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Research Article

IN VITRO STUDIES ON *SALVADORA OLEOIDES* DECNE USING NODAL EXPLANTS - A MULTIPURPOSE TREE

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ABSTRACT
This report describes *in vitro* shoot induction and plant regeneration from nodal segments of *Salvadora oleoides* on Murashige and Skoog's medium fortified with kinetin and BAP individually. MS medium supplemented with 2.5 mg l^{-1} BAP was the most effective treatment in inducing bud break and also in initiating multiple shoot proliferation. Individual optimized concentrations of cytokinins were tried with low concentrations of auxins (IAA and NAA). Among various combinations of cytokinins and auxins, 2.5 mg l^{-1} BAP + 0.25 mg l^{-1} NAA gave best results in terms of percent bud break, shoot length and number of shoot regenerated per explant. *In vitro* regenerated shoots were rooted on MS medium supplemented with 1 mg l^{-1} NAA. The *in vitro* raised plantlets with well developed shoots and roots were successfully established in pots containing sterilized soil and vermiculite (1:1) with 80% survival rate.

Keywords: *Salvadora oleoides, Nodal explants, Murashige and Skoog's medium, Cytokinins, Auxins*

INTRODUCTION
Salvadora oleoides Decne belonging to family Salvadoraceae is a drought - hardy species distributed in north -western Indian states. This plant is used for ethno-medicinal purposes. Leaves of this plant are used to treat cough and are fed to horses to treat constipation. The fruits are rich in glucose, fructose, sucrose and calcium (Duhan *et al.,* 1992). Fruits are given to cattle to boost milk yielding capacity. Fruits are also used to cure spleen disease and rheumatism. The seeds are rich source of non edible oil and their fat is used as a base of many ointments (Anonymous, 1972). Refined seed oil is a good raw material for soap, candle and detergent industries (Zodape *et al*., 1997). *Salvadora oleoides* is under threat due to its medicinal value, over exploitation, low rate of seed formation and poor viability of seeds. This species can not be propagated by vegetative methods (Khan, 1997; Khan and Frost, 2001 and Singh, 2004). Therefore objectives were set for *in vitro* propagation using nodal explants via optimization of basal media, growth regulators followed by successful outdoor establishment of regenerated plants. Biotechnological interventions for *in vitro* regeneration, mass propagation in woody species have been implemented successfully in last few decades. The present study has been designed to develop a reliable and reproducible protocol for *in vitro* regeneration of *Salvadora oleoides*.

MATERIALS AND METHODS
Plant Material and Source of Explants-
The Nodal segments were collected from mature tree growing at village Ajaib (Rohtak). Then explants were wrapped in moist filter paper in the form of bundle and were brought to laboratory. Nodal segments were cut into single node explants and thereafter were washed with detergent under running tap water to remove dust particles followed by washing in distilled water to remove all traces of detergent. Nodal explants were then kept in 90% ethanol for 2 minutes followed by treatment of freshly prepared 0.1% Mercuric Chloride solution (Hi Media Co. Mumbai) for 5-7 minutes under aseptic conditions. Finally explants were washed 5-6 times with sterile distilled water to remove all traces of mercury. The surface

sterilized nodal segments were then trimmed aseptically to smaller segments of approximately 1.5 cm size with the help of sterilized blade under laminar air flow cabinet.

Culture Media and Conditions-

MS medium (Murashige and Skoog, 1962) was used in all experiments with 3% sucrose. MS medium was supplemented with various concentrations of different PGRs individually as well as in combinations. Analytical grade chemicals were used (Hi Media Co. Mumbai).The medium was solidified with 0.8% agar-agar. pH of medium was adjusted to 5.8 before autoclaving for 20 minutes at 15 psi pressure. Cultures were maintained in a culture room at 25± 2^0C under 16 h light and 8 h dark period with 50 µmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes and 60% relative humidity.

Shoot Induction and Multiplication-

Nodal segments were inoculated in MS medium supplemented with different concentrations of cytokinins (Kn and BAP) either singly or in combination with auxins (IAA and NAA). MS medium without any growth regulator served as control. All observations were recorded regularly up to 35 days of culture. The regenerated shoots were transferred individually as well as in the form of small shoot clumps along with mother explants on a fresh medium for further growth and elongation once in thirty five days, maintaining the same concentration of hormones used in shoot induction culture.

In vitro rooting-

Healthy and well elongated *in vitro* regenerated shoots (2-4cm) were excised from culture and transfered to rooting media composed of MS medium supplemented with different concentrations (0.5 -2.0 mg l^{-1}) of IBA and NAA .

Acclimatization-

In vitro regenerated complete plantlets were taken out from medium and their roots were washed to remove traces of medium with the help of fine brush by dipping the roots in running tap water. Then these plantlets were transplanted in a pot containing sterilized soil and vermiculite (1:1). Initially the plantlets were kept in laboratory conditions. Potted plants were covered with transparent polyethene bags and inverted glass beaker to ensure high humidity. All plants were watered with quarter strength MS salt solution on alternate days for 2 week. Then plantlets were exposed for 3-4 hrs daily to the conditions of natural humidity and light to harden the plants followed by transfer to polyhouse. Finally plants were transfered to field conditions.

Statistical Analysis

Each experiment was repeated thrice containing ten replicates for each hormonal treatment. The data was analyzed (mean ±S.E.) for each treatment (Snedecor, 1956).

RESULTS AND DISCUSSION

Shoot formation from nodal segments is an important and easy aspect of micropropagation because nodal segments have intercalary meristem. During present investigation, nodal explants cultured on MS medium without any growth regulators produced single shoot per explant. Similar observations were made by Paal *et al*., (1981) and Cavallini and Lupi (1987). Role of growth regulators for multiple shoot regeneration was necessary during present work. Generally, cytokinins are required for *in vitro* axillary shoot induction and proliferation but type of cytokinins and its optimal concentration varies with the system (Park *et al*., 2008). Cytokinins are supposed to play a key role in DNA synthesis and organogenesis, which may be the reason for induction of multiple shoots. During present investigation, Bud break and development of shoots from nodal explants was also a function of cytokinins activity and a varied response was observed with supplementation of different concentrations (0.5-3.0 mg l^{-1}) of cytokinins (Kn and BAP). Of the two cytokinins tested, BAP was more effective than Kn in shoot formation (Table-1). Similar results were also reported by Choudhary *et al*., (2004), Goel *et al*., (2009), Kumar and Singh (2009) and Yadav and Singh in *Dendrocalamus strictus, Peganum harmala, Prosopis cineraria* and *Spilanthes acmella* respectively. There was a linear correlation between the concentration of cytokinins up to the optimum level (2.5 mg l^{-1}) and percentage of shoot development. Number of shoots regenerated per explant and shoots length also increased with increase in cytokinins concentration. In present study, the stimulatory effect of a singular

supplement of cytokinin (Kn and BAP) on bud break and multiple shoot formation in *Salvadora oleoides* is in accordance with earlier reports in other species such as *Chlorophytum borivelianum* (Purohit *et al*., 1994) and *Maerua oblongifolia* (Rathore *et al*., 2005) where lower and higher concentrations of growth regulators did not support the better results.

Table 1: Effects of different concentrations of cytokinins individually (Kn and BAP) on nodal explants taken from mature tree

Media	Concentratios of growth regulator (mg l^{-1})	Per cent bud break	No. of days required for bud break (±S.E.)	No. of shoots regenerated per explant (±S.E.)	Shoot length (cm) (±S.E.)	Callus growth
Control	-	30	16±0.35	1±0.45	0.8±0.36	No callus
MS+Kn	0.5	40	14±0.08	2±0.28	1.0±0.66	No callus
	1.0	50	14±0.42	2.±0.25	1.0±0.32	No callus
	1.5	60	13±0.25	3±0.54	1.0±0.39	No callus
	2.0	70	12±0.18	3±0.56	1.3±0.28	No callus
	2.5	70	11±0.65	5±0.75	1.5±0.48	No callus
	3.0	40	12±0.43	4±0.48	1.3±0.52	No callus
MS+BAP	0.5	50	14±0.88	2±0.36	1.1±0.78	No callus
	1.0	60	13±0.22	3±0.22	1.3±0.84	No callus
	1.5	60	12±0.18	4±0.43	1.4±0.27	No callus
	2.0	70	11±0.45	4±0.38	1.4±0.76	No callus
	2.5	80	10±0.72	6±0.26	1.6±0.36	No callus
	3.0	50	12±0.32	4±0.45	1.5±0.57	No callus

The synergistic effects of cytokinins (Kn and BAP) with auxins (IAA and NAA) were also evaluated to promote regeneration frequency. Optimised ratio of cytokinins to auxins has been shown to promote shoot formation in *Sapindus mukorossi* (Singh *et al*., 2010). In present studies, among two cytokinins (Kn and BAP) optimized, combined effect of BAP with IAA and NAA was more than Kn with IAA and NAA. Similarly NAA was noticed better than IAA when used in combination with cytokinins (Kn and BAP). The maximum number of shoots were produced in the presence of BAP (2.5 mg l^{-1}) +NAA (0.25 mg l^{-1}), so this medium was designated as best shoot induction and multiplication medium (Table-2). Similar results were obtained in *Dalbergia latifolia* (Swamy *et al*., 1992), *Sapindus mukorossi* (Singh *et al*., 2010) and *Salvadora oleoides* (Laura *et al*., 2012) Regular transfer of cultures was essential to maintain healthy shoot growth. Therefore *in vitro* regenerated small shoots were excised from mother explants in the form of small clumps and were implanted in same media for elongation and growth of shoots. Similar observations were made in many species like *Wedelia chinensis* (Kameri *et al*., 2005), *Coleus blumei* (Rani *et al*., 2006) where same composition of media and hormones were used for establishment and proliferation of culture.

In vitro raised shoots were transfered to full and half strength MS medium with and without growth regulators. Rooting was not achieved in half strength MS medium with and without growth regulators. Full strength MS medium without growth regulator also did not respond to root formation. Full strength MS medium augmented with different auxins supported roots formation. Among the two auxins used, NAA was found more effective for root induction than IBA. NAA (1.0 mg l^{-1}) produced eighty per cent rooting (Table-3). Superiority of NAA in root formation has been reported in many plants like *Alpinia galangal* (Anand and Hariharn, 1997) and *Mentha piperta* (Ghanti *et al*., 2004. Intermittent callus formation was noticed at the junction of root and shoot. To reduce the callogenesis, the sucrose

concentration was reduced to 20 g l^{-1} from 30 g l^{-1}. Lower concentration of sucrose has also been found beneficial in *Acacia auriculiformis* (Das *et al*., 1993).

Table 2: Combined effects of cytokinins (Kn and BAP) with different concentrations of auxins (IAA and NAA) on nodal explants taken from mature tree

Media	Concentrations of growth regulators (mg l^{-1})	Per cent bud Break	No. of days required for bud break (±S.E.)	No. of shoots regenerated per explant (±S.E.)	Shoot length (cm) (±S.E.)	Callus growth
MS+Kn+IAA	2.5+0.25	70	10±0.65	5±0.45	1.6±0.38	No callus
	2.5+0.5	70	11±0.94	4±0.38	1.3±0.35	No callus
	2.5+1.0	60	13±0.38	3±0.74	1.1±0.45	No callus
MS+Kn+NAA	2.5+0.25	80	10±0.68	6±0.72	1.7±0.95	No callus
	2.5+0.5	70	12±0.45	5±0.50	1.5±0.28	No callus
	2.5+1.0	60	12±0.27	4±0.68	1.2±0.35	No callus
MS+BAP+IAA	2.5+0.25	80	10±0.85	7±0.46	1.7±0.44	No callus
	2.5+0.5	70	10±0.82	6±0.62	1.5±0.25	No callus
	2.5+1.0	60	12±0.32	3±0.32	1.2±0.74	No callus
MS+BAP+NAA	2.5+0.25	90	9±0.98	8±0.08	1.8±0.66	No callus
	2.5+0.5	80	10±0.86	7.0±0.35	1.6±0.35	No callus
	2.5+1.0	70	12±0.64	4±0.35	1.5±0.52	No callus

Figure1: Shoot regeneration from nodal explant on MS +2.0 mg l^{-1} Kn

Figure 2: Effect of MS+2.5 mg l^{-1}Kn+0.25 mg l^{-1} IAA on shoot proliferation in nodal explant

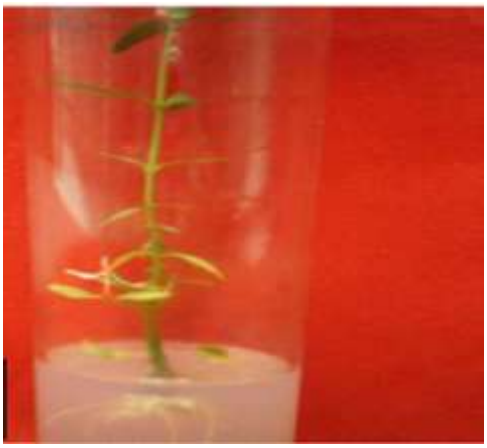

Figure3: The development of multiple shoots from nodal explants on MS+2.5 mg l^{-1} BAP+ 0.25 mg l^{-1} NAA

Figure 4: Rooting of *in vitro* regenerated shoot cultured on MS medium fortified with 1.0 mg l^{-1} NAA

Figure 4: Rooting of *in vitro* regenerated shoot cultured on MS medium fortified with 1.0 mg l^{-1} NAA

Figure 6: Establishment of plantlet under field conditions

Table 3: Effect of different concentrations of auxins (IBA and NAA) on *in vitro* regenerated shoots for root induction

	Auxins								
Auxins	Control	IBA				NAA			
Concentrations	-	0.5mg l^{-1}	1.0mgl^{-1}	1.5mg l^{-1}	2.0mg l^{-1}	0.5mg l^{-1}	1.0mg l^{-1}	1.5mg l^{-1}	2.0mg l^{-1}
Per cent root formation	-	-	-	40	30	40	80	60	60
Nature of roots	-	-	-	Delicate	Delicate	Healthy	Healthy	Healthy	Healthy

In vitro regenerated complete plants were transfered to small pots containing sterilized soil and vermiculite (1:1) and maintained under high humidity in culture room by covering them with inverted glass beaker and polythene bags. Equal ratio of soil and vermiculite has been used by Reddy *et al*., (2006) in *Azadirachta indica and Rao et al.,* (2006) in *Capsicum annum.* These pots were initially placed in the culture room. The pots were irrigated with quarter strength MS basal salts solution without sucrose on alternate days. After twenty days, the plantlets were started to expose for 3-4 hrs daily to the conditions of natural humidity and light to harden the plants. After about thirty days, plants were transfered to polyhouse and were maintained under natural conditions of day length, temperature and humidity. Finally plants were transfered to field condition with eighty per cent survival rate. The protocol reported in this study can be used for rapid and large scale multiplication of true to type plants and for conservation programmes of *Salvadora oleoides* Decne.

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