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SHORT COMMUNICATION

In Vitro Multiple Shoot Induction and Plant Regeneration from Male *Ephedra foliata*: A Potential Medicinal Gymnosperm

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Abstract *Ephedra foliata* Boiss. Ex. C.A. Mey, known as soma plant according to ancient Indian Ayurvedic system. It is known for its potential to cure many diseases. Review of literature revealed that *E. foliata* contains traces of alkaloids responsible for its therapeutic activity. In present study effect of different concentrations of plant growth regulator on nodal sector explants was checked for multiple shoot production. Maximum numbers of shoots were observed on Kinetin (2.5 mg/l) whereas, Kinetin (2.5 mg/l) with Indole acetic acid (2.5 mg/l) induced 9.04 ± 0.8 shoots in 22.46 ± 0.57 days and percentage of explants that showed response was more than any other combination. Out of the two plant growth regulators used for root induction from in vitro grown shoots 1-napthaleneacetic acid showed better response than indole-3-acetic acid.

Keywords *Ephedra foliata* · Nodal sector explants · Multiple shoots · Growth regulators

Ancient medicinal systems contain treasures of traditional knowledge about utilization of medicinal plants for the benefit of mankind. Modern allopathic medicine has its roots in ancient systems of medicine. It is likely that such traditional knowledge and experiences will lead to discovery of many new remedies by exploring many untapped resources in nature.

Ancient Indian Ayurvedic system revealed that *Ephedra* is known as a soma plant. It belongs to family Ephedraceae and contains over 50 species [1]. In Traditional Chinese medicine (TCM) it is known as Ma Huang for several thousand years [2] where dried stems of *Ephedra* species are used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hey fever.

Organs used in traditional medicine are dried green stems, which are usually boiled in water and administered as hot tea [3]. Aerial parts of different *Ephedra* species contain from 0.02 to 3.4% of alkaloids concentrated in Internodes [4]. The physiological effects of *Ephedra* have been attributed to six ephedrine alkaloids which include (−)-ephedrine, (+) pdeudoephedrine, (−)-*N*-methylephedrine, (−)-*N*-methylpseudoephedrine, (−)norephedrine, (+)-norpsudoephedrine [5]. The total contents and relative amounts of ephedrine alkaloids vary from species to species of *Ephedra.* This is because of other factors such as geographical distribution, growing conditions [6, 7]. Kasahara et al. [8] found that total alkaloid content in nodes of several Ephedra species is about 40% of total concentration in internodal parts.

Tissue culture would be a good means for understanding factors responsible for cell differentiation and organ formation [9–11].

Ephedrine and pseudo ephedrine are dominant and most pharmacologically useful alkaloids isolated from the stems of *Ephedra* species although related alkaloids such as norephedrine and norpseudoephedrine [12] methylephedrine [13] and normethylpseudoephedrine [14] are also present. Bhatnagar and Singh [15] carried out organogenesis of female gametophytes of *Ephedra foliata.*

In India, high drug yielding species, namely *E. gerardiana and E. nebrodensis* grow at higher altitudes. Due to over exploitation of the plant from wild populations, *E. gerardiana* is now listed as an endangered species [16].

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Table 1 **a** Effect of Cytokinins alone on Nodal sector explants of *Ephedra foliata*. **b** Effect of Cytokinins with auxins on nodal sector explants of *Ephedra foliata*. **c** Effect of auxins on rooting of in vitro raised shoots

(a)

Cytokinins (mg/l)	Nodal segment used as explants		
	Days required for shoot initiation ± S.D.	Percent of cultures showing response ± S.D.	Average no. of shoots per explants ± S.D.
Kin			
1	25 ± 1	41.31 ± 0.6	2 ± 0.1
1.5	24.31 ± 0.57	44.22 ± 0.7	2 ± 0.2
2	24 ± 0.12	58.33 ± 0.3	3.84 ± 0.4
2.5	22.33 ± 1.15	68.08 ± 0.6	7.52 ± 0.3
3	24 ± 1	60.12 ± 0.5	5.12 ± 0.2
BAP			
1	24.66 ± 0.57	35.23 ± 0.5	2.66 ± 0.1
1.5	23.33 ± 0.64	42 ± 0.2	2.04 ± 0.3
2	21.33 ± 0.5	45.40 ± 0.4	3 ± 0.4
2.5	23.62 ± 1.15	52.13 ± 0.7	4.76 ± 0.1
3	24.31 ± 1.23	61.84 ± 0.9	1.66 ± 0.6

(b)

Cytokinin + auxins (mg/l)	Nodal segment used as explants		
	Days required for shoot initiation ± S.D.	Percent of cultures showing response ± S.D.	Average no. of shoots per explants ± S.D.
Kin 2.5			
IAA1	23.94 ± 1.15	68 ± 1.96	1.66 ± 0.5
IAA 1.5	26.25 ± 2	72 ± 1.89	4.33 ± 0.2
IAA 2	22.49 ± 0.57	57.84 ± 0.57	6.12 ± 0.7
IAA 2.5	22.46 ± 0.57	82.02 ± 1.15	9.04 ± 0.8
IAA 3	23.21 ± 1.52	62 ± 1.73	3.66 ± 0.2
Kin 2.5			
NAA 1	25.09 ± 1.5	78.07 ± 3.21	2.44 ± 0.6
NAA1.5	24.13 ± 0.57	67 ± 2.71	1.07 ± 0.7
NAA 2	25.11 ± 1.15	73.36 ± 2.52	3 ± 0.3
NAA2.5	C	C	C
NAA 3	C	C	C
BAP 2.5			
IAA 1	26.94 ± 0.83	70 ± 1.72	3 ± 0.5
IAA 1.5	23.02 ± 1.29	53 ± 1.2	1.44 ± 0.4
IAA 2	25.32 ± 0.95	62 ± 3.21	2.33 ± 0.3
IAA 2.5	26.89 ± 1.37	55 ± 3.71	1 ± 0.2
IAA 3	23.22 ± 1.29	52 ± 2.67	1 ± 0.2
BAP 2.5			
NAA 1	22.33 ± 1.96	51 ± 0.57	2.84 ± 0.3
NAA1.5	26.56 ± 1.89	64 ± 0.57	1 ± 0.5
NAA 2	23.63 ± 0.57	54 ± 2.21	1 ± 0.4
NAA2.5	C	C	C
NAA 3	C	C	C

Table 1 continued

(c)

Rooting media	Shoots producing roots (%) ± S.D.	Number of roots/shoot ± S.D.
MS + 1 mg/l NAA	21.33 ± 1.5	2.33 ± 1.24
MS + 2 mg/l NAA	23 ± 2	2.66 ± 1.15
MS + 3 mg/l NAA	32.33 ± 2.5	6.33 ± 0.57
MS + 4 mg/l NAA	25 ± 1	3.66 ± 0.57
MS + 1 mg/l IAA	12.32 ± 2.5	1.33 ± 1.52
MS + 2 mg/l IAA	15.64 ± 2.08	2.33 ± 1.52
MS + 3 mg/l IAA	16.34 ± 2.3	1.33 ± 0.63
MS + 4 mg/l IAA	13 ± 1	1.33 ± 0.57

Results are mean of 14 replicates repeated thrice ± S.D.

C Explants produced callus

Results are mean of 10 replicates repeated thrice ± S.D.

There is another species *E. foliata* which contains traces of ephedrine [17]. It is a xerophytic plant and grows under adverse soil and climatic conditions such as high light intensity and high temperature. In 1993, O'Dowd and Richardson [18] carried out in vitro micro propagation of 11 species of *Ephedra* except *E. foliata.*

The present investigation aims to study the effect of different concentrations of plant growth regulators on nodal sector explants of Male *E. foliata* plant for the induction of multiple shoots.

Explants from *E. foliata* plant growing in Botanical garden of Department of Botany, Savitribai Phule Pune University, Pune, Maharashtra (India) was used for the present investigation.

Healthy branches (with 3-4 nodes) of *E. foliata* were collected and used as source of explant. The branches were thoroughly washed with running tap water and then with distilled water. Further the stem pieces were surface sterilized using Hi Clean solution (Liquid soap) for 5 min and again washing with distilled water. Then the explants were sterilized with 0.1% mercuric chloride (w/v) for 3 min followed by thorough rinsing with sterile distilled water. Explants were dipped in alcohol for 7–10 s and fresh cuts were made before inoculation.

The Murashige and Skoog's [19] medium was used as a basal medium for culturing of explants. Basal medium was fortified with different concentrations of cytokinins; Kinetin and BAP (1–3 mg/l) alone and in combination with auxins; indole-3-acetic acid (IAA) and 1-napthaleneacetic acid (NAA) (1–3 mg/l). Cultures were maintained in the culture laboratory and were incubated in dark and light for 8 and 16 h respectively at 25 ± 2 °C and 55–60% relative humidity.

All the experiments were designed as completely randomized design with minimum 14 replicates per treatment.

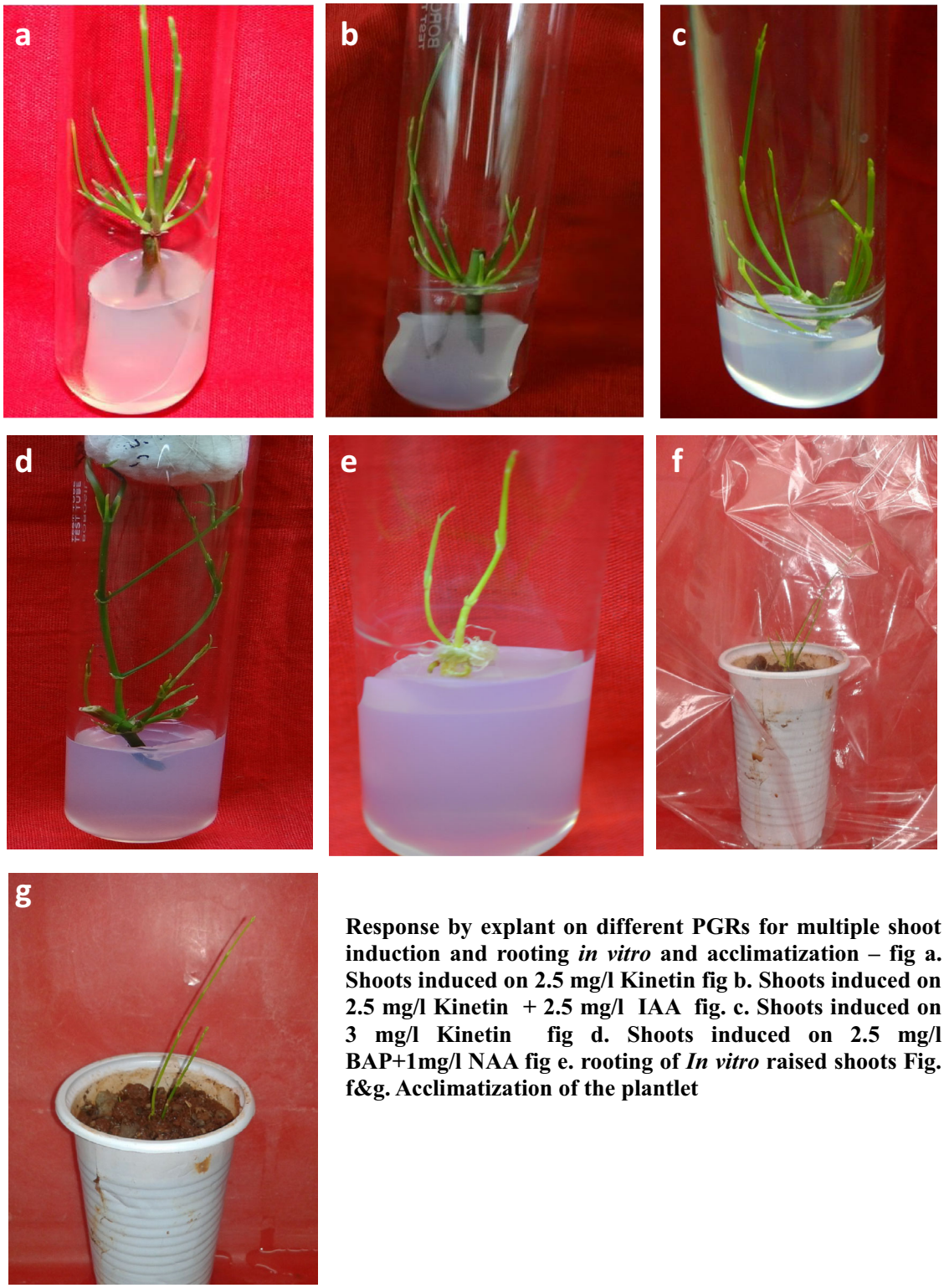
Well-developed shoots regenerated from nodal explants were excised and rooted on half and full strength MS basal medium supplemented with or without IAA, NAA slightly as well as in different concentrations (1–4 mg/l).

Plantlets with well-developed roots were removed carefully from culture tubes and washed with distilled water to remove medium sticking to the surface. Then the plantlets were dipped in 1 N aqueous solution of Bavistin for 3 min and washed with water. The treated plantlets were transferred to small plastic pots containing sterile soil: sand: Cocopeat (1:1:1). The pots were covered with plastic bags to maintain the high humidity for 16–18 days. Observations were made at the interval of 7 days and final observation was taken on 28th day.

Effect of various PGRs on nodal sector explants for multiple shoot induction was tested in the present study. Literature survey indicated that most of the work has been done on female *E. foliata* and there are no reports to show effect of PGRs on the male *E. foliata* plant making it a potential source of plant material for production of ephedrine alkaloids.

The results of effect of concentrations of Cytokinins alone on the nodal explants for the induction of shoots is given in Table 1(a).

Both Kinetin and BAP could induce shoots from nodal sector explants of *E. foliata.* However, Kinetin was found better than BAP for the multiple shoot induction. Maximum numbers of shoots (7.52 ± 0.3 shoots/explant) were observed on MS medium fortified with Kin (2.5 mg/l) in 22.33 ± 1.15 days. The response of explants for induction of shoots was also highest on the Kin (2.5 mg/l) where, 68.08 ± 0.6% explants showed shoot induction. Number of shoots decreased to 5.12 ± 0.2 and number of days for initiation increased to 24 ± 1 when concentration of kinetin was increased to 3 mg/l. Lower concentrations of kinetin (1 and 1.5 mg/l) were found to be less effective for



Response by explant on different PGRs for multiple shoot induction and rooting *in vitro* and acclimatization – fig a. Shoots induced on 2.5 mg/l Kinetin fig b. Shoots induced on 2.5 mg/l Kinetin + 2.5 mg/l IAA fig. c. Shoots induced on 3 mg/l Kinetin fig d. Shoots induced on 2.5 mg/l BAP+1mg/l NAA fig e. rooting of *In vitro* raised shoots Fig. f&g. Acclimatization of the plantlet

Fig. 1 Response by explant on different PGRs for multiple shoot induction and rooting in vitro and acclimatization—**a** Shoots induced on 2.5 mg/l Kinetin, **b** shoots induced on 2.5 mg/l Kinetin + 2.5 mg/l IAA, **c** shoots induced on 3 mg/l Kinetin, **d** shoots induced on 2.5 mg/l BAP + 1 mg/l NAA, **e** rooting of In vitro raised shoots, **f**, **g** Acclimatization of the plantlet

multiple shoot induction in *E. foliata* plant. BAP (2.5 mg/l) was found to be best concentration for shoot induction where, 4.76 ± 0.1 shoots per explants were produced but percentage of explants responded to shoot initiation was lower (52.13 ± 0.7) than that of BAP (3 mg/l) on which 61.84 ± 0.9% explants showed response for shoot initiation though the number of shoots was only 1.66 ± 0.6.

Experiments carried out by Mousavi et al. [20] found that BAP was better than kinetin for shoot multiplication in *E. procera*. Similarly, the results observed in the present investigation differs with those given by Lodha et al. [21] where basal medium used was MS medium fortified with 200 mg/l $(NH_4)_2$ SO_4; here BA was found better than Kin in terms of producing shoots per explant of *E. foliata* female plant. However, present investigation reports Kinetin as a better response for shoot induction than BAP in *E. foliata* male plant.

The results of effect of concentrations of Cytokinins (Kinetin and BAP) in combination with auxins (IAA and NAA) on the nodal explants for the induction of shoots are tabulated in Table 1(b). Different concentrations of Kinetin and BAP (2.5 mg/l) and auxins; IAA and NAA (1–3 mg/l) were tested for their effect on shoot induction and formation of multiple shoots (Fig. 1).

The most prominent response for shoot induction was observed on Kin (2.5 mg/l) with IAA (2.5 mg/l) where, 9.04 ± 0.8 shoots/explant were produced in 22.46 ± 0.57 days and percent cultures showing response was 82.02 ± 1.15. However, comparatively lower concentration of IAA (2 mg/l) with 2.5 mg/l Kinetin produced relatively less number of shoots (6.12 ± 0.7).

Similarly, Further increase in concentration of IAA (3 mg/l) reduced number of shoots to 3.66 ± 0.2. However, in case of effect of Kinetin with NAA, the lower concentrations of NAA (1–2 mg/l) induced shoots but further increase in concentration of NAA (2.5–3 mg/l) induced callus from explants. Kin(2.5 mg/l) with NAA (2 mg/l) induced 3 ± 0.3 shoots, but percent response of explants was less (73.36 ± 2.52) than that of Kin (2.5 mg/l) with NAA (1 mg/l) where response was 78.07 ± 3.21%; but number of shoots per explant was 2.44 ± 0.6.

BAP also showed better response when used in combination with IAA than with NAA. BAP (2.5 mg/l) along with IAA (1 mg/l) was most prominent where 3 ± 0.5 shoots per explant were observed, though number of days required were relatively more (26.94 ± 0.83). BAP (2.5 mg/l) when was used in combination with higher concentration of NAA (2.5–3 mg/l) showed formation of callus. According to Lodha et al. [21] NAA was less effective auxin than IAA for shoot induction.

The present study showed that NAA in higher concentration induced callus whereas IAA never induced callus when used in the same concentrations along with both Kinetin and BAP. Kinetin was more effective for induction of shoots along with IAA than with NAA.

A Lodha et al. [21] report that shoots obtained on BA together with Kin either alone or in combination with IAA show shoot tip necrosis and premature abscission of sorts, no such observations was made in the present study.

The results of rooting and acclimatization are given in Table 1(c). The in vitro raised shoots showed maximum rooting response on MS + 3 mg/l NAA on which number of roots produced was 6.33 ± 0.57 roots/shoot, on this concentration 32.33 ± 2.5% cultures showed rooting response. Least response in case of NAA was observed on MS + 1 mg/l NAA which produced 2.33 ± 1.24 roots/shoot. IAA at 2 mg/l could induce 2.33 ± 1.52 roots/shoot where response was 15.64 ± 2.08. IAA when used in higher concentrations (4 mg/l) induced less number of roots (1.33 ± 0.57 roots/shoot) and percentage of shoots producing roots was also very less (13 ± 1). Results therefore, indicated that NAA is better than IAA for rooting of in vitro raised shoots of *E. foliata*.

The male *E. foliata* is a potential medicinal gymnosperm as revealed by review of literature. The multiple shoots can be induced from nodal sector explants of male *E. foliata* plants using different concentrations of plant growth regulators. This plant species can be further exploited for secondary metabolite enhancement and commercial production of ephedrine.

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