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In vitro Assessment of Tolerance of *Orthosiphon stamineus* to Induced Water and Salinity Stress

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Abstract: The response of *in vitro* plantlets of *Orthosiphon stamineus* to water and salinity stress indicated potential tolerance to both stress, with better tolerance to water-stress detected. Survival and vegetative growth was not severely affected for plantlets under induced water or salinity stress. Tolerance to water and salinity stress might be attributed to the proline and total soluble proteins produced. The levels for both biochemical markers significantly increased and correlated positively to the increasing concentrations of polyethylene glycol (PEG) and sodium chloride (NaCl) used. However, the chlorophyll content in *Orthosiphon stamineus* were reduced with the increase in concentrations of NaCl. Therefore, *Orthosiphon stamineus* show better tolerance to water stress than salinity stress, as survival rate, growth (fresh weight), proline level, total soluble protein and chlorophyll content in plantlets under water stress were relatively higher than plantlets challenged with salinity stress.

Key words: Chlorophyll content, proline assay, total soluble proteins

INTRODUCTION

Orthosiphon stamineus or also known as cat's whiskers or Misai Kucing (Sumaryono *et al*., 1991), is an herbaceous shrub with a unique flower that looks like cat's whiskers, hence the name. This valuable medicinal plant can be found throughout South East Asia and tropical Australia. The growing interest in *O. stamineus* was initiated by their introduction to Europe as a popular herbal tea, attributed to their anti-allergic, anti-hypersensitive, anti-inflammatory and diuretic properties. As such, leaves of *O. stamineus* which contains terpenoids, polyphenols, sterols and antioxidants (Tezuka *et al*., 2000; Khamzah *et al*., 2006), is especially effective in treating diseases affecting the urinary tract, liver and other diseases such as hypertension, rheumatism, tonsillitis, gout and menstrual disorder (Awale *et al*., 2003a, b).

Although, *O. stamineus* has good potential for large scale cultivation in many South East Asian Countries, their importance pales in comparison to other major export commodities such as oil palm, rubber and cocoa. As such, cultivation of *O. stamineus* or any other medicinal plants for the matter is usually sidelined to non-arable lands or marginal lands. In these environments, drought (water stress) and salinity stress are the main limiting factors which affect plant survivability and crop productivity (Ashraf and Bashir, 2003; Flowers, 2004). Severe water stress interferes with the cellular metabolism (Mahajan and Tuteja, 2005), while salinity stress affects the plant growth, disrupts metabolism and reduce the production of photosynthates important for plant survival, growth and productivity (Munns and Termaat, 1986).

The tolerance to drought and salinity stress in most fruits, vegetables and ornamentals are well studied (Thomson *et al*., 1988). These plants are known to adapt themselves to the stresses by producing sugar alcohols, proline and glycine betaine (Flowers *et al*., 1977; Leach *et al*., 1990; Rhodes and Hanson, 1993; Serraj and Sinclair, 2002; Bartels and Sunkar, 2005) to function as osmolytes (Sivakumar *et al*., 2000), antioxidants (Ramajulu and Sudhakar, 2001) or scavengers that help plants to tolerate stresses (Singh *et al*., 1987). In contrast, little is known concerning the degree of tolerance of *O. stamineus* to water and salt stress.

As such, this study aims to investigate the tolerance of one such medicinal plant with commercial potential, the *O. stamineus*, to water and salinity stress induced *in vitro*. Preliminary evaluation on the mechanisms of tolerance exhibited by *O. stamineus* was also investigated and reported in this study to provide useful guidance on the cultural practices in cultivating *O. stamineus* on non-arable or marginal lands.

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MATERIALS AND METHODS

Media preparation for stress challenge: Water and salinity stress were induced by amending the MS (Murashige-Skoog) media with polyethylene glycol-6000 (PEG) and sodium chloride (NaCl), respectively. To induce water stress with concentration of 5 g L^{-1} of PEG (Treatment 1, T_1), 0.5 g L^{-1} of PEG (Duchefa Biochemie) was incorporated and dissolved completely in 50 mL of distilled water. The pH was adjusted to pH 5.7±0.1 and the final volume adjusted to 100 mL. Plain agar powder (Copper Scientific) was added at a rate of 0.8 g per 100 mL and dissolved in microwave for 2 to 4 min. The medium was stirred, evenly distributed into three culture tubes and autoclaved at 121°C, 15 psi for 15 min. The media was then cooled overnight prior to subculture. The procedure was repeated for preparation of media amended with 1.0 g and 1.5 g L^{-1} of PEG to achieve final concentrations of 10 g L^{-1} (T_2) and 15 g L^{-1} (T_3), respectively. MS media for salt-stress treatments were also prepared similarly, substituting PEG with NaCl (Fischer Scientific) at 0.29, 0.58 and 0.87 g to achieve concentrations of 50 mM (T_1), 100 mM (T_2) and 150 mM (T_3), respectively. MS media without PEG or NaCl amendments was designated as control (T_C).

Fourteen-day-old *in vitro* plantlets of *O. stamineus*, obtained from the collection of Dr. Anna Ling from Universiti Tunku Abdul Rahman, were then subcultured onto the amended media. The cultures were incubated in the culture room for 14 days, with 16 h light and temperatures between 22±3°C. After 14 days, vegetative parameters and mechanisms of tolerance were determined.

Parameters observed: Proline accumulation in tissues of *O. stamineus* plantlets was determined using methods modified from Bates *et al.* (1973). Approximately, 0.25 g of the grinded tissues was first placed into a 1.5 mL microcentrifuge tube and added with 5 mL of 3% sulfosalicylic acid (Agros Organics) to precipitate protein. The mixture was centrifuged at 10000 rpm for 10 min, the resulting supernatant (0.5 mL) pipetted and transferred to a new tube and the final volume adjusted to 1 mL with distilled water. The following chemicals were then added; 1 mL of glacial acetic acid (Fisher Scientific) and 1 mL of ninhydrin reagent [3% (w/v) ninhydrin (Fisher Scientific) in 60% (v/v) 6 M phosphoric acid (R and M chemicals)] and incubated at 90°C. After 1 h, the reaction was terminated on ice and the resultant product was extracted with 2 mL of toluene (Laboratory Reagent). The upper (toluene) phase was decanted into a glass cuvette and the absorbance read at a wavelength of 520 nm using a spectrophotometer (GENESYS 20). All readings were conducted in triplicates. Proline content for every 1 g of fresh weight of sample was determined from the standard curve constructed using L-proline.

Total soluble protein assay was performed using the method by Lowry *et al.* (1951). Harvested plantlets were grinded for 5 min in 5 mL of phosphate buffer (pH 6.5) (Fisher Scientific). The extract obtained was strained through cheese cloth and the filtrate was centrifuged at 9000 rpm, 4°C, for 40 min. The resulting pellet was resuspended with 5 mL of phosphate buffer (pH 6.5). To the 1 mL of sample (0.01+0.99 mL distilled water), 0.9 mL solution A [2 g potassium sodium tartrate (Fisher Scientific), 100 g Na_2CO_3 (Systerm) dissolved in 500 mL 1 M NaOH (Merck) and top up to 1 L] was added. The mixture was thoroughly mixed and incubated at 50°C for 20 min, followed by cooling to room temperature. Solution B [2 g potassium sodium tartrate, 1 g $CuS_4.5H_2O$ (Bendosen) dissolved in 90 mL distilled water and 10 mL 1 M NaOH] was then added (0.1 mL) and the mixture was left for 10 min at room temperature. The reagent Folin-Ciocalteau (MERCK) (3 mL, diluted 1:15 with distilled water) was then incorporated into the mixture and incubated at 50°C for 10 min Lowry *et al.* (1951). Finally, the tubes were cooled to room temperature and the absorbance was recorded at 750 nm using a spectrophotometer (GENESYS 20). Total soluble protein content was calculated using known concentrations of bovine serum albumin (BSA) (MERCK) diluted in varying concentrations of 0, 50, 100, 150, 200 and 250 mg mL^{-1}. The procedure was repeated for all three replicates.

Total chlorophyll content in leaf tissues of the plantlets was determined by first grinding 0.5 g of leaf tissues in 100 mL of 80% acetone (MERCK) for 5 min. The resulting homogenate was filtered and the filtrate was transferred into a 250 mL flask. The volume was then adjusted to 100 mL with 80% acetone. The mixture was centrifuged at 10000 rpm for 5 min. The pellet was discarded, while the absorbance value of the supernatant was read at a wavelength of 663 and 644 nm using a spectrophotometer (GENESYS 20). The chlorophyll a and b content in the plant tissues were calculated by the following formula and expressed in mg per g of $tissue^{-1}$ as follows (Arnon, 1949; Koski, 1950):

$$\text{mg chlorophyll a g tissue}^{-1} = 1.07\ (OD_{663}) - 0.094\ (OD_{644})$$

$$\text{mg chlorophyll b g tissue}^{-1} = 1.77\ (OD_{644}) - 0.280\ (OD_{663})$$

$$\text{Total chlorophyll} = \text{Chlorophyll a+Chlorophyll b}$$

In addition, the survival rate and fresh weight of the plantlets were also determined.

Statistical analysis: The experiment was conducted with each treatment having triplicates and repeated once. The Analysis of Variance (ANOVA) and mean comparison (Tukey's Studentized Range Test ($HSD_{0.05}$) was performed using SAS (Statistical Analysis System) version 6.2.

RESULTS

Proline levels increased in tissues of *O. stamineus* plantlets challenged with water and salinity stress. The higher the concentrations of PEG and NaCl, the more proline was derived, as observed in plantlets in T_3 (15 g L^{-1} PEG) and T_3 (150 mM NaCl) with 18.20 and 14.28 mg of proline g fresh weight of tissues^{-1}, respectively (Fig. 1a, b). These proline levels were significantly higher than levels from control plantlets (T_C) with only 8.79 mg of proline g fresh weight of tissues^{-1}. This indicated that *O. stamineus* plantlets produced higher amounts of proline as part of its tolerance mechanism.

Total soluble protein levels were also higher in plantlets challenged with higher concentrations of PEG and NaCl. Highest total soluble protein content was assayed from plantlets in T_1 (15 g L^{-1} PEG) and T_3 (150 mM NaCl), with 0.74 and 0.79 g soluble protein g fresh weight tissues^{-1}, respectively (Fig. 2a, b). The lowest total soluble protein content was obtained from plantlets in control (T_C) with 0.58 g soluble protein/g fresh weight of tissues. Again, results here suggested that total soluble proteins were produced by *O. stamineus* plantlets in response to water and salt stress as more total soluble proteins were produced with the increase in concentrations.

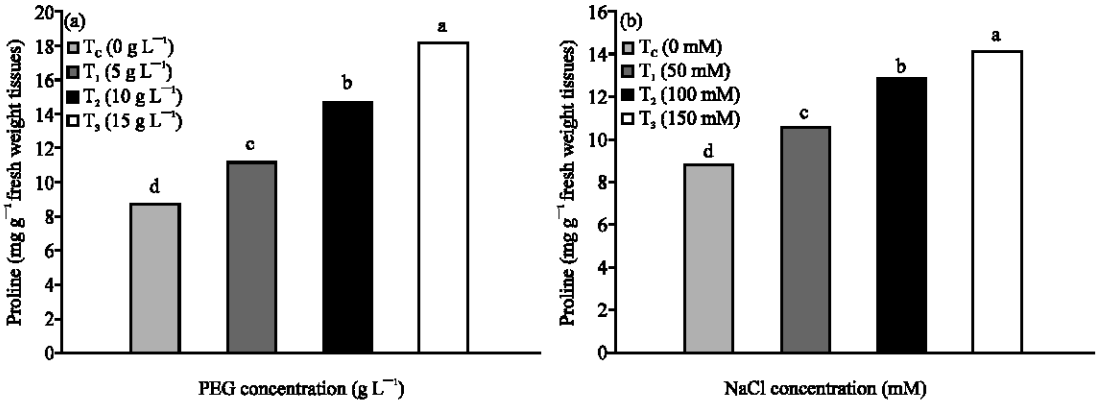

Fig. 1: Mean proline content (mg g^{-1} fresh weight tissue) in *Orthosiphon stamineus* plantlets cultured on (a) PEG-mediated water stress and (b) NaCl induced salinity stress at various concentrations. Mean values with same letter(s) are not significantly different ($HSD_{0.05}$)

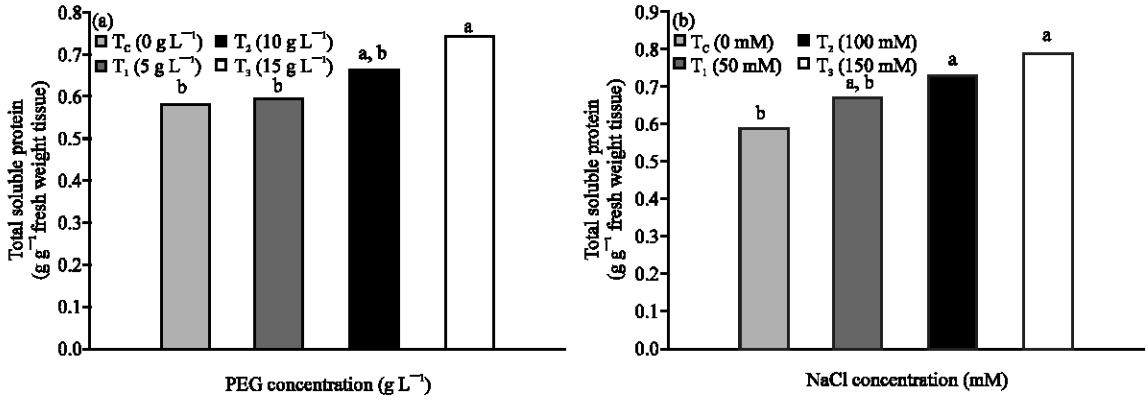

Fig. 2: Mean total soluble protein content (g g^{-1} fresh weight tissue) in *Orthosiphon stamineus* plantlets cultured on (a) PEG-mediated water stress and (b) NaCl induced salinity stress at various concentrations. Mean values with same letter(s) are not significantly different ($HSD_{0.05}$)

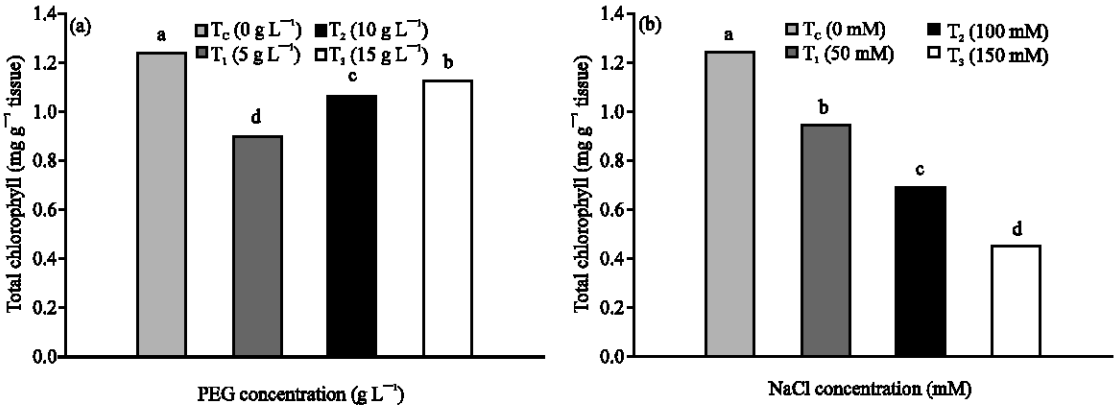

Fig. 3: Mean total chlorophyll content (mg g^{-1} tissue) in *Orthosiphon stamineus* plantlets cultured on (a) PEG mediated water stress (b) and NaCl induced salinity stress at various concentrations. Mean values with same letter(s) are not significantly different ($HSD_{0.05}$)

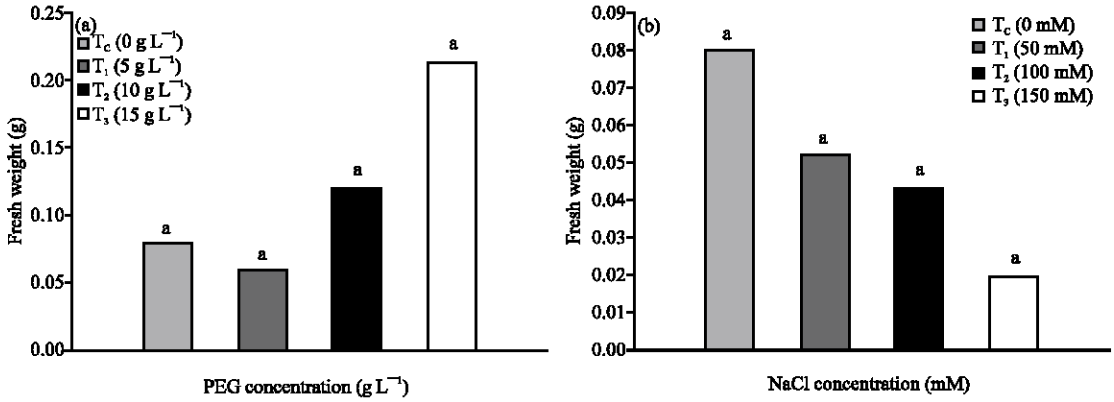

Fig. 4: Mean increase in fresh weight (g) of *Orthosiphon stamineus* plantlets cultured on (a) PEG mediated water stress and (b) NaCl induced salinity stress at various concentrations. Mean values with same letter(s) are not significantly different ($HSD_{0.05}$)

The total chlorophyll content recovered from plantlets challenged with water and salt stress, differed according to the type of stress treatments. For plantlets treated with PEG, total chlorophyll content produced correlated positively with the increasing concentrations of PEG, although levels from plantlets in T_1 to T_3 were all significantly lower than plantlets in control (T_C). In contrast, the higher the concentration of NaCl applied, the lesser the amount of total chlorophyll was recovered. In both stress assessments, the plantlets in control (T_C) produced the highest amount of total chlorophyll content with 1.24 mg total chlorophyll g tissues^{-1} (Fig. 3a, b).

All *O. stamineus* plantlets, in both stress-challenged conditions, recorded 100% survival rate (data not shown). The plantlets were however, more susceptible to salinity stress as salt-stress symptoms were observed in plantlets treated with 100 and 150 mM NaCl. Necrosis and defoliation appeared on the basal, oldest leaf of the plantlets at day 12. In contrast, plantlets did not show any drought-stress symptoms in all concentrations of PEG (data not shown).

The tolerance of *O. stamineus* plantlets to water stress was also evidently observed in the total fresh weight of plantlets. Mean increase in fresh weight for plantlets in water stress (T_1, T_2, T_3) was higher than plantlets in salinity stress (T_1, T_2, T_3) and even in control (T_C) (Fig. 4a, b). For plantlets challenged with drought-stress, the mean increase in fresh weight corresponds positively to the increasing concentrations of PEG. Plantlets were able to grow in water-stress conditions up to 15 g L^{-1} PEG (T_3) without any severe effect on fresh weight. This suggested that water-stress may have somewhat induced growth of *O. stamineus*, rather than inhibit growth. In contrast, fresh weight of plantlets was severely affected with the increase in concentrations of NaCl. Plantlets treated with 150 mM NaCl (T_3), recorded

mean increase of fresh weight of only 0.02 g. Although, statistical analysis showed that the mean of fresh weight was not significantly different for each treatment including control, fresh weight decreased by every increase in NaCl concentrations. At 50 mM NaCl (T_1), 100 mM NaCl (T_2) and 150 mM NaCl (T_3), fresh weight was reduced from a mean of 0.05, to 0.04 g and 0.02 g, respectively (Fig. 4b).

DISCUSSION

Results from this *in vitro* assessment showed that *O. stamineus* can tolerate both drought and salinity stress, although better tolerance to water stress was observed. *Orthosiphon stamineus* plantlets responded positively to PEG-induced water stress, with regards to vegetative growth, while salt-stress reduced fresh weight of plantlets. Growth-stimulation by PEG-induced water stress seen here is not uncommon, as certain plant species can respond positively to PEG-mediated water stress. Turkan *et al.* (2004) observed similar positive response in *Phaseolus acutifolius* which recorded better increment in fresh weight, root length and shoot length upon water stress stimulation.

From this study, the tolerance of *O. stamineus* plantlets to drought and salinity stress is shown to be associated with their production of proline and total soluble proteins as adaptive components to stress. Proline and total soluble protein levels were positively correlated with the increasing concentrations on PEG and NaCl. Accumulation of proline under stress protects the cells by balancing the osmotic strength of cytosol with that of vacuole and the external environment, thus protecting the enzyme structure, stabilizes the membranes and hydroxyl radicals (Igarashi *et al.*, 1997). Plantlets are therefore relieved from severe physical and metabolic alterations, enabling normal growth development, if not better than stress-free plantlets as seen in this study (increase in fresh weight). Total soluble proteins, on the other hand, help in enhancing osmotic and metabolic adaptations by providing storage forms of nitrogen, which are re-utilized when stress is over (Singh *et al.*, 1987; Sarhan and Perras, 1987; Chen and Plant, 1999). In many plants, both proline and total soluble protein levels were established to be generally higher in stress-tolerant than in stress-sensitive plants (Singh *et al.*, 1972; Ahmad *et al.*, 1981; Hurkman *et al.*, 1989; Fougere *et al.*, 1991; Ashraf and Tufail, 1995; Igarashi *et al.*, 1997; Zaifnejad *et al.*, 1997; Ashraf and Bashir, 2003; Teizera and Pereira, 2007). Therefore, we can conclude that *O. stamineus* could be categorized as stress-tolerant rather than stress-sensitive plants. They may have drought-tolerant (water stress) and salt-tolerant traits to enable adaptations to stress, due to the high levels of proline and total soluble proteins produced.

In addition to proline and total soluble proteins, chlorophyll content for *O. stamineus* under water stress was also relatively higher than levels in plantlets under salinity stress, although in both stress-induced conditions, the control plantlets recorded the highest chlorophyll content. This further established the tolerance of *O. stamineus* towards water-stress as their chlorophyll content, linked to photosynthesis potential is less severely affected compared to plantlets under salinity-stress. Generally, accumulation of salts in plantlets challenged with water and salinity stress, results in the loss of enzymatic activity and reduction of production of photosynthates, which leads to the reduction of photosynthesis rate (Munns and Termaat, 1986). In *O. stamineus*, adaptations enabled photosynthesis in water-stressed plantlets to continue to sustain growth.

The results from this study, albeit preliminary in nature, indicated that *O. stamineus* has better tolerance to PEG-mediated water stress compared to salinity stress induced by NaCl. Future studies can be conducted at the glasshouse stage to determine the response of *O. stamineus* to both stresses. It may also include investigations on the responses of different genotypes of *O. stamineus* towards drought and salinity stress, as this was not screened in this study. This may then provide useful guidance on the cultural practices in cultivating *O. stamineus* on non-arable or marginal lands.

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