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Callus Induction and Extraction of Ephedrine from *Ephedra alata* Decne. Cultures

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Abstract: An experiment was carried out to develop a protocol for callus induction and ephedrine production from *Ephedra alata* Decne. stem sections. The ecological conditions under which the plant naturally lives were also investigated. The highest callus induction and fresh weight of callus were shown on Murashige and Skoog (MS) medium supplemented with 1 mg/l of both 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn). The maximum ephedrine content was obtained from callus maintained on the same medium, it reached 14.06 mg/g dry weight and exceeded the ephedrine content in the stem of both wild and cultivated intact plants. Feeding the culture medium with different concentrations of L-phenylalanine (L-Phe), as a precursor, or casein hydrolysate (CH), as an elicitor, did not increase the ephedrine accumulation in callus cultures.

Key words: *Ephedra alata* · Ephedrine alkaloid · Callus · *In vitro* · Phenylalanine · Casein hydrolysate

INTRODUCTION

The genus *Ephedra* is a group of xerophytic, woody, perennial gymnosperm shrubs and the only genus in the family Ephedraceae. It is a non-flowering seed plant belonging to the order Gnetales, the closest living relative of the Angiosperms. These plants occur in dry climates over a wide area mainly in the northern hemisphere, across southern Europe, north Africa, southwest and central Asia, southwestern North America and in the southern hemisphere, in South America south to Patagonia. *Ephedra alata* Decne. is a species of *Ephedra*. It is a dioecious shrub to 1 m; stems richey branched, erect, not climbing; leaves are very short, more or less 3-6 mm; margins of leaf sheaths and bracts ciliate; male cones are arranged in dense auxiliary clusters; flowers with 4-6 anthers, distinctly stipitate; female cones 2-seeded, bracts with broad scarious margins at maturity [1]. The genus *Ephedra* is introduced as a medicinal plant [2], it has traditionally been used by indigenous people for a variety of medicinal purposes, including treatment of asthma, nose and lung congestion, hay fever, malaria, common cold and several other ailments. *Ephedra* has been used for many years in traditional Chinese medicine for over five thousand years to treat as a treatment for allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches and nasal congestion [3].

Ephedra is a source of valuable secondary metabolites such as antioxidants, antimicrobials and alkaloids but the main activity of this plant is related to its alkaloids [4, 5]. Alkaloids are the main active principles, of which is primarily ephedrine. The most principle activity of *Ephedra* extractions is in the existence of its alkaloid ephedrine [6]. Ephedrine was isolated in 1887, but it only came into extensive use during the last century. It has numerous biological activities; it is used for the relief of asthma and hay fever. Its action is more prolonged than that of adrenaline [7]. It stimulates the central nervous system and sympathetic nervous system, causes vasoconstriction; cardiac stimulation (increased cardiac output, heart rate and blood pressure). Ephedrine is also used for weight loss and it is claimed to be thermogenic [8]. In addition to pharmacological values of alkaloids, another source of interest in these compounds lies in their relevance to the mechanism of plant stress resistance [9]. The alkaloidal content of *Ephedra* varies considerably depending upon varieties, altitude, weather conditions and time of harvest. It generally contains about 0.5-2.5% of alkaloids. Of the total, ephedrine forms from 30 to 90% according to the species [10, 11]. Current advances in plant biotechnology demonstrate its potential to serve an excellent alternative to using the whole plant for isolation and extraction of secondary metabolites of commercial importance. Plant cell culture can be established from an

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impressive array of plant species, including most of those that produce secondary products of commercial interest. Production of a vital callus for certain biological activity has always been demanded by the researchers in this area of knowledge.

Many factors affect callus growth and development, the major ones are of genetic background and physiological status, the source, tissue, chemical composition and physical state of the culture medium and culture conditions. A common problem is that cultured cells produce only low levels of desired chemicals or do not produce the chemicals at all. Causes of this problem can be gene expression; pathway - regulation or precursor - availability levels. It was reported that the production level may sometimes be increased by adjusting the culture medium composition, including the salt, carbon source, growth regulators [12, 13] and vitamins or altering the culture condition. Exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. This approach is useful when the precursors are inexpensive. The concept is based on the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases; especially, amino acids [14-17]. Exogenous amino acids have proved useful in improving the yield of some cell products, particularly alkaloids. Also, elicitors are signals triggering the formation of secondary metabolites and use of elicitors has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites [18]. Biotic and abiotic elicitors which are classified on their origin are used to stimulate secondary metabolite formation in plant cell cultures, thereby reducing the process time to attain high product concentrations [19, 20]. Production of many valuable secondary metabolites using various elicitors has been reported by Mulabagal and Tsay [17], Lee and Shuler [21], Dong and Zhong [22] and Wang and Zhong [23]. Callus culture of the low alkaloid-yield *Epherda* species for the induction and elevation of these important secondary metabolites are becoming increasingly important for medicine and industry. Ephedrine producing callus cultures has been reported earlier in various *Ephedra* sp [4, 24-33].

The present study is the first study carried out to determine the yield of ephedrine in the callus tissue of *Ephedra alata*. L-Phenylalanine as a precursor and casein hydrolysate as an elicitor were examined for elevating the *in vitro* production of ephedrine in callus cultures and comparing its yield with that in the cultivated plant and the wild one. Moreover, the study was conducted to clarify the ecological conditions under which the plant naturally grown and its effect on the alkaloid content.

MATERIALS AND METHODS

Ecological Studies: The mean values of climatic factors for the studied area; Wadi El-Bagha, about 5 Km south west of Wadi Sudr, in Sinai Peninsula, were obtained from the Applied Agricultural Meteorological Laboratory, Ras Sudr Experimental Station of Desert Research Center, during the period of samples collection (2008-2009). Chemical and physical analyses of the soil supporting *E. alata* plant were also done [34].

Plant Material Collection: *Ephedra alata* Decne. actively growing shoots were collected from mature plants grown in their natural habitat (wild plant) and from El-Zohria Botanical Garden in Cairo (cultivated plant).

Callus Induction from *E. alata* and *In vitro* Production of Ephedrine

Disinfection of Plant Material: The terminal shoots of *E. alata,* obtained from El-Zohria Botanical Garden in Cairo, were washed under running tap water followed by a detergent for 5 min. The surface of stems were sterilized in 20% (v/v) commercial bleach solution (Clorox) (containing 1% sodium hypochlorite) for 20 min and rinsed 4-5 times with sterile distilled water. Subsequently, the wound sites exposed to the sterilization agent were trimmed and 0.8 ± 0.2 cm internodes were separated under a laminar flow hood.

Culture Conditions and Callus Induction: Murashige and Skoog (MS) basal medium (Duchefa, Haarlem, the Netherlands) [35] supplemented with 30 g/l sucrose and solidified with 2.5 g/l phytagel (Duchefa, Haarlem, the Netherlands) was used. A varied range of plant growth regulators (PGRs) (Sigma Cell Culture, min. 90%, St. Louis, USA); 2,4-dichlorophenoxy acetic acid (2,4-D), as an auxin, at concentrations of 1 and 2 mg/l and Kinetin (Kn) as a cytokinin at concentrations of 0.5 and 1 mg/l, were applied for callus induction and growth. The control medium free of PGRs acted as a control. The pH of the medium was adjusted to 5.7-5.8 with 0.1N HCl and 0.1N NaOH, before autoclaving at a pressure of 1.06 Kg cm^{-2} and 121°C for 15 min. Subcultures were continued every 60 days and the callus was reserved under fluorescent light (2500-3000 lux) at 25°C ± 2 with a 16 h photoperiod.

Precursor Feeding and Elicitation: An amino acid precursor; L-phenylalanine (L-Phe) (Sigma Cell Culture, St. Louis, USA) at concentrations of 12.5, 50 and 100 mg/l and an elicitor; casein hydrolysate (CH) (OXOID, OXOID Limited, Hampshire, England) at concentrations of 0.5, 1.5 and 2 g/l, were selected in order to improve the yield of ephedrine alkaloid in the callus cultures. The control medium was made without additives. Growth of the callus tissue and ephedrine content were measured. For the measurement of callus growth, callus tissues were oven dried at 35°C for 48 hours and the fresh and dry weights were recorded.

Extraction and Determination of Ephedrine: Ephedrine was extracted from 0.5 g dry powdered samples of callus and tissue of *E. alata* in 2.5 ml ethanol (HPLC grade). Acidic substances were removed by application of aliquots of plant extracts to Accell Plus QMA Sep-Pak cartridges (Waters) after dilution with an equal volume of water. Ephedrine level in the aqueous eluent was determined by HPLC (Agilent 1100 series), coupled with UV-Vis detector G1322A and G1315B DEGASSER, using a method based on that described by Barkan *et al.* [36]. Samples were chromatographed on a ZORBAX-EclipseXDB-C_{18} column (4.6 x 250 mm, particle size 5 µm) using 1% acetonitrile in 0.05 mol monobasic sodium phosphate at 1 ml/min. Substances responsible for peaks detected at 210 nm were identified by comparison of retention times and UV spectra with those of authentic compounds.

Statistical Analysis: The *in vitro* experiments were subjected to the completely randomized design. Variance analysis of data was carried out using ANOVA for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan´s multiple range test [37]. Means followed by the same letter are not significantly different.

RESULTS AND DISCUSSION

Investigation of the Ecological Conditions of Wadi El-Bagha: Wadi El-Baga, from which *E. alata* has been collected, is the third tributary of Wadi Wardan. It starts from the regional water divided with an east-west channel, which reflects downstream towards the northwest until it joins with Wardan main trunk. Total annual rainfall was 21.09 and 45.86 mm during the studied period of 2008 and 2009, respectively. The dry period extended to seven months, from April to October and the seasonal maximum temperature was recorded in July and August in both years. Relative humidity ranged between 46.6 and 76.88% during 2008 and between 46.6 and 71.62 during 2009.

Soil associated with *E. alata* at Wadi El-Bagha has loamy sand to sand loamy texture. The content of $CaCO_3$ ranged from 51.94 to 59.72%. Soil reaction (pH) was alkaline, its value ranged from 7.7 to 8. The soil extract electric conductivity (EC) showed a tendency to decrease with depth. Cl^- and Na^+ are the dominant anion and cation at upper and mid streams, respectively. Reviewing these data indicate that the climatic conditions of the studied habitat are of the arid type with high temperature especially during the dry period which extended to seven months. These conditions lead to increase the synthesis of secondary metabolites such as ephedrine to increase its capacity to tolerate theses adverse conditions. Living plants generally present various concentrations of active compounds, which involved in plant adaptation to environmental stresses, climatic changes and soil conditions [38]. Also, information about the influence of ecological conditions on the production of secondary compounds must be taken into consideration for a better production of biological active constituent [39], to select suitable habitats for the culture of these plants.

Callus Induction from *E. alata* and *In vitro* Production of Ephedrine

Callus Induction: The effect of PGRs on the callus induction of stem sections of *E. alata* is represented in table 1. No callus was induced on MS medium free of PGRs. According to preliminary studies, the selected ranges of PGRs had significant effects on the induction of pale, friable and loose callus (Fig 1). It was found that the addition of 1 mg/l Kn in combination with 2,4-D into the MS medium had prompt effect on callus formation and induced the maximum induction rate which recorded as 100%. The well grown callus were selected for subculture on MS medium supplemented with 1 mg/l 2,4-D in combination with 1 mg/l Kn, which had the maximum effect on callus induction and mean fresh weight (100% and 0.7 g, respectively). All tested *Ephedra* species could produce callus on modified MS medium supplemented

Table 1: The induction of callus from stem segments of *E. alata* using MS medium containing 2,4-D and Kn

PGRs conc. (mg/l)			
2,4-D	Kn	% of callus induction	Mean fresh weight of callus (g)
0	0	0	0
1	0.5	71.43^b	0.35^c
1	1.0	100.0^a	0.70^a
2	0.5	77.78^b	0.64^b
2	1.0	100.0^a	0.61^b

Table 2: Effect of different additives on fresh and dry weights of callus and the ephedrine content in callus of *E. alata*

L-Phe conc. (mg/l)	CH conc. (g/l)	Mean fresh weight of callus (g)	Mean dry weight of callus (g)	Ephedrine content (mg/g dry weight)	*Yield
-	-	1.40^{c}	0.11^{c}	14.06	1.547
12.5	-	5.09^{a}	0.47^{a}	3.24	1.523
50.0	-	2.49^{b}	0.21^{b}	6.52	1.369
100	-	1.64^{c}	0.09^{c}	6.22	0.559
-	0.5	1.32^{c}	0.21^{b}	9.91	2.081
-	1.5	0.91^{c}	0.16^{b}	7.64	1.222
-	2.0	2.31^{b}	0.18^{b}	1.02	0.184

*Yield = dry weight of callus x ephedrine content

Table 3: A comparison between the ephedrine yield in the intact cultivated and wild plants and callus of *E. alata*

Sample (stem)	Ephedrine content (mg/g dry weight)
Cultivated plant	0.209
Wild plant	2.923
Callus	14.06

Fig. 1: Callus of *E. alata* induced on MS medium supplemented with 1 mg/l 2,4-D and 1 mg/l Kn

with Kn and 2, 4-D or NAA [27]. Also, the highest callus induction and the highest fresh weight of *Ephedra strobilacea* callus were recorded using 1 mg/l 2,4-D combined with 1 mg/l Kn [33]. It was found that Kn as a cytokinin is more suitable than BA for callus and ephedrine production in *Ephedra procera* [4]. Previous studies showed that changing in physiological and morphological callus productions was related to primary and secondary metabolites and the auxin/cytokines ratio was always related to physiological and morphological changes and at the intermediate levels tissue grew as an undifferentiated callus [40, 41]. Auxin and cytokinins participate in regulation of cell cycle, but that auxin may regulate and even may lead to DNA replication, while cytokinin regulates event leads to mitosis which are highly correlated to phenotype changing and cells growing in *in vitro* cultures [42].

Effect of Precursor Feeding and Elicitation on Ephedrine Yield: Effect of L-Phe and CH on ephedrine yield in *E. alata* callus tissue was represented in Table 2. Callus growth did not correlate with ephedrine production on all tested treatments. The maximum fresh and dry weights of callus were recorded on the medium supplemented with 12.5 mg/l L-Phe. Comparing the effect of different concentrations of L-Phen on the callus growth, it could be noticed that the fresh weight was gradually decreased with increasing the concentration. The same observation was reported earlier, it was found that glutamic acid, tryptophane and phenylalanine inhibited the growth of callus tissue cultures of fenugreek [43]. Yield of ephedrine in callus tissue was influenced by increasing L-Phe concentration. Comparing the three tested concentrations of L-Phe, it was found that the highest ephedrine contents was recorded by using 50 and 100 mg/l. Phenylalanine appears to be the precursor of a large number of alkaloids and it is one the biosynthetic precursors of ephedrine, as it is directly incorporated into the nitrogen of ephedrine [7] and it was found to assist in maximum taxol production in *Taxus cuspidata* cultures [44]. It increased the ephedrine alkaloid accumulation in *Ephedra gerardiana* callus tissue [25]. The inability of L-Phe to further increase the alkaloid accumulation may be due to the fact that it supports the synthesis of proteins and is less effective in accumulating ephedrine in the callus tissue. Also, the tested concentrations of L-Phe may be sub- or supra-optimum for the stimulation of ephedrine content in the callus tissue.

The effect of CH (amino acids mixture) as a nitrogen source on callus growth and ephedrine accumulation was clearly shown in Table 3. The highest concentration of CH (2 mg/l) gave relatively the highest callus fresh weight comparing to the other tested concentrations. On the other hand, the maximum content of ephedrine (9.91 mg/g dry weight of callus) was recorded on the lowest concentration of CH (0.5 mg/l) and gradually decreased with the increase of CH concentration. Casein is a milk

protein and a rich source of amino nitrogen. Casein acid hydrolysate, a hydrochloric acid hydrolysate of casein has added to media primarily because of the organic nitrogen and growth factor components. The use of CH as an organic nitrogen source in the culture medium has been found to improve the growth of some tissues, such as *Taxus* spp. [45]. Addition of CH to basal media has increased the callogenesis frequency in deepwater rice [46]. Also, CH has promoted both callogenesis and alkaloid accumulation in *Catharanthus roseus* [47]. Similar response was recorded in *Stephania tetrandra* callus [48].

Among the two additives evaluated (L-Phe and CH), CH was found to be more favorable for the stimulation of bioaccumulation of ephedrine. The maximum amount of ephedrine accumulation was noticed in callus cultures grown on MS medium supplemented with 1 mg/l 2,4-D and 1 mg/l Kn without L-Phe or CH. Comparing the ephedrine content in callus tissue with that in stems of both wild and cultivated plants (Table 3), it was found that callus produced the highest content of ephedrine, it reached 14.06 mg/g dry weight of callus. This observation is in agreement with the previous studies reported that the growth regulators had an impact on the synthesis of secondary metabolites of cells culture. Table 6 also shows that the yield of ephedrine in wild plant stem exceeded that in the cultivated one, this may be due to its contribution in osmotic adjustment under stress conditions. Osmotic adjustment is considered as an important mechanism for adaptation of plants to drought. It assists in the maintenance of cell turgidity through the accumulation of solutes and may reduce the effects of water stress. In some plants, betaine, proline [49] or alkaloids function as osmolytes during stress [50]. In leaf tissues of acclimated periwinkle (*Catharanthus roseus*), polar water soluble alkaloids formed the major fraction of osmotica [51]. While, in *Ephedra alata*, ephedrine alkaloid was found to function as an osmolyte, it is partially contributes to the total osmotic potential of the plant (16%) [52]. Also, wild plants produce more secondary metabolites in nature as a defense mechanism against attack by pathogens [17].

CONCLUSION

The highest callus induction, fresh weight of callus and ephedrine yield in *E alata* callus tissue were shown on MS medium supplemented with 1 mg/l of both 2,4-D and Kn. A fairly high ephedrine callus culture of *E. alata* has been developed, which could be used as a natural source for commercial production of ephedrine. The callus may also be differentiated to produce better plant germplasm with high capacity to produce ephedrine and more tolerant to drought stress.

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