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Selenium modulates the activities of antioxidant enzymes, osmotic homeostasis and promotes the growth of sorrel seedlings under salt stress

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Abstract

Using physiological assays coupled with ultrathin tissue sections, we investigated the impacts of exogenous selenium (Se) on the growth, antioxidant enzymes, osmotic regulation and ultrastructural modifications of leaf mesophyll and root tip cells of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedlings. At low concentrations (1–5 μM), Se tended to stimulate the growth, the activities of superoxide dismutase and peroxidase enzymes, as well as the accumulation of water-soluble sugar in leaves of sorrel seedlings. At higher concentrations (10–30 μM), Se exerted diminished beneficial effects on growth and enzyme activities. CAT activity did not change with Se addition (1–30 μM). Electrolyte leakage of leaf cells declined, and K^+ and Na^+ ions increased in leaves with Se treatment, notably at 5 μM of Se. TEM observations revealed that treatment with 5 μM of Se positively promoted the integrity of membrane systems and cellular organelles, such as chloroplasts and mitochondria in leaf mesophyll and root tip cells. These results strongly suggest that an appropriate concentration of exogenous Se functions positively to promote the antioxidative and osmoregulatory capacity, and enhance the salt-resistance in sorrel seedlings.

Abbreviations: CAT – catalase; EDTA – ethylenediamine tetra acetic acid (disodium salt); NBT – nitroblue tetrazolium; POD – peroxidase; Se – selenium; SOD – superoxide dismutase

Introduction

Plant growth is limited by different unfavorable environmental conditions, among which salt stress is considered to be one of the most important worldwide agricultural problems. High salinity can cause hyperosmotic stress and ion disequilibrium in plant cells, producing oxidative stress (Hasegawa et al. 2000; Zhu 2001) and reducing plant growth. Salt tolerant plants can respond to salt stress via numerous modifications in cellular processes and morphological structure to alleviate ion toxicity and to maintain the balance between the generation and scavenging of oxygen radicals such as O_2^- and H_2O_2 (Gueta-Dahan et al. 1997; Ashraf and Harris 2004). Numerous investigations have shown that superoxide dismutase (SOD) is an integral part in the defense mechanisms against environmental stress, converting the superoxide radical (O_2^-) to a less harmful form – H_2O_2, which

is then converted by peroxidase (POD) or/and CAT into water (Gueta-Dahan et al. 1997; Ashraf and Harris 2004; Gómez et al. 2004). Another mechanism by which plants can adapt to growth reducing salt stress is through the ability to osmotically adjust by accumulation of either inorganic ions or low molecular weight organic solutes (Ashraf 1994; Bohnert et al. 1995; Gill et al. 2001; Ashraf and Harris 2004).

Most crops are salt sensitive or hypersensitive plants (glycophytes), having low resistance capability to salt stress (Yokoi et al. 2002). Although considerable effort has been directed into the selection and development of crop varieties resistant to salinity stress, progress can be limited due to inadequate understanding of the mechanism of tolerance (Dionisio-Sese and Tobita 1998), and finding genetic model systems suitable for study (Zhu 2001). It has been suggested that enhancement of nutrients can partially prevent some of the negative effects of salt stress on plant growth and development (Yu et al. 1998; Elkhatib et al. 2004). Currently, most of these investigations have focused on macronutrients, such as calcium and potassium (Yu et al. 1998; Cengiz et al. 2003; Cengiz and David 2003; El-Hamdaoui et al. 2003; Elkhatib et al. 2004).

Some data has indicated that Se is an essential micronutrient for both plants and animals and human, being an important component of glutathione peroxidase, a potential antioxidant system (Ramauge et al. 1996; Pallud et al. 1997; Gladyshev et al. 1998). Accumulating evidence suggested that Se was able to promote plant growth by increasing the antioxidative capacity and stress tolerance such as UV irradiation and senescence-related stress in lettuce, UV-induced stress in ryegrass and photooxidative stress in potato (Hartikainen et al. 1997; Hartikainen and Xue 1999; Hartikainen et al. 2000; Xue and Hartikainen 2000; Pennanen et al. 2002; Seppänen et al. 2003). However, to our knowledge, there has been limited effort to determine the role of Se in alleviating salt-induced damage.

The objective of this study was to determine if Se could enhance tolerance to salt stress of sorrel (*Rumex patientia* × *R. tianshanicus*), a glycophyte and forage crop of high quality. Here we report on the effects of Se on the activities of several antioxidant enzymes and osmolytes, along with an examination of leaf mesophyll and root tip ultrastructure.

Materials and methods

Plants and growth measurements

Sorrel (*R. patientia* × *R. tianshanicus*) seeds (20 grains) were planted in sand cultures in porcelain pots (30 cm diameter × 35 cm height) in a greenhouse at 18–26 °C and irrigated daily with complete Hoagland's nutrient solution containing 100 mM NaCl with or without Se. Se was added as sodium selenite, varying from 0 (control), 1, 3, 5, 10, to 30 μM. All treatments were replicated five times. To avoid Se contamination to the controls, sand was rinsed thoroughly with distilled water, and the nutrient solution was prepared with analytical reagents in distilled water. The cultures were irrigated to run-off with excessive nutrient solution to avoid accumulation of NaCl and Se. Five seedlings of similar size were selected in each pot 7 days after germination. All of the following measurements were carried out using samples collected around 43 days after seedling selection.

After harvest, samples from three replicates of the cultures were immediately oven dried at 70 °C for 48 h, and then the dry weight (DW) was determined and values were calculated to gram per pot.

Enzyme assays

SOD (EC 1.15.1.1) was assayed by the photochemical method described by Giannopolitis and Ries (1977) with some modifications. One gram of fresh leaf sample, washed with double distilled water, was finely ground in 50 mM phosphate buffer (pH 7.8) in ice bath. The homogenate was centrifuged at 13,000 × *g* for 15 min at 4 °C. The supernatant was collected for the total SOD activity assay. A 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 50 μM nitroblue tetrazolium (NBT), 30–80 μl of enzyme extract, and 1.3 μM riboflavin was illuminated for 15 min under a light bank consisting of six 15-W fluorescent lamps. Blanks non-illuminated and controls omitting the enzyme were run in parallel. The spectrophotomeric (Type 721, Shanghai, China) absorbance of the mixture was determined at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT photoreduction. SOD activity was expressed as activity per gram fresh weight fresh leaf.

POD (EC 1.11.1.7) activity was determined using the guaiacol oxidation method (Chance and Maehly 1955). Briefly, 1 g of fresh leaves was ground in 50 mM phosphate buffer (pH 6.4) in ice bath and then centrifuged at 13,000 × *g* for 15 min at 4 °C. The supernatant was used to determine enzyme activity. Absorbance of 3 ml reaction mixture containing 50 mM phosphate buffer (pH 6.4), 8 mM guaiacol, 100–200 μl enzyme extract and 2.75 mM H_2O_2 was recorded at 470 nm 180 s after 95 μl of 0.3% H_2O_2 was added. One unit of POD activity was defined as the change in absorbance per minute and specific activity as enzyme units per gram of fresh sample.

The enzymatic activity of catalase (CAT; EC 1.11.1.6) was determined using a modified method developed by Aeby (1984). One gram of fresh leaves was ground in 50 mM phosphate buffer (pH 7.0) in an ice bath and centrifuged at 13,000 × *g* for 15 min at 4 °C, collecting the supernatant. The 3 ml reaction mixture contained 10 mM H_2O_2 and 100 μl of enzyme extract in 50 mM phosphate buffer (pH 7.0). Enzyme activity was assayed by monitoring the decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. One unit of CAT activity (U) was defined as the decomposition of 1 μmol H_2O_2 per minute.

Measurements of electrolyte leakage

Twenty leaf discs (~1 cm in diameter) were excised and rinsed thoroughly with double distilled water to remove contamination caused by sampling. Samples were then transferred to tubes with 20 ml double dH_2O. The electrical conductivity (E_0) of the solution was immediately measured using an electrical conductivity meter (DDSJ-308A, Shanghai) at 25 °C. The tubes were incubated at 30 °C for 30 min, and the electrical conductivity (E_1) measured again. Subsequently, the tubes were placed in boiling water for 20 min, and the electrical conductivity (E_2) read after the tubes had cooled to room temperature. The electrolyte leakage (EL) of leaf cells was calculated accordingly: EL (%) = $(E_1-E_0)/(E_2-E_0) \times 100$.

Water-soluble sugar and K^+, Na^+ concentrations in leaves

Water-soluble sugar concentration was quantified colorimetrically with anthrone reagent according to Riazi et al. (1985). One gram of fresh leaf was ground, from which soluble sugars were extracted three times with 10 ml of 80% ethanol. The pooled extracts were centrifuged at 13,000 × *g* for 10 min. The amount of water-soluble sugars in the supernatant was determined spectrophotomerically at 500 nm using a standard glucose curve.

K^+ and Na^+ concentrations were determined using 2 g of fresh shoot or root oven dried at 70 °C to a constant weight. The dried material was finely powdered, and then subjected to a wet digestion with HNO_3 : $HClO_4$ (4:1) (Chapman and Pratt 1961). The resulting solutions were appropriately diluted and measured of K^+ and Na^+ concentrations made using a flame photometer (FP 640, Shanghai, China). Measurements were calibrated using NaCl or KCl solutions of known concentrations.

Electron microscopy

Leaf and root tip tissues from control and 5 μM Se-treated seedlings were fixed for 4 h in 2.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2). After washing in 100 mM sodium cacodylate, tissues were post-fixed in 1% osmium tetroxide for 2 h, dehydrated in an ethanol series, transferred to propylene oxide, and embedded in Epon812 resin. Ultrathin sections were cut using an LKB-V ultra-microtome, stained with 2% uranyl acetate (w/v) in 70% methanol (v/v), followed by 0.5% lead citrate, and observed under a TEM (JEM—1200EX, JEOL, Tokyo, Japan) at 80 kV.

Statistical analysis

All the data were subjected to an analysis of variance (ANOVA) using Microsoft Excel 2000, and given as the standard deviation (SD) of the means. The significance of differences between mean values were determined with a *t*-test. Differences at $P < 0.05$ were considered significant.

Results

Plant growth

Under 100 mM NaCl stress condition, the seedlings treated with different concentrations of Se grew better than the controls, and the

best-growing seedlings were consistently apparent with the 5 μM of Se treatment. Our results revealed that at low concentrations (1–5 μM), Se significantly promoted the growth of NaCl-stressed seedlings. At 5 μM Se plant biomass was double that of the control. At higher concentrations, Se had a negative effect with biomass accumulation declining (Table 1).

Activities of antioxidant enzymes and relative permeability of leaf cell membrane

The effects of different concentrations of Se on SOD and POD activities in the leaves of NaCl-stressed seedlings are presented in Figure 1. Data showed that the activities of these enzymes in leaves exhibited similar changes in response to exogenous Se. At low concentrations (1–5 μM), Se significantly increased SOD and POD activity (from 31 to 72 U g^{-1} FW and 0.73–1.56Δ OD_{470} min^{-1} g^{-1} FW, respectively). Both enzymes had maximal activities at 5 μM Se, and increased (in comparison to the control) by 133% ($P < 0.01$) and 100% ($P < 0.01$), respectively. As Se concentration increased, enzyme activity declined. CAT, another enzyme responsible for antioxidative processes, remained unaltered (0.257 ± 0.007 μmol $H_2O_2 min^{-1}$ g^{-1} FW) with respect to different Se treatments.

Table 1. Effect of different concentrations of Se on the biomass of 100 mM NaCl-stressed 43-days-old sorrel (*Rumex patientia* × *R. tianshanicus*) seedlings.

Se concentration (μM)	Biomass (leaf + root) (g DW pot^{-1})	Change %
0	9.44 ± 0.49a	–
1	13.22 ± 1.05b	40.04**
3	16.48 ± 1.07c	74.58**
5	19.70 ± 1.42d	108.69**
10	15.76 ± 0.94c	66.95**
30	10.34 ± 0.61e	9.53*

Values are means ± SD of 15 seedlings from 3 replicates. Significant difference between data was compared by *t*-test at the 5% level. Data sharing the same letter were not significantly different. The changes in biomass between control and various Se treatments were also tested by *t*-test: *$p \leq$ 0.01, **$p \leq 0.001$.

The extent of membrane damage was assessed indirectly by determining the amount of solute leakage from leaf cells. The conductivity measurements showed that at low concentrations of Se, the electrolyte leakage (EL) tended to decrease; notably at 5 μM Se, the EL decreased significantly, being about 22% ($P < 0.05$) lower than that of control leaf cells (Figure 1). At higher concentrations of Se, the EL progressively increased. The variation in EL of leaf cells in response to Se treatment was the inverse of seedling growth rate, SOD and POD activities; the negative correlation between EL and the activity of these two enzymes was $r = -0.95$ and $r = -0.98$, respectively.

Osmotic regulation

At 1–5 μM Se, total water-soluble sugar concentration gradually increased in leaves. As the Se

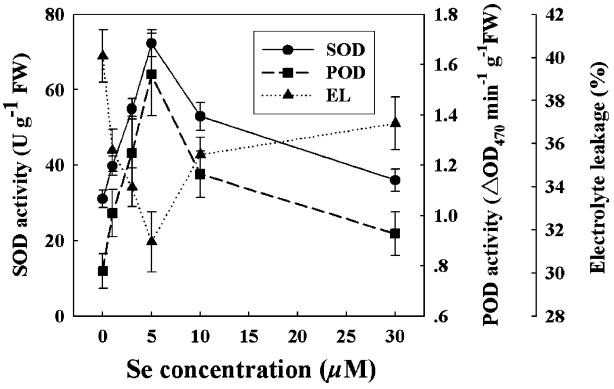


Figure 1. Effects of exogenous Se treatments on the SOD and POD activities and the eletrolyte leakage in leaf tissues of 100 mM NaCl-stressed 43 days old sorrel (*Rumex patientia* × *R. tianshanicus*) seedlings. Values are the means ± SD of five replicates.

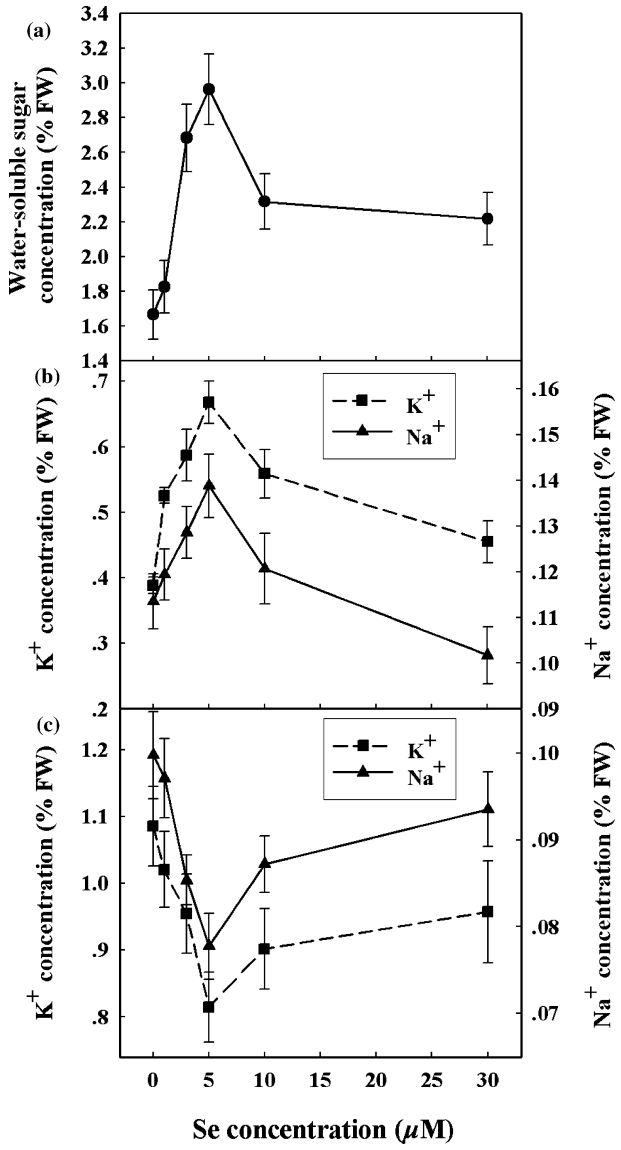


Figure 2. Effects of exogenous Se treatments on water-soluble sugar, K^+ and Na^+ concentrations in leaves of 100 mM NaCl-stressed 43-days-old sorrel (*Rumex patientia* × *R. tianshanicus*) seedlings. Values are the means ± SD of five replicates.

concentration increased, water-soluble sugars declined. The highest value (2.9% FW) was achieved at 5 μM Se, being 78% ($P < 0.01$) higher than that of the controls (Figure 2a). The change in water-soluble sugar concentration at different Se treatments correlated very well with changes in plant growth rate ($r = 0.89$) (Table 1), SOD ($r = 0.92$) and POD ($r = 0.92$) activities (Figure 1).

Under 1–5 μM Se conditions, K^+ and Na^+ concentrations gradually increased in leaf tissues. With further increases in Se, K^+ and Na^+ concentrations gradually declined (Figure 2b). While in roots, K^+ and Na^+ concentrations showed the reverse (Figure 2c). Although Se treatments did not block the uptake of Na^+, nor promote the uptake of K^+, both ions were inclined to accumulate in leaves. Notably in the treatment with 5 μM of Se, statistically significant increases in K^+ (72%, $P < 0.01$) and Na^+ (22%, $P < 0.05$) concentrations were detected.

Modifications in cellular ultrastructure

In control seedlings, NaCl (100 mM) impaired the cellular ultrastructure of leaf mesophyll and root tip cells of sorrel. Prominent ultrastructural damage to chloroplasts was characterized by disintegration of the envelope, structurally swollen, distorted, disrupted and irregularly shaped thylakoids, as well as fewer-stacked and irregularly arranged thylakoid grana (Figures 3a, d and e). All of the mitochondria of either leaf mesophyll or root tip cells were injured by 100 mM NaCl to varying degrees. Many mitochondria were dumbbell-shaped (Figure 3a), while for some mitochondria the cristae declined or totally disappeared, and the mitochondrial ectoblast was not clearly discernible (Figures 3a, e and h). In cytoplasm of the leaf mesophyll and root tip cells, numerous small vacuoles increased, inside which many membrane-like fragments or ring-shaped structures were recorded (Figures 3c and g). A common feature of nuclear and plasma membranes was that they became blurred, swollen, or disintegrated under 100 mM NaCl stress (Figures 3a, c, e, g and h).

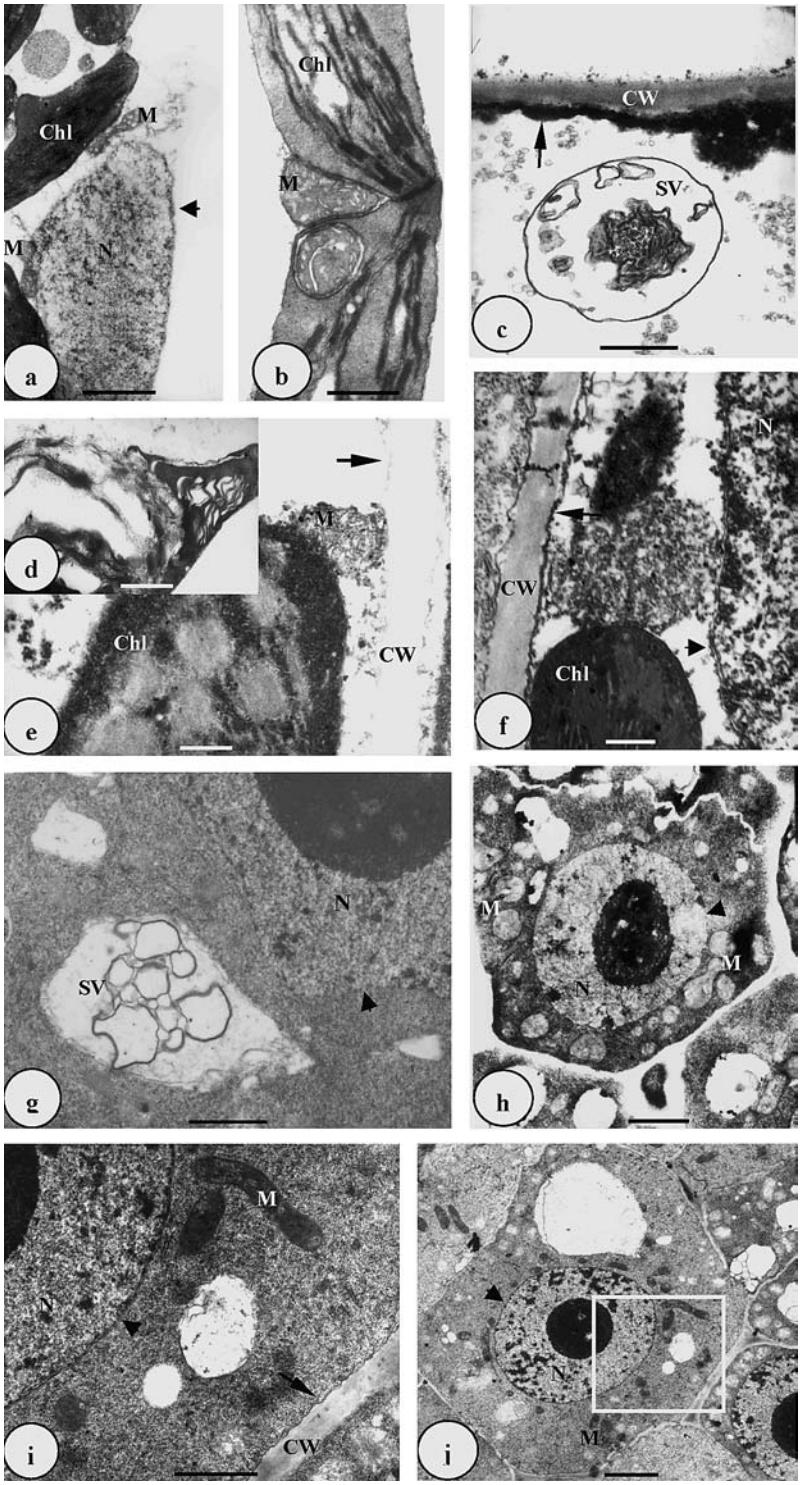
In saline environment, including Se (5 μM) in the irrigation solution, significantly alleviated the damage caused by 100 mM NaCl stress as observed in the controls. The integrity of cytoplasmic organelles, as well as the plasma and nuclear membranes were ameliorated in leaf cells (Figures 3b and f) and root tip cells (Figures 3i and j) by treatment with 5 μM of Se. In chloroplasts, grana dilation was abated and thylakoids became more regularly arranged (Figure 3b). The mitochondrial cristae in leaf mesophyll cells became more legible and their numbers increased in response to 5 μM Sev (Figure 3b). Although the mitochondrial cristae of root tip cells were not distinguishable, the mitochondria matrix was more heavily stained by lead (Figures 3i and j) than that in non-Se-treated plants (Figure 3h). It was apparent that the contact between chloroplasts, as well as, between chloroplasts and mitochondria were more intimate at 5 μM Se, with some mitochondria found inlaid into chloroplasts (Figure 3b).

Figure 3. Electron micrographs and ultrastructure of mosophyll cells and root tip cells of 100 mM NaCl-stressed 43-days-old sorrel (*Rumex patientia* × *R. tianshanicus*) seedlings with 5 μM or without Se treatment. Arrows indicate plasma membrane (PM); short arrows indicate nuclear membrane (NM). Bars: 1 μm (a and c), 0.5 μm (b, d, e and f), 2 μm (g and i), 5 μm (h and j). (a) Mesophyll cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling without Se treatment, showing the blurry nuclear membrane (NM) and dumbbell-shaped mitochondria. (b) Mesophyll cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedlings treated with 5 μm of Se, showing the intimate contact between chloroplast (Chl) and mitochondrion (M). (c) Mesophyll cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling without Se treatment, showing the swollen and heavily stained plasma membrane and small secondary vacuoles (SV) containing membrane-like fragments or ring-shaped structures. (d) Badly destroyed chloroplast by 100 mM of NaCl. (e) Mesophyll cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling without Se treatment, showing the damaged chloroplast, mitochondrion and plasma membrane. (f) Mesophyll cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling treated with 5 μM of Se. The plasma membrane and nuclear membrane were improved compared with that without Se treatment as shown in (e). (g) Root tip cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling without Se treatment, showing the indiscernible nuclear membrane and the secondary vacuole (SV) containing membrane-like fragments or ring-shaped structures. (h) Root tip cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling without Se treatment, showing the lightly stained mitochondria and the indiscernible nuclear membrane. (i and j) Root tip cell of 100 mM NaCl-stressed sorrel (*Rumex patientia* × *R. tianshanicus*) seedling treated with 5 μM of Se, showing the distinct plasma membrane and nuclear membrane compared with that in (g) and (h), as well as the electron-denser mitochondria compared without Se treatment (h). (i) is the magnified micrograph of the pane in (j).

Discussion

We observed that at lower concentrations (1–5 μM) of Se, growth was stimulated in salt-stressed sorrel seedlings, whereas at high concentrations (10–30 μM) the beneficial effect declined. The Se results agree with earlier reports for lettuce, ryegrass, and potato subjected to various oxidative stresses (Hartikainen and Xue 1999; Hartikainen et al. 2000; Xue and Hartikainen 2000; Xue et al. 2001; Pennanen et al. 2002; Seppänen et al. 2003).

The production of reactive oxygen species (ROS) is the important cause of damage to plants when subjected to salt stress, thus leading to the growth suppression (Dionisio-Sese and Tobita 1998; Zhu 2001). In our experiments, SOD and POD activity of salt-stressed seedlings increased when exposed to concentrations ranging 1–5 μM Se. At concentrations between 10 and 30 μM, there were adverse effects on both enzymes



compared with that at 5 μM Se. With different Se treatments the changes in plant growth correlated very well with that in SOD ($r = 0.98$) and POD ($r = 0.98$) activities. Considering the role of SOD and POD activities in scavenging oxygen radicals in various oxidative stress-resistant processes

(Ashraf and Harris 2004), we postulate an optimal concentration of Se may have positive consequences on seedling response to salt stress.

Electrolyte leakage has been thought to be an important index of the physiological functions of the cell. Adversities such as drought, salinity, and high and low temperatures initially damage the structure of the cell membrane, thereby affecting its function, leading to an increase in membrane permeability, and resulting in leakage of intracellular contents (Jia et al. 2002). To evaluate the degree of damage to membranes under salt-stress condition and to evaluate the roles of enhanced SOD and POD activities in protecting from stress, we measured solute leakage as an estimate of membrane damage. The resulting values were negatively correlated with the varying SOD and POD activities, which was highly consistent with what found in Citrus (Gueta-Dahan et al. 1997), rice seedlings (Dionisio-Sese and Tobita 1998), and others (Ashraf and Harris 2004). Therefore, we conclude that the reduced electrolyte leakage may be a direct consequence of Se treatment.

Water-soluble sugar is one osmoticum shown to be a good marker for selecting improved salinity or drought tolerance (Quick et al. 1989; Al Hakimi et al. 1995; Murakeozy et al. 2003). Sugars contribute up to 50% of the total osmotic potential in glycophytes subjected to saline conditions (Ashraf and Harris 2004). In this study, the accumulation of water-soluble sugar was observed in salt-stressed sorrel exposed to Se (Figure 2a).

It has been suggested that in NaCl-stressed plants, Na^+ is an energetically efficient osmoticum, which is always compartmentalized within the vacuole to minimize cytotoxicity (Blumwald et al. 2000). K^+, is one of the primary nutrients, playing multiple cellular roles; it is also energetically a cheaper osmoticum than organic metabolites (Ortiz et al. 1994). In our study, application of Se to sorrel induced the accumulation of K^+ and Na^+ in leaves and a reduction in roots under saline conditions. However, how the acclimated sorrel seedlings dealt with the increased Na^+ ion was unclear. One explanation may be that change(s) in cellular processes caused by the appropriate concentration of Se may favour compartmentation of Na^+ within vacuoles.

Some ultrastructural alterations are implicated in the acclimation of plants to a saline environment (Sam et al. 2003). Therefore, some ultrastructural modifications may reflect the protective effects of Se on NaCl-stressed plants. In this study, the integrity of cellular organelles and the membrane systems was found improved (in comparison with control) in leaf mesophyll and root tip cells treated with 5 μM of Se in saline environment. Additionally, the contact between cellular organelles was more intimate at 5 μM Se than control. The Se-induced higher growth rate may result from the benefits of well-preserved membrane systems, that are essential for photosynthesis and respiration, as well as from the intimate contact required to favorably improve structure–function relationship for scavenging oxygen radicals via the high chloroplasts SOD activity (Ogawa et al. 1997; Gómez et al. 2004).

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