The effect of pretreatment of Epibracinolide and Spermine on some growth, physiological parameters and SOS1 and NHX1 gene expressions in Pumpkin (*Cucurbita pepo* L.) under salinity stress

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Introduction:

Salinity stress causes changes in various physiological and metabolic processes and ultimately inhibits crop production (Rasool *et al*., 2022). The SOS1 gene, which encodes a plasma membrane Na^+/H^+ antiporter, is essential in regulating Na^+ efflux at the cell surface and also facilitates long-distance Na^+ transport from roots to shoots. Overexpression of this protein increases salt tolerance in plants (Shi *et al*., 2002). Intracellular NHX proteins are Na^+/H^+ or K^+/H^+ antiporters, which are important in K^+ homeostasis, endosomal pH regulation, and salt tolerance (Gupta & Huang, 2014). Brassinosteroids are important regulators of plant growth in multiple developmental processes and regulation and modulation of gene expression (Manghwar *et al*., 2022). Polyamines play an essential role in regulating plant defense responses to various environmental stresses, including metal toxicity, oxidative stress, drought, salinity, and cold stress (Liu *et al*., 2007). In this study, spermine was chosen because it is a tetra amine with a higher buffering capacity and less research has been done on spermine than the two polyamines putrescine and spermidine. The role of polyamines and brassinosteroids has been proven in reducing the effects of many environmental stresses, but there is limited information about their role in salinity stress resistance in pumpkin plants. Therefore, the aim of this study is to investigate the potential effect of spermine and epibrasinolide in reducing the effects of salinity in the medicinal pumpkin plant.

Materials & Methods:

Experiments were carried out in the greenhouse with the light/dark photoperiod of 16/8 h and 25/20 °C day/night temperature. The seeds of the pumpkin were germinated in Petri plates lined with two layers of filter paper moistened with sterile distilled water in the dark at 25 °C for 48 h. After emergence, seedlings were grown hydroponically in the plastic container filled with 6 L of one-half-strength Hoagland's nutrient solution and aerated using an air pump. After 5 days of growth, seedlings were pretreated with a different nutrient solution for 5 days: (a) control: one-half-strength Hoagland's nutrient solution; (b) EBL (Sigma–Aldrich, Germany) (0.01 μM); (c) EBL (0.1 μM); (d) Spm (0.1 mM (Sigma-Aldrich, Germany); (e) Spm (1 mM). After that, seedlings were treated with different concentrations of NaCl (0 mM as control, 40 mM, and 80 mM). The nutrient solution in each treatment was renewed every 2 days. The experiment was carried out in a randomized complete design with three replicates. Seven days after beginning of NaCl treatment, the fully expanded third leaves and the middle part of the roots were harvested, immediately frozen in liquid nitrogen, and stored at −80 °C until required for different analyses. The dry weight was obtained after drying at 70°C in an oven for 48 hours. Ions content were assayed by acid digestion method and with an atomic absorption Spectrometer (Spectra AA 220, Varian, Australia).

Analysis of gene expression was done by semi-quantitative RT-PCR. Total RNA was extracted from the fresh roots by RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Extracted RNA was quantified with a Nano Drop spectrophotometer and its quality was assessed by Agarose gel electrophoresis. To remove any contaminating genomic DNA prior to CDNA synthesis, total RNAs were treated with RNase-free DNaseI kit (Takara Bio Inc.) according to the manufacturer's instructions. In roots of pumpkin under different treatments, the expression pattern of SOS and NHX1 genes was analyzed by semi-quantitative RT-PCR with three repetitions. The EF1α housekeeping gene was used as reference gene to normalize the density of target genes bands for all semi-quantitative RT-PCR analyses. The relative density of bands was measured by ImageJ software. All data were subjected to ANOVA with SAS 9.1.3. Duncan's multiple range test was used to separate statistically different means at $P < 0.05$.

Results & discussion:

Salt stress decreased the dry weight of shoots and roots. Pretreatment with EBL and Spm increased the dry weight of shoots and roots, and 1 mM Spm and 0.1 µM EBL had a more significant effect on the dry weight of shoots and roots, respectively. Compared to the control, a significant increase in Na^+ accumulation was observed in the shoot and root under salt stress, and this increase was more in the root. Pre-treatment with EBL and Spm led to the reduction of shoot Na^+ under salt stress. 1 mM Spm and 0.01 µM EBL was more effective at 40 and 80 mM salinity, respectively. Pretreatment with both concentrations of EBL led to the reduction of root Na^+, and the effect of 0.01 µM EBL was more significant. Pretreatment with 0.1 mM Spm increased the Na^+ content of roots, which seems to be related to sodium transporter gene expression. Salinity stress significantly reduced shoot and root K^+ content compared to control, and this reduction was more in root. At 80 mM salinity, 0.1 mM Spm and both concentrations of EBL led to an increase in shoot K^+ content compared to control plants. Pretreatment with both EBL and Spm concentrations significantly increased root K^+ content at 80 mM salinity. Salt stress significantly reduced shoot and root Ca^{2+} compared to the control, and this reduction was greater in shoots. Pretreatment with EBL and Spm significantly increased the amount of Ca^{2+} in shoots and roots, and 0.01 µM EBL and 1 mM Spm had the greatest effect on shoot and root Ca^{2+}, respectively.

Salinity stress had no significant effect on NHX1 gene expression in roots compared to the control. Spm led to an increase in NHX1 gene expression, while EBL had no significant effect on the expression of this gene. Salt stress led to the upregulation of SOS1 gene expression in roots. Pretreatment of both EBL and Spm concentrations led to a significant decrease in the expression of this gene, and the effect of EBL was more significant.

Excessive absorption of salt causes osmotic stress, specific ionic toxicity and ionic imbalance and thus reduces plant growth (Rasool *et al*., 2022). Salinity stress not only reduces the accessibility of potassium and calcium, but also reduces the transfer and mobility of calcium and potassium to the growing areas of the plant (Zhou *et al*., 2019). The role of brassinosteroid on the improvement of growth parameters through the effect on the photosynthetic apparatus, Rubisco enzyme activity, the amount of photosynthetic pigments, stomatal conductance, antioxidant defense system, reduction of oxidative stress, increase in the correlation of biological membranes (reduction of ion leakage), nitrogen metabolism and mineral nutrition of the plant has been reported. (Fariduddin *et al*., 2014). Polyamines affect ion transport by interacting with plasma membrane phospholipids and promoting and maintaining membrane stability and affecting membrane ion transporters. Polyamines, especially Spm, appear to interact with numerous anionic molecules, such as DNA, RNA, proteins, and membrane lipids, due to their polycationic nature. Therefore, Spm may modulate the surface charge and consequently regulate membrane permeability and stability (Hussain *et al*., 2011).

Brassinosteroids have been shown to play a role in turgor or proton pump-induced cell development and modulating stress tolerance through increasing the activity of aquaporins (Morillon *et al*., 2001). SOS1 is essential for the homeostasis of both Na^+ and K^+ ions in salt stress. SOS1 encodes a plasma membrane Na^+/H^+ receptor that is responsible for the sodium release into the apoplast (Olias *et al*., 2009). It has been reported that the ability of wild tomato plants to retain Na^+ in stems and thus prevent Na^+ from entering the Photosynthetic tissues are largely dependent on SOS1 function (Hauser & Horie, 2010). Both changes in gene expression levels and activation of existing proteins involved in K^+ transport and coding are necessary to maintain cytosolic K^+ homeostasis in saline conditions (Zepeda-Jazo *et al*., 2008). Therefore, post-translational regulation and modulation of the activity of existing channels or transporters by multiple factors and secondary messengers (including polyamines) is of particular importance. Polyamines may act not only as scavengers of ROS, but also as activators of the expression of genes encoding Na^+/H^+ antireceptors.

Conclusion:

Salinity stress caused disturbances in the absorption and transfer of ions, decreased growth parameters and increased expression of SOS1 gene in pumpkin plants. Pretreatment with Spm and EBL has led to salinity resistance by maintaining the integrity of biological membranes, regulating ion absorption and improving the nutritional status of pumpkin plants. . Spm seems to lead to more vacuolar encoding of Na+, improvement of cytosolic K+/Na+ homeostasis and inhibition of sodium ion transport from roots to sensitive parts of shoots through increasing NHX1 gene expression and EBL have been effective in mitigating salinity stress by affecting the production of lateral roots and increasing the root surface.

Keywords: *Polyamine, Plant growth regulator, Osmotic stress, Sodium transporter gene*

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Statement on ethics: *All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.*