Efficient protocol for direct shoot organogenesis from \textit{in vitro} raised nodal explants of \textit{Solanum viarum} (Dunal) - An important anticancer medicinal plant

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Abstract: In the present study an attempt has been made to standardize a protocol for rapid direct plant regeneration from \textit{in vitro} derived nodal explants of \textit{S. viarum}, which promoted shoot bud regeneration and resulted in maximum number of multiple shoot induction in the medium supplemented with different concentrations of plant growth regulators such as cytokinins like BAP, Kn alone or in combination with auxins like NAA/IBA/IAA. Among the combinations tested BAP (2.0 mg l\textsuperscript{-1}) and IBA (0.5 mg/l\textsuperscript{-1}) produced maximum number of shoots (22.3±0.41) per explant. The maximum shoot length (8.2±0.14 cm) was observed with Kn (1.0 mg l\textsuperscript{-1}) and IBA (0.5 mg l\textsuperscript{-1}) combination. All the \textit{in vitro} raised shoots with a length of 3.0-5.0 cm were transferred to rooting medium supplemented with different concentrations of auxins such as NAA, IBA and IAA (0.2 – 1.0 mg l\textsuperscript{-1}). The best rooting response was observed on IBA (0.6 mg l\textsuperscript{-1}). 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 for maximum survivability.

Keywords: Multiple shoot induction; Nodal explants; Plant regeneration; \textit{Solanum viarum}.

Abbreviations: BAP- Benzyl aminopurine; IAA-Indole-3-acetic acid; IBA-Indole-3-butyric acid; Kn- Kinetin; NAA- \textgreek{a}-Naphthalene acetic acid.

Introduction

Multiplication of plants \textit{in vitro} through plant tissue culture methods have emerged in late 1980’s as a valuable approach to increase plant productivity (Gupta et al. 1993). In recent years, there has been an increased interest in \textit{in vitro} culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora and Bhojwani 1989; Sudha and Seeni 1994). \textit{Solanaceae} family comprises a number of plants widely known for the presence of variety of natural products of medicinal significance mainly steroidal lactones, glycosides, alkaloids and flavonoids. \textit{Solanum viarum} (D.) is an ample branched, prickly shrub 1-2 meters tall at maturity. It is a member of economically significant family \textit{Solanaceae}. The plant grown in India as a richest source of steroids (Mullahey et al. 1987), which yields an economically vital source of glycoalkaloiode solasodine which is present in the fruit is a pioneer for the steroid disogenin (Budhavari 1989). This is used in the production of steroid hormone for treating cancer, rheumatic arthritis, addison’s disease and for providing contraceptives (Srinivasan 2005). Since the high economical and pharmological values of secondary metabolites, industries are intensely attracted in utilizing the plant tissue culture technology for large scale production of these substances (Misawa 1994.). The present investigation was performed to determine the role of different plant growth regulators on direct shoot regeneration from \textit{in vitro} derived nodal explants of \textit{Solanum viarum}.

Materials and methods

Plant material and surface sterilization

Nodes of axillary branches were excised from the \textit{in vitro} regenerated shoots of \textit{S. viarum} were used as explants as a prerequisite for experiments. Therefore for the explant source the...
seeds were collected from mature and dried fruits of healthy plants of *S. viarum* that are cultivated in the herbal garden, Dravidian University, Kuppam, A. P., India. Matured seeds were first washed under running tap water for 30 mins. to take away any adherent fruit tissues and dried juice which might serve as an agent for fungal contagion. Then the seeds were rigorously washed with 0.4% bavistin (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% agar (w/v) supplemented with various concentrations of cytokinins BAP and Kn and auxins (NAA, IAA and IBA) previous 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 60-70% relative humidity. Light intensity was 3000 lux with a photoperiod of 18 hrs day light and 6 hrs in dark. The primary shoots formed *in vitro* were separated aseptically and cultured on MS medium supplemented with BAP (2.0 mg l$^{-1}$).

**Sub culturing**

Axillary bud 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 and gelled with 0.8% agar supplemented with various concentrations of cytokinins like BAP, Kn alone or in combination with auxins such as NAA, IAA and IBA were used for shoot proliferation. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 mins. at 121°C for 15 lbs pressure. The cultures were maintained by regular intervals of 21 days on fresh MS medium.

**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**

**Effect of cytokinins alone on direct shoot regeneration from *in vitro* derived axillary bud explants of *S. viarum***

Effect of two different cytokinins BAP and Kn on direct shoot regeneration was examined separately. After two weeks of inoculation shoot bud primordial was emerged along the cut portions of nodal explants, later they were developed as shoots. In BAP (0.5-3.0 mg l$^{-1}$) alone supplemented MS medium shoot initiation was observed and the mean number of shoots (10.3±0.31) was observed at (2.0 mg l$^{-1}$) (Figure 1a) and mean shoot length ranged from (2-4 cm) respectively. The optimum concentration of BAP was found to be (2.0 mg l$^{-1}$). The number of multiple shoots were decreased with further increase in BAP concentration (> 3.0 mg l$^{-1}$). In Kn (0.5-3.0 mg l$^{-1}$) alone supplemented MS medium shoot bud initiation was observed and the mean number of shoots ranged from (4.0-8.0) with mean shoot length (3.0-5.0 cm). The number of shoots were less compare to BAP supplemented medium (Table 1). Direct regeneration from nodal explant is another substitute step for clonal propagation and germplasm conservation is a well-established factor. *In vitro* regeneration of plants depends on the interaction between endogenous growth substances and the artificial growth regulators which may be added to the medium. The stimu-
In vitro direct shoot organogenesis from nodal explants of Solanum viarum

In vitro direct shoot regeneration depends on the nature of the plant organ from which the explant was derived and is extremely dependent on plant (George 1993a). Nodes contain axillary meristem which can generate direct proliferation of shoots. Preexisting meristems are the base of the two pathways: direct shoot organogenesis and direct shoot proliferation. Meristems are highly reactive cells and plants regenerated from them generally could show no variation in genotypic and phenotypic characters (Saeed et al. 1997). In the present study the cytokinin alone supplemented medium multiple shoots become visible after 2-3 weeks of inoculation this was well reported in Celastrus paniculatus (Lakshmi and Seeni 2001).

Table 1: Direct shoot organogenesis from nodal explants of in vitro raised S. viarum micro shoots.

<table>
<thead>
<tr>
<th>Plant growth regulators (mg l⁻¹)</th>
<th>Regeneration frequency (%)</th>
<th>Mean no. of shoots/explant</th>
<th>Mean shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP</td>
<td>Kn</td>
<td>NAA</td>
<td>IBA</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>0.5</td>
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<tr>
<td>3.0</td>
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<td>0.5</td>
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<td>0.5</td>
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<tr>
<td>1.0</td>
<td>-</td>
<td>0.5</td>
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</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>0.5</td>
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<tr>
<td>3.0</td>
<td>-</td>
<td>0.5</td>
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<tr>
<td>0.5</td>
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<td>0.5</td>
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</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>0.5</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

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

**Effect of BAP in combination with auxin on direct shoot regeneration from in vitro derived axillary bud explants**

BAP, Kn in combination with auxins (NAA or IBA or IAA) on direct shoot regeneration was examined. BAP (0.5-3.0 mg l⁻¹) in combination with NAA, IBA and IAA (0.5 mg l⁻¹) were used to produce direct shoot regeneration from the axillary bud explants of in vitro regenerated S. viarum micro shoots. After two weeks of inoculation shoot bud primordial was emerged along the cut portions of nodular explants, later they were developed as shoots. In BAP and NAA supplemented medium the mean number of shoots were ranged from (8.7-12.2) was obtained with shoot length (3.2-5.3 cm) respectively (Figure 1b). Whereas the lower concentration of cytokinin BAP (1.0 mg l⁻¹) and auxin IAA (0.5 mg l⁻¹) favored the mean shoot number (10.3±0.18). With further increase in concentration of BAP the shoot number decreased to (8.2±0.21). The highest number of shoots was obtained in the combination of BAP (2.0 mg l⁻¹) and IBA (0.5 mg l⁻¹) with (22.3±0.41) shoots (Figure 1c) with a shoot length (3.8±0.25 cm) (Table 1) but maximum shoot length was obtained on Kn (1.0 mg l⁻¹) in combination with IBA (0.5 mg l⁻¹). The shoot number were decreased with further increase in the BAP con-
concentration (> 2.0 mg l⁻¹). BAP preferred for multiple shoot induction and proliferation from nodal explants than Kn in the present study. In general, BAP is the most effective growth regulator for stimulation and shoot proliferation. BAP mimics as an inhibitor agent and drives beside apical dominance of shoot induction and shoot bud formation (Wang and Charle 1991). Advantage of BAP over any cytokinin in induction and proliferation of shoots has been very well standardized in Quercus rober (Puddephat et al. 1997) and Solanum nigrum (Sridhar and Naidu 2011).

Table 2: Root organogenesis of in vitro derived shoot lets of S. viarum supplemented with various concentrations of IAA, NAA and IBA using half strength MS medium.

<table>
<thead>
<tr>
<th>Plant growth regulators (mg l⁻¹)</th>
<th>Regeneration frequency (%)</th>
<th>Mean number of roots/shoot</th>
<th>Mean root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA 0.2 IBA - IAA 0.2</td>
<td>83</td>
<td>7.2±0.27b</td>
<td>4.5±0.26g</td>
</tr>
<tr>
<td>0.4 - - 0.6 -</td>
<td>85</td>
<td>9.3±0.34d</td>
<td>4.8±0.41g</td>
</tr>
<tr>
<td>0.8 - - 1.0 -</td>
<td>78</td>
<td>11.2±0.42f</td>
<td>3.7±0.52ef</td>
</tr>
<tr>
<td>0.2 - 0.4 - 0.6 -</td>
<td>87</td>
<td>14.5±0.35h</td>
<td>2.4±0.27a</td>
</tr>
<tr>
<td>0.8 - 1.0 - 0.2 -</td>
<td>90</td>
<td>8.3±0.31i</td>
<td>2.6±0.13ab</td>
</tr>
<tr>
<td>0.8 - 0.4 - 0.6 -</td>
<td>87</td>
<td>10.4±0.22e</td>
<td>7.0±0.12h</td>
</tr>
<tr>
<td>0.8 - 1.0 - 0.2 -</td>
<td>85</td>
<td>13.3±0.36g</td>
<td>4.6±0.32g</td>
</tr>
<tr>
<td>- - 0.4 - 0.6 -</td>
<td>92</td>
<td>19.2±0.22i</td>
<td>2.3±0.27a</td>
</tr>
<tr>
<td>- - 0.6 - 0.2 -</td>
<td>98</td>
<td>14.1±0.35h</td>
<td>3.5±0.15de</td>
</tr>
<tr>
<td>- - 1.0 - 0.4 -</td>
<td>85</td>
<td>10.3±0.21e</td>
<td>3.2±0.18d</td>
</tr>
<tr>
<td>- - 0.4 - 0.6 -</td>
<td>85</td>
<td>6.5±0.42a</td>
<td>3.6±0.28def</td>
</tr>
<tr>
<td>0.6 - 0.8 - 0.2 -</td>
<td>80</td>
<td>9.7±0.43d</td>
<td>3.3±0.25de</td>
</tr>
<tr>
<td>0.6 - 0.8 - 1.0 -</td>
<td>85</td>
<td>13.2±0.30g</td>
<td>4.0±0.16f</td>
</tr>
<tr>
<td>0.8 - 1.0 - 0.4</td>
<td>78</td>
<td>15.6±0.26i</td>
<td>3.0±0.29bc</td>
</tr>
<tr>
<td>1.0 - 0.8 - 0.2</td>
<td>70</td>
<td>11.6±0.51f</td>
<td>2.4±0.18a</td>
</tr>
</tbody>
</table>

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

Effect of Kn in combination with auxin on direct shoot regeneration from in vitro derived axillary bud explants

Direct shoot induction from the in vitro derived axillary bud explants of S. viarum was also observed on Kn alone and in combination with auxins like NAA, IBA and IAA but the number of shoot formation was less when compared with BAP. The optimal concentration for direct shoot regeneration using Kinetin was (2.0 mg l⁻¹) in combination with IBA (0.5 mg l⁻¹) produced (13.4±0.61) mean number of shoots. The maximum shoot length (8.2±0.15 cm) was observed at Kn (1.0 mg l⁻¹) and IBA (0.5 mg l⁻¹) (Table 1). In Kn and NAA supplemented medium the maximum shoot number (10.6±0.36) was obtained with a mean shoot length (3.2±0.13 cm) at Kn (2.0 mg l⁻¹) and NAA (0.5 mg l⁻¹). Whereas in Kn and IAA supplemented medium the maximum shoot number (11.8±0.53) was obtained with a mean shoot length (4.5±0.28 cm) at Kn (1.0 mg l⁻¹) and IAA (0.5 mg l⁻¹). With further increase in the concentration of kinetin number of shoots were decreased.

The combination of cytokinin and auxin seems to have a collaboration effect, as cytokinins enhance cell division, stimulate axillary bud and adventitious shoot proliferation and auxins regulate cell elongation, tissue swelling and shoot expansion. The effect of auxins and cytokinins on enhancing shoot regeneration has been reported in several spices, such as Echinacea purpurea (Koroch et al. 2002) and Catalpa ovate (Lisowska and Wysokinska 2000).
Figure 1: Direct regeneration of shoots from nodal explants of *in vitro* raised microshoots of *S. viarum*.

a) Shoot bud initiation from nodal explant after 7 days of inoculation on MS medium + BAP (2.0 mg l\(^{-1}\))
b) Initiation of multiple shoots from nodal explant on MS medium + BAP (2.0 mg l\(^{-1}\)) + NAA (0.5 mg l\(^{-1}\))
c) Multiplication of shoots from nodal explant on MS medium + BA (2.0 mg l\(^{-1}\)) + IBA (0.5 mg l\(^{-1}\))
d) Elongated multiple shoots regenerated from nodal explant on MS medium + BAP (3.0 mg l\(^{-1}\)) + IBA (0.5 mg l\(^{-1}\))
e) Initiation of roots from the regenerated shoots *in vitro* on MS medium + (IBA 0.6 mg l\(^{-1}\))
f) Plantlet showing elongated root system
g) Hardened plantlet in polycups containing soil and vermiculite in 1:1 ratio
h) Plantlet in field conditions.
In the present study *in vitro* derived nodal explants promoted shoot bud regeneration in presence of the cytokinin BAP, Kᵣ alone or in combination with auxins like NAA/IBA/IAA and resulted in maximum number of multiple shoot induction. Among the auxins tested (NAA, IBA and IAA) in combination with BAP, natural auxin IBA proved to be the better for providing maximum number of shoots. Similar response was studied earlier in *Platanus acerifolia* wild (Liu and Bao 2003). The next best auxin which performed well was NAA in combination with BAP. The effect of BAP and NAA on direct shoot regeneration was reported in *Gysophila paniculata* (Zukar et al. 1997) and *Spilanthes acmella* (Saritha and Naidu 2008). IAA in combination with Kᵣ also have showed the effect on direct shoot regeneration. These results are in line with the observations of *Stevia rebaudiana* (Preethi et al. 2011) and *Cajanus cajan* (Misra 2002).

In *vitro* rooting

The *in vitro* developed shoots were excised from clump of shoots and transferred on MS medium supplemented with different concentration of auxins such as NAA, IBA and IAA (0.2-1.0 mg l⁻¹). The best rooting response was observed on IBA (0.6 mg l⁻¹) supplemented medium, (Figure 1e) where maximum number of roots (19.2±0.22) were obtained followed by IAA (0.8 mg l⁻¹), NAA (0.8 mg l⁻¹) supplemented medium where (15.6±0.26), (14.5±0.35) number of roots were induced respectively. The number of roots decreased with further increase in auxin concentration (>0.8 mg l⁻¹) (Table 2). The excised shoots cultured on the MS medium containing different auxin concentrations for rooting in which the IBA performed well in producing the maximum number of roots/shoot with higher regeneration frequency these reports were similar with the study on *Mentha piperita* (Sujana and Naidu 2011). Rooted shoots showed the maximum percentage of survival. Among the plant growth regulators tested, in most of the combinations, the comeback of direct regeneration from nodal explants were resulted in prompting shoot regeneration directly from the nodal explants without forming callus. The excised nodes from *in vitro* derived shoots were used as explants for further regeneration as this is also true in *Cuminum cyminum* (Esmaeil Ebrahimie et al. 2007).

Acclimatization and hardening

Well-developed rooted plants were carefully removed from culture tubes and washed to remove the leftovers of agar (Figure 1f). These healthy shoots were transferred to tray containing autoclaved soil and vermiculate in 1:1 ratio for acclimatization (Figure 1g). For a period of 8-10 days the plants were kept in polythene membrane. After that the surviving plants were transferred to pots and allowed to grow under nursery shade conditions (Figure 1h). Finally these acclimatized plants were planted in field conditions with maximum survivability.

Acclimatization of regenerated plants to the outside environment is the last stage in plant tissue culture and its achievement depends on different factors as proposed by various researchers (George and Sherrington 1984b). Prior other investigation workers studied acclimatization of micropropagated plants in the greenhouse under field environments (Preece and Sutter 1991).

Conclusion

In the present study, regeneration of *S. viarum* was greatly influenced by different plant growth regulators supplemented in the media. It can be concluded that based on readily available nodal explants of *S. viarum* reproducible *in vitro* direct shoot regeneration and proliferation, has been described. The results obtained in the present study could be of enormous significance, which contribute to the development in the micropropagation of this economically important medicinal plant on commercial scale to light the current day demand.

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References


Srinivasan, R., Talekar, N.S., Uthamaswamy, S. 2005. Feeding Stimulants in *Solanum*
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