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EFFECT OF ADENINESULPHATE ON ORGANOGENESIS VIA LEAF CULTURE IN AN ORNAMENTAL PLANT: *THEVETIA PERUVIANA* (PERS.) SCHUM.

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ABSTRACT

The paper presents protocol for rapid, reliable and high frequency regeneration of plant via the culture of leaf explants in *Thevetia peruviana* (Pers.) Schum. *In vivo* leaves cultured on MS medium supplemented with a combination of 2,4-D (2.5 mg/l) and Kn (1.2 mg/l) produced stock callus after 20-28 days of inoculation. The obtained callus was dark green and friable. The small pieces of stock callus cultured on MS medium fortified with 2,4-D (2.0 mg/l), Kn (1.0 mg/l) and $AdSO_4$ (0.25 mg/l) for the morphogeneic responses. After 30 days of incubation period, emergence of 3-4 microshoots from the callus was observed. Multiple shoots separated from the cluster and subcultured for their elongation on the same medium used for the multiplication along with BAP (3.0 mg/l). *In vitro* elongated shoots rooted on MS medium supplemented with IBA (0.5 mg/l). The plantlets were hardened, acclimatized and transplanted in the field, where they showed 60% survival rate.

KEYWORDS

Thevetia peruviana (Pers.) Schum. Callus, organogenesis, leaf explants, MS medium.

Abbreviations

BAP- 6- Benzylamino purine, 2,4-D -2, 4-dichlorophenoxyacetic acid, Kn- 6-furfurylamino purine, IBA- Indole 3-butyric acid, $AdSO_4$ – Adenine sulphate, Medium- Murashige and Skoog medium.

INTRODUCTION

Plant tissue culture technology has been available to the plant breeders for nearly four decades and has been extensively employed for crop improvement in several crops. *Thevetia peruviana* (Pers.) Schum. (Family - Apocynaceae), commonly known as yellow oleander and Lucky nut. It is a small ornamental tree, which grows up to a height of about 10 to 15 feet. *Thevetia peruviana* is a plant probably native to Central and South America but now frequently grown throughout the tropical and sub- tropical region of the world. It is an evergreen small tree that bears yellow, trumpet like flowers and its fruit is deep red/black in color encasing a large seed that bears some resemblance to a Chinese "lucky nut." It contains a milky sap containing a compound - thevetin that is used as a heart stimulant but in its natural form it is extremely poisonous, as all parts of the plants, especially the seeds. It has immense medicinal

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properties like cardiotonic, oedema, diuretic, emetic and purgative, used in different kind of intermittent fever. This plant is particularly known for its ability to produce cardiac glycosides, such as nerrifolin and peruvoside, which have a relatively high therapeutic index compared to that of digoxin (Mantu and Sharma,1980). All parts of the plant are poisonous, especially the kernels of the fruit. Flavonol glycoside from leaves of *Thevetia peruviana* has inhibitory effect against HIV-1 reverse transcriptase and HIV-1 Integrase (Twetrakul *et al.*, 2002). It is one of the most poisonous plants that contain numerous toxic compounds which are proved deadly for humans and animals in even small quantities. Because of its poisonous nature it is only used externally. Few reports are available regarding the tissue culture of this medicinally potent plant species i.e. somatic embryogenesis (Kumar, 1992). However, plenty of work is done in response to explore its medicinal value by various scientists (Eddleston *et al.*, 2000; Dhanalakshmi and Lakshmanan, 1992).Keeping in view the potential action against various ailments and its regeneration problems in natural environment, micropropagation of this medicinally important plant was accomplished to provide stable supply of the *in vitro* raised plantlets for the procurement of anticancerous compounds.

MATERIALS AND METHODS

Plant Material

The plant of *Thevetia peruviana* (Pers.) Schum. grown in Mansarovar, Jaipur was used as an experimental material. The leaves (1-2 cm. in diameter) were used as explants for growth of callus from one and half year old field grown plant.

Surface Sterilization

Explants were treated with an appropriate sterilizing agent to kill contaminating microbes present on their surface. Leaves were thoroughly washed under running tap water for about 20 minutes and were pretreated with 1.0% (v/v) Teepol solution for 2-5 minutes. Priors to inoculation, explants were surface sterilized in 0.1% (w/v) aqueous mercuric chloride solution for 5 minutes followed by rinsing at least thrice with sterilized double distilled water.

Culture Media and Culture Conditions

Basal media consists of MS salts (Murashige and Skoog, 1962), 3.0% sucrose as carbon source and 0.8% agar as solidifying agent was prepared. The medium was supplemented with various concentrations (0.5- 5.0 mg/l) of different auxins (IAA,IBA,NAA and 2,4-D) and cytokinins (BAP and Kinetin).The pH of the medium was adjusted to 5.8±0.02 before autoclaving at 15 pounds per square inch (15 psi) for 20 minutes. 30 ml of medium was dispensed in to culture vials and it was allowed to solidify and the surface sterilized explants inoculated on MS medium with plant growth regulator under UV light treated laminar airflow chamber. The cultures were maintained at 26±2ºC, 16 hour's photoperiod in the growth chamber at 2000-2500 lux light intensity of cool, white and fluorescent light and 50±5% relative humidity. For all the experiments eight replicates were prepared and each experiment was repeated at least thrice.

Callus Induction and Shoot Regeneration

Leaf explants cultured on MS medium along with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mg/l) of 2,4-D and Kn either alone or in combination. Combinations of 2,4-D and Kn favoured the production of stock callus. The developed calli were dark green and friable.

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In vitro morphogenesis

Little amount of callus was subjected to see the effect of adenine sulphate along with optimized 2, 4-D and Kn for morphogenic responses. Adenine sulphate initiated shoot formation from the callus clumps, but it did not favour multiple shoot formation. For the attainment of multiple shoots, the initiated shoots were subcultured on MS medium fortified with different concentrations of BAP (1.0-5.0 mg/l) along with optimized hormones and $AdSO_4$. BAP was found to be favourable for the induction of multiple shoots.

These multiple shoots were then separated and transferred on root induction medium fortified with various concentrations of IBA (0.1- 4.0 mg/l). About 89% of rooting was achieved on MS medium supplemented with IBA (0.5 mg/l). The complete plantlets were then taken out from the culture vials and were washed delicately to remove adhering agar and potted in small polycups containing sterilized soil and vermicompost 3:1. The plantlets were then hardened by keeping covered the plantlets with inverted glass beaker onto the polycups to maintain high humidity. After 3-4weeks, the beakers were gradually removed and the pots containing plant exposed to partial shade for a short time, this time of exposure in the natural condition was increased simultaneously. Finally, the plants were transplanted in the natural environment.

RESULTS

1-2 cm. leaf explants inoculated on MS medium along with a combination of 2, 4-D and Kn for the production of stock callus. 2 4-D (2.5 mg/l) and Kn (1.2 mg/l) was found to be optimum for the induction of stock callus (Table-1, Fig-A, and B). The callus was maintained by subculturing on to the same medium along with hormones. The small pieces of the callus was cultured on MS medium supplemented with 2, 4-D (2.5 mg/l), Kn (1.2 mg/l) and $AdSO_4$ (0.25 mg/l) for the morphogenic responses (Table- 2, Fig-C). $AdSO_4$ only initiated microshoots emergence after 30 days of incubation, the number of shoots maximized up to (6.89±1.05) after the transfer of these initiated shoots on MS medium along with optimized hormone, $AdSO_4$ and BAP (3.0 mg/l). The incorporation of this BAP increased the number of shoots in *in vitro* conditions. These microshoots were elongated on the same medium, when the clump of regenerating shoots subsequently subcultured on the medium (Table-3, Fig-D). These shoots reached up to a length of 4-5 cm. on this medium. These *in vitro* elongated shoots were isolated from the cluster and transferred to root induction medium, which was composed with MS medium fortified with IBA (0.5 mg/l). Roots developed were lengthy, brownish in colour with root hairs (Table- 4, Fig-E). The presence of root hairs increased the frequency of absorption of the nutrients from the medium; so as to plant grew at faster rate. Complete plantlets were taken out from the culture vials, and hardened and acclimatized by the technique described in “materials and methods” (Fig-F).

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FIGURE

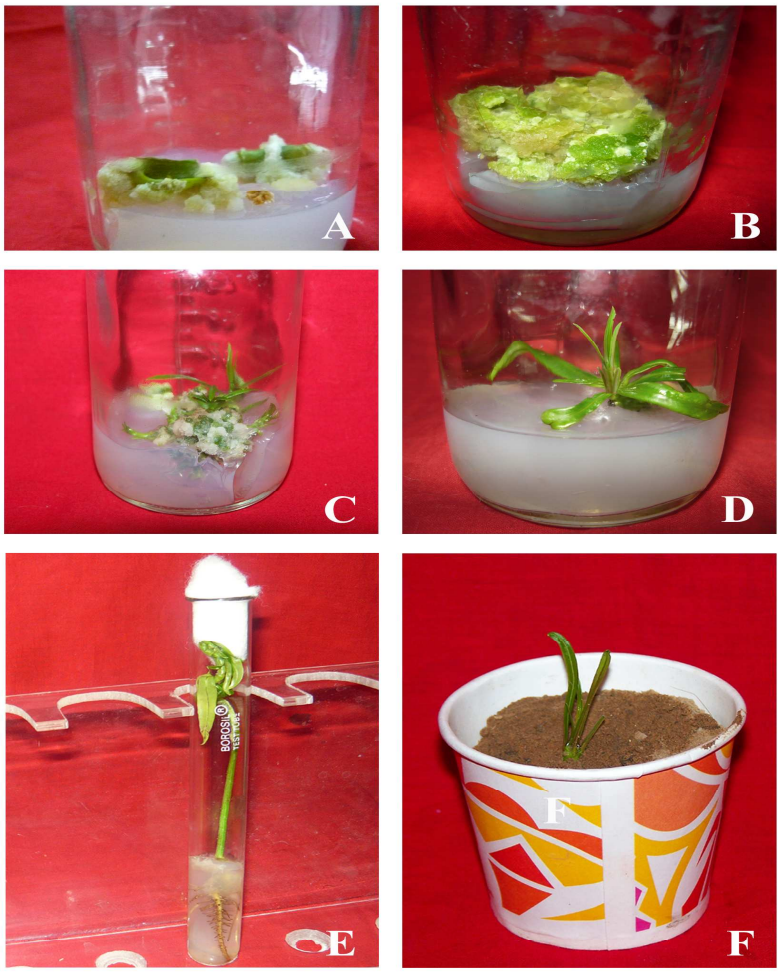

Fig. A: Initiation of callus from leaf explant on MS medium+ 2,4-D (2.5mg/l) and Kn (1.2mg/l).
Fig.B: Stock callus obtained with in 20-28 days on MS medium+ 2,4-D (2.5mg/l) and Kn (1.2mg/l).
Fig.C: Shoot formation from callus clumps on MS medium + 2,4-D (2.5 mg/l), Kn (1.2mg/l) and $AdSO_4$ (0.25mg/l).
Fig.D: Separated shoots on same MS medium along with BAP (3.0 mg/l) for their elongation.
Fig.E: *In Vitro* root induction on MS medium supplemented with IBA (0.5mg/l).
Fig. F: Hardened plantlet of *Thevetia peruviana*.

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Table 1.
Effect of different conc. of 2,4-D and Kn for the induction of callus from leaf explant in T.peruviana.

2,4- D (mg/l)	Kn (mg/l)	Intensity of callus induction	Nature of callus
0	-	-	No callus formation
0.1-0.5	-	-	No callus formation
2.0-2.5	-	+	White coloured, fragile
3.0-3.5	-	++	Light green coloured, fragile
4.0	-	-	Swelling of the leaf explant observed
0.1-0.5	1.0	-	No callus formation
2.0	1.0	++	Green coloured, fragile
2.5-3.0	1.0	++	Light green coloured, fragile
3.5	1.0	+	White coloured, fragile
4.0	1.0	-	No callus formation
0.1	1.2	-	No callus formation
0.5-2.0	1.2	+	White coloured, fragile
2.5	1.2	+++	Green coloured ,fragile
3.0	1.2	++	Light green coloured, fragile
3.5	1.2	+	White coloured, fragile
4.0	1.2	-	Swelling of the explant observed.

+: slight callusing;++: moderate callusing;+++: Maximum callusing

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Table 2.
Effect of different concentrations of PGRs added to MS medium On induction of callus and regeneration of shoots from leaf of T.peruviana after 30 days.

PGR (mg/l)			% Response of Callusing	% shooting Regeneration
2,4-D (mg/l)	Kn (mg/l)	$AdSO_4$(mg/l)		
3.5	0	0.25	15	2
3.0	0	0.25	25	5
2.5	1.0	0.25	30	4
2.0	1.2	0.25	72	6
1.5	1.2	0.25	60	3
1.0	1.2	0.25	12	1

*: Values are 95% confidence limits for Mean

Table 3.
Effect of different concentrations of BAP for multiple shoot regeneration from callus of T. peruviana

BAP (mg/l)	**Mean number of shoots produced per explants Mean± $t_{0.05}$ S.E. (X bar)**
1.0	0.12± 0.10
2.0	1.89 ± 0.45
3.0	6.89 ± 1.05
4.0	3.22 ± 0.21
5.0	1.87 ± 0.35

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Table 4.
Effect of different concentrations of IBA for root induction in T. peruviana

IBA (mg/l)	Days taken for root induction	Frequency of Rooting (%)
0.1	12	10
0.2	12	20
0.3	11	40
0.4	13	45
0.5	20	60
0.6	11	35
0.7	12	25

DISCUSSION

Callus is an unorganized mass of plant cells and its formation is controlled by growth regulating substances present in the medium (auxins and cytokinins). The specific concentration of plant growth regulators needed to induce callus, varies from species to species and even depends on the source of explant. In *T. peruviana,* green and friable callus was obtained on MS medium supplemented with 2, 4-D (2.5 mg/l) and Kn (1.2 mg/l) in combination. It has been demonstrated in many cases that 2, 4-D along with low concentration of cytokinin is usually the choice for *in vitro* callus induction in many plant species e.g. ryegrass (Bradley *et al.*, 2001). However, Singh *et al*., (2009) in *Rauvolfia serpentina* L. and Gul *et al.* (2008) in *Amsonia orientalis* Decne. reported BAP, IAA and BAP, NAA in combination was proved to be the best for *in vitro* callus induction, respectively.

The initiated callus were then allowed to grow on shoot induction medium with increasing concentration of 2, 4-D and Kn along with $AdSO_4$ as an additives. $AdSO_4$ was proved to be best for shoot differentiation was also shown by Hussain et al. (2009) in *Melia azedarach* L., Nandagopal *et al*, (2006) in *Cichorium intybus* L.cv. Focus and Beegum *et al*. (2007) in *Ophiorrhiza prostrate* but Salma *et al*. (2008) reported that for shoot initiation from callus in *Rauwolfia serpentina* L. Benth BAP and NAA was proved to be best.

The regenerated shoots were then multiplied on BAP (3.0 mg/l), which is found to be in consonance with the results obtained by Patil *et al*. (1997) in *Rauvolfia micrantha* and *Rauvolfia tetraphylla* but in

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Catharanthus roseus BAP along with NAA was proved to be the best for multiplication of shoots. Frabetti *et al.*, (2009) in *Teucrium fruticans* L. and Swanberg *et al*., (2008) in *Catharanthus roseus*.

The well developed shoots were then transferred to rooting medium supplemented with IBA (0.5 mg/l), was proved to be the optimum for *in vitro* rooting. Various other scientists also favoured the results obtained during the present study e.g. Faisal *et al*. (2006) in *Rauvolfia tetraphylla* L.; Raha *et al*. (2001) in *Holarrhena antidysenterica* wall and Sharma and Batra (2006) in *Withania somnifera*. In contrast to this, Sarma *et al.*, (1999) in *Rauvolfia tetraphylla* L. obtained rooting on NAA, on IAA in *Cardiospermum halicacabum* L. and *Azadirachta indica*, respectively (Anushi *et al*., 2009; Shekhawat *et al* ., 2009).

The rooted plantlets were taken out from the culture vials and washed with distilled water delicately to remove adhering agar medium to reduce the chances of contamination. Then, these plantlets were transferred to the polycups containing garden soil and vermicompost. They were hardened and acclimatized and are finally transplanted to natural surrounding.

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