2012 International Conference on Environmental, Biomedical and Biotechnology
IPCBEE vol.41 (2012) © (2012) IACSIT Press, Singapore

A Micropropagation System for Tylophora Indica and Extraction and Purification of Tylophorine from Cultures and in Vitro Regenerated Plants

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Abstract. A protocol has been established for the mass propagation of *Tylophora indica* followed by extraction of major secondary metabolite tylophorine from *in vitro* regenerated plants, callus and suspension cultures. Leaf explants formed green compact and fast growing callus when cultured on Murashige and Skoog's medium supplemented with 29.4 µM naphthalene acetic acid and 4.65 µM kinetin. High frequency shoot differentiation occurred from the calli when transferred onto 8.8 µM benzyladenine. Individual shoots were rooted on half strength basal MS medium and plantlets were successfully acclimatized and established in field conditions. Tylophorine was extracted from the leaves of regenerated plants, callus and suspension cultures using cold extraction with acetic acid in methanol followed by acid extraction with ethyl acetate : HCl and separation was done on high performance thin layer chromatography (HPTLC). Quantitative analysis showed maximum tylophorine (80µg/ml) in the leaves of *in vitro* regenerated plants, followed by suspension (28.30µg/ml) and callus cultures (24.46µg/ml).

Keywords: *Tylophora indica*, Tylophorine, High performance thin layer chromatography

1. Introduction

Tylophora indica (Burm.f) Merrill (Family Asclepiadaceae) is an important medicinal plant which is used as a folk remedy for the treatment of bronchial asthma, bronchitis, dysentery, inflammation, dermatitis and allergies (Singh *et al*., 2009). The roots and leaves contain pharmacologically active alkaloids like tylophorine, tylophorinine and anti cancerous tylophorinidine (Mulchandani *et al*., 1971 and Gopalakrishnan *et al*., 1980). The commercial cultivation of this important medicinal plant species is uncommon and only the wild populations are exploited for secondary metabolite extraction. The plant is propagated only through seeds which have low viability and germination and the destruction of plant caused by harvesting the roots as a source of drug has threatened the very existence of this plant (Faisal and Anis, 2003). It is therefore imperative to adopt alternative methods having high multiplication rates to produce plants on large scale. We present an efficient and reproducible protocol for mass propagation of this plant species under *in vitro* conditions and extraction and purification of tylophorine- the major secondary metabolite from callus and suspension cultures and *in vitro* regenerated plants.

2. Material and Methods

Leaves were collected from 3 years old healthy field grown plant of *T. indica* maintained at Thapar University campus, Patiala. After washing with teepol solution and bevistin (0.1% w/v), leaves were surface sterilized with 0.1% (w/v) aqueous solution of $HgCl_2$ and were cultured on MS (Murashige and Skoog's 1962) medium augmented with different growth regulators, 2% sucrose and 1% agar with pH adjusted to 5.8 before the addition of agar. All the inoculated cultures were incubated in growth room at a temperature of 25 ± 2ºC with a photoperiod of 16 hours per day with an illumination of 50 µmol/m^2/s^1 at the level of cultures.

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For suspension cultures, leaf callus (1g) was inoculated in 250 ml conical flask containing 100 ml of liquid MS medium supplemented with same hormone combination. The cultures were maintained on shaker at 120 rpm and regular biomass examination was done at an interval of 3 days starting from the sixth day after incubation till 33rd day. For tylophorine extraction, leaves collected from 1 year old field established plants, dried callus and suspension cultures were selected and fine grounded to powder with the help of mortar and pastle. Dried leaf, callus and suspension powders were processed using method of Rao and Brook, 1970. Extraction was done under cold conditions with acetic acid in methanol followed by acid extraction with ethyl acetate: HCl and separation of metabolite was done on high performance thin layer chromatography (HPTLC) using various solvent systems and were scanned at 258 nm. Presence of tylophorine in all the samples was confirmed by comparing the Rf values of test samples with that of standard tylophorine and for quantitative analysis, peak area and standard concentration were taken into consideration.

3. Results and Discussion

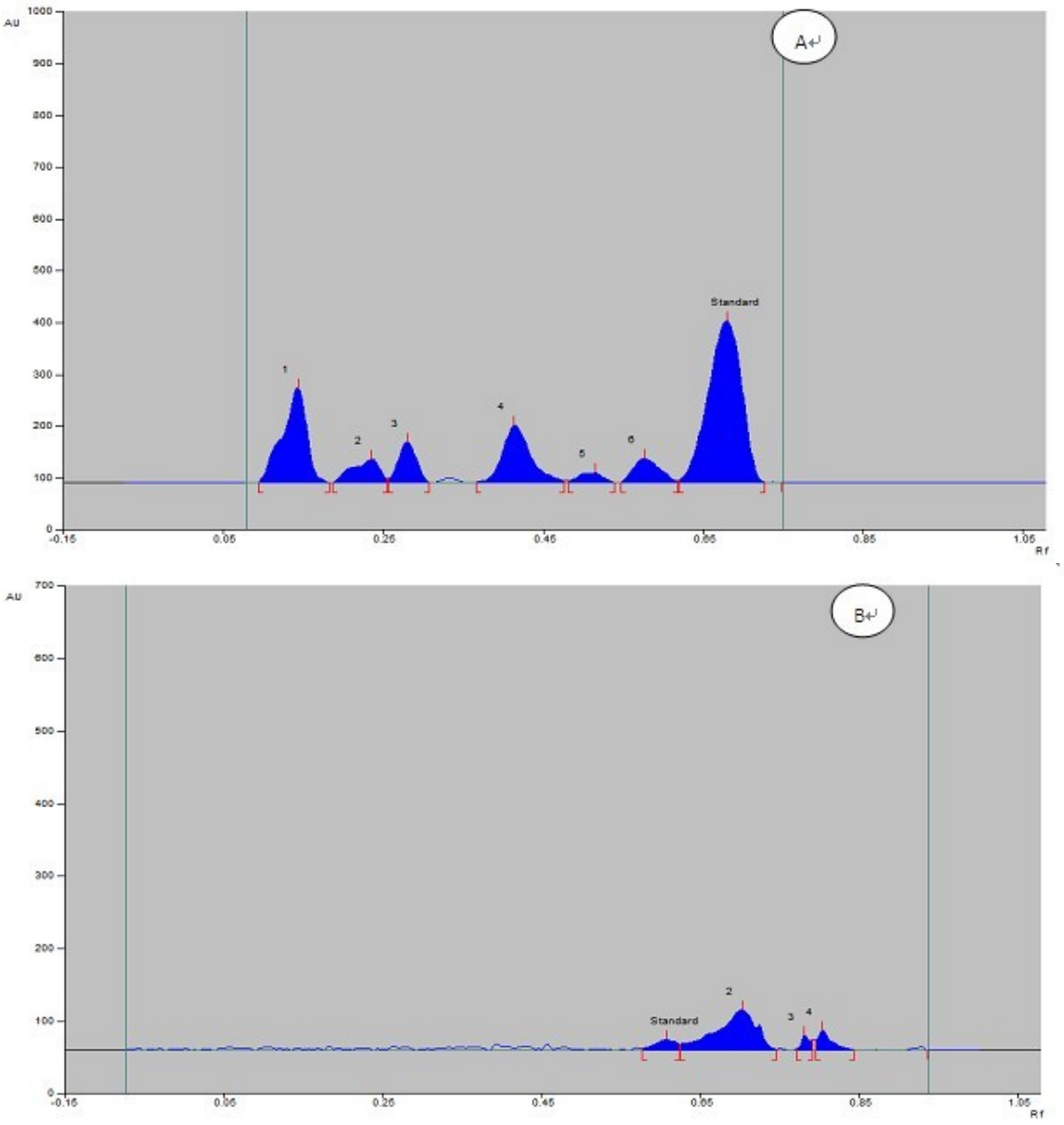
Callus was induced from cut surface of leaf lamina on MS medium supplemented with 29.4 µM naphthalene acetic acid with 4.65 µM kinetin where callusing initiated after 7-10 days and within 4 weeks the entire explant turned into a mass of callus. The callus was fast growing, green, compact and capable of sustained growth even on repeated subculturing. In earlier studies on *T.indica*, Faisal and Anis (2003) have reported the production of highly proliferative callus from the leaf explants on 2, 4, 5-trichlorophenoxy acetic acid, whereas Thomas and Philip 2005, obtained optimum callusing on 2, 4-dichlorophenoxy acetic acid and benzyladenine supplemented medium. High frequency shoot differentiation from leaf callus occurred on 8.8 µM benzyladenine where 86 % of the cultures resulted in excellent shoot induction with an average of 40.0 ± 1.45 shoots / culture. The stimulatory role of benzyladenine for shoot organogenesis from leaf callus was also advocated by Faisal and Anis, 2003 and Sahai *et al*., 2010. Microshoots, thus, formed were rooted on half strength MS medium where 4-6 healthy roots emerged in 90% of cultures after 10 days. In earlier reports, either indole 3 butyric acid or indole 3 acetic acid has been reported to be optimum for rooting in regenerated shoots of *Tylophora indica* (Bera and Roy 1993 and Thomas and Philip 2005). Complete plantlets were hardened, acclimatized and successfully transferred to natural environmental conditions with 90% survival rate.

Table 1: Tylophorine (µg/ml) in various tracks when analysed at 258 nm. Standard tylophorine applied at a working concentration of 20.0 µg/ml

Track ID	Solvent System	Peak	End Position (Rf)	Area (AU)	Area %	Tylophorine (µg/ml)
Track I (A)	Toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop)	7	0.68	9888.1	80.00	80.00
Track II (B)	Toluene: ethyl acetate: diethyl amine (7:2:1)	1	0.62	1200	3.26	24.46
Track III (C)	Toluene: ethyl acetate: diethyl amine (7:2:1)	1	0.62	376.4	10.37	28.30

Suspension cultures showed maximum biomass yield between 12th and 24th day and thereafter the biomass yield did not show any significant increase and remained almost stationary till 27th day. Suspension cultures were harvested at stationary phase and analysed for tylophorine content. This is perhaps the first report using HPTLC technique for purification of tylophorine. For the quantification of tylophorine, all the three samples were loaded along with standard tylophorine on pre coated silica gel HPTLC plates and developed using different solvent systems. For leaf extract, the solvent system comprised of toluene: chloroform: ethanol: ammonia (4:3.5:1.5: drop) whereas toluene: ethyl acetate: diethyl amine (7:2:1) were used for callus and suspension extracts and all the plates were scanned at 258 nm. Track I shows the presence of seven different peaks with seventh peak corresponding to tylophorine at Rf 0.68 while track II and III contain tylophorine peaks at Rf 0.62 (Fig. 1A, B & C). Spectra comparison of tylophorine peaks recorded at 200-400 nm showed quite similar UV absorbance. Visual impression of the plate captured under

UV 254 and 366 nm also showed tylophorine bands in different tracks. Quantitative analysis showed that leaf extract from *in vitro* regenerated plants yielded 80 µg/ml of tylophorine while leaf callus yielded 24.46 µg/ml and suspension extract contained 28.30 µg/ml of tylophorine (Table 1). In earlier reports, Chaudhari *et al*., 2004, 2005 have detected and analysed tylophorine using HPLC technique from the root regenerated plants and transformed root clones of *T. indica*.



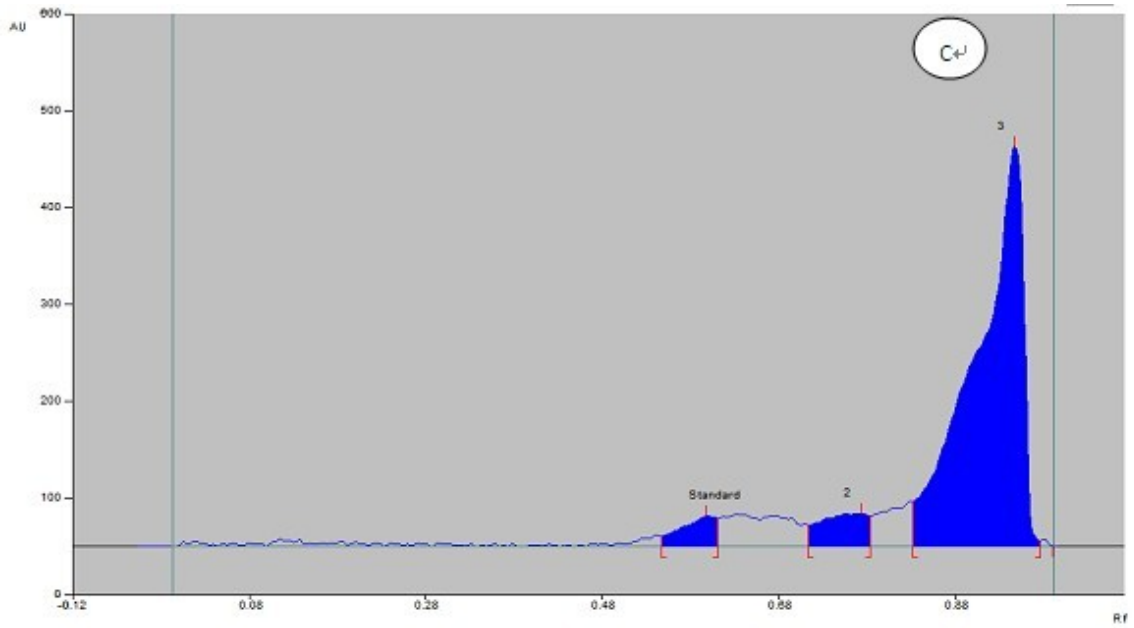


Fig. 1: A) HPTLC of crude leaf extract from in vitro raised plants showing tylophorine peak B & C) HPTLC of leaf callus extract and suspension extract harvested at stationary phase.

The present study demonstrates the high regeneration efficiency of *T. indica* for its mass multiplication and biosynthesis of tylophorine in cultures provide ample opportunity for further investigation into large scale production of tylophorine by standardizing different parameters.

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