# CHAPTER I

# I N T R O D U C T I O N

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When we consider the sequence of events leading to the formation of multicellular plant or animal organisms from the single cell of a zygote, it is relatively simple to trace the morphogenetic events that occur during the differentiation; but the pertinent changes which occur during the first few divisions are imperfectly understood. With the first division of the zygote, polarity is established. Possibly there are definite biochemical and biophysical factors which may have contributed towards the establishment of this polarity. Subsequent to this establishment of polarity and prior to the establishment of obvious morphological differentiation, certain cytological, chemical and enzymatic differences take place, which need elucidation. Such questions have arisen time and again and in the history of Biology we find several attempts to answer them. The complexity of the processes of cell and tissue differentiation, however, do not lend themselves to investigation by the traditional methods.

Cell is the basic structural and functional unit of all the living organisms. As has been outlined in the cell theory, the basic problem posed by multicellular

organisms is that an organism is both a sum total of activities of all cells taken together as also the activities of single cells on their own. To quote Schleiden (1838), "Each cell leads a double life, one independent pertaining to its own development alone, the other incidental as an integral part of a plant". The suggestions as to how such problems could be tackled have been put forth by Schwann (1839) "... if therefore we find that some of these elementary parts are capable of being separated from the organism and of continuing to grow independently, we can conclude that other elementary parts, each cell ... would be capable of developing independently if only there be provided the external conditions under which it exists in the organism" (c.f. Steward, 1968).

The experimental proof of this concept was attempted by G. Haberlandt in 1902 when he tried to culture isolated cells of flowering plants. Haberlandt attempted to grow palisade cells and spongy parenchyma of Lamium purpureum, staminal hair of Tradescantia virginica, glandular hair of Plumonaria mollisima etc., with a view to study the "inter-relationships and the reciprocal influences to which cells are subjected

within the multicellular organism". Haberlandt even recognised the tremendous potentialities of culturing free cells of plants and had the remarkable insight to 'prophesy' : ".... I believe, in conclusion, that it will be possible to grow, in this manner, artificial embryos from vegetative cells....". Thus, he visualised the method of growing isolated plant cells in nutrient solutions as a new experimental approach to various important problems. However, in this Haberlandt did not succeed, possibly due to the limitations of technique(s) then available.

Interestingly enough, the first successful plant tissue cultures were, however, not free cell cultures but cultures of isolated seedling roots. In 1922, Kotte, a student of Haberlandt, working in Germany, and Robbins, working in America, independently reported the growth of isolated plant roots. In their experiments excised roots were grown in media supplemented with inorganic salts, different complex substances to serve as growth promoters and glucose as a carbon source. However, by 1937, White succeeded in replacing the complex ingredient such as yeast extract by a mixture

of thiamine, niacin and pyridoxine, and was thus able to grow the excised roots of tomato in a completely defined medium. Then in 1939, three workers, White, Gautheret and Nobécourt working independently, reported the first continuous culture of plamt tissues. The prolonged culture of unorganised plant cells-callus- was thus achieved for the first time. Callus tissues derived from wide range of plants and different plant parts are subsequently grown in continuous culture on the surface of agar media, which contain utilisable sugar, inorganic salts and various organic growth factors.

Since 1939, a lot of significant work has been accomplished by employing plant tissue cultures for the studies of organisational, morphogenetic, nutritional and biochemical problems. For example, initiation of cell suspension cultures by Steward and Shantz (1955), growth of free cells on agar plates by Bergmann (1960), regeneration of entire plants from freely suspended carrot cells by Steward (1958), raising of haploid plants from pollen by Nitsch and Nitsch (1969), isolation of protoplasts from cultured cells as well as directly from intact leaves by Takebe et al. (1971), and finally the

fusion of isolated protoplasts and regeneration of somatic hybrid tobaco plant by Carlson et al. (1972). These and many more important contributions in the field of tissue culture which have been reviewed extensively (Gautheret, 1959; White, 1963; Street and Henshaw, 1966; Street, 1969) have amply demonstrated the advantages offered by the use of tissue culture techniques over the other traditional methods in experimental morphology and physiology. Moreover, in vascular cryptogams, the morphogenetic sequences of zygote to mature sporophyte and spore to fully developed gametophyte offered materials with ready access for experimental manipulation and analysis. For the type of work contemplated in our laboratory, the tissue culture methods, therefore, seemed more advantageous and were employed in the present investigation.

Almost a century ago Hofmeister elucidated the morphology of the life-cycle of Pteridophytes. In fact, this proved to be the key which unlocked the mysteries of life-cycle of the higher plants. Sexual reproduction invariably involved nuclear fusion, and at some subsequent stage reduction. In plants this nuclear cycle is often associated with an alternation of two dissimilar generations,

one of which the gametophyte, contains the haploid state of the nucleus, and the other, the sporophyte, the diploid. This regular alternation between gametophyte and sporophyte is shown unambiguously by the vascular cryptogams, both the generations being well-defined and capable of independent existence. A large amount of information on the alternation of generations has already been derived from the ferns (Bell, 1959).

Little insight is, however, gained in the nutrition and growth of ferns (Bristow, 1962). Moreover, the factors responsible for above said life forms have proved to be difficult to analyse. In vitro studies whereby the environment can be precisely controlled provide better opportunities for making a critical evaluation of such factors. In contrast to the numerous cultures of callus tissues isolated from Phanerogams, culture of tissues of vascular cryptogams has, however, been obtained in relatively few cases. Vascular cryptogams, specifically ferns on general grounds would not seem to be very favourable for tissue culture studies. They are in fact devoid of secondary activity and are almost entirely made up of highly differentiated tissues seemingly unlikely to proliferate masses of meristematic parenchyma, which tissue is condusive to growth in vitro (Morel and

Wetmore, 1951). These fears have, however, been belied as callus-like growths from gametophytic tissues of ferns and mosses have generally arisen spontaneously and have been photosynthetic and capable of autotrophic growth (Steeves, Sussex and Partanen, 1955; Ward, 1960; Lal, 1963; Kato, 1963b; DeMaggio, 1964). On the other hand, comparatively very little is known about either the mode of origin of sporophytic callus in ferns or its nutritional requirements. In fact, there are hardly any reports about induction of callus on the rhizome of ferns. Secondly, although ferns -both gametophyte and sporophyte have been extensively grown in vitro for various types of studies, the roots of these plants have as yet received very little attention as objects of study under these conditions. The technique of growing isolated organs is useful for studying problems of morphogenesis because of the experimental advantages it provides for precise environmental and nutritional control. Attempts were, therefore, made in the present investigation to remedy this situation.

Callus tissues obtained from the gametophytes of few ferns are investigated for morphogenetic responses (Kato, 1964; Mehra and Sulklyan, 1969). Both gametophytes and sporophytes are developed from the above callus

depending on the absence or presence of an energy source. Similarly, the callus obtained from the leaf (Bristow, 1962; Kato, 1965a) and roots (Mehra and Palta, 1971) are experimentally regulated to produce gametophytic or sporophytic forms. Thus ferns, in which the gametophytes and sporophytes are independent of each other are particularly suitable for investigating factors responsible for initiating two different kinds of morphogenesis. In a number of algae, Ulva and Dictyota gametophyte and sporophyte are distinguishable only by the nature of their reproductive organs. From this it must follow that the morphogenetic expression in these plants is independent of chromosome number. The successive formation of gametophyte and sporophyte in the typical fern life cycle produced both alternation of morphologies and levels of ploidy. The discovery of apogamy led to the conclusion that the diploid state is not the cause of the transition to sporophytic organisation (Näf, 1962). Investigation of polyploidy indicated that generally little or no change in phenotypic appearance accompanied diploidy (Stebbins, 1950). The morphogenetic problem intrinsic to the alternation of generations will remain as long as fern-life cycle can exist in cultivation and can be subjected to experimentation (Whittier, 1971).

In plants with heteromorphic life-cycles, however, the hereditary information is clearly expressed differently in each part of the life-cycle. Explanation of the ontogenetic control of the alternation have resolved around two possibilities as described by Lang (1909). He suggested that the different morphological expressions of each generation could be explained in terms of initial cells of each generation; the spore and zygote are inherently different which causes the two distinct developmental patterns, or second possibility is that the initial cells are equivalent and the environment in which they develop determine their structures.

Experiments were carried out first to germinate a spore in the condition in which the zygote begins its life and second, to liberate the zygote free from the surrounding gametophytic environment and observe how both develop and produce what kind of morphological expressions (Bell, 1959). Spore and zygote which initiate growth as different as that of gametophyte and sporophyte of ferns are structurally distinct. Cytochemistry and electron microscope studies have amply confirmed this expectation (Bell, 1970). There is a vast difference in the cytoplasm of the spore and zygote, so also in their nuclei. In their studies with Thelypteris palustris,

Jayasekera and Bell (1959) had been able to culture isolated archegonia containing fertilized eggs and found in every instance that the zygote yielded normal sporophyte. Moreover, the amount of gametophytic tissue surrounding the zygote was so little that its presence being? ~~was~~ essential for zygote to develop into a sporophyte was not convincing. DeMaggio and Wetmore (1961) who using suitably enriched media were able to extract young embryos, only 3-4 days old from archegonia of Todea barbara, found that the older embryo produced normal sporophyte the best. What The extremly young embryos could do was to produce prothallial outgrowths which resembled gametophyte rather than sporophytic tissue. Hence, DeMaggio (1963) concluded that for young embryo especially complex nutritive environment is provided by the archegonium. Bell (1961) working with polypodiaceous ferns, however, showed that nucleotides did not cross from gametophyte to sporophyte, but accumulated at the boundary of the embryo. With electron microscope studies in Todea and Osmunda, an additional osmophilic membrane round the mature egg of ferns was observed (Bell and Mühlethaler, 1962b). It seemed reasonable to attribute the failure of nucleotides to penetrate the young embryo to the impermeability of this layer to large molecules.

Besides the normal life cycle of fern, there are deviations in the life cycle such as apogamy and apospory due to which sporophyte and gametophyte can be on the same level of ploidy. The gametogenesis and fertilization, can be eliminated, meiosis can be replaced by mitosis and sporogenesis can be omitted. Hence apogamy and apospory are processes of considerable evolutionary significance. Their experimental induction provides an opportunity to investigate and learn about the factors governing the periodical expression of gametophytic and sporophytic generations.

Apogamy has been induced in a number of Pteridophytes, e.g. in Lycopodium species (Freeberg, 1957), in different strains of Pteridium aquilinum; Osmunda cinnamomea L.; Adiantum pedatum L.; Phlebodium aureum L.; Onoclea sensibilis L. etc. (Whittier and Steeves, 1960, 1962; Whittier, 1964a, 1966b). Apogamy offers the opportunity to study the initiation of sporophyte from a gametophyte without the complications of oogenesis and sexual reproduction. Induced apogamy has proved to be of great interest because it does not appear to involve specific genetic change, and its occurrence can to a degree at least be brought about under experimental control. Whittier and Steeves (1960) showed that prothalli grown on the medium without any source of

carbohydrate but their photosynthate as sole energy source produced no apogamous structures. On the other hand, induction of apogamy by high sugar concentration is a relatively widespread phenomenon. From in vitro studies, the factors responsible for the induction of apogamy appears to be a high level of carbohydrate (Bopp, 1968), as also undoubtedly is the prevention of fertilization because of unsuitable physical or physiological environment (Nayar and Kaur, 1971).

Successful attempts to induce apospory are limited to excised cotyledons, juvenile leaves upto certain order (Steil, 1939, 1951; Bell and Richards, 1958; Takahashi, 1962) beyond which the leaves regenerated into sporophyte (Morel, 1963) and on excised roots (Partanen and Partanen, 1963; Munore and Sussex, 1969). It seems in nature also apospory is a wide spread phenomenon the result of which is high level of polyploidy being observed among ferns. The replacement of sporogenesis by apospory would probably have more chance of success if the species grew in a moist habitat. With so many reports of apogamy and apospory, still the factors responsible for them are not yet well understood. The success of plants adopting these modified courses need proper evaluation and this aspect has been examined in the present investigation.

Alma G. Stokey (1951) proved that the fern gametophyte though simple in structure when compared to sporophyte affords dependable criteria for taxonomic and phyletic studies. Vast amount of information has been added to our knowledge of the gametophytes of the ferns, more especially of the various groups of the advanced ferns. This additional information serves materially to substantiate Stokey's conviction in the significance of gametophyte morphology for an understanding of the evolution and phylogeny of the ferns in the characterization of the major taxonomic groups (Nayar and Kaur, 1971). By a comparative study of gametophyte morphology in various taxonomic groups, Nayar and Kaur (1971) clearly indicated the trends of evolution of the gametophyte among homosporous ferns. As could be expected of such a vast and varied group of plants as the ferns, the gametophyte has undergone evolution along different lines of specilization. Besides, fern gametophyte has many advantages as research material being small and easy to handle in large numbers. Gametophytes are therefore, used to study growth correlations, hormonal effects, and also the effect of environmental gradients on their morphology (Miller, 1968).

Spore-morphology, its germination and the pattern of prothallial development as well as the morphology of adult prothalli of Adiantum trapeziforme L. and Thelypteris augescens (Link) Munz et Johnsdon are studied and the observations incorporated in the present thesis.

The present investigation is aimed to fill-in the lacunae in our knowledge concerning the nutrition and growth of the sporophytic and gametophytic callus tissues. Further, some insight into the physiology of regeneration from the sporophytic and gametophytic tissues might be gained if it proved possible to control the type of callus differentiation.

The results incorporated in the present thesis are based upon our studies with:

(1) Pteris vittata Linn.,
(Family: Pteridaceae);

(2) Adiantum trapeziforme Linn.
(Family: Adiantaceae); and

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

With Pteris vittata, studies are made for the induction of sporophytic callus from rhizomatic segments and leaflets of plants grown in a green house. Callus tissues are also induced from the sterile leaves of sexually produced and from apogamously derived sporophytes. Similarly, callus tissues are established from the gametophytes developed from spores.

The growth and nutritional requirements of the above tissues are examined and the effects of various cultural parameters such as auxins, carbohydrates and inorganic salts on regeneration of gametophyte and sporophyte from the callus tissues are investigated. The underlying histological changes in callus tissues subjected to sugar-auxin interactions are also examined.

Studies are also made of the conditions necessary for the regeneration of sporophytes and gametophytes from the free cells obtained from their suspension cultures.

Experiments are conducted with excised roots from sexually produced sporophytes for the production of laterals and induction of apospory.

All through the above said *in vitro* studies, it was noticed that hardly any contamination of cultures occurred. This coupled with the general observation that ferns are rarely infected by pathogens, prompted us to examine locally available ferns for the presence of antibacterial substances. The results obtained are published in *Planta Medica*, Volume 22, No.4, December, 1972 (see Appendix I for reprint). Fronds of *Pteris vittata* derived from callus cultures were also similarly screened and observations made are recorded in this thesis.