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Research Article

In vitro germination and bulblet and shoot propagation for wild edible *Eremurus spectabilis* M.Bieb.

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Abstract

Eremurus spectabilis M.Bieb is consumed as a vegetable because of its nutritious characteristics. The plants are also used for medicinal purposes, in the cut flower industry as an ornamental geophyte, and in industry as a natural adhesive. The aim of the present study was to improve the *in vitro* propagation protocol for germination and bulblet/shoot formation of *E. spectabilis*. For this purpose, *E. spectabilis* seeds were *in vitro* germinated in four different nutrient media: Murashige and Skoog (MS), Gamborg (B5), White (WH), and Shenk and Hildebrandt (SH). To stimulate bulblet and/or shoot regeneration, hypocotyls of 35-40-day-old *in vitro*-germinated plantlets were cut into 0.5-1.0 cm pieces, and the resultant explants were cultured in MS media containing 2,4-dichlorophenoxyacetic acid (2,4-D) (0.5, 1.0, 2.0, and 4.0 mg L^{-1}) + kinetin (0.5 mg L^{-1}), thidiazuron (TDZ) (0.5, 1.0, 2.0, and 4.0 mg L^{-1}) + 1-naphthylacetic acid (NAA) (0, 0.1, 0.5, and 1.0 mg L^{-1}), and 6-benzylaminopurine (BAP) (0.5, 1.0, 2.0 and 4.0 mg L^{-1}) + 2,4-dichlorophenoxyacetic acid (2,4-D) (0, 0.1, 0.5, and 1.0 mg L^{-1}). The best outcomes for germination ratio (57.5%) were obtained from the B5 medium. In the third set of *in vitro* propagation experiments, 100% bulblet formation was achieved in TDZ (0.5 mg L^{-1}) and NAA (0.5 and 0.1 mg L^{-1}) combinations of MS media, and this was followed by 0.5 mg L^{-1} BAP-containing medium (81.3%). Shoot formation ratios with the same media combinations varied from 60-70%, and the number of shoots per explant varied from 1.4-2.4 shoots. Further *in vitro* propagation research is planned with larger bulb sizes to develop a protocol for rooting bulblets and/or shoots.

Keywords: *Eremurus*; geophyte; *in vitro* regeneration; Liliaceae; plant growth; regulator

Introduction

E. spectabilis M.Bieb., a perennial herbaceous wild species, belongs to the *Eremurus* genus of the Liliaceae family, which includes about 50 species. *Eremurus* grows on dry and rocky slopes and is quite widespread, especially in Southern and Central Asia including Iran, Western Pakistan, Turkey, Palestine, Lebanon, Syria and the Caucasus (Wendelbo, 1982; Tuzlacı, 1985; Kamenetsky and Akhmetova, 1994). *E. spectabilis* has rosette-like, bare (non-hairy) leaves tapering toward the tip (Figure 1a). Plants have shallow roots, and rhizomes are short and bounded to the root bunch (Tuzlacı, 1987). The roots are succulent, juicy, thick, brownish-yellow and fusiform, tapering again toward the tip (Figure 1b). A hermaphrodite, light-yellow flower bunch is located

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over the pedicle. The fruit is green and has three united carpels (Figure 1c). The seeds are 3-4 mm long, trigonal in shape, with sharp edges, narrow wings, and a brownish colour (Figure 1d) (Matthews, 1986).

E. spectabilis is quite rich in antioxidants, phenolics, and minerals and thus, it is a highly nutritious plant (Tosun *et al*., 2012; Tuzcu *et al*., 2017). The shoots and leaves are cooked and consumed as vegetables. In addition, different sections of the plant are used for medicinal purposes, especially for the treatment of fungal diseases, diabetes, hepatitis, liver and stomach disorders and some cancers (Baytop, 1999; Tuzlacı and Doğan, 2010; Pourfarzad *et al*., 2014; Abubaker and Hidayat, 2015; Tuzcu *et al*., 2017). As well as medical significance, *E. spectabilis* is also used in cut flower production as a popular ornamental geophyte because of its long and alluring spikes (Schiappacasse *et al*., 2013; Ahmad *et al*., 2014). Plant roots are also dried and ground to produce a natural adhesive in industry (Dashti *et al*., 2005; Heshmatol Vaezin *et al*., 2010; Eghtedarnejad and Mansouri, 2016).

In vitro regeneration techniques are commonly used for the regeneration of valuable wild species, endangered species, endemic species, and hard-to-regenerate species. Only one study has been conducted on the *in vitro* regeneration of *E. spectabilis* worldwide (Tuncer, 2017). The researcher cultured *E. spectabilis* leaf and rhizome explants in BAP (1.0 and 2.0 mg L^{-1}) and 2,4-D (0.5, 1.0, and 2.0 mg L^{-1}) containing MS medium (Murashige and Skoog, 1962). The researcher reported that *in vitro* shoot regeneration was not achieved in either explant types. *In vitro* regeneration studies have also been conducted in different species of the Liliaceae family, including the *Aloe* (Molsaghi *et al*., 2014; Baskaran *et al*., 2015; Sharma *et al*., 2015; Suh *et al*., 2015; Razib *et al*., 2016), *Lilium* (Ault and Siqueira, 2008; Tang *et al*., 2009; Sun and Jin, 2011), *Chlorophytum* (Nurashikin *et al*., 2010; Pandey, 2016), *Fritillaria* (Çakmak *et al*., 2016), and *Scilla* (Kamaleswari *et al*., 2016) species. In these studies, different *in vitro* shoot and/or bulblet formation ratios were achieved based on the species and the nutrient media combinations.

The first stage of the protocol to be developed includes *in vitro* germination, *in vitro* bulblet and shoot formation. For this purpose, the aim of the present study was to investigate the possibilities of *in vitro* germination of *E. spectabilis* seeds in four different nutrient media: Murashige and Skoog (MS), Gamborg (B5), White (WH), and Shenk and Hildebrandt (SH), and *in vitro* bulblet and/or shoot regeneration from hypocotyl and bulblet explants developed under *in vitro* conditions with different plant growth regulator (PGR) combinations of MS medium. This study will be the first comprehensive report worldwide about the *in vitro* propagation of *E. spectabilis*.

Figure 1. *E. spectabilis*; (a) stem and leaves, (b) root and rhizomes, (c) fruits, (d) seeds (Tuncer, 2017; Tuncer, 2018) (Photographs: B. Tuncer and K. Keskiner)

Materials and Methods

Plant material and sterilization processes

The *E. spectabilis* seeds to be used in the experiments were supplied from the Gürpınar (38° 8′20.75″N, 43° 30′55.15″E, 1730 m) in Van province of Turkey in August 2017 (Figure 1d). The seeds were initially kept in 0.3% benomyl solution for an hour to remove fungal disease agents (Figure 2a), and then shaken and kept in distilled water for an hour. The seeds were then placed into a laminar flow sterile cabin, kept in 40% sodium hypochlorite (NaOCl) solution containing 1-2 drops of Tween-20 for 10 minutes, and then washed through sterile bidistilled water for five minutes three times (Figure 2b-c). The surface-sterilized seeds were placed over filter papers to remove excess water. The materials to be used in the germination and regeneration experiments (petri dishes, glass jars, forceps, bistoury, etc.) were sterilized in an autoclave at 121 °C for 1.5 hours, and the nutrient media were sterilized for 20 minutes.

In vitro germination trials

Tip-cut seeds were germinated in MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), B5 (Gamborg *et al*., 1968), and WH (White, 1943) media under *in vitro* conditions (Figure 2d-e). All nutrient media were supplemented with 7 g L^{-1}agar, 0.75 g L^{-1} GA_3, and 50 mg L^{-1}citric acid. The pH of all the media was adjusted to 5.8 (by adding 1 N NaOH or 1 N HCl). 20 g L^{-1} of sucrose was added to the B5 medium, while 30 g L^{-1} of sucrose was added to the MS and SH media. WH media did not contain sucrose. Following seeding, the petri dishes were wrapped in parafilm and preserved in a fridge at 4 °C until the initiation of germination to break the physiological dormancy of the seeds. Following the initiation of germination, the petri dishes were taken into an incubator set at 13-14 °C with dark conditions. The emergence of about 2 mm of radicle from the seed test was considered the criterion for germination (Figure 2e). The following equations were used to calculate the germination parameters (Abdul-Baki and Anderson, 1973; Murillo-Amador *et al*., 2002; Keskiner and Tuncer, 2019):

Germination ratio (%) = (G/T) × 100

Mean germination time (day) = [(1.day G × 1. day) + (2.day G × 2.day) +...+ (n.day G × n.day)] /Total G

Germination speed (day) = n1/t1 + n2/t2 +..... + nn/tn

n1, n2: number of germinated seeds, t1, t2: number of days for germination, G: germinated seed number; T: total number of seeds

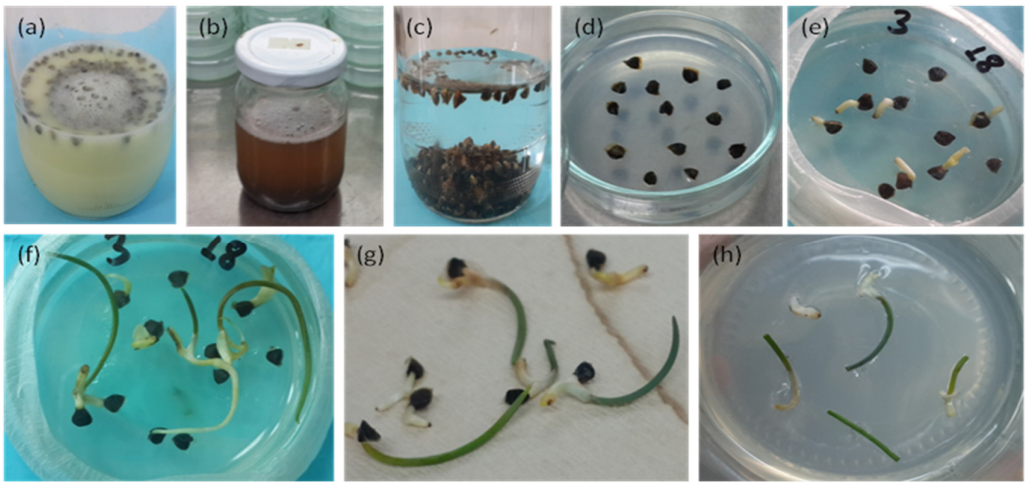

Figure 2. Seeds in (a) 0.3% benomyl solution, (b) 40% NaOCl solution, (c) bidistilled water, (d) *in vitro* seeding, (e) *in vitro* germinated seeds, (f-g) hypocotyls developed under *in vitro* conditions, (h) cultured of hypocotyls explants

In vitro bulblet and shoot regeneration

In the first set of experiments, hypocotyl sections of *in vitro*–germinated seedlings were cut into 0.5–1.0 cm lengths and used as explants (Figure 2f-h). For *in vitro* bulblet and shoot formation, explants were cultured in 36 different PGR in combination with MS media containing 30 g L^{-1} sucrose and 7 g L^{-1} agar as well as 2,4-D (0.5, 1.0, 2.0, and 4.0 mg L^{-1}) + Kinetin (0.5 mg L^{-1}), TDZ (0.5, 1.0, 2.0, and 4.0 mg L^{-1}) + NAA (0, 0.1, 0.5, and 1.0 mg L^{-1}), or BAP (0.5, 1.0, 2.0, and 4.0 mg L^{-1}) + 2,4-D (0, 0.1, 0.5, and 1.0 mg L^{-1}) (Figure 2h).

In the second and third sets of experiments, bulblet segment explants developed under *in vitro* conditions were used to stimulate bulblet and shoot formation. In the second set of experiments, explants were cultured in 17 PGRs in combination with MS media selected from the first set of experiments. In the third set of experiments, explants were cultured in 9 PGRs combinations selected from the second set of experiments. All the cultures were incubated at 25 °C ± 2 °C for 16/8 h photoperiod with a light intensity of 3000 lux.

Statistical analysis

The data were statistically analyzed using analysis of variance (ANOVA) with Statgraphics statistical software, followed by Duncan's multiple range test comparisons for significant differences. The germination experiments were conducted in a completely randomized block design with four replications and five petri dishes (12 seeds/petri dish) in each replication; the *in vitro* bulblet and shoot regeneration experiments were also set up in a randomized blocks design with three replications with 4-6 explants in the first set of experiments, 5-7 explants in the second set of experiments, and 5-8 explants in the third set of experiments for each replication.

Results

In vitro germination

The *in vitro* germination trial results are summarized in Table 1. The greatest germination ratio (57.5%) and the shortest germination time (3.7 day) were obtained from the B5 medium. The germination ratios of the other nutrient media (MS, WH, and SH) varied between 35.0-38.7%, and mean germination times varied between 4.4-5.7 days; they were placed into the same statistical group (Table 1).

Table 1. Results for *in vitro* germination of *E. spectabilis* seeds

Media	Germination ratio (%)	Mean germination time(day)	Germination speed (day)
MS	36.2 ± 2.1 b	5.7 ± 0.7 a	1.1 ± 0.2 c
SH	35.0 ± 2.8 b	5.1 ± 1.0 ab	1.4 ± 0.1 bc
B5	57.5 ± 1.7 a	3.7 ± 0.6 c	3.5 ± 0.9 a
WH	38.7 ± 2.4 b	4.4 ± 0.8 bc	1.8 ± 0.4 b
P Value	0.001**	0.003**	0.000**

The means indicated with different small letters in the same columns are significantly different (Duncan test, **: $p < 0.01$)

In vitro bulblet and shoot regeneration

For *in vitro* bulblet and shoot regeneration, hypocotyl explants (0.5-1.0 cm) of 35-40-day old seedlings germinated under *in vitro* conditions were cultured in 36 different PGR combinations in MS media (Table 2). The statistical analysis showed that there were significant differences among the 36 MS media containing different combinations of PGR ($P < 0.01$).

Table 2. *In vitro* bulblet formation, browning, and swelling ratios of hypocotyl explants of *E. spectabilis* in PGR combinations in MS media (The first set of experiment)

Media No	PGR (mg L^{-1})	Cultured explants	Bulblet ratio (%) ± S.E.	Browning ratio (%) ± S.E.	Swelling ratio (%) ± S.E.
2,4-D + Kinetin					
1	0.5 + 0.5	9	55.0 ± 1.1 c	22.5 ± 0.5 k	12.5 ± 0.5 j
2	1.0 + 0.5	8	37.5 ± 2.5 f	0.0 ± 0.0 ö	25.0 ± 0.4 ı
3	2.0 + 0.5	9	67.5 ± 0.5 b	22.5 ± 0.5 k	10.0 ± 0.3 j
4	4.0 + 0.5	10	0.0 ± 0.0 l	20.8 ± 0.8 l	25.0 ± 0.8 ı
TDZ + NAA					
5	0.5 + 0	9	45.0 ± 2.0 d	10.0 ± 0.7 n	45.0 ± 2.0 e
6	0.5 + 0.1	8	75.0 ± 2.0 a	0.0 ± 0.0 ö	25.0 ± 2.0 ı
7	0.5 + 0.5	11	36.6 ± 0.3 g	10.0 ± 0.5 n	53.3 ± 2.3 c
8	0.5 + 1.0	9	40.0 ± 2.1 ef	10.0 ± 0.7 n	20.0 ± 0.8 i
9	1.0 + 0	10	45.0 ± 1.0 d	20.0 ± 1.0 l	40.0 ± 1.1 f
10	1.0 + 0.1	9	36.6 ± 1.3 g	32.5 ± 0.5 h	32.5 ± 1.5 g
11	1.0 + 0.5	10	41.6 ± 1.7 e	29.1 ± 0.8 ı	29.1 ± 0.8 h
12	1.0 +1.0	9	12.5 ± 0.5 k	32.5 ± 1.5 h	55.0 ± 1.0 c
13	2.0 + 0	8	12.5 ± 0.5 k	62.5 ± 0.5 c	25.0 ± 1.0 ı
14	2.0 + 0.1	8	30.0 ± 0.5 h	50.0 ± 0.3 d	25.0 ± 0.4 ı
15	2.0 + 0.5	9	75.0 ± 1.4 a	12.5 ± 0.5 m	25.0 ± 2.0 ı
16	2.0 +1.0	9	37.5 ± 0.5 f	42.5 ± 0.9 e	12.5 ± 1.5 j
17	4.0 + 0	10	70.0 ± 2.0 b	20.0 ± 0.3 l	0.0 ± 0.0 k
18	4.0 + 0.1	9	12.5 ± 1.5 k	0.0 ± 0.0 ö	45.0 ± 1.0 e
19	4.0 + 0.5	11	25.0 ± 0.8 ı	26.6 ± 1.7 i	10.0 ± 0.3 j
20	4.0 + 1.0	8	25.0 ± 0.7 ı	25.0 ± 0.5 j	12.5 ± 0.5 j
BAP + 2,4-D					
21	0.5 + 0	9	37.5 ± 0.5 f	20.0 ± 0.7 l	55.0 ± 1.0 c
22	0.5 + 0.1	9	0.0 ± 0.0 l	22.5 ± 0.5 k	65.0 ± 1.0 b
23	0.5 + 0.5	9	10.0 ± 0.1 k	40.0 ± 1.1 f	25.0 ± 0.3 ı
24	0.5 + 1.0	11	22.5 ± 0.5 ıi	35.0 ± 0.9 g	0.0 ± 0.0 k
25	1.0 + 0	10	12.5 ± 0.5 k	50.0 ± 1.3 d	70.8 ± 1.2 a
26	1.0 + 0.1	8	25.0 ± 0.6 ı	25.0 ± 0.7 j	50.0 ± 1.0 d
27	1.0 + 0.5	8	25.0 ± 0.9 ı	25.0 ± 0.8 j	25.0 ± 0.6 ı
28	1.0 + 1.0	8	12.5 ± 0.5 k	75.0 ± 1.2 b	0.0 ± 0.0 k
29	2.0 + 0	9	0.0 ± 0.0 l	40.0 ± 0.9 f	12.5 ± 0.5 j
30	2.0 + 0.1	12	25.0 ± 1.7 ı	42.5 ± 0.5 e	28.3 ± 0.7 h
31	2.0 + 0.5	11	0.0 ± 0.0 l	33.3 ± 1.3 h	18.3 ± 0.7 i
32	2.0 + 1.0	9	20.0 ± 0.4 i	10.0 ± 0.3 n	12.5 ± 0.5 j
33	4.0 + 0	11	16.6 ± 1.7 j	10.0 ± 0.3 n	46.6 ± 0.3 e
34	4.0 + 0.1	7	16.6 ± 0.7 j	25.0 ± 0.9 j	12.5 ± 0.5 j
35	4.0 + 0.5	8	0.0 ± 0.0 l	87.5 ± 0.5 a	12.5 ± 0.5 j
36	4.0 + 1.0	8	25.0 ± 0.8 ı	0.0 ± 0.0 ö	37.5 ± 0.5 f
P Value			0.000**	0.000**	0.000**

The means indicated with different small letters in the same columns are significantly different (Duncan test, **: $p < 0.01$)

Bulblet formation ratios (%) in MS media varied between 0.0–75.0% based on PGR combinations. The best outcomes for bulblet formation ratios were obtained, respectively, from the 6th (0.5 mg L^{-1} TDZ + 0.1 mg L^{-1} NAA) medium (75%), the 15th (2 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA) medium (75%), the 17th (4 mg L^{-1} TDZ) medium (70%), and the 3rd (2 mg L^{-1} 2,4-D + 0.5 mg L^{-1} Kin) medium (67.5%) (Table 2). In general, in MS media, lower bulblet formation ratios (%) and greater browning ratios (%) were observed in BAP + 2,4-D

combinations than in the other PGR combinations. The greatest browning ratios of planted explants were observed in the 35th (4 mg L^{-1} BAP + 0.5 mg L^{-1} 2,4-D) and 28th (1 mg L^{-1} BAP + 1 mg L^{-1} 2,4-D) media (Table 2). The lowest bulblet formation ratios or no bulblet formation along with, the greatest swelling ratios (%) were observed, respectively, in the 25th medium (1 mg L^{-1} BAP) (70.8%) and the 22nd medium (0.5 mg L^{-1} BAP + 0.1 mg L^{-1} 2,4-D) (65%). In the first set of experiments, direct bulblet formation was provided from the hypocotyl explants planted into 36 PGR combinations in MS media (Table 2).

According to the results of the first set of experiments, 17 PGR combinations in MS media with more successful outcomes were selected (Table 3), and the second set of experiments were set up (35-40 days after the first set of experiments).

Table 3. Bulblet and shoot formation of *in vitro*–developed bulblet explants of *E. spectabilis* (The second set of experiment)

Media No	PGR (mg L^{-1})	Cultured explants	Bulblet ratio (%) ± S.E.	Browning ratio (%) ± S.E.	Shoot ratio (%) ± S.E.	Shoot/explant ± S.E.
2,4-D + Kinetin						
1	0.5 + 0.5	12	41.6 ± 1.3 g	0.0 ± 0.0 d	16.6 ± 0.5 i	0.5 ± 0.1 g
2	1.0 + 0.5	12	41.6 ± 1.3 g	0.0 ± 0.0 d	24.9 ± 1.3 h	0.5 ± 0.1 g
3	2.0 + 0.5	13	45.0 ± 0.9 f	38.7 ± 1.3 a	16.2 ± 1.2 i	0.7 ± 0.1 e
TDZ + NAA						
5	0.5 + 0	10	30.0 ± 2.0 i	0.0 ± 0.0 d	10.0 ± 0.3 j	0.4 ± 0.1 h
6	0.5 + 0.1	13	61.9 ± 1.8 c	0.0 ± 0.0 d	46.4 ± 0.6 f	1.0 ± 0.3 bc
7	0.5 + 0.5	11	81.6 ± 1.7 a	0.0 ± 0.0 d	63.3 ± 0.3 b	1.0 ± 0.3 bc
8	0.5 + 1.0	14	49.9 ± 0.9 e	0.0 ± 0.0 d	49.9 ± 0.1 e	0.9 ± 0.1 c
9	1.0 + 0	12	58.3 ± 1.3 d	0.0 ± 0.0 d	28.3 ± 0.7 g	0.8 ± 0.2 d
10	1.0 + 0.1	12	60.0 ± 1.5 cd	0.0 ± 0.0 d	28.3 ± 0.3 g	0.8 ± 0.2 d
11	1.0 + 0.5	12	33.3 ± 0.9 ı	0.0 ± 0.0 d	16.6 ± 0.7 i	0.6 ± 0.1 f
14	2.0 + 0.1	12	75.7 ± 0.7 b	0.0 ± 0.0 d	51.4 ± 1.4 d	0.9 ± 0.1 c
15	2.0 + 0.5	11	36.6 ± 1.3 h	0.0 ± 0.0 d	5.5 ± 0.4 k	0.4 ± 0.1 h
16	2.0 +1.0	11	28.3 ± 0.7 i	8.3 ± 0.3 b	16.6 ± 1.7 i	0.5 ± 0.1 g
17	4.0 + 0	12	75.7 ± 0.7 b	7.1 ± 0.1 c	48.5 ± 0.6 e	1.1 ± 0.2 b
19	4.0 + 0.5	10	20.0 ± 0.5 j	0.0 ± 0.0 d	20.0 ± 0.7 ı	0.4 ± 0.1 h
20	4.0 + 1.0	12	49.9 ± 0.7 e	0.0 ± 0.0 d	58.3 ± 0.3 c	1.0 ± 0.5 bc
BAP + 2,4-D						
21	0.5 + 0	12	83.3 ± 1.4 a	0.0 ± 0.0 d	83.3 ± 1.3 a	1.3 ± 0.4 a
P Value			0.000**	0.000**	0.000**	0.000**

The means indicated with different small letters in the same columns are significantly different (Duncan test, **: $p < 0.01$)

Bulblet pieces developed under *in vitro* conditions were used as explants. The differences in the investigated parameters of the 17 PGR combinations in MS media were found to be significant ($p < 0.01$). The best outcomes in terms of bulblet formation ratios were obtained, respectively, from the 21st (0.5 mg L^{-1} BAP) (83.3%), the 7th (0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA) (81.6%), the 14th (2 mg L^{-1} TDZ + 0.1 mg L^{-1} NAA) (75.7%), and the 17th (4 mg L^{-1} TDZ) (75.7%) media. Except for a few media, (3rd, 16th, and 17th media), browning was not observed in the explants (Table 3). In terms of shoot formation ratios, the 21st (0.5 mg L^{-1} BAP) (83.3%) and the 7th (0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA) media (63.3%) were the most successful. The greatest number of shoots per explant was obtained from the 21st and the 17th media, and they were followed by the 20th (4 mg L^{-1} TDZ + 1 mg L^{-1} NAA), the 6th (0.5 mg L^{-1} TDZ + 0.1 mg L^{-1} NAA), and the 7th (0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA) media, which were placed into the same statistical group (Table 3).

For the third set of experiments, 9 PGR combinations with better outcomes in terms of bulblet and shoot formation were selected. Bulblet pieces developed under *in vitro* conditions were used as explants, and the resulting data are provided in Table 4. In the third set of experiments, a 100% bulblet formation ratio was

achieved in TDZ (0.5 mg L^{-1}) and NAA (0.5 and 1.0 mg L^{-1}) combinations of MS media. These PGR combinations were followed by the MS medium containing only 0.5 mg L^{-1} BAP (81.3%) (Table 4). The shoot formation ratios of PGR combinations varied from 10.0–70.0%, and the number of shoots per explant varied from 0.5-2.4 shoots. The greatest shoot formation ratio (70%) was obtained from the MS medium containing 0.5 mg L^{-1} TDZ and 1.0 mg L^{-1} NAA and the greatest number of shoots per explant (2.4 shoots) was obtained from the MS medium containing 0.5 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA (Table 4). Figure 3 presents the *in vitro* bulblet and shoot regeneration in different PGR combinations of MS media.

Table 4. Bulblet and shoot formation of *in vitro*–developed bulblet explants of *E. spectabilis* (The third set of experiment)

Media No	PGR (mg L^{-1})	Cultured explants	Bulblet ratio (%) ± S.E.	Shoot ratio (%) ± S.E.	Shoot/explant ± S.E.
TDZ + NAA					
6	0.5 + 0.1	11	63.3 ± 1.3 c	28.3 ± 0.3 e	1.0 ± 0.1 b-d
7	0.5 + 0.5	11	100.0 ± 2.2 a	60.0 ± 1.0 b	2.4 ± 0.2 a
8	0.5 + 1.0	10	100.0 ± 1.9 a	70.0 ± 2.0 a	1.5 ± 0.5 a-c
9	1.0 + 0	10	60.0 ± 1.3 d	10.0 ± 0.8 g	0.5 ± 0.5 d
10	1.0 + 0.1	11	35.0 ± 2.0 g	16.6 ± 1.7 f	0.6 ± 0.3 cd
14	2.0 + 0.1	18	55.5 ± 1.4 e	33.3 ± 1.1 d	0.6 ± 0.2 cd
17	4.0 + 0	18	38.9 ± 0.6 f	16.6 ± 0.5 f	0.5 ± 0.1 d
20	4.0 + 1.0	15	66.1 ± 1.1 c	47.3 ± 0.8 c	1.7 ± 0.2 ab
BAP + 2,4-D					
21	0.5 + 0	16	81.3 ± 1.3 b	62.5 ± 0.5 b	1.4 ± 0.3 a-d
P Value			0.000**	0.000**	0.016*

The means indicated with different small letters in the same columns are significantly different (Duncan test, **: $p < 0.01$, *: $p < 0.05$)

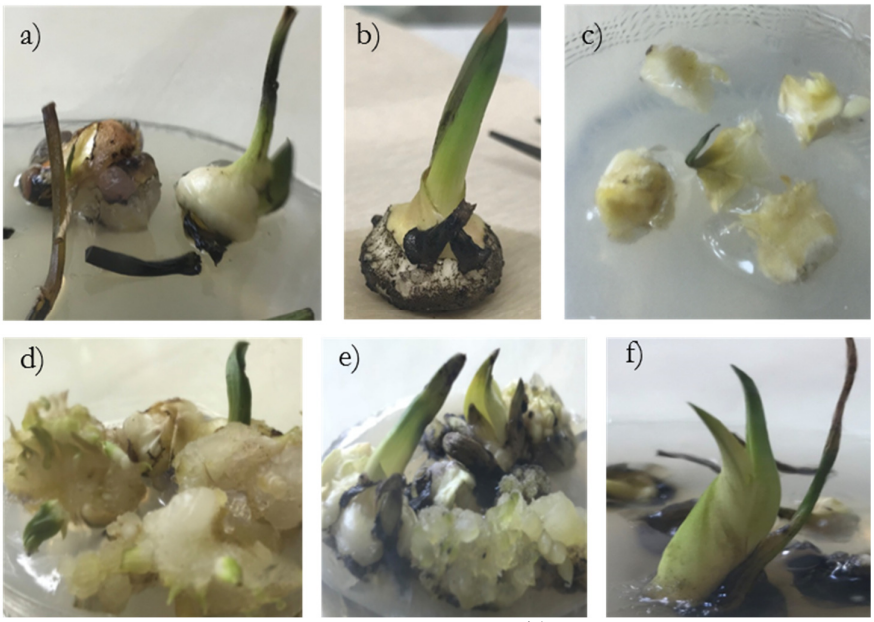

Figure 3. *In vitro* bulblets obtained from hypocotyl explants; (a) MS + 2 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA, (b) MS + 0.5 mg L^{-1} TDZ + 0.1 mg L^{-1} NAA, (c) cut into pieces and plantation of *in vitro*–developed bulblets into nutrient media, (d-e) MS + 0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} NAA, (f) MS + 0.5 mg L^{-1} BAP

Discussion

There are only two studies about *in vitro* germination of *E. spectabilis*; these were conducted by Keskiner (2017) and Akdağ (2019). Keskiner (2017) germinated 1,500 ppm GA_3–supplemented and unsupplemented *E. spectabilis* seeds in three different nutrient media (MS, WH, and B5) and reported quite low germination ratios (0.83%-1.50%) only in the WH medium. Akdağ (2019) germinated *E. spectabilis* seeds in three different nutrient media (MS, WH, and B5) under *in vitro* conditions, preserved the seeded petri dishes in a fridge (4 °C) for different durations (30, 50, 80, and 100 days), and reported the greatest germination ratio (5.88%) for 100-day cold storage with MS medium. However, it is remarkable that both Keskiner (2017) and Akdağ (2019) reported quite low germination ratios under *in vitro* conditions.

Other studies (not under *in vitro* conditions) have investigated the germination of *E. spectabilis*. Rahmanpour *et al*. (2005) subjected *E. spectabilis* seeds to mechanical abrasion followed by immersion into 0.01 M GA_3 solution for 45 minutes and reported a 53% germination ratio. Güngör (2002) reported the greatest germination ratio (65.3%) for 90-day moist-cold stratification treatments, and Keskiner and Tuncer (2019) reported the greatest germination ratio (73.3%) for 100-day moist-cold stratification treatments. Despite the high germination ratios, quite long moist-cold stratification durations (90 and 100 days) were used, and long mean germination times were observed in those studies. Akdağ (2019) cut the tips of *E. spectabilis* seeds, subjected them to moist-cold stratification treatments for different durations (30, 50, 80, and 100 days) and reported germination ratios from 45.4-60.6%; high germination ratios were achieved even with a short duration (< 30 days) of moist-cold stratification treatments of the tip-cut seeds. Akdağ's (2019) results were taken into consideration in this study, and thus the *in vitro* germination experiments were set up with tip-cut *E. spectabilis* seeds. Therefore, the present findings comply with the results of Akdağ (2019).

In previous studies conducted with other species of the *Eremurus* genus under non–*in vitro* conditions, Rahmanpour *et al*. (2007) reported the greatest germination ratio (80%) and germination speed (1.6 day) of *E. olgae* for seeds immersed in water for 24-48 hours, tip cut, seed testa abraded, and immersed into a 0.08 M GA_3 solution for 45 minutes; it was reported that tip cutting and abrasion of seed testa improved germination ratios and shortened mean germination times in *E. spectabilis* (Rahmanpour *et al*., 2005; Akdağ, 2019), in *E. olgae* (Rahmanpour *et al*., 2007). Present findings comply with the results of Rahmanpour *et al*. (2005), Rahmanpour *et al*. (2007) and Akdağ (2019). Sufficient germination ratios were achieved from tip-cut seeds preserved in a fridge (4 °C) for short durations (< 30 days) under in vitro conditions.

Only one study worldwide has investigated the in vitro propagation of *E. spectabilis*. Tuncer (2017) cultured leaf and rhizome explants of *E. spectabilis* in BAP (1.0 and 2.0 mg L^{-1}) and 2,4-D (0.5, 1.0, and 2.0 mg L^{-1}) combinations of MS media, but was not able to achieve any bulblet and shoot formations. In the present study, several different PGR combinations were experimented with in MS media, and sterile hypocotyl and bulblet explants obtained under *in vitro* conditions were used; thus, the findings of the present study were quite successful compared with Tuncer (2017). In the first set of experiments, *in vitro* direct bulblet formation was achieved from the hypocotyl explants; in the second and third set of experiments, both bulblet and shoot formations were achieved from bulblet segments developed under *in vitro* conditions with different PGR combinations on MS medium. In the third set of experiments, a bulblet formation ratio of 81.3–100% was achieved with TDZ (0.5 mg L^{-1}) + NAA (0.5 and 1.0 mg L^{-1}) combinations and 0.5 mg L^{-1} BAP-containing MS media; shoot formation ratios of 60-70% were also achieved with the same nutrient media combinations (Table 4).

Different researchers have used different cytokinin and/or auxin group hormones at different ratios for *in vitro* bulblet and shoot regeneration in species of the Liliaceae family. Nurashikin *et al*. (2010) obtained the greatest shoot regeneration (18.9 shoots/explant) from young shoot explants of *Chlorophytum borivilianum* from 3 mg L^{-1} BAP–supplemented MS medium. Choudhary *et al.* (2011) obtained the greatest shoot formation

ratio of shoot tip explants of *Aloe vera* from BAP (1.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) combination of MS medium. Razib *et al.* (2016) reported about 90% shoot regeneration from 2.0 mg L^{-1} BA + 0.5 mg L^{-1} NAA + 40.0 mg L^{-1} Ads (Adenine sulfide)–supplemented MS medium. Molsaghi *et al.* (2014) indicated successful outcomes for shoot regeneration with MS + 1 mg L^{-1} IAA + 4 mg L^{-1} BAP (58.88%) and from MS + 0.2 mg L^{-1} IAA + 0.8 mg L^{-1} BAP media (48.03%).

Jakhar *et al*. (2012) obtained the maximum shoot regeneration of micro shoots of *Aloe barbadensis* Mill. with young leaves with 0.5 mg L^{-1} BAP–containing medium; Baskaran *et al*. (2015) obtained 15.4 shoots per explant from shoot tip and leaf explants of the medicinal plant *Aloe pruinosa* in MS + 5 µM BA + 4 µM IAA medium; Kamaleswari *et al*. (2016) reported the maximum number of shoots per explant of leaf explants of *Scilla hyacinthina* with 2 mg L^{-1} BAP–supplemented media. Pandey (2016) was able to achieve maximum shoot regeneration of stem explants of bulbous *Chlorophytum borivilianum* from the MS media containing only 5 mg L^{-1} BAP. Tang *et al*. (2009) cultured leaf explants of *Lilium longiflorum* in MS + BAP (0.1 mg L^{-1}) + NAA (0.5 and 2.0 mg L^{-1}) media and reported direct bulblet formation in all media combinations. Complying with the results of Tang *et al*. (2009), in the first set of my experiments, direct bulblet formation was achieved from hypocotyl explants at varying ratios (0.0-75.0%) based on PGR combinations (Table 2). In the first set of experiments, greater bulblet formation ratios (%) were obtained from 2,4-D (0.5, 1.0, and 2.0 mg L^{-1}) + Kinetin (0.5 mg L^{-1}), TDZ + NAA combinations, and only 0.5 mg L^{-1} BAP–containing PGR combinations of MS media than from the other PGR combinations (Table 2).

In vitro bulblet and/or shoot formations were also reported in previous studies in which bulblet segments were used as explants. Haibin and Jiajun (2006) reported the greatest shoot regeneration of *Lilium lancifolium* in MS + BA (1.5 mg L^{-1}) + NAA (0.2 mg L^{-1}) media. Yang *et al*. (2010) reported the greatest shoot regeneration of *L. tsingtauense* in MS + 0.1 mg L^{-1} NAA + 2.0 mg L^{-1} BA combination. Sun and Jin (2011) reported the greatest shoot regeneration of *L. longiflorum* in MS + 2 mg L^{-1} BA + 0.2 mg L^{-1} NAA–supplemented media. Zhang and Jia (2014) reported the greatest shoot regeneration (88.91%) of *Lily* (cv. Siberia) in MS + 1.07 µM NAA + 4.44 µM BA-supplemented media. Çakmak *et al*. (2016) worked with bulblet explants taken from *in vitro*–developed *Fritillaria persica* seedlings and obtained the maximum bulblet regeneration ratio and number of bulblets per explant from 2.0 mg L^{-1} TDZ–supplemented MS medium. In another study, maximum number of shoots (13.2 shoots) of bulblet pieces of *Scilla hyacinthina* was obtained from 2 mg L^{-1} TDZ–supplemented MS medium (Kamaleswari *et al.*, 2016). Complying with the findings of the above-mentioned reports (Haibin and Jiajun, 2006; Yang *et al*., 2010; Sun and Jin, 2011; Zhang and Jia, 2014; Çakmak *et al*., 2016; Kamaleswari *et al*., 2016), in the second and third set of my experiments (Table 3 and 4), bulblet and shoot regeneration were achieved from *in vitro*–developed bulblet segments at varying ratios based on PGR combinations.

In previous *in vitro* propagation studies conducted with different species, more successful bulblet and shoot regeneration were generally achieved in BA + NAA (Haibin and Jiajun, 2006; Yang *et al*., 2010; Sun and Jin, 2011; Zhang and Jia, 2014; Razib *et al*., 2016), BAP + NAA (Tang *et al*., 2009; Choudhary *et al*., 2011), only BAP, (Nurashikin *et al*., 2010; Jakhar *et al*., 2012; Kamaleswari *et al*., 2016; Pandey, 2016) or TDZ–containing (Çakmak *et al*., 2016; Kamaleswari *et al*., 2016) MS media. Generally, in the second and third set of my experiments, 0.5 mg L^{-1} BAP–containing MS media and TDZ + NAA combinations yielded more successful bulblet and shoot formations (Table 3 and 4).

Conclusions

This study is the first comprehensive report worldwide about the *in vitro* propagation of *E. spectabilis*. In the present study, bulblet and shoot regeneration from *in vitro*–developed hypocotyl explants and bulblet segments were investigated in several PGR combinations of MS media. The most successful outcomes in terms of bulblet and shoot formation were achieved in only BAP (0.5 mg L^{-1})–containing and in TDZ (0.5 mg L^{-1}) + NAA (0.5 and 1.0 mg L^{-1}) combinations of MS media. In further studies of *in vitro* propagation of this species, besides TDZ + NAA combinations, BAP + NAA combinations could also be experimented with, bulb size could be increased, and a protocol for rooting the resultant bulblets and/or shoots could be developed.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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