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# Somatic Embryogenesis of *Tylophora indica* (Burm.f.) Merril., an Important Medicinal Plant

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**Abstract:** An efficient procedure has been developed for inducing somatic embryogenesis from mature leaves of *Tylophora indica* (Burm.f.) Merrill, an important medicinal plant. Leaf sections were initially cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) in addition with 2, 4-dichlorophenoxy acetic acid (2,4-D), particularly 0.5 µm TDZ along with 1.5 µm 2,4-D was very effective in inducing somatic embryos. Plants were regenerated from *in vitro* somatic embryos plated on semisolid medium devoid of growth regulators. Plantlets were obtained in 65% of the cultures with 2% Sodium alginate coated embryos and control embryos showed 90 % germination. The percent of survival during hardening was 65 to 70. Regenerated plantlets continued to grow after transfer to a greenhouse environment and were similar phenotypically to zygotic seedlings. This simple regeneration system through somatic embryogenesis may be beneficial for mass propagation of *Tylophora indica*.

**Keywords**: *Tylophora indica;* somatic embryogenesis; thidiazuron.

## 1. Introduction

*In vitro* clonal propagation of medicinal plants enables large-scale production of therapeutically high value taxa for commercialization and sustainable utilization in the industrial sector. *Tylophora indica* (Burm.f.) Merrill, previously called as *Tylophora asthematica*, a member of Asclepiadaceae is an important indigenous medicinal plant found in restricted localities in Indian sub continent. The roots have a sweetish taste turning acrid, aromatic odor and a brittle fracture. They possess stimulant, emetic, cathartic, expectorant, stomachic and diaphoretic properties and are used for the treatment of asthma [1], bronchitis, whooping cough, dysentery, diarrhoea and in rheumatic gouty pains [2]. The powdered leaves, stem, and root contain several alkaloids [3] including tylophorine ($C_{24}H_{27}O_4N$), tylophorinine ($C_{23}H_{25}O_4N$) which are pharmacologically active, and anticancer tylophorinidine ($C_{22}H_{22}O_4N$) has also been isolated from the roots of three-year old plant [4].

Apparently due to non-availability of sufficient quality planting materials, commercial plantations of this important aromatic and medicinal species have not been widely attempted and presently only the wild population is exploited for extraction purposes. Due to overexploitation and lack of organized cultivation, the wild populations have declined fast. There are a number of constraints for the propagation and conservation through conventional methods like vegetative and

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seed propagation. The major ones are variations in edaphic and climatic factors, low percentage of seed set and seasonal dormancy. The propagation in its natural habitat is a rare phenomenon evidenced by close field observation. In an attempt of vegetative propagation, the stem cuttings of the plants failed to root with the application of different hormones. The above mentioned causes prompted us to find an alternate method of rapid micropropagation of this species. Clonal mass propagation can be widely applied to shorten the long sexual cycle and other problems like limited seed availability and problems of seed physiology. It is difficult to collect the seed as they are dispersed by wind on attaining maturity in this system.

So in view of medicinal importance, there is an urgent need to conserve this species *ex situ* through *in vitro* methods. Reports on propagation are limited [5-8] so, in this study we are giving efficient and reproducible protocol for somatic embryogenesis in *Tylophora indica*.

## 2. Materials and methods

### 2.1. Plant material

Leaves collected from one year old mature plant were washed with distilled water for two times and then rinsed with 1% (v/v) detergent (Teepol) for 5 min, later were surface sterilized with 0.1% (w/v) aqueous solution of $HgCl_2$ for 5 min followed by 4-5 rinses in sterilized $ddH_2O$. Leaf explants were cut into 8 x 20mm bits at different regions and used in further studies.

### 2.2. Media and culture conditions

The glassware was subjected to chromic and sulphuric acid mixture (1:3) for 24 hours and washed thoroughly with teepol (10%) detergent solution. It was then cleaned under running tap water, further rinsed with distilled water and oven dried. The vessels were decontaminated by autoclaving them at 15 $lbs/in^2$ for 20 minutes then washed with detergent, tap water, distilled water, and finally oven dried. The basal medium used in the present study consisted of the salts and vitamins of MS medium [9]. Addition of plant growth regulators to medium was done with filter sterilization in aseptic conditions and unless it is specified, all media were fortified with 3% sucrose (w/v) and 0.8% agar (w/v; Qualizen, India). The pH of the medium was adjusted to 5.7 prior to the addition of agar. Molten medium (15 ml) was dispensed to 25x150 mm glass tubes (Borosil, India), plugged with non-absorbent cotton wrapped in a layer of cheese-cloth and autoclaved at 121°C for 15 min. All cultures were incubated at 25 °C ± 2 °C and under 16 h light/8 h dark and photoperiod with light intensity of 50 μE $m^{-2}$ $s^{-2}$ provided by cool-white fluorescent lamps.

#### [1] Callus and somatic embryo induction

Leaf bits were cultured facing the adaxial and abaxial side towards the medium. They were inoculated on MS medium containing 2,4-D (1.0, 1.5 and 2.5μM) or TDZ (0.25, 0.5 and 0.75 μM) or BA (0.5 and 1.0 μM) alone or in combinations. The different types of calli and globular somatic embryos initiated and were subcultured to fresh medium after every 3 weeks of interval. In each experiment 20 explants were cultured and all the experiments repeated thrice. 2.0 to 4.0 mm long somatic embryos (cotyledonary stage) were isolated from embryogenic cultures and transferred to solid MS basal medium without growth regulators for rooting.

#### [2] Encapsulation of somatic embryos and acclimatization

Encapsulation of the somatic embryos was done using 1, 2 and 3 % sodium alginate gel. Somatic embryos with sodium alginate dipping into 5% calcium nitrate solution. Drops

were dried and stored in a refrigerator for further long run use. In order to acclimatization, rooted plants were removed from the medium and transferred to cultured tubes containing sterile distilled water. After 10 days, plantlets were transferred to plastic pots containing sterile vermiculite covered with plastic bags and kept under the culture room conditions for 15 days. Plants with newly formed leaves were then transferred to the field and kept under shade for about a week before planting in the soil.

**Table 1.** Effect of plant growth regulators on direct somatic embryogenesis from leaf explants of *Tylophora indica.*

| Plant growth regulators | Concentrations (μM) | Percent of cultures showing response | Number of globular embryos/explant ± S.E. | Callusing |
|---|---|---|---|---|
| 2,4-D | 1.00 | 60.0 | - | ++++ |
| | 1.50 | 71.6 | - | +++ |
| | 2.50 | 68.3 | - | +++ |
| TDZ | 0.25 | 56.6 | - | ++ |
| | 0.50 | 71.6 | - | ++ |
| | 0.75 | 63.6 | - | ++ |
| BA | 0.50 | 62.3 | - | ++ |
| | 1.00 | 83.6 | - | + |
| 2,4-D + TDZ | 1.5 + 0.25 | 48.6 | 3.1 ± 0.81 | + |
| | 1.5 + 0.50 | 53.3 | 18.2 ± 0.91 | |
| | 1.5 + 0.75 | 41.6 | 6.8 ± 0.16 | + |
| 2,4-D + BA | 1.5 + 0.50 | 58.3 | - | +++ |
| | 1.5 + 1.00 | 51.6 | - | ++ |

Observation after 3 weeks. A mean of 20 replicates conducted thrice. Number of + indicates the degree of callusing SE - Standard Error.

## 3. Results and discussion

Non-embryogenic calli were induced with the leaf sections in most of the media containing 2,4-D, TDZ and BA alone concentrations. Alone concentrations of these hormones produced somatic embryos in different plant species [10-15] so, based on these reports we also tried initially with alone concentrations. The quantity and quality of callus formed was more in 2,4-D when compared to TDZ and BA. Since TDZ was effective in inducing multiple shoots in this species with axillary bud cultures (Unpublished data), we started investigation on its effect on callus cultures capable of somatic embryogenesis. When the leaf bits were cultured on a medium supplemented with various concentration of TDZ (0.25, 0.50 and 0.75 μM) in combination with 1.5 μM 2,4-D resulted embryogenic callus formation with friable nature. The combination of both 2,4-D and TDZ in medium significantly affected the number of embryos production. After two weeks of culture the globular embryos appeared as protuberances (Figure 1a and 1b) on both the surfaces of leaf explants growing in same combinations (Ta-

ble 1), particularly 1.5 μM of 2,4-D and 0.50 μM of TDZ combination resulted in on an average of 18.2 globular embryos. In this combination out of 60 replicates 53.3 % cultures showed 583 globular structures (Table 1), later they increased in size and formed distinctly. This reveals evidence for early protocol for a primary medium with an auxin source (Auxin plus) and a secondary medium devoid of growth regulators (Auxin minus), both containing a substantial supply of reduced nitrogen sources [16]. Higher concentration of 2,4-D along with TDZ resulted in formation of only compact, green non-embryogenic callus. Globular structures, passed through heart, torpedo and cotyledonary developmental stages. Finally 25 percent globular structures converted into cotyledonary embryos (146 embryos). Some of the cells are not converted and remained as globular structures and appeared as brownish structures. Similar observation was reported in *Pennisetum americanum* [17], that 2,4-D was basically involved at the early phases of embryo development. This concept was supported by many authors [18] by using 2,4–D for somatic embryogenesis, but 2,4-D in combination with TDZ is critical in the formation of somatic embryos in the present study supported the data in *Arachis hypogaea* [19] and *Paspalum scrobiculatum* [20]. The interaction between TDZ and 2,4-D for somatic embryogenesis was also observed in muskmelon [21] and Watermelon [22], where as BA along with 2,4-D combination resulted only shoot organogenesis. However, following an increase in the concentration more than 0.75 μM of TDZ in combination, showed cytokinin activity and formation of shoot organogenesis instead of somatic embryo conversion. This data supports geranium work [23], where very low concentration of TDZ works effectively for somatic embryogenesis. In addition to displaying cytokinin-like activity, it has been suggested that TDZ modulates endogenous levels of auxin. However it remains to be resolved weather it has auxin activity or is involved in auxin metabolism [24]. In all cultures, a wide variation in embryo morphology was noticed (Figure 1c and 1d). Somatic embryos separated from the parent leaf explant during late maturation stage. Further, embryos were encapsulated as artificial seeds with 1, 2 and 3 % (Table 2) with sodium alginate [25]. Artificial seeds are a potential method for delivering somatic embryos to the field. Different sodium alginate concentrations used in order to determine the effect of rigidity of bead on the conversion frequency of somatic embryos. Somatic embryos were encapsulated with 2 % sodium alginate resulted in 65 per cent germination. In general plant regeneration frequency decreased with an increase in sodium alginate concentration above 3%. On the other hand 1 and 3 % sodium alginate coated embryos exhibited less % germination. In contrast to this, somatic embryos of *Santalum album* encapsulated with 3 % sodium alginate gave the superior results [26] and 2 % sodium alginate failed in conversion of embryos into plantlets observed in loblolly pine [27]. Variability in per cent of germination is mainly due to sodium alginate concentration, bead preparation stage of somatic embryos (pre-maturation or maturation cotyledonary stage), medium factors like agar concentration and environmental factors like temperature and storing duration of artificial seeds(days or weeks).

After 6 weeks of time, encapsulated embryos were placed on MS basal medium for their germination (Figure 1e and 1f). Plantlets were obtained in 65% of the cultures with 2% coated embryos and control embryos showed 90 % germination (Table 2). The somatic embryos germinated after one week and emergence of radicle was noticed. However, shoot development was delayed for 5 to 8 days after radicle emergence.

The embryos resulted into 2 to 3 cm long plantlets with healthy leaves and washed well to remove remnants of agar from roots and transplanted to 10-cm diameter plastic pots containing autoclaved vermiculite.

**Table 2.** Effect of Sodium alginate concentrations on germination of encapsulated embryos of *Tylophora indica.*

| Alginate Con % | No of capsules cultured | No of capsules germinated | % of capsules germinated |
|---|---|---|---|
| Control | 20 | 18 | 90 |
| 1 | 20 | 11 | 55 |
| 2 | 20 | 13 | 65 |
| 3 | 20 | 10 | 50 |

Observation after 3 weeks.

**Table 3.** Percentage of survival during hardening of plant lets in *Tylophora indica.*

| Sample Number | No. of plants hardened | No. of plants survived | % Survived |
|---|---|---|---|
| 1 | 50 | 35 | 70 |
| 2 | 40 | 26 | 65 |
| 3 | 42 | 28 | 66.6 |

Observations after 3 weeks.

The plants were kept at high relative humidity (RH 90%) for one week. After one week they were brought to lower RH (60%) for further hardening for another two weeks. Plants were well irrigated with MS salt solution. Afterwards the hardened plants were repotted in 20 cm in diameter plastic pots containing pure garden soil and sand (1:1) and kept in the field for developing into mature plants (Table 3). The hardened plants were transferred to field with a survival rate of 70 %. All plants had normal leaf development and no morphological variation was noticed (Figure 1g).

## 4. Conclusion

We believe that this is the optimized and most suitable protocol for somatic embryogenesis, which helps to achieve consistently high multiplication rates for commercial practice of this particular medicinal plant.

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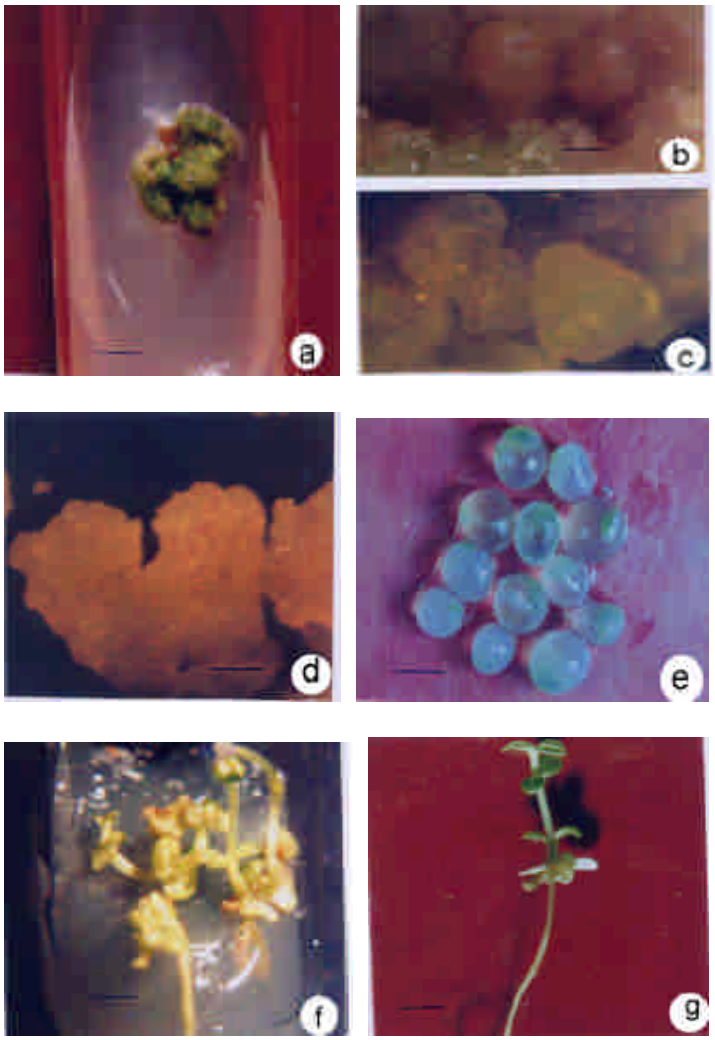


**Figure 1.** Somatic embryogenesis from leaf explants of *Tylophora indica.* (a) Initiation of somatic embryos on 1.5 μM of 2,4-D with 0.5 μM of TDZ (Bar = 4.1 mm). (b) Globular embryos (Bar = 1.2 mm). (c) Heart shaped embryos (Bar = 1.3 mm). (d) Early torpedo stage (Bar = 1.8 mm). (e) Encapsulated embryos (Bar = 6.1 mm). (f) Germination of somatic embryos (Bar = 3.4 mm). (g) Germinated plantlet from somatic embryo (Bar=6.5mm).

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