CHAPTER I

INTRODUCTION

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(1) The development of plant :

The development of plant begins fundamentally with a single cell, be it a spore, a fertilized egg or a generative cell. The development of a fertilized egg, in earlier stages, results in a more or less homogeneous mass of cells with several sites of differentiation. As the development proceeds, an embryo with the primordia of morphological structures which later develop into respective organs appears. Thus development is manifested in changes in the form of an organism, as also when it transforms from a vegetative to a flowering condition. This involves both growth and differentiation.

(a) Growth :

Growth is permanent, irreversible increase in size and dry matter during the course of plant development. Growth includes increase in cell size and also the process of cell division that leads to an increase in cell numbers and thus provides the units from which the plant is built up.

(b) Differentiation :

Differentiation is a precise and orderly process of sequence of changes by which derivatives of a cell, or a group of cells arising from a common progenitor form diverse structures to

carry out specialized functions. It is a dynamic process involving complex physiological, biochemical and biophysical changes which precede a discernible morphological change. The process of differentiation is common to all organisms and may be expressed in several ways. Growth and differentiation are thus two major developmental processes, both of which usually take place concurrently during development (Wareing and Phillips, 1978).

(2) Study of problems of development :

The problems of development of a plant can be studied in a number of different ways, but basically two major approaches can be made, viz. (1) the morphological and (2) the physiological and biochemical. Developmental morphology (and anatomy) was in the past mainly concerned with describing the visible changes occurring during development, but the present interest is largely directed to examine the factors and processes affecting plant form using experimental techniques like tissue culture, micro-surgery, autoradiography, etc. However, development cannot be fully understood without a study of the many biochemical and physiological processes which underlie and determine the morphological changes (Mehta, 1980a).

The term "morphogenesis", from Greek words meaning the origin of form, is used by experimental biologists to connote the inception and development of morphological characters.

The morphology of an organism or one of its constituent organs, may be regarded as a resultant, overall effect of all the physiological processes involved in growth and development. It is a process of higher magnitude than events occurring in single cells, wherein inter-tissue relationships and interactions among cells cannot be ignored, as they constitute profound influence on the collective fates of individual cells in their growth and development (Wardlaw, 1968).

The regulation of plant growth and development has, by far, remained perhaps the most mysterious and intricate problems in biology. What requires demonstration is, how a particular physiological process, or a system of biochemical reactions can be closely and directly related to the ultimate visible reality i.e. the manifestation of form. This is, of course, a very rigorous requirement, demanding many and varied experimental approaches. Consequently, tools and techniques of functional and analytical biochemistry, cytology and molecular biology are brought to bear (Wareing, 1971).

The developmental programme of a cell involves, among other things, the appearance and disappearance at specific times of specific proteins as detected by changes in enzyme activities, and by changes in the structure and content of organelles and membrane components. Further, different types of cell are characterized by different types of enzymes and

structural proteins. This illustrates, perhaps somewhat simplistically, that differentiation involves different gene expression. Even though specialised cells are characterised by the presence of specific gene products, many differentiated cells are totipotent, in that they contain all the genetic information necessary for growth of a whole plant. In final analysis, therefore, the morphological expression of a particular phenotype in unicellular as well as in multicellular organisms is the ultimate reflection of the genetic constitution. Regulation of physiological and biochemical activities, from the inception lead to differentiation of characteristic organs and tissues, with distinctive functions. Whether the biochemical investigations are carried out of cell free systems or studies are made of morphological features of whole plant, the objectives essentially remain the same : to understand how one cell gives rise to hundreds of thousands of specialized cells, partitioned into interdependent, interacting tissues and organs of a living entity (Wardlaw, 1968).

(3) Plant tissue culture as an experimental tool :

The organisation and morphogenesis of multicellular plants depends upon the integration and mutual interaction of various organs, tissues and cells which are separated from one another in space. Together they form a complex system for the analysis of which we need to know not only about the substances primarily involved in morphogenesis, but also about the

correlations which result from the differential capability of different parts of the plant to synthesize certain compounds and the transport of these substances. This system and its complex control mechanisms for organ, and/or embryo formation can be simplified by isolating cells, tissues and organs and their subsequent cultivation in vitro. Thus the complexity of the problems involved can be dissected and simplified in cultured tissues which permit interpretation and understanding at various levels of organization from free cells to callus to organs. Tissue culture is therefore, a tool which facilitates not only in understanding the activities of cells as cells, but also, their activities as components of a multicellular system.

(4) Advantage of tissue culture technique :

In addition, cell and tissue cultures offer the advantage of an almost perfect control of exogenous factors. This applies, for example, to the exclusion of biological contaminants which is attained by the use of axenic culture methods. Other advantages that tissue cultures offer over the intact plant include : (a) controlled environment, (b) better control of nutritional and hormonal requirements, (c) fairly definite, quick and reproducible response under deliberately manipulated experimental conditions, (d) permits distinction between initiation of organ primordia and their further growth, (e) free from the complex interactions occurring in the intact plant, etc.

The successes with indefinite maintenance in culture, first of roots (White, 1934), then of tissues (Nobecourt, 1939; Gautheret, 1939; White, 1939) and then the important progress made in techniques of single cell cultures and in synchronized suspension cell cultures (Muir et al., 1954; Street et al., 1968; Stuart and Street, 1971) have made possible the quantitative study of cellular metabolism during growth and differentiation.

(5) Earlier work on organogenesis :

The earliest report of controlled organ formation in vitro was by White (1939) and this observation was confirmed and extended by Skoog (1944). Further studies by Skoog and his coworkers culminated in the now classical finding that a basic regulatory mechanism underlying organised development in plants, by and large, involved a delicate balance between an auxin and a cytokinin. Both these substances are required for cell division and enlargement in tobacco callus, but a relatively high level of cytokinin to auxin resulted in shoot formation and the reverse favoured root formation (Skoog and Miller, 1957). Furthermore, other media constituents could alter the effectiveness of the phytohormones. These observations led them to suggest that quantitative interactions between diverse growth factors, rather than specific morphogenic substances, provided a common mechanism for the regulation of all types of morphogenetic phenomena in plants.

Though this mechanism cannot be universally demonstrated, a wide range of plant species respond in culture to variations of auxin and cytokinin concentrations, in the presence of other media components, by the production of shoots and roots (Narayanaswamy, 1977; Murashige, 1977; Thomas and Wernicke, 1978; Green, 1978).

However, since no specific organ forming substance has as yet been isolated and not all cultured tissues produce roots or shoot buds or embryos in response to known auxin/cytokinin treatments, Mehta (1975) suggested that other growth factors, as yet unidentified, might also be involved in hormonal interactions regulating organogenesis. Further, different plant parts respond similarly, including in recent years the epidermal explants consisting of epidermis and a few sub-epidermal layers (Tran Thanh Van et al., 1974; Tran Thanh Van, 1977; Bhatt et al., 1978).

The 'totipotency' view proposed by Steward and his co-workers (1958, 1964) envisaged that physical and/or physiological isolation of a cell from the correlative influences of other cells in a tissue system or organised structure is a prerequisite for a cell to express its developmental potential. Ultrastructural studies on cotton embryogenesis in vivo (Jensen, 1963) and carrot embryogenesis in vitro as demonstrated by Halperin and Wetherell (1964) have however, tended to cast doubt on the necessity of physical isolation of cells for differentiation to take place.

Steward et al. (1967) have further suggested that various growth regulating stimuli may need to be applied to cells, not only in the right amounts but also in the right sequence and under the right cultural conditions. Such a sequential treatment has since been used to induce shoot and root formation from a large number of cultured plant tissues (Chen and Galston, 1967; Yamada et al., 1967; Rao et al., 1970; Jagannathan et al., 1977; Wochok and Sluis, 1980).

(6) Factors influencing morphogenesis :

Among the many factors that influence morphogenesis, the main ones are : (a) hormonal and (b) nutritiona, besides of course, physical and environmental ones.

(a) Hormonal factors :

Growth of plant and their isolated cells and tissues is dependent upon the availability of growth hormones (Audus, 1972), as these substances are known to play important role in cell metabolism, cell membrane synthesis (Cocking, 1977), growth and differentiation (Butenko, 1968; Street et al., 1968; Crocomo et al., 1976).

Of these growth substances, auxins have been proved to be an essential supplement for establishing successful culture of many plant tissues (Morel, 1948; Gautheret, 1959; Simard, 1971; Jouanneau and Tandeau de Marsac, 1973) and also there

are numerous reports in literature that auxins regulate growth and differentiation (Rao and Narayanaswamy, 1972; Prabhudesai and Narayanaswamy, 1974; Street & Withers, 1974; Sunderland and Dunwell, 1974; Eliott, 1977; Johri, 1978). Also directly or indirectly auxin specifically affects the S phase of DNA synthesis (Péaud-Lenoël, 1977).

The effect of cytokinins on growth is mainly due to their effects on cell division, specific protein and nucleic acid metabolism (Skoog et al., 1967; Letham, 1968; Fletcher, 1969; Piesco and Alvarez, 1972; Fox and Erion, 1975; Klämbt, 1977; Vajranabhaiah and Mehta, 1976; 1977). Cytokinins, however, are not reported to affect the S phase of DNA synthesis (Péaud-Lenoël, 1977).

Similarly, gibberellic acid (GA_3), is also known for its multifarious effects on growth and metabolism of plants and their isolated tissues and cells. The promotory or inhibitory effects caused by GA_3 are either due to its influence on auxin metabolism (Kogl and Elma, 1960) or possibly it acts at the gene level to cause de-repression and/or repression of specific genes (Varner et al., 1965; Nitsch, 1968). GA_3 has also been demonstrated to induce inhibition of bud formation in Begonia (Schraudolf and Reinert, 1959), tobacco (Murashige, 1964) and Plumbago (Nitsch and Nitsch, 1967). GA_3 is further known to block the action of the cytokinin, 6-benzylaminopurine (6-BAP), and auxin, naphthalene acetic acid (NAA) which are ordinarily

potent stimulators of buds and roots respectively (Heide, 1969).

(b) Nutritional factors :

Of late, it is demonstrated that besides the influence of phytohormones on morphogenesis, media changes or nutritional modifications are also successful for organ induction in several conifers (Sommer et al., 1975; Cheng, 1975; Campbell and Durzan, 1975; Coleman and Thorpe, 1977).

(c) Environmental factors :

The physical environment is also important in studies on organogenesis, the major factors being the physical state of the medium, light, temperature, humidity etc. (Murashige, 1974; Tran Thanh Van et al., 1974).

Other parameters that require evaluation are source of explant, developmental phase of explant source, age of explant source, explant size, etc. (Murashige, 1977).

(7) Histological pathway leading to organogenesis :

Morphogenesis relies on the control and integration of the processes of cell division and cell differentiation. Histologic differentiation implies the types of cells and tissues that appear in callus.

Formation of vascular elements may be induced in

undifferentiated masses of callus tissue by the application of auxin and sucrose (Gautheret, 1966). An excellent control of differentiation of xylem and phloem has been achieved with callus derived from Syringa vulgaris by Wetmore et al. (1964) and with callus derived from Phaseolus vulgaris by Northcote and his associates (1966, 1967) and also callus tissues derived from seedling roots of tomato and Linum (Mehta, 1966). Cytodifferentiation in tissue cultures was reviewed by Bornman (1974) and more recently by Narayanaswamy (1981).

Mizuno and Komamine (1978) have demonstrated the role of cyclic AMP on tracheary element formation. While Lang and Kohlenbach (1975) and Torrey (1975) have studied the cyto-differentiation of specific cell types in cell suspensions, Fukuda and Komamine (1980 a, 1980 b) have shown direct evidence for cytodifferentiation to tracheary elements without intervening mitosis in a culture of single cells isolated from the mesophyll of Zinnia elegans.

Tracheal differentiation is followed in the cells by the development of "vascular nodules". Localized cell divisions around the vascular 'nests' lead to the organization of meristematic centres or "meristemoids" which are pre-requisite for the formation of root primordia depending upon the exogenous hormonal balance (Mehta, 1966; Reinert, 1973).

The developmental sequence leading to shoot differentiation began with the induction of radial tissue followed by

the appearance of surface meristems in which cell division activity led to the formation of tiny protuberances. These grew into leaf primordium-like structures and finally apical meristems were formed among them in rice callus (Nakano and Maeda, 1974 a, 1974 b) and also in tomato leaf disc system (Coleman and Greyson, 1977).

Thus in all cases, localized active cell divisions leading to the organization of a meristematic centre or meristemoid was the histological pathway which subsequently led to shoot or root primordium formation.

The differentiation of embryo, which at one time was considered to be exclusive to zygote, has now been demonstrated in the cultures of some vegetative and reproductive t tissues (Johri, 1965; Mohan Ram and Wadhi, 1965; Muralidhar and Mehta, 1980).

(8) Physiological and Biochemical changes underlying differentiation :

All above mentioned studies deal with the manipulation of organ formation by determining the optimum level of media additives, time of treatment, and/or the proper culture conditions and tell us very little about the regulation of organ initiation in the tissue.

Very little is known about what triggers this development in the cell or the groups of cells which participate

in organogenesis. How is organ initiation regulated at the tissue level? What are the physiological and biochemical changes associated with organogenesis?

Understanding the differentiation of cells in cultured tissues implies understanding the factors which regulate their biochemistry and physiology.

Though there are quite a good number of studies examining the physiological and biochemical parameters of growth in static and shake cultures (King and Street, 1973; Street, 1977; Everette et al., 1978), hardly any studies are there in literature on the physiological and biochemical events underlying organogenesis. As Thorpe (1978) has rightly pointed out the progress in this area of research has been slow and scanty for lack of a truly suitable experimental system; because the callus cultures used in such morphogenetic studies suffer from certain important drawbacks (Mehta, 1980b). The common drawback of all callus systems is that only a few cells in the callus mass are directly involved in the organ-initiation process. Another limitation is absence of synchrony in such callus masses. In this respect, recent studies with epidermal layers (Tran Thanh Van and Trinh, 1978), induction of somatic embryogeny on large scale (Ammirato, 1978) and achievement of partial synchrony in growth and embryogenesis (Komamine et al., 1978) offer more promising experimental approaches for morphogenetic studies.

(9) Hormones and differentiation :

To induce organogenesis (in tissue cultures) different concentrations of plant growth substances are required for different parts of different plant species. This is because of varying levels of endogenous hormones present in the tissues. Hence a critical balance of hormones must be attained within the plant tissue for the exogenously supplied hormones to be effective for the induction of organs. Since there is no data available at present on the endogenous levels of growth hormones in the tissues under study for morphogenesis, all the researchers have been trying a wide range of concentrations of various hormones. Thus, the approaches made in such morphogenetic studies tend to be purely empirical (Mehta, 1980).

However, during 1970s, some indirect studies have been made on the native indole-3-acetic acid levels in organ forming tissues through the estimation of the levels of peroxidase/indole-acetic acid oxidase (IAA oxidase) activity and changes in the isoperoxidase patterns during culture.

Peroxidase enzyme has been related with growth, differentiation and lignification (Harkin and Obst, 1973; Wolter and Gordon, 1975). Increases in total peroxidase activity before organ differentiation are reported by many

workers in tobacco (Rücker and Radola, 1971; Mäder, 1975; Thorpe and Gaspar, 1978) and endive (Legrand and Vasseur, 1972; Legrand, 1974). This increase in activity prior to organ initiation is indicative of a lower requirement of endogenous auxin to establish a favourable auxin-cytokinin balance as has been suggested previously by Lee and Skoog (1965a.) Changes in isoperoxidase patterns have been observed during root initiation in Ipomoea (Bhatt, 1977; Gaspar et al., 1977) and root and shoot initiation in tobacco callus (Rawal, 1979).

Comparative studies of peroxidase and IAA oxidase activities by Rawal (1979) in root/shoot forming callus with the non-root/shoot forming control showed characteristic differences both in diploid and haploid tobacco tissues (Rawal and Mehta, 1980). IAA oxidase was found markedly lower in root forming tobacco callus than in the shoot forming one, which might indicate higher endogenous auxin requirement for root differentiation than for shoot differentiation (Rawal and Mehta, 1980).

(10) Utilization of carbohydrates for growth and differentiation in cell and callus cultures :

Carbohydrates are of special significance in plants, since they represent important food reserves and are part of the structural framework of each cell (Albersheim, 1974;

Takeuchi and Komamine, 1978; Lamport, 1978). They comprise 50-80% of the total dry weight of most species. Besides being the main energy source, they also provide raw materials for important metabolites like nucleic acids.

(a) Carbohydrate nutrition :

Ever since the inception of plant cell culture, carbohydrate nutrition has been a focal point. In fact plants meet their energy requirements autotrophically via the photosynthetic fixation of carbon. The normal functions of chloroplasts are frequently absent or blocked (Laetsch and Stetler, 1965; Seyer et al., 1975; Kamínek and Luštinec, 1976; Dalton and Street, 1977) when the plant tissues are cultured aseptically. Therefore, in plant cultures, it is usually necessary to substitute suitable carbon sources for those carbohydrates that would otherwise have been generated photosynthetically in the intact plant. Sucrose is the usual form of sugar exported by the leaf in plants, loaded into the cells of the phloem, translocated, and deposited in meristematic and storage tissues. It seems reasonable then that sucrose or any carbohydrate source from which glucose or fructose can become readily available should serve best the energy needs of cells grown in culture.

The effects of certain carbon sources on the growth of tissue cultures have been extensively worked out by Street

and his coworkers (Dormer and Street, 1949; Ferguson et al., 1958 a, 1958 b; Morgan and Street, 1959).

Many monosaccharides, disaccharides and polysaccharides have been tested by Hildebrandt and Riker (1955) and Ball (1955); but generally sucrose, glucose or fructose were found to be the best for the growth of cultures (Gautheret, 1941; Hildebrandt and Riker, 1953; Nickell and Burkholder, 1950; Risser and White, 1964; Yatazawa et al., 1967; Nickell and Maretzki, 1969; Fadia and Mehta, 1973b; Subbaiah et al., 1974; Nash and Boll, 1975).

The ability of callus cultures isolated from a wide variety of plants to utilize different carbohydrates varies markedly. The divergence among species in their preference for different carbohydrates was demonstrated in a direct comparison of tissue isolated from five species (Hildebrandt and Riker, 1949), and the comparative effectiveness of hexoses, pentoses, disaccharides and polysaccharides for callus growth of different species, including those isolated from tumor tissue, has been more generally reviewed (Gautheret, 1959).

Dextrose, levulose and sucrose were excellent carbon sources for the tissues of marigold, paris-daisy, periwinkle, sunflower and tobacco (Hildebrandt and Riker, 1948). Mannose utilization is usually poor (Mathes et al., 1973; Meryl Smith

and Stone, 1973; Nickell and Maretzki, 1970). Spruce tumor tissue (Risser and White, 1964) could grow equally well on sucrose, glucose and raffinose. Yatazawa et al. (1967) found that glucose is better than fructose, sucrose and maltose for rice callus. Fridborg (1971) have also found glucose as the best carbon source for onion callus. Galactose was efficiently utilized by Vinca rosea (Hildebrandt and Riker, 1949, 1953), Sequoia sempervirens (Ball, 1955), and sugarcane (Nickell and Maretzki, 1969); while Yatazawa et al. (1967) reported no growth of rice callus tissue on galactose and lactose. Fructose at 3% concentration was found to be effective among the 13 carbohydrates tested for apple callus (Chong and Taper, 1974).

Disaccharides, including maltose (Simpkins et al., 1970; Thorpe and Laishley, 1974; Anstis and Northcote, 1973; Yatazawa et al., 1967), cellobiose (Nickell and Maretzki, 1970; Anstis and Northcote, 1973), trehlose (Nickell and Maretzki, 1970; Goris, 1968), and even lactose, have been reported to support growth. For carrot tissue 3% sucrose was found to be optimum (Gautheret, 1941). Hildebrandt et al. (1945) found 1% sucrose optimum for sunflower and tobacco tumor tissues. The optimum sucrose concentration for spruce tumor tissue (Risser and White, 1964) and for Citrus limon tissue (Murashige and Tucker, 1969) was 5%. Mathes (1967), Fridborg (1971) and Fadia and Mehta (1973b) found 2% sucrose optimum for Cucumis cultures. Superior growth of

plant cells on sucrose or one of its monosaccharide components was shown in early nutritional studies (Robbins, 1922; White, 1934) and continues to be demonstrated for more recently isolated species (Butenko et al., 1972; Koji et al., 1973). Invariably however, an apparent preference for sucrose over its constituent monosaccharides sometimes can be traced to sucrose hydrolysis, a problem that arises during autoclaving of media (Ball, 1953; Koblitz et al., 1965; Meryl Smith and Stone, 1973).

Raffinose, a trisaccharide containing galactose, has proved a suitable energy source for some species (Mathes et al., 1973; Nickell and Maretzki, 1970). Polysaccharides such as starch were effectively utilised by many tissues (Nickell and Burkholder, 1950; Karstens et al., 1960; Carpenter, 1961; Nickell and Maretzki, 1969; Hendre et al., 1975). Nash and Boll (1975) reported that Paul's scarlet rose cell suspension grew equally well on either glucose, fructose, sucrose or trehalose. Isolated maple (Acer rubrum and Acer saccharum) callus tissue could utilize a wide range of carbon sources such as lactose, melibiose, mannose, trehalose, cellobiose, raffinose, glucose, fructose, maltose and sucrose (Martin Mathes et al., 1973). They have shown that maple callus cultures grew as well on medium containing filter-sterilized sugars as on medium containing autoclaved sugars. Pentoses generally do not sustain cell growth, although Saccharum sp. can utilize ribose (Nickell and Maretzki, 1970).

(b) Utilization of sugar alcohols :

Although widely occurring, sugar alcohols are usually poor energy sources. Pollard et al. (1961) showed the presence of inositol in coconut milk and also reported that it enhances the growth of carrot and potato explants. Inositol has been shown to be an essential supplementary nutrient requirement for spruce tumor tissue (Risser and White, 1964), Fraxinus americana callus cultures (Wolter and Skoog, 1966), and Convolvulus arvensis cells (Earle and Torrey, 1965). Hendre et al. (1975) reported that growth of maize, wheat, rice and sorghum was not significantly affected when inositol concentration was varied from 0 to 200 mg/l.

Perhaps the most promising work has been that, callus cultures from Malus robusta were successfully isolated and maintained on a medium with sorbitol (D-glucitol) as the sole carbon source. The growth of the callus on sorbitol exceeded its growth on glucose or sucrose (Chong and Taper, 1972). Daucus carota (Goris, 1971), and Nicotiana tabacum (Thorpe and Meier, 1972) can also survive on sorbitol. Studies with Prunus persica suggested, however, that its growth on sorbitol may be adaptive, since the initial growth on the sugar alcohol was much lower than on sucrose and only with time did it come to equal that of the disaccharide.

Mannitol is usually either not available for growth (Nickell and Maretzki, 1970; Mathes et al., 1973; Goris, 1971; Homes, 1967) or sustains only poor growth (Hildebrandt and Riker, 1949). Yet, in Fraxinus americana utilization of chromatographically pure mannitol was excellent (Wolter and Skoog, 1966), and evolution of $^{14}CO_2$ from mannitol has been reported in this species (Trip et al., 1964).

Glycerol too has been reported to support growth of some species in culture (Gautheret, 1948; Scala and Semersky, 1971; Goris, 1971).

In view of the extensive studies reviewed above and the variety of carbohydrates which have been shown able to support growth in plant cell cultures, it is rather surprising that very few enzymological studies have been carried out. Such studies would provide intriguing insights into the potential enzymological repertoire of plant cells in the utilization of different carbon sources.

(11) Carbohydrates and differentiation :

Though the effect of different carbohydrates and their levels on both growth and differentiation has been extensively examined, it has been elucidated only in a few cases. Wright and Northcote (1972) found that in sycamore callus, any sugar which allowed good growth also allowed root formation. They demonstrated the presence of sucrose, glucose and fructose in

root-forming calli irrespective of the nature of added carbon source. Ferguson et al. (1958b) reported that galactose at low concentrations was inhibitory to the growth of excised tomato roots cultured in presence of 1% sucrose. The roots were able to recover from this galactose inhibition if transferred within 3 days to lactose-omitted medium and that the inhibitory action of concentrations of galactose up to 0.1% could be fully counteracted by the simultaneous presence in the medium of glucose at 5 X the concentration of galactose.

Maróti and Lévi (1977) working with meristem cultures of Dianthus caryophyllus, reported that keeping the hormonal level constant, the change in sucrose level in the medium would decide the formation of either unorganised callus or organised shoots and roots. Tran Thanh Van (1977) utilizing thin cellular layers of Nicotiana and Begonia has clearly demonstrated the importance of various sugars and their levels in the medium in controlling morphogenetic responses like the induction of flower or vegetative buds and roots (Rawal and Mehta, 1981 in press).

Even in lower vascular plants, alteration of sugar (sucrose) level marks a shift from the gametophytic to sporophytic generation (Kato, 1965; Mehra, 1972; Kshirsagar and Mehta, 1978) irrespective of the gametophyte or sporophyte origin of the fern callus.

Arabinose, xylose, ribose, rhamnose, sorbose, sorbitol, mannitol, myoinositol, tagatose, phytic acid and glucuronic acid did not support measurable growth or embryogenesis over a 2-week period in wild carrot cell suspensions (Verma and Dougall, 1977). On the other hand, fructose inhibited growth and embryogenesis when autoclaved, but not when filter-sterilised. They have also obtained significant correlation between dry weight and embryo number regardless of the carbohydrate source suggesting the involvement of a common intermediate in the metabolism of the various sugars. Rao and Narayanaswamy (1968) induced differentiation of shoots and roots or unorganised proliferation in tissue cultures of Solanum xanthocarpum by altering the ratio of auxin and meso-inositol. Kochba et al. (1978) working with embryogenic and non-embryogenic cell lines of Citrus have demonstrated the influence of various sugars on embryogenesis and growth. They have also demonstrated differential embryogenic responses towards increasing sugar concentrations. Embryogenesis in Citrus was very significantly stimulated by galactose, lactose, and raffinose in the 1 to 5% concentration range and by lactose also at 10 per cent. Sucrose markedly inhibited galactose-induced embryogenesis progressively with increasing concentrations in Citrus sp. The galactose and lactose induced embryogenesis is also very strongly inhibited by increasing IAA concentrations. This would be expected if these sugars

act by inhibition of auxin synthesis and if a reduced auxin level is inducive to embryogenesis in Citrus callus (Kochba and Spiegel-Roy, 1977 a; Kochba and Spiegel-Roy, 1977 b). Their results were also in strong support of Anker's findings (1974) and indicated a similar mode of action of galactose in a different plant system.

(12) Carbohydrate as an osmotic agent :

Besides serving as an energy source, an additional role of carbohydrate, during growth and differentiation, may well be osmotic in nature. Mild osmotic stress affected tobacco (Klenovska, 1973; Klenovska, 1976) and soybean tissue cultures (Kimball et al., 1975) by increasing callus growth, and modifying the cellular morphology. Furthermore, Maretzki et al. (1972) have observed that small changes in osmotic stress appear to cause a high turnover of amino acids, lower reducing sugar concentrations and a decrease in invertase activity in sugar cane cell cultures. The importance of osmotic conditions in the culture of zygote embryos of Datura (Rietsema et al., 1953), Capsella (Raghavan and Torrey, 1963) and Hordeum (Granatek and Cockerline, 1978) have also been documented and osmotic-hormonal interactions have been outlined by Doley and Leyton (1970) and Granatek and Cockerline (1978).

(13) Starch and Sucrose metabolism :

While sucrose can reach the cytoplasm intact, other di, oligo or polysaccharides are probably hydrolysed extracellularly. The main enzymes involved are amylases and invertases :

(a) Amylases : Extracellular amylases are essential for utilization of starch by cell cultures, and these enzymes are frequently released into the surrounding medium (Karstens and De Meester-Manger, 1960; Straus and Campbell, 1963; Marvin and Morselli, 1971). Extracellular α-amylases have been detected in cell-free media after the growth of Rumex acetosa wound virus tumor tissue (Brakke and Nickell, 1951) and Nicotiana crowngall tissue (Jaspars and Veldstra, 1965); both α- and β-amylases were present in the spent medium after Saccharum sp. growth (Maretzki et al., 1971).

The endogenous formation and utilization of starch reserves has been followed in Nicotiana tabacum callus (Thorpe and Meier, 1972). Conditions favouring bud formation greatly enhanced both build-up and breakdown of the starch. Light initiated an earlier starch accumulation, but in both light and dark, starch content increased sixfold before it decreased. Concomitantly higher specific activities of the appropriate anabolic and catabolic enzymes were reported in the shoot-forming, compared to the nonshoot-forming callus (Thorpe and Meier, 1974a).

Sucrose metabolism : Sucrose is the major mobile carbohydrate molecule of higher plants. As such great interest has attached in recent years to the manner of its transport within the plant body, the mechanism by which it enters into the cell following transport, the reactions through which glucose and fructose are released and the sites at which this occurs. Cells in culture can help elucidate the loading and subsequent metabolic processes in recipient cells.

Whether sucrose enters the cell as an intact molecule or not or whether it is completely degraded either passively or of necessity during passage into the cell is a matter of some discussion (Maretzki et al., 1974). In Daucus carota the evidence for a mechanism permitting sucrose uptake without prior hydrolysis is conclusive. Calli from a repressed and a fully expressed strains took up sucrose at similar rates. Labelling patterns of sucrose recovered from the tissues confirmed that no sucrose inversion took place in the free-space during uptake from the medium (Edelman and Hanson, 1971 a; Edelman and Hanson, 1971 b). Similarly, in Saccharum sp. the dynamics of sucrose uptake, controls over its storage, and recall from storage have been investigated extensively (Glasziou and Gayler, 1972; Sacher et al., 1963; Bowen, 1972; Maretzki and Thom, 1972).

(b) Invertases : Despite the extensive use of sucrose in plant cell cultures for well over 30 years, we do not

still have any comprehensive study on the activities of the enzymes involved in its degradation. Three major enzymes have been implicated in the degradation of sucrose in plant cells, invertase, sucrose phosphate synthetase and sucrose synthetase. Of these three, invertases have been the most studied. The multiplicity of invertases - and even their very existance - vary greatly, not only among species but also among single cell isolates from the same species (Straus, 1962). In Daucus carota (Ricardo et al., 1972) and in Convolvulus arvensis callus (Klis and Hak, 1972) only acid cell wall invertases have been found. Also two forms of the cell wall invertase, differing in temperature sensitivity and molecular weight, recently have been found in Convolvulus arvensis callus (Klis and Akster, 1974). That the acid invertase is present in vivo in the cell wall and this is not an artifact of preparation has been positively demonstrated by the work of Ueda et al. (1974) in studies with cultures of Daucus carota. In the case of neutral invertase and sucrose synthetase it seems probable that between them these two enzymes account for the cytoplasmic mobilisation of sucrose prior to its entry into the various metabolic pathways of the cell (Maretzki et al., 1974; Parr et al., 1976). Close correlations exist between their activities and the metabolic status of cells in different stages of growth.

(14) Pathways of carbohydrate oxidation :

One means of determining the general direction of carbohydrate metabolism in cell cultures is to follow their oxygen consumption. Measurements of respiration have been made in callus cultures of Nicotiana (Bellamy and Bielesky, 1966), Acer pseudoplatanus (Givan and Collin, 1967), Rosa sp. (Nash and Davies, 1972), carrot (Komamine et al., 1969) and potato (Akemine et al., 1975). In the initial phase of growth oxygen consumption increased two fold or more in each of these species. This was followed by a period of lessening oxygen demand in all the above mentioned systems. Two intermediate pathways of carbohydrate oxidation occur in plant cells : glycolysis which is generally regarded as universal to all living cells; and the pentose phosphate pathway (PPP) also widely distributed in nature.

Further information about the direction of energy consumption and carbohydrate metabolism can be determined from the relative preponderance of the Embeden-Meyerhoff-Parnas (EMP) or PP pathways during cell growth. Hexose production from incoming or stored sucrose provides a significant proportion of the substrate for respiration. Data from pea roots (ap Rees, 1977) indicated that some 40% of the incoming sucrose enter the respiratory pathways almost immediately in the cortex and tip of the apical 3 cm. The respiratory pathways may also receive both hexose from

the hydrolysis of polysaccharides and glucose-1-phosphate from the phosphorylysis of starch. Regardless of the immediate source of glucose-6-phosphate, it is clear that its consumption in respiration is a major drain on the carbohydrate content of the plant and that control of this consumption is of prime importance. So far, few have measured accurately the relative activities of these two pathways. Since in the PP pathway the #1 position, and in the EMP pathway the #1 and #6 positions, of glucose are preferentially oxidised, monitoring the ratio of $^{14}CO_2$ released from #1 and #6 carbon of glucose labelled with ^{14}C in either one or the other of these positions provides suggestive evidence for predominance of one pathway over the other. Using such measurements in Acer pseudoplatanus (Fowler, 1971) cells and Nicotiana tabacum (Thorpe and Laishley, 1973) callus it was demonstrated that PP pathway is favoured for a short period after cell inoculation. Also, of late evidence exists that PP pathway is activated during early germination in cotyledons of chick pea seeds (Nicolás and Aldasoro, 1979).

(a) Glycolytic pathway :

Hormonal control of glycolysis which favours oxidative phosphorylation has been demonstrated. In Acer pseudoplatanus cells enzyme control of the EMP pathway by exogenous substrate has been shown by Scala and Semersky (1971). In

Nicotiana tabacum, cytokinins have been reported to reduce the activity of EMP pathway enzymes (Bergmann, 1963).

(i) Fructose diphosphate aldolase :

Fructose diphosphate aldolase (FDPA) is an important key enzyme in the glycolytic pathway that clevages fructose-1,6-diphosphate into two 3-carbon molecules, dihydroxyacetone phosphate and 3-phosphoglyceraldehyde. It was shown in Saccharum sp. that aldolase to some extent is regulated in the absence of exogenous auxin (Maretzki et al., 1974).

(b) Tricarboxylic acid or Citric acid cycle :

Little has been done with the enzymatic regulation of citric acid cycle in vitro, other than to demonstrate their existance. Organogenesis, especially shoot formation, has been shown to be concomitant with high rate of respiration (Ross and Thorpe, 1973).

(i) NAD dependent Malate dehydrogenase :

Since most of the energy in aerobic systems is generated during terminal oxidation in the Krebs cycle and malate dehydrogenase (MDH) is the terminal enzyme in the pathway producing oxalacetic acid (OAA) which enters the cycle again by reacting with acetyl-CoA, the rate of OAA turn over can act as a very reliable pointer towards energy requirements for growth and differentiation.Moreover,

oxalacetic acid in higher amounts becomes rate limiting, because it causes inhibition of malate dehydrogenase, hence the oxalacetic acid turn over and MDH activity will again be determined by the rate of reaction of oxalo-acetic acid with acetyl-CoA. Also the study of MDH would give the overall picture of respiratory rate and ATP generation.

(c) Pentose Phosphate pathway (PP pathway) or Phosphogluconate oxidative pathway or Hexose monophosphate shunt :

In higher plants the oxidative pentose phosphate pathway produces NADPH, and intermediates for biosynthesis. As the intermediates may be formed by the non-oxidative branch of the pathway, NADP reduction must be regarded as the prime function of the complete PP pathway in plants. Some, but by no means all, of the reductive biosynthesis in higher plants use NADPH (ap Rees, 1974). The PP pathway may supply precursors for the biosynthesis of aromatic amino acids, nucleic acids, and NADPH - the source of reducing power for these and other biosynthetic routes. (Agrawal and Canvin, 1971; Street and Cockburn, 1972; Ashihara and Komamine, 1975; Ashihara and Matsumura, 1977). One role for the PP pathway could be the provision of reducing power for lignin synthesis; for several of the enzymes involved in lignin formation use NADP (Stafford, 1974). Higuchi and Shimada (1967 a, 1967 b) provided evidence of a correlation

between lignin synthesis and the activities of the two dehydrogenases of the pathway.

(i) Glucose-6-phosphate dehydrogenase (G-6-PDH) :

Glucose-6-phosphate dehydrogenase, the first enzyme in the pathway, catalyzes glucose-6-phosphate to 6-phosphogluconate. It has been suggested that its activity might participate in the regulation of the PP pathway (Ashihara and Komamine, 1974 a; 1974 b).

(15) Dark fixation of carbondioxide and organogenesis :

Since light is known to influence organogenesis (Murashige, 1977), some experiments in the present study were conducted in dark. Hence the role of dark carbondioxide (CO_2) fixation during organogenesis seemed worthy of examination. Moreover, CO_2 has been shown to be essential for the initiation of growth of cultured sycamore cells (Gathercole et al., 1976). Further, ethylene accumulation inhibited greening of spinach suspension cultures, and this effect of ethylene was antagonised when the culture gas phase was enriched with carbondioxide (Dalton and Street, 1976).

(a) Phosphoenolpyruvate carboxylase (PEPC) :

It has been clear for some time that some of the phosphoenolpyruvate produced during carbohydrate oxidation

of glycolysis must serve as a substrate for dark-fixation of carbondioxide. The manner in which internodes of Coleus blumei (Pryke and ap Rees, 1976) metabolize (3,4-^{14}C) glucose indicated that quite an appreciable proportion of the phosphoenolpyruvate was converted to oxaloacetate via phosphoenolpyruvate carboxylase. Davies (1973) has suggested that PEP carboxylase activity regulates the intracellular PH by generating organic acids through a process of dark CO_2 fixation.

(b) Malic enzyme (ME) :

Unlike PEP carboxylase, malic enzyme, exerted an opposite effect on intracellular PH by catalyzing the decarboxylation of malate. This is supported by work with Avena sativa coleoptiles which demonstrated the stimulation of coleoptile growth by CO_2 (Bown et al., 1974) and a correlation between malate accumulation and indole-acetic acid stimulated growth (Haschke and Lüttge, 1977).

(c) Glutamate-oxalacetate transaminase (GOT) :

Glutamate-oxalacetate transaminase catalyzes the transamination of L-aspartic acid and α-ketoglutaric acid to oxalacetic acid and L-glutamic acid. Studies on the activity of GOT during culture growth or organogenesis are scanty even though it is reported in intact plants.

The mechanism by which the isolated cells and tissues sense changes in their immediate environment and posses "switching network" that permits all or none-response is the essence of the problem of growth and differentiation. The primary aim of this study has been to gain some knowledge about the physiological and biochemical parameters associated with growth and differentiation.

The review made above formed the background for the present study to examine some aspects of carbohydrate metabolism in a differentiating (tobacco) and non-differentiating (cotton) tissue culture systems.

The results and observations are presented in the Chapter III as outlined below :

Section A : Initiation and establishment of callus cultures of Nicotiana tabacum L. var. Anand-2 and Gossypium hirsutum L., cv. Sankar-5.

Section B : Growth and accumulation of total and reducing sugars in callus cultures of Gossypium hirsutum.

Section C - I : Physiological studies with Amylase, Invertase, MDH, G-6-PDH and FDPA and total and reducing sugars and total starch during growth of callus tissues of tobacco and cotton.

Section C - II : Physiological studies with GOT, ME and PEPC and total and reducing sugars during growth of callus tissues of tobacco and cotton in the dark.

Section D : Hormonal effect on growth and development of Amylase, Invertase, MDH, G-6-PDH and FDPA and on total and reducing sugars in callus cultures of cotton.

Section E : Effect of carbohydrates on growth and development of Amylase, Invertase, MDH, G-6-PDH and FDPA and on total and reducing sugars in callus cultures of cotton.

Section F : Organogenesis in callus cultures of Nicotiana tabacum L.

Section G - I : Physiological studies with Amylase, Invertase, MDH, G-6-PDH and FDPA and total and reducing sugars and total starch in callus tissues of tobacco and cotton on shoot inducing medium.

Section G - II : Physiological studies with GOT, ME and PEPC and total and reducing sugars in callus tissues of tobacco and cotton cultured on shoot forming medium in the dark.

Section H - I : Physiological studies with Amylase, Invertase, MDH, G-6-PDH and FDPA and total and reducing sugars and total starch in callus tissues of tobacco and cotton cultured on root differentiating medium.

Section H - II : Physiological studies with GOT, ME and PEPC and total and reducing sugars in callus tissues of tobacco and cotton cultured on root differentiating medium in the dark.

Section I : Osmotic requirement for shoot and root formation in callus cultures of tobacco.

Section J : Effect of an inhibitor (Rifamycin) on organogenesis and carbohydrate metabolizing enzymes in callus cultures of tobacco.

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