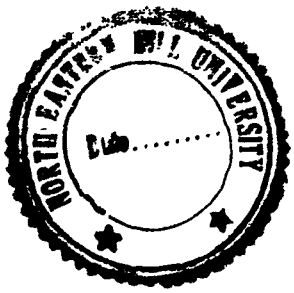
STUDIES ON SOME PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS OF GALL FORMATION IN SCHIMA



A.L.S RAJEE M.Sc

THESIS SUBMITTED IN FULFILMENT OF THE DEGREE OF

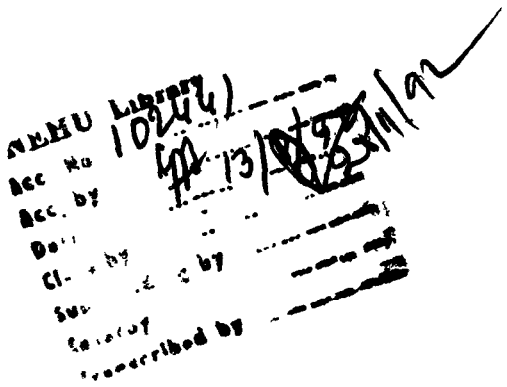
DOCTOR OF PHILOSOPHY IN BOTANY

To

THE NORTH-EASTERN HILL UNIVERSITY

SHILLONG, INDIA

AUGUST 1988



DEDICATED
TO
MY MOTHER

Cable : NEHU
Tel. Off. 23390
Res. 26049

North-Eastern Hill University

PRAMOD TANDON
Professor of Plant Physiology

DEPARTMENT OF BOTANY
SCHOOL OF LIFE SCIENCES
SHILLONG 793 014, INDIA

August 6,1988

I certify that the thesis entitled "**Studies on some physiological and biochemical aspects of gall formation in Schima**" 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.

Pramod Tandon
Suprevisor

Forwarded
6/8/88.
Head
Department of Botany
School of Life Sciences
N.E.H.U., Shillong-14

ACKNOWLEDGEMENTS

I wish to express my deep sense of gratitude to my teacher, Professor P. Tandon, Department of Botany, North-Eastern Hill University, Shillong, for not only supervising my work during the course of my investigation but also for rendering all kinds of necessary help.

I am grateful to Professor R.R. Mishra, Head of the Department of Botany, Professor M.K. Khare, Dean of the School of Life Sciences, the staff of the Library for providing me immense assistance within their capable authority.

I owe my sincere thanks, which really is difficult to express within this short space, to Dr. T.S. Rathore, Dr. S.C. Joshi, Dr. A.N. Verma, Dr. S.K. Sharma, Mr. E. Diengdoh and Mrs. S. Purkayastha, for their generous help in many ways during the preparation of the thesis.

I wish to acknowledge Mr. Godfrey Pathaw for typing the manuscript. I owe a good deal of thanks to my innumerable friends and colleagues within and outside the Department of Botany, for their encouragement and inspiration during the course of the work.

The Junior and Senior Research Fellowships from the University Grants' Commission is gratefully acknowledged.

Shillong,
July , 1988

A Rajee
(A.L.S. Rajee)

C O N T E N T S

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,

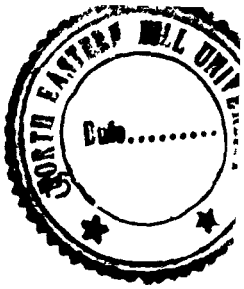
1966) and new unusual amino acids in crown-gall tissue (Lioret, 1957; Biemann et al., 1960) have been reported. The arginase activity was low in many plant tumors (Morel, 1971) and 'new' enzymes octopine dehydrogenase (Brinberg et al., 1983) and nopaline dehydrogenase (Otten and Schilperoort, 1978; Ondrej,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

Chapter II

Tissue culture of *Schima khasiana* and *S. wallichi* normal leaf and gall tissues : Studies of growth factor requirements

INTRODUCTION

One of the earliest applications of plant tissue culture with significant contribution to modern knowledge of plant cancer dealt with the study of plant tumor physiology. In addition to wound response, callus may develop in plant tissues following an invasion by certain microorganisms or by insect feeding. Tissue culture technique allowed callus formation in numerous plants, organs or tissues that do not usually develop callus in response to an injury (Street, 1969). The technique has not only supplemented coventional methods used in agriculture (Thorpe, 1981; Bhojwani and Razdan, 1983; Bajaj, 1986), forestry (Bonga and Durzan, 1982; Bajaj, 1986b) and breeding (Vasil et al., 1982; Dodds, 1985) but also as a model system for manipulating under closely controlled conditions, the physico-chemical, biochemical and molecular events that follow growth and differentiation of diseased plants (Sacristan and Melchers, 1977).

Trees exhibit chemical substances in free or bound forms associated with plant metabolic system which may be

considered as unwanted element for callus initiation (Monaco et al., 1977; Mulder-Krieger et al., 1982; Singh et al., 1982; Tisserat, 1984). On the other hand, a number of special considerations needed for callus initiation are selection of explant (Durzan and Chalupa, 1984), use of antioxidants (Murashige, 1974; Monaco et al., 1977; Tisserat, 1984), concentration of exogenous growth regulators (Davies, 1972; Shah et al., 1976; Singh et al., 1982), micro and macronutrients (Shah and Mehta, 1978; Mulder-Krieger et al., 1982) and selective physical conditions.

The development of gall involves factors that are released from the cells of the host surrounding the infected plant part. The insect feeding provokes several layers of highly nutritious cells to grow which enclose the insect in a sheltered chamber and become the insects sole source of food (Kant, 1967). Although there is little doubt that the insect provides the stimulus for gall formation, the factors involved and the mode of action of the stimulus is by no means clear. The insect acts through mechanical injury and may also apply cecidogenic active substances present in its saliva. Several chemicals, including auxin and other growth promoting substances have been detected in cecidozoan saliva (Boysen-Jensen, 1948).

The maintenance and regeneration into the whole plant (Wang *et al.*, 1981; Bonga and Durzan, 1982; Esenowo, 1986) and significant information associated with the technique and the factors involved in callus culture have been reviewed by many workers (Steward and Krikorian, 1979; Thorpe, 1981; Sharp *et al.*, 1984; Dodds, 1985; Bajaj, 1986a). The nutritional requirements for the initiation of callus vary considerably in primary explants of different organs. The majority of excised tissues require exogenous addition of growth factors in the medium in order to stimulate callus development (Yeoman and Macleod, 1977). Some tissues require both auxin and cytokinin whereas others only one of the two growth factors, and crown-gall neither of the growth factors (Gautheret, 1955; Braun, 1982; Meins, 1982). Synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) when provided at high concentrations replaced both auxin and CDF requirements of cultures (Witham, 1968).

Various tissues of insect-gall origin were isolated and studied *in vitro* (Hildebrandt, 1965; Arya ,1965; Kant, 1967; Rohfritsch, 1971; Tandon, 1976; Arora and Kant, 1980). Tumour formation is usually followed by simplification of the nutrient requirements (Butcher, 1973) and hormone independence (Braun, 1982). Crown-gall of sunflower showed autonomy for only auxin (refer Butcher, 1973). Growth

regulator independence has also been reported in habituated and genetic tumor tissues (Meins, 1982; Bayer, 1982).

Gautheret (1946) observed that a callus culture which originally required auxin in the medium for growth, after developing as out growth would survive indefinitely on medium lacking auxin. CDF habituated tissues when cultured on a medium containing high concentrations of kinetin do not require auxin. There is evidence that the autotrophic growth of the habituated cells results from changes in the production of specific growth factors rather than from a fundamental alteration in the cells requirement for these factors (Meins, 1982; Einset, 1985). Direct measurement showed that cells produce the growth factors for which they are habituated and in amounts sufficient to support the proliferation of non-habituated cells (Einset and Skoog, 1973; Pengelly and Meins, 1978; Pengelly, 1980).

Auxin is produced by actively growing regions of the plants (Sheldrake, 1973) and by proliferating non-habituated cells in culture. The proliferation of higher plant cells require the concerted action of specific growth factors. Differential requirement and competence to habituation may have an epigenetic basis in different type of tissues. During organogenesis some progeny of competents shift to a new stable state in which cells are unable to

respond to CDF by habituation (Meins and Lutz, 1980).

In spite of a great deal of research, the physiology of auxins in tumours is still unsolved. Various aspects of physiological differences in tumor cells with respect to normal cells have been well summarised (Braun, 1982). Higher amount of growth substances have been reported in crown-gall (Weiler and Spanier, 1981; Amasino and Miller, 1982; Kado, 1984; Nester et al., 1984) genetic tumours (Bayer, 1982); viral tumours (Black, 1982); fungus infected galls (Mahadevan, 1984) root nodules (Danger and Basu, 1984) mite and insect-incited galls (Purohit et al., 1980; Tandon and Arya, 1980a; Maden and Stone, 1984). However, the various manifestations caused by auxin even in normal growth are not clear. Braun (1962) has drawn attention to the similarities of the abnormal histological and cytological features observed in tumour tissues and those found in normal. He considered that these features can be accounted for in terms of abnormal growth hormone physiology which is characteristic of the tumour tissues.

Carbohydrates play a leading role in several metabolic processes of plants and are indispensable for tissue growth in vitro, because of their heterotrophic nature. The most preferred carbon source for most plant tissue cultures is sucrose. Glucose and fructose may be substituted in some

cases but most of the other sugars are poor carbohydrate sources for the plants. Various carbon sources such as mono-di-and poly-saccharides, sugar alcohols and organic acids have been used for a number of tissues grown *in vitro* (Ramawat and Arya, 1977; Tandon and Arya, 1979; Hu and Wang, 1986). Differences in the contents of starch, soluble sugars, carbohydrates, nitrogen, amino acid composition, aromatic amino-acids and steroids have also been shown between normal tissues and insect/mite-incited plant galls (Shekhawat *et al.*, 1978; Tandon and Arya, 1979; Rohfritsch and Shorthouse, 1982; Dixon, 1983).

MATERIALS AND METHODS

Schima belongs to the family ternstroemiaceae. Of the two species found in the North-East India, *S. wallichi* Choisy and *S. khasiana* Dyer (Fig. 1) are found at elevations of around 4,000 ft and 6,000 ft, respectively. Galling on these trees appear when the insect *Trioza* sp. (Homoptera) attack the leaf. The galls are present on very young to half mature leaf. Mani (1973) has described details regarding the distribution and type of galls associated with *Schima* spp. The leaf of *Schima* are heavily infested during the month of of May-June immediately after the leaf flush. The young galls that develop on the leaf of *S. khasiana* turn red at

Fig. 1. Schima khasiana (a) and S. wallichi (b) trees showing infected leaves.

Fig 1

(a)

(b)

maturity and attain size ranging from 4-6 cm and open up like flowers, whereas S. wallichi galls are green to yellow in colour and are found along the margin of the leaf and close at maturity. Infected leaves turn yellow-white with the loss of chlorophyll and clear diffusible matter tracts can be seen along the veins of the leaves.

i) **Tissue culture technique**

Corning glasswares were used for all experimentations. They were scrupulously cleansed with teepol a laboratory detergent and thoroughly washed with tap water. Further, they were rinsed 3-5 times with distilled water and dried in an oven at 100°C.

Either 0.1 N NaOH or 0.1 N HCl was used to adjust the pH of the medium at 5.7-5.8 and for gelling the medium 0.8% Difcobacto agar was used. About 15 ml of the medium was dispensed in the test tubes and 30 ml in 100 ml Erlenmeyer flasks and these were plugged with non-absorbent cotton and sterilized in an autoclave at 1.06 kg/sq cm. pressure for 20 min. The young leaves and galls were used as explants. After washing thoroughly with tap water, the explants were cut into 5-10 mm pieces in distilled water. The larvae present inside the galls were removed by a soft brush and washed repeatedly with distilled water. The tissues were

sterilized with 0.1% mercuric chloride solution for 5-6 min and washed at least ten times with sterilized double glass distilled water.

The explants were inoculated aseptically in test tubes containing modified Murashige and Skoog's (MS) medium (1962) in a laminar flow transfer table. The composition of the medium is given in Table 1. Before using, the working area of the transfer table was sprayed and thoroughly scrubbed with cotton soaked with ethanol (70% v/v) and sterilized by keeping the UV tube light on for 20 min. After exposure, transfer area was allowed to cool for at least 5 min for removal of ozone. Throughout inoculation, gentle flow of ultra-filtered sterile air of velocity 27±3 m/min across the working area was present in order to prevent air-borne contamination. The working parts of forceps, needles, surgical blades, etc. were sterilized by dipping in alcohol, flamed in a spirit lamp, cooled and returned to the jar containing alcohol. The rim of the test tubes were held in a flame provided by the spirit lamp during transfer of explants. After inoculation the test tubes were immediately plugged with cotton, covered with aluminium foil and kept again in UV light for 4-5 min. These were then transferred to a dark growth chamber held at 22°C temperature and 60-80% relative humidity.

Table 1. Murashige and Skoog's (MS) Medium (Modified)

Stock solution	Constituents	Conc. in stock solution (g/l)	Final conc. in medium (mg/l)
A	NH_4NO_3	82.5	1650.00
B	KNO_3	95.00	1900.00
C	H_3BO_3	1.24	6.20
	KH_2PO_4	34.00	170.00
	KI	0.166	0.83
	$Na_2MoO_4 \cdot 2H_2O$	0.05	0.25
	$CoCl_2 \cdot 6H_2O$	0.005	0.025
D	$CaCl_2 \cdot 2H_2O$	88.00	440.00
E	$MgSO_4 \cdot 7H_2O$	74.00	370.00
	$MnSO_4 \cdot 4H_2O$	4.46	22.30
	$ZnSO_4 \cdot 7H_2O$	1.72	8.60
	$CuSO_4 \cdot 5H_2O$	0.005	0.025
*F	Na_2EDTA	7.45	37.35
	$FeSO_4 \cdot 7H_2O$	5.57	27.85
G	Thiamine HCl	0.02	0.10
	Nicotinic acid	0.10	0.50
	Pyridoxine HCl	0.10	0.50
	Glycine	0.40	2.00

*$FeSO_4 \cdot 7H_2O$ was dissolved in approximately 200 ml double distilled water. Na_2EDTA was dissolved in approximately 200 ml double distilled water, heated and mixed (under continuous stirring) with $FeSO_4 \cdot 7H_2O$ solution. After cooling, the volume was adjusted to 1000 ml. Heating and stirring result in a more stable Fe-EDTA complex.

Addendum: Sucrose 30 g/l, myo-inositol 100 mg/l, agar 8 g/l, auxin and cytokinin (as mentioned in text). The stock solutions were prepared and stored in a refrigerator (for never more than 4-6 weeks) and mixed just before preparing the final volume.

The calli of about 1 cm in diameter grown over the explants were subcultured. Approximately 100 mg by fresh weight of calli were transferred to the fresh medium for callus proliferation.

ii) **Effect of antioxidants, other adjuvants and growth regulators** on callus initiation

Different concentrations of ascorbic acid (100 mg-800 mg/l), citric acid (100 mg/l) and polyvinyl pyrrolidone (PVP; 1000 and 2000 mg/l) were used separately for presoaking of the explants for 5 min to prevent their browning (Table 2). The presoaked explants were cultured on MS medium containing 2,4-D (2-10 mg/l) and kinetin (0.04 and 0.5 mg/l). 2,4-D,α-napthaleneacetic acid (NAA), indolepyruvic acid (IPA) and indolebutyric acid (IBA) were incorporated separately in concentrations ranging from 1-10 mg/l with either kinetin or benzylaminopurine (BAP) (0.04 and 0.5 mg/l) alone or their combinations in the medium for callus initiation. Activated charcoal (100 and 500 mg/l) was also incorporated in the medium and the explants used here were not treated with antioxidants. The cultures were incubated in dark.

iii) Effect of carbohydrates, strength of medium and growth regulators on callus tissue growth

Evaluation of the effects of carbohydrates, medium concentrations and growth regulators on callus growth were carried out. Different carbohydrates like sucrose, glucose, fructose and maltose in concentrations ranging from 10-60 g/l were incorporated separately in MS medium containing 2 mg/l NAA and 0.04 mg/l kinetin. Varying strengths of MS medium (1, 1/2, 1/4) containing 0.04 mg/l kinetin in conjunction with 2 and 8 mg/l of NAA were tried. 2,4-D, NAA, IAA and IBA (0.5-8 mg/l) were incorporated separately in MS medium along with kinetin (0.04 mg/l). Varying concentrations of kinetin 0.5-10 mg/l) in conjunction with NAA (2 mg/l) were also used in MS medium to study their influence on growth of both normal and gall tissues. These cultures were kept in fluorescent light of 3,000 lux.

For each experiment ten replicates (except for callus initiation) were taken and experiments were repeated thrice. The results are presented as mean with standard error (SE).

EXPERIMENTAL RESULTS

i) Effect of antioxidants, other adjuvants and growth regulators on callus initiation

Presoaking of explants in different solutions of antioxidants and PVP reduced the browning of the explants but

did not help in callus initiation (Table 2). The callus initiation did not occur on the explants cultured in MS medium containing activated charcoal.

Both normal and gall tissues of S. khasiana and S. wallichi showed callusing in 20-40 days time in MS medium containing 2,4-D (1-10 mg/l) in conjunction with kinetin alone or in combination with BAP (Table 3). However, better callusing occurred when 2,4-D (8-10 mg/l) was used along with kinetin (0.04 mg/l) alone. 2,4-D in conjunction with BAP did not result in callusing. While NAA (8 and 10 mg/l) with kinetin (0.04 mg/l) in the medium resulted in callusing on the explants (Table 4), both IAA and IBA alone or in combination with kinetin and BAP failed to do so.

The young gall tissue showed callusing from either its inner cut end or from its periphery whereas in normal young leaf it occurred from the midrib of the explants. The callusing was not observed in mature leaf and gall tissues. S. khasiana and S. wallichi normal and gall calli were compact and friable (Fig. 2). However, the gall tissues were more shining and translucent.

Table 2. Effect of presoaking of explants of Schima khasiana (SK) and S. wallichi (SW) in antioxidants and PVP on callus initiation

2,4-D mg/l	Kinetin mg/l 0.04		0.5		Antioxidants and PVP in one litre of distilled water	Remarks
	SK	SW	SK	SW		
2	+	+	+	+	100 mg ascorbic acid	Explant remained green but did not throw callus
2	+	+	+	+	200 mg ascorbic acid	- do -
2	+	+	+	+	400 mg ascorbic acid	- do -
2	+	+	+	+	800 mg ascorbic acid	- do -
4	+	+	-	-	100 mg ascorbic acid	- do -
4	+	+	-	-	400 mg ascorbic acid	- do -
8	+	+	-	-	100 mg ascorbic acid	- do -
8	+	+	-	-	200 mg ascorbic acid	- do -
10	+	+	+	+	100 mg citric acid	- do -
10	+	+	+	+	1000 and 2000 mg PVP	Explants as well as media turned brown

\+ tried
\- not tried.

Table 3. Effect of 2,4-D in conjunction with kinetin/BAP alone or their combinations on callus initiation of normal and gall tissues of *Schima khasiana* (SK) and *S. wallichi* (SW)

2,4-D mg/l	Kinetin mg/l				BAP mg/l				Kinetin + BAP mg/l				Remarks
	0.04		0.5		0.04		0.5		0.04		0.5		
	SK	SW	SK	SW	SK	SW	SK	SW	SK	SW	SK	SW	
1	-	-	+(10)	+(10)	-	-	-	-	+(5)	+(5)	+(5)	+(5)	Callus initiation but explant turned brown
2	-	-	+(15)	+(30)	-	-	-	-	+(10)	+(10)	+(10)	+(10)	- do -
4	+(50)	+(60)	+(50)	+(50)	-	-	-	-	+(30)	+(30)	+(35)	+(30)	Moderate callusing
8	+(80)	+(80)	+(70)	+(80)	-	-	-	-	+(60)	+(65)	+(40)	+(40)	Better callusing
10	+(80)	+(80)	+(60)	+(80)	-	-	-	-	+(60)	+(50)	+(60)	+(60)	- do -

- No response; + callusing

Figures in parenthesis represent the percentage of callus initiation on explants.

Table 4. Effect of NAA in conjunction with kinetin/BAP alone or their combinations on callus initiation of normal and gall tissues of *Schima khasiana* (SK) and *S. wallichi* (SW)

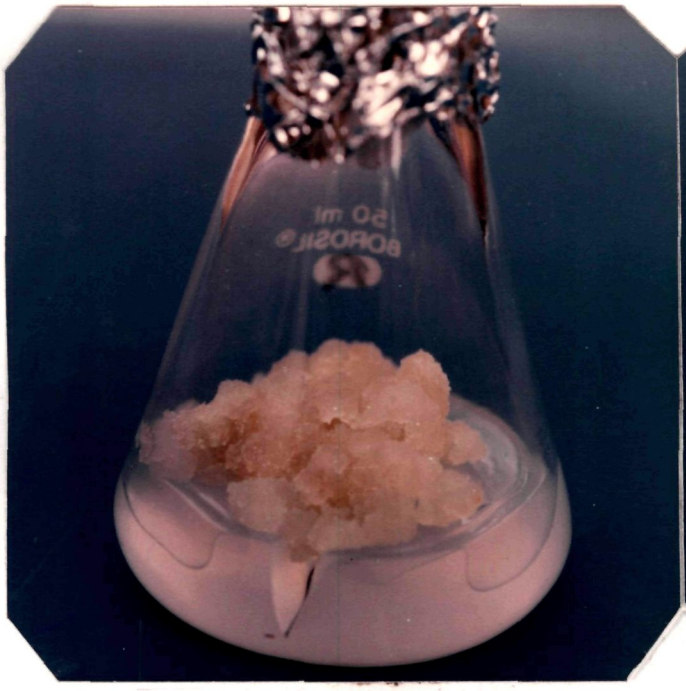
NAA mg/l	Kinetin mg/l				BAP mg/l				Kinetin + BAP mg/l				Remarks
	0.04		0.5		0.04		0.5		0.04		0.5		
	SK	SW	SK	SW	SK	SW	SK	SW	SK	SW	SK	SW	
1	-	-	-	-	-	-	-	-	-	-	-	-	Explants as well as media turned brown
2	-	-	-	-	-	-	-	-	-	-	-	-	- do -
4	-	-	-	-	-	-	-	-	-	-	-	-	- do -
8	+(15)	+(20)	+(30)	(40)	-	-	-	-	-	-	-	-	Callus initiation but explant turned brown
10	+(15)	+(20)	+(30)	(20)	-	-	-	-	-	-	-	-	- do-

- No response; + callusing

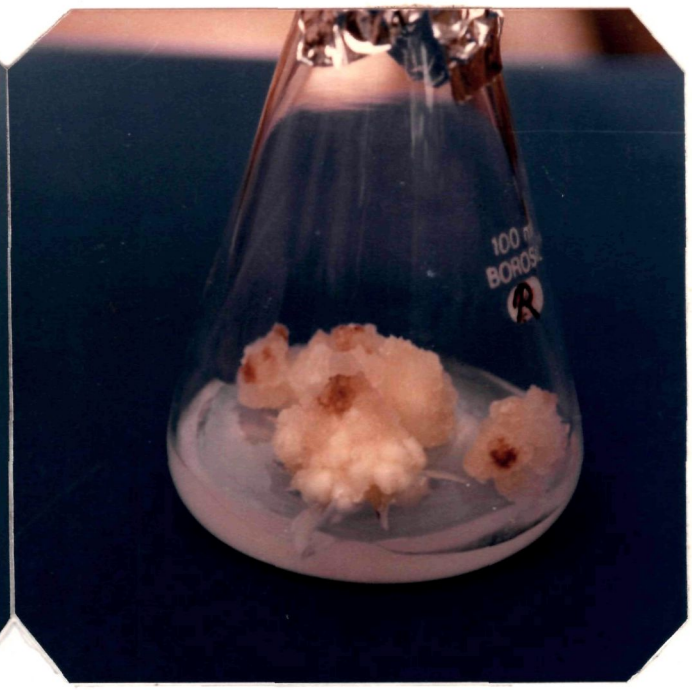
Figures in parenthesis represent the percentage of callus initiation on explants.

Fig. 2. Schima khasiana normal (1) and gall (2); S. wallichi normal (3) and gall (4) calli grown in MS medium.

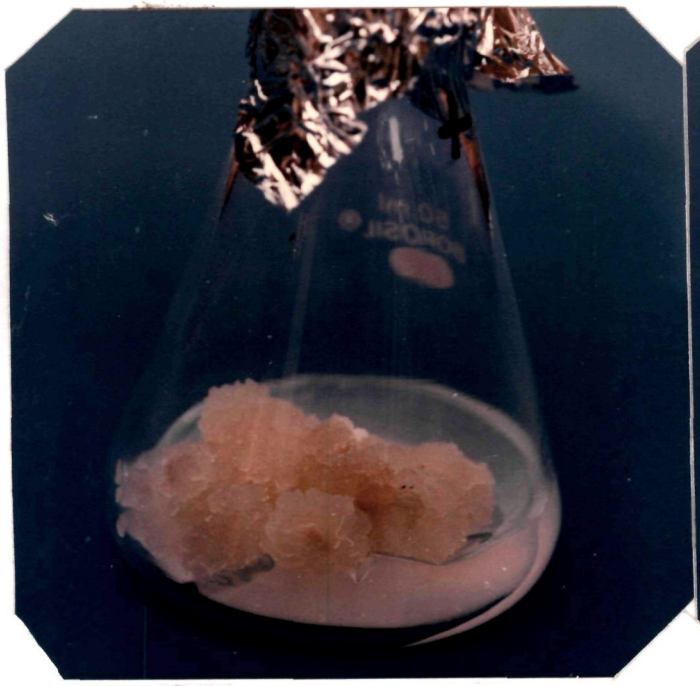
Fig 2



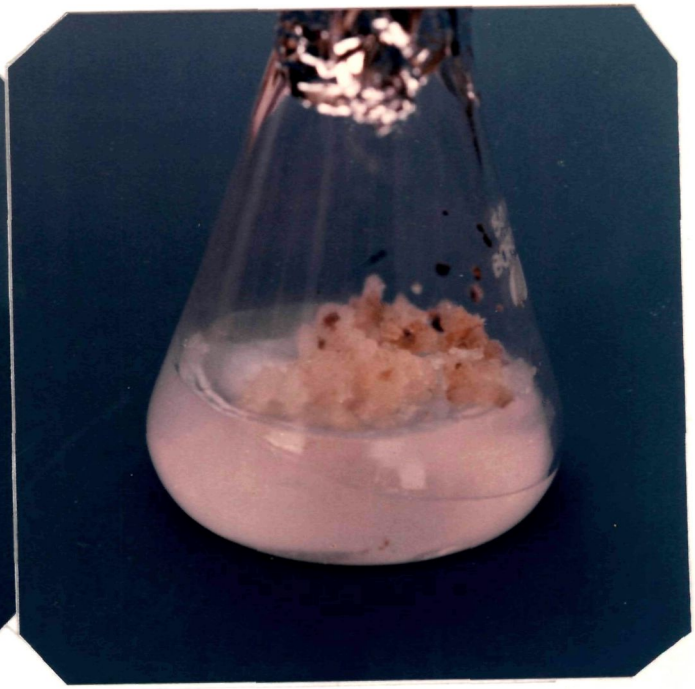
(1).



(2).



(3)



(4)

ii) Effect of carbohydrates, strength of MS medium and growth regulators on callus tissue growth

Of the various carbohydrates tested, sucrose at 30 g/l in the medium resulted in maximum callus weight of normal and gall tissues except normal tissue of S. wallichi where fresh weight further increased with higher concentrations of sucrose (Fig. 3). Fresh weights of the normal callus tissue in both the species was higher than corresponding gall. While using glucose in the medium, the optimum callus growth of normal and gall tissues was recorded in S. khasiana and S. wallichi at 30 g/l and 40 g/l of glucose, respectively, excepting normal leaf tissues of S. khasiana (Fig. 4). Fructose at 30 g/l in the medium resulted in maximum increase in callus weight of normal and gall tissues of both the species. The response was more pronounced in gall tissues as compared to normal (Fig. 5). Higher concentrations of fructose were found inhibitory for the callus growth. Maltose at 30 g/l in the medium exhibited maximum increase of callus fresh weight in normal and gall tissues of both the species except for S. khasiana normal tissue which showed maximum growth at 20 g/l (Fig. 6). Though at higher concentrations of maltose the fresh weight of normal and gall tissues of S. wallichi decreased, an increase in dry weight was recorded.

Fig. 3. Growth of *Schima khasiana* (A) and *S. wallichi* (B) normal (O) and gall (Δ) calli in response to different concentrations of sucrose in the medium.

Fig 3

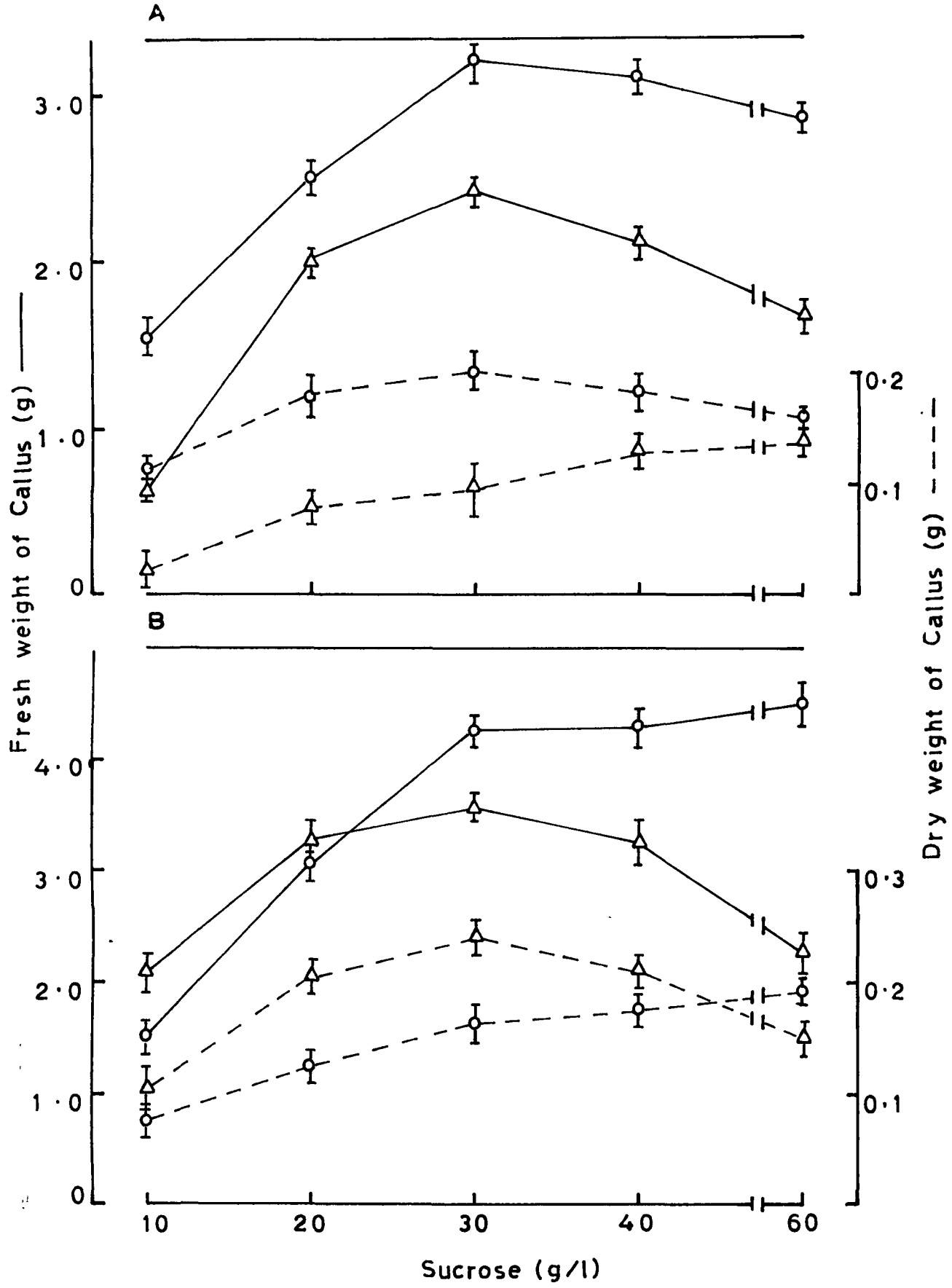

A
B
3·0
2·0
1·0
0
4·0
3·0
2·0
1·0
0
0·2
0·1
0·3
0·2
0·1
10
20
30
40
60
Sucrose (g/l)
Fresh weight of Callus (g) ——
Dry weight of Callus (g) – – –

Fig. 4. Growth of *Schima khasiana* (A) and *S. wallichi* (B) normal (O) and gall (Δ) calli in response to different concentrations of glucose in the medium.

Fig 4

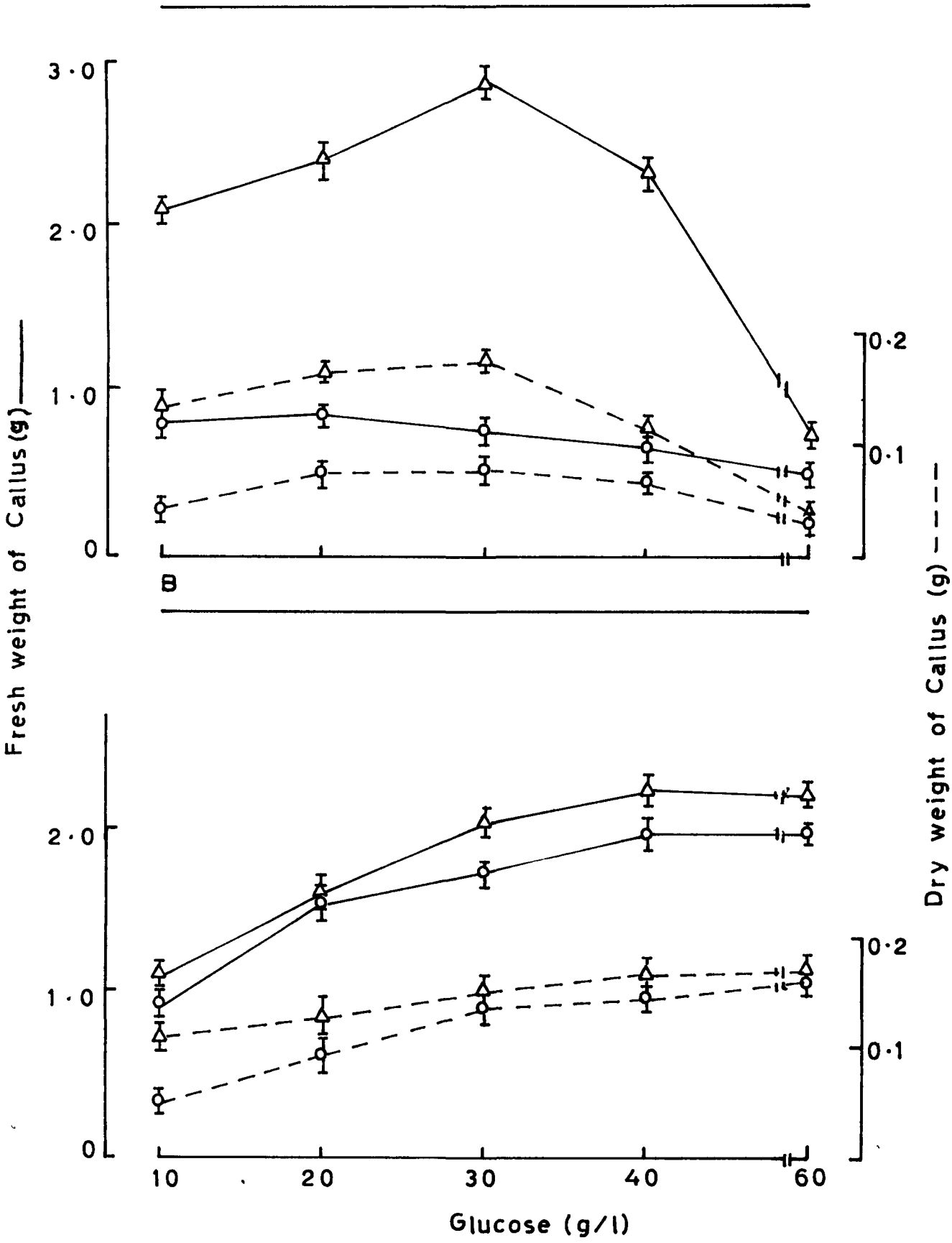

A
B
3·0
2·0
1·0
0
0·2
0·1
Fresh weight of Callus (g)
Dry weight of Callus (g)
10
20
30
40
60
Glucose (g/l)

Fig. 5. Growth of *Schima khasiana* (A) and *S. wallichi* (B) normal (O) and gall (Δ) calli in response to different concentrations of fructose.

Fig 5

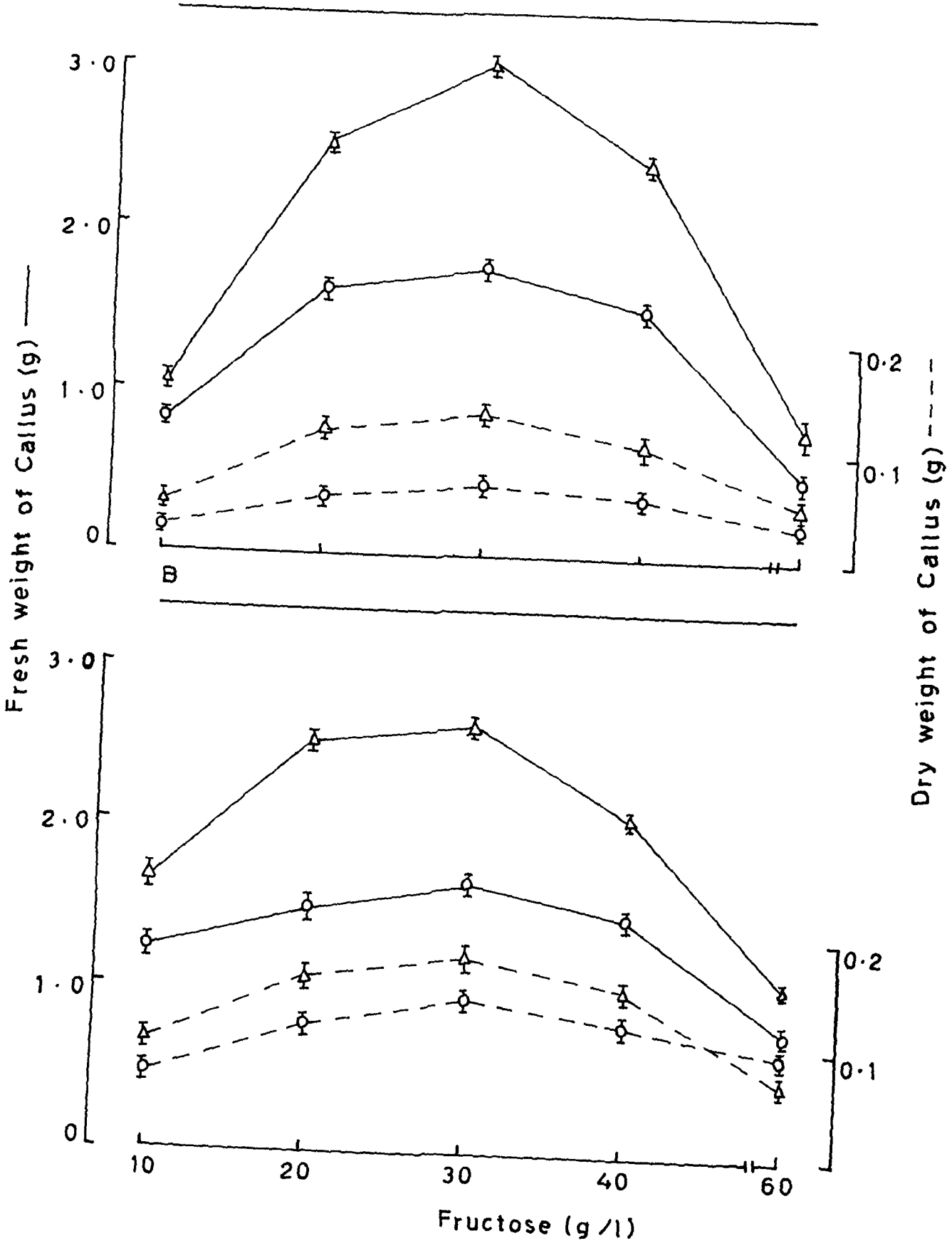
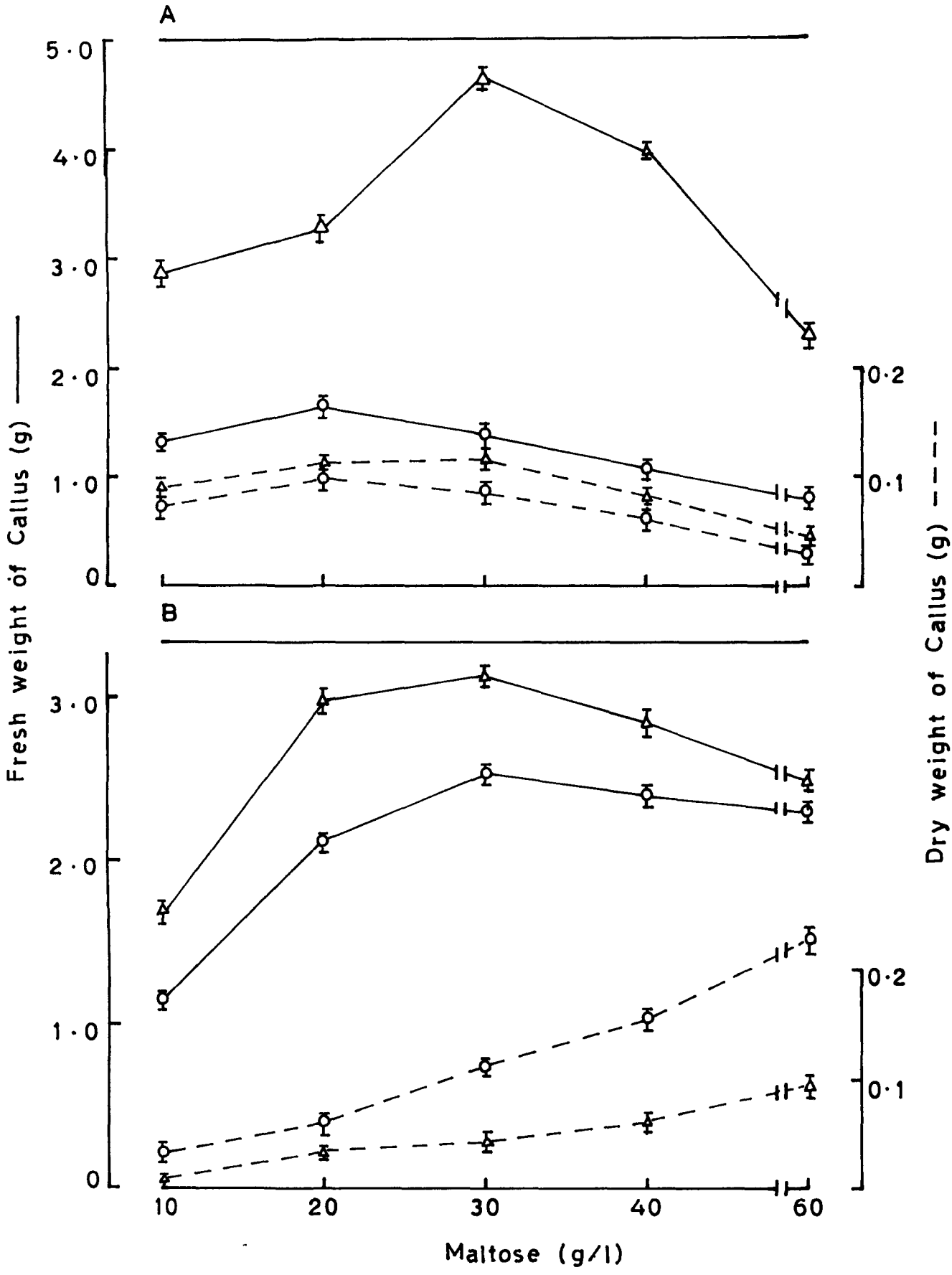

A
B
3·0
2·0
1·0
0
0·2
0·1
Fresh weight of Callus (g) ——
Dry weight of Callus (g) - - -
10
20
30
40
60
Fructose (g/l)

Fig. 6. Growth of Schima khasiana (A) and S. wallichi (B) normal (O) and gall (△) calli in response to different concentrations of maltose in the medium.

Fig 6


A
B
5·0
4·0
3·0
2·0
1·0
0
0·2
0·1
Fresh weight of Callus (g) ——
Dry weight of Callus (g) - - - -
10
20
30
40
60
Maltose (g/l)

By reducing the strength of the MS medium to 1/2 and 1/4 the normal and gall calli growth decreased in both the species (Figs. 7 and 8) except for the gall callus growth in media containing 8 mg/l NAA in conjunction with 0.04 mg/l kinetin.

The optimum growth of normal tissue of S. khasiana and S. wallichi resulted in MS medium containing 2,4-D at concentrations of 2 mg/l and 4 mg/l, respectively (Fig. 9). On the other hand, the gall tissue of S. khasiana and S. wallichi showed optimum growth in the medium containing 1 mg/l and 2 mg/l of 2,4-D, respectively. Higher concentrations of 2,4-D were inhibitory for the growth of both normal and gall calli. The best growth of normal tissue of both the species resulted in the medium containing 8 mg/l of NAA (Fig. 10). The growth of gall tissues of S. khasiana and S. wallichi were optimum in the media having 1 mg/l and 2 mg/l of NAA, respectively. Higher concentrations of NAA were found inhibitory for gall tissue growth. IAA and IBA served as poor sources of auxin for the growth of both normal and gall tissue (Figs. 11 and 12). 3 mg/l of kinetin in the medium resulted in optimum growth of normal tissue of both the species (Fig. 13). The optimum growth of all tissue of S. khasiana and S. wallichi, however, were recorded in media containing 4 mg/l and 5 mg/l of kinetin, respectively.

Fig. 7. Growth of Schima khasiana normal (O) and gall (Δ) calli in response to different concentrations of MS medium containing 2 mg/l (A) and 8 mg/l (B) of NAA.

Fig 7

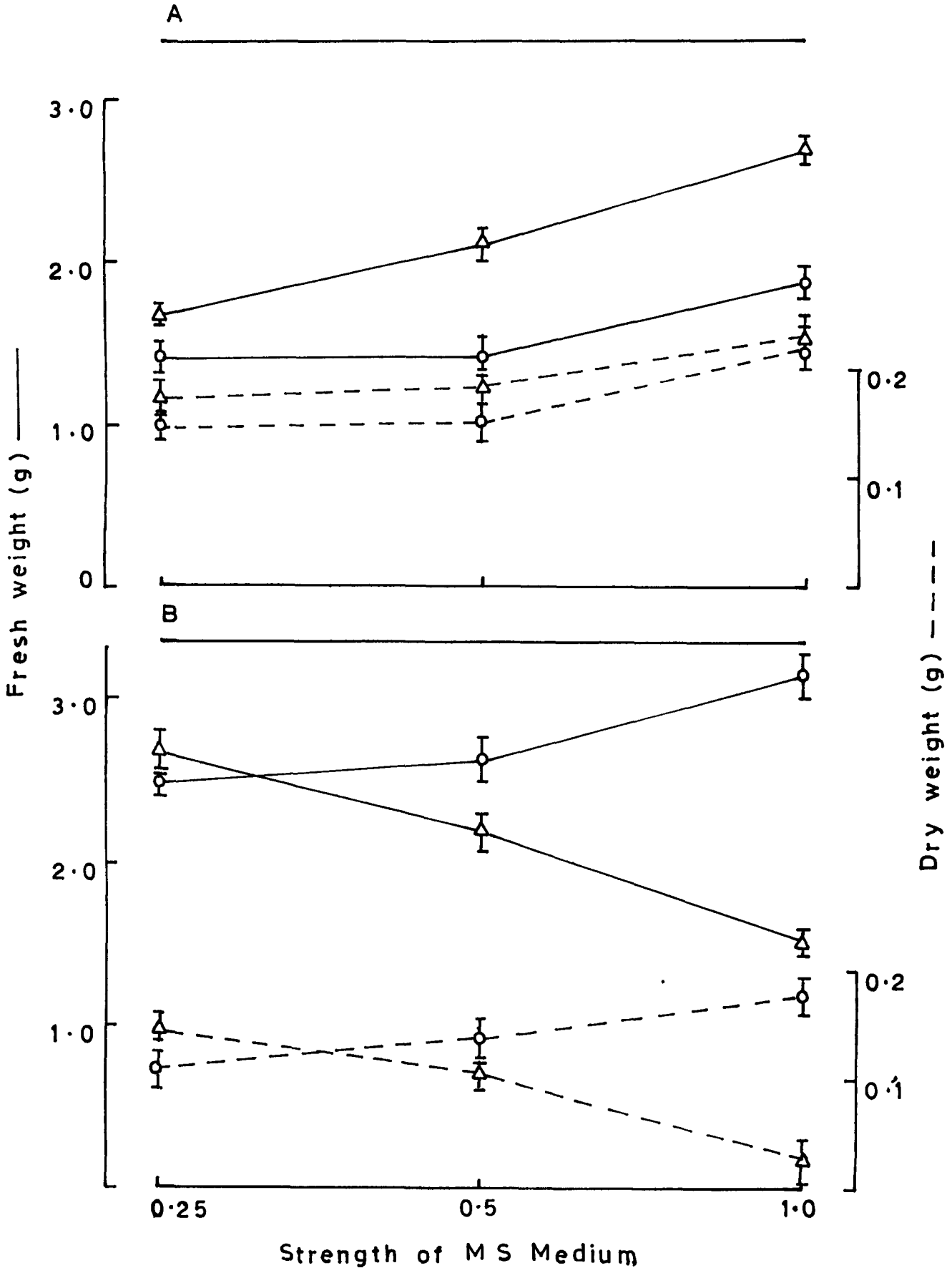

A
B
3·0
2·0
1·0
0
0·2
0·1
Fresh weight (g) ——
Dry weight (g) - - - -
0·25
0·5
1·0
Strength of M S Medium

Fig. 8. Growth of Schima wallichi normal (O) and gall (Δ) calli in response to different concentrations of MS medium containing 2 mg/l (A) and 8 mg/l (B) of NAA.

Fig 8

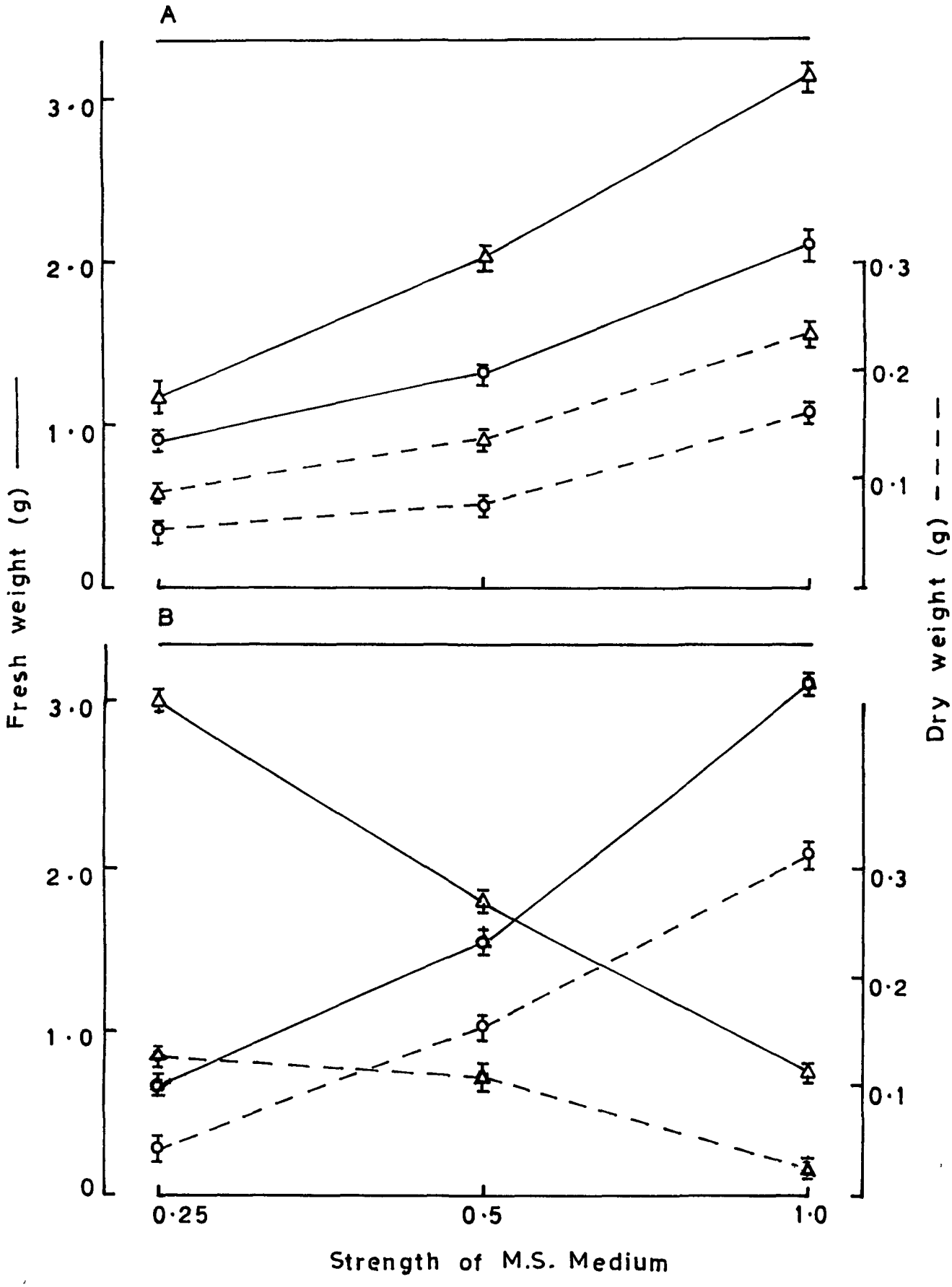

A
B
3·0
2·0
1·0
0
0·3
0·2
0·1
Fresh weight (g) ——
Dry weight (g) - - - -
0·25
0·5
1·0
Strength of M.S. Medium

Fig. 9. Effect of different concentrations of 2,4-D on fresh wt of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) calli grown on MS medium containing kinetin (0.04 mg/l)

Fig 9

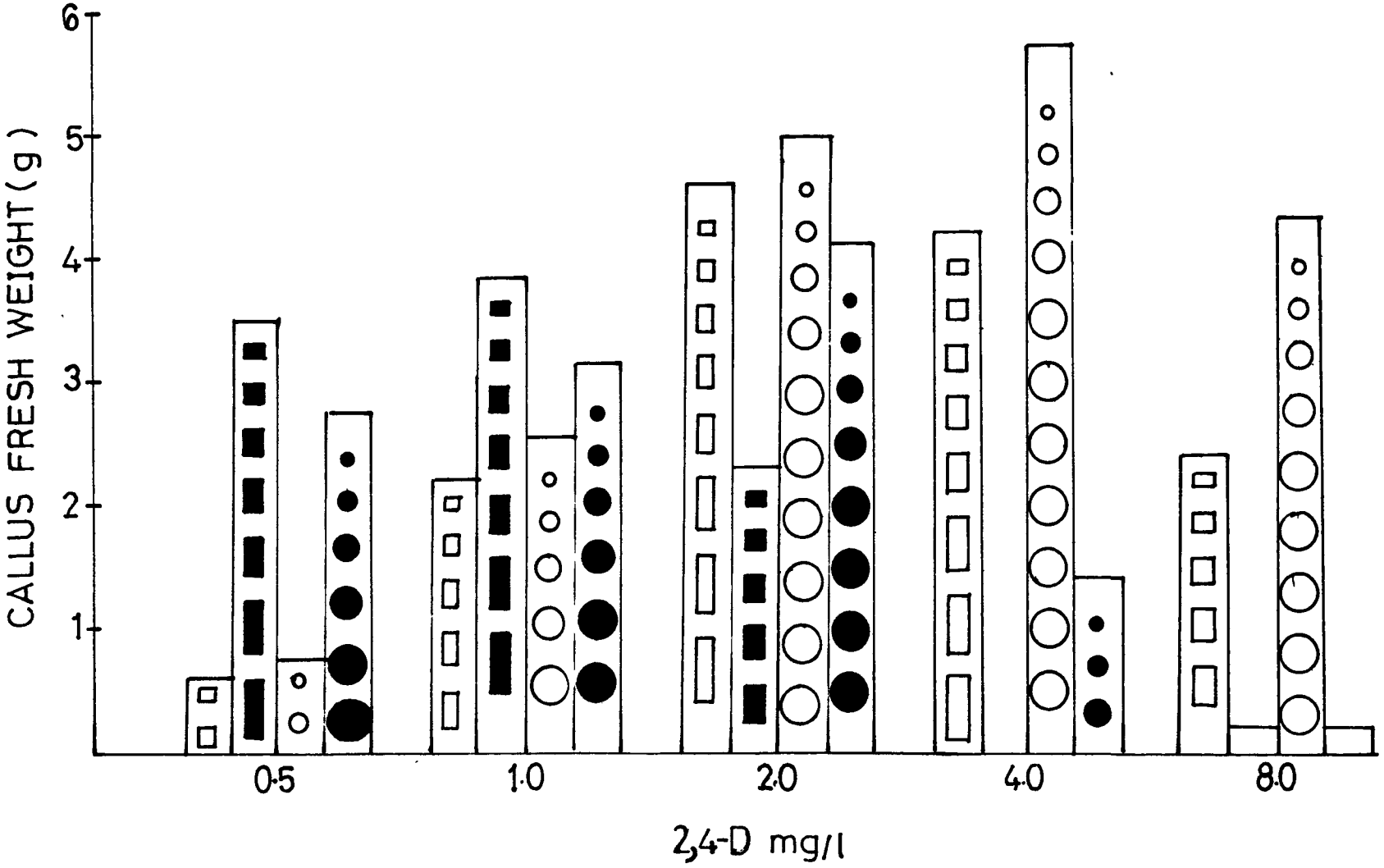


Fig. 10. Effect of different concentrations of NAA on fresh wt of *Schima khasiana* normal (□), and gall (■); *S. wallichi* normal (○) and gall (●) calli grown on MS medium containing kinetin (0.04 mg/l).

Fig 10

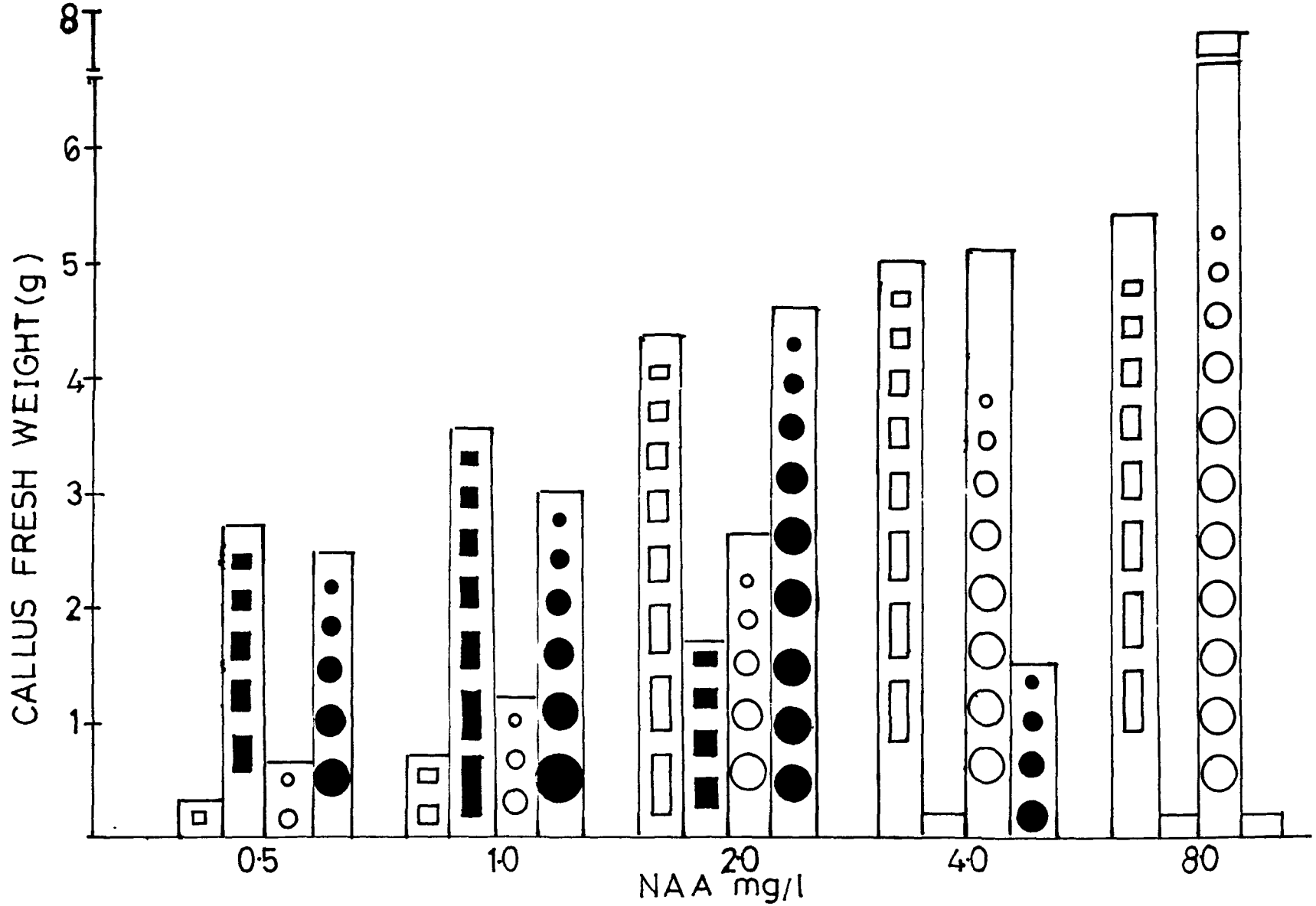


Fig. 11. Effect of different concentrations of IAA on fresh wt of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) calli grown on MS medium containing kinetin (0.04 mg/l).

Fig 11

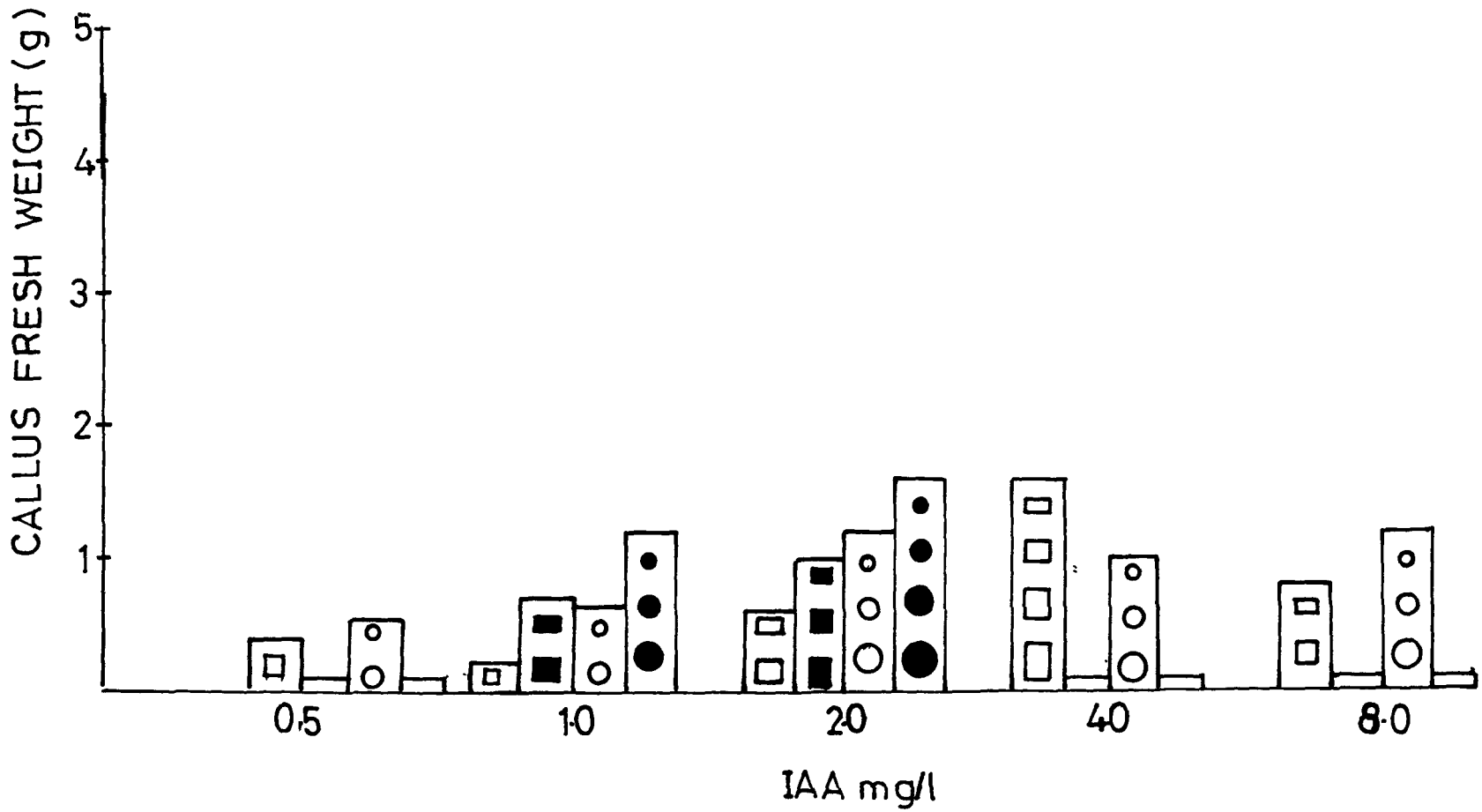


Fig. 12. Effect of different concentrations of IBA on fresh wt of Schima khasiana normal (□), and gall (■); S. wallichi normal (0) and gall (●) calli grown on MS medium containing kinetin (0.04 mg/l).

Fig 12

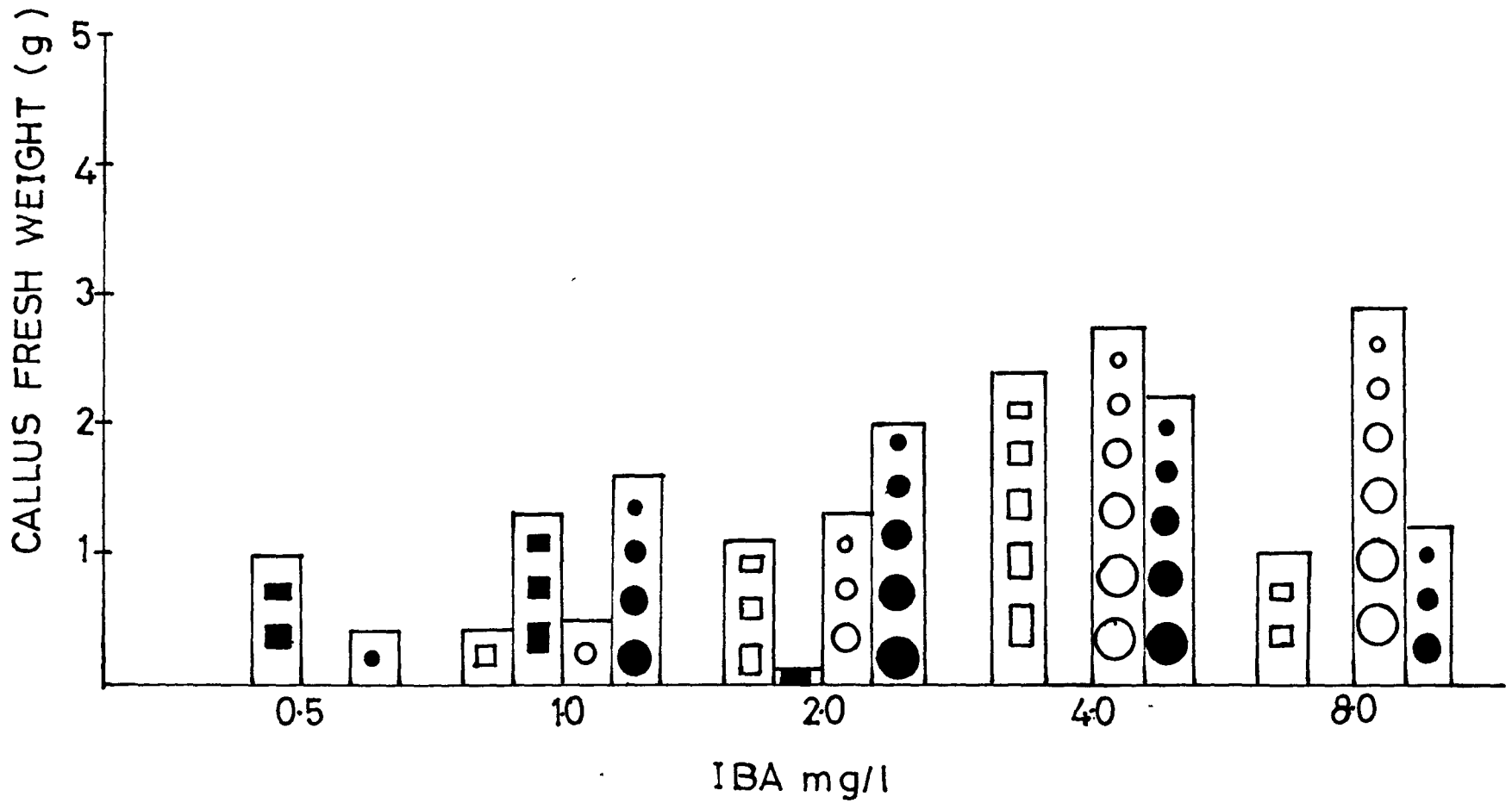
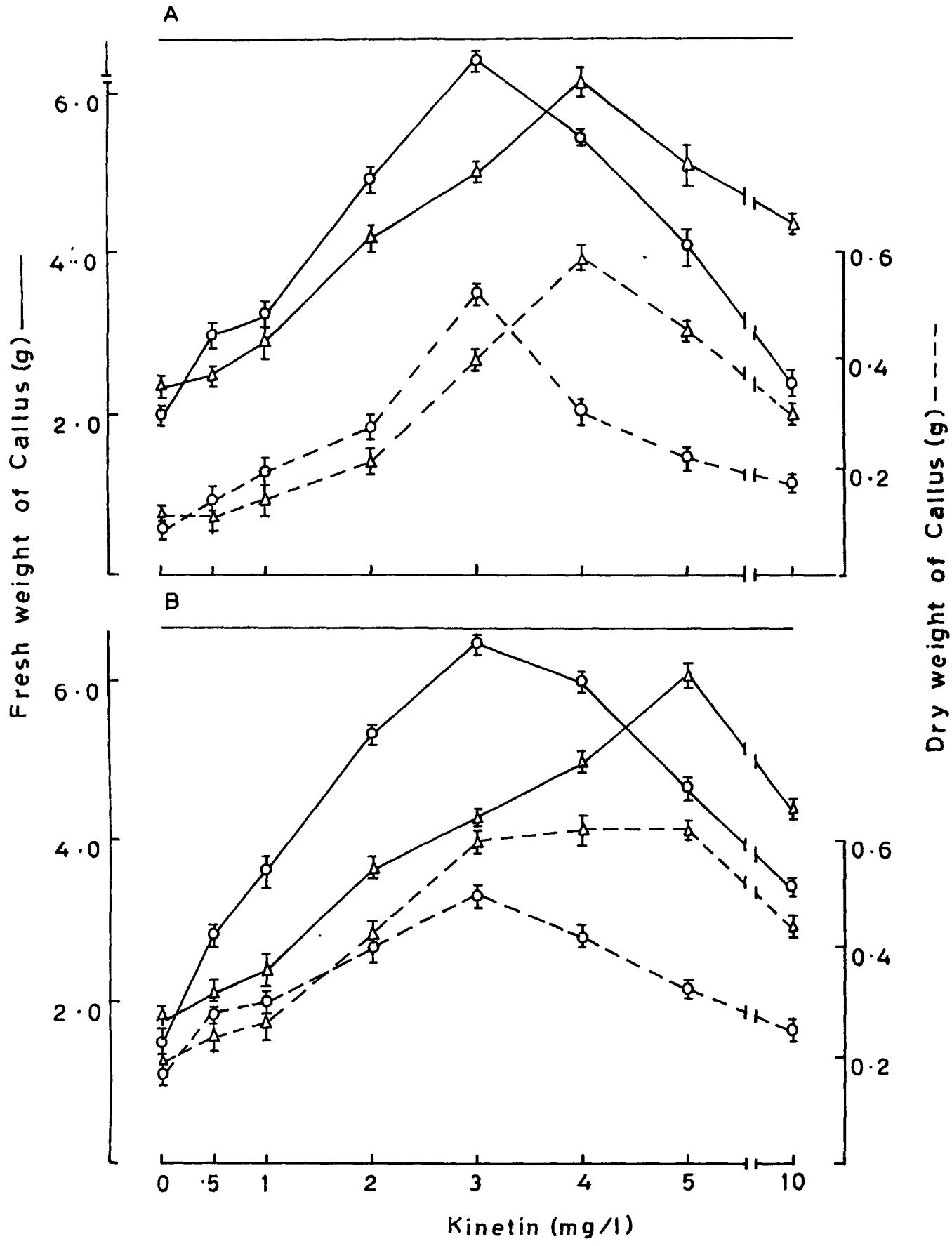


Fig. 13. Growth of *Schima khasiana* (A) and *S. wallichi* (B) normal (O) and gall (Δ) callus tissues in response to different concentrations of kinetin in the medium containing NAA (0.5 mg/l.)

Fig 13


A
B
6·0
4·0
2·0
0·6
0·4
0·2
Fresh weight of Callus (g)
Dry weight of Callus (g)
0
·5
1
2
3
4
5
10
Kinetin (mg/l)

DISCUSSION

Tissues and organs of most plants are susceptible to a variety of factors or stimulus resulting in pathological, abnormal or atypical growth. Many of the growths referred to as abnormal are characterised by extensive alterations and over growth of the affected area. An example of cecidia or gall induced by insects has long been considered as most complex of the abnormal plant growths.

All insect galls are initiated by tissue stimulating factor(s) supplied by the cecidozoan during feeding on the plant tissue. Insect galls are, therefore, a product of an interspecific association between a plant and an insect. In most abnormal growths, there are alterations in the mechanisms which regulate cell differentiation, tissue patterns and organogenesis.

How insect gall formers are able to control plant growth is far from understood. However, cecidogenetic hyperplasy and organization are thought to be determined by complex physico-chemical changes in the plant cells induced by behavioural and nutritional factors of the cecidozoan. Tissue differentiation in insect galls reveals all gradations of complexity, most are fundamentally histological differences from the normal organ.

The plant response to wounding or irritation is one of the reactivation or rejuvenation processes of the cells. The affected cells react with specific physiological, biochemical and histological processes (Rosenstock and Kahl, 1978). In vitro cultures of gall tissue require the same growth substances and other media constituents as do normal tissues (Arya et al., 1962; Rohfritsch, 1971). However, the nutritional requirements of crown-gall tissues differ fundamentally from those of the normal tissues. The normal and gall tissues of S. khasiana and S. wallichi showed differential response to auxin, cytokinin, carbohydrates, salt concentration etc. which signifies the ability of the gall tissues to metabolise differently. The acquisition of a capacity for unrestrained or autonomous growth within a host is the most important single characteristic feature of a plant or animal cell, since without this newly acquired property there would be no tumours. The plant tumour cell has been found to show autonomous growth as a result of the pesistent activation of certain normally repressed biosynthetic systems, the production of which are concerned with cell growth and division (Braun, 1982).

Many investigators have established a callus culture by transferring the plant meristems, organs or tissue fragments to various media (Bonga and Durzan, 1982; Sharp et al.,

1984; Esenowo, 1986). The growth and development of the plant tissues is controlled by the concerted interaction of various internal and external factors (Murashige, 1974) like auxin that control cell enlargement and chromosomal DNA replication, and cytokinins-cell division and physiological age (Tran Than Van, 1981; Zaerr and Mapes, 1982).

In the present investigation, callus isolation from normal and gall tissues of S. khasiana and S. wallichi was difficult due to browning of explants. Browning problem in tropical and subtropical woody plants has been noted in cultures like Eucalyptus (Conclave et al., 1977), Medinilla mangifera (Vande Casteele et al., 1981), coconut and other palms (Tisserat, 1984), coffee (Monaco et al., 1977), cinchona (Mulder-Krieger et al., 1982). In order to overcome and counteract browning of explants, number of antioxidants, L-cysteine, dithiothreitol, glutathione, β-mercaptoethanol (βME), ascorbic acid or ethyl-dithio-carbamate (Stonier, 1972; Monoco et al., 1977; Mulder-Krieger et al., 1982) and other substances like activated charcoal, PVP; dihydroxynaphthalene, dimethylsulphoxide, tyrosine, etc. (Reinert and White, 1956; Tisserat, 1984) have been used.

In the present findings, the use of different antioxidants and PVP for presoaking of explants prevented their browning. However, callusing could not be observed on these

explants (Table 2). The explants that were cultured on the medium containing activated charcoal also did not show callus initiation. The inhibition by activated charcoal (Friborg and Erikson, 1975) and PVP (Tisserat, 1984) on growth of callus cultures has been reported. The callusing occurred only on the explants (without presoaking) of young and half-mature leaf and gall tissues of S. khasiana and S. wallichi in media containing 2,4-D and NAA in conjunction with kinetin alone or in combination with BAP. It seems that the right combination and concentration of growth regulators was essential for callus isolation (Tables 3 and 4) rather than presoaking of the explants in solutions of antioxidants and PVP. The mature and old tissues of Schima spp. were recalcitrant and hard to culture. The problems encountered in callus initiation from mature explants from different plants have been discussed (Durzan and Chalupa, 1984; Thorpe and Biondi, 1984). Superiority of 2,4-D as the source of auxin for callus initiation has been reported in many plant species (Zaerr and Mapes, 1982; Esenowo, 1986). However, only higher concentrations of 2,4-D induced favourable proliferation of tissue in Holaulena autidysenterica and replacement of 2,4-D by IAA, NAA inhibited callus growth (Heble et al., 1976).

The proper/balances of growth regulators and the concentration of both micro- and macro-nutrients in the

medium are often critical for optimum growth of specific tissues. It is commonly observed that the explant and the media surrounding it turn brown and pH drifts to a lower concentration in improper combinations of the above, indicating that the tissue itself changes the pH of the medium (Hildebrandt, 1962).

Disease-tissue-clones as compared to normal ones have been reported to exhibit certain quantitative difference in their ability to use sugars (Arya et al., 1962). The normal and gall tissues of S. khasiana and S. wallichi showed differential growth response to different carbohydrates incorporated in the medium. While the gall tissue of both the species grew better in the medium supplemented with the maltose (Fig. 6), the normal tissues exhibited better growth in the medium containing sucrose (Fig. 3). With fructose in the medium, the growth of the tissues was moderate (Fig. 5). Glucose served as a poor source of carbon (Fig. 4). Ball (1963) while studying the effects of several sugars on normal Sequoia tissues found that fructose was the best carbon source followed by mannose, galactose and glucose. Sucrose has been found one of the best carbon sources which satisfied the requirement of most of the tissues in culture (Murashige and Skoog, 1962; Murashige, 1974).

It is also a known fact that plant tissues vary widely in their ability to utilise carbon sources. This variation in response of tissue is extended even to the behaviour of single cell clones of a particular plant tissue, and the different species of the same genus (Mathes et al., 1973; Coffin et al., 1976), individual cultivars of the same species (Chong and Taper, 1974), different organs of the same plant (Mathew et al., 1973) and even various clones derived from the same parent callus cultures (Arya et al., 1962, Sievert and Hildebrandt, 1965).

Plant tissue cultures have varied growth and nutritional requirements. Precise nature of such requirements is a prerequisite for nutritional and other studies of tissues in culture. The changes in the strength of MS salt have been reported to affect the tissue growth and differentiation in many plants. de Fossard (1978) observed better growth of callus and rooting in Eucalyptus ficifolia at lower concentration of nitrate in the medium. In the present investigation, the growth of both normal and gall tissues declined with the reduction in the strength of the MS medium (Figs. 7 and 8). However, use of higher concentrations of NAA in the media containing 1/2 and 1/4 strength of the medium resulted in better growth of the cultures.

The concentration of growth hormone in the medium help in predetermining the course of primary and secondary metabolism, like cell division, growth, differentiation, secondary product and cell hypertrophy (Street, 1969; Staba, 1977). A number of plant tissues *in vitro* synthesize hormones in sub-optimal amounts and, therefore, require addition of external phytohormones (Reinert and White, 1956; Arya, 1965; Horgan and Aitken, 1981). Variation of tissue reaction in response to the concentration of auxin was observed in many plant species. Both *S. khasiana* and *S. wallichi* showed better growth response when subcultured in optimal concentration of auxin (Figs. 9 and 10). However, with less concentrations of auxin and kinetin, the callus remained white over prolonged period with no appreciable growth. Palet *et al.*, (1960) reported that leaf gall callus grew in less amount of NAA and potassium in the medium. Growth of *Cassia fistula* callus tissue was slow when either too low or too high concentrations of 2,4-D were present in the medium (Shah *et al.*, 1976). A decrease in growth rate of hop callus was also recorded as the concentration of 2,4-D in the medium decreased (Itokawa *et al.*, 1980).

In the present study, the fresh weight of callus of both *S. khasiana* and *S. wallichi* normal tissues increased with increase in concentration of 2,4-D or NAA. A reverse

picture, however, was observed for the gall calli of both the species (Figs. 9 and 10). A relatively less increase in callus weight was recorded in the medium containing IAA and IBA (Figs. 11 and 12). In several instances it has been reported that the presence of higher amounts of 2,4-D help in better callus growth and prevent differentiation (de Fossard, 1976; Bhansali *et al.*, 1978). Murashige and Tucker (1969) observed variability of auxin requirements in higher group of plants. IAA and IBA alone do not favour callus growth and organogenesis (Bonga and Chakraborty, 1968; Lakshmi Sita *et al.*, 1979).

Tissue culture experiments have shown that exogenously applied cytokinin influence the auxin requirement of non-transformed tobacco callus tissues (Schmitz *et al.*, 1972), and grow without addition of auxin (Jordan and Skoog, 1971). In the present studies, it was observed that addition of kinetin to the medium at higher concentrations suppressed the requirement of auxin (Fig. 13). Kinetin (3-10 mg/l) with NAA (0.5 mg/l) in the medium resulted in sufficient growth of the callus in light. Syôno and Furuya (1972) demonstrated that cytokinin habituated tissues, which normally required auxin supplement can be forced to produce IAA by the addition of high levels of cytokinin in the medium. Thus inducing a plant metabolic system to form auxin enabling

the tissues to grow under an auxin autotrophic condition. One possible mechanism of habituation is the activation of host pathway for hormone synthesis. Several studies have demonstrated that habituated cells produce the expected growth factors (Meins, 1982). The relationship between cytokinin autotrophy and cytokinin production remains uncertain.

Chapter III

Studies on the enzymes of shikimic acid pathway, oxidative enzymes, and identification of phenolic compounds

INTRODUCTION

The building up of the end product of a biosynthetic pathway beyond a critical concentration acts as a signal that can slow down the rate of the reaction by which it is formed. It has become clear that certain key enzymes in the cell particularly those at the beginning of the reaction sequence or at a branch point in biological pathways function as regulatory enzymes, and are inhibited by the end product of the pathways.

The higher plants form many kinds of aromatic compounds such as aromatic amino acids, lignin, flavonoids, phenol glycosides, coumarins and alkaloids. Some of these, like the aromatic amino acids, are universally distributed in nature and others are found in a rather restricted number of plants. Higher plant cells convert amino acids into a wide variety of more complex aromatic compounds such as lignin, alkaloid and flavonoids, etc. The shikimic acid pathway is one of the longest and most complicated pathway so far established.

It is known that the metabolic activity of the cell is under genetic control and is influenced by environmental

factors. Changes in the biochemical and physiological responses of the host plants to pathogens usually reflex abnormal plant growth. Increased metabolic activities of the tissue is frequently connected with changes in the permeability of the cell wall or in the ultrastructure of the cell organelles (Wheeler and Hancheys, 1968). These processes change the compartmentalisation of substrates, often result in the concentration of the substrates, cofactors, inhibitors and metabolic stimulators which ultimately alter the metabolism of the affected plant part. Gall formation following infection by different pathogens brings about a number of changes both qualitative and quantitative at the level of growth hormones, enzymes and phenolics (Kado, 1976, 1984; Tandon, 1976; Purohit *et al.*, 1980; Kahl and Schell, 1982; Tandon and Arya, 1982; Dixon, 1983; Danger and Basu, 1984; Joshi and Tandon, 1984; Nester *et al.*, 1984; Joshi *et al.*, 1985).

During the last fifteen years the knowledge regarding "biochemical symptoms" of the diseased plants has increased considerably, especially in the area concerning oxidative enzymes. The role of oxidative enzymes in defence reactions has been tackled by many workers (Frič, 1976). The participation of phenol oxidase in IAA oxidation, host resistance and toxicity have been discussed in several

reviews (Pelet and Gasper, 1968; Kosuge, 1969; Mayer and Harel, 1979; Lazarowits and Ward, 1982; Vaughan and Duke, 1984). Phenol oxidase converts mono-phenol to diphenols to quinones, the last being toxic to pathogens. Higher PPO activity and phenol contents have been reported in crown-gall (Wegen and Glase, 1981), mite-incited stem gall in Zizyphus jujuba (Tandon and Arya, 1982) and leaf gall in Leea indica (Joshi and Tandon, 1984).

The phenylalanine and tyrosine biosynthetic pathways were first established in microorganism (Cotton and Gibbon, 1965). The same pathway of synthesis may operate in higher plants (Yoshida and Tower, 1963; Lynda and Thorpe, 1985). The biosynthesis of phenylpropanoids from phenylalanine and tyrosine is initiated through the action of ammonia lyase on phynylalanine (Koukol and Conn, 1961) and tyrosine (Neish, 1961). Since the discovery of phenylalanine ammonia lyase in higher plants, the physiological function of this enzyme has been the subject of extensive study (Camm and Tower, 1973). Much attention has been paid to its role in the secondary metabolism of plant phenolics. This enzyme catalyses the conversion of L-phenylalanine to transcinnamic acid and has an important role in the regulation of the general phenylpropanoid pathway (Hanson and Havir, 1981). On the other hand, TAL catalyses the

conversion of tyrosine to p-coumaric acid. Aromatic amino acids such as p-hydroxy-cinnamic acid, ferulic and salicylic acid were reported to act as plant growth inhibitors. These facts suggest a possible correlation between plant growth and PAL inhibitory activity. Dixon et al. (1980) were able to show modulation of the extractable PAL activity in the suspension cultures by feed-back and feed-forward effect of the pathway intermediates, possibly by the enzyme stabilisation. Shield et al. (1982) revealed the specific dual control of PAL activity over both production and removal of the enzyme by transcinnamic acid. Phenylalanine a ubiquitous aromatic aminoacid and its companion, tyrosine, serve as the initial substrates for many plant phenolics. They are utilized for the synthesis of the polypeptides, phenolics, flavonoids, lignin etc. The first step involving phenylalanine in most of these biosynthesis is catalysed by PAL.

The shikimic acid pathway involves not only phenylalanine and tyrosine synthesis but also the amino acid tryptophan, which, in addition to being a protein amino acid, is a precursor of number of secondary products. The role of oxidative enzymes and phenols in hyperauxinity and abnormal growth in many insect-incited galls are well studied (Bhansali et al., 1978; Purohit et al., 1979; Ramawat et al., 1979;

Tandon and Arya, 1982; Joshi and Tandon , 1984; Joshi et al.,, 1985)

The PPO (EC1.14.18.1) complex is perhaps best known for its diphenol oxidase activity demonstrated upon injury and when acqueous extracts are prepared from many higher plant tissues. In general, phenol oxidases catalyse the oxidation of phenolic substances with molecular oxygen. Phenol oxidase activity in higher plants are represented especially by two types of phenol oxidases (1) O-diphenol: oxygen-oxidoreductase (EC1.10.3.1). In general, it is known as the phenolase complex, as composed of two enzymes. One of the enzymes posses only catecholase activity, whereas the other shows cresolase and catecholase activity (2) p-diphenol: oxygen-oxidoreductase (EC 1.10.3.2). This enzyme known as laccase catalyses the aerobic oxidation of p-hydroxyquinone or p-phenylenediamines to p-quinones or p-quinonediamines.

Phenolase have differing substrate specificity that varies with plant species, organisation and ontogenetic state. The enzyme is often inactivated during the catalized oxidation of the diphenols. This inactivation or inhibition is generally attributed to the high chemical reactivity of the quinone produced by oxidation of dihydroxyphenol. Some

authors described the phenolase complex acting in plants as a hydroxylase, oxidising monophenol to O-diphenol. An increase of the phenolase activity in the host tissue, especially at and around the infection sites is a response which characterise a larger number of plant diseases. Taking into account the fact that biochemical symptoms of disease are determined not only by the genetical, physiological and biochemical characteristics of the host plants, but also by those of the parasites, the determination of the role of the phenolase within parasite-interaction is very difficult.

The Fe-porphyrin-enzyme peroxidase catalyses the oxidation of a wide variety of compounds peroxidatively with hydrogen peroxide (H_2O_2) or oxidatively with O_2 as oxidase. Peroxidase donor: hydrogen peroxide oxidoreductase (EC 1.11.1.7) generally catalyze, a redox reaction between H_2O_2 as electron acceptors and many kind of substrates (phenolic substances, aromatic amines, ascorbic acid; ferrycytochrome C, reduced nicotinamide adenine dinucleotide ($NADH_2$) etc.). Under certain circumstances, peroxidase also shows oxidase activity besides the peroxidase activity, i.e., it catalyzes the oxidation of different substances by atmospheric oxygen under aerobic conditions without exogenous peroxides, i.e. $NADH_2$, reduced nicotinamide adenine dinucleotide phosphate($NADPH_2$)

(Petrochenko and Kolesnikov, 1966), IAA (Platt et al., 1964), phenylpyruvate (Pelet and Gaspar, 1968; Jaynes et al., 1972).

Peroxidase, which is generally composed of a number of isozymes, is capable of catalysing several different reactions oxidatively. Peroxidase isozymes may differ in biochemical properties, such as specific activity, substrate affinity, cofactors, sensitivity to inhibitors, pH optima, etc. The reaction catalyzed by peroxidase may be influenced by the substances having either activator or inhibitor effects (Kosuge, 1969; Ku et al., 1970).

The role of peroxidase in plant resistance has been attributed to its ability to oxidise important metabolites either of the parasites or of the host plant, e.g., phenolics, enzymes, IAA, toxins, etc. (Farkas and Kiraly, 1962; Kosuge, 1969; Moustafa and Whittenbury, 1970). The increased peroxidase activity studied in connection with the oxidation of phenolic substances in the diseased plants and resistance in the host was attributed to the toxicity of these oxidation products.

Several malformations incited by plant pathogens have received attention in relation to the IAA oxidase activity. Hyperauxinity in root nodules (Banger and Basu,

1984), insect- and mite-incited plant galls (Purohit et al., 1980; Tandon and Arya, 1982) could be related with low IAA-oxidase activity. In many instances IAA-oxidase appeared to be of peroxidase function. A number of authors have reported that all peroxidase isozymes contained IAA-oxidase activity as well (Srivastava and van Hustee, 1973; Gove and Hoyle, 1975). A large variety of functions have been attributed to peroxidase in various biochemical pathways, for instance, relationship to IAA-oxidase and polyphenol oxidase (Pelet et al., 1970; Gove and Hoyle, 1975), as mediator in lignin synthesis, as index of ripening and senescence, biosynthesis of ethylene hormone balances, membrane integrity and respiration (ref. Vámos-vigyázó, 1981).

The distribution of growth inhibitors in plants has been well documented in literature. Out of a selection of 524 secondary plant constituents of known structures representing a range of structural types, 197 had singificant inhibitory activity. Much of the original work on this topic has been reviewed (Kefeli and Kadyrov, 1971; Thimann, 1972; Letham, 1978). Aromatic phenols form the main group of naturally occurring inhibitors among plants products. The possible role of such compounds in plant growth regulation has been discussed (Kefeli and

Kutacek, 1977; Cheng and Lynn, 1986; Stachel et al., 1985; 1986). Despite their frequency of occurrence, the origin and biological significance of phenols in higher plants is still obscure. It has been established that free phenols (catechol, resorcinol, caffeic, p-coumaric, p-hydroxybenzoic acid, etc.) could occur in living plant materials. These were discovered on many occasions as degradation product of low molecular weight materials. The presence of phenolic acids in the cell wall has been reported (Harris and Hartley, 1976; Cheng and Lynn, 1986). The cell-wall bound phenols may participate in resistance of plants against pathogens (Friend, 1979). Ferulic acid, and some other phenolics have been found to be cell wall components of higher plants (Kozlowska et al., 1983; Cheng and Lynn, 1986). There exists reasonable evidence, that not all natural phenolic compounds are growth inhibitors. Some of them possess stimulating properties and some are inert or participate in other processes such as respiration and photosynthesis (Kefeli and Kutacek, 1977).

The added phenolics are reported to have pronounced effects on in vitro enzyme system. Particular interest centers around the role of phenolic compounds in auxin metabolism (Lee et al., 1982; Kefeli, 1985). Various enzymes like IAA-oxidase (Felet, 1966; Kefeli, 1985), PPO (Robb et

al., 1966; Wong et al., 1981) and PRO (Lee et al., 1982) are influenced by these phenolics. Phenolics, both exogenously incorporated and synthesized by the cultures endogenously (Davies, 1972; Shah et al., 1976), regulate the growth of the in vitro grown tissues. Some investigators have proposed that IAA and other growth substances directly stimulate the production of phenolic compounds. Plant enzymes are also greatly influenced by growth hormones in both in vivo and in vitro tissue (Vernon and Straus, 1972; Barendse, 1983).

The naturally occurring IAA-oxidase inhibitors of phenolic nature range from low molecular weight compounds such as chlorogenic, ferulic and protocatechuic acids to the high molecular weight auxin protectors (Yoneda and Stonier, 1967; Novak and Galston, 1971; Atsumi and Hayashi, 1978; Tandon and Arya, 1980b,c). A common feature of the inhibition by these compounds is that they cause a delay in IAA-oxidation, after which the reaction resumes at the same rate as in the control (Lee et al., 1980).

The relationship between levels of auxins, oxidative enzymes and the phenolics compounds in both in vivo and in vitro gall and normal tissues may help in the understanding of the transformation process resulting in abnormal growth of gall tissues.

MATERIALS AND METHODS

i) Tissue extraction

Normal and gall tissues of both S. khasiana and S. wallichi were used for analyses. ~200 mg tissues were extracted at 4°C with 5 ml-prechilled 0.1 M phosphate buffer (pH 6.0-8.4), unless indicated otherwise.

Preparation of acetone powder

In vivo (1-2 cm pieces) and in vitro tissues were directly transferred to a blender containing prechilled acetone just enough to cover the tissue, and homogenized for 3-5 min at high speed. The homogenate was filtered through Buchner funnel using Whatman No.1 filter paper and washed at least 3 times with chilled acetone. The powdered residue was air dried for 1 hr, stored at 0°C in an air-tight capped container. This powder was used for enzyme assays.

ii) Analytical procedure

In vivo tissues

Assay of TAL and PAL

Fresh tissues or acetone powders were homogenized in a mortar with 5 ml each of different buffers separately (borate-HCl:pH 8.4; tris-HCl:pH 8.4 or phosphate:pH 8.4)

containing approximately 2 mM β-ME. The homogenates were then centrifuged at 15,000 g for 20 min. The supernatants were collected and passed through Sephadex G-150 column equilibrated with buffer to separate high molecular weight (mol. wt) peaks.

The method of Mahadevan and Sridhar (1982) was followed for measuring the amount of transcinnamic acid formed at 290 nm for PAL and the amount of p-coumarate formed at 333 nm for TAL using Hitachi Spectrophotometer Model 220.

To 1.0 ml of the above extract, 0.5 ml (0.05 M phenylalanine) and 2.5 ml of buffer were added. Zero time control and boiled enzymes without substrates were maintained as controls. After incubation of the mixture at 37°C for 1 hr the reaction was stopped by adding 0.1 ml of 5 N HCl. The acidified reaction mixture was then extracted twice with 6.0 ml e ther. The ether phase was pooled together in a 50 ml beaker and evaporated to dryness under a stream of air. The residue was dissolved in 5 ml of 0.05 N NaOH and the optical density was recorded. The reaction mixture used for assay of TAL was similar to PAL assay excepting the substrate which was replaced with tyrosine. The results are expressed as μg of cinnamic acid formed for PAL and p-coumaric acid for TAL/g fresh wt of the tissue/hr.

Assay of oxidative enzymes

Fresh tissue or acetone powders were homogenized separately with 5 ml prechilled sodium phosphate buffer- (pH 6.0). The homogenates were squeezed through double layers of cheese cloth and centrifuged at 18,000 g for 20 min. The supernatants were used for enzyme assays. The effects of the following additives on the extraction of oxidative enzymes were studied: (a) phenolic scavengers; phenol binding agent-PVP (0.1-0.5 g/g fresh wt of tissue) in phosphate buffer (b) anionic detergent:phosphate buffer with sodium dodecyl sulphate (SDS: 0.01-0.2 g/g fresh wt of tissue).

Peroxidase

The peroxidase (EC 1.11.1.7) activity was assayed by the method of Mahadevan (1974), where pyrogallol was used as a hydrogen donor. To a colorimetric tube, 2.5 ml pyrogallol (0.05 M in distilled water), and suitable amount of phosphate buffer and the enzyme extract were taken. The absorbance of the mixture was calibrated to zero. To this mixture, 0.5 ml H_2O_2 (1%) was added and the tube inverted immediately to mix the contents properly. The change in absorbance was recorded at 15 sec interval for 3 min at 420 nm. The enzyme activity is expressed as change in

absorbance/min/g fresh wt of tissue or acetone powder.

Polyphenol oxidase

The polyphenol oxidase (EC 1.10.3.1) activity was assayed using the method of Mahadevan and Sridhar (1982). In a 6 ml cuvette, 2.5 ml phosphate buffer and 0.5 ml catechol (0.1 M in 1N NaOH) were taken. The absorbance of the reaction mixture was calibrated to zero. Appropriate amount of the enzyme extract was added and the change in absorbance was recorded at 15 sec interval for 3 min at 420 nm. The activity is expressed as change in absorbance/min/g fresh wt of tissue or acetone powder.

IAA oxidase

The activity of this enzyme was measured by the method of Tandon and Arya (1982). In a reaction mixture, 1 ml 2,4-dichlorophenol (DCP), phosphate buffer, 1 ml manganese chloride, appropriate amount of enzyme, and lastly 1 ml IAA were added. The total volume of the reaction mixture was kept at 5 ml, and the final concentration of DCP, $MnCl_2$ and IAA were 0.2 mM. The reaction mixture was incubated at 37°C in a water bath. After 1 hr, 2 ml of Salkowaski reagent was added to each tube to terminate the reaction and the tubes were kept for cooling in dark. After 1 hr, the

absorbance was measured at 530 nm. The enzyme activity is expressed as mg IAA destroyed/g fresh wt of tissue or acetone powder/hr at 37°C using a standard curve prepared with IAA.

In all the experiments mean of standard error was taken and experiments were repeated thrice.

Estimation of phenols

The method of Mahadevan and Sridhar (1982) was used for phenol estimation. Two g each of the normal and gall tissue, were cut into 1-2 cm pieces and immediately plunged separately into 10 ml 96% boiling ethanol and boiled in a water bath for 8-10 min. After cooling, the tissue were crushed thoroughly in a mortar with pestle for 5-8 min and squeezed through cheese cloth. The extracts were collected in a 50 ml beaker. The residues were re-extracted with 3 ml 80% ethanol. Both the estracts were pooled together, filtered through Whatman No.1 filter paper. The final volume of the extract was made to 5 ml with 80% ethanol for every g of the tissue.

O-dihydroxy phenols

An appropriate amount of alcohol extract was diluted to 1 ml using distilled water. To this were added 2 ml of

0.05 N HCl, 1 ml of Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate dissolved in 100 ml distilled water; kept in amber-coloured bottle in the refrigerator), and 2 ml of 1 N NaOH. The final volume was made up to 10 ml with distilled water. The absorbance of the solution was recorded at 515 nm using a blank containing all the reagents minus Arnow's reagent. O-dihydroxy phenols were measured using a standard curve prepared with caffeic acid and are expressed as mg/g/fresh wt of tissue.

Total phenols

An appropriate amount of alcohol extract was diluted to 1 ml with distilled water and to this 1 ml of Folin-Phenol reagent (1 part diluted with 9 parts of distilled water) and 2 ml of 20% sodium carbonate (Na_2CO_3) solution were added. The reaction mixture was boiled exactly for a min and cooled. The final volume was made to 20 ml with the help of distilled water and intensity of blue colour was read at 650 nm. A blank containing all the reagents minus Folin-Phenol reagent, was used to adjust the absorbance to zero. The total phenols are expressed as mg/g/fresh wt of tissue using a standard curve prepared with catechol.

In vitro tissue

Both normal and gall calli grown in media containing 2.0 mg/l of different auxins (2,4-D, NAA, IAA, IBA) separately in conjunction with 0.04 mg/l kinetin were anlaysed. NAA grown calli served as the control. The oxidative enzymes and phenolic contents were estimated by the same procedures as mentioned above for *in vivo* tissues.

Identification of phenolic acid

100 g each of normal and gall tissue were chopped into 1-2 cm pieces and plunged into 500 ml of boiling 80% ethanol for 10 min. After cooling the tissue were transferred to a blendor, and blended for 10 min at high speed The homogenate was filtered and the residue was washed again with 100 ml 80% ethanol and filtered. Both the filtrates were pooled together and the alcohol was evaporated at 40°C. The acqueous fraction was divided into two parts. One part was hydrolysed over night in cold atmosphere with equal volume of 2 N NaOH. The other fraction was hydrolysed with equal volume of 2 N HCl for 1 hr in steam bath. Acid hydrolysates were directly extracted with ether for 6 hrs. Alkaline hydrolysates were acidified to

pH 2 with HCl. Both the extracts of normal and gall tissues were separately extracted with ether. While the ether extract was washed with 5% Na_2CO_3, the alkaline extract was acidified and re-extracted with ether. The final ether extract was taken to dryness, and the residue was dissolved in a small volume of ethanol for chromatography.

Chromatography on Whatman NO 1 filter paper was done in different solvents like - benzene: acetic acid: water-6: 1: 3 upper phase (Ibrahim and Tower, 1960); butanol: acetic acid: water-4: 1: 1 (Mahadevan, and Gridhar 1982); isopropanol: ammonia: water-10: 1: 1 (Mendez and Brown, 1971). The Rf values were determined for each phenol against a standard using the same solvent. The method of Bray *et al.* (1950) was used to compare the colour reaction of Rf value of unknown and standard phenols on the chromatogram employing diazotised p-nitroaniline and diazotised sulphanillic acid as spray reagents.

The remainder of the solvent extract was applied in a narrow band on Whatman NO 3 paper and separated with benzene: acetic acid: water solvent described above. The resulting bands were cut and the respective phenols eluted with 100% ethanol in separate test tubes. The aqueous

extract was poured off and centrifuged at 10,000 g for 30 min. The absorbance of the supernatant solutions containing the unknown phenols was determined and their wave length of maximum absorbance was compared with the absorbance of standard phenols using Beckman Recording Spectrophotometer Model Hitachi 220 under UV range from 210-360 nm.

Effect of different phenolics on the growth of normal and gall calli

Different phenolic like ferulic, caffeic, protocatechuic and p-coumaric acids, L-tyrosine and phloroglucinol at different molarities (1,2,4 and 6 mM) were incorporated in the medium containing 2 mg/l NAA and 0.04 mg/l kinetin to study their role on callus tissue growth. The wet and dry weights were taken as indices of growth.

Effect of different phenolics on IAA-oxidase

Different phenolics like ferulic, caffeic, chlorogenic, protocatechuic, p-coumaric and shikimic acids and L-tyrosine and phloroglucinol in different molarities (1,2,4and 10 mM) were added into the incubation mixture to study their influence on IAA-oxidase activity.

EXPERIMENTAL RESULTS

In vivo tissue

Assay of PAL and TAL

The activities of PAL and TAL were higher in gall tissues as compared to normal in both S. khasiana and S. wallichi (Table 5). Both normal and gall tissues of S. khasiana exhibited higher PAL and TAL activities as compared to respective tissues of S. wallichi.

Oxidative enzymes

Of the two additives incorporated in the extraction buffer for the assay of PPO and PRO activities in S. khasiana, SDS was found better than PVP (Figs. 14 and 15). The optimum activities of PPO and PRO were recorded in extracts prepared in extraction media containing 0.1 g of both SDS and PVP/g fresh wt. However, use of higher concentration resulted in the decrease in the enzyme activities. Using acetone powder, the normal tissue of both S. khasiana and S. wallichi showed higher IAA oxidase and PRO activities as compared to gall tissues. However, a reversed picture was obtained for PPO activity. Both o-dihydroxy and total phenol contents were higher in gall tissues of S. khasiana and S. wallichi as compared to corresponding normal tissues (Table 6).

Table 5. Phenylalanine ammonia lyase and tyrosine ammonia lyase activities in both normal and gall tissues of S. khasiana and S. wallichi

Tissue	Phenylalanine ammonia lyase (µg cinnamic acid/g fresh wt. of tissue/hr)		Tyrosine ammonia lyase (µg p-coumaric acid/g fresh wt. of tissue/hr)	
	290 nM	650 nM	333 nM	650 nM
S. khasiana				
Normal	18.0 ± 2.2	8.6 ± 1.0	7.6 ± 1.3	7.5 ± 1.2
Gall	31.4 ± 3.3	42.6 ± 4.7	12.2 ± 1.4	38.8 ± 4.1
S. wallichi				
Normal	13.0 ± 1.8	5.1 ± 0.4	8.2 ± 1.1	6.6 ± 1.0
Gall	26.2 ± 2.4	33.2 ± 6.6	10.4 ± 0.6	23.3 ± 3.4

± S.E.

Fig. 14 : Effect of PVP concentration on polyphenol oxidase (----) and peroxidase (——) activities in *in vivo* *S. khasiana* normal normal (O) and gall (Δ) tissues.

Fig. 15 : Effect of SDS concentration on polyphenol oxidase (----) and peroxidase (——) activities in *in vivo* *S. khasiana* normal (O) and gall (Δ) tissues.

Fig 14

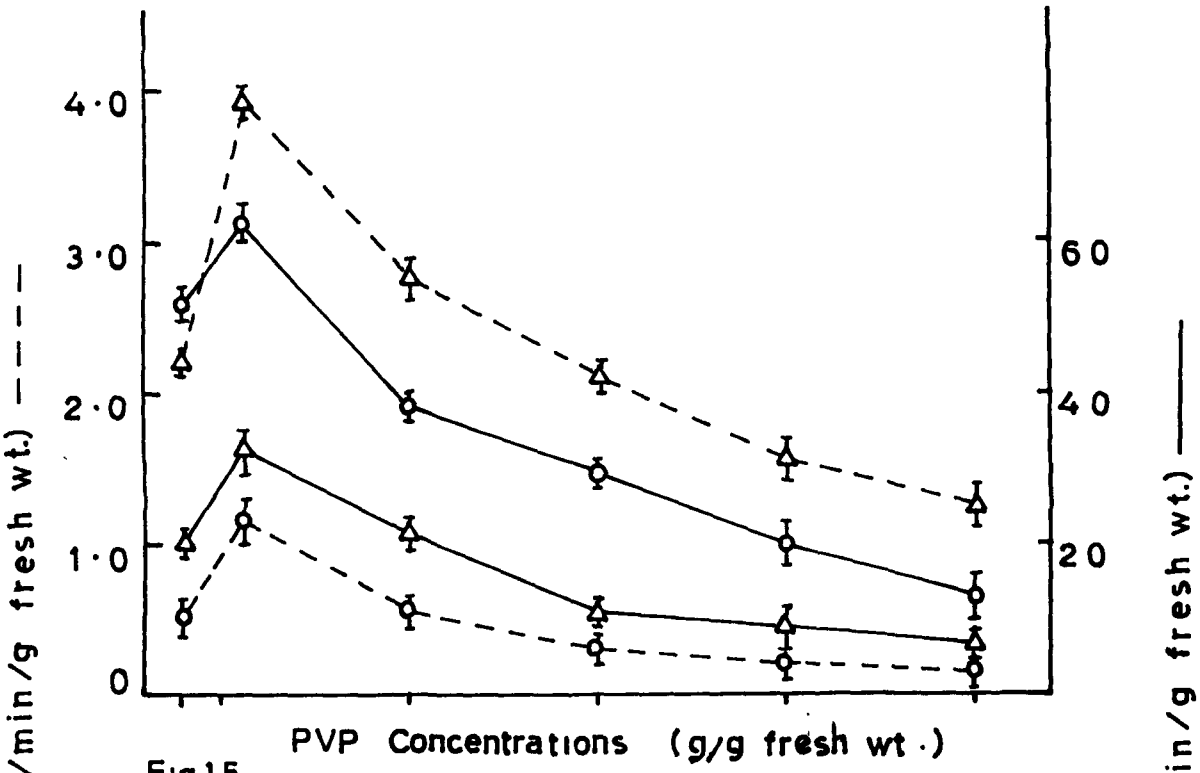

4·0
3·0
2·0
1·0
0
60
40
20
PVP Concentrations (g/g fresh wt·)
Polyphenol oxidase (ΔA/min/g fresh wt.) - - - -
Peroxidae (ΔA/min/g fresh wt.) ——

Fig15

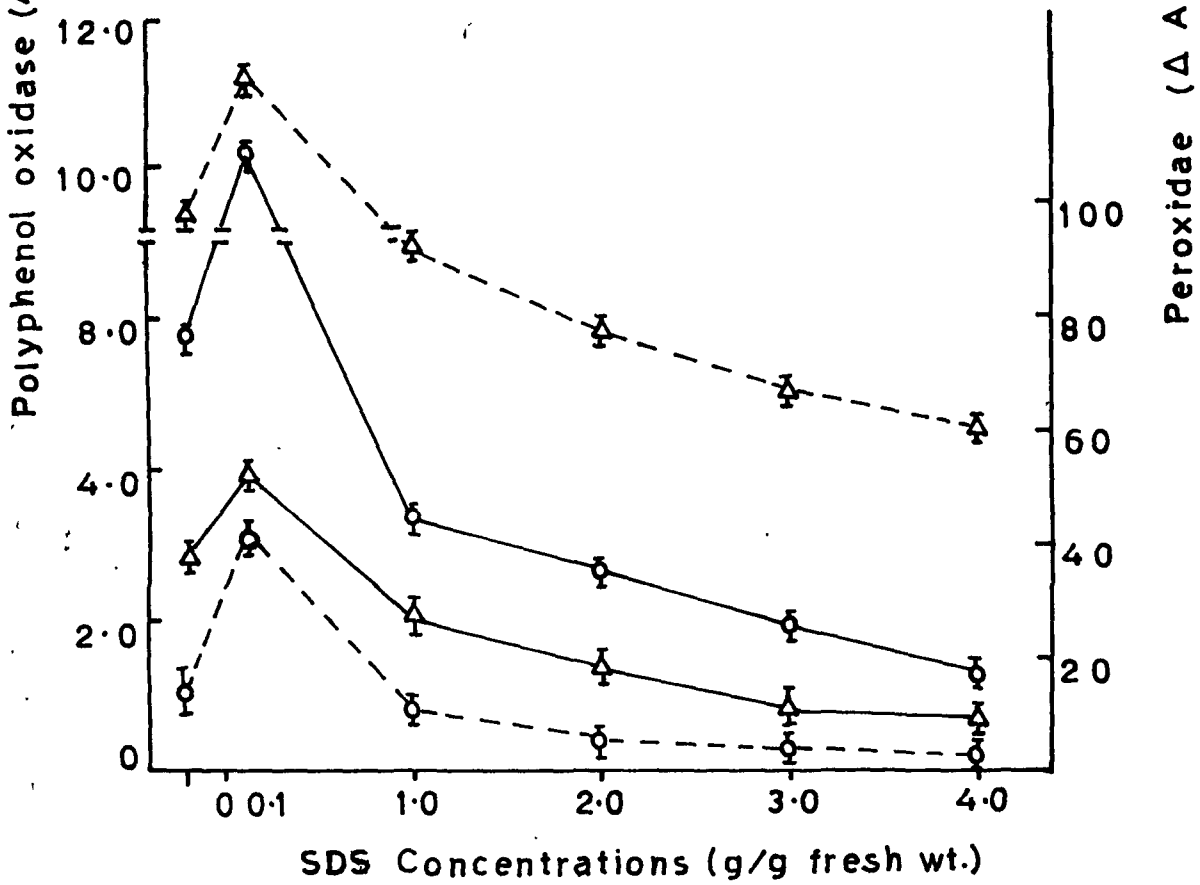

12·0
10·0
8·0
4·0
2·0
0
100
80
60
40
20
0 0·1
1·0
2·0
3·0
4·0
SDS Concentrations (g/g fresh wt.)
Polyphenol oxidase (ΔA/min/g fresh wt.) - - - -
Peroxidae (ΔA/min/g fresh wt.) ——

Table 6. Peroxidase, polyphenol oxidase, IAA-oxidase activities and contents of O-dihydroxy and total phenol in *in vivo* normal and gall tissues of *S. khasiana* and *S. wallichi*

	Peroxidase ΔA/min/g acetone powder	Polyphenol oxidase ΔA/min/g acetone powder	IAA-oxidase mg IAA destroyed/ h/g acetone powder	O-dihydroxy phenol mg/g fresh wt	Total phenol mg/g fresh wt
S. khasiana					
Normal	14.6 ± 0.33	0.25 ± 0.04	38.4 ± 1.10	0.039 ± 0.003	0.054 ± 0.004
Gall	1.4 ± 0.33	0.33 ± 0.06	26.2 ± 0.82	0.088 ± 0.004	0.144 ± 0.002
S. wallichi					
Normal	11.11 ± 0.23	0.16 ± 0.03	28.6 ± 0.82	0.024 ± 0.002	0.044 ± 0.003
Gall	0.83 ± 0.12	0.29 ± 0.05	18.0 ± 0.30	0.048 ± 0.004	0.084 ± 0.006

± S.E.

In vitro tissue

The in vitro grown gall tissue of both S. khasiana and S. wallichi exhibited higher PRO (Table 7) and PPO (Table 8) activities as compared to corresponding normal tissues in all treatments excepting 2,4-D for PPO activity. There was a remarkable decline in the PRO activity of the normal tissues of both the species in case of 2,4-D treatment. Conversely, the PPO activity was much higher in the normal tissues. The gall tissues of both the species cultured in media containing different auxins showed higher IAA-oxidase activity as compared to normal excepting IBA treatment where a reverse picture was obtained (Table 9).

The in vitro gall tissues showed more o-dihydroxy and total phenol contents as compared to normal in auxin treatments excepting IAA and 2,4-D in case of S. wallichi. In IAA and 2,4-D treatments, a marked decline in the phenolics was recorded in the gall tissues as compared to normal (Table 10).

Identification of phenolic acid

With the help of paper chromatography using different solvents and spray agents eight phenolic acids were detected in in vivo tissue of S. khasiana (Table 11). Out

Table 7. Peroxidase activity in *in vitro* normal and gall tissues of *S. khasiana* and *S. wallichi*

Treatment 2 mg/l	Δ A/min/g fresh wt of acetone powder			
	S. khasiana		*S. wallichi*	
	Normal	Gall	Normal	Gall
NAA (Control)	86.60 ± 0.89	320.94 ± 3.02	189.32 ± 1.01	879.12 ± 7.24
IBA	121.95 ± 1.00	179.77 ± 1.07	6.30 ± 0.12	8.67 ± 0.13
2,4-D	4.34 ± 0.14	250.02 ± 1.18	4.41 ± 0.12	98.26 ± 0.88
IAA	303.12 ± 4.13	422.22 ± 4.10	4.36 ± 0.12	26.78 ± 0.25

± S.E.

Table 8. Polyphenol oxidase activity in *in vitro* normal and gall tissues of *S. khasiana* and *S. wallichi*

Treatment 2 mg/l	△ A/min/g acetone powder			
	S. khasiana		*S. wallichi*	
	Normal	Gall	Normal	Gall
NAA (Control)	3.797 ± 0.108	8.986 ± 0.05	2.23 ± 0.03	6.38 ± 0.05
IBA	3.952 ± 0.13	12.124 ± 0.12	1.785 ± 0.06	2.673 ± 0.12
2,4-D	125.625 ± 8.13	6.65 ± 0.12	158.08 ± 7.64	6.652 ± 0.67
IAA	2.738 ± 0.43	38.88 ± 0.66	4.195 ± 0.12	4.964 ± 0.09

± S.E.

Table 9. IAA-oxidase activity in *in vitro* normal and gall tissues of *S. khasiana* and *S. wallichi*

Treatment 2 mg/l	mg IAA destroyed/hr/g acetone powder			
	S. khasiana		*S. wallichi*	
	Normal	Gall	Normal	Gall
NAA (Control)	13.2 ± 0.22	16.8 ± 0.33	16.9 ± 0.28	31.8 ± 0.15
IBA	30.4 ± 0.23	19.1 ± 0.32	116.0 ± 8.14	41.9 ± 0.26
2,4-D	14.5 ± 0.22	29.00± 0.23	10.1 ± 0.27	12.1 ± 0.21
IAA	1.59± 0.11	5.0 ± 0.21	14.9 ± 0.26	38.6 ± 0.17

± S.E.

Table 10. Phenolic contents in normal and gall calli of *S. khasiana* and *S. wallichi* grown in MS medium containing auxin (2 mg/l) and kinetin (0.04 mg/l)

Auxin	Tissue	O-dihydroxy phenol mg/g fresh wt	Total phenol mg/g fresh wt
NAA	*S. khasiana*		
	Normal	0.22 ± 0.03	1.26 ± 0.08
	Gall	0.67 ± 0.08	1.35 ± 0.08
	S. wallichi		
	Normal	1.10 ± 0.08	6.27 ± 0.52
	Gall	1.34 ± 0.13	6.60 ± 0.31
IAA	*S. khasiana*		
	Normal	0.35 ± 0.03	1.68 ± 0.08
	Gall	0.40 ± 0.02	2.43 ± 0.14
	S. wallichi		
	Normal	1.00 ± 0.06	5.48 ± 0.30
	Gall	0.18 ± 0.06	1.47 ± 0.06
IBA	*S. khasiana*		
	Normal	0.02 ± 0.00	1.01 ± 0.08
	Gall	1.23 ± 0.15	6.86 ± 0.26
	S. wallichi		
	Normal	0.49 ± 0.03	4.49 ± 0.51
	Gall	0.99 ± 0.10	4.71 ± 0.36
2,4-D	*S. khasiana*		
	Normal	0.38 ± 0.02	3.67 ± 0.33
	Gall	0.47 ± 0.03	0.94 ± 0.05
	S. wallichi		
	Normal	0.21 ± 0.02	0.60 ± 0.27
	Gall	0.10 ± 0.01	0.86 ± 0.08

± S.E.

Table 11. Phenolic acids present in *in vivo* normal and gall tissues of *S. khasiana*

Spot No.	Rf Value						Colour of the spot (Spray agents)		Absorption peak		Phenolic acids
	benzene: acetic: acid: water- 6:1:3	butanol: acetic acid: water - 4:1:1	isopropanol: ammonia: water - 10:1:1	Standard			diazotised p-nitroaniline	diazotised-sulphanilic acid	Tissue		
	Be	Bu	I	Be	Bu	I					
1	0.05	0.76	0.09	0.05	0.77	0.1	Brownish	Grey brown	N G	237 237	Caffeic acid (N & G)
2	0.12	0.78					Blue	Brown	N G	272 272	Unidentified (N & G)
3	0.23	0.78	0.18	0.21	0.78	0.16	Purple	Orange	N G	254 254	p-hydroxybenzoic acid (N & G)
4	0.28	0.88	0.25	0.30	0.87	0.26	Dark blue	Brown	N G	275 275	p-coumaric acid (N & G)
5	0.44	0.87	0.11				Pink	Yellowish brown	G	260	Unidentified (G)
6	0.56	0.88	0.12				Purple	Reddish brown	N G	260 260	Unidentified (N & G)
7	0.61	0.90	0.15	0.62	0.88	0.12	Violet	Purple brown	N G	235 235	Ferulic acid (N & G)
8	0.63	0.93	0.18				Blue	Blue green	G	272	Unidentified

N - Normal; G - Gall

of these spots, 4 were identified as caffeic, p-hydroxy-benzoic, p-coumaric and ferulic acids, present in both normal and gall tissues. The identified phenols showed similar absorpotion maxima as do the respective standards. Four distinct spots present in the tissue were not identified using standards.

Effect of different phenolics on the growth of normal and gall calli

The growth of normal tissues of S. khasiana and gall tissue of S. wallichi showed an increase with the increase in the concentration of ferulic acid in the medium (Fig. 16). However, the corresponding gall and normal tissues of the two species showed a decline in growth.

The growth of S. khasiana gall tissue increased with the increase of caffeic acid in the medium (Fig. 17). A reverse picture was obtained for normal tissue of both S. khasiana and S. wallichi. In all the concentrations of caffeic acid in the medium, the growth of S. wallichi gall tissue remained stationary except for a decline at 6 mM concentration.

The optimum growth of S. khasiana and S. wallichi gall and normal tissues resulted in the media containing 1 mM

Fig. 16 : Effect of different molarities of ferulic acid on the growth of Schima khasiana normal (□), and gall (■); S. wallichi normal (○) and gall (●) tissues grown in the medium containing NAA (2 mg/l) and kinetin (0.04 mg/l) Blackened portions of bars represent the dry weights.

Fig 16

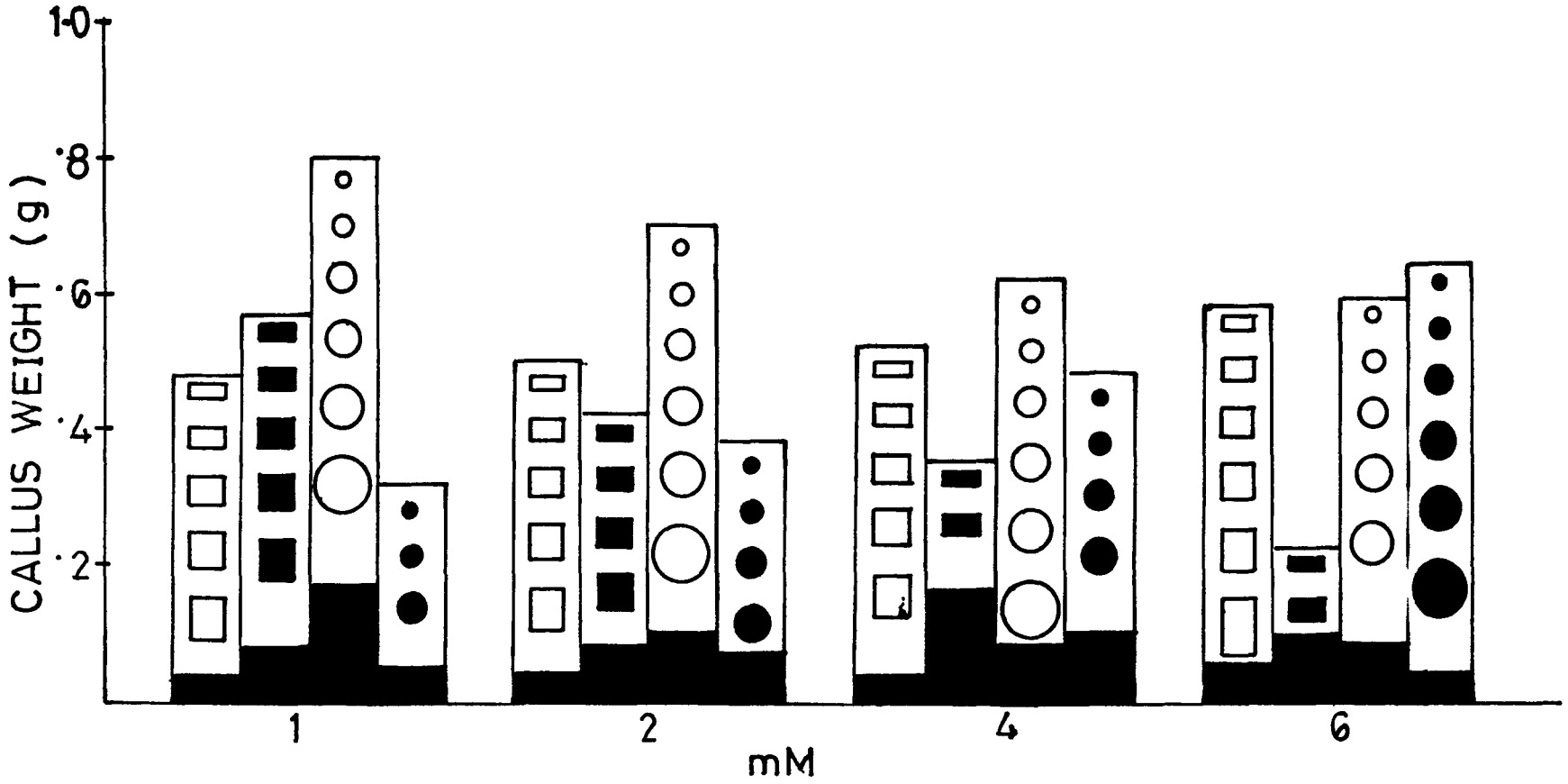
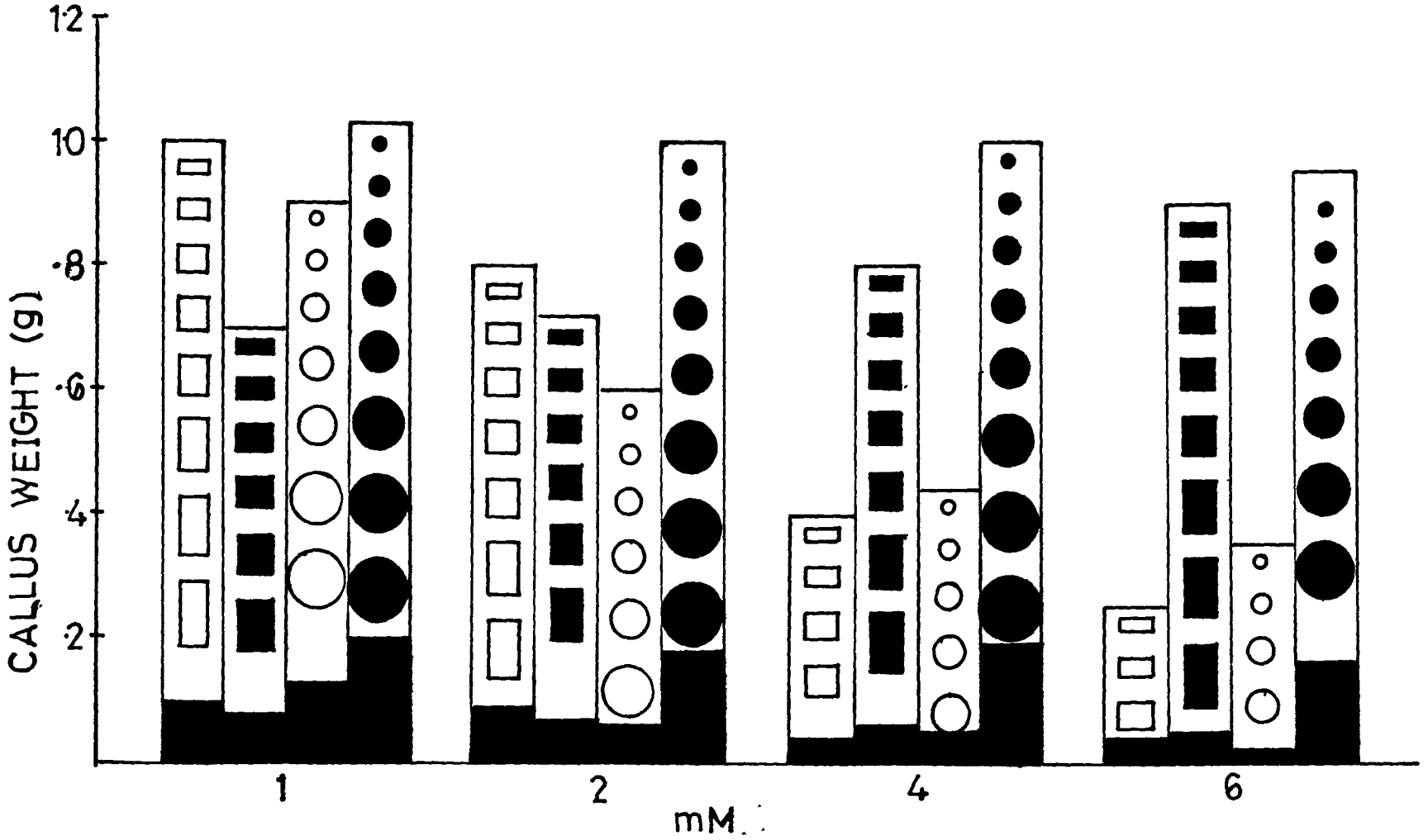


Fig. 17 : Effect of different molarities of caffeic acid on the growth of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) tissues grown in the medium containing NAA (2 mg/l) and kinetin (0.04 mg/l). Blackened portions of bars represent the dry weights.

Fig 17



of L-tyrosine (Fig. 18). The higher concentrations, however, were found inhibitory. On the other hand, the growth of S. wallichi normal tissue increased with increasing L-tyrosine concentrations.

At 1 mM protocatechuic acid in the medium the growth of normal and gall tissues of both the species exhibited optimum growth excepting S. khasiana normal tissue (Fig. 19). With increase in the concentrations of protocatechuic acid the growth declined. Conversely, the growth of S. khasiana normal tissue increased.

The growth of normal and gall tissues of both the species was optimum at 1 mM phloroglucinol in the medium (Fig. 20). However, higher concentration were inhibitory.

The growth of normal and gall tissues of S. khasiana was optimum in the media containing 1 mM of p-coumaric acid which declined at higher concentration (Fig. 21). However, a reverse picture was obtained for the growth of both normal and gall tissues of S. wallichi.

Effect of different phenolics on IAA-oxidase

Addition of 1-10 mM of protocatechuic, ferulic, caffeic, chlorogenic, p-coumeric and shikimic acids and L-tyrosine and phloroglucinol in the assay mixture enhanced

Fig. 18 : Effect of different molarities of L-tyrosine on the growth of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) tissues grown in the medium containing NAA (2 mg/l) and kinetin (0.04 mg/l). Blackened portion of bars represent the dry weights.

Fig 18

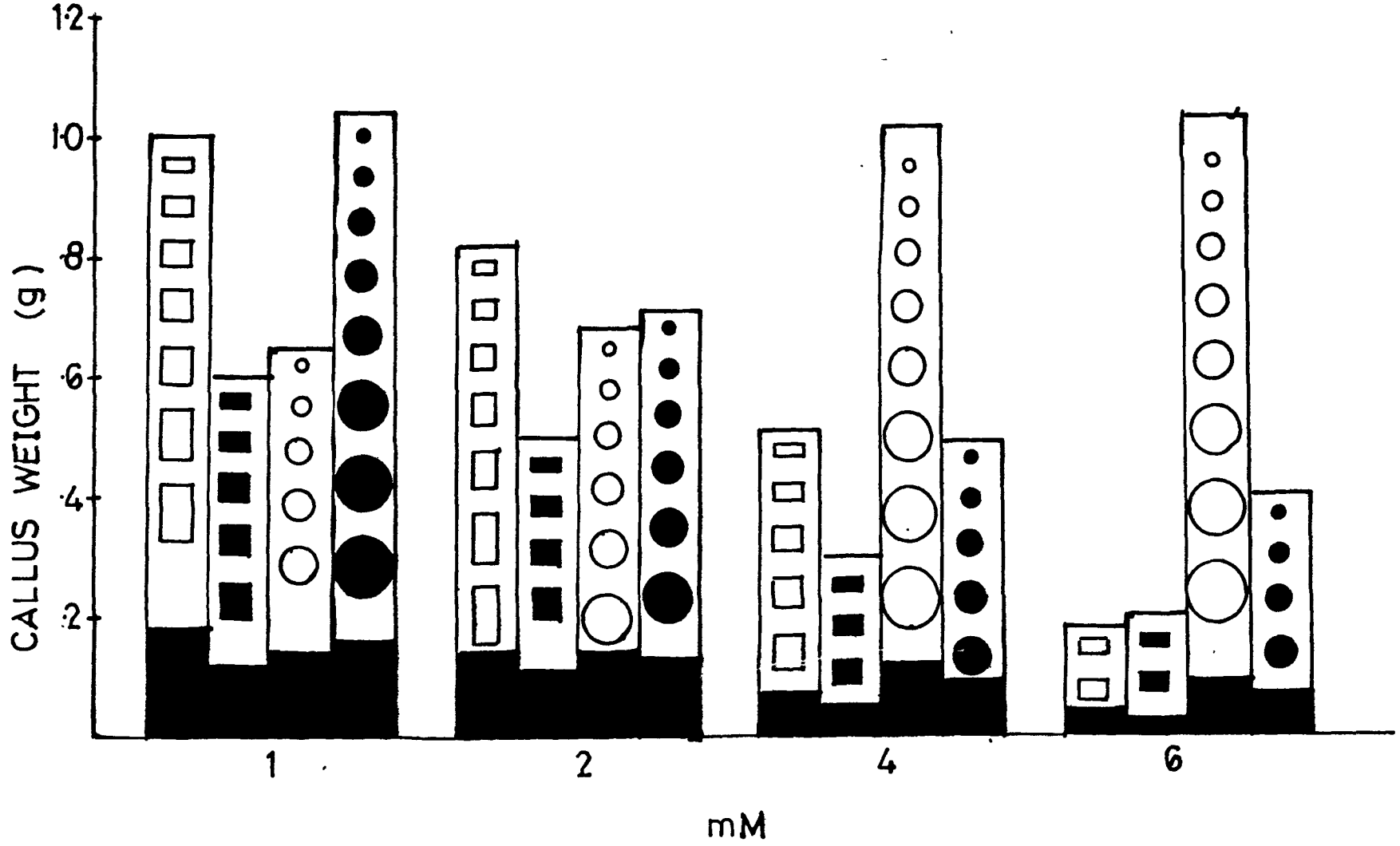


Fig. 19 : Effect of different molarities of protocatechuic acid on the growth of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) tissues grown in the medium containing NAA (2 mg/l) and kinetin (0.04 mg/l). Blackened portions of bars represent the dry weights.

Fig 19

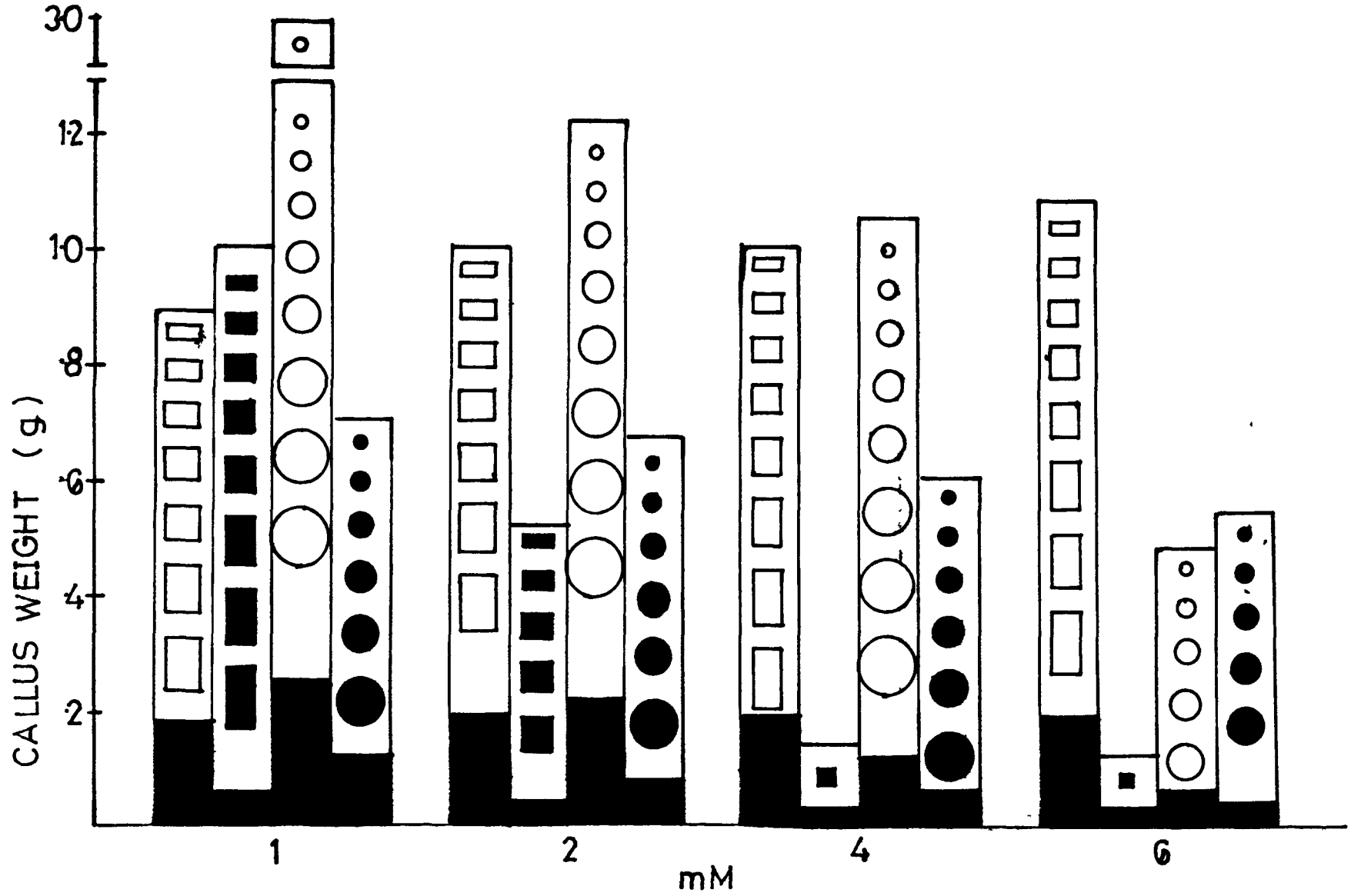


Fig . 20 : Effect of different molarities of phloroglucinol on the growth of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) tissues grown in the medium containing NAA (2 mg/l) and kinetin (0.04 mg/l). Blackened portions of bars represent the dry weights.

Fig 20

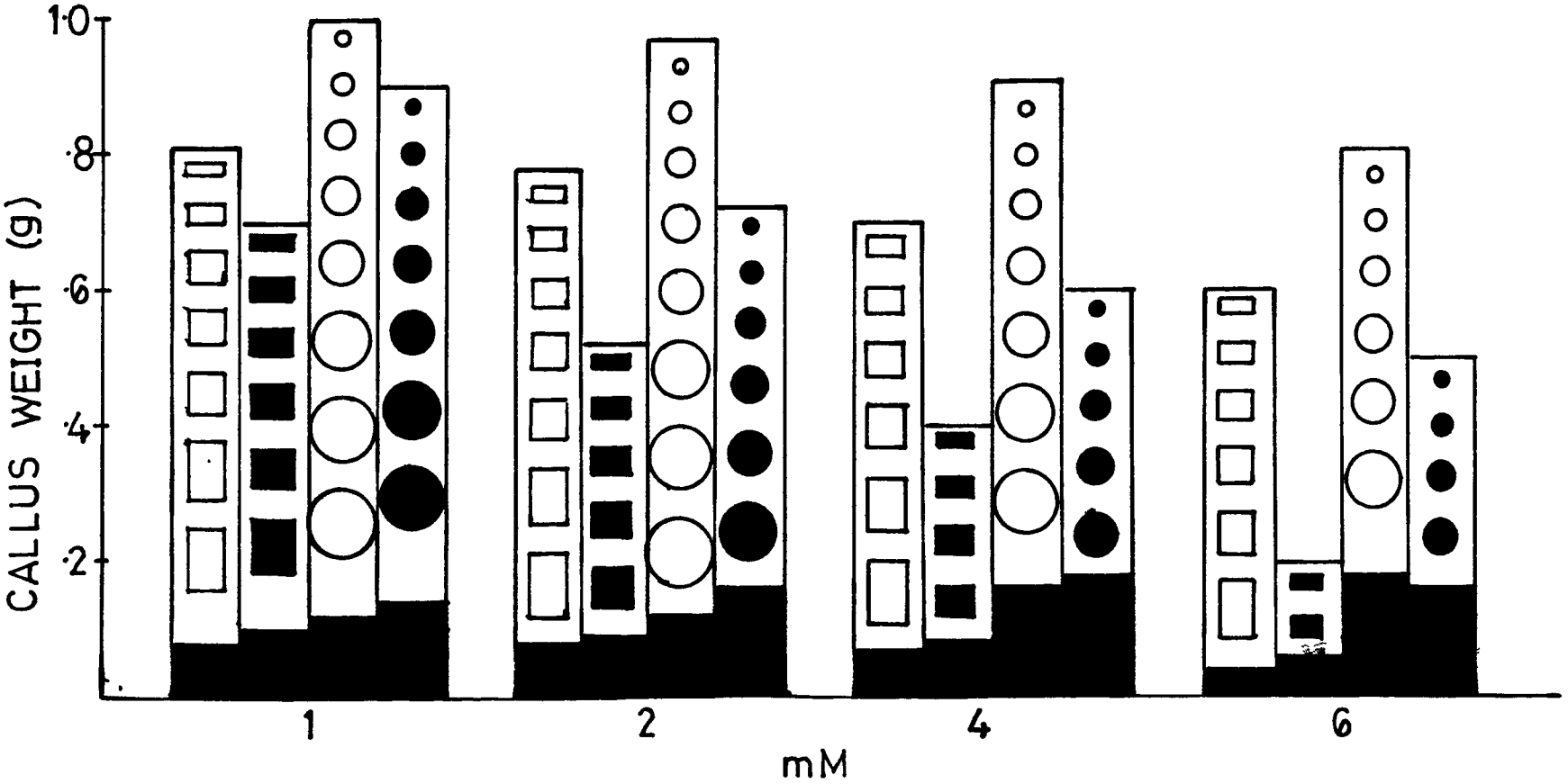


Fig. 21 : Effect of different molarities of p-coumaric acid on the growth of Schima khasiana normal (□) and gall (■); S. wallichi normal (○) and gall (●) tissues grown in the medium containing NAA (2 mg/l) and kinetin (0.04 mg/l). Blackened portions of bars represent the dry weights.

Fig. 22-25 : Effect of different molarities of protocatechuic acid (22 L-tyrosine (23), ferulic acid (24) and Caffeic acid (25) on the IAA-oxidase activities in both normal (O) and gall (Δ) tissues of S. khasiana

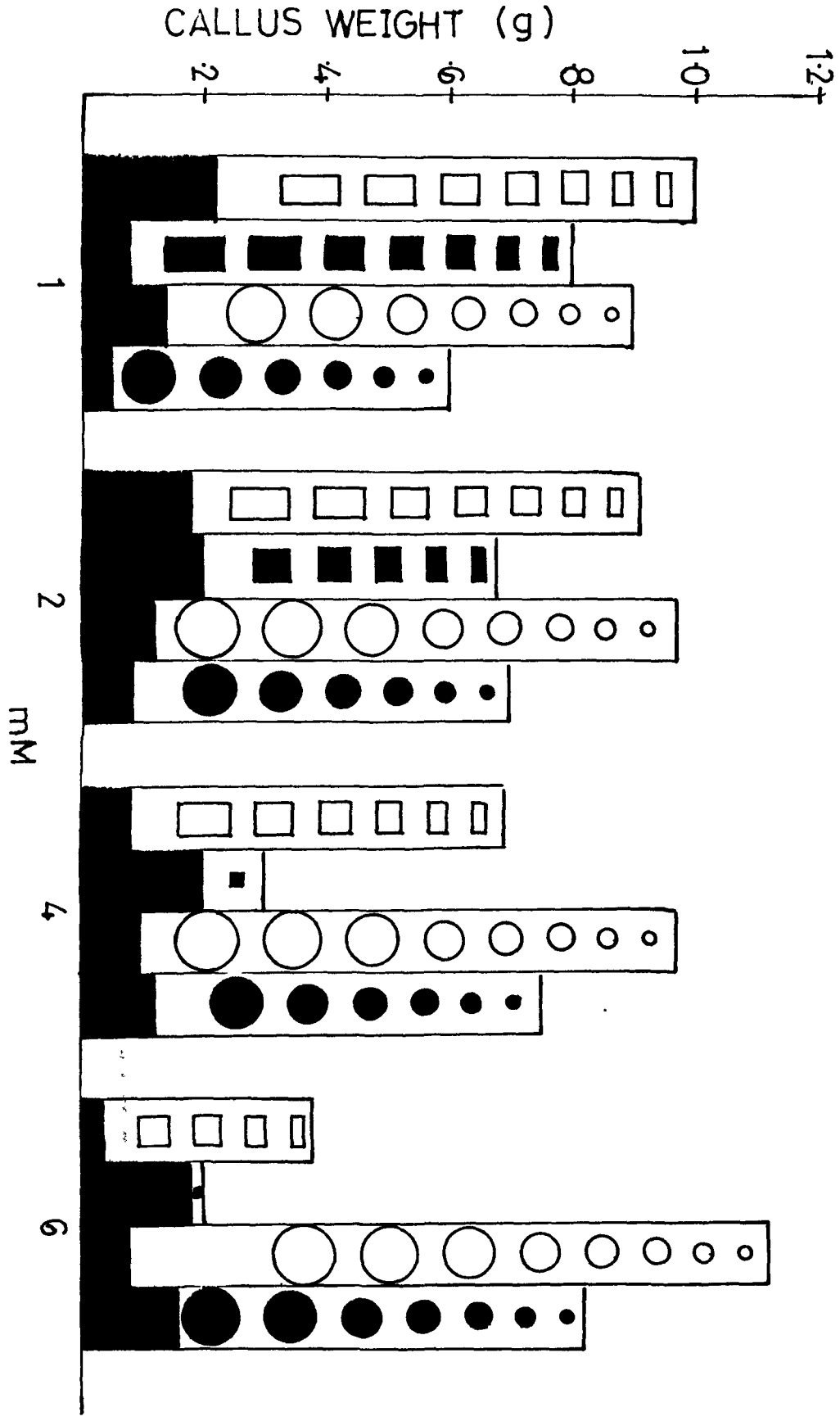


Fig 21

Fig 22

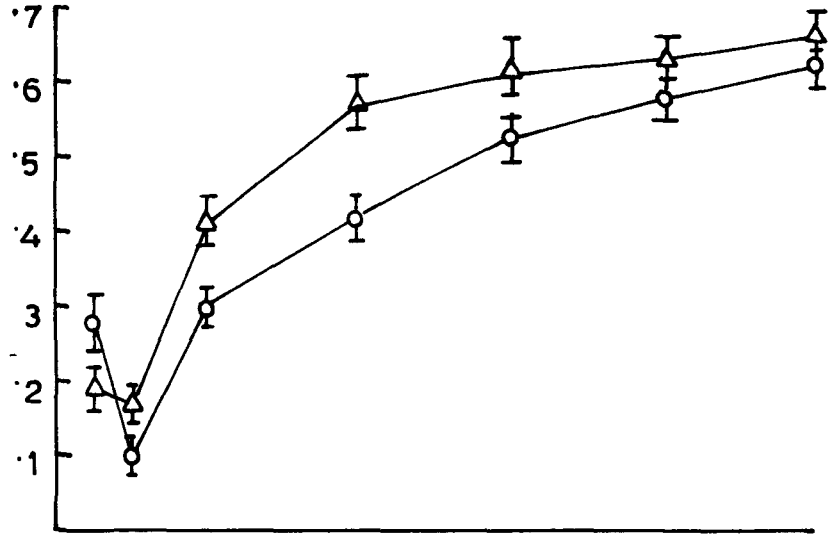


Fig 23

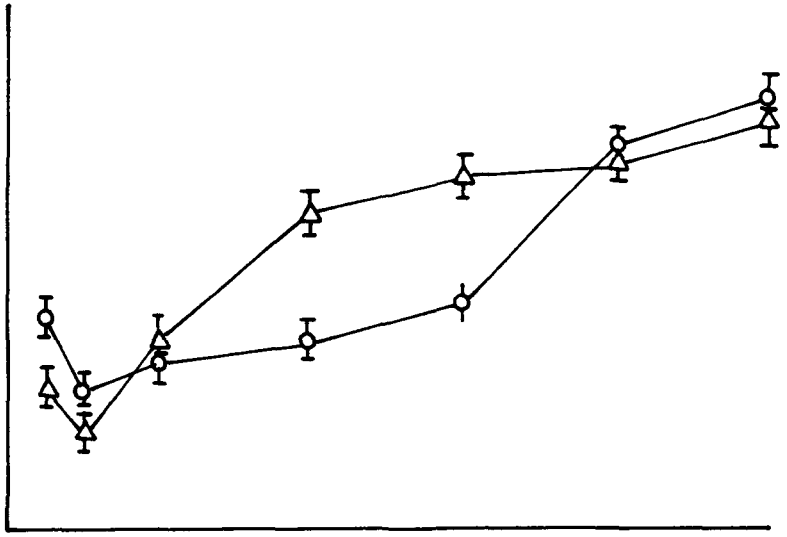


Fig 24

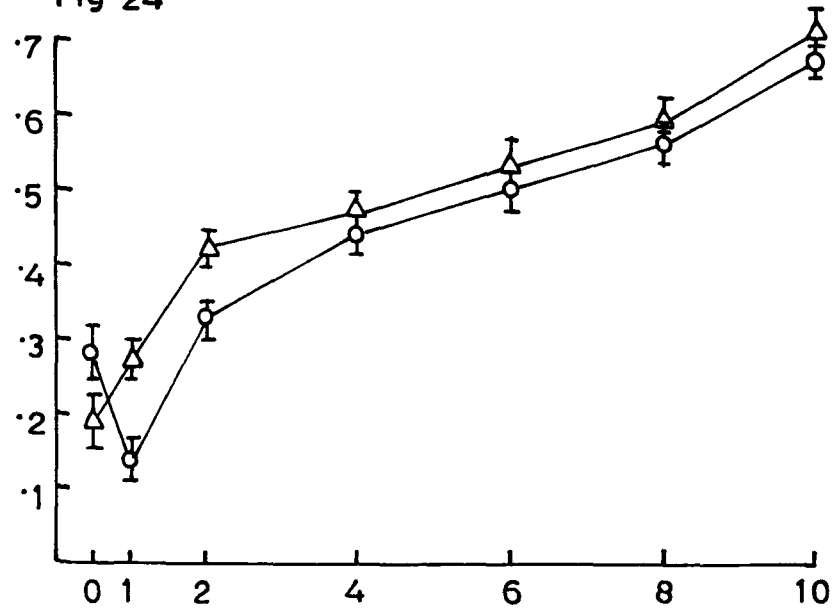
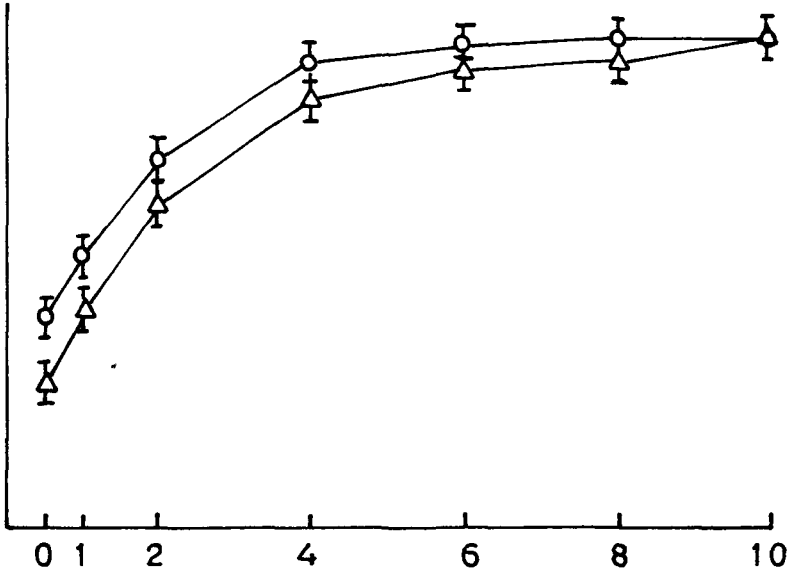


Fig 25



IAA Destroyed mg/h/g acetone powder

mM

Fig. 26-29 : Effect of different molarities of chlorogenic acid (26), phloroglucinol (27), p-coumaric acid (28) and shikimic acid (29) on the IAA oxidase activities on both normal (O) and gall (△) tissues of S. khasiana.

Fig 26

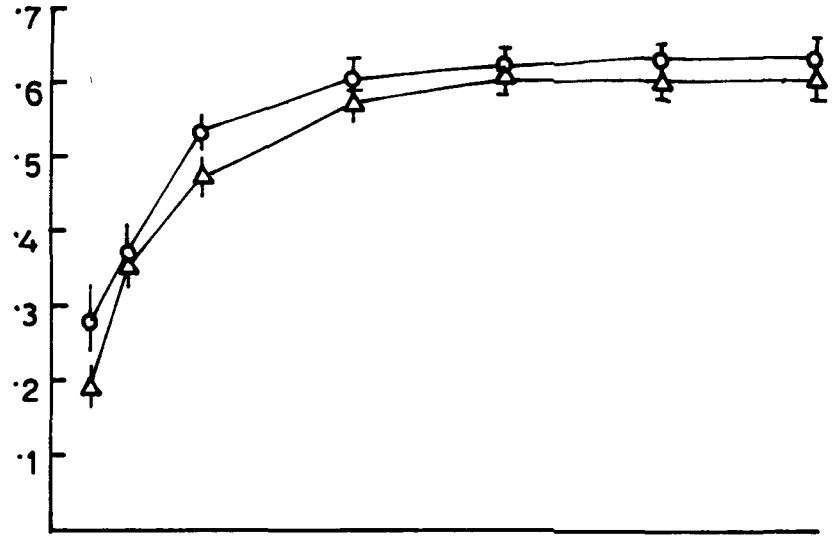


Fig 27

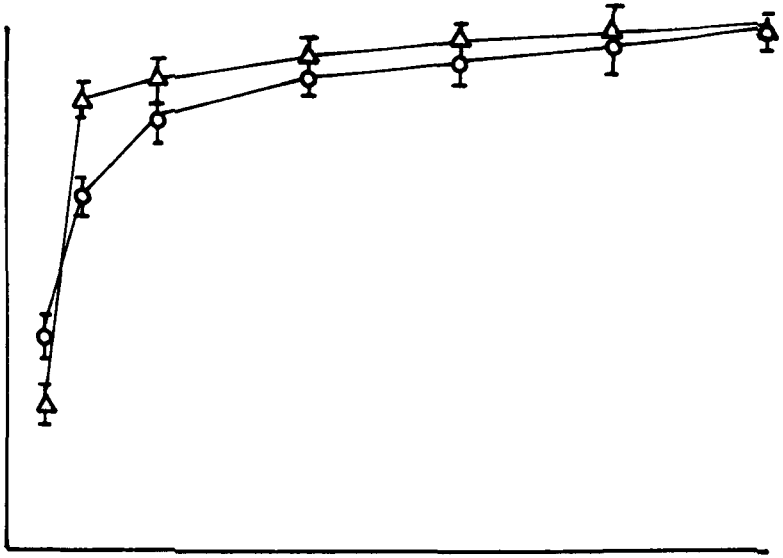


Fig 28

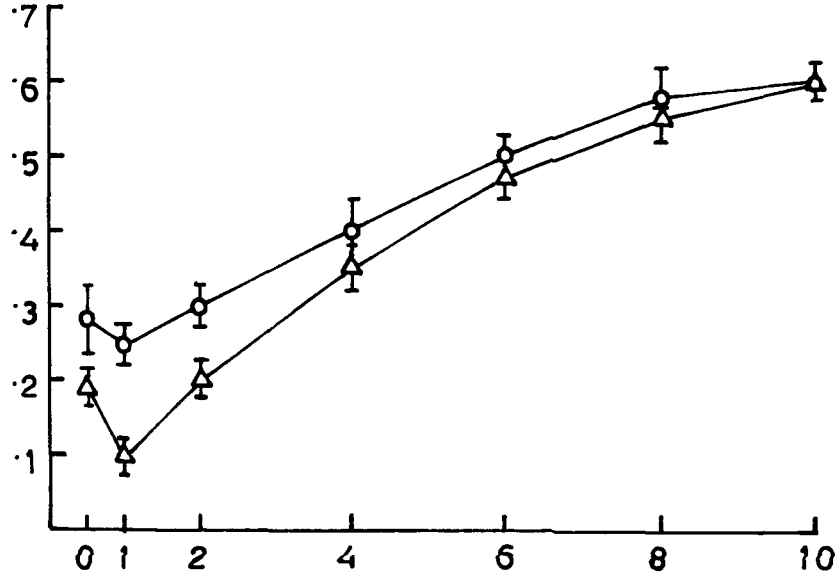
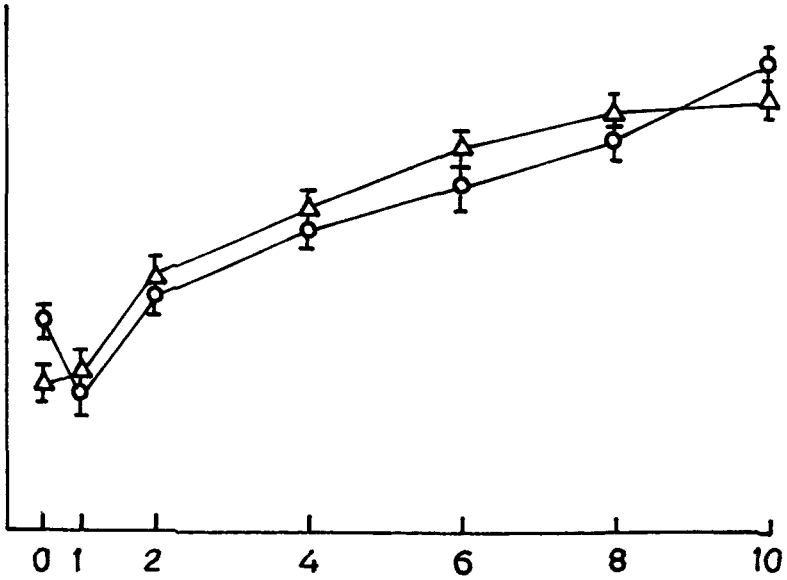


Fig 29



IAA Destroyed mg/h/g acetone powder

mM

IAA oxidase activities in normal and gall tissues of S. khasiana as compared to control excepting 1 mM of protocatechuic, ferulic, p-coumaric and shikimic acids and L-tyrosine (Fig. 22-29).

DISCUSSION

PAL and TAL are important enzymes in the biosynthesis of compounds of phenylpropanoid skeleton such as chlorogenic acid, caffeic acid and lignin. They are involved in diverting the flow of carbon from proteins into phenolic compounds. The enzyme activity data in normal and gall leaf tissue of S. khasiana and S. wallichi indicated the presence of these enzymes in both the tissues (Table 5). This suggests that aromatic amino acids like phenylalanine and tyrosine can enter secondary metabolism directly though their actual role in the process is not clear. TAL is sometimes believed to be a separate enzyme found mostly in monocotyledons. TAL activity could not be demonstrated without PAL preparation and often the activity if existed, was lost during preparation (Koukol and Conn, 1961). The two separate enzymes have been found in castor bean seed endosperms (Camm and Tower, 1973). Both PAL and TAL have also been reported in tobacco callus cultures (Lynda and Thorpe, 1985).

Higher plant cells respond to various exogenous influences for example wounding, with a dramatic increase in their extractable PAL activity. In gall tissues of Schima the increased activity of the enzyme may be due to the de novo synthesis of the enzyme protein which may be triggered by factor(s) that are produced or their levels increased after wounding. A variety of factors that influence PAL and TAL induction usually stimulate production of phenolic compounds.

The concept of protection of PAL from inactivation by phenylalanine suggested a complete hypothesis of the regulation of PAL in plant tissues (Stafford, 1974). Induction of PAL by various stimuli result in an increase in its rate of synthesis of inactivation. It is possible that this increase in activation is regulated by products of PAL (Engelsma, 1968) or by some other mechanism such as depletion of a pool of phenylalanine. The inactivation rate increases both because of a net increase in its activity and because of increase in content. Since it is of the first order reaction, a point is reached where synthesis is balanced by inactivation and no net change in activity occur until synthesis stops or inactivation is prevented. This imbalance caused a new steady state level which is maintained until some further metabolic events change the ratio of PAL synthesis and

inactivation. The net result is a system in which rapid changes can occur in the level of PAL in response to many external stimuli. With large changes in the state of utilisation of phenylalanine required for phenol biosynthesis, very sensitive controls of its level in plant must exist. This in turn suggested that the rate of production and not the concentration of phenylalanine is the controlling factor in phenolic biosynthesis.

Decrease in the activity of IAA oxidase associated with mite-incited galls has been reported (Purohit *et al.*, 1980; Tandon and Arya 1980; Joshi *et al.*, 1985). *Schima* gall tissue showed low IAA/activity as compared to normal (Table 6). IAA catabolism in plant tumours has been a subject of extensive investigation and there are number of conflicting reports (Boulillenne and Gaspar, 1970; Stonier, 1972; Butcher, 1973). The low IAA oxidase activity and more total phenols in gall tissue of *Schima* may be responsible for its hyperauxinity. The involvement of phenols in the oxidation may result in inhibition or stimulation of plant growth metabolism. The formation of IAA from tryptophan is accelerated under the effect of the polyphenol-polyphenolase system. IAA is formed from L-tryptophan in a rather basic medium by mere incubation of L-tryptophan with phenol (Kefeli, 1985).

Several hypotheses on the action of natural phenolic substances considered phenols as factors, regulating the activity of IAA oxidase, enabling IAA synthesis from L-tryptophan (Kefeli and Kutacek, 1977).

Higher auxin levels in crown-gall were reported (Kado, 1984). The genes directly involved in the IAA biosynthesis have been located in the crown-gall (Kado, 1984; Rausch et al., 1986). The virulent region of Ti-plasmid containing the iaap genes function in the plants to produce abnormally high levels of IAA, the concentration of which may vary with age and growth stage of tumours (Nakajima et al., 1981; Pengelly and Meins, 1982; Zofia, 1984a). Considerable evidence suggested that IAA-oxidase system is involved in the control of endogenous auxin levels and thus regulated various physiological processes as cell growth, differentiation and host-parasite interaction (ref. Sembdner et al., 1980). It is generally accepted that there might occur a negative correlation between growth rate (auxin content) and IAA-oxidase activity of various organs or tissues (Wareing and Phillips, 1982; Zofia, 1984b).

Certain Plant enzymes are also greatly influenced by growth hormones in in vitro tissues (Vernon and Strause, 1972; Singh et al., 1982; Barendse, 1983) and synthesis of

phenolic compounds is influenced by auxin level in the medium. In contrast to *in vivo* tissues, the *in vitro* tissues showed more IAA-oxidase activity in different auxin treatments. A decrease in auxin content has been reported in both normal and gall calli grown in media containing NAA, IAA and DL-tryptophan (Tandon and Arya, 1980b). IAA-oxidase activity was more in the gall as compared to normal of both *S. khasiana* and *S. wallichi* in the medium containing NAA, 2,4-D and IAA and vice versa in the medium containing IBA (Table 9). Bouillene and Gaspar (1970) have reported a high peroxidase and IAA oxidase activity in *Impatiens* crown-gall tissue homogenates free from inhibitors. IAA has been shown to stimulate the activity of PRO in pith cultures of *Pelargonium* (Lavee and Galston, 1968) but at higher levels inhibited the activity (Seigel *et al.*, 1960). The interaction between IAA and various soluble phenols with oxidase preparations have long been studied. Macháčková *et al* (1975) found that the peroxidase preparation from *Triticum* shoots will oxidise ferulate more easily than p-coumarate. The enzyme is not able to directly oxidize IAA, even with exogenous supplied peroxidase, but required a phenolic co-factor such as p-coumarate. It was also suggested that peroxidase degradation of phenolics may be regulated by auxins in the intact plants,

and further more, whether phenols or auxins are oxidised would depend on the concentration ratio of diphenols/ monophenol/auxin.

The phenolic compounds are presumed to act indirectly through their effect on IAA-oxidase. Thus monophenols are inhibitory because they promote decarboxylation of IAA, whereas diphenol are growth promoters by inhibiting IAA-oxidation. Higher concentrations, however, are inhibitory. Though it is generally accepted that peroxidase is responsible for some IAA-oxidase activity, it has not been clear whether a true oxidase exists distinct from peroxidase. Peroxidase isozymes have been demonstrated in many plants (Gove and Hoyle, 1975) and IAA-oxidase also occurred in multiple molecular forms. Several studies have shown that electrophoretically separated proteins possess peroxidase activity but no IAA-oxidase activity and the non-identity of IAA-oxidase with peroxidase was concluded (Endo, 1968).

In numerous incompatible host-parasite combinations, peroxidase activity is several times higher than in compatible ones (Daly et al., 1971; Loon and Geelen, 1971). On the other hand, however, peroxidase activity is greater in compatible host-parasite combinations than in the incompatible

ones (Wood and Barbara, 1971). Phytohormones are known to play a major role in growth and differentiation. The peroxidase functional to growth by its IAA oxidizing action and biosynthesis (Wheeler and King, 1968) regulation of relationship of IAA synthesis in normal and tumorous tissue with peroxidase has been studied by several workers (Ahuja and Gupta, 1974; Bayer, 1982). Basu and Tuli (1972) suggested that the oxidative transformation of IAA to other biologically active compounds in plants may contribute to the IAA activity rather than to its inactivation.

Peroxidases are the major components in the IAA oxidase system in plant. The rapid decline in the peroxidase activity from high to low level in the gall tissue might result in sparing the auxin from being oxidised. This sparing action of IAA-oxidation is provided by the low or high polymers of O-dihydroxyphenols. Interaction of IAA-oxidation by phenolics such as ferulic, hydroxycinnamic acid, and scopoletin add to the complexity of the system (Gelinas , 1973; Sirois and Miller, 1972). Peroxidase pathway relationship to polyphenol and IAA-oxidase has been suggested (Gove and Hoyle, 1975). In general peroxidase catalyses four type of reactions (i) peroxidative (ii) oxidative (iii) catalytic and (iv) hydroxylation (Vámos-Vigyázó,

1981). The gall tissues showed higher peroxidase activity as compared to normal, when grown in the medium containing NAA, IBA, 2,4-D and IAA (Table 7). In general, maximum activity was observed in the medium containing NAA followed by IAA and 2,4-D, whereas with IBA treatment the gall tissue showed the least peroxidase activity. Morel and Démétriades (1955) reported that, IAA and 2,4-D enhance the activity of PRO and PPO in cultures of Jerusalem artichoke. It is known that the oxidation of IAA takes place in several steps in which different intermediates of peroxidase are formed. Certain peroxidase intermediates rather than the native form are believed to be responsible for the breakdown of certain phenolic inhibitors (Gelinas, 1973; Lee and Chapman, 1977).

Higher PPO activity was observed in gall tissues of both the species of Schima as compared to normal (Table 8). The increase of activity in host tissues especially at and around the infection sites is a response which is characteristic to a large number of plant diseases. The phenolase activity is accompanied with increase in phenolic concentration which play a role in toxicity of pathogen (Kosuge, 1969). It was observed that callus showed black pigments if kept for longer periods in a medium containing auxin and kinetin without subculturing. Phenolase oxidise mono- and dihydroxyphenol to brown or black polymeric susbstances

(melanin pigments). Such darkening of tissues can also be caused by adverse environmental factors (Kosuge, 1969). Increased O-dihydroxy and total phenol concentrations in gall tissue as compared to normal (Table 10) could be due to higher activity of PPO in gall tissues.

It was thought that PPO enzyme may participate in redox reaction with in the plant cells. However, neither this nor localisation of this enzyme has been demonstrated definitely. Brigg and Ray (1956) stated that purified tyrosinase inactivates IAA in the presence of catechol. IAA is oxidised probably by the products of oxidation of catechol, i.e., by O-quinone. Another worker, however, suggested only a temporary formation of complex between IAA and the oxidised phenols (Pelet and Gasper, 1968).

The physiological functions of the enzymes are not well understood. PPO enzyme was involved in the IAA biosynthesis via indole pyruvic acid (Gordon and Paleg, 1961), and acted as a possible agent in the oxidation of auxin (Galston and Purves, 1960) at the wound site of the tissues and tissues in cultures (Wetmore and Morel, 1949). The increased polyphenol oxidase activity and phenolic contents has been reported in crown-gall (Wegen and Glase, 1981),

mite-incited stem galls (Tandon and Arya, 1982) and leaf roll galls (Joshi and Tandon, 1984). The gall tissues of both S. khasiana and S. wallichi show higher PPO activity than normal in the medium containing NAA, IBA and IAA. However, exceptionally high PPO activity was recorded in the normal tissues grown in the medium containing 2,4-D. contained less activity of PPO as compared to the normal tissues Zizyphus gall tissues under the influence of NAA, IAA, 2,4-D (Tandon and Arya, 1980). Interestingly, PPO activity decreased whereas IAA-oxidase and PRO increased in the gall tissues of both the species of Schima in the medium containing 2,4-D (Tables 7-9). This supported that the incorporation of growth regulators like 2,4-D in the culture medium decreased the auxin content in gall tissue (Tandon and Arya, 1980b). Both in vivo and in vitro cultures of gall tissues in the medium containing, NAA, IBA or IAA showed more PPO activity, thus it may be sugested that gall formation is followed with production of factor(s) essential for higher PPO activity by the growth regulators that could trigger switching off and on of IAA oxidase to regulate auxin contents. Though our knowledge of catbolic pathways of polyphenol in plants is still very meagre, various degradative and enzymatic studies have revealed some of the main biochemical parameter pertaining to this field. Many aspects of polyphenol metabolism and degradation have been studied

in cell suspension cultures because of the experimental advantages.

Phenolic compounds are widely distributed and spread in nature. These compounds play a great role in normal and abnormal plant growths (Kefeli and Kutacek, 1977; Rosenthal, 1986) by their effect on enzymatic oxidation reaction. Many natural phenols interfered with indole biosynthesis (Kefeli and Kutacek, 1977) and oxidation of IAA *in vitro*. The lowest oxidation products of phenolic substances - the quinones, are most toxic and are highly oxidised and polymerised products of polyphenol considered less toxic or non-toxic (Noveroski *et al.*, 1962).

Several workers have isolated phenolics from higher plants tissues (Ibrahim and Tower, 1960; Bohm and Tower, 1962; Van Lelyveld, 1974; Kozslowska *et al.*, 1983). There have been relatively few reports on the isolation of free cinnamic acid from tissue cultures in free and bound form. However, cinnamic acid, caffeic and ferulic acid (Brown and Tenniswood, 1974), chlorogenic acid (Stickland and Sunderland, 1972) and coumarin like scopolatin and four of its glycosides (Sargent and Skoog, 1961) have been isolated from callus and intact tissues of tobacco. Both normal and gall tissues of *S. khasiana* showed at least eight phenolic

compounds (Table 11). Interestingly, two additional spots were observed in gall extract alone, suggesting their formation due to gall induction.

Many investigators have suggested that IAA and other substances directly stimulated the production of phenolic compounds. However, others believed that the accumulation of phenolic compounds parallel, the increased synthesis of IAA because these compounds originated from the common biosynthetic pathway (Kosuge, 1969). In the present study, the accumulation of phenolic acid have been found to be influenced by the cultural conditions. The addition of 2,4-D (0.1 mg/l) caused a substantial refraction in the quantity of chlorogenic acid produced (Stickland and Sunderland, 1972). Low 2,4-D and high kinetin concentrations in the medium gave rise to increased caffeic acid formation (Ishikura et al., 1983). Sugano et al. (1975) reported that phenolic acids in carrot cells are markedly increased by lowering the concentration of 2,4-D. The synthesis of phenol compounds may also be influenced by the different auxins added in the medium (Davies, 1972; Shah et al., 1976), and/or the age of the culture medium. Relatively, only a few studies have been made on phenolic compounds in relation to growth period of cell cultures (Davies, 1972; Shailaja and Mehta, 1980). In the present studies,

the accumulation of phenolics during callus growth in cultures suggested that the synthesis of phenolic compounds may be essential for the mediation of callus growth. Low concentration of phenolic compounds (1 mM added to the MS medium containing NAA showed increase in wet and dry weight after 30 days from the result obtained with Schima.

It may be suggested that protocatechuic acid and phloroglucinol favour normal callus tissue growth whereas caffeic, ferulic and p-coumaric acid favour gall callus tissue growth (Figs. 22-29). Tyrosine did not contribute to callus tissue growth except at 1 mM concentration. Addition of caffeic acid and ferulic acid in tobacco callus promoted callus tissue growth (Lee and Skoog, 1965). The better growth of callus tissue occurred in the medium containing chlorogenic acid and catechins (Feucht and Schmid, 1980). Addition of tyrosine and phenylalnine into the medium enhanced the growth of number of normal and gall tissues but only moderately (Pelet et al., 1960). It was established that some phenolics are active stimulators of plant rhizogenesis and other morphogen. It was also showed that some compounds like caffeic acid, ferulic and chlorogenic acid act as cofactors of IAA-oxidase while p-coumaric acid inhibits it. Sequeira (1964) has shown a correlation between the rapid synthesis of scopoletin and chlorogenic acid and the decrease

of IAA-oxidase activity. Sirois and Miller (1972) suggested that such increase could completely inhibit IAA-oxidase and naturally affect growth.

The action of phenolic compounds on plant growth is frequently attributed to their interaction with IAA oxidase, regulating IAA levels *in vivo* (Thimann, 1972 ; Van Sumere., 1975; Le tham, 1978). Sinapic and ferulic acids are known to be inhibitors of IAA-oxidase activity, thereby showing an auxin sparing effect (Varga and Koves, 1959; Zenk and Muller, 1963). A similar observation was recorded in both normal and gall calli of *Schima* with the addition of ferulic acid in the medium. However, the results obtained indicated that the phenolic compounds tested show differential response to IAA-oxidase inhibition of which callus growth may be taken as an index of oxidation products.

From the results obtained it is clearly indicated that lower concentrations of phenols showed better effects on IAA-oxidase activity. Caffeic acid, chlorogenic acid phloroglucinol were less inhibitory as compared to protocatechuic, ferulic, p-coumaric, shikimic acids and tyrosine. Higher concentrations of most phenolics inhibit growth irrespective of hydroxylation pattern.

Inhibitory effects of coumarine on organogenesis and callus growth (Bagni and Fracasini, 1971) and promotion of root growth under specific conditions (Vazquez, 1973) have been reported. A possible explanation for such growth promotion based upon interference of O-dihydroxyphenols with IAA-oxidase has repeatedly been discussed in detail (Stonier and Yang, 1973; Kefeli and Kutacek, 1977). Phenolics may be subjected to rapid turn over and degradation involving steady state concentration of the various end products. The importance of phenolic compounds in controlling growth has been demonstrated in tobacco callus cultures by their action in promoting or inhibiting bud differentiation (Lee and Skoog, 1965). These effects were generally parallel to the cofactor or inhibitor activity of these compounds in the oxidation of IAA in vitro (Zenk and Muller, 1963; Tomasewski and Thimman, 1966).

Chapter IV

Studies on the biochemical changes in normal and gall tissues

INTRODUCTION

Cell growth is the simplest analysis that involves loosening of cell wall, expansion of the cell volume by increased water uptake, increase in the dry weight of total cell matters largely due to higher frequency of cell division, duplication of DNA and synthesis of cells phosphorylation. Continuity of cellular processes involved in replenishment of substrate required for necessary RNA and protein synthesis, by switching on and off old genes which result in morphological changes. Auxin produced in higher plants enter into nearly every facet of plant growth and development, and the recognisable essential feature of auxin action is the stimulation of cell enlargement that is expressed in a variety of ways among the tissue and organs of a growing plant.

Experiments with insects(Arya,1965) and plants (Key and Hansoon, 1961) have shown that the RNA and protein synthesis are involved in the action of a wide variety of hormones. DNA formation may be induced by kinetin in the absence of added IAA, and vice-versa causing mitosis (Naylor Sander and Skoog, 1954). Studies on the changes in RNA metabolism associated with cell expansion are usually complex with differential response (Key and Shannon, 1964).

The role of auxin seems to be associated with the regulation of the synthesis of RNA and protein. Therefore, enhanced nuclear activity must regulate the growth response to exogenous auxin. Auxins regulate the tissues, whereas cytokinin in conjunction with auxin regulate both the growth and morphogenesis of a variety of plant tissues in culture (Fosket, 1980). Although cytokinins alter enzyme activities (Kulaeva, 1980; Kaminek et al., 1981), stimulate protein synthesis and in some cases induce the appearance and disa-disappearance of specific polypeptides when applied to cultured tissues (Bevan and Northcote, 1981; Meyer and Charier, 1981; Wang et al., 1981), it is not known whether these changes reflect an early effect of the hormone induced changes in growth.

Auxin requirement for the stimulation of cells proliferation has been demonstrated for many types of tissues grown in vitro (Fosket, 1980). Tandon et al. (1976) reported that increase in concentrations of NAA increased the RNA content of Zizyphus jujuba normal tissue while no appreciable change was recorded in the gall tissue.. DNA content of the gall tissue was higher to that of normal tissue. Important relationship of the nucleic acids with the nature of the diseased and normal growth of the tissues was established. A large body of recent results has shown that

IAA, NAA and 2,4-D rapidly regulate the accumulation of a few mRNA in responsive plant tissues. This type of auxin effect has been reported for different plant species (Theologis and Ray, 1982; Meyer et al., 1984; Mohen et al., 1985; Walker et al., 1985) and for tissue showing major physiological responses including cell enlargement (Zurfluh and Guifoyle, 1980, 1982) or induction of mitosis (Beven and Northcote, 1981; Meyer et al., 1984; Mohen et al., 1985). However, the incorporation of oncogenic DNA into the host cell (Chilton et al., 1977; Yadav et al., 1980) results in biochemical changes that allow these cells to grow continuously on defined medium without exogenous phytohormones (Horgan and Aitken, 1981; Nester et al., 1984), and recently genes transferred to the host have been found to code for enzymes involved in the biosynthetic production of cytokinin (Akiyosh, 1984) and auxin (Thomashow et al., 1986). The transformed cells continuously produce these hormones and grow autonomously at a stage of differentiation specified by the bacterial strain (Kahl and Schell, 1982). The crown-gall tissues have the capacity to utilise abnormal amino acids termed opines (Karcher et al., 1984; Ellis et al., 1984; Komro et al., 1985). The octopine type strain of A. tumefacience frequently transferred two regions of T-DNA to plant cells

during tumorigenesis (Thomashow *et al.*, 1980).

Auxins have the capacity to alter the biosynthesis of particular proteins in various plant systems. If proteins have a role in initiating the biological phenomena known to be mediated by the hormone and constitute the primary hormonal response, then hormonally induced changes in the concentration of a particular protein should be detected prior to the onset of the phenomenon and shortly after induction of the hormones into the experimental system. Hormonally induced protein changes do not reveal the mechanism by which they are mediated. For example do protein changes result in difference in mRNA levels affected by the hormones at the transcriptional level, or are the effects of the hormones expressed at the translational or post translational level or on the stability of the protein or mRNA? Boulcombe *et al.* (1980) were the first to show differences among the *in vitro* translation products of polyadenylated mRNA isolated from 2,4-D untreated and treated tissue. Zurfluch and Guifoyle (1982) compared the *in vitro* translation product of IAA treated and untreated corn coleoptiles and found that rapid auxin induced changes at 50 μM concentration. *In vitro* translation experiments carried out by Meyer *et al.* (1984) have shown that the auxin induced increase in specific protein level in tobacco protoplast coincides with

an increase in corresponding mRNAs. It is obvious from the *in vitro* studies that the auxin rapidly and specifically potentiate certain mRNA sequences in various plant tissues. The role of cytokinin in mRNA is not well understood even though there have been number of studies on the effects of such modification in tRNA (Taller *et al.*, 1987).

MATERIALS AND METHODS

Extraction and estimation of auxin

The IAA extraction and quantitative measurement were done according to the method of Mahadevan and Sridhar (1982). Fresh normal and gall tissues were collected from the field brought on ice to the laboratory. 100 g each of the tissues was cleaned thoroughly with water, cut into pieces (1-2 cm), blended with prechilled methanol in mortar with pestle and squeezed through four layers of cheese cloth. The residue was extracted twice for complete recovery. The volume of the pooled filtrate was then reduced to 20 ml with reduced pressure evaporator at 40-50°C, and pH of the residue was adjusted to 3 with N HCl. The filtrate was then extracted three times with equal volume of peroxide-free diethyl ether over a period of an hour using the separatory funnel. The ether extracts were combined and partitioned

four times with 100 ml - 5% Na_2CO_3. The alkaline fraction was acidified to pH 3 with 6 N HCl and extracted four times with ether. The ether extract was evaporated to dryness and dissolved in 2 ml of methanol and separated by paper chromatography. All the operations described above were done in diffused light.

For separation of auxins 100 µl fractions were spotted on Whatman No.1 chromatographic paper. The descending chromatographs were run for 12 hrs separately in different solvents (isopropanol:ammonia:water-10:1:1; isopropanol:water 3:2; ethanol 70%; butanol:ethanol:water-4:1:1; pyridine:ammonia-3:1 v/v/v). A reference sample of IAA was also run simultaneously. The chromatograms were developed descendingly for 16 hrs and then dried at room temperature and sprayed with Ehrlich's reagent (2% p-dimethyl amino benzaldehyde in 2 N HCl). The chromatograms were dried in a ventilated oven at 100°C for 5 min. The area containing IAA from the unsprayed chromatogram (developed in isopropanol:ammonia:water) corresponding to the authentic IAA were removed and eluted in 2 ml of methanol (elution time, 30 min). For quantitative estimation, 1 ml of the eluant was mixed with 2 ml of Salkowski reagent (one ml of 0.5 M ferric chloride in 50 ml of 35% v/v perchloric acid, prepared freshly each time), followed by incubation of the

mixture in the dark for 1 hr. The absorbance was recorded at 530 nm against the solvent reagent blank and the concentration of IAA was measured using a standard curve and expressed as µg IAA/g fresh wt of the tissue.

Extraction of nucleic acid

Extraction of *in vivo* tissues was done according to the method of Mahadevan and Sridhar (1982). Equal amount of freshly collected normal and gall tissues were taken, cut into small pieces 1-2 cms and immediately extracted in hot ethanol (80%), macerated in a mortar with pestle. The homogenate was centrifuged at 4,000 g for 20 min. The supernatant was discarded and the pellet was suspended with 2 ml -5% trichloroacetic acid (TCA) at 0°C for 30 min in an ice bath. After 30 min, the sample was again centrifuged as above and the supernatant was discarded. This process was repeated twice and the pellet was reextracted once with absolute ethanol and twice with hot ethanol-ether (50/50-v/v) mixtures, every time discarding the supernatant after centrifugation. The pellet contained proteins and nucleic acid. The extraction of *in vitro* tissues was done using the same procedure described above. Both normal and gall tissues were subjected to growth regulator treatments described in Chapter III.

Nucleic acid

The pellet so obtained from the above extraction of normal and gall tissues were suspended separately in 3 ml of 0.3 N KOH and incubated overnight at 37°C. It was then centrifuged at 5,000 g at 0°C for 20 min and the supernatant was collected. To the residue, 2 ml distilled water was added, centrifuged as above and the supernatant was pooled together for clarification.

Separation of DNA and RNA

The above aliquot was acidified to pH 2 with perchloric acid to precipitate DNA and excess of potassium perchlorate ($KClO_4$), centrifuged at 5,000 g for 20 min. The supernatant contained RNA and the residue contained DNA. The RNA present in the supernatant was directly estimated while the residue was dissolved in perchloric acid to remove excess of perchlorate.

Estimation of RNA

The absorbance of the above extract was measured at 260 nm in UV-265 Shimadzu spectrophotometer and the concentration of RNA was calculated from a standard curve prepared with yeast RNA and expressed as µg/g fresh wt.

Clarification of DNA

The residue containing DNA was collected and suspended in suitable aliquot of 5% perchloric acid at 0°C, centrifuged at 5,000 g for 20 min and the supernatant was discarded. The process was repeated twice. The residue was washed with absolute ethanol-ether mixture, centrifuged at 2,000 g for 10 min and the supernatant was discarded and the residue was collected and suspended with a suitable volume 0.5 N perchloric acid and incubated at 90°C for 10 min in a constant temperature water bath. The solution was then centrifuged and the supernatant was collected. The residue was washed twice with water and the supernatant pooled together to make up the volume. To a known aliquot, equal volume of 1 N KOH was added to precipitate excess perchlorate as $KCLO_4$, centrifuged at 5,000 g for 20 min and the absorbance of the supernatant containing the DNA was measured at 260 nm. The concentration of DNA was calculated from a standard curve prepared with DNA and is expressed as µg/g fresh wt of tissue.

Colorimetric estimation of RNA

In a test tube, 0.2 ml of the extract containing RNA, 2 ml of orcinol reagent [10 ml orcinol (1% in water) was mixed with 40 ml conc HCl and 1 ml of ferric chloride (10%

solution)7 were taken and a reagent blank was also prepared. Each test tube was covered with aluminium foil and heated at 100°C constant temperature water bath for 10 min. The test tube was then cooled in a running water bath. The heavy intense coloured solution was diluted to 10 ml with n-butanol. The absorbance of the solution was measured at 665 nm in a colorimeter and concentration of RNA was determined utilizing the standard curve prepared with ribose. The pentoses present are expressed as µg/g fresh wt of tissue.

Colorimetric estimation of DNA

The extract was diluted to a suitable amount with 0.5 N perchloric acid, mixed with 4 ml of diphenylamine reagent (1.5 g of diphenylamine was dissolved in 100 ml glacial acetic acid followed by the addition of 1.5 ml conc H_2SO_4). The test tubes containing the mixture were incubated overnight at 30°C and the absorbance of these solutions was measured at 600 nm. The concentration of DNA is expressed as µg/g fresh wt of tissue.

Estimation of protein

The estimation of protein was done following Mahadevan and Sridhar (1982). The pellet so obtained from

previous extraction was suspended in 1 ml N-NaOH at 100°C for 5 min. 5 ml of alkaline cupper reagent [(50 ml 2% Na_2CO_3 (in 0.1 N NaOH) and 1 ml 0.5% $CuSO_4.5H_2O$ (in 1% sodium potassium tartarate)] freshly prepared. The mixture was allowed to stand at room temperature for 20 min, then 0.5 ml Folin-ciocalteu reagent (1 ml Folin-ciocalteu and 9 ml distilled water) was added quickly, shaken and incubated. Folin -ciocaltue reagent blank was used for callibrating the absorbance. After 30 min the absorbance was read in a colorimeter at 750 nm. The amount was calculated from the standard curve prepared with bovine serum albumin and is expressed as mg/g fresh wt of tissue.

Extraction of amino acids

The separation, identification and quantification of amino acids were done using the procedure of Mahadevan and Sridhar (1982). 10 g each of fresh normal and gall tissues were cut into small pieces (1-2 cm) and plunged into 100 ml boiling ethanol for 5 min, cooled and crushed in mortar with pestle. The pulp was then squeezed through 4 layers of cheese cloth. The residue was washed with 50 ml alcohol (80%) and squeezed out. The extracts were pooled together, partitioned twice with equal volume of petroleum ether to remove pigments using separatory funnel. The

petroleum ether fraction was discarded. The pigment free alcohol extract was evaporated to dryness at 40°C in a water bath. The alcohol-free residue was dissolved in 50 ml of distilled water and centrifuged for 20 min at 5,000 g. The supernatant was collected and the sediment was washed again with 10 ml distilled water. The washed aliquots were pooled together with the previous supernatant and the solution was allowed to pass through Dowex 50 WxH+ column (20-50 mesh). Before loading, the column was thoroughly washed several times with tap water followed by distilled water till the pH of the effluent was close to neutrality. The flow rate was adjusted to approximately 20 drops per min. The cation exchange column was washed with double distilled water and finally the amino acids present in the column were eluted with 50 ml of 2 M NH_4OH. The eluent was evaporated to dryness until no ammonia odour was detected. The residue was dissolved with 5 ml of distilled water and the pH was adjusted to 2.0 with 2 M formic acid to release remaining ammonia. The solution was evaporated to dryness and the residue dissolved in 5 ml water.

Quantitative estimation of amino acids

1 ml of above mentioned extract was taken in a test tube followed by addition of 1 ml of ninhydrin reagent

[800 mg of hydrated stannous chloride in 500 ml citrate buffer at pH 5.0 (21 g citric acid in 200 ml of 1 N NaOH) and 20 g of recrystallized ninhydrin in 500 ml of methyl cellosolve (prepared by mixing the two freshly before use)7. The contents in the test tube were heated in boiling water bath for 15 min. Then 5 ml diluent solution (equal volume of glass distilled water and n-propanol) was added to each test tube while still on water bath. Blank was maintained with 1 ml distilled water instead of the sample. The test tube was removed and cooled under running tap water, and contents were thoroughly mixed. The absorbance of the purple colour solution was measured in a colorimeter at 570 nm. The concentration of the amino acid was calculated using the standard curve prepared from glycine and is expressed as µg/g fresh wt of tissue.

Separation and identification of amino acids by paper chromatography.

The same extract prepared for quantitative estimation was used. 0.5 ml each of the sample of both gall and normal was spotted on No. 1 Whatman Chromatographic paper. The remaining extract was applied to the paper by streaking the sample across the paper and developed unidimensionally and descendingly.

The chromatographic paper with both standard and samples loaded on it was first equilibrated for 30 min in an air tight glass chamber saturated with the 100 ml solvent vapour (n-butanol: glacial acitic acid:water-4: 1:5 v/v/v upper layer organic phase)at room temperature. After 30 min, the paper was developed descendingly without disturbing the saturated atmosphere for 12 hrs. After developing the paper was dried for nearly 20 hrs under hood to get rid of solvent varpours. It was then sprayed with ninhydrin reagent (300 mg of commercial ninhydrin in 100 ml ethanol) dried at room temperature for 30 min and then heated at 100°C for 5 min in ventillated oven- Amino acids appeared as various shades of purple coloured spots. All the spots were marked with pencil and the Rf value of each spot was calculated by the formula :

$$Rf = \frac{\text{Movement of the spot from the origin}}{\text{Movement of the advancing liquid from the origin (solvent front)}}$$

The distance of the spot was taken from the origin to the center of the spot showing maximum intensity. The sample was identified by comparing with the standard spots.

EXPERIMENTAL RESULTS

Auxin contents

The galll tissues of both S. khasiana and S. wallichi contained more auxin than the normal tissues. The difference being more in the later species (Table 12).

Nucleic acid contents

The contents of DNA and RNA were more in the in vivo gall tissues of both S. khasiana and S. wallichi as compared to respective normal tissues (Table 13). The estimations of nucleic acids at 260 nm were comparable with colorimetric estimation.

The gall tissues of S. khasiana contained more DNA in 2,4-D and IAA treatments while in NAA and IBA treatments the normal tissue had higher DNA contents (Table 14). In case of S. wallichi the gall tissue possessed higher DNA contents compared to normal except IBA treatment. A general increase in RNA content was observed in the gall tissues compared with normal subjected to different growth regulator treatments.

Protein

The protein contents were higher in the gall tissues of both the species as compared to normal except for

Table 12. Auxin content in *in vivo* normal and gall tissues of *S. khasiana* and *S. wallichi*

µg/g fresh wt			
S. khasiana		*S. wallichi*	
Normal	Gall	Normal	Gall
0.028 ± 0.002	0.034 ± 0.001	0.016 ± 0.001	0.032 ± 0.002

± S.E.

Table 13. Nucleic acid contents in *in vivo* normal and gall tissues of *S. khasiana* and *S. wallichi*

Tissues	Nucleic acid µg/g fresh wt			
	260 nm		Colorimetric estimation	
	DNA	RNA	DNA	RNA
S. khasiana				
Normal	0.04 ± 0.002	0.10 ± 0.02	0.02 ± 0.001	0.08 ± 0.002
Gall	0.08 ± 0.02	0.15 ± 0.03	0.07 ± 0.002	0.15 ± 0.03
S. wallichi				
Normal	0.06 ± 0.001	0.14 ± 0.02	0.03 ± 0.001	0.10 ± 0.01
Gall	0.08 ± 0.004	0.16 ± 0.02	0.10 ± 0.03	0.12 ± 0.01

± S.E.

Table 14. DNA and RNA contents in in vitro normal and gall tissues of S. khasiana and S. wallichi grown in media containing different auxins

Treatment (2 mg/l)	Nucleic acid µg/g fresh wt							
	S. khasiana				S. wallichi			
	Normal		Gall		Normal		Gall	
	DNA	RNA	DNA	RNA	DNA	RNA	DNA	RNA
NAA (Control)	0.34±0.06	1.60±0.18	0.30±0.08	1.62±0.07	0.30±0.06	1.32±0.12	0.67±0.07	2.30±0.84
IBA	0.40±0.07	1.62±0.14	0.27±0.06	1.64±0.09	0.56±0.13	2.99±0.66	0.49±0.11	2.48±0.45
2,4-D	0.33±0.04	2.32±0.27	0.70±0.04	4.18±0.10	0.37±0.06	1.48±0.18	1.73±0.14	6.69±2.33
IAA	0.30±0.04	1.23±0.11	0.40±0.06	2.23±0.34	0.22±0.02	1.09±0.22	0.42±0.05	1.89±0.27

± S.E.

Table 15. Protein contents in normal and gall tissues of S. khasiana and S. wallichi grown in media containing different auxins

Treatment (2 mg/l)	Protein mg/g fresh wt			
	S. khasiana		S. wallichi	
	Normal	Gall	Normal	Gall
NAA (Control)	0.23 ± 0.03	0.47 ± 0.08	0.44 ± 0.03	0.68 ± 0.05
IBA	0.04 ± 0.001	0.56 ± 0.08	0.42 ± 0.02	0.72 ± 0.05
2,4-D	0.67 ± 0.06	0.18 ± 0.01	0.84 ± 0.05	0.03 ± 0.002
IAA	0.35 ± 0.04	0.10 ± 0.01	0.37 ± 0.02	0.15 ± 0.01

± S.E.

Table 16. Identification of amino acids present in S. khasiana normal and gall tissues

Amino acid	Rf value		
	Standard	Normal	Gall
Lysine monohydrochloride	0.023	0.026	0.026
Arginine monohydrochloride	0.05	0.04	0.04
Aspartic acid	0.08	-	0.08
Hydroxyproline	0.125	-	0.13
Tryptophan	0.17	0.18	0.18
DL-alanine	0.24	0.25	0.26
2-amino butyric acid	0.29	0.30	0.30
Methionine	0.39	-	0.38
Unidentified	-	-	0.45
Isoleucine	0.56	-	0.54
DL-nor-Leucine	0.60	-	0.61
Unidentified	-	-	0.75

Table 17. Amino acid contents in in vitro normal and gall tissues of S. khasiana and S. wallichi grown in media containing different auxins.

Treatment (2 mg/l)	Amino acids µg/g fresh wt			
	S. khasiana		S. wallichi	
	Normal	Gall	Normal	Gall
N&A (Control)	300.0 ± 1.2	350.0 ± 0.4	100.0 ± 0.2	450.0 ± 0.8
IBA	725.0 ± 0.6	535.0 ± 0.7	118.0 ± 0.2	111.0 ± 0.4
2,4-D	100.0 ± 0.1	187.0 ± 0.2	285.0 ± 0.4	400.0 ± 0.5
IAA	452.0 ± 0.4	1700.0 ± 1.3	100.0 ± 0.2	1000.0 ± 1.2

± S.E.

2,4-D and IAA treatments (Table 15).

Qualitative and Quantitative estimation of amino acids

In vivo normal tissue of S. khasiana contained 5 amino acids whereas the gall tissue contained 12 amino acids (Table 16).

The amount of amino acids in the in vitro gall tissues of S. khasiana were lower as compared to normal except for IAA treatment (Table 17). However, in case of S. wallichi the gall tissues exhibited higher amino acid contents as compared to normal in all treatments except for IBA.

DISCUSSION

The hormone autonomy of Schima gall tissues in culture and the tumorous outgrowth of transformed cells in the intact plant are due to higher levels of auxin and cytokinin. Higher amount of auxin content was observed in the gall tissue as compared to normal (Table 12). Similar results have been reported in crown gall (Meins, 1982), in insect/mite incited stem galls (Tandon and Arya, 1980a) and leaf gall (Madden and Stone, 1984). Variation in auxin levels during the growth of crown-gall tissues in culture has

also been shown (Nakajima et al., 1981; Pengelly and Meins, 1982; Zofia, 1984a).

Studies on tumour morphology lent support to the idea that hormones play a central role in maintaining the tumourous state. Chromosomal irregularities (Heinz and Mew, 1971) and a change in genetic expression (Heinz et al., 1977) have been reported for sugarcane culture induced and maintained on the media containing 2,4-D. The use of 2,4-D in tissue cultures caused cytological changes that result in the phenotypic differences between the donor plant and the plant regenerated from callus (Holgate, 1977). These may play a part in the occurrence of irregularities in cell division, the frequency of polyploidy increases and the frequencies of mitosis irregularities decreases. It may be argued that if an endogenous hormone has a certain effect on the process, additional hormones should enhance or promote the effect, provided that the endogenous hormone is not present at an optimal concentration. It is known that a certain process occurs at a particular stage of development and it is thought to be hormone dependent. It has now been established that hormone rarely determine the way in which a cell or tissue may respond but rather than in most cases their role is more likely to be that of inducing certain response the nature of which is pre-determined by factor(s)

intrinsic to the cell. In other words it is the programming of the cell.

It has remained a fact for about two decades that the physiological autonomy exhibited by the plant cancer cell reflects an abnormal growth hormone metabolism (Stonier, 1972). Excess auxins were found to be associated in various abnormal growths (Purohit et al., 1979; Tandon and Arya, 1982; Joshi and Tandon, 1984). Henderson and Bonner (1952) reported that normal tissues of sunflower contained an inhibitor that prevented the conversion of tryptophan to IAA while it was absent in tumor tissues. The presence of auxin protectors has also been reported (Tandon and Arya, 1980b). This favours excess accumulation of auxins in the gall tissue.

In plant cells, the accumulation of IAA could be regulated by three factors: (i) substrate availability (ii) the activity of IAA biosynthetic enzymes, (iii) the activity of the enzymes controlling the IAA catabolism or conjugation. The regulation of enzymes of IAA biosynthesis has proved difficult to establish because of the lack of substrate specificity (Truelsen, 1972). The spontaneous oxidative de-carboxylation of indole pyruvic acid to breakdown product, which included IAA, have led to the proposal that IAA

biosynthesis is not regulated by the change in catalytic activity but rather by the availability of the substrate (Sheldrake, 1973). The indole metabolising enzyme systems have not been observed in many tropical plants (Kunapuli and Vaidyanathan, 1985). There could be two possibilities, either the indole metabolising enzyme system is completely absent or if present, could be inactivated by polyphenols present in the tissues.

Increased auxin synthesising capacity of the gall was recognised (Bouillene and Gaspar, 1970). Tandon and Arya (1980a) suggested that Zizyphus gall tissues converted more auxin from tryptophan as compared to normal tissues. The increased levels of IAA in gall tissues may or may not be due to the expression of the new pathway. The elevated auxin in tumours are probably due to enhanced expression of the normal plant pathway for IAA biosynthesis (Joshi, 1986). Earlier studies in tumours have been reviewed (Nester et al., 1984) and subsequent work (Amasino and Miller, 1982; Pengelly and Meins, 1982, 1983; Weiler and Spanier, 1981) has provided further confirmation that IAA levels are elevated over those of untransformed tissues. However, there is little agreement on the time course of IAA concentration changes during tumour growth.

Schima gall tissues can grow on phytohormone-free medium a situation similar to crown-galls (Braun, 1982). Several results indicated that the phytohormone autotrophic growth of the transformed tissues is due to T-DNA induced elevation of the endogenous levels of auxin and cytokinin (Atsumi and Hayashi, 1978; Einset, 1980; Nakajima et al., 1981; Weiler and Spanier, 1981; Amasino and Miller, 1982). Based on the analogy between the effect of phytohormones in non-transformed plant tissues (Skoog and Miller, 1957) and the effect of mutation in the genes on the morphology of transformed tissues, the activity of the genes of T-DNA were predicted (Garfinkel et al., 1981; Leemans et al., 1982; Joos et al., 1983; Akiyoshi et al., 1984; Barry et al., 1984)

Phytohormone independent growth and suppression of differentiation are observed in Agrobacterium induced tumours on a very wide range of dicotyledonous plants (DeCleene and Deley, 1976) which might suggest that the introduced T-DNA (Nester et al., 1984) altered common basic plant control mechanisms. Two genes code for the synthesis of IAA from tryptophan via indole-3-acetamide (Thomashow et al., 1986; Schröder et al., 1984) and the third gene codes for an isopentanyl transferase which catalyses the first step of cytokinin biosynthesis (Barry et al., 1984). It is reported

that the morphology of non-transformed tobacco tissue in culture can be regulated by the ratio of auxin and cytokinin provided in the growth medium (Skoog and Miller, 1957). Direct measurement of hormone in crown-gall tumours that vary in morphology have shown auxin-cytokinin ratio in tumour as predicted by the hormone feeding experiment (Amasino and Miller, 1982; Akiyoshi et al., 1983; Van Onckelen et al., 1984). Enzyme catabolising cytokins have been investigated by several workers (Mc Craw and Horgan, 1983). This enzyme catalyses removal of the isopentanyl side chain of cytokinin. IAA exogenously applied to plant tissue is degraded with rate and degree of destruction which vary considerably with the tissue and physiological conditions (Davies, 1972; Thimann ,1972). Crown gall cells essentially grow because the T-DNA code for and direct the synthesis of auxin (Inze et al., 1984), and cytokinin (Barry et al., 1984). However, the regulating pattern of growth is totally unphysiological. Tumour containing the full component of T-DNA grown in a more or less undifferentiated manner and do not respond morphologically to added hormones (Cardarelli et al., 1987).

Several workers have shown effects of cytokinin on protein synthesis (Klambt, 1976). Other workers suggested

specific binding of cytokinin to ribosomes (Fox and Erion, 1975) and the acceleration of *in vitro* protein synthesis Klambt, 1976). The biosynthetic pathway of cytokinin is closely related to the metabolism of RNA, especially tRNA (Letham, 1978). Cytokinins have been reported in gall forming wasp larvae (Elzen, 1983). At present it is not clear whether the larvae are secreting cytokinin or accumulating them from the host plant.

Increased nucleic acid in gall tissues has been observed. Tissues cultivated *in vitro* exhibited a higher DNA and RNA content as compared to *in vivo* tissue of both normal and gall (Tables 13 and 14). Increase in DNA during tumour induction (Klein *et al.*, 1953), after wounding (Kupila and Stein, 1961) has been reported. The DNA thus synthesized play a role in tumour induction. Mathysse and Philips (1969) reported that RNA synthesis by tobacco or soybean nuclei was stimulated by addition of auxin 2,4-D provided that it was also included in the nuclear isolation medium. It was inferred that, in the absence of 2,4-D, some factors required for the auxin response was lost from the nuclei. Both DNA and RNA content of the tissue in the present studies are directly influenced by the external application of auxin in the medium. Highest amount of DNA and RNA was observed in the medium containing 2,4-D for gall tissue as

compared to normal. Higher DNA contents in Zizyphus gall tissues grown in NAA containing medium was reported (Tandon et al., 1976). On the other hand, the RNA content increased in the normal tissue by increasing the NAA content in the medium while no appreciable change was recorded in the gall tissue.

In the present findings higher RNA contents in the gall tissues than normal were recorded in response to auxins. Key and Vander hoef (1973) concluded that in tobacco pith callus different IAA concentration which exist induced different reaction in the tissue and a difference in nucleic acid contents of the cells. Thus low concentration of IAA (2 mg/l) a condition suitable for normal leaf callus to grow may induce cell division but little cell enlargement and increase the amount of RNA, while gall tissue which required less auxin in the treatment medium cause mainly cell enlargment and decrease in the RNA content of the cell due to higher levels of IAA.

The importance of RNA metabolism in auxin action has been confirmed by a number of workers employing various plant systems. Matsuda (1967) suggested that at least two types of RNA participated in the auxin induced expression of growth. One is responsible for the cell state preparatory

to the following auxin action, the other is produced by the action itself. There is little known about the initial interaction of hormone with cellular components, although there is more information available for auxin than for any other plant hormones. Nevertheless, there are reports that auxin-receptor complex possess biological activities. A receptor has been found in nuclei of tobacco and soybean tissue cultures (Mathysse and Philip, 1969) and auxin plus the factors stimulated RNA (Jacobson, 1977).

Proteins are known to play a key role in the metabolism directly resulting in the growth phenomena. RNA and protein metabolites are initiated in plant tissue in response to infection. Thus various kinds of proteins are synthesized from free amino acid that pre-exist and are hydrolysed from some storage proteins. Hormones are known to play a great role as endogenous factors in metabolism and are directly involved in the protein synthesis. A perusal of literature suggests that the effect of hormones on growth and differentiation are conditioned by an undisturbed protein synthesis. Growth expansion induced by auxin on plant tissue is strongly suppressed by inhibitors of protein synthesis in plants (Patterson and Trewavas, 1967) with differential increase or decrease in the level of enzymes. In many host parasite interactions changes in protein profile and isozyme

of many enzymes have been reported (Kado, 1976; Joshi and Tandon, 1984; Vanloon, 1985; Ebmerman and Lick, 1986). The isozymes served as unique markers in a wide range of plant species as they are directly linked with genes.

It is now accepted that continued RNA and protein synthesis are required if growth is to be sustained. Auxin could stimulate synthesis of protein and RNA in elongating tissues (Key and Ingle, 1964). This led to the proposition of the so called gene activation hypothesis which suggested that auxin depressed certain genes, the depression of which was necessary for the extension process. However, if inhibitors of protein synthesis could inhibit growth immediately or nearly so, inhibitors of RNA synthesis did not. It led to the idea of the existence of an unsuitable protein whose synthesis was dependent on auxin and which was pre-requisite of induction of growth. Most studies agree that DNA synthesis takes place prior to differentiation and that hormones affect this synthesis (Robert, 1976).

The amino acids present in Schima gall and normal plant part were mostly similar with the exception of the two unidentified spots present in the gall. However, the concentration of spots differed as noted by the colour intensity. Excess amount of amino acids present in the gall as compared to normal might be due to their particular role in the gall development.

Chapter V

Summary

Schima khasiana Dyer and S. wallichi Choisy are two economically important tree species of the North-East India. The trees are heavily infested with the insect Trioza sp. which results in the gall formation on the infected leaves.

Plant tissue culture methods utilising the exogenous application of nutrients, hormones, etc. are one of the powerful tools for understanding the physiological requirements of plants and also the mode of action of such substances. The general growth characteristics of callus involve a complex interation among plant materials used to initiate callus, the composition of the medium and the environmental conditions during the incubation period.

Browning of the explants is one of the major problems in tissue culture. Though pre-soaking of the explants with different antioxidants like PVP and incorporation of activated charcoal in the medium remarkably reduced the browning of explants of Schima normal and gall tissues, it did not help in callus initiation. Only the young normal and gall leaf tissue were able to throw callus, whereas mature explants were hard and recalcitrant.

In the present study, normal and gall tissues of S. khasiana and S. wallichi were successfully isolated on

MS medium containing 2,4-D (4-10 mg/l) and kinetin (0.04 mg/l), and maintained on the medium containing NAA (1-4 mg/l) and kinetin (0.04 mg/l). The optimum concentration of auxin for the growth of calli varied for the different type of tissues. It is generally accepted now that tumor formation is accompanied by a simplification of the nutrient requirements of the host cells. With sucrose as a source of carbohydrate maximum callus weight of normal and gall tissues resulted, of which normal was higher than gall. However, there was a differential growth response to different carbohydrates tested. By reducing the strength of MS medium, the growth of both normal and gall calli declined except for gall calli growth at higher concentration of NAA.

Maximum fresh weight of normal and gall calli was observed in the media containing different concentrations of either 2,4-D or NAA in conjunction with kinetin. Higher concentration proved more suitable for normal callus tissue growth whereas these were inhibitory for the growth of gall. Both IAA and IBA served as poor sources of auxins for the growth of normal and gall tissues. In vivo gall tissues contained higher concentrations of auxin which could account for their disorganised growth.

Important metabolic changes are involved during the transformation of normal to tumorous growth. Quantitative and qualitative variations in enzyme activities have been well documented. In vivo gall tissues of both the species of Schima as compared to normal, showed more PAL, TAL, PPO activities and O-dihydroxy and total phenol contents. A reverse picture was observed for IAA oxidase and PRO activities. In vitro tissues of both the species grown in media containing different auxins showed more, PRO, PPO and IAA oxidase activities in the gall tissue as compared to normal excepting 2,4-D and IBA treatments where the normal tissue showed more PPO and IAA oxidase activities, respectively. The concentration of enzymes in a cell at any given time is a function of the relative rate of its synthesis and the rate of its degradation. This rate may be affected by such factors as abrupt changes in the environment or administration of hormones. The mechanism underlying these changes are still not clear.

The presence of growth regulators of phenolic nature and their role in plant growth regulation has been a subject of recent studies. In vitro tissues cultured in media containing different auxins showed more phenol contents in the gall tissue as compared to the normal except for IAA and 2,4-D treatments. This may be attributed

to the high PPO activities in the former tissues. The concentration of natural growth regulators in plant tissues is determined by the activities of the two processes, synthesis and degradation. Since both hormone and natural inhibitors are synthesized from the common metabolic precursors, it has been suggested that the initial stages of growth regulation are realised at the level of biosynthesis of the natural growth regulators. Eight phenolic compounds were detected in in vivo tissues of S. khasiana. Out of these, four were identified as caffeic, p-hydroxy benzoic, p-coumaric and ferulic acids, two unidentified distinct spots were present only in the gall suggesting their formation following gall induction. Phenolic acids, both endogenous or exogenously incorporated in the medium influence the growth of in vitro grown tissue. With the 6 phenolic compounds tested for the growth of both normal and gall calli, differential results were obtained. The growth of S. khasiana normal and S. wallichi gall tissues showed an increase with the increase in the concentration of ferulic acid in the medium, and so was the case of S. khasiana gall tissue grown in the medium containing caffeic acid. However, a reverse picture was obtained for other phenolic compounds tested which showed a maximum growth of callus at very low

concentrations of phenolics. The addition of phenolic in the assay mixture enhanced the IAA oxidase activity in normal and gall tissues of S. khasiana as compared to control, suggesting more IAA destruction by higher concentrations of the phenols tested in the present investigation. However, with low concentrations of phenols differential responses were obtained. The callus growth increased when phenolics were incorporated at a suitable concentration. Perhaps, this affected IAA oxidase activities resulting in more auxin contents and unorganised growth of the callus in the medium.

In both in vivo S. khasiana and S. wallichi gall tissues, more auxins, nucleic acids, and protein were found as compared to normal. The in vitro grown tissues showed higher DNA and RNA contents than in vivo tissue. Of the four auxins tested, 2,4-D resulted in maximum DNA and RNA contents.

The more proteins and amino acid contents in the gall tissue as compared to normal suggested their formation following gall induction. The gall tissue contained 5 more amino acids as compared to normal. Hormones are known to play a significant role in the metabolism of amino acids. The natural auxin IAA resulted in the production of maximum amino acid contents than the other sources of auxins tested.

Lastly, from the results obtained in the present investigation, it can be concluded that the gall tissues showed hyperauxinity, simple media requirement, higher PAL, TAL, PPO activities, DNA, RNA, protein, amino acid and phenol contents as compared to normal tissues. A complex interplay of different enzymes, and metabolites seem to be involved in the process of gall induction in S. khasiana and S. wallichi.

BIBLIOGRAPHY

Amasino, R.M. and C.O. Miller. 1982. Hormonal control of tobacco crown gall tumor morphology. Plant Physiol. 69: 389-392.

Ahuja, M.R., and V.K. Gupta. 1974. Control of tumor associated peroxidases in a genetic tumour system in Nicotiana. Experientia 30: 1007-1008.

Akiyoshi, D.E., R.O. Morris, R. Hinz, B.S. Mischke, T. Kosuge, D.J. Carfinkel, M.P. Gordon and E.W. Nester. 1983. Cytokinin-auxin balance in crown gall tumours and regulated by specific loci in the T-DNA. Proc. Natl. Acd. Sci. U.S.A. 80: 407-411.

Akiyoshi, D.E., H.J. Klee, R. Amasino, E.W. Nester and M.P. Gordon. 1984. T-DNA of Agrobacterium tumeraciens codes an enzyme of cytokinin biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 81: 5994-5998.

Arora, D.K. and U. Kant. 1980. Auxin-kinetin interaction of the growth of gall and normal tissues of Emblica officinalis in vitro. Bull. Soc. Bot. Fr. Actual. Bot. 127: 210.

Arya, H.C. 1965. Extractability of nucleic acid from the callus tissue of Phyloxera gall and grape stem. Ind. Phytopathology, Vol. XVIII (3); 282-287.

Arya, H.C., A.C. Hildebrant, and A.J. Riker. 1962. Growth in tissue culture of single cell clones from grape stem and Phylloxera gall. Plant Physiol. 37: 387-392.

Arya, H.C., G.S. Vyas, and P. Tandon. 1975. The problem of tumor formation in plants, p.270-279. In H.Y. Mohan Ram. J.J. Shah, and C.K. Shah (eds.). Form, Structure and Function in Plants. Sarita Publication, Meerut, India.

Atsumi, S., and T.Hayashi.1978. The relationship between auxin concentration, auxin protection and auxin destruction in crown gall cells cultured in vitro. Plant Cell Physiol. 19: 1391-1397.

Bagni, N. and D. Fracassini. 1971. Effect of coumarin derivatives on organogenesis and callus growth in Cichorium intybus roots and Helianthus tuberosus tubers in vitro. Experientia 27: 1239-1241.

Bajaj, Y.P.S. 1986a. Biotechnology in Agriculture and Forestry 1. Trees. Springer-Verlag, Berlin Heidelberg, New York. 515p.

Bajaj, Y.P.S. 1986b. Biotechnology in Agriculture and Forestry 2. Crop Springer Verlag, Berlin Heidelberg, New York. 608p.

Ball, E. 1963. An optically efficient method for observing single plant cells from shake cultures during growth and division. Nature 197: 103-104.

Barendes, G.W.M. 1983. Hormonal regulation of enzyme synthesis and activity, p.1-68. In S.S. Purohit (ed), Aspects of Physiology and Bio-chemistry of plant Hormones. Kalyani Publishers, New Delhi, Ludhiana.

Barry, G.P., S.G. Roger, R.T. Fealy, and L. Brand. 1984. Identification of a cloned cytokinin bio-synthetic gene. Proc. Natl. Acad. Sci. U.S.A. 81: 4776-4780.

Basu, R.S. and V. Tuli. 1972. Auxin activity of 3-methyleneoxindole in wheat. Plant Physiol. 50: 499-502.

Baulcombe, D.C., J. Giongini and J.L. Key. 1980. The effect of auxin on the polyadenylated RNA of Soybean hypocotyls. In C.J. Leaver (ed.), Genome Organisation and Expression in Plants. Plenum Press, New York.

Bayer, M.H., 1982. Genetic tumors: Physiological aspects of tumors formations in inter-species hybrids. p.37-67 In: G .Kahl and J.S Schell (eds.),Molecular Biology of Plant Tumors..cademic Press,New York.

Beardsley, R.E. 1972. The inception phase in the crown gall disease. Progr. Exptt. Tumor, Res. 15: 1-75.

Beart, J.E., T.H. Lilley, and E. Haslam. 1985. Plant polyphenols-secondary metabolism and chemical defence: Some observations. Phytochem. 24: 33-38.

Bevan, M. and D.H. Northcote. 1981. Some rapid effects of synthetic auxin on mRNA levels in cultured plant cell. Planta. 152: 32-35.

Bhansali, R.R., A. Kumar and H.C. Arya. 1978. Polyphenols and related enzymes in normal and gall tissues of Ficus mysorensis Hyne. Indian J. Expt. Biol. 16: 850-851.

Bhojwani, S.S., and M.K. Razdan. 1983. Plant Tissue Culture: Theory and Practice. Elsevier Science Publishers, Amsterdam, 520 p.

Biemann, K., C. Lioret, J. Asselineau, E. Lederer, and J. Polonsky. 1960. On the structure of lysopine, a new amino acid isolated from crown-gall tissue. Biochim. Biophys. Acta 40: 369-370.

Binns, A. and F. Meins, Jr 1973. Habituation and tobacco pith cells for factors promoting cell division in heritable and potentially reversible (heritable differentiation/cytokinin habituation). Proc. Nat. Acad. Sci. U.S.A. 70: 2600-2662.

Black, L.M. 1982. Wound tumor disease, p.69-105. In G. Kahl and J.S. Schell (eds.), Molecular Biology of Plant Tumors. Academic Press, New York, London.

Bohm, B.A., and G.H.N. Towers. 1962. A study of phenolic compound in impatiens. Can. J. Bot. 40: 677-683.

Bolton, G.W., E.W. Nester and M.P. Gordon. 1986. Plant phenolic compounds induced expression of the Agrobacterium tumefaciens loci needed for virulence. Science 232: 983-985.

Bonga, J.M. and C. Chakraborty. 1968. In vitro culture of mistletoe, Arceuthobium pusillum. Can. J. Bot. 46: 161-164.

Bonga, J.M., and D.J. Durzan. 1982. Tissue Culture in Forestry. Martimus Nijhoff/Dr. W. Junk Publishers, The Hague Boston London, 420 p.

Bouckaert-Urban, A.M. and J.C. Vendrig. 1981. The influence of plant growth regulators on crown-gall initiation on cotyledonary leaves of Helianthus giganteus L. in vitro. Z. Pflanzenphysiol. 103: 75-81.

Bouillenne, C., and T. Gaspar. 1970. Auxin catabolism and inhibitors in normal and crown-gall tissues of Impatiens balsamina Can. J. Botany 48: 1159-1163.

Boysen-Jensen, P. 1948. Formation of galls by Mikiola fagi. Physiol. Plant. 1: 95-108.

Braun, A.C. 1958. A physiological basis for the autonomous growth of the crown-gall tumor cell. Proc. Natl. Acad. Sci. U.S.A. 44: 363-371.

Braun, A.C. 1962. Tumor inception and development in the crown gall disease. Ann. Rev. Plant. Physiol. 13: 533-558.

Braun, A.C. 1978. Plant tumors. Biochim. Biophys. Acta 516: 167-191.

Braun, A.C. 1982. A history of crown-gall problem, p.155-210. In: G. Kahl and J.S. Schell (eds.), Molecular Biology of Plant Tumors, Academic Press, New York.

Braun, A.C. and U. Näf. 1954. A non-auxinic growth-promoting factor present in crown gall tumor tissue. Proc. Soc. Exp. Biol. Med. 86: 212-214.

Braun, A.C. and T. Stonier, 1958. Morphology and Physiology of Plant Tumors. Protoplasmatologia 10: 5a-Springer Verlag, Vienna, pp.93.

Bray, H.G., W.V. Thorpe, and K. White. 1950. The fate of certain organic acids and amines in the rabbit. The application of paper chromatography to metabolic studies of hydroxybenzoic acid and amides. Biochem. J. 46: 271-275.

Briggs, W.R., and P.M. Ray. 1956. An auxin in-activation system involving tyrosinase. Plant Physiol. 31: 165-167.

Brinberg, P.R., S.S. Rao and J.A. Lippincott. 1983. Octopine dehydrogenase of a Vinca rosea crown gall tumor. Phytochemistry (oxf) 22(6): 1345-55.

Browder, L.E. and M.G. Eversmeyer, 1986. Parasites host specificity and resistance/susceptibility, two concepts, two perspectives. Phytop~athol. 7: 379-381.

Brown, S.A., and M. Tenniswood. 1974. Aberrant coumarin metabolism in crown-gall tissues of tobacco. Can. J. Botany 52: 1091-94.

Butcher, D.N. 1973. The origins, characteristics and culture of plant tumor cells, p.356-391. In: H.E. Street (ed.), Plant Tissue and Cell Culture. Botanical monographs, Vol. 11. Blackwell Scientific Publications, Oxford.

Butcher, D.N. 1977. Plant tumor cells, p.429-461. In: H.E. Street (ed.), Plant Tissue and Cell culture. Blackwell Scientific Publications, Oxford.

Byers, J.A., J.W. Brewer, and D.W. Denna. 1976. Plant growth hormones in pinyon insect galls. Marcellia. 39: 125-134.

Camm, E.L., and G.H.N. Tower. 1973. Phenylalanine amonia-lyase. Phytochemistry 12: 961-973.

Cadarelli, M., L. Spano, D. Mariotti, M.L. Mauro, M.A.V. Slys, and P. Constantine. 1987. The role of auxin in hairy root induction. Mol. Gen. Genet 208: 457-463.

*Chang, M.C. and D.G. Lynn. 1986. J. Chem. Ecol. 12: 561-579.

Chong, C., and C.D. Taper. 1974. Influence of light intensity on sorbitol metabolism, growth and chlorophyll content of malus tissue culture. Ann. Bot. 38: 359-362.

Chilton, M.D., M.H. Drumond, D.J. Merlo, P. Sciaky, A.L. Montoya, M.P. Gordon and E.W. Nester. 1977. Stable incorporation of plasmid DNA into plant cells: The molecular basis of crown-gall tumorigenesis. Cell. 11: 263-271.

Coffin, R., C.D. Taper, and C. Chong. 1976. Sorbitol and sucrose as carbon source for callus culture of some species of Rosacceae. Can. J. Bot. 54: 547-551.

Conclaves, A., M.A. Machado, L.S. Caldas, W.R. Sharp, and H.A. Mello, 1977. Tissue culture on Eucalyptus pp.509-526, In: W.R. Sharp, P.O. Larsen, E.F. Paddock and V. Raghavan (eds.), Plant cell and Tissue Culture Principle and application Ohio State University Press, Columbia.

Cotton, R.G., F. Gibbon. 1965. The biosynthesis of phenylalanine and tyrosine. Enzymes converting chorismic acid into prephenic acid and their relationship to prephenate dehydratase and prephenate dehydrogenase. Biochim. Biophys. Acta 100: 76-78.

Daly, J.M., B. Dudden, and P. Seevers. 1971. Biochemical comparisons of resistance to wheat stem rust disease controlled by the Sr 11 alleles. Physiol Plant Pathol. 1:397-407.

d'Amato, F. 1977. Cytogenetics of differentiation in tissue and cell cultures. p.343-357. In: J. Reinert and Y.P.S. Bajaj (eds.), Plant Cell, Tissue and Organ Culture. Springer-Verlag, Berlin, New York.

Dangar, T.K., and P.S. Basu. 1984. Seasonal changes and metabolism of plant hormones in root nodules of Lens sp. Biol. Plant. 26: 253-259.

Davies, M.E. 1972. Effects of auxin polyphenol accumulation and the development of phenylalanine ammonialyase activity in dark grown suspension cultures of Paul's Scarlet Rose. Planta 104: 66-67.

de, Fossard, R.A. 1976. Tissue culture for plant propagations. University of New England, Armidate, Australia 2351, viii-409.

de, Fossard, R.A. 1978. Tissue culture propagation of Eucalyptus ficifolia, pp.425-438. In: Proc. of Symp. on Plant Tissue Culture, May 25-30, Science Press, Peking.

Decleene, M. and H. Daley. 1976. The host range of crown gall. Botanical reviews. 40: 389-466.

Depicker, A., M. Van Montagu, and J. Schell. 1978. Homologous DNA. Sequences in different Ti-plasmids are essential for oncogenicity. Nature 275: 150-153.

Dixon, R.A., J. Browne, and M. Ward. 1980. Modulation of L-phenyl-alanine-ammonia-lyase by pathway intermediates in cell suspension cultures of dwarf French bean (Phaseolus vulgaris L.) Plant. 150: 279-285.

Dixon, A.F.G. 1983. Insect-induced phytotoxemias: Damage, tumors, and galls. p.297-314. In: K.F. Harris (ed.), Current Topic in Vector Research, Vol.1. Praeger Publisher, New York.

Dodds, J.D. 1985. Plant Genetic Engineering. Cambridge University Press, New York. 320 p.

Don, J.J.M., M. Valentyn, A. Schilperoort, and P. Van Duijn. 1974. Nuclear DNA content and phytohormone requirements of normal, crown gall, and habituated tissues of Nicotiana tabacum var. White. White Burley. Exp. Cell. Res. 89: 283-294.

Drummond, M.H., M.P. Gordon, E.W. Nester and M.D. Chilton. 1977. Foreign DNA of bacterial plasmid origin is transcribed in crown gall tumors. Nature 269: 535-536.

Durzan, D.J., and V. Chalupa. 1984. Special problem: Adults vs Juvenile explants, p.471-503. In: W.R. Sharp, D.A. Evans, P.V. Ammirato, and Y. Yamoda (eds.), Handbook of plant cell culture, Vol.2. Crop species. MacMillan Publication Company, New York.

Eberman, R., and E. Lick. 1986. The possible role of peroxidase isoenzymes in the infection of English oak by Loranthus europaeus. Phytopathol. 75: 1102-1104.

Einset, J.W. 1980. Cytokinins in tobacco crown-gall-tumors. Biochem. Biophys. Res. Communication 93: 510-515.

Einset, J.W. 1985. Cytokinin metabolism in autonomous and crown-gall tissue cultures. Biol. Plant. 27: 276-280.

Einset, J.W., and F. Skoog, 1973. Biosynthesis of cytokinins in cytokinin-autotrophic tobacco callus. Proc. Natl. Acad. Sci. U.S.A. 70: 658-660.

Ellis, J.C., M.H. Ryder, and M.E. Tate. 1984. Agrobacterium tumerfaciens TR-DNA encodes a pathway for agropine biosynthesis. Mol. Gen. Genet 195: 466-473.

Elzen, G.W. 1983. Cytokinins and Insect gall. Comp. Biochem. Physiol. A.76: 17-19.

Endo, T. 1968. Indoleacetic acid oxidase activities of horseradish and other plant peroxidase isozymes. Plant. Cell Physiol. 9: 333-341.

Engelsma, G. 1968. Photo-induction of phenylalanine diaminase in Gerkin seedling. iii. Effects of exicion and irradiation on enzyme development in hypocotyl segment. Planta 82: 355-368.

Esenowo, G.J. 1986. The initiation and maintenance of callus from tubers of Colocasia esculenta and Dioscorea rotundata. Environ. Exp. Bot. 26: 159-162.

Farkas, G.L., and Z. Kiraly. 1962. Role of phenolic compounds in the physiology of plant disease and disease resistance. Phytopath. Z. 44: 105-150.

Feucht, W., and P.P.S. Schmid. 1980. Effect of O-dihydroxy phenols on growth and protein pattern of callus cultures from Prunus avium. Physiologia Plant. 50(3): 309-313.

Flurley, W.H. 1986. Polyphenoloxidase in higher plants: Immunological selection and analysis of in vitro translation products. Plant Physiol. 86: 614-618.

Fosket, D.E. 1980. Hormonal control of morphogenesis in cultured tissues pp.363-369. In: Skoog, F. (ed.), Plant Growth Substance, Springer, Berlin Haidelberg N.Y.

Fox, J.E., and J.L. Erion, 1975. A cytokinin binding protein from higher plant ribosomes. Biochem. Biophys. Res. Commun. 64, 694-700.

Frič, F. 1971. Enzyme des Indolylessigsaureabbaues in Gerstenblattern. Biologia (Bratislava) 26: 677-688.

Frič, G., 1976. Oxidative enzymes, p.617-631. In R. Heitefuss and P.H. Williams (eds.), Physiological Plant Pathology, Encyclopedia of Plant Physiology, Vol.4, Springer-Verlag, Berlin Heidelburg New York.

Friend, J. 1979. Biochemistry of plant phenolics. p.557. In: T. Swain, J.B. Harborne and C.F.W. Sumere (eds.), Recent Advance in Phytochemistry Vol.12.

Fridborg, G., and T. Erikcson. 1975. Effect of activated charcoal on growth and morphogenesis in cell cultures. Physiol Plant. 34: 306-308.

Galston, A.W., and W.K. Purves. 1960. The mechanism of action of auxin. Ann. Rev. Plant Physiol. 11: 239-276.

Garfinkel, D.I., R.B. Simpson, L.W. Reaur, F.F.White, M.P. Gordon and E.W. Nester. 1981. Genetic analysis of crown-gall: Fine structure of the map of the T-DNA by site-directed mutagenesis. Cell 27: 147-182.

Gautheret, R.J. 1946. Plant tissue culture. Growth 10: 21-43.

Gautheret, R.J., 1947. Existe-til un cancer des plantes? Universite de Paris. Conference au Palais de la De'couvente. Paris, France.

Gautheret, R.J. 1955. Sur la varibilite des proporietes physiologiques des cultures de tissus vege'taux. Rev. Gen-Botan. 62: 1-106.

Gelinas, D.A. 1973. Proposed model for the peroxidase-catalysed oxidation of indole-3-acetic acid in the presence of the inhibitor ferulic acid. Plant Physiol. 51: 967-972.

Gordon, S.A. and L.G. Paleg. 1961. Formation of auxin from tryptophan through action of polyphenols. Plant. Physiol. 36: 838-45.

Gove, J.P., and M.C. Hoyle. 1975. The isozymic similarity of indoleacetic acid oxidase to peroxidase in birch and horseradish Plant. Physiol. 56: 684-687.

Guifoyle, J.J., C.Y. Lin, Y.M. Chen, R.T. Nagaad and J.L. Key. 1975. Enhancement of soybean RNA polymerase I by auxin. Proc. Natl. Acad. Sci. U.S.A. 72: 69-72.

Gurley, W.B., J.D. Kempt, M.J. Albert, D.W. Sutton, and J. Callis. 1979. Transcription of Ti plasmid derived sequences in three octopine type crown gall tumors lines. Proc. Natl. Acad. Sci. U.S.A. 76: 2828-2832.

Haard, N.F. 1978. Isolation and partial characterization of auxin protectors from Synchytrium endobioticum incited tumor in potato. Physiol. Plant Pathol. 13: 223-232.

Hanson, K.R., and E.A. Havir. 1981. Phenylalanine ammonia-lyase. p.577-625. In: E.E. Conn (ed.), The Biochemistry of Plants, Vol. 7. Secondary Plants Products. Academic Press, New York.

Harborne, J.B. 1980. Plant Phenolic. Encyclopedia of Plant Physiology, Vol. 8: 329-402.

Harris, P.J., and Hartley, R.D. 1976. Phenolic acid in cell walls. Nature (London) 259: 508.

Heble, M.R., S. Narayanswamy, and M.S. Chadha. 1976. Studies on growth and steroid formation in tissue cultures of Holarrhena antidysenterica. Phytochem. 15: 681-682.

Heinz, D.J. and G.W.R. Mew. 1971. Morphologic, cytogenetic and enzymatic variation in Saccharum species hybrid clones derived from callus tissue. Amm. J. Bot. 58: 257-262.

Heinz, D.J., M. Krishnamurthi, L.G. Nickell, and A. Maretzki. 1977. Cells tissue and organ culture in sugarcane improvement. p.3-17. In: J. Reinert and Y.P.S. Bajaj (eds), Applied and Fundamental Aspect of Plant Cell, Tissue and Organ Culture. Springer Verlag, Berlin.

Henderson, J.H.M. and J. Bonner. 1952. Auxin metabolism in normal and crown-gall tissue of sunflower. Am. J. Bot. 39: 444-451.

Hertel, R., T.L. Lomax, and W.R. Briggs. 1983. Auxin transport in membrane vesicules from Cucurbita pepo L. Planta 151: 193-201.

Hildebrandt, A.C. 1962. Tissue and single cell cultures of higher plants as a basic experimental method, p. p.383-421. In: H.F.Linskens and M.V. Tracy (eds.), Modernen Methoden de. Pflanzen Analyses Vol. 5. Springer-Verlag, Berlin.

Hildebrandt, A.C. 1965. Tissue culture as an aid in the study of plant disease, p. 584-597, In: Advances in agricultural sciences and their applications. Issue in Commemoration of the golden jubilee of the Madras Agricultural Journal.

Holgate, D.P. 1977. Propagation of ornamentals plants by tissue culture, p.18. In: J. Reinert, and Y.P.S. (eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and organ culture. Springer-Verlag, New York.

Holsters, M., B. Silva, F. Van Vliet, C. Genetello, M. De Block, P. Dhaese, A. Depicker, D. Inze's, G. Engler, R. Villarroel, M. Vontagu and J. Schell 1980. The functional organisation of the nopaline. A. tumefaciens plasmid. P. Ti C58. Plasmid 3: 212-230.

Horgan, K., and J. Aitken. 1981. Reliable plantlet formation from embryos and seedling shoots tips of radiata pine. Physiologia. Pl. 53(2): 170-5.

Hori, K. and O. Miller. 1977. Multiple plant growth promoting factors in the salivary glands of plant bugs. Marcellia 39: 399-400.

Hoyle, M.C. 1972. Indoleacetic acid oxidase : A dual catalytic enzyme. Plant Physiol. 50: 15-18.

Ibrahim, R.K., and G.H.N. Towers. 1960. The identification by chromatography of plant phenolic acids. Archives of Biochemistry and Biophysics 87: 125-128.

Inzes, D., A.V. Follin, M. Lysebetten, G. Simeons, G. Genetello, M. Von Montagu and J. Schell. 1984. Genetic analysis of the individual T-DNA genes of Agrobacterium tumefaciens; further evidence that two genes are involved in indole-3-acetic acid synthesis. Mol. Gen. Genet 194: 265-274.

Ishikura, M., M. Iwata, and S. Mitsui. 1983. The influence on some inhibitors of the formation of caffeic acid in cultures of Perilla U. Cell suspension. Bot. Mag. Tokyo 96(1042): 111-20.

Itokawa, H., N. Ebata, K. Takeya, and A. Ikuta. 1980. Studies on the tissue culture of Humulus lupulus and the chemical constituents. Shoyakugaku Zasshi 34: 196-199.

Jacobson, J.V. 1977. Regulation of ribonucleic acid metabolism by plant hormones. Ann . Rev. Plant Physiol. 28: 537-564.

Jaynes, T.A., F.A. Haskins, and H.J. Gorz. 1972. Oxidation of phenylpyruvate by sweet clover peroxidase. Phytochemistry 11: 563-569.

Joos, H., D. Inzes, A. Caplan, M. Sormann, M. Van Montagu, and J. Schell. 1983. Genetic analysis of T-DNA transcripts in nopaline crown galls. Cell. 32: 1057-1067.

Jordan, W.R. and F. Skoog, 1971. Effects of cytokinin on growth and auxin in coleoptiles of derooted Avena seedling. Plant Physiol. 48: 97-99.

Joshi, S.C. 1986. Studies on the biochemistry of gall formation in Cinnamomum tamala Fr. Nees with special emphasis on auxin metabolism. Ph.D. thesis, North-Eastern Hill University, Shillong, India.

Joshi, S.C., and P. Tandon. 1984. Studies on certain oxidases, their isozyme and phenols as related to gall formation in Leea indica. Biochem. Physiol. Pflanzen. 179: 711-715.

Joshi, S.C., P. Tandon and A.L.S. Rajee, 1985. Changes in certain oxidative enzymes and phenolics in Camellia sinensis and Eleocarpus lancifolius leaf roll-galls, p.51-58. In: Proc. Int. Symp on Importance of gall forming organisms in Agriculture and Medicine. Allahabad, India.

Kado, C.I. 1976. Tumor-inducing substances of Agrobacterium tumefaciens. Ann . Rev. Phopathol. 14: 265-308.

Kado, C.I. 1984. Phytohormone mediated tumorigenesis by plant pathogenic bacteria, p.311-336. In: D.P.S. Verma and Tho. Hohn (eds.), Plant Gene Research. Genes Involved in Microbe-Plant Interaction. Springer-Verlag, Wien, New York.

Kahl, G. and J.S. Schell (eds.), 1982. Molecular Biology of Plant Tumors. Academic Press, New York, 615p.

Kaminek, M., V. Hadacova, and J. Lustinec. 1981. Origin of cytokinin and auxin autonomy and changes in specific proteins in tobacco callus tissues. Biol. Plant 23: 228-236.

Kant, U. 1967. Studies on the insect and mite-induced plant galls. Ph.D. Thesis, Univ. of Rajasthan, Jaipur, India.

Karcher, S.J., V.J. Di Rita and S.B. Gelvin, 1984. Transcript analysis of Ti-DNA in octopine-type crown-gall tumors. Mol. Gen. Genet 194: 159-165.

Kefeli, V.I. 1985. Some phenolics as plant growth and morphogenesis regulators, p.89-102. In: S.S. Purohit (ed.), Hormonal Regulation of Plant Growth and Development, Vol. 2. Agro Botanical Publishers, Bikaner, India.

Kefeli, V.I., and Ch. Kadyrov. 1971. Natural growth inhibitors. Ann. Review of Plant Physiology. Acad. Press, 185-196.

Kefeli, V.I. and M. Kutacek. 1977. Phenolic substances and their possible role in plant growth regulation, p.181-188. In: Proc. 9th Intern. Conf. Plant Growth regulation. Springer Verlag, Berlin-Heidelberg New York.

Key, J.L., and J.B. Hanson. 1961. Some effect of 2,4-D on soluble nucleotides and nucleic acid of soybean seedling. Plant Physiol. 36: 145-152.

Key, J.L. and J.C. Shannon. 1964. Enhancement by auxin of ribonucleic acid synthesis in excised soybean hypocotyl tissue. Plant physiol. 39: 360-364.

Key, J.L. and L.N. Vander-Hoef. 1973. Plant hormones and developmental regulation: role of transcription and translation, p.49-83. In: S.J. Coward (ed.), Developmental Regulation: Aspect of Cell Differentiation, Academic Press, New York, London.

Key, J.L., and J. Ingle. 1964. Requirement for the synthesis of DNA-like RNA for growth of excised plant tissue. Proc. Nat. Acad. Sci. U.S.A. 52: 1382-1388.

Klambt, D. 1976. Cytokinin effects on protein synthesis of in vitro systems of higher plants. Plant and Cell Physiol. 17: 73-76.

Klein, R.M., E.M. Rasch, H. Swift. 1953. Nucleic acids and tumorigenesis in broad bean. Cancer. Res. 13: 499-502.

Komro, C.T., V.J. Dirita, S.B. Gelvin, and J.D. Kemp. 1985. Site-specific mutagenesis in the TR-DNA region of octopine-type Ti-plasmid. Plant Mol. Biol. 4: 253-263.

Kosuge, T. 1969. The role of phenolics in host response in infection. Ann. Rev. Phytopathol. 7: 195-222.

Koukol, J., E.E. Conn. 1961. Metabolism of aromatic compounds in higher plants. 4. Purification and properties of phenylalanine deaminase of Hordeum vulgare. J. Biol. Chem. 236: 2692-2698.

Kozlowska, H., D.A. Rotkiewicz, and R. Zadernowski. 1983. Phenolic acids in rapeseed and mustard. J. Am. Oil Chem. Soc. 60(6): 1119-22.

Ku, H.S., S.F. Yang, and H.K. Pratt. 1970. Inactivity of apoperoxidase in indoleacetic acid oxidation and ethylene formation. Plant Physiol. 45: 358-359.

Kulaeva, O.N. 1980. Cytokinin action on transcription and translation in plants. p.218-227. In: J. Guern and C. Pe' and-Lenoel (eds.), Metabolism and Molecular Activities of Cytokinins. Springer-Berlin, Heidelberg, New York.

Kunapuli, S.P., and C.S. Vaidyanathan. 1985. Indole metabolising enzyme systems in tropical plants. Phytochem. 24: 973-976.

Kupila, S. and H. Stein. 1961. DNA content of broadbean (Vicia faba) internodes in connection with tumor induction by A. tumifaciens. Plant Physiol. 36: 216-219.

Lakshmi Sita. G., N.V. Raghavaram and C.S. Vaidyanathan, 1979. Differentiation of embryoids and plantlet from shoot callus of Sandalwood. Plant. Sci. Lett. 15: 265-270.

Lavee, S., and A.W. Galston, 1968. Structural, physiological and biochemical gradients in tobacco pith tissue. Plant Physiol. 43: 1760-1768.

Lazarovits, G., and E.W.B. Ward. 1982. Polyphenoloxidase activity in soybean hypocotyls at sites inoculated with Phytophthora megasperma f. sp. glycinea. Physiol. Plant Pathol. 21: 227-236.

Lee, T.T. and F. Skoog. 1965. Effects of hydroxy benzoic acids in indole acetic acid inactivation by tobacco callus extracts. Physiol. Plant 18: 577-585.

Lee, T.T., and R.A. Chapman. 1977. Inhibition of enzymic oxidation of indole-3-acetic acid by metabolites of the insecticide carbofuran. Phytochem. 16: 35-39.

Lee, T.T., N. Albin. Starratt; John, J.Jevnikar, and Albert Stoesl. 1980. New phenolic inhibitors of the peroxidase-catalysed oxidation of IAA. Phytochemistry 19: 2277-2280.

Lee, T.T., N. Starratt, and J.J. Jevnikar, 1982: Regulation of enzymic oxidation of indole-3-acetic acid by phenols: Structure-activity relationships. Phytochemistry Vol. 21. No.3: 517-523.

Leemans, J., R. Deblaere, L. Willmitzer, H. De Greve, J.P. Henalsteins, M. Van Montagu and J. Shell. 1982. Genetic identification of function of TL-DNA transcripts in octopine crown gall. EMBO. J. 1: 147-152.

Letham, D.S. 1978. Naturally occurring plant growth regulators other than the principle hormones of higher plants, p.349-417. In: A.S. Letham, P.B. Goodwin and T.J.V. Higgin (eds.), Phytohormones and related compds. A comprehensive Treatise Vol. I. Elsevier/ North-Holland Biochemical Press, Amsterdam.

Lewis, I.F., and L. Walton. 1964. Gall formation on leaves of Geltis occidentalis L. resulting from material injected by Pachypsylla sp. Trans. Am. Microsc. Soc. 83: 62-78.

Lioret, C. 1957. Les acides amines libres des tissue de crown gall cultures in vitro mise en evidence d'un acide amine particulier a ces tissue. C.R. Acad. Sci. Paris, 244: 2171-2174.

Lippincott, J.A., and B.B. Lippincott. 1975. The genus Agrobacterium and plant tumorigenesis. Ann. Rev. Microbiol. 29: 377-405.

Lippincott, J.A., and B.B. Lippincott. 1976. Morphogenetic determinants as exemplified by the crown-gall disease, p.356-388. In: R. Heitefuss and P.H. Williams (eds.), Physiological Plant pathology, Springer-Verlag, Berlin, Heidelberg New York.

Loon, L.G., and J.L.M. Geelen . 1971. The relation of polyphenoloxidase and peroxidase to symptom expression in tobacco var, "Samsun NN" after infection with tobacco mosaic virus. Acta. Phytopathol. Acad. Sci. Hung 6: 9-20.

Lutz, A. 1971. Aptitudes morphogenetiques de cultures de tissus d'origine unicellulaire. Colloq. Intern. Centre Natl. Rech. Sci. (Paris), No.193: 163-168.

Lynda, D.B.E., and Y.A. Thorpe. 1985. Tyrosine and phenylalanine ammonia lyase activiries during shoot initiation in tobacco callus cultures. Plant. Physiol. 78: 438-441.

Macháčková, I., K. Ganceva, and Z. Zmrhal. 1975. The role of peroxidase in the metabolism of IAA and phenol in wheat. Phytochemistry 14: 1251-1254.

Madden, J.L., and C. Stone. 1984. Induction and formation of pouch and emergence galls in Eucalyptus pulchella leaves. Aust. J. Bot. 32: 33-42.

Mahadevan, A. 1974. Method in Physiological Plant Pathology. Sivakami Publications, Madras, 80 p.

Mahadevan, A. 1984. Growth Regulators, Microorganisms and Diseased plants. Oxford and IBH Publishing Company, New Delhi, Bombay, Calcutta, 466 p.

Mahadevan, A., and R. Sridhar, 1982. Methods in Physiological plant pathology. Sivakami Publication, Madras.

Mani, G.S. 1973. Plant Gall of India. M. MacMillan Company of India Limited, pp.354.

Masuda, Y. 1967. Auxin induced expansion growth of Jerusalem artichoke tuber tissue in relation to nucleic acid and protein metabolism. In Plant Growth Regulators, Ed. New York. Acad. Sci. New York.

Mathes, M.S., M. Morselli, and J.M. Markin. 1973. Use of various carbon sources by isolated maples callus cultures. Plant and Cell Physiol. 14: 797-801.

Mathysse, A.G. and C. Phillip. 1969. A protein intermediary in the interaction of a hormone with the genome. Proc. Natl. Acad. Sci. U.S.A. 63: 897-903.

Mayer, A.M., and E. Harel. 1979. Polyphenol oxidases in plants. Phytochem. 18: 193-215.

McGraw, B.A. and R. Horgan. 1983. Cytokinin catabolism and cytokinin oxidase. Phytochemistry. 22(5): 1103.

Meins, F. Jr. 1972. Progr. Exptl. Tumor Research 15: 93-109.

Meins, F. Jr. 1974. Mechanism underlying tumor transformation and tumor reversal in crown-gall, a neoplastic disease of higher plants, p.23-39. In: T.J. King (ed.), Developmental Aspects of Carcinogenesis and Immunity. Academic Press, New York.

Meins, F. Jr. 1982. Habituation of cultured plants cells, p.3-31. In: G. Kahl and J.S. Schell (ed.), Molecular Biology of Plant Tumors, Academic Press, New York.

Meins, F. Jr., and J. Lutz. 1980 . Epigenetic changes in tobacco cell culture: Studies of cytokinin habituation, p. 220-236. In: I. Rubenstein, R. Phillips, E. Green, and B. Gongenbach (eds.), Emergent Techniques for the Genetic Improvement.of Crops. Univ. of Minnesota Press, Minneapolis.

Mendez, J., and S.A. Brown. 1971. Changes in the phenolic metabolism of tomato plants infected by Agrobacterium tumefaciens. Can. J. Bot. 49: 2101-2105.

Meyer, Y., and Y. Chartier. 1981. Hormonal control of mitotic development in tobacco. Two-dimension distribution of newly synthesized proteins. Plant Physiol. 68: 1273-1278.

Meyer, Y., L. Aspart, and Y. Chartier, 1984. Auxin-induced regulation of protein synthesis in tobacco mesophyll protoplasts cultivated in vitro. I-Characteristics of auxin-sensitive proteins. Plant Phyiol. 75: 1028-1034.

Mohen, D., H. Shinshi, G. Felix and Jr. F. Meins. 1985. Hormonal regulation of 1,3-glucanase messenger RNA levels in cultured tobacco tissues. EMBO J. 4: 1631-1635.

Monaco, L.C., M.R. Sondhal, R. Corvalho, O.J. Crocamo, and W.R. Sharp. 1977. Applications of tissue culture in the improvement of coffee, p.109-129. In: J. Reinert and Y.P.S. Bajaj (eds.), Applied and Fundamental Aspects of Plant Cell, Tissue, and Organ Culture, Springer-Verlag, Berlin Heidelberg, New York.

Morel, G., 1971. Derivations du métabolisme azoté des tissus de crown-gall, p. 463-471. In: Colloques Internationaux du C.N.R.S. No. 193. Les cultures de Tissus de planets. Parais.

Morel, G., and S. Démétridís, 1955. Action de régulateurs de croissance sur l'activité Oxydosique de tissus de To pinambour. Année Biol. 31: 227-236.

Moustafa, F.A., and R. Whittenbury. 1970. Properties which appear to allow phytopathogenic Pseudomnas te counteract plant defence mechanisms. Phytopathol. Z. 67: 215-224.

Mulder-Krieger, T., R. Verpoorte, and A. Baerheimsvendsen. 1982. Tissue culture of Cinchona pubescens. Effect of media modification on growth. Planta Medica 44: 237-240.

Murashige, T. 1974. Plant propagation through tissue cultures. Ann. Rev. Plant Phyiol. 25: 135-166.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Murashige, T., and D.P.H. Tucker. 1969. Growth factor requirements of citrus tissue culture, pp. 1155-1161. In: H.D. Chapman (ed.), Proc. 1st Intern. Citrus symp. Vol.3, Riverside: Univ. California.

Nakajima, H.T., T. Yokota, T. Maisumoto, M. Noguchi, and N. Takahashi, 1979. Relationship between hormone content and autonomy in various autonomous tobacco cells cultured in suspension. Plant Cell Physiol. 20: 1489-1499.

Nakajima, H.T., T. Yokota, N. Takahashi, T. Matsumoto, and M. Noguchi. 1981. Changes in endogenous ribosyl-transzeatin and indole-3-acetic acid levels in relation to the proliferation of tobacco (Nicotiana tabacum cultivar Hicks-2) Crown gall cells. Plant Cell Physiol. 22: 1405-1410.

Naylor, J., G, Sander, and F. Skoog. 1954. Mitosis and cell enlargement with cell division in excised tobacco pith tissue. Physiol. Plant. 7: 25-29.

Neish, A.C. 1961. Formation of m & p-coumaric acid by enzymatic deamination of the corresponding isomers of tyrosine. Phytochem. 1: 1-24.

Nester, E.W., M.P. Gordon, R.M. Amasino and M.F. Yanofsky. 1984. Grown gall : A molecular and physiological analysis. Ann. Rev. Plant. Physiol. 35: 387-413.

Nitsch, J.P. 1956. Methods for the investigation of natural auxins and growth inhibitors, p.3-31. In: R.L. Wein and F. Wightman (eds.), The Chemistry and mode of action of plant growth substances. Butterworth's Scientific Publications, London New York.

Novak, J.E., and A.W. Galston. 1971. Studies on auxin protector substances, IAA oxidases and peroxidases in cotyledon of Prabitis nil. Plant Cell Physiol. 12: 931-94.

Noverosky, R.L., E.B. Williams, and J. Kuc. 1962. Phloridzin - its metabolism and role in resistance to Venturia inaequalis, incitant of apple scab. Phytopathology 52: 746.

Ondrej, M. 1983. Detection of Nopaline dehydrogenase and lysopine dehydrogenase activities in crown gall tumors of different plant species. Biologia Plantarum. 25(5): 378.

Otten L.A.B.M. and R.A. Schilperoort, 1978. A rapid microscale method for the detection of lysopine and nopaline dehydrogenase activity. Biochem. Biophys. Acta. 527: 497-500.

Parr, T.J., 1940. Asterocelanium variolosum Ratzeburg. a gall forming coccoid and its effect upon the host tree. Bull. Yale Univ. Sch. For. No. 46: 49.

Patterson, B.D., and A.J. Trewavas, 1967. Change in the pattern of protein synthesis induced by 3-indolylacetic acid. Plant. Physiol. 42: 1081-1086.

Pelet, F., A.C. Hildebrandt, A.J. Riker and F. Skoog, 1960. Growth in vitro of tissue isolated from normal stems and insect galls. Amer. J. Botany 47: 186-195.

Pelet, P.E. 1966. Auxin oxidase feedback effect. G.R. Acad. Sci. Ser. D. 263: 864-867.

Pelet, P.E. and T. Gaspar. 1968. Le catabolism auxinique, p. 1-148. Monographies de Physiologie vegetable, vol. 1. Paris, Mosan et Gie.

Pelet, P.E., P. Lavanchy, and S. Sevhonkian. 1970. Interaction between peroxidases, polyphenoloxidases and auxin-oxidases. Physiol. Plant 23: 800-804.

Pengelly, W.L. 1980. Studies on the variation in indole-3-acetic acid content of cultured crown-gall tissues of tobacco using a specific radioimmuno assay, Ph.D. Dissertation, Princeton University, Princeton, New Jersey.

engelly, W.L., and F. Jr. Meins. 1978. Variation in the a auxin content of crown-gall tumors tissue in culture. Plant Physiol. 61, Suppl. 72.

Pengelly, W.L. and F. Meins Jr. 1982. The relationship of indole-3-acetic acid content and growth of crown gall tumor tissues of tobacco in culture. Differentiation 21: 27-31.

Pengelly, W.L., and F. Jr. Meins. 1983. Growth, auxin requirment, and indole-3-acetic acid content of cultured crown-gall and habituated tissue of tobacco. Differentiation 25: 101-105.

Petrochenko, Y.E.I., and P.A. Kolesnikov. 1966. Oxidation of $NaDH_2$ by subcellular fractions with participation of peroxidase and molecular oxygen. Biokhimiya. 3: 1117-1120.

Platt, H., L. Shannon, and J. Lew. 1964. In vivo peroxidase inhibition in bush bean leaves. Nature 201: 1328.

Purohit, S.D., K.G. Ramawat, and H.C. Arya, 1979. Phenolic, peroxidase and phenolase as related to gall formation in some arid zone plant. Current Sci. 48(16): 714-716.

Purohit, S.D., N.S. Shekhawat, P. Tandon, and H.C. Arya. 1980. Hormonal profiles in some insect and mite-induced plant galls. Proc. Indian Natl. Sci. Acad. B.46: 892-900.

Ramawat, K.G., and H.C. Arya. 1977. Carbohydrate nutrition of Ephedra tissue grown in culture. Indian J. Exp. Biol. 15: 504-507.

Ramawat, K.G., S.D. Purohit, and H.C. Arya. 1979. Altered state of oxidising enzymes and phenolics in Cordia gall. Trans. Insdt and Ucds 4(1): 38-41.

Rausch. T., S.C. Minocha, W. Hilgenberg, and G. Kahl. 1985. L. Tryptophan metabolism in wound-activated and Agrobacterium tumefaciens - transformed potato tuber cells. Physiol. Plant. 63: 335-344.

Rausch, T., G. Kahl and W. Hilgenberg. 1986. Biosynthesis and conjugation of indole-3-acetic acid in tobacco crown gall. PHPLAI 68(3): Nov. 1986: 458.

Reddy, C.M., and E.H. Coe. 1986. Gene structure and function in higher plant; Oxford and IBH Publ. Co., New Delhi, p. 81.

Reinert, J., and R. White. 1956. The cultivation in vitro of tumor tissues and normal tissues of Picea glauca. Physiol. Plant. 9: 177-189.

Robb, D.A., T. Swain, and L.W. Mapron. 1966. Substrates and inhibitors of the activate tyrosinase of broad bean (Vicia faba L.). Phytochem. 5: 665-675.

Roberts, L.W. 1976. Cytodifferentiation in plants-xylogenesis as a model system. Cambridge University Press, Cambridge.

Rohfritsch, 1971. Culture *in vitro* de jeunes galles d'*Aulax glechomae* L. sur *Glechoma hederacca* L. Marcellia. 37: 151-161.

Rohfritsch, O. and J.D. Shorthouse, 1982. Insect-galls. p.131-152. In: G.Kahl and J.SSchell (eds) Molecular Biology of Plant tumors .. Academic Press, New York.

Rosenstrock, G. and G. Kahl. 1978. Phytohormones and the regulation of cellular processes in aging storage tissues. p.622-671. In: "Biochemistry of wounded Plant Tissues", de Cruyter, Berlin.

Rosenthal, C.A. 1986. The chemical defenses of higher plants. Am. Sci. 254: 94-99.

Sacristan, M.D. and G. Melchers. 1977. Regeneration of plants from 'Habituated' and '*Agrobacterium* - transformed' single-cell clones of tobacco. Mol. Gen. Genet. 152: 111-117.

Sargent, J.A. and F. Skoog, 1961. Scopoletin glycosides in tobacco tissues. Physiol. Plantarum 14: 504-519.

Schell, J., M. Van Montagu, M. De Beuckelerr, M. De Block, A. Depicker, M. De Wilde, G. Engler, C. Genetello, J.P. Hernalsteens, M. Holsters, J. Seurinck, B. Silva, F. Van Vliet, and R. Villarroel. 1979. Interactions and DNA transfer between *A. tumefaciens*, the Ti-plasmid and the plant host. Proc. R. Soc. London. Ser. B. 204: 251-266.

Schmitz, R.Y., F. Skoog, S.M. Hecht and N.J. Leonard. 1972. Comparison of zeatin indole acetate with zeatin and indole acetic acid in the tobacco bio-assay. Plant Physiol 50: 114-116.

Schröder, G., S. Waffenschmidt, E.W. Weiler, and J. Schröder. 1984. The T-region of Ti plasmids codes for an enzyme synthesizing indole-3-acetic acid. Eur. J. Biochem. 138: 387-391.

Seigel, S.M., P. Frost, and F. Porto. 1960. Effects of indole-acetic acid and other oxidation regulators on in vitro. Peroxidation and experimental conversion eugenol to lignin. Plant Physiol. 35: 163-167.

Sembdner, G., D. Cross, H.W., Liebisch, and G. Schneider. 1980. Biosynthesis and metabolism of plant hormone p.281-444. In: J. MaMillan (ed.), Hormonal Regulation of Development. Encyclopedia of Plant Physiology, New Series, Vol. 9. Springer-Verlag, Berlin Heidelberg New York.

Sequeira,L. 1964. Inhibition of indole-acetic acid oxidase in tobacco plants infested by Pseudamonas solanacearum. Phytopathol. 54: 1078.

Shah, R.R., K.V. Subbaiah, and A.R. Mehta. 1976. Hormonal effect on polyphenol accumulation in Cassia tissues cultured in vitro. Can. J. Bot. 54: 1240-1245.

Shah, R.R., and A.R. Mehta. 1978. Effect of nitrogen nutrition on growth and phenolic accumulation in Crotalaria tissues cultured in vitro. Indian J. Expt. Biol. 16: 1144-1147.

Shailaja, Rao, and A.R. Mehta. 1980. Synthesis of phenolics in Arachis hypogaea L. cell suspension. Ind. J. Expt. Bio. 18(12): 1423-1425.

Sharp, W.R., D.A. Evans, P.V. Ammirato, and Y. Yamada. 1984. Handbook of plant Cell Culture, vol.2, crop species. Macmillan Publishing Company, New York. 644 p.

Shekhawat, N.S., K.G. Ramawat, and H.C. Arya. 1978. Carbohydrate, protein, phenols and enzymes (PPO, PRO, and IAA-oxidase) in gall and normal tissues of Achyranthes aspera L. Current Sci. 47(20): 780-781.

Sheldrake, A.R. 1973. The production of hormones in higher plants. Bot. Rev. 48: 509-559.

Shields, S.E., V.P. Wingate, and C.J. Lamb. 1982. Dual control of phenylalanine amonialyase production and removal of the product cinnamic acid. Cur. J. Biochem. 123(2): 389-395.

Sievert, R.C., and A.C. Hildebrandt. 1965. Variation within single clones of tobacco tissue cultures. Amer. J. Bot. 52: 742-750.

Singh, B.D., G.S.R.L., Rao, and R.P. Singh. 1982. Polyphenol accumulation in callus cultures of cowpea Vigna sinensis. Indian J. Expt. Biol. 20: 387-389.

Sirois, J.C., and R.W. Miller. 1972. The mechanism of the scopoletin-induced inhibition of the peroxidase catalyzed degradation of indole-3-acetate. Plant Physiol. 49: 1012-1018.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symp. Soc. Exp. Biol. 11: 118-30.

Srivastava, O.P., and R.B. Van Huystee. 1973. Evidence for close association of peroxidase, polyphenol oxidase, and IAA-oxidase isoenzymes of peanut suspension culture medium. Can. J. Bot. 51: 2207-2215.

Staba, E.J., 1977. Tissue culture and pharmacy, p.699-715. In: J. Reinert and Y.P.S. Bajaj (eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, Berlin, Springer-Verlag.

Stachel, S.E.,E. Messens,M.Van Montagu, and P. Zambryski. 1985. Identification of the signal molecules produced by wounded plant cell that activate T-DNA transfer in Agrobacterium tumifaciens. Proc. Natl. 318: 624-629. Acad. Sci. U.S.A.

Stachel, S.E. and E.W. Nester. 1986. The genetic and transcriptional organization of the vir region of the A6Ti-plasmid of Agrobacterium tumefaciens EMBO J.5: 1445-1454.

Stafford, H.A. 1974. The metabolism of aromatic compounds. Ann. Rev. Plant Physiol. 25: 459-86.

Steward, F.C., and A.D. Krikorian. 1979. Problems and Potentialities of cultured plant cells in retrospect and prospect, p.221-262. In: W.R. Snarp, P.O. Larsen, E.F. Paddock and V. Raghavan (eds.), Plant Cell and Tissue Culture: Principles and Applications, Ohio State University Press, Columbus.

Stickland, R.G., and N. Sunderland. 1972. Production of anthocynanins, flavonols and chlorogenic acids by cultured callus tissues of Haplopappus gracilis. Ann. Botany 36: 443-457.

Stonier, T. 1972. The role of auxin protectors in autonomous growth, p.423-435. In: M.L. Hirth and G. Morel (eds.), Les cultures de tissus de plantes (Proc. Second Intern. Conf. on Plant Tissue Culture, Strasbourg, France, 1970), Calloq. Intern. C.N.R.S. (France) No.193.

Stonier, T., and H.M. Yang . 1973. Studies on auxin protectors XI. Inhibition of peroxidase-catalyzed oxidation of glutathione by auxin protectors and O-dihydroxyphenols. Plant Physiol. 51: 391-395.

Street, H.E. 1969. Growth in organized and unorganised systems knowledge gained by culture of organs and tissue explants, p.3-224. In F.C. Steward (ed.), Plant Physiology: A treatise, Vol.5 B. Academic Press, New York.

Sugano, N., R. Iwata, and A. Nishi. 1975. Formation of Phenolic acids in carrot cells in suspension cultures. Phytochemistry 14: 1205-1207.

Syôno, K. and T. Furuya. 1972. Effects of cytokinin on the auxin requirment and auxin content of tobacco calluses. Plant Cell Physiol. 13, 843-856.

Taller, B.J., N. Murai, and F. Skoog. 1987. Endogenous cytokin in the ribosomal RNA of higher plants. Plant Physiol. 83: 755-760.

Tandon, P. 1976. Further studies on the process of gall induction on Zizyphus and the factors involved. Ph.D. Thesis. Univ. of Jodhpur, Jodhpur, India.

Tandon, P. 1985. Hormonal regulation of abnormal growth in plants. p.45-64. In: S.S Purohit (ed.), Hormonal Regulation of Plant Growth and Development Vol. 2, Agro Botanical Publisher, India.

Tandon, P. and H.C. Arya. 1979. Effect of growth regulators on carbohydrate metabolism of Zizyphus jujuba gall and normal stem tissue in culture. Biochem. Physiol. Pflanzen. 74: 772-779.

Tandon, P., and H.C. Arya, 1980a. Auxin autotrophy and hyperauxinity of Eriophyes induced Zizyphus stem galls in culture. Biochem. Physiol. Pflanzen, 175: 537-41.

Tandon, P., and H.C. Arya. 1980b. Separation of auxin protectors from Zizyphus gall tissue by Sephedex gel filtration. Curr. Sci. 49: 864-865.

Tandon, P. and H.C. Arya, 1980c. Presence of auxin protectors in Eriophyes induced Zizyphus stem galls. Experientia 36(8): 958-959.

Tandon, P., and H.C. Arya. 1982. Association of auxin-protectors, peroxidase, indoleacetic acid oxidase and polyphenol oxidase in Zizyphus gall and normal stem tissues grown in culture. Biochem. Physiol. Pflanzen 177: 114-124.

Tandon, P. 1985. Peroxidaze catalysed-IAA-oxidation in presence of cofactors and auxin protectors isolated from Eriophyes incited Zizyphus gall tissue, p.69-81. In Proc. Intern. Symp. On Importance of gall forming organism in Agriculture and medicine Allahabad, India.

Tempe, J., and A. Petit. 1982. Opine utilization by Agrobacterium p.451-459. In: G. Kahl and J.S. Schell (eds.), Molecular Biology of Plant Tumors, Academic Press, New York, London.

Theologis, A., and P.M. Ray. 1982. Early auxin-regulated polyadehylated mRNA sequences in pea stem tissue. Proc. Nat. Acad. Sci. U.S.A., 79: 418-421.

Thimann, K.V. 1972. Physiology of development: The hormones. In: Steward ,F.C. (ed.), Vol. VIB .Plant Physiology New York.London.Academic Press.

Thomashow, M.F., R. Nutter, A.L. Montoya, M.P. Gordon, and E.W. Nester. 1980. Integration and organization of Ti-plasmid sequences in crown gall tumors. Cell 19: 729-739.

Thomashow, M.F., S. Hugly, W.G. Buchholz, and L.S. Thomashow. 1986. Molecular basic for the auxin-independent phenotype of crow gall tumor tissues. Science 231: 616-618.

Thorpe, T.A. 1981. Plant Tissue Culture: Methods and applications in Agriculture. Academic Press, New York, 392 p.

Thorpe, T.A., and S. Biondi. 1984. Conifers, p.435-470. In: W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada (eds.), Handbook of Plant Cell Culture. vol.2 Crop species. MacMillan Publishing Company, New York.

Tisserat, B. 1984. Date Palm, p.505-545. In: W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada (eds.), Handbook of Plant Cell Culture, vol. 2, Crop. Species. MacMillan Publishing Company, New York.

Tomaszewski, M., and K.V. Thimann. 1966. Interactions of phenolic acids, metallic ions and chelating agent on auxin-induced growth. Plant Physiol. 41: 1443-1454.

Tran Thanh Van, K.M. 1981. Control of morphogenesis in vitro cultures Ann. Rev. Plant Physiol. 32: 291-311.

Truelsen, T.A. 1972. Indole-3-pyruvic acid as an intermediate in the conversion of tryptophan to indole-3-acetic acid. I. Some characteristics of tryptophan transaminase from mung bean seedling. Physiol. Plant. 26: 289-295.

Vámos-Vigyázó, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. CRC. Critical reviews in Food Science and Nutrition 49-129.

Vande-Gasteele, K.L., M.J. Devw-Vankeymeulen, P.G. Deberch, L.J. Maene, M. Flame, and C.F. Van Sumere, 1981. The phenolics and a hydrolyzable tannin polyphenol oxidase of Medinilla mangifica. Phytochem. 25: 1105-1112.

Van-Lelyveld, L.J. 1974. Extraction and identification of some phenols from avocada leaves. Agrochemophysica. 6: 79-82.

Van Loon, L.C. 1985. Pathogenesis-related proteins. Plant. Mol. Biol. 4: 111-116.

Van Onckelen, H., P. Rudelsheim, R. Hermans, S. Horemans, E. Messens, J.P. Hernalsteens, M. Van Montagu and F. DeGreef. 1984. Kinetics of endogenous cytokinin, IAA, and ABA levels in relation to the growth and morphology of tobacco crown gall tissue. Plant Cell Physiol. 25: 1017-1025.

Van Sumere, G.F. 1975. Plant proteins and phenolics. p.211-264. In: Harborne, J.B., Van Summere, C.F. (eds.), Chemistry and Biochemistry of Plant Proteins. New York, London: Academic Press.

Varga, M. and K. Koves. 1959. Phenolic acids as growth and germination inhibitors in dry fruits. Nature (London) 183: 401.

Vasil, I.K., W.R. Scowcroft, and K.J. Frey. 1982. Plant Improvement and Somatic Cell Genetics. Academic Press, Orlando. 312 p.

Vaughan, K.C., and S.O. Duke. 1984. Function of polyphenol oxidase in higher plants. Physiol. Plant. 60: 106-112.

Vazquez, A. 1973. Effect of umbelliferone on rooting in bean cuttings. Plant Sci. Lett. 1: 433-438.

Vernon, S.L., and J. Straus. 1972. Effects of IAA and 2,4-D on polyphenol oxidase in tobacco tissue cultures. Phytochem. 11: 2723-2732.

Walker, J.G., J. Legocka, L. Edelman, and J.L. Key. 1985. An analysis of growth regulator interaction and gene expression during auxin-induced cell elongation using cloned complementary DNA to auxin-responsive messenger RNAs. Plant Physiol. 77: 847-850.

Walton, L. 1980. Gall formation and life history of Pineus floccus (Patch) (Homoptera: Adelgidae) in virginia. Virginia J. Sci. 31: 55-60.

Wang, K.J., P.F. Zhang, D.X. NI, and Z.H. Bao. 1981. Callus formation and organ regeneration in tissue culture of woody plants. Acta. Bot. Sin. 23: 97-103.

Wareing, P.F., and I.D.J., Phillips. 1982. Growth and differentiation in plants. Pergamon Press, Oxford, 343 p.

Warick, R.P., and A.C. Hildebrandt. 1966. Free amino acid content of stem and Phylloxera gall tissue cultures of grapes. Plant Physiology. Vol. 41, No.4: 573-578.

Wegen, H.W. and C. Clase, 1981. Investigations on tumor development in tobacco hybrids (Nicotiana clevelandii Gray x Nicotiana glutinosa L.) III. Changes in oxidase activities and phenolic compounds in extracts of tumor tissues, after infection with a tumor inducing factor (TIF). Phytopath. Z. 102: 60-77.

Weiler, E.W., and K. Spanier. 1981. Phytohormones in the formation of crown-gall tumors. Planta 153: 326-337.

Weiss, U., and J.M. Edwards. 1980. The biosynthesis of aromatic compounds. John Wiley and Sons, New York. 728 pp.

Westphal, E. 1980. Responses of some solanaceae to attack by the gall mite Eriophyte cladophthyrus. Plant Disease 64: 406-409.

Wetmore, R.H., and G. Morel. 1949. Polyphenol oxidase as problem in organ culture and auxin diffusion studies of horsetails and ferns. Am. J. Bot. 36: 830.

Wheeler, A.W., and H.G.C. King. 1968. Conversion of tryptophan to auxin by phenolics esters from leaves of dwarf-french bean (Phaseolus vulgaris L.). Phytochemistry 7: 1057-1063.

Wheeler, K. and P. Hanchey, 1968. Permeability phenomena in plant disease. Ann. Rev. Phytopathol. 6: 331-350.

Willmitzer, L., M. Debeucheleev, M. Lemmers, M. Van. Montagu and J.Schell. 1980. DNA-from Ti-plasmid present in nucleus and absent from plasmids of crown-gall plant cells. Nature 287: 359-361.

Witham, F.H. 1968. Effect of 2,4-dichlorophenoxyacetic acid on the cytokinin requirement of soybean cotyledon and tobacco stem pith callus tissues. Plant Physiol. 41: 1455-1457.

Wood, H.N. 1970. Revised identification of the chromophore of a cell division factor from crown-gall tumor cells of Vinca rosea L. Proc. Nat. Acad. Sci. U.S.A. 67: 1283-1287.

Wood, K.R., and D.J. Barbara. 1971. Virus multiplication and peroxidase activity in leaves of cucumber (Cucumis sativus L.) cultivars systematically infected with the W strain of cucumber mosaic virus. Physiol. Plant. Pathol. 1: 73-81.

Wullems, C.J., L. Molendijk, and R.A. Schilperoort. 1980. The expression of tumour marker in intraspecific somatic hybrids of normal and crown gall cells from Nicotiana tabacum. Theor. Appl. Genet. 56, 203-208.

Yadav, N.S., K. Postle, R.K. Saiki, M.F. Thomashow, and M.D. Chilton. 1980. T-DNA of a crown gall teratoma is covalently joined to host plant DNA. Nature 287: 458-461.

Yeoman, M.M., and A.J. Macleod. 1977. Tissue (callus) cultures - techniques, p.31-59. In: H.E. Street (ed.), Plant Tissue and Cell Culture Blackwell Scientific Publications, Oxford.

Yoneda, Y. and T. Stonier. 1967. Distribution of three auxin protector substances in seeds and shoots of the Japanese morning glory (Pharbitis nil). Plant Physiol. 42: 1017-1020.

Yoshida, S., and G.H.N. Tower. 1963. Synthesis of shikimic acid-C^{14} - in Pinus recinosa. Added from some C^{14} labelled compounds. Can. J. Biochem 41: 579-586.

Zaenen, I., N. Van Larebeke, H. Tenchy, M. Van Montagu, and J. Schell. 1979. Supercoiled circular DNA in crown gall inducing Agrobacterium strains. J. J. Molec. Biol. 86: 109-127.

Zaerr, J.B. and M.O. Mapes. 1982. Action of growth regulators, p.231-255. In: J.M. Bonga and D.J. Durzan (eds.), Tissue Culture in Forestry. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague/Boston, London.

Zenk, M.H., and G. Muller. 1963. In vivo destructtion of exogenously applied indolyl-3-acetic acid as influenced by naturally occurring phenolic acids. Nature 200: 761-763.

Zofia, C. 1984a. Changes in auxin levels in the course of growth of sunflower crown gall suspension cultures. Acta. Soc. Bot. Pol. 52: 285-294.

Zofia, C. 1984b. Dynamic of IAA-oxidase activity in suspension culture of sunflower crown gall. Acta. Soc. Bot. Pol. 53: 67-79.

Zurfluh, L.L., T.J. Guifoyle. 1980. Auxin-induced changes in the pattern of protein synthesis in soybean hypocotyl. Proc. Natl. Acad. Sci. U.S.A. 77: 357-361.

Zurfluh, L.L. and T.J. Guifoyle, 1982. Auxin-induced changes in the population of translatable messenger RNA in elongation section of soybean hypocotyl. Plant Physiol. 69: 332-337.

---

* Original not seen.