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Effects of Calcium and Nitrogen on the Growth and Antioxidative Enzyme Activity in Soybean (*Glycine max*) under Saline Condition

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ABSTRACT: Growth of *G. max* treated with NO_3^--N was decreased by high NaCl treatments, but NH_4NO_3-fed plants showed good growth with enhanced activity of antioxidative enzymes (SOD and APX). Especially, activity of APX was higher in 5 mM NH_4NO_3-fed plants than other types of N-supplied plants throughout the stress period. Higher SOD activity under salt stress was accompanied by increase in APX activity in 5 mM NH_4NO_3-fed plants. Similarly, application of calcium confirmed somewhat positive effects on growth. Salt-treated soybean plants showed the best growth response with the increase of SOD and APX activity at an additional 5 mM calcium treatment. Especially, the increase of SOD activity through the strengthened CuZn-SOD isoform was remarkable.

Key words: Antioxidative enzymes (SOD, APX), Calcium, Nitrogen, Salt treatments, Soybean

INTRODUCTION

Oxygen metabolism is very a dangerous process because of the generation of reactive oxygen species (ROS). The metabolic process along with an electron transfer, such as photosynthesis, respiration, and nitrogen fixation, creates an ROS (Asada 1994, Fridovich 1995). It is well known that stress conditions of abiotic factors including osmotic stress, UV, low temperature, dryness, ozone, and biotic factors induce the generation of ROS in plant cells (Scandalios 1997, Noctor and Foyer 1998). Additionally, salt stress is also known to cause oxidative stress (Hernandez et al. 1999, Zhu 2000). The earliest oxidative product is the superoxide radical (O_2^-), which generates a large reactive ROS, such as the hydroxyl radical ($OH^\cdot$) and singlet oxygen ($^1O_2$), which in turn lead to oxidative damage due to the induction of peroxidation of the cell membrane lipid (Fridovich 1986, Halliwell and Gutteridge 1989). Therefore, every life that uses oxygen, including higher plants, has a well-developed antioxidative defense system, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) to avoid the self-destruction caused by oxygen. What's most important in such antioxidative systems is that the increased activity of antioxidative enzymes must be maintained to protect cells from oxidative damage. For instance, if the degree of increment of H_2O_2 caused by increasing the activity of SOD exceeds the resolution capacity of the H_2O_2 detoxification enzyme including APX, a higher reactive $OH^\cdot$ radical will be created by the following mechanism.

$$O_2^- + H_2O_2 \rightarrow OH^\cdot + OH^- + O_2$$

In addition, the SOD (CuZn-SOD) itself will be suppressed by H_2O_2, so that it should be eliminated by APX or others (Asada 1994, Shalate and Tal 1998).

Nitrogen and calcium are required for growing plants in large amounts as essential inorganic nutrients. Calcium contributes to the stabilization of the cell membrane combined with phosphoric acid of the cell membrane lipid, carboxyl group and protein, and the outer surface of membrane (Legge et al. 1982). Moreover, many studies have been conducted in the fields of plant physiology and molecular biology on the function of calcium as a secondary messenger in the signal transduction system between environmental factors and the reactions of plant. The replacement of membrane-combined calcium is known as a major status related to the salt stress, including calcium deficiency caused by high concentrations of Na^+ in the salt environment (Loneragan et al. 1968, Loneragan and Snowball 1969, Lynch et al. 1987, Rengel 1992).

On the other hand, salt stress impedes growth, symbiotic development of root nodule bacteria, and nitrogen fixation of leguminous plants. According to several studies, it is reported that external nitrogen supply recovered the growth inhibition of salt-sensitive soybean plants by salt (Lauter et al. 1981, Tu 1981, Alston and Graham 1982, Yousef and Sprent 1983, Singleton and Bohlool 1984, Bekki et al. 1987).

The ionic form of available N, such as NH_4^+ and NO_3^-, influences the ionic equilibrium within plants, and in many cases also growth and productivity (Lewis and Chadwick 1983, Lips et al.

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1990, Cramer and Lewis 1993, Stivastava and Singh 1999). Under saline conditions, plants supplied with NH_4^+ show a more negative and sensitive growth response by the decrement of NH_4^+ assimilation rate at the root because a large part of the energy under the salt environments is demanded to control the osmoregulation (Lewis et al. 1989). In addition, salt environments not only reduce Vmax to absorb nitrogen in plants, but also suppress the transmission to the shoot (Lewis and Chadwick 1983, Hawkins and Lewis 1993a, b).

However, it is still unclear whether the additional supply of calcium or various types of nitrogen influences to the antioxidative defense mechanism and oxidative stress under salt environment. In this study, therefore, we investigated the effects of calcium and various nitrogen sources on the growth of salt-sensitive soybeans under salt environments and examined whether calcium and nitrogen effects on growth are related to the SOD and APX that are typical enzymes of antioxidative protective system.

MATERIALS AND METHODS

Experiment 1: Effects of Nitrogen Source and Concentration on Soybean Treated with Salt

1) Growing Condition and Nitrogen Treatment

We selected uniform seeds of soybean (*Glycine max* Merr. cv. Eunha) as a salt sensitive leguminous plant, and germinated them in a growth chamber (30/20℃ - day/night, relative humidity of 60/70% - day/night, 280 μmol photons $m^{-2}s^{-1}$, photoperiod of 12 hours) after soaking them distilled water for 2 hours. Individual plants were transplanted to a plastic pot (10cm × 15cm) filled with vermiculite. We supplied the supernatant of the soil solution including *Rhizobium* sp. of 100 ml per pot for 2 days after growth of the first leaf for the inoculation of *Rhizobium* sp. We supplied modified McKnight's medium (1949) (5 mM $CaSO_4 \cdot 2H_2O$, 1.47 mM KH_2PO_4, 4.02 mM KCl, 0.81 mM $MgSO_4$, 0.057 mM Fe-EDTA and trace elements). Seedlings were divided into nitrogen-free (control) and nitrogen-treatment group (NH_4NO_3-N, NO_3-N of 5 and 10 mM) and received modified McKnight's solution of 100 ml to which was added 0, 50, 100, and 150 mM of NaCl everyday.

2) Harvest

We harvested the leaves of 5 plants for each treatment group on the 12th day after nitrogen and salt treatments, and stored them in −70 ℃ after freezing in N_2 in order to analyze enzyme activity. The other plants are separated by each organ (leaf, stem and root) to analyze the growth. All experiments were replicated three times.

Experiment 2: Effects of Calcium on Soybean Treated with Salt

1) Growing Conditions and Treatments

The soybean plants were germinated and grown under the same methods and same conditions as in Exp. 1. We supplied modified McKnight's medium (1949) containing 5 mM KNO_3, and added NaCl of 0, 50, 100, and 150 mM for the control (5 mM Ca) and additional calcium of 5 mM (10 mM Ca) and 10 mM (15 mM Ca) by 100 ml of McKnight's medium everyday. All experiments were replicated three times.

2) Harvest

The harvest was done by the same methods as in Exp. 1 on the 21th day after the calcium and salt treatment. All experiments were replicated three times.

Analysis

1) Enzyme Extraction and Protein Content

The activities of antioxidative enzymes in leaves were investigated. For SOD (superoxide dismutase), all samples were ground with liquid nitrogen. These samples (50 mg) were homogenized with 1ml of 100 mM potassium-phosphate buffer (pH 7.8) including 100 mM EDTA, 1% PVP(w/v) and 0.5% Triton X-100. The extracts were centrifuged at 12,000 rpm for 20 min at 4℃ and then the supernatant was used for measurement of enzyme activity. For APX (ascorbate peroxidase), GR (glutathione reductase), CAT (catalase), samples were mixed and homogenized with 1 ml of 100 mM sodium-phosphate buffer (pH 7.8) containing 5 mM ascorbate and 1 mM EDTA, and were centrifuged in the conditions as with the supernatant. Extraction was prepared at 0 to 4℃ and measurement of enzyme activity was determined at 25℃. The contents of total soluble protein were obtained by the standard formula using BSA (Bovine Serum Albumin) by Lowry et al. (1951).

2) Measurement of The Enzyme Activity

SOD activity was measured using a reduction method of NBT (nitroblue tetrazolium) by Beyer and Fridovich (1987). The identification and activity for each isoform were measured by adding 3 mM KCN (CuZn-isoform inhibitor) or 5 mM of H_2O_2 (CuZn- and Fe-SOD inhibitor). One unit of SOD is presented by the decrement of 50% of SOD activity induced by NBT reduction at 560 nm.

APX activity was measured by the method of Jimenez et al. (1997) by using an spectrophotometer (Shimadzu UV-VIS spectrophotometer 1240) for 2 min with changes of absorption at 290 nm which are induced by the oxidation of 10 mM of ascorbate to monodehydroascorbate due to the addition of H_2O_2. The activity

was calculated by following formula.

$$\text{Activity} = 1{,}000 \times V \;/\; (\varepsilon \times d \times v \times p)$$

V: total assay volume (ml)
ε: extinction coefficient (2.8 mM^{-1} cm^{-1})
d: light path (cm)
v: volume of sample in total assay volume (ml)
p: protein content (mg)

3) Statistical Analysis

The results are the means ± SE of three independent replicates. Data of growth, SOD and APX were analyzed using ANOVA (completely randomized) to determined if significant differences were present among means. The multiple range test after Scheffe was carried out to determine if significant differences ($P<0.05$) occurred between N or Ca treatments (using SPSS 8.0 for Windows).

RESULTS

The effect of various nitrogen sources and concentration on growth of salt-treated soybeans was shown in Fig. 1. Soybeans showed decrement of growth by salt addition, and indicated severe growth reduction by KNO_3 in the high salt conditions. However, soybeans to which NH_4NO_3 was added showed a good growth response with high fresh weight in the salt condition. Moreover, the effects of the nitrogen source on the growth of soybean under salt condition showed a distinct appearance on the high concentration of salt above 100 mM NaCl. Especially, in the case of soybeans supplied with 5 mM of NH_4NO_3 at 100 mM NaCl the fresh weight of the shoots and roots was the highest.

In order to investigate whether nitrogen effect on growth has adirect relationship with antioxidant defense mechanism in the salt condition, we measured the activities of typical antioxidative enzymes, SOD and APX (Table 1).

The activities of SOD and APX were influenced by nitrogen supply under salt stress; especially, SOD showed significant changes of activities depending on the nitrogen sources and was significantly affected by salt and the interaction of N source and salt

Table 1. Changes of antioxidative enzyme activity (SOD, APX) and SOD/APX ratio in leaves of soybean treated with 100 and 150 mM NaCl according various nitrogen sources (12th day after treatment). Letters beside mean values denote significant differences between nitrogen treatments at each NaCl concentration (tested with a one-way ANOVA, multiple range test after Scheffe; $P<0.05$)

NaCl treatments	100 (mM)			150 (mM)		
Enzyme activity	SOD	APX	SOD/APX	SOD	APX	SOD/APX
N-Free	50.0 ± 4.6c	25.2 ± 3.8b	2.00 ± 0.11b	165.8 ± 16.3a	25.4 ± 5.0b	6.64 ± 0.65a
KNO_3 (5mM)	163.3 ± 8.0a	26.9 ± 5.4ab	6.25 ± 0.96a	130.7 ± 10.6b	26.8 ± 3.0b	4.89 ± 0.15bc
KNO_3 (10mM)	92.9 ± 9.7b	35.1 ± 7.3ab	2.70 ± 0.29b	115.3 ± 5.2b	24.5 ± 4.4b	4.82 ± 0.65bc
NH_4NO_3 (5mM)	74.3 ± 8.4b	44.2 ± 1.8a	1.68 ± 0.12b	172.2 ± 12.0a	39.3 ± 2.3a	4.39 ± 0.10c
NH_4NO_3 (10mM)	48.3 ± 2.6c	34.4 ± 5.5ab	1.43 ± 0.15b	142.6 ± 9.4ab	23.6 ± 3.5b	6.11 ± 0.52ab

* Two-way ANOVA of SOD, APX and SOD/APX by nitrogen sources (N) and salt treatments (S) are as follows. SOD: N^{***}, S^{***}, N X S^{***}; APX: N^{***}, S^{**}, N X $S^{n.s.}$; SOD/APX: N^{***}, S^{***}, N X S^{***}.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; n.s., not significant.

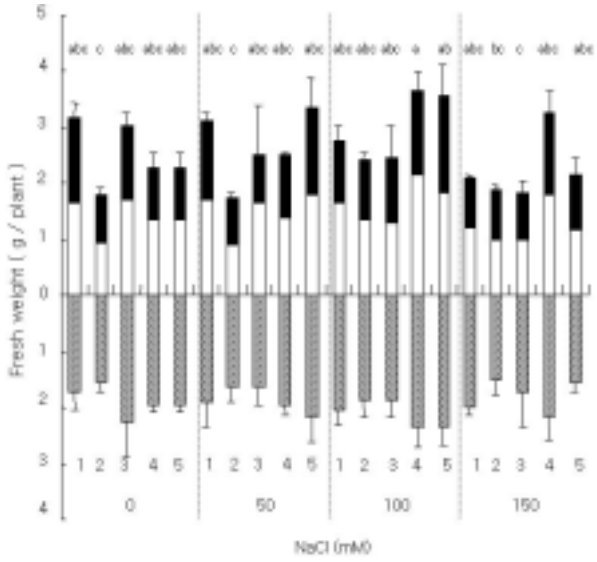

Fig. 1. Effects of various nitrogen sources and concentration on fresh weight (g FW/plant, means ± SD) in shoots and roots (□, stem; ■, leaf; ▨, root) of soybean treated with 0, 50, 100 and 150 mM NaCl (12th day after treatment). 1: N-free, 2: KNO_3 – N (5 mM), 3: KNO_3 – N (10 mM), 4: NH_4NO_3 – N (5 mM), 5: NH_4NO_3 – N (10 mM). Letters over bar denote significant differences between treatments ($4 \times 5 = 20$) in shoots (tested with a one-way ANOVA, multiple range test after Scheffe; $P<0.05$).

concentration. The KNO_3-fed soybeans showed high SOD activities with a serious growth inhibition at 100 and 150 mM NaCl. Meanwhile, APX activity showed differences according to the nitrogen source, but it was not significantly affected by the interaction of N source and salt concentration. Soybeans treated with 5 mM NH_4NO_3 showed the highest APX activity and increased SOD activity with the best growth response. Therefore, soybeans with 5 mM NH_4NO_3 maintained low ratio of SOD/APX compared with the control or KNO_3-added plants under the high NaCl concentration.

Soybeans under salt stress according to the addition of calcium showed growth inhibition according to salt concentration, but their growth inhibition by salt was relieved somewhat by an additional calcium supply. Soybeans with additional Ca supply showed a slight increment of shoot weight at 50 mM NaCl and clear increase of root weight at 100 mM NaCl.

The SOD activity of soybeans was increased by additional calcium supply under salt environments, and was the highest at additional 5 mM calcium. At 150 mM NaCl treatments, however, soybeans did not show significant differences in SOD activity. The soybeans contained 2 kinds of SOD (CuZn-SOD, Mn-SOD) and the major isoform was CuZn-SOD with a stronger activity than Mn-SOD.

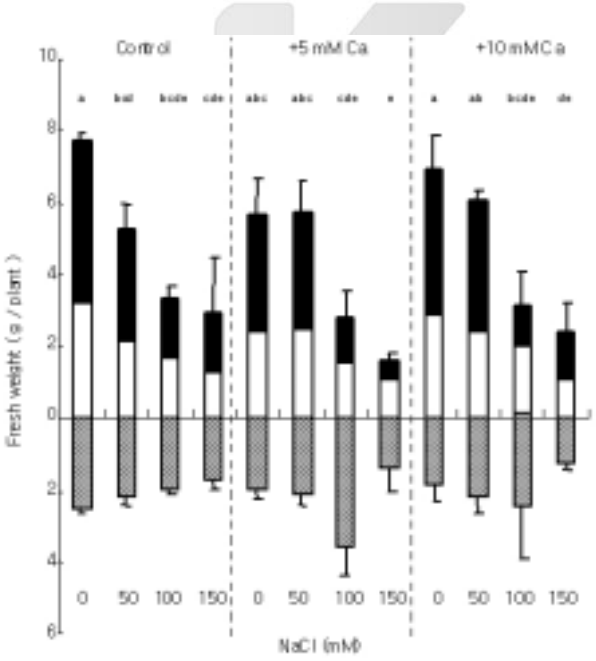

Fig. 2. Effects of additional calcium on fresh weight (g FW/plant, means ± SD) in shoots and roots (□, stem; ■, leaf; ▨, root) of soybean treated with 0, 50, 100 and 150 mM NaCl (21th day after treatment). Letters over bar denote significant differences between treatments (4 × 3 = 12) in shoots (tested with a one-way ANOVA, multiple range test after Scheffe; $P<0.05$).

Overall, soybeans supplied with additional 5 mM Ca showed the highest fresh weight and the highest ratio of CuZn/Mn-SOD, simultaneously.

Similarly, soybeans showed highest APX activity at additional 5 mM calcium treatments, but the decrement of APX activity was independent of calcium at a high salt level of 150 mM NaCl (Table 3). APX activity was affected by salt concentration, but was not significantly affected by Ca and the interaction of Ca and salt treatments.

Table 2. Total SOD activities and the ratio of individual isoforms (CuZn/Mn SOD) in leaves according to additional calcium of soybean treated with 100 and 150 mM NaCl (21th day after treatments). Letters beside mean values denote significant differences between calcium treatments at each NaCl concentration (tested with a one-way ANOVA, multiple range test after Scheffe; $P<0.05$)

SOD activity (unit/mg protein)	NaCl (mM) \ Ca (mM)	0	5	10
SOD	100	137.2 ± 7.5c	197.0 ± 12.0a	169.9 ± 15.0b
	150	205.9 ± 5.0a	218.9 ± 10.0a	214.3 ± 11.5a
CuZn/Mn-SOD ratio	100	0.24 ± 0.03	0.98 ± 0.07	0.54 ± 0.06
	150	1.01 ± 0.19	3.37 ± 0.13	1.87 ± 0.12

* Two-way ANOVA of SOD by calcium (C) and salt treatments (S) are as follows. SOD: C^{***}, S^{***}, C X S^{**}.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Table 3. APX activities and the ratio of SOD/APX in leaves according to additional calcium of soybean treated with 100 and 150 mM NaCl (21th day after treatments). Letters beside mean values denote significant differences between calcium treatments at each NaCl concentration (tested with a one-way ANOVA, multiple range test after Scheffe; $P<0.05$)

APX activity (mM AsA/mg protein/min)	NaCl (mM) \ Ca (mM)	0	5	10
APX	100	44.1 ± 5.6a	50.8 ± 6.1a	37.8 ± 4.6a
	150	27.4 ± 6.3a	31.1 ± 8.9a	26.0 ± 7.6a
SOD/APX ratio	100	2.79 ± 0.66a	3.47 ± 0.81a	4.02 ± 0.94a
	150	6.93 ± 2.29a	6.59 ± 2.42a	7.74 ± 2.86a

* Two-way ANOVA of APX and SOD/APX by calcium (C) and salt treatments (S) are as follows. APX: $C^{n.s.}$; S^{***}, C X $S^{n.s.}$; SOD/APX: $C^{n.s.}$; S^{***}, N X $S^{n.s.}$.

* $P<0.05$; ** $P<0.01$; *** $P<0.001$; n.s., not significant.

DISCUSSION

Recently, salt accumulation on farmland (esp. greenhouse) has become a serious problem that impedes productivity of salt-sensitive crops such as soybean. It is well known that the salt environments induce a decrement of nitrogen fixation by suppressing the growth of leguminous plants and the symbiotic mechanism of root nodule bacteria. In the case of salt sensitive species including soybean, the defense mechanisms against incoming of toxic ions like Na^+ and Cl^- by root and migration to shoots are important factors for their survival in saline environments (Yeo et al. 1977). There are abundant researches demonstrating that the additional supply of nitrogen in a saline environment has a relief effect for the impediment to the growth of leguminous plants (Lauter et al. 1981, Tu 1981, Alston and Graham 1982, Yousef and Sprent 1983, Singleton and Bohlool 1984, Bekki et al 1987).

According to this study, however, NO_3^-—N induced more stressful symptoms accompanied by a decrement of fresh weight, and exerted negative effects on the growth of soybeans under the high salt environment above 100 mM NaCl. By contrast, NH_4NO_3 was effective for relieving growth inhibition induced by salt treatment (Fig. 1). In general, most plant species show higher production of dry matter with nitrate and/or a mixed nitrate/ammonium nutrition. It is well known that nitrogen forms substantially influence ion uptake process and internal ion pattern in plants. On the whole, high NO_3-fed plants contained high concentration of cation and organic acids (Srivastava and Singh 1999). Therefore, high cation (esp. Na^+) accumulation under high salt conditions may have serious ionic problems such as ionic imbalance and toxicity in high NO_3^- fed plants.

The superoxide radical (O_2^-) that is the initial product among ROS is created by various environmental stresses including salt, and can lead to oxidative damage like lipid peroxidation of cell membrane. Therefore, most plants that use oxygen have developed various antioxidative systems. SOD is an important antioxidative enzyme that operates at the initial stage of the antioxidative mechanism, and that removes superoxide radical (O_2^-) among variety of oxygen free radicals. Therefore, many researchers investigated the relationship between removal of O_2^- by SOD and tolerance to oxidative stress. In addition, there are many studies of the protection of cell membrane according to the increment of the SOD activity.

However, H_2O_2, which is a kind of free oxygen radial produced by the SOD, has strong oxidative power and can inhibit SOD activity (especially, CuZn-SOD). Therefore, it is very important that H_2O_2 produced has to be removed by APX as soon as possible (Scandalios 1993). In general, the ratio of SOD/APX is kept low when SOD and APX operate effectively together (see Table 1). Therefore, a low SOD/APX ratio means that O_2^- and H_2O_2 are removed by SOD and APX at the same time, thus inhibiting creation of the hydroxyl radical ($OH^{\cdot}$) that is created by a chain reaction with O_2^- and H_2O_2. Hydroxyl radical ($OH^{\cdot}$) is the most powerful ROS and can cause damage to almost all molecules including nucleic acids that are very important in biological mechanisms (Charles and Halliwell 1981, Casano et al. 1994, Hagar et al. 1996). If an increment of APX is not accompanied with the increment of SOD, plants will suffer from more serious oxidative damage by creation of $OH^{\cdot}$ (Asada 1994, Shalata and Tal 1998). Actually, in the case of bacteria, the overexpression of SOD induces more serious oxidative stress by the excessive creation of H_2O_2 (Scott et al. 1987, Liochev and Fridovich 1991). Therefore, a balanced combination between SOD and APX is important to overcome oxidative stress.

In this study, soybeans supplied with NH_4NO_3 showed higher SOD and APX activity than plants of other nitrogen sources or nitrogen-free plants under salt environments, and maintained a lower SOD/APX ratio at the same time (Table 1). Therefore, the best growth response of NH_4NO_3-supplied plants under salt environment is considered as result of effective removal of oxidative products such as O_2^- and H_2O_2 by these two antioxidative enzymes (SOD, APX) interactively under the oxidative stress induced by the salt.

The interrelationship between the calcium deficiency reactions of a plant stressed by the salt and the salt tolerance of plants by external calcium is well established (Lynch et al. 1987). In the present experiment, soybeans showed slight relief of the salt-induced growth impediment by the addition of calcium (Fig. 2). From these results, we considered that the growth inhibition in soybeans may be caused by calcium deficiency and by some problems in signal transmission due to structural and functional destruction of the membrane by calcium deficiency under the salt environments (Lazof and Lauchli 1991, Rengel 1992).

Generally, the high level of salt (especially Na^+, Cl^-) in soil decrease the water potential of rhizosphere, and thus induces water stress by suppressing the absorption of water within plants. In addition, it causes ionic toxicity by the passive absorption of specific ions like Na^+ and Cl^- through the transpiration stream of plants. Especially, the suppression of inorganic ions like Ca^{2+} due to excessive absorption of Na^+ may disturb various physiological and biochemical metabolisms related with Ca^{2+} in plants, thus inhibiting growth in salt-sensitive plants under salt environment.

In the present study, soybeans showed an increment of the activity of antioxidative enzyme with a relief of the growth impediment by the additional supply of calcium in the salt environment. Especially, plants supplied with additional 5 mM calcium showed the

best growth response, with high fresh weight and strengthening of the expression of CuZn-SOD (Fig. 2 & Table 2). Increased CuZn-SOD alleviated the salt-induced growth suppression. Therefore, we considered that there is a relationship between relief of salt stress by additional calcium and strengthening of the expression of CuZn-SOD in soybean under the salt environments. However, APX activity was decreased and SOD/APX ratios were increased at high salt level regardless of any additional calcium. Therefore, it is considered that the growth impediment may be caused by the fact that APX did not make balance the increment of SOD activity to salt stress, which induces serious oxidative damage such as the peroxidation of membrane lipid.

It is concluded that relief effects by additional supply of NH_4NO_3 –N or calcium in soybean under salt stress were caused by protecting the cell from the salt-induced oxidative damage through increasing the balanced activity of SOD and APX in salt environment. In the future, further information related to the effects of nitrogen sources and calcium treatment would be advantageous to enhance the productivity of salt-sensitive crops such as rice and soybean.

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