Ornamental Horticulture
ISSN 2447-536X | HTTPS://ORNAMENTALHORTICULTURE.EMNUVENS.COM.BR/RBHO
SBFPO Sociedade Brasileira de Floricultura e Plantas Ornamentais

SCIENTIFIC ARTICLE

Micropropagation and assessment of somaclonal variation in *Galanthus transcaucasicus* in vitro plantlets

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Abstract

In vitro culture of twin-scaling explants of *Galanthus transcaucasicus* with different concentrations of plant growth regulators (PGRs) including 0.5, 1, 2, 3, 4, 6, 8, and 10 mg L^{-1} naphthaleneacetic acid (NAA) and 0.5, 1, 2, 3, and 4 mg L^{-1} benzyladenine (BA) was studied. After 18 weeks, the number of regenerated bulblets and intensity of callus was measured. Subsequently, bulblets were transferred to a medium with 0.5, 1, 2, 3, and 4 mg L^{-1} NAA and 0.5, 1, 2, 3, and 4 mg L^{-1} BA and, after 15 weeks, the bulblets length and diameter were measured. The highest intensity of callus was obtained on 4 mg L^{-1} NAA or 8 mg L^{-1} NAA with 1 mg L^{-1} BA. The highest number of regenerated bulblets was detected with 6 mg L^{-1} NAA and 2 mg L^{-1} BA. The highest diameter of bulblets occurred on four mgL^{-1} NAA (9.4 mm), while the lowest was observed on 0.5 mg L^{-1} BA (1.83 mm). The analysis of genetic variation using ISSR revealed that there was no somaclonal variation among the regenerated plants from BA and low level of NAA, but there was a significant somaclonal variation at high concentrations of NAA.
Keywords: callus, embryogenesis, histology, ISSR, micropropagation, snowdrop.

Resumo

Micropropagação e avaliação da variação somaclonal em plântulas de *Galanthus transcaucasicus* in vitro

Objetivou-se estudar o cultivo *in vitro* de explantes de dupla escala de *Galanthus transcaucasicus* com diferentes concentrações de reguladores de crescimento: 0,5; 1; 2; 3; 4; 6; 8 e 10 mg L^{-1} de ácido naftalenoacético (ANA) e 0,5; 1; 2; 3 e 4 mg L^{-1} de benziladenina (BA). Após 18 semanas foram avaliados o número de bulbilhos regenerados e a porcentagem do calo. Posteriormente, os bulbilhos foram transferidos para um meio com 0,5; 1; 2; 3 e 4 mg L^{-1} de ANA e 0,5; 1; 2; 3 e 4 mg L^{-1} de BA e após 15 semanas foram medidos o comprimento dos bulbos e diâmetro. A maior intensidade de calo foi obtida com 4 mg L^{-1} de ANA ou 8 mg L^{-1} de ANA com 1 mg L^{-1} de BA. O maior número de bulbilhos regenerados foi obtido com 6 mg L^{-1} de ANA e 2 mg L^{-1} de BA. O maior diâmetro dos bulbilhos ocorreu com 4 mg L^{-1} ANA (9,4 mm), enquanto o menor foi observado com 0,5 mg L^{-1} BA (1,83 mm). A análise da variação genética usando ISSR não revelou variação somaclonal entre as plantas regeneradas a partir de BA e baixa concentração de ANA, mas houve variação somaclonal significativa em altas concentrações de ANA.
Palavras-chave: calo, embriogênese, histologia, ISSR, micropropagação, snowdrop.

Introduction

Galanthus spp. (Snowdrops) are perennial bulbous plants belonging to the Amaryllidaceae tribe Galantheae whose seed germination is difficult in natural conditions (Larsen et al., 2010; Newton et al., 2013). The Amaryllidaceae family is potentially important from an economic viewpoint both for the chemical pharmaceutical industry and the ornamental plants market. It is easily identified because of its two leaves as well as pendant white flowers with six perianth segments; three outer and inner perianth segments (Rønsted et al., 2013). The bulb is formed very slowly over too long periods of time, especially in comparison with Liliaceae, Iridaceae and Amaryllidaceae families in the natural environment (Çiğ, 2015). Nevertheless, wild snowdrop reproduction is extremely slow and inefficient (Maślanka et al., 2013).

In vitro culture techniques are currently used tools to produce plants that are resistant against diseases, but are also necessary for rapid multiplication of scarce plant genotypes, plant genome transformation, and production

https://doi.org/10.1590/2447-536X.v27i4.2320

* Corresponding author: n.asadi82@yahoo.com
Received: Dec 02, 2020 | Accepted: July 21, 2021 | Available online: Aug 9, 2021

Area Editor: Fernanda Carlota Nery

of commercially important plant-derived metabolites (Espinosa-Leal et al., 2018). Propagation through in vitro techniques may reduce the required time for acquiring a large number of bulbs (Maślanka et al., 2013). The embryogenic callus is regarded as an appropriate tissue source for genetic variations, and somatic embryogenesis as one of the most competent micropropagation methods in genetic transformation (Dehestani-Ardakani et al., 2020). Induction of callus and subsequent somatic embryogenesis depend on several factors including type of explant, combination of PGRs concentration, and culture medium (Taghizadeh and Ganji-Dastjerdi, 2020; Nabieva and Gerasimovich, 2019).

The genetic and phenotypic diversity that exists in plants obtained from tissue culture is called somaclonal variation. In several tissue culture processes with cell division, the risk of somaclonal variation and contamination is high, which may be attributed to repeated manipulations (Campos et al., 2017). The use of molecular markers is one of the most effective strategies for monitoring somaclonal variations (Konar et al., 2019). Among molecular markers, both RAPD and ISSR markers markers are more practical because they do not need any preceding sequence information and use small amount of DNA (Baruah et al., 2019; Tikendra et al., 2019). *Galanthus transcaucasicus* is one of the lesser known species native to Iran (Rønsted et al., 2013). To date, there is no report on the callogenesis and somaclonal variation potentially occurred in micropropagated plantlets of *G. transcaucasicus*.

This study aimed to identify the effects of PGRs (NAA and BA) concentrations on *G. transcaucasicus* bulblet and callus regeneration. Moreover, the effects of PGR (NAA and BA) concentrations on bulblet growth were determined. The influence of PGR (NAA and BA) concentrations on bulblet development were also investigated. Another objective of this study was to evaluate the somaclonal variation in micropropagated plantlets.

Materials and methods

Plant material

Snowdrop (*Galanthus transcaucasicus*) plants were taken from Hirkani forests of Savadkuh region (36°18' N, 52°52' E) in Mazandaran province, Iran. The identification and authentication of plant was done by Dr. Dolatyari and voucher specimens (voucher number: 3487) have been deposited at the Herbarium of Iranian Biological Resource Center (IBRC), Tehran, Iran.

Bulb preparation and disinfection

Healthy bulbs of Snowdrops were selected and separated from their tunics and scale leaves displaying decoloration or brown markings. Basal bulb tissues were cut down to healthy white tissues with a scalpel. The bulbs were washed with detergent and then prepared for surface sterilization. Bulb scale explants were surface sterilized for 30 min followed by soaking of bulbs in fungicides for 30 min and 5% sodium hypochlorite for 20 min. The samples were finally washed with sterile ion-exchanged water three times.

In vitro establishment

Twin-scaling explants were cultivated on MS (Murashige and Skoog, 1962) basal medium containing 3% (w/v) sucrose and 0.8% (w/v) agar and various concentrations of NAA in combination with BA. The medium pH was set to 5.8 using 0.1 N NaOH or 0.1 N HCl before autoclaving at 121 °C for 20 min. Different concentrations of auxin (2, 4, 6, 8 and 10 mg L^{-1} naphthaleneacetic acid (NAA) in combination with cytokinin (1 and 2 mg L^{-1} benzyladenine (BA), auxin alone (0.5, 1, 2, 3 and 4 mg L^{-1} α-NAA) and cytokinin alone (0.5, 1, 2, 3 and 4 mg L^{-1} BA) were tested. Five explants were cultured in each growth vessel in five replicates. The explants were maintained at 22 ± 2 °C under 14/10 (light/dark) photoperiod (white fluorescent light, 45μmol m^{-2} s^{-1}) for 18 weeks. After 18 weeks, the number of regenerated bulblets and the intensity of callus formation)the fresh weight of callus(were measured. Subsequently, bulblets were transferred in media containing different levels of NAA and BA and media without PGRs (NAA and BA) as control. Growth regulator ranges were 0.5, 1, 2, 3 and 4 mg L^{-1} NAA and 0.5, 1, 2, 3 and 4 mg L^{-1} BA. The bulblets were kept for 15 weeks and then bulb length and diameter were measured.

Statistical analysis

Data obtained were evaluated by the analysis of variance. Significant differences were identified using Duncan's multiple range test at a significance level of $p \leq 0.05$ using SPSS (version 16.0).

Histological study

Cultures at different developmental stages including primary, embryogenic callus, immature bulblet and mature bulblet were prepared for histological studies. Samples were fixed in FAA (95% ethyl alcohol: glacial acetic acid: formaldehyde: water; 10:1:2:7), then dehydrated in an ethanol series (10%, 30%, 50%, 70%, 96%). After clearing with toluene-ethanol mixture and saturating with toluene-paraffin mixture, the samples were embedded in pure paraffin. Then they were cut with a microtome to a thickness of 10 μm. The incisions were glued to the slide with cytogenetic adhesive. Paraffin was removed by toluene as a solvent for paraffin. Hematoxylin and eosin were used to stain the sections. Then, the slides were observed by light microscope (Eclipse 80i, Nikon, Japan) and photographed.

Assessment of somaclonal variation

Genomic DNA extraction

Total genomic DNA was extracted from the leaves of both mother (culture in the PGR free basal medium) and in vitro regenerated plants (direct or indirect embryo organogenesis from different concentrations of PGRs used in this study) using the CTAB method (Bradaï et al., 2019). Qualitative and quantitative estimation of DNA samples were evaluated by spectrophotometer (LABTRONICS)

using 5 µL DNA aliquot at λ260/λ280 ratio and good quality of DNA samples (λ260/λ280 ratio in the range of 1.6–1.9) were selected. DNA purity was assessed on agarose 0.8% (w/v) gel electrophoresis stained with ethidium bromide. The bulk DNA samples were used for PCR amplification. An equal amount of mother plant and regenerated plants DNA from different concentrations of PGRs were bulked to prepare samples.

ISSR analysis

ISSR markers were used to detect the somaclonal genetic variations. Out of a total of 16 ISSR primers screened, 7 ISSR primers were chosen based on clear and polymorphic banding pattern they produced. ISSR amplification reactions by PCR were performed in a total volume of 12 µL volume, which consisted of genomic DNA (50 ng), Master Mix (Amplicon) (6.5 µL), primers (15 µmol L^{-1}), and ddH_2O (30 µL). The phases of PCR were as follows: initial denaturation phase; 3 min at 95 °C, followed by 36 cycles at 94 °C for 40 ***s***. Annealing phase; 55 °C for 1 min. Extension phase, 90 s at 72 °C. Final extension phase, 6 min at 72 °C. The reactions were preserved at 4 °C. The DNA amplification products were analyzed by electrophoresis in 1.5% agarose gel and 1 × TBE buffer (Tris–Acetate) and were stained with ethidium bromide. DNA ladder size (Thermo Scientific GeneRuler DNA Ladder Mix) was 100–10000 kb. The images of the gels were recorded by the gel documentation system.

Data analysis

The gel images were scored. The presence of a band was considered as '1'and the absence of a band was scored as '0'. Gel images analysis was performed using Totallab Tl120 Software. Clustering was performed by the Jaccard's coefficient of similarity, unweighted pair-group method analysis (UPGMA) using DARWIN-6 (6.0.17 version) software package. Also, STRUCTURE Software (version 2.3.4) used to access Bayesian model clustering. Suitable K was obtained through Structure Harvester.

Results

In vitro responses

The combinations of growth regulators used in *Galanthus* in vitro culture are shown in Tables 1, 2 and 3.

Table 1. Effect of growth regulator on *G. transcaucasicus* twin-scaling explants for 18 weeks.

Growth regulator composition (mg L^{-1})	Morphogenetic response	Number of regenerated bulblets/explant	Intensity of callus formation/ explant (g)
2 NAA+1 BA	callus developing bulblets	2.40^{de}	0.30^{ab}
2 NAA+2 BA	direct bulblets	0.67^{a}	0.00^{a}
4 NAA+1 BA	callus developing bulblets	1.54^{b}	0.45^{ab}
4 NAA+2 BA	direct bulblets	1.55^{b}	0.00^{a}
6 NAA+1 BA	callus developing bulblets	1.64^{bc}	0.82^{bc}
6 NAA+2BA	callus developing bulblets	3.00^{e}	0.11^{a}
8 NAA+1 BA	callus developing bulblets	1.92^{bcd}	1.70^{d}
8NAA+2 BA	callus developing bulblets	1.33^{ab}	0.78^{bc}
10 NAA+1 BA	callus developing bulblets	2.31^{cd}	1.37^{cd}
10 NAA+2 BA	callus developing bulblets	1.33^{ab}	1.22^{cd}

Data represent means of six replicate per treatment. Means followed by the same letter within columns are not significantly different at $p \leq 0.05$ level according to Duncan multiple range test (DMRT).

Table 2. Effect of different concentrations of NAA on *G. transcaucasicus* twin-scaling explants for 18weeks.

NAA (mg L^{-1})	Morphogenetic response	Number of regenerated bulblets/explant	Intensity of callus formation/ explant (g)
0.5	callus developing bulblets	2.70^{a}	0.50^{ab}
1	callus developing bulblets	1.85^{a}	0.14^{a}
2	callus developing bulblets	1.66^{a}	1.17^{bc}
3	callus developing bulblets	1.66^{a}	1.75^{c}
4	callus developing bulblets	1.66^{a}	2.67^{d}

Data represent means of six replicate per treatment. Means followed by the same letter within columns are not significantly different at $p \leq 0.05$ level according to Duncan multiple range test (DMRT).

Table 3. Effect of different concentrations of BA on *G. transcaucasicus* twin-scaling explants for 18weeks.

BA (mgL^{-1})	Morphogenetic response	Number of regenerated bulblets/explant	Intensity of callus formation/ explant (g)
0.5	direct bulblets	1.12^{a}	-
1	direct bulblets	1.16^{a}	-
2	direct bulblets	1.83^{b}	-
3	direct bulblets	2.00^{b}	-
4	direct bulblets	2.87^{c}	-

Data represent means of six replicate per treatment. Means followed by the same letter within columns are not significantly different at $p \leq 0.05$ level according to Duncan multiple range test (DMRT).

In the culture media containing NAA and BA, all cultures produced callus tissue with immature bulblets developed on calli (Figure1a) except the culture media containing 2 mg L^{-1} NAA + 2 mg L^{-1} BA and 4 mg L^{-1} NAA + 2 mg L^{-1} BA. In these media, bulblets direct organogenesis was observed (Table 1). The highest intensity of callus formation)the fresh weight of callus per explant(was obtained in the medium containing 8 mg L^{-1} NAA + 1 mg L^{-1} BA, while the lowest intensity was recorded on the medium supplemented with 6 mg L^{-1} NAA and 1 mg L^{-1} BA.

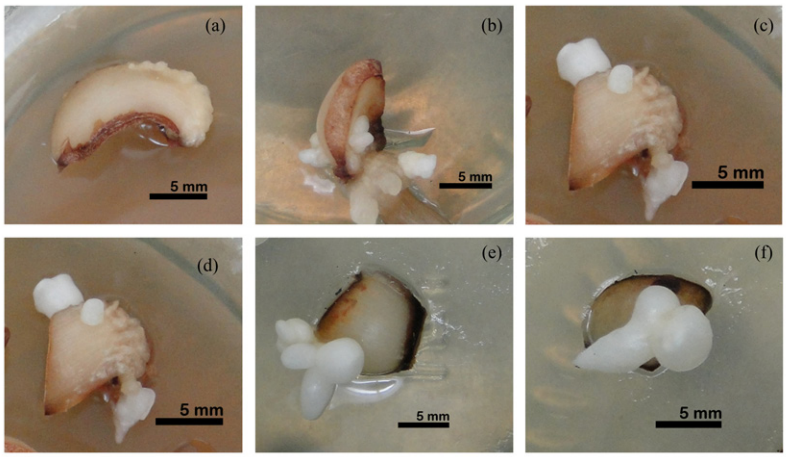

Figure 1. Morphogenic response of *Galanthus transcaucasicus* explants to combinations of growth regulators (NAA, BA) after 18 weeks: (a) Callus formation on the medium with combinations of 4 mg L^{-1} NAA + 1 mg L^{-1} BA; (b), (c) Callus and regeneration on medium with 0.5 mgL^{-1} NAA; (d) Callus formation on the medium with 3 mg L^{-1} NAA; (e), (f) direct bulblets regeneration on the medium with 4 and 3 mg L^{-1} BA.

The medium containing 6 mg L^{-1} NAA and 2 mg L^{-1} BA produced the maximum number of regenerated bulblets (3.0). The lowest number of regenerated bulblets was observed in the medium containing 2 mg L^{-1} NAA + 2 mg L^{-1} BA (0.67).

In the culture media containing NAA without BA, only indirect bulblet organogenesis was observed (Table 2 and Figures 1 b, c, d). The highest intensity of callus formation was observed in the medium supplemented with 4 mg L^{-1} NAA, while the lowest intensity was recorded on the medium containing 1 mg L^{-1} NAA. In addition, bulblets were observed but there was no significant difference between the number of regenerated bulblets and different levels of NAA. In the medium containing BA without NAA, no callus was observed but just direct bulblet regeneration was found (Table 3). In the medium with 4 mg L^{-1} BA, the highest number of regenerated bulblets was observed (Figures 1e, 1f).

Subsequently, bulblets were planted in the media containing different levels of NAA and BA. After 15 weeks, bulb length and diameter were measured (Table 4).

Cultures produced mature bulblets with immature shoots in presence of NAA (Figures 2d, 2e, 2f), while BA culture media produced a well-developed shoot with immature bulblets (Figures 2a, 2b, 2c).

Table 4. Effect of different growth regulator concentrations on growth characteristics of *G. transcaucasicus* bulblets were planted in the media containing different levels of NAA or BA after 15 weeks.

Growth regulator composition (mg L^{-1})	Average bulblet length (mm ± SD)	Median of bulblet length	Average bulblet diameter (mm ± SD)	Median of bulblet length diameter	Morphogenetic response
0 NAA, 0 BA	4.68± 0.39^{a}	4.7	1.84± 0.14^{a}	1.84	bulblets with well-visible shoot
0.5 NAA	5.18± 0.47 ^{a}	5.2	3.27± 0.59^{b}	3.27	bulblets with immature shoot
1 NAA	6.35±0.64 ^{bc}	6.475	3.46±0.73^{b}	3.48	bulblets with immature shoot
2 NAA	4.75±0.59^{a}	4.58	4.44±0.96^{c}	4.61	well-seen bulb scales and immature shoot
3 NAA	4.61±0.41^{a}	4.61	5.74±0.35^{d}	5.76	well-seen bulb scales and immature shoot
4 NAA	5.39±0.33^{ab}	5.28	9.40±0.58^{e}	9.295	well-seen bulb scales and immature shoot
0.5 BA	5.6±0.31^{abc}	5.62	1.83±0.87^{a}	1.535	immature bulblets with well-visible shoot
1 BA	6.59±0.73^{cd}	6.48	2.53±0.48^{ab}	2.35	mature bulblets with well-visible shoot
2 BA	6.54±0.44 ^{cd}	6.52	3.17±0.57^{b}	3.155	mature bulblets with well-visible shoot
3 BA	7.44±0.71^{d}	7.7	3.46±0.45^{b}	3.40	mature bulblets with well-visible shoot
4 BA	11.24±0.66^{e}	11.16	3.47±0.34^{b}	3.44	mature bulblets with well-visible shoot

Data represent means of six replicate per treatment; SD-standart deviation. Means followed by the same letter within columns are not significantly different at $p \leq 0.05$ level according to Duncan multiple range test (DMRT).

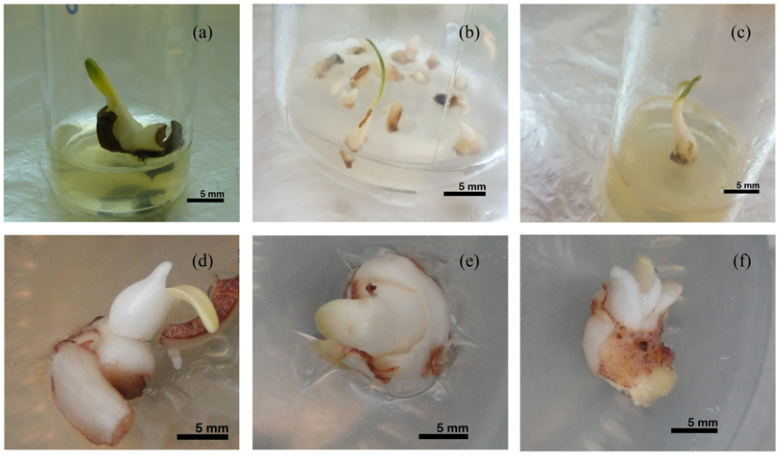

Figure 2. *Galanthus transcaucasicus* bulblet growth (bulblet diameter and length) 15 weeks after culture of the regenerated bulblets from previous experiments: (a) bulblet on the medium with 3 mg L^{-1} BA; (b), (c) mature bulblets with well-visible shoot on the medium with 4 mg L^{-1} BA; (d) bulblets with immature shoot on the medium with 1 mg L^{-1} NAA; (e), (f) well-visible bulb scales and immature shoot on the medium with 3 and 4 mg L^{-1} NAA.

The highest diameter of bulblet was observed in 4 mg L^{-1} NAA (9.4 mm), while the lowest was seen in 0.5 mg L^{-1} BA (1.83 mm). NAA had the greatest effect on increasing bulblets diameter but BA was effective on bulblet length. The highest and lowest lengths of bulblet were observed in 4 mg L^{-1} BA (11.24 mm) and 0.5, 2 and 3 mg L^{-1}NAA, respectively (Table 4).

Histological analysis

The histological analysis of different developmental stages of embryogenesis revealed the regeneration method in *Galanthus transcaucasicus* calli (Figures 3a, 3b). Thus, the primary steps of the development of somatic embryos were observed after 56 days of culture from callus (Figure 3c). Globular embryos on the surface of callus after 66 days of culture were also observed (Figure 3d). Immature bulblet with simple vascular bundles were observed (Figure 3e). After 6 weeks of subculture, some characteristics of whole bulb such as young foliage leaves and shoot primordia were visible in bulblets (Figure 3f).

Somaclonal variation

A total of 56 clear bands were obtained from seven ISSR primers ranging from 250 bp to 2500 bp in size (Table 5).

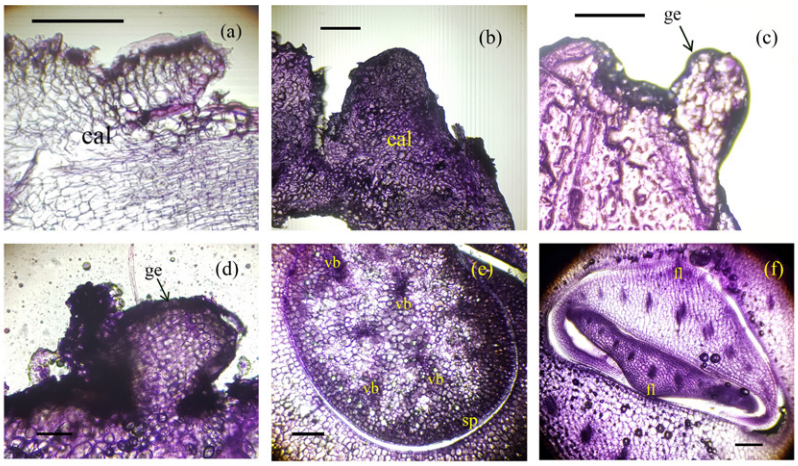

Figure 3. Histological analysis of somatic embryogenesis in callus of *Galanthus transcaucasicus* at different developmental stages: (a), (b) callus tissue (cal); (c), (d) globular embryos (ge) on the surface of callus; (e) cross-section of an immature bulblet containing vascular bundles (vb) and shoot primordia (sp); (f) cross-section of an immature bulblet with foliage leaves (fl). Scalebars: 100 µm.

Table 5. Specifications and data related to ISSR primers used to evaluate the genetic diversity in the in vitro regenerated plants of *G. transcaucasicus*.

Sl. No.	Primer Code	Sequence (5'-3')	Tm (ºC)	Total bands	No. of polymorphic bands	Polymorphism (%)	Range (bp)
1	ISSR-2	GAGAGAGAGAGAGAGAGAC	57	8	8	100.00	700-2500
2	ISSR-7	GAGAGAGAGAGAGAGAC	52	8	7	87.50	550-1750
3	ISSR-10	AGAGAGAGAGAGAGAGC	52	10	10	100.00	500-1500
4	ISSR-14	TCTCTCTCTCTCTCTCG	52	5	4	80.00	700-2000
5	ISSR-15	ACACACACACACACACG	52	13	10	79.92	300-2000
6	ISSR-17	ACACACACACACACACC	52	6	6	100.00	250-1750
7	ISSR-19	ATCATCATCATCATCATCC	52	6	4	80.00	600-1500
Total	-	-	-	56	49	-	-
Mean				8	7	89.63	

The highest and lowest number of bands were related to Primers ISSR-10 and ISSR-15 (5 to 13 bands) (Table 3); 49 bands were polymorphic (89.63%) and 6 bands were monomorphic (11%). Figure 4 shows ISSR banding pattern from the 20 somaclone samples and 1 mother plant using primer ISSR-9.

UPGMA cluster analysis placed samples into two major clusters (Figure 5). The first cluster involved 10 samples where the mother plant (Mo) was placed into this cluster. The second cluster contained 10 samples. The analysis revealed that p1, p2, p3, p4 and p5 were quite similar to the mother plant and p6, p7, p16, p17 and p18 was closer to mother plant while p14 and p15 were found to show maximum variability from the mother plant.

The best K value for ISSR markers based on highest ΔK value was seen at K = 2 in the given range of 1- 6 for K (Figure 6a). Bayesian clustering analysis based on K = 2 was done and the resulting clustering is reported in figure 6b.

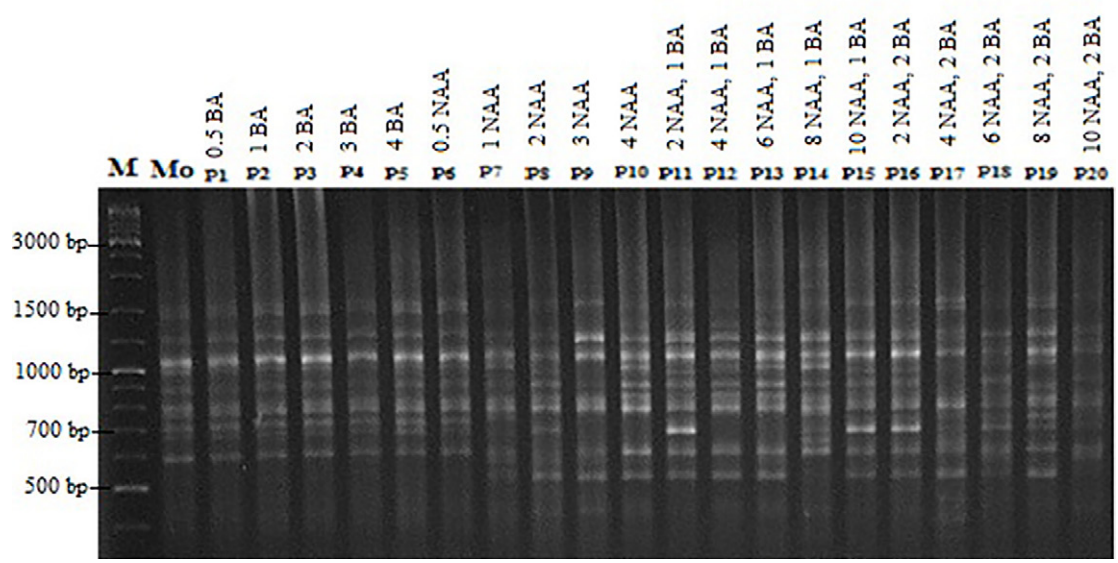

Figure 4. PCR amplified products pattern by primer ISSR-10: M (Molecular weight DNA Ladder); Mo (Mother plant); p1 to p20 (Plantlets regenerated from different concentrations of PGRs).

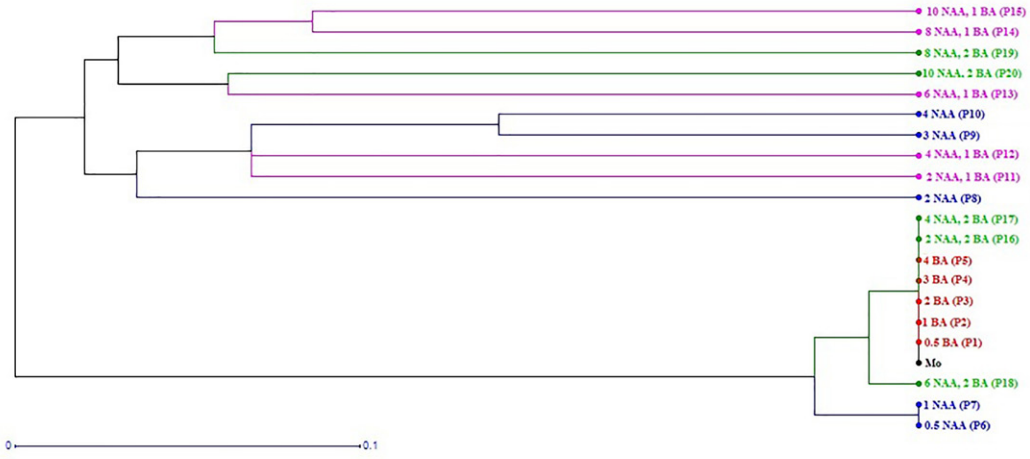

Figure 5. UPGMA dendrogram of *G. transcaucasicus* regenerated plants (p1 to p20) and their mother plant (Mo).

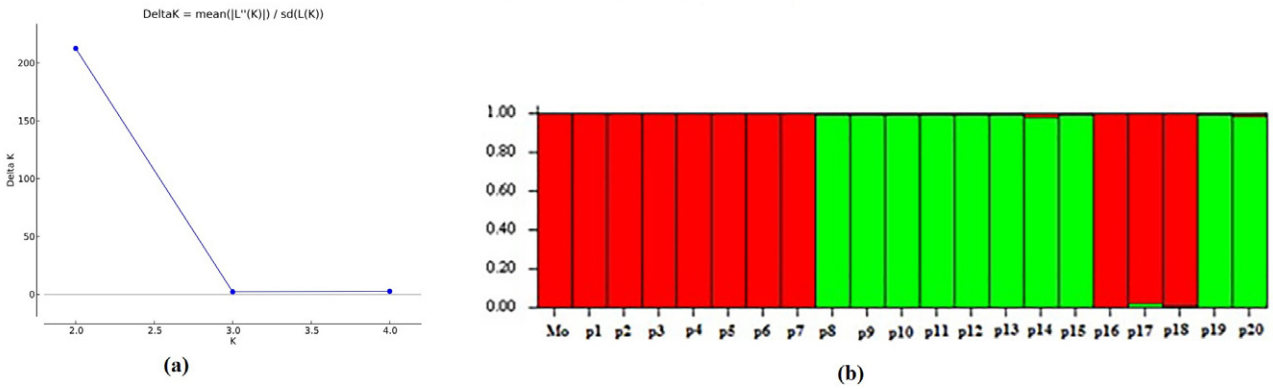

Figure 6. (a) Delta K diagram to determine the appropriate k using Structure Harvester software, (b) Clustering analyses of *G. transcaucasicus* regenated using STRUCTURE Software.

Discussion

Known as the most necessary factor for the regulation of embryogenesis, auxin is responsible for various effects in different phases of embryogenic processes. It is a crucial factor in order to induce embryogenesis and develop embryogenic cell clusters (Ren et al., 2018). The results obtained from the present study revealed that growth regulators, whether in combination or alone, significantly influenced the response of explants. NAA, whether alone or with BA, resulted in the formation of callus; however, NAA alone yielded a higher intensity of calli and showed a higher efficiency for callus induction (Table 2). This is in accordance with recent literature; for instance, MS medium containing a high amount of auxin was reported to be the most appropriate medium for the induction and proliferation of callus obtained from the bulb explants of *N. tazetta* var. *italicus* (Taleb et al., 2014). In vitro culture and micropropagation of *Lapiedra martinezii* Lag. resulted in the production of high concentrations of auxin compared with BAP or KIN, which were essential for attaining an optimal rate of callus stock (Juan-Vicedo et al., 2019). Our findings indicated that an increase in the biomass of callus is accounted for an increase in the concentration of NAA up to 8 mg L^{-1} (Table 1).

In a recent study, callus initiation was detected in a medium with a high level of auxin in *Narcissus pseudonarcissus* cv. Carlton (Ferdausi et al., 2020). Similar results were observed in studies on embryogenic cultures of *Galanthus nivalis* (Resetár et al., 2014) and *Narcissus pseudonarcissus* L.'Carlton' cultures (Malik and Bach, 2016). At concentrations higher than 8 mg L^{-1} callus biomass decreased, in line with a recent study on indirect regeneration of *Lilium ledebourii* Bioss (Ghanbari et al., 2018). Elevated rates of auxin concentration increased callus formation. After a certain level of auxin concentration callus formation decreased, and such inhibitory effect might be due to the endogenous hormones of explants. In the present study, a higher level of BA in combination with NAA reduced the callus formation and increased the bulblet regeneration from callus (Table 1). This is in agreement with results of tissue culture of *Griffinia liboniana* where a reduced response was found in the media with a higher absolute concentration of auxin (Ren et al., 2018).

BA has been proven to trigger bulblet regeneration, which is represented by either callus developing bulblets or direct bulblet organogenesis. Higher concentrations of BA guaranteed a higher number of regenerated bulblets (Table 3), whereas the highest number of bulblets were obtained when BA was added in combination with NAA. It seems that BA is more efficient in regeneration of bulblets when combined with NAA. A study on tissue cultures of *Narcissus pseudonarcissus* cv. Carlton indicated that a lower concentration of auxin in the medium influenced the initiation of small bulblets (Ferdausi et al., 2020). It is noteworthy that the largest number of somatic embryos of *Narcissus* L. 'Carlton' was obtained from the callus acquired in the regeneration medium with 5 µM BA plus 0.5 µM NAA (Malik and Bach 2017). Moreover, a study on *Hybanthus enneaspermus* (Linn.) F. Muell, found BA to be most effective for shoot formation when incorporated with NAA (Murugan and Kamaraj, 2018).

The current study revealed that when bulblets were transferred to a medium containing both BA or NAA, NAA increased bulblet diameter while BA increased its length (Table 4). On the other hand, use of NAA yielded larger bulblets. When auxin was incorporated in the culture medium of *Hippeastrum hybridum*, thicker bulblets were obtained, indicative that auxin concentration to a specific extent would increase bulblet diameter in scale explants (Ren et al., 2018). A recent study found that the best growth performance of bulblets in *Fritillaria imperialis* in terms of bulblet number and diameter was realized at higher concentrations of auxin (Saeed and Cömertpay, 2020). Bulblet diameters and root lengths were found to decrease at low concentrations of auxin, which might be explained by the higher carry-over of NAA that would bring about higher rates of bulblet growth and rooting (Staikidou and Selby, 2012). A direct relationship was detected between higher level of NAA and bulblet diameter. In addition, immature shoots appeared in the presence of NAA while BA resulted in the formation of intact shoots. Higher concentration of BA in the medium resulted in the formation of longer bulblets, which is consistent with the results obtained in a study on embryogenic cultures of *Galanthus nivalis* (Resetár et al., 2014) as well as in a research on in vitro propagation of *Hyacinthus orientalis* L. (Kizil et al., 2016).

Histological analysis revealed that callus was first developed on twin scales with basal bulb explants of *G. transcaucasicus* and the embryo subsequently emerged from the calli. Regardless of the totipotency theory, somatic embryos can be acquired either directly or indirectly. In the latter, an embryogenic callus is formed from which embryos are developed. In dicots, the morphogenetic stage is characterized by four distinct phases, namely globular, heart, torpedo and cotyledonary stage (Campos et al., 2017). Differentiation stages during somatic embryogenesis in *G. transcaucasicus* as reported in the present work are in accordance with the observations previously reported for *G. nivalis* (Resetár et al., 2014).

Molecular techniques are one of the recent methods for detecting somaclonal variation and ISSR markers have been used in many studies these days (Abdolinejad et al., 2020; Khan et al., 2018; Vitamvas et al., 2019). According to the results of genetic variation analysis by means of ISSR, no somaclonal variation was detected in the regenerated plants in media containing 0.5, 1, 2, 3 and 4 mg L^{-1} BA (Figure 5). Furthermore, monomorphic banding patterns were detected all over the ISSR markers system in this samples. Amplified products obtained from the regenerated plants from media incorporated with differing concentrations of cytokinins, BA, thidiazuron (TDZ) and zeatin were found to be monomorphic and similar to the mother plant in *Vaccinium arctostaphylos* L. (Bakhshipour et al., 2019). Likewise, plants regenerated from media with cytokinin (0.5, 1, 2, 3 and 4 mg L^{-1} BA) were found to come from direct bulblets without going through an intermediate callus phase (Table 3 and Figures 1e, 1f).

The regenerated plants obtained from the media with low concentration of NAA (p6 and p7) exhibited somaclonal variation, but they were comparable to the mother plant when lower levels of NAA were added. Thus, plantlets regenerated through indirect embryogenesis displayed somaclonal variation, while those derived from direct embryogenesis were entirely uniform. In a study on in vitro regenerated Chrysanthemum, it was also reported that plantlets from calli exhibited some level of variation, whereas those obtained from embryos were totally uniform (Jayanthi et al., 2018). When regeneration includes an intermediate callus phase somaclonal variation is more likely to happen as there is a large genetic or epigenic modification when callus is formed (Lee and Seo, 2018). Somatic embryogenesis protocols generally comprise a callus phase when embryogenic cultures are sustained and increased, but during which a high rate of genetic variability may occur. Hence, its duration is greatly influential on the frequency of somaclonal variation observed (Bradaï et al., 2019).

Analyses indicated that when higher concentrations of NAA are incorporated in the medium, somaclonal variation will increase and the similarity to the mother plant in the regenerated plants will decrease. The resultant regenerated plants in the present study were in fact indirect bulblets obtained through a callus phase. The highest rate of somaclonal variation was observed in regenerated plants derived from the culture media with the highest concentration of NAA (p14, p15, p19 and p20).

Several growth regulators, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and 6-benzylaminopurine (BAP) have been known as the most prominent factors causing genetic variability (Krishna et al., 2016). An elevated concentration of PGRs might increase the probability of genetic mutation through callus induction (Bakhshipour et al., 2019). Somaclonal variation could be regarded as an indispensable part of conservation strategies for endangered species because it affects the diversity of regenerated plants without genome multiplication (Żabicki et al., 2019).

Conclusions

Based on the results in this study, the most important factor that affects callus formation and intensity in *G. transcaucasicus* explant culture is concentration of NAA. Increasing the concentration of PGRs to a certain level has a positive effect, but further increases are negative. To produce calluses of *G. transcaucasicus* plants, MS medium supplemented with 4 mg L^{-1} NAA is recommended. Moreover, NAA increased bulblet diameter while BA culture media had the greatest effect on developing and increasing the elongation of shoots. It produced a well-developed shoot and the highest lengths of bulblet. Also, according to this study *G. transcaucasicus* plants that are formed through indirect regeneration from callus exhibit genetic diversity, and the higher the amount of callus and concentration of callus-stimulating PGRs, the greater this diversity, which could be useful for breeders.

Author Contribution

NA: conceptualization, obtaining and analyzing data, and writing original draft, **HZ:** review and editing, **SHH:** supervision, writing review and editing, **SJM:** review and editing.

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