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Influences of growth regulators on callus induction, ephedrine and pseudoephedrine contents and chemical analysis of mature embryo of *Ephedra strobilacea* Bunge

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Abstract. An experiment was carried out to develop a protocol for callus induction of *Ephedra strobilacea* Bunge from embryo. Different types and concentrations of plant growth regulators were tested in order to obtain the best callus formation, and the following hormonal ranges: 2, 4-D: 1 Kin: 1 (mg L^{-1}) and 2, 4-D: 1 BA: 0.1 (mg L^{-1}) had showed the highest callus induction and the highest fresh weight, respectively. Meanwhile, ephedrine and pseudo-ephedrine contents in callus were analyzed and we found that at NAA: 1 Kin: 1 (mg L^{-1}) range the ephedrine and pseudo-ephedrine contents had significantly increased compared to the other treatments.
Key word: *Ephedra strobilacea* Bunge, tissue culture, ephedrine, pseudo-ephedrine, chemical contents.

چکیده: آ زمایشی صورت گرفت به منظورارتقاء القاء کالوس در *Ephedra strobilacea Bunge* ازمنشاء جنین بذر. تیپ و مقادیر مختلف هورمونهای گیاهی استفاده شده به منظور دسترسی به بهترین فرم نشان دادند که تیمارهایی با رنج های هورمونی (mgl^{-1} 2,4-D: 1 Kin:1 و (mgl^{-1}) 2,4-D: 1 BA:0.1 بیشترین القاء کالوس و بیشترین وزن تر کالوس را باعث میشوند. در ضمن آزمایشات نشلن داد که بیشترین میزان تولید افدرین و پسوافدرین مربوط به تیماری با رنج هورمونی (mgl^{-1})NAA: 1 Kin: 1 بوده است.
کلمات کلیدی: افدرا، تولید افدرین و پسوافدرین، کشت بافت

Резюме: Эксперимент проводился с целью развития эмбриональных каллуса индукции в *Ephedra strobilacea Bunge* Для получения лучшего каллусообразования были протестированы различные типы и концентрации регуляторов роста. Высокий каллусогенез и свежий вес был получен при использывании гормонов: 2,4-Д: 1 Кин: 1 (мгл$^{-1}$) и 2,4-Д: 1 ба: 0.1 (мгл$^{-1}$), соответственно. В эмбриогенном каллусе были исследованны эфедрин и псевдо-эфедрин и при использывании гормонов НУК: 1 Кин: 1 (мгл$^{-1}$) содержание эфедрин и псевдо-эфедрин значительно возросла.
Ключевое слово: *Ephedra strobilacea*, эфедрин, псевдоэфедрин.

Introduction. Contemporary medicinal plant serves mans life in many aspects and subjects such as antioxidant, anti-inflammatory, anti-microorganisms or even anticancer drugs are covered by them. Undoubtedly the importance of medicinal plants exists in producing their priceless, valuable secondary metabolites. *Ephedra* is a genus of gymnosperm shrubs, the only genus in the family *Ephedraceae* and order of *Ephedrales* that is introduced as a medicinal plant (Friedman 1996). 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. Interestingly, *Ephedra* is a source of valuable secondary metabolites such as antioxidant, antimicrobials and alkaloids but the main activity of this plant is related to its alkaloids, 1-ephedrin and d-pseudoephedrin (Parsaeimehr et al 2010ab; Inoko et al 2007; Ganzera et al 2005). Various *Ephedra* species have had different reacts to the ranges of phytohormones in *invitro* studies but some general outcome were always been observed.

Velichko & Plihnskaya (2008) reported that, the callus of *Ephedra monosperma,* a source for ephedrine production had an optimum growth using NAA (α-Naphthalene acetic acid) and 6-BA (6-Benzylaminopurine) in the medium, whereas a rapid growth high alkaloid content callus were obtained from treatments. They have also mentioned that, the optimal sub-cultivation period was every 30-33 days. It is reported that, the callus of *Ephedra intermedia* was initiated from the segments of aseptically seedling on MS standard medium (Murashige & Skoog medium) containing 2 mg L^{-1} 2,4-D (2,4-Dichlorophenoxyacetic acid) and 0. 5 mg L^{-1} 6-BA and subcultures continued on MS medium supplemented with 0.5 mg L^{-1} 2,4-D and 0.2 mg L^{-1} NAA and 4% sucrose. Researchers had also induced callus from zygotic embryos of *Encephalartos dyerianus* and *E. natalensis* using a B5 modified medium supplemented with different combination of 2,4-D and Kin (kinetin) (Chavez et al 1992; Chavez & Litz 1999).

Paradoxically, there are scarce reports about embryo culture of this fabulous plant while the plant is one of the endangered species. Thus, comprehensive experiments feel to analyze this medicinal plant under *in vitro* conditions. This experiment tried to find out an appropriate range of plant growth regulators (PGR-s) for callus induction from the source of *Ephedra strobilacea* embryo; to estimate the contents of ephedrine and pseudo-ephedrine in callus and to compare the chemical contents of callus with mother plant.

Material and Methods. This experiment was conducted at the laboratory of plant tissue culture research center in Agriculture Natural Resources Center of Fars, Iran.

The seeds of *Ephedra strobilacea* Bunge were obtained from the Yazd, Iran Agricultural Research Center and used in this study. First of all, the seeds were washed under running tap water for 1 hour to remove dust and dirt and then they were soaked in mild detergent solution for 5 minutes, subsequently they were washed three times with distilled water under laminar air flow cabinet and finally seeds were surface sterilized and soaked in 70% (v/v) ethanol and 5% calcium hypochlorite for 1 min and 45 minutes respectively finally seeds were transferred into a sterile Petri dish glass (8 cm diameter) for operation and consequently well separated embryos were used for callus induction. Second of all, a MS (Murashige & Skoog's 1962) basal media (pH= 5.7; containing 30% sucrose and 8% agar) was used in the experiment. All cultures were incubated at 25 ± 2**°**C under completely dark condition. The callus growth was assessed between the first and fourth week and the treatments were sub-cultured to a fresh medium every 4 weeks using the same described conditions. For determination the influence of NAA (1-Naphthaleneacetic acid) and 2, 4-D (2,4-dichloro phenoxyacetic acid) combined with BA (Benzyladenine) on callus induction the embryos were cultured in a standard MS basal media which was supplied by NAA and 2,4-D in following ranges 0.0, 0.1, 0.5, 1.0 (mg L^{-1}) along BA with 0.01 mgL^{-1}. Finally a method described by Sheu et al (2001) was used to determine ephedrine and pseudo-ephedrine contents using a HPLC device (Germany) with a Eurosphere 100-C18, 5 µm, 250 × 4mm column was used as eluent A [50mM potassium dihydrogen phosphate buffer solution (H3PO4, pH=4)] and eluent B [H20-CH3CN (3:7, v/v)] for ephedrine and pseudo-ephedrine determination. Amount of 10 mg of each product were placed in a 10 mL flask and dissolved in methanol-water 5:5 (stock solvent). Six additional calibrations were prepared by 1:2 serial dilutions with methanol-water (50:50). Standard solutions were prepared 0-200 µg mL^{-1} stored in 4ºC. Finally two equations were determined in a range between 19 to 200 µg mL^{-1} as follow y=10512x+13130 (R^2=0.9911); y=10176x-25010 (R^2=0.9904) for ephedrine and pseudo-ephedrine, respectively. The plant and callus samples which shown ephedrine and pseudo-ephedrine accumulations were chemically analyzed according to Macro elements include Nitrogen (N), Phosphorus (P), Potassium (K), Magnesium (Mg), Calcium (Ca) and Micro elements include Iron (Fe), Manganese (Mn), Zinc (Zn) and copper (Cu) concentrations. The plant samples were pre-cleaned and dried in an oven in 60 ± 2 °C and subsequently they were rubbed by an electrical device until obtaining a soft, smooth powder shape. Finally by using a CX-2 device cells were freeze-dried, for 24 h, under -45°C (condenser temperature) and the pressure of 0.10 hectopascal (hPa), after a deep rapid freezing process at -80°C. Consequently 1g of samples (plant or callus) was dry-

ashed in a porcelain crucible at 550°C for 5 hours. Subsequently the ash samples were solved in 5 mL^{-1} HCl (Hydrochloride acid) 20% in a 150 mL volume metric Erlenmeyer flask, the samples were volumed up to 100 mL^{-1} using Distilled-Deionized water. Subsequently, an atomic device were used for determining the Zn, Fe, Cu, Mn, Ca and Mg. Whereas a flame photometer device were used for analysis the K content, relatively by proportion of 1/12.5 mL^{-1} of distilled-deionized water/ extract. Meanwhile for analysis the P element 5 mL^{-1} extract were combined with H4NO3V (Ammonium metavanadate) in a 250 mL^{-1} Erlenmeyer flask until the complex showed a yellowish color. Consequently the volumes were volumed up to 25 mL^{-1} to preserve the proportion of 1/25 mL^{-1} and finally the samples were analysis at 430 nm using a spectrophotometer device. For determining the N content, 0.3 g of plant or callus were mixed with 2.5 mL^{-1} sulphuric acid plus using selenium powder, while the combination process was continued at 2 levels of 120 and 360 ºC, finally the samples were analysis using a KjelTec 2400/2460 Auto Analyzer device.

Results and Discussion. Our results indicated that, the callus inductions were started at the presence of auxin in *Ephedra strobilacea* Bunge and neither BA nor Kin could induce the callus lonely in different levels of applied cytokines. Meanwhile, our data also revealed that, the types of applied auxins had significant influences on biomasses and qualities of the callus, but, between the applied auxines, 2,4-D achieved the highest rate of callus induction and also the best callus quality in compared to NAA (Tables 1-2). Obviously, where 2, 4-D was used in the MS medium, white callus were observed and induction rates significantly increased by enhance the 2, 4-D concentrations. Our results clearly showed that, the concentration of 0.01 (mgL^{-1}) of BA by combination with 1.0 (mgL^{-1}) 2, 4-D had the great influences on callus formation where it was recorded the maximum rate of callus induction and callus fresh weight. Relatively a light compact structure callus was observed from aforementioned treatment.

Table 1

Callus induction from embryos of *E. strobilacea Bunge* in the presence of various concentrations of PGR-s

Plant growth regulators mgL^{-1}			Callus induction rate (%)	Callus fresh weight (gr)	Callus quality
BA	NAA	2,4-D			
0	0	0	---------	---------	---------
0	0	0	---------	---------	---------
0	0	0	---------	---------	---------
0.01	0.1	0	36^{b}	0.42^{c}	Yellow, semi-
0.01	0.5	0	56^{ab}	1.31^{ab}	friable
0.01	1.0	0	60^{ab}	0.91^{abc}	
0.01	0	0.1	44^{ab}	0.36^{c}	Yellow-white,
0.01	0	0.5	72^{ab}	0.56^{bc}	compact
0.01	0	1.0	88^{a}	1.56^{a}	

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Use of NAA as an auxin along BA also had significant results and the highest fresh weight by amount of 1.31 g $explant^{-1}$ among the other treatments was achieved in NAA: 0.5 and BAP: 0.01 (mg L^{-1}) range. Even though, we have received a semi-friable callus using NAA but, color of the callus turned into brown and growth of the callus were significantly decreased during the time (Table 1).

Our results also indicated that, using Kin as a cytokine along with NAA and 2, 4-D had also significant influences on callus induction rates and enhance of the callus fresh

weight. In addition, treatment supplemented 1.0 (mg L^{-1}) 2, 4-D combined with Kin at 1.0 (mg L^{-1}) were chosen as the best hormonal range for callus induction of *E. strobilacea* from embryo (Table 2). Consequently, the highest callus quantity by amount of 1.08 g $explant^{-1}$ was obtained after 4 weeks (95% of explants produced callus in the medium). Generally our results clearly showed that, 2, 4-D as an auxine had more significant influences on callus induction rate in compared to NAA. In the counterpoint, several studies reported that, NAA was the best plant growth regulator for callus induction for *Ephedra* explants (Parsaeimeher et al 2010b; Velichko & Plihnskaya 2008; O'Dowd et al 1993).

Kiong et al (2008) have also reported about callus induction of *Cycas revoluta*, where callus culture was formed on a medium supplemented with 20 µM picloram. Much earlier, Webb et al (1983) reported about callus formation of *Zamia pumila* embryo and they have mentioned that high frequency of callus initiation occurred with 1.0 mg L^{-1} NAA combined with 0.01 or 1.0 mg L^{-1} BAP, and when the concentration of NAA was higher than BAP, friable callus was produced.

Table 2

Callus induction from embryos of *E. strobilacea Bunge* in the presence of various concentrations of PGR-s

Plant growth regulators mgL^{-1}			Callus induction rate (%)	Callus fresh weight (g)	Callus quality
KIN	NAA	2,4-D			
0	0	0	---------	---------	---------
0	0	0	---------	---------	---------
0	0	0	---------	---------	---------
1.0	0.1	0	39^{b}	0.65^{b}	Green,
1.0	0.5	0	73.6^{a}	1.04^{a}	semi friable
1.0	1.0	0	83.5^{a}	0.97^{a}	
1.0	0	0.1	90^{a}	0.56^{b}	Yellow-orange,
1.0	0	0.5	75^{a}	0.61^{b}	compact
1.0	0	1.0	95^{a}	1.08^{a}	

Values followed by the same letter are not significantly different ($p \leq 0.05$).

By analyzing the plant samples we realized that, ephedrine and pseudo-ephedrine were accumulated in the stems, and correspondingly amounts of 2282.33±125.7 µg g^{-1} dry weight and 1584±162.92 µg g^{-1} dry weight were determined for ephedrine and pseudo-ephedrine, respectively. Meanwhile, we succeeded to determine ephedrine and pseudo-ephedrine accumulations in some of our treatments where Kin had been used as a cytokine. We also realized that, plant growth regulators had significant influences on callus alkaloids contents, while the highest ephedrine content was achieved in NAA:1 Kin:1 treatment where it met amounts of 64.6±11.67 µgg^{-1} dry weight and 46±1.52 µgg^{-1} dry weight of ephedrine and pseudo-ephedrine, respectively. In the counterpoint, results also indicated that, the lowest quantities of ephedrine and pseudo-ephedrine were achieved in Kin: 1 and 2, 4-D: 0.5 mg L^{-1} treatment and, amounts of 51.6± 6.5 µgg^{-1} dry weight and 38 ± 45 µgg^{-1}dry weight for ephedrine and pseudo-ephedrine were recorded, respectively (Figure 1).

By the reports, *Ephedra* plant contains about 0.5 - 2.0% of alkaloids of the total ephedrine (and its isomers) forms from 30 to 90% depending on the species (Liu et al 2009). Several factors which can influence the accumulation of secondary metabolites in callus culture have been reported by researchers but definitely the most important are: the chemical constitution of the media and the ranges of plant growth regulators (PGR) (Nawa et al 1993), carbon source and its concentration (Decendit & Merillon 1996) and nitrogen source and its concentration (Mori & Sakurai 1994). Parsaeimehr et al (2010a)

claimed that, Kin as a cytokinin was more suitable than BAP for callus induction and ephedrine and pseudo-ephedrine productions in *E. procera*. They also mentioned that, the treatments containing 2mg L^{-1} NAA with 1mg L^{-1} Kin could produce the highest cell biomass, and amounts of 108.1 ± 0.6 µg g dry $weight^{-1}$ for ephedrine and 730.3±1µg g dry $weight^{-1}$ for pseudo-ephedrine had been received in this treatment.

The chemical contents of both plant and callus are presented in Tables 3-4. As it was predictable, among the treatments which had express accumulation of ephedrine and pseudo-ephedrine, the highest amount of chemical content was received from NAA: 1. Kin: 1 (mgL^{-1}) treatment.

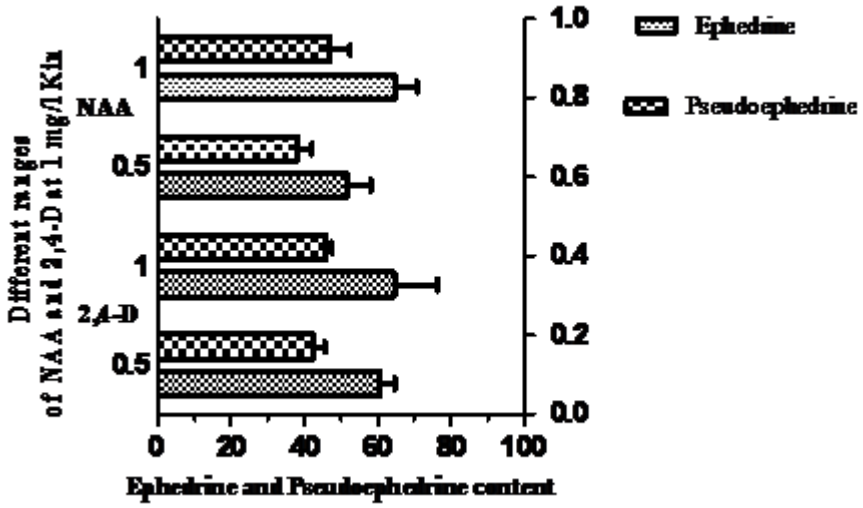

Figure 1. Influences of selected PGR-s on ephedrine and pseudoephedrine content in embryos callus of *E. strobilacea* Bunge.

Table 3

Results due chemical analysis of *E. strobilacea* Bunge plant

	Percent (%)					p.p.m			
Treatments	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
Plant	4.78	0.09	0.49	6.47	0.46	311	40	15	4

Meanwhile, we realized that the major element in our plant samples at Macro-element category was determined as calcium with quantity of 6.47% and this amount was recorded 1% for the callus at its highest level. In the counterpoint Phosphorus was determined as the lowest element in the plant while amount of 0.09 % was achieved for it. Meanwhile the highest level of Phosphorus in callus was recorded 0.21 % at NAA: 1 and Kin: 1 (mgL^{-1}) treatment.

Table 4

Results due chemical analysis of embryo callus of *E. strobilacea* Bunge at different ranges of PGR-s which shown ephedrine and pseudo-ephedrine contents

	Percent (%)					p.p.m			
Treatments	N	P	K	Ca	Mg	Fe	Mn	Zn	Cu
Callus									
NAA:1, Kin:1	5.3	0.21	2.09	1	0.14	857	438	136	3
NAA:0.5, Kin:1	3.8	0.14	1.65	0.6	0.1	532	346	78	2.3
2,4-D:1, Kin:1	5	0.17	1.72	0.8	0.12	754	411	113	3.3
2,4-D:0.5, Kin:1	3.2	0.17	0.66	0.5	0.1	635	406	122	2.6

Calcium is one of the macro elements that shapes and structuralizes the skeleton of cells in the plants, there for it was quit predictable that this element will be existed in the highest amount in compared to the other elements. According to our results, friability of callus in different treatments could be interpreted by mean of calcium usage by plant cells. Analysis of micro-element also showed significant results, as the highest amount in the plant was recorded for Iron (311 p.p.m), correspondingly the highest value for this element in the callus culture was achieved at NAA: 1, Kin: 1 (mgL^{-1}) range (857 p.p.m). In counterpoint, the lowest content was recorded for Magnesium. As amounts of 0.46 p.p.m was recorded for plant and amount of 0.1 p.p.m was recorded for callus at two ranges of NAA: 0.5, Kin: 1 and 2, 4-D: 0.5, Kin: 1 (mg L^{-1}). Interestingly, the accumulation of the Fe, Mn, Zn and Cu elements were significantly higher in all of the callus treatments in compared to their mother plant (Table 3 and Table 4). The chemical contents of plants have always been related to primary and secondary metabolites. As an instance, nitrogen and sulphur content can lead to amino acid expression one of the most necessary substances for evoke the alkaloids. Or even the chemical contents could illustrate the potential of samples due expression of flavonoids, anti-microorganism and antioxidant (Harisaranraj et al 2009; Balandrin & Klocke 1988).

Conclusion. Our results clearly revealed that, the 2, 4-D: 1; Kin: 1 (mgL^{-1}) was the best range for callus induction of *E. strobilacea* Bunge from embryo. Meanwhile, we understood that, NAA: 1 Kin: 1(mgL^{-1}) among the other treatments, showed the highest ephedrine and pseudo-ephedrine accumulations and finally the absorbance ratio of the elements could be varied under influences of different ranges of PGRs.

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