Phytoprotection

Humic acid may retard damages of cells in strawberry apices in high saline environment

L'acide humide peut retarder les dommages cellulaires au niveau des extrémités de la fraise dans un environnement hautement salé

Yong-Hong Lin and Yao-Tsung Chang

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Article abstract

Most studies were focused on the salt-resistance physiology by humic acid (HA) for strawberry in the past. For advancely verifying the remission of salt injury by humic acid, this study was conducted to evaluate K^+/Na^+ in the strawberry and observe cell morphology of strawberry after treatment of salt concentrations (0 and 50 mg kg^{-1}) and HA (0, 150 and 300 mg kg^{-1}). The results showed that the treatments of humic acid will increase the absorption of K^+ (potassium ion) and reduce Na^+ (sodium ion), and hence increase K^+/Na^+ in the root and leaf of strawberry. By the observing of SEM (scanning electron microscopy) and TEM (transmission electron microscopy), under no salt treatments, no matter the additive concentration of humic acid, the root apices of strawberries were normal and integrity. However, in the treatments of high salt concentration, the shrinking and cracking of cells in root apices of strawberries were serious and gradually getting integrity and normal after adding humic acids from 150 to 300 mg kg^{-1}. These results showed that the appropriate treating concentration of humic acid will inhibit the salt injury on root apices of strawberries.

Humic acid may retard damages of cells in strawberry apices in high saline environment

Yong-Hong Lin[1]✉, and Yao-Tsung Chang[2]

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Most studies were focused on the salt-resistance physiology by humic acid (HA) for strawberry in the past. For advancely verifying the remission of salt injury by humic acid, this study was conducted to evaluate K^+/Na^+ in the strawberry and observe cell morphology of strawberry after treatment of salt concentrations (0 and 50 mg kg^{-1}) and HA (0, 150 and 300 mg kg^{-1}). The results showed that the treatments of humic acid will increase the absorption of K^+ (potassium ion) and reduce Na^+ (sodium ion), and hence increase K^+/Na^+ in the root and leaf of strawberry. By the observing of SEM (scanning electron microscopy) and TEM (transmission electron microscopy), under no salt treatments, no matter the additive concentration of humic acid, the root apices of strawberries were normal and integrity. However, in the treatments of high salt concentration, the shrinking and cracking of cells in root apices of strawberries were serious and gradually getting integrity and normal after adding humic acids from 150 to 300 mg kg^{-1}. These results showed that the appropriate treating concentration of humic acid will inhibit the salt injury on root apices of strawberries.

Keywords: salt injury, K^+/Na^+, cell morphology, microscopy, humic acid.

[L'acide humide peut retarder les dommages cellulaires au niveau des extrémités de la fraise dans un environnement hautement salé]

La plupart des études antérieures sur la fraise se sont concentrées sur la résistance au sel par l'acide humique (AH). Pour vérifier l'atténuation des lésions salines par l'acide humique, cette étude vise à évaluer le ratio K^+/Na^+ chez la fraise et à observer la morphologie cellulaire de la fraise face à différentes concentrations de sel (0 et 50 mg kg^{-1}) et d'AH (0, 150 et 300 mg kg^{-1}). Les résultats indiquent que les traitements à l'acide humique augmentent l'absorption de K^+ (ion potassium), réduisent le Na^+ (ion sodium), et augmentent donc le ratio K^+/Na^+ dans la racine et la feuille de la fraise. Par l'observation en MEB (microscopie électronique à balayage) et en MET (microscopie électronique à transmission), sans aucun traitement au sel, quelle que soit la concentration additionnelle d'acide humique, les apex racinaires des fraises étaient normaux et intègres. Cependant, dans les traitements à forte concentration de sel, le rétrécissement et la fissuration des cellules dans les apex racinaires des fraises étaient graves et devenaient progressivement intègres et normaux après l'ajout d'acides humiques de 150 à 300 mg kg^{-1}. Ces résultats démontrent qu'une concentration appropriée en acide humique inhibe les dommages causés par le sel sur les apex racinaires des fraises.

Mots-clés : lésion saline, K^+/Na^+, morphologie cellulaire, microscopie, acide humique.

1. Department of Plant Industry, National Pingtung University of Science and Technology, Pingtung, Taiwan; yonghong@mail.npust.edu.tw
2. Division of Crop Environment, Kaohsiung District Agricultural Research and Extension Station, Pingtung, Taiwan

INTRODUCTION

The salt stress causes the most serious production loss of crops everywhere. The crops growth can be injured with the increase of salt in the medium, affecting the biomass and its production (Wani and Gosal 2011). Based on some research results, the active oxygen (ROS) may be induced by high salt concentration. The increase of ROS will cause cell necrosis and damaged pathology (Verma and Mishra 2005; Xipell *et al.* 2016). Furthermore, the leaf tissue was damaged and senescent (Tanou *et al.* 2009). The abscisic acid (ABA) produced obviously will accelerate the ageing of leaves as the main injury (Fahad, Hussain *et al.* 2015; Fahad, Nie *et al.* 2015). Other factors include the decrease of chlorophyll contents (Khosravinejad *et al.* 2008) and the permeability of cell membrane. In fact, plants will resist the salt injury by themselves. For example, the synthesis of organic solutes may maintain turgor pressure to resist high salt concentration (Yeo 2006). The soluble carbohydrate may induce cells to better permeability to resist environmental stress (Fahad, Hussain *et al.* 2015; Fahad, Nie *et al.* 2015). Another important salt tolerant index was K^+/Na^+ (Munns and Tester 2008). The K/Na ratio will be decreased if salt is added to the medium, causing imbalance between K^+ and Na^+ (Porcelli *et al.* 1995). Hence, the K/Na ratio was thought to be the salt tolerance index in horticultural crops (e.g., strawberry). The phenomenon mainly influences the steady of enzyme and membrane. The K/Na ratio in strawberry can be used to judge its salt tolerance (Sun *et al.* 2015; Yilmaz and Kina 2008).

Humic acid can promote the growth of crops, and it is commonly called biostimulant. It can induce the production of primary and secondary metabolism, and induce enzyme of plants to resist salt stress (Aydin *et al.* 2012). Other effective functions of humic acid include altering mineral uptake and inducing salt tolerance (Khaled and Fawy 2011). It can also increase the contents of protein and promote yield under salt stress (Antoun *et al.* 2010). These studies showed that the salt injury was decreased under the addition of humic acid to strawberry crops. In this study, K^+/Na^+ was estimated in the roots and leaves, and the cell morphology of strawberry root apices was observed by different salt environment with the salt resistance of different humic acid concentrations. The results may provide appropriate methods for reducing salt injury of strawberry when cultivating in the high concentration of salt environment.

MATERIALS AND METHODS

Cultivation of strawberries

The experimental cultivar of strawberry was *Fragaria ananassa*. After 5-6 leaves of strawberry were developed, the experiments were proceeded. Experiments were conducted in three replicates, three plants were planted for every replicate. The first factor was addition of salt including 0 (S_0) and 50 mM (S_{50}) of NaCl. Another factor was the addition of humic acid including 0 (HA_0), 150 mM (HA_{50}) and 300 mM (HA_{300}). It was designed for six treatments: S_0HA_0, S_0HA_{150}, S_0HA_{300}, $S_{50}HA_0$, $S_{50}HA_{150}$ and $S_{50}HA_{300}$. Strawberries were cleaned with ultrasonicator before being planted in a glass container (diameter is 10 cm and height is 15 cm) with a hydroponic solution (Hoagland and Arnon 1938) in triplicate. The pH was maintained at 6.5-7.0. Strawberries were planted in the container that was aerated by air pump, and then planted in growth chamber. The conditions of growth chamber was regulated at 27 °C in the day time (14 h, RH 65%) and 23 °C in the night-time (10 h, RH 85%). Hydroponic solution in the container was renewed every week. The experiments were proceeded for three weeks.

Estimation of K^+ and Na^+ in plants

The contents of K^+ and Na^+ were estimated as below briefly. At first, plants were grinded, and then 2 g sample was digested by sulfuric acid in 350 °C. After diluting, K^+ and Na^+ were analyzed by inductively coupled plasma spectroscopy (ICP-OES, Horiba JY-2000) for K^+ and Na^+.

Cell morphology of root apices in strawberries after different treatments

Observation of cell morphology of root apices with SEM

The root apices of strawberries were removed from the solutions and then observed using SEM (scanning electron microscopy). Each sample of root apices was placed on a glass container at 20 °C for 6 h and then fixed with 2% glutaraldehyde-p-for glutaraldehyde in sodium cacodylate at pH7.2 at 25 °C for 2 h. The sample was cleaned with sodium cacodylate buffer three times, each at 10 min. Then it was fixed again for 1 h with 1% osmium tetroxide in sodium cacodylate buffers and cleaned again with the buffer three times, each at 10 min as before. Then it was treated with a series of diluted alcohol for dehydration (separately, 30%, 50%, 70%, 80% and 90%, each at 10 min, and 100% at 15 min). This was followed by drying at supercritical carbon dioxide (HCP-2, Hitachi Kohi Co. Ltd., Tokyo, Japan). The dried sample was placed on an al-made platform and covered with 15 nm gold membrane with ion coater (IB-2, Giko Engineering Co. Ltd., Japan). Then the cell of root apices was examined with SEM (Hitchi S-570, Hitachi Co. Ltd, Tokyo, Japan) under 15 kV voltage.

Observation of root apices by TEM

Root apices (1 cm) of strawberries grown in different solutions for four weeks were observed with TEM (Transmission Electron Microscopy). Root apices of strawberries were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer at pH7.0 for 2 h, and then it was rinsed with 0.1 M sodium phosphate buffer and 5% sucrose mixture at pH7.0 for 10 min. It was fixed with 1% osmium tetroxide in 0.1 M sodium phosphate at pH7.0 for 1 h. After cleaning with distilled water, the subsequent alcohol concentrations (from 50% to 100%) and 100% acetone were added to make the sample be dehydrated. The dehydrated sample was then embedded in Spurr's resin before cutting with Ultramicrotome (Riecheat-Jung ultracut E) and then dyed with lead citrate uranyl acetate. The samples were ready for observation of the cells of root apices with TEM (Hitachi, H7100).

Statistical analysis of data

The completely randomized design was used for the experiment with three replicates. The factors were 0 and 50 mM salt concentrations, and 0, 150 and 300 ppm humic acid concentrations. Statistical analysis was performed using the SPSS software. The mean value ($P < 0.05$) was used for Duncan's multiple range test.

RESULTS AND DISCUSSION

K^+/Na^+ ratio of root and leaf in strawberry

The effect of the treatments of salt and humic acid concentrations on Na+, K+ and K+/Na+ in root and leaf of strawberry are presented in Table 1. After exposing to NaCl stress, concentrations of Na^+ in the leaf and root of strawberry were increased (Table 1). However, the addition of humic acid in the hydroponic solution containing NaCl reduced the Na^+ concentration in root and leaf. In the treatment of no-salt, the Na^+ and K^+ contents in root and leaf of strawberry were slightly increased after the concentration of humic acid was increased. However, in the treatments of 50 ppm NaCl, concentrations of Na^+ were reduced while that of K^+ were increased in root and leaf after the concentration of humic was increased. The results showed that K^+/Na^+ ratio was significantly reduced in both leaf and root after exposure of strawberries to salinity. However, the K^+/Na^+ ratio was increased when humic acid was at 300 mg L^{-1}.

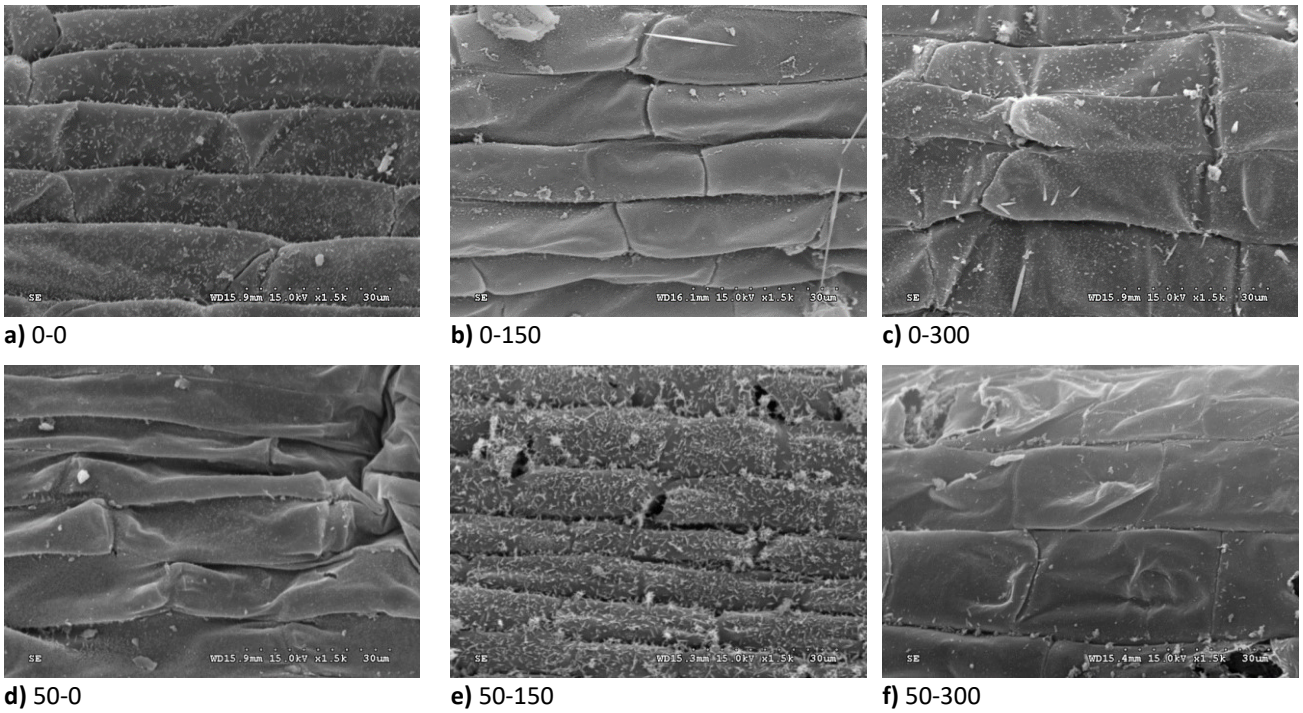
Table 1. Influence of NaCl stress and humic acid (HA) on Na^+, K^+ and K^+/Na^+ in the root and leaf of strawberry

Treatments	Root			Leaf		
	Na^+ (mg g^{-1} DW[1])	K^+ (mg g^{-1} DW)	K^+/Na^+	Na^+ (mg g^{-1} DW)	K^+ (mg g^{-1} DW)	K^+/Na^+
Control[3]	6.10 ± 0.13*c[2]	28.58 ± 0.89a	4.68 ± 0.18a	4.40 ± 0.11c	31.48 ± 0.33c	7.15 ± 0.21b
HA1	6.31 ± 0.15c	28.81 ± 0.31a	4.57 ± 0.11a	4.42 ± 0.13c	32.45 ± 0.21ab	7.34 ± 0.22ab
HA2	6.98 ± 0.17c	29.67 ± 0.55a	4.25 ± 0.19a	4.27 ± 0.04c	34.51 ± 0.18a	8.08 ± 0.06a
Salinity	12.18 ± 0.45a	19.19 ± 0.41c	1.58 ± 0.09c	16.71 ± 0.29a	26.11 ± 0.55d	1.56 ± 0.04d
Salinity+HA1	10.5 ± 0.10a	22.13 ± 1.08b	2.11 ± 0.08c	15.04 ± 0.35a	30.10 ± 0.08c	2.00 ± 0.06c
Salinity+HA2	8.88 ± 0.06ab	24.82 ± 0.23ab	2.80 ± 0.10ab	14.83 ± 0.30b	32.72 ± 0.12ab	2.20 ± 0.05c

[1] DW: Dry weight.

[2] Different letters in each cultivar indicate significant difference by LSD tests at $P < 0.05$. *Represent means ± SE.

[3] Control: 0 mM NaCL + 0 HA, HA1: 150 ppm humic acid, HA2: 300 ppm humic acid, Salinity: 50 mM NaCl.



a) 0-0 **b)** 0-150 **c)** 0-300

d) 50-0 **e)** 50-150 **f)** 50-300

Figure 1. Observation of root apex cells in strawberry by scanning electron microscopy. a) NaCl 0 mM, HA 0 mM. **b)** NaCl 0 mM, HA 150 mM. **c)** NaCl 0 mM, HA 300 mM. **d)** NaCl 50 mM, HA 0 mM. **e)** NaCl 50 mM, HA150 mM. **f)** NaCl 50 mM, HA 300 mM.

This experiment showed that high salinity decreases K^+ content of root and leaf in strawberries. However, the addition of HA increases K^+ content. After treating with salt, Na^+ in root and in leaf are both high, especially in the leaf. Na+ is absorbed by the roots and moved up by the transpiration of water from leaves (Watson *et al.* 2001). Saied *et al.* (2005) also observed that Na+ content increased in strawberry under high salinity. The results of this experiment showed that salt treatment increased Na+ concentration and decreased K^+ concentration in the root and leaf of strawberry. In root, salinity can increase Na^+ adsorptive rate and suppress K^+ uptake (Assaha *et al.* 2017; Sobahan *et al.* 2009; Wang *et al.* 2013). These results coincided with the research of Saidimoradi *et al.* (2019). They concluded that the Na^+ was increased and K^+ was decreased in the roots and leaves of two strawberry cultivars. When strawberries were planted at high salinity soil, the concentration of K^+ was much lower. However, the application of potassium will enhance the K^+ concentration of strawberries. Supplementary potassium can improve plant growth under highly saline conditions (Kaya *et al*. 2015). Na^+ concentration in tissues of Oso Grande and Camarosa strawberry cultivars was increased in the high NaCl treatment. Leaf concentrations of Ca^+, K^+, and N^+ were not significantly reduced in plants grown at high NaCl concentrations than in the no-sodium stressed treatment (Kaya *et al*. 2003). However, Al-Shorafa *et al.* (2014) demonstrated that K+ content in root and shoot of strawberry decreased dramatically with increasing salinity stress in Camarosa and Albino strawberry cultivars. These distinct results may be due to the physiological difference between cultivars. Under salinity stress, Na^+ may influence the cell membrane stability of root and change the selectivity of ions (Jamil *et al*. 2012). Addition of humic acid (HA) has a negative correlation value to soil exchangeable Na and SAR (sodium adsorptive ratio) and hence reduce the absorption by plants (Mindari *et al.* 2019). In this experiment, supplementation of humic acid in the hydroponic solution containing high salt stress caused lower concentration of Na^+ in the leaf and root of strawberry. HA can reduce soil Na^+ due to exchange with K^+. The main function is that humic acid contains abundant COOH groups (carboxylic acid) in metal ions bonding (Martyniuk and Więckowska 2003). Under application of HA, Na^+ adsorbed by humic complex is increased, and the availability of potassium for providing to root increases (Kumar *et al.* 2013). By the mediation of HA, result in the absorption of K^+ was increased and Na^+ was reduced, and led to K^+/Na^+ ratio in the root and leaf were increased (Table 1). Khaled and Fawy (2011) found that the application of HA under salinity stress will increase K^+ uptake of corn and Na^+ content and Na^+/K^+ ratio were both reduced.

Cell morphology of root apices in strawberries after different treatments

Observation strawberry root apices cells by SEM

SEM was an effective method to examine the morphology of plant cells (Neinhuis and Edelmann 1996). In this experiment, the cells of strawberry root apices were integrity and remaining normal under low salinity stress. The cells of root apices were still integrity under the increasing concentration of humic acid (Fig. 1a-c). Vaughan (1974) concluded that humic acid can promote cell elongation corresponding to substantial increase in cell wall bound with hydroxyproline. Under 50 mg kg^{-1} NaCl, the cells of root apice were shrinking and cracking seriously with no humic acid (Fig. 1d). The high salt concentration caused dehydration of the cells. However, when 150 mg kg^{-1} of HA was added, the cells were still slightly shrinking and cracking (Fig. 1e). With the treatment of 300 mg kg^{-1}, the root apice cells of strawberry were able to recover their integrity (Fig. 1f). Jindo *et al.* (2012) concluded that humic acid might release auxin-like plant growth promoters and enhance maize biochemical activities (Jindo *et al.* 2012; Trevisan *et al.* 2010). Ramos *et al.* (2015) suggested that some signaling pathways activated by humic acid are expressed at post-transcriptional level through calcium-dependent protein phosphorylation. These effects were mediated by hormonal signaling pathways and had been shown to be dominated by an upregulation of genes responsive to the synthesis of auxin in corn and tomato, as well as other genes that encode metabolic pathways for the uptake of nutrients. As a result, the salinity stress was relieved (Canellas and Olivares 2014).

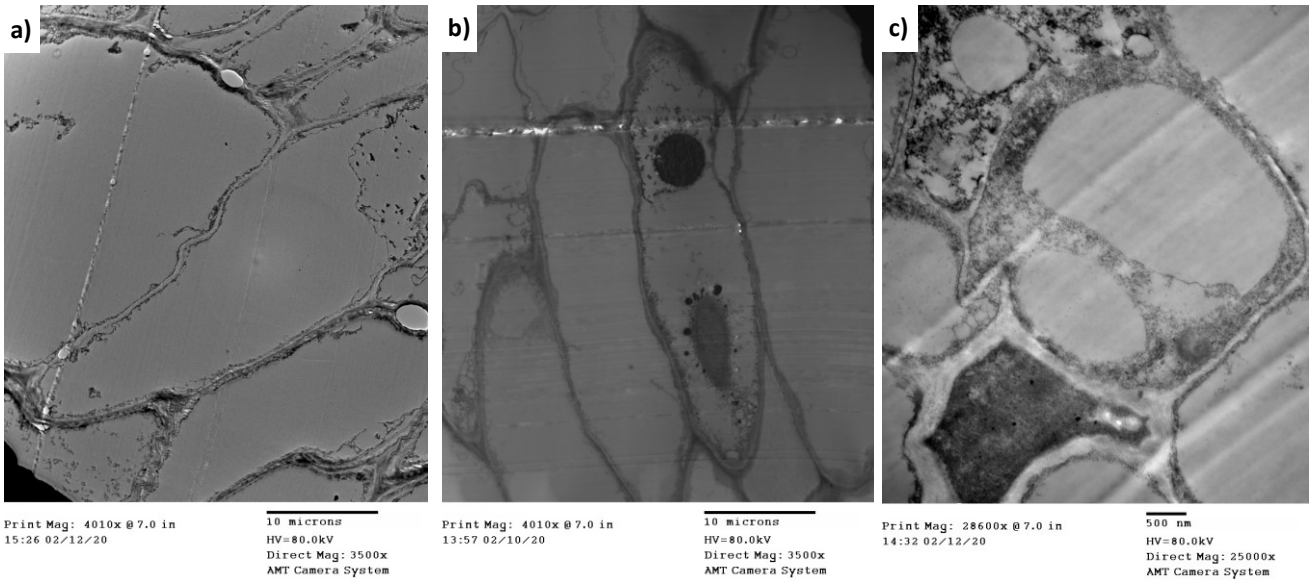


Figure 2. Observation of root apex cells in strawberry by transmission electron microscopy. a) NaCl 50 mM, HA 0 mM. **b)** NaCl 50 mM, HA 150 mM. **c)** NaCl 50 mM, HA 300 mM.

Observation of cells in root apices of strawberry by TEM

Through TEM observation, the vacuole in the cell was expanded and mitochondria were distributed around plasma membrane under the treatment of S50HA0 (Fig. 2a). However, the organs in cells were all normally distributed with normal size after treating with $S_{50}HA_{150}$ and $S_{50}HA_{300}$ (Fig. 2b, c). Cell wall hardening in the maintenance of cell integrity and elongation of root tips is an important reason for the resistance to high salinization (Neumann *et al*. 1994). Nardi *et al.* (2007) showed that humic acid could promote the enzyme activities that were related to glycolysis and the tricarboxylic acid cycle (TCA). The glycolysis enzymatic activities included glucokinase, phosphoglucose isomerase, PPi-dependent phosphofructokinase and pyruvate kinase. While those involved in the respiration process were citrate synthase, malate dehydrogenase and the cytosolic form of $NADP^+$-isocitrate dehydrogenase (Nardi *et al.* 2007). After the addition of humic acid to high salt environment, the mitochondria were concentrated at cell membrane. The phenomenon may increase the energy to resist Na^+ by glycolysis and TCA cycle. Miyasaka and Hawes (2001) showed that each vacuole was expanded in the root apices cells of *Picea rubens* when it encountered environmental stress. They discovered that vacuoles in the cells played a role in storing toxic substances and their detoxification. In this experiment, the salinity stress could make the cell vacuole enlarge, but it could return to recover normal size after treating with 300 mg kg^{-1} of humic acid.

CONCLUSION

Crops can be injured with the increase of salt in the soil environment. This study showed that more Na^+ was absorbed and accumulated in shoot and root under salt stress, and K^+ content was decreased. However, humic acid may reduce Na^+ and increased K^+ absorption under salinity environment. From the observation of SEM and TEM, the root apices of strawberry were injury. However, it will be remitted in the appropriate concentration of humic acid. This study can further prove the salt-remitting function of humic acid.

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