CHAPTER 2

MATERIALS AND METHODS

CONTENT OF CHAPTER 2: MATERIALS AND METHODS

2 MATERIALS AND METHODS

This chapter deals with the materials and methods used for the analytical and experimental studies on the selected samples.

BIOLOGICAL MATERIALS

The biological materials used for the present investigation are the three South Indian members of the family Phytolaccaceae, namely *Petiveria alliacea* L., *Phytolacca octandra* L. and *Rivina humilis* L. The family Phytolaccaceae is commonly known as Pokeweed family. All the members of the family are found to have some important medicinal uses. Plants collected from the natural habitat and those maintained in the Botanic garden of the Department of Botany, St. Teresa's college, Ernakulam, were used for different analyses in the study.

GLASSWARE AND CHEMICALS

All the glassware used for the analyses were of Borosil/Corning glass. They were first washed with detergent, then with tap water and finally rinsed in distilled water. If required, they were immersed in chromic acid for 24 hours and washed in tap water and rinsed in distilled water. They were drained and dried properly before use.

All the chemicals used in the analytical methods and reagents preparation were of analytical grade with maximum available purity supplied

by Hi-media (Bombay) E. Merck (Bombay) Loba chemie (Bombay), Sisco (Chennai) and Sigma (USA). RAPD primer kit was procured from Bio Gene Technologies USA. The PCR reagents and Taq polymerase were obtained from Finzyme (USA).

INSTRUMENTS USED

A number of instruments were used for conducting the work successfully. They are listed in table 2-1.

2.1 COLLECTION AND MAINTENANCE OF SAMPLES

The plant materials (samples) for the present study were collected from different localities of South India such as Munnar, Ootty, Kodaikanal, Trivandrum, Ernakulam and Muvattupuzha. All the collected plant materials were identified using standard floras (Hooker 1983 Gamble, 1967; Matthew 1999) and confirmed by comparing with authentic sheets in the herbaria in the Botanical Survey of India, Coimbatore, and Tropical Botanic Garden and Research Institute, Palode, Trivandrum. The name of the taxa and their locality of collection are given in table 2-2.

Table 2-1: List of Instruments Used

Name of Instruments	Make/Model
Light Microscope	Leitz, Germany
Trinocular photo microscope	Olympus, Germany
UV-VIS spectrophotometer - UV 2100	Schimadzu, Japan
High Speed centrifuge RC 5C	Sorwal Instrument USA
Micro centrifuge	Hitachi Germany
Weighing Balance	Metler, Switzerland
pH meter	Elico, India
Hot-air oven	Kemi, India
Microwave oven	Kenstar, India
Water bath	Siskin, Germany
Soxhlet apparatus	Borosil
Magnetic Stirrer	Kemi, India
Thermal cycler (PCR)	Perkin Elmer, USA
Vertical slab gen electrophoresis	Bio Rad, USA
Horizontal gel electrophoresis	Bio Rad, USA
Deep freezer	Blue Star
Refrigerator	Whirlpool, India
UV-illuminator	Genei Pvt. Ltd., India
Gel documentation system	Alpha Innotech Corp. USA
Distillation apparatus	Borosil
TLC Kit	Remi, India
Vortex Stirrer	Remi, India
Amino acid analyser (HPLC)	Shimadzu LC – 10 AS (Japan)
HPTLC System	CAMAG, Switzerland

Table 2-2: Locality of Taxa Under Study

Sl.No.	Name of Taxa	Locality
1	*Phytolacca octandra* L.	Munnar, Ootty, Kodaikanal
2	*Petiveria alliacea* L.	Trivandrum, Ernakulam, Muvattupuzha
3	*Rivina humilis* L.	Trivandrum, Ernakulam, Muvattupuzha

The materials for each and every aspect of the investigation were procured from the plants cultivated in the garden as well as from natural habitats. Fresh plant specimens were collected between 9.00 a.m. and 10.00 a.m. and kept in polythene bag and brought to the laboratory. The specimens were washed in running tap water to remove the adhered dirt and rinsed with distilled water before the analysis. The washed and air dried specimens were used for macroscopic and microscopic studies. Specimens collected from natural habitats and kept in ice gel were used for biochemical and molecular analyses.

2.2 BIOCHEMICAL STUDIES

2.2.1 ESTIMATION OF TOTAL CARBOHYDRATE

Phenol-Sulphuric acid method described by Dubois *et al.* (1956) was followed for the estimation of total carbohydrates.

MATERIALS

- 5% Phenol
- Concentrated sulphuric acid
- Standard glucose solution
- Acid alcohol reagent (80% ethyl alcohol in 0.1N per chloric acid)

PROCEDURE

The harvested fresh leaves were washed and blot dried. One gram leaf was homogenized in ice-cold acid alcohol reagent. The homogenate was centrifuged at 2500 rpm for 4 minutes. The supernatant was taken for assay.

Pipetted out 500 µl of adequately diluted sample into clean dry test tube and made up to 1 ml with water. One ml of Phenol solution was added to the test tube followed by 5 ml sulphuric acid. While adding sulphuric acid the tubes were kept in a water bath at 25-30^0 C for 20 minutes. The blank solution was prepared by taking 1 ml distilled water. The colour developed was read at 490 nm using a spectrophotometer.

2.2.2 ESTIMATION OF PHOTOSYNTHETIC PIGMENTS

The various chlorophyll pigments differ in their aliphatic side chains attached to the porphyrin nucleus. Higher plants contain chlorophyll *a* and chlorophyll *b*. In plants chlorophylls occur along with carotenoids. The quantity of each pigment was estimated spectrophotometrically by Arnon's (1949) method.

MATERIALS

- Acetone
- Distilled water

PROCEDURE

The fresh leaves were homogenized in 80% acetone (acetone and water in the ratio 8:2) in a mortar and pestle. The homogenate was centrifuged at 3000 rpm for 3 minutes. The supernatant was collected and the total volume was measured.

5 ml of the properly diluted extract was taken to measure the absorbance at 480 nm, 510 nm, 645 nm, 652 nm and 663 nm wavelengths using UV-VIS double beam spectrophotometer.

The amount of chlorophyll *a*, chlorophyll *b*, carotenoids and total chlorophyll were calculated according to the method of Arnon (1949). The formulae for estimating the photosynthetic pigments are as follows:

Total chlorophyll	=	D (652)x(1000/345) x V (/1000) x W
Chlorophyll *a*	=	(12.7xD663)-(2.69xD645)x(V/1000) x W
Chlorophyll *b*	=	(22.9xD645)-(4.68xD663)x(V/1000) x W
Carotenoids	=	7.6 (D480-1.49xD510)x(V/1000) x W

The ratio of chlorophyll *a* to chlorophyll *b* of all the samples was calculated to analyse the photosynthetic efficiency.

2.2.3 THIN LAYER CHROMATOGRAPHY ANALYSIS OF PHOTOSYNTHETIC PIGMENTS

The photosynthetic pigments were analysed by Thin Layer Chromatography (TLC) to study the different components.

MATERIALS

- Silica gel G
- Glass plates
- Extraction medium (petroleum benzene, acetone and chloroform in the ratio 3:1:1)
- TLC solvent system (petroleum benzene, acetone, chloroform and water in the ratio 7:2:1:1)

PROCEDURE

Clean, dry, grease free glass plates were wiped with alcohol and arranged on the applicator pad. The thickness of the applicator (spreader) was adjusted to 250 microns and placed it on the last plate of the applicator pad. Twenty five gm silica gel G was mixed with 50 ml water to make it a slurry. The mixture was shaken vigorously for thorough mixing. The slurry was quickly transferred to the applicatior and was drawn down smoothly over the plates up to the end of last plate. The coated plates were dried in a hot air oven at 80^0 C for 1 hour.

Five gm fresh leaf was washed, blot dried and homogenized in a mortar and pestle using the extraction medium. The extract was centrifuged at 1500 rpm for 2 min. and the supernatant was collected. The concentrated

extract was loaded on the activated silica gel G coated plates with the help of micro pipette.

The loaded sample was allowed to dry and the plates were developed in the running solvent system. The developed chromatogram was analysed and the Rf values of the different components separated were calculated.

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

2.2.4 ASSAY OF SPECIFIC ACTIVITY OF ENZYME AMYLASE IN GERMINATING SEEDS

Specific activity of the enzyme amylase was assayed by estimating the amount of substrate utilized and product formed in unit time. Amylase enzyme is catalysing the formation of maltose which on hydrolysis yield glucose. The enzyme activity was assayed according to Plummer (1988). The amount of glucose produced by hydrolysis of maltose was estimated by using 3, 5 – dinitro salicylate reagent.

MATERIALS

- 0.1M Phosphate buffer PH 6.7
- Dinitro salicylate reagent (DNS)
- 1% starch solution (substrate)
- 0.2M Citrate phosphate buffer (PH 4.5)
- Sodium hydroxide solution (2 mol/litre)
- Sodium chloride (10g/litre)

PROCEDURE

Seeds, soaked for 24 hrs, were homogenized in ice cold phosphate buffer of PH 7.0 and centrifuged in cold centrifuge tubes. The supernatant containing enzyme was collected and was kept in refrigerator.

1.5 ml of 1% starch solution was buffered by mixing with 1.5 ml of 0.2M citrate phosphate buffer (pH 4.5). 0.5 ml sodium chloride was added to this. 1 ml of the properly diluted enzyme was added to the reaction mixture and incubated for 3 minutes at 30^0 C. The reaction was arrested by adding 0.5 ml of 2 mol/litre sodium hydroxide solution. The reaction mixture was centrifuged at 2500 rpm for 5 minutes and the supernatant was collected for estimation. 100µl of the supernatant was taken in a test tube and made up to 1 ml by adding distilled water. 3 ml DNS reagent was added to this and mixed well. The solution was heated using water bath (100^0 C) for 5 minutes. 1 ml 40% potassium sodium tartrate solution was added to this warm solution and mixed well. The solution was cooled down to room temperature and the reddish brown colour developed was read at 540 nm in a spectrophotometer against a blank.

The amount of enzyme in 1ml extract was calculated according to the method of Lowry *et al*. (1951).

2.2.5 ESTIMATION OF TOTAL SOLUBLE PROTEIN

The method developed by Lowry *et. al.* (1951) is sensitive to give a moderately constant value and hence largely followed. The enzyme activity was expressed in terms of amount of product formed by the enzyme action in 1 minute at 30^0 C.

MATERIALS

- 1% Copper sulphate solution (A)
- 2% Sodium potassium tartrate solution (B)
- 2% Sodium carbonate in 0.1NaOH (C)
- Alkaline copper reagent (solution A, B and C in the ratio 1:1:98)
- Folin - Ciocalteau Reagent (diluted with equal volume of distilled water immediately before use)
- Protein standard [Bovine Serum Albumin (BSA)]
- 0.1 M Phosphate buffer of pH 7.4 (extraction buffer)
- 100% cold acetone.

PROCEDURE

The soluble protein was extracted in 0.1 M Phosphate buffer of pH 7.4. One gram fresh leaf was harvested from the plant and homogenized in the extraction buffer (pH 7.4) using mortar and pestle. The homogenate was centrifuged at 5000 rpm for 5 minutes. The supernatant was taken for assay. The pigment contents were removed by using cold acetone (100%). The precipitated protein was harvested by centrifugation at 3000 rpm for 3 minutes and dissolved in extraction buffer.

100 µl of adequately diluted sample was taken in clean dry test tube and made the volume up to 1 ml using distilled water. A test tube with 1 ml

of water and reagents served as the blank. Alkaline - copper reagent (4.5 ml) was added into each tube and mixed well immediately using Vortex stirrer. The solution was allowed to stand for 10 minutes. 0.5 ml Folin - Ciocalteau reagent was added and thoroughly mixed using stirrer and incubated at room temperature in the dark for 30 minutes. The blue colour developed by the reaction mixture was measured at 660 nm in a double beam spectrophotometer. The amount of protein was estimated using Bovine Serum Albumin (BSA) as standard.

2.2.6 ANALYSIS OF POLYPEPTIDE PROFILE BY SDS-PAGE

Analysis of polypeptides was carried out by Sodium Dodecyl Sulphate - Poly Acrylamide Gel Electrophoresis (SDS - PAGE). Vertical discontinuous electrophoresis was done to separate total proteins.

EXTRACTION OF TOTAL PROTEIN

500mg leaf tissue of each plant was weighed and ground with liquid nitrogen and 1ml extraction buffer (Table 2-3). The homogenate was centrifuged at 10000 rpm for 30 minutes at 4^0C. The supernatant was collected and stored at -20^0C. Sample buffer and samples were taken in the ratio 1:1 for gel loading.

Table 2-3: Extraction Buffer (100 ml)

No	Ingredients	Quantity
1	Tris(50mM)	0.606 gm
2	KCl(50mM)	0.373 gm
3	PMSF(1mM)	0.020 gm
4	EDTA(5mM)	0.190 gm
5	PVP (1%)	1.000 gm

The amount of protein was quantified by the method of Lowery *et al.* and compared with BSA standard.

CASTING OF POLYACRYLAMIDE GELS

The poly acrylamide gels were casted by mixing the required amounts of stock solutions. The details of stock solutions are recorded in Table 2-4.

SEPARATING GEL

Two slab gel plates were assembled in a casting mode using 1.5 mm thick spacers. Required amounts of stock solutions (Table 2-5) were mixed thoroughly except per sulphate and TEMED. The mixture was degassed in vacuum flask followed by addition of ammonium per sulphate and TEMED. The mixture was then gently swirled to mix. The gel solution after proper mixing was pipetted into the glass sandwich to a level of 4 cm from the top. A water layer of about 2 mm thick was applied over the solutions before polymerisation of the gel solution to make the gel surface uniform as evident

from the sharp water gel interface visible after polymerisation. The water was poured off after polymerisation and 1 ml of running gel overlay solution was applied on the gel surface.

Table 2-4: Stock Solutions for PAGE

Sl. No	Solutions	Ingredients	Quantity
1	Solution A Tris Buffer 1.5 M (pH 8.8)	Tris-HCl H_2O	18.17 gm 100 ml
2	Solution B Tris Buffer 0.5 M (pH 6.8)	Tris-HCl H_2O	3.03 gm 50 ml
3	Solution C Acrylamide : Bis Acrylamide Solution	Acrylamide Bis-Acrylamide H_2O	29.0 gm 1.0 gm 100 ml
4	Solution D Ammonium per sulphate (APS) (10%)	Ammonium per sulphate H_2O	0.1 gm 10 ml
5	Solution E 10% Sodium dodecyl sulphate (SDS)	SDS H_2O	10 gm 100 ml
6	Running Buffer (pH 8.3)	Tris Glycine SDS H_2O	7.25 gm 34.56 gm 24 gm 2.4 lit.
7	Fixative (10%) TCA	TCA Methanol H_2O	20 gm 80 ml 200 ml
8	Staining solution (0.25% Commassie brilliant blue solution)	Commassie brilliant blue R 250 Methanol Acetic acid H_2O	0.25 gm 45 ml 10 ml 45 ml
9	Destaining solution	Methanol Acetic acid H_2O	200 ml 70 ml 230 ml

STACKING GEL

Measured amount of stock solutions were mixed for stacking gels (Table 2-5). The mixture was degassed prior to the addition of ammonium per sulphate and TEMED. The running gel overlay solution was poured off and stacking gel solution was gently poured into the sandwich over the polymerised separating gel. A comb was inserted in to the sandwich to make wells in the stacking gel at regular intervals, for the application of the extracts. The stacking gel was allowed to polymerise for half an hour.

Table 2-5: Stock Solutions Required for Preparing Gels

Solutions	Separating gel	Stacking gel
Solution – A	7.5 ml	-
Solution – B	-	0.75 ml
Solution – C	12.0 ml	1.00 ml
Solution – D	0.3 ml	0.06 ml
Solution – E	0.3 ml	0.06 ml
TEMED	0.012 ml	0.006 ml
Distilled Water	9.9 ml	4.12 ml

ELECTROPHORESIS OF ENZYME EXTRACT

Poly acrylamide gel electrophoresis of the enzyme extracts was carried out using Bio Rad vertical dual slab gel electrophoresis system. The polymerised gel was fitted to the electrophoresis apparatus after removing the lower spacer of the separating gel. The comb was removed straight up from the stacking gel. Lower tank was filled with running buffer up to a certain

mark in such a way that the lower free surface of separating gel remained immersed in running buffer. The air bubbles at the interface of lower free end of separating gel and lower tank buffer coaxed away with a pipette.

The enzyme extracts were loaded in the wells of the stacking gel. Volume of each sample enzyme was adjusted in such a way that a fixed amount of enzyme extract, applied for different species for electrophoresis for a particular enzyme system, contained equal amount of proteins. Care was taken to avoid mixing of the samples with the reservoir buffer and cross contamination of samples in adjacent wells. Constant electric current supplied for 1.5 mm thick gel was 200 Volts. Initially it was 150 V, and increased to 200 V when dye front entered into the separating gel. With the completion of the electrophoresis the gels were fixed and stained as per staining protocol.

FIXATION OF DISSOCIATED POLYPEPTIDES

After electrophoresis the gels were released from glass plates, washed thrice in distilled water and immersed in fixative (Table 2-4) for over night in the staining box.

STAINING AND DESTAINING

The fixative was discarded and the staining solution (0.25% Commassie Brilliant Blue R-250) was poured over the gels and were shaken gently in a shaker for 45 min., then staining mixture was poured off and

destained in fast destaining solution. Stock solutions for all the reagents of SDS-PAGE are given in Table 2-4.

MOLECULAR WEIGHT MARKERS

The molecular weight of the dissociated polypeptides were determined by co-electrophoresis of molecular weight markers for SDS – PAGE (Promaga Corporation, USA), comprising Phosphorylase-B (97.4 KDa), BSA (66.2 KDa), Glutamate dehydrogenase (55.0 KDa), Ovalbumin (42.7 KDa), Aldolase (40.0 KDa), Carbonic anhydrase (31.0 KDa), Soyabean Trypsin Inhibitor (21.5 KDa) and Lysozyme (14.4 KDa).

2.2.7 ESTIMATION OF FREE AMINO ACIDS

Ninhydrin (tri keto hydrindene hydrate) method of Plummer (1988) was followed for the estimation of amino acids. Ninhydrin reacted with α-amino acids at a pH between 4 and 8 to give a purple coloured compound except for proline and hydroxy proline.

MATERIALS

- 80% Acid alcohol reagent – 80 ml of 100% ethyl alcohol was mixed with 20 ml 0.1 N Perchloric acid (PCA)
- 0.2% Ninhydrin in Acetone
- 50% Ethyl alcohol
- Standard Glycine solution

PROCEDURE

Young fresh leaves were harvested and washed thoroughly in running tap water and air dried. 1 gm leaf was homogenized in acid alcohol reagent

(80 per cent ethyl alcohol in 01.N perchloric acid). The homogenate was centrifuged at 2500 rpm for 3 minutes. The supernatant was collected for estimation.

100 µl of the adequately diluted sample was taken in a clean dry test tube and made the volume up to 1 ml. A test tube with 1 ml water served as the blank. Ninhydrin reagent (2ml) was added to each test tube, mixed well and heated in a boiling water bath for ten minutes. 3ml of 50% ethyl alcohol was added to the solution after cooling down to room temperature. The purple colour developed by the reaction was measured at 570 nm in the UV-VIS double beam spectrophotometer. The amount of amino acid was estimated using glycine as standard.

2.2.8 ESTIMATION OF PROTEIN AMINO ACIDS BY HPLC

Amino acids of the samples were estimated using the specially designed HPLC amino acid analyser (Model Schimadzu, LC – 10 AS Japan).

PROCEDURE

The samples for analysis of amino acids other than Tryptophan were prepared by acid hydrolysis. 100mg plant sample was hydrolysed using 10 ml 6N HCl at 110^0C for 24 hours in heat sealed evacuated tubes.

The acid hydrolysed samples were filtered through a Whatmann No.42 filter paper after breaking open the tubes. The filtrate was flash evaporated to

remove HCl and made up to a definite volume using Sodium citrate caprylic acid buffer of pH 2.2 (8 ml sodium perchloric acid ml and 50 ml n – caprylic acid). The buffered samples were again filtered using 0.45 µM membrane filter. 20µM of the filtrate of each sample was injected through the sample loop at 60^0C.

The HPLC column was packed with a strongly acidic cation exchange resin made up of Styrene divinyl benzene copolymer with sulfinic group. The column used was ISC – 07/S/1504/Na with a length of 19 cm and diameter of 5 mm. The guard column prevented the entry of impurities into the column. The amino acids were separated inside the column. The post column derivatisation of the components was carried out with O–phthalaldehyde and hypochlorite of individual amino acids at the reaction coil. These components were detected by spectrofluorometer.

The amino acids were eluted step-wise starting from acidic amino acids, then neutral and finally alkaline amino acids. The chromatogram was recorded by a data processor. Total nitrogen of the sample was estimated by Kjeldahl method and the amount of amino acids was expressed in gm amino acid per 16gN.

Tryptophan was estimated separately by colorimetric method after alkali hydrolysis. 200mg sample was hydrolysed with 100 ml of 5% NaOH at 110^0C for 24 hours in a sealed tube filled with pure nitrogen. The hydrolysate

was neutralised with 6N HCl. It was then filtered through Whatmann No.1 filter paper.

PROCEDURE OF TRYPTOPHAN ESTIMATION

0.1ml of 2.5% sucrose and 0.1 ml of 0.6% thioglycolic acid were successively added to test tubes containing 4 ml of 50% sulphuric acid and kept for 5 minutes in water bath at 45-50^{0}C and cooled. 0.1 ml of the alkali hydrolysed sample filtrate was added to this after cooling down to room temperature. The volume was made up to 5 ml with 0.1N HCl and mixed well. The absorbance at 500 nm wavelength was measured after 5 minutes using spectrophotometer. Commercial sample of tryptophan was used as standard.

2.3 PHARMACOGNOSTIC STUDIES

2.3.1 PRELIMINARY PHARMACOGNOSTIC EVALUATION

Pharmacognostic studies were conducted in plant specimens collected from natural habitats. Detailed macroscopic and microscopic analyses were conducted. Powder analysis, organoleptic characterization, phytochemical screening and *in vitro* bioactivity assay were also carried out.

MACROSCOPIC EVALUATION

Fresh specimens collected were used for the macroscopic studies. The external morphologic characters and structural peculiarities were studied.

Fresh plants were collected for the study of morphological characters. The foliar and floral features were studied morphologically and morphometrically. The foliar features included the shape, size and colour of lamina petiole, leaf base, leaf margin, leaf apex, venation and phyllotaxy. The floral characters such as type of inflorescence and features of flower, fruit and seed were also studied. Fresh materials were used for the study. Ten samples of each plant from 3 different localities were analysed qualitatively and quantitatively.

QUANTITATIVE MICROSCOPY

Microscopic determinations such as palisade ratio, stomatal index, vein-islet number and veinlet termination number were done according to the method of Evans (2002).

PALISADE RATIO

Palisade ratio is the average number of palisade cells beneath each upper epidermal cell.

PROCEDURE

Leaf pieces of about 2mm square were cleared by boiling with chloral hydrate solution using water bath till the pigments got bleached out. The bleached pieces were washed with distilled water, stained using 1% aquous safranin and mounted in glycerine. A group of four contiguous epidermal cells were selected and the palisade cells were counted separately. The

palisade cells in each group were counted. The average of the palisade cells of four epidermal cells was calculated (Hickey 1973; Evans 2002).

STOMATAL INDEX

The percentage proportion of the ultimate divisions of the epidermis of a leaf which have been converted into stomata is termed the stomatal index (Evans 2002)

$$Stomatal\ index\ (SI) = \frac{\mathrm{S\ x\ 100}}{\mathrm{E + S}}\ \mathrm{x\ 100}$$

where S = number of stomata per unit

E = number of ordinary epidermal cells in the same unit area

PROCEDURE

Epidermal peelings of pieces of leaf other than extreme margin or midrib were treated with alcohol to remove pigments. They were washed in distilled water and stained using 1% aquous safranin and mounted in glycerine. Counts were made of the number of epidermal cells and stomata within the square grid of the micrometer. The two guard cells and ostiole together considered as one unit. Successive adjacent fields were examined and average was calculated (Wallis 1985; Evans 2002).

VEIN-ISLET AND VEINLET TERMINATION NUMBERS

The number of vein-islets calculated from four continuous square millimetre in the central part of the lamina midway between the midrib and

the margin was termed as the vein-islet number. Veinlet termination number is the ultimate free termination of a veinlet in unit area of leaf.

PROCEDURE

The leaves were treated with the bleaching solution (Equal parts of IN KOH and H_2O_2) at 35-40^{0}C in a water bath till the pigments got bleached out. The bleached leaves were washed in distilled water, stained with safranin and mounted in glycerine. The vein-islets and veinlet terminations were observed under microscope. The area completely enclosed by veins was counted and the average vein-islet number per millimetre square area was calculated. The veinlet termination number was calculated by counting the ultimate free termination of a veinlet per millimetre square area of leaf (Melville 1976; Evans 2002).

POWDER ANALYSIS

The microscopic study of the plant powder was carried out after staining with phloroglucinol. A pinch of the plant powder was taken in a clean glass slide and 2 drops of phloroglucinol - ethanol reagent (1% solution in 90% ethanol) was added to the powder. It was allowed to dry for 1 minute. Then 2 drops of concentrated hydrochloric acid (HCl) was added. The stained acidified powder was fastened with a square cover glass and observed under light microscope (Evans 2002). Size of different powder components was measured using micrometer.

ORGANOLEPTIC STUDY

Fresh plant specimens were collected washed under running tap water and air dried in shade. The shade-dried samples were powdered. The whole plant powder characters such as colour, odour, texture and other peculiarities were evaluated.

2.3.2 PHYTOCHEMICAL SCREENING

QUALITATIVE ANALYSIS OF PHYTOCONSTITUENTS

The dried plant powder was used to analyse colour reactions with different selected chemicals. A pinch of dry powder was treated with different chemical reagents separately in clean dry test tubes. Each mixture was kept for 10 minutes and the colour developed was compared to the colour identification chart of Royal Botanic Garden, Edinburgh. The characteristic colour developed for each reagent was recorded.

FLUORESCENCE ANALYSIS

The crude plant powder was treated with different reagents in test tubes and kept undisturbed for 10 min. Each solution was then loaded on an activated silica gel G layered micro slide and observed the fluorescence under visible light and UV light (360 nm).

ASSAY OF ACTIVE INGREDIENTS

ESTIMATION OF TOTAL PHENOLIC COMPOUNDS

Phenolics were estimated according to the method of Harbone (1973).

Materials

- 80% Methanol
- Folin-ciocalteau reagent
- $Na_2 CO_3$

Procedure

1 gm leaf was chopped into fine pieces and refluxed in 80% methanol for 10 min and homogenized in the same solution. The homogenate was centrifuged at 6000 rpm for 5 minutes. The supernatant was collected. 0.05 ml of adequately diluted supernatant was taken in a test tube and made the volume to 3 ml using 80% methanol. A test tube with 3 ml 80% methanol served as blank. 0.5 ml folin - ciocalteau reagent was added to this solution and mixed well. The mixture was kept undisturbed for 3 minutes. 2.0 ml 20% Na_2CO_3 solution was added to the mixture and heated in a boiling water-bath for 5 min. The solution was cooled down to room temperature and centrifuged at 2000 rpm for 3 minutes to remove the white precipitate formed. The blue colour developed was measured at 650 nm in a UV-VIS double beam spectrophotometer.

ESTIMATION OF TOTAL ALKALOIDS

Materials

- 10% acetic acid is prepared in ethanol (10 ml acetic acid is mixed with 90 ml 100% ethyl alcohol)
- 100% Ammonium hydroxide (NH_4OH) solution
- 1% NH_4OH solution

Procedure

Alkaloids were analysed according to the method of Harbone (1973). 10 gm dried tissue was extracted with 10% acetic acid in ethanol solution for 10 hours. The extract was concentrated to one quarter of the original volume, by evaporation. The alkaloids present in the concentrated extract was precipitated by drop-wise addition of concentrated Ammonium hydroxide (NH_4 OH) . The precipitate was collected by centrifugation at 2000 rpm for 3 minutes. The precipitated alkaloids were washed with 1% NH_4OH by centrifugation. The residue was dissolved in a few drops of methanol. It was then evaporated to dryness and weighed.

$$Percentage\ of\ alkaloid = \frac{\text{Final weight of the flask} - \text{initial weight of the flask}}{\text{Weight of the powdered sample}}$$

ESTIMATION OF FLAVONOIDS

Materials

- 100 gm dried and powdered plant material
- Methyl alcohol
- 7% Hydrochloric acid
- Ethyl acetate
- Distilled water

Procedure

The flavonoids were estimated by the method (Harbone 1973). 100 gm dried and powdered plant material was used for solvent extraction. The extraction was carried out in soxhlet apparatus using the solvent methanol. The material was extracted for 24 hours at 65^0 C.

The extract in the bottom flask was collected and concentrated to dryness. 50 ml H_2O was added to the residue and hydrolysed in a water bath for 1 hour by adding 7% HCl. To the cooled hydrolysed extract ethyl acetate was added. The ethyl acetate soluble faction was separated using a separating funnel. The lower aquous layer was discarded and the upper ethyl acetate soluble fraction was collected. It was evaporated to dryness and weighed.

$$Percentage\ of\ flavonoids = \frac{\text{Final weight of the flask} - \text{initial weight of the flask}}{\text{Weight of the powdered sample}}$$

ESTIMATION OF RESIN

Materials

- Acetone
- Water bath
- Dilute HCl

Procedure

5 gm powdered plant material was refluxed in 50 ml acetone for 1 hour. It was then filtered and the filtrate obtained was concentrated in a water bath (100^0 C) for one hour. The concentrated sample was acidified with dilute Hydrochloric acid. The White precipitate thus obtained was centrifuged, washed with water and dried to a constant weight. The average of five readings was taken to calculate the percentage of resin (Harbone 1973).

$$Percentage\ of\ resin = \frac{\text{Final weight of the flask} - \text{initial weight of the flask}}{\text{Weight of the powdered sample}}$$

ESTIMATION OF TANNIN

Materials

- Plant powder
- Indigo – carmine reagent
- $KMnO_4$ (0.1N)
- NaCl
- 2% gelatine

Procedure

Tannin content was estimated by indigocarmine method (Daniel 1991). 5 gm plant powder was refluxed in 400 ml water for 30 minutes. The sample was then filtered and made up the volume to 500 ml litre using distilled water. 10 ml of this diluted filtrate was taken in a conical flask and 25ml of indigocarmine reagent (0.5% indigocarmine solution was prepared by dissolving 5 gm indigocarmine in 27.7ml conc. H_2SO_4 and made up to 1 litre using distilled water) was added and made up the volume to 800 ml using distilled water. It was titrated against 0.1N $KMnO_4$ taken in the burette. The volume of $KMnO_4$ titrated for the appearance of yellowish green colour was noted. The blank was calculated by titrating with tannin removed plant material. NaCl and 2% gelatine were used to remove tannin. 50 ml of the filtrate was mixed with 50 ml NaCl acid solution (5% H_2SO_4 solution saturated with NaCl) and 25 ml 2% gelatine solution. The mixture was shaken vigorously and the coagulated brown solid particles were removed by filtration. This tannin removed filtrate was titrated against 0.1N $KMnO_4$ to obtain blank value.

$$\boldsymbol{Percentage\ of\ tannin = (a - b) \times KMnO_4\ factor \times 1000}$$

Where 'a' and 'b' are volumes of $KMnO_4$ titrated against sample and blank respectively. $KMnO_4$ factor = 0.00415 g for 0.1N $KMnO_4$.

ESTIMATION OF SAPONIN

The total saponin in the plant was estimated according to the method of Harbone (1973).

Materials

- 90% ethyl alcohol
- Petroleum ether
- Chloroform
- Ethyl acetate
- Methanol
- Acetone

Procedure

5 gm dry sample powder was extracted with 25 ml 90% ethyl alcohol by refluxing for 2 hours. Extract was made solvent free by evaporation on water bath. The concentrated extract was treated with 25 ml petroleum ether (60-80^0 C) by refluxing for half an hour. The extract was cooled and the solvent was removed by decanting. The same process was repeated with 25 ml each of chloroform and ethyl acetate. The cooled solvent was poured off. The soft extract was dissolved in methanol (25 ml), filtered and concentrated. This methanolic extract was added drop by drop to 25 ml acetone while stirring, to precipitate the glycosides. The precipitate was filtered and concentrated in a water bath (100^{0}C) and the final weight was noted.

$$Percentage\ of\ saponin = \frac{\text{Final weight of the flask} - \text{initial weight of the flask}}{\text{Weight of the powdered sample}}$$

HPTLC ANALYSIS OF PHYTOCOMPOUNDS

The versatile separation technique, High Performance Thin Layer Chromatography (HPTLC), was conducted for the analysis of phytochemical compounds in the three samples studied. CAMAG HPTLC System (Switzerland) equipped with Linomat 5 Automatic Sample Spotter, CAMAG glass twin trough chamber and CAMAG TLC scanner 3 with software win CATS 1.30 planar chromatography manager was used to analyse the sample.

PROCEDURE

Pre-coated silica gel $60F_{254}$ TLC plates of thickness 0.2 mm and size 10x10 cm (Merck, Darmstadt, Germany) were used for the HPTLC analysis. The plates were activated by placing in oven at 110-120^{0}C for 30 minutes prior to sample loading. The sample prepared in methanol by solvent extraction method was evaporated to dryness and dissolved in known volume of the solvent. The standard (reference) also dissolved in the same solvent to ensure comparable distribution at starting zone.

Automatic sample applicator was used for loading the sample. 5 µl sample was loaded at the starting zone (6 mm bands with 12mm spacing between two tracks). The mobile phase was standardized by trial and error method. (Solvent system used for gallic acid was chloroform, ethyl acetate and acetone in the ratio 50:50:1. Solvent system used for benzoic acid was benzene and acetone in the ratio 80:20 containing 1% formic acid. The solvent system used for sterol was n-hexane and acetone in the ratio 24:2).

As soon as the development process was completed the plates were removed from the chamber and dried to remove the mobile phase completely. The developed plates were visualised under UV and scanned at their λ max. The Rf values of the compounds were recorded.

2.3.3 IN VITRO BIO ACTIVITY ASSAY

In order to understand the basis of traditional system of medicine and to find out new therapeutic activities, the plant extracts were subjected to *in vitro* bio activity studies on bacterial and fungal cultures.

PROCEDURE

Plants were collected from natural habitats. Roots and shoots were collected separately and washed thoroughly in running tap water and air dried in shades for one week. The dried samples were powdered and extracted the active principles by solvent extraction method.

The powdered plant materials were loaded in the soxhlet apparatus and exhaustively extracted for 7 hours with n-hexane, chloroform and methyl alcohol (methanol). The plant material taken in the soxhlet apparatus was dried well to remove the traces of the first solvent, before extracting with another solvent.

The extracts were harvested from the round bottom flask of the soxhlet apparatus and concentrated using rotary vacuum evaporator. The

concentrated residue was carefully packed in small clean bottles and stored at room temperature. A known volume of the extract was taken in a pre-weighed petridish. The weight of the petridish along with the extract was taken. The extract was allowed to dry in reduced pressure and ambient temperature. After complete drying, the weight was measured and the yield (concentration) of the content was calculated

ANTIBACTERIAL SCREENING

Antibacterial activity of the plant extract was tested against the Gram-positive and Gram-negative bacteria by the disc agar diffusion method. The bacteria were grown on Mueller-Hinton agar media (pH 7.3). Agar media were poured into the plates to a uniform depth of 5 mm and allowed to solidify. The microbial suspensions at $5x10^6$ cfu ml^{-1} were streaked over the surface of media using a sterile cotton swab to ensure confluent growth of the organism. The discs (6 mm in diameter) used were Whatmann No. 1 paper discs. 10µl aliquots of the extracts in DMSO were spotted on filter paper discs. The discs were then aseptically applied to the surface of agar plates at well-spaced intervals. The plates were incubated at 37^0C for 24 hours and the growth inhibition zones, including disc diameters were measured. Control discs, impregnated with 10 µl of the solvent DMSO and streptomycin (2 µg per disc) were used alongside the test discs in each experiment.

ANTIFUNGAL SCREENING

For the screening of antifungal properties, the fungi were obtained from MTCC, cultured in modified Sabouraud's agar. Suspensions at $5x10^6$ cfu ml^{-1} were used. 10 µl of extract (33.3% v/v) in DMSO, was impregnated on discs. 10 µl of the solvent DMSO and fluconazole (2µg per disc) were used as controls. The growth inhibition zone including disc diameters were measured.

Bacterial and fungal strains used for the analysis

The microbial strains for the antimicrobial screening were obtained as the Microbial Type Culture Collection (MTCC) from the Institute of Microbial Technology, Chandigarh (India). The following strains were used for the screening of antibacterial and antifungal activities.

Gram-positive bacteria :- *Staphylococcus aureus* (MTCC 96), *Bacillus cereus* (MTCC 430), *B. subtilis* (MTCC 441),

Gram-negative bacteria :- Serratia marcescens (MTCC 97), Pseudomonas fluorescens (MTCC 103), P. aeruginosa (MTCC 741), Klebsiella pneumoniae (MTCC 109), Proteus vulgaris (MTCC 426), Escherichia coli (MTCC 443), Salmonella typhi (MTCC 733)

Fungi :- *Candida albicans* (MTCC 227), *C. albicans* (MTCC 1637), *C. albicans* (MTCC 3017), *C. glabrata* (MTCC 3019).

2.4 ANALYSIS OF PHENOTYPIC VARIATION AMONG ACCESSIONS

The extend of morphological variability of the different accessions of the three samples collected from different localities in South India were investigated and statistically analysed.

A total of 90 specimens were collected from 9 localities of South India (Table 2.6). Each specimen was scored for 12 morphometric characters. Karl Pearson's correlation coefficient was calculated to test for association between pairs of morphometric characters of the accessions. The differences for morphological characters among samples were analysed by performing separate one-way analysis of variance (ANOVA). Correlation and ANOVA analyses were performed using SPSS Windows version 17.

Table 2-6 : Details of Sample Collection from Different Locations

Sl.No.	Name of Plant	Locality Name	Sample Size
1	*Phytolacca octandra* L.	Munnar Kodaikanal Ootty	10 10 10
2	*Petiveria alliacea* L.	Muvattupuzha Ernakulam Trivandrum	10 10 10
3	*Rivina humilis* L.	Muvattupuzha Ernakulam Trivandrum	10 10 10
Total	**3**	**9**	**90**

2.5 ANALYSIS OF GENETIC DIVERSITY AMONG ACCESSION

The accessions of the three plants of the family Phytolaccaceae (*Phytolacca octandra* L., *Petiveria alliacea* L. & *Rivina humilis* L.) were subjected to RAPD analysis with twenty primers (Table 2-7).

Table 2-7: Oligonucleotide 10 Mer Primers Used In RAPD

Primer	Primer Sequence (5'–3')
BG-A01	AGTCAGCCAC
BG-A02	CAGCACCCAC
BG-A03	AGCCAGCGAA
BG-A04	CAGGCCCTTC
BG-A05	GGAGGAAGGG
BG-A06	GGTGGTTGGG
BG-A07	CCCAACACCC
BG-C01	CCCAAGGTCC
BG-C03	CCAGATGCAC
BG-C04	GTGACATGCC
BG-C06	AAGACCCCTC
BG-C08	TCACCACGGT
BG-C10	CACCAGGTGA
BG-C14	TGCGGCTGAG
BG-D01	ACCGCGAAGG
BG-D03	GTCGCCGTCA
BG-D06	ACCTGAACGG
BG-D08	GTGTGCCCCA
BG-D10	GGTCTACACC
BG-D15	CATCCGTGCT

2.5.1 GENOMIC DNA EXTRACTION

Genomic DNA was extracted from tender leaves by CTAB method. 2.0 gm leaf of each sample was washed in distilled water and wiped with sterile filter papers. It was frozen in liquid nitrogen and ground to fine powder in mortar and pestle, mixed with 8 ml of CTAB extraction buffer with 2-β mercaptoethanol pH 8.0 (Table 2-8).

Table 2-8: Stock Solutions For Genomic DNA Extraction

Solutions	Composition	Amount
Tris Buffer pH 8	Tris 1 M H_2O	12.11 gm 100 ml
EDTA	Na_2 EDTA H_2O	18.61 gm 100 ml
CTAB Extraction Buffer pH 8.0 Stored at room temperature	CTAB 2% w/v Tris Buffer 100 mM Na_2 EDTA 20 mM PVP 1% NaCl 1.4 M H_2O	2 gm 10 ml 4 ml 1 gm 8.2 gm 100 ml
CTAB – NaCl Solution	CTAB 10% 0.7 M NaCl H_2O	2.5 gm 1.02 gm 25 ml
High Salt TE pH 8.0 Stored at room temperature	Tris Buffer 10 mM Na_2 EDTA 0.1 mM NaCl 1 M H_2O	1 ml 20 ul 5.85 gm 100 ml
CTAB Precipitation Solution pH 8.0 Stored at room temperature	CTAB 1% w/v Tris Buffer 50 mM Na_2 EDTA 10 mM H_2O	1 gm 5 ml 2 ml 100 ml
TE Buffer	Tris Buffer 10 mM Na_2 EDTA 1 M H_2O	1 ml 0.2 ml 100 ml
Sodium Acetate	Sodium Acetate 3 M H_2O	40.8 gm 100 ml

Mixtures were incubated at 65^0C for two hours in a water bath with gentle shaking at regular intervals. Equal volume of chloroform was added to

this and mixed well to make an emulsion, followed by centrifugation at 10,000 rpm for 5mins at 4^0C. To the upper aqueous phase, collected in a corex tube, 1/10 volume of CTAB / NaCl solution was added. The mixture was gently shaken followed by the addition of equal volume of chloroform.

The mixture was centrifuged at 10,000 rpm for 5 minutes at 4^0C. The upper aqueous phase was collected and added double volume of CTAB precipitation buffer (Table 2-8), mixed gently, sealed with parafilm and kept at 37^0C for overnight incubation in water bath.

Next day the mixture was centrifuged at 8,000 rpm for 10 minutes at 4^0C. Pellet containing nucleic acid was dissolved in 1 ml high salt TE buffer (Table 2-8). The solution was transferred to a fresh tube and 1ml isopropanol was added gently and kept at -20^0C for 30 minutes to precipitate nucleic acids. Nucleic acids were recovered as pellets by centrifugation at 8000 rpm for 10 minutes at 4^0C. The pellets were washed in 80% ethanol, and re-suspended in 0.5 ml TE, pH 8.0 (Table 2-8).

The solution was transferred to a fresh Eppendorf tube. RNA was removed from the isolated nucleic acids by incubating the solution with 3 µl RNAase A (Sigma) and the solution was incubated for 2 hours at 37^0C.

0.5 ml chloroform was added to this solution and centrifuged at 10,000 rpm for 5 minutes at 4^0C, and the supernatant was collected. This step was repeated twice. To the supernatant double volume of absolute alcohol and

50 µl 3 M solution of sodium acetate was added and kept for over night incubation at -20^0C.

The pellet was collected by centrifuging the solution at 12,000 rpm for 15 minutes at 4^0C, washed with 70% ethanol, air dried and re suspended in 100 µl 1X TE buffer pH 8.0.

2.5.2 RAPD- PCR ANALYSIS

RAPD assay was carried out in 25 µl reaction mixture containing 2.5 µl 10X amplification buffer (100 mM Tris HCl pH-8 at 25^0C, 15 mM $MgCl_2$, 500 mM KCl and 1.0% Triton X-100), 0.5 µl of dNTP mixture (10 mM each in 50 µl), 1.0 U of Taq DNA polymerase (Finzyme, Finland), 15 pmoles (1.2 µl) of 10-mer primer (Bio Gene Technologies Inc, USA) and 50 ng of genomic DNA. The reaction mixture was overlaid with 20 µl of mineral oil in order to avoid evaporation.

Amplification was performed in DNA Thermal Cycler (Perkin Elmer, USA). The sequential steps involved, 1 cycle of 2 min at 95^0 C, 2 min at 35^0 C and 2 min at 72^0 C followed by 39 cycles of 1 min at 93^0 C, 1 min at 36^0 C and 2 min at 72^0 C. The last cycle was followed by 7 min extension at 72^0 C.

2.5.3 AGAROSE GEL ELECTROPHORESIS

The amplified products (25 µl) were subjected to electrophoresis in a horizontal gel apparatus (Bio-Rad, USA) using 1.2% agarose gel (containing

0.5 mg/ml ethidium bromide) in 1XTBE buffer pH 8.0 (Table 2-9) at 100 volts for approximately 2.5 hours. Agarose powder (2.4 gm) (Gibco-BRL) was mixed with 200 ml of 1XTBE and melted in a microwave oven and mixed with ethidium bromide (Table 2-9). Gels were casted in the form of 0.5 cm thick, horizontal slab. Amplification products were electrophoresed with 5 µl of 10X loading buffer (Table 2-9). Amplified products were co-electrophoresed with pGM as a DNA marker.

Table 2-9: Stock Solutions for Agarose Gel Electrophoresis

Solutions	Compositions	Amount
TBE Buffer (10 X) pH 8	Tris Base Boric Acid Na_2 EDTA 0.5 mM H_2O	21.6 gm 11 gm 8 ml 100 ml
Gel Loading Buffer (10x)	Bromo Phenol Blue 0.25 % Xylene Cyanole 0.25 % Sucrose 40% (w/v) H_2O	250 mg 250 mg 40 gm 100 ml
Ethidium Bromide	Ethidium Bromide H_2O	1.0 gm 100 ml

2.5.4 ANALYSIS OF AMPLIFICATION PROFILE

Amplification profile was compared with each other and bands of DNA fragment were scored as present (1) or absent (0). The data for all the 20 primers were used to estimate the similarity on the basis of the number of shared amplification products (Nei and Li, 1979). A dendrogram based on similarity coefficient was generated by using the unweighted pair group method arithmetic means (UPGMA).

2.6 DATA ANALYSIS

2.6.1 CORRELATION

In order to study the relationship between the phenotypic characters among the different accessions of the three plants under study correlation coefficient was computed applying Karl Pearson's Coefficient of Correlation (r).

$$r = \frac{\sum(X - \bar{X})(Y - \bar{Y})}{\sqrt{\sum(X - \bar{X})^2 \sum(Y - \bar{Y})^2}}$$

where X and Y are the two variables under consideration.

2.6.2 ANALYSIS OF VARIANCE (ANOVA)

The variations in phenotypic characters of accessions of the three genus and the inter-plants variations in biochemical characters were studied by breaking down the total variances of a variable into additive components which may be attributed to regional factors and genus respectively, by applying the statistical method of analysis of variance (ANOVA).

The difference between the means is reflected in the value of the variance of the distribution of the sample means.

$$\sum_{j}^{k}\sum_{i}^{n_j}(Y_{ji} - \bar{Y})^2 = \sum_{j}^{k}\sum_{i}^{n_j}(Y_{ji} - \bar{Y}_j)^2 + \sum_{j}^{k} n_j (\bar{Y}_j - \bar{Y})^2$$

Total variation in Y	=	Sum of squares between groups	+	Sum of squares with groups

This expression shows how the total sum of squared deviations in Y (in all groups taken together) is partitioned into two parts: one part of the total variation of Y is due to the difference between the means (accessions or genus) and the other part is due to chance.

The significance of the difference between the means of the populations, $\mu_{1,}\mu_{2,}\mu_{3}$ is tested with the null hypothesis

H_0: $\mu_1=\mu_2=\mu_3$

Against the alternative hypothesis

H_1: μ_j not all equal.

If the three means are the same, that is, if the null hypothesis is true, the three populations may be considered as one large population with mean $\mu(\mu_1=\mu_2=\mu_3)$ and standard deviation σ, that is,

$$Y \sim N(\mu\sigma)$$

And the three samples may be considered as samples drawn from this one large population.

In order to study significance of the difference between the sample means, F ratio is applied. It may be noted that the two estimates are

independent, so that their ratio has an F distribution with v_1=k-1 and v_2=N-k degrees of freedom:

$$F^* = \frac{\frac{\sum_{j=1}^{k} n_j \, (\bar{Y}_j - \bar{Y}_J)^2}{k-1}}{\frac{\sum_j^k \sum_i^{n_j} (Y_{ji} - \bar{Y}_J)^2}{N-k}}$$

$$where\ n_j = size\ of\ the\ j^{th} sample$$

$$N = \sum_{j=1}^{k} n_j = size\ of\ the\ 'pooled'(enlarged\ sample)$$

$$k = number\ of\ samples$$

The variance ratio may be shown schematically as

$$\mathrm{F}^* = \frac{\text{estimated variance from 'between' the means variation}}{\text{estimated variance from 'within' the samples variation}}$$

When the sample means are not equal the estimated variance from 'between'-the-means differences will be large and hence the variance ratio F^* will become large.

The observed F^* variance ratio is compared with the theoretical values of F (with chosen level of significance, e.g., the 5 per cent level), which is found from the F-table with v_1 = (k-1) and v_2 = (N-k) degrees of freedom. The theoretical (or critical) value of F is the value of F that defines the critical region of the test at the chosen level of significance.

If $F^{*}>F$ the null hypothesis is rejected, i.e., it is accepted that the difference between means is significant. From this evidence, it is inferred that the populations from which the samples are drawn, do differ.

For the analysis of variance and estimation of correlations, SPSS Windows Version 17 is used. Computer programme used for genetic data analysis were POPGENE (Ver 1.0; Yeh and Boyle, 1998) and PCO3D (Ver 1.2; Adams). Chemiimager 4000I Ver 4.04 (Alpha Innotech Corporation, USA) was used to document and analyse, protein and RAPD gels.

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