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In Vitro Culture of *Eremurus spectabilis* (Liliaceae), a Rare Ornamental and Medicinal Plant, through Root Explants

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Abstract: *Eremurus spectabilis* M. Bieb, a perennial herbaceous wild species, is commonly used in the horticultural, ornamental, and pharmaceutical markets. Studies on the tissue culture systems for this species would be beneficial for mass multiplication as well as for future breeding programs. An in vitro propagation technique was established here using tuberous root explants as unique and responsive starting materials for culture initiation. The proliferated calli were sub-cultured on shoot proliferation media and regenerated microshoots were assessed. The shoot proliferation rate, leaf number, leaf length, and chlorophyll and carotenoid contents were recorded. The highest callus induction per explant (76.67%), callus dry weight (10.25 mg), callus firmness ratio (3.97), and callus color intensity ratio (2.83) were observed in explants inoculated on Murashige and Skoog (MS) medium supplemented with 10.0 mgL^{-1} 6-benzylaminopurine (BAP). The highest shoot proliferation rates were obtained when calli were sub-cultured on MS or Schenk and Hildebrandt (SH) basal media supplemented with 2.0 mgL^{-1} BAP. The half-strength MS medium fortified with 4.0% sucrose + 2.0 mgL^{-1} indole butyric acid (IBA) + 200 mgL^{-1} activated charcoal was a superior combination for root emergence and rooting parameters. Regenerated plantlets were then successfully adapted to ex vitro conditions. The reported protocol can be exploited at a commercial scale following minor modification, or could be beneficial in the production of secondary metabolites in bioreactors where callus is required as fresh plant material.

Keywords: callus induction; foxtail lily; micropropagation; proliferation rate; acclimatization

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1. Introduction

The foxtail lily (*Eremurus spectabilis* M. Bieb.) is a perennial and herbaceous wild plant species of the Liliaceae family (Figure 1). This rare species grows well on dry and rocky slopes and is most widespread in Southern and Central Asia, including Iran, western Pakistan, Turkey, Palestine, Lebanon, Syria, and Caucasus [1]. This plant is rich source of antioxidants, phenolic compounds, and minerals that is consumed as a vegetable in some countries, such as Turkey. Foxtail lily also has medicinal importance and is utilized to prepare a special type of plant-derived adhesive [2,3]. The leaves of this plant are frequently used against eczema, fungal infections, and diabetes [4], and recently the phytochemical composition, antioxidant, and antimicrobial capacities of *E. spectabilis* were examined in vitro [5]. Antioxidant, antimicrobial, and anticancer effects of different extracts from wild edible plant *E. spectabilis* leaves and roots have also already been reported by some Turkish scientists [3], where this plant is a wild vegetable, growing in spring in the east of Turkey.

Figure 1. Foxtail lily (*Eremurus spectabilis*) plant species under its natural habitat—Golestan National Park, Golestan province, Northern Iran (**right**); a close up view of inflorescence during full bloom ((**left**); Bar = 10 cm; Photo: Y. Basiri: April 2019).

Different parts of the plants are used to treat fungal diseases, diabetes, hepatitis, liver and stomach disorders, and some cancer types [5–8]. After drying and grinding, the roots of *E. spectabilis* have a highly useful application in adhesives [9]. In addition to all these beneficial nutritional, medicinal, and industrial properties, this plant species has a high ornamental value due to its long spike-type inflorescences and application as a cut flower [10,11].

In vitro mass micropropagation techniques have been considered a promising procedure for the multiplication of valuable, hard-to-propagate and endangered wild species [12]. Although in vitro regeneration studies have been performed on many genera from the Liliaceae family, such as *Aloe*, *Lilium*, *Chlorophytum*, *Feritillaria*, and *Scilla* [13–20], only a few studies have reported on the in vitro regeneration of *E. spectabilis* worldwide [21,22]. In a recent article [21], the leaf and rhizome explants of *E. spectabilis* were cultured on Murashige and Skoog (MS) media [23], supplemented with 6-benzylaminopurine (BAP) and 2, 4-dichlorophenoxyacetic acid (2, 4-D). However, the results showed no in vitro shoot regeneration regardless of explant types. For instance, Tuncer [22] cultured hypocotyls of 35–40-day-old in vitro-germinated seedlings on MS medium containing 2,4-D + kinetin, thidiazuron + NAA, and BAP + 2,4-D to stimulate bulblet and/or shoot regeneration. As such, the optimization of micropropagation methods and the establishment of proper protocols are required for such species. In the literature, it has been recommended that the micropropagation of some medicinal, endangered, wild, and endemic species must be performed through tissue culture techniques, and that consistent protocols must be optimized [21]. Therefore, such protocols may be utilized for mass multiplication as well as the genetic improvement of these valuable crops. According to such a statement, the importance of the in vitro propagation of Foxtail lily is more obvious. Consequently, the present study aimed to examine the effects of the disinfection method, root explant responses, and exogenous growth regulators on the in vitro propagation of Foxtail lily. This study investigates the possibility of the in vitro mass micropropagation of *E. spectabilis* based on the morphological and physiological responses of root explants. The utilization of root explants itself may be considered an innovation in the tissue culture of this species, as it is botanically a monocot plant and regeneration from leaf explants may not be considered a successful strategy. Moreover, the reported protocol would be significant for being the first in vitro optimization of micropropagation of this endemic species in northern Iran.

2. Materials and Methods

2.1. Plant Materials

The fleshy roots of *E. spectabilis* Clade II [24] were collected from its natural habitat (Figure 1), i.e., Golestan National Park, Golestan province, Iran (UTM zone: 37°32′49.8″ (37.5472°) N and 56°23′22.4″ (56.3896°) E), during the start of the dormant season. The Foxtail lily has tuberous roots. Each root clump was individually planted in a 5 L plastic pot. A blend of coco-peat: perlite (1:1) was used as a potting mixture. The pots were regularly irrigated using normal tap water with 7-day intervals. The pots were also irrigated with Hoagland solution (100 mL per pot) with 14-day intervals. The new, fresh leaves emerged in the spring season, three months after planting. The tuberous root segments were then used for in vitro culture initiation.

2.2. Explant Disinfection

In vitro cultures were initiated using root segments (about 1.0 to 1.5 cm long) obtained from root clumps (Figure 2) in March. Initially, the whole root clamp was kept in water for 3 h to soften and remove each and every soil particle; it was then thoroughly washed with tap water. The small root portions were dissected and were thoroughly washed with tap water supplemented with a few drops of Tween-80 for 30 min and were then agitated in a fungicide solution (4 gL^{-1} of carbendazim) for 1 h on a horizontal shaker. The root explants were rinsed with 70% alcohol (Ethanol) for 40 s prior to surface sterilization using either commercial bleach (NaOCl, 40% for 15 min) or $HgCl_2$ (0.1% for 7 min), followed by at least three-times rinsing with autoclaved double distilled water. Explants were then inoculated into 100 mL flasks containing culture initiation medium.

Figure 2. Tuberous root clump of Foxtail lily (*Eremurus spectabilis*) just before explant preparation (**left**); root explants inoculated for callus initiation ((**right**); Bar = 10 mm; Photo: Y. Basiri: May 2019).

2.3. Culture Initiation and Shoot Regeneration

The MS medium [23] and Schenk and Hildebrandt (SH) medium [25] were used as basal media for culture initiation and callus induction. A combination of NAA (Sigma-Aldrich, St. Louis, MO, USA; 0.2 mgL^{-1}) with either 5.0 or 10 mgL^{-1} BAP in both basal media was evaluated for callus induction (Table 1). The ability of the explants to develop calli was recorded two months after inoculation.

The callus' fresh and dry (60 °C for 24 h) weights were recorded. The firmness of the callus tissue was also scored from 1 to 4, where 1 = very soft, 2 = soft, 3 = firm, and 4 = very firm. The callus color intensity was also rated from 1 to 3, where 1 = pale, 2 = medium color intensity, and 3 = dark color.

The in vitro-grown produced plant pigments such as chlorophyll a and b and carotenoids were extracted with dimethyl sulphoxide (DMSO) and their concentrations were estimated using fresh callus samples according to the method described by Barnes et al. [26], which is a spectrophotometric procedure.

Table 1. Media compositions for callus initiation and shoot regeneration of Foxtail lily (*Eremurus spectabilis*).

Media	Basal Medium	Growth Regulators (mgL^{-1})	Other Modifications
Culture initiation (callus induction)	MS	NAA (0.1) + BAP (5.0)	-
		NAA (0.1) + BAP (10)	-
	SH	NAA (0.1) + BAP (5.0)	-
		NAA (0.1) + BAP (10)	-
Shoot regeneration	MS	IBA (0.1) + BAP (2.0)	AC [1] (200 mgL^{-1})
		IBA (0.1) + BAP (5.0)	AC (200 mgL^{-1})
	SH	IBA (0.1) + BAP (2.0)	AC (200 mgL^{-1})
		IBA (0.1) + BAP (5.0)	AC (200 mgL^{-1})
Root regeneration	MS	IBA (2.0) + NAA (1.0)	-
	SH	IBA (2.0) + NAA (1.0)	-
	$\frac{1}{2}$ MS	IBA (2.0)	4.0% sucrose + 200 mgL^{-1} AC
	$\frac{1}{2}$ SH	IBA (2.0)	4.0% sucrose + 200 mgL^{-1} AC

[1] Activated charcoal.

The proliferated calli were sub-cultured on shoot proliferation medium. The MS and SH basal media were supplemented with indole butyric acid (IBA; 0.1 mgL^{-1}) combined with either 2.0 or 5.0 mgL^{-1} BAP (Table 1). Two types of callus mass, i.e., whole uncut (intact) calli and divided callus explants, were evaluated. To obtain a sufficient biomass, the regenerated shoots were sub-cultured for at least three cycles on the same regenerated basal media. A mild concentration of activated charcoal (AC, 200 mgL^{-1}) was supplemented to the shoot proliferation media. Two months after inoculation, the ability of the callus tissues to regenerate shoots was assessed, and the shoot proliferation rate, leaf number, leaf length, and chlorophyll and carotenoid contents were recorded following the same procedure already mentioned for the estimation of pigments in callus tissues [26]. The growth chamber had controlled photoperiod (16/8 h) and temperature (26 ± 1 °C) conditions. It was equipped with cool-white fluorescent lights (227 µmol·m^{-2}·s^{-1}) 40 cm above the vessel cultures.

2.4. In Vitro Rooting

The regenerated shoots from the calli were sub-cultured on rooting media (Table 1). Both full and half-strength basal media were evaluated for root induction. The level of sucrose in the half-strength media was increased to 40 gL^{-1} (4.0%).

All media cultures utilized in the present experiment were solidified with 8.0 gL^{-1} agar and supplemented with 30 gL^{-1} sucrose. The pH was adjusted to 5.8 prior to autoclaving (1.05 kg·cm^{-2} for 15 min). The growth conditions in the rooting stage were exactly the same as the regeneration stage.

2.5. Acclimatization and Ex Vitro Conditions

The rooted plantlets were subjected to hardening and ex vitro transfer. The grapevine hardening strategy already reported by Alizadeh et al. [27] was evaluated for acclimatization of Foxtail lily in vitro-derived plantlets. The rooted plantlets (21-days-old) were transferred to glass jars with a polypropylene (PP) cap containing perlite:coco peat:vermiculite (2:1:1; volume). The hardening media was pre-soaked with either 1/2 MS or 1/2 SH solution (the media was devoid of vitamins and sucrose—only the mineral parts including macro/micro salts, calcium and iron-EDTA were taken). The pre-soaked substrate was subjected to autoclaving prior to plantlet transfer. A total of 10 replicates were used per treatment. The glass jars were then shifted to cool-white fluorescent lights (227 µmol·m^{-2}·s^{-1}) with controlled photoperiod (16/8 h) and temperature (26 ± 1 °C) conditions. After three weeks, the polypropylene caps were loosened gradually, and finally, removed completely. At regular intervals, the growing plantlets were then misted with sterile distilled water

containing 0.1% carbendazim (*w*/*v*) fungicide. Hardened plantlets were transferred to ex vitro glasshouse conditions at the 6 to 7th weeks of acclimation. Finally, at the end of this period, some growth characteristics such as root length, leaf number, leaf length, and biomass volume were recorded.

2.6. Statistical Analysis

All experiments were arranged in a completely randomized design, and the data were subjected to analysis of variance using SAS software (version 9.4, SAS Institute, Cary, NC, USA). Comparisons of means were tested using the least significant difference (LSD) test, at a 0.05 level of probability (*p*).

3. Results

3.1. Callus Induction

The analysis of variance of the in vitro callus induction data is presented in Table 2. The callus proliferation from the fleshy root explants of the Foxtail lily was also depicted in Figure 3A. The highest callus induction frequency per explant (76.67%), callus dry weight (10.25 g), concentration of chlorophyll a (10.23 mg·g^{-1} FW), chlorophyll b (19.81 mg·g^{-1} FW), and carotenoids (6.50 mg·g^{-1} FW), callus firmness ratio (3.97), and callus color intensity ratio (2.83) were found in explants grown in MS medium containing 10 mgL^{-1} BAP and 0.1 mgL^{-1} NAA (Table 2).

Table 2. Effects of basal medium, BAP levels and sterilization method on callus induction rate (C, %), callus dry weight (DW, mg), chlorophyll a (Chl a, mg·g^{-1} FW), chlorophyll b (Chl b mg·g^{-1} FW), chlorophyll a + b (Chl b mg·g^{-1} FW), carotenoids (Ctn, mg·g^{-1} FW), callus firmness (Cf), color intensity rate (CI), and callus color (CC) of Foxtail lily (*Eremurus spectabilis*).

Medium	Growth Regulators (mgL^{-1})	Sterilization Method	C	DW	Chl a	Chl b	Ctn	Cf	CI	CC
MS	NAA (0.1) + BAP (5.0)	NaClO, 40% for 15 min	36.67 ^{e}	6.21 ^{bc}	4.06 ^{c}	6.01 ^{b}	1.53 ^{c}	3.33 ^{bc}	1.25 ^{bc}	YG
		HgCl2, 0.1% for 7 min	25.00 ^{f}	5.19 ^{cd}	0.16 ^{e}	0.33 ^{c}	0.55 ^{d}	2.00 ^{f}	1.00 ^{c}	B
	NAA (0.1) + BAP (10)	NaClO, 40% for 15 min	76.67 ^{a}	10.25 ^{a}	10.23 ^{a}	19.81 ^{a}	6.50 ^{a}	3.97 ^{a}	2.83 ^{a}	G
		HgCl2, 0.1% for 7 min	55.00 ^{b}	8.16 ^{ab}	3.18 ^{d}	10.07 ^{b}	3.50 ^{b}	3.00 ^{cd}	1.25 ^{bc}	YG
SH	NAA (0.1) + BAP (5.0)	NaClO, 40% for 15 min	56.67 ^{b}	6.03 ^{bc}	4.82 ^{bc}	10.29 ^{b}	2.84 ^{b}	3.67 ^{ab}	1.33 ^{b}	YG
		HgCl2, 0.1% for 7 min	46.67 ^{cd}	4.15 ^{cd}	0.27 ^{e}	0.60 ^{c}	0.20 ^{d}	2.67 ^{de}	1.17 ^{bc}	B
	NAA (0.1) + BAP (10)	NaClO, 40% for 15 min	50.00 ^{bc}	5.04 ^{cd}	5.35 ^{b}	9.91 ^{b}	3.07 ^{b}	3.33 ^{bc}	1.33 ^{b}	YG
		HgCl2, 0.1% for 7 min	40.00 ^{de}	3.28 ^{d}	0.36 ^{e}	0.84 ^{c}	0.49 ^{d}	2.33 ^{ef}	1.00 ^{c}	B
	Source of variation									
	Medium (M)		25.00 ^{ns}	95.99 **	35.02 **	159.03 **	22.49 **	0.19 ^{ns}	1.69 **	
	Growth regulator (G)		2408.33 **	19.81 *	72.28 **	410.96 **	53.30 **	1.02 *	2.08 **	
	Sterilization method (S)		2133.33 **	34.24 **	315.09 **	875.43 **	63.43 **	15.19 **	4.08 **	
	M × G		5208.33 **	58.92 **	55.04 **	420.50 **	41.25 **	4.69 **	3.00 **	
	M × S		133.33 *	0.21 ^{ns}	1.48 ^{ns}	8.35 ^{ns}	1.17 *	0.19 ^{ns}	1.33 **	
	G × S		75.00 ^{ns}	0.66 ^{ns}	9.74 **	8.88 ^{ns}	2.83 **	0.02 ^{ns}	1.69 **	
	M × G × S		75.00 ^{ns}	1.10 ^{ns}	9.91 **	16.37 ^{ns}	3.23 **	0.02 ^{ns}	1.02 **	
	Error		45.83	3.33	0.55	15.49	0.33	0.19	0.06	

Means followed by the same letter are not significantly different by the LSD multiple range test at $p \leq 0.05$. YG, Yellowish green; B, Black; G, Green. * and **: Significant at 5% and 1% probability levels, respectively; ns: Non-significant.

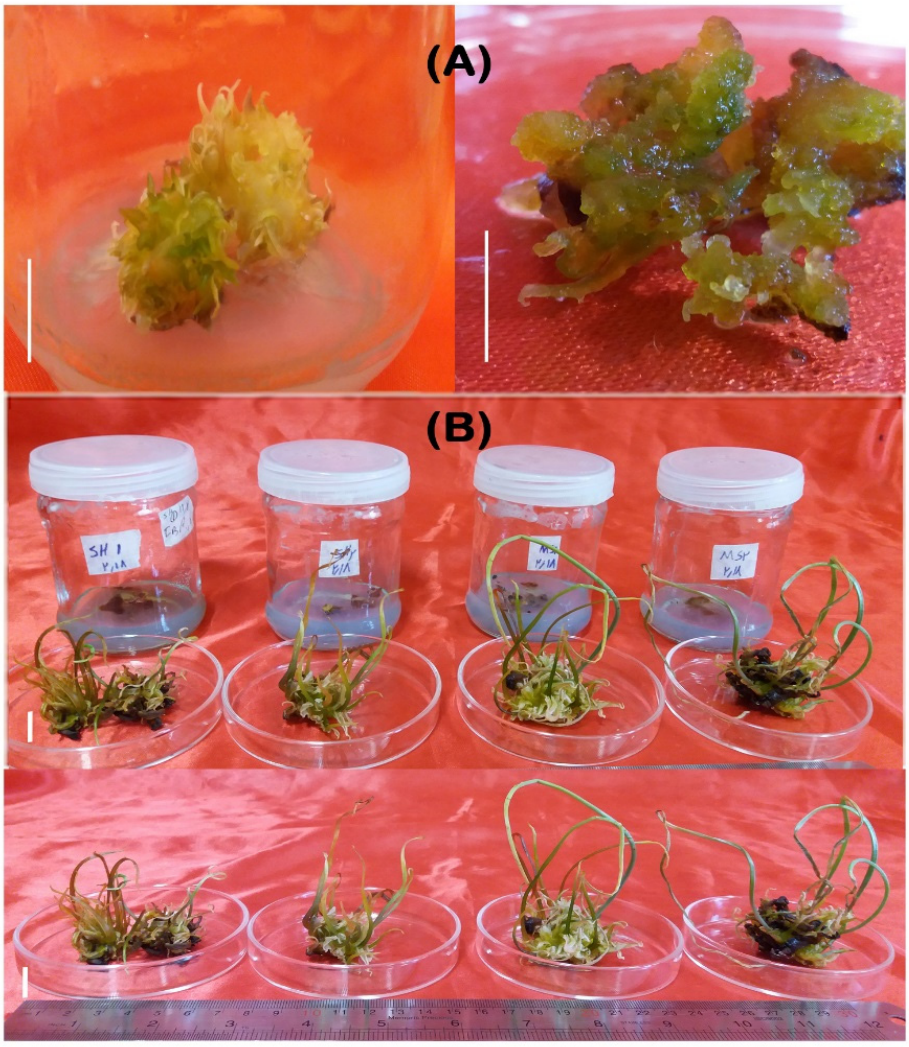

Figure 3. (**A**): Callus regenerated from Foxtail lily (*Eremurus spectabilis*) root explants inoculated on MS medium containing 10 mgL^{-1} BAP. (**B**): Shoot regeneration from callus on MS medium containing 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} IBA, two months after inoculation (Bar = 10 mm; Photo: Y. Basiri: June 2019).

3.2. Shoot Regeneration

The ANOVA table for the shoot regeneration data is shown in Table 3. According to this data, the culture medium and growth regulator had no significant effect on the number of leaves in regenerated shoots. The best proliferation rate was obtained from callus explants inoculated in either MS or SH media containing 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} IBA (Figure 3B). The application of higher concentrations of BAP (5.0 mgL^{-1}) in both basal media significantly reduced the shoot proliferation rate (Table 3). The highest number of leaves was obtained from uncut, intact explants on MS medium containing 5.0 mgL^{-1} BAP and 0.1 mgL^{-1} IBA, while the highest leaf length was observed with the same explant type and medium containing 2.0 mgL^{-1} BAP. The lowest number of leaves and the smallest leaves were observed in the cultures regenerated from calli sub-cultured on the SH medium containing 5.0 mgL^{-1}. The highest concentration of chlorophyll a, b, and carotenoids were recorded with the intact callus tissues sub-cultured on MS medium containing 2.0 mgL^{-1} BAP and 0.1 mgL^{-1} IBA. Conversely, shoots regenerated from divided callus tissues on SH medium containing either 2.0 or 5.0 mgL^{-1} BAP had the lowest levels of pigments (Table 3).

Table 3. Effects of basal medium, BAP levels and explant type on shoot proliferation (SP, %), leaf number (Ln), leaf length (Ll, cm), chlorophyll a (Chl a, mg·g^{-1} FW), chlorophyll b (Chl b mg·g^{-1} FW), and carotenoids (Ctn, mg·g^{-1} FW) of Foxtail lily (*Eremurus spectabilis*).

Medium	Growth Regulator	Callus Type	SP	Ln	Ll	Chl a	Chl b	Ctn
MS	IBA (0.1) + BAP (2.0)	Intact	6.33 ^{a}	39.17 ^{abc}	20.45 ^{a}	9.96 ^{a}	18.78 ^{a}	6.12 ^{a}
		Divided	5.17 ^{ab}	41.83 ^{ab}	16.42 ^{ab}	9.71 ^{ab}	16.82 ^{ab}	6.36 ^{a}
	IBA (0.1) + BAP (5.0)	Intact	3.67 ^{bcd}	47.17 ^{a}	5.83 ^{c}	7.45 ^{c}	13.64 ^{bc}	4.2 ^{b}
		Divided	4.17 ^{bc}	33.33 ^{bc}	6.28 ^{c}	8.33 ^{bc}	13.32 ^{bc}	4.53 ^{b}
SH	IBA (0.1) + BAP (2.0)	Intact	4.33 ^{ab}	36.67 ^{abc}	16.45 ^{ab}	7.47 ^{c}	14.34 ^{bc}	4.63 ^{b}
		Divided	3.17 ^{bcd}	37.33 ^{abc}	12.42 ^{b}	7.09 ^{cd}	12.75 ^{dc}	4.87 ^{b}
	IBA (0.1) + BAP (5.0)	Intact	1.83 ^{d}	42.17 ^{ab}	2.17 ^{c}	4.96 ^{e}	9.19 ^{d}	2.71 ^{c}
		Divided	2.16 ^{cd}	28.33 ^{c}	2.78 ^{c}	5.81 ^{de}	9.21 ^{d}	3.04 ^{c}
	Source of variation							
	Medium (M)		46.02 **	216.75 ^{ns}	172.52 **	76.68 **	218.62 **	26.65 **
	Growth regulator (G)		38.52 **	12.00 ^{ns}	1776.33 **	44.29 **	224.98 **	71.53 **
	Callus type (C)		1.69 ^{ns}	444.08 *	36.75 ^{ns}	0.90 ^{ns}	11.08 ^{ns}	0.99 ^{ns}
	M × G		0.02 ^{ns}	6.75 ^{ns}	0.52 ^{ns}	0.008 ^{ns}	0.003 ^{ns}	0.00 ^{ns}
	M × C		0.02 ^{ns}	3.00 ^{ns}	0.02 ^{ns}	0.02 ^{ns}	0.38 ^{ns}	0.00 ^{ns}
	G × C		7.52 ^{ns}	720.75 *	62.56 *	4.11 ^{ns}	7.92 ^{ns}	0.02 ^{ns}
	M × G × C		0.02 ^{ns}	3.00 ^{ns}	0.02 ^{ns}	0.008 ^{ns}	0.002 ^{ns}	0.00 ^{ns}
	Error		3.35	128.87	13.49	1.92	9.42	0.59

Means followed by the same letter are not significantly different, as determined by the LSD multiple range test at $p \leq 0.05$. * and **: Significant at the 5% and 1% probability levels, respectively; ns: Non-Significant.

3.3. Root Regeneration

The ANOVA table for the root parameters regenerated by in vitro microshoots (Table 4) revealed that the effect of the culture medium on all recorded parameters such as root length was statistically significant (Table 4). Figure 4 shows the emergence of roots from the basal part of shoot clumps. The best combination of basal medium and growth regulator for root length, number of leaves, and amount of chlorophyll *a* and *b* was found to be 1/2 MS + 2.0 mgL^{-1} IBA + 4.0% sucrose + 200 mgL^{-1} activated charcoal. MS + 2.0 mgL^{-1} IBA + 1.0 mgL^{-1} NAA also produced the highest chlorophyll *a* and *b* and carotenoid levels (Table 4).

Table 4. Effects of medium on root length (Rl, cm), biomass volume (Bv), leaf number (Ln), leaf length (Ll, cm), chlorophyll a (Chl a, mg·g^{-1} FW), chlorophyll b (Chl b, mg·g^{-1} FW), and carotenoids (Ctn, mg·g^{-1} FW) of Foxtail lily (*Eremurus spectabilis*).

Medium	Rl	Bv	Ln	Ll	Chl a	Chl b	Ctn
MS + 2.0 mgL^{-1} IBA + 1.0 mgL^{-1} NAA	0 ^{c}	37.30 ^{a}	16.77 ^{a}	4.70 ^{b}	8.92 ^{a}	15.19 ^{ab}	5.65 ^{a}
SH + 2.0 mgL^{-1} IBA + 1.0 mgL^{-1} NAA	0 ^{c}	37.70 ^{a}	6.71 ^{c}	4.30 ^{b}	7.23 ^{b}	12.60 ^{bc}	3.79 ^{b}
$\frac{1}{2}$ MS + 2.0 mgL^{-1} IBA + 4.0% sucrose + 200 mgL^{-1} AC	1.11 ^{a}	41.00 ^{a}	18.95 ^{a}	6.40 ^{a}	8.58 ^{a}	16.53 ^{a}	3.41 ^{b}
$\frac{1}{2}$ SH + 2.0 mgL^{-1} IBA + 4.0% sucrose + 200 mgL^{-1} AC	0.39 ^{b}	41.50 ^{a}	11.93 ^{b}	5.10 ^{ab}	5.85 ^{c}	11.00 ^{c}	2.04 ^{c}

Table 4. *Cont.*

Medium	Rl	Bv	Ln	Ll	Chl a	Chl b	Ctn
Source of variation							
Medium	2.73 **	47.56 ^{ns}	296.44 **	8.29 *	19.62 **	62.20 **	22.23 **
Error	0.062	140.35	17.21	2.65	1.60	12.38	1.03

Means followed by the same letter are not significantly different as determined by the LSD multiple range test at $p \leq 0.05$. * and **: Significant at the 5% and 1% probability levels, respectively; ns: Non-significant.

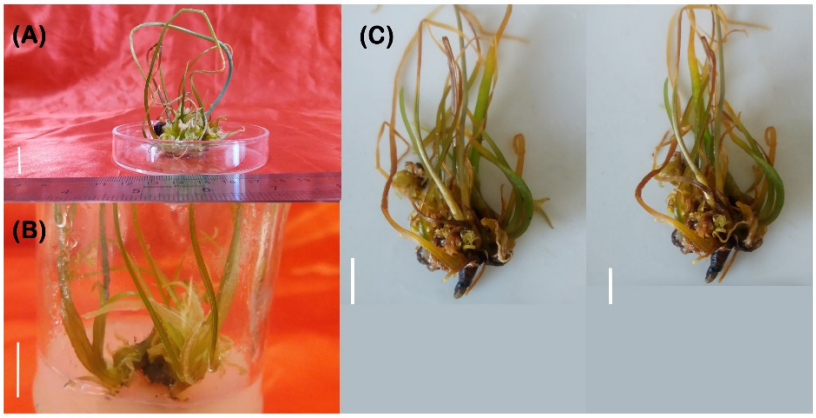

Figure 4. (**A**): Indirect shoot regeneration from callus explants. (**B**) Shoot clumps transferred to rooting medium. (**C**) Emergence of fleshy roots from the basal part of shoot clump three weeks after inoculation (Bar = 10 mm; Photo: Y. Basiri: July 2019).

3.4. Acclimatization and Ex Vitro Transfer

Plantlets comprising both healthy shoots and roots regenerated from different media were subjected to acclimatization. During the acclimation period, the plantlets fertigated with half MS solution had longer roots, bigger leaves, and higher biomass volume than plantlets that received the half SH solution (Table 5).

Table 5. Effects of nutrient solutions on root length (Rl, cm), leaf number (Ln), leaf length (Ll, cm) and biomass volume (Bv, cm^3) of Foxtail lily (*Eremurus spectabilis*) plantlets during acclimation and ex vitro transfer period.

Nutrient Solutions	Rl	Ln	Ll	Bv
$\frac{1}{2}$ MS	1.14 ^{a}	32.60 ^{a}	13.66 ^{a}	5.80 ^{a}
$\frac{1}{2}$ SH	0.66 ^{b}	27.80 ^{a}	6.98 ^{b}	3.70 ^{b}
Source of variation				
Nutrient solution	1.15 *	115.20 ^{ns}	223.11 **	22.05 *
Error	0.18	76.78	20.25	2.98

Means followed by the same letter are not significantly different as determined by the LSD multiple range test at $p \leq 0.05$. * and **: Significant at the 5% and 1% probability levels, respectively; ns: Non- significant.

4. Discussion

The *E. spectabilis* is an endemic wild species of the Iran/Turan region—especially in central Asia [21]. There is insufficient coverage of this species in the literature, especially with respect to its micropropagation. The fleshy roots of this species may be utilized as explant preparations; however, the contamination of explants is a significant and limiting factor when optimizing micropropagation protocols [28]. A surface-sterilization process aimed at eliminating all microorganisms could guarantee the explant's viability and regeneration capacity, which are affected by the concentration and application period [29].

Therefore, explant disinfecting was assumed as an essential step in the culture establishment of *E. spectabilis*. In the present study, the root explants were efficiently sterilized with NaClO (40% for 15 min). When these were cultured in MS medium supplemented with 10 mgL^{-1} BAP, they produced a green callus—but other treatments including media composition, disinfection methods, and BAP levels produced yellowish-green or blackish calli masses (Table 2). The success of the callus induction and the application of an appropriate disinfection method and growth regulators are in agreement with previous investigations [30–32]. Furthermore, Colgecen et al. [33] examined the potential of different basal media such as SH and MS in callus induction and the proliferation of *Arnebia* explants, and their results showed that the best friable callus was obtained with MS medium, which further supported our results regarding the superior effect of MS medium on callus induction in *E. spectabilis* explants. Regeneration of calli with various colors and structures from *E. spectabilis* explants is also in conformity with other results reported on various explant sources from other plant species [34,35]. Furthermore, it is well documented that there are several types of calli—especially in monocot plants—based on their regenerative and morphological characteristics [36]. However, these different types of callus often have significantly different plant regeneration capabilities [36]. Therefore, the details of the distinction and regenerative capacities of calli in *E. espectabilis* require further investigation in future studies.

The present study indicated significant interactions of basal medium, cytokinin concentration, and disinfection method on callus induction, carotenoid concentration, and chlorophyll content. The highest percentage of callus formation from potato explants had already been obtained using MS medium containing 5.0 [37] and 2.0 mgL^{-1} BAP [38]. The results of the present study corroborate the findings of Carsono and Yoshida [39] and Benderradji et al. [40], who also reported that medium composition could be a source of variation affecting callogenesis in different explants.

The degree of success in any technology employing cell, tissue, or organ culture is related to relatively few major factors. A significant factor is the choice of nutritional components and plant growth regulators [41]. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. There are several basal media reported in the literature [41,42]—among them, the MS medium [23] is the most widely used. Furthermore, auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures [42]. Hence, in the present study, a combination of NAA with BAP in two basal media (MS, SH) was evaluated for callus induction. Auxins are very widely used in plant tissue culture and usually form an integral part of nutrient media. Auxins promote, mainly in combination with cytokinins, the growth of calli, cell suspensions and division, and cell elongation. Since they are capable of initiating cell division, they are involved in the formation of meristems, giving rise to either unorganised tissue or defined organs [42]. BAP, either singly or combined with NAA, has a positive effect on shoot regeneration [22,27,42]. Based on a recent research work [43], it was revealed that the combination of BAP and NAA was a superior treatment for mico-bulb induction in Lily (*Lilium longiflorum*) explants. They also stated that when auxin or cytokines are exogenously added to culture media, they will trigger the further formation of micro tubers more quickly. This can increase the concentration of endogenous growth regulators in cells, helping the growing process in developing tissues.

The in vitro shoot regenerated from callus explants clearly showed a significant influence of medium type and BAP concentration. Hence, shoot proliferation, leaf length, and the concentrations of chlorophyll *a*, chlorophyll *b*, and carotenoids were also significantly affected. Tuncer [22] reported significant differences among MS media containing different combinations of plant growth regulators ($p < 0.01$) on direct shoot regeneration from hypocotyl explants of *E. spectabilis*. To our best of knowledge, the data presented here demonstrate a complete and applicable protocol for indirect shoot regeneration in callus cultures of Foxtail lily (Figures 3 and 4). The plant growth regulators might play a key role

in the shoot regeneration capacity of explants [44]. In most of the tissue culture studies, establishing a suitable ratio of auxin to cytokinin has been applied for high-frequency shoot regeneration [45]. Our study has demonstrated that the optimal level of BAP in shoot proliferation medium is 2.0 mgL^{-1}, which is satisfactory for superior shoot regeneration capacity. Furthermore, some physiological attributes, such as the chlorophyll and carotenoid contents of regenerated shoots, could be improved.

While the efforts of Tuncer [21] to regenerate shoots from rhizome and leaf explants of *E. spectabilis* have not been successful, we observed in vitro microshoots just 6 weeks after inoculation of the organogenic callus on media containing 2.0 mgL^{-1} BAP. Tuncer [21] ascribed phenolic compound-induced browning of cultured explants (rhizome and leaf) as a reason for the loss of the regeneration ability of the Foxtail lily. We supposed that one of the reasons that our proliferated callus tissues had satisfactory regenerating abilities may be due to their green appearance, as Iqbal et al. [46] has previously suggested that green callus tissues have good regeneration abilities and can be successfully applied for shoot proliferation. Irvani et al. [47] reported that the highest shoot regeneration from calli of *Dorema ammoniacum* D. Don plants was achieved on MS medium containing 2.0 mgL^{-1} BAP, which is equivalent to our results regarding the positive role of BAP at the same concentration. The results also showed that when the callus tissues were sub-cultured intact on MS medium containing 5.0 mgL^{-1} BAP, a higher number of leaves were recorded as compared to divided tissues. Furthermore, the most extended leaf length was also found with the same intact calli sub-cultured on medium containing 2.0 mgL^{-1} BAP. The number of leaves and their length are considered essential parameters to describe the leaf biomass and physiological functions in regenerated shoots due to photosynthesis and respiration [48]. Besides the Tuncer report [22], there is no other paper regarding the effects of different explant types and growth regulators on shoot regeneration in Foxtail lilies. The present study revealed that the micropropagation of Foxtail lily through callus culture may be practicable for the mass production of microshoots in this plant species.

The application of auxins for in vitro root induction is common in the micropropagation of different plant species [49,50]. In the present research, the effectiveness of IBA for root regeneration in Foxtail lily explants was also confirmed (Table 4). Auxins increase the expression of various genes involved in root regeneration [51]. The recorded root data revealed that the utilization of half-strength basal medium, combined with higher sucrose concentrations (4.0%) and AC (200 mgL^{-1}), are responsive modifications for root induction in Foxtail lily explants. The same may be followed in the commercial mass multiplication of this species.

The in vitro rooted plantlets must finally be transferred to ex vitro conditions. Consequently, a proper strategy for hardening and ex vitro transfer must be followed. In Foxtail lily, the ex vitro adaptation is slow, as has been already observed in many bulbous plants [52–54]. Interestingly, the grapevine hardening strategy already reported by Alizadeh et al. [27] was successfully exploited for the acclimation of in vitro-raised plantlets. However, from a practical point of view, this strategy may not be feasible in large-scale micropropagation, and needs to be standardized further. However, the fertigation with 1/2 MS solution followed in this strategy could efficiently avoid transplantation shock. Two months after hardening, the recorded morphological attributes, such as root length, leaf size, and biomass volume of the hardened and acclimatized Foxtail lily plantlets, demonstrated elongated roots that could easily penetrate the potting substrate (coco peat:perlite:vermiculite). The following result agrees with Ozel et al. [55], who observed similar results while acclimating *Muscari muscarimi* Medikus plants. In another study comparable to our findings, Sharma et al. [56] reported that the irrigation of rooted plantlets with a quarter strength of MS provided essential nutrients to the acclimatizing plant—thus increasing its chances of survival.

5. Conclusions

In the present study, in vitro callus induction and indirect shoot regeneration were developed from root explants of foxtail lily. Hereafter, the disinfection method and appropriate basal medium were reported for callus induction and further shoot regeneration. Surface sterilization of root explants with NaClO (40%, 15 min) was found to be an effective treatment for culture establishment. The inoculated explants had the highest callogenesis efficiency on MS medium supplemented with 10.0 mgL^{-1} BAP. The highest percentage of shoot regeneration from calli was obtained with 2.0 mgL^{-1} BAP. The half-strength MS medium was supplemented with 2.0 mgL^{-1} IBA + 4.0% sucrose + 200 mgL^{-1} AC, proving to be a suitable combination for root regeneration in micro-shoots. The regenerated plantlets were successfully adapted to ex vitro conditions. The reported in vitro regeneration protocol can be exploited at a commercial scale, following just minor modifications. Furthermore, it could be beneficial for producing valuable secondary metabolites at a large scale with the help of bioreactors, where calli would be required as input materials.

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