CHAPTER II

MATERIALS AND METHODS

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1. Plant Material

The work presented in the present thesis was carried out on three ferns which are grown in the University Botanical Gardens. They are:

(i) Pteris vittata L.
Family: Pteridaceae;

(ii) Adiantum trapeziforme L.
Family: Adiantaceae; and

(iii) Thelypteris augescens (Link) Munz et Johnsdon.
Family: Thelypteridaceae.

(A) Experiments were conducted on Pteris vittata L. sporophytic callus tissues initiated on:

(i) the rhizome segments and young leaflets obtained from the garden plants.

(ii) young leaf-lets and roots of aseptically produced sexual sporelings, and

(iii) the leaflets of sporophytes produced from the gametophytic callus.

(B) Spores were collected from the fertile fronds of all the three ferns mentioned above and the prothalli developed from them. The prothalli as well as the callus tissues induced on them were also used as experimental material for the present studies.

2. Culture Media

Three different culture media were used in the present investigations. They were:- White's (1954), Knudson's (1925) as modified by Steeves *et al*. (1955), and revised Murashige and Skoog's (1962). The detailed composition of the media are given in Tables 1, 2, and 3.

The sporophytic callus tissues of *Pteris vittata* L. were initiated and maintained on White's medium (Table 1). For the germination of spores, development of prothalli and gametophytic callus studies modified Knudson's medium (Table 2) was used. Murashige and Skoog's medium (Table 3) was tested for growth of sporophytic callus. The details of specific media and the supplements added are given for each experiment in the text.

A. Preparation of the Media

The chemicals used in the preparation of media were of research grade purity and were obtained from British Drug House (Analar grade) or E. Merck (Guaranteed reagent).

The basic media were prepared from concentrated stock solutions which had been stored at 2-4°C. Double

TABLE 1

White's (1954) Medium

I. Inorganic salts		concentration in 1 litre of complex medium expressed as Mg of hydrated salts.
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	288.00
Magnesium sulphate	$MgSO_4.7H_2O$	738.00
Potassium chloride	KCl	65.00
Potassium nitrate	KNO_3	80.00
Sodium sulphate	$Na_2SO_4.10H_2O$	454.00
Sodium dihydrogen orthoPhosphate	$NaH_2PO_4.2H_2O$	24.00
II. Microelements and Vitamins		
*Ferric citrate		2.00
Boric acid	H_3BO_3	1.50
Manganese sulphate	$MnSO_4.4H_2O$	6.65
Potassium iodide	KI	0.75
Zinc sulphate	$ZnSO_4.7H_2O$	2.68
Glycine		3.00
Nicotinic acid		0.50
Pyridoxine hydrochloride		0.10
Thiamine hydrochloride		0.11
III. Supplements		
Sucrose		20.00 gms
Casein hydrolysate		300.00 mg
Coconut milk		100.00 ml
2,4-dichlorophenoxyacetic acid (2,4-D)		2.00 mg
OR		
Napthalene acetic acid (NAA)		
Water (double glass distilled) to make		1 litre

*Iron was supplied as ferric citrate instead of ferric chloride.

TABLE 2

Knudson's (1925) medium as modified by Steeves et al. (1955)

I. Inorganic salts		Concentration in 1 litre of complete medium expressed as Mg of hydrated salts.
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	500.00
Ammonium sulphate	$(NH_4)_2SO_4$	250.00
Magnesium sulphate	$MgSO_4.7H_2O$	125.00
Dipotassium hydrogen phosphate	K_2HPO_4	125.00
Ferric citrate		10.00

II. Microelements (Nitsch, 1951)		
Sulphuric acid	H_2SO_4 (sp.gr. 1.83)	0.0005 ml
Manganese sulphate	$MnSO_4.4H_2O$	3.00
Zinc sulphate	$ZnSO_4.7H_2O$	0.5
Boric acid	H_3BO_3	0.5
Copper sulphate	$CuSO_4.5H_2O$	0.025
Sodium Molybadate	$Na_2MoO_4.2H_2O$	0.025
Cobalt chloride	$CoCl_2.6H_2O$	0.025

III. Supplements	
Sucrose	20.00 gms
Coconut milk	100.00 ml
2,4-dichlorophenoxyacetic acid (2,4-D)	2.00 mg
Water (double glass distilled) to make	1 litre

TABLE 3

Revised Murashige & Skoog's (1962) medium

I. Inorganic salts		Concentration in 1 litre of complete medium expressed as mg of hydrated salt.
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.2H_2O$	440.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Potassium dihydrogen phosphate	KH_2PO_4	170.00
II. Microelements and vitamins		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Sodium Molybadate	$Na_2MoO_4.2H_2O$	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Manganese sulphate	$MnSO_4.4H_2O$	22.3
Zinc sulphate	$ZnSO_4.7H_2O$	8.6
Copper sulphate	$CuSO_4.5H_2O$	0.025
*Ferric sulphate	$FeSO_4.7H_2O$	27.85
**Na_2EDTA		37.35
Thiamine hydrochloride		1.0
Nicotinic acid		1.0
Pyridoxine hydrochloride		1.0
Glycine		4.0
III. Supplements		
Sucrose		20.0 gms
Myo-inositol		100.0 mg
Kinetin		0.4 mg
2,4-D		2.0 mg
Agar		8.0 gms

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

**The Na_2EDTA was dissolved in 200 ml of double glass distilled water, heated and mixed (under continuous stirring) with the $FeSO_4.7H_2O$ solution. After cooling the volume was adjusted to 1000 ml. Heating and more stirring resulted in a more stable FeEDTA complex.

glass distilled water was used for preparing the medium as well as the stock solutions. Wherever hydrates of any salts were used appropriate corrections were made in weights. The constitutents of the three media were added in the order shown in the Tables 1, 2, and 3. The supplements to be incorporated in the basic media were added before the final adjustment of the volume. The pH of the medium was measured and adjusted to 5.5 with a Beckman pH meter with the help of 0.1N HCl or 0.1N NaOH as found necessary. The medium was solidified with 0.8% or 0.6% Difco-Bacto agar as per experimental requirement.

The green coconuts were obtained from a local dealer. The milk released from these coconuts was boiled and after cooling, it was filtered through Whatman No.1 filter paper in order to remove precipitated protein. This process was repeated until no more protein precipitated. 100 ml aliquots of the filtrate were placed in 150 ml capacity Corning tubes. The mouth of the tube was closed by using non-absorbent cotton. They were autoclaved at a pressure of 15 $lb/in.^2$ for 15 minutes and then stored in a deep freeze.

B. Culture Vessels

Erlenmeyer flasks (250 ml or 150 ml) and test-tubes (25 x 150 mm) made of Corning glass were used as culture vessels. The mouth of these flasks and tubes containing sterile cultures were covered with two layers of aluminium foil which had been sterilized in a flame.

All culture vessels and glassware used in the preparation of the media and other purposes were cleaned in chromic acid (Potassium dichromate in sulphuric acid). The acid was removed by prolonged rinsing with tap water. Next, the glassware was washed with detergent Teepol (B.D.H.) which was then removed by thorough washing with tap water. The glassware was finally rinsed twice with double glass distilled water and dried in an oven.

C. Sterilization of media and culture vessels

After the adjustment of pH, known volumes of media were distributed in culture vessels for autoclaving. The mouth of culture vessels was closed with non-absorbent cotton-wool plugs covered by gauze. Brown papers were wrapped to protect them from condensed water during autoclaving. The media and glassware were sterilized by autoclaving at a pressure of 15 lb/in^2 for 20 minutes. Instruments used were sterilized by flaming with absolute alcohol.

3. Aseptic Techniques

(A) Transfer cabinet

All inoculations and manipulations involving sterile cultures or media were carried out in a transfer cabinet. The working surface was cleaned with 4% formaldehyde. The interior of the cabinet was sprayed with absolute alcohol and irradiated with ultra-violet (= 2537A°) light for 60 minutes before use to reduce contamination.

(B) Surface sterilization of rhizome segments and leaflets

Rhizome segments and young leaflets were collected from Pteris vittata L. plants growing in the Botanical gardens. Rhizome segments were derooted and defoliated with a sharp blade, cut into pieces and then washed with tap water. The segments were next treated with 0.1% mercuric chloride solution for 5 minutes and then rinsed with sterile distilled water repeatedly. Smaller explants (1 cm in length) were cut from this material and transferred to culture vessels containing media.

(C) Sterilization of spores

Healthy fertile fronds were collected and thoroughly washed in running tap water in order to remove debris, foreign spores, etc. The fronds were then rinsed with distilled water and pressed between two sheets of blotting

paper. After 4-5 days, the spores released from sporangia were collected and surface sterilized with 2.5% sodium hypochlorite for five minutes and then rinsed 4 to 5 times with sterile distilled water using hand centrifuge. Next the volume of sterilized spore suspension was adjusted as desired with sterile distilled water and spore suspension inoculated on agar slants in test-tubes containing Knudson's medium (Table 2).

4. Culture Techniques

(A) Types of cultures

All the sterile tissues were maintained on agar media as Callus cultures. Some were also grown in liquid media as Suspension cultures.

(B) Initiation of callus culture

The excised segments of rhizome, roots, leaves or prothalli were transferred to culture vessels containing agar medium. The rhizome and leaf explants from garden plant were first sterilized as mentioned earlier in Materials and Methods 3, B. The medium used for callus induction, whether White's or Knudson's, was supplemented with coconut milk (10%) and an auxin, 2,4-Dichlorophenoxy-acetic acid (2,4-D). The culture vessels were incubated at 26±2°C in a culture room which was illuminated continuously with a fluorescent tube (40W).

(C) Initiation of suspension culture

Cell suspension was obtained by transferring pieces of callus tissues from 'Clonal' stocks maintained on agar media to the liquid media contained in Erlenmeyer flasks. The latter were continuously agitated on a horizontal rotary shaker in the culture room. Dissociation of the callus into free cells and cell aggregates of various sizes was obtained within a week or two after inoculation.

(D) Techniques of subculture

(a) Every 4-6 weeks callus mass was cut into 2-4 equal parts with a sterile scalpel and transferred to freshly made nutritive media in tubes or Erlenmeyer flasks. 'Clones' of callus cultures were established by selecting a healthy and fast growing callus and regularly subculturing it, until a large amount of callus tissue was obtained. From such 'Clones', weighed amounts or equal sizes of callus pieces were used for the inoculation of the experiment.

(b) Routinely, the suspension cultures were subcultured every four weeks. 5 ml aliquots of cell suspension were pipetted into 20 ml of freshly made liquid medium of same composition.

5. Measurements of Growth

Growth was measured by determining increase in fresh and dry weights in 6 to 8 replicate cultures. Standard error was calculated to ascertain statistical significance of the growth data.

(A) Fresh weights

In case of callus cultures growing on solid media, fresh weights were determined on Mettler balance (Zurich). For inoculation of experiments, callus pieces were weighed aseptically on a Torsion balance (Varmax, Poland).

(B) Dry Weights

Dry weights of callus tissues were determined by drying weighed mass of callus to a constant weight at 60°C in an oven.

6. Plating Techniques

Erlenmeyer flasks containing cell suspension was filtered aseptically through a nylon mesh of 300 µ pore size in order to obtain suspensions of single cells and small groups of (4 to 6) cells. 2 ml aliquots of cell suspension (less than 300 µ in diameter) were pipetted on the surface of fresh agar (0.8%) medium (15 ml) contained in petri dishes (10 cm in diameter). The cell suspension was then uniformly spread in a very thin layer

in the petri-dish which was sealed with a cloth insulating tape to prevent descication and contamination.

The plates were incubated for 4 to 8 weeks at a constant temperature at 26±2°C in light. The number of visible colonies developed and the morphogenetic responses invoked were recorded.

7. Histological Procedure

To examine the anatomical organization within the callus mass as a function of given cultural conditions and experimental treatments, histological preparations were made (Jensen, 1962). Small callus pieces with their outgrowths, where necessary, were fixed in FAA (5 ml formalin + 90 ml of 50% ethyl alcohol + 5 ml of glacial acetic acid) for 24 hours. Afterwards they were washed 3 to 4 times in 70% alcohol and dehydrated in the usual alcohol-xylene series and then embedded in paraffin wax. The blocks were cut at 10 µ on a rotary microtome and ribbons were stained with saffranin counterstained with fast green. Mounting was done in Canada balsam.

8. Cytological Procedures

(A) Chromosome preparations

The chromosome number of Pteris vittata L. and Adiantum trapeziforme L. were determined from garden

grown plants. The young leaftips pretreated with 0.2% colchicine for 6 to 8 hours, were fixed in acetic-alcohol (1:3) for 24 hours. After hydrolysing them with 1 N HCl at 60°C for 8 to 10 minutes, Feulgen (basic Fuschin) stain was added to the tubes until the leaftips were completely dipped in the stain. The tubes were kept in dark for 2 to 4 hours. Squashes were then made in acetocarmine. Permanent mounting was done in Euparal and chromosome number was counted from the metaphase plate. Callus tissues of different origin were similarly squashed for chromosome studies.

(B) Acetolysation of Spores

Spores collected from the fertile fronds were soaked in glacial acetic acid. The acid was removed by centrifugation followed by decantation. A mixture of acetic anhydride and concentrated sulphuric acid in the ratio of 9:1 was poured into the tube containing spores to make final volume 5 ml. The mixture was heated on a water bath to near boiling (80°C), cooled to room temperature, centrifuged and the autolysis mixture decanted off. Distilled water was then added to the sediment to make the volume 10 ml and the mixture thoroughly shaken until it foamed. The foam was removed by addition of a drop or two of alcohol. After centrifuging the sediment was washed with water and

50% glycerine added. After an hour spores from 50% glycerine were mounted in freshly prepared glycerine jelly (Erdtman, 1954).

9. Screening of Pteris vittata L. plants for antibacterial substances

The methodology employed is described in detail in the off-print of the paper entitled "Survey of Ferns in Gujarat State (India) for Presence of Antibacterial Substances" (See Appendix I).

10. Camera lucida diagrams

Camera lucida diagrams were drawn to illustrate the different stages of prothallial development in Adiantum trapeziforme L. and Thelypteris augescens (Link) Munz et Johnsdon.

11. Photomicrography

The photographs of the histological preparations were taken on Leitz photomicrography equipment of the reflex type. Exakta and/or Contax camera were used to photograph the culture vessels showing morphogenesis and differentiation. Orwo-Documentation Neg-film was used for most of the work. Developer used was Kodak DA-163. The Steindor microscope fitted with 6X, 10X, 15X projection eyepiece and 10X, 15X, 45X or 100X objective was used.