Discrimination between citrus genotypes

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Summary

Isozyme analysis was used to distinguish between genotypes and explore relationships within the genus *Citrus* . Two experimental studies were conducted, the first concerned with the identification of zygotic (sexual) from nucellar (asexual) seedlings of five polyembryonic citrus rootstocks resulting from open pollination, and the second with identification of mandarin types and investigation of the relationships amongst them.

Eight isozyme systems of six enzymes were utilized in the first study. 2.1 % of the seedlings were determined to be zygotic in rough lemon, 2.6 % in trifoliata orange, and 0.8 % in each of sweet orange and Troyer citrange, but none in Cleopatra mandarin. There was no correlation between isozyme genotype and any morphological character of the seedlings. The zygotic seedlings detected isozymically were not always located at the micropylar end of the seed as has been suggested previously nor were they characterized by weak growth, or as the single seedling produced by a seed.

Nineteen isozyme systems of sixteen enzymes were employed in the second study to discriminate between mandarin cultivars, hybrids and selections. Variability was observed at 12 loci, and all but three mandarin types could be differentiated from one another. Two of those which could not be differentiated are probably identical genotypes. The reported parentage was confirmed for seven cultivars, and disproved for five, with the rest undetermined. Relatedness within the tangelo and the tangor groups was high, probably reflecting their recent origin. Relatedness within the common mandarin group was low reflecting their multiple origins and long period in cultivation. Exceptions were Algerian and Beauty of Glen Retreat which differed from each other at only one locus. Relatedness between groups was generally low, with the least relatedness between the tangelos and the other groups, probably due to the grapefruit parent of the former. The Ellendale cultivars formed a particularly cohesive group, but contained two genotypes differing in isozyme

pattern, but both marketed as Ellendale. Five of the Ellendale type cultivars probably arose by self-pollination or by mutation of Ellendale.

This study has shown that isozyme techniques can be used successfully not only to discriminate between zygotic and nucellar seedlings, but also to investigate the parentage and relatedness of the cultivars. It has also shown that increasing the number of isozyme systems employed increases the probability of discriminating genotypes.

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by myself and does not incorporate any material previously submitted for another degree in any University. To the best of my knowledge and belief, it does not contain any material previously written or published by another person, except where due reference is made in the text.

Sumeru Ashari

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Discrimination between citrus genotypes

Chapter 1. Introduction

Citrus is one of the major commercial fruit-crops in the world. According to the FAO Production yearbook (1987), the average world production per year from 1983 to 1985 was 56.9 million metric tons, less than the production of grapes (66.4 million metric tons) , but greater than that of apples (37.5 million metric tons). The main producing countries were Brazil (13.0 million metric tons), USA (10.6 million metric tons), Mexico (2.6 million metric tons), India (1.8 million metric tons) and Israel (1.5 million metric tons). Australian production was 0.5 million metric tons.

Citrus is usually cultivated as an orchard crop, with selected superior scion cultivars with the desired fruit characteristics grafted or budded onto a rootstock with the required features such as vigour or disease tolerance . Both scion and rootstock material is generally clonally propagated. Genetic identification of citrus biotypes is very important, mainly in the fields of propagation and cultivar improvement programmes. In the nursery, apomictic (asexual, maternal or nucellar) citrus seedling populations apparently uniform in growth and morphology are used as rootstocks, but a proportion of the seedlings may be zygotic (sexual). It is desirable that the rootstocks should be of a uniform genotype ie. nucellar seedlings. If this is not the case, variation occurs between the trees following grafting and orchard establishment. The nursery industry needs a reliable method to discriminate zygotic seedlings from the nucellar population. The breeding of selected genotypes aims to provide new cultivars which have good fruiting, disease resistance , tolerance to unfavourable environmental conditions and other characteristics. In a cultivar improvement programme, information on the genetic background of the cultivar to be used as a parent is more valuable than morphological traits, because such traits are considerably influenced by environment.

There have been several attempts to discriminate between citrus genotypes. To assist discrimination between zygotic and nucellar seedlings, Pieringer and Edwards (1965) used infrared spectroscopy, by placing a leaf extract in the infrared absorption cell. and Teich and Spiegel-Roy (1972) analysed leaf shape. The distribution of chemical compounds between species has also been studied. Albach and

Redman (1969) investigated the flavonoids of some citrus species and related genera, Mackinney (1961) and Yokohama and White (1966) related the occurence of carotenoids to taxonomic boundaries, and Stanley (1963) studied coumarine. All these methods, however, have limitations, as they are empirical and only indirectly related to the genetic background of the species and progenies.

Analysis of proteins (direct products of genes) by electrophoresis offers a promising method for discrimination between genotypes (Iglesias *et al.*, 1974; Button *et al.*, 1976). Torres *et al.* (1978, 1982) determined the isozyme genotypes of some citrus cultivars and related genera with eight enzyme systems controlled by ten loci. This method can also be utilized to distinguish between related progenies (Soost *et al.*, 1980), and to assess inbreeding (Kawase and Hirai, 1985).

In the present project, it was proposed to employ isozyme techniques, together with an examination of morphological characters, to explore the feasibility of discriminating between zygotic and nucellar seedlings and to examine the genetic relationships between putative hybrids and clonal variants of citrus cultivars.

Chapter 2. Literature review

2.1 Citrus classification

Citrus has been classified in the order Geraniales, Sub-order Geraniaceae, Family Rutaceae and Sub-family Aurantioideae. (Swingle and Reece, 1967; Alexander, 1983). The Sub-Family Aurantioideae is divided further into Tribes, Sub-Tribes and Sub-Tribal groups (Table 2.1).

Swingle and Reece (1967) describe the characteristics of the genus *Citrus* as follows : "Small trees; young twigs angled, soon cylindrical with single spines in the axils of the leaves but older branches often spineless; leaves unifoliate; petioles more or less winged; flowers single in the axils of the leaves, axillary, perfect or staminate ; calyx cup-shaped with 4-5 lobes; petals 4-8, thick, linear ; stamens 4 times as many as the petals but some species have 6-10 times as many; ovary subglobose, fusiform or subcylindrical, locules 8-18; styles cylindrical; fruit a hesperidium with the segments containing seeds near the inner angle and the rest of the space filled with a very watery, large celled tissue; around the segments is a white endocarp, outside of which is the peel dotted with very numerous oil glands and turning yellow or orange at full maturity; seeds obovoid or flattened obovoid, more or less angular, containing single or multiple embryos ". Other important characteristics include the parthenocarpic tendency in fruit development and the production of apomictic seed. Citrus cultivars may be monoembryonic or polyembryonic. Monoembryonic seeds contain a single zygotic embryo. Polyembryonic seeds contain numerous embryos which are generally produced asexually and are of identical genotype to the maternal parent; they may or may not contain a zygotic embryo. In the propagation of scion cultivars, monoembryonic seeds are not recommended for use as rootstock material because the seedlings produced are sexual. The asexual embryos of polyembryonic cultivars, the nucellar seedlings, are genetically identical while the sexual embryo, the zygotic seedling, differs. In the propagation of citrus, nucellar seedlings are desirable

because they are identical to the maternal parent and scions budded on them will perform uniformly in the field. In this situation zygotic seedlings are undesirable. However, zygotic seedlings are required in plant breeding programmes because they are the source of genetic variability and the genetic improvement sought.

In the classification of the genus *Citrus*, until recently, taxonomists have based their model solely on morphological, physiological and geographical considerations (Swingle, 1928; Vardi and Spiegel-Roy,1978). Genetic relationships usually express themselves in similarities and differences in form and structure. Thus, a number of plants which have similarities in leaf shape, flower type and growth habit are assessed as belonging to a single group. Morphology alone does not suffice, however, particularly in the assessment of hybrids derived from parents which are morphologically similar, as frequently occurs in this genus. Sometimes fruit characters are used as a means of identification, which necessitates awaiting fruit bearing from 6 to 10 years, a very long and expensive procedure. Although groups of plants may also differ to a greater or lesser degree in physiological processes, such differences are often unstable and in response to changes in the environment, plants show greater changes in physiology than morphology. The limitation of geographical distribution as an aid to identification is the difficulty and uncertainty in ascertaining the centre of origin of most citrus cultivars. All of these limitations have led to disagreements between citrus taxonomists in numbering the species of citrus. The main reason for such disagreement is that citrus has been in cultivation since ancient times, hybridization between and within species occurs readily and self-pollination is possible. Furthermore, polyploids, mutations and apomixis add to the confusion. Eleven species were identified by Engler (1896, in Swingle and Reece, 1967), sixteen by Swingle and Reece (1967) and 159 by Tanaka (1969).

The most recent study on citrus taxonomy is that of Barrett and Rhodes (1976). They assessed a large number of citrus morphological characters (200), quantified the similarities among citrus biotypes and related these similarities to speciation and variation in citrus. In this study, *C. grandis* (L.) Osbeck, *C. medica* L. and *C. reticulata* Blanco were proposed as true biological species, while all others were proposed to be unique,

apomictically perpetuated genotypes of probable hybrid origin. The taxonomic classification of Swingle and Reece (1967), recognising sixteen species (see Table 2.1, sub-tribal), will be followed in this work because it is accepted by most citrus specialists (Alexander, 1983), but with the modification for mandarin species suggested by Hodgson (1967).

Attempts to reach agreement on citrus classification still continue, employing more reliable methods of identification. In recent years, chemical plant components, which are the products of a series of biosynthetic reactions in the plant, governed by genetic factors, have shown promise as additional characters for taxonomic and phylogenetic investigation. Essential oils are particularly well suited for such studies, because of the ease of collection, distribution in many plant families and wide chemical diversity. Pieringer *et.al.*. (1964) employed refractometer studies, infrared and ultrared spectrophotometry and gas-liquid chromatography of leaf oils obtained by steam distillation. A similar study was carried out by Kesterson *et al.* (1964). They came to the conclusion that this method was only useful as a supplementary tool for taxonomic study because the leaf oils were affected by tree variability, stage of plant growth and seasonal differences.

The flavonoids have also been considered. These are a numerous and widespread group of natural constituents, important in plant colour. The basic pattern of flavonoid synthesis is common to all higher plants. Swingle (1943) was the first to recognise the possible usefulness of flavonoid composition as an aid in making taxonomic decisions. Swingle and Reece (1967) found differences in these chemicals between some citrus species including *C.aurantium* L., *C. medica* L., *C. paradisi* Macf., *C. limon* L. Burm. F, *C. reticulata* Blanco, *Fortunella* species and *Poncirus trifoliata* L. Raf. Albach and Redman (1969) also reported variation in the flavonoid components of citrus in a thin layer-chromatographic survey, and discussed the probability of applying this method as a supplementary tool in citrus taxonomy. However, in any application to progeny assessment, the flavonones of the suspected parents must be sufficiently diverse to allow recognition of contributions from each parent in the progeny. The question also arises whether such components are stable or affected by changes in environmental factors and stage of plant growth. On this subject, Kefford (1959) claimed that variability in chemical fruit composition between and within citrus varities is influenced by a range of factors :

genetic background is dominant, but type of rootstock, degree of fruit maturity and orchard practice all influence the composition. Moreover, there is even variability in chemical composition within individual fruits.

A further method of identification is the isozyme technique, based on protein or enzyme analysis (Iglesias *et al.*, 1974; Esen and Soost, 1976; Torres *et al.*, 1978, 1982). Soost *et al.* (1980) proposed that the isozyme technique would provide an excellent single gene marker for citrus, because the individual isozymes are colinear with the controlling gene, codominant, little affected by environment and identical in the leaves of young and mature plants. This method has the potential to be utilized effectively in discrimination between citrus species and related genera and in identification of zygotic and nucellar seedlings. It has been reported to be relatively ineffective in discriminating between citrus cultivars and mutant seedlings (Torres *et al.*, 1978, 1982; Hirai *et al.*, 1986). This failure in discrimination between cultivars may have been due to the genetic differences being too small to detect by enzyme methods. It would seem that a combination of all available methods would be most useful in reaching agreement between citrus taxonomists in solving the problems of citrus taxonomy.

Table 2.1 Botanical classification within Sub-Family Aurantioideae

Sub-Family	Tribes	Sub-Tribes	Sub-Tribal groups
Aurantioideae	**Clauseneae:** -very remote and remote citroid fruit trees, fruit are very small, semi-dry and inedible. -3 sub-tribes, 5 genera and 79 species.	**Triphasiinae:** -minor citroid fruit trees -3 sub-tribal groups, 8 genera and 46 species.	**A. Primitive citrus fruit trees:** -5 genera: *Severina* *Pleiospermium* *Burkillanthus* *Limnocitrus* *Hesperethusa*
		Balsamocitrinae: -hard-shelled citroid fruit trees -3 sub-tribal groups 7 genera and 13 species	
	Citreae: -true citroid fruit trees -3 sub-tribes, 9 sub-tribal groups, 28 genera and 124 species.	**Citrinae:** -true citroid fruit trees -3 sub-tribal groups, 13 genera and 65 species.	**B. Near citrus fruit trees:** -2 genera: *Citropsis* *Atalantia*
			C. True citrus fruit trees: -6 genera: *Fortunella* *Eremocitrus* *Poncirus* *Clymenia* *Microcitrus* *Citrus*

2.1.1 Classification of rootstock

In the intensive culture of citrus the contribution of the rootstock is very important. The performance of the scion grafted onto the rootstock is affected in many ways including time of fruiting, tree size, cropping, fruit quality and tolerance to unfavourable environmental conditions including salinity, poor drainage, *Phytophthora* root rot infection and nematode susceptibility (Wutscher, 1979).

In the citrus classification systems of Tanaka (1954, in Swingle and Reece, 1967) and Swingle and Reece (1967) rootstock cultivars are not separated from scion cultivars. For example, Cleopatra mandarin (rootstock) and Satsuma mandarin (scion) are included in the one species i.e *Citrus reticulata* Blanco. Similarly the sweet orange species includes both scion and stock cultivars. Alexander (1983) listed scion and rootstock species in separate groups. However, some cultivars within *C. sinensis* L. Osbeck, *C.aurantium* L. and*C. reticulata* Blanco species are present in the scion and rootstock groups. The only species used solely as rootstocks are *Poncirus trifoliata* L. Raf., *C. macrophylla* Wester,*C.jambhiri* Lush., citrange (*C.sinensis* X*P. trifoliata*), and citrumello (*C. paradisi* X *P. trifoliata*). Although the use of citrus species or cultivars as rootstock or scion is not relavent to their taxonomic distinction, the inclusion of such information in descriptions would be useful. The two groups differ significantly in many aspects such as seediness, juice content and tolerance to unfavourable conditions and these characters are believed to be controlled genetically.

Many genera in the sub-family Aurantioideae are used as rootstocks. Within the sub-tribal group of True citrus fruit trees (see Table 2.1) the genus *Citrus* and *Poncirus* are of major importance while *Clymenia*, *Eremocitrus* and *Microcitrus* are being evaluated (Wutscher, 1979). Hybridization between species and genera has also been used to produce new cultivars which will be useful to the citrus industry. Examples of hybrid rootstock cultivars include Smooth Flat Seville a hybrid between sour orange (*C.aurantium*) and grapefruit (*C.paradisi*) (Hodgson, 1967); citrange (*C.sinensis* X *P. trifoliata*) and citrumelo (*C. paradisi* X*P. trifoliata*) (Alexander, 1983). A possible problem arising in hybridization between genera by controlled pollination is incompatibility, a barrier to the production of a

zygotic embryo. Recently, however, this handicap has been partly circumvented by protoplast fusion between different genera (Ohgawara *et al.*, 1985; Grosser *et al.*, 1988).

The choice of rootstock is governed by the particular needs of the industry such as salinity tolerance, *Phytophthora* resistance, or nematode tolerance, and hence each major producing area may use several rootstocks in different localities. In this work, five polyembryonic citrus rootstock cultivars were considered : rough lemon, sweet orange, trifoliata orange, Troyer citrange and Cleopatra mandarin. These rootstocks are commonly used both in Australia and other citrus-producing regions (Thorntorn and Dimsey, 1987). The characteristics of these rootstocks is given in Table 2.2, from Wutscher (1979).

Table 2.2 Characteristics of rootstocks used in this study

Rootstock cultivar	Characteristics
Rough lemon	Produces large trees; high yields; large fruit; low quality; poor cold hardiness; deep rooted; susceptible to foot rot; very susceptible to blight and excess soil moisture; tristeza tolerant; suitable for orange and grapefruit.
Sweet orange	Produces large trees; good crops and good quality; poor drought tolerance; shallow rooted; little affected by blight;resistant to tristeza, exocortis,xyloporosis; susceptible to *Phytophthora*.
Trifoliata orange	Smaller than standard trees; high yields and high fruit quality;good cold hardiness and foot rot tolerant; tristeza tolerant; salt, boron, and exocortis sensitive; suitable for mandarins, oranges, and kumquats.
Troyer citrange	Trees standard size; high yields; large fruit and good quality; foot rot and tristeza tolerant; low salt tolerance; moderate cold tolerance; susceptible to exocortis; suitable for oranges, grapefruit, Lisbon lemon and mandarins.
Cleopatra mandarin	Large trees; small fruit size; high fruit quality; slow growth in the nursery; tristeza, salt, and cold tolerant; suitable for tangerines, tangelos, oranges and grapefruit.

2.1.2 Classification of mandarins

Agreement has not been achived between citrus taxonomists in numbering species within the mandarin group. This is partly due to the morphological methods employed, as it is well known that morphology is influenced by the environment where the mandarin is planted. Tanaka (1954, in Hodgson, 1967) recognised 43 species, Swingle (1943) listed three species and Singh and Nath (1969) proposed only a single species, i.e. *C. reticulata* Blanco. The classification which is most generally accepted is that of Swingle and Reece (1967) but with the modification by Hodgson (1967) in which four species are recognised including Satsuma mandarin (*C.unshiu* Marcovitch); King mandarin (*C. nobilis* Loureiro); Mediterranean mandarin (*C. deliciosa* Tenore), and Common mandarin (*C.reticulata* Blanco) (Forsyth, 1987).

Accurate identification of cultivars, hybrids and selections based on genetic diversity rather than on morphological markers is most important, not only in mandarin improvement and breeding programmes but also to elucidate the taxonomy of the group. In this work 19 mandarin cultivars were investigated. They were classified into five groups namely *C. unshiu*, *C. deliciosa*, *C.reticulata* , *C. reticulata* X *C. sinensis*, and *C. reticulata* X *C. paradisi* (see Table 3.1).

2.2 Variation

Environmental factors such as light, temperature, nutrition and disease cause variation in the performance of citrus trees (Cameron and Frost, 1968). Within the same orchard, unusual growth habit of the trees are frequent. They may vary in flowering time, number of seeds per fruit, fruit colour and fruit shape (Tolley, personal communication, 1988). In crop improvement programmes, reliable methods of identification of unusual characters are needed, and genetic traits are prefered rather than variation in morphology.

2.2.1 Genetic variation

2.2.1.1 Self-incompatibility

Progeny of self-incompatible plants would be expected to show more variation than those of self-compatible individuals due to the invariable introduction of genes from other plants in the former as compared with the high degree of segregation within the parental genome in the latter.

The flowers of citrus are perfect, and most cultivars are capable of both selfing and crossing. When pollination occurs followed by fertilization, one haploid sperm cell and one haploid egg cell fuse to form a diploid cell which gives rise to the zygotic embryo. This contains a combination of genes from both parents. Some citrus cultivars are self-incompatible, and do not set seeds after self-pollination. Minneola (*C. paradisi* Macf. x *C. reticulata* Blanco), Orlando (*C. paradisi* Macf. x *C. reticulata* Blanco), Clementine mandarin (*C. reticulata* Blanco), and pummelo (*C. grandis* [Linn.] Osbeck) are reported to be self-incompatible varieties (Mustard *et al.*, 1956; Krezdorn and Robinson, 1958; Soost, 1969).

Soost (1969) proposed the hypothesis that a series of incompatibility (S) alleles determines gametophytic incompatibility in citrus as in many other plant species, and suggested that self-compatible genes were dominant over self-incompatible. He stated that self-incompatible progeny derived only from crossing between self-incompatible parents. Dominance of the self-compatible genes over the self-incompatible may not be complete, however, because the self-compatible taxa generally have been derived from self-incompatible relatives (Stebbins, 1957 ;Mulcahy, 1984). The S-alleles involved in this self-incompatibility system apparently exhibit only partial or incomplete dominance (White, 1940).

There are two types of pollen grains in the angiospermae (Brewbaker, 1956), binucleate and trinucleate. Binucleate grains contain a generative and a vegetative cell, the generative cell dividing during pollen tube growth to form the male gametes. A

trinucleate grain is formed when the division of the generative cell occurs before the pollen is shed. Most *Citrus* species are reported to have binucleate pollen grains. However, the pollen grain of Orlando tangelo and its parent *C. paradisi* Macf. has been reported to be trinucleate by Banerji (1954) but binucleate by Frost and Soost (1968). There are also two types of self-incompatibility, gametophytic and sporophytic. In the gametophytic type, the pollen interaction is controlled by the allele present in the haploid pollen grain, while in the sporophytic system, the reaction is determined by parental proteins on the pollen grain surface. The gametophytic system is regularly associated with binucleate grains and the sporophytic with trinucleate grains (Brewbaker, 1956).

Attempts have been made to localize the site of the incompatibility reaction in plants. In a study on *Petunia hybrida* , a species which is typically gametophytic (Herrero and Dickinson, 1980), the velocity of pollen tube growth was measured and it was found that the incompatibility reaction was located in the style. Brewbaker (1956) suggested that the incompatibility inhibition generally occurs at some stage during pollen tube growth in the style of gametophytic species (with binucleate pollen grains). In the sporophytic system (trinucleate pollen grains), on the other hand, inhibition occurs at the stigma. In citrus, Ton and Krezdorn (1966) reported that the factor or factors inhibiting the growth of the pollen tubes of the self-incompatible cultivar, Orlando tangelo (*C.paradisi* Macf. x *C. reticulata* Blanco) occured at the base of the style or in the upper portion of the ovary. However, Kahn and DeMason (1985) criticized this conclusion and claimed that the site was in the stigma as a large number of pollen tubes ceased growth in the surface of the stigma. Some pollen tubes were also found in the upper style region, however. In the avocado, Sedgley (1977) reported that pollen grain germination was affected by temperature, and Ter-Avanesian (1978) reported that there was competition between pollen grains during growth in the stigma such that many ceased growth even in a compatible cross. Thus, the cessation of growth in the stigma by a large number of pollen tubes reported by Kahn and DeMason (1985) may have been due to competition or to environmental factors rather than to the location of the self- incompatibility reaction.

2.2.1.2 Variation due to segregation

If cultivars are largely homozygous, segregation following self-pollination is unlikely to be frequent, but its occurence will increase with increasing heterozygosity. In citrus , however, the apparent rate of segregation is particularly low, as most embryos which survive are vegetatively produced from nucellar tissue. Such embryos compete strongly with the zygotic embryo during development and the apparent rate of segregation can be increased if very young embryos are cultivated *in vitro* to minimize competition between embryo types (Rangan *et al.*,1969).

Despite these problems in recognizing and retrieving segregants, a variant of *Poncirus trifoliata* (L.) Raf. was found by chance in Japan (Kawase and Hirai, 1985). The aberrant was clearly identified by isozyme analysis as resulting from selfing. Thornlessness was the only morphological marker which could be used to distinguish the variant from nucellar *Poncirus* seedlings.

2.2.1.3 Variation due to mutation

Mutation is a change in genetic constitution which is not due to normal recombination or segregation of genes. It can be caused by chromosomal alteration, by abnormal segregation of the chromosomes, leading to the loss, duplication or rearrangement of genes or to structural changes in individual genes (Cameron and Frost, 1968). Under natural conditions, the occurence of a mutant which produces a dominant advantageous effect is rare (Brock,1971), although the induction of mutant alleles may be worthwhile in a breeding programme.

There are three important aspects to a plant improvement programme, the assembly of an adequate gene pool, manipulation of the selected gene pool and finally, comparative tests to demonstrate the superiority of the selected genotypes (Brock, 1971). Plant introduction and plant hybridization are the conventional methods to enhance variation in genotype, but mutation offers an alternative way to create a new genetic population.

The occurence of natural citrus mutants and their artificial induction have been matters of interest to citriculturists. Cells formed by division of a mutant cell will have the mutant characteristic inherited from their abnormal parent. Such mutations may be confined to discrete cell layers of the plant, and plants with tissues of two or more genetic constitutions are known as chimeras. The term is restricted to forms in which the genetic types grow together, side by side (Cameron and Frost, 1968) and a simple budded or grafted plant is not considered a chimera as the scion and rootstock maintain their individual genotypes in separate parts of the plant. Mutants have been found showing modifications in leaf shape and size together with sectorially corrugated or banded fruit. Generally these changes are undesirable, but occasionally a mutant with special virtue, such as late or early maturing fruit is found (Anon., 1968). In Japan, numerous "wase " or early ripening types have arisen from common Satsuma mandarin (*C. unshiu*), originating from limb sports (mutations). Many of them revert to the ancestral common type, but others are stable in the mutant character (Iwamasa and Nishiura, 1970). In addition, chimeras such as Kobayashi-mikan (*C. natsudaidai* X *C. unshiu*) which originated from the junction of Satsuma mandarin scion and Natsudaidai (*C. unshiu* Hayata) stock are found. This chimera resembles both stock and scion genotypes in the isozyme pattern of esterase and peroxidase (Yamashita, 1983). The pink-fleshed Thompson grapefruit (*C. paradisi* Macf.) and the white Marsh grapefruit (*C. paradisi* Macf.) are also reported to be chimeras (Cameron *et al.*, 1964). It seems that most mutations occur in actively growing meristems.

Tissue culture provides a means of increasing the variability of citrus genotypes. Navarro *et al.* (1985) suggested that variant citrus types arising *in vitro* from nucellus tissues of monoembryonic cultivars were not the result of mutation during embryogenesis, because plants produced from individual cultured nucelli were either uniformly normal or abnormal. This conclusion may not be entirely true. Genetically aberrant plants occur commonly in tissue culture and maintenance of plant tissues , especially callus, for long periods *in vitro* results in increased variability. In many cases the abnormality takes the form of polyploid plants arising from diploid plants (D'Amato, 1965; Murashige, 1974; Evans *et al.*, 1984). Genetic variability among plants resulting from tissue organ culture has also been reported in *Asparagus officinalis* (Malnassy and Ellison,

1970), *Brassica oleracea* (Horak *et al.*, 1971) , *Oriza sativa* (Nishi *et al.*, 1961) , and *Saccharum sp.* (Heinz and Mee, 1971).

There are few examples of mutation breeding in citrus. Russo *et.al.*. (1981) and Hearn (1984) irradiated citrus seeds with gamma-rays in an attempt to obtain seedlessness. In addition, a compact plant and early fruiting has been obtained in Shamouti orange (*C. sinensis* L. Osbeck) as a result of the irradiation of budwood with gamma-rays (Vardi and Spiegel-Roy, 1978).

as compared with other woody genera

The *Citrus* genus is particularly prone to mutation (Soost and Cameron, 1975) and this can present problems in citrus cultivation as abberant forms may reduce productivity and must be removed from the orchard.

2.2.2 Non genetic variation

2.2.2.1 Variation due to age

Citrus seeds have no dormant period and do not store well. Within a few days of sowing the seed swells due to absorption of water and the seed germinates. The first leaves produced are paired and cordate in shape. They lack petiole wings and are generally unifoliate even in trifoliate orange varieties. Like most other perennial crops, citrus has a juvenile period, during which flowering does not occur. The length of the juvenile period varies in woody plants from one month to 40 years (Hackett, 1985). The transition from the juvenile to the mature phase (flowering and fruiting) has been referred to as a phase change in morphological and developmental attributes including bark characteristics, leaf shape and thickness, phyllotaxis, thorniness and shoot orientation, branch numbers, branching pattern and vigour (Frost and Soost, 1968; Hackett, 1985).

2.2.2.2 Variation due to climatic conditions

Climate is the most important single factor influencing variation in fruit maturity and quality in citrus. Various climatic factors determine growth and development

and give rise to variation even within a single variety. In this work only general aspects of climate are discussed, but this does not mean that microclimatic factors such as soil type, soil temperature, wind velocity, rainfall and water status are less important.

The growth habit of the citrus shoot is influenced by the thermal environment during its growth. Shoots produced in cool coastal climates tend to have shorter internodes, thicker leaves and leaves more closely appressed to the stem than comparable shoots produced in the warm and intermediate valley climates of California (Reuther and Rios-Castanos, 1969). In warmer conditions, budded citrus trees grow faster and take a shorter time to reach a marketable size than those grown in cool areas, because they have no dormant period (Mendel, 1969). In the lowlands of Bolivia, where the temperature is higher than in the highlands , sweet orange produces larger and heavier fruit (Scorza *et al.*, 1982). This information suggests that the warm temperature affects cell division with a resulting increase in leaf and fruit size and shoot number. Mendel (1969) has suggested that this may be due to a decrease in the accumulation of growth inhibiting phenolic substances although there is little direct evidence for this. Under very high temperatures such as 50° C however, the leaves are small and abnormal, with a cupped morphology, water soaked spots and cessation of growth of the shoot tip (Ketchie, 1969; Reuther *et al.*, 1979). These symptoms are similar to those caused by infection with the pathogen *Spiroplasma citri* (Markam *et al.*, 1974).

Mandarin or tangerine (*C. reticulata* Blanco) is one of the species in the genus *Citrus* which is particularly sensitive to climatic variation (Tolley, personal communication, 1988). Some clones within varieties such as Clementine and Ellendale develop more fruit colour when grown in desert climates than in the tropical zone of Australia. In addition, lower altitudes gives less fruit colour than higher altitudes in the same region. Ellendale is the most important export variety of the mandarin types in Australia. In 1976, 1980 and 1982 about 90 % of Australian mandarin exports were Ellendale (McAlpin, 1983). Unfortunately, the quality of this Australian selection is greatly influenced by environment and plantings at different latitudes give different results. Ellendale planted in Gayndah, Queensland (approximately 25° S) fruits very early and has

poorer colour but larger fruit size than the crop produced in Renmark, South Australia ,where the latitude is about 35° S. Even within the same block, variation in fruit size, fruit colour, and seediness occurs (Tolley, personal communication, 1988). Moreover, the seed number per fruit varies according to season (McAlpin, 1983).

There are many mandarin varieties in Australia, but commercial production is limited to only 12, with two Australian local cultivars, Ellendale and Imperial the most important (Forsyth,1987). Bearing time varies within the mandarin groups in this country and is commercially important, with Imperial, Orlando, and Algerian mandarin being early season, Thorny, Hickson and Beauty of Glen Retreat mandarin mid-season and Kara, Seminole and Ellendale mandarin late-season (Bowman, 1956; Hodgson, 1967; Alexander, 1983). These variations in time of fruiting are due to genetic differences and it is desirable to investigate the genotypes of the mandarins in general since the information is needed in cultivar improvement programmes.

2.3 Citrus propagation

Plant propagation is the multiplication of plants by sexual or asexual means. Sexual propagation of crop plants is desirable only when plants are homozygous as in cereal and vegetables or where variability is acceptable, as in some garden flowers. By contrast, most fruit tree cultivars, including citrus are heterozygous. With these plants, sexual propagation cannot maintain the desired characters of the parent tree due to segregation in the progeny. The superiority of the parent can be perpetuated in heterozygous cultivars only by vegetative propagation. Even with vegetative propagation, some variation between individuals can occur through mutation. This usually takes place in the limbs and can be avoided by careful selection of the budwood to be used in propagation.

2.3.1 Scion propagation

Scions can be propagated in several ways, depending on the purpose of propagation, the number of trees required and the plant material available. The oldest

methods, which are still used in Southeast Asia, consist of marcotting and taking cuttings (Wutscher, 1979). These methods are both simple and rapid but only a small number of trees can be produced due to the large size of the propagule. Cutting materials may be obtained from roots, stems or leaves (Halma, 1931; Salomon and Mendel, 1965; Wutscher, 1979; Yelenosky, 1987).

A persistent horticultural theory argues that fruit trees derived from cuttings are inferior to those produced by alternative methods because cuttings produce shallow root systems with no taproot. Halma (1947) found in the first few years after planting that lemon trees which were either own-rooted or budded on grapefruit or sweet orange seedlings were similar in growth rate. Later, however, the budded lemon trees showed more vigour, a higher yield and greater hardiness than those grown from cuttings. Indeed, Wutscher (1979) reported that the horticultural performance of scions was influenced by the rootstock in many ways, including reduction of the juvenile period resulting in early fruiting, production of a uniform tree size, regulation of cropping, control of fruit quality, tolerance to unfavorable soil factors (salinity, high pH, poor drainage), and to *Phytophthora* root rot, nematodes and viruses.

Tissue culture has been used for the rapid propagation of scions (Bitter *et al.*, 1969; Bhansali and Arya, 1978) but regenerated plants often show reversion to the juvenile stage, and excessive variation (Navarro *et al.*, 1985). These weaknesses make the use of budding or grafting techniques preferable to propagation by cuttings or tissue culture. In special circumstances and in experimental work, however, own-rooted plants may give rapid and satisfactory results.

Micropropagation is a further method which may overcome these problems.

2.3.2 Rootstock propagation

In the citrus industry, most rootstocks are propagated by seed. This is because most commercial citrus rootstocks are highly polyembryonic (Wutscher, 1979), and the technique is most suitable for the propagation of a large number of plants. In some areas, however, and for special conditions, such as the slow growth and hence low seed yield of Trifoliata orange in Indonesia, propagation by cuttings is also used. In those

rootstock varieties which are monoembryonic e.g. shaddock, cuttings are preferable as seedlings have a variable, different genetic constitution from the maternal parent.

The tissue culture method, which potentially provides faster multiplication is unsuitable for rootstock propagation due to the occurence of genetic alterations during the callus phase (D'Amato, 1965; Navarro *et al.*, 1985). Such resultant variation may affect scion performance following budding and planting in the orchard.

Micropropagation is a further method which may overcome these problems.

2.4 Growth, development, and discrimination of zygotic and nucellar seedlings

2.4.1 Pollination and fertilization

Pollination is the transfer of pollen from the anther to the stigma. After the pollen grains land on the stigma, they absorb liquid secreted by the stigmatic cells which results in pollen hydration followed by germination and pollen tube formation (Knox, 1982). The pollen tubes, which carry the sperm cells, grow down the style to reach the embryo sac via the micropyle.

It is a widely accepted concept that the pollen tube reaches the egg and central cell via a degenerated synergid cell . The initiation of synergid degeneration differs between plant species. Wylie (1941) in a study of *Vallesnerima* and Schulz and Jensen (1968) with *Capsella bursa pastoris* L. suggested that the degeneration of the synergid cell occured after the pollen tube contacted the cell. In other plant species, such as cotton (*Gossypium hirsutum*) (Jensen and Fisher, 1968); barley (Cass and Jensen, 1970), and an orchid (Coccuci and Jensen, 1969) the synergid cell degenerated after pollination but before the pollen tube contacted the synergid cell. Unpollinated flowers do not show degeneration of the synergid cell, degeneration being characterized by swelling and darkening of the organelle membranes, collapse of the vacuole and disappearance of the plasma membrane.

When the pollen tube reaches the embryo sac and delivers its contents, one of the two cellular sperm cells fuses with the egg cell and forms the embryo (double fusion), while the other fuses with the two polar nuclei and forms the endosperm (triple fusion). The

endosperm provides a site for food storage and transfer to nourish the embryo. In most cases, embryo and endosperm formation are preceded by both pollination and fertilization. In citrus, however, there has been some debate as to whether pollination alone, both pollination and fertilization, or neither, are essential for the initiation and development of vegetative embryos. The first view was that the vegetative embryos occured without pollination and fertilization. Webber (1930) emasculated flowers of Navel orange and fruit developed with a few small rudimentary seeds. This indicated that pollination and fertilization were not strictly necessary for the formation of adventive embryos, but the lack of endosperm may have resulted in their limited development.

Another suggestion is that both pollination and fertilization are essential. Kobayashi *et al.* (1979, 1981) in a study of the polyembryonic cultivars, Trovita orange, Satsuma mandarin, and Natsudaidai mandarin found embryoid formation in the ovule both before and at the anthesis stage. This strongly suggested that neither pollination nor fertilization was required for the initiation of nucellar embryos in these polyembryonic varieties. However, a recent study of Wakana and Uemoto (1987) found no developed embryos in the unpollinated flowers of Valencia orange, Hayashi unshiu and Duncan grapefruit, but these were present in pollinated flowers of the fertile polyembryonic varieties examined. This suggests that initiation of nucellar embryos may occur without pollination and fertilization but that these processes are required for the sustained development and maturation of the embryos.

Although seeds of monoembryonic cultivars do not normally contain nucellar embryos at maturity, they nevertheless have the potential to produce such embryos. Rangan *et al.* (1968, 1969) successfully developed embryos in an artificial medium (Murashige and Skoog, 1962) using nucellar tissues of pollinated flowers of the monoembryonic cultivars Pong yau pummelo (*C.grandis* L. Osbeck) , Ponderosa lemon (*C. limon* Burm. F) and Temple orange (*C. reticulata* Blanco x *C. sinensis* L. Osbeck). As the nucellar tissue was cultured 100 days after pollination, it is suggested that pollination and perhaps fertilization provided the stimulus for the initiation of nucellar embryos in these normally monoembryonic cultivars. In cross-pollinated flowers of monoembryonic varieties *in vivo* the adventive embryo may be initiated at the same time as the zygotic embryo because the

sperm reaches the ovule within 9-12 days after pollination and adventive embryos have been found to be present 8 days after pollination (Geraci *et al.*, 1978; Kahn and DeMason, 1985). As nucellar embryos do not reach maturity, the rate of cell division of these embryos in monoembryonic varieties is worth further examination. In polyembryonic cultivars, Esen and Soost (1977) reported that small, globular, adventive embryos as well as one-celled embryos were frequently observed in seeds whose zygote had not divided, indicating that cell division in adventive embryos was more rapid than that of the zygotic embryo.

2.4.2 The site of zygotic and nucellar embryos within the embryo sac

As in most plant species, the egg cell in citrus is located in a central position at the apex of the embryo sac. When the pollen tube delivers its content by the way of the micropyle the sperm cell then fuses with the egg cell. Thus the zygotic embryo is always located at the apex of the embryo sac at the micropylar end (Esen and Soost, 1977). In the case of polyembryonic cultivars, the zygotic embryo may be displaced, due to proliferation of the adventive embryos, into the embryo sac with resulting competition for space and nutrients. Hence, the location of the embryo may not be a reliable marker of the zygotic embryo in polyembryonic cultivars. Similarly, the presence of embryo stalks or suspensors is an unreliable indication since both zygotic and adventive embryos consistently possess suspensors (Esen and Soost, 1977). Further, Maheshwari and Ranga Swamy (1958) reported that both mono and polyembryonic seeds may occur in the same fruit and even in the same locule.

Nucellar embryos provide genetically uniform seedlings which reproduce the seed parent genotype without the variation caused by segregation during sporogenesis or recombination during fertilization. There are different views concerning the site of adventive embryo initiation. Maheshwary and Ranga Swamy (1958) reported that adventive embryos arose from the nucellus cells situated at the micropylar end of the embryo sac but not from those towards the chalaza. Frost and Soost (1968) also found that these embryos arose mainly near the micropylar end of the embryo sac and within one or two cell layers of it. An alternative suggestion is that supernumerary embryos are formed by

proliferation of nucellar cells at all locations surrounding the embryo sac (Frost, 1926). Esen and Soost (1977) and Kobayashi *et al.* (1981) stated that adventive embryos arose mostly from the micropylar end of the nucellus, particularly at and around the apex of the embryo sac but that a few arose from the chalazal end of the nucellus. However, most of those initiated from deep within the nucellus or from the chalazal end did not develop further. Possibly they are too far away from the nourishment provided by the endosperm located within the embyo sac. The investigation of Maheshwari and Ranga Swamy (1958), which was confined to the end of the period of adventive embryo development , may have been conducted after the loss of embryos originating from these areas.

2.4.3 The time of zygotic and nucellar embryo initiation

The time at which the two embryo types initiate is important in relation to their later success in growth and development. Embryos initiated earlier may have a better chance to survive in competition with those initiated later. The rate of cell division, however, may be another independent determining factor. Frost (1943) , and Bitter *et al.* (1969) suggested that the initiation of adventive embryos occured soon after the fertilized egg first divided. However, Esen and Soost (1977) reported that the initiation of adventive embryos appeared to be independent of pollination and fertilization. Moreover, Kobayashi *et al.* (1979; 1981) found that adventive embryos were present both before and at anthesis in polyembryonic varieties. This strongly suggested that adventive embryos were initiated earlier than the zygotic embryo in such cultivars.

2.4.4 The proportion of zygotic and nucellar embryos

The proportion of zygotic and nucellar embryos varies between different citrus species and cultivars. There are many factors that determine the number of embryos per seed in citrus, including the source of pollen, chemicals and climate (Tisserat *et al.*, 1979). Frost and Soost (1968) suggested that the final proportions of each embryo type in the mature seed was the result of competition during development within the embryo sac. In

addition, they proposed that the relative success in competition depends on the time of initiation, the position, and the number of nucellar embryos produced. Due to the initiation of nucellar embryos earlier than the zygotic embryo (Kobayashi *et.al.*, 1979;1981) and the comparative rates of cell division thereafter (Esen and Soost, 1977; Watanabe, 1985a) some zygotic embryos are lost. As a result, the proportion of zygotic embryos is much lower than that of adventive embryos. The proportion of seeds of rough lemon with zygotic embryos was 0 -4 %, around 20 % in sweet orange, mandarin, trifoliata orange, sour orange and grapefruit, up to 50 % in cultivated lemon, and 100 % in shaddock (Bowman, 1956), which is monoembryonic (Hodgson, 1967). These figures were determined by crossing to a parent with a distinctive morphological marker, such as the trifoliate leaf character.

The high percentage of nucellar embryos in most commercial citrus varieties is a handicap to breeding programmes. Consequently, methods to control their development within the ovule have been of interest to citrus breeders. DeLange and Vincent (1977) reported that the application of chemicals such as naphthalene acetic acid (NAA),maleic hydrazide (MH),coumarine and gibberellin were effective in reducing embryo numbers. But, in *Fortunella,* Watanabe (1985a) reported that continuous gamma-irradiation was more effective as a method to inhibit the development of nucellar embryos.By this means 87-100 % of progeny resulting from a cross between *C. unshiu* and *P. trifoliata* were marked by the trifoliate character, indicating that the zygotic embryo tolerated a higher dose of gamma-irradiation than the adventive embryos (Watanabe, 1985b). The reduction in nucellar embryos per seed was accompanied by an increase in the number of small embryos which degenerated beyond the small globular stage, indicating that gamma-irradiation was more effective when used during early development.

2.4.5 Discrimination between zygotic and nucellar seedlings.

There are two major areas of the citrus industry where discrimination between zygotic and nucellar seedlings is necessary. Propagation of citrus rootstocks depends upon the production of clonal plants from nucellar seedlings. Uniformity of rootstock genotype is essential for reliable plant performance following budding and orchard establishment. In this situation, the zygotic seedlings are undesirable and early identification

and removal of these seedlings types would be very beneficial. In contrast, the zygotic seedlings are the source of genetic variability required for plant breeding. Therefore, methods are needed to detect and preserve the zygotic seedlings so that the clonal nucellar seedlings can be discarded from the selection programme. Several methods of identification have been attempted. Morphological methods are the simplest and are effective when the male and female parents differ significantly in morphology (Teich and Spiegel-Roy, 1972). When the morphology of both parents is similar, as is the case with most citrus cultivars, separation is more difficult and may be inaccurate (Roose and Traugh, 1988). Biochemical methods have also been used in attempts to distinguish between zygotic and nucellar seedlings. Furr and Reece (1946) applied a colour test to detect differences in the organic acid content of hybrids resulting from crossing. Pieringer and Edwards (1965) analysed leaf oils by infrared spectroscopy, and Weinbaum *et al.* (1982) applied gas chromatographic methods to genotype discrimination. In general, these methods were not sufficiently reliable for the accurate detection of the zygotic seedlings. This is due to the fact that plant chemical compounds such as oils, pigments and other classes of compounds are the products of a series of biosynthetic reactions and their presence in plants is greatly influenced by changes in environmental conditions. The most promising method for the identification of zygotic and nucellar seedlings of citrus varieties is the isozyme technique (Torres *et al.*, 1978; 1982).

2.4.6 Isozyme analysis

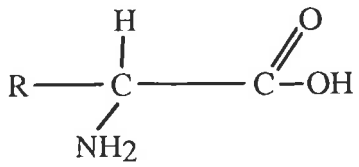
2.4.6.1 Enzymes

Plant metabolism, whether it be the synthesis or degradation of organic materials, is under the control of enzymes which are products of the genes. Enzymes work specifically and act on only a single substrate (reactant) or group of closely related substances. The same enzymes may be found in different parts of the plant.

The molecular weights of enzymes are large, many being over 10,000 (Hart and Schuetz, 1966). They are composed of a protein moietysometimes anon-protein

organic (prosthetic) group and frequently another organic compound, metal ion or both (co-enzyme). The prosthetic group and co-enzyme function as protein activators.

Proteins are composed of smaller molecules called amino acids, so that composition and size depends upon the kind and numbers of the amino acid sub-units . Amino acids may be represented by the general formula (Fig. 2. 1)



(α)

Figure 2.1 The general formula of an amino acid

NH_2 forms the amino group responsible for the basic properties, while COOH (carboxyl or acidic group) is responsible for the acidic properties. R represents the remainder of the molecule and is different for each amino acid.

The formation of proteins from amino acids occurs when the carboxyl group of one amino acid and the amino group of another combine with the elimination of a water molecule, producing a peptide bond (the primary structure). The individual peptide chains making peptides and polypeptides.
are further extensively coiled into sphere-like shapes with hydrogen bonds between the amino acids (secondary structure) and various other kinds of bonds cross-linking one chain to another (tertiary structure) and forming a polypeptide chain. Hence, each enzyme differs in the structure and number of polypeptide chains. The individual polypeptide chains may function singly or as polymers of various sizes and can be shortened by proteolysis to produce functionally different molecules.

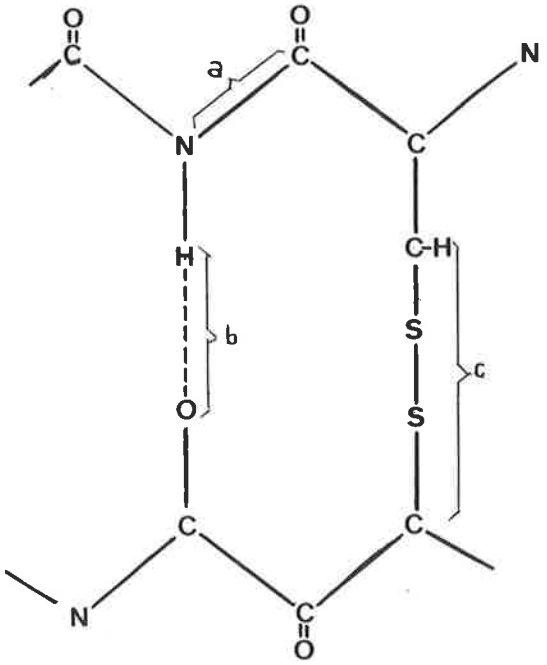


Figure 2.2 Diagram of structure of protein

a. primary structure (peptide bond)

b. secondary structure (hydrogen bond)

c. tertiary structure

2.4.6.2 Enzyme separation

There are several ways to separate enzymes from other cellular constituents. Dialysis, molecular sieve chromatography and ultra centrifugation methods separate enzymes on molecular size, fractional precipitation separates them on the ease of solution in certain solvents, column chromatography on the ability of certain chemicals to absorb the enzymes and electrophoresis on the electrical charge (Salisbury and Ross, 1969). The electrophoresis method is discussed further here because it has the potential to separate closely related proteins.

2.4.6.3 Electrophoresis

Due to the presence of amino and carboxyl groups, enzymes may have either a net positive or net negative charge (amphoteric) at the surface, depending on the pH. Under conditions of low pH, they will have a positive charge from the amino groups, while at high pH more carboxyl groups will ionize and a negative charge will result. This property is important in enzyme separation by electrophoresis. In the electrical field, negatively charged molecules (anions) migrate towards the positive electrode (anode) and positively charged molecules (cations) migrate towards the negative electrode (cathode). The speed of migration is related to the size of the net charge on the enzyme molecule (governed by the pH of the buffer solution) and the electrical field strength applied through the electrodes (determined by the voltage setting on the power supply) (Harris and Hopkinson, 1976).

A variety of different supporting matrices in which the electrophoretic separation of enzymes can be carried out are now available. Among them are starch gel, acrylamide gel, agarose gel and cellogel. The supporting matrices function as molecular filters. Hence, starch gel electrophoresis separates enzymes on both charge and molecular weight (Shields *et al.*, 1983). To some extent, the choice of method is simply a matter of inclination and convenience, but for some enzymes clear separation is only obtainable using particular support matrices. In this work, starch gel was used because it is a simple method with the capacity to load a large number of samples.

When the enzymes have been resolved by means of electrophoresis in a solid matrix such as starch gel, they are then stained with the appropriate reactant to locate their positions as an array of bands known as a "zymogram" (Shield *et al.*, 1983). The substrates and other solutes of the reactant diffuse into the gel. The product of enzymatic reaction then reacts with a reagent such as a diazonium salt, a tetrazolium salt

and forms a coloured precipitate, as in most cases the primary product of the enzyme reaction cannot be detected visually. An example is given in Fig.2. 3, the detection of phosphatase activity (Vallejos, 1983).

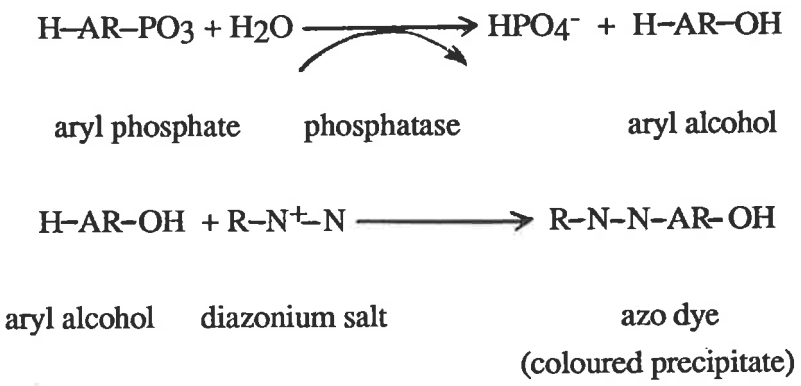


Figure 2. 3 Detection of phosphatase enzyme

2.4.6.4 Genetics of isozymes

Isozymes are variant molecular forms of enzymes that are readily separated and detected by standard electrophoretic and staining techniques (Tanksley, 1983). The precipitation of enzymes in a supporting matrix following the appropriate staining procedure will give a zymogram related to the genotype of the individual. Variation in banding patterns between individuals can be analysed genetically. Based on the individual alleles, Torres *et.al.* (1978) proposed three basic isozyme types in *Citrus* : S, indicated the allele which specified a slowly migrating enzyme or subunit, F the fast and M (and I) as intermediates. For PGI-1 (Phosphoglucoisomerase) isozymes, two further alleles W and P were named for Wilial and Papeda respectively. The W subunit migrates more rapidly towards the anode than that specified by F, and P migrates more slowly than that specified by S. Similarly, for the isozyme of GOT-1 (Glucose oxaloacetate transaminase), Torres *et al.* (1978) specified P (for *Poncirus*) which migrated faster than F. P (for *Poncirus*) isozymes of PGM (Phosphoglucomutase) migrated slightly faster than isozymes encoded by M. Plants homozygous for an isozyme allele will display a single band. If an individual is heterozygous, there will be two bands for a monomeric enzyme, three for a dimeric, four for a trimeric and five fo a tetrameric enzyme (Crawford, 1983), as a dimeric enzyme for instance has two separately coded polypeptide chains. SS and FF genotypes in Fig. 2. 4 have similar molecular weights (one band identical to one polypeptide chain) but they differ in net electrical charge due to the contribution of other parts of the molecule and hence may be separated electropheretically (Markert, 1963; 1974). Hybrids of homozygous parents may be empirically distinguished from their parents by the presence of hybrid bands (Peirce and Brewbaker, 1973). The second generation (F-2) of crossing will provide three possible genotypes. One each of the P-1 and P-2 parental genotypes and the hybrid genotype. In Figure 2.4 is presented a diagram of segregation of the citrus isozymes at the PGI locus from Soost *et al.* (1980).

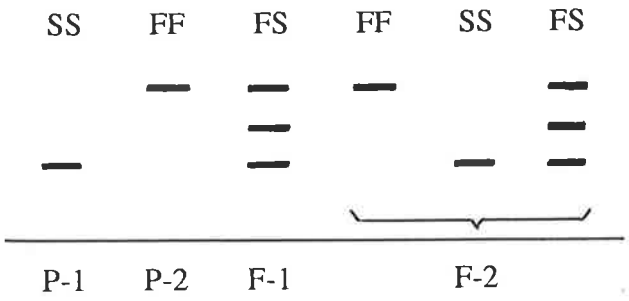


Figure 2. 4. The segregation of citrus isozymes at the PGI locus.

Chapter 3. Materials and Methods

This work consists of two sections, the first concerning discrimination between zygotic and nucellar seedlings of citrus rootstocks resulting from open pollination, and the second identification of mandarin types, hybrids and selections.

3.1 Plant material

3.1.1 Rootstock study

Seeds of five polyembryonic citrus rootstocks, rough lemon (*C.jambhiri* Lush.), trifoliata orange cv. William's strain (*Poncirus trifoliata* [L.] Raf.), sweet orange cv. White Siletta No. 1 (*C. sinensis* [L.] Osbeck), Troyer citrange (*P.trifoliata* x *C. sinensis*) and Cleopatra mandarin (*C.reticulata* Blanco) were obtained from Tolley's Nurseries, Renmark, South Australia. Mature leaves from the maternal parent and neighbouring trees were also investigated. The aim was to analyse the maternal parent genotypes, and to check the genotype of the nucellar seedlings which should be identical to that of the maternal parent. Progeny which had a different genotype to the maternal parent were presumed to be zygotic in origin. The male parents of these zygotic seedlings may have been the same tree (selfing) or the neighbouring trees (crossing). In addition, mature leaves of Carrizo citrange were included in the experiments to test the report that it is similar to Troyer citrange (Savage and Gardner, 1965). Healthy, fully expanded leaves were sampled and held on crushed ice during transfer by road to Adelaide (approx. 269 km). All rootstocks analyzed are regularly used in Australia and overseas (Thornton and Dimsey, 1987).

Seed coats were removed to enhance germination, and the seeds were soaked in distilled water for two hours and germinated in pots containing sterilized sand. Seedlings were grown in a growth cabinet with a photoperiod of 9 h, and radiant flux density of 450 μmole m^{-2} S^{-1} provided by sodium vapour lamps, and a 25^{o} /20^{o} C day/night temperature. Five weeks after germination, the multiple groups of seedlings arising from individual seeds

were separated and surviving seedlings were planted individually in five cm diameter pots containing sterilized sand. The seedlings were labelled according to their position of emergence commencing at the micropylar end of the seed. The seedling at or closest to the micropylar end was designated by the letter A and the others were labelled in sequence. The seedlings were treated at weekly interval, with "Mancozeb" fungicide, "White Oil" insecticide and "Aquasol" NPK 23;4;19 fertilizer. The number of seedlings emerging from each seed was recorded at germination and eight weeks later. Plant height was measured 20 weeks after germination and the seedlings were scored for leaf shape, presence of thorns and winged petioles. At this time the seedlings were transferred to larger pots (10 cm) and placed in a glass house with a temperature range from 25° to 35° C in summer and autumn, and between 15° and 23° C from late autumn till early spring.

3.1.2 Mandarin study

The procedure of sampling and transport described for section 3.1.1 were applied when mature leaves of mandarin citrus were collected, including three types of Ellendale (here designated Ellendale 1, Ellendale 2, Ellendale 3), Kara, Imperial, Thorny, Hickson, Silverhill, Murcott and Beauty of Glen Retreat. Leaves of Algerian tangerine were obtained from the New South Wales Department of Agriculture at Dareton, four further Ellendale types (Robinson, Koster, Herps, and Burndale) and Wallent mandarin were obtained from the Bundaberg Research Station, Queensland. These leaves were air transported from the source to the Waite Institute. The mature leaves were stored at -20° C. The parentage of these mandarin types where known is listed in Table 3.1. Leaves of Seminole, Minneola and Orlando tangelo were obtained from the Waite Agricultural Research Institute orchard at Glen Osmond. Sweet orange (unknown cultivar), which is believed to be one of the Ellendale mandarin parents, was also tested, and leaf material was obtained from the Waite orchard. The isozyme profiles of parents which were not available to this study were obtained from the work of Torres *et al.* (1978, 1982).

The rest of the experimentation was identical for sections 3.1.1 and 3.1.2.

Table 3.1 Reported parentage of the mandarins

Species and group	Cultivar	Reported parentage	Country or area of origin	Reference*
C. unshiu (Satsuma mandarin)	Silverhill	Identical to Owari satsuma	U.S.A	4
C. deliciosa (Mediterranean mandarin)	Thorny	Identical to Willowleaf	Mediterranean	2, 4
C. reticulata (Common mandarin)	Imperial	Parentage unknown, possibly Willowleaf X Emperor	Australia	1, 2, 4
	Kara	Owari X King	U.S.A	4
	Beauty of Glen Retreat	Parentage unknown, one parent probably a mandarin, possibly Dancy	Australia	4
	Algerian	Parentage unknown, one parent probably a mandarin, possibly Willowleaf	North Africa	4
C. reticulata X *C. sinensis* (Tangor)	Hickson **	Unknown, possibly a hybrid between sweet orange and mandarin	Australia	4
	Murcott **	Unknown, possibly a hybrid between sweet orange and mandarin	U.S.A	4
	Ellendale 1**	Hybrid between sweet orange and mandarin	Australia	4, 6
	Ellendale 2**	Hybrid between sweet orange and mandarin	Australia	4, 6
	Ellendale 3**	Hybrid between sweet orange and mandarin	Australia	4, 6
	Wallent	Seedling selection of Ellendale	Australia	1
	Burndale	Seedling selection of Ellendale	Australia	5
	Koster	Bud selection of Ellendale	Australia	5
	Robinson	Bud selection of Ellendale	Australia	5

	Herps	Bud or seedling selection of Ellendale	Australia	3
C. reticulata X *C. paradisi* (Tangelo)	Seminole	Hybrid between Duncan grapefruit and Dancy tangerine	U.S.A	4, 7
	Minneola	Hybrid between Duncan grapefruit and Dancy tangerine	U.S.A	4, 7
	Orlando	Hybrid between Duncan grapefruit and Dancy tangerine	U.S.A	4, 7

*1: Alexander (1983); 2: Bowman (1956); 3: J. B. Forsyth (personal communication, 1988); 4: Hodgson (1967); 5: L.S. Lee (personal commonication, 1988); 6: McAlpin (1983); 7: Soost (1969).

** Parent cultivars not recorded.

3.2 Method of leaf extraction

Three methods of leaf extraction were compared, namely : freezing with liquid nitrogen in potassium phosphate buffer (Torres, 1984); homogenizing followed by centrifugation (Arulsekar and Parfitt, 1986) and the plier method (see later) described by Torres *et al.* (1978). All three methods gave similar results and as the plier method was the simplest, this was used subsequently in all experiments. Young and old leaves, ie. leaves in the first flush and in the third flush of the maternal parents, were also compared. The results of band resolution were similar, so both young and old leaves were included in the experiments due to limitations in the number of leaves available for sampling.

Approximately two square cm of leaf blade was folded around a 5 x 7 mm wick of Whatman No. 30 filter paper. The folded leaf was then squeezed with a pair of pliers with the jaws covered by transparent plastic tape. In order to avoid contamination from other leaf samples, a new piece of tape was used for each extraction. The wicks were then inserted into the starch gel prior to electrophoresis. Extraction of the leaves was conducted at 2-3^{o} C.

3.3 Gel preparation

Gel preparation was conducted at room temperature. Twenty four grams of starch was suspended in 200 ml of buffer solution in a 500 ml vacuum flask. In order to facilitate the determination of the location of the anodal front, 1 ml of 0.1 % bromphenol blue (in water) was added to the suspension. The suspension was mixed thoroughly by swirling, and then boiled. While boiling, the suspension was swirled until it became viscous when it was degassed using a water-pump for 60 second and poured on a gel plate (with frame, 6 mm thickness). A glass plate was placed on the gel frame and pressed with a 1 kg weight. The gel was then kept overnight at room temperature to ensure its solidity. The next morning the cover glass was lifted off and the gel was cut 4 cm from one end to insert the wicks .

Table 3.2 Composition of starch and electrode bridge buffer solutions

Enzyme	Starch buffer	Electrode bridge buffer	Reference
1. GOT	0.03 M tris citrate pH 8.2	0.34 M sodium borate pH 8.7	4
2. PGI	0.019 M tris citrate pH 7.7	0.34 M sodium borate pH 8.7	4
3. PGM	1:10 electrode bridge buffer : water pH 7.4	0.1 M tris; 0.1 M maleate 0.01 M Na_2EDTA; 0.01 M magnesium chloride pH 7.4	1
4. IDH	1:9 electrode bridge buffer : water	0.3 M tris citrate pH 7.0	6
5. LAP	1:14 electrode bridge buffer : 0.07 M tris citrate pH 8.1	0.3 M lithium borate pH 7.9	6
6. MDH	16mM tris citrate pH 6.9	48mM tris citrate pH 6.9	5
7. ME	1:9 electrode bridge buffer : water	0.3 M tris citrate pH 7.0	6
8. PER	0.05 M tris ; 0.09 M boric acid ; 0.0016 M Na_2EDTA pH 8.0	0.5 M tris ; 0.6 M boric acid ; 0.016 M Na_2EDTA pH 8.0	2
9. SOD	0.05 M tris ; 0.09 M boric acid ; 0.0016 M Na_2EDTA pH 8.0	0.5 M tris ; 0.6 M boric acid ; 0.016 M Na_2EDTA pH 8.0	2
10. CAT	1:28 electrode bridge buffer : water pH 7.2	0.223 M tris ; 0.069 M citric acid pH 7.2	3
11. SkHD	1:28 electrode bridge buffer : water pH 7.2	0.223 M tris ; 0.069 M citric acid pH 7.2	3
12. 6-PGD	1:28 M electrode bridge buffer : water pH 7.2	0.223 M tris ; 0.069 M citric acid pH 7.2	3
13. APH	0.045 M tris ; 0.007 M citric acid ; 0.004 M lithium hydroxide; 0.019 M boric acid pH 8.3	0.038 M lithium hydroxide ; 0.188 M boric acid pH 8.3	3
14. LAC	0.045 M tris ; 0.007 M citric acid ; 0.004 M lithium hydroxide; 0.019 M boric acid pH 8.3	0.038 M lithium hydroxide ; 0.018 M boric acid pH 8.3	3

15. GDH	1:28 M electrode bridge buffer : water pH 7.2	0.223 M tris + 0.069 M citric acid pH 7.2	3
16. F1,6DP	0.08 M tris; 0.002 M citric acid pH 7.2	0.223 M tris; 0.069 M citric acid pH 7.2	3

1: Harris and Hopkinson (1976); 2: Shaw and Prasad (1970);3 : Soltis *et al.* (1983); 4: Torres *et al.* (1978); 5: Torres and Bergh (1980); 6: Torres *et al.* (1982).

3.4 Electrophoresis

The gel was connected to the electrode bridge buffer compartment of the electrode vessel through a fibre sheet (wettex) (Fig. 3.1). Two hundred and fifty ml of the appropriate electrode bridge buffer solution was poured into each compartment of the electrode vessel. The electrode bridge buffer solution for each enzyme system is given in Table 3.2. The gel was run at 2-3° C, and after 30-45 minutes the power was switched off and the wicks removed from the gel with forceps. The surface of the gel which was in direct contact with the wick was then wiped free from adhering debris with cotton wool. Following removal of the wicks, the electric current was renewed. The time of electrophoresis and the current (mA) differed between enzyme systems (Table 3.3). A problem in the electrophoresis process was breakage of the gel during a long period of running. When this occured, migration of the anodal front was interrupted, resulting in uneven positioning of the band front. Another cause of gel breakage during running and uneven positioning of the band front was the high current employed. Therefore in this experiment, some of the gels were charged at low current for a longer time of running (10-24 hours). The systems used are presented in Table 3. 3 and followed various sources with little modification.

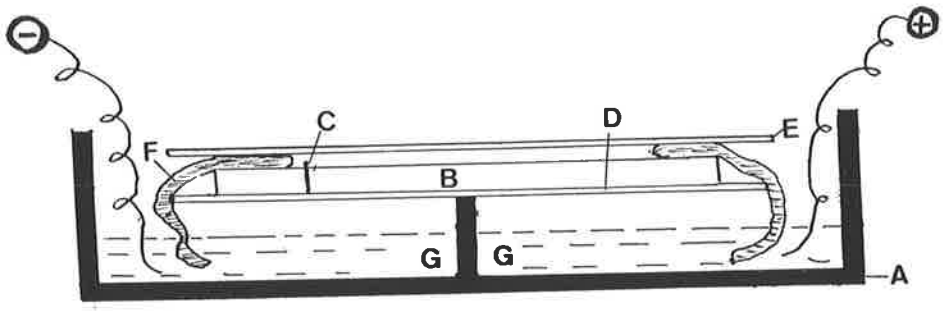


Figure 3.1 Assembly of starch gel electrophoresis system.
A : electrode vessel, B : starch gel,
C : wicks, D : glass plate, E : glass cover,
F : wettex, G : electrode bridge vessel.

Table 3.3 Current and duration of electrophoresis used in the present study.

Enzyme	Current (mA /cm^2 gel cross section)	Duration (h)	Reference
1. GOT	3.5	6	2
2. PGI	2.0	6	2
3. PGM	2.5	24	1
4. IDH	4.7	11	4
5. LAP	3.5	5	4
6. MDH	1.0	8	3
7. ME	4.7	11	4
8. PER	4.7	10	1
9. SOD	3.4	20	1
10. CAT	3.0	8	1
11. SkHD	3.0	8	1
12. 6-PGD	1.5	20	1
13. APH	1.5	20	1
14. LAC	1.5	20	1
15. GDH	1.5	20	1
16. F1,6DP	2.5	20	1

1 : Harris and Hopkinson (1976); 2 : Torres *et al.* (1978); 3 : Torres and Bergh (1980);4 : Torres *et al.* (1982)

3.5 Gel staining

Following electrophoresis, the gel was sliced into three pieces, and each slice was immersed in 50 ml of pre-prepared staining solution following standard methods formulated by different authors (Table 3.4). The upper region of the sliced gel was not usually as clear in band resolution as were the middle and lower regions. In some cases, the starch and electrode buffers for the enzymes tested were similar and each slice of the gel could be stained for the different enzyme systems (for example GDH, 6-PGD and APH). Two general methods of staining were utilized, "ordinary" (without agar) and "overlay" (with agar). The agar overlay method was applied when the band was soluble. To prevent diffusion of the band the staining solution was incorporated into a solid agar matrix (Vallejos, 1983). The agar overlay method was used for the ME, PGM and SOD enzymes. The following enzyme systems were utilized; the enzyme code follows that of Harris and Hopkinson (1976) and Soltis *et al.* (1983).

1. GOT : glutamate oxaloacetate transaminase (E.C.2.6.1.)
2. PGI : phosphoglucoisomerase (E.C. 5.3.1.9)
3. PGM: phosphoglucomutase (E.C.2.7.5.1)
4. IDH: isocitrate dehydrogenase (E.C. 1.1.1.42)
5. LAP: leucine aminopeptidase (E.C. 3.4.11.1)
6. MDH : malate dehydrogenase (E.C. 1.1.1.37).
7. ME : malic enzyme (E.C. 1.1.1.40)
8. PER : peroxidase (E.C. 1.11.1.7)
9. SOD: superoxide dismutase (E.C. 1.15.1.1)
10. CAT: catalase (E.C. 1.11.1.6)
11. SkHD: shikimate dehydrogenase (E.C. 1.1.1.25)
12. 6-PGD: 6 phosphogluconate dehydrogenase (E.C.1.1.1.44)
13. APH: acid phosphatase (E.C. 3.1.3.2)
14. LAC: laccase (E.C. 1.10.3.2)

15. GDH: glutamate dehydrogenase (E.C 1.4.1.2)

16. F1,6DP : fructose-1,6-diphosphatase (E.C. 3.1.3.11)

For the rootstock experiments, the enzyme systems GOT, PGI, IDH, LAP, MDH and ME were used, while in the mandarin experiments all systems were employed. as six systems were insufficient to discriminate the cultivars Staining was carried out in the dark at 30° - 35° C for 30 - 60 minutes, or until bands appeared. The gel was then fixed with 50 % methanol for one hour and rinsed with distilled water before recording and photographing. In some cases methanol caused gel shrinkage and obscured the separation of the bands. When this occurred, the gel was dipped into distilled water as soon as possible to avoid further shrinkage.

All the enzyme systems used except superoxide dismutase gave positive staining (coloured bands on a white or clear background). Superoxide dismutase showed negative staining (clear bands on a coloured background). Most band colours were blue on a white background with the exception of the peroxidase, glutamate oxaloacetate transaminase, acid phosphatase and laccase enzymes. Peroxidase stained red on a brown background, glutamate oxaloacetate transaminase reddish on a white background , acid phosphatase red on a white background and laccase pale on a yellowish background. The peroxidase stain was very soluble and bands disappeared quickly following staining presenting difficulties in recording the results. The other difficult stain was that for the catalase enzyme where soon after the bands stained the gel changed from white to dark blue and the stained bands became indistingushable from the background. Phosphoglucomutase, which was stained by the agar overlay method, also differed from the others. As the bands tended to diffuse into the agar, the bands could be recorded through the agar.

The chemicals used in the experiments, and their sources are listed in Table 3.5.

Table 3.4 Composition of enzyme staining systems

Enzyme	Composition	Reference
1. GOT	Sol. A: 50 ml 0.1 M tris HCl pH 8.5; 50 mg a- ketoglutaric acid; 100 mg L-aspartic acid Sol. B : 5 mg pyridoxal-5-phosphate; 75 mg fast blue BB salt	7
2. PGI	0.4 ml 0.01 M PMS ;2.5 ml 1 M tris HCl pH 8.0; 2.5 ml 0.5 M magnesium chloride; 2.5 ml 0.018 M fructose-6-phosphate; 57.5 ml water; 25 units glucose-6-phosphate dehydrogenase;5 mg NADP.	4
3. PGM	25 ml 0.05 M tris HCl pH 8.0; 20 ml 0.025 M magnesium chloride; 25 ml boiled liquid agar (2 %,prior to staining); 140 units glucose-6-phosphate dehydrogenase; 50 mg glucose-1-phosphate; 5 mg NADP in 1 ml water; 5 mg MTT in 1 ml water; 5 mg PMS	1
4. IDH	50 ml 0.1 M tris HCl pH 7.5; 0.5 ml 1 M magnesium chloride; 50 mg DL-isocitric acid; 5.5 mg NADP; 10 mg MTT; 2 mg PMS	6
5. LAP	60 ml 0.05 M phosphate buffer pH 6.0; 10 mg black K salt; 10mg L-leucine b-naphthyl amide HCl dissolved in 1 ml N, N dimethyl formamide.	6
6. MDH	6 ml1.0 M trisHCl pH 8.8; 0.8 ml 0.01 M NAD; 0.8 ml 0.01 M PMS;4 ml 0.01 M NBT; 3 ml 2 M DL-malic acid pH 7.0 with sodium hydroxide; 50 ml water.	5
7. ME	2 ml 0.2 M magnesium chloride; 25 ml boiled liquid agar (2 %, prior to staining);100 mg DL-malic acid in 20 ml 0.1 M tris HCl pH 7.0 readjusted to pH 7.0 with sodium hydroxide; 5 mg NADP in 1 ml water; 5 mg MTT in 1 ml water; 0.5 mg PMS in 1 ml water.	6
8. PER	5 ml 1.0 M sodium acetate pH 4.7 with glacial acetic acid; 30 ml 95 % ethanol; 65 ml water; 0.5 ml 30 % hydrogen peroxide; 250 mg p-phenylenediamine; 50 mg manganese sulphate.	7
9. SOD	25 ml 0.05 M tris HCl pH 8.0; 25 ml boiled liquid agar (2%, prior to staining); 5 mg MTT in 1 ml water; 5 mg PMS in 1 ml water.	1

10. CAT	Sol. A: 100 ml water; 2 ml acetic acid; 2 g potassium iodide Sol. B: 100 ml water; 1 ml 30% hydrogen peroxide.	2
11. SkHD	10 ml 1.0 M tris HCl pH 8.5; 90 ml water; 100 mg shikimic acid; 10 mg NADP; 20 mg MTT; 2 mg PMS	3
12. 6-PGD	10 ml 1.0 M tris HCl pH 8.2; 90 ml water; 2 ml 1.0 M magnesium chloride; 40 mg 6-phosphogluconic acid; 10 mg NADP; 10 mg MTT; 2 mg PMS.	3
13. APH	100 ml 0.05 M sodium acetate pH 5.0; 0.5 ml magnesium chloride; 100 mg b-naphthyl acid phosphate; 80 mg fast garnet GBC salt	3
14. LAC	100 ml 0.1 M potassium phosphate bufferpH 6.8; 15 mg pyrogallol; 50 mg sulphanilic acid	3
15. GDH	10 ml 1.0 M tris HCl pH 8.0; 70 ml water; 20 ml 1.0 M L-glutamic acid pH 8.0; 20 mg NAD; 10 mg MTT ; 2 mg PMS	3
16. F1,6DP	10 ml 1.0 M tris HCl pH 8.0; 90 ml water; 1.0 ml 1.0 M magnesium chloride; 50 units phosphoglucoisomerase; 50 units glucose-6-dehydrogenase; 250 mg fructose-1,6-diphosphate; 10 mg NADP; 5 mg MTT; 1 mg PMS	3

1: Harris and Hopkinson (1976); 2: Shaw and Prasad (1970); 3: Soltis *et al.* (1983); 4: Torres *et al.* (1978); 5: Torres and Bergh (1980); 6: Torres *et al.* (1982); 7 : Vallejos (1983).

Table 3.5 Chemicals used in this study and their sources

Chemical	Source
1. Acetic acid (glacial)	Ajax
2. Agar	Difco
3. L-aspartic acid	Sigma
4. Boric acid	Sigma
5. Bromphenol blue	Andrews
6. Citric acid	Sigma
7. N,N dimethyl formamide	BDH
8. [3-(4,5-dimethylthiazol-1-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)	Sigma
9. Disodium hydrogen orthophosphate	Univar
10. Ethanol	Univar
11. Ethylenediamine tetraacetic acid (Na_2EDTA)	BDH
12. Fast black K salt	Sigma
13. Fast blue BB salt	Sigma
14. Fast garnet GBC salt	Sigma
15. D-fructose-6-phosphate	Sigma
16. D-fructose-1,6-diphosphate	Sigma
17. Glucose-6-phosphate dehydrogenase	Sigma
18. D-glucose-1-phosphate	Sigma
19. D-fructose-1,6-diphosphate	Sigma
20. L-glutamic acid	Sigma
21. Hydrochloric acid (HCl)	Univar
22. Hydrogen peroxide	Merck
23. DL-isocitric acid	Sigma
24. a-ketoglutaric acid	Sigma
25. L-leucine-b-naphthyl amide HCl	Sigma
26. Lithium hydroxide	BDH
27. Magnesium chloride	Univar
28. DL-malic acid	Sigma
29. Manganese chloride	Univar
30. Manganese sulphate	BDH
31. b-naphthyl acid phosphate	Sigma
32. b -nicotinamide adenine dinucleotide (NAD)	Sigma
33. b -nicotinamide adenine dinucleotide phosphate (NADP)	Sigma

34. Nitro blue tetrazolium (NBT)	Sigma
35. Phenazine methosulfate (PMS)	Sigma
36. p-phenylenediamine	Sigma
37. 6-phosphogluconic acid	Sigma
38. Phosphoglucoisomerase	Sigma
39. Potassium iodide	BDH
40. Potassium dihydrogen orthophosphate	BDH
41. Pyridoxal-5-phosphate	Sigma
42. Pyrogallol	Sigma
43. (-)-Shikimic acid	Sigma
44. Sodium acetate	Sigma
45. Sodium hydroxide	BDH
46. Starch	Electrostarch, Sigma
47. Sulphanilic acid	Sigma
48. [Tris(hydroxymethyl) amino methane] (Tris)	Sigma

CHAPTER 4. RESULTS

4.1 Discrimination between zygotic and nucellar seedlings of citrus rootstocks resulting from open pollination.

4.1.1 Seed germination

The number of germinated seedlings per seed varied between cultivars (Table 4.1). Rough lemon gave the highest number of germinated seedlings per seed, as well as the most seedlings surviving 8 weeks after germination, while trifoliata orange cv. William's Strain gave the least. Trifoliata orange, however, had the lowest seedling loss due to competition between the embryos in the seed or between the germinated seedlings, (5.8 %) whereas the death rates of the other cultivars were similar (23-25 %).

Table 4.1 Seedling numbers of five polyembryonic citrus rootstocks

Rootstock	Total number of seeds sown	Total number of germinated seedlings	Mean number of germinated seedlings per germinated seed	Total number of surviving seedlings at 8 weeks after germination	Percentage death of seedlings
Rough lemon	90	188	2.1	141	25.0
Trifoliata orange	92	120	1.3	113	5.8
Sweet orange	95	156	1.6	119	23.7
Troyer citrange	83	159	1.9	122	23.3
Cleopatra mandarin	113	163	1.4	123	24.5

4.1.2 Isozyme genotypes of the maternal parents and neighbouring trees

4.1.2.1 Glucose oxaloacetate transaminase (GOT)

The zymograms of GOT consisted of two zones of activity. The first zone, closest to the origin on the gel was termed GOT-1, while the faster zone was termed GOT-2 according to the nomenclature of Torres *et al.* (1978).

There were four alleles present at the GOT-1 locus, namely F, S, M and P (Table 4.2) . The FS genotypes were found in rough lemon (Plate 4.1) and Prior lemon. MP occured in trifoliata orange cv. William's strain. The homozygous genotype, SS was observed in Smooth Seville, Ellendale, Imperial and Cleopatra mandarin, sweet orange cv. White Siletta No. 1, Valencia and Washington navel orange. Red blush grapefruit had no detectable bands. Troyer citrange, which is reported to be a hybrid between *P.trifoliata* and *C. sinensis,* showed the expected genotype, PS. The genotype of Carrizo citrange was similar to that of Troyer citrange (Table 4.3).

At the GOT-2 locus, the alleles F, S and M were involved. The heterozygous genotype, FS occured in rough lemon and the heterozygous genotype FM was found in the mandarins (Ellendale, Imperial and Cleopatra). The other heterozygous genotype was MS found in trifoliata orange cv. William's strain and Prior lemon. The rest of the genotypes were homozygous; Smooth Seville, Red blush grapefruit, Troyer and Carrizo citrange, sweet orange cv. White Siletta No. 1, Valencia and Washington navel orange.

4.1.2.2 Phosphoglucoisomerase (PGI)

The zymogram of PGI showed three zones of activity, but only one, situated in the central zone could be satisfactorily resolved. The PGI locus had three alleles, F, S and W. Two cultivars, Prior lemon and Smooth Seville had the WS genotype. Rough lemon, trifoliata orange cv. William' strain (Plate 4.4, 4.5), sweet orange cv. White Siletta No. 1 (Plate 4.6), Valencia and Washington navel orange had FS. The homozygous

genotype, FF was found in the mandarins (Ellendale, Imperial and Cleopatra). Red blush grapefruit, Troyer and Carrizo citranges had the homozygous genotype SS.

4.1.2.3 Isocitrate dehydrogenase (IDH)

IDH enzyme activity occured in two zones, and it appeared that more than one enzyme was involved, as was also found in cherimoya (Ellstrand and Lee, 1987) and in apple (Weeden and Lamb, 1985). The zone with the fastest mobility, however, was stained weakly and only the slower zone was used. The IDH enzyme of the maternal parents and neighbouring trees gave either one or three bands, indicating a dimeric form (Torres *et al.*, 1982). The enzyme was coded by four alleles, F, S, M and I. The heterozygous genotype, FM was only found in Troyer (Plate 4.7) and Carrizo citrange. Four cultivars tested had the heterozygous genotype, MI i e. rough lemon, Ellendale mandarin, Valencia and Washington navel orange, whereas only one cultivar (Prior lemon) showed the heterozygous genotype SI. Two homozygous genotypes, FF and II were recorded. FF was found only in trifoliata orange cv. William' strain, while II occured in Smooth Seville, Red blush grapefruit, Imperial and Cleopatra mandarin (Table 4.2).

4.1.2.4 Leucine aminopeptidase (LAP)

The zymogram of LAP demonstrated a single zone of activity, the enzyme consisting of either one or two doublet bands. A doublet band consists of two bands which lie very close to each other and are counted as a pair. A cultivar with one doublet band has been proposed to be homozygous and those with two heterozygous (Torres *et al.*, 1982). Two alleles were found, F and S. The heterozygous genotype FS was found in Smooth Seville, Red blush grapefruit, Troyer and Carrizo citrange, sweet orange , Valencia and Washington navel orange. The homozygous genotype FF was found in rough lemon, trifoliata orange, Ellendale, Imperial and Cleopatra mandarin, and Prior lemon (Table 4.2).

4.1.2.5 Malate dehydrogenase (MDH)

Three zones of activity were found for the MDH enzyme on the gel, but only one, situated in the middle, showed both band variation, and better resolution than the others. There were two types of banding pattern, similar to those reported by Torres *et al.* (1982), MDH-1 and MDH-2. Both loci have two alleles, F and S. There were three genotypes in the MDH-1 locus, FS found in rough lemon (Plate 4.2), Troyer and Carrizo citranges and Prior lemon, FF in Smooth Seville, the mandarins (Ellendale, Imperial and Cleopatra), Red blush grapefruit, sweet orange cv. White Siletta No. 1, Valencia and Washington navel orange. The other genotype was homozygous, SS which was only observed in trifoliata orange cv. William's strain.

At the MDH-2 locus, two genotypes were found, FS and FF. The FS genotype occured in trifoliata orange cv. William's strain, Troyer and Carrizo citranges, while FF occured in rough lemon, Smooth Seville, Ellendale, Imperial and Cleopatra mandarin, Red blush grapefruit, sweet orange cv. White Siletta No. 1, Valencia and Washington navel orange, and Prior lemon (Table 4.2).

4.1.2.6 Malic enzyme (ME)

The zymogram of ME showed activity in a single zone, and is thought to be encoded by a single gene (Torres *et al.*, 1982). This enzyme consisted of one or two bands, M and I indicating a monomeric enzyme. The MI genotype was found in rough lemon and Troyer and Carrizo citranges. The II genotype was found in Smooth Seville, Ellendale, Imperial and Cleopatra mandarins, Red blush grapefruit, Prior lemon, sweet orange cv. White Siletta No. 1, Valencia and Washington navel orange. A difference was found in the genotype of trifoliata orange cv. William's strain from that reported by Torres *et al.* (1982). In this study trifoliata orange cv. William's strain had the MM genotype (Plate 4.3) whereas Torres *et al.* (1982) found RF (R band migrated slower than S).

4.1.3 Troyer and Carrizo citranges.

The isozymic bands of all the enzymes tested, i.e. GOT-1, GOT-2, PGI, IDH, LAP, MDH-1, MDH-2 and ME gave similar results in Troyer and Carrizo citrange (Table 4.3).

Table 4.2 Isozyme genotypes of maternal parents and neighbouring trees of five polyembryonic citrus rootstocks

Rootstock	Neighbouring tree	Enzyme systems *							
		GOT-1	GOT-2	PGI	IDH	LAP	MDH-1	MDH-2	ME
Rough lemon		FS	FS	FS	MI	FF	FS	FF	MI
	Smooth Seville	SS	MM	WS	II	FS	FF	FF	II
	Ellendale mandarin	SS	FM	FF	MI	FF	FF	FF	II
	Red blush grapefruit	-	M	SS	II	FS	FF	FF	II
Trifoliata orange cv. William's strain		MP	MS	FS	FF	FF	SS	FS	MM
	Imperial mandarin	SS	FM	FF	II	FF	FF	FF	II
	Troyer citrange	PS	MM	SS	FM	FS	FS	FS	MI
Sweet orange cv. White Siletta No.1		SS	MM	FS	MI	FS	FF	FF	II
	Valencia orange	SS	MM	FS	MI	FS	FF	FF	II
Troyer citrange		PS	MM	SS	FM	FS	FS	FS	MI
	Trifoliata orange	MP	MS	FS	FF	FF	SS	FS	MM
	Washington navel orange	SS	MM	FS	MI	FS	FF	FF	II
Cleopatra mandarin		SS	FM	FF	II	FF	FF	FF	II
	Imperial mandarin	SS	FM	FF	II	FF	FF	FF	II
	Prior lemon	FS	MS	WS	SI	FF	FS	FF	II
	Red blush grapefruit	-	MM	SS	II	FS	FF	FF	II

*GOT : Glutamate oxaloacetate transaminase; IDH : Isocitrate dehydrogenase; PGI : Phosphoglucose isomerase; LAP : Leucine amino peptidase; MDH : Malate dehydrogenase; ME : Malic enzyme
- not detected

Table 4.3 The isozyme genotypes of Troyer and Carrizo citranges

Cultivar	Enzyme systems*							
	GOT-1	GOT-2	PGI	IDH	LAP	MDH-1	MDH-2	ME
Troyer citrange	PS	MM	SS	FM	FS	FS	FS	MI
Carrizo citrange	PS	MM	SS	FM	FS	FS	FS	MI

*GOT: Glucose oxaloacetate transaminase; PGI: Phosphoglucoisomerase;
IDH : Isocitrate dehydrogenase; LAP : Leucine amino peptidase;
MDH : Malate dehydrogenase; ME : Malic enzyme.

4.1.4 Detection of zygotic seedlings by isozyme techniques

The percentage of zygotic seedlings detected was low; 2.1 % in rough lemon, 2.6 % in trifoliata orange cv. William's strain , 0.8 % each in sweet orange and Troyer citrange and none in Cleopatra mandarin (Table 4.4). The majority of the seedlings were identical in genotype with the maternal parent and were presumed to be nucellar in origin. More zygotic seedlings may have been detected if more enzyme systems were used.

Six out of the eight loci tested were useful in discriminating zygotic from nucellar seedlings of the five polyembryonic citrus rootstocks while two others, LAP and MDH-2 showed no variation in genotype between these seedling types. The isozymes in the GOT-1, GOT-2 and MDH-1 systems gave different banding patterns for the three seedlings of rough lemon (Table 4.4). In Plate 4.1 and Plate 4.2 the variants of the bands from these seedlings are presented. The GOT-2, PGI and ME enzymes could be used to differentiate the three zygotic seedlings of trifoliata orange cv. William's strain (19 B, 25 and 48 A). The isozymic ME bands of the zygotic seedling 19 B are shown in Plate 4.3. Plate 4.4 and Plate 4.5 show the isozymic PGI bands of zygotic seedlings 25 and 48 A. The isozymes of the PGI system differed between the two seedling types of sweet orange cv. White Siletta No. 1 (Plate 4.6) and the single zygotic seedling of Troyer citrange could be differentiated from nucellar seedlings by the IDH enzyme (Plate 4. 7).

4.1.5 Parentage of zygotic seedlings

Seedlings 48 A and 80 A of rough lemon had the genotype SS at the GOT-1 locus. Crossing with the neighbouring trees, smooth Seville, Ellendale mandarin and Red blush grapefruit was unlikely, since no M and F alleles from these cultivars were present. It appeared, therefore, that these seedlings resulted from self-pollination of the maternal tree. Seedling 89 C could also have resulted from selfing, since neighbouring trees had no F allele of the GOT-1 enzyme. It was concluded, accordingly, that the three zygotic seedlings of rough lemon originated from self-pollination (Table 4.5).

One of the zygotic seedlings of trifoliata orange cv. William's strain (19 B) could have resulted from cross-pollination with the neighbouring trees, Imperial mandarin or Troyer citrange, because these trees possesed the M and I alleles at the ME locus. Self-pollination was unlikely since the maternal parent was homozygous MM at the ME locus. Seedlings 25 and 48 A may have resulted from self-pollination as cross-pollination by either Imperial mandarin or Troyer citrange was unlikely due to the presence of different alleles at the GOT-2 locus.

The single zygotic seedling of sweet orange (50 B) could have resulted from selfing or from crossing with Valencia orange, because both the maternal parent and Valencia orange have the F and S alleles at the PGI locus. Seedling 85 A of Troyer citrange could also have resulted from selfing or from crossing with Washington navel orange as both have the M allele of the IDH enzyme. Cross-pollination by trifoliata orange was unlikely since the M allele is not present in this cultivar.

Table 4.4 Isozyme genotypes of surviving seedlings of five polyembryonic citrus rootstocks.Genotypes differing from the maternal parent are underlined.

Rootstock	Presumed origin of seedlings	Enzyme system* GOT-1	GOT-2	PGI	IDH	LAP	MDH-1	MDH-2	ME	Percentage of zygotic seedlings detected
Rough lemon	Nucellar	FS**	FS	FS	MI	FF	FS	FF	MI	2.1
	Zygotic:									
	48 A	FS	SS	FS	MI	FF	FF	FF	MI	
	80 A	FF	SS	FS	MI	FF	SS	FF	MI	
	89 C	FF	FS	FS	MI	FF	SS	FF	MI	
Trifoliata orange cv. William' strain:										
	Nucellar	MP	MS	FS	FF	FF	SS	FS	MM	2.6
	Zygotic :									
	19 B	MP	MS	FS	FF	FF	SS	FS	MI	
	25	MP	SS	SS	FF	FF	SS	FS	MM	
	48 A	MP	SS	SS	FF	FF	SS	FS	MM	
Sweet orange cv. White Siletta No.1:										
	Nucellar	SS	MM	FS	MI	FS	FF	FF	II	0.8
	Zygotic:									
	50 B	SS	MM	SS	MI	FS	FF	FF	II	
Troyer citrange										
	Nucellar	PS	MM	SS	FM	FS	FS	FS	MI	0.8
	Zygotic :									
	85 A	PS	MM	SS	MM	-	-	-	MI	
Cleopatra mandarin										
	Nucellar	SS	FM	FF	II	FF	FF	FF	II	0.0

*GOT : Glutamate oxaloacetate transaminae; IDH : Isocitrate dehydrogenase; PGI : Phosphoglucoisomerase; LAP : Leucine amino peptidase; MDH : Malate dehydrogenase; ME : Malic enzyme.

- : not tested

** F : fast- migrating band
S : slow- migrating band
M: medium- migrating band
I : intermediate-migrating band
W: allele named for "Willowleaf" variety
P : allele named for *Poncirus* genus

Plate 4.1 Zymogram of GOT-1 and GOT-2 enzymes showing segregation amongst rough lemon seedlings

Origin (O) at the bottom, anode at the top.

Channels 1-10, 12-24 : nucellar seedlings, FS/GOT-1; FS/GOT-2.
Channel 11 : zygotic seedling (80 A) , FF/GOT-1; SS/GOT-2.

Note that a heterodimer of intermediate migration is produced by the heterozygotes.

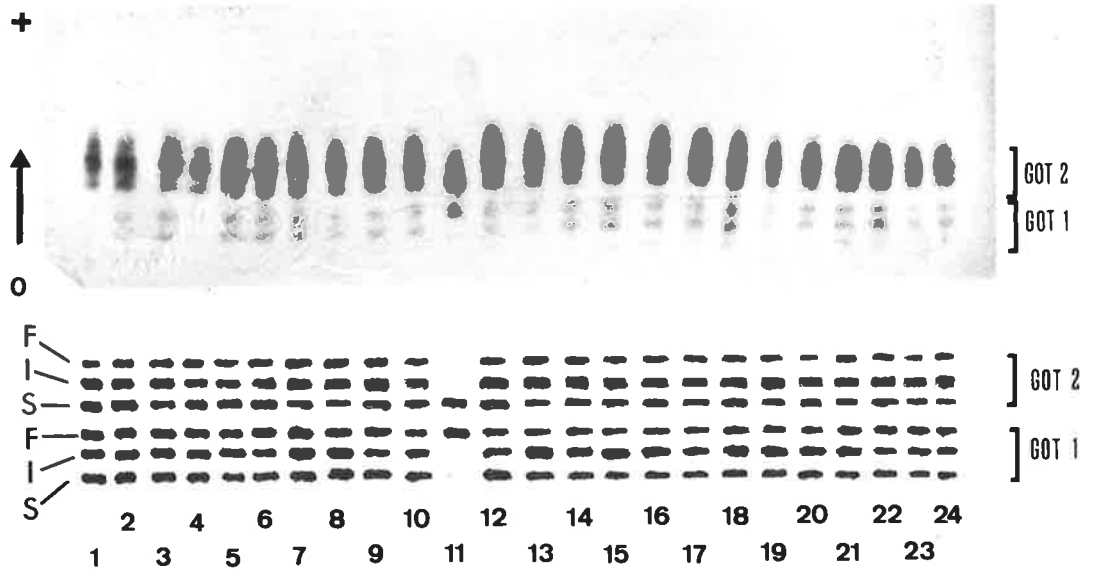
F: fast-migrating band, S: slow-migrating band, I: intermediate-migrating band.

Plate 4.2 Zymogram of MDH-1 enzyme showing segregation amongst rough lemon seedlings.

Origin (O) at the bottom, anode at the top.
Channels 1-13, 18-23, 26-30 : nucellar seedlings, FS
Channel 14 : maternal parent, FS
Channel 15 : Smooth Seville, FF
Channel 16 : Ellendale mandarin, FF
Channel 17 : Red blush grapefruit, FF
Channel 24 : zygotic seedling (80 A), SS
Channel 25 : zygotic seedling (89 C), SS

Note that a heterodimer of intermediate migration is produced by the heterozygotes.

F: fast-migrating band, S: slow-migrating band, I: intermediate migrating band.


+
0
GOT 2
GOT 1
F
I
S
F
I
S
GOT 2
GOT 1
2 4 6 8 10 12 14 16 18 20 22 24
1 3 5 7 9 11 13 15 17 19 21 23

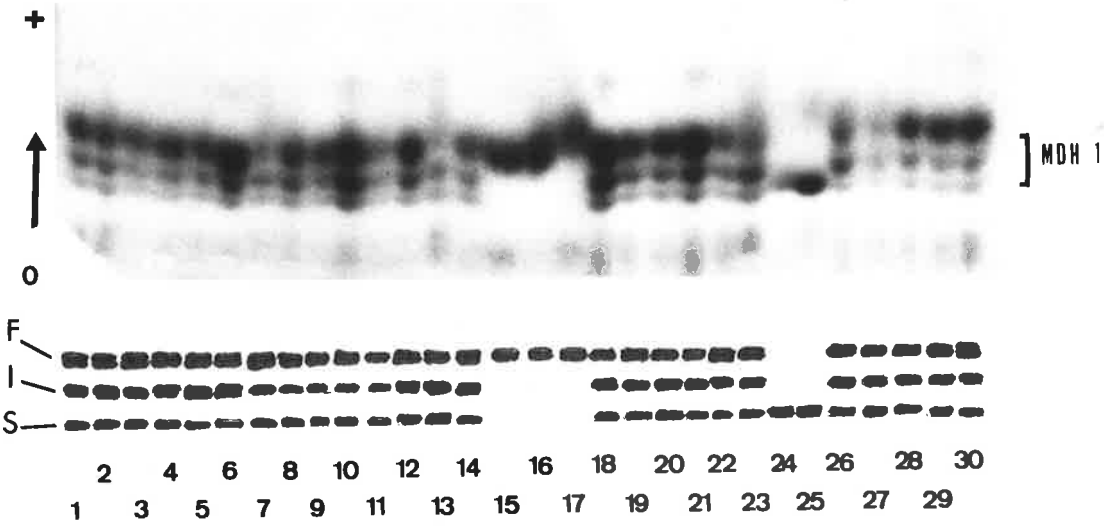

+
0
MDH 1
F
I
S
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29

Plate 4.3 Zymogram of ME enzyme showing genotype variation in trifoliata orange seedlings.
Origin (O) at the bottom, anode at the top.

Channels 1-7, 9-23 : nucellar seedlings , MM
Channel 8 : zygotic seedling, MI

I: intermediate-migrating band, M: medium-migrating band.

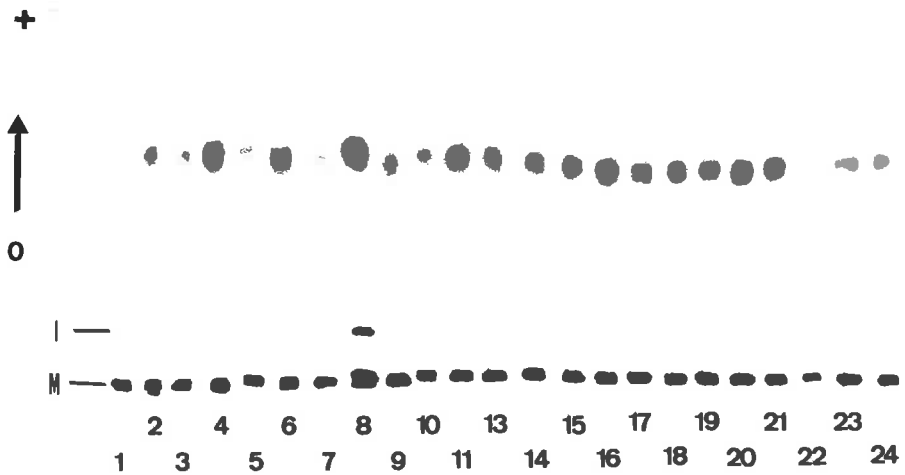
Plate 4.4 Zymogram of PGI enzyme showing variation in genotype amongst trifoliata orange seedlings.

Origin (O) at the bottom, anode at the top.

Channels 1-5, 7-13, 19-21 : nucellar seedlings of trifoliata orange, FS
Channel 6 : zygotic seedling (25) , SS
Channel 14 : maternal parent , FS
Channel 15 : Imperial mandarin, FF
Channel 16 : Troyer citrange, SS
Channel 17 : Carrizo citrange, SS
Channel 18 : Washington navel orange ,FS
Channel 22-25 : nucellar seedlings of Troyer citrange, SS

Note that a heterodimer band of intermediate migration is produced by the heterozygotes.

F: fast-migrating band, S: slow-migrating band, I: intermediate-migrating band.


+
0
I
M
2 4 6 8 10 13 15 17 19 21 23
1 3 5 7 9 11 14 16 18 20 22 24

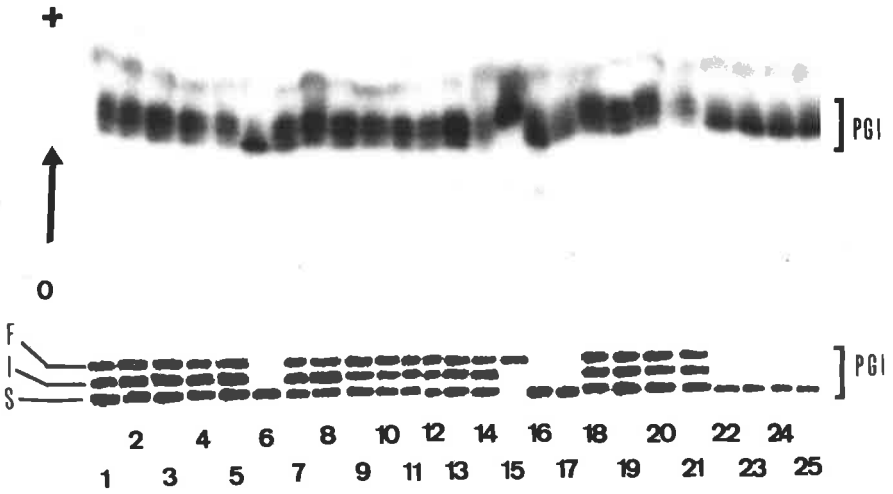

+
PGI
0
F
I
S
PGI
2 4 6 8 10 12 14 16 18 20 22 24
1 3 5 7 9 11 13 15 17 19 21 23 25

Plate 4.5 Zymogram of PGI enzyme showing genotype variation in trifoliata orange seedlings. Origin (O) at the bottom, anode at the top.

Channels 1-13, 18-20, 22-27 : nucellar seedlings, FS
Channel 14 : maternal parent, FS
Channel 15 : Imperial mandarin , FF
Channel 16 : Troyer citrange, SS
Channel 17 : Carrizo citrange, SS
Channel 21 : zygotic seedling (48 A), SS.

Note that a heterodimer band of intermediate migration is produced by the heterozygotes.

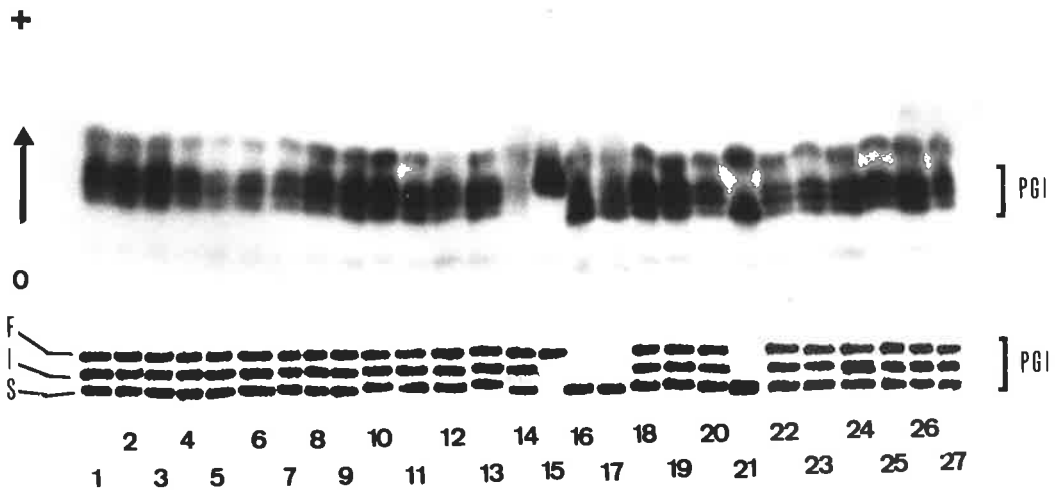
F: fast-migrating band, S: slow-migrating band, I: intermediate-migrating band.

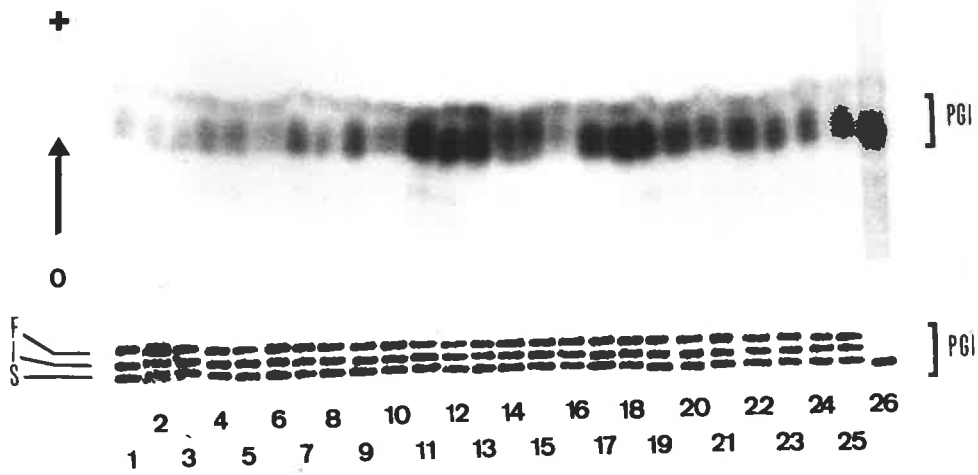
Plate 4.6 Zymogram of PGI enzyme showing genotype variation in sweet orange seedlings. Origin (O) at the bottom, anode at the top.

Channels 1-13, 16-25 : nucellar seedlings, FS
Channel 14 : maternal parent, FS
Channel 15 : Valencia orange, FS
Channel 26 : zygotic seedling (50 B) , SS

Note that a heterodimer band of intermediate migration is produced by the heterozygotes.

F: fast-migrating band, S: slow-migrating band, I: intermediate-migrating band.


+
0
F
I
S
PGI
PGI
2 4 6 8 10 12 14 16 18 20 22 24 26
1 3 5 7 9 11 13 15 17 19 21 23 25 27


+
0
F
I
S
PGI
PGI
2 4 6 8 10 12 14 16 18 20 22 24 26
1 3 5 7 9 11 13 15 17 19 21 23 25

4.1.6 Position of the zygotic seedlings within the seed

The zygotic seedlings identified by isozyme analysis were not always located at the micropylar end of the seed. Two zygotic seedlings of rough lemon (48 A and 80 A) were located at the micropylar end, but seedling 80 C was not. Seedling 48 A of trifoliata orange cv. William's strain was located at the micropylar end but seedling 19 B was not, and seedling 25 was from a monoembryonic seed. The one zygotic seedling of sweet orange cv. White Siletta No. 1 was not at the micropylar end (50 B), but the zygotic seedling of Troyer citrange (85 A) was. It can be concluded that there is little relationship between position in the seed and the occurence of zygotic seedlings.

4.1.7 Morphological characters of the zygotic seedlings

The morphological characters of the surviving seedlings varied little between zygotic and nucellar seedlings or within the zygotic group. All the nucellar seedlings in each rootstock group were similar to each other and to the maternal parent. The only zygotic seedlings which could be distinguished on morphology were those of sweet orange and Troyer citrange. The zygotic seedling of sweet orange differed from the maternal parent in lack of the winged petiole. The zygotic seedling of Troyer citrange had unifoliate leaves and no thorns, in contrast to the maternal parent which had trifoliate leaves and thorns. Therefore, morphological markers could not be used reliably to select zygotic seedlings (Table 4.5). The characteristics of the zygotic seedlings are given in Table 4.6. In Plate 4.8 the morphology of the zygotic seedling of Troyer citrange is presented and in Plate 4.9 the leaf morphology of other zygotic seedlings are shown.

The height of the zygotic seedlings of the four polyembryonic citrus rootstocks fell within the normal binomial height distribution of the nucellar seedlings (Figure 4.1). None of the zygotic seedlings fell in the smallest size of seedling class. One zygotic seedling of rough lemon was in class 25-50 mm, one in 50-75 mm and one in 100-125 mm. Two zygotic seedlings of trifoliata orange were vigorous, belonging to the classes

75-100 mm and 100-125 mm. Two zygotic seedlings of sweet orange and Troyer citrange fell in the middle range.

Table 4.5 Summary of characteristics of zygotic seedlings identified from populations of nucelllar seedlings of four polyembryonic citrus rootstocks.

Rootstock		Discriminated from nucellar seedlings on the basis of :		Presumed origin of zygotic seedling
		Isozyme genotype	Morphology	
Rough lemon :				
	48 A	yes	no	self-pollination
	80 A	yes	no	self-pollination
	89 C	yes	no	self-pollination
Trifoliata orange cv. William's strain :				
	19 B	yes	no with Troyer citrange	cross-pollination
	25	yes	no	self-pollination
	48 A	yes	no	self-pollination
Sweet orange cv. White Siletta No.1:				
	50 B	yes	yes	self-pollination or cross-pollination with Valencia orange
Troyer citrange:	85 A	yes	yes	self-pollination or cross-pollination with Washington navel orange

Table 4.6 Morphological characters of surviving seedlings of five polyembryonic citrus rootstocks

Rootstock	Presumed origin of seedlings	Leaf shape	Character Winged-petiole	Presence of thorns
Rough lemon	Nucellar	unifoliate	+	+
	Zygotic :			
	48 A	unifoliate	+	+
	80 A	unifoliate	+	+
	89 C	unifoliate	+	+
Trifoliata orange cv. William's strain				
	Nucellar	trifoliate	+	+
	Zygotic:			
	19 B	trifoliate	+	+
	25	trifoliate	+	+
	48 A	trifoliate	+	+
Sweet orange cv. White Siletta No.1				
	Nucellar	unifoliate	+	-
	Zygotic:			
	50 B	unifoliate	-	-
Troyer citrange	Nucellar	trifoliate	+	+
	Zygotic:			
	85 A	unifoliate	+	-
Cleopatra mandarin	Nucellar	unifoliate	-	-

+ character present, - character not present

Plate 4.7 Zymogram of IDH enzyme showing segregation amongst Troyer citrange seedlings. Origin (O) at the bottom, anode at the top.

Channels 1-17, 19-20: nucellar seedlings, FM
Channel 18 : zygotic sedling (85 A), MM

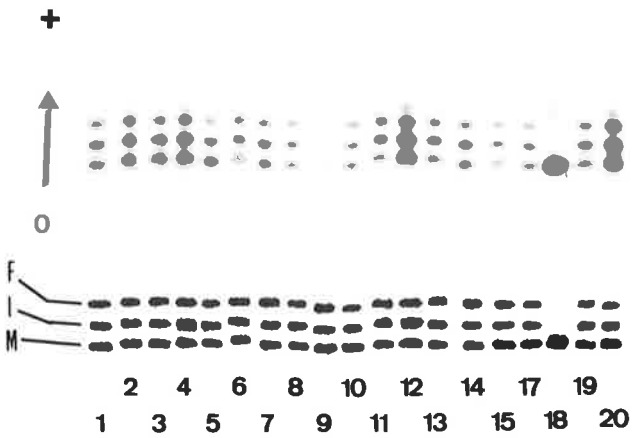
Note that a heterodimer band of intermediate migration is produced by the heterozygotes.

F: fast-migrating band, M: medium-migrating band, I: intermediate-migrating band.

Plate 4.8 Morphology of nucellar and zygotic seedlings of Troyer citrange.

A : nucellar seedling of Troyer citrange
B : zygotic seedling of Troyer citrange (85 A)
C : nucellar seedling of sweet orange

Note the unifoliate leaves of seedling B indicating that it arose from selfing or from crossing with Washington navel (sweet orange).


+
O
F
I
M
2 4 6 8 10 12 14 17 19
1 3 5 7 9 11 13 15 18 20


A
B
C

Plate 4.9 Leaf morphology of zygotic and nucellar seedlings of sweet orange, rough lemon and trifoliata orange rootstocks.

Sweet orange : A. leaf of zygotic seedling (50 B), lacking winged petiole
B. leaf of nucellar seedling (20 A), with winged petiole

Rough lemon : A, B, C : leaves of zygotic seedlings (80 A, 89 C and 48 A).
D : leaf of nucellar seedling (105 A)
Leaves of all seedlings have similar morphology

Trifoliate orange : A, B, C : zygotic seedlings (19 B, 25, and 48 A)
D : nucellar seedling (21 A).
All leaves have similar morphology.

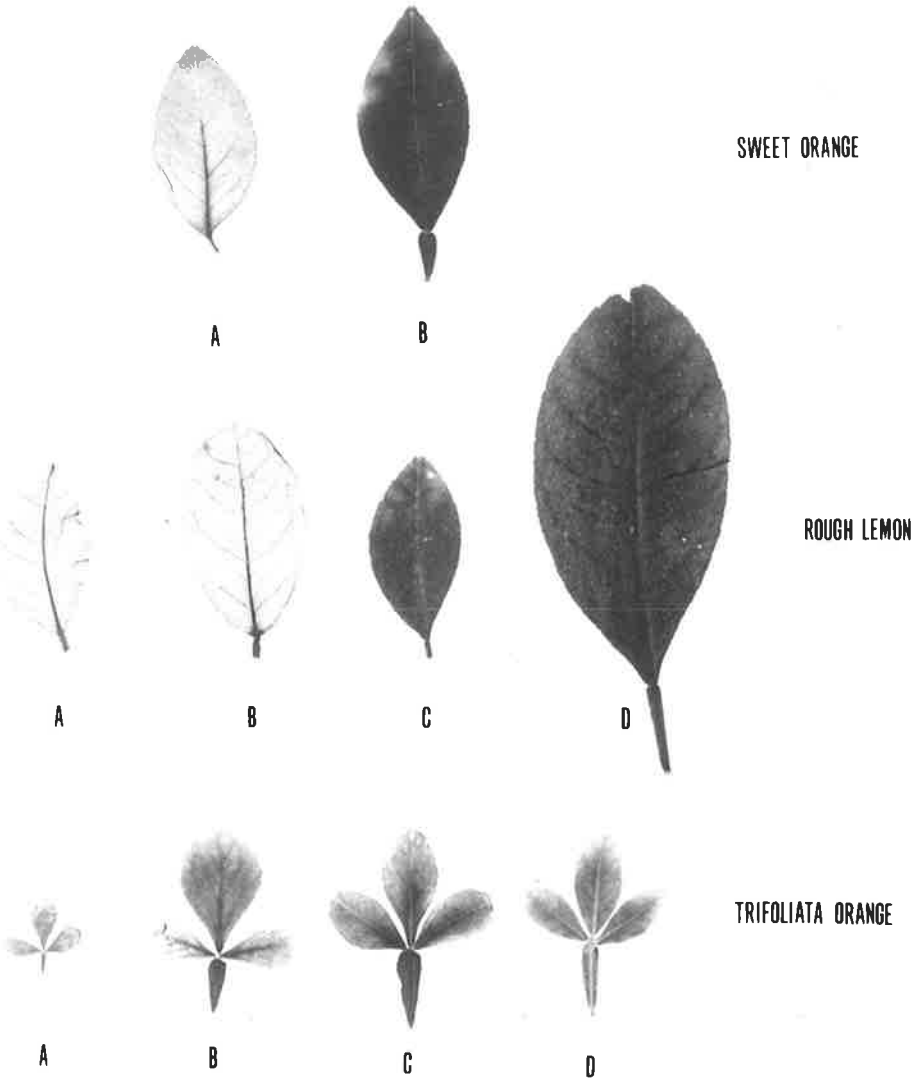
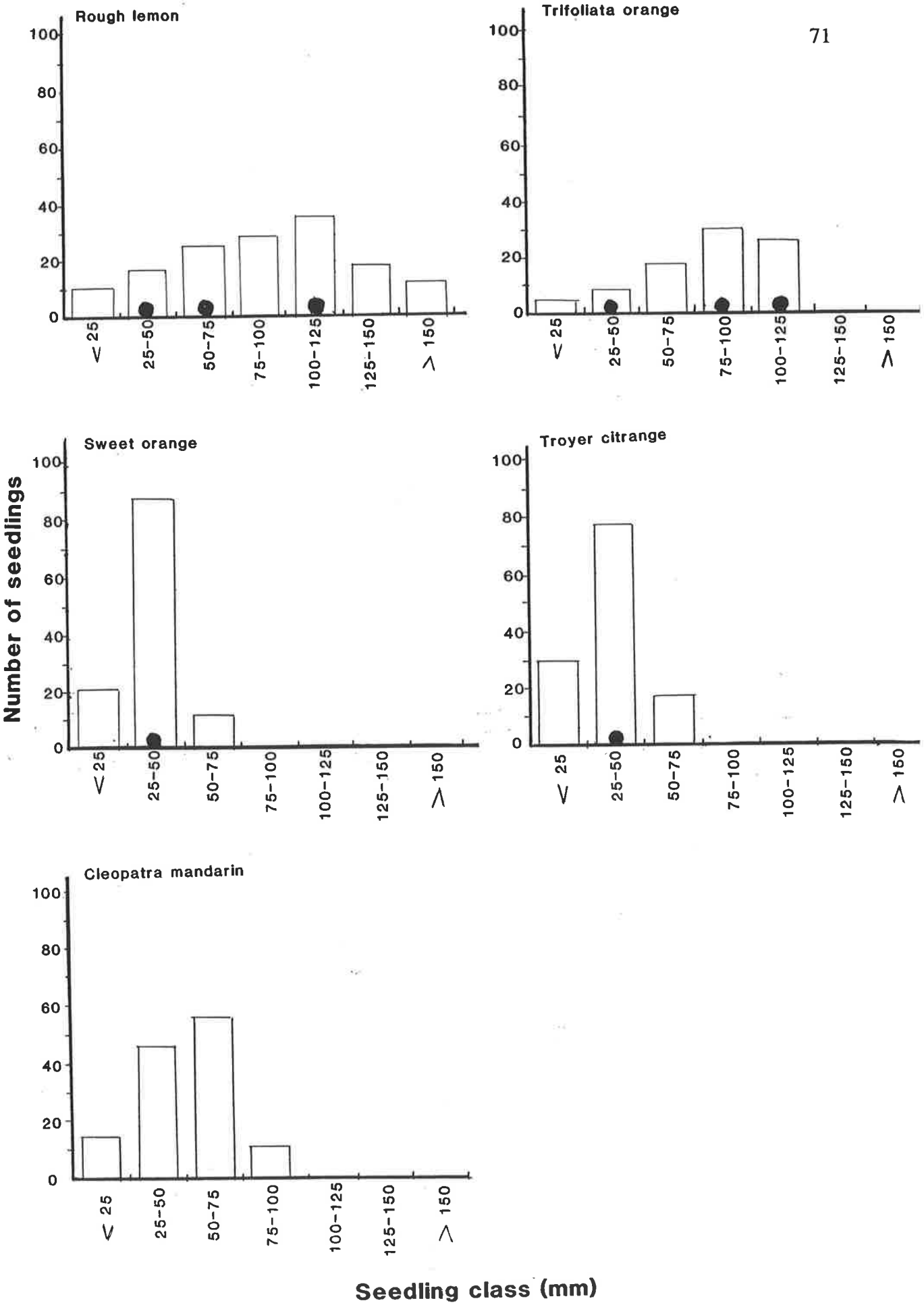

SWEET ORANGE
A
B
ROUGH LEMON
A
B
C
D
TRIFOLIATA ORANGE
A
B
C
D

Fig. 4.1 Distribution of plant heights of nucellar and zygotic seedlings of five polyembryonic citrus rootstocks at 20 weeks after germination. (▭) nucellar seedlings; (●) zygotic seedlings.


Rough lemon
Trifoliata orange
Sweet orange
Troyer citrange
Cleopatra mandarin
Number of seedlings
0
20
40
60
80
100
< 25
25-50
50-75
75-100
100-125
125-150
> 150
Seedling class (mm)

4.2 Identification of mandarin types, hydrids and selections

4.2.1 Isozyme genotypes of the mandarins

The isozyme banding patterns and number of loci found for enzymes GOT, PGI, IDH, LAP, MDH and ME in the mandarins are consistent with those described in section 4.1. In Plate 4.10 and 4.11 the isozyme profile of the mandarins for the IDH and MDH enzymes are presented. The genotypes of the mandarins based on these isozymes are listed in Table 4.7.

4.2.1.1 Phosphoglucomutase (PGM)

Only a single zone of activity was resolved on the gel of the PGM enzyme. This isozyme locus appeared to be controlled by two alleles, F and S and there was no heterodimer band in the hybrids. This indicates a monomeric enzyme. The FS genotype was found in Silverhill, Kara, Hickson, Seminole, Minneola, Orlando and sweet orange (Table 4.7), whereas the SS genotype occured in nine of the *C.reticulata* X *C. sinensis* group and in Thorny, Imperial, Beauty of Glen Retreat and Algerian.

4.2.1.2 Peroxidase (PER)

At least a single zone of activity occured in each of the PER+ (anodal migration) and PER- (cathodal migration) positions. The PER+ zone consisted of either one or two bands, indicating a monomeric structure with two isozymes controlled by F and S alleles. The homozygous genotype FF occured in thirteen cultivars (Silverhill, Thorny, Imperial, Kara, Beauty of Glen Retreat, Algerian, Ellendale 3, Burndale, Robinson, Herps, Seminole, Minneola and Orlando) and the heterozygous genotype FS in the remainder apart from Koster which was the only cultivar with the SS genotype (Table 4.7).

The PER- zone also consisted of one or two bands,with two isozymes coded by F and S alleles. Silverhill, Thorny, Imperial, Beauty of Glen Retreat, Algerian, Hickson, Ellendale 3, Burndale and Koster had the SS genotype, while the remainder possesed the FS genotype.

4.2.1.3 Superoxide dismutase (SOD)

The zymogram of SOD consisted of one or two bands. Two genotypes were observed, the homozygous SS and the heterozygous FS. Silverhill, Thorny, Imperial, Beauty of Glen Retreat, Algerian, Hickson, Murcott, Ellendale 1, 2, 3, Wallent, Burndale and Robinson were heterozygous, and Kara, Koster, Herps, Seminole, Minneola and sweet orange were homozygous.

4.2.1.4 Catalase (CAT)

At the CAT locus, the majority of the cultivars were homozygous FF, including the *C. reticulata* X *C. sinensis* group, Minneola and Orlando. Other cultivars had the FS genotype (Kara, Beauty of Glen Retreat, Algerian, and Seminole), or the SS genotype (Thorny and Imperial).

4.2.1.5 Shikimate dehydrogenase (SkHD)

There was variation at the SkHD locus: with isozyme combinations FM, FF, FS, MS and MM. Six cultivars had the FM genotype, Silverhill, Thorny, Kara, Beauty of Glen Retreat, Algerian and Murcott. Imperial was FF and Hickson was FS while eight cultivars were MS (Ellendale 1, 2, 3, Wallent, Koster, Robinson, Herps and sweet orange) and four cultivars were MM (Burndale, Seminole, Minneola and Orlando).

4.2.1.6 6-Phosphogluconate dehydrogenase (6-PGD)

The 6-PGD enzyme was dimeric as one or three bands were present. The MI genotype occured in Silverhill, Thorny, Kara, Beauty of Glen Retreat, Hickson, Murcott and Minneola; the FI genotype in Imperial and Algerian; the II genotype in the *C. reticulata* X *C. sinensis* group (except Hickson and Murcott) together with sweet orange and the MS genotype was observed in Seminole and Orlando.

4.2.1.7 Acid phosphatase (APH)

APH enzyme activity was found in a single region and appeared to be controlled by two alleles, F and S. The FS genotype occurred in Silverhill, Kara, Beauty of Glen Retreat , Algerian and three cultivars within the *C. reticulata* X *C. paradisi* group, and the FF genotype occured in Thorny, Imperial and all cultivars within the group of *C.reticulata* X *C. sinensis*.

4.2.1.8 Laccase (LAC) and Glutamate dehydrogenase (GDH)

The zymogram of LAC showed a single region of activity, and GDH showed two. However, there was no variation in the banding patterns associated with these loci, and all cultivars were designated as SS genotypes.

4.2.1.9 Fructose-1,6-diphosphatase (F1,6DP)

A single zone of activity was found on the gel of the F1,6DP enzyme. Three alleles, F, M and S were present at this locus. The heterozygous genotype MS was found in Silverhill, FM in Thorny, Murcott, Minneola ; FS in Imperial, Beauty of Glen Retreat, Hickson, Seminole and Orlando and the homozygous genotype MM in Kara, Ellendale 1, 2, 3 and sweet orange. Algerian, Wallent, Burndale, Koster, Robinson and Herps were not tested.

4.2.2 Discrimination between the mandarin genotypes

Of the sixteen enzyme systems tested three were encoded at two loci giving a total of nineteen loci. Seven of the loci were monomorphic, and showed no variability amongst the nineteen mandarin cultivars, hybrids and selections tested. All but three of the mandarin types could be discriminated using twelve polymorphic loci (Table 4.7). Only Ellendale 1, 2 and Wallent were identical at all isozyme loci, whereas three genotypes differed from each other at eleven out of the possible twelve loci (Table 4.8).

4.2.3 Genetic relationships between the mandarin types

The genetic relationships between and within the mandarin groups are varied. The greatest mean differences in isozyme banding patterns were observed between the tangelo and *C.deliciosa* groups and between the tangelo and the tangor groups both of which differed at nine loci (Table 4.9). The greatest variation in isozyme genotype within a group was found amongst the common mandarins (*C. reticulata*) and the least was found in the tangelos which only differed at two loci. In addition, differences at only three loci were found within the tangor group.

4.2.4 Determination of parentage of the mandarin types

Silverhill has been reported to be identical to Owari (Table 3.1) but demonstrated different alleles at the PGM locus, with FS in Silverhill and FF in Owari (Table 4.10). Similarly, Thorny differed from the reported identical cultivar Willowleaf at both the PGI and PGM loci. At the PGI locus the genotype of Thorny was FF and Willowleaf WF and at the PGM locus Thorny was SS and Willowleaf FI. Similarly, Imperial did not demonstrate the expected genetic combination of the reported parents, Willowleaf and Emperor at the PGM locus, Beauty of Glen Retreat did not have any

contribution of the proposed parent, Dancy at the PGM locus and the allele of the proposed parent, Willowleaf, at the PGM isozyme locus was not found in the Algerian tangerine. From the isozyme genotype of sweet orange (unknown cultivar) tested (Table 4.7), it is possible that the tangors (Hickson, Murcott, Ellendale 1, 2 and 3) could have arisen from a similar but not identical parent (*C. sinensis* X *C. reticulata*) (Table 4.7). Seminole, Minneola and Orlando possessed the expected hybrid bands from the combination of the parent genotypes of Duncan grapefruit and Dancy tangerine. Wallent, Burndale, Herps, Koster and Robinson showed genotypic segregation from Ellendale.

Table 4.7 Isozyme genotypes of the mandarin types and reported parents

Cultivar	Isozymes																		
	GOT-1	GOT-2	PG1	PGM	IDH	LAP	MDH	ME	PER+	PER-	SOD	CAT	SkHD	6PGD	APH	LAC	GDH-1	GDH-2	F1,6DP
"Silverhill"	SS	FM	FS	FS	II	FM	FF	II	FF	SS	FS	FF	FM	MI	FS	SS	SS	SS	MS
"Thorny"	SS	FM	FF	SS	II	FF	FF	II	FF	SS	FS	SS	FM	MI	FF	SS	SS	SS	FM
"Imperial"	SS	FM	FF	SS	II	FF	FF	II	FF	SS	FS	SS	FF	FI	FF	SS	SS	SS	FS
"Kara"	SS	FM	FF	FS	II	FF	FF	II	FF	FS	SS	FS	FM	MI	FS	SS	SS	SS	MM
"Beauty of Glen Retreat"	SS	FM	FS	SS	MI	FS	FF	II	FF	SS	FS	FS	FM	MI	FS	SS	SS	SS	FS
"Algerian"	SS	FM	FS	SS	MI	FS	FF	II	FF	SS	FS	FS	FM	FI	FS	SS	SS	SS	-
"Hickson"	SS	FM	FF	FS	II	FF	FF	II	FS	SS	FS	FF	FS	MI	FF	SS	SS	SS	FS
"Murcott"	SS	FM	FF	SS	II	FF	FF	II	FS	FS	FS	FF	FM	MI	FF	SS	SS	SS	FM
"Ellendale"-1	SS	FM	FF	SS	MI	FF	FF	II	FS	FS	FS	FF	MS	II	FF	SS	SS	SS	MM
"Ellendale"-2	SS	FM	FF	SS	MI	FF	FF	II	FS	FS	FS	FF	MS	II	FF	SS	SS	SS	MM
"Ellendale"-3	SS	FM	FF	SS	MI	FF	FF	II	FF	SS	FS	FF	MS	II	FF	SS	SS	SS	MM
"Wallent"	SS	FM	FF	SS	MI	FF	FF	II	FS	FS	FS	FF	MS	II	FF	SS	SS	SS	-
"Burndale"	SS	FM	FF	SS	II	FF	FF	II	FF	SS	FS	FF	MM	II	FF	SS	SS	SS	-
"Koster"	SS	FM	FF	SS	MI	FF	FF	II	SS	SS	SS	FF	MS	II	FF	SS	SS	SS	-
"Robinson"	SS	FM	FF	SS	MI	FF	FF	II	FF	FS	FS	FF	MS	II	FF	SS	SS	SS	-
"Herps"	SS	FM	FF	SS	MI	FF	FF	II	FF	FS	SS	FF	MS	II	FF	SS	SS	SS	-
"Seminole"	SS	FM	FS	FS	II	FS	FF	II	FF	FS	SS	FS	MM	MS	FS	SS	SS	SS	FS
"Minneola"	SS	FM	FS	FS	II	FS	FF	II	FF	FS	SS	FF	MM	MI	FS	SS	SS	SS	FM
"Orlando"	SS	FM	FS	FS	II	FS	FF	II	FF	FS	SS	FF	MM	MS	FS	SS	SS	SS	FS
Possible parents																			
Sweet orange, unknown cultivar	SS	MM	FS	FS	II	FS	FF	II	FS	FS	SS	FS	MS	II	FS	SS	SS	SS	MM
*"Owari	SS	FM	FS	FF															
*"King"	SS	FM	FF	FS															
*"Dancy"	SS	FM	FF	FF															
*"Willowleaf"	SS	FM	WF	FI															
*"Emperor"	SS	FM	FF	FF															
*"Duncan"	FS	MM	SS	SS															

-: not tested; *: From Torres *et al.* (1978)

Table 4.8 Genetic relationships between the mandarin types based on the number of loci at which the isozyme pattern differs

	*Sil	Tho	Imp	Kar	BGR	Alg	Hick	Mur	El-1	El-2	El-3	Wal	Bur	Kos	Rob	Her	Sem	Min	Orl
Sil	0	6	8	6	5	5	6	7	10	10	8	9	6	9	8	9	7	5	6
Tho		0	3	6	6	6	5	3	7	7	5	6	3	6	5	6	10	8	10
Imp			0	8	7	6	5	6	7	7	5	6	3	6	5	6	9	10	9
Kar				0	7	7	7	6	8	8	8	8	7	8	7	6	5	5	6
BGR					0	1	8	8	9	9	7	8	7	8	7	8	6	7	7
Alg						0	9	8	8	8	6	8	7	8	7	8	6	7	7
Hick							0	4	6	6	6	5	4	6	6	7	9	8	8
Mur								0	4	4	6	3	4	6	4	5	10	7	9
El-1									0	0	2	0	4	3	1	2	11	10	10
El-2										0	2	0	4	3	1	2	11	10	10
El-3											0	2	2	2	1	2	11	10	10
Wal												0	4	3	1	2	10	9	9
Bur													0	4	3	4	8	7	7
Kos														0	3	2	10	9	9
Rob															0	1	9	8	8
Her																0	8	8	7
Sem																	0	3	1
Min																		0	2
Orl																			0

*Sil: Silverhill; Tho: Thorny; Imp: Imperial; Kar: Kara; BGR: Beauty of Glen Retreat; Alg: Algerian; Hick: Hickson; Mur: Murcott; El-1: Ellendale-1; El-2: Ellendale-2; El-3: Ellendale-3; Wal: Wallent; Bur: Burndale; Kos: Koster; Rob: Robinson; Her: Herps; Sem: Seminole; Min: Minneola; Orl: Orlando

Table 4.9 Summary of the genetic relationships between the mandarin species groups

	C. unshiu	*C. deliciosa*	*C. reticulata*	*C. reticulata X C. sinensis*	*C. reticulata X C. paradisi*
C. unshiu	0.0±0.0	6.0±6.0	6.0±1.4	8.2±1.5	6.0±1.0
C. deliciosa		0.0±0.0	5.3±1.5	5.3±1.4	9.3±1.2
C. reticulata			6.0±2.5	7.1±1.3	7.0±1.6
C. reticulata X C. sinensis				3.2±1.9	9.0±1.2
C. reticulata X C. paradisi					2.0±1.0

* Mean ± standard deviation of number of locus difference recorded in Table 4.8 averaged for each species group.

Table 4.10 Assessment of the parentage of the mandarin types

Cultivar	Confirmation of parentage (in Table 3.1)	Reason for discrepancy
Satsuma mandarin:		
Silverhill	No	Differs from Owari at PGM locus
Mediterranean mandarin:		
Thorny	No	Differs from Willowleaf at PGI and PGM loci
Common mandarin:		
Imperial	No	Willowleaf and Emperor both lack S allele at PGM locus
Kara	Yes	
Beauty of Glen Retreat	No	Dancy lacks S allele at PGM locus
Algerian	No	Willowleaf lacks S allele at PGM locus
Tangors:		
Hickson	Possible	
Murcott	Possible	
Ellendale 1	Possible	
Ellendale 2	Possible	
Ellendale 3	Possible	
Wallent	Yes	
Burndale	Yes	
Koster	Possible	
Robinson	Possible	
Herps	Yes	
Tangelos:		
Seminole	Yes	
Minneola	Yes	
Orlando	Yes	

Plate 4.10 Zymogram of IDH enzyme showing variation in the genotype of the mandarins. Origin (O) at the bottom, anode at the top.

Channel 1 and Ch. 21: Kara, II; Ch. 2: Seminole, II; Ch. 3: Minneola, II;
Ch. 4: Orlando, II; Ch. 5: Imperial, II; Ch. 6: Thorny, II; Ch. 7: Silverhill, II;
Ch. 8: Hickson, II; Ch. 9: Murcott, II; Ch. 10: Ellendale 1, MI;
Ch. 11 and 13 : Burndale, II; Ch. 12: Ellendale 2, MI; Ch. 14: Wallent, MI;
Ch. 15: Koster, MI; Ch. 16: Robinson, MI; Ch. 17: Herps, MI;
Ch. 18: Beauty of Glen Retreat, MI; Ch.19: Algerian, MI; Ch. 20: sweet orange,II.

Note that a heterodimer band of intermediate is produced by the heterozygotes.

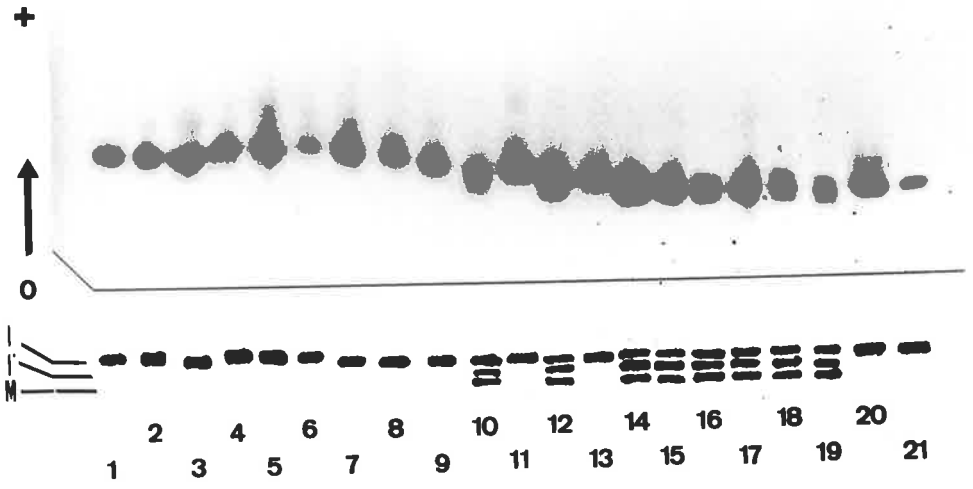
I: intermediate-migrating band, I' : intermediate-migrating band for mandarins, M:medium-migrating band.

Plate 4.11 Zymogram of MDH enzyme showing lack of variation for this enzyme in the genotype of the mandarin cultivars tested.

Origin (O) at the bottom, anode at the top.

Channel 1: Kara, FF; Ch. 2: Seminole, FF; Ch. 3: Minneola, FF; Ch. 4: Orlando, FF;
Ch. 5: Imperial, FF; Ch. 6: Thorny, FF; Ch. 7: Silverhill, FF: Ch. 8: Hickson, FF;
Ch. 9: Murcott, FF; Ch. 10: Ellendale 1, FF; Ch. 11: Ellendale 2, FF;
Ch. 12: Ellendale 3, FF; Ch. 14: Burndale, FF; Ch. 15: Koster, FF; Ch. 16:
Robinson, FF: Ch. 17: Herps,FF; Ch. 18: Beauty of Glen Retreat,FF; Ch. 19: Algerian,FF;
Ch. 20: Clementine, FF; Chs. 21: and 22: sweet orange, FF

F: fast-migrating band.


+
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I
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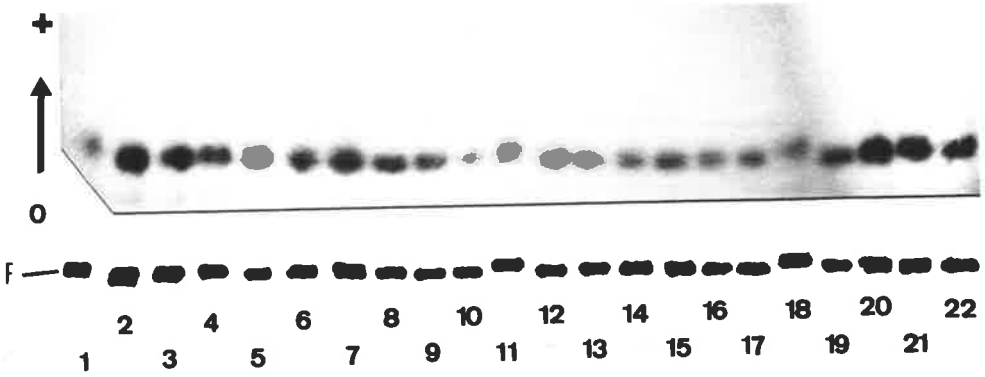
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Chapter 5. Discussion

5.1 Discrimination between zygotic and nucellar seedlings of citrus rootstocks resulting from open pollination

5.1.1 Isozyme identification of the zygotic and nucellar seedlings

The ability to produce nucellar embryos in the seeds of citrus rootstocks is genetically controlled (Parlevliet and Cameron, 1959; Iwamasa *et al.*, 1967), with the polyembryonic character being dominant over the monoembryonic. Cross pollination of a monoembryonic cultivar with polyembryonic varieties produced numerous of nucellar embryos in the progeny (Esen and Soost, 1977; Wakana and Uemoto, 1987). The production of a large number of nucellar embryos poses a problem for the development of the zygotic embryo, as the zygotic and nucellar embryos compete for space and nutrition during proliferation within the embryo sac (Cameron and Frost, 1968). Because the nucellar embryos are formed earlier, they often lead in the competition and depress the growth of the zygotic embryo, leading in the extreme case to loss of the zygotic embryo. This may explain the low number of zygotic seedlings detected in the present study. In the weakly polyembryonic cultivar "Yuma", where presumably inter-embryo competition is minimal, 36 % of the seedlings were identified as of zygotic origin, but in polyembryonic cultivars only a few zygotic seedlings were identified by isozyme analysis (Moore and Castle, 1988). This also supports the suggestion that competition between embryos during growth and development limits the survival of zygotic embryos. The effects of competition also can be seen in the distribution of seedling height (Figure 4.1). Seedlings varied in size even though they germinated at the same time in the same environment. The death rate of the seedlings after germination was also high (approximately 25 % for all but trifoliata orange) and this may also have contributed to the low presence of zygotic seedlings as some may have died before isozyme analysis was carried out. Moore and Castle (1988) reported a similar situation. Cultivation of all the embryos produced may be important in genetical studies. In this situation embryo culture techniques may be necessary.

and variable embryo size.

Alternatively, the low number of zygotic seedlings detected may have been due to the limited number of enzyme systems employed.

The only cultivar in which zygotic seedlings were not detected was Cleopatra mandarin. This cultivar was found to be homozygous at seven out of the eight isozyme loci tested, and consequently there was little chance that segregation or recombination of alleles would occur following self pollination. Roose and Traugh (1988) also found no zygotic seedlings of Cleopatra mandarin in their electrophoretic survey of rootstocks using an extract of bark material, but none of the loci they used were heterozygous for this cultivar.

The results of the experiment were similar to earlier reports (Torres *et al.*, 1978, 1982). One difference was found in the isozyme profile of Red blush grapefruit at the GOT-1 locus, and in that of trifoliata orange at the ME locus. Red blush grapefruit consistently showed no activity associated with the GOT-1 locus while Torres *et al.* (1978) reported a genotype of FS using similar material. It is possible that the GOT-1 enzyme was inactive in this study, so this cannot be taken to indicate a difference in genotype between the cultivars used in the two studies. The isozyme genotype of the trifoliata orange in this experiment was MM but Torres *et al.* (1978) reported RF, where R migrated slower than the S band and F migrated faster than S. The trifoliata cultivar used in this study was William's strain, but Torres *et al.* (1978) reported on seven trifoliata orange strains none of which were similar to William's strain with respect to isozyme pattern. This indicates that different cultivars were tested in the two investigations.

5.1.2 Origin of zygotic seedlings

The zygotic seedlings found in rough lemon resulted from self pollination (Table 4.5), as cross-pollination from neighbouring trees can be ruled out due to the different alleles which would have been present in the progeny. Seedlings 25 and 48 A of trifoliata orange also probably resulted from self-pollination for the same reason but seedling 19 B of trifoliata orange must have derived from cross pollination as it carried the MI genotype at the ME locus, in contrast to the maternal parent which had the MM genotype. The pollen donor presumably was a neighbouring tree, but of the two possibilities, Imperial mandarin or Troyer citrange, the former can be eliminated due to the presence of different alleles at other loci. The zygotic seedling found in the sweet orange progeny (50 B) could

have arisen either from selfing or from crossing with Valencia orange as the isozyme patterns do not discriminate between these two possibilities. Similarly, the genotype of seedling 85 A of Troyer citrange was MM at the IDH locus which could have arisen either following selfing or crossing with Washington navel orange. The leaf shape of this seedling was unifoliate (Plate 4. 8). The trifolate leaf character is dominant over unifoliate (Soost and Cameron, 1975), but Troyer citrange is heterozygous for this character, so again this character cannot be used to discriminate between a selfing or crossing origin for this seedling.

5.1.3 The morphology of zygotic seedlings

A further objective of the work reported here was to ascertain whether zygotic seedlings could be consistently identified by morphological characters as well as by isozyme patterns. If this is the case then roguing of off-type seedlings in the nursery would be very easy. However, only two out of eight seedlings identified as zygotic by isozyme analysis could also be detected on the basis of morphology (Table 4.5). In the sweet orange cultivar the zygotic seedling had no winged petiole, and in Troyer citrange the zygotic seedling had a unifoliate leaf and no thorns. A thornless variant of trifoliata orange resulting from spontaneous inbreeding in the normal thorny trifoliata orange was reported by Kawase and Hirai (1985) and its origin supported by isozyme analysis. It is concluded, however, that morphological traits are not a reliable method to discriminate between zygotic and nucellar seedlings in the seedling population in the nursery (Figure 5.1 a). By comparison Figure 5.1 b, shows the number of zygotic seedlings, detected by dominant trifoliate leaf shape following crossing with *P. trifoliata* for the eleven rootstock cultivars i.e rough lemon, Mexican, Kusaie and Red limes, *C. taiwanica* Tan. & Shimada, *C. macrophylla* Hook, *C. amblicarpa* Ochse, *C.ichangensis* Swing., two Yuzu cultivars and Ichang hybrid (Frost and Soost, 1968) . The number of zygotic seedlings in this report is very high (approximately 32 % of the total seedlings produced).

It has been suggested that seeds with a single embryo should be rogued out as they are likely to be zygotic, however this is not reliable. 20 % of rough lemon seeds

were monoembryonic, 50 % of the trifoliata orange, 34 % of sweet orange, 23 % of Troyer citrange and 53 % of Cleopatra mandarin. However, only one out of eight zygotic seedlings detected by isozyme analysis originated from a single embryo seed (seedling 25 of trifoliata orange), and the remainder came from polyembryonic seeds. The position of the seedling in relation to the micropyle is also not a reliable marker for zygotic seedling determination. Four out of eight detected zygotic seedlings were located in the micropylar region i.e seedlings 48 A and 80 A of rough lemon; seedling 48 A of trifoliata orange and seedling 85 A of Troyer citrange, while the rest were located elsewhere (seedling 89 C of rough lemon, 19 B of trifoliata orange, 50 B of sweet orange and seedling 25 of trifoliata orange which was the only seedling produced by the seed). Further, zygotic seedlings were not necessarily weak as has been claimed (Webber, 1948; Cameron and Frost, 1968). None of the eight detected zygotic seedlings fell in the smallest seedling size class, while seedling 25 of trifoliata orange was in the most vigorous class and the other detected seedling in the middle range of plant size. Khan and Roose (1988) in an isozyme survey of progeny resulting from open pollination of three cultivars of trifoliata orange, Pomeroy, Rubidoux and Flying Dragon, also found that variation in seedling vigour was not correlated with seedling origin (zygotic or nucellar), and that some zygotic seedlings grew more vigorously than nucellar seedlings. Therefore, it is concluded that selection on seedling size would not be useful.

Troyer citrange is a hybrid made by Savage and Swingle in 1909 (Savage and Gardner, 1965) from a cross between Washington navel orange and trifoliata orange. One of the progeny, CPB 45019, was named Troyer citrange. Further propagation of this cultivar was poorly documented, and a new name, Carrizo citrange, was given by Swingle in about 1938 (Savage and Gardner, 1965). This presupposes a distinction between Troyer and Carrizo, perhaps arising through zygotic seedling propagation, and both the Troyer and Carrizo citrange names are still in use. From the isozyme analysis of eight loci, these two cultivars appear to be similar, as also reported by Moore and Castle (1988) who employed seven isozyme loci. This accords with evidence on the physiological effects of these citrange cultivars. The growth and yields of Ellendale tangor grafted on Troyer or Carrizo

citrange rootstocks were not significantly different (Bevington and Duncan, 1978). Similarly, juice qualities of four mandarin types, Imperial, Emperor, Dancy and Ellendale were similar when grafted onto these citranges (El-Zeftawi and Thornton, 1978). Similar results from a rootstock trial were reported by Thornton and Dimsey (1987).

The plants were sprayed regularly to control pests and diseases which are particularly prevalent under glasshouse conditions. This was to ensure the survival of all the seedlings from the polyembryonic seed, including those which were small and weak. In commercial citrus propagation only the most vigorous embryo is allowed to grow, and such regular spraying is unnecessary. Isozymes are under genetic control and are not influenced by environmental variations such as pesticide application.

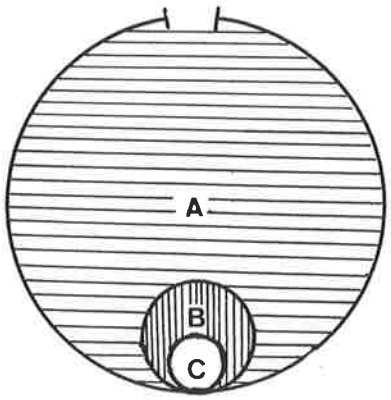

Figure 5.1a. Accuracy of isozyme analysis compared with morphological methods in the discrimination of zygotic from nucellar seedlings of five polyembryonic citrus rootstocks resulting from open pollination

A: Total population of seedlings tested: 614

B: Zygotic seedlings detected by isozyme analysis: 8

C: Zygotic seedlings detected by morphological characters: 2

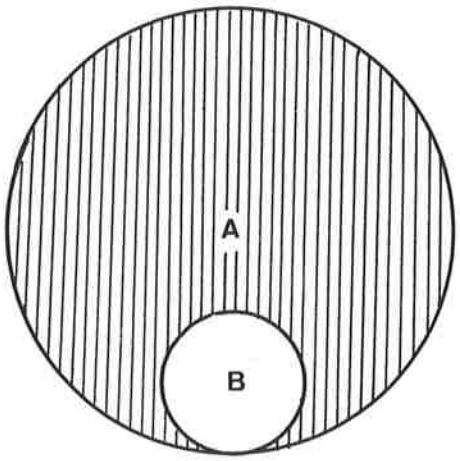

Figure 5.1b. Zygotic seedlings detected by controlled crossing to a parent with a morphological marker e.g.trifoliate leaf.

A: Total seedling population: 2176

B: Total zygotic seedlings: 696

5.1.4 Screening of zygotic seedlings in the nursery and following controlled pollination in a breeding programme.

Routine isozyme testing of seedlings in the production of nucellar seedling rootstocks in the nursery situation should be considered. The first criterion in the selection of enzymes to test is that the maternal parent is heterozygous. Based on the results in section 5.1, the GOT, IDH, PGI, MDH-1 and ME enzymes are suggested for the rough lemon, trifoliata orange, sweet orange and Troyer citrange rootstocks. However, more heterozygous loci are needed, for use with Cleopatra mandarin.

In a breeding programme where cross-pollination is controlled carefully, the number of isozyme loci needed is different from the number required in open-pollination. If both parents have homozygous but different alleles at a single locus then all progeny will be heterozygous giving a one hundred percent of chance of discrimination. This case is very rare, however. When the genotype of the seed parent is homozygous and the male parent is heterozygous at the same locus, then 50 % of the progeny would be heterozygous and could be discriminated from the nucellar seedlings, but the remainder would be indistinguishable. By further analysis using additional heterozygous locus, 50 % of the zygotic seedlings undetected at the first locus could be detected by the second locus, giving a 75 % chance that zygotic seedlings will be discriminated from the nucellar seedlings. In a further case, when both parents have similar heterozygous alleles at one locus then again 50 % of the zygotic seedlings would not be discriminated from the nucellar. If a further heterozygous locus was assessed then 25 % of the zygotic seedlings would still be indistinguishable from nucellar seedlings. In Table 5.1 a calculation of the chance of determining zygotic seedlings from nucellar is presented. Based on this calculation it is apparent that when both parents are homozygous for different allele at one locus, this one locus is sufficient for identification of zygotic seedlings. Where when one parent is homozygous (FF) and the other heterozygous (FS), six heterozygous loci would be reliable, because it will give a high chance (98.4 %) of identifying the zygotic progenies. It is apparent that a detailed knowledge of the isozyme profiles of both parents prior to embarking upon a crossing programme is essential to

identify suitable enzyme systems and to minimize the work needed to identify zygotic seedlings.

Table 5.1 Probability of discriminating zygotic from nucellar seedlings following controlled pollination.

Number of heterozygous loci	(♀) SS X (♂) FF Nucellar : SS Zygotic : FS		(♀) FF X (♂) FS Nucellar : FF Zygotic : FF or FS		(♀) FS X (♂) FS Nucellar : FS Zygotic : FF, SS or FS	
	Chance of zygotic seedling (%)					
	Disc.*	Undisc.	Disc.	Undisc.	Disc.	Undisc.
1	100	0	50	50	50	50
2			75	25	75	25
3			87.5	12.5	87.5	12.5
4			93.7	6.2	93.7	6.2
5			96.8	3.1	96.8	3.1
6			98.4	1.5	98.4	1.5

*Disc.: Discriminated; Undisc. : Undiscriminated

5.1.5 Survey of variant rootstocks and scions in the citrus orchard.

Sometimes, variation in the performance of the scion occurs (in a citrus tree population). If the variant has superior characteristics then it may be desirable to identify and perpetuate it. Variation may be in flowering time, cropping, quality of the fruit, or vigour. If this is the case, it may be caused by different genotypes of the rootstock being used, as it is well known that the rootstock may affect the performance of the scion in many ways. Even though some aberrant rootstocks may have been visually rogued from the nursery at the time of grafting, this does not guarantee that all zygotic seedlings have been totally removed. The situation of screening rootstcoks of zygotic origin in the orchard is similar to that in the nursery. Roose and Traugh (1988) investigated rootstock variants in a citrus orchard with between 9 and 19 year old rootstocks by isozyme analysis. They used eight isozyme loci, PGI, IDH, PGM-1, PGM-2, GOT-1, GOT-2, MDH-1 and MDH-2, and found several zygotic seedlings of 24 rootstock cultivars. Based on the investigation of zygotic seedlings reported in section 5.1, it is suggested that at least four loci should be used to identify zygotic rootstocks in the citrus orchard.

The number of isozyme loci required to screen superior bud sports in orchard trees would be much greater than that needed for zygotic and nucellar seedling determination. Spontaneous mutations are of frequent occurence in citrus, and valuable mutations have been found recently, especially with Satsuma mandarin in Japan and Shamouti orange in Israel (Vardi and Spiegel-Roy, 1978). Because scion cultivars are propagated clonally and their possible mutants will be closely related. it is suggested that application of a wide range of isozyme loci will be needed to positively identify a bud sport isozymically. In this situation, more than twelve heterozygous loci is preferable based on experience with the Ellendale bud sports in this study.

5.2 Identification of mandarin cultivars, hybrids and selections

5.2.1 Isozyme genotype of the mandarins

Innumerable enzymes are involved in plant metabolism, and are distributed throughout the plant. Of these enzymes, as many 57 enzymes have been used for detection of plant genotypes (Vallejos, 1983). There are two types of isozyme pattern coded by genes, monomorphic and polymorphic. In monomorphic enzymes, variation in the individual genotype does not occur, but in the polymorphic enzyme small variations in the proteins occur although function is unaffected. The more polymorphic enzymes that are used in determination of a plant cultivar, the more the chance of discriminating between cultivars. A cultivar which cannot be discriminated at one enzyme locus may be discriminated at other polymorphic loci. In the mandarins, several attempts have been made to discriminate between the member cultivars of this group by isozyme analysis. However, most reports have come to the conclusion that genetic variation within the group is low, as most of the cultivars tested were not distinguishable one from another (Torres *et al.*, 1978; Hirai *et al.*, 1986; Hirai and Kajiura, 1987). The limited number of enzymes used by these workers was the most probable cause of this conclusion. Torres *et al.* (1978) used four loci, Hirai *et al.* (1986) five and Hirai and Kajiura (1987) only three. In contrast, in apple, Weeden and Lamb (1985) utilized six enzymes coded by nine loci and were able to discriminate between individual cultivars which had arisen from intercultivar crossing. However, no intracultivar variation in isozyme phenotype was observed. It is possible that by application of an even wider range of isozyme loci, intracultivar variation could be detected.

In this work, mandarin cultivars originating as hybrids or selections were investigated for their genotype background by isozyme analysis. Within a group, variation would be expected to be smaller than between groups or species. Some of the material tested has been reported to have originated from bud variants or sports. As such variants

may be expected to differ only slightly in genotype, as many as sixteen enzymes, coded by nineteen loci were investigated to maximize the possibility of detecting differences.

Of the 19 mandarin types tested, most could be distinguished isozymically. Three cultivars, Ellendale 1, 2 and Wallent were not discriminated one from another, but it is likely that Ellendale 1 and 2 are identical, possibly being propagated clonally from the same parent tree; and Wallent is reported to be a seedling selection of Ellendale. Thus, isozyme analysis offers a powerful technique for the identification of mandarin types, even though some of them originated from seedling selections.

5.2.2 Genetic relationships between the mandarin types

The differences in genotype based on isozyme patterns between individual mandarin cultivars varied from 1-11 loci. This indicated that some of them were very closely related while others were widely separated. Within the collection, the greatest relatedness was found within the tangor and tangelo groups. This relatedness is to be expected, since Seminole, Minneola and Orlando originated from similar crosses between Duncan grapefruit and Dancy tangerine in a recent breeding programme in the USA, and were named and released as recently as 1931 (Hodgson, 1967). The tangor group, although mainly consisting of natural hybrids, is also a group of relatively recent derivation. With the exception of Murcott, all have arisen within Australia during the last 120 years. Within this group (tangor) Hickson and Murcott show relatively high deviation from the other members of the group. Hickson originated in Queensland, Australia and Murcott in the USA. It is possible that the ancestors of these tangor cultivars are not closely related to those of the other tangors. There appeared to be little relatedness within the common mandarin group (except for Beauty of Glen Retreat which only differed from Algerian tangerine in one locus). The tangelo group tended to diverge from the other groups at more loci, probably reflecting the grapefruit parentage. It is interesting to note that only where cultivar parentage was reported from breeding programmes, could the parentage be confirmed from isozyme analysis. None of the speculated parentage of older cultivars which has been based on morphological characters could be confirmed. Apparently, it is hazardous to speculate on

parentage from morphological characters alone. The exception to this case was the tangor group where the isozymic patterns at most loci tested was consistent with the speculated parentage of sweet orange and mandarin. The Ellendale types in particular are considered to form a closely related group. The three experimental Ellendale 1, 2 and 3 are all budded trees marketed as Ellendale mandarin (Tolley, personal communication, 1988). In fact, Ellendale 3 differs at two loci from Ellendale 1 and 2. There are some possible reasons to explain this condition, firstly the Ellendale cultivar probably has multiple origins, secondly, mutation has occured over the last 120 years in cultivation, thirdly, mislabelling of the cultivar may have occured. The most reasonable explanation however, is that Ellendale 3 has arisen from a zygotic seedling of Ellendale, since its isozyme profile showed segregation from Ellendale 1 or Ellendale 2 at two loci, PER+ and PER- (Table 4.7). Burndale and Wallent are reported to be seedling selections of Ellendale, and results of the isozyme analysis support this view. Koster and Robinson have been reported to be bud selections (sports) of Ellendale, and their isozyme genotypes are consistent with them having arisen by mutation. Similarly, Herps is reported to be either a bud or seedling selection of Ellendale, and again appears to be the result of mutation or self-pollination. Citrus cultivars are particularly prone to mutation (Soost and Cameron, 1975).

5.3 General discussion

5.3.1 Further application of isozyme analysis

In sections 5.1 and 5.2 the use of isozymes in cultivar identification, zygotic and nucellar seedling differentiation, elucidation of the pollen source for zygotic seedling formation, determination of the relatedness of cultivars within the mandarin group and elucidation of the parentage of mandarin types has been described. There is still a range of other applications of isozyme analysis in the field of horticulture including taxonomic research in the genus *Citrus* and plant breeding.

5.3.1.1 Classification of the genus *Citrus*

Agreement between citrus taxonomists regarding the number of species in this genus has not been achieved. Species have been distinguished primarily on morphological characters alone. As it is known that environment and physiology affect the morphology, this is an insecure criterion. The genus *Citrus* has been divided by different authorities into from 3 to 159 species. The main reason for this lack of agreement is that citrus has been cultivated for a long time in different countries around the world without a precise knowledge of its centre of origin. In addition, hybridization can occur easily between the species and some species produce nucellar embryos with identical genetic constitution to the maternal parent. This last characteristic can lead to the indefinite propagation of heterozygous genotypes which can confuse the classification of the genus.

Isozyme analysis, on the other hand, offers a promising method to assist classification. Isozymes are colinear with the genes, codominant, little affected by environment and identical in leaves of young and mature plants (Soost *et al.*, 1980) and can be used to differentiate between taxa. The application of isozymes in relation to citrus taxonomy has been reported. Esen and Soost (1976) in a study of peroxidase isozymes claimed that some cultivars which were proposed to be true species were disproved by this method, but that *C. medica* and *C. grandis* had a unique peroxidase isozyme. They also reported variation in the isozyme patterns of different taxa. Another isozyme analysis of amylase reported by Esen and Scora (1977) confirmed the distinctive genotype of *C.medica* , and further proposed that *C. paradisi* was a hybrid between*C.grandis* and *C.sinensis*. Moreover, Torres *et al.* (1978) employed four enzyme loci and found unique alleles in some citrus species including*C. media, C. grandis* and *C. micrantha.* Torres *et al.* (1978) also reported that some isozyme loci were characteristic for certain species or taxa, *P. trifoliata* had a specific P isozyme at the PGM and GOT-2 loci, and Willowleaf had a specific W isozyme at the PGI-1 locus. These unique alleles were not found in other species. Hirai *et al.* (1986) reported that some pummelo and mandarin cultivars which were proposed to be true species (Tanaka, 1969) were shown to be hybrids by isozyme analysis.

In addition, they hypothesize that pummelo and mandarin form the basis of the genetic resources of the citrus industry in Japan.

Among citrus biotypes, the mandarin group has the greatest genetic variability, possibly reflecting the different centres of origin of the species. Hirai and Kajiura (1987) proposed the hypothesis that the genetic resources of mandarins in Japan are contributed from mainland China, India and Japan. This was based on an isozyme survey of the superoxide dismutase (SOD), polyphenol oxidase (PPO) and glucose oxaloacetate transaminase (GOT) enzymes.

The use of 19 isozyme systems in the current study gave much greater discrimination of mandarin cultivars than reported by previous authors using fewer systems (Torres *et al.*, 1978; Hirai *et al.*, 1986; Hirai and Kajiura, 1987). Although small numbers of cultivars were involved in this study, wide genetic variability was found. Within the common mandarin group (*C. reticulata*) the cultivars varied at between 1 and 8 loci. The fact that the genetic variation within this group is as great as the variation between it and other groups suggests that the currently-accepted classification of mandarins may be open to question. However, further investigation is still necessary since the number of cultivars used in this work is limited. The situation could be further clarified, and the classification of *Citrus* assisted by the testing of a wider range of cultivars using more enzyme systems.

5.3.1.2 Breeding of citrus cultivars

Citrus and its relatives are heterozygous and display wide variation. Hybridization and mutation are common occurences and create more heterogeneity within the genus. This genetic diversity is important in citrus improvement programmes as raw material for plant breeding. The genetic identification of cultivars, therefore, is very important. Some investigations to identify cultivars have been attempted. Many could be distinguished isozymically, but cultivars derived from bud or nucellar seedling mutations were not (Ueno, 1976). This study has shown that the application of more heterozygous loci increases the probability of identifying cultivar types. This will have important implications

in the future use of isozyme analysis for cultivar identification in plant varietal rights (Bailey, 1983).

Isozymes appear to have limited value in inheritance studies of citrus progeny resulting from controlled crossing. The segregants of the F-1 generation often produce unexpected genetical ratios. This is partly due to the death of the embryos or seedlings during growth and development in the polyembryonic citrus cultivars. For this reason Torres *et al.* (1985) concluded that distortion segregation is common in citrus.

Breeding of citrus scion and rootstock cultivars via conventional breeding and selection techniques is hampered by the long generation cycle. Apomixis, heterozygosity and incompatibility add further difficulties in controlled breeding, with the result that progress in citrus breeding and selection is slow (Grosser and Chandler, 1987). Somatic hybridization has been reported to successfully bypass the barriers to sexual hybridization. Grosser *et al.* (1988) reported that somatic fusion of different genera which display sexual incompatibility have been obtained. The regenerated plants showed isozyme banding patterns contributed by both parents. Weeden and Gottlieb (1979) reported that the isozyme profile of the haploid cell (pollen) and diploid cell (somatic) of apple were different. This indicates that isozyme analysis can be used in ploidy studies. Determination of triploid cultivars in citrus is important as triploid cultivars produce little or no seed and are also being considered as dwarfing rootstocks due to their slow growth rate. However, the ploidy level in Willowleaf mandarin was undetected by four isozyme loci (Torres *et al.*, 1978).

Isozyme analysis has also been applied in the gene mapping of several annual crops such as tomato, maize and wheat (Tanksley, 1983). In citrus, the linkage of genes has been reported by Torres *et al.* (1985). Isozyme analysis offers a rapid method for linkage studies which would otherwise take many years to develop due to the long generation time. The long-term application of gene mapping lies in the possible location and transfer of desirable genes by genetic engineering techniques.

5.4 Conclusions

1. Zygotic seedlings could be differentiated isozymically from nucellar seedlings in the four polyembryonic citrus rootstock cultivars including rough lemon, trifoliata orange, sweet orange and Troyer citrange resulting from open pollination. No zygotic seedlings were detected in Cleopatra mandarin due to the lack of heterozygous isozymes exhibited by the maternal parent. The frequency of the detected zygotic seedlings in this work was low, probably due to the limited number enzyme systems used.

2. There was no apparent correlation between morphological traits and the origin of the seedlings. Some of the zygotic seedlings were the only seedling produced by the seed, but others were one of multiple seedlings. Some zygotic seedlings were located at the micropylar end of the seed, but others were not. Weak seedlings were not always zygotic, and some zygotic seedlings were vigorous. In addition, most of the zygotic seedlings exhibited the same morphology as the nucellar seedlings. Isozyme analysis detected more zygotic seedlings than morphological characters.

3. The speculated parentage of some mandarin cultivars was disproved isozymically but was confirmed for those which originated from a breeding programme. Therefore it is hazardous to speculate on the parentage of cultivars based solely on morphological traits.

4. The greatest genetic variability within the mandarin group was found amongst the common mandarins and the least was found in the tangelos. The genetic differences between the tangelos and *C. deliciosa* groups and between the tangelo and the tangor groups were the greatest.

5. Isozyme analysis offers great value in some horticultural applications such as classification in the genus *Citrus*, breeding and selection programmes, ploidy and linkage studies and gene mapping.

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Glossary of terms

Allele	: the form of the gene present at a locus.
Apomixis	: the production of seeds with the absence of fertilization.
Band	: results from the precipitation of coloured product by an enzyme in the gel. The bands result from differences in mobility of the enzymes towards the anode or the cathode.
Dimer	: enzyme form consisting of two polypeptide chains.
Genotype	: the genetic composition of the individual.
Heteromer	: enzyme form consisting of two or more polypeptide chains where the amino acid sequence within the polypeptide chains are different.
Heterozygote	: an organism with chromosome pairs carrying dissimilar genes.
Homozygote	: an organism with chromosome pairs carrying identical genes.
Isozyme	: multiple molecular form of an enzyme which can be separated by electrophoresis and detected by specific staining technique.
Locus	: the position of the gene on a chromosome.
Monoembryonic	: one seed contains a single embryo.
Monomer	: enzyme form which consists of only one polypeptide chain.
Nucellar embryo	: embryo derived from nucellus tissue in the seed.
Polyembryonic	: one seed contains more than one embryo.
Tetramer	: enzyme form which consists of four polypeptide chains.
Zymogram	: an isozyme banding pattern in an electrophoretic gel.