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EFFECT OF PHOTOPERIOD AND GROWTH REGULATORS ON PHYSIOLOGY AND ROOTING OF MICROPROPAGATED *EUSTOMA GRANDIFLORUM* (RAF.) SHINN

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ABSTRACT

Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn] is an important ornamental plant with high potential for Romanian cut flower market. The plant material tested and evaluated in this research included three cultivars of *Eustoma grandiflorum*. The purpose of our study was to evaluate the effect of photoperiod and plant growth regulator concentrations in growing medium on physiology and rooting of micropropagated Lisianthus. The shoot tip explants from *Eustoma grandiflorum* were cultured on half strength macro and micro salts of Murashige and Skoog basal medium supplemented with different concentrations of indole–3 butyric acid (IBA), indolyl-3-acetic acid (IAA), and naphthalene acetic acid (NAA). The plantlets were treated with four concentrations of each growth regulator in 16-hour and 12-hour photoperiod conditions. The main physiological parameters and characteristics of rooting during *in vitro* phase were analyzed in order to assess the proper photoperiod and growing media. The experiment was carried out in a randomized complete block design with three replications per treatment. For each treatment we used five vessels. The statistical analysis was performed using the analysis of variance – Duncan test and t-test for independent samples with SPSS 16.0 software. The percentage of shoots in all cultivars that produced roots increased with higher IBA concentrations, ranging from 0.2 mg l^{-1} to 0.6 mg l^{-1} for both levels of photoperiod. The longest roots formed on the *in vitro* plantlets in the rooting phase were recorded on the growing media supplemented with 0.3 mg l^{-1} NAA. The increase of NAA concentration from 0.1 mg l^{-1} to 0.3 mg l^{-1} positively influenced the growth of the roots for all the varieties in both types of photoperiods. Our outcomes emphasized that there were no statistical differences in photosynthetic pigments between the plantlets treated with 12-hour or 16-hour photoperiod ($p>0.05$). According to these results, the testing of different photoperiods led to the conclusion that the operation of the growth chamber in 12-hour of light had stronger effects in specific conditions, also allowing a 4-hour light energy saving, which reduces energy costs by about 25% in the *in vitro* rooting phase. The results indicated an optimization of lisianthus *in vitro* micropropagation and demonstrated the efficiency of 12-hour photoperiod. Therefore, the present study introduces a useful and an effective protocol for in vitro propagation of lisianthus.

Keywords: growing media, growth regulators, *in vitro* culture, physiology and photoperiod, Lisianthus [*Eustoma grandiflorum* (Raf.) Shinn].

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INTRODUCTION

Biotechnologies, indifferent of their nature, industrial, medical, pharmaceutical, food or agricultural, is a leading area of research. Thus, the biotech industry is a rapidly growing segment that has its application in various sectors of activity. In this respect, it is necessary to improve and continuously develop plant biotechnology techniques through research and development studies.

Eustoma grandiflorum, a member of the Gentianaceae family and originating in the Southern United States and Northern Mexico, is an ornamental plant that has recently entered on the floral market in Romania, being highly appreciated by consumers for its outstanding aesthetic qualities. Although it is relatively new to the world floral market, this species is among the most popular cut flowers in the world (Azadi *et al*., 2016), having its place in the top 10. Cut flowers constitute the main segment in the global ornamental plant market and tissue culture techniques is more applied for rapid micropropagation of ornamental plants (Yumbla-Orbes *et al*., 2017).

In recent years, in Romania, *E. grandiflorum*, commonly known as lisianthus or prairie gentian, has become a popular flower. The demand for this species in Romania is increasing, necessitating the development of improved plant propagation through *in vitro* cultures of Lisianthus varieties. Conventional propagation technologies for lisianthus are represented by vegetative propagation through cuttings and sexually by seeds (Rezaee *et al*., 2012; Abou-Dahab *et al*., 2019). However, the vegetative methods are not very suitable for commercial production. Nowadays, *in vitro* propagation is a very useful tool for mass propagation, much more efficient, being applied for a many species (Kumari *et al*., 2018; Faisal *et al*., 2018; Ilyas *et al*., 2019).

Micropropagation of *E. grandiflorum* by tissue culture technique is relatively low (Abou-Dahab *et al*., 2019). Moreever, few studies have addressed to *in vitro* multiplication of this species (Paek and Hahn, 2000; Kaviani, 2014; Winarto *et al*., 2015) and very few studies were accomplished to evaluate the effects of growing media on plant physiology (Kaviani, 2014). Yumbla-Orbes *et al.* (2017) demonstrated rapid lisianthus micropropagation using root segments as explant sources. Islam *et al.* (2005) examined the effects of different photoperiod on floral initiation, development and growth of *E. grandiflorum*. The authors reported that Eustoma is a quantitative long-day plant. Wang *et al.* (2014) analyzed the effects of different photoperiods on the growth and flowering of lisianthus and suggested that the day-length of 14-hour is recommended in order to promote the flowering of lisianthus.

Plant growth regulators (PGRs) play an essential role in the indirect regeneration in the procedure for plant propagation, such as shoots development, stimulation and callus formation, and root induction (Singh *et al.*, 2016; Hesami *et al.,* 2018). Singh *et al.* (2016) reported that the highest rooting percentage in *Santalum album* L. and survival was achieved on growing media with 1.5 mg 1^{-1} indole-3-butyric acid. Hesami *et al.* (2018) mentioned that the effect of plant growth regulators on root induction was maximum in MS medium consisting of 2.0 mg l^{-1} indole butyric acid in combination with 0.1 mg l^{-1} naphthalene acetic acid. Venkatachalam *et al.* (2015) found that plant growth regulators influenced shoot bud induction and the percent of plantlets rooted *in vitro* of *Bambusa arundinacea*.

Rooting is an important step of a successful micropropagation. Auxins promote cell division and root induction in *in vitro* plant propagation. The most of plant growth regulators that have been used in rooting tissue culture for micropropagation were naphthalene acetic acid (NAA), indolyl-3-acetic acid (IAA) and indole butyric acid (IBA) in different concentrations on growing media based mainly on Murashige and Skoog (1962) modified basal medium. Patel *et al.* (2014) found that half strength of MS salts in medium is appropriate for *in vitro* rooting. Raigond *et al*. (2019) reported that standard photoperiod used in tissue culture is 16-hour light and 8-hour dark. The photoperiod level used for micropropagation of Eustoma is 16 hours of light and 8 hours of darkness and it is generalized worldwide, while some authors use an even longer period of 16-hour of photoperiod (Paek and Hahn, 2000; Rezaee *et al*. 2012; Kaviani, 2014). The general hypothesis of the present study is that plant growth regulators and 12-hour photoperiod improve the *in vitro* propagation of *E. grandiflorum.* The novelty of our study is the development of a useful and effective protocol for in vitro propagation of *E. grandiflorum* represented by the reduction of photoperiodism. Previous studies seem did not report any concerns about reducing the photoperiod at 12-hour. Thus, the purpose of this study was to evaluate the possible beneficial effects of this photoperiod and that of specific plant growth regulator concentrations in the growing medium on the physiology and the rooting of micropropagated lisianthus.

MATERIALS AND METHODS

Plant material and growth conditions: The study was conducted at the laboratories and greenhouse of the University of Pitesti, Romania. The plant material tested and evaluated in this research included three cultivars of *Eustoma grandiflorum* (Raf.) Shinn.: Echo White with white, double flowers, Magic Blue with double flowers, blue-purple, and Asenka with simple, blue-purple flowers. Explant of the Asenka, Magic Blue and Echo White cultivars used for *in vitro* culture initiation were taken from shoot tips harvested in the vegetative growth phase. The packaging of the biological material was made in polyethylene bags immediately after harvesting and they were stored in the refrigerator at 4°C until the explants were taken to initiate the cultures. The biological material used for the *in vitro* rooting was represented by microshoots removed from the shoot culture. For root induction under in vitro conditions, multiplied shoots were transferred on half strength macro and micro salts of MS (Murashige and Skoog, 1962) supplemented with different PGRs concentrations. Rooting of micro-shoots was accomplished in growth chambers at 25±2˚C with 16-hour photoperiod and 12-hour photoperiod at a light intensity of 33.78 PPDF (µmol m^{-2} s^{-1}). The light intensity was provided by white fluorescent lamps. Sterilization was accomplished under laminar air flow. Micro-shoots were rooted on half strength macro and micro salts of MS basal medium supplemented with vitamins LS (Linsmaier and Skoog, 1965), 0.3 g l^{-1} active charcoal, 38 mg l^{-1} sodium iron ethylenediaminetetra acetate (NaFeEDTA), 0.1 mg l^{-1} 6-benzylaminopurine (BAP), 30 g l^{-1} glucose, 7 g l^{-1} agar – agar and different concentrations of PGRs according to the data presented in Table 1.

In our experiment we used three auxins as PGRs, with the following concentrations:

- 2 mg l^{-1}, 0.4 mg l^{-1}, 0.6 mg l^{-1}, and 0.8 mg l^{-1} indole 3– butyric (IBA);
- 0.5 mg l^{-1}, 1.0 mg l^{-1}, 1.5 mg l^{-1}, and 2.0 mg l^{-1} indolyl–3–acetic acid (IAA);
- 0.1 mg l^{-1}, 0.2 mg l^{-1}, 0.3 mg l^{-1}, and 0.4 mg l^{-1} naphthalene acetic acid (NAA).

In the present study, the evaluation of physiological parameters was performed on the plantlets with highest rooting capacity on the growing media supplemented with 0.6 mg l^{-1} indole – 3 butyric acid.

Prior to distribution to culture vessels, the pH of the growing media was adjusted to 5.7 and solidified with 7 g l^{-1} agar-agar. The growing media distributed in the culture vessels were sterilized by autoclaving at 121°C, 1 atmospheric pressure for 20 minutes.

Data and Measurements: The effects of photoperiod and different growth regulators on physiology and rooting of micropropagated *E. grandiflorum* were evaluated using the following measurements: rooting formation (%), number of roots per micro-shoots, length of roots (cm), photosynthesis rate [µ mol (CO_2) m^{-2} s^{-1}], respiration rate [µ mol (CO_2) m^{-2} s^{-1}], dry weight content (%), total water content (%), content of chlorophyll a (mg g^{-1} fresh weight), chlorophyll b (mg g^{-1} fresh weight), and carotenoids content (mg g^{-1} fresh weight).

Leaf gas exchange parameters were measured with a portable plant CO_2 analysis package. Photosynthesis and respiration rate was determined with a S151 Infrared CO_2 portable plant analyzer (IRGA from Qubit Biology Inc., Ontario, Canada). During testing, the photosynthetic photon flux density was 2500 µmol m^{-2} s^{-1}, and the air temperature was 25±2°C. Photosynthetic pigments were determined using a spectrophotometer (BOECO S-20VIS from Boeckel & Co, Hamburg, Germany). The amounts of chlorophyll a, chlorophyll b and carotenoid pigments were calculated according to the Holm's formulas (1954):

- Chlorophyll a = $9.78\ A_{662} - 0.99\ A_{644}$;
- Chlorophyll b = $21.4\ A_{644} - 4.65\ A_{662}$;
- Carotenoids = $4.69\ A_{440.5} - 0.267$ (Chl a + Chl b).

Data analysis: A multifactorial experiment with three factors was designed: genotype, plant growth regulator, and photoperiod. The experiment was carried out in a randomized complete block design with three replications per treatment. Each treatment consisted of five vessels. The statistical analysis was performed using the analysis of variance in the SPSS 16.0 software (IBM Corporation, Armonk, New York, USA) and means of each parameter were compared using Duncan's multiple range test (DMRT) and t-test, at $p<0.05$ level of significance. All data in the study are expressed as mean ± standard error (SE).

RESULTS AND DISCUSSION

The results of the root formation under the effect of photoperiod and growing media supplemented with different concentrations of indole-3-butyric acid growth regulator are presented in Figure 1. The results showed that Echo White cultivar cultured in 16-hour photoperiod had the highest rooting percentage on the growing media supplemented with 0.6 mg l^{-1} indole – 3 butyric acid (A3), while the lowest rooting percentage was achieved on the growing media supplemented with 0.2 mg l^{-1} indole – 3 butyric acid (A1). However, the Echo White recorded higher rooting percentages in the *in vitro* culture condition of 12-hour photoperiod than under a 16-hour photoperiod. The percentage of shoots in all cultivars that produced roots increased with higher IBA concentrations from 0.2 mg l^{-1} to 0.6 mg l^{-1} for both levels of photoperiod: 16-hour and 12-hour. Paek and Hahn (2000) reported that the increase of indole – 3 butyric acid concentrations in rooting medium favored root formation. In the case of growing media supplemented with 0.2 mg l^{-1} indole – 3 butyric acid, we did not find any statistically significant difference between plantlets cultured in conditions of 16-hour or 12-hour photoperiod. Plantlets of Echo White and Asenka treated with 16-hour photoperiod and 12-hour photoperiod were statistically different in the case of growing media supplemented with 0.6 mg l^{-1} IBA. *In vitro* culture of lisianthus is a modern technique for rapid propagation (Nhut *et al.,* 2006; Wang *et al*., 2009, Zhou *et al*., 2014, Winarto *et al*., 2015, Ruffoni and Bassolino, 2016). The cultures from these studies were placed in a growth chamber with a 16-hour photoperiod. Winarto *et al.* (2015) reported that micro - shoots rooted easily on MS medium containing 0.1 mg l^{-1} BA and 0.02 mg l^{-1} NAA with 3.9 roots per shoot. The results of the present study are in agreement with other reports that mentioned the efficacy of IBA over other auxins for root induction. Hesami *et al.* (2018) reported that MS medium consisting of 2.0 mg l^{-1} IBA in combination with 0.1 mg l^{-1} NAA had a maximum positive effect for root induction (96.66 %) and number of roots per shoot (5.50) as well as root length (4.83 cm). In other study, the maximum percent of rooted plantlets (85%) was noticed on MS medium with 3.0 mg l^{-1} IBA (Venkatachalam *et al.,* 2015). Faisal *et al.* (2018) reported that the highest rhizogenesis was achieved on half-strength MS medium having 0.5 mg l^{-1} IBA with 16/8 h light/dark cycle.

The root formation in the growing media supplemented with indolyl-3-acetic acid from 0.5 mg l^{-1} to 2.0 mg l^{-1} was analyzed in Figure 2. Root formation on shoots cultured on a rooting medium containing 1.5 mg l^{-1} IAA was higher for all the studied cultivars than in other treatments. In the case of Echo White and Asenka, there were no statistical differences between photoperiods for plantlets treated with growing media supplemented with 1.5 mg l^{-1} indolyl-3- acetic acid (B3) and 1.5 mg l^{-1} indolyl-3- acetic acid (B4). Increasing IAA concentration from 0.5 mg l^{-1} to 1.5 mg l^{-1} in all cultivars for both level of photoperiod had a positive effect in promoting rooting. The 12-hour photoperiod induced better rooting capacity in all cultivars on all growing medium tested. The Magic Blue registered statistically significant differences between the values obtained under the influence of the 16-hour and 12-hour photoperiods, both on the B1 and

B3 culture media. Dong *et al.* (2002) found that MS medium supplemented with 0.1 mg l^{-1} IAA was more suitable for plantlet rooting. Kaviani (2014) noted that *E. grandiflorum,* when cultured on a half-strength MS medium supplemented with 2 mg l^{-1} IAA with a photoperiod of 16-hour per day, promoted root formation. The best induction for rooted shoots of *Ficus religiosa* was found in MS medium containing 2 mg l^{-1} IBA and 0.5 mg l^{-1} IAA (Siwach and Gill, 2011).

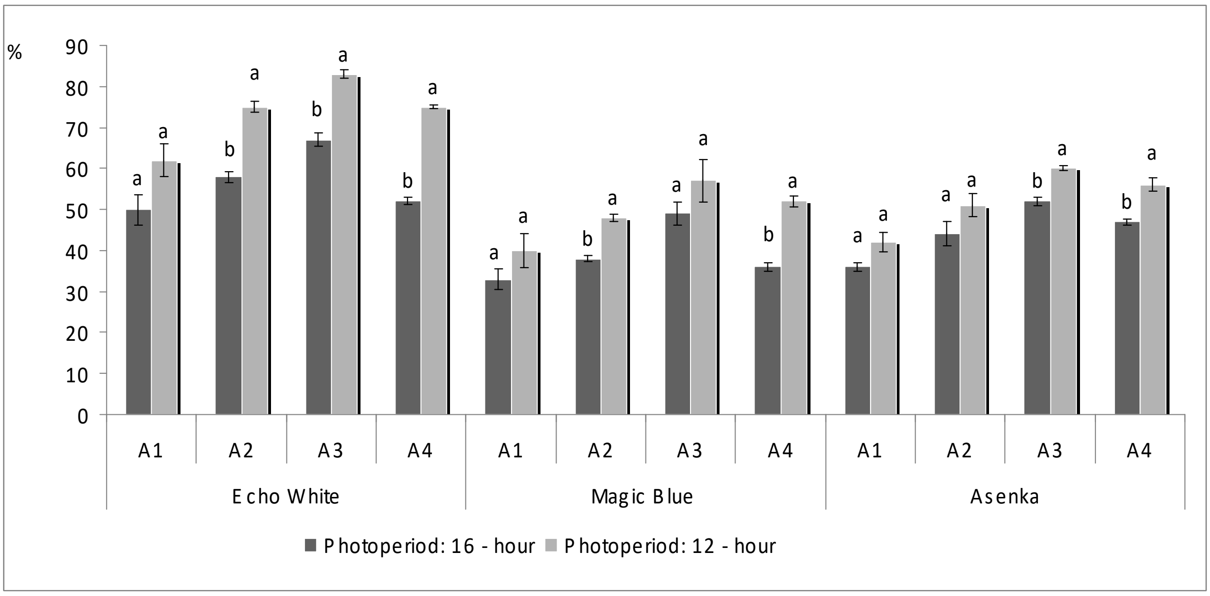

Fig. 1. Effect of photoperiod and growing media containing IBA on root formation (%) (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within growing media are not significantly different according to t-test ($p > 0.05$). A1: growing media supplemented with 0.2 mg l^{-1} indole – 3 butyric acid; A2: growing media supplemented with 0.4 mg l^{-1} indole – 3 butyric acid; A3: growing media supplemented with 0.6 mg l^{-1} indole – 3 butyric acid; A4: growing media supplemented with 0.8 mg l^{-1} indole – 3 butyric acid)**.**

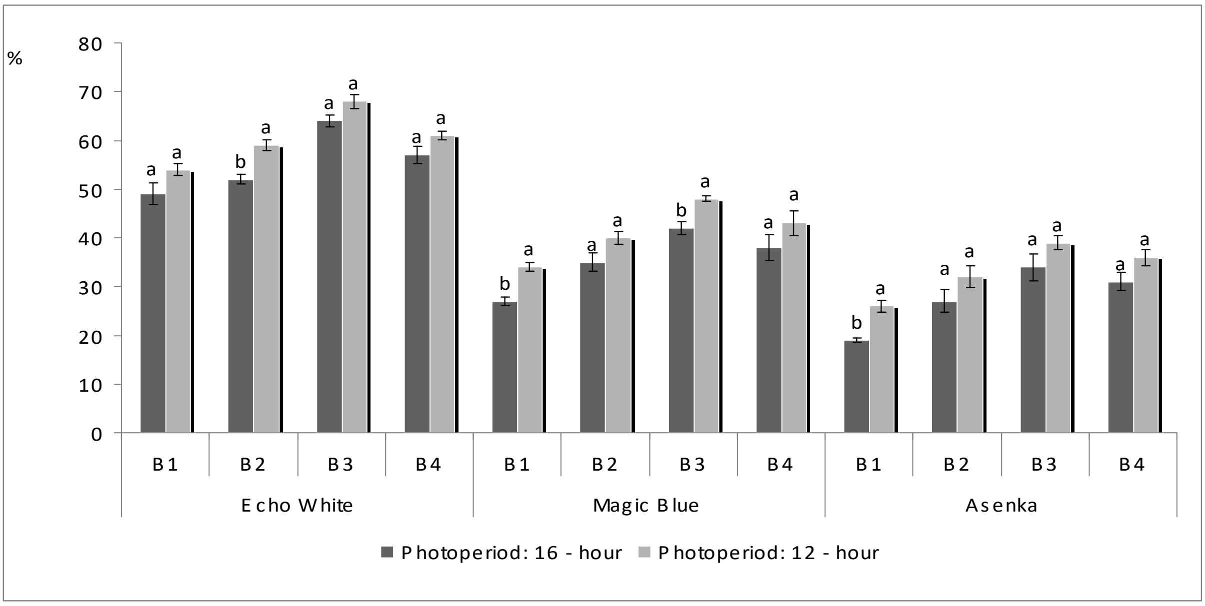

Fig. 2. Effect of photoperiod and growing media containing IAA on rooting percentage (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within growing media are not significantly different according to t-test ($p > 0.05$). B1: growing media supplemented with 0.5 mg l^{-1} indolyl-3- acetic acid; B2: growing media supplemented with 1.0 mg l^{-1} indolyl-3- acetic acid; B3: growing media supplemented with 1.5 mg l^{-1} indolyl-3- acetic acid; B4: growing media supplemented with 2.0 mg l^{-1} indolyl-3- acetic acid (IAA))**.**

All three studied cultivars had a better rooting capacity under the influence of the 12-hour photoperiod than under 16-hour photoperiod for growing media supplemented with naphthalene acetic acid (Figure 3). Increasing NAA concentration did not favor root formation from the shoot tip culture. These results regarding the effect of NAA concentration for root formation are in agreement with data reported by Paek and Hahn (2000). Root formation on shoots cultured on medium containing 0.4 mg l^{-1} naphthalene acetic acid was the lowest. The largest number of micro-shoots with roots was obtained on medium containing 0.1 mg l^{-1} naphthalene acetic acid. Asenka showed no significant difference between treatments with 12-hour and 16-hour photoperiod on rooting media C2, C3 and C4. Rooting capacity was stimulated by photoperiod conditions. Our results are in accordance to the earlier reported results. Ordogh *et al*. (2006) reported that the highest rooted plantlets were found on growing medium supplemented with 1.0 mg l^{-1} NAA. Tao (2006) demonstrated that half strength MS medium supplemented with 0.5 mg l^{-1} IAA, or 0.5 mg l^{-1} IBA or 0.3 mg l^{-1} NAA was suitable media for promoting root formation. Our result emphasized that half strength of MS salts in medium is appropriate for *in vitro* rooting. Half strength MS medium containing 1.5 mg l^{-1} indole-3-butyric acid was optimal for root induction under *in vitro* conditions (Patel *et al.,* 2014).

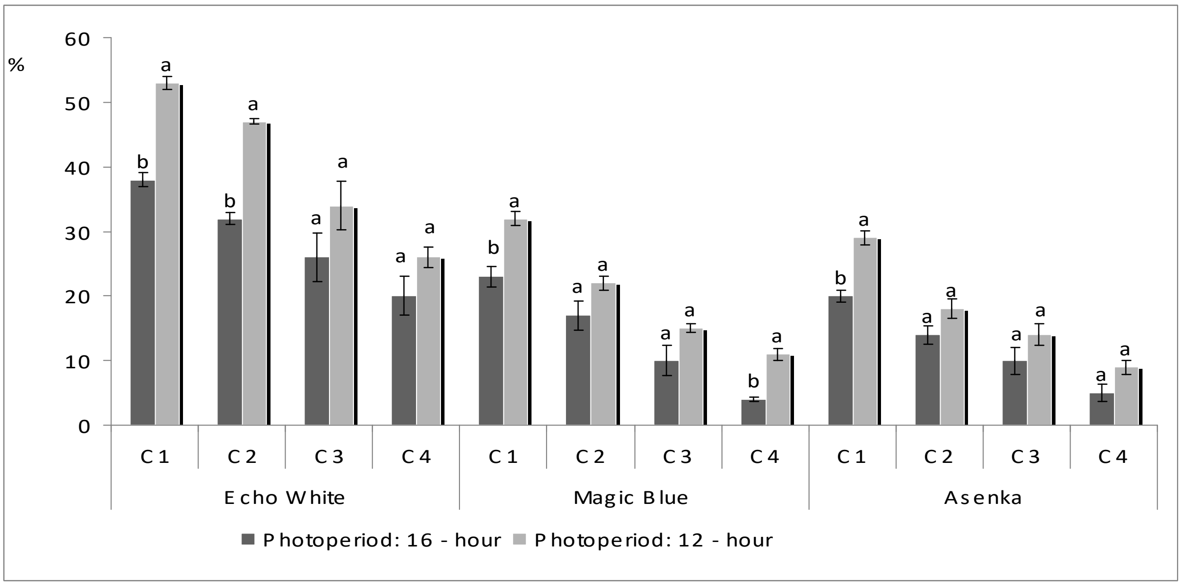

Fig. 3. Effect of photoperiod and growing media containing NAA on rooting percentage (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within growing media are not significantly different according to t-test ($p > 0.05$). C1: growing media supplemented with 0.1 mg l^{-1} naphthalene acetic acid; C2: growing media supplemented with 0.2 mg l^{-1} naphthalene acetic acid; C3: growing media supplemented with 0.3 mg l^{-1} naphthalene acetic acid; C4: growing media supplemented with 0.4 mg l^{-1} naphthalene acetic acid (NAA)).

The effect of auxins on root formation for studied cultivars is presented in table 2. Rooting capacity was more pronounced in cultivars grown in medium supplemented with 0.6 mg l^{-1} indole – 3 butyric acid than in medium with other concentration of indole – 3 butyric acid. When testing cultivars, root formation response was strongly influenced by concentration and type of auxins. No statistical differences in roots formation on micro-shoots for Magic Blue were observed between the vitroplants grown in media with 0.2 mg l^{-1}, 0.4 mg l^{-1} and 0.6 mg l^{-1} IBA treated with 16-hour photoperiod and between the plantles treated with 0.6 mg l^{-1} IBA and 0.8 mg l^{-1} IBA under the influence of 12-hour photoperiod. The same cultivar showed significant differences between values obtained on growing media supplemented with different concentration of NAA in 16 – hour photoperiod. Most of the values obtained under the influence of auxins differ statistically. Among the auxins, indole – 3 butyric acid showed the highest activity for the induction of root formation (Table 2). These results confirms the important role of auxin type in the promotion of root formation.

Root formation response on the growing medium supplemented 0.2 mg l^{-1} IBA was strongly influenced by genotype with a range of variation from 33% in Magic Blue to 50% in Echo White under 16-hour photoperiod or from 42% to 62% in the case of plantles treated with 12-hour photoperiod (Table 3). On medium supplemented with different concentration of IAA genotypes treated with 16-hour photoperiod or 16-hour photoperiod showed significant differences. No statistical differences in roots formation in Magic Blue and in Asenka were observed plantlets grown in media with different concentration of NAA for both photoperiod, except auxin 0.2 mg l-1 NAA under the conditions of a 12-hour photoperiod.

Regarding the average length of roots formed on the *in vitro* plantlets in the rooting phase, the highest values were recorded on the growing media supplemented with 0.3 mg l^{-1} NAA, under a photoperiod 16-hour (Table 4). The values obtained under a 12-hour photoperiod are smaller, but there are no significant differences. The increase of NAA concentration from 0.1 mg l^{-1} to 0.3 mg l^{-1} positively influenced the growth of the roots for all the varieties in both types of photoperiods. In most cases, there were no significant differences between the roots length values obtained under the effect of a 16-hour and 12-hour photoperiods. Winarto *et al.* (2015) noted that the biggest root length was recorded on growing media supplemented with 0.1 mg l^{-1} BA+0.1 mg l^{-1} NAA. The rooting media containing MS medium supplemented with 2 mg l^{-1} IAA, 2 mg l^{-1} NAA, or 2 mg l^{-1} IBA under light conditions with 16-hour photoperiod was applied for micropropagation and there was no significant difference on number of roots per plantlet for the tested varieties (Shintiavira *et al.*, 2015). The growing media containing 2 mg l^{-1} IAA was the appropriate medium for inducing root formation (1.9 roots per plantlet and 1.85 cm root length).

The average number of roots formed on micro-shoots according to variety, photoperiodism, and growing media supplemented with 0.2 mg l^{-1} indole – 3 butyric acid, varied from 3 to 5 (Figure 4). The number of roots grown on the plantlets is higher in conditions of 16-hour photoperiod, but the differences are not significant, except for Echo White cultured on media supplemented with 0.2 mg l^{-1} IBA. The highest number of roots was observed in the Echo White and Magic Blue varieties on the A3 culture medium and the lowest number of roots was found for Magic Blue variety on growing media supplemented with 0.2 mg l^{-1} IBA.

The increase of the auxin concentration has positively influenced the number of roots, up to a 0.6 mg l^{-1} threshold, higher concentration reducing the number of roots formed on micro-shoots. Winarto *et al.* (2015) reported that the number of roots produced per shoot was higher in growing media supplemented with 0.1 mg l^{-1} BA+0.01 mg l^{-1} NAA.

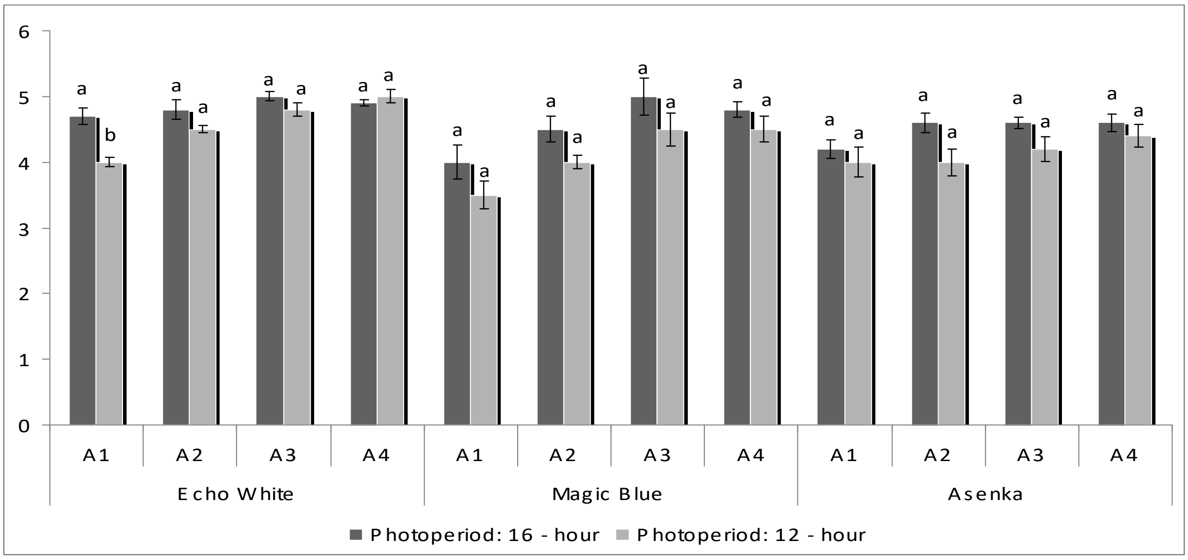

Fig. 4. Effect of photoperiod and growing media containing IBA on number of roots per micro-shoot (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within growing media are not significantly different according to t-test (p >0.05). A1: growing media supplemented with 0.2 mg l^{-1} indole – 3 butyric acid; A2: growing media supplemented with 0.4 mg l^{-1} indole – 3 butyric acid; A3: growing media supplemented with 0.6 mg l^{-1} indole – 3 butyric acid; A4: growing media supplemented with 0.8 mg l^{-1} indole – 3 butyric acid).

Regarding the number of roots formed on plantlets depending on photoperiod level and growing media supplemented with indolyl-3- acetic acid in concentration from 0.5 mg l^{-1} to 2.0 mg, we found that the Echo White recorded the highest values on the culture media with 1.5 mg l^{-1} indolyl-3- acetic acid concentration (Figure 5). No statistical differences in the number of roots formed on micro-shoots for Echo White and Asenka were observed between the *in vitro* plantlets treated with 16-hour photoperiod and 12-hour photoperiod on growing medium supplemented with IAA. Magic Blue showed significant differences between photoperiod treatments. Raigond *et al.* (2019) noted that root length, number of roots and the number of leaves of potato were higher in short photoperiod (12-hour) than in standard photoperiod (16-hour).

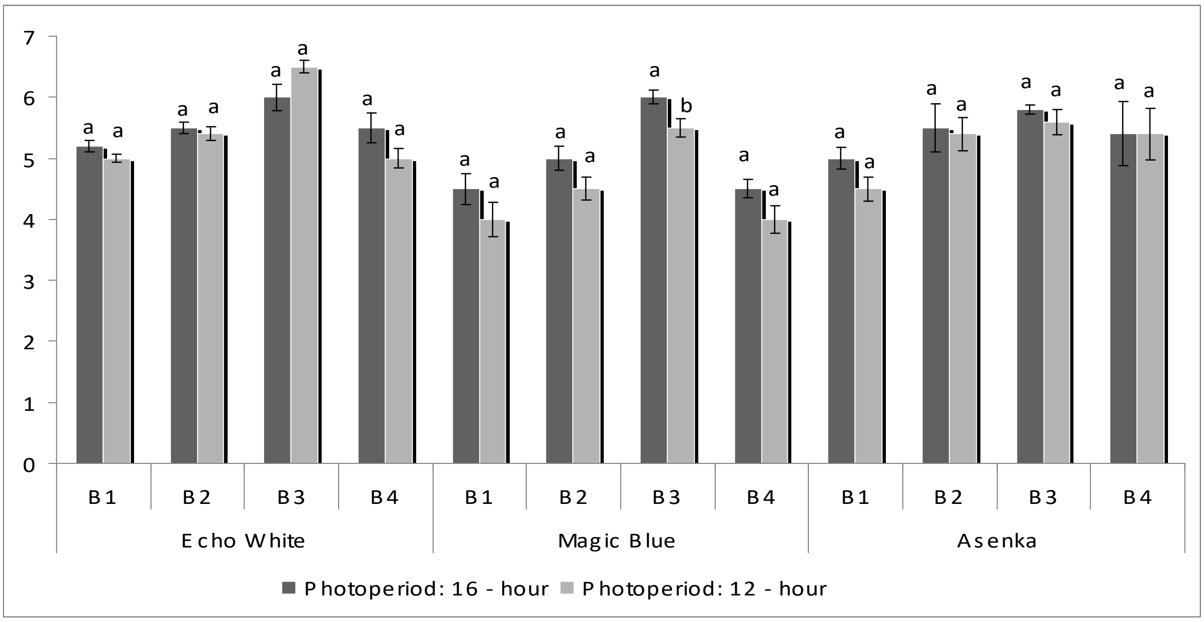

Fig. 5. Effect of photoperiod and growing media containing IAA on the number of roots per micro-shoot (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within growing media are not significantly different according to t-test (p >0.05). B1: growing media supplemented with 0.5 mg l^{-1} indolyl-3- acetic acid; B2: growing media supplemented with 1.0 mg l^{-1} indolyl-3- acetic acid; B3: growing media supplemented with 1.5 mg l^{-1} indolyl-3- acetic acid; B4: growing media supplemented with 2.0 mg l^{-1} indolyl-3- acetic acid (IAA)).

The effects of photoperiod and growing media containing NAA on number of roots per micro-shoot is presented in Figure 6. In the case of growing media based on naphthalene acetic acid it was found that the increase in auxin concentrations induced the increase in number of roots. The Echo White developed the most roots on growing media supplemented with 0.4 mg l^{-1} NAA.

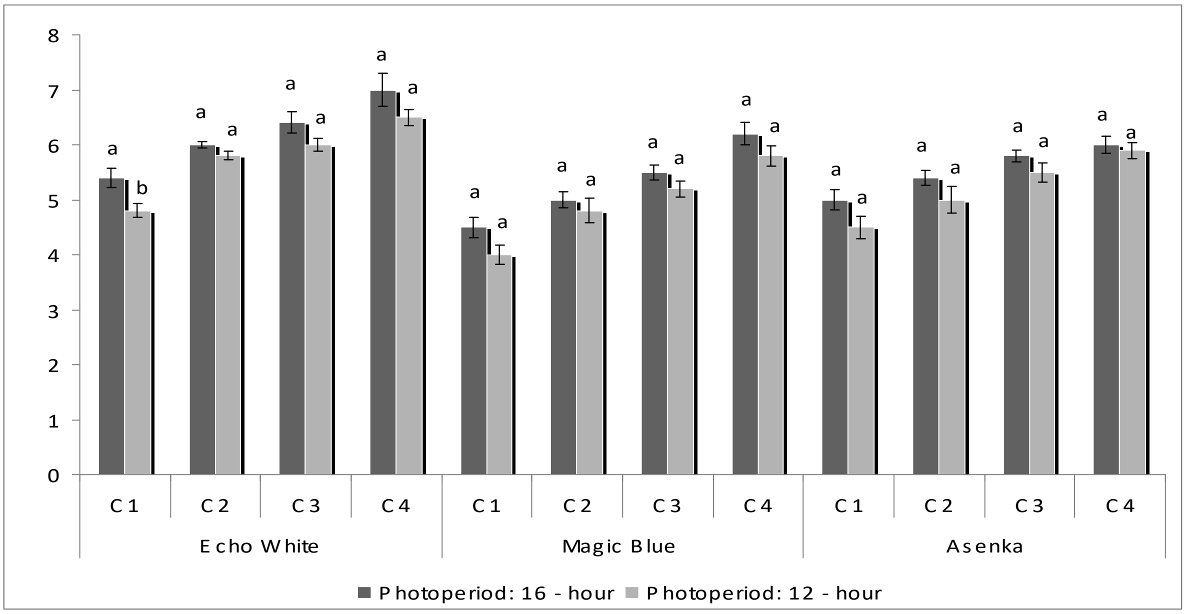

Fig. 6. Effect of photoperiod and growing media containing NAA on number of roots per micro-shoot (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within growing media are not significantly different according to t-test (p > 0.05). C1: growing media supplemented with 0.1 mg l^{-1} naphthalene acetic acid; C2: growing media supplemented with 0.2 mg l^{-1} naphthalene acetic acid; C3: growing media supplemented with 0.3 mg l^{-1} naphthalene acetic acid; C4: growing media supplemented with 0.4 mg l^{-1} naphthalene acetic acid (NAA)).

The number of roots formed on the micro-shoots was higher in the condition of 16-hour photoperiod, but the differences were not significant, except for the Echo White cultured on media supplemented with 0.1 mg l^{-1} NAA. According to Ordogh *et al*. (2006), 2 and 3 mg l^{-1} NAA concentrations reduced the number of roots at all of the cultivars. The highest root number per micro-shoot was found in the rooting medium supplemented with 0.5 mg l^{-1} NAA (Esizad *et al*., 2012). Kaviani (2014) reported that the highest root number was observed in medium supplemented with 2 mg l^{-1} kinetin + 0.5 mg l^{-1} NAA. The presence of kinetin + NAA in growing media had no significant effect on the root number (Kaviani, 2014).

In the current experiment, the effects of photoperiod and growth regulators in culture medium were evaluated by determining the following parameters: photosynthesis rate [µ mol (CO_2) m^{-2} s^{-1}], respiration rate [µ mol (CO_2) m^{-2} s^{-1}], photosynthetic pigments, dry weight content (%) and total water content (%).

Photosynthetic pigments were expressed by chlorophyll a, chlorophyll b and carotenoids content (Figure 7). In the rooting medium, Asenka recorded the highest content of chlorophyll a and carotenoid pigments, both in the 16-hour and the 12-hour photoperiod, the amounts being higher for the 16-hour photoperiod. Magic Blue has the lowest content of chlorophyll b. Regarding the results obtained for assimilatory pigments, our outcomes emphasize that there is no statistical differences in photosynthetic pigments between the plantlets treated with 16-hour and 12-hour photoperiods. Kaviani (2014) found that the highest chlorophyll content were in plantlets grown on media containing 2 mg l^{-1} KIN + 1 mg l^{-1} NAA.

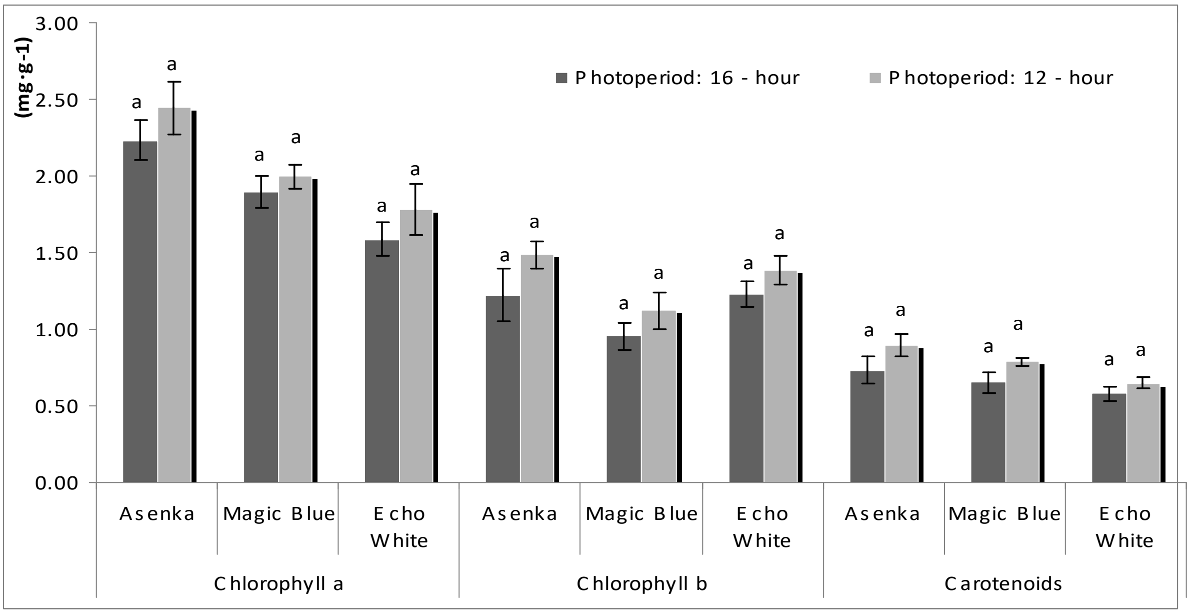

Fig. 7. Effect of photoperiod on photosynthetic pigments (mg l^{-1} fresh weight) (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within genotype are not significantly different according to t-test ($p > 0.05$)).

The total water content recorded the highest values for the Echo White (90.7%: 12-hour photoperiod and 89.2%: 16-hour photoperiod), and the lowest for the Asenka (88.5%: 12-hour photoperiod and 87.3%: 16-hour photoperiod). The total water content was higher under a 12-hour photoperiod condition, while the dry matter content was higher for 16-hour photoperiod. However, the difference in total water and dry water content treated with 12-hour and 16-hour photoperiod was not significant (Figure 8). The photoperiod effect on photosynthesis and respiration was more pronounced for all cultivars in 16-hours photoperiod conditions than in 12-hour photoperiod, but we did not find significant differences. Asenka had the highest intensity of photosynthesis, while Magic Blue recorded the slightest intensity (Figure 9). This shows that the application of a 12-hour photoperiod does not adversely affect physiological processes. Kaviani (2014) reported that the highest dry weight was found in plantlets cultured on media containing 1 mg l^{-1} KIN + 0.5 mg l^{-1} NAA.

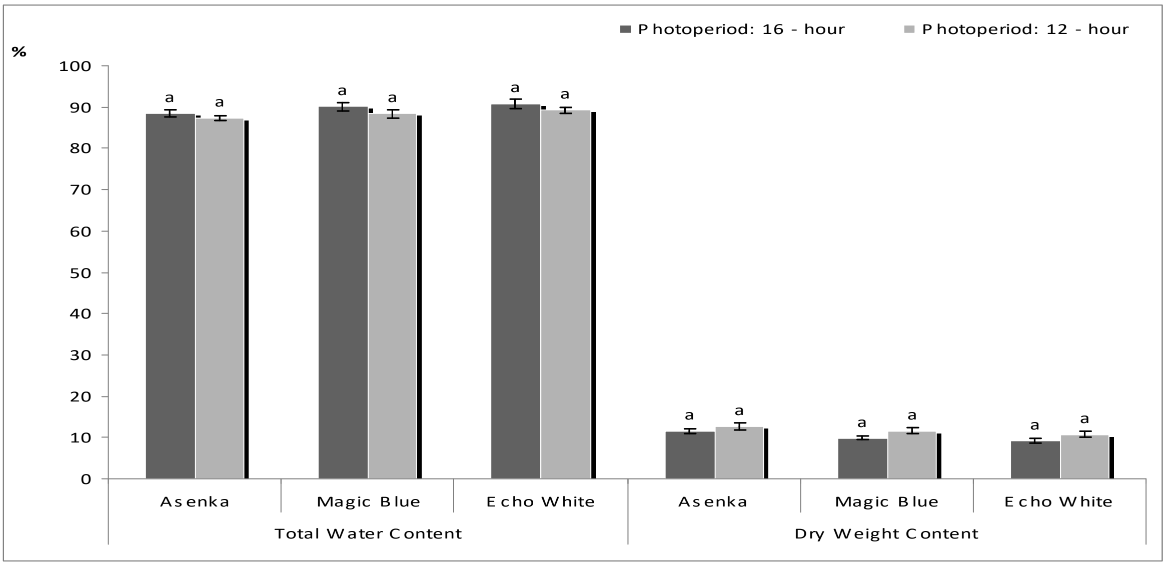

Fig. 8. Effect of photoperiod on dry weight content (%) and total water content (%) (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within genotype are not significantly different according to t-test ($p > 0.05$)).

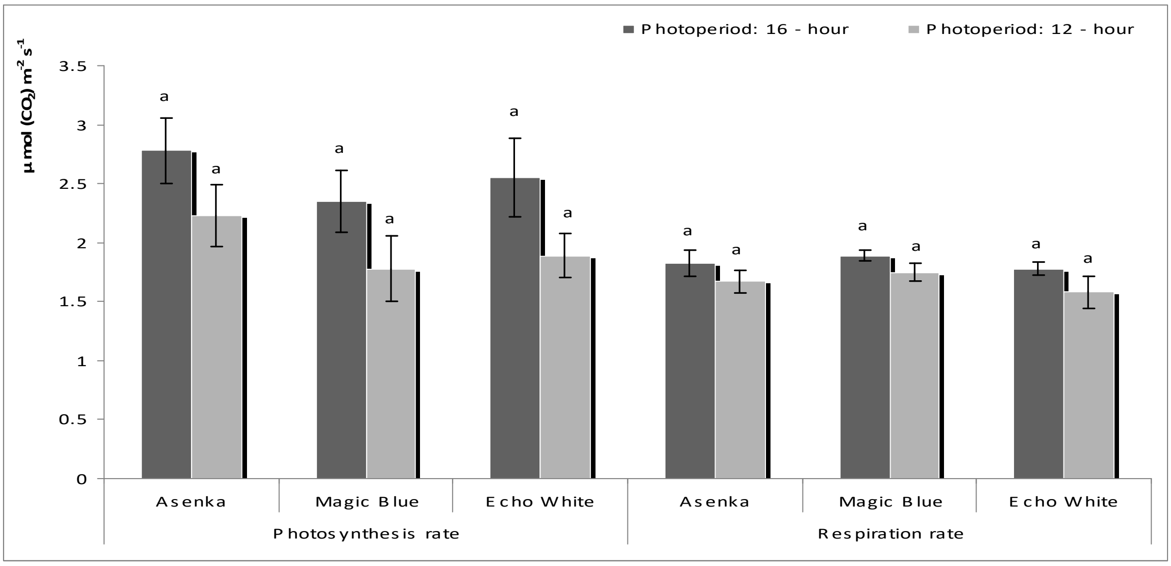

Fig. 9. Effect of photoperiod on photosynthesis rate [µ mol (CO_2) m^{-2} s^{-1}] and respiration rate [µ mol (CO_2) m^{-2} s^{-1}] (Data presented as mean ± standard error (SE). Error bars represent SE. Mean values followed by the same letter within genotype are not significantly different according to to t-test ($p < 0.05$)).

Table 1. Growing media composition for rooting phase of *in vitro* culture.

Components	Rooting growing medium (treatments)											
	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4
MS macrosalts (mg l^{-1})	1/2	1/2	½	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
MS microsalts (mg l^{-1})	1/2	1/2	½	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2	1/2
LS vitamins (mg l^{-1})	1	1	1	1	1	1	1	1	1	1	1	1
Active charcoal (g l^{-1})	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
NaFeEDTA (mg l^{-1})	38	38	38	38	38	38	38	38	38	38	38	38
BAP (mg l^{-1})	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

IBA (mg l^{-1})	0.2	0.4	0.6	0.8	-	-	-	-	-	-	-	-
IAA (mg l^{-1})	-	-	.	.	0.5	1.0	1.5	2.0	-	-	-	-
NAA (mg l^{-1})	-	-	-	-	-	-	-	-	0.1	0.2	0.3	0.4
Glucose (g l^{-1})	30	30	30	30	30	30	30	30	30	30	30	30
Agar-agar (g l^{-1})	7	7	7	7	7	7	7	7	7	7	7	7

Table 2. Effect of auxins on root formation (%).

Auxin type	Echo White	Magic Blue	Asenka	Echo White	Magic Blue	Asenka
	16-hour photoperiod			12-hour photoperiod		
0.2 mg l^{-1} IBA	50±3.701 c	33±2.509 b	36±1.140 c	62±4.062 c	40±4.086 b	42±2.345 c
0.4 mg l^{-1} IBA	58±1.341 b	38±0.836 b	44±2.983 b	75±1.264 b	48±0.948 ab	51±1.974 b
0.6 mg l^{-1} IBA	67±1.581 a	49±2.683 a	52±2.366 a	83±1.048 a	57±5.089 a	60±3.847 a
0.8 mg l^{-1} IBA	52±0.894bc	36±0.948 b	47±4.560 ab	75±0.447 b	52±1.264 a	56±3.162 ab
0.5 mg l^{-1} IAA	49±2.236 c	27±0.836 c	19±0.447 c	54±1.140 c	34±0.894 c	26±1.140 c
1 mg l^{-1} IAA	52±1.048 c	35±1.897 b	27±2.323 b	59±1.095 b	40±1.224 b	32±2.190 b
1.5 mg l^{-1} IAA	64±1.140 a	42±1.378 a	34±2.756 a	68±1.378 a	48±0.547 a	39±1.483 a
2 mg l^{-1} IAA	57±1.816 b	38±2.683 ab	31±1.843 ab	61±0.836 b	43±2.607 b	36±1.673 ab
0.1 mg l^{-1}NAA	38±1.140 a	23±1.643 a	20±0.894 a	53±1.048 a	32±1.048 a	29±1.048 a
0.2 mg l^{-1}NAA	32±0.894ab	17±2.280 b	14±1.414 b	47±0.447 a	22±1.048 b	18±1.516 b
0.3 mg l^{-1}NAA	26±3.741bc	10±2.345 c	10±2.097 b	34±3.741 b	15±0.707 c	14±1.673 b
0.4 mg l^{-1}NAA	20±3.033 c	4±0.316 d	5±1.378 c	26±1.516 c	11±0.894 d	9±1.095 c

Data presented as mean ± standard error (SE). Mean values followed by the same letter within columns, for the same auxin, are not significantly different according to Duncan's multiple range test ($p > 0.05$).

Table 3. Effect of genotype on root formation (%).

	16-hour photoperiod				12-hour photoperiod			
	0.2 mg l^{-1} IBA	0.4 mg l^{-1} IBA	0.6 mg l^{-1} IBA	0.8 mg l^{-1} IBA	0.2 mg l^{-1} IBA	0.4 mg l^{-1} IBA	0.6 mg l^{-1} IBA	0.8 mg l^{-1} IBA
Echo White	50±3.701 a	58±1.341 a	67±1.581 a	52±0.894 a	62±4.062 a	75±1.264 a	83±1.048 a	75±0.447 a
Magic Blue	33±2.509 b	38±0.836 b	49±2.683 b	36±0.948 b	40±4.086 b	48±0.948 b	57±5.089 b	52±1.264 b
Asenka	36±1.140 b	44±2.983 b	52±2.366 b	47±4.560 a	42±2.345 b	51±1.974 b	60±3.847 b	56±3.162 b
	0.5 mg l^{-1} IAA	1 mg l^{-1} IAA	1.5 mg l^{-1} IAA	2 mg l^{-1} IAA	0.5 mg l^{-1} IAA	1 mg l^{-1} IAA	1.5 mg l^{-1} IAA	2 mg l^{-1} IAA
Echo White	49±2.236 a	52±1.048 a	64±1.140 a	57±1.816 a	54±1.140 a	59±1.095 a	68±1.378 a	61±0.836 a
Magic Blue	27±0.836 b	35±1.897 b	42±1.378 b	38±2.683 b	34±0.894 b	40±1.224 b	48±0.547 b	43±2.607 b
Asenka	19±0.447 c	27±2.323 c	34±2.756 c	31±1.843 c	26±1.140 c	32±2.142 c	39±1.483 c	36±1.673 c
	0.1 mg l^{-1} NAA	0.2 mg l^{-1} NAA	0.3 mg l^{-1} NAA	0.4 mg l^{-1} NAA	0.1 mg l^{-1} NAA	0.2 mg l^{-1} NAA	0.3 mg l^{-1} NAA	0.4 mg l^{-1} NAA
Echo White	38±1.140 a	32±0.894 a	26±3.741 a	20±3.033 a	53±1.048 a	47±0.447 a	34±3.741 a	26±1.516 a
Magic Blue	23±1.643 b	17±2.280 b	10±2.345 b	4±0.316 b	32±1.048 b	22±1.048 b	15±0.707 b	11±0.894 b
Asenka	20±0.894 b	14±1.414 b	10±2.097 b	5±1.378 b	29±1.048 b	18±1.516 c	14±1.673 b	9±1.095 b

Data presented as mean ± standard error (SE). Mean values followed by the same letter within columns, for the same PGR concentration, are not significantly different according to Duncan's multiple range test ($p > 0.05$).

Table 4. Effect of photoperiod and growing media containing IBA, IAA, and NAA as plant growth regulators on length of roots.

	A1		A2		A3		A4	
	H 16	H12	H 16	H12	H 16	H12	H 16	H12
Echo White	6.4±0.202a	6.0±0.121a	6.7±0.130a	6.4±0.117a	4.3±0.255a	4.3±0.182a	5.2±0.273a	5.2±0.105a
Magic Blue	6.9±0.154a	6.8±0.175a	6.7±0.084a	6.5±0.117a	4.8±0.229a	4.7±0.244a	5.3±0.188a	5.2±0.110a
Asenka	6.5±0.183a	6.2±0.164a	6.8±0.055a	6.4±0.136b	4.4±0.136a	4.3±0.198a	5.3±0.136a	5.0±0.150a
	B1		B2		B3		B 4	
	H 16	H12	H 16	H12	H 16	H12	H 16	H12

Echo White	6.3±0.172a	6.0±0.159a	6.7±0.152a	6.1±0.112b	4.8±0.117b	5.4±0.049a	5.5±0.108b	5.9±0.087a
Magic Blue	6.9±0.160a	6.8±0.141a	7.3±0.120a	7.1±0.207a	6.4±0.139a	6.5±0.258a	6.3±0.130a	6.1±0.171a
Asenka	6.9±0.239a	6.7±0.120a	7.4±0.172a	7.0±0.157a	5.8±0.213a	5.5±0.141a	6.2±0.105a	6.2±0.114a
	C1		C2		C3		C4	
	H 16	H12	H 16	H12	H 16	H12	H 16	H12
Echo White	7.1±0.100a	6.9±0.093a	7.3±0.103a	7.1±0.068a	7.6±0.136a	7.2±0.117b	6.0±0.103a	5.7±0.203a
Magic Blue	7.3±0.195a	7.2±0.183a	7.5±0.114a	7.8±0.075a	8.0±0.180a	7.8±0.205a	6.8±0.144a	6.5±0.218a
Asenka	7.5±0.130a	6.8±0.128b	7.8±0.132a	7.3±0.093b	8.9±0.147a	7.4±0.120b	6.7±0.129a	6.4±0.211a

Data presented as mean ± standard error (SE). Mean values followed by the same letter within a row, for the same growing media and genotype, are not significantly different according to t-test ($p > 0.05$).
H 16: 16-hour photoperiod; H 12: 12-hour photoperiod.
A1: growing media supplemented with 0.2 mg l^{-1} IBA; A2: growing media supplemented with 0.4 mg l^{-1} IBA; A3: growing media supplemented with 0.6 mg l^{-1} IBA; A4: growing media supplemented with 0.8 mg l^{-1} IBA. B1: growing media supplemented with 0.5 mg l^{-1} IAA; B2: growing media supplemented with 1.0 mg l^{-1} IAA; B3: growing media supplemented with 1.5 mg l^{-1} IAA; B4: growing media supplemented with 2.0 mg l^{-1} IAA. C1: growing media supplemented with 0.1 mg l^{-1} NAA; C2: growing media supplemented with 0.2 mg l^{-1} NAA; C3: growing media supplemented with 0.3 mg l^{-1} NAA; C4: growing media supplemented with 0.4 mg l^{-1} NAA.

Conclusions: The *in vitro* propagation protocol we conceived may be efficiently used for the large scale propagation of this valuable ornamental plant. The photoperiod and plant growth regulators in growing media influenced the physiology and the rooting of micropropagated *E. grandiflorum*. Rhizogenesis was observed in all treatments, but the percentage response varied. Among the auxins, indole – 3 butyric acid showed the highest activity for the induction of root formation. Increase of IBA concentrations in rooting medium favored root formation. However, increasing the concentration of auxin to 0.8 mg l^{-1} determined the reduction of plantlets rooting capacity. The results confirm the different reactivity of varieties in the *in vitro* rooting phase. Physiological response confirm that media containing indole – 3 butyric acid is more suitable for lisianthus *in vitro* propagation than growing media with others auxins. The photoperiod of 12-hour is a good approach, inducing better rooting capacity in all cultivars on all tested growing medium, and resulting in 4 hour light energy saving (25%) during the *in vitro* rooting phase when compared to the usual 16-hour photoperiod. Therefore, the present study introduces a useful and an effective protocol for *in vitro* propagation of lisianthus.

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