly acts as a downstream molecule in the ABA signaling pathway [Freschi 2013]. It has been described that there is an interaction between nitric oxide and GAs [Bethke et al. 2007]. NO has been served to act upstream of GA₃ regulating both GA₃ biosynthesis and perception/transduction [Bethke et al. 2007, Leon and Lozano-Juste 2011, Freschi 2013]. A number of studies reported that there is an antagonism between the NO and GAs in many physiological processes in which both of these signaling compounds participate [Leon and Lozano-Juste 2011, Fernandez-Marcos et al. 2012, Freschi 2013]. According to Zhu. et al. [2012], GA₃ application causes a decrease in NO level and this situation is regulated by GSNO (S-nitroglutathion). Unlike the literature, we determined that GA₃ application increases the NO levels in the leaf and root tissues. It is believed that application of NaCl or SNP provided regulator effect on GA₃ application. Sufficiently high concentrations of ABA can be

blocked by NO and GA signaling cascade during seed dormancy breaking [Bethke et al. 2006, Sarath et al. 2006, Dong et al. 2012]. Increased NO production has often been observed after exogenous auxin application [Tun et al. 2001, Correa-Aragunde et al. 2004, Hu et al. 2005, Lombardo et al. 2006]. Indeed, auxins are not directly effective on NO production in some particular experimental conditions or cell types and thus suggesting that NO production via auxins may occur especially under specific timewise and spatial contexts [Hu et al. 2005]. There is adequate information on the interaction between NO and auxins in root tissues but for the other plant parts information is less [Freschi 2013]. NO might also modulate auxin metabolism, transport and signaling under stress conditions. For instance, NO can enhance IAA level in *Medicago truncatula* root under cadmium stress [Xu et al. 2010]. Besides, the high concentration of NO inhibits acropetal auxin transport in Arabidopsis [Fernández-Marcos et al. 2011]. The present study showed that IAA + SNP application increased NO level in root and leaf tissues of sunflower. NaCl + GA₃ treatment generated an increase in NO level for sunflower root and leaf tissues. This increase is higher than NaCl treatment but it is lower than NaCl + SNP and NaCl + GA₃ applications. These results demonstrated that GA₃ effects on NO metabolism. As stated above, a number of studies reported that there is antagonism between NO and GAs [Leon and Lozano-Juste 2011, Fernandez-Marcos et al. 2012, Freschi 2013]. NaCl + IAA treatment vaguely affected NO level when compared to NaCl treatment. NaCl + ABA application caused an increase in NO level when compared to NaCl treatment. Interestingly, NaCl + ABA application did not significantly increase NO level, whereas literature mentions that NO and auxin have synergistic effects during the regulation in a series of plant responses including root organogenesis [Pagnussat et al. 2004, Lanteri et al. 2006] gravitropic responses [He et al. 2012], formation of root nodule [Pii et al. 2007], root responses to iron deficiency, activation of cell division and embryogenic cell formation [Ötvös et al. 2005]. Our results pointed out that ABA + SNP application caused high NO level at 24 and 72 h for sunflower plants. Moreover, our findings also suggested that the positive effects of ABA on NO level depends on stress conditions. Figure 2 shows that NaCl, NaCl + SNP and NaCl + IAA applications decreased IAA levels of leaves and root at 24 h and 72 h compared to control group. Only the combination of IAA and SNP provided highest IAA level at 72 h in root tissues when compared to control group. In a different study, it was revealed that IAA induces the accumulation of NO in Arabidopsis roots [Yu et al. 2007]. Researchers concluded that the amount of IAA was increased by NO in Arabidopsis [Correa-Aragunde et al. 2004, Hu et al. 2005]. Besides, the high concentration of NO inhibits acropetal auxin transport in Arabidopsis [Fernandez-Marcos et al. 2011]. Recent studies have demonstrated that NO might modulate IAA metabolism and transport [Xu et al. 2010]. According to Figure 3, NaCl treatment caused an increase in the level of ABA. Plants produce ABA under stress conditions [Yu et al. 2007, Zhao et al. 2009, Yarra et al. 2012]. ABA is an important mediator in order to trigger the plant responses to stress. Water stress provides a 40-fold increase in ABA level in the roots [Neill and Horgan 1985]. Zörb et al. [2013] concluded that ABA concentration significantly increased the resistance of maize leaves under salt stress. We have observed the similar increases in ABA level in NaCl treatment. The combination of SNP and NaCl provided increase ABA level when compared to control group and NaCl application. This result shows

that SNP is effective on ABA level. Numerous studies indicated that NO and ABA are important stress-related molecules against environmental changes [Desikan et al. 2004, Tossi et al. 2009, Hancock et al. 2011]. On the other hand, NaCl + ABA treatment caused significant increase in ABA level for both roots and leaves of sunflower. The combination of ABA and NaCl increased ABA level. Only NaCl application provided a slight increase in the level of ABA. Exogenous ABA increased the amount of total ABA. Control group has the highest GA₃ level than other applications (fig. 4). NaCl treatment decreased GA₃ level. According to Achard et al. [2006], GA₃ level decreased under salt stress. Colebrook et al. [2014] reported that GA₃ level decreased under salt stress condition. We obtained similar results (fig. 4). NaCl + SNP application has made enhancing the effect on GA₃ level in sunflower. However, this increase was not higher than the control group. Our result suggested that SNP has positive effect on GA₃ metabolism and there are positive interactions between them. Similar result was obtained from GA₃ + SNP treatment. Moreover, GA₃ + SNP treatment provided so high results as the control group

CONCLUSION

In light of these results, NO and plant hormones (IAA, GA₃ and ABA) are important molecules and are acting together. Plant growth regulators are effective not only in routine metabolic processes but also against environmental change. In salinity stresses, sodium nitroprussid is involved may use NO as a mediator. Generally, when the findings of leaves and roots were analysed comparatively, an increase in NO level was determined with NO donor SNP plus plant growth regulators treatment. When NaCl and/or SNP were treated, GA₃ levels decreased compared with control but IAA and ABA levels increased, and we could evaluate that SNP and NO may have protective effects against the negative effects of NaCl in the leaves and roots of sunflower via elevating plant growth regulators levels. Future studies should be evaluated other physiological and biochemical parameters in sensitive and tolerant cultivars whether beneficial effects of SNP in which cultivars under salt stress are.

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RELACJE MIĘDZY TLENKIEM AZOTU A HORMONAMI ROŚLINNYMI SŁONECZNIKA W OBECNOŚCI SNP W WARUNKACH STRESU SOLNEGO

Streszczenie. Tlenek azotu (NO) oraz nitroprusydek sodu (SNP) to molekuly odgrywające ważną rolę u zwierząt i roślin. SNP służy jako dawca tlenu azotu w obydwu grupach. NO może działać jako wolny rodnik i pogarszać ważne funkcje biomolekuł, poza tym ma dobry wpływ na zdrowienie po zasoleniu, suszy, itp. NO i SNP są dobrowolnymi ochronnymi molekułami, które radzą sobie z warunkami stresu. U roślin molekuly te są bardzo ważne i regulują wiele zjawisk fizjologicznych. W doświadczeniu badano poziomy kwasu abscysynowego (ABA), kwasu giberelinowego (GA₃) oraz NO w liściach i korzeniach słonecznika (*Helianthus annuus* L.), na którego działano NaCl, SNP oraz regulatorami wzrostu roślin. Zawartość NO była większa w liściach roślin pod wpływem GA₃ + SNP w 72 godzinie. Największy poziom IAA stwierdzono w korzeniach roślin pod wpływem IAA + SNP w 72 godzinie. W liściach roślin traktowanych za pomocą NaCl + ABA, ABA był większy w 72 godzinie, a poziomy GA₃ były równe lub mniejsze niż w grupie kontrolnej. Wnioskuje się, że jednoczesne zastosowanie SNP i regulatorów wzrostu pomaga sprostać stresowi zasolenia poprzez podniesienie poziomu wewnętrznego hormonu i NO w porównaniu z zasoleniem.

Słowa kluczowe: tlenek azotu, nitroprusydek sodu, zasolenie, słonecznik

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