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Effect of Photoperiod, Plant Growth Regulators and Explant Size on *In Vitro* Bulblet Formation in Snowdrop

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Abstract

Plant tissue culture techniques have a widespread usage in rapid, easy and continuous propagation of bulbous and tuberous plants, called geophytes. The genus *Galanthus* (commonly called snowdrops), perennial bulbiferous geophytes belonging the *Amaryllidaceae* family, contain several high-value alkaloids with pharmaceutical use to treat Alzheimer's disease. The objective of this study was to assess the effect of photoperiod, plant growth regulators and explant size on *in vitro* bulblet formation in snowdrop (*Galanthus woronowii*). Bulb twin-scale explants were cultured on MS medium containing different combinations of BAP with various concentrations of NAA, IAA and IBA under 16/8 h light/dark and 24 h dark conditions. Effect of plant growth regulators was significant on the number of bulblet, bulblet diameter, bulblet weight, and the number of bulblet bigger than 5 mm in diameter. Continuous dark application resulted in highly significant increases in bulblet formation, as compared to light treatment. The maximum number of bulblet (4.83) was recorded from 4-piece explants treated with 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA hormone combination kept in 24 h dark condition. Dark treatment resulted in an increase of 57.33% in the number of bulblet bigger than 5 mm in diameter. Explant size showed no effect on any of the attributes studied. In conclusion, this study revealed that 24 h dark treatment has a very important positive effect on *in vitro* bulblet formation in snowdrop.

Keywords: bulbous plants, galanthus woronowii, geophyte, micropropagation, tissue culture

Fotoperyot, Bitki büyüme Düzenleyicileri ve Eksplant Büyüklüğünün Kardelende *İn Vitro* Soğancık Oluşumuna Etkisi

Öz

Geofit olarak adlandırılan soğanlı ve yumrulu bitkilerin hızlı, kolay ve seri üretiminde bitki doku kültürü teknikleri çok yaygın olarak kullanılmaktadır. *Amaryllidaceae* familyası içinde yer alan *Galanthus* (Kardelen) cinsi, Alzheimer hastalığının tedavisinde kullanım potansiyeli olan yüksek değerli alkaloitler içermektedir. Bu araştırma, fotoperyot, bitki büyüme düzenleyicileri ve eksplant büyüklüğünün *Galanthus woronowii* kardelen türünde *in vitro* soğancık oluşumu üzerine etkilerini belirlemek amacıyla yürütülmüştür. Eksplant kaynağı olarak kullanılan çift soğan pulları BAP'ın farklı dozlarda NAA, IAA ve IBA ile oluşturduğu hormon kombinasyonları ilave edilen MS ortamında, 16/8 saat aydınlık/karanlık ve 24 saat karanlık şartlarda kültüre alınmıştır. Bitki büyüme düzenleyicilerin soğancık sayısı, soğancık çapı, çapı 5 mm ve üzerinde olan soğancık sayısı ve soğancık ağırlığı üzerine etkileri önemli bulunmuştur. Sürekli karanlık uygulaması soğancık oluşumunda, 16/8 saat aydınlık/karanlık uygulamasına göre, çok önemli artışlara yol açmıştır. En yüksek soğancık sayısı (4.83) 24 saat karanlıkta tutulan ve 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA ilave edilen MS ortamındaki dört parçalı eksplantlardan alınmıştır. Karanlık uygulaması çapı 5 mm ve üzerinde olan soğancık sayısında %57.33'lük bir artışa yol açmıştır. Eksplant büyüklüğü çalışmada incelenen hiçbir özellikte önemli farklılık oluşturmamıştır. Sonuç olarak, bu çalışma 24 saat karanlık uygulamasının kardelende *in vitro* soğancık oluşumunda çok önemli olumlu etki yaptığını ortaya koymuştur.

Anahtar Kelimeler: doku kültürü, galanthus woronowii, geofit, mikroçoğaltım, soğanlı bitkiler

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Introduction

Türkiye, located at the intersection point of the three basic floristic regions, has a very remarkable plant diversity due to its versatile soil types and climatic conditions (Avcı, 2005). It has been stated that, Türkiye's flora comprises of around 12.000 plant species with a 34.5% of endemism (Özhatay, 2005). Bulbous and tuberous plants, known as geophytes, have a considerable importance in this plant richness of the country (Kahraman, 2020; Şekeroğlu et al., 2013; Yüzbaşıoğlu, 2012).

The genus *Galanthus* (commonly known snowdrops), a member of the *Amaryllidaceae* family and often called as Kardelen in Turkish, is one of naturally grown geophytes in most part of Türkiye, with the species of *Galanthus elwesii* and *Galanthus woronowii* showing a great economic importance (Demir, 2010). *Galanthus* species are of commercial and medicinal importance as ornamental plants due to their splendid flowers and the presence of *Amaryllidaceae* alkaloids such as galantamine, tazettin and licorine (Babashpour-Asl et al., 2016b; Bores & Kosley, 1996). Galantamine alkaloid has been recently considered a good source for curing Alzheimer's disease (Ay et al., 2018; Heinrich & Teoh, 2004).

In Türkiye, geophytes are commonly collected from the natural flora, as in the most majority of medicinal and aromatic plants. Their bulbs are removed from nature habitat when the leaves are still green and usually before seed formation by removing the entire plant, leaving no plant parts that will provide further vegetative growth (Entwistle et al., 2002). Therefore, the effect of plant collection from nature has been much more negative, particularly on bulbous plants, causing their populations to gradually decrease and face with danger of extinction (Koyuncu & Ekim, 1984). *Galanthus woronowii*, native to the north-east Türkiye and the west and central Caucasus, is classified under the category of Vulnerable (VU), facing a high risk of extinction, in the Red Data Book of Turkish Plants (Ekim et al., 2000). In Türkiye, a number of legislative regulations have long been undertaken for conservation and sustainability of bulbous plants (Resmi Gazete, 2017) and a bulletin has been published in each year with a quota limitation for exporting natural bulbs (Resmi Gazete, 2021).

Snowdrop is a plant that can reproduce by seeds or vegetatively by the formation of new bulblet. It takes 4 to 5 years for snowdrop bulbs produced from seed to reach flowering size, about 4-5 cm circumference (Atay, 1996; Tıpırdamaz, 2003). Since the life cycle of snowdrop is long and its reproduction rate is low, it is of considerable importance using propagation methods that will provide easy, fast and mass production (De Klerk, 2012). In this context, tissue culture techniques are widely used in rapid and mass production of endangered plants with some problems in propagation by seed (Karaoğlu, 2010; Seydi et al., 2019).

In vitro propagation has previously been reported for various bulbous plant species such as *Galanthus elwesii* (Zencirkıran & Mengüç, 2004), *Eucomis zambesiaca* (Cheesman et al., 2010), *Galanthus woronowii* (Yüzbaşıoğlu & Dalyan, 2017), *Fritillaria imperialis* (Seydi et al., 2019), *Cyclamen persicum* (Cengiz, 2019) and *Narcissus tazzeta* (Khonakdari et al., 2020).

Various explant sources such as immature embryos, ovaries, leaves, bulb scales and flowers have been reported for snowdrops and bulb-twin scales are the most commonly preferred explants (Babashpour-Asl et al., 2016a; Nasırcılar & Karaguzel, 2006; Tıpırdamaz et al., 1999). In snowdrop tissue culture studies, generally the combinations of benzyl amino purine (BAP) and naphthalene acetic acid (NAA) hormones at different doses have been used, whereas the number of studies examining effect of indole-3-acetic acid (IAA) and indole-3-butiric acid (IBA) is limited (Staikidou et al., 2006; Staikidou & Selby, 2012; Zencirkıran & Mengüç, 2004). On the other hand, it is noteworthy that tissue culture studies in snowdrops were carried out in 16/8 hour photoperiod conditions in the literature, but there were no studies on bulblet formation in continuous dark environment. In accordance with these reasons, this study was carried out to determine the effects of photoperiod, plant growth regulators and explant size on *in vitro* bulblet formation in snowdrop (*Galanthus woronowii*).

Materials and Methods

Galanthus woronowii bulbs purchased from a private commercial company dealing with the trade of bulbous plants were used as plant materials in the study. Healthy bulbs with no discolorations or scars were selected and their outer tunics were carefully removed. For surface sterilization, snowdrop bulbs were washed using running tap water for 30 min, kept on filter paper for one night and treated with 70% ethanol solution for 2 min. Then, the bulbs were placed in 2,5% sodium hypochlorite (NaOCl) solution including 2-3 drops of Tween-20 and put in a magnetic stirrer (140 rpm) for 30 min continuous stirring (Khonakdari et al., 2020). Finally, the bulbs were rinsed three times with distilled water and dried on filter paper for 10 min. All equipment and culture media used in the study were autoclaved at 1.2 atmospheric pressure and 121°C for 20 min.

Outer scales of surface-sterilized bulbs were peeled off by a scalpel and the remaining twin bulb scales were used as explants for the establishment of *in vitro* culture. Twin scale explants were divided vertically into two and four pieces of equal size and they were cultured separately. The medium used in the study was MS (Murashige & Skoog, 1962) with 30 g l^{-1} sugar and 8 g l^{-1} agar. The pH of the medium was adjusted to 5.60-5.80. Two- and four-pieces twin-bulb scales were cultured on MS media containing different combinations of BAP (1 and 2 mg l^{-1}) with different concentrations of NAA (0.1 and 1 mg l^{-1}), IAA (0.1 and 1 mg l^{-1}), and IBA (0.05 and 2 mg l^{-1}), resulting a total of 12 combinations. The bulb-scale explants with two- and four-pieces were then transferred into petri dishes containing prepared MS media (36 ml) supplemented with various doses of plant growth regulators. Four explants were placed in each petri dish and the explants were cultured in triplicates per treatment. After placing twin-bulb scales in petri dishes, the lids of petri dishes were closed and wrapped with cling film. The cultures were incubated in a growth chamber at 22±1°C under two different photoperiod conditions; a) 16/8 h light/dark photoperiod from white florescent lamb and b) complete dark (24 h dark) kept in petri dishes covered by aluminum folio. The number of bulblet, bulblet diameter, the number of bulblet bigger than 5 mm in diameter, and bulblet weight were measured after six months of culture.

The data obtained from the research were subjected to the analysis of variance according to a completely randomized factorial design with three replications, and the differences between the treatment means were compared by the Tukey test. Before variance analysis, all data were transformed into √X+1, since there were zero values among the data (Yurtsever, 1984). Statistical analysis and mean comparisons were carried out using the JUMP statistical package program.

Results

Snowdrop twin-scale explants cultured on MS media fortified with various concentrations of BAP, NAA, IBA, and IAA under 16/8 h light/dark and 24 h dark conditions showed variation in bulblet formation. The results of variance analysis revealed that photoperiod conditions and plant growth regulators had significant effects on bulblet number, bulblet diameter, bulblet number with ≤ 5 mm diameter, and bulblet weight (Table 1). The effect of explant size, however, was not significant on any of the characters studied. Most of the interaction effects among photoperiod (P), plant growth regulator (PGR), and explant size (ES) were also significant. *In vitro* bulblet formation occurred in all cultured explants in the study, whereas four explants produced no bulblet with ≤ 5 m diameter.

Table 1. Analysis of Variance of the Effect of Photoperiod, Plant Growth Regulators and Explant Size on Measured Characters in *Galanthus woronowii*

Source of Variations	df	Means of Squares			
		Number of Bulblet	Number of Bulblet with ≤ 5 m Diameter	Bulblet Diameter	Bulblet Weight
Photoperiod (P)	1	0.582*	0.562*	1.862*	39.093*
Plant growth regulator (PGR)	11	0.422*	0.117*	0.258*	5.054*
Explant size (ES)	1	0.087	0.014	0.067	2.664
P x PGR	11	0.375*	0.141*	0.164*	2.521
P x ES	1	1.746*	0.776*	2.330*	18.101*
PGR x ES	11	0.244*	0.098	0.117	3.981*
P x PGR x ES	11	0.282*	0.129*	0.123	1.359
Error	96	0.074	0.046	0.069	1.940

**: Significant at the 0.05 probability level.*

The used photoperiod treatments (16/8 h light/dark and 24 h dark) produced very significant differences in all characters evaluated and bigger values were obtained under 24 h dark condition. The average number of bulblet and bulblet with ≤ 5 mm in light and dark environments were 1.63-2.09 and 0.61-0.96, respectively (Table 2 and 3). Continuous dark culture resulted in an increase of 28.2%, 57.4%, 40.2%, and 50.5% in bulblet number, number of bulblet with ≤ 5 mm diameter, bulblet diameter, and bulblet weight compared to 16/8 h light/dark culture, respectively (Table 4 and 5). The results indicated that there was a significant difference (P<0.05) among 12 PGR treatments in all attributes studied and the highest number of bulblet (2.72) and bulblet with ≤ 5 mm diameter (1.27) were noted from the explants cultured on MS media fortified with 2.0 mg l^{-1} BAB+0.1 mg l^{-1} NAA (Table 2 and 3). The maximum bulblet diameter (3.07 mm) and bulblet weight (34.79 mg) were obtained from the PGR treatment of 1.0 mg l^{-1} BAB+2.0 mg l^{-1} IBA.

Table 2. Number of Bulblet in Two- and Four-pieces Snowdrop Twin Scale Explants Treated with Different Plant Growth Regulators at 16/8 Light/dark and 24 h Dark Conditions

Plant Growth Regulator		16/8 h light/dark			24 h dark			PGR Mean[3]
		Two Piece[1]	Four Piece[1]	PGR - Light Mean[2]	Two Piece[1]	Four Piece[1]	PGR - Dark Mean[2]	
1 mg l^{-1} BAP	0.1 mg l^{-1} NAA	2.83 a-f	1.75 a-f	2.29 A-D	0.83 e-f	1.50 a-f	1.16 BCDE	1.72 ABC
	1.0 mg l^{-1} NAA	3.00 a-f	2.00 a-f	2.50 ABC	2.00 a-f	2.58 a-f	2.29 A-D	2.39 AB
	0.05 mg l^{-1} IBA	0.75 f	0.50 f	0.62 DE	1.25 b-f	2.25 a-f	1.75 A-E	1.18 BCD
	2.0 mg l^{-1} IBA	1.25 a-f	2.00 a-f	1.62 A-E	2.83 a-f	2.33 a-f	2.58 ABC	2.10 ABC
	0,1 mg l^{-1} IAA	0.83 ef	2.25 a-f	1.54 A-E	0.66 f	1.00 c-f	0.83 CDE	1.18 BCD
	1.0 mg l^{-1} IAA	2.00 a-f	1.16 def	1.58 B-E	4.58 abc	3.08 a-f	3.83 A	2.70 A
2 mg l^{-1} BAP	0.1 mg l^{-1} NAA	4.08 a-e	0.50 f	2.29 A-D	1.50 a-f	4.83 a	3.16 AB	2.72 A
	1.0 mg l^{-1} NAA	4.58 abc	1.50 a-f	3.04 AB	0.66 f	1.66 a-f	1.16 B-E	2.10 ABC
	0.05 mg l^{-1} IBA	1.25 a-f	0.33 f	0.79 CDE	2.50 a-f	3.00 a-f	2.75 ABC	1.77 ABC
	2.0 mg l^{-1} IBA	2.00 a-f	1.91 a-f	1.95 A-D	1.33 a-f	2.75 a-f	2.04 A-D	2.00 ABC
	0.1 mg l^{-1} IAA	0.25 f	0.25 f	0.25 E	1.08 def	0.75 f	0.91 CDE	0.58 D
	1.0 mg l^{-1} IAA	0.58 f	1.75 a-f	1.16 B-E	0.50 f	4.75 ab	2.62 A-D	1.89 ABC
Photoperiod Mean[4]				1.63 B			2.09 A	
Photoperiod-size Mean[5]		1.95 ^{AB}	1.32 ^{C}		1.64 ^{BC}	2.54 ^{A}		

Means with different letters on the column and the row with the same number are significantly different (p<0.05).

Although explant size caused significant differences in none of the attributes tested, its interaction effect with photoperiod treatments was significant for all studied traits. The data obtained in the study clearly indicated that, four-pieced explants performed so much better than two-pieced explants in continuous dark environment. The number of bulblet, bulblet diameter, and bulblet weight in 24 h dark condition was approximately twice than 16/8 h light/dark culture. The number of bulblet with ≤ 5 mm diameter recorded in continuous dark was 2.6 times greater than that of light condition. On the other hand, two-pieced explants responded more positively to the treatment of 16/8 h light/dark photoperiod compared to continuous dark.

Table 3. Number of Bulblet (≤ 5 mm circumference) in Two- and Four-pieces Snowdrop Twin Scale Explants Treated with Different Plant Growth Regulators at 16/8 Light/dark and 24 h Dark Conditions

	Plant Growth Regulator	16/8 h Light/Dark			24 h Dark			PGR Mean[3]
		Two Piece[1]	Four Piece[1]	PGR - Light Mean[2]	Two Piece[1]	Four Piece[1]	PGR - Dark Mean[2]	
1 mg l^{-1} BAP	0.1 mg l^{-1} NAA	1.91 a-d	0.33 bcd	1.12 abc	0.50 a-d	0.66 a-d	0.58 abc	0.85 AB
	1.0 mg l^{-1} NAA	1.00 a-d	1.00 a-d	1.00 abc	1.00 a-d	0.91 a-d	0.95 abc	0.97 A
	0.05 mg l^{-1} IBA	0.41 bcd	--	0.20 c	0.75 abcd	0.66 a-d	0.70 abc	0.45 AB
	2.0 mg l^{-1} IBA	0.66 a-d	0.75 a-d	0.70 abc	0.91 a-d	1.16 a-d	1.04 abc	0.87 AB
	0,1 mg l^{-1} IAA	0.58 a-d	1.00 a-d	0.79 abc	0.16 bcd	0.41 bcd	0.29 bc	0.54 AB
	1.0 mg l^{-1} IAA	0.58 a-d	--	0.29 bc	2.16 abc	1.75 a-d	1.95 a	1.12 A
2 mg l^{-1} BAP	0.1 mg l^{-1} NAA	1.91 a-d	--	0.95 abc	0.41 bcd	2.75 a	1.58 ab	1.27 A
	1.0 mg l^{-1} NAA	1.08 a-d	0.58 a-d	0.83 abc	0.33 bcd	1.08 a-d	0.70 abc	0.77 AB
	0.05 mg l^{-1} IBA	0.41 bcd	0.33 bcd	0.37 bc	1.41 a-d	1.08 a-d	1.25 abc	0.81 AB
	2.0 mg l^{-1} IBA	0.41 bcd	0.25 bcd	0.33 bc	0.83 a-d	1.00 a-d	0.91 abc	0.62 AB
	0.1 mg l^{-1} IAA	--	0.08 cd	0.04 c	0.25 bcd	0.58 a-d	0.41 bc	0.22 B
	1.0 mg l^{-1} IAA	0.33 bcd	1.00 a-d	0.66 abc	0.08 cd	2.16 ab	1.12 abc	0.89 AB
Photoperiod Mean[4]				0.61 B			0.96 A	
Photoperiod-size Mean[5]		0.77 ^{B}	0.44 ^{B}		0.73 ^{B}	1.18 ^{A}		

Means with different letters on the column and the row with the same number are significantly different (p<0.05).

Table 4. Bulblet Diameter (mm) in Two- and Four-pieces Snowdrop Twin Scale Explants Treated with Different Plant Growth Regulators at 16/8 Light/dark and 24 h Dark Conditions

	Plant Growth Regulator	16/8 h Light/Dark			24 h Dark			PGR Mean[3]
		Two Piece[1]	Four Piece[1]	PGR -Light Mean[2]	Two Piece[1]	Four Piece[1]	PGR - Dark Mean[2]	
1 mg l^{-1} BAP	0.1 mg l^{-1} NAA	3.09	1.67	2.38 abc	2.09	2.45	2,27 abc	2.32 AB
	1.0 mg l^{-1} NAA	2.94	2.41	2.68 ab	2.85	2.65	2.75 ab	2.71 A
	0.05 mg l^{-1} IBA	1.95	0.83	1.39 bc	2.32	3.12	2.72 ab	2.06 AB
	2.0 mg l^{-1} IBA	2.83	2.20	2.51 ab	2.89	4.37	3.63 a	3.07 A
	0,1 mg l^{-1} IAA	2.25	4.26	3.25 ab	2.00	3.25	2.62 ab	2.94 A
	1.0 mg l^{-1} IAA	2.40	0.63	1.52 bc	3.24	4.54	3.89 a	2.70 A
2 mg l^{-1} BAP	0.1 mg l^{-1} NAA	3.27	0.50	1.88 abc	1.76	3.93	2.85 ab	2.36 AB
	1.0 mg l^{-1} NAA	2.39	2.00	2.19 abc	1.41	3.01	2.21 abc	2.20 AB
	0.05 mg l^{-1} IBA	1.75	1.29	1.52 bc	2.62	3.54	3.08 ab	2.30 AB
	2.0 mg l^{-1} IBA	2.63	1.75	2.19 abc	2.61	3.49	3.05 ab	2.62 A
	0.1 mg l^{-1} IAA	0.41	0.66	0.54 c	1.72	2.29	2.00 abc	1.27 B
	1.0 mg l^{-1} IAA	1.87	1.86	1.86 abc	1.12	3.79	2.45 ab	2.16 AB
Photoperiod Mean[4]				1.99 B			2.79 A	
Photoperiod-size Mean[5]		2.31 ^{B}	1.67 ^{C}		2.22 ^{C}	3.37 ^{A}		

Means with different letters on the column and the row with the same number are significantly different (p<0.05).

In the study of triple interaction effect of PGRs, light conditions and explant sizes, the highest number of bulblet (4.83) and bulblet with ≤ 5 mm diameter (2.75) were recorded in four-pieced explants cultured on MS medium fortified with 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA under 24 h dark condition. The highest bulblet diameter (4.54 mm) was obtained from four-pieced explants treated with 1 mg l^{-1} BAP+1 mg l^{-1} IAA under 24 h dark. The twin-scale explants with four pieces cultured on medium with 1 mg l^{-1} BAP+2.0 mg l^{-1} IBA under continuous dark produced the highest bulblet weight (63.33 mg).

Table 5. Bulblet Weight (mg) in Two- and Four-pieces Snowdrop Twin Scale Explants Treated with Different Plant Growth Regulators at 16/8 Light/dark and 24 h Dark Conditions

Plant Growth Regulator		16/8 h light/dark			24 h dark			PGR Mean[3]
		Two Piece[1]	Four Piece[1]	PGR -Light Mean[2]	Two Piece[1]	Four Piece[1]	PGR - Dark Mean[2]	
1 mg l^{-1} BAP	0.1 mg l^{-1} NAA	33.33	15.83	24.58	32.50	20.83	26.66	25.62 AB
	1.0 mg l^{-1} NAA	20.83	25.00	22.91	22.50	30.00	26.25	24.58 AB
	0.05 mg l^{-1} IBA	10.00	8.33	9.16	24.16	24.16	24.16	16.66 AB
	2.0 mg l^{-1} IBA	31.66	17.50	24.58	26.66	63.33	45.00	34.79 A
	0,1 mg l^{-1} IAA	20.00	45.83	32.91	13.33	34.16	23.75	28.33 AB
	1.0 mg l^{-1} IAA	30.83	5.00	17.91	36.66	35.83	36.25	27.08 AB
2 mg l^{-1} BAP	0.1 mg l^{-1} NAA	25.83	5.00	15.41	22.50	27.50	25.00	20.20 AB
	1.0 mg l^{-1} NAA	13.33	20.83	17.08	17.50	27.50	22.50	19.79 AB
	0.05 mg l^{-1} IBA	15.00	20.00	17.50	23.33	32.50	27.91	22.70 AB
	2.0 mg l^{-1} IBA	10.00	11.66	10.83	20.00	30.00	25.00	17.91 AB
	0.1 mg l^{-1} IAA	2.50	7.50	5.00	15.83	34.16	25.00	15.00 B
	1.0 mg l^{-1} IAA	18.33	20.00	19.16	10.00	28.33	19.16	19.16 AB
Photoperiod Mean[4]				18.09 B			27.22 A	
Photoperiod-size Mean[5]		19.30 B	16.87 B		22.08 B	32.36 A		

Means with different letters on the column and the row with the same number are significantly different ($p<0.05$).

Discussion

Galanthus woronowii, a bulbous perennial plant commonly distributed in north eastern Türkiye, has a high medicinal and ornamental importance (Ay et al., 2018). But the populations of snowdrops are gradually decreasing in native habitat and facing the thread of extinction as their bulbs are excessively collected from the wild. Besides that, snowdrops require a period of 4-5 years for the formation of a new-mature bulb in its natural environment (Yüzbaşıoğlu & Dalyan, 2017). Therefore, rapid and easy reproduction methods are needed for the mass production of these precious species (Ulus & Seydioğlu, 2006). Over the last 25 years, it has proven that *in vitro* techniques have a great potential in the production of bulbous and tuberous plants (Kahraman, 2020; Özdemir et al. 2016). Previous studies in bulbous plants revealed that growth regulators, photoperiod, and explant type are the factors with the greatest effect (Akyüz, 2018; Khonakdari et al., 2020).

Photoperiod is one of the most important environmental factors in tissue culture studies, and its effect may differ depending on the explant source and type of plant growth regulator. In geophytes, the response of plants to the photoperiod can generally be in three ways; 1) dark inhibits bulb-tuber development, 2) dark encourages bulb-tuber development, and 3) both light and darkness are required for bulb-tuber development (Ascough et al., 2008). The findings of the present study reveal that there are very significant increases in all measured attributes in the dark environment as compared to the light environment. In the dark environment, the number of bulblet increased by 28.22% compared to the 16/8 h light/dark condition, while the increase in the bulblet diameter, the number of bulblet with ≤ 5 mm diameter, and bulblet weight were 40.20%, 57.33% and 23.28%, respectively. These findings indicate that continuous dark application promotes *in vitro* bulblet formation in *Galanthus woronowii.*

Since there is no study conducted on snowdrops in continuous dark in the literature, it was not possible to make a comparative evaluation of the data obtained from our study with the literature findings. In *Eucomis zambesiaca* Baker species, a bulbous plant in the family *Asparagaceae*, it was reported that the dark environment prevented the formation of bulblet and 8-hour light condition produced the highest bulblet number (Cheesman et al., 2010). In contrast, continuous dark application in *Hyacinthus orientalis* promoted bulblet formation and resulted in an increase in the number and diameter of bulblet (Economou & Read, 1987). A study in *Narcissus Tazetta* L., a bulbous plant from the *Amaryllidaceae* family, showed that 16/8 h light/dark photoperiod treatment resulted in significant increases in bulblet and leaf number, leaf length, and galantamine content compared to 24 h dark (Khonakdari et al., 2020). Similarly, there are some research results indicating that photoperiod application promotes bulblet formation in geophytes (Rice et al., 2011). On the contrary, in *Hyacinthus orientalis* (Kim et al., 1981) and in *Lilium longiforum* (Kumar et al., 2006) species, the highest number of bulblet was obtained from the explants cultured in complete darkness, supporting the findings of our study. Differences between research results may be caused by a number of factors such as plant species, genotype, plant growth regulator used and explant type. Therefore, it is of considerable importance to conduct further studies to fully reveal the effect of photoperiod and continuous dark treatments on bulblet formation in snowdrops and other geophytes.

The type and concentration of PGRs are supposed to be the most second important factor affecting *in vitro* bulblet formation in bulbous plants (Khonakdari et al., 2020). The effects of growth regulators on bulblet formation may be low when used alone, but their effects increase when several of them are used together (Öztürk, 2021). According to literature reports, different plant hormone combinations containing benzyl amino purine (BAP), naphthalene acetic acid (NAA), indole butyric acid (IBA), and indole acetic acid (IAA) were commonly used in studies for the formation of bulblet in snowdrops (Nasırcılar & Karagüzel 2006; Staikidou et al. 2006; Tıpırdamaz et al. 1999). It has been reported that using BAP as cytokinin and NAA as auxin source in snowdrops had higher effect on *in vitro* bulblet formation due to the synergetic effect between them, which is consistent with the results of this study (Staikidou & Selby, 2012; Yüzbaşı & Dalyan, 2017). Similarly, it has been suggested that the auxin type has a very important role in *G. nivalis* and that bulblet formation is encouraged especially in high doses of NAA alone or in combination with IAA (Resetár et al., 2014). On the other hand, Zencirkıran and Mengüç (2004) report that IBA is more suitable than IAA as an auxin in *G. elvesii* species. In a study conducted in the *G. transcaucasicus* species, the highest bulblet number was obtained from explants in MS medium supplemented with 2.0 mg l-1 BAP and 2.0 mg l-1 IBA (Babashpour-Asl et al., 2016a).

The results of the presents study revealed that explant size (with two and four pieces) did not have a significant effect on any of the traits examined, but the interactions with photoperiod and plant growth regulators were important in some traits. In our study, two-pieced explants responded more positively to the treatment of 16/8 h light/dark photoperiod compared to continuous dark. In the contrary, four-pieced explants performed interestingly so much better than two-pieced explants in continuous dark environment. The positive enhancing effect of continuous dark on particularly the number of bulblet, bulblet diameter and bulblet weight is more evident in cultures with IAA and IBA compared to NAA applications. The interactions of explant size with photoperiod and plant growth regulator can be a complex phenomenon. Thus, further studies need to be carried out to optimize the interactive effect of explant size with photoperiod and PGR.

Conclusion

Commercial and medicinal importance of snowdrops are accounted for their beautiful flowers and naturally occurring bioactive compounds, *Amaryllidaceae* alkaloids. Life cycle of snowdrops is long and production rate is rather slow, so plant cell and tissue culture provides an appealing alternative for optimizing maximum production of these plants in a relatively short time. Thus, this study aimed at describing an efficient production protocol for *in vitro* bulblet formation in *Galanthus woronowii*. The results of the present study revealed that photoperiod and plant growth regulators showed a significant effect on *in vitro* bulblet formation in *Galanthus woronowii*. In contrary, explant size caused no significant variation in any of the attributes studied. The highest number of bulblet were obtained from four-pieced bulb-scale explants cultured on MS medium fortified with 2 mg l^{-1} BAP+0.1 mg l^{-1} NAA under 24 h dark condition. Continuous dark treatment resulted in highly significant increases in bulblet formation, as compared to 16/8 light/dark photoperiod, with an increase of %28.2 and %57.3 in the number of bulblet and bulblet with ≤ 5 mm diameter, respectively. It is concluded that 24 h dark treatment has a very important positive effect on *in vitro* bulblet formation in snowdrop. However, it would be worthwhile to carry out further studies in order to fully optimize the effect of light and dark applications on *in vitro* bulblet formation in snowdrops.

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Author Contributions

Mehmet Muharrem Özcan, prepared and maintained experimental conditions. *Betül Başeli*, carried out the experiment, performed statistical analysis. *Şevket Metin Kara*, supervised experimental process, wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

Ethics statement

There are no ethical issues concerning the publication of this article.

Conflict of Interest

The authors state that there is no conflict of interest.

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References

Akyüz, E. (2018). Bazı *Fritillaria* türlerinde *in vitro* soğancık üretimi ve dış koşullara alıştırma çalışmaları [Yayımlanmamış yüksek lisans tezi]. Hacettepe Üniversitesi.

Ascough, G. D., Erwin, J. E., & Van Staden, J. (2008). *In vitro* storage organ formation of ornamental geophytes. *Horticultural Reviews*, *34*, 417–445. https://doi.org/10.1002/9780470380147.ch7

Avcı, M. (2005). Çeşitlilik ve endemizm açısında Türkiye'nin bitki örtüsü. *İstanbul Üniversitesi Fen Edebiyat Fakültesi Coğrafya Dergisi,* *13*, 27–55. https://dergipark.org.tr/tr/pub/iucografya/issue/25062/264581

Ay, E. B., Gül M., Açıkgöz M. A., Yarılgaç, T., & Kara, S. M. (2018). Assessment of antioxidant activity of giant snowdrop (*Galanthus elwesii* Hook) extracts with their total phenol and flavonoid contents. *Indian J of Pharmaceutical Education and Research, 51*(4S), 128-132. https://doi.org/10.5530/ijper.52.4s.88

Atay, S. (1996). *Soğanlı bitkiler; Türkiye'den ihracatı yapılan türlerin tanıtım ve üretim rehberi*. Doğal Hayatı Koruma Derneği Yayınları.

Babashpour-Asl, M., Movafeghi, A., & Zare, K. (2016a). *In vitro* production of bulblet in *Galanthus transcaucasicus* Fomin, an endangered medicinal plant. *Journal of Plant Physiology and Breeding, 6*(2), 1-8. https://www.researchgate.net/publication/329058764

Babashpour-Asl, M., Zakizadeh, H., Nazemiyeh, H., & Motallebi-Azar, A. (2016b). *In vitro* micropropagation and alkaloid production of *Galanthus transcaucasicus* Fomin. *Pharmaceutical Sciences*, 22(4), 267-271. https://doi.org/10.15171/PS.2016.41

Bores, G., & Kosley, R. (1996). Galanthamine derivatives for the treatment of Alzheimer's disease. *Drugs Future*, 21(6), 621–635. https://eurekamag.com/research/031/534/031534046.php

Cengiz, Ö. (2019). *Cyclamen coum* ve *Cyclamen persicum*'da farklı büyüme düzenleyicileri, eksplant tipleri ve genotipin organogenesis üzerine etkisi (Yayımlanmamış yüksek lisans tezi). Çukurova Üniversitesi.

Cheesman, L., Finnie, J., & Van Staden, J. (2010). *Eucomis zambesiaca* Baker: Factors affecting *in vitro* bulblet induction. *S Afr J Bot, 76*(3), 543–549. https://doi.org/10.1016/j.sajb.2010.04.004

De Klerk, G. J. (2012). Micropropagation of bulbous crops: Technology and present state. *Floriculture and Ornamental Biotechnology*, *6*(1), 1-8. https://library.wur.nl/WebQuery/wurpubs/530926

Demir, A. (2010). Türkiye'de kardelen ticareti ve politik yaklaşımlar. *Biological Diversity and Conservation*, *3*(3), 111-120. https://dergipark.org.tr/en/pub/biodicon/issue/55900/766065

Economou, A. S., & Read, P. E. (1987). Light treatments to improve efficiency of *in vitro* propagation systems. *HortScience*, *22*, 751–754. https://agris.fao.org/agris-search/search.do?recordID=US880555288

Ekim, T., Koyuncu, M., Vural, M, Duman, H., Aytaç, Z., & Adıgüzel, N. (2000). *Türkiye bitkileri kırmızı kitabı*. Türkiye Tabiatını Koruma Derneği.

Entwistle, A., Atay, S. Byfield, A., & Oldfield, S. (2002). Alternatives for the bulb trade from Türkiye: A case study of indigenous bulb propagation. *Oryx*, *36*, 33-341. https://doi.org/10.1017/S0030605302000674

Heinrich, M., & Teoh, H. L. (2004). Galanthamine from snowdrop--the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. *J Ethnopharmacol*, *92*(2-3), 147-162. https://doi.org/10.1016/j.jep.2004.02.012

Kahraman, O. (2020). Use of sucrose in propagation of snowdrop bulb. *Yuzuncu Yil University Journal of Agricultural Science*, *30*(2), 379-385. https://doi.org/10.29133/yyutbd.680442

Karaoğlu, C. (2010). Soğanlı bitkiler ve *in vitro* hızlı çoğaltım. *Tarla Bitkileri Merkez Araştırma Enstitüsü Dergisi*, *19*(1-2), 24-29. https://dergipark.org.tr/en/download/article-file/118447

Khonakdari, M. R., Rezadoost, H., Heydari, R., & Mirja, M. H. (2020). Effect of photoperiod and plant growth regulators on *in vitro* mass bulblet proliferation of Narcissus tazzeta L. (*Amaryllidaceae*), a potential source of galantamine. *Plant Cell, Tissue and Organ Culture*, *142*, 187–199. https://doi.org/10.1007/s11240-020-01853-y

Kim, Y., Hasegawa, P., & Bressan, R. (1981*). In vitro* propagation of hyacinth (*Hyacinthus orientalis*). HortScience, *16*, 645–647. https://agris.fao.org/agris-search/search.do?recordID=US19830840843

Koyuncu, M. & Ekim, T. (1984). *Türkiye'nin ihraç ettiği geofitler ve bunların ekonomik önemi*. V. Bitkisel İlaç Hammaddeleri Toplantısı Tebliği, Ankara, Türkiye.

Kumar S., Kanwar, J., & Sharma, D. (2006). *In vitro* propagation of Lilium. *Adv Hortic Sci*, *20*(2), 181-188. https://www.jstor.org/stable/42882479

Murashige, T., & Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Plant Physiology*, *15*, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nasırcılar, A. G. & Karagüzel, Ö. (2006). *Galanthus elwesii* Hook. bitkisinin olgunlaşmamış embriyolarından *in vitro* soğan üretimi. *Akdeniz Üniversitesi Ziraat Fakültesi Dergisi*, 19(2), 159-164. https://dergipark.org.tr/en/download/article-file/18141

Özdemir, F. A., Yıldırım, M. U, Kahriz M. P., & Kılıç, Ö. (2016). *In vitro* bulblet regeneration from *Scilla Siberica* Haw. subsp. armena (Grossh.) mordak peduncle. *Propagation of Ornamental Plants*, *16*(1), 14-18. https://dergipark.org.tr/en/download/article-file/18377

Özhatay, N., Byfield, A. ve Atay, S. (2005). Türkiye'nin 122 önemli bitki alanı. WWF Türkiye Doğal Hayatı Koruma Vakfı Yayını, İstanbul.

Öztürk, G. (2021). Kardelenin (*Galanthus woronowoii*) *in vitro* rejenerasyonu. *MAS Journal of Applied Sciences, 6*(4), 1027–1033. http://dx.doi.org/10.52520/masjaps.152

Resetár, A., Demeter, Z., Ficsor, E., Balázs, A., Mosolygó, A., Szőke, E., Gonda, S., Papp, L., Surányi, G., & Máthé, C. (2014). Growth *regulator requirement for in vitro embryogenic cultures of snowdrop (Galanthus nivalis* L.) suitable for germplasm preservation. *Acta Biologica Hungarica*, *65*(2), 165-177. http://doi.org/10.1556/ABiol.65.2014.2.5

Resmi Gazete. (2017, 21 Mart). Doğal çiçek soğanlarının doğadan toplanması, üretimi ve ihracatına ilişkin yönetmelik (Sayı: 30014). https://www.resmigazete.gov.tr/eskiler/2017/03/20170321-5.htm adresinden 21 Ağustos 2022 tarihinde alınmıştır.

Resmi Gazete. (2021, 31 Aralık). Doğal çiçek soğanlarının 2022 yılı ihracat listesi hakkında tebliğ (Tebliğ No: 2021/46). https://www.resmigazete.gov.tr/eskiler/2021/12/20211231-10.htm adresinden 21 Ağustos 2022 tarihinde alınmıştır.

Rice, L., Finnie, J., & Van Staden, J. (2011). *In vitro* bulblet production of *Brunsvigia undulata* from twin-scales. *S Afr J Bot*, *77*(2), 305–312. https://doi.org/10.1016/j.sajb.2010.08.011

Seydi, S., Sedaghathoor, S., & Kaviani, B. (2019). Plants regeneration by organogenesis from bulbous explants in Fritillaria imperialis L., a wild rare ornamental species at the risk of extinction. Adv. Hort. Sci., *33*(4), 503510. https://doi.org/10.13128/ahsc8128

Staikidou, I., Selby, C., & Hanks, G. (2006). Stimulation of *in vitro* bulblet growth in *Galanthus* species with sucrose and activated charcoal. *Acta Horticulturae*, *725*, 421–426. https://doi.org/10.17660/ActaHortic.2006.725.57

Staikidou, I., & Selby, C. (2012). Effects of growth regulators and activated charcoal on *in vitro* bulblet multiplication and growth in *Galanthus nivalis* "Flore Pleno". *The Journal of Horticultural Science and Biotechnology*, *87*, 527–530. https://doi.org/10.1080/14620316.2012.11512906

Şekeroğlu, N., Aydın, K., Gözüaçık, H. G., & Kulak, M. (2013). Kilis ilinde yetişen geofitler. *Türk Bilimsel Derlemeler Dergisi,* *6*(1), 199-201. https://www.derleme.gen.tr/index.php/derleme/article/view/146

Tıpırdamaz, R., Ellialtıoğlu, Ş., & Çakırlar, H. (1999). Kardelenin (*Galanthus ikariae* Baker.) doku kültürü yoluyla çoğaltımı: Eksplant tipi, ortam pH'sı ve karbonhidrat kaynağının soğancık oluşumuna etkisi. *Turkish Journal of Agriculture and Forestry, 23*(4), 823–830. https://search.trdizin.gov.tr/yayin/detay/25280

Tıpırdamaz, R. (2003). Rooting and acclimatization of *in vitro* micropropagated snowdrop (*Galanthus ikariae* Baker.) Bulbletes. *Akdeniz Üniversitesi Ziraat Fakültesi Dergisi*, *16*(2), 121-126. https://dergipark.org.tr/en/download/article-file/18261

Ulus, A., & Seyidoğlu, N. (2006). Bazı doğal geofitlerin doku kültürü ile üretimi. *İstanbul Üniversitesi Orman Fakültesi Dergisi*, 56(1), 71-80. https://dergipark.org.tr/en/pub/jffiu/issue/18713/198798

Yurtsever, N. (1984). *Deneysel istatistik metotlar (Yayın no. 121)*. Tarım ve Orman Bakanlığı Köy Hizmetleri Genel Müdürlüğü Yayınları.

Yüzbaşıoğlu, S. (2012). Morphological variations of *Galanthus elwesii* in Türkiye and difficulties on identification. *Bocconea*, *24*, 335-339. https://www.herbmedit.org/bocconea/24-335.pdf

Yüzbaşıoğlu, E., & Dalyan, E. (2017). Büyüme hormonları ve aktif kömürün *in vitro* koşullarda kardelen (*Galanthus woronowii* Losinsk.) soğancık oluşumuna etkisi. *Mediterranean Agricultural Sciences*, *30*(3), 239-243. https://doi.org/10.29136/mediterranean.359870

Zencirkıran, M., & Mengüç, A. (2004). *In vitro* propagation of *Galanthus elwesii* Hook (Snowdrop). *Biotechnology & Biotechnological Equipment*, *18*(1), 19-22. https://doi.org/10.1080/13102818.2004.10819224