CHAPTER - 2

MATERIALS & METHODS

MATERIALS AND METHODS

1. PLANT MATERIAL

The experimental work incorporated in the present thesis has been carried out on two leguminous tree species *viz.*,

(i) *Dalbergia sissoo* Roxb.

Family : Leguminosae

Sub family : Papilionaceae

(ii) *Albizzia lebbeck* Benth.

Family : Leguminosae

Sub family : Mimosae

About fifty year old plants of *D.sissoo* and *A.lebbeck* were selected from the M.S. University Arboratum and other localities. Fertile twigs of each plant were collected, Herbarium sheets were prepared and maintained in the departmental Herbarium. Explants *viz.*, axillary buds, stem segments and leaves of both these plants were collected for the experimental work. Bark and leaf samples were also collected for their chemical analysis.

2. CHEMICALS

The chemicals used in the preparation of media as well as for chemical analysis were of Analar grade and were obtained from Sisco Laboratories India, British Drug House and E.Merck, England. Fine chemicals including quercetin were obtained from Sigma Chemicals, U.S.A.

3. CHEMICAL ANALYSIS

Healthy stem-bark and leaves from *D.sissoo* and *A.lebbeck* were collected, washed under running tap water and dried in an oven at 60° C. Such an air dried, moisture free material was finely powdered separately in a grinder and stored in glass bottles.

(i) Extraction of flavonoid/s

The powders of stem-bark/ leaves of both these plants were used for the extraction of flavonoids-quercetin in particular. About 40 gm of powder of each sample was taken in a soxhlet, mixed with 200 ml of aqueous methanol (80%) and extracted for 48h continuously on a water bath at 50°C. After completion of extraction,the solvent was distilled off

and the extract was concentrated to dryness on a water bath. To the viscous mass which remained in the flask, few ml. of water was added and water soluble compounds were filtered out. The filtrate was hydrolysed with HCl (7%) by keeping it in a water-bath. Ethylacetate soluble aglycones were separated from the water soluble glycoflavones with the help of a separating funnel and subsequently concentrated.

(ii) Identification of flavonoid/s

The ethylacetate soluble fraction was concentrated and subjected to paper chromatography on Whatman No.1 paper in the form of a band. They were run in acetic acid : water ; 30 : 70 solvent system in a chromatographic chamber from where the chromatograms were taken out, dried and ascertained by their colour under UV light and by spraying on them with 10% sodium carbonate. Identification of the compound was done by co-chromatography of the sample with authentic sample of quercetin. Presence of quercetin was confirmed by Gas chromatography carried out at R&D section of I.P.C.L.

(iii) Quantification of flavonoid/s

Quantification of the flavonoid was done according to the procedures described by Block and Durrum (1958). The concentrated aglycone fraction was loaded on paper with a banding and compound separated in paper chromatogram was eluted with methanol. The volume of the eluate containing the compound was adjusted to 10 ml with the help of methanol and 3 ml of aluminium chloride (0.1 M) was added to it. Further, these mixtures were incubated at room temperature for 20 min and readings were taken at 440 nm wavelength using Spectrophotometer (Shimadzu). The quantification of quercetin was done by referring to the standard graph of quercetin and it was expressed on percentage dry weight basis.

4. CULTURE VESSELS

Culture flasks (100/150/250/500 ml), tubes (25 x 150 mm) and all other glassware used were of Borosil brand. They were washed with chromic acid (40%) and thoroughly cleaned under running tap water until the acid was removed. Subsequently

they were washed with teepol, rinsed with distilled water and dried in an oven (60° C) for 24h.

5. COMPOSITION AND PREPARATION OF MEDIA

The culture media used were : Wood and Braun (1961); Murashige and Skoog (1962); Gamborg's B_5 (1968), Schenk and Hilderbrandt (1972) and Lloyd and McCown (1981) whose compositions have been given in Table I (a, b, c, d and e). The stock solutions required for the preparation of culture media were prepared in double glass distilled water and kept at 5°C and those of vitamins and hormones at -4°C in deep freeze. Supplements *viz.*, cytokinins & auxins were added to the basal medium before final adjustment of the volume. The pH of the medium was adjusted to 5.8 using 0.1N NaOH/HCl by pH meter (Global). The medium was solidified with difco bacto agar (0.8%).

6. STERILIZATION OF MEDIA AND CULTURE VESSELS

Culture media were distributed into tubes (25 x 150 mm) and Erlenmeyer flasks (150 ml) in 20 and 30 ml aliquots respectively. In culture tubes for giving necessary support to shoots, Whatman no.1 filter paper bridges were used in the case of

TABLE - 1 (a)
Composition of Wood and Braun (1961) Medium

			Concentration of salts in mg/l of medium
I.	**MACROELEMENTS**		
	Sodium nitrate	$NaNO_3$	1800
	Potassium chloride	KCl	910
	Ammonium sulphate	$(NH)_2SO_4$	790
	Magnesium sulphate	$MgSO_4.7H_2O$	720
	Calcium nitrate	$Ca(NO_3)_2.4H_2O$	300
	Sodium sulphate	Na_2SO_4	200
	Potassium nitrate	KNO_3	80
	Sodium dihydrogen phosphate	$NaH_2PO_4.2H_2O$	316.5
II.	**MICROELEMENTS**		
	Manganese sulphate	$MnSO_4.4H_2O$	7.0
	Zinc sulphate	$ZnSO_4.7H_2O$	2.7
	Boric acid	H_3BO_3	1.5
	Potassium iodide	KI	0.7
III.	**IRON STOCK**		
	Ferrous sulphate	$FeSO_4.7H_2O$	22.8
	Sodium salt of Ethylene diamine tetra acetic acid	Na_2EDTA	37.25
IV.	**VITAMINS**		
	Thiamine hydrochloride		0.1
	Nicotinic acid		0.5
	Pyridoxine hydrochloride		0.1
	Glycine		3.0
	Myo-inositol		100.0
V.	**SUPPLEMENTS**	Sucrose, Phytohormones	

TABLE - I (b)

Composition of Murashige and Skoog (1962) Medium

			Concentration of salts in mg/l of medium
I.	**MACROELEMENTS**		
	Potassium nitrate	KNO_3	1900
	Ammonium nitrate	NH_4NO_3	1650
	Calcium chloride	$CaCl_2.2H_2O$	440
	Magnesium sulphate	$MgSO_4.7H_2O$	370
	Potassium dihydrogen phosphate	KH_2PO_4	170
II.	**MICROELEMENTS**		
	Manganese sulphate	$MnSO_4.4H_2O$	22.3
	Zinc sulphate	$ZnSO_4.7H_2O$	8.6
	Boric acid	H_3BO_3	6.2
	Potassium iodide	KI	0.83
	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
	Cobalt chloride	$CoCl_2.6H_2O$	0.025
	Copper sulphate	$CuSO_4.5H_2O$	0.025
III.	**IRON STOCK**		
*	Ferrous sulphate	$FeSO_4.7H_2O$	27.85
**	Disodium ethylene diamine tetra acetic acid	Na_2EDTA	37.25
IV.	**VITAMINS**		
	Glycine		2.0
	Nicotinic acid		0.5
	Pyridoxine hydrochloride		0.5
	Thiamine hydrochloride		0.1
	Myo-inositol		100.0
v.	**SUPPLEMENTS**	Sucrose, Phytohormones	

* The $FeSO_4.7H_2O$ was dissolved in approximately 200 ml of double distilled water.

** The Na_2EDTA was dissolved in 200 ml double distilled water, separately heated and mixed with $FeSO_4.7H_2O$ under continuous stirring until it dissolved completely. The mixture was boiled for 5 minutes. After cooling, the volume was adjusted to 1000 ml. Heating and stirring resulted in more stable Fe-EDTA complex.

TABLE - I (c)
Composition of Gamborg's B_5 (1968) Medium

			Concentration of salts in mg/l of medium
I.	**MACROELEMENTS**		
	Potassium nitrate	KNO_3	2527.5
	Magnesium sulphate	$MgSO_4.7H_2O$	246.5
	Calcium chloride	$CaCl_2.2H_2O$	150
	Ammonium sulphate	$(NH_4)_2SO_4$	134
	Sodium dihydrogen orthophosphate	$NaH_2PO_4.H_2O$	150
II.	**MICROELEMENTS**		
	Manganese sulphate	$MnSO_4.H_2O$	10
	Boric acid	H_3BO_3	3
	Zinc sulphate	$ZnSO_4.7H_2O$	2
	Potassium iodide	KI	0.75
	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
	Copper sulphate	$CuSO_4.5H_2O$	0.025
	Cobalt chloride	$CoCl_2.6H_2O$	0.025
III.	**IRON STOCK**		
	Ferrous sulphate	$FeSO_4.7H_2O$	27.85
	Disodium ethylene diamine tetra acetic acid	Na_2EDTA	37.25
IV.	**VITAMINS**		
	Thiamine hydrochloride		10
	Nicotinic acid		1
	Pyridoxine hydrochloride		1
	Myo-inositol		100
V.	**SUPPLEMENTS**	Sucrose, Phytohormones	

TABLE - I (d)

Composition of Schenk and Hilderbrandt (1972) Medium

			Concentration of salts in mg/l of medium
I.	**MACROELEMENTS**		
	Potassium nitrate	KNO_3	2500
	Magnesium sulphate	$MgSO_4.7H_2O$	400
	Calcium chloride	$CaCl_2.2H_2O$	200
	Ammonium dihydrogen phosphate	$NH_4H_2PO_4$	300
II.	**MICROELEMENTS**		
	Manganese sulphate	$MnSO_4.H_2O$	10.0
	Boric acid	H_3BO_3	5.0
	Potassium iodide	KI	1.0
	Zinc sulphate	$ZnSO_4.7H_2O$	1.0
	Copper sulphate	$CuSO_4.5H_2O$	0.20
	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.10
	Cobalt chloride	$CoCl_2.6H_2O$	0.10
III.	**IRON STOCK**		
	Ferrous sulphate	$FeSO_4.7H_2O$	15
	Sodium salt of Ethylene diamine tetra acetic acid	$Na_2EDTA.2H_2O$	20
IV.	**VITAMINS**		
	Thiamine hydrochloride		5.0
	Nicotinic acid		5.0
	Pyridoxine hydrochloride		0.5
	Myo-inositol		100
V.	**SUPPLEMENTS**		Sucrose, Phytohormones

TABLE - I (e)

Composition of Lloyd and McCown (1981) Medium

			Concentration ofsalts in mg/l of medium
I.	**MACROELEMENTS**		
	Calcuim nitrate	$Ca(NO_3)_2.4H_2O$	556
	Ammonium nitrate	NH_4NO_3	400
	Magnesium sulphate	$MgSO_4.7H_2O$	370
	Calcium chloride	$CaCl_2.2H_2O$	96
	Potassium dihydrogen phosphate	KH_2PO_4	170
II.	**MICROELEMENTS**		
	Manganese sulphate	$MnSO_4.H_2O$	22.3
	Zinc sulphate	$ZnSO_4.7H_2O$	8.6
	Boric acid	H_3BO_3	6.2
	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
	Copper sulphate	$CuSO_4.5H_2O$	0.25
III.	**IRON STOCK**		
	Ferrous sulphate	$FeSO_4.7H_2O$	27.8
	Sodium salt of ethylene diamine tetra acetic acid	$Na_2EDTA.2H_2O$	37.3
IV.	**VITAMINS**		
	Thiamine hydrochloride		1
	Nicotinic acid		0.5
	Pyridoxine hydrochloride		0.5
	Myo-inositol		100.0
V.	**SUPPLEMENTS**		Sucrose, Phytohormones

liquid media. The mouths of the culture tubes as well as flasks were plugged with non-absorbent cotton plugs covered with cheese cloth. Once again, they were wrapped with brown paper to avoid condensation of water during autoclaving.

Erlenmeyer flasks (150/250 ml) containing distilled water/media, culture tubes, pipettes, petridishes and millipore filter units were autoclaved at 15 lb/sq.in. pressure for 20 min at 120°C. Gibberellic acid was filter sterilized using millipore membrane (0.22 μ) and added to the sterilized media under aseptic conditions.

7. ASEPTIC TECHNIQUES

Laminar flow cabinet (Klenzaids, India) was used to carry out all inoculations involving axenic cultures. The working table was cleaned with dettol and irradiated with UV light (2537 A°) for 20 mins before use. The stainless steel instruments such as scalpel, spatula and forceps were kept in a jar containing alcohol and sterilized by flaming before use.

(i) Surface sterilization

The explants such as nodal segments with single axillary bud, young leaflets and stem segments from selected *Dalbergia sissoo* and *Albizzia lebbeck* plants were collected in a flask containing distilled water and washed under running tap water. They were further washed with teepol and distilled water. Subsequently, they were surface sterilized by mercuric chloride solution (0.1% w/v) for 2-5 min and rinsed with sterile distilled water. Each of the explant was cut into desired size and inoculated on the culture medium. Culture flasks were maintained in culture room at 25±2°C under white fluorescent light for 16h photoperiod (1000 lux).

(ii) Culture techniques

Surface sterilized nodal segments with an axillary bud were excised and inoculated vertically on the culture medium. Similarly, surface sterilized leaflets from young leaves and stem segments were inoculated on the test media. Culture flasks were incubated in culture room at 25±2°C for 16h photoperiod (1000 lux).

(iii) Technique of subculture

Axillary buds which sprouted into shoot-buds were separated into single shoot-bud or a unit consisting of two or three shoot-buds together and were subcultured on fresh medium (30 ml) of the same composition, at four weeks interval. Leaflets which induced adventitious buds were also subcultured on fresh medium at four weeks interval. Callus cultures were subcultured on fresh medium as per experimental requirements.

8. MEASUREMENT OF GROWTH

Callus growth measurements were made as a function of increase in fresh and dry weights of callus tissues.

(i) Fresh weight

Callus tissues grown on agar media were carefully transferred on preweighed aluminium foil after removing agar adhering to them. Six replicate cultures were harvested at a fixed interval of four weeks and weighed on a Sartorius micro balance.

(ii) Dry weight

Preweighed fresh callus tissues were dried in an oven at 60° C till a constant weight was recorded.

9. HISTOLOGICAL STUDIES

For histological studies, *in vitro* cultured axillary buds showing proliferation into shoot-buds, stem and leaflet of *D.sissoo* with induced callus, leaflets with adventitious buds of *A.lebbeck* and nodular callus were cut into small segments. They were fixed in FAA (40% formalin : glacial acetic acid : 50% ethyl alcohol; 5:5:90) for 24h. Dehydration was done using TBA (Tertiary butyl alcohol) series. Gradual infiltration of paraffin wax (m.p., 58° C) was carried out and blocks were prepared. Serial sections were cut using rotary microtome at 10μ and mounted on slide using egg albumin. Sections were stained with toluidine blue and mounted in Dextrine Plasticizer of Xylene (DPX).

10. CYTOLOGICAL STUDIES

Fresh root tips from regenerated plants were excised and pretreated with Para dichlrobenzene(PDB) for three and a half hours. They were washed with distilled water and fixed in acetic

acid and alcohol mixture (1:3) for 24h. Squash preparations of the root tips with aceto-orcein were made. Cells with metaphase plate were observed and the chromosome number was noted. This number indicated the ploidy of regenerated plant.

11. PHOTOGRAPHY

The histological and cytological peculiarities noticed were photographed using ORWO-22 black and white negative. Photomicrographs of the serial sections were taken using Carl-Zeiss Microscope.