3. MATERIALS AND METHODS

In vitro propagation protocols in *Adenia hondala* and *Baliospermum montanum* were carried out through standard techniques at the Plant Tissue Culture Laboratory, Department of Botany, Sacred Heart College, Thevara, during the period 2000 - 2003.

3.1 Source and Choice of Plant materials

3.1.1 *Adenia hondala* (Gaertn.) de Wilde

One year old plants of *Adenia hondala* grown and maintained in the greenhouse, Department of Botany, Sacred Heart College, Thevara, Kochi, were used as the source of explants. Excised shoot tips, nodal segments (3-4 cm) tender leaves (2nd or 3rd) from the apex and internodal segments (2.0-3.0 cm) were used as explants.

3.1.2 *Baliospermum montanum* Muell. Arg.

One year old plants of *Baliospermum montanum* grown and maintained in greenhouse, Department of Botany, Sacred Heart College, Thevara, Kochi, were used as the source of explants. Excised shoot tips, nodal segments (3-4 cm) tender leaves (2nd or 3rd) from the apex and internodal segments (2-3 cm) were used as explants.

3.2 Establishment of Cultures

3.2.1 Glassware and instruments

Glassware Borosil grade consisting of beakers, conical flasks, petridishes, standard flasks, pipettes, measuring cylinder etc were used. Explants were cultured in wide neck Erlenmeyer's conical flask (100 ml, 150 ml, 50 ml), culture tubes (150 mm long and 25 mm diameter) and bottles (20 cm³, 60 cm³).

All the glasswares in use were regularly cleaned to ensure no contamination. Glasswares were thoroughly washed in running water using 'Labolene' detergent and finally rinsed in distilled water. All the cleaned glasswares were placed in hot air
oven for 2hrs at 100°C to make them dry. Once used culture vessels were first autoclaved and washed following the earlier procedure.

Accessories used like scalpels, forceps, spatula, needles, holder were made of stainless steel and were sterilized every time before use. The equipment and instruments used in the laboratory included Laminar Air Flow Cabinet (Yorko horizontal, Scientific industries), Hot Air Oven (Kumar), Refrigerator (Allwyn), Distillation unit (Magnum) Electronic Monopan balance (Sartorius), Digital electronic pH Meter (Systronics), Autoclave, Hotplate, Microscope (Labex), Rotary Microtome (ERMA), Steel racks, Cooling centrifuge (C24-Remi) etc.

3.2.2 Basal Media

MS medium (Murashige and Skoog, 1962), B5 medium (Gamborg et al., 1968), SH medium (Schenk-Hildebrandt, 1972), White’s medium (White, 1963), Nitsch’s medium (Nitsch and Nitsch, 1969) with different constituents and concentrations were used (Table. 1).

3.2.3 Preparation of Culture Media

Standard procedures were followed for the preparation of media. The media strength, plant growth regulators and other supplements used is depicted in the table 2 and 3. Stock solutions of major and minor salts, vitamins and growth regulators were prepared by dissolving required quantity of chemicals in distilled water. The stock solutions of nutrients were stored in amber coloured bottles and of vitamins in tightly lidded volumetric flasks, both under chilled conditions in refrigerator. The stock solutions of nutrients were prepared fresh every month and vitamins and growth regulators every fortnight.

The glassware used for the preparation of the media were washed with dilute ‘Labolene’ and rinsed with double glass-distilled water. The stock solution in the required quantity were pipetted out into a standard flask containing distilled water. 3% sucrose and 100 mg L-1 myo-inositol, as per the specification of the media
(Table 1) were added and dissolved in the media. All plant growth regulators; additives for the different combinations were added before making up the media to the required volume. With an electronic digital pH meter, the pH of the medium was appropriated between 5.6 and 5.8 using 0.1N NaOH or 0.1 N HCl. 0.8% w/v difco bacterial grade agar (in the case of solid medium) was then added to the medium and mixed well.

The solution was then heated on a hot plate and stirred thoroughly, till the agar dissolved uniformly. The medium was then poured into pre-sterilized culture vessels. 15 ml was taken in culture tubes (25mm X 150 mm) and 50 ml was taken in flasks (250 ml). The culture tubes containing the medium were plugged tightly with non-absorbent cotton wool plugs and the flasks with autoclavable lids and sealed tight with sealing film.

3.2.4 Sterilization

The sterilization of the culture medium was carried out in an autoclave for 15 minutes at 121° C and 15 Lbs pressure. After sterilization, the culture tubes and flasks were stored in an air-conditioned culture room until further use.

All metal and glass instruments and other accessories used in the inoculation cabinet were wrapped in aluminium foil and sterilized in an autoclave at 1.06 kg cm² pressure for 15-20 minutes at a temperature of 121° C. Scalpels, scissors, forceps etc. used were again dipped in alcohol and flamed on a spirit lamp at the time of use.

3.2.5 Explant Preparations and Surface Sterilization

The explants collected from the source plants were coarsely trimmed to a size of 3 cm. and washed in running tap water for 5 minutes followed by washing in distilled water with a few drops of 'Labolene'. After washing these explants in double distilled water, they were immersed in 0.1 % Mercuric Chloride solution and incubated for 5 - 20 minutes. The liquid was stirred by swirling to give proper contact of chemical to the explant. The treated explants were washed three to five
times in sterile distilled water. After a final wash in sterile distilled water, the explants were spread on the pre-sterilized petridishes lined with sterile blotting paper inside a laminar airflow chamber. They were then trimmed finely to the appropriate size (1-1.5 cm)

3.2.6 Inoculation and Incubation

Single nodes (1.0 - 2.0 cm) leaf disc (1cm²) and internodal segments (1.0 - 2.0cms) were dissected out and all the inoculation operations were carried out under strict aseptic condition inside a Laminar Air Flow chamber, which was made sterile by the incessant exposure of germicidal U.V. rays for half an hour before use. All operations were carried out using pre-sterilized instruments and glassware. Explants were then aseptically introduced into culture vessels. In order to curtail contamination during drying and inoculation, only a few explants were treated at a time.

The cultures were maintained in the culture room at 25±1°C at under 16/8 hr photoperiod of 2000 lux light intensity provided by white fluorescent tubes with 55%-60% relative humidity or in darkness, as per the treatment. 8 replicates were made in each treatment and all trials were carried out three repeats.

3.2.7 Subculture

Cultures were regularly transferred to fresh medium or regeneration medium as per requirement after every 30 days.

3.2.8 Experiments & Observations

All experiments were conducted in 8 replicates twice and data on number of shoots obtained through axillary bud proliferation as well as regeneration of adventitious shoots were analysed using standard ANOVA procedures and the difference between the means were compared using the Fischer’s least significant difference test (LSD).
3.3 Direct Regeneration

3.3.1 Explants

Excised shoot tips, nodal segments (3.0-4.0cm) tender leaves (2nd or 3rd) from the apex and internodal segments (2.0-3.0cm) were used as explants in the case of direct regeneration.

3.3.2 Culture medium

Murashige and Skoog media was used as basal medium for induction of shoot buds (Table 1). Basal media strength, different combinations and concentrations of growth regulators, cytokinins (BAP, Kn, 2iP) and auxins (NAA, IAA, 2,4-D) and additive supplementation were tested to see their effect on direct shoot induction and shoot bud elongation in Adenia hondala and Baliospermum montanum (Table 2,3).

3.3.3 Callus Proliferation Scoring

Observations on the amount of the callus proliferated (Scoring method ‘-‘) no callusing ‘+‘, meager (0.5-1.0 cm width of callus), ‘++‘ moderate (1.0-1.5 cm width of callus), ‘++++‘ intense (>1.5 cm width of callus) and the colour of the callus were recorded.

3.3.4 Data Presentation

The data pertaining to the percentage of cultures showing shoot induction response in terms of the number of shoots per culture and shoot length (cm) were recorded and analyzed statistically using completely randomized design.

3.4 Callogenesis

3.4.1 Explants

Excised nodal segments (3.0-4.0cm) tender leaves (2nd or 3rd) from the apex and internodal segments (2.0-3.0cm) were used as explants.
3.4.2 Culture medium

The culture medium used to induce callus consisted of MS basal salts and vitamins supplemented with auxin (2,4-D, NAA, IAA) alone or in combination with cytokinins (Kin, BAP or 2iP).

3.4.3 Subculture

The Calli were subcultured at 15-20 days interval for proliferation.

3.4.4 Data collection and Presentation

3.4.4.1 Callusing Frequency

Cultures were scored for callus induction at the end of the fourth week (30 days) Frequency of callus induction was computed as the ratio of the number of explants responding to that of total number of explants involved and was expressed as percentage as shown below.

\[
\text{Frequency of response} \, (\%) = \frac{\text{Number of explants responding}}{\text{Total no. of explants cultured}} \times 100
\]

3.4.4.2 Growth measurements

Growth rates were determined on a fresh weight basis during the initiation and growth phase by measuring the weight increase over two weeks subculture period on the medium tried.

The fresh weight as a measure of callus growth at an interval of 15 days were recorded. Moisture was removed from the sample callus by gently blotting with a filter paper and then transferred to a pre-weighted Aluminium foil for the determination of fresh weight.

3.4.4.3 Callus Index

Growth index was calculated by the following equation:

\[
\text{Growth index} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}}
\]
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3.4.4.4 Nature of Callus

Visual features of the callus developed were recorded and photographed.

3.5 Indirect Regeneration

3.5.1. Explants

Excised shoot tips, nodal segments (3.0-4.0 cm) tender leaves (2nd or 3rd) from the apex and internodal segments (2.0-3.0 cm) were used as explants for regeneration via callus. 500mg of green compact calli was used as explant for indirect regeneration.

3.5.2 Culture medium

MS media was used as basal medium for induction of shoot buds. Different combinations and concentrations of growth regulators cytokinins (BAP, Kn, 2iP) and auxins (NAA, IAA, 2,4-D) in 19 treatments were tested for direct shoot induction and shoot bud elongation. Additives were supplemented for shoot bud enhancement studies.

3.5.3 Data presentation

The data pertaining to the percentage of cultures showing shoot induction response in terms of the number of shoots per culture and shoot length (cm) after 45 days were recorded and analyzed statistically using completely randomized design.

3.6 Root Initiation

In vitro rooting was attempted on liquid MS medium supplemented with IBA, NAA and IAA. Full, half and quarter strength MS media were also tried. The shoots were cultured on filter paper bridges. The culture conditions were same as that of multiplication phase. Eight microshoots were used for each treatment. The percentage of shoots rooted, the number of roots formed and the root lengths were recorded. Data on the percentage frequency of root induction and the first order
interaction of various factors were statistically analysed by F-test at 5 % level of significance.

3. 7 Somatic Embryo Induction

3. 7. 1 Explants

Leaf explants and juvenile callus derived from internodal segments rachis were used as explants.

3. 7. 2 Culture medium

MS medium was used for somatic embryogenesis. The effects of plant growth regulators Kin, BAP, 2iP, IAA, NAA and 2,4-D were tested by incorporating them alone and in combinations. 15 treatments with different combinations and concentrations of these plant growth regulators, in 3 trials each of 8 replicates were tried for both direct and indirect somatic embryogenesis. To obtain enhancement of embryogenesis, additive supplementation was tried.

3. 7. 3 Culture Conditions

Cultures for somatic embryogenesis were maintained in a light regime consisting of 16h light at 25 ° ± 2 ° C temperature.

3. 8 Somatic Embryo Maturation

The effects of plant growth regulators - Kin, BAP, 2iP, IAA, NAA and 2,4-D - were tested by incorporating them alone and in combinations. 23 treatments with different combinations and concentrations of these plant growth regulators and additives in 3 trials each of 8 replicates were tried for somatic embryo maturation.

3. 8. 1 Carbon source

Different levels of sucrose (20, 30, 40, 50 and 60 gm/l) were used as carbon source for shoot induction.

3. 8. 2 Data collection and Presentation

Data were recorded as the mean of cultures initiating embryogenesis (induction of proembryo), mass and number of embryoids produced per cultures by
90 days of inoculation. The percentage of surviving cultures after 150 days and the number of embryoids, which survived, were also recorded.

3.8.3 Growth measurements

The size, length and breadth of somatic embryos formed were observed by placing them on a glass slide in which a graph paper was pasted and the slide was observed under the stereomicroscope and the number of squares were counted. One square was equivalent to 1 mm.

3.9 Hardening and Potting

The rooted plantlets were hardened for 60 days in liquid half strength basal medium on filter paper bridges. The plantlets that regenerated were thoroughly washed in tap water to remove sucrose and transferred to 6.5 cm diameter plastic cups (6.5cm diameter) containing planting substrates like sand: soilrite, soil: manure, sand: soil: farmyard manure and soilrite (Kelttec Energies Ltd., Banglore). The plantlets were irrigated with 10% MS on alternate days. For maintaining high humidity the plants were transferred to mini green house with a polythene roof. The survival percentage and the plantlet development in different substrates were monitored for 4 months before transplanting to the open garden soil.

3.10 Statistical Analysis

The results were analysed statistically using the analysis of variance (Windows excel 2000). When ANOVA showed treatment effects (p<0.05), the least significance difference (Fishers LSD) was applied to make comparison between means at the 5% level of significance and grouped into several classes.

3.11 Histology, Histochemistry and Histoenzymology

3.11.1 Collection and Fixation of Samples

To study in vitro ontogeny, samples were collected from organogenic, nonorganogenic, and embryogenic calli at an interval of 15-30 days. In vitro regenerated plant parts and in vivo explants were also collected. The collected
samples were fixed in FAA (Formalin: Acetic Acid: Alcohol) (O'Brien and Macully, 1981) to a minimum time of 48 hrs and preserved in 70% alcohol. They were dehydrated in a graded series of tertiary butyl alcohol and then infiltrated with and embedded in paraffin wax 56-58° C (Beryln and Miksche, 1976).

3. 11. 2 Sectioning
Serial sections of paraffin embedded materials were taken on a rotary microtome. Sections of 8 to 10 μm thickness were cut at transverse and longitudinal planes. Ribbons were spread in 2% Formalin solution on micro slides smeared with Haupt's adhesive.

Free hand sections of samples of 10 - 20 μM thickness were also used for histological studies. For histochemical and histoenzymological preparations, only fresh free hand sections were used.

3. 11. 3 Staining
The staining methods followed for general, histology, histochemistry and histoenzymology are summarized in Table 4. Controls for enzyme localization were prepared by boiling the sections in water 6 -10 minutes before incubation.

3. 11. 4 Photomicrography
Photomicrographs with transmitted light were taken on Carl Ziess photomicroscope with planochromatic objectives using appropriate filter combinations using ORWO ND%, 100 ASA colour negative film.

3. 11. 5 Presentation of Results
For general histology photographs were used to illustrate the structure and organization of cells, tissue and organs. For histochemistry and histoenzymology the intensity of localization of metabolites and enzymes were represented as visual grading-intense, moderate and meagre.
3.12 Biochemical Quantification

Total protein, soluble sugar and starch were quantified from in vivo samples (the source of explants) and samples collected during different in vitro developmental stages, following the procedures mentioned.

3.12.1 Sample Collection

Samples from organogenic, non-organogenic and embryogenic cultures were collected at an interval of 5 days from the day of inoculation. 500 mg. fresh samples were taken for the estimation of protein and 20 mg dry samples were used to estimate the soluble sugar and starch.

3.12.2 Estimation of Proteins

Total proteins were estimated following the method of Lowry et al. (1951). In this method protein was estimated in trichloroacetic acid precipitates, which had been treated, to remove contaminants (Khanna et al., 1969). Bovine serum albumin (Sigma) was used as standard. All determinations were carried out 3 times to permit standard evaluation.

3.12.3 Estimation of Soluble Sugars

Tissue samples were extracted with 5ml of methanol. The residue was extracted again with 5 ml of methanol and the combined extract was reduced to dryness. This was resuspended in 5ml of distilled water. An aliquot 0.1 to 0.5 ml of this solution was employed for the determination of soluble sugars by Anthrone Calorimetric method (Mc Cready et al., 1950).

3.12.4 Estimation of Starch

Starch was extracted from the residue of the sample after extracting the soluble sugar in methanol. This residue was boiled in water to extract the starch and estimated by I2 KI method (Mc Cready et al., 1950).
3.12.5 Presentation of Results

The quantities of metabolites in respective stages were estimated in mg/g samples. Results obtained were presented graphically, X-axis showing the days of culture and Y-axis showing mg/g of the specified constituent.

3.13 Quality analysis

3.13.1 Chromatographic Separation of Constituents

3.13.1.1 Sample Collection

25 g. powdered sample of in vivo and in vitro plant parts was macerated and refluxed with 100 ml 70 % ethanol for 30 min. The extract was filtered and the filtrate concentrated to 10 ml. (Daniel, 1990).

3.13.1.2 Thin Layer Chromatography

TLC was carried out to separate the plant constituents and to identify them as per the procedure suggested in the Ayurvedia Pharmacopeia of India, (Anonymous, 2001). Silica gel was used as the absorbant and the ethanol extracts of the plant samples were used for TLC. 5 microlitres of sample was applied on the plate using a capillary tube in a horizontal line about 2cms from the lower end. Spots were made at 1 to 2 cms distance. The plate was then transferred to a rectangular glass chamber saturated with the solvent system containing toluene: ethyl acetate (9:1). Care was taken to keep the level of solvent slightly below the level of spots. Plates were kept slanting on the walls of the chamber and the chamber was closed tightly with the lid. The solvent was allowed to run up to 2/3rd portion of the plate, after which, it was taken out and allowed to dry in air. The chamber was kept undisturbed till the run was complete.

3.13.1.3 Result Analysis

Detection of samples was done under UV (366 nm). The plates were exposed to iodine vapour. Rf value was calculated by spraying the plates with 50% methanolic - sulphuric acid reagent and heating the plate for 10 min at 110°C. The
colour of the bands was noted and the Rf values were calculated and compared (Sadasivam and Manickam, 1992). Photographs were also used to illustrate the results.

3.13.2 Phytochemical Tests.

50 g of samples were successively extracted with different solvents like, Hexane, Benzene and chloroform, ethyl alcohol and water and the different extracts were tested for Saponins, triterpenoids, alkaloids, steroids, reducing sugars, flavanoids and tannins following the procedure of Brinda et al. (1981).

3.13.2.1 Sample preparation

Air-dried tuber, two-months-old stem and leaves of in vivo plant, two month old stem and leaves of regenerated plants and callus derived from the stem were used as sample.

3.13.2.2 Extraction and Isolation of Saponins

50 g powdered sample was refluxed with 100 ml of 70 % ethanol for 30 min. The extract was filtered and the filtrate was concentrated to 10 ml and applied to silica gel TLC plates. The chromatograms were developed in chloroform: methanol: water (65: 50: 10). Saponins were visualized using vanillin-sulphuric spray reagent. The presence of saponins was indicted by blue violet or yellow spots on plants heated to 100°C for few minutes.

3.13.2.3 Data presentation

Rf values of the spots were calculated and compared. Colour of the spots was noted. Colour Photographs were taken.

3.14 Physiochemical Analysis

Physiochemical chemical studies were conducted to determine the quality of the callus and regenerated plant and compared it with the in vivo plant.
3.14.1 Sample Collection

Samples were collected from two-months-old *in vivo* plant and two-months-old regenerated plant for quality analysis.

3.14.2 Preparation of Sample

*In vivo* and *in vitro* extracts were prepared by macerating 5 gms of air-dried powdered sample with 100 ml solvent by continuous shaking for 6 hrs and allowed to stand still for 18 hrs. It was then filtered and 25ml filtrate was evaporated to dryness at 105°C in a tarred flat bottom dish and weighed.

3.14.3 Solvent Extractions

3.14.4 Determination of Alcohol Soluble Extractive

The extract of the samples was prepared as mentioned in procedure 3.14.2 using alcohol as the solvent.

3.14.5 Determination of Water Soluble Extractive

The extract of the samples was prepared as mentioned in procedure 3.14.2 using water as the solvent.

3.14.6 Determination of Ether Soluble Extractive

The extract of the samples was prepared as mentioned in procedure 3.14.2 using ether as the solvent.

3.14.7 Determination of chloroform Soluble Extractive

The extract of the samples was prepared as mentioned in procedure 3.14.2 using chloroform as the solvent.

3.15 Ash Tests

3.15.1 Determination of Total Ash

3 g of air dried powder sample was taken in a silica dish and incinerated at a temperature not exceeding 450°C until free carbon was obtained and weighed.
3.15.2 Determination of Acid Insoluble Ash.

The ash obtained in 3.15.1 was boiled with 25 ml hydrochloric acid for 5 minutes and the insoluble matter is collected in a gooch crucible or an ashless filter paper. It was then washed with hot water and ignited to content weight.

3.15.3 Determination of Water Soluble Ash.

The ash obtained in 3.15.1 is boiled with water the insoluble matter was collected in the gooch crucible or on ashless paper. It was then washed with hot water and ignited for 15 min at room temperatures not exceeding 450 °C. The weigh of the insoluble matter was subtracted from the weight of the ash; the difference in weight represents the water-soluble ash.

3.15.4 Presentation of Results

Results were presented as the percentage of the extractor ash obtained with reference to the air-dried samples.

3.16 Isozyme Studies

Isozyme analysis was carried out using Polyacrylamide Gel Electrophoresis following essentially, the method of Davis (1964) and using the buffer systems (Brewbaker et al., 1968). Nine percent gels of Cyanogum 41 (gelling agent electrophoresis grade Sigma USA) in 9:1 v/v of lithium hydroxide boric acid pH 8.1 and Tris citrate pH 8.2 were used for electrophoresis. Lithium borate buffer of pH 8.3 was used as the tank buffer in case of enzymes analysed. Electrophoresis was carried out at 6 to 10 °C and 150 volts for first 15 minutes and then at 200 volts for remaining period (3 to 4 mA) per tube till the indicator dye (Bromophenol blue) migrated to the end of the tube.

3.16.1 Sample Preparation

100 mg samples from the three different developmental stages, explant, non-organogenetic callus and embryogenic callus, were selected for isozyme analysis. Extracts were prepared by grinding the fresh samples in Tris citrate buffer at pH 7.8.
The extraction was carried out at 4°C. (Rao et al., 1992). The extracts were centrifuged in a cooling centrifuge and the supernatants were collected. Three to five drops of marker dye (Bromophenol blue prepared in concentrated sucrose solution) was added to the extract till bright violet colour was obtained.

3.16.2 Staining

For the isozymes, Acid Phosphatase, Peroxidases and Esterases staining procedures of Shaw and Prasad (1970) were carried out. After staining the gels were fixed in a mixture of ethanol: distilled water: acetic acid at the ratio 5: 5: 2.

3.16.3 Presentation of Results

In order to make definitive comparisons among the patterns obtained from different tissues, an Rf value was calculated for each band based upon the migration of the band relative to the front.

The numbering of bands followed the standard procedure that is the fastest (anodic) moving band being the first. The relative intensities of the bands were judged visually. The zymograms and photographs were used to illustrate and analyse the results. The Rf value for the bands were calculated as follows:

\[
Rf\ value = \frac{\text{Distance traveled by the band of Gel}}{\text{Distance traveled by indicator}}
\]