Chapter 7

In Vitro Regeneration of Endangered Medicinal Plant *Heliotropium kotschyi* (Ramram)

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Abstract

Heliotropium kotschyi (Ramram) is an important endangered medicinal plant distributed in the Kingdom of Bahrain. Plant tissue culture technique is applied for ex situ conservation study. Nodal stem segments are cultured in modified MS media supplemented with various combination and concentration of plant growth regulators (PGRs). Plants are regenerated via shoot organogenesis from the nodal meristems. Plants are regenerated in three different steps: initial shoot development, shoot multiplication, and rooting. After 4 weeks of culture, 100 % explants respond to shoot initiation on the medium containing 8.88 μM BAP and 5.71 μM IAA. The highest frequency of shoot regeneration is observed in the same media after second subculture of shoots. The highest rooting frequency is observed in the presence of 2.85 μM IAA. After root development, the plantlets are transferred to pots filled with soil and 60 % of plants survived after 45 days. This plant regeneration protocol is of great value for rapid desert plant propagation program.

Key words Endangered, Ex situ conservation, *Heliotropium kotschyi*, Organogenesis, Plant regeneration

1 Introduction

Desert plants in dry ecosystems owe their importance to a long history of evolution and adaptation in dry and hot deserts. They are important as a source of good gene pool in food chain, extreme environmental adaptability, herbal medicine for human health, etc. Also, they prevent soil erosion for maintaining soil fertility. In Bahrain, 81 plant species are indigenous and reported to be used in traditional herbal medicine [1]. The importance of medicinal plants both in drug research and genetic biodiversity conservation is now well recognized.

Heliotropium kotschyi (local name Ramram) belongs to Boraginaceae family, and is an endangered, important medicinal

S. Mohan Jain (ed.), *Protocols for In Vitro Cultures and Secondary Metabolite Analysis of Aromatic and Medicinal Plants, Second Edition*, Methods in Molecular Biology, vol. 1391, DOI 10.1007/978-1-4939-3332-7_7,

plant distributed in Bahrain [2]. The plant is in general used as an antidote for snake venom; it is used so either by drinking the water extract of leaves or by applying leaf paste on the snake bite [3]. Global worry about the loss of valuable plant genetic resources has stimulated many advanced programs to conserve genetic diversity using either in situ or ex situ conservation strategies [4]. The application of in vitro culture as an important conservation tool of the rare, threatened, and endangered plants has gained a huge drive in the last two decades and is considered as one of the greatly applicable program of ex situ conservation strategy [5–8]. Different techniques of plant tissue culture are used for propagation, rapid proliferation, preservation, and storage of several medicinally important endangered and threatened plants [8, 9]. Considering the multipurpose importance of endangered desert plants, the biotechnological tool of plant tissue culture is applied to multiply them rapidly for conservation purposes [2, 9]. Here, we describe a highly efficient in vitro plant regeneration protocol for *H. kotschyi*.

2 Materials

2.1 Explant

1. Collection of apical shoots of 1-year-old Ramram (*Heliotropium kotschyi*) plants (Fig. 1a) from Al-Areen Wildlife Park in the Kingdom of Bahrain (*see* **Note 1**)

2.2 Chemicals

1. Murashige and Skoog (MS) medium [10] (*see* **Note 2**).
2. Stock solutions of vitamins (*see* **Note 3**).
3. Stock solutions of plant growth regulators (*see* **Note 4**).
4. 1 % (v/v) Lux solution (Soap).
5. 0.5 % mercuric chloride solution.
6. Tween 20.
7. 0.1 % Copral solution.
8. 50 % (v/v) Clorox solution.
9. 70 % (v/v) ethanol.
10. 0.1 M hydrochloric acid (HCl).
11. 0.1 M sodium hydroxide (NaOH).
12. Casein hydrolysate.
13. Sucrose.
14. Agar.

2.3 Equipment

1. Balance.
2. Measuring cylinder.

Fig. 1 Stages of micropropagation of *Heliotropium kotschyi*. Field-grown plants (**a**); stem segment for surface sterilization (**b**); explants in media 1 after 4 weeks (**c**); explants show shoot initiation response in media 4 after 2 weeks (**d**); initiated shoots in media 4 after 4 weeks (**e**); multiplication of initially developed shoot in first transferred media after 4 weeks (**f**); multiplication of shoots in second transferred media after 4 weeks (**g**); micro-shoots developing roots (**h**); plantlet growing in pots containing soil (**i**)

3. Magenta vessels.
4. pH meter.
5. Autoclave.
6. Millipore water, autoclaved.
7. Laminar flow air cabinet.
8. Refrigerator.
9. Plant culture room.

3 Methods

3.1 Preparation of Culture Media

1. Dissolve 4.4 g MS powder in 800 ml autoclaved water and supplement with 0.3 % casein hydrolysate, 3 % sucrose, 0.1 % nicotinic acid, 0.1 % pyridoxine–HCl, 1 % thiamine–HCl. Adjust pH of the medium to 5.8 by adding NaOH, make up the volume to 1 L, solidify by adding 0.9 % agar, and autoclave at 121 °C, for 20 min at 15 psi.
2. Add different concentrations and combinations of filter-sterilized plant growth regulators (PGRs) such as 6-benzylaminopurine (BAP), kinetin (KI), 3-indoleacetic acid (IAA), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA) in the autoclaved modified MS media for culture initiation, shoot multiplication, and rooting (Tables 1 and 2).

3.2 Explant Sterilization

1. Use apical shoots of *H. kotschyi* (Fig. 1b) for surface sterilization (*see* **Note 5**).

3.3 Culture Initiation and Plant Regeneration

1. Culture initiation experiments follow completely randomized design (CRD) with three replicates and 3–5 explants per replication.
2. Transfer surface-sterilized nodal stem segments to culture initiation media containing various concentrations and combinations of PGRs (Table 1).
3. Incubate cultures in culture room for 4 weeks to see the initial response. The culture room should provide 40–50 μmol/m^2 s^{-1} fluorescent light intensity for 16 h photoperiod, 24 °C± 2 °C temperature, and 70–80 % relative humidity.
4. MS media without any PGRs show no shoot initiation response (Fig. 1c).
5. The nodal explants start shoot initiation in media containing PGRs within 2–4 weeks of culture initiation (Fig. 1d).
6. Initially developed shoots show good growth after 4 weeks of culture and 100 % explants respond to initiate shoot development in the presence of 8.88 μM BAP with 5.71 μM IAA after 4 weeks (Fig. 2) (*see* **Note 6**).
7. A comparison of shoot initiation frequencies in different PGRs combinations and concentrations is shown in (Table 3) (*see* **Note 7**). The highest shoot initiation frequency has been noticed in the presence of 8.88 μM BAP with 5.71 μM IAA (Fig. 1e).
8. Transfer initially developed shoots to different multiplication media (Table 4) and observe the best performance in subculture media containing 8.88 μM BAP with 5.71 μM IAA (Fig. 1f) (*see* **Note 8**).

Table 1
Concentration and combination of PGRs in modified MS media for culture initiation and multiplication

Name of media	Concentration of PGRs (μM)
1	Medium without hormone
2	4.44 μM BAP + 2.85 μM IAA
3	6.66 μM BAP + 2.85 μM IAA
4	8.88 μM BAP + 5.71 μM IAA
5	13.3 μM BAP + 5.71 μM IAA
6	4.44 μM BAP + 2.68 μM NAA
7	6.66 μM BAP + 2.68 μM NAA
8	8.88 μM BAP + 5.37 μM NAA
9	13.3 μM BAP + 5.37 μM NAA
10	4.65 μM KI + 2.85 μM IAA
11	6.97 μM KI + 2.85 μM IAA
12	9.29 μM KI + 5.71 μM IAA
13	13.9 μM KI + 5.71 μM IAA
14	4.65 μM KI + 2.68 μM NAA
15	6.97 μM KI + 2.68 μM NAA
16	9.29 μM KI + 5.37 μM NAA
17	13.9 μM KI + 5.37 μM NAA
18	0.89 μM BAP
19	2.22 μM BAP
20	4.44 μM BAP
21	8.88 μM BAP

Table 2
Concentration and combination of PGRs in modified MS media for subculture and rooting

Name of media	Concentration of PGRs (μM)
22	8.88 μM BAP + 1.14 μM IAA
26	1 % charcoal + 7.4 μM IBA
27	1 % charcoal + 9.84 μM IBA
28	0.93 μM KI + 5.71 μM IAA
30	2.85 μM IAA

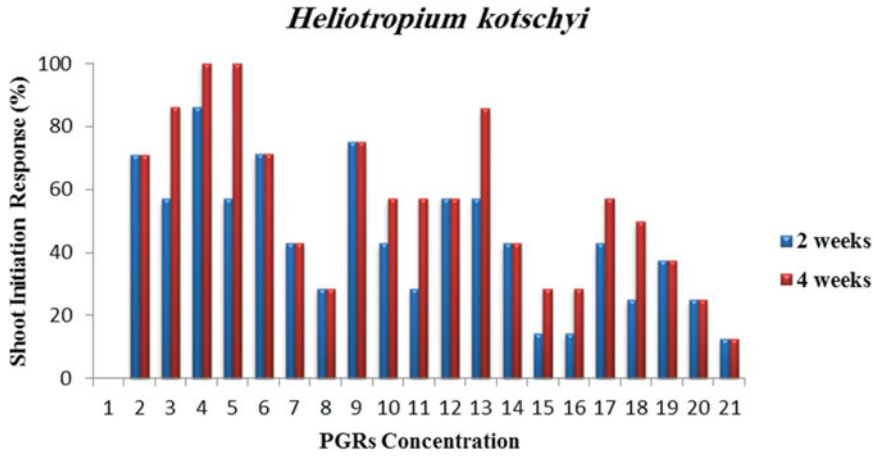

Fig. 2 Effect of various plant growth regulators supplemented to modified MS medium on in vitro shoot initiation from nodal segments of explants of *H. kotschyi* after 2 and 4 weeks of culture. PGR concentration in Table 1

Table 3
Effect of various plant growth regulators supplemented to modified MS media on in vitro shoot initiation response from shoot apex and nodal explants of *H. kotschyi* after 4 weeks of culture. Results are means of shoots developed per explant. Means followed by the same letter are not significantly different at $P \leq 0.05$

Name of media	PGR concentrations (µM)	Mean
4	8.88 µM BAP + 5.71 µM IAA	10.66 A
5	13.3 µM BAP + 5.71 µM IAA	8 B
3	6.66 µM BAP + 2.85 µM IAA	5.33 C
13	13.9 µM KI + 5.71 µM IAA	4 D
10	4.65 µM KI + 2.85 µM IAA	3.66 D E
14	4.65 µM KI + 2.68 µM NAA	3.66 D E
9	13.3 µM BAP + 5.37 µM NAA	3 D E F
11	6.97 µM KI + 2.85 µM IAA	3 D E F
12	9.29 µM KI + 5.71 µM IAA	3 D E F
2	4.44 µM BAP + 2.85 µM IAA	2.66 E F G
19	2.22 µM BAP	2.66 E F G
6	4.44 µM BAP + 2.68 µM NAA	2 F G
15	6.97 µM KI + 2.68 µM NAA	2 F G
17	13.9 µM KI + 5.37 µM NAA	2 F G
20	4.44 µM BAP	2 F G

continued

Table 3
(continued)

Name of media	PGR concentrations (μM)	Mean
7	6.66 μM BAP + 2.68 μM NAA	1.66 G
18	0.89 μM BAP	1.66 G
8	8.88 μM BAP + 5.37 μM NAA	1 H
16	9.29 μM KI + 5.37 μM NAA	1 H I
21	8.88 μM BAP	1 H I
1	Medium without PGRs	0 I

Table 4
Effect of BAP, KI, IAA, IBA, and NAA on percentage of shoot multiplication frequency after first transfer of *H. kotschyi*. PGR concentration in Tables 1 and 2

Name of media	Percentage of proliferated shoot	Shoot multiplication frequency
1	0	0
3 → 4	100	15
4 → 4	92.3	12.07
5 → 4	75	2.5
6 → 4	87.5	8.1
7 → 4	33.3	3.33
7 → 28	100	12.5
8 → 4	50	7.5
9 → 4	100	5
20 → 22	100	7.6

9. Calculate plant regeneration capacity of *H. kotschyi* based on shoot initiation frequency of explants and multiplication frequency of initially developed shoots after first and second transfer (Fig. 3) (*see* **Note 9**).
10. Transfer newly formed micro-shoots, 1–2 cm long to rooting media containing 2.85 μM IAA (Fig. 1h) (*see* **Note 10**).
11. Transfer plantlets to pots containing soil (Fig. 1i) (*see* **Note 11**).

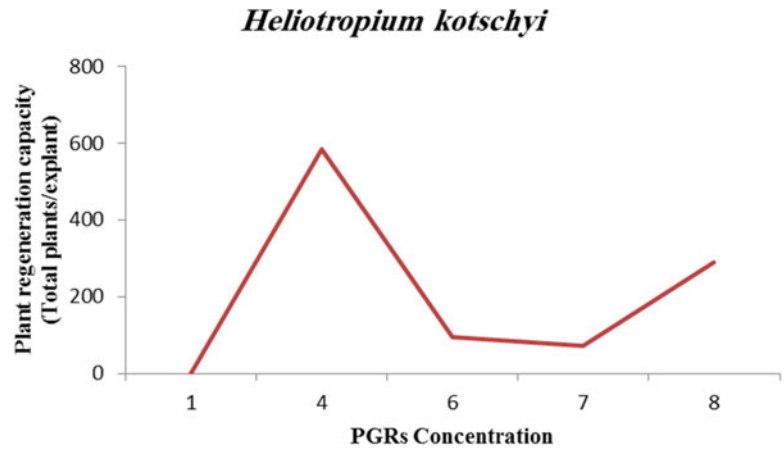

Fig. 3 Plant regeneration capacity of *H. kotschyi* after 2nd transfer in media 1 while initial shoots develop from culture of nodal explants in different media [1, 4, 6–8] and their multiplication in media 4. PGR concentration in Table 1

4 Notes

1. Collect apical shoots (12–15 cm long) from 1-year-old actively growing field-grown plants for experiments. Cut them into pieces and store in plastic bags at 4 °C for future use.
2. Use MS powder for all experiments.
3. Make stock solutions of vitamins: 1 mg/ml nicotinic acid, 1 mg/ml pyridoxine–HCl, and 10 mg/ml thiamine–HCl, then filter-sterilize with sterile Acrodisc 0.45 μm, and store in a refrigerator (4 °C) until future use.
4. For the preparation of PGR stock solutions at 2 mg/ml concentration, weigh 100 mg IAA, NAA, IBA, KI, BAP, separately, dissolve in 2 ml 100 mM NaOH solution, add autoclaved water to raise the volume to 50 ml, then filter-sterilize using sterile Acrodisc 0.45 μm. Store them at −20 °C for future use and keep at 4 °C for routine use.
5. Surface-sterilize the stem segments by using subsequent mixtures of 1 % Lux solution (soap solution), 0.5 % mercuric chloride with few drops of Tween 20, 0.1 % Copral, 50 % (v/v) Clorox (containing 2.625 % hypochlorite), 70 % ethanol. Then slice the stem segments into smaller segments (1–1.5 cm), each containing one node, for use as explants for culture initiation.
6. The nodal explants initiate highest 100 % direct multiple shoot formation on the modified MS medium containing 8.88 μM BAP with 5.71 μM IAA after 4 weeks of culture.

7. The results show significant differences ($P \leq 0.05$ level) by ANOVA according to Duncan's multiple range test (DMRT) using JMP (version 9) statistical software. After 4 weeks of culture, the highest shoot initiation frequency is 10.6 on MS medium supplemented with 8.88 μM BAP and 5.71 μM IAA.
8. 100 % shoot proliferation in association with the highest shoot multiplication frequency of initially developed shoots is observed during subculture in media containing 8.88 μM BAP with 5.71 μM IAA.
9. The highest plant regeneration capacity is observed while explants are cultured subsequently in media with higher concentration of BAP (8.88 μM) and lower concentration of IAA (5.71 μM) than other combinations and concentrations of PGRs. Similarly, several studies report that higher concentrations of BAP with lower concentrations of IAA induce multiple shoots in *Salvia africana-lutea* L. [11], *Melissa officinalis* L. [12]. The stages of in vitro plantlet regeneration of *H. kotschyi* are shown in Fig. 1.
10. Gently remove culture media sticking to roots by washing with autoclaved distilled water and transfer rooted plantlets (Fig. 1h) to plastic pots containing autoclaved compost soil (1:1 mixture of peat substrate and potting soil); and keep them in a small transparent covered chamber to maintain humidity. The plants acclimatize in room conditions at 25 °C ± 3 °C, 16/8 h photoperiod and by regular watering at 3 days interval.
11. Expose gradually well-developed rooted plantlets (Fig. 1i) to normal growing conditions and 60 % plantlets survive after 45 days.
12. This is the first report on the micropropagation of this endangered plant in the Kingdom of Bahrain.
13. The establishment of in vitro culture and efficient plant regeneration protocol is a crucial initial step of ex situ conservation strategy.

Acknowledgements

The work was supported by College of Graduate Studies, Desert and Arid Zone Sciences Program, Arabian Gulf University, Manama, Kingdom of Bahrain.

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