IN VITRO STUDIES IN TRACHEARY ELEMENT DIFFERENTIATION IN CITRUS JUICE VESICLES

ABSTRACT

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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 vesicles cultures and an optimal osmotic concentration is essential for better callus growth and cytodifferentiation. Stage of fruit development 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 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 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.

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