Alteration ofMedia Enables Efficient In-vitro Regeneration in Antamool (*Tylophora indica*): AThreatened Medicinal Plant

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Abstract:
Tylophora indica commonly known as antamool is a threatened medicinal plant. Native species of this plant is decreasing very rapidly due to complete lack of organized cultivation and over-exploitation. Therefore, an efficient protocol for large-scale T. indica multiplication needs to be established. Current study on T. indica is conducted to develop a beneficial, consistent & feasible method for mass multiplication in in-vitro controlled conditions. Murashige and Skoog medium was used as a basal medium to culture T. indica and to studyorganogenesis and somatic embryogenesis. Further, the presence of secondary metabolite was detected by Thin Layer Chromatography technique. Callus was developed on Murashige and Skoog medium augmented with varying concentrations of 6-benzylaminopurine (BAP), naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) with 3% sucrose and 50 mg/l casein hydrolysate. Highest rate of organogenic and embryogenic calli was observed at 0.5 mg/l NAA +1.0 mg/l BAP and 1.0 mg/l 2,4-D + 0.5mg/l BAP. Differentiation of shoot buds was best achieved from the callus surface after transferring into shooting media. The highest rate (100%) of shoot bud initiation was observed on MS medium containing 0.5mg/l NAA +1.0mg/l BAP and 1.0mg/l NAA +1.5mg/l. BAP.Similarly, somatic embryos were induced on Murashige and Skoog medium supplemented with 1.0mg/l 2,4-D and 0.1% potassium nitrate.The present research provides evidence of significant organogenic and embryogenic potency of T.indica as its exhibits callus formation and shoot buds formation at higher rate. The above method will allow this medicinally important plant to be produced on a large scale for the vegetation, nurseries and for the pharmaceutical corporations.

Keywords:*Tylophora indica, In-vitro* propagation, Callus, Regeneration, Organogenesis, Somatic embryogenesis

Introduction

Tylophora indica commonly known as antamool is a threatened medicinal plantthat belongs to familyAsclepiadaceae. It is a perennial, small, slender, and climbing plant, native to sub-Himalayan tract, Deccan Plateau, Western Ghats, and Eastern Ghats. Historically, this plant has been used as a traditional medicine to treat respiratory diseases, bronchitis, rheumatoid arthritis, swelling, infection, and redness, immune-modulatory function with allergies and dermatitis, anti-inflammatory properties, anti-cancer, and anti-amoebic operation [1].

Native species of this plant is decreasing very rapidly due to complete lack of organized cultivation and over-exploitation. Therefore, an efficient protocol for large-scale *T.indica* multiplication needs to be established. Some researchershave established mass cloning of *T.indica* by *denovo* adventitious shoot multiplication primarily using leaf as explants. Regenerated plantlets were checked for antioxidant activity. 75% antioxidant activity was observed at 1000 µg/ml concentration in methanolic root extract of *in vitro* regenerated plants [2].In another study by Najar *et.al.*2018*in-vitro* culture of *T.indica* by direct shoot regeneration with nodal explants were found to have the maximum regeneration rate in Murashige and Skoog medium augmented with different PGR regimes [3].The study demonstrated that for shoot multiplication Thidiazuron was very efficient

in comparison to other cytokinins with 90% regenerated plant survival rate. Kaur *et.al.,*2016reported that chromatographic determination confirmed tylophorine availablity inregeneratedplants of *T.indica*[4]. Quantitative analysis of in-vitro regenerated plant of *T.indica* indicated the highest tylophorine concentration of 80µg/mLof which 71 µg/mL of tylophorine was derived directly from plants. Direct regeneration was reported varying with hormone concentrations and mixture, illumination and incubation, genotype and part of plant used for regeneration [5,6].

Due to the medicinal value of *Tylophora indica*, there is a vital need to preserve the plant ex situ by means of in-vitro methods. Very less reports are available on multiplication of this plant [7,8]. So, current study on *T. indica* is conducted to develop a beneficial, consistent & feasible method for mass multiplication in *in-vitro* controlled conditions and to check the presence of secondary metabolites in naturally grown plant and *in-vitro* raised calli.

Materials and Methods

Plant Material

Tylophora indica plants were sourced from the nursery of Kapoor Chandra Kulish Smriti Van, Jaipur and plants were grown in pots. Leaves were taken as an explant from these plants. Leaves were rinsed with running tap water to eliminate the dust particles. Surface of leaves was swabbed with 70% (v/v) alcohol. For surface sterilization, three treatments utilizing differentconcentration of sodium hypochlorite & mercuric chloride were tested, as shown in Table 1.

Leaves were surface sterilized via the method established in section 4.5 and 1cm^2 size explants were cut from treated leaves. These explants were subjected to two different types of media treatments for initiating cultures for regeneration via organogenesis or via somatic embryogenesis.

Table 1: Surface Sterilization of Explants

Treatment	Sodium hypochlorite Concentration (v/v), TT: 3 mins.	Mercuric chloride Concentration (w/v), TT: 3 mins.
T1	5%	0.075%
T2	10%	0.09%
T3	20%	0.1%

Media and Culture Conditions

Murashige and Skoog medium (1962) (MS medium) was used as a basal medium to culture *T.indica.* Stock solutions were prepared with major elements, minor elements, and organic constituents. Such stock solutions were kept at 4°C and mixed in appropriate quantity before use.

Definite amount of all constituents of MS medium except agar were mixed. The pH of solution was adjusted to 5.8± 0.2 with 0.1N HCl or NaOH. Required amount of agar was added and media vessels were plugged with cotton before autoclaving. All the media used in this study were autoclaved at 121ºC, 15 psi for 16 minutes.

All the cultures have been incubated at controlled temperature of 25±2°Cin laboratory. Fluorescent tubes are the source of illumination. The illumination rate was 800-1000 lux at culture level and photoperiod of 16 hrs. was maintained.

Organogenesis

Callus formation: Treated explants were inoculated in MS(1962) basal salts, 2x MS-vitamins, casein hydrolysate, 3% sucrose, myo-inositol100mg/L, 0.8% agar augmented with varying concentrations of NAA and BAP at pH 5.8 (Table 2)

Organogenic calli formation: Calli derived from all four treatments were transferred to a second stage media containing MS(1962) basal salts, 2x-MS vitamins, casein hydrolysate, 3% sucrose, 100 mg/l myo-inositol, 0.8% Agar supplemented with 0.2mg/l NAA and 0.5mg/l BAP, for organogenic callus formation.
Shoot bud formation: For this, organogenic calli derived from stage 2 cultures were transferred to a third stage media containing MS (1962) basal salts, 2x-MS vitamins, casein hydrolysate, augmented with NAA 0.2mg/L and BAP 1.0mg/L, for shoot bud formation and further elongation of shoots.

Table2: Induction of callus with various concentrations and conjunction of plant growth regulators (Organogenesis mode)

PGR Treatment Codes	NAA(mg/l)	BAP(mg/l)
PD-1	0.5	1.0
PD-2	1.0	1.0
PD-3	0.5	1.5
PD-4	1.0	1.5

Somatic Embryogenesis
Callus Induction: Explants were inoculated onto MS (1962) medium with Gamborg B5 vitamins, 0.1% potassium nitrate, augmented with varying concentration of BAP and 2,4-D at pH 5.8(Table 3).
Embryogenic calli formation: Calli derived from all four treatments were transferred to a second stage media containing MS (1962) medium with Gamborg B5 vitamins, 0.1% potassium nitrate, 3% sucrose, 100mg/l myo-inositol, Agar 0.8% augmented with 0.1 mg/l BAP at pH at 5.8 for embryogenic calli formation.
Embryo formation: For this, embryogenic calli derived from stage 2 cultures were transferred to a third stage media containing MS(1962) medium with Gamborg B5 vitamins, 0.1% potassium nitrate, 3% sucrose, myo-inositol100mg/l, 0.8% Agar for embryo formation.

Table 3: PGR treatments used for callus induction (Somatic Embryogenesis mode)

PGR Treatment Codes	2,4-D(mg/l)	BAP(mg/l)
SPD-1	0.5	0.1
SPD-2	1.0	0.1
SPD-3	0.5	0.5
SPD-4	1.0	0.5

Detection of secondary metabolites
Sampling and extraction:Two types of samples were taken for extraction of secondary metabolites**;** Leaves were taken from pot-grown plants of *Tylophora indica*&*In-vitro* raised calli from leaf explants.
Leaves of *T.indica* were washed with tap water 2-3 times. In-vitro raised calli were taken from cultures and rinsed with sterile water after removing adhered media. Both leaves and calli were dried in oven at a temperature of 50°C for 30 minutes, later left for shade drying at the laboratory bench for 2 days. After drying, both types of samples were pulverized using mortar and pestle.
One gram of powdered leaf was taken in two separate containers and soaked in 5ml each of two solvents- ethyl acetate and ethanol. Similarly, 0.1g of powdered calli were taken in two separate microcentrifuge tubes and soaked in 0.5ml ethyl acetate and 0.5ml ethanol. These four samples were left at room temperature with proper

agitation. After 24 hrs fresh solvent (equal in volume) were added and allowed to stand with frequent agitation. After 48 hrs extracts of leaves and calli were filtered using Whatmann filter paper no. 1. Filtrate was reduced by evaporation in a water bath at 50°C and resultant extracts were dissolved in 500 µl and 50 µl of solvents (ethyl acetate and ethanol) for leaf sample and callus sample, respectively.

Secondary metabolites analysis by thin layer chromatography(TLC)

Precoated TLC plates were used. Mobile phase, i.e., Chloroform: methanol: ethyl acetate: toluene (2:1.5:4:5) was prepared in the TLC chamber to develop 5-10 mm depth. Chamber was closed and left for vapor saturation at room temperature. Sample application was done by using capillary tubes. In chamber, TLC plates were inserted, covered with glass-plate, and left undisturbed for some time (until mobile phase reached close to the end of the plate). Plates were taken out and dried in oven for 1 minute and sprayed with Dragendroff's reagent and after spraying were again dried in oven for 2 minutes. The TLC plates were visualized under UV-light.

Results

Surface sterilization treatments

To find an optimized protocol for sterilization of a specific tissue, three factors were taken into consideration *viz*., sterilant, its concentration and the treatment duration. Leaf explants were treated by sodium hypochlorite and mercuric chloride at different concentrations. Explants treated at 5%NaOCl (v/v), 0.075%$HgCl_2$(w/v) (T1) showed fungal contamination with 70 % survival rate of explants. All explants are free from contamination with 100% viability when treated at 10%NaOCl(v/v), 0.09% $HgCl_2$ (w/v) (T2). Thus, results suggest that 10%NaOCl, 0.09% $HgCl_2$ are the best suitable concentrations for explant sterilization (T2) (Table 4). At concentration 20%NaOCl (v/v), 0.1% $HgCl_2$(w/v) explants are contamination free but yellowing of explants were observed with less than 50% survival rate(T3) as shown in figure 1A.

Table-4: Response of explants to surface sterilization treatments in terms of contamination and explants viability

Treatments	(+/-) Contaminating Agent	Explant died/ Explant growing	Explants showing growth/ Total no. of explants
T1 (5%NaOCl, 0.075% $HgCl_2$)	+ Fungal - Bacterial	1/5 1/5 1/5 Avg. = 1/5	15/18
T2 (10%NaOCl, 0.09% $HgCl_2$)	- Fungal - Bacterial	0/9 0/5 0/7 Avg. = 0/7	21/21
T3 (20%NaOCl, 0.1% $HgCl_2$)	- Fungal - Bacterial	3/5 2/5 3/8 Avg. = 2.7 / 6	10/ 18 (8 explants turned yellow and didn't show any growth)

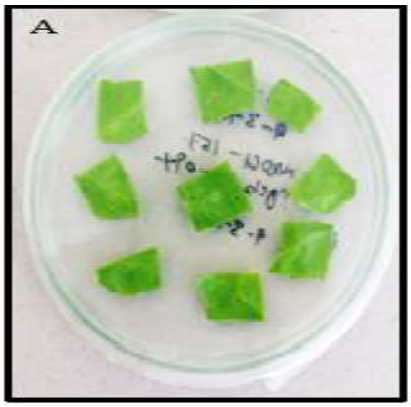


Figure 1A: Response of explants to Surface sterilization treatments T1, T2, T3.

Organogenesis

Inoculation of leaf explants was carried out on MS (1962) medium augmented with varying concentration of NAA (0.5 mg/l, 1.0mg/l) and BAP (1.0 mg/l, 1.5 mg/l) for the induction of callus. Each media combination was supplemented with casein hydrolysate (50 mg/l) and 3% sucrose. Callusing induced either from the ends or from the complete explant after 15 days. After sub culturing, callus was green and compact in the center and light green in the periphery. Optimum callus for organogenesis was obtained after four weeks of culture(Table5, Figure1B).After addition of casein hydrolysate in the callus induction medium the growth rate of callus was increased and the 'fast growing calli' were obtained. The experimental study confirmed regeneration of the shoots by direct organogenesis. Upon switching to 1/2 MS media with IBA, approximately 95% of the in vitro shoots formed roots, acclimatized, and developed in the soil successfully.

Table-5: Response of explants to different PGR regimes for Callus induction

Plant growth regulators(mg/L)	**No. of Explants inoculated**	**Number of explants showing callus induction (* No. of explants which got contaminated)**	**Frequency of callus Induction**	**Morphology**
PD-1 (0.5 mg/l) NAA + (1.0 mg/l) BAP	24	19(5*)	100%	Green & compact
PD-2 (1.0 mg/l) NAA+(1.0 mg/l) BAP	29	24(5*)	100%	White, green & compact
PD-3 (0.5 mg/l) NAA + (1.5 mg/l) BAP	24	21(3*)	100%	yellowish green & compact
PD-4 (1.0 mg/l) NAA + (1.5 mg/l) BAP	23	17(6*)	100%	Creamish, green & compact

Figure 1 B: Induction of callus on different combination with varying concentration of BAP and NAA.

Compact callus was taken and inoculated on MS medium consisting NAA 0.2mg/l and BAP 0.5mg/l with casein hydrolysate (50 mg/l) to initiate regeneration. Miniature shoot buds were developed after 21 days (Figure1 C)
Callus with shoot buds were transferred to fresh medium containing NAA 0.2 mg/l and BAP 1.0 mg/l and shoot buds were developed from the entire surface of callus after three weeks (Figure D)
Shoot buds initiation with 100% frequency was observed from PGR combination 0.5 mg/l NAA and 1.0 mg/l BAP, when transferred to media containing intermediate concentration (0.2mg/L NAA and 0.5mg/l BAP) of both PGR whereas shoot bud formation requires low concentration of auxin (0.2 mg/l NAA). Low concentration of NAA(auxin) than BAP (cytokinin) showed best results.

Somatic Embryogenesis
For induction of callus, inoculation of leaf explants was done on medium augmented with varying concentration of 2,4-D + BAP (0.5 mg/l, 1.0 mg/L). Each media combination was supplemented with 0.1% potassium nitrate and 3% sucrose. Callus with compact nature were obtained from all combinations. (Table 6, Figure1, E)

Table 6: Response of explants to PGR treatments for Callus induction (somatic embryogenesis mode)

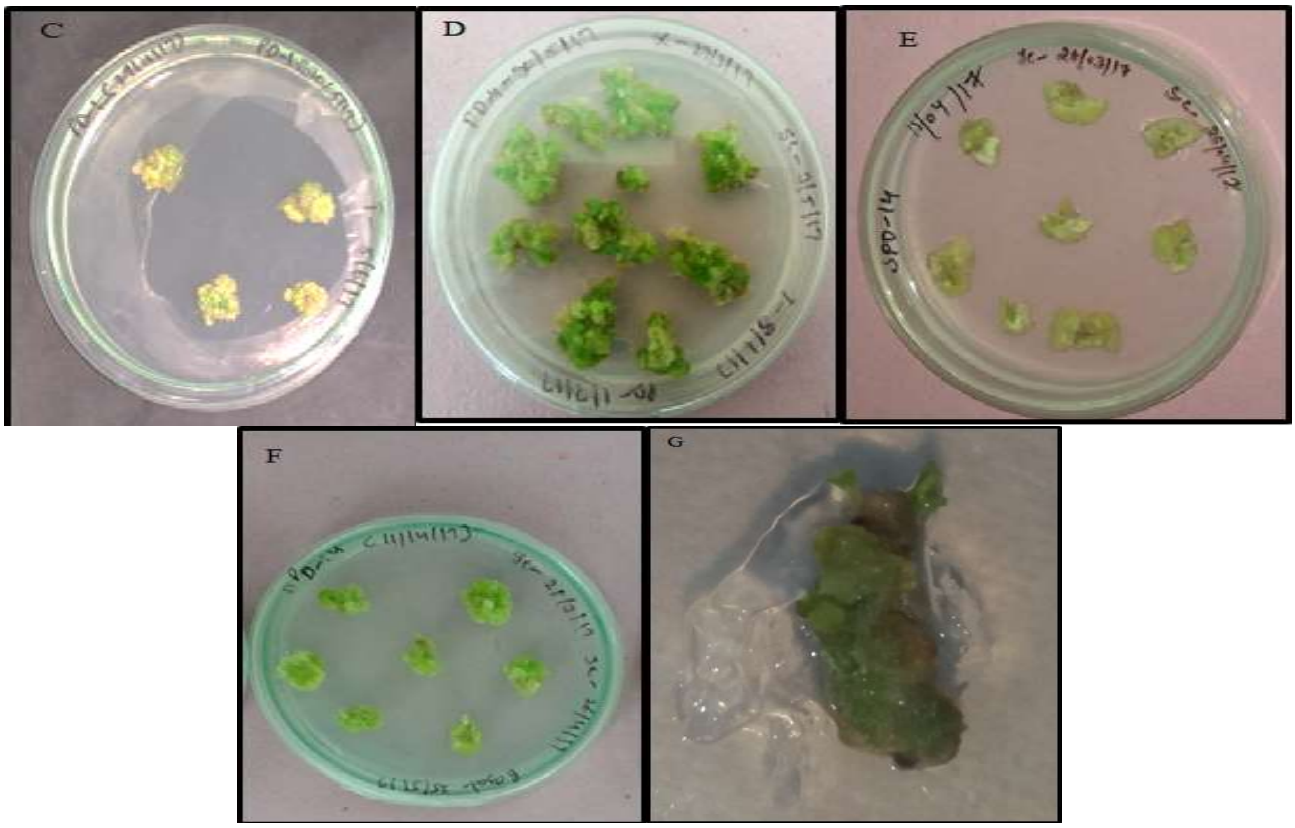
Concentration of Plant growth regulators(mg/l)	No. of explants inoculated	Number of explants showing induction of callus(* No. of explants which got contaminated)	Frequency of callus Induction	Morphology
SPD-1 2,4-D(0.5)+BAP(0.1)	25	22(3*)	100%	Green & compact
SPD-2 2,4-D(1.0)+BAP(0.1)	27	21(6*)	100%	White & compact
SPD-3 2,4-D(0.5)+BAP(0.5)	24	18(6*)	100%	Green& compact
SPD-4 2,4-D(1.0)+BAP(0.5)	25	20(5*)	100%	Green, yellowish & compact

After 21 days of culture, calli were sub-cultured supplemented with BAP 0.1mg/l resulting into the formation of friable callus and found optimum for initiation of somatic embryos. (Figure1, F)
After three weeks of incubation, friable callus was sub-cultured to medium having only MS basal salts for production of somatic embryos. Calli derived from combination (1.0 mg/l 2,4-D + 0.5 mg/l BAP) resulted into the initiation of somatic embryos. Globular and heart shaped embryos stages were identified at the apex of callus after three weeks of incubation. (Figure1,G)

Detection of secondary metabolites
Extraction of major alkaloid was carried out from the powderedleaf collected form field grown plant and powdered callus obtained from *in-vitro* raised calli (3months old). The excised leaves were powdered and processed using optimized extraction protocol and the final extract obtained was analyzed using TLC. TLC was performed by utilizing solvent system-chloroform: methanol: ethyl acetate: toluene (2:1.5:4:5) and visualized under UV-light of 254 nm. Three spots were visualized in ethanolic extract (leaf) and one spot was observed in ethanolic extract (calli) with Rf value of sample A (Leaf sample) is 0.43 and Rf value of sample B (Calli sample) is 0.72. No spots were observed in ethyl acetate extract in both (leaf and calli) samples(Table 7,Figure1 H).

Table 7: Separation of compounds from *T. indica* leaves and callus on TLC plate.

Sample	Number of Bands	Color	R_f value	hR_f value
Ethanolic extract - A(Leaf)	3	Pink	0.43	43
Ethanolic extract-B(Callus)	1	Pink	0.72	72
Ethyl acetate extract-C(Leaf)	-	-	-	-
Ethyl acetate extract -D(Callus)	-	-	-	-



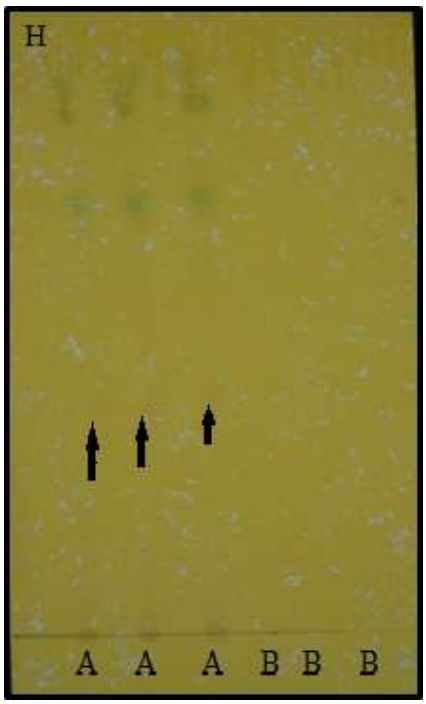


Figure 1 C: Organogenic callus formation and Shoot buds initiation.
D: Shoot buds formation in culture derived from different combinations of NAA and BAP.
E: Induction of callus on various combinations containing different concentrations of BAP and 2,4-D
F: Friable callus formation on MS-Basal and BAP.
G: Somatic embryos formation
H: Separation of compounds on TLC

A: Ethanolic Leaf extract
B: Ethanolic Calli extract

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

$$hRf = Rf \times 100$$

Rf of sample A = 6.2/14.5 = 0.43
Rf of sample B = 10.4/14.5 = 0.72
hRf of sample A = 0.43×100 = 43
hRf of sample B = 0.72×100 = 72

Discussion

Sterilization of explants is a necessary step for carrying out any plant tissue culture strategy. To find an optimized protocol for sterilization of a specific tissue, three factors were taken into consideration *viz*., sterilant, its concentration and the treatment duration. Results suggest that 10%NaOCl, 0.09% $HgCl_2$ are the best suitable concentrations for explant sterilization. Some other studies showed surface sterilization of young leaves and stem explants with teepol, Bavistin (1%) (v/v), alcohol and $HgCl_2$ at different concentrations [9,10].

For organogenesis, callusing was induced after 15 days. After sub culturing, callus was green and compact. Also, the experimental study confirmed regeneration of the shoots by direct organogenesis. Similar results have been observed in a study conducted by Saritha & Naidu 2008 [11]. In a study by Pathak *et.al* (2019), MS media containing sucrose (3%), mixtures of 6-BA and KIN individually and in conjunction with NAA were used for inoculation leaf explants [12].

Shoot buds initiation with 100% frequency was observed from PGR combination 0.5 mg/l NAA and 1.0 mg/l BAP. Low concentration of NAA (auxin) than BAP (cytokinin) showed best results. A study conducted by Arora & Mishra (2019) demonstrates that in *Monodopsis subterranea*, IBA (100 µM) was reported to have the highest biomass yield on the late stable stage [13]. Introduction of 6-BAP (1 µM) revealed a small increase in biomass precipitation. Biomass revealed a correlation with increased concentration of 6-BAP. The synergistic effect of BAP and kinetin was reported by Sompornpailin and Khunchuay (2016) [14].The lower BAP concentration in CIM1 (NAA 2 mg/l and BA 0.5 mg/l) resulted in low regeneration efficiency and lower shoots per callus given the high concentration of 2 mgl^{-1} BAP and 2 mg/l kinetin in the PRM3 regeneration medium. Nevertheless, the calli grown in CIM2 lacked kinetin but with a sufficient level of BAP was maintained to regenerate at higher rate in PRM2 and PRM3 containing 1 and 2 mg/l kinetin, respectively.

Reports on the growth of organogenic callus and shoot are available using combinations of hormones at a concentration of 1.0 mg/l 2,4-D and 1.0 mg/l BAP [15]. BAP I conjunction with diverse auxins like 2,4-D, IBA and NAA for growth of stock calli has been reported by Verma *et.al.,*2010[16]. Shoot bud differentiation documented by Failsal and Anis (2005) in *Tylophora indica,*revealed that the total number of shoots reduced with the increase of BAP concentration[17]. Nema *et.al.,*2007 reported that in *Tylophora indica,* BAP at higher concentration inhibits shoot bud formation [18]. The synergistic impact of BAP and Auxins has been reported among members of family Asclepiadaceae like*Ceropegia candelabrum* [19], *Hemidesmus indicus* [20], *Holestemma annulare* [21], and *Gymnema sylvestre* [22].

Several investigations suggest the use of casein hydrolase (CH) and proline for induction of embryogenic calli in *Oryza sativa* [23]. Presence of casein hydrolase allows for rapid organogenesis over auxin and cytokinin levels. In plant tissue culture, addition of CH is more beneficial than the introduction of amino acids because it is agood source of micronutrients, vitamins, calcium, and combination of 18 Amino acids. In this present investigation, a novel method is used for organogenic callus induction and shoot buds development with CH.

For somatic embryogenesis, calli derived from combination of 1.0 mg/l 2,4-D and 0.5 mg/l BAPresulted into the initiation of somatic embryos. Several investigations demonstrated the regeneration via somatic embryogenesis in *Tylophora indica*. Callus initiation using stem as an explant were carried out on MS (1962) media augmented with 2,4-D and BAP with L-glutamine[24]. Addition of potassium nitrate benefits celldevelopment during callus growth stage through somatic embryogenesis in culture of date palm [25]. 2, 4-D independently is not able to produce enough somatic embryos.Use of cytokinins in medium containing 2, 4-D has been reported to be effective for somatic embryos production [26,27]. Reports are available on the production of Somatic embryos using various auxin and cytokinin like 2,4-D and TDZ [28].

Extraction of major alkaloid was carried out from the powdered leaf collected form field grown plant and powdered callus obtained from *in-vitro* raised calli. The excised leaves were powdered and processed and the final extract obtained was analyzed using Thin Layer Chromatography.Presence of spots showed the presence of alkaloids. A spot may indicate the presence of 2 or 3 compounds (Tylophorine, Tylophorindine, Tylophrinidine). Qualitative analysis of plant extract for secondary metaboliteswas done, which showed the presence of secondary metabolites like nitrogen containing compounds- alkaloids, flavonoids, phenols, saponins, steroid and terpenes[29]. Alkaloids, flavonoids, steroids and triterpenoids were reported by Vanitha *et.al.,*2019 from the extracts of *T.indica* [30]. In a quantitative analysis high amount of tylophorine (80 µg/ml) was reported in plants regenerated from leaf callus whereas plants obtained directly without any intervening callus stage contained 71 µg/ml of tylophorine [4].Cyriac *et.al.,*2020 reported biotechnological production of tylophorine which was fulfilled by inducing hairy roots mediated by *Agrobacterium rhizogenes* (A4 strain)[31]. They reported its growth in liquid suspension culture that could yield maximum biomass and tylophorine production. This type of liquid suspension culture yielded9.8 ± 0.21 mgL−1 tylophorine within 4–6 weeks of incubation.

Conclusion and Future Directions

This current research illustrates an efficient method for in-vitro regeneration and mass propagation of *T. indica* an endangered plant with therapeutic value. A methodology for high frequency regeneration and the establishment of plants from leaf derived callus has been developed. Experiments conducted to develop an effective, consistent & reproducible approach for rapid multiplication of *T.indica* mass propagation through *in-vitro* plant tissueculture. Secondary metabolites were also identified in the form of alkaloids and flavonoids

from naturally grown plants and *in-vitro* raised calli. *T.indica* is a good source for gene transfer studies, and has the potential for high-frequency shoot regeneration. Thus, the above method will allow this medicinally important plant to be produced on a large scale for the vegetation, nurseries and for the pharmaceutical corporations.

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