J. Indian bot. Soc. ISSN - 0019 - 4468 Vol. 93 (1 & 2) 2014 : 42-49

PHYTOCHEMICAL INVESTIGATIONS AND MICROPROPAGATION OF *TYLOPHORA INDICA* (BURM. F.) MERILL FROM NODAL EXPLANTS

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Tylophora indica (Burm. F.) Merill is commonly known as asthma herb. It is a threatened medicinal plant (climber) of the family Asclepiadaceae. The present paper deals with phytochemical studies in *Tylophora indica.* Plant extracts were analysed for phytochemical constituents. The qualitative and quantitative analysis confirmed the presence of various phytochemicals like Alkaloids, Flavonoids, Phenols, Saponins, Steroids, Tannins and Terpenoids. Some compounds were also estimated quantitatively (Alkaloids, Flavonoids and Saponins). *In vitro* clonal propagation is an alternative method of propagation. Multiple shoot buds were regenerated from nodal explants on MS medium supplemented with different concentrations and combinations of growth regulators and efficiently rooted on half-MS medium supplemented with IBA/IAA. The regenerated plantlets were successfully transferred to the glasshouse, acclimatized and transferred to the field.

Key words: Axillary meristem, micropropagation, phytochemicals, qualitative analysis, shoot buds.

Tylophora indica is a threatened perennial twining medicinal herb belonging to family Asclepiadaceae. It is commonly known as an antamool. The plant is distributed in Assam, West Bengal and peninsular India. It is used as a traditional folk medicine and also used as an ingredient in Ayurvedic and Unani preparations in certain regions of India for treatment of various diseases like asthma, inflammation, bronchitis, diarrhoea, allergies, rheumatism, dermatitis, syphilis, fever, eye diseases, urinary disorders (Gupta *et al.* 1979), burning sensation and also used in antitumor treatment (Donaldson *et al*. 1968).

The herb contains certain bioactive compounds like Alkaloids, Flavonoids, Tannins and Saponins, (Rao *et al.* 1971, Benjamin and Mulchandani 1973, 1976). There is a great demand for *Tylophora indica* for production of traditional and modern medicine in pharmaceutical industries. Due to the over exploitation from its natural habitat, it has been listed as a threatened plant.

Due to the importance of phytochemicals of *Tylophora indica,* certain qualitative and quantitative analysis was undertaken in the present study. Micropropagation or clonal propagation is an alternative method for large scale multiplication of germplasm of *Tylophora indica* (Tapan and Satyesh 1993). Previous studies on tissue culture of *Tylophora indica* concentrated mostly on adventitious shoot regeneration (Sharma and Chandel 1992, Rao *et al.* 1970, Rao and Narayanaswamy 1972, Jayanthi and Mandal 2001, Chandudhuri *et al.* 2004, Thomas and Philip 2005). Therefore, micropropagation of *Tylophora indica* was taken up to improve the conservation strategies by development of efficient protocols.

MATERIALS AND METHODS

Tylophora indica plants were collected from Herbal garden, N. G. Ranga Agricultural University at Hyderabad and planted in the Botanical Garden at Department of Botany, Osmania University, Hyderabad. These plants were subjected to phytochemical analysis (both qualitative and quantitative) for the presence of several medicinally important compounds. Further, a good protocol for micropropagation was developed to aid in its multiplication and conservation.

Qualitative Analysis: The plant extract was prepared by grinding 0.5 gm of the plant part (leaf, shoot or root etc) in 100 ml distilled water. This extract was filtered through a fine mesh into a test tube. This crude extract was used for the qualitative tests given below (Karthikeyan *et al.* 2009, Lozoya *et al.* 1989) and the tests were carried out in triplicate.

Test for identification of Alkaloids: The leaf extract was prepared (ground in 100 ml of water). It was dissolved in dilute HCl solution and clarified by filtration. The filtrate was tested with Drangendroff's and Mayer's reagent. The treated solution was observed

Received on November 30, 2012 Accepted on December 27, 2013

for precipitation of white or creamy colour.

Test for identification of Flavonoids: Ethyl acetate (5 ml) was added to the leaf extract and the mixture was shaken and allowed to settle. Production of green colour is taken as positive for Flavonoids.

Test for identification of Phenols: The leaf extract was taken in a test tube (0.5 gm of roots ground in 100 ml of water) and warmed. To this 2 ml of ferric chloride was added and observed for formation of green or blue colour.

Test for identification of Saponins: The root extract was taken in a test tube and shaken vigorously for about 30 sec and allowed to stand in vertical position and observed for 30 min. If honey comb froth above the surface of the liquid persists after 30 min, it indicates the presence of Saponins.

Test for identification of Steroids: The extract was mixed with 2 ml of acetic anhydride. To this 1 or 2 drop of concentrated sulphuric acid was added slowly along the sides of the test tubes. An array of colour change shows the presence of phytosterols.

Test for identification of Tannins: The leaf extract was prepared and the solution was clarified by filtration. 10 % ferric chloride solution was added to the clear filtrate, and it was observed for a change in colour to blue.

Test for identification of Terpenoids: 5 ml of the extract was mixed with 2 ml of chloroform and concentrated sulphuric acid to form a layer. A reddish brown coloration of the interface showed the presence of Terpenoids.

Qualitative Analysis: Quantitative analysis was carried out for total Alkaloids, Saponins, and total Flavonoids. The plant extract was prepared as explained above.

Determination of Alkaloids (Harborne 1973): To 5 gm of the sample in 250 ml beaker, 200 ml of 10 % acetic acid in ethanol was added, covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the Alkaloid, which was dried and weighed.

Determination of total Flavonoids: Aluminium chloride colorimetric method (Chang *et al.* 2002) with some modifications was used to determine Flavonoids content. 1.0 ml plant extract (0.5 gm of root extract in 100 ml of water) was mixed with 1.0 ml methanol, 0.5 ml aluminium chloride (1.2 %) and 0.5 ml potassium acetate (0.1176 %). The mixture was allowed to stand for 30min at room temperature. Later, the absorbance was measured at 415 nm. Quercetin was used as standard. Flavonoid content is expressed in terms of quercetin equivalent (mg/g of extracted compound).

Determination of Saponins: (Obadoni and Ochuko, 2001): The samples were ground and 20 gm of each were put into a conical flask and 100 ml of 20 % aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55º C. The mixture was filtered and the residue reextracted with another 200 ml 20 % ethanol. The combined extracts were reduced to 40 ml over water bath at about 90º C. The concentrate was transferred into a 250 ml separator funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5 % aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The content of Saponins was calculated as percentage.

Micropropagation Studies : The micropropagation studies comprised the *in vitro* culture of nodal explants of *T. indica* on defined culture media under standard growth conditions. The nodal explants were collected from mature and healthy field grown plants

grown in the botanical garden of Osmania University, Hyderabad. They were washed under running tap water for 20 min followed by soaking in 0.1 % (v/v) liquid detergent tween-20 for 5 min and then subsequently washed with tap water. The explants were then soaked in 70 % ethanol for 5 min followed by washing with water. Finally the explants were surface sterilized with 0.1 % solution of mercuric chloride for 3 to 5 min followed by thorough rinsing in sterile distilled water. The explants were inoculated in culture tubes containing MS medium (Murashige and Skoog, 1962) augmented with 2 % sucrose and 0.8 % agar and combinations and concentrations of various plant growth regulators. Prior to that, the pH of the medium was adjusted to 5.8, autoclaved at 121°C for 15 lbs / cm^2 for 15 min and allowed to cool before inoculation. The culture media comprised of the following:

MS + BAP (0.5 / 1.0 / 2.0 mg/l)
MS + KN (0.5 / 1.0 / 2.0 mg/l)
MS + AS (0.5 / 1.0 / 2.0 mg/l)
MS + BAP (1.0 mg/l + 2,4-D 0.5 mg/l)
MS + KN (1.0 mg/l + 2,4-D 0.5 mg/l)
MS + AS (1.0 mg/l+ 2,4-D 0.5 mg/l)

All the inoculated cultures were incubated in sterile growth room under controlled conditions of 22 ± 1°C temperature 75 % humidity and 2000 lux illumination of 16 hr / 8 hr L/D cycle. The 2 cm long regenerated shoots were transferred to root inducing media comprising half MS medium supplemented with IAA or IBA (1.0 mg/l). The regenerated plantlets were later transplanted to pots and moved to the glasshouse through a process of gradual acclimatization and by maintaining high humidity.

RESULTS AND DISCUSSION

Tylophora indica is a slender climber with twining woody stem and opposite petiolate leaves, which are entire, smooth, shiny, varying in shape and size according to their age. Flowers are small, in auxiliary and sessile racemes. The root is long, rigid and cylindrical.

The present study contributes valuable information of bioactive compounds in *T. indica.* Qualitative analysis of plant extract was carried out for Alkaloids, Flavonoids, Phenols, Saponins, Steroids, Tannins, and Terpenoids. All of the phytochemicals like Alkaloids Flavonoids, Phenols, Saponins, Steroids and Terpenoids were present in *Tylophora indica* except Tannins (Table-1). The plant extracts were quantitatively analyzed for secondary metabolites like Alkaloids, Flavonoids and Saponins. The quantitative analysis portrays that all three secondary products were present in the plant material (Table-2). Whereas, our study reports the absence of Tannins, Meera *et al.* (2009) and Kumar (2011) indicated that Tannins were present in *T. indica* in the aqueous extract. Several medicinal properties have been attributed to Tannins by Okwu and Okwu (2004) and Kumar (2011) but surprisingly, Tannins were not found in the present study. Alkaloids are however reported in the present study which agrees with the findings of Meera *et al.* (2009) and Kumar (2011) who has attributed analgesic, anti-spasmodic and bactericidal effects. The present study also reports Saponins, similar to the report of Meera *et al.* (2009) and Kumar (2011).

Table 1. Qualitative analysis of the aqueous extracts of *Tylophora indica* to screen for phytochemicals.

S.No	Aqueous extract of	Test for Phytochemicals	Test result
1.	Leaf	Alkaloids	Positive
2.		Flavonoids	Positive
3.		Phenols	Positive
4.		Steroids	Positive
5.		Tannins	Negative
6.		Terpenoids	Positive
7.	Root	Saponins	Positive

Table 2. Quantitative analysis of the aqueous extracts of *Tylophora indica* for estimation of phytochemicals.

S.No	Plant extract	Phytochemicals	*Average Estimated value (mg/gm) (Mean± S.E)
1	Alkaloids	Leaf	1.46 ± 0.7
2	Flavonoids	leaf	4.5 ± 1.1
3	Saponins	Root	2.0 ± 0.6

* The value is the average of studies conducted in triplicate.

Table-3 Effect of plant growth regulators on shoot regeneration from nodal explants and root induction of *Tylophora indica*

Shoot regeneration		
S. No	**Culture Medium**	***Percentage response**
1	MS + BAP (0.5 mg/l)	20
2	MS + BAP (1.0 mg/l)	40
3	MS + BAP (2.0 mg/l)	40
4	MS + KN (0.5 mg/l)	20
5	MS + KN (1.0 mg/l)	35
6	MS + KN (2.0 mg/l)	30
7	MS + AS (0.5 mg/l)	60
8	MS + AS (1.0 mg/l)	70
9	MS + AS (2.0 mg/l)	65
10	MS + BAP (1.0 mg/l + 2,4-D (0.5 mg/l)	55
11	MS + KN (1.0 mg/l) + 2,4-D (0.5 mg/l)	40
12	MS + AS (1.0 mg/l) + 2,4-D (0.5 mg/l)	75
Root induction		
13	MS + IAA (1.0 mg/l)	65
14	MS + IBA (1.0 mg/l)	55

Alkaloids and Saponins are known to be effective for the treatment of syphilis and other venereal diseases. Oliver-Bever (1986) and Okwu (2004) had earlier reported that Saponins have antibiotic properties and so help the body to fight infections and microbial invasion. Also, it is used as a mild detergent and in intracellular histochemistry staining to allow antibody access to intracellular proteins, These proteins were also reported in hyperchlolesterolaemia, hyperglycaemia, antioxidant, anticancer, anti-inflammatory and weight loss and have anti-fungal properties. Singh (2012) reported the presence of tylophorine alkaloids in *T. asthamatica*. Investigation of *T. indica* for the presence of Tylophorine is therefore needed in exclusive studies .The presence of flavonoids are reported in *T. indica* in present study which is in agreement with Meera *et al.* (2009) who also reported the diuretic property of extracts of *T. indica*. The present report indicates the presence of flavonoids and also reports its quantitative value. Further, the quantitative estimation of alkaloids and saponins is also reported which underlines the fact that presence of alkaloids, flavonoids and Saponins in suitable quantities in *T. indica* is very valuable information.

Nodal explants were cultured on MS medium supplemented with different concentrations and combinations of growth regulators. A high percentage of shoot buds could be regenerated from the explants on the culture medium MS + AS (1.0 mg/l) which was overtaken by the most efficient regeneration response on MS + AS (1.0 mg/l) + 2, 4-D (0.5 mg/l) of 75 % (Table-3). In terms of inducing the production of a high efficient shoot regeneration, the growth regulator BAP was the best followed by AS both singly as well as in combination with 2,4-D. The regenerated shoots were transferred to the root inducing medium. Better rooting was achieved with half MS + IAA (1.0 mg/l) (Table – 2) and the plantlets were transferred to glasshouse after acclimatization. A total of 7 plants survived out of 10 regenerated plants with a survival percentage of 70 %.

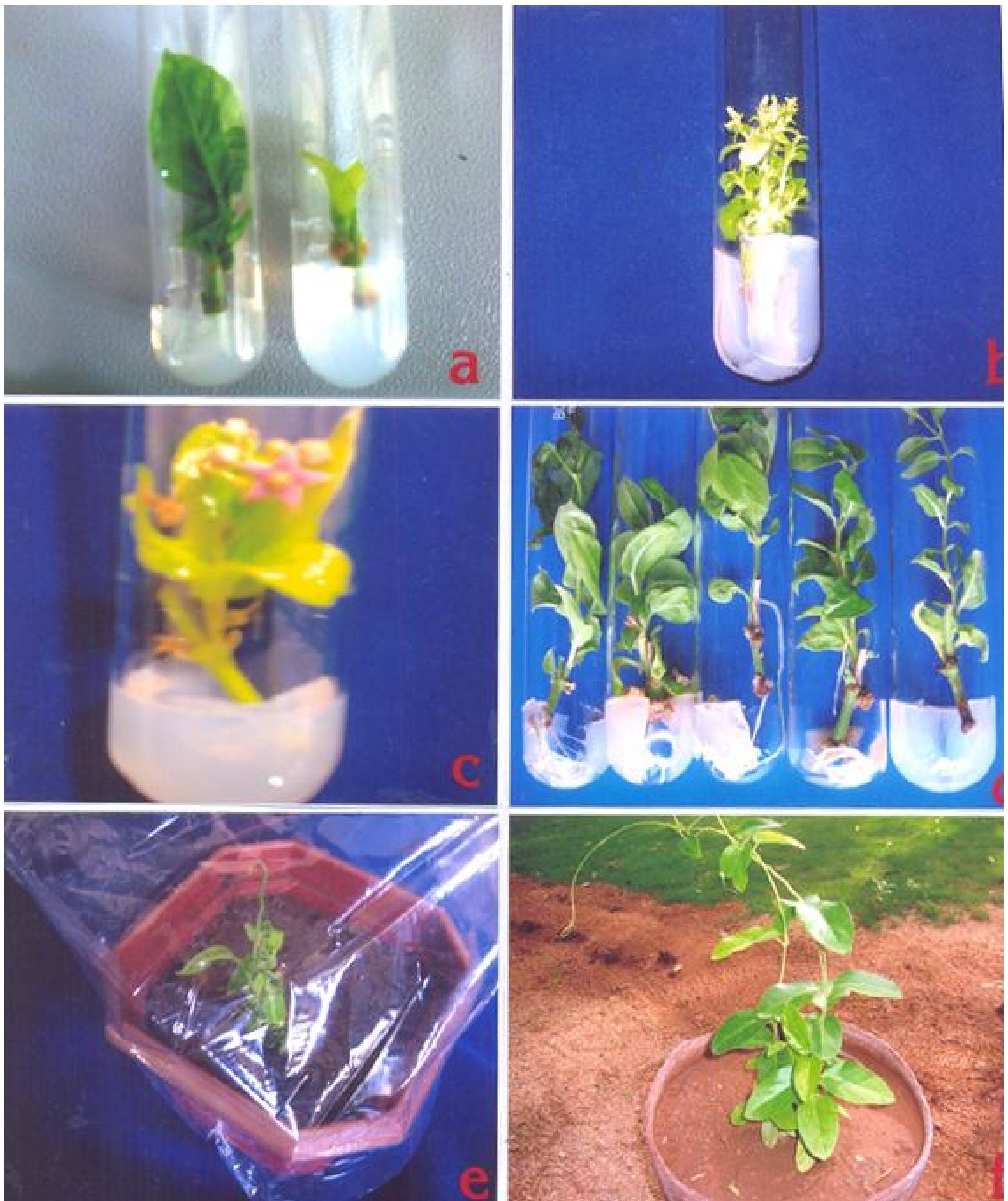

Figure 1 (a-f): Micropropagation of *Tylophora indica*. a. Initiation of shoot buds. b. Multiple shoots. c. In vitro flowering. d. Root induction. e. Acclimatization of regenerated plantlets. f. Regenerated plant.

The results show that AS elicited a very high response both singly and in combination with 2, 4-D. Hence the AS + 2, 4-D is the best combination of growth regulators that can be used for efficient plantlet regeneration in *T. indica.*

Whereas the present study reports maximum response of plant regeneration with AS, Faisal *et al.* (2006) reported maximum regeneration with TDZ (Thidiazuron). By using only some concentrations of BAP, KN, or TDZ (Thidiazuron), Faisal *et al.* (2005) also reported callusing, whereas we did not record callusing with any of the culture media used in the present study. Our findings of good response with AS is similar to those of Bera and Roy (1993). The best rooting response presently was achieved by IAA which is similar to the report of Bera and Roy (1993). However, we could achieve good rooting response with a low concentration of 1.0 mg/l IAA whereas Bera and Roy (1993) reported similar response with 3.0 mg/l IAA. Faisal *et al.* (2005) and Sulekha and Rana (2010) however reported encouraging rooting with IBA. Further, Sulekha and Rana (2010) reported maximum root length of 5.2 cm. Present study reports a higher root length of 10.1 cm achieved with a high concentration of IAA (1.0 mg/l) and also better acclimatization of the regenerated plantlet and improved success in the production of micropropagated plants of *Tylophora indica* .

CONCLUSION

It is concluded that *Tylophora indica* is a plant with a variety of ethnic medicinal uses. The qualitative and quantitative analysis of *T. indica* shows the presence of bioactive compounds such as Alkaloids, Flavonoids, Phenols, Saponins, Steroids, Tannins and Terpenoids. This is valuable information for preparation of drugs in pharmaceutical industry and stress the need for more intensive research since they play a great role in healthcare. The present study describes the successful development of rapid micropropagation protocol of *Tylophora indica.* This protocol provides a successful technique for multiplication and conservation of the valuable medicinal plant which is used in treating various disorders.

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