CHAPTER VIII

GENERAL DISCUSSION

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In the present studies an attempt was made towards the establishment of callus cultures from the following members of Filicales:

(1) Pteris vittata L.
Family: Pteridaceae

(2) Adiantum trapeziforme L.
Family: Adiantaceae

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

Maintenance of the tissues in continuous culture on appropriate media and experimental regulation of sporophytic or gametophytic life forms were further examined. The various sporophytic parts used for callus initiation of Pteris vittata L. were:

(1) rhizome segments,
(2) leaves of the garden plants,
(3) leaves from sexually produced sterile plants,
(4) leaves of sporophytes produced apogamously from gametophytic callus, and
(5) roots from sterile sexually produced plants.

Rhizome Callus

Rhizome segments 1 cm in length were excised from healthy Pteris vittata L. plants growing in garden. For callus induction, White's medium (Table 1) with sucrose, coconut milk, casein hydrolysate and supplemented with different concentrations of 2,4-D or NAA were tried. At none of the concentrations of NAA tested, callus was induced on rhizome explants. However, 2,4-D at the concentration 2 mg/l showed maximum callus initiation in almost all the replicates (Expt. No. 3-1). Callus is reported to develop from stem or rhizophore segments of Seglaginella and stem segments including intercalary meristem of Equisetum in sterile culture (Morel, 1956a); but in both the genera the callus was rather ephemeral. Callus induction from the rhizome of Pteris vittata is rather unusual in that such growths on fern rhizome have, to our knowledge, been rarely reported. Laetsch and Brigg's (1961) induced callus on shoot-apices of Marsilea vestita incubated in Knop's medium with Berthelot's trace elements and supplemented with kinetin. In vivo callus induction was observed by Peterson (1967) in Ophioglossum petiolatum at the cut ends of the rhizome explants without the application of externally supplied hormone.

In our studies,in place of White's medium, Knudson's medium was also tried to observe if the salts (nutrients) of Knudson's medium with sucrose, 2,4-D and CM supplied at the same concentrations (i.e. 2%, 2 mg/l and 10% respectively) were capable of callus induction. Callus did develop on the rhizome segments of Pteris vittata incubated on Knudson's above medium which lacked vitamins. This emphasized the relatively simple nutrient requirements of fern sporophytic tissues in culture. DeMaggio and Wetmore (1961) working with Todea barbara presented evidence that single ingredient of the medium may exert profound influences on the morphology of the growing plant or plant part. Callus initiation in Pteris vittata rhizome explants was observed in the presence of sucrose and 2,4-D; either one of them being incapable of inducing callus (Expt. 3-2). Auxin alone was incapable of producing callus in the absence of sucrose probably because of the absence of an adequate supply of energy. A dependence upon an exogenous energy source for auxin activity has been observed not only in cultured cells and tissues of higher plants (Street, 1966), but also in their excised root cultures (Butcher and Street, 1964). Callus also initiated on Pteris vittata rhizome explants incubated on media containing 2% sucrose with lower (1.0 mg/l) 2,4-D concentration; however, some

organised growth persisted on this medium resulting in the formation of few leaves.

Growth of the callus was examined on (1) White's medium supplemented with coconut milk, (2) Murashige and Skoog's (MS) completely defined medium, and (3) a combination of White's macroelement salts with MS micro-elements and vitamins along with coconut milk. The growth measured in terms of fresh and dry weights showed that White's medium (1) supported maximum growth (fresh weight increase 10 fold, dry weight increase 9 fold) in 4 weeks time (Expt. 3-3). The maximum amount of growth on White's medium and on the combination of White's and MS medium, both of which contained 2,4-D and CM indicated synergism between 2,4-D and coconut milk. There is ample evidence of the same in the tissue cultures of higher plants (Steward et al., 1969).

The effect of macronutrients and micronutrients either by lowering or doubling their levels was studied on growth of callus. Neither complete omission nor doubling of their levels enhanced callus growth as measured in terms of fresh and dry weights (Expt. 3-5). It was clear that for the growth of sporophytic callus obtained from

rhizome explant of Pteris vittata, the doses of macro-elements and microelements as present in White's medium are at optimal level.

Some insight into the physiology of regeneration from sporophytic tissues of ferns might be gained if it proved possible to control the type of callus differentiation. Experiments were, therefore, set up to determine whether the type of callus differentiation could be influenced by nutritional or hormonal supplements to the basal medium. Callus was subjected to sucrose auxin (2,4-D) interactions (Expt. 3-6). Callus in complete absence of 2,4-D showed a range of responses ranging from the formation of sporophytic leaves to cylindrical structures according to the carbohydrate level in the medium. Before the morphogenetic change appeared, callus mass was covered with profuse hairs. In presence of sucrose and 2,4-D the callus mass increased, the growth enhancement being proportional to the sucrose content (upto 4%) of the medium when 2,4-D was supplied at 2 mg/l. At low concentration of 2,4-D (0.2 mg/l) in absence of sucrose or at its low concentration, callus showed the formation of spherical nodules. The nodules represented the response of the callus to a supply of appropriate

biochemicals supplied once at a designated place or simultaneously at several places (Wetmore and Rier,1963). But on this medium no organogenesis occurred except the formation of hairs over the nodules. However, when sucrose level was high (4%) with 0.2 mg/l 2,4-D, roots were produced. Thus at lower sucrose concentrations with 0.2 mg/l 2,4-D, though the root primordia were differentiated, they failed to develop further, probably due to lack of sufficient energy source (Expt. 3-6). When histological studies were carried out of the above callus subjected to sucrose-auxin interaction, the nodules formed showed concentric layers of cells which behaved as localised growth centres (Expt. 3-7).

While comparing the angiosperm callus isolated from aerial bulbs of Allium cepa var. proliferum, Fridborg (1971) reported that presence of 2,4-D in the medium promoted callus growth but strongly inhibited the formation of roots and to a lesser degree the formation of buds. There are many much instances where 2,4-D is found to suppress organogenesis and promote unorganised growth of tissues grown in vitro. On the other hand, the callus subjected to sucrose - NAA interaction showed that in the presence of sucrose but in low concentration of NAA (0.1 mg/l) leaves were differentiated; while on high NAA (1.0 mg/l) concentration only roots were produced (Expt. 3-8).

Aposporous gametophytes, were produced from the callus, when it was subjected to sucrose, auxin and coconut milk free medium after very prolonged incubation (Expt. 3-6).

It seemed that the carbohydrate nutrition determines the type of differentiation the rhizome callus would undergo. In its total absence prothalli were produced, while its presence and concentration determined the degree and number of sporophytic leaves.

Suspension culture provides useful techniques to deal with problems concerning growth, differentiation and metabolism of plants at cellular level (Mehta, 1973). The rhizome callus appeared to be well suited for suspension culture by virtue of its friability, high growth rate and clean healthy appearance, all of which are the basic requirements of suspension cultures. The sporophytic callus suspension when plated on sucrose-auxin (2,4-D) medium showed the formation of colonies. These colonies when subcultured on sucrose medium showed formation of shoot apices (Expt. 3-9). Incorporation of 2,4-D in the plating medium resulting again in enhanced callus growth, but inhibition of organ primordia.

Segments excised directly from rhizomes behaved no different from rhizome callus; as revealed in Experiment 3-2 where rhizome explants of Pteris vittata were subjected to different sucrose concentrations. Complete sporophyte regenerated on the rhizome explant in presence of 2% sucrose. In 1% sucrose also sporophytes were produced, but fewer in number; while in still lower concentration of sucrose, cylindrical structures were produced. Further, in absence of sucrose, auxin and coconut milk, gametophytes (aposporous) were produced on the rhizome segment (Expt. 3-2). Working with Phlebodium aureum rhizome segments or sections in culture, Ward (1963) found the development of sporophytic and gametophytic forms at random and often adjacent to each other. Occurrence of the two forms side by side on the same sporophytic segment and under the same apparent conditions as well as development of intermediate forms at slightly varying sucrose concentrations in present investigation suggest the identity of origin of the two generations. The pattern of origin of these forms, however, is different; gametophytic forms appeared by filamentous uprising, while the sporophytic form is regularly preceded by an accumulation of cellular mass. In Ampleopteris prolifera Mehra and Sulklyan (1969)

reported that the concentration of sucrose in the medium and the size of the explant profoundly affected the nature of regeneration. In all these studies, therefore, the carbohydrate content of the medium proved to be the deciding factor for the type of regenerative outgrowths provoked.

Root Callus

Roots of ferns in general have received very little attention in experimental studies as mentioned in the Introduction (Chapter 1). Callus was induced on the primary and lateral meristems (Expt. 4-4). The callus showed the capacity of differentiation into roots on 4% sucrose medium and also on 2% sucrose medium after some more time. After prolonged incubation on Knudson's basal medium, callus showed the formation of aposporous gametophytes. The callus mass had already turned black and along its periphery surface in contact with agar medium few gametophytes were produced (Expt. 4-5).

In Cyclosorus dentatus, Mehra and Palta (1971) induced callus on excised roots. The suspension of this callus when plated on basal medium regenerated gametophytes, on medium with 0.5% sucrose, structures with characteristic

of both gametophyte and sporophyte were observed and on 2% sucrose medium, after the callus had produced sufficient mass, roots were differentiated. Thus in case of Cyclosorus dentatus too, the sucrose seemed to be the triggering factor which brought about the sporophytic or gametophytic expressions.

In our present investigation, studies were also directed towards a definition of growth conditions and media suitable for actively growing root cultures. Excised roots 1 cm in length from the sexually produced plants were grown in liquid White's medium containing sucrose and coconut milk. This medium supported active growth of roots. Knudson's liquid medium with low concentrations of Indole-acetic-acid (IAA) was also tested. The growth of the main axis ceased, but profuse laterals were produced (Expt. 4-2). Partanen and Partanen, (1963) had earlier found that Knudson's medium with IAA was better suited for active growth of roots of Pteridium aquilinum.

Experimental regulation of growth form revealed that kinetin in low concentration stimulated the formation of aposporous gametophytes on the excised roots of

Pteris vittata (Expt. 4-3). When excised roots were grown in Knudson's basal medium as well as in Knudson's medium supplemented with different concentrations of kinetin, in light and dark, it was observed that in the basal medium containing kinetin (0.02 mg/l) numerous root cells produced aposporous gametophytes in light. In Pteridium aquilinum, Munore and Sussex (1969) also observed increased number of aposporous gametophytes from excised roots. The stimulatory effect of kinetin is of particular interest both because of its known effect on the maintenance of chlorophyll and protein levels in detached leaves (Osborne, 1962), and in callus tissues (Vajranabhaiah, 1969), and because of its known effect on the stimulation of cell division in tissue cultures (Miller et al., 1955).

The capacity to produce gametophytes aposporously is general in ferns (Bell and Richards, 1958). Moreover, apospory is reported to be induced by continued maltreatment and malnutrition of the organs concerned. For instance, Takahashi (1962) working with Pteridium aquilinum, Kuhn reported occurrence of apospory on the detached root, the aposporous outgrowth developing from root-epidermis. In case of rhizome as well as root callus grown on basal

media, it was noted earlier that the gametophytes were regenerated rather late when the callus had turned dark. It is, in fact, interesting that the apospory occurred, only after active growth has largely, if not entirely ceased. This suggested that the gametophytic expression may be an alternate to the sporophytic when conditions no longer permit, or are conducive for relatively normal growth in the sporophytic pattern. For gametophyte is certainly the most directly fully potential form in ferns. In this case the requirement of light is probably not related entirely to photosynthetic production of carbohydrate since addition of 0.1% sucrose reduced the percentage of roots which formed gametophytes. Moreover, it was further found in present studies that if the roots were kept on conditions for maximal growth, this alternate expression never manifested.

Leaf Callus

In Pteris vittata callus initiation was tried on leaves from garden growing plants and also other types of leaves as mentioned earlier (Expt. 4-6). The requirement for callus development from apogamously produced ones was compared to that from leaves of garden plants. The former

showed initiation of callus even at lower concentrations of sucrose and 2,4-D; while the latter needed more of both sucrose and 2,4-D in the medium (Expt. 4-6). The younger the sporophyte, the stronger was its ability for callus initiation. Not only the amount of growth but even the type of growth is implicated with the age of the plant part studied. Bell (1959) reported regeneration of both gametophytic and sporophytic tissue from the fifth leaf of the sporeling of Thelypteris palustris. However, in no case had any control been exerted over the type of regenerate obtained and the physiology of regeneration remained obscure. Morel (1963) observed that the juvenile leaves of Adiantum pedatum regenerated gametophytes, whereas adult leaves regenerated sporophytes. This indicated that during the process of aging the factor leading to the production of gametophytic tissue still present in the juvenile leaves had been lost. Similarly, Baur (1963) reported that the morphological nature of the tissue regenerating from sporogonium of Moss was dependent upon the physiological age of the regenerating zone.

The callus initiated on the leaves of garden growing Pteris vittata was subjected to the action of sucrose. It was found that the callus in medium containing 2%

sucrose produced leaves, while from the callus in basal medium after prolonged period - when the cells turned black - gametophytes were regenerated. This seemed to offer indications that the intracellular changes may be similar to those in meiocytes, because gametophytic outgrowths appeared only after the cells of the sporophytic callus had yellowed and senesced. Bristow (1962) working with Pteris cretica described the differentiation of the leaf callus into sporophytes or gametophytes according to the presence or absence of carbohydrates in the medium. Hence, here again differentiation of the two morphological forms was in direct response to the cultural conditions, particularly sugar, provided.

Spore Germination and Development of Prothalli

Difficulties are often encountered in culturing fern prothalli as the spores show variable viability such as are observed in species of Anemia and Mohria by Atkinson and Stokey (1964). In our studies with Adiantum trapeziforme and Thelypteris augescens, the spores germinated readily when they were freshly collected. In case of Pteris vittata L., it was still easier to obtain spore germination as the plants were fruiting

through most part of the year. It was further noticed that the spores of these species stored in refrigerator even for few weeks showed markedly less viability both in the number of spores germinated and the vigor of the prothalli produced. Ward (1954a) also found in Phlebodium aureum J. sm. that spores germinated best when freshly shed from sporangia.

Germination requires adequate moisture which can be provided by moist substrate. Wetting the surface of agar with sterile distilled water appeared in the present studies to facilitate spore-germination. Moreover, 0.5% agar concentration of the medium was found better for prothallial cultures than other higher concentrations tried. Steeves et al. (1955) working with Pteridium aquilinum observed that the mortality rate in subculturing was greatly reduced due to softer medium. The spore germination in all the three species examined varied from one sowing to another; for which one cause may be as suggested by Bell (1958), the quality of agar used.

Out of the pH levels of the medium tested 5.5 gave optimal response for spore germination. Various workers have reported different pH for different species (Convay, 1949; Mohr,1956a; Miller, 1968). Some thalli

grow on very acid media, some only under alkaline conditions. A great many, however, tolerate a wide range of pH. Some differences in pH preference could be correlated with the habitat of fern, since among different species of Notholaena, Pellaea and Cheilanthus spores from limestone species have higher pH optima (Hevly, 1963).

In present studies Knudson's medium was found satisfactory for the prothallial cultures of three species investigated. In fact, except for Kato (1963b, 1965a) who used Moore's medium, most other workers (Ward, 1954a; Bell, 1958; Whittier, 1962, 1964a; Partanen and Partanen, 1963; Munore and Sussex,1969; Mehra and Palta, 1971) have reported vigorous growth of prothalli on Knudson's medium.

Gametophytic Callus

Callus was induced on the developing prothalli of Pteris vittata L. (Expt. 5-1) and Adiantum trapeziforme L. (Expt. 6-5). In both the cases callus initiation was possible only at certain stage of development when incubated on Knudson's medium containing sucrose (2%), coconut milk (10%) and 2,4-D (2.0 mg/l). The callus initiated at the

points where the prothalli came in direct contact with the medium. Mehra and Sulklyan (1969) also induced calli from the germinating spores of Ampleopteris prolifera by culturing them on Knudson's medium containing 0.5% sucrose and higher 2,4-D concentrations. There are, however, instances where the gametophytic callus has been initiated in absence of the auxin. Kato (1963b) induced callus on 4-5 days old germinated sporelings of Pteris vittata grown in complete darkness on Moore's medium with a carbohydrate. Darkness perhaps acted as a selector for callus initiation, for on bringing back to light the callus regenerated into gametophytes. However, Kato (1969) later succeeded in inducing callus in presence of light by treatment with 2,4-D and yeast extract in the medium. In Osmunda cinnamomea Morel and Wetmore (1951) reported the spontaneous formation of callus when the gametophytes were grown on Knudson's medium with 2% dextrose. Steeves et al. (1955) obtained callus from the gametophytes of Pteridium aquilinum when they were grown on Knop's or Knudson's medium with different dextrose concentrations. DeMaggio (1966) working with Lycopodium obscurum produced callus on 4% sucrose with 10% coconut milk.

For the production of callus well developed gametophytes were found to be best suited; this may probably be due to an age dependent change in the capacity of the cells composing the gametophytes to respond to the exogenous auxin. It is more likely that the different responses of the gametophyte cells, result from a change in the responsiveness of cells on account of their varying endogenous auxin levels. Miller and Miller (1964) have presented evidence which supports the proposed relationship of the developmental stage of a cell and its response to auxin. It was further noticed that the aging prothalli incubated on the same medium without any subculture gave rise to a whole spectrum of abnormalities at one extreme of this spectrum, masses of intertwined filaments were observed which occassionally bore sex organs and retained the ability to give rise to normal prothalli. At the other extreme were formed callus like compact parenchyma with a limited amount of vascular differentiation and only exceptional regeneration of organised structures.

In our present studies, the gametophytic callus obtained from Pteris vittata L. and Adiantum trapeziforme L. was subjected to the action of varying sucrose concentrations.

When callus was grown on basal Knudson's medium in light, gametophytes were regenerated. Numerous sporophytes were produced on Pteris vittata gametophytic callus grown on 4% sucrose medium. The number of sporophytes differentiated decreased with the decreasing sucrose level in the medium. On 1% sucrose medium at first few leaves were produced but later roots developed. On 0.5% sucrose medium an intermediate structure was produced (Expt. 5-2). After a prolonged period of culture on 1% sucrose medium, when there was no more production of sporophytes, possibly due to the depletation of carbohydrates in the medium, gametophytes started regenerating. This could be explained on the assumption that the more complex sporophytic forms required more of energy source from the medium for their growth and development; and when it was depleted to an extent autotrophic gametophytic forms alone could develop. In case of Adiantum trapeziforme callus sporophytes were produced at higher sucrose concentrations and only gametophytes on basal medium (Expt. 6-6). Scattered tracheids were observed in the gametophytic callus grown in the sucrose containing media of both the fern species studied; however, the presence of tracheids

did not by itself indicate that apogamy would result. The gametophytic callus of Ampleopteris prolifera also produced sporophytes on higher sucrose level and gametophytes on basal medium. Their capacity for differentiation was, however, found to be dependent upon how long they were allowed to grow on the previous auxin containing medium (Mehra and Sulklyan, 1969). Clearly, these induced callus tissues by virtue of their capability to produce either sporophyte or gametophyte, in presence or absence of sugar respectively, or to revert to callus growth when returned to normal conditions (i.e. when transferred to sucrose-auxin medium) shows that it is a response of genetically unaltered cells to prevailing environmental conditions. In fact, Partanen (1972) has used this behaviour to distinguish environmentally induced callus from the spontaneous or radiation induced tumor tissues of Pteridium aquilinum.

While examining the regenerative capacities of small fragments (less than 300 μ in diameter) of gametophytes of Pteris vittata and Adiantum trapeziforme, it was found that complete gametophytes were regenerated quite easily from the free cells (Expts. 5-4 and 6-4).

Kato (1964) and Ito (1962) also reported the regeneration of gametophytes from single cells of Pteris vittata gametophytes. The basic stimulus which seemed to underlie such examples of regeneration was the mechanical or physiological isolation of cells from the rest of the prothallus. The cell or cells which are cut off from the communication from its neighbours assumed spore-like behaviour, resumed growth and often recaptulated the pattern of growth by germinating spores. This is comparable to Steward's (1968) hypothesis, based upon his pioneering work on carrot cultures, concerning the totipotency of cells.

Apogamy

Apogamy occurs frequently in ferns. It has also been induced in large number of Pteridophytes. The occurrence of this phenomenon has been attributed to a number of different factors by earlier workers. In Doodia caudata it was attributed by Heim (1896) to the upright growth of the prothalli in response to light, while in Phegopteris polypodioides Brown (1923) thought it a response to the reduced vitality of the prothalli

resulting from malnutrition. In Cystopteris fragilis the occurrence of apogamy was related to seasonal variation, being more frequent in the high light intensities prevailing in the summer (Heilbronn, 1910). Lang (1898, 1929) considered prevention of fertilization, the ability of certain prothallial cells to organise meristems and direct illumination to be the factors responsible for the induction of apogamy. Induced apogamy is of interest because it does not appear to involve a specific genetic change and its occurrence can to a degree at least be brought under experimental control. The ease with which it can be induced in vitro may be due to their higher chromosome number coupled with genetic imblance brought about by hybridization (Manton, 1950).

In Pteris vittata and Adiantum trapeziforme apogamy was induced in their prothalli by subculturing them on sucrose containing medium (Expts. 5-3 and 6-3). No apogamous structures were noted in those prothalli grown on media lacking sucrose. Whittier and Steeves (1962) working with Pteridium aquilinum found that the prothalli grown in the absence of glucose (i.e. when they had only their photosynthate as an energy source)

produced no apogamous structures. The same was true in his work with other strains of Pteridium, Osmunda cinnamomea and Adiantum pedatum.

No apogamous structures were visible until the formation of thick cushion of cells on which multicellular hairs were initiated. This formation of hairs on the surface of the prothallus was the first external indication of the occurrence of an apogamous development. Moreover, the leaf was the first organ to be formed at these thickened portion. Similar response was observed by Whittier (1962, 1964a) in Pteridium aquilinum, by Mehra and Sulklyan (1969) in Ampleopteris prolifera and by Freeburg (1957) in Lycopodium cernnum L. and Lycopodium selago. In case of Lycopodium Freeburg (1957) noted that varying the sucrose concentration had no influence on apogamous shoots formed. We, however, found that the increase in the level of carbohydrates did result in the increase in number of sporophytic structures upto an optimal sucrose (4%) level in Pteris vittata and Adiantum trapeziforme. Whittier (1964a) too reported that the number of apogamous sporophytes per Pteridium aquilinum

culture steadily increased with the concentrations of glucose to a peak of over 12 at optimal 2.5%. Mehra and Sulklyan (1969) reported 4% sucrose as optimal for induction of apogamy in Ampleopteris prolifera; whereas in the presence of 2% and 3% sucrose apogamy was delayed. In our present studies a correlation was in fact apparent between the decreasing sucrose concentrations and the time taken for apogamy to appear. Also in obligate apogamous species of Cheilanthus tomentosa and Cheilanthus alabamensis, Whittier (1965) observed the acceleration of apogamy with increase in sugar content of the medium. In absence of light no apogamy was recorded in the species we examined nor in Pteridium aquilinum (Whittier, 1964a). Kato (1970) reported apogamy in Pteris vittata L. in dark, but in the presence of sugars, yeast extract and tryptophan. Apogamous structures seemed to develop in this case from the callus initiated in the dark rather than from the prothallus directly. Since no apogamy occurred on any prothalli grown on mineral medium, the role of sugar is in some way implicated in the induction of apogamy. Moreover, once prothallial cells have already matured in Pteris vittata and

Adiantum trapeziforme, they were unable to produce apogamous structures even when supplied high doses of carbohydrates. Once the prothallial cells had matured, they were apparently unable to respond to conditions which cause the gametophytic cells of the sinus region to become sporophytic.

Whittier (1964a) tested whether apogamy is due to high osmotic concentrations; but he failed to get apogamous structures in Pteridium with mannitol. Parallel experiments using mannitol to replace glucose proportionately and achieve equivalent concentrations have shown that the effect of the glucose is not due to increased osmotic pressure in the medium. Evidence therefore, confirms that apogamy is not due to increased osmotic pressure in the medium.

Inorganic nutrition had no effect on apogamy; when in Pteris vittata different levels of macroelement salts were tested with optimum sucrose concentration (4%); it was found that neither reduction nor doubling of these salts resulted in increased apogamous response (Expt. 5-3). In fact, vigorous growth of the prothallus

which could occur in the presence of optimal concentrations of macroelements was the basic need of apogamy. The thickened tissue of the prothallus was produced only by vigorous gametophytes growing in light on a medium supplied with all the essential minerals and sugars. A reduction in mineral nutrition did not induce apogamy in Pteridium (Treanor and Whittier, 1969). The prothalli of Osmunda cinnamomea did not produce any apogamous plants on a nitrogen free medium, but these gametophytes formed many apogamous sporophytes under conditions which promoted vigorous growth (Whittier and Steeves, 1962). This was in aggrement with Schwabe (1951) who reported that mineral deficiencies reduced the growth of fern gametophytes and apogamy was not promoted by elimination or decrease of these macroelements from the nutrient medium.

Sporulation

Besides its role in the regulation of regeneration into sporophyte or gametophyte, sugar also influenced the growth and development of the fronds and formation

of the spores. When sexually produced plants of Pteris vittata were allowed to grow in various sucrose concentrations, the growth of these plants above 4% sucrose was inhibited. These plants were well developed in 4% sucrose alone and more so in 4% sucrose and 0.5 gm/l yeast extract. The plants grown in 2%, and 1% sucrose were quite thin compared to those in 4%. They were also smaller in size. This indicated that 4% sucrose is optimal carbohydrate concentration in the medium for the growth of these plants. Still there was no formation of sporangia in any of the above treatments. No sporangial structures appeared even after 12-18 months of culture period. In any case, the leaves produced by these in vitro plants were of smaller dimensions than in vivo plants.

Possible interpretations of small size of the cultured leaves in relation to their precocious development is offered by Steeves and Sussex (1957). The more available carbohydrate, the more cell-division activity there was and hence the more complex was the leaf. The simple juvenile leaf forms developed in low concentration of sugar, the adult leaf form developed in higher sucrose concentration (Sussex, 1958).

Caponetti and Steeves (1970) working with Osmunda cinnamomea presented evidence that the final size of the cultured leaves, which is very much less than natural field leaves, was on account of reduced number of cells in the developing primordia in culture rather than reduced size of the cells. Further, the primordia of Osmunda cinnamomea under the influence of 4% and 6% sucrose showed significant and progressive increase in the final height of the leaves (Caponetti, 1972). Working with Marsilea, Allsopp (1963a, 1964) concluded that at a suitable sugar concentration (4% or 5% glucose) sporelings developed the differentiated structure characteristic of land forms even in liquid media; whereas at lower sugar concentrations (2% or 1% glucose) the less differentiated water forms were obtained.

Attempts by Rashid (1971) to induce the formation of sporangia and spores on isolated leaves of Lygodium flexuosum and Ceratopteris thalictroides were unsuccessful. In Osmunda cinnamomea and Todea barbara Sussex and Steeves (1958) showed the formation of sporangia by increasing sucrose concentration upto 12%; but there were no indications that meiosis and spore formation had occurred.

When Clutter and Sussex (1965) incubated Osmunda cinnamomea excised pinnae bearing sporangia that have already attained pre-meiotic spore mother cell stage and grew them in culture, some viable, haploid spores developed. Harvey and Caponetti (1972) working with Cinnamon fern cataphyll found that increasing levels of sucrose resulted in increased fertility; the highest percentage of fertility being obtained under conditions of continuous dark at 26°C with 6% sucrose. In Todea barbara DeMaggio (1968) was successful to complete the entire life-cycle from spore to spore in vitro. Plants produced were kept in 2% sucrose medium without transfer for 16 months. He interpreted that as the nutrients become reduced there was a general breakdown of chlorophyll protein transport of nitrogenous material from the leaf to other parts of the plant and stimulation of synthesis of new proteins associated with sporogenesis; sometimes during these events sporogenesis was initiated. But all the above examples where sporogenesis could to a certain degree be achieved, belonged to Family: Osmundaceae alone. It appears that sucrose provides a necessary pre-requisite for the sporangial development but that

stimuli must be present also and these appear to be provided from the vegetative plant (Clutter and Sussex, 1965). That nutrition and environment, especially photoperiod are important may be inferred but the critical studies have not been done (White, 1971).

Alternation of Generations

It is evident from the foregoing that with the advent of tissue culture technique it has now become possible to break the barriers between the sporophytic and gametophytic generations of ferns and fern allies, at will. It is strange that a simple substance like sugar (with or without auxin) can do this trick. At optimal concentration this substance can stimulate the gametophyte to develop a complete sporophyte or any of its parts like isolated roots, leaves or shoots. Conversely any part of the sporophyte can be made to develop gametophyte when the medium is devoid of sugar, thus obviating spore formation. The ploidy level of the species is clearly no consideration in respect of such experimental manipulation. How exactly the sugar or lack of it can bring about such responses, however, is not known. The process of ontogeny in ferns involves

a shift from the autotrophy and totipotency of gametophytic cells to the heterotrophy of the sporophytic cells, since the sporophytic tissues require an exogenous supply of sugar for growth in vitro. Bristow (1962) working with Pteris cretica L. presented evidence that the role of sugar is in some way to induce this shift.

Mehra (1973), on the other hand, postulated a Gene Block Hypothesis. It visualises that from any cell of the plant, be it of the gametophytic or sporophytic generation, it is possible after suitable manipulation to trigger into activity a gene block controlling the formation of root, leaf or gametophyte. Four gene blocks are proposed in a fern system, any of which could be stimulated. However, it is difficult to trigger any of the sub-divisions of a gene block without activating the master gene of that block. For instance, it is difficult to have antheridia formed on the leaves or mesophyll cells or sporangia appearing directly on the gametophyte.

Another important postulate that emerges from such a study concerns the phenomenon of alternation of generations. Neither the Antithetic Theory nor the

Homologous Theory of alternation of generations is adequate to explain the phenomenon in light of modern knowledge. The best approach would be the Genetic Theory of alternation of generations.

Bell (1959, 1970) proposed that the alternation of morphological levels in the life cycle of the Pteridophytes is a reflection of different states and properties of cytoplasm in each generation. Hence the gametophyte and the sporophyte may be looked upon as two levels of morphological complexity; not as two almost unrelated parts, but an oscillation between one form of growth and the other. The two forms of growth in the heteromorphic life cycle depend upon extra-chromosomal factors which influence the activities of the genes. In apogomous ferns Dryopteris borreri and and Adiantum seemannii, in a position corresponding closely to that in which one would expect archegonia, a sporophytic embryo was generated directly. This suggested that although oogenesis and direct production of an embryo appear superficially very different, the two phenomenon are, nevertheless basically similar. Furthermore, there is the implication that the organisms' genetic information for the less nutritionally demanding

gametophytic stage may be easily called forth in adverse conditions and permits a special kind of regenerative capacity apparently not found in higher plants (Expt. 3-6).

Unfortunately nothing is yet known of the changes in cell metabolism that occur during the prolonged period which appears to be necessary before the formation of gametophytes (Expt. 4-5), but it seems resonable to conclude that it involves some autolysis and redistribution of metabolites in the cells and between cells (Munore and Bell, 1970). Most of the cells producing aposporous gametophytes appear to degenerate through the breaking up of the cytoplasmic organellels and membranes. Gametophytic outgrowths appeared only after the cells of the sporophyte have yellowed and senesced to the extent that most of them are no longer viable (Expt. 4-3 and Expt. 4-7). Nevertheless, autolysis resulting from the experimental conditions is presumably bringing at least a few of the cells to a condition similar to that of the spore, with identical effect upon the kind of growth of which they are capable (Bell, 1970).

The investigations described in the foregoing lead us to picture the Pteridophyte life cycle as involving two occasions at which there are substantial modifications of gene action and cytoplasmic structure. These occasions are recognizable developmentally as oogenesis and sporogenesis. The resultant egg and the spore which are structurally quite distinct share one very significant characteristic, i.e. both are isolated cells. The mature egg is admittedly not released as is the spore, but the absence of plasmodesmata between it and the parent gametophyte and the conspicuous osmophilic layer exterior to the plasmalemma, showing little, if any contact with the boundary of the archegonial chamber (Bell, 1970).

This interpretation of the pteridophyte life cycle differs from those current hitherto in no longer regarding the zygote and the spore as two distinct points in the cycle at which there is an abrupt change in cellular behaviour. Instead, the genetic and cytoplasmic conditions, of which sporophytic and gametophytic growth are the inevitable consequences, are generated in differentiation of these particular cells. The macro-molecular preparations for each form

of growth, the consequence of a changing activation of the genes takes place over a period of time in the preceding generation.

Triple control mechanism can be proposed for the regulation of regeneration, as differentiation is an outcome of the action and interaction between (1) exogenous factors, in tissue culture - the constitution of nutrient medium, (2) cytoplasmic factors and the (3) nuclear factor. The cytoplasmic factors dance to the tune of nucleus and also to that of exogenous environment. Though the cytoplasm is not the carrier of hereditary particles, it is the medium through which the nuclear genes find expression and that the genes alone determine the development of heritable characters. To be sure, the nucleus determines what sort of a pattern of differentiation shall be established, perhaps by a genic production of substances like those so commonly employed by experimental morphologists, but the construction of a pattern with its precise location of specific cellular differentiation is apparently the prerogative of the cytoplasm alone.. It is as though the cytoplasm contained the plan of the organism and the nucleus its specifications

(Sinnott, 1960). Thus differentiation is the outcome of a form of dialogue between an unchanging genome and a labile cytoplasm, the cytoplasm at any one period determining the patterns of gene expression and itself suffering consequent modification in due course of time. Not only is the cytoplasm an effective buffer between the nucleus and extrachromosomal environment, but it may wield enough delegated nuclear authority or hold enough by its own right the genetic system of organelles to be a significant independent factor in differentiation (Heslop-Harrison, 1967).

From results of studies from microbial system and knowledge of genetic basis of enzyme synthesis, models for genetic regulatory circuits have been postulated which would selectively and differentially control gene expression according to environmental and cytoplasmic conditions (Stange, 1965). The regulating gene activity comes from outside the genotype and their origin, nature and mode of action has to be studied.

The various calli obtained in our studies from different sporophytic parts such as rhizome segments (Expt. 3-1), roots (Expt. 4-4), leaves (Expts. 4-6 and 7-3), and from gametophytes (Expts. 5-1 and 6-5) behave

the same way. This means irrespective of their origin, when the medium on which these calli are cultured contain a source of carbohydrate then differentiation of sporophytic form occurs; while on carbohydrate free medium gametophytes are regenerated (Expts. 3-6, 4-5, 4-7, 5-2 and 6-6). Possibly, the amount of carbohydrate supply to the callus brings about such change in their macromolecular constituents of which the sporophytic growth are possible; while the reverse is true in the absence of carbohydrates in the medium. In order to determine whether this developmental approach to understanding the alternation of sporophytic and gametophytic generations is correct, a broader sampling of different fern species is needed (White, 1971).