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Somatic Embryogenesis and Plant Regeneration in *Ephedra foliata* (Boiss.); a non Coniferous Gymnosperm

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Abstract

Embryos from mature seeds of *Ephedra foliata* were excised and used as explant after longitudinal splitting. Half embryos were cultured *in vitro* to assess their morphogenic potential. When 2 - 10 μM 2,4-D with 2 - 10 μM Kn was used in MS with 10% coconut milk callus, roots and somatic embryos were induced. Lower concentration of 2 and 5 μM 2,4-D with 2 to 15 μM Kn gave best responses in terms of percentage of cultures showing somatic embryos. Greater degree of callusing was obtained in a combination of 8 - 10 μM 2,4-D with Kn, but frequency of somatic embryos was low. Substituting BAP for Kn in the medium containing 2,4-D showed almost the same response as that in 2,4-D and Kn. The percentage of cultures showing root formation was low as compared to Kn combinations. Somatic embryos developed, but with a lesser frequency. When different concentrations of NAA were added with Kn, only rooting and callusing occurred. On this medium, there was a total absence of somatic embryos. When somatic embryos were transferred to a hormone free medium, they grew within 10 - 15 days. The fully grown somatic embryos then germinated and developed into plantlets. Chromosomal count confirmed retention of ploidy level. Plantlets from somatic embryos were transferred to pots containing sterilized mixture of coarse sand and garden soil in equal proportions. They survived well and formed new nodes and internodes after nearly one month.

Introduction

Ephedra (Joint fir) is an interesting genus of the seed bearing non flowering plants belonging to the highly evolved order of Gymnosperm, Ephedrales (Bhatnagar and Moitra 1996). Ancient Indian Ayurvedic literature reveals *Ephedra* as the Soma plant and in China, it is being used as TCM (Traditional Chinese Medicine)

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and is popularly known as Ma Huang. *Ephedra* has been used for more than 5,000 years to treat conditions such as cold, fever, flu, headache, asthma, wheezing, and nasal congestion. It has also been an ingredient in many dietary supplements and used for weight loss, increased energy, and enhanced athletics performance. Due to a heavy demand and consumption, Japanese market began to search the crude drug from different species of *Ephedra* from other countries (Kakiuchi et al. 2007).

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 (Gupta and Sethi 1983). There is another species, *E. foliata*, which contains traces of ephedrine (O' Dowd et al. 1993). It is a xerophytic plant and grows under adverse soil and climatic conditions such as high light intensity and high temperature. This species can be exploited for medicinal purposes by increasing the alkaloid content through biotechnology. Present report deals with induction of somatic embryos and plant regeneration from embryonal explant of *Ephedra foliata*.

Most of the *in vitro* work carried onto *Ephedra* is related to alkaloid contents of the callus (Khanna and Uddin 1976, Uddin 1977, Ramawat and Arya 1977, 1979a, b, c, d). Female gametophyte cultures of *Ephedra foliata* for aiming the haploids has been done by Konar and Singh 1979, Singh et al. 1981, Singh and Konar 1981, Bhatnagar and Singh 1984. O'Dowd and Richardson (1993a) carried out *in vitro* micropropagation of 11 species of *Ephedra* excluding *Ephedra foliata.* None of the reports in any of *Ephedra* species described somatic embryogenesis. Survey of literature also reveals that no attempt has been made to develop an efficient protocol for *in vitro* micropropagation of *Ephedra foliata*, which contains the traces of alkaloid.

Materials and Methods

Seeds of *Ephedra foliata* were collected from plants growing in Botanical Garden of University of Delhi, Delhi. Seeds were rinsed in 0.2% solution (v/v) of Tween-20 and washed thoroughly in running tap water for 30 min. These seeds were soaked in sterile distilled water for 20 - 25 hr. Prior to inoculation, seeds were surface sterilized with 0.2% $HgCl_2$ solution (w/v) for two min and then repeatedly washed in sterile distilled water. Embryos were excised from seeds and cut into two longitudinal halves. Each half was placed horizontally with their cut surface facing the medium.

Explants were cultured onto MS having various plant growth regulators. For each treatment, a minimum of 24 cultures were raised and each experiment was repeated at least twice. The cultures were examined periodically and the morphological changes noted on the basis of visual observations. Results are

expressed as per cent cultures responded and number of somatic embryos differentiated per culture. The number was counted under a stereoscopic binocular microscope. Standard error was calculated in experiments dealing with the number of somatic embryos regenerated.

For transplantation, 6 - 10 cm long plantlets, 20 - 25 days after rooting, were transferred to plastic pots containing autoclaved coarse sand and garden soil in equal proportions and irrigated with sterile tap water. Initially the plants were covered with a small plastic chamber for 20 - 25 days for acclimatization.

For cytological studies, root tips were pretreated with a saturated solution of 1, 4-diclorobenzene for 4 hr, washed with ethanol and then used for squash preparations the same day without fixing and preserving. Before squashing, the material was hydrolysed in 1N HCl for 15 min at 60^0C followed by a thorough rinsing in water. The material was squashed in 1% acetocarmine. Only such of the metaphase plates in which the individual chromosomes were clearly visible were utilized for making counts.

Unfixed embryogenic cultures and specimens representing the early stages of somatic embryo development were stained with acetocarmine and were temporarily mounted on glass slides for observation. The material was slightly squashed or gently teased.

Results and Discussion

Embryos from mature seeds were excised aseptically, cut into two longitudinal halves and each half was placed horizontally with its cut surface facing the medium.

In order to assess the effect of 2, 4-D together with Kn on the morphogenic behaviour of embryo explants, various concentrations of 2, 4-D was chosen, and their interaction with different concentration of Kn was studied (Table 1).

At 2 μM 2,4-D, in combination with Kn (2 - 10 μM) the embryo explants enlarged approximately twice their original size within three days of culture. Subsequently, greening of explants occurred within next two days. Callus initiation was observed after eight - ten days of implantation. Callus was shiny white to yellowish green in colour. At 2 μM Kn, 60% of the cultures exhibited callusing, after three weeks. Some roots also appeared on the surface of the callus in 40% of cultures. After five weeks, 20% cultures showed roots and somatic embryos (Fig. 1A). On this medium, average number of somatic embryos was 17 per culture. These were transferred to hormone-free medium for further elongation. Increase in the Kn concentration to 5 μM caused callusing in 80% of the cultures, but the callus formation was reduced. Roots were produced in 30% cultures. Only 20% cultures showed somatic embryo formation with an average

number of 8.5 per culture. Percentage of cultures forming callus was higher (90%) when 8 μM Kn was used and all cultures showed root formation. Somatic embryos were produced in 40% cultures. On still higher concentration of Kn (10 μM) callus and root formation was observed in 75% cultures; 50% cultures showed high frequency regeneration of somatic embryos. When transferred to BM, somatic embryos did not grow further. New roots were also formed.

Table 1. Effect of 2, 4-D and Kn onto embryo culture studies of *Ephedra foliata*.

2, 4-D conc. (μM)	Kn conc. (μM)	% culture showing callusing	% culture showing rooting	% culture showing somatic embryos	Average No. of somatic embryos	Maximum No. of somatic embryos
2	2	60	40	20	17.0 ± 1.32	19
	5	80	50	20	8.5 ± 0.91	11
	8	90	90	40	14.5 ± 1.13	16
	10	75	75	50	11.5 ±4.88	21
5	2	100	20	-	-	-
	5	100	20	10	8.0 ± 0.62	11
	8	100	26	26	4.3 ± 0.41	5
	10	100	30	30	4.0 ±0.33	6
	15	100	17. 5	25	18.5 ±1.21	21
8	2	100	10	-	-	-
	5	100	20	10	4.1 ± 0. 37	5
	8	90	20	20	3. 2 ± 0. 34	4
	10	90	20	15	4 .2 ± 0.39	6
	15	80	18	-	-	-

Initial response of the explant at 2 - 15 μM was same as in the first set (1a) of experiment. Callus was initiated within two2 weeks in 100% cultures on all the combinations tried. On 2 and 5 μM Kn, root formation occurred in 20% cultures while 10% cultures on 5 μM Kn produced somatic embryos. Kinetin at 10 μM produced roots, and somatic embryos (4.0/culture) in 30% cultures. Further increase in Kn concentration (15 μM) led to a decrease in rooting (17.5%). At this concentration, 25% cultures showed somatic embryo differentiation. When callus bearing regenerated somatic embryos was transferred to the basal medium, somatic embryos grew further.

In order to study the effect of Kn in BM with 8 μM 2,4-D, Kn was added in concentrations ranging from 2 - 20 μM, the degree of callus formation was very high, and shiny, yellowish callus developed vigorously on all concentrations of Kn tried. At lower Kn concentration (2 - 5 μ M), the percentage of culture forming callus was 100 percent, but at higher Kn concentrations (8 - 20 μ M), 90 - 80% cultures formed callus after four weeks. Percentage of culture showing root formation was low (10 - 20%). Somatic embryos were produced only on medium

containing 5 to 10 μM Kn within 45 days. At 5 μM Kn, 10% of cultures differentiated somatic embryos (4.1/culture). At 8 μM Kn combination although callusing occurred in 90% cultures, 20% exhibited roots, somatic embryos. At 10 μM Kn, 15% of cultures showed an average of 4.2 somatic embryos per explant. The portion of the root which was in contact with the medium callused again. The callus with somatic embryos was transferred to BM. Somatic embryos remained stunted.

Explants callused profusely 2,4-D (10 μM) was tried with various concentrations of Kn. Rooting occurred in a few cultures (5 - 10%) grown on Kn (2 - 5 μM), however no somatic embryos were formed.

Table 2 suggests effect of 2, 4-D with BAP onto somatic embryogenesis in *Ephedra foliata*. Substituting Kn with BAP in medium containing 2, 4-D resulted in callus initiation and its growth. But the percentage of the cultures showing root formation was less as compared to Kn. BAP alone could not induce any regeneration.

Table 2. Effect of 2, 4-D and BAP onto embryo culture studies of *Ephedra foliata*.

2, 4-D conc. (μM)	BAP conc. (μM)	% culture showing callusing	% culture showing rooting	% culture showing somatic embryos	Average No. of somatic embryos	Maximum No. of somatic embryos
2	2	40	32	10	6.0 ± 0. 43	9
	5	80	30	30	7.2 ± 1.43	12
	8	80	26	20	7. 66 ± 0.69	8
	10	70	26	-	-	-
5	2	8 0	10	40	3.2 ± 0. 31	6
	5	9 0	10	45	8.6 ± 0.42	11
	8	9 0	16	50	8.0 ± 0.40	9
	10	80	16	10	3.2 ± 0.17	5
8	2	90	16	-	-	-
	5	100	16	-	-	-
	8	100	14	40	8.0 ± 1.51	14
	10	100	12	40	2. 6 ± 0. 11	4
	15	100	12	30	2.0 ± 0.16	3
	20	100	-	24	3 .2 ± 0.19	4
10	10	100	-	20	1.2 ± 0.09	3
	15	100	-	20	1.4 ± 0.13	3

When 2 - 10 μM BAP was added with 2 μM 2, 4-D in the basal medium, explant grew twice its original size within three days of inoculation. Callus was initiated within two weeks of culture but its growth was not much. Callus was shiny white to yellowish green. In all the combinations tried, percentage of

cultures showing root formation was between 25 and 32. On medium with 2 μM 2, 4-D + 2 μM BAP, 40% cultures exhibited callusing. Somatic embryos (7.2/culture) developed in 30% cultures after four weeks. At 8 μM BAP, callusing was observed in 80% cultures. Only 20% cultures formed somatic embryos (7.66/culture).

Initial response of the embryos was same as in previous set of experiment but the degree of callusing was higher. At BM + 5 μM 2,4-D + 2 μM BAP, 80% cultures showed callus formation. Root differentiation was observed in only 10% cultures. However, 40% cultures initiated somatic embryos with an average number of 3.2/culture. When 5 μM BAP was added to BM + 5 μM 2, 4-D, callus formation was observed in 90% cultures but rooting was limited to only 10%. At this concentration, 25% cultures showed regeneration of somatic embryos (8.6/culture) (Fig. 1B). On increasing the concentrations of BAP (8 - 15 μM), callusing was noticed in 80 - 90% cultures and rooting was observed in 14 - 16% cultures. At 8 μM BAP, 50% cultures differentiated somatic embryos (8.0/culture) (Fig. 1C). However, at 10 μM BAP, 10% cultures produced somatic embryos (3.2/culture).

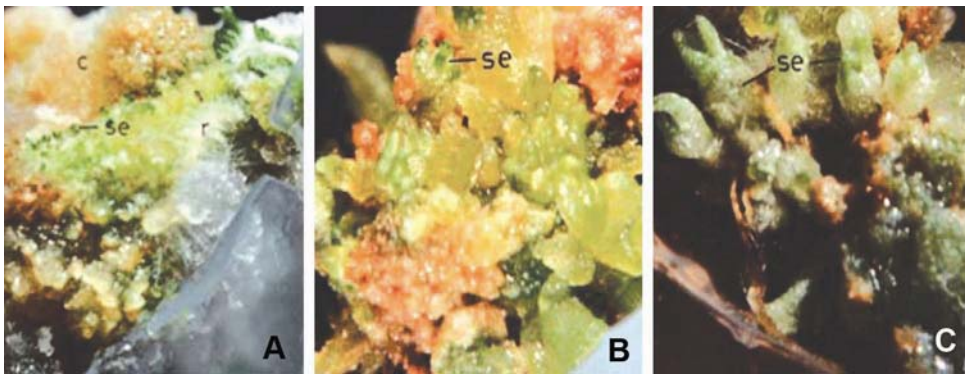

Fig. 1. A. Induction of somatic embryos onto half embryo explants of *Ephedra foliata* cultured onto BM + 2 μM 2, 4-D + 2 μM Kn after five weeks of culture. Roots and callus are also visible (× 2.4). B. Somatic embryos onto BM + 5 μM 2, 4-D +5 μM BAP after 45 days of culture (× 3.4). C. Somatic embryos produced onto BM + 5 μM 2, 4-D + 8 μM BAP after 45 days of culture (× 3.3). (c- callus, se-somatic embryos).

At 8 μM 2,4-D with various concentrations of BAP, profusely growing callus was obtained within two weeks in 90 - 100% cultures. On media containing 2 and 5 μM BAP, only roots were produced in 15% cultures. On higher concentrations of BAP, ranging from 8 - 15 μM, there was not much change in the number of cultures showing root formation. However, at 8 μM BAP, 40% cultures initiated somatic embryos (8.0/culture). A further increase in BAP concentration to 10 μM resulted in the formation of somatic embryos (2.6/culture) in 60% cultures. On still higher concentration of BAP (15 μM), there was a decline in percentage of

culture showing somatic embryos. The number of somatic embryos produced per culture was also less. No rooting was observed at 20 μM BAP. However, 24% cultures produced somatic embryos (3.2/culture).

On all concentrations of 10 μM 2,4-D with BAP, 100% cultures showed profuse callusing. On medium containing 2 - 8 μM BAP only rooting occurred in few cultures. At higher concentration of BAP (10 μM), 20% cultures formed somatic embryos (1.2/culture). Root formation occurred in only 10% cultures. Somatic embryos grew further. At 15 μM BAP, differentiation of somatic embryos was limited to only 20% cultures. However, BAP at 20 μM could not promote any regeneration.

When somatic embryos were transferred to basal medium, they grew into fully developed dicotyledonous embryos within 10 - 15 days of transfer (Fig. 2A). These somatic embryos germinated onto same medium (Fig. 2B). After three to four weeks of germination, fully developed plantlets were obtained.

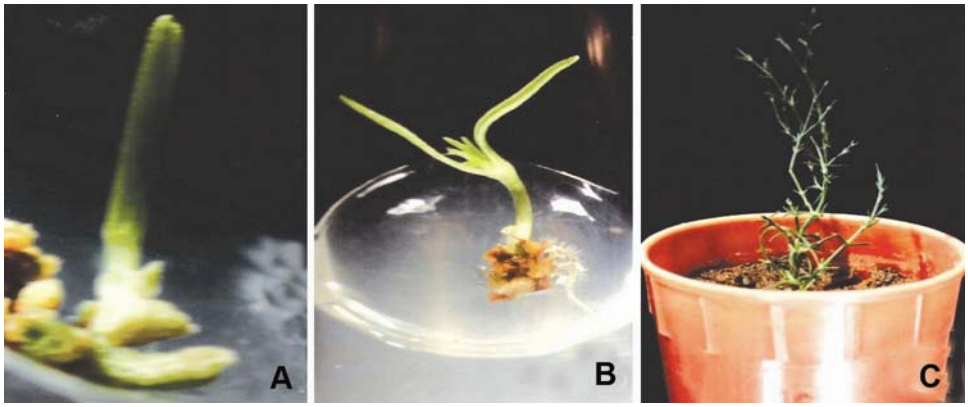

Fig 2. A-B. Different stages of germination of somatic embryos after one and three weeks of transfer, respectively onto BM (2A × 2.7, 2B × 2.0). C. Regenerated plant after six weeks of transplantation (× 0.69).

The plantlets thus produced through somatic embryogenesis were transferred to pots containing sterilized mixture of coarse sand and garden soil in equal proportion. The pots were covered with a plastic transparent box having proper space for aeration in order to facilitate hardening of the plantlets and kept at 25^0C. Nearly 83% plants survived after transplantation and reached a height of 8 - 10cm. after 20 - 25 days. At this stage, plastic box was removed and pots were kept at room temperature. Within two week, they elongated further and formed new nodes and internodes (Fig. 2C).

Squash preparations of *in vitro* differentiated roots from plantlets were used for chromosome counts. The cells were diploid (2n=14) confirming that the plants retained their ploidy level (Fig. 3A).

The rapidly proliferating embryogenic callus consisted of densly stained groups of small cytoplasmic rich cells. Some cells were elongated and other formed a cluster of cells (Fig. 3B). Embryogenesis occurred primarily by the development of these embryogenic cell clumps into precotyledonary heart and torpedo shaped somatic embryos. Provascular strands were also evident within the hypodermal region (Fig. 3C). In fully developed embryos, these strands extended from the base of leaf primordium through the hypocotyl up to the radicular end. At later stages (after nearly 40 - 45 days) the cotyledons were large and well developed; radicular end was also clearly demarcated.

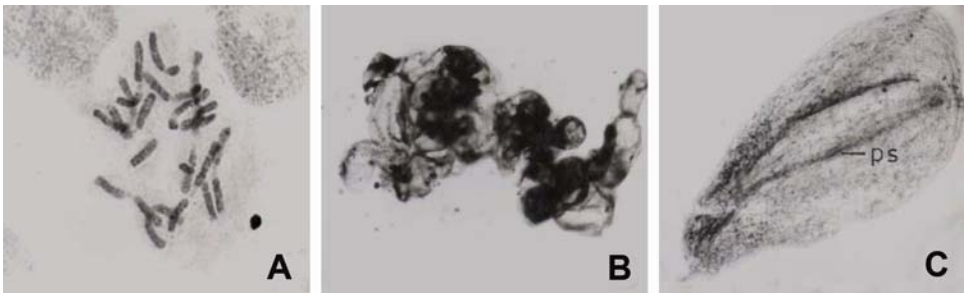

Fig. 3. A. Squash preparation from *in vitro* differentiated roots showing diploid (2n=14) number of chromosomes. B. Squash preparation of two-week-old embryogenic callus showing dense cytoplasmic cell cluster (×60). C. Precotyledonary embryo with distinct provascular strands (×15).

In gymnosperms, there are reports dealing with the culture of proembryos, immature embryos and embryonic explant as in conifers and cycads (Sommer et al. 1975, Von Arnold and Eriksson 1981, Chavez et al. 1992a,c, Rinaldi and Leva 1990, 1995, Jager and Van Staden 1996a,b). However, *Ephedra* embryo cultures studies have not been paid much attention (Sankhla et al. 1967b). Somatic tissues such as stem portion have also been neglected (O' Dowd and Richardson 1993a,b).

In case of *Ephedra gerardiana* nodal cuttings, addition of CM to the medium showed improved shoot growth (Banerjee 1991). Singh (1981) also reported improvement in the rate of shoot multiplication by addition of 10% CM. Therefore, in the present investigation also 10% CM was used for *Ephedra foliata* embryo. Cytokinin alone could bring about organogenic response in some gymnosperms such as conifers (Aitken et al. 1981, Patel and Thorpe 1984, Noh et al. 1988, Toivonen and Kartha 1988). However, in the present work, both cytokinin and auxin were found to be essential to elicit morphogenesis as in *Pinus taeda* (Mott and Amerson 1981) and *P. strobus* (Kaul 1987). Results obtained in present study show that low levels of 2,4-D (2 μM) when added with Kn (ranging between 2 and 10 μM), 60 - 90% cultures showed callusing and rooting. At higher concentration of 2,4-D (5 - 10 μM), callus formation improved

substantially to 100%, whereas only 30% cultures showed rooting. Higher concentration of 2,4-D (8 - 10 μM) with Kn had an adverse effect on somatic embryogenesis. However, 2 and 8 μM 2, 4-D led to initiation of somatic embryos in nearly all the Kn combinations. When BAP was substituted for Kn in medium containing various concentrations of 2,4-D, it was observed that although the response of callus formation was nearly the same as that with Kn, but there was a significant decrease in the percentage of cultures (0 - 30%) showing rooting in all combinations.

In the present work, a complete protocol has been developed for induction of somatic embryogenesis and plant regeneration from *Ephedra foliata*. As already mentioned, *Ephedra* is one of the source of the alkaloids mainly l-ephedrine and d-pseudoephedrine. Callus cultures can be possibly used for ephedrine biosynthesis in reactors. It may also be feasible to screen *Ephedra* populations and then clonally propagate the elite plants. Herbal extracts of certain species of *Ephedra* are known as stimulating beverage to the tribals in America and Indo-China. The development of a protocol for mass scale propagation of *E. foliata* through somatic embryogenesis opens up the prospect of using this methodology for other species of *Ephedra* which yield pharmacologically active principles.

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