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Original Contribution

FACTORS AFFECTING THE SHOOT MULTIPLICATION OF *EPHEDRA PROCERA* IN IRAN

B. Mousavi[1,2*], Y. A. Saadat[2], M. Solouki[3]

[1]Previous Master student at the Field of Plant Biotechnology, Zabol University, Iran
[2]Fars Research Centre for Agriculture and Natural Resources- Shiraz, Iran.
[3]Department of Biotechnology Science, University of Zabol, Iran.

ABSTRACT
A number of experiments were conducted to identify suitable procedure for *in vitro* shoot multiplication of *Ephedra procera*. Two different nutrient media (MS, DCR) and two different explants (Axillary and axial buds) were studied in first experiment. Murashige and Skoog (MS) medium was found optimum. Per formance of explants was better on MS medium than on DCR, axillary buds was significantly better than axial buds in whole measured of indexes. In another experiment different concentration of BA were studied. Medium containing 0.05 mgl^{-1} BA and 0.01 mgl^{-1} IBA was the best, although medium containing 0.15 and 0.3 was also successful. Different concentrations of Kinetin were studied. Medium containing 0.1 mgl^{-1} Kinetin and 0.01 mgl^{-1} IBA was the best, in all of indexes. In general explants did not grow in concentrations 0.3 and 0.4 mgl^{-1} Kinetin. In general BA was better than Kinetin for shoot multiplication of *Ephedra procera*.

Key words: *Ephedra procera*; Nutrient medium; Explants; Shoot multiplication.

INTRODUCTION

Ephedra botanically belongs to chelamidosperm class (advanced gymnosperms with several angiosperm characteristics). *Ephedra*, also known as ma huang, belong to the family Ephedraceae that is an evolutionarily primitive plant family. *Ephedra* species favor dry, sandy or rocky environments, and are found in the temperate and subtropical regions of China, Mongolia, India, parts of the Mediteranean and Afghanistan as well as regions of North and Central America (1); the genus *Ephedra* consists of more than 50 species (2), *Ephedra* species are short, evergreen and almost leafless shrubs that grow about 60 to 90 cm high (23.5 to 35.5 inches high). The stems are green in color, slender, erect or reclining, small ribbed and channeled, about 1.5 mm in diameter and usually terminating in a sharp point. Nodes are 4 to 6 cm apart, and small triangular leaves appear at the stem nodes. The nodes are characteristically reddish brown. The stems usually branch form the base. They bear minute, yellow-green flowers and fruits, and emit a strong pine-like odor and have an astringent taste. The six optically active alkaloids that have been isolated from *Ephedra* species are (-)-ephedrine, (+)-pseudoephedrine, (-)-*N*-methylephedrine, (+)-*N*-methylpseudoephedrine, (-)-norephedrine, (+)-norpseudoephedrine. Usually, (-)- ephedrine is the major isomer that comprises 30 to 90 percent of total alkaloid fraction accompanied by (+)-pseudoephedrine, with trace amount of other ephedrine type alkaloids (1). In traditional Chinese medicines, dried stems of *Ephedra* species are used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hay fever. They were also used for a treatment of arthritis, fever, hives, lack of perspiration, headache, aching joints and bones, wheezing, and low blood pressure (3). Besides their medicinal value, *Ephedra* species have potential as both indoor and outdoor foliage plants. *Ephedra* species are traditionally propagated by seed or vegetatively by dividing old plants (4-5). Conventional plant-breeding

***Correspondence to**:*Badrossadat Mousavi, badri.mousavi@gmail.com, Tel.:+98-711-7204959, Fax:+98-711-7205107.*

methods can improve both agronomic and medicinal traits. *In vitro* propagation for the production of high-quality plant-based medicines. This can be achieved through different methods including micropropagation (6). The use of conventional cutting of *Ephedra* gives poor results (7). Micropropagation offers the opportunity for rapidly increasing elite strains of special horticultural value or those which give high yields of alkaloids. It can also speed up the production of plants from conventional hybridization or mutagenesis (8). Clonal propagation by organogenesis is a tissue culture preparation method. It allows for *in vitro* regeneration of conifers, and is an efficient tool for mass production of genetically improved material. However, seed production in natural stands is limited. Therefore, there is a need to develop an alternative propagation method.

MATERIAL AND METHODS

Plant materials

Shoots of *Ephedra procera* were collected from shrubs in a natural stand located on area of Qanaby and Meimand in Fars, Shiraz, Iran. The explants consisted of fresh green offshoots harvested from the new growth on mature branches as well as from basal sprouts. These were collected at march, 2007. All culture media were adjusted to pH 5.7 before autoclaving and sterilized by autoclave at 121°C and 1.0 kg cm^{-2} pressure for 20 min. All media were solidified with 8.5 g l^{-1} agar. Cultures were grown at 27 ± 1ºC in a 16h photoperiod with fluorescent light (75 µmol m^{-2} s^{-1}). Plant stems were surface sterilized by 70% ethanol for 1 min, then treated with 4% Sodium hypoclorite solution for 20 min. These steps were followed by three rinses with sterile distilled water in a laminar air flow hood.

In this study, five jars were used for each experimental treatment and each jar was inoculated with five explants. All statistical analyses were performed using the programme on SAS (9). Mean comparisons were performed by Duncan' s studentised range test. Results were analyzed by the method of analysis of variance (ANOVA).

Experiment 1

In vitro propagated of *Ephedra procera* from shoots of many elite tree from collection of Iran. All treatments contained 0.05 mgl^{-1} BA, 0.01 mgl^{-1} IBA. The experiment was laid out as a factorial experiment with two different nutrient media (MS and DCR) as factor A and two different explants (axillary and axial buds) as factor B. Treatments were arranged in a completely randomized design with 5 replications, with each replication containing five explants. All culture were transferred (the same) fresh medium after 4 weeks, and then after 5 weeks data were collected.

Experiment 2

In vitro propagated axillary buds of *Ephedra procera* were used as explants. The experiment was laid out as a completely randomized design with seven treatments including seven levels of BA (0.05, 0.1, 0.15, 0.2, 0.3, 0.5 and 1.0 mgl^{-1}). All media were supplemented MS with 0.01 mgl^{-1} IBA. Each treatment consisted of five replications, with each replication containing five explants. All cultures were transferred to the same fresh media after 4 weeks and then after 1 month data were collected.

Experiment 3

In vitro propagated shoot with axillary buds of *Ephedra procera* were used as explants. MS medium was used as basal medium. The experiment was laid out as a completely randomized design with five treatments including five levels of Kinetin (0.02, 0.1, 0.2, 0.3 and 0.4 mgl^{-1}), All media were MS supplemented with 0.01 mgl^{-1} IBA. Each treatment consisted of five replications, with each replication containing five explants.

RESULTS AND DISCUSSION

Experiment 1

Results of this experiment inducted that a MS medium with axillary buds were best for shoot multiplication of *Ephedra procera*. The MS and DCR media were significantly different for all the chracterstics **(Table 1).** Axillary buds were significantly better than axial buds. The best results for percent of growing explants, shoot length, shoot fresh weight and number of axillary shoots was observed on MS medium supplemented axillary buds **(Table 1)**. This was significantly better than DCR medium with axial buds. Vitrutually non growing explant on DCR with axial buds, DCR was very poor medium for this species.

Among media tested in this experiment MS medium with axillary buds produced the great growth in all the characteristics and DCR with axial buds produced the smallest growth. The

interaction of nutrient media and explants was not significantly different for number of buds per explant, but a significant interaction was observed between them for percent of explant growth shoot length, shoot fresh weight and a number of axillary shoots per replication differently at each explant **(Table 1).**

Cultures on DCR media with axillary buds had a lower growth rate and showed chlorosis.

Table 1. *Responses of Ephedra procera to different basal nutrient and explants*

Nutrient medium	% of explant growth	Average no. of buds per explant	Average shoot length (cm)	Average shoot fresh weight (g)	Average no. of axillary shoots per replication
MS	68.00[a]	2.31[a]	2.91[a]	0.07[a]	9.20[a]
DCR	12.00[b]	1.00[b]	0.95[b]	0.02[b]	1.30[b]
Explants					
Axillary shoot	66.00[a]	2.76[a]	2.59[a]	0.07[a]	9.30[a]
Axial shoot	22.00[b]	0.55[b]	1.27[b]	0.02[b]	1.20[b]
Interaction	P<0.05	NS	P<0.01	P<0.05	P<0.01

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

Experiment 2

This experiment was conducted to find the optimum concentration of BA for shoot multiplication of *Ephedra procera*. **Table 2** clearly shows that the greatest percent of growing explants was obtained on medium containing 0.3 mgl^{-1} BA and this was significantly different from treatments containing 0.15, 0.5 and 1.0 mgl^{-1} but this was no significantly differences with other treatments. The treatment with 0.05 mgl^{-1} BA produced maximum main shoot lengths and was significantly different from treatments containing 0.3, 0.5 and 1.0 mgl^{-1} BA, but was no significantly differences with other treatments. Explants cultured on medium containing 0.05 mgl^{-1} BA were significantly different from treatments containing 0.3, 0.5 and 1.0 mgl^{-1} BA for shoot fresh weight. There was no significant difference between a medium containing 0.05 mgl^{-1} BA and the other treatments containing 0.1, 0.15 and 0.2 mgl^{-1} BA. Explants cultured on a medium containing 0.05 and 0.15 mgl^{-1} BA produced the greatest number of axillary shoots and showed a significant difference from treatments containing 0.5 and 1.0 mgl^{-1} BA, but there were no significant differences with other treatments.

Table 2. *In vitro growth of Ephedra procera explants on different concentrations of BA (all treatments containing 0.01 mg l^{-1} IBA).*

BA concentration (mg l^{-1})	% of explant survival	% of explant growth	Average no. of buds per explant	Average shoot length (cm)	Average shoot fresh weight (g)	Average no. of axillary shoots per replication
0.05	100[a]	88[bc]	3.52[ab]	3.28[a]	0.11[a]	16.00[a]
0.1	83[ab]	93.33[c]	2.90[bc]	3.04[abc]	0.08[ab]	11.00[ab]
0.15	96[a]	72[ab]	4.52[a]	3.22[ab]	0.09[ab]	15.00[a]
0.2	90[ab]	90[bc]	3.19[ab]	2.94[abc]	0.09[ab]	11.5[ab]
0.3	100[a]	96[c]	2.72[bc]	2.67[bcd]	0.06[bc]	13.20[ab]
0.5	72[b]	64[a]	1.50[c]	2.53[cd]	0.04[c]	2.80[c]
1.5	87[ab]	73[ab]	2.70[bc]	2.28[d]	0.06[bc]	7.17[bc]

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

Experiment 3

In this experiment, the effect of five different concentrations of Kinetin in combination with 0.01 mgl^{-1} IBA on shoot multiplication of *Ephedra procera* were studied. The greatest percent of explants survival was obtained on medium containing 0.1 mgl^{-1} Kinetin and this was significantly different from treatments containing 0.02, 0.3 and 0.4 mgl^{-1} but this was no significantly differences with 0.2 mgl^{-1} Kinetin.

The treatment with 0.1 mgl^{-1} Kinetin produced maximum main shoot lengths and was significantly different from treatment containing 0.2 mgl^{-1}, but was no significantly differences with 0.02 mgl^{-1} Kinetin. Explants cultured on medium containing 0.1 mgl^{-1} Kinetin were significantly different from treatment 0.02 mgl^{-1} Kinetin for shoot fresh weight. There was a significant difference for number of axillary shoots between the treatment containing 0.1 mgl^{-1} Kinetin with other treatments.

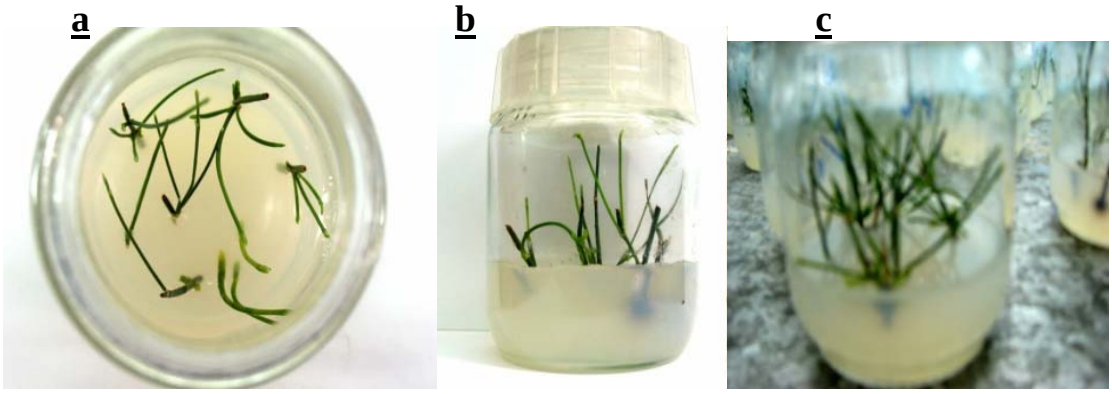

Figure 1. *Shoot multiplication* of *Ephedra procera* (a) Shoot proliferation from nodal explants after 4 weeks ; (b) growth axillary buds explants cultured on modified MS medium containing 0.05 mg l^{-1} BA and 0.01 mg l^{-1} IBA; (c) shoot multiplication in *Ephedra procera*.

DISCUSSION

Results of this study indicated that Axillary buds were significantly better than axial buds. The best results for percent of growing explants, shoot length, shoot fresh weight and number of axillary shoots was observed on MS medium supplemented axillary buds **(Table 1)**. This was significantly better than DCR medium with axial buds. Vitrutually non growing explant on DCR with axial buds, DCR was very poor medium for this species. Many studies have demonstrated that MS medium was a suitable medium for *Ephedra* was confirmed (4-10-11-12-13-14). Literature reviews (15-16-17) indicated that DCR medium in orders of gymnosperms was superior to MS for shoot multiplication that in contrast with the results in this experiment. Generally resting axial meristems of *Ephedra* cause to stimulate and develop into axillary buds that confirms the results reported by the other experts (18-19). In this study, in general, it seems that the treatment containing 0.05 mgl^{-1} BA and 0.01 mgl^{-1} IBA is a suitable medium for length, fresh weight and number of axillary shoots **(Table 2)**, the treatment 0.5 mgl^{-1} BA and 0.01 mgl^{-1} IBA is not a suitable for all of characteristics. Media containing 1.0 mgl^{-1} is not good for shoot elongation, because by increasing the concentration of BA, the main shoot lengths decreased. Many experts reported that kinetin was the best cytokinin for shoot multiplication that is in contrast with this results (8-20-21-22). In this study on high concentrations of BA, after 3-4 subcultures, many shoots began to appear pale and slightly swollen above the nodes and confirms the results reported by O' Dowd and Richardson (8). In general, the treatment containing 0.1 mgl^{-1} kinetin and 0.01 mgl^{-1} IBA is the most suitable medium in whole measured of indexes. The best concentration of kinetin for shoot multiplication of *Ephedra procera* was kinetin at 0.1 mgl^{-1} **(Table 3)**. This confirms the results reported by O' Dowd and Richardson (8). The maximum main shoot lengths was 0.1 mgl^{-1} kinetin and in contrast with the results of O' Dowd and Richardson (8).

Table 3. *In vitro growth of Ephedra procera explants on different concentrations of kinetin (all treatments containing 0.01 mg l^{-1} IBA)*

Kinetin concentration **(mg l^{-1})**	% of explant survival	Average shoot length (cm)	Average shoot fresh weight (g)	Average no. of axillary shoots per explant
0.02	12.00[b]	0.36[ab]	0.02[b]	0.60[b]
0.1	32.00[a]	0.68[a]	0.05[a]	1.80[a]
0.2	16.00[ab]	0.30[b]	0.03[ab]	0.60[b]
0.3	0.00[b]	0.00	0.00	0.00
0.4	0.00[b]	0.00	0.00	0.00

Values followed by different letters are significantly different at P<0.05 according to LSD test.

CONCLUSION

The best results for percent of growing explants, shoot length, shoot fresh weight and number of axillary shoots was observed on MS medium supplemented axillary buds **(Table 1)**. MS medium with axillary buds supplemented 0.05 mgl^{-1} BA and 0.01 mg^{-1} IBA is the optimum for shoot multiplication **(Table 2)**. In general BA was better than kinetin for shoot multiplication of *Ephedra procera*.

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Abbreviations: BA– 6-benzyladenine; DCR – Gupta and Durzan, 1985; IBA– indole-3-butyric acid; MS–Murashige and Skoog, 1962.

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