Plant Regeneration from Protoplasts of *Lilium ledebourii* (Baker) Boiss

S. K. Tahami¹, E. Chamani¹*, and N. Zare¹

ABSTRACT

For half a century, the limitations of obtaining cross-combinations in lilies because of the incompatibility and incongruity between different varieties have been known. Somatic hybridization is one of the most powerful tools for achieving distant interspecific hybrids. For this purpose, protoplast preparation is a first and important step in efficient system for the regeneration of plants from protoplasts. Protoplast isolation method was previously developed in *Lilium ledebourii* (Baker) Boiss. In this study, several valuable experiments were done based on completely randomized design with 3 replications and also each experiment was repeated twice. The results revealed that cell wall and colony formation were better in a liquid medium than those on a semi-solid medium. The highest plating efficiency ($1.34 \times 10^6$ per gr FW) and callus formation was obtained by using a medium containing 1 mg L⁻¹ 2,4-D, 0.2 mg L⁻¹ Kin and 2 g L⁻¹ Yeast extract. Micro calli were formed after one month of culture. Many plantlets were formed on the calli after transfer of the proliferated calli to regeneration medium. The highest plantlet regeneration (91.66%) was obtained by using a medium containing 0.5 mg L⁻¹ NAA, 1.5 mg L⁻¹ BA. Means comparison revealed that the semi-solid MS medium containing 0.5 mg L⁻¹ NAA and 1.5 mg L⁻¹ BA had the highest percentage of regeneration (91.66%), bulb number (8.83), and length (0.7366 cm), root length (0.421cm) and leaf number (13.66) and length (0.5052cm).

Keywords: Callus formation, In-vitro culture, Medium, Proliferated calli.

INTRODUCTION

Among various types of lilies, asiatic, oriental and *L. longiflorum* hybrids have premium potentialities in the florist trade (Kumar *et al*., 2006). *Lilium ledebourii* Boiss is one of the important ornamental and odorant plants. This plant has high adaptability to Iran environmental condition. It has a wide applicability in floral industry as cut flower and potted plant. *Lilium ledebourii* is grown in the eastern slopes of Alburz Mountains, where it is now protected on a small area of degraded beech forest around Damash village, Khangah of Ardabil province and Kalardasht (Rechinger, 1989). Wild populations are currently at risk of rapid eradication because of irregular grazing and poaching (Azadi and Khosh-Khui, 2007).

Somatic hybridization is one of the most powerful tools for achieving distant interspecific hybrids (reviewed in Evans, 1983). In lilies, efficient methods for isolating large quantities of protoplasts from calli initiated from bulb scale tissues (Simmonds *et al*., 1979), pollen (Tanaka *et al*., 1987), and generative cells (Tanaka, 1988) have been reported. Plant regeneration from protoplasts has been achieved (Mii *et al*., 1994), and a modified protocol for the regeneration of plants subsequently established (Godo *et al*., 1996, 1998). Mii *et al*., 1994) first succeeded in regenerating fertile plants derived from the protoplasts of *Lilium formolongi* through

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successful cell division in a solidified medium containing picloram. However, we were not able to obtain divided cells from our preliminary culturing of protoplasts isolated from various kinds of oriental hybrid cultivars according to their protocol. Protoplasts can be isolated from plant tissues or cultured cells. The success of protoplasts isolation depends especially on the condition of the tissue and the combination of enzymes being used. The individual cell or tissue source may require special conditions for successful isolation or for culturing. Protoplasts isolated from cell suspension cultures, generally, are readily obtained and usually regenerate into dividing cells at a reasonable frequency. Moreover, if the plant regeneration is possible from the cells of the suspension culture, the same is often true for the cells regenerated from the protoplast derived from the culture (Tan et al., 1987). Leaf mesophyll cells of a wide range of plants also have been used as a protoplasts source with success by Keller et al. (1982) and Gledie and Keller (1989).

Protoplasts have served as recipient hosts for DNA transformation and are required in somatic hybridization by protoplasts fusion (Stephen, 1995). Protoplasts have been isolated from various genotypes of petunia hybrida (Izhar and Power, 1977), as well as from P. inflata, P. violacea and P. axillaris (Dulieu et al., 1983). Also, there are reports on isolation and culture of protoplasts from leaves and suspension in species such as Allium cepa (Karim and Adachi, 1997), Spathiphylion and Anthurium (Duquenne et al., 2007), Lotus corniculatus (Raikar et al., 2008).

To our knowledge, no report exists on protoplast technology in Lilium ledebouri (Baker) Boiss. In the present study, we report the induction of callus formation from protoplasts in Lilium ledebouri for the first time. To optimize the preparation of protoplasts and cell division frequency, factors such as donor material, composition of enzyme solutions, duration of incubation period, and culture system and the flowering of the regenerates through subsequent protoplast culturing were studied.

**MATERIALS AND METHODS**

Protoplast from young leaves of L. ledebouri were isolated from leaf explants, digested with different enzyme solutions and also purified as described previously (Chamani et al., 2012).

**Protoplast Culture**

Protoplasts were cultured at a density of 1×10⁶ protoplasts ml⁻¹. Two culture systems were tested: liquid culture and semi-solidified culture. For the semi-solidified culture, protoplasts were plated as a thin layer on a MS medium containing 9% mannitol and solidified with 8 g L⁻¹ agar in small petri dishes. For liquid culture (MS without agar, with 9% mannitol), protoplasts were suspended in 4 ml of media in small Petri dishes (5.5 cm diameter).

Five days after protoplast culture, the cells were transferred to Erlenmeyer flasks containing MS liquid medium and incubated at 120 rpm on a rotary shaker in the dark at 25 ± 2°C. After 10 days, every time, 5 ml of fresh medium was added to the culture medium. Star shaped microcalli developed within 15 days of culture. After the development of microcalli visible by naked eye, the cultures were transferred to the light. The plating efficiency was defined and measured as the ratio of cell number undergoing division to the total cultured protoplast number. After one month when the calli attained sizes of 0.5–1.0 mm in diameter, they were transferred to the semi-solidified MS medium at 23°C under fluorescent light (40 µmol per m² s⁻¹) in a 16:8 hour of day/night regime in the culture cabinets.

**Experimental Designs, Data Collection, and Analysis**

In this study, three separate experiments were done and each experiment was repeated twice. In the first experiment, in order to optimize the medium for protoplast
Plant Regeneration and Lilium ledebourii

Table 1. Analysis of variance of the effects of yeast extract and different plant growth regulators on the proliferation and growth of Lilium ledebourii protoplasts.

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>df</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinetin</td>
<td>1</td>
<td>2.659×10^6***</td>
</tr>
<tr>
<td>2,4-D</td>
<td>1</td>
<td>9.191×10^5**</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1</td>
<td>8.911×10^5**</td>
</tr>
<tr>
<td>2,4-D×Kinetin</td>
<td>1</td>
<td>2.025×10^5**</td>
</tr>
<tr>
<td>Yeast extract × Kinetin</td>
<td>1</td>
<td>9.460×10^4**</td>
</tr>
<tr>
<td>Yeast extract × 2,4-D</td>
<td>1</td>
<td>1.381×10^4***</td>
</tr>
<tr>
<td>Yeast extract × 2,4-D × Kinetin</td>
<td>1</td>
<td>1.152×10^4**</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>3.552×10^3</td>
</tr>
<tr>
<td>CV%</td>
<td></td>
<td>1.74</td>
</tr>
</tbody>
</table>

ns, *, **: Non significant and significant at probability level of 5% and 1%, respectively. a Degree of Freedom, b Mean of Squares.
ml), and 2 mg L$^{-1}$ 2,4-D and 1 mg L$^{-1}$ Kin (8.28×10$^5$ cells per ml), respectively (Figure 1). However, other MS suspension media containing 2 mg L$^{-1}$ 2,4-D and 0.2 mg L$^{-1}$ Kin and 2 gr L$^{-1}$ Yeast extract, 2 mg L$^{-1}$ 2,4-D and 0.2 mg L$^{-1}$ Kin, 2 mg L$^{-1}$ 2,4-D and 1 mg L$^{-1}$ Kin and 2 gr L$^{-1}$ Yeast extract and as well as 2 mg L$^{-1}$ 2,4-D and 1 mg L$^{-1}$ Kin produced significantly lower density of cells. Hence, the latest mentioned media was not used in the next experiments (Figure 1).

Thus, the best treatment for proliferation and growth of *Lilium ledebourii* (Baker) Boiss cells was MS medium supplemented with 1 mg L$^{-1}$ 2,4-D, 0.2 mg L$^{-1}$ Kin and 2 gr L$^{-1}$ Yeast extract. The first cell divisions were observed 48 hours after protoplast culture. Cell density was measured every five days and the first density measurement was done 15 days after protoplast culture (Figures 2 and 3).

**Second Experiment: Callus Mass Formation from Plating of Cell Suspension on Solid MS Medium**

The results of ANOVA showed that growth of plated cells and formation of calli (detectable by naked eye) on semi-solidified medium were significantly (P≤0.01) influenced by different combinations of plant hormones and yeast extract (Table 2). Means comparison revealed that the highest and lowest callus induction from plated cell on semi-solidified MS medium were produced on media containing 1 mg L$^{-1}$ 2,4-D and 0.2 mg L$^{-1}$ Kin with 2 gr L$^{-1}$ Yeast extract (29.66) and 2 mg L$^{-1}$ 2,4-D and 0.2 mg L$^{-1}$ Kin (0), respectively (Table 3, Figure 4).

**Figure 1.** The mean effect of different combinations of hormones on density of cells in *Lilium ledebourii*. Columns with different letters are significantly different at P≤ 0.05.

**Table 3.** The effects of different treatments on callus formation from plated cells of *Lilium ledebourii*.

<table>
<thead>
<tr>
<th>2,4-D (mg L$^{-1}$)</th>
<th>Yeast extract (gr L$^{-1}$)</th>
<th>Kin (mgL$^{-1}$)</th>
<th>Number of callus mass formed in each petridish$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>0.2</td>
<td>11$^b$</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>1</td>
<td>7$^d$</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>0.2</td>
<td>29.66$^a$</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1</td>
<td>22.66$^b$</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.2</td>
<td>4$^c$</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1.66$^d$</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>0.2</td>
<td>0$^f$</td>
</tr>
</tbody>
</table>

$^a$ Means followed by different letters in each column are significantly different at P≤ 0.05.
**Figure 2.** Growth and development of *Lilium ledebourri* protoplast in suspension culture: (a) The protoplast release; (b) The cell wall formation of protoplasts; (c) Initiation of cell division after 48 hours; (d) and (e) Subsequent cell divisions, and (f) Formation of cell aggregates.

**Figure 3.** Developmental stage of protoplast in culture suspension: (A) Culture suspension contain release protoplast; (B) Cell proliferation and growth after two days and turbid suspension medium; (C) Formation of cell masses After 14 days, and (D) Cell mass enlargement and callus formation 20 days after culture.

**Figure 4.** Callus induction of *Lilium ledebourri* plated cell suspension on solid MS medium: (A) Plate of cell suspension and callus formation can be detected with the naked eye after 10 days; (B) Formed callus differentiated into plantlets after 29 days, and (C) Regeneration of plantlet (arrow) from callus.
Table 2. Analysis of variance of the effects of different treatments on growth efficiency of plated cells of *Lilium ledebourii* on solidified MS medium and formation of callus.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>MS&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>6</td>
<td>378.762**</td>
</tr>
<tr>
<td>Error</td>
<td>14</td>
<td>2.571</td>
</tr>
<tr>
<td>CV%</td>
<td>14.77</td>
<td></td>
</tr>
</tbody>
</table>

** Significant at probability level of 1%.<sup>a</sup> Degree of Freedom, <sup>b</sup> Mean of Squares.

Third Experiment: Plant Regeneration

The results showed that there was no significant difference (P< 0.01) between initial treatments of cells proliferation suspension culture in all of the measured traits, except bulb length and number of leaves (Table 4). However, regeneration medium significantly (P< 0.01) affected the percentage of regeneration, bulb number and length, leaf number and length, and root number and length (Table 4). Means comparison (Table 5) revealed that the semi-solidified MS medium containing 0.5 mg L<sup>-1</sup> NAA and 1.5 mg L<sup>-1</sup> BA produced the highest percentage of regeneration (91.66%), bulb number (8.83) and length (0.7366 cm), root length (0.421 cm) and leaf number (13.66) and length (0.5052 cm). The results also showed that the highest root number was produced from MS medium containing 0.5 mg L<sup>-1</sup> NAA and 0.5 mg L<sup>-1</sup> BA (4.5). Moreover, hormone free MS medium had the lowest effects in all measured traits (Table 5). No significant differences were found between hormones free MS medium and suspension medium in case of bulb number and length, and root and leaf number. However, these two media significantly differed from each other in terms of root and leaf length and regeneration percent. MS medium supplemented with 0.5 mg L<sup>-1</sup> NAA+1.5 mg L<sup>-1</sup> BA was the best medium for plant regeneration from *L. ledebourii* (Baker) Boiss protoplast-derived calli (Table 5).
**Table 5.** The effect of different treatments on plant regeneration and measured traits from cultured protoplasts in *Lilium ledebourii.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Regeneration %</th>
<th>bulb length(cm)</th>
<th>bulb number</th>
<th>root length(cm)</th>
<th>root number</th>
<th>leaf length(cm)</th>
<th>leaf number</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS media</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>The suspension medium</td>
<td>13.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.123&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.333&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.115&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.125&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5mg/l NAA+0.5mg/l BA</td>
<td>41.66&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.363&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.421&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.355&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5mg/l NAA+1.5mg/l BA</td>
<td>91.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.736&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.583&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.405&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.502&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*a* Means followed by different letters in each column are significantly different at $P \leq 0.05$. 

**DISCUSSION**

No reports have been published on protoplast culture and regeneration in *Lilium ledebourii* (Baker) Boiss. In this study, plants were regenerated from *Lilium ledebourii* (Baker) Boiss protoplasts. The efficiency of protoplast isolation and growth depends on many factors, such as the enzyme mixture, the presence and type of growth regulator, and *in vitro* culture (Assani *et al*., 2001). A liquid medium was better than an agarose-solidified PCA medium for further growth of isolated protoplasts. Although in many crops agarose-solidified media were used. They showed that more frequent browning occurred in an agarose-solidified medium than in a liquid medium. This browning is probably caused by the oxidation of phenolic compounds, which are released from plant cells cultured into the medium (Saxena and Gill, 1986). Oxidation causes severe damage to plant cells or tissue and, consequently, stops their growth. In a liquid medium, this toxic compound might be diluted, thus showing less browning than an agarose-solidified medium does. Ochatt and Power, (1988a) reported that casein hydrolysate is needed for sustaining protoplast division of Williams pear as a source of amino acids. In these experiments, we used yeast extract instead of casein hydrolysate. These results are supported with the findings of Ochatt and Power (1988b) who used protoplasts of several woody fruit crops. Therefore, the positive effect of casein hydrolysate or yeast extract and amino acids on cell division proved its successful effect in protoplast cultures.

This result indicated that combination of Kin and 2,4-D in high concentration inhibited protoplast division, consistent with earlier findings that the combined optimal auxin and cytokinin were relatively effective for cell division in petal protoplast of Petunia hybrid (Oh and Kim, 1994), and in cell suspension protoplast of *Allium cepa* (Karim and Adachi, 1997). Another important factor for protoplast culture is the culture system. In these experiments, protoplasts were cultured either in liquid or solid MS medium comprising $1\times10^5$ and $1\times10^5$ protoplasts ml$^{-1}$. Division of protoplasts obtained in liquid MS medium at optimal density was $1.34\times10^6$ protoplasts ml$^{-1}$. The density of protoplasts influenced the initiation of cell divisions, as has been reported in oat by Hahne *et al.* (1990). The suspension-derived protoplasts of vetiver did not divide on gelrite-solidified medium. In contrast to published data (Kisaka *et al*., 1998) the same gelrite was successfully used for protoplast culture. There are some reports that agarose and phytagel have been used to improve protoplast culture in *Medicago* sp. and *Garcinia atroviridis* Griff., respectively (Te-chato, 1997).

During the present study, cell-wall regeneration, cell division, and callus formation were obtained (Figure5). Among the plant growth regulators we tested, only the combination of 2,4-D and Kin induced cell division. In earlier studies on rose mesophyll protoplasts, NAA and BA were the most efficient growth regulators for the regeneration of microcalli (Marchant *et al*., 1997). In lily protoplasts, the addition of
Figure 5. Callus growth stages and plantlet regeneration from protoplast culture in *lilium ledebourii*. (A, B, C) Growth and differentiation of callus and formation of small bulblet; (D and E) Growth and development of bulblet, and (F) Regenerated plantlets.

picloram to the culture medium was critical of development of microcalli (Horita *et al.*, 2002).

The number of microcalli we obtained was close to those obtained in earlier studies in banana (Assani *et al.*, 2001). Auxin is involved in cell division and callus formation. The high concentration of auxin, does not induce root formation but callus formation (Pierik, 1998).

Shoot organogenesis depends on many parameters, including the genotype, protoplast-derived material, plant growth regulators, culture system, and exposition time of protoplasts on nurse cells (Chabane *et al.*, 2007). Previous investigations showed the impact of genotype on plant regeneration from protoplasts in apple and banana (Assani *et al.*, 2002).

Chang (1999) reported that optimum callus formation from inflorescence explants of *lilium* was obtained in medium containing 3 mg L\(^{-1}\) 2,4-D and 0.25 mg L\(^{-1}\) BA. In another experiment, Naik and Nayak (2005) reported that callus induction in scale explants of *ornithogalum virens* was obtained in medium containing 1-4 mg L\(^{-1}\) 2,4-D and 2 mg L\(^{-1}\) BA. Chen (2005) also stated that, the highest percentage of callus induction from another culture of Narcissus, was obtained in medium containing 1 mg L\(^{-1}\) 2,4-D and 0.5 mg L\(^{-1}\) BA.

The main plant growth regulators such as auxin and cytokinin, alone or in combination, are generally essential for efficient protoplast division in plant systems (Davey *et al.*, 2005). Plant growth regulator concentrations and combinations need to be optimized for each protoplast development step. The following plant growth regulators were tested in our preliminary experiments: 2,4-D, Kin, BA, NAA, and Yeast extract. Only the combination of 2,4-D and Kin induced sustained cell divisions and callus formation. None of the plant growth regulators induced plant regeneration, which may be related to the negative interaction between those plant growth regulators and some metabolites produced by callus tissues. Nagata and Takede (1984) succeeded in
isolating of protoplasts from *Nicotiana tabacum* L. leaves using enzyme solution. They isolated $10^7$ protoplasts from 1 gr fresh weight tobacco leaves. After 3 weeks, shoots were induced in the colonies by transferring them into differentiation medium containing NAA and BA at 4 mg L$^{-1}$ and Kin at 2.56 mg L$^{-1}$. Shoots were transferred to hormone free MS-medium to induce root formation.

Concentration of 0.2 mg L$^{-1}$ 2,4-D, 1 mg L$^{-1}$ NAA and 0.5 mg L$^{-1}$ Zeatin, produced the highest protoplast regeneration and cell division (Pongchawee et al., 2006). According to Tamura et al. (1992) report, high concentration of glucose (0.5M) requested the best outcome for protoplast culture.

They also proved that addition of Zeatin (1 mM) and NAA (10 mM) gives the normal size of the colonies formed. Changing the protoplast culture medium to 5.4 mM NAA and 2.3 mM Zeatin was suitable for protoplast regeneration, therefore, that was the appropriate density of cells in the medium (Tian et al., 1999). In case of bulb number, Takayama et al. (1991) also achieved similar results. They reported that high ratio of cytokinin in culture medium produced high bulb number from liliium scale explants. Nhu (1998) reported that there was a direct relationship between the concentration of cytokinin and bulb formation. However, the use of cytokinin alone was more effective in bulb formation than the combination of cytokinin with auxin.

It is reported that solid MS medium containing 2 mg L$^{-1}$ BA produced the optimal bulb number from scale of *L. pyrenacium* and *L. longiflorum* (Hassey, 1976). Azadi and kosh-khui (2007) revealed that the highest bulb number of *lilium ledebourii* was produced from solid MS medium containing 0.1 mg L$^{-1}$ NAA and 1 mg L$^{-1}$ BA. Hence, the mentioned results confirm our experimental findings, too. However, the ratio of Auxin to Cytokinin was related to root and shoot regeneration. In case of root number, it seems that decreasing BA concentration in the medium, increased the root number of explants. This result is consistent with the results of Izadi *et al.* (2011). Nhu (1998) also reported application of NAA significantly increased the root number in *L. longiflorum* compared to control. Pierik (1998) reported that cytokinin in low concentrations stimulates cell division, but usually the higher concentrations prevent the formation of root. The result of our experiment revealed that auxin by influencing cell elongation was able to produce more elongated shoots and also leaf length. Pierik *et al.* (1975) reported that auxins increased shoots length in hyacinth flower. Asoa (1992) showed that leaves formation occurred under *in vitro* culture of *Lilium japonicum* when the bulb was cultivated in a medium containing 2 mg L$^{-1}$ BA or 9 mg L$^{-1}$ GA3, but was stopped when transferred to a medium containing a low concentration of NAA (0.01 mg L$^{-1}$) and BA (0.001 mg L$^{-1}$), indicating that leaves emergence was directly affected.

**CONCLUSIONS**

In conclusion, we described here, for a first time, an efficient protocol for plant regeneration from protoplasts of *L. ledebourii* (Baker) Boiss. In this protocol, the efficiency of viable protoplast recovery, cell proliferation and callus formation, and finally plant regeneration were relatively high and reproducible. Thus, this protocol can be utilized in protoplast fusion projects, and also genetic manipulation of *Lilium* species.

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پازرایی گیاه سوسن چلعراغ از طریق کشت بروتوبلاست

چکیده

نیم فرن است که محدودیت‌های موجود در تلاقی بین سوسن‌ها (دگرگونه‌افشانی) بدلیل وجود ناسازگاری و عدم تقارن زننگی بین وارنه‌های مختلف شناخته شده است. هیری بر سوماتیکی با انتزاع بروتوبلاست‌ها یکی از ابزارهای اصلی در دورگاه گیری بین گونه‌ای است، برای این منظور، اولین قدم وجود یک سیستم بهینه برای آماده‌سازی و پازرایی گیاه از بروتوبلاست‌ها بانست. روش جداسازی بروتوبلاست در سوسن چلعراغ قبل بهینه شده است. این آزمایش بر اساس طرح کاملاً تعادلی با تکرار انگشت گرفت و هر آزمایش دو بار تکرار شد. نتایج حاصل از آزمایش نشان داد که تشکیل دیواره سلولی و کلوتوی در محیط باعث بهینه جامد آگازه بهتر بود. بیشترین تراکم کشت و تشکیل کالوس در محیط کشت حاوی یکسال میلی‌گرم در لیتر-D و 2-4، 20 میلی‌گرم در لیتر کیتنین به همراه دو گرم در لیتر عصاره مخمر بسته آمد (10×10×3). کالوس‌های کوچک به از یک ماه کشت تشکیل شدند. گیاه‌های زیادی پس از انتقال کالوس‌های رشد یافته به محیط کشت حاوی NAA تنظیم کننده‌های رشد گیاهی تشکیل شدند. محیط حاوی 5/0 میلی‌گرم در لیتر BA به همراه با NAA 91/66 درصد بیشترین پازرایی گیاهی می‌یافت. مقایسه میانگین‌های BA و NAA در لیتر کشت حاوی 75/66 میلی‌گرم در لیتر به همراه با 15/0 میلی‌گرم BA در لیتر کشت حاوی 75/66 میلی‌گرم NAA و 75/66 میلی‌گرم BA 86/80 درصد، تعداد پازرچه با میانگین 76/34 طول بیشتر با میانگین 67/44 طول برگ با میانگین 13/46 درصد، تعداد برگ با میانگین 76/44 طول برگ با میانگین 13/46 و تولید شد.