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Micropropagation of *Erodium sibthorpianum* subsp. *sibthorpianum*, an endemic threatened species of Uludağ Mountain (Bursa-Turkey)

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Abstract: Uludağ Mountain is one of the Important Plant Areas of Turkey. Its native plant species are threatened by various human and environmental factors. In this study, 2 different micropropagation techniques, with Murashige Skoog (MS) and modified MS (ModMS) media, were used for *Erodium sibthorpianum* Boiss. subsp. *sibthorpianum*. Mineral composition was developed according to the plant requirements. Shoot tips were cultured on these media. Seedlings, obtained from in vitro germinated seeds, were used as explant sources in micropropagation studies. The highest percentage of shoot induction (11.33 shoots/explant) was determined in 1 ppm 6-benzyl amino purine and plant growth regulator-free MS medium (35.97 mm shoot length). Elongated shoots were successfully rooted in the ModMS medium with different naphthalene acetic acid concentrations. Shoots with regenerated roots were transferred to the greenhouse. This protocol could be used for conservation studies of endemic and endangered plant species.

Key words: *Erodium sibthorpianum*, endangered, endemic, micropropagation

1. Introduction

Native plants are important components of the global biological diversity and are often highly adapted to specific environmental and climatic conditions (Sudhersan et al., 2003). Thus, many endemic plant species are becoming endangered because of the increasing human activity and consequent habitat deterioration (Güleryüz et al., 2011). Hence, there is a need to develop efficient techniques that allow large-scale multiplication and preservation of rare endemic plant species (Kapai et al., 2010). Besides the conventional propagation methods, in vitro methods provide an important opportunity for the propagation and preservation of endemic and endangered plant species (Fay, 1992; Rao, 2004).

Erodium sibthorpianum Boiss. subsp. *sibthorpianum* (Geraniaceae) is an endemic species of Uludağ Mountain in Turkey, the highest mountain in the Marmara region, including Thrace and the northwestern side of the Anatolian peninsula. It has a geomorphologically interesting structure with steep southern slopes of calcareous rocks (Ketin, 1983). The climate of the mountain varies with elevation, resulting in high biological diversity. This high plant diversity has led to the designation of Uludağ Mountain as one of the Important Plant Areas (IPAs) of Turkey (Güleryüz et al., 2010). *Erodium sibthorpianum* grows on limestone rocks in poor soils and prefers calcareous soils. The species is spread among the alpine communities (*Acantholimon ulucinum* Boiss. and *Festuca punctoria* Sm. communities) at upper altitudes of ridges and slopes above 2200 m. It is a densely caespitose perennial, 5–12 cm, forming hard wide hummocks; basal leaves 2-pinnatisect, canescent, blade ovate, laciniae shortly oblong, acute; stems erect or ascending and flowers pale lilac. *Erodium sibthorpianum* shares its habitats with other endemic species including *Veronica caespitose* Boiss. var *caespitose* Boiss., *Astragalus hirsutus* Vahl., *A. sibthorpianus* Boiss., *Arabis drabiformis* Boiss., *Festuca punctoria* Sm., *Linum olympicum* Boiss., *Muscari bourgaei* Baker, *Galium olympicum* Boiss. Flowering time is July to August (Güleryüz, 2000).

The habitat of this species has been damaged by heavy recreational and winter sports activities. In alpine environments, natural or human-induced disturbances are fairly common. Damage to plant diversity on Uludağ Mountain has been primarily caused by the construction of hotels and ski lift areas, recreational activities, and the use of artificial snow (Pintar et al., 2009; Güleryüz et al., 2011). *Erodium sibthorpianum* is classified as an Endangered

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(EN) species according to the IUCN classification and it is considered under threat of extinction according to the IPA Project (Ekim et al., 2000; Güleryüz, 2010).

This species occupies less than 10 km^2 and its habitat is severely fragmented. An inferred decline in the area and/or quality of habitat leads to the assumption that the population size of approximately <250 mature individuals will decline. It is estimated that it will decline by at least 25% within 3 years. For this reason, the development of a viable micropropagation protocol is essential. It must be an effective and rapid method for clonal propagation and could prove useful for the conservation of many rare and endemic species (Sudha et al., 1998).

In vitro multiplication efficiency is widely dependent on culture medium, growth regulators, and genotypes (Ansar et al., 2009; Farsi et al., 2012). The deficiency of minerals in plants can cause biochemical, physiological, and morphological changes according to nutrients and the level of deficiency (Monteiro et al., 2000). The interaction of nutrient salts and plant growth regulators (PGRs) in the culture medium is also important and has long been recognized (Murashige and Skoog, 1962). Optimizing the culture medium is difficult because of interactions between different medium components, leading to the creation of various tissue culture media (Nas and Read, 2004).

Until now no study has been initiated on in vitro propagation of *Erodium sibthorpianum*. It is hypothesized that developing a medium according to the results of soil analyses of the plant habitat will result in the best nutritional conditions for the growth of shoot tips in the culture medium.

2. Materials and methods

2.1. Plant material and sterilization

Mature seeds of the *Erodium sibthorpianum* were collected from the alpine belt between 2000 and 2400 m on Uludağ Mountain during July–August 2009. Seeds were dried and cleaned in the laboratory before the experiment. Surface sterilization of the seeds was carried out with 0.5%, 1%, and 2% sodium hypochlorite (NaOCl) solution containing a few drops of surfactant Tween 20 for 10 min. This was followed by 3 rinsing steps with sterile distilled water.

2.2. Culture media and isolation of explants

Throughout the study, different strengths of Murashige Skoog (MS) medium were used for experiments. Full-strength MS basal medium with different concentrations of 6-benzyl amino purine (BAP), kinetin (Ki), and modified MS (ModMS) medium with different combinations of BAP/naphthalene acetic acid (NAA) and auxins [indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), and NAA] were used as the initiation media for in vitro multiplication responses. MS medium was modified according to the soil analyses of the plant habitat (Table 1). The amounts of NH_4^+, NO_3^-, Ca^{++}, and Cl^- ions and vitamins, including nicotinic acid and thiamine-HCl, were changed (Table 2). Sucrose (30%) was used as the carbon source in all the combinations. All media combinations were solidified by adding 0.7% agar.

Seeds were germinated in a growth chamber at 21/16 °C for a 16/8 h photoperiod, respectively. The pH of the nutrient medium was adjusted to 5.8 before autoclaving the medium at 121 °C and 1.1 atm for 20 min. Seeds were aseptically transferred into Magenta vessels containing medium with 0 (control) and 100 ppm of gibberellic acid (GA_3) (filter-sterilized and added to the growth media after autoclaving) (Kocaçalışkan, 2008). Each trial consisted of 5 magenta vessels containing 10 seeds. Three replicates were used for each treatment.

At the end of 30–40 days (Figure 1), *Erodium sibthorpianum* shoot tips were placed on MS media with various concentrations of BAP (0, 1, 2, 4, 6, and 8 ppm), Ki (0.5, 1, 2, and 3 ppm), and ModMS with various concentrations of BAP/NAA (5/1, 2/0.04, 2/0.1, 4/0.1, and 2/2 ppm) for 8 weeks. Shoots were subcultured 3 times for each application.

The regenerated shoots (2 to 3 cm) were excised and individually transferred to MS medium without plant growth regulators (control), with various concentrations of IBA and IAA (0.5, 1, 1.5, and 2 ppm) and ModMS medium control, and with various concentrations of NAA (0.1, 0.5, 1, and 2 ppm) in 50-mL Magenta vessels to test the rooting potential. The number of roots per shoot, root lengths, and rooting percentages were determined after 8 weeks from the culture initiation. Rooted plantlets were acclimatized in a growth chamber for several days. After that, plantlets were transferred to 16-cm pots containing 2:1 turf and perlite and were grown under greenhouse conditions.

2.3. Statistical analysis

Each treatment had 3 replicates containing 5 explants in each culture vessel. Collected data were subjected to t-tests and one-way analysis of variance (ANOVA, SPSS for Windows 18.0), and post-hoc analysis was performed using Duncan's multiple range test. Statistical analysis was performed separately for MS and ModMS media.

3. Results

In this study, 0.5%, 1%, and 2% NaOCl were used to identify the effect of sterilization on the decontamination and germination of *Erodium sibthorpianum* seeds. When 3 different sterilization treatments were compared, a high level of contamination (45%) was observed at the lower percentage of NaOCl (0.5%). According to this result, 1% sodium hypochlorite treatment (67.33%) was selected as the optimum concentration for the decontamination and germination of *Erodium sibthorpianum* seeds (Table 3).

The highest number of shoots per explant was obtained with 1 ppm BAP (11.33; Table 4), but the highest shoot

Table 1. Nutritional composition of MS basal and modified medium.

Elements	Basal MS medium (mg/L)	Modified MS medium (mg/L)
NH_4NO_3	1650	2000
$CaCl_2.2H_2O$	440	660
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
KNO_3	1900	1900
H_3BO_3	6.2	6.2
$CoCl_2.6H_2O$	0.025	0.025
$CuSO_4.5H_2O$	0.025	0.025
$MnSO_4.4H_2O$	22.3	22.3
KI	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25
$ZnSO_4.4H_2O$	8.6	8.6
Na_2EDTA	37.3	37.3
$FeSO_4.7H_2O$	27.8	27.8
Vitamins		
Glycine	2.0	2.0
myo-Inositol	100	100
Nicotinic acid	0.5	0.025
Pyridoxine-HCl	0.5	0.5
Thiamine-HCl	0.1	0.5
Saccharose	30 g/L	30 g/L

length was determined in PGR-free MS medium (Table 4; Figure 1). The shoot explants of *Erodium sibthorpianum* cultures were also successfully established in modified MS medium. The combination of cytokinin with auxin (4 BAP/0.1 NAA) in modified MS medium showed a higher response in shoot length (29.40 mm; Table 4). According to the results given in Table 5, while MS medium had significant impact on stem length and on number of leaves and shoots, ModMS medium had an impact on rooting parameters of *Erodium sibthorpianum*.

Table 2. Alterations in MS basal medium for preparing ModMS nutritional composition.

Ions	Modifications
Ammonium (NH_4^+)	+4.38 mmol
Nitrate (NO_3^-)	+4.38 mmol
Calcium (Ca^{++})	+1.98 mmol
Chloride (Cl^-)	+3.96 mmol
Vitamins	
Nicotinic acid	–0.475 mg
Thiamine-HCl	+0.4 mg

+: Increased values; –: decreased values.

In vitro regenerated shoots of *Erodium sibthorpianum* showed different behaviors during the processes of rooting and acclimatization. According to the results of this study, the presence of auxins, including IBA, IAA, and NAA, in the culture medium is generally necessary to promote rooting of *Erodium sibthorpianum* in vitro cultures. The number of roots per shoot (10.57 roots/shoot), root length (16.40 mm), and the percentage of rooting (84.17%) changed significantly with a concentration of 0.5 ppm IBA (Table 6). The addition of 0.5 ppm IAA to the MS culture medium increased the number of roots (8.47 roots/shoot), root length (18.87 mm), and rooting percentage (80.37%), but not as much as IBA. We compared the effect of MS and ModMS media on the rooting of seedlings and the results showed that ModMS medium with 0.1 ppm NAA was superior compared to MS medium with IBA and IAA (Table 6; Figure 1).

Well-rooted *Erodium sibthorpianum* shoots were rinsed with sterilized water to remove residual rooting medium, were transplanted to pots containing a 2:1 mixture of turf and perlite, and were grown in a growth chamber. After that, regenerated *Erodium sibthorpianum* plantlets were transferred to the greenhouse (Figure 1).

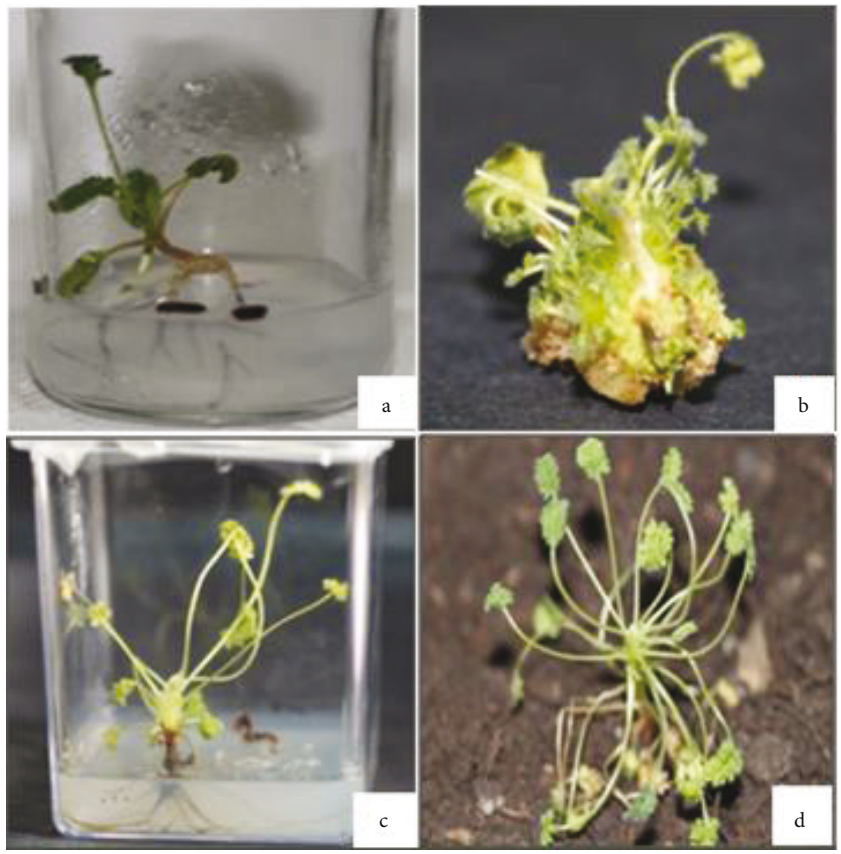

Figure 1. Seed germination, shoot regeneration, rooting, and acclimatization of *Erodium sibthorpianum* subsp. *sibthorpianum*. **a**- Seedlings of 30–40 days old cultured on MS medium, **b**- in vitro shoot regeneration from a shoot tip explant cultured on MS medium with 1 ppm BAP, **c**- microshoots rooted on ModMS medium with 0.1 ppm NAA, **d**- acclimatized plantlets grown in a greenhouse.

4. Discussion

The main objective of this study was to establish a rapid and proper micropropagation system for *Erodium sibthorpianum*, which is a rare and threatened endemic plant of Uludağ-Bursa. The most important and rather difficult aspect of the in vitro techniques is the requirement to carry out various operations under aseptic conditions. Contamination with microorganisms, including viruses, bacteria, yeast, and fungi, is considered the most important reason for plant losses during in vitro culturing (Sarasan et al., 2006; Omamor et al., 2007). During seed formation, embryos and associated tissues tend to exclude pathogens and foreign materials. The contents of the seed are essentially aseptic and the resultant seedlings can be maintained in aseptic conditions if the outer surface of the seed (seed coat) is sterilized with sodium hypochlorite (or other surface sterilants) (Mineo, 1990).

In our study, different concentrations of NaOCl were tested; 1% NaOCl showed significant reduction

Table 3. Effects of different concentrations of NaOCl on decontamination and germination of *Erodium sibthorpianum* subsp. *sibthorpianum* seeds.

Treatments	% of seeds germinated	% of seeds contaminated
0.5% NaOCl × 10 min	51.00 ± 2.08 b	45.00 ± 2.89 a
1.0% NaOCl × 10 min	67.33 ± 0.92 a	34.33 ± 1.48 b
2.0% NaOCl × 10 min	28.00 ± 1.04 c	33.00 ± 0.29 b

Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$); ±SE.

Table 4. Effect of different culture media (MS and ModMS) and growth regulators (BAP, Ki, BAP, and NAA) on shoot formation of *Erodium sibthorpianum* subsp. *sibthorpianum*.

Medium	BAP	Ki	Number of	Shoot length	Shoot	Callus-forming
	(ppm)	(ppm)	shoots/explant	(mm)	explant %	explant %
MS	0	0	5.27 ± 0.37 de	35.97 ± 0.55 a	100.00 ± 0.0 a	0
	1	0	11.33 ± 0.44 a	27.87 ± 0.45 b	100.00 ± 0.0 a	100.00 ± 0.0 a
	2	0	7.83 ± 0.44 b	25.37 ± 0.32 c	100.00 ± 0.0 a	100.00 ± 0.0 a
	4	0	6.80 ± 0.42 bc	16.73 ± 0.37 ef	100.00 ± 0.0 a	100.00 ± 0.0 a
	6	0	4.70 ± 0.35 ef	14.77 ± 0.39 g	87.03 ± 0.58 c	100.00 ± 0.0 a
	8	0	7.47 ± 0.29 b	20.00 ± 0.58 d	93.73 ± 0.67 b	100.00 ± 0.0 a
	0	0.5	1.43 ± 0.23 g	9.8 ± 0.42 h	73.17 ± 0.44 d	100.00 ± 0.0 a
	0	1	4.17 ± 0.20 f	20.60 ± 0.38 d	86.90 ± 0.23 c	100.00 ± 0.0 a
	0	2	5.77 ± 0.39 cde	18.00 ± 0.58 e	100.00 ± 0.0 a	100.00 ± 0.0 a
	0	3	6.17 ± 0.18 cd	16.57 ± 0.38 f	100.00 ± 0.0 a	100.00 ± 0.0 a
Medium	BAP	NAA	Number of	Shoot length	Shoot	Callus-forming
	(ppm)	(ppm)	shoots/explant	(mm)	explant %	explant %
ModMS	5	1	6.17 ± 0.20 b	22.90 ± 0.29 b	75.00 ± 0.58 c	100.00 ± 0.0 a
	2	0.04	3.93 ± 0.23 c	19.03 ± 0.20 d	78.67 ± 0.88 b	100.00 ± 0.0 a
	2	0.1	3.72 ± 0.25 c	22.77 ± 0.15 b	81.33 ± 0.67 a	100.00 ± 0.0 a
	4	0.1	6.88 ± 0.20 a	29.40 ± 0.26 a	79.33 ± 0.33 b	0
	2	2	5.90 ± 0.26 b	21.97 ± 0.26 c	69.67 ± 0.67 d	0

Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$); ±SE.

in contamination of *Erodium sibthorpianum* seeds and healthy plants were obtained. These results are very much in conformity with other previous studies on various rare, endangered, and endemic plants including *Gentiana cerina* Hook.f., *G. corymbifera* Kirk, *Centaurea tchihatcheffii* Fisch. et Mey., *Linum usitatissimum* L., and *Arabis drabiformis* Boiss. (Morgan et al., 1997; Özel et al., 2006; Yıldız et al., 2010; Akın and Kocaçalışkan, 2011).

For multiplication, many plant species require specific in vitro conditions. A range of cytokinins (kinetin, BAP, 2-iP, and zeatin) have been used in micropropagation studies (Babaoğlu et al., 2001; Sarasan et al., 2006; Bektaş et al., 2013). If in vitro grown plantlets are to be used as a source of explants for the induction of organogenesis or embryogenesis, well-developed plantlets may also be of significant importance (Monteiro et al., 2000). The nutrient composition of in vitro culture medium may play a decisive role in research of in vitro culturing of new species and development of industrial applications (production of secondary metabolites) and horticulture (micropropagation). The formulation of in vitro culture media can be adjusted to meet the various requirements of plant species (Nas and Read, 2004). In this study, shoot tips were tested for shoot organogenesis. The highest multiplication rate of *Erodium sibthorpianum* shoot tips was achieved on MS medium with 1 ppm BAP, but the highest shoot length was obtained in PGR-free medium. BAP also significantly induced the auxiliary shoots when it was compared to Ki. Among the cytokinins, the superior effect of BAP over Ki on multiplication was reported by Sudha et al. (1998), Gümüşçü et al. (2008), and Akın and Kocaçalışkan (2011). However, as shown in different studies, high BAP levels caused necrosis in various plant species (Bhatia et al., 2002; Gümüşçü et al., 2008). In our research, MS medium proved to be superior to ModMS medium for shoot multiplication.

Table 5. Comparison of MS and ModMS medium for seedling growth.

Measured parameters	MS	ModMS
Stem length (mm)	28.93 ± 0.23*	25.60 ± 0.21
Root length (mm)	13.37 ± 0.27	39.17 ± 0.28*
Number of leaves/seedling	11.03 ± 0.20*	5.67 ± 0.35
Number of shoots/seedling	5.67 ± 0.28*	2.77 ± 0.19
Number of roots/seedling	3.40 ± 0.23	7.40 ± 0.21*

* t ($P < 0.05$); ±SE.

Table 6. Effect of different culture media (MS & ModMS) and growth regulators (IBA, IAA & NAA) on rooting of in vitro regenerated shoots after 8 weeks of rooting treatments.

Medium	IBA (ppm)	IAA (ppm)	Number of roots/ shoot	Root length (mm)	Rooting (%)
	Control		3.10 ± 0.31 e	12.83 ± 0.44 d	32.13 ± 0.41 i
	0.5	0.0	10.57 ± 0.30 a	16.40 ± 0.46 b	84.17 ± 0.60 a
	1.0	0.0	8.97 ± 0.26 b	13.27 ± 0.49 d	77.83 ± 0.38 c
	1.5	0.0	8.57 ± 0.35 b	10.07 ± 0.41 e	72.70 ± 0.35 d
MS	2.0	0.0	4.37 ± 0.35 d	6.33 ± 0.28 f	65.40 ± 0.61 h
	0.0	0.5	8.47 ± 0.32 b	18.87 ± 0.49 a	80.37 ± 0.69 b
	0.0	1.0	6.30 ± 0.44 c	14.90 ± 0.46 c	70.93 ± 0.47 e
	0.0	1.5	5.47 ± 0.38 c	10.70 ± 0.35 e	69.33 ± 0.35 f
	0.0	2.0	4.00 ± 0.10 de	10.30 ± 0.15 e	67.63 ± 0.47 g
Medium	NAA (ppm)		Number of roots/ shoot	Root length (mm)	Rooting (%)
	Control		7.87 ± 0.20 d	39.17 ± 0.41 b	43.10 ± 0.55 d
	0.1		21.17 ± 0.43 a	44.03 ± 0.58 a	100.00 ± 0.00 a
ModMS	0.5		20.80 ± 0.40 ab	40.77 ± 0.43 b	100.00 ± 0.00 a
	1		17.00 ± 0.29 c	22.83 ± 0.52 c	90.03 ± 0.58 c
	2		19.83 ± 0.44 b	23.33 ± 0.85 c	92.17 ± 0.62 b

Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P < 0.05$); ±SE.

In vitro shoot formation may have resulted in different success levels depending upon the explant types used (Zobayed and Saxena, 2003). Our results confirm those of Pevalek-Kozlina et al. (1999), Makowczynska and Andrzejewska-Golec (2003), and Gümüşçü et al. (2008) showing the achievement of shoot induction from shoot tips, which is the best source for the highest shoot induction.

The rooting parameters were changed significantly with different concentrations of IBA. The best rooting was achieved in the MS medium containing 0.5 ppm IBA. IBA is by far the most commonly used auxin to obtain root initiation. Similarly, IBA, as a synthetic auxin, was found to be the most favorable root inducer and was preferred based on the results of prior studies (Mikulík, 1999; Pretto and Santarém, 2000; Prasad et al., 2004; Akbar and Roy, 2006; George et al., 2008; Gümüşçü et al., 2008; Yasodha et al., 2008; Akın and Kocaçalışkan, 2011; Okumuş et al., 2011; Chalageri and Babu, 2012). However, when we compared the effects of MS and ModMS media on the rooting of seedlings, ModMS medium was superior to MS medium. The best rooting was obtained when ModMS with 0.1 ppm NAA was added to culture medium. ModMS medium contains a higher level of nitrogen and calcium. Because nitrogen affects the rate of plant growth and is an essential element in the molecular make-up of nucleic acids, proteins, chlorophyll, amino acids, alkaloids, and some plant hormones, it has a great influence on the rooting of shoot explants in vitro. Additionally, calcium is an integral component of plant cell walls and helps in the formation of pectin, a substance that bonds cell walls together. It also promotes root development (Wu et al., 2005; Sathyanarayana and Varghese, 2007). As a result, rooted plants were transplanted into pots in a greenhouse for further acclimatization studies.

The in vitro techniques reported here offer a powerful tool for mass multiplication of this threatened endemic plant species. The possibilities of plantlet regeneration through tissue culture technology were explored in this study. Several plantlet regeneration protocols were developed for the first time for this species. The regeneration system described here can be used in studies dealing with in vitro conservation of *Erodium sibthorpianum*. It is concluded that mineral salt of media and PGRs are very important in the success of plant tissue cultures.

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