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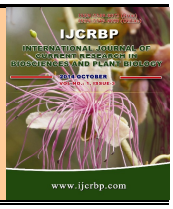


**Original Research Article**

# *In Vitro* Studies and *Agrobacterium* Mediated Transformation in *Tylophora indica* L. (Burm. F.) Merr.

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**A b s t r a c t**

*In vitro* culture of transformation was carried out by co-cultivation of economically important Indian medicinal plant *Tylophora indica* L. (Burm. F.) Merr. During the infection process of the *Agrobacterium rhizogenes* transferred a heritable change in the DNA of the plant resulting in the formation of tumor by the plant. Different explants of *T. indica* were cultured on various concentrations of hormones (IAA, NAA, 2,4-D, IBP and BAP). Out of different concentrations (0.5, 1, 2 and 5mg/l) of 2, 4-D used, the maximum growth in terms of fresh and dry weight was observed at 5mg/l and the minimum growth was observed at 0.5mg/l. IAA at 5mg/l was found to be enormously promoting growth from the nodal explants of *T. indica*. Among different explants of *T. indica*, nodal explants showed more growth response in culture.

**K e y w o r d s**

*Agrobacterium rhizogenes*

*In vitro*-culture

Medicinal plant

Tissue culture

*Tylophora indica*

## Introduction

The important of plants in human life is not to be numbered. From the primitive ages we are directly dependent on plants. Long before the scientific era, man knew the enumerable of the plant life. India is the leading country which produces plant based drugs and drug intermediates. The plants which are not growing wildly are introduced from the places through co-ordinates projects on experimental/commercial basis. Recent advances in the techniques of plant biotechnology including genetic engineering has opened up a new era in secondary product research through production of the plants and plant constituents In the laboratory rather than in the field.

The production of secondary metabolites is the expression of a plant genome under development control. On the other hand, secondary metabolites accidentally appear as result of stress and disease. Such metabolites may be synthesized de novo, (i.e., by gene activation) and are referred to as phytoalexins. Invite genetic manipulation to better understand the biology of secondary metabolites in plants and to increase yields.

Some of the most valuable plant species are facing extinction. Suitable agro technology has to be adapted to cultivate medicinal plant, which is in bulk demand, and modern methods of propagation inducing plant tissue culture should be evolved to multiply the medicinal plants so as to meet the demand from within and across our country. Secondary metabolites are commercially feasible and as such for enhancing the *in vitro* production of natural products many approaches have been propounded. Plant extracts and their constituents had always been a part of various types of herbals and therapeutic systems. In fact, plants are the self-governed factories producing such a vast array of compounds like carbon dioxide, water and inorganic ions.

It has been estimated that higher plants produce more than 100,000 secondary metabolites of which only 15-20% have been chemically characterized (Wink, 1998). Plant tissue culture technique has been successfully utilized on generating genetic variability for selecting better genotypes in many crops in plants.

Herbs are staging a comeback and an herbal renaissance is blooming across the world. *Agrobacterium* mediated plant transformation is a highly complex and evolved process involving genetic determinants of both the bacterium and the host plant cell. *Agrobacterium* mediated transformation (Xiang et al., 2000; Alagumanian et al., 2003; Lavanya et al., 2004) is the easiest and most simple plant transformation (Chaudhuri et al., 2005). *Agrobacterium tumefaciens* and related *Agrobacterium* species have been known as plant pathogens since the beginning of the 20$^{th}$ century.

*Tylophora indica* L. is an Indian species (Fig. 1), also called as *T. asthmatica* L. and *T. vomitoria* L., which belongs to the family Aslepiadaceae. It is used as herbal medicine for asthma, high blood pressure, diarrhea (loose bowel movements), allergies, upset stomach, and inflammation (red, swollen joints) (Bone, 2000).

Crown gall disease is of interest to plant molecular biologist and to biotechnologist, because it is an example of naturally occurring genetic engineering and has led to the development of useful tools for directing genetic transformation to standardize genetic transformation technique for induction of secondary metabolites in *T. indica* (Gupta et al., 2010).

## Materials and methods

### Plant materials

The major plant source for this study was *T. indica* (Asclepiadaceae). The plants were collected from the Herbal Garden, Department of Plant Science, Bharathidasan University, Tiruchirapalli, for tissue culture studies.

### Strain collection

Lyophilized form of *Agrobacterium tumefaciens* (MTCC-532) obtained from the Microbial Type Culture Collection (MTCC), The Institute of Microbial Technology, Chandigarh, India, was cultured in B-3 media.

### Extraction of explants

Leaf explants and shoot apices were used for the present study. The first fully expanded leaves in the shoot apex were collected from the garden grown plant. The explants were excised with the help of sterile forceps and blade. The nodes were cut into 0.5-1.0cm sized segments and care was taken that each explant is included with midrib portion. Apical shoot buds measuring 10-15mm in length with 2-3 leaf primordial attached were also used.

### Surface sterilization of the explants

Surface sterilization was done by using mercuric chloride and alcohol. The explants were treated with 0.1% mercuric chloride for 1-2 min and washed twice with sterile distilled water. Then the materials were rinsed in 50% alcohol for 2-3 min. then the explants were thoroughly washed twice with sterile distilled water.

### Inoculation

Before starting inoculation, culture tubes containing media, instruments like sprit lamp, sterilized forceps, scissors, Petri dishes and sterilized distilled water were transferred to UV chamber and there were exposed to UV light for 30 min. After that the surface sterilized explants were inoculated. The leaf explants were implanted on the medium with abaxial surface in contact with nutrient medium.

### *Agrobacterium* Mediated Transformation Studies (Chaudhuri et al., 2005)

#### Media and solutions

Y EP (yeast extract peptone) medium was prepared and autoclaved. MS (Murashige and Skoog´s) medium was prepared with and without hormones.

*Antibiotics used:*

Cefotaxime 50mg/l
Kanamycin 50mg/l

- *Agrobacterium* was grown in 5ml of YEP medium overnight (12 h) at 28°C.
- The tissues were chopped into small pieces (about 0.5cm long).
- Several pieces of plant tissues were placed in a small Petri dish (50mm) and then 4ml of callus induction medium was added to it.
- 50µl of an overnight grown culture of *Agrobacterium* was added.
- The Petri plate was sealed with parafilm and the bacteria was cocultivated with plant tissues for 2 days at 28°C in the dark.
- The plant tissues were washed with MS medium and blotted for dryness with sterile filter papers.
- The tissues were pre-cultured for a few days on a non-selective callus inducing medium containing 100µg/ml cefotaxime.
- After 5-7 days, the tissues were transferred to selective medium (shoot inducing) containing 200µg/ml kanamycin and 100µl/ml cefotaxime.

#### Confirmation tests for transformation

Basically, two separate experiments were carried out to confirm the transformation of *A. tumefaciens* having *Ti* plasmid into *T. indica*. These two experiments were broadly based on the transformation capacity of *Ti* plasmid.

#### *In vitro* studies

In this experiment, callus was excised from transformed callus of *T. indica* in the shoot inducing medium (IAA 0.5mg/l + 1.0mg/l) with 200mg/ml kanamycin and 100mg/ml cefotaxime. Transformed tissues were cultured after the heat or antibiotics treatments. The bacteria selectively have acquired new properties that are stably inherited and by which the tissue can be distinguished from normal callus, when inoculated in the medium without growth regulators, auxins and cytokinin.

## Results

#### Determination of optimal concentration of hormones

Out of different concentrations (0.5, 1, 2 and 5mg/l) of 2, 4-D used, the maximum growth in terms of fresh and dry weight was observed at 5mg/l and the minimum growth was observed at 0.5mg/l. The growth responses of 2, 4-D was parallel to the concentration (Table 1, Figs. 2 and 3).

**Table 1. Effect of 2,4-D on the growth of nodal explants of *Tylophora indica* L.**

| Concentration of 2,4-D (mg/l) | Formation of callus (%) | Weight at harvest (mg) | |
|---|---|---|---|
| | | Fresh wt. | Dry wt. |
| 0.5 | 36 | 220 ± 64.5 | 11.6 ± 0.64 |
| 1.0 | 37 | 230 ± 62.4 | 12.0 ± 0.59 |
| 2.0 | 68 | 456 ± 112.8 | 25.0 ± 2.8 |
| 5.0 | 79 | 792 ± 196.7 | 44.4 ± 5.7 |

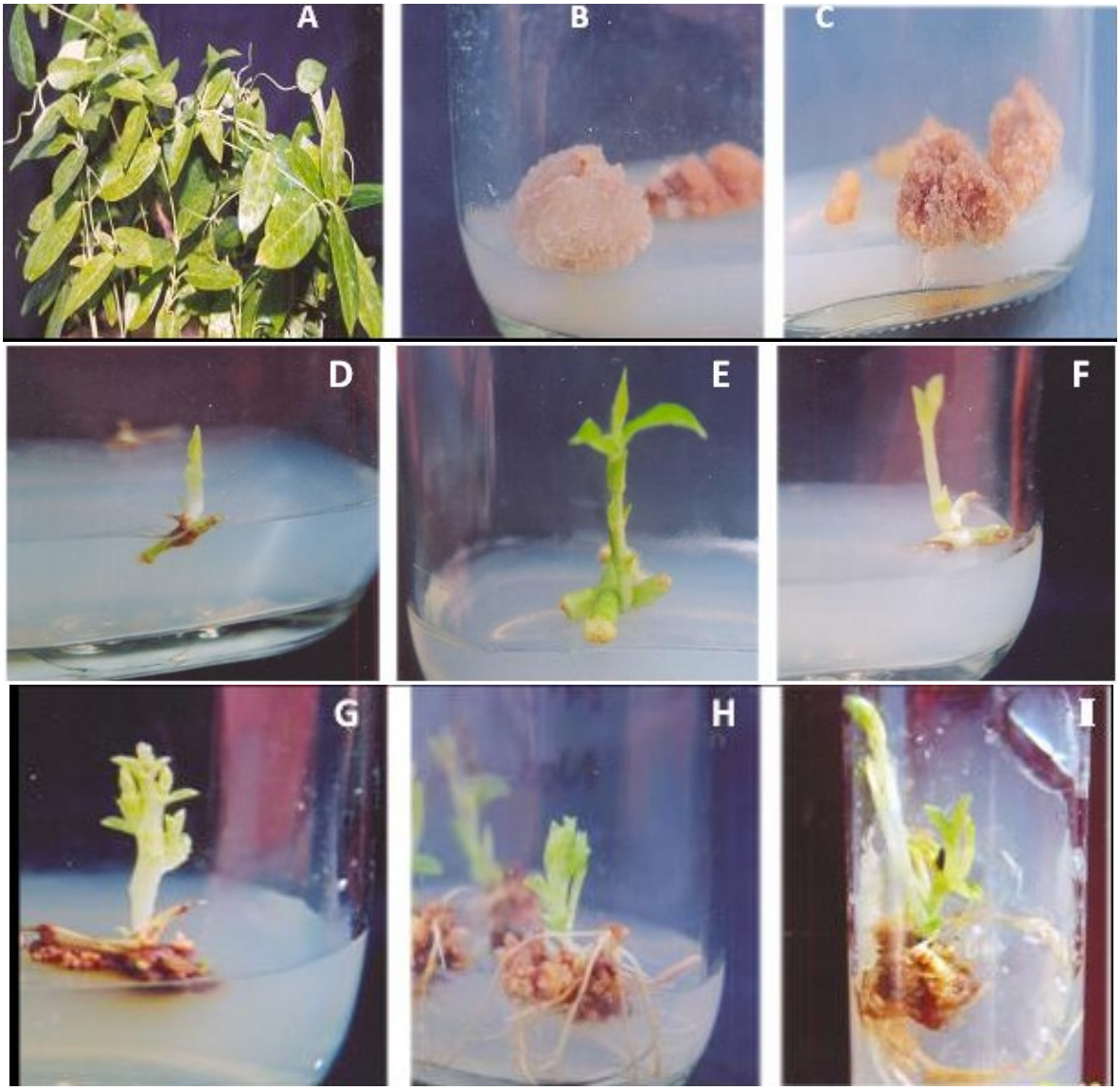
Incubation period: 6 weeks; Initial fresh weight: 18.7 ± 4.6mg; Initial dry weight: 3.5 ± 0.9 mg;

**Table 2. Effect of IAA on the growth 1of nodal explants of *Tylophora indica* L.**

| Concentration of IAA (mg/l) | Formation of callus (%) | Weight at harvest (mg) | |
|---|---|---|---|
| | | Fresh wt. | Dry wt. |
| 0.5 | 2 | 220.1 ± 3.12 | 1.14 ± 0.39 |
| 1.0 | 14 | 690.5 ± 2.22 | 3.89 ± 0.54 |
| 2.0 | 75 | 867.6 ± 3.3 | 50.8 ± 5.9 |
| 5.0 | 90 | 3250.3 ± 364.11 | 177.5 ± 12.8 |

Incubation period: 6 weeks; Initial Fresh weight: 18.7 ± 4.6mg; Initial dry weight: 3.5 ± 0.9 mg;

**Fig. 1: *In vitro* regeneration of *Tylophora indica* L. mediated by *Agrobacterium rhizogenes*.**




[A. Habit of *Tylophora indica*; B. Friable and light brown callus in the fourth week on 0.5mg/l of 2,4-D; C. Colour change of callus from white into brown in the sixth week on 2mg/l of 2,4-D; D. Development of etiolated shoot buds in 0.5mg/l of BAP; E. Well developed green shoots with few leaves in 5mg/l of BAP; F. NAA 0.5mg/l induced shoots with few numbers of leaves in 5mg/l of BAP; G, H and I. Well developed roots formed in different concentrations and combinations of IAA 0.5 + BAP 0.5mg/l: IAA + BAP 2mg/l.]

**Table 3. Effect of NAA on the growth of nodal explants of *Tylophora indica* L.**

| Concentration of NAA (mg/l) | Formation of callus (%) | Weight at harvest (mg) | |
|---|---|---|---|
| | | Fresh wt. | Dry wt. |
| 0.5 | 60 | 850.8 ± 94.5 | 47.2 ± 8.6 |
| 1.0 | 64 | 992.6 ± 101.7 | 55.1 ± 11.2 |
| 2.0 | 80 | 1364.4 ± 123.8 | 75.7 ± 16.9 |
| 5.0 | 88 | 1821.5 ± 162.5 | 101.2 ± 21.4 |

Incubation period: 6 weeks; Initial fresh weight: 18.7 ± 4.6mg; Initial dry weight: 3.5 ± 0.9 mg;

**Table 4. Effect of BAP on the growth of nodal explants of *Tylophora indica* L.**

| Concentration of BAP (mg/l) | Formation of callus (%) | Weight at harvest (mg) | |
|---|---|---|---|
| | | Fresh wt. | Dry wt. |
| 0.5 | 78 | 2112.4 ± 112.5 | 117.3 ± 34.7 |
| 1.0 | 76 | 1498.6 ± 88.9 | 83.2 ± 27.3 |
| 2.0 | 58 | 1009.8 ± 67.5 | 56.0 ± 17.5 |
| 5.0 | 55 | 504.6 ± 42.3 | 28.0 ± 8.4 |

Incubation period: 6 weeks; Initial fresh weight: 18.7 ± 4.6mg; Initial dry weight: 3.5 ± 0.9 mg;

**Table 5. Effect of IBA on the growth of nodal explants of *Tylophora indica* L.**

| Concentration of IBA (mg/l) | Formation of callus (%) | Weight at harvest (mg) | |
|---|---|---|---|
| | | Fresh wt. | Dry wt. |
| 0.5 | - | 22.6 ± 1.2 | 3.4 ± 0.8 |
| 1.0 | - | 21.7 ± 1.1 | 3.3 ± 0.7 |
| 2.0 | - | 21.6 ± 1.3 | 3.5 ± 0.8 |
| 5.0 | - | 23.9 ± 1.5 | 3.4 ± 0.9 |

Incubation period: 6 weeks; Initial fresh weight: 18.7 ± 4.6mg; Initial dry weight: 3.5± 0.9 mg;

Of the different concentrations of IAA (0.5, 1, 2 and 5mg/l) used, 5mg/l was found to be enormously promoting growth from the nodal explants. The other concentrations of this hormone were also induced maximum callus (Table 2, Figs. 7, 8 and 9). Among the four concentrations of BAP (0.5, 1, 2 and 5mg/l), 5mg/l was proved to be most effective than other concentrations. Generally this hormone has very good performance in this study (Table 3, Fig. 4).

Out of different concentrations (0.5, 1, 2 and 5mg/l) of NAA used, the maximum growth in terms of fresh and dry weight was observed at 0.5mg/l and minimum growth was observed at 5mg/l. Hormone concentrations induced maximum amount of callus but higher concentrations induced minimum amount of callus (Table 4, Fig. 6). Not all concentrations of IBA (0.5, 1, 2, 5 mg/l) had promoted callus and other morphogenetic responses (Table 5).

### Effect of factorial combination of IAA and BAP

Factorial combination of different concentrations and combinations of IAA and BAP was tried to elicit morphogenetic potential of nodal explants. Friable nature, generally white colour but rarely light brown colour callus was observed in all the combinations of IAA and BAP. 0.5mg/l of IAA and 1mg/l of BAP combination gave the high calluligenetic responses, which was 1:2. 0.5mg/l and 1mg/l of IAA with all concentrations of BAP (0.5, 1, 2, 5mg/l) showed only callus. Shoot induced from the nodal explants of *T. indica* were observed in the combinations of IAA 1mg/l with 0.5 and 2mh/l of BAP. The shoots grew only 1cm height, but has not elongated. Minimum amount of calluses were induced on the media containing combinations of 2 and 5mg/l of NAA with all concentrations of BAP (0.5, 1, 2 and 5mg/l) (Table 6).

## Discussion

Among different explants of *T. indica*, nodal explants showed more growth response in culture. The young node of *T. indica* has succulent in nature and contains more meristamatic tissues than any other explants. Nodal explants are more sensitive to 2,4-D when compared to IAA, IBA, NAA and BAP. Minimum concentration of 2,4-D (5mg/l) was able to induce maximum callus when compared to IAA, NAA and IBA, among the auxins. The sensitivity of plant tissues to 2,4-D in culture is well documented in earlier works.

The callus inducing ability of the three kinds of auxins on root explants of *T. indica* was in the following order 2,4D > BAP > IAA > NAA > IBA. The preference of specific hormone and concentration of tissue explants of various plant species for callus induction is known already (Rao et al., 1973; Kansara et al., 2013). Nodal explants of *T. indica* produced maximum callusing potential in the medium supplemented with 2,4-D at 5mg/l. Various factorial combination of hormones elucidated different morphogenetic potential of root explants. Morphogenetic potential of tissue explants is also altered by genetic and physiological age of the mother plant (Chaudhuri et al., 2004; Thomas and Philip, 2005; Sivakumar et al., 2006).

**Table 6. Effect of auxins and cytokinins on the nodal explants of *Tylophora indica* L.**

| Concentration hormones IAA + BAP (mg/l) | Formation of callus (%) | Formation of Shoot (%) | Formation of Root (%) | Fresh wt. (mg) | Dry wt. (mg) |
|---|---|---|---|---|---|
| 0.5 + 0.5 | 62 | - | - | 900 ± 45.9 | 50.1 ± 11.2 |
| 0.5 + 1.0 | 80 | - | - | 1200 ± 88.2 | 66.6 ± 14.5 |
| 0.5 + 2.0 | 58 | - | - | 700 ± 39.3 | 38.8 ± 18.5 |
| 0.5 + 5.0 | 61 | - | - | 850 ± 46.2 | 47.2 ± 21.3 |
| 1.0 + 0.5 | 36 | 45 | - | 230 ± 21.3 | 12.7 ± 1.9 |
| 1.0 + 1.0 | 38 | - | - | 600 ± 37.2 | 33.2 ± 9.2 |
| 1.0 + 2.0 | 58 | 42 | - | 1008 ± 98.3 | 55.2 ± 12.4 |
| 1.0 + 5.0 | 75 | - | - | 1009 ± 96.8 | 55.4 ± 12.2 |
| 2.0 + 0.5 | 60 | - | - | 1003 ± 96.4 | 55.7 ± 13.1 |
| 2.0 + 1.0 | 62 | - | - | 1520 ± 112.7 | 84.2 ± 21.6 |
| 2.0 + 2.0 | 64 | - | - | 2010 ± 157.5 | 111.0 ± 27.3 |
| 2.0 + 5.0 | 80 | - | - | 2507 ± 280.8 | 139.0 ± 32.6 |
| 5.0 + 0.5 | - | - | - | 18.5 ± 4.1 | 3.2 ± 0.9 |
| 5.0 + 1.0 | - | - | - | 18.9 ± 4.2 | 3.5 ± 0.8 |
| 5.0 + 2.0 | - | - | - | 18.5 ± 3.9 | 3.2 ± 0.9 |
| 5.0 + 5.0 | - | - | - | 18.0 ± 3.8 | 3.1 ± 0.8 |

Incubation period: 6 weeks; Initial fresh weight: 18.7 ± 4.6mg; Initial dry weight: 3.5± 0.9 mg;

Node explants of *T. indica* produced shoots directly from explants (IAA 1mg/l + BAP 0.5mg/l and IAA 1ml/l + BAP 2mg/l). Only 1 cm length of shoots was proliferated from both combinations of the hormones. Similar observations were made in *Eclipta prostrata* and *Wedelia chinensis* by Kamalam (1998). The shoots in the present study appear to develop from growth centers formed in the compact and meristematic callus mass. Formation of embryoids was not observed. The shoots with vascular cambium were formed directly from explants, originating from the cut ends as observed in *Enicostemma axillare* (Suthersan, 1998).

## Conclusions

In the present study, transformation was carried out by co-cultivation and regeneration of an economically important Indian medicinal plant *T. indica*. Tissue explants different parts of an important Indian medicinal plant, *T. indica* were cultured *in vitro* and their morphogenetic potential was elucidated in the present investigation. Explants from leaf, stem and node were cultured on MS medium supplemented with different concentration and combination of plant hormones like IAA, NAA, 2,4-D and IBA. It was observed that nodal explants have maximum morphogenetic potential than explants of *T. indica*. The optimum concentration of individual hormones for the growth of the node explants was determined. It was found to be 5mg/l for 2,4-D and NAA, 2mg/l for IAA and 1mg/l for BAP. No response in all the concentrations of IBA was observed.

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