MATERIALS AND METHODS

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Experimental Materials

Two ferns viz; *Marsilea minuta* and *Ceratopteris thalictroides* were selected for present investigations. Fern *Marsilea minuta* is an aquatic heterosporous fern belonging to the family Marsileaceae, while, fern *Ceratopteris thalictroides* is an aquatic homosporous fern and belongs to the family Parkeriaceae.

Classification

Division	Pteridophyta	Pteridophyta
Class	Fillicinae	Fillicinae
Series	Leptosporangiate	Leptosporangiate
Family	Marsileaceae	Parkeriaceae
Genus	*Marsilea*	*Ceratopteris*
Species	*minuta*	*thalictroides*

Source of materials

Sporophytes of *Marsilea minuta* were collected from the pond water (pond located in the village Surajpur, a place near by Chandigarh), while, mature spores of *Ceratopteris thalictroides* were collected from National Botanical Research Institute, Lucknow.

METHODS

Glassware used

The glassware used for the purpose of raising experiments, comprised 6"x1" corning test tubes; 100 ml, 250 ml, 500 ml and 1000 ml borosil or corning flasks. The nature of the experiment determined the choice of a particular type of glassware. The glassware first treated with hot chromic acid (mixture of $K_2Cr_2O_7$ + H_2SO_4 + H_2O). It was then brushed with a detergent teepol and finally washed in running tap water. The glassware was dried by keeping it in inverted position. Glass distilled water (approximately 5 ml) was poured into each culture vessel and then each vessel was plugged with an absorbant, surgical cotton wrapped in muslin. The glassware was then steam sterilized in an autoclave at a pressure of 15 lbs./sq. inch and at the temperature of 120°C for 20 minutes.

Culture media

For the present experimentations two nutrient media viz; Knop's medium and basal Murashige & Skoog's medium with the following composition were used.

1. Knop's medium

KNO_3	200 mg/l
$MgSO_4.7H_2O$	200 mg/l
KH_2PO_4	200 mg/l
$Ca(NO3)_2.4H_2O$	300 mg/l

$FeCl_3$	Traces
Distilled Water	1000 ml
Agar	8 gm/l

2. Murashige and Skoog's medium (1962)

Murashige & skoog's (MS) medium consists of (a) major / macro elements, (b) minor / micro elements and (c) organic/ vitamins constituents.

(a) Major elements	mg/l
KNO_3	1900
NH_4NO_3	1650
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
$FeSO_4.7H_2O$	27.81
Na_2-EDTA	37.31

(b) Minor elements	mg/l
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
$ZnSO_4.7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$,	0.025

(c) Organic constituents	mg/l
Glycine	2.0
Mesoinositol	100.0
Nicotinicacid	0.5
Pyridoxine-HCL	0.5
Thiamine-HCL	0.1
Agar	8 gm/l
Sucrose	20 gm/l
Distilled water	1000 ml

Preparation of media

Different media were prepared by adding appropriate quantity of the constituents as mentioned earlier. Each salt was dissolved one by one in order to avoid precipitation. The pH of the medium was adjusted between 5.5 to 5.8 with the help of digital pH meter by adding 1N HCl or 1N NaOH as required. 8 gm/l agar (Bacteriological grade) was added to the medium. Medium was then heated till the agar dissolved fully. The accurately measured amount of medium was poured into the culture vessels depending on their capacity. Usually the test tubes contained 25 ml; 100, 250 and 500 ml flasks contained 50, 100 and 200 ml of the media respectively. Various growth regulators were added into the medium as required in different concentrations. The culture vessels were plugged before sterilization and then

autoclaved for 20 minutes at a pressure of 15 lbs./sq. inch and at the temperature of 120°C. After sterilization the medium was allowed to cool at room temperature in order to let the medium solidified. Test tubes containing sterilized medium were kept in tilted position to make the slants.

Inoculation

For the purpose of raising cultures from spores, filter papers (Wattman's filter paper No.1) were placed in a petridish which was then wrapped in tin foil. It was sterilized for 20 minutes at 15 lbs/sq. inch.

All the experiments were carried out under strictly sterile conditions in an inoculation chamber/ laminar (air) flow (thermadyne). The floor and walls of the inoculation chamber were scrubbed with cotton dipped in alcohol. Culture vessels containing medium and accessories (i.e. forceps, scissors, spatula, spirit lamp, alcohol container, flasks containing sterilizing solution and sterile distilled water etc.) needed were placed in the chamber after cleaning them with cotton dipped in alcohol. The living material kept in the chamber was always covered with a black paper to protect it from deleterious effects of U.V. rays. Lastly, the alcohol was sprayed in the chamber with the help of an atomizer. It was also sprayed on the gloves which were then rolled up. The U.V.tube was kept on for 2 hours. Hands were surface sterilized by alcohol before taking them in through

gloves. All surgical instruments required were sterilized by dipping them in alcohol and flaming them later on. This process was repeated again and again to ensure complete sterilization of the required instruments and accessories. The rims and sides of the culture vessels were flame sterilized.

Besides inoculation chamber, laminar (air) flow was also used. In this instrument, normal air is sucked in, passes through the filters and finally sterilized air blows out on the working area, which provides the aseptic conditions required for experimentations. In order to raise cultures in laminar (air) flow, its floor as well as walls were cleaned with cotton dipped in alcohol and then switched on both light and air sucker systems. All the accessories and culture vessels required were kept inside (after cleaning them with cotton dipped alcohol) over the working area of the instrument and waited for 25 to 30 minutes. Hands were surface sterilized by alcohol and afterwards same process was repeated as described above.

Rhizome segments of _Marsilea minuta_ were taken from the plants growing in nature. These plants were derooted, leaves were excised and rhizome segments were cut with node. They were surface sterilized with 0.25% solution of mercuric chloride for 5-8 minutes, washed thoroughly for 3-4 times and then inoculated.

Spores of fern *Ceratopteris thalictroides* were surface sterilized in 1.5% solution of calcium-hypochlorite for 5-8 minutes. The spore suspension was filtered and the spores after thorough washing in sterile distilled water were inoculated in each test tube.

In order to induce callus, different plant parts of both the ferns (viz; *Ceratopteris thalictroides* and *Marsilea minuta*) were excised from aseptically growing sporophytes and inoculated on both Knop's + 0.5% sucrose and basal MS medium supplemented with various growth regulators. Gametophytic callus (of *Ceratopteris thalictroides*) was induced from the actively growing gametophytes *in vitro*.

Culture conditions

All the cultures were maintained in an air conditioned room where temperature was controlled at 25±2°C. The culture tubes were placed on wooden racks in the shelves. Each shelf had two fluorescent tubes (40 watts) fitted at 1 1/2 meter height above the cultures. All the cultures were provided with 12 hours photoperiod by the fluorescent tubes with a light intensity of 120 foot candles.

Culture studies

Each experiment was repeated twice with four replicates in each treatment in order to confirm the results obtained. Morphological changes of explants were noted regularly.

Comparative growth of callus on different media was observed. Differentiation of callus to root/shoot or both was also studied at regular intervals.

Histological studies

Temporary slides of the callus were prepared in sterilized distilled water to study the color, nature and type of callus. The material was fixed in FAA (5 ml formaline, 5 ml acetic acid and 90 ml of 50% ethanol).

Cytological Studies

Callus induced from different plant parts of both ferns viz; Marsilea minuta and Ceratopteris thalictroides on basal MS medium containing and growth regulators (viz; 2,4-D; NAA; Kinetin and BAP alone) was fixed in carnoy's reagent (choloroform 1 : acetic acid 3 : alcohol 6) for one hour. It was then transferred to 70% alcohol for preservation. Slides were made and stained in aceto-carnine and examined under light microscope.