STUDIES ON SOME PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF GALL FORMATION IN SCHIMA

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I certify that the thesis entitled "Studies on some physiological and biochemical aspects of gall formation in <u>Schima</u>" submitted by Mr. A.L.S. Rajee for the degree of Doctor of Philosophy in Botany of the North-Eastern Hill University, Shillong, embodies the record of original investigation carried out by him under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. degree. This work has not been submitted for any degree of any other University.

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Chapter I

General Introduction

Abnormal growths are characterized by extensive alterations and over growth due to the plant organ losing control over the growth potentials of the affected area. "Galls" or "cecidia" induced by insects, are pathologically developed tissues mostly by hypertrophy or hyperplasia. The gall tumor tissue proliferation is a unique phenomenon of autonomous growth of the diseased cells.

Natural biological assemblage systems are subjected to high disturbance frequencies. Galls offer ample scope for micro-habitat and sustain a number of arthropods, bugs, wasps etc. for feeding or for their requirement of plant in any plausible way to complete the life cycle. The adaptive strategies that lead to synchronized systems between the hosts and inducing agents are concerned with the multiplicity of diverse physical, chemical and biological agents - phytocecidia, zoocecidia and plasmatocecidia (Rohfritsch, 1971) and circumstances of life (Arya _et al_., 1975; Bayer, 1982) - that can transform a normal cell into a tumor cell. Association can be dynamic between highly specialized feeding behaviour and adaptive strategies. However, the correlation of development in the highly intricate cecidogenic system and what contributed to form has not yet been solved. The complex interactions involve deviation of the dynamic equilibrium related to metabolically normal plant. Changes of mutational type involving the loss of substitutions or permanent rearrangement of the genetic information in a cell could lead to the establishment of the tumorous state. It may be noted that plant cell transformation does not always mean whole plant transformation. The neoplastic growth strikingly illustrates the ability of an infectious agent to interfere normal plant morphogenic processes and re-establish growth in a new stable state, potentially beneficial to the pathogen as in the case of crown-gall caused by *Agrobacterium tumefaciens*.

Autonomy exhibited by neoplastic growth has many gradations. At one extreme are located the benign (self-limiting) tumors which grow and remain localized in the hosts, at the other end are the most malignant (non-self-limiting) cancers that invade neighbouring tissues and spread throughout an organism by metastasis (Braun and Stonier, 1958).

Mani (1973) has described about 817 galls on more than 400 species of plants belonging to about 84 natural orders. Approximately, 15,000 gall forming insects have been recorded from around the world (Rohfritsch and Shourthouse, 1982). The subject of insect-gall formation has been extensively reviewed (Arya *et al*., 1975; Purohit *et al*., 1980; Rohfritsch and Shorthouse, 1982; Tandon, 1985). Gall forming insects are considered to be host specific, though a few of them invade closely related species within a genus (Rohfritsch and Shorthouse, 1982) while others like *Eriophyes cladophthyrus* attack different species of plants (Westphal, 1980). Pathogenic insects while harbouring on particular plant, release a toxin, so called, "host specific substance", which damage or destroy the host tissue susceptible to it. However, the toxin has little or no effect on other plants, microorganisms or animals (Browder and Eversmeyer, 1986). Parr (1940) reported that the chemical gall-initiator was injected by the insect into the plant together with its saliva during feeding. Many workers have detected and showed the presence of hormones, steroids, amino acids and amides as well as numerous digestive enzymes in the saliva of cecidozoans (Byers *et al*., 1976; Hori and Miller, 1977; Rohfritsch and Shorthouse, 1982; Dixon, 1983). The association of viruses with plants and their role in tumor formation has been well established. Parr (1940) reported that insect transmitting virus *Aureogenus magnivena* can produce tumors in plants. The virus like material (Walton, 1980) cecidogen present in the insect and gall tissue (Lewis and

Walton , 1964) is a crystal or granular-amorphous mass that multiply in the insects but not in the plants.

Wounding appeared indispensable to all types of plant tumors-viral, bacterial and genetically induced. The normal cells surrounding a wound are stimulated to intense mitotic activity and invade the wound site. When the wound is healed, the involved cells again become quiscent. Tumor appears to be uncontrolled wounding response, since the cell of crown-gall tumors rather closely resemble those in normal wound healing (Beardsley, 1972).

Decleene and Daley (1976) reported that at least 643 host plants from 331 genera were susceptible to crown-gall. Undoubtedly the molecular basis for this naturally occurring host range variations is not clear. The finding that the crown-gall tumor cells develop a capacity for autonomous growth as the result of the persistent activation of certain normally repressed biosynthetic system led to attempts to determine how these systems are regulated in the cells.

Plant-galls have been reported to contain higher levels of growth hormones (Purohit _et_ _al_., 1980; Tandon and Arya, 1980a; Weiler and Spanier, 1981; Braun, 1982; Pengelly and Meins, 1983; Dixon, 1983; Kado, 1984; Maden and Stone, 1984; Nester *et al*., 1984) which presumably contribute to the abnormal growth. Auxin requirement is the expression of the committed autonomous cells which may have an epigenetic basis (Braun, 1978). However, several factors in addition to auxin may mediate many of the exaggerated growth reponses characteristic of plant diseases (Bouckaert-Urban and Vendrig, 1981). The hyperauxinic habit in gall tissue has led to three major hypotheses: i) increased synthesis, ii) decreased destruction and iii) both increased synthesis and less destruction. Several workers are of the opinion that the transformation of normal plant cells to tumor cells is followed by rapid cell division with increase in amount of cell division substances and other metabolites essential for the maintenance of autonomous growth of the tumor cell (Stonier, 1972; Bouckaert-Urban and Vendrig, 1981; Amasino and Miller, 1982; Braun, 1982; Kado, 1984). It is not clear whether the cells responding to auxin are auxin deficient tumor cells that are promoted to grow as long as auxin is supplied or partially transformed cells whose complete conversion requires added auxin.

Crown-gall tissues, in contrast to normal plant callus tissue, can be grown *in vitro* in the absence of exogenous auxin or cytokinin (Gautheret, 1947; Braun, 1962) and it is now generally accepted that tumor formation is accompanied by a simplification of the nutrient requirements of the host cells (Butcher, 1973). Differential autonomous requirement of the nutrients vary from species to species, for instance, fully transformed crown-gall tissues of Vinca rosea became more autonomous as compared to normal tissue. Although the number of growth factors involved varies, the attainment of auxin independence apprears to be a consistent feature of tumor formation. The tumor tissue were shown to have an enhanced capacity for indole-3-acetic acid (IAA) synthesis either from tryptophan (Henderson and Bonner, 1952) or other indole precursor molecules (Nitsch, 1956).

The involvement of non-auxinic growth factor in crown-gall tumorigenesis was implied (Braun and Naf, 1954) when extract of crown-gall tumor tissues in combination with auxin caused much better growth of normal plant tissue than auxin alone. After the discovery of cytokinin it was found that auxin and cytokinin in the growth medium cause non-tumorous tissue to grow as rapidly as tumor tissue cultured on medium without phytohormones (Braun, 1958). It was concluded that crown-gall tumorigenesis results from th production of both auxin and cell-division factors (CDF) by tumor cells. Many workers have shown that crown-gall tumo tissues of various species contain virtually all of the known forms of naturally occurring cytokinins (Nakajima *et al.*, 1981; Einset, 1980; Weiler and Spanier, 1981; Amasino and Miller, 1982) which play a role in cell division. The growth hormone autonomy of crown-gall cells is apparently a result of the production of auxin and cytokinin by those cells.

Many plant tissues require cytokinin and auxin for continuous growth in culture on an otherwise complete medium (Street, 1969). It was recognised that auxin habituation occurs widely in cultured plant tissue and that similar changes are encountered for cytokinins and certain vitamins as well (Gautheret, 1955; Street, 1969). Stable habituated tissues can exhibit the same nutritional autonomy and lack of organizational capacity as crown-gall tumors in culture and in several cases of grafting of crown-gall to normal plants. Reversal of habituated tissues to auxin or cytokinin dependency also occurs in culture (Lutz, 1971). Binns and Meins (1973) showed that stable unorganized variants derived from cultured crown-gall teratoma tissues can form organized structures when grafted to normal plants. This indicated a favourable *in vitro* phenotypic change.

Comparison of the properties of habituated and crown-gall cells led to the conclusion that habituation is a form of neoplastic transformation involving heritable progressive change in cell phenotype that can result in autonomous growth. The significance of this conclusion lies in the fact that habituation occurs in the absence of a recognizable infective agent and appears to have an epigenetic basis (Meins, 1972). Physiological differences between habituated and crown-gall tissue cell reside in the mechanism of the turning off the IAA production (Pengelly, 1980). Plant tumors are also known to produce a complex substituted purine derivative (Wood, 1970). The fact that cytokinin habituation occurred in absence of any recognizable production of CDF an essential feature of tumor state, did not necessarily result from foreign genes introduced during tumor inception (Meins, 1974). All cytokinin stimulated cells at the time of division were thought to produce CDF. Plant cells exhibit the process of partial or complete habituation. The epigenetic changes were believed to be due to the partial or complete switching on of the endogenous genes and were also partially reversible (Meins, 1974). Although there is correlation between growth factor autotrophy and chromosomal constitution, it is unlikely that these changes provide a mechanism for habituation (Butcher, 1977). Plant cells serially propagated in culture usually undergo progressive heritable changes in gross chromosomal constitution (d'Amato, 1977). Habituated cells have higher nuclear deoxyribo-nucleic acid (DNA) content and presumably higher chromosome number than non-habituated cells (Don et al., 1974).

Studies on changes in ribo-nucleic acid (RNA) metabolism associated with cell expansion are usually complex with differential responses (Key and Shannon, 1964). The increase in nucleic acid content during tumor induction (Klein et al., 1953) after wounding (Kupila and Stein, 1961) and chromosomal abnormalities with changes in auxin level (d'Amato, 1977) in several plant species has been reported. Auxin induced increase in RNA synthesis in soybean (Guifoyle et al., 1975), formation of specific proteins in tobacco (Meyer et al., 1984), and the regulation of auxin and RNA in different species (Meyer et al., 1984; Walker et al., 1985) are well documented. All these evidences suggest that enhanced nuclear activity must regulate the growth response to exogenous auxin.

In *A. tumefaciens*, the process of transformation is associated with the presence of tumor inducing Ti-plasmid in the causative organism (Zaenen et al., 1979). Multiple copies of the part of the Ti-plasmid, are subsequently found in the transformed plants cells (Chilton et al., 1977). It has been shown that during the inception of crown-gall, the T-DNA sequences from the oncogenic Ti-plasmid are transferred to the nucleus of the normal plant cells, incorporated, maintained (Chilton *et al*., 1977; Schell *et al*., 1979; Willmitzer *et al*., 1980; Yadav *et al*., 1980) and transcribed (Drummond *et al*., 1977; Gurley *et al*., 1979) in the transformed cells. In addition to its oncogenic properties, T-DNA is essential for the maintenance of the tumorous state (Depicker *et al*., 1978; Holsters *et al*., 1980) and for the biosynthesis (Holtster *et al*., 1980) of a group of unusual plant metabolites called opines (Schell *et al*., 1979; Tempe and Goldman, 1982) which served as a source of energy and nutrient for the bacteria (Tempe and Petit, 1982). The extensive body of evidence indicated that auxin and cytokinin over production, is important for the maintenance of the transformed state. Several reports indicated that the phytohormone autotrophic growth of the transformed tissue of *A. tumefaciens* is due to T-DNA induced elevation of the endogenous level of auxin and cytokinin (Atsumi and Hayashi, 1978; Einset, 1980; Nakajima *et al*., 1981; Amasino and Miller, 1982). Elevation of endogenous levels of growth hormones in insect-galls has also been reported (Tandon and Arya, 1980a). On the other hand, it is also pointed out that the increased phytohormone levels are not the only factors that are involved in gall formation (Nakajima *et al.*, 1979; Weiler and Spanier, 1981).

The increased levels of IAA in diseased tissue have given rise to a question with regard to their origin. Transformed bacterial DNA encodes two genes involved in the pathway of auxin biosynthesis, thereby abolishing the requirement of exogenously supplied auxin for growth of transformed cells (Hertel *et al.*, 1983; Schröder *et al.*, 1984). The genes responsible for IAA biosynthesis from crown-gall cells have been isolated (Kado, 1984; Rausch *et al.*, 1985, 1986). Endogenous T-DNA directed synthesis of plant-hormones both auxin (Inzes *et al.*, 1984) and cytokinin (Barry *et al.*, 1984), is the basis for the homones autotrophic growth of crown-gall cells.

Totipotency in plant cells provides clue to reactions to investigate the reversal of tumorigenesis. In addition to its bearing on plant tumor experimentation, it provides information on the central mechanism that restrain and regulate growth of normal plant tissue. There are now a number of cases where phenotypic reversal of the tumorous state has been achieved (Sacristan and Melchers, 1977; Wullems *et al.*, 1980). Cells from an organized tumor line when fused to normal cells can become teratomas (Wullems *et al.*, 1980). The different morphologies in crown-gall tumor correspond to the pattern of growth observed by Skoog and Miller (1957) in normal tissue of tobacco by manipulating auxin and cytokinin levels. Similarly, the endogenously produced levels of auxin and cytokinin appear to control the morphology of crown-gall tumor (Amasino and Miller, 1982).

Differential biochemical metabolism of normal and gall tissue *in vitro* has been reviewed (Arya *et al.*, 1975; Kado, 1976; Lippincott and Lippincott, 1976; Shekhawat *et al.*, 1978; Braun, 1982; Rohfritsch and Shorthouse, 1982; Dixon, 1983; Tandon, 1985). There are two fundamental suggestions, firstly change in energy metabolism resulting in more permeability (Lippincott and Lippincott, 1975) and secondly the interaction and inter-dependence of metabolites and gene action affecting internal metabolic system resulting in abnormal growth. One of the most puzzling aspects of tumor problem generally over the years, has been, concerned with the multiplity of diverse physical, chemical and biological agents that can transform a normal cell into a tumor cell. All these agents lead to the same end result.

Metabolic control of disease reaction can occur by depression or promotion of genetic control of protein synthesis. Qualitative and quantitative variations in free amino acids and nitrogen constituents (Warick and Hildebrandt,

1966) and new unusual amino acids in crown-gall tissue (Lioret, 1957; Biemann et al., 1960) have been reported. The arginase activity was low in many plant tumors (Morel, 1971) and 'new' enzymes octopine dehydrogenase (Brinberg et al., 1983) and nopaline dehydrogenase (Otten and Schilperoort, 1978; Ondrej, 1983) were reported which led to the production of abnormal amino acid octopine and nopaline.

The metabolic pathways responsible for biosynthesis of aromatic amino acids and for vitamin-like derivatives is a major enzyme network in nature (Weiss and Edwards, 1980). In higher plants, this pathway plays even larger role since it is the source of precursors for numerous phenylpropanoid compounds, lignin, auxins, tanins and an enormous variety of other secondary metabolites (Stafford, 1974). Such secondary metabolites may originate from the amino acid end products or from intermediates in the pathway. The aromatic pathway interfaces with the carbohydrate metabolism at the reaction catalysed by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase the condensation of erythrose-4-phosphate and phosphoenol pyruvate to form the 7-carbon sugar DAHP. It has been assumed that the universally occurring aromatic amino acid in higher plants followed essentially the same pathway as in microorganisms

(Yoshida and Tower, 1963) and that the fundamental aromatic amino acids or intermediate aromatic compounds restricted to certain species might be derived secondarily from their biosynthesis through the action of phenylalanine ammonia lyase (PAL) and tyrosine ammonialyase (TAL). Lynda and Thorpe (1985) reported the presence of both PAL and TAL in tobacco callus tissues.

Phenolic compounds are widely distributed in plant kingdom (Harborne, 1980), and play a role in normal and abnormal plant growths (Kefeli and Kutacek, 1977; Lee *et al.*, 1982; Beart *et al.*, 1985; Kefeli, 1985; Rosenthal, 1986). Tissue of higher plants accumulate a wide variety of aromatic phenols which form the main group of naturally occurring inhibitors among plant products. The possible role of such compounds in plant growth regulation has been discussed (Kefeli and Kutacek, 1977).

Endogenous phenolic acids as well as exogeneously applied in the medium play a significant role in *in vitro* growth of tissue (Feucht and Schmid , 1980). Gene expression involving phenolics has been reported (Stachel *et al.*, 1985; Bolton *et al.*, 1986). The genetic control of anthocyanin biosynthesis in maize has been of great interest since it provided a model system for studying the gene action and gene regulatory mechanism (Reddy and Coe, 1986).

The role of oxidative enzymes and phenols in abnormal growth has been well documented (Frič, 1976; Bhansali et al., 1978; Purohit et al., 1979; Ramawat et al., 1979; Tandon and Arya, 1982; Tandon, 1985; Joshi and Tandon, 1984; Beart et al., 1985; Rosenthal, 1986). The participation of polyphenol oxidase (PPO) in IAA oxidation (Tandon and Arya, 1980b; Mayer and Harel, 1979; Lazarovits and Ward, 1982; Vaughan and Duke, 1984); the relationship between peroxidase (PRO), PPO and IAA-oxidase (Gove and Hoyle, 1975; Tandon and Arya, 1982); peroxidase function with IAA-oxidase (Frič, 1971; Hoyle, 1972), IAA oxidation by phenolics (Gelinas, 1973; Sirois and Miller, 1972); increase in phenolic acids with higher PPO and the factors involved (Kosuge, 1969; Tandon and Arya, 1982; Mayer and Harel, 1979; Flurley, 1986) in tumor development are well documented. The auxin protectors of phenolic nature prevent PRO or IAA oxidase catalized oxidation of IAA and alter auxin metabolism in tumor tissue (Atsumi and Hayashi, 1978; Haard, 1978; Tandon and Arya, 1980b). Extremely high levels of these substances are sufficient to explain the autonomy even when gall tissue contain more auxin destroying enzymes (Stonier, 1972).

Auxin and cytokinin regulate growth and morphogenesis in plant tissue cultures, induce the synthesis of specific polypeptides in cultured tissues (Bevan and Northcote, 1981; Wang et al., 1981; Meyer and Chartier, 1981) by altering enzyme activities (Kulaeva, 1980; Kaminek et al., 1981). Protein and isozyme patterns change in many enzyme systems (Joshi and Tandon, 1984; Vanloon, 1985; Eberman and Lick, 1986). The major net work of enzyme systems of the cells play a great role in the regulation and synthesis of primary and secondary products, amino acids, vitamins (Weiss and Edwards, 1980), etc. essentially required for each system of life and to maintain their equilibrium dynamic. From a phytochemical stand point plants are producers of chemicals and insects are consumers. Insect-and mite-incited plant galls offer an ideal system to study abnormal proliferation of plant cells in response to a stimulus. Though the crown-gall formation in plants has been an area of extensive research, the physiology and biochemistry of insect-and mite-incited galls has not received due attention by the researchers.

The present investigation deals with the following studies:

1) Isolation of both normal and gall tissues of *Schima khasiana* and *S. wallichi* in culture and studies of their growth factor requirements.

2) The assay of some enzymes of skikimic acid pathway and oxidative enzymes and the formation of phenolic

compounds as related to gall formation.

3) Aminoacids, protein, nucleic acids and metabolism of growth hormones in normal and gall tissues.

Understanding the facts underlying growth and development patterns covering normal and gall tissues will be of immense value in case of Schima an economically important tree species of the North-Eastern region of India.