Micropropagation of *Salvadora oleoides* Decne through shoot tip explants

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Abstract: In vitro multiple shoot regeneration of *Salvadora oleoides* has been accomplished on MS medium utilizing shoot tip explants. Direct multiple shoots differentiated with in 5 weeks when explants were cultured on MS medium containing Kn and BAP individually as well as in combination with auxins. Among various concentrations of cytokinins tested, maximum shoots induction from shoot tip explants was obtained on MS medium supplemented with 2.5 mgl^{-1} BAP individually. 2.5 mgl^{-1} BAP+0.25 mgl^{-1} NAA gave better results than all other treatments used in combinations. The regenerated shoots were rooted on MS full strength medium augmented with 1.0 mgl^{-1} NAA .The regenerated plantlets were established successfully under field conditions.
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Key words- Shoot tip, *Salvadora oleoides* , Micropropagation, Cytokinins, auxins

Introduction-

Salvadora oleoides is a multipurpose tree distributed in the arid and semi-arid tracts in the state of Punjab, Haryana, Gujrat, Madya Pradesh, Rajasthan and south-western parts of Uttar Pradesh in India. It is locally known as Chhotapilu, Jhal and Pilu. The plant has a great ethno-medicinal value. Leaves of this plant are used to relieve cough and are given to horses as purgative. The fruits contain glucose, fructose, sucrose and are good source of calcium (Duhan *et al*., 1992). Fruits are fed to cattles to increase the milk yield. Fruits are also used in the treatment of enlarged spleen, rheumatism and low fever. The seeds are rich in non edible oil and their fat is used in the treatment of rheumatic pains and as a base of ointment (Anonymous, 1972). This species is decreasing very rapidly due to over exploitation, indiscriminate collection, low rate of seed set, poor viability and inefficiency to propagate by vegetative means (Khan, 1997, Khan and Frost, 2001 and Singh, 2004). Therefore, keeping in view the importance of this plant species efforts were made to develop an efficient method for rapid *in vitro* propagation using shoot tip explants via optimization of basal media, growth regulators and followed by successful outdoor establishment of regenerated plants.

Material and Methods-

Shoot tip explants were collected from young branches of mature tree. The explants were trimmed into small pieces and thereafter explants were washed with detergent under running tap water to remove dust particles followed by a dip in 90% ethanol for 2 minutes. Finally the explants were treated with freshly prepared 0.1% mercuric chloride (Hi Media Co. Mumbai) for 5 minutes under aseptic conditions and subsequently washed 5-6 times with sterile distilled water to remove all traces of mercury. MS medium (Murashige and Skoog, 1962) was used in all experiments with 3% (w/v)sucrose supplemented with various concentrations of different PGRs individually as well as in combinations. All the chemicals used were of analytical grade (Hi Media Co. Mumbai).The medium was solidified with 0.8% (w/v) extra pure agar-agar and the pH of medium was adjusted to 5.8 before autoclaving for 20 minutes at 1.06 K Pa pressure and 121^0C temperature. Culture conditions were maintained in a culture room at 25± 2^0C temperature under 16/8 hours (light/dark) period with 50 µmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes (Philips, India) and 60% relative humidity. Shoot tip explants were inoculated in MS medium supplemented with various cytokinins (Kn and BAP) at different concentrations (0.5-3.0 mgl^{-1}) either singly or in combination with auxins (IAA and NAA) in different concentration (0.25-1.0 mgl^{-1}). MS medium without any growth regulator served as control. The frequencies of explants for percent bud break, number of shoot regenerated per explant and shoot length were recorded after 35 days of culture. The multiple shoots regenerated were transferred individually as well as in the form of small shoot clumps along with original explants on a fresh medium for further growth and elongation once in 35 days, maintaining the same concentration of hormones used in shoot induction culture. Healthy and well elongated *in vitro* regenerated shoots (2-5cm) were excised from culture and transferred to rooting media composed of MS medium supplemented with different concentrations (0.5 -2.0 mgl^{-1}) of IBA and NAA. Data on percentage of rooting, mean number of roots and root length were recorded after 5weeks of shoot

transfer in rooting media. *In vitro* regenerated complete plants were taken out from medium and then these plants were transplanted in a pot containing sterile soil and vermiculite (1:1) mixture. Initially the plants were kept in laboratory conditions. All plants were watered with quarter strength MS salt solution on alternate days for 2 weeks and finally plants were shifted to polyhouse followed by field conditions with 80% survival rate.

Results and discussion-

Emergence of multiple shoots bud from shoot tip explants on MS medium supplemented with Kn and BAP was observed without an intervening callus phase in present investigation. Shoot tip explants inoculated in MS basal medium did not show any sign of shoot differentiation and morphogenesis. Addition of cytokinins (Kn and BAP) had a positive effect on shoot formation. Shoot formation from the shoot apical meristem was achieved in almost all treatments of Kn and BAP (0.5-3.0 mgl^{-1}). Among individual concentration of cytokinins, both Kn and BAP favored shoot bud differentiation but BAP was more efficient than Kn with respect to initiation and subsequent proliferation of regenerated shoots .Supermacy of BAP over Kn has been reported in *Pterocarpus marsupium* (Chand and Singh ,2004), *Mucuna pruriens* (Faisal et al.,2006),*Aegle marmelos* (Nayak *et al.,* 2007) and *Clitoria ternatea*(Singh and Tiwari ,2010). It was observed that higher percentage and higher number of shoot regeneration were found in medium supplemented with 2.5 mgl^{-1} BAP (5 shoot) followed by 2.5 mgl^{-1} Kn (4 shoots) individually. Further increase in concentration of BAP reduced the morphogenetic response in terms of percent bud break, number of days required for bud break, number of shoot regenerated per explants and length of regenerated shoots. So enhancement in concentration of growth regulators indicated an upper optimum limit beyond which morphogenetic response decreased considerably (Table-1). Higher concentration of BAP than optimum leval (2.5 mgl^{-1}) showed decline in shoot induction frequency. Similar reports have been made in *Ocimum americanum* and *Ocimum santum* (Pattnaik and Chand,1996), *Coleus blumei*(Rani et al.,2006*), Musa spp*.(Rahman et al., 2006) .

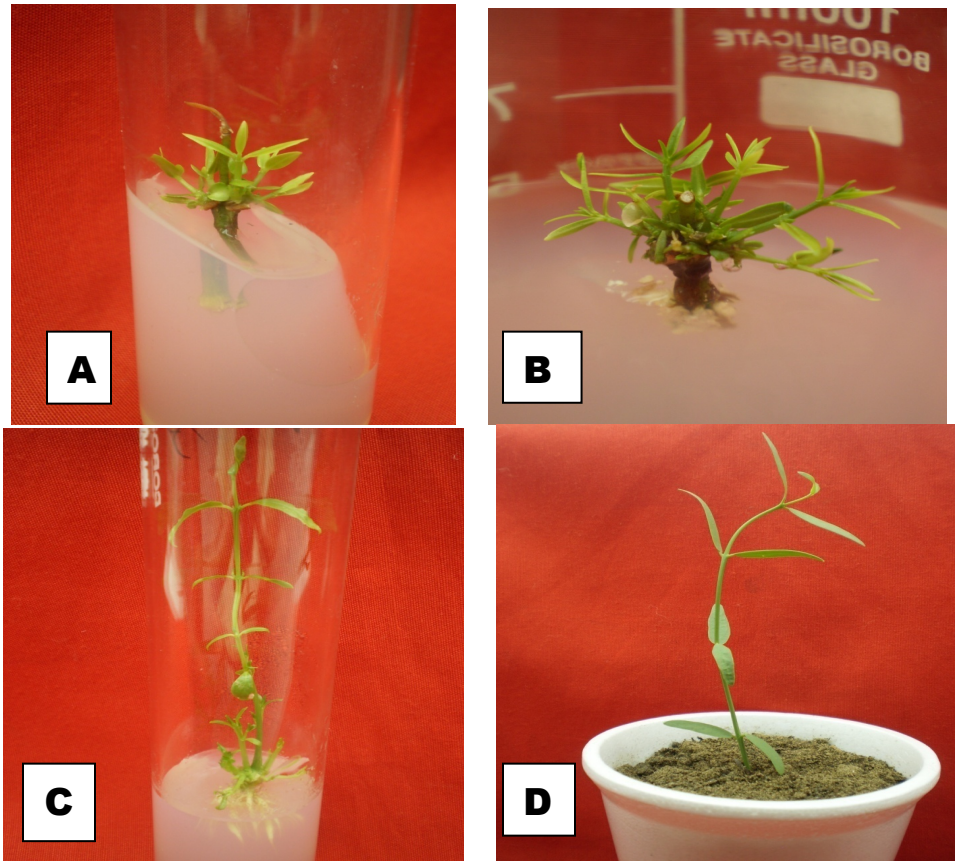
A combined effect of Cytokinins (Kn and BAP) with auxins (IAA and NAA) was also evaluated to accelerate multiple shoot induction. MS medium blended with cytokinins and auxins resulted in a significant increase in shoot length and number of shoot formed. Supplementation of NAA gave better results than IAA when used in combination with cytokinins irrespective of concentration of cytokinins (Kn and BAP).Similarly BAP showed superior morphogenetic response than Kn when both these cytokinins were used in combination with auxins (IAA and NAA). Addition of NAA along with BAP to the medium enhanced the shoot induction rate most significantly. As an optimum concentration for maximum shoot bud induction ,Kn and BAP at 2.5 mgl^{-1} with different auxins (IAA and NAA) at 0.25-1.0 mgl^{-1} were added to the media. Low levels of IAA and NAA (0.25 mgl^{-1}) in combination with cytokinins had a promotional effect on shoot bud induction whereas further higher concentrations (0.5 and 1.0 mgl^{-1}) were not found beneficial. Among all combinations of cytokinins and auxins, response in terms of percent bud break and number of shoot produced per explants was highest in MS medium supplemented with 2.5 mgl^{-1} BAP +0.25 mgl^{-1} NAA where cent percent cultures responded with highest 8 shoots per explant, and the shoots attended an average length of 2.5 cm. Since the maximum number of shoot buds was initiated in the presence of 2.5 mgl^{-1} BAP +0.25 mgl^{-1} NAA, this medium was designated as best shoot induction and multiplication medium. (Table-2) .The best stimulatory results of BAP and NAA in combination for shoot induction and proliferation were obtained in *Dalbergia latifolia* (Swamy et al.,1992), *Ficus carica* (Kumar *et al*.,1998) and *Syzygium travancorium* (Ajith *et al.,*1999). Shoots in such culture were healthy with well developed leaves. All cultures were transferred to fresh medium after regular interval of 5 weeks to get 2-5 cm long shoots. In general, it is widely accepted that apical meristems are strong zones for synthesis of auxins(IAA) therefore exogenous application is often found deleterious. So very low concentration of NAA can be better alternative of IAA in combination with cytokinins. Based upon finding of present investigation we can authentically state that exogenous application of NAA is more fruitful for morphogenetic response as compared to IAA in combination with cytokinins.

The *in vitro*-regenerated shoots (2-5 cm long), having at least two leaves and two to three nodes were excised from shoot clumps and transferred to rooting medium. Full strength MS medium without growth regulator did not respond to root formation. Presence of NAA (0.5, 1.0,1.5 and 2.0 mgl^{-1}) in MS medium facilitated better rhizogenesis as compared to similar concentration of IBA (Table-3). There was satisfactory improvement in rooting as 80% of shoots responded to rhizogenesis on full strength MS medium containing 1.0 mgl^{-1} NAA with fairly good length and number of roots. Naik *et al.,* (2000) and Chand and Singh(2004) reported that NAA was very effective auxins for rhizogenesis in *Punica granatum* and *Pterocarpus marsupium* respectively. During acclimatization and hardening, shoot elongated and leaves turned green and expanded. *In vitro* regenerated complete plants were transferred to small thermocol cups containing sterilized soil and vermiculite (1:1) mixture and

maintained under high humidity in culture room by covering them with inverted glass beaker and polyethene 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.* Then plants were transferred to poly house for 20 days to ensure acclimatization. After acclimatization in polyhouse, plants were transferred to the field conditions with 80 % survival rate. The protocol reported in this study can be used for rapid and large scale multiplication of true to type plants.

Table-1.Effects of different cytokinins (Kn and BAP) on shoot tip explants.

Media	Concentration of growth regulators(mgl^{-1})	Percent bud break	No. of shoot regenerated per explants (±S.E.)	Shoot length (cm) after35 days(±S.E.)
Control	-	-	-	-
MS+Kn	0.5	30	1±0.06	0.8±0.42
	1.0	50	1.5±0.24	1.0±0.024
	1.5	50	2±0.08	1.4±0.28
	2.0	60	3±0.24	1.5±0.16
	2.5	70	4±0.15	1.8±0.22
	3.0	50	3±0.18	1.6±0.18
MS+BAP	0.5	40	2±0.04	1.2±0.36
	1.0	60	3±0.05	1.3±0.30
	1.5	70	3±0.32	1.7±0.08
	2.0	70	4±0.43	1.8±0.14
	2.5	80	5±0.45	2.0 ±0.46
	3.0	70	4±0.64	1.7±0.34



(A) Multiple shoot formation from shoot tip explants on MS medium fortified with 2.5mg l^{-1} BAP. **(B)** Multiple shoot proliferation from shoot tip explant on MS medium supplemented with 2.5mg l^{-1} BAP + 0.25 mg l^{-1} NAA. **(C)** Rooting of *in vitro* regenerated shoot on MS medium fortified with 1.0 mg l^{-1} NAA . **(D**) Acclimatization of *in vitro* regenerated complete plant in soil and vermiculite mixture.

Table-2. Combined effects of different cytokinins (Kn and BAP) with auxins(IAA and NAA)on shoot tip explants

Media	Concentration of growth regulators(mgl^{-1})	Percent bud break	No. of shoot regenerated per explants after 35 days(±S.E.)	Shoot length (cm) after35 days.(±S.E.)
MS+Kn+IAA	2.5+0.25	80	5 ±0.46	2.0±0.38
	2.5+0.5	70	5±0.12	1.7±0.18
	2.5+1.0	60	4±0.18	1.6±0.32
MS+Kn+NAA	2.5+0.25	90	6±0.04	2.2±0.75
	2.5+0.5	80	5±0.27	1.9±0.42
	2.5+1.0	60	4±0.44	1.7±0.52
MS+BAP+IAA	2.5+0.25	90	7±0.36	2.4±0.58
	2.5+0.5	80	6±0.52	2.3±0.38
	2.5+1.0	70	5±0.32	2.1±0.26
MS+BAP+NAA	2.5+0.25	100	8±0.48	2.5±0.76
	2.5+0.5	90	7±0.53	2.3±0.54
	2.5+1.0	70	6±0.36	2.3±0.65

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

Auxins	Concentrations (mgl^{-1})	Percentage of root formation	No. of regenerated Root per shoot(±S.E.)	Root length(cm) after 35days(±S.E.)
Control	MS full strength	-	-	-
MS+IBA	0.5	-	-	-
	1.0	-	-	-
	1.5	40	3±0.40	1.3±0.32
	2.0	30	3±0.42	1.2±0.65
MS+NAA	0.5	40	4±0.54	1.2±0.62
	1.0	80	6±0.16	1.5±0.15
	1.5	60	4±0.45	1.4±0.10 1ef
	2.0	60	4±0.45	1.3±0.16

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