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In vitro micropropagation of summer snowflake (*Leucojum aestivum* L.) from bulb scale and immature embryo explants

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Abstract

Leucojum aestivum is an important medicinal and ornamental plant due to the its alkaloid contents and beautiful flowers. However, this species suffers from low propagation rate under natural conditions and is threatened by excessive collection from nature. In this study for the purpose of rapid in vitro propagation of *L. aestivum,* two and four bulb scale, and immature embryo explants were cultured on Murashige and Skoog (MS) medium containing different concentrations of 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA). The concentration of plant growth regulators, their combinations, and explant type had major influence on *in vitro* bulblet regeneration. The highest mean number of bulblets per explant (6.17) on bulb scales was obtained from MS medium containing 1 mg/l BAP and 1 mg/l NAA after 9 months in culture. Bulblet regeneration was much lower on immature embryos than bulb scales, and the highest number of bulblets per embryo explant (2.27) was achieved on MS medium supplemented with 0.5 mg/l BAP and 4 mg/l NAA after 5 months of culture initiation. In vitro regenerated bulblets were successfully rooted on MS medium with 1 mg/l NAA and finally transferred to potting mixture for acclimatization to external conditions.

Introduction

Leucojum aestivum L. (the summer snowflake), which belongs to the Amaryllidaceae family, is a bulbous plant that grows widely in England, Continental Europe, especially Southeast Asia, and the Mediterranean countries such as Türkiye (Darlington and Janika-Ammal, 1946; Tubives, 2024). In Türkiye, *L. aestivum* grows in many parts of Marmara, Central and Eastern Anatolian Regions at altitudes of 0-1100 m and likes wet habitats, including damp woodland, riversides, and swamps (Mill, 1984). It bears attractive white colored scented flowers with green or occasionally yellow spots at the end of petals and therefore can be used as cut flower and border plants (Kohut et al., 2007). Galantamine and lycorine are two the most important alkaloids obtained from *L. aestivum*. Galantamine is an acetylcholinesterase inhibitor utilized to cure Alzheimer, and valuable alkaloid lycorine can be used as a strong antiviral, cytotoxic, and antimitotic agent (Diop et al., 2007). Although it is possible to artificially synthesize galantamine, *L. aestivum* and narcissus are the main sources for the extraction of these alkaloids for use in the pharmaceutical industry (Diop et al., 2007).

Wild populations of *L. aestivum* are rapidly eroding and are not sufficient to meet increasing market demand due to limited availability. In addition, the wild plants are heterozygous, which results in supply of non-

uniform material to pharmaceutical industry. This can cause many problems in the extraction of these alkaloids and maintaining quality of the products obtained from these plants. Traditional methods of *L. aestivum* propagation are very slow, time-consuming and labor-intensive. Development of *in vitro* micropropagation methods can ensure rapid and uniform multiplication of *L. aestivum* plants, which may serve as secure chain for extraction of alkaloids and use of these plants in the ornamental plants growing industry by maintaining the quality.

Few studies have been previously carried out on in vitro micropropagation of *L. aestivum*. Ptak and Cierniak (2003) obtained a maximum of 1.5 bulblets per explant from *L. aestivum* bulb scales cultured on MS medium containing BAP, NAA and 2,4-D, but they did not obtain plants from these bulblets. Although Kohut et al. (2007) achieved 11.77 shoots per explant from in vitro cultured bulb scales on MS medium supplemented with 1 mg/L BAP and 0.1 mg/L NAA, they could not produce bulblets large enough to be transferred to soil from these shoots. Similarly, Ptak et al. (2013) did not develop bulblets from the embryos regenerated at high rates from *L. aestivum* leaf explants cultured on MS nutrient medium containing 50 µM of 2.4-D. In another study conducted by Ptak (2014) on in vitro bulblet production in *L. aestivum* using growth retardants, sucrose, GA_3, solid and liquid medium, 100% bulblet formation was achieved, but no information was given regarding the number of bulblets obtained per explant. Recently, Abedinimazraeh and Kalatehjari (2021) cultured two and four bulb scale segments on MS medium supplemented with 10 different combinations of BAP, NAA and Kinetin, and obtained the highest rate of bulb regeneration (90%) and the number of shoots per explant (2.8) from four bulb scales on nutrient medium containing 1 mg/L BAP and 0.5 g/L NAA. In this study, bulblet production remain low and it was stated that the development of bulblets transferred to soil was also very slow.

Low in vitro bulblet production in previous studies and failures in transferring these bulblets to soil indicated the need for new studies on micropropagation of L. *aestivum*. On the other hand, in our previous studies, we were able to achieve high frequencies and high numbers of in vitro bulblets per explant from immature embryos and bulb scales of the bulbous plants *Sternbergia fischerina* (Mirici et al., 2005), *Muscari muscarimi* (Uzun et al., 2014) and *Fritelleria imperialis* (Çakmak et al., 2016*)*. The bulblets obtained in these studies were transferred to the soil after hardening using activated charcoal, different gelling agents and temperatures, and were successfully grown under external conditions. Therefore, present study aimed to develop an efficient in vitro propagation protocol using bulb scales and immature zygotic embryos for *L. aestivum.*

Materials and Methods

Plant material and surface-sterilization

Bulbs and immature fruits of *L. aestivum* were collected at the same time from the natural flora in May, around Beysehir Lake, Konya province of Türkiye. Soon after collection, the bulbs were washed in running tap water, dried, and stored at cool dry place in the dark at room temperature for 45 days. After removing the roots and outer dry scales, bulbs and immature fruits were washed and surface-sterilized with 80% commercial bleach (Axion 5%) for 20 min. Thereafter, bulbs and fruits were rinsed with sterile distilled water three times each with 5 min.

Culture of bulb scales

The bulbs were longitudinally sliced in laminar flow cabinet to obtain two and four scale explants with 4-5 mm wide and 8-10 mm long and containing approximately 2 mm of basal tissue. After isolation, bulb scales were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 0.5-4.0 mg/l 6-benzylaminopurine (BAP), 0.25–2.0 ms/1 α-naphthaleneacetic acid (NAA), 3% sucrose, and %0.8 agar in glass Petri dishes. The pH of all media was adjusted to 5.7 before autoclaving at 121 °C under 1.4 kg/cm^2 pressure for 20 minutes. Unless otherwise stated, all cultures were incubated at 24 °C in growth cabinets under white fluorescent light (35 µmol photons m^{-2} s^{-1}) with a 16-hour photoperiod.

Culture of immature zygotic embryos

In the culture of immature embryos, the four-stage method developed by Bronsema et al. (1997) and Mirici et al. (2005) was used with significant modifications. After surface-sterilization, seeds of *L. aestivum* were cut open, and the 3 mm long immature embryos were isolated in a laminar flow cabinet under a stereo microscope. The isolated embryos were then cultured on N6 medium containing 0.5-4.0 mg/l BAP, 0.25–4.0 mg/1 NAA, 2.3 g/l L-proline, 200 mg/l casein hydrolysate, 2% sucrose, and 0.25% gelrite in glass Petri dishes and incubated in the dark. After two weeks, embryo explants were transferred to the same media with the addition of 30 g/l mannitol and subcultured at two-week intervals. Then, the explants were transferred to MS medium supplemented with 6% sucrose and 7% agar and gradually exposed to the light by wrapping the Petri dishes with towel paper for the first three days. Finally, the cultures were transferred to MS medium containing 2% sucrose and 7% agar, and subcultured on this medium every 2 months.

Rooting and ex vitro culture of bulblets

The regenerated bulblets on both types of explants were rooted on MS medium containing 0.5, 1 and 2 mg/l NAA, 6% sucrose, and 7% agar. Acclimatization was carried out carefully by removing agar adhering to the roots of bulblets under running tap water. Bulblets

between 5-10 mm diameter were transferred to the potting mixture in wooden cases for acclimatization in the greenhouse at 22-24 °C under natural light.

Statistical Analysis

The experiments were set up according to the randomized plot design and consisted of 100 x 10 mm petri dishes or Magenta vessels with 3 replicates, with 5–10 explants in each treatment. Before statistical analysis, Arcsin transformation was performed to percentage values (Snedecor and Cochran, 1976). The significance between treatments was determined by analysis of variance at the ≤ 0.05 significance level using (ANOVA), and the difference between the means was compared with the Duncan test.

Results

Bulblet regeneration from bulb scales

Bulb scale explants of *L. aestivum* containing two or four scale segments were cultured on MS medium supplemented with various combinations of BAP and NAA. One week after the bulb scales were cultured on medium, significant increases in their volume were observed. Callus formation was visible at the base of the majority of the bulb scales 15–20 days after culture initiation. Bulb scales turned brown and died completely 3-4 months after culture. However, this did not affect regeneration and growth at basal plates that attached scales. A large number of shoots developed on the callus developed from basal plate after 4 months (Fig. 1a), but not all of these shoots turned into bulblets. Direct bulblet formation was also seen on some explants, especially on four scales. Fully developed bulblets were obtained 6 to 8 months after the culture initiation on bulb scales in Magenta culture vessels (Fig. 1b and c).

Shoot regeneration and bulblet formation after 4 and 9 months in culture, respectively, from two or four bulb scale explants on MS medium supplemented with various concentrations of BAP and NAA are given in Table 1. Since the effect of explant type and media x explant interaction on shoot regeneration and bulblet formation are statistically insignificant, the Duncan test was performed on the average of explants. As can be seen from Table 1, the majority of the explants regenerated 100% shoots on most of the media tested. Reduced shoot regeneration was noted on MS medium containing 0.5 and 4 mg/l BAP. Considering the explant average, the highest mean number of shoots per explant (11.02) was obtained from the MS medium containing 1 mg/BAP and 1 mg/l NAA. However, this medium was statistically in the same group with the medium containing 2 mg/l BAP and 2 mg/l NAA (8.47), and the medium supplemented with 2 mg/l BAP and 1 mg/l NAA (8.14). The lowest number of shoots per explant, with an average of 0.83, were obtained from the medium containing 4 mg/l BAP and 0.25 mg/l NAA.

The mean number of bulblets per explant according to 2 and 4 scales average varied between 0.83 and 6.17 on MS medium containing different concentrations of BAP and NAA (Table 1). As in shoot regeneration, the highest mean number of bulbs per explant (6.17) was achieved from the medium containing 1 mg/l BAP and 1 mg/l NAA. However, this medium was statistically in the same group with the medium containing 2 mg/l BAP and 0.5 mg/l NAA (5.75), and the medium supplemented with 2 mg/l BAP and 1 mg/l NAA (4.50). The lowest bulb formation was obtained on MS medium supplemented with 4 mg/l BAP and 0.25 mg/l NAA, which was the same in shoot regeneration.

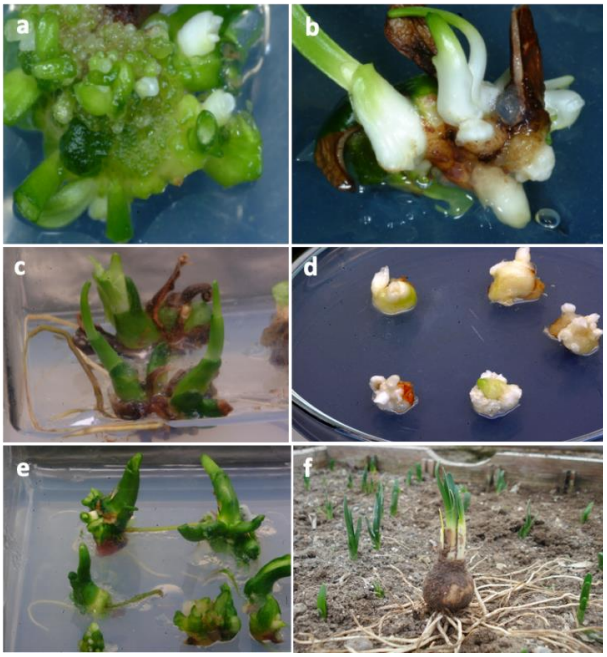

Figure 1. In vitro bulblet regeneration from bulb scale segments and immature embryos of *Leucojum aestivum*. (**a**) Prolific shoot regeneration on a medium containing 1 mg/l BA and 1 mg/l NAA on bulb scales after 4 months in culture. Development of shoots into bulblets after 6 (**b**) and 8 months (**c**) in culture on bulb scales. Shoot regeneration after 3 months (**d**) and bulblet formation after 5 months (**e**) on immature embryos on MS medium supplemented with 0.5 mg/l BAP and 4 mg/l NAA. (**f**) Acclimatized plant growing in wooden containers in greenhouse.

Bulblet regeneration from immature zygotic embryos

Callus initiation started within 10 days on immature embryos, and there was a significant increase in callus formation after they were transferred to the second medium containing mannitol. When the explants were transferred to MS medium containing 6% sucrose and 7% agar, shoot formation increased in the light conditions (Fig 1d). The transformation of shoots into bulbs occurred 4-5 months of culture on MS nutrient medium containing 2% sucrose and 7% agar in magenta culture vessels (Fig. 1e).

The majority of immature embryos cultured on nutrient medium containing different amounts of BAP and NAA formed shoots (Table 2). The lowest shoot formation rate was obtained from the nutrient medium containing 4 mg/l BAP and 0.25 mg/L NAA. However, the difference between the media used in terms of shoot

Table 1. Shoot regeneration and bulblet formation after 4 and 9 months in culture respectively from two or four bulb scale explants of *L. aestivum* on MS medium supplemented with various concentrations of BAP and NAA

Growth regulators (mg/l)		Explants producing shoots (%)			Mean number of shoots per explant after 4 months*			Mean number of bulblets per explant after 9 months*		
BAP	NAA	2 scales	4 scales	Explant Average	2 scales	4 scales	Explant average	2 scales	4 scales	Explant average
0.5	0.25	100	100	100 ^{a}	3.61	3.75	3.68 ^{cde}	2.72	2.89	2.80^{cd}*
0.5	0.5	100	100	100 ^{a}	6.17	4.00	5.08 ^{bcde}	3.83	2.83	3.33^{cd}
0.5	1	100	100	100 ^{a}	2.13	1.87	2.00 ^{de}	2.07	1.87	1.97^{de}
0.5	2	100	100	100 ^{a}	3.33	5.30	4.32 ^{bcde}	3.00	5.30	4.15^{bc}
1	0.25	100	100	100 ^{a}	3.97	3.60	3.78 ^{bcde}	3.90	2.50	3.21^{cd}
1	0.5	100	100	100 ^{a}	4.07	3.07	3.57 ^{cde}	2.07	3.07	2.57^{cd}
1	1	100	100	100 ^{a}	10.17	11.87	11.02 ^{a}	5.67	6.67	6.17^{a}
1	2	100	100	100 ^{a}	4.95	4.31	4.63 ^{bcde}	3.89	4.31	4.10^{bc}
2	0.25	67	100	83 ^{ab}	3.50	4.00	3.75 ^{bcde}	3.50	4.00	3.75^{cd}
2	0.5	100	100	100 ^{a}	5.83	7.33	6.58 ^{bcd}	5.83	5.67	5.75^{ab}
2	1	100	100	100 ^{a}	8.17	8.11	8.14 ^{abc}	4.00	5.00	4.50^{abc}
2	2	93	100	97 ^{a}	9.07	7.87	8.47 ^{ab}	4.33	4.20	4.27^{b}c
4	0.25	33	33	33 ^{c}	0.67	1.00	0.83 ^{e}	0.67	1.00	0.83^{e}
4	0.5	100	100	100 ^{a}	8.07	3.80	5.93 ^{bcd}	3.57	3.13	3.35^{cd}
4	1	100	100	100 ^{a}	4.63	5.27	4.95 ^{bcde}	3.43	4.27	3.85^{cd}
4	2	50	83	66 ^{b}	3.00	8.72	5.86 ^{bcd}	3.00	4.92	3.96^{bc}
Average		90	95		5.24	5.08		3.47	3.85	

Values within a column followed by different letters are significantly different at the 0.05 level.
*From bulb scales which produced shoots or bulblets.

formation rate was found to be statistically insignificant. The highest number of shoots per explant (6.15) was achieved on MS medium containing 1 mg/l BAP and 1 mg/l NAA. However, the difference between this medium and the medium containing 2 mg/l BAP and 1 mg/l NAA, 0.5 mg/l BAP and 1, 2 or 4 mg/L NAA was found to be statistically insignificant (Table 2). Although the number of bulbs per explant varied between 1.17 and 2.27, the highest number of bulbs was achieved from the medium containing 0.5 mg/l BAP and 4 mg/l NAA. The lowest bulb number per explant was obtained from the medium supplemented with 4 mg/l BAP and 0.25 mg/l NAA (Table 2).

Rooting and acclimatization of bulblets

The regenerated bulblets were separated from the mother explant and cultured on MS medium containing 0.5, 1 and 2 mg/l NAA in Magenta vessels to induce root formation. In this medium, the diameters of the bulbs increased, and 100% rooting was achieved. The highest number of roots per bulb (7.0) was obtained from the nutrient medium containing 1 mg/L NAA, and this value was found to be statistically significant compared to the number of roots obtained from the nutrient medium containing 0.5 mg/L (2.67) and 2 mg/L NAA (3.67). All rooted bulblets were transferred to pots for acclimatization. Irrespective of the medium used and the length of roots, all bulblets grew well in the potting mixture (Fig. 1f).

Discussion

Plant biotechnology provides an opportunity to introduce techniques through many approaches such as

Table 2. Shoot regeneration and bulblet formation after 3 and 5 months in culture respectively from immature embryo explants of *L. aestivum* on MS medium supplemented with various concentrations of BAP and NAA

Growth regulators (mg/l)		Explants producing shoots (%)	Mean number of shoots per explant after 4 months*	Mean number of bulblets per explant after 5 months*
BAP	NAA			
1	0.25	90	2.62 ^{c}	1.27 ^{ab}
2	0.25	87	2.51 ^{c}	1.33 ^{ab}
4	0.25	50	2.61 ^{c}	1.17 ^{b}
1	1	100	6.15 ^{a}	1.44 ^{ab}
2	1	90	5.03 ^{ab}	1.71 ^{ab}
4	1	100	3.25 ^{bc}	1.40 ^{ab}
0.5	1	93	4.17 ^{abc}	2.12 ^{ab}
0.5	2	100	5.67 ^{a}	2.23 ^{ab}
0.5	4	100	4.21 ^{abc}	2.27 ^{a}

Values within a column followed by different letters are significantly different at the 0.05 level.
*From bulb scales which produced shoots or bulblets.

plant regeneration (Ochatt et al., 2018), gene transfer (Barik et al., 2005), somaclonal variation (Roy et al., 2001) and plant multiplication. The present study reports in vitro bulblet induction in high-valued ornamental and medicinal plant *L. aestivum* using bulb scale and immature embryo explants. *L. aestivum* has low propagation rate in nature, and its population is continuously under threat due to a number of socio-economic reasons. Establishment of a suitable *in vitro* propagation system will help in conservation of this plant. It will also ensure production of high-quality plants that could also be used for the extraction of industrial metabolites favorably. If the bulblet regeneration in terms of the number of bulblets per explant on the two types of explants is compared, bulb scales had more potential to induce bulblets compared to immature zygotic embryos as reported in previous studies on *Urginea marititima* (Aasim et al., 2008), *L. Candidum* (Khawar et al., 2005, Sevimay et al., 2005), *Ornithogalum ulophyllum* and *Muscari macrocarpum* (Ozel and Khawar, 2007; Ozel et al., 2007).

BAP and NAA combination had variable effect on regeneration behavior of two and four bulb scale explants, resulting in promotion of a variable number of bulblets regeneration in each case. At the start of study, it was assumed that four scale explants, owing to their number would induce a higher number of bulblets than two scale explants. If the results of the two experiments are compared, a number of bulblets regenerated on two scale explants are not statistically different from those induced on four scale explants using MS medium containing the same variants of BAP and NAA. It was further understood that there was possibility to induce more bulblets per explant if necrosis could be inhibited. Ethylene synthesis in *L. aestivum* (Adkins et al., 1993; George et al., 2008; Ptak et al., 2009; Ptak et al., 2010) due to use of auxin-NAA (Chatfield and Raizada, 2008) leading to precisely transcription of specific isogenes of the ethylene biosynthetic enzyme could be possible reason of necrosis (George et al., 2008). There is possibility that this phenomenon was more active on scales close to or in touch with regeneration medium. This was very visible on two and four scale explants. Two scale explants showed necrosis everywhere except on binding segments of scales that regenerated bulblets. The same was noted on four scale explants, where outer scales close to or in touch with culture medium induced full or partial necrosis compared to internal two scales that were not in touch with culture medium.

Previous studies have shown that an efficient in vitro micropropagation system for *L. aestivum* could not be established. Ptak and Cierniak (2003) did not obtain plants from the low number of bulblets (1.5 bulblets per explants) regenerated from bulb scales of *L. aestivum* cultured on MS medium containing 10 µM NAA and 0.5 µ BAP. Kohut et al. (2007) reported that they obtained a high number of shoots (11.77) per explant from bulb scales of *L. aestivum* cultured on MS medium containing 1 mg/L BAP and 0.1 mg/L NAA, but they stated that these shoots did not develop into bulblets large enough to be transferred to the soil. Moreover, Ptak et al. (2013) also could not achieved bulblet regeneration from the embryos developed on leaf explants of *L. aestivum* cultured on MS supplemented with 50 µM of 2.4-D. In another study carried out by Ptak (2014) to determine the effects of growth retardants, sucrose, GA_3, solid and liquid media on in vitro regeneration of *L. aestivum*, 100% bulblet formation was obtained from the explants cultured but no information about the number of bulblets per explant was provided. In a study conducted by Abedinimazraeh and Kalatehjari (2021) using bulb scales, 10 different combinations of BAP, NAA and Kinetin, they obtained the highest frequency of bulblet formation (90%) and the highest number of bulblets per explant (2.8) from MS medium with 1 mg/L BAP and 0.5 g/L NAA. However, in this study, the number of bulblets obtained per explant was quite low and the development of bulbs transferred to soil was also very slow.

In previous studies on in vitro micropropagation of *L. aestivum* given above, no bulblets were obtained from embryos and shoots obtained in high numbers. In addition, in the studies where bulblets were obtained, the number of bulblets per explant remained low. Moreover, since bulblets large enough to be transferred to soil could not be obtained, there were also failures in adaptation to external conditions. On the other hand, in the current study, shoot regeneration was achieved from majority of the bulb scales cultured on MS medium containing different concentrations of BAP and NAA, and more than 50% of these shoots developed into bulblets. The highest bulblet regeneration frequency (%100) and the number of bulblets per explant (6.17) were obtained from the MS medium containing 1 mg/L BAP and 1 mg/L NAA as the average of two and four scale leaves. These bulblets, rooted and hardened in vitro, continued to develop by adapting to external conditions at a rate of 100% when they were transferred to the soil.

Conclusion

The results obtained from the current study in *L. aestivum* provided significant advantages over previous studies in terms of in vitro bulblet production and adaptation of bulblets to external conditions. This work also showed that *L. aestivum*, an important medicinal and ornamental plant and is threatened by excessive collection from nature, can be propagated efficiently by in vitro techniques. It is further concluded that use of this novel methodology would favorably facilitate its use in genetic transformation and functional genomic studies in *L. aestivum.*

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