Response of Increasing NaCl Concentrations on Growth and Proline Content of Tacca leontopetaloides cultured in vitro

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Abstract

The effects of increasing NaCl concentrations on growth and proline content of *Tacca leontopetaloides* cultured in vitro were investigated. *T. leontopetaloides* were suspected to have high tolerance against salinity, thus the purpose of this research was to investigate the effect of increasing NaCl concentrations added on growth medium on growth and proline content of T. leontopetaloides grown in vitro. In vitro corms were cultured on MS medium supplemented with NaCl at concentrations of 0, 10, 25, 50, 75, 100, 150 and 200 mM, respectively. After six weeks in culture, shoots height, shoots number, leaves number, fresh weight, as well as their proline content were recorded. The results showed that fresh weight of shoots grown on MS medium supplemented with 10, 25 and 75 mM NaCl was higher compared to the control treatment. Fresh weight decreased when shoots were cultured on MS medium supplemented with NaCl at more than 100 mM. Proline content increased along with the increase of NaCl concentrations. Meanwhile, the height of shoots, number of shoots, and number of leaves decreased along with the increase of NaCl concentrations.

Keywords: *Tacca leontopetaloides*, NaCl, salinity stress, proline content, *in vitro*

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Introduction

Salinity is a major stress limiting the increase in the demand for food crops. More than 20% of cultivated land worldwide is affected by salt stress and the amount is increasing day by day (Gupta & Huang, 2014). Salinization can be managed by changed farm management practices. In irrigated agriculture, better irrigation practices, such as drip irrigation to optimized use of water can be employed. In rain-fed agriculture, practices such as rotation of annual crops with deep-rooted perennial species may restore the balance between rainfall and water use, thus preventing rising water tables bringing salts to the surface (Munns, 2002). Salt stress leads to the suppression of plant growth and development, membrane leakage, ion imbalance or disequilibrium, enhances lipid peroxidation and increases production of reactive oxygen species like superoxide radicals, hydrogen peroxide and hydroxy radicals, which are scavenged by both enzymatic and non-enyzmatic reactions (Roychoudhury *et al*., 2008).

In order to maintain homeostasis during salt stress condition, plants need to have special mechanism for adjusting internal osmotic conditions and changing in osmotic pressure inside the cells, this process is called osmotic adjustment (OA). Stressed plants diminish osmotic potential by accumulating low molecular weight, and osmotically active compounds called osmolytes (Summart *et al*., 2010). One of the osmolytes was proline. Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and essential for primary metabolism. Proline accumulation was reported during conditions of drought, high salinity, high light and UV radiation, heavy metals, oxidative stress and in response to biotic stresses (Szabados & Savouré, 2010). Proline accumulation is frequently reported in salt-stressed plants.

Proline is often considered to act as a compatible solute involved in osmotic adjustment at the plant cell level, although the precise role of this accumulation in osmotic adjustment is still debated, proline is accumulated in cytoplasm without having a detrimental effect on cytosolic enzyme activities (Hasegawa *et al*., 2000).

Polynesian arrowroot (*Tacca leontopetaloides (L.*) Kuntze Syn. *T. pinnatifida* Forst, *T. involucrata* Schum and Thonn.) is a species of flowering plant belongs to family Taccaceae (Caddick *et al*., 2002). The tubers contain 20-30 % of starch. The amylose content of Tacca starch was found to be 22.5%, which is in the same range as the amylase content of potato, cassava and some other root starches. Physicochemical tests show that properties of *T. leontopetaloides* starch are similar to those of potato and maize starch, even though Tacca starch was relatively more resistant to compression. This could be concluded that Tacca starch can be used as pharmaceutical excipient comparable to maize starch in tablet formulation (Kunle *et al*., 2003). Tacca were often found in dappled shade behind sandy beaches and therefore this plant was suspected tolerant against salinity. The plant remains wild and under-utilized in Indonesia, although some of the region in Indonesia (Karimunjawa and Cikelet) have utilized this plant for emergency food. T. leontopetaloides was suspected tolerant against salinity, thus the aim of this study was to investigate the effect of increasing NaCl (high salinity) added on growth medium on growth and proline content of T. leontopetaloides grown in vitro.

Materials and Methods

Plant Culture Materials and NaCl Treatment

Plant materials of *T. Leontopetaloides* used were corms of two month olds shoots originated from *in vitro* shoots grown in MS medium (Murashige & Skoog, 1962), supplemented with 0.5 ppm Benzyl Amino Purine (BAP), solidified with 8 g/L of agar, containing 30 g/L sucrose. Corms were placed on MS solid medium supplemented with NaCl at 0 (control), 10, 25, 50, 75, 100, 150 and 200 mM. Each treatment consisted of four replicates, on which four corms were planted on each replicate. The pH medium was adjusted to 5.8 and 8 g/L agar was added prior to autoclaving at 121^{o}C and 103 kPa for 15 min. All plant materials were cultured at 26 ± 2^{o}C under continuous light provided by cool white fluorescent tube with 1000-1400 lux light intensity.

Growth Parameters

The height of the explants, total number of shoots, and total number of leaves per explant were recorded every week until 6 weeks after culture. The shoot fresh weight per explant was recorded after 6 weeks of culture.

All data were analyzed by variance analysis (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at 5% level of probability from mean comparison.

Determination of Proline Concentration

After six weeks of culture, whole parts of plantlets were harvested for proline analysis. Purified proline was used as standard for proline quantification. The proline assay was determined as described by Bates *et al.* (1973). The acid-ninhydrin reagent was prepared by warming 1.25 g ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid, agitated and dissolved. Approximately 0.5 g of plant material was homogenized in 10 mL of 3% sulfosalicylic acid and filtered by Whatman no.2 filter paper. Two mL of filtrate was reacted with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100^{o}C, and the reaction was terminated in ice bath. The reaction mixture was extracted with 4 mL toluene, mixed with stirrer for 15-20 sec. The chromophore containing toluene was aspirated from the aqueous phase, warmed to a room temperature and read the absorbance at 520 nm with toluene as a blank. The proline concentration was determined based on a standard curve and calculated on a fresh weight basis as follows: [(µg proline/mL x mL toluene)/115.5 µg/µmole]/[(g sample)/5] = µmoles proline / g of fresh weight material.

Results and Discussion

Results

Growth Parameters

After six weeks in culture, the fresh weight of *T. leontopteloides* shoot culture was tend to increase along with the increase of NaCl concentration up to 75 mM, except for data in 50 mM NaCl. The fresh weight was tend to decrease on NaCl concentration from 100 up to 200 mM NaCl (Figure 1 and 2).

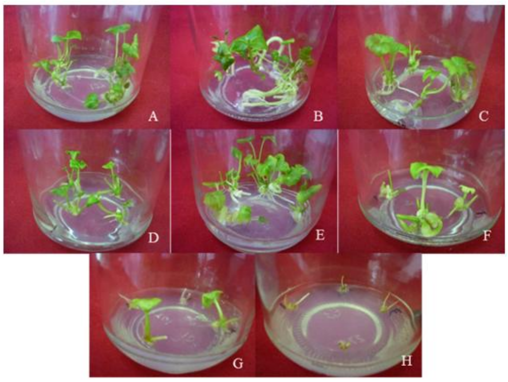

Figure 1. *T. leontopetaloides* culture after six weeks in the treatment medium: (A) MS medium(control); (B) 10 mM NaCl; (C) 25 mM NaCl; (D) 50 mM NaCl; (E) 75 mM NaCl; (F) 100 mM NaCl; (G) 150 mM NaCl; (H) 200 mM NaCl.

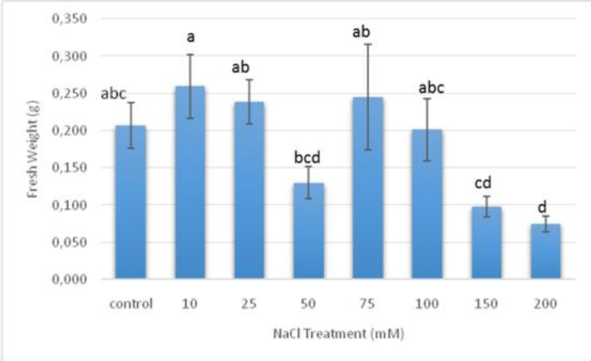

Figure 2. The effect of NaCl on fresh weight of *T. leontopetaloides.* Bar with different letter is significantly different (*P*=0.05) according to DMRT.

The shoot height, shoot number and leaves number showed different results. Shoot height, shoot number and leaf number were decreased along with the increase of NaCl concentrations and the number even lower when comparing with control treatment (Table 1). The highest shoot height were obtained only on the medium supplemented with 10 mM NaCl (2.106 ± 0.226).

Table 1. Rate of shoot height, shoot number and leaf number of 6 weeks old culture of *T. leontopteloides* in the treatment medium at different concentration of NaCl.

NaCl Treatment (mM)	Shoot height (cm)	Shoot number	Leaf number
Control (0)	1.850 ± 0.148 ab	1.938 ± 0.213 a	3.125 ± 0.482 a
10	2.106 ± 0.226 a	1.563 ± 0.203 ab	3.063 ± 0.588 a
25	1.458 ± 0.248 bc	1.333 ± 0.188 bc	1.250 ± 0.494 bc
50	1.514 ± 0.283 bc	1.143 ± 0.097 bc	1.429 ± 0.429 bc
75	1.450 ± 0.186 bc	1.333 ± 0.256 bc	2.167 ± 1.014 ab
100	1.140 ± 0.132 c	1.067 ± 0.067 bc	0.800 ± 0.200 bc
150	1.178 ± 0.141 c	1.000 ± 0.000 c	0.889 ± 0.200 bc
200	0.000 ± 0.000 d	0.000 ± 0.000 d	0.000 ± 0.000 c

For each column, Mean ± s.e. followed by letter(s) are significantly different (*P*=0.05) according to DMRT.

Proline Concentration

Proline concentration increased along with the increase of NaCl concentration. The highest proline concentration was achieved by shoots grown on medium supplemented with 200 mM NaCl (16,883 µmol/g) (Figure 3).

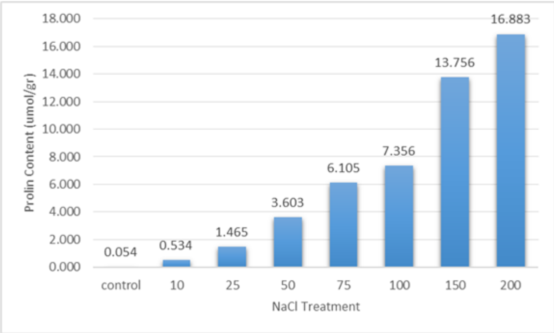

Figure 3. Proline concentration on *T. leontopetaloides* culture under NaCl treatment.

Discussion

According to He and Cramer (1993), growth analysis is fundamental to the characterization of plant's response to an environmental stress. Shonjani (2002) observed the inhibition of root and in particular shoot growth with NaCl treatments for sugar beet, rice and cotton seedlings and a decrease in length of shoots was more pronounced at higher salt treatment.

As data shown in figure 2, the fresh weight data, tend to increase along with the increase in NaCl concentration up to 75 mM (except for 50 mM NaCl treatment). Dimassi-Theriou (1998) reported that the fresh weight of peach (*Prunus cerasifera*) cultured *in vitro* were increased along with the increasing of NaCl concentration up to 20 mM. Similar result also reported by Mane *et al*. (2011) in *Pennisetum alopecoroides* seedlings, whereas the growth parameter such as fresh weight, shoot length and root length were increase in NaCl treatment up to 100 mM. Ceyhan and Ali (2002) observed an increase in fresh weight of lettuce plant in high salinity, and Ruiz *et al*. (1999) also reported this for orange leaf and attributed this to increased water content in plant. In our present study, fresh weight of *T. leontopetaloides* planlet increased by 25% at 10 mM NaCl (Figure 2). Mane *et al*. (2011) suggest that the increase of fresh weight in lower levels of NaCl might be the adaptation of plants to osmotic adjustment which maintains water uptake and turgor with the accumulation of organic solutes.

According to Volkmar *et al*. (1998), higher level salts can produce decreased water uptake in plants. In our present study, salt stress had an inhibiting effect on *Tacca* plantlet fresh weight. The plantlet fresh weight was reduced with increasing salinity concentration from 100 mM NaCl to 200 mM NaCl. Salinity-stressed plantlets possessed lower fresh weight in comparison to the control planlet (Figure 2). Chauhan and Prathapasenan (1998) reported that the dry weight of the rice callus reduced in 200 mM NaCl treatment. Ahmad *et al.* (2007) also noticed that NaCl treatment reduced the indica rice callus fresh weight. Similar result was also reported by Saygideger and Deniz (2008) on biomass reduction of *spirulina* by NaCl stress treatment, and reduction of pea (*Pisum sativum*) biomass on NaCl treatment from 25 mM up to 75 mM NaCl (Shahid *et al*., 2011). The reduction of plantlet fresh weight because of osmotic stress due to lowering of external water potential or the effect of ion toxicity on metabolic processes (De Herralde *et al*., 1998) and thus leads to decreases in the plantlet growth.

The growth parameter such as shoot height, shoot number and leaf number starting reduced in 10 mM NaCl treatment and stronger inhibition effect were recorded on higher concentration of NaCl. According to McFarland *et al.* (2014) salinity threshold for salt-sensitive plant is about 1 dS/m on irrigation water (10 mM NaCl equal to 0,91 ds/m). Even though shoot height parameter in 10 mM NaCl higher than control, other parameters such as shoot number and leaf number were lower than control. This data (Table 1) clearly indicated that shoot culture of *T. leontopetaloides* was sensitive to NaCl treatment.

Many studies showed that the plant growth were associated with photosynthesis, and the effect of high salinity on photosynthesis have been reported on many studies (Ahmed *et al.*, 2008; Rajasekaran *et al.*, 1997). Salinity stress causes reduction the plant capacity for CO_2 fixation and causes stomata closures which lead to decrease in photosynthetic activity (Rajasekaran *et al.*, 1997). The decrease in photosynthetic activity lead to reduction in metabolism and eventually will reduce the growth of NaCl-stressed plant. In salt-sensitive species, salt is not effectively excluded from transpiration stream, therefore, salt will have to build up to toxic levels in the leaves (Munns, 2002), resulting in progressive losses of the leaves numbers as data shown in Table 1. Similar result also reported by D'onofrio and Morini (2002), on shoot regeneration of quince leaves (*Cydonia oblonga*) treated with increasing NaCl concentration up to 80 mM.

Proline is distinguished from other amino acids in several ways. The most fundamental is that proline is the only one of proteogenic amino acids where the α-amino group is present as secondary amine. On numerous of studies there were several type of plant stresses caused proline to accumulate to high levels in many plant species (Verslues & Sharma, 2010). Proline accumulation primarily occurs in response to the stresses such as drought, salinity and freezing that cause dehydration of the plant tissue (Verslues & Bray, 2006), in addition it can also occur in response to heavy metal toxicity even if at low level of toxicity (Sharma & Dietz, 2009), plant pathogen interaction (Fabro *et al*., 2004) and other biotic and abiotic stresses. The large accumulation of proline which occurs during drought is related to its basic chemical properties: proline is the most water soluble of the amino acids and exists much of the time in a zwitterionic state having both weak negative and positive charges at the carboxylic acid and nitrogen groups, respectively (Verslues & Sharma, 2010). As data shown in Figure 3, proline biosynthesis clearly induced by NaCl stress as reported by Jiang and Deyholos (2006) in *Arabidopsis*. Genes regulating proline biosynthesis (*P5CR* and *P5CS*) were induced and proline level increased during NaCl stress. Proline is known to maintain a hydration sphere around the bio-polymers and maintain their native state, thereby regulating growth under drought and salinity stresses (Gangopadhyay & Basu, 2000). Our studies confirmed that proline level was increase along with the increase in NaCl concentrations (Figure 3). From our growth data indicated that *Tacca* is a salt-sensitive plants. In green house experiment, addition of NaCl at low concentration inhibited root growth of *Tacca* (data is not presented). Therefore, this indicated that *Tacca* is a salt-sensitive plant.

In our previous work, *Dioscorea alata* shoot culture (salt-moderate tolerant) treated with 50 mM NaCl resulted in 8.3 µmol/g of proline and increased almost 3 fold after treated with 100 mM NaCl (Martin *et al.*, 2012). In our present work, *Tacca* treated with 50 mM NaCl only resulted in 3.6 µmol/g of proline and addition of 100 mM NaCl resulted in 7.3 µmol/g, respectively (Figure 3). These showed that compared to *Dioscorea, Tacca* had lower response to NaCl treatment as it indicates by lower proline concentration at *Tacca* compared to *Dioscorea*. According to Lawlor (2002), there is potential links between photosynthetic activity and proline synthesis and metabolism, therefore, further investigation is required. From this we could also assumed that *T. leontopetaloides* grows in coastal area might have tolerate against salinity since there is enough photosynthetic activity and lead to enough proline synthesis in leaves area, eventually lead to tolerance against salinity.

Conclusions

Shoot height, shoot number and leaf number of *T. leontoptealoides* shoot culture decreased along with the increase of NaCl concentrations. Proline content increased along with the increase of NaCl concentration. Low proline level found in *T. leontopetaloides* shoot culture indicated that this plant is sensitive to NaCl treatment.

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