The Effects of Exogenous Pyridoxal-5-phosphate on Seedling Growth and Development of Wheat under Salt Stress

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Salt stress is one of the major abiotic stress which severely limits plant growth and reduces crop productivity across the world. In the present study, the effects of exogenous pyridoxal-5-phosphate (vitamin B\textsubscript{6}, VB\textsubscript{6}) on seedling growth and development of wheat under salt stress were investigated. The results showed that exogenous application of pyridoxal-5-phosphate (VB\textsubscript{6}) significantly increased the RWC, biomass, the concentration of photosynthetic pigments, proline, the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), together with decreasing the content of Malondiadehyde (MDA) and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) in wheat leaves under salt stress. Meanwhile, the transcript level of \textit{P5CR}, \textit{P5CS}, \textit{SOD}, \textit{TaSOS1} and \textit{TaSOS4} were also up-regulated after treatment with pyridoxal-5-phosphate. VB\textsubscript{6} acts as a signal in regulating the activities of plant antioxidant enzymes and SOS pathway to improve resistance to salt stress. The current study results may give an insight into the regulatory roles of VB\textsubscript{6} in improving salt stress and VB\textsubscript{6} could be an easily and effective method to improve salt-stress tolerance to wheat in the field condition. It is urgency to understand the molecular mechanism of VB\textsubscript{6} to enhance the salt tolerance of wheat in the next work.

\textbf{Keywords:} pyridoxol-5-phosphate, vitamin B\textsubscript{6}, salt stress, wheat, \textit{Triticum aestivum}

\section*{Introduction}

Salinity soil is a worldwide problem threatening crop production and leads to reduction in the economic yield of a wide variety of crops in mostly sea costal area across the world. (Askari et al. 2006; Munns and Gilliham 2015). In previous study, it was estimated that 150 million ha cultivated land out of 0.77 billion ha (about 5\%) was affected by soil salinity (Askari et al. 2006). On the other hand, about 27 million ha of salted land in China, out of these 0.06 billion ha is cultivated land (accounting for 8.5\%) affecting by salt stress (Li et al. 2005; Wang et al. 2011).

Plant growth and development can be affected by salinity stress at any time during the crop life cycle (Arzani 2008). Salt soil will disrupt homeostasis, physiological and biochemical processes in cells (Gururani et al. 2013). It also impacts cell membrane stability, photosynthesis, the concentration of chlorophyll, protein content and relative water con-

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tent (RWC) in plants (Talaat and Shawky 2014). Excessive amounts of sodium (Na\(^+\)) and Cl\(^-\) in soil can adversely affect metabolism of plant cells (Shalata and Neumann 2001). Salt-stress conditions also lead to accumulation of reactive oxygen species (ROS) and lipid peroxidation products in plant roots, stems and leaves (Shalata and Neumann 2001). ROS like superoxide radical (O\(^2-\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals (OH\(^-\)) cause lipid peroxidation of plant cell membranes (Gururani et al. 2013). To mitigate its (ROS) damage to cells, plants activate a range of enzymatic and non-enzymatic defense systems to alleviate cellular damage under salinity stress conditions (Hasanuzzaman et al. 2011).

Many studies have shown that applying suitable concentration of endogenous substances (plant growth regulator) can improve salt tolerance in plants. Such as jasmonic acid (JA) (Qiu et al. 2014), Salicylic Acid (SA) (Arfan et al. 2007), ascorbic acid (AsA) (Shalata and Neumann 2001), mannitol (Seckin et al. 2009), and 24-epibrassinolide (Dong et al. 2017) can inhibit accumulation of active oxygen species (ROS) and alleviate NaCl damages to plants. Therefore, exogenous application of plant growth regulator gives an effective way to enhance plant resistance in abiotic stress, but the regulating mechanism of such endogenous substances in plant growth remains unclear.

Vitamin B\(_6\) is one of the essential cofactors for numerous metabolic enzymatic reactions and is considered as a potent antioxidant in plant organisms (Tambasco-Studart et al. 2005; Titiz et al. 2006). VB\(_6\) includes pyridoxine-5-phosphate (PLP) which constitutes the active form of VB\(_6\) (Herrero and Daub 2007). As the active intracellular form, PLP has multiple roles as a versatile cofactor that almost exclusively functions in the metabolism of amino compounds. Wang et al. (2004) reported that pyridoxal kinase is a salt tolerance determinant important for the regulation of Na\(^+\) and K\(^+\) homeostasis in plants. The new feature of VB\(_6\) as a ROS scavenger, and its potential ability to increase resistance to both biotic and abiotic stresses, has opened up new directions of plant VB\(_6\) research in photosynthesis and pathogen-response (Mooney and Hellmann 2010). Wheat (Triticum aestivum L.) is the second major crop planted in the world (Bhardwaj 2010). Saline soil limits wheat production by reducing plants biomass, RWC, photosynthetic pigments, proline, the activities of peroxidase in wheat leaves as other crops (Egamberdieva 2009; Qiu et al. 2014). Therefore, the objective of this study was to investigate the regulatory functions and mechanism of exogenous VB\(_6\) in wheat seedlings under salt stress.

**Materials and Methods**

The wheat seeds (Triticum aestivum L.) used in this study is Chuanyu23 (Fig. S4*) from Chengdu Institute of Biology, Chinese Academy of Sciences, which is one of the varieties certificated by Sichuan province in 2003. Wheat seeds were surface sterilized with 10% (v/v) sodium hypochlorite solution for 10 mins, then vigorously rinsed with distilled water (>200 ml/per time) for 5 times. Sterilized seeds were sown in plug tray (10 cm × 12 cm pot) and arranged in illumination incubator with 25 °C 16 h light (600 μmol m\(^{-2}\) s\(^{-1}\)),

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.
18 °C 8 h darkness, 65% relative humidity (Dong et al. 2017). Seven days after sowing, plants at the same growth stage were selected and transplanted to containers filled with Hoagland solution. Plants were added with water or with pyridoxine-5-phosphate (VB₆). The treatments is given as follows: (1) 0 mM NaCl + 0 mM VB₆ (CK); (2) 200 mM NaCl + 0 mM VB₆ (NaCl); (3) 0 mM NaCl + 100 mM VB₆; (4) 200 mM NaCl + 100 mM VB₆ (NaCl). The nutrient solution was adjusted to pH 6.5–6.8. The treatment solution was changed daily to maintain constant NaCl concentration. The plants were sampled at 7 ds after treatment.

Measurement of biomass and physiological index in wheat seedlings

The current study measured height of seedlings, root length, fresh and dry weight of Chuanyu23 seedlings after 14 d of germination in Figure S1. The seedlings were dried at 80 °C for 2 days for dry weight measurement, and their final dry weights were measured in Figure S2. For relative water content was calculated (Fig. S3) as follow: RWC(%) = (FW – DW)/FW × 100 (Dong et al. 2017). The chlorophyll content (Song et al. 2016) was determined (Fig. S3). Lipid peroxidation was evaluated by measuring malondialdehyde content (MDA). According to the method (Rao and Sresty 2000), MDA content was determined (slightly modified). H₂O₂ content was determined according to Shi et al. (2005). Proline concentration (Bates et al. 1973) was determined. Superoxide dismutase (SOD) activity (Giannopolitis and Ries 1977) was measuring at 560 nm. Peroxidase (POD) activity (Kochba et al. 1977) was measured by the increase in absorbance at 470 nm. Catalase (CAT) activity (Cakmak and Horst 1991) was measured as the decline in absorbance at 240 nm. RNA extraction use TransZol™ Up Plus RNA kit (from TransGen Biotech). The cDNA was synthesized from total RNA using a first strand cDNA synthesis kit (from TransGen Biotech). Primers used for the relative quantification of biosynthetic gene transcripts in Table S1.

Statistical analysis

SPSS 20.0 statistical analysis software, Graphpad software 5.0 and Microsoft Excel 2013 software were used to analyze all the experimental data in this study. One-way analysis of variance (ANOVA) was conducted to evaluate the variance and significance between groups. Differences between treatments were separated by the least significant difference (LSD) test at 0.05 level.

Result

The biomass index of wheat seedling

The current study resulted that salt stress suppressed the growth of the wheat seedlings. The biomass of wheat roots and shoots were reduced (Fig. 1, Figs S1, S2) under salt stress. However, adding 200 mM VB₆ were mitigate wheat plant growth inhibition by salt.
Figure 1. The growth of wheat seedlings under VB₆, salt treatment and control.
Figure 2. The contents of H$_2$O$_2$ and MDA in wheat leafs, bars with different letters are significantly different at 5% level (p < 0.05).

Figure 3. The enzyme activity of POD, CAT and SOD in the wheat seedlings, and the SOD expression patterns, bars with different letters are significantly different at 5% level (p < 0.05).
stress (Figs S1, S2). This results showed that exogenous application of VB₆ effectively alleviated growth suppression in the wheat seedlings under salt stress.

Photosynthetic pigments and relative water content of wheat seedling

With salt treatment the content of chlorophyll a, chlorophyll b, carotenoid and RWC significantly decreased in wheat seedlings (Fig. S3). The VB₆ + Salt treatment increased photosynthetic pigments and RWC content. These results suggest that exogenous VB₆ can alleviate the degradation of chlorophyll and the loss of cell water in wheat under salt stress.

Wheat seedling lipid peroxide

The content of H₂O₂ and MDA in plant tissues is an important indicator to measure the reactive oxygen species and damage of plant cells under salt stress condition. The contents of H₂O₂ and MDA increased significantly in Chuanyu23 seedlings with salt or salt + VB₆ treatment (Fig. 2). For the salt treatment, the H₂O₂ and MDA contents were about 2 and 2.8-fold higher compare with control. But significantly decreased with the application of VB₆ (Fig. 2). The result demonstrates that application VB₆ under salt stress is beneficial to reduce the reactive oxygen species in plant cell and diminish the damage of plant cell membrane.

Activity of peroxidase in wheat seedling

After application of VB₆, the enzyme activity of POD, CAT and SOD in the wheat seedlings was higher than that in the salt treatment alone (Fig. 3). When the wheat seedlings were treated with the 200 mM NaCl, the enzyme activity of POD, CAT and SOD increased. But the increase was even more significant after application of VB₆. The results indicated that application VB₆ under salt stress can effectively increase the activity of POD, CAT and SOD enzymes, and remove more free radicals under salt stress, then reduce cell membrane damage, and improve the plant resistance to salt stress.

To further explore the mechanism of VB₆ in improving plant resistance to salt stress, RT-qPCR was conducted to analyze the expression patterns of the antioxidant gene SOD. Interestingly, the transcript level of SOD increased significantly by 2.4-fold in the salt + VB₆ treatment compared to the salt treatment (Fig. 3), which is consistent with the result of SOD enzyme activity. And application of VB₆ significantly up-regulated the SOD gene expression, when without salt stress conditions in wheat plants. So we infer that VB₆ might act as a signal in regulating the expressions of some peroxidase genes, such as SOD under salt stress.
Osmotic regulator of wheat seedling

Wheat seedling growth in salt stress has higher proline content (about 2.8 times) than control (Fig. 4). Similarly, after application of VB₆ the content of proline in salt-stress also increased by 2.5 times compared with non-salt group. VB₆-treated seedling accumulated more proline than salt stressed alone, however, it did not increase significantly. High content of proline can protect wheat seedlings and alleviated the growth inhibition caused by salt stress.

The expression of proline biosynthesis genes (P5CR and P5CS) were also investigated by quantitative real-time PCR (RT-qPCR). The P5CR and P5CS are two of key enzymes in the proline synthesis, which can reflect the changes of proline content in wheat plants. The expressions of P5CR and P5CS in the wheat seedlings with VB₆ treatment were significantly up-regulated under salt stress (Fig. 4), which is consistent with the above result that application of VB6 can promote the proline synthesis by up-regulating the expression of P5CR and P5CS. For same genes, the expression level of VB₆ treated seedlings has

![Graph showing the content of proline and the expression of P5CR and P5CS](image)

*Figure 4. The content of proline in wheat seedlings and the expression of P5CR and P5CS, bars with different letters are significantly different at 5% level (p<0.05)*

*Cereal Research Communications 47, 2019*
changed not significantly compared to the control plants (Fig. 4). Therefore, we suggest that VB₆ pretreatment will up-regulate the expression of P5CS and P5CR in wheat seedlings exposed to 200 Mm NaCl treatments.

Expression of TaSOS1 and TaSOS4 in wheat

SOS1 and SOS4 are two genes related to Salt Overly Sensitive (SOS) pathway, which plays a vital role in exclusion Na⁺ at cellular level, and mitigated osmotic stress caused by high salt condition. SOS1 is a Na⁺/H⁺ antiporter that regulate the Na⁺ transport in salt stress (Xu et al. 2008). SOS4 is a vital cofactor for enzymes in the cell, and pyridoxal kinase encodes by SOS4 to participate in pyridoxal-5-phosphate biosynthesis in the cell. It also involved in regulation of ion transport of cells in salt stress (Mahajan et al. 2008). The quantitative expressions of TaSOS1 and TaSOS4 genes in wheat leaves under salinity stress were investigated in current study (Fig. 5). VB₆ treated seedlings has up-regulate in TaSOS1 and TaSOS4 expression (Fig. 5), but not significant. Applied VB₆ in salt-stressed nutrient solution, seedlings recorded about 2.1-fold higher TaSOS1 expression compared to the salt-stressed plants. Similar to the TaSOS1 gene response, wheat plants increased significant (about twice) in the expression levels of TaSOS4 in salt stress. VB₆ enhanced the expression of TaSOS1 and TaSOS4 in wheat leaves under salt stress.

![Figure 5](image)

*Figure 5. The expression of TaSOS1 and TaSOS4, bars with different letters are significantly different at 5% level (p<0.05)*

Discussion

The biomass and growth development in wheat

The responses of cultivated crop species to salinity in terms of growth and yield are the ultimate expression of several interacting physiological and biochemical parameters (Almansouri et al. 2001). High concentrations of salinity in the soil severely restrict plant growth and development (Ashraf and Foolad 2013). The current study found similar
results that the length of wheat roots and seedlings were significantly decreased at 200 mM NaCl-stress. However, application of VB₆ were mitigate growth inhibition under salt stress (Figs S1, S2).

Photosynthesis is the most important process that directly or indirectly influencing the growth and survival of every organism (flora and fauna) (Gururani et al. 2013). Therefore, it cannot be examined simply as an isolated phenomenon but must be studied within the context of whole-plant regulation. Salinity-stress effects on crop growth are manifested by impairment of photosynthetic capacity (Brini et al. 2007). Salinity also reduced wheat relative growth rate, photosynthetic rate in stems and leaves during whole crop cycle from seed germination to crop maturity (El-Hendawy et al. 2005). An interesting aspect of PLP-dependent enzymes is also impact on the biosynthesis of phytohormones which are key regulators in plant development (Mooney and Hellmann 2010). The current study found that the content of chlorophyll and carotenoid were significantly reduced in wheat seedlings (Fig. S3). Photosynthetic pigments degradation was relieved by VB₆ treatment.

Regulate osmotic system in wheat

Osmotic adjustment can alters the relationship between cell protoplast volumes (approximated by RWC) (Brini et al. 2007). The VB₆ salt treatment showed increased RWC content compared with CK (Fig. S3). Proline has been shown to accumulate in plant tissues under various stress conditions (Gururani et al. 2013). The proposed function of the accumulated proline in osmosis regulation has an adaptive mechanism to environmental stress and salinity (El-Sayed et al. 2014). Previous research reported that the proline concentration significantly increased in the leaf of all cultivars under increasing salt tolerance (Moradi and Ismail 2007). The current results show that wheat seedling growth in salt stressed condition have higher proline content (Fig. 4). VB₆ treated seedling accumulation more proline. Two key enzymes in proline biosynthesis Δ¹-pyrroline-5-carboxylate syntheses (P5CS) and Δ¹-pyrroline-5-carboxylate reductase (P5CR) (Porcel et al. 2004; Zhang et al. 2014) were estimated in the current study. And the result suggested that VB₆ enhances the tolerance of wheat seedlings to salt stress, synthesizes more proline by increasing the expression of proline synthesis gene (P5CS and P5CR), and enhances the osmotic regulation ability of plants to protect them from damage.

Activate peroxidase system in wheat

Cellular redox state is an important factor which regulates the key process in growth and development as well as stress tolerance (Jisha et al. 2013). In plant, the salt effect on the enzymes might be involved in the dark reactions of photosynthesis and could result in the generation of excess ROS with subsequent damage to lipid membranes, as demonstrated by the high levels of MDA and severe leaf damage in this cultivar (Moradi and Ismail 2007). The current study suggests that salinity-induced oxidative damage in wheat seedlings, as indicated by the higher levels of H₂O₂ and MDA, is probably due to the inhibi-
tion or insufficient induction of the antioxidant defense (Hasanuzzaman et al. 2011). The current study showed that 200 mM NaCl stress increased the content of H$_2$O$_2$ and MDA in seedlings. But significantly reduced after the application of VB$_6$ (Fig. 2). Hydrogen peroxide levels in wheat were significantly higher in control group under all the applied salt-stress treatments than in the VB$_6$ treated seedlings. VB$_6$ enabled wheat seedlings to maintain a higher proline content and lower MDA, H$_2$O$_2$ accumulation in wheat. The present study results indicated that use VB$_6$ can effectively reduce the damage of NaCl stress to wheat seedlings by reducing H$_2$O$_2$ and MDA content.

The antioxidant enzymes of the cell have the ability to remove the free radicals produced during abiotic stress conditions. These enzymes also protect the membranes and DNA from damage (Gururani et al. 2013). The specific activity of SOD significantly increased in the VB$_6$ treated seedlings growing under NaCl stresses. Plant tolerance to these stresses correlated with the increased expression levels of ROS-scavenging enzymes SOD, CAT and POD, suggesting that VB$_6$ treated wheat seedling triggered salt stress related defense pathways under high salinity conditions. The mRNA expression of SOD in wheat plants growing under salt-stress with VB$_6$ conditions increased. The present experimental result was consistent with the RT-qPCR results where the relative mRNA expression levels of the anti-oxidative pathway genes were more pronounced in the VB$_6$ applied wheat than in the non-VB$_6$. Based on the finding, it was suggested that VB$_6$ could play an important role in oxidative stress injury of wheat leaves grown in stressed condition. Possibly, the protective effect of VB$_6$ is more related to reduced active oxygen species (ROS) damage to essential proteins and/or nucleic acids. The current study suggest that exogenous application of 200 mM VB$_6$ could alleviate salt-induced oxidative damage by enhancing antioxidant enzyme activities in the seedling.

**Activate SOS pathway in wheat**

High Na$^+$ concentration in the cytosol is detrimental to plant growth and leaves are usually more susceptible to Na$^+$ toxicity. Further to control the transport of Na$^+$ and Cl$^-$ is very critical for salinity tolerance in plants. On the other hand, the excess ions will enter in cell and accumulate to toxic levels in the older transpiring plant leaves (Munns et al. 2010). The Current research had reported that the Na$^+$/H$^+$ antiporters in cytoplasmic membrane plays an important role in plant resistance to salt stress (Chen et al. 2008). Salt Overly Sensitive (SOS) pathway plays a vital role in exclusion Na$^+$ at cellular level by regulate Na$^+$/H$^+$ antiporters, and mitigated osmotic stress caused by high salt condition (Shi et al. 2003; Xu et al. 2008). Previous study found that over expression of SOS1 can reduce the accumulation of sodium ions and enhance the salt tolerance in transgenic Arabidopsis (Shi et al. 2003). The TaSOS1 protein is induced by salt treatment and contributes to plasma membrane Na$^+$/H$^+$ exchange (Xu et al. 2008). Whereas the present study measured the expression of TaSOS1 and TaSOS4 in wheat that are related to SOS pathway. The current study result shown that application of salinity stress lowered TaSOS1 and TaSOS4 genes expression compared with VB$_6$ treated in wheat plants (Fig. 5). With the salt stress, application of VB$_6$ markedly enhanced the expression of
TaSOS1 and TaSOS4 in wheat leaves. It means VB₆ can improve the salt tolerance of wheat plants by active SOS pathway in plant and regulate the ion transporters to exclude the excessive ion to protect plant in salt stressed conditions. SOS4 have also been characterized and reported that SOS4 may be involved the pyridoxal-5-phosphate biosynthesis which encodes a pyridoxal kinase (Ramezani et al. 2013). It is an active form of vitamin B₆ (Mahajan et al. 2008). Like previous reports, the application of VB₆ markedly enhanced the expression of TaSOS4 in wheat leaves in the present study. Further adding the VB₆ to up-regulate the expression of SOS4 in plant cells suggested that treatment with VB₆ could be an easily and effective method to improve salt-stress tolerance to wheat.

In conclusion, the current result found that exogenous VB₆ effectively alleviated plant growth inhibition. Application of VB₆ is beneficial to reduce the reactive oxygen species in plant cell and diminish the damage of plant cell membrane under salt stress by increasing the activity of plant antioxidant enzymes (POD, CAT and SOD) to remove more free radicals caused by salt stress. Also, VB₆ might act as a signal in regulating the expressions of some peroxidase and osmotic regulator genes such as SOD, P5CS, P5CR, TaSOS1 and TaSOS4 under salt stress. The current study results give an insight into the regulatory roles of VB₆ in improving salt stress by cross talking with peroxidase system, proline synthesis and SOS pathway.

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References


Cereal Research Communications 47, 2019


Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at https://akademiai.com/loi/0806

Electronic Supplementary Table S1. The primers for RT-qPCR

Electronic Supplementary Figure S1. Root length and shoot height of wheat seedlings in each treatment, bars with different letters are significantly different at 5% level (p<0.05)

Electronic Supplementary Figure S2. Fresh/dry weight of wheat seedling roots and shoots, bars with different letters are significantly different at 5% level (p<0.05)

Electronic Supplementary Figure S3. The content of chlorophyll a, chlorophyll b, carotenoid and relative water content in wheat seedlings, bars with different letters are significantly different at 5% level (p<0.05)

Electronic Supplementary Figure S4. The pedigree of the Chuanyu23

Cereal Research Communications 47, 2019