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Research article

In vitro micropropagation of *Tinospora cordifolia* (Willd.) Miers from shoot tip explants

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Abstract

An efficient *in vitro* micropropagation protocol from shoot tip explants was established for *Tinospora cordifolia* (Willd.) Miers. Young and mature shoot tip (YST and MST, respectively) explants from two different-aged plant sources (15 d and 3 yr) were treated simultaneously to develop and compare their efficacy in the micropropagation protocol. Among the cytokinins and synergetic treatments, 6-benzyladenine (BA; 2.0 mg/L) and kinetin (KN; 1.0 mg/L) responded better in bud break and shoot development, at both individual concentrations and in combination. Addition of the auxin indole-3-acetic acid (IAA; 0.5 mg/L) to the optimal cytokinin concentrations of BA (2.0 mg/L) with KN (1.0 mg/L), enhanced multiple shoot induction and shoot growth. Further supplementation of gibberellic acid (0.1 mg/L) and an antioxidant (ascorbic acid at 100 mg/L) significantly enhanced the shoot bud induction, shoot number and shoot length. Assessment of different basal media using the best plant growth hormone complex confirmed that the Murashige and Skoog (MS) medium best supported shoot bud break and shoot development in both the shoot tip explants. The best rooting response was from half-strength MS medium fortified with indole-3-butyric acid (0.5 mg/L), resulting in approximately 80% of the plantlets successfully acclimatized both at *in vitro* and *in vivo* conditions. In conclusion, the YST explants responded better than the MST explants in all aspects of growth and development and proved that the age of explants was an important determinant for efficient micropropagation in *T. cordifolia*.

Introduction

Medicinal plants have been used fundamentally in all cultures from ancient times, as remedies and cures for humans. An increasing reliance on the use of medicinal plants in the industrialized societies has been traced to the extractions and development of several drugs and chemotherapeutics from these plants as well as from several traditionally used herbal remedies (UNESCO, 1994). In the interim, medicinal plant reserves in developing countries are decreasing and in danger of extinction due to increasing trade demands for cheaper healthcare products to more target specific drugs and biopharmaceuticals (Debnath et al., 2006). Micropropagation or tissue culture technology can provide a continuous, reliable source of pharmaceuticals and could be used for the large-scale culture of plant cells from which important metabolites can be extracted (Hussain et al., 2012).

Tinospora cordifolia (Willd.) Miers. (Menispermaceae), a large glabrous climbing shrub, is distributed throughout tropical India upto an altitude of 300 m (Pradhan et al., 2013). It has been used

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in Ayurvedic Rasayanas to improve the immune system, provide resistance against infections and also to general debility, dyspepsia, fever and urinary diseases (Pradhan et al., 2013). A variety of phytoconstituents belong to different classes (including the alkaloids, diterpenoid lactose, glycosides, steroids, sesquiterpenoids, phenolics, aliphatic compounds and polysaccharides) and have been isolated and their structures elucidated and reviewed in *T. cordifolia* (Singh et al., 2003; Mittal et al., 2014). Biotechnological advances have established various medicinal properties from different plant parts of *T. cordifolia* such as inhibition of *in vitro* growth of Mycobacterium tuberculosis at 1:50,000 diluter (Gupta and Viswanathan, 1956), anti-allergic (Nayampalli et al., 1986), anti-spasmodic, anti-pyretic (Vedavathy and Rao, 1991), phagocytic and intra cellular killing capacities of polymorphs in rats (Thatte et al., 1994), protection in cholestatic patients against *E. coli* infection (Dhuley, 1997), anti-neoplastic agent (Jagetia et al., 1998), anti-oxidant action in alloxan diabetes rats (Prince et al., 1999), anti-inflammatory (Jana et al., 1999), hypoglycemic activity in rabbits (Prince and Menon, 2000), *in vitro* inactivating property against Hepatitis B and E surface antigen (Mehrotra *et al*., 2000), anti-leprotic (Asthana et al., 2001), immune stimulation activity in memory deficit rats (Agarwal et al., 2002), anti-osteoporotic potential (Kapuret al., 2008), anti-diabetic activity (Patel and Mishra, 2012), immunomodulatory activity (Sharma et al., 2012; Sachdeva et al., 2014), uroprotective activity (Hamsa and Kuttan, 2012), nephroprotector activity (Uppuluri et al., 2013), gastroprotective activity (Antonisamy et al., 2014), protective effect against asthmatic inflammation and other lung inflammatory conditions (Tiwari et al., 2014).

Conventional methods of propagation using vegetative cuttings and seed germination are hampered due to poor productivity, low seed viability and high susceptibility to infections in *T. cordifolia*. *In vitro* propagation methods could possibly enhance the commercial supply of plant materials. Earlier works on *in vitro* propagation of this plant, primarily used only node and leaf segments (Bhalerao et al., 2013; Bhat et al., 2013; Sivakumar et al., 2014). The current study for the first time applied efficient *in vitro* micropropagation of *T. cordifolia* using shoot tip explants.

Materials and Methods

Explant source and sterilization

The sources of explants were healthy, wild mature plants (aged 3 yr; Fig. 1A) and *in vitro* seed germinated young seedlings (aged 15 d) of *T. cordifolia*, established in the field of Department of Botany, Bharathidasan University campus, Tiruchirappalli, India. Shoot tips collected from these young plantlets (young shoot tip; YST) and mature plants (mature shoot tip; MST) were used as explants (Figs. 1B and 1C).

The collected explants were washed under running tap water for 2–5 min and then washed with Teepol solution for 30 s. The explants were surface sterilized with 70% alcohol for 30 s followed by 0.1% $HgCl_2$ for 2–4 min. The explants were then sheared either side (1.0–1.5 cm long) before being transferred to the nutrient medium.

Culture medium and culture conditions

Shoot bud induction, multiplication and shoot elongation were experimented on Murashige and Skoog (MS) medium (1962) based on macro, micro nutrients at full or half strength, 3% sucrose, myo-inositol (100 mg/L), 0.8% agar (Himedia; India) fortified with different plant growth regulators (PGRs)—cytokinins (6-benzyladenine, BA; kinetin, KN) at 0.5–3.0 mg/L; auxins (indole-3-acetic acid, IAA; indole-3-butyric acid, IBA; and 1-naphthalene acetic acid; NAA) at 0.1–1.0 mg/L; additives (adenine sulphate (AdS) at 5–15 mg/L and gibberellic acid (GA_3) at 0.01–0.5 mg/L); and antioxidants (polyvinylpyrrolidone, PVP; ascorbic acid, AA; and activated charcoal, AC) at 10–100 mg/L.

The best resultant combination on shoot bud induction of BA (2.0 mg/L), KN (1.0 mg/L), IAA (0.5 mg/L), GA_3 (0.1 mg/L) and AA (100 mg/L) was tested on different basal media of MS, mMS and woody plant medium (WPM) (Lloyd and Mc Cown, 1981). Finally, healthy and elongated shoots of 6–7 cm length were transferred to half-strength MS basal medium supplemented with IBA (0.1–1.0 mg/L) or NAA (0.1–1.0 mg/L) for rooting.

The pH of the medium was adjusted to 5.7±0.2 using 0.1 N NaOH or 0.1 N HCl before adding molten agar. The culture tubes were then sterilized by autoclaving at 104 KPa and 121°C for 20 min. All cultures were maintained under controlled conditions of 25±2°C at 16 hr photoperiod

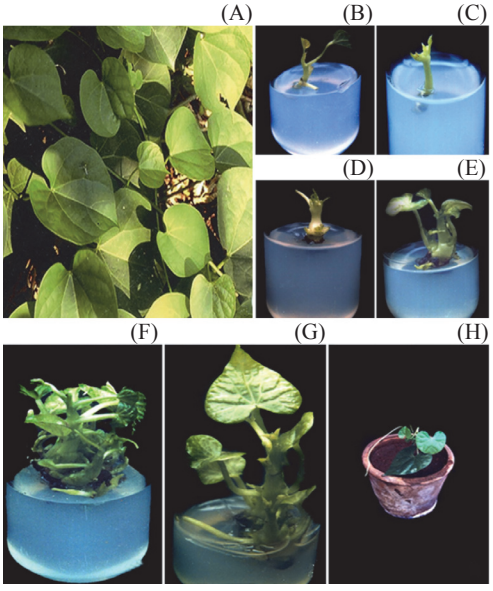

Fig. 1 *In vitro* micropropagation of *T. cordifolia* from shoot tip explants: (A) habit; (B) young shoot tip explants (2.0×); (C) mature shoot tip explants (2.5×); (D) shoot bud break from young shoot tip explant on kinetin (KN) 1.0 mg/L (2.5×); (E) shoot bud break from mature shoot tip explant on 6-benzyladenine (BA) 2.0 mg/L + KN 1.0 mg/L (2.0×); (F) multiple shoot induction from young shot tip explants on MS medium containing BA (2.0 mg/L), KN (1.0 mg/L) and 1-naphthalene acetic acid (NAA; 0.75 mg/L) (1.5×); (G) multiple, elongated shoots from mature shoot tip explant on MS medium with BA (2.0 mg/L), KN (1.0 mg/L), IAA (0.5 mg/L), gibberellic acid (GA_3; 0.1 mg/L) and ascorbic acid (100 mg/L) (2.0×); (H) rooted plantlets (on indole-3-butyric acid 0.5 mg/L) on hardening (3.5×)

under cool white fluorescent lights (35 μE/M^2/S^1) and 55–66% relative humidity.

Acclimatization

In total, 54 well-rooted plantlets from cultures aged 6 wk were removed from the culture tubes and washed free of agar. The plantlets were then transferred to paper pots (4 cm × 6 cm; height × width) containing red soil and vermiculite (1:3) and maintained at 25±2°C with 16 hr day length (35–50 μE/M^2/S^1) and 75–80% relative humidity. The potted plants were irrigated with half-strength MS basal medium devoid of sucrose and myoinositol every 4 d for 2 wk. Then, the plants were transplanted to earthen pots (25 × 25 cm; height × width) containing natural soil and farmyard manure and kept under shade for 2 wk and then moved to the garden.

Statistical analysis

The percentage of explants responding, shoot bud induction, number of shoots, shoot length and root initiation were recorded at the end of 45 d of culture. All experiments were repeated in triplicate with 10 replicates for each treatment. The experimental design was random and factorial with cytokinins and auxins as independent variables. Data on frequencies of shoot proliferation, shoot elongation and rooting were analyzed using Duncan's new multiple range test (Duncan, 1955) at the 5% level of significance.

Results

Shoot bud break

In plant growth regulator-free MS medium, the shoot tip explants failed to initiate shoot buds even after 30 d of culture, despite being green and fresh. In contrast, shoot bud initiation occurred on MS medium supplemented with PGRs, after 2 wk of culture with both YST and MST explants (Figs. 1D and E). There was a significant decrease in the frequency of bud break when the concentration was below or above the optimum concentration.

Effect of cytokinins on shoot production

On MS medium containing cytokinins, the inoculated shoot tips and their bases produced shoot primordia varyingin number and length at all the concentrations within 15 d of inoculation. Basal MS medium containing 2.0 mg/L BA produced the greatest number of shoots/explant (2.3), whereas KN was less effective (Table 1). Varying the concentration of BA and KN individually did not increase either the shoot number or shoot length. Assuming that KN was important in promoting shoot elongation, it was combined with BA in equal proportions at different concentrations. On the smallest improvement was observed in the number of shoots produced as well as their length. There was no callus formation on any of the explants at any of the concentrations. Multiple shoot formation occurred at 15–20 d of culture.

Table 1 Shoot induction and proliferation after 15 d of *Tinospora cordifolia* shoot tip explants on Murashige and Skoog medium (1962) supplemented with cytokinins and auxins

Plant growth regulators (mg/L)	Young shoot tip			Mature shoot tip		
	Bud break (%)	Number of shoots	Shoot length (cm)	Bud break (%)	Number of shoots	Shoot length (cm)
BA 0.5	86.6^{cd}	1.9 ± 0.35^{d}	1.5 ± 0.15^{f}	85.1^{cd}	1.4 ± 0.06^{d}	0.9 ± 0.05^{gh}
1.0	86.0^{d}	2.0 ± 0.22^{cd}	1.9 ± 0.06^{d}	85.9^{c}	1.9 ± 0.15^{c}	1.5 ± 0.15^{de}
2.0	88.3^{b}	2.3 ± 0.35^{b}	2.5 ± 0.22^{a}	87.3^{b}	2.1 ± 0.22^{b}	2.0 ± 0.18^{b}
3.0	88.0^{bc}	2.1 ± 0.36^{c}	1.8 ± 0.08^{de}	84.0^{de}	2.0 ± 0.18^{bc}	1.7 ± 0.05^{cd}
KN 0.5	83.7^{e}	1.4 ± 0.38^{g}	1.7 ± 0.06^{e}	83.3^{e}	1.0 ± 0.06^{f}	1.4 ± 0.15^{e}
1.0	90.1^{a}	1.6 ± 0.46^{f}	2.0 ± 0.14^{cd}	86.0^{bc}	1.1 ± 0.25^{ef}	1.6 ± 0.04^{d}
2.0	77.9^{fg}	1.0 ± 0.20^{i}	1.3 ± 0.30^{g}	78.0^{g}	0.7 ± 0.15^{h}	1.0 ± 0.12^{g}
3.0	71.5^{i}	0.9 ± 0.15^{ij}	0.8 ± 0.04^{i}	68.4^{i}	0.5 ± 0.05^{i}	0.5 ± 0.08^{i}
KN (1.0) + BA 1.0	85.1^{de}	2.2 ± 0.35^{bc}	2.1 ± 0.25^{c}	91.7^{a}	2.2 ± 0.35^{ab}	1.8 ± 0.15^{c}
2.0	87.5^{c}	2.5 ± 0.28^{a}	2.3 ± 0.24^{b}	84.1^{d}	2.3 ± 0.16^{a}	2.4 ± 0.16^{a}
3.0	78.9^{ef}	1.8 ± 0.05^{de}	1.6 ± 0.15^{ef}	80.1^{f}	1.2 ± 0.15^{e}	1.3 ± 0.20^{ef}
4.0	76.1^{h}	1.2 ± 0.15^{h}	1.1 ± 0.08^{h}	75.0^{h}	0.9 ± 0.05^{fg}	0.7 ± 0.15^{i}
BA+KN+IAA 0.10	88.2^{b}	5.4 ± 0.22^{e}	3.5 ± 0.25^{bc}	84.8^{e}	5.0 ± 0.38^{de}	3.0 ± 0.16^{c}
0.50	89.7^{ab}	8.2 ± 0.55^{a}	4.1 ± 0.22^{a}	87.6^{ab}	6.0 ± 0.52^{a}	3.6 ± 0.24^{a}
0.75	83.1^{d}	6.9 ± 0.45^{c}	3.3 ± 0.25^{c}	84.0^{cd}	5.5 ± 0.35^{c}	2.9 ± 0.15^{cd}
1.00	76.6^{g}	4.7 ± 0.26^{f}	2.4 ± 0.18^{e}	71.0^{g}	4.3 ± 0.26^{g}	2.1 ± 0.09^{g}
BA+KN+NAA 0.10	80.1^{ef}	5.0 ± 0.68^{ef}	2.9 ± 0.24^{d}	79.6^{e}	4.6 ± 0.28^{f}	2.5 ± 0.10^{f}
0.50	85.3^{c}	5.7 ± 0.55^{d}	3.1 ± 0.09^{cd}	83.3^{d}	5.1 ± 0.20^{d}	2.7 ± 0.08^{e}
0.75	90.0^{a}	7.6 ± 0.48^{b}	3.7 ± 0.15^{b}	89.1^{a}	5.9 ± 0.16^{ab}	3.3 ± 0.14^{b}
1.0	81.0^{e}	5.5 ± 0.68^{de}	2.0 ± 0.24^{f}	76.0^{f}	4.1 ± 0.25^{gh}	2.0 ± 0.21^{gh}

BA = 6-benzyladenine; KN = Kinetin; IAA = indole-3-acetic acid; NAA = 1-naphthalene acetic acid.
Values (mean ± SE) with the same superscript are not significantly different at 5% probability level according to Duncan's new multiple range test.

Effect of auxins on shoot production

The optimized concentration of cytokinins (BA 2.0 mg/L + KN 1.0 mg/L) was combined with different auxins (IAA and NAA) to enhance shoot formation. There was a significant increase in shoot number on YST (8.2 shoots/explant) and MST (6.0 shoots/explant) on MS medium fortified with BA (2.0 mg/L) + KN (1.0 mg/L) + IAA (0.5 mg/L) within 15–20 d of culture (Table 1, Figs. 1F and G).

Effect of additives on shoot production

Addition of AdS (5–15 mg/L) to the culture medium considerably reduced both the shoot induction and number of shoots, whereas GA_3 (0.01–0.5 mg/L) significantly enhanced the shoot formation and development (Table 2). GA_3 (0.1 mg/L) in combination with BA (2.0 mg/L) + KN (1.0 mg/L) + IAA (0.5 mg/L) produced highest response of 92.1% and 91.3% on YST and MST, respectively. The highest number of 11 shoots/explant with an average length of 6.6 cm was observed.

Effect of antioxidants on shoot production

In order to control phenolic exudation from cut ends which considerably reduced the shoot number and quality of shoots, various antioxidants (PVP, ascorbic acid and charcoal) at different concentrations were used. Among the treatments, ascorbic acid at 100 mg/L considerably reduced phenolic exudation followed by PVP (Table 3). The highest number of YST explants (98.3%) responded with the highest number of 16 shoots/explant on medium containing 100 mg/L of AA. Charcoal at 10 mg/L produced a good response but increased concentration greatly affected shoot number. PVP and charcoal were least effective and were inhibitory for shoot induction and multiple shoot formation, respectively.

Effect of various culture media on shoot production

Of the different media tested (MS, mMS, WPM), the MS medium produced the highest numbers of 98% and 98.3% shoot bud break and multiple shoot formation, respectively, on YST and MST explants, respectively within 30 d of culture (Table 4). The media mMS and WPM were less supportive of bud break and shoot formation at the same PGR treatments compared to the MS medium.

Table 2 Effect of additives on shoot induction and proliferation after 30 d of *Tinospora cordifolia* explants on Murashige and Skoog medium (1962)

Additive (mg/L)	Young shoot tip			Mature shoot tip		
	Bud break (%)	Number of shoots	Shoot length (cm)	Bud break (%)	Number of shoots	Shoot length (cm)
AdS5	65.0^{d}	6.0 ± 0.40^{e}	4.9 ± 0.11^{cd}	63.6^{d}	5.5 ± 0.18^{c}	3.6 ± 0.20^{e}
10	69.5^{cd}	7.1 ± 0.34^{d}	5.2 ± 0.09^{c}	67.3^{cd}	5.1 ± 0.20^{cd}	4.8 ± 0.25^{c}
15	70.9^{c}	6.6 ± 0.25^{de}	4.3 ± 0.18^{e}	69.1^{c}	5.0 ± 0.24^{d}	4.1 ± 0.14^{d}
GA_3 0.01	90.0^{ab}	9.1 ± 0.28^{bc}	6.3 ± 0.15^{ab}	90.0^{ab}	8.6 ± 0.06^{ab}	6.0 ± 0.15^{ab}
0.1	92.1^{a}	11.0 ± 0.36^{a}	6.6 ± 0.22^{a}	91.3^{a}	9.3 ± 0.35^{a}	6.1 ± 0.09^{a}
0.5	87.6^{b}	9.7 ± 0.51^{b}	6.2 ± 0.30^{b}	85.0^{b}	8.0 ± 0.14^{b}	5.7 ± 0.10^{b}

AdS = adenine sulphate; GA_3 = gibberellic acid.

MS medium contained 6-benzyladenine (2.0 mg/L), kinetin (1.0 mg/L), indole-3-acetic acid (0.5 mg/L).

Values (mean ± SE) with the same superscript are not significantly different at 5% probability level according to Duncan's new multiple range test.

Table 3 Effect of antioxidants on shoot induction and proliferation after 30 d of *Tinospora cordifolia* explants on Murashige and Skoog medium (1962)

Antioxidant (mg/L)	Young shoot tip			Mature shoot tip		
	Bud break (%)	Number of shoots	Shoot length (cm)	Bud break (%)	Number of shoots	Shoot length (cm)
PVP 10	81.0^{d}	5.3 ± 0.22^{ef}	4.6 ± 0.15^{de}	78.3^{e}	4.9 ± 0.41^{de}	4.4 ± 0.25^{de}
50	84.1^{cd}	6.0 ± 0.10^{e}	4.9 ± 0.24^{d}	83.6^{cd}	5.4 ± 0.36^{d}	4.8 ± 0.14^{b}
100	86.7^{c}	9.7 ± 0.15^{d}	5.3 ± 0.15^{c}	86.0^{c}	8.6 ± 0.25^{bc}	5.1 ± 0.30^{c}
AA 10	97.1^{b}	11.7 ± 0.05^{bc}	6.3 ± 0.18^{ab}	95.3^{b}	9.3 ± 0.35^{b}	6.2 ± 0.22^{b}
50	98.0^{ab}	13.0 ± 0.08^{d}	6.6 ± 0.06^{ab}	97.9^{ab}	11.1 ± 0.32^{b}	6.4 ± 0.12^{ab}
100	98.3^{a}	16.0 ± 0.12^{a}	6.9 ± 0.04^{a}	98.0^{a}	12.9 ± 0.27^{a}	6.7 ± 0.36^{a}
AC 10	75.1^{e}	3.3 ± 0.15^{g}	5.0 ± 0.12^{cd}	73.0^{e}	3.0 ± 0.32^{f}	5.0 ± 0.20^{cd}
50	72.6^{ef}	2.9 ± 0.20^{gh}	3.9 ± 0.20^{f}	71.1^{ef}	2.0 ± 0.14^{fg}	3.7 ± 0.15^{f}
100	69.1^{f}	2.6 ± 0.14^{h}	2.7 ± 0.05^{g}	67.1^{g}	1.9 ± 0.05^{g}	2.4 ± 0.25^{g}

PVP = polyvinylpyrrolidone; AA = ascorbic acid; AC =activated charcoal.

MS medium contained 6-benzyladenine (2.0 mg/L), kinetin (1.0 mg/L), indole-3-acetic acid (0.5 mg/L), gibberellic acid(0.1 mg/L).

Values (mean ± SE) with the same superscript are not significantly different at 5% probability level according to Duncan's new multiple range test.

Table 4 Shoot induction and proliferation after 30 d of *Tinospora cordifolia* shoot tip explants on different media

Medium	Young shoot tip			Mature shoot tip		
	Bud break (%)	Number of shoots	Shoot length (cm)	Bud break (%)	Number of shoots	Shoot length (cm)
MS	98.0^{a}	16.0 ± 0.21^{a}	6.9 ± 0.34^{a}	98.3^{a}	12.9 ± 0.16^{a}	6.7 ± 0.18^{a}
mMS	81.0^{b}	11.0 ± 0.32^{b}	6.6 ± 0.25^{ab}	80.3^{b}	9.1 ± 0.18^{ab}	6.5 ± 0.25^{ab}
WPM	76.7^{bc}	6.3 ± 0.16^{bc}	6.5 ± 0.32^{b}	76.1^{bc}	6.0 ± 0.20^{c}	6.4 ± 0.12^{b}

MS = Murashige and Skoog medium (1962); mMS = modified Murashige and Skoog medium (1962); WPM = woody plant medium (Lloyd and Mc Cown, 1981).

Media contained 6-benzyladenine (2.0 mg/L), kinetin (1.0 mg/L), indole-3-acetic acid (0.5 mg/L), gibberellic acid (0.1 mg/L) and ascorbic acid (100 mg/L).

Values (mean ± SE) with the same lowercase superscript are not significantly different at 5% probability level according to Duncan's new multiple range test.

Rooting of shoots

Auxins were essential for rooting in *T. cordifolia*, since the explants failed to develop roots in medium devoid of PGRs. Micropropagated shoots of about 6 cm of length were cut and placed on rooting medium. Half-strength MS medium supplemented with IBA and NAA (0.1–1.0 mg/L) was effective for rooting, where root induction was observed with 15 d of culture. IBA at 0.5 mg/L produced maximum rooting responses of 79.1% and 77% and averages of 5.8 and 5.3 roots, respectively, on YST-derived and MST-derived shoots, respectively (Table 5). In addition, the highest root lengths of 4.3 cm and 4.1 cm roots YST-derived and MST-derived shoots, respectively, were observed on the same medium (Fig. 1H). However, some very low instances of callus formation were also observed on all rooting treatments; the concentration of auxins increased the frequency of callus formation.

Acclimatization and hardening of plantlets

Plantlets (6–7 cm long) developed with fully expanded leaves and well-developed roots were successfully transferred to plastic containers containing red soil and vermiculite (1:3) and kept at 25±2°C for 2 wk (Fig. 1H). The hardened plantlets were then transferred to earthen pots containing red soil and vermiculite (1:3), for another 2 wk and the potted plants were then placed under shade. Later potted plants were transferred to field conditions. About 80% (43 plants) of the plants survived during the transfer from the *in vitro* to the *in vivo* conditions. Normal morphology and growth characteristics were noted in the regenerated plants. Normal growth of the potted plants was observed within 15–20 d after transfer to field conditions.

Discussion

Nutrient media comprising mineral nutrients, together with the qualitative and quantitative aspects of PGRs play a major role in micropropagation (Vardja and Vardja, 2001). In the same way, the nature and concentration of cytokinins used in the current study greatly influenced the shoot proliferation in the axillary shoots of the mature and *in vitro*-raised plants. BA was more effective in shoot proliferation (2.3 shoots/YST explant and 2.1 shoots/MST explant) than KN (1.6 shoots per YST explant and 1.1 shoots/MST explant) from both types of shoot tip explants of *T. cordifolia* (Table 1). A similar effect of BA (BAP 1.0 mg/L) over KN was reported from nodal explants of *T. cordifolia* (Kumari, 2012). However, higher shoot formation (12.3 shoots/explant and 4.81 shoots/explant) from 0.2 mg/L and 2.0 mg/L BA was reported from the same nodal explants of *T. cordifolia* (Bhat et al., 2013; Sivakumar et al., 2014). Raghu et al. (2006) reported that BA was more effective in axillary shoot proliferation (6.3 shoots/explant), while KN was superior to BA in shoot elongation. However, both the shoot number and shoot length were higher on media supplemented with BA than with KN.

The combined effect of BA along with KN is often reported to enhance the axillary shoot induction and shoot growth from nodal explants of *T. cordifolia*, albeit, KN alone (at 8 µM or 13.94 µM) was sufficient to generate the maximum frequency (100%) of shoot induction, followed by increased shoot proliferation combined with BAP or BA (Gururaj et al., 2007; Khanapurkaret al., 2012; Kumari, 2012; Bhaleraoet al., 2013). The maximum number (14.9) of shoots per nodal explant was reported on the MS medium containing BAP (2.0 mg/L) and KN (4.0 mg/L) (Handique and Choudhury, 2009). The superiority of BA over other cytokinins regarding better bud break and multiple shoot formation is well documented in other species such as *Curculigo orchioides* Gaertn (Wala and Jasrai, 2003) and *Rauvolfia serpentina* (L.) Benth. (Ahmed et al., 2002). In the current study, the BA and KN combinations produced no improvement in shoot production from both types of shoot tip explants, indicating that beside the tissue type, the PGR requirement for improved shoot production varies with different explants of the same plant.

The synergistic effect of an auxin (IAA/NAA) with the cytokinins (BA + KN) in *T. cordifolia* was pronounced in axillary shoot proliferation from the shoot tip explants. Bhat et al. (2013) observed thatBA in combination with NAA supported multiple shoot formation, while rooting was achieved with IAA from the nodal explant shoots of *T. cordifolia*. However, in the current study the inclusion of either IAA or NAA, along with BA and KN, enhanced shoot production to more than two–fold (8.2 shoots/YST explant and 6.0 shoots/MST explant) with more improved shoot growth than bud break possibly because the advantages of adding auxin at low concentration nullified the effect of higher concentrations of the cytokinin on axillary shoot elongation (Hu and Wang, 1983). Previous reports on other plants including *Solanum trilobatum* L., *Rauvolfia serpentina* (L.) Benth. and *Santolina canescens* Lagasca were consistent with the current results of multiple shoot formation using BA with IAA combinations (Ahmed et al., 2002; Arockiasamy et al., 2002; Casado et al., 2002). In addition, low concentration of auxins in combination with the cytokinins positively modified the frequency of shoot induction and

Table 5 Root induction after 15 d of *in vitro* shoots of *Tinospora cordifolia* on half-strength Murashige and Skoog medium (1962) supplemented with auxins

Auxin (mg/L)	Young shoot tip				Mature shoot tip			
	Rooting (%)	Number of roots	Root length (cm)	Callus formation (%)	Rooting (%)	Number of roots	Root length (cm)	Callus formation (%)
IBA 0.1	77.0^{bc}	3.7 ± 0.23^{bc}	4.0 ± 0.15^{bc}	3.0^{de}	75.1^{bc}	3.4 ± 0.12^{c}	4.0 ± 0.26^{ab}	3.0^{d}
0.5	79.1^{a}	5.8 ± 0.30^{a}	4.3 ± 0.09^{a}	5.0^{c}	77.0^{a}	5.3 ± 0.15^{a}	4.1 ± 0.32^{a}	4.0^{c}
1.0	76.1^{c}	3.5 ± 0.18^{c}	4.1 ± 0.16^{b}	6.0^{ab}	74.3^{c}	3.3 ± 0.08^{cd}	3.8 ± 0.45^{c}	6.2^{a}
NAA 0.1	75.0^{cd}	3.2 ± 0.24^{cd}	3.6 ± 0.18^{d}	2.6^{e}	73.7^{cd}	3.0 ± 0.22^{d}	3.5 ± 0.28^{e}	2.0^{e}
0.5	77.8^{b}	4.1 ± 0.20^{b}	3.9 ± 0.35^{c}	3.6^{d}	75.3^{b}	4.0 ± 0.16^{b}	3.7 ± 0.30^{cd}	3.4^{cd}
1.0	73.2^{e}	3.1 ± 0.16^{d}	3.5 ± 0.24^{de}	6.2^{a}	71.9^{e}	2.9 ± 0.24^{de}	3.1 ± 0.25^{f}	6.0^{ab}

IBA = indole-3-butyric acid; NAA = 1-naphthalene acetic acid.

Values (mean ± SE) with the same lowercase superscript are not significantly different at 5% probability level according to Duncan's new multiple range test.

growth, yet an increased concentration of auxins facilitated more callus formation on the shoot base in *T. cordifolia*. Premature leaf fall was seen in some plants during *in vitro* multiplication even with the addition of KN.

In order to improve the quality of shoots, GA_3 and AdS were added to the medium. The addition of AdS did not enhance shoot proliferation as reported by Neeta Mishra et al. (2003), whereas GA_3 (0.1 mg/L) considerably enhanced the frequency of bud break as well as shoot proliferation and shoot length. In the micropropagation of *Vitex negundo* L., Sahoo and Chand (1998) reported on the synergetic effect of GA_3 and BA producing similar results of faster bud break coupled with an enhanced frequency of shoot development and internode elongation.

The current study also showed the potential of antioxidants for enhanced shoot proliferation in *T. cordifolia*. AC and PVP virtually failed to support the explants for enhanced shoot production. Conversely, ascorbic acid increased the shoot production at 10–100 mg/L of concentration. At the highest concentration of 100 mg/L AA, the greater reduction of phenolic exudation supported the shoot tip explants to produce their highest numbers of shoots (16 for YST explants and 12.9 for MST explants). In general, antioxidants protect explants from browning as they act as reducing agents by decreasing the redox potential of phenols in the medium. This is achieved by reverting quinones that are formed by the oxidation of the phenolic compounds produced in damaged tissue or by competing with free radicals and removing them from the reaction (Debergh and Read, 1993).

The effect of juvenility on explant performance was seen with the ability of the young shoot tip explants to provide better results than the mature shoot tip explants. This was best illustrated in *Gymnema elegans* (Komalavalli and Rao, 1997) and in *Asimina triloba* (L.) Dunal (Finneseth et al., 2000) where only 4% of mature explants survived and they failed to produce shoot buds, whereas 88% of seedling explants produced expanded shoots. The rate of multiplication was high and stable up to the third subculture and then declined in subsequent subcultures. This might have been due to the balancing of the endogenous and exogenous PGRs and the ionic concentration of nutrient salts as reported in other plants (Rout and Das, 1997).

The composition of the basal medium significantly affected the bud break and shoot production, whereas there was no significant effect on shoot length. The percentage of bud break, number of shoots per explant and average shoot length were comparatively higher on MS medium than for mMS and WPM, albeit the latter was reported to be superior to MS medium for mature nodal explants in multiple shoot induction from *T. cordifolia* (Raghu et al., 2006). However, the current results on MS medium from both YST and MST explants were much better than reported from mature nodal explants by Raghu et al. (2006). Nitrogen is well known to serve as a constituent of many plant cell components and its deficiency inhibits plant growth (Giehl and von Wirén, 2014). In addition, the total nitrogen content and the ratio of nitrate to ammonium are very important aspects in nitrogen nutrition, since the ratio strongly influences the pH of the medium, which in turn determines the absorption of various other nutrients. Thus, as in most plant species, the relatively higher supply of nitrate-nitrogen within the MS medium could have had a profound effect on the shoot growth of this plant species as observed with *Amomum krervanh* Pierre (Tefera and Wannakrairoj, 2004).

MS medium with either full strength or half strength macro nutrients, has been used for rooting in *T. cordifolia*. In the current study, half-strength MS medium fortified with IBA was better than NAA for *in vitro* rooting. Several reports on this species have supported that IAA, IBA or NAA either alone or in combination with BA were suitable for effective root induction to shoots derived from nodal explants. Bhat et al. (2013) and Raghu et al. (2006) reported that minimal concentrations of 0.2 to 0.5 mg/L of IAA supplied on half-MS medium were suitable for rooting. Furthermore, IBA levels as low as 0.1 mg/L or about 1.3 mg/L were reported to produce the best response for root number and length within 15–27 d of culture (Gururaj et al., 2007; Sivakumar et al., 2014). NAA from 0.4 mg/L to 2.5 mg/L individually or in combination with BA has been reported as best for *in vitro* rooting of micro shoots (Singh et al., 2009; Khanapurkar et al., 2012; Kumari, 2012; Bhalerao et al., 2013). In the current study, the percentile response of root induction was similar in IBA and NAA, whereas IBA produced a considerably greater number of roots along with better root length compared to NAA.

In conclusion, shoot tip explants from young plantlets are recommended in preference to those from mature plants for *in vitro* mass production of *T. cordifolia* via micropropagation. Full-strength MS medium fortified with BA (2.0 mg/L), KN (1.0 mg/L), IAA (0.5 mg/L), GA_3 (0.1 mg/L) and AA (100 mg/L) induced the greatest numbers (16.0 and 12.9) and lengths (6.9 cm and 6.7 cm) of shoots from YST and MST explants, respectively. Root induction with the greatest root numbers (5.8 and 5.3) for YST and MST explants, respectively, was best achieved on one-half MS medium fortified with IBA (0.5 mg/L). Rooted plantlets can be hardened and acclimatized in a red soil and vermiculite mixture (1:3) to produce the maximum (80%) survival rate. The efficient protocol developed for this plant produced multiple shoots *in vitro* and effective hardening and acclimatization produced healthy normal shoots *in vivo*; both procedures should be very helpful in further study on the medicinally important compounds of *T. cordifolia*. This protocol could also be applied to other medicinally important genera.

Conflict of Interest

The authors declare no conflicts of interest.

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