Journal of Medicinal Plants Research Vol. 5(14), pp. 3002-3006, 18 July, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Cytological study and anti-microbial activity of embryogenic callus induced from leaf cultures of *Tinospora cordifolia* (Willd.) Miers

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Accepted 10 February, 2011

The present cytological study of callus, induced from mature leaves of *Tinospora cordifolia* revealed the presence of somatic embryos at different stages. Induction of embryogenic callus was observed on both MS basal medium and MS supplemented with growth regulators 2, 4-D and BAP. Semisolid as well as liquid growth regulator free medium at higher concentration of sucrose and lower strength of salts favoured precoucious germination of somatic embryos. Anti-microbial activity of aqueous leaf and callus extracts was studied against Gram positive and Gram negative bacteria. The phytochemical contents of callus and that of leaf extract were found to be different. The presence of additional compounds in callus extract may attribute to its activity against Gram positive organisms.

Key words: *Tinospora cordifolia* (Willd.) Miers, somatic embryos, callus extract, antimicrobial activity.

INTRODUCTION

*Tinospora cordifolia (*Willd.) Miers ex Hook. F. and Thoms is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. It is distributed throughout tropical Indian subcontinent and China (Anonynmous, Wealth of India , 1976). The notable medicinal properties reported are anti-diabetic, anti-periodic, anti-spasmodic, anti microbial, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, hepatoprotective, immunomodulatory and anti-neoplastic activities (Krishna et al., 2009; Panchabhai et al., 2008; Upadyay et al., 2010).

The root, stem, leaves and starch of the plant are used for medicinal purpose, externally. The medicated oil of the plant is effectively used to reduce the pain and oedema, in gout and skin diseases. It is a natural blood purifier and is very useful for skin problems like acne, psoriasis, eczema and others. It is immensely helpful in the digestive ailments like hyperacidity, colitis, worm infestations, and loss of appetite, abdominal pain, excessive thirst, vomiting and liver disorders like hepatitis. Leaves of this plant are rich in protein (11.2%) and are fairly rich in calcium and phosphorus (Wadood et al., 1992) and containing anti-oxidant activity *in vitro* models (Premanath and Lakshmidevi, 2010).

In vitro propagation in *T. cordifolia* was reported through nodal segments through axillary shoot proliferation (Raghu et al., 2006; Gururaj et al., 2007) and an efficient system of plant regeneration through somatic embryogenesis from seedling leaf explants was also reported (Reddy et al., 2003). The anti-microbial activity of *T. cordifolia* was observed in root, stem and leaf extracts on pathogenic microorganisms (Jeychandran et al., 2003; Mahesh and Satish, 2008; Samy, 2005).

In this report, we describe the procedure for proliferation, maintenance of stable embryogenic callus, both on growth regulator free semisolid and liquid medium. Its cytological study, to reveal different stages of somatic embryogenesis, anti-microbial activity of aqueous extract of mature leaves of *T. cordifolia* as well as its embryogenic callus.

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MATERIALS AND METHODS

The mother plants were collected from medicinal plants conservation center, Kadki, Pune. India and were maintained in shade net of Bharati Vidyapeeth University, Rajiv Gandhi Institute of IT and Biotechnology. The experimental materials such as leaves were obtained from such six-months-old plants. For raising cultures, the leaves (Figure 1a) of *T. cordifolia* were surface sterilized with 0.1% mercuric chloride for 4 min. After repeated washing in sterile distilled water, 5 mm diameter leaf discs were made aseptically and inoculated in culture tubes (150 mm length, 25 mm diameter) containing 15 ml of Murashige and Skoog's (MS) medium7 and in the flasks (100 ml) containing 30 ml with 3.0% sucrose and 0.8% Difco-bacto agar alone and with 1.0, 2.0 and 5.0 mg/lit of 2,4-D, BAP individually and in their combination. Analytic reagent grade chemicals from Hi media and Borosilicate glassware were used. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl using digital pH meter before sterilization. Illuminated rotary shaker was used for maintaining cultures in liquid medium and cell suspension cultures. For callus subculturing, 100 + 10 mg, friable callus was regularly transferred to fresh semisolid and liquid medium with 4 weeks intervals. All the cultures were maintained under diffused light (1000 lux) for 10 h daily at 28 + 2°C temperature and 50 to 60% relative humidity.

For the study of antimicrobial activity, the fresh leaves were collected and were washed under running tap water. The leaves and calli induced from leaf explants were dried at 37°C for 24 h, powdered and stored in air tight container. 10 g of each powdered samples were soaked in 35 ml of sterilized distilled water for 3 days and filtered through Whatman No.1 filter paper. Further extraction of the residue was repeated 3 times until a clear colourless supernatant extraction liquid was obtained. The combined filtrates were concentrated by rotary evaporator at 70°C to make the final volume ½ of original volume and evaporated in water bath maintained at 70°C till the dried extracts were obtained. Both the extracts were dissolved in 5% DMSO to give final concentration of 100 mg/ml. The extracts were kept under sterile conditions at 4°C.

Antimicrobial susceptibility test was carried out by agar disc diffusion method. Four different bacterial cultures were used for testing antimicrobial activity. The cultures, *Escherichia coli* (NCIM 2065), *Staphylococcus aureus* (NCIM 5021), *Pseudomonas aeruginosa* (NCIM 5029) and *Bacillus subtilis* (NCIM 2813) were procured from National Collection of Industrial Microorganism (NCIM), National Chemical Laboratory (NCL), Pune, India. 100 µl of overnight grown cultures spread over the surface of nutrient agar plates. The paper discs (6 mm in diameter) impregnated with each extract were placed on seeded agar plates. A paper disc soaked in 5 µl ampicillin (100 mg/ml) was used as a positive control and 10 µl of 5% DMSO used as a negative control. The plates were incubated at 37°C for 24 h and zones of inhibition were measured.

RESULTS

The induction of friable, light brownish mass of embryogenic callus occurred from leaf explants on MS basal medium (Figure 1b) and nodulated brownish mass of callus in presence of 2,4-D and BAP individually and in a combination within 6 weeks. Embryogenic callus proliferated readily and retained its globular appearance even after subculturing on semisolid growth regulator free MS medium up to eight folds within 4 weeks showing proembryos (Figures 1c, d and e). However, isolated somatic embryos were observed on liquid medium (Figures 1f and g). Somatic embryo development was promoted on both the media containing higher concentration of sucrose. Globular embryos developed gradually up to bipolar structures. The increased concentration of sucrose and decreased strength of stock solutions stimulated the precuacious germination of somatic embryos (Figures 1h, i and Table 1).

The aqueous extracts of both leaves and that of embryogenic callus induced from such leaves of *T. cordifolia* were assayed against four different bacterial cultures. The inhibition zones were obtained for *E. coli* (NCIM 2065) and *S. aureus* (NCIM 5021) (Table 2). At 1 and 2 mg concentration of callus extract inhibition was obtained for both the organisms. However, equal concentration of leaf extract showed inhibition of *E. coli* only. GC-MS analysis of leaf and callus extracts revealed the presence of 12 and 14 compounds respectively (data not shown). Six compounds viz. 7-Tetradecene, hydroxylamine, decocyl acrylate, isopropyl myristate, squalene and phenol-2,4 bis (1,1-dimethylethyl) were found to be present in both the extracts.

DISCUSSION

Information about *in vitro* propagation (Raghu et al., 2006) and micropropagation (Gururaj et al., 2007) by axillary shoot proliferation in *T. cordifolia* is restricted to the production of plantlets through nodal segments' and plant regeneration through somatic embryogenesis from leaf explants obtained from seedlings parts (Reddy et al., 2003). The present study is explaining indirect somatic embryogenesis from mature leaves. Accumulation of protoberberin alkaloids from callus and cell suspension cultures of *T. cordifolia* was reported from stem explants (Chintalwar et al., 2003). We are reporting an efficient procedure for initiating stable embryogenic leaf callus and their maintenance on growth regulator free medium. Which will be useful for initiating suspension cultures, micropropagation for large scale propagation of selected high yielding plant, their conservation (Sharma et al., 2010), transformation studies and for advanced techniques by using plant cell reactors.

The aqueous, alcoholic and chloroform extracts of the leaves of *T. cordifolia* exerted a significant hypoglycemic effect in normal as well as in alloxan-treated rabbits indicating that leaves have an insulin-like action (Wadood et al., 1992). The antibacterial activity of aqueous, ethanolic and chloroform stem extract of *T. cordifolia* against number of Gram negative and Gram positive bacteria was reported by suggesting significant antimicrobial activity of ethanolic extract (Jeychandran et al., 2003). In the present work, aqueous extracts of *T. cordifolia* callus induced from leaf explants were used. The callus extract has shown inhibition of both Gram positive and Gram negative organisms while leaf extract has shown inhibition of only Gram negative bacteria

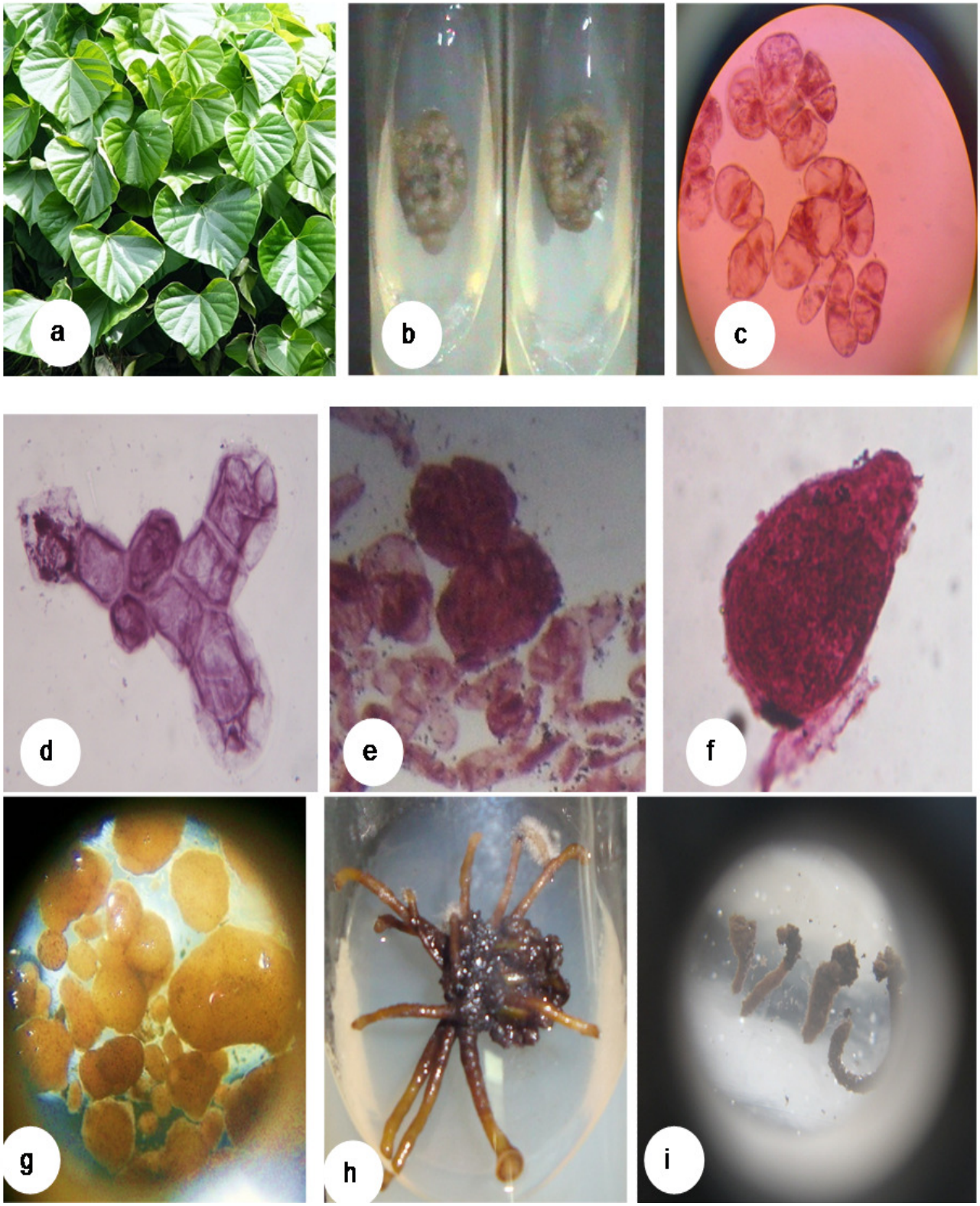

Figure 1. Indirect somatic embryogenesis in *T. cordifolia* leaf disc cultures. (a) Portion of a plant showing healthy leaves (b) Friable embryogenic brownish mass of callus all over the leaf disc. (c) Cytological study of such callus showing proembryos (d) One of the cell of multicellular proembryo showing totipotency (e and f) Microscopic observation of callus showing somatic embryos of late globular and torpedo stage (g) Somatic embryos on liquid medium (h and i) Precuocious germinaation of somatic embryos.

Table 1. Somatic embryogenesis in leaf induced callus of *T. cordifolia*.

Induction of embryogenic callus		
Nutrient medium	**% of explants showing callus**	**Response**
MS	80	Friable light brown callus
MS+2,4-D(1.0 mg/l)	70	Whitish friable callus
MS+BAP(1.0 mg/l)	30	Nodulated dark brown callus
MS+2,4D(2.0 mg/l)+BAP(1.0 mg/l)	60	Llight brownish nodulated callus
Proliferation of callus and development of somatic embryos		
Nutrient medium	**% of callus showing proliferation**	**Response**
MS (semisolid)	100	Globular somatic embryos
MS (liquid)	100	Separated bipolar somatic embryos
Maturation and germination of somatic embryos		
Nutrient medium	**% of cultures showing response**	**Response**
½ MS + 40 g sucrose (liquid)	80	Precuacious germination
MS + 40 g sucrose (semisolid)	50	Precuacious germination

Table 2. Antimicrobial activity of leaf and callus induced from leaf explants of *T. cordifolia*

Pathogen	**Zone of inhibition**				
	Leaf extract (mg)		**Callus extract (mg)**		**Ampicillin (µl)**
	1	**2**	**1**	**2**	**5**
E. coli (NCIM 2065) (mm)	10	17	6	15	27
S. aureus (NCIM 5021) (mm)	-	-	12	15	35
P. aeruginosa (NCIM 5029) (mm)	-	-	-	-	20
B. subtilis(NCIM 2813) (mm)	-	-	-	-	7

indicating the significant activity of callus extract than leaves.

Conclusion

T. cordifolia is a widely used in ayurvedic medicine because of its multipotent bioactive molecules and most of their pharmacological evaluation by modern test have been reported. This is the first report of antimicrobial activity of aqueous extract of leaf induced callus in *T. cordifolia.* The chemical analysis of callus extract varies in their phytochemical contents as compared to leaf extract. Thus it can be used as a source for developing new drugs or valuable metabolites for commercialization through biotechnological approaches.

ACKNOWLEDGEMENTS

The authors are thankful to Hon. Dr. Shivajirao Kadam, V.C. Bharati Vidyapeeth University for providing financial support and facilities to undertake this work. The authors also thank Doctors` Analytical Laboratories Pvt Ltd. Mulshi, Pune for GC-MS analysis of the sample.

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