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In Vitro Production of Bulblet in *Galanthus transcaucasicus* Fomin, an Endangered Medicinal Plant

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Abstract
To optimize conditions for micropropagating *Galanthus transcaucasicus* Fomin, the effect of explant type, different concentrations of sucrose, type and different concentrations of auxin were examined on bulblet production. For the induction of bulblets, three types of bulb scales were usesd as explants. A two-step sterilization procedure was applied. Assessments were made after incubation for 20 weeks at 23±1ºC with 16h photoperiods. Highest number of bulblets were proliferated culturing bulb scales on modified MS medium supplemented with 2.0 mg.l^{-1} BA and 2.0 mg.l^{-1} IBA. About 27% of bulblets were bigger than 5 mm in diameter, suitable for acclimatization. With the increase of sucrose concentration, number of bulblets decreased, but their size increased. This protocol provided a basis for future study on large-scale multiplication system for commercial nurseries of *Galanthus transcaucasicus.*

Keywords: Micropropagation; Snowdrop; Tissue culture

Introduction

Propagation of bulbous plants from seeds will show wide variation in morphological features, whereas separation of bulbs is a very slow process for obtaining a large number of plants in a short time. Normally, a plant produces two or three bulbs in a year of growth (Zayed *et al*. 2011). A more acceptable method for the rapid propagation of the bulbous plants with stable morphological features would be micropropagation.

Galanthus L. commonly known as snowdrops, are perennial bulbous petaloid monocots, belonging to Amaryllidaceae, tribe Galantheae Parl. (Meerow *et al*. 2006; Larsen *et al*. 2010). As currently indicated, *Galanthus* includes 20 species, which are distributed from the Pyrenees (France, Spain) in the west, to the Caucasus, Talysh and Alborz (Iran) in the east, and extending south to Sicily, the Peloponnese and Aegean (Greece), southern Turkey, Lebanon and Syria (Davis 1999, 2001; Zubov and Davis 2012; Ronsted *et al.* 2013). The northern distribution limit cannot be assessed due to human introduction and cultivation (Davis 2001).

A particular characteristic of the Amaryllidaceae family is a consistent presence of an exclusive group of isoquinoline alkaloids, which have been isolated from all genera of this family (Berkov *et al.* 2009a). Some snowdrop species are threatened in their wild habitats in most countries, so it is now illegal to collect bulbs from the wild. *Galanthus transcaucasicus* Fomin is an endemic medicinal plant of Iran and is threatened by complete extinction (Jalili 1999).

In vitro propagation has been reported previously for many geophytes such as *Narcissus bulbocodium* (Santos *et al.* 1998), *Fritillaria thunbergii* (Peak and Murthy 2002), *Allium*

sativum (Kim *et al.* 2003), *Sternbergia fischeriana* (Mirici *et al*. 2005), *Muscari azureum* (Uranbey 2011), *Brunsvigia undulata* (Rice *et al*. 2011) and *Muscari muscarimi* (Ozel *et al.* 2015) from a range of explants, such as bulb scales, stem nodes, leaves, mature seeds and thin cell layers. For the Amaryllidaceae, twin scale explants which comprise of two adjacent scales with a piece of basal plate tissue have been successfully used for bulblet regenaration (Fennel and Van Staden 2004). In this Family, it is necessary to include the basal plate as part of the explant as no bulblets are regenarated if not present (Fennel *et al.* 2001). When the basal plate cut, apical dominance is overcome and out-growth of pre-existing axillary meristems are stimulated (Fennel and Van Staden 2004). There are many factors which affect organogenesis in *in vitro* cultures. Some of these factors e.g. explant type, plant growth regulators and carbohydrate source and their concentrations were investigated in this study. Perhaps the most important of these, plant growth regulators, generate different types of organogenesis depending on the type and ratio used (Yeoman 1986). As sucrose is energetically the most suitable carbon source for plant *in in vitro* systems (Pavlov and Bley 2006), this source was selected in this study. Our work undertook with the aim of developing a method for the *in vitro* production of bulblets of endangered wild species of *Galanthus transcaucasicus* Fomin.

Materials and Methods

Plant material

Galanthus transcaucasicus Fomin plants were collected from Oak (*Quercus* spp.) woodland edges in Andabil region (37° 38' N, 48° 33' E) in the Ardebil province, Iran, in May 2014.

Bulb preparation and disinfection

Healthy bulbs were selected and their tunics and scale leaves showing discoloration or brown markings were removed by hand. With a scalpel, basal bulb tissues were cut away down to healthy white tissues, care being taken not to remove more base plant tissues than was necessary. Apical bulb tissues were also cut away 1 mm below the region of scale leaf senescence so that only healthy white tissues remained. The bulbs washed in detergent and were then ready for surface sterilization. A two-step sterilization procedure was applied: after the consequent soaking of bulbs in 70% ethanol for 1 min and 5% sodium hypochlorite for 20 min, they were halved and put in 2.5% sodium hypochlorite for a further 10 min. After the usual three rinse with sterile distilled water, three types of explants prepared. A) individual 5- mm wide bulb scales including a 2 mm section of basal plate, B) bulb twin scales, C) bulb divided vertically into four equal segments consisting three or four scales (Figure 1).

Media preparation and culture conditions

The basic nutrient medium consisted of the salt mixture of MS medium (Murashige and Skoog 1962), organic substances that their amount were determined according to Girman (1986) and Tipirdamaz (2003), 100 mg/l myo-inositol, 0.5 mg/l thiamin-HCl , 0.5 mg/l pyridoxine-HCl, 2.0 mg/l glycine and 1.0 mg/l nicotinic acid. This medium was designataed as modified Murashige and Skoog (MMS). In this study two different experiments were carried out. In experiment 1, in order to investigate effect of explant type, three types of explants cultured on MMS medium with 2 $mg.l^{-1}$ BAP and 0.5 mg.l-1 NAA, 3% sucrose solidified with 8% agar. Based on the result of the

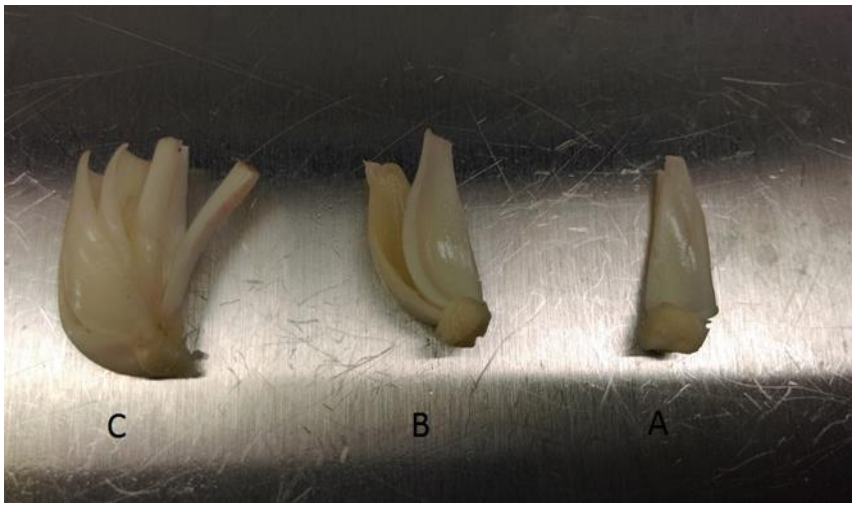


Figure 1. Three types of explant (A: Individual 5- mm wide bulb scales including a 2-mm section of basal plate, B: Bulb twin scales, C: Bulb divided vertically into four equal segments consisting three or four scales)

experiment 1, for experiment 2 the explant C was cultured on MMS with 2.5%, 3% and 6% sucrose solidified with 0.8% agar. To determine the effect of auxin on bulblet formation, three types of auxin [2,4-dichlorophenoxy acetic acid (2,4-D), indole butyric acid (IBA), α-naphtalene acetic acid (NAA)] with three concentrations (0.5, 1, 2 mg.l $^{-1}$) were combined systematically with 2 mg.l^{-1} benzylaminopurine (BA) and included in the medium. In all cultures the medium pH was adjusted to 5.7 with 1N NaOH or 1N HCl before autoclaving at 121°C for 20 min. The explants were placed vertically in the culture media with the basal plate tissue. Cultures were maintained in a growth chamber at 23±1ºC with a 16h photoperiod provided by LED lights (2000 lux) for 20 weeks. Experiments were carried out as a completely random design. Each treatment had three replicates containing 4 explants.

Measurments and statistical analysis

Number of bulblets per explant and bulblet diameter were scored after four months of culture. After analysis of variance, the differences between the means were compared by Duncan's multiple range test at the 5% level using SPSS software version 15.0.

Results and Discusion

Experiment 1

In spite of the difficulties in their sterilization, bulb section explants are the most commonly used, due to their high regenerative capacity. In the present study, three bulb explant types were tested for their regenerative capacity. For the explant C, bulbs were divided vertically into four equal segments (Figure 1), formed bulblets with highest number (3.25 bulblets/explant) and largest diameter (4.80 mm). Also, 92.59% of the explants produced bulblets (Table 1). According to the results of Tipirdamaz *et al*. (1999) with *G. ikariae*, bulb segment explants (explant C, in this study) formed the most bulblets at both NAA levels (0.2 and 0.4 mg/l). This was due to the smaller meristem tissues of the single- and twin-scales in comparison with the bulblet sectors (explant C).

Table 1. Effect of explant type on bulblet production in *Galanthus transcaucasicus* Fomin

Explant type	Explant producing bulblet (%)	Mean number of bulblet/explant	Mean bulblet diameter (mm)
A*	36.11 [c]	0.67 [c]	2.27 [c]
B	79.63 [b]	1.12 [b]	3.32 [b]
C	92.59 [a]	3.25 [a]	4.80 [a]

Values within a column followed by different letters are significantly different at the 0.05 level.
*A) Individual 5- mm wide bulb scales including a 2-mm section of basal plate, B) Bulb twin scales, C) Bulb divided vertically into four equal segments.

Experiment 2

Sucrose concentration

According to Table 2 the effect of sucrose concentration on bulblet regeneration was significant. After 16 weeks of culture, the highest number of bulblet per explant (2.963) was achieved in the MMS medium with 3% sucrose (Figure 2). Doubling of the sucrose quantity (6%) led to an increase in the size of bulblets, but their number decreased (Figure 2). This is in agreement with previous results where the addition of sucrose to the nutrient medium improved bulb development in *Narcissus* cultivars (Chow *et al.* 1992; Staikidou *et al.* 2005) and in *Leucojum aestivum* (Berkov *et al.* 2009b). The use of 3% or 6% sucrose, therefore depended on the aims considered in the experiment. Formation of numerous bulblets was performed in a medium containing 3% sucrose. Bulblet intended for *ex vitro* adaptation or subcultivation were transferred on a medium supplemented with 6% sucrose for faster enlargement.

Table 2. Analysis of variance for the effect of sucrose and auxin on bulblet regeneration in MS medium from bulb scale explant of *Galanthus transcaucasicus* Fomin after 16 weeks of culture

Source	Trait	df	Sum of squares	Mean squares	F	Sig.
Sucrose	No. of bulblet/explant	2	5.358	2.679	6.576	0.003*
	Bulblet diameter	2	33.434	16.717	16.977	0.000*
Auxin (A)	No. of bulblet/explant	2	7.728	3.864	9.485	0.000*
	Bulblet diameter	2	11.011	5.505	5.591	0.006*
Concentration of auxin (CA)	No. of bulblet/explant	2	2.247	1.123	2.758	0.072
	Bulblet diameter	2	11.204	5.602	5.689	0.006*
A*CA	No. of bulblet/explant	4	2.938	0.735	1.803	0.142
	Bulblet diameter	4	6.774	1.693	1.720	0.159
Error	No. of bulblet/explant	54	22.000	0.407		
	Bulblet diameter	54	53.173	0.985		
Total	No. of bulblet/explant	81	605.000			
	Bulblet diameter	81	2340.270			

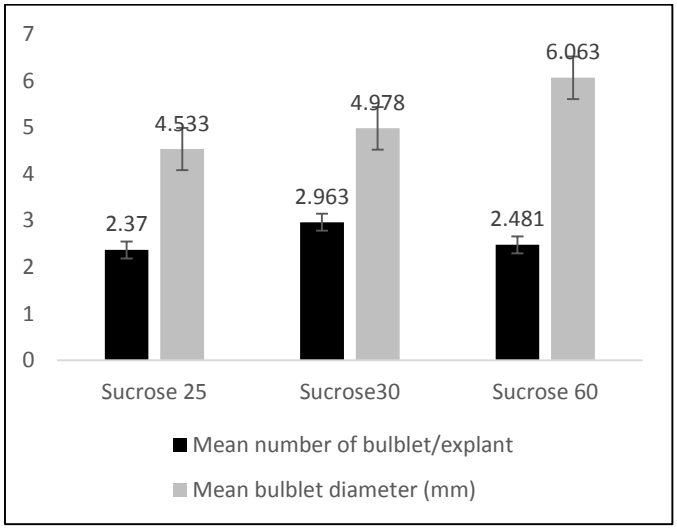
*Significant at $p \leq 0.05$.

Auxin type and concentration

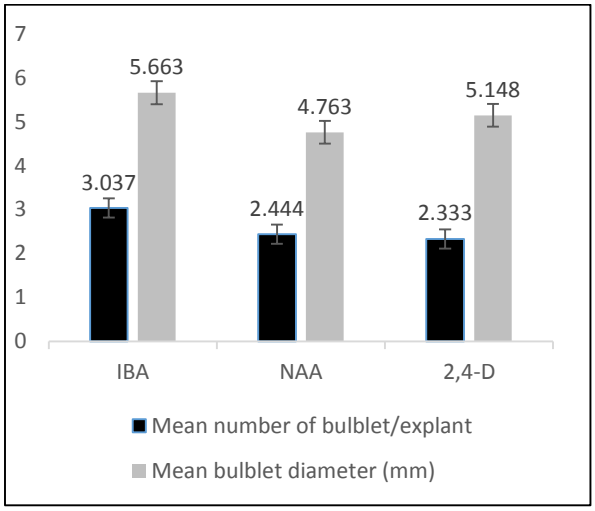
The effect of auxin type on bulblet formation was significant (Table 1) and the highest number of bulblets (3.037) and the largest ones (5.663 mm) were observed in MMS medium enriched with IBA in combination with 2 $mg.l^{-1}$ BA (Figure 3). Interaction between auxin type and its concentration was found insignificant for number of bulblet per explant and the bulblet diameter (Table 1). The best concentration for bulblet

diameter regardless of auxin type was found at 2mg.l^{-1} (Figure 3). Tipirdamaz *et al.* (2003) reported the formation 0f 7.1 adventitious bulblets from a single quarter-bulb chip explant (similar to the explant C in this study) of *G. ikariae*, on a MMS medium supplemented with 0.2 mg/L NAA and 2.0 mg/L BA. This can be attributed to the differences exist between the genotypes used in each experiment.

Previous studies showed that addition of growth regulators to nutrient medium have promoted bulblet regeneration from bulb scales of many geophytes (Ault 1995; Ulrich *et al.* 1999; Wawrosch *et al.* 2001; Paek and Murthy 2002). In the present study three or four scale segments of *G. transcaucasicus* were cultured on MMS medium supplemented with various concentration of IBA, NAA and 2,4-D with 2mg.l^{-1} BA. Although the concentrations of growth regulators influenced bulblet regeneration from scale sections, the frequency of bulblet development remained low (maximum 3.03 bulblets/explant). Wawrosch *et al.* (2001) and Paek and Murthy (2002) reported higher frequencies of bulblets regeneration from bulb scales of *Lilium nepalense* (7 bulblets/explant) and *Fritillaria thunbergii* (13.7 bulblets/explant), respectively. These contrasting results may be related to the genotypes, explants and concentrations of growth regulators tested in the current study.



Figrure 2. Effect of sucrose concentration on bulblet regeneration in MS medium from the bulb scale explant of *Galanthus transcaucasicus* Fomin after 20 weeks of culture



Figrure 3. Effect of auxin type on bulblet regeneration in MS medium from the bulb scale explant of *Galanthus transcaucasicus* Fomin after 20 weeks of culture

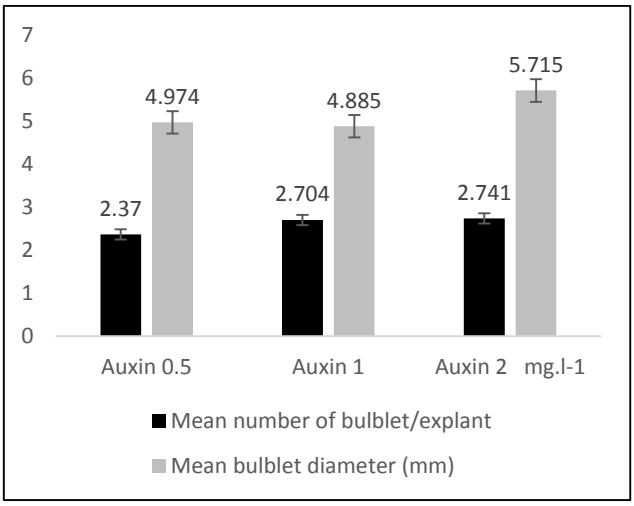


Figure 4. Effect of auxin concentration on bulblet diameter in MS medium from the bulb scale explant of *Galanthus transcaucasicus* Fomin after 20 weeks of culture

Conclusion

The natural propagation rate of most geophytes including *G. transcaucasicus* is relatively low. This often hampers the large-scale cultivation of these plants. This problem can be overcome by tissue culture techniques. In the micropropagation protocols, the multiplication phase is perhaps the most important part in terms of practical use of tissue culture. Although in the present study we developed a protocol for the regeneration system, detailed research work needed to be carried out on the multiplication stage. In conclusion, to our knowledge the present study is the first report for *in vitro* bulblet production from endangered medicinl species, *G. transcaucasicus*. The procedure described here provides a prolific bulblet regeneration system that may form the basis of micropropagation of *G. transcaucasicus*.

Acknowledgements

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