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RESEARCH ARTICLE

IN VITRO CALLUS INDUCTION STUDIES IN *EPHEDRA FOLIATA* BOISS. EX. C.A. MEY

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ABSTRACT

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years. *Ephedra foliata* Boiss. Ex. C.A. Mey is a xerophytic plant and grow sunder adverse soil and climatic conditions such as highlight intensity and high temperature. Total 5 PGRs (Plant Growth regulators) viz. IAA (indole-3-acetic acid), NAA (1-napthaleneacetic acid), 2,4D (2,4-Dichlorophenoxyaceticacid), Kinetin and BAP (6 Benzyl aminopurine) were used. NAA was found to be effective for callus yield than IAA and 2,4D when used alone whereas Kinetin was proved to be more effective in comparison with BAP and callus induction increased when NAA was used as an auxin along with kin as cytokinin.

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INTRODUCTION

Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and it is being continued to provide mankind with new remedies. Modern allopathic medicine has its roots in ancient systems of medicine and it is likely that many important new remedies will be discovered and commercialized in the future by following the leads provided by traditional knowledge and experiences. The principles of Ayurvedic medicine and the medicinal plants uses of herbs are contained in thousands of poetic hymns in the Rig Veda. In the search for alternatives for the production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2002). Callus is unorganized mass of cells usually produces on wounds of differentiated tissue and organs called explants in *in vitro* culture. The production of callus by division of differentiated tissues or excised plant cells, organs is known as Callogenesis. The cell division usually occurs in parenchymatous cell by the process of dedifferentiation. Callus is recognized as an efficient alternative for some of the intact plants for metabolite production. The main benefit of this is that, it may provide potential, renewable, year-round and reliable source for large scale production and extraction of secondary metabolites (Ramawat and Arya, 1979). *Ephedra foliata* is climbing shrub belonging to family Ephedraceae. In traditional Chinese medicines dried stems of *Ephedra* species are used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hey fever. They are being used for the treatment of arthritis, fever hives, lack of perspiration, headache, aching joints, low blood pressure (Leung and foster, 1996).

Organs used in traditional medicine are dried green stems, which are usually boiled in water and administered as hot tea (Leung, 1999). Almost all commercial products of *Ephedra* species is derived from ephedrine alkaloid. In 1993, O'Dowd and Richardson carried out *in vitro* micro propagation of 11 species of *Ephedra* except *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 (Gupta and Sethi, 1983). There is another species *E. foliata* which contains traces of ephedrine (O' Dowd *et al.,* 1993). It is axerophytic plant and grow sunder adverse soil and climatic conditions such as highlight intensity and high temperature. This species can be exploited for medicinal purposes by increasing the alkaloid content through biotechnology.

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MATERIAL AND METHODS

Chemicals

Growth regulators and antibiotics were obtained from Sigma, Chemical Co., USA. Sucrose, gelling agent – agar –agar, yeast extract and beef extract were obtained from Hi-Media, India.

Culture media

The Murashige and Skoog (1962) (MS) media was used for culturing of explants.

Plant material

Ephedra foliata plant growing in Botanical garden of Department of Botany, University of Pune was used for the present investigation. The herbarium was prepared and it was identified and authenticated by Botanical Survey of India, Western Circle, Pune – 411 001. The voucher specimen number is BSI/WRC/Tech./2014.

Surface sterilization and establishment of explants

Healthy branches (with 3-4 nodes) of *Ephedra foliata* from Botanical garden, Department of Botany 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 liquid soap (Hi Media) for 5 minutes and again washing with distilled water. Then the plants were sterilized with 0.1% mercuric chloride (w/v) for 3 minutes followed by thorough rinsing with sterile distilled water. Explants were dipped in alcohol for 7-10 seconds and fresh cuts were made before inoculation.

Before starting the process of inoculation, the platform of laminar air flow chamber was cleaned with alcohol and the UV light was switched on for 10 minutes. The autoclaved culture vessels with medium, sterilized petri plates, forceps and blade holder with surgical blade were kept in laminar air flow chamber and exposed to UV light for 15-20 min. Scalpels and forceps were flame sterilized prior to inoculation and also between the work by dipping in absolute alcohol. Surgical blades (No. 24) were used for excision of stem (~0.5-1cm) explants.

Culture conditions

All the cultures were maintained in the culture laboratory. The cultures were incubated in either dark or under 40µmol $m^{-2}s^{-1}$ light (provided by philip's cool white fluorescent tube lights) with 16 hours light period at 25±2°C. All the cultures were monitored daily and the observations were taken periodically.

Statistical analysis

Statistical methods were used for comparison of treatment means during optimization of parameters. All the experiments were setup in completely randomized design. Experiments were conducted at least three times with minimum 14 replicates per treatment. Means and standard deviation for the data were calculated and were analyzed using Microsoft Excel package.

Growth measurement of callus

After six weeks of incubation initiation, the calli obtained on all the culture treatments were harvested. Fresh weight was taken and then the calluses were dried in oven at 60°C until constant weight was obtained (Sestak, *et al.* 1971). The growth of callus was determined on the basis of dry weight.

Callus proliferation

Approximately300mg of callus induced from different explants was inoculated on MS medium containing different concentration of auxins and cytokinins alone and in combinations. The cultures were incubated in culture room at controlled temperature and photoperiod as mentioned above.

RESULTS AND DISCUSSION

Effect of Auxins alone on explants for production of callus

The results of effect of various concentrations of auxins (IAA, NAA and 2,4D) alone on internodal segment as explants are given in Table 1. The number of days required for the initiation of callus, fresh weight of callus after 6 weeks and dry weight were observed. Effect of same range of concentrations of auxin was recorded for all the three auxins tested i.e. IAA, NAA and 2,4D. The minimum number of days required were 19.88±1.73 days on NAA 2.5 mg/L whereas 28.45±1.2days was the maximum time required for callus initiation on 2,4D 3 mg/L. However, fresh and dry weight in case of NAA 1.5 mg/L was better than any other concentration of auxin used where fresh weight was 3.05±0.07gm and dry weight was 0.91±0.01 gm but number of days required were 22.84±0.48 days. 2,4D (3 mg/L) produced 1.17±0.02gm callus in terms of fresh weight and 0.3 ± 0.01 gm in terms of dry weight and number of days required for this were 28.45±1.2 days. Effect of IAA for callus induction was maximum on IAA (2 mg/L) which took **22.27±1.52** days for 2.80±0.09gm and 0.72±0.02gm as fresh and dry weight of callus respectively. Callus produced on IAA and NAA was more fragile than on 2,4D. Overall NAA was found to be effective for callus yield than IAA and 2,4D.

Table 1: Effect of Auxins alone on Internodal explants of *Ephedra foliata*

MS+Auxins (mg/L)		Callus Derived from Internodal explants		
		Callus Initiation Days after inoculation ±S.D.	Fresh wt. callus(gn ±S.D.	Dry wt. of the callus(gm) ±S.D.
IAA 2	1	23.30±0.72	2.13±0.02	0.61±0.01
IAA	1.5	22.43±0.89	2.33±0.02	0.60±0.01
	2	22.27±1.52	2.80±0.09	0.72±0.02
	2.5	24.31±0.77	1.76±0.02	0.53±0.02
	3	25.92±1.0	1.6±0.15	0.44±0.03
NAA	1	21.14±0.81	1.91±0.07	0.50±0.02
NAA	1.5	22.84±0.48	3.05±0.07	0.91±0.01
	2	20.13±0.57	2.67±0.03	0.81±0.01
	2.5	19.88±1.73	2.25±0.04	0.72±0.03
	3	23.24±1.15	2.46±0.04	0.43±0.01
2,4D 2,4D	1	25.23±0.96	1.48±0.02	0.42±0.01
	1.5	23.14±0.51	1.13±0.11	0.39±0.02
	2	23.89±0.67	1.63±0.01	0.49±0.01
	2.5	21.36±0.59	1.74±0.04	0.62±0.02
	3	28.45±1.2	1.17±0.02	0.35±0.01

Results are mean of 14 replicates repeated thrice ±S.D.
Dry weight taken after 48 hours of drying at 60° C

Fig. 1-3 . Callus grown on Auxins alone

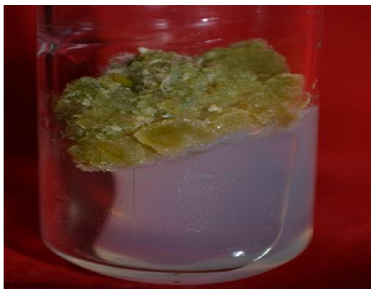

Fig.1 NAA 1.5 mg/L

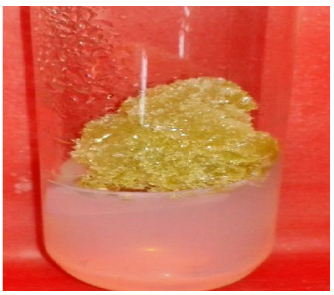

Fig. 2 IAA 2 mg/L

Fig. 3 2,4D 2.5 mg/L

Fig. 4-7 . Callus grown on Auxins along with cytokinins

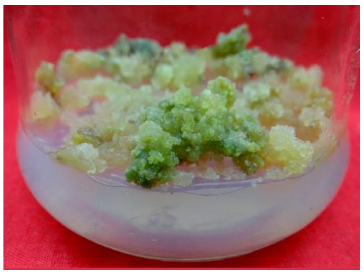

Fig. 4 IAA 2 + Kin 2 mg/L

Fig. 5 NAA 1.5 + Kin 1.5 mg/L

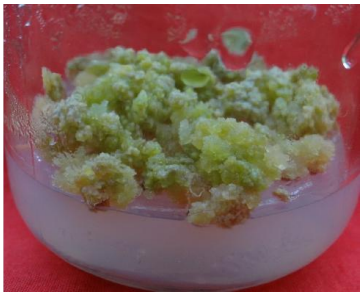

Fig. 6 NAA 1.5+ BAP1.5 mg/L

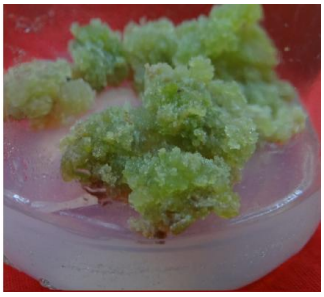

Fig 7 IAA 1.5 + BAP 1 mg/L

Fig. 8-9 Morphogenesis in callus

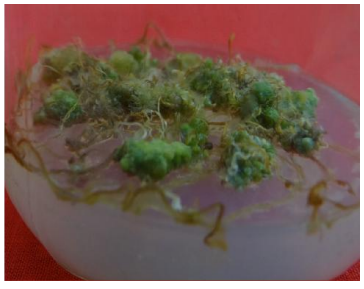

Fig. 8 . 3 mg/L NAA

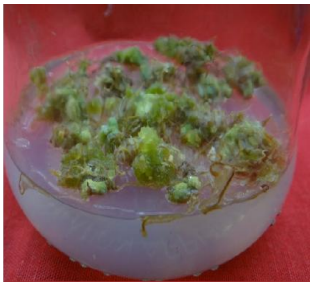

Fig 9. 2.5 mg/L NAA

Table 2: Effect of Auxins in combination with cytokinins on Internodal Explants of *Ephedra foliata*

MS+Auxins (mg/L)		Callus Derived from Internodal explants		
		Callus Initiation days after inoculation ±S.D.	Fresh wt. of callus(gm) ±S.D.	Dry wt. of the callus(gm) ±S.D.
IAA 2	Kin0.5	25.57±1.0	2.12±0.02	0.62±0.02
	Kin 1	23.48±1.52	2.34±0.01	0.65±0.01
	Kin 1.5	21.84±1.52	2.13±0.01	0.72±0.06
	Kin 2	21.84±0.57	1.80±0.01	0.59±0.05
	Kin 2.5	20.08±0.48	2.33±0.02	0.5±0.01
IAA 2	BAP0.5	24.12±0.63	1.91±0.02	0.48±0.03
	BAP 1	26.66±1.72	2.13±0.01	0.61±0.06
	BAP 1.5	24.33±1.52	1.82±0.04	0.42±0.03
	BAP 2	23.48±0.57	1.71±0.05	0.41±0.04
	BAP 2.5	25.69±0.57	1.63±0.01	0.38±0.02
NAA 1.5	Kin0.5	24.89±0.57	2.94±0.04	0.76±0.08
	Kin 1	20.94±0.52	3.28±0.05	0.97±0.04
	Kin 1.5	21.84±0.57	3.49±0.05	1.1±0.02
	Kin 2	22.57±0.63	3.11±0.04	0.95±0.01
	Kin 2.5	26.69±1.57	3.33±0.03	0.98±0.03
NAA 1.5	BAP0.5	22.47±0.57	2.42±0.02	0.66±0.04
	BAP 1	21.36±0.57	2.11±0.02	0.68±0.03
	BAP 1.5	23.78±0.63	2.70±0.02	0.8±0.01
	BAP 2	25.08±0.42	1.92±0.01	0.25±0.01
	BAP 2.5	25.12±0.42	2.12±0.01	0.35±0.02
2,4D 2.5	Kin0.5	23.33±0.52	2.00±0.01	0.42±0.02
	Kin 1	21.10±0.60	1.73±0.02	0.41±0.03
	Kin 1.5	25.33±0.58	1.98±0.04	0.48±0.01
	Kin 2	24.41±0.58	1.86±0.03	0.46±0.02
	Kin 2.5	24.66±1.72	2.24±0.02	0.56±0.04
2,4D 2.5	BAP0.5	26.33±1.57	1.47±0.01	0.32±0.03
	BAP 1	28.45±1.72	1.69±0.03	0.37±0.01
	BAP 1.5	29.21±1.72	1.56±0.03	0.40±0.03
	BAP 2	24.32±1.57	1.23±0.01	0.3±0.01
	BAP 2.5	23.86±1.52	1.24±0.01	0.3±0.01

Results are mean of 14 replicates repeated thrice ±S.D. ; Dry weight taken after 48 hours of drying at 60^{o} C

Effect of Cytokinins in Combination with Auxins on Callus production

The results of effect of concentrations of auxins (IAA, NAA and 2,4D) in combination with Cytokinins (Kinetin and BAP) on internodal segment as explants are given in Table 2. The number of days required for initiation of callus, fresh weight of callus after 6 weeks and dry weight were observed. In this case combinations the concentrations of auxins on which maximum fresh and dry weights of callus were obtained (IAA 2 mg/L; NAA 1.5 mg/L; 2,4D 2.5 mg/L) were kept constant and effect of varying concentrations of cytokinins were observed. The optimum callus was obtained on NAA 1.5 mg/L with Kin 1.5 mg/L where fresh weight of callus was 3.49±0.05 gm and dry weight was 1.1±0.02gm. However, NAA (1.5 mg/L) with BAP (1.5 mg /L) produced callus with 2.70±0.02 gm and 0.8±0.01gm as fresh and dry weight respectively.

In present investigation, dry weight of callus produced on IAA (2 mg/L) with Kin (1.5 mg/L) was 0.72±0.06 gm. 2,4D (2.5 mg/L) in combination with Kin (2.5 mg/L) could produce more callus 2.24±0.02gm as fresh weight and 0.56±0.04 as dry weight.On2, 4 D 2.5 mg/L with BAP 1.5 mg/L, results of callus obtained were 0.40±0.03 as dry weight and 1.56±0.03 as fresh weight.The results indicated that, Kinetin was proved to be more effective than BAP when used in combination with auxins for production of callus. These results are in consonant with results by Parsaeimehr *et al*. (2010) according to him NAA 1.5 mg/L with Kin 1 mg/L was best for callus induction in *Ephedra strobiliacea*, NAA 2mg/L with Kin 1mg/L for *Ephedra procera* and NAA 2mg/L with Kin 0.5mg/L was best for *Ephedra pachyclada*. In the same report by Parsaeimehr *et al*. (2010). Kin was proved to be more effective in comparison with BAP and callus induction increased when NAA was used as an auxin along with kin as cytokinin.

Morphogenesis in callus

Morphogenic response of callus depends on the growth regulators present endogenously at time of culture. The requirement of growth regulator varies from species to species and culture of explants (Narayanswamy, 1994). Regenerative ability of callus depends upon donor tissue. Cells in callus vary in their potential to regenerate because of differences in the chromosomal compliment. In the present investigation callus derived from internodal explants was cultured on Cytokinins (0.5-2.5 mg/L) alone and in combination with auxins NAA/IAA (0.2-0.5 mg/L) failed to regenerate in to organs. However NAA alone at higher concentration (>2.5mg/L) could develop roots as a morphogenic response. Such a formation of roots in case of high concentrations of NAA was found after 3 sub culturing on same concentrations of PGRs in the medium. However, IAA at higher concentrations (1.5-3 mg /L) never showed any morphogenic response.

Conclusion

Present study shows response of *Ephedra foliata* to the various PGRs. As callus provides a reliable source for extraction of secondary metabolites, these callus induction studies can be used further for cell suspension studies as well as for enhancement of secondary metabolites

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