# CHAPTER I

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

# INTRODUCTION

It is now an established fact that the cell is the basic structural and functional unit in all organisms. When the cells are developing within the frame work of organised plant body, it is difficult to study their precise nutrition and the factors that influence the course of their growth and differentiation. A frequently employed technique, therefore, is to remove from the plant a group of cells constituting either an entire organ or a particular kind of tissue. These cells can then be transferred to a nutrient medium where their growth, nutrition and morphogenetic patterns may be studied under a wide variety of deliberately varied conditions. These techniques are referred to collectively as "tissue culture".

Successful development of tissue culture was necessiated by physiological problems which clearly demanded for their solution some extreme form of isolation of the tissue being studied. Although real success first came with animal tissues, the botanist Gottlieb Haberlandt (1902) clearly setforth the purposes and potentialities of plant cell culture. Though not entirely successful, Haberlandt foresaw the use of

cell cultures as an elegant means of studying physiological and morphological problems.

The first successful plant tissue cultures were, however, not such free cell cultures but cultures of isolated seedling roots (White, 1934) and, very quickly following this, of the callus tissue which arises from cambial tissue and certain other plant tissues in response to appropriate chemical stimuli (Gautheret, 1939; Nobécourt, 1939; White, 1939). Callus tissues derived from a wide range of plants and different plant parts are subsequently grown in continuous culture on the surface of agar media, which contain a utilisable carbohydrate, inorganic salts and various organic growth factors (Gautheret, 1959).

Tissue cultures so derived provide excellent research tools for determining the precise nutritional and metabolic characteristics of cells, tissues and organs after liberating them from the influence of the organism as a whole. By programming media or the physical conditions for growth, it is now possible to measure the important factors which influence growth and differentiation, and at the same time correlate these findings with underlying metabolic changes taking

place in the tissues. Besides their use for studying orgnisational nutritional, cytological and biochemical problems the use of this research technique is finding more and more applications in a wide variety of fields.

Many workers have employed it to clarify certain basic problems of infection, propagation and multiplication of certain plant viruses (Wu et al., 1960; Hansen et al., 1966), others (Holsten et al., 1965; Eriksson, 1965; Steward et al., 1967; Klein, 1967; Bajaj, 1970) have used it for investigating the direct and/or indirect effects of radiation. The prospectus of biosynthesizing secondary products from plant tissue cultures have been advocated (Street, 1966; Staba, 1969); and are being used to produce antimicrobial substances (Mathes, 1963; Campbell et al., 1965; Khanna and Staba, 1970) and alkaloids (Kaul and Staba, 1965; Staba and Jindra, 1968; Mehta and Staba, 1970; Thomas and Street, 1970).

Surface sterilization followed by aseptic removal of tissues or embryos has been used to obtain callus tissues (Gautheret, 1959; Street and Henshaw, 1966). For the initiation of callus, the explants are transferred onto the surface of agar media which contain

inorganic salts, a sugar as energy source and various organic growth factors (White, 1954).

The need to provide in plant culture media those macronutrient elements which are known to be required for the nutrition of the whole plant was established by pioneer work of White (1943) with root culture and of Heller (1953) with callus culture. Though these media have been found most suitable to support the growth of a wide range of tissues, many attempts have been made to modify the composition of the inorganic solutions used in the above media to suit particular tissues (Shigemura, 1958; Murashige and Skoog, 1962; Norstog and Smith, 1963). So far, however, most of these reports have not contributed anything substantial to our knowledge of uptake of these macronutrient elements and their involvement in metabolism. Studies with the micronutrient elements, on the other hand, have provided more useful data in the field of mineral nutrition of plant tissue and organ cultures (Butcher and Street, 1964).

The carbohydrate requirement of callus culture was first studied by Gautheret (1941, 1945) who examined the ability of various sugars to support the growth of

carrot tissue cultures. Similar studies involving tissues from number of dicotyledons were subsequently made by Hildebrandt and Riker (1949, 1953), Nickell and Burkholder (1950), Henderson (1954), Straus and LaRue (1954), Brakke and Nickell (1955), Arya *et al.* (1962), and Simpkins *et al.* (1970). The above studies revealed that most tissues grow best when supplied with sucrose, dextrose or laevulose.

Working with excised roots, White (1934) had, however, observed that tomato roots grew ten times more in sucrose medium compared to glucose grown ones. Similar superiority of sucrose as a carbon source for the growth of certain root cultures is also reported by Street (1966). However, a complete understanding of the superior activity of sucrose as a carbon source for the excised root cultures as well as callus cultures has yet not been achieved. It was, therefore, intended in the present study to test various sugars for their ability to support the growth of callus tissues and to investigate the superiority of one over the other, if any.

Plant tissues growing *in vitro* are autotrophic with regard to nitrogen metabolism. Nitrates are most

commonly used as source of nitrogen for tissue growth and their substitution with ammonium salts or nitrites has not been very successful (Riker and Gutsche, 1948; Burkholder and Nickell, 1949; Heller, 1953, 1954). However, a balanced supply of nitrates and ammonium salts has been shown to support better growth of certain callus cultures than the nitrates alone (Steward, Bidwell and Yemm, 1958; Shantz and Steward, 1959). The effects of urea, alanine, glutamic acid, aspartic acid, arginine and asparagine on the growth of tissue cultures are also examined (Riker and Gutsche, 1948) and the growth promoting activities of some amino acids are reported by several workers (Street, 1955; Steward *et al.*, 1958; Street *et al.*, 1961). Casein hydrolysate either acid or enzyme hydrolysed has also been extensively used in tissue culture media, but with varying results. Its toxic effect on the tissues has been reported by Nitsch and Nitsch (1957), Babcock and Carew (1962), Staba (1962), Steinhart, Anderson and Skoog (1962) and Vasil and Hildebrandt (1966); while Henderson *et al.*, (1952), Steward and Shantz (1954), Steward and Pollard (1959), Hildebrandt (1963), Arya (1965), and Tiwari and Arya (1967) and Fadia (1971) found that addition of casein hydrolysate enhanced the growth of tissues *in vitro*.

Besides normal nutritional requirements growth of plants and their isolated tissues is dependent upon the availability of growth substances (Audus, 1963). They are known to play important role in cell metabolism, predetermining course of cell division, growth, differentiation and cell hypertrophy (Butenko, 1968). Of these growth substances, auxin is an essential supple ment which needs to be added to a basic medium for the successful culture of a number of normal callus tissues. The entire plant synthesize sufficient amount of auxin to enable them to grow and develop, completing the normal cycle of life. But isolated tissues and cells synthesize auxins in suboptimal amounts needing an additional supply of auxin in nutrient medium for their continuous growth in culture (Reinert and White, 1956; Paris and Buhamet, 1958; Gautheret, 1959).

Over and above auxin, for many callus cultures, and for most suspension cultures, the culture medium must also contain vitamins or vitamins and reduced nitrogen compounds or other complex growth factors such as are present in coconut milk, yeast extract or other natural fluids (Street, 1966). The later are found to act synergistically with auxins and enhance growth considerably (Steward and Shantz, 1955; Torrey and Shigemura,

1957; Mehta, 1966; Rao and Mehta, 1968). Such complex supplements, however, limit the usefulness of tissue culture technique when analysis of chemical changes are desired. Attempts have been made, therefore, to replace the natural fluids and other supplements of incompletely known composition by known mixture of amino acids, purines and vitamins.

Many synthetic media which are evolved in the above pursuit contain cytokinins, another class of growth substances. Kinetin which is the most widely used of the cytokinins is known to possess growth stimulating property (Butcher and Street, 1960; Miller, 1961). Incorporation of kinetin into nutrient medium has led therefore to substantial enhancement in growth of a number of callus tissues (Murashige and Skoog, 1962; Steward, 1969; Vajranabhaiah, 1969; Lalchandani, 1970).

Just as callus is only initiated on explants above a critical size (Jablonski and Skoog, 1954), so the maintenance of growth in callus tissues by subcultures requires the transfer on each occasion of a piece of callus tissue above a certain critical mass. There is a critical minimum inoculum size for any fixed volume of the medium, below which growth in subculture normally

fails. When the size of the callus mass is not markedly above this critical level, a lag phase intervenes before the culture embarks upon a period of rapid growth. The effect of initial size on the ability to grow has been recognised by Steward and Caplin (1954) and the influence of the volume of the medium on the growth of the tissues was studied by Gadgil and Roy (1961). Henshaw, Jha, Mehta, Shankeshaft and Street (1966) have established the close relationship between the inoculum-volume ratio on one hand, with the final growth attained on the other.

Further, our knowledge regarding the patterns of growth and cell division in plant tissue and cell cultures has advanced considerably in the past decade. Studies by Torrey, Reinert and Merkel (1962) on Convolvulus and Haplopappus cultures, by Street and Henshaw (1963) in Rubus cultures and by Mehta, Henshaw and Street (1967) on Phaseolus and Linum cultures demonstrated that the peak of mitotic activity was attained by the cells within the first seven or ten days after inoculation into a fresh medium. They further showed that during this initial period of high mitotic activity, there was hardly any increase in cell dry weight. In all the above cases investigated the period of maximum mitotic activity

has been of very short duration. It is particularly important that further information is obtained about the cause of rapid fall in the mitosis so that, if possible, cells can be maintained for longer period in the state of active cell division. The short duration of phase of high mitotic activity could be the result, either of the accumulation of a cell division inhibitor, or of the depletion of nutrient. These possibilities were examined by Henshaw et al. (1966) in their studies with Parthenocissus tricuspidata Crown gall tissue. They have shown that the main factor which operated to limit cell division was the supply of an essential nutrient and not the accumulation of endogenous inhibitors. They have further demonstrated that this limiting nutrient was neither sucrose, nor one of the microelement salts of the Heller's medium, but the nitrate supply. Whether it is depletion of these nutrients which supervenes to check mitotic activity in other tissue cultures alo, however, remained to be examined.

The above observations formed the background for consideration of experimental approaches to the studies on growth in culture of Datura callus described in the first part of the present thesis.

The major phase of any culture system is the period of most rapid growth, and the most important transitions are those which mark the beginning and end of that period. It is obvious that the metabolism associated with cell multiplication must be switched on at the beginning of this period, and switched off at the end. It was, therefore, thought worthwhile to examine the changes in cellular nitrogen and carbohydrates associated with the growth of tissues in culture for three weeks. This period would cover the major phase of the culture system as mentioned above.

Givan and Collin (1967) examined the relationship between growth, respiration and nitrogen content in Acer pseudoplatanus cells in suspension cultures. They demonstrated that nitrogen content and rate of oxygen uptake rose sharply in first few days of culture, during which time no significant increase in dry weight and only a small increase in cell number were registered. While in the subsequent period of rapid cell division a decline in both respiration rate and nitrogen content per mg dry weight or per cell was discernable. Pronounced rise in respiration rate and cell nitrogen were thus found to occur prior to the period of rapid cell

division. Filner (1965) had earlier reported that pronounced rise in protein content preceded growth by increase in dry weight in Nicotiana tobacum cells in liquid suspension.

As mentioned earlier, many plants absorb most of their nitrogen in the form of nitrates. Since in nitrates the nitrogen is in a highly oxidised state, reduction of nitrate is one of the steps in the synthesis of amino acids and other organic nitrogenous compounds. The enzyme or enzyme systems which catalyzes the reduction of nitrate to nitrite is called nitrate reductase. Evans and Nason (1953) were the first to demonstrate the presence of nitrate reductase in cell free extracts of soybean leaves. Nitrate reductase is an inducible enzyme and it can be induced by nitrate and possibly by nitrite was first reported by Hewitt et al. (1956). It has been similarly found to be inducible by nitrate in rice (Tang and Wu, 1957), wheat (Rijven, 1958), cauliflower and mustard (Hewitt and Afridi, 1959), corn (Hugeman and Flesher, 1960), tobacco (Filner, 1965), and probably in virtually all other species. In cauliflower both molybdenum and nitrate are found to be required for the induction of

nitrate reductase (Afridi and Hewitt, 1964), and the level of nitrate reductase is further reported to increase with the increasing nitrate concentration (Beevers et al., 1965; Afridi and Hewitt, 1962). Candella et al. (1957) observed that the activity of the enzyme obtained from cauliflower which was given ammonium sulphate was much lower. Filner (1966), on the other hand, reported the repression of nitrate reductase by casein hydrolysate in cultured tobacco cells.

Moreover, it has been established with considerable certainty that nitrate is assimilated via ammonia in higher plants. Evidence for this comes from several different investigations in which $N^{-15}$ labelled compounds have been utilized. Firstly, it has been shown that nitrate is reduced to ammonia (Delwiche, 1951; and Mendle and Visser, 1951). Secondly, it has been demonstrated that nitrate and ammonia have essentially the same effect on the production of free amino acids and proteins (Mendel and Visser, 1951; Yemm and Willis, 1956). Finally ammonia has been shown to be directly incorporated into organic nitrogenous substances via glutamate and glutamine (Cocking and

Yemm, 1961). The other amino acids could arise secondarily from glutamic acid or glutamine by transamination or other inter conversion. An interconversion of glutamic to other amino acids of barley seedlings has already been shown in experiments of Folkes and Yemm (1958).

Besides the measurement of cellular nitrogen, the progressive changes in the pattern of activities of nitrate reductase and glutamic oxaloacetic transaminase are also followed in the present investigation to examine correlation with growth of Datura callus tissues. The progress of enzyme activity of acid β-glycerophosphatase too was examined during the course of culture. The effect of 2,4-D and kinetin on the phosphatase activity was further studied and these observations are also incorporated in the present thesis.

The aims of the present investigation were: First, to establish tissue cultures from the anthers of Datura, to study the nutritional requirements and to examine the effects of various cultural parameters on the growth of callus tissues; and second, to investigate the physiological and biochemical changes associated with growth of the tissue culture.