ABSTRACT

Seedlessness is a desirable horticultural attribute in *Citrus* and is positively associated with triploidy. The conventional cytological method for triploid identification is a laborious technique as it involves the preparation of root-tips for chromosomal analysis. Isozymes and digital densitometry, however, offer the facility to distinguish triploid *Citrus* from large populations of seedlings both quickly and cheaply. Where there are no gene-dosage regulation effects, greater band density, reflecting increased enzyme activity, should be evident in the allozyme contributed by the diploid gamete for a heterozygous locus.

To achieve this, appropriate methods of sample preparation, isozyme electrophoresis and digital densitometry were established. The isozymes of four enzymes, malate dehydrogenase, 6-phosphogluconate dehydrogenase, shikimate dehydrogenase, and phosphoglucose isomerase were investigated for band density differences between allozymes. Polyacrylamide gel electrophoresis was employed to study the isozymes of these four enzymes and band density was measured using a digital densitometer.

Of the 4 enzymes investigated only allozymes for shikimate dehydrogenase exhibited consistent differences over a wide range of *Citrus* cultivars. Greater band density was evident in the allozyme contributed by the diploid gamete. The band density ratio between allozymes for triploid *Citrus* was close to 0.5, while for diploid *Citrus* band density ratios were close to 1.0. This effect is due to the extra protein coded by the additional gene dose and was not observed in diploids. Shikimate dehydrogenase proved to be an accurate molecular marker for distinguishing between diploid and triploid *Citrus*.

MOLECULAR TECHNIQUES FOR THE IDENTIFICATION OF TRIPLOID CITRUS

by

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A thesis submitted in fulfilment of the requirements

for the award of the degree

Master of Applied Science

from

The Central Queensland University

Rockhampton, Queensland

Department of Biology

September 1995

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ACKNOWLEDGMENTS

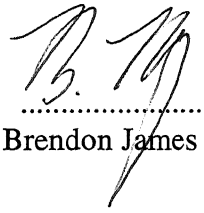
The financial support of the Queensland Department of Primary Industries and the Central Queensland University for the grant and scholarship that has made this research possible is gratefully acknowledged.

Thanks to Dr L. Slade Lee, my principal supervisor at the QDPI, for the initial recognition of the significance of this research, his assistance and guidance in the field and laboratory, as well as critical review of the manuscript. Also to Dr. Paul Scott and Dr. Kerry Walsh my CQU supervisors for their assistance in this research and critical review of this manuscript.

Thanks to Mr Geoffrey Rackemann for his technical expertise in electrophoresis and laboratory management, Dr. Helen Wallace for her critical review of the manuscript, John Taske for his photography, David Gillespie, Garry Fullelove, Lynton Vawdrey and Jason Olsen for technical field advice and computer assistance, Pam Cass, Karen Bates for advice in editing, Margaret Kelly and Iain Kay for advice in thesis compilation and the rest of the Bundaberg Research Station Staff, Bevan Zischke, John Maltby, Reg Gounder, James Schaefer, Leeola Schaefer, Jeff Barnes, Lyn Wakefield, Paul Farrell and Jerry Lovatt for their advice and constant support.

Declaration

I declare that this thesis is my own work and has not been submitted in any form for another award at any other university or institution of tertiary education. Information from the published or unpublished work of all other persons has been acknowledged in full in the text, and a complete list of references is provided.



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Brendon James King

CHAPTER ONE

INTRODUCTION

The citrus industry is a significant horticultural industry in Australia. Total Australian citrus production was around 803kT in 1992-93 (Australian Citrus Growers Federation-ACGF) (Sykes *et al.,* 1994). After sweet oranges, which account for around 82% of Australian citrus production, the mandarin or easy-peel group at approximately 8% of production is the second most important citrus type produced. In 1991/92, mandarin production had a gross value of $41.9m or around 15% of the gross value of citrus production, which was $272m. The higher value of mandarins on a return per tonne basis was emphasised further in the ABARE (Australian Bureau of Agriculture and Resource Economics) Australian Farm Surveys report for 1992. In terms of cash received per hectare for different citrus types averaged over all citrus growing regions, mandarins were worth $16,756, navel oranges $5,866 and valencia oranges $3,776 (Sykes, *et al.,* 1994).

Mandarins are produced in all citrus growing states and territories of Australia. Queensland produces the largest volume, approximately 57% in 1991-92, followed by South Australia (21%), New South Wales (10%), Victoria (9%) and Western Australia (3.0%). In recent years, many new mandarin plantings have been established and mandarins now account for over 10% of all citrus trees in Australia (Sykes *et al.,* 1994). In 1991/92 77% of the Australian mandarin crop was absorbed by the domestic fresh market, 17% was processed and 6% was exported. Export of mandarins should continue to increase over the next decade,

especially with an increasing tonnage of mandarins becoming available on the domestic market (Lee pers. comm.).

Genetic improvement programs aimed at developing new mandarins or easy-peel types are being conducted by Queensland Department of Primary Industries and CSIRO (Commonwealth Scientific and Industrial Research Organisation) using conventional and molecular based approaches. A primary objective in these genetic improvement programs is the development of new seedless types (Sykes *et al.*, 1994). In the mandarin breeding program at Bundaberg Research Station, it has been recognised that there is great potential for seedless mandarin cultivars; firstly in creating new markets both domestically and internationally (especially aimed at the Asian market, where a sweet seedless mandarin would be in high demand); and secondly by solving problems such as seediness in existing cultivars such as Murcott.

Triploids are seedless due to gametic sterility which is a result of irregular chromosome pairing during meiosis. Examples of commercial citrus cultivars which are seedless include "Oroblanco" and "Melogold" which are the progeny of pummelo × grapefruit crosses. If triploid citrus are to be exploited in breeding programs, then a method is needed that can quickly screen large numbers of seedlings to determine their ploidy level. The conventional cytological technique (the preparation of root-tips for chromosome counts) for differentiating between diploids and triploids of citrus is very laborious. Furthermore, citrus chromosomes are very small and difficult to count. Small seed size is associated with triploid embryos and has been used as a means of selecting potential triploids. However, this method is not

accurate, and a more reliable method to select the triploids out of these small-seed populations would be desirable.

Currently the mandarin breeding program at Bundaberg Research Station has over 500 plants in the field, the progeny of crosses made in order to produce seedless triploids, as well as hundreds of seedlings being produced yearly. The man hours required with conventional cytological techniques to check the ploidy of this population is a significant impediment in this project. Plants in the field which are not triploids are using valuable resources in the breeding program. Therefore an efficient method is required which will enable mass screening of citrus seedling populations. This project investigates whether isozyme electrophoresis and densitometry can be utilised for this purpose.

Hybrid triploid citrus may exhibit distinctive band pattern polymorphisms in electrophoresed isozymes, and in particular, characteristic differences in band densities within isozyme tracks. It is hypothesised that, where no gene dosage regulation effects occur, greater band density will be evident in the allozyme contributed by the diploid (usually maternal) gamete relative to that of the haploid gamete for a heterozygous locus. This effect is due to the extra protein coded by the additional gene dose and would not be observed in diploids. This project aims to investigate within-track differences in band densities between diploids and triploids for certain isozymes.

To achieve this, the project was undertaken in three stages. The first involved the development of good isozyme band resolution and activity so enzyme bands could be easily quantified using a densitometer. This required an understanding of associated problems with

enzyme denaturation and inactivation and problems associated with enzyme electrophoresis. The second stage of the project, required demonstrating linear relationships between isozyme band density (as measured by a densitometer) and isozyme activity. A linear relationship between isozyme activity and band density is necessary if any gene dosage effects were to be detected on the polyacrylamide gel. The final stage of the investigation examined possible differences in quantitative aspects of gene expression between citrus diploids and triploids, and determined the accuracy of this method in comparison with the conventional cytological technique.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Citrus* Polyploidy

To develop a rapid and reliable method of triploid identification in mandarins, there is a need to understand polyploidy in *Citrus*. It is therefore necessary to review what ploidy is, types of polyploids found in *Citrus*, their potential as cultivars, methods of reproduction, their origins as well as methods used for estimation of ploidy levels in other crops.

2.1 (i) What is ploidy?

Ploidy refers to the number of individual genomes (complete chromosome sets) found in a cell (Sanford, 1983). Individuals with more than two genomes are called polyploids, while monoploid, diploid, triploid and tetraploid refer to the presence of one, two, three and four sets of chromosomes per cell, respectively (Sanford, 1983).

Polyploid cells arise at low frequency as the result of a "mistake" in meiosis or mitosis in which the chromosomes divide but the cell does not (Raven *et al.*, 1986). This results in a cell with more than the usual number of individual genomes. If such cells continue to divide, or in the case of a germ cell become fertilised, they give rise to polyploid progeny (Sanford, 1983). Stebbins (1950) presented evidence that polyploidy in nature is most often associated with hybridisation, either between species or between different subspecies or races of the

same species. A well known example of this, is the evolution of a vigorous polyploid hybrid (2n=122) which was derived from natural hybridisation between the salt-marsh grass *Spartina maritima* (2n=60) and *Spartina alterniflora* (2n =62) introduced to Great Britain from North America in 1890 (Raven *et al.*, 1986).

Polyploidy is known to some degree in all groups of plants (Stebbins, 1950). Many valuable crop plants are polyploids and polyploidy is an essential consideration in the breeding of fruit crops (Table 2.1). Introgression between ploidy levels will continue to be of value in crops with more than one ploidy level or in crops with related species of other ploidy levels (Sanford, 1983). In *Citrus* there are different levels of ploidy which offer considerable potential for manipulations in breeding programs.

2.1 (ii) Polyploidy in *Citrus*

Frost (1925_a) and Longley (1925) identified the haploid chromosome number of *Citrus* as n=9. Diploidy with the somatic chromosome number 2n=18 is the norm for *Citrus* and closely related genera (Frost and Soost, 1968). However, various euploids (i.e. exact multiples of the basic chromosome number of the genus) have been reported. Monoploid plants of several *Citrus* species (Chen *et al.*, 1980; Chen, 1985) have been derived from pollen culture, and also from seeds exposed to gamma irradiation (Karasawa, 1971). Zhang (1985) derived monoploid cultures from mutant somatic cells. Rare occurrences of pentaploids were reported by Krug (1943), Esen and Soost (1973_a) and Esen *et al.*, (1978). One octoploid was recovered by Barrett and Hutchison (1981). Hexaploids have also arisen in *Citrus* from open pollinations (Krug, 1943) and from diploid × tetraploid crosses (Esen and

Soost, 1973_a; Oiyama *et al.,* 1981). The occurrence of spontaneous *Citrus* aneuploids (i.e not exact multiples of the basic chromosome number; some chromosomes are duplicated more than others) has long been recognised (Krug, 1943).

Table 2.1

Common ploidy levels of some valuable crop plants

Crop	(n)	Common Ploidy Levels	References
Apple	x=7	2x, 3x, 4x	Einset, 1945. Dermen, 1965.
Banana	x=11	2x, 3x	Dodds and Simmonds, 1948.
Blackberry and Raspberry	x=7	2x, 3x, 4x, 6x, 7x, 9x, 10x,	Darrow, 1955.
Cherries	x=8	2x, 3x, 4x	Fogle, 1975.
Cotton	x=13	2x, 4x	Beasly, 1940
Grapes	x=19	2x, 4x	Olmo, 1952.
Peach	x=8	2x, 3x, 4x	Dermen, 1938.
Pear	x=8	2x, 3x, 4x	Dermen, 1938.
Strawberry	x=7	2x, 4x, 6x, 8x	Evans, 1977
Tobacco *(N. tabacum)* *(N. sylvestris)* *(N. tomentosa)*	x=12 2n=48 2n=24 2n=24	amphidiploids	Greenleaf, 1941.
Wheat	x=7	2x, 4x, 6x	McFadden and Sears, 1946.

Esen and Soost (1972_a) showed that almost all *Citrus* aneuploids derive from polyploid parents. Triploids and tetraploids are the most common polyploids of *Citrus*.

2.1 (iii) The potential of polyploids as cultivars in *Citrus*

The potential of polyploidy as a means of achieving improved *Citrus* cultivars has long been recognised (Frost 1925_b, 1926). There are two approaches to the use of polyploids for *Citrus* cultivar improvement. (i) The exploitation of selected polyploid cultivars because of their horticulturally desirable characteristics, and (ii) as parents in breeding programs usually aimed at deriving other desirable polyploid cultivars (Lee, 1988).

Growth rate reduction with increasing ploidy is a general characteristic of many plant species (Lindstrom, 1936). Frost (1925_b) first reported the distinctive appearance of *Citrus* tetraploids and noted their slower growth. Tree size of tetraploids is generally smaller than diploids (Frost 1938; Frost and Krug, 1942; Barrett and Hutchison, 1978), and roots of tetraploid plants are shorter and stouter, with fewer laterals than their diploid counterparts (Furusato 1953_a; Barrett and Hutchison, 1978). Barrett and Hutchison (1978) recognised the potential of such tetraploids as agents for tree size control. However, the chief use of *Citrus* tetraploids has been as parents in breeding programs aimed at producing seedless triploids (Cameron and Soost, 1969; Soost and Cameron, 1969, 1980, 1985; Tachikawa, 1973; Cameron and Burnett, 1978; Esen *et al.*, 1978; Oiyama *et al.*, 1981). As specific cultivars, early recognition was given to seedless triploids (Krug and Bacchi, 1943). However, except for "Oroblanco" and "Melogold" (Soost and Cameron, 1980, 1985) few have attained commercial significance (Lee, 1988).

2.1 (iv) Apomixis and poly/monoembryony

There are two distinct modes of origin of *Citrus* polyploids - sexual and somatic (Lee, 1988). Apomixis in *Citrus* is characteristic of *Citrus reticulata* but is not universal within this group (Frost and Soost, 1968) (Table 2.2). Some varieties are obligate apomicts, some entirely sexual and others display partial apomixis (Frost and Soost, 1968). Apomixis in *Citrus* occurs as nucellar embryogenesis in which adventitious embryos develop from the nucellus and results in polyembryony. Except in varieties that are strictly sexual (monoembryonic), the zygotic embryo usually competes for space and nutrients unsuccessfully with one or more nucellar embryos. Embryo sacs may contain a few embryos developing normally together with others that are partially suppressed (Lee, 1988)

Table 2.2

The embryogenesis of commercial mandarin varieties grown in Australia.

Variety	Embryogenesis
Clementine	Monoembryonic
Dancy	Polyembryonic
Elenor	Polyembryonic
Ellendale	Monoembryonic
Emperor	Polyembryonic
Fremont	Monoembryonic
Glen	Polyembryonic
Hickson	Monoembryonic
Imperial	Monoembryonic
Kara	Polyembryonic
Murcott	Polyembryonic
Nova	Polyembryonic
Ortanique	Polyembryonic
Satsuma	Polyembryonic
Sunburst	Polyembryonic

2.1 (v) The origin of triploid *Citrus*

Triploid progeny from ***monoembryonic*** *seed parents*

Triploid progeny have been derived from cross pollinations where the ploidy relationship of monoembryonic pistillate parent to staminate parent have been (4X x 2X), (2X x 4X), and (2X x 2X) (Lee, 1988).

4X x 2X

Where the pistillate parent was tetraploid, Cameron and Burnett (1978) recovered 85% triploid progeny, the remainder were tetraploids and near triploids [26,28,29 chromosomes]. The high triploid recovery arises from normal sexual fertilisation of the diploid female gamete with a haploid male gamete (18 + 9 chromosomes) (Cameron and Soost, 1969_b; Cameron and Burnett, 1978) and subsequent development of the triploid zygotic embryo. Esen and Soost (1971) noted small seed size associated with triploid embryos from (4X x 2X) crosses, and postulated that it was due to premature termination of the pentaploid endosperm development. This was attributed as the cause of observed early initiation and termination of triploid embryo development, resulting in small seed size. This provides a useful characteristic for selecting probable triploid progeny (Esen and Soost, 1971; Geraci *et al.*, 1975).

2X x 4X

Cameron and Soost (1969_b) recorded a much poorer recovery of triploid progeny from the reciprocal crosses, and a high incidence of empty and poorly developed seeds. Only 9% recovery of triploids was observed by Esen *et al.*, (1979); the remainder were presumed to be

tetraploids. Esen and Soost (1973_b, 1973_c) determined an abortion rate of 92-99% for triploid embryos from 2X x 4X crosses.

The 3:4 triploid embryo to endosperm chromosome ratio which results from 2X x 4X crosses was found to be responsible for the abortions. Normal diploid embryos display a ratio of 2:3 [0.667]. Ratios below 0.667 are claimed to cause no impairment to embryo viability, even for aneuploids (Esen and Soost 1972_a). Hence while seed size is reduced when the ratio is 3:5 [0.60] from 2X x 4X crosses, embryos are still viable, but poor viability is characteristic when the ratio is 3:4 [0.75].

2X x 2X

Triploid progeny can arise from a diploid monoembryonic pistillate parent crossed with a diploid pollen parent. As the progeny is zygotic, one of the gametes has become diploid prior to fertilisation (18 + 9 or 9 + 18 chromosomes). Esen and Soost (1971) concede both possibilities, but point out the improbability of the latter due to triploid embryo abortion (see above). In the majority of cases, triploid progeny from 2X x 2X crosses are derived from diploid megagametes (Esen and Soost, 1972_b; $1973_{a,b,c}$; Geraci *et al.*,1975; Esen *et al.*, 1979). Breeding triploids from 2X x 2X crosses eliminates the need for tetraploid parents, thus overcoming the problem of aneuploidy which can result from irregular meiosis in tetraploids (Esen and Soost, 1971; Geraci *et al.*, 1975).

*Triploid progeny from **polyembryonic** seed parents*

Crosses of both diploid and tetraploid polyembryonic seed parents with diploid pollen parents have produced triploid progeny (Tachikawa, 1973). Geraci *et al.*, (1975) obtained triploid

progeny from 2X x 2X crosses using both monoembryonic and polyembryonic seed parents, and accredited their origins to diploid megaspore formation. Viable zygotic embryos can develop in polyembryonic *Citrus* varieties and triploids arising from polyembryonic parents would be zygotic.

2.1 (vi) Origin of *Citrus* tetraploids

Tetraploid progeny from monoembryonic seed parents

Tetraploid progeny from monoembryonic seed parents have arisen from crosses of 2X x 4X and 4X x 2X. With the 2X x 4X crosses Esen and Soost (1973_c) claim that tetraploid progeny arise from the diploid male gametes fertilising diploid megagametes (18 + 18 chromosomes). Tetraploid progeny have been derived from 4X x 2X crosses with monoembryonic seed parents. Cameron and Burnett (1978) obtained high frequencies of triploids [85%] from this cross as would be expected. They concluded that the few tetraploids produced originated from chance development of nucellar embryos.

Tetraploid progeny from 4X x 4X crosses are not reported in the literature. This is because most available tetraploids are highly polyembryonic (Esen and Soost 1972_a; Starrantino and Recupero, 1981). If such a cross were to be conducted with a monoembryonic seed parent, tetraploid progeny would be expected as a result of normal sexual union.

Tetraploid progeny from polyembryonic seed parents

Tetraploid offspring have arisen from all combinations of diploid and tetraploid parentage using polyembryonic seed parents (2X x 4X), (2X x 2X), (4X x 2X), (4X x 4X). Tachikawa

(1973) recovered one tetraploid out of eleven progeny from a 2X x 4X cross. The remainder were all nucellar diploids. The author indicated the possibility of a diploid megagamete occurring, in which the tetraploid would be zygotic. Despite this, the possibility of spontaneous nucellar tetraploid embryogenesis must also be considered.

Tetraploid progeny from 2X x 2X crosses using polyembryonic parents have been attributed as spontaneous somatic polyploids of nucellar origin (Barrett and Hutchison 1978; Wakana *et al.*, 1981). Tetraploid progeny arising from tetraploid polyembryonic seed parents crossed with diploid pollen parents [4X x 2X] are normal nucellar offspring (Tachikawa 1973; Esen *et al.*, 1978). Similarly, Esen *et al.*, (1978) assumed that tetraploid progeny from 4X x 4X crosses were also nucellar.

2.1 (vii) Methods of estimation of ploidy levels

Counting chromosome numbers in meristematic cells is considered to be the unequivocal method for ploidy determination. A variety of techniques documented for chromosome staining of root tips and other tissues are used in *Citrus* (Sharma and Bal, 1957; Dyer, 1963; Storey and Mann, 1967; Frost and Soost, 1968; Vardi, 1981).

Counting chloroplast numbers in guard cells and measuring pollen grain diameter offer fast and reliable alternatives to chromosome count in some plant species (Singsit and Ozias-Akins, 1992). Chloroplast number in guard cells has routinely been employed as a convenient, rapid and accurate procedure for determining ploidy status in many crop species (Bingham, 1968; Jacobs and Yoder, 1989; Ho *et al.*, 1990). Bingham (1968) determined that

diploid, triploid, tetraploid, and hexaploid alfalfa plants had 9.2, 9.4, 12.8, and 15.2 chloroplasts per pair of stomatal guard cells respectively, and used this technique to screen alfalfa populations for plants with desired ploidy levels.

The size of pollen grains has been used to estimate the ploidy level for crop species of the donor parents (Bamberg and Hanneman, 1991). Pollen size was found to be positively correlated with the number of chromosome sets in a cell. Rapid screening of a population of pollen grain for size can be applied for the characterisation of interspecific hybrid populations, where ploidy levels and unreduced (2n) gamete formation may vary among individuals (Singsit and Ozias-Akins, 1992). This approach is not feasible in tree crops which take many years to commence flowering, therefore early determination of ploidy is not possible by this method.

Citrus tetraploids possess distinctive vegetative morphology (Frost, 1938; Frost and Krug, 1942; Furusato 1953_a, Tachikawa, 1973; Barrett and Hutchinson, 1978). Morphological characters such as leaf size can therefore be used as markers for ploidy detection. However, triploid *Citrus* do not possess very distinctive morphology from their diploid counterparts. Small seed size associated with triploid embryos however does provide a useful characteristic for selecting probable triploid progeny (Esen and Soost, 1971; Geraci *et al.*, 1975). These methods though faster than chromosome counting do not provide an exact method of ploidy determination for all individuals screened.

Flow cytometry has been used to characterise large populations of cells for ploidy (Costich *et al.*, 1983; Ramulu and Dijkhuis, 1986). DNA flow cytometry measures the fluorescence of a

large number of stained nuclei within seconds, and provides an estimate of nuclear DNA within somatic plant tissue (Glabraith *et al.,* 1983; Arumuganathan and Earle, 1991). Although a rapid and reliable technique, equipment required is extremely expensive. While the immediate technique is rapid cells must be obtained from tissue culture stocks, therefore this would be a laborious process when ploidy determination has to be made from *Citrus* seedlings and plants in the field.

Isozymes however offer the ability to quickly distinguish large numbers of polyploid plants both quickly and cheaply. Whereas increased isozyme numbers are frequently observed in allopolyploids, no differences exist in the isozyme banding patterns between autopolyploids and their diploid counterparts (Gottlieb, 1982). However, the presence of additional genes may increase the overall rate of transcription, and result in an increase in staining intensity of certain allozyme bands (De Maggio and Lambrukos 1974). This may provide a fast and reliable method for distinguishing triploid and diploid *Citrus* progeny. This project aimed to develop such a method.

2.2 Isozymes

To use isozymes to distinguish between triploid and diploid *Citrus*, it is necessary to review what isozymes are and what they have been used for, their genetics and variation within and between species, enzyme structure, and their expression in polyploid plants. As well it is important to review the function and distribution of individual isozymes which may prove useful in distinguishing triploid and diploid *Citrus*.

2.2 (i) What are isozymes?

Since their discovery by Hunter and Markert (1957), isozymes have played a key role in many branches of biology. They were originally defined by Markert and Moller (1959) as different variants of the same enzymes, having identical or similar functions, and present in the same individual. Today the precise use of the term isozyme or isoenzyme has shifted in accordance with recommendations of the International Union of Biochemistry Nomenclature (Webb, 1984) and with conventions that have arisen in subdisciplines making widespread use of electrophoresis (Kephart 1990). The relatively broad use of the term in some publications (Brewer and Sing, 1970; Harris and Hopkinson, 1976) differs from the more restrictive definitions of recent years (Gottlieb, 1982; Weeden and Wendel, 1989). In the former, isozymes included electromorphs (i.e the isozyme pattern on the gel for any given genotype) generated by different loci, different alleles of the same loci, and post-translational modifications of the enzymes (secondary isozymes). These secondary enzymes are no longer treated as isozymes, in part because they can arise through molecular changes that result from assay conditions. In practice, the term isozymes is applied more broadly to electromorphs than "allozyme", which is reserved for bands known by genetic analysis to result from alleles of one locus (Kephart, 1990).

2.2 (ii) What have isozymes been used for?

With the discovery of genetic polymorphism for isozymes within a population of *Drosophilia* (Lewontin and Hubby, 1966) and humans (Harris, 1966), population geneticists were able to make precise quantitative estimates of genetic variability based upon one parameter of the

molecular structure of the primary products of genes themselves (Gottlieb, 1977). Isozymes have since been used to provide useful information on hybridisation and gene duplication (Yahara *et al.,* 1989; Grosser *et al.,* 1992), the recognition of species boundaries (Wake, 1981; Tilley and Schwerdtferger, 1981; Hanken, 1983; Gastony, 1988), phylogenetic relationships (Hirai and Kozaki 1981; Hirai *et al.,* 1986; Hirai and Kajiura, 1987; Ashari *et al.,* 1989; Benzie, 1990), population structure analysis (Antonovics, 1971; Warwick and Briggs, 1978; Turkington and Harper, 1979), gene flow (Loveless and Hamrick, 1984; Chepko-Sade and Halpin, 1987; Ryman and Utter, 1987), and rates of evolution (Rosen and Buth, 1980).

2.2 (iii) Genetics of isozymes

An isozyme locus is defined as the structural gene for an enzyme capable of catalysing a specified biochemical reaction (Wendle and Weeden, 1989). Allozymes generally exhibit Mendelian inheritance, codominant expression, complete penetrance (i.e the proportion of genotypes that show an expected phenotype) and absence of pleiotropic (i.e multiple phenotypic effects of a single gene) and epistatic interactions (i.e the effect of one gene changing or modifying that of another) (Hubby and Lewontin, 1966; Lewontin and Hubby, 1966; Peirce and Brewbaker, 1973; Tanksley and Rick, 1980; Brown and Weir, 1983). The observation of codominant expression in nearly all cases provides compelling evidence that both alleles are transcribed, and both transcripts are translated.

2.2 (iv) Gene number and conservation

For enzymes with well defined biochemical roles, and especially those acting in pathways of primary metabolism (e.g. pentose phosphate pathway, Krebs cycle) a definite number of genes will be expressed in most diploid plants (Wendle and Weeden, 1989; Kephart, 1990). Each of the isozymes expressed have a specific subcellular location and a conserved subunit structure. In contrast, enzyme systems such as acid phosphatase, esterases, and peroxidase display considerable variation in total number of isozymes expressed, number of isozymes per subcellular compartment and number of subunits per enzyme (Wendle and Weeden, 1989).

Isozyme number and subcellular distribution are open to wide variation, therefore specific isozymes may not be strongly expressed in certain species or tissues. Several recent papers have stressed that significant variability in isozyme number exists when tissue specificity and plant age are considered (Jones, 1984; Murray and Ayre, 1987; Pedersen and Simonsen, 1987).

2.2 (v) Enzyme structure

Interpretation of the banding patterns evident on stained electrophoresis gels is aided considerably by the somewhat predictable quaternary structure of many enzymes and their localisation within particular organelles or cellular compartments (Wendle and Weeden, 1989; Kephart, 1990).

Most enzymes assayed for electrophoretic mobility exist as monomers, dimers or tetramers. Since most allozymes of a single gene are codominantly inherited, the polypeptide subunits encoded by each allele are visualised as phenotypes (coloured bands) on a gel after electrophoresis; with heterozygotes showing multiple bands (Richardson *et al.,* 1986; Wendle and Weeden, 1989).

In a monomer, heterozygotes display two bands, one from each of the contributing parental alleles, whereas in dimers and tetramers, the products of the alleles usually associate at random in the cells of each heterozygote. This results in three subunit combinations (1a, 2ab, 1b) of dimeric proteins (two homodimers and one heterodimer) and five combinations (1aaaa, 4aaab, 6aabb, 4abbb, 1bbbb) of tetrameric proteins (two homotetramers and three heterotetramers). Each combination of subunits will migrate at different rates under the appropriate electrophoretic conditions (Richardson *et al.,* 1986; Wendle and Weeden, 1989; Hillis *et al.,* 1990; Kephart, 1990).

Because the number of compartments in which an isozyme may be found is predictable, the anticipated number of isozyme loci for a given enzyme is highly conserved between different taxa (Gottlieb, 1982). Deviations from the predicted number of isozyme loci suggest possible duplication events, polyploidy, or technical procedures that may be causing enzyme degradation (Kephart, 1990).

2.2 (vi) Isozyme expression in polyploid plants

Increased numbers of isozymes are frequently observed in polyploid plants (allopolyploids) and are a predictable consequence of their mode of origin (Gottlieb, 1982). However, there will be no difference in the isozyme banding pattern of a polyploid in comparison to the diploid counterpart if the polyploid is an autopolyploid. But the presence of additional genes may increase the overall rate of transcription, and result in an increase in activity (De Maggio *et al.*, 1974). Isozyme staining intensities may therefore be used to investigate ploidy levels. Danzmann and Bogart (1982) and Dessauer and Cole (1984) found that gene dosages, and thus ploidy levels (2n, 3n, 4n), could be inferred accurately from staining intensities because subunit interaction was additive.

Albuzio *et al.*, (1978) found no polymorphism for isozyme bands tested in the autotetraploid as compared to the diploid of *Lycopersicon esculentum.* However, it was found that there was increased activity of malate dehydrogenase, acid invertase, glutamate dehydrogenase and nitrate reductase, and decreased activity of peroxidase. These results suggested that gene expression of the double gene dose is enhanced in some cases and depressed in others. Changes in the enzyme multiplicity are unlikely in the case of autopolyploids and metabolic consequences of polyploidization can be ascribed to the quantitative level of expression of the genes (Scandalios, 1974).

2.2 (vii) Isozymes investigated (Function and distribution)

In selecting specific isozymes for this project the following factors were considered;

(i) Only enzymes coded by nuclear DNA were used. This is the case for the vast majority of enzymes, but supporting evidence had to be available. Only isozymes widely reported in the genetics literature to demonstrate Mendelian segregation (indicating nuclear origin) were used. Preferably genetic information regarding *Citrus* should be available.

(ii) Satisfactory band density and resolution in each isozyme band had to be achievable.

Malate Dehydrogenase (NAD) - MDH (E.C.1.1.1.37)

Malate dehydrogenase (NAD) E.C.1.1.1.37, catalyses the reversible oxidation of malate to oxaloacetate (Stryer, 1988). In this reaction NAD^+ is the hydrogen acceptor, which is reduced to NADH (Salisbury and Ross, 1991). This reaction is involved in a number of essential biochemical pathways occurring in the cytosol, peroxisomes of leaves and mitochondria (Tolbert, 1981). Cytosolic MDH (NAD) takes part in the malate/oxaloacetate shuttle where oxaloacetate formed in the cytosol is transported into the chloroplast for reduction to malate (Salisbury and Ross, 1991). Cytosolic MDH (NAD) also supports a malate shuttle to the peroxisomes where peroxisomal MDH (NAD) supplies NAD(H) to the glycerate pathway (Tolbert, 1981). The glyoxylate pathway utilises MDH (NAD) in the glyoxysomes of germinating seeds (Tolbert, 1981; Raven *et al.*, 1986). Mitochondrial MDH (NAD) catalyses the final step in the Krebs cycle (Stryer, 1988). The inner mitochondrial membrane is impermeable to oxaloacetate. Therefore, a series of bypass reactions is needed. The first step entails reduction of oxaloacetate to malate by NADH. This reaction is catalysed by malate dehydrogenase in the cytosol (Stryer, 1988).

Boulter and Laycock (1966) found chloroplast and mitochondrial MDH (NAD) isozymes in *Vicia faba* which occupied the same band location on acrylamide gels. Quiros (1987) showed in *Brassica* species that MDH had both plastid and cytosolic isozymes, and that the faster (anodal) isozyme was of plastid origin. MDH is dimeric and has been reported for a wide range of *Citrus* cultivars (Hirai and Kozaki, 1981; Ben-Hayyim *et al.*, 1982; Hirai *et al.*, 1986; Xiang and Roose, 1988; Ashari *et al.*, 1989). In *Citrus* three regions of MDH (NAD) have been identified by starch gel electrophoresis, the middle one of which shows two overlapping isozymes of considerable variability (Soost and Torres, 1981; Torres *et al.*, 1982). These two *Citrus* MDH (NAD) loci have been widely reported (Torres, 1983; Torres *et al.*, 1985; Ashari *et al.*, 1988; Moore and Castle, 1988; Roose and Traugh, 1988). The slower moving (cathodal) isozyme is designated MDH-1, and the faster MDH-2 (Torres *et al.*, 1982). Torres *et al.*, (1982) demonstrated Mendelian segregation for MDH-1 and MDH-2 in *Citrus*, indicating nuclear coding. Australia's major mandarin cultivars Murcott, Imperial and Ellendale have all been reported to be homozygous for both MDH-1 and MDH-2 loci.

Shikimate dehydrogenase - SkDH (E.C.1.1.1.25)

SkDH catalyses the reversible reduction of 3-dehydroshikimate by NADP to shikimate in the shikimate pathway (Stryer, 1988). It is the principal one of only three major pathways of aromatic amino acid biosynthesis (Lourenco *et al.*, 1991). The shikimate pathway exists in fungi, bacteria and plants, but not in animals (Lourenco and Valdir, 1984; Salisbury and Ross, 1991). This pathway leads to the production of chorismate from D-glucose. Chorismate is the precursor of the three aromatic amino acids, L-phenylalanine, L-tyrosine, and L-tryptophan (Stryer, 1988). Furthermore, in higher plants the synthesis of many secondary metabolites relies on intermediates and end-products of the shikimate pathway

(Salisbury and Ross, 1991). These secondary metabolites include most known plant alkaloids and pigments. This pathway also provides the building blocks for the biosynthesis of lignin and several vitamins.

Shikimate dehydrogenase is monomeric, with 1 or 2 loci (Kephart, 1990). Both cytosolic and chloroplastic forms exist (Weeden and Gottlieb, 1980; Kephart, 1990). Mendelian segregation for chloroplast-specific SkDH allozymes in higher plants was demonstrated by Weeden and Gottlieb (1980) indicating coding by a nuclear gene. Two areas of SkDH activity were observed upon electrophoresis, the faster (anodal) being the chloroplastic enzyme (Weeden and Gottlieb, 1980). Moore and Castle (1988) identified one monomeric SkDH isozyme in *Citrus*. Shikimate dehydrogenase is homozygous in Imperial mandarin, and heterozygous in Murcott and Ellendale (Ashari *et al.*, 1989).

6-Phosphogluconate dehydrogenase - 6PGD (E.C.1.1.1.44)

The NADP-linked decarboxylating form of 6PGD catalyses the third reaction of the oxidative pentose phosphate pathway - conversion of 6-phosphogluconate to ribulose 5-phosphate (Stryer, 1988). Two isozymes of 6PGD, one cytosolic, the other chloroplastic have been reported (Schnarrenberger *et al.*, 1973; Herbert *et al.*, 1979). Schnarrenberger *et al.*, (1973) showed that the two isozymes were nearly identical in molecular weight, but electrophoretic separation suggested charge difference. The chloroplastic isozyme migrates fastest (anodal) in spinach (Schnarrenberger *et al.*, 1973) and in *Brassica* (Quiros, 1987). Weeden (1981) reported the chloroplastic form is coded by a nuclear gene.

In higher plants the two isozymes are reportedly dimeric (Weeden and Wendel, 1989; Kephart, 1990). This is possibly the case in *Citrus* (Hirai and Kozaki, 1981). Ashari *et al.*, (1989) reported a heterozygous locus in Murcott.

Phosphoglucose Isomerase - PGI (E.C.5.3.1.9)

Phosphoglucose Isomerase (NADP) (D-glucose-6-phosphate Keta-isomerase, E.C.5.3.1.9; PGI) is a dimeric enzyme that catalyses the isomerization of the six-carbon pyranose ring of glucose-6-phosphate to the 5-carbon furanose ring of fructose-6-phosphate in the glycolysis pathway (Lumaret, 1986; Wendle and Weeden, 1989; Stryer, 1988). PGI is found in both the cytoplasm and chloroplasts (Schnarrenberger and Oeser 1974; Schnarrenberger *et al.*, 1973; Weeden and Gottlieb, 1980) and each form is specified by a distinct nuclear gene (Weeden and Gottlieb, 1980). PGI is reportedly dimeric (Richardson *et al.*, 1986). Ashari *et al.*, (1989) reported Murcott, Ellendale and Imperial to be homozygous at this locus.

2.3 Electrophoresis

To reveal isozymes on a gel, extracts must be electrophoresed and then histochemically stained. To achieve scorable zymograms, the process of electrophoresis must be understood, and therefore it is necessary to review what electrophoresis is, mediums used in electrophoresis, the advantages and properties of polyacrylamide gel electrophoresis, and the effects of buffer systems on isozyme activity, separation and resolution.

2.3 (i) What is electrophoresis?

Protein electrophoresis is the migration of proteins under the influence of an electrical field (Murphy *et al.*, 1990). Electrophoresis was developed as an analytical technique for chemical and biological research (Tiselius, 1937). Its scope of application has broadened in recent years because of its reproducibility and simplicity (Richardson *et al.*, 1986). Zone electrophoresis is a modification of the procedure whereby a mixture of molecules to be separated is placed in a narrow band at suitable distance from the electrodes such that during electrophoresis, proteins of different mobilities travel as discrete zones which gradually separate from each other as electrophoresis proceeds (Hames and Rickwood, 1990). It has become one of the three most common methods for fractionating molecular mixtures along with liquid chromatography and ultracentrifugation (Gordon, 1975).

Electrophoresis, coupled with the zymogram technique (Hunter and Market, 1957), has been the tool of choice for studies of heritable variation by geneticists, systematists, and population biologists (Gottlieb, 1977; Crawford, 1985). The zymogram technique involves the location of specific proteins on areas of the gel using specific histochemical staining techniques in which some functional property of the protein is used to detect its presence. Since enzymes catalyse a specific reaction, any enzyme can be histochemically localised, provided that either a substrate, or more commonly a product involved in it's reaction can be made visible (Richardson *et al.*, 1986). The stained gels produced by these procedures are termed zymograms.

2.3 (ii) Media used in electrophoresis

In practice there are disadvantages to zone electrophoresis being carried out in free solution. Heating caused by electrophoresis can result in convective disturbance and disruption of the separating zones (Richardson *et al.*, 1986). Furthermore, the effects of diffusion are to constantly broaden the protein zones and this continues after electrophoresis has been terminated. To minimise these effects, zone electrophoresis of proteins is rarely carried out in free solution, but instead is performed in a solution stabilised within a supporting medium (Hames and Rickwood, 1990).

Electrophoretic separation of complex mixtures of proteins can be accomplished in several types of support media (Wendle and Weeden, 1989). The media available fall into two main classes (i) paper, cellulose acetate, and thin-layer materials and (ii) gel media. These thin-layer media are relatively inert and serve mainly for support and to minimise convection (Smith, 1976; Wendle and Weeden, 1989; Hames and Rickwood, 1990). Hence separation of proteins using these materials is based largely upon the charge density of the proteins at the pH selected, as with electrophoresis in a free solution (Richardson *et al.*, 1986). In contrast, the various gels such as agarose, starch and polyacrylamide not only prevent convection and minimise diffusion but in some cases they actively participate in the separation process by interacting with the migrating particles (Maurer, 1971; Gordon, 1975; Richardson *et al.*, 1986; Wendle and Weeden, 1989, Hames and Rickwood, 1990). These gels can be considered as porous media in which the pore size is the same order as the size of the protein molecules such that molecular sieving occurs and the separation is dependent on both charge and size of the protein (Maurer, 1971; Gordon, 1975; Hames and Rickwood, 1990).

The extent of molecular sieving depends on how close the gel pore size approximates the size of the migrating particle. The pore size of agarose gels is large enough that molecular sieving of most protein molecules is minimised and separation is mainly based on charge density (Hames and Rickwood, 1990). In contrast, starch and polyacrylamide gels have pores of the same order of size as protein molecules and these do contribute to a molecular sieving effect (Maurer, 1971; Gordon, 1975; Richardson *et al.,* 1986; Wendle and Weeden, 1989; Hames and Rickwood, 1990).

The success of starch gel electrophoresis is highly dependant on the quality of the starch gel itself, which, being prepared from a biological product, can be difficult to reproduce, and may contain contaminants. On the other hand, polyacrylamide gel electrophoresis can always be prepared from highly purified reagents in a reproducible manner provided that polymerisation conditions are standardised (Hames and Rickwood, 1990).

2.3 (iii) Properties of the polyacrylamide gel

The chemical structure

Polyacrylamide gels result from the polymerisation and cross linking product of the acrylamide monomer, CH_2=CH-CO-NH_2, and a cross-linking comonomer, usually N,N'-methylene-bis-acrylamide (Maurer, 1971; Hames and Rickwood, 1990). The concentration of monomer and comonomer in the gelating solution and the degree of polymerisation (chain length) and cross-linking determine the density, viscosity, elasticity and mechanical strength of the gel (Maurer, 1971; Gordon, 1975; Smith, 1976).

Polymerisation catalysts

The polymerisation of acrylamide is initiated by the addition of either ammonium persulphate or riboflavin (Hames and Rickwood, 1990). In addition, N,N,N,N'-tetramethylethylenediamine (TEMED) or, less commonly, 3 dimethylamino-propionitrile (DMAPN) are added as accelerators of the polymerisation process (Maurer, 1971; Gordon, 1975; Hames and Rickwood, 1990).

Advantages of the polyacrylamide gel

Polyacrylamide gels have the advantage of being chemically inert, and stable over a wide range of pH, temperatures and ionic strengths. Their uniformity and transparency facilitate densitometric quantification of the product (Chrambach and Rodbard, 1971; Chrambach, 1980). Furthermore polyacrylamide is better suited to a size fractionation of proteins since gels within a wide range of pore sizes can be readily made, whereas the range of pore sizes which can be made with starch is limited.

2.3 (iv) Buffer Systems

Gel and electrode buffer

An electrophoresis buffer system consists of a gel buffer used in preparing the gel and an electrode buffer, which is an ionic solution that conducts current through the gel during electrophoresis (Kephart, 1990). Proteins, which are zwitterions, carry positive charges as a result of ionised amino groups and negative charges contributed by ionised carboxyl groups (Richardson *et al.*, 1986). Their net charge, and thus their migration in the electrical field of the gel, depends on the pH of the buffer system (Morris, 1974; Schulz and Schirmer, 1979;

Kephart, 1990). At its isoelectric point a molecule is electrically neutral and will not migrate (Richardson *et al.*, 1986). Because different enzymes are ionised under different pH conditions, a researcher usually selects two to four different buffer systems with pH ranging from 5 to 9, to find which is most suitable buffer for the isozyme being studied (Kephart, 1990).

Buffer systems may be continuous (gel and electrode buffer of the same chemical composition) or discontinuous (buffers differing in chemical composition) (Maurer, 1971; Gordon, 1975; Smith, 1976; Richardson *et al.*, 1986; Hills *et al.*, 1990; Hames and Rickwood, 1990). Discontinuities in buffers tend to compact the protein bands, thereby sharpening resolution (Smith, 1976). Molarity may differ between gel and electrode buffer. Low ionic strength buffers (gel and electrode) lead to more of the electric current being carried by the protein rather than by the buffer. This provides faster migration rates and lower heat production, whereas high ionic strength buffers promote the stabilisation of zones and low migration rates but cause high heat production which can lead to enzyme denaturation problems in isozyme analysis (Richardson *et al.*, 1986).

Selecting the appropriate buffer systems involves experimentation (Kephart, 1990). The pH affects the charge and separation of proteins but composition and molarity which can affect band resolution, separation and enzyme activity are also critical factors especially for enzymes with broad pH tolerance (Pasteur *et al.*, 1988).

Extraction buffers

Tissues which are to be subjected to isozyme analysis need to be buffered extracts (Wendle and Weeden, 1989). In the living plant cell, the protoplasm is protected from potentially damaging compounds by compartmentalisation (Loomis and Battaile, 1966; Anderson *et al.,* 1968; Loomis, 1969, 1974). When plant tissue is homogenised the compartmentalisation is destroyed, and enzyme inactivation or precipitation may result (Loomis and Battaile, 1966; Anderson and Rowan, 1967; Loomis 1974).

Each taxon and tissue poses its own set of problems regarding the difficulty of cell breakage and associated problems caused by endogenous tannins, phenols, proteinases, phenoloxidases, and other (mostly unidentified) cellular constituents (Soltis and Soltis, 1989). Reactions catalysed by the phenol enzyme complex (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase E.C.1.14.18.1) and subsequent reactions are primarily responsible for the loss of enzymatic activity in plant extracts (Pierpoint, 1966; Anderson, 1968; Kelly and Adams 1977). As a result, when leaf material containing high concentrations of phenolic compounds is prepared using standard techniques, electrophoresis of the resultant samples yield negligible enzyme activity (Soltis *et al.,*1980). Therefore it is important to use an extraction buffer which will maintain optimal enzyme activity, for sharper resolution and minimal streaking (Soltis and Soltis, 1989).

The general approach in preparing samples is to isolate phenolic compounds and other secondary plant products as soon as possible, at the same time preventing their formation in covalent complexes with enzymes (Anderson, 1968; King, 1971; Iglesias *et al.,* 1974; Loomis, 1974; Wilson and Hancock, 1978; Mitton *et al.,* 1979; Soltis *et al.,* 1983; Kephart,

1990). Prevention of damage due to phenolics and quinones is best accomplished by adding absorbent or protective agents such as polyvinyl-pyrrolidone (PVP) that can compete with the plant enzymes in reacting with the phenolics and quinones, and at the same time prevent oxidation of phenols (Loomis 1969, 1974; Pitel and Cheliak, 1984). Methods of eliminating these problems in protein extraction have been outlined by Loomis and Battaile (1966), Anderson and Rowan (1967), and Loomis (1974). However, since the production of phenolics is a function of growing conditions as well as the species and variety of plant, it cannot be expected that only one procedure for enzyme extraction will be equally effective with all material (Loomis, 1969; Soltis and Soltis, 1989). Therefore extraction buffers need to be investigated for each type of tissue being used and for each taxon being studied, to achieve optimal enzyme activity and resolution with fewer buffer ingredients (Kelly and Adams, 1977). For a full description on the process of extraction buffer development, refer to Chapter 3 in the results and discussion section "Preparation of Extracts from Citrus Leaves for Electrophoresis".

2.4 Densitometry

To be able to quantify band density differences which occur between allozyme bands, densitometry must be employed. The technique of polyacrylamide gel electrophoresis and isozyme analysis separates and reveals discrete forms (allozymes) of isozyme proteins as bands on the gel medium. These bands are the result of chromophores (dyes) precipitated on the gel as a product of isozyme-catalysed reactions (Vallejos, 1983; Richardson *et al.*, 1986). If a surfeit of substrates and other necessary reagents is available, the concentration of

chromophore in a band will be a function of enzyme activity (Rosalki and Foo, 1984; Ros Barceló, 1987). The darkness of these bands (*i.e* their *density*) can be measured with a densitometer.

2.4 (i) The use of densitometry, and its reliability in comparison with other quantification methods.

Assessment of density of electrophoretic bands has been widely applied to quantify enzymes and other proteins. At the simplest level, this can be achieved by assigning visual rating systems to band density (Chyi and Weeden, 1984; Dessauer and Cole, 1984; Sidhu *et al.,* 1984). Detailed discussions on the densitometric characteristics of general protein stains applied to electrophoretic gels are presented by Cornell (1989), and Neuhoff *et al.,* (1990). No such information is available regarding stains produced by *in situ* enzymatic reactions employed to visualise isozymes.

Nonetheless, as shown in the Table 2.3, densitometric instruments have been used to quantify numerous isozymes and other proteins in gel electrophoresis studies. All such studies are based on the assumption of a linear relationship between band density and the protein concentration or enzyme activity being measured. Because a direct relationship between band density and isozyme activity forms the foundation of the main technique employed in this project, particular attention has been paid to reviewing publications in which similar techniques were used.

In the case of general proteins, dyes are used which bind to the proteins on the gel. The greater the protein concentration the greater the amount of dye binding and hence the darker the band. However, the optical properties of the dye, the nature of the gel matrix and the affinity of the particular protein for the dye necessitate calibration procedure to ensure reliable determinations (Cornell, 1989; Neuhoff *et al.*, 1990). Gambert *et al.*, (1988) found that lipoprotein values determined by gel densitometry were strongly correlated (r = 0.99) with the same samples quantified by the cholesterol assay. Tichy (1985) quantified monoclonal immunoglobulins by densitometry of cellulose acetate electrophoreograms and compared the results with values derived by single radial immunofusion, laser nephelometry and sedimentation analysis. The results indicated that densitometry correlated well with the

Table 2.3

Examples of studies entailing measurement of isozyme activity or protein concentrations using densitometric quantification of electrophoretic bands.

Enzyme/protein	Reference
Acid phosphatase	Fobes (1980)
Alcohol dehydrogenase	Mitra and Bhatia (1971)
Alkaline phosphatase	Burlina and Galzigna (1976) Rosalki and Foo (1984) Rosalki and Foo (1989) Secchiero *et al.*, (1989) Steinberg and Rogers (1987)
Aspartate aminotransferase	Rosendahl *et al.*, (1989)
Esterase	Fobes (1980) Mitra and Bhatia (1971) Rosendahl *et al.*, (1989) Timko *et al.*, (1980)
Glutamate dehydrogenase	Mitra and Bhatia (1971)
Isocitrate dehydrogenase	Mitra and Bhatia (1971)
Lactate dehydrogenase	Triveni and Rao (1986) Kim and Yum (1985) Yum and Kim (1989)

Malate dehydrogenase	Danzmann and Bogart (1982) Mitra and Bhatia (1971)
Peptidase	De Maggio and Lambrukos (1974) Fobes (1980) Ros Barceló (1987)
6-Phosphogluconate dehydrogenase	Gasperi *et al.*, (1983)
Phosphoglucose Isomerase	Gottlieb and Higgins (1984) Linde *et al.*, (1990) Lumaret (1986)
Ribulose biphosphate carboxylase	Tarczynski and Outlaw (1987) Tarczynski et al.,(1989)
Superoxide dismutase	Sevilla *et al.*, (1984) Sevilla *et al.*, (1989)
Other proteins	Autran and Galterio (1989_a) Autran and Galterio (1989_b) Bauer *et al.*, (1985) Csiba and Szecsenyi-Nagy (1989) Dannenberg and Kessler (1986) Gambert *et al.*, (1988) Graml *et al.*, (1989) Hillier (1976) Honeycutt *et al.*, (1989) Ramos *et al.*, (1985) Sato *et al.*, (1986) Sullivan and Johnson (1989) Tichy (1985)
General proteins	Autran and Galterio (1989^a) Autran and Galterio (1989^b) Bauer *et al.*, (1985) Birchler and Newton (1981) Csiba and Szecsenyi-Nagy (1989) DeMaggio and Lambrukos (1974) Ferguson and Grabe (1986) Honeycutt *et al.*, (1989) Kusama *et al.*, (1984) Mansur - Vergara *et al.*, (1984) Neuhoff *et al.*, (1990) Ohmori *et al.*, (1985) Romagnolo *et al.*, (1990) Timko *et al.*, (1980)

other quantification methods. Bauer *et al.*, (1985) compared densitometric analysis and chemical methods for quantification of total proteins, albumin and globulins in equine sera, and in each case recorded correlation coefficients of r = 0.99. These reports clearly

demonstrate that densitometry of electrophoretic bands is a very reliable method of quantification of proteins.

In the case of electrophoresed enzymes, the chromophore is an end-product of a reaction catalysed *in situ* by the enzyme. A substrate and all other necessary reagents must be supplied as well as a conducive environment for the reaction to proceed (Vallejos, 1983). Under optimal conditions the concentration of chromophore precipitated in a band will be a function of enzyme activity, thus allowing densitometry of bands to be used to measure enzyme activity. Rosalki and Foo (1984) demonstrated that alkaline phosphatase activity determined by sequential heat-activation was strongly correlated ($r = 0.98$) with band density on cellulose acetate gels. Similarly, the densities of four different peroxidase bands were all strongly correlated ($r \geq 0.99$ in each case) with peroxidase activity as determined by the benzidine assay (Ros Barceló, 1987).

Electrophoretic band density of carbonic anhydrase was shown to be highly correlated ($r^2 > 0.99$) with carbonic anhydrase concentration loaded on the gel (Tarczynski and Outlaw, 1987). Mitra and Bhatia (1971) measured isozyme band densities for alcohol dehydrogenase, malate dehydrogenase and glutamate dehydrogenase from *Triticum* species of four different ploidy levels. Activity of the three enzymes for each of the four species was also determined by spectrophotometric methods. With both methods, increasing activity with higher ploidy level was observed for all three enzymes.

The extracellular environment of the gel is hostile to isozymes and their activity is relatively short-lived (Richardson *et al.*, 1986; Wendel and Weeden, 1989). During sample preparation

and electrophoresis, every effort must be made to minimise enzyme degradation through strict adherence to defined procedures. Catalysis, and the resultant chromophore precipitation, precedes only while an enzyme is active. Because they degrade quickly, activity of isozymes is synonymous with concentration - the more enzyme present the more substrate that can be catalysed before degradation occurs, and hence the more chromophore precipitated. Accordingly the density of an isozyme band will be proportional to isozyme concentration and to its activity.

By far the most common use of densitometry of electrophoresed proteins entails direct comparison between samples in adjacent tracks within a single gel. This approach has been employed to differentiate between genotypes or species (Mitra and Bhatia, 1971; Kusama *et al.*, 1984; Mansur - Vergara *et al.*, 1984; Ferguson and Grabe, 1986; Autran and Galterio, 1989_{ab}; Graml *et al.*, 1989; Honeycutt *et al.*, 1989; Rosendahl *et al.*, 1989; Linde *et al.*, 1990), and between different tissues (Rosalki and Foo, 1984; Secchiero *et al.*, 1989; Yum and Kim, 1989).

The most conservative approach taken to densitometric quantification of bands on electrophoretic gels is the comparison of bands within a single track, *i.e* from the one sample. This method overcomes the need for stringent control over sample size. The method has been employed with general protein stains where, within each track there are numerous bands available for calculation of density ratios. It has been used to characterise cultivars of wheat (Autran and Galterio, 1989_a), perennial rygrass (Ferguson and Grabe, 1986), soybeans (Honeycutt *et al.*, 1989) and rice (Kusama, 1984).

A number of different approaches have been employed to ensure reliability of densitometric methods. Most entail some standardisation procedure (Hiller, 1976; Rosalki and Foo, 1984, 1989; Bauer *et al.*, 1985; Ramos *et al.*, 1985; Tichy, 1985; Sato *et al.*, 1986; Gambert *et al.*, 1988; Sullivan and Johnson, 1989). Comparative densitometry of electrophoresed isozyme bands, has proven to be a useful technique for investigating dosage effects of duplicated gene loci in polyploids and polysomics. Isozymes studied include, glutamate, isocitrate and malate dehydrogenases, acid phosphatase, esterase, peroxidase, phosphoglucose isomerase and also general proteins (Table 2.3). This procedure entails comparing band densities of adjacent isozyme tracks of samples of different ploidy levels. If the samples are quantitatively equivalent the band densities of different ploidy levels can be compared directly.

The relationship between the concentration of a protein or activity of an enzyme, and the density of its electrophoresed bands should be assessed over a range of concentrations for each application of the densitometric quantification technique. Differing response slopes were observed for the relationship between band densities and concentrations of various proteins (Hiller, 1976; Ramos *et al.*, 1985). Linearity and variability of the response will indicate the reliability of the method for each situation. Dilution series experiments have shown linear responses for alcohol dehydrogenase (Mitra and Bhatia, 1971), albumins and lactoglobulins (Hiller, 1976), general proteins (Birchler and Newton, 1981), heme-protein (Ohmori *et al.*, 1985) caseins (Ramos *et al.*, 1985), carbonic anhydrase (Tarczynski and Outlaw, 1987), peroxidases (Ros Barceló, 1987), alkaline phosphatase (Rosalki and Foo, 1989) and neutral endopeptidase (Sullivan and Johnson, 1989).

Densitometric instruments represent band densities as a bell shaped curve, with band width indicated by the curve width and band darkness indicated by the peak height of the curve. Therefore quantification of band density from densitometric scans can be derived in two ways, (a) the peak height of the curve and (b) the area under the curve. The majority of studies utilise curve area. This approach poses a problem which is usually overlooked. Rarely are adjacent bands on gels clearly separated and the resultant densitometer curves are fused. Often however, merged band curves are simply separated by a vertical line coinciding with the trough between the peaks, hence truncating one tail of each curve. Areas determined from such truncated curves will underestimate the band densities. Recent examples of this occur in Gambert *et al.*, (1988) and Secchiero *et al.*, (1989).

Although curve areas have been shown to increase linearly with protein concentrations, peak height may be a more reliable estimator of band density than area because it overcomes the problem discussed above. Linear relationships have been demonstrated between densitometric peak height and concentrations of various animal proteins (Hiller, 1976; Ramos *et al.*, 1985; Csiba and Szecsenyi-Nagy, 1989). Peak height data was also shown to be well correlated with esterase, aspartate aminotransferase and peptidase activity in mycorrhizal fungi (Rosendahl *et al.*, 1989).

CHAPTER THREE

PREPARATION OF EXTRACTS FOR ELECTROPHORESIS FROM CITRUS LEAVES

Summary

Extracting proteins from vegetative tissues while maintaining good enzyme activity and electrophoretic resolution presents numerous problems due to the presence of phenols, quinones, proteases and other components released during cell disruption. To overcome this problem in *Citrus* leaves, an extraction buffer was developed which contained EDTA, potassium chloride, magnesium chloride hexahydrate, PVP-40, 2-mercaptoethanol and bovine serum albumin in a Tris-HCl buffer pH 7.5. This extraction buffer was used in association with liquid nitrogen for sample preparation. Buffers used in previous studies for *Citrus* isozyme extraction for PAGE were found to provide unsatisfactory resolution and activity for the three enzyme systems investigated (malate dehydrogenase, 6-phosphogluconate dehydrogenase and shikimate dehydrogenase). This extraction buffer maintains high enzyme activity and provides good resolution in PAGE gels suitable for densitometric analysis.

3.1 Introduction

Isozymes have been widely used for phylogenetic studies in *Citrus* (Esen and Soost, 1976; Esen and Scora, 1977; Torres *et al.*, 1978; Hirai and Kozaki, 1981; Hirai *et al.*, 1986; Hirai

and Kajiura, 1987; Ashari *et al.*, 1988; Ashari *et al.*, 1989) and in distinguishing sexual and apomictic seedlings (Iglesias *et al.*, 1974; Torres *et al.*, 1982; Xiang and Roose, 1988). Much of this work has used starch gel electrophoresis (Torres *et al.*, 1978; Torres *et al.*, 1982; Ashari *et al.*, 1988; Roose and Traugh, 1988; Xiang and Roose, 1988; Ashari *et al.*, 1989). Enzyme extracts from leaf samples in starch gel electrophoresis are often prepared directly by crushing the leaves onto filter paper with pliers and transferring the extract to wicks which are inserted into the starch gel and then subjected to electrophoresis (Torres *et al.*, 1978; Torres *et al.*, 1982).

Studies involving quantitative analysis of isozymes however, require the use of polyacrylamide gel electrophoresis (PAGE) (Wendle and Weeden, 1989), and in this system buffer extracted samples are loaded directly into wells of the gel and electrophoresed (Esen and Soost, 1976; Esen and Scora, 1977; Hirai and Kozaki, 1981; Hirai *et al.*, 1986; Hirai and Kajiura, 1987). Plant tissue samples require homogenisation to achieve cell disruption and enzyme release (Wendle and Weeden, 1989). In a living cell, the protoplasm is protected from potentially damaging compounds by compartmentalisation (Loomis, 1974). During plant tissue homogenisation, the compartmentalisation is destroyed releasing phenols, quinones, proteases and other components from vegetative tissues which may result in decreased enzyme activity and poor resolution during analysis in PAGE (Loomis and Battaile, 1966; Anderson and Rowan, 1967; Loomis, 1974; Pitel and Cheliak, 1984; Wendle and Weeden, 1989). These substances may act to inhibit, inactivate or precipitate enzymes (Anderson and Rowan, 1967; Pitel and Cheliak, 1984). Reactions catalysed by phenolase, the phenol enzyme complex (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase.

E.C.1.14.18.1) and subsequent reactions are primarily responsible for the loss of enzyme activity in plant extracts (Pierpoint, 1966; Kelly and Adams, 1977).

It has been previously reported that improved efficacy of extraction is attributed to the removal of endogenous tannins and/or preventing the oxidation of endogenous phenolics (Anderson, 1968). In addition, methods for eliminating these compounds during protein extraction have been outlined by Loomis and Battaile (1966), Anderson (1968), and Loomis (1974). However since the production and level of phenolics is a function of growth conditions, plant species and/or plant variety, it cannot be expected that any one procedure for enzyme extraction will be equally effective with all types of plant material (Loomis, 1969; Soltis and Soltis, 1989).

The following work which has been published (King *et al.*, 1994; Appendix 3) describes a rapid technique for obtaining highly active enzymes with good electrophoretic resolution from *Citrus* leaves. Increased electrophoretic resolution will facilitate future densitometric analysis of *Citrus* isozymes. Current methods for enzyme extraction in *Citrus* for PAGE have involved the use of Sepharose columns, however, this method has the drawback of expense and problems of enzyme denaturation (Hirai and Kozaki, 1981; Hirai *et al.*, 1986; Hirai and Kajiura, 1987). We evaluated 5 extraction buffers, 2 previously used in *Citrus* and 3 general extraction buffers developed for ferns (Soltis *et al.*, 1983; Soltis and Soltis, 1989). The *Citrus* extraction buffers included were used for isozyme extraction from *Citrus* callus (Ben-Hayyim *et al.,* 1982), and in combination with Sepharose columns for enzyme extraction from *Citrus* leaves (Handa *et al.*, 1986). Extraction buffers suitable for achieving high resolution PAGE isozymes from crude *Citrus* leaf tissue preparations have not been

previously reported. Alteration of extraction buffer ingredients were also assessed to achieve optimal activity and resolution for the enzymes examined.

3.2 Materials and Methods

3.2 (i) Sample collection

Fresh mature leaves were collected from *Citrus reticulata* (L.) Blanco cv. Imperial, and extracted immediately upon collection.

3.2 (ii) Basic extraction buffers

Extraction buffers A, B, C, D, and E (Table 3.1) were prepared fresh and used within 48 hours. All buffer ingredients were dissolved in deionised water and adjusted to the required pH. Mercaptoethanol (an inhibitor of *o*-diphenoloxidase) was added after final volume adjustment.

Buffer A: 0.1M Tris-HCl buffer, pH 7.5, 1mM ethylene diamine tetraacetic acid (EDTA), 0.01M potassium chloride, 0.01M magnesium chloride hexahydrate, 4% (w/v) PVP-40 (Polyvinyl-pyrrolidone), 0.1% (v/v) 2-mercaptoethanol.

Buffer B: 0.05M Tris-HCl, pH 7.5, 5% (w/v) sucrose, 0.1% (v/v) 2-mercaptoethanol.

Buffer C: 0.1M Tris-HCl, pH 7.5, 0.2M sodium tetraborate, 0.25M L-ascorbic acid sodium salt, 0.26 M sodium diethyl dithiocarbamate, 0.1M maleic acid, 4% (w/v) PVP-40, 0.1% (v/v) 2-mercaptoethanol.

Buffer D: 0.14M sodium chloride, 23mM sodium nitrate, pH 7.5, 20% (w/v) sucrose.

Buffer E: 0.05M Tris-HCl, pH 7.5, 1mM EDTA, 0.2M sodium chloride, 20% (w/v) PVP-40, 0.1% (v/v) 2-mercaptoethanol.

3.2 (iii) Modifications of extraction buffer A

Six modified extraction buffers were based on extraction buffer A which included the addition of the following components in various combinations (see Table 3.2): bovine serum albumin (BSA) 2mg/ml; dimethyl sulphoxide (DMSO) 10%; nicotinamide adenine dinucleotide (NAD) 0.5 mg/ml; nicotinamide adenine dinucleotide phosphate (NADP) 0.25 mg/ml; sodium metabisulphite 0.02M; Triton X-100 0.1% (v/v); and buffer A without the salts: EDTA; potassium chloride; and magnesium chloride hexahydrate were incorporated before pH adjustment with the exception of Triton X-100.

3.2 (iv) Extraction procedure

Citrus foliage (0.3g) was ground immediately following harvest in a cold mortar with liquid N_2 until a very fine green powder was obtained. Frozen samples were then transferred to Eppendorf tubes containing 0.6ml of the extraction buffer. The samples were kept cool at 0-2°C in an ice/water bath for 30 minutes. Following incubation the samples were centrifuged at 12000 rpm for 2 minutes.

3.2 (v) Electrophoresis

Samples were electrophoresed immediately after extraction using polyacrylamide gel electrophoresis. 30µl samples of supernatant were loaded into the wells, and electrophoresed at less than 10°C for 4-5 hours at 70V with an initial current of 50mA using a 0.34M sodium borate buffer (pH 8.7). Electrophoretic parameters were the following: 8.7% acrylamide (30 : 0.8, acrylamide : N, N'-Methylene-bis-acrylamide), 1.5%(w/v) ammonium persulphate, 0.06%(v/v) TEMED, 1X gel buffer which contains 0.37M Tris-HCl pH 7.5. Gels could be stored between 0-4°C up to 5 days.

3.2 (vi) Gel staining procedures

Gels were stained for malate dehydrogenase, 6-phosphogluconate dehydrogenase and shikimate dehydrogenase following the method of Richardson *et al.,* (1986). For full stain recipes refer to Appendix One.

3.2 (vii) Densitometry

Enzyme activity was measured by band density using a Novaline Gel Documentation System with Q-gel-1D, slab gel imaging and analysis system version 1.46 software (QuantiGel Corp.). Activity was rated into four categories based on peak height: +++, high activity (>60); ++, medium activity (30-60); +, low activity (<30); -, no activity. Resolution was rated visually; S - Sharp, M - Moderate, and P - Poor.

3.3 Results

The results of electrophoresis of samples prepared with 5 different extraction buffers (A-E) are summarised in Table 3.1. Extraction buffer A gave the best electrophoretic band resolution and activity for the three enzyme systems investigated. Using buffer A it was observed that both malate dehydrogenase (MDH) and 6-phosphogluconate dehydrogenase (6PGD) showed high levels of activity, whereas shikimate dehydrogenase (SkDH) showed a satisfactory level of activity. Resolution of all enzyme bands in PAGE were satisfactory. Extraction buffer B showed moderate levels of activity for MDH and 6PGD, but a low level of activity was recorded for SkDH. With the use of this buffer resolution for all three enzymes was satisfactory. Extraction with buffer C resulted in low levels of activity for both SkDH and 6PGD (see Figures 3.1& 3.2), however high levels of activity for MDH (Figure 3.3).

Table 3.1

Enzymatic activity and resolution of electrophoretic bands from *Citrus* leaf tissue samples prepared using 5 basic extraction buffers

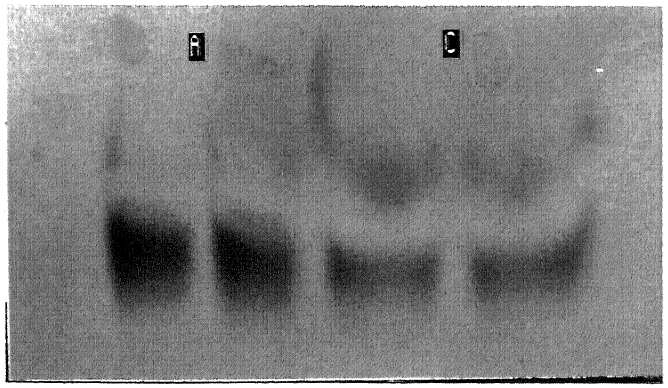
	Isozymes					
Extraction Buffer	**Malate Dehydrogenase**		**6-Phosphogluconate Dehydrogenase**		**Shikimate Dehydrogenase**	
A	M	+++	M	+++	M	++
B	M	++	M	++	M	+
C	P	+++	P	+	P	+
D	N	-	N	-	N	-
E	P	++	P	+	P	+

1) Buffer A: 0.1M Tris-HCl buffer, pH 7.5, 1mM ethylene diamine tetraacetic acid (EDTA), 0.01M potassium chloride, 0.01M magnesium chloride hexahydrate, 4% (w/v) PVP-40, 0.1% (v/v) 2-mercaptoethanol; Buffer B: 0.05M Tris-HCl, pH 7.5, 5% (w/v) sucrose, 0.1% (v/v) 2-mercaptoethanol; Buffer C: 0.1M Tris-HCl, pH 7.5, 0.2M sodium tetraborate, 0.25M L-ascorbic acid sodium salt, 0.26 M sodium diethyl dithiocarbamate, 0.1M maleic acid, 4% (w/v) PVP-40, 0.1% (v/v) 2-mercaptoethanol; Buffer D: 0.14M sodium chloride, 23mM sodium nitrate, pH 7.5, 20% (w/v) sucrose; Buffer E: 0.05M Tris-HCl, pH 7.5, 1mM EDTA, 0.2M sodium chloride, 20% (w/v) PVP-40, 0.1% (v/v) 2-mercaptoethanol.

2) Resolution in PAGE: S - Sharp resolution of bands; M - Satisfactory resolution, some streaking; P - Poor resolution, considerable streaking. N - no bands visible.

3) Enzyme activity (Arbitrary units): +++, high (>60); ++, moderate (30-60); +, Low (<30); -, no activity.

Figure 3.1 Shikimate dehydrogenase - a comparison of enzyme activity and band resolution for the extraction buffers A and C.

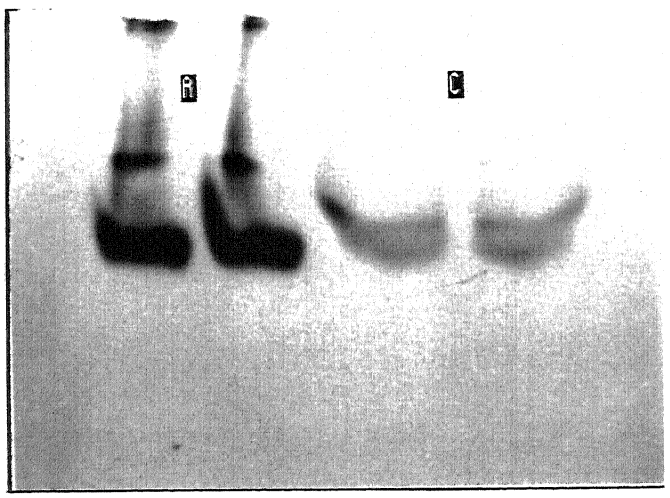


Anode

↓

+

Figure 3.2. 6-Phosphogluconate dehydrogenase - a comparison of enzyme activity and band resolution for the extraction buffers A and C.

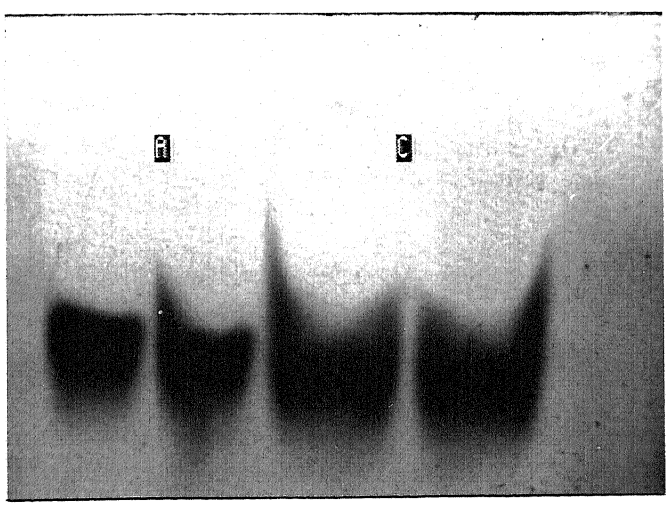


Anode

↓

+

Figure 3.3. Malate dehydrogenase - a comparison of enzyme activity and band resolution for the extraction buffers A and C.



Anode

↓

+

This buffer seriously affected the resolution in PAGE for all enzymes studied resulting in very poor resolution. No enzyme activity was observed for any of the enzyme systems when extraction buffer D was used. MDH showed moderate enzyme activity when extraction buffer E was used, whereas the activity for both SkDH and 6PGD were low and resolution for all these enzymes when extracted with buffer E was poor. Electrophoresis of samples prepared using modifications of extraction buffer A (1-7) are summarised in Table 3.2. The inclusion of BSA in buffer A2 resulted in sharper resolution for all enzymes investigated (see Figures 3.4, 3.5, & 3.6). However no increase in enzyme activity was observed. The addition of DMSO in buffer A3 did not result in any increase in enzyme activity or resolution for any of the enzymes. The combination of DMSO and BSA in buffer A4 failed to increase resolution or activity beyond that observed when BSA was added alone.

Addition of the co-enzymes NAD and NADP in extraction buffer A5 had no effect on increasing either the resolution nor the activity of the enzymes. Buffer A6 included sodium metabisulphite and Triton X-100 in combination with BSA, although resolution was sharp it did not surpass that of buffer A2, which contained BSA alone. The absence of the salts (EDTA, KCl, $MgCl_2.6H_20$) from grinding buffer A7 resulted in a loss of enzymatic activity for all enzymes, however there was no effect on resolution.

3.4 Discussion

Enzyme activity is impaired by phenolic compounds, proteases and other compounds to which enzymes are exposed after tissue homogenisation. As summarised by Anderson

Table 3.2

Enzymatic activity and resolution of electrophoretic bands from *Citrus* leaf tissue samples using modifications of extraction buffer A

	Isozymes		
Extraction Buffer	**Malate Dehydrogenase**	**6-Phosphogluconate Dehydrogenase**	**Shikimate Dehydrogenase**
A1) A	M +++	M +++	M ++
A2) A + BSA	S +++	S +++	S ++
A3) A + DMSO	M +++	M +++	M ++
A4) A + BSA + DMSO	S +++	S +++	S ++
A5) A + NAD + NADP	M +++	M +++	M ++
A6) A + BSA + sodium metabisulphite + Triton X-100	S +++	S +++	S ++
A7) A - EDTA, KCL, $MgCl_2.6H_20$	M ++	M ++	M +

1) Buffer A: 0.1M Tris-HCl buffer, pH 7.5, 1mM ethylene diamine tetraacetic acid (EDTA), 0.01M potassium chloride, 0.01M magnesium chloride hexahydrate, 4% (w/v) PVP-40, 0.1% (v/v) 2-mercaptoethanol;

2) Resolution in PAGE: S - Sharp resolution of bands; M - Satisfactory resolution, some streaking; P - Poor resolution, considerable streaking. N - no bands visible.

3) Enzyme activity (Arbitrary units): +++, high (>60); ++, moderate (30-60); +, Low (<30); -, no activity.

Figure 3.4 Shikimate dehydrogenase - the effect of bovine serum albumin (BSA) in combination with extraction buffer A on band resolution.

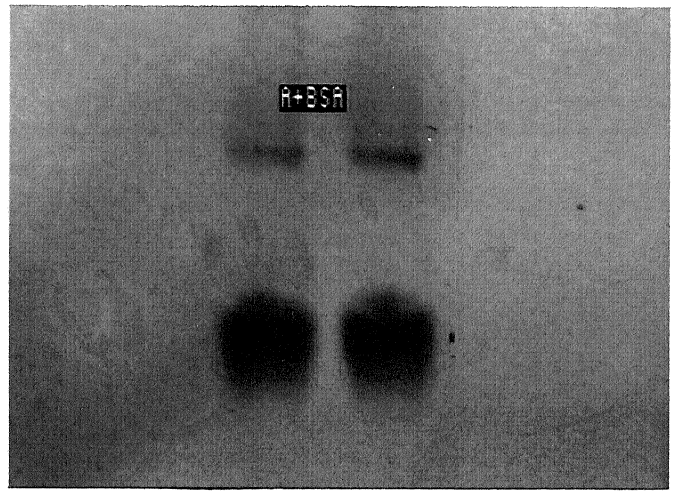


Figure 3.5 6-Phosphogluconate dehydrogenase - the effect of bovine serum albumin (BSA) in combination with extraction buffer A on band resolution.

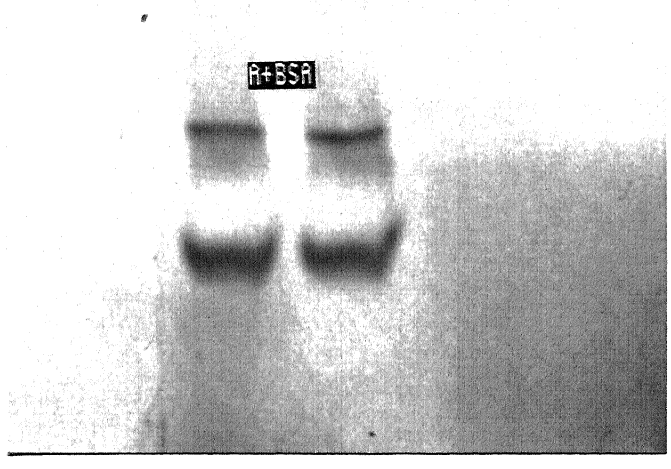
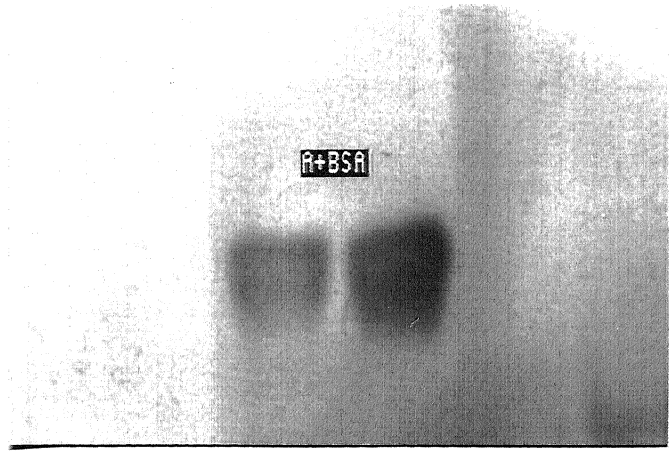


Figure 3.6 Malate dehydrogenase - the effect of bovine serum albumin (BSA) in combination with extraction buffer A on band resolution.



(1968) and Loomis (1974), the formation of oxidation products of the phenolase complex and the subsequent reactions can be inhibited in a variety of ways: removal of the phenolic substrates; inhibition of the phenolase complex activity; and the removal of quinones by reduction back to *o*-diphenols or by condensation with compounds that yield a product which will not be inhibitory to enzymes. Inhibition of proteases will further guard against loss of enzyme activity. In order to reduce the effects of these compounds in *Citrus* foliage it was necessary to include EDTA, potassium chloride, magnesium chloride hexahydrate, polyvinylpyrrolidone (PVP), Bovine serum albumin (BSA) and 2-mercaptoethanol in a 7.5 pH Tris-HCl buffer. All of which by their nature should help combat stated problems of extraction alone or in combination. These buffer components resulted in increased enzyme activity; the addition of BSA resulted in the sharpest band resolution for the three enzyme systems investigated in PAGE.

The increased resolution was attributed to the addition of BSA in the extraction buffers. BSA may function to absorb free fatty acids and is known to efficiently bind phenolic compounds and provides a competitive substrate for protease activity which may thereby reduce the degradation of isozymes. It also reverses uncoupling of low molecular weight phenolics unlike PVP which is less effective (Anderson, 1968; Loomis, 1974; Kephart, 1990).

2-mercaptoethanol acts by inhibiting *o*-diphenoloxidase and by reducing quinones as they are formed. Additionally, 2-mercaptoethanol acts to reduce disulfide bonds resulting from oxidation, thereby restoring the enzyme's native conformation (Anderson, 1968; Kephart, 1990). Buffer D was the only buffer which did not contain 2-mercaptoethanol, and showed no enzyme activity for the three enzyme systems investigated. EDTA, present in buffers A

and E, inhibits phenol oxidase by acting on the copper containing active centre of this enzyme.

It was concluded that the PVP based buffers provide superior protection compared to the sucrose-based buffers. PVP was included in most buffers because of the previous knowledge that its hydrogen atoms bond to phenolics forming insoluble compounds, thereby limiting the ability of phenols to hydrogen bond with oxygen atoms in the peptide bonds of proteins (Anderson, 1968; Loomis, 1969; Pitel and Cheliak, 1984; Kephart, 1990). However excessive amounts of PVP (greater than 20%) in the preparation of grinding buffer can result in a decrease or complete loss of enzymatic activity (Loomis, 1969), which may explain the dramatic reduction in enzymatic activity when the concentration of PVP was increased in buffer E.

Kelly and Adams (1977) found that removal of DMSO from the extraction buffer used for *Juniper* had little effect. DMSO is added to buffers because of its ability to stabilise enzyme extractions during prolonged storage (Kelly and Adams, 1977). The results presented support Kelly and Adams in that the addition of DMSO resulted in no increase in either activity or resolution of any of the enzymes and its exclusion had no more adverse effect than that of other components.

NAD and NADP are electron acceptors essential in the reactions of many enzymatic pathways (Stryer, 1988). They were included in an attempt to increase the activity of the enzymes. However no increase in the activity was observed, which may in part be due to an increase in activity of proteases responsible for the breakdown of the enzymes being investigated.

Triton X-100 and other detergents may be added to enhance the release of membrane bound enzymes from organelles (Kephart, 1990). However its addition to the extraction buffer had no beneficial effect on activity or resolution.

Kelly and Adams (1977) found that when sodium metabisulphite was excluded from their extraction buffer, there was a reduction in both resolution and activity of enzymes under investigation in *Juniper* foliage. Its omission from the extraction buffer appeared to have no detrimental effect on either activity or resolution.

Citrus leaves were ground in liquid nitrogen because it allows fracture of the waxy leaf tissue and cools the extract while providing an oxygen free atmosphere during the initial cellular destruction of the extraction procedure (Kelly and Adams, 1977). The absence of molecular oxygen, required for the conversion of monophenols to diphenols and quinones by the phenolase complex (Kelly and Adams, 1977), may have served an initial benefit to this system.

Loomis's publication (Loomis, 1974) described the "bag of tricks" needed to tackle the special problems of plant enzymology. In support of that conclusion this investigation revealed that it was necessary to employ a similar strategy in our extraction buffer to achieve good enzyme activity and resolution in PAGE of *Citrus* leaf samples. Buffers used by Ben-Hayyim *et al.,* (1982)(Buffer D), and Handa *et al.,* (1986)(Buffer E), proved to be unsatisfactory in the resolution of the three enzyme systems investigated. Overall, the modification of a Soltis buffer, buffer A (Soltis *et al.*, 1983) with the addition of BSA, proved to be highly satisfactory. There remains, however the need for further alterations in the

parameters of gel and electrode buffer systems, for optimisation of *Citrus* isozyme gel band resolution and separation.

CHAPTER FOUR

ELECTROPHORETIC CONDITIONS FOR HIGH RESOLUTION CITRUS ISOZYMES IN POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE).

Summary

Electrophoretic conditions including electrode and gel buffers, acrylamide concentration, use of stacking gels, voltage, current, and run time were investigated in order to produce isozyme bands of high resolution which would facilitate densitometric quantification of enzyme activity following polyacrylamide gel electrophoresis (PAGE). Electrode buffers which provided optimal conditions for gels stained for the isozymes of malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), and shikimate dehydrogenase (SkDH) were 0.02M Tris-glycine pH 8.5, 0.1M sodium borate pH 6.0, 0.1M sodium borate pH 8.7, and 0.07M sodium borate pH 7.0, respectively. A 0.5M Tris-HCl pH 7.5 gel buffer was optimal for gels stained for the isozymes of 6PGD, PGI and SkDH. A 0.5M Tris-HCl pH 8.5 gel buffer was best for gels stained for MDH. Stacking gels were found to be detrimental to enzyme activity and showed no improvement in resolution for any of the enzymes. Acrylamide concentration for gels stained for MDH were 8.7%, gels stained for 6PGD and PGI were 7.5%, while gels stained for SkDH had an acrylamide concentration of 5.0%. Higher concentrations above these levels caused a reduction and in

some cases loss of band activity, while below this concentration there was a decrease in band resolution. Gels stained for MDH yielded best results when run for 6.5 hours at constant current of 5mA/gel and initial voltage of 40V. Gels stained for 6PGD were best after 10 hours at an initial current of 8mA/gel and a constant voltage of 140V. Gels stained for PGI were run for 22 hours at an initial current of 9mA/gel and a constant voltage of 34V and gels stained for SkDH were run for 10 hours at an initial current of 5mA/gel and a constant voltage of 60V. Tris-citrate buffers used widely in *Citrus* and other taxons on both starch and polyacrylamide gels were found to be unsatisfactory. Higher molarity buffers with lower current and longer run times were found to provide superior resolution and band separation in comparison to lower molarity buffers with higher current and shorter run times. Zones of activity previously reported in *Citrus* but not in mandarin cultivars were revealed for both MDH and PGI. Our interpretation of the alleles for SkDH and 6PGD were not in agreement with those previously reported for the cultivars studied. These electrophoretic conditions provide isozyme bands of high resolution on PAGE, which will be suitable for densitometric analysis.

4.1 Introduction

As already stated in the previous chapter most isozyme investigations in *Citrus* have employed starch gel electrophoresis (Torres *et al.*, 1978; Torres *et al.*, 1982; Ashari *et al.*, 1988; Roose and Traugh, 1988; Xiang and Roose, 1988; Ashari *et al.*, 1989). Fewer studies have used polyacrylamide gel electrophoresis (PAGE) (Ben-Hayyim *et al.*, 1982; Esen and Soost, 1976; Protopapadakis, 1987) and consequently, less developmental work has been

undertaken on gel and electrode buffer systems for PAGE of isozymes. The majority of isozyme work on *Citrus* has been used for phylogenetic analysis (Esen and Soost, 1976; Esen and Scora, 1977; Torres *et al.*, 1978; Ashari *et al.,* 1988; Roose and Traugh, 1988; Xiang and Roose, 1988; Ashari *et al.*, 1989). PAGE electrode and gel buffers which can produce isozyme bands of high resolution are required for the quantitative analysis of electrophoretic band densities using densitometric devices. While starch gels provide isozymes of high resolution, variation in the background of gels and their uneven thickness make them unsuitable for densitometric studies. Because of the clarity and consistency of the gel medium, and capacity to precisely control sample size, polyacrylamide is therefore the medium of choice for studies involving densitometric analysis (Hames and Rickwood, 1990).

Factors which have to be taken into account in the selection of an appropriate buffer system include the chemical composition, pH and molarity of both electrode and gel buffers. The pH of a buffer system affects the charge and separation of proteins as well as their activity, but the chemical composition and molarity can also affect band resolution, separation and enzyme activity (Kephart, 1990). Other parameters which also have to be considered in an electrophoretic run include the use of stacking gels, run times, voltage and current, as well as the acrylamide concentration of the gel (Maurer, 1971; Hames and Rickwood, 1990; Hillis *et al.,* 1990; Kephart, 1990).

The following work which has been published (King *et al.*, 1995) describes the development of electrode and gel buffers which would provide high resolution isozymes for PAGE systems for *Citrus*. This would facilitate the investigation of quantitative differences in aspects of gene expression between *Citrus* diploids and their triploid progeny. Modifications

of a variety of buffer systems used in *Citrus* isozyme analysis on starch (Torres *et al.*, 1978; Torres *et al.*, 1982; Ashari *et al.*, 1988; Roose and Traugh, 1988; Xiang and Roose, 1988; Ashari *et al.*, 1989) and polyacrylamide gels (Ben-Hayyim *et al.*, 1982; Protopapadakis, 1987) were investigated as well as buffers developed on starch systems (Soltis *et al.*, 1983; Benzie, 1990) and cellulose acetate (Benzie, 1990) for other taxa. Modification of electrophoretic conditions for the 4 enzyme systems being investigated were also undertaken.

4.2 Materials and Methods

4.2 (i) Sample collection

Fresh leaves for isozyme resolution work were collected from 3 *Citrus* mandarin cultivars, Imperial, Murcott, Ellendale and a *Poncirus* hybrid rootstock, Troyer. Leaves were also collected from progeny of the three mandarin cultivars for genetic analysis of isozyme banding patterns. Crude sample extracts were prepared on the same day. Electrophoresis was conducted on all samples immediately after the extraction process.

4.2 (ii) Extraction buffer

Extraction buffer A2 was used. [see chapter 3 section 3.2(ii) and (iii)]

4.2 (iii) Extraction procedure [see chapter 3, section 3.2 (iv)]

4.2 (iv) Electrophoresis

Vertical slab PAGE was employed using 1.5mm thick gels (gel dimensions 80mm×100mm). TEMED 0.06% (v/v) and ammonia persulphate 1.5% (w/v) were used as initiators for the polymerisation process (Hames and Rickwood, 1990). Gels were cast and allowed to polymerise for 30 minutes. They were then used straight away or stored at 2-4°C for a maximum of 5 days. Samples were loaded into wells, and electrophoresis was carried out at 2-4°C. Various electrode and gel buffer compositions, molarities and pHs were investigated. Different acrylamide concentrations, electrical run conditions and the use of stacking gels to optimise isozyme resolution, activity and separation were also investigated.

4.2 (v) Densitometry

Enzyme activity was measured by band density using a Novaline Gel Documentation System with Q-gel-1D, slab gel imaging and analysis system version 1.46 software (QuantiGel Corp.).

4.2 (vi) Electrode buffer investigations

Chemical composition. Six electrode buffers were tested for their effect on isozyme band resolution, activity and separation, on gels stained for malate dehydrogenase (MDH) (E.C.1.1.1.37).

(a) 0.034M sodium borate pH 8.7 (Torres *et al.*, 1978; Ben-Hayyim *et al.*, 1982)

(b) 0.04M Tris-citrate pH 7.0 (Torres *et al.*, 1978; Torres *et al.*, 1982; Ashari *et al.*, 1988; Ashari *et al.*, 1989)

(c) 0.1M Tris-citrate pH 8.0 (Soltis *et al.*, 1983)

(d) 0.2M Tris-citrate pH 7.5 (Soltis *et al.*, 1983)

(e) 0.01M Tris-glycine pH 8.5 (Protopapadakis, 1987)

(f) 0.05M Tris-maleate pH 7.8 (Benzie, 1990)]

Only electrode buffers (a) - (d) were tested on the gels stained for the other enzymes, 6-phosphogluconate dehydrogenase (6PGD) (E.C.1.1.1.44), phosphoglucose isomerase (PGI) (E.C.5.3.1.9) and shikimate dehydrogenase (SkDH) (E.C.1.1.1.25).

Molarity. Sodium borate pH 8.7 electrode buffer was tested over a range of molarities (0.034M, 0.07M, 0.1M, 0.15M, 0.2M) for its effect on isozyme band resolution, activity and separation on gels which were stained for 6PGD, PGI, and SkDH. Tris-glycine pH 8.5 electrode buffer was tested over a range of molarities (0.01M, 0.015M, 0.02M, 0.03M) and stained for MDH.

pH. The pH of the following electrode buffers, 0.1M sodium borate (gels stained for 6PGD and PGI), 0.07M sodium borate (gels stained for SkDH) and 0.02M Tris-glycine (gels stained

for MDH) were tested over a pH range between 5.8 and 9.0 (see Table 4.1). Gel buffers which were used during the chemical composition, molarity and pH investigations were 0.37M Tris-HCl pH 7.5. The acrylamide concentration used for all gels in this investigation was 8.7%. Electrophoresis was carried out between 2-4°C. For time, voltage and current of the electrophoretic run see *run conditions* in the Materials and Methods section.

4.2 (vii) Gel buffer investigations

Chemical composition. To assess the effect of gel buffers on isozyme band resolution, activity and separation, four gel buffers were tested.

(a) 0.37M Tris-HCl pH 7.5 (Ben-Hayyim *et al.*, 1982)

(b) 0.015M Histidine-HCl pH 7.0 (Soltis *et al.*, 1983)

(c) 0.015M Tris-citrate pH 7.0 (Torres and Bergh, 1980)

(d) 0.03M Tris-citrate pH 7.5 (Torres *et al.*, 1978; Soltis *et al.*, 1983)

These gel buffers were used in combination with a variety of electrode buffers as described in Table 4.2, and stained for 6PGD, PGI, SkDH and MDH. The acrylamide concentration used for all gels in this investigation was 8.7%. Electrophoresis was carried out between 2-4°C. For time, voltage and current of the electrophoretic run see *run conditions* in the Materials and Methods section [4.2 (x)].

Molarity. Tris-HCl gel buffers were tested over a range of molarities (0.2M, 0.37M, 0.5M, 0.6M) to assess the affect of gel buffer molarity on enzyme band resolution, activity and separation. Electrode buffers used were as follows: 6PGD 0.1M sodium borate pH 6.0, PGI 0.1M sodium borate pH 8.7, SkDH 0.07M sodium borate pH 7.0, and MDH 0.02M Tris-

Table 4.1

The pH of the electrode buffers sodium borate and Tris-glycine, for which gels were stained for the enzymes 6PGD, PGI, SkDH and MDH

6PGD(a)	PGI(a)	SkDH(b)	MDH(c)
5.8	8.0	6.5	7.5
6.0	8.5	7.0	8.0
6.2	8.7	7.5	8.5
6.5	9.0	8.7	8.7
7.0		9.0	9.0
8.7			

Legend

(a) Electrode buffer used sodium borate 0.1M

(b) Electrode buffer used sodium borate 0.07M

(c) Electrode buffer used Tris-glycine 0.02M

Table 4.2

Gel buffers investigated, for which gels were stained for the enzymes 6PGD, PGI, SkDH, and MDH

	Isozymes			
Electrode buffers	**MDH**	**6PGD**	**PGI**	**SkDH**
0.02M Tris-glycine pH 8.5	(a)	nil	nil	nil
0.1M Tris-citrate pH 8.0	(b-d)	nil	nil	nil
0.1M sodium borate pH 6.0	nil	(a), (d)	nil	nil
0.1M sodium borate pH 8.7	nil	nil	(a), (d)	nil
0.07M sodium borate pH 7.0	nil	nil	nil	(a), (d)
0.1M Tris-citrate pH 8.0	nil	(d)	(d)	(d)

Legend

Gel buffers investigated

(a) 0.37M Tris-HCl pH 7.5

(b) 0.015M Histidine-HCl pH 7.0

(c) 0.015M Tris-citrate pH 7.0

(d) 0.03M Tris-citrate pH 7.5

nil = investigation not conducted

glycine pH 8.5. The acrylamide concentration used for all gels in this investigation was 8.7%. Electrophoresis was carried out between 2-4°C. For time, voltage and current of the electrophoretic run see *run conditions* in the Materials and Methods section [4.2 (x)].

pH. A range of gel buffer pHs were tested (6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5) to assess the effect of gel buffer pH on isozyme resolution, activity and separation. Electrode buffers used were the same as used in the gel buffer molarity trials. The gel buffers which were used for these investigations were 0.5M Tris-HCl. The acrylamide concentration used for all gels in this investigation was 8.7%. Electrophoresis was carried out between 2-4°C. For time, voltage and current of the electrophoretic run see *run conditions* in the Materials and Methods section.

4.2 (viii) Stacking gel investigations

A 0.1M Tris-HCl pH 6.7 stacking gel was tested for all enzymes. Electrode buffers used were the same as in the two previous investigations. Acrylamide concentration of all gels was 8.7%. The gel buffer used for gels stained for the enzymes 6PGD, PGI and SkDH was 0.5M Tris-HCl pH 7.5. For gels to be stained for MDH a 0.5M Tris-HCl pH 9.0 gel buffer was used.

4.2 (ix) Acrylamide investigations

After optimisation of gel and electrode buffers using an acrylamide concentration of 8.7%, other acrylamide concentrations were tested for all enzymes, between 5.0% and 10.0%. The gel buffers and electrode buffers were the same as those used in the Stacking gel tests.

4.2 (x) Run conditions

Run times for all enzymes were tested over a period between 4 and 26 hours. Both voltage and current were raised and decreased (30-160V, 3-25mA/gel) to achieve optimal conditions for each enzyme system. Constant current versus constant voltage was tested as well.

4.2 (xi) Gel staining procedures

Gels were stained for malate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase and shikimate dehydrogenase following the method of Richardson *et al.*, (1986). For a full description see Appendix One "Isozyme Stain Protocols".

4.3 Results and Discussion

4.3 (i) Electrode buffer results

Chemical composition. Sodium borate electrode buffer had previously been used in *Citrus* on starch gels by Torres *et al.,* (1978) and with polyacrylamide by Ben-Hayyim *et al.,* (1982). It proved to give the best band resolution, separation and activity for the enzymes for 6PGD, SkDH and PGI, while it also gave good resolution and activity for MDH. However, Tris-glycine buffer used by Protopapadakis (1987) and Esen and Soost (1976) with polyacrylamide gels provided the best results for MDH. A variety of Tris-citrate buffers were tested on gels which were stained for all enzymes. Tris-citrate buffers have been widely used in starch gel electrophoresis of isozymes for a wide variety of taxa (Soltis *et al.*, 1983; Torres and Bergh, 1980) including *Citrus* (Torres *et al.*, 1978; Torres *et al.*, 1982; Ashari *et al.*, 1988; Ashari *et al.*, 1989). However with PAGE under the conditions described, these buffers resulted in a reduction in enzyme band resolution (increased streaking), and enzyme activity. In the case of MDH the faster moving (anodal) isozymes did separate better, but these bands were badly streaked. A Tris-maleate buffer was used for MDH (Benzie, 1990), but resulted in no enzyme activity, and was not used for any of the other enzymes.

Molarity. Large increases in molarity resulted in much improved band resolution without any loss in enzyme activity for 6PGD, PGI and SkDH. However at higher molarities band separation was reduced which necessitated greatly increased electrophoretic run times (refer to section on run times). High ionic strength buffers promote the stabilisation of protein zones, due to more of the electric current being carried by the buffer instead of the protein.

These buffers have slower migration rates and therefore longer electrophoretic runs are required (Kephart, 1990). A problem associated with high ionic strength buffers is the increase in heat production, therefore electrophoresis must be performed at a low temperature (2-4°C). The 0.034M electrode buffer used by Torres *et al.,* (1978) and Ben-Hayyim *et al.,* (1982) with shorter run times was found to be inferior to the electrode buffers with higher molarity using longer run times. A 0.1M sodium borate electrode buffer provided optimal enzyme activity, separation and band resolution for 6PGD and PGI. Increases in molarity above 0.1M resulted in decreases in both enzyme activity and separation for these enzymes. For shikimate dehydrogenase a 0.07M sodium borate electrode buffer provided optimal conditions. Molarity trials were also run on Tris-glycine which resulted in increased resolution up to 0.03M, however at this level there was a sharp decrease in band separation and a total loss of enzyme activity of the slowest moving (cathodal) isozyme bands. A 0.02M Tris-glycine electrode buffer provided optimal conditions.

pH. The pH appeared in all cases to have little effect on band resolution. However it did strongly influence enzyme activity and band separation. Because different enzymes are ionised under different pH conditions, 6PGD, SkDH, PGI and MDH were tested over a pH range between 5.8 and 9.5 to find the most suitable pH for each of the isozymes. Enzyme activity for 6PGD was satisfactory over this range, however band separation was dramatically reduced above pH 6.0. Enzyme activity for SkDH increased with a pH decrease from 8.7 to 7.0. SkDH band separation was reduced at a pH higher than 7.0. PGI activity was dramatically reduced when the pH was decreased below 8.7, but band separation was satisfactory over the pH range. MDH activity for the faster migrating isozymes was satisfactory over a wide pH range. However at a pH less than 8.5 there was a total loss in

enzyme activity for the slower migrating isozymes of MDH. Band separation was satisfactory over the pH range.

4.3 (ii) Gel buffer results

Chemical composition. The Tris-HCl gel buffer used by Ben-Hayyim *et al.,* (1982) and Protopapadakis (1987) provided superior resolution for MDH. The Tris-citrate and Histidine-HCl buffers used by Torres *et al.,* (1978) and Soltis *et al.,* (1983) produced enzyme bands with a greater degree of streaking. For gels stained for enzymes 6PGD, PGI and SkDH only Tris-HCl and Tris-citrate buffers were tested. The Tris-HCl buffers provided the best resolution and activity, whereas Tris-citrate buffers showed a greater degree of streaking of the enzymes. The continuous Tris-citrate system used by Torres *et al.,* (1978) and Soltis *et al.,* (1983) showed increased streaking and reduced enzyme activity compared to the discontinuous system of a sodium borate electrode buffer and a Tris-HCl gel buffer.

Molarity. Increasing gel molarity from 0.37M to 0.5M resulted in improved resolution, but did not alter activity, or band separation. Increases above 0.5M resulted in decreases in both enzyme activity and band separation and in some cases total loss of isozyme bands. A decrease in molarity below 0.37M, resulted in poorer resolution and streaking.

pH. For gels stained for the three enzymes 6PGD, PGI and SkDH, a gel pH of 7.5 was found to be optimal. At higher pH there was a reduction in band separation for 6PGD and SkDH, while the fastest moving band for PGI became streaked. At a lower pH there was a dramatic reduction in enzymatic activity for these three enzymes. For gels stained for MDH activity it

was found that a decrease below a gel pH of 9.0 resulted in the loss of the slower moving bands. A higher pH resulted in an over-intense stained reaction.

4.3 (iii) Stacking gels

The Tris-HCl 0.1M pH 6.7 stacking gels resulted in decreased enzymatic activity, increased streaking and no increase in band resolution for all enzymes.

4.3 (iv) Acrylamide concentration

The optimal concentration of acrylamide for gels to be stained for MDH was 8.7%, for gels stained for 6PGD and PGI, the best acrylamide concentration was 7.5%, and 5% acrylamide was found to be best for gels stained for SkDH. At higher concentrations there was a decrease in enzymatic activity and in some cases a loss of the slower moving isozyme bands. At concentrations lower than described here there was a decrease in band resolution, and a greater degree of streaking evident.

4.3 (v) Run conditions

The optimal run conditions for the enzymes investigated were as follows: 6PGD, 10 hours, an initial current of 8mA/gel and a constant voltage of 140V; PGI, 22 hours, an initial current of 9mA/gel and a constant voltage of 34 V; SkDH, 10 hours, an initial current of 5mA/gel and a constant voltage of 60 V; and MDH 6.5 hours, a constant current of 5mA/gel and a initial voltage of 40V. These run conditions are significantly different from those used in past investigations, which have been 3 to 8 hours in length at a much higher voltage and current (Esen and Soost, 1976; Esen and Scora, 1977; Torres *et al.*, 1978; Torres and Bergh, 1980; Torres *et al.*, 1982; Ashari *et al.*, 1988; Ashari *et al.*, 1989; Ben-Hayyim *et al.*, 1982; Protopapadakis, 1987; Kephart, 1990). Because of the increased molarity of the gel and electrode buffers, migration rate of the enzymes was reduced and therefore run times were increased. Current was kept low since the enzymes were prone to denaturing with increased heat production. All optimal electrophoretic conditions for the four enzyme systems are summarised in Table 4.3.

4.3 (vi) The densitometric studies of enzyme bands

For future densitometric analysis of MDH the slowest migrating bands will be of use since they have good separation and resolution (Figure 4.1). The heavier staining and faster migrating bands may not be as useful since there is no definite separation of these bands and resolution is poorer (Figure 4.2). These highly active isozymes appear to exhaust the available substrate or other reagents thus limiting the potential band density. However these faster migrating bands may be useful at much lower dilutions.

Table 4.3

Electrophoretic conditions which provide high resolution isozyme bands for the 4 enzyme systems investigated.

Isozyme	Electrode Buffer	Gel Buffer	Run Conditions	Acrylamide
MDH	0.02M Tris-glycine	0.5M Tris-HCl	6.5 hours	8.7%
	pH 8.5	pH 9.0	*Amp = 5mA	
			Volt. = 40 V	
6PGD	0.1M $NaBO_3$	0.5M Tris-HCl	10 hours	7.5%
	pH 6.0	pH 7.5	Amp = 8mA	
			*Volt. = 140 V	
SkDH	0.07M $NaBO_3$	0.5M Tris-HCl	10 hours	5.0%
	pH 7.0	pH 7.5	Amp = 5mA	
			*Volt. = 60 V	
PGI	0.1M $NaBO_3$	0.5M Tris-HCl	22 hours	7.5%
	pH 8.7	pH 7.5	Amp = 9mA	
			*Volt. 34 V	

Legend

*Volt = Constant Voltage

*Amp = Constant Current

Volt = Initial Voltage

Amp = Initial Current

Figure 4.1 The slowest migrating MDH isozyme bands, MDH-a (slowest) and MDH-b, for the mandarin cultivar Murcott.

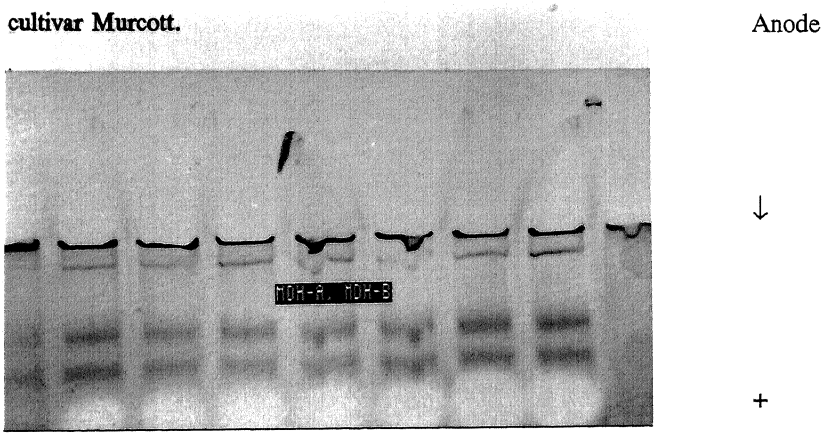
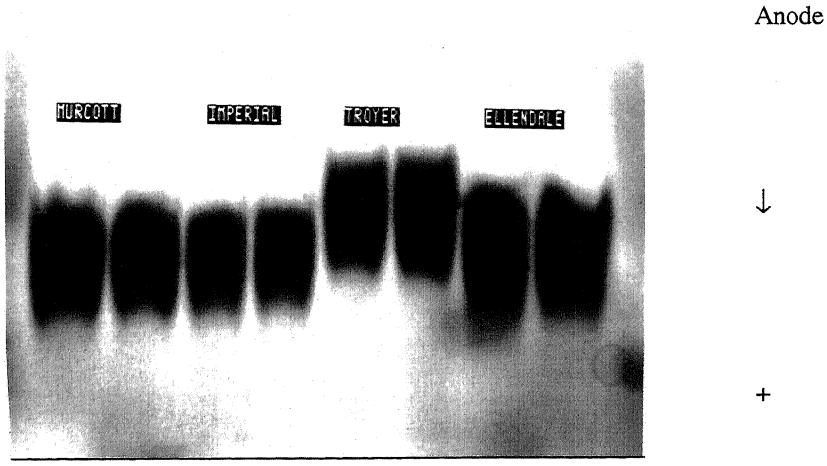


Figure 4.2 The fast migrating and overlapping MDH isozyme bands, MDH-1 and MDH-2 for the cultivars (from left to right) Murcott, Imperial, Troyer and Ellendale.



All bands for 6PGD especially the darker staining bands may be useful for densitometric studies (Figure 4.3). The slower migrating PGI bands have sharp definition and will also be useful for densitometric analysis (Figure 4.4). However, the faster migrating PGI bands have excessive activity (Figure 4.5) as in the case of MDH. Also there is a problem with the faster moving PGI bands with the formazan stain only precipitating on the surface of the gel. This problem appears to be caused by the lack of penetration of the linking enzyme used in the reaction into the pores of the acrylamide gel (Hames and Rickwood, 1990). Unfortunately, when gel pore size was increased to allow greater penetration of the linking enzyme, band resolution deteriorated considerably. Therefore the faster migrating PGI bands may not be useful for densitometric studies. All bands for SkDH will be useful for densitometric studies (Figure 4.6).

4.3 (vii) Genetic interpretation of banding patterns

MDH is dimeric and has been reported for a wide range of *Citrus* cultivars (Hirai and Kozaki 1981; Hirai *et al.*, 1986; Xiang and Roose, 1988; Ashari *et al.*, 1989). {For a full review of the literature relating to MDH refer to pages 20-21 of the Literature review}. Using the densitometer we were able to distinguish two large overlapping areas of activity on the polyacrylamide gel, which were deemed to be MDH-1 and MDH-2 (Figure 4.2). We were also able to distinguish two slower migrating areas of activity which had not been reported for mandarin cultivars (Ashari *et al.*, 1989). These two areas of isozyme activity were termed MDH-a (slowest migrating) and MDH-b (fastest migrating) (Figure 4.1). Both MDH-a and MDH-b were present in all three mandarin cultivars, but appeared to be absent in the *Poncirus* hybrid rootstock Troyer.

Figure 4.3 The allozyme of 6-PGD for the cultivars (from left to right) Troyer, Imperial, Ellendale and Murcott.

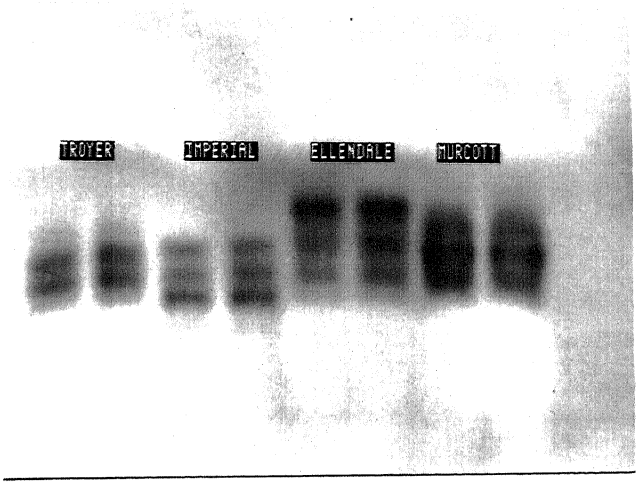


Figure 4.4 The slow migrating bands of PGI, PGI-1 for the cultivars (from left to right) Troyer, Murcott, Imperial and Ellendale.

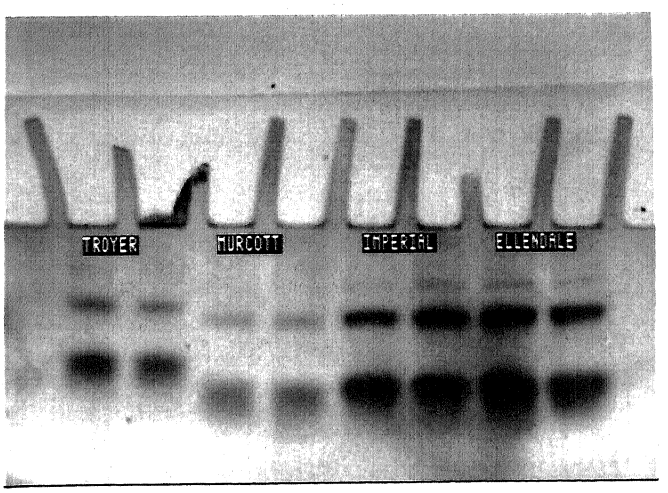


Figure 4.5 The isozymes PGI-1 and PGI-2 for the cultivars (from left to right) Troyer, Murcott, Imperial and Ellendale.

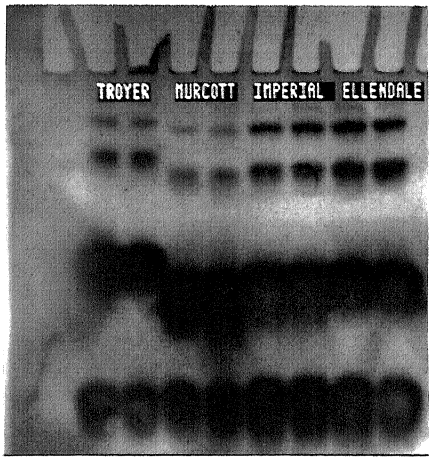
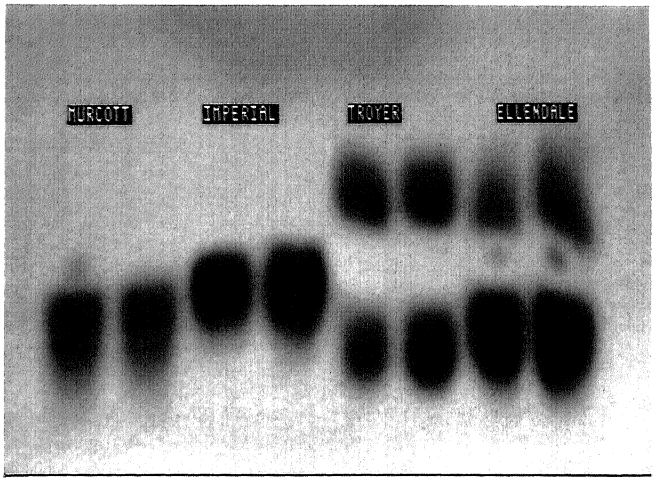


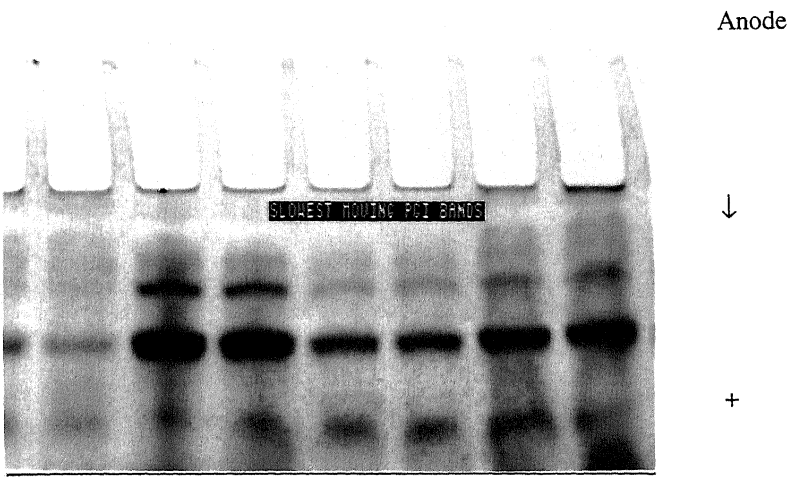
Figure 4.6. The allozymes of SkDH for the cultivars (from left to right) Murcott, Imperial, Troyer, and Ellendale.



PGI is also reportedly a dimeric enzyme (Richardson *et al.*, 1986). Torres *et al.*, (1978) reported that PGI isozymes occur in two zones. The slower migrating set number one or three bands and are specified by a gene called PGI-1 having four alleles. The faster migrating set as designated PGI-2. Ashari *et al.*, (1989) only reported on one band which they termed as FF for the 3 mandarin cultivars and one band which they termed SS for Troyer.

Polyacrylamide gels run under the conditions described here, and stained for PGI showed two clear zones of activity for all three mandarin cultivars (Figure 4.5). The slower migrating zone was PGI-1 while the faster migrating zone was PGI-2. For the slower migrating zone, PGI-1, two alleles were clearly visible and were determined to be homozygous at both loci for the three mandarin cultivars (FF-a, slower) (FF-b, faster) investigated and the *Poncirus* hybrid rootstock Troyer (SS, SS) (Figure 4.4). A slower migrating and very weakly staining set of three isozymes were present, and more evident when the voltage of the electrophoretic run was raised from 34V to 60V. The zymogram of Ellendale mandarin progeny suggest that these bands were also homozygous (Figure 4.7). These electrophoretic conditions however were unsuitable for the other isozymes of PGI. For the faster migrating zone two alleles were visible and were determined to be homozygous (FF-a, FF-b) at both loci for the three cultivars Imperial, Murcott and Ellendale and homozygous (FF, SS) for the Poncirus hybrid rootstock Troyer (Figure 4.5).

Figure 4.7 The slowest migrating bands of PGI (PGI-1) of the progeny of the maternal parent Ellendale, after increasing Voltage from 34 to 60V.



Shikimate dehydrogenase is monomeric and Moore and Castle (1988) identified one SkDH isozyme in *Citrus*. Ashari *et al.*, (1989) reported Imperial mandarin to be homozygous (FF), and Ellendale and Murcott to be heterozygous (MS and FM respectively) at this locus. While our investigations found homozygosity and heterozygosity commensurate with the findings of Ashari *et al.,* (1989) for these cultivars, we are not in agreement with the interpretation of the different alleles. Our findings indicated that the alleles were, MM (Imperial), FS (Ellendale) and FM (Murcott) while Troyer was determined to be (FS) (Figure 4.6). However these genotypes are only putative.

In higher plants 6PGD is reportedly dimeric (Torres *et al.*, 1982; Kephart, 1990). This is possibly the case in *Citrus* (Hirai and Kozaki, 1981). Ashari *et al.,* (1989) reported a heterozygous locus in both Imperial (FI) and Murcott (MI), and a homozygous locus in Ellendale (II). However our results indicate that all three mandarin cultivars and the Poncirus hybrid rootstock Troyer are heterozygotes (Figure 4.3). While allozyme band migration distances for the cultivars Ellendale, Murcott and Troyer are similar, there are slight differences in their migration rates. Differences also existed between the level of activity of the individual allozymes for each of the cultivars under the same electrophoretic conditions, suggesting that these three cultivars posses different alleles.

CHAPTER FIVE

THE RELATIONSHIP BETWEEN ISOZYME BAND DENSITY AND ISOZYME ACTIVITY

Summary

A linear relationship was shown to exist between sample load (reflecting enzyme activity) and isozyme band density for the enzymes, malate dehydrogenase, phosphoglucose isomerase, shikimate dehydrogenase, and 6-phosphogluconate dehydrogenase. Peak height was determined to be a more reliable estimate of band density than area under the curve. The fast migrating bands of PGI-2 were the only bands which were analysed and not found to exhibit a linear relationship between sample load and isozyme band density. Although bands of all other isozymes increased linearly with sample load, at higher concentrations some of these bands exhibited a down-trend suggesting a limitation in the enzymatic reaction. Since a linear relationship exists between band density and isozyme activity at lower sample concentrations, densitometry could be applied to study isozyme activity differences between diploids and triploids.

5.1 Introduction

Polyacrylamide gel electrophoresis was employed to investigate differences in quantitative aspects of gene expression between *Citrus* diploids and their triploid progeny. This technique separates and reveals discrete forms of isozyme proteins (allozymes) as bands on the gel

medium. These bands are the result of chromophores (dyes) precipitated on the gel as a product of isozyme-catalysed reactions (Vallejos, 1983; Richardson *et al.*, 1986). If a surfeit of substrates and other necessary reagents is available, the concentration of chromophore in a band will be a function of enzyme activity (Rosalki and Foo, 1984; Ros Barceló, 1987). The darkness of these bands (i.e. their density) can be measured with a densitometer. If it could be shown that there is a linear relationship between band density and isozyme activity, then these techniques could be applied to study isozyme activity differences between diploids and triploids. We expected that hybrid triploid *Citrus* should exhibit characteristic differences in relative band densities within isozyme tracks compared to their diploid progenitors. It was hypothesised that, where no gene dosage regulation effects occur, greater band density would be evident in the allozyme contributed by the diploid gamete (usually maternal) relative to that of the haploid gamete. This effect is due to the extra protein coded by the additional gene dose in the triploid.

For densitometry to provide a valid method of studying quantitative aspects of gene expression, a direct relationship must be shown to exist between measured band density and isozyme activity. Assessment of density of electrophoretic bands has been widely applied to quantify enzymes and other proteins (For full review of literature relating to quantification of enzymes and proteins refer to the densitometry section [2.4] in the literature review of Chapter 2).

The purpose of this work was to show that such a relationship between band density and sample load (reflecting isozyme activity) existed for the 4 enzymes, malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), shikimate dehydrogenase (SkDH), and 6

phosphogluconate dehydrogenase (6PGD) in the 3 mandarin cultivars Imperial, Ellendale and Murcott. Two parameters, peak height (measurement of maximum band darkness) and area under the curve (measurement of total enzyme present in the band) were investigated to determine which was the most reliable estimate of band density.

5.2 Materials and Methods

For extraction procedure see Chapter 3, section 3.2 (iv), for sample collection, extraction buffer, and gel staining procedures see Chapter 4, sections 4.2(i), 4.2(ii), 4.2(iii), 4.2(xi), respectively and for electrophoretic conditions see Chapter 4 Table 3.

5.2 (i) Dilution series

Samples were weighed out and 800µl of extraction buffer was added. Initial extracts were diluted with extraction buffer to produce a linear series of various sample concentrations. If enzyme activity was excessive a linear response of band density to increasing sample load was not achieved. Excessive activity resulted in a response plateau indicating a limitation in the enzymatic reaction. In such cases extracts were further diluted to obtain a linear response. Four replications were performed for each treatment, with samples randomly assorted within a gel. Different leaf samples were taken for each replication. Outermost wells were left free of sample extracts and loaded with extraction buffer and marker dye.

5.2 (ii) Densitometry

Sample load (reflecting enzyme activity) was measured by band density using a Novaline Gel Documentation System with Q-gel 1-D-slab gel imaging and analysis system version 1.46 software (Quantigel Corp). Densitometer software produced a bell-shaped curve representing band density. Two parameters, peak height (amplitude of the curve) and area under the curve were investigated to determine which parameter was the most reliable estimate of band density.

5.2 (iii) Statistical analysis of data

Linear regressions were performed on each area of isozyme activity on the darker bands, which would provide the most reliable densitograms (Table 5.1). Regressions were performed on all enzymes for each cultivar. Each treatment (cultivar × enzyme) had four replicates each comprising a separate gel. The response slope of the linear regression for each replicate was analysed for significant differences. Data from all replicates were pooled if no significant difference was found between the slopes of the replicates. A significant difference between the intercepts of the linear regressions of the replicates was ignored since this investigation does not examine differences between gels, but differences within a gel only. Significant differences between intercepts only indicate a block effect which is expected, since there is no stringent control over enzyme staining reagents (due to agar overlay method), and sample size of the replicates may have varied as would the moisture content of the different leaves used in each replicate.

Table 5.1

Isozyme bands investigated for a linear relationship between band density (measured by a densitometer) and sample load (reflecting enzyme activity) for the Mandarin cultivars Imperial, Ellendale and Murcott.

Isozyme	Cultivar		
	Imperial	**Ellendale**	**Murcott**
Malate Dehydrogenase	MDH-b MDH-1, MDH-2	MDH-b MDH-1, MDH-2	MDH-b MDH-1, MDH-2
Phosphoglucose Isomerase	PGI-1, (FF-a, FF-b) PGI-2, (FF-a, FF-b)	PGI-1, (FF-a, FF-b) PGI-2, (FF-a, FF-b)	PGI-1, (FF-a, FF-b) PGI-2, (FF-a, FF-b)
Shikimate Dehydrogenase	(MM)	(FF, SS)	(MM)
6 Phosphogluconate Dehydrogenase	(MM, MF)	(RR, RI, II)	(SI, II)

Legend: (Band Migration)

R : Retarded

S : Slow

M : Medium

I : Intermediate

F : Fast

a : Slower migrating isozyme

b : Faster migrating isozyme

5.3 Results and Discussion

5.3 (i) Determination of band density using peak height and area under the curve.

For the determination of enzyme band density, two parameters were investigated, peak height and area under the curve (mean of four replicates). The r^2 values for the linear relationship between area under the curve and sample load (reflecting enzyme activity) were consistently lower than those for the linear relationship between sample load and peak height (Table 5.2). This is due to bands within tracks not being well separated and the resultant curves being fused. These merged band curves are separated by a vertical line coinciding with the trough between the peaks, hence truncating one tail of each curve. Those areas determined from such truncated curves underestimate the band density of isozyme bands, therefore resulting in a poorer linear relationship between band density and sample load. Therefore peak height was determined to be a more reliable estimate of band density than area under the curves.

5.3 (ii) Linear regression of sample load and peak height.

Whereas response slopes differed significantly between isozymes there were no significant differences between the response slopes for each of the replicates and therefore data from all replicates were pooled. The slower migrating band, MDH-b, in the cultivars Imperial, Ellendale and Murcott showed a significant linear relationship ($P<0.01$) between band density and sample load (Figures 5.1, 5.2, 5.3). This band should be useful for triploid-diploid investigations in *Citrus* for the three cultivars in question. The heavier staining and faster migrating bands of the isozymes MDH-1 and MDH-2, were highly active isozymes which

Table 5.2

Mean r^2 values of sample load against band peak height and area under the curve

Isozyme Band	Cultivar		
	Imperial	**Ellendale**	**Murcott**
MDH-b	0.98**, ***0.90***	0.92**, ***0.74****	0.97**, ***0.82***
MDH-1	0.72*, ***0.70****	0.89**, ***0.74****	0.97**, ***0.71****
MDH-2	0.83**, ***0.71****	0.86**, ***0.72****	0.96**, ***0.72****
PGI-1			
(FF-a)	0.86**, ***0.86***	0.93**, ***0.86***	0.80**, ***0.72****
(FF-b)	0.89**, ***0.82***	0.91**, ***0.86***	0.82**, ***0.70****
SkDH			
(F)		0.97**, ***0.91***	
(M)	0.87**, ***0.82***		0.92**, ***0.80***
(S)		0.96**, ***0.84***	
6PGD			
(MM)	0.99**, ***0.99***		
(MF)	0.98**, ***0.90***		
(RI)		0.89**, ***0.88***	
(SI)			0.96**, ***0.89***
(II)		0.87**, ***0.86***	0.96**, ***0.93***

Legend

** $P < 0.01$

* $P < 0.05$

normal text = Regression of band height against sample load

Bold, italics text = Regression of area under the curve against sample load

Figure 5.1 Response slope of band density (peak height) to increasing sample load for the isozymes of Malate dehydrogenase in the *Citrus* cultivar Imperial.

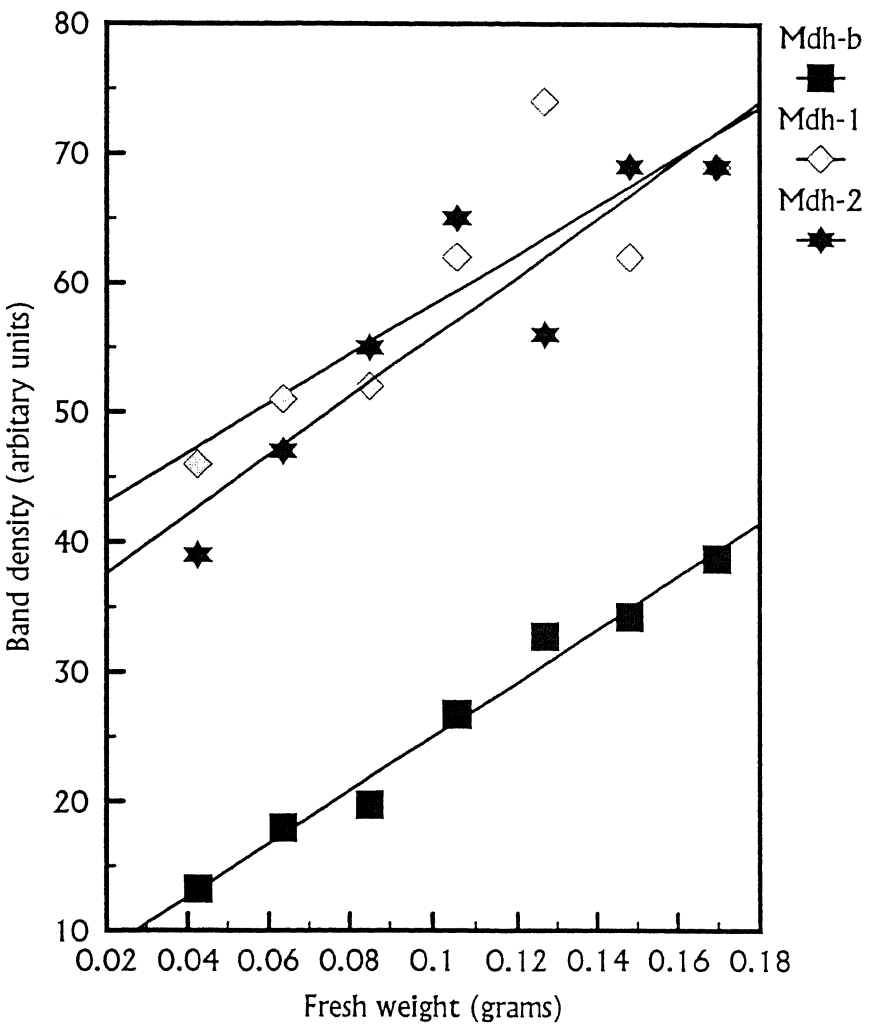


Figure 5.2 Response slope of band density (peak height) to increasing sample load for the isozymes of Malate dehydrogenase in the *Citrus* cultivar Ellendale.

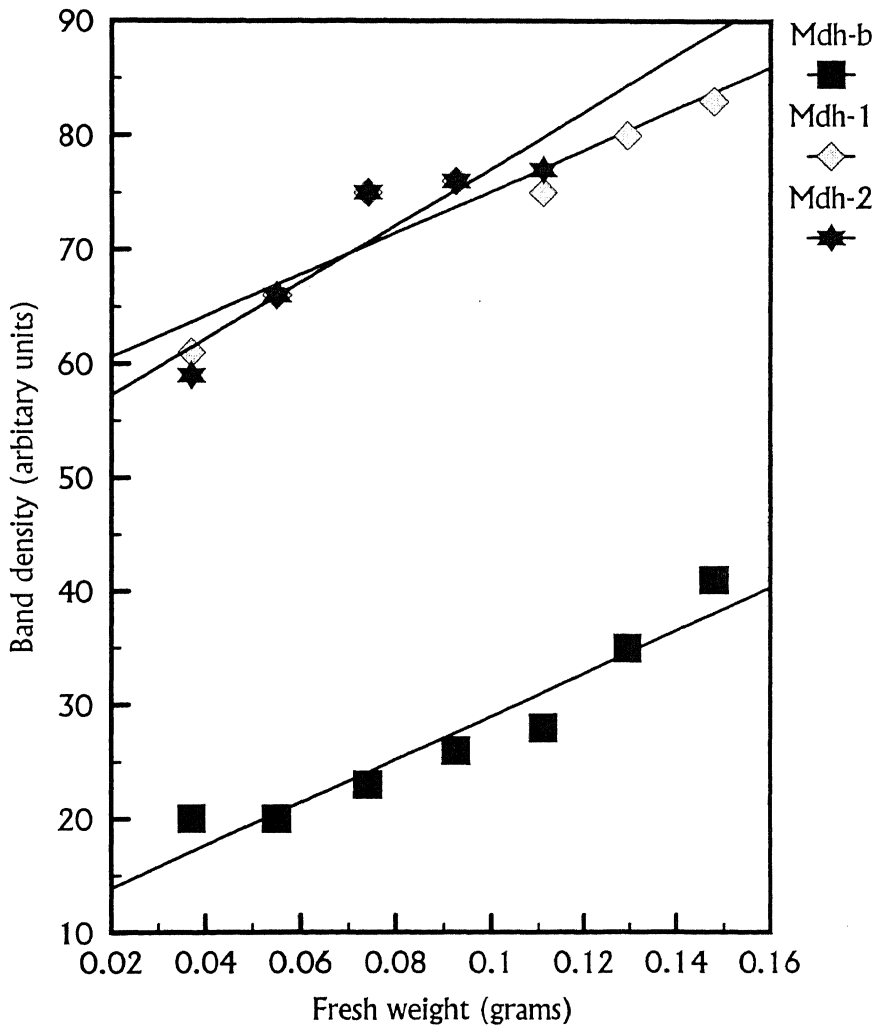
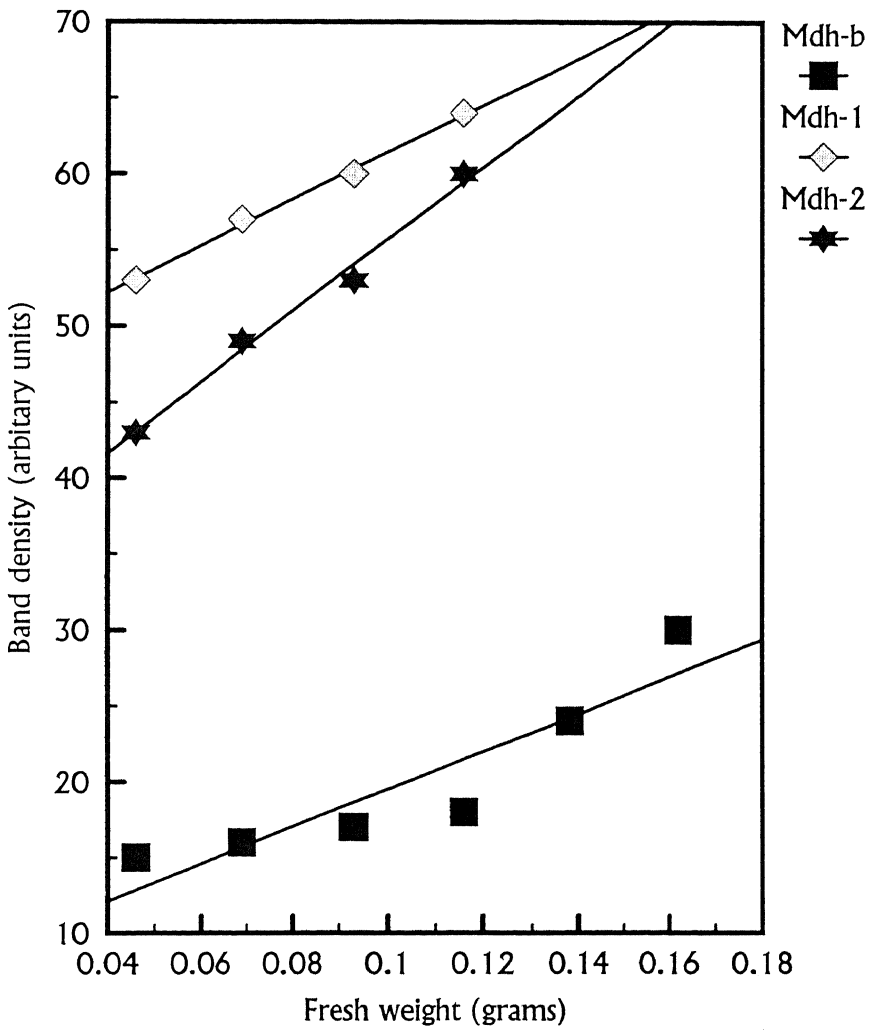


Figure 5.3 Response slope of band density (peak height) to increasing sample load for the isozymes of Malate dehydrogenase in the *Citrus* cultivar Murcott.



appeared to exhaust the available substrate or other reagents thus limiting the potential band density. A limit in the capacity of the gel matrix to accumulate chromophore may have also existed and therefore resulted in a nonlinear relationship. Therefore while the isozymes of MDH-1 and MDH-2 should be useful for triploid-diploid investigations at high dilution levels, the slower migrating band MDH-b should be useful at lower dilution levels.

The slower migrating PGI-1 bands showed a significant linear relationship ($P<0.01$) between band density and sample load (Figures 5.4, 5.5, 5.6). These bands should therefore be useful for triploid-diploid investigations. However for the thicker and faster migrating bands for PGI, PGI-2 (FF-a, FF-b), a linear regression was not performed between sample load and band density, since band densities indicated a strong plateau effect, even at high dilution levels (Figures 5.7, 5.8, 5.9). This problem is due to the precipitation of the formazan stain only on the surface of the gel, which appears to be caused by the lack of penetration of the linking enzyme used in the *in situ* reaction for PGI into the pores of the gel, therefore limiting potential band density. These fast migrating bands will not be useful in our triploid-diploid investigations.

All bands which were analysed for SkDH, (Figures 5.10, 5.11, 5.12) showed a significant linear relationship ($P<0.01$) between sample load and band density. Therefore all bands of SkDH should be useful in quantifying any band density differences which exist between triploid and diploid *Citrus* in the cultivars Imperial, Ellendale and Murcott.

Figure 5.4 Response slope of band density (peak height) to increasing sample load for the isozymes (FF-a, FF-b) of Phosphoglucose Isomerase-1 (PGI-1) for the *Citrus* cultivar Imperial.

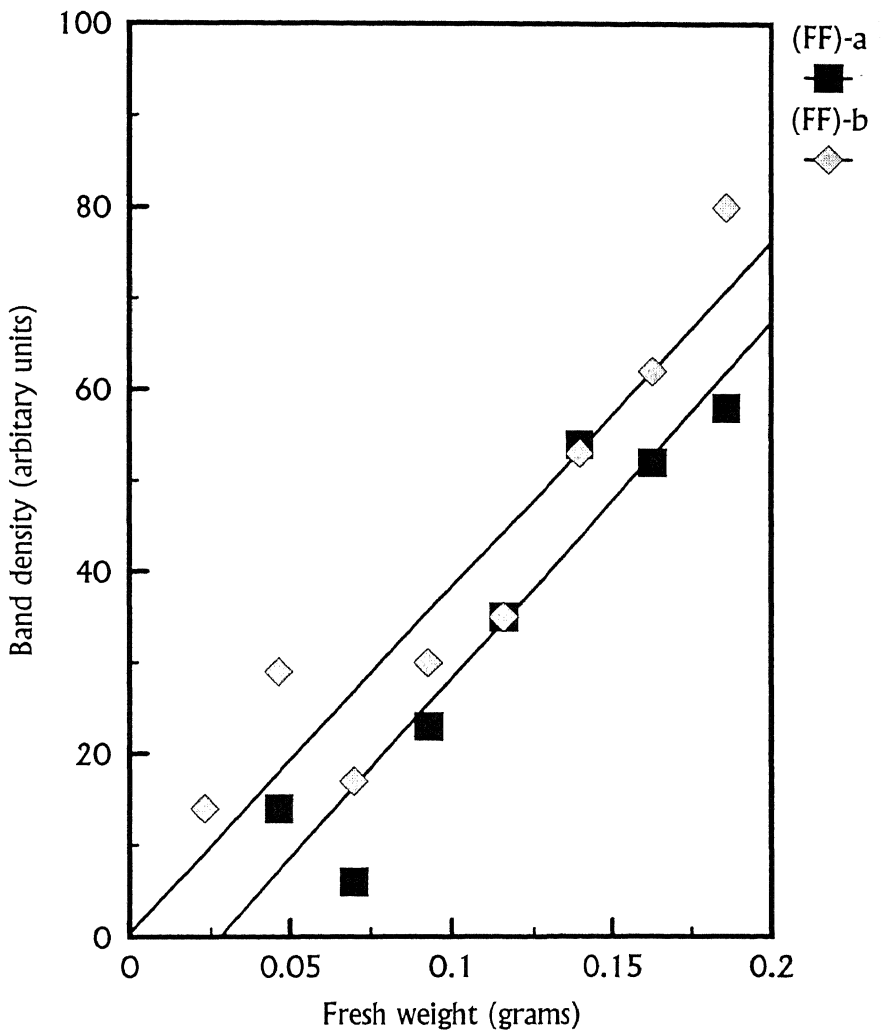


Figure 5.5 Response slope of band density (peak height) to increasing sample load for the isozymes (FF-a, FF-b) of Phosphoglucose Isomerase-1 (PGI-1) for the *Citrus* cultivar Ellendale.

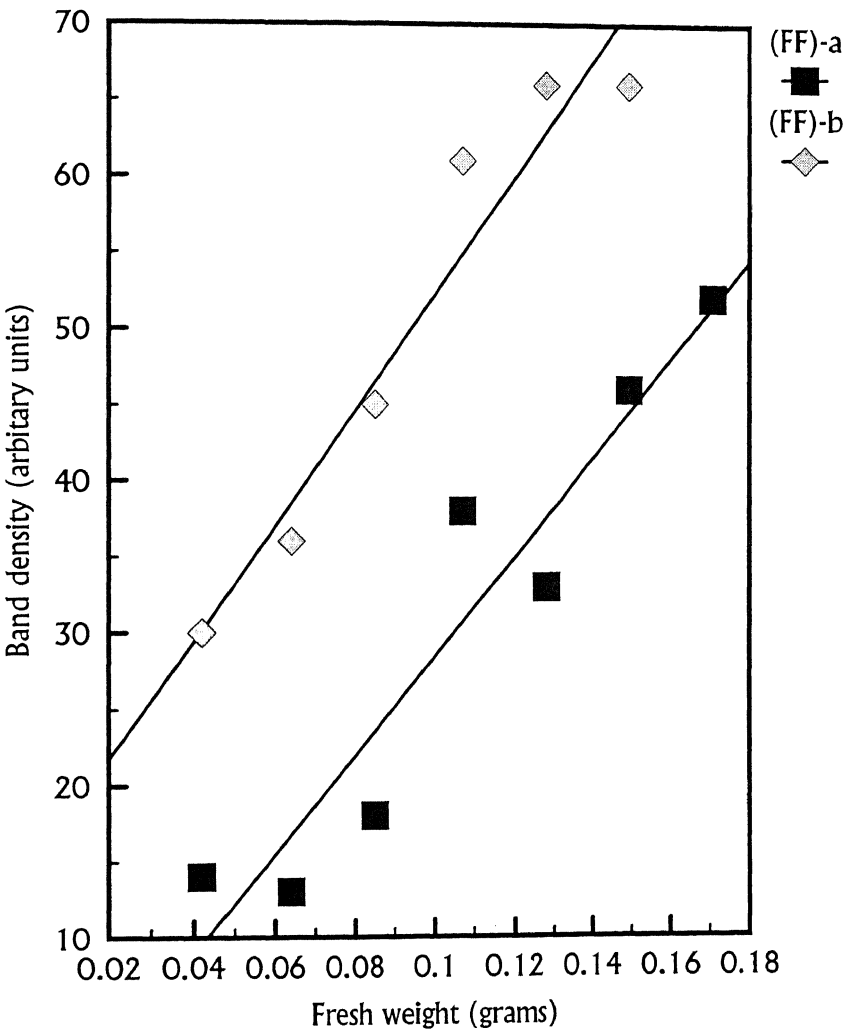


Figure 5.6 Response slope of band density (peak height) to increasing sample load for the isozymes (FF-a, FF-b) of Phosphoglucose Isomerase-1 (PGI-1) for the *Citrus* cultivar Murcott.

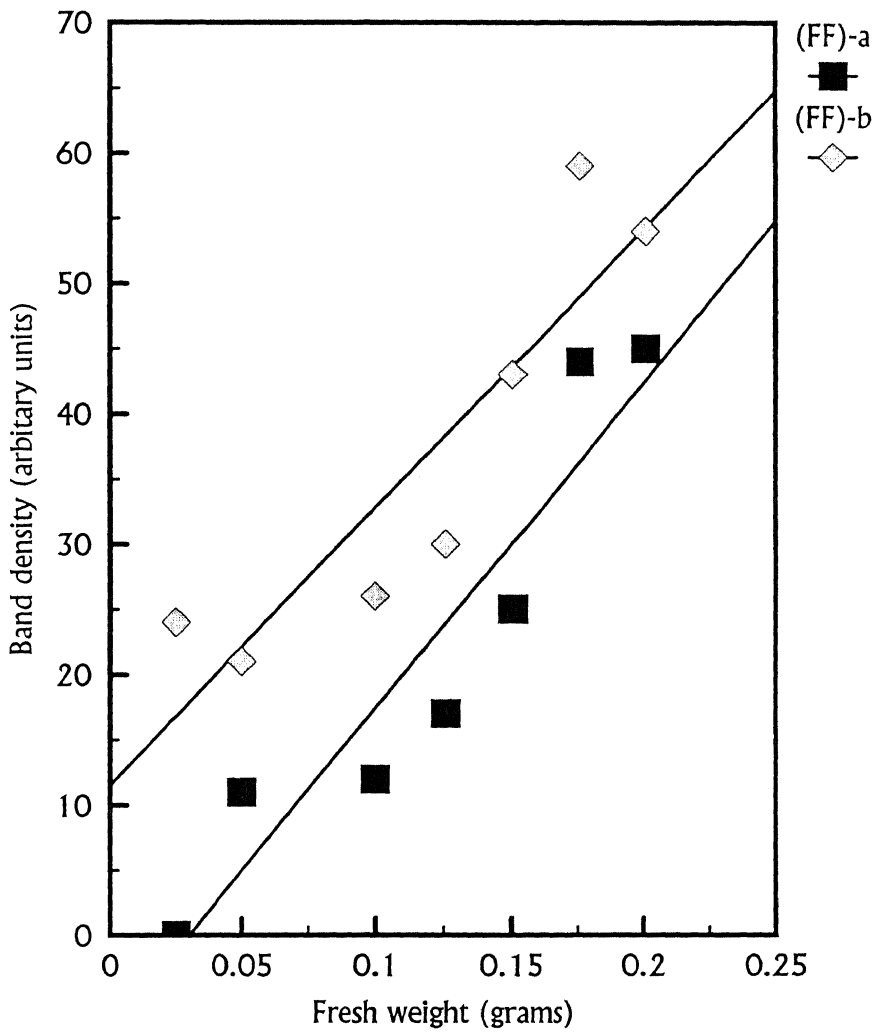


Figure 5.7 Response slope of band density (peak height) to increasing sample load for the isozymes (FF-a, FF-b) of Phosphoglucose Isomerase-2 (PGI-2) for the *Citrus* cultivar Imperial.

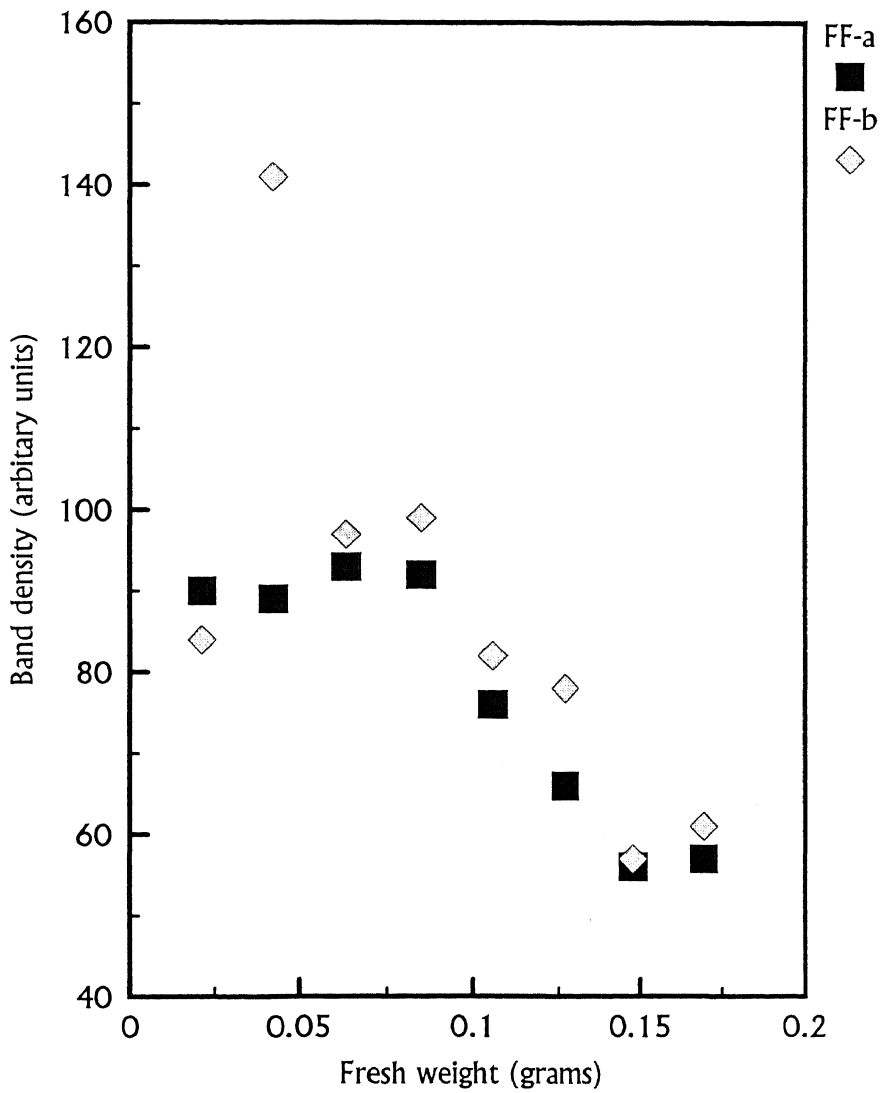


Figure 5.8 Response slope of band density (peak height) to increasing sample load for the isozymes (FF-a, FF-b) of Phosphoglucose Isomerase-2 (PGI-2) for the *Citrus* cultivar Ellendale.

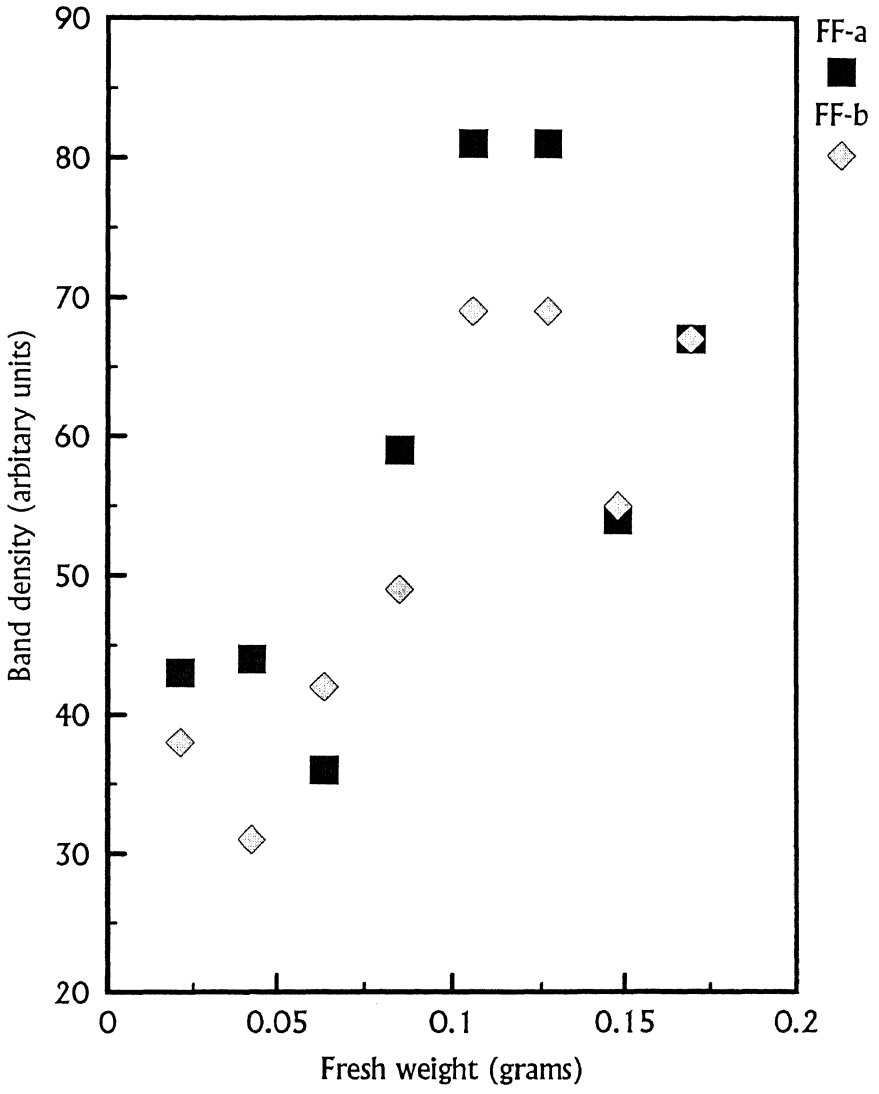


Figure 5.9 Response slope of band density (peak height) to increasing sample load for the isozymes (FF-a, FF-b) of Phosphoglucose Isomerase-2 (PGI-2) for the *Citrus* cultivar Murcott.

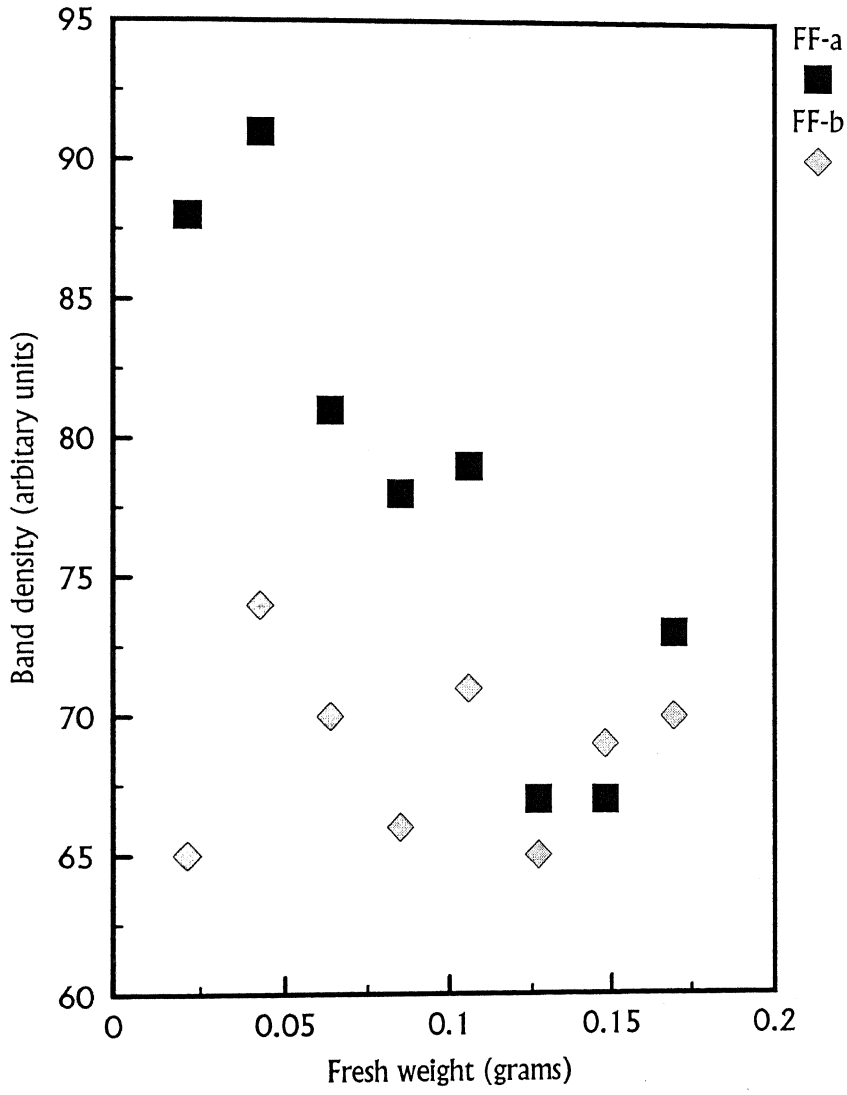


Figure 5.10 Response slope of band density (peak height) to increasing sample load for the allozymes of Shikimate dehydrogenase for the *Citrus* cultivar Imperial.

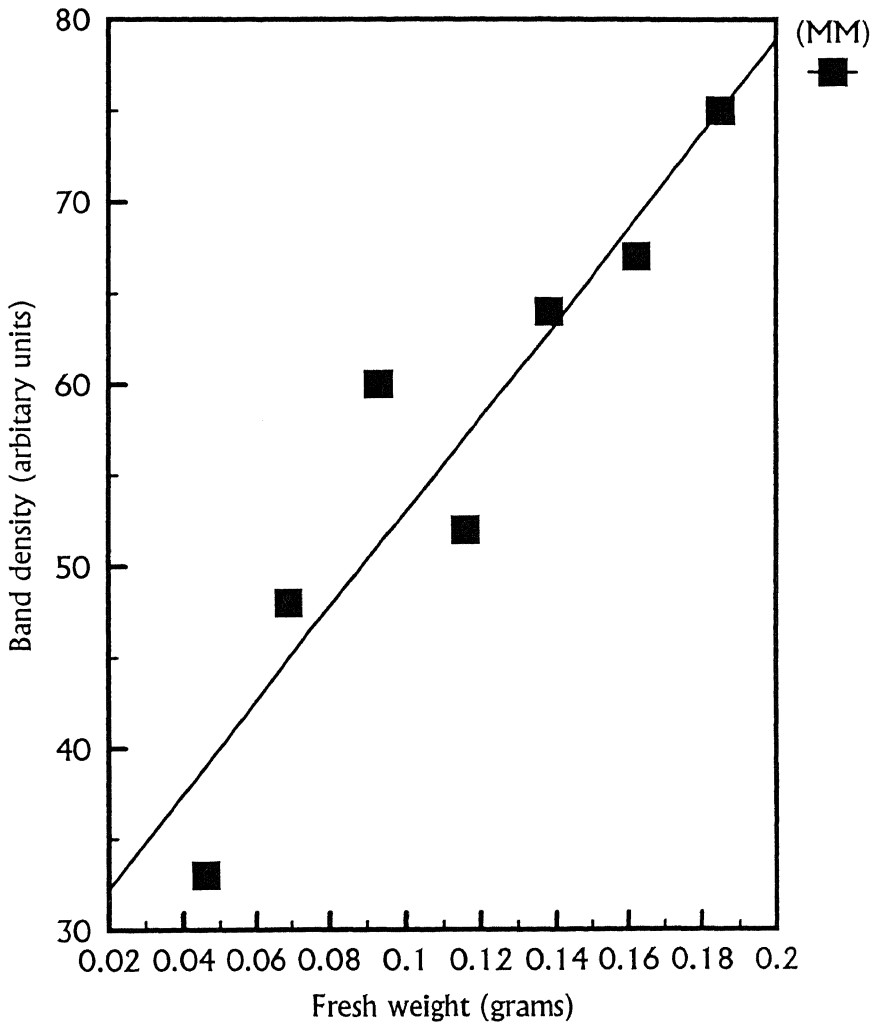


Figure 5.11 Response slope of band density (peak height) to increasing sample load for the allozymes of Shikimate dehydrogenase for the *Citrus* cultivar Ellendale.

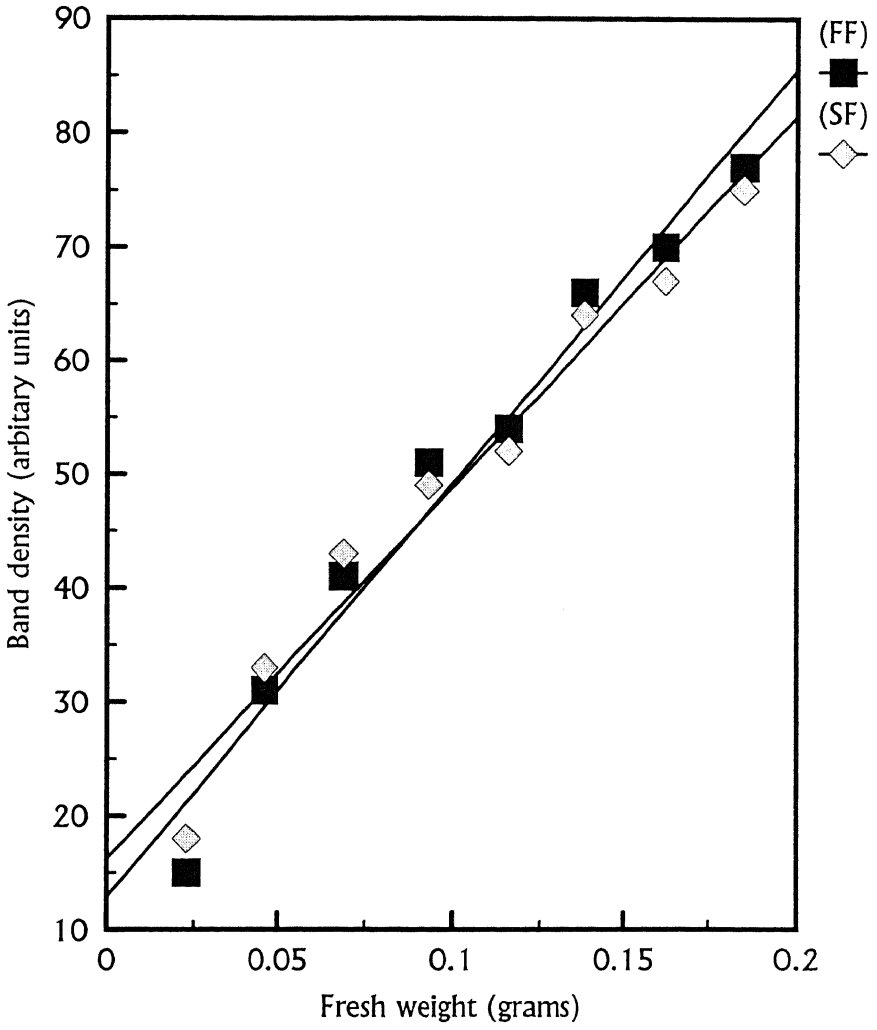
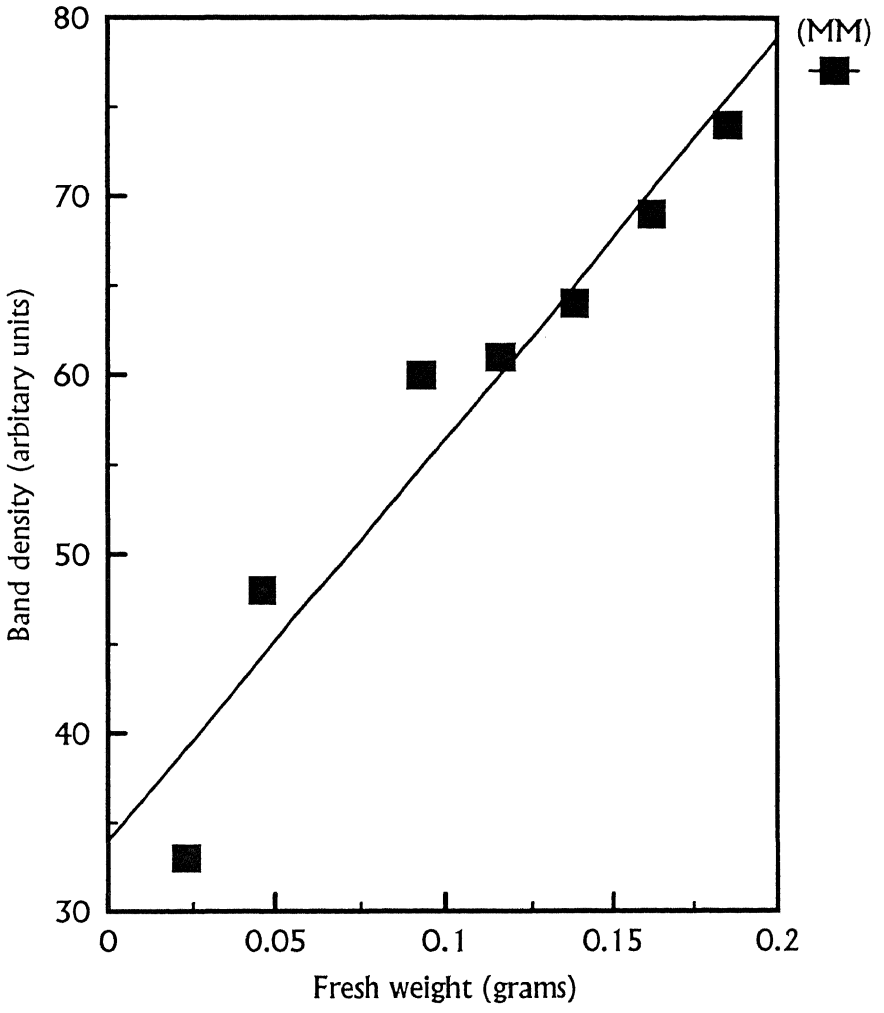


Figure 5.12 Response slope of band density (peak height) to increasing sample load for the allozymes of Shikimate dehydrogenase for the *Citrus* cultivar Murcott.



All bands which were analysed for 6PGD for the three cultivars showed a significant linear relationship ($P<0.01$) between band density and sample load (Figures 5.13, 5.14, 5.15). Therefore all bands of 6PGD should be useful for the triploid-diploid investigations.

5.3 (iii) Exhibition of a downtrend

Although for most bands band density increased linearly with sample load reflecting enzyme activity, at higher levels after an initial positive gradient, many bands exhibited a downtrend in the response slope, resulting in a plateau (Figure 5.16). This plateau indicates a limitation to the enzymatic reaction. The cause was probably associated with the exhaustion of the substrate, or some other reagent in the staining formula under conditions employed. It may have been, as discussed with MDH-1, MDH-2 and PGI-2, a limit in the capacity of the gel matrix to accumulate the chromophore (formazan). The sample load at which the downtrend point occurred was deemed to be maximum sample concentration which should be used in isozyme quantification experiments. Ideally, sample loads should correspond to a site on the initial gradient below the downtrend point, yet be sufficiently high enough to ensure adequate band density to achieve reliable densitograms. All linear regressions which were performed were done so below the downtrend points. In addition to a response plateau, some enzyme bands showed a decrease in enzymatic activity which may be caused by a lack of protective agents in the extraction buffer, which is proportionately reduced at the higher sample loads.

Figure 5.13 Response slope of band density (peak height) to increasing sample load for the allozymes of 6 Phosphogluconate dehydrogenase in the *Citrus* cultivar Imperial.

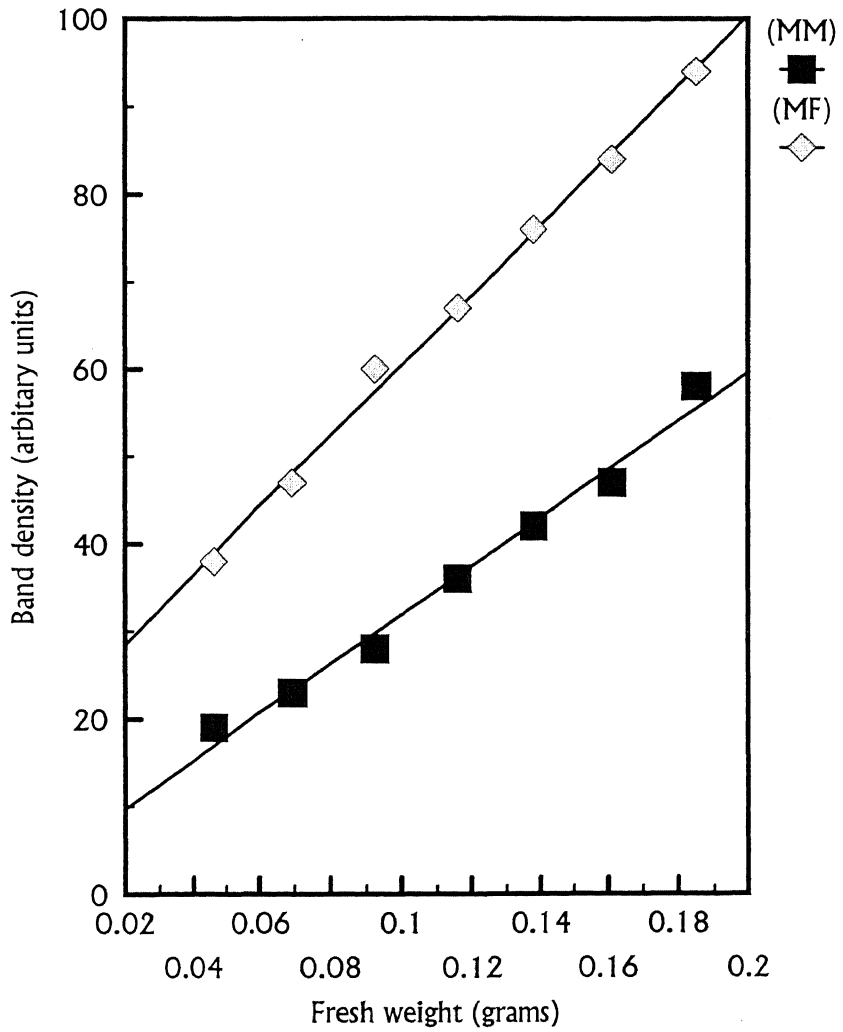


Figure 5.14 Response slope of band density (peak height) to increasing sample load for the allozymes of 6 Phosphogluconate dehydrogenase in the *Citrus* cultivar Ellendale.

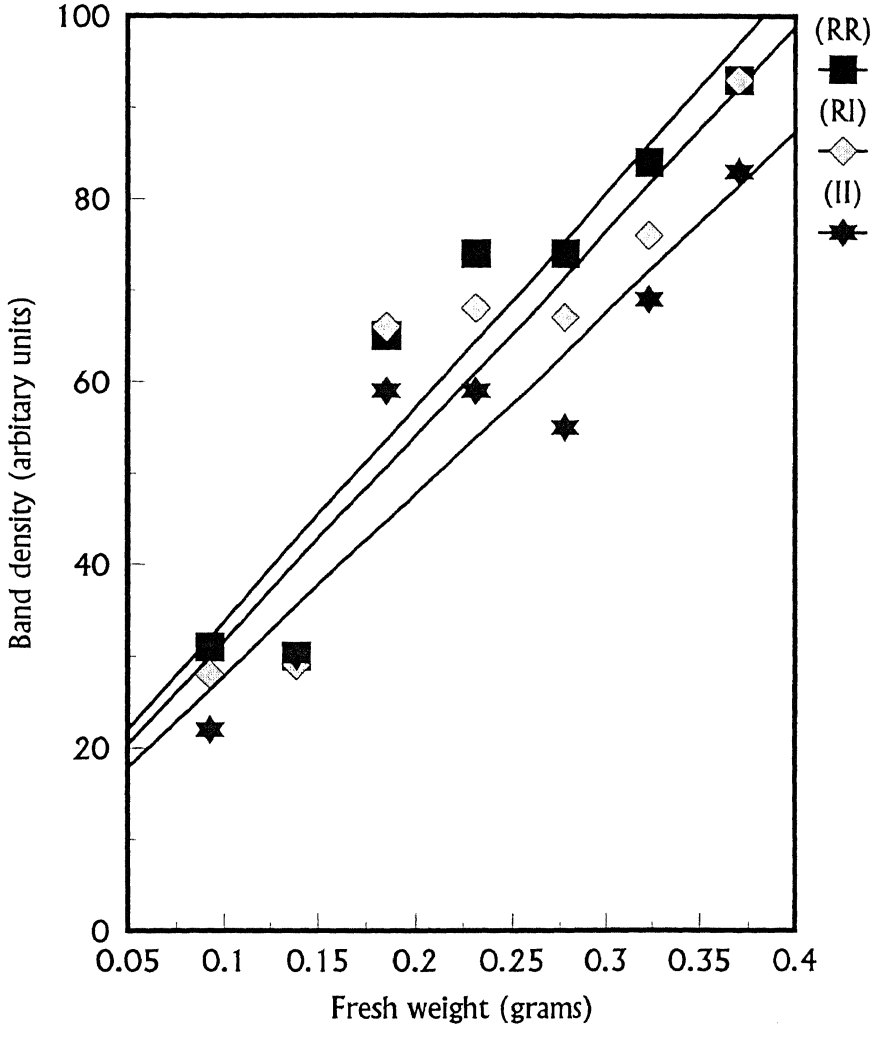


Figure 5.15 Response slope of band density (peak height) to increasing sample load for the allozymes of 6 Phosphogluconate dehydrogenase in the *Citrus* cultivar Murcott.

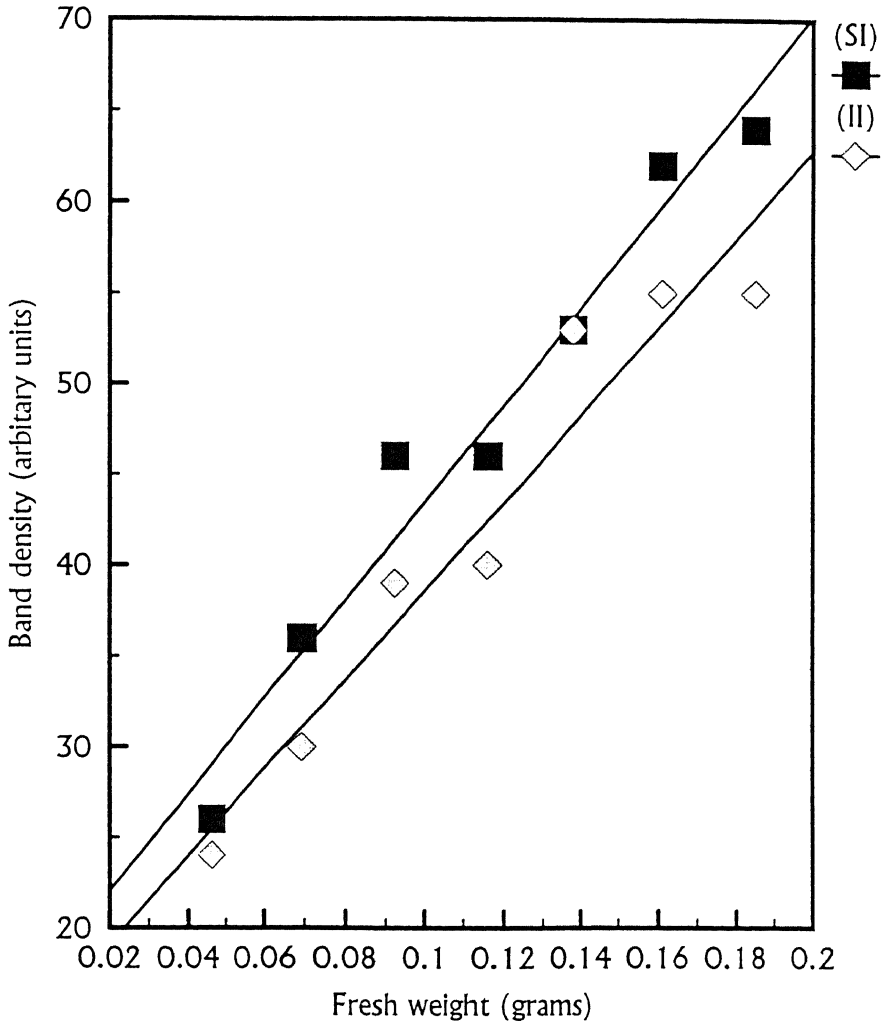
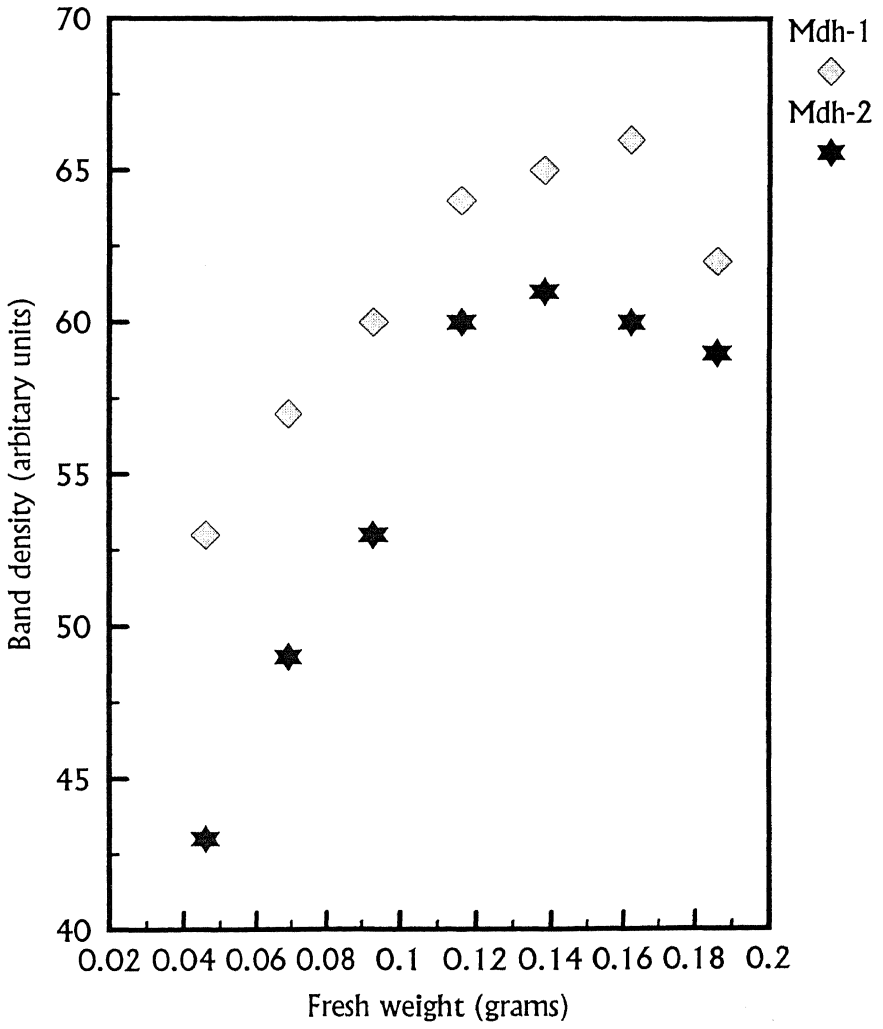


Figure 5.16 Response slope of band density (peak height) to increasing sample load for the isozymes MDH-1& MDH-2 of Malate dehydrogenase in the *Citrus* cultivar Murcott exhibiting a response plateau.



5.3 (iv) The relationship for slopes between varieties

For the MDH bands MDH-b, MDH-1, MDH-2 and the PGI-1 bands FF-a and FF-b, there was no significant difference between the mean slopes of the response curves for the three cultivars investigated (Table 5.3). The mean slopes for the response curves of Murcott and Ellendale for 6PGD for the Intermediate band II also showed no significant difference (Table 5.3) as did the mean slopes of the response curves for the medium band (M) for the cultivars Murcott and Imperial for SkDH. Since the response slopes are the same for the allozymes between cultivars this method should be useful not only for investigating triploid-diploid band densities for the progeny of selfs, but also for hybrids between these three cultivars for all 4 enzymes.

Table 5.3

Response slopes for of identical allozymes of the three cultivars, Imperial, Ellendale and Murcott

Isozyme Band	Cultivar			
	Imperial	Ellendale	Murcott	
MDH-b	206	189	251	N.S.
MDH-1	193	181	134	N.S.
MDH-2	227	248	203	N.S.
PGI-1				
(FF-a)	379	385	213	N.S.
(FF-b)	392	326	258	N.S.
SkDH				
(M)	202	nil	206	N.S.
6PGD				
(II)	nil	234	268	N.S.

Legend

N.S no significant differences

nil = no corresponding allozyme band for that cultivar

values shown are for the slope (y axis / x axis)

CHAPTER SIX

IDENTIFICATION OF TRIPLOID *CITRUS* BY ISOZYME ANALYSIS

Summary

Seedlessness is a desirable horticultural attribute in *Citrus* and is positively associated with triploidy. The conventional cytological method for triploid identification is a laborious technique as it involves the preparation of root-tips for chromosomal analysis. Isozymes and digital densitometry, however, offer the facility to distinguish triploid *Citrus* from large populations of seedlings both quickly and cheaply. Where there are no gene-dosage regulation effects, greater band density should be evident in the allozyme contributed by the diploid gamete for a heterozygous locus. The isozymes of 4 enzymes; malate dehydrogenase, 6-phosphogluconate dehydrogenase, shikimate dehydrogenase, and phosphoglucose isomerase, were investigated with polyacrylamide gel electrophoresis. Band densities of these isozymes for triploid *Citrus*, their diploid siblings and diploid progenitors were measured using a digital densitometer. Of the 4 enzymes investigated only allozymes for shikimate dehydrogenase exhibited consistent differences over a wide range of *Citrus* cultivars. Greater band density was evident in the allozyme contributed by the diploid gamete. The band density ratio between allozymes for triploid *Citrus* was close to 0.5, while for diploid *Citrus* band density ratios were close to 1.0. This effect is due to the extra protein coded by the additional gene dose and was not observed in diploids. Shikimate

dehydrogenase proved to be an accurate molecular marker for distinguishing between diploid and triploid *Citrus*.

6.1 Introduction

Seedlessness is considered to be a desirable horticultural attribute in *Citrus*, therefore triploid *Citrus* are potentially useful cultivars due to their gametic sterility which can result in seedlessness (Lee 1988). Triploid *Citrus* have been derived from both monoembryonic and polyembryonic seed parents. For monoembryonic seed parents triploid progeny have been derived from cross pollinations where the ploidy relationships of monoembryonic pistillate parent to staminate parent have been 4X × 2X, 2X × 4X, and 2X × 2X. For triploid progeny derived from polyembryonic seed parents, the ploidy relationship between parents has been 4X × 2X, and 2X × 2X (Cameron and Soost$_a$, 1969; Esen and Soost, 1971; Geraci *et al.*, 1975; Cameron and Barrett, 1978.)

In *Citrus* breeding programs, the use of both staminate and pistillate diploid parents eliminates the need for tetraploid parents, which are rare, and overcomes the problem of aneuploidy which can result from irregular meiosis in tetraploids (Esen and Soost, 1971; Geraci *et al.*, 1975). As triploid progeny are zygotic in such crosses, one of the gametes has become diploid prior to fertilisation. Esen and Soost (1971) concede both possibilities, of either maternal or paternal gamete being the diploid but point out the improbability of a diploid paternal gamete due to triploid embryo abortion caused by an embryo:endosperm ratio greater than 0.667 which generally results in a non-viable seed.

If triploid *Citrus* are to be exploited in breeding programs then a method is needed that can quickly screen large numbers of seedlings to determine their ploidy levels. The conventional cytological technique which involves the preparation of root-tips for chromosome counts is a very laborious way of differentiating between diploid and triploid *Citrus*. Furthermore *Citrus* chromosomes are small and difficult to count (Ito *et al.*, 1992). While tetraploids possess distinctive morphology (Frost, 1938; Frost and Krug, 1942; Furusato 1953a; Tachikawa, 1973; Barrett and Hutchinson, 1978), triploids do not. Small seed size however is associated with triploid embryos (Esen and Soost, 1971; Geraci *et al.*, 1975), and has been used as a means of selecting potential triploids. This is not entirely reliable however and a need exists to establish a rapid method of selecting triploids out of these populations.

Isozymes offer the ability to quickly distinguish large numbers of polyploid plants both quickly and cheaply. Triploid *Citrus* are not expected to show isozyme polymorphisms in comparison to their diploid hybrid siblings, since only the maternal genome has been doubled and there is no addition of a different genome as with allotriploids. However it is hypothesised that, where no gene dosage regulation effects occur, greater band density will be evident in the allozyme contributed by the diploid (usually maternal) gamete for a heterozygous locus. This effect would be due to the extra protein coded by the additional gene dose, and would not be observed in diploids. This work aimed to investigate such differences in band densities between triploid and diploid *Citrus* for certain allozymes and to determine the effectiveness of isozyme analysis as a method for screening triploids from seedling populations.

6.2 Materials and Methods

For the extraction procedure see Chapter 3, section 3.2 (iv), for extraction buffer, and gel staining procedures see Chapter 4, sections 4.2, (ii), (xi), for electrophoretic conditions see Chapter 4 Table 4.3, and for densitometry see Chapter 5, section 5.2 (ii).

6.2 (i) Sample collection

Fresh leaves for allozyme band investigations were collected from the following diploid mandarin cultivars; Clementine, Ellendale, Hickson, Imperial, Kara, Murcott, Nova, Page, and from an orange cultivar Hamlin. Samples were also collected from nine known triploids; six progeny from Ellendale open pollinations, two progeny from Murcott open pollinations, and Eloise, a seedless triploid cultivar from a cross of Imperial(maternal)×Murcott.

Leaves were also collected from plants raised from small seeds and suspected of being triploids; (i) 35 seedlings (1.5 year old) from Murcott open pollinations, (ii) 20 seedlings (6 months old) from Murcott open pollinations, and (iii) small seeds from crosses of Murcott×Clementine (3), Murcott×Kara (2), Murcott×Page (1), Imperial×Hamlin (2) and Murcott×Nova (3). All plants from the groups (i), (ii) and (iii) were analysed both cytologically and electrophoretically. Leaf samples were also collected from Ellendale × Murcott (20), and Ellendale × Kara (20) crosses for analysis of isozyme banding patterns.

6.2 (ii) Sample dilution levels

Dilutions were made on all crude sample extracts to achieve a linear response of band density to enzyme activity (Chapter 5). *Citrus* foliage (0.3g) was ground in liquid nitrogen as previously described, and 0.8 ml of extraction buffer was added. After centrifugation 0.2ml of supernatant was extracted, this was further diluted with extraction buffer according to the isozyme being investigated to optimise sample concentrations. Crude sample extracts for the investigation of MDH-a, MDH-b, and 6PGD were further diluted with 0.2ml of extraction buffer, whereas sample extracts for the investigation of MDH-1, MDH-2, PGI and SkDH were diluted with 0.4ml of extraction buffer. Aliquots 10µl were loaded into the wells of the polyacrylamide gels which were to be stained for SkDH because this provided a clear distinction between the medium (M) and fast (F) migrating allozymes of SkDH. For other isozymes, 30µl aliquots were used.

6.2 (iii) Cytological ploidy determination

Roots were washed with tap water, and actively growing meristematic root-tips were removed and placed in 0.05% colchicine for 2 hours. Root-tips were then washed with distilled water and fixed in 3:1 ethanol (95%) and propionic acid for 1 hour. After being washed with distilled water, roots were placed on slides and immersed in approximately 1N HCl for 5 minutes. Root-tips were blotted dry with filter paper and stained with a propionic/lactic/HCl orcein stain for 20 minutes at room temperature. After blotting dry, root-tips were immersed in 75% glycerol and squashed. The orcein stain mixture was as follows; 22.5ml of propionic acid and 22.5ml of lactic acid were combined and made up to

100ml with distilled water. This mixture was heated to near boiling, and 1g of orcein was added, stirred, and then filtered. Nine parts of the 1% orcein mixture was added to 1 part of 1N hydrochloric acid, to prepare the working stain solution.

6.2 (iv) Raising seedlings

Small seeds were placed in peat at a depth of 1cm in a growth cabinet at 27°C. Three weeks after germination seedlings were placed in the *Citrus* nursery where they were transferred to individual pots containing a complete fertiliser mixture.

6.2 (v) Statistical analysis of data

For gels stained for SkDH, treatments (diploid cultivars, triploid progeny) had a minimum of 4 independent replications. Data was analysed for significant differences using Analysis of Variance (Table 6.1).

6.2 (vi) Calculation of band density ratios

Band density ratios were always calculated by the ratio of the lighter staining allozyme over the heavier staining allozyme.

6.3 Results and Discussion

6.3 (i) Initial band density studies for the enzymes MDH, PGI, SkDH, and 6PGD between diploid and triploid *Citrus*.

Initial band density studies for the 4 enzymes MDH, PGI, SkDH, and 6PGD were conducted on 9 known triploids (section 6.2 (i)). Band density studies were also conducted on the diploid parents Imperial, Ellendale and Murcott.

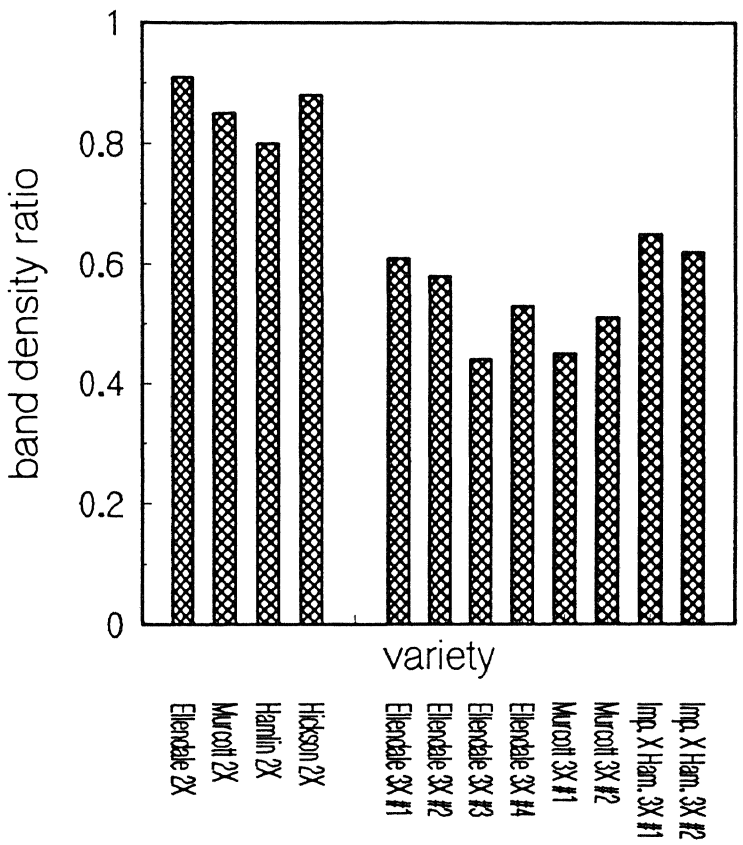
For the malate dehydrogenase bands MDH-a and MDH-b, band density differences between allozymes were not investigated since these bands were homozygous loci therefore precluding allozyme comparisons, and also identical for the three cultivars Imperial, Ellendale and Murcott (King *et al.,* 1995). However they were included in the investigation in case novel isozyme products resulting in the absence or presence of bands in the zymogram were produced which may differentiate diploids from triploids. Such novel isozyme products have been reported by De Maggio and Lambrukos (1974), who found several novel peroxidase bands in an autotetraploid fern. Spettoli and Cacco (1976) reported specific isozyme bands characterised the invertase of the triploid variety of sugar beet. Button *et al.* (1976) reported the absence of one weak peroxidase band from each of 3 nucellar tetraploid "King" mandarins, and Yamashita (1977) found an additional minor peroxidase band in a nucellar tetraploid "Natsudaidai" mandarin. However no such differences were exhibited here, and it was concluded that these bands would not be useful for distinguishing between diploid and triploid *Citrus* for the progeny of these cultivars.

For the fast migrating isozymes of malate dehydrogenase, MDH-1 & MDH-2, which were heterozygous loci, no differences between diploids and triploids were observed. This however does not mean a gene dosage effect does not exist for these bands, since the allozymes of these two loci overlap making it impossible to distinguish individual allozyme bands. These bands may be useful if better resolution could be obtained. Better band resolution may be achieved through isoelectric focusing or the use of other electrophoresis systems such as cellulose acetate for these malate dehydrogenase bands (Lee 1994).

Phosphoglucose isomerase is homozygous at all loci and identical for 3 cultivars investigated, Imperial, Ellendale and Murcott (King *et al.*, 1995). Therefore, since there are no heterozygous loci, band density differences between allozymes could not be investigated. However this enzyme was included in the initial investigations in case of the formation of novel isozyme products which could distinguish between diploid and triploid *Citrus*. No such differences were observed, and it was concluded that these bands would not be useful for the cultivars studied.

Shikimate dehydrogenase is a heterozygous locus for both Ellendale (FS) and Murcott(FM) mandarins, and is homozygous in Imperial (MM) mandarin (King *et al.*, 1995). Therefore it is expected that any progeny which are heterozygous at this locus and are triploids will exhibit band density differences between the two allozymes. For the diploid mandarin cultivar Ellendale, the band density ratio between the two allozymes for SkDH was close to one as expected (Figure 6.1). Of the six Ellendale open pollinated triploids four were heterozygous for the SkDH locus, and two were homozygous. Of these four heterozygous triploids, two showed a greater band density for the slower migrating allozyme (S) (Figure

Figure 6.1 Ratios of SkDH allozyme band densities for diploid and triploid *Citrus*.



6.2), whereas the other two showed a greater density for the fast migrating allozyme (F) (Figure 6.3). These bands which show a greater density are assumed to be the maternal allozyme contributed by a diploid gamete. The band density ratios between the two allozymes in these four heterozygous triploids were close to 0.5; these were significantly different ($P<0.01$) to their diploid maternal parents band density ratio (Figure 6.1). This is the expected ratio in a triploid for a monomeric enzyme (Figure 6.4). The two triploid progeny which were homozygous at this locus could not be differentiated from other progeny which were homozygous and diploid (Figure 6.5).

For the diploid mandarin cultivar Murcott, the band density ratio between the two allozymes was close to one as expected (Figure 6.1). The two open pollinated triploid Murcott progeny were both homozygous for the SkDH loci, and therefore could not be distinguished from other diploid progeny which may be homozygous at this locus as well. The seedless triploid cultivar Eloise, an Imperial × Murcott cross, was homozygous (MM), and therefore could not be differentiated from other diploid progeny which would be homozygous for the SkDH locus. Therefore SkDH shows strong band density differences between allozymes for a heterozygous locus for triploid progeny in comparison to diploid progeny. However, progeny which are not heterozygous for the SkDH locus were not distinguishable from other diploid progeny homozygous for this locus.

6PGD is dimeric (Torres *et al.*, 1982; Kephart, 1990) and is heterozygous for the three mandarin cultivars Ellendale, Imperial and Murcott (King *et al.*, 1995). A dimeric enzyme should yield a band density ratio of 1:2:1 between the three bands (Figure 6.6). While this allozyme band density ratio exists in Murcott, this was not the case for either Ellendale or

Figure 6.2 Greater band density for the slower migrating allozyme (S) of SkDH for a triploid progeny of an Ellendale open pollination.

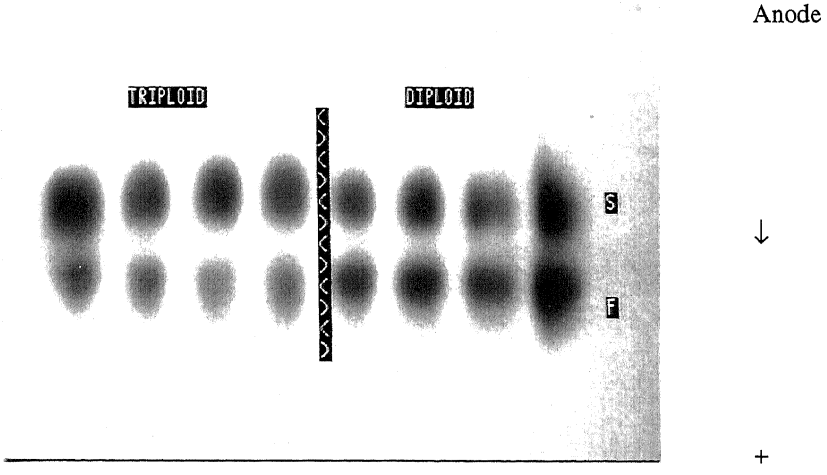


Figure 6.3 Greater band density for the faster migrating allozyme (F) of SkDH for a triploid progeny of an Ellendale open pollination.

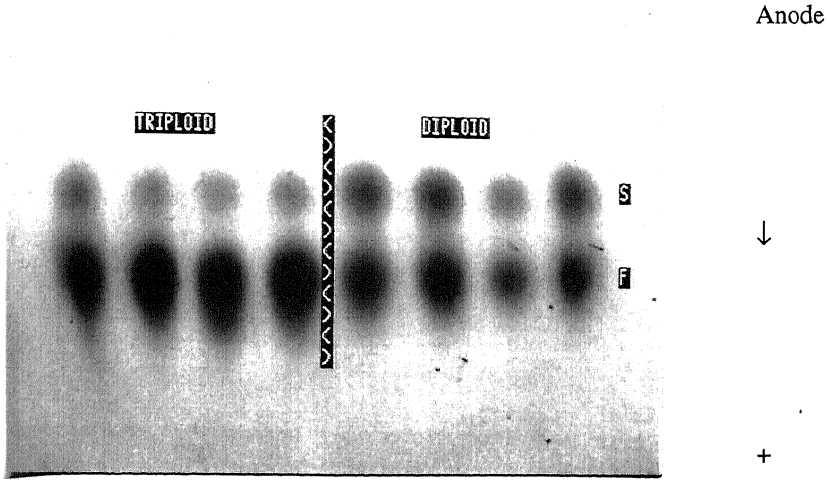


Fig. 6.4.

Zymogram banding patterns for monomeric enzymes for diploid and triploid *Citrus*

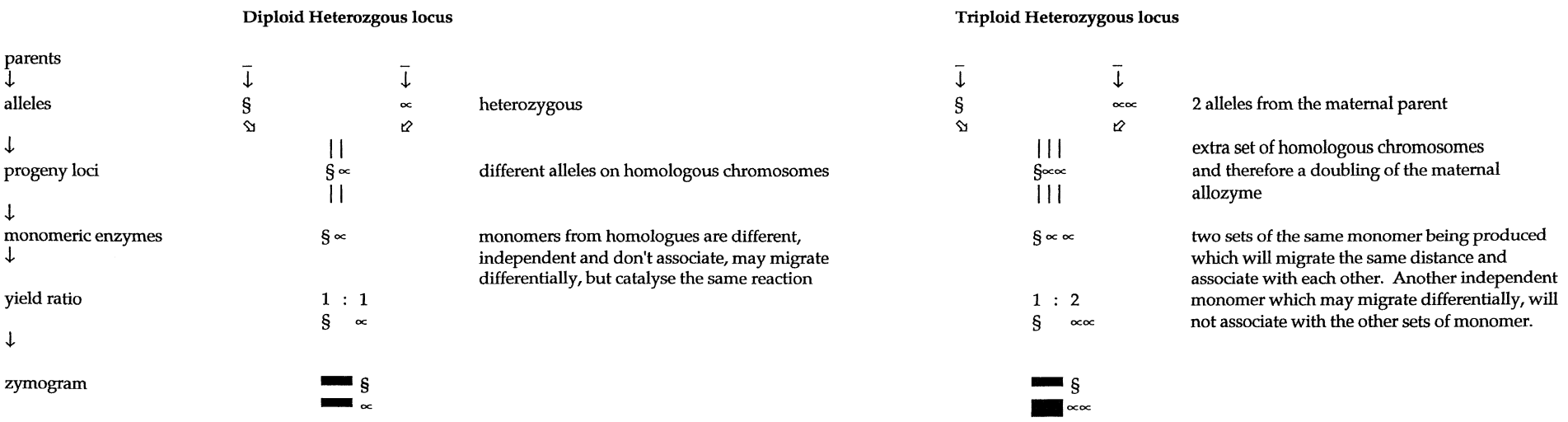
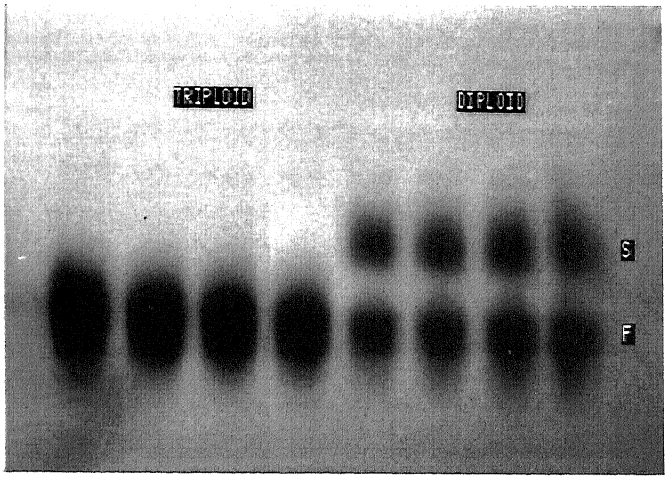


Figure 6.5 Homozygous triploid progeny for the isozyme SkDH from an Ellendale open pollination.



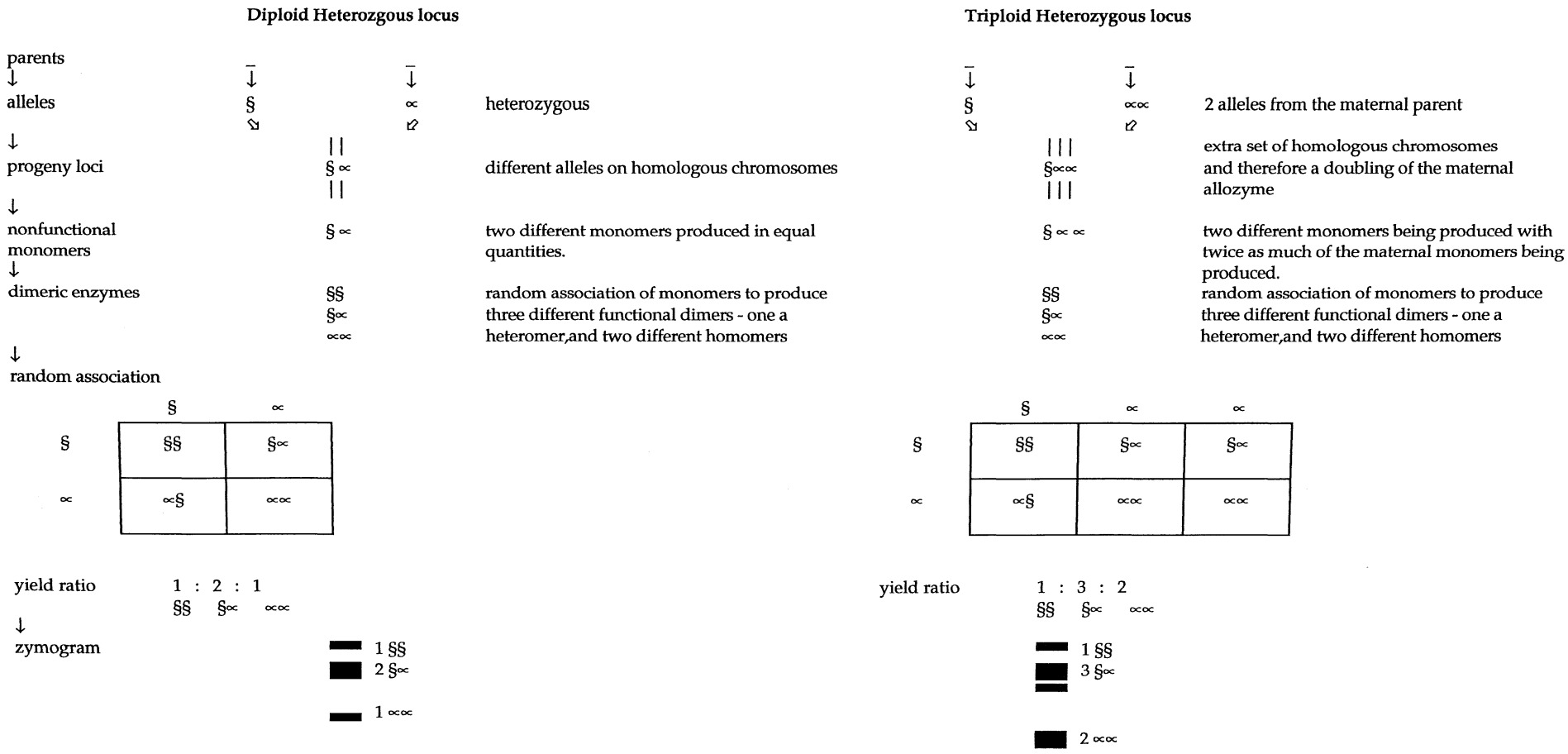
Anode

↓

+

Fig. 6.6

Zymogram banding patterns for dimeric enzymes for diploid and triploid *Citrus*



Imperial mandarin. Ellendale exhibited a band density ratio close to 1:1:1 and Imperial mandarin exhibited a band density ratio of 1:1:2. Complex electrophoretic conditions may have caused degradation of some allozymes. However, if this were the case it would be expected that the effect would be similar for the different cultivars as for SkDH where the fastest migrating allozyme was consistently weaker in density than its slower migrating counterpart for all diploid cultivars and progeny. Secondly there may be a second 6PGD locus producing an isozyme migrating the same distance as the first isozyme and underlying one of the bands, therefore producing a stronger densitogram than expected for that band.

While band density ratios between allozymes were not as expected, initial studies between diploid and triploid *Citrus* were conducted with the expectation that there would be an increase in the band density of two of the bands (Figure 6.6). This was the case for two Ellendale open pollinated triploids which showed two bands of similar density and a third band which hardly produced a visible densitogram (Figure 6.7). However, other Ellendale triploids which were heterozygous for the locus showed no band density differences between triploid and diploid *Citrus* (Figure 6.8). This was also the case for the Murcott open pollinated progeny which showed identical band patterns and band density ratios to their maternal parent. These results suggest that a gene dosage effect may occur in some cultivars, therefore no increase in the staining intensity occurs for two of the bands as a result of the doubling of the maternal 6PGD locus. Birchler reported a negative gene dosage effect in maize for 6PGD as well as for the enzymes G6PD, IDH and EST (Birchler, 1983). While a gene dosage effect may occur in some progeny, it appears that a doubling of the maternal locus does increase the density of two of the bands in other progeny. Because different cultivars show different band density ratios, the band density ratios for diploids produced

Figure 6.7 Heterozygous triploid progeny for the isozyme 6PGD from an Ellendale open pollination, with the progeny showing a strong two banded pattern.

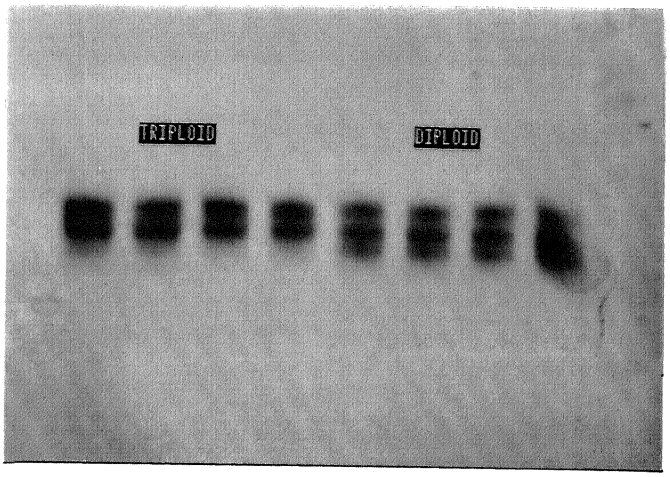
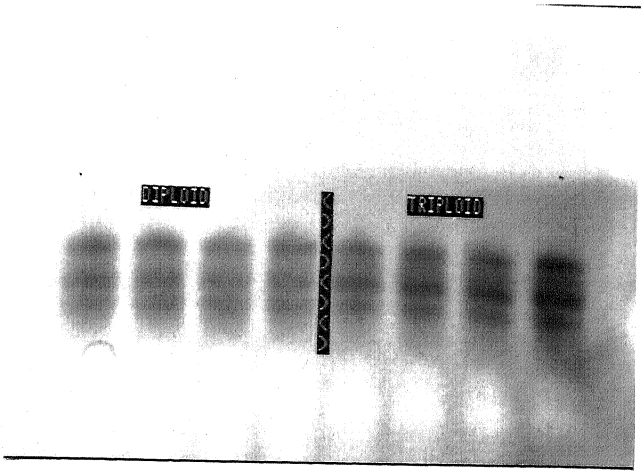


Figure 6.8 Heterozygous triploid progeny for the isozyme 6PGD from an Ellendale open pollination, with the progeny showing an identical banding pattern to its maternal parent.

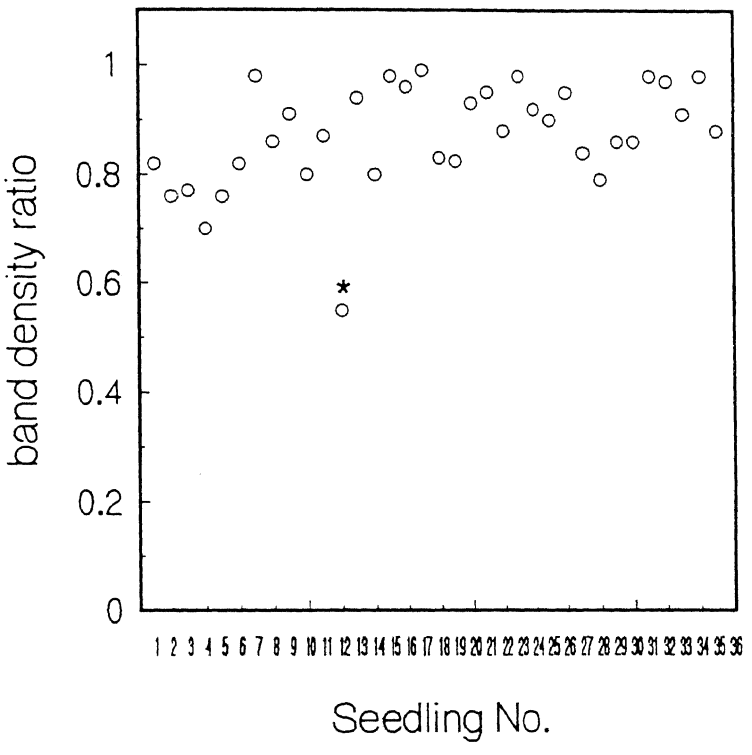


from various crosses would be difficult to predict, especially if gene dosage regulation exists in certain cultivars and not in others. The complexities of 6PGD therefore preclude the use of this enzyme as a suitable molecular marker for distinguishing between diploid and triploid *Citrus*.

6.3 (ii) Suitability of shikimate dehydrogenase as a molecular marker for triploidy

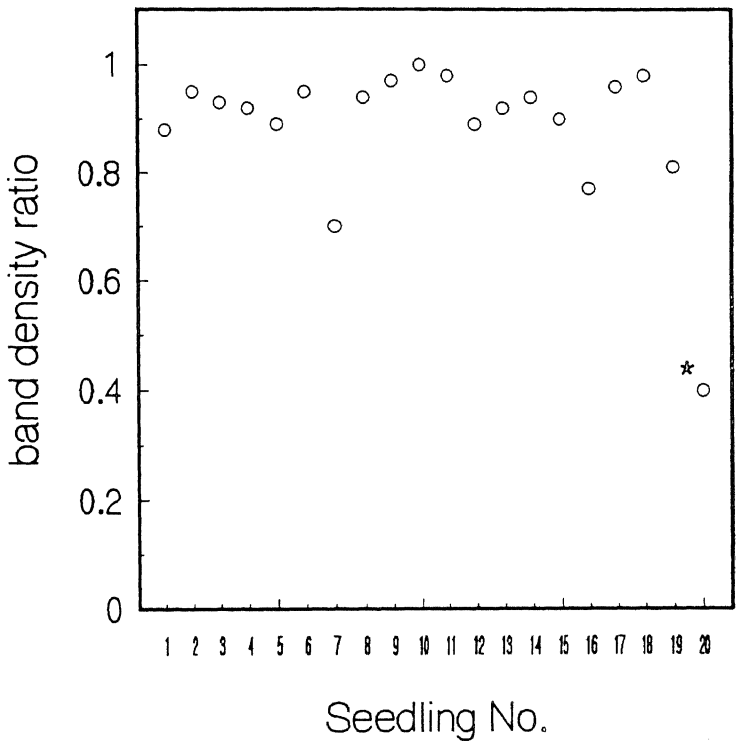
Although strong band density differences between the allozymes for a heterozygous locus have been shown to exist for SkDH in triploid *Citrus*, there is a need to establish its suitability as a molecular marker. Therefore 3 populations of seedlings raised from small seeds and suspected of containing some triploids were analysed to determine the accuracy of this technique. A population of 35 seedlings (1.5 year old) from Murcott open pollinations were analysed electrophoretically using SkDH. All 35 progeny were heterozygous for the SkDH locus. Out of this population 34 exhibited band density ratios between the two allozymes (F and M) close to one (Figure 6.9). These were analysed cytologically and determined to be diploid. One seedling (Murcott 3X #1) (Figure 6.9) exhibited a ratio between the two allozymes close to 0.5 (Figure 6.1). The seedling was also analysed cytologically and determined to be triploid. A second population of 20 seedlings (6 months old) from Murcott open pollinations were also analysed both cytologically and electrophoretically. One seedling (Murcott 3X #2) (Figure 6.10) exhibited a ratio between the two allozymes (F and M) close to 0.5, and was determined cytologically to be a triploid (Figure 6.1). The other 19 were all heterozygous, showing band densities close to one and were determined cytologically to be diploids (Figure 6.10).

Figure 6.9 Ratios of SkDH allozyme band densities for diploid and one triploid* *Citrus* for Murcott (1.5 years old) open pollinated progeny.



* - Murcott 3x#1 (cf. Figure 6.1)

Figure 6.10 Ratios of SkDH allozyme band densities for diploid and one triploid* *Citrus* for Murcott (6 months old) open pollinated progeny.



Murcott 3x#2 (cf. Figure 6.1)

A population of small seeded crosses of Murcott×Clementine (3), Murcott×Kara (2), Murcott×Page (1), Imperial×Hamlin (2) and Murcott×Nova (3) were also checked both cytologically and electrophoretically. All were heterozygous for the SkDH locus and exhibited band densities close to 1 with the exception of the Imperial×Hamlin crosses which both exhibited band density ratios close to 0.5 and were determined to be triploids (Imp. x Ham. 3X #1, Imp. x Ham. 3X #2) (Figure 6.1). Extremely significant differences were found to exist between the two ploidy levels (Table 6.1). Significant differences were also found to exist between various diploid varieties and also between triploid progeny. The differences in varieties may be genotypic but could also contain a strong environmental component.

The differences between triploid and diploid band density ratios for the allozymes of a heterozygous SkDH locus were quite distinct. Occasionally there was some variation in ratios for diploid progeny, with some progeny showing ratios as low as 0.7. Usually however, ratios were much closer to one (0.8-1.0). However band density ratios for triploid *Citrus* were consistently low and never above 0.65. These anomalous results for diploid *Citrus* are most likely due to an electrophoretic factor or factors involved in sample preparation. It must also be considered there could be slight environmental effects, which influence the production of one allozyme product to a greater degree in comparison to the other allozyme product. It was noted that the faster migrating allozyme for the SkDH locus was nearly always less intense then the slower migrating allozyme in a heterozygous diploid, suggesting that the further an allozyme travelled through a polyacrylamide gel the more it became degraded.

Table 6.1

Analysis of Variance Table for triploid and diploid *Citrus* band density ratios between allozymes of Shikimate dehydrogenase.

Source	df	MS	F
d	3	0.0178109	5.276006 **
t	7	0.0273125	8.090603 **
p	1	1.802870	534.052366 **
e		0.00337583	

d = diploids
t = triploid
p = ploidy effect (differences between the diploid group and triploid group)
e = error term

This technique appears to be a very powerful method to distinguish diploid and triploid *Citrus* at a heterozygous locus for SkDH. For open pollinated Murcott progeny there appears to be a high number of heterozygous loci, therefore diploids in this population can be effectively and quickly discriminated. The proportion of triploids in these small seeded populations appears to be a lot smaller than expected. Murcott is polyembryonic and therefore small seeds which are extracted from either open pollinations or crosses using Murcott as the maternal parent are likely to be asexual diploid embryos with a very small percentage being sexual triploid and diploid embryos.

While only two crosses using a monoembryonic seed parent were analysed (Imperial×Hamlin) seed of both were found to be triploids, which suggests that this avenue may be worthwhile pursuing. It is expected that a high proportion of the progeny from these small seeds will be triploids. Maternal parents which are monoembryonic and which will be used in breeding programs are Clementine, Ellendale, Hickson and Imperial. Cultivars which are polyembryonic and which will be used as pollen parents are Hamlin, Kara, Murcott, Nova and Page. The allozyme banding patterns of these cultivars were analysed so the proportion of heterozygotes which will be produced could be determined and, therefore, the percentage of progeny that could be distinguished as being either diploid or triploid using this technique. The allozyme banding patterns for SkDH for the cultivars studied were the following; Clementine (MM), Ellendale (FS), Hamlin (FS), Hickson (FS), Imperial (MM), Kara (MM), Murcott (FM), Nova (MM), and Page (MM). For Hamlin and Hickson the band density ratio between the two allozymes (F, S) was close to 1 as expected (Figure 6.1).

As already mentioned this technique has the ability to distinguish between triploid and diploid *Citrus* progeny which are heterozygous for the SkDH locus. However it is not able to distinguish triploids from diploids which are homozygous at this locus. Three types of genotype exist for the SkDH locus in the cultivars analysed, FS, FM, and MM. Therefore the types of crosses made determine the proportion of heterozygotes produced and hence the screening ability of SkDH as a molecular marker for triploidy (Table 6.2). For crosses made between the genotypes FS and MM all progeny are heterozygous and therefore all triploids produced will be distinguishable from their diploid counterparts. However for the cross MM × MM all progeny produced will be homozygous for the SkDH locus, and no triploids will not be distinguished from their diploid counterparts. This technique is very powerful for certain crosses and is a significant improvement over the arduous method of chromosome root-tip analysis. However for other crosses it has no screening ability (Table 6.3). Therefore there is a need for a wider array of enzymes, which would make this method an extremely powerful technique.

Table 6.2

The proportion of heterozygotes produced from crosses between the three genotypes FS, FM and MM for the shikimate dehydrogenase locus

Cross	Total % of Heterozygotes	Genotypes of the progeny produced
FS × FS	50%	FF 25%, FS 50%, SS 25%
FS × FM	75%	FF 25%, FM 25%, FS 25%, MS 25%
FS × MM	100%	FM 50%, SM 50%
FM × FM	50%	FF 25%, FM 50% MM 25%
FM × MM	50%	FM 50%, MM 50%
MM × MM	0%	MM 100%

Legend: (Band migration)

F: Fast migrating allozyme

M: Medium migrating allozyme

S: Slow migrating allozyme

Table 6.3

The proportion of heterozygous SkDH loci produced from *Citrus* crosses using monoembryonic cultivars as the maternal parent.

100%	75%	50%	0%
Ellendale×*Clementine*	*Ellendale*×Murcott	*Ellendale*×*Ellendale*	*Clementine*×*Imperial*
Ellendale×*Imperial*	*Hickson*×Murcott	*Ellendale*×Hamlin	*Clementine*×Kara
Ellendale×Kara		*Ellendale*×*Hickson*	*Clementine*×Nova
Ellendale×Nova		*Hickson*×Hamlin	*Clementine*×Page
Ellendale×Page		*Hickson*×*Hickson*	*Imperial*×Kara
Hickson×*Clementine*		*Clementine*×Murcott	*Imperial*×Nova
Hickson×*Imperial*		*Imperial*×Murcott	*Imperial*×Page
Hickson×Kara			
Hickson×Nova			
Hickson×Page			
Clementine×Hamlin			
Imperial×Hamlin			

Legend

Italics =Monoembryonic

Normal text =Polyembryonic

CHAPTER SEVEN

CONCLUSION

For the 4 enzymes studied, MDH, PGI, 6PGD and SkDH, only SkDH was shown to consistently exhibit characteristic differences between allozymes for a heterozygous locus over a wide range of *Citrus* cultivars. Greater band density was evident in the allozyme band contributed by the diploid (usually maternal) gamete relative to that of the haploid gamete for a heterozygous locus. This effect is presumably due to the extra protein coded by the additional gene dose and was not observed in diploids. This enzyme proved to be a useful molecular marker for distinguishing between diploid and triploid *Citrus*. However this technique has limitations in only being able to distinguish triploid and diploid progeny which are heterozygous for the SkDH locus, and is unable to distinguish between progeny homozygous for this locus. Therefore there is a need for a wider array of enzymes which can be used to screen potential triploid progeny. A short selection critique for initial enzyme selection can be established;

1) Preferably enzymes should be monomers, because differences between allozymes should be readily quantified. Although dimers and tetramers may prove useful, quantifiable differences between bands in these enzymes may be very slight when compared to those differences exhibited by monomers. Therefore a degree of inaccuracy may be introduced when trying to distinguish between diploid and triploid *Citrus* for a heterozygous locus using such enzymes.

2) There should be polymorphism for that enzyme in *Citrus*. If the enzyme is homozygous for an allozyme, and there are no differences between cultivars, then all progeny from such crosses will be homozygous, and triploids will not be distinguished from their diploid counterparts. The greater the percentage of heterozygotes produced from a certain cross for a particular enzyme, the greater the screening ability for that enzyme.

3) There should be no overlapping loci from other isozymes, because this could cause errors when quantifying band densities for what may have been assumed to be an individual band.

If enzymes meet these criteria then the investigator has to develop good enzyme band resolution and activity, so enzymes can be easily quantified using a densitometer.

Extracting proteins from vegetative tissues while maintaining good enzyme activity and electrophoretic resolution presents numerous problems due to the presence of phenols, quinones, proteases and other components released during cell disruption. To solve this problem an extraction buffer was developed which maintained high enzyme activity in PAGE gels suitable for densitometric analysis. To produce isozyme bands of high resolution, electrophoretic conditions including electrode and gel buffers, acrylamide concentration, use of stacking gels, voltage, current and run time were investigated. If new enzymes are to be investigated, similar problems may be encountered: modifications of the current extraction buffer may be needed, or new buffers may be implicated. Investigation of electrophoretic parameters may also be needed especially if conditions for starch

systems or cellulose acetate systems do not provide satisfactory resolution under a PAGE system.

A linear relationship between isozyme activity and band density must be shown if any gene dosage effects are to be detected on the polyacrylamide gel. For the four enzymes studied all showed linear relationships between isozyme activity and band density on the polyacrylamide gel, but at higher concentrations some of these bands exhibited a declension point suggesting a limitation in the enzymatic reaction. Therefore appropriate dilution levels for any enzyme being studied must be used if a correct gene dosage effect is to be registered on the polyacrylamide gel. Isozymes using a linking enzyme in their staining protocol may present some special problems as encountered with PGI. For the faster migrating bands of PGI (PGI-2) there was precipitation of the formazan stain only on the surface of the gel, therefore limiting potential band density. This was especially so at higher activity levels because some or all the formazan precipitated off the gel into the reagent mixture, creating unreliable band density measurements for enzyme activity.

After linear relationships between enzyme activity and band density have been demonstrated for the new enzyme being investigated, the investigation can then proceed to examine possible differences in quantitative aspects of gene expression between diploid and triploid *Citrus*. This thesis provides an essential foundation for the application of this technology.

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APPENDIX ONE

Isozyme Stain Protocols

The isozyme stain protocols used here are based on those presented by Richardson *et al.* (1986) with occasional minor modifications. In all cases an agar overlay method of reagent application was used. This was found to give superior band resolution on PAGE gels compared to the liquid reagent application method of Richardson *et al.* (1986). For the PAGE gels (80mm×100mm gel dimensions) 1 volume of stain reagent was added to 1 volume of 1% hot agar (70°C) and then poured over each gel in a dark oven (37°C) and allowed to solidify. Bands were allowed to develop for a period of 1.5 - 2 hours.

The following abbreviations are used-

NAD - β-nicotinamide adenine dinucleotide.

NADP - β-nicotinamide adenine dinucleotide phosphate

MTT - methyl thiazolyl tetrazolium blue

PMS - phenazine methosulphate

Malate Dehydrogenase (NAD)-MDH E.C.1.1.1.37

DL-malic acid	1ml	out of 50mg/ml pH 8.0 stock
NAD	200µl	out of 5mg/ml stock
MTT	200µl	out of 6mg/ml stock
PMS	200µl	out of 2mg/ml stock
$MgCl_2$	200µl	out of 0.1M stock
0.1M Tris-HCl pH 8.0	4ml	

6-Phosphogluconate Dehydrogenase 6PGD E.C.1.1.1.44

6-Phosphogluconic acid	10mg
NADP	200µl out of 5mg/ml stock
MTT	200µl out of 6mg/ml stock
PMS	200µl out of 2mg/ml stock
$MgCl_2$	200µl out of 0.1M stock
0.1M Tris-HCl pH 8.0	4ml

Shikimate Dehydrogenase SkDH E.C.1.1.1.25

Shikimic acid	20mg
NADP	200µl out of 5mg/ml stock
MTT	200µl out of 6mg/ml stock
PMS	200µl out of 2mg/ml stock
$MgCl_2$	200µl out of 0.1M stock
0.1M Tris-HCl pH 8.0	4ml

Phosphoglucose Isomerase PGI

Fructose-6-phosphate 10mg

NADP	200µl out of 5mg/ml stock
MTT	200µl out of 6mg/ml stock
PMS	200µl out of 2mg/ml stock
$MgCl_2$	200µl out of 1M stock
0.1M Tris-HCl pH 8.0	5ml (part A) & 4ml (part B) = 9ml total

Linking enzyme Glucose-6-phosphate 10µl out of 5000units in 2ml of buffer.

For PGI a post-coupling technique was used where all reagents apart from MTT and PMS were added in 5ml of stain buffer without agar overlay and poured onto the gel which was allowed to incubate in the dark for 30 minutes at 30°C before the addition of the MTT and PMS in 4ml of stain buffer with the addition of 1 volume of agar overlay, bands were then allowed to develop at 37°C.

APPENDIX TWO

Origins of Cultivars

Clementine

According to Trabut (1902_a, 1902_b, 1926) this highly important North African variety originated as an accidental hybrid in a planting of mandarin seedlings, presumably of the common or Mediterranean mandarin, made by Father Clement Rodier in the garden of the orphanage of the Peres du Saint-Espirt at Misserghin, a small village near Oran, Algeria. There is some doubt about the parentage of this mandarin. Trabut (1902_a, 1902_b, 1926) concluded that the seed parent was a willow-leafed ornamental variety of *C. aurantium* known as *Granito*. Chapot (1963_a) however refuted this with convincing evidence, and concluded that Clementine was of oriental origin, and was indistinguishable from the Canton Mandarin.

Ellendale

This Australian variety is reported (Bowman, 1956) to have originated about 1878, when seed from fruit grown at the penal colony at Baffle Creek was taken by Mr E. A. Burgess and planted on his property "Ellendale" on the Burrum River. One of these seedlings became the parent tree of the "Ellendale" cultivar. Ellendale has some orange-like characteristics (tight peel and rich flavour) and may be a hybrid of a tangerine and orange (McAlpin, 1967), and Ashari *et al.,* 1989 indicated this with isozyme evidence. Ellendale is a mid-season fruit

maturing in June and July in Queensland. The fruit is large, orange in colour, moderately seedy with a tight but readily peeled rind. The juice has high sugars but the acid remains high well into the season.

Imperial

Imperial is the most popular mandarin on the Australian market. This cultivar is claimed to have originated about 1890 at Emu plains some thirty miles west of Sydney, N.S.W. and is believed to be a chance hybrid of Mediterranean or Willow leaf, to which it bears considerable resemblance (Reuther *et al.,* 1967). However there is some evidence that it may be of oriental origin (possibly Sha Tza), introduced by early immigrant Chinese (pers. comm. J C. Chapman). Because of it's early maturity, attractive appearance, and pleasant flavour, it commands a premium in the markets.

Kara

This very late ripening and richly flavoured variety is an Owari satsuma´King mandarin hybrid created in 1915 by H.B. Frost (1935) of the University of California Citrus Research Centre, Riverside, and named and introduced in 1935. Though outstanding in flavour, Kara has not achieved commercial importance, presumably because of the competition provided by oranges at its season of maturity and its seediness.

Murcott

The origin of Murcott is obscure but is believed to have arisen in a USDA breeding program in Florida from a cross made late last century (Reuther *et al.,* 1967). It is known to be a tangor (*C. reticulata ´ C. sinensis*). Murcott first appeared in Queensland in the 1960's apparently as an illegal budwood importation from the U.S.A. Legal seed imports were subsequently made but the earlier introduction has become universally accepted in Australia (pers. comm. L.S Lee). Murcott fruit is seedy with a firm skin which is difficult to peel. It is a large-fruited cultivar with a good tangerine colour and is one of sweetest of citrus fruit.

Nova

This very early ripening variety resulting from a Clementine mandarin and an Orlando tangelo cross made in 1942 by Gardner and Bellaus of the U.S. Department of Agriculture at Orlando, Florida, and described and released in 1964 (Reuther *et al.,* 1967). Seed was imported by the Queensland Department of Primary Industries in 1977 from a registered source in Florida. The fruit is medium sized, early maturing and is a yellow-orange colour at maturity. It is moderately seedy, easy to peel and the rind is thin with a pebbled character.

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This early ripening, high quality variety, the fruit of which has considerable resemblance to a sweet orange originated from a Minneola tangelo´Clementine mandarin cross made by Gardner and Bellows of the U.S. Department of Agriculture in 1942. Page was described and released in 1963 by P.C. Reece and F.E. Gardner at the U.S. Horticultural Field Station,

Orlando Florida. Parentage is 3/4 mandarin and 1/4 grapefruit (Reuther, Webber and Batchelor, 1967).

Hamlin

Hamlin is a common orange. This variety originated as a chance seedling in an orchard near Glenwood, Florida, which was planted in 1879, and named for the owner A.G. Hamlin. Currently, it is a major variety in Florida, of considerable importance as an export variety in Brazil, and of limited importance in South Africa and elsewhere, and possibly the world's principal variety of very early maturing common sweet orange.

Hickson

The Hickson mandarin is understood to have originated as a sport branch on an Ellendale tree on the property of Mr. Hickson at Roma, Queensland. In 1941 the cultivar was brought to the attention of officers of the Department of Agriculture and Stock who arranged for its propagation by Mr. Oscar Obrist, nurseryman of Rochedale. Hickson fruit is similar to Ellendale but it has a small nipple at the stem end. Fruit is large and good quality, easy to peel and a pleasant flavour. After a slump in popularity it is again finding favour with growers, but still remains a minor cultivar (pers. comm L. S Lee and J. C. Chapman).

APPENDIX THREE

Papers Published

The following are papers which have been published from work performed in this thesis.

(A) King, B.J., Lee, L.S., Rackemann, R.G., Scott, P.T. (1994) The preparation of extracts for electrophoresis from *Citrus* leaves. *Journal of Biochemical and Biophysical Methods* **29**, 295-305.

(B) King, B.J., Lee, L.S., Rackemann, R.G., Scott, P.T. (1995) Electrophoretic conditions for high resolution *Citrus* isozymes on polyacrylamide gel electrophoresis (PAGE). *Electrophoresis* **16,** 32-38.

(C) King, B.J., Lee, L.S., and Scott, P.T. (1996) Identification of triploid *Citrus* by isozyme analysis. *Euphytica* "In Press".