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A Comparative in vitro Propagation Studies on Different Explants of *Salvadora oleoides* Decne-An Endangered Plant

Surender Kumar[1,2]*, Jitender Singh Laura[1] and Narender Singh[3]

[1]Department of Bio-Sciences (Environmental Science), M. D. University Rohtak, India
[2]Department of Botany, K.M. Govt. P.G. College Narwana, India
[3]Department of Botany, K. U. Kurukshetra, India
**Corresponding author*

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A B S T R A C T

Salvadora oleoides Decne belonging to family Salvadoraceae was selected for present study. This species is declining very rapidly due to various man made adverse activities like indiscriminate collection, over exploitation and loss of habitat. Therefore, in order to conserve and propagate this species, all modern micropropagation strategies were employed for rapid multiplication of this species. In this reports biotechnological technique especially micropropagation showed significant results from propagation point of view. Various explants like shoot tips, Nodal segments and cotyledonary nodes were cultured on Murashige and Skoog's medium. There was a diverse response of different explants regarding concentration of different growth regulators. However, growth regulators used in combination supported better results in terms of per cent regeneration and number of shoots. The different explants obtained from in vitro grown seedlings, gave better results under higher concentration of growth regulators whereas explants obtained from field grown mature tree showed better results supplemented with lower concentration of growth regulators. Furthermore, explants obtained from in vitro grown seedlings bore out better in terms of per cent regeneration of shoots and number of shoot regenerated. Rhizogenesis was achieved on full strength MS medium fortified with auxins. The present research strongly recommends the maximum use of micropropagation techniques for rapid multiplication of true to type plants of *Salvadora oleoides* Decne.

Introduction

Salvadora oleoides is a multipurpose tree species found in the arid areas of north-western Indian states. It is locally known as, Jhal and Peelu Jhal. This is also known for its ethno-medicinal properties. Decoction and powder of leaves are very effective in diseases like cough, enlarged spleen and are given to horses being purgative.

The fruits are sweets in taste and rich in calcium, glucose, fructose, and sucrose content. The fruits are also effective in the treatment of piles, tumors, bronchitis, low fever and spleen diseases. The fruit are consumed by many species of insects, birds and rodents. Leaves and fruits are brought into play during child birth. The seeds are

loaded with non edible oil and their fat is utilized in the therapy of rheumatic pains and as a base of liniment (Anonymous, 1972; Kirtikar and Basu, 2012). Seed oil is widely employed in commercial production of cosmetics, paints, varnish and lubricants. Oil of this plant has key role as mosquito repellants and very proficient in killing of larvae of mosquitoes like *Culex fatigans* and *Anopheles stephensi* (Tare and Sharma, 1991). This species is ecologically very vital being drought hardy, salt tolerant and soil binder.

Besides this the species is known for restoration of fragile arid tracts, stabilization of sand dunes. Afforestation programmes on degraded lands are accompanied with *Salvadora oleoides* as dominant species. *Salvadora oleoides* endows with ecological services such as territory, victuals and cover for array of mammals, reptiles, birds and hence maintains biological equilibrium (Khan, 1996).

This species is diminishing very speedily due to unsystematic utilization, little pace of seed formation, poor seed viability (Laura *et al*., 2014). This species gives inadequate results by traditional vegetative and sexual propagation methods (Khan, 1997; Kumar *et al*., 2014a).

Consequently, keeping in observation the economic and ecological magnitude of this plant species; investigations were carried out to explore a competent system for rapid *in vitro* propagation using different explants like shoot tips, nodal segments and cotyledonary node. The investigation was focused on optimization of medium supplemented with different growth regulators individually and in combination followed by successful outdoor establishment of regenerated plants finally paving different strategies for conservation and propagation of this endangered species.

Materials and Methods

Experiments were conducted to develop protocols using various explants of *Salvadora oleoides.* Nodal explants were taken from field grown mature tree. Shoot tip explants were obtained from mature tree as well as from in vitro aseptically raised seedlings whereas cotyledonary node explants were taken from in vitro aseptically raised seedlings only. In all cases average length of explants was 1-1.5 cm except cotyledonary node explants.

All the glasswares used in this investigation were of the borosilicate quality. All the chemicals were of superior quality and analytical grade. The chemicals were procured from Merck, S D Fine, Glaxo and Hi-media laboratories. MS (Murashige and Skoog, 1962) medium was used for the present investigation. Stock solutions of major salts, minor salts, vitamins and Iron-EDTA for this medium were prepared and stored in a refrigerator. Standard methods were followed for preparation of the stock solutions. The medium was autoclaved at 15 psi (1.06 kg/cm^2) pressure for 15-20 minutes for the sterilization. After autoclaving, the culture vessels were kept at room temperature for 48 hours to ensure the aseptic conditions and then were used for culture of various explants.

The explants obtained from field grown mature tree like nodal segments and shoot tip were trimmed and then washed with liquid detergent under running tap water to remove dust particles followed by washing with distilled water to eliminate traces of detergent. The Explants were then awarded a dip in 90% ethanol. Afterward, the explants were treated with 0.1% mercuric chloride for 5 minutes under sterilized conditions and later the same were washed 4-5 times with sterile double distilled water to eradicate all traces of mercuric chloride.

For in vitro seed germination purpose, seeds were depulped and then seeds were rinsed with liquid detergent in running tap water to confiscate traces of fruit pulp, followed by assortment of healthy seeds. The seeds were exposed to natural conditions at least for a day. The seeds were then surface sterilized in 90% ethanol for 3 minutes trailed by a treatment of freshly prepared 0.1% $HgCl_2$ solution for five minutes. Lastly, seeds were washed 4-5 times in sterilized double distilled water to make seeds free from traces of mercuric chloride and inoculated in MS medium. During later stage of seedlings development, if any sign of contamination occurred; then whole culture was discarded. Laminar air flow cabinet was sterilized by UV radiation for 35 minutes before use. The explants like shoot tips and cotyledonary nodes which were taken from in vitro raised seedlings were 12-15 days old. The different explants were inoculated in culture vessels containing medium supplemented with different concentration of cytokinins (Kn and BAP) alone in combination with auxins (IAA and NAA). Individual standardized treatments of cytokinins which proved best in different explants were subsequently used in combination with auxins. So in present manuscript, emphasis has been given for documentation of standardized optimal concentrations of different growth regulators for different explants.

Culture conditions were regulated in a culture room at 25± 2^0C temperature characterized by 16 hours photoperiod and 8 hours dark period with 50 µmol m^{-2} s^{-1} photon flux density provided by cool white fluorescent tubes (Philips, India) and 60% relative humidity.

For rooting, single and multiple shoots (3-5cm length) regenerated under *in vitro* conditions were cut out aseptically and set in MS medium containing different concentrations of auxins (IBA and NAA). The regenerants with well developed roots were pulled out from the culture vessels and implanted in pots. These pots were initially kept in the culture room. High humidity was maintained for initial 20 days by covering them with inverted glass beaker and polythene bags. The pots were irrigated with quarter strength (salts only) MS medium on alternate days. Then, the plantlets were exposed to 3-4 hours daily to the conditions of natural humidity and light. Finally plants were shifted to bigger pots in greenhouse followed by transfer to the field conditions permanently.

A mean of ten replicates was assessed per treatment and each experiment was repeated thrice. All Cultures were shifted to fresh medium at regular period of four to five weeks to retain healthy shoots development on same media and hormonal concentration. Statistical analysis was done to assess the standard error (Snedecor, 1956).

Results and Discussion

Present investigation was worked out to develop a suitable protocol using different explants viz. nodal segments, shoot tip and cotyledonary node for the micropropagation of *Salvadora oleoides* Decne. Growth regulators applied externally during *in vitro* studies may disturb the internal polarity and change the genetically programmed physiology of explants resulting in the differentiation of meristematic tissue leading to callus formation or to promote direct organogenesis from the explants. That is why different explants need variable amount of growth regulator for a reproducible protocol as endogenous level of phytohormones differs from species to species and in different explants of a species also. Consequently, explants used in this

research showed mixed optimized concentration for better results in terms of per cent plant regeneration and number of shoots regenerated. In other words, application of exogenous growth regulators in present investigation had variable effects which varied with type of growth regulators, their concentration and nature of the explants.

Table.1 Standarized concentrations of plant growth regulators (cytokinins) for different explants of *Salvadora oleoides*

Explants	Concentration of plant growth regulators (kinetin)	Per cent regeneration	No. of shoots	Concentration of plant growth regulators (BAP)	Per cent regeneration	No. of shoots
Shoot tip (from mature tree)	MS+ 2.5 mg L^{-1}	70	4±0.15	MS+ 2.5 mg L^{-1}	80	5±0.45
Nodal segment (from mature tree)	MS+ 2.5 mg L^{-1}	70	5±0.75	MS+ 2.5 mg L^{-1}	80	6±0.26
Shoot tip (from in vitro grown seedlings)	MS+ 3.0 mg L^{-1}	100	6±0.28	MS+ 3.0 mg L^{-1}	100	8±0.36
Cotyledonary node (from in vitro grown seedlings)	MS+ 4.0 mg L^{-1}	100	9±0.54	MS+ 4.0 mg L^{-1}	100	10±0.58

Table.2 Standarized concentrations of Kinetin +IAA and Kinetin +NAA for different explants of *Salvadora oleoides*

Explants	Concentration of plant growth regulators (kinetin+ IAA)	Per cent regeneration	No. of shoots	Concentration of plant growth regulators (kinetin + NAA)	Per cent regeneration	No. of shoots
Shoot tip (from mature tree)	MS+ 2.5 + 0.25 mg L^{-1}	80	5 ±0.46	MS+ 2.5 + 0.25 mg L^{-1}	90	6±0.04
Nodal segment (from mature tree)	MS+ 2.5 + 0.25 mg L^{-1}	70	5±0.45	MS+ 2.5 + 0.25 mg L^{-1}	80	6±0.72
Shoot tip (from in vitro grown seedlings)	MS+ 3.0 + 0.5 mg L^{-1}	100	7±0.72	MS+ 3.0 + 0.5 mg L^{-1}	100	8±0.58
Cotyledonary node (from in vitro grown seedlings)	MS+ 4.0+0.5 mg L^{-1}	100	11±0.33	MS+ 4.0+0.5 mg L^{-1}	100	13±0.35

Table.3 Standarized concentrations of BAP + IAA and BAP +NAA for different explants of *Salvadora oleoides*

Explants	Concentration of plant growth regulators (BAP+IAA)	Per cent regeneration	No. of shoots	Concentration of plant growth regulators (BAP+NAA)	Per cent regeneration	No. of shoots
Shoot tip (from mature tree)	MS+ 2.5 + 0.25 mg L^{-1}	90	7±0.36	MS+ 2.5 + 0.25 mg L^{-1}	100	8±0.48
Nodal segment (from mature tree)	MS+ 2.5 + 0.25 mg L^{-1}	80	7±0.46	MS+ 2.5 + 0.25 mg L^{-1}	90	8±0.08
Shoot tip (from in vitro grown seedlings)	MS+ 3.0 + 0.5 mg L^{-1}	100	9±0.46	MS+ 3.0 + 0.5 mg L^{-1}	100	10±0.36
Cotyledonary node (from in vitro grown seedlings)	MS+ 4.0+0.5 mg L^{-1}	100	13±0.23	MS+ 4.0+0.5 mg L^{-1}	100	14±0.43

Figure.1 Multiple shoots originated from shoot tip explant taken from in vitro grown seedlings supplemented with 3.0 mg L^{-1} kinetin. **Figure-2**. The development of multiple shoots from shoot tip explant taken from in vitro grown seedlings on 3.0 mg L^{-1} BAP. **Figure-3**. Root formation in medium fortified with 1.0 mg L^{-1} NAA. **Figure.4** Acclimatization of plant under natural conditions.

Table.4 Rhizogenetic effects of optimized concentration of IBA and NAA

Auxins	Concentrations of auxins (mg L^{-1})	Percentage of root induction
MS+IBA	1.5	40
MS+NAA	1.0	80

After the development of MS medium, the technique of plant tissue culture has been successfully used for mass multiplication of many economically important and endangered plant species. The proliferation of shoots might be due to the supply of adequate amount of cytokinins in the nutrient medium which nullify the effect of apical dominance and enhanced the proliferation of lateral buds (Hu and Wang, 1983). The present study also showed that of the two cytokinins (Kn and BAP) used, BAP was more effective than Kn for organogenesis (Table-1). Supremacy of BAP over Kn has been reported in many species like *Leucaena leucocephala* (Nangia and Singh, 1996); *Prosopis cineraria* (Kumar and Singh, 2009); *Salvadora oleoides* (Laura *et al*., 2012).

The synergistic effects of cytokinins (Kn and BAP) with auxins (IAA and NAA) were also evaluated to promote regeneration frequency. Combined effect of cytokinins with auxins has been demonstrated in many plants such as *Gymnema sylvestre* (Reddy *et al*., 1998), *Salvadora oleoides* (Singh *et al*., 2012). Shoots regeneration in the medium equipped with auxin (NAA) along with cytokinins has been established in *Berberis thunbergii* (Karhu and Hakala, 1991), *Salvadora oleoides* (Kumar *et al*., 2014b). Higher concentration of auxins in the medium did not improve the response of shoot formation. This inhibition of shoot formation may be attributed to the action of accumulated auxins at the base end of the explant (Mark and Simpson, 1994). Low levels of IAA and NAA in combination with cytokinins had a promotional effect on shoot bud induction whereas further higher concentrations were not found beneficial indicating the threshold level of exogenous supply of hormones. These results presumably indicated a threshold level of endogenous auxins in the explants also (Julliard *et al*., 1992). Among two cytokinins optimized, combined effect of BAP with IAA and NAA was more than Kn with IAA and NAA. Similarly, NAA was evaluated better than IAA when used in combination with cytokinins (Table 2 and 3). Combined effect of cytokinins with auxins has been demonstrated in *Salvadora oleoides* (Kumar *et al*., 2014c).

During rhizogenesis, NAA was reported more effective than IBA (Table-4). This may be due to the variation in the route of auxins uptake (De-Klerk *et al*., 1997). Similarly, Singh and Lal (2007) also achieved root induction on MS medium fortified with 1.0 mg L^{-1} of NAA in *Leucaena leucocephala*. Finally, the regenerated plantlets after sufficient root length were acclimatized by gradual shifting to small pots under high humidity. The complete plants were then shifted to field condition with 80% survival rate. So this research indicates the role of plant tissue culture in multiplication of *Salvadora oleoides* using different explants.

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