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In vitro propagation of *Tinospora cordifolia* and estimation of berberine content by Chromatographic analysis

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ABSTRACT

The present study was designed to establish a propagation method of *Tinospora cordifolia* using tissue culture technique. Nodal explants of field grown mature plants of *T. cordifolia* were used to initiate the cultures. Conditions were standardized to establish contamination free shoot cultures on nutrient medium fortified with plant growth regulators. *In vitro* raised cultures and shoots from mother plant were assessed for presence of the secondary metabolite. Thin layer chromatographic analysis was done and presence of berberine in shoot cultures was established.

Key words : *Tinosopra cordifolia, Berberine, Thin layer chromatographic analysis, Secondary metabolite, Tissue culture technique.*

Introduction

Tinospora cordifolia (Willd) Miers is a deciduous, climbing shrub belonging to the family Menispermaceae, often found in the wild throughout the tropical India ascending upto an altitude of 300m. It is well known as 'amrita' in Sanskrit and Hindi which refers to the property of providing longevity and youthfulness (Choudhary *et al*., 2013). It is a key constituent in many ayurvedic formulations like *Amritharishtam*, *Dhanwanthara thailam* etc for its anti-inflammatory, anti-diabetic, anti-spasmodic, anti-allergic and many other properties (Singla, 2010). The plant possesses wide range of active compounds like berberine, protoberberine, palmatine, magnoflorine, tinosporin etc. Berberine is an isoquinoline alkaloid mainly found in the stem and in minor quantities in roots (Kumar *et al.,* 2000; Singh *et al.,* 2010). It is used to cure diabetes, fever, jaundice, diabetics respiratory and neurological disorders, rheumatism, and several other ailments (Albert, 2012; Sharma *et al.,* 2015). Diverse bioactive components under the classes of alkaloids, diterpenoid, lactose, glycosides, steroids, sesquiterpenoids, phenolics, aliphatic compounds and polysaccharides have also been identified in *T. cordifolia* (Musliarakathbacker and Azadi, 2004).

The drug Guduchi is the major ingredients of about 68 Ayurvedic formulations and demand of this drug has increased up to 2000 to 5000 MT with 9.1% annual growth rate. Of around the 960 species of traded medicinal plants, 178 species are con-

Abbrevations
MS: Murashige and Skoog (1962); MT: metri ton; mL: Milliter; m: metres l: Litre; BAP: 6-Benzylaminopurine; Kn: 6-furfuryl aminopurine; NAA: Naphthalene Acetic Acid; kg: Kilogram; Rs: Rupee; R_f: Retention factor.

sumed over 100 metric tons annually. The total annual requirement of raw materials is about 2000 tons, of which the demand for *T. cordifolia* has been estimated to be 191.64 tons per annum for the period 2006–2011, with a steady growth rate of 5.70% per annum (Albert, 2012; Sen and Chakraborty, 2017). Of late, interest in giloy has increased tremendously due to its proven effect in increasing platelets count in dengue infection, the latter being responsible for several untimely deaths. Different pharmaceutical companies have now developed several medicinal preparations containing giloy as an ingredient; leading to a paradigm shift in *Tinospora* demand and supply. Moreover, the plant continues to be exploited for local traditional usage as medicine.

Consequently, the natural stands of the plant are suffering from huge load of unscientific harvesting leading to a significant decline in population in the wild. Owing to its high demand, the National Medicinal Plant Board (NMPB) of India has recently prioritized *T. cordifolia* for mass multiplication. The NMPB has also listed it among the 178 medicinal plant species in high Volume Trade (Mittal *et al.*, 2014).

Therefore, the present study was designed to establish a system for large scale propagation of important medicinal plant *Tinospora cordifolia* and also to assess the active ingredient in tissue-raised cultures.

Materials and Methods

In vitro culture

Explant collection and surface disinfection

Twigs and leaves were collected from mature plant of *Tinospora cordifolia* growing in Graphic Era (Deemed to be University), Dehradun campus (Fig. 1) for initiation and establishment of cultures.

For sterilization of explants mercuric chloride was tried at 0.1% concentration for different time durations for the sterilization of explants. Following procedure was adopted:

Nodes were cut into appropriate size (2.0-2.5 cm) from twigs and washed with 70% alcohol for 6 minutes (stem) and 3 minutes (leaf). Explants were then rinsed with running tap water. Explants were washed with liquid detergent (Teepol) for 10 minutes (leaf) and 15 minutes (stem) in different wash bottles by gentle agitation. This was followed by

Fig. 1. Mother plant of *Tinospora cordifolia*

treatment with surfactant (Tween -20) and fungicide (Bavistin) for 20 minutes each. The explants were now transferred to laminar air flow cabinet and were treated with mercuric chloride (0.1%) for 5, 10, 15 minutes to find out the best treatment for explants. To remove the traces of steriliant, explants were washed in sterilized distilled water at least 4 to 5 times. Prior to inoculation proximal and distal ends (of nodes) were trimmed slightly to remove dead tissue.

Culture Media and culture conditions

For establishment of cultures MS basal (Murashige and Skoogs, 1962) medium (containing sucrose as a carbohydrate source) supplemented with various different concentrations of cytokinins and auxins were used. The medium was gelled with 0.7% bacteriological agar and pH was maintained at 5.8. The culture medium was autoclaved at 121 °C and 14 psi pressure for 15 minutes. All the cultures were incubated in a culture room and maintained at (25±2) °C for 16 hours in light and 8 hours in dark.

Berberine identification

Sample Preparation

Three type of samples were tested in the study. For first sample *in vitro* raised shoots were removed

from the flask and dried at room temperature. For second sample, mature shoots were collected from the mother plant and were also dried at room temperature. Samples were prepared by dissolving 10 mg of dried sample (*In vitro* shoots and field grown shoots) in 10 mL methanol and the solution was used for TLC profiling. Third sample was prepared with Patanjali Giloy tablets (500 mg).

Solvent system

The solvent system toluene: acetic acid: water (5:15:1) was used for the separation of berberine.

Sample application

Sample was applied on a preheated aluminium precoated silica gel 60 F TLC plate (E.Merck) of uniform thickness (0.2 mm). A 100 µL syringe was used and the sample was loaded. O grown (First sample: *in vitro* grown shoots, Second sample: shoots from mother plant, Third sample: Patanjali Giloy tablets)

Development of Chromatogram and Separation of band

After application of the spots the chromatogram was developed in Twin through glass chamber 20 x 10 cm saturated with solvent toluene, acetic acid, water (5:15:1).

Visualization

The plates were viewed at 256 nm and 366 nm and value of berberine was found to be: 0.553.

Results and Discussion

Culture establishment

Effect of steriliant

When the explants were treated with fungicide Bavistin (1% W/V) followed by treatment with 0.1% mercuric chloride solution for 5 minutes, 70 % of the cultures were green and contamination free. It was observed that extending this period resulted in blackening and death of the plant whereas reducing the time period resulted in fungal and bacterial contamination in the cultures (Table 1).

Establishment and multiplication of culture

Different combinations of auxins and cytokinins were tried in different concentrations to initiate axillary shootsin nodal explants. Cytokinin BAP (1–2mg/L) alone and in combination with kinetin (0.5-1 mg/L) or NAA (0.5 mg/L) was tried for the purpose.

Table 1. Effect of 0.1% mercuric chloride in surface sterilization of the nodal explant

Duration	% Survival
5 min	70%
7 min	55%
10 min	35%

However, high incidence of bacterial contamination was observed in about 90 % cultures. This was attributed to presence of bacterial infection in mother plant. To remove the contamination different concentrations of antibiotics gentamicin (40 mg/100 mL) and amoxicillin (50 mg/100 mL) were used, either during explants sterilization or in the medium.

Of the different combinations tried with and without antibiotics, best results of shoot induction were observed with MS medium supplemented with BAP (2 mg/L) + NAA (0.5 mg/L) + gentamicin (40 mg/100 mL) (Fig. 2). On this combination an average of 5 shoots per explants could be obtained. The same medium was used for further culture multiplication.

Effectiveness of BAP in establishing *in vitro* shoot cultures in *T. cordifolia* has been reported earlier (Mittal *et al.,* 2017; Pillai and Siri, 2019). The present

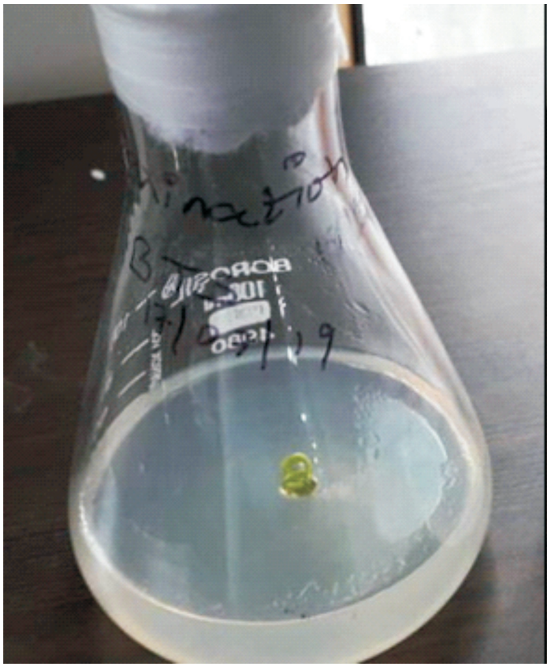

Fig. 2. *In vitro* culture establishment from nodal explants

results are in contrast with previous studies where kinetin has given better results than BAP (Pradhan *et al*, 2009).

In our study a combination of cytokinin and auxin in MS medium resulted in development of strong healthy shoots from the explants.

TLC analysis

TLC was done to assess the presence of berberine in the *in vitro* raised shoots and mature plants as present in commercially available berberine tablets (Fig. 3). Different aqueous mediums and solvent systems were used for the purpose. The aqueous mediums used were acetone, ethanol, 100% methanol, 90% methanol, chloroform and petroleum ether. The different solvent systems used were butanol: acetic acid: water (BAW), toluene: acetone: water (TAW), butanol: ethyl acetate: acetic acid: water and chloroform: methanol in the different ratios (Khatoon *et al.*, 2014, Mohind *et al.*, 2017). Comparisons were done on the basis of Rf values (Rf of berberine - 0.553 (Dabur *et al.*, 2012)).

Of the different combinations tried, best results were observed with 100 % methanolic extract and toluene: acetone: water (TAW) in the ratio of (5: 15:1). Therefore, it was further used for comparing berberine content in shoots (both *in vitro* and *ex vitro* grown) with the tablets which are considered as standard.

Rf value of 0.55 was obtained in all the three samples as assessed from specific bands obtained on TLC plate (Fig 4). This confirmed the presence of berberine in tissue-culture raised shoots suggesting that tissue-culture raised plants can be used for

Fig. 3. Commercially available giloy tablets

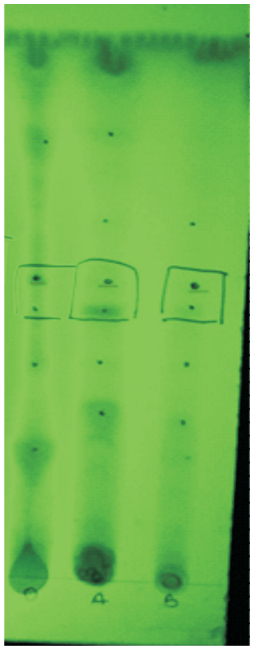

Fig. 4. TLC analysis O: Tablet sampleA:*In vitro* shoot sample B: Mother plant shoot sample

Table 2. Observations of Bands on TLC plate.

Extract prepared from Giloy tablet		Extract prepared from shoots of mother plants		Extract prepared from *in vitro* raised shoots	
Values	R_f	Values	R_f	Values	R_f
3.5	0.31	2.6	0.23	2.7	0.24
4.5	0.40	4.5	0.40	4.0	0.36
5.6	0.50	5.5	0.49	**6.2**	**0.55**
6.2	**0.55**	**6.2**	**0.55**	6.7	0.73
7.4	0.66	9	0.80	8.8	0.78
9.2	0.82				

large scale production to meet the pharmaceutical demand of berberine.

Conclusion

The present study concludes that mass propagation of *T. cordifolia* can be done via tissue culture technology and the tissue-raised plants have the active ingredient berberine which can be further utilized for pharmaceutical preparations.

Recommendations

Priority plants suitable for large scale cultivation in Uttarakhand regions and having a high export demand are medicinal plants and flowers [*as identified by Agri Export Zone (AEZ)*]. The region has a conducive climate for growth of *Tinospora cordifolia.* The details on cultivation economics of the species also sound remunerative as defined by the National Medicinal Plants Board that, for an area of 1 acre, with a harvest period of one year, yield of 4 quintals of dry stem could be achieved. The capital investment per stem is Rs. 2.50 while the output amounts to Rs. 6.00. The stems and the extract of *T. cordifolia* yield returns of about Rs. 60,000 per hectare from second year onwards. The price prevailing in the market is around Rs.15-20 per kg. Average yield of 2 kg extract is achieved out of 100 kg stem and priced at Rs.100/ kg. The statistics has led to NMPB prioritizing *T. cordifolia* for mass multiplication. It is hereby suggested that farmers in Uttarakhand region take up giloy cultivation as a means of additional source of income through supply to pharmaceutical industry.

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