Cleome gynandra L. (C₄ plant) shows higher tolerance of salt stress than its C₃ close relative, C. viscosa L.

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Abstract. The effect of salt stress on some physiological and anatomical parameters in two species of Cleome, C. gynandra (C₄ plant) and C. viscosa (C₃ plant) was investigated. Seedlings were grown in potted soil in greenhouse for 3 months, before being subjected to salinity stress. Salinity was induced by adding sodium chloride solution at the concentration of 75 mM for 15 days. Growth and physiological parameters were recorded every three days after addition of NaCl. The results showed that salinity caused slightly higher reduction in plant height of C. gynandra than C. viscosa. A small reduction in chlorophyll content was observed in C. viscosa after 15 days of NaCl treatment. Proline content in roots, stems and leaves of both species was generally unaffected by NaCl treatment. The amount of hydrogen peroxide, malondialdehyde and electrolyte leakage increased in response to NaCl. These parameters, which indicate secondary oxidative stress response, were more affected in C. viscosa than C. gynandra. Significant reductions in leaf thickness, petiole and root diameters, and root vascular cylinder were observed in NaCl-treated plants of C. viscosa compared to control plants. The results suggested that C. gynandra was more tolerant of salt stress compared to C. viscosa.

Key words: C₃ plant, C₄ plant, Cleome, salt stress, electrolyte leakage.

Introduction. Cleome (Capparaceae), a genus closely related to Arabidopsis, contains species spanning a developmental progression from C₃ to C₄ photosynthesis and provides a potentially excellent new model plant to increase the understanding of C₄ photosynthesis (Marshall et al 2007). The C₃ plant, Cleome viscosa and the C₄ plant, C. gynandra are utilized as vegetables in some tropical countries and have many medicinal applications often in rubificant and irritant preparation (Songsak & Lockwood 2002). The essential oil extracted from the seeds of C. gynandra is occasionally used as an insecticide (Edeoga et al 2009). In general, C₄ plants are distinctive from C₃ plants in having a particular leaf anatomy due to the presence of: (i) a well defined bundle sheath; (ii) chloroplast dimorphism; (iii) two carboxylation pathways; (iv) negligible photorespiration due to CO₂ concentrating mechanism in bundle sheath cells (Nayyar & Gupta 2006). Moreover, C₄ plants have advantages when limitation on carbon acquisition is imposed by high temperature, drought and salinity stress while C₃ plant is relatively inefficient because oxygen competes with CO₂ for the active sites of Rubisco enzyme and some of the fixed carbon is lost by photorespiration (Voznesenskaya et al 2001).

Salinity is one of the major environmental factors limiting crop production. Saline environment imposes three major kinds of stress on plants: osmotic stress, specific ion toxicity and nutrient deficiency thus affecting a range of physiological processes involved in cell metabolism (Munns 2002). Many studies indicate that salt stress leads to secondary stress i.e. the production of active oxygen species (AOS) such as superoxide (O₂⁻), hydroxyl radicals (OH⁻), hydrogen peroxide (H₂O₂), and singlet oxygen (¹O₂), a process referred to as oxidative stress (Jungklang et al 2004). Gambarova & Gins (2008) who studied the characteristics of plants with C₃ and C₄ photosynthesis during salinization
reported that wheat (C₃) and amaranth (C₄) plants displayed a different sensitivity and response of the antioxidant systems to the effect of salt stress. Amaranth plants, unlike wheat, were able to detoxify the superoxide radicals with the participation of the enzyme superoxide dismutase and amaranthin, to effectively oppose the accumulation of active oxygen species and to reduce the tension of lipid peroxidation processes. Not only the physiological processes are affected by salt stress, but some anatomical structures of stressed plants are also changed. Raising the concentration of NaCl in hydroponic solution resulted in greater leaf succulence and greater mesophyll thickness for bean (Longstreth & Nobel 1979). Recently, Junghans et al (2006) discovered that the salt-resistant Populus euphratica and salt-sensitive P. x canescens showed decreases in vessel lumina associated with increases in wall strength in response to salt. Most of comparative studies on C₃ and C₄ plants responding to abiotic stress were done with plants in the same family (Nayyar & Gupta 2006) or differ in taxonomic levels (Gambarova & Gins 2008). However, studies on responding to salt stress of closely related C₃ and C₄ plants which belong to the same genus is still meager. The purpose of this study is to compare physiological and anatomical responses of C. gynandra (C₄ plant) and C. viscosa (C₃ plant) to salt stress.

**Material and Method.** Seeds of C. gynandra and C. viscosa were germinated in potted soil and maintained in greenhouse under natural photoperiod and temperature. Three-month-old seedlings were then subjected to NaCl stress by watering with 75 mM NaCl solution for 15 days. The controlled plants were treated with tap water. During the period of NaCl stress, plant height was measured at days 0, 3, 6, 9, 12 and 15. For physiological analysis, fresh samples of roots, stems and leaves were taken on days 6 and 15 after salinity treatment. Fresh samples were immediately used for determination of electrolyte leakage, for other physiological parameters samples were frozen in a -20 °C freezer for later analyses.

Chlorophyll content was determined according to Arnon (1949). Approximately 0.1 g of sampled tissues were extracted in 10 mL of 80 % acetone and filtered through filter paper. After recording the volume of filtered extract, the absorbance of the filtrate was estimated at 645 nm and 663 nm.

Free proline content was determined by using the method of Bates et al (1973), sampled tissues (0.1 g) were homogenized in 3 % sulfoalicylic acid and filtered through filter paper. After addition of acid ninhydrin and glacial acetic acid, the resulting mixture was heated at 100 °C for 1 h. in water bath. Reactions were stopped by placing the tubes on ice bath. The mixture was then added with toluene, and the absorbance of fraction with toluene aspired from upper liquid phase was read at 520 nm. The proline concentration was determined from the standard curve and calculated on a fresh weight basis (µg g⁻¹FW).

Malondialdehyde (MDA) content was determined using the thiobarbituric acid (TBA) method (Health & Packer 1968). Sampled tissues (0.1 g) were cut into small pieces, added with 1.4 mL of distilled water, and mixed by a vortex mixer. Thiobarbituric acid reagent (1.5 mL of 0.5% TBA in 20 % TCA) was added. The mixture was heated at 95 °C for 25 min and then quickly cooled in an ice bath. After centrifugation at 10000 x g for 10 min, the absorbance of the supernatant was read at 532 and 600 nm. The correction for unspecific turbidity was done by subtracting the absorbance at 600 nm from the absorbance at 532 nm. MDA content was calculated according to its extinction coefficient of 155 mM⁻¹ cm⁻¹. The concentration was expressed as nmol g⁻¹ FW.

Electrolyte leakage was assessed as described by Dionisio-sese & Tobita (1998). Fresh samples were washed with deionized water. Sampled tissues (0.1g) were cut into small pieces, placed in closed vials containing 10 mL of deionized water, and incubated at 32°C for 2 h. After that, electrical conductivity of the solution (EC₁) was determined. Samples were then autoclaved at 120 °C for 20 min and the electrical conductivity (EC₂) was obtained after equilibration at 25 °C. The electrolyte leakage (EL) was defined as follows: EL (%) = (EC₁ / EC₂) x 100.

Hydrogen peroxide (H₂O₂) content was determined according to Velikikova et al (2000). Sampled tissues (0.5 g) were homogenized at 4 °C with 5 mL of 0.1% (w/v) AAB Bioflux, 2011, Volume 3, Issue 1.
trichloroacetic acid. The homogenate was centrifuged at 12,000 x g for 15 min and 0.5 mL of supernatant was added to 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide. The absorbance of reaction mixture was read at 390 nm. The H$_2$O$_2$ content was calculated from a standard curve and expressed as µmol g$^{-1}$ FW.

For anatomical studies, fresh leaf blades and petioles (from the fully expanded leaves at the third internode from the top), stems (the middle part of the stem) and tap roots (0.5 cm below ground) were collected from both control and NaCl-treated plants at day 15 of salinity stress. These specimens were fixed in 70% FAA. The fixed materials were dehydrated, embedded in paraffin and sectioned transversely using a rotary microtome at 10 µm thickness following a method of Thammathaworn (1995). The sections were deparaffinized, rehydrated and stained with 1% safranin followed by 1% fast green and mounted with DePeX mounting medium. The thickness of leaf blade, the diameters of petiole stem and tap root, and the wideness of mesophyll, petiole’s vascular bundle and root’s xylem were investigated under light microscope.

The experiment was carried out in 2 x 2 x 3 factorial in completely randomized design with 5 replications. Significant differences of means of physiological parameter measured between species were determined by independent sample t-test; and those between treatments, and those among sampling dates were determined by Duncan’s multiple range test (DMRT) at p ≤ 0.05.

**Results.** NaCl stress had an inhibitory effect on plant growth causing 31.35% and 27.31% reduction in height of plants, exposed to NaCl for 15 days, in *C. gynandra* (C$_4$ plant) and *C. viscosa* (C$_3$ plant) respectively (Figure 1). During the earlier period up to day 6 of stress, the overall morphology of both species was not different from the controls. After 10 days of stress, the first sign of salt stress symptoms appeared in *C. viscosa* including the yellowing and wilting of lower leaves starting from the lowest node. On day 15, the stress symptoms progressed so that the leaves up to the fourth node from the top yellowed and those at the lower nodes started to drop off. Most leaves of *C. gynandra*, however, remained green; only those at the lowest node became yellow.

In both plant species, there was a slight non-significant increase in chlorophyll content of the third leaf from the top after 6 days of exposure to NaCl. At 15 days after stress treatment there was only a slight reduction in chlorophyll in *C. gynandra* leaves in stressed plants compared with the controls, whereas greater reduction was was observed in *C. viscosa* (Figure 2). Both species of *Cleome* did not significantly accumulate proline, a common osmolyte, in any of the tissues investigated (Figure 3).
Figure 2. Effects of NaCl stress on chlorophyll content of *C. gynandra* and *C. viscosa* after addition of 75 mM NaCl solution to the culture soil for 6 and 15 days. Bars indicate means ± SE of 5 replications.

Figure 3. Effects of NaCl stress on the amount of proline in root (A), stem (B) and leaf (C) of *C. gynandra* and *C. viscosa* after addition of 75 mM NaCl solution to the culture soil for 6 and 15 days. Bars indicate means ± SE of 5 replications.

Salinity treatment caused marked increase in electrolyte leakage values, an indicator of cellular membrane integrity, in all types of tissue. The most profound effect was observed in roots, stems and leaf tissues of *C. viscosa* after 15 days under salt stress (Figure 4 A-C).

Sharp increase (100% increase from control) in hydrogen peroxide, an indicator of oxidative stress, was observed only in leaves of *C. viscosa* after 15 days in salinity stress (Figure 4 F), whereas the amount in leaves of *C. gynandra* increased only slightly (9.24%). Pattern of response in term of hydrogen peroxide content in roots and stems are inconsistent compared to that in leaves. In general, tissues of *C. viscosa* tended to accumulate more H$_2$O$_2$ in response to NaCl than *C. gynandra*.

Sodium chloride stress caused an increase in the amount of malondialdehyde, an indicator of lipid peroxidation process, in all tissues tested in both plant species. Leaf tissue normally has several folds higher amount of MDA than root and stem. The most significant increase in MDA content (54.72% higher than control) was observed in leaf tissue of *C. viscosa* after 15 days in NaCl treatment (Figure 4 I), whereas MDA in the leaf of *C. gynandra* increased only slightly. Similar observation was found in stem tissues (Figure 4 H).
Salt stress for 15 days imposed more negative effects on anatomical structures of *C. viscosa* than those of *C. gynandra*. The dimensions of four anatomical features of *C. viscosa* were significantly decreased in salt-stressed *C*₃ plants including leaf thickness, petiole and root diameters and diameter of root vascular cylinder (composing mainly of secondary xylem). On the other hand, most anatomical features of *C. gynandra* were unaffected by salt stress except for the decrease in the width of the stem cortex (Table 1).

**Discussion.** It can be concluded that *C. gynandra* (*C*₄) was more tolerant of salt stress and less suffered from secondary oxidative stress than *C. viscosa* (*C*₃) as indicated by smaller increase in the amount of *H₂O₂*, MDA and electrolyte leakage. Stepień & Klobus (2005) also observed the lower level of lipid peroxidation in maize (*C*₄) than wheat (*C*₃) under salt stress, which was due to higher activity of antioxidative enzymes. Sudhakar et al (2001) who studied the effect of NaCl stress on two genotypes of mulberry found that MDA content was high in sensitive variety, while the tolerant variety showed no change in...
### Table 1

<table>
<thead>
<tr>
<th>Anatomical characteristics *</th>
<th>C. gynandra (C₄)</th>
<th>C. viscosa (C₃)</th>
</tr>
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<tbody>
<tr>
<td>Leaf thickness (µm)</td>
<td>178.75±10.68a</td>
<td>178.12±11.33a</td>
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<tr>
<td>Petiole diameter (µm)</td>
<td>860.00±22.73a</td>
<td>857.50±25.96a</td>
</tr>
<tr>
<td>Diameter of petiole vascular bundle (µm)</td>
<td>200.00±7.07a</td>
<td>195.00±11.90a</td>
</tr>
<tr>
<td>Stem diameter (mm)</td>
<td>5.50±0.295a</td>
<td>5.41±0.13a</td>
</tr>
<tr>
<td>Width of stem cortex (µm)</td>
<td>532.00±39.68a</td>
<td>415.00±30.13b</td>
</tr>
<tr>
<td>Root diameter (mm)</td>
<td>4.63±0.54a</td>
<td>4.38±0.22a</td>
</tr>
<tr>
<td>Diameter of root vascular cylinder (mm)</td>
<td>2.39±0.037a</td>
<td>2.290±0.074a</td>
</tr>
</tbody>
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* For each species, mean ± SD of the control and salt-treated group for each parameter followed by the same letter are not significantly different at p <0.05. (n=6)

MDA content, lower amount of electrolyte leakage and high activity of antioxidative enzymes. Zhang & Kirkham (1996) reported that sunflower (*Helianthus annuus* L., C₃) had much higher MDA contents than sorghum (*Sorghum bicolor* (L.) Moench, C₄), indicating that sunflower membranes are more injured by oxidative stress than sorghum under drought stress. Sreenivasulu et al (1999) reported the effect of NaCl on lipid peroxidation of two genotypes of *Setaria italica* L. They found that the tolerant cultivar (Prasad) had lower MDA content than the susceptible one (Lepakshi) during salt stress. Chaparzadeh et al (2004) who studied long term salt-induced oxidative stress in marigold (*Calendula officinalis* L.) reported that high salinity caused reductions in growth parameters, lipid peroxidation and hydrogen peroxide accumulation. Maize shoots under stress showed higher water potential, osmotic adjustment and less electrolyte leakage than wheat shoots (Nayyar 2003). Nayyar & Gupta (2006) reported the effect of water stress in wheat (*Triticum aestivum*, C₃) and maize (*Zea mays*, C₄). They discovered that moderate and high stress levels caused more damage to wheat as compared to maize. This was accompanied by more loss of water and chlorophyll in wheat relative to maize at these stress levels. The oxidative damage in terms of malondialdehyde and H₂O₂ content was markedly higher in wheat as compared to maize at moderate and high stress levels.

Proline accumulation in plants occurs after a wide range of abiotic stress including salt, drought, high temperature, low temperature, anaerobiosis and UV radiation and the levels of accumulation varies from species to species (Verbruggen & Hermans 2008). Although, accumulation of proline in shoots and roots is a common physiological response to NaCl stress found in many halophytes and glycophytes (Sairam & Tyagi 2004), no significant accumulation of proline was observed in *Cleome* spp. under the condition of this study. Kumar et al (1984) reported that *C. gynandra* (C₄) was more resistant to water stress than *C. speciosa* (C₃) because leaf osmotic potential of *C. speciosa* was more decreased than *C. gynandra*. Moreover, proline content of *C. speciosa* was higher than *C. gynandra* under stress condition.

NaCl stress affected not only the physiology but also the anatomy of stressed plants. The present study indicated that NaCl induced differential anatomical changes in
leaf of both species of *Cleome*. However, it was evident that leaf and root tissues of *C. gynandra* were more tolerant of salt stress than *C. viscosa*. The petiole diameter and thickness of *C. gynandra*’s leaf were unaffected, while those of *C. viscosa* were significantly reduced. Longstreth & Nobel (1979) found that raising the concentration of NaCl in hydroponic solutions resulted in greater leaf succulence and thickness for bean and cotton. Such salt-induced succulence could lower the resistance to CO₂ uptake and thus increase photosynthetic rates by increasing the amount of internal leaf surface area across which gaseous exchange can occur per unit leaf area. Moreover, NaCl significantly inhibited growth of *C. viscosa* by reducing root diameters (22% reduction) and root vascular cylinder diameter (41% reduction). These root anatomical features in *C. gynandra* were only slightly reduced. Salinity also affected root growth of cotton seedlings causing a reduction in the width of the cortex (Kurth et al 1986).

**Conclusion.** Results from physiological and anatomical studies indicated that *C. gynandra* (C₄) was more tolerant of salt stress and less suffered from secondary oxidative stress than *C. viscosa* (C₃). A better understanding of the mechanism involved in the responses and adaptation of plants to salinity may help the introduction of environmental and genetic manipulations aimed at increasing crop salinity resistance.

**Acknowledgements.** Financial support made available to the first author by Graduate School, Khon Kaen University, Khon Kaen, Thailand is gratefully acknowledged.

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Received: 15 April 2011. Accepted: 29 May 2011. Published online: 30 May 2011.

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How to cite this article: