D I S C U S S I O N

DISCUSSION

The culture of angiosperm cells and tissues excised from intact plant, has now become a powerful tool, which is being widely exploited. Recent advances in tissue culture techniques have made possible the investigations of growth and morphogenesis. The present research endeavour concentrated on : initiation of diploid and haploid callus cultures of Nicotiana tabacum L. var. Anand-2; the examination of growth responses and production of phenolics in the above mentioned callus cultures; organogenetic responses as influenced by growth substances; the physiological changes during callus initiation and its growth, and lastly the study of certain enzymes and isoperoxidase patterns during root and shoot differentiation.

A. Initiation of Diploid and Haploid callus tissues of tobacco.

Callus was induced from the floral buds of N. tabacum L. var. Anand-2 as described in Chapter III, Section A, Expt. 1. The callus was fast growing and was maintained in 30 day cultural cycles. Chapter III, Section A, Expt. 2, describes the induction of haploid tobacco plantlets and the pathway followed during pollen embryogenesis. Microspores at uninucleate stage were found suitable for androgenesis, since

binucleate pollen followed the normal course of development and germinated. The microspore nucleus divided to give rise to two identical nuclei. Further divisions of the cell occur, resulting in the formation of globular embryo which is released from the exine. The embryos grew further and finally emerged from the anther. The path of pollen embryogenesis was essentially as observed by Nitsch (1974 a) and Reinert et al. (1975) for N. tabacum. The anther derived plantlets on transfer to NM with 1% sucrose developed a good root system, as suggested earlier by Nitsch (1969). Higher sucrose levels in the medium did not promote the development of root system.

Contrary to the reports of Nitsch and Norreel (1972), Nitsch (1974 b), Wang et al. (1974), Wenzel et al. (1977), Sangwan - Norreel (1977), Sunderland and Roberts (1977) and Nitsch (1977), low temperature pretreatment (0-15°) of anthers for a period of 2-72 hr neither stimulated the frequency of pollen embryogenesis nor enhanced developmental responses.

The minimal medium required for androgenesis was NM with 0.2 mg/l IAA or 15% (v/v) coconut milk and 2% sucrose. In the present study the nutritional requirements are not as simple as reported by Nitsch (1969) for N. tabacum and by Rashid and Street (1973) for Atropa. Since the addition of IAA (0.2 mg/l) and coconut milk (15%) in combination enhanced embryogenesis, the nutritional requirements seemed to be more complex than envisaged.

In Chapter III, Section A, Expt. 3, are given the details of callus initiation from shoot explants of haploid plantlets. The medium used was the same as for floral buds. Thus both the diploid and haploid callus was maintained under identical cultural conditions.

B. Growth and Accumulation of Phenolics in Diploid and Haploid callus cultures.

For the culture of a number of callus tissues, auxin is an essential supplement which needs to be added to the basal medium supplying inorganic ions and carbohydrates. Among the natural and synthetic auxins, indoleacetic acid (IAA), naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and indolebutric acid (IBA) are most widely used for the culture of various normal tissues (Gautheret, 1955). The above four auxins were tested for their ability to support growth and synthesis of phenolics. The results are given in Chapter III, Sections B-I, B-II.

A glance at tables 5 and 8 revealed that floral bud callus and haploid callus could grow in absence of exogenous hormonal supply. However, the growth was more (4.8 fold on dry wt. basis) of diploid callus than of haploid callus (2.8 fold on dry wt. basis). In diploid callus, incorporation of 2,4-D into the basal medium antagonized growth (as was

obvious from comparison between increase of dry wt. on basal medium and the media containing different levels of 2,4-D. See Table 5). Haploid callus tissues responded only to low level (0.5 mg/l) of 2,4-D, higher concentrations resulted in decreased growth values. NAA (2.0 mg/l) supported maximum growth of both diploid and haploid callus. The growth of haploid callus was more (15.1 fold on dry wt. basis) than that of diploid callus (9.8 fold on dry wt. basis).

An interesting aspect of auxin stimulated growth of tobacco callus cultures became obvious by comparing the growth of diploid and haploid callus in response to the native auxin IAA. At all concentrations of IAA tested (0.5, 2,0 and 4.0 mg/l) growth of diploid callus on dry wt. basis was nearly twice as much as that of haploid callus. The same, however, does not hold true for synthetic auxins i.e. 2,4-D, NAA and IBA (Tables 5 and 8). Higher growth response of diploid callus than haploid, as influenced by IAA, can be explained by any of the following statements. In the haploid tissues where one of the chromosome homologues is absent, the growth rate was less by a factor of almost 2, as compared with the diploid callus; suggesting thereby that each of the homologues contributes equally towards the synthesis of endogenous hormone. When both diploid and haploid callus are supplied exogenously with identical levels of IAA, the better growth

response of diploid tissue may be due to favourable endogenous IAA level, which when supplemented with the exogenous IAA gives better results. Secondly, the destruction of IAA in haploid callus may be much more rapid than in diploid callus, thereby resulting in sub-optimal IAA level in haploid callus. Moreover, Moyed and Tuli (1968) have demonstrated that low levels of 3-methylenoxindole - the degradation product of IAA - stimulated growth, whereas its higher concentrations antagonized it. Low 3-methyleneoxindole level can be achieved by either low IAA Oxidase activity or by high 3-methyleneoxindole reductase activity. Hence, in haploid callus it may be that the rate of IAA destruction was high, resulting in low endogenous IAA and high 3-methyleneoxindole levels, which did not favour growth. On the other hand, the reverse situation should hold good for diploid callus. Further, the IAA Oxidase activity is greatly influenced by the quality of phenolics. Monophenols stimulate IAA Oxidase activity resulting in low growth values; whereas diphenols inhibit IAA Oxidase activity thus stimulating growth (Thimann et al., 1962; Furuya et al., 1962; Lee and Skoog, 1965 a, 1965 b; Van Overbeek, 1966; Tomaszewski and Thimann, 1966).

Poor growth response of diploid and haploid callus in response to 2,4-D can be explained by the following arguements. Firstly, the degradation of 2,4-D in the plant cells is known to be slow, it being shown to metabolize ten times slower than

IAA (Bendana et al., 1965), due to possible lack of 2,4-D oxidizing enzyme system. Secondly, the rate of 2,4-D transport is very slow (McCready, 1963). Thirdly, 2,4-D is known to interfere with glucose metabolism (Bourke et al., 1962) and increases its catabolism via pentose phosphate pathway (Humphreys and Dugger, 1957). Furthermore, 2,4-D at low levels can stimulate growth and metabolic rates, but at higher concentrations inhibits respiration, invertase and phosphate absorption (Wedding et al., 1967; Palmer, 1968).

The maximum growth response attained with the use of NAA in the medium could be explained by either or both of the two points. NAA is reported to stimulate the invertase activity which will result in better utilization of carbohydrate source i.e. sucrose (Glaszion et al., 1966). It is also known to enhance RNA content by as much as 50% (Leaver, 1966), which would result in increased synthesis of structural and functional proteins. NAA is found to accumulate rapidly in plant tissues and is then metabolized slowly to a series of unidentified derivatives (Glaszion et al., 1968). The optimum level of NAA in the system can thus be maintained resulting in rapid growth of callus.

The indispensibility of kinetin for growth of callus tissues has been demonstrated by Murashige and Skoog (1962) and Steward (1969). Kinetin induced growth is acredited to

enhanced RNA and protein synthesis (Skoog et al., 1967; Letham and Williams, 1969; Kende and Gardner, 1976). Kinetin has, further, been reported to have additive effect to that of auxin for growth promotion of Spruce and Scymore callus cultures (Steinhart et al., 1961; Digby and Wareing, 1966). In the present study kinetin stimulated growth of floral bud and haploid callus (Tables 6 and 9). MAP (6-methylaminopurine) at low level (0.1 mg/l) was promotory to the growth of diploid callus but not so to the haploid callus. Growth inhibition due to MAP was also much pronounced in haploid callus than in diploid callus.

Murashige (1964, 1965) working with tobacco callus, and Nickell and Tulecke (1959) working with various monocots and dicots, found GA_3 to inhibit growth. In the present study, diploid as well as haploid callus responded marginally to GA_3 at high concentration (100 mg/l). In the present study GA_3 requirement is obviously quite high to produce growth response.

Of all the auxin and kinetin combinations tested, 2.0 mg/l each of IAA, NAA and KN in combination with 2% sucrose supported maximum growth of both diploid and haploid callus. Though on fresh wt. basis, 2.0 mg/l each of IAA and NAA in combination with 0.4 mg/l KN supported maximum growth, during the subsequent sub-cultures the growth was found to decline gradually due to certain inexplicable reasons.

The accumulation of phenolics in diploid and haploid tobacco callus was influenced by exogenous hormonal supply. Maximum phenolic accumulation in diploid callus was supported by 0.5 mg/l IAA in the medium. Increasing IAA concentrations led to increased growth and decreased phenolic accumulation. With the exception of IBA, increasing auxin concentrations resulted in decrease of phenolic accumulation. In haploid tobacco callus maximum phenolics accumulated with the incorporation of 4.0 mg/l 2,4-D in the medium, which supported minimum growth. Higher levels of IAA and IBA also supported higher phenolic accumulation. On both the auxins, varying levels of auxin in the medium did not bring about substantial changes in growth values. Repression of phenolic synthesis in diploid callus in response to higher auxin level is in agreement with studies conducted with Haplopappus (Constabel et al., 1971), Paul's Scarlet Rose (Davies, 1972), Cassia (Shah et al., 1976) and Crotalaria (Shah, 1976). Increasing concentrations of IAA led to decreasing phenolics and increasing growth of diploid callus; whereas increasing IAA levels led to increased phenolic accumulation without any substantial change in growth values. The biosynthesis and accumulation of phenylpropanoid compounds in plants is controlled by specific genes (Feenstra, 1960; Harborne, 1965). Tobacco callus tissues in the present study responded differentially towards various auxins and their concentrations. The possibility of indirect effects, such as

influence of auxin levels on sucrose metabolism cannot be ignored (Wiggans, 1954). The final concentration of extractable phenolics in a system can also be regulated by the activity of polyphenol oxidase and lignin turnover.

Increasing concentrations of kinetin (KN) resulted in less accumulation of phenolic compounds in diploid and haploid callus at the same time stimulating growth. Similar trends were observed with Crotalaria callus cultures (Shah, 1976). However, quite opposite results were also obtained in our laboratory in case of Cassia (Shah et al., 1976) and Datura (Subbaiah, 1974). MAP favoured synthesis and accumulation of phenolics in diploid callus but not in haploid callus, though growth of both the tissues was inhibited by MAP.

Increasing GA_3 concentrations resulted in decreasing phenolic accumulation and stimulation of growth in both diploid and haploid callus. GA_3 effect on production of phenolic compounds in tobacco callus cultures is similar to that observed for Crotalaria (Shah, 1976). The GA_3 effect on depressed phenolic synthesis may be a consequence of its influence on PAL inactivating protein (Walton and Sondheimer, 1968; Zucker, 1968). Further, GA_3 is known to influence the synthesis of nucleic acids and proteins, which suggest its role at gene level (Cleland, 1969).

Of all the auxin and kinetin combinations tested, maximum

phenolic accumulation in diploid callus was achieved by incorporating 2.0 mg/l IAA + 0.4 mg/l KN and 4% sucrose into the basal medium. At the same hormonal level decrease of sucrose concentration resulted in decline of phenolic accumulation and improvement of growth of both diploid and haploid callus. In haploid callus maximum phenolic accumulation was, however, achieved on the medium containing 2.0 mg/l IAA, NAA and KN. Sucrose stimulated PAL activity and enhanced phenolic synthesis has been demonstrated in strawberry leaf discs (Creasy, 1968), Paul's Scarlet Rose cell suspensions (Davies, 1972), Cassia and Datura (Shah, et al., 1978) callus. It has been suggested to increase the synthesis of aromatic amino acids through shikimic acid pathway.

C. Organogenesis in Diploid and Haploid callus of tobacco.

Experiments conducted with the diploid callus (derived from floral buds) and haploid callus (derived from shoot explants of haploid plantlets) to invoke morphogenetic responses are described in Chapter III, Section C-I, C-II. Various concentrations and combinations of growth hormones and growth regulators were used in the basal medium to study their influence on organogenesis. The growth hormones and growth regulators used were IAA, NAA, 2,4-D, IBA, KN, MAP, GA_3, adenine sulfate, t-cinnamic acid, caffeic acid, ferulic

acid and p-hydroxybenzoic acid. Moreover, the effect of various sucrose levels on differentiation was also examined.

Both diploid and haploid callus responded essentially in an identical fashion to the stimulus of auxins (IAA, IBA, NAA and 2,4-D), cytokinins (KN and MAP) and adenine (Chapter III; Section C-I, C-II; Expts. 12, 18). Except IAA, none of the above tested growth hormones induced organogenesis. In both diploid and haploid callus, low IAA concentrations (0.175 - 0.3 mg/l) favoured shoot differentiation; whereas higher levels (0.5 - 2.0 mg/l) favoured root differentiation. Still higher concentrations of IAA (3.0 - 4.0 mg/l) brought about complete suppression of organogenesis. The concentration of sucrose in the medium had marked influence on organogenesis. However, the sucrose mediated effect on organogenesis was apparently restricted to low IAA concentration (0.3 mg/l). Sucrose at 1% in the medium (IAA - 0.3 mg/l) did not support organogenesis, it, however, supported shoot differentiated at 3% sucrose and roots at 6% sucrose levels. With higher level of IAA (2.0 mg/l) in the medium, the variation in sucrose concentrations (2-6%) did not influence organogenesis, since roots were invariably differentiated. At low (0.3 mg/l) as well as high (2.0 mg/l) IAA levels in the medium, the increase of sucrose concentration affected temporal responses. For instance, IAA (0.3 mg/l) with 2% sucrose induced shoots in four weeks from diploid callus

and in 15 days from haploid callus. However, with the increase of sucrose level to 3% in the same medium shoots were differentiated within 15 and 9 days respectively in diploid and haploid callus. Similarly, IAA (2.0 mg/l) with 2% sucrose induced roots from diploid callus in four weeks. However, with the increase of sucrose level to 3 and 6% in the same medium root differentiation occurred within 12-15 days. Haploid callus also responded in a similar manner.

In lower vascular plants the influence of sucrose levels on the differentiation of gametophyte and sporophyte in vitro has been demonstrated (Mehra, 1972; Kshirsagar, 1974). The influence of sucrose in inducing morphogenetic shift and in reducing the time taken for organogenesis in tobacco seems to be a complex process which might involve many biochemical pathways. As will be pointed out in the forthcoming sections, higher sucrose level leads to higher MDH activity. At the same IAA (0.3 mg/l) level, high sucrose (6%) which favours root differentiation demonstrated higher MDH activity than the shoot forming system on 3% sucrose medium. Thus suggesting higher energy requirements for rhizogenesis than caulogenesis. Secondly, the IAA Oxidase activity was low in root differentiating system than in shoot differentiating system. This seemed to suggest higher endogenous auxin requirement for root differentiation than for shoot differentiation. Thirdly, high sucrose levels have already been shown to support higher

phenolic synthesis. Hence, a phenomena of auxin-phenolic synergism and auxin-phenolic antagonism can be envisaged. Since higher sucrose levels lead to higher synthesis of aromatic amino acids phenylalanine, tyrosine and tryptophan - the last one being the precursor for IAA, it could also affect the endogenous IAA synthesis and its final active level. The carbohydrate besides being the source of energy and an osmotic agent in organ-forming callus (Thorpe, 1978), will provide more reducing power (NADH or NADPH) at higher levels, thus bringing about early organogenetic response. The sucrose effect is not entirely osmotic has been convincingly demonstrated by Thorpe (1978) for tobacco callus tissues.

IAA induced root and shoot differentiation from tobacco callus cultures has been reported earlier. Low IAA favouring shoot differentiation has been reported earlier for tobacco (Skoog and Miller, 1957), Pergularia (Prabhudesai and Narayanaswamy, 1974), Anagallis (Bajaj and Mader, 1974), Cyclamen (Geier, 1977). IAA induced root differentiation has been reported from carrot (Nobecourt, 1939), Rumex (Nickell, 1952), wheat and barley (Gamborg and Eveleigh, 1968), Petunia (Rao et al., 1973), Pergularia (Prabhudesai and Narayanaswamy, 1974), Anagallis (Bajaj and Mader, 1974), Ipomoea batatas (Sehgal, 1975), Antirrhinum (Rao et al., 1976), Ipomoea biloba (Bhatt, 1977), Cyclamen (Geier, 1977), Linum (Mehta, 1975).

Innumerable reports exist, demonstrating organogenesis in tissue cultures as influenced by synthetic auxins like 2,4-D, NAA and IBA. 2,4-D induced embryogenesis and shoot bud formation has been reported in carrot (Halperin and Wetherell, 1964; Nakajima, 1963; Kato and Takeuchi, 1963), Petunia (Rao et al., 1973), Antirrhinum (Sangwan and Harada, 1975; Rao et al., 1976), Tylophora (Rao et al., 1970; Rao and Narayanaswamy, 1972), Pergularia and Asclepias (Prabhudesai and Narayanaswamy, 1974), Cyclamen (Geier, 1977). Likewise 2,4-D can induce rhizogenesis in Solanum xanthocarpum (Rao and Narayanaswamy, 1968), Petunia (Rao et al., 1973), Cyclamen (Geier, 1977). Root formation as a consequence of NAA and IBA has been recorded in various plant species in culture, to name a few, Hyoscymus (Bohnke and Kohlenbach, 1978), Tapioca (Prabhudesai and Narayanaswamy, 1975), Antirrhinum (Rao et al., 1976), Petunia (Rao et al., 1973), Linum (Mehta, 1975), Sweet potato (Yamaguchi and Nakajima, 1974); Sehgal, 1975), Cyclamen (Geier, 1977), Salix alba, Ulmus campestris, Acer pseudoplatanus, Populus nigra, Populus canescens (Chalupa, 1977), Pinus radiata (Smith and Thorpe, 1975). However, in the present study with diploid and haploid callus of tobacco, all the three synthetic hormones - 2,4-D, NAA and IBA - failed to induce organogenesis at all the concentrations tested. The possible explaination for this failure could be due to accumulation of these synthetic auxins in the callus tissues, resulting in

concentrations exceeding the requirements for organogenesis. Such an arguement is substantiated by the fact that synthetic auxins are accumulated and not destroyed by plant species at the same rate as IAA (Gamborg et al., 1968; Thomas et al., 1964; Bendana et al., 1965).

Adenine induced bud formation was reported by Skoog (1954) in tobacco and Ipomoea batatas (Sehgal, 1976). It is also reported to influence organogenesis in nucellar callus cultures of Citrus (Rangaswamy, 1961; Kochba and Spiegel-Roy, 1973; Mitra and Chaturvedi, 1972). With diploid and haploid tobacco callus used in the present study adenine at the concentration tested proved totally ineffective in inducing organogenesis.

GA_3 at 10.0 ppm was effective in inducing root differentiation from diploid callus, though roots were thin, fragile and small. No response was obtained from haploid callus. GA_3 is reported to induce rhizogenesis in Lycopersicon (Coleman and Greyson, 1977 a, 1977 b) and Trianthema (Ravishankar and Mehta, 1978). The limited and poor rooting response in diploid callus and its failure in haploid tobacco callus could be due to GA_3 blocked organized cell divisions leading to either root or shoot formation (Heide, 1969). In Begonia it has been clearly shown to block root and shoot differentiation (Schrandolf and Reinert, 1959; Heide, 1969). In a similar fashion shoot differentiation from tobacco callus is inhibited by 5×10^{-7}M GA_3 (Thorpe and Meier, 1972, 1973).

Usually cytokinins are known to promote bud formation in cultured tissues (Skoog and Miller, 1957; Heide, 1965; Nitsch and Nitsch, 1967; Skoog et al., 1967; Skoog and Leonard, 1968; Kavi Kishore and Mehta, unpublished). Contrary to these observations cytokinins tested (kinetin 0.02 - 2.0 mg/l; MAP 0.4 - 2.0 mg/l) over a wide range of concentrations did not induce bud or even root formation in either diploid or haploid tobacco callus tissues. Experimental evidence is accumulating where cytokinins have no effect on organogenesis (Miller, 1961, 1962; Harris and Hart, 1964; Fox, 1969; Applegreen and Heide, 1972; Rao and Harada, 1974). It has been also shown to antagonize auxin induced embryogenesis in Antirrhinum (Rao et al., 1976), retard bud development in Petunia (Rao et al., 1973) and root differentiation in Ipomoea (Bhatt, 1977).

Shoot bud formation in diploid and haploid callus of N. tabacum appears to be a function of IAA or IAA-KN together, since all other auxins and KN alone, did not induce shoot bud differentiation. IAA tested in a wide range (0.03 - 2.0 mg/l) in combination with KN (0.175 - 2.0 mg/l) induced shoot from both diploid and haploid callus tissues. The significant finding of the present experimentation was that IAA and KN were required in 1 : 1 ratio to induce shoot differentiation. A high IAA to KN ratio of 10 : 1 failed to prove effective in inducing organogenesis. Furthermore, higher levels of IAA and KN (0.75 - 2.0 mg/l) had tendency to initiate shoots which did not

grow well and remained stunted. High concentrations of IAA (4.0 mg/l) in combination with KN (0.4 - 4.0 mg/l) suppressed organogenesis from both the types of callus cultures (Results in Section.C-I and C-II of Chapter III).

Bud formation in cultured tissues as a function of auxin-cytokinin interactions has been reported for an array of plant species. Almost similar results have been reported for Lycopersicon esculantum (Padmanabhan et al.,1974), horse radish (Sastri, 1963), Antirrhinum majus (Sangwan and Harada, 1975), Petunia inflata (Rao et al., 1973), Nicotiana tabacum (Thorpe and Murashige, 1968; Ross and Thorpe, 1973; Thorpe and Laishley, 1973; Gaspar et al., 1977; Wardel and Skoog, 1969), Arabidopsis thaliana (Negrutin et al., 1978), Cyclamen persicum (Geier, 1977), Ipomoea quamoclit (Bhatt, 1977). Stimulation of shoot and root formation by auxin is well known in many plants, provided cytokinin is present at a high level in comparison with auxin level (Bornman, 1974; Negrutin et al., 1978). Auxin to cytokinin dependent root and shoot regeneration has been vindicated in many a species (Tran Thanh Van et al., 1974; Sheridan, 1974; Bui Dang Ha et al., 1975). Most of the workers generalize that high auxin to kinetin ratio leads to root differentiation, while high kinetin to auxin ratio leads to shoot differentiation. Such a generalization, however, cannot be made in the present study. The definite levels of auxin and cytokinin rather than their ratio was the determining factor

for organogenesis in the present study. Furthermore, the effect of varying levels of sucrose on organogenesis can not be ignored. Paulet (1965) summarizing his findings stated that low sucrose in combination with high auxin favoured root differentiation; whereas high sucrose, low auxin and high cytokinin, in combination, favours shoot differentiation. Identical situation was demonstrated for tobacco tissue cultures (Tran Thanh Van, 1977; Gaspar et al., 1977). Maroti and Levi (1977) working with Dianthus caryophyllus, however, demonstrated improvement in organized shoot differentiation with increasing sucrose concentrations. Our results with both diploid and haploid tobacco callus project an entirely different picture. Sucrose at low level (1%) when used in combination with IAA or with IAA and KN, failed to invoke any morphogenetic response. Moreover, it was effective in bringing about shifts of organogenetic response from shoot to root differentiation and vice versa only when low IAA (0.3 mg/l) concentration was used. In IAA-KN combinations which differentiated shoots from callus cultures, the reduction of sucrose level instead of favouring the shift of organogenetic response, completely inhibited morphogenesis.

Studies with substituted phenolic acids on shoot and root differentiation in diploid and haploid tobacco callus, showed that they too influenced growth and organogenesis markedly. All

the phenolic acids tested - t-cinnamic acid, caffeic acid, ferulic acid and p-hydroxybenzoic acid - blocked IAA induced root or shoot differentiation in diploid callus. t-Cinnamic acid (500 μM) and caffeic acid (100 μM) when incorporated into IAA (0.3 mg/l) containing medium blocked shoot differentiation from haploid callus and favoured instead, the differentiation of roots. All phenolic acids with the exception of t-cinnamic acid (100 μM) and caffeic acid (500 μM) inhibited root differentiation from haploid callus in response to IAA (2.0 mg/l). Like-wise incorporation of caffeic acid (100 μM) into IAA (0.5 mg/l) and KN (0.5 mg/l) containing medium shifted organogenetic response from caulogenesis to rhizogenesis. Working with tobacco callus cultures LaMotte (1960) reported the influence of quite a few phenolic substances on organized development of roots and buds. Lee and Skoog (1965 a, 1965 b) observed promotion of root and/or bud formation with tyrosine, ferulic acid, caffeic acid and p-hydroxybenzoic acid.

Gautheret (1959, 1965) had observed that the hormonal requirements for shoot and/or root differentiation varied with the plant species and even with different tissues of the same species. Consequently in the present study root, stem and leaf explants of diploid and haploid tobacco plants were cultured on IAA (2.0 mg/l) and KN (0.4 mg/l) containing medium.

With the exception of haploid stem explants all other organ explants gave rise to shoot buds either directly or preceded by callus formation. The results obtained thus do not support the views that different hormonal requirements exist for different tissues. Shoot buds have likewise been obtained from : root explants of tomato (Norton and Boll, 1954), Brassica (Bajaj and Nietsch, 1975), Convolvulus (Bounett and Torrey, 1965), Ipomoea batatas (Gunckel et al., 1972); shoot explants of Linum (Murray et al., 1977; Gamborg et al., 1974), Antirrhinum (Sangwan and Harada, 1975), Tamarix (Kavi Kishore and Mehta, unpublished); leaf explants of Begonia (Shigematsu and Matsubara, 1972), Saintpaulia (Rao and Morel, 1974), Echeveria (Raju and Mann, 1971), tobacco (Prabhudesai and Narayanswamy, 1973) Gupta et al., 1966), Macleaya (Kohlenbach, 1965), Torenia (Bajaj, 1972), Anagallis (Bajaj and Mader, 1974) and Crassula (Murashige, 1977).

D. Physiological studies during callus induction from floral buds, and growth of diploid and haploid callus of tobacco.

Metabolic changes associated with wounding or aging of plant organs have been investigated by many authors. However, few details are available which picturize changes in metabolism, the extent and timing of enzyme synthesis and their activities during callus initiation. Striking changes in metabolism are known to occur during callús formation from plant tissues in

culture. Hardly any study has been carried out to investigate spectral changes of isoenzyme patterns during callusing *in vitro*. On the other hand, substantial data is available regarding metabolism of cultured cells and tissues during their growth cycle.

In the present investigation with callusing from floral buds of tobacco, two distinct growth phases were apparent. Lag phases appeared between days 0 to 3 and days 15 to 18, followed in both cases by exponential growth period. Callus induction was observed on day 18 i.e. at the end of second lag phase in growth.

The specific enzyme activities of peroxidase and IAA Oxidase, remained low during the pre-callusing phase of growth. Peroxidase activity increased with the advent of callusing and so did IAA Oxidase. However, the activity of both the enzymes declined immediately after callus formation. MDH and PAL activities reached peak values many days before actual callusing was observed. MDH declined with callus initiation and PAL remained more or less stable (Chapter III, Section D, Table 17, Fig. 79).

Increase in peroxidase activity during callusing from floral buds, is in agreement with the results of Kanazawa *et al*. (1965) working with sweet potato tuber slices, Baslin and Unluer (1972) working with Jerusalem artichoke tuber slices

and the results of Lavee and Galston (1968) and Gaspar et al. (1977) working with tobacco tissue cultures. Failure to detect IAA Oxidase activity in cell free extracts till day 18 of culture i.e. the day of callus formation, implies the presence of IAA Oxidase inhibitors and the high requirement of endogenous auxin for callus initiation. IAA Oxidase activity was in high amounts during the second lag period of growth i.e. days 18 and 21, and decayed immediately after callus initiation and its growth. Wright (1961), Van Overbeek (1962) and Nitsch (1968) have demonstrated sequential requirement and utilization for kinetin and auxin, in that order. Kinetin being required during the phases of early nuclear and cell division and auxin required during the later phases of cell division and cell enlargement. Similar correlation is evident in the present study during callus initiation where IAA Oxidase activity remained low during active period of growth and was high during the second lag period when mitotic activity would be high leading to callus initiation. Reports by Yeoman et al. (1965), Komamine et al. (1969), Akemine et al. (1970), Komamine ahd Shimizu (1975) that the respiratory rate and the activity of related enzymes increases during early stages of callus induction agree with the results obtained in the present study. MDH activity increased tremendously before the callus was induced and declined after callus formation. The pattern of PAL activity was supported by its synthesis of phenolic acids,

both having inverse correlation with IAA Oxidase.

No anodic isoperoxidases were detectable on day 0 of culture. However, during the culture period of 30 days, a total of 23 slow and fast migrating anodic isoperoxidases were synthesized. Only three of them A_{17}, A_{21} and A_{15} - in order of their mobility - were detected in the callus. A_{17} and A_{21} appeared on the day of callusing i.e. day 18, and A_{15} appeared 3 days earlier i.e. on day 15. Rest of the isoperoxidases appear to be associated with growth and development of the floral bud in culture and also may have role to play in callus initiation. On the cathodic scale three isoperoxidases - C_1, C_2 and C_3 - were initially present. The callus also had three cathodic isoperoxidases - C_4, C_5 and C_9 - which were slow migrating. All the three cathodic isoperoxidases found in the callus, were synthesized many days before the appearance of callus.

The diploid tobacco callus exhibited only lag and exponential phases of growth, the stationary phase was absent. The activity of enzymes peroxidase, IAA Oxidase, MDH and PAL was high during the lag period. The activities declined during the exponential period of growth. In diploid callus the activity of all enzymes increased slightly at the onset of postexponential period but declined thereof. The phenolic content in diploid callus increased during the lag period and

on entering the exponential period it declined.

Unlike the growth of diploid callus, no lag phase was noticed in the growth cycle of haploid callus. The exponential phase lasted from day 0 to day 9 and was followed by post-exponential phase upto day 30. The peroxidase activity/unit protein increased during the exponential phase and then was on decline for the entire duration of the culture period. IAA Oxidase activity increased sharply during the early exponential phase and then declined during the period of rapid growth. The MDH activity on the other hand, was on increase during the exponential period and recorded peak activity during the post-exponential phase which was characterized by rapid growth. As in the case of peroxidase the results with MDH in haploid callus were also in contrast with diploid callus. The PAL activity registered highest activity during the early exponential phase and then declined.

Few investigations have reported activity levels of enzymes during the culture cycle of cell and tissue cultures from higher plants. Such studies showed that the activities of enzymes during the lag, exponential and stationary phases varied. Simola and Sopanen (1970), reported peak peroxidase activity during the stationary phase of the growth cycle of Acer cell suspensions. Nash and Davies (1975) recorded peak peroxidase activity during the mid-exponential phase of Paul's

Scarlet Rose cell suspension cultures. Likewise high peroxidase activity was recorded during the exponential growth phase of Arachis (Arnison and Boll, 1976a, 1976b) as also in the present study with haploid callus. All these findings are in agreement with earlier studies (De Jong, 1967; De Jong et al., 1977; Makinen, 1968; Galston et al., 1968). However, the results obtained with diploid tobacco callus are not in agreement with the earlier findings, since high activity was recorded during the lag period of growth.

Peroxidase activity is closely associated with IAA Oxidation and IAA Oxidase (Ockerse et al., 1966; Pilet et al., 1970; Lee, 1971b; Legham and Galston, 1971; Fowler and Morgan, 1972; Srivastava and Van Huystee, 1977a). Hence an increase in peroxidase activity would mean concomitant increase of IAA Oxidase activity, unless such specific inhibitors as diphenols come into play. Lee (1971a, 1971c) demonstrated peak IAA Oxidase activity during the lag period of growth of tobacco callus and its subsequent decay with the progress of growth. The present results obtained with diploid and haploid tobacco demonstrate high IAA Oxidase activity during the early periods of growth and its decline during the expenential and/or post-exponential phases of growth. It clearly reflects the requirement of IAA during cell growth and its apparent non-requirement during the period of mitotic activity.

Studies with Nicotiana (De jong, 1967) and Paul's Scarlet Rose (Nash and Davies, 1975) cell suspension cultures have shown a rapid rise in total MDH activity till the mid-exponential phase of growth followed by a very rapid decline during the remainder of the exponential period. Similarly in the present study with haploid callus MDH activity was high during the exponential period and then declined during the postexponential phase. Givan and Collins (1967) have demonstrated high respiratory rate during the lag phase of growth of Acer pseudoplatanus. The respiratory rate declined with the progress of growth as also did cellular nitrogen content. The authors suggested close association between respiration and protein content. In the present study with diploid tobacco callus the enzyme activity increased during the early growth phase and post-exponential period. However, the enzyme activity at the termination of growth period of 30 days had declined again. It can be concluded that the energy required for exponential growth is produced earlier than its onset.

Shah et al. (1978) demonstrated peak PAL activity during the post-exponential growth phase in Cassia tissue cultures, whereas maximum phenolic accumulation was attained during the early exponential period of growth. On the contrary, diploid and haploid tobacco callus tissues showed maximum PAL activity

during the early periods of growth and was on decline during the exponential and/or post-exponential periods of growth. Peak phenolic accumulation in both callus cultures was also attained during the early period of growth cycle. The present results with diploid and haploid callus are in accord with those obtained with parsley callus by Hahlbrock et al. (1971) and Hahlbrock and Wellmann (1973). The amount of phenolics seemed to have direct relation with growth as low levels of phenolics were detected during the exponential period.

The present study with diploid and haploid callus of tobacco showed differences in number and development, during the standard culture period, for anodic and cathodic isoenzymes of peroxidase. Isoenzymes could be categorized into those which appeared during the lag, exponential and post-exponential phases of growth. The anodic isoperoxidases A_2-A_5 were synthesized during the lag phase. The original ones A_1, A_2 and A_3 were also synthesized during the post-exponential period of growth. A_4, however, also appeared during the early period of exponential phase. A_6-A_9 were strictly restricted to the exponential period, either early or late. On the cathodic side the isoperoxidase C_1 was synthesized throughout the culture period. C_2 appeared during the early - and post- exponential phases. C_3 was limited to the post exponential growth phase. C_6 - C_8 were restricted to the exponential and post-exponential growth periods. C_4 was synthesized during the lag and early-

exponential periods. C_5, was restricted to the lag phase. In haploid callus the anodic isoperoxidases A_1 and A_7 were limited to the post-exponential growth phase. However, A_7 made a brief appearance during the early-exponential phase. A_2 and A_3 were synthesized during the lag and post-exponential growth phases only. A_6, A_8 and A_9 were isoenzymes of the exponential period. A_4 was synthesized during the lag phase only. A_5 was restricted to the lag and early-exponential period of growth. On the cathodic scale C_1 appeared right through the culture period. C_2 and C_3 were essentially limited to the post-exponential and lag phases, except for a brief appearance by C_2 during the exponential growth phase. C_6 - C_9 were present during the exponential and C_{10} - C_{12} during the post-exponential periods of growth.

Though Rucker and Radola (1970) challanged qualitative and quantitative changes of isoperoxidases in Nicotiana callus cultures, suggesting that the non-occurrence of some bands of activity is a problem of resolution rather than a case of absence. However, the results obtained in the present study are in agreement with the results of Nash and Davies (1975), Arnison and Boll (1974, 1976b, 1978), Lee (1972) and Wolter and Gordon (1975) who have demonstrated spectral changes in the isoperoxidase patterns during the growth of tissues in culture.

E. Physiology of root differentiation in diploid and haploid tobacco callus.

The medium used for root differentiation from diploid and haploid tobacco callus was MS basal supplemented with 2.0 mg/l IAA and 3% sucrose. Roots from diploid callus were differentiated between days 13 and 15, and on day 6 from haploid callus. Further studies were carried out to examine the influence of different sucrose levels, added phenolic acids and Mn^{++} levels in the above mentioned root inducing medium. Use of low (1%) sucrose level in the medium inhibited root differentiation from diploid callus, whereas high (6%) sucrose level only reduced the frequency of its response. Manipulation of sucrose level in the root inducing medium did not effect rhizogenic expression from haploid callus. However, the rhizogenetic expression was delayed by 3 to 6 days and also the frequency of response was comparatively low. Addition of all the phenolic acids tested (t-cinnamic acid, 100, 500 µM; caffeic acid, 100, 500 µM; ferulic acid, 100, 500 µM; p-hydroxybenzoic acid, 1, 10 µM), into the rhizogenic medium inhibited root differentiation from diploid callus. The above mentioned phenolic acid with the exception of 100 µM t-cinnamic acid and 500 µM caffeic acid, suppressed root differentiation from haploid callus also. With the lowering of Mn^{++} level to $\frac{1}{2}$ X or increasing it to 2 X level in the standard root inducing

medium resulted in total repression of rhizogenesis in haploid as well as diploid callus tissues (Chapter III, Results; Section C-I, C-II).

The reduction of sucrose level to 1% in the medium resulted in enhanced growth of diploid callus, whereas its increase to 6% resulted in marginal inhibition. The growth of diploid callus was stimulated with the addition of phenolic acids into the medium. The growth was also inhibited by altering the Mn^{++} level in the medium. The growtho of haploid callus on 3% and 6% sucrose containing media on day 9 was almost identical, but on 1% sucrose medium it was less. With the exception of caffeic acid (500 μM) and p-hydroxybenzoic acid (1.0 μM), the addition of all other phenolic acids inhibited growth. The use of $\frac{1}{2}$ X and 2 X Mn^{++} ion levels in the medium also inhibited growth.

The peak enzyme activity per mg protein of all the enzymes studied - Peroxidase, IAA Oxidase, MDH and PAL - in diploid callus was higher on the non-root differentiating media than on the root differentiating media. The exception to this generalization being the root differentiating medium containing 6% sucrose, where upon high enzyme activities were accompanied by root differentiation. An examination of the enzyme activities on the day immediately preceding root differentiation and the day of root differentiation reveals interesting information. Irrespective of the fact whether roots

are differentiated on 3% or 6% sucrose containing medium (MS + 2.0 mg/l IAA), the peroxidase activity on the day immediately preceding root differentiation was in the range of 0.71 - 0.79 units; whereas on the day of rhizogenesis the enzyme activity on both the media was 0.83 units/mg protein. The IAA Oxidase activity/mg protein likewise remained more or less stable during this period, though the activity was nearly four times higher on the 6% sucrose medium than on 3% sucrose medium. The MDH activity/mg protein increased from 0.26 units on the day preceding root initiation to 2.66 units on the day of root initiation on 3% sucrose medium. On the contrary, on 6% sucrose medium during the corresponding period the activity declined from 3.0 to 1.93 units. It thus becomes obvious that the MDH activity during root differentiation remains in the vicinity of 2.0 units. PAL activity could not, however, act as a pointer towards rhizogenesis. It seemed to be more dependent on the exogenous sucrose supply. The activity on 6% sucrose medium during rhizogenetic period was 5 to 25 more than on 3% sucrose medium during the corresponding period. The content of total polyphenols was, however, a good marker of rhizogenesis. On both the rhizogenic media the phenolic content on the day preceding root differentiation was in the range of 3.36 - 3.8 mg% and in the range of 2.64 - 2.8 mg% on the day of rhizogenesis (Chapter III, Results; Section E; Tables 20 - 32; Figs. 82 - 94).

In the haploid tobacco callus the peroxidase activity per mg protein increased with the increase of sucrose level to 6% in the medium. Likewise, increased activity appeared with the addition of all ; phenolic acids, except t-cinnamic acid, and with the use of ½ X and 2X Mn^{++} levels in the medium. Use of 1% sucrose or incorporation of t-cinnamic acid (100, 500 μM) in the medium resulted in decreased peroxidase activity. The IAA Oxidase activity increased substantially over the control with the incorporation of 6% sucrose, caffeic (100 μm) and ferulic acid (100 μM) into the standard root inducing medium. Alteration of Mn^{++} ion level in the medium to ½ X or 2 X resulted in tremendous increase of enzyme activity. High IAA Oxidase activity was also recorded on 6% sucrose containing medium. By and large, with the exception of the standard root inducing medium containing 100 μM ferulic acid and altered Mn^{++} ion levels, the MDH activity per unit protein remained higher in haploid callus tissues on root differentiating media than on non-root differentiating media. PAL activity per mg protein varied with the treatment. On the standard root inducing medium the peak activity of 0.99 units was recorded. On other root inducing media like the ones with 1 and 6% sucrose, with 100 μM t-cinnamic and with 500 uM caffeic acid, the peak PAL activity recorded was 6 - 104 times more than the standard. In general, high sucrose (6%), addition of phenolic acid in the medium and alteration of Mn^{++} level to ½ X or 2 X resulted in high PAL

activity. The phenolic content of haploid callus also changed with the treatment. Maximum value was reached with incorporation of 6% sucrose in the medium. Incorporation of phenolic acid or the use of $\frac{1}{2}$ X or 2 X Mn^{++} level in the medium resulted in higher polyphenol levels than the control.

The peroxidase activity during the period of root differentiation in haploid callus on control medium supplemented with either 1 or 6% sucrose or 100 μM t-cinnamic acid either increased or decreased but the value attained on the day of root differentiation was in the range of 0.35 - 0.38 units/mg protein. On the other two root inducing media, i.e. the standard root inducing medium and the standard medium with 500 μM caffeic acid, the enzyme activity during the root inducing phase came within the range of 0.84 - 1.26 units/mg protein. Likewise, IAA Oxidase activity on the root inducing media, with the exception of standard root inducing medium and 6% sucrose medium, either increased or decreased during the root differentiating period to remain within the range of 0.93 - 1.28 units/mg protein. On both standard root inducing medium and the root inducing medium with 6% sucrose, the IAA Oxidase activity increased just before root initiation and was substantially higher than rest of the rhizogenic systems. MDH activity increased during the root differentiation phase on 1% sucrose medium and the activity was 4 - 36 times more than on other root inducing media. On standard root inducing medium also the

MDH activity was high and increasing during the pre- root differentiation period. Irrespective of the fact, whether the enzyme activity increased or decreased on other media the value reached on the day of root differentiation was in the range of 3.06 - 5.0 units/mg protein. The changes in the PAL activity on all the root inducing medium during the period of root differentiation were too varied to arrive at any generalization. Same was the case with spectral changes of polyphenol content during the root inducing phase (Chapter III, Results; Section E; Tables 33 - 45; Figs. 95 - 107).

From the above discussion the facts which assume prominence are that though differences occur in enzyme activities between diploid and haploid callus tissue, the actual values of enzyme activity (in most of the rhizogenic systems)determines root differentiation in callus tissues. Quoirin et al. (1974) working with Prunus, Chandra et al. (1971) with Phaseolus, Van Hoof and Gaspar (1973, 1976) with Zea and Asparagus, Simola (1973) with Atropa and Gaspar et al. (1977) with tobacco, reported increased peroxidase activity concomitant with rhizogenesis. The anther in the present investigation using various root inducing media, for both diploid and haploid callus, recorded variations in the developmental patterns of peroxidase activity. However, the enzyme activity, within reasonable limits, attains a certain value with the root differentiation. To attain that particular

value the enzyme activity either decreases or increases. Van Raalte (1954) and Gorter (1962) suggested that low IAA Oxidase activity, either by itself or regulated by exogenous supply of phenolic compounds promotes root differentiation. However, in the present study root differentiation occurred at low as well as high IAA Oxidase activity. For instance, in diploid callus the enzyme activity during root differentiation on 3% sucrose medium was at least 4 times less than on 6% sucrose medium. Nevertheless, both media induced rhizogenesis. Likewise, though low activity of IAA Oxidase was encountered during root differentiation from haploid callus on most of the root inducing media, the same did not hold true for the enzyme activity during root differentiation on standard root inducing medium and the medium containing 6% sucrose. Except for these abarant results obtained during rhizogenesis from both diploid and haploid callus our results are in agreement with those of the above named authors. Basu (1970) reported lack of correlation between rhizogenesis and IAA Oxidase activity in Phaseolus. The inhibition of root differentiation due to altered levels of Mn^{++} in the medium could be due to high rate of IAA destruction. Mn^{++} influenced IAA Oxidation has been extensively studied by Morgan et al. (1966, 1976) and Taylor et al. (1968). Moreover, Abbott (1963) has demonstrated disruption of vascular differentiation due to Mn^{++} deficiency. It is also known to influence most of the dehydrogenases, kinases, oxidases and

decarboxylases (Nason and McElory, 1963). The MDH and PAL activity can not be used with any confidence as indicators of rhizogenesis. The PAL activity is highly mosaic when the activity of all root differentiating systems is compared. The MDH activity in the diploid callus has entirely different values during root differentiation than in haploid callus. Though phenolics seem to be good indicators of rhizogenesis in diploid callus, the same is not true for haploid tissue.

Multiplicity of peroxidase isoenzymes was observed during the unorganized as well as organized growth of tobacco tissue cultures and the components showed varying degree of intensity. Under each of the different cultural conditions, some new isoperoxidase appeared while the original ones disappeared. In maize (Scandalios, 1964) and pea (Siegel and Galston, 1967) similar patterns are reported. These suggest that the synthesis of enzymes is directly controlled by genes which operate themselves right from the early stages of development and the others are being switched on at appropriate stages during development to produce new isoperoxidases.

Studies with diploid callus during root differentiation on the control root inducing medium (MS + 2.0 mg/l IAA + 3% sucrose) and the same medium with 6% sucrose revealed that the differentiation of roots was preceded by the synthesis of a

slow migrating anodic isoperoxidase PR (R_m = 0.3). The differentiation of roots on both the media was also preceded by the total suppression of the anodic isoperoxidase A_3. No such correlation was observed with cathodic isoperoxidases which changed in intensity during the culture period. Use of high (6%) or low (1%) sucrose in the medium resulted in the repression of slow migrating anodic as well as cathodic isoperoxidase (Chapter III, Figs. 82a - 84a).

The addition of the phenolic acids t-cinnamic acid, caffeic acid and ferulic acid, into the control root inducing medium suppressed the synthesis of isoperoxidase PR as well as rhizogenesis in diploid callus. Moreover, the addition of the above named phenolic acids inhibited the synthesis of the original anodic isoperoxidases A_2 and A_3 for the entire culture period. Ferulic acid (100, 500 μM) and p-hydroxybenzoic acid (10.0 μM) banished the synthesis of initial anodic isoperoxidase A_1, while other phenolic acids blocked its synthesis only during the early phase of culture period. p-Hydroxybenzoic acid at 10 μM blocked the synthesis of isoperoxidase A_3 for the entire culture period and of A_2 during the early and late phases of culture. All the phenolic acids besides bringing about the synthesis of some slow migrating anodic peroxidase, were instrumental in promoting the synthesis of a number of fast migrating ones. On the cathodic scale the synthesis of original

isoperoxidases C_1, C_2 and C_3 was by and large inhibited with the addition of phenolic acids into the medium. All phenolic acids brought about the synthesis of certain slow migrating cathodic isoperoxidase, however, the synthesis of fast migrating one was stimulated by only ferulic acid (100, 500 μM) and p-hydroxybenzoic acid (1, 10 μM) (Chapter III, Figs. 85a - 92a).

Both low (½ X) and high(2 X) Mn^{++} ion levels in the root inducing medium resulted in total suppression of the synthesis of anodic isoperoxidase A_1. The synthesis of A_3 was completely blocked at high (2 X) Mn^{++} ion level whereas A_2 was affected by low (½ X) Mn^{++} level. The synthesis of Isoperoxidase PR and root differentiation was blocked at both the levels of Mn^{++}. On the cathodic scale the original isoperoxidases C_1 and C_2 were not affected whereas, C_3 was completely blocked (Chapter III, Figs. 93a, 94a).

As was the case with diploid callus, haploid callus tissue also exhibited the synthesis of anodic isoperoxidase PR (R_m = 0.3) prior to rhizogenesis on all the root inducing media i.e. the control root inducing medium, the root inducing media with 1% and 6% sucrose, the medium with 100 μM t-cinnamic acid and the medium with 500 μM caffeic acid. On all the above mentioned rhizogenic media, the original anodic isoperoxidases A_2 and A_3 were suppressed. The cathodic isoperoxidase did not give any clue to rhizogenesis (Chapter III, Figs. 95a - 97a).

All phenolic acids, with the exception of ferulic acid (100, 500 μM) and p-hydroxybenzoic acid (10 μM) inhibited the synthesis of original anodic isoperoxidase A_1. Likewise, the synthesis of original isoperoxidase A_2 was blocked by all phenolic acids with the exception of 1.0 μM p-hydroxybenzoic acid. The isoperoxidase A_3 was suppressed only by t-cinnamic acid and caffeic acid, while ferulic acid and p-hydroxybenzoic acid inhibited its synthesis during the early culture period. Ferulic acid (100, 500 μM) and p-hydroxybenzoic acid (1, 10 μM) in the medium brought about the synthesis of fast migrating anodic isoperoxidases. On the cathodic scale, the original isoperoxidase C_2 and C_3 were repressed by t-cinnamic acid and caffeic acid. Furthermore, the cathodic isoperoxidase patterns on these two media were essentially similar. The synthesis of the original cathodic isoperoxidase C_1 was not blocked by any of the phenolic acids tested. The isoperoxidase C_2 was repressed by ferulic acid and p-hydroxybenzoic acid also. C_3 was suppressed by p-hydroxybenzoic acid for the entire duration of the culture period. Ferulic acid at 100 μM suppressed it at the early stages and 500 μM suppressed it during the later stages of culture period. Ferulic acid and p-hydroxybenzoic acid were instrumental in the synthesis of fast migrating cathodic isoperoxidases (Chapter III, Figs. 98a - 105a).

As in diploid callus, in haploid callus too the synthesis

of original anodic isoperoxidase A_1 was blocked with the alteration of Mn^{++} levels ($\frac{1}{2}$ X and 2 X) in the rhizogenic medium. Likewise the synthesis of isoperoxidase A_3 was inhibited. A_2 was, however, inhibited only by high (2 X) Mn^{++} ion level. Five new anodic isoperoxidases were synthesized on $\frac{1}{2}$ X Mn^{++} medium. On 2 X Mn^{++} medium six new isoperoxidases appeared. Both levels of Mn^{++} ($\frac{1}{2}$ X and 2 X) in the medium blocked the synthesis of isoperoxidase PR and root differentiation. On cathodic scale the synthesis of original isoperoxidase C_1 was not affected by either of the two Mn^{++} ion levels. C_3 was blocked totally at $\frac{1}{2}$ X Mn^{++} level and C_2 appeared only at the end of culture period. At 2 X Mn^{++} level C_3 was synthesized on day 15 of culture, whereas C_2 was not inhibited except on day 3 (Chapter III, Figs. 106a, 107a).

A correlation between peroxidase activity and morphogenesis was reported in pea nut cell suspension cultures (Verma and Van Huystee, 1970a, 1970b). The peroxidase activity was shown to increase with the degree of differentiation. Similarly, studies with whole plants or plant organs have shown both quantitative and qualitative increases in peroxidase with increasing morphological development (Conklin and Smith, 1971; Parish, 1969 and Gordon, 1971). Rucker and Radola (1971), however, have challanged such observation, suggesting that the appearance or non-appearance of some bands is a problem of resolution rather than gene control. Chandra et al. (1971),

Van Hoof and Gaspar (1973), Quoirin et al. (1974) demonstrated that rhizogenesis was accompanied by accentuation of the fast migrating anodic peroxidases. Quoirin et al. (1974) and Van Hoof and Gaspar (1976) suggested the decline of the activity of cathodic peroxidase concomitant with root differentiation. Gaspar et al. (1977) have demonstrated continuous increase in the number of anodic isoperoxidase with rhizogenesis and the intensification of the cathodic ones. In the present study such a generalization can not be made, because more than one medium was used to differentiate roots from haploid and diploid callus. However, the appearance of the isoperoxidase PR prior to rhizogenesis in diploid and haploid callus on all the root inducing media is a subtle indicator of root differentiation. Similar results were obtained during rhizogenesis froml leaf discs of Ipomoea by Bhatt (1977). The synthesis of isoperoxidase PR in haploid callus in response to ferulic acid (100 uM) and the non-appearance of roots on the same medium, may be due to the formation of incipient root primordia, which did not develop further due to one or more metabolic factors becoming limiting. Srivastava and Van Huystee (1977) have demonstrated that phenolics bind through both hydrogen and covalent bonds to peroxidase proteins. Similar results were obtained by Fieldes and Tyson (1973). The phenolics- protein bonds have also been demonstrated by (Thomson, 1964; Evans, 1969; Moore, 1973; Van Huystee, 1976).

The phenol peroxidase complex was shown to be electrophoretically fast migrating in pea nut cells and the uncoupling of phenolic from peroxidase resulted in the conversion of that peroxidase to a slow migrating one (Srivastava and Van Huystee, 1977). Such a scheme might hold good for tobacco tissue cultures also.

F. Physiology of shoot differentiation in diploid and haploid tobacco callus.

The standard medium for shoot differentiation from diploid and haploid tobacco callus was MS basal supplemented with 0.3 mg/l IAA and 3% sucrose. Shoot differentiation from diploid callus occurred between days 13 and 15, whereas from the haploid callus shoot differentiation occurred on day 9 of culture. During the process of shoot differentiation the parameters examined were growth, peroxidase, IAA Oxidase, MDH, PAL and total phenolic content. Further to understand the physiology of shoot initiation, the hormonal level was kept fixed (0.3 mg/l IAA) whereas the level of sucrose and Mn^{++} ions in the medium were changed. The influence of phenolic acids on caulogenesis was also examined by incorporation of various phenolics into the above mentioned shoot inducing medium.

The growth of both diploid and haploid tobacco callus was enhanced with either reducing the level of sucrose to 1% or

its increase to 6% in the medium (Tables 46 - 48; Figs. 108 - 110; Tables 59 - 61; Figs. 121 - 123).

The addition of phenolic acids - t-cinnamic acid (100, 500 μM), caffeic acid (100, 500 μM), ferulic acid (100, 500 μM) and p-hydroxybenzoic acid (1, 10 μM) - into the standard shoot inducing medium brought about enhancement of growth (Tables 49 - 56; Figs. 111 - 118). The growth of haploid tobacco callus was inhibited by 100 and 500 μM t-cinnamic acid and 500 μM caffeic acid. p-Hydroxybenzoic acid at 10 μM did not interfere with the growth of haploid tobacco callus. All other phenolics stimulated growth over the control (Tables 62 - 69; Figs. 124 - 131).

Lowering of Mn^{++} ion level to $\frac{1}{2}$ X or its increase to 2 X in the control shoot inducing medium brought about growth inhibition of diploid callus (Tables 70, 71; Figs. 132, 133). The growth of haploid callus was also inhibited with alteration of standard Mn^{++} level in the medium (Tables 70, 71; Figs. 132, 133).

Shoot differentiation occurred in diploid callus between days 13 - 15 on standard shoot inducing medium. Decrease of sucrose level to 1% in the medium inhibited organogenesis. With the increase of sucrose level to 6% in the same medium a shift occurred favouring rhizogenesis on day 12 of culture. No

shoot differentiation occurred. All phenolic acids blocked the differentiation of shoots from diploid callus. Same was the fate with alteration of standard Mn^{++} ion level to either $\frac{1}{2}$ X or 2 X in the control medium.

From haploid callus shoot differentiation occurred on day 9 of culture on control shoot inducing medium. Decrease of sucrose level to 1% suppressed caulogenesis. On the contrary, the increase of sucrose level to 6% resulted in root differentiation on day 12 of culture. The addition of t-cinnamic acid (500 μM) and caffeic acid (100 μM) into the control shoot inducing medium also resulted in a shift favouring rhizogenesis. Root differentiation on t-cinnamic acid (500 μM) containing medium occurred on day 6 of culture and on caffeic acid (100 μM) containing medium on day 9 of culture. All other phenolics inhibited organogenesis. Alteration of standard Mn^{++} level also suppressed shoot differentiation.

The peroxidase activity in diploid callus increased from 0.66 units to 1.01 units between days 12 and 15 - the days of shoot induction. These values of activity were not matched for the corresponding period by any other treatment. In haploid tobacco callus also the enzyme activity increased from 1.08 to 1.11 units/mg protein between days 6 and 9 and shoot differentiation occurred on day 9. Increase in peroxidase

activity during the process of shoot differentiation was observed by other workers using tobacco (Rucker and Radola, 1971; Mader, 1975; Thorpe and Gaspar, 1978; Gaspar et al., 1977) and Cichorium intybus (Legrand and Vasseur, 1972; Legrand, 1974). Bajaj et al. (1973), Thorpe and Gaspar (1978) and Gaspar et al. (1977) have further demonstrated that specific peroxidase activity is higher in non-differentiating systems than in the differentiating systems. Gaspar et al. (1977) and Thorpe and Gaspar (1978) working with tobacco have demonstrated that the specific peroxidase activity strikes a plateau at the time of the initiation of organized development upto the time of primordia emergence. The results obtained in the present study are, however, quite different. The peroxidase activity per mg protein increased during the inductive phase of shoot differentiation in both the diploid and haploid calluses, instead of either declining or remaining stable. Corresponding with these observations was the fact that during the same period of shoot induction the IAA Oxidase activity was on decline. The inductive phase was preceded by increase in IAA Oxidase activity in both diploid and haploid calluses. In both the systems the IAA Oxidase activity per mg protein attained was 2.48 units for diploid callus and 2.72 units for haploid callus. Lee and Skoog (1965a, 1965b) reported bud induction in tobacco in response to monophenols. They argued that since monophenols enhanced the IAA Oxidase activity

which resulted in low levels of endogenous IAA; thus creating an atmosphere conducive for shoot differentiation. In the present study also high IAA Oxidase activity was encountered during the pre-inductive phase of shoot differentiation in both diploid and haploid callus tissues. However, since the IAA Oxidase activity declined during the inductive phase - days 12 to 15, for diploid callus and days 6 to 9 for haploid callus - it became obvious that native auxine must attain a certain level before shoot primordia can develop and emerge as shoots. Since at the same IAA level (0.3 mg/l) in the medium, IAA Oxidase activity in both diploid and haploid callus attained almost identical values at the time of shoot differentiation, it was evident that a subtle endogenous IAA level was required for the final phase of shoot development.

Thorpe and Murashige (1968) reported heavy starch accumulation in tobacco callus cultures prior to shoot differentiation and postulated that caulogenesis was a high energy requiring process. This postulate was confirmed by the subsequent studies of Thorpe and Meier (1972, 1973, 1974), Thorpe and Laishley (1973) and Thorpe (1977). These authors recorded higher enzyme activities of certain EMP and PPP pathway enzymes, and higher respiratory rate in shoot forming callus than in non-shoot forming callus. Present investigation with diploid and haploid callus showed high and increasing MDH activity during the shoot inducing phase, thus the results are

in agreement with those of the above named authors.

Though PAL has been vindicated in the development, maturation and ripening of fruits (Melin et al., 1977; Melin-Moulet and Hartmann, 1978) and in floral morphogenesis (Sheen, 1973), to the best of my knowledge, this enzyme has not been studied in relation with shoot differentiation. In both the above instances PAL activity was reported to increase with the degree of differentiation. Sheen (1973) had recorded increase in phenolic content with the development of tobacco flowers. In the present study though PAL activity increased during the pre-inductive phase of shoot differentiation in both haploid and diploid callus, it nevertheless, was on decline during the inductive phase i.e. the days immediately preceding shoot differentiation. Similar trend was followed by the phenolic content in the diploid and haploid callus (Chapter III, Tables 46, 59;Figs. 108, 121).

Any alteration made with the constituents of the standard shoot inducing medium reflected on enzyme activities. Reduction of sucrose level to 1% in the control shoot inducing medium resulted in higher activity of all enzymes studied in diploid callus as compared with the control. The accumulation of phenolics was, however, much less (Chapter III; Table 47; Fig. 109). The decrease of sucrose level in the medium also resulted in total suppression of shoot differentiation.

Increase of sucrose to 6% in the control medium brought about phenomenal increase in the activity of all the enzymes. The phenolic content was also high (Chapter III; Table 48; Fig. 110). Moreover, instead of shoot differentiation, a shift occurred favouring rhizogenesis. The peroxidase activity recorded here during rhizogenesis was comparable to the activity recorded during rhizogenesis from diploid callus on the standard root inducing medium. As in the previous instance the IAA Oxidase activity declined slightly during the root differentiation period. Both MDH and PAL activities were markedly higher than during the corresponding period of root differentiation on standard root inducing medium. The amount of phenolics was, however, comparable to the rhizogenic tissue grown on standard root inducing medium. In the present instance the phenolics were in the range of 3.13 - 2.36 mg%, whereas on standard root inducing medium it was in the range of 3.8 - 2.8 mg% (For comparison see Chapter III, Section E and Chapter IV, E).

The incorporation of 500 µM t-cinnamic acid and 100 µM caffeic acid, and the increase of sucrose level to 6% in the control shoot inducing medium resulted in the differentiation of roots instead of shoots. On all the three media the decline of peroxidase activity occurred concomitant with root differentiation. However, the value reached on the day of root differentiation on 6% sucrose medium was higher (0.99 units/mg

protein) than on either 500 μM t-cinnamic acid medium (0.38 units/mg protein) or 100 μM caffeic acid containing medium (0.36 units/mg protein). The IAA Oxidase activity on all the three media attained nearly identical values on the day of root i differentiation irrespective of the developmental pattern of enzyme activity. The enzyme values were in the range of 0.93 - 1.06 units/mg protein. The enzyme values on the day of rhizogenesis were essentially equal as noted earlier (Chapter III, Section E and Chapter IV, E). The MDH activity on the above mentioned root differentiating media was on decline during the rhizogenic phase and was in the range of 3.63 - 5.5 units/mg protein on the day of root initiation. Though the patterns of development of the enzyme activity were not similar, the values were essentially those observed during rhizogenesis from haploid callus on the root inducing medium and other rhizogenic media listed in Section E of the Chapter III and Chapter IV, E. The variations in the PAL activity were too mosaic to compare. On 6% sucrose medium the activity increased from 1.52 to 2.03 units/mg protein during the period of root initiation i.e. day 9 to 12. The enzyme activity on 500 μM t-cinnamic acid medium increased from 3.39 to 11.28 units/mg protein and on 100 μM caffeic acid medium it increased from 9.40 to 12.22 units/mg protein. Though on all the three media the enzyme activity was on increase during the period of root differentiation, it, however,

was higher on the media containing the phenolic acids. Earlier recorded details in Chapter III, Section E and Chapter IV, E, also demonstrated low PAL activity during rhizogenesis from haploid callus on the media which lacked added phenolic acids, and high PAL activity during rhizogenesis on the media containing the phenolic acids. The values of phenolic content in the haploid callus during root differentiation on the above mentioned three media differed with the treatment.

The alterations of the control shoot inducing medium which resulted in the differentiation of roots from haploid callus rather than shoots, also resulted in drastic changes in the enzyme activities. On the media which facilitated root differentiation, the activity of peroxidase in haploid callus was less as compared with shoot differentiating haploid callus. Likewise, the activities of IAA Oxidase and MDH were low in root differentiating systems than in the shoot differentiating one. PAL activity and the phenolic content, however, were high in the rhizogenic systems than in shoot inducing systems.

The incorporation of t-cinnamic acid (500 μM) into the control shoot inducing medium brought about marginal inhibition of the peroxidase activity in diploid callus tissues. The decrease of sucrose level to 1% or its increase to 6%, the incorporation of phenolic acid other than 500 μM t-cinnamic acid

and the use of ½ X and 2 X Mn^{++} ion levels in the control shoot inducing medium resulted in the increase of not only peroxidase activity but also of IAA Oxidase, MDH and PAL. Comparatively less phenolic accumulation occurred with the use of 1% sucrose, 100 μM t-cinnamic acid, 10 μM - p-hydroxy-benzoic acid and of ½ X and 2 X Mn^{++} ion levels.

In haploid callus tissues the incorporation of t-cinnamic acid (100, 500 μM) and caffeic acid (100, 500 μM) into the control shoot inducing medium brought about inhibition of peroxidase, IAA Oxidase and MDH activity. MDH activity was also blocked by ferulic acid (100, 500 μM). Inhibition of IAA Oxidase activity was also brought about by either lowering of sucrose level to 1% or the incorporation of 1.0 μM p-hydroxy-benzoic acid in the medium. PAL activity, on the other hand, was stimulated over the control by all the treatments (Chapter III, Section F; Tables 46 - 58; Figs. 108 - 120; Tables 59 - 71; Figs. 121 - 133).

Regulation of differentiation to give rise to various organized structures starting from the same material must represent master control at the genome level, in which localized derepression and repression must occur. This is reflected by changes in the patterns of enzymes, in which some enzymes not originally present appear and others present in small amount or low activity, show increase in content and/or

activity. As Bonner (1965) has pointed out such changes must represent the cause rather than the result of differentiation.

The isoenzyme system of peroxidase has been genetically defined in maize (Scandalios and Sorenson, 1977) and has been studied in several systems cultured *in vitro* including tobacco (Lee, 1974; Rucker and Radola, 1971; Mader, 1975), *Asparagus* (Van Hoof and Gaspar, 1976) and *Prunus* (Quoirin *et al.*, 1974).

Compared to the inoculum, the number of anodic and cathodic isoperoxidases have been shown to increase during shoot inducing process (Gaspar *et al.*, 1977; Thorpe and Gaspar, 1978; Legrand and Vasseur, 1972). These observations were contradicted by Mader (1975) who observed a decrease in the number of fast migrating anodic bands.

In diploid and haploid callus of tobacco the differentiation of shoots was preceded by the synthesis of three anodic isoperoxidases PS_1 (R_m = 0.41), PS_2 (R_m = 0.44) and PS_3 (R_m = 0.46). In diploid callus the original anodic isoperoxidases, A_1 - A_3, of the inoculum were present throughout the culture period. In haploid callus, however, the isoperoxidase A_2 was suppressed. The above mentioned isoperoxidases were also detected in the differentiated shoots. On the cathodic scale the initial isoperoxidase C_3 was suppressed during the shoot differentiation period; however, the other two isoperoxidases C_1 and C_2 were not affected. A fast migrating cathodic

isoperoxidase C_7 also appeared during the period of shoot initiation. In haploid callus also the original isoperoxidases C_1 and C_2 were not affected, but the third one C_3 was inhibited. Two slow migrating ones C_4 and C_5 appeared concomitant with shoot induction (Figs. 108a, 121a).

The culture of diploid callus on 1% sucrose containing medium resulted in the suppression of anodic isoperoxidase A_1 during the early culture period and of A_3 for the entire culture period.Though some new isoenzymes were synthesized, the synthesis of isoperoxidases PS_1, PS_2 and PS_3 was completely blocked. In haploid callus the anodic isoperoxidases A_2 and A_3 were inhibited along with the shoot peroxidases PS_1, PS_2 and PS_3. On the cathodic scale the synthesis of isoperoxidase C_1 in diploid callus was blocked for most of the days of culture period except day 15. The fast migrating isoperoxidase (C_5) which was synthesized during the differentiation of shoots on the control medium was synthesized on 1% sucrose containing non-inducive medium also (labelled C_4) (Fig. 109a). The decrease of sucrose level to 1% in the control medium resulted in the suppression of slow migrating cathodic isoperoxidases and also the lack of synthesis of isoperoxidase C_2 (Fig. 122a).

The increase of sucrose level to 6% in the control shoot inducing medium caused a shift from shoot differentiation to root differentiation. As in the previous experiments with

rhizogenesis (Chapter III, Section E), here also the root differentiation was preceded by the synthesis of isoperoxidase PR (R_m = 0.3). The shoot inducing isoperoxidases PS_1, PS_2 and PS_3 were inhibited. Moreover, the original isoperoxidases A_2 and A_3 were suppressed. On the cathodic scale the original isoperoxidase C_3 was repressed, whereas, new isoperoxidases C_6 and C_7 were synthesized (Fig. 110 a).

The incorporation of either 6% sucrose or 500 μM t-cinnamic acid or 100 μM caffeic acid into the control shoot inducing medium created an atmosphere conducive to root differentiation rather than shoot induction from haploid callus. In all the three instances rhizogenesis was preceded by the synthesis of anodic isoperoxidase PR (Rm = 0.3). On all the three media new isoperoxidases - both anodic as well as cathodic - were synthesized; however, no close correlation could be found with rhizogenesis (Figs. 123 a, 125 a, 126 a). The synthesis of isoperoxidase PR was also noted on incorporation of 500 μM ferulic acid into the medium, though no rhizogenesis occurred (Fig. 129 a). The phenomenon of root differentiation and its relation with isoperoxidase patterns has already been discussed earlier in the present chapter (Chapter IV, E).

Addition of t-cinnamic acid (100, 500 μM) into the control shoot inducing medium resulted in the block of synthesis of shoot isoperoxidases PS_1, PS_2 and PS_3 in diploid

callus. Both levels of t-cinnamic acid tested inhibited the synthesis of original isoperoxidases A_2 and A_3. The third original one A_1, was suppressed only in the early culture period. Upto eight new isoenzymes were synthesized during the culture period. On the cathodic scale the synthesis of all the three original isoperoxidases C_1 - C_3 was inhibited by the high concentrations of t-cinnamic acid (500 μM) though 3 new isoperoxidases were synthesized (Figs. 112 a, 113 a).

The incorporation of caffeic acid (100, 500 μM) into the control shoot inducing medium resulted in total suppression of the original anodic isoperoxidases A_1 - A_3 in the diploid callus. The synthesis of shoot isoperoxidases PS_1, PS_2 and PS_3 was also blocked. Both the levels of caffeic acid inhibited the synthesis of cathodic isoperoxidases C_1 - C_3. C_1 and C_3, however, appeared only at the beginning of the culture period (Figs. 113 a, 114 a).

The synthesis of isoperoxidase A_1 was inhibited for the first 12 days of culture in diploid callus on ferulic acid (100, 500 μM) containing medium. The isoperoxidases PS_1 - PS_3 responsible for caulogenesis were suppressed. Many fast migrating anodic and cathodic isoperoxidases were synthesized (Figs. 115 a, 116 a).

Like ferulic acid, incorporation of p-hydroxybenzoic acid (1, 10 μM) in the control shoot inducing medium brought about a spurt in the synthesis of fast migrating anodic isoperoxidases in diploid callus. The synthesis of original anodic isoperoxidases A_1 - A_3 remained by and large inhibited. The original cathodic isoperoxidases C_1 - C_3 were also repressed. There was reduction in the number of slow migrating isoperoxidases. A fast migrating one (C_7) was, however, synthesized. The synthesis of shoot isoperoxidases PS_1 - PS_3 was totally blocked (Figs. 117 a, 118 a).

The use of $\frac{1}{2}$ X or 2 X Mn^{++} ion levels in the control shoot inducing medium resulted in total suppression of the original anodic isoperoxidase A_1. A_3, however, was not affected. It also brought about the synthesis of a number of slow migrating isoperoxidases. The cathodic isoperoxidase C_3 was suppressed, whereas C_1 and C_2 were not influenced (Figs. 119 a, 120 a).

In haploid callus the use of t-cinnamic acid (100, 500 μM) blocked the synthesis of anodic isoperoxidases A_1 - A_3. The synthesis of shoot isoperoxidases PS_1 - PS_3 was also inhibited. 500 μM t-cinnamic acid favoured the synthesis of root peroxidase PR and root differentiation. On the cathodic scale low level (100 μM) t-cinnamic acid proved inhibitory to the synthesis of isoperoxidases C_2 and C_3. C_1 was synthesized only

on day 9. High (500 μM) cinnamic acid level repressed the isoperoxidases C_2 and C_3 but had no influence on C_1 (Figs. 124 a, 125 a).

Caffeic acid at 100 and 500 μM in the control shoot inducing medium inhibited the synthesis of original anodic isoperoxidases A_1 - A_3 and of shoot isoperoxidases PS_1 - PS_3. Caffeic acid at 100 μM favoured the synthesis of root isoperoxidase PR and root differentiation. On the cathodic scale the original isoperoxidases C_2 and C_3 were inhibited C_1, however, was not influenced. Caffeic acid brought about drastric reduction in the number of isoperoxidases (Figs. 126 a, 127 a).

The synthesis of original anodic isoperoxidase A_1, in haploid callus, was not influenced by 100 μM ferulic acid; whereas, at higher level (500 μM) its synthesis was delayed. Likewise, the synthesis of isoperoxidase A_2 was delayed on 100 μM ferulic acid but totally blocked by 500 μM ferulic acid in the medium. A_3 was suppressed entirely by the incorporation of ferulic acid into the medium. Likewise, the synthesis of shoot isoperoxidases PS_1 - PS_3 was inhibited. Ferulic acid at 100 μM favoured the synthesis of root isoperoxidase PR but not the differentiation of roots. The synthesis of 2 fast migrating isoperoxidases was favoured at both the levels of ferulic acid. The original cathodic isoperoxidases C_1 and C_3 were not

influenced by ferulic acid, whereas, C_2 was completely suppressed (Figs. 128 a, 129 a).

p-Hydroxybenzoic acid at both the levels (1, 10 μM) blocked the synthesis of anodic isoperoxidase A_3. It exhibited sparing influence on the synthesis of isoperoxidase A_2 at 1.0 μM level. A_1 was suppressed only during the later part of culture period. Fast migrating anodic isoperoxidases (R_m = 0.5 - 0.6) were synthesized. On the cathodic scale the original isoperoxidase C_2 was inhibited, partial inhibition of C_3 was also exhibited. C_1, however, was not influenced much (Fig. 130 a, 131 a). The synthesis of shoot isoperoxidases PS_1 - PS_3 was blocked here also.

Both ½ X and 2 X Mn^{++} ion levels in the control medium reflected drastically on the original anodic isoperoxidases A_1 - A_3 in haploid callus. These remained suppressed either for the entire culture period or for major part of the culture period. However, both levels of Mn^{++} ion facilitated the synthesis of certain slow migrating isoperoxidases, but the shoot isoperoxidases PS_1 - PS_3 were inhibited. The original cathodic isoperoxidase C_1 was not affected at all. On the other hand, C_2 was repressed at 2 X Mn^{++} level and C_3 remained suppressed at ½ X Mn^{++} level (Fig. 132 a, 133 a).

On taking cognizance of the entire data obtained through experimentation in the present study, the following observations come into focus :

1. In haploid tobacco callus on most of the media used to induce rhizogenesis, the total peroxidase activity during the root inducing period declined. In majority of the cases the enzyme activity on the day of root differentiation was in the range of 0.35 to 0.38 units/mg protein. In a few cases higher activity was encountered. Also on standard root inducing medium (MS + 2.0 mg/l IAA + 3% sucrose) and on containing 6% sucrose the activity increased instead of declining during the root induction period. In diploid callus the total peroxidase activity either increased or decreased during the period of root inoduction. However, the activity reached on the day of rhizogenesis was in the range of 0.83 to 0.88 units/mg protein.

2. In both haploid and diploid callus of N. tabacum the peroxidase activity/mg protein increased during the shoot inducing period and was in the range of 1.01 to 1.11 units/mg protein.

3. The total IAA Oxidase activity/mg protein either increased or decreased during the root inducing period in

haploid callus. However, the activity on the day of rhizogenesis was in the range of 0.93 to 1.28 units/mg protein. Comparatively higher activity was recorded during rhizogenesis on standard root inducing medium and root inducing medium containing 6% sucrose.

In diploid callus the IAA Oxidase activity during rhizogenesis on different media was rather mosaic to draw a generalization. However, the activity declined during the root inducing period.

4. In both haploid and diploid tobacco callus, the IAA Oxidase activity increased during the early culture periods, but declined with the advent of the time phase during which shoot differentiation occurred. In both the cases the activity decayed and was registered (to be) in the range of 2.48 to 2.72 units/mg protein. Moreover, by and large the enzyme activity in shoot inducing systems was higher than in the root inducing systems. The exception to this generalization was encountered with the use of 6% sucrose in the medium. Such a phenomenon seemed to suggest higher requirement of endogenous auxin for rhizogenesis than for caulogenesis. The aspects of auxin requirement for root and shoot differentiation have been discussed in Chapter IV, C.

5. In both haploid and diploid callus the total MDH

activity during rhizogenesis on different media did not follow any definite developmental pattern. In haploid callus, the activity subsequent to an increase or decrease reached the value of 3.06 to 5.63 units/mg protein on the day of rhizogenesis. The exception to this generalization was high (3 - 10 times) enzyme activity during rhizogenesis on standard root inducing medium (MS + 2.0 mg/l IAA + 3% sucrose) and the medium containing only 1% sucrose. In diploid callus the MDH activity on the day of rhizogenesis on different media was too varied to be placed within a specific narrow range.

6. Caulogenesis in both diploid and haploid callus occurred concomitant with increasing MDH activity. The activity was 6-7 times higher in the haploid callus than in diploid during the shoot induction period. In diploid callus the MDH activity was by and large substantially higher during rhizogenesis than during caulogenesis. In haploid callus comparatively lower MDH activity was registered during the period of root differentiation than during the period of shoot differentiation. Nevertheless, in most of the rhizogenic systems high MDH activity was recorded during the early culture periods. These results substantiate the earlier observation (Chapter IV, C) that requirements for energy (ATP) and

reducing power (NADH or NADPH) are higher for rhizogenesis than for shoot differentiation.

7. Speaking in generalized terms, in both haploid and diploid callus rhizogenesis occurred concomitant with increasing PAL activity. The PAL activity on the day of rhizogenesis on different media varied.

8. In both haploid and diploid tobacco callus shoot differentiation occurred concomitant with declining PAL activity. In all the cases the PAL activity/mg protein was higher in root differentiating callus than in shoot differentiation callus. Higher PAL activity is known to lead to higher synthesis of phenolics, which can play a subtle role in organogenesis as a consequence of auxin-phenolic synergism or auxin-phenolic antagonism (see Chapter IV, C).

9. In haploid callus the phenolic content increased in the root inducing systems but in diploid callus it declined. Moreover, in both haploid and diploid callus the phenolics declined during shoot initiation.

10. In diploid callus the phenolic content during the period of root initiation remained in the range of 2.3 to 3.8 mg%. However, during shoot differentiation also it

was in the range of 2.3 to 2.8 mg%. In haploid callus on the other hand, the phenolic content varied with the medium used. Though phenolics may have a definite hand to play in organogenesis, it is desirable to make qualitative studies rather than gross quantitative ones. Further work along these lines could yield useful information on this aspect.

11. In both haploid and diploid callus rhizogenesis on any medium was preceded by the synthesis of anodic isoperoxidase PR (R_m = 0.3), which was later detected in the regenerated roots also. The objection to this generalization eminates from the synthesis of PR (R_m= 0.3) in haploid callus cultured on standard root inducing medium (MS + 2.0 mg/l IAA) containing 100 uM ferulic acid. No roots were developed on the said medium.The arguments for this abnormality are listed in Chapter IV, E.

12. In haploid and diploid callus caulogenesis was preceded by the synthesis of anodic isoperoxidases PS_1 (R_m = 0.41), PS_2 (R_m = 0.44) and PS_3 (R_m = 0.46).

From the present study it becomes quite apparent that organogenesis from unorganized cells is under strict gene control and is accompanied by the synthesis of definite

functional proteins. It will be worthwhile to examine the spectral changes in the isoenzyme patterns of various enzymes involved in auxin metabolism (IAA Oxidase), phenolic metabolism (PAL, Coumaric CoA ligase), enzymes of the respiratory pathway (enzymes of EMP, PPP and TCA cycle) and many more. Also the changes in the molecular species of RNA can not be ignored. Future efforts following this approach would help to elucidate further the process of differentiation.

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