CHAPTER III

STUDIES ON INITIATION, GROWTH AND DIFFERENTIATION OF RHIZOME TISSUES OF PTERIS VITTATA L. IN VITRO

STUDIES ON INITIATION, GROWTH AND DIFFERENTIATION OF RHIZOME TISSUES OF PTERIS VITTATA L. IN VITRO

Experiments described in the present chapter were aimed at successful establishment of callus tissues from the rhizome of Pteris vittata L. White's (1954) medium containing inorganic salts, sucrose, coconut milk, casein hydrolysate and supplemented with growth hormones was used for callus initiation. Knudson's (1925) medium as modified by Steeves et al. (1955), with and without coconut milk, was also tested for callus initiation.

To carry out experiments on the differentiation of rhizomatic callus, an adequate supply of callus was essential. Experiments were, therefore, conducted first to study the conditions promoting continuous growth of the callus in culture. Effect of inorganic salts from White's (1954), and Murashige and Skoog's (1962) revised media and their combinations on growth parameters was studied. The morphogenetic and histogenetic pattern of callus differentiation was observed by varying the levels of sucrose and 2,4-D in the culture media. The rhizome segments too were subjected to above sucrose-auxin interactions to examine regenerative products provoked.

Experiment No. 3-1 : Initiation of Callus from Rhizome segments of Pteris vittata L.

Healthy, and vigorously growing rhizome segments of Pteris vittata L. (Figs. 1, 2) were collected from the University Botanical garden. After derooting and defoliating, they were thoroughly washed with tap water. The rhizome segments were then sterilized with 0.1% mercuric chloride for 5 minutes and rinsed with sterile distilled water 3 to 4 times to remove the sterilizing agent. Then the rhizome segments were cut into 1 cm long explants with a sharp sterile scalpel. The explants were transferred to 150 ml Erlenmeyer flasks containing 30 ml of White's medium (Table 1) containing 2% sucrose, 10% coconut milk (CM), 300 mg/l casein hydrolysate (CH) and supplemented with varying concentrations of 2,4-D or NAA. The medium was solidified with 0.8% Difco-Bacto agar. Five replicates were prepared for each treatment. All the cultures were maintained at 26±2°C in culture room illuminated with a white flourescent tube (40W).

After incubation for 8 weeks callus initiation was not observed on the rhizome segments in (no-auxin) control and in any of the concentrations of NAA tested (Table 4). Low concentration of 2,4-D (0.1 mg/l) also

Fig. 1. Herbarium specimen of Pteris vittata L.

Fig. 2. Metaphase plate of leaftip of Pteris vittata L. (2n = 58) (1500X)


Pteris vittata Linn.
Det. - G. Panigrahi.
HERBARIUM
No. MKC 4
Family Pteridaceae
Genus Pteris
Species Vittata (Linn).
Local Name
Locality
Remarks
Date : December 93
Collected by
M. Krishnaraju

Fig.1

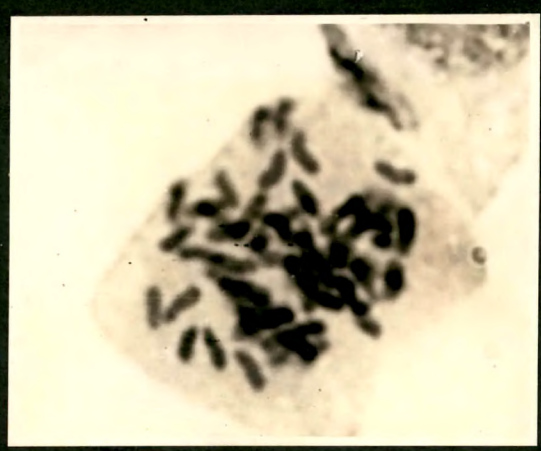

Fig.2

Table 4: Initiation of callus from Rhizome segments of *Pteris vittata* L.

Inoculum : 1 cm rhizome segments in White's medium containing 2% sucrose, 10% CM, 300 mg/l CH and supplemented with different concentrations of either 2,4-D or NAA.

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

No.	Auxin conc. (mg/l)	Callus initiation
1	Control (no auxin)	nil
2	0.1 2,4-D	nil
3	1.0 "	poor
4	2.0 "	maximum
5	0.1 NAA	nil
6	1.0 "	nil
7	2.0 "	nil

Results are representative of five replicates.

did not induce callus initiation, while callus development was maximum at 2 mg/l 2,4-D (Figs. 3,4). Initially the callus, which developed at the cut ends of the explant and then proliferated all over the explant, was deep green in colour. After 6 weeks, however, its colour changed to light brown.

Experiment No. 3-2 : Regeneration and controlled Differentiation from Rhizome segments of Pteris vittata L.

Rhizome segments after derooting and defoliating them from garden plants were sterilized as described in Chapter II, Materials and Methods 3,B. Excised segments were inoculated in 150 ml Erlenmeyer flasks containing 60 ml of White's medium (Table 1) with 10% CM, 300 mg/l CH, and a range of sucrose and 2,4-D concentrations, singly and in combinations, as shown in the chart below:

2,4-D (mg/l) \ Sucrose (%)	0.1	0.1	1.00	2.00
0.0	(1)	(5)	(9)	(13)
0.1	(2)	(6)	(10)	(14)
1.0	(3)	(7)	(11)	(15)
2.0	(4)	(8)	(12)	(16)

Bracketed Nos. indicate Treatment Number.

Fig. 3. Callus initiation on rhizome explants grown on White's medium supplemented with 10% CM, 2% sucrose, and 2,4-D concentrations (0.1, 1.0 and 2.0 mg/l)

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

Fig. 4. Metaphase plate of above callus (1500X)

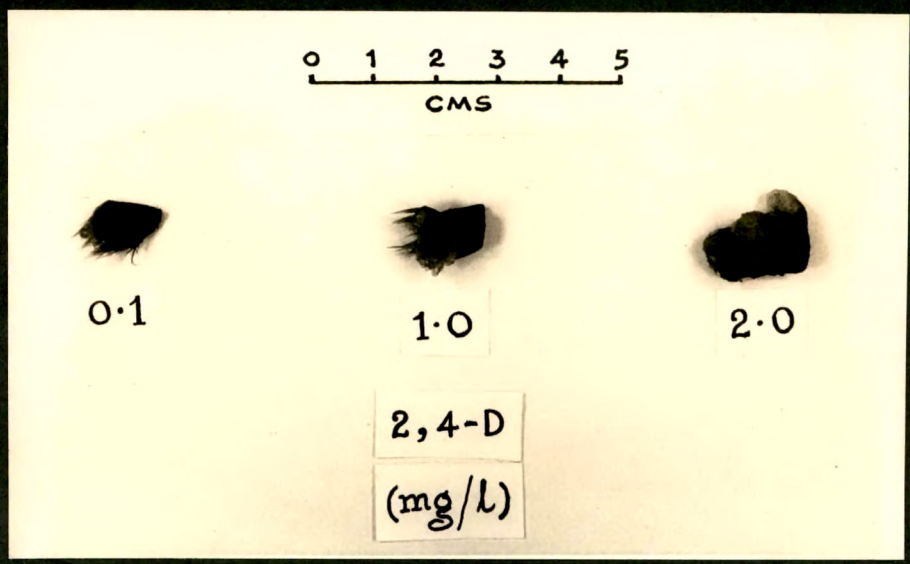

Fig.3

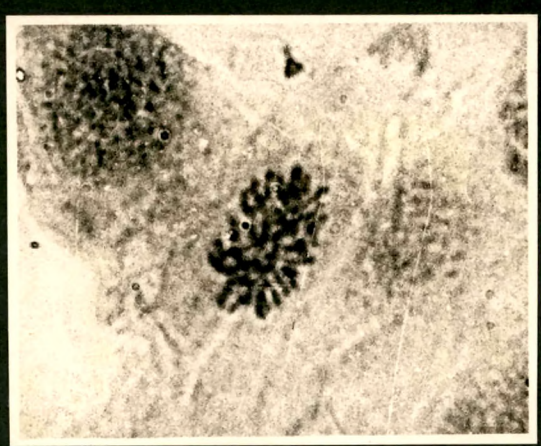

Fig.4

Observations were made after incubation at 26±2°C in lighted culture room for 8 weeks. Since certain treatments elicited responses after prolonged period of incubation, observations were made upto 16 weeks.

In complete absence of sucrose and auxin, in presence of 2,4-D either without sucrose or with low concentration (0.1%) of sucrose (treatment nos. 1,2,6), regenerative outgrowths were not observed. After prolonged incubation (16 weeks), however, in the absence of sucrose, coconut milk and 2,4-D, formation of aposporous gametophytes was noticed in some (20%) cases. These gametophytes were first filamentous, and then became cordate. The filamentous and cordate forms often clustered together to form green pad-like structure.

Apparently, 2,4-D in absence of sucrose (treatments 2,3,4) was not capable of initiating callus development on excised segments. Incorporation of low sucrose level (treatments 7, 8), however, resulted in limited cell proliferations giving rise to callus which failed to grow continuously.

In complete absence of 2,4-D (treatments 9, 13), on the other hand, transition was noticed from cylindrical

structures (Fig. 5) to many sporophytic shoots (Fig.7) with increase in sucrose level (treatments 5, 9, 13). The number of sporophytes formed and their developmental pattern fully corresponded with the level of sucrose in the media. Initially on the rhizome segments, a kind of outgrowth in the form of hemispherical cushion was seen on which scales were differentiated. Continuous proliferations at these hemispherical cushions subsequently resulted into the organization of many sporophytic apices. These apices later on developed into complete sporophytes. Thus the establishment of many primordia often resulted in a dense sporophytic population on the rhizome segments (Figs. 6, 7).

At 1% and 2% sucrose with 1 mg/l 2,4-D (treatments 11,15), callus developed on the rhizome segment from where the outgrowth shoots originated (Fig. 8). The callus thus formed was smooth, 'nodular' and sloppy mass (Fig. 9) when subcultured on the same medium. With 2% sucrose and 2 mg/l 2,4-D (treatment 16), maximum callus development occurred in all the replicate cultures. The callus growth was rapid and it was highly granular in appearance and friable in nature (Fig. 10). The above

Fig. 5. Formation of cylindrical structures on rhizome explant grown on White's medium containing 10% CM and 0.1% sucrose

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

Fig. 6. Formation of many sporophytic apices on rhizome explant grown on White's medium containing 10% CM and 2% sucrose

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

Fig. 7. Development of many sporophytic shoots from the rhizome explant

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

Fig.5

Fig.6

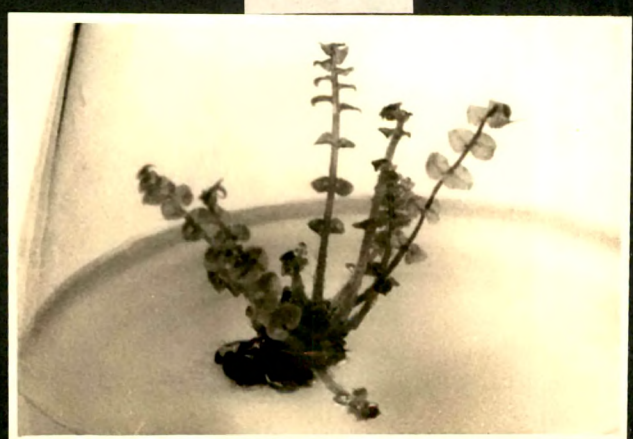

Fig.7

Fig. 8. Development of callus and shoots on rhizome explants grown on White's medium containing 10% CM, 2% sucrose and 1.0 mg/l 2,4-d

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

Fig. 9. Formation of nodules on the callus developed on rhizome explants grown on White's medium containing 10% CM, 2% sucrose and 1.0 mg/l 2,4-D

Fig. 10. Callus initiated from rhizome explant grown on White's medium containing 10% CM, 2% sucrose and 2.0 mg/l 2,4-D showing profuse growth when subcultured on the same medium

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

Fig.8

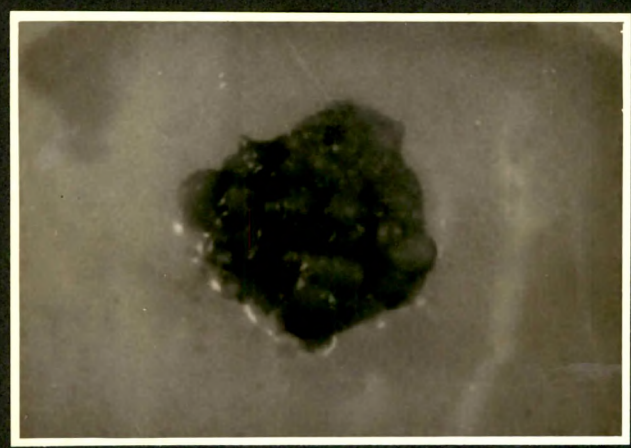

Fig.9

Fig.10

difference in callus morphology might be ascribed to the relative amount of 2,4-D in the medium.

Experiment No. 3-3 : Effect of Macroelements as present in different culture media on Growth of Pteris vittata L. (rhizome) callus

This experiment was undertaken to evolve a medium which would support rapid and continuous growth of rhizome callus. For the support of growth of rhizomatic callus relative merits of White's (1954) medium (A) containing 10% CM with Murashige and Skoog's (1962) completely defined medium (B) was examined. A third medium (C) which constituted a combination of macro-elements as present in White's medium with micro-elements and vitamins as present in Murashige and Skoog's medium was also tested.

Weighed pieces (100±10 mg) of rhizomatic callus were inoculated in 150 ml Erlenmeyer flasks containing 30 ml of culture medium A, B, or C. Five replicates were kept for each treatment in culture room at 26±2°C in continuous light.

The growth measurements were made (as described in Chapter II, Materials and Methods 5, A & B) after 4 weeks,

Table 5: Effect of Macroelements as present in different culture media on growth of Pteris vittata L. rhizome callus

Inoculum : 100±10 mg callus (Dry weight: 6.023 mg) in
Medium A: White's medium (Table 1) + 2% sucrose + 10% CM + 300 mg/l CH + 2 mg/l 2,4-D
Medium B: Murashige and Skoog's medium (Table 3) + 2% sucrose + 2 mg/l + 2,4-D
Medium C: White's macroelements + Murashige and Skoog's microelements and vitamins + 2% sucrose + 10% CM + 2 mg/l 2,4-D
Incubation: 4 weeks at 26±2°C in light

Medium	Fresh wt. (mg)	Dry wt. (mg)
A	1042.0 (±3.12)	56.5 (±1.32)
B	528.0 (±5.2)	28.0 (±1.3)
C	823.7 (±3.5)	36.5 (±2.2)

Figures in parentheses represent standard error.

showed that the highest growth measured by fresh (10 fold increase) and dry (9 fold increase) weights was obtained on White's medium (A) containing coconut milk (Table 5, Fig. 11). Medium C, a combination of both A and B media with coconut milk supported good growth (fresh wt : 8 fold; and dry wt : 6 fold). In Murashige and Skoog's complete synthetic medium (B) growth was relatively poor. Coconut milk appeared to be essential for rapid growth of callus tissues.

Experiment No. 3-4 : Growth curve of Pteris vittata L. rhizome callus

The progress in growth of callus grown in optimal medium (Experiment No. 3-3) was examined in this experiment. Callus pieces weighing 100±10 mg were inoculated in culture vessels containing 30 ml of White's medium with 2% sucrose, 10% CM, 300 mg/l CH and supplemented with 2 mg/l 2,4-D. The culture vessels were incubated in light at 26±2°C for six weeks. Five of the replicate flasks were harvested after every two weeks in order to measure growth by increase in fresh weight and dry weight (as described in Chapter II, Materials and Methods 5, A & B).

Fig. 11 : Effect of Macroelement salts as present in different culture media on growth of Pteris vittata L. callus.

Experimental details as given in Table 5.

The results obtained are shown in Table 6 and Fig. 12A and B.

The graph of growth parameters plotted against time showed the typical sigmoid curve (Fig. 12A). At the end of incubation period of six weeks, 24 fold increase in fresh weight and 21 fold increase in dry weight was observed. Data plotted on semilogarathmic basis (Fig. 12B) indicated that growth as measured by fresh and dry weights was most rapid during the first two weeks after which it maintained rather a steady rise.

Experiment No. 3-5: Effect of inorganic nutrients on Growth of Pteris vittata L. callus

Effect of macro- and microelement salts supplied at different levels in culture medium on growth of rhizomatic callus was studied.

Weighed pieces (100±10 mg) of callus were transferred to culture vessels containing White's medium supplemented with 2% sucrose, 10% CM and 300 mg/l CH. The level of macro- and micro-element salts was raised from zero to double the standard level.

Table 6: Growth curve of Pteris vittata L. rhizome callus in culture

Inoculum : 100±10 mg callus (Dry weight: 6.023 mg) in White's medium containing 2% sucrose, 10% CM, 300 mg/l CH and 2 mg/l 2,4-D

Incubation: 6 weeks at 26±2°C in continuous light

Time (weeks)	Fresh wt. (mg)	Dry wt. (mg)
0	100 (±10.0)	6.023 (±0.9)
2	501.8 (±8.5)	30.2 (±0.25)
4	1045.0 (±5.34)	56.5 (±1.2)
6	2401.5 (±5.4)	128.0 (±0.7)

Figures in parentheses represent standard error.

Fig. 12A. Growth curve of Pteris vittata L. callus culture

100±10 mg tissue in 30 ml of White's medium (Table 1).

Other experimental details as given in Table 6.

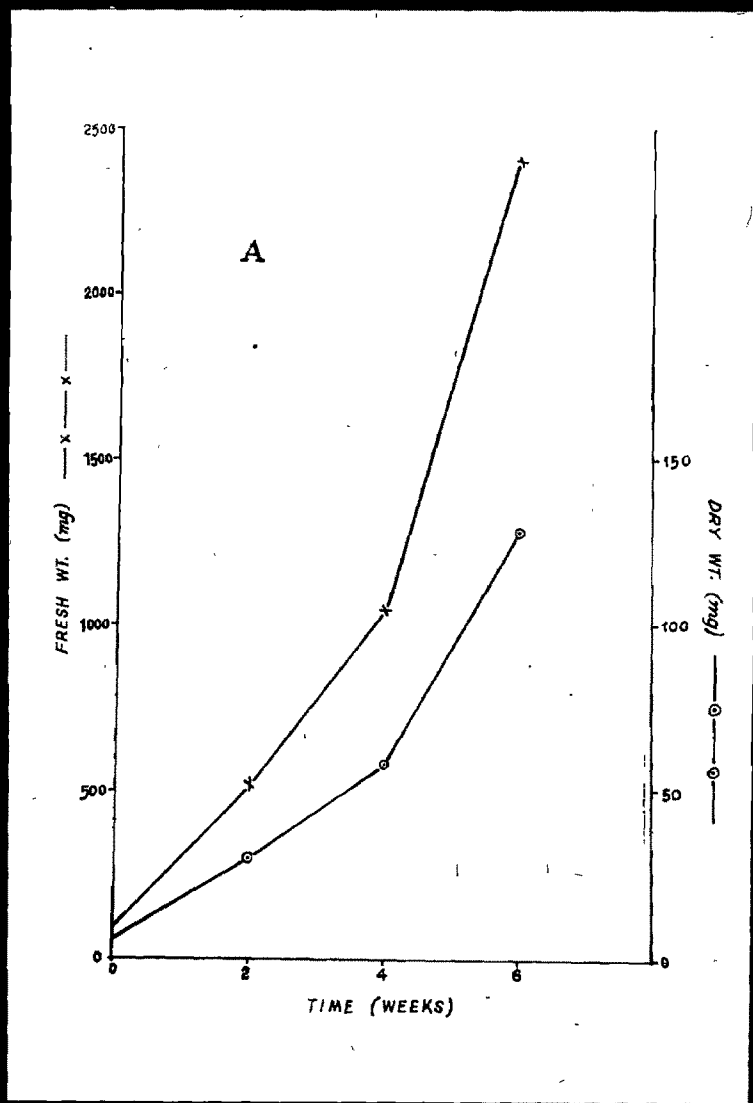
A
2500
2000
1500
1000
500
0
FRESH WT. (mg) —x—x—x—
150
100
50
0
DRY WT. (mg) —⊙—⊙—⊙—
0
2
4
6
TIME (WEEKS)

Fig.12A

Fig. 12B. Growth curve (on semilogarithmic basis) of *Pteris vittata* L. callus culture

100±10 mg tissue in 30 ml of White's medium (Table 1).

Other experimental details as given in Table 6.

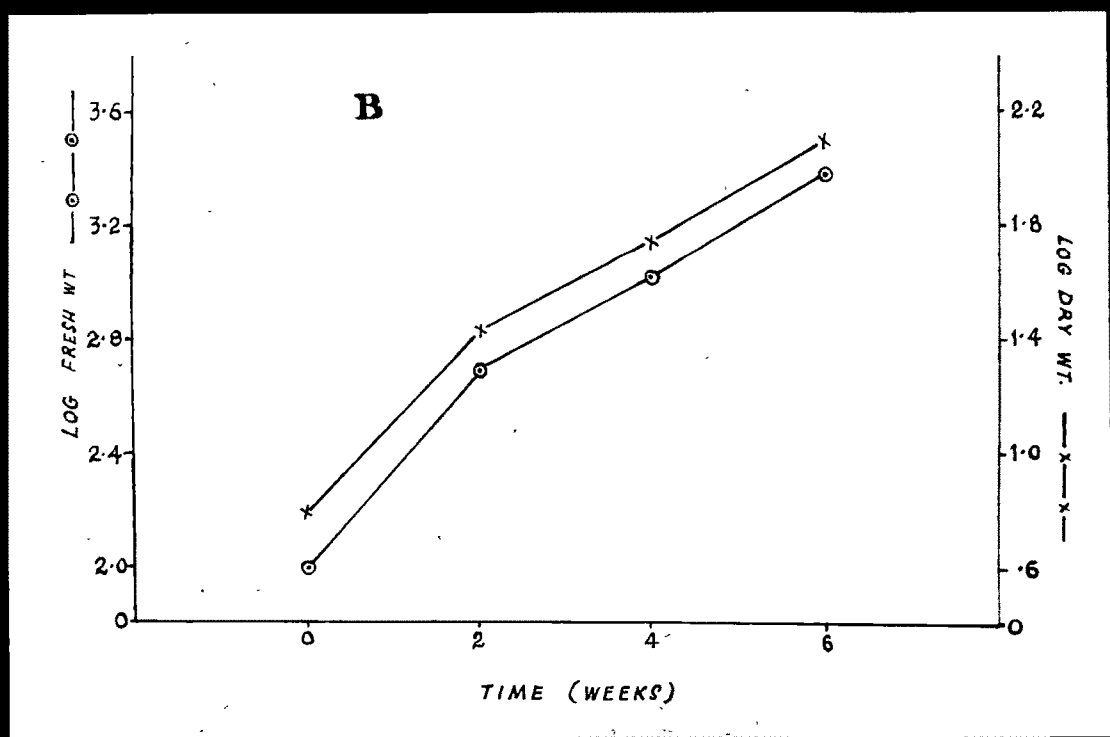
B
LOG FRESH WT
3·6
3·2
2·8
2·4
2·0
0
LOG DRY WT.
2·2
1·8
1·4
1·0
·6
0
0
2
4
6
TIME (WEEKS)

Fig.12B

The data presented in Tables 7 and 8 and Fig.13A and B, showed that growth as measured by increase in fresh (10 fold) and dry weights (9 fold) was maximum at single dose of macro-elements and also single dose of micro-elements, i.e. both as present in the standard medium (control). Doubling the concentrations of either showed marked reduction in growth as measured by fresh and dry weights; and total absence of either micro- or macro-element salts caused drastic retardation of growth.

Experiment No. 3-6 : Morphogenetic effect of Sucrose and 2,4-D Interactions on Pteris vittata L. rhizome callus

The callus was transferred to sucrose and auxin free medium for a week before being used as inoculum in this experiment in order to minimise any carry-over effect White's medium containing 10% CM and 300 mg/l CH was supplemented with various combinations of sucrose and 2,4-D at concentrations as shown in the chart below:

Table 7 : Effect of different levels of macro-element salts on growth of Pteris vittata L. callus

Inoculum : 100±10 mg callus (Dry weight: 6.023 mg) in 30 ml culture medium containing 2% sucrose, 10% CM, 300 mg/l CH, 2 mg/l 2,4-D, White's micronutrients and X0, X1, or X2 White's macroelement salts.

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

Macroelement level	Fresh wt. (mg)	Dry wt. (mg)
X0	102.0 (±1.4)	6.68 (±0.15)
X1 (control)	1042.5 (±3.1)	56.5 (±1.2)
X2	369.0 (±4.3)	18.6 (±2.6)

Figures in parentheses represent standard error.

Fig. 13A. Effect of different levels of macroelement salts on growth of Pteris vittata L. callus

Experimental details as given in Table 7.

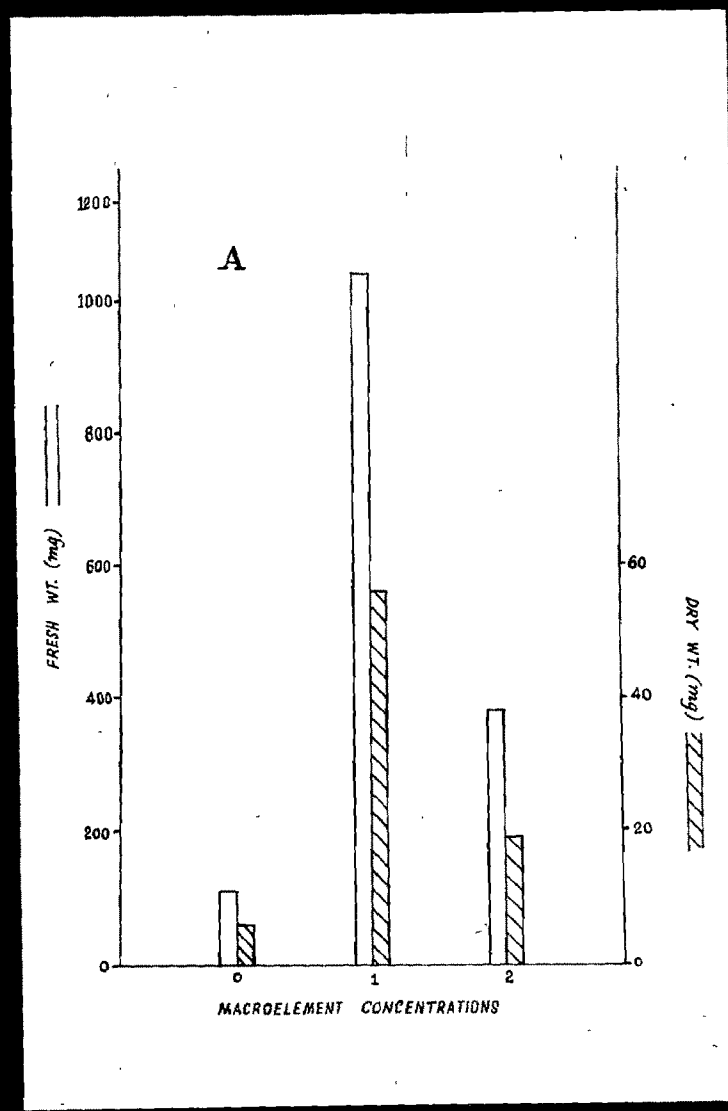
A
1200
1000
800
600
400
200
0
FRESH WT. (mg)
60
40
20
0
DRY WT. (mg)
0
1
2
MACROELEMENT CONCENTRATIONS

Fig.13A

Table 8 : Effect of different levels of micro-element salts on growth of Pteris vittata L. callus

Inoculum : 100±10 mg callus (Dry weight: 6.023 mg) in 30 ml culture medium containing 2% sucrose, 10% CM, 300 mg/l CH,2 mg/l 2,4-D, White's macronutrients and X0, X1 or X2 White's microelement salts.

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

Microelement level	Fresh wt. (mg)	Dry wt. (mg)
X0	126.0 (±2.12)	7.2 (±0.05)
X1	1042.5 (±3.1)	56.5 (±1.2)
X2	506.0 (±2.06)	30.5 (±0.4)

Figures in the parentheses represent standard error.

Fig. 13B. Effect of different levels of microelement salts on growth of Pteris vittata L. callus

Experimental details as given in Table 8.

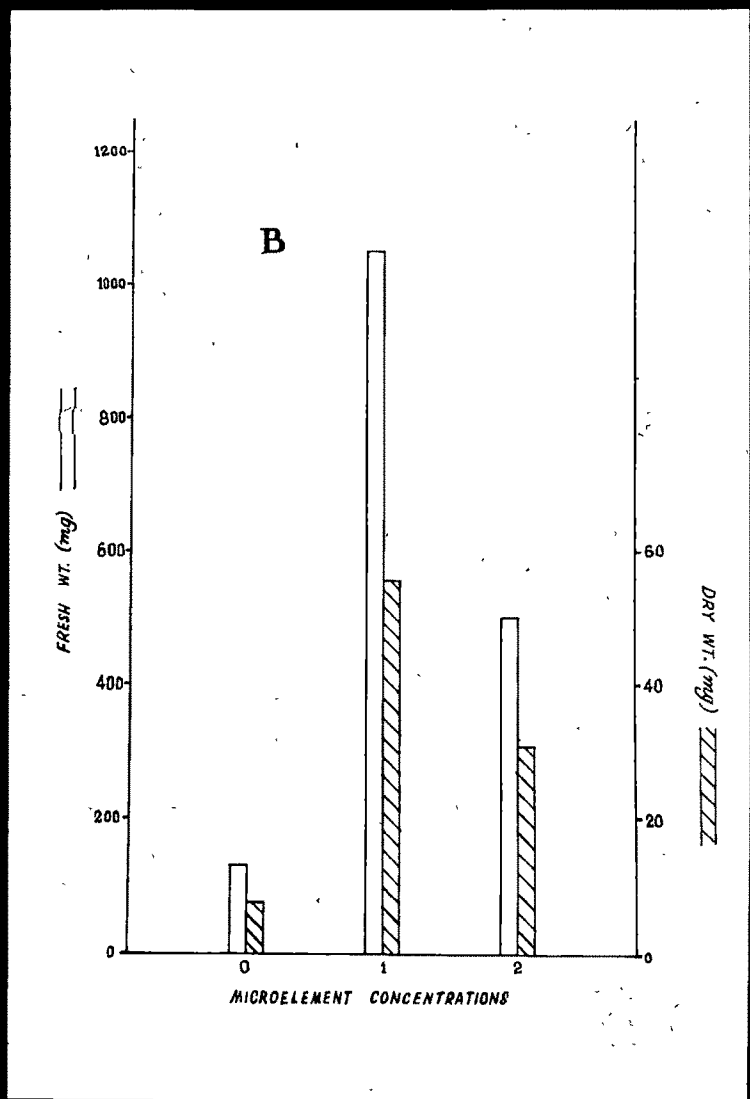
B
1200
1000
800
600
400
200
0
FRESH WT. (mg)
60
40
20
0
DRY WT. (mg)
0
1
2
MICROELEMENT CONCENTRATIONS

Fig.13B

		Sucrose (%)			
		0.0	0.1	1.0	4.0
2,4-D (mg/l)	0.0	(1)	(4)	(7)	(10)
	0.2	(2)	(5)	(8)	(11)
	2.0	(3)	(6)	(9)	(12)

Bracketed nos. indicate Treatment number

Callus tissues measuring about 1.0 cm in diameter were inoculated in 60 ml of the culture medium. Observations noted after 4 weeks were continued upto 12 weeks. Pattern of morphogenesis during this period was observed and representative replicates photographed.

In the absence of 2,4-D, increasing sucrose level did not promote callus growth (treatments 1, 4, 7, 10). However, after scanty hair development, cylindrical structures were differentiated on callus in low sucrose medium (1% sucrose, treatment 7) within 4 weeks (Fig.14). Similar structures appeared on very low sucrose medium (0.1% sucrose, treatment 4) after 8 weeks. In presence of high sucrose level (4% sucrose, treatment 10), though the callus turned brown, it developed profuse hairs over it in patches. After 6 weeks, sporophytic leaves

developed on such spots (Figs. 15, 16, 17). Fig. 18 shows the metaphase plate in the leaftip squash of leaves so regenerated. By the end of 12 weeks, the number of sporophytic leaves had increased and some roots also were produced (Fig. 19).

In presence of high (2.0 mg/l) 2,4-D concentration growth of the callus enhanced with increasing sucrose level in the medium (treatments 3, 6, 9, 12). In absence of sucrose as well as at its low concentration (treatments 3, 6), the callus growth was poor, but the tissue became profusely hairy (Fig. 20). On the other hand, callus growth was vigorous at higher level of sucrose (treatments 9, 12) without any hair formation (Fig. 21).

In low auxin (0.2 mg/l 2,4-D) medium, increasing sucrose concentration (treatments 2, 5, 8) turned the callus distinctly nodular without any marked effect on its growth (Fig. 22). In presence of high sucrose level (4% sucrose, treatment 11) callus growth was accompanied by development of hairs in patches and after incubation for 8 weeks, root primordia emerged from the callus mass. After 12 weeks not only many roots were produced but some sporophytic leaves also were developed (Figs. 23, 24).

Fig. 14. Callus grown on White's medium containing 10% CM and 1% sucrose differentiated into cylindrical structures

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

Fig. 15. Callus grown on White's medium containing 10% CM and 4% sucrose (no auxin) covered with hairs and sporophytic leaves developed at such spots

Incubation: 6 weeks in light at 26±2°C

Fig. 16. Callus grown on White's medium containing 10% CM and 4% sucrose (no auxin) covered with hairs and more leaves developed at such spots

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

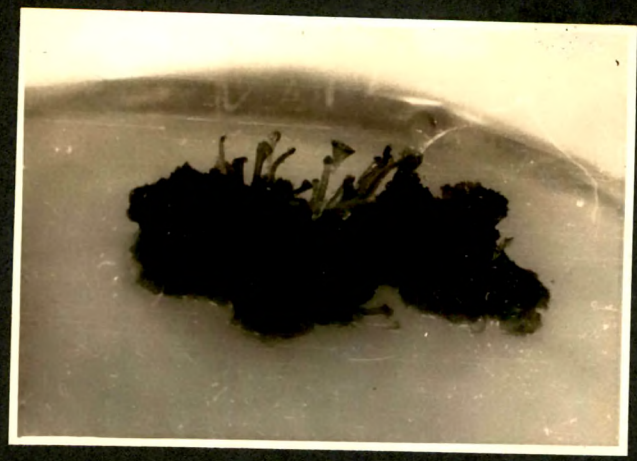

Fig.14

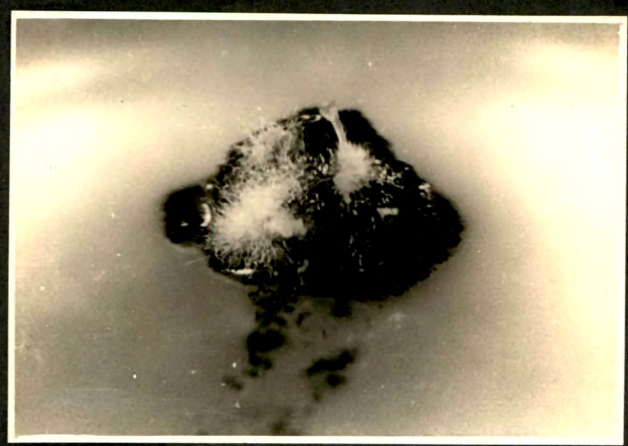

Fig.15

Fig.16

Fig. 17. Callus grown on White's medium containing 10% CM and 4% sucrose (no auxin) showing many leaves produced

Incubation: 10 weeks in light at 26±2°C

Fig. 18. Metaphase plate of the above leaftip (1500X)

Fig. 19. Formation of leaves and roots from callus grown on White's medium containing 10% CM and 4% sucrose (no auxin)

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

Fig.17

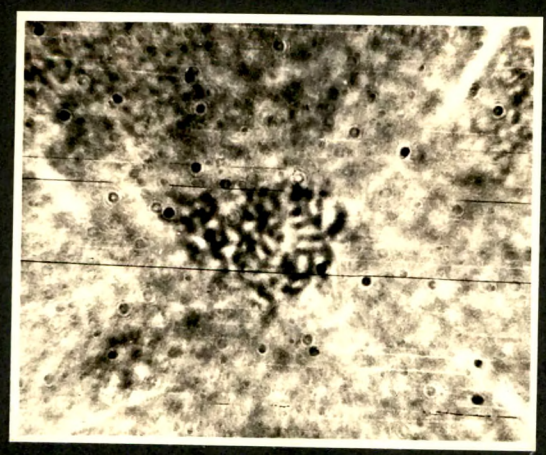

Fig.18

Fig.19

Fig. 20. Callus grown on White's medium containing 10% CM and 2.0 mg/l 2,4-D showing formation of hair over it

Incubation: 8 weeks in light at $26 \pm 2°C$

Fig. 21. Callus grown on White's medium containing 10% CM, 4% sucrose and 2.0 mg/l 2,4-D showing unorganised growth

Incubation: 8 weeks in light at $26 \pm 2°C$

Fig.20

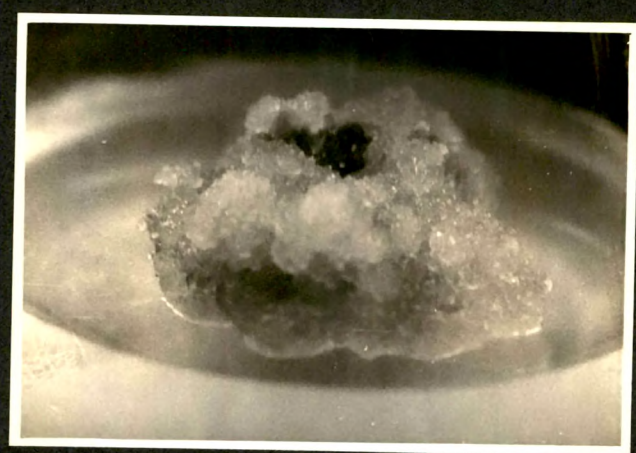

Fig.21

Fig. 22. Development of nodules on callus grown on White's medium containing 10% CM and 0.2 mg/l 2,4-D

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

Fig. 23. Callus grown on White's medium containing 10% CM, 4% sucrose and 0.2 mg/l 2,4-D covered with hairs from where roots were differentiated

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

Fig. 24. Development of roots and leaves on the callus grown on White's medium containing 10% CM, 4% sucrose and 0.2 mg/l 2,4-D

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

Fig.22

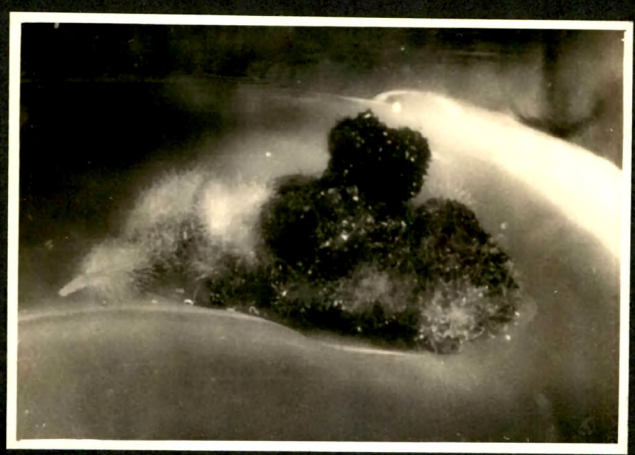

Fig.23

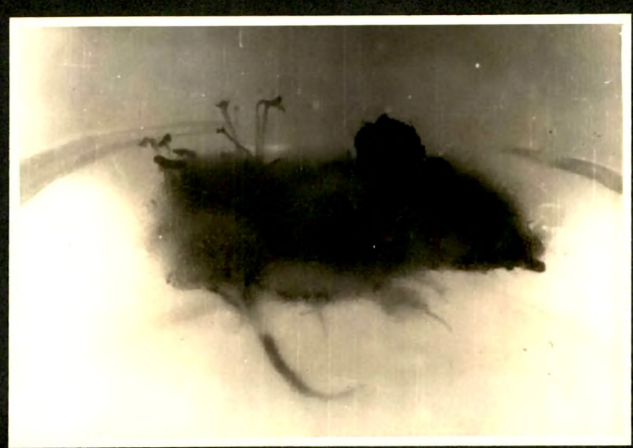

Fig.24

There was no formation of gametophytes in any of the treatments given, including no-auxin no-sucrose control (treatment 1). This was possibly on account of sugars present in the CM which was added to the medium. The callus was, therefore, transferred to White's basal medium without CM in an attempt to regenerate gametophyte. It was observed after growth for 12 weeks on this medium that the callus which had turned black did differentiate gametophytes (Fig. 25).

Experiment No. 3-7 : Histological studies of callus subjected to sucrose-2,4-D Interactions

Histological examination was made in the callus tissues treated with different concentrations and combinations of sucrose and 2,4-D for morphogenetic (organogenic) responses in the previous experiment.

Development of hair on (rhizome) callus was found to give a prior indication of organogenic differentiation. This was confirmed when the hairy callus was serially sectioned (see Chapter II, Materials and Methods 7 for detailed procedure). Callus which became hairy when grown for 4 weeks in high sucrose medium (4% sucrose,

Fig. 25. Callus grown on White's medium (no CM, no sucrose and no auxin) showing formation of aposporous gametophytes

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

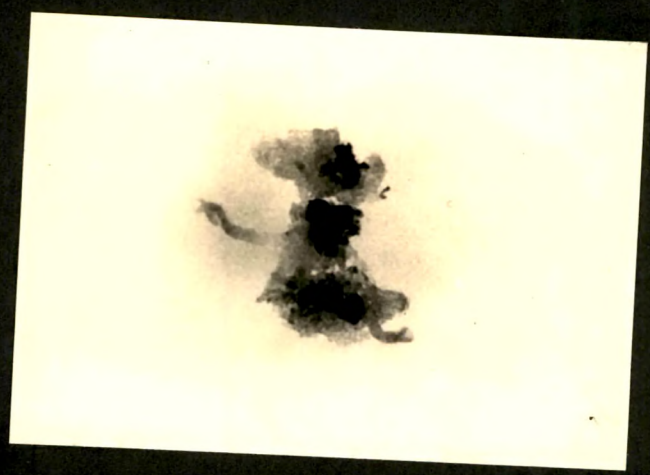

Fig.25

Fig. 26. Photomicrograph of a section of callus showing nodules with meristematic cells (100X)

Incubation: 4 weeks in light at 26±2°C on White's medium containing 10% CM and 4% sucrose (no auxin)

Fig. 27. Photomicrograph of a section of callus showing well organised nodules with meristematic cells (150X)

Incubation: 4 weeks in light at 26±2°C on White's medium containing 10% CM and 0.2 mg/l 2,4-D

treatment 10) which lacked auxin, showed islets or nodules of meristematic cells in superficial layers (Fig. 26). Such nests or nodules appeared better organised and defined when auxin was present in low concentration (treatments 2, 8, 11; Fig. 27). These nodules seem to behave like embroys and depending upon the concentrations of exogenously supplied sucrose and/or auxin, they gave rise to shoots (Fig. 17) or roots (Fig. 23) or both (Fig. 19).

Leaf-primordia developed from such embryo-like structures after 8 weeks in callus grown on high sucrose medium (4% sucrose, treatment 10). In the same callus after prolonged incubation (12 weeks) root primordia developed from the opposite side of the embryo-like structure (Figs. 28, 29). Thus in high sucrose medium the leaves were produced first, and roots were produced much later.

In presence of 2,4-D, on the other hand, first roots were differentiated from the embryo-like structures (4% sucrose and 0.2 mg/l 2,4-D, treatment 11, Fig. 30) and the leaves developed at a much later stage (after 12 weeks). In low sucrose medium (1% sucrose, treatment 8) though the root primordia were differentiated (in

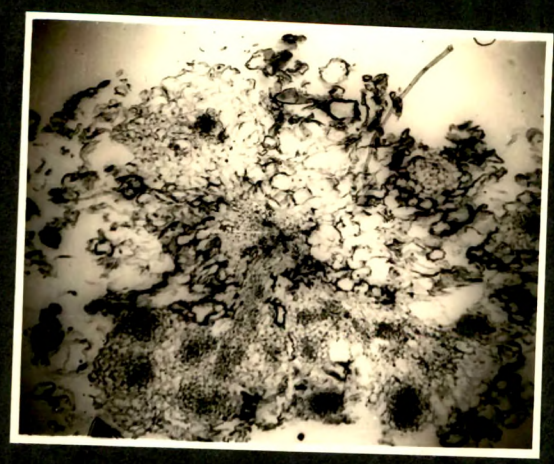

Fig.26

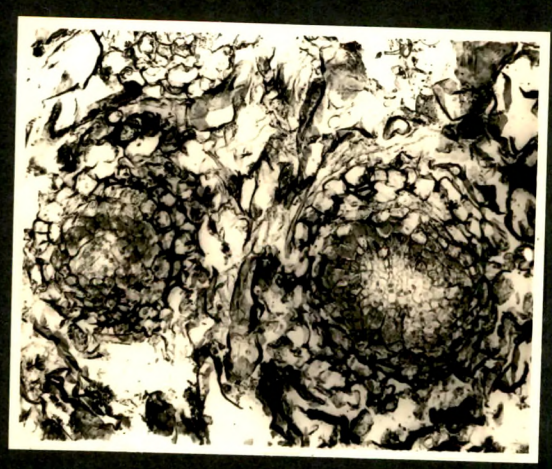

Fig.27

Fig. 28. Photomicrograph of a section of callus showing formation of leaf-primordia (100X)

Incubation: 8 weeks in light at 26±2°C on White's medium containing 10% CM and 4% sucrose

Fig. 29. Photomicrograph of a section of callus showing differentiation into leaf (L) and root (R) (100X)

Incubation: 12 weeks in light at 26±2°C on White's medium containing 10% CM and 4% sucrose

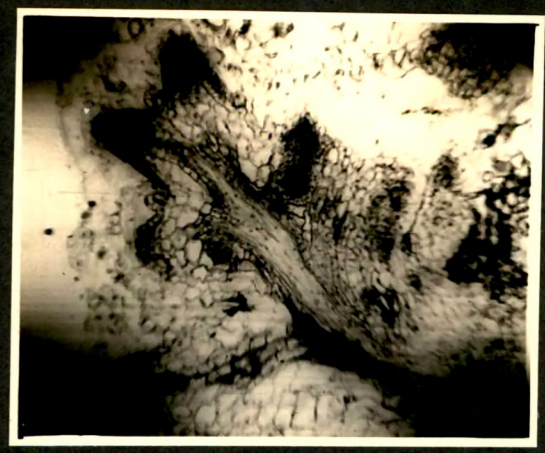

Fig.28

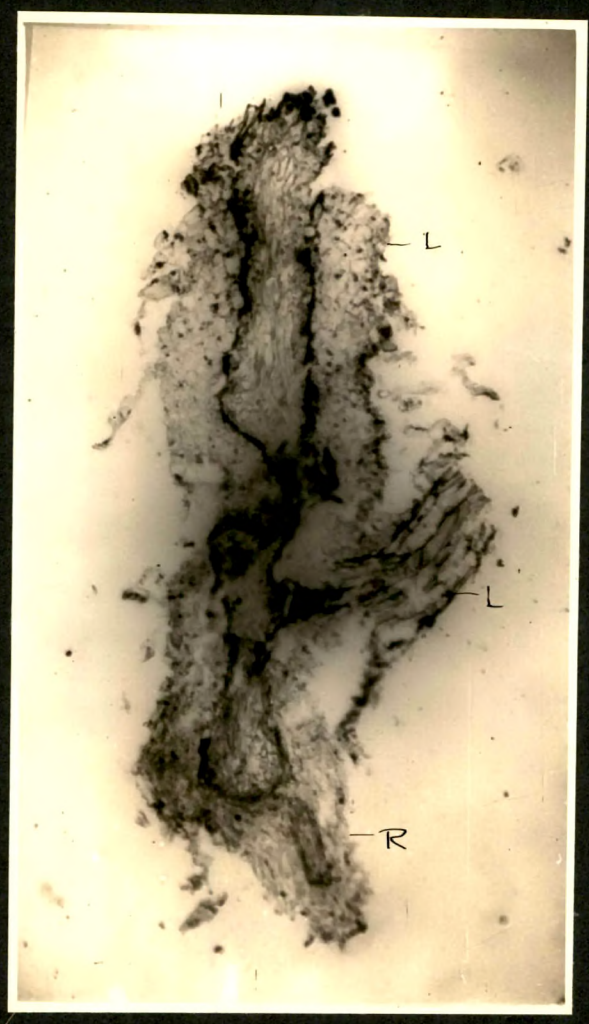

Fig.29

Fig. 30. Photomicrograph of a section of callus showing root formation (150X)

Incubation: 8 weeks in light at 26±2°C on White's medium containing 10% CM, 4% sucrose and 0.2 mg/l 2,4-D.

Fig. 31. Photomicrograph of a section of callus showing root-primordia (100X)

Incubation: 12 weeks in light at 26±2°C on White's medium containing 10% CM, 1% sucrose and 0.2 mg/l 2,4-D

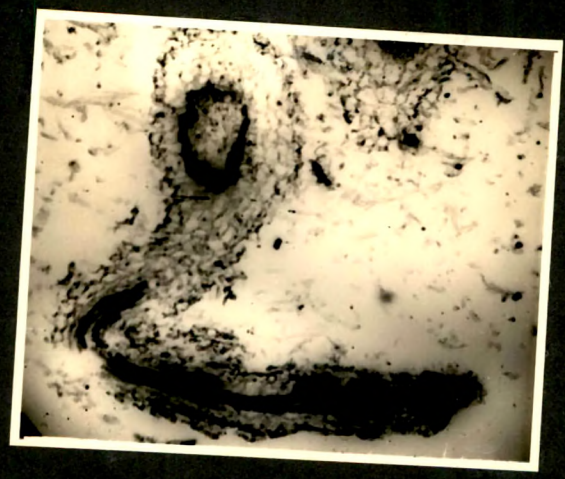

Fig.30

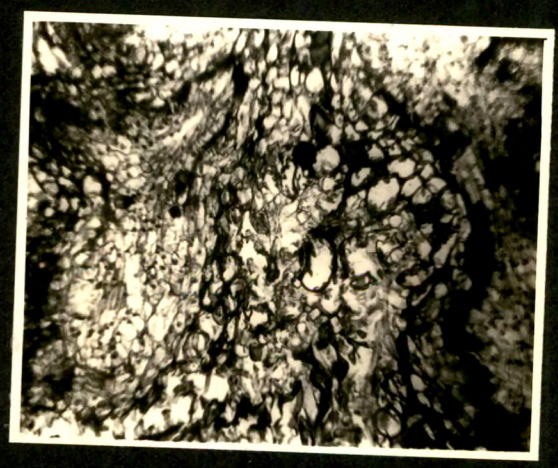

Fig.31

presence of 0.2 mg/l 2,4-D), they failed to develop further and emerge out of the callus body even after 12 weeks (Fig. 31). In total absence of sugar (treatment 2), the embryo-like structures although well defined and having established axial polarity did not differentiate into any organ primordia.

Experiment No. 3-8: Effect of NAA on Pteris vittata L. rhizome callus

Callus tissues previously maintained on White's medium containing 2 mg/l 2,4-D and 1% (A), 2% (B), or 4% (C) sucrose were ~~was~~ subjected to NAA treatment in presence or absence of sucrose. Prior to the inoculation of the experiment, the callus grown on standard medium i.e. containing 2% sucrose and 2 mg/l 2,4-D (Medium B here) was transferred to auxin and sucrose free medium for a period of one week to eliminate carry-over effect. The sucrose/NAA combinations tested for morphogenetic responses are shown in the chart below:

NAA (mg/l)	Sucrose (%)	
	0.0	1.0
0.1		
1.0		

It was observed that the callus previously grown on medium A (1% sucrose and 2 mg/l 2,4-D) formed numerous roots after growth for 8 weeks on medium containing 1 mg/l NAA, with or without sucrose (Fig.32). On the other hand, the callus previously grown on medium C (4% sucrose and 2 mg/l 2,4-D) did not give any morphogenetic response on the NAA containing media. However, the callus maintained on the standard medium B (2% sucrose and 2 mg/l 2,4-D) and kept on sucrose-auxin free medium for a week produced leaves and roots in media containing 1% sucrose, and low (0.1 mg/l) or high (1.0 mg/l) NAA respectively (Figs. 33, 34). After a prolonged period of culture roots were also formed on leaf bearing callus in low auxin medium; but on high auxin medium only roots developed.

Experiment No. 3-9 : Colony formation from Sporophytic Cell Suspensions of Pteris vittata L.

Suspension cultures were established by transferring healthy looking callus mass to liquid medium of the same composition (Table 1) as described in Chapter II, Materials and Methods 4, C. The friable callus produced a thick suspension of cells after agitation for two weeks on a

Fig. 32. Profuse root formation on callus from medium A: (Expt. 3-8) grown on White's medium containing 10% CM, 1% sucrose and 1.0 mg/l NAA

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

Fig. 33. Formation of leaves and roots on callus from medium B: (Expt. 3-8) grown on White's medium containing 10% CM, 1% sucrose and 0.1 mg/l NAA

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

Fig. 34. Formation of roots on callus from medium B: (Expt. 3-8) grown on White's medium containing 10% CM, 1% sucrose and 1.0 mg/l NAA

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

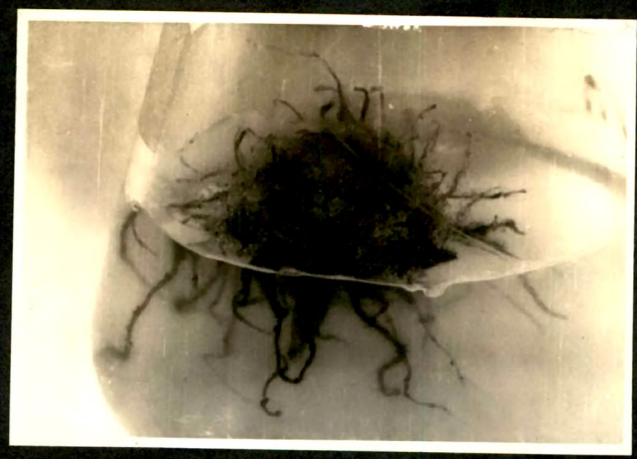

Fig.32

Fig.33

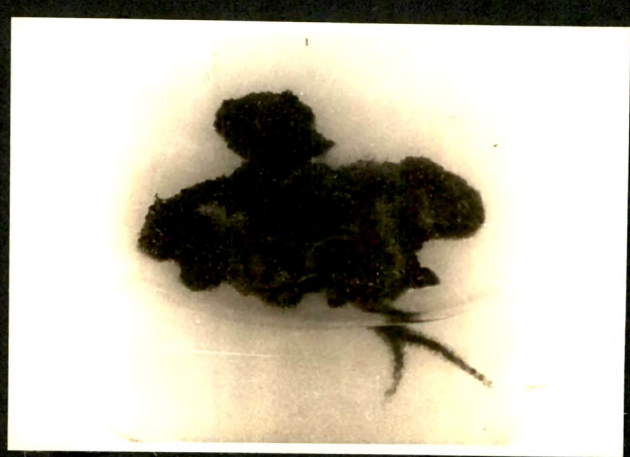

Fig.34

horizontal rotary shaker installed in the culture room. The suspension which consisted of free cells and cell aggregates of various sizes, was filtered aseptically through a nylon mesh of 300 μ pore size. The filtrate consisted of single cells and groups of upto 6 cells (Fig. 35). 2 ml of this filtrate was spreaded on 15 ml culture medium of the same composition, but solidified with 0.8% agar.

Visible colonies developed by eight weeks (Fig.36). Such colonies were transferred to culture media which contained sucrose (2%) but no auxin. Within 4 weeks shoot apex and leaf primordia were differentiated on the colonies (Fig. 37). Vascular tissue had developed deep inside such colonies.

DISCUSSION

It was quite evident from Experiments No. 3-1 and 3-2 that initiation of sporophytic callus was possible from the rhizome explants of *Pteris vittata* L. *in vitro*. Out of the various combinations of sucrose and 2,4-D tried, White's medium containing 2% sucrose and supplemented with 2 mg/l 2,4-D and 10% coconut milk showed maximum response for callus initiation. NAA at the concentrations

Fig. 35. Photomicrograph of suspension showing cells (150X)

Incubation: 4 weeks in light at 26±2°C in White's medium containing 10% CM, 2% sucrose and 2.0 mg/l 2,4-D

Fig.35

Fig. 36. Colony formation from the cell suspension plated on White'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. 37. Photomicrograph of a section of the above colony showing organisation of shoot-apex (100X)

Incubation: 4 weeks in light at 26±2°C on White's medium containing 10% CM and 2% sucrose (no auxin)

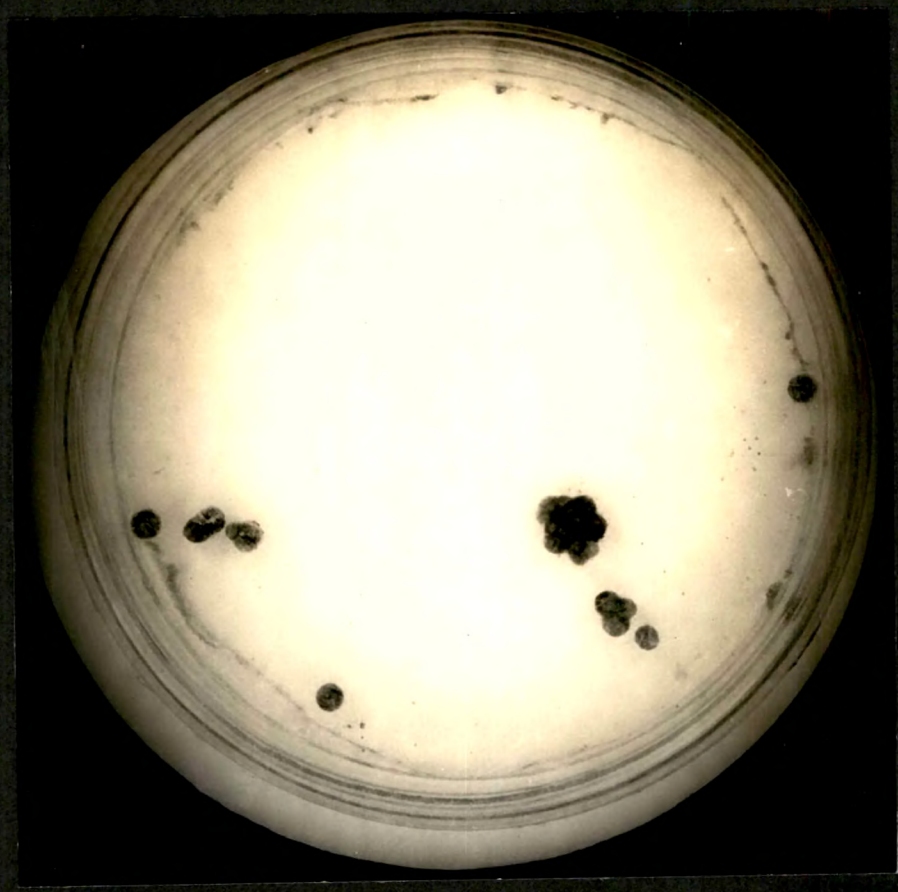

Fig.36

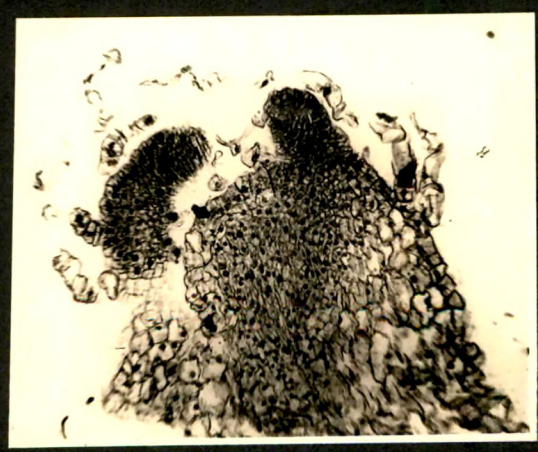

Fig.37

tested failed to induce callus from rhizome segments. It was observed in almost all the cases that an increase in sucrose concentration enhanced the effect of 2,4-D for the initiation of callus. This seemed to indicate the dependence upon an exogenous energy source for auxin activity. Substituting Knudson's medium in place of White's medium, also produced callus from the rhizome explants. Moreover, the callus initiation was rapid if the medium contained coconut milk. Callus initiation from rhizome explants in Knudson's medium, which lacked vitamins indicated the relatively simple nutrient requirements of fern sporophytic tissue in culture.

Rhizome segments in the presence of sucrose showed regenerative outgrowths ranging from cylindrical structures in low sucrose containing media, to the formation of complete sporophytes at higher sucrose level (Expt. 3-2). Aposporous gametophytes were formed when the medium lacked sucrose, auxin and even coconut milk. This aposporous development occurred after prolonged period of culture (16 weeks), by which time the rhizome explant had turned black and its growth had almost ceased. When comparision of three different media to support the growth of callus was made (Expt. 3-3), White's medium proved to be the

best (10 fold increase in fresh weight and 9 fold increase in dry weight). Next best growth (8 fold increase in fresh weight and 6 fold increase in dry weight) was obtained in the coconut milk containing medium having combination of White's macro-elements with micro-elements and vitamins as present in Murashige and Skoog's medium. Doubling the level of macro- or micro-elements in White's medium did not enhance the callus growth but reduced it, when compared to the growth of the callus in the standard medium (Expt. 3-5). This indicated that the salts as present in the standard medium were at optimal level for the callus growth (Expt. 3-4).

Callus when subjected to sucrose-auxin interactions showed differentiation into sporophytic or gametophytic structures. In the auxin-free media containing sucrose the callus mass was first covered with hairs. Later cylindrical structures developed in presence of low (0.1%) sucrose, while leaves were differentiated in high (4%) sucrose medium (Expt. 3-6). However, in presence of 0.2 mg/l 2,4-D, the high (4%) sucrose level invoked the root formation; whereas in low sucrose level the hairy callus became distinctly nodular. Histological examination of such nodular callus revealed organ primordia which were

inhibited in their further development, probably on account of adequate carbohydrate energy source (Expt. 3-7). At higher concentration of 2,4-D (2 mg/l), enhanced unorganised (callus) growth occurred with increasing sucrose level. Aposporous gametophytic outgrowths were observed from the margin of the callus incubated in the medium which lacked sucrose, 2,4-D and coconut milk. Furthermore the callus too, like the rhizome explant, by this time (16 weeks), had turned completely black in colour and stopped growing.

Callus treated with sucrose and NAA combinations produced leaves or roots depending upon the concentration of NAA. In presence of low level (0.1 mg/l) of NAA leaves were formed first and after prolonged incubation, few roots also were organised. On the other hand, only roots were differentiated in media containing high concentration (1.0 mg/l) of NAA (Expt. 3-8).

The callus being friable produced a thick suspension of free cells and cell aggregates on mechanical agitation. When plated on sucrose 2,4-D medium, such suspension gave rise to colonies. On transfer to auxin-free medium containing 2% sucrose, the colonies showed organisation of shoot-apices (Expt. 3-9).