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Growth and Proline Accumulation in Response to Osmotic Stress Induced by Polyethylene Glycol Treatment in *Tacca leontopetaloides* Cultured *In Vitro*

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Abstract *Tacca leontopetaloides* known as Polynesian arrowroot is a wild tuberous perennial herb. In addition, *T. leontopetaloides* is a valuable plant not only as staple food but also as a medicinal herb. Nonetheless, there are very few literatures and research on the potential of this plant. In this study, the effect of polyethylene glycol (PEG) as stress osmoticum in *T. leontopetaloides* cultured *in vitro* on growth and proline accumulation was investigated. The shoot culture of *T. leontopetaloides* was subjected to six levels of PEG concentrations (2.5; 5; 7.5; 10; 12.5 and 15%). Growth parameter was evaluated by observing shoot height, number of shoots, number of leaves and number of roots for 6 weeks of culture. After 6 weeks of culture, fresh weight and proline content were determined. The results showed that number of shoots and number of leaves increased after 2.5% PEG treatment while number of roots increased after 5% PEG treatment. Conversely, higher concentrations of PEG inhibited growth. In line with this, culture fresh weight decreased along with the increase of PEG concentrations. To summarize, the growth of culture decreased along with the increase of PEG concentrations. In contrast, proline concentration increased along with the increase of PEG concentrations.

Keywords: Growth, *in vitro*, polyethylene glycol (PEG), proline content, *Tacca leontopetaloides*

Introduction

Plant growth and metabolism are influenced by abiotic and biotic factors. Plants are frequently exposed to stress condition such as heavy metal toxicity, nutrient availability, salinity, temperature and water stress (Búfalo *et al.*, 2016). Water stress is a major abiotic factor that limits crop productivity, restrict plant distribution, growth, and yield (Silva *et al.*, 2016). The insufficient water status in plant leads to water-deficit stress or commonly known as drought stress. Moreover, drought or water-deficit stress is not only caused by water shortage

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but can also be influenced by an environmental change such as low temperature and salinity. Hence, plants are share multitude of molecular substance in response to these stresses (Hirt and Shinozaki, 2004). Basically, there are two principal strategies in response to water stress. The first one is that the plant synthesizes osmoprotectant molecules to prevent damage during water stress and the second one is repair mechanism during rehydration (Hirt and Shinozaki, 2004).

Osmoprotectant molecule could be characterized in three types. First, osmoprotectant containing ammonium compounds such as polyamines, *b*-alanine, betaine, and choline-*O*-sulfate. Second, osmoprotectant containing sugar and sugar alcohols such as trehalose, fructan, mannitol, and sorbitol. Lastly, osmoprotectant containing amino acids such as proline and ectoine (Singh *et al.*, 2015). Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and essential for primary metabolism. Proline accumulated in the cytoplasm and was reported to accumulate during conditions of drought, high salinity, high light and UV radiation, heavy metals, oxidative stress and in response to biotic stresses (Szabados and Savouré, 2010). In addition, proline regulates different metabolic processes such as stabilizing sub-cellular structure including membranes and proteins (Singh *et al.*, 2015) and relieve cytoplasmic acidosis (Hoque *et al.*, 2008).

Numerous studies have been conducted by using polyethylene glycol (PEG) as osmotic agent in tissue culture such as in wheat (Bajji *et al.*, 2002), atriplex (Martínez *et al.*, 2005), soybean (Hamayun *et al.*, 2010), pigeonpea (Kumar *et al.*, 2011), rice (Wu *et al.*, 2015), tomato (George *et al.*, 2015) and castor bean (Silva *et al.*, 2016). PEG is a neutral polymer and is available in different molecular mass (Zyl and Kennedy, 1983), and it has been used for some research on plant water stress (Ahmad *et al.*, 2007; Bajji *et al.*, 2002; Dami and Hughes, 1997; George *et al.*, 2015; Hamayun *et al.*, 2010; Kumar *et al.*, 2011; Martínez *et al.*, 2005; Meneses *et al.*, 2011; Wu *et al.*, 2015; Xu *et al.*, 2013; Zyl and Kennedy, 1983). PEG is frequently used in plant culture research because it is chemically inert, does not enter the cell, does not cause toxicity. Hence it is satisfactorily simulating the drought effects (Rai *et al.*, 2011). The molecular weight of PEG used for the study of water stress were various i.e. PEG-4000 (Wu *et al.*, 2015; Zyl and Kennedy, 1983), PEG-6000 (George *et al.*, 2015; Meneses *et al.*, 2011) and PEG-10000 (Hamayun *et al.*, 2010; Martínez *et al.*, 2005; Mohamed and Tawfik, 2006).

Polynesian arrowroot (*Tacca leontopetaloides (L.*) Kuntze Syn. *T. pinnatifida* Forst, *T. involucrata* Schum and Thonn.) is tropical flowering plant species and recently were grouped into D*ioscoreaceae* family (Caddick *et al.*, 2002; Zhang *et al.*, 2011). Physicochemical analysis from *T. leontopetaloides*

starch revealed that the starch is relatively more resistant to compression compared with those of potato and maize starch. This peculiar attribute of Tacca starch could make it be used for pharmaceutical excipient comparable to maize starch in tablet formulation (Kunle *et al.*, 2003). *T. leontopetaloides* is often found behind seashore and associated with beach vegetation, savannas, secondary forest, grassy slopes and under the shade of coconut plantations (Lim, 2016). Therefore, this plant is suspected to be more tolerant against osmotic stresses such as drought and salinity. For this reason, it is essential to develop bioassays to screen *T. leontopetaloides* genotypes for drought tolerance. The tissue culture methodology of *T. leontopetaloides* has been successfully done (Martin *et al.*, 2012b) and thus the objective of this study was to investigate the effect of PEG addition as osmotic agent on growth and proline content of *T. leontopetaloides in vitro* culture.

Materials and methods

Plant culture materials and PEG treatment

Two-months old *T. leontopetaloides* shoots were excised, and the corms were isolated. The corms were subcultured in MS medium (Murashige and Skoog, 1962) added with 0.5 ppm Benzyl Amino Purine (BAP), 30 g/L sucrose and supplemented with various concentration of PEG (MW 4000) at 0; 2.5; 5; 7.5; 10; 12.5 and 15%, respectively. The medium was solidified with 8 g/L agar and pH was adjusted to 5.8 with 1N KOH. The medium was autoclaved at 121 ^{0}C and 103 kPa for 15 min. Four shoots were placed in each culture jar per treatment with 6 replicates. The cultures were incubated at 25 ± 2 ^{0}C under continuous light provided by cool white fluorescent tube with 1000-1400 lux light intensity.

Growth Parameters

The height of shoots, number of shoots, and number of roots per explant were recorded every week until 6 weeks after culture. The shoot fresh weight per explant was also recorded after 6 weeks in culture. All data were analyzed by variance analysis (ANOVA), followed by Duncan's Multiple Range Test (DMRT) at 5% probability from the mean comparison.

Determination of Proline concentration

After six weeks of culture, whole parts of explants were harvested for proline analysis. Pure L-Proline (Phytotech, product id: p698) was used as a

standard for proline quantification. The proline assay was determined as described by Bates *et al.* (1973) with slight modification. Acid-ninhydrin reagent was made by warming 1.25 g ninhydrin in 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid until it dissolved. Roughly weigh 0.5 g of plant material and homogenized using mortar 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 reagent and 2 mL of glacial acetic acid in a test tube for 1 h at 100 ^{o}C and the reaction was terminated in an ice bath. Four (4) mL toluene was added to the reaction tube, stirred for 15-20 seconds, and the chromophore containing toluene was aspirated. Warm the chromophore to room temperature and read 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

The addition of PEG into culture medium caused an inhibiting effect on shoot height of *T. leontopetaloides* culture. Table 1 showed that the shoots height decrease was noticeable in the first week of culture. In the first week of culture, shoot height of control culture was already significantly different with PEG treatment. This data trend was continued until the end of the observation (6^{th} week).

Table 1. The effect of PEG with various concentrations on shoot height (cm) of *T. leontopetaloides in vitro* culture.

PEG treatment	Week(s)					
	1	2	3	4	5	6
Control	1.20 ± 0.07 ^{a}	1.47 ± 0.13 ^{a}	1.60 ± 0.12 ^{a}	1.83 ± 0.14 ^{a}	2.05 ± 0.16 ^{a}	2.42 ± 0.17 ^{a}
2.50%	1.10 ± 0.05 ^{ab}	1.24 ± 0.06 ^{bc}	1.44 ± 0.09 ^{ab}	1.65 ± 0.11 ^{a}	1.84 ± 0.13 ^{ab}	1.98 ± 0.14 ^{b}
5%	1.07 ± 0.03 ^{b}	1.41 ± 0.08 ^{ab}	1.52 ± 0.10 ^{a}	1.57 ± 0.09 ^{ab}	1.61 ± 0.09 ^{bc}	1.66 ± 0.09 ^{c}
7.50%	1.02 ± 0.02 ^{b}	1.02 ± 0.01 ^{d}	1.08 ± 0.05 ^{c}	1.15 ± 0.07 ^{cd}	1.18 ± 0.07 ^{de}	1.29 ± 0.09 ^{de}
10%	1.02 ± 0.01 ^{b}	1.11 ± 0.04 ^{cd}	1.23 ± 0.07 ^{bc}	1.34 ± 0.09 ^{bc}	1.45 ± 0.10 ^{cd}	1.49 ± 0.11 ^{cd}
12.50%	1.00 ± 0.00 ^{b}	1.05 ± 0.04 ^{d}	1.10 ± 0.04 ^{c}	1.13 ± 0.05 ^{cd}	1.17 ± 0.06 ^{de}	1.21 ± 0.07 ^{de}
15%	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{d}	1.01 ± 0.01 ^{c}	1.04 ± 0.02 ^{d}	1.05 ± 0.02 ^{e}	1.09 ± 0.04 ^{e}

Description: Mean ± s.e. followed by the same letter in the same column are not significantly different by Duncan's multiple range test at $\alpha = 5\%$.

The numbers of shoots were increased with the addition of 2.5 to 5% of PEG treatment and noticeable in the third week of culture although it is not significantly different compared to other treatment (Table 2). The number of

leaves was noticeably higher on plantlet supplemented with 2.5% PEG in the first week of culture and reach the highest number at the end of observation (3.50 ±0.57) although it is not significantly different to the control (Table 3).

Table 2. The effect of PEG with various concentrations on number of shoots of *T. leontopetaloides in vitro* culture.

PEG treatment	Week(s)					
	1	2	3	4	5	6
Control	1.00 ± 0.00 ^{b}	1.08 ± 0.06 ^{a}	1.08 ± 0.06 ^{a}	1.08 ± 0.06 ^{bc}	1.13 ± 0.07 ^{ab}	1.13 ± 0.07 ^{bc}
2.50%	1.08 ± 0.06 ^{a}	1.13 ± 0.07 ^{a}	1.13 ± 0.07 ^{a}	1.33 ± 0.14 ^{a}	1.38 ± 0.15 ^{a}	1.42 ± 0.15 ^{a}
5%	1.00 ± 0.00 ^{b}	1.08 ± 0.06 ^{a}	1.17 ± 0.10 ^{a}	1.29 ± 0.19 ^{bc}	1.33 ± 0.19 ^{a}	1.33 ± 0.19 ^{ab}
7.50%	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{a}	1.00 ± 0.00 ^{a}	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{b}	1.08 ± 0.06 ^{bc}
10%	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{a}	1.04 ± 0.04 ^{a}	1.04 ± 0.04 ^{bc}	1.04 ± 0.04 ^{b}	1.00 ± 0.00 ^{c}
12.50%	1.00 ± 0.00 ^{b}	1.04 ± 0.04 ^{a}	1.00 ± 0.00 ^{a}	1.04 ± 0.04 ^{bc}	1.04 ± 0.04 ^{b}	1.00 ± 0.00 ^{c}
15%	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{a}	1.00 ± 0.00 ^{a}	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{b}	1.00 ± 0.00 ^{c}

Description: Mean ± s.e. followed by the same letter in the same column are not significantly different by Duncan's multiple range test at α = 5%.

Root formation was firstly recorded in the second week of observation on 5% PEG treatment, while other treatment, root formation was recorded on the 3rd and 4th weeks (Table 4). Similar data trend was observed on the roots parameter that the number of roots increased from 2.5 to 5% PEG treatment compared to the control treatment. The highest numbers of roots per explant were obtained at 5% PEG treatment significantly different compared to the control (Table 4). PEG treatment from 7.5 up 15% inhibited the root formation significantly. The roots were not formed in the 12.5% PEG treatment as shown in Table 4.

Table 3. The effect of PEG with various concentrations on number of leaves of *T. leontopetaloides in vitro* culture.

PEG treatment	Week(s)					
	1	2	3	4	5	6
Control	0.50 ± 0.17 ^{b}	0.79 ± 0.19 ^{b}	1.13 ± 0.21 ^{b}	1.63 ± 0.26 ^{abc}	2.00 ± 0.32 ^{ab}	2.42 ± 0.32 ^{ab}
2.50%	1.00 ± 0.30 ^{a}	1.58 ± 0.28 ^{a}	2.08 ± 0.42 ^{a}	2.50 ± 0.50 ^{a}	2.92 ± 0.47 ^{a}	3.50 ± 0.56 ^{a}
5%	0.50 ± 0.12 ^{b}	0.96 ± 0.24 ^{b}	1.38 ± 0.36 ^{b}	1.79 ± 0.47 ^{ab}	2.63 ± 0.52 ^{a}	2.75 ± 0.62 ^{a}
7.50%	0.04 ± 0.04 ^{b}	0.08 ± 0.06 ^{c}	0.21 ± 0.08 ^{c}	0.46 ± 0.15 ^{d}	0.79 ± 0.19 ^{c}	1.00 ± 0.27 ^{c}
10%	0.25 ± 0.11 ^{b}	0.63 ± 0.13 ^{b}	0.79 ± 0.18 ^{bc}	1.25 ± 0.20 ^{bcd}	1.25 ± 0.23 ^{bc}	1.63 ± 0.25 ^{bc}
12.50%	0.21 ± 0.08 ^{b}	0.50 ± 0.13 ^{bc}	0.67 ± 0.14 ^{bc}	0.63 ± 0.18 ^{d}	0.67 ± 0.21 ^{c}	0.75 ± 0.20 ^{c}
15%	0.42 ± 0.10 ^{b}	0.58 ± 0.10 ^{bc}	0.71 ± 0.13 ^{bc}	0.83 ± 0.13 ^{cd}	0.92 ± 0.16 ^{c}	0.88 ± 0.16 ^{c}

Description: Mean ± s.e. followed by the same letter in the same column are not significantly different by Duncan's multiple range test at α = 5%.

Table 4. The effect of PEG with various concentrations on number of roots of *T. leontopetaloides in vitro* culture.

PEG treatment	Week(s)					
	1	2	3	4	5	6
Control	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.04 ± 0.04 ^{b}	0.04 ± 0.04 ^{c}	0.42 ± 0.22 ^{bc}
2.50%	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.04 ± 0.04 ^{b}	0.04 ± 0.04 ^{b}	0.58 ± 0.21 ^{ab}	0.79 ± 0.23 ^{ab}
5%	0.00 ± 0.00 ^{a}	0.21 ± 0.13 ^{a}	0.17 ± 0.10 ^{a}	0.50 ± 0.19 ^{a}	0.67 ± 0.19 ^{a}	1.08 ± 0.28 ^{a}
7.50%	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{c}	0.08 ± 0.06 ^{c}
10%	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.13 ± 0.09 ^{b}	0.25 ± 0.15 ^{bc}	0.29 ± 0.15 ^{c}
12.50%	0.00 ± 0.00 ^{a}	0.04 ± 0.04 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{c}	0.00 ± 0.00 ^{c}
15%	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{c}	0.04 ± 0.04 ^{c}

Description: Mean ± s.e. followed by the same letter in the same column are not significantly different by Duncan's multiple range test at $\alpha = 5\%$.

After six weeks in culture (Tables 1-4 and Figure 1), the growth of *T. leontopetaloides* shoots cultures were reduced along with the increase of PEG concentrations. The declining of the growth rate was noticed in the second to third weeks of culture. In the final week of observation, the growth of *T. leontopetaloides* cultures was qualitatively observed to reduce in the presence of PEG from 7.5 up to 15 %.

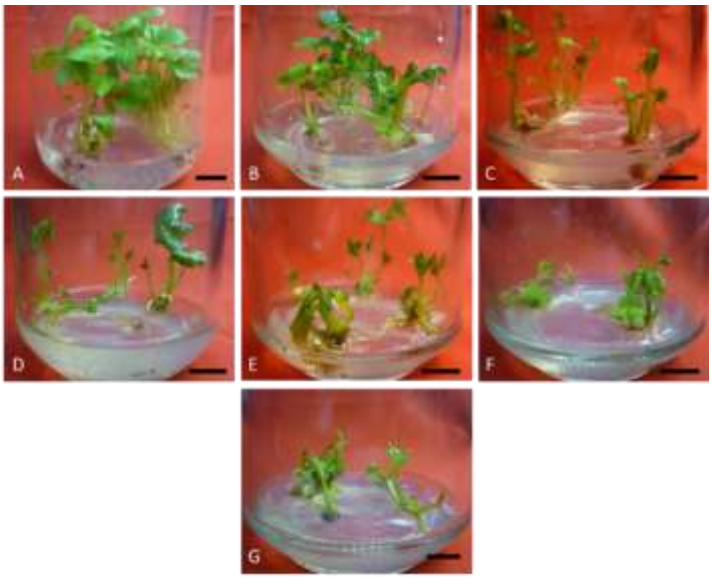

Figure 1. Visual performance of *T. leontopetaloides* culture after six weeks in the treatment medium: (A) MS medium (control); (B) 2.5% PEG; (C) 5% PEG; (D) 7.5% PEG; (E) 10% PEG; (F) 12.5% PEG; (G) 15% PEG. The black bar indicates 1 cm.

Fresh weight of *T. leontopetaloides* explant decreased along with the increase of PEG concentrations (Fig. 2). The decrease of FW in 2.5 and 5% PEG are not significantly different compared to the control, correspondingly the decrease of FW was significantly different in 7.5 up 15% PEG treatment compared to the control.

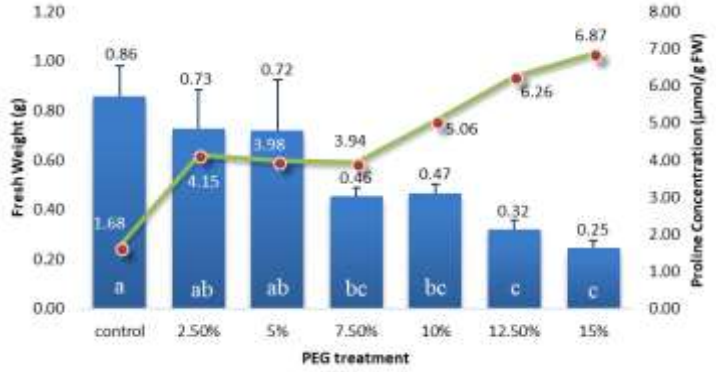

Figure 2. The effect of PEG on fresh weight and proline of *T. leontopetaloides* in the sixth week after subculture. Bar with different letter is significantly different (*P=0.05*) according to DMRT.

Discussion

The addition of PEG inhibited shoot height (Table 1) and number of shoots (Table 2) of *T. Leontopetaloides.* The shoot height decrease in the presence of PEG treatment was also reported by Gopal and Iwama (2007) on *Solanum tuberosum in vitro* culture; Said *et al.* (2015) on Banana plantlet and Siaga *et al.* (2016) on *Solanum melongena* plantlet. The decreasing water potential caused by PEG greatly suppressed cell elongation as a result of low turgor pressure (Piwowarczyk *et al.*, 2014) eventually, resulting in the decrease in shoot height. At the end of observation, control, 2.5% and 5% PEG treatment were significantly different compared to another PEG treatment. As data shown in Table 1, the addition of 2.5% and 5% PEG had already decreased the shoot height of *T. leontopetaloides* culture significantly in the first week of culture. In the final weeks of observation, the lowest shoot height was achieved at culture treated with 15% PEG treatment at 1.09 ± 0.04 cm while the highest shoot height was recorded from control treatment at 2.42 ± 0.17 cm.

In our research, similar to number of shoots parameter (Table 2), number of leaves increased with the addition of 2.5 up to 5% PEG (Table 3). Siaga *et al.*, (2016) also reported an increase of eggplant leaves number treated with 10% PEG. This phenomenon could be a physiological response of the plant because

PEG lowered the osmotic pressure, thus the plant may try to cope the osmotic stress with more transpiration by forming more leaves and root. The growth inhibition effect on number of leaves was strongly seen at 7.5 up to 15% PEG treatments. The lowest numbers of leaves recorded were from 12.5% PEG treatment. Piwowarczyk *et al.* (2014) reported that on several genotypes of *Lathyrus* had increased shoot multiplication rate at 5% PEG treatment. At the end of observations in the sixth week of culture, culture with PEG treatment from 10 to 15% inhibited the formation of new shoots, while the highest number of shoots was recorded from 2.5% PEG treatment significantly different from the control treatment. The lowest number of shoots was recorded from 10, 12.5, and 15% PEG treatment at 1.00 ± 0.00. Additionally, limited changes were recorded during the whole period of observation at 10–15% PEG treatments, indicating there are no new shoots formed in high PEG concentration treatment. High PEG concentration induces drought stress to the plant and could lead to a decrease of IAA (indole-3-acetic acid) levels in the plant (Cui *et al.*, 2016; Li *et al.*, 2011), thus resulting in inhibition of shoot and root formation as indicated in our present study (Tables 2 and 4).

The growth of *T. leontopetaloides* was reduced after PEG treatment. Our results were confirmed by previous study on using PEG for inducing water stress (Al-Bahrany, 2002; Joshi *et al.*, 2011; Kumar *et al.*, 2011; Rao and FTZ, 2013; Silva *et al.*, 2016). Polyethylene glycol added to the medium would mimic the effect of water stress because it reduces the water potential of the medium (Rai *et al.*, 2011). This condition would lead to the reduction of nutrient and water absorption by the root (Suharjo, 2012). Since the explants were depending on sucrose as the only source of carbon in the medium, the reduced water uptake and nutrient will reduce carbon intake and eventually will lead to reduce the growth of explants significantly.

The decrease in *T. leontopetaloides* biomass indicated that the culture has the ability to sustain their water content to mild stress, whereas this ability lost under severe stress treatment. A similar result was reported in pigeon pea (Kumar *et al.*, 2011) where the plant was able to maintain growth under PEG mild stress. This result also confirmed the previous study reported that an increase in drought stress by PEG was accompanied by a steep decline in moisture content of the tissues (Joshi *et al.*, 2011). The highest fresh weight was obtained from control treatment (0.86 ± 0.12 g) while the lowest fresh weight was obtained from 15% PEG treatment (0.25 ± 0.03 g). In contrary, proline content increased along with the increase of PEG concentrations. The highest proline content was obtained from 15% PEG treatment (1.68 µg/g FW), while the lowest proline content was obtained from control treatment (6.86 µg/g FW) (Figure 2).

Growth analysis is a fundamental characterization to assess plant's responses to environmental stress (He and Cramer, 1993). The high-molecular-weight PEG has been used to study *in vitro* induction water deficit because it reduces the osmotic potential of the medium (Rai *et al.*, 2011), thus inducing water stress that adversely affected the growth of the plantlets. In our present study osmotic stress induced by PEG from 7.5 to 15% resulted in decrease of growth significantly as data shown in Tables 1-4 and Figure 2. In lower level of PEG (at 2.5 – 5% PEG) the cultures were able to maintain growth as shown in Tables 1-4. Number of shoots and number of leaves were slightly higher compared to the control, although it was not significantly different. Interestingly the root numbers increased significantly in the present of 5% PEG. This result was similar to research finding in germination of cotton cultivar that had radicle length increased in the presence of low level of PEG (Meneses *et al.*, 2011).

It has been shown that proline has a key role in stabilizing cellular protein and membranes in the presence of high concentration of osmoticum (Rudolph *et al.*, 1986). In our previous work, proline concentrations increased in the presence of stress osmoticum (NaCl) (Martin *et al.*, 2012a; Martin *et al.*, 2015). In our present work proline concentrations began to increase at 2.5% and at higher than 15% PEG (Figure 2). Similar result was also reported in rice calli (Joshi *et al.*, 2011) and pigeon pea (*Cajanus cajan*) (Kumar *et al.*, 2011). The accumulation of proline during drought is related to its basic chemical properties whereas proline is the most water-soluble amino acids and exists much of the time in zwitterionic state having weak negative and positive charges at the carboxylic acid and nitrogen groups, respectively (Verslues and Sharma, 2010). In our present work, it seems that proline was effective in maintaining growth from 2.5 to 5% PEG as shown in Table 1-4. This was also because of characteristic of proline whereas proline capable of maintaining a hydration sphere around the biopolymers and maintaining their native state, thereby regulating growth under drought and salinity stresses (Gangopadhyay and Basu, 2000). Thus, it can be concluded that addition of PEG into the medium clearly indicated water stress effect on *T. leontopetaloides* culture. Even though *T. leontopetaloides* culture could maintain growth from 2.5 to 5% PEG, *T. leontopetaloides* culture was not able to tolerate higher PEG concentrations which were from 7.5 to 15%.

Conclusion

PEG concentrations affected *T. leontopetaloides* grown *in vitro.* Shoot height and fresh weight of *T. leontopetaloides* culture decreased along with the increase of PEG concentrations, whereas the number of shoots, number of

leaves and number of roots slightly increased in the presence of 2.5 up to 5 % PEG then decreased at high level of PEG (from 7.5 to 15%). Proline level increased along with the increase of PEG concentrations.

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