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In vitro shoot and callus induction andalkaloid contents of *Ephedraintermedia*(Schrenket) of Iran

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Abstract: *Ephedra intermedia*(Schrenket) isan evergreen shrub belongs to family Ephedraceae.The present study deals with *Invitro* callus induction, multiple shoots regeneration and alkaloid production of *E.intermedia*. Callus andmultiple shoots formation were initiated on MS medium supplementedwith different concentrations of 1-Naphthalene Acetic Acid (NAA)and Kinetin (Kn)hormonescombination. Alkaloid analysis was done by spectrophotometry, TLC, HPLC methods.Alkaloid was found in all tissue cultures and *E. intermedia* organs. Total alkaloids were higher in roots and some shoots cultures than of ephedra shoots and seeds, cultures shoots and callus. The ephedrine and pseudoephedrine were higher in ephedra roots than in ephedra shoots and seeds, all callusculturesand some shoots cultures but they balancedin roots with shoots cultures of 15 mgl^{-1} NAA supplemented with 10 mgl^{-1} Kn.This study can be utilized to initiate multiple shoots culture of *Ephedra*that may provide a good source of pharmacologically active constituents.

Keywords: Ma-Haung, Multiple Shoots, Ephedrine, Pseudoephedrine

1. Introduction

Ephedraintermedia fromEphedraceaefamily is a genus of nonfloweringseed plants belonging to the Gnetales, the closest living relativesof the Angiosperms [1].Most of the 50 *Ephedra*species worldwide [2,3] are shrubsadapted to semiarid and desert conditions [4]. 10 species of *Ephedra* are found in Iran. *E. intermedia*is an evergreen shrub also called as Ma-Haung and in Iran it is called as ormak. It is a xerophytic plant and grows under adverse soil and climatic conditions such as high light intensity and high temperature[5].Almost all commercial drugs of *Ephedra* extracts derivefrom the ephedrine and pseudoephedrine alkaloids found in the shoots in many Eurasianspecies. The best-documented drug made from *Ephedra* is Ma-huang,used in Chinese medicine, as a treatment forfever, nasal congestion, and asthma [6]. Ma-huang isalso an effective respiratory sedative and cough remedy. Herbalmixtures containing Ma-huang are sold in health food storesin the West as nutritional supplements under such names as HerbalEcstasy and Escalation accompaniedby dubious claims that they have energizing value or assistin dieting [7]. Ma-huang was traditionallyobtained from the dried stems of *E. sinica, E. equisetina,* and*E. intermedia*[8],species found in the drier regions of China, North West India,Pakistan and Iran. These plants also showed antimicrobial, antioxidant activities [9, 10, 11, 12]. Gelatin micro particle loaded with *E.gerardiana* extracts have enhanced proliferation and growth of human lung epithelial cells. This result indicating that extract could be a bioactive component that can be utilized in tissue engineering and regenerative medicine [13]. Ephedra's primary ingredients consist of the phenol and alkaloids ephedrine and pseudoephedrine[14]. The stem consists of 1-3% total alkaloids, with ephedrine comprising 30-90% of the total.This all depends upon the type of ephedra plant species. Ephedrine stimulates the central nervous system (CNS), dilates the bronchial tubes, raises blood pressure, and increases pulse rate. Pseudoephedrine, in its synthetic form, is a standard over-the-counter treatment for the relief of nasal congestion [15, 16, 17, 18]. Plant cell culture can be established from an 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 effect 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; pathwayregulation or precursoravailability levels. It was reported that the production level may sometimes be increased by adjusting the culture medium composition, including the salt [19], carbon source, growth regulators [20] and vitamins or altering the culture condition. It is suggested that the rate of callus growth and its metabolites are inversely related to its chlorophyll content besides nutrients and plant growth promote regulators that are usually supplied in cell culture [21]. Some amino acids is produced by*E.foliata* suspension cultures [22].Stems of *Ephedra* respond to infection by *Agrobacterium rhizogenes* by producing roots and tumors*in vivo* and *in vitro*. Excised tumor tissues were cultured for more than 2 years in the absence of exogenous plant-growth regulators without any deterioration in growth rate. *In vivo*tumors of *E.fragilis* and *E. minima* contained up to 0.3% dry weight *l*-ephedrine, and slow-growing *in vitro* cultured tumors of *E. fragilis* contained up to 0.01% ephedrine, but alkaloid was not detected in faster growing isolates [23]. The effects of a range of plant growth regulators on callus production in various *Ephedra* species were examined. Species examined were *E. andina, E. distachya, E. equisitina, E. fragilis*var, *camplyopoda, E. gerardiana, E. intermedia, E. major, E. minima* and *E. saxatilis*. All species produced callus on modified MS medium supplemented with 0.25 µM kinetin(Kn) and 5.0 µM 2,4-dichlorophenoxyacetic acid(2,4-D) or 1-naphthaleneacetic acid(NAA).Neither parent plants nor*in vitro* cultures of *E. distachya, E. fragilis* or *E. saxatilis* produced alkaloids. Trace quantities of 1-ephedrine and trace-0.14% dwt d-pseudoephedrine were produced by *in vitro* cultures of other species. The ability to produce alkaloid diminished to zero with successive subcultures [24]. The female gametophyte of *E.foliata*has used as an explant for the production of haploids as it is composed of haploid cells, all of the same genotype. The regeneration of roots is dependent upon the presence of NAA, while BAP had a modifying effect. Meristemoids were located on the surface or embedded in the callus tissue. The deep seated meristemoids organized only root primordia, but the peripheral ones gave rise to root as well as shoot bud primordia. Initially, there was no vascular connection between the shoot-bud and the callus [25]. *In vitro* cultures of inter nodal and nodal explants of *E. gerardiana* have produced somatic embryos and buds in MS medium containing TDZ [26].The highest callus induction and ephedrine content were shown on MS medium supplemented with2, 4-D and Kn[27] by in vitro stem culture of *E.alata. E.intermedia* can be exploited for medicinal purposes by increasing the alkaloid content through biotechnology.

2. Experimental

2.1. Plant Material and Induction of Shoots and Callus

Ephedra intermedia **(**Schrenk) plants was collected from Saveh region of central province and Tehran of Iran andidentified by central herbarium of Tehran University (herbarium number15307-THU).

*E. intermedia*were cultured by modified methods of Dixon (1987) [28].The nodal explants(2 cm segments) were uninfected by hypochlorite 5% for 45 min and were washed by sterile 0.250% tetracycline. The explants were cultured on MS agar medium[30], supplemented different concentration of 1-naphthalene acetic acid (NAA) and kinetin (Kn) treatments (Table 1) and were placed in a growth chamber at dark period for first of six weeks and 8 h dark and 12 h light period (fluorescent, cool white light, 7 W/m2) for second of 4 weeks at 25ºC.After 10 weeks; the callus and some new shoots from stem explants of *E. intermedia* used for alkaloid assay. The remain shoots in them first mediums were incubated in a growth chamberat8 h dark and 12 h light period(fluorescent, cool white light, 7 W/m2)and25ºC, for 2 years(24 months). One time of intervals of 6 months, 5 ml sterile water were added to cultures mediums without subculture.

2.2. Alkaloid Extraction

Alkaloid was extracted by boiling water (crude water alkaloid) and ethanol (crude ethanol alkaloid). 3-5 g dry weightofplant organs, callus and new shoot were homogenized in 60-100 ml boiling water or ethanol for 5 minand were set in bathroom 60ºC for 60 min. Following filtrated, crude ethanol alkaloids were extracted purified according modified method as described byDixon (1987) [28]. For alkaloid isolation, ethanol extracts were dried by vacuum evaporator at 60ºC and used. Alkaloids isolation stages from crud water and ethanol extracts were: 1. Acidic phase was isolated by sulfuric acid (5%) and diethyl ether (50/50; v/v) in a decanter. 2. Acidic phase separated and made basic (pH 10) with 10 N NaOH and extracted with 60 - 100 ml chloroform in decanter. 3. Chloroform phase were concentrated by vacuum evaporator at 60ºC. The obtained alkaloids extracts were dissolved in 1 ml methanol used for HPLC alkaloids assay. Before injection to HPLC device, all samples for HPLC were filtered through 0.45 *µm* nylon membrane filters (Sigma-Aldrich).

2.3. Alkaloids Assay

The existence and content of total alkaloids, ephedrine and pseudoephedrine of intact plant organs and in *vitro* callus and shoots of*E. intermedia* analyzed by spectrophotometry, TLC and HPLC.

2.4. Total Alkaloid

Amount of 10 mg of ephedrine and pseudoephedrine alkaloid standards were placed in a 10 ml flask and dissolved in 10 ml ethanol. Six additional calibrations were prepared by 1:2 serial dilutions with ethanol-water (50:50). Standard solutions were prepared 0 -200 µg ml^{-1}. Total alkaloids were measured at 254 nm by spectrophotometry (UV- Visible Shimatzu) method.

2.5. Thin Layer Chromatography (TLC)

TLC did according to modified methods of Morris et al. as described by Dixon (1987) [28]. 200µl of crude alkaloids of plants organs, callus and shoots of different treatments were applied on to TLC plates. TLC solvent systems routinely used was water: ethanol (1:3) and TLC plates were 0.2 mm thick silica gel aluminum backed plates. Alkaloids were identified using TLC and Dragendorff Reagent. The mobility and characters of each of alkaloids analyzed by TLC were compared with ephedrine and pseudoephedrine alkaloid standards. The standards were from Caspian Tamine Drugs Company of Iran and 0.0025 mg from each of standard alkaloids were dissolved in 1ml ethanol and used for alkaloids assay.

2.6. High Performance Liquid Chromatography(HPLC)

HPLC was performed with a Shimatzu LC-6A chromatography system using a programmed a phosphate gradient elution system described by Sheu et al. (2001) [29] was used as eluent A [50 mM potassium dihydrogen phosphate buffer solution (H3PO4, pH = 4)] and eluent B [H20-CH3CN (3:7 v/v)] for ephedrine and pseudoephedrine determination on a reversed-phase column was µm bond pack C18, and 4.6 µm (15 cm × 2.5 mm, I.D) maintained at 30°C. A guard column with 37-50 µm bond pack C18/CO reversed-phase protected the column against contamination from tissue extracts. In HPLC analysis, alkaloids were detected by retention time at 254 nm.In HPLC analyses, for obtaining carefully results, the 20 µl alkaloids were injected three times.

3. Statistical Analysis

Statistical significance of differences was determined by one-way analysis of variance (ANOVA) followed by Duncan's post-hoc analysis. The data were presented as the mean ± standard error mean (SEM). Difference was considered significant at $p < 0.05$.

4. Results

4.1. Shoot and Callus Induction

Shoot induction: After 10 weeks incubation, callus and shoots were producedby nodalexplantsof *E. intermedia*in the presence 0.5,2.5, 5, 10 and 15mgl^{-1} NAA supplementedwith 0.5,2.5, 5, 10 and 20 mgl^{-1}Kn. New shoots regenerated in the some mediaincludeKn=0.5 with NAA=10,15, Kn=2.5 with NAA=0.5,5,10, Kn=5 with NAA=10, Kn=10 with NAA=0.5,10 and Kn=20 with NAA=10mgl^{-1}(Table1). The highest shoots regeneration found in mediacontaining15mgl^{-1}NAA supplemented with 0.5, 2.5, 5, 10 and20 mgl^{-1}Knand 0.5 and 10mgl^{-1} NAA with 2.5mgl^{-1}Kn (Figure 1b) after 10 weeks(Figure 1 a).The medium contain 10 mgl^{-1} NAA supplemented with2.5 mgl^{-1} Kn was the best organogenesis treatment. In this medium, all explants produced 3,4,5 new shoots (Figure1b).The explants without subculture grew in growth chamber and they are produced some new shoots and callus after 6 -24month (Figure 1c-e).

Figure 1. *Shoots and multiple shoots regeneration and growth and Callus induction of Ephedra shoot explants: Shoot regeneration after 10 weeks: (a) Two new shoots from stem nod in MS medium supplemented with Kn = 10 mgl^{-1} and NAA =0.5 mgl^{-1}. (b) Multi new shoots from stem nod in MS medium supplemented with Kn = 2.5 mgl^{-1} and NAA = 10 mgl^{-1}. multiple shoots regeneration and Growth: Multiple shoots growth and regenerated from stems nods in MS medium supplemented with Kn = 2.5 mgl^{-1}, NAA = 10 mgl^{-1}, after 6, 12, 18 , 24 months: (c) after 6 months. (d) after 12 months.(e) after 24 months(2 years). Callus induction: (f) Callus produced from stems nods in MS medium supplemented with Kn = 5 mgl^{-1}, NAA =5 mgl^{-1}. (g) Callusproduced stems nods in MS medium supplemented with Kn = 10 mgl^{-1}, NAA = 0.5 mgl^{-1}*

Table 1.*Shoot (Sh) and Callus (Ca) production from stem explants of ephedra in NAA supplemented with Kntreatments*

N.	Kn(mgl^{-1})	NAA(mgl^{-1})	%Ca[1]	%Sh[2]	Callus Quality[3]	Shoot number
1	0.5	0.5	75b	0g	b. wbr.a.	-
2	0.5	5	50c	0g	s.w.a.	-
3	0.5	10	25e	25e	s.w.a.	1,1,1
4	0.5	15	0g	100a	-	1,2,2
5	2.5	0.5	0g	100a	-	1,1,1
6	2.5	5	33d	33d	s.w.	1,2,1
7	2.5	10	0g	100a	-	3,4,5
8	2.5	15	33d	100a	s.w.	-
9	5	0.5	5f	0g	s.w.	-
10	5	5	100a	0g	b,wbr.a.	-
11	5	10	50c	37d	b,w.a	1,2,5
12	5	15	0g	100a	-	-
13	10	0.5	75b	25e	b.wb.	1,1,2
14	10	5	0g	0g	-	-
15	10	10	0g	0g	-	1,1,1
16	10	15	0g	100a	-	-
17	20	0.5	0g	0g	-	-
18	20	5	0g	0g	-	-
19	20	10	0g	0g	-	1,1,2
20	20	15	0g	100a	-	-

[1]Ca= Callus,[2]Sh= Shoot,[3](b= big, s=small, br=brown, w=white, a= aggregate)

Calluses induction: Calluses were produced in some treatments but in5 mgl^{-1}NAA with 5 mgl^{-1}Knwith 100% callus genesis and 0.5 mgl^{-1}NAA with 0.5 and 10 mgl^{-1}Kn; with75% callus genesis were the higher than other treatments(Table1 and Figure 1f,g). In some treatments didn't produce callus. Calluses in all treatments were white and white- brown but in some mediums were big and in some other were small (Table1).

4.2. Alkaloid Assay Results

a- Total Alkaloid

Purified alkaloid was extracted from organs of wild ephedra organs and explants in different treatments (Table 2). The total alkaloid content of ephedra shoots, seeds and roots were1.29, 3.22 and 4.82%.Roots had the highest alkaloid contents. There was significant difference between alkaloid contents ofthe ephedra organs. The level of total alkaloids of all treatments explants were low(0.15- 2.61%) but in medium containing 15 mgl^{-1} of NAA supplemented 10 mgl^{-1} of Kn with multiple shootsregeneration,alkaloid contents were more than of ephedra seeds and shoots. In all treatments containing 20 mgl^{-1} of Kn with and without shoots regeneration, total alkaloids were more than plant shoots (Table 2).

Table 2. *Total alkaloid (T.Alk), Ephedrine (E) and Pseudoephedrine (PE) in ephedra organs and explants in different concentration of Kn and NAA*

N.	Kn(mgl^{-1})	NAA(mgl^{-1})	%T.Alk***	%PE	%E
1	0.5	0.5	0.15 ±0.024	0.07 ±0.002	0.04 ±0.001
2	0.5	5	0.20 ±0.023	0.14 ±0.003	0.05 ±0.002
3	0.5	10	0.22 ±0.021	0.14 ±0.000	0.57 ±0.003
4	0.5	15	0.10 ±0.015	0.08 ±0.003	0.00±00.000
5	2.5	0.5	0.08 ±0.002	0.05 ±0.001	0.00±00.000
6	2.5	5	0.44 ±0.023	0.23 ±0.002	0.13 ±0.002
7	2.5	10	0.42 ±0.025	0.21 ±0.001	0.13 ±0.002
8	2.5	15	0.36 ±0.024	0.21 ±0.004	0.07 ±0.003
9	5	0.5	0.09 ±0.022	0.05 ±0.001	0.01 ±0.002
10	5	5	0.15 ±0.026	0.07 ±0.000	0.04 ±0.001
11	5	10	0.50 ±0.025	0.30 ±0.005	0.12 ±0.003
12	5	15	0.18 ±0.024	0.10 ±0.001	0.03 ±0.001
13	10	0.5	0.50 ±0.001	0.30 ±0.001	0.10 ±0.002
14	10	5	0.40 ±0.021	0.24 ±0.002	0.08 ±0.001
15	10	10	0.45 ±0.003	0.29 ±0.001	0.11 ±0.008
16	10	15	3.53 ±.015	1.97 ±0.05	0.90 ±0.008
17	20	0.5	2.58 ±0.020	1.45 ±0.008	1.01 ±0.003
18	20	5	2.43 ±0.039	1.2 ±0.009	0.9 ±0.002
19	20	10	2.58 ±0.022	1.32 ±0.003	0.83 ±0.003
20	20	15	2.61 ±0.015	1.3 ±.0007	0.99 ±0.004
Root	-	-	4.82 ±0.016	1.93 ±0.001	0.92 ±0.001
Shoot	-	-	1.29 ±0.034	0.65 ±0.002	0.45 ±0.008
Seed	-	-	3.22 ±0.017	1.22 ±0.004	0.89 ±0.004

b- Ephedrine and Pseudoephedrine

- Crude water alkaloid and crude ethanol alkaloid of plants organs, callus and shoots of different treatments were compared by TLC analysis. Result of TLC showed the pattern of ephedrine and pseudoephedrinefrom ethanoland only pseudoephedrine fromwater extracts(Figure 2), were similar to the pattern of TLC analysis of standards ephedrine and pseudoephedrine.

- HPLC analysis of purified alkaloid extract of plants organs, callus and shoots culturesshowed 5- 8alkaloids peaks. The HPLC retention time of each of alkaloids were compared with ephedrine andpseudoephedrine standards.The sequence of retention time of ephedrine and pseudoephedrine were 8.8 and 9.50 minutes (Figure 2).Ephedrine and pseudoephedrine exist in some samplesbut in plant organs and treatments of containing 15 mgl^{-1} NAA supplemented 10 mgl^{-1} Kn with shoot regeneration and in all treatments containing 20 mgl^{-1} Kn with and without shoots regeneration were more than other treatments (Table 2). Pseudoephedrinein plant roots and in treatment containing 10 mgl^{-1} Kn supplemented with 15 mgl^{-1} NAA, was 0.05- 1.97%.Ephedrine in plant roots and seeds, and in samples oftreatments containing 10 and 20 mgl^{-1} Kn supplemented 15 mgl^{-1}NAA with shoot induction, 20 mgl^{-1}Kn supplemented 0.5, 5 and 10 mgl^{-1} NAA without callus and organogenesis was 0-1.01% and was higher than other treatments(table 1, 2). There were some treatments with 100% shoots induction(containing 0.5 and 2.5 mgl^{-1}Kn supplemented 15 and 0.5 mgl^{-1} NAA) but none ephedrine and little pseudoephedrine (table 1, 2).

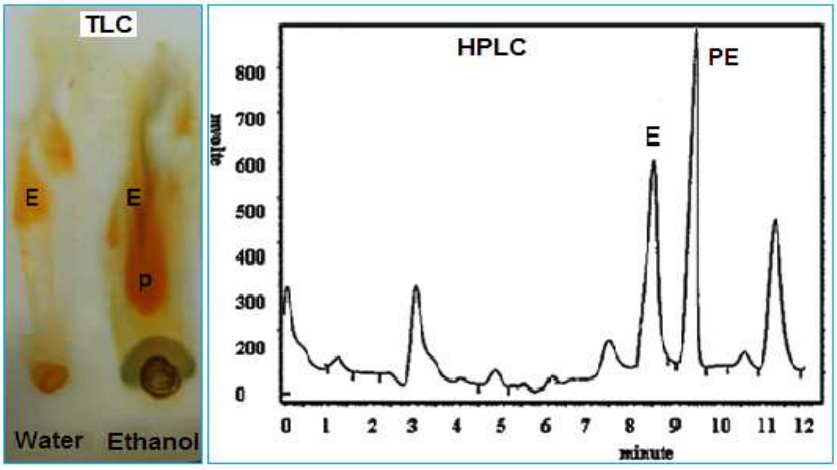

Figure 2.*Thin Layer Chromatography (TLC)and High Performance Liquid Chromatography(HPLC)analysis of Ephedra alkaloids:TLC: Ephedrine (E) and Pseudoephedrine (P) found in TLC plate of water and ethanol extract of ephedra shoot after Dragendorff Reagent spray.HPLC: HPLC analysis of the alkaloid extract of Ephedra explants with shoots emerging in the medium containing 15 mgl^{-1} of NAA supplemented with 20 mgl^{-1} of Kn: ephedrine (E)retention time was 8.8 minand pseudoephedrine (PE) retention time was 9.50 min.*

5. Discussion

Cytokinins and auxins could bring about organogenesis response in some gymnosperms such as conifers [31, 32]. Cytokinin and auxin were used to be essential to elicit morphogenesis as in *Pinustaeda*and *P. strobus.*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 in *P. taeda*[33] and *P. strobus*[34]. The callus, roots and somatic embryos were induced from half embryos of mature seeds of *E.foliata*were cultured inMS medium with 2 - 10 µM 2, 4-D supplemented with 2 - 10 µMKnand 10% coconut milk[35]. *In vitra* culture of *E.gerardiana*nodal cuttings, addition of coconut milk to the medium has improved shoot growth and improvement in the rate of shoot multiplication by addition of 10% coconut milk [36]. *E. foliate* embryogenesis was increased by 10% coconut milk [37,38]. The embryo genic callus has produced using of *Iphionamucrona* seedling cultured for nine months on MS medium supplemented with NAA.Transfer of developed embryos to MS medium supplemented with Kn induced shoot formation [39]. The highest callus has produced from nodal culture of*E.strobilacea*in MSmedium containing2, 4-D supplemented with Kn or BA butthe ephedrine and pseudo-ephedrine contents have increased significantly in MS medium *containing*NAA supplemented with Kn, compared to 2, 4-D supplemented with Kn or BA treatments[40].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[41].Therefore, in the present investigation also NAA with Kn was used for *E. intermedia* shoots and multiple shootsregenration.After 10 weeks incubation, callus and shoots were produced from shoot explants of *E. intermedia*but in the medium contain 10 mgl^{-1}NAA supplemented with 2.5 mgl^{-1}Kn was the best organogenesis treatment for multiple shoots regeneration. In this medium, all explant produced 3, 4, 5 new shoots. Also support this fact *in vitro* regeneration of some *Ephedra* species reported.Explants obtained from seedlings of *E.*

intermedia were cultured on media containing different combinations of plant growth regulators. The results indicated that MS medium supplemented with 2 mgl^{-1} 2, 4-D and 1 mgl^{-1} 6-BA was the best for callus induction, with the highest induction frequency from hypocotyls [42].In *E.gerardiana,* auxin mainly NAA and 2, 4-D was required for callus initiation. 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. These all results were also supported by O'Dowd and Richardson (1993) [23] and O'Dowd et al. (1998) [43], when they used stem tissues as explant.The highest callus induced in MS medium supplemented with 1mg/l of both 2, 4-D and Kn. The casein hydro lysate in medium, didn't ephedrine accumulation in callus culture [27]. 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 explants and its generation capacity, length and morphology were observed [44].In the present investigation,calluses were produced in some treatments by NAA with Knbut in5 $mgl^{-1}$of NAA with 5 $mgl^{-1}$of Kn(100% callus genesis) and 0.5 $mgl^{-1}$of NAA with 0.5 and 10 $mgl^{-1}$of Kn(75%) were the higher than other treatments. Also support this fact *in vitro* callus induction of some *Ephedra* species reported.*Ephedra* is one of the source of the alkaloids mainly l-ephedrine andd-pseudoephedrine. Alkaloid components were found in callus and cell suspension culture[42].The ephedra shoots consists of 1-3% total alkaloids, with ephedrine comprising 30-90% of the total. This all depends upon the type of ephedra plant species[15, 16, and 17]. The concentration of ephedrine alkaloids can vary from 0.02 to 3.40% in the aerial parts of the Ma-Huang [45].*E. intermedia*contained ephedrine alkaloids,althoughvariation in content was observed. The total content ofephedrine alkaloids varied from 0.46% to 1.81 %[46]. The treatments containing 2 mg l^{-1} NAA with 1 mg l^{-1}kn can produce the highest callus (68%), ephedrine (0.0108%) and pseudoephedrine (0.0730%) from *E.procera*explants culture [47]. In the present study,total alkaloid content of ephedra shoots, seeds and roots of *E. intermedia*sequenced 1.29, 3.22 and 4.82%. Roots had the highest alkaloid contents. There was significant difference between alkaloid contents of ephedra organs. The level of total alkaloids of all treatments explants were low(0.15-2.61%) but in medium containing 15 mgl^{-1} NAA in combination 10 mg/l Kn with multiple shoots regeneration, alkaloid contents were more than of ephedra seeds and shoots. In all treatments containing 20 mgl^{-1}Kn with and without shoots regeneration, total alkaloids were more than plant shoots.High level of Kn was effective factors for *in vitro* alkaloid increasing in *E. intermedia*explants. Maximum bud (90 %) from*in vitro* culture of nodal explants of *E. foliata* has obtained on MS medium supplemented with 1.5 mg l^{-1} of benzyl adenine (BA). Explant produced 5.3 shoots from single node with 3.25 cm length [48].The tissue cultures of *Ephedra foliata* has doneon MS medium containing 0.5 mg l−1 each of 2, 4-D and Kn. Maintained callus cultures exhibited regeneration potential and maximum number of shoots with an average height has achieved on MS medium containing combination of 0.25 mg l^{-1} each of Kn, BA and 0.1 mg l^{-1} of NAA. Highest alkaloid content has recorded in callus culture on MS medium having 0.5 mg l^{-1} each of 2, 4-D and Kn, 100 mg l^{-1} l-phenylalanine and 5 mg l^{-1}IBA [49].

Ephedrine and pseudoephedrine existed in some cultures of *E. intermedia* but in plant organs and treatments with shoots regeneration and in all treatments containing 20 mg/l Kn with and without shoots regeneration were more than other treatments. Pseudoephedrine (0.05- 1.97%) in plant roots and in treatment containing 10 mgl^{-1}Kn supplemented with 15 mgl^{-1}NAA, and ephedrine (0- 1.01%) in plant roots and seeds, and in treatments containing 10 and 20mgl^{-1}Kn supplemented with 15 mgl^{-1}NAA with shoot induction, 20 mgl^{-1}Kn supplemented 0.5, 5 and 10 mgl^{-1} NAA without callus and other organogenesis were higher than other treatments. There were some treatments with 100% shoots induction but none ephedrine and little pseudoephedrine. Also support this fact *in vitro* callus induction of some *Ephedra* species reported [49].

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