IN VITRO STUDIES IN TRACHEARY ELEMENT DIFFERENTIATION IN CITRUS JUICE VESICLES

ABSTRACT

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

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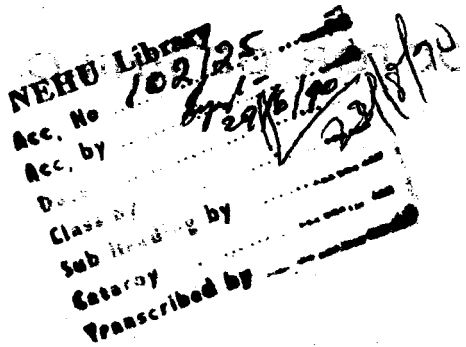

Study of development and differentiation in plants at cell and tissue level **in vitro** is unique as it provides an occasion to understand the mechanism of hormonal stimulation of dedifferentiation of a mature cell and its redifferentiation into a different type of mature cell (Shininger, 1978). Because of typical structural characteristics the tracheary cells are readily distinguishable in cultured calli. Thus using tissue culture techniques the cytodifferentiation of these cells has been achieved in many systems (Roberts, 1976), but our current understanding of the mechanism of vascular tissue differentiation and development is extremely rudimentary (Shininger, 1979). The cultured **Citrus** vesicle is an interesting system for the study of cytodifferentiation because tracheary element differentiation is localized in a callus layer confined to the upper end of the stalk adjacent to the basal end of the juice sac (Kulshrestha **et al.**, 1982). Therefore, an attempt was made to investigate the followings using **C. limon** juice vesicle cultures:

1) Nutritional and hormonal requirements for callusing and xylogenic response;
2) Effect of fruit development, juice vesicle position inside fruit and different regions of the juice vesicle on cytodifferentiation;
3) pH effects on xylogenesis;
4) Effect of variable carbohydrate sources on xylogenesis;
5) Essentiality of nitrogen for xylogenesis;
6) Effects of juices of various **Citrus** species growing in the Northeastern region of India and various organic acids (maleic, α-ketoglutaric, pyruvic and citric acid), on the xylogenic response of juice sacs;
7) Effect of ionizing radiation and light conditions on xylogenesis including histological and histochemical investigations in the control and irradiated material.

Histological and histochemical investigations were also undertaken to investigate the granulation disorder in the pummelo (**C. grandis**) juice vesicles to understand the process of granulation. During granulation the juice in the juice sacs gets gelatinized and thickwalled cells differentiate in the sac region causing deterioration of the fruit quality.

In the present investigation the effects of diverse tissue culture media on callusing and cytodifferentiation in **C. limon** juice vesicle cultures were investigated by using five different nutrient media (MS, B_5, White, Heller and Roberts). Best growth of the callus and cytodifferentiation was evoked by MS medium while Heller and White meida were least effective. Effectiveness of B_5 and Roberts media ranged intermediate between MS and Heller and White media. The differential effectiveness of the various nutrient media could be due to qualitative and quantitative differences in their chemical composition. Effects of varying osmotic concentration of the nutrient medium on cytodifferentiation revealed that osmotic concentration of the medium has a bearing on cytodifferentiation in juice vesicle cultures and an optimal osmotic concentration is essential for better callus growth and cytodifferentiation. Stage of fruit development influences potentiality of juice vesicle to callus and cytodifferentiation. The juice vesicles from the fully developed fruits are excellent experimental material for callusing and xylogenesis while juice vesicles excised from green immature fruits and senescent fruits do not develop callus. Callusing in the vesicles obtained from partially ripe fruits was also less compared to the vesicles excised from fully developed green fruits. Therefore it thus suggests that the developmental stage of the fruit is crucial for the induction of cell divisions in juice vesicle cultures. Kordan (1984) also suggested the use of mature fruits for culturing since the lemon fruits have short life history in vivo. Amongst the various

regions of the juice vesicle usually callus develops only from the neck region and ordinarily no callusing occurs in the sac part of the juice vesicles. This thus suggests that mitotically, the neck region is most sensitive region of the juice vesicle explant. Similar were the findings of Kordan (1965) who also obsereved that in **Citrus** culture experiments, vesicle region usually degenerates and collapses while growth occurs in the neck region. Better growth in the neck region has been attributed to the differences in the acidity of sap present in neck and sac regions (Bartholomew and Sinclair, 1951). Compared to sap present in vesicle, the sap found in neck region is less acidic. The difference in the acidity of the sap may be due to differences in the concentration of the citric acid in sap of the two regions (Kordan, 1965). In the present investigations cell divisions were completely inhibited if pH of the nutrient medium was below pH 3.0.

Essentiality of nitrogen for cytodifferentiation and differences in the efficacy of ammonium and potassium form of nitrogen in inducing cytodifferentiation was also investigated in **C. limon** juice vesicle cultures. The investigations revealed that nitrogen is not essential for cytodifferentiation in **C. limon** juice vesicle cultures. But incorporation of nitrogen sources (NH_4NO_3 and / or KNO_3) in the nutrient medium influenced differentiation. These obserevations are in agreement with the findings of Phillips and Dodds (1977) which suggest that inorganic nitrogen influences TE differentiation and reduction of nitrogen content

of the medium promotes differentiation. In the present study both ammonium and potassium nitrogen inhibited differentiation of tracheid, fibers and sclereids but degree of inhibition differed with the type of nitrogen source used.

Role of carbon source on cytodifferentiation in **C. limon** juice vesicle culture was studied by using different carbohydrates. The investigation revealed that callusing and cytodifferentiation occurred only in presence of a carbon source in the medium suggesting necessity of exogenous sugar in the medium for successful xylogenesis. This supports conclusions of Wetmore and Sorokin (1955). However, in cultured explants of **Helianthus tuberosus** (Minocha and Halperin, 1974) and lettuce pith (Roberts, 1982) callus development and differentiation of TE occurs even in the absence of exogenous carbon source. Thus different species differ in their carbon requirement for cytodifferentiation. Amongst different carbon sources, used in the present study, glucose supported best callus growth while sucrose induced best cytodifferentiation. Myoinositol which is most effective in inducing cytodifferentiation in lettuce pith cultures (Roberts 1982), was least effective in the present study. This thus further supports the conclusions that different species have different carbon source preferences. Different sugars influence cytodifferentiation differently in juice vesicle cultures. These findings are in conformity with the conclusions of Ball (1955) and Jeff's and Northcote (1967). In **Coleus** stems low sucrose levels (1.5-2.5%) induce strong xylem differentiation while higher sucrose levels (3-4%) prefer phloem differentiation

(Wetmore **et al.**, 1964; Wetmore and Rier, 1963). But experiments with callus cultures contradict these findings since in these experiments the number of xylem elements increased with the increasing concentration of sucrose, at least upto 8% (Rier and Beslow, 1967). A similar situation existed in excised **Coleus** internodes (Beslow and Rier, 1969) and in cultured tuber tissue of **Helianthus** (Minocha and Halperin, 1974). Aloni (1980), on the other hand, could not find any correlation between sucrose concentrations and the differentiation of vascular elements and concluded that sucrose concentration in the nutrient medium does not determine the differentiation of xylem and phloem in tissue cultures. But in **C. limon** juice vesicle cultures, sucrose concentration of the medium has a bearing on the differentiation of tracheid, fibers and sclereids. Since the sucrose concentrations upto 4% level promoted differentiation of tracheid, fibers and sclereids, while higher concentrations (above 4%) inhibited their differentiation. However, differentiation of phloem did not occur even at the highest concentration of sucrose (12%), used in the present study. These findings thus while support the observations of Wetmore and Rier (1963) also suggest that a threshold concentration of sucrose is necessary for phloem differentiation may vary with the species.

Plant growth hormones influence differentiation in experimental material. In the present study besides auxin, GA, Kn, C_2H_4 and ABA influenced cytodifferentiation in juice vesicle cultures. Amongst auxins IAA was most effective while

IBA was least effective in evoking differentiation of tracheid, fibers and sclereids. 2,4-D and NAA evoked intermediate responses. The present findings thus corroborate the conclusions that different auxins differ in their effectiveness in inducing cytodifferentiation and the responses are dependent on the species and auxin being used for experimentation (Dalessandro and Roberts, 1971; Dalessandro, 1973a,b; Minocha and Halperin, 1974; Phillips and Dodds, 1977). Synergistic effects of auxin and cytokinin (Sorokin et al., 1962; Minocha and Halperin, 1974; Dalessandro, 1973a,b; Haddon and Northcote, 1975), auxin and GA (Wareing, 1958; Neiten, 1957; Roberts and Fosket, 1966) and auxin, cytokinin and gibberellic acid (Dalessandro, 1973) are reported in literature. In the present study also the combinations of various growth hormones revealed synergism. The combination of IAA, Kn and GA had most effective synergistic effect on cytodifferentiation in juice vesicle cultures.

In the present study methionine, an ethylene precursor, promoted cytodifferentiation while $CoCl_2$, an inhibitor of C_2H_4 biosynthesis, inhibited callusing and cytodifferentiation. The present findings thus further support the conclusions that ethylene is involved in TE differentiation (Abeles and Abeles, 1972; Roberts, 1976; Roberts and Miller, 1982; Miller and Roberts, 1984).

Abscisic acid, a plant growth hormone, inhibits TE differentiation (Minocha and Halperin, 1974; Minocha, 1984; Haddon and Northcote, 1976). But in the present investigations

lower concentrations of ABA improved TE differentiation while higher concentration inhibited cytodifferentiation. Further, ABA induced effects on TE differentiation were more pronounced in presence of growth hormones IAA and Kn than in their absence which suggests an interaction between the three growth hormones.

Most of the evidence for and against cell division, as a prerequisite for differentiation, was accumulated with studies on TE differentiation. Evidence in support of the hypothesis that cell division must precede differentiation comes from the studies on **Coleus** stem (Fosket, 1970) and pea root (Shininger, 1975) explants. In the present investigation, using colchicine, it was found that in explants, which did not develop callus, no differentiation occurred. Thus, in **C. limon** juice vesicle cultures also, cell division is a prerequisite for cytodifferentiation. Similar were the findings of Fosket (1968) Dodds and Phillips (1977) and Malawer and Phillips (1979). In the present investigations higher concentrations of colchicine induced differentiation of abnormal tracheid and fibers which could be due to the effects of colchicine on microtubules and microfibril orientation (Taylor, 1965; Falconer and Seagull, 1985).

During culturing acidification of the medium occurs. The pH of the medium influences callusing and cytodifferentiation in juice vesicle cultures (Khan et al., 1986). Highly acidic medium inhibit cytodifferentiation while moderately acidic medium (pH 5) is most suitable for both callusing and differen-

tiation of tracheids, fibers and sclereids. The differentiation of tracheids is relatively more sensitive to changes in medium pH.

The modified MS medium devised (Table- 8), on the basis of the findings of the present investigation, improved callus growth and induced better cytodifferentiation, in **C. limon** juice vesicle cultures, in comparison to the MS medium.

Analysis of the effects of different organic acids (maleic, α-ketoglutaric, pyruvic and citric acid) on cytodifferentiation in **C. limon** juice vesicle cultures revealed that all the organic acids used improved differentiation. α-ketoglutaric and maleic acids had most pronounced effects while pyruvic acid was least effective. The effectiveness of citric acid was intermediate. However, Gamborg and Skylak (1982) found that amongst Kreb's Cycle, citric acid is most effective in soyabean cell suspension cultures. Thus the responses of different species differ. This is also evident by the fact that in **C. hassaku** juice vesicle cultures citric/maleic acid incorporation in the medium inhibited growth (Kato, 1980). Citric acid which is a major component of **Citrus** fruit juices stimulates cytodifferentiation in **C. limon** juice vesicle cultures (Kulshreshtha **et al.**, 1982). Orange juice also has stimulatory effects in **C. limon** cultures (Murashige and Tucker, 1969). Therefore, effects of fruit juice, from five different **Citrus** species (**C. limon, C. grandis, C. aurantifolia, C. reticulata** and **C. jambhiri**), were also investigated on cytodifferentiation

in **C. limon** juice vesicle cultures. Orange juice induced best xylogenic response while Assam lemon juice was least effective. The other juices evoked intermediate responses. Erner (1975) (1975) also found that orange fruit juice is more effective than grape fruit and lemon fruit juice. Thus, juices from different fruits are differentially effective in inducing differentiation which may be due to variation in the chemical composition of their juices. Since citric acid can substitute partly the effects of orange juice it may be considered that atleast some of the growth activity of the orange juice is due to citric acid present in it. But besides citric acid some other components of the juice must also be responsible for the responses evoked by various **Citrus** fruit juices. Recently presence of some endogenous plant growth substances have been reported in young fruit of seeded and seedless clementine mandarin (Garcia-Papi and Garcia-Martinez, 1984), which could be true for **C. limon** as well since even in the absence of growth hormones juice vesicle cultured on MS basal medium supplemented with **Citrus** fruit juice /differentiated fibers. Aloni (1980) reported that IAA and GA are the limiting and controlling factors in the differentiation of fibers.

In the present investigation, white (fluorescent) light, had detrimental effect on the quality and quantity of differentiation, in comparison to dark conditions. These findings support the conclusions of Phillips and Dodds (1977) and Yeoman and Davidson (1971). Low doses of gamma rays promote callus growth and differentiation while higher doses, which inhibited

callus growth (cell division) also inhibited differentiation. Similar were the findings of Dodds and Phillips (1977), Phillips and Arnott (1983). The radiation induced stimulation of differentiation was associated with the presence of more protein and nucleic acid in the irradiated juice vesicles. Conversely radiation induced inhibition of differentiation was associated with less protein and nucleic acid in the irradiated juice vesicles. This may suggest that radiation effect on differentiation of trachied, fibers and sclereids are mediated through their effect on protein and nucleic acid metabolisms of the irradiated juice vesicles.

The normal and granulated juice vesicles of **C. grandis** differ in their morphology. Compared to the normal juice vesicle, the granulated juice vesicles are hard, granular in appearance and heavier than normal juice vesicles. The hardening of the granulated juice sac is due to gelatinization of the cell contents and lignification of the cell wall of the cells present in the sac region (Bartholomew et al., 1941).

The normal and granulated juice vesicles also differ in their anatomical characteristics. Compared to the normal juice vesicles, the cell walls of the vesicle membrane cells are thicker in granulated juice vesicles. Further while the normal juice vesicle is parenchymatous in nature, the granulated juice vesicles reveal differentiation of thick-walled pitted cells in the vesicle region. The extent of differentiation of these thick walled cells increases with the progression

and increase of granulation so much so that the sac region gets fully filled with these cells. In the later stages of granulation the granulated juice vesicles collapse due to disintegration of cells in the sac region. The granulated juice vesicles are biger than the normal juice vesicles. A similar situation exists in granulated juice vesicles of valencia oranges (Turrel and Bartholomew, 1939; Bartholomew et al., 1941). The normal and granulated juice vesicles differ histochemically also. Compared to the normal juice vesicles, the granulated juice vesicles have more insoluble polysaccharides but protein and nucleic acid content is much less. Thus, histochemically granulation is associated with a rapid loss of protein and nucleic acid contents while a simultaneous increase in their insoluble polysaccharide content occurs. Further in comparison to the cells of normal juice vesicles rapid and pronounced lignification of the cell walls takes place in the cells of granulated juice vesicles. All these could be manifestations of metabolic differences in the two types of juice vesicles. Thus it may be suggested that metabolic disorders are the reasons for granulation disorder.

Table 8: A comparison of composition of MS and MS medium modified for *C. limon* juice vesicle culture (mg/l).

Component	MS	Modified MS
NH_4NO_3	1,650	-
KNO_3	1,900	-
$CaCl_2.2H_2O$	440	440
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
$MnSO_4 4H_2O$	223	223
$ZnSO_4.7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
Myoinositol	100.0	-
Nicotinic acid	0.5	0.5
Pyridoxin-HCl	0.5	0.5
Thiamine-HCl	0.1	0.1
Glycine	2.0	2.0
Sucrose	30,000	40,000
Agar	10,000	10,000
IAA	1.0-30.0	10.0
Kinetin	0.04-10.0	0.2
pH	5.7-5.8	5.0

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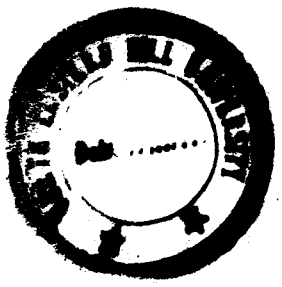

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I certify that the thesis entitled "IN VITRO STUDIES IN TRACHEARY ELEMENT DIFFERENTIATION IN CITRUS JUICE VESICLE CULTURE" *submitted by Mrs. Aysha Khan for the Degree of Doctor of Philosophy of the North-Eastern Hill University, Shillong embodies the record of original investigation carried out by her under my supervision. She has been duly registered and the thesis presented is worthy of being considered for the award of the Ph.D. Degree. This work has not been submitted for any degree of any other University.*

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CONTENTS

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ABBREVIATIONS USED

ABA	abscisic acid
IAA	indole-3-acetic acid
IBA	indole butyric acid
NAA	γ-naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
GA	gibberellic acid
Kn	kinetin
$Na_2FeEDTA$	sodium salt of ferric ethylene diamine tetra acetic acid
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
TE	tracheary element
TBA	tertiary butyl alcohol
WVM	wound vessel member

CHAPTER I
INTRODUCTION

Study of development and differentiation in plants at cell and tissue level **in vitro** is unique as it provides an occasion to understand the mechanism of hormonal stimulation of dedifferentiation of a mature cell and its redifferentiation into a different type of mature cell (Shininger, 1978). Because of typical structural characteristics, the tracheary cells are readily distinguishable in culture calli. Thus using tissue culture techniques the cytodifferentiation of these cells has been achieved in many systems (Roberts, 1976), but our current understanding of the mechanism of vascular tissue differentiation and development is extremely rudimentary (Shininger, 1979).

The nutritional requirements for cytodifferentiation varies with the cells, tissues or organ being cultured since it is dependent on the endogenous nutrient present in the experimental material and chemical ingredient of the nutrient medium (Hildebrandt **et al.**, 1954). The quality of vascular tissue differentiation in callus cultures depends on relative concentration of auxin and sucrose used in the experiment (Wetmore and Rier, 1963). Essentiality of exogenous sugar for xylogenesis has been demonstrated time and again (Wetmore and Sorokin, 1955; Fosket and Roberts, 1964). The sugar induced response varies with the alteration of sugar used (Ball, 1955; Jeff's and Northcote, 1967). Shininger (1979) considers that the effects of alternative carbohydrate supplies on xylogenesis is still not resolved beyond doubt and specific effects of various carbohydrate sources on xylem differentiation cannot be explained satisfactorily at present.

have been emphasized, the study of possibility of sequential requirements of growth regulators in xylogenesis has remained neglected (Roberts, 1976). Indoleacetic acid, α-naphthaleneacetic acid and 2,4-dichlorophenoxyacetic acid, when used individually or in combination with the same cytokinin, induced different patterns of xylem formation (Dalessandro and Roberts, 1971). A similar response was also found when same auxin was used in combination with different cytokinins. Besides, the auxin, cytokinin effect was modified by gibberellic acid (Dalessandro, 1973b). Also, there is dearth of literature on the possible effects of mixtures of the same type of growth regulators (auxin, cytokinin) (Roberts, 1976). It is reported that a synergism exists between gibberellin and other growth regulators in the stimulation of cambial activity and secondary xylem formation, but why this effect is not evident in the primary xylem differentiation is ill-understood (Roberts, 1976). Further, both stimulation (Gautheret, 1961; Dalessandro, 1973b) and inhibition (Minocha and Halperin, 1974) of cytodifferentiation is induced by incorporation of gibberellin in auxin-cytokinin medium having variable carbohydrate source. But no attempt has been made to investigate relative effectiveness of various gibberellins in altering the initiation and morphology of tracheary elements (Roberts, 1976). Thus much work needs to be done on the effects of gibberellins on cytodifferentiation in isolated tissue to understand the gibberellin involvement in xylogenesis. Investigations on the role of pressure in secondary xylem differentiation suggest

that stress induced ethylene production may be involved in cytodifferentiation (Brown and Sax, 1962; Brown, 1964). Roberts (1976) stated that ethylene plays an important role in xylogenesis. Abscisic acid influences differentiation of tracheids without affecting cell division (Minocha and Halperin, 1974). Thus hormone ABA is extremely useful in providing additional culture conditions in which the process of differentiation can be controlled independently of growth (Minocha, 1984).

The controversy whether or not cell division is an essential prerequisite of TE differentiation still remains unresolved (Aloni, 1987). That it is an essential prerequisite for cytodifferentiation has been demonstrated in **Coleus** and **Phaseolus** using mitotic inhibitors (Fosket 1968, 1970; Torrey and Fosket 1970; Comer, 1978 and Jeff's /Northcote, 1967). But in **Zinnia elegans** differentiation occurs even without cell division (Fukuda and Komamine, 1980b). Colchicine, which also affects development of secondary wall thickenings in TE (Green, 1963; Roberts and Baba, 1968; Hepler and Fosket, 1971), is a useful diagnostic tool in the studies aimed at to find out the essentiality of cell division for cytodifferentiation (Fukuda and Komamine, 1980b).

In general, most of plant tissue culture media are buffered prior to autoclaving within the range of pH 5-6. Media vary considerably in regard to their buffering capacity. Sometimes, the pH may shift dramatically during a tissue culture experiment (Martin, 1980). Almost no work has been done on the possible

effects of pH on the cytodifferentiation of tracheary elements (Roberts, 1983). Acidity of the media was shown to be a factor in secondary xylem formation in **Plumeria** culture (Datta and 1975)/ tracheary element differentiation in **citrus** juice vesicles (Khan **et al.**, 1986) besides being an important factor in the regulation of morphogenetic pathways in thin layer explants of tobacco (Van **et al.**, 1985).

Tracheary element differentiation is influenced by a number of physical factors (Roberts, 1976). Ionizing radiation affect differentiation of TE in gamma irradiated plants (Foard and Haber, 1961; Fosket and Miksche, 1966; Chauhan and Singh, 1975) and cultured explants of **Helianthus tuberosus** (Phillips, 1981b; Phillips and Hawkins, 1985) and **Zinnia elegans** (Sugiyama **et al.**, 1986). Besides, light generally inhibits xylogenesis (Phillips and Dodds, 1977). But in carrot culture light, which is a requirement for xylogenesis, could be replaced by cytokinin (Mizuno and Kamamine, 1978; Mizuno **et al.**, 1971).The xylogenic response varies with the quality of light also (Phillips and Dodds, 1977). Therefore, investigations on the light effect on xylogenesis need to be emphasized (Shininger, 1979).

The citrus juice vesicles are composed of an enlarged juice sac and a slender stalk. The juice vesicle in **Citrus** arises as stalked outgrowth from the endocarp (Shininger, 1968). The early literature on the anatomy of juice vesicles of **Citrus** fruits shows that these structures are devoid of vascular elements (Ford, 1942; Bartholomew and Reed, 1948; Bartholomew and Sinclair,

1951). The presence of tracheary elements in the stalk region of various juice vesicles has been reported in many **Citrus** species (Webber, 1948; Kordan, 1964; Chauhan and Roberts, 1978). Tracheary elements have also been induced to differentiate in isolated juice vesicles of **Citrus** fruit during **in vitro** culture (Kordan, 1964; Kato, 1980). Kulshrestha **et al.** (1982) reported that citric acid has a xylogenic effect similar to auxin. In previous experiments even though citric acid was incorporated in medium its xylogenic effects could not be observed (Kordon, 1965). Erner **et al.** (1975) suggested that orange juice induced stimulation in albedo tissue cultures of **C. sinensis** could be due to citric acid present in the juice. However, Einset (1978) considers organic acids other than citric acid may be involved in the growth stimulation of citrus cultures. Further, the possible xylogenic properties of orange juice have neither been examined nor experiments have been undertaken to investigate in detail the possible role of citric acid in xylogenesis in different **Citrus** cultures. It is hoped that such studies may also lead to understand the granulation disorder of **Citrus** juice sacs, since in granulation affected citrus juice sacs thick walled tracheary element like cells form in the juice sacs (Bartholomew **et al.**, 1941). The cultured **Citrus** vesicle is an interesting system for the study of cytodifferentiation because tracheary element differentiation is localized in a callus layer confined to the upper end of the stalk adjacent to the basal end of the juice sac (Kulshrestha **et al.**, 1982).

In view of the above, the following aspects of xylogenesis have been investigated using juice vesicles of Assam lemon as experimental material.

1) Nutritional and hormonal requirements for callusing and xylogenic response as these are not known for cultured **Citrus** vesicles (Kulshrestha **et al.**, 1982).

2) Effects of fruit development, juice vesicle position inside fruit and different regions of the juice vesicles on cytodifferentiation.

3) pH effects on xylogenesis.

4) Effect of variable carbohydrate sources on xylogenesis.

5) Phillips and Dodds (1977) reported that xylogenesis in tuber is stimulated if nitrogen concentration of the medium is reduced. Therefore, an attempt has been made to find out whether nitrogen is obligatory for xylogenesis.

6) Effects of juice of various **Citrus** species growing in this region and organic acids e.g., citric acid etc. on the xylogenic response of juice sacs.

7) Effect of ionizing radiation and light conditions on xylogenesis including histological and histochemical investigations in the control and irradiated material to correlate the structural and histochemical changes occurring during radiation induced xylogenic response.

8) Histological and histochemical studies in the pummelo (**C. grandis**), which exhibits granulation disorder, to understand the process of granulation.

CHAPTER II

REVIEW OF LITERATURE

The cytodifferentiation of vascular tissue induced by wounding was first described by Vochting (1892), and subsequently studied by Simon (1908), Freundlich (1908), Kaan Albert (1934), Jost (1940, 1942), Sinnott and Block (1944, 1945). The differentiation of tracheary elements in tissue explants, from storage organs of plants, has been used as a model system for the study of the regulation of cytodifferentiation in plants (Roberts, 1976; Lamb, 1933). Mode and factors controlling cytodifferentiation **in vivo** are little understood. Understanding of cellular differentiation requires an investigation into the explants responsible for the differentiation of strikingly different cell types, both biochemically and morphologically, from similar cells (Roberts, 1976; Phillips, 1980). The ultimate objective of this approach is therefore to understand the mechanism of differential gene expression responsible for diverse type of cytodifferentiation (Roberts, 1976; Comer, 1978; Dodds, 1981). DNA synthesis precedes cytodifferentiation (Stockdale and Topper, 1966; Torrey and Fosket, 1970; Malawer and Phillips, 1979). The genes for cytodifferentiation could be freed from their existing repressors and become available for programming during the period when both DNA and histone synthesis occur (Ebert and Sussex, 1970). Cell division is an essential prerequisite for cytodifferentiation (Shininger, 1975; Simpson and Torrey, 1977; Malawer and Phillips, 1979). Investigations in the wound xylem differentiation reveal that the initiation of tracheary element formation occurs only during some phase of the mitotic

cycle suggesting a close relationship between cell division and cytodifferentiation. But in pea root segment and soyabean callus tissue cultures cell division follows the differentiation of tracheary elements (Torrey and Fosket, 1970; Fosket and Torrey, 1969). Taylor (1965) found the role of microtubules in microfibril orientation using of colchicine. This substance inactivates the microtubules by combining with a protein subunit of these structures. Esau (1965) demonstrated that during the formation of tracheary elements, secondry wall microfibrils are deposited in localized bands to form patterns described as annular, spiral, reticulate, scalariform or pitted. In the presence of microtubule inhibitors, secondary wall is not deposited in the pattern consisting of discrete bands but rather is found smeared over a wide area of the primary wall (Brower and Hepler, 1976). Using fluorescence microscopy and immuno-cytochemical techniques Falconer and Seagull (1985) described the changes in wall deposition and microtubule organisation during xylogeneis of **Zinnia elegans**. Relationship between DNA synthesis and differentiation process has also been established using inhibitors. The requirement of DNA synthesis for cytodifferentiation in cultured coleus stem segments has been demonstrated by using fluorodeoxyuridine and mitomycin (Fosket, 1968). In coleus stem segments colchicine blocked differentiation suggesting the need of mitosis for cytodifferentiation. Torrey (1975) found that single cells of **Centaurea cyanus** L. stem corn produce tracheary element **in vitro** on xylogenic medium. The increase in tracheary element number followed the increase in total cell number.

Although great majority of investigations reveal a close relationship between cell division and xylogenesis (Torrey and Fosket, 1970, Comer, 1978), direct differentiation of certain cell types into tracheary elements without cell division has also been observed in few cases (Torrey, 1975; Fukuda and Komamine, 1980a,b; Dodds, 1980; Phillips, 1981; Kohlenbach, Korber and Li, 1982). In several investigations certain parenchymatous cells either undergo a direct transformation or redifferentiation into a tracheary element without an immediately preceding cell division (D'Amato, 1953; Sachs, 1969; Dalessandro and Roberts, 1971; Gee, 1972; Basile et al., 1973). In lettuce pith explants, xylogenesis was reported in the presence of 10^{-5}m FUDR, apparently in the absence of DNA synthesis and mitosis (Turgeon, 1975). In cultured artichoke explants large tracheary elements differentiate following exposure of the tuber to high levels (15 KRad) of ionizing radiation prior to explant excission (Dodds, 1978). Fukuda and Komamine (1980a,b) demonstrated that mesophyll cells isolated from young leaves of **Zinnia elegans** may differentiate directly, without intervening mitosis or cell division. Phillips (1981), Phillips and Hawkins (1985) reported that immature tuber of Jerusalem artichoke can differentiate directly into tracheary element **in vitro**, when treated with 15 KRad dose of gamma rays.

Nitrogen effect on the cytodifferentiation are little studied. Tracheary elements differentiation in the Jerusalem artichoke explants is stimulated by reducing the nitrogen content

of the medium (Phillips and Dodds, 1977). White and Gibley (1966) reported that the type of nitrogen compounds incorporated in the nutrient medium, can influence tracheary element differentiation in cultured tissues. Fukuda and Komamine (1980a) found that higher concentration of potassium nitrate (20 mM) and lower concentration of ammonium chloride (0-1mM) are essential for tracheary element differentiation. They also found that the number of tracheary elements decreased with the decreasing concentration of potassium nitrate. Nutrient medium devoid of potassium nitrate do not support differentiation of tracheary elements in the cultured tissues. They also stated that ammonium chloride does not induce differentiation of tracheary elements (Fukuda and Komamine, 1980a). Conversely, Shiraishi, Baba and Roberts (1982) stated that only ammonium nitrate is sufficient to stimulate differentiation of tracheary elements in lettuce pith. Certain amino acids inhibit growth of tobacco, tomato, carrot and soyabean cell cultures if nitrate of urea are the nitrogen sources but when nitrogen source is ammonium, the inhibition is not evident (Behrend and Mateles, 1975). Tobacco cells are capable of growth on medium having ammonia as a sole nitrogen source if succinate, malate, fumarate, citrate, α-ketoglutarate, glutamate or pyruvate is added to the growth medium (Behrend and Meteles, 1976).

A variety of plant growth regulators have been implicated in the control of tracheary element differentiation in different tissues (Roberts, 1969, 1976; Torrey, Fosket and Hepler, 1971; Dalessandro, 1973a; Minocha and Halperin, 1974; Torrey, 1975;

Barnett, 1979; Fukuda and Komamine, 1981). Cell division in the presence of appropriate inducing substance was a necessary prerequisite for the induction of cytodifferentiation of tracheary element (Torrey and Fosket, 1970; Comer, 1978; Dodds, 1981). Consequently, any factor affecting cell division will also affect the number of tracheary element formed (Roberts, 1976; Malawer and Phillips, 1979; Dodds and Phillips, 1977). Such factor, which particularly include auxin, cytokinin and the carbohydrate source (Roberts, 1971, 1976) may have direct effect on cytodifferentiation of tracheary elements.

Incorporation of exogenous sugar in the medium is a necessity for successful xylogenesis **in vitro** (Wetmore and Sorokin, 1955; Fosket and Roberts, 1964) and the response varies with the type of sugar used (Ball, 1955; Jeff's and Northcote, 1967). Medium having a dissaccharide containing an α-glucosyl radical at the non-reducing end of the molecules differentiated vascular nodules having organized xylem in bean callus experiments while all other sugars induced the formation of scattered tracheary element (Jeff's and Northcote, 1967). Sucrose, maltose and trehalose are more effective than a variety of other sugars in the induction of vascular nodules (Jeff's and Northcote, 1967). In some investigations, however, glucose was found to be as effective as sucrose, and trehalose relatively ineffective (Minocha and Halperin, 1974; Phillips and Dodds, 1977). When two equally effective sugars (sucrose and glucose) were incorporated in the medium inhibition occurred (Minocha and Halperin, 1974), suggesting

that sugars have specific effects which go beyond a generalized role as osmotic reagent or carbon sources (Jeff's and Northcote, 1967). Doley and Leyton (1970) consider that some of the carbohydrate effects on xylogeneis may be due to variation in water potential because addition of biologically inert substance polyethylene glycol in the nutrient medium also increased the amount of xylem in **Fraxinus** stem segments. Glycerol and Myoinositol also can be used as exogenous carbon source for the study of xylogenesis in tissue culture experiments (Roberts and Baba, 1982).

In long term callus cultures, on media having both IAA and sucrose, while low sucrose concentration favours differentiation of vascular nodules having mainly xylem elements, the higher concentrations of sucrose (4 to 6%) favour differentiation of phloem (Wetmore and Rier, 1963; Jeff's and Northcote, 1967). In **Parthenocisscus** culture, high levels of sucrose favoured xylogeneis (Rier and Beslow, 1967) although this is not a regular feature when other plant species are used (Minocha and Halperin, 1974; Phillips and Dodds, 1977). Aloni (1980), however, considers that rather than sucrose, auxin concentration in the nutrient medium determines the differentiation of xylem and phloem in tissue cultures. Aloni (1980) demonstrated that low concentrations of IAA induces differentiation of sieve elements with no tracheary elements while higher concentrations of IAA favours differentiation of both tracheary element and phloem. The sucrose concentration, however, influences the amount of callose deposited on the sieve plates (Aloni, 1980).

The discovery that auxin applied to decapitate plants induced differentiation of a mass of WVM led to the enquiry into hormonal regulation (Blum, 1941; Whiting and Murray, 1946; Beal, 1951). Plant growth hormones, auxin, cytokinin, gibberellin and ethylene play important role in the differentiation of xylem elements (Roberts, 1976). Auxin plays a central role in the process of xylogenesis since, supply of exogenous auxin is required for the induction of xylem differentiation in pith parenchyma of lettuce heads (Clutter, 1960; Earle, 1968; Dalessandro and Roberts, 1971) and callus cultures (Gautheret, 1961; Wetmore and Rier, 1963; Bergmann, 1964; Jeff's and Northcote, 1966; 1967; Rier and Beslow, 1967; Cronshaw, 1967; Torrey, 1968; Fosket and Torrey, 1969). However, the mechanism determining the position where vascular tissue will differentiate are little understood (Roberts, 1971; Wilson, Roberts, Gresshoff and Dircks, 1982). The surface form of the plant body, its polarity, the position of existing vascular tissues, and the location of sources and sinks, for certain transported substances, seem to influence differentiation of tracheary elements (Wetmore, De Maggio and Rier, 1964; Sachs, 1978, Wilson, 1978; Mitchison, 1980). Using bipolar gradient technique the influence of morphogenetic gradients on vascular differentiation patterns; information on the mobility of particular morphogens; and on the dependence of callus formation and tracheary element differentiation in their presence was investigated by Wilson, et al. (1982). An exogenous supply of auxin is usually essential for xylogenesis in all the culture

experiments, except crown gall tumor callus cultures which are autotrophic for their auxin requirement (Basile et al., 1973). The arrangement of tracheary element presumably reflects the direction of auxin flows (Sachs, 1978; Mitchison, 1980). Investigations on wood formation in decapitated pine trees suggest that both cambial activity and differentiation of tracheids including their cell wall thickness and radial diameter are influenced by auxin (Wodzicki, et al., 1982). Auxin production by leaves has often been implicated as one of the morphogenetic factors influencing xylem differentiation within leaf traces (Jacobs, 1979; DeGroote and Larson, 1984). The possibility that auxin may mediate cell enlargement has been entertained (Roberts, 1976; Aloni and Zimmermann, 1983) and considerable indirect empirical evidence has accumulated suggesting that auxin may regulate xylary element size (Digby and Wareing, 1966; Doley and Leyton, 1968). However, the direct involvement of auxin in cell enlargement has been questioned recently (Hanson and Trelwaves, 1982). The effectiveness of various auxins and their optimum concentration changes with the species investigated (Dalessandro and Roberts, 1971; Dalessandro, 1973 a,b; Minocha and Halperin, 1974; Phillips and Dodds, 1977). Different auxins and different combinations of growth hormones (IAA+K_m/IAA+GA/K_m+GA/ IAA+K_m+GA) induce different patterns of tracheary elements distribution in culture experiments (Dalessandro and Roberts, 1971; Dalessandro, 1973a) which could be due to differences in the polar transport characteristics of the auxin being tested/used

(Roberts, 1976). Various auxins (IAA,α-NAA and 2,4-D), when used in combination with some cytokinin, induce diverse type of xylem differentiation in **lettuce** pith explants (Roberts, 1971). A similar situation existed when a given auxin was used in combination with different cytokinins (Roberts, 1971). The primary role of auxin appear to be in the stimulation and maintenance of cell division (Phillips, 1980; Yajima **et al.**, 1980). Tracheary elements are not known to occur in the absence of auxin and a threshold concentration seems to be essential for their differentiation (Aloni, 1980). Bergmann (1964) first reported that kinetin may increase the number of tracheids in cultured tobacco tissue. Auxin and cytokinin are necessary for both cell division (Das **et al.**, 1956) and differentiation of tracheary elements (Dalessandro and Roberts, 1971; Kohlenbach **et al.**, 1982). Cell divisions in soyabean callus cultures occur without cytodifferentiation in presence of cytokinin but addition of auxin in the medium is essential for tracheary element differentiation (Fosket and Torrey, 1969). However, in Jerusalem artichoke tissue, auxin is essential for cell division (Yeoman and Evans, 1967; Yasuda, Yajima and Yamada, 1974; Minocha, 1979a,b; Yajima, Yasuda and Yamada, 1980), while cytokinin benzylaminopurine, specifically induces differentiation of tracheary elements without any effect on cell division (Minocha and Halperin, 1974). Direct involvement of auxin in tracheary element differentiation is further evidenced by investigations on explanted interfascicular tissue of **Ricinus** hypocotyle (Siebers, 1971a). Observations revealed that in the presence of relatively high levels of IAA

the presumptive procambial cells differentiated directly into tracheary elements without undergoing any division while incubation on hormone free medium resulted into normal cambial activation (Siebers, 1971b). For cytodifferentiation of tracheary element **in vitro** exogenous supply of cytokinin is an essential requirement (Dalessandro, 1973b; Dalessandro and Roberts, 1971) but xylogenesis occurs only when both auxin and cytokinin are incorporated in the medium (Torrey, 1968; Fosket and Torrey, 1969). Combinations of auxin and cytokinin although have synergistic effect on xylem differentiation (Sorokin **et al.**, 1962) in isolated **coleus** explants mixture of IAA and Kinetin were either inhibitory (Fosket and Roberts, 1964) or ineffective in stimulating xylogenesis (Earle, 1968). Cytokinin induces xylogenesis in the prothalli of various Ferns and Lycopods (De Maggio, 1967) and increased tracheary differentiation and lignification in **Nicotiana tobacum** (Bergmann, 1964). Artichoke (Dalessandro, 1973b; Minocha and Halperin, 1974), **lettuce** pith (Dalessandro, 1973a) and bean callus (Haddon and Northcote, 1975) explants undergo division and differentiation with auxin alone but addition of a cytokinin is stimulatory. The addition of a 5 mg/l BAP in the medium containing 1 mg/l NAA resulted in a fourfold increase in tracheary element differentiation compared to control which containedonly NAA (1 mg/l) suggesting involvement of cytokinin in tracheary element differentiation (Minocha and Halperin, 1974). Aloni (1982) has shown that cytokinin can be a limiting factor in the early stages of fiber differentiation in cultured hypocotyl segment of **Helianthus tuberosus**. The cytokinin requirement

varies with the experimental material and the variation may be due to differences in the endogenous production (Mizuno et al., 1971). The xylogenic response differ with the type of cytokinin used. For example, in experiments involving lettuce pith, zeatin was more effective than kinetin or BAP (Dalessandro and Roberts, 1971; Dalessandro, 1973a). But in experiments using artichoke as experimental material, BAP was more effective than other cytokinins. Minocha (1984) presented a hypothetical scheme for the role of benzyladenine in the differentiation of tracheary element in artichoke tuber explants. It is suggested that during one or more critical stages of cell division, in the presence of optimal level of benzyladenine, a proportion of cells are induced or committed for later differentiation into tracheary elements. Cawthon (1972) examined the sequential hormonal (auxin and cytokinin) requirements for xylogenesis. According to Cawthon, explants received auxin prior to cytokinin were completely lacking in tracheary elements but number of dividing cells were higher as compared to those explants which received cytokinin before auxin and revealed tracheary elements. In lettuce and pea the number of tracheary elements increased with the increasing concentration of kinetin (Fosket and Torrey, 1969; Shininger and Torrey, 1974).

The effects of GA on xylem differentiation varies with the species and culture conditions. GA concentrations induce both stimulation (Gautheret, 1961; Dalessandro, 1973b; Phillips and Dodds, 1977) and inhibition (Minocha and Halperin, 1974; Minocha, 1984) of xylogenesis. Application of GA stimulates cambial activity in appricot spur shoot (Bradley and Crane, 1957).

GA treatment of **Lycopersicon esculantum** stem increases length of annular, spiral tracheary elements and pitted scalariform cells (Davis and Holmes, 1962). It also increased the frequency of pitted scalariform tracheary elements (Davis and Holmes, 1962). Internodes of **Mercurialis annua** treated with GA have more secondary xylem compared to control (Tranchet, Bressat, Marchand and Blane, 1961). GA_3 applied to the storage roots of carrot significantly reduced the amount of secondary phloem production and decrease phloem/xylem ratio for parenchyma cells (Mckee and Morris, 1986). An interaction between GA and IAA influences formation of xylem by cambium (Wareing, 1958) and differentiation of xylem/WVM in explants of **Helianthus tuberosus** (Neiten, 1957) and stem internode tissue slices of Coleus (Roberts and Fosket, 1966). The interaction of auxin, cytokinin and gibberellin influences cell division and xylem differentiation in cultured explants of Jerusalem artichoke (Dalessandro, 1973b). Roberts and Fosket (1966) also found an interaction of gibberellic acid and indole-acetic acid which increased the formation of wound vessel members in cultured **Coleus blumei** stem segment. Wareing (1958) postulated an interaction between GA and IAA in xylem formation resulting from cambial activity. Digby and Wareing (1964, 1966) have shown that the ratio of exogenous GA to IAA is important in regulating cambial activity and xylem differentiation.

Several investigations suggested that ethylene can regulate cytodifferentiation (Apelbaum, Fisher and Burg, 1972;

Neel, 1970; Shain and Hillis, 1973). The possible involvement of wound induced ethylene production in cytodifferentiation is reported (Abeles and Abeles, 1972). Enhanced xylem differentiation in wound coleus shoots subjected to clinostat treatment provides evidence for involvement of stress-induced ethylene in cytodifferentiation (Roberts and Fosket, 1962). The unusual effects of carbon dioxide on cytodifferentiation may involve ethylene antagonism (Bradley andDahmen, 1971). In cultured radish roots the induction of vascular cambium on application of exogenous auxin and cytokinin is attributed to the endogenous ethylene production by the excised organ (Loomis and Torrey, 1964). Exogenous proline may stimulate xylogenesis in cultured explants of **Coleus** under certain experimental conditions (Roberts and Baba, 1968). Linkins, Lewis and Palmer (1973) examined the relative effects of auxin and ethylene on cytodifferentiation in explants of **Phaseolus**. Indoleacetic acid stimulated ethylene production in the explants, and cell division and cytodifferentiation occurred. Roberts (1976) considers ethylene to be hidden hormone during the initiation of xylem differentiation. Xylogenesis in **lettuce** pith parenchyma and soybean cotyledon explants is stimulated by incorporation of L-methionine, an ethylene precursor, in the nutrient medium (Roberts and Baba, 1978; Miller and Roberts, 1982). Tracheary element formation is, however, inhibited by high levels of ethylene (Zobel and Roberts, 1978). Silver inhibits stimulatory effects of ethylene (Miller and Roberts, 1982; Beyer, 1978). This inhibition can be partially reversed by the addition of ethylene releasing agents (Miller and Roberts, 1984) suggesting the

possibility of involvement of ethylene, a plant growth hormone, in xylogenesis (Roberts and Miller, 1982). Xylem formation in cultured tissue has been correlated with increased levels of phenylalanine ammonia lyase (PAL) (Fukuda and Komamine, 1982; Haddon and Northcote, 1976; Rubery and Fosket, 1969), wall bound peroxidase activity (PO) (Fukuda and Komamine, 1982) and two marker of enzymes of lignin biosyntheis (Goldberg, Thoan and Catesson, 1985). Ethylene treatment stimulates the activity of PAL (Chalutz, 1973; Hyodo, Kuroda and Yang, 1978; Rhodes and Wooltorton, 1973) PO (Matsuno and Uritani, 1972; Morgan and Fowler, 1972) and lignin deposition (Rhodes and Wooltorton, 1973). Miller, Crawford and Roberts (1985) therefore suggested that ethylene may control xylogenesis by modulating wall bound PO activity. When undifferentiated cells begin/start differentiating into tracheids and xylem vessels, a new acid phosphatase isoenzyme appears (De and Roy, 1984). The transformation of living cells into dead empty tracheids during cellular differentiation and the biosynthesis of the acid phosphatase enzyme are functionally related to the autolysis of the cell contents and lignin synthesis (De and Roy, 1984).

Abscisic acid, a growth inhibitory phytohormone (Addicott and Lyon, 1969; Milborrow, 1966, 1974), although promotes cell division and DNA synthesis in Jerusalem artichoke tuber explants (Minocha, 1979a,b), the tracheary element formation is inhibited (Minocha and Halperin, 1974; Minocha, 1984). In bean callus, abscisic acid inhibits both vascular nodule formation and PAL

activity simultaneously (Haddon and Northcote, 1976). However, Haddon and Northcote (1976) did not investigate the effects of ABA on cell division.

The effects of pH on xylogenesis have received little attention. Shininger and Torrey (1974) reported a clear optimum for xylogenesis at pH 6.0, although total cell number was constant between pH 4.5 and pH 7.0. Acidity of the medium influences secondary xylem formation in **Plumeria** culture (Datta **et al.**, 1975). The pH of the medium is also important in the regulation of morphogenetic pathway in thin cell layer explants of tobacco (Van **et al.**, 1985). Cytodifferentiation of different cell types in **Citrus** cultures is influenced by the pH of the medium (Khan **et al.**, 1986).

In the presence of 2% sucrose with citric, pyruvic and maleic acid the marigold, paris daisy, periwinkle or sunflower crown gall tissue and normal tobacco callus cultures reveal growth. However, fumaric acid have little or no effects (Hildebrandt **et al.**, 1954). Presence of citric acid in the nutrient medium stimulates callus development from cultured juice vesicles (Erner et 1975). Addition of citric acid (2 gm/l) in the medium induces xylogenic response in cultured juice vesicles of Assam lemon suggesting that citric acid effect is similar to auxin (Kulshrestha **et al.**, 1982). Incorporation of orange juice (10% w/v) in the medium stimulates growth in culture of **Citrus medica, Citrus limon, Citrus paradisi, Citrus sinensis** and **Citrus reticulata**

juice vesicles (Einset, 1978). The role of organic acid is neither connected with ammonium transport nor the relief of ammonia toxicity, but may be related to the need for additional skeleton for synthesis of amino acids (Behrend and Mateles, 1976).

Light usually inhibits cytodifferentiation. White light inhibits cell division in explants of Jerusalem artichoke (Yeoman and Davidson, 1971). However, the response is evident only in the presence of 2,4-D prior to DNA synthesis. Cytokinin overcomes light requirement for cytodifferentiation (Mizuno et al., 1971). Different wave lengths of light affect cell wall growth by altering microtubules orientation (Miller and Stephan, 1971). Stafford (1948); Williams, Preston, and Ripley (1955) and Thomson and Miller (1963) found that the pattern of secondary wall deposition in differentiating xylem is not influenced by light. Goodwin (1942); Stafford (1948); Shields and Settler (1952) and Thomson and Miller (1963), however, reported a shift from spiral to annular type of secondary wall thickening in xylem elements with increasing light intensity. Kleiber and Mohr (1967) reported that the formation of tracheary elements in the hypocotyl of **Sinapis alba** L. seedlings are photo-induced by red light and reversed by far-red light. A short exposure of green light ($0.02/m/m^2$) during incubation increases the cell and tracheary element number in Jerusalem artichoke tubers. The exposure to continuous white light ($4.81\ 1m/m^2$, Universal white fluorescent) throughout the 72 hour culture period while has no effect on cell number, the number of tracheary elements is reduced (Phillips and Dodds, 1977).

The components that are clearly involved in tracheary element formation are auxin, cytokinin and sucrose. Both auxin and cytokinin are essential for the activation of the cell cycle (Patau et al., 1957). Deoxyribonucleic acid synthesis precedes tracheary element differentiation in explants of pea root (Torrey and Fosket, 1970) and jerusalem artichoke tuber (Malawer and Phillips, 1979). Studies with mitotic inhibitors have shown that high rates of xylogenesis are dependent on corresponding high rates of cell division (Malawer and Phillips, 1979; Dodds and Phillips, 1977).

Foster (1944) described ontogenic development of foliar sclereids in **Camellia japonica** and observed that these sclerenchymatous idioblasts originate from parenchymatous cells of the leaf during the late stage of leaf maturation, subsequent to the ceasing of all cell division activity. The first detectable changes evident in the parenchymatous sclereid initials are increased nuclear and nucleolar sizes. There is a period of localized primary wall growth in the form of tubular branches, and this is followed by the formation of a massive secondary wall. Foard (1960) demonstrated that the cytodifferentiation of these sclereids could be experimentally induced by leaf wounding. Parenchyma cells, that usually differentiate into photosynthesizing mesophyll cells, were made to develop into foliar sclereids without undergoing cell division. Foard (1970) has reviewed various aspects of cytodifferentiation and organogenesis in the absence of cell division.

The differentiation of fibers, concurrently with the conducting elements in the vascular bundles (Esau, 1969; Fahn **et al.**, 1972), raises the questions as to whether there is a common limiting or controlling factor for fibers and vascular elements. Leaves influence differentiation of fibers in **Coleus** (Aloni and Gad, 1982; Sachs, 1972; Aloni, 1976a,b, 1978; Gad and Aloni, 1984). In **Coleus**, role of the leaves in the differentiation of primary phloem fibers can be replaced by combined exogenous application of indole-3 acetic acid and gibberellic acid (Aloni, 1979). Thus, both IAA and GA are limiting and controlling factors in the fiber differentiation (Aloni, 1979). IAA, when administered alone, induces the differentiation of only a few phloem fibers, whereas GA_3 by itself does not exert any effect on fiber differentiation. But when both the hormones are applied together they induce differentiation of a considerable number of fibers (Aloni, 1979). This synergistic effect demonstrates the usefulness of the combined administration of auxin and gibberellin as a perfect substitute, both qualitatively and quantitatively for the aforementioned role of leaves in **Coleus** (Aloni, 1979). However, the type of response is dependent on the IAA and GA concentration used. It is reported that while high levels of IAA stimulate rapid differentiation of fibers with thick secondary walls, high levels of GA_3 result in long fibers with thin secondary walls (Aloni, 1979).

The combined presence of both growth regulators (IAA and GA) is also a requirement for differentiation of secondary

fibers in the xylem of **Populus**. In **Populus** GA_3 in the presence of IAA effected alongation of the xylem fibers (Digby and Wareing, 1966). In **Phascalus** combination of IAA and GA_3 produced differentiation of xylem made up almost entirely of secondary fibers (Hess and Sachs, 1972). However in **Xanthium**, the combination of auxin and gibberellin did not influence the differentiation of secondary xylem fibers (Shininger, 1971). The experiments with IAA and GA_3 suggest that the differentiation of fibers alongwith the conducting elements in the vascular bundles results from their common dependence on the polar movement of auxin (Aloni, 1987). Thus auxin becomes a limiting factor in the differentiation of both xylem and fiber. But the differentiation of the conducting cells and fiber differs in one aspects i.e., while the conducting cells can differentiate only in presence of auxin, the differentiation of fibers requires an additional stimulus gibberellin (Aloni, 1987). Cytokinin also influences fiber differentiation (Maity **et al.**, 1976; Saks, **et al** 1984; Aloni, 1979). But, cytokinin induces fiber differentiation, in the explants, only in the presence of IAA plus GA_3. Cytokinin has been found to be both a limiting and controlling factor in the early stages of fiber differentiation, when many nuclear divisions take place in the young fiber cells (Aloni, 1982). Kinetin has been found to increase the length of secondary xylem fibers in **Adhatoda** (Maity **et al.**, 1976). In tissue culture experiments just as xylem and phloem can be induced to differetiate from parenchyma cells (Aloni, 1980; Wetmore and Rier, 1963), the parenchyma cells can be induced to redifferentiate

into fibers also (Aloni, 1982). The discovery has thus made possible the study of fiber differentiation under controlled conditions using tissue culture techniques (Aloni, 1982).

CHAPTER III

NUTRIENT MEDIUM

Introduction

Cytodifferentiation is a unique morphogenetic phenomena among plant cells. Factors influencing the cytodifferentiation are all fundamental and can be probed into, under controlled conditions, using tissue culture techniques. Basic nutrition including mineral salts, vitamins, hormones, sources of carbon and nitrogen are some of the important ingredients of culture medium for differentiation of tracheids, fibres and sclereids. The nutritional requirements for cytodifferentiation vary with the cells, tissue or organ being cultured as it is dependent on the endogenous nutrient present in the experimental material and chemical ingredients of the nutrient medium (Hildebrandt **et al.**, 1954). A number of nutrient media, differing in their chemical composition, have been developed for general and specific uses. The most common media in vogue for cytodifferentiation studies are Murashige and Skoog (1962), Roberts (Shiraishi, **et al.**, 1982), White (1963), Heller (1953) and B_5 (Gamborg **et al.**, 1968).

Since no information is available on the nutrient requirements for culturing of Assam lemon juice vesicles the present investigation was taken up to find out which one of the aforementioned medium is most suitable for culturing of **C. limon** juice vesicles as also inducing cytodifferentiation of tracheids, fibers and sclereids.

Materials and Methods

Peeled mature fruits of Assam lemon were surface steri-

lized by immersing them in a 10% aqueous solution of sodium hypochlorite for 10 min. Further processing of the surface sterilized fruits was done under aseptic conditions. The fruits were rinsed three times with sterile double glass distilled water to remove excess sodium hypochlorite, and cut open to expose the juice vesicles. After carefully removing individual vesicles, the sac of each vesicle was slit open longitudinally with glass needles. The ruptured vesicles were rinsed briefly in sterile double distilled water, blotted on Whatman No.I filter paper and placed individually in corning culture tubes of 16x75 mm dimensions having 25 ml of the semisolid medium. The media used were MS, B_5, White, Heller and Roberts. The cultured vesicles were incubated in dark in a B.O.D incubator maintained at 25±1°C. Per treatment/20 culture tubes were maintained.

Nutrient media

Chemical compositions and pH of the various nutrient media (MS, B_5, White, Heller and Roberts) used for culturing juice vesicles are given in Table-3.1. Twenty explants were cultured for every medium used. The media were sterilized by autoclaving for 20 minutes at 121°C and 15 lb/in^2 pressure. After having selected the best medium, from amongst the above media, for successful callusing of the juice vesicles and cytodifferentiation, subsequent experiments were conducted to find out the optimal concentration of the nutrient medium for callusing of the cultured juice vesicles and cytodifferentiation of tracheids, fibres and sclereids. The three concentrations (full, half and

Table 3.1: Compositions of some plant tissue culture media (mg/l)

Ingrediants	MS	B_5	White	Heller	Roberts
$(NH_4)_2SO_4$		134			
$(NH_4)NO_3$	1,650				165
$NaNO_3$				600	
KNO_3	1,900	2,500	80		
$Ca(NO_3)_2$			300		
$CaCl_2.2H_2O$	440	150		75	
$MgSO_4.7H_2O$	370	250	720	250	
Na_2SO_4			200		
KH_2PO_4				125	
$NaH_2PO_4.H_2O$		150	16.5		
KCl			65	750	
$FeSO_4.7H_2O$	27.8	27.8			
Na_2EDTA	37.3	37.3			37.3
$FeCl_3.6H_2O$				1.0	
$Fe_2(SO_4)_3$			2.5		
$MnSO_4.4H_2O$	22.3		7	0.01	22.3
$MnSO_4.H_2O$		10			
$ZnSO_4.7H_2O$	8.6	2	3	1	8.6
H_3BO_3	6.2	3	1.5	1	6.2
KI	0.83	0.75	0.75	0.01	
$Na_2MoO_4.2H_2O$	0.25	0.25			0.25
$CuSO_4.5H_2O$	0.025	0.025		0.03	
$CoCl_2.6H_2O$	0.025	0.025			2.5
$NiCl_2.6H_2O$				0.03	
$AlCl_3$				0.03	
Myo-inositol	100	100			100
Nicotinic acid	0.5	1.0	0.5		0.5
Pyridoxine-HCl	0.5	1.0	0.1		
Thiamine HCl	0.1	10.0	0.1	1.0	
Glycine	2.0		3.0		
Ca D-panthothenic acid			1.0		
Sucrose	30,000	20,000	20,000	20,000	20,000
Kinetin	0.04-10	0.1			0.1
2,4-D		0.1-1.0	6.0		
IAA	1.0-30				10
pH	5.7-5.8	5.5	5.5		5.5

Table 3.2: Effect of different media on callusing and cytodifferentiation in C. limon juice vesicle culture.

Treatment	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
MS	4	100	9.8 ±1.16	7.0±2.12	13.8±2.38	24.4±4.44	32.2 ±3.83	44.6±4.15	67.6±6.84	79.6±6.22	116.4±5.81	55.8±6.30	105.0±6.32	182.0±7.38	197.8 ±9.67
B_5	6	100	6.0[b]±0.8	3.4±1.34	13.0±4.06	16.8±3.03	21.6[b]±3.28	12.5±4.27	55.0±4.84	88.6±5.63	123.6[b]±4.77	0	38.6±5.72	86.8±6.41	136.8[b]±5.26
Roberts	6	100	6.6[b]±1.01	3.2±3.16	9.0±3.16	12.4±3.04	26.6±5.22	17.2±4.96	59.4±5.41	78.0±4.30	132.6[bc]±3.36	4.4±2.70	23.8±4.91	30.6±3.64	99.0[bc]±4.06
White	8	10	2.6[bd]±0.8	0	0	0	0	0	36.6±4.77	42.8±3.33	48.8[bd] ±3.19	0	0	0	0
Heller	8	45	3.2[b]±0.74	0	0	0	0	0	21.4±2.70	49.0±5.70	66.2[be] ±2.86	0	0	0	0

Values are means (n=5) ± s.d.

b. Significantly different from MS medium.

c. Significantly different from B_5 medium.

d. Significantly different from Roberts medium.

e. Significantly different from White medium.

Significance tested by student's 't' test (p=0.05).

quarter) of the best medium (MS in this case) were tested for optimal results.

Data recording

The cultures were examined regularly for visible signs of callus initiation. At the conclusion of the experiments (30 days) juice vesicles with associated callus were fixed in F.A.A. for 24 hrs. and subsequently cleared in NaOH (4% w/v) at 55°C for approximately 12 hrs. The cleared vesicles and associated calli were first stained with an aqueous solution of safranin 'O' (0.03% w/v) for 30 min at 55°C and subsequently differentially destained giving several changes of HCl (1 N) over a 2 hr. period. The stained experimental material was stored in glycerine. Later these were dissected and examined microscopically for the presence of tracheid, fibre and sclereid which were scored using a Leitz ortholux 2 microscope equipped with a projection attachment.

Results

Initiation of callus occurred earliest in the juice vesicles cultured on MS medium (Table-3.2). The juice vesicles grown on B_5 and Roberts media produced calli next (Table-3.2). Juice vesicles cultured on White and Heller media developed calli last, i.e. 8 days after culturing (Table-3.2). Whereas all the juice vesicles (100%) cultured on MS, B_5 and Roberts media produced calli, only 45% juice vesicles grown on Heller medium developed calli. However, only 10% of the juice vesicles

Plate 3.1

(a) Effect of different nutrient media on callusing in **C. limon** juice vesicle cultures:

A-MS; B-B_5; C-Heller; D-White and E-Roberts.

(b) Effect of different strengths of MS medium on callusing in **C. limon** juice vesicles cultures:

A-Full strength; B-half and C-Quarter strength.

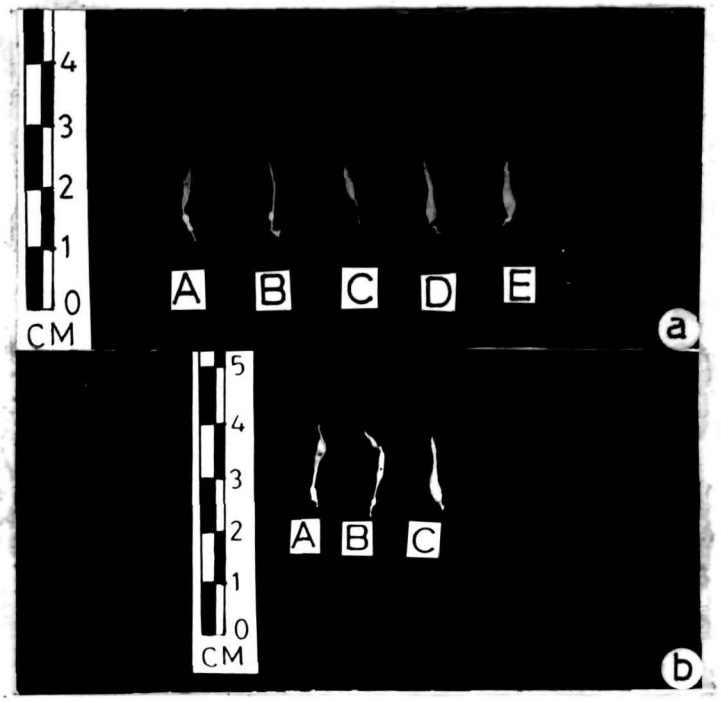
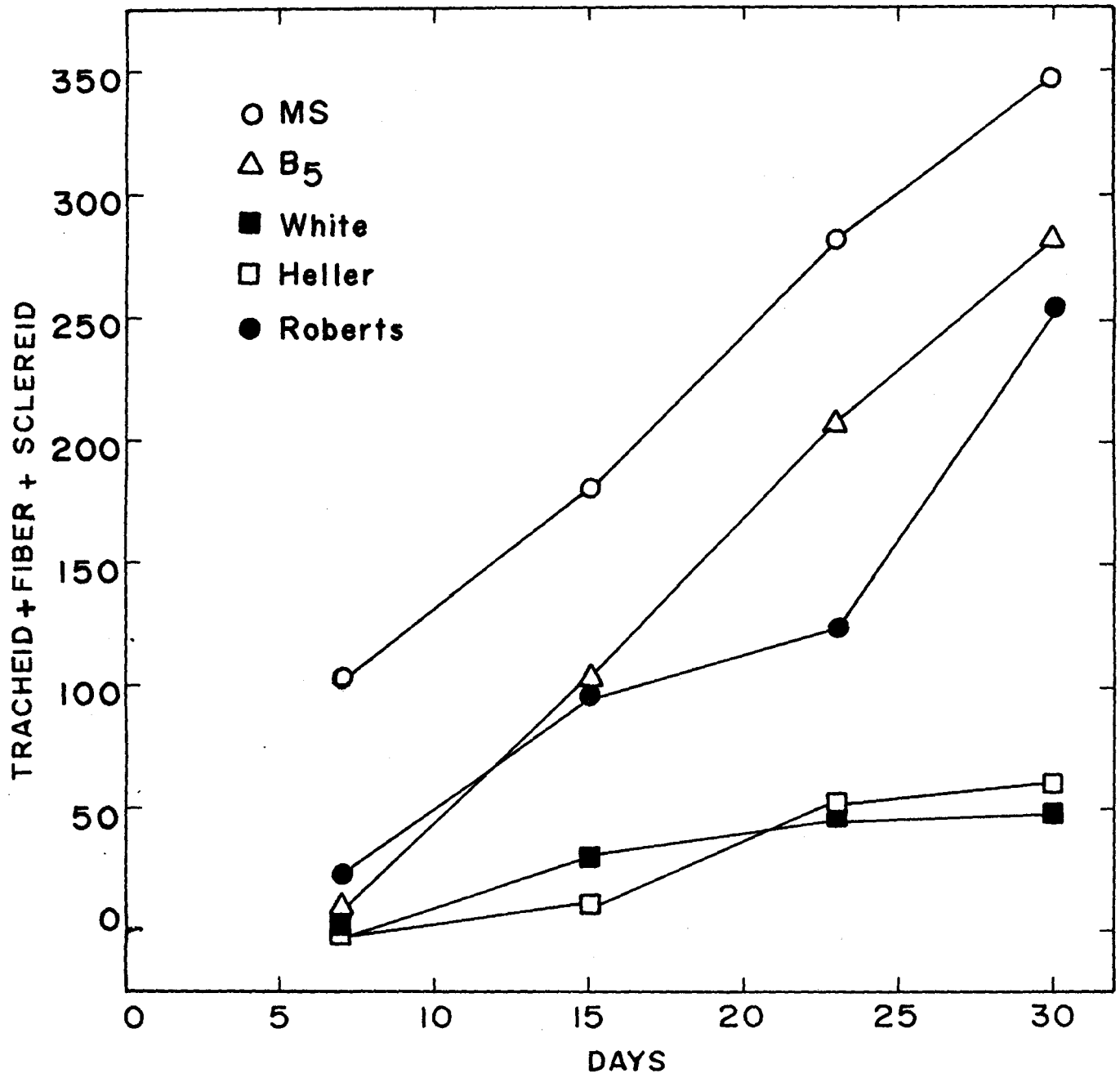


PLATE 3.1

Fig.3.1 Effect of different media on cytodifferentiation in **C. limon** juice vesicle culture

Fig.3·1


MS
B5
White
Heller
Roberts
TRACHEID + FIBER + SCLEREID
350
300
250
200
150
100
50
0
0
5
10
15
20
25
30
DAYS

cultured on the White medium produced calli (Table-3.2). Callus growth, as evident by the fresh weight of calli, also revealed a similar pattern. Thus, the suitability of various media for callus initiation and callus growth decreased in the following order: MS > Roberts > B_5>Heller > White (Plate 3.1a). Cytodifferentiation of tracheids, fibres and sclereids (Plate 3.2a-c) occurred first (5th day) in the explants cultured on MS medium, while it was evident only on the 7th day in vesicles cultured on Roberts and B_5 media. In B_5 medium only trachieds and fibres differentiated on the 7th day while sclereids were observed on the 15th day (Table-3.2). However, in explants cultured on Roberts medium differentiation of tracheids, fibres and sclereids occurred simultaneously on the 7th day (Table-3.2). In explants cultured on White and Heller's media only fibres differentiated (Table-3.2). Tracheids which differentiated in vesicles cultured on MS, B_5 and Roberts media exhibited scalariform type of secondary wall-thickenings (Plate 3.2a). But tracheids with both scalariform thickenings and reticulate secondary wall pittings differentiated in explants cultured on B_5 medium (Plate 3.2d). Cytodifferentiation of fibres (Plate 3.2b) in juice vesicles cultured on White and Heller media became evident on the 15th day of inoculation (Table-3.2). The medium did not induce differentiation of tracheids and sclereids (Table-3.2). The degree of cytodifferentiation was highest in the explants cultured on MS medium (Fig. 3.1). B_5 and Roberts media were the 2nd and 3rd best medium to support cytodifferentiation (Fig. 3.1). White and Heller's media were least effective for inducing cytodifferentiation in cultured

Plate 3.2

a. Tracheid with scalariform thickenings. X 470

b. Fibers. X 190

c. Sclereids. X 120

d. Reticulate pitted tracheids. X 300

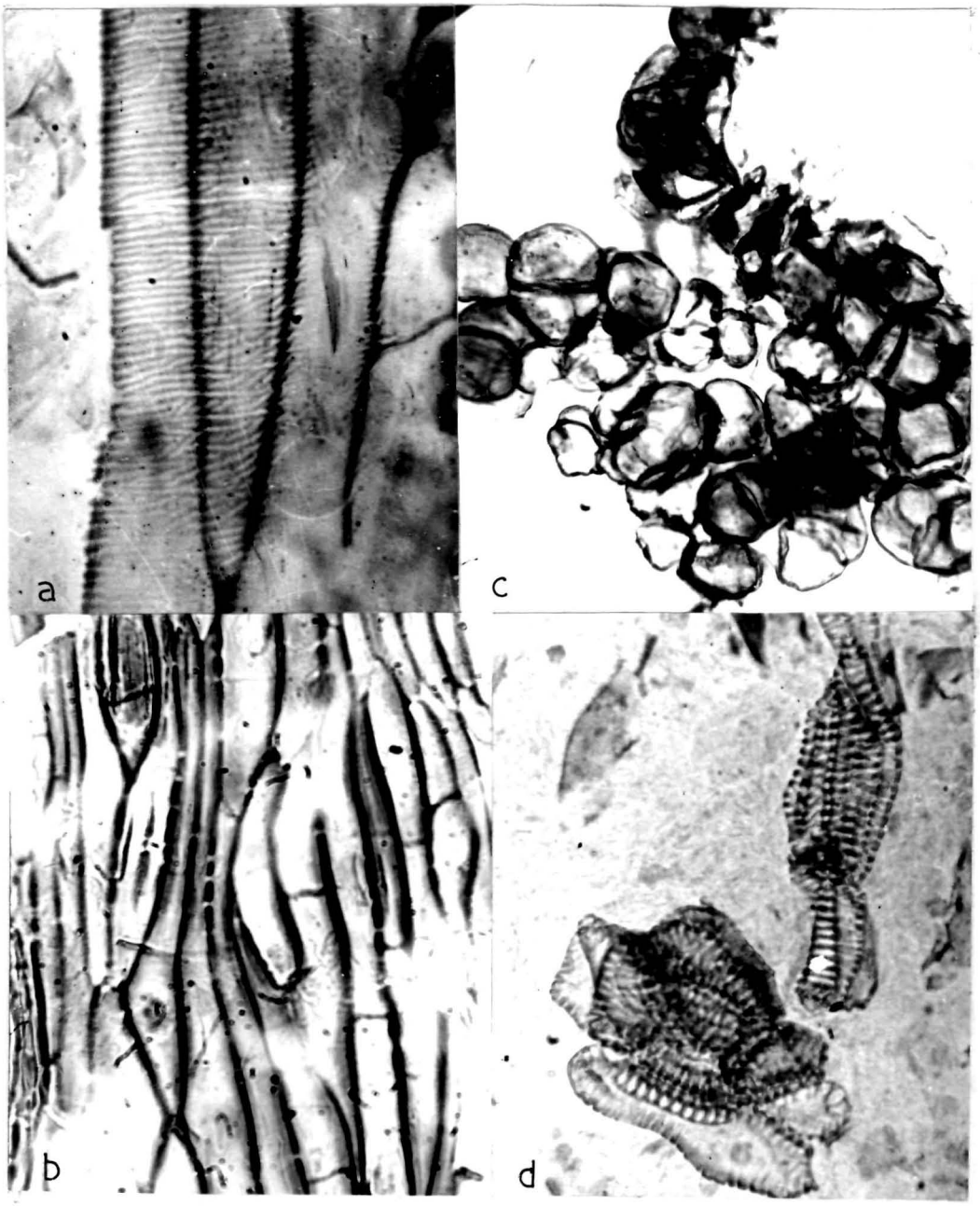


PLATE 3.2

Table 3.3: Effect of full, half and quarter strength MS medium on cytodifferentiation in cultured C. limon juice vesicles.

Treatment	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
MS (Full strength)	4	100	6.6 ±0.80	7.8±2.28	13.0±3.16	20.6±2.88	31.2 ±3.03	30.0±2.54	53.0±4.30	95.4±4.61	101.4 ±6.10	43.8±5.40	91.0±6.89	104.0±6.5	148.6 ±4.72
MS (half strength)	4	75	4.4^{b}±1.01	4.4±2.07	6.8±1.92	10.0±2.23	23.0^{b}±4.12	17.0±1.58	38.6±5.72	50.8±2.28	78.0^{b}±4.30	23.6±2.30	38.8±4.60	59.4±2.70	70.6^{b}±5.59
MS(quarter strength)	9	40	3.6^{b}±1.01	0	0	3.1±2.55	9.0^{bc}±3.16	0	20.2±3.49	39.0±3.16	58.4^{bc}±2.70	0	0	0	0

Values are means (n=5) ± s.d.

b. Significantly different from full strength medium.

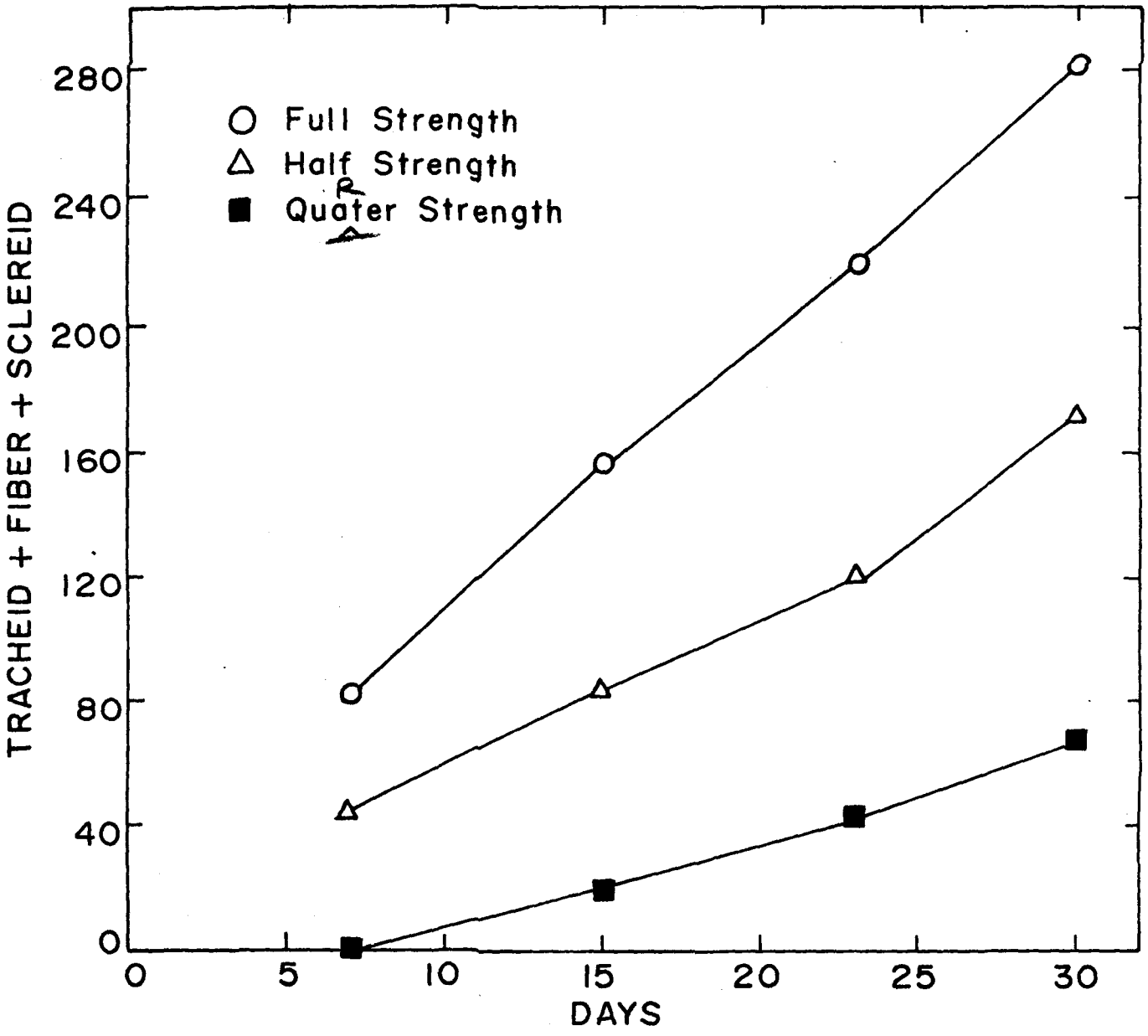
c. Significantly different from half strength medium.

Significance tested by student's 't' test (p=0.05).

Fig.3.2 Effect of full, half and quarter strength of MS medium on cytodifferentiation in **C. limon** juice vesicle culture.

How this fig. is different from Table 3.3?

Fig.3·2



Assam lemon juice vesicles (Table-3.2, Fig. 3.1).

Effects of full, half and quarter strength of MS medium on the growth of callus and differentiation of tracheids, fibres and sclereids was also studied. Vesicles cultured on full and half strength of the MS medium callused in 4 days while vesicles cultured on quarter strength of MS medium developed callus only 9 days after inoculation (Table-3.3).

Full strength MS medium induced callusing in all the juice vesicles cultured (100%). But only 75% and 40% juice vesicle cultured callused on half and quarter strength MS medium, respectively (Table-3.3). Likewise, the callus weight was maximum in vesicles cultured on full strength MS medium which decreased with the decreasing strength of the MS medium (Plate 3.1b, Table-3.3). Cytodifferentiation was delayed in vesicles cultured on the quarter strength MS medium since differentiation of fibers and tracheids occurred only on the 15th and 23rd day respectively (Table-3.3). This medium, however, did not support differentiation of sclereids (Table-3.3). Maximum differentiation occurred in vesicles cultured on full strength MS medium but the cytodifferentiation decreased with the decreasing concentration of the MS medium (Table-3.3, Fig. 3.2). The tracheids, fibers and sclereids which differentiated at different concentrations of MS medium were, however, alike (Plate 3.2a-c).

Discussion

In the present study MS medium induced best cytodifferen-

tiation in comparison to all other media used. Roberts and B_5 media proved next best. However, the effectiveness of the three media in inducing cytodifferentiation was more or less same which may be due to qualitative and quantitative similarity in their chemical composition. The White and Heller media had inhibitory effect on cytodifferentiation in Assam lemon juice vesicle cultures in comparison to MS medium. Since both the media differed considerably from MS medium in their chemical composition, the negative effect of these media could be attributed to these differences. The White medium has following chemicals which are absent in MS medium: Ca $(NO_3)_4$, Na_2SO_4, $Na_2H_2PO_4.H_2O$, KCl, $Fe_2(SO_4)_3$, Ca D-Panto-thenic acid, 2,4-D. Similarly, $NaNO_3$, KH_2PO_4, KCl, $FeCl_3.6H_2O$, $NiCl_2.6H_2O.AlCl_3$, which are present in Heller's medium, are also absent in MS medium. Additionally some other components NH_4NO_3, $FeSO_4.7H_2O$, Na_2EDTA, $MnSO_4.H_2O$. $Na_2MoO_4.2H_2O$, $CuSO_4.5H_2O$, $CoCl_2.6H_2O$, myoinositol, kinetin and IAA are present in the MS medium although absent in White medium. Further, KNO_3, $MnSO_4.H_2O$, $CoCl_2$, myoinositol, nicotinic acid, pyridoxine-HCl, glycine, kinetin and IAA are absent in Heller medium, but present in MS medium. Complete absence of any growth hormone in Heller medium and presence of high concentration of auxin (2,4-D) in White medium could also be the reason for detrimental effect of these media on cytodifferentiation in cultured juice vesicles. Roberts (1971) tested the effect of different concentration of 2,4-D on xylogenesis in lettuce pith culture and found that low concentration of 2,4-D (0.7 mg/l) was best for xylogenesis in the presence of 0.1 mg/l kintin while

higher concentration of 2,4-D (5.0 mg/l) was inhibitory for xylogenesis in his experiments. Similar were the observations of Murashige & Tucker(1969) who also found that low concentration of 2,4-D ($3x10^{-6}M$) was good for growth of C.limon culture while 2,4-D concentrations higher than this concentration were inhibitory. In the present investigation, Heller's medium, which lacks growth hormones, could not well support cytodifferentiation. Thus, the present study provides further support to the finding that growth hormones are essential for inducing cytodifferentiation (Jacobs, 1954; Torrey, 1966; Roberts, 1969; Gautheret, 1966). The lesser effectiveness of Roberts and B_5 media, in comparison to the MS medium, for induction of cytodifferentiation may be attributed to their lower sucrose levels. The Roberts and B_5 media have 2% sucrose which is lower than the sucrose concentration (3%) in the MS medium. Murashige &Tucker(1969) also found that higher concentrations of sucrose (4-6%) are required for good growth of Citrus culture. When MS concentration was varied (full, half and quarter) cytodifferentiation was also affected. Half and quarter strength of the MS medium adversely affected the capacity of the medium to support callus growth and induce cytodifferentiation in C. limon juice vesicle cultures suggesting that an optimal osmotic concentration is 'essential for optimal cytodifferentiation'.

Since amongst the different media used in the present study MS medium induced best cytodifferentiation in the cultured juice vesicles the same will be used for further studies.

CHAPTER IV

EFFECT OF FRUIT DEVELOPMENT, JUICE VESICLE POSITION INSIDE FRUIT AND DIFFERENT REGIONS OF THE JUICE VESICLE ON CYTODIFFERENTIATION

Introduction

The controversy whether an immediately preceding mitosis is a necessary prerequisite for induction of cytodifferentiation still remains unresolved. It has been suggested that changes in the pattern of gene expression that lead to cytodifferentiation might only occur at some specific stage of the cell cycle, giving a plausible, if largely intested, rationale for the link between cell division and differentiation (Shininger, 1975; Stockdale and Topper, 1966). Recently, Phillips (1981) demonstrated that parenchyma cells of immature tuber of **Helianthus** differentiate directly into TE without undergoing cell division. With tuber maturity the capacity for direct differentiation also declined in irradiated tissues since TE differentiation occurred only in non-irradiated control following a period of cell division. In **Citrus** fruits cell division in juice vesicles ceases very early and subsequent increase in fruit size is due to the enlargement of cells (Ford, 1942). Thus fruits attaining the maturity are no longer mitotically active (Bartholomew and Sinclair, 1951). The author is not aware of any investigation where the relationship between fruit maturity and differentiation of tracheids, fibers and sclereids in **Citrus** juice vesicles has been investigated using tissue culture techniques.

The present investigation, on cytodifferentiation of trachied, fiber and sclereid in **Citrus** juice vesicle **in vitro**, attempts to determine the following:

1) Response of juice vesicles from Assam lemon fruit of different developmental stages.

2) **Response of juice vesicles from different locations inside the Assam lemon fruit.**

3) Variation, if any, in the responses of different regions (Neck/Sac region) of the juice vesicles.

Materials and Methods

Assam lemon fruits of four developmental stages (1) Young fruit (green), (2) Developed fruit (green-yellow), (3) Ripe fruit (pale-yellow) and (4) Senescent fruit (lemon-yellow), were used.

The fruits of the above developmental stages were collected and data on fruit diameter, size of the juice vesicles and pH of the juice were recorded. For culturing, juice vesicles were prepared, as described in the Chapter III. The juice vesicles were then grown **in vitro** in culture tubes having 25 ml of MS basal medium (1962). The basal medium was prepared as in Chapter III and the pH of the medium was adjusted to 5.7, prior to autoclaving. Three sets of experiments were performed as detailed below:

1) Juice Vesicles from fruits of different developmental stages mentioned above were cultured to find out if cytodifferentiation is influenced by the developmental stage of fruits.

2) Explants from different locations inside the fruit were cultured individually to find out if juice vesicle position inside fruit has any bearing on cytodifferentiation.

3) To investigate the differences in the cytodifferentiation potential of different regions of the juice vesicles; whole vesicles, neck of the vesicle and sac of the juice vesicles were cultured separately.

In all experiments explants were cultured in dark in a

Table 4.1: Morphological features of the citrus fruits and juice vesicles and pH of the juice at different developmental stages of C. limon fruit.

Developmental stage of fruit	Fruit diameter (cm)	Size of the explants(mm)	pH
Young	4.8	6.0	2.5
Developed	6.0	14.0	2.7
Ripe	6.1	14.0	2.75
Senescent	6.0	13.8	2.75

Plate 4.1

Effect of fruit development on callusing in **C. limon** juice vesicle cultures:

A - Young fruit; B - Developed fruit; C - Ripe fruit and D - Senescent fruit.

whether D increased in length as Tukey data does not match with prints.

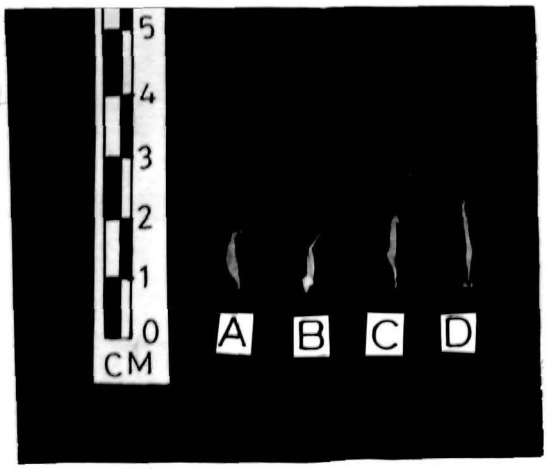


PLATE 4.1

B.O.D. incubator maintained at 25±1°C. Twenty culture tubes were maintained for each treatment. At the expiry of the experiments (30 days) explants were fixed in F.A.A. The fixed juice vesicles were cleared, stained and stored for microscopic observation as in Chapter III.

Results

The juice vesicles of young fruits are filled with juice of low pH (pH 2.5) compared to the vesicles in developed (pH 2.7) and ripe fruits (pH 2.75). Fruit diameter and juice vesicle size are given in Table-4.1.

Juice vesicles isolated from young fruits and senescent fruits did not callus (Plate 4.1). On the otherhand, all the juice vesicle explants obtained from developed fruits callused on the 4th day of inoculation while 73 per cent explants obtained from the ripe fruits, produced callus on the 6th day of inoculation.

Like callusing, differentiation of tracheid and sclereid also started earlier in the developed fruits compared to ripe fruits (Table-4.2). The frequency of fibers, tracheids and sclereids differentiated was higher in explants obtained from developed fruits than ripe fruits (Table-4.2, Fig. 4.1). But the type of secondary wall thickenings of tracheids were common (scalariform) at both the fruit stages (Plate 4.2a). The only difference between the tracheids differentiated in the explants from developed and ripe fruits was that while tracheids differen-

Table 4.2: Effect of developmental stage of citrus fruit on callusing and cytodifferentiation in juice vesicles.

Developmental stage of fruit	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Young	0	0	4.4 ±1.01	0	0	0	0	0	0	0	0	0	0	0	0
Developed	4	100	12.4[b] ±1.85	4.2±1.48	10.4±2.97	14.4±2.40	17.6±5.72	10.4±2.6	23.0±3.08	50.2±2.38	72.8 ±4.32	4.6±1.67	9.4±2.30	20.8±2.8	30.6 ±3.28
Ripe	6	73	9.8[b][c] ±1.48	0	5.2±3.03	8.2±2.38	11.6±2.40	4.0±2.0	14.8±3.49	32.2±5.26	51.0[c] ±7.0	0	0	11.4±2.28	21.0[c] ±3.87
Senescent	0	0	6.0[b][c] ±0.0	0	0	0	0	0	0	0	0	0	0	0	0

Values are means (n=5) ± s.d.

b. Significantly higher than young fruit.

c. Significantly lower than developed fruit.

Significance tested by student's 't' test (p=0.05).

Fig.4.1 Effect of fruit development on cytodifferentiation in **c. limon** juice vesicle culture.

Fig. 4·1

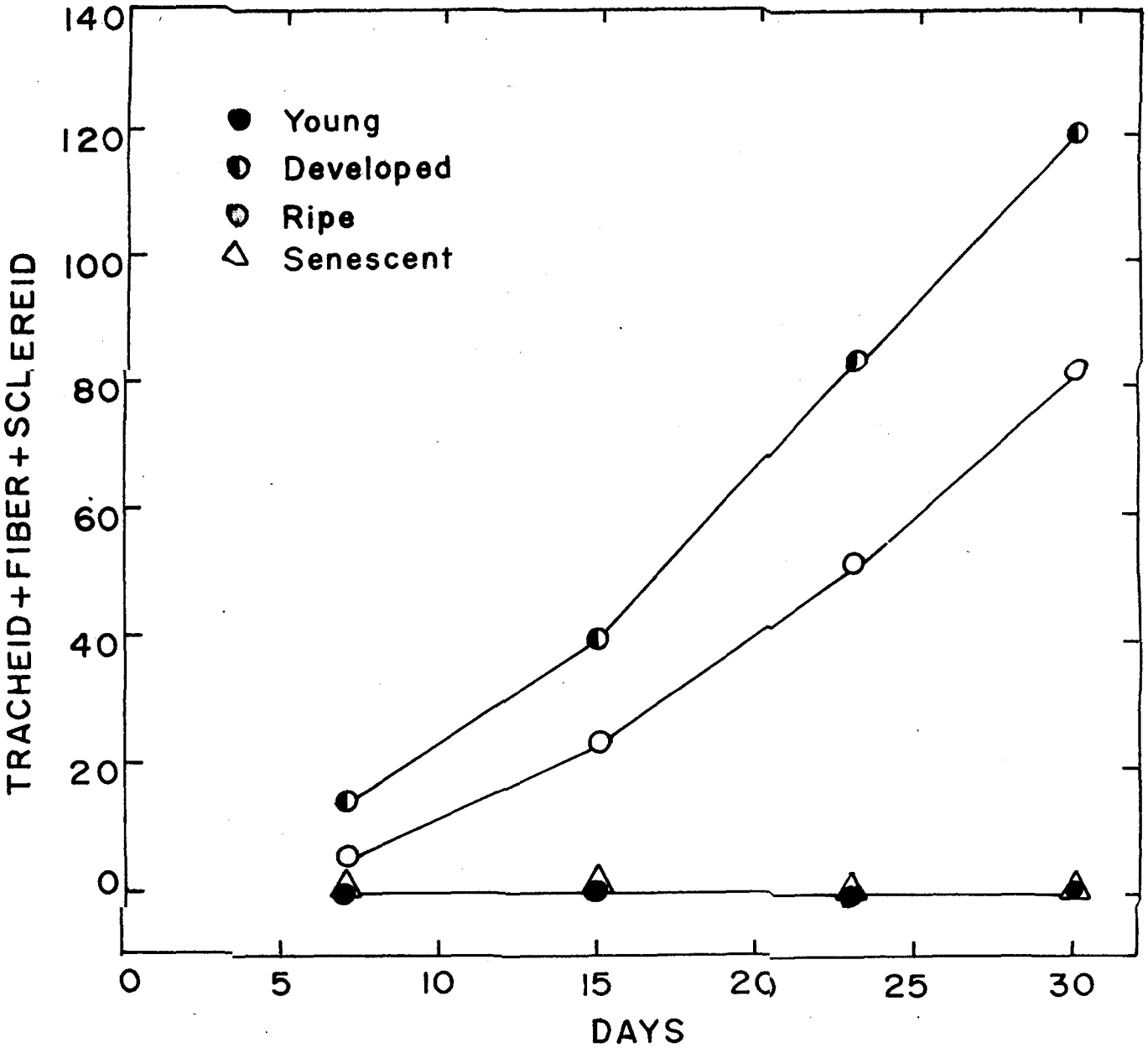

140
120
100
80
60
40
20
0
TRACHEID+FIBER+SCLEREID
Young
Developed
Ripe
Senescent
0
5
10
15
20
25
30
DAYS

Plate 4.2

(a) Tracheid with scalariform secondary wall thickenings. X 100

(b) Tracheid exhibiting wavy cell wall. X 310

(c) Fibers. X 190

(d) Macro sclereids. X 470

(e) Micro sclereids. X 120

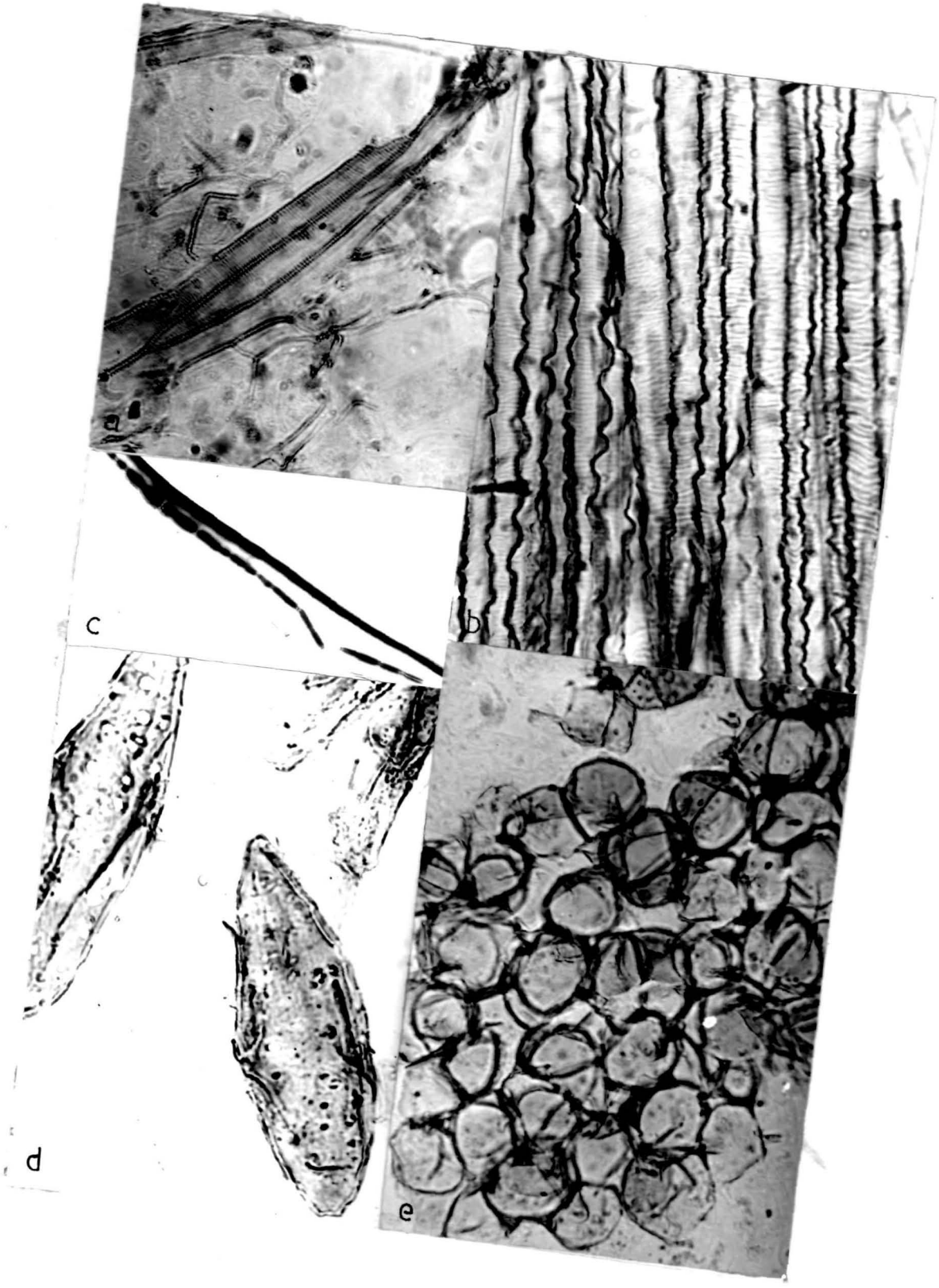


PLATE 4.2

tiated in the juice vesicles explants of developed fruit had smooth and straight wall, tracheids in the explants from ripe fruit revealed wavy cell wall (Plate 4.2a,b). No difference was evident in the fibres which differentiated in the explants from either developed or ripe fruits (Plate 4.2c). In juice vesicle explants obtained from ripe fruit the frequency of macro sclereids (Plate 4.2d) was more than micro-sclereids (Plate 4.2e). However, reverse was true in the case of explants obtained from developed fruit. For further studies juice vesicle explants were obtained only from developed Assam lemon fruits.

Juice vesicles present in the middle part of the Assam lemon fruits are bigger compared to the vesicles present at the proximal and distal ends of the fruit (Plate 4.3). The stalk of the juice vesicles in the middle part of the fruit is bigger than the stalk of the juice vesicles present towards the two ends of the fruit (Plate 4.3). But size of the sac does not vary much with the location of the juice vesicles in fruit. The juice vesicles from middle part of the fruit callused earlier (on 4th day) than juice vesicles present at the two ends of the fruit (Table-4.3). Weight of the calli and the frequency of its formation were higher in explants procured from the middle region of the fruit than those located at both the ends of the fruit.

Although cytodifferentiation was first evident in explants from middle part of the fruit, it was not unusually delayed in the explants obtained from proximal and distal ends of the

Plate 4.3

Effect of position of juice vesicle inside **C. limon** fruits on callusing in juice vesicle cultures:

A - Middle; B - Anterior; C - Posterior part of the fruit.

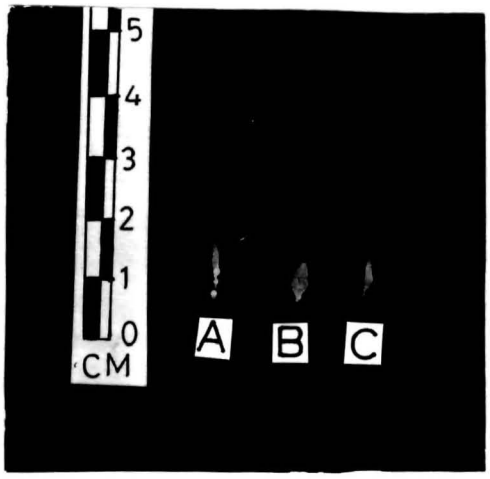


PLATE 4.3

Table 4.3: Effect of position of juice vesicles in C. limon fruit on callusing and cytodifferentiation.

Position of the juice vesicle inside fruit	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Anterior part	5	83	8.4 ±1.35	3.2±1.78	5.4±1.51	10.4±2.96	11.0 ±2.12	12.0±4.0	27.4±4.62	54.0±3.80	89.2 ±3.34	0	6.2±2.48	17.8±2.86	23.6 ±3.01
Middle part	4	100	14.6^{b}±1.49	9.4±2.30	12.5±2.08	18.2±2.38	22.4^{b}±1.94	23.0±3.08	31.4±2.70	73.0±1.58	95.4^{b}±4.61	7.4±2.40	21.0±2.54	28.4±3.97	54.3^{b}±3.16
Posterior part	4	100	12.2^{c}±1.16	4.6±1.67	8.6±3.84	10.0±2.23	13.2^{c}±6.18	7.3±3.16	68.0±2.40	80.0±3.16	91.2^{c}±2.86	0	4.4±2.70	21.0±3.87	30.8^{b,c}±5.49

Values are means (n=5) ± s.d.

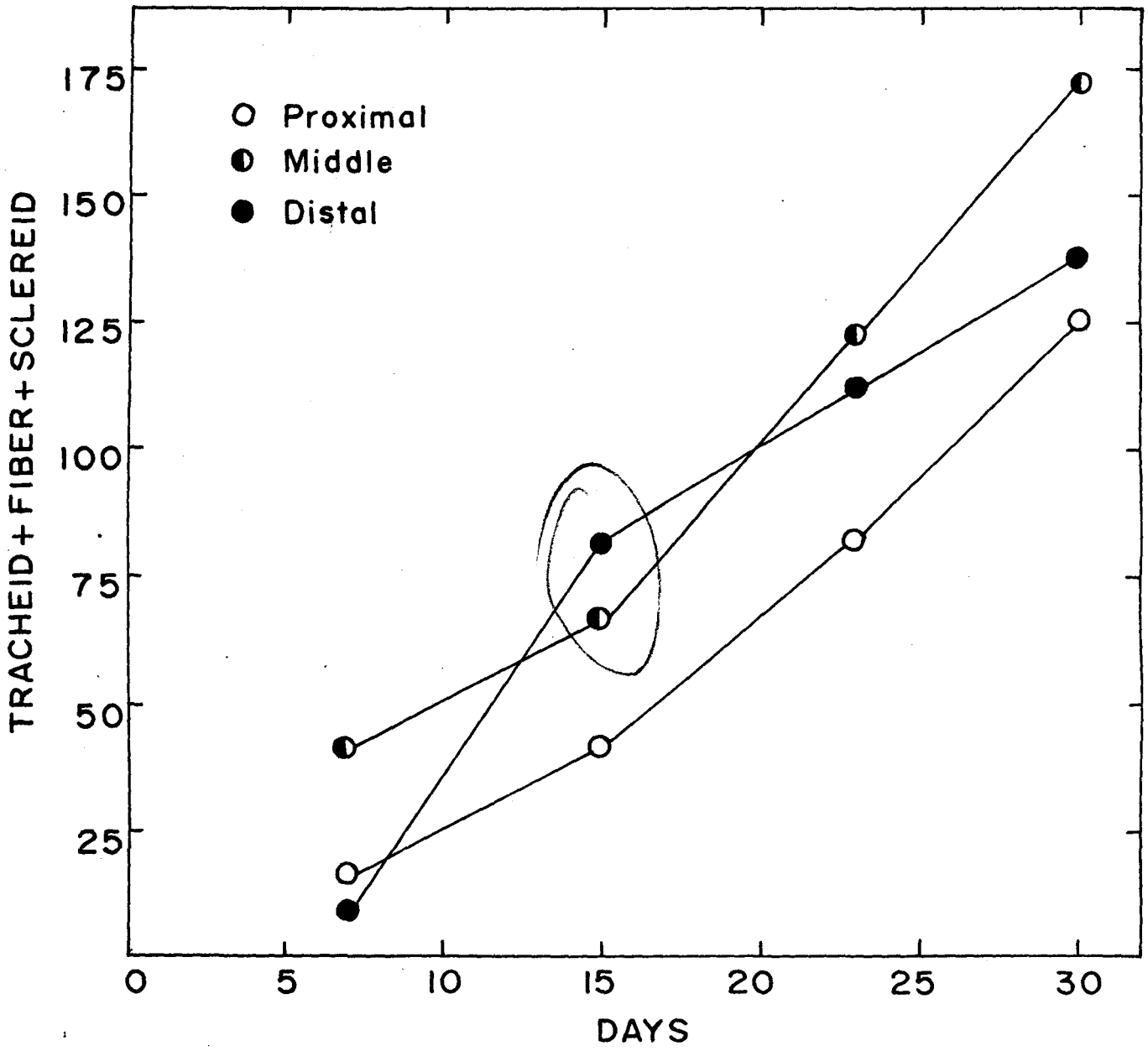
b. Significantly higher than anterior part.

c. Significantly lower than middle part.

Significance tested by student's 't' test (p=0.05).

Fig.4.2 Effect of position of juice vesicles inside fruit on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 4·2


175
150
125
100
75
50
25
TRACHEID + FIBER + SCLEREID
Proximal
Middle
Distal
0
5
10
15
20
25
30
DAYS

fruit (Table 4.3). However, the number of differentiating tracheid, fiber and sclereids were much different (Table 4.3, Fig. 4.2). Although large number of micro- sclereids (Plate 4.2e) were observed in explants obtained from middle parts of the fruit, the number of fibers (Plate 4.2c) and tracheids (Plate 4.2a) formed did not much depend on the position of the vesicle inside fruit (Table-4.3). The differentiating elements: fibers, tracheids with scalariform-reticulate secondary wall thickening and sclereids were of the same type in explants from all parts of the fruits.

Both complete juice vesicles and neck region of the vesicle when cultured callused on the 4th day of inoculation but no callusing occurred if only vesicle region was cultured (Table-4.4, Plate 4.4a). Callusing in intact juice vesicle explants was higher (100%) than in explants of neck region alone (54%). The weight of the callus formed by the neck region explants was less compared to the callus developed by the complete juice vesicles (Table-4.4). Further, when complete juice vesicles were cultured the first sign of callusing appeared in the form of nodules in the neck region which in the latter phase of the experiments extended towards the sac region in some juice vesicles (Plate 4.4b).

The differentiation of tracheids and fibers occurred simultaneously on the 5th day of culturing in the entire juice vesicle and neck region explants (Table-4.4). The diversity of differentiation, however, increased on the 7th day when tracheids

Table 4.4: Response of different regions of the citrus juice vesicles on callusing and cytodifferentiation.

Treatment	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Intact juice vesicle	4	100	12.2 ±1.24	5.4±2.60	10.0±2.23	16.4±2.70	19.2±2.86	18.6±3.84	32.4±2.30	59.4±2.70	75.4 ±3.20	4.6±1.67	10.8±3.19	26.6±2.07	33.0 ±2.91
Neck region	4	54	10.2[b] ±1.16	3.8±2.58	8.6±2.30	14.0±2.23	17.0±1.58	7.8±3.71	21.6±2.40	41.4±2.70	65.6[b] ±4.61	0	6.0±2.23	10.4±4.78	25.4[b] ±6.69
Vesicle region	0	0	4.0[b,c] ±0.63	0	0	0	0	0	0	0	0	0	0	0	0

Values are means (n=5) ± s.d.

b. Significantly different from intact juice vesicles.

c. Significantly different from neck region.

Significance tested by student's 't' test (p=0.05).

Plate 4.4

(a) Effect of different regions of **C. limon** juice vesicles on callusing in juice vesicle cultures:

A - Intact juice vesicles; B - Neck and C - Vesicle region of the juice vesicle.

(b) Progression of callusing in **C. limon** juice vesicle culture with aging:

A - Callus development during early phase; B - Callus development in the later phase.

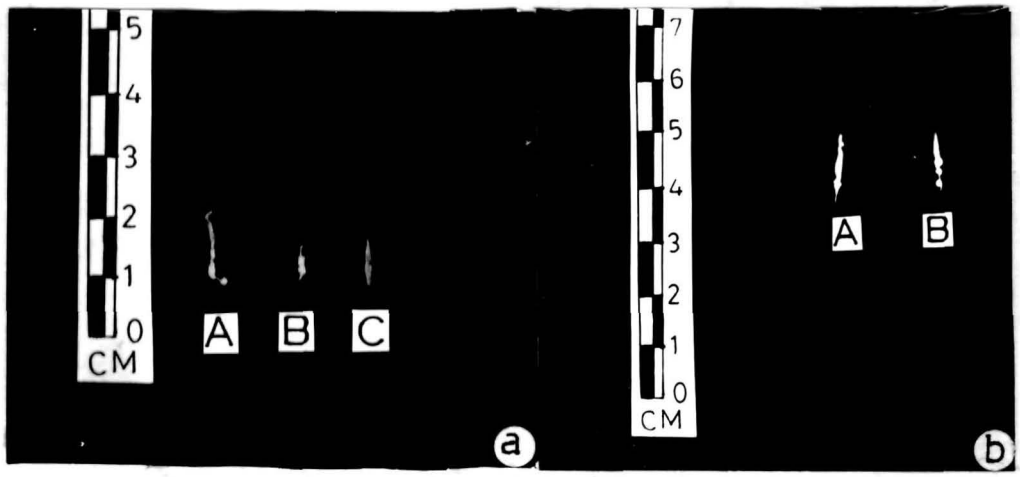
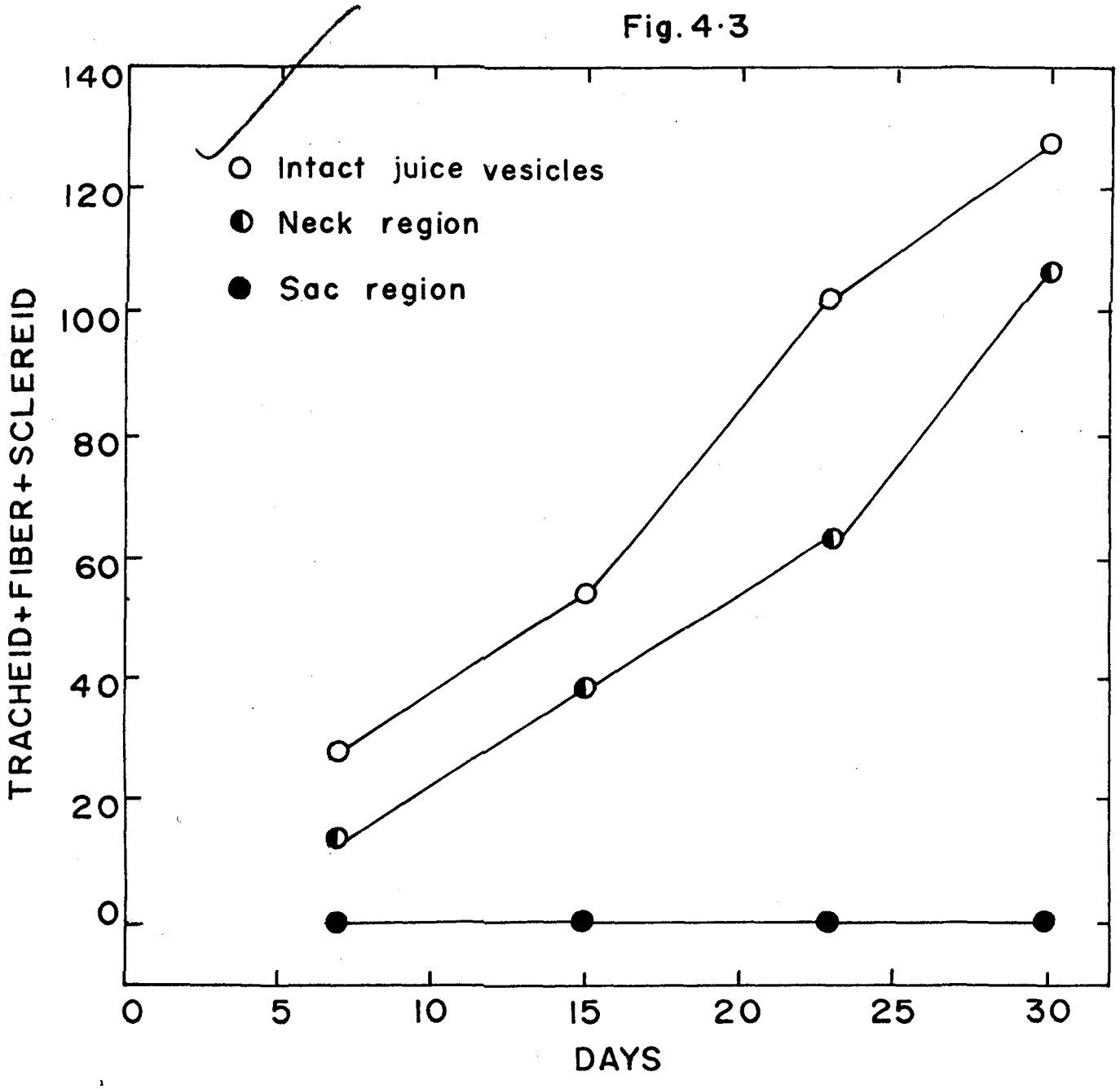


PLATE 4.4

Fig.4.3 Response of different regions of the juice vesicles on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 4·3


Intact juice vesicles
Neck region
Sac region
TRACHEID+FIBER+SCLEREID
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
DAYS

with scalariform reticulate thickening and sclereids also differentiated. The number of tracheids, fibers and sclereids was more in the callus formed by entire juice vesicle explants than explants of neck region alone (Table-4.4, Fig.4.3). The intensity of cytodifferentiation increased with the aging of the callus.

Discussion

The present investigations reveal that juice vesicles from immature and senescent fruit are incapable of forming callus while those from developed (mature) and partially ripe fruits develop callus. Opinions differ about the developmental stage of **Citrus** to be used for juice vesicle culture since, **Citrus** fruits remain active mitotically for a brief period only (Ford, 1942). Kordan (1984) suggested the use of mature fruits because the lemon fruits have short life history **in vivo** and only mature fruits, which have high juice contents, are easily available in the market. The data collected in the present investigation supports this contention since cell division was faster and cytodifferentiation was better in explants from mature fruit than explant from partially ripe fruit.

In the present investigation, the explants which were obtained from the middle part of the fruit had bigger neck and exhibited early callusing and best growth of the callus suggesting that the cells of the neck region can very easily become active mitotically. Kordan (1965) also stated that in culture experiments, vesicle region usually degenerates and collapses while growth occurs in the neck region. Better growth in the

neck region may be attributed to the differences in the acidity of the sap present in neck and sac regions (Bartholomew and Sinclair, 1951). Compared to sap present in vesicle, the sap found in neck region is less acidic, which may be due to differences in the concentration of the citric acid in saps of two regions (Kordan, 1965).

In the present investigation in spite of the consistency and precautions in techniques, while some neck regions from a given fruit failed to grow, others, also obtained from the same individual fruit, yielded positive results. These results are in full agreement with those of Kordan (1962) who ascribed these obserevations to: (i) a possible difference in the extent of damage to different neck regions; extent of damage being greater for stalks which failed to form calli, and (ii) possible subtle physiological and/or structural variations among various stalks within a single fruit.

CHAPTER V

(a) CARBON SOURCE

Introduction

Cytodifferentiation of vascular tissue using tissue culture techniques has been studied by several workers (Bergmann, 1964; Clutter, 1960; Haccius and Lakshmann, 1965; Skoog and Miller, 1957; Wetmore and Sorokin, 1955; Rier, 1970). Using gradient techniques it has been shown that in **Parthenocissus tricuspidate** var. Veitchi differentiation of vascular tissue is induced by combinations of IAA and sucrose (Wetmore and Rier, 1963). However, the differentiation of xylem/phloem in these experiments is regulated by sucrose concentrations. While relatively low sucrose concentrations (1-2%) favour differentiation of xylem, higher sucrose concentrations (3-4%) favoured phloem differentiation (Wetmore and Rier, 1963; Jeff's and Northcote, 1966). Lamotte and Jacobs (1963) reported that rather than sucrose, IAA limits phloem regeneration in **Coleus** internodes. Thompson and Jacobs (1966) found that IAA is the common controlling factor for both phloem and xylem regeneration. On the other hand, in **Parthenocissus tricuspidate** var. Veitchi, **Coleus** stem and **Helianthus** tuber cultures the number of xylem elements differentiated was directly proportional to the sucrose concentration (Rier and Beslow, 1967; Beslow and Rier, 1969; Minocha and Halperin, 1974). In **Phaseolus** tissue cultures both xylem and phloem differentiated when 2% sucrose was incorporated in the medium (Jeff's and Northcote, 1966). Discussing the role of IAA and sucrose in vascular tissue differentiation Aloni (1980) stated that auxin concentration is critical for the differentiation of vascular elements (xylem/phloem) while sucrose concentration determines

only callose deposition. But according to Roberts (1976), auxin, cytokinin and sucrose are essential metabolites for vascular tissue differentiation.

All the sugars are not equally effective in inducing cytodifferentiation of vascular tissues. For example, while maltose, trehalose and sucrose induce xylem and phloem differentiation, other sugars are not able to induce differentiation of vascular tissues (Jeff's and Northcote, 1967). Using monosaccharides, Minocha and Halperin (1974) obtained similar result, but monosaccharides could induce differentiation of xylem only. They could establish a clear correlation between cell number and the proportion of TE formed in response to a variety of sugars. These findings were further confirmed by Dodds (1978). Recently it has been shown that glycerol and myoinositol can also serve as carbon source for the induction of xylogenesis in **Lactuca** (Roberts and Baba, 1982). Murashige & Tucker (1969) demonstrated that higher concentrations of sucrose (4-6%) are favourable for good growth of **Citrus** tissue culture. In all likelihood, any carbohydrate that permits rapid cell proliferation in a particular tissue will effectively support xylogenesis (Phillips, 1980).

The present study attempts to find out the most effective carbon source and its optimum concentration for differentiation of tracheid, fiber and sclereid in cultured Assam lemon juice vesicles.

Table 5a.1: Effect of different Carbon sources on cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sucrose	4	100	21.4 ±2.24	4.0±2.00	10.0±2.23	18.2±4.81	29.8 ±4.14	13.0±3.16	29.4±3.84	41.0±5.09	53.6 ±3.50	15.2±4.14	29.2±6.49	43.8±4.65	45.8 ±4.76
Glucose	4	100	26.0^{b}±1.41	2.0±1.41	5.4±2.60	11.6±2.40	16.4^{b}±2.70	11.2±3.89	23.0±4.30	30.4±4.77	46.0^{b}±4.74	5.0±3.80	17.2±4.14	23.2±6.57	28.6^{b}±5.12
Glycerol	12	75	11.0^{b}±1.09	0	3.8±2.58	10.0±2.23	14.0^{b}±2.23	0	11.8±1.92	13.2±2.86	21.4^{bc}±3.13	0	0	20.6±6.50	24.8^{b} ±5.44
Myoinositol	9	55	7.2^{bd}±0.74	0	0	0	4.0^{bd}±1.58	0	12.4±3.97	21.0±4.18	24.4^{b}±4.97	0	0	0	1.4^{bd} ±1.34

Values are means (n=5) ± s.d.

b. Significantly different from sucrose.

c. Significantly different from glucose.

d. Significantly different from glycerol.

Significance tested by student's 't' test (p=0.05)

Materials and Methods

For culturing, the juice vesicles were prepared as described in the chapter III. The juice vesicles were then cultured **in vitro** in culture tubes having 25 ml of MS basal medium. For finding out the most suitable carbon source for inducing differentiation of tracheid, fiber and sclereid in cultured Assam lemon juice vesicles, two per cent concentration of sucrose, glucose, glycerol and myoinositol (w/v) were incorporated in the basal medium. Sucrose was found to be the best carbon source. Subsequently, different sucrose concentrations (1, 2, 3, 4, 6, 8, 10 and 12%) were also tested to find out the optimal sucrose concentration for optimal differentiation of these elements. Control explants were cultured on MS basal medium, devoid of exogenous carbon source. The juice vesicles were incubated in dark at 25±1°C. Twenty culture tubes were maintained for each treatment. At the expiry of the experiments explants were fixed in F.A.A. The fixed juice vesicles were cleared, stained and stored for microscopic observationsas in Chapter III.

Results

Juice vesicles cultured on the MS medium devoid of carbon source neither revealed callusing nor cytodifferentiation. The minimum time for the initiation of callusing in the explants cultured on medium having either 2% (w/v) sucrose or glucose was 4 days while in the presence of myoinositol, it started on the 9th day (Table-5a.1). Explants cultured on glycerol supplemented medium callused only after 12 days of culturing (Table-5a.1).

Plate 5a

1. Development of callus in **C. limon** juice vesicles cultured on MS medium differing in carbon source:

   A - Control (devoid of carbon source); B - Sucrose; C - Glucose, D - Glycerol and E - Myoinositol.

2. Tracheid with scalariform secondary wall thickenings. X 100

3. Sclereids. X 240

4. Fibers. X 120

5. Tracheid with reticulate secondary wall thickenings. X 780

6. Development of callus in **C. limon** juice vesicle cultures on MS medium differing in sucrose concentration:

   A - Control (devoid of sucrose); B - 1%; C - 2%; D - 3%; E - 4%; F - 6%; G - 8%; H - 10% and I - 12%.

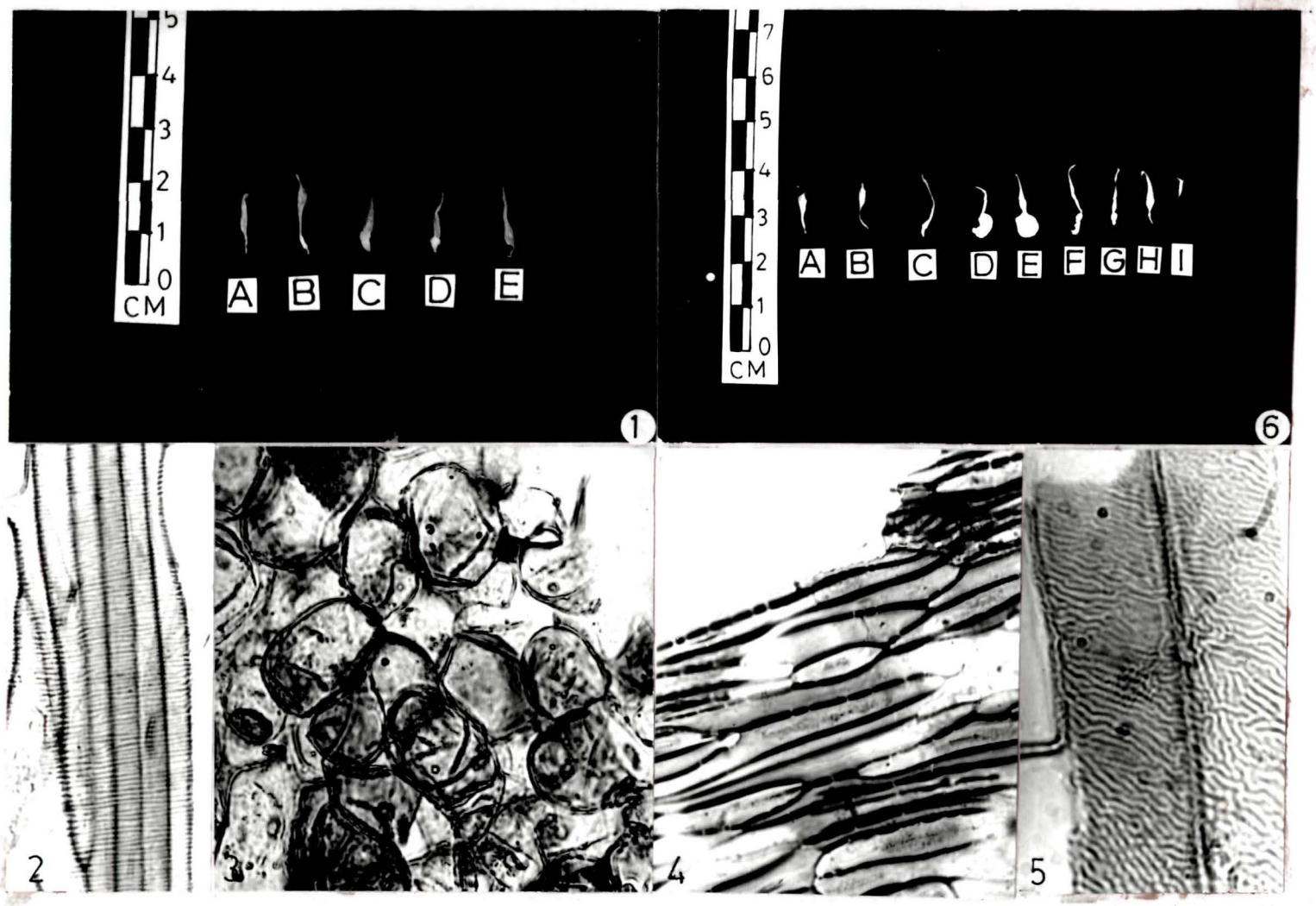


PLATE 5a

All the juice vesicles (100%) cultured on the sucrose and glucose supplemented medium developed callus while only 75% and 55% juice vesicles callused in the presence of glycerol and myoinositol, respectively (Table-5a.1). The minimum (7 mg) and maximum (25.95 mg) growth of the calli were evident in the explants cultured on myoinositol and glucose containing medium, respectively (Table-5a.1, Plate 5a.1). The differences observed in the weight of the calli could be indicative of differences in the rate of cell divisions. But no relationship was evident between intensity of cell division, as expressed by increase in callus weight, and differentiation of tracheid, fibers and sclereids. The weight of the callus developed by juice vesicles cultured on glucose supplemented medium was, though highest, the maximum number of tracheid, fibers and sclereids differentiated in the calli developed by the juice vesicles cultured on sucrose supplemented medium (Table-5a.1, Fig. 5a.1). Juice vesicles cultured on the glycerine incorporated medium revealed delayed cytodifferentiation in comparison to glucose supplemented medium (Table-5a.1). But the number of differentiating tracheids (Plate 5a.2) and sclereids (Plate 5a.3) were not much different in these treatments (Table-5a.1). In explants cultured on glucose supplemented medium the number of fibers (Plate 5a.4) differentiated was double than the number of fibers which differentiated in juice vesicles cultured on glycerine supplemented medium (Table-5a.1). The differentiation of tracheid, fibers and sclereids in calli developed by juice vesicles cultured on medium containing myoinositol was much delayed and their number was also very

Fig.5a.1 Effect of different carbon sources on cytodifferentiation in **C. limon** juice vesicle culture.

Fig.5a.1

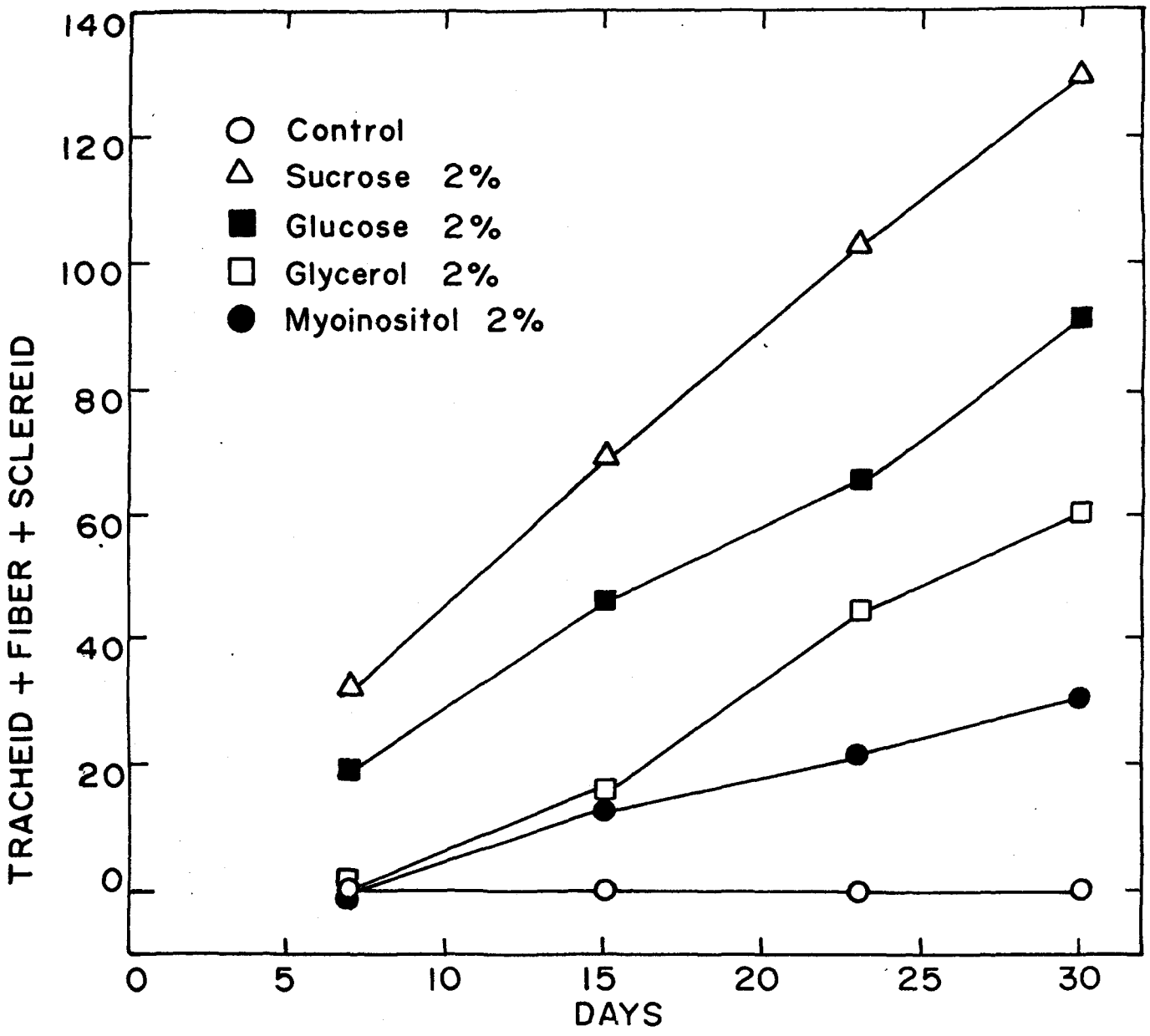

140
120
100
80
60
40
20
0
TRACHEID + FIBER + SCLEREID
Control
Sucrose 2%
Glucose 2%
Glycerol 2%
Myoinositol 2%
0
5
10
15
20
25
30
DAYS

Table 5a.2: Effect of sucrose concentrations on cytodifferentiation in C. limon juice vesicles.

Sucrose concentration (Percent)	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	5	45	10.2 ±1.6	0	4.4±2.07	8.4±2.40	11.2 ±2.38	23.6±2.30	49.6±2.40	55.0±3.16	68.6 ±2.70	11.4±2.07	14.4±3.36	28.0±3.80	40.0 ±4.12
2	5	100	11.6 ±1.35	4.2±1.92	8.0±2.23	10.6±2.88	12.4 ±3.43	37.2±5.58	82.4±2.88	103.6±2.96	105.8^{b}±6.14	18.6±3.84	38.6±3.36	62.6±2.40	71.8^{b}±2.30
3	4	100	21.2^{c}±1.16	5.0±2.23	9.2±3.19	13.8±3.34	27.0^{c}±3.16	43.8±5.40	91.2±5.97	110.4±6.98	148.6^{c}±4.72	20.8±2.58	32.4±2.30	59.4±2.70	72.8 ±4.32
4	4	100	22.2 ±1.92	7.4±2.40	9.2±2.86	21.0±2.54	28.4 ±3.97	50.6±5.31	91.0±6.89	104.0±6.51	153.0 ±5.24	29.6±3.64	43.2±2.86	67.2±4.14	82.2^{d}±3.76
6	6	80	15.4^{e}±1.49	2.0±1.41	4.4±2.70	10.8±3.19	13.2^{e}±2.86	11.6±2.40	55.6±6.58	90.0±4.47	20.6^{e}±3.64	2.8±1.92	19.0±2.91	26.8±4.20	32.33^{e}±2.73
8	6	75	13.0^{f}±1.67	0	4.2±2.38	6.0±2.23	11.4±2.40	10.0±2.23	43.6±5.12	70.6±5.59	92.6^{f}±7.89	0	0	3.0±2.00	9.2^{f}±2.28
10	7	75	10.0^{g}±0.89	0	2.0±1.41	4.8±1.30	10.6±1.94	0	0	0	7.0^{g}±2.12	0	0	0	4.6^{g}±2.79
12	7	70	4.2^{h}±0.74	0	0	0	5.8^{h}±1.78	0	0	0	3.2^{h}±1.64	0	0	0	2.2 ±1.48

Values are means (n=5) ± s.d.
b. Significantly different from 1%.
c. Significantly different from 2%.
d. Significantly different from 3%.
e. Significantly different from 4%.
f. Significantly different from 6%.
g. Significantly different from 8%.
h. Significantly different from 10%.

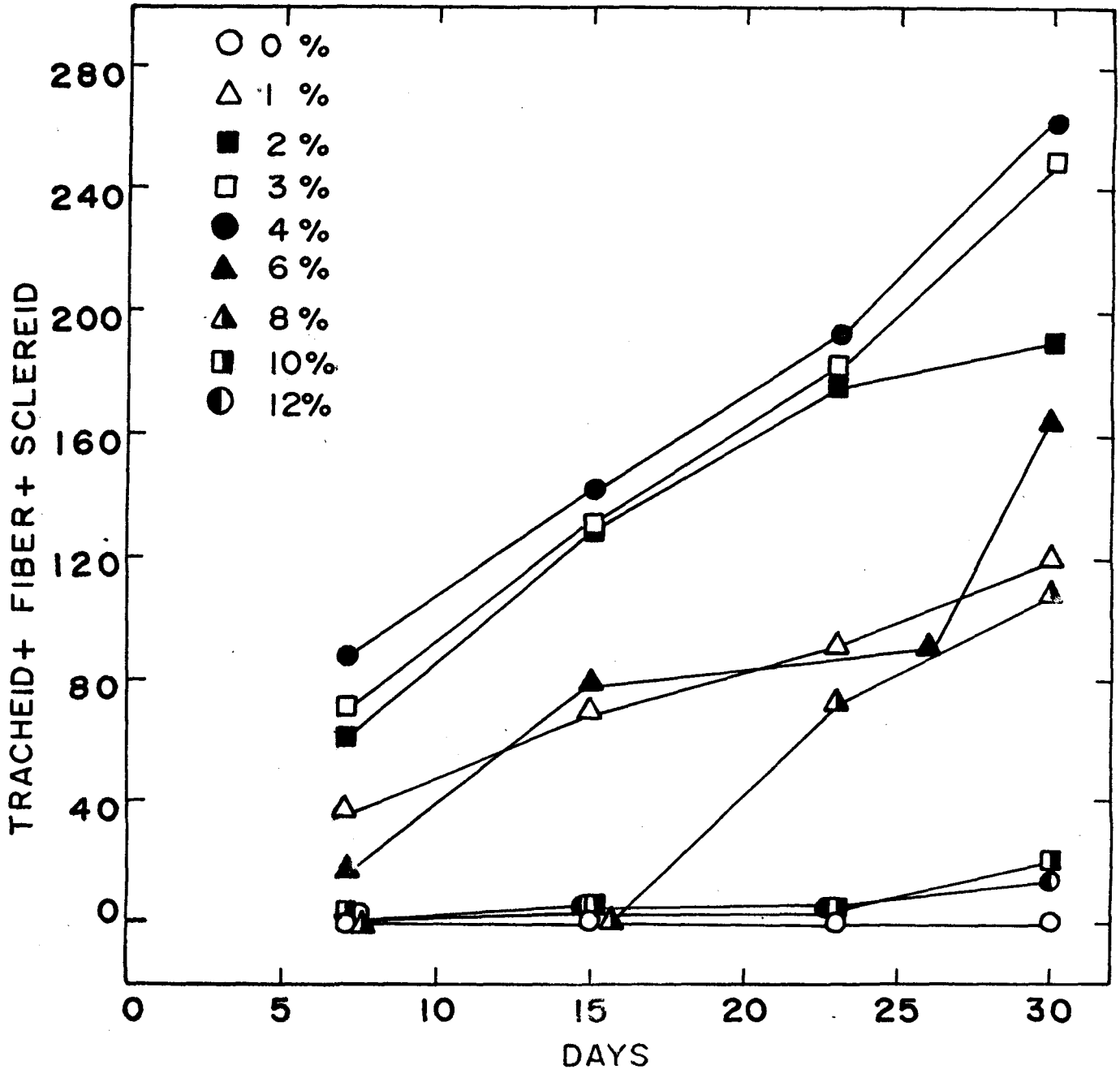
Significance tested by student's 't' test (p=0.05).

less (Table-5a.1). The elements differentiated were tracheids with reticulate thickening (Plate 5a.5) and fibers (Plate 5a.4). The number of sclereids differentiated was very low (Table-5a.1) suggesting an inhibitory effect of myoinositol on sclereid differentiation. Unlike tracheids which differentiated in explants cultured on myoinositol supplemented medium, the tracheids in explants cultured on glucose, sucrose and glycerine incorporated media revealed only scalariform secondary wall-thickening (Plate 5a.2).

To find out the optimum concentration of sucrose for differentiation of tracheid, fibers and sclereids eight concentrations of sucrose (0.1-12.0%) were tested (Table-5a.2). The MS medium devoid of carbohydrate served as control. This medium did not support callusing and revealed no cytodifferentiation in the cultured Assam lemon juice vesicles. Sucrose concentrations (1-12%) induced callusing and differentiation of tracheid, fibers and sclereids in the cultured juice vesicles (Table-5a.2). All the juice vesicles cultured on medium having 2-4% sucrose callused but vesicles cultured on medium having 3 and 4% sucrose required least time for callus initiation (Table-5a.2). The explant cultured on medium having 6-12% sucrose required more time to callus and the per cent of explants developing callus also decreased with the increasing concentration of sucrose, in comparison to 2-4% sucrose (Table-5a.2). The growth of callus increased with the increasing sucrose concentration in the medium upto 4% and thereafter a decreasing trend became evident (Table-5a.2, Plate 5a.6). The number of differentiated tracheid, fibers and

Fig.5a.2 Effect of sucrose concentrations on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 5a·2


0 %
1 %
2 %
3 %
4 %
6 %
8 %
10%
12%
TRACHEID+ FIBER+ SCLEREID
280
240
200
160
120
80
40
0
0
5
10
15
20
25
30
DAYS

sclereids also increased with the increasing sucrose concentration upto 4 per cent and thereafter their number declined (Table-5a.2, Fig. 5a.2). The degree of decrease, increased with the increasing concentration of sucrose (Table-5a.2).

Discussion

In the present study no callusing and cytodifferentiation occurred in the Assam lemon juice vesicles cultured on the medium devoid of exogenous carbon source. However, even in the absence of exogenous carbon source cultured explants of **Helianthus tuberosus** (Minocha & Halperin,1974) and **lettuce** pith (Roberts & Baba, 1982) develop callus and differentiate TE. Thus either the carbohydrate present in **C.limon** juice vesicles is inadequate to support cell division required for callusing and cytodifferentiation or carbon source is not limiting for callusing and cytodifferentiation in **Helianthus tuberosus** and lettuce pith tissue cultures. But according to Roberts (1976) a carbohydrate source in the medium is essential requirement for TE differentiation. Further, Murashige & Tucker (1969) and Kato (1980) also found that **C. limon** and **C. hassaku** juice vesicle cultures require a carbohydrate source for callusing and cytodifferentiation. Therefore the first possibility i.e. lack of adequate carbyhydrates in the Assam lemon juice vesicle ,to support cytodifferentiation, seems to hold good in case of **C. limon** juice vesicle cultures. Similarly, amongst different sugars, glucose supported best callus growth while sucrose induced best cytodifferentiation.

In the present study sucrose, a disaccharide, induced differentiation of tracheid and fibers in vascular nodules while tracheids and fibers which differentiated in explants cultured on medium having glucose, a monosaccharide, were scattered throughout the callus. This is in agreement with the findings of Jeff's and Northcote (1967). Glucose is as effective as sucrose in inducing growth and differentiation of TE in cultured explants of **Helianthus tuberosus** (Minocha and Halperin, 1974; Phillips and Dodds, 1977). But in the present investigation glucose was relatively less effective than sucrose. Amongst the various carbon sources used in the present investigation myoinositol proved to be the poorest. But Roberts and Baba (1982) found it to be the most effective carbon source for xylogenesis in lettuce pith. Thus it may be concluded that different species differ in their requirement of carbon source. In the present investigation, 2% myoinositol in the medium was, however, inhibiting only to callus formation although previous studies reported stimulatory effect. This discrepancy could be due to the much lower concentration of myoinositol (10 to 100 mg/l) used in these investigations (Torrey and Loomis, 1967; Kaul and Sabharwal, 1975; Goforth and Torrey, 1977). Thus the myoinositol induced responses may be dependent on the experimental material and the level of myoinositol used. Compared to myoinositol, glycerol is a relatively better carbon source for callus development and cyto-differentiation in **C. limon** juice vesicle. Glycerol is also effective in supporting the **in vitro** growth of **Rumex** virus tumor tissue (Nickell and Burkholder, 1950), **Daucus** and **Helianthus** (Gautheret, 1948,1959). Gautheret (1959) also observed that glycerol is the only tested polyalcohol with any appreciable effectiveness as a carbon source for the

growth of plant tissue cultures. However, findings of Hildebrandt and Riker (1949) and Baba and Roberts (1982) in marigold, Paris-daisy, periwinkle crown gall tissue, tobacco and lettuce do not support this.

Low sugar concentrations favour xylem formation, while higher sugar concentrations favour phloem differentiation (Wetmore and Rier, 1963). Rier and Beslow (1967) demonstrated a proportional increase in the number of differentiating cells with increasing concentration of sucrose upto 8 per cent. In contrast, Aloni (1980) could find no correlation between sucrose concentrations and the differentiation of vascular element. He, however, stated that low IAA levels favour differentiation of phloem while high auxin concentrations favour xylem differentiation and sucrose concentrations influence the amount of callose deposited on the sieve plates. In the present study the differentiation of tracheid, fibers and sclereids increased with the increasing concentration of sucrose upto 4% but beyond this it decreased. No sieve element differentiated even at the highest concentration used although number of tracheid, fibers and sclereids differentiated at these concentrations was very few. The present investigation thus provides evidence to suggest that low concentrations of sucrose upto a threshold level promote differentiation of tracheid, fibers and sclereids but with the further increase in sucrose level the number of differentiating tracheid, fibers and sclereids diminishes. Besides, the threshold level of sucrose for phloem differentiation varies with the species.

CHAPTER V

(b) NITROGEN SOURCE

Introduction

Analysis of cellular and metabolic changes preceding and accompanying TE differentiation is an important step towards understanding of the mechanism regulating cytodifferentiation. Phillips and Dodds (1977) observed that complete omission of inorganic nitrogen from the MS medium stimulated cytdifferentiation in Jerusalem artichoke tuber explants. Shiraishi et al, (1982) tested the effects of both the inorganic nitrogen components (NH_4NO_3 and KNO_3) present in MS medium on cytodifferentiation in lettuce pith culture and concluded that only NH_4NO_3 is essential for TE differentiation. According to some workers, however, the nitrogen source required for growing plant cells is a mixture of ammonium and nitrate (Filner, 1966; Erikson, 1965 and Gamborg 1968).

The purpose of this study is to investigate whether or not both the nitrogen sources present in the MS medium are essential for cytodifferentiation of tracheid, fibers and sclereids in Assam lemon juice vesicle cultures.

Materials and Methods

The procedure for the culturing of Assam lemon juice vesicles has been described in chapter III. Four sets of MS medium were used to study the effects of nitrogen source on cytodifferentiation. The MS medium having both the inorganic nitrogen sources (NH_4NO_3 and KNO_3) served as the control. In the remaining three sets of the MS medium one had only NH_4NO_3,

Table 5b: Requirement of nitrogen source (NH_4NO_3/KNO_3) for cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing	Percentage of juice vesicles showing callus development	Final fresh weight (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	4	100	11.9 ±0.67	2.8±0.83	11.0±2.12	22.4±1.94	30.2 ±3.96	32.2±5.26	93.4±7.02	121.8±7.04	141.6 ±3.50	10.4±2.60	51.0±2.54	61.2±3.03	78.6 ±3.57
MS-(KNO_3)	4	100	12.4 ±0.88	4.8±0.89	13.8±2.28	20.8±0.83	32.4 ±3.36	24.4±3.04	72.6±7.98	104.0±12.94	136.8 ±5.26	10.8±2.28	40.6±1.51	72.0±4.30	94.6[b] ±3.97
MS-(NH_4NO_3)	4	100	12.0 ±0.82	3.2±1.78	12.0±1.58	24.0±3.16	29.0 ±5.09	17.8±5.26	60.8±4.76	90.8±6.97	123.6[b] ±4.77	15.2±3.49	52.0±3.53	72.0±2.12	89.2[b] ±3.34
MS-(NH_4NO_3 and KNO_3)	3	100	13.0 ±0.92	5.4±1.67	14.5±2.81	27.6±2.40	36.2[b] ±3.80	26.2±3.56	81.2±7.12	116.0±4.69	147.4 ±4.56	21.0±2.44	61.6±3.41	82.4±2.60	101.8[b] ±6.05

a. Values are means (n=5) ± s.d.

b. Significantly different from control.

Significance tested by student's 't' test (p=0.05).

while the other had KNO_3 alone. The third set of MS medium was devoid of both the nitrogen sources. Twenty culture tubes were maintained for each treatment. The cultured juice vesicles were incubated in dark in a B.O.D. incubator maintained at 25±1°C. Cultured juice vesicles were collected at weekly intervals and fixed in F.A.A. The experiment was terminated at the end of one month. The fixed juice vesicles were cleared, stained and stored for microscopic observations as in Chapter III.

Results

Juice vesicle explants cultured on the MS medium having either both or only one nitrogen source revealed callusing on the 4th day of inoculation while it was observed on the 3rd day in the juice vesicles cultured on the medium devoid of both the nitrogen sources (Table-5b, Plate 5b.1). In All the treatments though the frequency of calli development was hundred per cent, very little difference was observed in the fresh weight of calli in different treatments (Table-5b). Even the maximum weight of the callus (13 mg) obtained at the end of the experiment was not significantly different from the weight of the calli (11.85 mg) developed by control explants (Table-5b).

The differentiation of tracheid, fibers and sclereids occurred on the 7th day in all the treatments (Plate 5b.2-4). The type of secondary wall thickening exhibited by differentiating tracheids was scalariform in all the treatments (Plate 5b.2). The largest number of tracheid, fibers and sclereids differentiated

Plate 5b

1. Effect of nitrogen source on development of callus in **C. limon** juice vesicle cultures:

   A - MS medium having NH_4NO_3 + KNO_3; B - MS medium having KNO_3; C - MS medium having NH_4NO_3 and D - MS medium lacking in both NH_4NO_3 and KNO_3.

2. Tracheid with scalariform secondary wall thickening. X 780;

3. Fibers. X 240

4. Sclereids. X 470

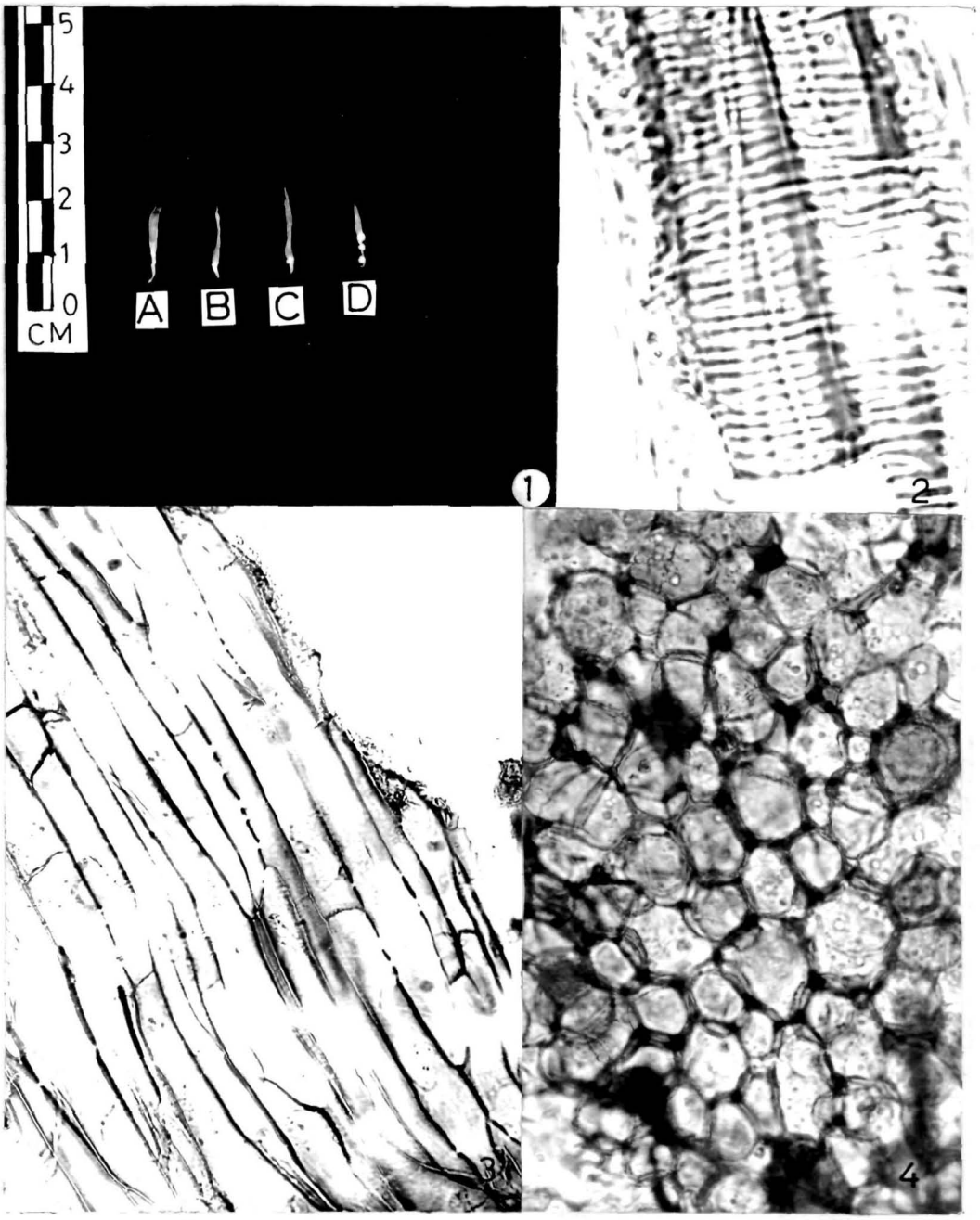
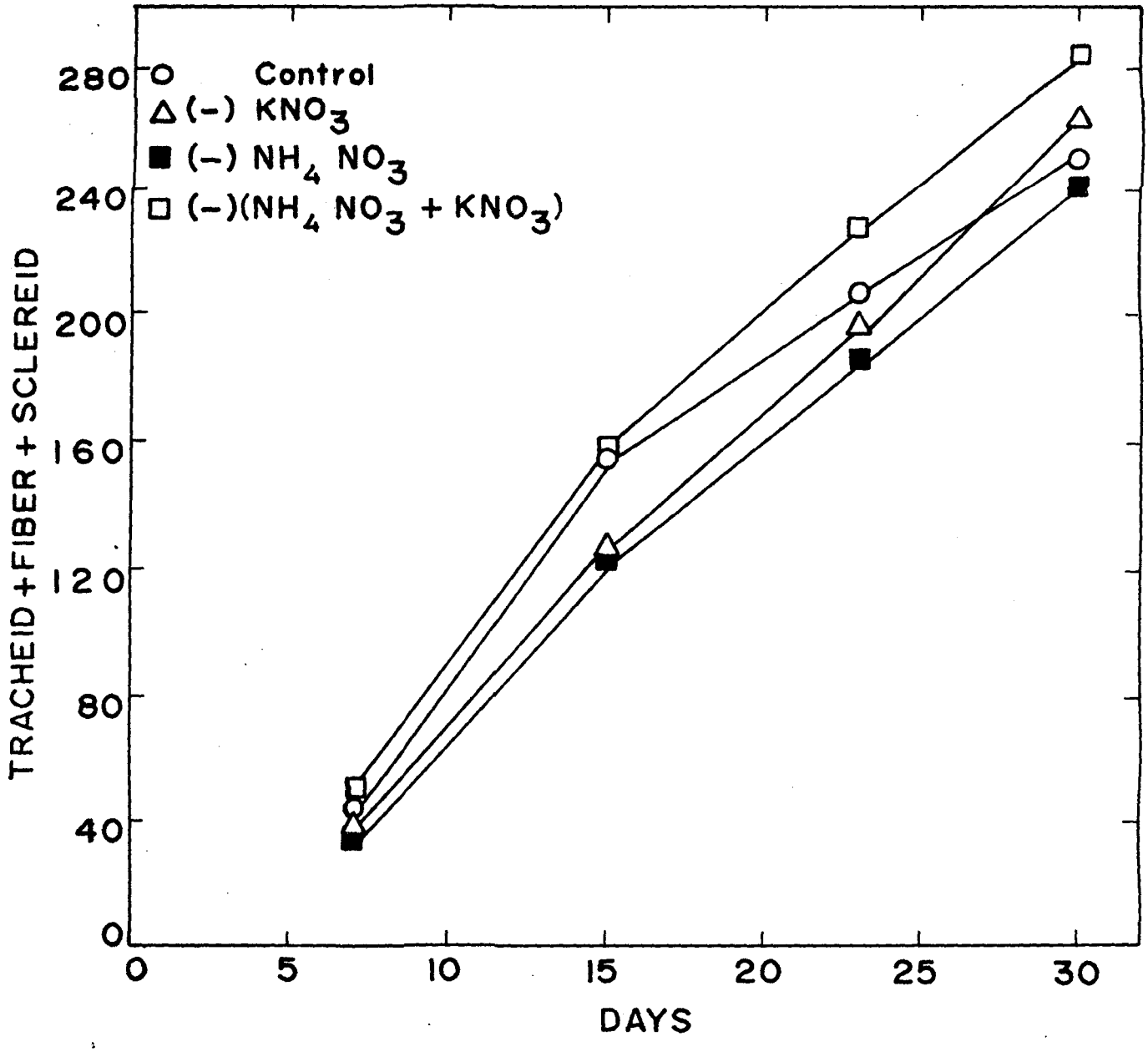


PLATE 5b

Fig.5b Effect of nitrogen source (NH_4NO_3/KNO_3) on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 5b


Control
(-) KNO3
(-) NH4 NO3
(-)(NH4 NO3 + KNO3)
TRACHEID + FIBER + SCLEREID
0
40
80
120
160
200
240
280
0
5
10
15
20
25
30
DAYS

in the absence of both the nitrogen sources, while least number of these elements differentiated in presence of only one nitrogen source i.e., KNO_3 (Table-5b, Fig.5b).

Discussion

In the present investigation the MS medium devoid of both the inorganic-nitrogen sources is the best medium for supporting callusing and cytodifferentiation. Conversely, MS medium containing both the nitrogen sources (NH_4NO_3 and KNO_3) induced differentiation of lesser number of tracheid, fibers and sclereids. Thus, MS medium devoid of any nitrogen source induced better cytodifferentiation which is in agreement with the findings of Phillips and Dodds (1977). Since in the present investigation cytodifferentiation occurred even in the absence of any nitrogen, it may be suggested that nitrogen is not essential for the differentiation of tracheid, fibers and sclereids in cultured **C. limon** juice vesicles. In cell-suspension cultures of sycamore the incorporation of a nitrogen source inhibits secondary metabolism involved in phenolics biosynthesis by promoting an upsurge in protein synthesis which competes strongly for common precursors (Phillips and Henshaw, 1977) which may be the reason for nitrogen induced inhibition of cytodifferentiation in **C. limon** juice vesicle culture, as well.

In the present investigation incorporation of NH_4NO_3 alone in the medium improved cytodifferentiation over the cytodifferentiation evident in explants cultured on medium having KNO_3. However, Fukuda and Komamine (1980a) have reported that

in cultures of **Zinnia elegans** mesophyl cell, incorporation of KNO_3 is a necessity to stimulate TE differentiation. Shiraishi **et al.**,(1982) found that incorporation of KNO_3 in the medium does not stimulate differentiation of xylem in lettuce pith. Thus it seems that different nitrogen (ammonium/nitrate) influence cytodifferentiation differently and the response is species dependent.

CHAPTER V

(c) GROWTH HORMONES AND INHIBITORS

Introduction

Recent survey of literature on xylem differentiation brings out that a variety of substances promote or substantially modify cytodifferentiation process in experimental system (Minocha, 1984; Dodds, 1981; Barnett, 1979; Comer, 1978; Roberts, 1976).

IAA being a limiting factor for xylem differentiation was demonstrated by Jacobs (1952). Many reports reveal that an exogenous supply of auxin is usually required for xylogenesis in culture explants, the only exception being explants autotrophic for auxin (Clutter, 1960; Earle, 1968; Dalessandro and Roberts, 1971; Gautheret, 1961; Wetmore and Rier, 1963; Bergmann, 1964; Jeff's and Northcote, 1966, 1967; Rier and Beslow, 1967; Cronshaw, 1967; Torrey, 1968; Fosket and Torrey, 1969). The low level of differentiation which occurs in the whole stem slices cultured on basal medium devoid of auxin could be due to residual endogenous auxin (Fosket and Roberts, 1964). The effectiveness of various auxins and their optimum concentration varies with the experimental material (Dalessandro and Roberts, 1971; Dalessandro, 1973a,b; Minocha and Halperin, 1974; Phillips and Dodds, 1977). Different auxins (Dalessandro and Roberts, 1971) and combination of various hormones (Dalessandro, 1973b) induce different patterns of TE distribution in cultured explant. This may be because of differences in the polar transport characteristics of various auxins (Roberts, 1976).

Together with auxin, cytokinin is required for TE differentiation in cultured soyabean callus (Fosket and Torrey,

1969), pea root cortex (Phillips and Torrey, 1973) and Jerusalem artichoke tuber tissue (Phillips and Dodds, 1977). Bergmann (1964, 1965) demonstrated that kinetin may increase WVM formation in cultures of **Nicotiana tabacum**, and this increased xylogenesis is concomitant with an increased synthesis of lignin. Addition of kinetin in an auxin supplemented medium resulted in a four-fold increase in TE differentiation in Jerusalem artichoke tuber suggesting a direct role for cytokinin in the differentiation of TE (Minocha and Halperin, 1974). However, cytokinin are not always essential for TE differentiation (Dalessandro, 1973a; Haddon and Northcote, 1975). Cytokinin requirement is dependent on the explant thickness, culture conditions and level of endogenous cytokinin in explant (Mizuno **et al.**, 1971).

Gibberellin induced xylem differentiation is dependent on the species cultured and culture conditions (Bradley and Crane, 1957; Wareing, 1958; Doley and Leyton, 1968; Davis and Holmes, 1962; Roberts and Fosket, 1966; Netien, 1957). GA treatment increases the length of annular and spiral elements and pitted scalariform cells in **Lycopersicon esculantum** stem (Davis and Holmes, 1962). The interaction of GA with other hormones, i.e. IAA or kinetin has both stimulatory (Netien, 1957; Gautheret, 1961; Dalessandro, 1973b; Phillips and Dodds, 1977) and inhibitory (Minocha and Halperin, 1974) effects on differentiation of TE. The effectiveness of different cytokinins varies with the experimental material (Dalessandro and Roberts, 1971; Dalessandro, 1973a; Dodds, 1978; Minocha, 1984). Roberts and Fosket (1966) investigated

the interaction of GA and IAA on the differentiation of WVM in stem internode tissue slices of **Coleus**. In combination treatments of GA+IAA, low concentration of GA (0.5 or 0.05 ppm) had stimulatory effects while higher concentration of GA (5.0 ppm) proved inhibitory, and revealed incompletely differentiated secondary xylem elements having thin and incomplete secondary wall striations and WVM (Roberts and Fosket, 1966; Hansen, 1966). Similar observations (incompletely differentiated TE) were made in debudded shoots of **Populus robusta** and **Vitis vinifera** following the application of GA (500 ppm) and IAA (100 ppm) (Digby and Wareing, 1966; Wareing, Haney and Digby, 1964). Murashige and Tucker (1969) suggested that the gibberellin may be an optional nutrient medium ingredient for lemon tissue culture.

Ethylene, a gaseous plant hormone also plays an important role in the differentiation of xylem (Roberts, 1976). Low levels of methionine, an ethylene precursors, in the medium stimulates xylogenic response of cultured **Lactuca** pith (Roberts and Baba, 1978), while high levels of ethylene blocks TE formation (Zobel and Roberts, 1978). Ethylene precursors stimulate TE differentiation, in the presence of auxin and kinetin, in soyabean (Miller and Roberts, 1982) and **Lactuca** (Roberts and Miller, 1982; Miller and Roberts, 1984; Miller **et al.**, 1984 and 1985) cultures.

Abscisic acid, which is also a plant hormone, now ranks in importance with the auxin, gibberellin and cytokinin as a controlling factor in physiological processes (Addicott

and Lyon, 1969). ABA although does not induce cell division, it substantially promotes the number of cells in the explants that respond to auxin for cell division. The stimulatory effects of ABA are negated by the presence of cytokinin in the medium (Minocha, 1979a,b). The most striking effect of ABA, however, was that it completely inhibited differentiation of tracheids without affecting cell division in Jerusalem artichoke tuber tissue (Minocha and Halperin, 1974; Minocha, 1984). The hormone ABA are extremely useful in providing additional culture conditions in which the process of differentiation can be controlled independently of growth (Minocha, 1984).

Kordan (1964) observed differentiation of WVM in 30 month old proliferating subcultures of lemon fruit vesicles. However, enough work has not been done to find out the hormonal requirement of xylogenesis in cultured **Citrus** juice vesicles (Kordan, 1965; Kato, 1980; Kulshrestha **et al.**, 1982). The present investigation deals with the effect of different growth hormones, various auxins in varying concentration and interaction of different growth hormones (auxins, kinetin, gibberellic acid, abscisic acid and ethylene) on callusing and differentiation of tracheid, fibers and sclereids in **Citrus** juice vesicles **in vitro.**

Whether or not the cell division is a prerequisite for TE differentiation is an important but controversial problem of cytodifferentiation. By using mitotic inhibitors, Fosket and Torrey (1968-'70, 1969), Comer (1978) and Jeff's and Northcote (1967) have reported that cell division is a prerequisite for

cytodifferentiation of TE. But Fukuda and Komamine (1980b) found that cell division is not a pre-requisite for TE formation in **Zinnia elegans**. Colchicine is a useful diagnostic tool to determine if cell divisions are prerequisite for cytodifferentiation of TE. Colchicine also affects secondary wall thickening in TE (Hepler and Fosket, 1971; Pickett-Heaps, 1967; Roberts and Baba, 1968; Green, 1963). In the present investigation, colchicine was therefore used to find out if cell division is a prerequisite for cytodifferentiation in cultured Assam lemon juice vesicles.

Materials and Methods

The detailed procedure for the isolation, preparation and culturing of vesicles from mature fruits of Assam lemon has been described in Chapter III. Each culture tube contained 25 ml of MS basal medium. To study the effects of different auxin on callusing and cytodifferentiation in cultured citrus juice vesicles the basal medium was supplemented with varying concentrations of IAA, IBA and NAA (0.1, 0.5, 1.0, 5.0, 10.0 and 15.0 mg/l) and 2,4-D(0.1, 0.5 and 1.0 mg/l). Juice vesicles cultured on MS medium devoid of auxin served as control.

Having selected the best auxin, IAA in this case, the effect of different plant hormones IAA (10 mg/l), Kn (0.2 mg/L) and GA (0.2 mg/l) on tracheid, fibers and sclereids differentiation was also investigated by incorporating these hormones in the basal medium, one at a time, and comparing the effects. Hormones, IAA, Kn and GA were also used in combinations to investigate

effects of combination treatments on the differentiation of above elements. The combinations used were IAA+Kn (10 mg/l + 0.2 mg/l); IAA+GA (10 mg/l + 0.2 mg/l); Kn+GA (0.2 mg/l + 0.2 mg/l) and IAA+Kn+GA (10 mg/l + 0.2 mg/l + 0.2 mg/l).

For investigating ethylene effects its precursor (methionine, 3.5 µg/l) and inhibitor (cobalt chloride 1 mM) were used. Methionine and $CoCl_2$ were incorporated in MS basal medium having IAA (10 mg/l) and Kn (0.2 mg/l). MS basal medium devoid of methionine and $CoCl_2$ served as control.

Effect of ABA on callusing and cytodifferentiation was investigated by incorporating ABA (0.001, 0.005, 0.01 and 0.05%) in MS basal medium. ABA was incorporated in the basal medium both in the presence and absence of plant hormones IAA (10 mg/l) and Kn (0.2 mg/l).

The effects of colchicine (a mitotic inhibitor) on cytodifferentiation were investigated by incorporating different colchicine concentrations (0.005, 0.01, 0.05, 0.1 and 0.5%) in the MS basal medium having IAA (10 mg/l) and Kn (0.2 mg/l).

Per treatment forty culture tubes having one juice vesicle each were maintained. Daily observations were made for visible sign of callusing. Materials were collected every day after culturing till expiry of experiment on the 30th day. Fixing, clearing and staining of the explants was done as described in Chapter III. The stained material, stored in glycerine, was dissected and examined under microscope for the presence of

Table 5c.1: Effect of different auxins on cytodifferentiation in C. limon juice vesicles.

Treatment (Concentration mg/l)	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	8	10	1.7 ±0.34	0	0	0	0	0	0	0	13.2 ±2.86	0	0	0	0
IAA															
0.1	6	35	3.0^{b} ±0.89	0	0	2.2±1.92	7.4 ±1.14	4.15±2.07	13.4±2.07	25.2±3.49	30.8^{b} ±5.49	0	0	0	6.8 ± 1.48
1.0	6	67	4.0^{b} ±0.63	0	4.2±2.38	6.0±2.23	8.0 ±1.58	5.4±1.51	14.8±2.86	23.6±2.70	$38.4^{b,c}$ ±3.20	0	8.0±1.58	17.0±1.58	20.4^{c} ±2.96
5.0	5	100	5.4^{b} ±1.01	5.1±1.58	7.0±2.23	11.4±2.40	14.4^{d} ±2.40	7.0±1.58	15.8±3.96	27.4±3.64	$58.4^{b,d}$ ±4.21	0	9.0±3.0	15.6±3.97	28.4^{d} ±2.70
10.0	4	100	7.6^{b} ±1.01	9.6±2.70	11.2±3.03	16.2±2.58	24.0^{e} ±2.54	20.8±3.19	32.4±3.97	44.4±3.50	$76.6^{b,e}$ ±5.31	26.6±2.07	37.0±2.54	58.2±1.92	66.0^{e} ±2.23
15.0	5	100	5.6^{b} ±1.01	5.2±1.92	10.2±1.92	13.2±2.86	20.2 ±3.96	17.0±1.58	28.4±3.20	38.4±4.66	$65.6^{b,f}$ ±4.61	21.8±3.49	34.8±3.11	51.0±3.87	54.0^{f} ±3.16
IBA															
0.1	7	50	1.8 ±0.97	0	0	0	0	0	0	2.0±1.87	5.33^{b} ±1.52	0	0	0	0
1.0	7	72	2.2 ±1.01	0	3.4±2.70	7.0±3.16	9.0 ±2.13	0	3.2±2.58	8.6±3.36	11.2 ±3.70	0	0	0	0
5.0	5	100	3.2^{b} ±0.74	4.2±1.92	6.2±2.38	9.0±2.91	13.4^{g} ±2.30	5.6±2.40	8.8±3.03	12.8±3.03	$19.4^{b,g}$ ±3.84	0	0	0	0
10.0	5	100	4.2^{b} ±1.46	4.6±2.40	9.0±3.16	10.0±2.23	14.2 ±2.39	7.6±2.40	11.0±3.16	15.2±3.49	$25.0^{b,h}$ ±3.60	7.0±3.16	9.0±3.0	12.4±1.67	18.0 ±4.58
15.0	4	100	5.0^{b} ±1.41	5.6±2.40	8.8±3.03	12.8±3.03	$19.4^{e,i}$ ±3.84	8.2±2.60	15.2±3.07	20.2±1.92	$30.0^{b,i}$ ±2.23	4.2±2.77	10.8±1.78	13.4±2.70	15.2 ±3.07
NAA															
0.1	8	83	2.2 ± 1.32	0	0	0	0	0	0	0	0	0	0	0	0
1.0	7	90	4.0^{b} ±0.63	0	0	0	2.6 ±1.81	0	0	0	8.4^{b} ±2.40	0	0	0	0
5.0	7	100	4.8^{b} ±1.32	0	1.8±1.78	5.0±1.58	11.6^{j} ±2.40	0	15.0±3.16	22.6±4.92	$24.8^{b,j}$ ±3.96	0	10.6±1.51	16.4±3.57	20.8 ±3.54
10.0	6	100	6.0^{b} ±1.41	4.2±1.01	9.0±3.16	13.0±2.23	16.0^{k} ±2.91	16.4±5.45	20.2±3.11	30.6±3.36	$46.6^{b,k}$ ±6.76	0	13.0±3.16	18.2±1.92	32.0^{k} ±5.43
15.0	7	100	4.6^{b} ±0.8	0	6.5±2.16	10.2±3.04	14.0 ±3.16	0	9.8±4.20	19.8±2.86	41.0^{b} ±2.64	0	11.6±2.40	22.2±2.86	30.0 ±3.16
2,4.D															
0.1	5	100	3.4^{b} ±1.01	0	9.4±1.51	11.2±1.92	13.6 ±2.30	8.6±3.84	12.4±1.94	21.6±3.04	34.6^{b} ±5.02	10.8±3.19	13.2±2.86	15.8±3.27	31.0 ±4.94
0.5	4	100	6.4^{b} ±1.01	5.4±2.60	10.4±2.96	11.8±2.38	18.2 ±4.97	15.0±3.80	23.6±3.04	42.8±3.34	$54.0^{b,l}$ ±3.80	20.0±4.12	26.8±4.81	37.6±5.12	56.8^{m} ±4.30
1.0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Values are means (n=5) ± s.d. b. Significantly different from control. c. Significantly different from the value at 0.1 mg/L IAA
d. Significantly different from the value at 1.0mg/L IAA. e. Significantly different from the Value at 5.0mg/L IAA.
f. Significantly different from the Value at 10.0mg/L IAA. g. Significantly different from the Value at 1.0mg/L IBA.
h. Significantly different from the Value at 5.0mg/L IBA. i. Significantly different from the Value at 10.0mg/L IBA.
j. Significantly different from the Value at 1.0mg/L NAA. k. Significantly different from the Value at 5.0mg/L NAA.
l. Significantly different from the Value at 0.1mg/L 2,4-D.

Significance tested by student's 't' test (p=0.05).

tracheid, fibers and sclereids. Cell counts from squash preparation of calli sampled at weekly intervals were made with a Leitz Ortholux-2 microscope equipped with a projection attachment.

RESULTS

Effects of different auxins

The juice vesicles cultured on medium devoid of auxins callused very late (8th day of inoculation). Addition of different concentrations of various auxins: IAA, NAA, IBA (0.1, 1.0, 5.0, 10.0 and 15.0 mg/l) and 2,4-D (0.1 and 0.5 mg/l), in the medium decreased the lag period for callusing (Table-5c.1). Stimulation of callusing occurred with the increasing concentration of auxin in the medium (Table-5c-1). However, optimal concentration differed with the auxin used: IAA and NAA (10 mg/l); IBA (15 mg/l), and 2,4-D (0.5 mg/l). One mg/l 2,4-D suppressed callusing of the juice vesicles (Table-5c.1). As shown in Table-5c.1, the highest fresh weight of calli (7.6 mg) was obtained in the presence of 10 mg/l IAA while calli developed by juice vesicle cultured on medium having 15 mg/l IBA exhibited very little callus growth (5 mg) (Table-5c.1).

In the juice vesicles cultured on medium having no growth hormones differentiation of fibers (Plate 5C.1) was evident on the 30th day of inoculation. However, their number was very less (Table-5C.1). IAA concentrations induced differentiation of tracheid, fibers and sclereids. Fibers differentiated earliest followed by tracheids and sclereids (Table-5C.1). The differentia-

Plate 5c

1. Fibers differentiated in control medium (devoid of hormones). X 120

2. Trachieds with scalariform thickening. X 240

3. Pitted tracheids. X 470

4. Fibers. X 380

5. Micro sclereids. X 470

6. Development of callus in **C. limon** juice vesicle cultures on MS medium having different plant growth hormones:

   A - Control (devoid of growth hormone); B - IAA; C - Kn; D - GA; E - IAA + Kn; F - IAA + GA; G - Kn + GA and H - IAA + Kn + GA.

7. Sclereids. X 780

8. Stone cells. X 240

9. Reticulate pitted tracheids. X 960.

10. Tracheid. X 470

11. Tracheids with reticulate secondary wall thickenings. X 1200

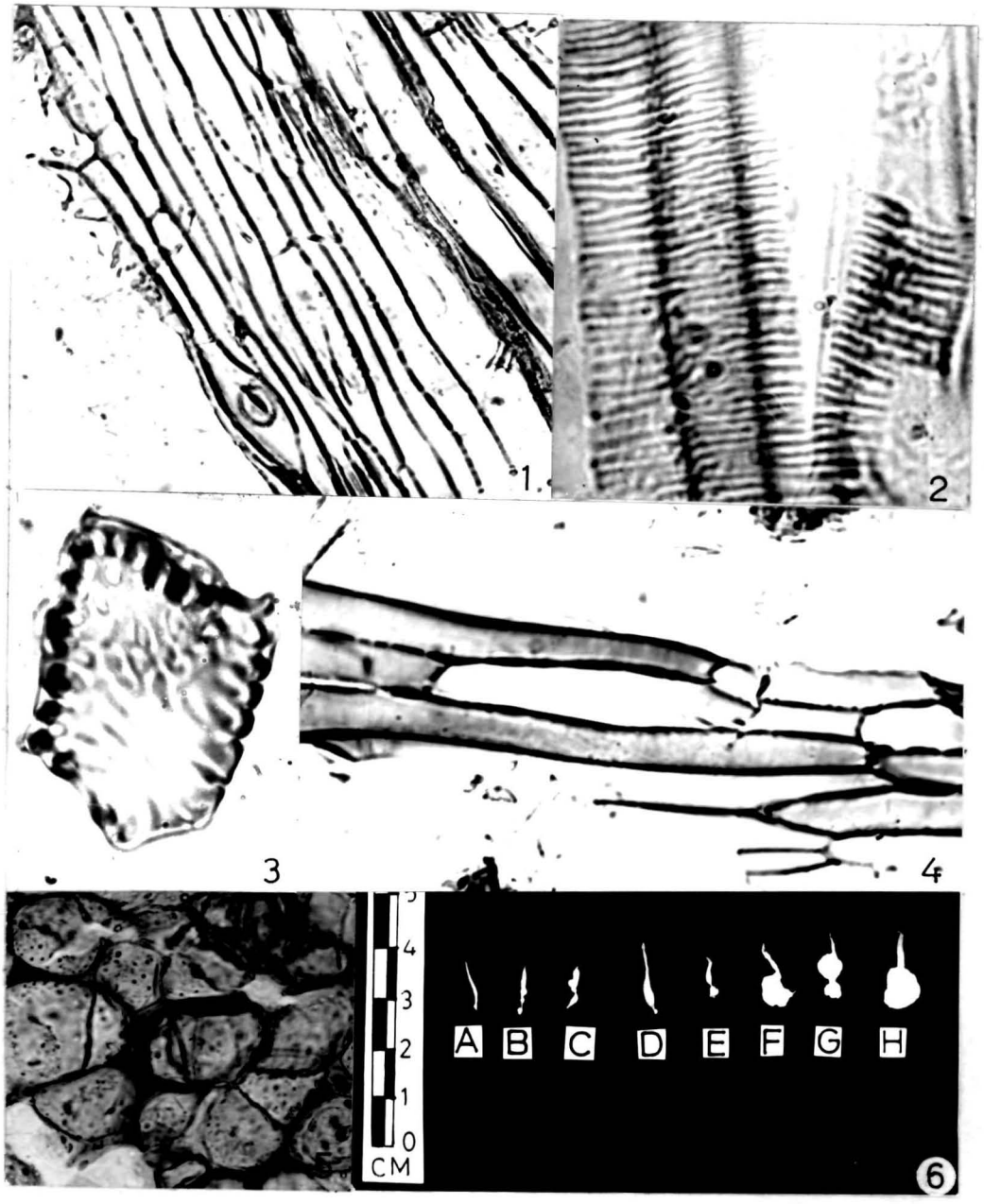


PLATE 5c

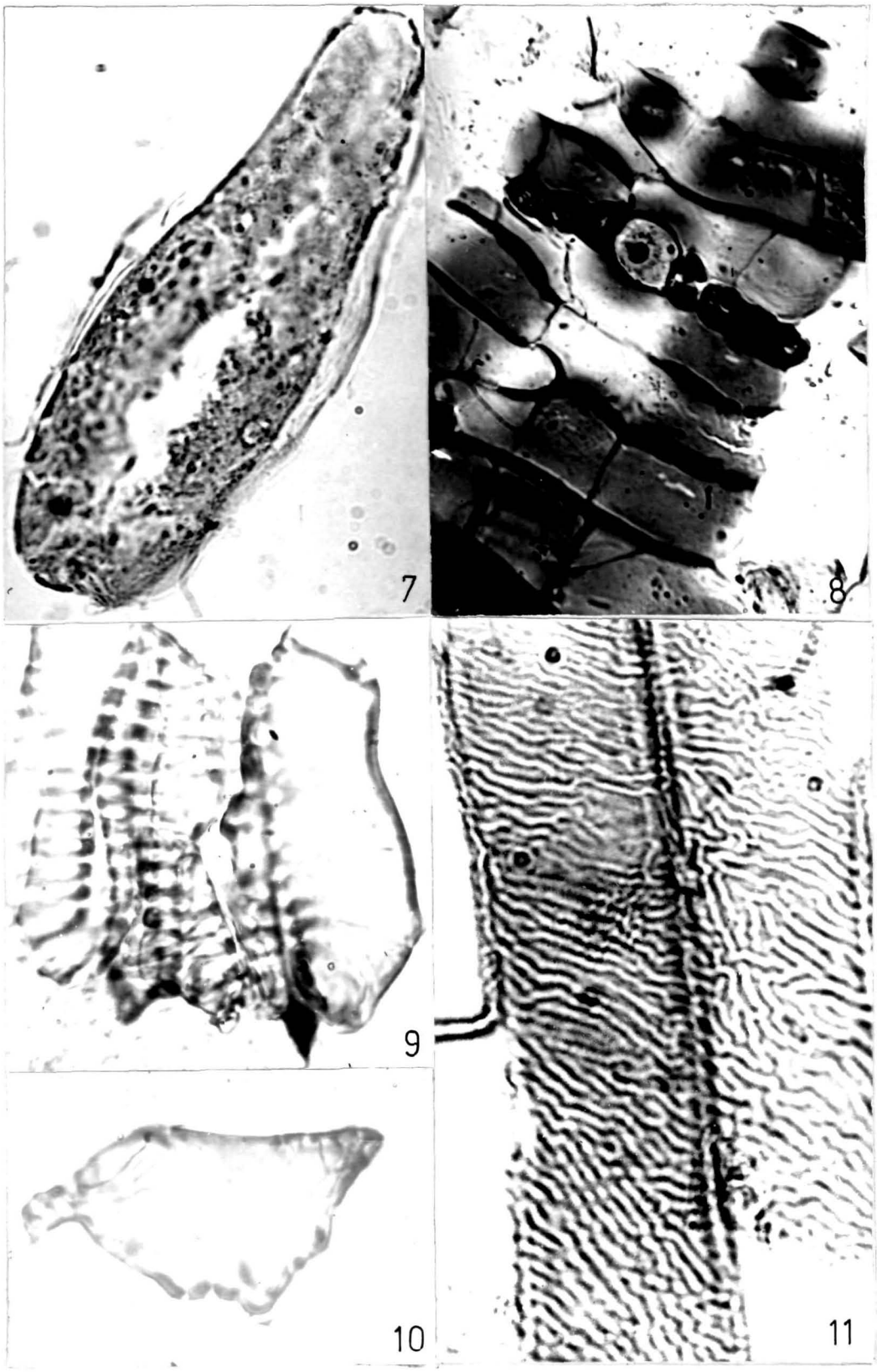


PLATE 5c (Contd..)

tion of fibers was highest in number while tracheids were least in number (Table-5C.1). With the progression of time the intensity of differentiation increased at all concentrations of IAA. The IBA concentrations also induced differentiation of fibers, tracheids and sclereids. Differentiation of fibers occurred at all the concentration of IBA while trachieds did not differentiate at 0.1 mg/l concentration of IBA (Table-5C.1). Sclereids, however, differentiated only at 10-15 mg/l (Table-5c.1). Lowest concentration of NAA (0.1 mg/l) did not induce any cytodifferentiation in the cultured juice vesicles but 1 mg/l of NAA differentiated tracheids and fibers towards the end of the experiments. The sclereids did not differentiate even at this concentration of NAA (Table-5c.1). At other concentrations of NAA (5-15 mg/l) differentiation occurred in the following order: fibers, sclereids and tracheids (Table-5c.1). Fibers and tracheids differentiated earlier than sclereids at 10 mg/l NAA concentration (Table-5c.1). The number of tracheid, fibers and sclereids increased with the aging of culture at 1-15 mg/l of NAA.

Low concentrations of 2,4-D (0.1 and 0.5 mg/l) stimulated the differentiation of tracheid, fibers and sclereids but higher concentration (1 mg/l) proved inhibitory to both callusing and cytodifferentiation (Table-5c.1). With ageing of culture the number of differentiating trachied, fibers and sclereids increased at both the concentrations (0.1 and 0.5 mg/l). However, the largest number of tracheid, fibers and sclereids differentiated at 0.5 mg/l of 2,4-D.

Table 5c.2: Effect of different growth hormones (IAA, Kn and GA) and their combinations on cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	7	100	2.6 ±1.05	0	0	0	0	0	14.8±2.86	22.8±2.38	27.4 ±2.07	0	0	0	0
IAA (10mg/l)	5	100	20.8^{b} ±3.05	5.2±2.86	10.0±2.23	15.2±1.92	20.4±3.64	20.4±2.07	41.0±3.16	51.0±2.91	71.0^{b} ±2.91	15.2±2.86	29.0±3.16	32.8±3.70	40.0 ±4.12
Kn(0.2mg/l)	7	83.4	$14.2^{b,c}$ ±2.78	0	4.0±2.00	11.6±2.40	14.6^{c} ±3.20	0	16.8±3.04	22.4±3.64	$47.0^{b,c}$ ±3.16	0	9.0±2.73	12.6±2.40	24.6^{c} ±3.20
GA(0.2mg/l)	4	100	$23.6^{b,d}$ ±2.72	8.0±2.23	12.6±2.07	12.8±3.34	30.4^{d} ±3.97	18.6±3.04	39.4±3.64	69.2±3.03	$85.4^{b,d}$ ±3.20	2.2±1.92	16.4±2.70	29.8±2.77	31.4^{d} ±2.70
IAA+Kn (10mg/l+ 0.2mg/l)	6	100	$22.2^{b,e}$ ±1.92	2.6±2.60	14.8±3.49	21.6±2.40	30.0±4.12	12.2±1.92	35.4±3.71	93.6±4.97	$130.2^{b,e}$ ±3.96	10.0±2.23	23.4±2.70	47.0±3.16	78.0^{e} ±2.54
IAA+GA (10mg/l+ 0.2mg/l)	5	100	$29.4^{b,f}$ ±1.74	10.0±2.23	24.2±3.83	38.8±3.03	54.2^{f} ±2.28	41.8±2.86	78.2±2.58	172.8±3.11	$280.2^{b,f}$ ±3.96	29.0±3.16	60.2±3.96	108.8±3.89	136.8^{f} ±2.86
Kn+GA (0.2mg/l+ 0.2mg/l)	4	100	27.0^{b} ±2.09	6.4±2.07	18.4±4.56	28.6±3.04	41.4^{g} ±2.70	40.8±3.19	62.8±3.34	162.8±3.64	$211.4^{b,g}$ ±3.84	30.6±3.36	77.4±4.61	119.0±3.16	120.2^{g} ±3.96
IAA+Kn+GA (10mg/l +0.2mg/l +0.2mg/l)	4	100	$33.8^{b,h}$ ±2.71	10.0±2.23	25.2±2.68	40.8±6.22	58.2^{h} ±2.38	54.8±3.12	99.0±4.06	249.0±3.52	$307.7^{b,h}$ ±2.21	55.6±3.84	83.0±2.16	132.6±3.36	160.6^{h} ±3.84

Values are means (n=5)±s.d.

b. Significantly different from control. c. Significantly different from IAA.
d. Significantly different from Kn. e. Significantly different from GA.
f. Significantly different from IAA+Kn. g. Significantly different from IAA+GA.
h. Significantly different from Kn+GA.

Significance tested by student's 't' test (p=0.05)

GA postponed the callussing either alone or in combinations compared to other PGRs, why?

Unlike IAA, IBA and NAA which induced differentiation of elangated tracheids having scalariform secondary wall thickening (Plate 5c.2), 2,4-D induced differentiation of short round to oval shaped pitted tracheid having reticulate secondary wall-thickenings (Plate 5c.3). All the auxins, however, induced differentiation of similar type of fibers (Plate 5c.4) and scleredids (Plate 5c.5).

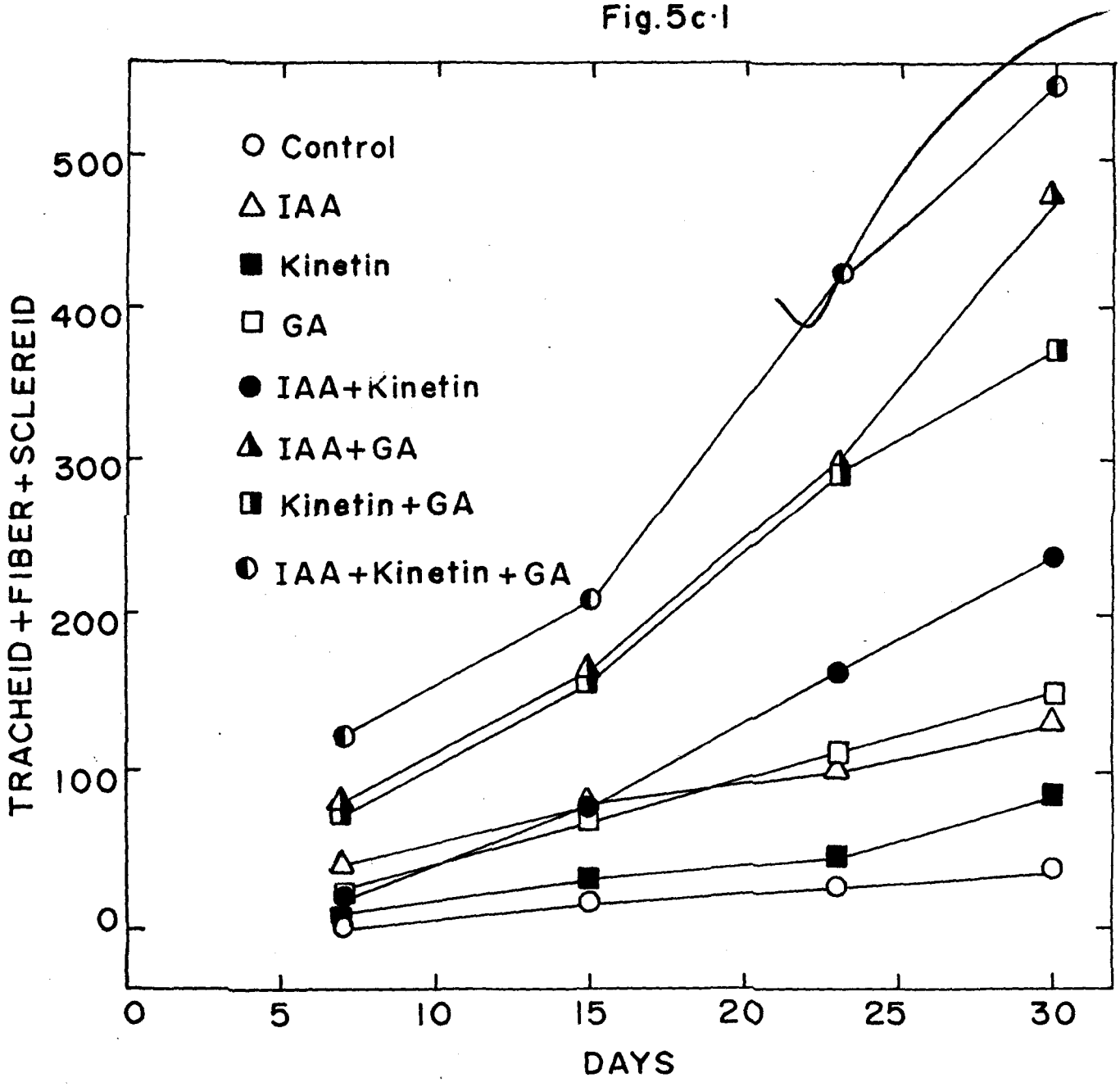
Effects of various growth hormones (IAA, Kn and GA) and their combinations

Juice vesicles cultured on MS medium having no hormone, developed callus in 7 days. Incorporation of growth hormone IAA (10 mg/l) and GA (0.2 mg/l) in the medium reduced callusing time to 5 and 4 days, respectively. But addition of Kn (0.2 mg/l) into the medium did not influence callusing of the juice vesicles (Table 5c.2). Amongst juice vesicles cultured on growth hormone supplemented media maximum growth of the callus was evident on GA supplemented medium while least growth occurred on Kn incorporated medium (Table-5c.2, Plate 5c.6). The callus growth in explants cultured on auxin supplemented medium was intermediate (Table-5c.2, Plate 5c.6).

Addition of both IAA (10 mg/l) and Kn (0.2 mg/l) in the medium improved the percentage of cultured juice vesicles forming callus and calli growth compared to the vesicles cultured on medium having only Kn (Table-5c.2, Plate 5c.6). The juice vesicles cultured on medium having combination of IAA (10 mg/l) and GA (0.2 mg/l) callused on the 5th day of inoculation (Table-5c.2).

Fig.5c.1 Effect of different growth hormones (IAA, Kn and GA) and their combinations on cytodifferentiation in **C. limon** juice vesicle culture.

Fig.5c·1


Control
IAA
Kinetin
GA
IAA+Kinetin
IAA+GA
Kinetin+GA
IAA+Kinetin+GA
TRACHEID+FIBER+SCLEREID
0
100
200
300
400
500
0
5
10
15
20
25
30
DAYS

The growth of calli was also very good on this medium since substantial increase in the fresh weight of the calli occurred at the end of the experiment (Table-5c.2). The juice vesicles grown on the Kn (0.2 mg/l) plus GA (0.2 mg/l) supplemented medium required 4 days to callus and the fresh weight of the calli measured 27 mg at the end of the experiment (Table-5c.2). Callusing of the juice vesicles cultured on medium having combination of IAA (10.0 mg/l), Kn (0.2 mg/l) and GA (0.2 mg/l) occurred on the 4th day of inoculation and the calli produced on this medium had maximum fresh weight at the end of experiment (Table-5c.2).

The juice vesicles cultured on the control medium (devoid of hormones) exhibited delayed cytodifferentiation compared to the vesicles cultured on hormone supplemented medium (Table-5c.2). Further the number of tracheary element was also less compared to their number observed in calli formed on hormone supplemented media (Table-5c.2, Fig.5c.1). In control calli only fibers differentiated (Plate 5c.1). Addition of hormones (IAA, Kn and GA) to the medium greatly enhanced differentiation and induced interesting pattern of differentiation (Table-5c.2, Fig.5c.1). Amongst the calli developed on medium supplemented with any of the three growth hormones (IAA/GA/Kn) maximum cytodifferentiation occurred in calli formed by juice vesicles cultured on GA supplemented medium while minimum cytodifferentiation occurred in the calli developed on Kn supplemented medium (Table-5c.2, Fig. 5c.1). The calli formed by the vesicles cultured on IAA supplemented medium were intermediate in their response (Table-5c.2, Fig.5c.1). The calli which developed

on media having either IAA or Kn revealed tracheid, sclereids and fibers in 1:2:3 ratio (Table-5c.2). But in the calli formed on medium having GA although the number of tracheid and sclereids differentiated was almost equal, the number of differentiated fibers was three fold higher than sclereids and tracheid (Table-5c.2). All the three growth hormones used induced differentiation of fibers (Plate 5c.4), tracheid having scalariform secondary wall thickenings (Plate 5c.2) and sclereids (Plate 5c.5,7). Stony cells were also observed in the calli developed by juice vesicles cultured on GA supplemented medium (Plate 5c.8).

Compared to single hormone, incorporation of two or more hormones in the medium improved cytodifferentiation. Amongst the combination of IAA+Kn; IAA+GA and; GA+Kn the best differentiation of tracheid, fibers and sclereids was evident in calli developed by juice vesicles cultured on the medium having combination of IAA+GA (Table-5c.2, Fig. 5c.1). The frequency of cytodifferentiation was least in the calli developed by vesicles cultured on the medium having both IAA+Kn (Table-5c.2, Fig.5c.1). The tracheid differentiated in calli cultured on the medium having Kn+GA were small of various shapes and reticulately pitted (Plate 5c.9-10). However, elongated tracheids with reticulate thickenings also differentiated (Plate 5c.11). IAA+Kn treated explants while differentiated tracheid only with scalariform secondary wall thickening (Plate 5c.2), tracheid with spiral (Plate 5c.12) and scalariform secondary wall thickenings differentiated (Plate 5c.2) in explants given IAA+GA treatment.

Plate 5c (continued)

12. Tracheids exhibiting helical secondary wall thickenings. X 310

13. Macro sclereids. X 300

14. Elongated sclereids. X 380

15. Effect of ethylene on the development of callus in **C. limon** juice veiscle cultures:

    A - Control (normal MS); B - Methionine; C - $CoCl_2$

16. Development of callus in **C. limon** juice vesicle cultures on ABA supplemented medium (in absence of IAA and Kn):

    A - Control; B - 0.001%; C - 0.005%; D - 0.01% and E - 0.05% ABA.

17. Development of callus in **C. limon** juice vesicle cultures on ABA supplemented medium (in presence of IAA and Kn):

    A - Control; B - 0.001%; C - 0.005%; D - 0.01% and E - 0.05% ABA.

18. Development of callus in **C. limon** juice vesicles cultures on MS medium having colchicine:

    A - Control (no colchicine); B - 0.005%; C - 0.01%; D - 0.05% and E - 0.1% colchicine.

19. Fibers. X 190

20. Tracheids with ill-developed secondary wall thickenings. X 300

21. Colchicine induced fibers. X 240

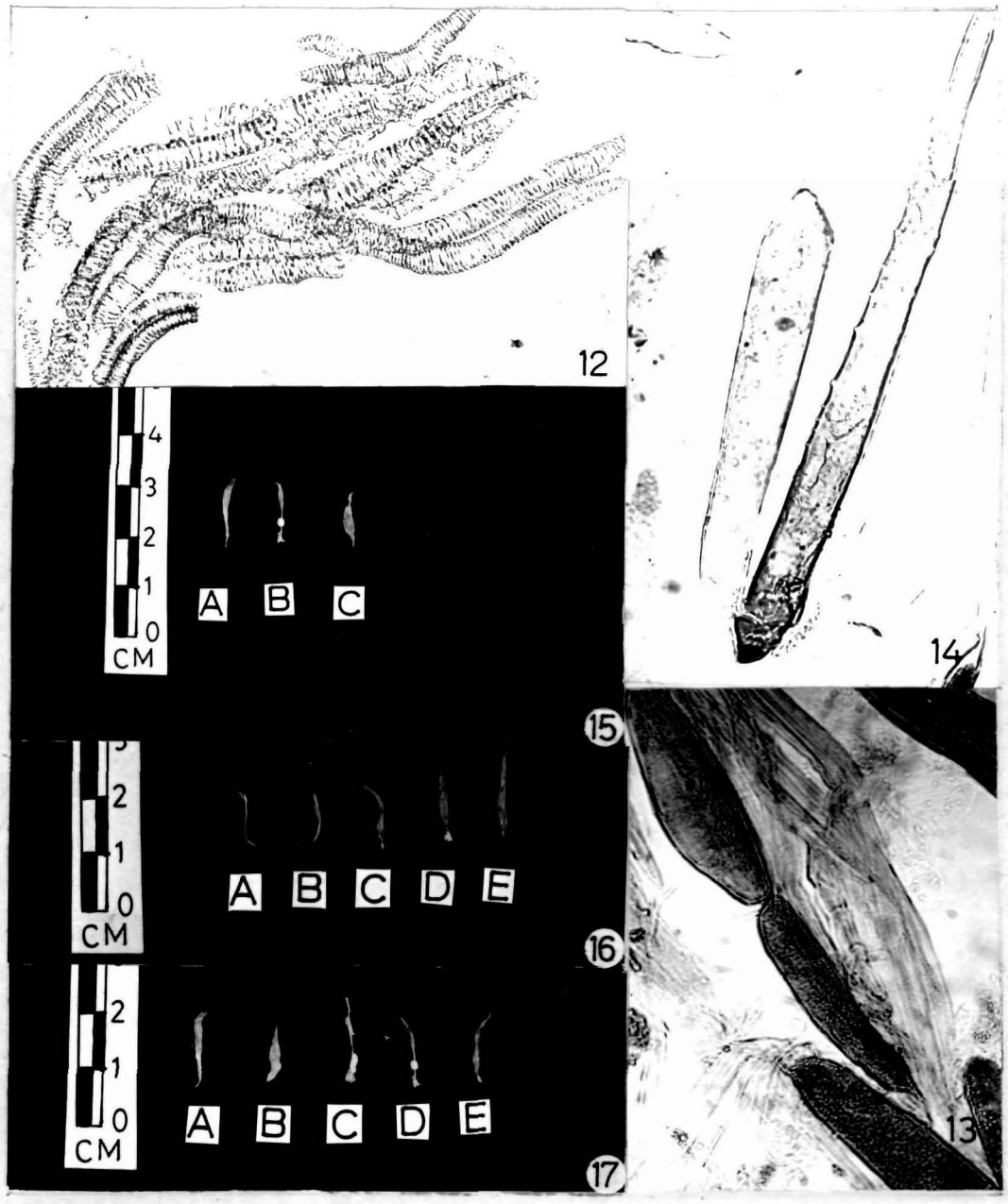


PLATE 5c (Contd..)

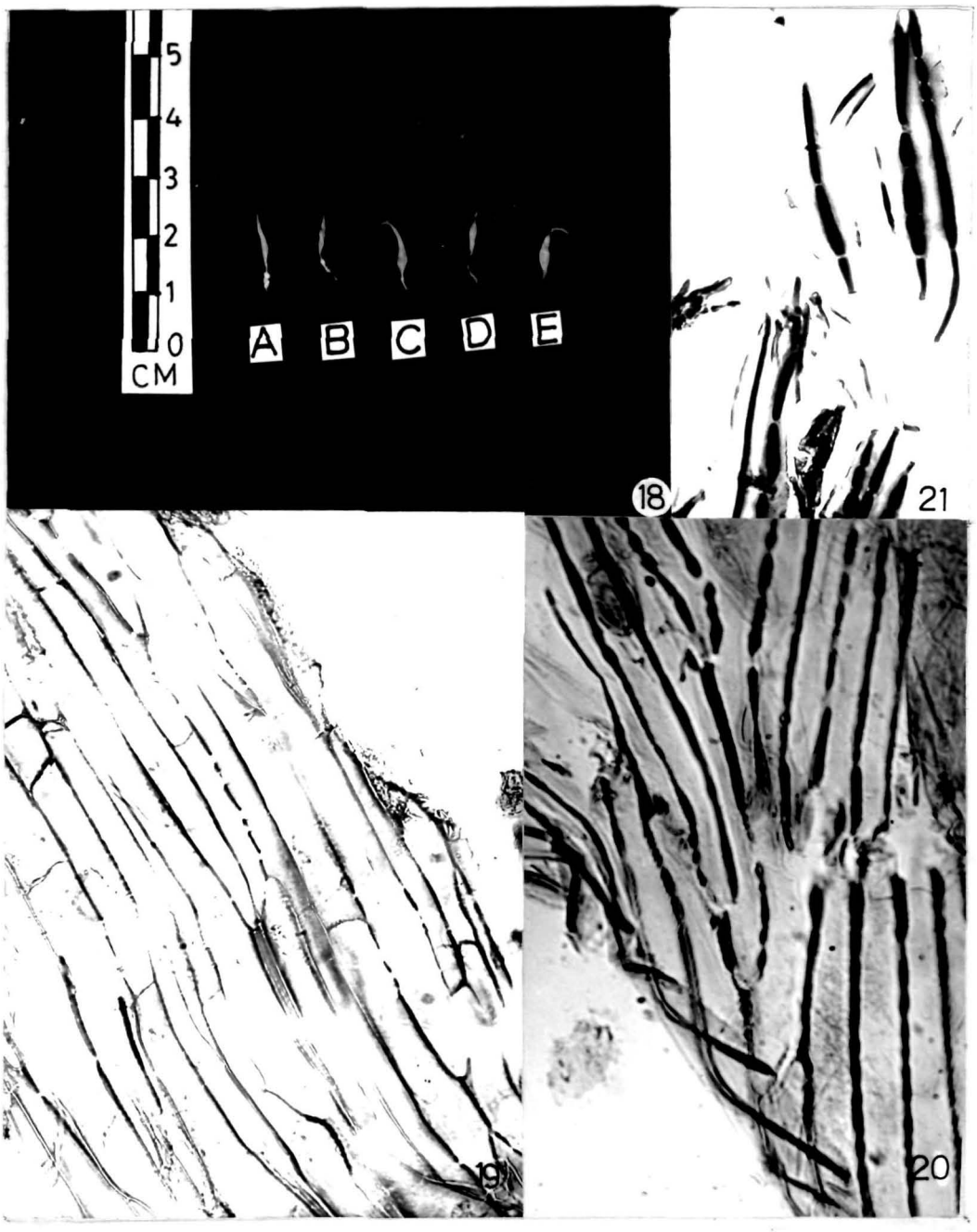


PLATE 5c (Contd...)

Differentiation of fibers (Plate 5c.1) and sclereids (Plate 5c.5) was induced by all the treatments (IAA+Kn; Kn+GA and IAA+GA) but diversity of sclereids was more in the explants cultured on Kn+GA supplemented medium. In these explants two more types of macro-sclereids differentiated (Plate 5c.13-14). The incorporation of IAA+GA+Kn in the medium induced best cytodifferentiation of tracheid, fibers and sclereids (Table-5c.2; Fig.5c.1). The fibers, tracheid and sclereids differentiated in 6:1:3 ratio. The tracheids exhibited reticulate (Plate 5c.11), scalariform (Plate 5c.2) and spiral (Plate 5c.12) thickenings. Different types of sclereids also differentiated in the presence of all three hormones (Plate 5c.7,13-14).

Effect of ethylene

Effect of plant hormone C_2H_4 was studied by incorporating methionine in the MS basal medium. Juice vesicles cultured on basal medium callused 7 days after culturing while callusing of juice vesicles cultured on methionine (3.5 µg/l) supplemented medium occurred on the 3rd day of culturing (Table-5c.3). The calli developed on methionine supplemented medium had higher fresh weight compared to the control calli (Table-5c.3, Plate 5c.15) No callusing could be observed when $CoCl_2$ (1 mM) was added to the medium (Plate 5c.15).

Juice vesicles cultured on control medium differentiated fibers (Plate 5c.1), tracheid with scalariform thickening (Plate 5c.2) and sclereids (Plate 5c.5) in a lesser number. On the other hand in methionine incorporated medium, the cytodifferen-

Table 5c.3: Effect of ethylene on cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	7	100	7.4 ±1.01	0	4.6±1.67	8.8±1.78	10.0 ±2.23	0	11.6±2.40	19.6±2.70	20.2 ±3.04	0	0	5.0±1.01	12.3 ±2.21
Methionine (3.5 mg/l)	3	100	12.2[b]±1.72	2.2±1.48	10.8±3.19	24.0±2.73	29.8[b]±2.38	9.2±2.58	49.0±3.16	51.6±3.84	79.6[b]±2.40	55.8±4.15	106.2±5.54	201.6±6.26	204.8[b]±9.33
$CoCl_2$(1mM)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

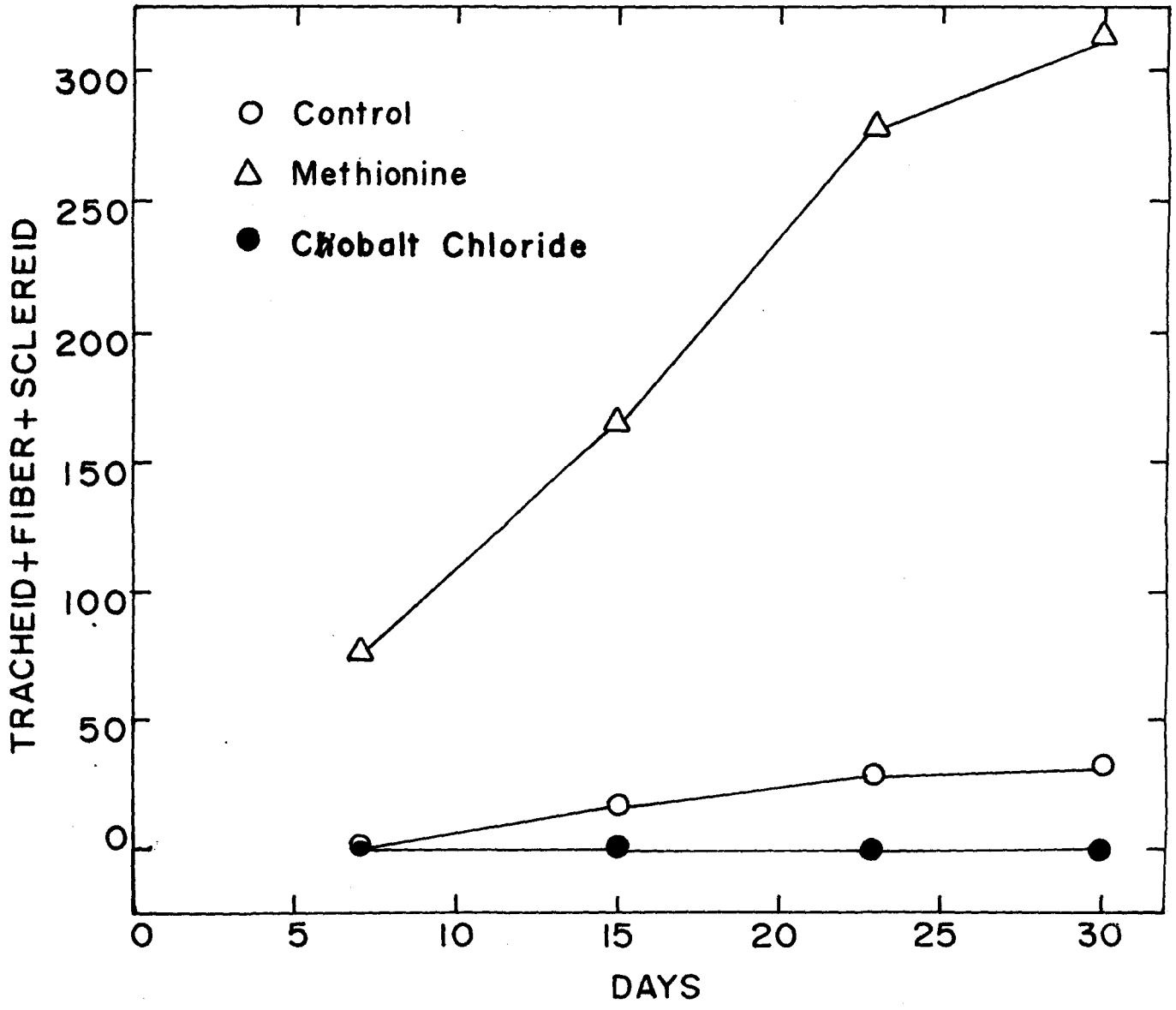
Values are means (n=5) ± s.d.

b. Significantly different from control

Significance tested by student's 't' test (p=0.05).

Fig.5c.2 Effect of ethylene on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 5c·2


TRACHEID+FIBER+SCLEREID
300
250
200
150
100
50
0
Control
Methionine
Cobalt Chloride
0
5
10
15
20
25
30
DAYS

tiation started earlier and differentiation of tracheid, fibers and sclereids was excellent compared to control (Table 5c.3, Fig. 5c.2). Methionine also induced differentiation of tracheids with reticulate secondary wall thickenings (Plate 5c.11), fibers (Plate 5c.1) and sclereids (Plate 5c.5). Frequency of occurrence of fibers was double the number of differentiating tracheid but number of sclereids was almost ten fold higher than that of tracheid (Table 5c.3). No cytodifferentiation was evident in explants grown on $CoCl_2$ supplemented medium (Table-5c.3, Fig. 5c.2).

Effects of ABA

Effects of abscisic acid on cytodifferentiation were investigated both in the presence and absence of growth hormones IAA and Kn. The details are as follows:

In this experiment juice vesicles cultured on MS basal medium devoid of hormones and having no ABA served as control. The control explants developed callus within 4 days of inoculation (Table 5c.4). Addition of 0.001% ABA to the above basal medium did not influence the lag period involved in the development of callus although higher concentrations of ABA delayed callusing (Table-5c.4). The degree of delay increased with the increasing concentration of ABA (Table-5c.4). Callusing was evident in the 68% control explants (Table-5c.4). ABA concentrations marginally improved the incidence of callusing in treated explants. Maximum frequency of callusing was evident

Table 5c.4: Effect of ABA on cytodifferentiation in *C. limon* juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control (MS basal medium devoid of hormones)	4	68	2.0 ±1.8	0	0	0	0	9.0±3.16	14.4±2.96	20.0±3.39	31.2 ±3.03	0	0	0	0
Abscisic acid (%) 0.001	4	70	3.2 ±0.74	0	0	0	0	6.0±2.0	14.0±3.16	30.0±2.23	47.8^{b} ±1.92	4.6±1.94	13.2±2.86	30.8±3.19	57.4 ±3.20
0.005	5	70	5.2^{b}±0.74	0	0	0	0	11.8±2.16	24.8±3.49	42.4±3.36	$60.6^{b,c}$±2.40	0	6.8±2.38	28.8±3.96	50.0^{c}±4.63
0.01	7	81	6.6^{b}±1.01	0	0	0	0	7.4±2.6	19.4±2.70	29.2±6.31	$74.6^{b,d}$±4.56	0	0	12.8±3.34	37.4^{d}±3.84
0.05	7	73	6.0^{b}±1.41	0	0	0	0	4.6±1.94	13.4±2.60	30.8±3.19	$41.8^{b,e}$±4.56	0	0	0	7.8^{e}±2.58

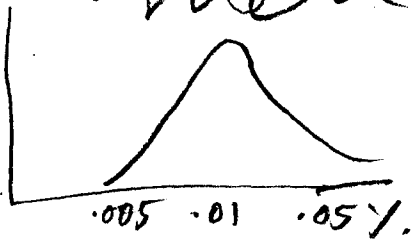
Values are means (n=5) ± s.d.

b. Significantly different from control.
c. Significantly different from 0.001%.
d. Significantly different from 0.005%.
e. Significantly different from 0.01%.

Significance tested by student's 't' test (p=0.05).

What may be the cause of such behaviour?

Callus



in explants grown on medium having 0.01% ABA (Table-5c.4). The growth of callus was also better than control even at higher concentrations of ABA (Table-5c.4, Plate 5c.16).

Differentiation of fibers only occurred in control explants, 7 days after culturing (Plate 5c.1). Control explants did not differentiate tracheid and sclereids (Table-5c.4). Incorporation of ABA in the medium, although did not induce differentiation of tracheid it induced differentiation of fibers and sclereids (Table-5c.4; Plate 5c.1). Maximum number of sclereids differentiated at 0.001% ABA (Table-5c.4). The differentiation of sclereids decreased with the increasing concentration of ABA (Table-5c.4). Differentiation of fibers was, however, stimulated by increasing concentration of ABA upto 0.01% and thereafter it diminished (Table-5c.4).

Interaction of ABA with IAA and Kn was investigated by incorporating ABA concentrations in MS basal medium having IAA (10 mg/l) and Kn (0.2 mg/l). The control explants were cultured on MS basal medium without incorporating ABA. The control explants developed callus on the 4th day of inoculation (Table-5c.5). Incorporation of lower concentrations of ABA (upto 0.005%) in the basal medium reduced callusing time to 3 days while higher concentrations of ABA (0.01-0.5%) delayed callusing (Table-5c.5). Further while frequency of explants developing callus did not differ from control at lower concentrations of ABA it was reduced at higher concentrations of ABA (Table-5c.5). The callus growth was improved over control by 0.001-0.01% concentrations of ABA (Table-5c.5, Plate 5c.17).

Table 5c.5: Effect of ABA on cytodifferentiation in C. limon juice vesicles in presence of IAA (10mg/l) and kinetin (0.2mg/l).

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control (MS Basal medium having IAA (10mg/l) and Kn (0.2mg/l)	4	100	16.8 ±1.32	0	4.2±1.92	8.4±1.67	13.4 ±2.70	4.6±1.94	13.2±2.86	24.2±2.70	56.8 ±2.86	5.2±3.03	11.0±3.08	26.6±2.70	52.8 ±3.34
Abscisic acid (%) 0.001	3	100	18.0 ±2.82	0	5.4±2.50	8.4±4.08	19.5^{b} ±3.20	7.0±1.87	16.0±3.74	45.2±3.11	70.0^{b} ±2.23	20.9±6.80	43.2±2.86	71.6±4.15	96.2^{b} ±6.72
0.005	3	100	25.4^{b} ±2.05	3.4±1.67	7.8±2.28	13.0±3.16	20.6^{b} ±2.88	13.4±2.60	23.2±2.86	41.6±2.70	72.2^{b} ±2.58	24.6±4.15	53.0±3.53	94.6±5.92	$127.4^{b,c}$ ±5.54
0.01	5	73	18.4 ±1.01	0	0	4.75±1.5	14.0^{d} ±2.23	6.8±1.78	13.8±3.70	29.0±2.64	$49.6^{b,d}$ ±3.28	7.4±1.81	18.4±3.20	45.0±3.53	$70.6^{b,d}$ ±3.36
0.05	6	70	10.8^{b} ±1.32	0	0	0	$4.2^{b,e}$ ±2.77	8.6±3.28	13.4±2.70	23.2±2.86	$42.0^{b,e}$ ±4.64	6.4±1.81	12.6±2.70	18.8±5.54	$30.0^{b,e}$ ±2.23

Values are means (n=5) ± s.d.

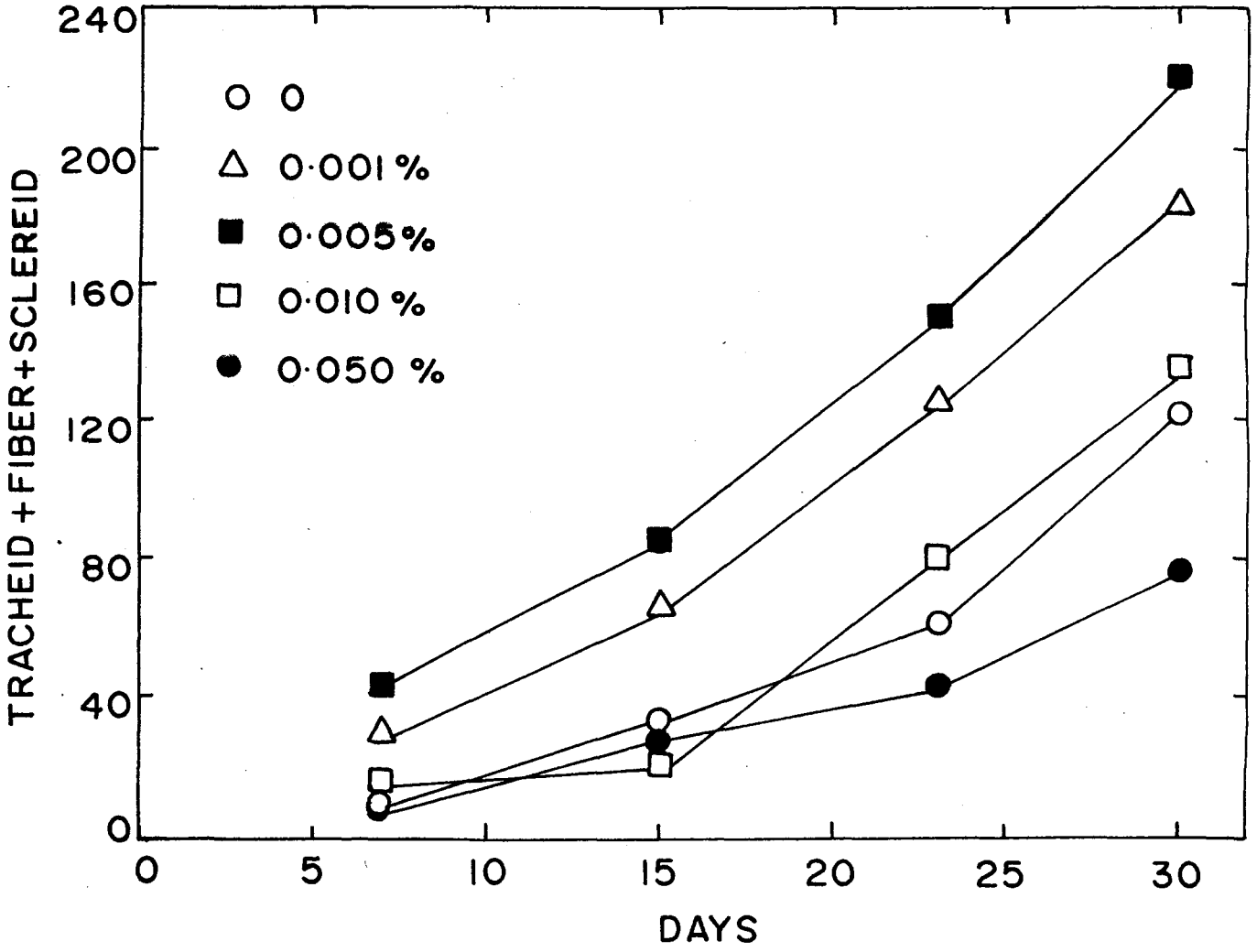
b. Significantly different from control.
c. Significantly different from 0.001%.
d. Significantly different from 0.005%.
e. Significantly different from 0.01%.

Significance tested by student's 't' test (p=0.05).

Both are similar, how to explain it?

Fig.5c.3 Effect of ABA on cytodifferentiation in **C. limon** juice vesicles in presence of IAA (10 mg/l) and Kn (0.2 mg/l).

Fig.5c·3


240
200
160
120
80
40
0
TRACHEID + FIBER + SCLEREID
0
0·001 %
0·005 %
0·010 %
0·050 %
0
5
10
15
20
25
30
DAYS

But it was adversely affected by 0.05% ABA (Table-5c.5, Plate 5c.17).

The control explants revealed differentiation of fibers (Plate 5c.1), tracheid with scalariform thickening (Plate 5c.2) and sclereids (Plate 5c.5). The differentiation of tracheid, fibers and sclereids was improved over control by 0.001-0.005% concentration of ABA (Table-5c.5, Fig.5c.3). The highest concentration of ABA (0.05%), used in the present investigation, inhibited cytodifferentiation (Table-5c.5, Fig. 5c.3). Tracheid, fibers and sclereids which differentiated in ABA treated explants were, however, similar to the control (Plate 5c.1,2 and 5).

Effect of colchicine

Juice vesicles grown on MS basal medium having IAA (10 mg/l) and Kn (0.2 mg/l) served as control. Callusing was, however, delayed in explants grown on colchicine supplemented medium and the delay increased with the increasing concentration of colchicine (Table-5c.6). The frequency of explants developing callus decreased with the increasing concentration of colchicine, so much so that no explants treated with 0.5% colchicine developed callus (Table-5c.6). A similar trend was evident in the growth of the calli as well (Table-5c.6, Plate 5c.18).

Like callusing, cytodifferentiation was relatively faster in the control explants. Differentiation of fibers started on the third day while tracheid (Scalariform thickening) and

Table 5c.6: Effects of colchicine on the cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	3	95	18.0 ±2.82	5.2±3.03	13.2±2.28	23.0±2.73	29.4 ±2.30	10.6±1.94	19.2±2.86	45.4±3.50	101.4 ±6.10	20.8±2.77	42.8±3.34	73.0±1.58	96.2 ±6.72
Colchicine (%) 0.005	4	81	14.8 ±2.03	4.2±1.48	14.4±2.40	20.2±1.92	21.0[b] ±2.44	9.0±3.16	21.0±2.44	44.0±2.64	75.4[b] ±3.20	8.2±2.6	16.0±2.0	24.0±3.87	41.4[b] ±2.70
0.01	4	65	7.4[b,c]±1.01	3.8±3.03	8.2±2.38	11.4±2.70	15.2[b,c]±2.12	7.8±1.92	16.8±2.86	33.0±2.91	60.4[b,c]±3.04	0	6.2±2.86	7.4±2.07	20.2[b,c]±1.92
0.05	5	30	5.4[b,d]±1.01	0	2.0±1.94	7.2±1.92	11.6[b,d]±2.40	5.8±1.78	15.2±3.07	35.2±3.42	41.4[b,d]±2.70	0	5.6±1.67	11.4±2.28	15.2[b,d]±3.07
0.1	6	21	3.8[b,e]±0.74	0	0	2.0±1.41	4.8[b,e]±1.64	0	6.2±2.86	17.6±2.96	23.2[b,e]±2.73	0	0	4.8±1.30	7.4[b,e]±1.81
0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Values are means (n=5) ± s.d.

b. Significantly different from control.
c. Significantly different from 0.005%.
d. Significantly different from 0.01%.
e. Significantly different from 0.05%.

Significance tested by student's 't' test (p=0.05).

sclereids differentiated on the 7th day of inoculation in control explants. Lower concentrations of colchicine (0.005 and 0.01%) induced late cytodifferentiation: fibers on the 5th day, tracheid on the 6th day and sclereids on the 7th day in explants treated with 0.005% colchicine while these differentiated on the 12th day in explants given 0.01% colchicine. Incorporation of higher concentrations of colchicine (0.05 and 0.1%) also delayed cytodifferentiation (Table-5c.6). Highest concentration of colchicine (0.5%) completely inhibited cytodifferentiation in cultured Assam lemon juice vesicles (Table-5c.6, Fig.5c.4).

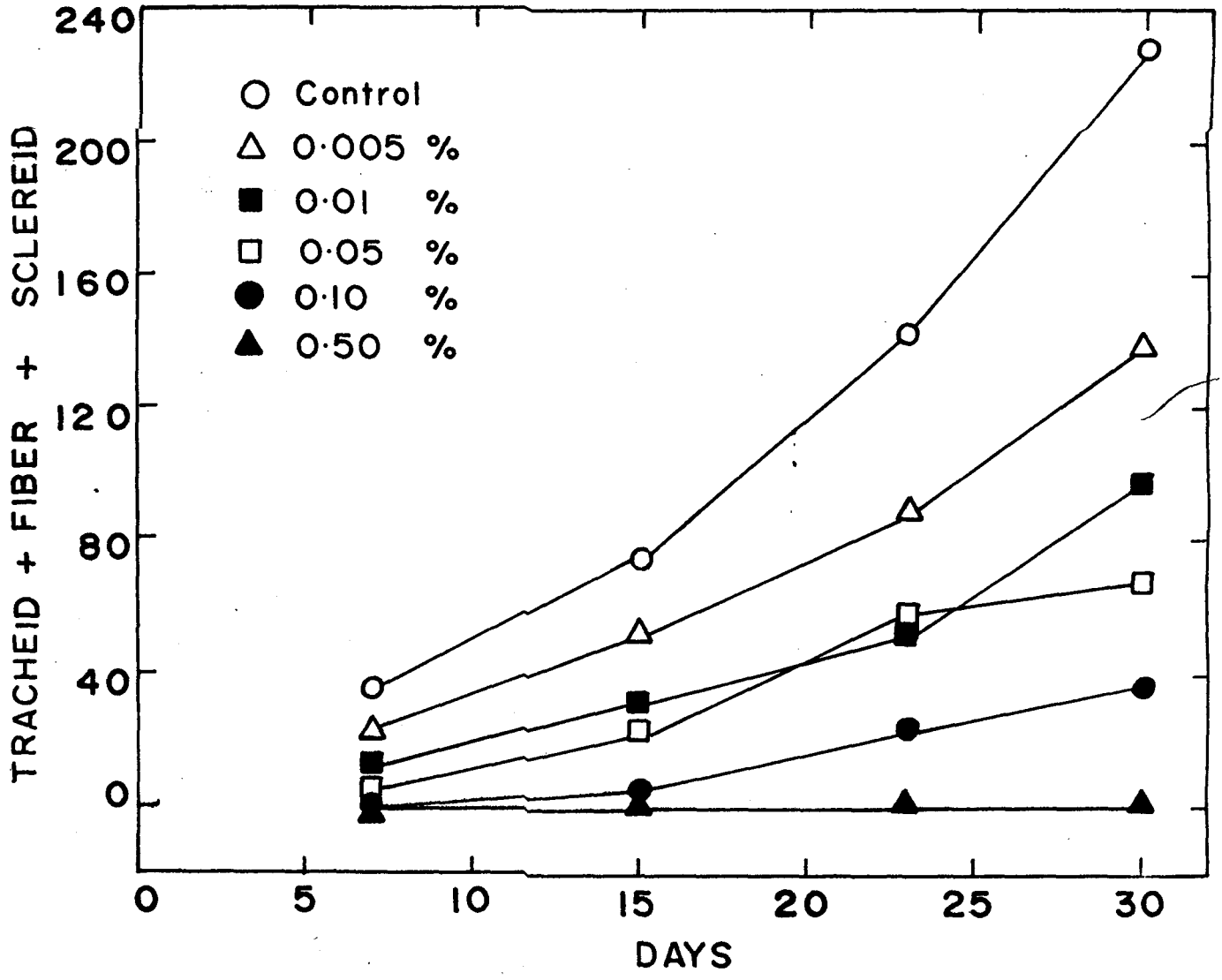
In the control explants the fibers were elongated with moderate thick pitted walls (Plate 5c.19). On the other hand, short fibers having very thick walls with large number of pits were detected in colchicine (0.05 and 0.1%) treated vesicles (Plate 5c.20). Tracheids differentiated in the control explants had scalariform secondary wall thickening (Plate-5c.2) as against the tracheids formed in presence of colchicine (0.05 and 0.1%) which exhibited incomplete secondary wall thickenings (Plate 5c.21). These thickenings were incomplete and stained heavily with safranin 'O' near the cell wall of the tracheid (Plate 5c.21).

Discussion

A variety of plant growth regulators have been implicated in the control of TE differentiation (Roberts, 1969, 1976; Torrey, 1975; Dalessandro, 1973a; Barnett, 1979). Differentiation occurs

Fig.5c.4 Effect of colchicine on the cytodifferentiation in **C. limon** juice vesicle culture.

Fig.5c·4


240
200
160
120
80
40
0
TRACHEID + FIBER + SCLEREID
Control
0·005 %
0·01 %
0·05 %
0·10 %
0·50 %
0
5
10
15
20
25
30
DAYS

only in the cells which undergo mitotic cycle in the presence of a specific hormonal milieu (Fosket, 1968; Fosket and Torrey, 1969). Auxin produced by leaf is considered a morphogenetic factor in the differentiation of xylary elements in leaf traces (Jacobs and Morrow, 1957; Sachs, 1968, 1978; DeGrocte and Larson, 1984). In the present investigation also auxin improved callusing and cytodifferentiation in the cultured juice vesicles suggesting a role for auxin as a need of threshold level of auxin for better cytodifferentiation. Similar were the conclusions of Aloni (1980). In the present study even in control explants some cytodifferentiation did occur which may be due to the presence of some growth substances in the Assam lemon fruits. Occurrence of endogenous growth substances is reported in the young fruits of seeded and seedless clementine mandarin (Garcia-Papi, and Garcia-Martinez, 1984).

In the present investigation different auxins differed in their effectiveness in inducing cytodifferentiation in cultured Assam lemon juice vesicles. Whereas IAA was most effective in inducing TE differentiation, IBA was least effective; 2,4-D and NAA were intermediate. The range of concentration for inducing cytodifferentiation also differed with the auxin used. The present findings thus corroborate conclusions that different auxins have differential effectiveness for inducing cytodifferentiation and that this is dependent on the species being used for experimentation (Dalessandro and Roberts, 1971; Dalessandro, 1973,a,b; Minocha and Halperin, 1974; Phillips and Dodds, 1977).

Further, in the present investigation while best callus growth was evident in 2,4-D treated juice vesicles the best cytodifferentiation was evident in IAA treated explants. Thus the auxin requirement for optimal callus growth and TE differentiation may not be same. The present study also revealed that combinations of any two or more hormones always induced better cytodifferentiation than when the hormone was used individually. In other words when combinations of two or three hormones were used a synergism occurred. Synergistic effect of auxin and cytokinin (Sorokin et al., 1962; Minocha and Halperin, 1974; Dalessandro, 1973a,b; Haddon and Northcote, 1975) auxin and GA (Wareing, 1958; Netien, 1957; Roberts and Fosket, 1966) and auxin, cytokinin and GA (Dalessandro, 1973b) are reported in literature. In the present study the combinations of the three growth hormones IAA, Kn and GA had most effective synergistic effect on cytodifferentiation.

Roberts (1971) reported that in lettuce pith explants various auxins when used in combination with some cytokinin induced diverse type of xylem differentiation. In the present study, however, tracheid, fibers and sclereids differentiated when IAA was used in combination with Kn and/or GA. Thus both Kn and GA can induce differentiation of all the three types of elements, mentioned above, in Assam lemon juice vesicles.

Recent studies have suggested possible involvement of ethylene in cytodifferentiation (Abeles and Abeles, 1972). Roberts (1976) suggested that ethylene is a hidden hormone in

xylem differentiation. In the present investigation effects of ethylene on differentiation of tracheid, fibers and sclereids were studied using methionine, an ethylene precursor, and $CoCl_2$, an ethylene inhibitor. The study revealed that cytodifferentiation was improved by methionine, while $CoCl_2$ inhibited both callusing and cytodifferentiation. The present findings thus further support the conclusions that ethylene is involved in the process of cytodifferentiation of xylem.

Abscisic acid, a growth inhibiting plant hormone, is known to induce inhibition of TE differentiation (Minocha and Halperin, 1974; Minocha, 1984; Haddon and Northcote, 1976). In the present investigation, however, lower concentrations of ABA improved cytodifferentiation while higher concentrations inhibited the process. Further, ABA induced effects on cytodifferentiation were more pronounced in presence of growth hormones IAA and Kn than in their absence.

Cell division is considered an essential pre-requisite for cytodifferentiation (Shininger, 1975; Malawer and Phillips, 1979). Drug colchicine is a mitotic inhibitor and mitotic inhibitors have been used to elucidate the role of cell division in TE differentiation (Dodds and Phillips, 1977). In the present study colchicine concentrations inhibited callus growth and cytodifferentiation. The degree of inhibition to both the processes increased with the increasing concentration to the extent that at the highest concentration neither callusing occurred nor any differentiation was evident. Callusing is dependent on the

initiation of cell divisions in the cultured explants. Since no callusing occurred in the explants cultured on medium incorporated with 0.5% colchicine it can be assumed that this concentration of colchicine completely inhibits cell division in these explants. Incidentally, no cytodifferentiation was also evident in these explants. Thus it may be concluded that cell division is a pre-requisite for cytodifferentiation of tracheid, fibers and sclereids in Assam lemon juice vesicles. Similar were the findings of Fosket (1968), Dodds and Phillips (1977) and Malawer and Phillips (1979). Abnormal differentiation of xylem in colchicine treated explants (Roberts and Baba, 1968) could be due to the effect of colchicine on microtubules and microfibril orientation (Taylor, 1965; Falconer and Seagull, 1985). In the present study abnormal type of fibers differentiated, at the higher concentrations of colchicine (0.05 and 0.1%), which were short thick walled and had a large number of pits. Hepler and Fosket (1971) also observed that the pitting pattern of xylem elements of Zea was altered by colchicine treatments (Stein, Rowly and Lockhart, 1971). Trachied differentiated in colchicine treated explants had a unique secondary wall pattern i.e., instead of sharply defined scalariform thickening of secondary wall, the thickenings were incomplete and present only adjacent to the cell walls. The data of this experiment therefore supports the findings of Pickett-Heaps (1967), Roberts and Baba (1968) and Hepler and Fosket (1971) who also found that colchicine influences development of secondary wall thickenings in TE.

CHAPTER V

(d) ORGANIC ACIDS AND FRUIT JUICE

Introduction

Growth is perhaps the most basic of biological phenomenon. Factors encouraging callus growth **in vitro** and differentiation of xylem elements are all fundamental, and the basic nutrition is obviously important, including mineral salts, vitamins, hormones and sources of carbon or nitrogen. For vigorous growth, orange (**Citrus sinesis**) tissue cultures require a supply of orange juice in the medium (Erner **et al.**, 1975). But the requirement of orange juice in the medium can be overcome by incorporating pure citric acid in the medium. The growth of subcultured citrus albedo explants, from species other than **Citrus limon**, is dependent on the supply of orange juice in the medium (Murashige & Tucker, 1969). This requirement of orange juice can not be replaced by IAA, 2,4-D, GA_3 or Kn. Thus it may be assumed that orange juice contains a special unknown growth factor which cannot be synthesized by the subcultured callus of citrus albedo (Murashige & Tucker, 1969). Therefore, experiments with the acids of Kreb's cycle are of special interest because **Citrus** juice contain citric acid and other organic acids. Succinic, maleic and malonic acid although do not inhibit tissue growth, citric acid does (Kordan, 1965). It is suggested that the stimulation of the growth of albedo tissue culture of **Citrus sinensis** could be due to citric acid components of the juice (Erner **et al.**, 1975). But Einset (1978) considers that organic constituents other than citric acid may be involved in the growth stimulation of **Citrus** cultures. **In vitro** growth of the juice vesicles of **Citrus hassaku** is inhibited by incorporation of citric/maleic

acid in the medium (Kato, 1980). Kulshrestha **et al.** (1982) reported that in **Citrus limon** juice vesicle cultures, citric acid has xylogenic response similar to auxin. Citric acid stimulates **in vitro** growth of marigold and sunflower tissues (Hildebrandt **et al.**, 1954). Gamborg and Shyluk (1970) found that citric acid was the most effective acid among Kreb's cycle acids, which interact with ammonia, to stimulate growth of soyabean cell suspension culture.

The present investigation deals with the effects of 1) different organic acids including citric acid and (2) juice of different **Citrus** species on **in vitro** growth and xylogenesis in **C. limon** juice vesicles.

Materials and Methods

The preparation and culture procedures for the Assam lemon juice veiscles are described in Chapter III. Explants were inoculated in culture tubes having 25 ml of MS basal medium. Different organic acids, i.e. citric, maleic, pyruvic and α-keto-glutaric acids were added (2 gm/l) individually to two sets of MS medium, differing in the presence and absence of plant hormones IAA (10 mg/l) and Kn (0.2 mg/l). For examining the effects of the juice of different **Citrus** species, the juice was squeezed from peeled **Citrus** fruits of orange (**Citrus reticulata**), Assam lemon (**Citrus limon**), pumello (**Citrus grandis**), Soh-jhalia (**Citrus jambhiri**) and Kagzi (**Citrus aurantifolia**) separately. The extracted juice was filtered through cloth and added separately (10% v/v) to the MS medium differing in their hormonal

Table 5d.1: Effects of various exogenous organic acid in absence of IAA and Kn on cytodifferentiation in c. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	8	20	2.0 ±0.83	0	0	0	0	0	4.2±2.1	11.4±2.76	15.2 ±2.82	0	0	0	0
Maleic acid	8	35	5.0[b] ±1.41	0	0	5.0±2.23	11.6 ±2.40	0	8.2±2.77	18.4±5.72	22.0[b] ±6.04	0	9.2±3.19	27.2±5.26	34.4 ±4.82
α-keto-glutaric acid	7	41	7.2[b,c]±1.16	0	7.0±3.16	7.2±2.86	13.2 ±6.18	0	8.8±3.03	27.8±5.49	34.2[b,c]±5.80	0	15.2±3.76	37.2±3.34	56.4[c]±5.22
Pyruvic acid	12	24	2.0[d]±0.63	0	0	0	8.8 ±3.03	0	5.0±3.80	12.5±4.27	17.6[d] ±5.72	0	0	0	10.8[d]±1.78
Citric acid	18	34	3.2[b,e]±0.74	0	0	1.4±1.34	15.4[e]±4.27	0	0	10.0±4.12	26.6[b,e]±5.22	0	0	9.2±3.19	18.4[e]±5.72

Values are means (n=5) ± s.d.

b. Significantly different from control.
c. Significantly different from maleic acid.
d. Significantly different from α-ketoglutaric acid.
e. Significantly different from Pyruvic acid.

Significance tested by student's 't' test (p=0.05).

composition mentioned above. As usual the pH of the medium was adjusted to 5.7. Twenty culture tubes were maintained per treatment and usual procedures of tissue culture techniques described in Chapter III were followed. Cultures were incubated in dark at 25±1°C in a B.O.D. incubator. Samples were collected at weekly intervals and fixed in F.A.A. The fixed juice vesicles were cleared stained and stored for microscopic observation as in Chapter III.

Results

Callusing occurred on the 8th day of inoculation in the explants cultured on MS medium devoid of hormones and organic acids (Table-5d.1). Explants cultured on media supplemented with any of the organic acids(citric, maleic, α-ketoglutaric and pyruvic) at 2 gm/l concentration callused between 7-18 days of culturing. The earliest callusing was evident in explants cultured on medium having α-ketoglutaric acid while explants cultured on medium having citric acid callused last (Table-5d.1).

The percentage of explants developing callus and growth of the callus was highest on medium having α-ketoglutaric acid but it was least in explants grown on control medium (Table-5d.1, Plate 5d.1).

Cytodifferentiation started simultaneously in explants cultured on media supplemented with either α-ketoglutaric acid/ maleic acid/or critic acid and it occurred last in the explants cultured on medium having pyruvic acid (Table-5d.1). Compared

Plate 5d

1. Development of callus on organic acids supplemented MS medium (in absence of IAA and Kn) in *C. limon* juice vesicle cultures:

   A - Control; B - Maleic acid; C - α-ketoglutaric acid; D - Citric acid and E - Pyruvic acid.

2. Fibers. X 310

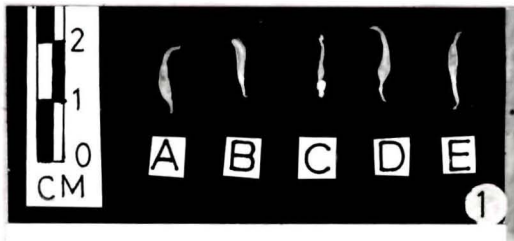
3. Tracheid having scalariform secondary wall thickenings. X 470

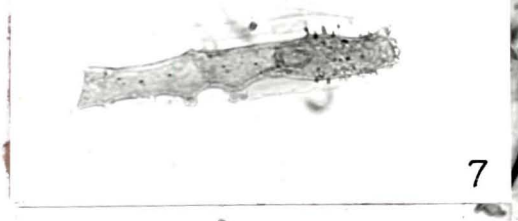
4. Micro-sclereids. X 470

5. Macro-sclereids. X 380

6. Elongated sclereids. X 380

7. Elongated sclereids. X 310





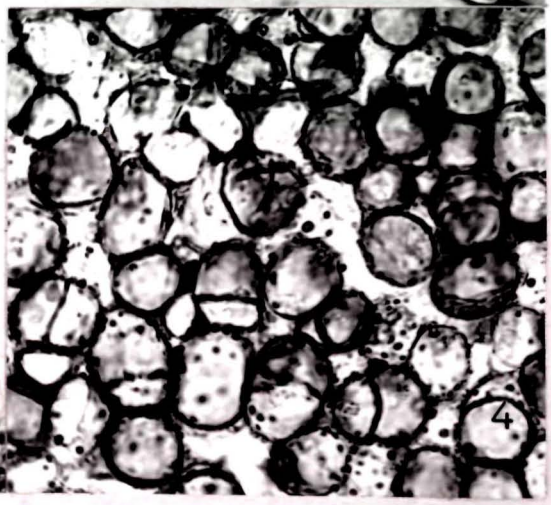


PLATE 5d

to control explants better cytodifferentiation occurred in organic acid treated explants (Table-5d.1, Fig.5d.1). In juice vesicles cultured on organic acid supplemented medium, maximum number of tracheid, fibers and sclereids differentiated in presence of α-ketoglutaric acid while least differentiation occurred in presence of pyruvic acid (Table-5d.1, Fig.5d.1). Other organic acids induced intermediate responses (Table-5d.1, Fig.5d.1). All the organic acids used induced differentiation of fibers (Plate 5d.2), tracheids with scalariform thickenings (Plate-5d.3) and micro-, macro- and elongated sclereids (Plate 5d.4-7). The fibers differentiated in the explants cultured on control medium resembled fibers which differentiated in explants given treatments of different organic acids. However, differentiation of tracheid and sclereids was totally lacking in the control explants (Table-5d.1).

Juice vesicles cultured on MS medium having plant hormones (10 mg/l IAA+0.2 mg/l Kn) revealed callusing on the 4th day of inoculation. The callusing time was reduced to 3 days if α-ketoglutaric acid or maleic acid was added to this medium (Table-5d.2). But addition of pyruvic acid or citric acid delayed the time taken to callus (Table-5d.2). In this experiment all the explants (100%) treated with different organic acids callused-except where pyruvic acid was used (Table-5d.2). Pyruvic acid could induce callusing only in 95% explants (Table-5d.2). Whereas most of the treatments improved callus growth over control, the growth of callus was inhibited by pyruvic acid (Table-5d.2, Plate 5d.8). Cytodifferentiation commenced on the 7th day in

Fig.5d.1 Effect of various organic acids (in absence of IAA and Kn) on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 5d·1

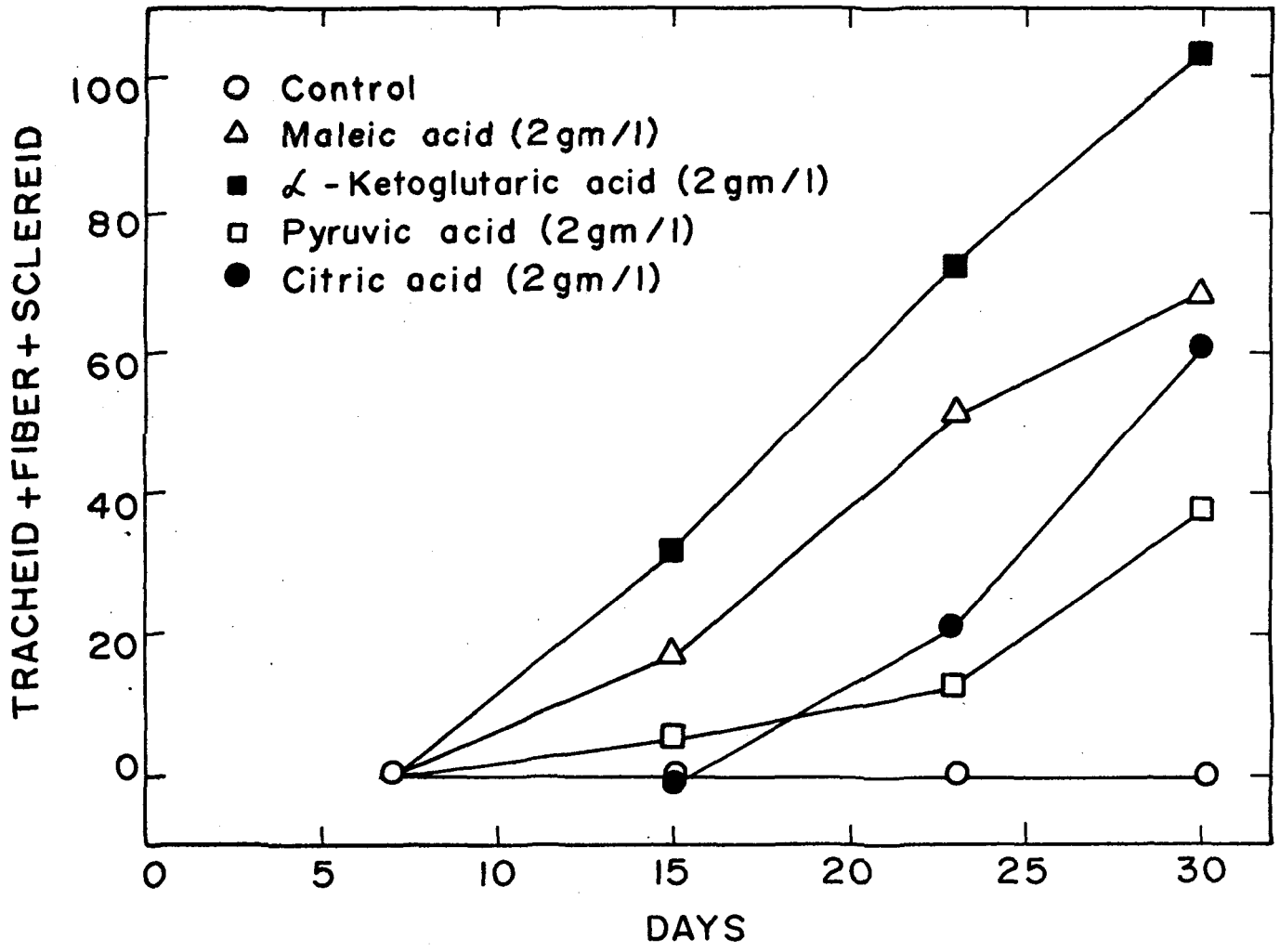

TRACHEID + FIBER + SCLEREID
100
80
60
40
20
0
Control
Maleic acid (2gm/l)
α-Ketoglutaric acid (2gm/l)
Pyruvic acid (2gm/l)
Citric acid (2gm/l)
0
5
10
15
20
25
30
DAYS

Table 5d.2: Effect of various exogenous organic acids in presence of IAA and Kn on cytodifferentiation in Assam lemon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	4	100	7.4 ±1.01	6.2±1.92	11.6±2.40	14.0±3.16	15.2 ±3.03	12.0±2.91	20.0±1.58	28.8±3.03	41.4 ±3.04	0	5.0±2.23	15.0±3.80	21.8 ±5.94
Maleic acid	3	100	14.4^{b} ±1.74	8.2±2.77	11.0±4.47	15.4±4.27	20.2 ±3.96	12.2±2.58	20.0±2.23	40.4±2.96	69.6^{b} ±2.96	22.4±4.08	65.6±4.61	93.8±4.65	101.2^{b} ±4.57
α-keto-glutaric acid	3	100	14.0^{b} ±2.44	7.4±2.07	11.2±3.03	20.8±3.19	$25.0^{b,c}$ ±2.16	20.6±3.36	33.0±2.54	68.0±2.40	$80.0^{b,c}$ ±3.16	23.2±4.08	48.8±7.36	78.8±5.97	$80.8^{b,c}$ ±5.11
Pyruvic acid	6	95	$3.4^{b,d}$ ±1.01	0	7.0±1.58	13.0±2.54	13.4^{d} ±2.70	4.2±2.77	38.4±2.40	47.6±3.20	$52.6^{b,d}$ ±3.64	0	13.0±4.06	21.6±3.28	$30.6^{b,d}$ ±3.64
Citric acid	5	100	$10.6^{b,e}$ ±1.01	6.8±1.92	12.0±2.91	17.4±2.30	$27.4^{b,e}$ ±2.07	16.0±2.23	31.4±2.07	58.4±2.40	$75.4^{b,e}$ ±2.07	3.8±2.58	48.2±6.05	55.6±5.59	$71.2^{b,e}$ ±8.07

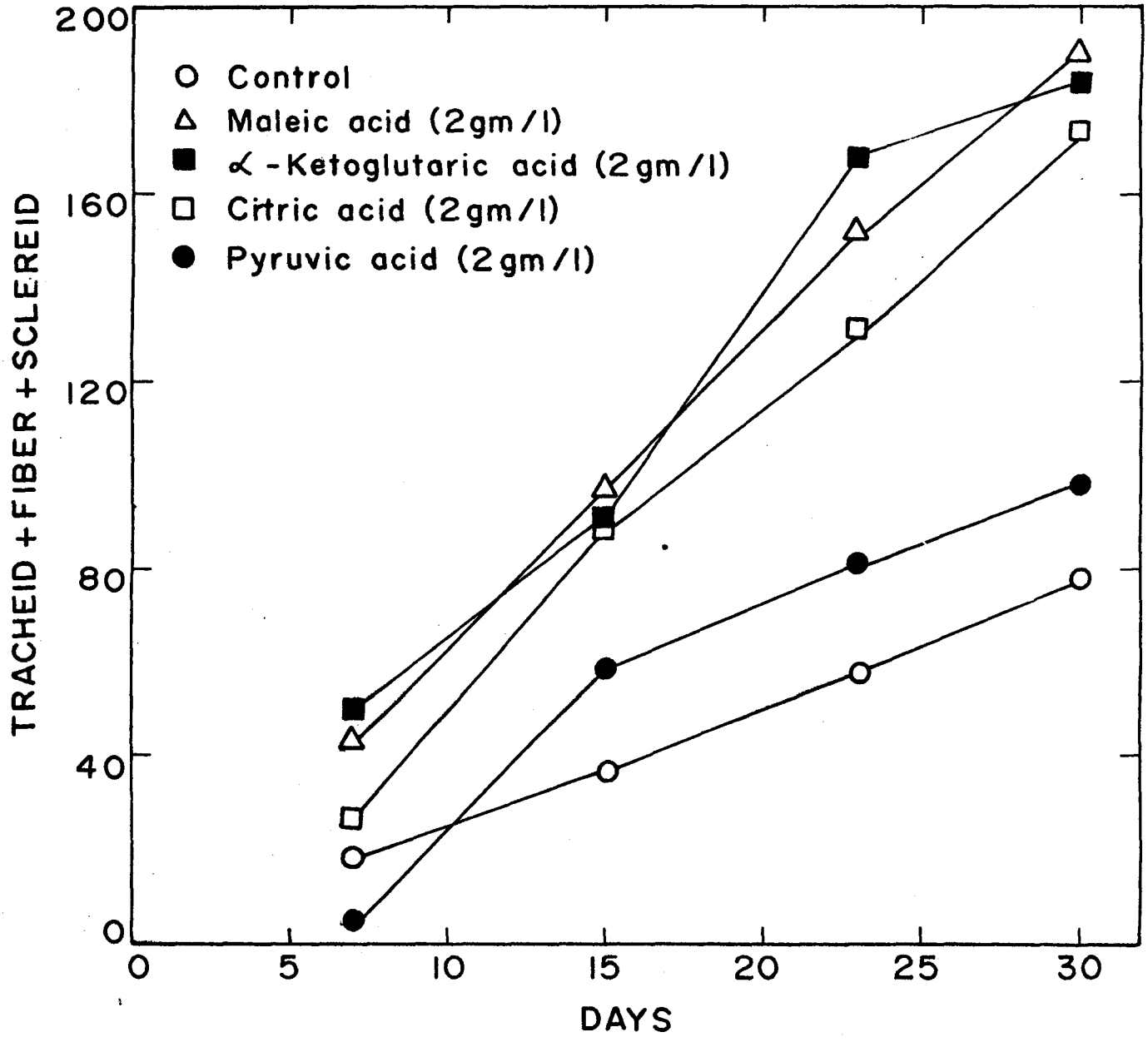
Values are means (n=5) ± s.d.

b. Significantly different from control.
c. Significantly different from maleic acid.
d. Significantly different from α-ketoglutaric acid.
e. Significantly different from pyruvic acid.

Significance tested by student's 't' test (p=0.05).

Fig.5d.2 Effect of various exogenous organic acids (in presence of 10 mg/l IAA and 0.2 mg/l Kn) on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 5d·2


200
160
120
80
40
0
TRACHEID + FIBER + SCLEREID
Control
Maleic acid (2gm/l)
∝-Ketoglutaric acid (2gm/l)
Citric acid (2gm/l)
Pyruvic acid (2gm/l)
0
5
10
15
20
25
30
DAYS

all the treatments including control (Table-5d.2). Compared to control explants, organic acids treated explants have better cytodifferentiation (Table-5d.2, Fig.5d.2). Amongst organic acids treated explants best cytodifferentiation was evident in explants treated with α-ketoglutaric acid (Table-5d.2, Fig.5d.2). The least differentiation occurred in pyruvic acid treated explants (Table-5d.2, Fig.5d.2). The largest number of fibers differentiated in the presence of α-ketoglutaric acid (Table-5d.2). But differentiation of maximum number of tracheid was induced by both α-ketoglutaric acid and citric acid (Table-5d.2). However, maximum number of sclereids differentiated in the presence of maleic acid and least number of sclereids differentiated in control explant (Table-5d.2). All the organic acids induced differentiation of fibers, tracheid with scalariform thickenings and sclereids (Plate 5d.2-7). The selecreids differentiated were, however, of different types: micro (Plate 5d.4); macro (Plate 5d.5) and elongated (Plate 5d.6-7). In the presence of citric acid, vessels also differentiated (Plate 5d.9). Differentiation of vessels was not evident in explants of other treatments and control. In control explants only fibers, tracheid with scalariform thickenings and microsclereids differentiated (Plate 5d.2-4).

Since citric acid is the main component of **Citrus** juice the effects of four concentrations of citric acid (1, 2, 2.5 and 3 mg/l) were studied in the presence of growth hormones IAA (10 mg/l) and Kn (0.2 mg/l). MS medium devoid of citric acid served as control. It was observed that early callusing (on the 4th day) occurred in the explants cultured on control medium

Plate 5d (contd.)

8. Development of callus on organic acids supplemented MS medium (in presence of IAA and Kn) in **C. limon** juice vesicle cultures:

   A - Control; B - Maleic acid; C - α-ketoglutaric acid; D - Citric acid and E - Pyruvic acid.

9. Vessel. X 310

10. Development of callus in **C. limon** juice vesicle cultures on MS medium having citric acid:

    A - Control (devoid of citric acid); B - 1 gm/l; C - 2 gm/l; D - 2.5 gm/l; and E - 3 gm/l citric acid.

11. Development of callus in **C. limon** juice vesicle cultures on MS medium supplemented with fruit juice of different **Citrus** species:

    A - Control (devoid of any fruit juice); B - **C. limon**; C - **C. jambhiri**; D - **C. reticulata**; E - **C. grandis** and F - **C. aurantifolia.**

12. Tracheid with scalariform secondary wall thickenings. X 380.

13. Fibers. X 310

14. Micro-sclereids. X 470

15 to 17. Macro-sclereids. X 310

18. Elongated sclereids. X 780

19. Tracheids exhibiting reticulate secondary wall thickenings. X 470

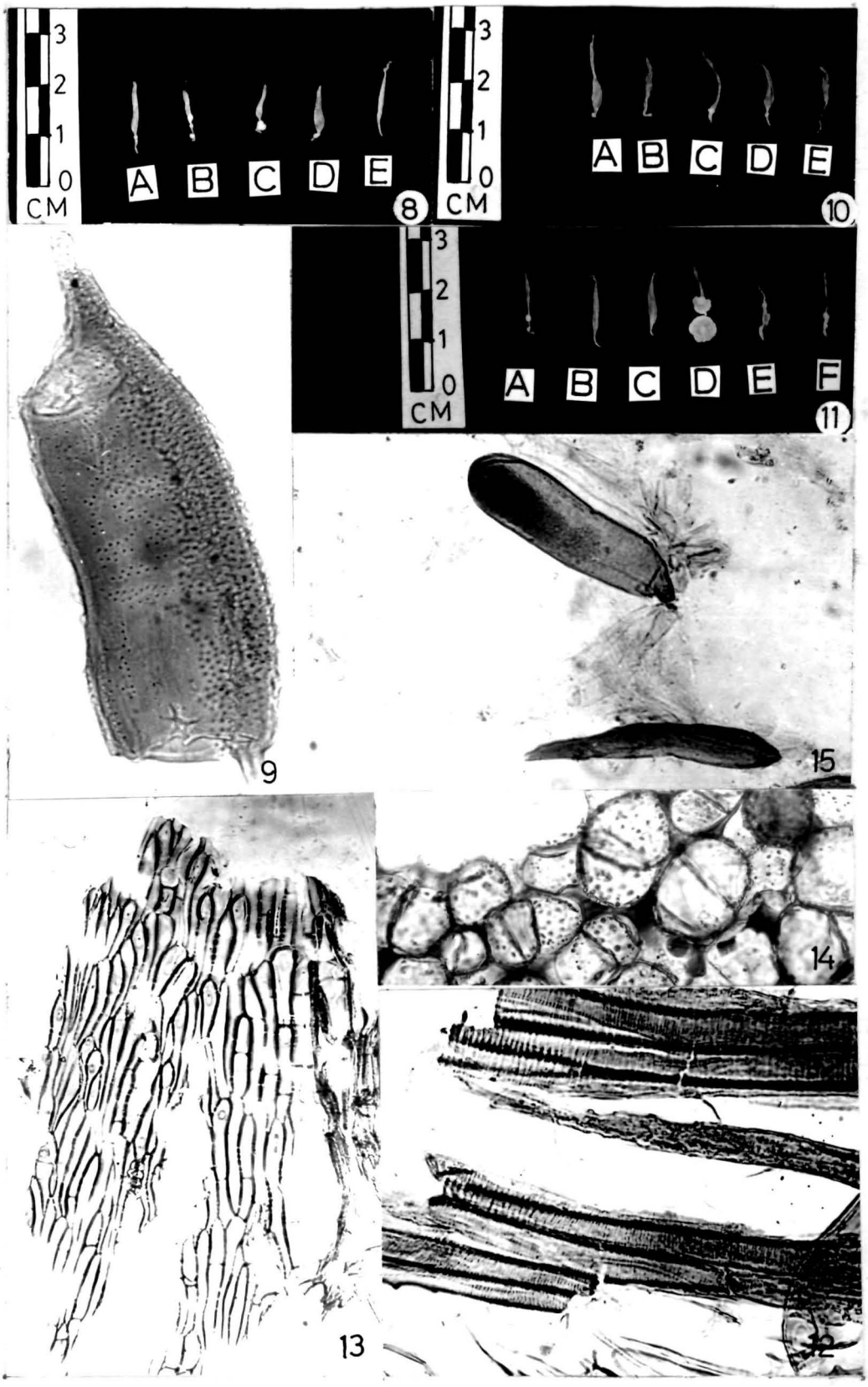


PLATE 5d (Contd..)

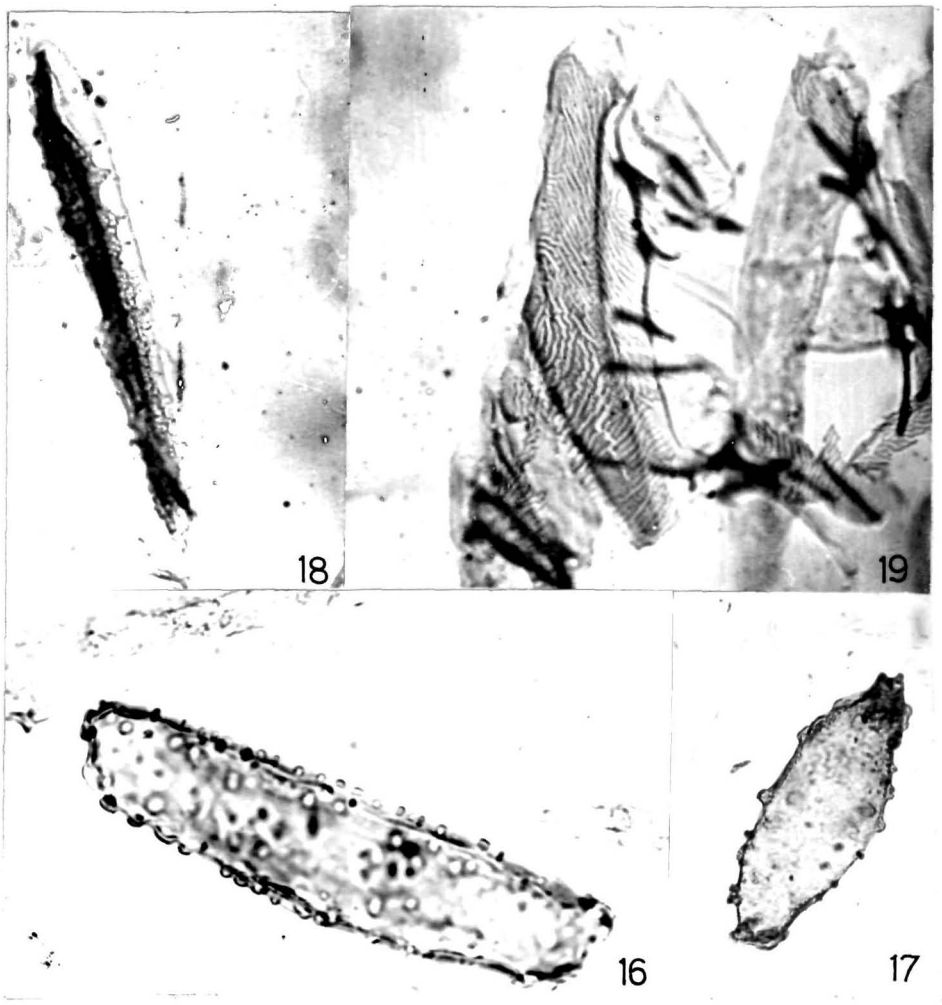


PLATE 5d

Table 5d.3: Effects of different concentrations of citric acid on cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	4	100	5.8 ±0.74	4.3 ±2.31	8.8±2.78	10.2±2.58	17.8 ±5.26	17.0±2.91	28.4±2.40	37.8±1.92	50.2 ±2.38	12.0±4.00	20.2±4.60	23.0±3.08	51.2 ±3.97
1 gm	4	100	6.6 ±1.01	11.4 ±2.70	13.6±2.40	19.2±2.58	20.2 ±3.96	20.8±2.77	30.6±3.04	60.8±3.19	68.8^{b} ±3.89	15.0±3.16	27.0±2.23	37.4±3.04	42.8^{b}±3.34
2 gm	6	100	$10.6^{b,c}$±1.35	8.4 ±2.40	18.2±2.38	20.4±3.64	$25.2^{b,c}$±2.26	9.8±1.92	31.4±2.70	49.2±2.86	73.0^{b} ±1.58	0	19.2±2.86	31.0±2.91	40.0^{b}±4.12
2.5 gm	8	20	$2.8^{b,d}$±0.74	0	0	0	0	0	3.8±1.48	9.0±3.16	$10.0^{b,d}$±2.23	0	0	0	0
3 gm	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Values are means (n=5) ± s.d.

b. Significantly different from control.
c. Significantly different from the value at 1 gm/l.
d. Significantly different from the value at 2 gm/l.

Significance tested by student's 't' test (p=0.05).

and medium having 1 gm/l citric acid (Table-5d.3). An increase in the lag period was observed with the increasing concentration of citric acid (Table-5d.3). No callusing, however, occurred at citric acid concentration higher than 2.5 gm/l (Table-5d.3). Callusing was evident in all the explants cultured on control medium and medium incorporated with 2 gm/l citric acid. But the calli produced by control explants were of lesser weight compared to the calli produced by explants cultured on 2 gm/l citric acid supplemented medium (Table-5d.3). Maximum growth of the calli was evident in calli produced by explants cultured on medium having 2 gm/l citric acid (Table-5d.3, Plate 5d.10). In the presence of 2.5 mg/l citric acid in the medium, only twenty per cent explants exhibited callusing and the growth of the calli was poorer than the control (Table-5d.3, Plate 5d.10).

Cytodifferentiation in control and treated explants occurred on the same day (7th day) upto 2 gm/l citric acid. The elements differentiated included fibers, tracheid with scalariform thickening and micro-sclereids (Plate 5d.2-4). Highest number of tracheid and fibers differentiated in explants cultured on medium having 2 gm/l citric acid but differentiation of sclereids was better in explants cultured on control medium (Table-5d.3). At higher concentration of citric acid (2.5 gm/l) an inhibitory effect was evident on cytodifferentiation since only few fibers differentiated, 15th day onwards (Table-5d.3).

Effect of different **Citrus** fruit juices on cytodifferentiation in Assam lemon juice vesicle was investigated by supple-

Table 5d.4: Effect of different citrus juices on cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	8	50	2.2 ±1.32	0	0	0	0	0	10.2±1.92	22.8±2.38	27.0 ±2.54	0	0	0	0
MS+ C. limon juice	13	10	1.8 ±0.97	0	0	0	4.6 ±1.67	0	11.2±3.70	20.4±3.64	20.2^{b} ±3.96	0	0	0	0
MS+ C. reticulata juice	5	100	$18.4^{b,c}$±1.01	11.6±2.40	28.6±3.36	35.2±3.70	48.8^{c}±3.03	20.2±3.34	30.4±2.70	48.6±3.84	$70.4^{b,c}$±3.04	34.0±3.87	38.0±5.0	42.8±3.96	49.0 ±3.16
MS+ C. Jambhiri juice	9	45	$6.2^{b,d}$±1.16	0	9.4±2.30	13.2±2.86	19.0^{d}±3.16	0	27.6±1.94	38.6±3.36	$48.4^{b,d}$±3.64	0	8.5±3.08	13.2±2.86	20.2^{d}±3.96
MS+ C. grandis juice	6	100	$15.2^{b,e}$±1.32	4.2±2.77	12.5±2.08	27.2±2.86	37.8^{e}±3.27	11.2±3.89	27.6±2.40	38.8±3.03	$59.8^{b,e}$±5.71	0	24.8±3.34	29.6±2.70	32.4^{e}±3.84
MS+ C. aurantifolia	7	100	$13.4^{b,f}$±1.01	0	14.8±3.34	23.0±2.54	35.2^{f}±3.03	0	22.0±3.16	31.0±3.16	$45.0^{b,f}$±3.80	0	11.6±2.40	17.0±3.16	23.4^{f}±4.15

Values are means (n=5) ± s.d.
b. Significantly different from control.
c. significantly different from the value in C. limon juice.
d. Significantly different from the value in c. reticulata juice.
e. Significantly different from the value in C. jambhiri juice.
f. Significantly different from the value in c. grandis juice.

Significance tested by student's 't' test (p=0.05).

menting MS medium (devoid of plant hormones) with fruit juice (10% v/v) of various citruses (orange, Soh-Jhalia, Assam lemon, pummelo and Kagzi). Explants cultured on the MS medium (devoid of plant hormones and without adding any **Citrus** juice) served as control. Callusing occurred in all the treatments. Initiation of callusing occurred earliest in the juice vesicles cultured on medium having orange juice while callusing in vesicles cultured on medium having lemon juice occurred last (Table-5d.4).

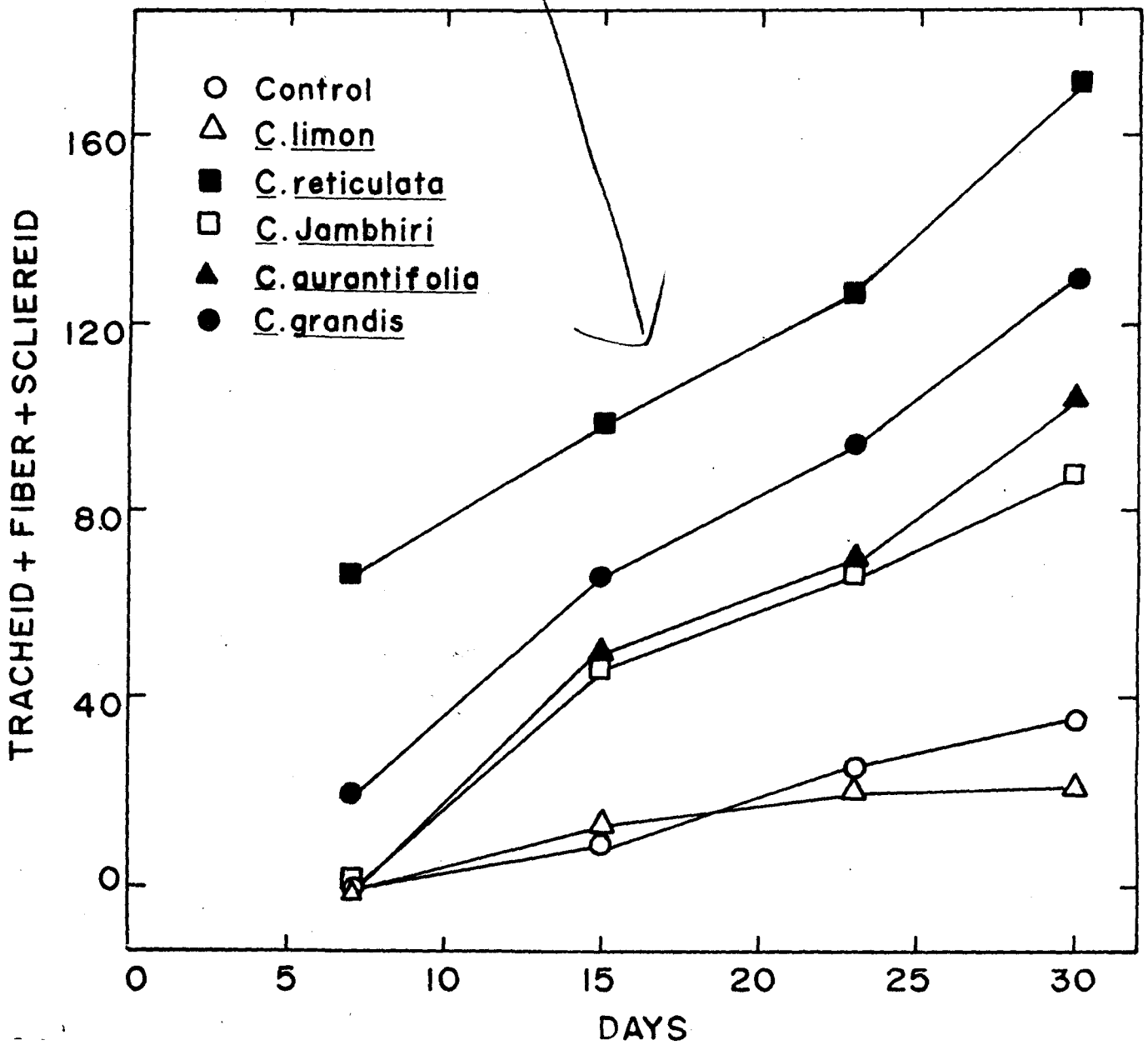
Fifty per cent control explants developed callus and revealed cytodifferentiation on the 15th day (Table-5d.4). Medium supplemented with Assam lemon juice induced callus only in 10% explants while **C. reticulata, C. grandis** and **C. aurantifolia** juice caused callusing in all the explants (100%) (Table-5d.4). Fruit juice of **C. jambhiri** induced callusing in only 45% treated explants (Table-5d.4). The medium having Assam lemon fruit juice supported minimal callus growth, which was not different from control, while medium having juice of **C. reticulata** induced maximal callus growth (Table-5d.4). Other **Citrus** juices induced intermediate responses (Table-5d.4, Plate 5d.11).

Cytodifferentiation occurred earliest in the juice vesicles cultured on media supplemented with **C. reticulata** and **C. grandis** juice (Table-5d.4). But delayed cytodifferentiation was evident in vesicles cultured on control medium and media supplemented with **C. jambhiri, C. limon** and **C. aurantifolia** juices (Table-5d.4). The pattern of cytodifferentiation also differed. In the juice vesicles cultured on the control medium only fibers differentiated while in juice vesicles cultured on medium having Assam lemon juice, both tracheid and fibers differentiated

Fig.5d.3 Effect of different fruit juices of different **Citrus** species on cytodifferentiation in **C. limon** juice vesicle culture.

What may be the reason for diff. behaviour?

Fig.5d.3



(Plate 5d.12,13). No differentiation of sclereids was evident in these treatments. However, in the juice vesicles cultured on media supplemented with **C. reticulata, C. jambhiri, C. grandis** and **C. aurantifolia** juice besides the tracheid, fibers and sclereids (micro- and macro-) also differentiated (Plate 5d.14-18). In all the treatments, the patterns of secondary wall thickenings observed in the differentiated tracheids was scalariform (Plate 5d.12). Tracheids with reticulate thickenings (Plate 5d.19) were, however, observed in explants cultured on medium supplemented with **C. jambhiri** juice.

The degree of differentiation was higher than the control in all the treatments except where Assam lemon juice was used (Table-5d.4, Fig.5d.3). The best differentiation was evident in presence of **C. reticulata** juice followed by **C. grandis, C. aurantifolia** and **C. jambhiri** juices, in decreasing order (Table-5d.4, Fig.5d.3).

Discussion

In order to test the effect of Kreb's cycle acid for cytodifferentiation, maleic, α-ketoglutaric, pyruvic and citric acid were incorporated in MS basal medium at a concentration of 2 gm/l. It was observed that all the four organic acids significantly improved cytodifferentiation in cultured juice vesicles. Maleic, α-ketoglutaric and citric acids were almost equally effective while pyruvic acid proved less effective in inducing cytodifferentiation, Kato (1980) found that in **C. hassaku** juice vesicle cultures both citric and maleic acids caused inhibition

of explant growth. But in the present investigation citric acid improved callus growth while pyruvic acid inhibited callus growth. Thus the organic acid induced responses are species dependent.

In agreement with the findings of Kulshrestha **et al.** (1982) The present studies also revealed that addition of citric acid at low concentration (2 gm/l) in the MS medium is stimulatory for cell division and cytodifferentiation. The data reveal that low concentration (2 gm/l) of citric acid improves callus growth (cell division) and cytodifferentiation while higher concentrations of citric acid (2.5 gm/l and above) inhibit both the processes. The inhibitory effect is so pronounced that in the present investigation both the processes stop at 3 gm/l citric acid concentration. Hildebrandt **et al.** (1954) also found that growth of marigold and sunflower tissue culture was stimulated by low concentrations of citric acid (0.15 to 1.25 gm/l). Erner **et al.** (1975) found that an optimum concentration of citric acid was 2 gm/l for growth stimulation of **Citrus** albedo culture, while concentrations higher than 2.5 gm/l proved inhibitory. The inhibitory effect could be due to toxicity of supraoptimal concentration.

Effects of fruit juices of five different local **Citrus** species (**C. limon, C. grandis, C. aurantifolia, C. reticulata** and **C. jambhiri**) were investigated on cytodifferentiation in cultured Assam lemon juice vesicles. The concentration of the fruit juice incorporated in the MS basal medium was 10% v/v. Orange juice was most effective in inducing cytodifferentiation while Assam lemon juice proved least effective. Juices of other

Citrus species evoked intermediate responses. Thus the juice of different **Citrus** species differ in their effectiveness in inducing callusing and cytodifferentiation which could be due to the difference in their chemical composition. Erner et al., (1975), also found that juice from orange fruits is more effective than juices of grape fruit and lemon fruit. Murashige & Tucker (1969) has observed that organge juice stimulatory effects on **C. limon** culture. It seems therefore that orange juice contains some growth factors. Orange juice requirement although could not be replaced by IAA, 2,4-D, GA_3, or Kn (Murashige & Tucker, 1969), citric acid can replace this requirement (Erner **et al.**, 1975). Thus atleast some of the growth activity of the orange juice is due to citric acid present in it. Nitsch (1970) also considers that the growth activity of the orange juice is due to citric acid and not cytokinin. However, the presence of additional growth factor in the juice cannot be ruled out because Einset (1978) could not increase growth by addition of appropriate concentration of citric acid in the basal medium. Therefore some components other than citric acid may also be responsible for responses evoked by **Citrus** fruit juices. Recently, occurrence of some endogenous plant growth substances, auxin, gibberellin and ABA like, is reported in young fruit of seeded and seedless clementine mandarin (Garcia-Papi and Garcia-Martinez, 1984).

CHAPTER V

(e) EFFECT OF pH

Introduction

Xylem differentiation has been studied in tissue culture for a number of years and reviewed recently (Roberts, 1976; Aloni, 1987). But factors other than hormones have not been studied extensively in relation to induced xylogenesis (Shininger 1979). Acidity of the medium influences secondary xylem formation in **Plumeria** cultures (Datta **et al.**, 1975) and is an important factor in the regulation of morphogenetic pathways in thin cell-layer explants of tobacco (Van **et al.**, 1985). In investigations dealing with the TE differentiation although the pH of the tissue culture medium is adjusted between 5-6 prior to autoclaving, the media differ considerably in their buffering capacity. Further the pH may shift dramatically during experimentation (Martin, 1980). Besides, the xylogenic effect of citric acid on **Citrus** cultures suggested the need to investigate the possible effects of media pH on cytodifferentiation. The present investigation thus deals with the effects of pH on cytodifferentiation in cultured juice vesicle explants of **C. limon**.

Materials and Methods

The effects of varying pH on cytodifferentiation in Assam lemon juice vesicles were investigated by ranging the pH of the media between 2-7. Themedium used was MS basal medium having IAA (10 mg/l) and Kn (0.2 mg/l). The tubes contained 25 ml of the autoclaved MS basal medium. Each culture tube contained a single vesicle placed on a filter paper bridge of Whatman No.1 filter paper (Dodds and Roberts, 1982). The

Table 5e-1: Effect of pH on cytodifferentiation in C. limon juice vesicle culture.

Treatment	Days taken for cellu-sing	Percen-tage of juice vesicles showing callus develop-ment	Final fresh weight of callus (mg/ex-plant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
pH															
3	6	78	5.0 ±1.41	0	0	0	0	11.2±3.89	31.2±4.43	72.8± 4.32	103.0 ±10.0	0	41.8± 6.45	121.8± 6.05	173.0 ± 11.0
4	5	100	8.2[b]±1.16	4.2±1.78	21.0±2.54	34.2±4.18	51.0 ±7.0	10.8±3.19	60.6±4.15	106.2±10.52	186.0[b]±25.0	0	106.6±12.62	392.2±16.88	522.0[b]±23.0
5	4	100	13.4[c]±0.94	7.2±1.92	30.6±3.28	88.4±3.04	101.0[c]±12.0	45.4±3.64	85.4±5.94	145.4± 4.50	190.0 ±10.0	87.4±10.55	254.6±5.77	529.4±6.19	710.0[c]±23.0
6	5	100	10.0[d]±1.04	2.8±1.92	7.8±2.58	21.0±3.87	53.0[d]±10.0	19.4±3.28	100.8±5.89	187.5± 4.54	204.0[d]± 7.0	31.2± 3.03	102.4± 7.89	362.8± 7.32	700.0 ±21.0
7	7	93	4.2[e]±1.07	0	6.2±2.48	16.2±2.86	29.0[e]±6.0	0	81.2±2.77	197.2± 4.81	294.0[e]±19.0	0	24.4± 3.84	69.75± 2.50	98.0[e]±10.0

Values are mean (n=5) ± s.d.

b. Significantly different from pH 3.
c. Significantly different from pH 4.
d. Significantly different from pH 5.
e. Significantly different from pH 6.

juice vesicles were extracted and prepared for culturing following the process detailed in Chapter III. Twenty culture tubes were maintained per treatment and cultured juice vesicle explants were incubated at 25±1°C. All the experiments were terminated at the end of 30 days and cultures were fixed in F.A.A., cleared in NaOH and stained with Safranin 'O' for microscopical observations as in Chapter III.

Results

The Table 5e.1 gives data on effects of media pH on callusing and cytodifferentiation in **C. limon** juice vesicle cultures. Initiation of callusing occurred first in the juice vesicles cultured on media having pH 5 while it occurred last in the juice vesicles cultured on medium having pH 7. Neither callusing nor cytodifferentiation was evident in the explants cultured on media having pH less than 3. All the explants cultured at pH 4-6 developed callus but only 93 and 78 per cent explants formed callus at pH 7 and 3, respectively (Table -5e.1). Best growth of callus was evident at pH 5 and it decreased in decreasing order at pH 6,4,3 and 7 (Table- 5e.1, Plate 5e.1). Cytodifferentiation commenced simultaneously in explants cultured at pH 3-6 but was delayed in explants grown at pH 7. Explants grown at pH 3 did not differentiate tracheid although fibers and sclereids differentiated (Table- 5e.1). However, in explants grown at pH 4-7, besides fibers and sclereids, tracheids also differentiated. The microsclereids differentiated were spherical and exhibited thickwalls (Plate 5e.2) while elongated tapered

Plate 5e

1. Development of callus in **C. limon** juice vesicle cultures of different medium pH:

   A - 3; B - 4; C - 5; D - 6 and E - 7 pH.

2. Sclereids. X 470.

3. Fibers. X 780

4. Tracheids with scalariform secondary thickenings. X 470

5. Tracheids with helical secondary wall thickenings. X 780

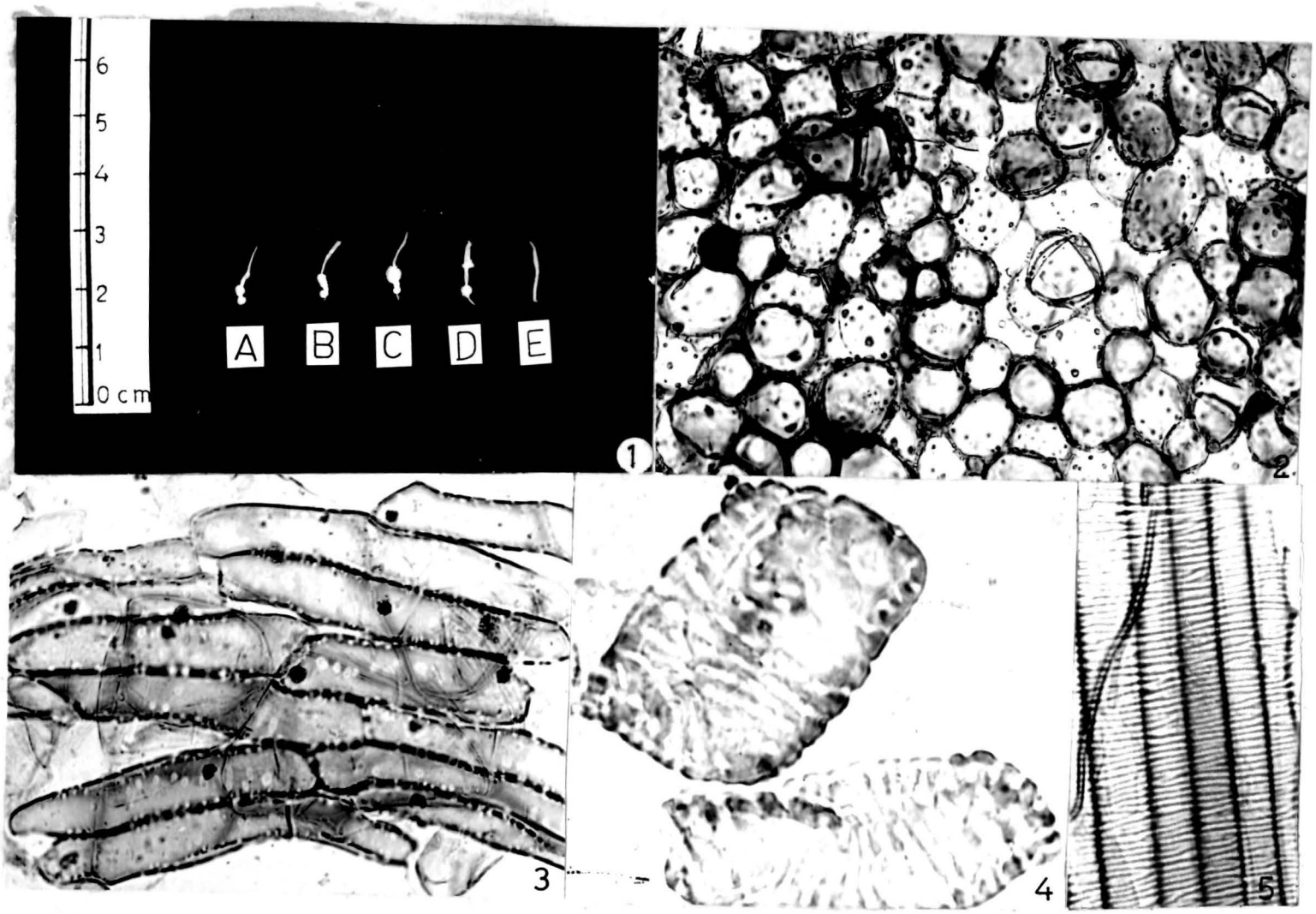
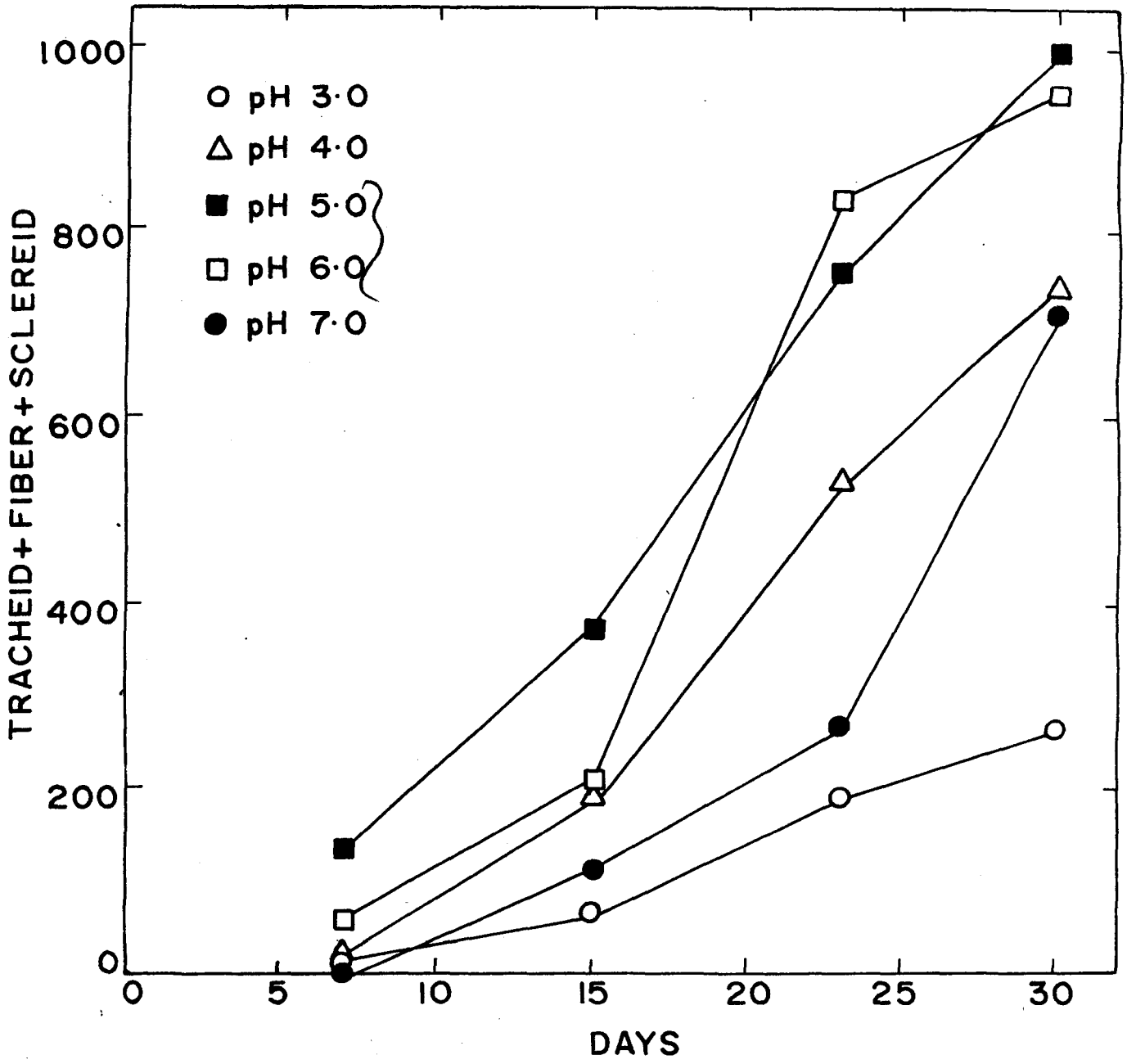


PLATE 5e

Fig.5e Effect of pH on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 5e


1000
800
600
400
200
0
TRACHEID + FIBER + SCLEREID
O pH 3·0
Δ pH 4·0
■ pH 5·0
□ pH 6·0
● pH 7·0
0
5
10
15
20
25
30
DAYS

cells with thick pitted walls resembled xylem fibers (Plate 5e.3). The tracheids exhibited scalariform and helical type of secondary wall thickenings (Plate 5e.4,5). The intensity of differentiation increased with the increasing pH upto pH 5 and thereafter it declined (Table 5e.1, Fig.5e). However maximum tracheid and sclereids differentiated at pH 5-6 while maximum fibers differentiated at pH 7 (Table-5e.1).

Analysis of pH towards the end of experimentation revealed a decrease in the pH of the medium in all the treatments (Table- 5e.2). The media having the higher initial pH revealed greater decreases in pH during experimentation.

Discussion

In the present investigation neither callusing nor cytodifferentiation occurred below pH 3 suggesting that such low pH of the medium is not suitable for either of the processes. Moderately acidic medium (pH 5) was most suitable for callusing, callus growth and cytodifferentiation of the tracheids, fibers and sclereids. But in highly acid medium differentiation of tracheid does not occur although fibers and sclereids differentiated. This thus suggests that differentiation of tracheids is relatively more sensitive to changes in medium pH.

During culturing acidification of the medium occurred in the present study. Similar were the findings of Martin (1980). Acidification occurs due to preferential ion assimilation. Dougall (1981) reported that in the presence of both nitrate and ammonium ions between pH 5-6, a preferential uptake of

Table 5e.2: Change in pH of the medium during 30 days culturing of C. limon juice vesicles.

Initial pH	Final pH
3.0	2.9
4.0	3.9
5.0	4.5
6.0	4.7
7.0	4.5

ammonium ions takes place decreasing the pH of the external medium. But Khan et al., (1986) consider that fluctuation in the pH of the culture medium could be more dependent on the plant tissue than the medium.

CHAPTER VI

EFFECTS OF PHYSICAL FACTORS

Introduction

The physiological control of TE differentiation has evolved since siluaraian time but understanding of control mechanisms are still incomplete. Various factors affecting TE differentiation are physical, chemical, genetical and metabolic. As these factors overlap it is very difficult to understand the central mechanism involved in the differentiation of xylem cells (Berlyn, 1979). A number of physical factors influence TE differentiation (Roberts, 1976) but in the present study only effects of ionizing radiation (gamma rays) and light and dark conditions during experimentation have been investigated.

Radiation influence **in vivo** differentiation of TE in gamma irradiated plants (Foard and Haber, 1961; Haber and Foard, 1964; Fosket and Miksche, 1966; Chauhan and Singh, 1975). Gamma radiation influences differentiation of TE in cultured explants of **Helianthus tuberosus** (Phillips, 1981; Phillips and Hawkins, 1985) and **Zinnia elegans** (Sugiyama **et al.**, 1986). Gunkel (1957, 1965) observed that irradiation also enhanced xylem differentiation.

Light usually inhibits cytodifferentiation (Roberts, 1976). Continuous white light reduces the TE differentiation in cultured jerusalem artichoke tuber tissue (Phillips and Dodds, 1977). No reports are available to reveal the effect of radiation and presence and absence of light during experimentation and cytodifferentiation in **Citrus** juice vesicle cultures. Therefore in the present study besides investigating the general

effects of continuous light and dark conditions on differentiation of TE in **C. limon** juice vesicle culture, the effects of radiation have also been studied.

Materials and Methods

Effect of light on cytodifferentiation in Assam lemon juice vesicle explants was studied by culturing these on MS basal medium, having IAA (10 mg/l) and Kn (0.2 mg/l). The explants were incubated under continuous light and dark conditions in B.O.D. incubator maintained at 25±1°C. The intensity of fluorescent light available was 2410 lux. Per treatment 20 culture tubes were maintained. The experiments continued for 30 days. The cultured explants were collected at weekly intervals; fixed in F.A.A. and processed for observation as described in Chapter III.

The effects of gamma rays on cytodifferentiation in Assam lemon juice vesicles were also investigated by irradiating developed fruits of Assam lemon with 2.5, 5, 10, 50, 100 and 150 Gy of gamma rays using a 60 cobalt source emitting γ rays at a rate of 10.749 rad/second. Subsequent to irradiation juice vesicles were extracted from the irradiated fruits (for detail please see chapter III) and cultured on MS basal medium having IAA (10 mg/l) and Kn (0.2 mg/l). Forty culture tubes were maintained for each treatment and cultured explants were incubated in dark in a B.O.D. incubator maintained at 25±1°C. The cultured explants were collected at weekly intervals, fixed and processed for observation as described previously in Chapter III. The experiment continued for 30 days.

Histological and Histochemical technique - The histological and histochemical manifestations of cytodifferentiation in cultured juice vesicle explants were investigated using histological and histochemical techniques. For every treatment five juice vesicles were collected per collection. The fixed juice vesicles were dehydrated using tertiary butyl alcohol series and embedded in paraffin wax. Serial sections 12 micron in thickness were cut using Leitz rotary microtome. For histological studies, safranin-haematoxylin staining combination was used (Johansen, 1940) while histochemical localization of insoluble carbohydrates, proteins and nucleic acids was done, in the cultured explants, using following techniques.

(a) Insoluble polysaccharides (Jensen, 1962): First deparafinized sections were brought to water for 3-5 min. Next slides were incubated at 25°C in 0.5% aqueous solution of periodic acid for 30 min. At the expiry of incubation period the slides were washed for 5 min in running water and then stained with Schiff's reagent for 25 min. at 4°C. The stained sections were first rinsed in water and then placed in 2% sodium metabisulphite for 1-2 min. The slides were again washed in running water for 2-3 min, dehydrated and mounted in D.P.X. The insoluble polysaccharides and starch stain purplish-pink.

(b) General proteins (Mazia et al., 1953): The deparafinized sections were first transferred to insoluble alcohol and then placed for 30 min in 0.1% bromophenol blue solution prepared in 95% alcohol having 10% mercuric chloride. Subsequent to

Table 6.1: Effect of light on cytodifferentiation in C. limon juice vesicles.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Dark	3	100	12.0±1.01	1.4±1.34	4.3±2.31	10.2±4.43	13.4±3.20	5.2±1.92	23.6±2.70	58.4±2.70	70.6 ±3.84	0	17.0±1.58	28.4±3.20	58.4 ±4.21
Light	5	100	20.0[b]±1.20	0	0	0	0	20.9±6.80	45.4±3.50	75.4±3.20	101.4[b]±6.10	0	9.0±3.0	15.6±3.97	20.4[b]±2.96

Values are means (n=5) ± s.d.

b. Significantly different from dark. ($p = 0.05$)

staining in bromophenol blue slides were first washed in 0.5% aqueous acetic acid for 3-5 min. and then dipped for 3 min. in Sorenson buffer. The sections were quickly dehydrated through T.B.A., cleared in xylene and mounted in D.P.X. Proteins stain blue.

(c) Nucleic acids (Flax and Himes, 1952): The deparafinized sections were hydrated and placed in Azur B solution (0.25 mg/ml), prepared in citrate buffer (pH 4.0) for 2 hr at 50°C. Once staining was over, the slides were washed in water, placed in pure T.B.A. for 30 min. and then passed through xylol and mounted in D.P.X. The different nucleic acids stain differently. DNA stains green blue while RNA appears either purple or dark blue.

The differences in the concentrations of above constituents in the cultured control and treated juice vesicles were reflected by the differences in the intensity of stain.

Results

Light: Juice vesicles cultured under continuous light exhibited delayed callusing in comparison to the juice vesicles incubated in dark (Table- 6.1). Percentage of explants developing callus did not differ during presence or absence of light although a remarkable difference was evident in the growth of callus (Table- 6.1). The explants grown under dark condition developed callus which weighed 12 mg at the end of experiment but under continuous light the calli weighed 20 mg. Further, while under the dark condition the calli developed only from stalk portion

Plate 6a

1. Development of callus in **C. limon** juice vesicle cultures in: A - Dark and B - Light conditions.
2. Sclereids. X 470
3. Fibers. X 75
4. Tracheids with scalariform secondary wall thickenings. X 240
5. Fibers. X 310
6. Sclereids. X 470

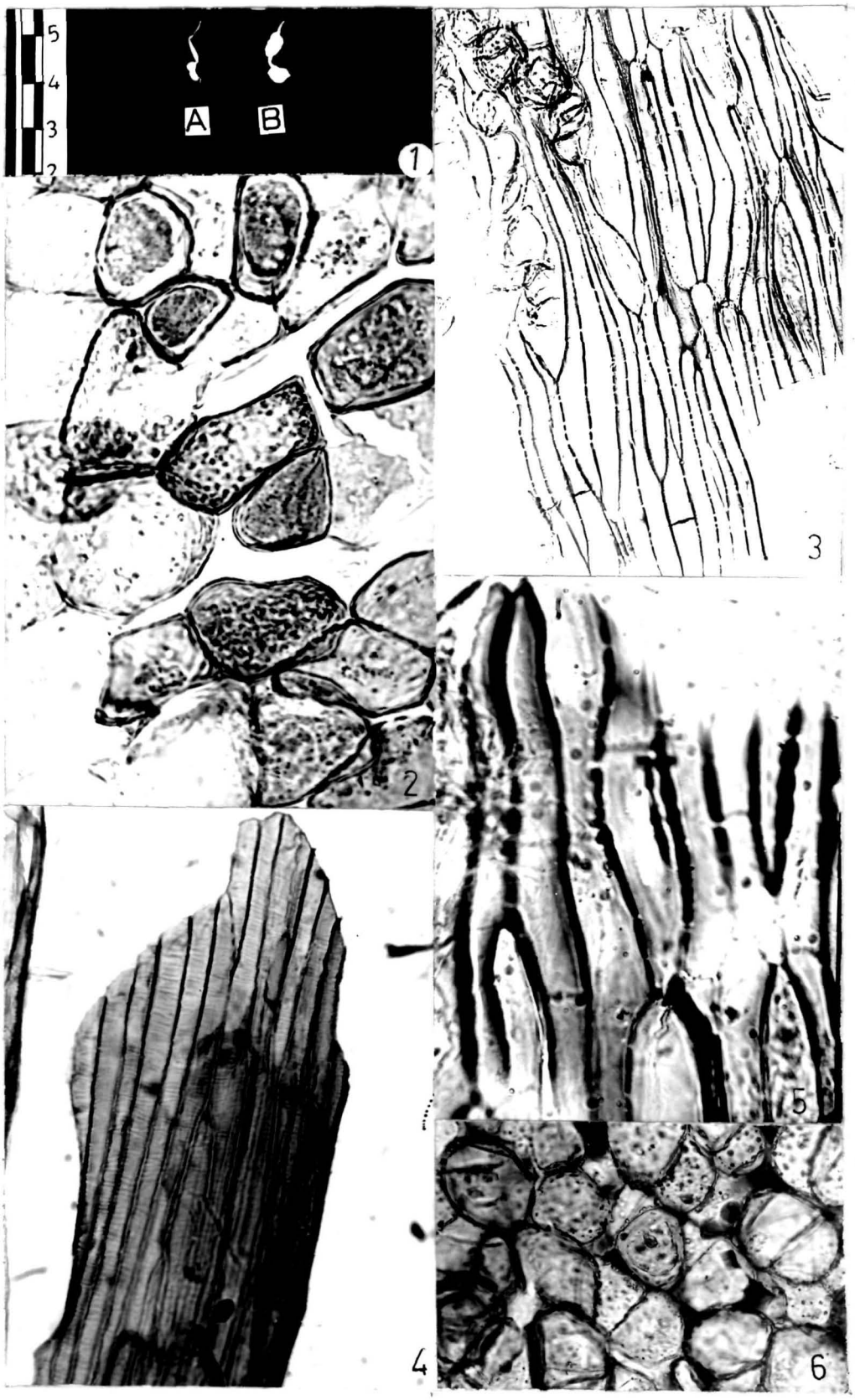
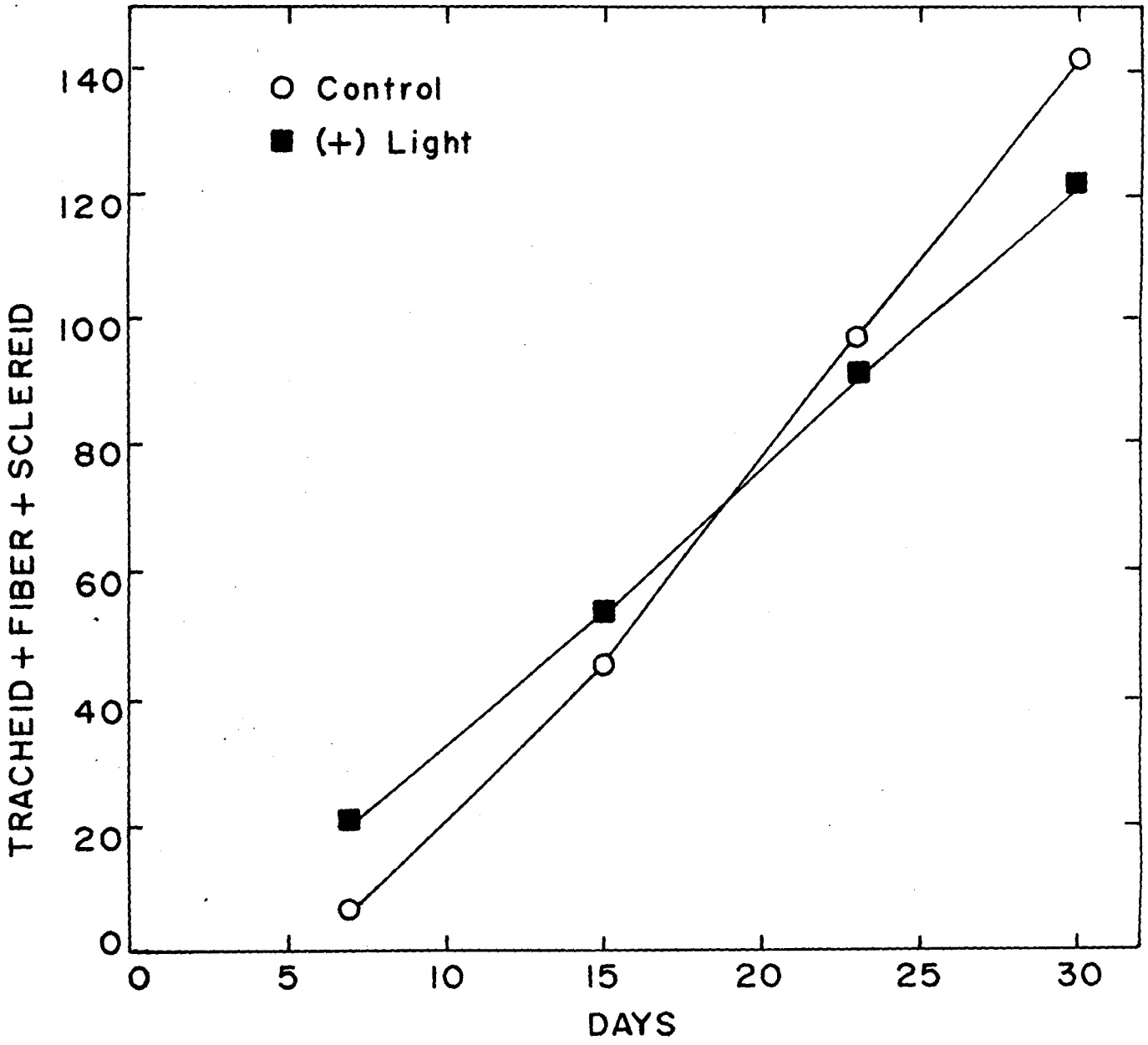


PLATE 6a

Fig.6.1 Effect of dark and light conditions on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 6·1


Control
(+) Light
TRACHEID + FIBER + SCLEREID
0
20
40
60
80
100
120
140
0
5
10
15
20
25
30
DAYS

of the explants, in presence of light the calli developed from both stalk and sac regions of the juice vesicle (Plate 6a.1).

Cytodifferentiation in the explants cultured in continuous light occurred later than in the explants cultured under dark condition (Table- 6.1). In the explants grown in light fibers and sclereids (Plate 6a.2-3) differentiated on the 7th and 15th day respectively. No tracheid differentiated in these explants. Conversely in explants cultured in dark condition besides fibers, tracheid with scalariform and reticulate thickening also differentiated (Table- 6.1, Plate 6a.4-5). With the passage of time, differentiation of sclereids (Plate 6a.6) also became evident in these explants (Table- 6.1). At the expiry of the experiment, number of differentiating tracheid, sclereids and fibers were more in dark cultured explants than in the explants given light conditions (Fig. 6.1).

Radiation: Control (unirradiated) explants revealed callusing on the 5th day of inoculation. The callusing time decreased in explants irradiated with 5.0 and 10.0 Gy radiation doses but it increased, with the increasing radiation doses, in explants given 50-150 Gy radiation (Table- 6.2). Callusing of all the juice vesicles was evident in control explants and explants irradiated with 2.5 -10 Gy radiation doses. The percentage of juice vesicles developing callus decreased with the increasing radiation doses in explants treated with 50-150 Gy radiation doses (Table- 6.2). The growth of callus increased with increasing radiation dose upto 10 Gy and thereafter it decreased (Table- 6.2,

Table 6.2: Effect of Radiation on cytodifferentiation in C. limon juice vesicle culture.

Treatment	Days taken for callusing.	Percentage of juice vesicles showing callus development.	Final fresh weight of callus (mg/explant)	Tracheid				Fibre				Sclereids			
				Days				Days				Days			
				7	15	23	30	7	15	23	30	7	15	23	30
Control	5	100	6.6 ±1.01	1.4 ±1.34	3.1±2.55	9.0±3.16	14.0 ±2.64	17.0±1.58	39.0±3.16	58.4±2.70	89.2 ±3.34	10.2±4.43	31.6±2.40	47.0±2.54	33.4 ±3.13
2.5 Gy	5	100	10.0^{b} ±1.25	1.6 ±1.14	10.0±2.23	13.2±3.56	13.2 ±2.86	30.0±2.54	90.2±3.83	98.6±2.70	106.8^{b} ±2.86	9.0±3.16	20.2±3.49	31.2±2.86	69.2^{b} ±3.34
5.0 Gy	3	100	$14.2^{b,c}$ ±1.67	2.16±2.04	6.8±1.92	16.8±2.86	$19.0^{b,c}$ ±3.16	53.0±4.30	99.6±4.50	180.8±4.30	$146.4^{b,c}$ ±5.07	16.4±2.70	29.4±2.07	39.0±3.16	$46.6^{b,c}$ ±3.20
10.0 Gy	3	100	$23.4^{b,d}$ ±0.78	4.4 ±2.07	11.6±2.40	23.0±4.12	$37.4^{b,d}$ ±3.84	50.8±2.28	95.4±4.61	126.6±2.70	$201.8^{b,d}$ ±0.28	17.0±2.54	30.6±3.04	58.4±2.70	$70.6^{b,d}$ ±3.84
50.0 Gy	6	95	8.8^{e} ±1.94	0	4.8±2.38	9.4±2.30	10.8^{e} ±3.19	29.2±2.86	56.8±4.14	91.2±2.86	$101.4^{b,e}$ ±1.10	0	10.8±3.19	18.6±3.36	$23.6^{b,e}$ ±3.01
100.0 Gy	6	73	$4.8^{b,f}$ ±0.74	0	0	0	$2.2^{b,f}$ ±1.48	20.4±3.64	37.2±3.49	41.4±3.48	$50.6^{b,f}$ ±3.36	0	13.4±3.20	16.0±3.80	25.6^{b} ±3.64
150.0 Gy	12	50	$2.2^{b,g}$ ±1.01	0	0	0	0	0	13.2±2.07	20.2±3.96	$23.6^{b,g}$ ±2.70	0	2.6±2.6	6.0±2.23	$9.4^{b,g}$ ±2.30

Values are mean (n = 5) ± s.d.

b. Significantly different from control.
c. Significantly different from 2.5 Gy.
d. Significantly different from 5.0 Gy.
e. Significantly different from 10.0 Gy.
f. Significantly different from 50.0 Gy.
g. Significantly different from 100.0 Gy.

Significance tested by student's 't' test (p=0.05).

Fig.6.2 Effect of γ-rays on cytodifferentiation in **C. limon** juice vesicle culture.

Fig. 6·2

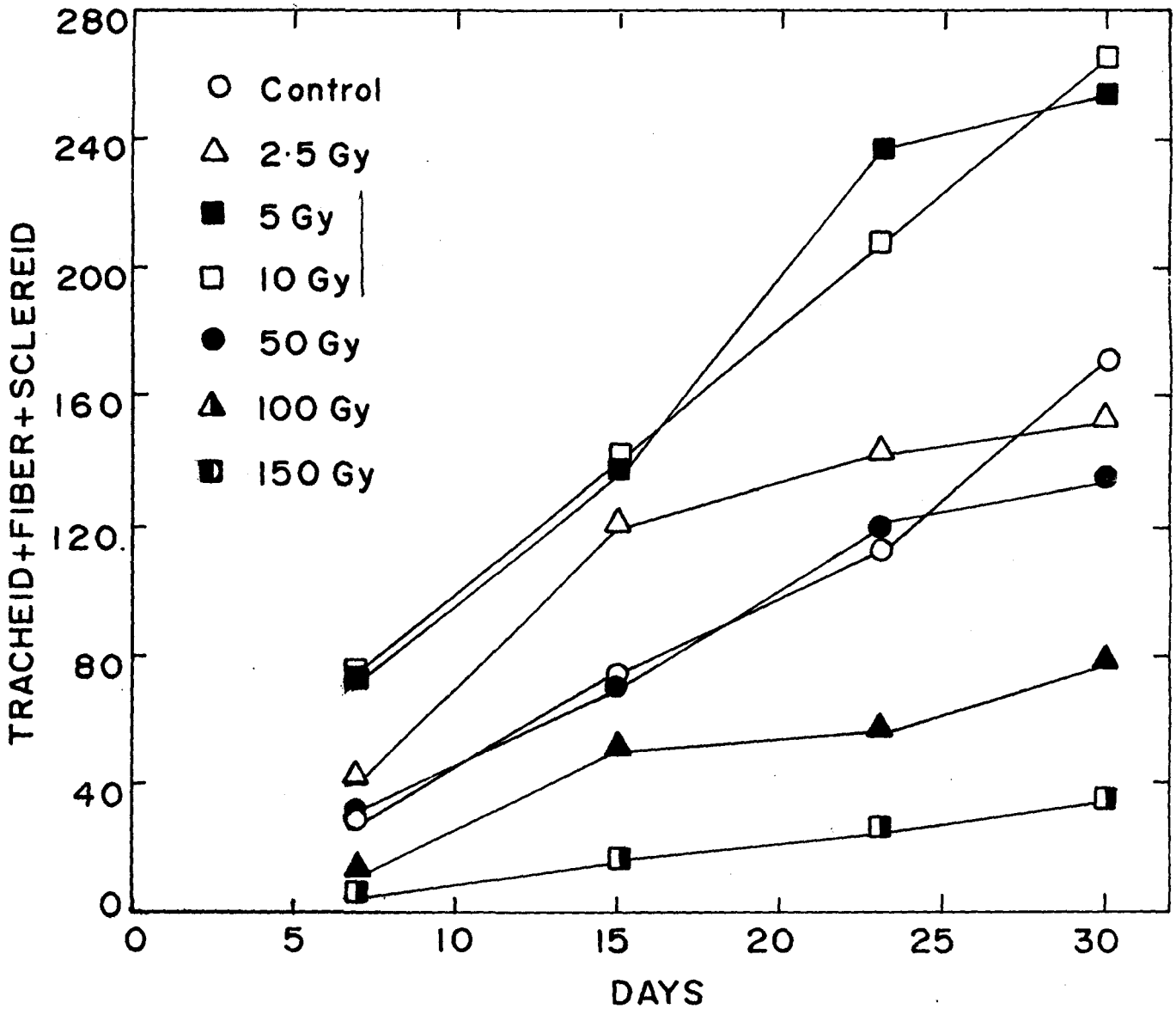

280
240
200
160
120
80
40
0
TRACHEID+FIBER+SCLEREID
Control
2·5 Gy
5 Gy
10 Gy
50 Gy
100 Gy
150 Gy
0
5
10
15
20
25
30
DAYS

Plate 6c.1). Maximum weight of the callus was found in explants exposed with 10 Gy radiation dose and minimal weight of the callus was evident in explants irradiated with 100 and 150 Gy radiation doses (Table- 6.2). Cytodifferentiation started simultaneously both in control and irradiated explants given upto 100 Gy radiation doses (Table- 6.2). But radiation dose 150 Gy delayed cytodifferentiation (Table-6.2).

With the progression of time some of the cells in callus, developed by irradiated juice vesicles (2.5-150 Gy), differentiate into tracheids, fibers and sclereids (Plate 6c.2-12). The process of differentiation in irradiated explants resembled differentiation in control. In all the treated explants fibers differentiated first followed by differentiation of tracheid and sclereids. The cytodifferentiation started on the 7th day of incubation in explants given upto 100 Gy while it commenced on the 15th day in explants treated with 150 Gy radiation dose. The number of tracheid, fibers and sclereids increased with the increasing radiation doses upto 10 Gy and thereafter the number declined sharply (Fig. 6.2). Maximum differentiation of tracheids, fibers and sclereids occurred at 10 Gy radiation dose, while least cytodifferentiation occurred at 150 Gy radiation dose (Table- 6.2, Fig. 6.2). In explants given 150 Gy dose, cytodifferentiation was less than the control and only fibers and sclereids differentiated (Table- 6.2). The differentiation of tracheid was completely inhibited in these explants (Table 6.2). Maximum diversity of secondary wall thickening of tracheid was noticed

Plate 6b

1. Distribution of insoluble polysaccharides on the day of culturing in cells of the control juice vesicles. X 120

2. Distribution of nucleic acids on the day of culturing in cells of the control juice vesicles. X 100

3. Distribution of general protein on the day of culturing in cells of the control juice vesicles. X 75

4. Distribution of insoluble polysaccharides on 3rd day of culturing in cells of control juice vesicles. X 75

5. Distribution of nucleic acids on 3rd day of culturing in cells of the control juice vesicles. X 120

6. Distribution of general protein on 3rd day of culturing in cells of the control juice vesicles. X 190

7. Distribution of insoluble polysaccharides in cells of the control juice vesicles during callus development. X 190

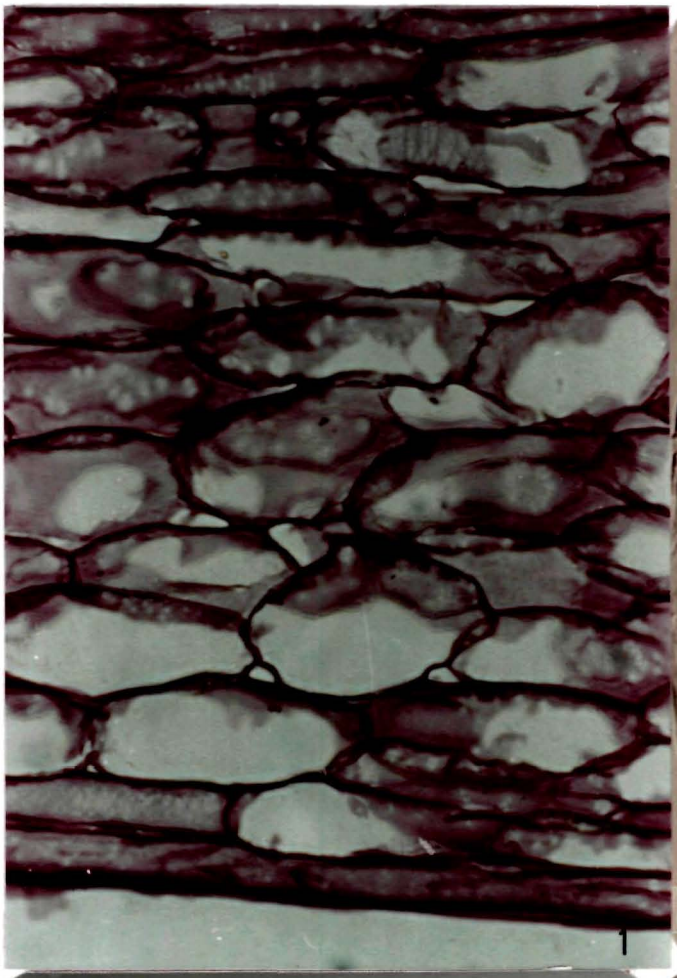
8. Distribution of nucelic acids in differentiating cells of the control juice vesicles. X 300

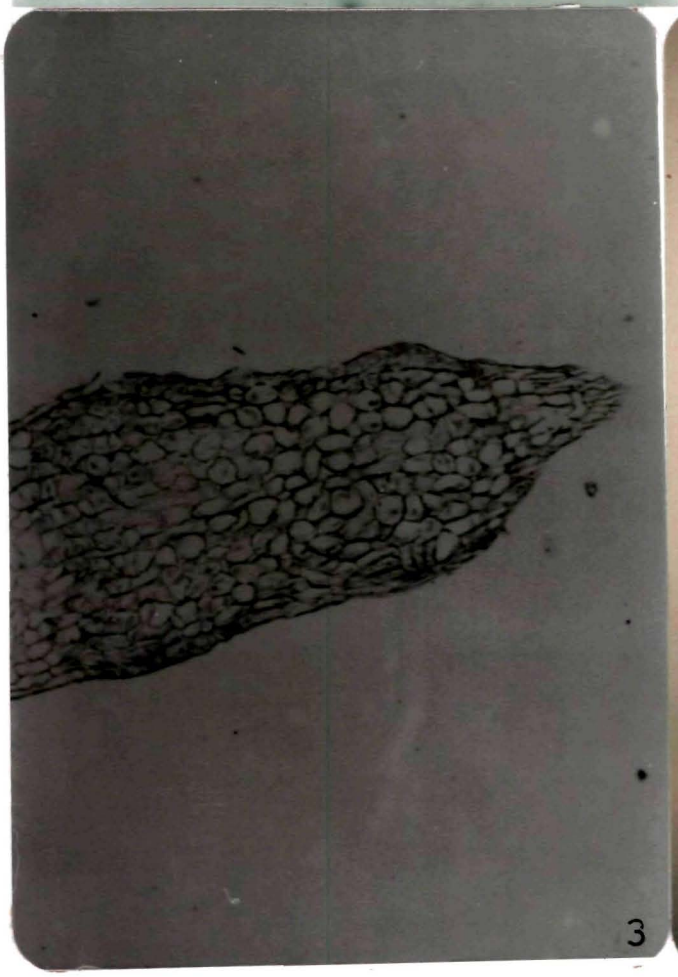
9. Distribution of general protein in differentiating cells of the control juice vesicles. X 470

10. Distribution of insoluble polysaccharides in differentiated and differentiating cells of the control juice vesicles. X 310

11. Distribution of general protein in cells of 2.5 Gy irradiated juice vesicles. X 240

12. Distribution of nucleic acids in cells of 2.5 Gy irradiated juice vesicles. X 240





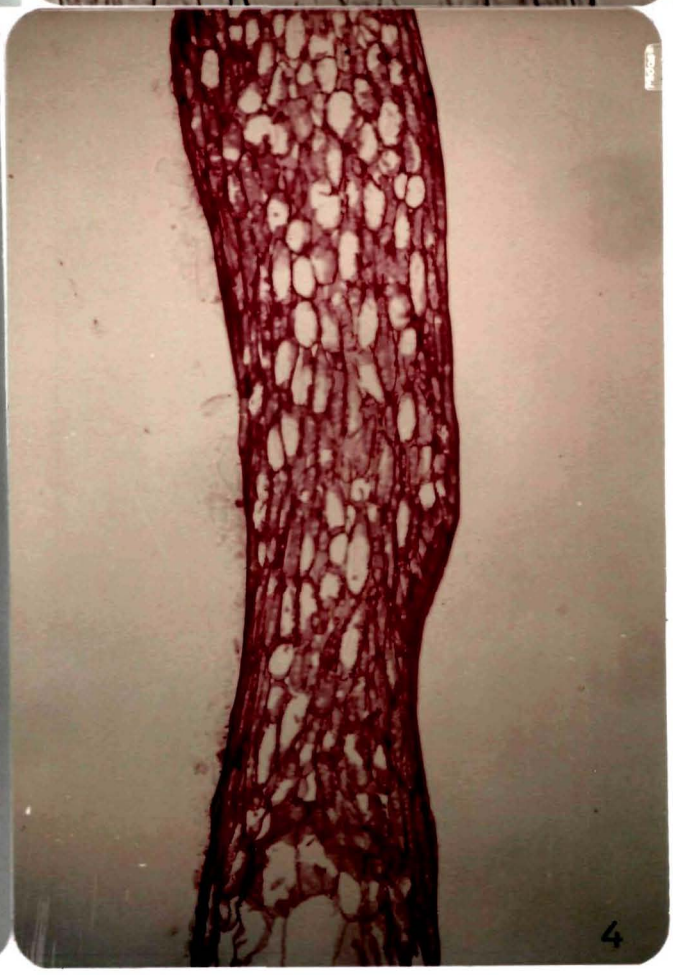


PLATE 6b

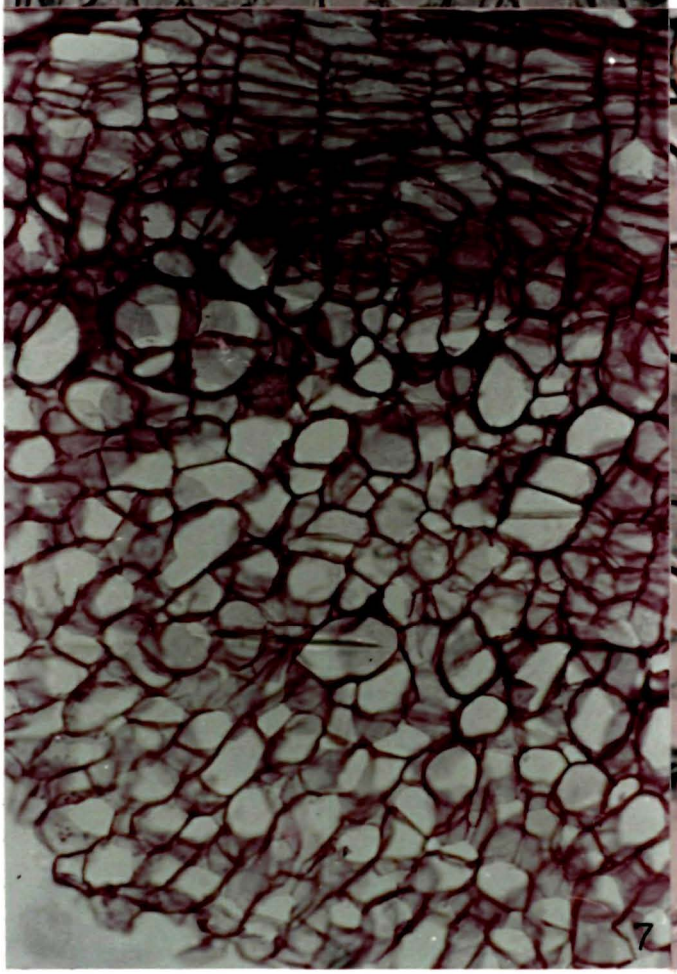
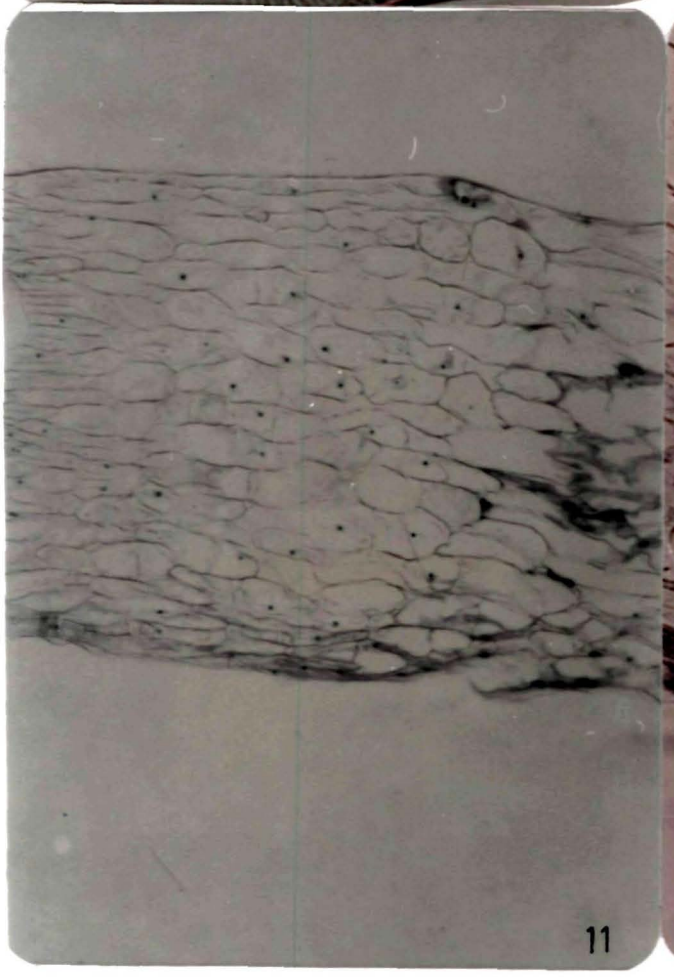


PLATE 6b (Contd...)



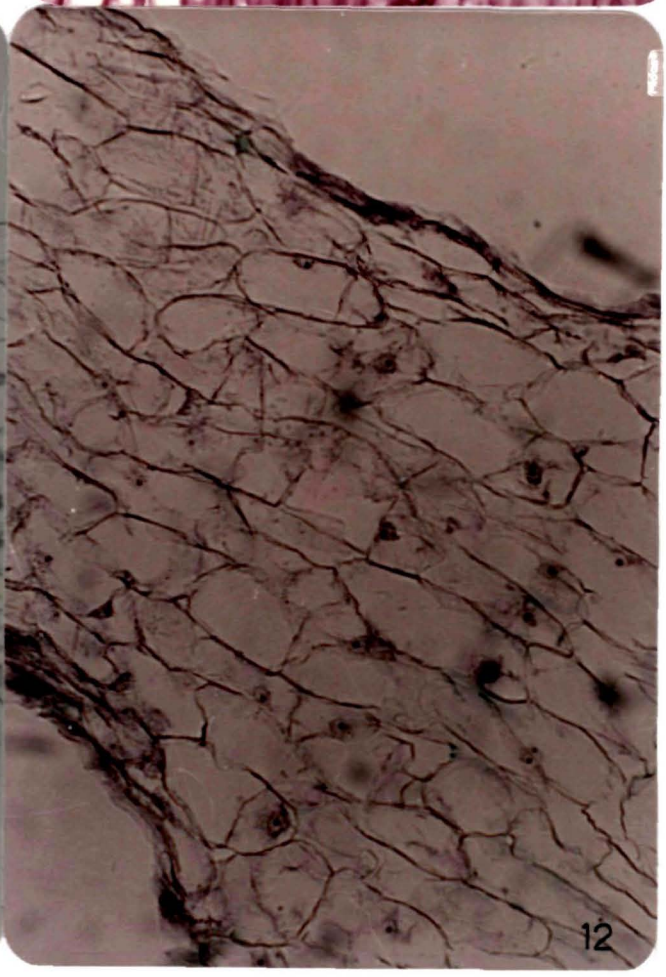


PLATE 6b (Contd.)

Plate 6b (contd)

13. Distribution of general protein in cells of 10 Gy irradiated juice vesicles X 300

14. Distribution of nucleic acids in cells of 10 Gy irradiated juice vesicles. X 300

15. Distribution of general protein in cells of 150 Gy irradiated juice vesicle X 480

16. Distribution of nucleic acids in cells of 150 Gy irradiated juice vesicles X 480

17. Distribution of insoluble polysaccharides in differentiated and non-differentiated cells of control juice vesicles. X 75

18. Distribution of insoluble polysaccharides in cells of 2.5 Gy irradiated juice vesicles at the commencement of differentiation. X 100

19. Distribution of insoluble polysaccharides in cells of 150 Gy irradiated juice vesicles at the commencement of differentiation. X 120

20. Distribution of general protein in the differentiating cells of the 10 Gy irradiated juice vesicles. X 380

21. Distribution of general protein in differentiating cells of 5 Gy irradiated juice vesicles. X 480

22. Distribution of general protein in differentiated tracheid and normal cells of 10 Gy irradiated juice vesicles. X 480

23. Distribution of nucleic acids in differentiated sclereid and normal cells of the 2.5 Gy irradiated juice vesicles. X 310

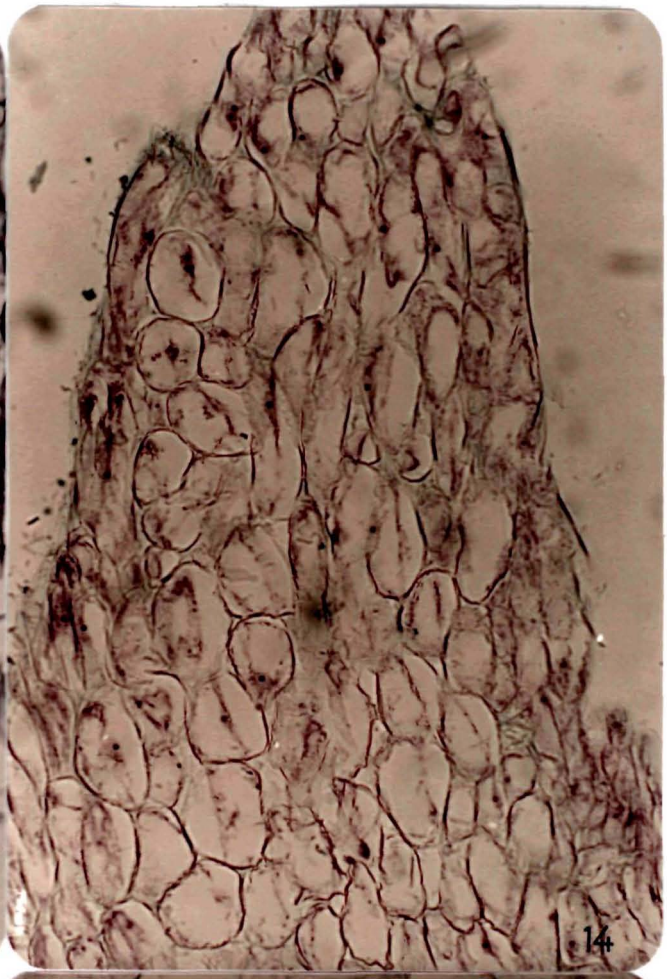
24. Distribution of nucleic acids in differentiated and differentiating fibers of 10 Gy irradiated juice vesicles. X 240

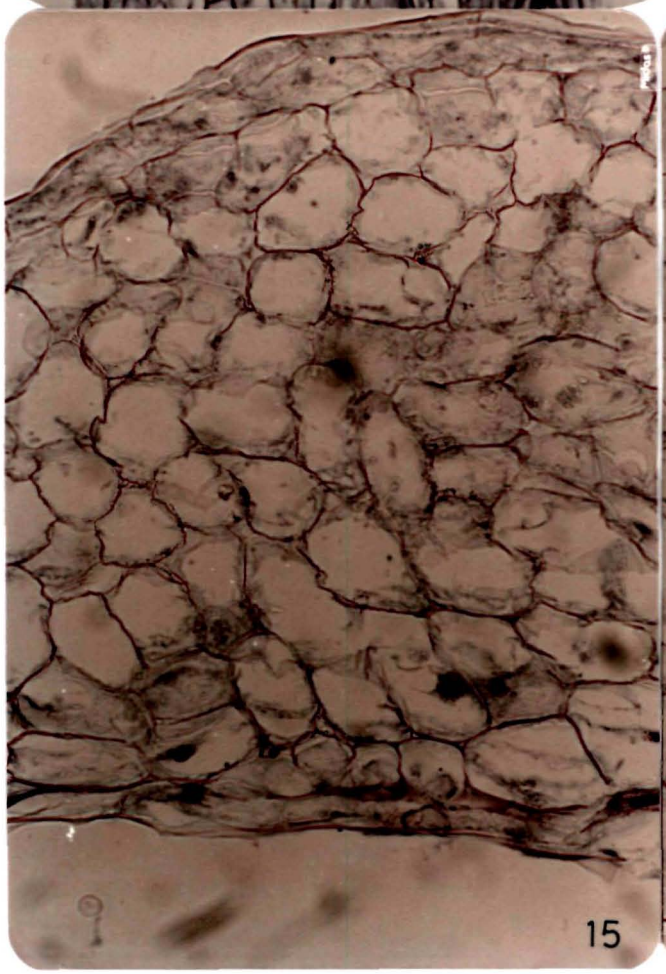
25. Distribution of general protein in differentiating cells of the 150 Gy irradiated juice vesicle. X 380

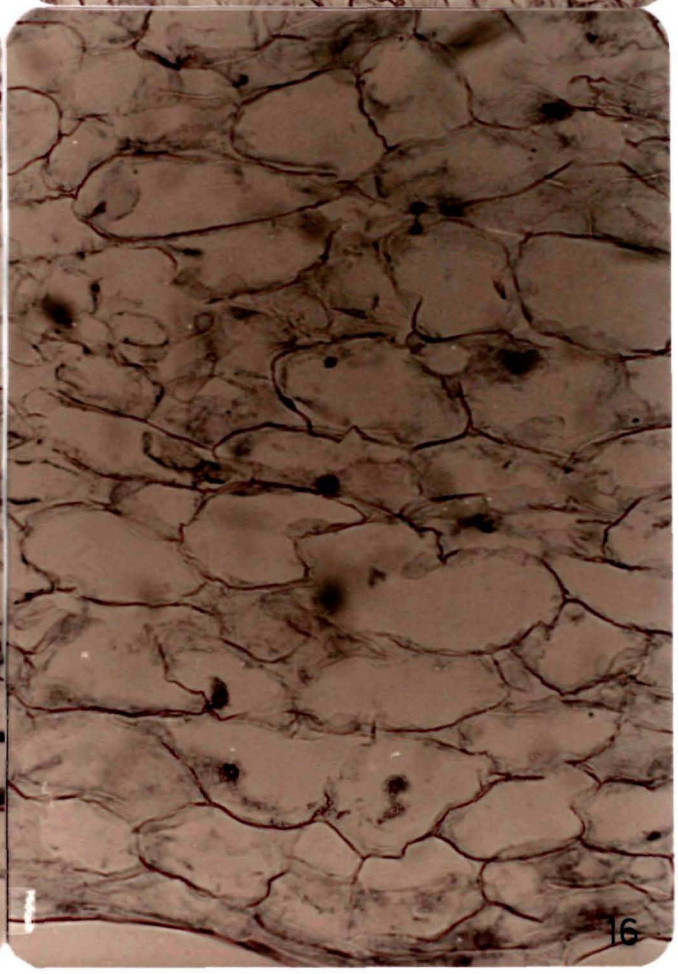
26. Distribution of nucleic acids in differentiating cells of 150 Gy irradiated juice vesicles. X 480

27. Distribution of nucleic acids in differentiating and normal cells of 10 Gy irradiated explants. X 240

28. Distribution of insoluble polysaccharides in differentiating and normal cells of 10 Gy irradiated juice vesicles. X 480







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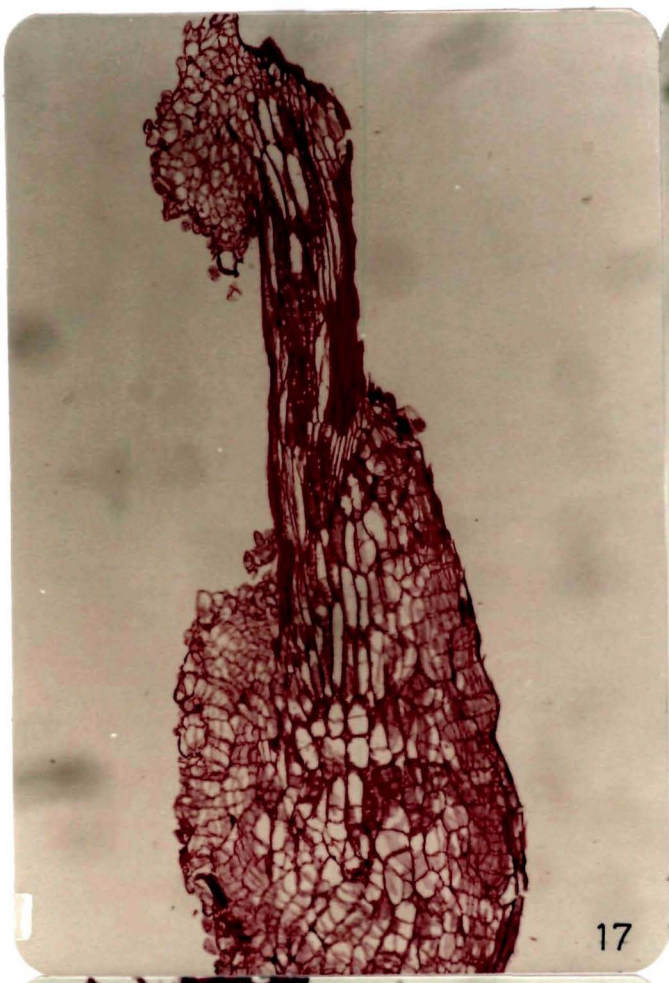

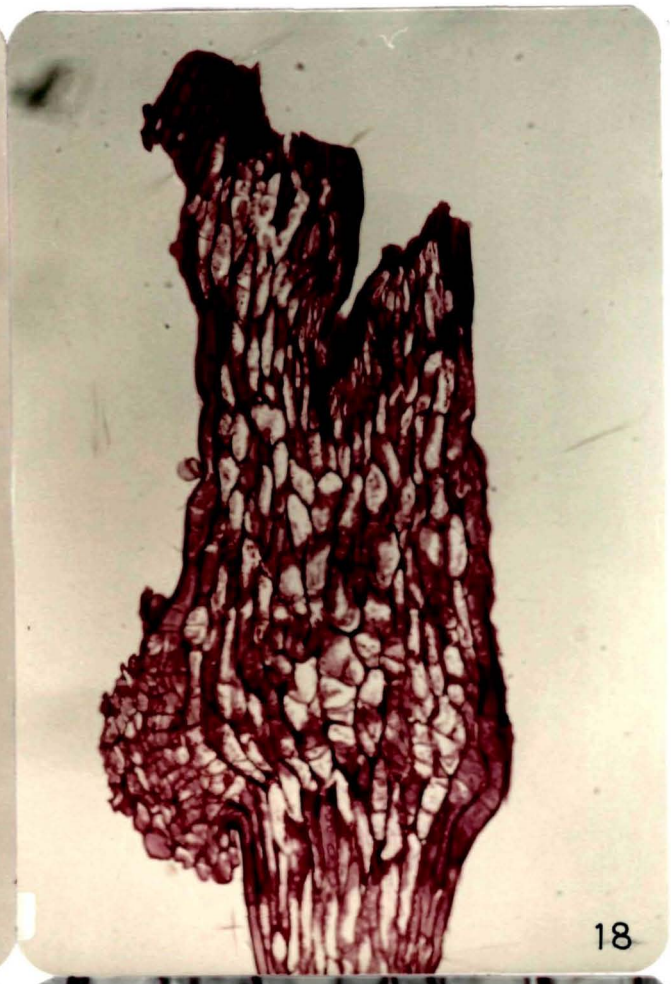

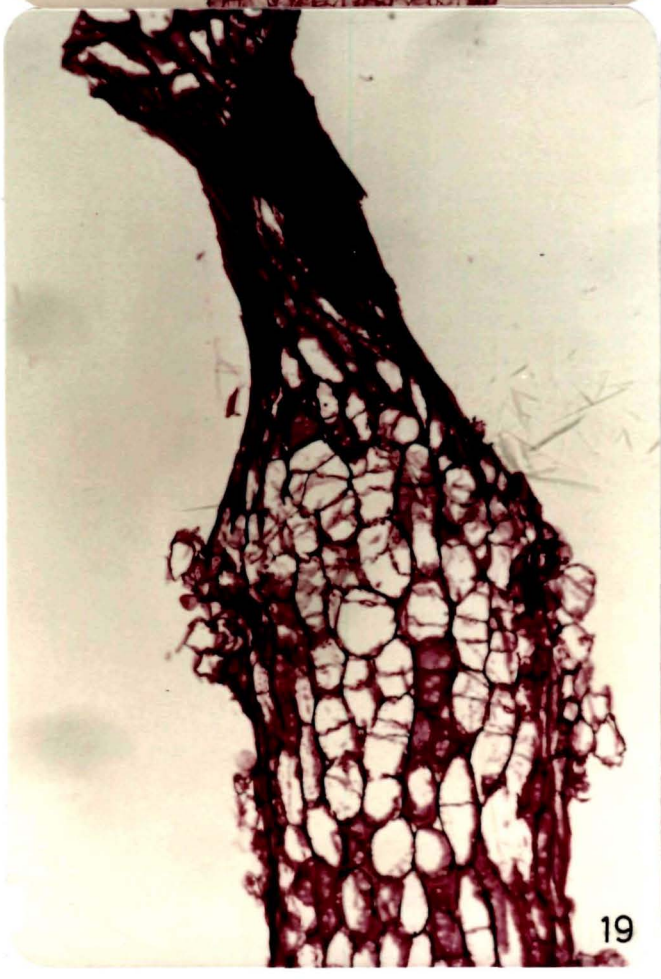

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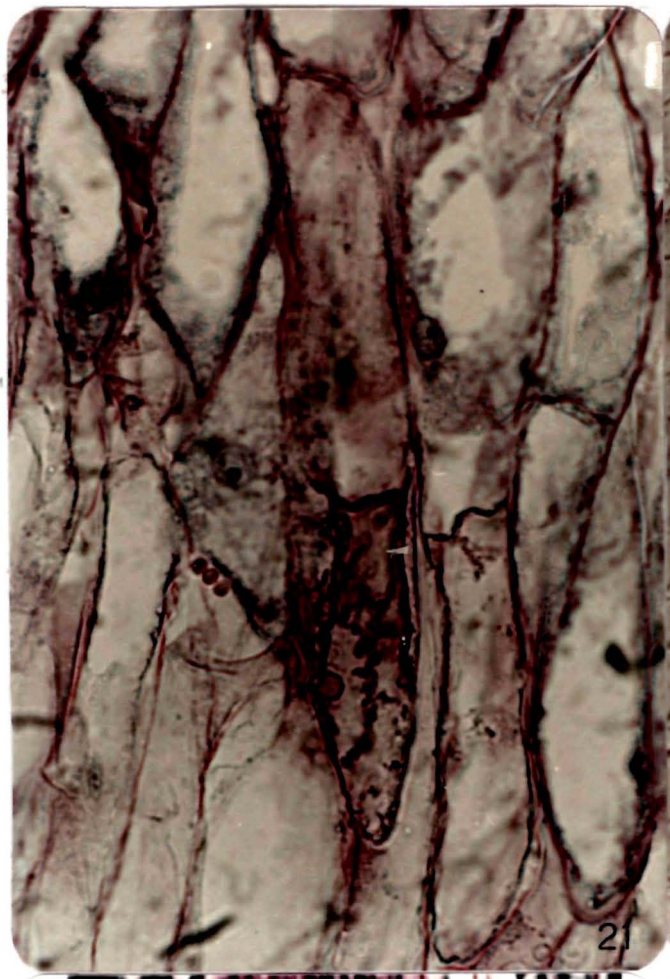
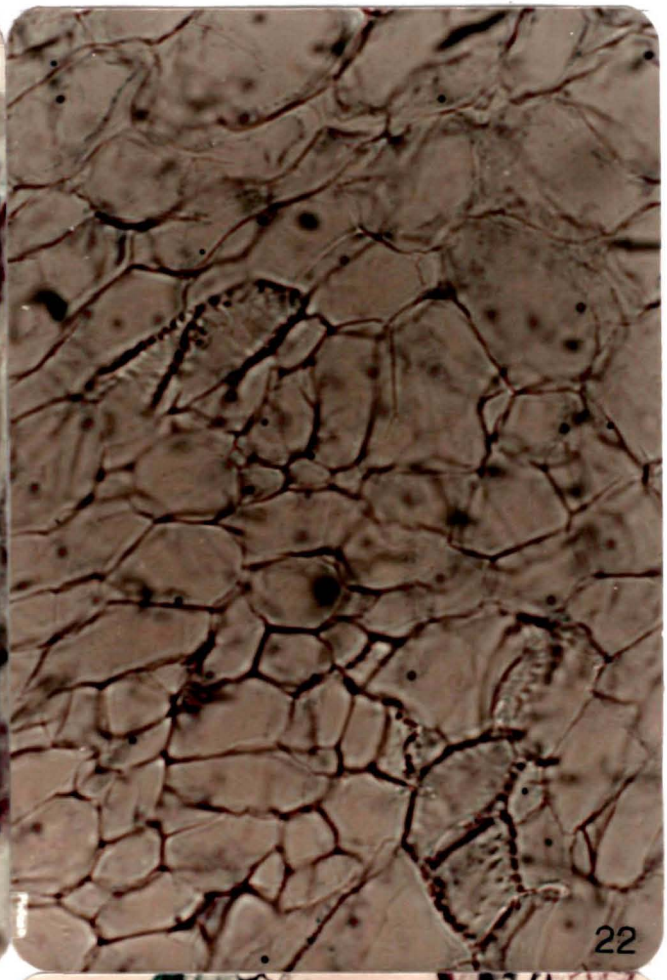




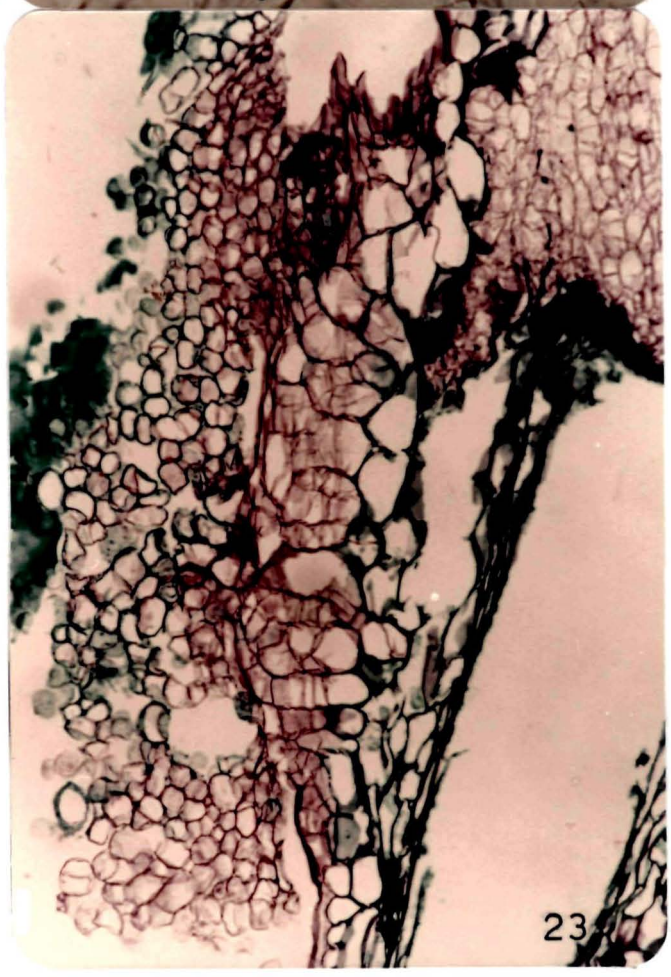
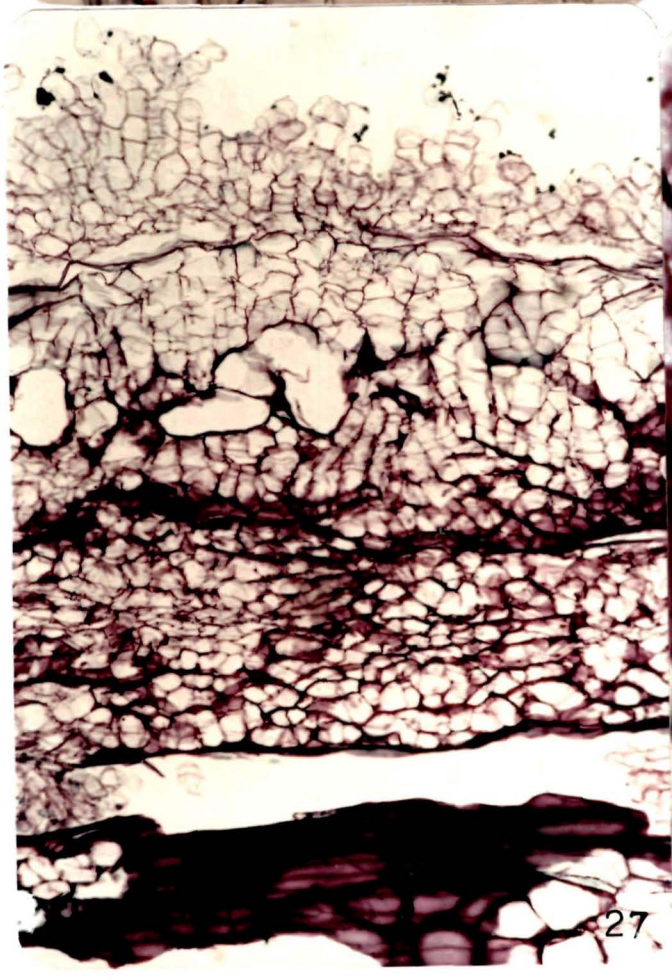


PLATE 6b (Contd...)



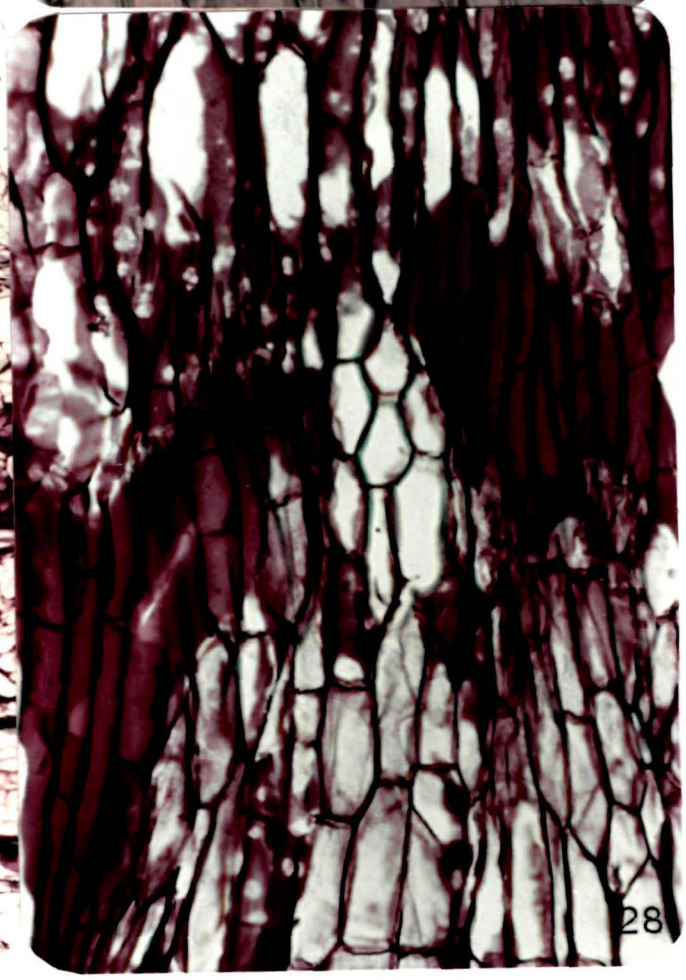


PLATE 6b (Contd...)

Plate 6c

1. Effect of radiation on development of callus in **C. limon** juice vesicle cultures:

   A - Control; B - 2.5; C - 5; D - 10; E - 50; F - 100 and G - 150 Gy.

2. Tracheids with scalariform thickenings. X 1200

3. Trachieds showing scalariform pitting. X 240

4. Tracheids with helical secondary wall thickenings. X 100

5. Trachieds with annular secondary wall thickenings. X 190

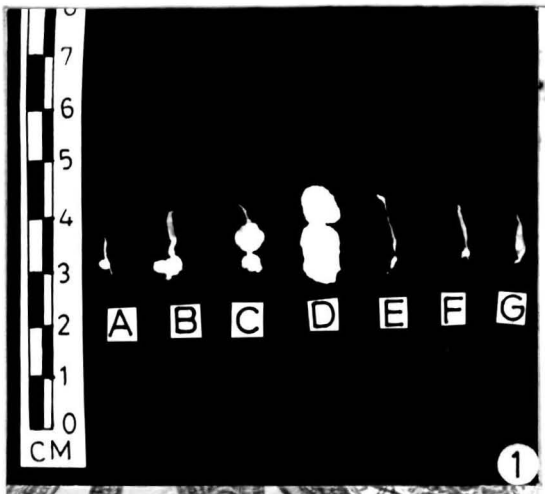
6. Pitted tracheids with reticulate secondary wall thickenings. X 240

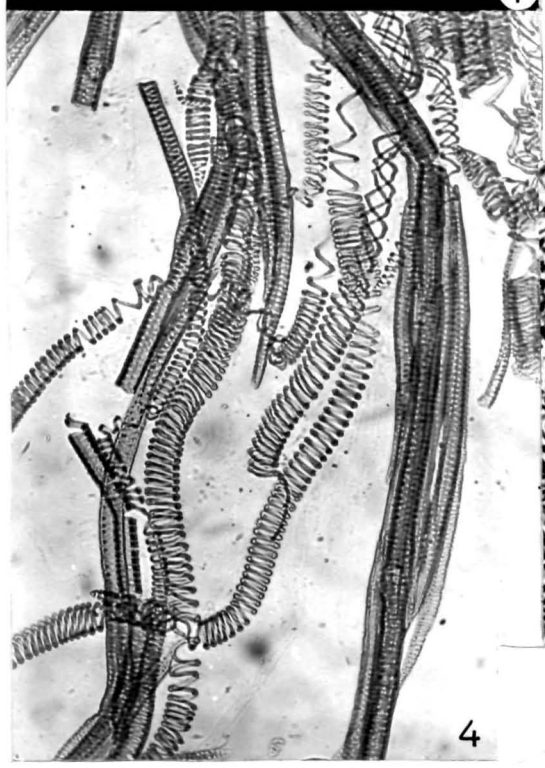
7. Branched tracheids with reticulate pitting. X 240

8. Fibers. X 190

9. Micro-sclereids. X 310

10. Macro-sclereids. X 300





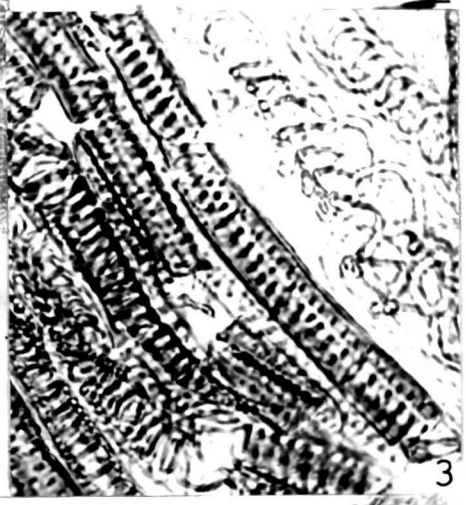


PLATE 6c

in explants given 5-10 Gy radiation dose. These tracheids revealed scalariform, reticulate, annular and spiral secondary wall thickenings (Plate 6c.2-5). Besides, some rounded pitted tracheids (GA like, Roberts 1976) and few branched tracheids (Datta et al., 1975) also differentiated (Plate 6c.6,7). However, in explants given 2.5 Gy and 50-150 Gy radiation dose tracheids with only scalariform thickening differentiated (Plate 6c.2). Both control and irradiated explants exhibited differentiation of fibers (Plate 6c.8) and microsclereids (Plate 6c.9), but their number varied in different treatments. In 5 and 10 Gy irradiated explants besides microsclereids some macrosclereids also differentiated (Plate 6c.10-12) but these were not observed in other treatments.

Histology and Histochemistry

Control: On the day of culturing all the cells of the juice vesicle have a small nucleus, little cytoplasm and large number of vacuole. Both cell wall and cytoplasm reveal presence of polysaccharides and nucleic acids (Plate 6b.1-3). But compared to cytoplasm, the cell walls stain more intensely. The nucleus of these cells, however, stains feebly for protein and DNA (Plate 6b.5,6). By 3rd day of inoculation some cells present at the base of the stalk start dividing. Such cells have relatively more dense cytoplasm and bigger nucleus (Plate 6c.13). However, no change takes place in the remaining cells of the stalk and all the cells of the vesicle region (Plate 6c.13).

The early divisions are predominantly periclinal (Plate 6c.13) but occasionally anticlinal divisions also occur (Plate

Plate 6c (contd)

11. Macro-scleries. X 380

12. Macro-sclereids. X 310

13. Longitudinal section of the juice vesicle revealing cell divisions. X 380

14. Longitudinal section of the juice vesicle showing differentiating and normal juice vesicle cells. X 240

15.q Longitudinal section of the juice vesicle revealing differentiation of tracheids. X 190

16. Differentiating fibers revealing presence of nucleus. X 100

17. Longitudinal section of the juice vesicle exhibiting differentiation of sclereids. X 310

18. Longitudinal section of the juice vesicle showing differentiating sclereids X 190

19. Longitudinal section of the juice vesicle revealing tracheids. X 300

20. Longitudinal section of the juice vesicle revealing fibers. X 300

21-23. Longitudinal sections of the juice vesicles revealing development of callus. X 100

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d - diffeentiating cells; nc - normal cells of the juice vesicle.

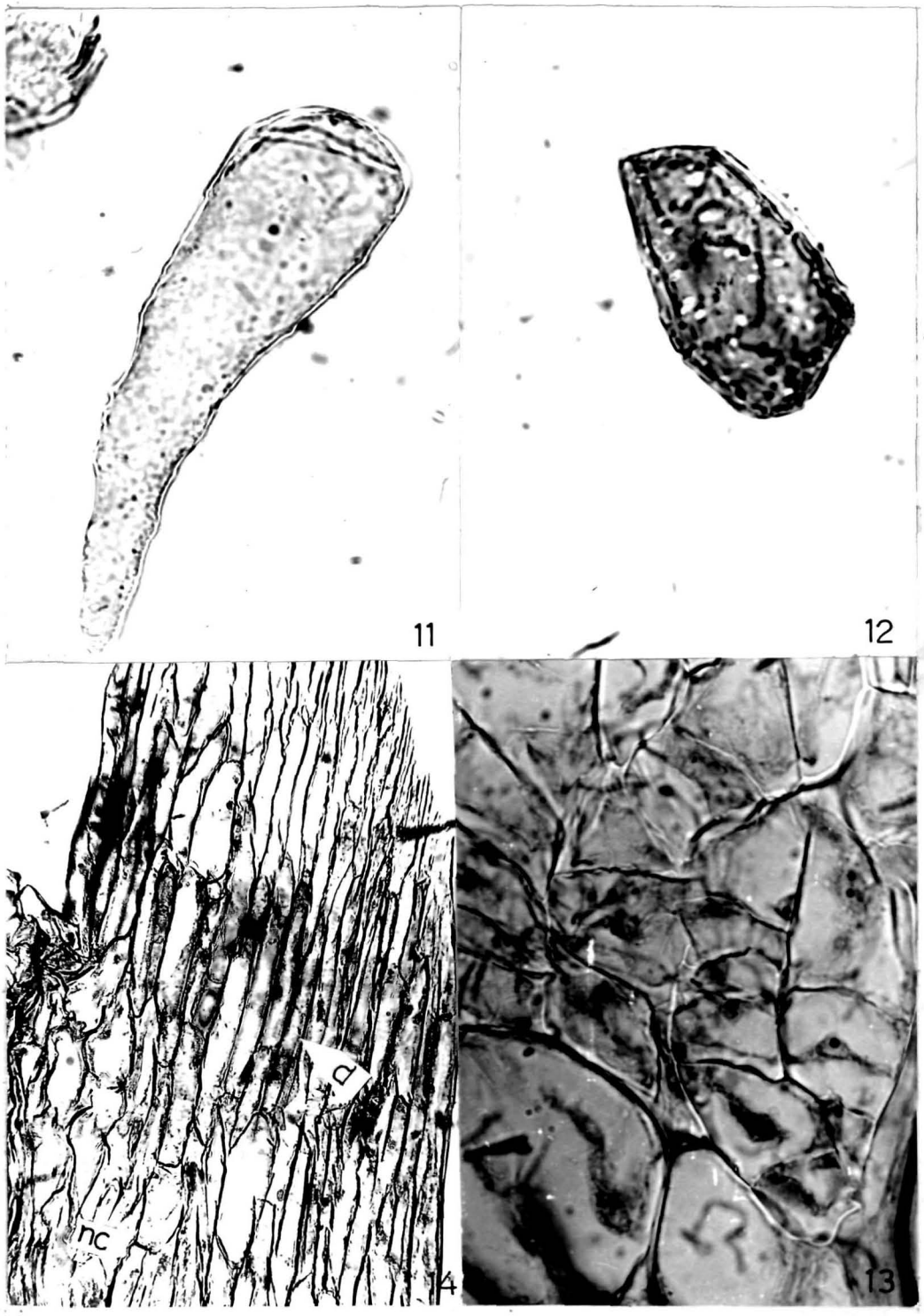

PLATE 6c (Contd...)

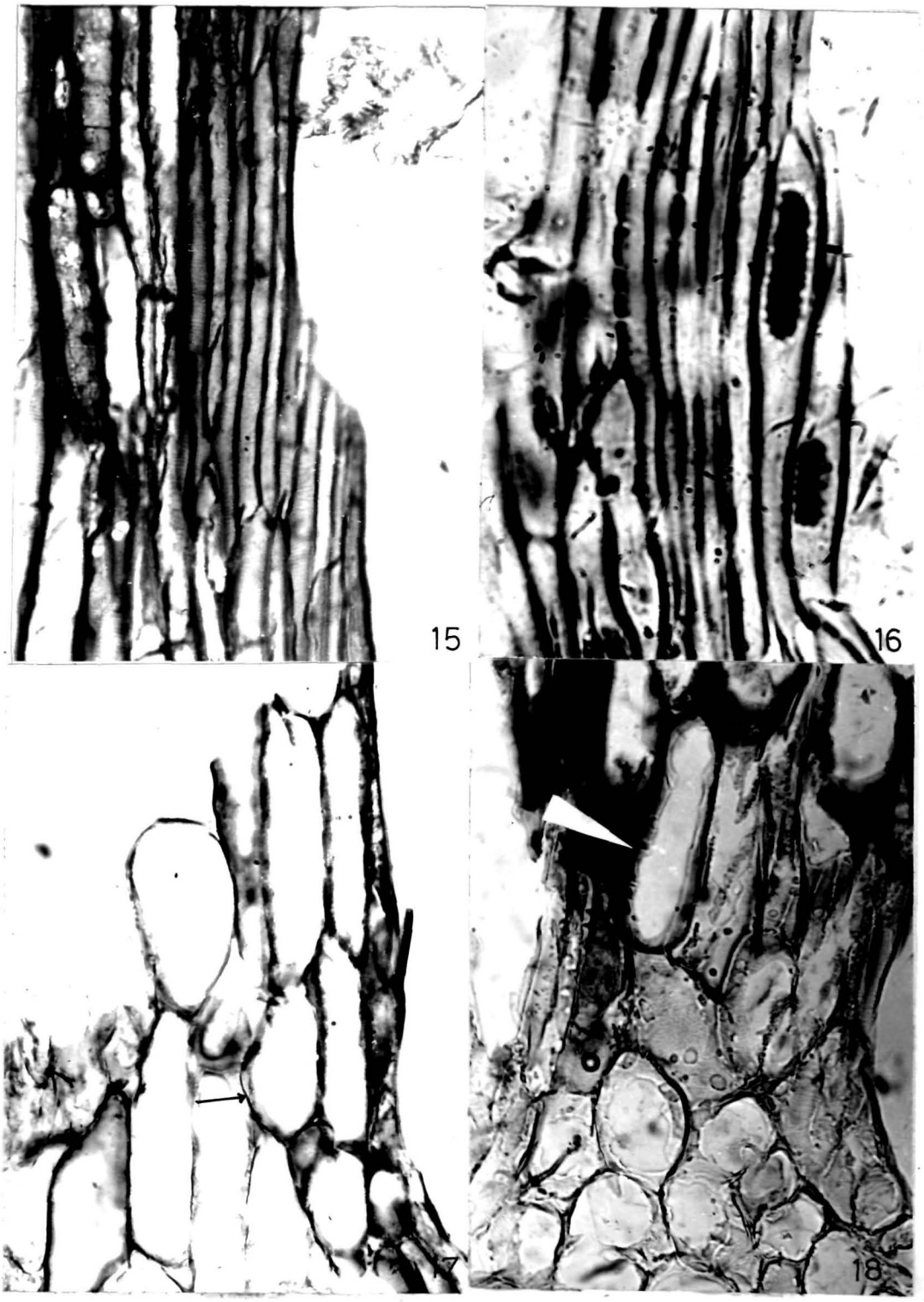

PLATE 6c (Contd...)

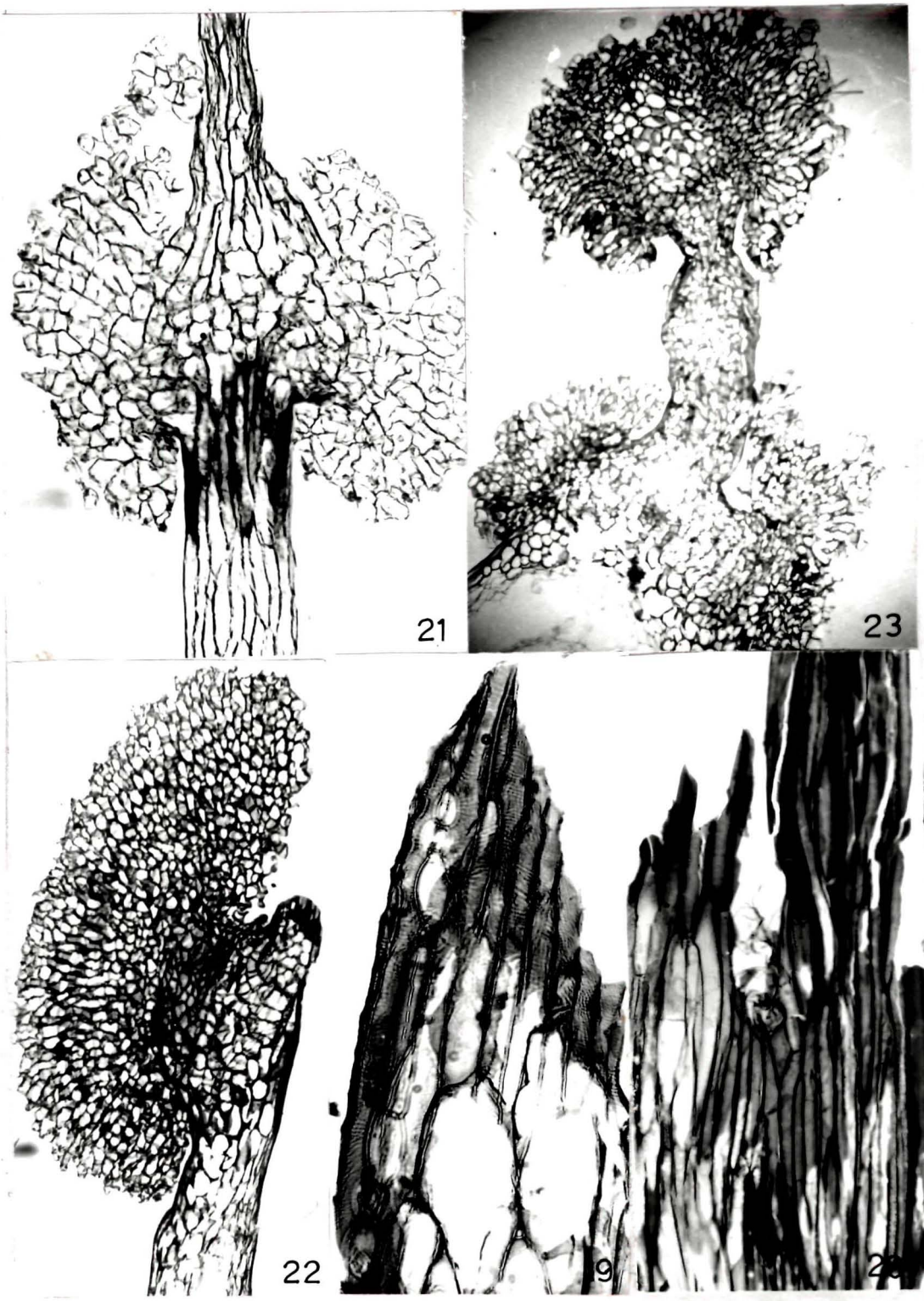

PLATE 6c (Contd...)

6c.13)/ and ultimately a callus develops. There is no change in the insoluble polysaccharide content of the dividing cells during early phases of meristematic activity (Plate 6b.7). In the callus some cells differentiate into tracheid, fibers and sclereids while others remain parenchymatous (Plate 6c.2, 8,9). These differentiating cells present different anatomical characteristics than the non-differentiating cells. Initially, size and vacuolation increases in the differentiating cells (Plate 6c.14). Next secondary wall deposition and lignification starts in the differentiating elements (Plate 6c.15,16) and by the time secondary wall thickenings are fully laid;complete autolysis of cytoplasm and nucleus, present in the early stages of differentiation, occurs (Plate 6c.19,20). Therefore, cytoplasm and nucleus are not found in differentiated tracheid and fibers (Plate 6c.19,20). During sclereids development, with the increase in cell size, the cytoplasm moves towards the periphery of these cells and a big vacuole develops in the centre of the cells (Plate 6c.17). With the progression of differentiation developments of pits in the wall and lignification occurs in differentiating sclereids (Plate 6c.18).

During early ontogeny, the cells of differentiating elements have moderate RNA staining in cytoplasm but their nucleus stains feebly (Plate 6b.8). However, a reverse situation existed in case of proteins (Plate 6b.9). As these cells complete differentiation, staining of nucleic acids and protein completely disappears from these cells suggesting their absence in differen-

tiated tracheid, fibers and sclereids. Conversely the differentiating fibers, tracheid and sclereids have more intense PAS staining in cell wall in comparison to non-differentiated cells (Plate 6b.10) and the intensity of PAS staining in these cells further increased with the progression of differentiation.

Irradiated: At the time of culturing irradiated explants also revealed histological and histochemical characteristics similar to control juice vesicles. Like control, in explants given 2.5 Gy dose some cells of the stalk undergo divisions on the 3rd day of culturing but in explants given 5 and 10 Gy radiation doses all the cells of the stalk region start dividing even on the 2nd day of culturing. But only few cells of the stalk divide on the 5th day of incubation in the juice vesicles irradiated with 50 and 100 Gy radiation doses and on the 10th day in explants given 150 Gy dose. At the time of initiation of cell divisions the distribution pattern of insoluble polysaccharide in the dividing and non-dividing cells of the irradiated juice vesicles was similar to that evident in central explants of corresponding stage (Plate 6b.4). At this stage, amongst irradiated explants, the intensity of protein and nucleic acid staining was similar to control in explants given 2.5 and 50-150 Gy (Plate 6b.11,12) but their intensity was more in explants given 5 and 10 Gy irradiation doses (Plate 6b.13,14). The irradiated explants given 2.5 and 50-150 Gy radiation doses, revealed relatively less protein in cytoplasm than nucleus. But in the explants given 5 and 10 Gy radiation doses intense protein

staining was observed both in nucleus and cytoplasm (Plate 6b.13). The intensity of protein and nucleic acid staining which increased with the increasing dose upto 10 Gy became less in explants given 50-150 Gy doses (Plate 6b.15,16).

The degree of cell division increases with the increasing radiation dose upto 10 Gy and thereafter it starts declining very fast as is evident by the callus growth (Table- 6.2, Plate 6c.1). In the beginning and towards the end of cell divisions periclinal divisions are dominant but in between anticlinal divisions became predominant. Thus a nodule like structure develops in the stalk region. The position and the size of the nodule depends on the orientation and degree of cell division. For example, while some juice vesicles reveal a big nodule at the distal end of the stalk surrounding stalk from both the side (Plate 6c.21), others reveal only one nodule on any one side of the stalk either near the tip or middle of the stalk (Plate 6c.22), still others may develop nodules both in the stalk and vesicle region (Plate 6c.23). Compared to the control growth of the callus was more in explants given upto 10 Gy radiation dose. At higher doses, however, growth of the callus declined (Table-6.2). Smallest calli were produced by 150 Gy irradiated explant (Plate 6c.1). In such explants the calli were smaller than control. In 10 Gy irradiated explants few peripheral cells of the vesicle region also undergo both periclinal and anticlinal divisions leading to the development of nodule like structures in the vesicle region (Plate 6c.1 and 6c.21).

At the commencement of cytodifferentiation, cytoplasm and cell wall of the cells of the irradiated explants had more insoluble polysaccharides than control (Plate 6b.17-19). The intensity of insoluble polysaccharides increased with increasing radiation doses in differentiating cells (Plate 6b.18,19) and maximum polysaccharides were evident in cell walls of the cells of explants treated with 150 Gy (Plate 6b.19). However, compared to control, the protein and nucleic acid contents were higher in differentiating cells at low radiation doses (2.5, 5 and 10 Gy, Plate 6b.20-24) which declined at higher doses in the differentiating cells of the irradiated explants (50-150 Gy, Plate 6b.25,26). In comparison to the non-differentiating cells initially differentiating cells reveal more polysaccharides, protein and nucleic acid (Plate 6b.22,27,28). But in the final stages of differentiation while protein and nucleic acids disappear in the differentiated cells, the polysaccharides further increase as in control. The differentiated elements revealed the presence of lignin since in Ajur B test the fully differentiated cells stained green (Plate 6b.23-25).

Discussion

In the present study white (fluorescent) light although stimulates cell division it is unable to improve the differentiation of tracheid, fibers and sclereids in cultured **C. limon** juice vesicles. Besides, compared to dark conditions, white light delayed and induced inhibition of cytodifferentiation.

Thus white light does not favour differentiation of tracheid, fibers and sclereids in cultured **C. limon** juice vesicle. This is in agreement with the findings of Phillips and Dodds (1977) and Yeoman and Davidson (1971) on TE differentiation in cultured jerusalem artichoke tuber tissues. Further, the diversity of differentiation was more under dark conditions than in white light since no tracheid differentiation occurred in explants given white light during culturing.

In the present investigation low doses of radiation (2.5-10 Gy) stimulated both cell division and cytodifferentiation of tracheid, fibers and sclereids. But radiation doses above 10 Gy inhibited both the processes. The diversity of differentiation including patterns of secondary wall depositions in tracheids was more, compared to control, in explants given low radiation doses. Higher radiation doses, however, had inhibitory effect. Both stimulation and inhibition of growth and differentiation by low and high doses of radiation are reported in literature (Saric **et al.**, 1961; Kahan **et al.**, 1972; Kahan, 1974). Moreover as in the present study, Dodds and Phillips (1977), Malawer and Phillips (1979) and Phillips and Arnott (1983) also found that radiation doses which inhibit cell division inhibit differentiation of TE as well.

In the present investigation an attempt was made to investigate the histological and histochemical manifestations of radiation effect on cytodifferentiation. It was found that juice vesicles which exhibit stimulated differentiation also

reveal more protein and nucleic acid in comparison to control. Conversely juice vesicles with inhibited cytodifferentiation have less protein and nucleic acids. However, the polysaccharide content increased in the irradiated explants irrespective of the dose used and intensity of differentiation. Thus while the stimulatory effects of radiation on cytodifferentiation are associated with the presence of more protein and nucleic acids in the irradiated explants, the reverse is true where cytodifferentiation was inhibited by the radiation doses. It may therefore be assumed that stimulatory effects of radiation on differentiation of tracheid, fibers and sclereids are mediated through their effect on the protein and nucleic acid metabolism of the irradiated explants.

Scanty Discussion

CHAPTER VII

HISTOLOGY AND HISTOCHEMISTRY OF GRANULATION DISORDER IN *C. GRANDIS*

Introduction

Granulation disorder of **Citrus** species was first discovered by Turrel and Bartholomew (1939) in **C. sinensis** osbeck. They found that due to granulation the juice in the cells of the sac becomes more or less gelatinized, rendering juice sacs hard. Later in granulated juice vesicles some of its cells may become thickwalled and pitted. Finally, softening and collapse of the granulated juice vesicles occurs due to the disintigration of the cells in the sac region. The disintigration of juice sac cells progresses centrifugally. The granulated juice vesicles also become juice-less resulting in deterioration of the quality of fruits. Since granulation disorder is very frequent in **C. grandis** the present study attempts to investigate and correlate histological and histochemical manifestations of granulation disorder during juice vesicle development of this species.

Materials and Methods

Developing **C. grandis** fruits were collected every month from May to January, when they ripened. Data on fruit size, size and weight of the juice vesicles, and pH of the juice were recorded every month. Juice vesicles collected from fruits of different developmental stages were fixed in F.A.A. for 24 hr and stored in 70% ethanol. Squash preparation of the juice vesicles, cleared in 4% NaOH (w/v) for 24 hr at 30°C and, stained with safranin 'O' (0.03%, w/v) for 10 min. were also made to study granulation. Differential staining of thickwalled abnormal cells

and normal cells of juice vesicles was achieved by destaining safranin stained juice sacs in 1 N HCl. These differentially stained juice vesicles were stored in glycerine for future use and dissected when required for microscopic observations.

For histological and histochemical investigations of granulations, juice vesicles were fixed in F.A.A. (24 hr), dehydrated through tertiary butyl alcohol series and embedded in paraffin wax for microtomy. Serial sections 12 µm in thickness were cut using a Leitz rotary microtome. for histological investigations safranin-haematoxylin staining combination was used (Johansen, 1940) while histochemical localization of insoluble polysaccharides, general proteins and nucleic acids in the juice vesicles was done using following techniques:

Insoluble polysaccharide (Jensen, 1962): First deparafinized sections were brought to water for 3 - 5 min. Next slides were incubated in 0.5% aqueous solution of periodic acid for 30 min. at 25°C. At the expiry of incubation period the slides were washed for 5 min. in running water and then stained with Schiff's reagent for 25 min. at 4°C. The stained sections were first rinsed in water and then placed in 2% sodium metabisulfite for 1-2 min. The slides were again washed in running water for 2-3 min., dehydrated and mounted in D.P.X. The insoluble polysaccharides and starch stain purplish-pink.

General protein (Mazia **et al.**, 1953): The deparafinized sections were first transferred to absolute alcohol and then placed for

Table 7: Morphological features of the citrus fruits and pH of the juice at different developmental stages of C. grandis fruit.

Developmental stages of fruit	Fruit diameter (cm)	Length of the vesicle (mm)	Weight of the vesicles (mg/ explant)	pH of the juice
May	2.84	1.25±0.03	1.1±0.02	2.5
June	3.40	2.04±0.08	2.1±0.14	2.5
July	5.56	5.04±0.10	10.9±0.19	2.5
August	10.45	9.12±0.24	19.8±0.77	2.5
September	12.40	18.80±1.16	28.8±1.16	2.5
October	13.17	20.06±0.08	40.2±0.97	2.72
November	13.43	20.34±0.24	51.8±1.60	2.95
December	14.34	20.64±0.10	52.6±2.33	3.23
January	15.16	20.64±0.20	52.6±1.49	3.24

30 min. in 0.1% solution of bromophenol blue prepared in 95% alcohol having 10% mercuric chloride. Subsequent to staining in bromophenol blue, slides were first washed in 0.5% aqueous acetic acid for 3-5 min. and then dipped for 3 min. in Sorenson buffer. The sections were quickly dehydrated through TBA, cleared in xylene and mounted in D.P.X. Proteins stain blue.

Nucleic acid (Flax and Himes, 1952): The deparafinized sections were hydrated and placed in Azur B solution (0.25 mg/ml) prepared in citrate buffer (pH, 4.0) for 2 hr at 50°C. Once staining was over, the slides were washed in water, placed in pure T.B.A. for 30 min, and then passed through xylene and mounted in D.P.X. Both the nucleic acids stain differently. DNA stains green blue while RNA appears either purple or dark blue.

Results

Table-7 gives data on size of the fruit, size and weight of the juice vesicles and pH of the juice at different stages of fruit development. During May when fruits of **C. grandis** have a diameter of 2.84 cm juice vesicles are not differentiated. The juice vesicles become apparent in the month of July when developing fruits have attained a diameter of 4.61 cm. At this stage the pH of the juice present in the juice vesicle is 2.5. With the fruit development the fruit size increases and at maturity (during January) the diameter of the fruits is 15-16 cm and the juice vesicles,which are 20.05 mm long, weigh 54.4 mg. The pH of the juice of the ripe fruit is 2.97 (Table-7).

Plate 7a

1. Longitudinal section of the normal juice vesicle in the month of May. X 75

2. Longitudinal section of the normal juice vesicle during the month of May. X 190

3. Longitudinal section of the normal juice vesicle in the month of June. X 190

4. Longitudinal section of the normal juice vesicle in the month of September. X 190

5. Longitudinal section of the normal juice vesicle in the month of September showing neck region. X 120

6. Longitudinal section of the normal juice vesicle in the month of December. X 190

7. Longitudinal section of the granulated juice vesicle in the month of July showing presence of TE in the neck region. X 310

8. Tracheids with annular secondary wall thickenings and scalariform pittings. X 300

9. Trachieds with alternate pittings. X 120

10. Vessels. X 470

11. Branched tracheid. X 310

12. GA trachied. X 120

13. Longitudinal section of the granulated juice vesicle towards periphery exhibiting thick walled cells of vesicle membrane. X 300

14. Longitudinal section of the granulated juice vesicle showing differentiation of thick walled pitted cells. X 470

15. Longitudinal section of the granulated juice vesicle towards the distal end of the vesicle revealing differentiation of fibers. X 240

16. Longitudinal section of the granulated juice vesicle in the month of January exhibiting disintegration of the cells of the middle region. X 310

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vm - vesicle membrane, m - middle region, c - central region, s - neck region.

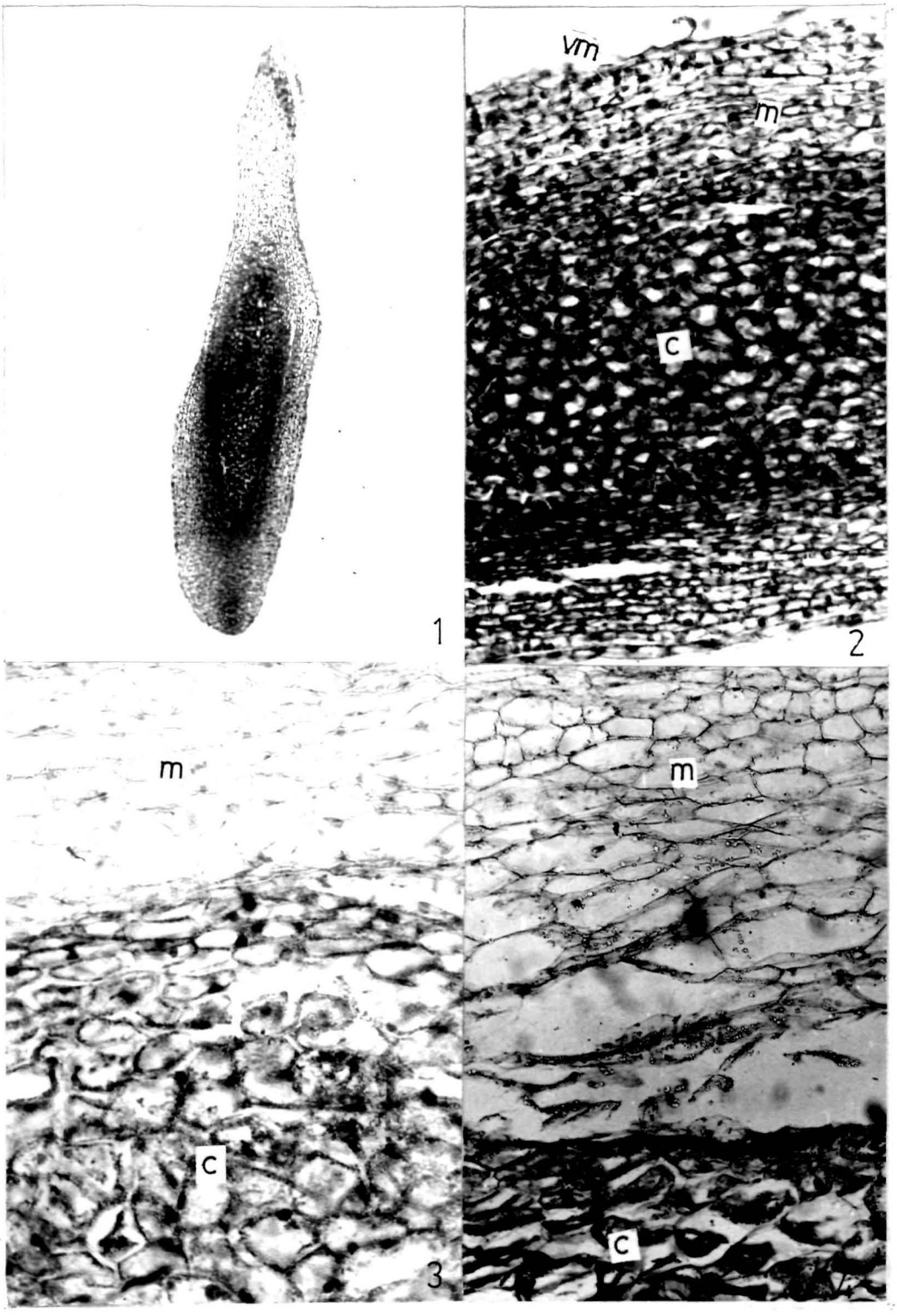


PLATE 7a

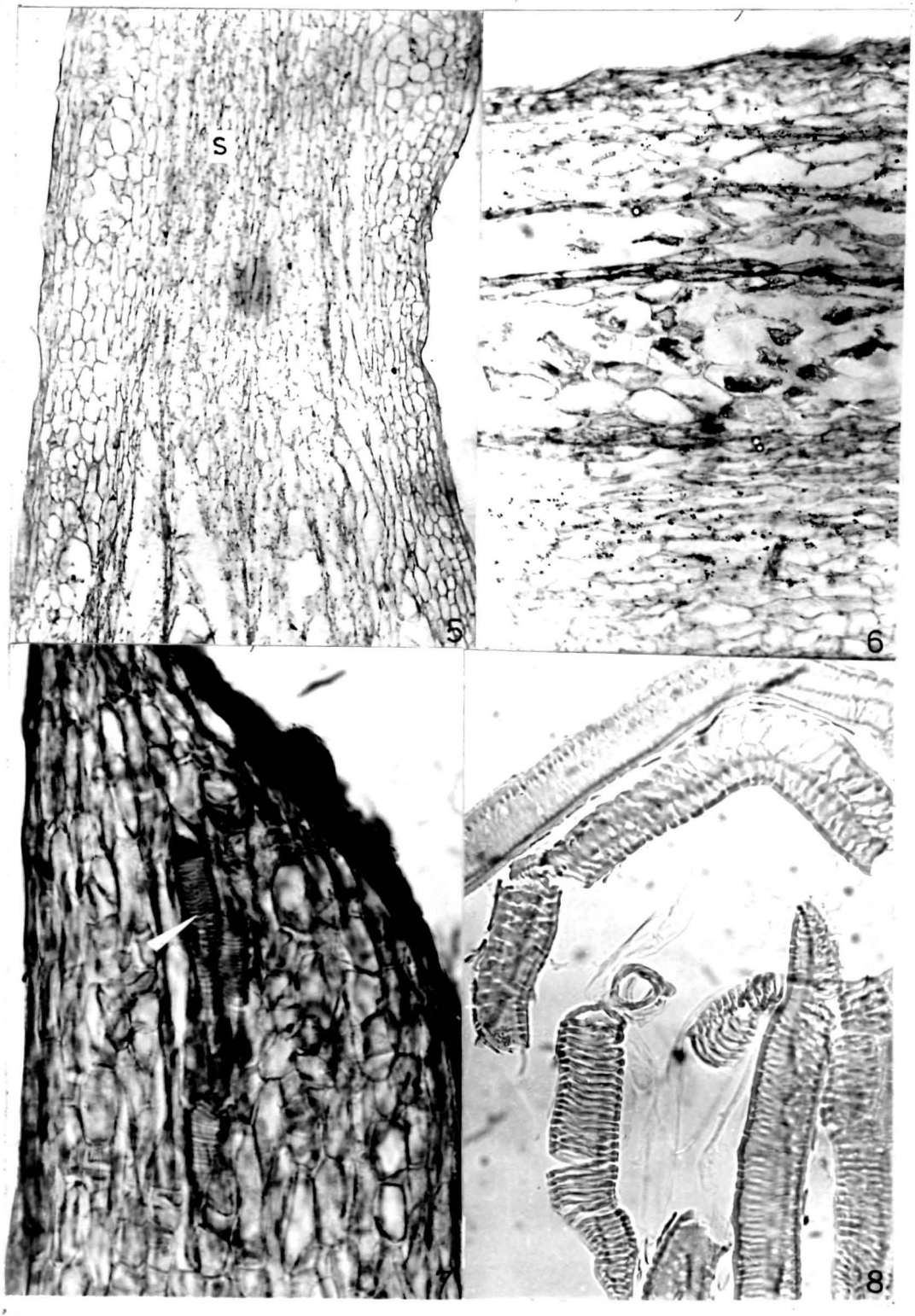
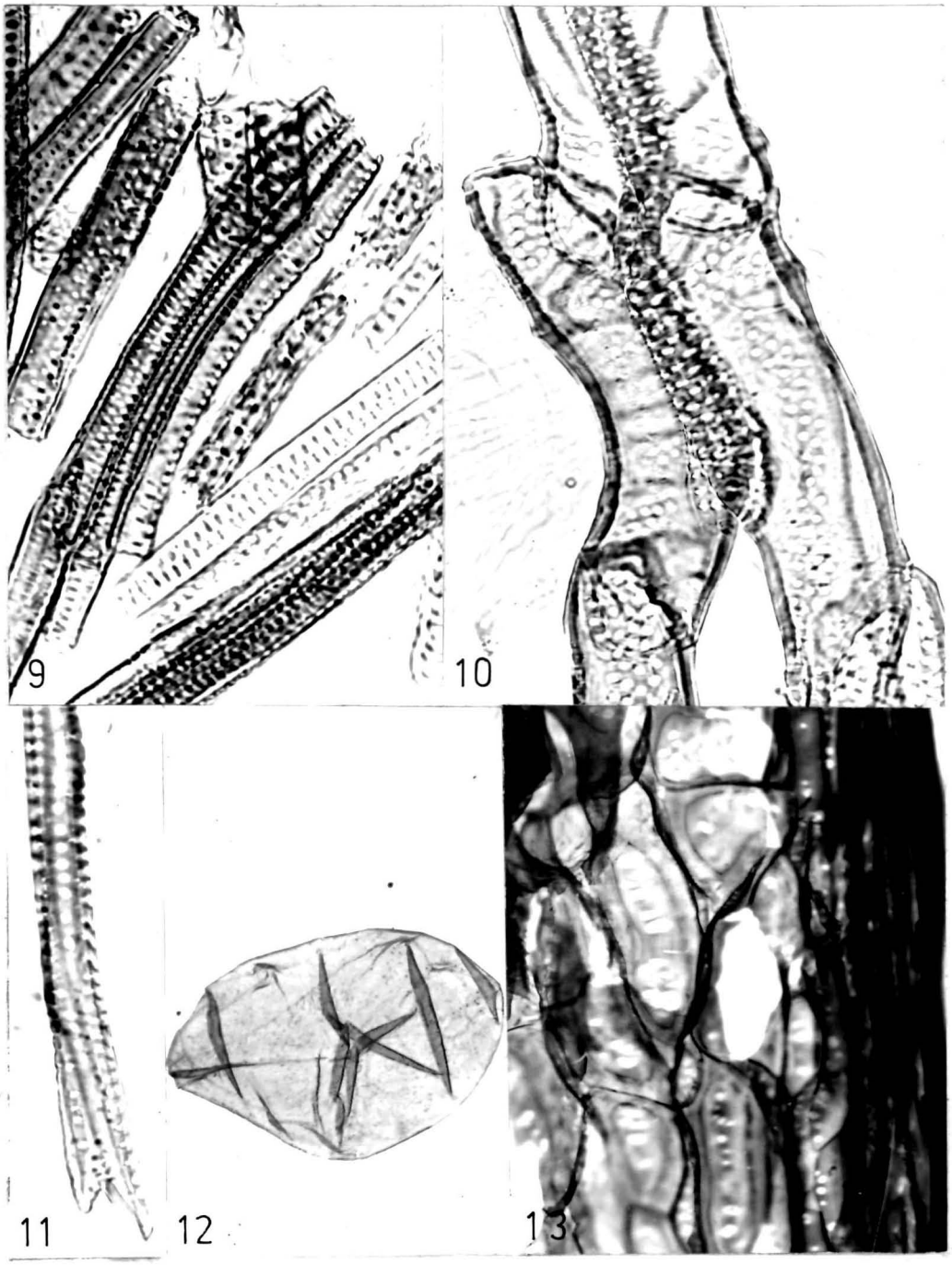


PLATE 7a (Contd...)



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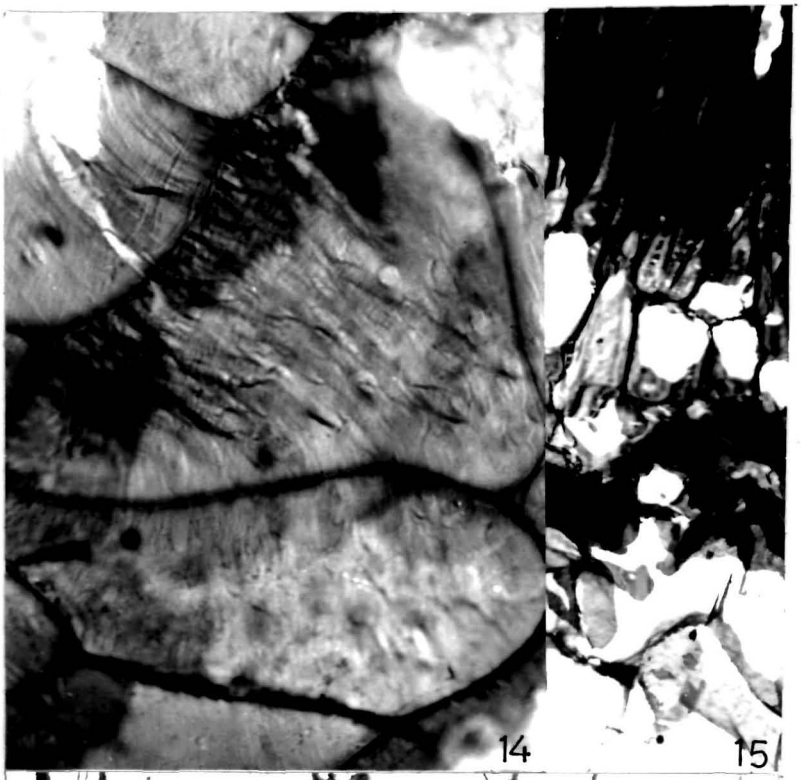


PLATE 7a (Contd...)

Normal juice vesicle development: The development of juice vesicle in developing fruit of **C. grandis** commences in the month of May. Initially the juice vesicle is made up of parenchyma cells which are relatively isodiametric and non-vacuolated (Plate 7a.1). At this stage, in the juice vesicle ,neck is ill differentiated but the sac region is well demarcated. Anatomically the juice vesicle is distinguishable into epidermis (vesicle membrane), middle region and a central region (Plate 7a.2). The cells of the outer epidermal layer of the juice vesicles (vesicle membrane) are parenchymatous and longer than broad. Inner to the vesicle membrane, four to five layers of small parenchyma cells constitute middle region of the sac. In the centre of the sac two to three layers of large parenchyma cells are present. All the cells of the juice vesicles exhibit prominent nucleus. Central cells have relatively more cytoplasm and bigger nucleus as compared to the cells of the middle region and vesicle membrane (Plate 7a.2). In the juice vesicle, cells of the central region have high nucleic acid contents both in cytoplasm and nucleus (Plate 7b.1). The cells of the central region reveal maximum accumulation of RNA and DNA in cytoplasm and nucleus respectively (Plate 7b.1). Accumulation of RNA and DNA in cells of the juice vesicle decreases gradually towards periphery. Epidermal cells and cells of middle region adjacent to it exhibit moderate RNA staining in the cytoplasm while nuclei have high DNA content. Distribution of protein in the juice vesicle also follows nucleic acid pattern; maximum being in the central region and least in the epidermal cells while cells of the middle region have

Plate 7b

1. Normal juice vesicle showing differences in nucleic acids content of different regions. X 75

2. Distribution of general proteins in different regions of the normal juice vesicles. X 75

3. Distribution of insoluble polysaccharides in the normal juice vesicles. X 75

4. Distribution of general protein in the juice vesicle cells of the stalk region. X 120

5. Distribution of nucleic acids in the normal juice vesicle. X 75

6. Distribution of general protein in the sac region of normal juice vesicle X 190

7. Distribution of insoluble polysaccharides in the normal juice vesicle. X 75

8. Sac region of normal juice vesicle showing distribution of insoluble polysaccharide and presence of starch grains. X 190

9. Sac region of normal juice vesicle showing distribution of insoluble polysaccharide during September. X 190

10. Sac region of normal juice vesicle showing distribution of insoluble polysaccharide in decline and the starch grain content of the cells. X 190

11. Sac region of normal juice vesicle showing distribution of insoluble polysaccharides during ripening. X 120

12. Sac region of juice vesicle showing distribution of general protein in the normal juice vesicle during maturation in the month of October. X 190

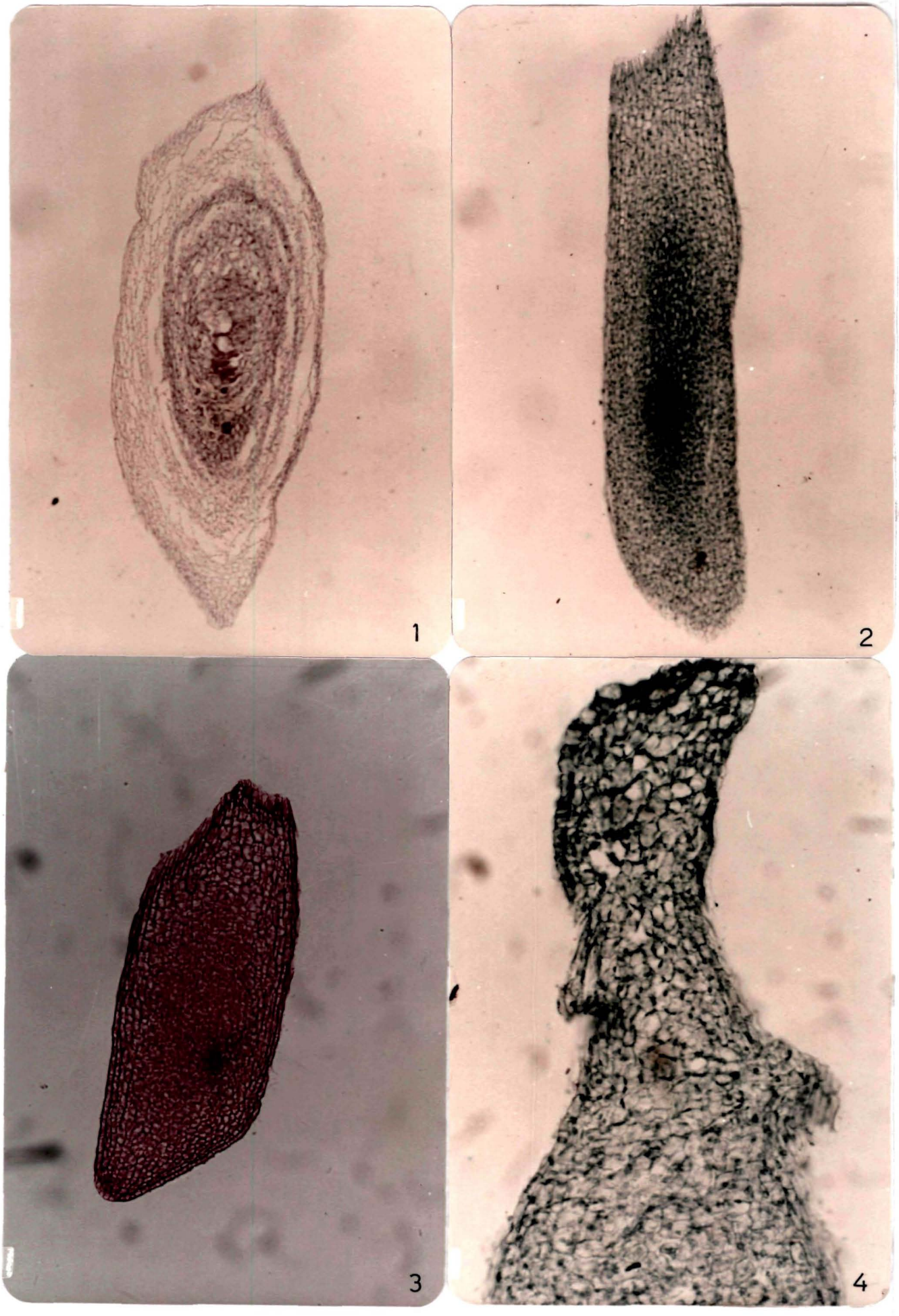
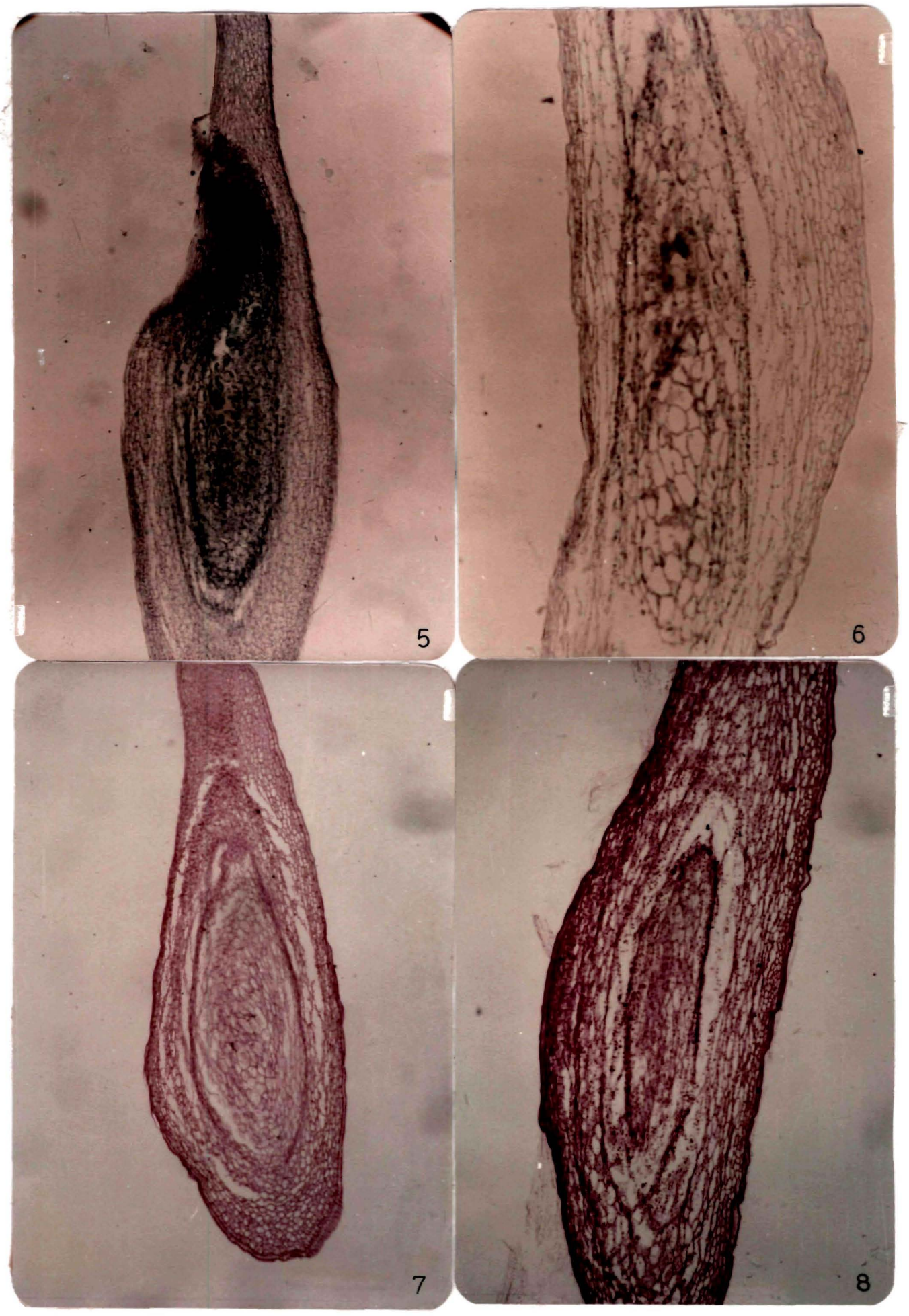


PLATE 7b



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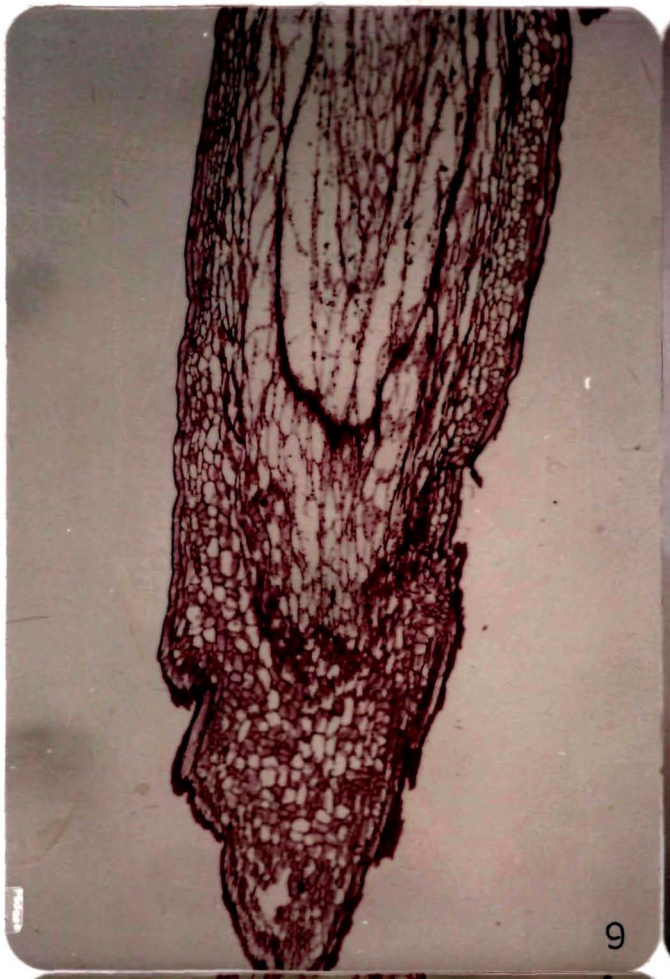
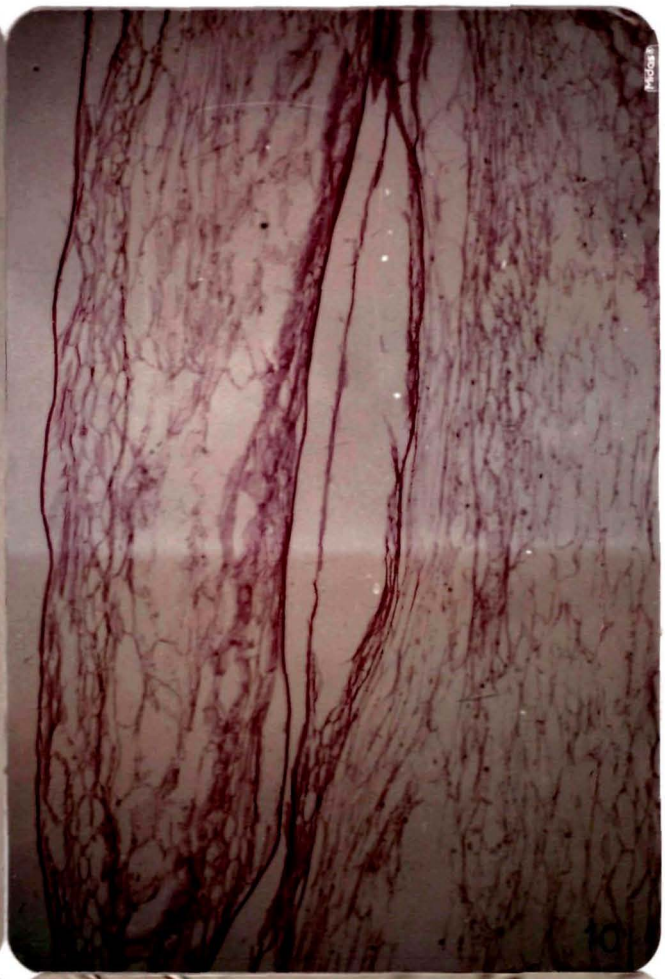




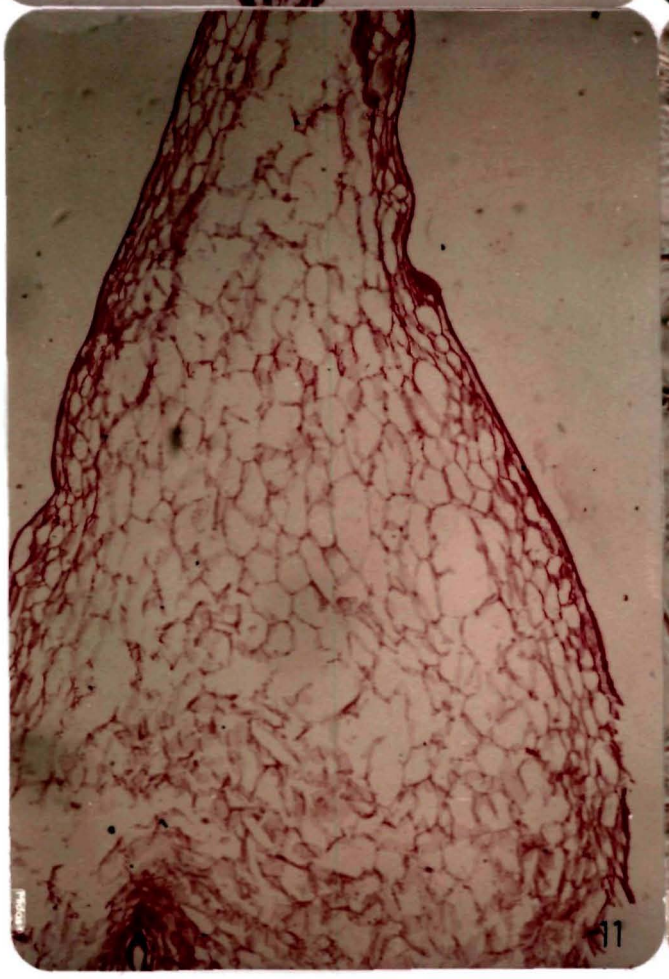


PLATE 7b (Contd...)

intermediate concentration of protein (Plate 7b.2). Nuclei of all juice vesicle cells reveal presence of protein by staining intense blue with bromophenol blue. Cell wall and cytoplasm of central cells are also highly protein positive. But cells of epidermis and middle region reveal moderate intensity of protein staining in cytoplasm and faint staining in cell wall (Plate 7b.2). Distribution pattern of insoluble polysaccharides in juice vesicle cells was different, as compared to protein and nucleic acid patterns, since cell walls and cytoplasm of cells of all the regions (epidermis, middle and central) of the juice vesicles stained almost equally with PAS (Plate 7b.3).

During the month of June some changes occur in the vesicle region. There is an increase in the central cells making middle portion of the vesicle more broad and swollen. However, the increase in the cell number is relatively less towards anterior and posterior ends causing vesicle to gradually taper at the two ends. Further, at this stage cells of the central region start loosing contact with each other (Plate 7a.3). With the progression of the juice vesicle development, cells of the stalk region also increase in number and size. Therefore, the juice vesicles, at this stage of development, can be differentiated into a narrow elongated stalk region and a broad vesicle region. Stalk of the juice vesicle is made of parenchymatous cells which are bounded by a vesicle membrane or epidermis towards the periphery (Plate 7b.4). Compared to the cell wall, cytoplasm of the cells of the stalk region have very little protein. However,

7a.5-6). At this stage, the cells of the middle region became loose and cell wall of the juice vesicle cells appears very thin (Plate 7a.6). Once the juice vesicles have developed fully the starch grains accumulation start diminishing and by the month of October all the starch grains have almost vanished (Plate 7b.10). In ***C. grandis*** *fruit, which have either matured* or are ripening, the intensity of PAS staining does not vary (Plate 7b.10,11). But a decreasing trend was evident in protein and nucleic acid staining (Plate 7b. 12 - 15). The intensity of protein staining decreased very sharply during October (Plate 7b.12). Very little protein was noticed in cytoplasm and nucleus of the central cells while cells of the other regions revealed absence of protein. Similar trend was noticed for nucleic acid also. Although very faint staining revealed presence of RNA in the cytoplasm of the central cells, cells of the other region have no nucleic acids in cytoplasm (Plate 7b.14). During December-January (Senescent stage) all the cells of the juice vesicle exhibited little protein and nucleic acids in cytoplasm and nucleus of the cells (Plate 7b.13,15).

Granulated juice vesicle: During early ontogeny (May-June) both normal and granulated juice vesicles are similar histologically and histochemically. First signs of granulation appear in the month of July with the differentiation of abnormal cells (tracheary cells with spiral thickenings) in the neck region (Plate 7a.7). The number and diversity of TE in the neck region increased during October-November when tracheids with annular and scalariform

Plate 7b (contd.)

13. Sac region of normal juice vesicle showing distribution of general protein during December-January. X 150

14. Sac region of normal juice vesicle showing distribution of nucleic acids during maturation in the month of October. X 190

15. Sac region of normal juice vesicle showing distribution of nucleic acids during December-January. X 150

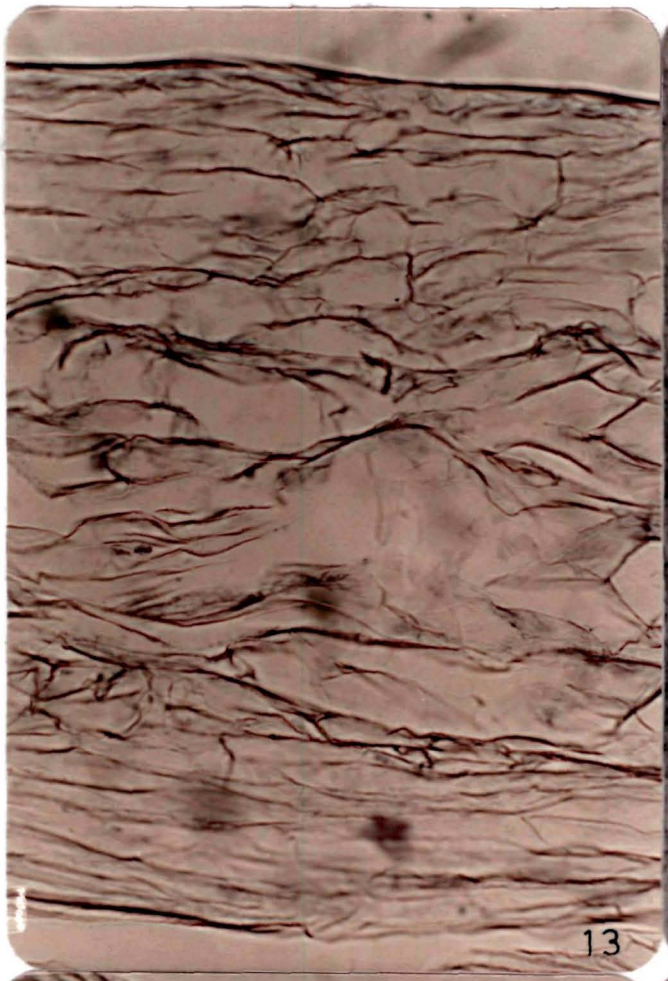
16. Distribution of nucleic acids in granulated juice vesicle in the month of July (lignified abnormal cells take green colour of lignin with Azur B test). X 240

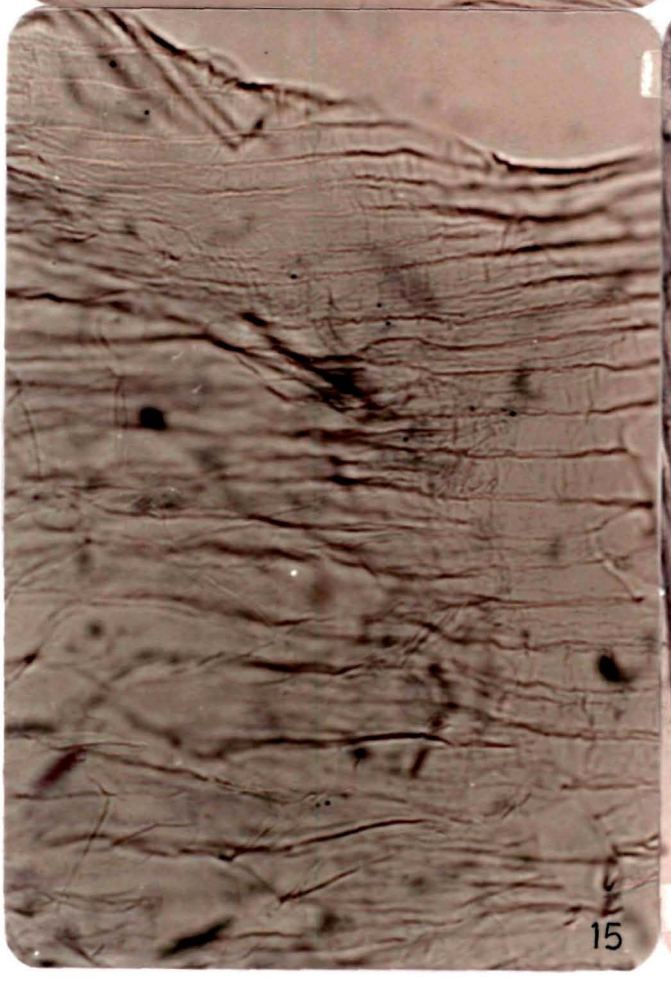
17. Sac region of the granulated juice vesicle showing distribution of general protein during fruit maturation. X 150

18. Sac region of granulated juice vesicle showing distribution of nucleic acids during fruit maturation. X 150

19. Sac region of granulated juice vesicle showing distribution of insoluble polysaccharides during fruit maturation. X 240

20. Sac region of granulated juice vesicle showing distribution of insoluble polysaccharides in ripe fruit. X 120





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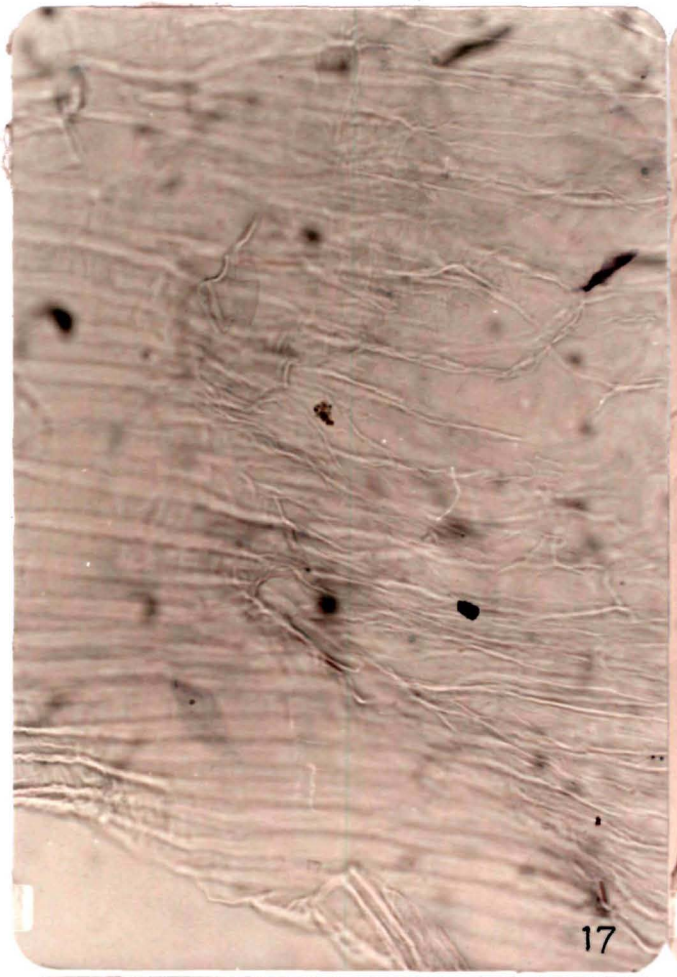
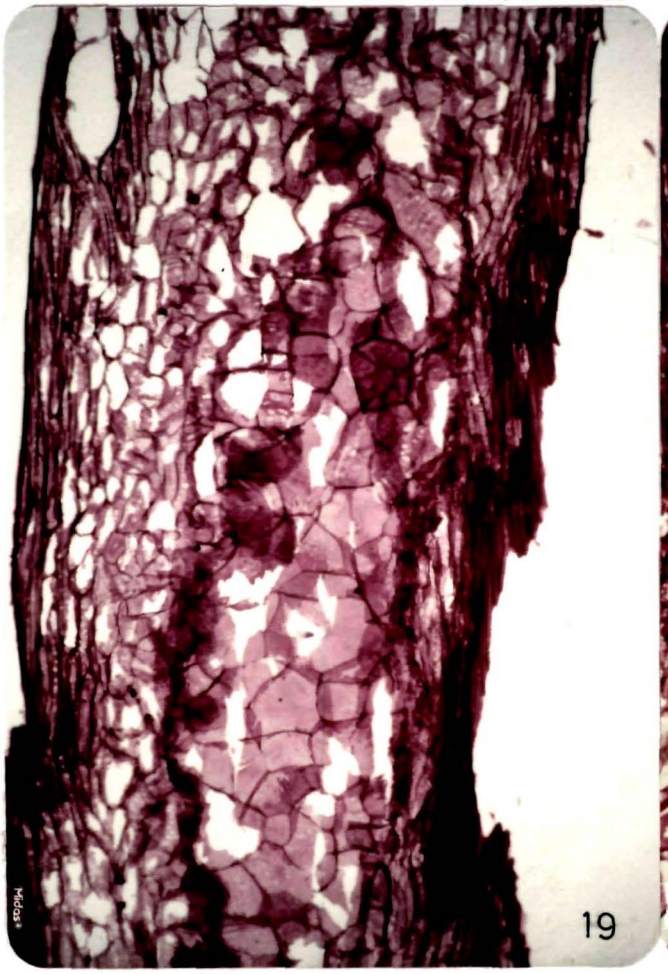




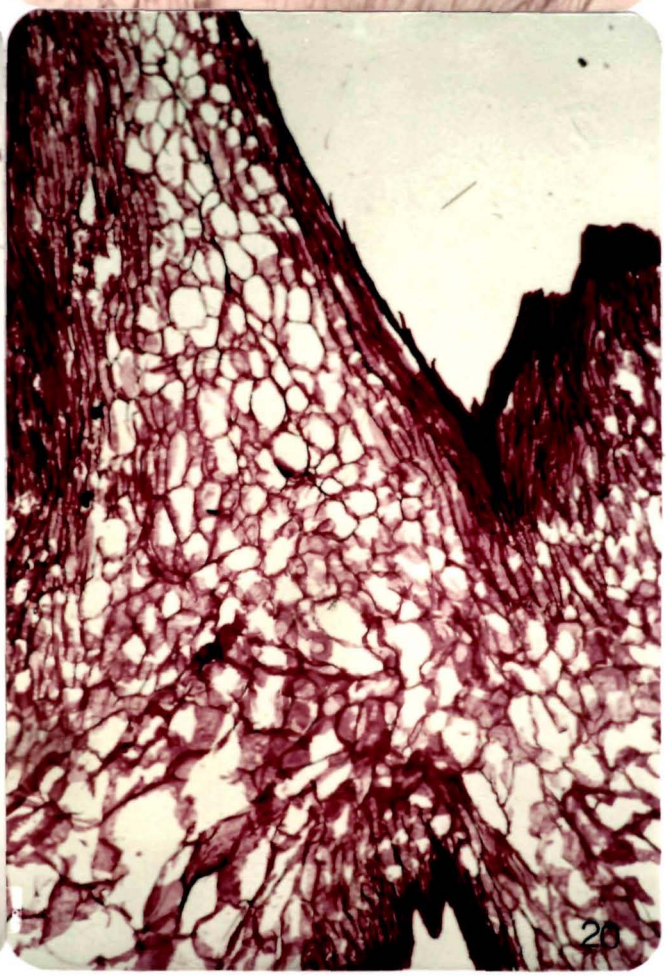


PLATE 7b (Contd..)

thickenings (Plate 7a.8,9) and vessels differentiated (Plate 7a.10). Besides, a few branched tracheids were also observed (Plate 7a.11). These TE-like cells while do not stain for protein the Azur B test reveals presence of lignin (green colour) but no nucleic acid in these cells (Plate 7b.16). The wall of these thick walled cells stain darkly with PAS in comparison to the cell wall of other cells of the juice vesicles. In the month of September, signs of granulation appear in the sac region also. Meanwhile thick walled pitted cells (Gibberellin tracheids, Roberts, 1976) develop in the cells of the central region (Plate 7a.12).

During maturation of the fruit, a sharp decline in protein and nucleic acid contents occur in normal as well as thick walled cells of the granulated juice vesicle (Plate 7b. 17,18). But intensity of PAS staining increased in the cell wall of the juice vesicle. During october cells of the outer epidermis of the juice vesicle become very thick walled (Plate 7a.13) and the number of rounded thick walled pitted cells increases in the vesicle region (Plate 7a.14). In the month of November, some fibers also differentiate towards the distal end of the vesicle (Plate 7a.15). These remain adhered to the vesicle wall. Thus in the granulated juice vesicles, except cells of middle parenchymatous layer, all the cells become thick-walled and later due to pressure of these thick walled cells the parenchymatous cells of the middle region also collapse (Plate 7a.16) leaving only thick walled cells and a cavity in

the sac region of the vesicle. Both cell wall and cytoplasm of these thick walled cells stain more intensely with PAS than parenchyma cells of the middle layers (Plate 7b.19). In granulated juice vesicles intensity of PAS staining increases continuously from September (mature fruit) upto December (ripe fruit) when maximum number of thick walled cells had differentiated in the juice vesicle (Plate 7b.20).

Degree of granulation depends on the number of thick walled cells formed. In less granulated juice vesicles only few thick walled rounded pitted cells develop in the central region while epidermal cells remain thin walled. In fully granulated juice vesicles, however, most of the juice vesicle cells become thick walled. Due to the presence of the thick walled cells and cavity in the sac region of granulated juice vesicle the area having thick walled cells becomes elevated while depressions occur in the cavity region making juice vesicle surface uneven.

Discussion

Morphologically the normal and granulated juice vesicles are different. Compared to the normal juice vesicle, the granulated juice vesicles are hard and have granular appearance. The granulated juice vesicles are heavier than normal juice vesicles. The hardening of the granulated juice sac is due to gelatinization of the cell contents and lignification of the cell wall of the cells present in the sac region (Bartholomew et al., 1941).

During development normal and granulated juice vesicles, reveal differences in their anatomical characteristics. Compared to the normal juice vesicles, the cell walls of the vesicle membrane cells are thicker in granulated juice vesicles. Further, while the normal juice vesicles are parenchymatous in nature, the granulated juice vesicles reveal differentiation of thick walled pitted cells in the vesicle region. The extent of differentiation of these thick walled pitted cells in the vesicle region increases, with the progression and increase of granulation, so much so that the sac region gets fully filled with these cells. In the later stages of granulation the granulated juice vesicles collapse due to disintegration of cells in the sac region. The granulated juice vesicles are bigger than the normal juice vesicles. Similar were the observations of Turrel and Bartholomew (1939) in valencia oranges. These findings are thus in agreement with the observations of Bartholomew **et al.**, (1941) in **C. sinensis.**

Histochemically normal and granulated juice vesicles are similar during early stages of ontogeny. But differences appear with the start of granulation disorder. Compared to the normal juice vesicles, the granulated juice vesicles have more insoluble polysaccharides but protein and nucleic acid contents are much less. Thus while histochemically granulation is associated with the rapid loss of protein and nucleic acid content, a rapid increase in insoluble polysaccharids takes place. Further, in comparison to the cells of normal juice

vesicles, rapid and pronounced lignification of the cell walls takes place in the cells of granulated juice vesicles. Similar were the observations of Turrel and Bartholomew (1939) in valencia oranges. The above hiostochemical differences in the normal and granulated juice vesicles are manifestations of metabolic differences in the two types of juice vesicles. Thus it may be suggested that metabolic disorders are the reasons of granulation disorder.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

In the present investigation the effects of diverse tissue culture media on callusing and cytodifferentiation in **C. limon** juice vesicle cultures were investigated by using five different nutrient media (MS, B_5, White, Heller and Roberts). Best growth of the callus and cytodifferentiation was evoked by MS medium while Heller and White meida were least effective. Effectiveness of B_5 and Roberts media ranged intermediate between MS and Heller and White media. The differential effectiveness of the various nutrient media could be due to qualitative and quantitative differences in their chemical composition. Effects of varying osmotic concentration of the nutrient medium on cytodifferentiation revealed that osmotic concentration of the medium has a bearing on cytodifferentiation in juice vesicles cultures and an optimal osmotic concentration is essential for better callus growth and cytodifferentiation. Stage of fruit develoomment influences potentiality of juice vesicles to callus and /cytodifferentiation. The juice vesicles from the fully developed fruits are excellent experimental material for callusing and xylogenesis while juice vesicles excised from green immature fruits and senescent fruits do not develop callus. Callusing in the vesicles obtained from partially ripe fruits was also less compared to the vesicles excised from fully developed green fruits. This thus suggests that the developmental stage of the fruit is crucial for the induction of cell divisions in juice vesicle cultures. Kordan (1984) also suggested the use of mature fruits for culturing since the lemon fruits have short life history **in vivo**. Amongst the various

regions of the juice vesicle usually callus develops only from the neck region and ordinarily no callusing occurs in the sac part of the juice vesicles. This thus suggests that mitotically the neck region is most sensitive region of the juice vesicle explant. Similar were the findings of Kordan (1965) who also obsereved that in **Citrus** culture experiments, vesicle region usually degenerates and collapses while growth occurs in the neck region. Better growth in the neck region has been attributed to the differences in the acidity of sap present in neck and sac regions (Bartholomew and Sinclair, 1951). Compared to sap present in vesicle, the sap found in neck region is less acidic. The difference in the acidity of the sap may be due to differences in the concentration of the citric acid in sap of the two regions (Kordan, 1965). In the present investigations cell divisions were completely inhibited if pH of the nutrient medium was below pH 3.0.

Essentiality of nitrogen for cytodifferentiation and differences in the efficacy of ammonium and potassium form of nitrogen in inducing cytodifferentiation was also investigated in **C. limon** juice vesicle cultures. The investigations revealed that nitrogen is not essential for cytodifferentiation in **C. limon** juice vesicle cultures. But incorporation of nitrogen sources (NH_4NO_3 and / or KNO_3) in the nutrient medium influenced differentiation. These observations are in agreement with the findings of Phillips and Dodds (1977) which suggest that inorganic nitrogen influences TE differentiation and reduction of nitrogen content

of the medium promotes differentiation. In the present study both ammonium and potassium nitrogen inhibited differentiation of tracheid, fibers and sclereids but degree of inhibition differed with the type of nitrogen source used.

Role of carbon source on cytodifferentiation in **C. limon** juice vesicle culture was studied by using different carbohydrates. The investigation revealed that callusing and cytodifferentiation occurred only in presence of a carbon source in the medium suggesting necessity of exogenous sugar in the medium for successful xylogenesis. This supports conclusions of Wetmore and Sorokin (1955). However, in cultured explants of **Helianthus tuberosus** (Minocha and Halperin, 1974) and lettuce pith (Roberts, 1982) callus development and differentiation of TE occurs even in the absence of exogenous carbon source. Thus different species differ in their carbon requirement for cytodifferentiation. Amongst different carbon sources, used in the present study, glucose supported best callus growth while sucrose induced best cytodifferentiation. Myoinositol which is most effective in inducing cytodifferentiation in lettuce pith cultures (Roberts 1982), was least effective in the present study. This thus further supports the conclusions that different species have different carbon source preferences. Different sugars influence cytodifferentiation differently in juice vesicle cultures. These findings are in conformity with the conclusions of Ball (1955) and Jeff's and Northcote (1967). In **Coleus** stems low sucrose levels (1.5-2.5%) induce strong xylem differentiation while higher sucrose levels (3-4%) prefer phloem differentiation

(Wetmore **et al.**, 1964; Wetmore and Rier, 1963). But experiments with callus cultures contradict these findings since in these experiments the number of xylem elements increased with the increasing concentration of sucrose, at least upto 8% (Rier and Beslow, 1967). A similar situation existed in excised **Coleus** internodes (Beslow and Rier, 1969) and in cultured tuber tissue of **Helianthus** (Minocha and Halperin, 1974). Aloni (1980), on the other hand, could not find any correlation between sucrose concentrations and the differentiation of vascular elements and concluded that sucrose concentration in the nutrient medium does not determine the differentiation of xylem and phloem in tissue cultures. But in **C. limon** juice vesicle cultures, sucrose concentration of the medium has a bearing on the differentiation of tracheid, fibers and sclereids. Since the sucrose concentrations upto 4% level promoted differentiation of tracheid, fibers and sclereids, while higher concentrations (above 4%) inhibited their differentiation. However, differentiation of phloem did not occur even at the highest concentration of sucrose (12%), used in the present study. These findings thus while support the observations of Wetmore and Rier (1963) also suggest that a threshold concentration of sucrose necessary for phloem differentiation may vary with the species.

Plant growth hormones influence differentiation in experimental material. In the present study besides auxin, GA, Kn, C_2H_4 and ABA influenced cytodifferentiation in juice vesicle cultures. Amongst auxins IAA was most effective while

IBA was least effective in evoking differentiation of tracheid, fibers and sclereids. 2,4-D and NAA evoked intermediate responses. The present findings thus corroborate the conclusions that different auxins differ in their effectiveness in inducing cytodifferentiation and the responses are dependent on the species and auxin being used for experimentation (Dalessandro and Roberts, 1971; Dalessandro, 1973a,b; Minocha and Halperin, 1974; Phillips and Dodds, 1977). Synergistic effects of auxin and cytokinin (Sorokin et al., 1962; Minocha and Halperin, 1974; Dalessandro, 1973a,b; Haddon and Northcote, 1975), auxin and GA (Wareing, 1958; Neiten, 1957; Roberts and Fosket, 1966) and auxin, cytokinin and gibberellic acid (Dalessandro, 1973) are reported in literature. In the present study also the combinations of various growth hormones revealed synergism. The combination of IAA, Kn and GA had most effective synergistic effect on cytodifferentiation in juice vesicle cultures.

In the present study methionine, an ethylene precursor, promoted cytodifferentiation while $CoCl_2$, an inhibitor of C_2H_4 biosynthesis, inhibited callusing and cytodifferentiation. The present findings thus further support the conclusions that ethylene is involved in TE differentiation (Abeles and Abeles, 1972; Roberts, 1976; Roberts and Miller, 1982; Miller and Roberts, 1984).

Abscisic acid, a plant growth hormone inhibits TE differentiation (Minocha and Halperin, 1974; Minocha, 1984; Haddon and Northcote, 1976). But in the present investigations

lower concentrations of ABA improved TE differentiation while higher concentration inhibited cytodifferentiation. Further, ABA induced effects on TE differentiation were more pronounced in presence of growth hormones IAA and Kn than in their absence which suggests an interaction between the three growth hormones.

Most of the evidence for and against cell division, as a prerequisite for differentiation, was accumulated with studies on TE differentiation. Evidence in support of the hypothesis that cell division must precede differentiation comes from the studies on **Coleus** stem (Fosket, 1970) and pea root (Shininger, 1975) explants. In the present investigation, using colchicine, it was found that in explants, which did not develop callus, no differentiation occurred. Thus, in **C. limon** juice vesicle cultures also, cell division is a prerequisite for cytodifferentiation. Similar were the findings of Fosket (1968) Dodds and Phillips (1977) and Malawer and Phillips (1979). In the present investigations higher concentrations of colchicine induced differentiation of abnormal tracheid and fibers which could be due to the effects of colchicine on microtubules and microfibril orientation (Taylor, 1965; Falconer and Seagull, 1985).

During culturing acidification of the medium occurs. The pH of the medium influences callusing and cytodifferentiation in juice vesicle cultures (Khan **et al.**, 1986). Highly acidic medium inhibit cytodifferentiation while moderately acidic medium (pH 5) is most suitable for both callusing and differen-

Table 8: A comparison of composition of MS and MS medium modified for C. limon juice vesicle culture (mg/l).

Component	MS	Modified MS
NH_4NO_3	1,650	-
KNO_3	1,900	-
$CaCl_2.2H_2O$	440	440
$MgSO_4.7H_2O$	370	370
KH_2PO_4	170	170
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
$MnSO_4 4H_2O$	223	223
$ZnSO_4.7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
Myoinositol	100.0	-
Nicotinic acid	0.5	0.5
Pyridoxin-HCl	0.5	0.5
Thiamine-HCl	0.1	0.1
Glycine	2.0	2.0
Sucrose	30,000	40,000
Agar	10,000	10,000
IAA	1.0-30.0	10.0
Kinetin	0.04-10.0	0.2
pH	5.7-5.8	5.0

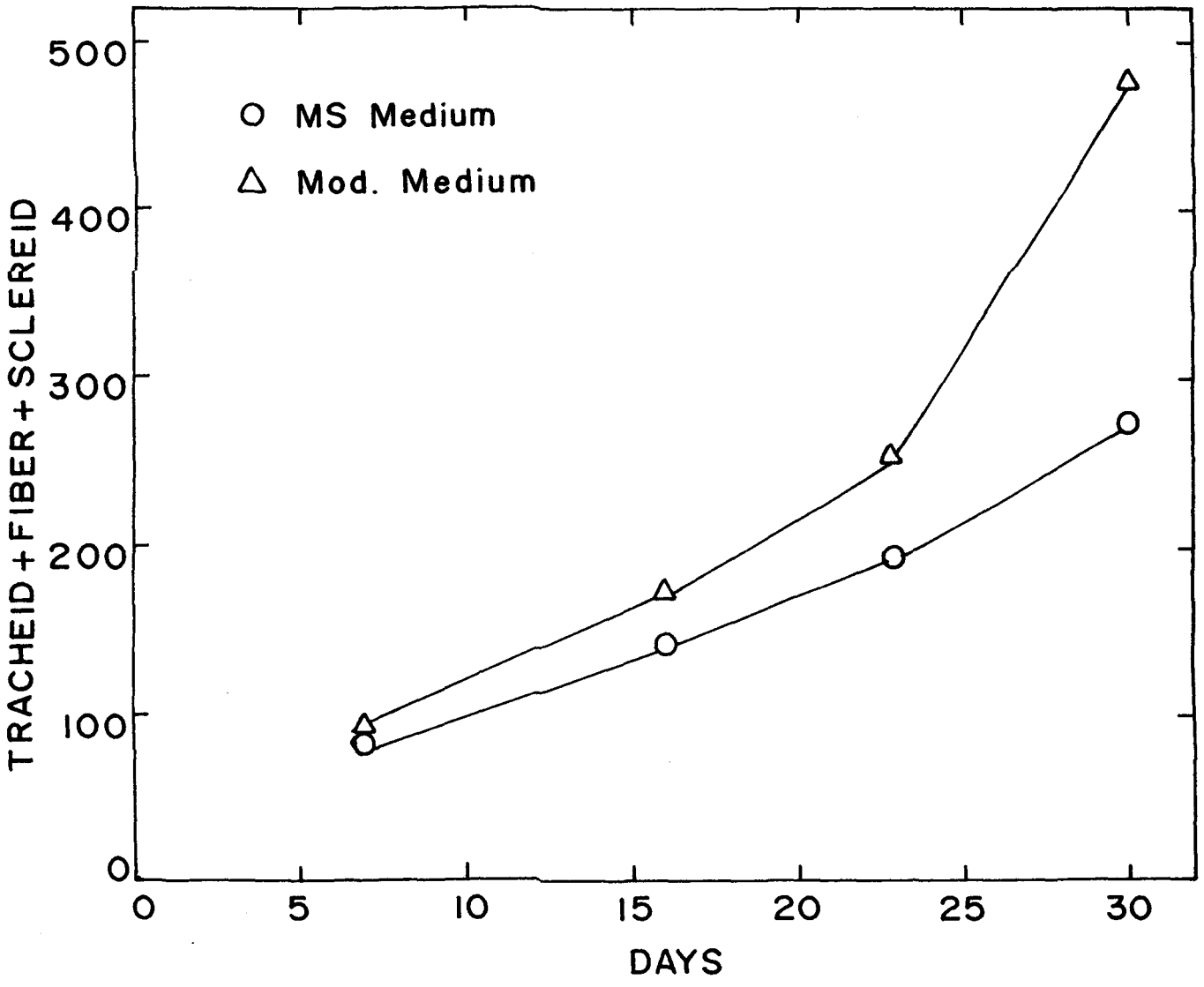
tiation of tracheids, fibers and sclereids. The differentiation of tracheids is relatively more sensitive to changes in medium pH.

The modified MS medium devised (Table-8), on the basis of the findings of the present investigation, improved callus growth and induced better cytodifferentiation, in **C. limon** juice vesicle cultures, in comparison to the MS medium (Fig. 8).

Analysis of the effects of different organic acids (maleic, α-ketoglutaric, pyruvic and citric acid) on cytodifferentiation in **C. limon** juice vesicle cultures revealed that all the organic acids used improved differentiation. α-ketoglutaric and maleic acids had most pronounced effects while pyruvic acid was least effective. The effectiveness of citric acid was intermediate. However, Gamborg and Skylak (1970) found that amongst Kreb's Cycle, citric acid is most effective in soyabean cell suspension cultures. Thus the responses of different species differ. This is also evident by the fact that in **C. hassaku** juice vesicle cultures citric/maleic acid incorporation in the medium inhibited growth (Kato, 1980). Citric acid which is a major component of **Citrus** fruit juices stimulates cytodifferentiation in **C. limon** juice vesicle cultures (Kulshreshtha **et al.**, 1982). Orange juice also has stimulatory effects in **C. limon** cultures (Murashige and Tucker, 1969). Therefore, effects of fruit juice, from five different **Citrus** species (**C. limon, C. grandis, C. aurantifolia, C. reticulata** and **C. jambhiri**), were also investigated on cytodifferentiation

Fig.8 Cytodifferentiation in **C. limon** juice vesicle cultured on MS and modified MS medium.

Fig. 8


500
400
300
200
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TRACHEID + FIBER + SCLEREID
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DAYS

in **C. limon** juice vesicle cultures. Orange juice induced best xylogenic response while Assam lemon juice was least effective. The other juices evoked intermediate responses. Erner (1975) (1975) also found that orange fruit juice is more effective than grape fruit and lemon fruit juice. Thus, juices from different fruits are differentially effective in inducing differentiation which may be due to variation in the chemical composition of their juices. Since citric acid can substitute partly the effects of orange juice it may be considered that atleast some of the growth activity of the orange juice is due to citric acid present in it. But besides citric acid some other components of the juice must also be responsible for the responses evoked by various **Citrus** fruit juices. Recently presence of some endogenous plant growth substances have been reported in young fruit of seeded and seedless clementine mandarin (Garcia-Papi and Garcia-Martinez, 1984), which could be true for **C. limon** as well since even in the absence of growth hormones juice supplemented with **Citrus** fruit juice vesicle cultured on MS basal medium /differentiated fibers. Aloni (1980) reported that IAA and GA are the limiting and controlling factors in the differentiation of fibers.

In the present investigation, white (fluorescent) light, had detrimental effect on the quality and quantity of differentiation, in comparison to dark conditions. These findings support the conclusions of Phillips and Dodds (1977) and Yeoman and Davidson (1971). Low doses of gamma rays promote callus growth and differentiation while higher doses, which inhibited

callus growth (cell division) also inhibited differentiation. Similar were the findings of Dodds and Phillips (1977), Phillips and Arnott (1983). The radiation induced stimulation of differentiation was associated with the presence of more protein and nucleic acid in the irradiated juice vesicles. Conversely radiation induced inhibition of differentiation was associated with less protein and nucleic acid in the irradiated juice vesicles. This may suggest that radiation effect on differentiation of trachied, fibers and sclereids are mediated through their effect on protein and nucleic acid metabolisms of the irradiated juice vesicles.

The normal and granulated juice vesicles of **C. grandis** differ in their morphology. Compared to the normal juice vesicle, the granulated juice vesicles are hard, granular in appearance and heavier than normal juice vesicles. The hardening of the granulated juice sac is due to gelatinization of the cell contents and lignification of the cell wall of the cells present in the sac region (Bartholomew **et al.**, 1941).

The normal and granulated juice vesicles also differ in their anatomical characteristics. Compared to the normal juice vesicles, the cell walls of the vesicle membrane cells are thicker in granulated juice vesicles. Further while the normal juice vesicle is parenchymatous in nature, the granulated juice vesicles reveal differentiation of thick-walled pitted cells in the vesicle region. The extent of differentiation of these thick walled cells increases with the progression

and increase of granulation so much so that the sac region gets fully filled with these cells. In the later stages of granulation the granulated juice vesicles collapse due to disintegration of cells in the sac region. The granulated juice vesicles are biger than the normal juice vesicles. A similar situation exists in granulated juice vesicles of valencia oranges (Turrel and Bartholomew, 1939; Bartholomew et al., 1941). The normal and granulated juice vesicles differ histochemically also. Compared to the normal juice vesicles, the granulated juice vesicles have more insoluble polysaccharides but protein and nucleic acid content is much less. Thus, histochemically granulation is associated with a rapid loss of protein and nucleic acid contents while a simultaneous increase in their insoluble polysaccharide content occurs. Further in comparison to the cells of normal juice vesicles rapid and pronounced lignification of the cell walls takes place in the cells of granulated juice vesicles. All these could be manifestations of metabolic differences in the two types of juice vesicles. Thus it may be suggested that metabolic disorders are the reasons for granulation disorder.

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~~28 x 9 = 252~~

Total Ref = 250

Not Cited (NC) = 9

$\frac{9}{250} \times 100 = 3.6\%$