GENERAL DISCUSSION

The main objectives of the present investigation were, as mentioned in the Introduction (Chapter 1), to establish tissue cultures from Datura metel L., to examine the nutritional requirements and the effects of various cultural parameters on growth of the tissues. Studies were also designed to investigate the physiological and biochemical changes associated with growth. To what extent the evidences obtained in the present investigation have realised the aim now remains to be assessed.

It was clear from the Experiment 1 that callus could be induced on the excised anther segments of Datura in White's medium containing an auxin, coconut milk and casein hydrolysate. Only mature anthers with fully developed pollen grains proliferated to form a callus mass. Microscopic examination of histological preparations revealed that the callus initiated from the connective region first and later wall cells of the anther also joined in.

It was found that the presence or absence of auxin had a marked effect on the growth of the tissue

(Experiment 2). Of the auxins tested, Datura callus cultures grew best in the 2,4-D containing medium, the optimal concentration being 2.0 mg/l. The same concentration of 2,4-D has been reported to be optimal for the growth of callus tissues derived from different plant parts, such as seed, root, stem or leaf. Clearly, the anther origin of callus in present case did not reveal any different auxin requirement.

Growth was measured of Datura anther tissue on the Datura medium (Table 4), which was evolved by combining the macroelement salts as given in Heller's medium (Table 2) with the microelement salts and vitamins according to White (Table 1) and supplementing it with coconut milk (10%), casein hydrolysate (300 mg/l) and 2,4-D (2.0 mg/l), (Experiment 3). The growth curve illustrated in Figures 5A and B showed that after a slow start the growth was most rapid during second and third weeks after which it slowed down considerably (Experiment 4). The most rapid growth proceeded for 7 days (between 2 and 3 weeks) during which there was registered about 8 fold increase in the total cell number. This corresponded to almost 3 cell generations in 7 days with a mean generation

time of little over 2 days. These findings compare favourably with Lamport's (1964) results with Acer pseudoplatanus suspension cultures. He found from the determinations of cell dry weight and packed-cell volume that during the exponential phase the mean generation time was of two days in his cultures. His data were, however, obtained by sampling large suspension cultures involving 2-5 litres of culture medium agitated in 5-10 litre culture flasks. Using batch culture methods, Henshaw et al. (1966) reported a 7 fold increase in cell density, corresponding to between 3 and 4 cell generations in 14 days, with a mean cell generation time of 4 days.

Studies with Kinetin, alone and in combination with 2,4-D, showed that the maximum callus growth was attained at 0.5 mg/l kinetin alone or with 0.5 mg/l kinetin and 2.0 mg/l 2,4-D (Experiment 6). At this optimal combination of kinetin and 2,4-D, the growth was appreciably more than when both were added separately at the same concentration. Witham (1968) has suggested that 2,4-D may be a weak cytokinin as well as an auxin, or that 2,4-D may cause the tissue to produce its own cytokinin. The present

studies with Datura tissues seemed to support Witham's suggestion; for the growth attained in 0.05 mg/l kinetin and 2.0 mg/l 2,4-D containing medium was markedly higher than in the medium containing 0.5 mg/l kinetin alone.

Besides hormones, many callus cultures are known to require other complex growth factors as stated in the Introduction (Chapter 1). Datura callus tissues presently investigated also showed pronounced growth enhancement when coconut milk was added to the auxin containing medium (Experiment 1). The synergism between auxin and coconut milk is well documented in literature (Steward and Caplin, 1951; Steward and Shantz, 1955; Street, 1966; Rao and Mehta, 1968; Lalchandani, 1970; and Fadia, 1971).

Studies of the carbohydrate requirement of Datura callus cultures (Experiment 5) showed that sucrose at 2 per cent concentration supported the highest growth as measured in terms of increase in dry weight and total cell number. The equimolar mixture of glucose and fructose did not support any higher growth than when glucose and fructose were supplied separately. Though much less as compared with sucrose, soluble

starch did support some growth of the tissues. Nickell and Burkholder, (1950) had found starch to be an excellent carbon source for the growth of virus tumor tissues from the roots of Rumex acetosa. Whether the utilisation of starch as the source of carbon by Datura tissues was accomplished by the production and secretion by the tissues of an α-amylase was not ascertained in the present investigation. Similarly, in our present studies the various sugars were supplied separately (except where the equimolar mixture of glucose and fructose was incorporated ~~separately~~ and no antagonism, therefore, had been observed as was demonstrated in case of excised root cultures (David, 1954; Ferguson, Street and David, 1958; Morgan and Street, 1959).

Growth was found to enhance with increasing level of sucrose upto 2 per cent, but at 4 per cent there was sharp decline in growth. Calculated on per cell basis it was, however, noticed that the dry weight per cell enhanced with increasing sucrose concentration from 0.5 to 4 per cent. Similar increase in mean dry weight per cell without enhancing either cell number or packed-cell volume was noticed by

Henshaw et al., (1966) in Parthenocisus tricuspidata crown-gall tissue and by Simpkins et al., (1970) in Acer pseudoplatanus tissue when they enhanced the sucrose level in the medium.

The present studies clearly showed the superiority of sucrose as carbon and energy source for the growth of Datura tissue cultures, as was also indicated in the brief survey of the carbohydrate requirements of callus and root cultures in the Introduction (Chapter 1). Attempts were made in the present study to elucidate the superiority of sucrose over other carbohydrates tested by examining the bound and free sugars, cellular nitrogen contents and patterns of invertase and glutamic oxaloacetic transaminase in tissues cultured in sucrose or glucose media.

Studies with total and free sugars in Datura callus cultures incubated on different carbohydrate sources revealed that the tissues contained more of free (reducing) sugars than bound (non reducing) sugars, except in tissues grown on media containing sucrose where the bound sugars were consistently more (Experiment 11). Perhaps this might explain the

highest growth attained on sucrose medium. Further, with the increase in concentration of sucrose there was progressive increase in bound sugars over free sugars (Experiment 12). The growth of the tissue as measured by increase in dry weight enhanced, however, only upto 2 per cent sucrose, and at 4 per cent sucrose the growth declined which may possibly be on account of the supra-optimal ratio between bound and free sugars. The differential growth values obtained on media containing various carbohydrates examined cannot be arising from differences in the osmotic pressures of the solutions; for in the control where the osmotic pressure was minimum, there was least growth and in soluble starch where the osmotic pressure was maximum, the growth was poor as compared with sucrose which supported the highest growth. Dormer and Street (1949) had presented evidence to show that the contrast in growth promoting activity between sucrose and dextrose could not be explained in terms of impurities in sugars nor as resulting from differences in the osmotic pressures in the two solutions.

The progress of invertase activity, which increased with the age of the tissue cultures upto day 15

before declining, was markedly higher in tissues grown on medium containing sucrose than in those grown on glucose or fructose media (Experiment 11). The higher levels of bound sugars noticed in tissues grown on sucrose medium could possibly be on account of rapid utilization of the monosaccharides formed by the enhanced invertase activity. Presence of higher amount of free sugars than bound sugars in equimolar mixture of glucose and fructose probably suggests preferential rapid utilization of monosaccharides formed by enzymatic hydrolysis of sucrose rather than the utilization of monosaccharides supplied as such. Moreover, there was higher protein synthesis and enhanced glutamic oxaloacetic transaminase activity in Datura tissues grown on sucrose containing medium as compared with glucose grown tissues (Experiments 14 and 15). However, a more detailed investigation involving the uptake of sugars and their secretion into the medium needs to be undertaken to gain better understanding of the superior effect of sucrose over other sugars.

Examination of the relationship between invertase activity and growth of Datura tissue (Figure 16) showed that the enzyme activity enhanced rapidly during the initial two weeks of culture when

the dry weight increase was slight and that during the subsequent week the enzyme activity declined when pronounced increase in dry weight was recorded (Experiment 11). It was further noticed that the invertase activity enhanced with the increasing level of sucrose, but the growth declined (Experiment 12). These findings seemed to suggest that there was no clear correlation between growth and invertase activity.

Examination of changes in cellular nitrogen associated with growth of Datura callus cultures showed that during the initial lag phase, in which there was little increase in cell number and dry weight, there was marked increase in the nitrogen contents. Before the cells embarked on rapid cell proliferation and the dry weight commenced very rapidly inccrease, there was sharp rise in soluble and insoluble nitrogen. The maximum values for the nitrogen fractions were attained on day 12 before declining. The growth measured by increase in dry weight and cell number continued to increase after the cellular nitrogen had passed its peak values. Thus, a pronounced rise in nitrogen per unit dry weight and per cell occured before the cells began to grow rapidly

and during the subsequent period of rapid growth the nitrogen content per unit dry weight and per cell declined. The data presented clearly indicated that long before the cells began to divide rapidly they were very active in synthesizing nitrogen compounds, particularly the insoluble ones. The insoluble nitrogen compounds (presumably mainly protein) synthesized prior to the on set of rapid cell division might well include mitotic proteins, though this is purely conjunctural at present.

Summarising previous work, Mazia (1961) has indicated that in a number of organisms and tissues there are significant increases in protein synthesis and respiratory activity prior to cell division, although these processes may be markedly reduced during the division process itself. Stern and Hotta (1963) had presented evidence that there was a synthesis of protein prior to division and that this protein was essential for the cell division to occur.

The present findings support the above observations and also the work of Givan and Collin (1967) which is mentioned in detail in the Introduction (Chapter I). Working with Nicotiana tobacum cells in

liquid suspensions, Filner (1965) presented interesting evidence showing that the increase in protein during the growth cycle represented changes in specific proteins and not merely a generalised increase. Accompanying the general protein increase, he demonstrated that there was much larger relative increase in nitrate reductase, whereas the activity of acid phosphatase declined. Reexamination of above postulate using Datura callus cultures did not yield supporting evidence; for the pattern of acid phosphatase simulated the curve for nitrate reductase, particularly during the initial two weeks of culture (Experiment 21, Figure 22).

A number of workers (Loustalot et al., 1953; Berezovsky and Kurochkina, 1957; and Switzer, 1957) have made observations showing the relation between auxin and phosphorous, lending further support to the possibility of phosphorous metabolism as the sight of auxin action as suggested by Bonner and Bandursky (1952) and by Van Overbeek (1952).

The studies on acid phosphatase activity described in Experiment 21 (Chapter IV) clearly showed that

the activity changed during the course of culture and was considerably influenced by auxin and kinetin treatments. Both 2,4-D and kinetin caused similar patterns of effect on acid phosphatase activity; the effect of kinetin being, however, more pronounced than that of 2,4-D. The same concentration of 2,4-D and kinetin, singly and in combination, which supported the highest growth of Datura tissues, also promoted the maximum acid phosphatase activity. These findings agree with the previous reports that the phosphatase activity increased in potato (Turian, 1956) and in roots of Zea mays and Vigna seedlings (Oslen, 1960) treated with auxin. The stimulatory effect on acid phosphatase by NAA and kinetin in Cucumis cell suspensions was demonstrated by Vajranabhaiah (1969) working in this laboratory.

The inoculum/volume ratio had considerable influence on total growth attained by Datura callus tissues (Experiment 7). In a fixed volume (20 ml) of medium, the highest growth as measured by increase in fresh and dry weights and total cell number occured when the inoculum was low (50 mg), and with the increasing inoculum size there was gradual decline in

growth values (Experiment 7A). At a fixed inoculum size (100 mg), on the other hand, the growth values (in terms of increase in fresh weight, dry weight and total cell number) enhanced with increasing volumes of the medium (Experiment 7B).

Buchasbaum (1932) in his studies with embryonic cells of the chick observed that cultures of smaller initial weight tended to grow relatively more rapidly. Working with carrot root cultures, Gauthret (1942) had presented evidence that the ratio of final to initial weight of a culture varied inversely as the initial weight. Subsequent work by other researches has led to recognization of the importance of initial size on the capacity of the tissue to grow. Further, Henshaw et al. (1966), Synno, Kunihiko and Tustomu Furuya (1968), Lalchandani (1970) and Fadia (1971) observed that the different growth responses by small and large inocula, in a fixed volume of the medium, were also reflected on the duration of the initial lag period of growth; the lag being longer when the inoculum is small and vice versa.

This close relationship between the volume of the medium and the inoculum size, on one hand, with

total growth attained on the other, strongly suggested that the growth of the callus tissues was limited by the supply of some essential nutrients. This possibility was examined in the present investigation with Datura callus tissues; and it was found that it was neither sucrose (Experiment 5), nor one of the microelement salts (Experiment 8A) that limited the growth of Datura tissues. However, when the level of macroelement salts was doubled there was pronounced enhancement in growth. Alteration of the concentrations of separate macroelement ions indicated that the level of nitrate was the factor limiting growth; doubling the concentration of nitrate ion reproduced the effect of doubling the concentration of the whole of the macroelement supplement. The enhanced growth on higher levels of nitrate could not, however, be maintained during subsequent culture passages (Experiment 8C). The 4 fold nitrate in the medium drastically reduced the growth during the second culture passage, while the 2 fold nitrate level as well as the double the level of complete macroelement salts in the medium had severe adverse effect on growth during the third passage.

The above findings are consistent with observations made by others with cultured plant cells. Harris (1956) found that the growth of cultured oat embryos was nitrogen limited. Murashige and Skoog (1962) showed that the nitrogen supply was the major growth limiting factor for tobacco cells on a defined medium. The evidences produced by Hensahw et al. (1966) also clearly demonstrated that the total cell production per culture of Parthenocissus tricuspidata crown-gall tissue was also limited by nitrate supply.

Studies of the nitrogen requirement of Datura callus tissues showed that when sodium nitrate was replaced by equimolar concentration of ammonium nitrate, the fresh weight and total cell number of the tissues enhanced (Experiment 9A). Similar high rate of growth with ammonia as the sole source of nitrogen was observed in excised tomato roots by Hannay (1959) and in Cucumis callus cultures by Fadia (1971). Steward et al. (1958) and Shantz and Steward (1959) however, presented an evidence that when 10 per cent of nitrate were replaced by ammonium salts, the growth of the carrot and potato tissues in media containing coconut milk was better than in presence of nitrate alone.

Casein hydrolysate has been shown to be capable of meeting the nitrogen requirement of some cultured tissues such as sunflower crown gall tissue (Riker and Gutsche, 1948) maize-endosperm tissue (Straus and LaRue, 1954; Tamaoki and Ullstrup, 1958), mature embryos of Hordeum (Harris, 1956), immature embryos of Datura (Sanders and Burkholder, 1948) and Capsella bursa-postoris (Rijven, 1952) and Cucumis callus cultures (Fadia, 1971). It was, however, found to be ineffective as sole nitrogen source for Datura tissues. But it enhanced the growth of its tissues when added as a supplement in the medium containing nitrate. Murashige and Skoog (1962) who investigated the activities of casein hydrolysate rather extensively, suggested that responses may differ depending upon the nitrogen level in the medium. Vasil and Hilderbrandt (1966) found casein hydrolysate highly inhibitory to growth when incorporated in Murashige and Skoog (1962) medium. On the other hand, Tiwari (1968) reported that casein hydrolysate enhanced growth of Pennisetum typhoides Stapf and Hubb. grown on White's basal medium. The above results clearly indicated that the effects of casein hydrolysate were stimulatory or inhibitory, when the level of nitrate ions was low (3.2 m. moles/l

as in White's medium) or high (39.4 m. moles/l as in Murashige and Skoog medium). In present studies with Datura tissues, which were grown on relatively low (7.1 m moles/l) NO_3 ions containing Datura medium (Table 4), the addition of casein hydrolysate promoted growth; which further supports Murashige and Skoog's suggestion. Recently, Simpkins, Collin and Street (1970) observed that the growth, in presence of nitrate, of Acer pseudoplatanus cells in synthetic liquid medium was significantly enhanced by supply of urea. Addition of casein hydrolysate enhanced growth, particularly when nitrate was omitted; as has also been found with Cucumis callus studied by Fadia (1971).

Pursuing the studies of nitrogen requirement of Datura callus tissues, experiments were conducted to examine the patterns of nitrate reductase and glutamic oxaloacetic transaminase in Datura callus during the course of culture and to determine the effects of different levels and sources of nitrogen on their activities.

The results obtained in Experiments 16 to 20 revealed that the enzyme nitrate reductase was almost

entirely induced during first 3 days of culture. The subsequent increase in the specific activity of the enzyme roughly paralleled the rise in cellular nitrogen particularly soluble nitrogen (Figures 17 and 18), and the maximum enzyme activity was recorded on day 12 when highest protein per cell occurred. After day 12 the enzyme activity declined as also the nitrogen content per cell (Tables 28 and 31).

Similar trend was observed with glutamic oxaloacetic transaminase which was also entirely induced during the initial 3 days and reached the peak value on day 12 before declining (Experiment 16).

The nitrate reductase activity was further influenced by the level of nitrate in the medium (Experiment 17). The activity increased with the enhanced levels of nitrate; though very high (x4) level of nitrate was toxic for the growth of Datura callus tissue. The glutamic oxaloacetic transaminase responded differently in that it followed a pattern very similar to that of growth. In other words, though higher (x4) level of nitrate enhanced nitrate reductase activity it repressed the glutamic oxaloacetic transaminase activity as well as growth.

In so far as comparable, the present findings are consistent with the observations made by Filner that the nitrate reductase activity in Nicotiana tobacum cell suspensions increased with the age of the culture and reached the highest value when the protein content per cell was maximum. He further found that nitrate reductase not only depended on nitrate to be induced, but in addition the enzyme decayed in absence of nitrate. The cells ceased to grow and devide at about the time when nitrate was completely depleted and nitrate reductase almost completely decayed.

Studies with the influence of different nitrates on nitrate reductase and glutamic oxaloacetic transaminase activities revealed that the presence of ammonia in the medium repressed nitrate reductase activity, whereas the glutamic oxaloacetic transaminase was stimulated (Experiment 18). Similar inhibition of the enzyme nitrate reductase in cells grown with ammonia as the nitrogen source was shown by Morris and Syrett (1964) and by Afridi and Hewitt (1964).

The patterns of nitrate reductase and glutamic oxaloacetic transaminase in Datura tissues grown in presence of increasing amounts of casein hydrolysate

in Datura medium (Table 4) were also quite different (Experiment 19). The data showed that there was progressive repression of induction and subsequent development of nitrate reductase with increasing levels of casein hydrolysate in the medium. In contrast, the glutamic oxaloacetic transaminase activity enhanced with the increasing level of casein hydrolysate. Thus casein hydrolysate markedly repressed the induction and development of nitrate reductase without inhibiting growth, whereas both the induction and development of glutamic oxaloacetic transaminase was stimulated.

Furthermore, the presence of molybdenum in trace amounts (0.001 mg/l) which promoted the growth of Datura callus tissues (Experiment 10) also enhanced the nitrate reductase activity (Experiment 20). The requirement for molybdenum (besides nitrate) for the induction of nitrate reductase activity in excised leaf tissues of Cauliflower was demonstrated by Afridi and Hewitt (1964).

In view of the above observations, it may be concluded that one of the environmental changes that brings about major alteration in cell growth is the

level and type of nitrogen available from the medium. The findings in the present investigation that the growth of Datura cultured cells might be experimentally regulated by the exogenous nitrogen supply, accompanied by the fact that the endogenous organic nitrogen level is regulated through feed-back control of nitrate reductase suggests the possibility that the regulation of endogenous nitrogen may be used by the plant as a device for regulating and co-ordinating growth.

There is a growing body of evidence that amino acids can upset the normal development of a plant, and that nitrate reductase level is related to developmental processes. Filner (1965) has presented evidences concerning the role of amino acids in the regulation of nitrate reductase. Waris (1959) induced neomorphs by amino acid treatments. Sanders and Burkholder (1948) observed altered morphogenesis in Datura embryos cultured in the presence of amino acids. LaMotte and Skoog (1960) showed a morphogenetic effect of tyrosine on tobacco pith callus cultures. Nakashima (1964) found that amino acid inhibited flower formation in Lemna.

Nitrate reductase level has been correlated with differentiation and development in several cases. Candella, Fisher and Hewitt (1957) found that the nitrate reductase level in the leaves depended on the position of leaf on the plant. Zieserl and Hageman (1962) found that nitrate reductase in maize depended upon the developmental stage of the plant. Sanderson and Cocking (1964) noted a marked difference between the nitrate reductase levels in the leaves and roots of six species. Raghavan and Torrey (1964) found that orchid embryos in culture could not produce nitrate reductase activity until a certain stage of growth.

More intensive studies of the critical role of nitrate reductase regulation in the growth of cultured plant cells needs to be undertaken to prove conclusively that the induction and repression of this enzyme are important factors in plant growth and development.