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Influence of Plant Growth Regulators on Callogenesis and on the Biomass of Cell Suspensions in Lily (*Lilium ledebourii* and *Lilium* Regal)

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Abstract

Introduction: Lily is often described as one of the most widespread, commercial crops in the floriculture industry. Commercially grown cultivars are mostly propagated by bulb scales which it cost effective and uniformity in tissue culture conditions. Plant tissue culture techniques can effectively provide far-reaching implications in micro-propagation.

Materials and Methods: This study was conducted to investigate the effects of various concentrations of cytokines and auxins on callogenesis and Biomass under *in vitro* conditions. In this experiment, the evaluations were aimed at measuring different characteristics in two lily cultivars, namely, Lilium ledebourii and *Lilium regal*.

Results: The results showed significant values in all of the measured characteristics. The highest percentage of callogenesis was caused by 2 µm picloram (PIC) plus 1 µm kinetin (KIN) in *L. ledebourii* (88.66%) and *L. regal* (88.66%). Also, the callus weight in both cultivars was obtained by applying the same combination of treatments. In the second experiment, the highest fresh biomass of the cell suspension occurred by applying 2 µm PIC plus 4 µm KIN. The maximum amount of fresh biomass in *L. ledebourii* (88.93 g/L) and *L. regal* (41 g/L) occurred on the 24th and 20th day of the culture, respectively.

Conclusions: An efficient and fragile callus induction was developed by naphthalene acetic acid (NAA), which nonetheless made the condition more suitable for cell suspension culture. These lily cultivars need high amounts of PIC as auxin to grow well in cell suspensions. By increasing the PIC level, the biomass accumulates more. These results can moderately optimize large-scale production of both fragile calli and cell suspension biomass.

Keywords: Callus Formation, Cell Suspension Culture, Lilium

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Introduction

Lilium belongs to the Liliaceae family which has 220 genera in total. There are 85 species in the Liliaceae family, some of which are found as wild in natural habitats.[1] These plants have great commercial value.[2] Lily is mostly propagated in Southeast Asian countries such as China, Korea and Japan, while North America maintains also a strong market for this ornamental plant.[3] Plants in the *Lilium* genera have large and attractive flowers. They are among the top ten targets of flower production in the world.[4]

An important lily species is *Lilium ledebourii* which is widespread in Iran.[5] It grows specifically in the northern parts of the country.[6] *L. ledebourii* flowers each have 2–15 long clusters which are important in breeding programs.[3] Another species of lily is *L. regal* which is native to China. It has white flowers that are horn shape and yellowish on the inside.[7] In bulbous plants, traditional propagation methods sometimes prove to be inefficient,[8] while the danger of extinction continues to threaten wild lily species. Nonetheless, tissue culture and plant cell culture are important tools for genetic engineering and physiological studies. These techniques allow large-scale productions with acceptable qualities that can be aimed at export to global flower markets.[5] One of the best and most successful methods in this regard is tissue culture technology.[1] The first step in achieving a peak in high performance and productivity is to establish good quality callus in vitro.[1] Many factors can affect callus formation: genotypes, growth regulators, culture medium, carbohydrate type, explant type, explant age and environmental conditions such as light and temperature.[2,4]

Plant growth regulators (PGRs) can inhibit or promote growth, depending on the developmental stage of the bulb and the concentration used.[9] Controlling the differentiation process depends on the presence of auxin and cytokinins which perform best in a quantitative balance.[10] Different concentration of PGRs have noticeable effects on callus induction, proliferation and morphology.[11] Cytokinins play a key role in cell division and the differentiation of shoots from callus. Different combinations of auxins and cytokinins impart different effects in terms of inducing the formation of

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roots and shoots. Cytokinins which can be used in treating callus formations are, namely, zeatin, benzyl adenine (BA), Thidiazuron (TDZ), kinetin (KIN) and benzyl amino purine (BAP). The list of auxins include indole acetic acid (IAA), indole butyric acid (IBA), 2,4-Dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)[12] and Picloram.[13] The IAA and NAA are often used for rooting or, when used in combination with a cytokinin, can induce shoots. Also, 2,4-D and 2,4,5-T are efficient PGRs that promote callus growth.[14] The impact of cytokinins on the tissue culture of lily species usually varies. In *L. ledebourii* they cause branching, whereas in *L. longiflorum* they stimulate embryogenesis and regeneration.[15]

Chamani et al[16] examined the effects of 2, 4-D and IAA treatments on the regeneration and callus formation of *L. longiflorum*, suggesting that the different concentrations of IAA have no significant effect on callus formation.[17] However, it was noted that a gradual increase in the concentration of 2, 4-D (up to 2 mg/L) caused a decrease in the number and length of roots, whereas the fresh weight of callus increased. Chamani et al evaluated the effects of different concentrations of benzyl adenine and picloram on callus formations of *L. ledebourii*. The report indicated that various concentrations of picloram and benzyl adenine can have significant effects on callus formation and callus diameter. Combining the benefits of callus production and of uniform plant material in tissue culture is a necessity for the future breeding and culture of lilies (e.g. by somatic embryogenesis, organogenesis and hybridization).[18]

A cell suspension culture is normally initiated by transferring pieces of undifferentiated and friable calli to a liquid medium.[19] This system consists of cells and cell aggregates that are dispersed in a moving liquid medium and thus grow accordingly.[20] Cell suspensions are used for generating large amounts of cells for several purposes. These can be aimed at producing transgenic plants in many monocotyledonous plant species,[21] producing secondary plant products,[22] and discovering potential, organic herbicides.[23] In addition, cell suspension culture is necessary for somatic hybridization in distant interspecific hybrids by the development of single cell suspensions,[23] especially in species like lily which sometimes encounter cross incompatibility.[3] Cells dispersion in a liquid culture medium is usually increased by combining a relatively high concentration of auxin with a low concentration of cytokinin.[24] The aim of the current study was to establish optimal hormone levels so as to induce callus formation and obtain high-quality calli in two lily cultivars in cell suspension medium.

Material and Methods

Plant Material and Explant Preparation

The bulbs of *L. ledebourii* were collected from the natural habitat in Khangah village, Ardabil province, Iran, in October 2018. Also, fresh bulbs of *L. regale* were prepared from the Chinese Academy of Agricultural Sciences, China. The bulbs were kept in the refrigerator at 4°C for four weeks and were then used as an explant source in the subsequent experiment. The bulbs were washed with a few drops of washing-up liquid followed by 30 minutes tap water. The bulbs surfaces were disinfected with 70% ethanol for 30 seconds followed by immersion in 5 % commercial bleach solution containing few drops of Tween 20 for 30 minutes and then soaked three times in the sterile distilled water. Lower portions (0.5 × 0.5 cm) of aseptic inner scales were excised from the mother bulbs and served as explants.

Callogenesis Experiment

The objective of the first experiment was to evaluate the effects of different PGRs on the callogenesis and regeneration capacity of two *Lilium* species. Based on literature survey, eight combinations of PGRs including 4 µm 2, 4-D + 2 µm BA, 8 µm 2, 4-D + 2 µm BA, 1 µm TDZ + 2 µm NAA, 0.5 µm TDZ + 1 µm NAA, 1 µm BA + 2 µm NAA, 2 µm BA + 4 µm NAA, 2 µm PIC + 1 µm KIN and 4 µm PIC + 1 µm KIN were selected for callus induction. Aseptic explants were incubated horizontally on the B5 medium supplemented with the above mentioned PGRs combinations and 20 g/L sucrose. Six bulb scale explants were placed on 200 ml glass jar containing 25 mL medium, and each jar served as a replicate. The experiment was conducted in a completely randomized design with a factorial arrangement (2 species × 8 PGRs combination) with six replications. All cultures were maintained in a growth chamber at 25±1°C in the dark for six weeks. At the end of the culture period, callogenesis percentage, color and texture of calli, percentage of organogenesis, and the number of regenerated plantlets and roots were recorded.

Cell Suspension Cultures Experiment

To establish the cell suspension cultures, one gram of friable callus obtained from the previous experiment transferred into 100 mL Erlenmeyer flasks containing 40 mL B5 liquid medium fortified with 2 µm PIC + 4 µm KIN and 40 g/L sucrose. The culture flasks were kept on a rotary orbital shaker at 100 rpm and incubated at 25 ± 2°C under cool-white light illumination with the photoperiod of 16 hours. The cultures were then sub cultured every 14 days by transferring 10 mL of culture medium to 30 mL of fresh medium with the same composition and continued for three months until uniform cell suspension culture was achieved. The growth curve of the cultures was depicted by plotting the fresh weight (FW) and dry weight (DW) accumulation in cultures against the culture period. For this purpose, the cells were harvested from three flasks at four days intervals during the 28 days of culture, and the FW and DW of cells were recorded.

Results and Discussion

In the present experiment, FW of callus, percentage of root induction, number of branches and percentage of regeneration were significantly affected by the interaction of cultivar and hormonal treatments. As shown in Table 1, there were no significant differences between two cultivars (Ledebourii and Regal) except for callus FW. According to the obtained results, there was a significant interaction between cultivar and treatments for some of the traits. The highest and lowest percentage of callus formation was observed at 2 µm PIC plus 1 µm KIN treatment in Ledebourii (88.66%) and

Table 1. Effect of Different Treatments on Traits Measured in Ledebeourii and Regal lily Cultivars

Cultivar	Treatment	Regeneration (%)	No. of Branches	No. of Roots	Percent Rooting	Callus Induction (%)	Fresh Weight of Callus (mg)
Regal	Control	66.3^{ab}	5^{bc}	1.3^{c}	27.66^{cd}	16.66^{c}	13.3^{c}
	4 µm 2,4,D + 2 µm BA	0^{c}	0^{c}	0^{c}	0^{d}	30.33^{bc}	136^{b}
	8 µm 2,4,D + 2 µm BA	20.3^{b}	3.3^{c}	0^{c}	0^{d}	75.33^{ab}	210^{b}
	1 µm TDZ + 2 µm NAA	88.6^{ab}	11^{c}	10^{ab}	70^{b}	44.3^{b}	44.3^{c}
	0.5 µm TDZ + 1 µm NAA	77.6^{ab}	6^{cd}	13.3^{a}	100^{a}	77.66^{ab}	33.3^{c}
	1 µm BA + 2 µm NAA	100^{a}	22.3^{b}	3.3^{bc}	52.3^{bc}	40^{b}	44b
	2 µm BA + 4 µm NAA	75^{ab}	36a	3.3^{bc}	35^{c}	33^{bc}	72.3b
	2 µm PIC + 1 µm KIN	0^{c}	0^{c}	0^{c}	0^{d}	88.66^{a}	613^{a}
	4 µm PIC + 1 µm KIN	20^{b}	3.6^{cd}	0.66^{c}	8.3^{d}	83.3^{ab}	510^{a}
Ledebourii	Control	27.66^{c}	1.66^{d}	0^{c}	0^{d}	0^{b}	0^{c}
	4 µm 2,4,D + 2 µm BA	5^{cd}	2.33^{d}	0.33^{c}	6.66^{d}	56.66^{a}	181^{bc}
	8 µm 2,4,D + 2 µm BA	0^{d}	0^{d}	0^{c}	0^{d}	48.33^{a}	22.6^{bc}
	1µm TDZ + 2 µm NAA	91.66^{ab}	24^{ab}	17^{b}	78.33^{b}	55^{a}	24.33^{bc}
	0.5 µm TDZ + 1 µm NAA	100^{a}	30.66^{a}	41.66^{a}	100^{a}	77.33^{a}	336.6^{b}
	1 µm BA + 2 µm NAA	51^{bc}	8^{cd}	3.33^{c}	11^{d}	88.66^{a}	72.33^{bc}
	2 µm BA + 4 µm NAA	83.33^{abc}	15.6^{bc}	2.66^{c}	38.33^{c}	58^{a}	210.3^{bc}
	2 µm PIC + 1 µm KIN	66.33^{abc}	4^{cd}	0^{c}	8.33^{d}	88.66^{a}	690^{a}
	4 µm PIC + 1 µm KIN	89^{abc}	7^{cd}	0^{c}	0^{d}	70.66^{a}	750.3^{a}

Mean letters in each column did not differ statistically at the 5% level of probability.

Regal (88.66%) as well as at the control treatment respectively (Figure 1a and 1b). Also, the maximum FW of callus in both cultivars was recorded in the same treatment (Figure 1a and 1b). Ghanbari et al[25] stated that the highest callus induction, weight and diameter from bulb scales of *L. ledebourii* was obtained in medium amended by 3 µM 2,4-D + 0.5 µM KIN. Chamani et al[16] reported that the highest amount and diameter of callus was obtained from lily anthers at concentrations of 0.2 mg/L PIC and 1 mg/L BA. Based on our findings, different concentrations of picloram provided the highest percentage of callus induction and FW of callus among other hormonal treatments in the both cultivars.

The mode of interaction between auxins and cytokinins is dependent on the type of explant and plant species, but the most plant explants required auxin and cytokines treatment for callus formation under in vitro conditions.[26] Of course, auxins have more effects on callus formation than cytokines because of their greater effect on cell division.[19] The most callus induction processes described to date employ transcriptional regulators that induce universal changes in gene expression.[27] The signaling of Auxin-induced callus formation is transduced via auxin response factor transcription factors and a family of transcription factors that plays a central role in cell cycle reentry.[28] Also, among the studied treatments, TDZ displayed the highest regeneration, either direct or indirect. Actually, TDZ accelerated the rate of endogenous cytokinin production and inhibited the cytokinin oxidase activity.[29] It also made better absorption of nutrients and increased regeneration.[30] According to our results, the treatments with NAA increased rooting percentage. Auxins in PGRs are a prerequisite for adventitious rooting. It has been shown that the first step in root initiation depends on the presence of endogenous or synthetic auxin such as NAA. The internal auxin production varies according to the plant species, tissue and developmental stages. Our findings indicated that increasing the NAA concentration reduced the adventitious root formation and higher number of roots was formed on medium amended with 1 µm NAA. These observations have been verified with Hentig and Gruber's citation who reported that hormonal doses could induce the best rooting when being just below the toxic level.[31]

Correlation Coefficient

The correlation coefficient was employed to measure the linear association between evaluated traits in this research. According to the results, some of the studied characteristics were significant. A positive significant correlation was observed between traits such as; the number of roots and branches, percentage of roots and regeneration as well as FW callus per plant ($P<0.05$ and $P<0.01$) under different

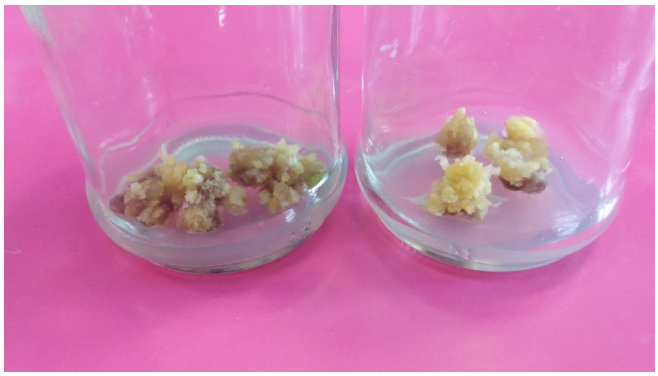

Figure 1. The Callus FORMATION of (a) Ledebourii and (b) Regal in 2 µM PIC and 1 µM KIN.

hormone combinations presented in Table 2. In this study, the number of roots and percentage of roots (r= 0.86**) as well as the number of shoots with percentage of roots (r= 0.76**) and number of roots (r= 0.72**) exhibiting a positive correlation. The regeneration percentage had a positive and significant correlation with callus formation percentage (r= 0.44*), rooting percentage (r= 0.50**), number of roots (r= 0.43*) and shoots (r= 0.70**). According to a research conducted by Chamani et al, there was a significant positive correlation between the percentage of regeneration and number of roots, which was in accordance with our results. They reported that with increasing the amount of auxin, regeneration percent, was increased.[12] In another study by Hadizadeh et al on chicory (*Cichorium intybus*) plant, between regeneration percentage, mean number of shoots, callogenesis percentage and FW of callus exhibiting a positive correlation.[32]

In the study, a weak positive correlation around 0.11 was found between the percentage of regeneration and FW callus. Al-Khayri et al cited that an inverse relationship exists between callus weight and plant regeneration capacity.[33] Groenewald et al. stated that with increasing callus formation, the frequency of organogenesis decreased.[34] Callus formation is often considered to inhibit plant regeneration.[35] Auxin and cytokinin control callus formation as well as differentiation (regeneration) from callus. The latter process was triggered by a high concentration of cytokinin and a low concentration of auxin.[36]

According to the results shown in the Table 3, different concentrations of NAA produced more fragile calluses than other treatments. There are multiple types of callus tissue in terms of their character such as friable, soft and compact callus. It is also known that different types of callus affected not only by genotype and the type of explant but also by the composition of the culture medium including PGRs, and by the culture conditions.[33,37] It has been shown that friable calluses fall apart easily and are most suitable for establishing cell suspension cultures.[38] Therefore, callus cultures obtained from the best treatments (2 µm BA plus 4 µm NAA) were used to establish the cell suspension cultures of *L. ledebourii* and *L. regal*. Parsaeimehr and Mousavi reported that NAA induced high amounts of friable calluses.[39]

Cell Suspension Culture

In the present experiment, cell suspension culture of *L. ledebourii* and *L. regale* were established using a friable calli in B_5 medium fortified with different PGR combinations. The growth curve of cultures was depicted based on fresh biomass weight (Figures 2 and 3). The biomass growth of cell suspension cultures typically undergoes four stages, namely lag, exponential, declaration, and stationary phases.[40] In the present study, *L. ledebourii* suspension cultures in all treatments represented the four distinctive phases, which indicated that suspension cultures established successfully (Figure 4). Cultures treated with 2 µm PIC + 4 µm KIN and 6 µm PIC + 2 µm KIN remained in the initial lag phase for 12 days, and then went into the exponential phase until 24 days after incubation. Then, the rate of growth was stable, followed by a gradual reduction in cell density and cultures entered in a stationary phase. The exponential phase is a stage of cell suspension culture where, in it the most proliferation and biomass accumulation of cells occur.[41] Several factors such as nitrogen, phosphate, carbon source and PGRs levels

Table 2. Correlation Between Measured Traits

Source of Variations	Callus Induction (%)	Percent Rooting	No. of roots	No. of Branches	Regeneration (%)	Weight Callus
Callus Induction (%)	1					
Percent rooting	0.139	1				
No. of roots	0.216	0.86**	1			
No. of branches	0.205	0.76**	0.72**	1		
Regeneration (%)	0.44*	0.50**	0.43*	0.70**	1	
Weight callus	0.50**	0.123	0.027	0.274	0.112	1

* Significant at the 5% level; **significant at the 1% level.

Table 3. Effect of Different Concentrations of Plant Growth Regulators in MS Medium on Callus Quality

	Control		Ledebourii		Regal	
Treatment	Callus Quality	Callus Color	Callus Quality	Callus Color	Callus Quality	Callus Color
4 µm 2,4,D + 2 µm BA	-	-	-	-	CC	Y
8 µm 2,4,D + 2 µm BA	-	-	CC	YG	CC	Y
1 µm TDZ + 2 µm NAA	-	-	SC	Y	SC	YG
0.5 µm TDZ + 1 µm NAA	-	-	FC	Y	SC	Y
1 µm BA + 2 µm NAA	-	-	SC	Y	FC	Y
2 µm BA + 4 µm NAA	-	-	SC	Y	FC	LY
2 µm PIC + 1 µm KIN	-	-	FC	YG	CC	Y
4 µm PIC + 1 µm KIN	-	-	CC	Y	CC	Y

Y: Yellow, LY: Light yellow, YG: Yellow green. Callus quality: FC: Friable callus, SC: Soft callus, CC: Compact callus

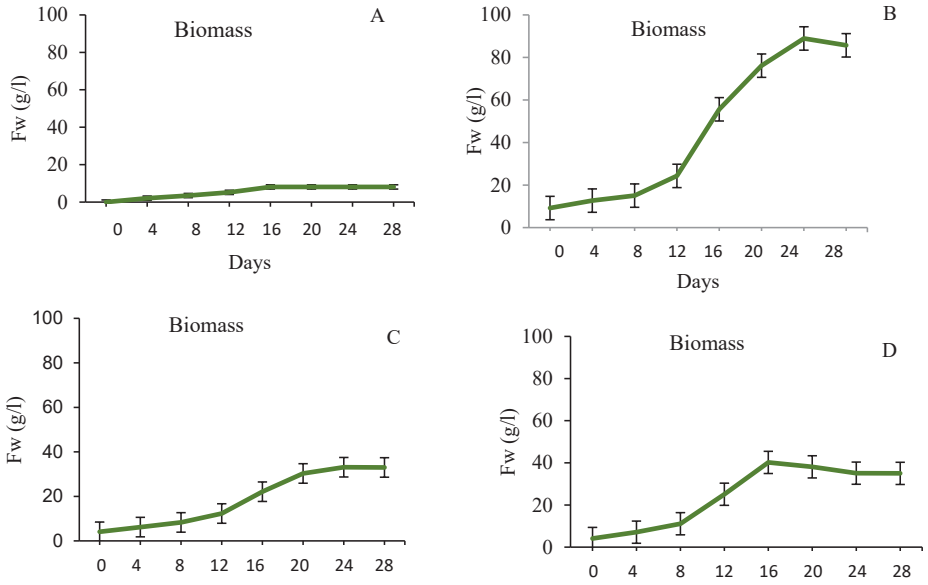

Figure 2. Growth Curve and Biomass Production of *L. Ledebourii* Cell Suspension Culture Based on the Fresh Weight (A) 1 µm PIC + 1 µm KIN, (B) 2 µm PIC + 4 µm KIN, (C) 4 µm PIC + 2 µm KIN, (D) 6 µm PIC + 2 µm KIN.

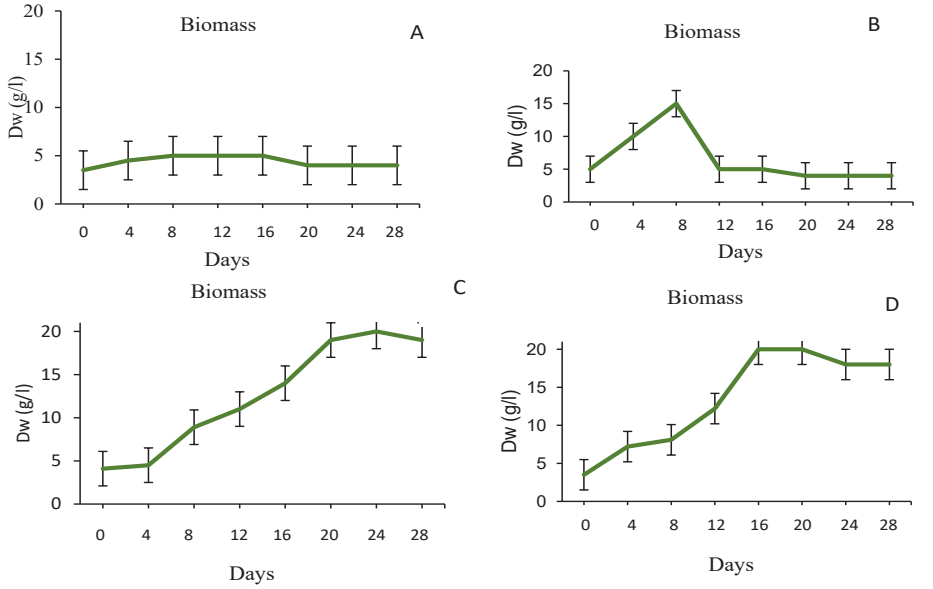

Figure 3. Growth Curve and Biomass Production of *L. regal* Cell Suspension Culture Based on the Fresh Weight. (A) 1 µm PIC + 1 µm KIN, (B) 2 µm PIC + 4 µm KIN, (C) 4 µm PIC + 2 µm KIN, (D) 6 µm PIC + 2 µm KIN.

could influence cell division and proliferation in this phase.[42] Among these factors, the PGRs are a key factor responsible for cell division and expansion.[43] A proper PGRs combination in optimum concentration led to enhancement of cell division during the exponential phase and provided the highest biomass accumulation.[44] A study conducted by Fan et al[45] on *Lilium brownii* indicated that PGRs combination had an important role in the establishment of its suspension culture. They reported that however 0.5 mg/L BA+ 2 mg/L 2,4-D + 0.2 mg/L NAA treatment was the best combination for callus induction, the 1 mg/L BA+ 2 mg/L 2,4-D + 0.2 mg/L NAA combination produced maximum FW in suspension culture. Modarres et al.[46] reported that *Salvia leriifolia* suspension culture produced higher FW and phenolic content in medium supplemented with 5 mg/L NAA + 5 mg/L BA which was the same as callus induction medium and significantly differed from other treatments.

Our results revealed that increasing in Pic level in the culture medium from 1 to 6 mg/L resulted in 4-fold raised in fresh biomass accumulation. Previous research on some Lilium species such as *L. longiflorum*, *Lilium* Oriental hybrid 'Crimson Beauty' and *L. speciosum* showed that picloram is a better auxin for callus induction and proliferation than others.[47] A study undertaken by Stella et al showed that culture

Figure 4. The Fresh Weight of *L. Ledebourii* Cell Suspension Culture With 2 µm PIC + 4 µm KIN During Stationary Phase.

supplemented with picloram has been successfully used for the maintenance of totipotent callus lines.[48] Figueiredo et al showed that *Rollinia mucosa* cultures supplemented with picloram displayed the fastest growth rate and reduced lag phase as compared to medium supplemented with 2,4-D or NAA. Those reports are consistent with our findings.

In the *L. regale* cell suspension culture, when Pic concentration was raised to a 6 mg/L, the highest accumulation of fresh biomass with 18.52 g/L was achieved. In this species, culture incubated with 1 µm PIC + 1 µm KIN could not establish successful suspension culture, because four distinctive phases were not observed in the growth curve (Figure 3a). As the concentration of picloram in the culture medium increased, the cultures went into the exponential phase from the lag phase and the cell suspensions were successfully established. These results indicated that concentrations of PGRs especially auxin, is a key factor in the establishment of *L. regale* cell suspension culture. Exogenous PGRs type and concentration altered endogenous hormone levels in plant cells and in this way, it affected the division potential of cells.[49] Wu et al[50] reported that raising 2, 4-D concentration from 0.45 to 6.79 µM and lowering NAA levels from 5.37 to 1.07 µM in *Lilium brownii* var. *giganteum* callus cultures resulted in a loss of callus growth and FW through the drop in endogenous zeatin, IAA and abscisic acid (ABA) levels. Therefore, it can be concluded that in the present study, the failure to establish the suspension culture in 1 µm PIC + 1 µm KIN treatment was due to low levels of endogenous hormones in cells due to low concentrations of exogenous kinetin and picloram.

Unlike *L. ledebourii* in the *L. regale* cell culture, the highest fresh weight was recorded in the 2 µm PIC + 4 µm KIN treatment and no significant change in fresh weight was observed with increasing picloram levels above 2 mg/L. Also, the biomass accumulation in *L. ledebourii* suspensions was significantly higher than in the *L. regale* cell culture. In accordance with our results, in other plants such as *Azadirachta indica*,[51] Salvia nemorosa[52] *Lippia gracilis*,[53] *Lycium barbarum*,[54] *Allium hirtifolium*[55] and Fritillaria *imperialis*[9] different morphological responses of different species and cultivars of a plant to the same types and concentrations of hormones have been reported. Among several recognized classes of growth regulators or plant hormones, including auxins, cytokinins, gibberellins, ethylene and ABA, using appropriate concentrations of auxins and cytokinins is by far the most effective for a successful establishment of cultures and in vitro mass propagation of lilies, depending on the stage of in vitro cultures.[47] Different species and genotypes of Lilly, required different combinations of PGRs for callus initiation and proliferation.[56,57]

Conclusions

In the present research, an efficient protocol has been developed for callus induction and plantlet regeneration in two species of Lily (*L. regale* and *L. ledebourii*). Our results indicated that PGRs combination is an essential factor in callogenesis and organogenesis of Lilium. Also, findings revealed that different species of Lilium showed a different response to the same PGRs combinations. Moreover, in the present investigation, we stablished cell suspension cultures of *L. regale* and *L. ledebourii* successfully. *L. ledebourii* needs a high amount of picloram as auxin, and with increasing in Pic level, the biomass accumulation enhanced. Also, cell suspension culture establishment in lilies is important to single cell isolation due to protoplast fusion.

Authors' Contributions

AE and ZK contributed to the conception of the study, in vitro cultures establishment, data extraction statistical analysis, and writing of the manuscript. EC supervised the whole experiments.

Conflict of Interest Disclosures

The authors declare that they have no conflicts of interest.

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