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Full Research Article

Efficient Stable *in Vitro* Micropropagation and Conservation of *Tinospora cordifolia* (Willd.) Miers: an Anti-diabetic Indigenous Medicinal Plant

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Abstract

An efficient protocol is described for the rapid *in vitro* multiplication of *Tinospora cordifolia* (Willd.) Miers., an important indigenous medicinal plant, via enhanced shoot bud proliferation from shoot tip explants. This plant has great importance to different pharmaceutical industry for its medicinal value and mainly harvested from the wild population is resulting an over-exploitation of natural habits. To avoid this alarming situation and to ensure its continuous supply, plant tissue culture technique was applied. The explants cultured on to MS medium containing different concentrations of BAP, Kn and NAA either alone or in combination for investigation of morphogenetic response. The best shoot proliferation (16.5±0.25) was observed MS media supplemented with 5.0 mg l^{-1} BAP alone. The highest shoot elongation observed MS medium containing 1.0 mg l^{-1} BAP in combination with 0.6 mg l^{-1} GA_3 (12.5±1.5 cm). The maximum rooting frequency (82.00±3.0%) obtained from the MS medium in the combination 1.0 mg l^{-1} IBA. For *in vitro* conservation, the best response (95%) was achieved using 2% sorbitol and 2% mannitol in combination with MS medium at 10 °C temperature up to 8 months by *in vitro* slow growth technique. Regenerated plantlets were transferred to greenhouse conditions and observed 90.00±0.4% survival frequencies. The diploid status (2n=24) of regenerated plantlets as well as mother plant was determined using chromosome counts of root-tips. Berberine content (0.218%) in regenerated plants was determined by using HPTLC and HPLC methods. It is concluded that the regenerated plants were stable in respect to their morphology, chromosome number and berberine content, which offers a unique opportunity to obtain true-to-type plants as source plant.

1. Introduction

Tinospora cordifolia (Willd.) Miers. ex. Hook. F. and Thomas, that claimed to have well known Indian medicine, belongs to Meninspermaceae family, found through out India, especially the tropical part of India (Gururaj et al., 2007). This plant is well-known to us as the name of giloya and guduchi Sharma et al., 2010). This plant is the rich source of alkaloid and terpenes (Sharma et al., 2010). One of the important chemical components is berberine have been isolated from the stem of *T. cordifolia* (Rao et al., 2008; Singh et al., 2003). Berberine is bioactive alkaloids. Berberine belongs to the protoberberine alkaloids and although several alkaloids of this type are of pharmaceutical importance most attention has been paid to berberine; it is widely used, especially in the Asian market (Berlin, 1997). Modern science has confirmed that berberine has impressive benefits for several different health problems. It has been frequently used for the treatment of diabetes, hypertension, hyperlipidemia etc (Lan et al., 2015). The drug is well known Indian bitter and prescribed in fevers, dyspepsia, diabetes, urinary problems, jaundice, skin disease, gout, gonorrhea, rheumatism, snake bites, leucorrhoea etc (Gururaj et al., 2007). During the last decades considerable progress has been achieved regarding this plant biological activity and medicinal applications such as anti-diabetic, anti- periodic, general tonic, anti-arthritic, antioxidant and anti fertility activities (Gulati, 1980; Stanely et al., 2000 and 2001; Gupta and Sharma, 2003). The National Medicinal Plant Conservation Board has considered as an important plant species for sustainable utilization, cultivation and trade of *T. cordifolia* (Richa et al., 2013). Due to the high pharmaceutical importance, these plants are collected and used as raw material for large scale medicinal industry. The genetic biodiversity of this plant may be a threatened condition due to population growth, urbanization, the unrestricted collection of these plants from the natural habits (Sharma et al., 2010). To cope up with this alarming situation, it is very essential

to immediate conservation of these important plant species through biotechnological approach like plant tissue culture technique (Hassan, 2012). Due to less seed germination with poor viability in nature normal propagation of this plant species is very difficult. Conventional propagation cannot meet the increasing demand and will ultimately lead to extinction if no attention is given to its conservation and propagation (Amoo et al., 2009). Tissue culture techniques offer a viable tool for mass multiplication and germplasm conservation of elite medicinal plants while at the same time facilitating pharmaceutical and other commercial needs (Sahoo and Chand, 1998; Anis and Faisal, 2005). Genetic improvement is another approach to augment the drug yielding capacity of the plant (Tejavathi and Shailaja, 1999). Slow growth is usually achieved by reducing the culture temperature, by modifying culture media with supplements of osmotic agents, growth inhibitors, or by removing growth promoters (Lata et al., 2010).

The cytological stability of micropropagated plants needs to be checked before using this protocol at the commercial level (Bhojwani and Razdan, 1996; Landey et al., 2015; Regalado et al., 2015; Tomiczak et al., 2015). Joshi and Rao (1935), in their study, have counted the haploid chromosome numbers (n=12) of *Tinospora cordifolia*.

Evaluation of phytochemical content through HPTLC and HPLC techniques are very popular in herbal medicine and pharmaceutical industry due to easy learns and is not limited by the volatility or stability of the sample compound (Dubey et al., 2004). HPLC and HPTLC methods have emerged as an efficient tool for phytochemical evaluation of herbal drugs (Liang et al., 2004).

In order to the flow of loss of biodiversity, an attempt to conserve through *in vitro* technique of *T. cordifolia*, has been made in this study. Shoot tip explants were introduced for establishing *in vitro* culture, induced to multiply. The objective of the present study based on to develop a simple, efficient protocol via shoot tip culture for large scale uniform plant production, *in vitro* conservation using a slow growth technique of *T. cordifolia* and also to assess genetic stability of regenerants by chromosomal status, morphological character analysis and berberine content evaluation.

2. Materials and Methods

2.1. Plant material

Tinospora cordifolia (Willd.) Miers. ex Hook. F. and Thomas were collected from various locations in West Bengal, India, were grown under identical environmental conditions of the experimental garden of the RKMVC College, Rahara, Kolkata, W.B., India. The identification of collecting specimen was confirmed by the authority of Botanical Survey of India (Reference No.- CNH/2014/Tech.II/78/200).

2.2. Multiple shoots induction and plantlet regeneration

Excised shoot tip segments (8–10 mm) were surface sterilized (with 2% w/v Bavistin®, 5% v/v Tween-20 and 0.1% w/v $HgCl_2$ solution) and after that inoculated on to MS (Murashige and Skoog, 1962) medium alone as control and MS medium with different concentrations and combinations of cytokinins (BAP and Kn) either individually or with auxin (NAA). All the cultures were incubated at 24±2 °C temperature and 55±5% relative humidity under 16/8 h photoperiod. The effects of different plant growth regulators were tested and were recorded periodically. All cultures were subcultured at 15 days interval.

2.3. Shoot elongation

After 60 days of culture, the induced multiple shoots were excised individually and subcultured on to MS medium supplemented with BAP (1.0 mg l^{-1}) and varying concentrations of gibberellic acid (GA_3) (0.2, 0.4, 0.6, 0.8, and 1.0 mg l^{-1}) for elongation of shoots. A control group (basal medium without any PGR) was also maintained. The cultures were maintained at 25±2 °C and 16 h photoperiod with light intensity of 30 µmol $m^{-2}s^{-1}$.

2.4. In vitro root induction

For rooting, *in vitro* grown micro shoots (2–3 cm) with 2–4 young light green leaves were excised and implanted on both MS and ½ MS basal media, which served as a control. The individual micro shoots were subcultured with different concentrations of IBA and IAA (0.2, 0.5 and 1.0 mg l^{-1}) in MS medium along with 3% sucrose (Merck, India).

2.5. In vitro conservation

A standard slow growth procedures with minimal nutrient media and long gap between subculture as well as cold temperature storage methods were followed in this experiment. Mannitol and sorbitol (1.0%–3.0%, w/v) with 1.5% sucrose (w/v) and 0.8% (w/v) agar were used as slow growth regulators. The effects of MS medium in combination with different concentration of sorbitol and mannitol, on growth of *T. cordifolia* cultures were studied. Proliferated shoots were rooted on MS medium containing IBA and IAA.

2.6. Acclimatization and field experiments

Prior to field transfer hardening is must and for hardening, the tissue culture derived regenerated plantlets were removed from the agar medium and transferred to earthen pots containing soilrite (Keltech Energies Ltd, Bangalore, India) and covered with transparent polythene bag for 30–35 days and finally were transferred to the experimental field.

2.7. Morphological study

Through morphological study of conventionally grown plant as well as regenerated plants of *T. cordifolia,* were analysed by some parameters like petiole length, stem diameter, plant

length, number of leaves, leaf size and seed size.

2.8. Mitotic chromosome study

Mitotic chromosome studies of mother plant as well as regenerated plants were carried out from root tips of young and healthy plant of *T. cordifolia* using aceto-orcine staining method. Root tips were perpetrated in a saturated solution of *p*-dichlorobenzene (PDB) for 6 hrs at 14 °C and fixed with dehydrated ethanol and glacial acetic acid (3:1, v/v) for 24 hours at 4 °C. Fixed root tips were stained with 2.0% aceto-orcein: 1(N) HCl (9:1, v/v) mixture, followed by incubating for 2 h at room temperature. Metaphase plates were observed in Leica DM750 microscope and photographed with Leica DFC 295 camera.

2.9. Evaluation of berberine content

The major active compound berberine from the stems of *T. cordifolia* of mother plant as well as regenerated plants were extracted and analyzed. Berberine percentage was quantified through high performance thin layer liquid chromatography (HPTLC) technique (Camag, Muttenz, Switzerland) and high performance liquid chromatography (HPLC) technique (Waters 1525). A stock solution of standard berberine (Merck, India) was prepared (1 mg l^{-1}) which was black yellow in color. The dried stems were powdered and 1 g coarsely powdered drug samples were extracted with 10 ml methanol solvent by soxhlet apertures for 16 hours. The extracts were filtered by Whatmann no. 42 filter paper. TLC Aluminum pre coated plate with Silica gel $60F_{254}$ (20×10 cm^2; 0.2 mm thick) was used for HPTLC analysis. Methanolic extract of samples and standard berberine were applied on TLC plate by using Linomat 5 automated TLC applicator. The camag twin trough glass chamber was saturated with mobile phase 1 Butanol: Acetic acid: Water (7:2:1, v/v/v) for 30 minutes.TLC plate was developed to 8 cm distance above the position of the sample application. The plate was removed from the chamber and air dried at room temperature. The plate was scanned before derivatization using Camag TLC Scanner 4. Wincats Software 4.02 was used for the detection as well as for the evaluation of data. In HPLC method was used for qualitative and quantitative study of berberine from the stem of *T. cordifolia*, using C18 column and mobile phase was used acetonitrile: water (60:40, v/v). The calibration curve was plotted using single level calibration, made by preparation the solution of 1mg l^{-1} standard berberine. The plates were activated for 1 hr in hot air oven at 100 °C before used.

2.10. Statistical analysis

All the experiments were conducted with three replications. Means and standard errors were carried out for each experiment and data was analyzed using one way Analysis of Variance (ANOVA) to detect significant differences between means. Means differing significantly were compared using Tukey's multiple range test at a 5% probability level. Data analysis was performed using SPSS v 16.0 software.

3. Results and Discussion

3.1. Role of plant growth regulators on in vitro shoot induction and multiplication

The earliest visible sign of green shoot bud induction from shoot tip explants was noticeable after 12 days of culture on MS medium supplemented with BAP (1.0 mg l^{-1}). MS medium in combinations with BAP and Kn or BAP and NAA in different concentrations was found to be insignificant responding. Maximum response of shoot multiplication observed 62.50±0.23% and number of multiple shoots were 5.8±0.35, when MS medium supplemented with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} Kn. Highest 47.50±1.19% response of shoot multiplication with 3.3±0.15 multiple shoots were observed in MS medium in combination with 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA. In the presence of auxin, shoots remained stunted and the length did not improve even after repeated subcultures on the same fresh medium. Whereas, among the concentrations of BAP alone, ranging from 0.5 mg l^{-1} to 5.0 mg l^{-1}, gave the best results and it has been observed that the higher concentrations of BAP induced large amounts of white-greenish callus at the base of the shoot tip explants. As a result, the shoot become with deformed rosette leaves. The thick layer of the basal callus might also have prevented uptake of nutrients from the medium resulting in abnormal growth of the shoots or shoot growth become stunted (Rathore et al., 2007). Among the varying concentrations, MS medium supplemented with 1.0 mg l^{-1} Kn alone gave the better result and shoot multiplication rate was 50.0±0.10% with maximum 5.6±0.22 number of multiple shoots after 45 days of culture. Moreover, among the different concentrations of BAP tested, 5.0 mg l^{-1} was found to be the best in terms of yielding a high number of healthy shoots (16.5±0.25) with maximum number of leaves (17.0±0.45) and shoot multiplication observed 95.00±0.11% (Table 1) (Figure 1A and 1B) after 45 days of culture.

The inhibitory effect of BAP alone on shoot induction was also reported in *Tinospora cordifolia* (Raguhu et al., 2006), *Sidacordifolia* (Sivanesan and Jeong, 2007), *Cucurbita maxima* (Mahzabin et al., 2008) and *Gloriosa superba* (Chatterjee and Ghosh, 2015). Therefore, MS medium supplemented with 5.0 mg l^{-1} of BAP was selected as the optimal shoot multiplication medium and used for further study. And by repeated sub-culturing of shoot tip segments from newly formed axenic shoots, a high-frequency rapid shoot culture protocol was established.

3.2. Shoot elongation

Proliferated multiple shoots with an average height of 1.5 cm were carefully excised and transferred for shoot elongation on

Table 1: Effect of different cytokinins and auxins on *in vitro* shoot multiplication of *Tinospora cordifolia* after 45 days. (Each value represents the mean±SE 10 replicates and each experiment was conducted thrice.)

Medium (3% sucrose)	Concentrations (mg l^{-1})		% of shoot tip explants showing shoot multiplication	No. of multiplied shoots from shoot tip explant^{-1}	Length of micro shoots (cm)	No. of leaves micro shoot^{-1}
	Cytokinins	Auxins				
MS	0	0	31.66±0.10^{a}	1.8±0.20^{a}	10.5±0.40^{h}	9.6±0.70^{ab}
MS	BAP (0.5)	0	62.50±0.30^{c}	13.8±0.20^{cd}	6.0±0.25f^{g}	10.8±0.72^{abc}
MS	BAP (1.0)	0	65.00±1.2^{ab}	7.0±0.29^{g}	4.2±0.22^{g}	12.1±0.60^{ab}
MS	BAP (2.0)	0	72.50±0.12^{e}	10.1±0.43^{f}	4.5±0.25^{ef}	13.0±0.70^{gh}
MS	BAP (3.0)	0	87.50±0.75^{cd}	14.1±0.34^{cf}	4.6±0.25^{de}	14.8±0.75^{efg}
MS	BAP (5.0)	0	95.00±0.11^{e}	16.5±0.25^{def}	5.5±0.15^{abcd}	17.0±0.45^{bcde}
MS	BAP (6.0)	0	57.50±2.10^{c}	5.7±0.30^{de}	3.4±0.21^{ab}	11.2±0.50^{abc}
MS	BAP (7.0)	0	47.50±1.30^{g}	5.2±0.32^{cde}	3.1±0.08^{a}	8.9±0.40^{a}
MS	Kn (0.5)	0	32.50±0.40^{a}	9.0±0.21^{abc}	4.2±0.10^{bcd}	13.3±0.36^{cde}
MS	Kn (1.0)	0	50.00±0.10^{f}	5.6±0.22^{ab}	4.3±0.09^{bcd}	14.1±0.43^{def}
MS	Kn (2.0)	0	47.50±0.31^{ab}	4.3±0.15^{a}	4.1±0.09^{abcd}	12.1±0.38^{bcd}
MS	BAP (1.0)	Kn (0.5)	47.50±0.36^{d}	4.8±0.20^{ab}	3.8±0.13^{abcd}	10.1±0.31^{ab}
MS	BAP (2.0)	Kn (0.5)	62.50±0.23^{bc}	5.8±0.35^{cd}	4.4±0.10^{cd}	11.9±0.38^{bcd}
MS	BAP (3.0)	Kn (0.5)	57.50±0.70^{cd}	5.1±0.23^{abc}	3.8±0.13^{abcd}	16.0±0.45^{fgh}
MS	BAP (1.0)	NAA (0.5)	47.50±1.19^{e}	3.3±0.15^{a}	3.5±0.17^{abc}	7.0±0.33^{abc}
MS	BAP (2.0)	NAA (1.0)	32.50±2.21^{ef}	2.2±0.13^{a}	3.1±0.20^{a}	5.6±0.54^{ab}

MS medium containing different concentrations of Gibberellic acid (GA_3) (0.1, 0.2, 0.6, 0.8, and1.0 mg l^{-1}) with 1.0 mg l^{-1} BAP . The result suggests that 0.6 mg l^{-1} GA_3 in combination with 1.0 mg l^{-1} BAP was effective in stimulating *T. cordifolia* shoot elongation. Shoots attained a maximum height of 12.5±1.5 cm during GA_3 (0.6 mg l^{-1}) with BAP (1.0 mg l^{-1}) treatments after 30 days of culture (Table 2). Whereas, shoots attained a maximum height of 5.6±0.2 cm on to MS medium supplemented with GA_3 (1.0 mg l^{-1}) in combination with 1.0 mg l^{-1} BAP. The same result showed for shoot elongation for the effect of GA_3 in *Plumbago zeylanica* (Chatterjee and Ghosh, 2015) and *Artemisia vulgaris* (Sujatha and Kumari, 2007).

Table 2: Effect of GA_3 on shoot elongation of *T. cordifolia* from regenerated shoots cultured on MS medium supplemented with GA_3 and BAP (1.0 mg l^{-1}). (Data collected after 30 days of culture) (Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

BAP (mg l^{-1})	GA_3 (mg l^{-1})	Shoot elongation response (%)	Mean shoot (cm)
1.0	0.2	75.0±1.5^{c}	7.2 ±1.0^{c}
1.0	0.4	80.0±2.0^{a}	9.3 ±0.5^{b}
1.0	0.6	83.0±0.3^{bc}	12.5 ±1.5^{a}
1.0	0.8	70.0±1.6^{c}	6.0 ±1.0^{ab}
1.0	1.0	63.0±0.2^{e}	5.6 ±0.2^{ef}

3.3. Induction of roots from in vitro grown micro shoots

Root induction was achieved on both the MS and ½ MS basal media, but it was observed that in MS medium, roots were induced quickly than in ½ MS medium. In ½ MS medium, number of roots induced from micro shoots 6.6±0.22 after 24.2±0.49 days, but in MS medium these numbers were 9.0±0.36 after 14.3±0.30 days. Therefore, the only MS medium was selected for subsequent studies. To facilitate the induction of maximum number of roots within a short period of time, MS medium was supplemented with different concentrations of IBA and IAA (0.2, 0.5 and 1.0 mg l^{-1}). IBA gave better results than IAA for root induction of *T. cordifolia*. Maximum 75% root were induced with maximum number (11.0±0.45) of roots per explants was observed on MS medium in combination with 1.0 mg l^{-1} IAA after 19.2±0.31 days. The earliest (12.4±4.0 days) and highest percentage (82%) of root were induced with maximum number (11.0±0.45) of roots per explants (Figure 1C) was achieved on MS medium containing 1.0 mg l^{-1} of IBA (Table 3). The enhancing role of IBA among different auxins on *in vitro* root induction has been reported to be the most effective in different plant species, like *Hagenia abyssinica* (Bruce) (Feyissa et al., 2005); *Eclipta alba* (L) (Husain and Anis, 2006); *Acacia mangium* (Willd.) (Monteuuis and Bon, 2000).

Table 3: Effect of different auxins on *in vitro* root induction of *T. cordifolia*. (Each value represents the mean±SE 10 replicates and each experiment was conducted thrice.)

Nutrient medium (3% Sucrose)	Concentrations of auxins (mg l^{-1})	% of micro shoots showing root induction	No. of roots induced micro $shoot^{-1}$	Days taken for root induction
½ MS	0	68.00±1.1^{c}	6.6±0.22^{b}	24.2±0.49^{a}
MS	0	70.00±0.3^{a}	9.0±0.36^{a}	14.3±0.30^{b}
MS	IBA (0.2)	75.00±0.1^{fg}	7.5±0.23^{d}	22.4±3.6^{a}
MS	IBA (0.5)	78.00±1.7^{cd}	7.5±0.31^{a}	16.8±0.20^{b}
MS	IBA (1.0)	82.00±3.0^{a}	11.0±0.45^{c}	12.4±4.0^{c}
MS	IAA (0.2)	70.00±0.2^{b}	6.5±0.33^{a}	13.6±0.23^{b}
MS	IAA4 (0.5)	74.00±0.5^{ef}	7.0±0.43^{c}	10.4±0.30^{c}
MS	IAA (1.0)	75.00±0.7^{c}	8.3±0.36^{d}	19.2±0.31^{a}

3.4. In vitro conservation

Regeneration and successful propagation of genetically stable plants from cultures are perquisite in any *in vitro* conservation effort (Dube et al., 2011). The cultures maintained in good health conditions in controlled medium (MS medium with 3% sucrose, without PGR) with 100% survival rate maximum up to 6 weeks without subculture at 25±2 °C. But when cultures were maintained in manitol and sorbitol at 10 °C survived up to 8 months without subculture.

At 4 °C temperature, all the cultures showed poor performance (78.0±3.0%) and culture did not survive as in case of control temperature. At 18 °C, the survival rate of the cultures was 83.0±1.2%. This experiment suggests that cultures growth could be reduced at 18 °C, but storage period could not be increased to maintain healthy cultures. But at10 °C temperature regime the cultures grew healthy with reduced growth in comparisons to control and the survival rate was 94.0±1.4% (Table 4) and storage period could be increased up to 6 months. The addition of 2% sorbitol (w/v) and 2% mannitol (w/v) in combination with MS medium has increased survival rate 90% at 10 °C and storage period could be increased up to 8 months (Table 5). But MS medium without sorbitol and mannitol, the survival rate was 65.0±0.06% up to 4 months, 50.0±0.44% up to 6 months, 40.0±0.01% up to 8 months at 10 °C. The addition of osmotic agents to the culture has been proved to be efficient in reducing growth and increasing the storage life of different *in vitro* grown plant species (Wilson et al., 2000). Growth

Table 5: Effect of different osmotic on *in vitro* slow growth storage conditions of *T. cordifolia* at 10 °C. (Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

Medium	Plant height (cm)	Survival %	Storage period (month)
Full MS	9.3±0.46^{a}	65.0±0.06^{a}	4
	11.7±0.73^{c}	50.0±0.44^{bc}	6
	11.4±1.02^{c}	40.0±0.01^{ab}	8
MS+1% sorbitol	6.3±0.36^{b}	75.0±0.02^{a}	4
	8.4±0.33^{d}	68.0±1.01^{abc}	6
	8.5±0.02^{cd}	55.0±0.07^{b}	8
MS+2% sorbitol	6.3±0.40^{a}	85.0±0.40^{a}	4
	8.3±0.43^{c}	79.0±0.02^{cd}	6
	8.4±0.37^{bc}	65.0±0.34^{b}	8
MS+3% sorbitol	5.3±0.87^{b}	70.0±0.40^{cd}	4
	5.7±0.73^{bc}	61.0±0.01^{de}	6
	5.8±0.02^{c}	52.0±0.14^{c}	8
M+1% manitol	5.3±0.56^{b}	77.0±0.12^{ef}	4
	5.7±0.70^{c}	71.0±0.05^{cd}	6
	5.7±1.12^{cd}	63.0±0.56^{def}	8
M+2% manitol	4.3±0.16^{b}	82.0±0.03^{ab}	4
	5.3±0.73^{c}	77.0±0.21^{d}	6
	5.4±0.02^{b}	68.0±1.32^{b}	8
M+3% manitol	4.3±0.46^{a}	71.0±0.08^{ab}	4
	4.7±1.73^{bc}	62.0±0.12^{cd}	6
	4.9±0.05^{c}	48.0±0.24^{cd}	8
2% sorbitol+2% manitol	8.12±0.26^{a}	82.0±0.06^{ab}	4
	8.42±0.73^{ac}	85.0±0.09^{abc}	6
	8.0±1.22^{b}	90.0±0.02^{ab}	8

Table 4: Effect of low temperature on *in vitro* slow growth storage conditions of *T. cordifolia*. (Each value represents the mean±SD of 10 replicates and each experiment was repeated thrice)

Storage condition	Re-growth±SE after storage 4 mo	Survival (%)	Re- growth±SE after storage 6 mo	Survival (%)	Re-growth±SE after storage 8 mo	Survival (%)
Low temperature						
4 °C	3.0±1.0^{b}	78.0±3.0^{bc}	1.07 ±1.0^{c}	53.0±1.0^{cd}	1.9 ±1.7^{bc}	55.0±1.6^{bc}
10 °C	16.5 ±0.3^{a}	94.0±1.4^{ab}	5.5±1.0^{cd}	95.0±5.0^{b}	22.1±0.5^{ab}	95.0±1.5^{ab}
18 °C	7.3 ±1.0^{c}	83.0±1.2^{abc}	1.10 ±0.5^{a}	75.0±1.4^{ab}	3.2±1.9^{c}	65.0±0.4^{b}

suppression had positively reduced the labor during culture maintenance in the tissue culture laboratory and also promoted uniformity of growth among the converted plantlets (Lata et al., 2010). No signs of shoot or root growth was observed during storage. Sucrose is a major component of tissue culture media. Sucrose has ability to reduce the growth of *in vitro* plant (Moges et al., 2003). However, adding sucrose to the media has prevented dehydration in storage but it did not improve shelf-life of germplasm (Orlikowska, 1992). So, in this present study, less amount of sucrose was used (1.5%) for growth reduction of the culture and subculture interval also increased (8 months). All the cultures in storage condition were able to form roots during re-growth and successfully acclimatized in soilrite. Similar reports have documented post–storage beneficial effect in *Withania somnifera* (Chatterjee and Ghosh, 2012) and *Plumbago zeylanica* (Chatterjee and Ghosh, 2015). Our studies provided an effective protocol for storage of this medicinal plant under slow growth conditions. Germplasm can be stored effectively for 8 month without subcultures.

3.5. Acclimatization

Acclimatization is the final step in a successful micropropagation system. Successful establishment of *in vitro* regenerated plant lets in field conditions requires great care (Hoagland and Arnon, 1950). The successfully rooted plantlets were transfer to a small earthen pot containing soilrite and covered with transferent polytheen bags for hardening (Figure 1D). Finally the plantlets were transferred to Poly House and maintained 3–4 weeks (Figure 1E) and then it transferred to the experimental field condition (Figure 1F) and there was 90% survival rate (Table 6). After 8–10 months old all field growing regenerated plants produce flower as well as fertile seeds.

Table 6: Field acclimatization of *T. cordifolia* (Each value represents the mean±SE 15 replicates and each experiment was conducted thrice.) (After 30 days)

Parameters	No. of plants	Plants survived	Response %
Transferred for hardening	50.00±1.8^{a}	42.00±1.3^{c}	84.00±0.7^{ab}
Transferred for acclimatization	42.00±0.5^{bc}	40.00±2.1^{c}	90.00±0.4^{ab}

3.6. Morphological study

The plant, *T. cordifolia* exhibited extreme morphological and physiological variability in different ecological regions; including branching habits (branched or non-branched), degree of branching, leaf morphology within an individual and within a population (Richa et al., 2013). But in this *in vitro* experiment, there were no noticeable morphological changes in regenerated plants in respect to the source plant (Table 7). So, regenerated plants are morphological stable. Such mode of preservation can be utilized in other medicinal and economically important plants. Similar observation was reported in *Aloe vera* L. (Gantait et al., 2010).

Table 7. Morphological evaluation of the plant and tissue culture raised plants of *T. cordifolia* (Each value represents the mean±SE 15 replicates and each experiment was conducted thrice.) (Result taken after 90 days)

Parameters		Mother plants	Tissue culture raised plants
Petiole length (cm)		6.58±0.45^{a}	6.66±0.47^{a}
Stem diameter (cm)		2.9±0.37^{a,b}	3.2±0.36^{a,b}
Plant length (cm)		450.0±0.52^{b,c}	500.0±0.12^{b,c}
No. of leaf		76.0±0.32^{a,c}	90.0±0.35^{a,c}
Leaf size (cm)	Length	12.34±0.12^{b,d}	11.6±0.13^{b,d}
	Breadth	10.38±0.25^{b,d}	9.26±0.24^{b,d}
Seed size (cm)		0.83±0.37^{a}	0.84±0.37^{a}

3.7. Mitotic chromosomal study

Chromosomal analysis in the present studies from randomly selected root tips from the source plant showed 2n=24; cytological preparations from the *in vitro* derived plantlets (25 root tips and 5–10 metaphase/root tip) showed a predominantly diploid number (2n= 24) of chromosomes. All 15 regenerates so far analysed and same number of mitotic chromosome of mother plants and tissue culture raised plants (Figure 1G) was observed remain the same without any structural alterations. Thus the somatic chromosome complements of *in vitro* generated *T. cordifolia* plants remained stable even after passing through three cycles of multiplication. It also confirms that no chromosomal variation was induced from the short term conserved plants among the regenerates. It indicates that reduced growth conditions applied in our studies along with the inherent genotypes potentiality and the stability of the species helped to maintain germplasm over such a short period of time in maintained and chromosomal uniformity among the regenerates.This present findings are corroborated by some other studies where regenerants are found cytological stable (Ghosh and Sen, 1992; Ghosh and Sen, 1994).

3.8. Evaluation of berberine content

In connection with this study, a simple and accurate HPTLC and HPLC methods has been developed for the quantification of berberine from the stem of *T. cordifolia*.Various solvent compositions were used to determine the suitable mobile phase to obtain sharp, well resolved peaks for precise HPTLC analysis. The UV spectra measured for the peak showed maximum absorbance with mobile phase 1 Butanol: Acetic acid: Water (7:2:1, v/v/v) and detected at a wavelength of

Figure 1a and 1b: *In vitro* shoot multiplication

Figure 1c: *In vitro* complete plant with root
Figure 1d: Hardening

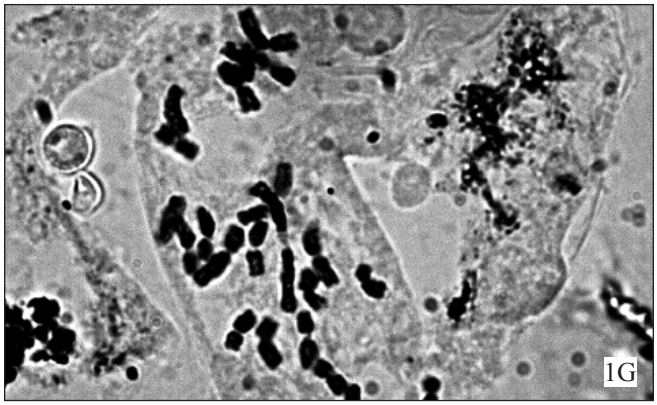

Figure 1e: Hardening; Figure 1f: Tissue culture raised plant;
Figure 1g: Chromosome (2n=24)

220 nm. Sharp peaks of berberine (standard and samples) were obtained when the plate was scanned at wavelength 220 nm and black yellow colorb and of berberine was obtained at R_f0.77. Quantities of berberine found in samples were obtained automatically via graph. The accuracy and reproducibility of the method were established by means of recovery experiment. The mean recovery was close to 100% which indicates the accuracy of the method. The robustness of the method was studied, during method development, by determining the effect of small variation, of mobile phase composition (±2.0%), chamber saturation period, development distance, derivatization time and scanning time (10% variation of each). No significant change of R_f or response to berberine was observed, indicating the robustness of the method.

A sharp and symmetric peak of berberine was obtained with good baseline resolution and minimal tailing, thus facilitating the accurate measurement of peak area. Satisfactory retention times and good resolution of berberine was achieved using C18 column with mobile phase acetonitrile: water (60:40, v/v). The HPLC analysis was carried out in isocratic condition and a retention time (Rt) of 5.15 min and detected at a wavelength of 265 nm was obtained for standard berberine.The purity of the isolated constituents was confirmed by TLC.

It was observed that concentration of berberine of crude extract was 0.218% of mother plant same as tissue culture raised plants. In this present study, the proposed phytochemical analysis technique may be used as an alternative method for quantification and standardization of berberine from the stem of *T. cordifolia*. The similar technique reported by Sivakumar and Dhanarajan (2011); Balajee et al. (2012).

4. Conclusion

Present studies provide a simple and effective protocol of *in vitro* propagation and conservation of *T. cordifolia* up to 8 months without subcultures. The adopted HPTLC and HPLC techniques are used indicates that the methods were efficient for qualitative identification and quantitative determination of berberine. This in vitro method also provides an important methodology which determines the genetic-morhogenetic-phychemical stable quality plantlets. So anyone can adopt such protocol for future use.

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