CHAPTER V

IN VITRO STUDIES ON GAMETOPHYTES AND GAMETOPHYTIC CALLUS OF *PTERIS VITTATA* L.

IN VITRO STUDIES ON GAMETOPHYTES AND GAMETOPHYTIC CALLUS OF PTERIS VITTATA L.

Experiments described here were aimed at the establishment of gametophytic callus from prothalli derived from spores. A suitable developmental stage of prothallus at which callus initiation occurred was established. The culture medium employed was Knudson's (1925) as modified by Steeves et al. (1955) with Nitsch trace elements (Table 2). The effect of different sucrose concentrations was examined for morphogenetic potentiality of the gametophytic callus. Effect of light intensity on callus differentiation was also studied. Suspension culture established from healthy gametophytic callus was used for plating experiments. Regenerative capacity of free cells and cell-aggregates measuring less than 300 μ in diameter was examined and compared with that of prothallial cells of the same dimension.

The influence of mineral nutrients on induction of apogamy was studied to understand the role, if any, of mineral nutrition in the apogamous production of shoots. Effect of varying levels of macroelement salts on the fresh weight of gametophytic colony and the number of visible apogamous shoots per culture was determined. Sporophytes were produced after normal fertilization which was brought

about by flooding the mature prothalli with sterile distilled water. The resultant plants were treated with different concentrations of sucrose to examine their developmental pattern.

Experiment No. 5-1: Initiation of callus on prothalli

Gametophytes were developed aseptically in Knudson's medium (Table 2) from the spores as described in Chapter II, Materials and Methods 3 C. Such prothalli which were either 2, 4 or 6 weeks old were inoculated separately onto 20 ml Knudson's media containing 2% sucrose and supplemented with 1 mg/l or 2 mg/l 2,4-D. Sterilized spores were also similarly inoculated in culture tubes. Five replicates of each of the treatments were incubated in continuous light at 26±2°C for 8 weeks and the ability for callus initiation by the prothalli at different developmental stages was determined.

No callus formation was noticed directly from the spores nor on prothalli which were very young, filamentous or two dimensional. Callus initiation occurred on prothalli which were 4 and 6 weeks old (Fig. 52), initiation being

Fig. 52. Gametophytes of Pteris vittata L. grown on Knudson's medium containing 10% CM, 2% sucrose and 2.0 mg/l 2,4-D showing callus initiation

Incubation: 4 weeks in light at 26±2°C

Fig. 53. Growth of callus grown on Knudson's medium containing 10% CM, 2% sucrose and 2.0 mg/l 2,4-D

Incubation: 8 weeks in light at 26±2°C

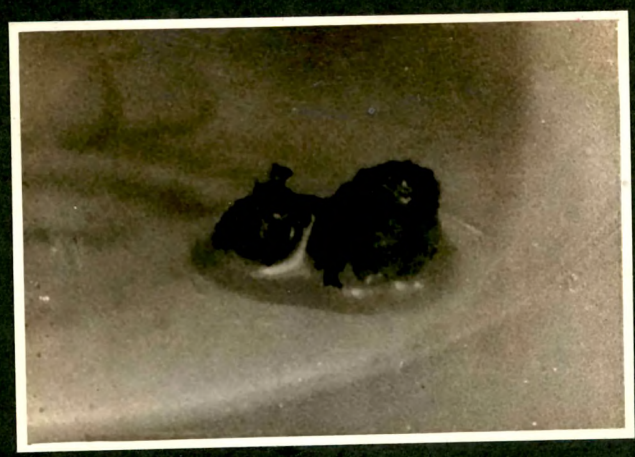

Fig.52

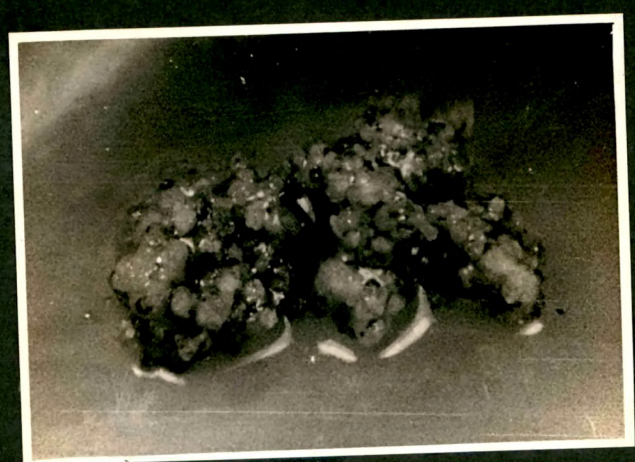

Fig.53

more rapid on 2 mg/l 2,4-D containing medium than on the other (1 mg/l 2,4-D containing medium). To establish large callus stock, the callus was regularly subcultured every four to six weeks on Knudson's medium containing 10% coconut milk. Coconut milk was incorporated into medium as it enhanced callus growth markedly (Fig. 53).

Experiment No. 5-2: Effect of sucrose level in the medium on the differentiation of callus

Pieces of callus tissue, about 1.0 cm in diameter, were inoculated in 150 ml Erlenmeyer flasks containing 60 ml of the culture medium. The media tested were Knudson's basal medium (Table 2) alone, or containing 0.5, 1.0, 2.0 or 4.0 per cent of sucrose. The culture vessels were incubated in continuous light (300 Lux) in culture room at 26±2°C. Five culture vessels containing media with and without 2% sucrose, were incubated at 25±1°C in an environmental chamber with light intensity of 5000Lux. Detailed observations were made every four weeks and the morphogenetic changes (if any) were photographed.

Sporophytic shoots were observed on callus tissues incubated on media containing 1% and higher sucrose. On media containing 4% sucrose the apogamous shoots produced were more in number as compared to those produced on 2% and 1% sucrose media (Figs. 54, 55, 56). Clearly, the number of apogamous shoot enhanced with the increase in sucrose level in the medium. After incubation for 12 weeks, more apogamous shoots but few roots were formed on 4% and 2% sucrose media, while more of roots were produced from the apogamous shoots on medium containing 1% sucrose (Figs. 57, 58).

In the case of media containing no sucrose and 0.5% sucrose, there was noticed regeneration of gametophytes (Fig. 59). Forms intermediate between gametophyte and sporophyte along with many regenerated gametophytes were observed on the callus cultured on medium containing 0.5% sucrose (Fig. 60). To begin with, the intermediate form was a flat thick cushion with multicellular scales at its margin (Fig. 61). Most of its cells were found rich in starch when squashed and stained with iodine.

Fig. 54. Formation of well developed many apogamous shoots on callus grown on Knudson's medium containing 4% sucrose (no auxin)

Incubation: 4 weeks in light at 26±2°C

Fig. 55. Formation of apogamous shoots on callus grown on Knudson's medium containing 2% sucrose (no auxin)

Incubation: 4 weeks in light at 26±2°C

Fig. 56. Formation of very few apogamous shoots on callus grown on Knudson's medium containing 1% sucrose (no auxin)

Incubation: 4 weeks in light at 26±2°C

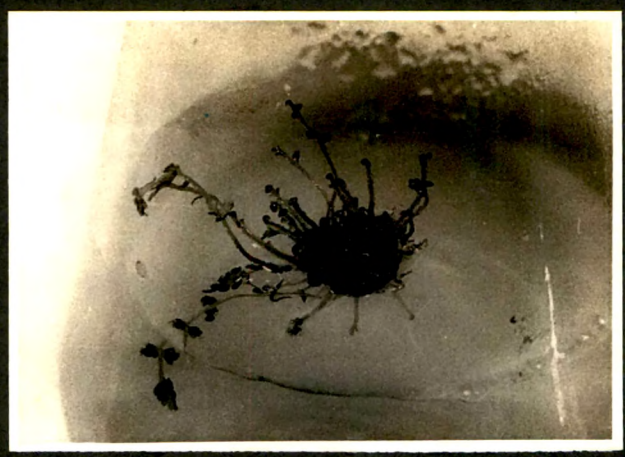

Fig.54

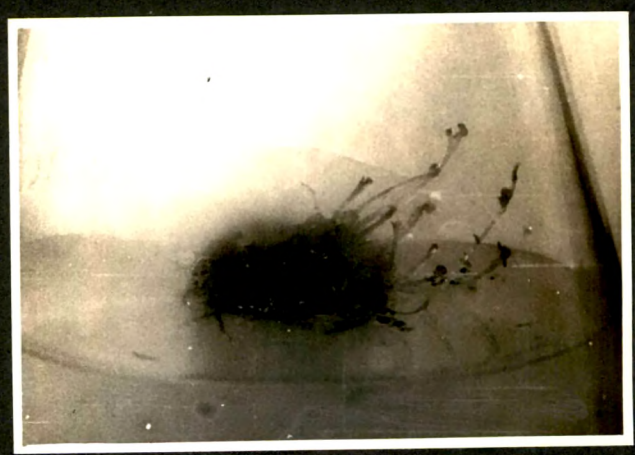

Fig.55

Fig.56

Fig. 57. Many more apogamous shoot formation on callus grown on Knudson's medium containing 2% sucrose (no auxin)

Incubation: 12 weeks in light at 26±2°C

Fig. 58. Few apogamous shoots along with root formation on callus grown on Knudson's medium containing 1% sucrose (no auxin)

Incubation: 12 weeks in light at 26±2°C

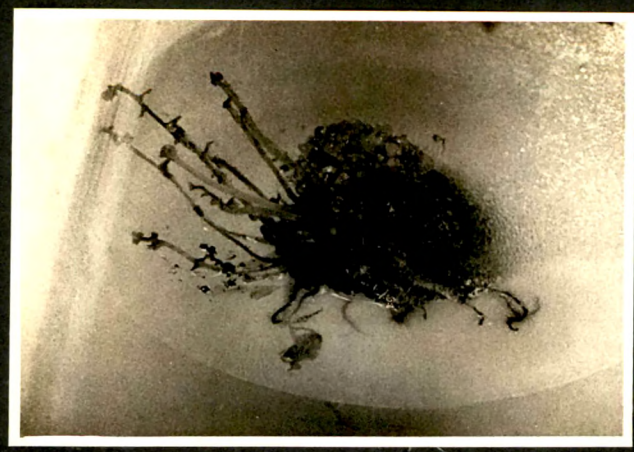

Fig.57

Fig.58

Fig. 59. Regeneration of gametophytes (G) from callus grown on Knudson's medium (no sucrose, no auxin)

Incubation: 12 weeks in light at 26±2°C

Fig. 60. Callus grown on Knudson's medium containing 0.5% sucrose showing formation of intermediate (I) structure and gametophyte (no auxin)

Incubation: 8 weeks in light at 26±2°C

Fig. 61. One of the intermediate structures with multicellular scales at its margin

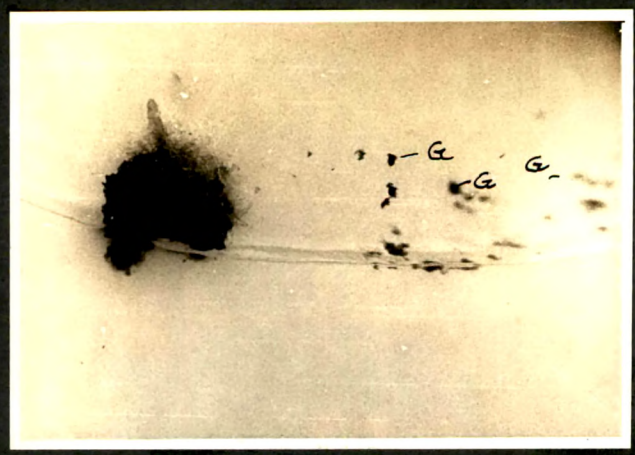

Fig.59

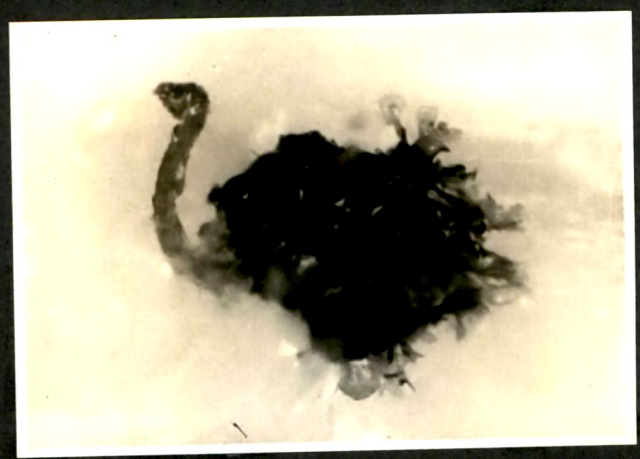

Fig.60

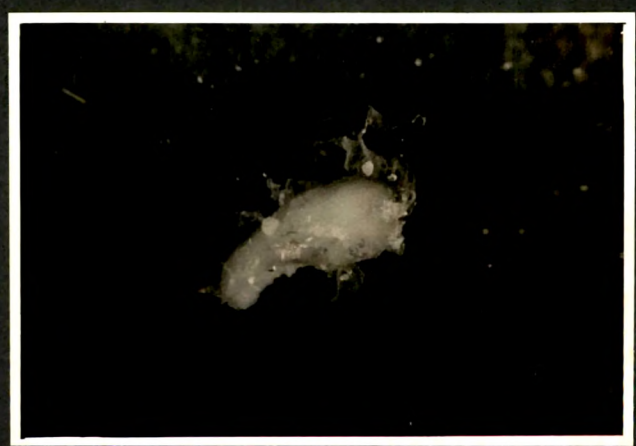

Fig.61

Comparison of cultures incubated in moderate (300 Lux) and bright light (5000 Lux) revealed that the regeneration of gametophytes and production of apogamous shoots was much earlier in the latter treatment. Though there was no apparent increase in the number of gametophytes/sporophytic shoots in bright light, the growth of both was markedly enhanced in this higher light intensity.

Experiment No. 5-3: Effect of mineral nutrition on apogamy in gametophytes

In this experiment the effect of different levels of macroelement salts in Knudson's medium was studied. The concentrations of the macroelements tested in the media were adjusted to 1/10th, 1/2, 1 and 2 times the concentrations of macroelements present in Knudson's basal medium (Table 2). The ionic concentrations of the media were adjusted by proper addition of sodium or chloride ions. The level of sucrose was raised to 4% as it has been found in the previous experiment to induce initiation of sporophytic shoots. Young gametophytic colonies of about equal sizes were inoculated separately in 150 ml Erlenmeyer flasks containing 60 ml of the

culture media. The flasks were incubated at 26±2°C in light for 12 weeks and then the number of observable apogamous sporophytes was counted. The gametophytes along with apogamous shoots were weighed after pressing them gently between two sheets of blotting paper to remove excess of moisture.

Of the four treatments tested, the basal medium with standard concentrations of macroelements showed maximum number of apogamous shoots and the highest fresh weight (Table 9, Fig.62). In low concentrations of macroelements, there was no apogamous shoot formation. Doubling the concentrations of macroelement salts also did not invoke the apogamous response nor any increase in fresh weight. This suggested that at 4% sucrose, standard macroelement level supported optimal growth and favoured apogamous production of sporophytes (Fig. 63).

Experiment No. 5-4: Regeneration of gametophytes from free cells isolated from gametophytic suspension culture and obtained directly from the prothalli

Suspension culture was prepared from friable gametophytic callus by transferring a measured mass of callus to 20 ml of liquid Knudson's medium supplemented with 2% sucrose and

Table 9: Effect of Mineral nutrients on Apogamy in gametophytes of Pteris vittata L.

Medium : The concentrations of macroelements adjusted to 1/10th, 1/2, 1 and 2 times the concentrations as present in Knudson's basal medium containing 4% sucrose.

Inoculum : Several gametophytes of about equal sizes.

Incubation: 12 weeks at 26±2°C in continuous light.

Level of macroelement salts (X Standard)	Fresh wt. (mg)	No. of visible apogamous shoots
X1/10	369.0 (±8.6)	nil
X 1/2	512.5 (±5.90)	2
X 1.0	573.0 (±4.90)	7
X 2.0	478.5 (±1.57)	3

Figures in parentheses represent standard error.
No. of replicates = 5.

Fig.62: Effect of Mineral nutrients on Apogamy in gametophytes of Pteris vittata.

Experimental details as given in Table 9.

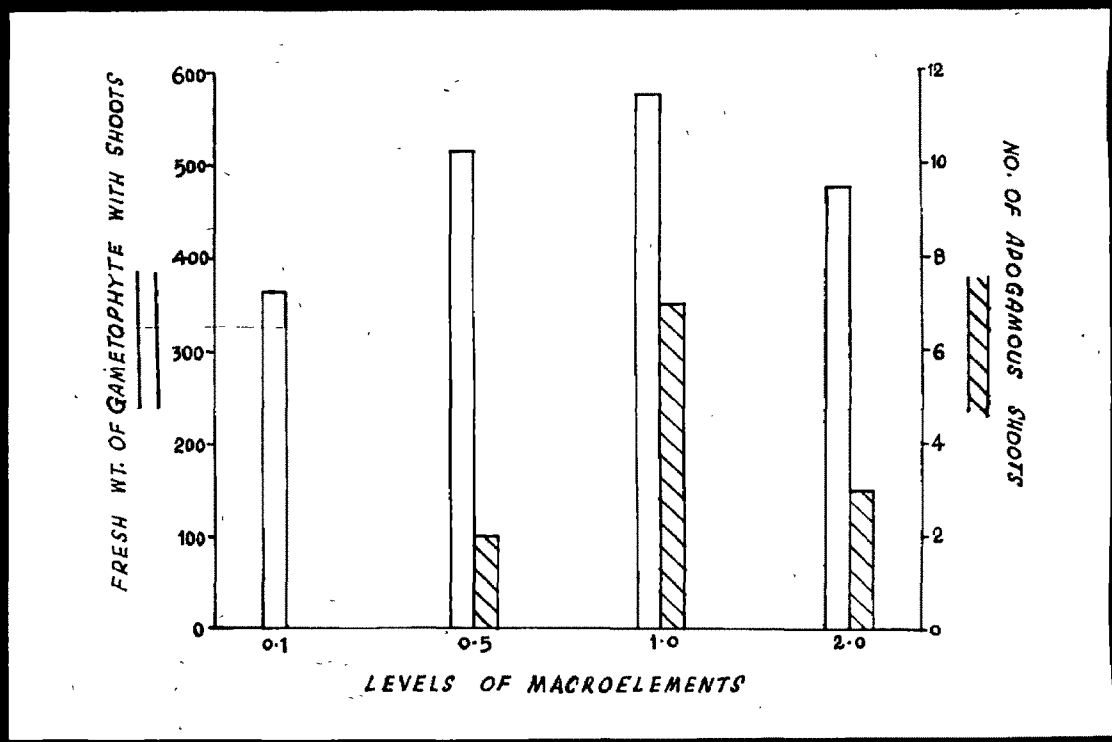
FRESH WT. OF GAMETOPHYTE WITH SHOOTS
600
500
400
300
200
100
0
NO. OF APOGAMOUS SHOOTS
12
10
8
6
4
2
0
0·1
0·5
1·0
2·0
LEVELS OF MACROELEMENTS

Fig.62

Fig. 63. Gametophytes grown on Knudson's medium containing 4% sucrose showing apogamy

Incubation: 12 weeks in light at 26±2°C

Fig.63

2 mg/l 2,4-D (Chapter II, Materials and Methods 4,C). The liquid culture was constantly agitated on a horizontal rotary shaker in a lighted culture room at 26±2°C. A thick suspension of free cells and cell-aggregates was obtained and it was serially propagated by transferring 5 ml aliquots of the suspension into 20 ml of the above fresh medium every 4 weeks. After 4 such transfers the suspension was utilized for plating experiment. Free cells were also obtained directly from the prothalli. Sterile prothallial colony was immersed in 10 ml of sterile distilled water and gently crushed by hand, care being taken not to give too much pressure. Cells and cell-aggregates measuring less than 300 μ in diameter were separated from the prothallial cell suspension by sterile filtration through a nylon mesh of 300 μ pore size. Similarly, the suspension culture of callus was aseptically filtered through a nylon mesh of 300 μ pore size. The less than 300 μ in diameter fractions of above suspensions contained single cells and groups of upto 6 cells (Fig.64). They were (2 ml) plated separately on 15 ml of Knudson's basal medium solidified with 0.6% agar in petri dishes. The sealed petri dishes were incubated at 26±2°C in light.

Fig. 64. Photomicrograph of suspension culture showing groups of cells (150X)

Incubation: 4 weeks in light at 26±2°C in Knudson's medium containing 2% sucrose and 2.0 mg/l 2,4-D

Fig. 65. Photomicrograph of regenerated gametophytes from callus - suspension culture plated on Knudson's medium

Incubation: 8 weeks in light at 26±2°C

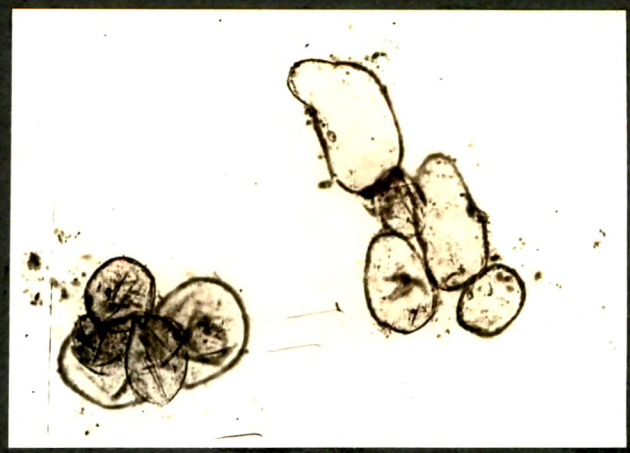

Fig.64

Fig.65

Fig. 66. Photomicrograph of regenerated gametophytes from prothallial cell-suspension plated on Knudson's medium

Incubation: 8 weeks in light at 26±2°C

Fig.66

Results were recorded after incubation for 8 weeks. Regeneration of gametophytes was observed in both the cases. The regeneration from prothallial cells was more rapid than the regeneration from liquid cell cultures. Moreover, the capacity to regenerate gametophytes was more pronounced in cells derived directly from the prothalli when compared with that of cell suspension culture (Figs. 65, 66).

Experiment No. 5-5 : Effect of different concentrations of sucrose on the development of sporophytes

Mature gametophytes of Pteris vittata L. were flooded with sterile distilled water to ensure fertilization. After the appearance of sporelings, they were transferred to Knudson's medium containing 2, 4, 6 or 8% sucrose with and without 0.5 gm/l yeast extract. Sporelings were transferred to culture vessels each containing 60 ml of the medium and incubated at 26±2°C in light (300 Lux). Observations were made at regular intervals of time for almost a year.

Plants did not grow at higher sucrose concentrations of 6% and 8% in presence and absence of yeast extract (Fig.67).

Fig. 67. Growth of sporelings on Knudson's medium containing different sucrose concentrations (4.0%, 6.0% and 8.0%)

Incubation: 4 weeks in light at 26±2°C

Fig. 68. Growth of sporeling on Knudson's medium containing 4% sucrose

Incubation: 8 weeks in light at 26±2°C

Fig. 69. Growth of sporeling on Knudson's medium containing 4% sucrose and 0.5% yeast extract

Incubation: 8 weeks in light at 26±2°C

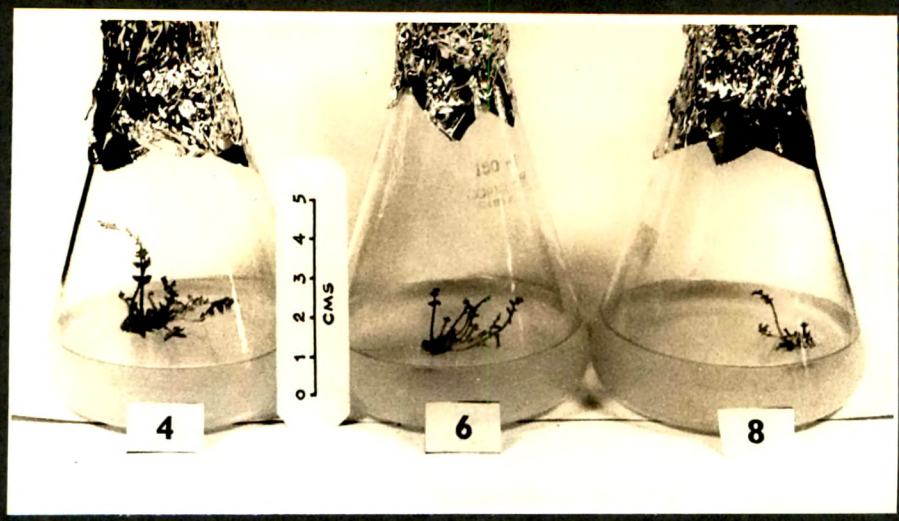

Fig.67

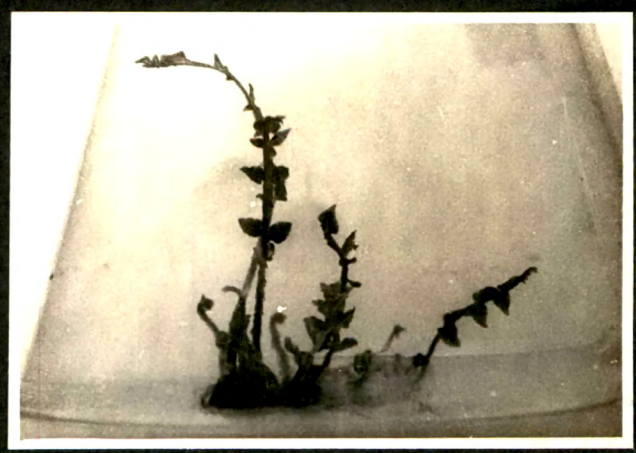

Fig.68

Fig.69

Fig. 70. Well developed Pteris vittata plants grown on Knudson's medium containing sucrose 4% and 0.5% yeast extract

Incubation: 16 weeks in light at 26±2°C

Fig.70

While maximum growth was observed in the medium containing 4% sucrose both with and without yeast extract (Figs. 68, 69, 70). In none of the treatments, however, fruiting was observed on the leaves, even after incubation for over an year.

Experiment No. 5-6: Screening of In vitro plants for the presence of Antibacterial substances

An experiment was set up to find out any active antibacterial principle(s) present in the plants produced in vitro. The fronds of sexually and apogamously produced sporophytes were dried at room temperature, powdered and their methanol extracts prepared as mentioned in Chapter II, Materials and Methods, 9. The filter-paper disks impregnated with the extracts and dried were screened against Bacillus cereus NCTC 9946 and Staphylococcus aureus B-4B-5. The same bacterial strains were selected which gave positive results with in vivo plants. No zones of inhibition were, however, observed after incubation for 24 hours. These in vitro plants were grown for 12 months in culture without any sign of contamination.

DISCUSSION

Callus initiated on mature, cordate gametophytes, but not on the gametophytes at earlier stages of development (Expt. 5-1). This might be due to an age dependent change in the capacity of cells composing gametophyte to respond to auxin. The optimal concentration of sucrose and 2,4-D required for cell-multiplication and enlargement were probably different from cell to cell, almost dependent upon the age of the plant material in question. This probably is on account of variation in endogenous auxin content of the gametophyte cells.

Differentiation of callus occurred only when it was transferred to media without 2,4-D (Expt. 5-2). The callus when subcultured on various sucrose concentrations showed the formation of apogomous shoots. The time required for the initiation of these apogamous sporophytes and also the number of apogamous sporophytes was directly correlated with the sucrose content of the media. It was observed first on 4% sucrose and with decreasing sucrose content it took correspondingly longer time for the development of apogamous sporophytes. On the other hand, in cultures grown in 1% sucrose the formation of roots occurred earlier than in cultures grown on higher sucrose

media. After prolonged period of incubation (12 weeks), in all the treatments, when there was no more formation of sporophytic structures, the callus gave rise to gametophytes. The autotrophic prothalli were formed first from the callus grown on the basal medium which lacked sucrose. In sucrose containing media too, the prothalli were produced, but much later, after 12 weeks, by which time most of the energy source must have been exhausted from the medium.

Cells and cell-aggregates of less than 300 μ in diameter were capable of regenerating entire prothalli. The capacity for regeneration into prothalli was more pronounced among prothallial cells than the callus cells (Expt. 5-4). This might be due to slight carry-over of auxin by cells grown as suspension culture.

In no case did the decrease or increase of macro-element level in the nutrient medium increased gametophytic growth. Reduced growth of gametophytes was found to be less condusive to the apogamous shoot formation (Expt. 5-3). Though the number of sporophytic shoots and their growth enhanced with increasing sucrose level upto 4%, no sporangia were noticed even after incubation for over a

year. Incorporation of yeast extract also did not induce sporogenesis (Expt. 5-5).

When the sexually produced as well as apogamously produced plants were screened for any antibacterial principle, the results were negative. The in vivo plants, however, had earlier shown the presence of antibacterial substance(s). One possibility for their absence in plants produced in vitro might be that the active substance(s) could have diffused i.e. leached out into the medium. This possibility needs to be explored in future work.