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In Vitro Propagation of Lisianthus (*Eustomagr andiflurom*)

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Abstract
Nowadays, the most rapid method for producing healthy and disease-free Lisiantus is micropropagation. With respect to high economic value of this plant which is regarded among the 10 top cutting flowers in the world, this research was carried out to suggest a suitable protocol for its *in vitro* propagation, using nodal sections as an explant. To carry out this object, the effects of the pH (5.5, 5.6, 5.7, 5.8), culture vessel (small glass bottle, large glass bottle, polypropylene container), the concentration of macro elements, including NH_4NO_3 (1.45, 1.65, 1.85 g L^{-1}), KNO_3 (1.7, 1.9, 2.1 g L^{-1}), $CaCl_2.2H_2O$ (0.66, 0.44, 0.24 g L^{-1}), $MgSO_4.7H_2O$ (0.43, 0.37, 0.31 g L^{-1}), KH_2PO_4 (0.13, 0.17, 0.21 g L^{-1}), and the concentration of sucrose (25, 30, 35, 40 g L^{-1}) were investigated in four independent experiments. The effects of the different studied factors were significant on the shoot regeneration. Results showed that pH 5.7 and the use of 35 g L^{-1} sucrose in MS medium were the best treatments for improving the number of shoots per explants (2.25 and 2 shoots, respectively). Moreover, increasing KH_2PO_4 concentration in MS medium produced the highest number of shoots per explant (3.5 shoots). The polypropylene container was also the best culture container for the lisianthus micropropagation (7.5 shoots per explant).

Keywords: Culture container; Lisianthus; Macro elements; Micropropagation; pH

Introduction
Lisianthus (*Eustoma grandiflurom*) belongs to the family of Gentianaceae and originates from the warm regions of Mexico, Southern United States, the Caribbean and the northern parts of South America. Lisianthus plants are herbaceous annuals and this is a popular ornamental plant that is also commonly used as a cut flower (Rob and Lawson 1984; Kuntake *et al*. 1995). *E. grandiflorum* is commonly propagated by seed. A large number of seedlings can be produced by seed culture but the quality is not uniform due to variations in flowering time, plant height and the number of flowers. In some cultivars, such as those with marginal variegation, the seedlings show a wide range of variation due to their heterozygous characteristics (Furukawa *et al.* 1990). This problem could be overcome by *in vitro* propagation of this plant. Micropropagation has been extensively utilized for the rapid production of many plants. The success in the micropropagation method depends on several factors, including genotype, culture media, plant growth regulators, type and concentration of carbohydrate source and culture container (Edwin *et al.* 2008). Effects of the plant growth regulators on micropropagation of *E. grandiflorum* have been already investigated in some studies (Mousavi *et al*. 2012; Ghaffari Esizad *et al*. 2012; Kaviani *et al*. 2014; Jamal Uddin *et al*. 2017).

The lid of the culture container protects the culture medium from microbial infections and also

prevents excessive evaporation of water from the culture medium. Type of container and lid also affect the gaseous composition inside the container as well as light penetration (Islam *et al.* 2005). Therefore, the growth and the development of tissues in culture (shoot regeneration, proliferation, elongation, fresh weight and possibly the hyperhydric degradation processes) are also affected by culture vessels (Islam *et al.* 2005). The pH of culture media has also been known as a very important factor in many aspects of explant growth and development. Sensitivity or tolerance to medium pH changes the *in vitro* responses of the explants according to specific requirements of individual species (Harbage *et al*. 1998; Shinohara *et al*. 2006; George *et al*. 2008). Medium pH level may influence nutrient uptake (Ramage and Williams 2002), cellular pH (Ballarin-Dentiand Antoniotti 1991) and root formation (De Klerk *et al.* 2008). Carbohydrate is another important ingredient in the culture media and sucrose is commonly used carbohydrate. Sucrose acts as an enhancer of osmotic potential and plays a vital role in shoot and root induction (Demo *et al.* 2008). Generally, for tissue culture, Murashige and Skoog (1962) stated that the use of 3% sucrose was better than 2 or 4%. Moreover, Lakes and Zimmerman (1990) observed the highest rooting percentage in apple on a medium with high osmolarity. Hyndman *et al.* (1982) obtained more and larger roots with an increase in sucrose concentration from 30 to 60 g L^{-1}. Optimum mineral composition in culture medium is necessary for obtaining normal growth and development in *in vitro* conditions. Therefore, the concentrations of the microelements and macro -elements should be optimized for any species and genotype.

The aims of the current study were to determine optimal pH, container type, sucrose concentration and the concentration of some macro elements such as NH_4NO_3, KNO_3, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, and KH_2PO_4 in shoot regeneration medium of lisianthus (*E. grandiflurom*).

Materials and Methods

In the present study for the micropropagation of lisianthus, the donor plants were obtained from seed culture in plastic pots (peat moss/perlit, 1:2). Plants grew in a glass greenhouse under a 16 h photoperiod at 25 °C. The nodal explants were provided from 30-day seedlings. Initially, the leaves subtending each bud were cut at the base of the petiole; the plant materials were then washed with liquid detergent (common dishwashing liquid), diluted in water and were placed under running tap water for 30 min. Then, the plant materials were first treated with 2% (w/v) sodium hypochlorite for 10 min, followed by rinsing with sterile distilled water. Plant materials were then rinsed with 70% (v/v) ethanol for 30 s, followed rinsing three times with sterile distilled water.

In the present study, the effects of the pH (5.5, 5.6, 5.7, 5.8), culture container (small glass bottle, 55 mm in diameter, 80 mm in height), large glass bottle (160 × 75 mm), polypropylene container (90 × 90 ×110 mm) and the concentration of the macro elements, including NH_4NO_3 (1.45, 1.65, 1.85 gL^{-1}), KNO_3 (1.7, 1.9, 2.1 gL^{-1}), $CaCl_2.2H_2O$ (0.66, 0.44, 0.24 gL^{-1}), $MgSO_4.7H_2O$ (0.43, 0.37, 0.31 gL^{-1}) and KH_2PO_4 (0.13, 0.17, 0.2 gL^{-1}), and the

different concentrations of sucrose (25, 30, 35, 40 gL^{-1}) were investigated in the independent experiments. Each experiment was carried out using a completely randomized design with four replications. The base medium was the MS medium supplemented with 4 mg L^{-1} BAP and solidified by 7 gL^{-1} agar-agar (Murashige and Skoog 1962). Glass bottles with autoclave-resistant plastic caps and polypropylene containers, containing 50 ml medium were autoclaved for 20 min (121 °C and 1.2 bar). Each replication comprised four explants per container and the cultures were incubated at 24±1 °C in a controlled growth chamber under a 16 h photoperiod with a light intensity of 3000 lux (provided by tube fluorescent). After 30 days, the shoot number, shoot length (cm) and leaf number per explant were recorded.

For *in vitro* rooting of lisianthus, some healthy shoots were selected and transferred to root induction medium supplemented with NAA (0 , 0.2, 0.5, 1 mg l^{-1}) and activated charcoal (0, 3 gl^{-1}). Experiment of rooting was carried out as factorial based on completely randomized design with four replications. After 30 days the mean root number per explant was recorded. Primary statistical analyses such as normality test (Kolmogorov-Smirnov test) and the homogeneity of variances (Levene's test) were carried out. The treatment means were compared by the Duncan's Multiple Range Test at the 1% probability level. All of the above statistical analyses were performed by the SPSS software, version 14 (SPSS Institute 2004).

Results and Discussion

The effect of pH on *in vitro* propagation of lisianthus

The results of analysis of variance (ANOVA) indicated a significant difference between various pH at 1% probability level for the mean shoot number per explant, but the effect of studied pH levels was not significant on the mean shoot length and mean leaf number per explant. The mean comparison (Figure 1) showed that pH 5.7 produced the highest mean shoot number per explant (2.25 shoots).

Although pH 5.7 produced the highest number of abnormal shoots per explants, some shoots had yellowish leaves, while other shoots were vitrificated along with callus formation in the site of connection with the medium. The pH 5.5 represented the normal shoots, but these shoots had small leaves. Previous researches had indicated that pH 5.7 and 5.8 could be the most suitable pH for lisianthus micropropagation (Semeniuk and Griesbach 1987; Kee and Eun 2000). The pH of a medium may be used as a diagnostic tool for some abnormal growth symptoms, such as necrosis, that are caused by the low pH induced nutrient deficiency (Singha *et al.* 1987). Uptake of components by the explants may be directly influenced by pH of the medium (De Klerk *et al.* 2008). The change in the medium pH may have various effects that may influence the performance and development of the explants (George *et al.* 2008).

The effect of the type of container on *in vitro* propagation of lisianthus

Analysis of variance indicated a significant difference between container types at 1% probability level for the mean shoot number per explant (Figure 2) and mean shoot length (Figure 3). The use of polypropylene container produced the highest shoot number per explant (7.5 shoots). Furthermore, the polypropylene container and large glass bottle (160 × 75 mm) produced the largest shoot (0.57 and 0.47 cm, respectively). Polypropylene container had the unique capacity of continuous gas-exchange between the inner volume of the container and the outside environment. There was a microscopic and continuous supply of fresh air in the container and no accumulation of volatile compounds. Another benefit was the minimal volume of condensation. Due to the special labyrinth-closure construction, it is absolutely impossible for microorganisms to penetrate into polypropylene container. It looks like that the polypropylene container was the most appropriate container for enlarging the shoot and increasing its number. The results of a research on peach-almond hybrid GF 677 had shown that the best conditions for rhizogenesis expressed in the percentage of rooting, stem height, the number of roots and their length could be achieved when growing the micro plants in the square vessels made of polypropylene (Kornova and Popov 2009).

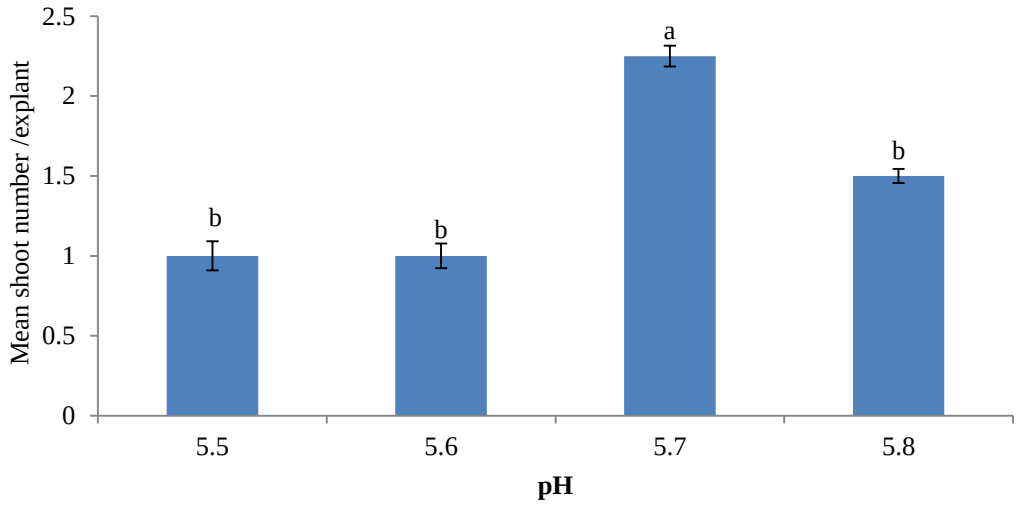

Figure 1. Mean comparison of different pH for the mean shoot number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

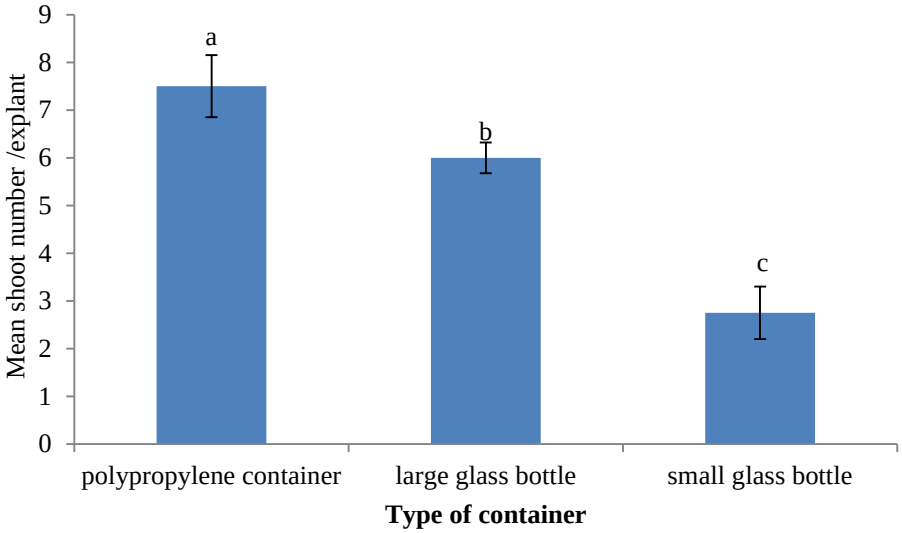

Figure 2. Mean comparison of different containers for the mean shoot number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

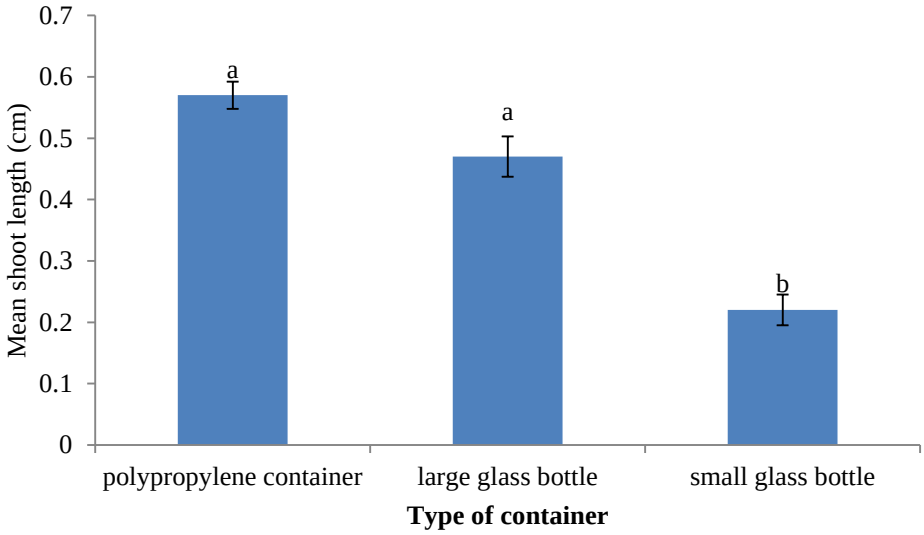

Figure 3. Mean comparison of different containers for the mean shoot length in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

The effect of sucrose concentration on *in vitro* propagation of lisianthus

The analysis of variance of this experiment showed that there was a significant difference between the treatments. The mean comparison (Figures 4-6) indicated that the use of 35 gL^{-1} sucrose produced higher shoot number per explant (2 shoots) and number of leaves per shoot (9.25 leaves). Furtheremore, the use of 30 gL^{-1}sucrose produced the larger shoot (0.52 cm) and the use of 25, 30 and

40 gL^{-1} sucrose produced lower shoot number, mean shoot length (cm) and mean leaf number per explant. The optimum sucrose concentration in micropropagation is species-specific and its importance has been well documented in the different reports (Gabryszewska 1996; Kozai *et al*. 2002; Hazarika *et al*. 2004; Rahman and Alsadon 2007).

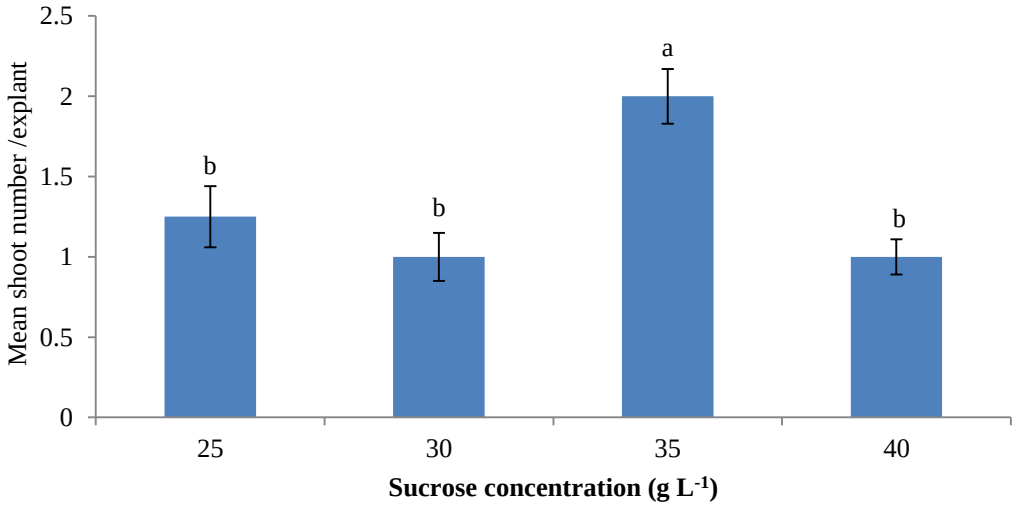

Figure 4. Mean comparison of different sucrose concentrations for the mean shoot number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

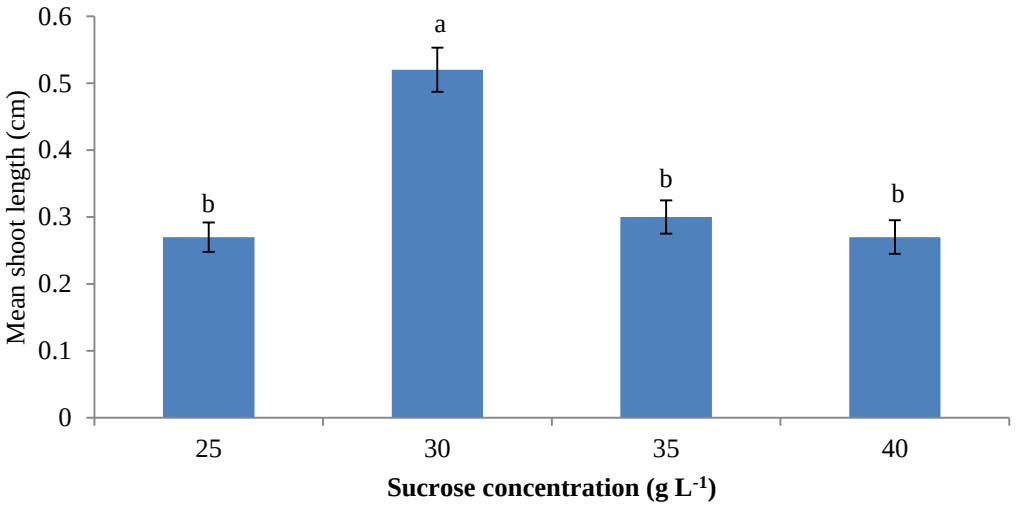

Figure 5. Mean comparison of different sucrose concentrations for the mean shoot length in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

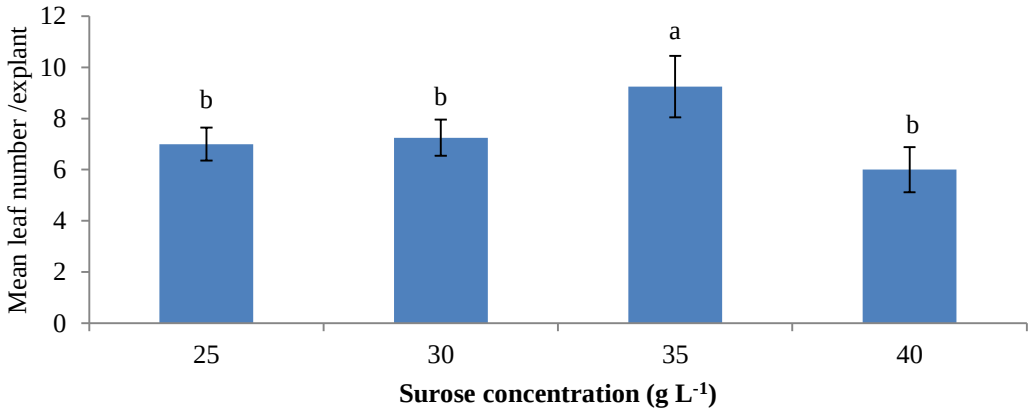

Figure 6. Mean comparison of different sucrose concentrations for the mean leaf number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha=0.01 probability level

The effect of different concentrations of medium macro elements on *in vitro* propagation of lisianthus

The results of ANOVA indicated a significant difference between concentrations of NH_4NO_3 at 1% probability level for the mean shoot number per explant and mean leaf number per shoot. The mean comparison (Figures 7 and 8) revealed that utilization of 1.65 gL^{-1} NH_4NO_3 produced the highest shoot number per explant (2.75 shoots) and the mean leaf number per shoot (12 leaves). The results revealed that the use of 1.85 $gL^{-1}$and 1.45 gL^{-1} NH_4NO_3 produced the lowest shoot number per explant and the mean leaf number per shoot, respectively. Moreover, the results of ANOVA indicated a significant difference between concentrations of KNO_3 at 1% probability level for the mean shoot number per explant and the mean shoot length. The mean comparison (Figure 9 and 10) showed that the use of 1.9 gL^{-1} KNO_3 produced the highest shoot number per explant (4.5 shoots) and the mean shoot length (1.32 cm). The use of 2.1 and 1.75 gL^{-1} KNO_3 produced the lowest shoot number per explant and mean shoot length, respectively.

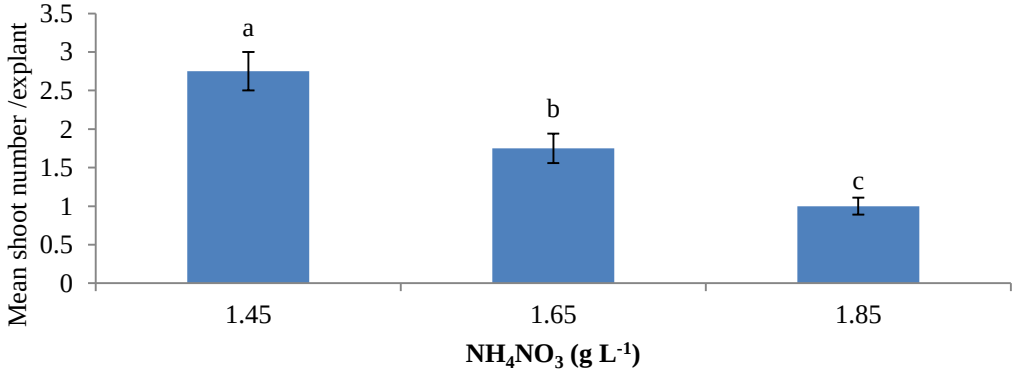

Figure 7. Mean comparison of different NH_4NO_3 concentrations for the mean shoot number per explants in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

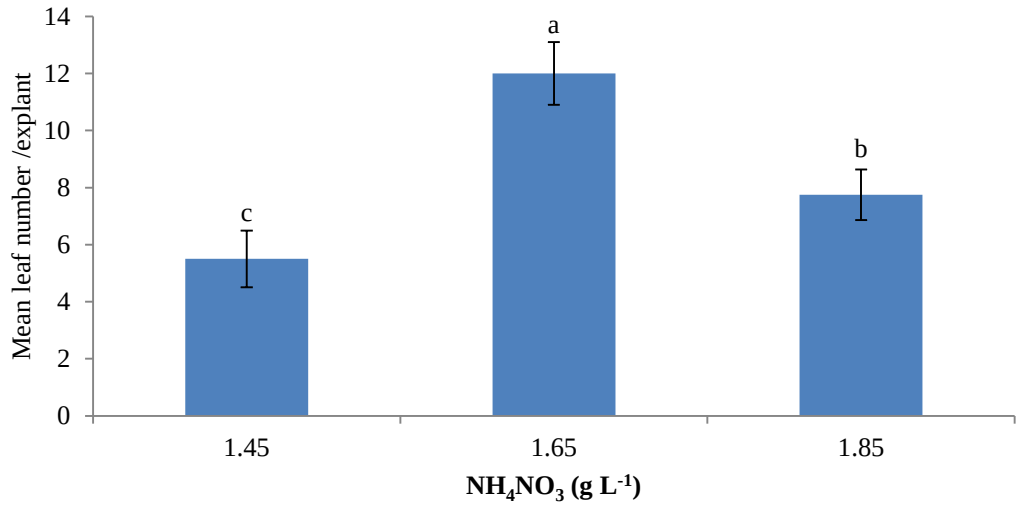

Figure 8. Mean comparison of different NH_4NO_3 concentrations for the mean leaf number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

The effects of concentration and source of nitrogen on important traits of plant tissue culture have already been reported in different plants such as tomato (Gerenda and Sattelmacher 1999), wild cherry (Hajnajari *et al.* 2008) and Prunus (Alanagh *et al.* 2014). The nitrogen supply, as well as other nutrients, usually affects the *in vitro* growth and micropropagation of plants although their effects, depend on the cultivar and the hormonal balance (Danci and Danci 2008). Furthermore, the results of ANOVA indicated a significant difference between concentrations of $CaCl_2.2H_2O$ at 1% probability level for the mean leaf number/shoot. The mean comparison (Figure 11) indicated that the use of 0.66 gL^{-1} $CaCl_2.2H_2O$ produced the highest leaf number per shoot (10 leaves) and 0.44 gL^{-1} $CaCl_2.2H_2O$ produced the lowest leaf number/shoot. There was also significant difference between various concentrations of $MgSO_4.7H_2O$ at 1% probability level for the mean shoot length. The mean comparison (Figure 12) showed that the use of 0.43 gL^{-1} $MgSO_4.7H_2O$ produced the largest shoot (0.5 cm) and 0.37 gL^{-1} $MgSO_4.7H_2O$ produced the shortest shoot. The results of a research on the micropropagation of lisianthus (O'Brien and Lindsay 1993) had shown that when the $MgSO_4$ concentration was reduced to half or increased twice, there was no change in the plant growth and only the leaf area increased when the $MgSO_4$ concentration was doubled.

The results of ANOVA also indicated a significant difference between concentrations of KH_2PO_4 at 5% probability level for the mean shoot number/explant. The mean comparison (Figure 13) revealed that the use of 0.2 gL^{-1} KH_2PO_4 produced the highest shoot (3.5 shoots), while 0.13 gL^{-1} KH_2PO_4 produced the lowest shoot number.

According to the analysis of variance, NAA × activated charcoal interactions were significant for the mean root number / explant. Mean comparison of treatment combinations (Figure 14) indicated that medium containing 0.5 mg L^{-1} NAA without

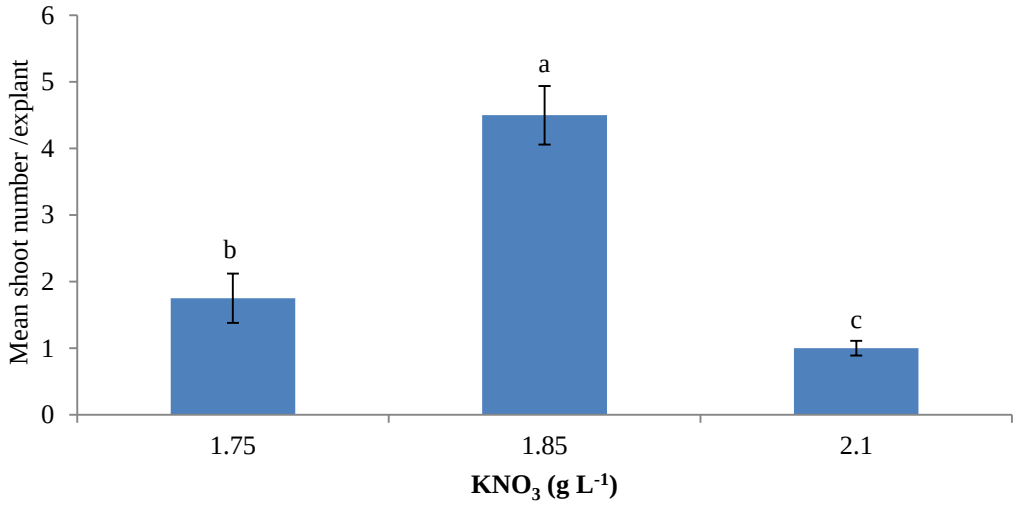

Figure 9. Mean comparison of different KNO_3 concentrations for the mean shoot number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

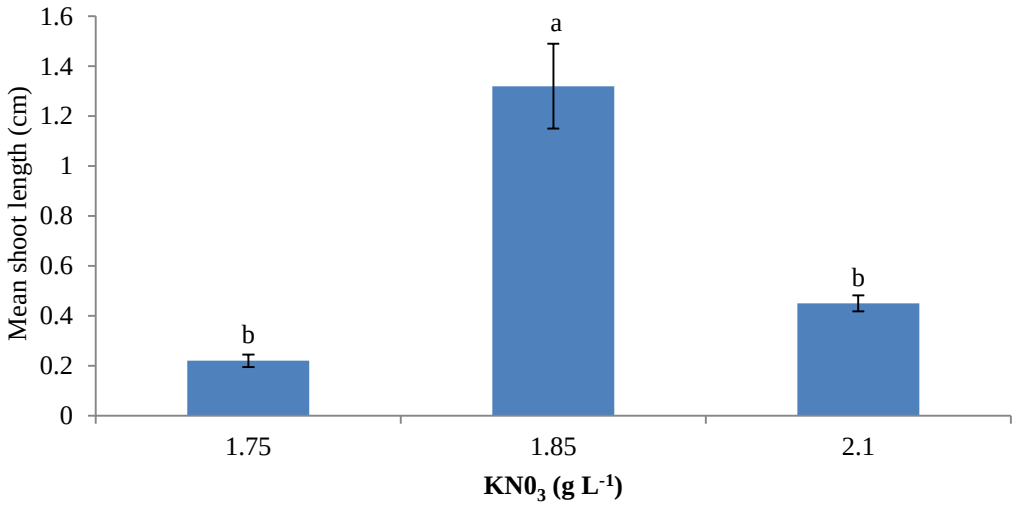

Figure 10. Mean comparison of different KNO_3 concentrations for the mean shoot length in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

activated charcoal had the highest root number.

The above-mentioned experiments were carried out under similar conditions. Therefore, the best means for the studied characteristics were compared in the different experiments. In general, the highest shoots/explant were obtained from a polypropylene container (7.5 shoots), and the highest leaves/shoot were produced by the use of 1.65 mgL^{-1} NH_4NO_3 (12 leaves). Moreover, the largest shoot (1.32 cm) produced when 1.85 gL^{-1} KNO_3 was used. Overall, all new findings obtained in the current study could be utilized in complementary experiments in the future.

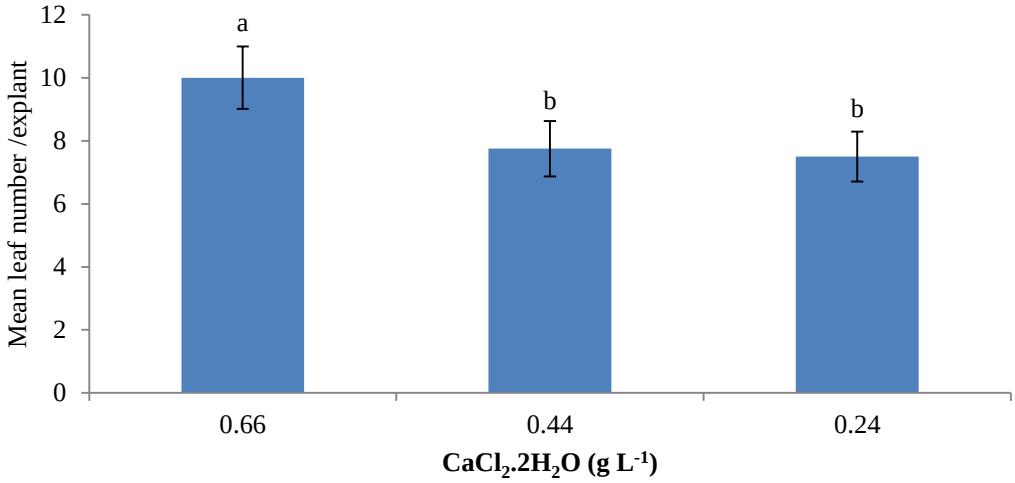

Figure 11. Mean comparison of different $CaCl_2.2H_2O$ concentrations for the mean leaf number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

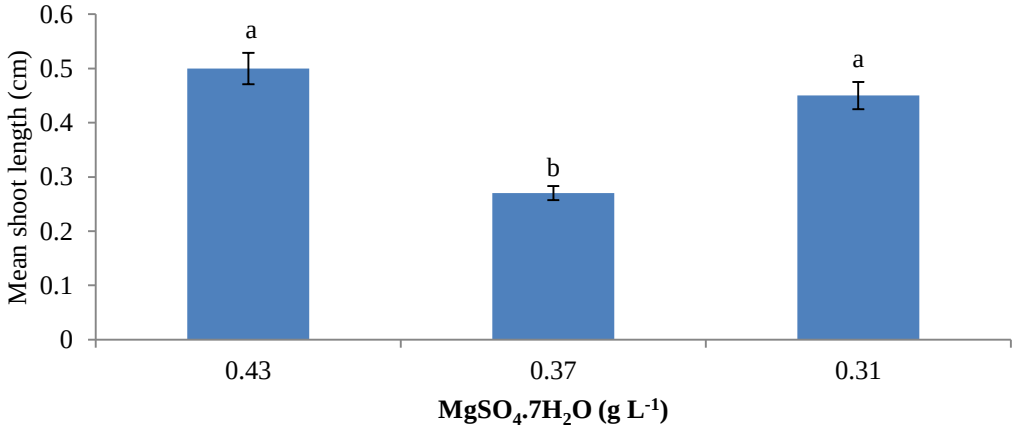

Figure 12. Mean comparison of different $MgSO_4.7H_2O$ concentrations for the mean shoot length in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

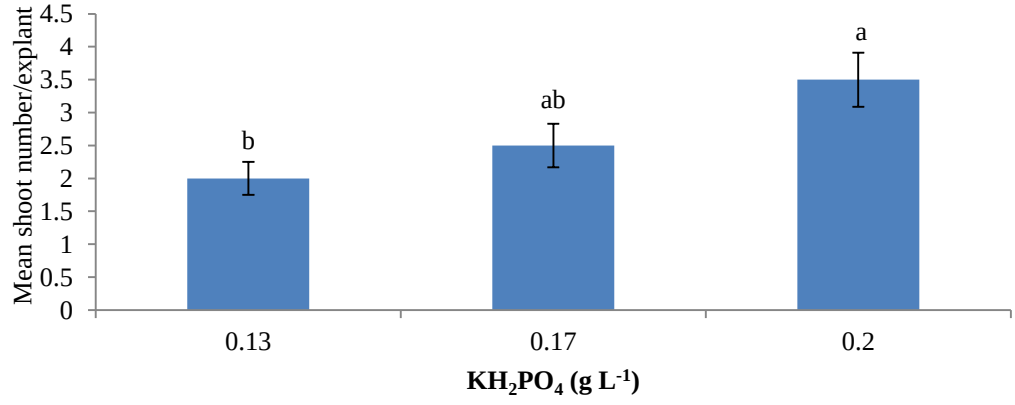

Figure 13. Mean comparison of different KH_2PO_4 concentrations for the mean shoot number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

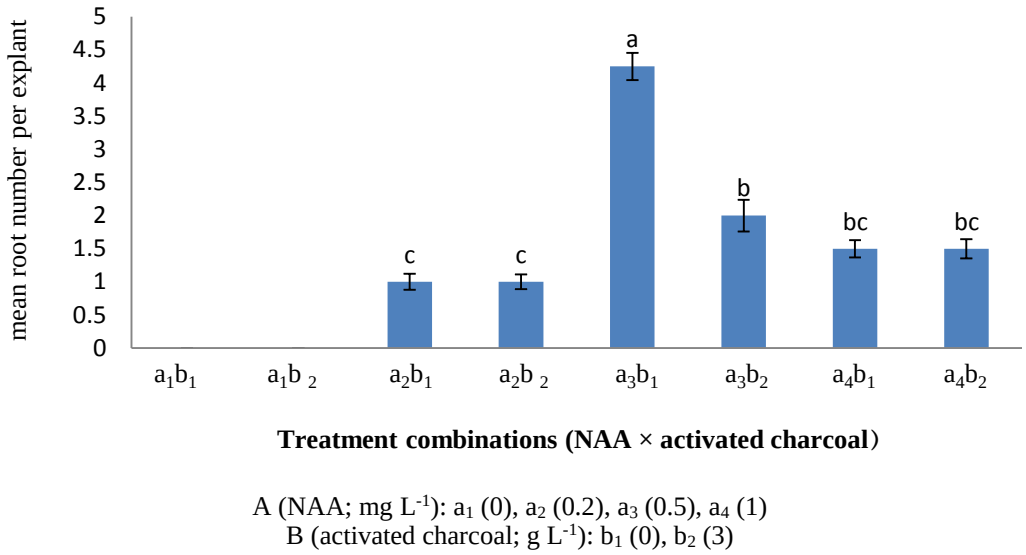

Figure 14. Mean comparison of different combination treatments for the mean root number per explant in lisianthus micropropagation by using Duncan's Multiple Range Test at alpha= 0.01 probability level

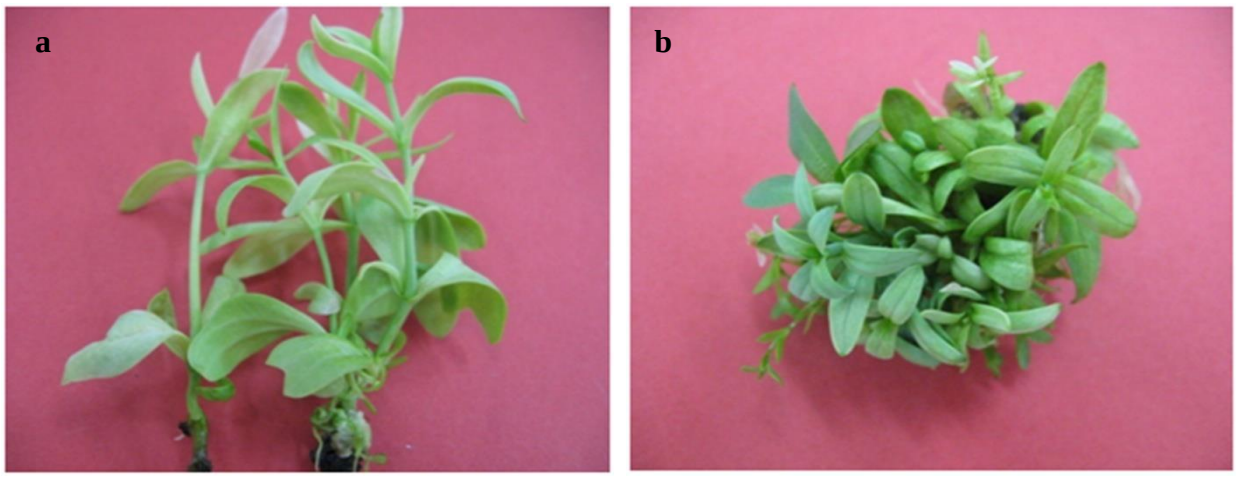

Figure 15. Shoots (a) and shoot cluster (b) obtained from nodal explant culture of lisianthus in a polypropylene container

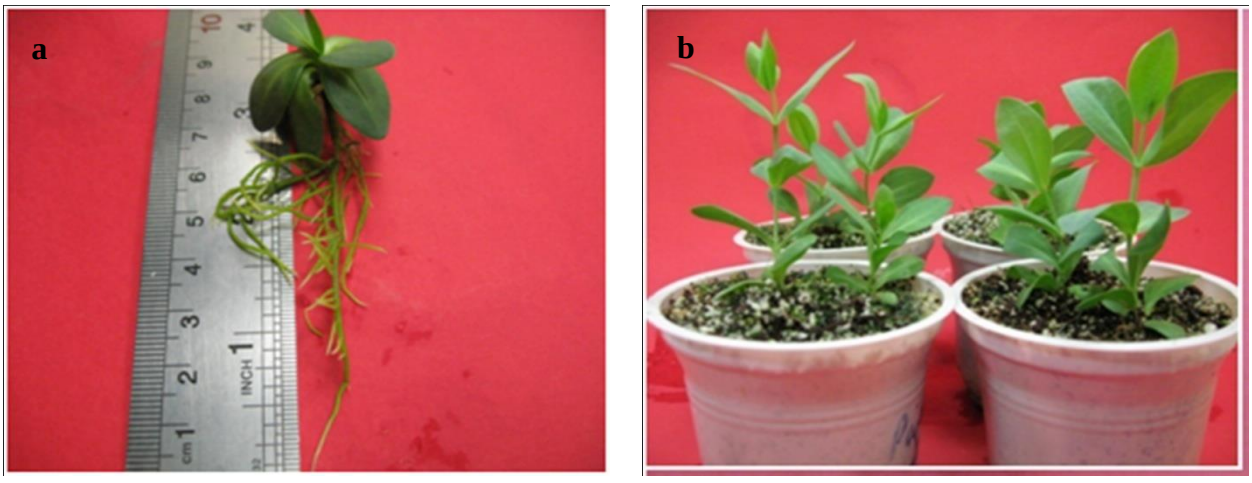

Figure 16. Shoot rooting of lisianthus in the medium containing 0.5 mg L^{-1} NAA and without activated charcoal (a), acclimated *in vitro* plants after 30 days (b)

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