In *vitro* plant regeneration through callus in Giloy (*Tinospora cordifolia* (Willd.) Miers ex Hook. f & Thoms.)

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Publication History
Received: 20 November 2014
Accepted: 28 December 2014
Published: 21 January 2015

Citation
Hemant Sharma, Vashistha BD. In *vitro* plant regeneration through callus in Giloy (*Tinospora cordifolia* (Willd.) Miers ex Hook. f & Thoms.). *Indian Journal of Science*, 2015, 12(34), 59-68

ABSTRACT

A simple and efficient plant regeneration system was developed for Giloy (*Tinospora cordifolia*) through callus development. Leaf explants were inoculated on MS medium and woody plant medium supplemented with different concentrations (0.5, 1.0, 2.0 and 4.0 mg/l) of growth regulators IAA, NAA; 2, 4-D, BAP and Kn. Activated charcoal (5 g/l) was also added in the medium. Leaf segments did not give any response in control cultures and cultures supplemented with BAP and Kn. Explants produced callus at all the four concentrations of IAA, NAA and 2, 4-D on both the media. Maximum average fresh and dry weights were obtained from the callus at 4.0 mg/l 2,4-D on WPM. The leaf derived callus was transferred to media with 0.5, 1.0, 2.0 and 4.0 mg/l of BAP and Kn. The maximum shoots were developed on the callus cultured on WPM supplemented with 2.0 mg/l BAP and 5 g/l AC. Rooted plants were grown in the Polyhouse and acclimatized successfully with 70% survival rate.

Key words: Callus, Leaf explants, WPM, Giloy

Abbreviations
MS- Murashige and Skoog, WPM- Woody plant medium, AC- Activated charcoal, BAP- 6 benzylaminopurine, Kn- Kinetin, IBA- Indole-3-butyric acid, IAA- Indole-3-acetic acid, NAA- a-Naphthalene acetic acid; 2,4-D- 2,4-Dichloro phenoxy acetic acid, ANOVA- Analysis of Variance.

1. INTRODUCTION

Micropropagation technique offers a viable tool for mass multiplication of plants. The technique involves plant regeneration via direct and indirect organogenesis. There are few reports of direct organogenesis of *Tinospora cordifolia* from nodal explants (Kumar et al., 2003; Raghu et
al., 2006; Gururaj et al., 2007). We have developed a procedure for whole plant regeneration via indirect organogenesis. This is the report of successful callus induction and plant regeneration through leaf explants of Tinospora cordifolia.

Tinospora cordifolia is a large, woody climbing shrub belongs to the family Menispermaceae. It is commonly known as Giloy. It is a well-known medicinal plant, used in Ayurvedic and other systems of medicine. The drug obtained from stem and leaves have anti-pyretic, anti-inflammatory, anti-allergic and anti-diabetic properties (Anonymous, 1992; Singh et al., 2003). It is prescribed in case of diabetes, fever and skin diseases. It is also used to improve the immune system of the body. The conventional methods of propagation of Giloy such as stem cuttings have limited potential for large scale production. Because the plant has poor seed producing capacity. Therefore, in the present investigation, a simple and efficient plant regeneration system is developed for T. cordifolia.

2. MATERIALS & METHODS
2a. Source of explant
Giloy plants were collected from Kurukshetra University campus, Kurukshetra, Haryana. Leaf segments were used as explants for the in vitro culture work.

2b. Sterilization
The explants were washed in running tap water to remove all the dust particles. It was followed by washing with liquid detergent (Teepol) for 10 minutes and again washed several times with tap water to remove all the traces of detergent. Then, in laminar air flow chamber surface sterilization was carried out by treating with 0.1% (w/v) mercuric chloride solution for 2-5 minutes and subsequently washed 3-4 times with sterile double distilled water to remove all the traces of mercuric chloride. Again explants were treated with 70 % (v/v) ethyl alcohol for 1 min. The medium was sterilized by autoclaving at 121 °C temperature and 15 psi pressure for 20 min.

2c. Culture medium and conditions
MS basal medium (Murashige and Skoog, 1962) and Woody Plant Medium (Lloyd and McCown, 1980) with 3 % (w/v) sucrose and solidified with 0.8 % (w/v) agar were used. Moreover 5 g/l activated charcoal was also used in the present investigation. All the cultures were maintained at 25±2°C under a 16 hours photoperiod with 30 μmol m⁻² s⁻¹ irradiance provided by cool white fluorescent tubes (40 w-Philips, India).

2d. Callus induction and growth
Leaf segments were cultured on MS and WPM supplemented with different concentrations (0.5, 1.0, 2.0 and 4.0 mg/l) of growth regulators IAA, NAA; 2,4-D, BAP and Kn, individually. Visual observations like percent response of explant, number of days required for callus induction, colour and texture of callus were periodically recorded. The callus was collected after 60 days of their initiation and growth was measured in terms of fresh and dry weights. Fresh weight of callus was taken after removing the excess of moisture on the surface using blotting paper. Dry weight was determined by drying the callus in a hot air oven at 60°C for 24 hours.

2f. Shoot regeneration
Callus developed from the leaf segments on induction medium was separated and cut into small pieces and transferred to the MS and WPM supplemented with 0.5, 1.0, 2.0 and 4.0 mg/l of BAP and Kn for shoot initiation. Observations like the percentage of callus cultures forming shoots, number of days required for shoot induction, number of shoots per culture and shoot length were recorded.

2g. Rooting of in vitro shoots
After in vitro regenerated shoots attained a height of 2-3 cm, they were excised and planted on half strength MS medium supplemented with different concentrations of IAA, IBA, NAA and 2,4-D for rooting.

2h. Hardening and acclimatization of plantlets in Soil
The rooted plantlets were gently pulled out of the medium. They were washed in running tap water to remove the medium sticking to the root. The plantlets with well-developed roots were transferred to sterilized soil and sand mixture (1:1) in small plastic pots. For initial 15 days maintained high humidity around the plants. So, they were covered with transparent polythene bags and made small holes in them for air circulation. Plants were watered with ¼ MS or WP salt solution on alternate days. Then pots were transferred in Polyhouse.

2i. Statistical Analysis
The data were collected after 60 days of inoculation of leaf explants. Each experiment was repeated three times with 10 explants per treatment. All the data were analyzed by ANOVA followed by Duncan Multiple Range Test at P = 0.05.

3. RESULTS AND DISCUSSION
3a. Callus initiation and growth
Callus induction from leaf explants were reported in many plant species such as Hypericum perforatum (Pretto and Santarem, 2000), Coleus forskohlii (Reddy et al., 2001), Echinacea purpurea (Koroch et al., 2002) and Vanilla planifolia (Janarthanam and Seshadri, 2008). Further, young
leaf explants of *T. cordifolia* were more responsive for callus induction than older ones. This could be attributed to high plasticity of cells at younger age. The younger tissues are physiologically and biochemically more active as well as they have less rigid cell walls. Therefore, they show better callusing potential. In *Vanilla planifolia*, juvenile leaf explants were more responsive for callus induction than nodal explants (Janarthanam and Seshadri, 2008). Leaf segments cultured on basal MS and WPM served as control. In control cultures, no any response of explant was observed. This is probably due to the insufficient level of endogenous growth regulators in explants to induce callus and therefore it required an exogenous supply. Further, calli formations from leaf explants were depended on medium type and auxin added to the medium. The results suggested that WPM was better as a basal medium for callus induction and differentiation in *T. cordifolia*. The suitability of WPM over MS medium might be due to its low ionic strength which counteracts salt sensitivity of woody species (Lloyd and McCown, 1980). The WPM has been found suitable for many other species like *Quercus floribunda* (Purohit et al., 2002), *Echinacea purpurea* (Mechanda et al., 2003), *Prunus avium* (Bhagwat and Lane, 2004), *Ziziphus jujuba* (Feng et al., 2010) and *Ficus religiosa* (Siwach and Gill, 2011). Further, leaf explants produced callus at all the four concentrations -0.5, 1.0, 2.0 and 4.0 mg/l of IAA, NAA and 2,4-D in *T. cordifolia*. Among these, 2,4-D was most effective for the induction of callus. According to Murashige (1974); 2,4-D is a most potent auxin and it stimulates callus formation. Presence of 2,4-D has been effective in inducing callus in *Paspalum scrobiculatum* (Arockiasamy et al., 2001), *Calamus tenuis* (Sett et al., 2002), *Vernonia cinerea* (Baig and Shahzad, 2003), *Momordica charantia* (Agrawal and Kamal, 2004) and *Vanilla planifolia* (Janarthanam and Seshadri, 2008). Callus formation started at 0.5 mg/l and increased with increase in concentration of auxins from 0.5 to 4 mg/l (Figure 3). Therefore, the concentration of 4 mg/l of IAA, NAA and 2,4-D was most suitable for callus induction as maximum fresh and dry weights were observed at this concentration (Table 1). Further, the callus was initiated near the cut ends of leaf segments. The colours and textures of different calli are summarised in Figure 1 & 2.

### 3b. Shoot regeneration

The leaf derived calli were divided into small pieces and cultured on WPM supplemented with 0.5, 1.0, 2.0 and 4.0 mg/l concentrations of BAP and Kn individually for shoot formation. The results are summarised in Table 2 & Figure 4. From the table, it is clear that the concentration of 2 mg/l BAP as well as Kn was optimum for callus organogenesis. BAP at this level resulted in 63.3 % response with 3.3 ± 0.15 shoots per culture whereas Kn resulted in 36.7 % response with 1.5 ± 0.07 shoots per culture. Further of the two growth regulator tried, BAP was found to be more effective than Kn for shoot regeneration. Similar observations were made in several plant species including *Capsicum annuum* (Singh and Shukla, 2001), *Geoderum purpureum* (Mohapatra and Rout, 2005), *Solanum melongena* (Solanki et al., 2006), *Curculigo orchioides* (Nagesh, 2008), *Rubus* (Wy et al., 2009), *Vanilla planifolia* (Tan et al., 2011) and *Citrus jambhiri* (Savita et al., 2011). In the present investigation in *T. cordifolia*, addition of 5 g/l AC in the medium prevented browning of medium and explants and increased percent shoot induction from callus (Figure 4). It is due to the fact that AC has a very fine network of pores with large surface area that gives it a unique adsorption capacity (Baker et al., 1992). Positive effect of AC on plant regeneration has been reported by many workers (Chang et al., 2001; Wang et al. 2005 and Guo et al., 2007).

### 3c. Rooting of *in vitro* shoots

In the present report, roots failed to appear in *in vitro* plants on MS basal medium. Roots were induced on half-strength MS medium supplemented with IAA, IBA, NAA and 2,4-D. Among these, IBA was most effective in rooting as it induced healthy and elongated roots (Figure 5A). The promotive effect of IBA on rooting has been reported in *Pterocarpus marsupium* (Chand and Singh, 2004), *Bacopa monnieri* (Mohapatra and Rath, 2005), *Chlorophyllum borivilianum* (Sharma and Mohan, 2006), *Datura metel* (Khan et al., 2010) and *Cinnamomum camphora* (Sharma and Vashishta, 2010). IBA at 0.5 mg/l concentration was optimum for rooting which resulted in 46.7 % response with 2.1 ± 0.11 roots initiated in MS medium (Table 3). At higher concentration of IAA, IBA, NAA and 2,4-D; response decreased and inhibited rooting and callus formation was observed at the base of shoots.

### 3d. Hardening and acclimatization of plantlets in soil

In the present investigation, in *T. cordifolia*, for successful acclimatization to natural conditions and normal growth a careful and gradual transfer of *in vitro* regenerated plantlets was necessary. Therefore, after about a month, the plantlets with well-developed roots were shifted to pots in Polyhouse where they grew normally with 70 % survival rate (Figure 5B).

**REFERENCES**

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Values are means ± S.E. of three independent experiments, each consisted of 10 replicates per treatment. Data from 60 days old culture.
Means followed by the same letter within columns are not significantly different at $P = 0.05$ according to Duncan’s Multiple Range Test.
(-) No response.

Table 3
Effect of different concentrations of growth regulators on rooting of in vitro regenerated shoots of *T. cordifolia* cultured on MS medium

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentrations (mg/l)</th>
<th>% root induction</th>
<th>Time taken for root induction (Days)</th>
<th>Average number of roots</th>
<th>Average root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0</td>
<td>-</td>
<td>-</td>
<td>0.0 ± 0.00e</td>
<td>0.0 ± 0.00e</td>
</tr>
<tr>
<td>IAA</td>
<td>2.0</td>
<td>23.3</td>
<td>35</td>
<td>1.0 ± 0.05c</td>
<td>1.2 ± 0.05c</td>
</tr>
<tr>
<td>IBA</td>
<td>0.5</td>
<td>46.7</td>
<td>25</td>
<td>2.1 ± 0.11a</td>
<td>2.3 ± 0.12a</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>36.7</td>
<td>30</td>
<td>1.3 ± 0.06b</td>
<td>1.5 ± 0.07b</td>
</tr>
<tr>
<td>NAA</td>
<td>0.5</td>
<td>20.0</td>
<td>35</td>
<td>1.0 ± 0.00c</td>
<td>0.6 ± 0.05d</td>
</tr>
<tr>
<td>2,4-D</td>
<td>0.5</td>
<td>20.0</td>
<td>35</td>
<td>1.0 ± 0.00c</td>
<td>0.5 ± 0.03d</td>
</tr>
</tbody>
</table>

Values are means ± S.E. of three independent experiments, each consisted of 10 replicates per treatment.
Means followed by the same letter within columns are not significantly different at $P = 0.05$ according to Duncan’s Multiple Range Test.
(-) No response.
Figure 1
Effect of different concentrations of growth regulators on leaf explants of *T. cordifolia* cultured on MS medium- (A) Percent callus induction (B) Time taken for callus induction
Effect of different concentrations of growth regulators on leaf explants of *T. cordifolia* cultured on WPM- (A) Percent callus induction (B) Time taken for callus induction
Figure 3
Effect of different growth regulators on leaf explants of *T. cordifolia*: (A) Callus initiation on WPM + 2 mg/l IAA; (B) Callus on WPM + 4 mg/l IAA; (C) Callus initiation on WPM + 2 mg/l NAA; (D) Callus on WPM + 4 mg/l NAA; (E) Callus initiation on WPM + 2 mg/l 2,4-D; (F) Callus on WPM + 4 mg/l 2,4-D.
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http://www.discovery.org.in/ijs.htm

**Figure 4**
(A) Callus from leaf segments on WPM + 4 mg/l 2,4-D + 5 mg/l AC; (B)-(C) Shoot differentiation from leaf derived callus of *T. cordifolia* on: (B) WPM + 2 mg/l KIN + 5 g/l AC, (C) WPM + 2 mg/l BAP + 5 g/l AC

**Figure 5**
(A) Rooting of *in vitro* regenerated shoot of *T. cordifolia* on MS + 0.5 mg/l IBA; (B) *In vitro* regenerated plant in plastic container