CHAPTER I

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

INTRODUCTION

Plant tissues contain a large number of substances with phenolic functions, such as anthocyanins, the flavones and their derivatives, the tannins, and the phenolic acids whose wide distribution in plants has recently been recognized. The history of these compounds of plant origin has its beginnings in the industries. The tannins have been employed since ancient times in tanning skins and the manufacture of ink, and some of their properties have been made use of in the fining of wines. Botanists of all persuasions, systematics, cytologists and physiologists, became interested in these substances. They are increasingly utilized in chemotaxonomy. Their intracellular occurrence was examined : the observation of cells containing coloured phenolic compounds gave valuable insights into the origin and evolution of vacuoles. Eventually, studies were undertaken to elucidate the origin of these compounds and to understand their physiological significance.

Though phenolic compounds have received the attention of phytochemists and botanists for a long time; their physiological functions are uncertain. Do these constituents act as sexual hormones, as growth factors, as sensitisers

in photosynthesis or other photobiological phenomena, as intermediates or regulators of oxidation-reduction phenomena, as constituents of cell membranes or organelles, as bactericides or fungicides ... or are they merely secondary metabolites for storage or excretion of aromatic rings, true "by-products" whose function is no more? However, their almost universal occurrence and abundance in vascular plants suggest that they play some part in biological processes. Their pathological functions, particularly their potential toxicity and their possible role in certain plant diseases of viral and fungal origin (Cadman, 1960; Cruickshank and Perrin, 1964) and their role in inhibition of seed germination (Roubaix and Lazar, 1960; Sumere Van, 1960) are known. The functions - both physiological and pharmaceutical - of phenolic constituents in animal organisms (Rainwell et al., 1964), their role in vascular resistance mechanisms (vitamin P activity) (Lavollay and Neumann, 1959), and their bactericidal action (Masquelier, 1959) are also reported. However, most of the valid results were obtained by the method of ^{14}C labelling and these results so far have scarcely had any physiological bearing. Thus, the role of phenolic substances still remains uncertain.

Plant tissue and cell cultures provide admirable material for the study of metabolic events and growth. Their growth, nutrition, physiological and biochemical changes can conveniently be studied under a wide variety of conditions. The value of this technique is that cell and tissue system can be subjected to direct experimental control, to some extent at least free from the complex inter-relationships and interactions occurring in the intact plant. A particular convenience of the technique is the high measure of control that can be exerted over the experimental material. It was with this idea that the present studies on polyphenols and few related enzymes in plant tissues cultured in vitro were initiated.

Though the potentialities of growing isolated plant cells in culture was visualized at the turn of the present century by G. Heberlandt (see Krikorian and Berquam, 1969); plant tissue began to be successfully cultured about forty years ago, as a consequence of the development of suitable nutrient media. In 1939, Gautheret, White and Nobecourt working independently succeeded in initiating continuous culture from tobacco and carrot. Since then there are now established tissues from a wide range of plants in

continuous cultures. Callus tissues derived from many plants can now be grown on the surface of agar media which contain a utilizable sugar, inorganic salts and various organic growth factors (Gautheret, 1959). Such cultures can be serially subcultured by sub-division of the tissue masses provided that the tissue fragment transferred on each occasion is above a critical size (Street, et al., 1965; Mehta, 1965).

Plant callus as well as cell cultures, the latter derived by transferring callus pieces to agitated liquid medium (Steward and Shantz, 1955) have been used in a variety of problems, including the study of cell division (Muir et al., 1954; Torrey, 1957; Steward et al., 1958 a), susceptibility of tissues to virus infection (Hilderbrandt, 1958; Bergman, 1959), cellular differentiation (Steward, et al., 1958 b), the production of cell metabolites (Tulecke and Nickell, 1960), relationship between growth, respiration and protein synthesis (Givan and Collin, 1967) and relationship between cell division, cell expansion and cell separation (Henshaw, et al., 1966). Further, the potentialities of tissue culture preservation technique by freezing for the conservation of plant genetic resources has been recently pointed out by Henshaw (1975). However, the establishment of many plant

cultures has been achieved mainly with complex media containing coconut milk, yeast extract or others which limit the usefulness of the technique where analysis of chemical changes is desired. Considerable progress has been made by several workers (Torrey and Reinert, 1961; Heller, 1963; Linsmear and Skoog, 1965; Henshaw *et al.*, 1966; Stuart and Street, 1969; Raj Bhandari *et al.*, 1969) in evolving simple, completely defined, synthetic media for studying growth, cell differentiation and associated physiological changes. In the present studies, a completely defined medium has been used, avoiding coconut milk and other complex compounds, for studying growth, polyphenol synthesis and related enzymes in *Crotalaria* tissues.

Crotalaria juncea L. (sub-family Papilionaceae of Leguminosae), commonly called sunn or sunhemp, is well-known for its industrial and agricultural value as it yields the bast fibre - the common hemp of commerce - and also for its green matter which forms an excellent green manure as well as fodder for cattle. The species of *Crotalaria* have been extensively screened for a toxic alkaloid, monocrotaline, by Atal and his co-workers (Kumari et al., 1966; Suri and Atal, 1967) at Regional Research Laboratory, C.S.I.R., Jammu-Tawi. The species examined in the present investigation,

however, is not toxic. To our knowledge, monocrotaline is not reported in this species. However, the seeds are rich in phenolic compounds (Shah, et al., 1976a) and very prompt in germination. The callus tissue derived from the seedling grew quite satisfactorily and responded in a consistent way to a variety of external stimuli. This prompted us to take up this plant for the study of growth, polyphenol accumulation and few allied enzymes under different cultural conditions.

Carbohydrate nutrition has been a focal point ever since the inception of plant cell culture. Intact plants meet their energy requirements autotrophically via the photosynthetic fixation of carbon. When plant tissues are cultured, the normal functions of chloroplasts are frequently absent or partially or totally blocked. Therefore, in plant culture, it is necessary to substitute carbon sources for those carbohydrates that would otherwise have been generated photosynthetically in the intact plant. The carbohydrate requirement of callus cultures from a number of dicotyledons were studied by many workers (Gautheret, 1945; Hilderbrandt and Riker, 1949, 1953; Arya et al., 1962; Fadia and Mehta, 1973; Subbaiah et al., 1974). These studies revealed that

most tissues grow best when supplied with sucrose, dextrose or laevulose as a carbohydrate source.

Like carbohydrates, nitrogen is another most important nutrient for higher plants and for the cultured tissues. The capacity of plants and cultured tissues to synthesize their nitrogenous compounds using inorganic and organic nitrogen source is one of the fundamental phenomena of life. Organic and inorganic nitrogen compounds incorporated in the nutrient medium have marked effect on growth. Nitrates are most commonly used by cultured tissues as source of nitrogen for their growth. Ammonium nitrate supported maximum growth of soybean cell cultures (Gamborg et al., 1968), while wheat cells grew best in a medium containing calcium and potassium nitrates (Gamborg, 1970). However, for many tissues the most suitable inorganic nitrogen source is a mixture of ammonium salts with nitrate (Steward et al., 1958 a; Filner, 1966; Bhatt et al., 1973). Substitution by nitrites or ammonium salts has not been very successful. Effects of different amino acids as a source of nitrogen on growth and metabolism have been discussed in detail (Filner, 1965; Street, 1966). Casein hydrolysate, either acid or enzyme hydrolysed, has also been incorporated into culture media by various workers to test its ability to support growth (Nitsch and Nitsch, 1957; Staba, 1962; Steinhart et al., 1962; Vasil and Hilderbrandt, 1966; Subbaiah, 1974).

Vitamins are reported to be essential for or markedly stimulatory to the growth of plant cultures. They are, particularly thiamin (vitamin B_1) and pyridoxine (vitamin B_6), involved as prosthetic groups or co-enzymes of enzymes essential to all living cells(Lichstein *et al.*, 1945; Racker *et al.*, 1953). Attempts have been made, therefore, to incorporate these vitamins in the synthetic medium for successful growth of callus cultures.

Among the growth substances, which are known to play an important role in cell metabolism, cell division, growth, differentiation and cell hypertrophy (Butenko, 1968), auxin is an essential supplement in the nutrient medium for the successful culture of many excised plant tissues. Examples of such tissues are cited by Gautheret (1942, 1959), Morel (1948) and Ball (1950). Media employed for the successful culture of plant tissues have contained one of the following auxins - indole-3-acetic acid (IAA), napthaleneacetic acid (NAA), 2-4-dichlorophenoxyacetic acid (2,4-D), or para-dichlorophenoxyacetic acid. A comprehensive summary of the auxin requirements of normal callus cultures is presented in Gautheret's review (1955).

That kinetin, the most widely used cytokinin, has an

additive effect on growth to that of auxin was demonstrated by Steinhart (1961) in spruce and by Digby and Wareing (1966) in sycamore cultures. Incorporation of kinetin into the nutrient medium has led to substantial enhancement in growth of a number of callus and suspension cultures (Murashige and Skoog, 1962; Steward, 1969; Lalchandani, 1970; Subbaiah, et al., 1974). However, in the present studies, maximum growth was registered without the addition of kinetin.

Plant cells cultured in vitro can synthesize and accumulate a wide variety of secondary metabolites (Krikorian and Steward, 1968; Staba, 1969; Becker, 1969; Koblitz, 1969; Davies, 1972 a; Chokshi, 1975; Shah et al., 1976 b). Nickell (1962), Staba (1963) and Street (1965) reviewed the possible adaption of plant cell cultures as an important tool for the production of useful compounds by industry. Tulecke (1966) stated that if tissue culture as a technique continues the same rate of advance as in recent years, it is reasonable to predict that some commercial use will be made in the next decade. Recent advances in plant culture technique suggest that aseptically grown cell cultures of higher plants might be used for the commercial production of natural products. This subject and the problem involved with this method have been comprehensively reviewed (Furuya, 1968; Puhan and

Martin, 1971; Teuscher, 1973). Recently, Misawa et al. (1974) pointed out that application of plant cell cultures to industrial production of physiologically active substances will before long be realized. Supporting this statement, Zenk et al. (1975) stated that world-wide increased shortage of raw material, independence of climatic factors and plant epidemics, as well as constant yield and quality of material produced, would favour the exploration of aseptic methods for the production of metabolites. The report of Atal group (Grewal and Atal, 1976) on maximum growth rate of the cultured tissues is also promising in this field.

Methods of inducing the cells to accumulate an increased concentration of secondary metabolites would be very useful. Moreover, in many cases it is clear that the quantitative and qualitative performance of the tissue in forming these products may be greatly influenced by the cultural conditions and the nutrient status of the medium on which they are grown (Siegelman, 1964; Staba, 1969). Anthocyanin accumulation is inhibited or delayed at high auxin concentrations in Haplopappus gracilis tissue cultures (Blakeley and Steward, 1961; Constabel et al., 1971) and is influenced by the nitrogen/carbohydrate ratio in cultured red cabbage

embryos (Szweykowska, 1959). Flavone glycoside synthesis in suspension cultures of Petroselium hortense has been reported to be light dependent (Hahlbrock and Wellman, 1970), and synthesis of a range of polyphenolic material in tea callus cultures is increased on illumination (Forrest, 1969). On the other hand, tannin synthesis in juniper callus cultures is reported to be reduced by light (Constabel, 1963) and enhanced at high sugar concentrations (Constabel, 1968). Polyphenol synthesis in Paul's scarlet rose (Davies, 1972 b) and in Acer (Westcott and Henshaw, 1974) cell suspension cultures is influenced by auxin concentration in the medium. Deoxyisoflavone synthesis in soybean callus tissue is reported to be stimulated by 2,4-D and NAA, although the latter is only effective in the presence of kinetin (Miller, 1969). Our studies with Cassia (Shah et al., 1976 b) and Datura (Subbaiah, 1974; Chokshi, 1975) also revealed that production and accumulation of secondary metabolites by the tissues cultured in vitro is regulated by the cultural conditions and the nutrient status of the medium on which they are grown.

In the present study a survey for total phenolics was made in tissue cultures of Crotalaria. Influence of various nutrients and hormones on accumulation of phenolic compounds was further examined under different cultural conditions.

Although plant tissue cultures have been screened by many workers for the presence of phenols, coumarins, flavonoids, and related compounds (Chan and Staba, 1965; Gamborg, 1966; Gamborg and Keeley, 1966; Ellis and Towers, 1970; Schafer and Wender, 1970; Fritig et al., 1970), few studies have been designed to study the effect of these compounds on growth and basic metabolism. Naturally occurring benzoic and cinnamic acids derivatives can inhibit ion uptake (Glass, 1974) ultimately effecting the growth of the callus. Lee and Skoog (1965 a, 1965 b) reported inhibitory effect of trans-cinnamic acid on tobacco callus growth. However, chlorogenic acid was found inhibitory only at relatively high concentration (Danks et al., 1975). Experiments were conducted to study the effect of phenolic acids, in presence or in absence of auxin, on growth and accumulation of phenolic compounds.

The enzyme peroxidase (EC. 1.11.1.7) prevails in plants and has been implicated in a number of diverse phenomena observed in plants, including disease resistance (Uritani and Stahmann, 1961; Yu and Hampton, 1964), respiration (Chance, 1954; Nicholls, 1965), IAA breakdown (Hinman and Lang, 1965) and lignin biosynthesis (Freudenberg et al., 1958; Higuchi and Ito, 1958; Freudenberg, 1959). It has also been implicated in several reactions related to phenolic biosynthesis. Thus, horseradish peroxidase can

catalize the hydroxylation of a number of aromatic compounds in the presence of oxygen and dihydroxyfumaric acid (Mason et al., 1957). The findings of Mazelis (1962) revealed that peroxidase in the presence of Mn^{++} and pyridoxal phosphate catalizes the oxidative decarboxylation of amino acids and may well have significance in the biosynthesis of certain phenolic compounds.

The enzyme IAA oxidase, of widespread occurrence in plants, oxidatively converts the plant growth hormone, IAA, to a physiologically inactive and as yet uncharacterized product (Tang and Bonner, 1947; Wagenknecht and Burris, 1950). Since the elongation of plant cell is known to be hormonally regulated by IAA, and since the amount of growth accomplished by the cell is quantitatively dependent on IAA concentration, the presence of an IAA destroying system obviously offers a mechanism for a regulation of growth rate within each cell, tissue or organ. The oxidase system appears to consist of a light-activable flavoprotein coupled through hydrogen peroxide to peroxidase, the latter enzyme accomplishing the actual destruction of IAA (Goldacre, 1951; Galston et al., 1953). The activity of the system appears to be limited by the quantity of phenolic cofactor.

With this background, IAA oxidase and peroxidase activities are investigated in the present studies under different cultural conditions and their probable role in the physiology of growth and the regulation of phenolic compounds is discussed.

L-Phenylalanine ammonia-lyase (EC 4.3.1.5) (PAL) is the first and limiting enzyme in the pathway of phenolic biosynthesis. It catalyzes the formation of trans-cinnamate from L-phenylalanine by non-oxidative deamination. Cinnamic acid is a precursor for many plant secondary constituents and PAL, a key enzyme in the metabolism of aromatic compounds, has been assigned a number of roles including lignification (Cheng and Marsh, 1968; Higuchi, 1966; Rubery and Northcote, 1968; Rhodes and Wooltorton, 1973), flavonoid synthesis (Creasy, 1971; Maier and Hasegawa, 1970), and phytoalexin production (Hadwiger and Schwochau, 1971 a). This enzyme has been of interest not only for its role in plant phenolic metabolism, but because its activity fluctuates significantly in plant tissues in response to a variety of physical and chemical stimuli. Thus, light induced increases in PAL activity have been reported for a variety of tissues including etiolated seedlings (Durst and Mohr, 1966; Amrhein and Zenk, 1971), potato tuber discs (Zucker, 1965) and

cultured parsley cell suspensions (Hahlbrock and Wellman, 1970). The other factors shown to regulate PAL activity include ethylene (Hyodo and Yang, 1971), Y-irradiation (Riov et al., 1968), UV light (Hadwiger and Schwochau, 1971 a), gibberellic acid (Cheng and Marsh, 1968), sucrose (Creasy, 1971; Subbaiah, 1974), auxin (Subbaiah et al., 1974) and a variety of DNA intercalating agents including actinomycin-D (Hadwiger and Schwochau, 1971 b).

PAL has been found to be widely distributed whereas the enzyme performing an analagoas reaction with tyrosine as substrate, tyrosine ammonia-lyase (TAL) is restricted to certain plants only. TAL catalyzes the deamination of L-tyrosine to p-coumaric acid and ammonia. Thus, far no TAL has been purified separate from PAL activity. It is for this reason that at present there is no enzyme classification number for this enzyme. Although most PALs have bisubstrate activity for L-phenylalanine and L-tyrosine not all do as is exemplified by the potato tuber enzyme, which deaminates only phenylalanine (Havir and Hanson, 1968 a).

The effects of sucrose, auxins, gibberellic acid, cycloheximide, light and kinetin concentrations on PAL and TAL enzyme patterns are further examined in the present investigation.

The synthesis of many phenolic compounds in plants includes oxidation, reduction and condensation stages, in which the side-chain of cinnamic acid or its hydroxylated derivatives is involved. These stages are presumed to require activation of the carboxylic group, probably by formation of the CoA ester (Neish, 1964). The formation of CoA thiol ester has been postulated to be involved in the conversion of cinnamic acids to flavonoids (Grisebach, 1967), lignin (Gross and Zenk, 1966), benzoic acids (Vollmer et al., 1965), and other products in higher plants. Recently, extracts from leaves of spinach beet have been prepared, which activated cinnamate and acetate providing that CoA was added (Walton and Butt, 1971). The enzyme was also demonstrated in cell suspension cultures of parsley (Hahlbrock and Grisebach, 1970) and soybean (Lindl et al., 1973). Attempts are made in the present investigation to demonstrate coumarate : CoA ligase (acid : CoA ligase, EC 6.2.1.2) activity in Crotalaria callus cultures.

Phenylalanine and tyrosine are the initial material leading to the formation wide variety of secondary metabolites. The initial stages of the degradation of these two compounds are very interesting. A sequence involving transaminati on of

phenylalanine, reduction of the keto acid so formed to phenyllactic acid and dehydration of the latter to form cinnamic acid was proposed by Neish (1960). An analogous sequence was also proposed for the conversion of tyrosine to p-hydroxycinnamic acid with the final step, the dehydration of p-hydroxyphenyllactic acid, being limited in its occurrence to the Graminae. However, evidence (Koukol and Conn, 1961; Neish, 1961) of an enzymatic nature has cast doubt on this reaction sequence and instead suggest that cinnamic acid and p-coumaric acid are produced by direct deamination of the corresponding amino acids. Whatever the case may be, the transamination of phenylalanine and tyrosine reaction sequence can exert its influence on the production of secondary metabolites by limiting the rate of direct deamination of these amino acids. In the present studies experiments were, therefore, carried out on reactions involving transamination of phenylalanine and tyrosine with a view to examine the regulatory role of these enzymes on the degradation of phenylalanine and tyrosine via other pathways.

PAL has been extensively purified from potato tubers (Havir and Hanson, 1968 a), maize shoots (Marsh et al., 1968)

and wheat seedlings (Nari et al., 1972). The enzyme preparations from different sources exhibit considerable variations in kinetic behaviour. The purified preparations from potato tubers (Havir and Hanson, 1968 b), maize shoots (Marsh et al., 1968) and wheat seedlings (Nari et al., 1974) show significant deviations from Michaelis-Menten kinetics, whereas those obtained from sweet potato roots (Minamikawa and Uritani, 1965) and others obey the classical Michaelis-Menten kinetics. Further, PAL from potato and Maize (Havir and Hanson, 1973) is made up of four identical subunits whereas the wheat enzyme (Nari et al., 1972) with a molecular weight of 330,000 is composed of two pairs of unidentical subunits each having a molecular weight of 75,000 and 85,000 respectively. Havir and Hanson (1968 a) have purified the enzyme PAL more than 300-fold from extracts of potato tubers. They estimated molecular weight of the enzyme as 330,000 and found that sulfhydryl reagents failed to inhibit the enzyme whereas carbonyl reagents were potent inhibitors, whose action could be blocked by cinnamate. This showed a catalytically essential dehydroalanyl moiety as the active site of phenylalanine ammonia-lyase.

To our knowledge, no attention has been paid on the purification and properties of PAL extracted from plant

tissues cultured *in vitro*. Experiments were, therefore, carried out on PAL extracted from callus tissues of *Crotalaria* to study certain properties of the enzyme such as : substrate specificity, molecular weight, subunit composition and kinetic behaviour. This was compared with the PAL extracted from the hypocotyls of *Crotalaria* seedlings.

The above discussed aspects formed the background for the present work. The results obtained are incorporated under the following broad heads :-

I. Growth, polyphenol accumulation and the development of peroxidase, IAA oxidase, PAL and TAL enzymes in the germinating seeds of *Crotalaria*.

II. Initiation and growth of seedling callus cultures.

III. Nutritional studies of the tissues on defined medium for growth and production of phenolic compounds.

IV. Studies on the progressive changes in phenolic content and the development of peroxidase, IAA oxidase, PAL, TAL, p-coumarate :CoA ligase, phenylalanine transaminase and tyrosine transaminase enzymes in callus cultures of *Crotalaria* under varied cultural conditions.

V. Purification and properties of PAL extracted from callus tissues and from hypocotyls of *Crotalaria* seedlings.

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