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RESEARCH ARTICLE

# Efficient protocol for *in vitro* callus induction and indirect plant regeneration of *Solanum viarum* (Dunal) - An important anticancer medicinal plant

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**Abstract**: In the present investigation, an attempt has been made to standardize a protocol for rapid callus induction and plantlet regeneration from young leaves and in nodal explants of *Solanum viarum* (D) was described. For *in vitro* callus induction auxins such as IAA, NAA and 2, 4-D in combination with cytokinins like BAP and Kn were used. High frequency of greenish colored, fragile to nodular organogenic calli was obtained in nodal explants cultured on MS medium supplemented with NAA (3.0 mg/l) and BAP (0.5 mg/l). Indirect shoot organogenesis was achieved from the callus using BAP (2.0 mg/l) and NAA (0.5 mg/l) induced maximum frequency of shoot regeneration (96%) with the maximum shoot number (16.2±0.14). All the *in vitro* raised shoots with the length of 3.0-5.0 cm were transferred to rooting medium supplemented with different concentrations of auxins such as IAA, NAA and IBA (0.5-2.0 mg/l). The best rooting response with high rooting frequency (96%) with the highest number of roots (18.3±0.21) were obtained on IBA (1.0 mg/l). The well rooted plantlets were transferred to polycups containing soil and vermiculite in 1:1 ratio for hardening. Finally the hardened plantlets were transferred to field conditions. Rooted shoots showed the (98%) of survivability.

**Keywords**: Callus induction; indirect organogenisis; 2, 4-D; *Solanum viarum*.

**Abbreviations**: BAP: Benzyl amino purine; Kn: 6-Furfuryl amino purine; 2,4- D: 2, 4-Dichloro phenoxy acetic acid; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; NAA: Naphthalene acetic acid.

## Introduction

Plant tissue culture has been identified as an excellent surrogate method to overcome the problems connected with utilization and conservation of medicinal plants (Bajaj et al. 1988). Micropropagation is a substitute method to the conventional methods of vegetative propagation with the unbiased of improving the rate of multiplication (Kaur 1998). For increasing in production and productivity plant tissue culture has been noticed as an important technology for enhancing the competence of selected best high yielding varieties.

A callus culture system offers many advantages as a model system for several biological investigations. Callus cultures have been used widely in various physiological and related studies in the genus *Rosa* (Weinstein et al. 1962) and in the genus *Citrus* (Altman et al. 1982). Even callus has proved better for the synthesis of alkaloids in several cases (Bhat 1995)

*Solanum viarum* (Dunal) belongs to the family Solanaceae, commonly referred as Tropical soda apple is one of the multipurpose medicinal plant. Because of its alkaloid substances, the solution of the boiled-leaf of this wild plant is used as a drug in Senegal by early adolescences. In another part in West Africa, Chad, a solution of *S. viarum* roots is locally used for tooth treatment (Aliou Diongue et al. 2005). In India it was moreover regarded as a medicinal crop (Talekar 1999). Solasodine existent in the fruits is used as a precursor to yield complex steroidal compounds and contraceptive pills (Babu and Hepper 1979). Steroids formed by the plant have been used for the treatment of rheumatic arthritis, addison's disease, cancer, leukemia, chronic asthma, obesity and additional skin diseases (Pingle and Dhyansagar 1980). Considering its high economical and pharmacological importance of secondary metabolites industries are intensely fascinated in exploiting the plant tissue culture technology for large scale produc-

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tion of these substances (Misawa 1994). Indirect shoot regeneration using leaf explants was well explained in *Spilanthes acmella* (Saritha et al. 2003), *Azadirachta indica* (Reddy and Naidu 2008). In the present investigation was performed to determine the role of different plant growth regulators on *in vitro* callus induction, multiple shoot regeneration using young leaf, nodal explants of *S. viarum*.

## Materials and methods

### *Plant material and surface sterilization*

Young leaf and nodal segments excised from the *in vitro* regenerated shoots of *S. viarum* were used as explants as a prerequisite for experiments. Hence the seeds were collected from mature and dried fruits of healthy plants of *S. viarum* that are erudite in the herbal garden, Dravidian University, Kuppam, A. P., India. Matured seeds were first washed under running tap water for 30 mins to eliminate any adherent fruit tissue and dried juice which might serve as an agent for fungal infection. Then the seeds were thoroughly washed with bavistin 0.4% (w/v) a fungicide under sterile conditions for an hour and washed thrice with sterile distilled water. This was followed by surface sterilization with 5% Tween-20 (v/v) for 15 mins and the seeds were sterilized in 70% alcohol (v/v) for 2 mins. Then the seeds were washed with 0.1% $HgCl_2$ (w/v) for 2 mins. Finally the seeds were washed thrice with sterile distilled water to remove the traces of $HgCl_2$.

### *Culture medium and culture conditions*

The seeds were inoculated on MS medium (Murashige and Skoog 1962) containing 0.8% (w/v) agar supplemented with various concentrations of cytokinins (BAP and Kn) and auxins (NAA, IAA and IBA) prior to autoclave, the medium was adjusted to the pH of 5.8 and sterilized for 20 mins at 121˚C for 15 lbs pressure. The culture room conditions maintained for *in vitro* cultures were 26˚C ± 2˚C and 60 to 70% relative humidity. Light intensity was 3000 lux with a photoperiod of 18 hours day light and 6 hours in dark.The primary shoots formed *in vitro* were separated aseptically and cultured on MS medium supplemented with BAP (2.0 mg/l).

### *Sub culturing*

Young leaf and nodal explants (1.0-2.0 cm) were excised from the *in vitro* regenerated shoots of *S. viarum* were inoculated on MS medium containing 3.0% sucrose (w/v) and gelled with 0.8% agar (w/v) supplemented with various concentrations of auxins such as (NAA, 2,4-D and IAA) in combination with cytokinins like (BAP and Kn) were used for callus initiation and shoot proliferation. The cultures were sub cultured by regular intervals of 21 days on fresh MS medium for the development of shoot proliferation and *in vitro* rooting.

The frequency of callus induction was calculated by applying the formula

$$\text{Callus induction frequency (\%)} = \frac{\text{No.of internodal explants produced calli}}{\text{No.of internodal explants cultured}} \text{X100}$$

### *Data analysis*

Visual observations were recorded on the frequency in terms of number of cultures responding for axillary shoot proliferation, shoot development, number of shoots per explant, average length of the regenerated shoots, number of roots per shoot and average root length.

### *Statistical analysis*

All the experiments were conducted with a minimum of 20 explants. All assays were repeated at least three times. The experimental data were statistically analyzed by one-way ANOVA using the DMRT (Duncan's Multiple Range Test) ($P < 0.05$) and were presented as the mean ± standard error (SE).

## Results and Discussion

Young leaf and nodal explants of *S. viarum* were cultured on MS medium supplemented with various concentration of auxins such as (NAA, 2,4-D and IAA) in combination with cytokinins like (BAP and Kn). Callus tissues were initiated from both leaf and nodal explants after two weeks of inoculation.

*Influence of auxin: cytokinin on callus induction*

*Effect of NAA, 2,4-D and IAA in combination with BAP on callus induction from leaf and nodal explants***.**

Callus induction was observed on MS medium supplemented with different concentrations of NAA 2, 4-D and IAA in combination with BAP. Callus initiation was achieved from the explants within 10-15 days of inoculation, a wide range of variation in frequency of callus formation and nature of callus was observed. Initially leaf folding and bulging of nodal was observed. At lower concentration of NAA in combination with BAP the leaf explant produced high intensity, brown color callus, whereas at higher concentrations dark green callus which are compact and organogenic in nature was formed. Nodal explants produced the greenish color, complexity, nodular in nature of callus on NAA (3.0 mg/l) and BAP (0.5 mg/l) supplemented medium. (Table1). Callus initiation was also observed on MS medium supplemented with 2, 4-D and IAA in combination with BAP from the young leaf and nodal explants at lower concentrations of auxins light brown fragile in nature and at higher concentration light green, compact nature of callus was observed.

**Table 1**: Effect of different concentrations of auxins and cytokinins on callus induction from *in vitro* cultured leaf and nodal explants of *S. viarum*.

| Plant growth regulators (mg/l) | | | | | Type of explant | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | Leaf | | Node | |
| NAA | 2,4-D | IAA | BAP | Kn | Intensity of callus formation | Nature of callus | Intensity of callus formation | Nature of callus |
| 0.5 | - | - | 0.5 | - | ++ | Light brownish, fragile | + | Brownish , fragile |
| 1.0 | - | - | 0.5 | - | ++ | Yellowish green, compact | ++ | Light brownish green, fragile |
| 2.0 | - | - | 0.5 | - | ++ | Light green, compact | +++ | Green, nodular |
| 3.0 | - | - | 0.5 | - | +++ | Dark green, compact | +++ | Greenish, organogenic |
| 0.5 | - | - | - | 0.5 | + | Cremish green, fragile | + | Cremish brown, fragile |
| 1.0 | - | - | - | 0.5 | ++ | Light green, nodular | + | Light brownish green, fragile |
| 2.0 | - | - | - | 0.5 | ++ | Light green, fragile | ++ | Light green, nodular |
| 3.0 | - | - | - | 0.5 | +++ | Dark green, nodular | ++ | Green, nodular |
| - | 0.5 | - | 0.5 | - | + | Light brownish, fragile | + | Whitish green, fragile |
| - | 1.0 | - | 0.5 | - | + | Light brown, fragile | + | Light brown, fragile |
| - | 2.0 | - | 0.5 | - | ++ | Light brown, fragile | ++ | Light brown, compact |
| - | 3.0 | - | 0.5 | - | ++ | Light green, compact | ++ | Light brown, compact |
| - | 1.0 | - | - | 0.5 | ++ | Light brown, fragile | + | Light green, fragile |
| - | 2.0 | - | - | 0.5 | ++ | Light brown, fragile | ++ | Light green, fragile |
| - | - | 1.0 | 0.5 | - | ++ | Light green, fragile | + | Light brown, fragile |
| - | - | 2.0 | 0.5 | - | ++ | Light green, nodular | ++ | Light brown whitish, fragile |
| - | - | 1.0 | - | 0.5 | + | Light green, nodular | + | Cremish white, fragile |
| - | - | 2.0 | - | 0.5 | ++ | Dark green, nodular | ++ | Yellow green, fragile |

Callus is an undifferentiated and unorganized mass of parenchyma cells formed by the propagation of parent tissue. Callus tissue is a good basis of genetic variability and adventitious shoot formation (Dodds and Roberts 1982). Callus started from the cut portions of the explant, where cells at the cut ends undergo mitosis, which leads to callus formation. It may be due to wound reaction or effect of exogenous growth regulator. The surface of callus varied according to the nature of cytokinin and also on auxin: cytokinin ratio (Martin 2002). For the establishment of optimal concentration of growth hormones for the production of callus various combinations of auxin: cytokinin were tested, among the different combinations tested, NAA and BAP proved to be the better in terms of inducing high frequency of greenish organogenic callus formation. Similar results were reported in *Stevia rebaudiana* (Preethi et al. 2011). Higher concentration of NAA and BAP induced callus formation compare to lower concentration. Similar observations were reported in *Biophytum sensitivum* (Shivanna 2009).

In 2,4-D and BAP supplemented MS medium explants showed the light brown to dark brown color callus development. Brown color of callus showed compassion to plant tissues to 2, 4-D. The color is being mostly influenced by the site of phenolic secondary metabolites in cells. If the deposit of the phenolics in the cytoplasm it undergoes oxidation and polymeriza-

tion and oxidized products appear in brown color (Lukas 2000).

*Effect of NAA, 2,4-D and IAA in combination with Kn on callus induction from leaf and nodal explants.*

Callus induction was also observed on MS medium supplemented with different concentrations of NAA 2,4-D and IAA in combination with Kn. Both the leaf and nodal explants induced the moderate callus formation. The leaf explants at the higher concentration of NAA (3.0 mg/l) and Kn (0.5 mg/l) induced the high intensity of callus with color complex of dark green, nodular in nature. IAA in combination with Kn induced the low and moderate intensity of callus formation with the color complex ranging from whitish to yellow and callus was fragile in nature. Among the different combination of auxin: cytokinin tested, NAA and BAP proved to be the better in terms of inducing high frequency of green, compact and organogenic nature of callus using nodal explants of *S. viarum.*

Callus initiation was also observed on MS medium supplemented with different auxins such as 2,4-D and IAA in combination with cytokinin like Kn. where green nodular to organogenic callus was formed. Similar response of callusing report was noted in *Justicia gendarussa* (Agastian 2006) and *Erythrina variegata*. (Shasthree 2009).

*Indirect shoot organogenesis from nodal derived callus.*

Induction of callus was essential for the vegetative plant proliferation. Organogenic callus was observed on nodal explants only. Callus initiation was attained from the explants within 10 days of incubation on MS basal medium with any one of the auxins like NAA, IAA and in combination with either cytokinin BAP or Kn.

Plant propagation through callus required the induction of organogenic callus and it is the prerequisite for adventitious shoot formation and also for other *in vitro* genetic improvement including induction of somaclonal variations and embryoids. The presence of cytokinin along with auxin is necessary for indirect shoot induction was noted (Skoog and Miller 1957).

*Effect of BAP on indirect shoot regeneration from callus*

The presence of cytokinin along with auxins is essential for indirect adventitious shoot induction. The induction of callus and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators such as BAP, Kn, NAA and IAA in the culture medium. Among the growth regulators tested BAP (2.0 mg/l) and NAA (0.5 mg/l) were induced maximum frequency of shoot regeneration (96%) with the maximum shoot number (16.2±0.14). But the maximum shoot length (6.3±0.24 cm) was observed on BAP (0.1 mg/l) and IAA (0.5 mg/l). The minimum regeneration frequency (60%) and shoot number (1.5±0.23) were noted on (BAP 0.5 mg/l). Minimum shoot length (2.3±0.30 cm) was noted at BAP (3.0 mg/l) alone. Kn when used alone showed the poor performance or no shoots. In the present study BAP along with NAA exhibited better morphogenesis (Table 2).

**Table 2**: Effect of different concentrations of cytokinins and auxins on indirect shoot organogenesis of *in vitro* derived callus cultures of *S. viarum*

| Plant growth regulators (mg/l) | | | | Regeneration frequency (%) | Mean no. of shoots per callus | Mean shoot length (cm) |
|---|---|---|---|---|---|---|
| BAP | Kn | IAA | NAA | | | |
| 0.5 | - | - | - | 60 | $1.5\pm0.23^{a}$ | $3.6\pm0.12^{def}$ |
| 1.0 | - | - | - | 75 | $3.0\pm0.35^{b}$ | $3.3\pm0.23^{cd}$ |
| 2.0 | - | - | - | 80 | $3.7\pm 0.41^{c}$ | $2.8\pm0.21^{ab}$ |
| 3.0 | - | - | - | 75 | $4.5\pm0.28^{d}$ | $2.3\pm0.30^{a}$ |
| 1.0 | - | 0.5 | - | 63 | $3.8\pm0.50^{c}$ | $6.3\pm0.24^{j}$ |
| 2.0 | - | 0.5 | - | 67 | $4.3\pm0.31^{d}$ | $5.5\pm0.41^{g}$ |
| 3.0 | - | 0.5 | - | 70 | $5.4\pm0.43^{e}$ | $4.0\pm0.35^{f}$ |
| - | 1.0 | 0.5 | - | 70 | $6.5\pm0.63^{f}$ | $5.7\pm0.42^{gi}$ |
| - | 2.0 | 0.5 | - | 75 | $8.3\pm0.32^{h}$ | $3.8\pm0.52^{def}$ |
| - | 3.0 | 0.5 | - | 85 | $10.2\pm0.27^{j}$ | $3.4\pm0.19^{cde}$ |
| 1.0 | - | - | 0.5 | 82 | $9.3\pm0.28^{i}$ | $5.7\pm0.46^{gi}$ |
| 2.0 | - | - | 0.5 | 96 | $16.2\pm0.14^{l}$ | $3.6\pm0.27^{def}$ |
| 3.0 | - | - | 0.5 | 92 | $12.4\pm0.20^{k}$ | $3.0\pm0.18^{bc}$ |
| - | 1.0 | - | 0.5 | 70 | $5.4\pm0.34^{e}$ | $6.0\pm0.34^{ij}$ |
| - | 2.0 | - | 0.5 | 81 | $7.0\pm0.18^{g}$ | $5.4\pm0.22^{g}$ |
| - | 3.0 | - | 0.5 | 75 | $8.6\pm0.42^{h}$ | $3.8\pm0.25^{ef}$ |

Data represent treatment means ± SE fallowed by different letter(s) within column indicate significant differences according to ANOVA and DMRT test ($P < 0.05$).

In the present study the addition of auxins (IAA and NAA) to the culture medium in combination with cytokinin BAP and Kn enhances the frequency of shoot initiation and shoot number. These results were similar with the reports *Rawolfia tetraphylla* (Ghosh and Banerjee 2003) and *Withania somnifera* (Kannan 2005). Among the growth regulators tested BAP in combination with NAA exhibited maximum frequency of shoot regeneration. Similar *in vitro* response was reported in *Asteracantha longiflia* (Panigrahi 2007), *Solanum nigrum* (Sridhar and Naidu 2011) and *Mentha piperita* (Sujana and Naidu 2011). BAP mimics as an inhibitor agent and functions against apical dominance of shoot induction and shoot bud formation (Wang and Charle 1991).

*Effect of different auxins on rooting of in vitro regenerated plantlets from callus*

*In vitro* derived shoots with a length of 2.0-4.0 cm were excised and transferred to MS medium supplemented with different concentrations of auxins such as NAA, IAA and IBA (0.5-2.0 mg/l). In all the concentrations tried, exogenous supply of auxins favored the root formation and root primordial appeared between 8-10 days of inoculation. High rooting frequency (96%) with the highest number of roots (18.3±0.21) was obtained on IBA (1.0 mg/l). The maximum root length (4.3±0.26 cm) was observed on IBA (0.5 mg/l) (Table 3).

**Table 3**: Effect of different concentrations of auxins on rooting of *in vitro* derived micro shoots of *S. viarum* (D.) on half strength MS medium.

| Plant growth regulators (mg/l) | | | Regeneration frequency | Mean number of | Mean root length |
|---|---|---|---|---|---|
| IAA | NAA | IBA | (%) | roots/shoot | (cm) |
| 0.5 | - | - | 80 | $6.3\pm0.18^{a}$ | $2.6\pm0.26^{b}$ |
| 1.0 | - | - | 83 | $9.5\pm0.21^{d}$ | $3.5\pm0.32^{e}$ |
| 1.5 | - | - | 78 | $12.6\pm0.31^{g}$ | $3.0\pm0.23^{bcd}$ |
| 2.0 | - | - | 65 | $7.4\pm0.22^{b}$ | $2.0\pm0.16^{a}$ |
| - | 0.5 | - | 82 | $14.3\pm0.26^{h}$ | $3.6\pm0.13^{bc}$ |
| - | 1.0 | - | 90 | $17.3\pm0.31^{i}$ | $3.0\pm0.22^{cde}$ |
| - | 1.5 | - | 78 | $12.5\pm0.17^{g}$ | $3.4\pm0.34^{de}$ |
| - | 2.0 | - | 70 | $8.2\pm0.22^{c}$ | $2.0\pm0.17^{a}$ |
| - | - | 0.5 | 88 | $10.0\pm0.18^{e}$ | $4.3\pm0.26^{f}$ |
| - | - | 1.0 | 96 | $18.3\pm0.21^{j}$ | $3.2\pm0.18^{de}$ |
| - | - | 1.5 | 90 | $14.5\pm0.28^{h}$ | $2.6\pm0.21^{bc}$ |
| | - | 2.0 | 80 | $11.6\pm0.32^{f}$ | $1.7\pm0.26^{a}$ |

Data represent treatment means ± SE fallowed by different letter(s) within column indicate significant differences according to ANOVA and DMRT test ($P < 0.05$).

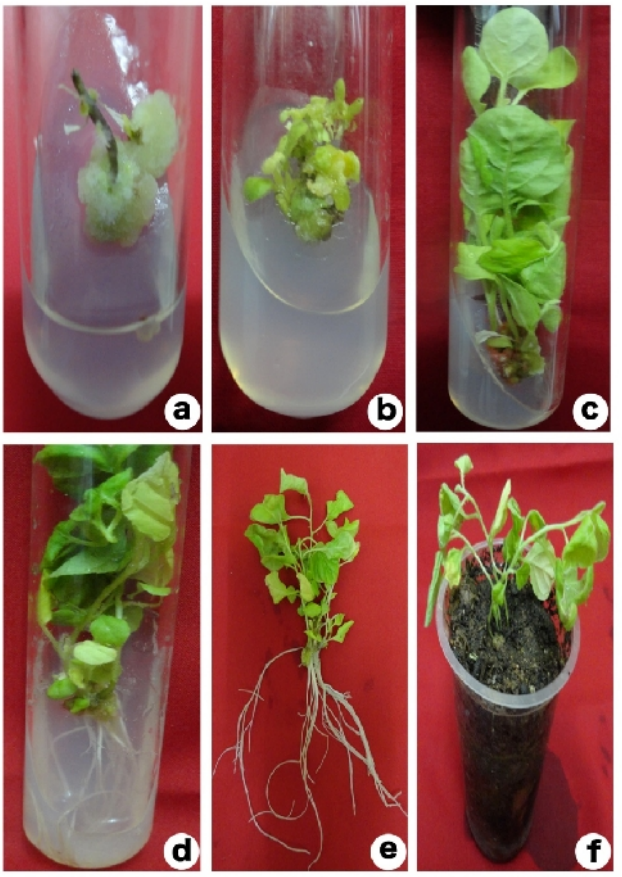


**Figure 1**: *In vitro* callus induction from nodal explants and regeneration form the calli of *Solanum viarum*.

a) Callus formation from the nodal explant of *in vitro* grown *S. viarum* on MS medium + (NAA 3.0 mg/l) + (BAP 0.5 mg/l)

b) Indirect shoot regeneration from node derived callus after 10 days of inoculation on MS medium + (BAP 2.0 mg/l)

c) Proliferation of multiple shoots from *in vitro* derived callus from nodal explant on MS medium + (BAP 2.0 mg/l) +(NAA 0.5 mg/l)

d) Initiation of roots from the regenerated shoots *in vitro* on MS medium + (IBA 1.0 mg/l)

e) Plantlet showing elongated root system

f) Hardened plantlet in polycup containing soil and vermiculite in 1:1 ratio.

Among the auxins used for *in vitro* rooting IBA showed the best rooting response than the IAA and NAA. Similar *in vitro* rooting response was reported in *Anisochilus carnosus* (Jayachandran 2004), *Centella asiatica* (Chandra Sekhar et al. 2014) and *Quisaqualis indica* (Poornima and Shivamurthy 1998). The gentle movement and slow degradation of IBA assists

its localization near the site of application and therefore it functions better in inducing roots (Nickell Kirk-Othmer 1982).

*Acclimatization and Hardening*

The well-developed rooted plantlets were transplanted to the polycups containing soil and vermiculate mixture (1:1) ratio for hardening. Finally the hardened plantlets were transferred to field conditions. Rooted shoots showed the 98% of survivability and regenerated plants did not show any detectable variation in morphology or growth characteristics when compared with the field grown plants.

## Conclusion

In the present study of *in vitro* propagation of *Solanum viarum* through indirect regeneration from vegetative plant parts especially nodal explants we have achieved a clear, simple and reliable protocol for large scale multiplication. Callus culture system offer many advantages as a model system for several biological investigations. In view of the medicinal properties and increased demand of this plant in pharmaceutical industry, the outline protocol offers a potential system for improvement, conserving and mass propagation of this important medicinal plant.

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