SUMMARY

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The work is described in five sections. This includes : experiments with germinating Crotalaria seeds in Section A; initiation and growth of seedling callus cultures in Section B; growth and polyphenol accumulation as influenced by different cultural parameters in Section C; growth, production of phenolic compounds and the development of allied enzyme activities at various levels of sucrose, 2,4-D, NAA, GA_3, kinetin and cycloheximide and also light effect in presence of auxin or GA_3 in Section D; and isolation, purification and characterization of seedling and callus PAL enzyme in Section E.

The studies on growth responses, polyphenol accumulation and the development of related enzymes during germination of Crotalaria juncea L. seeds are presented in Section A. Observations showed that imbibition rate of water by seeds was fast and the germination was prompt. Progressive changes in polyphenol accumulation in relation to growth in different parts of the seedling revealed that maximum rate of polyphenol synthesis was restricted to the period of most rapid growth phase. Close correlation between the accumulation of phenolic compounds and the development of peroxidase activity in individual parts of the seedling suggested an important role of peroxidase in the

synthesis of phenolic materials. On the other hand, IAA oxidase activity was maximum in the part where minimum phenolics was registered. The results further indicated that endogenous levels of phenolic compounds could play regulatory role in the development of this oxidase activity. Like peroxidase, the development of PAL (phenylalanine ammonia-lyase) activity in individual parts of the seedling showed correlation with the accumulation of phenolic compounds. TAL (tyrosine ammonia-lyase) is also reported in the present studies. The report is few of its kind from dicotyledonous plants. The activity of TAL terminated a day earlier than the PAL in all the parts of the seedling but the level of TAL in root was higher than PAL. This suggested that though phenylalanine pool is deaminating in Crotalaria for polyphenol biosynthesis, tyrosine pool is also preferentially utilized.

Of the different media tested for the induction of callus from the seedling of Crotalaria, MS medium supplemented with 2,0 mg/l 2,4-D, 2.0 mg/l IAA and 2.0 mg/l kinetin was found to be the best (Chapter III, Section B, 1). Further studies clearly established that tissue initiated on the above medium registered higher growth when transferred to MS medium supplemented with only 2,4-D at 2.0 mg/l level. The callus grown on a medium containing 2.0 mg/l 2,4-D and subcultured at monthly intervals

on similar auxin enriched media continued growth indefinitely with undiminished vigour.

Among the auxins, 2,4-D was superior to IAA and NAA for the growth of the callus (Section B, 2). Addition of kinetin to the medium suppressed the growth. The possible reasons for the superiority of 2,4-D over other auxins and the inhibitory effect of kinetin on growth are discussed.

The faster growth was registered in the callus cells than in the suspension cells (Section B, 3). However, the typical sigmoid type of curve was observed for both the cultures. When the data was replotted on semilog basis according to the kinetic formula $K = \frac{1}{T} \times \log \frac{X}{X_o} \times Ke$ (Price, 1970), very brief period of lag phase was observed and 2-10 days period constituted exponential growth phase.

Section C, 1 of Chapter III clearly showed the superiority of sucrose over other carbohydrates tested both for the growth of the tissue and also for the maximum production of phenolic compounds. Different requirement of nitrogen sources was observed for the maximum rate of growth and polyphenol accumulation. Potassium salt supported maximum growth while ammonium salt supported maximum polyphenol synthesis (Section C, 2 B). However, a balanced supply of ammonium and potassium nitrates was more

effective than other sources both for the growth and for the polyphenol production (Section C, 2 C). Among the organic nitrogen sources tested, (Section C, 2 D) yeast extract at 5 g/l concentration supported maximum growth and accumulation of phenolic materials. Further, though yeast extract as a sole nitrogen source was found superior to other organic and inorganic nitrogen sources, inorganic nitrogen source was preferred over yeast extract for further experiments because of its chemically defined constitution. Urea at all the concentrations tested failed to enhance growth and phenolic formation (Section C, 2 E).

L-Phenylalanine and L-tyrosine, the well-known precursors in the pathway leading to the synthesis of phenylpropanoid compounds, were tested for their ability to support growth and polyphenol production (Section C, 3). Both these amino acids, singly or in combinations, failed to stimulate growth over the control. Though the capacity of cells to accumulate polyphenols increased with L-phenylalanine medium, overall production of phenolic compounds was less than in the control. On the other hand, L-tyrosine at 0.1% concentration promoted maximum polyphenol synthesis. This opposite effect of L-phenylalanine and L-tyrosine was clearly due to the pronounced difference in their effect on growth.

The results obtained in the experiments with the effect of six phenolic acids on growth and polyphenol production (Section C, 4) showed that these acids were inhibitory to growth and production of phenolic materials in presence of 2,4-D. However, in absence of 2,4-D only ferulic and cinnamic acids were effective inhibitors at low concentrations. Caffeic acid stimulated polyphenol production, both per culture as well as on unit basis, over the control at the highest level tested. The possible mechanism of action of these phenolic acids on growth and the production of phenolic compounds is discussed.

In Section D of Chapter III are described the progressive changes in growth, polyphenol synthesis and the development of peroxidase, IAA oxidase, PAL, TAL, p-coumarate : CoA ligase, phenylalanine transaminase and tyrosine transaminase enzymes under different cultural conditions.

Increased production of phenolic compounds was registered during the maximum rate of growth at all the levels of all the substances tested. Experiments in Section D, 1 clearly indicated that synthesis of phenolic materials was dependent on the availability of carbohydrate and that the depletion of sucrose from the medium led to the decrease in the production of phenolic compounds. 2,4-D (Section D, 2) and Kinetin (Section D, 5)

affected the initiation of polyphenol synthesis in that the initiation was delayed at higher levels, while GA_3 (Section D, 4) and NAA (Section D, 3) showed no such adverse influence on initiation. Further, both the auxins tested (2,4-D and NAA) showed marked variation in their effect on growth. NAA containing medium influenced tissue growth more markedly than the polyphenol formation. GA_3 supported maximum phenolic content both on total and relative amount basis. Cycloheximide (Section D, 6) inhibited growth and polyphenol synthesis at all the levels tested.

Examination of the interaction between light and different hormones (Section D, 2, 3 and 4) clearly revealed that light induced the synthesis of phenolic compounds. The promotory effect of light could be substituted by higher GA_3 levels. The growth was inhibited by all the parameters tested in presence of light, except at higher GA_3 concentrations where reversal of inhibitory effect of light was recorded. The mode of action of these hormones and their interactions with light for their effects on growth and polyphenol synthesis are reviewed with the help of results obtained in the present investigation.

Studies on peroxidase, IAA oxidase, PAL and TAL enzyme activities in relation to polyphenol synthesis under different cultural conditions showed correlation either throughout the culture period or during the part of it. A close correlationship

was recorded between peroxidase activity and phenolic accumulation under all the cultural parameters tested, suggesting that polyphenol production could be controlled through the regulation of peroxidase activity. Though PAL and TAL showed relation with the synthesis of phenolic materials only during the part of culture period, the steep increase in their activities at the time of maximum rate of phenolic synthesis indicated their undoubtable key roles. IAA oxidase showed both discripancy and similarity with the synthesis of phenolic compounds and its role in the phenolic biosynthesis can be traced only with the role of peroxidase. The latter point is dealt with and discussed in detail. Usual inhibition of all the enzymes by cycloheximide was observed (Section D, 6). However, specific activity of all the enzymes studied showed increase over the control, suggesting that cycloheximide affected structural proteins more severely than the enzymic ones.

Interaction of 2,4-D, NAA and GA_3 with light for their effects on the development of above mentioned enzymes was examined (Section D, 2, 3 and 4). Light induction of all the enzymes was registered, the induction being less marked at higher level of hormone tested. Further, the reversal of light induction was observed with peroxidase at higher level of 2,4-D and GA_3; IAA oxidase at higher level of all the hormones tested and PAL and TAL at the higher level of GA_3.

Concomitant large increases in p-coumarate : CoA ligase and PAL and TAL activities are evident from the results obtained in experiments described in Section D, 2 and 7. The activity of ligase enzyme was picked up only with the maximum increase in the enzymes PAL and TAL. It seemed hence that the development of p-coumarate : CoA ligase depended on the activities of PAL and TAL which could provide substrate necessary for the ligase activity. Similar observation was made with the enzymes phenylalanine transaminase and tyrosine transaminase on one hand and PAL and TAL on the other. The coordination obtained in the development of these enzymes during the growth cycle clearly indicated that the synthesis of phenylpropanoid compounds in Crotalaria followed the usual pathway via the well established shikimic acid pathway (Chart I). However, the presence of TAL enzyme is few of its kind in dicotyledonous plants and suggested that both the phenylalanine and tyrosine pools are involved in the biosynthesis of phenolic compounds in Crotalaria callus cultures.

Phenylalanine ammonia-lyase (PAL) has been purified to homogeneity from both the Crotalaria seedling and the callus derived from it (Section E, 1). The purified enzyme catalyses the deamination of both L-phenylalanine and L-tyrosine. As the ratio of PAL to TAL was nearly equal throughout the purification

procedure it is proposed that the activities of both the enzymes resided in the same protein. A similar pH profile with an optimum around 8.8 was recorded for PAL from both the origins. The carbonyl reagents were found to bring about irreversible inactivation of PAL. Further, there were indications that PAL in this plant was a sulphydryl enzyme. Effects of substrate concentrations on PAL activity exhibited deviation from Michaelis-Menten equation. Instead, the Linweaver-Burk plots were biphasic and the Hofstee plots were curvilinear. Two Km values were determined, K_m^H was 4.2 mM and K_m^L was 0.8 mM for seedling PAL and K_m^H was 5.0 mM and K_m^L was 0.4 mM for callus PAL. Electrophoretic pattern and gel filtration studies revealed only one isoenzyme of PAL with molecular weight around 326,000. The Rs values were 86 and 88.48 for seedling and callus enzymes respectively. Hill coefficient was determined to be 0.2 for the PAL from both the origins. Sodium dodecyl sulphate (SDS) electrophoresis of purified PAL revealed two unidentical subunits with molecular weights around 72,000 and 90,000. Thus, Crotalaria enzyme is made up of two pairs of unidentical subunits. Activation energy of seedling PAL was 7,700 cal. per mol and that of callus PAL was 4,800 cal. per mol. All these studies, together with the tested effects of aromatic compounds on PAL, clearly suggested that PAL in Crotalaria exhibited negative homotropic cooperativity and is a regulatory enzyme. This further suggested the significance

of PAL in the regulation of phenolic biosynthesis.

The findings of the present investigation explored the potentialities of cultured tissues to synthesis and accumulate phenolic compounds. Methods of inducing these compounds for increased production by manipulating cultural conditions were further presented. The enzymatic studies revealed the biochemical regulation of phenolic compounds in Crotalaria. The suggested mode of action of growth hormones, nutrient factors and environmental factor (light) was strengthened by providing additional evidences obtained from the experiments on their effects on polyphenol synthesis and related enzymes. Finally, the isolation, purification and characterization studies on phenylalanine ammonia-lyase (PAL) revealed in-depth regulatory mechanism of this enzyme in the biosynthesis of phenolic compounds.