CHAPTER 3

MATERIALS AND METHODS

The present study focuses on two important medicinal plants of Asclepiadaceae family i.e. *Leptadenia reticulata* (Retz.) Wight and Arn and *Tylophora indica* (Burm. F.) Merrill. The experimental work is divided into two parts: Development of *in vitro* cultures and phytochemical analysis. First aim was to establish regeneration protocol utilizing leaf and nodal explants for both the plants. The second aim was qualitative and quantitative analysis of selected secondary metabolites for different *in vitro* cultures using HPTLC. Lastly *in vitro* cultures were treated with different elicitors for the enhancement of targeted metabolites in both the plants.

3.1 Development of *In vitro* Cultures

3.1.1 Plant Material

Leptadenia reticulata (Retz.) Wight and Arn plants were procured from Directorate of Medicinal and Aromatic Plants Research, Boriyavi, Anand and maintained in green house in the Botanical Garden of The Maharaja Sayajirao University of Baroda. They were regularly irrigated and monitored, after a year they were utilised for the experimental studies. Whereas *Tylophora indica* (Burm. F.) Merrill. plants were already growing in the Botanical Garden.

3.1.2 Glasswares and Plasticwares

Different glasswares (Borosil and Duracil, Vadodara, India) and plasticwares (Tarsons, Vadodara, India) were used in the experimental studies and are listed below:

- Beakers (100, 250 and 500 ml)
- Culture tubes (25 × 150 mm, 55 ml)
- Erlenmeyer flasks (50, 100, 150, 250, 500 and 1000 ml)
- Measuring cylinders (10, 50, 250 and 500 ml)
- Petriplates (100 × 15 mm)
- Culture tube caps

All the glasswares were thoroughly washed before use in experiments. First they were dipped in chromic acid solution (mixture of concentrated H_2SO_4 + $K_2Cr_2O_7$ + water) for overnight and then cleaned with detergent under running tap water followed by rinsing with D/W. They were kept in hot air oven at 60 ºC for drying.

3.1.3 Instruments

Several instruments which were used in the experimental studies are:

- Autoclave (M. Shah and Co., Mumbai, India)
- Balance (Sartorius, Mumbai, India and Scasen, Mumbai, India)
- Hot air oven (Modern Scientific Industries, Mumbai, India)
- Hot plate (Jay Scientific Instrument, Vadodara, India)
- Laminar air flow (Klenzaids Bioclean Devices Pvt. Ltd., Mumbai, India)
- Micropipette (Fischer Scientific Ltd., Mumbai, India)
- pH meter (Analab Scientific Instrument Pvt. Ltd., Vadodara, India)
- Leica microscope (Leica Microsystems, Mumbai, India)

3.1.4 Chemicals

Regeneration of plants under *in vitro* conditions requires additions of different macro and micronutrients, sucrose as a carbon source and plant growth regulators (PGRs). All the chemicals of Murashige and Skoog's (MS, 1962) stocks were procured from SRL, Mumbai, India.

3.1.4.1 MS Salts Stock

To establish *in vitro* cultures initially different stocks of MS medium containing different macro and micronutrients, irons, vitamins were prepared. MS medium mainly consist of stock A (macronutrients), stock B (micronutrients), stock F (iron source) and stock G (vitamins) and is documented in Table 2.

- Stocks A, B and G were prepared by dissolving the chemicals sequentially one after the other in 100 ml D/W.
- Whereas for stock F, $Na_2EDTA.2H_2O$ and $FeSO_4.7H_2O$ were added separately in two different flasks containing 100 ml D/W, boiled and mixed after dissolution.
- All stocks were filled in amber colour bottles and stored in refrigerator (4 ^oC).

3.1.4.2 Plant Growth Regulators (AR grade, SRL, Mumbai, India)

To establish cultures different types of PGRs like cytokinins and auxins were used.

- Cytokinins

  Adenine sulphate ($AdSO_4$), N^6-benzyladenine (BA) and kinetin (Kn)

- Auxins

  2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthalene acetic acid (NAA)

- Gibberellins

  Gibberellic acid (GA_3)

Table 2: Murashige and Skoog's (MS, 1962) basal stock composition.

Chemicals	Normal stock (mg/l)	Concentrated stock (gm/l)
Stock A (500 ml)	**1X**	**10X**
$MgSO_4.7H_2O$	370	03.70
KH_2PO_4	170	00.17
KNO_3	1900	19.00
NH_4NO_3	1650	16.50
$CaCl_2.2H_2O$	440	04.40
Stock B (400 ml)	**1X**	**200X**
H_3BO_3	6.2	1.240
$MnSO_4.4H_2O$	22.3	4.460
$ZnSO_4.7H_2O$	8.6	1.720
$Na_2MoO_4.2H_2O$	0.25	0.050
$CuSO_4.5H_2O$	0.025	0.005
$CoCl_2.2H_2O$	0.025	0.005
KI	0.83	0.166
Stock F (200 ml)	**1X**	**100X**
$FeSO_4.7H_2O$	27.8	2.780
$Na_2EDTA.2H_2O$	37.3	3.730
Stock G (400 ml)	**1X**	**200X**
Thiamine HCl	0.5	0.1
Pyridoxine HCl	0.5	0.1
Nicotinic acid	0.5	0.1
Myo-inositol	10.0	20
Glycine	2	0.4

KH_2PO_4 of stock- A and myo-inositol of stock- G were added at time of medium preparation to avoid precipitation.

3.1.4.3 Stocks of Plant Growth Regulators (PGRs)

1 mM stocks of all the PGRs were prepared in D/W as shown in Table 3 and stored in refrigerator (4 ºC).

Table 3. Preparation of PGR stock solutions (100 ml).

PGRs	Quantity (gm)	Dissolve
$AdSO_4$	0.0368	HCl
BA	0.0225	HCl
Kn	0.0215	HCl
2,4-D	0.0221	NaOH
IAA	0.0175	NaOH
IBA	0.0203	NaOH
NAA	0.0186	NaOH
GA_3	0.0346	Cold D/W

3.1.4.4 Antibiotics

- Streptomycin (SRL, Mumbai, India)
- Tetracyclin (SRL, Mumbai, India)

3.1.4.5 Other Chemicals

- Agar (Bacteriology grade, SRL, Mumbai, India)
- Alcohol (Local make)
- Bavistin (Local make)
- Dettol (Reckitt Benckiser, Mumbai, India)
- Hydrochloric acid (HCl) (Loba Chemie, Mumbai, India)
- Labolene (Fisher Scientific, Mumbai, India)
- Mercury chloride ($HgCl_2$) (Qualigens, Mumbai, India)
- Polyvinylpyrrolidone (PVP) (SRL, Mumbai, India)
- Potassium dichromate ($K_2Cr_2O_7$) (SRL, Mumbai, India)
- Sodium hydroxide (NaOH) (S.D. Fine Chem. Ltd., Mumbai, India)
- Sucrose (SRL, Mumbai, India)
- Sulphuric acid (H_2SO_4) (Loba Chemie, Mumbai, India)

3.1.5 MS Media Preparation (1 l)

Steps followed for MS media preparation:

- 50 ml D/W was filled in flask and then 50 ml stock A was added
- 2 ml each of stock B, F and G were added
- 0.170 gm KH_2PO_4 was added and dissolved
- Then 0.100 gm myo-inositol was added and dissolved
- Sucrose (3% w/v) as a carbon source was added to the medium
- Different PGRs were added and their calculations was done using following formula:

$$\frac{\text{PGR concentration } (\mu M) \times \text{Volume of media (ml)}}{\text{Concentration of PGR stock } (\mu M)}$$

- pH of the medium was adjusted to 5.80 using 1 N NaOH and 1 N HCl
- Agar (0.8% w/v) was used as a gelling agent and the medium was boiled till it dissolved
- Then the medium was poured into culture tubes/flasks and their mouths were closed using cotton plugs/caps
- Media, D/W, headgear-mask, forceps, scalpel handle, scooper and petriplates wrapped in paper were sterilized in autoclave at 121 ºC (15 psi) for 25 min

3.1.6 Establishment of *In vitro* Cultures

The methodology for establishment of *in vitro* cultures of both the plants are as below.

3.1.6.1 Antibacterial Assay

Selection of suitable antibiotic for the explants treatment was done as follows:

- first step was to isolate bacteria from contaminated cultures of leaf/node
- Bacteria were grown on petri dishes of solidified nutrient agar medium without antibiotic
- Detectable bacterial contaminants were individually isolated and subcultured onto the fresh medium for 2 to 4 days after incubation
- The isolates were cultivated by streaking onto nutrient agar, then different concentrations antibiotics containing disc was placed onto the centre of the petridish and incubated at 30-32 °C for four to five days
- The cultures were daily observed for the zone of inhibition

3.1.6.2 Plant Material and Treatment

Entire leaves and nodes were excised from healthy twigs of *L. reticulata* and *T. indica* and washed as follow-

- The explants were kept under running tap water for 1 h
- Then 2-3 drops of labolene was added to form lather and stirred for 5 min. Labolene was removed thoroughly using tap water and then rinsed twice with D/W
- The explants of *L. reticulata* were given additional treatment of cold PVP solution (0.01% w/v) for 5 min followed by rinsing with D/W
- Further surface sterilization treatments were given to the explants in the laminar air flow cabinet

3.1.6.3 Aseptic Manipulations

All the aseptic manipulations were carried out in the laminar air flow cabinet and it was cleaned as follow-

- Working table and glasswares were swabbed with freshly prepared aquoes dettol solution followed by alcohol (70% v/v)
- All the culture tubes/bottles, sterile D/W, head gear-mask, forcep, scalpel handle, blade, petriplates, match box, marker and coupling jar containing alcohol (70% v/v) were kept in the chamber and were irradiated with U.V. light for 45 min
- Leaves and nodes were treated with bavistin (0.01% w/v), streptomycin (0.01% w/v) and $HgCl_2$ (0.1% w/v) solutions for 4 min each, and rinsed with sterile D/W after each treatment
- Leaves were cut in square piece of lamina (1 cm^2) containing midrib and inoculated abaxially on the medium
- Whereas intermodal segments of stem with one node (2-3 cm long) were excised and the lower portion was given an oblique cut and then placed vertically into the medium

3.1.6.4 Culture Media

MS basal medium fortified with sucrose (3%) was used as a control and it was fortified with different concentrations of cytokinins and auxins individually and in combinations.
The following media were used for developing the *in vitro* cultures.

❖ ***Media used for L. reticulata* and *T. indica***

A. Leaf explants

- MS + sucrose (3%) - Control

Individual cytokinins:

- MS + sucrose (3%) + BA (5-20 µM)
- MS + sucrose (3%) + Kn (5-20 µM)

Individual auxins:

- MS + sucrose (3%) + IAA (0.1-1 µM)
- MS + sucrose (3%) + NAA (0.1-1 µM)
- MS + sucrose (3%) + 2,4-D (0.1-1 µM)

Combination of cytokinins:

- MS + sucrose (3%) + BA (5-20 µM) + Kn (5-20 µM)
- MS + sucrose (3%) + BA (5-20 µM) + $AdSO_4$ (5-20 µM)
- MS + sucrose (3%) + Kn (5-20 µM) + $AdSO_4$ (5-20 µM)

Combination of cytokinins and auxins:

- MS + sucrose (3%) + BA (5-20 µM) + IAA (0.1-1 µM)
- MS + sucrose (3%) + BA (5-20 µM) + NAA (0.1-1 µM)
- MS + sucrose (3%) + BA (5-20 µM) + 2,4-D (0.1-1 µM)
- MS + sucrose (3%) + Kn (5-20 µM) + IAA (0.1-1 µM)
- MS + sucrose (3%) + Kn (5-20 µM) + NAA (0.1-1 µM)
- MS + sucrose (3%) + Kn (5-20 µM) + 2,4-D (0.1-1 µM)

Development of somatic embryos

Leaves of *L. reticulata* which differentiated somatic embryos (SEs) were transferred to static and liquid media supplemented with different PGRs combinations for further development:

- MS + sucrose (3%) + BA (15 µM) + $AdSO_4$ (15 µM)
- MS + sucrose (3%) + GA_3 (0.5-2.0 µM)
- MS + sucrose (3%) + CW (4%)

Whereas in *T. indica* somatic embryos differentiated from leaves were germinated in the same medium but for further development of plantlets and its elongation they were transferred to static and liquid media supplemented with different PGR combinations-

- MS + sucrose (3%) + Kn (5 µM) + IAA (0.1 µM)
- MS + sucrose (3%) + Kn (15 µM) + NAA (0.1 µM)
- MS + sucrose (3%) + BA (5 µM) + $AdSO_4$ (20 µM)
- MS + sucrose (3%) + GA_3 (1.0 µM)
- MS + sucrose (3%) + CW (4%)

B. Nodal explants

- MS + sucrose (3%) – Control

Individual cytokinins:

- MS + sucrose (3%) + BA (5-20 µM)
- MS + sucrose (3%) + Kn (5-20 µM)

Combination of cytokinins:

- MS + sucrose (3%) + BA (5-20 µM) + Kn (5-20 µM)
- MS + sucrose (3%) + BA (5-20 µM) + $AdSO_4$ (5-20 µM)
- MS + sucrose (3%) + Kn (5-20 µM) + $AdSO_4$ (5-20 µM)

Elongation of shoots for *L. reticulata*:

- MS + sucrose (3%) + CW (2-8%)

C. Rooting of Shoots

Eight weeks old *in vitro* shoots (≥ 3 cm) of both the plants were placed into different strength (MS/ ½MS/ ¼MS) of MS medium fortified with different concentrations of IBA and NAA, and media without PGR served as a control for the study. Following methodology was used for rooting studies.

- Healthy shoots were harvested from mother explant and leaves from last 2-3 nodes were removed
- The shoots were initially dipped in sterile D/W followed by bavistin solution (0.01% w/v) for 1 min each
- After treatments, shoots were inserted in the center of the filter paper bridge. The bridge with single shoot was placed in such a manner that the last two to three nodes remained immersed in medium
- The base of the culture tubes were covered with aluminium foil till the root induction started

Media used for rooting of *L. reticulata* shoots

- MS + sucrose (1%) – Control
- MS + sucrose (1%) + IBA (2 and 4 µM)
- ½MS + sucrose (1%) – Control
- ½MS + sucrose (1%) + IBA (2 and 4 µM)

Media used for rooting of *T. indica* shoots

- ½MS + sucrose (1%) – Control
- ½MS + sucrose (1%) + IBA (2-8 µM)
- ½MS + sucrose (1%) + NAA (2-8 µM)
- ¼MS + sucrose (1%) – Control
- ¼MS + sucrose (1%) + IBA (2-8 µM)
- ¼MS + sucrose (1%) + NAA (2-8 µM)

Number of SEs/explants, no. of shoots/explant, no. of roots/shoots and % response was calculated for each combinations.

3.1.6.5 Hardening and Acclimatization of Plantlets

Hardening was carried out only for *T. indica*, plantlets developed from shoot cultures and plantlets derived from SEs. Rooted shoots were removed from medium and gently washed with D/W. The plantlets were transferred in thermocol/plastic cups filled with cocopeat:sand (1:1) which were then covered with plastic bags to maintained humidity and kept in lab condition for four weeks. They were watered every alternate day and regularly monitored for the growth. Then plants were transferred to the pots filled with same substrates to greenhouse for another 4 weeks where they gradually acclimatised. The plants were moved to garden in soil under full sunlight.

3.1.7 Culture Conditions

All the cultures were kept in culture room at 26 ± 2 ºC under 16/8 h (dark/light) photoperiod of 40 µmol/m^2/s provided by cool white fluorescent lights (Philips India Ltd., India).

3.2 Phytochemical Analysis

Different *in vitro* samples were analysed qualitatively and quantitatively for their biosynthetic potential in comparison to shoots from *in vivo* plants. The qualitative analysis was done using HPTLC fingerprint for both the plants. Quantitative analysis was done for selected metabolites i.e. *p*-coumaric acid in *L. reticulata* and lupeol in *T. indica*. The selected samples in which maximum content was recorded, then treated with different elicitors to observe their effects on the content of compounds.

3.2.1 Glasswares and Plasticwares

- Beakers (50 ml)
- Eppendorff tubes (1 ml)
- Erlenmeyer flask (50 ml)
- Evaporation dishes (80 × 45 mm)
- Funnels (50 mm)
- Measuring cylinders (10 ml)
- Pipettes (0.1, 1, 2 and 5 ml)

3.2.2 Instruments

- Linomat V sample applicator (Camag, Muttenz, Switzerland)
- Mortar-pastel
- Orbit shaker (Scigenics Biotech Pvt. Ltd., Chennai, India)
- Syringe (100 µl, Anchrom Enterprises Pvt. Ltd., Mumbai, India)
- TLC scanner (Camag, Muttenz, Switzerland)
- Twin through glass chamber (Camag, Muttenz, Switzerland)
- UV chamber (Camag, Muttenz, Switzerland)
- Water-bath (Durga Scientific Ltd., Ahmedabad, India)

3.2.3 HPTLC Fingerprint of *In vitro* Samples

In vivo shoots and *in vitro* cultures of both the plants were harvested from different PGRs supplemented media and evaluated using HPTLC fingerprint. The study required different chemicals, reagents and the methodology that was adopted is described below.

3.2.3.1 Chemicals and Reagent Preparation

(AR Grade, S.D. Fine Chem. Ltd., Mumbai, India)

- Ethyl acetate
- Formic acid
- n-Hexane
- Methanol
- Toluene
- Anisaldehyde-sulfuric acid reagent (100 ml)-

  Anisaldehyde- 0.5 ml

  Glacial acetic acid- 10 ml

  Methanol- 85 ml

  H_2SO_4- 05 ml

The reagent was allowed to cool at room temperature and stored in amber colour bottle in refrigerator (4 ºC).

3.2.3.2 Plant Material

Healthy *in vivo* shoots of *L. reticulata* and *T. indica* were collected from the plant grown in the Botanical Garden. They were thoroughly washed using running tap water followed by D/W and oven dried (40 ºC).

- For *L. reticulata, in vitro* cultures harvested from different media like-

  L1 - *In vivo* shoots of *L. reticulata*

  L2 - *In vitro* shoots from nodal explant in BA (5 µM) + Kn (5 µM)

  L3 - *In vitro* shoots from nodal explant in BA (10 µM) + $AdSO_4$ (10 µM)

  L4 - *In vitro* shoots from nodal explant in CW (4%)

  L5 - Callus from leaf explant in BA (15 µM) + 2,4-D (1.0 µM)

  L6 - Callus from leaf explant in BA (20 µM) + NAA (0.5 µM)

- For *T. indica, in vitro* cultures harvested from different media like-

  T1 - *In vivo* shoots of *T. indica* from the Botanical Garden

  T2 - *In vitro* shoots from leaf explant in BA (10 µM) + Kn (15 µM)

  T3 - Shoots of SEs derived plantlets from leaf explant in GA_3 (1 µM)

  T4 - Shoots from hardened plants

  T5 - *In vitro* shoots from nodal explant in BA (10 µM) + Kn (5 µM)

T6 - *In vitro* shoots from nodal explant in BA (15 µM) + $AdSO_4$ (10 µM)

T7 - *In vitro* shoots from nodal explant in Kn (10 µM) + $AdSO_4$ (15 µM)

The shoots were harvested and washed with D/W and kept in oven (40 ºC) for drying, whereas callus samples were removed from mother explant, agar were removed and dried in oven. All the dried samples were stored in freezer (0 ºC) until use.

3.2.3.3 Preparation of Extract

1 gm dried *in vivo* and *in vitro* samples were powdered using mortar-pestle and extracted from optimized methodology (Pathak et al., 2017). In brief, 10 ml hexane was added to the samples and refluxed in water bath at 50 ºC for 10 min. The extracts were then filtered and collected in evaporation dish, and the procedure was repeated twice using same solvent. The same procedure was repeated three times for ethyl acetate and methanol too. All the extracts were kept overnight at room temperature for evaporation and next day the marc was reconstituted to 1 ml using the same solvent and stored in freezer (0 ºC).

3.2.3.4 Mobile Phases and TLC Plate Development

All the extracts were spotted as a band (8 mm length and 12 mm distance between tracks) on a precoated Silica gel G60 F_{254} (20 x 20 cm, 0.2 mm thick, E. Merck Ltd., Mumbai, India) with the help of Linomat V sample applicator.

Following were used for different extracts-

a) *L. reticulata*-

- Hexane extract- toluene:ethyl acetate:formic acid (7.2:2.5:0.3 v/v)
- Ethyl acetate extract- toluene:ethyl acetate:methanol (6.4:2.7:0.9 v/v)
- Methanol extract- toluene:ethyl acetate:formic acid:methanol (6.0:2.7:0.6:0.7 v/v)

b) *T. indica*-

- Hexane extract- toluene:methanol (9:1 v/v)
- Ethyl acetate extract- toluene:ethyl acetate:formic acid (6:3:1 v/v)
- Methanol extract- toluene:ethyl acetate:formic acid (6.5:3.0:0.5 v/v)

Freshly prepared mobile phases were poured in CAMAG twin through glass chamber and chamber was saturated for 45 min. The plates loaded with samples were allowed to develop in respective mobile phase and after development, they were air dried. Scanning was done in CAMAG TLC scanner 3 linked to winCATS software at different UV wavelengths e.g. 254, 366 and 525 nm (after derivatization using anisaldehyde-sulfuric acid reagent).

3.2.4 Standard Curve Preparation

3.2.4.1 Standard Curve of p-Coumaric Acid and HPTLC Method Validation

1 mg/ml stock solution was prepared in methanol and was serially diluted to prepare various standard solutions with six different concentrations ranging from 100 to 600 µg. A calibration curve was made by spotting a fixed volume (10 µl) of different concentrations of standard solution in triplicates on precoated TLC plates. They were developed in a mobile phase toluene:ethyl acetate:methanol (7.2:2.5:0.7 v/v), dried and scanned at 254 nm and peak area of each concentration was recorded using the CAMAG TLC scanner.

HPTLC method was validated according to International Conference on Harmonisation (ICH) guidelines (ICH 1997) for linearity, limit of detection (LOD) and limit of quantification (LOQ), precision and specificity.

- Peak areas were plotted against the pre determined concentrations of standards and linearity of the method was measured by calculating correlation coefficient (R^2) value. Peak area and concentration were subjected to linear regression analysis to calculate the calibration equation and correlation coefficient.
- The variability was studied by analyzing one sample and three different concentrations of the standard, three times at regular intervals on the same day (intra-day precision) and at a particular time period in 3 different days (inter-day precision). Detection was done at 254 nm and RSD was calculated to determine the precision. The repeatability was assessed by analyzing the known concentration of a standard spotted multiple times (n = 6) on TLC plate and calculating the relative standard deviation (RSD).
- LOD and LOQ values were calculated based on signal-to-noise (S/N) ratio which should be 3:1 and 10:1 respectively.

3.2.4.2 Standard Curve of Lupeol

1 mg/ml stock solution of Lupeol was prepared in methanol and was serially diluted to give different concentrations ranging from 100 to 600 ng. A calibration curve was prepared by

spotting different concentrations in triplicates on precoated TLC plates. They were developed in a mobile phase toluene:methanol (9:1 v/v), dried, derivatised and scanned at 525 nm using the CAMAG TLC scanner.

3.2.5 Quantification of Selected Metabolites

3.2.5.1 p-Coumaric Acid

Quantitative analysis of *p*-coumaric acid was done by densitometry scanning at 254 nm using HPTLC scanner. All the samples were applied (in triplicate) on the TLC plates and developed in aforementioned optimized mobile phase. The peaks in samples corresponding to the standard were validated on the basis of similar Rf values and spectral analysis. For quantification, area under the curve (AUC) was recorded and concentration of compounds and was calculated using the regression equation obtained for the standard curve.

3.2.5.2 Lupeol

10 µl of standard and samples were applied as a band (8 mm length and 12 mm distance between tracks) on TLC plates. The chambers were saturated with respective mobile phases for 45 min. The plates were developed in toluene:methanol (9:1 v/v), air dried and scanned at 525 nm for quantification. AUC for respective peak areas were recorded in all the samples.

3.2.6 Elicitation of Metabolites

3.2.6.1 Chemicals

- Yeast extract (SRL, Mumbai, India)
- Salicylic acid (SRL, Mumbai, India)

3.2.6.2 Elicitor Stock Preparation

In elicitation experiment, YE was directly added into the media whereas for SA stock was prepared as follows:

- **Yeast extract (YE):**
  - Yeast extract was added at 25, 50, 100 and 200 mg/l concentration in medium

- **Salicylic acid (SA) (M.W.: 138.12):**
  - 138.12 mg of SA was dissolved in 100 ml D/W to prepare 1 mM stock

- It was stored in amber colour bottle in refrigerator (4 ºC)
- Following formula was used to calculate SA quantity in the medium:

$$\frac{\text{SA concentration (µM)} \times \text{Volume of media (ml)}}{\text{Concentration of SA stock (µM)}}$$

- Different concentrations like 25, 50, 100 and 200 µM were used for the study

Elicitation study for *L. reticulata* was done in static cultures and the elicitors were added in the optimised culture media from the start of the culture. For *T. indica*, elicitors were supplemented in the medium during subculture after four weeks. For both the plants, medium devoid of elicitor served as a control.

3.2.6.3 Culture Establishment

Leaf derived callus in *L. reticulata* and nodal derived shoots in *T. indica* were used for elicitation studies. The methodology for elicitation is described below:

- ***L. reticulata***
  - Callus growth determination study was performed for callus biomass and it was harvested from three to six weeks
  - Leaf explant were inoculated in static optimised medium i.e. MS + sucrose (3%) + BA (20 µM) + NAA (0.5 µM) which served as a control
  - For elicitation, YE and SA were added in the media from the beginning i.e. MS + sucrose (3%) + BA (20 µM) + NAA (0.5 µM) + YE (25-200 mg/l) and MS + sucrose (3%) + BA (20 µM) + NAA (0.5 µM) + SA (25-200 µM)
  - Control as well as treated callus was harvested from mother explant after fourth and fifth weeks and kept in an oven (40 ºC)
  - The biomass of callus in terms of fresh and dry weight was recorded for all the samples

- ***T. indica***
  - Nodal explants were inoculated in optimised medium i.e. MS + sucrose (3%) + Kn (10 µM) + $AdSO_4$ (15 µM)
  - They were subcultured after four weeks in static medium having same PGR combination which served as a control

- Further, shoots were subcultured in static media supplemented with YE and SA i.e. MS + sucrose (3%) + Kn (10 µM) + $AdSO_4$ (15 µM) + YE (25-200 mg/l) and MS + sucrose (3%) + Kn (10 µM) + $AdSO_4$ (15 µM) + SA (25-200 µM)
- Control as well as treated shoots were harvested at four, five and six weeks, washed thoroughly using D/W and oven dried
- The biomass of these shoots in terms of fresh and dry weight was recorded for all the samples of different concentrations

3.2.6.4 Extraction and Quantification

Methanol extracts for *L. reticulata* and hexane extracts for *T. indica* were prepared for cultures treated with different elicitors using above mentioned methodology for extraction. All the samples were analysed using optimized method for HPTLC quantification of *p*-coumaric acid in *L. reticulata* and lupeol in *T. indica*.

3.3 Statistical Analysis

Total 10 replicates were used for regeneration studies, 5 replicates were used for biomass of cultures and 3 replicates for secondary metabolites quantification. Means and standard errors (SE) were calculated for each experiment followed by ANOVA ($\alpha = 0.05$). The significant means were further analysed using Tukey's test using GraphPad Prism 6.01.