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# Spermidine alleviates the growth of saline-stressed ginseng seedlings through antioxidative defense system

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ABSTRACT

Protective effects of exogenous spermidine (Spd), activity of antioxygenic enzymes, and levels of free radicals in a well-known medicinal plant, *Panax ginseng* was examined. Seedlings grown in salinized nutrient solution (150 mM NaCl) for 7 d exhibited reduced relative water content, plant growth, increased free radicals, and showing elevated lipid peroxidation. Application of Spd (0.01, 0.1, and 1 mM) to the salinized nutrient solution showed increased plant growth by preventing chlorophyll degradation and increasing PA levels, as well as antioxidant enzymes such as CAT, APX, and GPX activity in the seedlings of ginseng. During salinity stress, Spd was effective for lowering the accumulation of putrescine (Put), with a significant increase in the spermidine (Spd) and spermine (Spm) levels in the ginseng seedlings. A decline in the Put level ran parallel to the higher accumulation of proline (Pro), and exogenous Spd also resulted in the alleviation of Pro content under salinity. Hydrogen peroxide ($H_2O_2$) and superoxide ($O_2^-$) production rates were also reduced in stressed plants after Spd treatment. Furthermore, the combined effect of Spd and salt led to a significant increase in diamine oxidase (DAO), and subsequent decline in polyamine oxidase (PAO). These positive effects were observed in 0.1 and 1 mM Spd concentrations, but a lower concentration (0.01 mM) had a very limited effect. In summary, application of exogenous Spd could enhance salt tolerance of *P. ginseng* by enhancing the activities of enzyme scavenging system, which influence the intensity of oxidative stress.



## 1. Introduction

Salinity is a major abiotic stressor that limits plant growth and yield. Growth inhibition of plants exposed to salinity is associated with the reduction of water availability and ion accumulation, causing an imbalance of minerals that leads to morphological, physiological, and metabolic modifications in plants. Salinity also induces the generation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals in plants (Leshem et al., 2007). These ROS interrupt normal metabolism in plants by lipid peroxidation of membrane, denaturation of proteins and nucleic acids. Salt stress alters the critical balance between the production of ROS and the quenching activity of antioxidants, resulting in oxidative stress that causes damage to plants (Hernandez et al., 1999). Plants have evolved three main strategies to counteract salt stress: detoxification, re-establishment of homeostasis, and growth regulation. The relevant responses involve the accumulation of compatible solutes (proline, amino/organic acids, betaines, and polyamines), the up-regulation of antioxidant enzymes and $Na^+/H^+$ antiporters, the re-translocation of $Na^+$ from the photosynthetic organs back to the roots, the $K^+$ retention in root and leaf cells (Shabala and Cuin, 2007), the increased activity of plasma membrane ($H^+$-ATPase) and tonoplast $H^+$ pumps (Hasegawa and Bressan, 2000), and the expressions of different sets of genes that are part of plant signaling and defense system against salinity (Sairam and Tyagi, 2004). Antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX), and non-enzymatic antioxidants, such as ascorbic acid (ASC), glutathione (GSH), α-tocopherol, carotenoids, phenolics, and proline, which play a key role in quenching ROS, are implicated in stress tolerance (Hernandez et al., 1999).

Polyamines (PAs), including spermidine (Spd, a triamine), spermine (Spm, a tetramine), and their obligate precursor, putrescine (Put, a diamine), are polybasic aliphatic amines that are ubiquitously distributed in all living organisms. Their positive charges at physiological pH levels enable PAs to interact electrostatically with polycationic macromolecules such as DNA, RNA, proteins, and phospholipids (Bouchereau et al., 1999). Most of these processes are positively correlated with changes in the intracellular levels of PAs. Put can be synthesized by two carboxylases

*Abbreviations:* Spd, (Spermidine); Put, (Putrescine); Spm, (Spermine); Pro, (Proline); RT-PCR, (Reverse Transcriptase-Polymerase Chain Reaction); PA, (Polyamine); ROS, (reactive oxygen species); (SOD), Superoxide dismutase; (APX), Ascorbate peroxidase; (CAT), Catalase; (GPX), Guaiacol peroxidase; (ASC), Ascorbic acid; (GSH), Glutathione; (ODC), ornithine decarboxylase; DAO, (Diamine oxidase); PAO, (Polyamine oxidase).

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in plants: ornithine decarboxylase (ODC) and arginine decarboxylase (ADC). Spermidine (Spd) and spermine (Spm) are formed from Put by the subsequent addition of an aminopropyl moiety from decarboxylated s-adenosylmethionine. PAs modulate numerous biological processes including cell proliferation, growth and development, morphogenesis, senescence, and response to abiotic stresses (Galston and Sawhney, 1990). Several mechanisms have been postulated for the protective nature of PAs, which include scavenging free radicals, stabilizing membranes and cellular structures, maintaining a cation–anion balance (Bouchereau et al., 1999). Both mono- and dicotyledonous plants increase the accumulation of endogenous PAs under salt stress, and the pattern of PA metabolism in response to salinity seems to be dependent on plant defense systems and/or duration of exposure to salt stress. Exogenous PA application has been proposed as a convenient and effective approach for combating the salt tolerance of plants and ultimately improving crop productivity under high salinity (Chattopadhayay et al., 2003). Put content is temporally correlated with the accumulation of Pro under salinity stress, because they are both competing for glutamate as a precursor. On the basis of these observations, our goal was to elucidate the protective role of exogenous PA, especially Spd, in salt stressed ginseng plant.

*Panax ginseng* (Korean ginseng) is a perennial herb of the family Araliaceae. Its dried roots are used for medicinal purposes, and the major active component of ginseng roots is ginsenosides, a triterpenoid saponin. Ginseng has pharmacological effects that can be used to normalize the human metabolic system and biological activities (Lee et al., 2011). However, ginseng cultivation is difficult, because ginseng is a shade loving crop and its cultivation requires a long period (4–5 years) to produce the highly valued roots. During long cultivation time, the ginseng plant can be easily exposed to different environmental stresses, which may cause drastic effects in its growth such as salt stress. Our earlier report showed that higher cellular levels of Spd facilitate the transcription of *PgSPD* (spermidine synthase gene in *P. ginseng*) in response to different abiotic stresses (Parvin et al., 2010). The present study is aimed at determining whether exogenous Spd enhanced salt tolerance, and whether Spd affects antioxidant properties in the seedlings of *P. ginseng*. In addition, changes in chlorophyll, carotenoid, PA, PA degradative enzyme activity, Pro, and lipid peroxidation were also assessed with regard to salt stress, along with or without Spd.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Korean ginseng (*P. ginseng* C. A. Meyer) cv. "Yunpoong" seeds were immersed in 70% ethanol for 1 min, surface-sterilized in 2% NaOCl for 15 min, rinsed three times with sterilized distilled water, and then the inner zygotic embryos were dissected out since seed is not germinating easily. Intact zygotic embryos were placed on MS (Murashige and Skoog, 1963) basal medium containing 3% sucrose and 0.7% phytoagar (Purev et al., 2010). Cultured plantlets were planted in glass bottles that contained a 70 ml MS medium with 3% (w/v) sucrose and 0.7% phytoagar under controlled conditions at 25 °C with a 16-h photoperiod. The pH of the MS medium was maintained at 5.7 ± 0.1.

### 2.2. Chemical treatments

Healthy 2-week-old seedlings were used for chemical treatments in different nutrition solutions. Spermidine (Sigma Chemical Co.) in different concentrations (0.01, 0.1, and 1 mM), and in combination with 150 mM NaCl was treated in MS medium as specified in the figure legend. Seedling samples were collected after 0, 1, 3, 5, and 7 d of treatment, and were immediately frozen in liquid nitrogen and stored at −70 °C until required for the subsequent total RNA isolation and estimation of polyamines (PA), proline (Pro), antioxidants, and antioxygenic enzyme activity.

### 2.3. Relative water content, chlorophyll and carotenoid estimation

After 7 d of treatment, 50 plants per treatment were collected for the determination of plant growth and relative water content (RWC). For the determination of fresh weight, shoots and roots were separated and weighed after being washed with sterile distilled water. The dry weight was obtained after drying in an oven for 72 h at 65 °C. RWC, indicating the level of water stress in leaves, was estimated according to the following formula:

$$\text{Fresh weight} - \text{dry weight} \times 100/(\text{fresh weight at full turgor} - \text{dry weight}).$$

The chlorophyll (Chl) and carotenoid (CAR) contents in the leaves of ginseng seedlings were estimated according to previous method (Kirk and Allen, 1965).

### 2.4. Free polyamines and proline determination

PAs (Put, Spd, and Spm) were estimated as dansyl-derivatives by reversed phase HPLC using salt stressed ginseng seedlings as described previously [14]. Free proline (Pro) content was determined according to the previously reported method (Bates et al., 1973).

### 2.5. Polyamine catabolizing enzymatic activity assay

The activity of polyamine catabolizing enzymes was determined as described previously (Su et al., 2005), by using Put (for DAO) and Spd (for PAO) as substrates. Ginseng seedling samples were extracted in 100 mM K-phosphate buffer (pH 6.5) containing 5 mM dithiothreitol. The extract was centrifuged at 10,000 *g* for 20 min at 4 °C, and the supernatant was used for the enzyme assay. The reaction mixture contained 2 ml of potassium phosphate buffer (100 mmol $l^{-1}$), 0.2 ml 4-aminoantipyroine/N (5 mmol $l^{-1}$), N-dimethylaniline reaction solutions, 0.1 ml of horseradish peroxide (50 mmol $l^{-1}$), and 0.2 ml of the enzyme extract. The reaction was initiated by the addition of 15 μl of Put (10 mM) for DAO determination, and Spd (10 mM) for PAO determination. A 0.001 absorbance unit of change in the optical density at 555 nm $min^{-1}$ was considered one unit of enzyme activity.

### 2.6. Selection of antioxidant genes from the EST database, RNA isolation, and qRT-PCR

The selection of antioxidant EST genes was performed using the ginseng EST database (Sathiyamoorthy et al., 2009) and the BLAST program at the Plant Genome Database server [http://www.plantgdb.org/cgi-bin/blast/PlantGDBblast]. The blastn and tblastx programs were used with exception values (E-values) less than $10^{-4}$. Using ginseng EST searches, we identified and selected various candidates of antioxidant genes such as *PgCAT*, *PgAPX*, and *PgGPX*, based on their open reading frames (ORF), which encoded a specific protein via the blastx program. Specific gene primers (Table 1) were designed for each of the antioxidant genes based on the gene sequences obtained. RNA isolation and cDNA

**Table 1**
List of antioxidant genes used in the present study and their specific primers.

| No. | Antioxidant genes | EST No. | Primers (5′-3′) |
|---|---|---|---|
| 1. | PgCAT | EU327037 | F: CAAGGATGGGAAAGCACA<br>R: TGGTTACATCGAGTGGGTCA |
| 2. | PgAPX | DC03contig333 | F: CTGGACCAACAACCCTCTCA<br>R: CTCAGCAAATCCCAATCCCAGTTCA |
| 3. | PgGPX-1 | DC04010_C01 | F: GTCAAGGATGCTAGAGGGAATG<br>R: GTCCCTGGCTCCTGTGCT |
| 4. | Actin | DC03003B12 | F: CGTGATCTTACAGATAGCTTGATGA<br>R: AGAGAAGCTAAGATTGATCCTCC |