Journal of Agricultural Technology 2012 Vol. 8(5): 1839-1854
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

In vitro plantlet regeneration from cotyledonary node explants of *Salvadora persica* L. a medicinally important desert tree

Suman Kumari and Narender Singh*

Department of Botany, Kurukshetra University, Kurukshetra, Haryana, India

Suman Kumari and Narender Singh (2012) *In vitro* plantlet regeneration from cotyledonary node explants of *Salvadora persica* L. a medicinally important desert tree. Journal of Agricultural Technology 8(5): 1839-1854.

An efficient and reproducible *in vitro* propagation protocol has been developed for rapid micropropagation of *Salvadora persica* L. a medicinally and economically important desert tree. The present investigations were carried out with an aim for the development of a tissue culture method for the clonal propagation of this tree. The seeds were germinated on MS half strength medium devoid of growth regulators. The cotyledonary node explants were excised from twenty days old seedlings and inoculated on MS medium supplemented with different concentrations (0.25-2.0 mg l^{-1}) of auxins (NAA, IAA and 2,4-D) and cytokinins (BAP & Kinetin) individually as well as in various combinations. The frequency of shoot regeneration from cotyledonary node was affected by various concentrations of auxins, cytokinins and successive transfer of mother explant. Maximum numbers of shoots (17.5±0.02) were obtained on MS medium containing BAP (2.0 mg l^{-1}) in combination with IAA (0.5 mg l^{-1}). The *in vitro* raised shoots (>3cm long) were excised aseptically and implanted on MS half and full strength medium fortified with auxins (IAA, NAA and IBA) at the concentrations of 0.5, 1.0 and 2.0 mg l^{-1} for root formation. Maximum per cent roots induction and length was obtained on MS full strength medium supplemented with 1.0 mg l^{-1} IAA. Seventy per cent plantlets were successfully established in earthen pots containing soil and sand mixture (3:1). The plantlets were then transferred to the field conditions. The regenerated plants were morphologically uniform and exhibited similar growth characteristics.

Key words: Cotyledonary node, *Salvadora persica*, Micropropagation, *in vitro*.

Abbreviations: BAP-6-benzylaminopurine, Kn-kinetin, IAA-Indole-3-acetic acid, IBA-indole-3-butyric acid, NAA-α-naphthalene acetic acid, 2, 4-D-2,4-Dichlorophenoxyacetic acid.

Introduction

Salvadora persica L. (family Salvadoraceae), commonly known as tooth brush tree, is a branched evergreen small tree. It is distributed mainly in tropical regions of Asia and Africa. This species has wide adaptability from sand dunes

* Corresponding author: Narender Singh; e-mail: nsheorankuk@yahoo.com

of deserts to heavy soils, non-saline to highly saline soils and dry regions to and waterlogged areas (Rao *et al*., 1999).The tree is able to tolerate very dry environment with mean annual rainfall of less than 200 mm.

The plant contains several biologically active compounds such as alkaloids, flavonoides, steroids, volatile oils, tarpenoides, saponins, and carbohydrates (Kamil *et al*., 1999; Abdillahi *et al*., 2010). Traditionally, the twigs of this plant are widely used as toothbrush in the Middle East, Africa and India to relieve toothache. Leaves are used as mouthwash and also used as purgatives for curing asthma and cough. Roots of this plant were found to contain Salvadourea, which is a urea derivates (Al-Quran, 2008). It also contain a fairly large amount of alkaloidal constituents including trimethyleamine (Nadkarny, 1954) which has a stimulating effect on the gums and sulphur like compounds lend antibacterial activity (Watt and Breyer-Brandwijk, 1962). Ezmirly *et al.* (1979) reported that its roots indicated anti-inflammatory, anti-bacterial and hypoglycemic activities. Seeds contain non edible oil that has over 50% lauric and myristic acids, which is used as an alternative source of oil in soap industries (Makwana *et al*., 1988). *Salvadora persica* is one of the important species noticed with high salt tolerance and drought resistance and can be used to reclaim salt effected black soils and it also act as an effective soil binder (Zodape and Indusekhar,1997; Sher *et al*., 2004).

The exhaustive use of the plant, low viability and poor germination of seeds has contributed the endangered status of the plant in this region. The seeds lose their germination viability soon after harvesting. Micropropagation, which is often used successfully for the multiplication of several woody plants, represents an interesting alternative for this species. Considering the benefits of tissue culture, we focused on the *in vitro* study of plant growth regulators to establish a protocol for clonal propagation of this species from cotyledonary segments because it avoids the chance of somaclonal variation. We anticipated that this research work will lead to conservation and exchange of germplasm, and commercial plant production. Some preliminary work on micropropagation of this species has been done by a few workers (Mathur *et al.*, 2002, 2008; Phulwaria *et al*., 2011). But efforts are still needed to make the protocol more practical and reproducible. The present communication has been designed with an aim to develop high frequency regeneration protocol from cotyledonary node explants.

Materials and methods

Explants source

Mature fruits of *Salvadora persica* were collected from an old tree growing at Hisar which is a study site of Central Soil Salinity Research Institute, Karnal. After removing the pulp of the fruits, the seeds were washed under running tap water for 15 - 20 minutes and dried under shade for 5-6 days and then used as a source material for the present studies. These seeds were immersed in an aqueous solution of liquid detergent for 15 minutes followed by 3-4 rinses in sterile distilled water. Surface sterilization of seeds was done in 0.1% $HgCl_2$ solution for 4-5 minutes followed by washing with sterile double distilled water 3-4 times under aseptic conditions. The surface disinfected seeds were inoculated on MS half strength medium. The cotyledonary node explants were excised from twenty days old seedlings.

Media preparation and cultural conditions

MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 0.8% (w/v) agar-agar with different concentrations (0.25-2.0 mg l^{-1}) of growth regulators individually as well as in combinations was selected for the present investigation. Explants were evaluated in terms of per cent bud break, number of shoots per explants and average shoot length after 8 week of culture. The pH of the medium was adjusted to 5.8 prior to autoclaving for 20 min. at 15 psi and 121ºc temperature. The cultures were incubated in a culture room maintained at a temperature of 25±2ºC and a photoperiod of 16hrs (2000 Lux) and 8hrs of dark period. Each treatment consisted of 24 explants and repeated three times.

Bud break and Shoot induction

Cotyledonary nodes were excised aseptically and inoculated on MS medium supplemented with various concentrations (0.25-2.0 mg l^{-1}) of auxins (IAA, NAA, 2, 4-D) and cytokinins (BAP, Kn) alone and in various combinations. Addition of auxins and cytokinins to the medium had a positive effect on shoot formation. Explants were evaluated in terms of per cent shoot regeneration, number of shoots per explants and average shoot length after 8 week of culture.

Root development

Elongated and well developed shoots were aseptically excised and inoculated on full and half strength MS medium supplemented with auxins (IAA, NAA and IBA) at different conc. (0.5-2.0 mg l^{-1}) for root formation. Rooting response was evaluated in terms of percentage of shoots forming roots and number of roots per rooted shoot and average root length after 8 week of culture.

Acclimatization of regenerated plantlets

Complete regenerated plantlets with sufficient roots were taken out from the cultural tubes and washed several times with sterile distilled water to remove the traces of medium by dipping the roots in water with the help of fine brush. The *in vitro* regenerated plantlets were then transplanted to small plastic cups containing sterilized soil and sand mixture (3:1).

Statistical analysis

All the experiments were conducted with a minimum of 24 replicates per treatment and repeated three times. The data was analyzed statistically using one way analysis of variance (ANOVA). The significances of difference among means were carried out using Duncan's multiple range test (DMRT) at ($P<0.05$). The results are expressed as a mean±SE of three repeated experiments.

Results

Shoot multiplication

Ninety per cent of the cultured seeds germinated in the MS half strength basal medium. Cotyledonary nodes (1.0-1.5 cm) from the 20 days old seedlings were cultured on MS medium supplemented with various concentrations of BAP and Kn individually or in combination with auxins (NAA, IAA, 2,4-D) for shoot regeneration (Table-1). Cotyledonary nodes did not show any morphogenetic response in the medium devoid of growth regulators (Table-1). Both BAP and Kn favored shoot bud differentiation, but BAP was more efficient than Kn with respect to the initiation and subsequent proliferation of shoots. Of the two cytokinins used, BAP induced higher number of shoots per explants (Table-1).

The frequency of shoot bud development and number of shoots per explants increased with increasing concentration of BAP up to 1.0 mg l^{-1}. Further increase in the concentration of BAP resulted in reduction of number of shoots. MS medium supplemented with 1.0 mg l^{-1} BAP produced higher number (13.5±0.07) of shoots (Table-1, Fig.1A). Different concentrations of Kn were also able to induce shoots, but the number of shoots was lower than that in BAP and was not encouraging. However Kn has a profound effect on shoot length compared with BAP.

Supplementation of auxins at lower concentrations could not produce multiple shoots whereas 3-4 shoots were observed at higher concentration. However complete plantlet development was observed from cotyledonary node explants inoculated on media supplemented with 1.0 and 2.0 mg l^{-1} of IAA and all the concentration (0.25- 2.0 mg l^{-1}) of NAA (Table-1, Fig.1C). Among the different growth regulators tested individually, BAP at the concentration of 1.0 mg^{-1} indicated the most promising results.

In the present study, it was found that BAP and Kn fortified individually at 1.0 and 2.0 mg l^{-1} concentration were more effective for multiple shoot induction. Therefore, a combined effect of these concentrations of BAP and Kn with auxins (IAA, NAA, 2, 4-D) was also tried to study the shoot induction efficiency of cotyledonary node explants (Table-2&3). Addition of IAA along with BAP increased the number of shoots per explants. Maximum per cent explant response (100) and the number of shoots (17.5±0.02) produced per explant was reported on the medium supplemented with BAP (2.0 mg l^{-1}) in combination with IAA (0.5 mg l^{-1}) (Table-2, Fig.1B).The combinations of BAP and IAA supplemented with MS medium resulted in the best shoot growth. With the increasing concentration of auxins along with BAP (1.0 mg l^{-1}), the number of shoots declined. But at higher concentration of BAP (2.0 mg l^{-1}), the increase in number of shoots was recorded. Callus formation was also observed from the cut ends of the cotyledonary node on the medium supplemented with BAP in combination with IAA. As the concentration of IAA in combination with BAP increases, the callus growth at the cut ends of cotyledonary node also increases, which might inhibit shoot number and shoot elongation.

Supplementation of Kn in place of BAP with auxins resulted in reduction of number of shoots (12.0±0.63) (Table-3). Whereas no basal callusing was observed on Kn supplementation. The medium containing Kn in combination with NAA elicited less number of shoots whereas MS media fortified with Kn (2.0 mg l^{-1}) and NAA (2.0 mg l^{-1}) produced higher number of shoots (Table-3). However, the supplementation of IAA in place of NAA with Kn could produce only 2-5 average numbers of shoots per explants (Table-3). Longer shoots were

obtained on media supplemented with cytokinins (BAP and Kn) individually than their combined effect with auxins (Table 1, 2 and 3).

Table 1. Effect of different growth regulators on cotyledonary node explants of *Salvadora persica*

Medium and conc. of growth regulator mg l^{-1}	Days required for response (mean value)	Per cent Response*	No. of shoots per explant*	Shoot length* (cm)
Ms Medium	0.00	0.00±0.00	0.00 ± 0.00^{l}	0.00 ± 0.00^{i}
MS+IAA (0.25)	8.0	90.2±1.93	1.8 ± 0.07^{ij}	1.6 ± 0.07^{gh}
(0.5)	8.0	87.5±0.00	1.7 ± 0.07^{ij}	1.3 ± 0.06^{h}
(1.0)	8.0	94.4±1.97	1.8 ± 0.05^{ij}	4.7 ± 0.14^{bc}
(2.0)	8.4	90.5±1.97	2.4 ± 0.14^{gh}	4.5 ± 0.27^{bc}
MS+NAA(0.25)	9.0	75.0±0.00	1.5 ± 0.10^{jk}	2.0 ± 0.14^{fg}
(0.5)	9.6	77.7±1.93	1.8 ± 0.12^{ij}	1.6 ± 0.11^{gh}
(1.0)	9.0	100±0.00	3.6 ± 0.05^{fg}	7.6 ± 0.07^{a}
(2.0)	9.0	90.2±1.93	4.3 ± 0.18^{f}	7.3 ± 0.29^{ab}
MS+2,4-D(0.25)	0.00	0.00±0.00	0.00 ± 0.00^{l}	0.00 ± 0.00^{i}
(0.5)	21.0	54.1±0.00	1.0 ± 0.11^{kl}	1.2 ± 0.13^{hi}
(1.0)	21.0	59.7±1.97	1.6 ± 0.16^{jk}	1.7 ± 0.17^{gh}
(2.0)	20.0	60.3±2.92	2.1 ± 0.11^{hi}	1.3 ± 0.07^{h}
MS+BAP (0.25)	8.0	81.9±1.97	4.3 ± 0.24^{f}	1.3 ± 0.09^{h}
(0.5)	8.6	93.0±0.00	7.6 ± 0.19^{c}	3.5 ± 0.10^{cd}
(1.0)	7.0	100±0.00	13.5 ± 0.07^{a}	2.9 ± 0.04^{de}
(2.0)	7.0	90. ±1.93	9.1 ± 0.36^{b}	1.6 ± 0.08^{gh}
MS+KN (0.25)	7.6	81.9±1.97	4.5 ± 0.26^{e}	2.4 ± 0.14^{ef}
(0.5)	7.0	83.3±0.00	7.7 ± 0.41^{c}	3.2 ± 0.19^{d}
(1.0)	7.0	100±0.00	5.7 ± 0.07^{de}	5.0 ± 0.03^{b}
(2.0)	6.0	95.8±0.00	6.1 ± 0.15^{d}	3.4 ± 0.09^{cd}

* Values are Means ±S.E of three independent experiments. Data were recorded after 8 week of culture, Means values within a column sharing the same subscript are not significantly different at $P<0.05$ according to Duncan's Multiple Range Test.

Table 2. Effect of auxins and cytokinins on cotyledonary node explants of *Salvadora persica*

Medium and conc. Of growth regulator (mg l^{-1})	Days required for response (mean value)	Per cent Response*	No. of shoots per explant*	Shoot length* (cm)
MS+BAP(1.0)+IAA(0.5)	8.0	88.8±1.93	8.3 ± 0.33^{ef}	1.2 ± 0.05^{fg}
(1.0)	8.0	94.4±1.97	9.6 ± 0.28^{d}	1.6 ± 0.06^{de}
(2.0)	8.4	100±0.00	7.6 ± 0.04^{fg}	1.4 ± 0.03^{ef}
MS+BAP(2.0)+IAA(0.5)	9.6	100±0.00	17.5 ± 0.02^{a}	2.7 ± 0.04^{ab}
(1.0)	9.0	90.2±1.93	13.8 ± 0.54^{b}	2.2 ± 0.13^{ab}
(2.0)	9.0	91.6±0.00	12.8 ± 0.46^{c}	3.2 ± 0.14^{a}
MS+BAP(1.0)+NAA(0.5)	11.0	91.6±0.00	3.6 ± 0.14^{jk}	1.9 ± 0.08^{cd}
(1.0)	10.9	88.8±1.93	5.0 ± 0.21^{hi}	2.1 ± 0.11^{bc}
(2.0)	11.5	84.7±1.97	6.1 ± 0.31^{gh}	1.1 ± 0.07^{fg}
MS+BAP(2.0)+NAA(0.5)	8.6	100±0.00	4.1 ± 0.14^{ij}	2.0 ± 0.09^{bc}
(1.0)	7.0	93.0±1.97	6.0 ± 0.04^{gh}	1.7 ± 0.04^{de}
(2.0)	7.0	87.4±3.38	9.3 ± 0.42^{de}	2.0 ± 0.10^{bc}
MS+BAP(1.0)+2,4D(0.5)	7.0	83.3±3.42	6.9 ± 0.36^{g}	1.5 ± 0.09^{ef}
(1.0)	7.0	100±0.00	8.2 ± 0.03^{ef}	1.7 ± 0.06^{de}
(2.0)	6.0	94.4±1.97	4.0 ± 0.12^{ij}	1.0 ± 0.04^{fg}
MS+BAP(2.0)+2,4D(0.5)	15.2	56.9±1.97	3.3 ± 0.35^{jk}	0.99 ± 0.10^{g}
(1.0)	16.5	55.5±1.97	2.9 ± 0.3^{k}	1.6 ± 0.18^{de}
(2.0)	0.00	0.00±0.00	0.00 ± 0.00^{l}	0.00 ± 0.00^{h}

*Values are Means ±S.E of three independent experiments. Data were recorded after 8 week of culture, Means values within a column sharing the same subscript are not significantly different at $P<0.05$ according to Duncan's Multiple Range Test.

Table 3. Effect of auxins and cytokinins on cotyledonary node explants of *Salvadora persica*

Medium and conc. of growth regulator (mg l^{-1})	Days required for response (mean value)	Per cent Response*	No. of shoots per explant*	Shoot length* (cms)
MS+KN(1.0)+IAA(0.5)	6.5	98.6±1.97	5.9 ± 0.09^{c}	2.5 ± 0.05^{b}
(1.0)	6.5	100±0.00	4.1 ± 0.04^{ef}	1.6 ± 0.05^{de}
(2.0)	7.0	100±0.00	4.2 ± 0.04^{ef}	1.7 ± 0.04^{cd}
MS+KN(2.0)+IAA(0.5)	6.5	91.6±3.38	1.9 ± 0.07^{hi}	3.5 ± 0.14^{ab}
(1.0)	6.0	90.2±1.93	1.8 ± 0.07^{hi}	3.8 ± 0.15^{a}
(2.0)	6.0	91.6±0.00	1.8 ± 0.06^{hi}	3.4 ± 0.13^{ab}
MS+KN(1.0)+NAA(0.5)	4.0	90.2±1.93	1.8 ± 0.07^{hi}	1.6 ± 0.08^{de}
(1.0)	0.0	0.00±0.00	0.00±0.00	0.00±0.00
(2.0)	4.5	91.6±0.00	1.7 ± 0.07^{hi}	1.9 ± 0.08^{c}
MS+KN(2.0)+NAA(0.5)	4.5	83.3±0.00	12.0 ± 0.63^{a}	1.4 ± 0.10^{ef}
(1.0)	4.5	80.5±1.97	10.0 ± 0.58^{bc}	1.0 ± 0.08^{fg}
(2.0)	4.5	79.1±0.00	11.9 ± 0.72^{ab}	1.4 ± 0.10^{ef}
MS+KN(1.0)+2,4D(0.5)	11.0	72.2±1.97	5.2 ± 0.37^{cd}	1.8 ± 0.13^{cd}
(1.0)	10.0	70.8±0.00	4.3 ± 0.33^{ef}	1.5 ± 0.13^{de}
(2.0)	10.0	70.8±0.00	4.7 ± 0.36^{de}	1.8 ± 0.15^{cd}
MS+KN(2.0)+2,4D(0.5)	10.5	69.4±1.97	2.6 ± 0.21^{gh}	1.8 ± 0.15^{cd}
(1.0)	10.0	66.6±0.00	3.3 ± 0.28^{fg}	1.3 ± 0.12^{ef}
(2.0)	0.00	0.00±0.00	0.00 ± 0.00^{j}	0.00 ± 0.00^{h}

* Values are Means ±S.E of three independent experiments. Data were recorded after 8 week of culture, Means values within a column sharing the same subscript are not significantly different at $P<0.05$ according to Duncan's Multiple Range Test.

Root development

For root induction, *in vitro* raised shoots (2-3 cm long) were aseptically excised and inoculated on MS half and full strength medium supplemented with 0.5-2.0 mg l^{-1} of IBA, IAA and NAA. None of the shoots cultured on auxin free medium formed roots. Root induction was observed after 16.5 days after culture in IAA supplemented media. Depending upon auxin type and concentration, root number ranging from 6.8-17.5 were observed in the auxin supplemented medium (Table- 4). Auxin concentration also played a significant role in root induction and root length was significantly affected by the type of auxins. Supplementation of NAA in the medium induced small roots (Table-4 & Fig.1F). Whereas the number of roots produced per shoot was maximum in NAA (2.0 mg l^{-1}). However, the roots were short and no further elongation was observed (Table-4). Whereas the shoots cultured on IAA and IBA showed elongated roots. Out of the three auxins tested, IAA proved to be better than NAA and IBA in terms of per cent root formation and root length. Maximum

per cent roots induction and length was obtained on MS full strength medium supplemented with 1.0 mg l^{-1} IAA (Fig.1 D and E).

Table 4. Effect of different growth regulators on root induction of regenerated shoots of *Salvadora persica*

Medium and conc. of growth regulator (mg l^{-1})		Days required for root induction (mean value)	Per cent Response*	No. of roots per shoot*	Root length* (cms)
MS Medium	NAA+0.5	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
(Half Strength)	1.0	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	2.0	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	IAA +0.5	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	1.0	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	2.0	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	IBA +0.5	22.5	70.8±3.41	6.8 ± 0.52^{fg}	2.4 ± 0.21^{e}
	1.0	21.6	63.8±3.94	7.0 ± 0.63^{ef}	3.1 ± 0.29^{d}
	2.0	22.5	61.0±3.94	8.1 ± 0.77^{de}	4.0 ± 0.38^{c}
MS Medium	NAA+0.5	20.5	63.8±3.94	16.8 ± 1.51^{ab}	1.2 ± 0.13^{gh}
(Full Strength)	1.0	21.9	58.3±0.00	17.5 ± 1.76^{a}	1.9 ± 0.19^{ef}
	2.0	19.0	55.5±1.97	15.8 ± 1.69^{bc}	1.4 ± 0.16^{fg}
	IAA+ 0.5	17.4	73.6±1.97	10.2 ± 0.72^{cd}	5.9 ± 0.42^{bc}
	1.0	16.5	76.3±1.93	11.3 ± 0.75^{c}	6.6 ± 0.43^{a}
	2.0	18.3	70.8±3.41	8.8 ± 0.70^{d}	6.2 ± 0.47^{ab}
	IBA +0.5	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	1.0	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}
	2.0	0.00	0.00±0.00	0.00 ± 0.00^{h}	0.00 ± 0.00^{i}

* Values are Means ±S.E of three independent experiments. Data were recorded after 8 week of culture, Means values within a column sharing the same subscript are not significantly different at $P<0.05$ according to Duncan's Multiple Range Test.

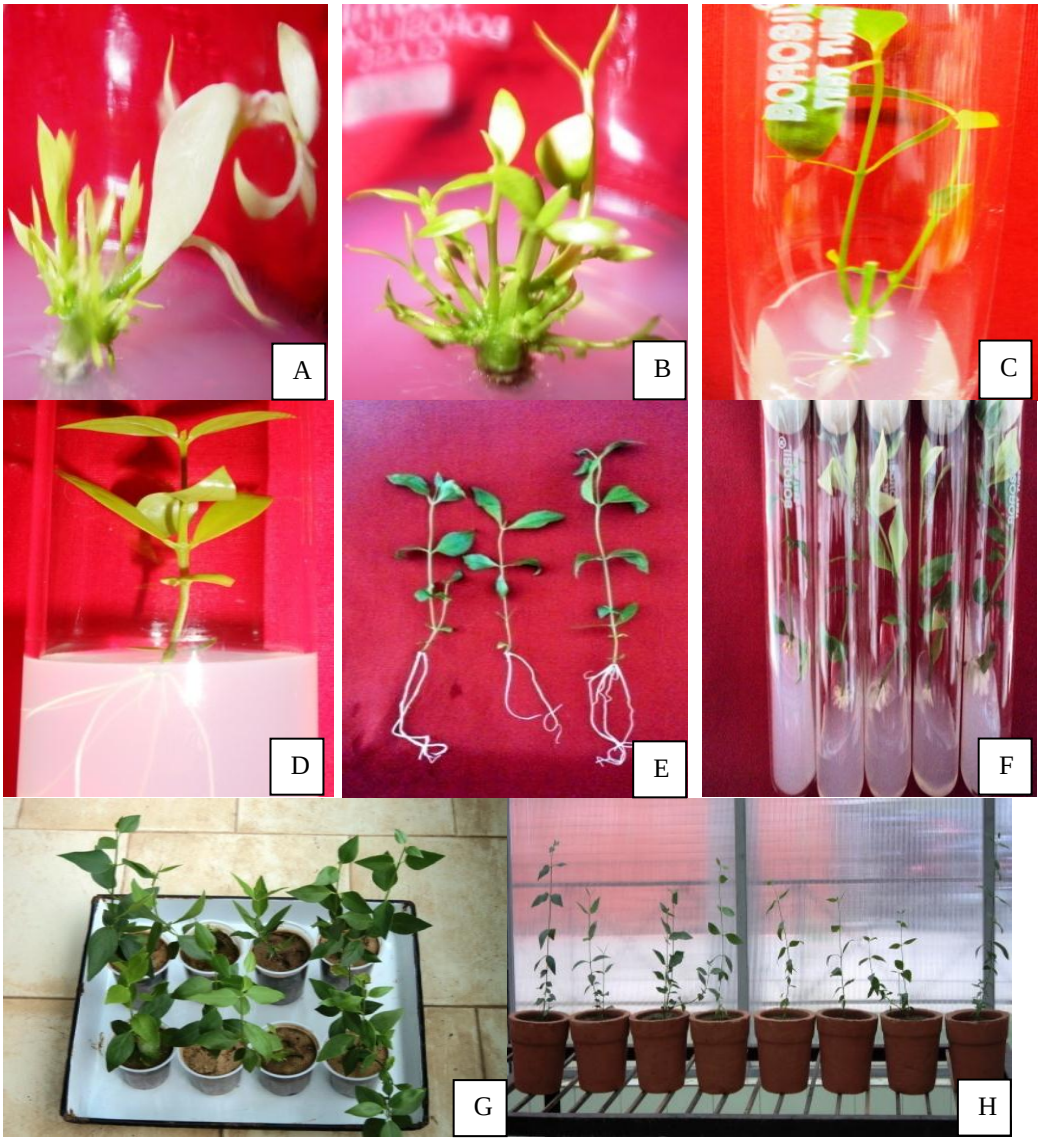

Fig. 1. (A) Multiple shoot formation from cotyledonary node explant of *Salvadora persica* on MS medium supplemented with 1.0 mg l^{-1} of BAP (B) Maximum number of shoot formation from cotyledonary nodes on MS media supplemented with BAP(2.0 mg l^{-1}) in combination with IAA (0.5 mg l^{-1}). (C) Complete plantlet formation from cotyledonary node explant of *Salvadora persica* on MS medium supplemented with 1.0 mg l^{-1} IAA after 8 week of culture. (D) Root formation from *in vitro* regenerated shoots of *Salvadora persica* on half strength MS medium supplemented with 1.0 mg l^{-1} IAA. (E) Rooted plantlets in IAA 1.0 mg l^{-1} (F) Root formation on NAA 1.0 mg l^{-1}. (G) *In vitro*-raised plants of *Salvadora persica* one month after transfer to plastic cups just before transfer to earthen pots (H) Well-established plants in earthen pots three months following transfer to soil (one month in plastic cups followed by two months in earthen pots).

Acclimatization of regenerated plantlets

This experimental step was undertaken with the aim of exploring and evaluating the micropropagated plantlets in terms of their adaption and survival potential with respect to the acclimatization environment. The regenerated plantlets were carefully taken out of the glass tubes and their roots thoroughly washed to remove any adhering agar medium. They were than transplanted in to plastic cups containing a soil: sand mixture (3:1). The transplanted plantlets were irrigated on alternate days for two weeks with a nutrient solution composed of half concentrations of MS macro and micro nutrients. High humidity was maintained for initial 15 days with the help of polythene bags and thereafter, these cups were exposed to natural conditions for 3-4 hours daily in an attempt to acclimatize the plantlets to natural conditions. After a month, these plants were transferred to small earthen pots and shifted to glasshouse where they grew normally with seventy per cent survival rate. After 8 weeks of glass house period, the plants were transferred to fields. The survived plants grew normally.

Discussion

During the past three decades, Plant tissue culture technique has emerged as an important tool for solving the increasing number of problems in plant sciences, viz. cell and tissue developmental biology, biochemistry, genetics, molecular biology and for general applied aspects such as clonal propagation, haploid production, and embryo culture for improving medicinally and economically important plant species. Clonal multiplication basically depends on the proliferation and induction of axillary or apical shoot meristem or through callus differentiation. By using tissue culture technique, the nodal meristem can be stimulated to produce multiple shoots through differentiations and organogenesis using appropriate auxin cytokinin ratio. Through this method of multiplication large numbers of plants have been produced in diverse group of families (Nugent *et al*., 1991).The main problems encountered in developing *in vitro* system for woody plant regeneration are establishment of explants in culture due to the oxidation of polyphenols from them, absence of juvenility in the explants of mature trees and root induction in culture. However, explants from mature trees are more difficult to establish *in vitro* because of rapid necrosis of the explants. The purpose of this study was to develop an *in vitro* propagation method from cotyledonary node explant as they are more responsive than mature explants.

Supplementation of auxins and cytokinins individually and in various combinations to MS medium favored bud break as well as shoot growth. Earlier

plant regeneration from seedling explants of woody trees was also reported in including *Anogeissus sericea* (Kaur *et al*., 1992), *Prosopis cineraria* (Nandwani and Ramawat, 1993), *Sterculia urens* (Purohit and Dave, 1996), and *Dalbergia sissoo* (Pradhan *et al*., 1998), *Syzygium cumnii* (Jain and Babber, 2000), *Termanilia chebula* (Shymakumar *et al*., 2003), *Termanilia bellirica* (Sadanandam *et al*., 2005), *Boswellia ovalifoliolata* (Chandrasekhar *et al*., 2005), *Pterocarpus santalinus* (Rajeshwari and Paliwal, 2006), *Aegle marmeolus* (Nayak *et al*., 2007), *Stereospermum personatum* (Shukla *et al*., 2009), *Commiphora wightii* (Kant *et al*., 2010), *Sapindus trifoliatus* (Asthana *et al*., 2011).

In the present investigation, seed germination was observed on MS half strength medium without growth regulators. Similar results were reported by Nayak *et al*., (2007) in *Aegle marmeolus,* Khalafalla and Daffallia (2008) in *Acacia senegal* and Rani *et al*., (2010) in *Murraya koengii.* In the present study, it was found that the medium without growth regulator failed to induce bud break but addition of cytokinins proved to be better in multiple shoot induction from cotyledonary node explants. The role of cytokinins in shoot multiplication or differentiation from seedling has been emphasized in other woody trees species by (Mittal *et al*., 1989). After 6-8 days in induction medium containing growth regulators, cotyledonary node explants showed adventitious bud formation. Frequency of shoot induction and multiplication were generally influenced by the concentration of auxins and cytokinins in the medium.

Among various concentrations of BAP tested, the maximum number of shoots (13.5±0.07) per explants was observed on medium containing 1.0 mg l^{-1} BAP (Table-1, Fig.1A) as compared to other concentrations of BAP. Superiority of BAP in inducing shoots from cotyledonary nodes has been described previously in various woody species like *Dalbergia sissoo* (Pradhan *et al*., 1998), *Simmondsia chinesis* (Agrawal *et al*., 2002) *Cassia angustifolia* (Agrawal and Sardar, 2003), *Albizia odoratissima* (Rajeshwari and Paliwal, 2006) *Melia azadirachta* (Hussain and Anis, 2009) and *Bauhinia cheilantha* (Gutierrez *et al*., 2011).

Kinetin seemed to be more effective than the BAP for the elongation of the shoots. In the present study, higher concentrations of BAP resulted in reduction of shoot number as well as shoot length. Similarly, Hu and Wang (1983) reported that higher concentrations of cytokinin reduced the number of shoot formation. However, the supplementation of auxins (NAA and IAA) at higher concentration (2.0 mg l^{-1}) found more effective for multiple shoot induction with better shoot growth as compared to lower concentration (0.25-1.0 mg l^{-1}) (Table-1).

Inclusion of BAP in combination with IAA, NAA and 2, 4-D (0.5-2.0 mg l^{-1}) was not only effective in enhancing shoot proliferation, but also supported prolific callus growth at the basal end of cotyledonary node explants. Combination of BAP and IAA considerably enhanced shoot bud differentiation and the development of axillary shoots from the cotyledonary node explants was accompanied by basal callusing of the explants. However this callus remained undifferentiated. Vengadesan *et al*., (2002) also reported that when cotyledonary nodes of *Acacia sinuata* were cultured on MS medium containing a combination of BAP and auxins (IAA, NAA, and IBA), the number of shoots was reduced but in turn produced basal callus. Callus formation was also obtained at the basal end of the explants in case of *Balanites aegyptiaca* (Ndoye *et al*., 2003) *Acacia chundra* (Rout *et al*., 2008), *Sapindus trifoliatus* (Asthana *et al*., 2011). The formation of callus may be due to the accumulation of auxin at the base of the explants (Marks and Simpson, 1994).

Supplementation of Kn in place of BAP with auxins resulted in reduction of number of shoots and did not produce callus at the basal portions of cotyledonary nodes. Supplementation of BAP and Kn in combination with NAA was proved useful for regeneration of shoots in *Moringa pterygosperma* (Mohan *et al*., 1995). Similar results were observed by Martin (2003) in *Rotula aquatica* and Asthana *et al*., (2011) in *Sapindus trifoliatus*. Highest number of shoots (4.0) was observed on explants cultured on MS medium supplemented with Kn (1.0 mg l^{-1}) in combination with 0.01 mg l^{-1} of NAA was reported by Okere and Adegeye (2011) in *Khaya grandifolia.*

In vitro parameters offer a better control of environmental conditions such as temperature and light that influence root development. In addition to this auxins and minerals supply is also crucial for adventitious root system formation in micro shoots (George, 1993). Among the auxins used, IAA was found to be best for root development. MS half strength media with IAA failed to develop roots but MS full strength medium with IAA (1.0 mg l^{-1}) developed maximum per cent root induction and root length after 16.5 days of implantation. The roots were long and healthy. Similar results were also obtained by Nanda and Dhaliwal (1974) when MS medium supplemented with 1.0 mg l^{-1} alone was most effective in rooting of hypocotyl cuttings with intact cotyledons of *Impatiens balsamina*. Sixty per cent rooting was observed on MS half strength medium supplemented with IAA by Peternel *et al*., (2009) in *Populus tremula*. Supplementation of IAA was also found to be effective on root development in *Impatiens campanulata* (Balakrishnan *et al*., 2009), *Murraya koengii* (Rani *et al*., 2010). Among auxins, NAA failed to induce roots on half strength medium however at full strength maximum frequency of roots

were produced with small length however supplementation of IBA also produced roots but with lesser frequency and per cent root induction than IAA.

For acclimatization, plantlets were removed from rooting medium after complete development of roots, were then transplanted to small plastic cups containing sterilized soil and sand mixture (3:1). Similarly in case of *Acacia senegel*, (Khalafalla and Daffalla, 2008), *Prosopis cineraria* (Kumar and Singh, 2009) *Commiphora mukul* (Singh *et al*., 2010) *Aegle marmelos* (Yadav *et al*., 2011) also used soil and sand (3:1) for hardening of regenerated plantlets. After a month these plants were shifted to glasshouse where they grew normally with seventy per cent survival rate (Fig.G & H.) About 3-4 month old soil established plants were successfully transferred to the field. All the regenerated plantlets were morphologically uniform.

In conclusion, an efficient protocol was developed for successful mass multiplication of this important endangered plant species and this can be utilized for rapid and large scale propagation of this plant.

Acknowledgement

The authors are grateful to Kurukshetra University, Kurukshetra for providing financial support and the laboratory facilities.

References

Abdillahi, H. S., Stafford, G. I., Finnie, J. F., Staden, J. W. (2010). Ethanobotany, Phytochemistry and Pharmacology of *Podocarpus sensus* latissimo (S. I.) South African Journal of Botany, 76(1) pp. 1-24.

Agrawal, V. and Sardar, P. (2003). *In vitro* organogenesis and histomorphological investigations in Senna *Cassia angustifolia* -a medicinally valuable shrub. Physiology and Molecular Biology of Plants, 9:1-10.

Agrawal, V., Prakash, S., Gupta, S. C. (2002). Effective protocol for *in vitro* shoot production through nodal explants of *Simmondsia chinensis*. Biologia Plantarum, 45: 449-453.

Al- Quran S. (2008). Taxonomical and Pharmacological Survey of Therapeutic Plants in Jordan. Journal of Natural Products, 1: 10-26.

Asthana, P., Jaiswal, V. S. and Jaiswal, U. (2011). Micropropagation of *Sapindus trifoliatus* and assessment of genetic fidelity of micropropagated plants using RAPD analysis. Acta Physiologiae Plantarum, 33:1821-1829.

Balakrishnan, V., Konganapuram, C., Ravindran and Jayachandran P. Robinson. (2009). Effective regeneration of *Impatiens campanulata* Wight a Valuable Medicinal Plant from Western Ghats of Tamil Nadu, India. Global Journal of Pharmacology, 3 (1): 41-49.

Chandrasekhar, T., Hussain, T.M., Jayanand, B. (2005). *In vitro* micropropagation of *Boswellia ovalifoliolata*. Natl. Cent. Biotechnology Inf., 60:505–507.

Ezmirly, S.T., Cheng, J.C. and Wilson S.R. (1979). Saudi Arabian Medicinal Plants: *Salvadora persica*, Planta Medica, 35: 191-192.

George, E.F. (1993). Plant Propagation by Tissue Culture, Vol 1. Exegetics Ltd., England.

Gutierrez, I.E.M., Nepomuceno, C.F., Ledo, C.A.S. and Santana J.R.F. (2011). Micropropagation and acclimatization of *Bauhinia cheilantha* (an important medicinal plant). African Journal of Biotechnology, vol. 10(8) pp. 1353-1358.

Hu, C.Y., Wang, P.J. (1983). Handbook of Plant Cell Culture. In: Evans DA, Sharp WR, Ammirato P.V., Yamada, Y. (eds.) Meristem shoot tip and bud culture. New York, Macmillan, pp. 177–227.

Hussain, M. K. and Anis, M. (2009). Rapid *in vitro* multiplication of *Melia azedarach* L. (a multipurpose woody tree). Acta Physiologiae Plantarum, 31:765–772.

Jain, N., and Babber, S. B. (2000). Recurrent production of plants of black plum *Syzgium cumini* (L.) Skees, a myrtaceous fruit tree, from *in vitro* cultured seedling explants. Plant Cell Reports 19: 519-524.

Kamil, M., Ahmad, F., Jayaraj, A.F., Guna-sekhar, C., Thomas, S., Habibullah, M., Chan, K. (1999). Pharmacognostical and Phytochemical studies on *Salvadora persica* L. 42: 64-75.

Kant, T., Prajapti, S., Parmar, A. K. (2010). Efficient micropropagtion from cotyledonary node cultures of *Commiphora wightii* (ARN.) Bhandri, an endangered medicinally important desert plant. Journal of Plant Development, 17:37-48.

Kaur, G., Singh, R.P., Rathore, T.S., Shekhawat, N.S. (1992). *In vitro* propagation of *Anogeissus sericea*. Indian Journal of Experimental Biology, 30:788-791.

Khalafalla, M.M. and Daffalla, H.M. (2008). *In vitro* micropropagation and micrografting of gum tree (*Acacia senegal*) L. (Wild). International Journal of Sustainable Crop Production, 3(1): 19-27.

Kumar, S. and Singh, N. (2009). Micropropagation of *Prosopis cineraria* (L.) Druce-a multipurpose desert tree. Researcher, 1:28-32.

Makwana, M.T., Patolia, J.S., Iyengar, E.R.R. (1988). *Salvadora* plant species suitable for saline coastal wastelands. Transactions of Indian Society of Desert Technology, 2: 121-131.

Marks, T.R. and Simpson, S.E. (1994). Factors affecting shoot development in apically dominant Acer cultivars *in vitro*. Journal of Horticultural Science, 69: 543-551.

Martin, K.P. (2003). Rapid *in vitro* propagation and *ex vitro* rooting of *Rotula aquatica* Lour. a rare rhoeophytic woody medicinal plant. Plant Cell Reports, 21: 415- 420.

Mathur, S., Shekhawat, G.S., Batra, A. (2002). Micropropagation of *Salvadora persica* via cotyledonary nodes. Indian Journal of Biotechnology, 1:197–200.

Mathur, S., Shekhawat, G.S., Batra, A. (2008). Somatic embryogenesis from cotyledon explants of *Salvodora persica* L. Phytomorphology, 58(1-2):57-63.

Mittal, A., Aggarwal, R., Gupta, S.C. (1989). *In vitro* development of plantlets from axillary buds of *Acacia auriculiformis*, leguminous tree. Plant Cell Tissue and Organ Culture, 19:65–68.

Mohan, V., Purohit, M. and Srivastava, S.P. (1995). *In vitro* micropropagation of *Moringa pterygosperma*. Phytomorphology, 45 (3&4) pp. 253-261.

Murashige, T. Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue scultures. Physiologia Plantarum, 15:473–497.

Nadkarny, A.K. (1954). Indian Materia Medica, 3rd ed.

Nanda, K.K., and Dhaliwal, G. (1974). Interaction of Auxin, Nutrition and morphological nature in rooting hypocotyle cuttings of *Impatiens balsamina*. Journal of Experimental Biology, January. pp. 82-84.

Nandwani, D., Ramawat, K.G. (1993). *In vitro* plantlet formation through juvenile and mature explants in *Prosopis cineraria*. Indian Journal of Experimental Biology, 31: 156-160.

Nayak, P., Bahera, P.R., Manikkannan, T. (2007). High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In vitro* cellular and Developmental Biology of Plants, 43: 231-236.

Ndoye, M., Diallo, I., Gassama, Dia. Y.K. (2003). *In vitro* multiplication of the semi-arid forest tree *Balanites aegyptiaca* (L.) Del. African Journal of Biotechnology, 2(11): 421-424.

Nugent, G., Wardley, T., Lu, C. (1991). Plant regeneration from stem and petal of Carnation (*Dianthus caryophyllus*). Plant Cell Reports, 10: 477-480.

Oker, A.U. and Adegeye, A. (2011). *In vitro* propagation of an endangered medicinal timber species *Khaya garandifolia* C. Dc. African Journal of Biotechnology, vol 10(17), pp. 3335-3339.

Peternel, S., Gabrovsek, K., Gogala, N. Regvar, M. (2009). *In vitro* propagation of European aspen (*Populus tremula* L.) from axillary buds via organogenesis. Scientia Horticulturae, 121(1): 109-112.

Phulwaria, M., Ram, K., Gahlot, P. and Shekhawat, N.S. (2011). Micropropagation of *Salvadora persica* – a tree of arid horticulture and forestry. New Forest. DOI 10.1007/s11056-011-9254-z.

Pradhan, C., Kar, S., Chand, P.K. (1998). Propagation of *Delbergia sisso* Roxb. through *in vitro* shoot proliferation from cotyledonary nodes. Plant Cell Reports, 18:122-126.

Purohit, S.D. and Dave, A. (1996). Micropropagation of *Sterculia urens* Roxb. an endangered tree species. Plant Cell Reports, 15, 704-706.

Rajeswari, V. and Paliwal, K. (2006). *In vitro* propagation of *Albizia odoratissima* L. F. (Benth.) from cotyledonary node and leaf nodal explants. *In Vitro* Cellular and Developmental Biology of Plant, 42:399-404.

Rani, U., Ismail, N., Zibbu, G. And Batra, A. (2010). High frequency shoots regeneration from *in vitro* raised seedlings of *Murraya koengii*. International Journal of Pharma and Bio Sciences, pp 107.

Rao, G.G., Polra, V.N., Ravindran, B.V. (1999). Salt tolerance studies in *Salvadora persica*: a facultative halophyte. Indian Journal of Soil Conservation, 27: 55-63.

Rout, G.R., Senapati, S.K., Aparajeta, S. (2008). Micropropagation of *Acacia chundra* (Roxb.) DC. Scientia Horticulturae (Prague), 35 (1): 22–26.

Sadanandam, A., Ramesh, M., Umte, P., Rao, V.K. (2005). Micropropagation of *Terminalia bellirica*. - A sericulture and medicinal plant. *In Vitro* Cellular and Developmental Biology of Plants, 41: 320-323.

Sher, H., Khan, Z.D., Khan, A.U., Hussain, F. (2004). Ethanobotanical study on some plants species in Village Tigdri, District Swat. Journal of Acta Botanica Yunnanica, 25: 42-54.

Shukla, S., Shukla, S.K., Mishra, S.K. (2009). *In vitro* plant regeneration from seedling explants of *Stereospermum personatum* D. C. a medicinal tree. Trees: Structure and Function, Vol. 23, No. 2 pp. 409-413.

Shyamkumar, B., Anjaneyulu, C., Giri, C.C. (2003). Multiple shoot induction from cotyledonary node explants of *Terminalia chebula* Retz. A tree of medicinal importance. Biologia Plantarum, 47: 585-588.

Singh, N., Garg, A., Yadav, K. and Kumari, S. (2010). Influence of growth regulators on the explants of *Commiphora mukul* (Hook. ex Stocks) Engl. under *in vitro* conditions. Researcher, 2(7):41-48.

Vengadesan, G., Ganapathi, A., Premanand, R., Ramesh, A.V. (2002). *In vitro* propagation of *Acacia sinuta* (Lour.) Merr via cotyledonary nodes, Agroforestry Systems, 55: 9-15.

Watt, J.M. and Breyer-Brandwijk M.G. (1962). The Medicinal and Poisonous Plants of Southern and East Africa. 2nd ed., Edinburg and London.

Yadav, K. and Singh, N. (2011). *In vitro* propagation and biochemical analysis of field established Wood apple (*Aegle marmelos* L.). *Analele Universităţii din Oradea – Fascicula Biologie*, 18(1): 23-28.

Zodape, S.T. and Indusekhar, V.K. (1997). *Salvadora persica*: A boon to wasteland development. Journal of Scientific & Industrial Research, 56:657-661.

(Published in September 2012)