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In-vitro callus induction and shoot regeneration in *Ephedra* – A medicinal Plant

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ABSTRACT

The present paper deals with In-vitro callus induction and shoot regeneration in Ephedra gerardiana from nodal explant. Ephedra gerardiana an evergreen shrub also called as Ma-Haung and in India it is called as Somlata, belongs to family Gnetaceae. It is mostly grow at higher altitudes. Ethnobotanical information showed that this plant has tremendous medicinal value for cure out different diseases. Extracts of Ephedra have been used in traditional Chinese medicine for over five thousand years to treat asthma, nose and lung congestion, hay-fever, malaria and several other ailments. The present study highlights the importance of plant tissue culture in order to be used for large-scale production of drug at cost affordable levels. This study can be utilized to develop a standard protocol to initiate multiple shoot culture of plant that may provide a good source of pharmacologically active plant constituents. Callus culture were initiated from nodal explants on Murashige and Skoog medium (MS medium) supplemented with different concentrations of hormone(s), alone or in combination, for rapid initiation of callus and biomass production. Shoot cultures were regenerated from these three explants and its generation capacity, length and morphology were observed.

Keywords: *Ephedra gerardiana*, Explant, Gnetaceae, MS medium, hormone.

INTRODUCTION

Indian is geography rich for its biodiversity of medicinal herbs [1], but these medicinal plants species decline very rapidly in last few decades [2]. After decades of serious obsession with the modern medicinal system, people have started lookng at the ancient healing system like Ayurveda, Siddha, and Unnani. This is because of the adverse effect of the synthetic drugs.

Herbal drugs play an important role in health care program, especially in developing countries. So there is a need for doing extensive research mostly on traditional medicines [3].

Ephedra gerardiana is an evergreen shrub also called as Ma-Haung [4] and in India it is called as Somlata, belonging to the family Gnetaceae. It is widely distributed in North India, West Central China, Southern Siberia, Japan, Nepal, Pakistan, Sicily, Spain and Afghanistan [5, 6, 7,8]. Several *Ephedra* species have been cultivated experimentally in various countries but commercially important species are available mostly in China, northwestern India and northern Pakistan. *Ephedra* is quite rich in species and genetic diversity with extreme morphological proliferations [9].

Amongst the *Ephedra* plants, there are ten *Ephedra* species which has been reported from North America which includes *E. trifurca* or *E. viridis*, *E. nevadensis* and *E. Americana* [10]. Hence, only 9 species (*E. ciliate*, *E. regeliana*, *E. pacyclada*, *E. wallichii*, *E. gerardiana* Syns E. *distachya*, *E. przewalskyi*, *E. procera* Syn E. *Nebrodensis*, *E. sarcocarpa*, *and E. monosperma*) were provisionally recognized from Pakistan [11]. 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 [12]. Most of the *in vitro* work carried out on *Ephedra* is related to alkaloid contents of the callus [13, 14, 15, 16, 17, 18, 19]. The chief constituents of *Ephedrine* are alkaloids viz. l-ephedrine and d-pseudoephedrine. Its active alkaloid, ephedrine, was first used in western medicine as an asthma treatment in the 1930s. *Ephedra* has been used in China to treat respiratory conditions like bronchial asthma, cold, flu edema, typhoid, cough and various pains for over 5,000 years. It has also been an ingredient in many dietary supplements, used for weight loss, increased energy, and enhanced athletics performance [20]. Medicinally the tincture is cardiac and circulatory stimulant. Liquid extract is used for controlling asthmatic attack [21]. Decoction of stem and root is considered a remedy for rheumatism and syphilis in Russia. In Pakistan and India it is used to relieve bronchial asthma [21]. Due to a heavy demand and consumption, Japanese market began to search the crude drug from different species of *Ephedra* from other countries [22]. Due to the over-exploitation of *Ephedra* for medicinal values the demand of *Ephedra* plant increasing day-by- day so few of the species of this plant are becoming endangered. So, plant tissue culture techniques were found to be one of the best methods in order to fulfill the demand of this medicinal plant.

MATERIALS AND METHODS

Plant Material

Ephedra giardiana plant parts used as explants in the present study were collected from District Knnaur (H.P.). As the *Ephedra giardiana* doesn't have leaf. So only Nodal region is used as explants for the callus establishment.

Expant Sterilization

The sterilization of nodal explants is a crucial step in micro propagation. It was done by using different chemicals for different time intervals.

In vitro establishment and multiplication of Callus

The sterilized nodal explant was cultured on agar solidified MS medium supplemented with different concentrations of combinations of NAA, BAP, Kn, IBA and 2, 4-D alone or in combination.

Callus obtained from nodal explants was used for shoot culture on MS medium supplemented with different concentrations of Kinetin and BAP. In this, 30 tubes were inoculated with desired explants i.e. nodal region for callus induction and incubated under optimal conditions like light, temperature, sucrose (carbon source), Agar (gelling agent) etc. were applied for the initiation of callus. All the cultures after inoculation were incubated in growth room at 25± 2ºC. Illumination of growth room was maintained at 1.5 lux using cool white fluorescent tubes under 16 hours photoperiod. After 30 days, the experiment was terminated and shoot generation capacity, its length and morphology were recorded.

RESULTS AND DISCUSSION

Expant Sterilization

The surface sterilization of nodal explants was carried out using Bavistin (0.2 %) and $HgCl_2$ (0.1%) respectively at varying time intervals. The data obtained from the explant of nodal segment is given in Table 1, and it reveals that the percent survival of nodal explants were highest (96.20%) with treatment T_3 (0.2% Bavistin for 10-15 min. and 0.1% $HgCl_2$ for 30 seconds) and was significantly higher than all other treatments. The least survival (16.33%) was recorded with 0.2% Bavistin for 10-15 minutes and 0.1% $HgCl_2$ for 2-3 minutes. It was also observed that there was a considerable reduction in the survival of the explants when the treatment time with the $HgCl_2$ was increased to 2-3 minutes in combination with Bavistin for 10-15 minutes. Therefore treatment of T_3 was considered best for sterilization of nodal segment and used in further experimentation. Therefore, based on the above results, it was observed that Bavistin plays a significant role in reducing contamination and increasing survivability of the explants from the nodal segments and took 10- 15 minutes with 0.2% Bavistin and 30 seconds with 0.1% $HgCl_2$ for sterilization (Table 1).

Table 1. Effect of sterilants on surface sterilization of explants of *Ephedra gerardiana*

T_1	Bavistin 0.2% $HgCl_2$ 0.1%	10-15 2-3	16.33 %
T_2	Bavistin 0.2% $HgCl_2$ 0.1%	10-15 1-2	60.17 %
T_3	Bavistin 0.2% $HgCl_2$ 0.1%	10-15 0.30	96.20 %

Callus Induction

Initially explants from nodes, were cultured on MS medium supplemented with different concentration of growth hormones, (2, 4-D, kinetin and NAA) alone or in combination for callusing. The maximum callus induction was observed in medium (MS_4) after 16 days, which was supplemented with growth regulator NAA (1.5g/l).Whereas, minimum callus induction was observed in medium (MS_3) in 7.33 days, which was supplemented with 2, 4-D (1 mg/l). In MS_2

and MS_3 the callus developed was green and friable whereas in medium MS_5 the callus developed was pale green and compact. (Table 2). (Fig.1).

Table 2. Concentrations of growth regulators used for establishment of callus culture

Medium code	plant growth regulators(mg/l)		Callus initiation time (days)	Callus quality
	2,4-D	NAA		
MS_1	0.50	--------	11.17	pale yellow, friable
MS_2	0.75	--------	8.23	pale-green, friable
MS_3	1.00	--------	7.33	green, friable
MS_4	--------	1.5	16	Brown, compact
MS_5	--------	3.5	12.33	Pale-green compact
MS_6	--------	5.0	9.0	green, compact

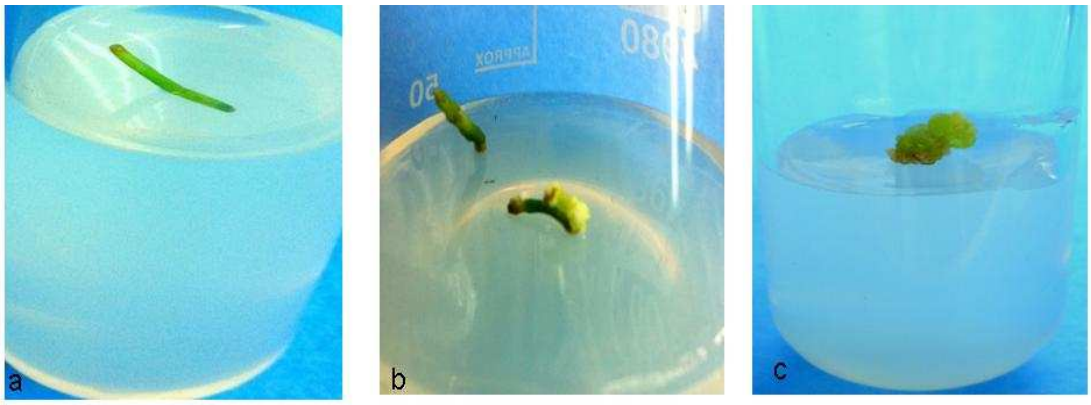

Fig. 1. Callus induction on MS4 medium containing growth regulator NAA(1.5g/l) after 1 (a), 8 (b) and 16 (c) days of incubation.

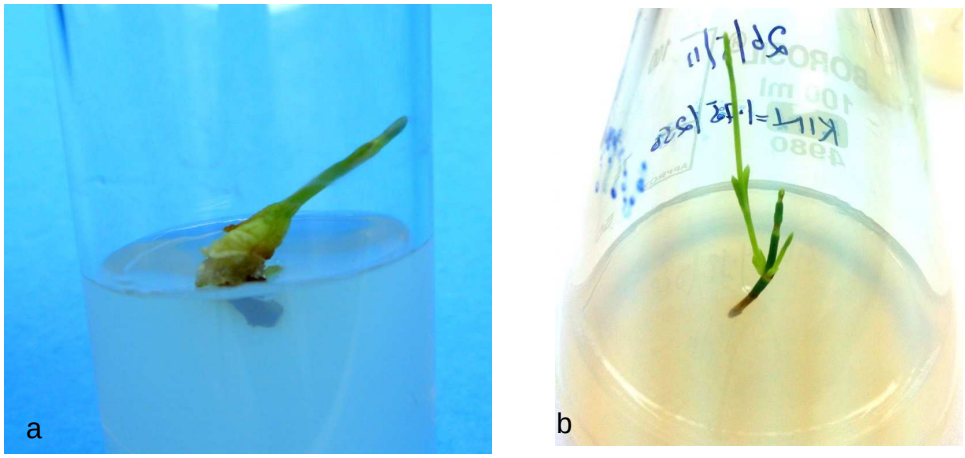

Fig. 2. Shoot multiplication on medium MM7 containing KN (7mg/l) after 10 days (a) and 30 day (b) of incubation

Shoot Regeneration

Small pieces of callus were cultured on MS medium supplemented with BAP (1 to 5mg/l) and Kn (3 to 7mg/l) alone and BAP (5mg/l) in combination with 2,4-D (1mg/l) to induce shoot buds from the callus **(**Table 3 **)**. The results reviled that the medium (MM_7) containing growth regulator Kn alone have resulted in maximum number of shoots per explant (5.40). The minimum number of shoots per explant (1.20) was observed when medium (MM_1) was supplemented with BAP (1mg/l) alone as a growth regulator. (Fig. 2). The medium (MM_7) containing 7mg/l Kn have resulted in maximum number of shoots per explant so this medium was selected for further multiplication of shoots via subculturing at an interval of two weeks.

Table3: *In vitro* multiplication of shoots from multiplied callus

Medium code	Plant growth regulators(mg/l)			Number of shoots/exp
	BAP	Kn	2,4-D	
MM_1	1.0	------	------	1.20
MM_2	3.0	------	------	1.93
MM_3	5.0	------	------	4.33
MM_4	5.0	------	1.0	3.83
MM_5	------	3.0	------	1.96
MM_6	------	5.0	------	3 .33
MM_7	------	7.0	------	5.40

DISCUSSION

Ephedra giardiana an evergreen shrub and in India it is known as Somlata, is an important medicinal plant [4]. *Ephedra* has been studied regarding micro propagation as well as secondary metabolite production in cell culture. It is used in China to treat respiratory conditions like bronchial asthma, cold, flu edema, typhoid, cough and various pains. It has also been an ingredient in many dietary supplements, used for weight loss, increased energy, and enhanced athletics performance. *Ephedra gerardiana* due to over exploitation for medicinal purposes and other has been assessed as endangered species. Also, *in vivo* propagation of *Ephedra* is slow and time consuming and also depends on season which is unable to meet the ever increasing demand. Thus, micro propagation is proposed to be an alternative for vegetative propagation, allowing for multiplication of selected genotypes and chemiotypes.

In the present investigation the nodal segments were surface sterilized with Bavistin 0.2 percent for 10-15 minutes and then sterilized with 0.1 percent $HgCl_2$ for 30 seconds following rinsing with sterile distilled water. The best sterilization of the nodal tissue of the *Ephedra* was done by sodium hypochlorite (0.5%) containing a few drops of Tween 20 for 7 min and rinsed 4-5 times with distilled water by Parsaeimehr *et al.* [23].

In *Ephedra gerardiana,* auxin mainly NAA and 2, 4-D was required for callus initiation. The results obtained from the present investigations revealed that *Ephedra* (*Ephedra giardiana)* took less number of days for callus initiation when grown in medium MS_3 supplemented with 2, 4-D (1 mg/l) as compared to the medium MS_6 supplemented with NAA (5mg/l) which took more days and the callus friability was less and more compact in comparison to 2, 4-D. It was observed that by increasing the concentration of NAA as an auxin the rate of callus induction was increased and time of induction decreased significantly. Also by subsequent increase in the

concentration of NAA the color of callus become green but the nature remained compact. The cultures established much easier when MS medium was supplemented with 1mg/l 2, 4-D. This may be due to the fact that 2, 4-D promotes the callogenesis. Explants cultured on MS medium growth regulators such as NAA also induced callus formation but callogenesis was strongly stimulated by the addition of 2, 4-D at a concentration of 1mg/L as an auxin. 2, 4-D gave a more green and friable callus whilst NAA tended to give more compact callus. These all results were also supported by Dowd *et al.* 24 and Ramawat and Arya [25], when they used stem tissues as explant. Kn played a significant role in the regeneration and multiplication of shoots in *Ephedra*. Medium supplemented with Kn was more effective in promoting shoot development than other growth regulators such as BAP+2,4-D and BAP. In the medium with KN the adventitious buds were induced on callus pieces, resulting in normal shoots. The best results were obtained at the concentration of 7mg/L Kn. Mungole et al., [26], also support this fact *in vitro* regeneration of *Physalis minima*.

CONCLUSION

From the above study, it is concluded that the callus and multiple shoot cultures of *Ephedra giardiana* were established from nodal explants on MS medium supplemented with growth regulator NAA and BAP & Kn, respectively. This study aims to develop a standard protocol to initiate multiple shoot culture and standardization of media and hormonal concentration of plant that may provide a good source of pharmacologically active plant constituents.

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