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Investigation of Direct Regeneration in *Lilium ledebourii* Bioss through bulblet explant

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ABSTRACT

Lilium ledebourii Bioss is a wild species of Lilium that naturally grows in the Damash heights of Roudbar, Iran. This flower is one of Liliums delicate species and is endangered of extinction, while it has a high potential for being introduced as corrective plans. Proliferation and mass production are the first steps of this plant correction and since the common methods have a low speed of the proliferation, tissue culture propagation has been examined through bulbs. Establishment of bulb explants were investigated on three mediums (Murashige and Skoog) MS include: medium lacking the growth regulators, medium with NAA (0, 0.5, 1, 1.5 $mg.l^{-1}$) and finally a medium with BA (0, 0.5, 1, 1.5, 2 $mg.l^{-1}$). This research has been accomplished on a triplicate factorial experiment and was totally random. Only in one treatment (0 $mg.l^{-1}$ or hormone free media) bulbing and rooting have been observed. The largest number and percentage of bulb and also the largest number and percentage of root were obtained from the medium lacking the growth regulators. The content of growth regulators is one of the most significant ($P \leq 0.01$) factors of bulblets regeneration, so that the growth of bulblets in the represent mentioned medium suggests that the endogenous hormones available in explants were sufficient for making physiological responses.

Key words: Bulb, Growth regulators, *Lilium ledebourii* Bioss, Regeneration, Tissue culture.

INTRODUCTION

Iran is one of the main centers of native as it possesses 8000 plant species plant species (Jalili and Jamzad 1999; Rechiner 1990). Lilium genus with 120 species is one of the important bulbous flowers in which widely are used in flower's world market (Maesato *et al*. 1994). *Lilium ledebourii* Bioss form Liliaceae family is the only plant species of this kind that it' transmittance is reported from Damash (wendelbo 1978) dorfak (kazemi and saberi 2004) Gilan, Kelardasht Mazandaran (Ghahreman 1997), Khanghah Ardebil (Padasht Dahkaei 2005) to Lankaran Azerbaijan (Wendelbo 1978). *L. ledebourii* has a high potential as a new flower and is appropriate for marketing in Iran and the whole world. Additionally, this plant has proper characteristics such as high stalk length which gives the flower, the proper durability of the flower with amazing and eye catching appearance

(Ghahreman 1991). Proliferation of lilium via bulb shooting is considered as one of the efficient ways in proliferation of different species of Lilium. In this method it is produced 3 to 5 bulblets from every bulb. These bulblets produce bulbs in commercial size in the period of 2 years (Miller and Wiliam 1993; Roh 1996). In this flower the middle and external bulbs tend to produce more bulblets rather than the internal ones (Matsue 1972; Choi 1982). Liliums bulb is the storage organ with no cover and is made from bulbs and head. Head is a compressed stalk in which creates stalk and root. Bulbs are modified, with inflated leaves that contain stored nutrition. The size of the bulbs is to a great extent depends on the number and the degree of their compaction (Hartman *et al.* 1997; Roh 1999). Many factors such as explant source, concentration of sucrose, PH and growth regulators affect the Liliums which grown under in vitro conditions (Jeong 1996). Most of achieved successes in Liliums tissue culture was related to *L. longiflorum* genotypes and little successes has reported from the other types of Lilium (Bahr and Compton 2004). In other studies about Liliums micropropagation, some factors such as, growth regulators, temperature, sucrose concentration and bulb parts have been evaluated. In most of the experiments, high concentration of Cytokinin and low concentration of auxin was necessary for the production of scallion (Maesato et al. 1994), while the concentration of 1 to 10 $mg.l^{-1}$ NAA (Naphthaleneacetic acid) would accelerate the production of callus. While uder low concentration of NAA (0.01 $mg.l^{-1}$) along with BAP (Benzylaminopurine) at the level of 0.1 $mg.l^{-1}$ the production of scallions was increased (Jeong 1996). Azadi (2003) and Tavassolian (2001) have reported the highest number of regenerated bulblets from the bottom of main bulb. Proliferation via leaf's explants, bulb, stalk, petal and peduncle is extensively reported in Lilium. For example, it has been proved that parts of bulbs of lily bulbs are appropriate explants for the proliferation via tissue culture (Niimi 1995). Amaury *et al.* (2007) in an investigation on *L. Maculatum* concluded that, the best results were achieved for the production of bulblets in the MS medium containing 2 $mg.l^{-1}$ NNA with 0.5 $mg.l^{-1}$TDZ (Thidiazuron). El-Nagar *et al*. (2012) in an investigation on *L. Prato* showed that the highest percentage of organogenesis was produced in MS medium containing 0.5 $mg.l^{-1}$ BA while the treatment of 2 $mg.l^{-1}$ BA caused the reduction of organogenesis. Saetiew and Umamanit (2015) in a survey on *L. formelongo* found that in MS medium containing 1 $mg.l^{-1}$ NAA and 0.5 $mg.l^{-1}$ TZD the highest amount of callusing was formed after 4 weeks. Furthermore MS medium containing 0.5 $mg.l^{-1}$ IAA (Indole-3-acetic acid) is effective on root induction. Skoric *et al.* (2012) in an investigation on *L. martagon* found that, using MS medium containing BAP 0.2 $mg.l^{-1}$ with NAA 0.25 $mg.l^{-1}$ leads to the formation of organogenesis in bulb. Azadi *et al.* (2007) have shown that, the best hormone combination for direct regeneration in *L. ledebourii* is using 0.1 $mg.l^{-1}$ BA + 0.1 $mg.l^{-1}$ NAA. Chang *et al*. (2000) concluded that, the best medium for callus initiation in *L. speciosum* is MS medium containing 3 mg/l 2,4-D including with 0.25 $mg.l^{-1}$ BA. Kanchanapoom *et al.* (2011) concluded that, the best compound for *L. longiforum* organogenesis is 1.5 µM NNA and 15 µM BA, and for rooting is 5 µM IBA. Kumar *et al*. (2007) in a research on the eastern Lilium have shown that, the best medium for direct regeneration is the MS containing 0.5 $mg.l^{-1}$ NAA with 1 $mg.l^{-1}$ BA. In the present study, bulb explant in MS media was used with different hormonal treatments for Lilium tissue culture. The purpose of conducting this research was to obtain the best combination of tissue culture media for direct organogenesis in *L. ledebourii* of Damash.

MATERIALS AND METHODS

The scallion of *L. ledebourii* was collected from the Damash heights and was transferred to Iranian Biological Resource Center. For disinfection of plants, the damaged or contaminated bulbs were excluded in early stages. The intact bulbs were isolated from the lower parts and were placed under the water flow for the period of 6 hours. Afterwards, these bulb parts were left in benomyl solution of 5% for 75 minutes. Then bulbs were washed and were placed in 70% alcohol for 1 minute. Again these bulbs are washed with distilled water and were disinfected in a solution containing 5% sodium hypochlorite for 25 minutes. Next, the utensil containing the explants was washed for 3 times with distilled water and under sterile condition.

For medium preparation: basal medium used in this experiment was MS (Murashige and skoog 1962). The bulbs were divided into 5 mm parts and put inside the glass. In order to evaluate direct organogenesis, MS basal medium containing 30 $g.l^{-1}$ sucrose and 7 $g.l^{-1}$ agar with 5 levels of (0, 0.5, 1, 1.5, 2 $mg.l^{-1}$) BA and 4 levels of (0, 0.5, 1, 1.5 $mg.l^{-1}$) NAA were prepared. Disinfection has been performed by autoclave in the temperature of 121 °c and the pressure of 1.5 $kgcm^{-2}$. The medium pH was set by NaOH between 5.6-5.8. Explants were cultivated in jars of jam which contained regeneration medium. Glass doors were blocked with parafilm and they were exposed to a photoperiod of 8-16 hours of light-dark in the temperature of 25±2 °c. These researches have been done as factorial experiments in a totally random way in 3 frequencies (every 3 glasses of tissue culture as one frequency). After 20 days, the percentage of bulbing, number of new bulbs, the number of roots and their percentage were recorded every eight days. All the data have analyzed by

SAS 9.1, and means comparison were done based on Duncan's multiple range test.

RESULTS

The result of this study has shown that, there was internal contamination in used bulbs. Because this contamination hasn't scattered in all parts of the tissues at least some parts remained free of any contaminations and elimination and renewed disinfection (multi stages) of explants, some intact same intact samples of bulb's tissue have obtained. Mohamadi-Dehcheshmeh (2005) and Gholami (2007) despite of performing different types of treatments for disinfection such as alcohol, sodium hypochlorite, mercuric chloride and thermal treatments couldn't separate the intact explants from the bulb explant of Fritillaria, eventually petal explants have been used. They stated that the reason of such condition was the internal contamination of bacteria so that they emphasized on non-bulb explants in micropropagation of Fritillaria. The results of variance analysis table (Table 1) have shown that hormone has a significant effect on formation of bulb. The results suggest the difference in formation of bulb according to the presence or absence of hormone. So that in MS medium of no hormone had yielded the highest number of bulbs, while in mediums with hormone there were no bulb anymore (Table 2). According to (Table 1), hormone had a meaningful effect on the number of root, its percentage, and the percent of bulb, as the highest root, its percentage and the percent of the bulb was obtained in a medium with no hormone (basal medium without hormone) (Table 2). Interactions of 2 hormones are also meaningful for every traits at the level of 1 %. Results have shown that the hormone free media has interacted for all traits.

Table 1: variance analysis of the combination of NAA and BA hormones on studied traits

S.O.V	df	Mean Square Number of Bulb	Percent of Bulb	Number of Root	Percent of Root
NAA	3	70.42**	582.81**	123.26**	735.00**
BA	4	70.42**	582.81**	123.26**	735.00**
NAA*BA	12	70.42**	582.81**	123.26**	735.00**
Error	40	0.02	0.32	0.02	0.20
C.V	-	11.92	18.05	9.01	12.77

**: Significant at 1%

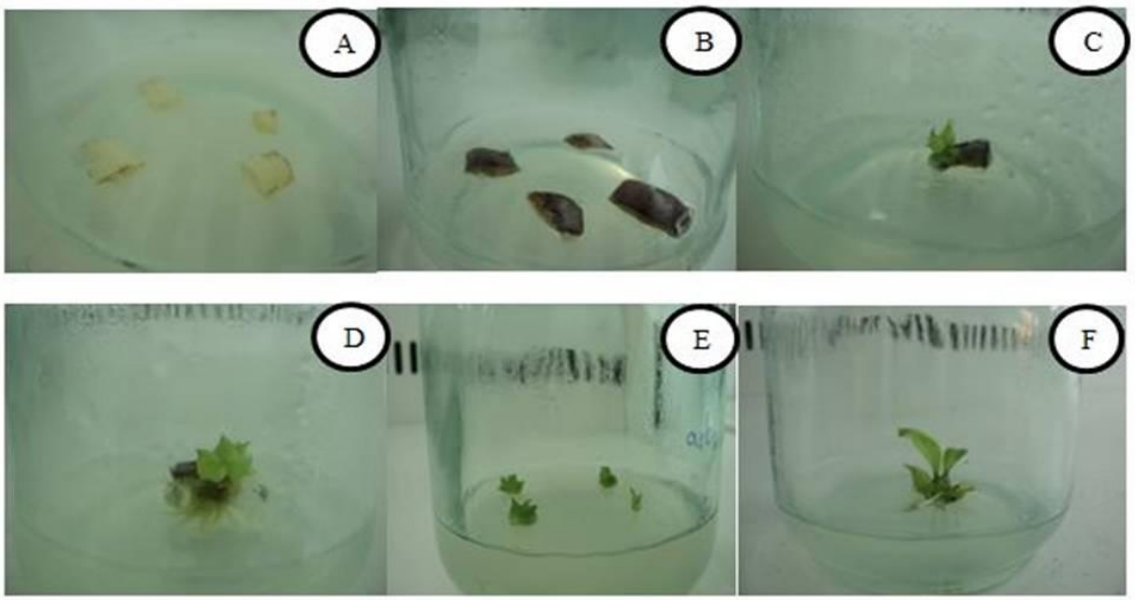

Fig. 1: Direct organogenesis formation steps (formation of bulblet and root) from *Lilium ledebourii* explant, A: *Lilium ledebourii* bulb cultivation, B: Changes in color and becoming inflated of *Lilium ledebourii* in which shows the survival of the *Lilium ledebourii* C: Formation of the first bulbs of *Lilium ledebourii* D: Formation of the first root from regenerated explants in *Lilium ledebourii*, E and F: Total organogenesis from the *Lilium ledebourii* bulb.

Table 2: Comparison of interaction means of studied traits in *Lilium ledebourii* Bioss.

NAA (mg/l)	BA (mg/l)	Number of Bulb	Number of Root	Percent of Bulb	Percent of Root
0	0	21.66a	22.66a	62.33a	70a
0.5	0	0.0b	0.0b	0.0b	0.0b
1	0	0.0b	0.0b	0.0b	0.0b
1.5	0	0.0b	0.0b	0.0b	0.0b
0	0.5	0.0b	0.0b	0.0b	0.0b
0	1	0.0b	0.0b	0.0b	0.0b
0	1.5	0.0b	0.0b	0.0b	0.0b
0	2	0.0b	0.0b	0.0b	0.0b
0.5	0.5	0.0b	0.0b	0.0b	0.0b
0.5	1	0.0b	0.0b	0.0b	0.0b
0.5	1.5	0.0b	0.0b	0.0b	0.0b
0.5	2	0.0b	0.0b	0.0b	0.0b
1	0.5	0.0b	0.0b	0.0b	0.0b
1	1	0.0b	0.0b	0.0b	0.0b
1	1.5	0.0b	0.0b	0.0b	0.0b
1	2	0.0b	0.0b	0.0b	0.0b
1.5	0.5	0.0b	0.0b	0.0b	0.0b
1.5	1	0.0b	0.0b	0.0b	0.0b
1.5	1.5	0.0b	0.0b	0.0b	0.0b
1.5	2	0.0b	0.0b	0.0b	0.0b

Any two means with different litters are significantly different 1% probability level.

DISCUSSION

The results of this study have shown that the presence of growth regulators on studied traits was not effective, in fact the growth regulators and have inhibitory effects on regeneration which corresponds with Niimi (1985) results. It seems that *L. ledebouri* Bioss explants contains adequate amounts of cytokinin hormone and didn't need any external cytokinin and addition of external cytokinin to medium imbalanced hormones. Different effects of BA on the formation of scallion might be due to physiological difference, growing stages, age of leaves and bulbs as the explant and being different genotype (Niimi 1985). Actually; BA did not stimulate the production of bulblets, but also in comparison with control treatment prevented the production of bulblets. in this survey, zero concentration of hormone has produced the largest number and percentage of bulbs. These results agreed with Dabrowski *et al*. (1992) experiments in which have reported the highest frequency of bulblets in a medium of without any growth regulators. Niimi (1985) showed that, adding growth regulators doesn't have any meaningful effect on the number of bulblets in which is compatible with the results of this study.Desirable regeneration of bulblets in a medium without any growth regulator represents those endogenous hormones available in explants will suffice physiological responses. This subject has reported in various types of Lilium (Mizuguchi and Ohkawa 1994). The contents of growth regulators are one of the determinant factors of bulblets regeneration. seasonal changes of endogenous growth regulators previously was reported in bulblets of eastern hybrid Lilium (Kim and Kim 2005). Accordingly, in regeneration of Lilium, requirement or lack of requirement of cytokinin could not be generalized to all types of Lilium, because the amount of cytokinin available in every tissue depend of various factors. According to the existing reports, high concentration of cytokinin leads to the formation of abundant callus (Wozniewski 1991), but the small amount of NAA in the medium causes the improvement of bulbs formation (Takayama 1980), as auxin usually causes cell elongation, swelling of the tissues and meiosis (Bagheri and Safari 2004). In this study in a medium without any growth regulators, rooting have been done to a large extent that these results conflicts with the results of wawrosch *et al.* (2001) who contrasted our results as they reported no rooting in mediums lacking any growth regulators. The results of the number and percentage of root doesn't correspond with the results of Mizuguchi and Ohkawa (1994) Saetiew and Umamanit (2015) Padasht Dahkaei (2005) Kawarabayashi (1993). The existence of some differences in obtained results, in comparison with the results of other researchers could be due to the type of variety. Also, it was reported that due to the seasonal differences of sun angle in different seasons, the red light emitting to the earth is subsequently different. Various researches have shown that such light reaction in plant changes the content of auxin (Tian and Reed 2001). In an experiment conducted by Memar Moshrefi *et al.* (2002) who compared media with different levels of BA with the hormone free medium have shown that the hormone free medium has the highest amount of rooting. While using the different levels

of NAA (0.05 and 0.1 $mg.l^{-1}$) with the medium without any growth regulators caused the highest amount of rooting. Increasing the number of roots with NAA doesn't have corresponded with the effect of functioning auxins in rooting. It seems that Liliums scallion has an adequate amount of endogenous hormone and adding external auxin disrupts the hormone's balance. in a medium lacking hormone firstly regenerating of bulblets and then formation of root was observed, this matter is an advantage for plant in vitro so that the explant can generate firstly bulblets and secondly root.

Conclusion

Using tissue culture technique we succeeded to find the best hormone (only media without hormones) compound in order to regeneration the plant and prevent it from extinction.

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