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Effects of NaCl Stress on Plant Growth and Antioxidative Defense of Kumquat Seedlings *In Vitro*

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Abstract

This experiment was conducted to determine the effect of salt stress (0, 50, 100, 150 and 200 mM NaCl) on plant growth and antioxidative defense of Kumquat (*Fortunella margarita*) seedlings *in vitro*. Stem diameter, leaf number, plant height, root morphology, leaf superoxide dismutase (SOD) and catalase (CAT) activities, and leaf soluble protein content were determined. The Kumquat germination percentage and plant growth significantly reduced with the increasing of NaCl concentrations, and only keeping vitality and no germination in seeds were at 200 mM NaCl concentration. Root biomass and morphological traits (length, projected area, surface area, average diameter, volume, and tips) gradually decreased with the increasing of NaCl concentrations. The leaf soluble protein content and CAT activities of *Fortunella margarita* significantly decreased with the increasing of NaCl concentrations *in vitro*, but leaf SOD activities had the highest activity at 50 mM NaCl. It may be suggest that NaCl stress heavily inhibited seed germination at 200 mM level and plant growth and root morphology, and only the kumquat plants possessed an antioxidant enhancement in 50 mM NaCl.

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Keywords

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Introduction

Kumquat (*Fortunella margarita*) is a kind of high nutritional value of citrus fruit and originated from china. Just like other fruit trees, kumquat production and development are usually influenced by biotic or abiotic factors conditions.

Salt stress in nature is one of the major abiotic factors limiting seed germination (Zhao et al., 2014) and plant productivity (Wu et al., 2010). As a result, enhanced antioxidant responses lead to the breeding programs (Li et al., 2011). Plants show low solute potential in salt stress (Sebei et al., 2007; Wang et al., 2016; Parihar et al., 2015), resulting in physiological disorder or death. Salt stress often leads to the burst of reactive oxygen species (ROS) (Li et al., 2011), such as hydrogen peroxide (Almansa et al., 2002), superoxide radical and hydroxyl radical (Ei-Mashad, 2012) in cell, thereby triggering protein degradation (Wang et al., 2014) and the enzymatic antioxidant defense system abnormal (Li et al., 2011; Ei-Mashad, 2012; Keyster et al., 2013). Salt stress may lead to a wide range of adverse metabolic responses within plants (Yasar et al., 2008) and even death (Kan et al., 2015).

Among antioxidant enzymatic defense system components, SOD (superoxide dismutase) and CAT

(catalase) play a significant role in ROS elimination under salt stress (Almansa et al., 2002; Wang et al., 2014; Keshavkant et al., 2012). SOD is a major enzyme in catalysing the disproportionation of superoxide radicals convert into molecular oxygen and hydrogen peroxide (Almansa et al., 2002). CAT could decompose hydrogen peroxide into water and oxygen (Wang et al., 2014), thus against oxidative damage. Interestingly, antioxidant enzyme activities show different capacity in salt tolerance (Yasar et al., 2008; Wang et al., 2014). Perveen et al. (2016) revealed that SOD and CAT activities of mung bean variety NM-2006 significantly increased under 75 mM NaCl stress. The activities of SOD in the root of *Kandelia candel* were reduced but enhanced CAT activity under different salinity stress (150 and 300 mM), and SOD activities had shown a trend from ascent to descent with the extension of time (Wang et al., 2014). It indicated that the activities of SOD and CAT were essential for elimination ROS under salt stress.

Soluble protein content has a positive correlation relationship with antioxidative enzyme activity in salt stress, and always influences the plant sustainable in osmoregulation (Gulen et al., 2006). Interestingly, plants have a storage form of nitrogen in plant growth and development, and the nitrogen can re-utilized when salinity stress was over, leads to the accumulation of soluble proteins (Singh et al., 1987). Perveen et al. (2016) revealed that soluble protein content significantly increased at 75mM NaCl stress in mung bean.

Earlier works showed that culture techniques can provide uniform environments, reduce morphological and genetic variability to study the salt stress response of seedlings has been attempted *in vitro* (Miki et al., 2001; Aghaleh et al., 2009; Mills et al., 2004).

In this paper, we aimed to study the effect of NaCl stress (0, 50, 100, 150 and 200 mM) on plant growth, leaf SOD and CAT activities, and leaf soluble protein content of *Fortunella margarita* seedling *in vitro*.

Materials and methods

Experimental materials

The kumquat (*Fortunella margarita*) seeds were offered by Professor Qiang-Sheng Wu (Yangtze University, China). The Murashige and Tucker (MT) medium (pH 5.8 and no hormone was add) consists of a large number of elements and organic matter. The flat tubes (15-cm height and 4-cm diameter) were used.

Experimental design

The experiment was conducted with one-single factor in randomized design. The factor is NaCl concentrations containing 0, 50, 100, 150 and 200 mM, a total of five treatments with six replicates. Each tube is a replicate and inoculated with one seed. These NaCl treatments were designed in MT medium.

Kumquat seeds were sterilized and cultured in MT medium at darkness for a period of time (usually 1~2 weeks) on MT medium *in vitro*, and subsequently placed a bright environment. After 52 days of the NaCl stress, kumquat seedlings were harvested. Growth parameters such as stem diameter, leaf number and height per plant were recorded before harvesting. Seedlings were divided into the shoot and the root, whose fresh biomass was determined immediately.

The WinRHIZORoot analyzer was used to analyze root morphology, leaf CAT activity was expressed by permanganate titration during 1 min from 1g fresh sample (mg/g.min) (Li, 2000). SOD activity was determined by Nitroblue tetrazolium method (Li, 2000). Soluble protein content in leaves was determined by G-250 Coomassie brilliant blue method (Li, 2000).

Statistical analysis

The data were statistically analyzed with one-factor of variance in SAS (8.1) software, and the significant differences between the treatments were compared with the LSD at $p<0.05$.

Results

Plant growth

As shown in Table 1, the kumquat seedlings with different NaCl treatments resulted in significant reduces in plant growth traits, compared with the no-NaCl treatment, and nip it in the bud when the NaCl treatment up to 200 mM. There was no significant difference in plant leaf number of kumquat seedlings between the 50 and 100 mM NaCl treatments. In kumquat seedlings, plant height, stem diameter, leaf number, shoot and root dry weight notably reduced with the increasing of NaCl concentration *in vitro*.

Table 1. Effects of different salt stress on plant biomass of *Fortunella margarita* seedling *in vitro.*

NaCl (mM) treatments	Plant height (cm)	Stem diameter (cm)	Leaf number	Shoot dry weight (g)	Root dry weight (g)
0	4.9±0.3a	0.14±0.01a	5±1a	0.14±0.01a	0.07±0.00a
50	3.7±0.4b	0.13±0.01b	4±1b	0.10±0.01b	0.04±0.00b
100	3.0±0.4c	0.12±0.00c	4±1b	0.09±0.00c	0.03±0.00c
150	0±0d	0±0d	0±0c	0±0d	0.02±0.00d
200	0±0d	0±0d	0±0c	0±0d	0±0e

Note: different letters indicate significant differences (p<0.05) in the same column of figures between treatments. "0±0": No seed germination *in vitro*.

Root morphology

Root morphology of the kumquat seedlings was significantly reduced by NaCl treatments, compared with the no-NaCl treatment (Table 2). Kumquat seedlings represented significantly lower root length, projected area, surface area, average diameter, volume and tips than no-NaCl seedlings: 9.73, 19.32, 18.61, 13.79, 34.52 and 23.08% lower in leaf under 50 mM NaCl; 23.26, 35.23, 37.23, 20.69, 52.38 and 23.08% lower in leaf under 100 mM NaCl; 55.50, 60.23, 59.85, 24.14, 66.67 and 46.15% lower in leaf under 150mM NaCl; 100, 100, 100, 100, 100 and 100% lower in leaf under 200 mM NaCl, respectively (Table 2).

Table 2. Effects of different salt stress on root morphology of *Fortunella margarita* seedling *in vitro.*

NaCl (mM) treatments	Length (cm)	Projected areas (cm^2)	Surface area (cm^2)	Average Diameter (mm)	Volume (cm^3)	Tips
0	7.91±0.58a	0.88±0.04a	2.74±0.12a	1.16±0.08a	0.084±0.016a	13±3a
50	7.14±0.48b	0.71±0.03b	2.23±0.12b	1.00±0.06b	0.055±0.005b	10±5ab
100	6.07±0.53c	0.57±0.05c	1.72±0.17c	0.92±0.08bc	0.040±0.006c	10±2ab
150	3.52±0.71d	0.35±0.08d	1.10±0.26d	0.88±0.08c	0.028±0.008d	7±3b
200	0±0e	0±0e	0±0e	0±0d	0±0e	0±0c

Note: different letters indicate significant differences (p<0.05) in the same column of figures between treatments. "0±0": No seed germination *in vitro*.

Leaf CAT and SOD activity

Compared with the no-NaCl treatment, the kumquat seedlings with NaCl treatments showed a significantly (p<0.05) lower CAT activities: 59.66, 75.28, 100 and 100% lower in leaf under 50, 100, 150 and 200 mM NaCl treatments, respectively (Fig. 1). However, the NaCl treatments notably increased SOD activities by 30.79% in leaf under 50 mM NaCl treatment, whereas it decreased SOD activities by 36.45, 100 and 100% under 100, 150 and 200 mM NaCl treatments, respectively (Fig. 2).

Leaf soluble protein content

The kumquat seedlings exposed to NaCl stress exhibited significantly lower soluble protein content than the seedlings of the no-NaCl treatment: 35.78, 38.73, 100 and 100% lower in leaf under 50, 100, 150 and 200 mM NaCl treatments, respectively (Fig. 3).

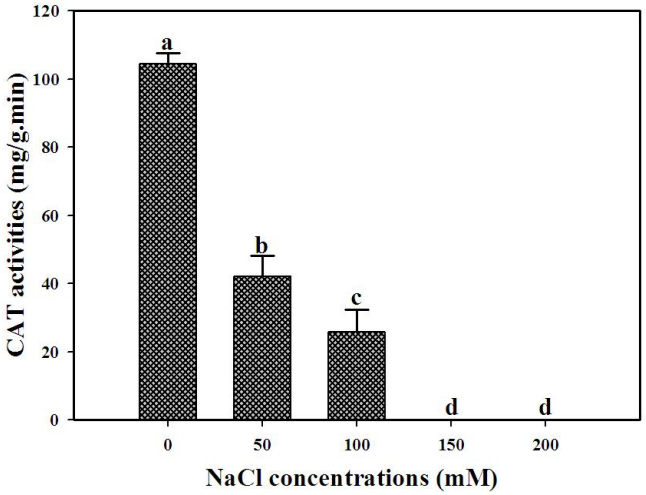

Fig. 1: Effect of salt stress on leaf catalase (CAT) activities of *Fortunella margarita* seedling *in vitro*. **Note:** different letters indicate significant differences (p<0.05) between treatments.

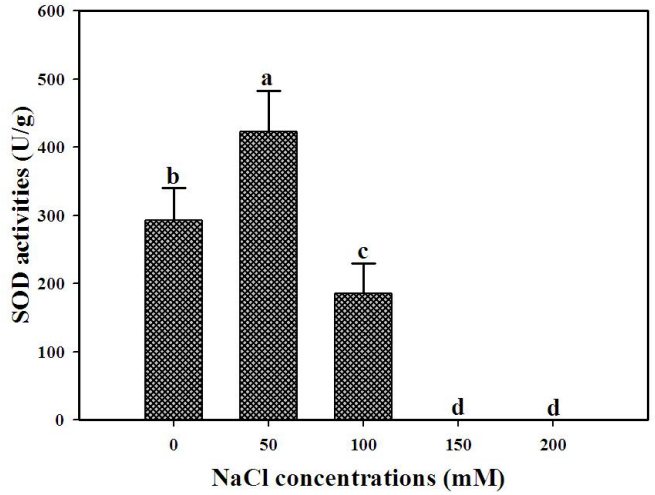

Fig. 2: Effect of salt stress on leaf superoxide dismutase (SOD) activities of *Fortunella margarita* seedling *in vitro*. **Note:** different letters indicate significant differences (p<0.05) between treatments.

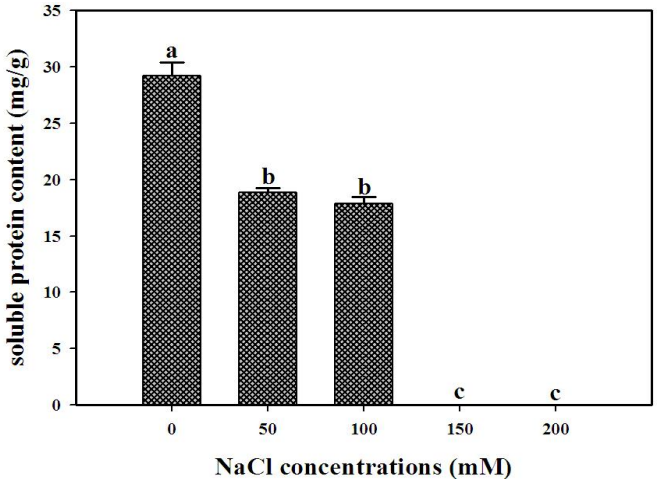

Fig. 3: Effect of salt stress on leaf soluble protein content of *Fortunella margarita* seedling *in vitro.* **Note:** different letters indicate significant differences (P<0.05) between treatments.

Discussion

In our study, the seed germination and growth traits of *Fortunella margarita* seedlings dramatically restricted under NaCl stress condition. This is in agreement with earlier studies reported that salt stress induced ion imbalance (Parihar et al., 2015), destruction of membrane permeability (Jabeen et al., 2014; Wang et al., 2016), and oxidative stress (Li et al., 2011), leading to seed germination and plant growth dramatically restricted. If the salt stress exceeded the limits of salt-tolerant, plants will not germinate, as seen at 200 mM NaCl concentration, in this study, which is in agreement with earlier works (Murkute et al., 2005; Li et al., 2016).

The present study also showed that root morphological traits (length, area, volume, diameter, tips) dramatically more lower accompany with NaCl concentrations increasing. In salt stress condition, plant root suffer from adversity stress signal and inhibited morphological growth and development (Wu et al., 2010). And as well as plant root can produce the corresponding abnormal physical reaction and reduced the absorption of nutrients and water, which in turn effect aboveground growth and photosynthesis (Cramer et al., 1982). Plant root acted as a variety of material assimilation, transformation and synthesis of vital organs (Guo, 2003). A better root morphology directly affects the growth of plants, salt stress on the aboveground growth by inhibition of more obvious. This is in agreement with our study results.

NaCl treatments caused oxidative stress and adverse growth processes at high concentrations, thus results in ROS overproduction and plant damage (Li et al., 2011; Keyster et al., 2013). In this study, compared with the no-NaCl treatment, 50 mM NaCl treatment significantly decreased leaf CAT activities of *Fortunella margarita* seedlings, but significantly increased leaf SOD activities. Leaf SOD and CAT activities markedly reduced under 100, 150, 200 mM NaCl treatments. It is possible exactly that plant in order to maintain the balance of ROS metabolism in the plant cell only under 50 mM NaCl. Nevertheless, SOD can catalyze disproportion reaction and produce the non-toxic of hydrogen peroxide and molecular oxygen, thus avoiding plant cell damage under lower salt stress (Song et al., 2006; Gulen et al., 2006). Sorkheh et al. (2012) reported that SOD activity was significantly increased under 40, 80, and 120 mM NaCl stress in *Prunus scoparia*, whereas CAT activity was significantly higher at 40 mM NaCl stress in *Prunus scoparia*, and reduced at 80, 120 mM NaCl stress. It suggested that the activity of SOD to alleviate the oxidative damage in leaf of *Fortunella margarita* seedlings under salt stress is very important.

Protein is an extreme important impact on the tolerance mechanisms of plant under adverse situation (Gulen et al., 2006). Wang et al. (2014) considered that although salt stress brings about the osmotic potential reduction, whereas the effect in some protein is not always. Salt stress has a direct influence on protein and cellular membranes (Lahav et al., 2004). In this paper, soluble protein content of *Fortunella margarita* seedlings was significantly lower in leaf under 50-200 mM NaCl treatment condition than under 0 mM NaCl conditions. Such reduction of soluble protein may be

due to pH decrease (Zhao et al., 2014). This is in accordance with Behera et al. (2009).

In short, although growth traits, leaf soluble protein content, CAT and SOD activities of *Fortunella margarita* seedlings dramatically decreased in accompany with NaCl concentrations increasing, but the kumquat has a certain response to salt resistance under 50 mM NaCl.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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