The Effect of Increase in NaCl Concentration on Growth and Proline Content of Purple Yam (*Dioscorea alata* L.) Grown *In Vitro*

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Abstract

Tuber of purple yam (*Dioscorea alata* L.) has been used as an alternative food in some areas in Indonesia. The tuber contains high carbohydrate, low glycemic index and gluten free, therefore, study on genetic improvement of this species is needed to increase the productivity and to find out new cultivars which can be cultivated in marginal lands. This research was aimed to investigate the effect of NaCl concentration on growth and proline content of purple yam grown *in vitro*. Shoot tips were cultured on MS (Murashige and Skoog) medium supplemented with NaCl at concentrations of 25, 50, 100, 200 and 250 mM. After six weeks in culture, height of shoots, number of nodes, number of leaves, as well as proline content were recorded. The results showed that shoots grown on MS medium supplemented with NaCl at 25 and 50 mM had better growth compared to control. The best medium for its growth was MS containing 50 mM of NaCl. Increase in NaCl level's resulted in decrease of growth. The LD_{50} value was obtained at 183 mM of NaCl. The highest proline concentration was achieved by shoots grown on the medium supplemented with 100 mM of NaCl. This result indicated that purple yam was tolerant to the increase of NaCl concentration up to 100 mM, on MS medium without addition of plant growth regulators.

Keywords: purple yam, *Discorea alata* L., NaCl, *in vitro* growth, proline content

Introduction

Dioscorea alata L. (purple yam) is one of tuberous plant species potential as flour source useful for alternative food crop as well as functional food. *Dioscorea alata* L. species has two different color of tubers which is yellowish white tuber (yellow) and purple tuber. Purple yam has advantages compared to yellow yam because it has high content of beta-carotene, vitamins, minerals and carbohydrate content. It has low glycemic index, gluten-free and low sugar levels, making it safe for diabetic's patient. Gluten-free character of purple yam tuber were beneficial for people with celiac disease. Purple yam flour can be utilized as materials for making snacks and as raw flour for cakes, noodles, ice cream and so on (Ubi Production Techno Guide, *www.da.gov.ph*).

Salinity is one of the major abiotic stresses limiting agricultural production in many areas of the world. At the whole plant level, salinity stress frequently induces an increase in Na and Cl contents as well as decreases in K, Ca, NO_3 and P concentrations. In salt resistant cultivars, Na is frequently accumulated in oldest leaves, thus preserving young photosynthetically active tissue (Yeo & Flower, 1986). 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 (Roychoudury *et al.,* 2008).

In order to maintain homeostasis during stress condition, plants need to have special mechanism for adjusting internal osmotic conditions and changing in osmotic pressure inside the cell. 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 has been reported during conditions of drought, high salinity, high light and UV radiation, heavy metals, oxidative

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stress and in response to biotic stresses (Szabados & Savoure, 2010). Proline accumulation is frequently reported in salt-stressed plants. Although the precise role of this accumulation is still debated, proline is often considered to act as a compatible solute involved in osmotic adjustment at the plant cell level. Proline is accumulated in cytoplasm without having a detrimental effect on cytosolic enzyme activities,meanwhile toxic ions which is mainly Na, is accumulated in vacuoles (Munss, 1993).

Tissue culture is a useful technique for producing transplant which is seasonal independent, mass propagation can be done in a relatively short of time, useful for genetic manipulations, and for *in vitro* conservation. Mutation of the plant varieties can also be done by several techniques to produce mutant with high productivity. This plant trait can also be selected *in vitro* to produce a new tolerant variety to stress conditions with manipulation of culture medium and *in vitro* growth conditions.

The aim of this research was to investigate the effect of increase in NaCl concentration on growth and proline content of purple yam grown *in vitro*. This technique will be useful to find out the genotype tolerant to salt.

Materials and Methods

Plant culture material. Shoots culture of purple *Dioscorea alata* used in this experiment were *in vitro* shoots grown in hormon free Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) solidified with 8 g/L of agar, containing sucrose at 20 g/L. Shoot cultures were sub-cultured every month. All plant materials were cultured at 26±2°C under continuous light provided by cool white fluorescent tube with 1,000–1,400 lux light intensity.

Treatment with NaCl. The shoot of purple yam was cut into 1–1.5 cm long with 2–3 nodes, having 2–3 leaves. The shoots were placed on MS solid medium supplemented with various concentration of NaCl (0/control treatment; 25, 50, 100, 200 and 250 mM). The medium pH was adjusted to 5.8. Medium was solidified with 0.8% agar before autoclaving at 121°C and 103 kPa for 15 min. Four shoots were placed in each culture jar per treatment with 12 replicates. Cultures were incubated at 26±2°C under continuous light. Every week, the height of the explants, total number or nodes and total number of leaves per explants were recorded until 6 weeks of culture. The shoot fresh weight per explants was recorded after 6 weeks of culture. Survival shoot was also recorded for determination of LD_{50} NaCl (NaCl concentration in the nutrient medium that is lethal to 50% of the population). 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 (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°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×mL toluene)/115.5 µg/µmole]/[(g sample)/5] = µmoles proline/g of fresh weight material.

Results and Discussion

After six weeks in culture (Figure 1), growth of *D. alata* shoot culture was significantly reduced by the increase in NaCl concentration in the culture medium. The addition of NaCl was significantly reduced the fresh weight of *D. alata* plantlet (Figure 2). Salt stress induced an inhibiting effect on plantlet fresh biomass. The planlet fresh weight means clearly indicated that plantlet fresh weight were reduced with the increase in salinity from lower level to higher levels (from 25 mM to 250 mM of NaCl). Salinized plantlet possessed lower fresh weight in comparison to the control (Figure 1). This result also indicated that *D. alata* was salt-sensitive plant

because plantlet biomass were significantly reduced when they were treated with lower level of NaCl. Similar result was also reported on rice (Chauhan *et al.*, 1988; Ahmad *et al.*, 2007), *Spirulina* (Saygideger *et al.*, 2008) and pea (Shahid *et al.*, 2011). The reduction of biomass production by the plant culture might be due to the chlorosis and necrosis of the leaves that reduce the photosynthetically active area (De Herralde *et al.*, 1998), thus it reduces metabolism and it leads to decreases in plant growth. The biomass reduction occurred on the purple yam cultured on the increase in NaCl concentrations.

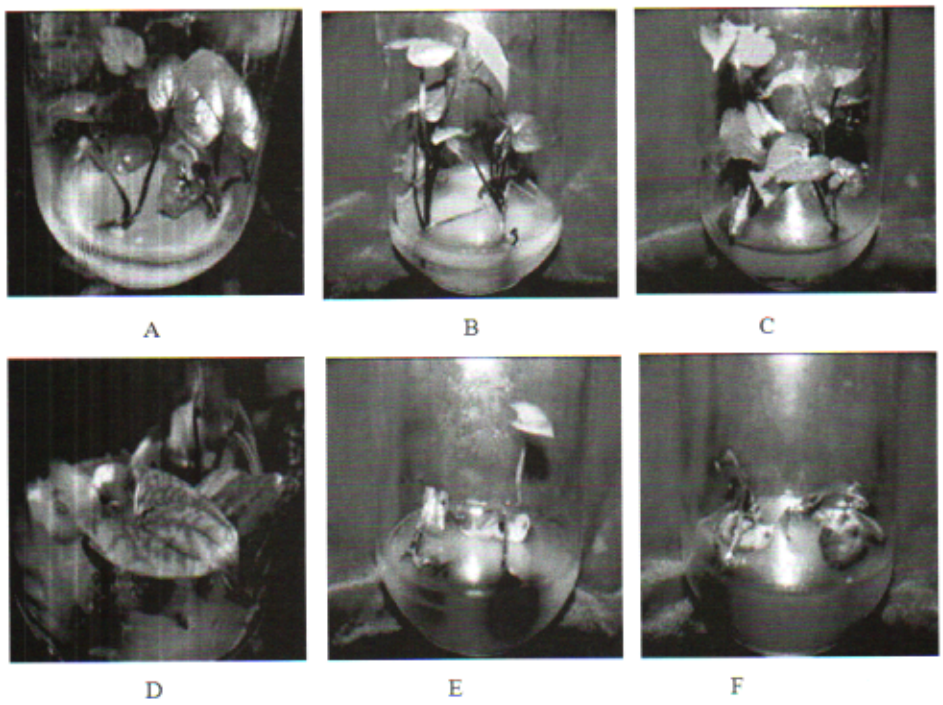

Figure 1. *D. alata* L. after six weeks in the treatment medium: (A) MS medium; (B) 25 mM of NaCl; (C) 50 mM of NaCl; (D) 100 mM of NaCl; (E) 200 mM of NaCl; and (F) 250 mM of NaCl.

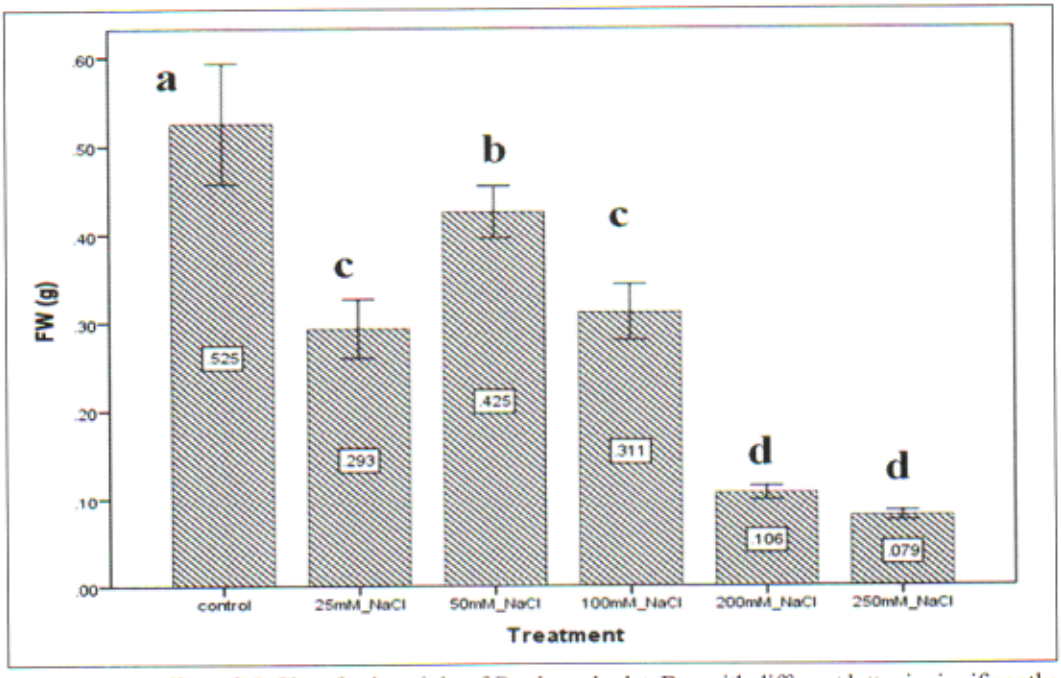

Figure 2. The effect of NaCl on fresh weight of *D. alata* planlet. Bar with different letter is significantly different (P=0.05) according to DMRT.

Different from fresh weight, shoot height, leaves number and nodes number showed different results. After 6 weeks in culture the highest shoot height, leaves number and nodes number of *D. alata* were identified on the medium supplemented with 25 and 50 mM of NaCl. The height of *D. alata* shoot culture treated with 50 mM of NaCl (3.99 ± 0.18) and 25 mM of NaCl (3.65 ± 0.21) were significantly different compared to the other treatment (Table 1). Similar result was also obtained on leaves number and nodes number. High leaves number was found on the culture treated with 25 mM of NaCl (5.28 ± 0.24) and 50 mM of NaCl (5.48 ± 0.25), meanwhile the number of nodes also gave similar result on culture treated with 25 mM of NaCl (4.31 ± 0.19) and 50 mM of NaCl (4.25 ± 0.19). The results (Table 1–3) showed that growth increased in culture treated with NaCl from 25 to 50 mM. Significant decreases of growth were obtained in the culture treated with 100–250 mM of NaCl. Similar results were reported by Mane *et al.* (2011) that the lower level of salinity stimulated the growth rate of grass *Pennisetum alopecuroides*, but, in higher level of salinity decreased growth and gave negative correlation with increasing level of NaCl. According to Bohnert *et al.* (1995) high level of salinity in solution initially establishes a water potential imbalance between the apoplast and symplast which leads to the decrease in turgor which may cause the growth reduction. In our study the decrease in plant height, leaves number and nodes number of *D. alata* culture treated with 100–250 mM of NaCl might be due to high salinity and toxicity of sodium chloride to the shoots.

Table 1. The effect of NaCl with various concentrations on shoot height of *D. alata*. Data were recorded after 6 weeks in culture.

Treatment with NaCl (mM)	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
control	1.52±0.08^{bc}	1.73±0.10^{c}	1.92±0.10^{c}	2.01±0.11^{c}	2.28±0.16^{c}	2.70±0.19^{c}	2.86±0.23^{c}
25	1.69±0.09^{c}	1.59±0.08^{bc}	2.01±0.13^{c}	2.38±0.14^{d}	2.92±0.18^{d}	3.31±0.20^{d}	3.65±0.21^{d}
50	1.55±0.11^{bc}	1.56±0.10^{bc}	1.85±0.10^{c}	2.52±0.11^{d}	3.33±0.15^{d}	3.71±0.16^{d}	3.99±0.18^{d}
100	1.29±0.08^{b}	1.32±0.08^{b}	1.43±0.09^{b}	1.48±0.09^{b}	1.67±0.11^{b}	1.91±0.14^{b}	2.16±0.19^{b}
200	1.32±0.10^{b}	1.39±0.10^{b}	1.43±0.11^{b}	1.45±0.11^{b}	1.53±0.17^{ab}	1.52±0.16^{ab}	1.59±0.19^{ab}
250	1.01±0.08^{a}	1.00±0.09^{a}	1.02±0.09^{a}	1.00±0.08^{a}	1.08±0.14^{a}	1.16±0.17^{a}	1.12±0.18^{a}

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

Table 2. The effect of NaCl with various concentrations on shoot leaves number of *D. alata.* Data were recorded 6 weeks in culture.

Treatment with NaCl (mM)	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
control	2.09±0.14^{b}	2.49±0.16^{c}	3.41±0.24^{c}	3.64±0.22^{c}	3.97±0.24^{c}	4.43±0.28^{c}	4.63±0.29^{c}
25	1.96±0.11^{ab}	2.47±0.13^{c}	2.95±0.14^{bc}	3.58±0.17^{c}	3.98±0.17^{c}	4.50±0.21^{c}	5.28±0.24^{c}
50	1.81±0.09^{ab}	2.29±0.13^{bc}	3.08±0.15^{bc}	3.60±0.17^{c}	4.04±0.18^{c}	4.65±0.21^{c}	5.48±0.25^{c}
100	1.83±0.11^{ab}	2.31±0.13^{bc}	2.68±0.16^{b}	2.80±0.18^{b}	2.95±0.18^{b}	3.08±0.20^{b}	3.15±0.20^{b}
200	1.88±0.08^{ab}	1.98±0.11^{ab}	2.14±0.12^{a}	2.37±0.14^{b}	2.40±0.21^{ab}	2.44±0.22^{ab}	2.60±0.26^{ab}
250	1.65±0.09^{a}	1.75±0.10^{a}	1.82±0.10^{a}	1.84±0.11^{a}	1.79±0.15^{a}	1.90±0.18^{a}	1.89±0.20^{a}

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

Table 3. The effect of NaCl with various concentrations on shoot nodes number of *D. alata.* Data were recorded 6 weeks in culture.

Treatment with NaCl (mM)	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
control	2.26±0.14^{b}	2.43±0.15^{c}	2.95±0.20^{b}	3.21±0.20^{d}	3.33±0.20^{d}	3.51±0.19^{c}	3.71±0.22^{c}
25	2.15±0.15^{ab}	2.19±0.12^{bc}	2.59±0.15^{b}	2.90±0.16^{cd}	3.23±0.15^{cd}	3.72±0.18^{c}	4.31±0.19^{c}
50	1.88±0.12^{ab}	1.94±0.13^{ab}	2.50±0.13^{b}	2.79±0.14^{cd}	3.02±0.13^{cd}	3.63±0.15^{c}	4.25±0.19^{c}
100	2.25±0.16^{b}	2.10±0.12^{bc}	2.53±0.16^{b}	2.63±0.18^{bc}	2.74±0.18^{bc}	2.82±0.19^{b}	2.72±0.18^{b}
200	1.81±0.13^{a}	1.82±0.12^{ab}	2.05±0.16^{a}	2.21±0.16^{ab}	2.40±0.22^{b}	2.40±0.22^{b}	2.30±0.28^{b}
250	1.85±0.12^{ab}	1.63±0.11^{a}	1.87±0.13^{a}	1.92±0.14^{a}	1.64±0.17^{a}	1.60±0.16^{a}	1.56±0.18^{a}

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

The LD_{50} of *D. alata* treated with NaCl was 183 mM. The LD_{50} value was obtained by extrapolating equation obtained from linear regression on percentage survivability curve against NaCl concentration (Figure 3). The LD_{50} was used to asses plant survival to salt stress and to select shoot culture that tolerance to NaCl stress. The shoot culture survived from LD_{50} doses could have possibility to tolerate the NaCl stress.

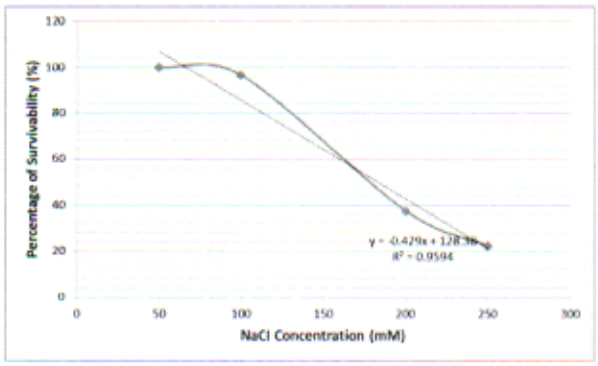

Figure 3. Linear regression on standard curve for LD50 determination.

Proline biosynthesis is clearly induced by NaCl stress as reported by Jiang & Deyholos (2006) in *Arabidopsis*. Genes regulating proline biosynthesis (*P5CR, P5CS*) were induced and proline level was increased during NaCl stress. Proline is known to function as an osmoregulatory molecule preventing cellular dehydration through osmotic adjustment. In addition, it may interact with crucial macromolecules of the cell to maintain their biological activity under stress conditions. Therefore, 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 increased along with the increase in NaCl concentrations (Figure 4). In the culture treated with 100 mM, proline content increased about 20 fold compared to the control. In higher level of NaCl concentration *i.e* 200 and 250 mM NaCl, proline content was significantly decreased. These suggested that in higher level of NaCl stress, the plants were unable to tolerate the stress. Therefore, the metabolism decreased and leads to the cells death, resulting in lower level of proline content. It is possible that proline accumulation in excessive amount under stressful condition could be due to its stimulated synthesis from glutamate, lower rate of its oxidation and slowed incorporation of proline into proteins (Hanson & Hitz, 1982).The higher levels of proline at higher levels of salinity (NaCl) and drought (PEG) are thought to regulate growth through osmotic adjustment and to reduce the acidity of the cytoplasm (Vanekamp *et al.*, 1989). It also could be concluded that proline accumulation is not just a sign of cellular injury as a result of the response to drought or salt stress, but also a marker of stress tolerance having a definite osmoregulatory role, thereby regulating growth under stressful environments.

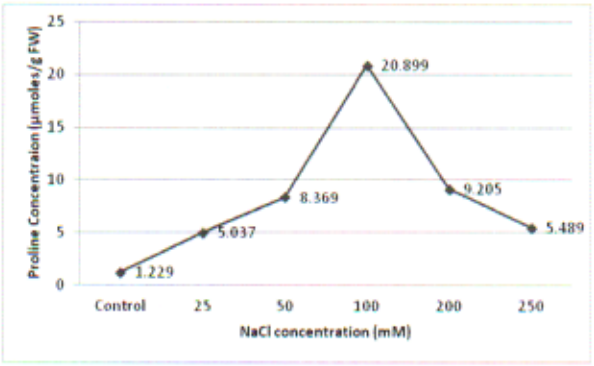

Figure 4. Proline concentration on *D. alata* L. culture under NaCl treatment.

Conclusions

The results indicated that shoot height, leaves number and nodes number of the *D. alata* were stimulated by lower levels of salinity, however higher levels salinity inhibited those growth. It was observed that lower levels of salt concentration *i.e.* 25 and 50 mM stimulated and enhanced the growth rate of *D. alata* culture, indicating that lower levels salt concentration was more favorable for culture medium of *D. alata*. It appears that *D. alata* species exhibit a moderate salinity tolerance as far as linear growth is concerned. The best medium for its growth was MS containing 50 mM of NaCl, while on higher level of NaCl concentration levels resulted in decrease of growth. LD_{50} value of NaCl was obtained at 183 mM of NaCl. These LD_{50} value could be used for selecting shoot culture that tolerate to salt stress. Highest proline concentration was achieved by shoots grown on the medium supplemented with 100 mM of NaCl. This result indicated that on MS medium without addition of plant growth regulators, the purple yam was tolerant to the increase of NaCl concentration up to 100 mM.

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