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Original Paper

Indirect organogenesis in *Ephedra foliata*

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Abstract

Ephedra foliata Boiss. ex C.A. Mey (Ephedraceae) is a well-known source of the alkaloid ephedrine used for the treatment of chronic asthma and associated respiratory ailments. This investigation was conducted to standardize an efficient regeneration protocol for *E. foliata* via indirect organogenesis through intermodal explants. Maximum callus induction (80.3%) was achieved on Murashige and Skoog (MS) medium supplemented with 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid + 0.5 mg L^{-1} kinetin and additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} citric acid). The maximum shoot regeneration (n = 5.27) was achieved on MS medium containing 6.0 mg L^{-1} kinetin, followed by 5.0 mg L^{-1} 6-benzylamino purine (n = 4.27). MS half strength medium with 3.0 mg L^{-1} α-naphthalene acetic acid resulted in the highest rooting percentage (32%). Sixty percent of the plantlets survived during acclimatization and were successfully transferred under field conditions. These plants and callus will be a suitable source of plant material for mass multiplication, genetic modification for enhanced bioactive constituents, and germplasm conservation.

Key words: callus induction, *Ephedra foliata, ex vitro* acclimatization, growth regulators, organogenesis.
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid; KIN, kinetin; MS, Murashige and Skoog; NAA, α-naphthalene acetic acid.

Introduction

Ephedra foliata Boiss. ex C.A. Mey (Ephedraceae), commonly known as 'unthphog' and 'shrubby horsetail', is widely distributed in deserts of Africa, Arabian Peninsula and India. In India, it is found in arid and semi-arid regions of the north-western (Bhandari 1990; Lodha et al. 2014). It is the only gymnosperm that grows sporadically on sand hills in arid and semiarid areas in the Thar Desert of India (Shekhawat et al. 2012). At present, *E. foliata* is considered to be a threatened species in India (IUCN 2017; Meena et al. 2019). The plants are woody climbers with highly reduced scale-like foliage leaves arranged in a decussate pattern on nodes. The branches and seeds of this plant are an important component of the diet of camel, sheep, goat and insect species surviving in the nutrient poor arid desert of Rajasthan. It also acts as a major soil binder (Singh et al. 2007).

The species is known to contain the alkaloids ephedrine and pseudoephedrine, which are of great importance for their biological and pharmacological potential (Ghiasvand et al. 2019). It is used to treat bronchial asthma, hypersensitivity, fever, influenza, chills, colds, hack, cerebral pains, nasal blockage and other respiratory problems (Elhadef et al. 2020). It possesses antimicrobial, antioxidant, antidiabetic, hepatoprotective and cardiovascular activity (Al-Snafi 2017).

The conventional propagation of this plant is through seeds. The percentage seed germination and establishment is low due to abiotic stressors like high ambient temperature and soil alkalinity, which are common severe environmental conditions of the region. Pre-dispersal seed predation and post-dispersal seed predation by insects groups, rodents and other burrowing animals is a serious issue in restricting recruitment of new *E. foliata* population (Singh et al. 2007). Also, anthropogenic activities also have impact on the dwindling population of *E. foliata* (Singh 2004; Lodha et al. 2014).

Micropropagation offers an efficient method for mass propagation of threatened medicinal plants via direct and indirect organogenesis under *in vitro* conditions for *ex situ* conservation, genetic improvement and commercial applications, without any seasonal limitations (Yadav, Singh 2012; Yadav et al. 2012; Groach et al. 2014). Although there are many reports on *in vitro* propagation of *E. foliata* (Lodha et al. 2014a; Lodha et al. 2014b), considerable efforts are still required to make it more economical and practical. Therefore, the development of an efficient micropropagation protocol for *E. foliata* is urgently needed for both germplasm conservation and to expand pharmaceutical prospects. The present investigation deals with indirect organogenesis through callus-mediated induction of internodal explants of *E. foliata* as an alternative to naturally grown plants.

Materials and methods

A mature healthy plant of *E. foliata* growing in the Herbal Garden of the Department of Botany, Kurukshetra University, Haryana (India) was used as an explant source. Healthy internodal explants (1.0 to 1.5 cm) were surface sterilized by washing with 5% liquid detergent (Tween 20) followed by washing under running tap water in a plastic sieve for ten minutes to remove the adhering dust particles. Thereafter, the explants were disinfected using 0.1 % (w/v) of mercuric chloride (Hi-Media, India) for 3 to 5 min followed by a brief rinse with 70% ethanol and five times washing with sterilized double distilled water to remove the traces of mercuric chloride under aseptic conditions.

Explants after trimming the ends were inoculated on Murashige and Skoog (MS; 1962) medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, additives (50 mg L^{-1} ascorbic acid and 25 mg L^{-1} citric acid) supplemented with various concentrations of auxin-type growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) and cytokinin-type growth regulators 6-benzylaminopurine (BAP), kinetin (KIN) individually or in different combinations (0.5 to 2.0 mg L^{-1}) for callus induction. After explanting, the culture tubes, flasks capped with non-absorbent cotton plugs, were incubated at 25 ± 2 °C and 60 to 70 % relative humidity under a 16 h photoperiod at 40 µmol m^{-2} s^{-1} photon flux density of photosynthetically active radiation provided by Philip's cool white fluorescent tube lights. The pH of the medium was adjusted to 5.8 and autoclaved at 1.5 kg cm^{-2} and 121 °C for 20 min.

After 4 weeks of incubation, the calli formed from intermodal explants were periodically sub-culturing for multiplication and maintenance on various callus proliferation media (Table 1 and 2) and finally, a mass of calli was harvested. Visual observations like number of days required for callus induction, frequency of the callus induction (%) and nature of callus (colour, texture) were also recorded. For shoot regeneration, the best *in vitro* regenerated calluses from the 3rd successive sub-culture were excised aseptically and implanted on the different shoot induction medium (Table 3).

For root induction, the regenerated shoots (2.5 to 3.0 cm) were excised and cultured on half-strength MS media supplemented with various concentrations (1.0 to 4.0 mg L^{-1}) of IAA, NAA and indole-3-butyric acid (IBA) alone. The well-rooted plantlets were gradually pulled out from the culture tubes and gently washed with a soft brush under running tap water to remove the adhering agar with minimum injury.

After washing these plantlets, they were then transferred to small plastic cups containing autoclaved vermiculite/sand (3:1) potting mixture. In order to maintain the elevated humidity around the plants, the plantlets were covered with a glass jar. They were supplied with half-strength MS salt solution on alternate days. In the third week, the glass jars were removed for 3 to 4 h daily to expose the plants to the natural field conditions. After 4 weeks, these plants were transferred to bigger pots and were maintained in a greenhouse for acclimatization. Finally, the plants were transferred to field conditions.

All the experiments were repeated thrice with a

Table 1. Effect of plant growth regulators on callus induction of *E. foliata* recorded after four weeks on MS medium. (–) no response, (+) poor growth, (++) moderate growth, (+++) good growth. Data shown are mean ± SE of 45 replicates .Mean values followed by different letters within a column do not differ significantly at $P = 0.05$ according to Duncan's Multiple Range Test

Growth regulator (concentration mg L^{-1})	Time required for callus induction (days)	Callus induction (%)	Nature of callus	Visual growth of callus
Control	–	–	–	–
2,4 D (0.25)	–	–	–	–
2,4 D (0.5)	28	20.0 cd	Creamish yellow	+
2,4 D (1.0)	27	40.3 cb	Creamish yellow	+
2,4 D (2.0)	21	65.1 a	Fluorescent green	+++
NAA (0.25)	–	–	–	–
NAA (0.5)	–	–	–	–
NAA (1.0)	25	45.2 b	Yellowish brown	++
NAA (2.0)	26	47.1 ab	Yellowish brown	++
BAP (0.25)	–	–	–	–
BAP (0.5)	27	22.1 c	Light green	++
BAP (1.0)	26	15.0 cd	Light green	+
BAP (2.0)	–	–	–	–
KIN (0.25)	21	5.1 d	Green	+
KIN (0.5)	17	40.4 cb	Dark green	+
KIN (1.0)	16	45.1 b	Dark green	++
KIN (2.0)	16	42.2 cb	Dark green	++

Table 2. Effect of growth regulators in combinations on callus induction recorded after four weeks on MS medium. (–) no response, (+) poor growth, (++) moderate growth, (+++) good growth. Data shown are mean ± SE of 45 replicates. Mean values followed by different letters within a column do not differ significantly at $P = 0.05$ according to Duncan's Multiple Range Test

Growth regulator (concentration mg L^{-1})	Time required for callus induction (days)	Callus induction (%)	Nature of callus	Visual growth of callus
Control	–	–	–	–
KIN (0.25) + 2,4D (0.5)	32.1	10.0 e	Brownish	+
KIN (0.25) + 2,4D (1.0)	27.2	13.2 de	Brownish	++
KIN (0.25) + 2,4D (2.0)	35.7	21.1 d	Brownish	+
KIN (0.5) + 2,4D (0.5)	21.4	80.3 a	Dark green	+++
KIN (0.5) + 2,4D (1.0)	25.2	45.2 c	Dark green	+
KIN (0.5) + 2,4D (2.0)	25.9	42.1 c	Dark green	+
KIN (1.0) + 2,4D (0.5)	35.2	38.1 cd	Brownish green	++
KIN (1.0) + 2,4D (1.0)	36.3	34.1 cd	Brownish green	+
KIN (1.0) + 2,4D (2.0)	38.0	28.4 cd	Brownish green	+
KIN (0.25) + NAA (0.5)	32.0	62.0 b	Brown friable	++
KIN (0.25) + NAA (1.0)	31.5	62.7 b	Brown friable	++
KIN (0.25) + NAA (2.0)	31.0	63.1 b	Brown friable	++
KIN (0.5) + NAA (0.5)	32.1	55.1 bc	Brown friable	++
KIN (0.5) + NAA (1.0)	32.3	55.6 bc	Brown friable	++
KIN (0.5) + NAA (2.0)	30.0	56.0 bc	Brown friable	++
KIN (1.0) + NAA (0.5)	33.0	47.0 c	Brown friable	+
KIN (1.0) + NAA (1.0)	32.8	47.7 c	Brown friable	++
KIN (1.0) + NAA (2.0)	33.1	48.5 c	Brown friable	++
BAP (0.25) + NAA (0.5)	30.0	46.0 c	Light green	++
BAP (0.25) + NAA (1.0)	30.6	46.3 c	Light green	+
BAP (0.25) + NAA (2.0)	30.8	45.1 c	Light green	+
BAP (0.5) + NAA (0.5)	29.8	46.2 c	Light green	++
BAP (0.5) + NAA (1.0)	29.0	46.0 c	Light green	+
BAP (0.5) + NAA (2.0)	28.0	45.4 c	Light green	+
BAP (0.5) + NAA (0.5)	30.1	46.0 c	Light green	+
BAP (0.5) + NAA (1.0)	30.0	45.1 c	Light green	+
BAP (0.5) + NAA (2.0)	30.0	44.4 c	Light green	+

minimum of fifteen replicates per treatment and one explant per replicate. The statistical calculations were all carried out using SPSS (V. 16.0) statistical software. The difference between means was analyzed by one-way analysis of variance (ANOVA) using Duncan's multiple range test at a significance level $p = 0.05$.

Results

All of the tested auxins showed a better callogenic response over cytokinins (Table 1). MS medium supplemented with 2,4-D (2.0 mg L^{-1}) resulted in the highest per cent callus induction (65%) within 21 days of inoculation (Fig. 1A). The callus obtained was fluorescent green in colour. Among auxins, 2,4-D was found to be superior over NAA regarding the percent callus induction in a lower number of days.

Since MS medium supplemented with auxins resulted in better results, the effect of 2,4-D and NAA in combination with KIN and BAP was studied on callogenesis in intermodal explants (Table 2). The best callus induction percentage (80%) with higher growth was noticed in media supplemented with KIN (0.5 mg L^{-1}) + 2,4-D (0.5 mg L^{-1}) (Fig. 1B). All of the different concentrations of KIN + NAA supplemented media resulted in the production of brownish friable callus while light green was observed with all of the concentrations of BAP + NAA. The different concentrations of KIN + 2,4-D showed different nature of calli ranging from brown, brownish green to dark green.

For differentiation of shoots from intermodal derived callus, various concentrations (1.0 to 6.0 mg L^{-1}) of BAP and KIN were tested (Table 3). Shoots bud formation was occurred in both BAP and KIN fortified medium. Vigorous growth of callus was visible in the form of green patches after two weeks of transfer on various shoot induction media. After three weeks, these green patches further developed into multiple shoots.

Both full and half-strength MS medium devoid of growth regulator failed to produce roots (Table 4). Among NAA and IBA as root inducers, both NAA and IBA resulted in significantly better results. However, the best root

Table 3. Effect of plant growth regulators on shoot regeneration of *E. foliata* from callus recorded after four weeks on MS medium. (–) no response. Data shown are mean ± SE of 45 replicates. Mean values followed by different letters within a column do not differ significantly at $P = 0.05$ according to Duncan's Multiple Range Test

Growth regulator (concentration mg L^{-1})	Time required for formation of green patches (days)	Number of shoots per explant
Control	–	–
BAP (0.5)	–	–
BAP (1.0)	16.0	1.13 de
BAP (2.0)	16.2	1.19 de
BAP (3.0)	15.8	1.82 d
BAP (4.0)	15.9	3.45 c
BAP (5.0)	15.5	4.27 b
KIN (1.0)	–	–
KIN (2.0)	–	–
KIN (3.0)	17.3	1.86 d
KIN (5.0)	17.1	3.21 c
KIN (6.0)	16.9	5.37 a
2,4D (1.0) + BAP (1.0)	–	–
2,4D (1.0) + BAP (3.0)	20.0	0.98 e
2,4D (1.0) + BAP (5.0)	16.8	3.75 bc

Table 4. Effect of plant growth regulators on root development of *E. foliata* recorded after four weeks on 0.5 MS medium. (–) no response. Data shown are mean ± SE of 45 replicates. Mean values followed by different letters within a column do not differ significantly at $P = 0.05$ according to Duncan's Multiple Range Test

Growth regulator (concentration mg L^{-1})	Root-producing shoots (%)	Number of roots per shoot
Control	–	–
NAA (1.0)	–	–
NAA (2.0)	23.00 ± 0.66 b	1.20 ± 0.01 c
NAA (3.0)	32.00 ± 0.78 a	2.10 ± 0.04 a
NAA (4.0)	25.00 ± 0.05 ab	1.30 ± 0.02 bc
IBA (1.0)	–	–
IBA (2.0)	–	–
IBA (3.0)	16.00 ±0.07 bc	1.50 ± 0.02 b
IBA (4.0)	13.00 ± 0.39 c	1.30 ± 0.00 bc

formation was observed with 3.0 mg L^{-1} NAA (Table 4; Fig. 1D).

The period of acclimatization is one of the most important stages, where the plant self-fixes the abnormalities to ensure survival under *ex vitro* conditions. The in vitro raised plantlets were then transferred to small plastic cups containing autoclaved vermiculite: sand (3:1) potting mixture (Fig. 1E). Sixty per cent of the plantlets survived during acclimatization and were successfully transferred to field conditions.

Discussion

Indirect organogenesis involves the formation of callus from cultured explants followed by shoot bud differentiation. Callus is an undifferentiated proliferative mass of cells, obtained by culturing explants aseptically on nutrient medium under controlled experimental conditions (Hussain et al. 2012).

The MS medium devoid of any plant growth regulators did not show any callogenic response in the tested explants, which may be due to the inadequate level of endogenous growth hormones in explants to induce callusing and requirement of an external contribution of growth regulators to trigger cell division (Huang et al. 2012). 2,4-D is the most commonly used plant growth regulator in callus induction (Zang et al. 2016; Mostafiz, Wagiran 2018; Carsono et al. 2021). The resulting colour variation in callus might be due to the type and concentration of the growth regulator. Compared to only auxin, a combination of auxin and cytokinin resulted in a greener callus, caused by cytokinin, which tends to promote chlorophyll formation (George et al. 2008). Other factors like pigment, nutrients and exposure to light also account for changes in callus colour (Evans et al. 2003).

Explants of *E. foliata* on MS medium fortified with KIN

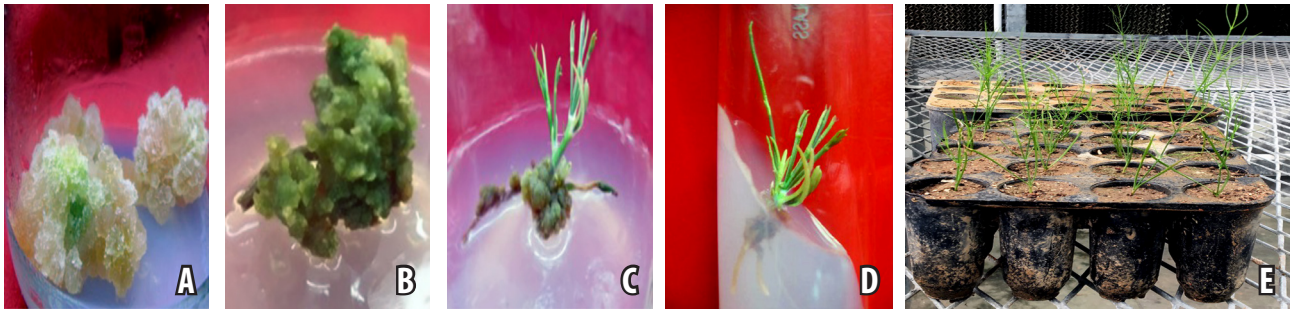

Fig. 1. *In vitro* plant regeneration of *E. foliata*. A, callus from internodal explants on MS medium supplemented with 2,4-D (2.0 mg L^{-1}). B, callus formation on MS + KIN (0.5 mg L^{-1}) + 2,4-D (0.5 mg L^{-1}). C, shoot differentiation on MS + KIN (6.0 mg L^{-1}). D, root induction on ½ MS medium + NAA (3.0 mg L^{-1}). E, *in vitro* raised plantlets before transfer to field conditions.

(6.0 mg L^{-1}) produced 5.37 shoots per callus culture within 16.9 days (Fig. 1C). Kinetin was found to be more potent over other cytokinins with or without the combination of auxin for the differentiation of multiple shoots from callus in *Ananas comosus* (Akbar et al. 2003), *Oryza sativa* (Libin et al. 2012), *Plectranthus rotundifolius* (Asha et al. 2013) and *Plectranthus bourneae* (Elangomathavan et al. 2017). The addition of 2,4-D in lower concentration also facilitated better morphogenesis and enhanced the rate of shoot bud differentiation in different plant species (Parveen et al. 2012; Mehaboob et al. 2019; Putri et al. 2020). In contrast, BAP was found to be more effective than kinetin for shoot multiplication in *Albizia lebbeck* (Yadav, Singh 2011), which may be due to the ability of BAP to induce and produce natural hormones, such as zeatin, within the tissue through the natural hormone system (Sharma, Wakhlu 2003).

Rooting is necessary to generate the whole plantlet from *in vitro* regenerated shoots. The plantlets have difficulty in survival under *ex vitro* conditions without an appropriate root system (De Klerk 2002). MS medium without any growth regulators proved ineffective for root induction in *Prosopis cineraria* (Kumar, Singh 2009) and *Stevia rebaudiana* (Verma et al. 2011). The effectiveness of half-strength medium over full strength medium in inducing *in vitro* rooting has also been reported by many researchers (Yadav et al. 2012; Kumar et al. 2013; Groach et al. 2014). The rooting of elongated shoots on MS medium supplemented with NAA or IBA has been well documented in *Stevia rebaudiana* (Verma et al. 2011), *Simmondsia chinensis* (Kumar et al. 2013) and *Vitex negundo* (Groach et al. 2014). In the culture laboratory, *in vitro* raised plants are constantly maintained under a controlled environment of high humidity, low light intensity, photoperiod, optimum temperature, supplementary sugar supply and growth regulators (Hazarika, Bora 2006; Yadav et al. 2013).

The efficient protocol developed for *Ephedra foliata* in the present study could help in using this plant material for mass multiplication, genetic modification for enhanced bioactive constituents and germplasm conservation.

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