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SHORT COMMUNICATION

# Exogenous salicylic acid-induced nitric oxide regulates leaf water condition through root osmoregulation of maize seedlings under drought stress

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**Abstract** Under drought stress, the role of nitric oxide (NO) in the regulation of leaf water condition by salicylic acid (SA) through root osmoregulation of maize (*Zea mays* L.) seedlings was investigated. The results showed that drought stress markedly increased the contents of NO, soluble sugar, proline, soluble protein, $Na^+$, $K^+$ and $Ca^{2+}$, as well as the activity of plasmalemma $H^+$-ATPase in roots, compared with control. However, drought stress significantly decreased root hydraulic conductivity and leaf relative water content (RWC). Exogenous SA under drought stress significantly increased above indicators, compared with drought stress alone. Above effects of SA were significantly inhibited by the pretreatment with NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). Overall, the findings indicated that SA-induced NO participated in the regulation of leaf water condition through root osmoregulation of maize seedlings under drought stress.

**Keywords** Drought tolerance · Osmotic adjustment · Signal molecule · Water balance · *Zea mays*

## Introduction

Drought stress usually causes osmotic stress, which disturbs the water balance of plants (Yang et al. 2015). Under osmotic stress, plants can maintain their water balance by increasing the contents of osmolytes, such as soluble sugar, proline, $Na^+$ and $Ca^{2+}$ (Yang et al. 2015). It is well known that roots are the important and foremost organs that respond to water deficit conditions. It has been reported that plants could enhance their osmotic adjustment ability by increasing the contents of osmolytes in roots (Barcia et al. 2014). Maize is very important crop in the world as a staple food. However, drought stress severely disrupted the growth and production of maize (Ali and Ashraf 2011). Up to now, the studies related to the osmoregulation in maize roots by exogenous use of growth regulators under drought stress are still very limited.

Salicylic acid (SA) is regarded as an important plant growth regulator. It can regulate plant growth and development not only under non-stressed conditions, as well as under stressful environments (Kang et al. 2013; He et al. 2014; Khoshbakht and Asgharei 2015). It has been documented that SA enhanced the drought tolerance in wheat by increasing the contents of osmolytes (Loutfy et al. 2012). However, the signal regulatory mechanism of the osmoregulation by SA is still unclear. Therefore, the present study was aimed to find out the signal regulatory mechanism of the osmoregulation in maize roots by exogenous SA.

Nitric oxide (NO) is an important gaseous regulator in plants. Many studies showed that NO could regulate the drought tolerance of plants (Fan and Liu 2012; Xiong et al. 2012; Shan et al. 2015). Previous studies about the regulation of drought tolerance by NO were focused on the regulation of antioxidant systems by NO (Fan and Liu 2012; Xiong et al. 2012; Shan et al. 2012, 2015). There are also few studies about the osmoregulation in plants by NO under drought stress, which indicated that NO mainly regulated the water relation of plants through the proline accumulation (López-Carrión et al. 2008; Xiong et al. 2012). However, there is still very little knowledge for the root osmoregulation by NO. It has been reported that NO

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participated in the signal transduction of SA in regulating the stomatal movement (Liu et al. 2003). However, whether NO participates in the osmoregulation of maize by SA is unclear, especially in root osmoregulation. Thus, it will be necessary to study the role of NO in the osmoregulation of maize root by exogenous SA under drought stress.

In the present study, we measured the contents of NO, soluble sugar, soluble protein, proline, $Na^+$, $K^+$ and $Ca^{2+}$, the activity of plasmalemma $H^+$-ATPase in roots, root hydraulic conductivity and leaf RWC of maize seedlings under drought stress induced by 10% polyethylene glycol (PEG)-6000. The aim of this study was to provide new insight into the role of NO in root osmoregulation by exogenous SA under drought stress.

## Materials and methods

**Plant culture conditions and treatments** – Seeds of *Zea mays* L. (maize) cultivar Xindan 29 were germinated in distilled water and grown in the artificial climate chamber. The culture conditions were as follows. The temperature was 25/15 °C (day/night). The available photosynthetic active radiation was 500 μmol m$^{-2}$ s$^{-1}$. The photoperiod was 10-h. Eight days after germination, seedlings were transferred to plastic boxes filled with full-strength Hoagland's solution. The full-strength Hoagland's solution was exchanged every other day. Eighteen days old after germination, seedlings with similar growth vigor and height were selected for further experiments.

To study the effect of drought stress, the roots of seedlings were placed in beakers containing 50 ml 10% (W/V) PEG (molecular weight 6000) solution for 48 h under above conditions. To study the effects of SA, the roots of seedlings were pretreated with 10 μM SA for 24 h and then exposed to PEG for 48 h. To study the effect of NO scavenger [cPTIO], the roots of seedlings were pretreated with 100 μM cPTIO or 10 μM SA + 100 μM cPTIO for 24 h and then exposed to PEG for 48 h. The roots of control seedlings were treated with full-strength Hoagland's solution alone. After treatment of 24 and 48 h, the contents of NO, soluble sugar, soluble protein, proline, $Na^+$, $K^+$ and $Ca^{2+}$, the activity of plasmalemma $H^+$-ATPase in roots, root hydraulic conductivity and leaf RWC were determined. In order to study the effects of SA, cPTIO, cPTIO + SA on the root morphological characters, such as root surface area, root volume and root length per plant, were investigated by using root scanner (STD1600 Epson, USA) and root analysis system (WinRhizo Regent Instruments, Canada) after treatment of 144 h. After treatment of 144 h, the root dry weight was also measured after oven-drying at 80 °C for 48 h.

**Determination of NO, soluble sugar, soluble protein, proline contents** – NO content was estimated by measuring the absorption value at 540 nm by using Greiss assay following Zhou et al. (2005). Soluble sugars were determined following the method described by Wei (2009). Soluble protein content was measured by using bovine serum albumin as standard according to the method of Bradford (1976). Proline content was determined following the method of Bates et al. (1973).

**Determination of $Na^+$, $K^+$ and $Ca^{2+}$** – Each root sample (100 mg dry weight) was digested using the digestion mixture and the final volume (50 ml) was maintained in a volumetric flask with distilled water. The contents of $Na^+$, $K^+$ and $Ca^{2+}$ were determined by using an atomic absorption spectrophotometer following Jamil et al. (2015).

**Determination of plasmalemma $H^+$-ATPase activity** – Plasmalemma vesicles were purified by sucrose gradient centrifugation methods according to the method described by Du et al. (2015). $H^+$-ATPase activity was assayed by measuring inorganic phosphate from ATP following the method of Qiu and Su (1998).

**Determination of root hydraulic conductivity and leaf RWC** – Root hydraulic conductivity was measured according to the method of Shimizu et al. (2005). Leaf RWC was determined following the method of Barrs and Weatherley (1962). RWC was calculated using the equation: RWC = [(FW − DW)/(TW − DW)] × 100.

**Statistical analysis** – The data presented is the mean of six replications. Means were compared by one-way analysis of variance and Duncan's multiple range test at the 5% level of significance.

## Results

**The selection of suitable SA treatment concentration under drought stress** – Compared with control, drought stress significantly decreased the root hydraulic conductivity and leaf RWC (Table 1). Different concentrations of SA under drought stress significantly increased the root hydraulic conductivity and leaf RWC, compared with drought stress alone. The application of 10 μM SA under drought stress significantly increased root hydraulic conductivity and leaf RWC, compared with other SA concentrations. These results suggested that 10 μM SA was a suitable concentration to study the effect of SA on the root hydraulic conductivity and leaf RWC of maize seedlings under drought stress.

**The selection of suitable cPTIO treatment concentration under drought stress with or without SA** – There was a dose–response for the effects of different cPTIO concentrations on root hydraulic conductivity and leaf

**Table 1** Effects of different concentrations of SA on maize root hydraulic conductivity and maize leaf RWC under drought stress

| Treatments | Root hydraulic conductivity ($\times 10^{-7}$ ms$^{-1}$ MPa$^{-1}$) | Leaf RWC (%) |
|---|---|---|
| Control | $7.5 \pm 0.86^a$ | $93.8 \pm 10.36^a$ |
| Drought | $4.1 \pm 0.50^d$ | $85.0 \pm 8.57^d$ |
| 5 μM SA + drought | $5.0 \pm 0.46^c$ | $87.3 \pm 7.11^c$ |
| 10 μM SA + drought | $6.1 \pm 0.63^b$ | $90.2 \pm 9.75^b$ |
| 30 μM SA + drought | $6.3 \pm 0.71^b$ | $89.6 \pm 8.05^b$ |

Values are presented as mean ± standard deviations (SD) of six replicates for each treatment. Different letters with means indicate statistical difference at $P < 0.05$

RWC (Table 2). Compared with other cPTIO concentrations, the application of 100 and 150 μM cPTIO with or without SA significantly increased the root hydraulic conductivity and leaf RWC under drought stress. There was no significant difference in root hydraulic conductivity and leaf RWC between 100 and 150 μM cPTIO. These results suggested that 100 μM cPTIO was a suitable concentration for this study.

**Effects of SA and cPTIO on NO content in roots under drought stress** – Drought stress markedly increased the NO content in roots, compared with control (Fig. 1). Compared with drought stress alone, exogenous SA under drought stress significantly induced the production of NO in roots. Pretreatment with cPTIO significantly reduced NO content induced by drought stress alone or drought stress plus SA. These results suggested that exogenous SA plus drought stress could enhance the accumulation of NO in roots.

**Effects of SA and cPTIO on the contents of organic osmolytes in roots under drought stress** – Drought stress increased the contents of soluble sugars, soluble proteins and proline in roots, compared with control (Fig. 2). Compared with drought stress alone, SA plus drought stress markedly increased the contents of soluble sugars, soluble

proteins and proline in roots. The application of cPTIO markedly decreased the contents of soluble sugars, soluble proteins and proline in roots under drought stress alone or drought stress plus SA. These results suggested that NO participated in the regulation of the contents of organic osmolytes by exogenous SA under drought stress.

**Effects of SA and cPTIO on the contents of inorganic osmolytes in roots under drought stress** – Drought stress markedly increased the contents of $Na^+$, $K^+$ and $Ca^{2+}$ in roots, compared with control (Table 3). Drought stress plus SA further increased the contents of $Na^+$, $K^+$ and $Ca^{2+}$ in roots, compared with drought stress alone. The application of cPTIO with or without SA markedly decreased the contents of $Na^+$, $K^+$ and $Ca^{2+}$ in roots under drought stress. These results indicated that NO played an important role in regulating the contents of $Na^+$, $K^+$ and $Ca^{2+}$ in roots under drought stress alone or drought stress plus SA.

**Effects of SA and cPTIO on the activity of plasmalemma $H^+$-ATPase in roots, root hydraulic conductivity and leaf RWC under drought stress** – Drought stress increased the activity of plasmalemma $H^+$-ATPase in roots, compared with control (Table 4). Compared with drought stress alone, drought stress plus SA significantly increased the activity of plasmalemma $H^+$-ATPase in

**Table 2** Effects of different concentrations of cPTIO on maize root hydraulic conductivity and maize leaf RWC under drought stress

| Treatments | Root hydraulic conductivity ($\times 10^{-7}$ ms$^{-1}$ MPa$^{-1}$) | Leaf RWC (%) |
|---|---|---|
| Control | $6.8 \pm 0.57^a$ | $92.5 \pm 9.95^a$ |
| Drought | $3.7 \pm 0.40^c$ | $85.0 \pm 7.06^d$ |
| 50 μM cPTIO + drought | $3.1 \pm 0.30^d$ | $84.3 \pm 8.57^d$ |
| 100 μM cPTIO + drought | $2.5 \pm 0.22^e$ | $82.5 \pm 9.34^e$ |
| 150 μM cPTIO + drought | $2.3 \pm 0.25^e$ | $83.0 \pm 8.33^e$ |
| 10 μM SA + drought | $5.3 \pm 0.61^b$ | $89.7 \pm 10.25^b$ |
| 50uM cPTIO + 10 μM SA + drought | $4.0 \pm 0.36^c$ | $87.4 \pm 10.48^c$ |
| 100 μM cPTIO +10 μM SA + drought | $3.1 \pm 0.36^d$ | $84.8 \pm 7.90^d$ |
| 150 μM cPTIO + 10 μM SA + drought | $2.9 \pm 0.27^d$ | $85.2 \pm 8.50^d$ |

Values are presented as mean ± standard deviations (SD) of six replicates for each treatment. Different letters with means indicate statistical difference at $P < 0.05$
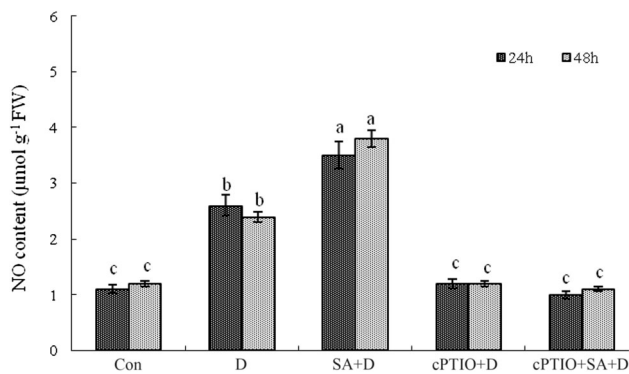
Fig. 1 Effects of exogenous SA and cPTIO on NO content in roots of maize seedlings under drought stress. Values are presented as mean ± standard deviations (SD) of six replicates for each treatment. *Different letters* with means indicate statistical difference at $P < 0.05$

roots. Application of cPTIO with or without SA markedly decreased the activity of plasmalemma $H^+$-ATPase in roots under drought stress, compared with drought stress alone. These findings suggested that SA-induced NO plays an important role in regulating the activity of plasmalemma $H^+$-ATPase in roots.

Drought stress decreased the root hydraulic conductivity and leaf RWC, compared with control (Table 4). Compared with drought alone, drought stress plus SA significantly increased root hydraulic conductivity and leaf RWC under. Application of cPTIO with or without SA markedly decreased root hydraulic conductivity and leaf RWC under drought stress, compared with drought stress alone. These findings suggested that SA-induced NO played an important role in maintaining leaf water conditions.

**Effects of SA and cPTIO on the root morphological structure and dry weight of maize seedlings under drought stress** – Drought stress increased the root surface area, root volume, root length and root dry weight per plant, compared with control (Table 5). Compared with drought stress alone, drought stress plus SA significantly further increased the root surface area, root volume, root length and root dry weight per plant. Application of cPTIO with or without SA markedly decreased the root surface area, root volume, root length and root dry weight per plant under drought stress, compared with drought stress alone. These findings suggested that SA-induced NO plays an important role in regulating the root growth of maize seedlings under drought stress.

## Discussion

Drought stress is a condition when the soil water potential is lower than the plant cell water potential. The direct effect of drought stress on plants is the limitation of root water absorption, which disturbs the plant water balance and
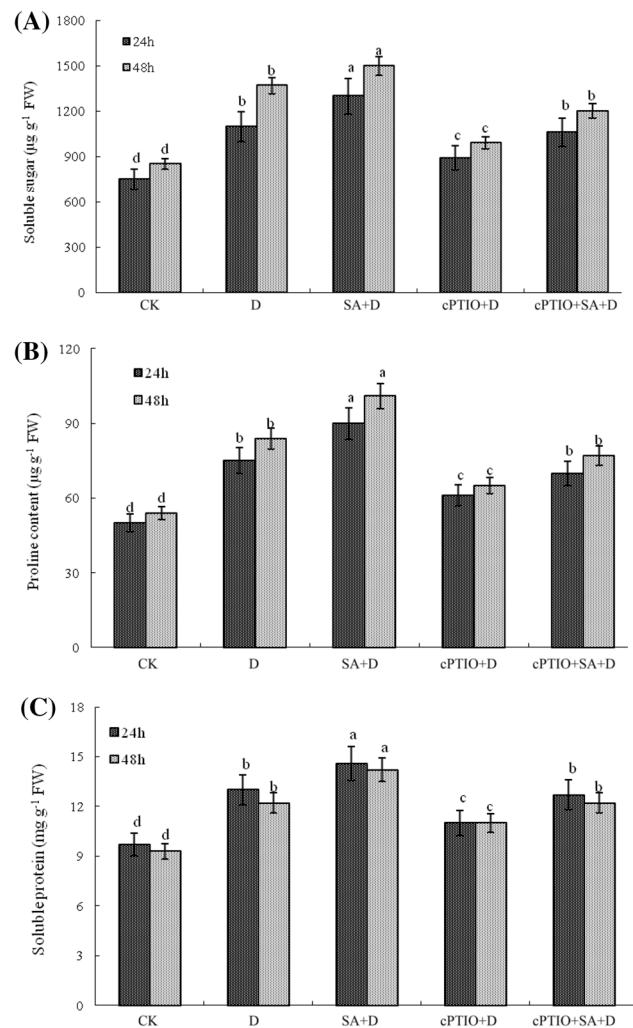
Fig. 2 Effects of exogenous SA and cPTIO on the contents of soluble sugar, soluble protein and proline in maize roots under drought stress. Values are presented as mean ± standard deviations (SD) of six replicates for treatment. *Different letters* with means indicate statistical difference at $P < 0.05$

inhibits plant growth. It has been documented that plants could reduce the cell water potential of roots by increasing the contents of inorganic osmolytes ($Na^+$, $K^+$ and $Ca^{2+}$) and organic osmolytes (soluble sugars, soluble proteins and proline) under drought stress. However, the osmotic adjustment ability of plants is very limited only through their own responses to drought stress. Now, increasing studies showed that exogenous regulating substances could further enhance the osmotic adjustment ability by increasing the contents of osmoregulatants under drought stress, such as glycinebetaine and gibberellins (Yang et al. 2010; Gupta et al. 2014). In the present study, our results showed that SA could enhance the osmotic adjustment ability of plants exposed to drought stress by increasing the contents of osmoregulatants in roots, which, in turn, increased root hydraulic conductivity and leaf RWC.

**Table 3** Effects of exogenous SA and cPTIO on the contents of Na$^+$, K$^+$ and Ca$^{2+}$ in maize roots under drought stress

| Treatments | Time (h) | Na$^+$ content (mg g$^{-1}$ DW) | K$^+$ content (mg g$^{-1}$ DW) | Ca$^{2+}$ content (mg g$^{-1}$ DW) |
|---|---|---|---|---|
| Control | 24 | 1.52 ± 0.21[f] | 6.25 ± 0.72[e] | 1.21 ± 0.14[f] |
| | 48 | 1.80 ± 0.18[f] | 7.05 ± 0.64[e] | 1.42 ± 0.15[e] |
| Drought | 24 | 3.91 ± 0.38[d] | 10.40 ± 1.19[c] | 2.05 ± 0.17[d] |
| | 48 | 5.38 ± 0.47[b] | 13.09 ± 1.32[b] | 2.49 ± 0.23[c] |
| 100 μM cPTIO + drought | 24 | 2.90 ± 0.25[e] | 8.25 ± 0.80[d] | 1.38 ± 0.12[e] |
| | 48 | 3.55 ± 0.33[d] | 9.00 ± 0.83[d] | 1.49 ± 0.16[e] |
| 10 μM SA + drought | 24 | 5.64 ± 0.52[b] | 14.65 ± 1.50[a] | 2.73 ± 0.30[b] |
| | 48 | 7.36 ± 0.66[a] | 17.37 ± 1.63[a] | 3.35 ± 0.38[a] |
| 100 μM cPTIO + 10 μM SA + drought | 24 | 3.53 ± 0.40[d] | 10.04 ± 0.95[c] | 1.59 ± 0.14[e] |
| | 48 | 4.44 ± 0.38[c] | 11.17 ± 1.06[c] | 1.89 ± 0.20[d] |

Values are presented as mean ± standard deviations (SD) of six replicates for each treatment. Different letters with means indicate statistical difference at $P < 0.05$

**Table 4** Effects of exogenous SA and cPTIO on maize root plasmalemma H$^+$-ATPase activity, maize root hydraulic conductivity and maize leaf RWC under drought stress

| Treatments | Time (h) | Plasmalemma H$^+$-ATPase activity (μmol mg$^{-1}$ min$^{-1}$) | Root hydraulic conductivity (×10$^{-7}$ ms$^{-1}$ MPa$^{-1}$) | Leaf RWC (%) |
|---|---|---|---|---|
| Control | 24 | 0.31 ± 0.03[d] | 6.0 ± 0.63[a] | 93.1 ± 9.07[a] |
| | 48 | 0.29 ± 0.03[d] | 6.5 ± 0.75[a] | 92.2 ± 9.58[a] |
| Drought | 24 | 0.55 ± 0.08[b] | 3.3 ± 0.36[c] | 85.5 ± 8.11[c] |
| | 48 | 0.52 ± 0.06[b] | 4.0 ± 0.35[c] | 84.9 ± 9.12[c] |
| 100 μM cPTIO + drought | 24 | 0.39 ± 0.05[c] | 2.3 ± 0.30[d] | 82.0 ± 8.05[d] |
| | 48 | 0.37 ± 0.04[c] | 3.0 ± 0.34[d] | 82.7 ± 7.29[d] |
| 10 μM SA + drought | 24 | 0.73 ± 0.10[a] | 4.5 ± 0.54[b] | 88.8 ± 9.31[b] |
| | 48 | 0.79 ± 0.08[a] | 5.3 ± 0.62[b] | 89.5 ± 8.06[b] |
| 100 μM cPTIO + 10 μM SA + drought | 24 | 0.44 ± 0.06[c] | 3.0 ± 0.37[c] | 84.5 ± 7.36[c] |
| | 48 | 0.43 ± 0.04[c] | 3.7 ± 0.37[c] | 85.0 ± 9.30[c] |

Values are presented as mean ± standard deviations (SD) of six replicates for each treatment. Different letters with means indicate statistical difference at $P < 0.05$

Therefore, our results suggested that SA may be used as a regulator to improve the osmotic adjustment ability of plants. However, Loutfy et al. (2012) reported that SA decreased the content of proline in wheat roots, which is not consistent with our results in the present study. This difference may be due to the difference in species (Loutfy et al. 2012).

It has been shown that drought stress and exogenous SA can induce the production of NO and endogenous NO is involved in the signal transduction of SA in the process of stomatal closure (Liu et al. 2003; Shan et al. 2012). In the present study, we found that exogenous SA and drought stress also induced the production of NO in regulating the contents of osmoregulatants. Meanwhile, NO induced by SA increased the contents of osmoregulatants, root hydraulic conductivity and leaf RWC under drought stress, compared with drought stress alone. Above results

indicated that NO played an important role in SA signaling in regulating the osmotic adjustment ability of roots under drought stress.

Plasmalemma H$^+$-ATPase has important roles in regulating plant growth and responses to stresses. Particularly, the stress tolerance of plants is closely associated with the activity of plasmalemma H$^+$-ATPase. It has been documented that drought stress increased the activity of plasmalemma H$^+$-ATPase in the developing embryos of wheat (Du et al. 2015). In the present study, our results also showed that drought stress increased the activity of plasmalemma H$^+$-ATPase of maize roots. Besides, our results showed that SA could further increase the activity of plasmalemma H$^+$-ATPase under drought stress, compared with drought alone. So, the results of the present study indicated that the improvement in the osmotic adjustment ability of roots regulated by SA was due to the

**Table 5** Effects of exogenous SA and cPTIO on the morphological structure and dry weight of maize seedlings roots per plant under drought stress

| Treatments | Root surface area (cm$^2$) | Root volume (cm$^3$) | Root length (cm) | Root dry weight (g) |
|---|---|---|---|---|
| Control | 26.7 ± 3.11[c] | 0.92 ± 0.10[c] | 72.7 ± 6.35[c] | 0.19 ± 0.02[c] |
| Drought | 34.0 ± 3.73[b] | 1.12 ± 0.13[b] | 89.2 ± 8.33[b] | 0.26 ± 0.03[b] |
| 100 μM cPTIO + drought | 28.3 ± 2.69[c] | 0.95 ± 0.11[c] | 75.3 ± 6.08[c] | 0.20 ± 0.02[c] |
| 10 μM SA + drought | 40.6 ± 3.91[a] | 1.33 ± 0.15[a] | 100.8 ± 11.74[a] | 0.32 ± 0.04[a] |
| 100 μM cPTIO + 10 μM SA + drought | 29.0 ± 3.04[c] | 1.01 ± 0.11[c] | 78.7 ± 9.31[c] | 0.20 ± 0.03[c] |

Values are presented as mean ± standard deviations (SD) of six replicates for each treatment. Different letters with means indicate statistical difference at $P < 0.05$

enhancement of plasmalemma H$^+$-ATPase activity in roots under drought stress, compared with drought stress alone. In the present study, lower water uptake from root to shoot was happened which induced less availability of nutrient in the rooting medium for maize seedlings. However, the nutrient uptake was increased in drought-stressed maize plants. This phenomenon might be due to the increase in the activity of plasmalemma H$^+$-ATPase in roots under drought stress, which, in turn, enhanced the absorption of the inorganic osmolytes, including Na$^+$, K$^+$ and Ca$^{2+}$.

Previous studies of other researchers showed that signal molecules H$_2$O$_2$, Ca$^{2+}$ and protein kinases participated in the signal transduction of SA in regulating the stomatal movement of plants (Dong et al. 2001; Liu et al. 2003). In the present study, we found that NO participated in the signal transduction of SA in regulating the osmotic adjustment ability of roots under drought stress. However, whether there are relationships between NO and other signal molecules in the signal transduction of SA in regulating the osmotic adjustment ability of roots remains unclear. Therefore, it is very interesting to investigate the crosstalks between NO and other signal molecules in regulating the osmotic adjustment ability of roots by SA, which will provide more new insight to the role of SA in regulating the osmotic adjustment ability of roots under drought stress.

In conclusion, our findings indicated that SA-induced NO participated in the regulation of the osmotic adjustment ability and plasmalemma H$^+$-ATPase activity in roots, which, in turn, maintained root hydraulic conductivity, leaf RWC and water balance under drought stress. These findings provide new insight to the osmotic adjustment ability regulated by SA under drought stress.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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