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Effects of NaCl salinity on leaf water status, proline and mineral ion content of four *Cucurbitaceae* species

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Abstract

Members of *Cucurbitaceae* are salt-sensitive plants and continuous fertilization without sufficient leaching may create saline environment that consequently reduce their growth and yield. A study was carried out to evaluate the physiological responses of four selected *Cucurbitaceae* species to NaCl salinity stress. Four types of *Cucurbitaceae* viz. cucumber (*Cucumis sativa*), squash (*Cucurbita moschata*), bitter gourd (*Momordica charantia*) and bottle gourd (*Lagenaria siceraria*) were subjected to four levels of NaCl (0, 25, 50, 75 mM) and data on leaf relative water content, proline content and concentration of Na^+ and Cl^- for leaf, stem and root were collected. Given that the dominant salt in saline soils is NaCl, both Na^+ and Cl^- ions will occur naturally in high concentrations. However, degree of increment was different between species. As salinity levels increased from 0 to 75 mM, Na^+ concentrations in roots in bitter gourd had the highest increase; while in leaf had the lowest increase compared to other species. Increment of Cl^- in leaf, stem and roots was the lowest in bitter gourd and highest in cucumber. Increase of proline content in cucumber was 3.55 times higher compared to control whereas in squash, bottle gourd and bitter gourd, the increase were respectively 2.00, 1.47 and 2.03 higher compared to their respective control. Proline content in cucumber was negatively correlated with relative water content, RWC ($r = -0.83$, $p \leq 0.01$), whereas in other species no correlation was recorded. In conclusion, based on Na and Cl ion concentration, RWC and proline content, bitter gourd was least salt-sensitive while cucumber was most salt-sensitive Cucurbitaceae species.

Keywords: Salinity stress, Osmotic stress, Ionic stress, Proline, Salt tolerance, *Cucurbitaceae*.
Abbreviations: RWC_relative water content, EC_electrical conductivity, FW_Freash weight, TW_Turgid weight, DW_Dry weight

Introduction

Salinity is one of the most serious abiotic stresses that limit the productivity of agricultural crops, with adverse effects on germination, plant vigour and crop yield (Munns and Tester, 2008). Salinity affects more than 6% of the world's land. Of the 230 million hectares of irrigated land, 45 million hectares is salt-affected (19.5%) while for the 1500 million hectares under dryland agriculture, 32 million is salt-affected to varying degrees (2.1%) (Ashraf et al., 2008).
In general, soil salinity is known to affect plant growth in the form of osmotic stress which is then followed by ion toxicity (Munns and Tester, 2008). Initially, the osmotic stress happened due to reduction in soil water potential following the increase in root zone solute concentration. This will create in low soil water potential around the root zone area and consequently interferes with the ability of the plants to extract water and remain turgid. Hence, in some species salt stress may have similar symptoms of drought stress. When salinity environment is prolonged or the concentration is increased, ion toxicity phase will follow. The high concentration of Na^+ and Cl^- ions may disturb membrane integrity and function as well as interfering with internal solute balance that also affects nutrient uptake; this gives the resemblance of nutrient deficiency symptoms which similar to those that occur in the absence of salinity (Munns and Tester, 2008). The combinations of both osmotic stress and ion toxicity will collectively affect all the major processes in plants such as growth, photosynthesis, water relations and uptake of mineral.
Cucurbitaceae comprises species consumed as food worldwide that thrives well in tropical, subtropical, arid deserts and temperate regions (Rai et al., 2008). In arid and semi-arid regions, salinity problem is particularly profound due to hot and dry weather combined with scarce water resources that further causing elevation of salinity concentrations (Yadav et al., 2011). In Malaysia, intensive agricultural methods such as high application of fertilizer and manure contribute to increasing soil salinity level. Major *Cucurbitaceae* production sites such a Cameron Highlands was reported to have EC level increased from the normal level to as high as 6.4 dS/m due to intensive rain shelter farming system that did not provide natural rainfall leaching (Wong et al., 2002). With approximately 130 genera and 800 species (Jeffrey, 2005), genetic diversity within *Cucurbitaceae* family is tremendous, and the response

against salinity stress may differ greatly among the species in the family. The mechanisms that glycophytes plants developed for absorbing, transporting and utilizing mineral nutrients from non-saline substrates may not operate as efficiently or as effectively under saline compared to non-saline conditions. Na^+ and/or Cl^- concentrations often exceed those of most macronutrients by one or two orders of magnitude, and by even more in the case of micronutrients. Therefore, high concentrations of Na^+ and Cl^- ions in the soil solution may depress nutrient-ion activities and produce extreme ratios of Na^+/Ca^{2+}, Na^+/K^+, Ca^{2+}/Mg^{2+} and Cl^-/NO_3^- (Shafieizargar et al., 2015). As a result, the plant becomes susceptible to osmotic and specific-ion injury as well as to nutritional disorders that may result in reduced yield or quality. Being one of compatible solutes, proline adjusts plant osmotolerance by numerous ways, defending enzymes from denaturation, stabilising membrane or macromolecules or mediates osmotic adjustment (Ashraf and Foolad, 2007). Prolonged salinity environment will lead to ion toxicity in salt-stressed plants. The high concentration of Na^+ and Cl^- ions may disturb membrane integrity and function; interfering with internal solutes balance and also affecting nutrients uptake, giving the plants resemblance of nutrient deficiency symptoms. These combinations of osmotic stress and ion toxicity will affect all the major processes in plants such as growth, water relations, and mineral uptake (Munns and Tester, 2008). This study explores the response of four Malaysian *Cucurbitaceae* species to saline conditions at vegetative stage in order to determine their tolerance level by using physiological responses; Na^+ and Cl^- concentrations, RWC and proline concentration as indicators.

Results and discussion

Leaf water status and osmolyte activity

Both main effects of Cucurbit species and salinity had strong significant effect (P<0.01) on RWC and proline. Significant interaction was recorded between the main effects for RWC (P<0.05) and proline (P<0.01) (Table 1).

In squash and bottle gourd, increased in salinity had no significant effect on RWC. RWC of cucumber and bitter gourd were both reduced significantly at 50 mM NaCl with reduction percentage of 23.4 and 10.2% respectively, compared to control. Further increase of salinity from 50 to 75 mM NaCl did not change RWC of bitter gourd whereas in cucumber, the RWC was significantly reduced with reduction percentage of 34.4% compared to control (Fig. 1).

Exclusion of Na^+ and Cl^- ions is not the only mechanisms controlling salt tolerance in plants. Other mechanisms, such as osmotic tolerance, which is closely related to osmolyte activity such as proline should be considered in selecting salt tolerance species (Travakolli et al., 2010). Results in this study showed that at 75 mM salinity, increase of proline content in cucumber was 3.55 times higher compared to control whereas in squash, bottle gourd and bitter gourd, the increase were respectively 2.00, 1.47 and 2.03 higher compared to control (Fig. 2). Proline has been reported to increase greatly under salt stress (Munns, 2002) and this is in accordance with result in this experiment. Proline which is made of amino acid is one of compatible solutes that are synthesized and accumulated within the cytoplasm of salt-stress plants (Carillo et al., 2011). As a compatible solute, proline adjusts plant osmotolerance by numerous ways, defending enzymes from denaturation, stabilising membrane or macromolecules or mediates osmotic adjustment (Ashraf and Foolad, 2007). Zhu et al. (2008) reported that the proline content of relatively salt-tolerant cucumber cultivar, Zaoduojia under NaCl stress increased significantly, whereas that of salt-sensitive cultivar, Jinchun No. 2 was unaffected. Further correlation analysis between osmotic potential and proline content proved that Zaoduojia can alleviate NaCl stress by accumulation of proline for osmotic adjustment. In this experiment, correlation analysis revealed that proline in cucumber was significantly and negatively correlated with relative water content, RWC (r= -0.83, p ≤ 0.01), whereas in other species no significant relationship was recorded. This suggested that cucumber leaf water status is very prone to salinity stress compared to other species studied.

Differences of Na^+ and Cl^- ions increment in the species

The Na^+ concentration was affected significantly (P<0.01) among species in all parts of plant measured. Salinity significantly affected concentration of Na in all parts of plant measured (P<0.01), with highly significant interactions between the main effects recorded in leaf and stem, (P<0.01) and in root (P<0.05) (Table 2).

Significant interaction recorded indicated that increase pattern of Na^+ was different between the species. For cucumber, concentration of Na^+ in leaf was significantly increased at every salinity interval and at 75 mM NaCl, the concentration of Na was 11.5 times higher than control. In bottle gourd and squash, concentration of Na^+ was significantly increased at 50 mM of NaCl and at 75 mM NaCl, the concentration was respectively 9.50 and 19.0 times higher compared to control. Concentration of leaf Na^+ in bitter gourd was affected significantly at 25 mM NaCl. However, further increased in salinity did not significantly affect concentration of Na^+ in leaf and at 75 mM NaCl, the concentration of Na^+ in leaf was only 8.00 times higher compared to control (Fig. 3A). Concentration of Na^+ in stem for all species was significantly increased at 25 mM NaCl. Further increased to 50 mM NaCl resulted in significant increase of Na^+ concentration in stem of all species except cucumber. From 50 to 75 mM NaCl, no significant increase of Na^+ concentration in stem was recorded in all species except squash (Fig. 3B). Concentration of Na in root was significantly increased at 25 mM NaCl in squash and bottle gourd whereas in bitter gourd and cucumber, at 75 mM NaCl. Comparing control and 75 mM NaCl, the concentration of Na^+ in root was 2.89, 2.56, 2.57 and 5.71 times higher in squash, cucumber, bottle gourd and bitter gourd respectively (Fig. 3C).

High root Na^+ along with low leaf Na^+ in bitter gourd suggests that it has the ability to store relatively high Na^+ ions in its root thus preventing excessive transport to shoot. This strategy of combating salinity stress is described as compartmentation of ions at organ level (Davenport et al., 2005). Leaf blade is the main site of Na^+ toxicity in most plants since after Na^+ ions have been deposited in the transpiration system, they accumulates in the leaf rather than the roots (Munns, 2002). Preventing Na^+ ions from reaching the leaf blades is critical in avoiding Na^+ ion toxicity

Table 1. Main and interaction effects of four Cucurbit species and four NaCl salinity concentrations on relative water content and proline.

Factor		Relative water Content (%)	Proline (µmol/g FW)
Species	Squash	78.97b	1.28a
	Cucumber	79.52b	0.35d
	Bottle gourd	74.51b	0.77b
	Bitter gourd	91.53a	0.69c
NaCl (mM)	0	87.33a	0.54d
	25	84.97ab	0.66c
	50	77.71bc	0.83b
	75	74.53c	1.08a
Species		**	**
NaCl		**	**
Species*NaCl		*	**

**Significant at 1% probability level, *Significant at 5% probability level. Means in each column with the different letters within each factor indicate significant differences at P≤0.05% level according to Tukey's HSD.

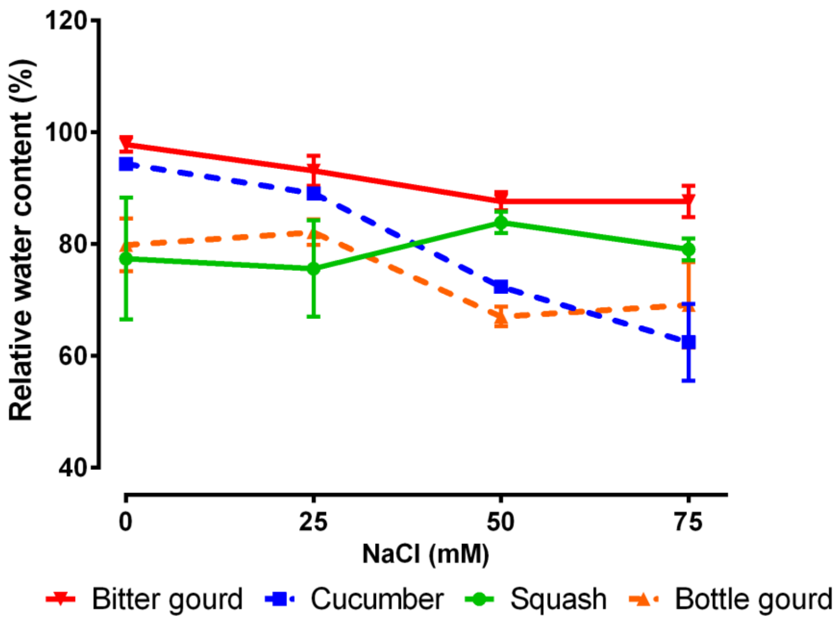

Fig. 1. Effects of salinity and species on relative water content (RWC) (Mean ± S.E; n=3)

Table 2. Main and interaction effects of four Cucurbit species and four NaCl salinity concentrations on Na and Cl contents of leaf, stem and root.

**Significant at 1% probability level, *Significant at 5% probability level, ns: Not significant. Means in each column with the different

Factor		Na(%) Leaf	Na(%) Stem	Na(%) Root	Cl(%) Leaf	Cl(%) Stem	Cl(%) Root
Species	Squash	0.10c	0.29bc	0.46a	3.70a	5.25b	2.21ab
	Cucumber	0.26b	0.47a	0.35b	3.23a	6.70a	3.39a
	Bottle gourd	0.09c	0.41b	0.44a	2.58b	6.66a	2.68ab
	Bitter gourd	0.33a	0.38c	0.20c	1.82c	2.94c	2.07b
NaCl (mM)	0	0.03d	0.11d	0.15b	1.21c	2.36d	1.31c
	25	0.17c	0.45c	0.41a	2.72b	4.67c	2.37bc
	50	0.25b	0.53b	0.41a	3.17b	6.05b	2.98ab
	75	0.33a	0.56a	0.47a	4.22a	8.78a	3.69a
Species		**	**	**	**	**	*
NaCl		**	**	**	**	**	**
Species*NaCl		**	**	*	ns	*	ns

letters within each factor indicate significant differences at P≤0.05% level according to Tukey's HSD.

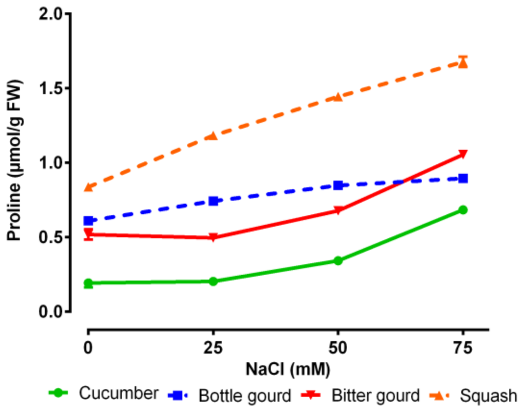

Fig. 2. Effects of salinity and species on proline content (Mean ± S.E; n=3)

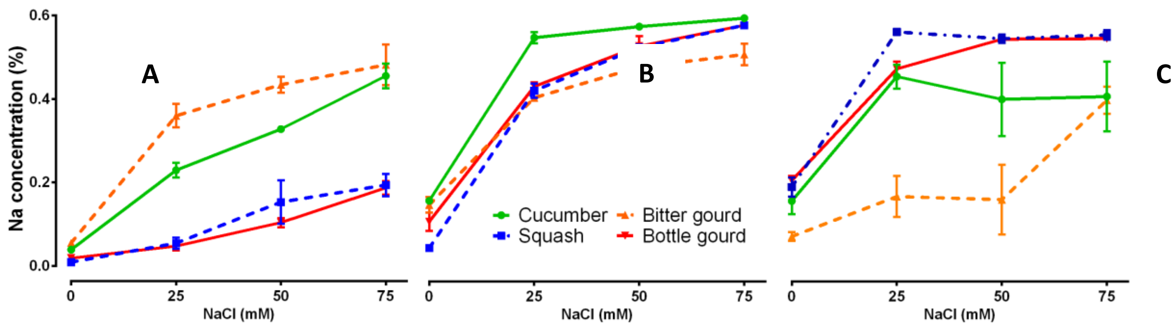

Fig 3. Effects of salinity and species on concentration of Na in leaf (A), stem (B) and root (C). (Mean ± S.E; n=3).

since plants in general transpire 50 times more water than they retain in leaves (Munns et al., 2006). Otherwise, Na^+ ions will accumulate in older non-expanding leaves and necrosis symptom will first appear at tip and margin of the leaf and extends towards the center of the leaf as Na^+ concentration increases.

Apart from toxicity in Na^+ ion, toxicity in Cl^- ion is also deleterious in salt-stressed plants. Regulation of Cl^- ions transport and Cl^- ions exclusion from shoots is correlated with salt tolerance in many species in various genus such as *Legumes, Glycine, Citrus* and *Vitis* (Travakkoli et al., 2010). In this study, bitter gourd has the lowest leaf Cl^- concentrations compared to other species. For root Cl^-, squash and bottle gourd were ranked lowest together with bitter gourd, with bitter gourd having relatively lower concentration, 2.07% among them. Together with low leaf Na^+, bitter gourd also had significantly lowest leaf Cl^- compared to other species. Significant interaction in stem Cl^- indicated that the increase pattern was different in all species. Concentration of Cl^- in stem of cucumber and squash were significantly higher at 25 mM salinity compared to control whereas in bitter gourd and bottle gourd, the increments were significant at 50 and 75 mM of NaCl compared to control, respectively.

Materials and methods

Plant materials and growth condition

The plant materials used in this study were MARDI MT1cucumber (*Cucumis sativa*), Green Eagle 155 squash (*Cucurbita moschata*), Green Eagle B19 bitter gourd (*Momordica charantia*) and Green Eagle B88 bottle gourd (*Lagenaria siceraria*). Cucumber seeds were provided by MARDI while the rest were bought from a local seed supplier. Seeds were sown in plug trays filled with peat at University's Agriculture Park nursery, Universiti Putra Malaysia. The 4-days old germinated seedlings were then transplanted into 1.5 litre pot. The growing media used were cocopeat with 6 g/L N:P:K - 15:15:15 compound fertilizer, 2 g/L ground magnesium limestone and 1 g/L mixture of micronutrients. The plants were grown under 50% shade on a 1.2 meter bench and prior to salt treatment, approximately 300 ml of tap water were given to each plant every day.

Salinity treatments

When the first true leaf had fully expanded, four different levels of NaCl concentrations; 0, 25, 50 and 75 mM were given by manually drench the media approximately 300 ml of the solutions once per day for the first 10 days and twice per day for the following days as the plants grew. EC of the media was determined using the pour-through method (Cavins et al. 2000), which were 2.35, 4.12, 6.40 and 8.73 dS m^{-1} for 0, 25, 50 and 75 mM NaCl, respectively. The EC of solutions were checked by the EC meter (5061 Pen SHSX).

Relative water content

At 18 days of treatment, samples from the top fully expanded leaves were taken for RWC determination. Fresh weight (FW) of five leaf discs were measured before floated on deionized water for 4 hours. The wet surface of the turgid leaf discs were blot dried quickly before weighing (TW). The leaf discs were then dried for 72 hours at 70°C in oven and dry weight (DW) was then measured. The RWC was calculated and expressed in percentage based on the formula: RWC = (FW – DW / TW – DW) × 100

Proline assay

Free proline content was extracted from the leaf tissues according to the method described by Bates et al. (1973). The samples were frozen in liquid nitrogen prior to analysis. The samples were weighed for 0.5 g and homogenized in 10 mL of 3% aqueous sulfosalicylic acid. The mixture was then filtered through filter paper. Two mL of the filtrate was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The reaction mixture was extracted with 4 mL toluene and the chromophore containing toluene was aspirated. The absorbance was quantified spectrophotometrically at 520 nm and proline concentration was estimated using the standard curve.

Mineral ion content

At 20 days of treatment, the top fully expanded leaf, stem and roots samples were harvested and washed with deionized water prior to drying at 70°C for 72 h. Briefly 0.25 g of the dry sample was transferred to a 100 mL digestion flask and 5 mL concentrated H_2SO_4 was added. The flasks were then heated for 7 minutes at 450°C and 10 ml of 50% H_2O_2 was added to complete the process. The flasks were then removed from the digestion plate, cooled to room temperature and then made up to 100 mL with distilled water. Concentration of sodium (Na^+) was quantified using an atomic absorption spectrophotometer (Perkin Elmer, Model 3110, USA) while Chloride (Cl^-) was extracted from the dried samples using silver ion titration method (Richards, 1954).

Experimental design and data analysis

The treatments comprising 4 salinity levels and 4 *Cucurbitaceae* species were arranged in a RCBD with three replications; 6 plants per replication. The data obtained was analyzed using ANOVA in the SAS software (Version 9, SAS Institute Inc. Cary, North Carolina, USA) and differences between treatment means were compared using Tukey's Honest Significant Difference (HSD) at $P \leq 0.05\%$. Pearson correlation coefficient (r) was determined between the variables in each species at $P \leq 0.05\%$.

Conclusion

Increased in salinity significantly impaired the plant water status and increased the accumulation of proline in all species studied. Accumulation of Na^+ and Cl^- ions in leaves was prevalent in cucumber and the least in bitter gourd. Compartmentalization of Na^+ ions in the bitter gourd root served as mechanism in maintaining ion homeostasis in the plant and reducing salinity stress. Based on Na^+ and Cl^- ion concentration, RWC and proline content, bitter gourd was classified as least salt-sensitive while cucumber was most salt-sensitive.

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