THE USE OF ISOZYMES AS AN AID IN DISTINGUISHING *IN VITRO* PRODUCED ZYGOTIC AND NUCELLAR GENOTYPES IN *CITRUS SINENSIS* AND *CITRUS LIMON* SEEDLINGS

JESSE SIMIYU MACHUKA

A Thesis submitted in part fulfilment of the degree of

MASTER OF SCIENCE

UNIVERSITY OF NAIROBI

FEBRUARY 1989

Dedicated to my parents, Harrison and Leah Machuka

DECLARATION

This Thesis is my original work and has not been submitted for a degree in any other university

JESSE SIMIYU MACHUKA

This Thesis has been submitted with our approval as University Supervisors

PROFESSOR H. N. B. GOPALAN

PROFESSOR KIMANI WAITHAKA

ACKNOWLEDGEMENTS

I am greatly indebted to my first supervisor, Professor H.N.B. Gopalan of the Department of Botany for his interest in my studies and for patiently guiding me through all the stages in this work. I thank my second supervisor, Professor Kimani Waithaka of the Department of Crop Science for suggesting the problem and providing plant material and tissue culture facilities for this work. I thank Dr. T.K. Mukiama of the Department of Botany for introducing me to electrophoresis and availing his laboratory for the work. I am very grateful to Mr. S. Obukosia of the Department of Crop Science , Miss. M. Njogu and Mr. K. Kimani of the Tissue Culture Laboratory in Crop Science Department for their help in initiating and maintaining the cultures.

I am also indebted to Dr. Osir of ICIPE for assisting in protein analysis and Mr. H. Khayo of the Department of Botany for his help in photography. The assistance offered by Mrs. R. Antipa in typesetting the thesis is also highly appreciated. Lastly, I thank the University of Nairobi for offering me the scholarship to pursue an M.Sc. degree programme in genetics.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Five isozyme systems, esterase (EST), glutamate oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), malic enzyme (ME) and peroxidase (PXD) were assayed using embryos, calli, roots, cotyledons and leaves of *Citrus sinensis* cv. "Valencia" and *Citrus limon* cv. "Rough Lemon" by polyacrylamide gel electrophoresis. Isozyme profiles of these five enzyme systems were used to distinguish nucellar from zygotic genotypes of *in vitro* produced seedlings. The frequency of zygotic seedlings resulting from open-pollination was found to vary between 0.0% and 6.3%. Embryo culture significantly enhanced the recovery of zygotic seedlings in "Rough Lemon" but not in "Valencia". All, except one, of 86 plantlets produced via *in vitro* nucellar embryogenesis were found to be genetically uniform. Tissue and age specific PXD isozyme patterns were observed.

Isolation and culture of preformed embryos resulted in the production of upto 13 and 9 seedlings per seed in "Valencia and "Rough Lemon", respectively. The use of isozymes and tissue culture as tools for the identification of nucellar and zygotic genotypes in polyembryonic *Citrus* species is discussed. Isozymes can be used for the preliminary identification of potential new genotypes resulting from cross-pollination and tissue culture manipulation.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Species of the genus Citrus belong to the large dicotyledonous family Rutaceae. They are believed to be native to tropical and sub-tropical Asia and the Malay archipelago (Webber, 1967). Currently, the cultivated varieties, which include tangerines, sweet and sour oranges, grapefruit, lemons and limes, are grown in over fifty countries (Burke, 1967; Button and Kochba, 1977). In Kenya, citrus fruits account for the largest proportion (tonnage) of the overall horticultural production, after pineapples, bananas and mangoes.

There is a very high demand for citrus fruits both for local and export markets. In fact, they are being imported from Tanzania. To satisfy this demand and to reduce importation, it is necessary to carry out research in areas such as breeding and selection of new cultivars with respect to disease resistance, fruit colour and size, juice quality, flavour, and other characteristics that are useful for the full exploitation of the potential of this important horticultural crop.

Cultivated Citrus species are valued mainly for their

juicy, edible fruits. The trees are also important for the production of essential oils from fruits, flowers, leaves and stems. The oils differ in composition depending upon the species from which they have been obtained and the tissue from which they have been extracted (Cobbley, 1975). Such oil is used industrially in the production of organic chemicals, perfumes and toilet waters and to flavour drinks and confectionery.

Owing to the demand for more food and a better nutritious diet in Kenya, the Ministry of Agriculture has set up a fruit tree seedling programme aimed at supplying farmers with seedlings in all districts of the Republic. The government aims not only to establish more orchards, but also to enhance the current soil conservation and afforestation efforts. It also hopes to encourage rural industrialization and employment through the development of agro-industries based on fruit processing (Report of the Committee on Greening in Citrus and Fruit Tree Nursery System, 1982).

In most government, prison and private seedling multiplication or propagation nurseries, "Rough Lemon" (Citrus limon) is the recommended rootstock. The scion is provided from other mother trees e.g. sweet orange (C. sinensis). The programme involving the production of seedling material through budding and grafting has been under implementation in Kenya for some time now and has had a positive impact on the

citrus industry. Three major problems, however, still remain unresolved: First, the lack of disease indexing procedures has meant there is no guarantee that the material produced and supplied to farmers is disease-free; Secondly, the propagation of some mother trees, e.g. sweet oranges, is known to be difficult; The third major problem is related to the lack of a reliable method that can be employed for the early selection of desirable seedling genotypes needed for the establishment of genetically uniform citrus orchards. Some cultivated Citrus species are polyembryonic and apomictic. Consequently, two or more seedlings are produced when polyembryonic seeds are germinated in soil (Frost and Soost, 1968). It is largely because of the unknown genetic origin of such seedlings (that is, whether derived from zygotic or nucellar embryos) that budding is resorted to as a method of propagation in Citrus.

Tissue culture techniques can be used to solve the first two problems related to the propagation of citrus scions and rootstocks and the dissemination of disease-free material (Navarro, 1984). This approach can be further augmented by the use of isozyme gene markers as "fingerprints" for checking the genetic uniformity or heterogeneity of in vitro propagated trees in seedling multiplication nurseries. Currently, citrus breeders at the various horticultural research stations in the country are under pressure to select for fruit trees that are disease-free,

high yielding and generally better suited for the local market, export and processing. In such breeding exercises, knowledge of Citrus isozymes and their genetics will be useful, as these can be employed as a tool to distinguish sexual (zygotic) from asexual (nucellar) seedlings of polyembryonic varieties in their early stages of development. Such early identification could greatly help to cut down on the cost, time and effort normally spent in growing plants with undesirable gene combinations (Torres et al., 1978).

Button et al., (1976) suggested the use of in vitro generated seedlings obtained through embryo culture as sources of tissue for isozyme analysis. The use of cultured embryos might not only allow zygotic and nucellar progenies to be distinguished at their very early stages but may also enhance the recovery of zygotes which can, in turn, be used as Citrus breeding material. With this in mind, and other problems alluded to in the foregoing account, the current study was carried out with the following objectives:

1. To produce seedlings of "Rough Lemon" (Citrus limon) and sweet orange cv. "Valencia" (C. sinensis) through embryo culture;
2. To identify zygotic and nucellar genotypes in in vitro generated seedlings using isozyme gene markers;
3. To check for genetic uniformity or heterogeneity among plantlets derived from nucellar tissue culture; and
4. To assess the degree of polyembryony in "Rough Lemon"

and "Valencia" and the effect of embryo culture on the recovery of zygotic seedlings.

1.2. Literature Review

There are sixteen species in the genus Citrus. Those cultivated for their fruits belong to the sub-genus Eucitrus (Cobbley, 1975). The cultivated species are all diploids (2n = 18). Due to ancestral mutation and outcrossing, almost all Citrus forms are heterozygous. Cultivated varieties, in particular, are highly heterozygous and have remained unchanged for many generations because they are clonally propagated (Button and Kochba, 1977). Most species within the genus hybridize easily, producing considerably stable, fertile F1 hybrids (Cameron and Frost, 1968).

1.2.1. Polyembryony in Citrus

The development of two or more embryos from a seed is known as polyembryony (Maheshwari, 1950). Except for a few taxa, notably Citrus and Mangifera, polyembryony occurs only as an abnormal feature (Litz et al., 1982). Polyembryony was first reported in some orange seeds by Antonni van Leeuwenhoek in 1719 (cited by Bhojwani and Bhatnagar, 1983). Following fertilization and the growth of an embryo from the zygote, asexual embryos may develop in the same ovule from the diploid somatic cells of the nucellus, a phenomenon referred to as adventive or nucellar embryony (Ernst, 1918; Sharp, 1934). The embryos that develop from the nucellus are called nucellar embryos in

contrast to those which arise from the fertilized egg, referred to as zygotic embryos.

Some Citrus species are monoembryonic (e.g. C. grandis and C. medica). Most, however, are polyembryonic (e.g. C. limon, C. reticulata, C. sinensis, etc.). Frusato et al., (1957) reported as many as forty embryos in the nucellus of a single seed. The induction of nucellar embryos in vivo appears to be dependent upon a stimulus. Except for two reports (Webber, 1930; Wright, 1937), pollination appears to be essential for seed formation (Coit, 1915; Ikeda, 1904; Nagai and Tanikawa, 1928; Swingle, 1927). Frost and Soost (1968), more specifically, reported that a stimulus for nucellar embryo induction is provided by pollination, fertilization and possibly the early development of the zygote. The extent of polyembryony has been shown to be influenced by the nutritional status of the fruit (Traub, 1936), the age of the tree and orientation of its branches (Frusato et al., 1957), the pollen parent and environmental factors (Frost and Soost, 1968).

Nucellar embryos are known to compete with one another and with the zygotic embryo for food and space within the seed. Because of this competition, one, two or rarely, three seedlings emerge when such seeds germinate (Frost and Soost, 1968). Since nucellar plants arise by ordinary mitotic divisions from the cells of the nucellus, they are expected to be genetically identical to the mother tree, barring

mutation. In practice, however, marked phenotypic variability among nucellar offsprings has been observed (Bitters and Murashige, 1967; Maheshwari and Rangaswamy, 1958). The explanation for this variation is not known for certain, though some workers have suggested that the variation may be due to the presence of chimeric nucellar cells in some Citrus clones (Bitters and Murashige, 1967; Cameron et al., 1964; Frost and Krug, 1942; Frost et al., 1957). Zygotic progeny are also known to be highly variable in all polyembryonic Citrus forms (Cameron and Frost, 1968).

The genetics of polyembryony is not very well understood. Initially, polyembryony was believed to be a recessive hereditary trait controlled by a series of multiple genes, as suggested by Maheshwari and Rangaswamy (1958). These genes were thought to regulate the synthesis of volatile (e.g. ethanol) and non-volatile (e.g. IAA, IBA and GA3) substances that act as embryogenic inhibitors in the ovule of monoembryonic Citrus species (Esan, 1973). Such inhibitors are believed to be absent in polyembryonic species. The current view is that the production of seeds bearing nucellar embryos is inherited as a dominant qualitative trait (Cameron, 1979; Cameron and Soost, 1980; Iwamasa et al., 1967).

1.2.2. Embryo and nucellar culture in Citrus

In vitro culture techniques have proved an invaluable tool in the study of isolated plant cells, tissues and organs (Narayanaswamy, 1977). In Citrus, the techniques have already been used for breeding purposes (Ohgawara et al., 1985; Rangan et al., 1969; Vardi et al., 1975), clonal rejuvenation and virus elimination (Navarro, 1984), studies involving the morphology and physiology of Citrus tissues (Altmann and Goren, 1974; Bar-Akiva et al., 1974; Kochba et al., 1982), clonal propagation and in investigating various aspects of nucellar embryony (Button Kochba, 1977; Moore, 1985).

The nucellus represents the wall of the megasporangium or ovule. Because of its natural tendency to form embryos by adventive embryony, most studies involving artificial induction of embryos in Citrus have been largely confined to this tissue. The embryo is the beginning of the new sporophytic generation of the plant. Sobrinho and Gurgel (1953) were the first to attempt culturing preformed Citrus embryos on an artificial nutrient medium. Stevenson (1956) and Hurosvilli (1957) later reported the growth of shoots and roots when small embryos were cultured on agar and Tukey's medium (Tukey, 1933), respectively. The first successful culture of Citrus embryos was accomplished on White's medium (White, 1943) by Ohta and Frusato (1957). After this achievement, many reports pertaining to embryo culture began to appear,

including cases where whole plants were obtained (Button and Bornmann, 1971; Gritter, 1986; Kochba et al., 1972, 1974; Maheshwari and Rangaswamy, 1958; Rangaswamy, 1958a, 1958b; Sabharwal, 1963).

With these accomplishments, much of the research attention since the mid 70s has shifted to the more applied aspects of embryo culture, e.g. in vitro mutant induction and selection (Ben-Hayyim and Kochba, 1982; Kochba et al., 1982; Spiegel-Roy and Ben- Hayyim, 1985; Spiegel-Roy et al., 1983), protoplast culture (Kobayashi, 1987; Vardi et al., 1975) and protoplast fusion (Ohgawara et al., 1985). Wakana and Vemeto (1987), using light and electron microscopy, have recently demonstrated that pollination and fertilization may not be essential for in vivo adventitious embryogenesis. If this observation is proved to be true, it will nullify the widely held view that the induction of nucellar embryos requires a stimulus (Esan, 1973).

Since virus particles are generally restricted to the host's vascular system, seedlings derived from zygotic or nucellar embryos in vitro are largely virus-free (Bitters and Murashige, 1967; Childs and Johnson, 1966; Navarro, 1984). In monoembryonic species the trees can be freed from viruses and rejuvenated through artificial embryo induction via nucellar tissue culture (Kochba et al., 1972; Navarro, 1984; Rangan et al., 1969).

1.2.3 The role of tissue culture in the identification nucellar and zygotic seedlings

The establishment of a successful tree nursery/orchard requires careful selection of the trees in order to attain genetic uniformity. In polyembryonic *Citrus*, the major problem is to distinguish between nucellar and zygotic seedlings (Frost and Soost, 1968).

Rangan *et al.*, (1969) were the first to correctly identify and culture zygotic embryos from the seeds of polyembryonic sour orange (*C. aurantium*). The most mature embryos (120 days after anthesis), in their case, turned out to be zygotic. They were, however, not successful in culturing immature embryos. Their results suggest that a vigorous zygotic embryo may retard or even inhibit the development of nucellar embryos. In seeds of open-pollinated sour orange, it is known, however, that nucellar embryos appear earlier and are more vigorous than the zygotic embryo (Button and Kochba, 1977; Ohta and Frusato, 1957). The pollen parent is also known to exert an influence on the pace of development of the zygotic embryo (Frost and Soost, 1968). Moreover, some *Citrus* species produce exclusively nucellar embryos (Kochba *et al.*, 1972). Therefore, tissue culture *per se* cannot be used with absolute certainty to solve the age-old problem of identifying zygotic and nucellar embryos and seedlings. If it is used in combination with isozyme gene markers, more

accurate identification may be possible.

1.2.4. Isozymes and their utilization in Citrus tissue culture and breeding.

Although nucellar seedlings represent an ideal method for propagating and maintaining a desirable genotype, the breeder is usually interested only in recombinant genotypes for horticultural evaluation. In practice, nucellar and zygotic seedlings have to be grown for up to five or more years until fruiting before the two kinds of seedlings can be identified basing on vegetative (morphological) characters. Various approaches have been used by earlier workers to avoid such an expensive and time consuming procedure. Parameters such as leaf position, leaf morphology and the analysis of essential oil components were initially employed for the identification and selection of zygotic and nucellar seedlings (Cameron and Scora, 1973; Iwamasa et al., 1967; Teich and Spiegel-Roy, 1972). These methods are not widely used because the genetic control of the compounds and markers employed is not well understood.

Multiple molecular forms of enzymes (isozymes) provide a much more promising approach to address the problem. They have been extensively used in plant genetics and breeding (Tanksley and Orton, 1983). In perennial fruit and nut trees, the use of isozymes is limited owing to the difficulties of obtaining good isozyme gel preparations. This is because compounds such as polyphenols and

mucopolysaccharides interfere and prevent the clear resolution of gels in these plants (Wendel and Park, 1983). Despite these limitations, isozyme gene markers provide a powerful tool for studying codominant alleles at single marker loci that can be used in a number of ways, including characterization of cultivars, assessment of genetic purity in cultivar multiplication nurseries, estimation of outcrossing rates, verification of genetic stability in cell and tissue cultures and the characterization of somaclonal variants through the detection of spontaneous electrophoretic mutants (Bretell et al., 1986; Evans and Sharp, 1986; Jacobs, 1974b; Orton, 1983; Scandalios and Sorensen, 1977; Shaw and Allard, 1982).

Work on Citrus isozymes has been carried out mainly with respect to their utilization in the identification of zygotic and nucellar seedlings and different cultivars. Using polyacrylamide gel electrophoresis, Iglesias et al., (1974) employed leaf peroxidase and esterase isozymes to distinguish zygotic and nucellar seedling progeny of known parentage. They reported variability at the peroxidase locus even among nucellar seedlings derived from the same seed. Further work needs to be done to confirm or establish the basis of such unexpected variability. The studies of Iglesias et al., (1974) were limited by the small number of isozyme bands observed, especially for peroxidases. Esen and Soost (1976) employed peroxidases extracted from mature leaves to characterize Citrus taxa and two related genera.

They observed qualitative and quantitative differences between the isozyme profiles of the species studied and suggested that more studies be done to establish possible tissue and age specific isozyme patterns of peroxidase in Citrus. Button et al., (1976) were able to characterize five Citrus clones using peroxidase isozymes of root. Owing to their high degree of polymorphism, peroxidase and esterase gene/enzyme systems can act as very reliable markers for the detection of genetic differences. Their disadvantage, however, lies in the fact that they are highly non-specific (Jacobs, 1974a; Lagrimini and Rothstein, 1987).

Torres et al., (1978, 1982) studied the genetic control of the isozymes of glutamate oxaloacetate transaminase (GOT), phosphoglucose mutase(PGM), phosphoglucose isomerase (PGI), malic enzyme(ME), malate dehydrogenase (MDH), hexokinase (HK), isocitrate dehydrogenase (IDH) and leucine aminopeptidase (LAP). They established reliable criteria for the identification of zygotic and nucellar genotypes and suggested that one or more gene loci can be used for such identification. Zygotic seedlings resulting from self-pollination can be distinguished from nucellars if they are homozygous at one or more loci at which the seed parent is heterozygous. Thus a total of eight genetically defined gene/enzyme systems are now available for use in the identification of nucellar and zygotic seedlings in Citrus. ME, IDH, HK, and LAP have not

so far been employed for identification. The other four enzymes have been used (Hirai et al., 1986; Khan and Roose, 1988; Soost et al., 1980). The frequency of zygotics in seedlings grown from open-pollinated varieties is less than 10%, according to Roose and Traugh (1988).

It is to be pointed out that except one (Hirai et al., 1986), all of the studies involving the use of genetically defined isozyme gene markers have employed only starch gel electrophoresis. In some cases, the resolution of some isozyme bands on the gels was so poor that some of the affected loci could not be used for identification purposes (Khan and Roose, 1988). Except for one study (Iglesias et al., 1974), all investigations involved the use of either greenhouse or field grown plant material for enzyme extraction.

In the current study, immature, preformed embryos were cultured in vitro to generate mature embryos and seedlings of Citrus limon cv. "Rough Lemon" and C. sinensis cv. "Valencia" for electrophoretic analysis. The genetic uniformity of plantlets derived through nucellar tissue culture was also confirmed, since Citrus plantlets regenerated in vitro may not always be true to type (Juarez et al., 1976; Khuroshvili and Takidze, 1979; Navarro, 1984). Polyacrylamide gel electrophoresis was employed for all the enzymes that were screened, since it is simpler and generally superior to starch gel electrophoresis (Feret and Bergmann, 1976). It is also known that the two

electrophoretic techniques (starch and acrylamide) do not always yield the same isozyme patterns (Bergmann, 1971). Because isozymes are known to be dependent on tissue type and age (Lagrimini and Rothstein, 1987; Pedersen and Simonsen, 1987), studies were carried out using different tissues for enzyme extraction.

Initially, peroxidase (PXD) and glutamate oxaloacetate transaminase (GOT) were selected for this study. PXD was chosen because of the high degree of polymorphism of PXD loci. GOT was selected because the genetic control of its isozymes is perhaps the best understood among all the gene/enzyme systems so far unravelled in Citrus (Torres et al., 1978, 1982). GOT appeared to be homozygous at both of its known loci (Got-1 and Got-2) in "Valencia", and was also very difficult to resolve on the gels. Hence three other gene/enzyme systems, malate dehydrogenase (MDH), malic enzyme (ME) and esterase (EST) were screened.

CHAPTER 2

MATERIAL AND METHODS

2.1. Plant material

Fruits (7-21 weeks old) were collected from mature "Rough Lemon" (C. limon) and "Valencia" (C. sinensis) trees growing in a large Citrus orchard at the Field Experimental Station, Kabete Campus, College of Agriculture and Veterinary Sciences, University of Nairobi. The orchard contains several other species of Citrus. thus permitting open-pollination to occur. The mother trees from which the fruits were collected were marked and records maintained throughout the period of study.

2.2. Methods

2.2.1 Embryo and nucellus culture

The outermost coats of immature seeds of Citrus sinensis and C. limon were first removed and the resultant seeds with an intact inner coat surface sterilized in a 0.5% sodium hypochlorite solution for 10-15 min. This was followed by thorough rinsing in three or four changes of sterile deionized water.

The seeds were then inoculated onto a modified MS medium (Murashige and Skoog, 1962). The medium contained MS salts at full strength supplemented with sucrose (50 g/l), myo-

inositol (0.1 g/l), glycine (4.0 mg/l), nicotinic acid (1.0 mg/l), thiamine-HCl (1.0 mg/l), pyridoxine-HCl (1.0 mg/l), and casein hydrolysate (0.4 g/l). The pH of the medium was adjusted to 5.7 ± 1 with 1.0 N sodium hydroxide and 1.0 N hydrochloric acid. The medium was solidified with 10 g/l of Difco-Bacto agar and autoclaved at a temperature of 121 °C and pressure of 15 Lbs p.s.i. Nucellar tissue was isolated from the micropylar end of the seed (after removal of the integuments), sterilized and cultured on the same medium. Cultures were grown at 26 °C in continuous white light from fluorescent lamps (51 μmol. m^{-2} s^{-1}). The quantum flux density was measured by a Portable Infra-Red Gas Analyzer (ADC) at sample level.

Two kinds of treatments were given to the preformed embryos during culture. In some cultures, proembryos from the same seed were aseptically teased apart and left to grow without contact with one another. The second category of cultures consisted of embryos that were not separated. At 2-4 week intervals, the developing embryos and seedlings were either transferred to a fresh medium to continue growing or were harvested for isozyme analysis. Records of the numbers of seeds used and seedlings obtained were maintained throughout the investigations. Fresh cultures were initiated four times in the course of the study to provide a continuous supply of seedlings at different stages of development for analysis.

2.2.2. Enzyme analysis

2.2.2.1 Sample preparation

Samples for enzyme extraction were obtained from *in vitro* generated embryos or seedlings of the two *Citrus* species studied. The following tissues were sampled: calli, roots, cotyledons, young and mature leaves. Seedlings of different ages ranging from 2 weeks to 7 months were used. Fresh, frozen or freeze-dried tissue sample (100 mg) was ground in 1 ml of 0.1 M potassium phosphate buffer (pH 6.8) using a small pestle and mortar. Where smaller or larger tissues were available, the same ratio of sample weight to buffer volume was maintained. The homogenate was then transferred to a sample tube and centrifuged at 4,000 x g for 10 min. The resultant, clear supernatant was used as the enzyme source. All operations were carried out at 4 °C. No differences in isozyme band resolution were observed whether fresh, frozen or freeze-dried samples were used.

2.2.2.2 Peroxidase assay

Pronounced quantitative differences in the isozyme profiles of different tissues and at different ages were observed in the initial experiments. Hence, peroxidase assay was done to assess the relative activity of the enzyme in different tissues and at different growth stages. Samples were prepared as described in section 2.2.2.1. The enzyme activity was assayed in 0.05 M sodium phosphate buffer

(pH 6.8), 1.25% pyrogallol and 0.125% hydrogen peroxide (100 Vols) according to the method of Sathaiah and Reddy (1984). For each tissue, 1 mg per ml of the extract was used and the absorbancies recorded at 420 nm using a Beckmann-DN-50-Spectrophotometer. The activities were expressed in terms of absorbancy units per mg protein (see section 2.2.2.2.1).

2.2.2.2.1 Protein assay

The determination of protein content in the samples was carried out according to the bicinchoninic acid (BCA) method (Pierce Chemical Company). Using bovine serum albumin (BSA) as the standard protein, a standard curve of absorbance (at 562 nm) against protein concentration was first prepared. Then 0.1 ml of the enzyme extract was mixed with the BCA working reagent and incubated at 37°C for 30 min. After incubation, each tube was cooled under running tap water and the absorbance read at 562 nm using a Beckmann-DN-50-Spectrophotometer. The protein concentration in the sample was determined using the standard curve obtained with BSA.

2.2.3 Isoenzyme analysis

2.2.3.1 Preparation of acrylamide gels

Stock solutions were prepared and adapted for use in the formulation of acrylamide gels by altering the porosity of the gel. This was done either by increasing the concentration of acrylamide and reducing the volume of water

Table 1 Quantity of reagents and pH of resolving buffer used in the preparation of gels for electrophoresis of enzymes

Enzyme	E.C. NO.*	Resolving buffer pH	Gel %	Volume of dH_2O
Esterase	3.1.1.2	8.6	7.5	16.95
Peroxidase	1.11.1.7	8.6	5.0	19.45
Glutamate Oxaloacetate Transaminase	2.6.1.1	8.0	7.5	16.95
Malate Dehydrogenase	1.1.1.37	7.6	8.5	15.95
Malic Enzyme	1.1.1.40	7.6	8.5	15.95

* Enzyme Classification Number.

or vice versa (Hames, 1982). The percentage of acrylamide and the pH of the resolving buffer used for each of the five enzyme systems studied are shown in Table 1. The amounts of acrylamide and water indicated in Table 1 were mixed with 3.75 ml of resolving buffer (tris-HCl), 0.05 ml TEMED (tetraethylmethylene diamine), and 1.5 ml of 1.5% ammonium persulfate solution. Tris-glycine (0.04 M) buffer (pH 8.3) served as the reservoir buffer.

2.2.3.2 Electrophoresis

Gels of high acrylamide concentrations (>5%) were stacked with 5% gel (see Table 1). The top of every gel contained thirteen wells made with prior insertion of a comb. Into each of the thirteen wells, 25-30 µl aliquots of the sample solutions were then loaded. Gel electrophoresis was conducted at 4 °C. A current of 25 mA was delivered. The voltage varied from about 50 V to 300V at the end of every run which lasted about 5 hr. A tracker dye (bromophenol blue) was used to monitor the progress of each experiment. Experiments were terminated when the dye had come within a few millimetres from the edge of the gel.

2.3.3.3 Gel staining and analysis of electrophoresis data

Isozyme bands of the five enzyme systems were located by mixing the stain components and buffers as shown in Table 2. After staining, the distances migrated by the bands were

Table 2. Staining technique for enzyme detection

Enzyme*	Stain Components		Buffer used	Reference
PXD	1g benzidine 3% H_2O_2	10ml 50ml	none	Tuikong and Gupta (1985)
EST	20mg α-naphthyl acetate 20mg β-naphthyl acetate acetone 100mg fast blue RR salt	 4ml	0.2M sodium phosphate (pH 6.8) 100ml	Coppens and Gillis (1987)
GOT	130mg aspartic acid 85mg α-ketoglutaric acid 1.5mg pyridoxal-5' - phosphate 180mg fast blue RR salt 100mg D-gluconic acid		tris-citrate (pH 8.0) 60ml	Scandalios and Sorensen (1977)
MDH	10mg MTT (methyl thiazol tetrazolium) 20mg NAD (nicotinamide adenine dinucleotide) 0.54mg malic acid 5mg PMS (phenzaine methosulfate)		tris-HCl (pH 7.6) 35ml	Steiner and Joslyn (1979)
ME	10mg MTT (methyl thiazol tetrazolium) 5mg NADP (nicotinamide adenine dinucleotide phosphate) 0.8mg malic acid 25mg $MgCl_2$ 5mg PMS		tris-HCl (pH 7.6) 35ml	Steiner and Joslyn (1979)

* PXD - Peroxidase
EST - Esterase
GOT - Glutamate oxaloacetate transaminase
MDH - Malate dehydrogenase
ME - Malic enzyme

measured. In some cases, photographs were taken using Ilford Pan F film (50 ASA). Peroxidase and malate dehydrogenase bands faded away quickly with time, hence gels pertaining to these enzymes were not stored. Other gels were stored in a solution of 10% glycerol and 8% acetic acid, in plastic bags for subsequent analysis.

2.2.4 Comparison of mean numbers of seedlings generated through seed and embryo culture

The mean numbers of seedlings obtained per seed cultured with and without the separation of preformed embryos were compared using the Mann-Whitney U-test (normal approximation with tie correction) to calculate Z values according to the formula shown below (Roscoe, 1975):

$$Z = \frac{U-(n_1 n_2/2)}{\sqrt{\left(\frac{n_1 n_2}{N^2-N}\right)\left(\frac{N^3-N}{12} - \Sigma T\right)}}$$

where $N = n_1 + n_2$

$$T = \frac{f^3 - f}{12}$$

$$Ua = nanb + \frac{nb(nb+1)}{2} - \Sigma Rb$$

$$Ub = nbna + \frac{na(na+1)}{2} - \Sigma Ra$$

where ΣRa = Sum of the ranks for sample A

Σ Rb = Sum of the ranks for sample B

Ua = Number of times a rank from sample A is preceded by a rank from sample B

Ub = Number of times a rank from sample B is preceded by a rank from sample A

The smaller value between Ua and Ub is considered as U and used in the formula above.

$$\text{Rank} = \text{cumulative frequency (cf)} - \frac{\text{frequency (f)}}{2} + 0.5$$

The two treatments (samples) in this study were the situations with and without embryo separation (see section 3.2).

2.2.5. Criteria for the identification of zygotic and nucellar seedlings

Since the genetic control of peroxidase and esterase isozymes is unknown, the designation of a seedling as being either nucellar or zygotic was solely based on a comparison of the isozyme profiles for samples from seedlings originating either from the same seed or from different seeds. Seedlings were suspected to be zygotic if they gave rise to an isozyme band(s) that was not expressed by samples from the mother tree or from other seedlings either derived from the same seed or from different seeds.

The genetic control of GOT, MDH, and ME in Citrus is

now well known (Torres et al., 1978, 1982). In this study, gene loci were assigned to the bands belonging to these three gene/enzyme systems as described in sections 4.2.3, 4.2.4 and 4.2.5 (also see Table 13). The identification of zygotic and nucellar genotypes was based on the observed genotype of each individual seedling relative to the genotype of the parent nucellar tree. The criterion here assumes that pollination from a Citrus tree other than C. limon ("Rough Lemon") and C. sinensis ("Valencia") mother trees from which the seeds were collected for culture is the same as self-pollination, and that the loci studied are unlinked (Torres et al., 1982; Khan and Roose, 1988). Zygotic seedlings resulting from outcrossing had an allele not expressed by the seed parent tree. The Yates' Correction was applied in the calculation of Chi Square tests of heterogeneity owing to the low frequency of zygotic seedlings identified (section 3.4.2).

Rf values for peroxidase isozyme bands were calculated as:

$$\frac{\text{distance from origin travelled by isozyme band}}{\text{distance from origin travelled by the standard isozyme band.}}$$

This technique has been used before for the characterization of peroxidase isozyme bands (Button et al., 1976; Feret and Bergmann, 1976; Siegel and Galston, 1967).

CHAPTER 3

RESULTS

3.1 In vitro initiation of seedlings from preformed embryos

The results are summarized in Table 3 and Table 4. Seeds or embryos that failed to develop into plantlets were classified together as "stranded" seeds in Table 3.

3.1.1 Growth of Valencia proembryos

Growth of proembryos commenced with the enlargement of cotyledons. This was followed by the development of a shoot and finally a root. Larger proembryos formed seedlings earlier than smaller ones (Fig.1a). More seedlings were observed when the embryos were separated than when they were left to grow together (Fig.1b, Fig.2a, Fig.2b). In the latter case, they aggregated together into clumps. Fully formed seedlings appeared 8 or more weeks after the culturing of the seeds or embryos onto the medium in "Valencia", as opposed to 2 weeks in "Rough Lemon".

3.1.2 Growth of Rough Lemon proembryos

The *in vitro* growth of "Rough Lemon" embryos and the numbers of seedlings obtained per seed are shown in Fig.3a, Fig.3b and Fig.3c. More seedlings were obtained when the

Table 3. Number of seeds cultured on the MS medium and the total number of "Rough Lemon" and "Valencia" seedlings obtained.

Cultivar	No.seeds cultured	No.seeds lost through contamination	No.stranded seeds	Total no. seedlings obtained
Rough Lemon	486	124	95	477
Valencia	319	78	144	401

Table 4: Comparison of the *in vitro* germination response between seeds of "Rough Lemon" and "Valencia".

Cultivar	Total No. seeds	seeds not germinated	% germination	X^2
Rough Lemon	362	95	73.7	
				67.89-S*
Valencia	241	144	40.2	

S* - Significant at 1% level.

Fig. 1a

Growth of separated preformed embryos of "Valencia in culture. Notice seven seedlings that have already developed directly from embryos. A second category of embryos (arrows) can be seen to be differentiating into large cotyledonous bodies known as "pseudobulbils" that originate in part from callusing proembryos (9 weeks in culture).

Fig. 1b

Fully formed "Valencia" seedlings all derived from the same seed. Thirteen seedlings were produced in this culture bottle. (seedlings are 12 weeks old).

1a

1b

Fig.3a

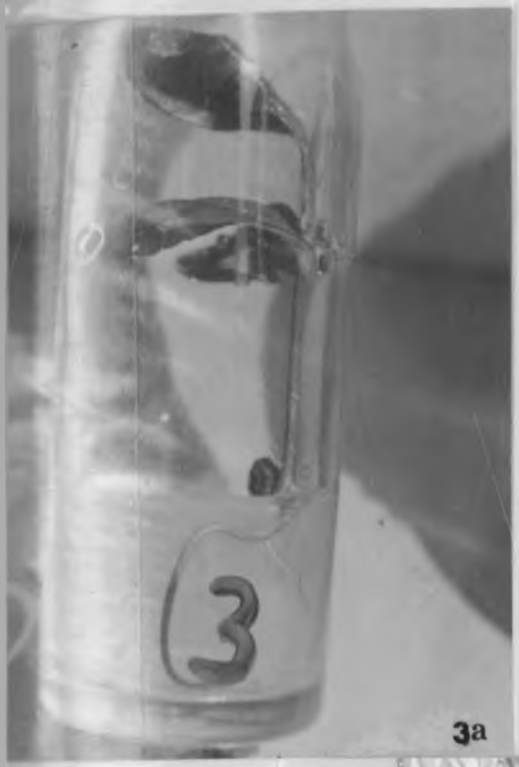
A single "Rough Lemon" seedling derived from a seed whose outer coat had been initially removed to facilitate sterilization prior to inoculation of the naked seed onto the culture medium (7 weeks in culture).

Fig. 3b

Photograph showing inhibition by a single "Rough Lemon" seedling upon the development of other embryos from the same seed (3 weeks in culture).

Fig.3c

Photograph showing seven different "Rough Lemon" seedlings all derived from the same seed. Notice the size difference betweeen the seedlings and the absence of stranded embryos. Embryos were separated during culture (3 months in culture).



3a

3b

3c

embryos were separated than when they were allowed to grow together (Fig.3a, Fig.3c, Fig.4a, Fig.4b).

3.2 Comparison of the mean numbers of seedlings

The mean numbers of seedlings per seed obtained with and without embryo separation were compared using the Mann-Whitney U-test (section 2.2.4). The results are summarized in Table 5 and Table 6.

3.3 Electrophoresis

3.3.1 Peroxidase

Blue bands appear immediately after flooding the gel with saturated benzidine and 3% hydrogen peroxide. The bands soon turn brown and become faint, therefore it was not possible to store the gels. Variation in the pH of the resolving buffer and the duration of the electrophoretic run had no effect on the number of isozyme bands, but markedly altered the distance migrated by the bands. Hence Rf values were calculated (Table 7). Differences between "Rough Lemon and "Valencia" leaf peroxidase isozymes are shown in Fig.5a.

3.3.1.1 Tissue and age specific peroxidase activity

The results obtained from quantitative (spectrophotometric and isozyme) analysis of peroxidases in different tissues of the two Citrus species are presented in Fig.5b, Fig.6, Table 8 and Table 9. Fig.7 is

Fig. 4a

Frequency distribution of the number of seedlings obtained per seed in the absence of embryo separation in "Rough Lemon".

Fig. 4b

Frequency distribution of the number of seedlings obtained per seed in the presence of embryo separation in "Rough Lemon".

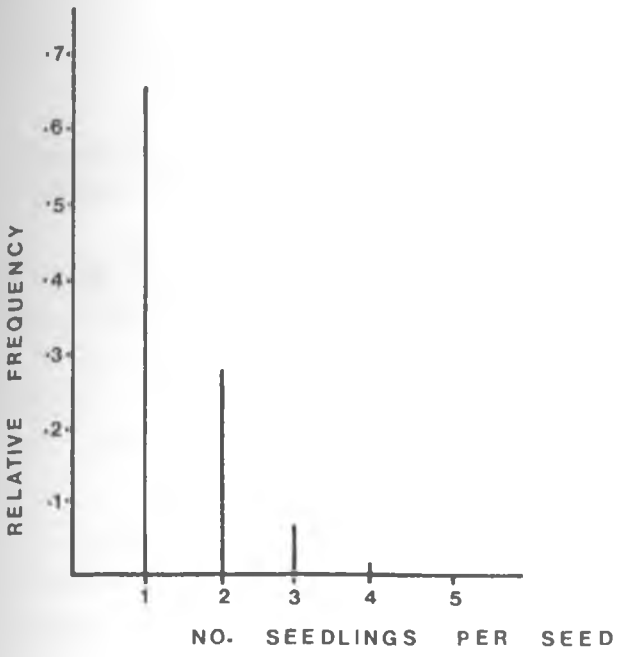


Figure 4a

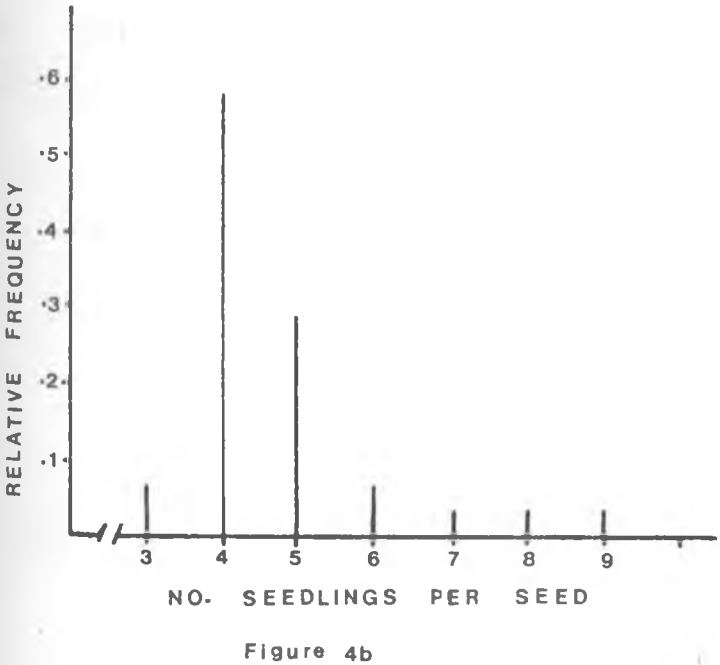


Figure 4b

Table 5. Comparison of numbers of seedlings obtained per seed with and without the separation of cultured embryos of "Valencia".

Embryos separated	Total no. seeds	Total no. seedlings obtained	mean no. seedlings per seed	Z*
Yes	38	234	6.15	
				8.19-S*
No	59	155	2.62	

S* = Significant at 1% level

Z* = Approximate normal deviate

Table 6. Comparison of numbers of seedlings obtained per seed with and without the separation of cultured embryos of "Rough Lemon".

Embryos separated	Total no. seeds	Total no. seedlings obtained	Mean no. seedlings per seed	Z*
Yes	31	138	4.45	
				-10.00-S*
No	236	339	1.44	

S* = Significant at 1% level
Z* = Approximate normal deviate

Table 7: Rf values for peroxidase isozyme bands of "Rough Lemon" and "Valencia" seedlings.

Isozyme band	Rf value*	
	Rough Lemon	Valencia
PX2	0.04	0.04
PX5	0.104	0.09
PX9	0.19	0.16
PX13	0.27	0.23
PX15	0.31	0.27
PX18	0.38	0.34
PX24	0.50	0.43
PX26	0.54	-
PX36	0.75	0.64
PX40	0.83	0.71
PX48	1.00	-
PX56	-	1.00
PX72	1.50	-
PX94	1.96	-
PX96	2.00	1.71

* - = bands absent

a diagrammatic representation of all the isozyme bands detected under the experimental conditions of this study using leaf, root, cotyledon and callus.

3.3.2 Esterase

Esterase bands were resolved by incubating the gels in 0.5 M boric acid for 15 min to lower the pH to about 6.0 before staining in 1:1 α and β-naphthyl acetate. Bands appear within 15 min and become clear after one hour of incubation at 37 °C. The isozyme bands visualized are shown in Fig.8.

3.3.3 Glutamate oxaloacetate transaminase

Dark blue bands appear within 10 min and are fully developed after 30 min. The bands are clear but faint owing to the brownish background conferred to the gel by the staining reagents. Therefore, photographic prints were poor. The isozyme bands obtained are shown in Fig.9 and Fig.10.

3.3.4 Malate dehydrogenase

The isozyme bands visualized are shown in Fig.11. The distances travelled by the bands were dependent on the pH of the resolving buffer and the acrylamide concentration in the gel, as exemplified by two pH levels (7.6 and 8.0) which also affected the number of bands (Table 10, Fig.11, and Fig.18).

Fig.5a

Differences between isozymes of mature leaves of "Rough Lemon" (slow band phenotype-S shown in lanes 2,8 and 11) and "Valencia" (fast band phenotype-F shown in other lanes).

Fig.5b

Tissue specific peroxidase profiles from root callus, cotyledon and leaf of "Rough Lemon" (RL) and "Valencia" (V). Lane 1,9: (RL) root; Lane 2,13: (RL) leaf (9 weeks old); Lane 3: (RL) (mature leaf); Lane 4,11: (RL) callus; Lane 5,7: (V) root; Lane 8: (V) mature leaf; Lane 10: (V) callus; Lane 12: (V) young leaf (9 weeks old). The bands shown were obtained by applying twice diluted samples of enzyme extract prepared from 100mg fresh weight of tissue in the wells on the gel. The bands designated PX2, PX5, PX9, PX48 and PX56 can be seen in this photograph. O= point of sample insertion (origin). Migration is anodal.

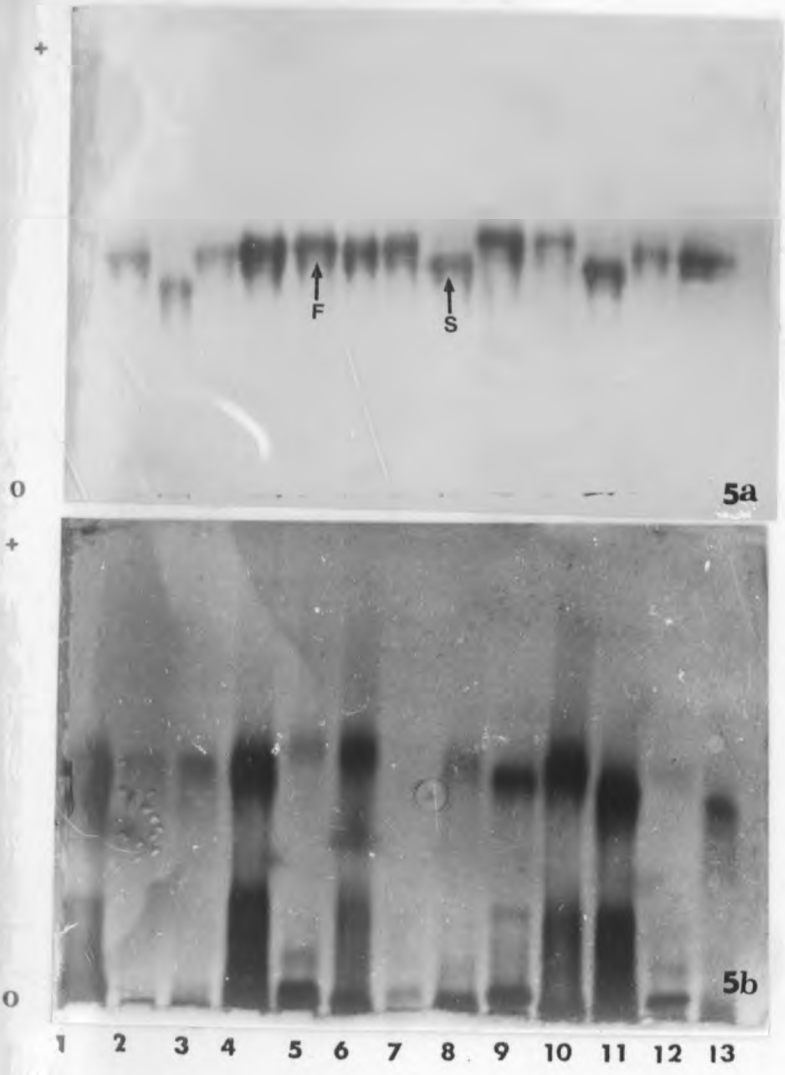
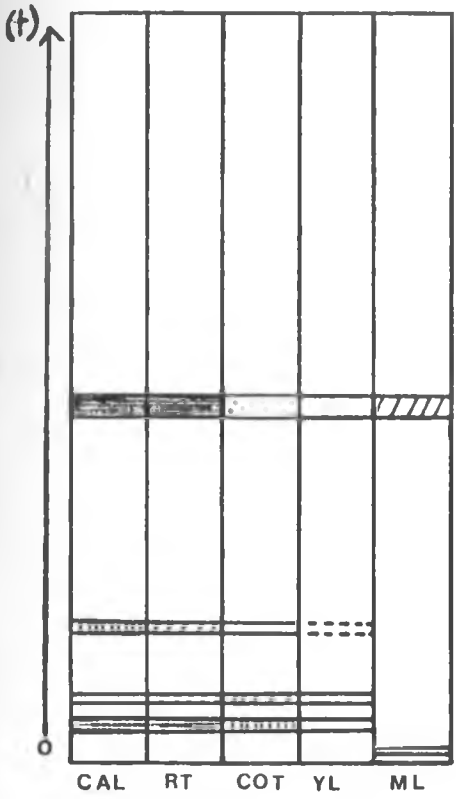

+
F
S
0
5a
+
0
5b
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 6

Zymogram showing electrophoretic patterns of peroxidase in callus (CAL), root (RT), cotyledon (COT) and young leaf (YL) of 9 week-old "Rough Lemon" seedlings. The mature leaf obtained from the seed parent (>5 years old) gives the pattern designated ML.



KEY:

Staining intensity

— Very dark

— Dark

— Medium

— Light

— Very light

— Detectable in traces

O Origin

Figure 6

Table 8: Protein content and peroxidase activity in four different tissues of "Valencia".

Tissue	Age+	(a) peroxidase activity*	(b) Protein content**	(a/b) Peroxidase activity per mg protein
Callus	9 wks	0.73	105.00	6.95
Root	9 wks	0.53	100.00	5.30
Cotyledon	9 wks	0.62	125.00	4.96
Leaf	9 wks	0.42	70.00	6.57
Leaf	>5 yrs	0.46	112.50	4.08

* Peroxidase activity = ΔA_{420}^{nm}/mg fresh weight/ml enzyme extract.
** Protein content = ug protein/mg fresh weight.
+ Refers to the age of the seedling or mature tree from which tissue was derived.

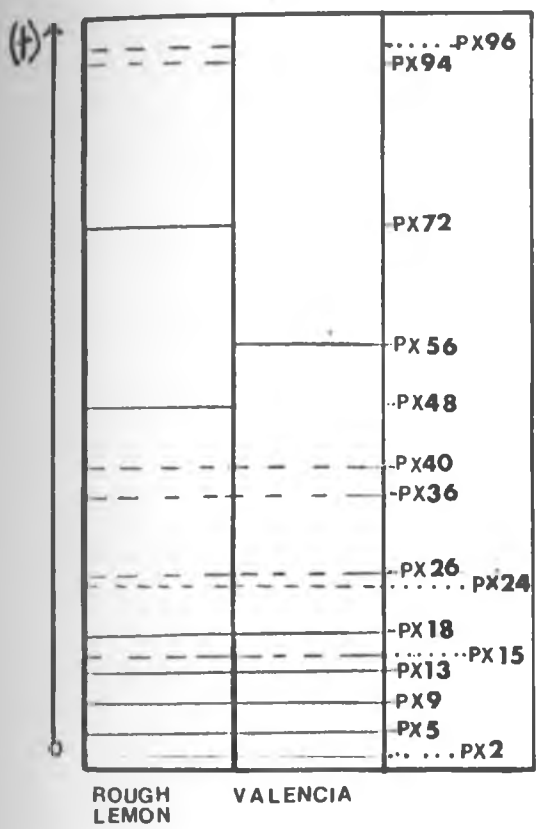
Table 9: Protein content and peroxidase activity in four different tissues of "Rough Lemon".

Tissue	Age+	(a) Peroxidase activity*	(b) Protein content**	(a/b) Peroxidase activity per mg protein
Callus	9 wks	0.70	111.5	6.27
Root	9 wks	0.56	90.00	6.22
Cotyledon	9 wks	0.62	115.00	5.39
Leaf	9 wks	0.43	75.00	5.60
Leaf	>5 yrs	0.48	125.00	3.80

Peroxidase activity = ΔA_{420} nm/mg fresh weight/ml enzyme extract.
Protein content = ug protein/mg fresh weight.
Refers to the age of the seedling or mature tree from which tissue was derived.

Fig. 7

Composite zymogram of peroxidase(PX) bands of "Rough Lemon" and "Valencia" seedlings aged between 2 weeks and 6 months. PX15 was only detected in root and cotyledon and PX94 in root.

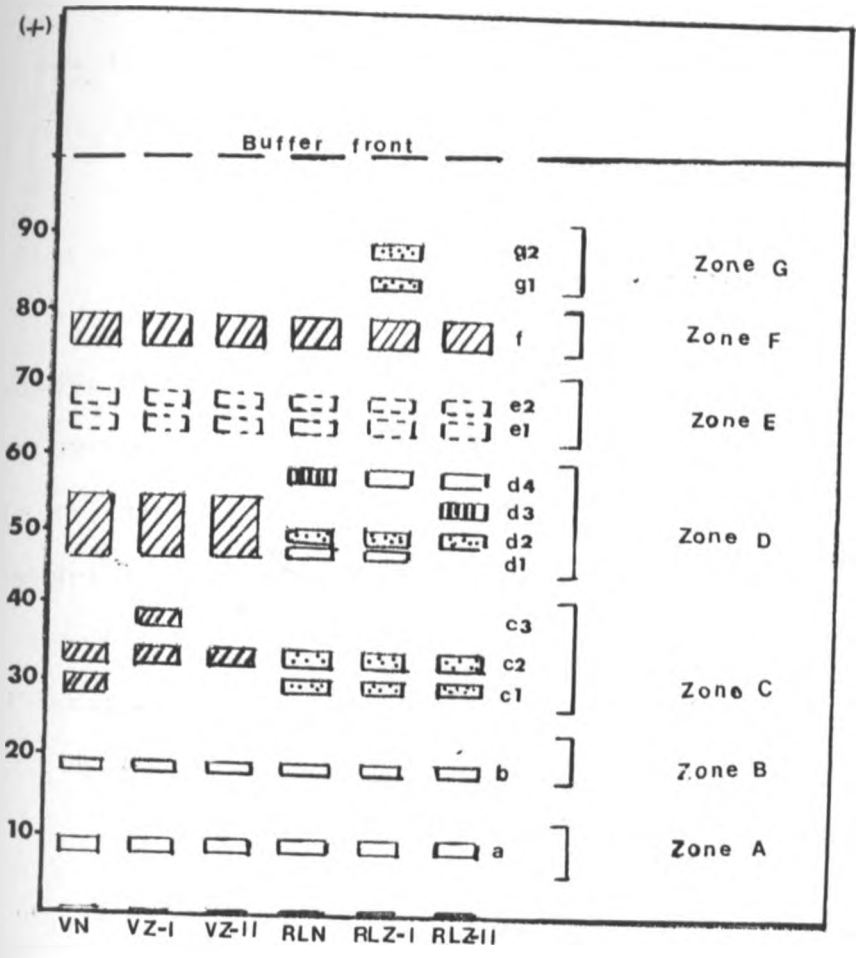


— — — Bands detected in traces
O Origin

Figure 7

Fig.8

Generalized zymogram showing electrophoretic patterns of esterase in leaf of "Rough Lemon" and "Valencia" seedlings aged under 6 months. A total of 14 bands (a, b, c1, c2, c3, d1, d2, d3, d4, e1, e2, f, g1 and g2) were observed in 7 zones (A, B, C, D, E, F and G). Two types (I&II) of zymogram were observed for putative zygotic seedlings of each species. Zone D bands were sometimes visualized as a single diffuse band. (isozyme patterns were similar in leaf from seedlings aged 2-6 months). VN = nucellar seedlings of "Valencia": VZ = zygotic seedlings of "Valencia": RLN = nucellar seedlings of "Rough Lemon": RLZ = zygotic seedlings of "Rough Lemon".



KEY:

Staining intensity

- Dark
- Medium
- Light
- Very light
- Detected in traces

Figure 8

Fig.9

Electrophoretic patterns of glutamate oxaloacetate transaminase in "Rough Lemon " (RL) and "Valencia" (V). IA: (RL) tissues other than embryo;IB: (RL) embryonic pattern; IIA: (V) tissues other than embryo;IIB: (V) embryonic pattern. The bands corresponding to Got-1 and Got-2 loci are indicated.

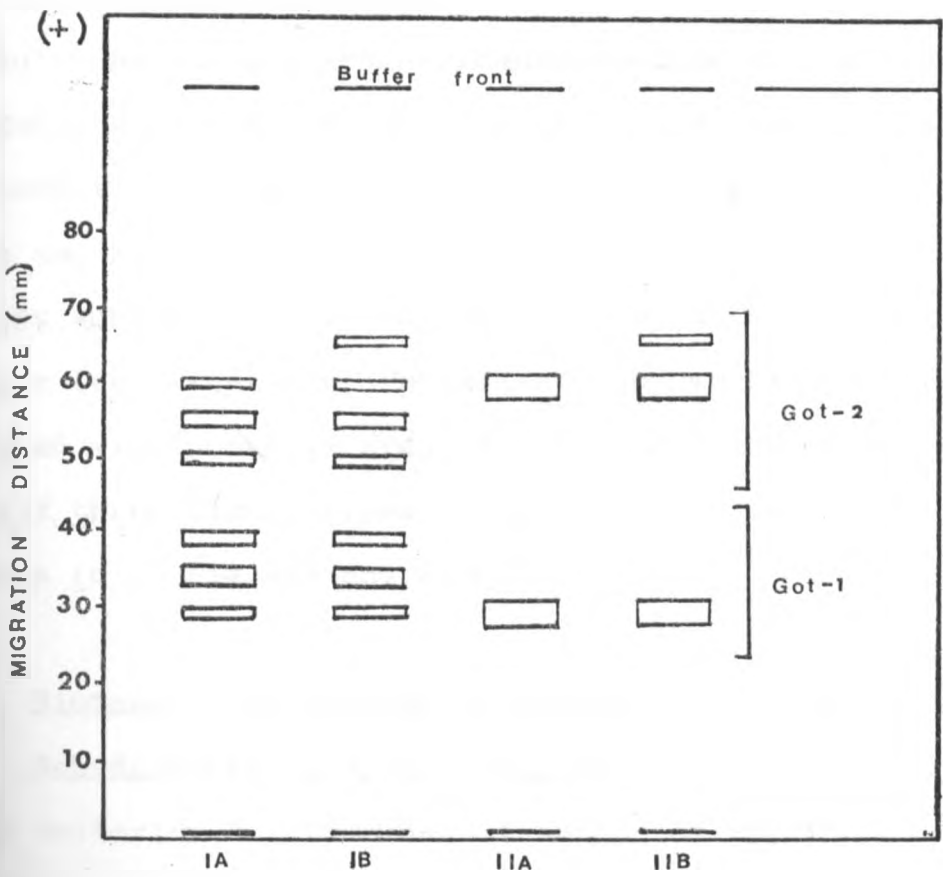

(+)
Buffer front
80
70
60
50
40
30
20
10
MIGRATION DISTANCE (mm)
Got-2
Got-1
IA
IB
IIA
IIB

Figure 9

3.3.5 Malic enzyme

Bands were visualized in two well separated regions of the gel as shown in Fig.12 and Fig.13.

3.4 Identification of nucellar and zygotic seedlings

3.4.1 Peroxidase and esterase

Results pertaining to the identification of nucellar and zygotic seedlings using peroxidases and esterases are summarized in Table 11, Table 12, Fig.14, Fig.15a, Fig.15b, Fig.16a and Fig.16b. All "Rough Lemon" and "Valencia" seedlings analyzed using leaf peroxidases were also analyzed for their leaf esterases. Peroxidase isozyme bands associated with putative zygotic seedlings were detected using all three tissue types, that is, leaf, root and cotyledon (c.f. Fig.15a and Fig.15b).

3.4.2 Glutamate oxaloacetate transaminase, malate dehydrogenase and malic enzyme

The criterion for the identification of zygotic and nucellar seedlings is shown in Table 13 (also section 2.2.5), Fig.9, Fig.13, Fig.17, and Fig.18. Data pertaining to the frequency of zygotic seedlings is presented in Table 14 and Table 15. The effect of embryo separation (or lack of it) on the recovery of zygotic seedlings was tested with Chi Square analyses as summarized in Table 16.

Fig.10

Electrophoretic patterns of glutamate oxaloacet transaminase (GOT) in leaf of "Rough Lemon" (la 1-8) and "Valencia" (lane 9-13). S is the slow band phenotype representing the homozygous Got-: SS genotype. S, I and M are the three band isoz phenotypes representing the heterozygous Got-2 S genotype which was only observed in "Rough Lemo O= point of sample insertion (origin). Migratio is anodal.

Fig.11

Leaf malate dehydrogenase (MDH) isozymes of "Rou "Lemon" seedlings resolved with pH 8.0 buffer an 8.5% acrylamide. The arrow indicates the positi of the slowest migrating band that only appears after overnight destaining of the gel. (see also Fig.18). O= point of sample insertion (origin). Migration is anodal.

Fig.12

Leaf malic enzyme (ME) isozymes of "Valencia". Two well separated zones are prominent. Streaking of ME bands was sometimes observed during electrophoresis, as seen for the fast (F) and slow (S) migrating isozyme forms in this photograph. O= point of sample insertion. (origin) Migration is anodal.

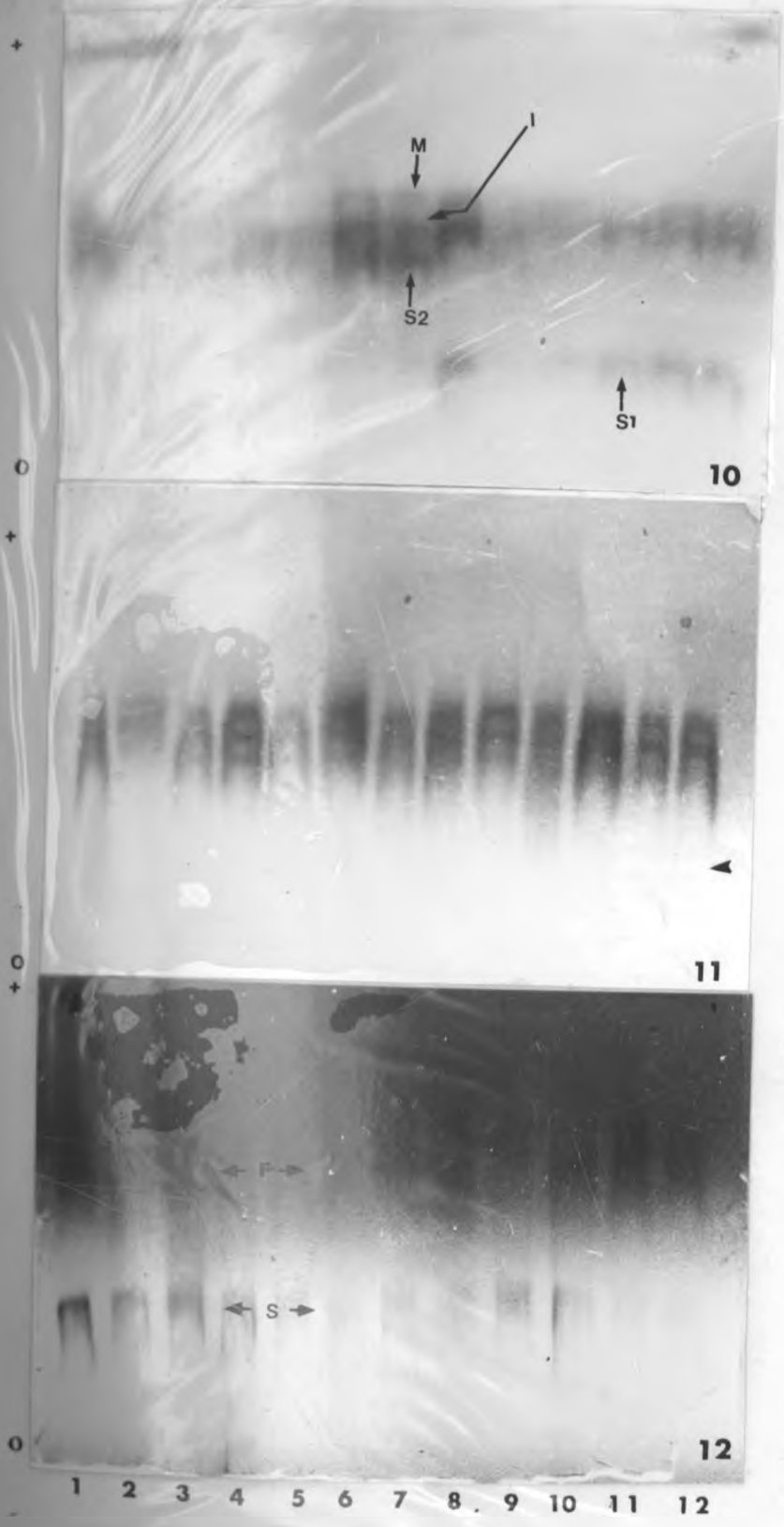

+
M
I
S2
S1
10
O
+
11
O
+
P
S
12
O
1 2 3 4 5 6 7 8 9 10 11 12

Table 10: Migration distances and numbers of MDH isozyme bands of "Rough Lemon" and "Valencia" at resolving buffers of 7.6 and 8.0 and 8.5% acrylamide concentration.

MDH locus	pH of resolving buffer	Number and distance (mm) travelled by band	
		Rough Lemon	Valencia
Mdh-1	7.6	3 bands at 81,78 and 75	2 bands at 80 and 82
	8.0	3 bands at 66,59 and 53	1 band at 66
Mdh-2	7.6	1 band at 60	1 band at 66
	8.0	1 band at 42	1 band at 42

Fig.13

Zymogram showing the electrophoretic patterns of malic enzyme in leaf of "Rough Lemon" and "Valencia". The pattern for nucellar genotypes was identical in both species (pattern A). Patterns B and C were observed in zygotic seedlings of "Valencia".

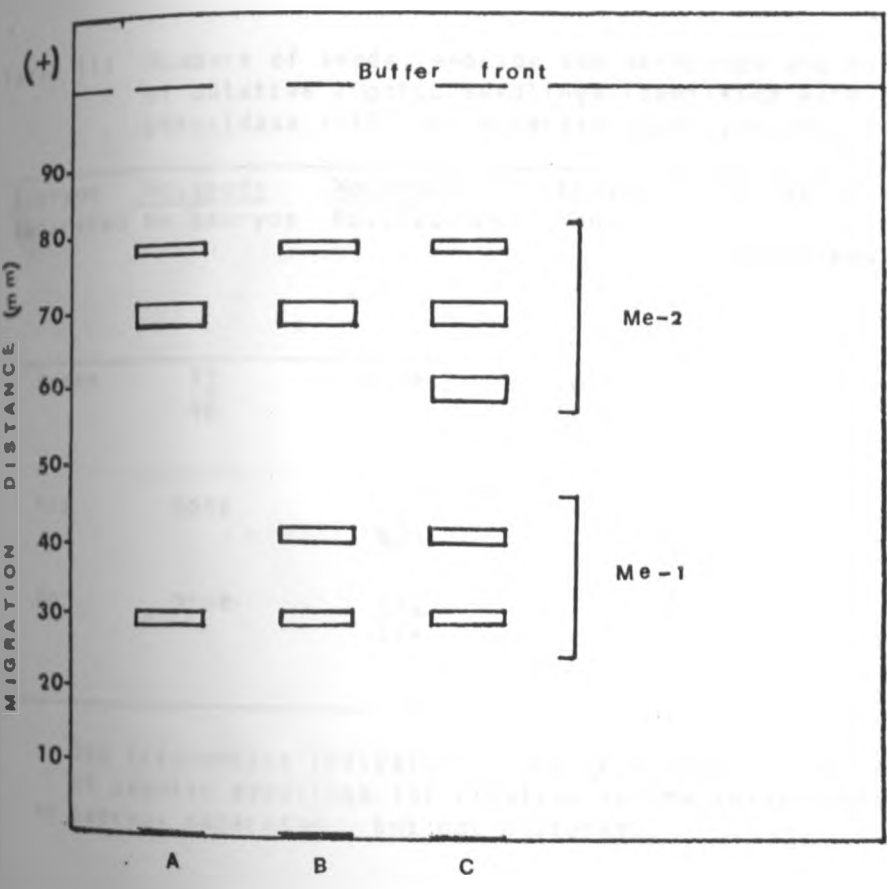

(+)
Buffer front
90
80
70
60
50
40
30
20
10
MIGRATION DISTANCE (mm)
Me-2
Me-1
A
B
C

Figure 13

Table 11: Numbers of seeds, embryos and seedlings and the frequency of putative zygotic seedlings identified with the aid of peroxidase (PXD) and esterase (EST) isozymes in "Rough Lemon".

Embryos separated	No.seeds / No.embryos	No.seeds / No.seedlings	Enzyme source	No.putative zygotic seedlings PXD	No.putative zygotic seedlings EST	%(frequency) of zygotic seedlings*
Yes**	92 / 92	none	embryo	-	1	1.08 (1.08)
Yes	none	19 / 83	leaf	2	5	8.4 (36.8)
No	none	102 / 144	leaf	0	2	1.39 (1.96)

* The frequencies indicated in brackets were calculated as the proportion of zygotic seedlings (%) relative to the total number of seeds.

** Embryos separated but not cultured.

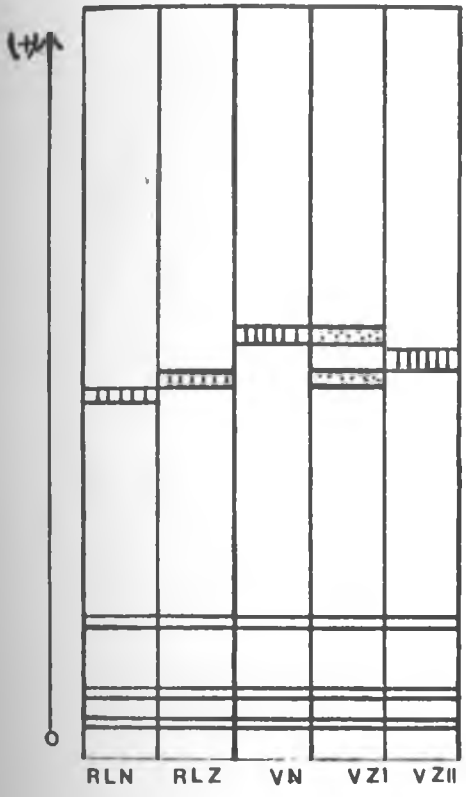
Table 12: Numbers of seeds and seedlings and the frequency of putative zygotic seedlings identified with the aid of peroxidase (PXD) and esterase (EST) isozymes in "Valencia"

Embryos separated	No. seeds	No. seedlings	Enzyme source	No. putative zygotic seedlings		%(frequency) of zygotic seedlings*
				PXD	EST	
Yes	17	94	leaf	2	3	5.3 (29.4)
No	35	81	leaf	3	4	4.9 (11.4)

* The frequencies indicated in brackets were calculated as the proportion of zygotic seedlings (%) relative to the total number of seeds.

Fig.14

Identification of zygotic and nucellar seedlings with peroxidase isozymes in "Rough Lemon" (RL) and "Valencia" (V). (the staining intensity for cathodal bands is not shown owing to tissue specific differences). Two types of zymograms were were observed for putative zygotic seedlings of "Valencia" (V-Z I and V-Z II). Z = zygotic; N = nucellar.



KEY:

Staining	intensity
	Dark
	Light
O	Origin

Figure 14

Fig. 15a

Leaf peroxidase isozymes of 3 month-old "Rough Lemon" seedlings. Lane 6 shows a faster migrating band representing a putative zygotic seedling (arrow). Notice the darkly stained bands (lane 4 and 5) of leaf from the mature seed parent.

Fig. 15b

Root peroxidase isozymes of "Rough Lemon" (11 weeks old) and "Valencia" (12 weeks old). Lane 3 shows two lightly stained anodic bands (arrows) representing the pattern of a putative zygotic seedling. Lane 1-8: "Valencia" seedlings from the same seed; Lane 9-13: "Rough Lemon" seedlings from the same seed. O= point of sample insertion (origin). Migration is anodal.

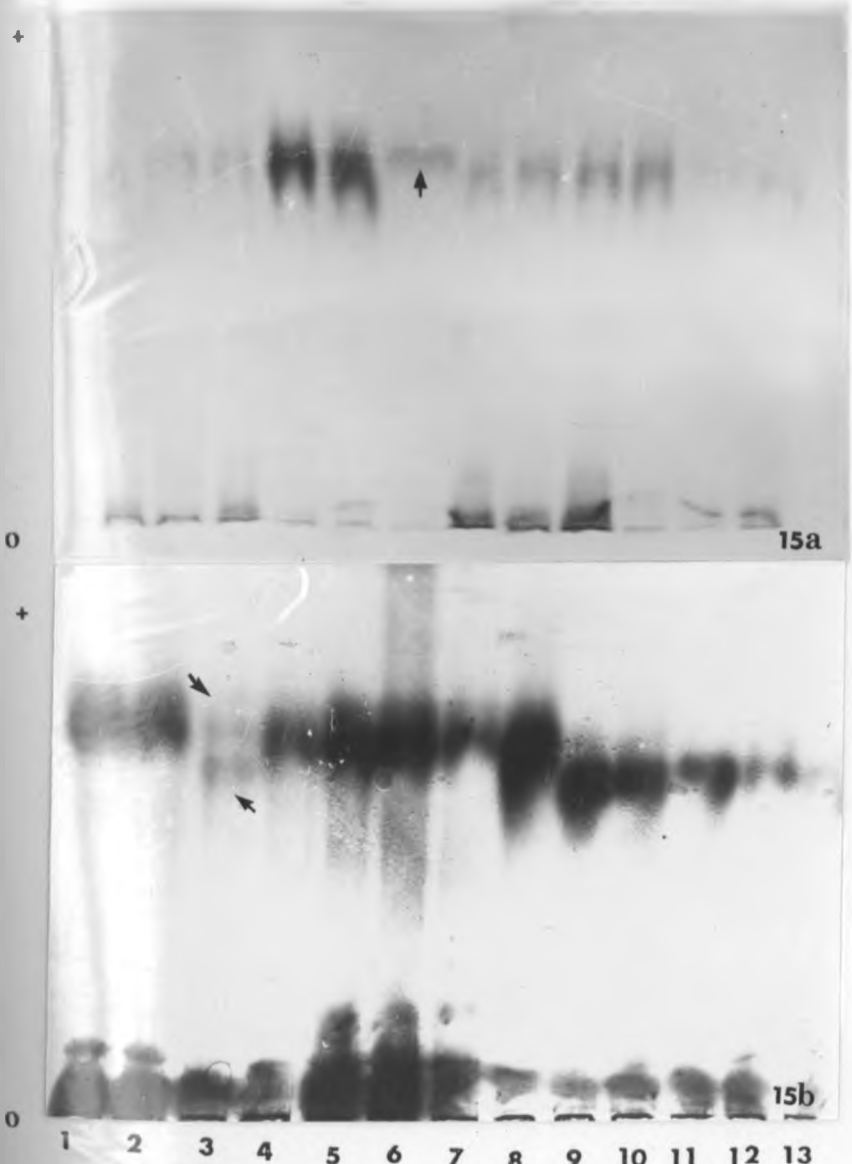

+
0
15a
+
15b
0
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig.16a

Electrophoretic patterns of esterase in leaf of "Rough Lemon" and Valencia" seedlings. Lane 2-5: nucellar "Rough Lemon" seedlings; Lane 6 and 7: putative zygotic seedlings of "Rough Lemon" ; Lane 8-9: putative zygotic seedlings of "Valencia"; Lane 10-12: nucellar seedlings of "Valencia"; Lane 1 and 13: mature seed parent patterns of "Rough Lemon" and "Valencia" respectively.

Fig. 16b

Electrophoretic patterns of esterase in embryos of "Rough Lemon" and "Valencia". Lane 1-10: nucellar "Rough Lemon"; Lane 11: three combined nucellar embryos of "Valencia"; Lane 12 and 13: putative zygotic embryo of "Rough Lemon". All "Rough lemon" embryos were derived from different seeds. O= Point of samle insertion (origin). Migration is anodal.

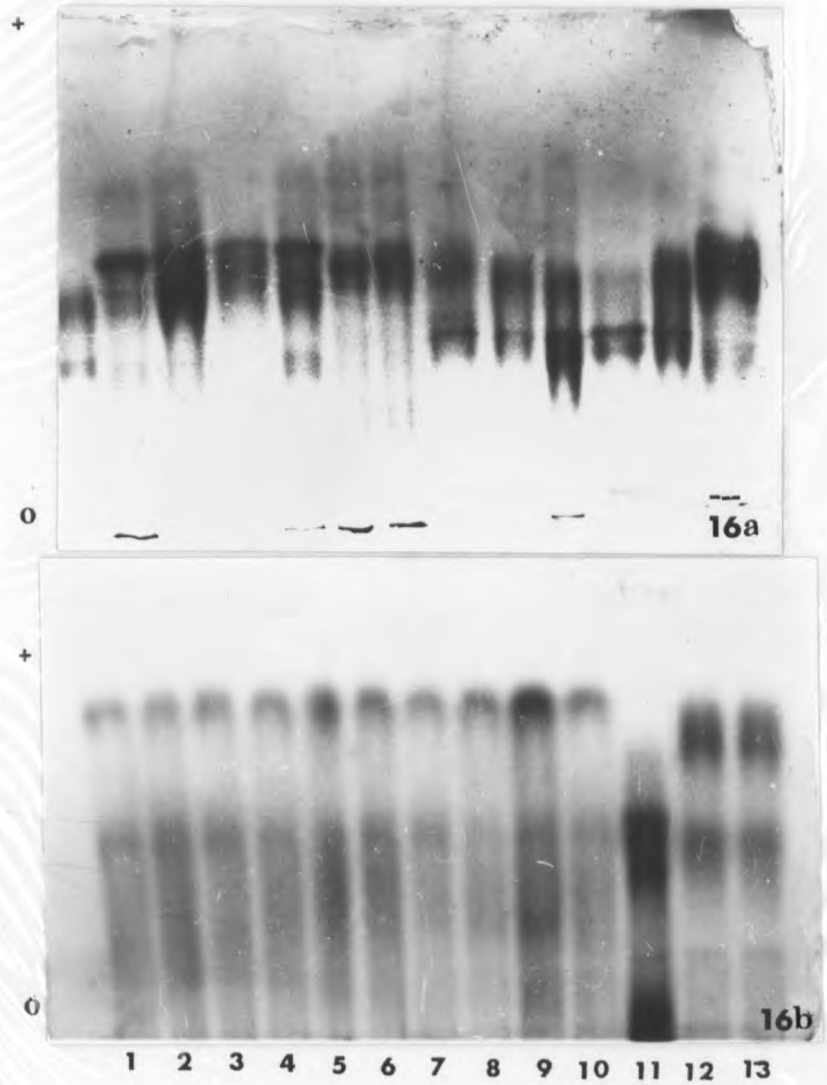

+
0
16a
+
0
16b
1 2 3 4 5 6 7 8 9 10 11 12 13

Fig. 17

Electrophoretic patterns of malic enzyme in leaf of 4 month-old "Valencia" seedlings. Lane 5 shows a unique band (R) detected in one seedling out of five that were derived from the same seed. F1 and S are fast and slow band phenotypes, respectively (Me-1 FS genotype). F2 and M1 and M2 are fast and slow band phenotypes, respectively (Me-2 FS genotype). The bands M1 and M2 (doublets) were frequently visualized as one single band. O= point of sample insertion (origin). Migration is anodal.

Fig. 18

Electrophoretic patterns of malate dehydrogenase in leaf of 2 month-old "Rough Lemon" seedlings. S2 = slow band phenotype (Mdh-2 SS genotype); S1 = slow band phenotype (Mdh-1 SS genotype); T= three band phenotypes representing Mdh-1 FS genotype. The slow band (S2) stains weakly and was only visible after destaining the gel. The bands shown were resolved with pH 7.6 buffer and 8.5% acrylamide. O= point of sample insertion (origin). Migration is anodal.

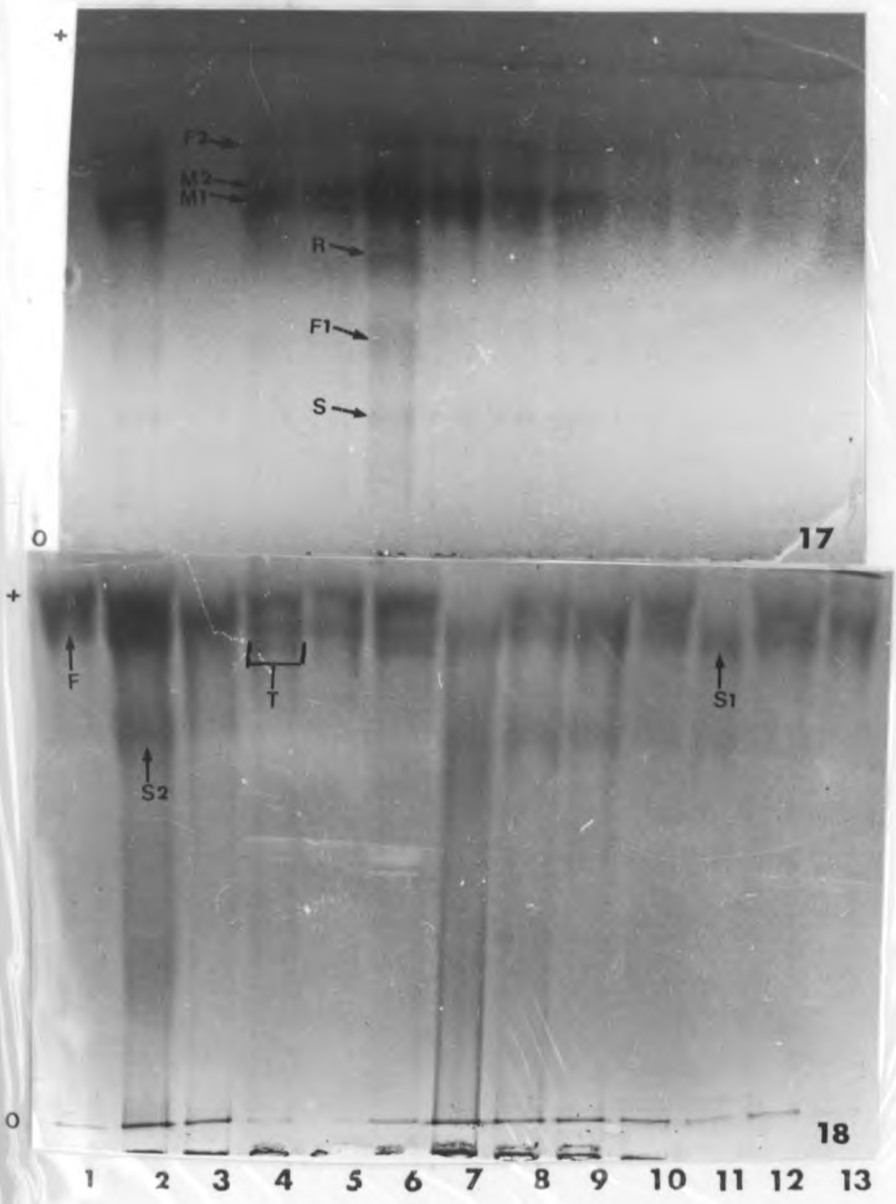

+
F2
M2
M1
R
F1
S
0
17
+
F
T
S1
S2
0
18
1 2 3 4 5 6 7 8 9 10 11 12 13

Table 13: Mdh, Got and Me genotypes of "Rough Lemon" and "Valencia" nucellar and zygotic seedlings.

Enzyme system	Enzyme locus	Rough Lemon*		Valencia*	
		Parent or nucellar seedlings	Zygotic seedlings	Parent/ nucellar seedlings	zygotic seedlings
MDH	Mdh-1	FS	FS,FF,SS	SS	SS
	Mdh-2	SS	SS	SS	SS
GOT	Got-1	FS	FS,FF,SS	SS	SS
	Got-2	SM	SM,MM,SS	MM	SS
ME	Me-1	SS	SS	SS	SS
	Me-2	FS	FS,FF,SS	FS	FS,FF,SS

* Migration speed of F, M and S subunits is in the order:F>M>S during electrophoresis.

Table 14: Numbers of seeds and seedlings and the frequencies of zygotic genotypes identified using Me-1 and Me-2 loci in "Valencia".

Embryos separated	No.seeds / No.seedlings	No.seedlings and genotype								% (frequency) of zygotic seedlings
		Me-1			Me-2					
		FF	FS	SS	FF	FS	SS	SR		
Yes	18 / 111	0	4	108	2	107	1	1		6.3 (38.8)
NO	11 / 34	0	0	34	1	33	0	0		2.9 (9.09)

Table 15: Numbers of seeds, embryos and seedlings and the frequencies of zygotic genotypes identified using Got-2 and Mdh-1 heterozygous enzyme loci in "Rough Lemon".

Embryos separated	No.seeds / No.seedlings		No.seeds / No.embryos		No.putative zygotic seedlings						% (frequency) of zygotic seedlings
					Got-2			Mdh-1			
	GOT	MDH	GOT	MDH	MM	MS	SS	FF	FS	SS	
Yes	11 / 51	11 / 51	none	none	1	50	0	0	49	2	5.8 (27.2)
	none	none	84 / 84	none	0	84	0	-	-	-	0.0
No	62 / 73	125 / 180	none	none	0	73	0	4	175	1	2.8 (4.0)

Table 16: Chi Square values and significance for the difference in frequencies of nucellar and zygotic seedlings identified using PXD, EST, ME, MDH and GOT gene/enzyme systems (comparison between numbers of zygotic seedlings recovered with and without embryo separation).

Cultivar	Gene/ enzyme system**	Frequency of zygotic seedlings (%)		X^2
		ES*	ENS*	
	PXD	10.50	0.0	18.35-S*
Rough	EST	26.30	1.96	13.24-S*
Lemon	MDH	18.20	4.0	16.60-S*
	GOT	9.09	0.0	16.50-S*
Valencia	PXD	11.70	8.60	0.02-NS*
	EST	17.60	2.90	1.74-NS*
	ME	38.80	9.09	1.72-NS*

** - PXD- peroxidase; EST-esterase; MDH-malate dehydrogenase; ME-malic enzyme; GOT-glutamate oxaloacetate transaminase.

S* - Significant at 1% level

NS* - Not significant at 1% level

ES* - Embryos separated.

ENS*- Embryos not separated

3.5 Analysis of genetic uniformity of Valencia plantlets obtained through nucellar tissue culture

Peroxidase, esterase and malic enzyme gene/enzyme systems were employed for the analysis of a total of 86 plantlets derived from *in vitro* culture of the nucellus tissue (Fig. 19). Some of the plantlets analysed also originated from budding of enlarged cotyledons or "pseudobulbils" (Fig.1a, Fig.20). One seedling was found to be variable. The rest were genetically uniform (Fig.21).

Fig. 19

Nucellar embryogenesis in "Valencia". Notice the many plantlets that arise from cultured nucellar tissue.

19

Fig.20

Two-year old plantlets of "Valencia" obtained through nucellar tissue culture. Thirty nine such plantlets were analyzed for genetic uniformity using peroxidase, esterase and malic enzyme isozymes.

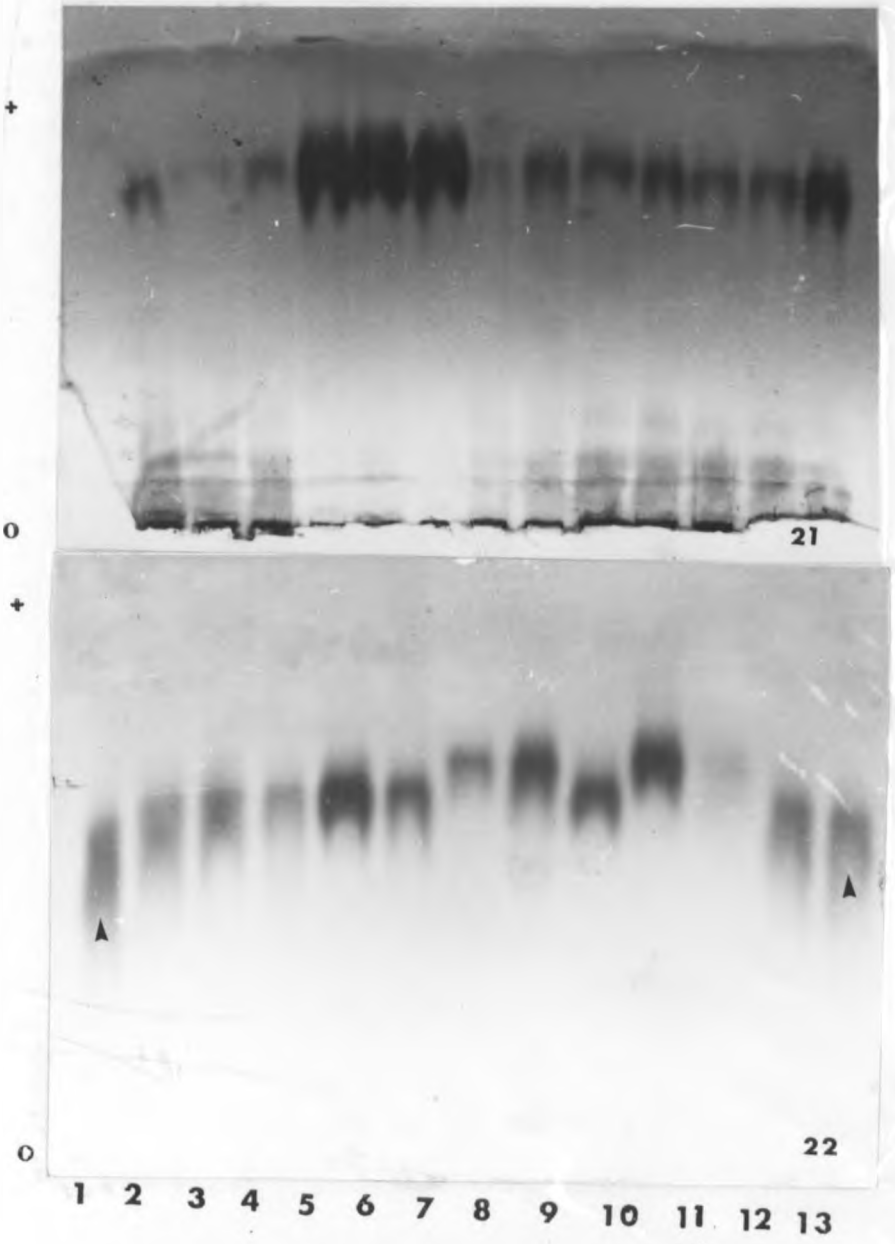

C. sinensis
Nucellar
seedling
20

Fig. 21

Peroxidase isozyme patterns in leaf of 12 nucellar plantlets of "Valencia". Lane 1-3, 7-12: Six month-old plantlets from two different calli, respectively; Lane 4-6: Two year-old plantlets from the same callus; Lane 13: seed parent. O= point of sample insertion (origin). Migration is anodal.

Fig. 22

Photograph showing "edge effects" caused by unequal distribution of heat in the gel (arrows). Bands in the centre of the gel migrate faster than those at the edges. Lane 7, 8, 10, 11 = "Valencia"; Other lanes = "Rough Lemon". O= point of sample insertion (origin). Migration is anodal.


+
0
21
+
0
22
1 2 3 4 5 6 7 8 9 10 11 12 13

CHAPTER 4

DISCUSSION

4.1 *In vitro* generation of seedlings from redundant preformed embryos

Out of a total of 805 cultured seeds of *C. limon* and *C. sinensis*. 202 (25%) were discarded owing to bacterial and fungal contaminations. Other 239 seeds (29.7%) failed to produce seedlings due to lack of a germination/growth response on the MS (Murashige and Skoog, 1962) medium. The percentage of seeds that germinated was significantly higher in "Rough Lemon" than "Valencia" (Table 4). The embryos showed very marked differences in size while still in the seed. Larger sized proembryos developed into seedlings earlier than the smaller ones (Fig.1a, Fig.3c). Similar size-dependent differences in growth between embryos were reported by Rangan *et al.*, (1969).

The average number of seedlings per seed reported in previous studies is 1.31 and 2.00 in *Citrus sinensis* and *C. limon*. respectively (Frost and Soost, 1968). These figures refer to soil sown seeds. In this study, the mean numbers of seedlings per seed were 2.62 and 1.44 for *in vitro* sown "Valencia" and "Rough Lemon" seeds, respectively, in the absence of embryo separation. These

numbers were significantly enhanced (Z = -8.19; -10.00) through embryo separation (section 2.2.1, Table 5, Table 6). A maximum of 13 "Valencia" and 9 "Rough Lemon" seedlings were obtained per seed when embryos were separated (Fig.1b, Fig.3c, Fig.2a, Fig.2b, Fig.4a, Fig.4b). Though a detailed study was not carried out in connection with embryo numbers, isolated and random counts of embryos from mature seeds revealed upto 18 embryos per seed in "Valencia" and 11 in "Rough Lemon".

4.2 Polyacrylamide gel electrophoresis

The numbers and patterns of isozyme bands of GOT, MDH and ME obtained using polyacrylamide gel electrophoresis (PAGE) in this study were similar to those reported earlier using starch gel electrophoresis (Torres *et al.*, 1978, 1982). The report by Iglesias *et al.*, (1974) on *Citrus* esterases (EST) did not contain any information about the actual number and distribution of esterase bands. Therefore the present report is the first on this subject. The number and distribution of peroxidase (PXD) bands was largely in agreement with that previously observed using PAGE (Button *et al.*, 1976; Iglesias *et al.*, 1974).

4.2.1 Peroxidase

Peroxidase bands were named using the prefix PX and a number that represents the average distance migrated by

the band relative to the standard isozyme band (Esen and Soost, 1976; Lagrimini and Rothstein, 1987). Under the electrophoretic conditions described in section 2.2.3.2, the standard isozyme bands occur at 56 and 48 mm from the origin of the gel for sample extracts derived from "Valencia" and "Rough Lemon" tissues, respectively. Quantitative and qualitative differences were consistently observed between the peroxidase zymograms of root, callus, cotyledon, young and mature leaves (Fig.5b, Fig.6, Fig.7). The total peroxidase activity per mg fresh weight in tissues decreased in the order: callus > cotyledon > root > mature leaf > young leaf (Table 8 and Table 9). This order was different when total peroxidase activity per mg of protein was considered, decreasing in the order: callus > root > cotyledon > young leaf > mature leaf. Low PXD activity was reflected either as fewer number of isozyme bands or as lighter staining bands (Fig.5b). Such correlation between the levels of expression of peroxidase in different tissues and its isozymes has been shown in several plant systems (Gasper et al., 1982).

Peroxidase isozymes have been extensively used to study tissue specificity, developmental regulation and the effects of tissue culture (Bassiri and Carlson, 1979; Scandalios and Sorensen, 1977). In this study, the overall tissue and developmental expression of isozyme bands of "Rough Lemon" and "Valencia" was very similar. Fig.7 shows all the

isoperoxidase bands detected under the electrophoretic conditions described in section 2.2.3.1 using leaf, cotyledon, root and callus. A total of 14 bands were detected in "Rough Lemon" as compared to 11 in "Valencia" samples. Bands designated PX15, PX24, PX26, PX36, PX40, PX72, PX94 and PX96 were detected in traces and at very low frequencies (<5%). This observation was also noticed by Esen and Soost (1976) who have discussed in detail the limitations of PXD profiles in connection with definability and resolution. Their study showed that not all isozymes in profiles are readily definable following one electrophoretic run. The anodic bands reported by them, were, however, not detected in this study. Because the isozyme phenotype on electrophoretic gels is affected by many factors such as modifier genes, degradation and other factors that alter the electrophoretic mobility and/or number of isozymes (Jacobs, 1974a), PXD bands detected in traces and low frequencies were not considered for use in the identification of zygotic and nucellar seedlings.

4.2.2 Esterase

Esterase bands were observed in seven zones on the gels and named according to these zones (Fig.8). Zones A, B and F show single bands and do not demonstrate any electrophoretic variability. These bands are probably coded by monomorphic loci. Zone C stains much more intensely for "Valencia" leaf

samples than "Rough Lemon" (Fig.16a). Bands of zone D were visualized either as a diffuse single band or as three separate bands in "Valencia". The F band (zone F) is more prominent in embryonic than in leaf tissue (Fig.16a, Fig. 16b). These differences may allude to much more prominent quantitative and probably qualitative differences in isozyme patterns between tissues and ontogenetic stages and when different concentrations of enzyme extract, pH levels of resolving buffer, specific enzyme substrates and other factors are considered. Electrophoretic conditions in this study were kept constant in all experiments pertainig to esterases, thus obviating the need to calculate Rf values.

4.2.3 Glutamate oxaloacetate transaminase (GOT)

Six GOT bands, occuring in two sets of triplets, were resolved in two regions of the gel. The slow migrating set was composed of three bands at 29, 34 and 39 mm, respectively, from the origin. These three bands (genotype FS) were assigned to the Got-1 locus according to the nomenclature of Torres et al., (1978). They were clearly resolved in embryos, but very poorly in leaf (Fig.10). So they were not used in the identification of zygotic and nucellar seedlings. The faster migrating set of bands, located at 50, 55, and 60 mm, respectively, (genotype SM) were assigned to the Got-2 locus (type IA, Fig.9). Only two bands at 29 (genotype SS) and 60 mm (genotype MM) were

obtained using "Valencia" leaf extract (type IIA, Fig.9). Extracts from embryos gave rise to a fourth band at 66 mm (types IB and IIB, Fig.9).

The GOT zymograms obtained in this study were also observed by Hirai et al., (1986) and Kawase and Hirai (1985). This study therefore provides further evidence for the dimeric nature of both GOT-1 and GOT-2. The poor resolution of GOT-2 bands may be attributed to the age of the leaves used, as reported by of Wu and Jwo (1986). They showed that the clearness of GOT isozyme bands is largely influenced by the age of the leaf.

4.2.4 Malate dehydrogenase (MDH)

Two, three or four bands were visualized on gels stained for MDH (Table 10). After several electrophoretic runs using several acrylamide concentrations (6.0, 6.5, 7.0, 7.5, 8.0 8.5 %), 8.5% was selected and a pH of 7.6 or 8.0. Only pH 7.6 was, however, routinely employed. The three bands at 81, 78 and 75 mm (genotype FS) were assigned to the Mdh-1 locus (Torres et al., 1982). The slowest band at 42 mm (genotype SS) shows very weak activity and was assigned to the Mdh-2 locus (Fig.18). The distance between bands reported here is less than that obtained using starch gel elctrophoresis (Torres et al., (1982) . Therefore further work need to be done in order to standardize the conditions for resolution of MDH bands using PAGE by trying

other pHs, buffer systems, and ionic strengths.

4.2.5 Malic enzyme (ME)

ME bands occur in two well separated zones on the gel. Two bands were visualized at the anodal end at 69 and 78 mm (Me-2), respectively. Either one or two faint bands were located at the cathodal end (Fig.13) at 27 and/or 40 mm (Me-1 locus). The slower migrating one of the pair of the faster migrating set was occassionally seen as two very close, darkly stained bands (Fig.17), referred to as doublets. The reason for the occurance of these doublets is not clear, but they may have been due to slight variations in electrophoretic conditions during the electrophoretic run e.g. current applied, duration of the run or, most likely, the higher resolution attributed to PAGE as opposed to starch gel electrophoresis (Hames, 1982).

4.3 Identification of nucellar and zygotic seedlings and the analysis of nucellar plantlets for genetic uniformity

The frequency of zygotic seedlings identified using GOT and PXD was nil in situations where embryos were not separated in culture. Only two "Rough Lemon seedlings were identified as "putative" zygotics using PXD isozymes (Fig. 14, Fig.15a). A unique electrophoretic pattern for leaf esterases (EST), observed in zone D (type II, Fig.8,

Fig.16a) was present in a total of four "Rough Lemon" seedlings. Three other seedlings had the type I pattern (Fig.8, Table II). Four "Valencia" seedlings had the type I PXD zymogram while one other seedling had the type II pattern (Fig.14, Fig.15b)

Zygotic seedlings identified using PXD and EST isozyme bands were referred to as "putative" because the genetic control of these bands is not known. It has previously been difficult to characterize PXD bands largely because of the absence of a reproducible method of resolving them (Esen and Soost, 1976). Moreover, both PXD and EST isozymes have only been analyzed using PAGE (Button et al., 1976; Esen and Soost, 1976; Iglesias et al., 1974). It would be useful in future to assess the effectiveness of starch gel in the resolution of PXD and EST bands. In this study, bands were taken to represent the "putative" zygotic pattern on the basis of their uniqueness and reproducibility (Fig.8, Fig.14, Fig.15a, Fig.15b, section 2.2.5).

All 84 embryos analyzed at Got-2 showed the standard (nucellar) SM genotype (type IB, Fig.9). The embryos used were obtained directly from uncultured seeds. Only the largest redundant embryos were used for enzyme extraction. PXD bands did not appear when such embryos were used as the source of enzyme for electrophoresis, except the band designated PX5, which, nevertheless, shows very weak

activity. One isolated embryo out of 92 analyzed for EST isozymes yielded a zymogram that appeared to represent an embryo of hybrid origin (Fig.16b). These results, using GOT and EST, indicate that the identification of zygotic and nucellar genotypes can be accomplished as early as at the embryonic stage of development, assuming that the zygotic isozyme pattern will still appear, albeit in a different form, in later stages of development. Such an exercise (of early genotype identification) can be useful in studies involving tissue culture manipulation of embryos, especially in applied aspects of embryo culture e.g. *in vitro* nucellar budding and the recognition of hybrid cells in culture (Raghavan, 1977; Scandalios and Sorenson, 1977).

Four "Valencia" seedlings produced the type I esterase zymogram (Fig.8, Fig.16a), while three seedlings yielded the type II zymogram using leaf tissue as the enzyme source. It is likely that these patterns represent cases of outcrossing since similar isozyme patterns had been observed earlier in situations where controlled crosses between different *Citrus* species were employed (Esen and Soost, 1976; Iglesias *et al*., 1974). Though "Rough Lemon" and "Valencia" are highly heterozygous and are known to hybridize easily with other *Citrus* species, "Valencia" has been reported to have more pollen parents than "Rough Lemon" (Cameron and Frost, 1968; Frost and Soost, 1968).

The frequency of zygotic seedlings identified with ME,

MDH and GOT is shown in Table 14, Table 15 and Table 16. ME bands, however, deserve further mention here. With a probability of 50% that zygotic seedlings resulting from self-pollination could be detected, 7 out of 111 seedlings from 18 seeds were found to be zygotic. Four of the seedlings, however, were heterozygous at the Me-1 locus and therefore must have necessarily arisen from outcrossing. Torres *et al*., (1982), using starch gel electrophoresis, reported that the faster migrating isozymes stain very faintly and show no obvious polymorphism. Because FF and SS genotypes were detected in this study, it may be true to say that the Me-2 locus is in fact polymorphic. In addition, one seedling possessed a unique genotype (pattern C, Fig.13, Fig.17). The occurance of this unique pattern and the doublets (Fig.17) probably indicated the presence of a third ME locus. Generally, however, ME bands showed the isozyme patterns typical of a monomeric enzyme as established by Torres *et al*., (1982).

The separation of cultured embryos was shown to enhance the recovery of zygotic seedlings significantly in "Rough Lemon". This was not the case in "Valencia" (Table 16). The situations in which embryos were not separated may be assumed to correspond to the common practice of acquiring seedlings by sowing the seeds in soil. In such situations, cases where zygotic seedlings have not been detected have been reported by Khan and Roose (1988) in various *Citrus*

species. Roose and Traugh (1988) observed substantial variations in the frequency of zygotic seedlings of the same cultivars obtained from different localities and between years. For example, the frequency of zygotic trees of "Valencia" had shot up from nil to 4.5% within an interval of three years.

Despite early embryo separation, the frequencies of zygotic seedlings, are still low in this study. Interestingly, the frequencies obtained using PXD and EST (38.8% and 27.2% in "Rough Lemon" and "Valencia", respectively - Table 11, Table 12) and with ME and MDH and GOT (36.8% and 29.4% in "Valencia" and "Rough Lemon", respectively - Table 14, Table 15) are very similar. This finding probably indicates that the isozymes of PXD and EST are under definite genetic control. It is therefore suggested here that the genetics of these enzyme systems be studied in Citrus by first characterizing species and cultivars. Subsequently, controlled crosses and detailed genetic analyses of progeny resulting from such crosses can be carried out.

The low frequency of zygotic seedlings may be attributed to the use of preformed embryos that were already advanced in age and may therefore have outgrown other embryos. In this study, embryos were derived from fruits that were 7-21 weeks old. This assumption is supported by the recent observations of Chen and Wang (1987) on the development of citrus embryos. They were

able to correctly isolate and culture zygotic embryos on modified White's medium (White, 1943). Their observations showed that there is only one zygotic embryo in the embryo sac at approximately 50 days after the beginning of development. In this study, it was observed that one or, rarely, two embryos rapidly develop into plantlets earlier than the other embryos derived from the same seed (Fig.3a, Fig.3b). The evidence from analyses of such seedlings in both "Valencia" and "Rough Lemon" showed that they are not necessarily zygotic. These findings probably indicate that the two Citrus species largely produce seedlings of nucellar origin. This is in conformity with the report of Cameron and Soost (1969) who, in their summary of the characteristics of Citrus species with respect to the genetic origin of embryos, stated that species which form nucellar embryos are generally facultative apomicts and polyembryonic.

Environmental and genetic factors are known to affect the frequency of zygotic seedlings. These factors include tree age, crop load, nutrient status, locality, seasonal effects, the male (pollen) parent, and differential susceptibility of nucellars vs. zygotics to seedling diseases (Esen, 1973; Frusato et al., 1957; Khan and Roose, 1988). Such factors operate either singly or in combination to exert their influence on the development of nucellar vs. zygotic seedlings.

The analysis of nucellar plantlets (Fig.19) of "Valencia" for genetic uniformity using ME, PXD and EST gene/enzyme systems showed that all, except one of the 86 plantlets were genetically uniform (Fig.21). The exceptional seedling exhibited the FF genotype (Me-1 locus). This was one out of 39 two year-old seedlings (Fig. 20) produced in the Tissue Culture Laboratory, College of Agriculture and Veterinary Sciences, Faculty of Agriculture University of Nairobi, under the auspices of the Citrus Project funded by the International Foundation for Science. The seedling is suspected to be either an electrophoretic variant arising from the tissue culture cycle (Evans and Sharp, 1986), or it may have arisen from the nucellar tissue of a seed derived from a different female (seed) parent. An anomalous genotype of this kind has also been reported by Roose and Traugh (1988) for the Got-1 locus. In the latter case, however, no explanation was given for the presence of the aberrant genotype.

4.4 Conclusions and Recommendations

This study shows that *in vitro* embryo culture greatly enhaces the production of both nucellar and zygotic seedlings of two polyembryonic species. The recovery of zygotic seedlings using isozyme gene markers was also significantly augmented by embryo culture. It is suggested that the two approaches can complement each other even

more effectively through isolation and culture of embryos in their early stages of development and the use of genetically defined heterozygous isozyme loci. Peroxidase and esterase isozymes showed a higher degree of polymorphism though inconsistent, compared to GOT, ME, and MDH and can therefore serve as appropriate gene/enzyme systems for future studies on gene expression in Citrus.

For the methodology of PAGE outlined in this study to be adopted for use in the identification of zygotic and nucellar seedlings, it will require the investigator to first establish the genotype of the seed parent. The use of genetically undefined enzyme systems such as PXD and EST is not recommended until their genetics has been fully elucidated. Careful analysis of isozyme bands must also be done to avoid misclassification of band patterns, some of which may be due to the problems inherent in the technique of PAGE. For example, it was sometimes difficult to compare PXD isozyme bands on the same gel because of the problem of "edge" effects that occur as a result of unequal distribution of heat in the gel during an electrophoretic run (Fig.22). In such cases, the samples must be analyzed for a second time.

Another suggestion is that more enzymes should be screened for heterozygous loci which can then be used for identification of zygotic and nucellar genotypes. The tissue culture approach adopted in this study is

recommended for use especially in seedling multiplication nurseries and by breeders, although more inputs in terms of the workforce and resources will be required e.g. for the generation of the seedlings *in vitro*. Such seedlings, however, have an advantage over conventionally produced seedlings since the former are known to be largely disease-free (Navarro, 1984). This study was part of an on-going *Citrus* Project aimed at producing disease -free (especially from greening disease) plants for use by farmers in the establishment of disease-free orchards. The findings of this study make it possible to generate and select genetically defined seedlings without necessarily following the nucellar embryogenesis pathway. This is because the two species are polyembryonic. The extent of polyembryony is indeed likely to be much higher than was revealed in this study.

"Rough Lemon" and "Valencia" are commonly used as rootstock and scion material, respectively, in Kenya. From this study, it is clear that seedlings obtained from the seeds of these two apomictic species cannot be assumed to be genetically uniform, as has been the case before. In breeding practice, inbred seedlings normally show poor growth and therefore are usually discarded through the exercise known as "roguing". Such seedlings can be correctly identified with the aid of isozymes and used for breeding purposes. Zygotic progeny resulting from

open-pollination can serve as either sources of, or can themselves be used as, new genotypes for commercial evaluation.

LITERATURE CITED

Altmann, A. and Goren, R. (1974): Growth and dormancy levels in Citrus bud cultures and their hormonal control. Physiol. Plant. 30 : 240-245.

Bar-Akira, A., Sogiv, J. and Reuveni, O.: Physiological approaches to plant nutrition problems. Proc. 7th Intern. Colloq. Plant Analysis and Fertilizer Problems, Hanover, W. Germany, 1974.

Bassiri, A. and Carlson, P.S. (1979): Isozyme patterns in tobacco plant parts and their derived calli. Crop Sci. 19: 909-914.

Ben-Hayyim, G. and Kochba, S. (1982): Growth characteristics and stability of tolerance of citrus callus cells subjected to NaCl tolerance. Plant Sci. Lett. 27: 87-94.

Bergmann, F. (1971): Genetische Untersuchungen bei Picea abies mit Hilfe der Isoenzym-Identifizierung. I. Moglichkeiten fur genetische Zertifizierung von Forstsaatgut. Allg. Forst U. J. Ztg. 142: 278-280.

Bhojwani, S.S. and Bhatnagar, S.P. (1983): The Embryology of Angiosperms. 4th rev. edition, Vani Educational Books, New Delhi, India.

Bitters, W.P. and Murashige, T. (1967): A place for tissue culture in Citrus. Calif. Citrograph 52: 226-228.

Bretell, R.I.S., Dennis, E.S., Scowcroft, W.R. and Peacock, W.J. (1986): Molecular analysis of a somaclonal variant of maize alcohol dehydrogenase. Mol. Gen. Genet. 202: 235-239.

Burke, H.T. (1967): The commercial citrus regions of the world. In: Reuther, W., Webber, H.J. and Baxter, L.D. (Eds.), The Citrus Industry, Vol. I, pp. 40-189, Univ. Carlifornia, Riverside.

Button, J. and Bornmann, C.H. (1971): Development of nucellar plants from unpollinated and unfertilized ovules of the Washington Navel orange in vitro. J. South Afr. Bot. 37: 127-134.

Button, J., and Kochba, J. (1977): Tissue culture in the citrus industry. In: Reinert, J. and Bajaj, Y.P.S. (Eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, Springer-Verlag, Berlin, pp. 70-92.

Button, J., Vardi, A. and Spiegel-Roy, P. (1976): Root

peroxidase isoenzymes as an aid in citrus breeding and taxonomy. Theor. Appl. Genet. 47: 119-123.

Cameron, J.W. (1979): Sexual and nucellar embryony in F1 hybrids and advanced crosses of Citrus with Poncirus. J. Amer. Soc. Hort. Sci. 104: 408-410.

Cameron, J.W. and Frost, H.B. (1968): Genetics, Breeding and Nucellar Embryony. In: Reuther, W., Batchelor, L.P. and Webber, H.J., (Eds.), The Citrus Industry, Vol. 11, pp. 335-370, Univ. California, Riverside.

Cameron, J.W. and Scora, R.W. (1973): Leaf oils and fruit characters in relation to genetic identity among twin and triplet citrus hybrids. Lloydia 36: 410-415.

Cameron, J.W. and Soost, R.K. (1969): Citrus (Citrus spp.). In: F.P. Ferwerda and F. Wit (Eds.), Outlines of perennial crop breeding in the tropics, p. 129-162, Univ. Agriculture, Wageningen, The Netherlands.

Cameron, J.W. and Soost, R.K. (1980): Mono- and polyembryony among tetraploid citrus hybrids. HortScience 15: 23-28.

Cameron, J. W., Soost, R.K. and Olson, O.E. (1964): Chemical basis for colour in pink and red grapefruit. J. Hered. 55: 23-28.

Chen, Z.G. and Wang, D.F. (1987): Citrus culture of zygotic embryos at an early stage of development. J. Fuj. Agr. Coll. 15: 271-276.

Childs, J.F.L. and Johnson R.E. (1966): Preliminary report on seed transmission of psorosis virus. Plant Disease Reporter 50: 81-83.

Cobbley, L.S. (1975): An Introduction to the Botany of Tropical Crops. Rev. by W.M. Steele, second edition, Longman, London.

Coit, J.E. (1915): Citrus fruits. The Mcmillan Publishing Co. Inc., New York.

Coppens, L. and Gillis, E. (1987): Isoenzyme electrofocusing as a biochemical marker system of embryogenesis and organogenesis in callus tissues of Hordeum vulgare L. J. Plant Physiol. 127: 153-158.

Ernst, A. (1918): Bastardierung als ursache der Apogamie im Pflazenreich. J. Gustav Fischer, Jena, 668 pp.

Esan, E.B. (1973): A detailed study of adventive embryogenesis in Rutaceae. Ph.D. Thesis, Univ. California,

Riverside.

Esen, A. and Soost, R.K. (1976): Peroxidase polymorphism in Citrus. J. Hered. 67: 199-203.

Evans, D.A. and Sharp, W.R. (1986): Applications of somaclonal variation. Biotechnology 4: 528-532.

Feret, P.P. and Bergmann, F. (1976): Gel Electrophoresis of Proteins and Enzymes. In: Miksche, J.P. (Ed.), Modern Methods in Forest Genetics , Proc. in Life Sci., Springer-Verlag, Berlin.

Frost, H.B., Cameron, J.W. and Soost, R.K. (1957): Diversity among nucellar seedling lines of Satsuma mandarin and differences from the parental old line. Hilgardia 27: 201-222.

Frost, H.B. and Krug, C.A. (1942): Diploid-tetraploid periclinal chimeras as bud variants in Citrus. Genetics 27: 619-634.

Frost, H.B. and Soost, R.K. (1968): Seed Reproduction: Development of Gametes and Embryos. In: Reuther, B.W., Batchelor, L.D. and Webber, H.J. (Eds.), The Citrus Industry, Vol.11, Univ. California, Riverside.

Frusato, K., Ohta, Y. and Ishibashi, K. (1957): Studies on polyembryony in Citrus. Report of the Kihara Institute for Biological Research (English Summary). No. 8: 40-48, Numazu, Japan.

Gasper, T., Penel, C. Thorpe, T. and Greppin, H. (1982): Peroxidases: A survey of their biochemical and physiological roles in higher plants. Univ. Geneva Press, Geneva, Switzerland.

Gritter, F.G. (1986): Citrus embryogenesis in vitro: Culture initiation, plant regeneration and phenotypic characterization. Dissert. Abstr. Intern., B (Science and Engineering) 47: 13578.

Hames, B.D. (1982): An introductin to polyacrylamide gel electrophoresis. In: Hames B.D. and Rickwood, O., (Eds.), Gel Electrophoresis: a practical approach, Oxford University Press, Oxford.

Hirai, M., Kozaki, I. and Kajiura, I. (1986): The rate of spontaneous inbreeding of trifoliate orange and some charateristics of inbred seedlings. Jpn. J. Breeding 36: 138-146.

Hurosvilli, K.G. (1957): Cultivation of isolated embryos

and tissues as a method of citrus breeding. Bull. Vses. Nauch. Issl. Inst. Chaia i Subtrop. Kul't. 1: 178-197.

Iglesias, L., Lima, H. and Simon, J.P.S. (1974): Isoenzyme identification of zygotic and nucellar seedlings in Citrus. J. Hered. 65: 81-84.

Ikeda, T. (1904): On the parthenocarpy of Citrus. J. Sci. Agr. Soc. (Tokyo) 60: 19-30.

Iwamasa, M., Ueno, I. and Nishiura, M. (1967): Inheritance of nucellar embryony in citrus. Bull. Hort. Sta., Japan (Ser. B), 7: 1-8.

Jacobs, M. (1974a): Isozymes and a Strategy for Their Utilization in Plant Genetics: Genetic and Epigenetic control. In: Ledoux, L. (Ed.), Genetic Manipulations with Plant Material - NATO Advanced Study Institute on Genetic Manipulations with Plant Material, Liege.

Jacobs, M. (1974b): Isozymes and a Strategy for Their Utilization: Isozymes as a tool in Plant Genetics. In: Ledoux, L. (Ed.), Genetic Manipulations with Plant Material - NATO Advanced Study Institutes on Genetic Manipulations with Plant Material, Liege.

Juarez, J., Navarro, L. and Guardiola, J.L. (1976): Obtenzion de plantes de divers cultivars de clementiniers au moyen de la culture de nucelle in vitro. Fruits 31: 751-762.

Kawase, K. and Hirai, M. (1985): Thornless trifoliate orange from self pollination. HortScience 20: 395-396.

Khan, I.A. and Roose, M.L. (1988): Frequency and characteristics of nucellar and zygotic seedlings in three cultivars of trifoliate orange. J. Amer. Soc. Hort. Sci. 113: 105-110.

Khuroshvilli, K.G. and Takidze, M.I. (1979): Some data from cytological analysis of plants obtained by means of artificial in vitro embryo culture. "Subtropicheskie" Kul'tury 5: 55-58 (RU).

Kobayashi, S. (1987): Uniformity of plants regenerated from orange (Citrus sinensis Osb.) protoplasts. Theor. Appl. Genet. 74: 10-14.

Kochba, J., Spiegel-Roy, P. and Sufran, H. (1972): Adventive plants from ovules and nucelli in Citrus. Planta 106: 237-245.

Kochba, J., Spiegel-Roy, P. and Lavee, S. (1974): Embryoid

differentiation in nucellar callus of "Shamouti" orange (Citrus sinensis). In: 3rd Intern. Congr. Plant Tissue and Cell Culture, Abstr. 114, Leicester, University of Leicester.

Kochba, J., Spiegel-Roy, P., Neumann, H. and Saad, S. (1982). Effects of carbohydrates on somatic embryogenesis in sub-cultured nucellar callus of Citrus cultivars. Z. Pflazenphysiol. 105: 359-368.

Lagrimini, M.L. and Rothstein, S. (1987): Tissue specificity of tobacco peroxidase isozymes and their induction by wounding and tobacco mosaic virus infection. Plant Physiol. 84: 438-442.

Litz, R.E., Knight, R.L. and Gazit, S. (1982): Somatic embryos from cultured ovules of polyembryonic Mangifera indica L. Plant Cell Reports 1 : 264-266.

Maheshwari, P. (1950): An Introduction to the Embryology of Angiosperms, McGraw-Hill, New York, Toronto, London.

Maheshwari, P. and Rangaswamy, N.S. (1958): Polyembryony and in vitro culture of embryos of Citrus and Mangifera. Indian J. Hort. 15: 275-282.

Moore, G. (1985): Somatic embryogenesis in Citrus (Abstract). In: Biotechnologia en las Americas II. Applications en agricultura tropical, San Jose, Costa Rica.

Murashige, T. and Skoog, F. (1962): A medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant 15: 474-487.

Nagai, K. and Tanikawa, T. (1928): On citrus pollination. Third Pan-Pacific Sci. Congr. (Tokyo, 1926) Proc. 2: 2023-2029.

Narayanaswamy, S. (1977): Regeneration of plants from tissue cultures. In: Reinert, J. and Bajaj, Y.P.S. (Eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, Springer-Verlag, Berlin.

Navarro, L. (1984): Citrus tissue culture. In: Micropropagation of Selected Rootcrops, Palms, Citrus, and Ornamental Species. Proc. FAO/NORWAY Sympos. on plant tissue culture technology and utilization, Norway, pp.113-154.

Ohgawara, T., Kobayashi, S., Ohgawara, E., Uchimlya, H. and Ishii, S. (1985): Somatic hybrid plants obtained by protoplast fusion between Citrus sinensis and Poncirus trifoliata. Theor. Appl. Genet. 71:1-4.

Ohta, Y. and Frusato, K. (1957): Embryo culture in Citrus. Report of the Kihara Institite for Biological Research. No. 8: 49-54, Namazu, Japan.

Orton, J.J. (1983): Spontaneous electrophoretic and chromosomal variability in callus cultures and regenerated plants of celery. Theor. Appl. Genet. 67: 17-24.

Pedersen, S. and Simonsen, V. (1987): Tissue and developmental expression of isozymes of barley (Hordeum vulgare L.). Hereditas 106: 59-66.

Raghavan, V. (1977): Applied Aspects of Embryo Culture. In: Reinert, J. and Bajaj, Y.P.S. (Eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, Springer-Verlag, Berlin.

Rangan, T.S., Murashige, T. and Bitters, W.P. (1969): In vitro studies of zygotic and nucellar embryogenesis in citrus. In: Chapman, M.D. (Ed.), Proc. Intern. Citrus Symp., pp. 225-229, Univ. California, Riverside.

Rangaswamy, N.S. (1958a): Culture of nucellar tissue of Citrus in vitro. Experientia: 14: 111-112.

Rangaswamy N.S. (1958b): In vitro culture of nucellus and embryos of citrus. In: Maheshwari, P. (Ed.), Proc. Modern Devel. Plant Physiol., pp.1o4-106, Delhi University, Delhi.

Report of the Committee on Greening in Citrus and Fruit Tree Nursery System, (Presented to the Director of Agriculture), Kenya, April, 1982.

Roose, M.L. and Traugh, S.N. (1988): Identification and performance of citrus trees on nucellar and zygotic rootstocks. J. Amer. Soc. Hort. Sci. 113: 100-105.

Roscoe,J.T. (1975): Fundamental Research Statistics for the Behavioural Sciences. Second Edition. Holt, Rinehart and Winston inc., New York, U.S.A.

Sabharwal, P.S.(1963): In vitro culture of ovules, nucelli and embryos of Citrus reticulata Blanco var. Nagpuri. In: Maheshwari, P. and Rangaswamy, N.S. (Eds.), Plant Tissue and Organ Culture. A Symposium. Intern. Soc. Plant Morphologists, New Delhi.

Sathaiah, V. and Reddy, T.M. (1984): Qualitative and quantitative analysis of peroxidase and esterase enzymes in castor (Ricinus communis L.). Indian J. Bot. 7: 68-73.

Scandalios, J.G. and Sorenson, J.C. (1977): Isozymes in Plant Tissue Culture. In: Reinert, J. and Bajaj, Y.P.S. (Eds.), Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture, Springer-Verlag, Berlin.

Sharp, L.W. (1934): Introduction to Cytology. Third Edition. McGraw-Hill Book Co., New York and London.

Shaw, C.R. and Allard, R.W. (1982): Estimation of outcrossing rates in Douglas fir using isozyme gene markers. Theor. Appl. Genet. 62: 113-120.

Siegel, B.Z. and Galston, R.W. (1967): The isoperoxidases of Pisum sativum. Plant Physiol. 42: 221-226.

Sobrinho, J.S. and Gurgel, J.T. (1953): Poliembrionia e embrionia adventicia em Citrus. Mangiferae. Myrtaceae frutiferas. Dusenia 4: 421-428.

Soost, R.K., Williams, T.E. and Torres, A.M. (1980): Identification of nucellar and zygotic seedlings of citrus with leaf isozymes. HortScience 15: 728-729.

Spiegel-Roy, P. and Ben-Hayyim, G. (1985): Selection and breeding for salinity tolerance in vitro. Plant and Soil 89: 243-252.

Spielgel-Roy, P., Kochba, J. and Saad, S. (1983): Selection and tolerance of 2,4-dichlorophenoxyacetic acid in ovular callus of orange (Citrus sinensis). Z. Pflazenphysiol. 109: 41-48.

Steiner, W.W.M. and Josyln, D.J. (1979): Electrophoretic techniques for the genetic study of mosquitoes. Mosquito News. 39: 35-52.

Stevenson, F.F. (1956): The behaviour of citrus tissues and embryos in vitro. Diss. Abstr. 16: 2292-2293.

Swingle, W.T. (1927): Seed production in sterile citrus hybrids, its scientific explanation and practical significance. New York Hort. Sci. Mem. 3: 19-21.

Tanksley, S.D. and Orton, T.J. (1983): Isozymes in Plant Genetics and Breeding. Part A and B. Elsevier Science Publishers BV, Amsterdam, The Netherlands.

Teich, A.H. and Spiegel-Roy, P. (1972): Differentiation between nucellar and zygotic citrus seedlings by leaf shape. Theor. Appl. Genet. 42: 314-315.

Torres, A. M., Soost, R. K. and Diedenhofen, V. (1978): Leaf isozymes as genetic markers in citrus. Am. J. Bot. 65:

869-881.

Torres, A. M., Soost, R.K. and Mau-Lastovicka, T. (1982): Citrus Isozymes: Genetics and distinguishing nucellar from zygotic seedlings. J.Hered. 73: 289-294.

Traub, H.B. (1936): Artificial control of nucellar embryony in citrus. Science 83: 165-166.

Tuikong, A.R. and Gupta, V.K. (1985): Electrophoresis as an aid in identifying different commercial clones of pyrethrum, Chrysanthemum cinerarinefolium Vis. Kenya J. Science and Technology (Series B) 6: 123-128.

Tukey, H.B. (1933): Artificial culture of sweet cherry embryos. J. Hered. 24: 7-12.

Vardi, A., Spiegel-Roy, P. and Galun, E. (1975): Citrus cell culture: Isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. Plant Sci. Lett. 4: 231-236.

Wakana, R. and Vemeto, S. (1987): Adventive embryogenesis in Citrus. I. The occurrence of adventive embryos without pollination or fertilization. Am. J. Bot. 74: 517-530.

Webber, H.J. (1930): Influence of pollination on the set of fruit in Citrus. Calif. Citrograph 15: 322-323.

Webber, H.J. (1967): History and development of the Citrus industry. In: Reuther, W., Webber, H.J. and Baxter, L.D. (Eds.), The Citrus Industry, Vol. I pp. 1-39, Univ. California, Riverside.

Wendel, J.F. and Park, C.R. (1983): Genetic control of isozyme variation in Camella japonica L. J. Hered. 73: 197-204.

White, P.R. (1943): A Handbook of Plant Tissue Culture. Lancaster, Jacques Catell.

Wright, N. (1937): Pollination and the seedlings of marsh grapefruit. Proc. Agr. Soc. Tob. 37: 51-60.

Wu, M.T. and Jwo, J.M. (1986): Studies on isozymes of citrus leaves. I. Gel electrophoresic analysis of six important citrus species in Taiwan. J. Agric. Rs. China 35: 484-494.