CHAPTER 3

MATERIALS AND METHODS

The present experimental work is mainly divided into two sections: regeneration and secondary metabolite studies. First aim of the study was to optimize regeneration protocol utilizing leaf and nodal explants of *H. indicus* to establish the shoot cultures. After optimization of media, shoot cultures regenerated from nodal explants were qualitative and quantitative analyzed for secondary metabolite content. The shoots were then utilized for elicitation studies and quantification of lupeol and rutin were done for the same. These elicited shoot cultures were taken up for gene expression analysis for flavonoid biosynthetic pathways.

3.1 REGENERATION STUDIES

3.1.1 Plant Material

Hemidesmus indicus (L.) R. Br. plants were procured from Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand and grown in Arboretum and green house in the Botanical Garden of The Maharaja Sayajirao University of Baroda. They were regularly watered and used for the experimental studies.

3.1.2 Glasswares and Plasticwares

Different glasswares and plasticwares were used in the experimental studies. The galsswares were washed by dipping them in chromic acid solution (Conc. H_2SO_4 + $K_2Cr_2O_7$) overnight. Next day they were thoroughly washed with detergent and rinsed with D/W followed by drying in oven (60 ºC).

All the glasswares were of Borosil and Duracil whereas plasticwares were of Tarson

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

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)

3.1.4 Chemicals

The plants under *in vitro* conditions are in heterotrophic mode of nutrition and hence carbon source is added to medium. All the chemicals of Murashige and Skoog's (MS, 1962) stocks were procured from SRL, Mumbai, India.

3.1.4.1 Plant growth regulators (AR grade, SRL, Mumbai, India)

To establish shoot cultures different types of plant growth regulators like cytokinins and auxins were used.

- Cytokinins

  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)

3.1.4.2 Others

There are several other chemicals which were used for establishing shoot cultures:

- 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)

- 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 Stock and Media Preparation

The first step was preparation of stocks for MS medium containing different macro and micronutrients, irons, vitamins and plant growth regulators which were then used for media preparation.

3.1.5.1 MS salts stock

- MS medium consist mainly four stocks: stock A (macronutrients), stock B (micronutrients), stock F (iron source) and stock G (vitamins), as shown in Table 2.
- All the chemicals for stock A, B and G were dissolved in 100 ml D/W sequentially one after the other.
- Whereas for preparation of stock F, both $Na_2EDTA.2H_2O$ solution and $FeSO_4.7H_2O$ were boiled in two different flasks (100 ml D/W), and were mixed in the boiling condition and allowed to cool at room temperature.
- All stocks were stored in amber colored bottle in refrigerator (4 ºC).

3.1.5.2 Stocks of plant growth regulators (PGRs)

- Cytokinins and auxins were two important groups of plant growth regulators which were used in the regeneration studies.
- 1 mM stocks of all the PGRs were prepared in D/W as shown in Table 3 and stored in refrigerator (4 ºC).

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.

Table 3: Preparation of PGR stock solutions

PGR	gm	Dissolve	Volume
Cytokinins			
BA	0.02252	HCl	100 ml
Kn	0.02152	HCl	100 ml
Auxins			
2,4-D	0.02210	NaOH	100 ml
IAA	0.01752	NaOH	100 ml
IBA	0.02032	NaOH	100 ml
NAA	0.01862	NaOH	100 ml

3.1.5.3 MS media preparation (1 l)

The following steps were used to prepare MS medium:

- Initially 50 ml D/W was taken and 50 ml stock A was added to this
- Then 2 ml each of stock B, F and G were added sequencially
- KH_2PO_4 (0.170 gm) was added and dissolved completely
- Later myo-inositol (0.100 gm) was added and dissolved

- Sucrose (3% w/v) was added to the medium as a carbon source
- Different PGR(s) were added as per requirement and 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 HCl and 1 N NaOH
- Agar (0.8% w/v) was added as a gelling agent and the medium was boiled till it completely dissolved
- Then the medium was poured into culture tubes/bottles and the mouth of the flask was enclosed by non-absorbent cotton plugs or caps.
- Media, D/W, headgear-mask, blunt forceps, scalpel handle and petriplates wrapped in paper were autoclaved
- Sterilization of the media and instruments was done in autoclave at 121 ºC (15 psi) for 20 min

3.1.6 Establishment of Shoot Culture

To carry out the regeneration experiments, following methodology has been used to establish shoot cultures and to induce rooting in shoots.

3.1.6.1 Plant material

Entire leaf and nodal explants, were excised from healthy twigs of *H. indicus* and washed as follow before being placed on medium-

- The explants were kept under running tap water for 1 h
- After 1 h, 2-3 drops of labolene was added to form lather and stirred for 5 min. Then labolene was thoroughly removed using tap water and then rinsed twice with D/W
- Further surface sterilization treatments were given in the laminar air flow cabinet

3.1.6.2 Aseptic manipulations

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

- Working table was swabbed with freshly prepared dettol solution followed by alcohol (70%), whereas glass pomes were cleaned with dettol

- 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%) were kept in the chamber and were irradiated with U.V. light for 45 min

3.1.6.3 Establishment of shoot culture using leaf and nodal explant

To establish shoot cultures, leaves and nodes were surface sterilized and then inoculated as described below.

- Leaves and nodes were treated with bavistin solution (0.01% w/v) for 4 min
- After that they were rinsed with sterile D/W for 1 min
- Surface sterilization was carried out using $HgCl_2$ solution (0.1% w/v) for 4 min followed by rinsing with sterile D/W twice for 1 min each
- Leaves having midrib were cut in square piece of lamina (1 cm^2) and inoculated abaxially on the medium
- Whereas for nodes, 2-3 cm long stem contained node and having oblique cut at lower portion were inoculated vertically into the medium

3.1.6.4 Culture Media

MS basal medium fortified with sucrose (3%) was used as a control for the regeneration studies and it was fortified with different concentrations of cytokinins and auxins individually and in combinations.

The following media were used for developing shoot cultures.

<u>Media used for leaf explant</u>

- **Individual cytokinins:**
  - MS + sucrose (3%) + BA (5-30 µM)
  - MS + sucrose (3%) + Kn (5-30 µM)
- **Individual auxins:**
  - MS + sucrose (3%) + IAA (0.1-2 µM)
  - MS + sucrose (3%) + NAA (0.1-2 µM)
  - MS + sucrose (3%) + 2, 4-D (0.1-2 µM)
- **Combination of cytokinins:**
  - MS + sucrose (3%) + BA (5-30 µM) + Kn (5-30 µM)

- **Combination of cytokinins and auxins:**
  - MS + sucrose (3%) + BA (5-30 µM) + IAA (0.1-2 µM)
  - MS + sucrose (3%) + BA (5-30 µM) + NAA (0.1-2 µM)
  - MS + sucrose (3%) + Kn (5-30 µM) + IAA (0.1-2 µM)
  - MS + sucrose (3%) + Kn (5-30 µM) + NAA (0.1-2 µM)

Media used for nodal explant

- **Individual cytokinins:**
  - MS + sucrose (3%) + BA (5-20 µM)
  - MS + sucrose (3%) + Kn (5-20 µM)
- **Individual auxins:**
  - MS + sucrose (3%) + IAA (0.5-2 µM)
  - MS + sucrose (3%) + NAA (0.5-2 µM)
- **Combination of cytokinins:**
  - MS + sucrose (3%) + BA (5-20 µM) + Kn (5-20 µM)
- **Combination of cytokinins and auxins:**
  - MS + sucrose (3%) + BA (5-20 µM) + IAA (0.5-2 µM)
  - MS + sucrose (3%) + BA (5-20 µM) + NAA (0.5-2 µM)
  - MS + sucrose (3%) + Kn (5-20 µM) + IAA (0.5-2 µM)
  - MS + sucrose (3%) + Kn (5-20 µM) + NAA (0.5-2 µM)

3.1.6.5 Establishment of shoot culture using in vitro nodes

Regeneration utilizing *in vivo* nodes was also tried in presence of individual and combinations of cytokinins were tried. MS basal medium fortified with sucrose (3%) served as a control. Following PGR combinations were tried. Eight weeks old *in vitro* shoots were taken up for this study and they were excised and inoculated as follows:

- Healthy *in vitro* shoots were harvested
- Single *in vitro* nodes were excised, leaves were removed and placed vertically in the following media

Media used for *in vitro* nodes

- **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)

3.1.6.6 Rooting of shoots

Eight weeks old healthy *in vitro* shoots (≥ 3 cm) were selected for the rooting studies and three different strengths of MS medium i.e. full, half and quarter, fortified with different concentrations of IBA and NAA were used for optimization. Media without PGR served as a control for the study. Following procedure was done for rooting studies.

- Leaves from last 2-3 nodes were removed and the shoots were dipped in sterile D/W for 1 min
- The shoots were then treated with bavistin solution (0.01% w/v) for 1 min
- After treatments, the shoots were inserted into the hole in filter paper bridge in such a manner that two to three nodes were immersed in medium
- The base of the culture tubes were covered with aluminium foil till the root induction started

Media used for rooting of shoots

- MS/ ½MS/ ¼MS + sucrose (1%) + IBA (1-25 µM)
- MS/ ½MS/ ¼MS + sucrose (1%) + NAA (1-25 µM)

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^{-1} provided by cool white fluorescent lights (Philips India Ltd., India).

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

3.2 SECONDARY METABOLITE STUDIES

In secondary metabolite studies, first experiment was to assess the biosynthetic potential of *in vivo* as well as *in vitro* regenerated shoots and they were compared. After this qualitative analysis, quantification of lupeol and rutin was done and the shoots were also treated with different elicitors to observe their effects on these compounds.

3.2.1 Glasswares and Plasticwares

- Beakers (50 ml)
- Eppendorff tubes (1 and 2 ml)
- Evaporation dishes (80 × 45 mm)
- Funnels (50 mm)
- Measuring cylinders (10, 25 and 50 ml)
- Pipettes (0.1, 1, 2, 5 and 10 ml)

3.2.2 Instruments

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

3.2.3 Qualitative Analysis of Shoots

In vivo and *in vitro* regenerated shoots regenerated from different media were assessed for their ability to synthesize secondary metabolites by HPTLC fingerprinting and chemicals, reagents and methodologies used are 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 *H. indicus* were collected from Green house in the Botanical Garden, thoroughly washed with running tap water, wrapped in filter paper and kept in oven for drying at 40 ºC.

Nodes from the same plants were inoculated in optimized medium i.e. MS + sucrose (3%) + BA (10 µM) + Kn (5 µM). After eight weeks the shoots were subcultured on another medium fortified with IAA (2 µM) for another eight weeks. Hence *in vitro* shoots from two different media were used:

(1) MS + sucrose (3%) + BA (10 µM) + Kn (5 µM) (MS-1, eight weeks old shoots) and

(2) MS + sucrose (3%) + IAA (2 µM) (MS-2, sixteen weeks old shoots)

Shoots from both the media were harvested, washed with D/W and kept in oven (40 ºC) for drying. All the dried shoots were kept in zip lock pouch and stored in freezer (0 ºC) until use.

3.2.3.3 Extract preparation

1 gm dried *in vivo* and *in vitro* shoots were powdered using mortar-pestle and extracted using hexane, ethyl acetate and methanol. Hexane (10 ml) was added to the powder and refluxed in water bath for 10 min at 50 ºC. Then the extract was filtered and the procedure was repeated twice using same solvent. The extract was collected in evaporation dish and the same procedure was repeated three times for ethyl acetate and methanol. All the extracts were kept overnight at room temperature (28 ºC) for evaporation and next day the marc was reconstituted to 1 ml using the same solvent and stored in freezer (0 ºC) till the time of analysis.

3.2.3.4 Mobile phases and development of TLC plates

The extracts of *in vivo* and *in vitro* shoots 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 automatic sample applicator and the following mobile phases were used for different extracts:

- Hexane extract- toluene:methanol (9:1 v/v)
- Ethyl acetate extract- toluene:ethyl acetate:formic acid (6.5:2.5:1 v/v)
- Methanol extract- toluene:ethyl acetate:formic acid (6.8:2.5:0.7 v/v)

Freshly prepared mobile phases were poured in CAMAG twin through glass chamber and saturated for 45 min and then the plates were allowed to develop in respective mobile phase. After development, they were air dried and these plates were derivatized with anisaldehyde-sulfuric acid reagent and scanned using CAMAG TLC scanner 3 linked to winCATS software.

3.2.4 Standard Curve Preparation

- **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 made by spotting different concentrations in triplicates (band width 8 mm and distance between tracks 12 mm) on precoated TLC plates. They were developed in a mobile phase toluene:methanol (9:1 v/v), dried and derivatized with anisaldehyde-sulfuric acid solution followed by scanning at 525 nm using the CAMAG TLC scanner.

- **Standard curve of rutin**

1 mg/ml stock solution of rutin was prepared in methanol and was serially diluted to give different concentrations ranging from 100 to 500 ng. A calibration curve was made by spotting different concentrations in triplicates (band width 8 mm and distance between tracks 12 mm) on precoated TLC plates. They were developed in a mobile phase ethyl acetate:butanol:formic acid:water (5:3:1:1 v/v), dried and then scanned at 254 nm using the CAMAG TLC scanner.

3.2.5 Elicitation of Lupeol and Rutin

3.2.5.1 Chemicals

Yeast extract (SRL, Mumbai, India), salicylic acid (SRL, Mumbai, India) and methyl jasmonate (Sigma-Aldrich, USA).

3.2.5.2 Elicitor stock preparation

In elicitation experiment, YE was directly added into the media whereas for SA and MJ stocks were 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):**
  - 1 mM stock was prepared and stored in amber colour bottle in refrigerator (4 ºC)
  - 138.12 mg of SA was dissolved in 100 ml D/W
  - SA was added in µM concentrations as per the following formula:

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

  - 25, 50, 100 and 200 µM concentrations were fed to the shoot cultures

- **Methyl jasmonate (MJ) (M.W.: 224.30):**
  - MJ was filter sterilized and hence this was added at the time of inoculation in the medium. Two stocks (100 and 200 mM) were prepared in ethanol and 25 µl from these working stocks were added to 50 ml medium which gave final concentration of 50 and 100 µM respectively. Following formula was used for calculating the volume taken from working stock:

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

  - 50 and 100 µM concentrations were used in the experiment

Elicitation was done in 150 ml flask containing 50 ml liquid medium and the flasks were kept on shaker at 100 rpm in the culture room in same conditions mentioned above.

3.2.5.3 Shoot culture establishment for elicitor feeding

Nodal explant of *H. indicus* established in static optimised medium for shoot culture i.e. MS + sucrose (3%) + BA (10 µM) + Kn (5 µM) were harvested after eight weeks and transferred to liquid medium with same PGR concentration. Control as well as treated shoots was harvested at weekly intervals (till three weeks), washed thoroughly

using D/W followed by drying in an oven at 40 ºC. The biomass of these shoots in terms of fresh and dry weights was recorded for all the samples of different concentrations.

For YE and SA elicitation, medium devoid of elicitor was served as a control whereas medium fortified with 25 µl ethanol served as control for elicitation using MJ.

3.2.5.4 Lupeol and rutin quantification

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. To quantify lupeol, the plates were developed in toluene:methanol (9:1 v/v), air dried and derivatized using anisaldehyde-sulfuric acid reagent followed by scanning at 525 nm. Whereas for rutin quantification, the plates were developed in ethyl acetate:butanol:formic acid:water (5:3:1:1 v/v) air dried and scanned at 254 nm. The respective peak areas were recorded for both the compounds in all the samples.

3.2.6 Gene Expression Analysis for Rutin Biosynthesis in Shoots

3.2.6.1 Plant material

Shoots from *in vivo* plant, *in vitro* control medium (MS-1) and three weeks old elicited samples from YE (50 mg/l) and SA (50 µM) were utilized for gene expression studies.

3.2.6.2 Plasticwares

All were procured from Tarsons

- Measuring cylinders
- Micro centrifuge tubes (1.5 ml)
- PCR tubes

3.2.6.3 Instruments

- Centrifuge (Universal 320 R, Hettich, Massachusetts, USA)
- Dry bath incubator (Yorco, Mumbai, India)
- Electrophoretic unit (Medox Biotech India Pvt. Ltd., Chennai, India)
- Gel doc (Syngene, Bangalore, India)
- Micropipette (Fischer Scientific Ltd., Mumbai, India)

- Thermal cycler (Veriti Applied Biosystems, Mumbai, India)
- Vortex mixer (Bio-lab, Bangalore, India)

3.2.6.4 Chemicals and reagents

- Agarose (Invitrogen, CA, USA)
- Chloroform (Himedia, Mumbai, India)
- EtBr (Thermo Scientific, Mumbai, India)
- Ethanol
- iScript reverse transcription supermix for RT-PCR (Bio-Rad, Mumbai, India)
- Isopropyl alcohol
- PCR master mix (Himedia, Mumbai, India)
- RNA loading dye (Thermo Scientific, Mumbai, India)
- RNase Away reagent (Ambion, life technologies, CA, USA)
- TAE buffer (1 l, 50X, pH 8.0)

Tris base-	242 gm
Glacial acetic acid-	57.1 ml
EDTA-	100 ml (0.5 M)

This solution was sterilized by autoclaving and stored at 4 ºC.

- TE buffer (100 ml, 1X, pH 8.0)

Tris base-	1 ml (1 M)
EDTA-	200 µl (0.5 M)

3.2.6.5 RNA isolation

RNA isolation was performed using RNeasy plant mini kit (Qiagen, New Delhi, India) and according to the manufacturer's protocol with slight modification as given below. All the plasticwares and glasswares were first rinsed with chloroform to destroy any RNase present.

- 100 mg samples were macerated using mortar-pastel in ice-box and 500 µl buffer RLC was added and vigorously mixed
- The lysates were transferred to a QIAshredder spin column and centrifuged at 10000 rpm for 2 min (4 ºC)
- The supernatants of the flow-through were transferred to a new tube followed by addition of 0.5 volume of absolute ethanol and mixed properly
- The samples were transferred to an RNeasy Mini spin column and centrifuged at 10000 rpm for 1 min (4 ºC) and the remaining flow-through was discarded

- 500 µl buffer RW1 was added to the columns followed by centrifugation at 10000 rpm for 1 min (4 ºC) and the flow-through was discarded
- 500 µl buffer RPE was added to the column and centrifuged at 10000 rpm for 1 min (4 ºC). The step was repeated and the flow-through was discarded
- The column were then kept open and centrifuged at 10000 rpm for 1 min (4 ºC) for air drying
- 10 µl RNase-free water was added to a spin column membrane and kept in dry bath for 5 min at 60 ºC. Centrifugation was done at 10000 rpm for 1 min (4 ºC).
- The above step was repeated and the eluted RNAs were transferred to a new tube and stored at -20 ºC until use

3.2.6.6 Agarose gel electrophoresis

1% agarose gel was prepared by dissolving 1 gm agarose in 100 ml of 0.5X TAE buffer and heated until the complete dissolution of agarose. EtBr was mixed when the gel was cooled down to approximately 45 ºC. The comb was inserted into the gel cast tray and gel was poured into it and left for solidification. After 30 to 45 min comb was removed and gel was kept into the buffer tank filled with 0.5X TAE buffer. RNA samples were mixed with bromophenol blue tracking dye and added into the wells. Gel was run on 50 volts for 30 to 60 min. RNA was documented with the help of gel documentation system. Similarly for PCR products, 2% gel was prepared and above procedure was followed.

3.2.6.7 Qualitative and quantitative analysis of RNA

The isolated RNAs were qualitatively analyzed by electrophoresis on 1% agarose gel in 0.5X TAE followed by staining with EtBr and documented using gel documentation unit. O.D. at 230, 260 and 280 nm was recorded for all samples in UV spectrophotometer and the quantity was calculated using following formula:

RNA concentration (µg/ml) = O.D. 260 × dilution factor × 40 µg/ml

Ratio of 260/280 was calculated for RNA purity and 260/230 ratio for contaminants.

3.2.6.8 cDNA synthesis from isolated RNA

cDNA synthesis was done using 1 µg each of total RNA sample and mixed with anchored oligo-dT in a 20 µl system using iScript reverse transcription supermix cDNA

synthesis kit (Bio-Rad, Mumbai, India). The reaction mixture and PCR conditions are given in Table 4.

Table 4: Reaction mixture and PCR conditions for cDNA synthesis

Components	Volume (µl)
mRNA	10
Master mix	4
RNase free water	6
Total	20

Steps	Temperature (ºC)	Time
Pre-cycle denaturation	25	5 min
Denaturation	42	30 min
Primer annealing	85	5 min

3.2.6.9 cDNA analysis using β-actin amplification

Each of the cDNA preparations was then amplified for 35 cycles in a thermal cycler with β-actin specific primers (forward: 5'-GGCTGGATTTGCTGGAGATGATGC-3' and reverse: 5'-CAATTTCTCGCTCTGCTGAGGTGG-3'.) by taking 2 µl of cDNA in a 12.50 µl system. Reaction mixture and PCR conditions for the same are given in Table 5.

The PCR amplified products were analyzed by electrophoresis on 2% agarose gel in 0.5X TAE followed by staining with EtBr and viewed in gel doc.

Table 5: Reaction mixture and PCR conditions for β-actin amplification

Component	Volume (µl)
cDNA	2
Master mix	6.25
Forward primer (10 pmol)	1
Reverse primer (10 pmol)	1
Nuclease free water	2.25
Total	12.50

Steps	Temperature (ºC)	Time	No. of cycles
Pre-cycle denaturation	95	5 min	1
Denaturation	95	15 sec	35
Primer annealing	56	30 sec	
Primer extension	72	30 sec	
Final extension	72	2 min	1

3.2.6.10 Gene expression analysis

The first step in gene expression study was to synthesize primers for genes of flavonoid pathway i.e. Flavonoid 3',5'-hydroxylase (*F3'5'H*) and flavonoid 3'-monooxygenase (*F3'H*) (Fig. 6).

- These genes were searched in KEGG pathway for flavonoids
- Nucleotide sequences were downloaded in FASTA format followed by multiple sequence alignment using CLUSTALW
- Similarly genes were compared using 'Run BLAST' tool of NCBI and the plants with more than 95% indent were selected
- Sequences for primer synthesis were selected from the regions with the highest sequence similarity
- Primer sequences were selected from exonic regions highlighted using 'highlight sequence feature' in NCBI
- Primers were verified using different parameters like optimum Tm, GC content and self-complementarities using Oligo Calc software (vs. 3.27)
- Sequences of forward and reverse primers were finally checked using Primer3 software (vs 0.4.0)

Two pairs of each primer set for both the genes were synthesized (Xcelris Labs Ltd., Ahmedabad).

The cDNAs were checked for gene expression analysis using different primers of respective genes and PCR mixture and conditions are given in Table 6. The PCR amplified products were analyzed by electrophoresis on 2% agarose gel in 0.5X TAE followed by staining with EtBr and viewed in gel doc.

Table 6: Reaction mixture and PCR conditions for gene amplification

Component	Volume (µl)
cDNA	2
Master mix	6.25
Forward primer (10 pmol)	1
Reverse primer (10 pmol)	1
Nuclease free water	2.25
Total	12.50

Steps	Temperature (ºC)	Time	No. of cycles
Pre-cycle denaturation	95	5 min	1
Denaturation	95	15 sec	
Primer annealing	56	30 sec	35
Primer extension	72	30 sec	
Final extension	72	5 min	1

3.3 STATISTICAL ANALYSIS

Total 12 replicates were used for regeneration studies whereas 5 repicates were used for biomass analysis and 3 replicates for secondary metabolites quantifications. Means and standard errors (SE) were calculated for each experiments followed by ANOVA ($\alpha = 0.05\%$). The significant means were further analysed using Tukey's test for regeneration from leaf and *in vitro* nodes, rooting of shoots and secondary metabolite studies. Whereas Duncan's multiple range test was used for regeneration from *in vivo* nodal explant. MS Excel, XLSTAT v2017.02 and GraphPad Prism 6.01 were used for statistical analysis.