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Growth and Anatomical Adaptations in Response to Salinity Stress in *Cucurbita moschata* Duchesne 'Butternut' (Cucurbitaceae)

(Adaptasi Pertumbuhan dan Anatomi *Cucurbita moschata* Duchesne 'Butternut' (Cucurbitaceae) dalam Gerak Balas terhadap Tekanan Kemasinan)

Worasitikulya Taratima[1,*], Sununta Sudjai[1] & Pitakpong Maneerattanarungroj[2]

[1]*Salt Tolerance Rice Research Group, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand*
[2]*Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand*

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ABSTRACT

Pumpkin or squash is an economically important crop that is moderately sensitive to salinity but how its growth is affected by soil salinity is poorly understood. Salinity stress on physiological and anatomical traits of *Cucurbita moschata* 'Butternut' was investigated under hydroponic culture using Hoagland's solution with various NaCl concentrations (0, 25, 50, 75, 100, and 150 mM) for four weeks. The results showed that pumpkin growth characters decreased after cultured in various NaCl concentrations. Leaf number, leaf width, leaf length, root number, stem height, stem diameter, green intensity in terms of SPAD units, chlorophyll fluorescence (Fv'/Fm', Fv/Fm), total chlorophyll, chlorophyll a and chlorophyll b contents significantly ($p < 0.05$) decreased after culture in various NaCl concentrations. Salinity stress impacted fiber layer thickness, vascular bundle size and vessel diameter of treated plants but did not affect cuticle thickness. Physiological and anatomical traits significantly correlated with salinity gradients, except for chlorophyll b and chlorophyll fluorescence, in both light and dark condition. Results provide significant data to improve the understanding of adaptation mechanisms of tolerant pumpkin cultivars under salinity stress condition.

Keywords: Chlorophyll content; free-hand sectioning; pumpkin; salinity; stem anatomy

ABSTRAK

Labu adalah tanaman yang penting daripada segi ekonomi serta agak sensitif terhadap kemasinan tetapi bagaimana pertumbuhannya dipengaruhi oleh kemasinan tanah masih kurang difahami. Tekanan kemasinan pada ciri fisiologi dan anatomi *Cucurbita moschata* 'Butternut' dikaji dalam kultur hidroponik menggunakan larutan Hoagland dengan pelbagai kepekatan NaCl (0, 25, 50, 75, 100 dan 150 mM) selama empat minggu. Hasil kajian menunjukkan bahawa pertumbuhan labu menurun setelah dikultur dalam pelbagai kepekatan NaCl. Bilangan daun, lebar daun, panjang daun, nombor akar, ketinggian batang, diameter batang, keamatan hijau daun dari segi unit SPAD, pendarfluor klorofil (Fv'/Fm', Fv/Fm), jumlah klorofil, klorofil a dan kandungan klorofil b menurun dengan ketara ($p<0.05$) selepas dikulturkan dalam pelbagai kepekatan NaCl. Tekanan kemasinan mempengaruhi ketebalan lapisan serat, saiz berkas vaskular dan diameter salur tanaman yang dirawat tetapi tidak mempengaruhi ketebalan kutikul. Ciri fisiologi dan anatomi berkorelasi dengan kepekatan kemasinan, kecuali klorofil b dan pendarfluor klorofil, dalam keadaan cahaya dan gelap. Keputusan memberikan data yang signifikan untuk meningkatkan pemahaman mekanisme penyesuaian kultivar labu yang toleran terhadap keadaan tekanan kemasinan.

Kata kunci: Anatomi batang; kandungan klorofil; kemasinan; labu; pembahagian bebas tangan

Introduction

Soil salinity is one of the most severe global stressors, affecting approximately 20% of irrigated land and drastically reducing crop yields (Okon 2019). Plants are extremely susceptible to salinity during both the vegetative and reproductive stages (Hussain et al.

2017). Salinity weakens plant growth by decreasing soil osmotic potential, leading to loss of water in plant cells, while sodium and chloride ions are toxic toward plant nutrients (Kurum et al. 2013; Martins et al. 2013). Plants that are unable to adapt to salinity wither and eventually die. However, numerous plants species can adapt under salinity stress by utilizing biochemical pathways that promote growth and development through water management as a response to environmental situations (Albuquerque et al. 2016; Brito et al. 2014; Kurum et al. 2013; Oliveira et al. 2015). Many studies have reported plant adaptation under salinity conditions including lettuce (*Lactuca sativa*) (Oliveira 2013), watermelon (*Citrullus lanatus*) (Martins et al. 2013), *Sporobolus arabicus* (Hameed et al. 2013), squash (*Cucurbita maxima*) (Oliveira et al. 2014), beet (*Beta vulgaris*) (Oliveira et al. 2015), cucumber (*Cucumis sativus*) (Albuquerque et al. 2016), *Salvadora persica* (Parida et al. 2016), and pumpkin (*Cucurbita moschata* or *Cucurbita pepo*) (Bischoff 1999).

Pumpkin or squash is an important crop belonging to the family Cucurbitaceae. Numerous well-known cultivars include *C. moschata*, *C. pepo,* and the squash *C. maxima*. Cucurbitaceae species comprise the second highest global crop value (FAO 2014; Resende et al. 2013). Winter squash and pumpkin have been used to graft many cultivars of watermelon, melon and cucumber (Salehi et al. 2008). Winter squash and pumpkin varieties can reduce sodium ion poisoning (Balkaya et al. 2016). Pumpkins are moderately sensitive to salinity because the fairly deep root system increases the absorption of groundwater (Bischoff 1999). There are many varieties of pumpkin and squash but how their growth is affected by soil salinity is poorly perceived and understood. No previous reports have considered the physiology and anatomy of pumpkin *C. moschata* 'Butternut' under salinity cultivation. Here, the growth and stem anatomy of pumpkin 'Butternut' were investigated under salinity stress. Results can be applied to adapt other plant species or pumpkin cultivars in soil salinity situations.

Materials and Methods

PLANT MATERIALS AND HYDROPONIC CULTURE BY DEEP FLOW TECHNIQUE (DFT)

Cucurbita moschata 'Butternut' seeds were individually germinated in 3 × 5 cm soil pots. Fourteen-day seedlings with 1-2 leaves were used as explants for hydroponic culture. Rectangular tubes 10 × 25 cm with a foam sheet were used as planting materials for hydroponic culture. Each tube was filled with one liter of Hoagland's solution (pH 5.5) and aerated using an air pump (model ACQ-007, 75 watts, 100 l/s). Selected healthy pumpkin seedlings were cultured in Hoagland's solution with 0 as the control, 25, 50, 75, 100, and 150 mM NaCl for four weeks at 27 ± 2 °C under 40 μmol $m^{-2} s^{-1}$ light intensity. Leaves more than 2 cm long, leaf size, healthy roots more than 2 cm long, plant height and stem diameter were counted and recorded.

SPAD MEASUREMENT, CHLOROPHYLL AND CHLOROPHYLL FLUORESCENT CONTENT

Green leaf intensity was measured using a SPAD-502 Plus Chlorophyll Meter in three areas (leaf base, middle and leaf apex) of mature leaf blades. SPAD unit averages were calculated. Chlorophyll content was assessed in terms of total chlorophyll, chlorophyll a and chlorophyll b. Mature leaves (0.1 g) were ground in a mortar with 5 mL of 80% acetone. This step was repeated until all the green material was dissolved. The solution was filtrated through filter paper. When all the green material had dissolved, a further 20 ml of 80% acetone was added. The supernatant was detected by absorbance measurements at 645 and 663 nm using a spectrophotometer (Spectronic 20), with 80% acetone as the blank. Chlorophyll contents were calculated based on Arnon (1949) as follows (1), (2) and (3):

$$\text{Total chlorophyll (mg/g tissue)} = 20.2\,(A645) + 8.02\,(A663) \times V / (1{,}000 \times W) \quad (1)$$

$$\text{Chlorophyll a (mg/g tissue)} = 12.7\,(A663) - 2.69\,(A645) \times V / (1{,}000 \times W) \quad (2)$$

$$\text{Chlorophyll b (mg/g tissue)} = 22.9\,(A645) - 4.68\,(A663) \times V / (1{,}000 \times W) \quad (3)$$

where V is the total volume of solution (mL) and W is the weight of leaves (g).

To investigate chlorophyll fluorescence, mature leaves were measured using a Chlorophyll Fluorometer Handy PEA as dark-adapted leaves (30 min dark) (Fv'/Fm' units) and light condition (Fv/Fm units) (Theerakulpisut 2016). All measurements were conducted in triplicate.

ANATOMICAL ANALYSIS

Explants were removed from mature stems of all treatments at 1-3 cm from the root. Each explant was carefully chosen and cut off before soaking in 100 mL of FAA70 fixative (70% ethyl alcohol, acetic acid, formaldehyde; 90:5:5) (Johansen 1940). The explants were transversely sectioned using a free-hand technique. Samples were stained with 1% (w/v) Safranin O solution before dehydration with successive ethyl alcohol and xylene and mounted with DePeX.

Anatomical characteristics including fiber thickness, cuticle thickness, width, and length of vascular bundle and widest vessel cell/vascular bundle diameter were observed under a light compound microscope (ZEISS Axiocam ERc 5s) and scored based on Taratima et al. (2019) using the Axio Vision LE64 program.

STATISTICAL ANALYSIS

Completely randomized design (CRD) was used and at least three replicates were tested in every treatment. Statistical analysis was checked using one-way analysis of variance (One-way ANOVA), while comparative analysis of mean values was examined using the post hoc test (Duncan's test) at 95% confidence level.

RESULTS AND DISCUSSION

Measurement of butternut squash plants after treatment with various concentrations of NaCl exhibited a dramatic decrease in leaf number, leaf size, root number, root length, plant height, and stem diameter when cultured in higher NaCl concentrations (Table 1). The control, 25 and 50 mM NaCl treatments showed no significant differences in leaf number, leaf width, root number, root length and stem diameter. The decrease in leaf number, leaf size, root number, root length, plant height, and stem diameter of butternut pumpkin concurred with previous reports, indicating that salinity stress impacted pumpkin rootstock growth, while root length, leaf length, plant height and fresh and dry weight decreased after treatment with high NaCl concentrations (Kurum et al. 2013).

TABLE 1. Growth responses of butternut squash after treatment with various NaCl concentrations under hydroponics for four weeks

NaCl concentration (mM)	Leaf number per plant	Leaf width (cm)	Leaf length (cm)	Root number per plant	Root length (cm)	Plant height (cm)	Stem diameter (cm)
0	8.0±1.0[a]	11.7±2.0[a]	9.3±0.5[a]	22.5±2.2[a]	21.8±3.0[a]	53.1±8.7[a]	0.57±0.09[a]
25	7.3±0.5[a]	10.3±0.2[ab]	7.9±0.3[ab]	16.0±3.0[ab]	22.0±2.5[a]	32.5±5.2[b]	0.49±0.05[ab]
50	6.6±0.5[a]	9.1±1.6[b]	6.8±1.1[b]	15.3±2.5[ab]	19.0±1.1[ab]	30.8±7.8[b]	0.49±0.03[ab]
75	4.3±1.3[b]	6.4±1.2[c]	4.8±1.0[c]	12.3±3.9[b]	14.5±2.7[ab]	22.8±9.1[bc]	0.46±0.04[ab]
100	3.3±0.5[b]	3.1±1.5[cd]	3.0±0.6[cd]	9.0±2.7[b]	9.0±1.7[b]	14.9±3.0[c]	0.40±0.06[bc]
150	1.0±0.0[c]	3.0±1.4[d]	2.8±0.8[d]	12.0±2.5[b]	10.1±1.2[b]	11.1±4.3[c]	0.33±0.04[c]

Mean ± SE values followed by a different letter in the same column are significantly different according to ANOVA and Duncan's multiple range test ($p < 0.05$)

Pumpkins or squash are glycophytes. They do not grow well under salinity but can adapt after culture in a saline medium (Zhu et al. 2008). Planting in saline soil affects physiological characteristics that subsequently inhibit growth and development (Tang et al. 2018). In a saline environment, soil salinity affects plants in many ways and causes different osmotic, ionic, and oxidative stresses. Excessive accumulation of toxic Na^+ and Cl^- in plants negatively impacts physiological and biochemical pathways, resulting in inhibition of membrane disorder and photosynthesis activity that produces toxic metabolites and reactive oxygen species

(ROS) leading to physiological drought, chlorosis, necrosis, and eventually plant death (Okon 2019). Climate change is an important factor affecting food security, with related increases in levels of sodium chloride in the soil (Omovbude & Hamadina 2018).

Total chlorophyll, chlorophyll a, chlorophyll b and green intensity in terms of SPAD units of treated plants decreased after culture in higher NaCl concentrations, while chlorophyll fluorescence in 30 min dark condition (dark-adapted leaves) and light condition of 25, 50, and 75 mM NaCl treatments was not significantly different compared to the control (Table 2).

TABLE 2. Some physiological traits of butternut squash after treatment with various NaCl concentrations under hydroponics for four weeks

NaCl concentration (mM)	Total chlorophyll (mg/g FW)	Chlorophyll a (mg/g FW)	Chlorophyll b (mg/g FW)	SPAD (units)	Fv'/Fm' (units)	Fv/Fm (units)
0	3.39±0.2[a]	1.79±0.2[a]	1.59±0.3[a]	38.73±2.9[a]	0.75±0.04[a]	0.65±0.08[a]
25	1.93±0.1[ab]	1.18±0.2[ab]	0.75±0.1[b]	34.90±0.4[b]	0.76±0.03[a]	0.63±0.06[a]
50	1.72±0.2[b]	1.11±0.1[ab]	0.61±0.2[b]	36.13±1.0[b]	0.74±0.04[a]	0.64±0.06[a]
75	1.42±0.1[b]	0.85±0.1[b]	0.57±0.0[b]	27.70±1.1[c]	0.76±0.02[a]	0.63±0.04[a]
100	1.34±0.3[b]	0.70±0.2[b]	0.64±0.1[b]	19.49±1.4[d]	0.49±0.07[b]	0.40±0.08[b]
150	0.00±0.0[c]	0.00±0.0[c]	0.00±0.0[c]	0.00±0.0[e]	0.00±0.00[c]	0.00±0.00[c]

Mean ± SE values followed by a different letter in the same row are significantly different according to ANOVA and Duncan's multiple range test ($p < 0.05$)

Reduction in green intensity, total chlorophyll, and chlorophyll a after treatment with higher NaCl concentrations was similar to Sevengor et al. (2011) who found that 100 mM NaCl impacted chlorophyll content in pumpkin seedlings after culture for 7 days. However, in our study, chlorophyll b contents of the 25, 50, 75, and 100 mM NaCl treatments were not significantly different. This result concurred with Gong et al. (2018) who stated that salinity stress did not affect the photosynthetic pigment of *Kalidium foliatum*, especially chlorophyll b but impacted total chlorophyll and chlorophyll a. This phenomenon occurred because the free radicals formed during the salinity stress process destroyed the thylakoid membrane, causing chlorophyll damage and loss of properties, producing a colorless substance as chlorophyll bleaching (Theerakulpisut 2016). The butternut squash leaves in this study turned pale green after culture on higher NaCl concentrations. Chlorophyll a presents as a dark green color and is easily destroyed by free radicals, while chlorophyll b is a light green color and more resistant to stress than chlorophyll a. Chlorophyll a can be synthesized from chlorophyll b by oxidation of the methyl to formyl group (Ito et al. 1996). Chlorophyll fluorescence measurement of 30 min dark condition (dark-adapted leaves) and light condition delivers important information involving the quantum efficacy of photochemistry, and is normally used for photosystem efficiency, especially Photosystem II (PSII) (Theerakulpisut 2016). In this study, chlorophyll fluorescence of both light and dark condition of 25, 50, and 75 mM NaCl was not significantly different compared to the control group, indicating that PSII was unaffected (Zhao et al. 2019). By contrast, the 100 mM NaCl treatment was significantly different from the other treatments. This result agreed with Zhong et al. (2019) who found that chlorophyll fluorescence of cucumber plants significantly decreased after treatment with high NaCl concentrations. Many factors affect plant growth performance but decrease in photosynthesis efficiency is a major reason for growth inhibition under high salt stress (Bose et al. 2017).

Salinity stress affected stem anatomy and fiber layer thickness of treated butternut pumpkin. Concentrations higher than 25 mM NaCl showed significantly thicker fiber layers than the control. However, salinity stress in our experiment did not affect cuticle thickness (Table 3, Figure 1). Excessive salt was linked to the sclerenchymatic and lignified ring of butternut pumpkin stem, supporting Akcin et al. (2014) and Hameed et al. (2010) who found that sclerenchymal thickness of *Spergularia marina* and *Cynodon dactylon* significantly increased under high salinity. Lignin has also been suggested as a cellular resistance factor against high osmotic pressure inside the plant body (Grigore & Toma 2007). This trait may provide some resistance to water deficit within the stem as an important adaption to adverse environments (Hameed et al. 2010). Vascular bundle size and vessel diameter were influenced by salinity stress (Atabayeva et al. 2013; Farhana et al. 2014; Sellami et al. 2019). Our results showed that sodium chloride inhibited the expansion of the vascular bundle. Width and length of vessel bundle and vessel diameter decreased when sodium chloride concentrations increased (Table 3, Figure 2). Decreasing vascular bundle size and vessel diameter under NaCl treatment found here were consistent with Younis et al. (2014). They reported that xylem and phloem sizes of root and stem of *Gazania harlequin* decreased after treatment with higher NaCl concentrations, while *Astragalus gombiformis* vascular bundle size and vessel diameter also decreased when treated with 300 mM NaCl (Boughalleb et al. 2017). Reduction of vascular bundle and vessel size reduced the water absorption of plants (Hampson & Simpson 1990). Under a salinity stress situation, reducing vascular tissue leads to decreased absorption of Na^+ and Cl^-. Vessel size decrease under salinity stress may be related to cell wall deposition. In xylem fibers, lignin deposition did not increase (Sellami et al. 2019). Consequently, vessels are less likely to resist higher tensions within xylem generated by high soil osmotic pressure under a salinity stress situation (Venturas et al. 2017). However, cellulose and hemicellulose content alterations in salt-treated plants may advocate additional cell wall flexibility, thus compensating for the lignin shortfall to some extent (Sellami et al. 2019).

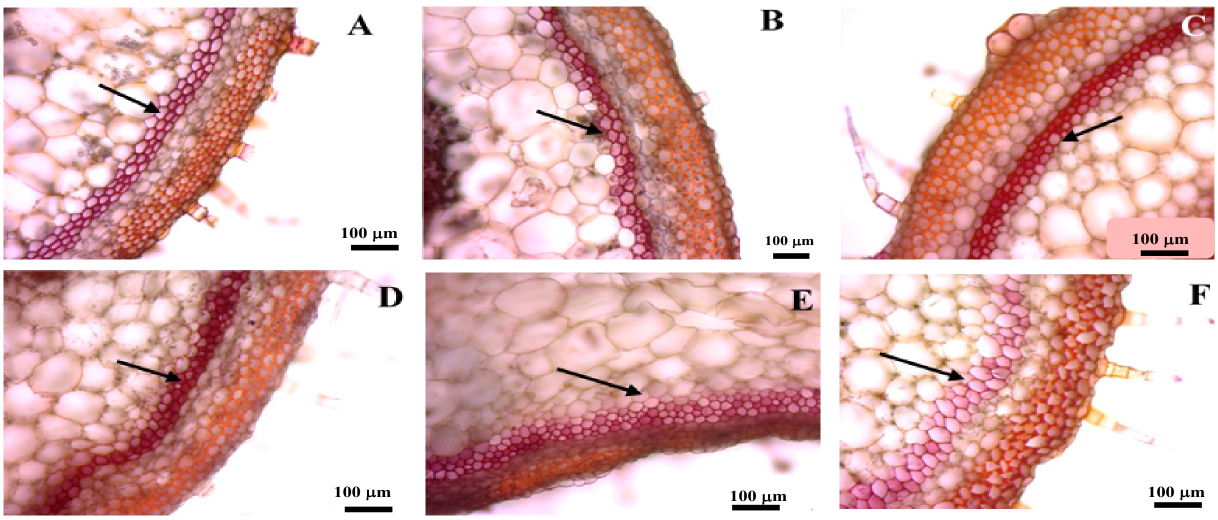

FIGURE 1. Fiber layer thickness of butternut squash stem (arrows) after treatment with various NaCl concentrations under hydroponics for four weeks; (A) 0 mM NaCl, (B) 25 mM NaCl, (C) 50 mM NaCl, (D) 75 mM NaCl, (E) 100 mM NaCl and (F) 150 mM NaCl

Non-significant differences in cuticle thickness of the control and NaCl treatments in our study did not concur with previous studies in soybean, where cuticle thicknesses of higher NaCl concentration treatment plants were greater than in lower NaCl concentration treatment plants (Dolatabadian et al. 2011). Increasing the cuticle layer is one of the most important mechanisms to reduce water loss of plant cells (Taratima et al. 2020, 2019; Zhang et al. 2015). Stressful salinity condition results in water loss by plant cells, causing wilting and eventual death (Kosma & Jenks 2007; Samuels et al. 2008). Moreover, cell wall integrity of plant cells is an important aspect that encourages plant growth and salt stress tolerance (Feng et al. 2018; Zhao et al. 2020).

TABLE 3. Some anatomical characteristics of butternut squash stem after treatment with various NaCl concentrations under hydroponics for four weeks

NaCl concentration (mM)	Fiber thickness (µm)	Cuticle thickness (µm)	Width of vascular bundle (µm)	Length of vascular bundle (µm)	Vessel diameter (µm)
0	55.35±7.03[b]	4.24±0.51[a]	147.93±80.41[a]	598.67±98.96[a]	901.83±20.28[a]
25	53.95±6.23[b]	3.97±0.77[a]	95.45±51.09[a]	593.77±139.08[b]	706.54±25.31[b]
50	58.33±3.11[b]	3.37±0.67[a]	102.33±40.54[b]	458.23±137.6[b]	671.17±17.02[b]
75	57.87±11.23[b]	3.43±0.76[a]	64.42±59.24[b]	379.96±104.49[c]	475.34±16.33[c]
100	61.56±4.65[ab]	4.34±0.86[a]	56.65±50.38[bc]	431.05±78.77[c]	477.62±20.04[c]
150	70.65±8.58[a]	3.75±0.41[a]	44.29±43.77[c]	271.33±44.78[c]	383.21±7.56[c]

Mean ± SE values followed by a different letter in the same column are significantly different according to ANOVA and Duncan's multiple range test ($p < 0.05$)

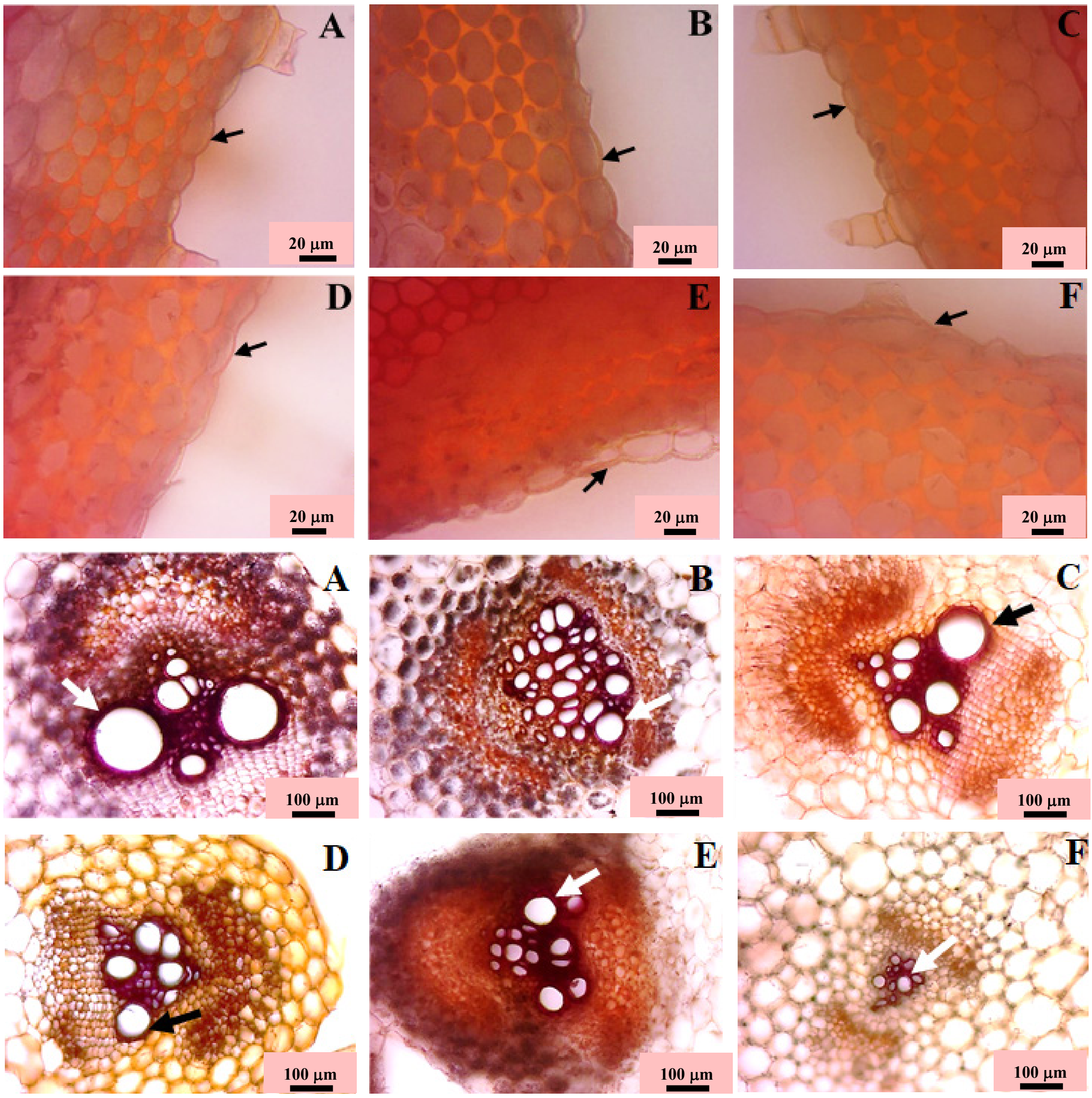

FIGURE 2. Cuticle thickness and stem vascular bundle of butternut squash stem (arrows) after treatment with various NaCl concentrations under hydroponics for four weeks; (A) 0 mM NaCl, (B) 25 mM NaCl, (C) 50 mM NaCl, (D) 75 mM NaCl, (E) 100 mM NaCl and (F) 150 mM NaCl

Conclusions

Current studies suggest that butternut pumpkin responds to salinity stress, especially in high NaCl concentration treatments. Physiological traits decreased, except for chlorophyll fluorescence under both light and dark condition. Anatomical characteristics (fiber layer thickness, vascular bundle size and vessel diameter) were altered, except for cuticle thickness. This finding provides a theoretical basis for further research concerning plant salt tolerance.

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*Corresponding author; email: worasitikulya@gmail.com