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-benzylaminopurine (*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 ‘shruby 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 (Elhafed 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 intermodal 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\(^{-1}\) 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 internodal 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 3\(^{rd}\) 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.

<table>
<thead>
<tr>
<th>Growth regulator (concentration mg L(^{-1}))</th>
<th>Time required for callus induction (days)</th>
<th>Callus induction (%)</th>
<th>Nature of callus</th>
<th>Visual growth of callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2,4 D (0.25)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2,4 D (0.5)</td>
<td>28</td>
<td>20.0 cd</td>
<td>Creamish yellow</td>
<td>+</td>
</tr>
<tr>
<td>2,4 D (1.0)</td>
<td>27</td>
<td>40.3 cb</td>
<td>Creamish yellow</td>
<td>+</td>
</tr>
<tr>
<td>2,4 D (2.0)</td>
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<td>65.1 a</td>
<td>fluorescent green</td>
<td>+++</td>
</tr>
<tr>
<td>NAA (0.25)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NAA (0.5)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NAA (1.0)</td>
<td>25</td>
<td>45.2 b</td>
<td>Yellowish brown</td>
<td>++</td>
</tr>
<tr>
<td>NAA (2.0)</td>
<td>26</td>
<td>47.1 ab</td>
<td>Yellowish brown</td>
<td>++</td>
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<td>BAP (0.25)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>27</td>
<td>22.1 c</td>
<td>Light green</td>
<td>++</td>
</tr>
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<td>26</td>
<td>15.0 cd</td>
<td>Light green</td>
<td>+</td>
</tr>
<tr>
<td>BAP (2.0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>KIN (0.25)</td>
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<td>5.1 d</td>
<td>Green</td>
<td>+</td>
</tr>
<tr>
<td>KIN (0.5)</td>
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<td>40.4 cb</td>
<td>Dark green</td>
<td>+</td>
</tr>
<tr>
<td>KIN (1.0)</td>
<td>16</td>
<td>45.1 b</td>
<td>Dark green</td>
<td>++</td>
</tr>
<tr>
<td>KIN (2.0)</td>
<td>16</td>
<td>42.2 cb</td>
<td>Dark green</td>
<td>++</td>
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</table>
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 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>
<thead>
<tr>
<th>Growth regulator (concentration mg L(^{-1}))</th>
<th>Time required for callus induction (days)</th>
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<th>Visual growth of callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>KIN (0.25) + 2,4D (0.5)</td>
<td>32.1</td>
<td>10.0 e</td>
<td>Brownish</td>
<td>+</td>
</tr>
<tr>
<td>KIN (0.25) + 2,4D (1.0)</td>
<td>27.2</td>
<td>13.2 de</td>
<td>Brownish</td>
<td>+</td>
</tr>
<tr>
<td>KIN (0.25) + 2,4D (2.0)</td>
<td>35.7</td>
<td>21.1 d</td>
<td>Brownish</td>
<td>+</td>
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<tr>
<td>KIN (0.5) + 2,4D (0.5)</td>
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<td>80.3 a</td>
<td>Dark green</td>
<td>+++</td>
</tr>
<tr>
<td>KIN (0.5) + 2,4D (1.0)</td>
<td>25.2</td>
<td>45.2 c</td>
<td>Dark green</td>
<td>+</td>
</tr>
<tr>
<td>KIN (0.5) + 2,4D (2.0)</td>
<td>25.9</td>
<td>42.1 c</td>
<td>Dark green</td>
<td>+</td>
</tr>
<tr>
<td>KIN (1.0) + 2,4D (0.5)</td>
<td>35.2</td>
<td>38.1 cd</td>
<td>Brownish green</td>
<td>+</td>
</tr>
<tr>
<td>KIN (1.0) + 2,4D (1.0)</td>
<td>36.3</td>
<td>34.1 cd</td>
<td>Brownish green</td>
<td>+</td>
</tr>
<tr>
<td>KIN (1.0) + 2,4D (2.0)</td>
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<td>28.4 cd</td>
<td>Brownish green</td>
<td>+</td>
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<tr>
<td>KIN (0.25) + NAA (0.5)</td>
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<td>62.0 b</td>
<td>Brown friable</td>
<td>++</td>
</tr>
<tr>
<td>KIN (0.25) + NAA (1.0)</td>
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<td>62.7 b</td>
<td>Brown friable</td>
<td>++</td>
</tr>
<tr>
<td>KIN (0.25) + NAA (2.0)</td>
<td>31.0</td>
<td>63.1 b</td>
<td>Brown friable</td>
<td>++</td>
</tr>
<tr>
<td>KIN (0.5) + NAA (0.5)</td>
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<td>55.1 bc</td>
<td>Brown friable</td>
<td>++</td>
</tr>
<tr>
<td>KIN (0.5) + NAA (1.0)</td>
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<td>55.6 bc</td>
<td>Brown friable</td>
<td>++</td>
</tr>
<tr>
<td>KIN (0.5) + NAA (2.0)</td>
<td>30.0</td>
<td>56.0 bc</td>
<td>Brown friable</td>
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<td>KIN (1.0) + NAA (0.5)</td>
<td>33.0</td>
<td>47.0 c</td>
<td>Brown friable</td>
<td>+</td>
</tr>
<tr>
<td>KIN (1.0) + NAA (1.0)</td>
<td>32.8</td>
<td>47.7 c</td>
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<td>++</td>
</tr>
<tr>
<td>KIN (1.0) + NAA (2.0)</td>
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<td>48.5 c</td>
<td>Brown friable</td>
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<tr>
<td>BAP (0.25) + NAA (0.5)</td>
<td>30.0</td>
<td>46.0 c</td>
<td>Light green</td>
<td>++</td>
</tr>
<tr>
<td>BAP (0.25) + NAA (1.0)</td>
<td>30.6</td>
<td>46.3 c</td>
<td>Light green</td>
<td>++</td>
</tr>
<tr>
<td>BAP (0.25) + NAA (2.0)</td>
<td>30.8</td>
<td>45.1 c</td>
<td>Light green</td>
<td>+</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (0.5)</td>
<td>29.8</td>
<td>46.2 c</td>
<td>Light green</td>
<td>++</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (1.0)</td>
<td>29.0</td>
<td>46.0 c</td>
<td>Light green</td>
<td>+</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (2.0)</td>
<td>28.0</td>
<td>45.4 c</td>
<td>Light green</td>
<td>+</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (0.5)</td>
<td>30.0</td>
<td>46.0 c</td>
<td>Light green</td>
<td>+</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (1.0)</td>
<td>30.0</td>
<td>45.1 c</td>
<td>Light green</td>
<td>+</td>
</tr>
<tr>
<td>BAP (0.5) + NAA (2.0)</td>
<td>30.0</td>
<td>44.4 c</td>
<td>Light green</td>
<td>+</td>
</tr>
</tbody>
</table>
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.

![Fig. 1](image-url)

**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.

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.

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 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.
Regenerated shoots. The plantlets have 12: 503–508. conditions without
Oryza (Verma et al. 2011), Zingiber officinale
12: 127–133. Oryza sativa
12: 1–3. Al-Snafi A.E. 2017. Medicinal plants possessed antioxidant and
regulators (Hazarika, Bora 2006; Yadav et al. 2013).
high humidity, low light intensity, photoperiod, optimum
al. 2014). In the culture laboratory, 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
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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