Pollination Studies in Almond

by

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Almond orchard at Waite institute
Photo. Ali Vezvaei (January 1992)

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Summary

This research into almond pollination embraces several aspects of the subject. The work can be divided into two areas; identification of cultivars by isozyme analysis, PCR markers and pollen morphology, and use of these identifying characters for pollination studies.

For the first time commercially important Australian bred almond cultivars have been characterized by molecular genetic markers and compared with the better known Californian varieties. The assignment of alleles at the various loci was aided by examination of the inheritance of these markers by crossing Nonpareil with pollen from 17 Australian and Californian cultivars. This demonstrated for the first time, null genes in Price at LAP-1 and in Drake at AAT-1. The Australian cultivars differed from the Californian at seven loci. A comparison was made between these two sets of cultivars by pollen ultrastructure studies as well. The results showed that the ultrastructure parameters were useful in distinguishing cultivars and six of these cultivars can be identified with one or two characters. For the remaining cultivars a combination of ultrastructure parameters was necessary for identification. Pollen morphology and exine characteristics were therefore only found to be useful for the present pollination studies when used in combination with other morphological and genetics markers. Genetic linkage between several isozyme loci has been established in almond for the first time, namely AAT-1 linked with IDH and LAP-1 with PGM-2, both of which had a highly significant association (P-value < 0.001). In addition, a significant association (P-value < 0.001) was observed between LAP-1 and GPI-2 when pollen sources were Fritz, Mission or Price, but could not be tested for the remaining five pollen sources due to homozygosity at these loci. If LAP-1 is linked with GPI-2 and PGM-2, it might be expected that there should be linkage between GPI-2 and PGM-2. The lack of a significant association between these two isozymes suggest that LAP-1 is located centrally on the chromosome. The results of other studies not using hand pollination (e.g. limited bee induced self-pollination and bee induced cross-pollination in isolated cages) supported these pairs of linkages as well.

Isozyme diversity in the Iranian almond population has also been investigated. The results showed that there is a high level of gene diversity between genotypes and between almond species gathered from different locations. Some new isozyme alleles not reported previously in almond were observed. Gene diversity in cultivated almonds was greater than in the wild species and these species from the north of Iran showed greater diversity than those from the south. Among wild species *P. lysioides* and *P. scoparia* showed the highest polymorphism possibly due to the relatively wide distribution of these species.

Identification of twelve wild and cultivated almonds by randomly amplified polymorphic DNA (RAPD) has been carried out. All 12 species and cultivated almond were distinguished by seven primers. The dendogram showed that among the three wild species, *P. lysioides* is more closely related to *P. scoparia* than to *P. reticulata.* Australian cultivars were separated from Iranian genotypes. Comparison of banding patterns between cultivated almonds and wild species shows that the variability of bands was lower for wild than cultivated almonds. We found that for the pollination studies isozyme markers are more convenient to use than RAPD markers.

Pollination of the Nonpareil almond cultivar at 6 succeeding stages (days) of flower development showed that newly opened flowers were more fertile than at the other 5 stages of development. Pollen from 8 other almond cultivars was examined for differences in proportion of pollinated Nonpareil flowers which set nuts. Pollen from cultivars Peerless, Fritz, Keane, and Price set highest numbers of nuts, Grant, Mission and Ne Plus Ultra intermediate numbers, and Carmel set the lowest numbers of nuts on Nonpareil. There were no significant differences in physical and chemical traits of nuts produced with pollen from the different sources. When 5 different pollen donors were used on Price and Keane cultivars as female recipients, only pollen from Keane cultivar produced nuts on Price which were significantly different from others (they were heavier), while pollen donors did not influence kernel weight on nuts set on Keane cultivar. IXL seedling was cross-incompatible with Nonpareil and Ne Plus Ultra was found to be incompatible with Price.

Pollen tube growth observations were carried out varying pollen genotype and the effect of temperature and competition between mixed pollen types investigated. Pollen germination on the stigma surface was found to be related to temperature. Pollen tube

penetration in the upper part of the style began for only Peerless pollen after 12 hours at 22° C, and after 24 hours for other treatments. The percentage of pollen tubes in the ovule under field conditions was always higher than in the controlled growth room experiments at 22° C and 15° C respectively. As to the competition experiments a comparison of 5 different isozyme loci showed that the frequency of pollen genes in the embryo was higher for Peerless, being twice that for Keane genes.

An investigation of gene flow by pollen in a commercial orchard was also undertaken and interpretation of these assisted by a study of gene flow by pollen between two almond cultivars isolated in a cage with a hive of bees. Overall, these studies confirmed that gene flow by pollen is generally highest between nearest neighbour trees and suggest that Price, Keane and Peerless cultivars are the preferred pollinizers of Nonpareil. The present studies suggest that cultivars should be planted in a 1:1 design (pollinizer : Nonpareil), using alternating rows of different pollinizer trees with Nonpareil rows. Pollinizer trees alternating with Nonpareil trees in the same row should give even higher nut yields.

Declaration

I hereby declare that this work was conducted in the Department of Horticulture, Viticulture and Oenology, within the University of Adelaide and contains no material which has been previously accepted for the award of any other degree or diploma at any University. To the best of my knowledge this thesis contains no material previously published or written by another person except where due references is made in the text.

I consent to this thesis being made available for loan and photocopying if it is accepted for the award of the degree.

Ali Vezvaei

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I thank my wife, for her support and encouragement during my study.

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Publications

Part of the work described in this thesis has been reported in the following publications:

(a) Conference papers presented

Vezvaei, A. and Jackson, J.F. (1993) Pollen ultrastructure of ten Australian and Californian almond cultivars. *Proceedings of the First Scientific Seminar of Iranian Student in Sydney, Australia.*

Vezvaei, A. and Jackson, J.F. (1994) Evaluation of Keane cultivar as a pollinizer for Australian almond cultivars. *Second Horticultural Industry Technical Conference, Wentworth NSW, Australia.*

(b) Papers *in press*

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Vezvaei, A. and Jackson, J.F. (1994) The effect of pollen parent and stage of flower development on almond nut production. *Australian Journal of experimental Agriculture.*

Vezvaei, A.; Clarke, G.R. and Jackson, J.F. (1994) Characterization of Australian almond cultivars and comparison with Californian cultivars by isozyme polymorphism. *Australian Journal of experimental Agriculture*, **34.**

Vezvaei, A.; Hancock, T; Giles, L. Clarke, G.R. and Jackson, J.F. (1994) Linkage and inheritance of isozyme loci in almond. *Theoretical Applied and Genetics.*

(c) Papers *in preparations*

Vezvaei, A. and Jackson, J.F. Cyanogenic compounds in almond pollen. *phytochemistry.*

Vezvaei, A. and Jackson, J.F. Gene flow in an almond orchard as determined by isozyme analysis of individual kernels and honey bee pollen load. *Australian Journal of Experimental Agriculture.*

Vezvaei, A.; Giles, L. and Jackson, J.F. Pollen tube growth in Nonpareil almond related to pollen genotype, temperature and competition among mixed pollen. *Sexual plant Reproduction.*

Abbreviations

AAT	asparatate amino transferase
ACO	aconitate hydrogenase
ACP	acid phosphatase
ADH	alcohol dehydrogenase
APS	acid phosphatase
CAT	catalase
CTAB	hexadecyl trimethyl-ammonium bromide
EDTA	ethylenediamine-tetra-acetic acid
EPP	effective pollination period
EST	esterase
FDP	fructose-1,6-diphosphatase
G6PD	glucose-6-phosphate dehydrogenase
GAL	galactosidase
GDH	glutamate dehydrogenase
GOT	glutamate-oxaloacetate transaminase
GPI	glucose-phosphate isomerase
6GPD	6-glucosephosphate dehydrogenase
ICPS	inductively coupled plasma spectrometry
IDH	isocitrate dehydrogenase
LAP	leucine amino-peptidase
MDH	malate dehydrogenase
ME	malic enzyme
MTT	methyl thiazolyl blue
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine diucleotide phosphate - reduced form
PCR	polymerase chain reaction
PER	peroxidase
PGD	phosphogluconate dehydrogenase
PGI	phospho- gluco isomerase
PGM	phosphoglucomutase
PMS	phenazine methosulphate
PRX	peroxidase
RAPD	random amplification polymorphic DNA
RFLP	restriction fragment length polymorphism
SEM	scanning electron microscopy
SKDH	shikimate dehydrogenase
TBE	tris-borate
TPI	triose-phosphate isomerase
TRIS	tris (hydroxymethyl) aminomethane
UPGMA	Un-weighted pair-group method with arithmetical average

Chapter 1

General introduction and literature review

Chapter 1

General introduction and literature review

1.1 General introduction

In flowering plants pollination, which leads to fertilization, determines the success of fruit formation. Pollination mechanisms influencing effective pollination are extremely important for seed set, where seed is required. In varieties having self-pollination mechanism fruit is produced when the flowers are pollinated with their own pollen, but in those requiring cross-pollination fruit is only produced when flowers are pollinated by pollen from another cultivar. When cultivars are produced for horticulture by vegetative propagation (as for almonds) this can cause problems where an out-crossing mechanism such as self-incompatibility is present, particularly when they are planted out in a solid block of single cultivars. In this situation, lack of adequate fertilization can seriously limit production.

One such example of the above occurs in almond *Prunus dulcis (Mill.) D.A. Webb [Prunus amygdalus Batsch.; Prunus communis (L.) Arcangeli.]*, which is a member of the *Rosaceae* family with strong self-incompatibility characteristics. Almost all almond cultivars used commercially are self-incompatible and cross-pollination is necessary for seed set and nut production. While other nut crops (e.g. pistachio, walnut, chestnut, filbert and pecan) are anemophilous, almond is entomophilous and requires insects for transfer of pollen from one cultivar to another for successful fertilisation and nut set. The honeybee is the only pollinator vector used commercially for almond production. Other factors affecting pollination in almond are flower characteristics and environmental conditions.

In order to maximize nut production as many flowers as possible should be pollinated, which requires a high bee population and suitable weather to encourage bee flight. For Australian conditions we require knowledge about the best overlapping of flowering time of pollinizers with Nonpareil, the most desirable commercial cultivar. High nut set is not a problem with the almond, thinning is not even necessary. This is different from apple, where 5% fruit set is commercially viable; higher than this requires thinning. For almond however any flower not successfully fertilized reduces production by that much.

The aim of this study is primarily to evaluate factors affecting almond nut set under Australian conditions so as to be able to increase nut set in Australian orchards. Factors studied include gene flow by pollen from cultivar to cultivar within the orchard, pollen tube growth, pollen donor effects, selection of the best pollinizers for Nonpareil among the various cultivars, the possibility that cross-incompatibile pairs of cultivars are being used commercially in same orchards and honeybee behaviour through observation on caged trees. Coming out of these studies also is the possibility of conclusions about genetic inheritance and linkage between genetic traits used for gene flow studies, means of identifying cultivars for plant variety rights purposes, and determining relationships between cultivars and with a selection of almond cultivars and wild species from Iran. Pollen ultrastructure, isozyme polymorphism, and random amplification polymorphic DNA (RAPD) will be investigated as identifying traits for these studies.

1.2 Literature review

1.2.1 Almond - botanical classification and domestication

The botanical name proposed for the cultivated sweet almond is *Prunus dulcis [Mill.] D.A. Webb*. according to Webb (1967). Synonyms listed include *Prunus amygdalus Batsch* (1801) and *Prunus communis (L.) Archangeli* (1882). The almond belongs to the family *Rosaceae* and sub family *prunoidae* (Kester and Asey, 1975). The *Prunus* genus is divided into three sub genera; 1) *Amygdalus*, including almond, peach, and nectarine; 2) *Prunophora*, which includes European and Japanese plum and apricot; 3) *Cerasus* which includes sweet and sour cherries. All members have a haploid chromosome number of eight.

Grassely (1976) and Denisov (1988) classified the wild species of almond into five sections: 1)*Section euamygdalus Spach.*, the ancestry of cultivated almond lies in this section e.g. *Prunus communis (L.) Archangeli.* and *Prunus fenzliana Fritsch.* 2) *Section spartioides Spach.*, this section represents a complex number of species that have similar morphological characters adapted for the extreme xerophytic conditions. A typical species is *Prunus scoparia Spach.* 3) *Section lycioides Spach.*, typical species include *Prunus*

lycioides Spach. 4) *Section Leptopus Spach.*, included here is *Prunus pedunculata Pall.* 5) *Section Chamaeamygdalus*, e.g. *Prunus nana Stock.*

Domestication of the almonds probably took place during the third millennium BC (Spiegel-Roy, 1986). The cultivated almonds spread from Central-Asia, toward the Mediterranean sea, and then to both North and South America and eventually to Australia (Socias i Company and Felipe, 1992a). Wild species of almond trees are found in Central Asia (Popov *et al.*, 1929). Many wild species have naturally hybridized with cultivated almond (Grassely, 1976). Among these species, probably *Prunus fenzliana Fritsch.*, *Prunus Kuramica Korsh.*, and *Prunus bucharica (Korsh.) Feldisch.* have evolved in this way (Kester *et al.*, 1990). Furthermore when almond passed from Mediterranean countries other new hybridizations may have take place, especially with the wild Mediterranean species *Prunus webbii (Spach.)Vierh.* (Socias i Company, 1990). This has resulted in a particular type of almond population along the northern border of the Mediterranean sea, from Greece and the Balkans to Spain and Portugal. As domestication took place many years ago, it is presumed that it was introduced to the other countries (from Central Asia to the Mediterranean countries) through the seeds. Later the migration from the old world to the newly explored countries continued the expansion of these crops to regions where the climate was also favorable for its growth, including California and Australia. This movement was mainly through seeds.

In Australia almond was probably imported before 1900. Quinn (1928) recognized that it may have been brought to Kangaroo Island in 1836, as seed. In 1842 George Stevenson had two varieties growing in his garden in North Adelaide. Sir Samuel Davenport and Dr. R. Schomburg imported almond nuts of the Jordan type from Spain.

In South Australia it is possible that there are two different distinct sources. The first source comes from the offspring of Spanish hard shelled varieties called Jordan (which could have been parents for the local varieties such as Brandis, Chellaston, Johnson's prolific and Stockman's paper shell), the second source from those of Californian origin. The latter include Nonpareil (Callifornian paper shell), Ne Plus Ultra, IXL, Golden State and Languedoc.

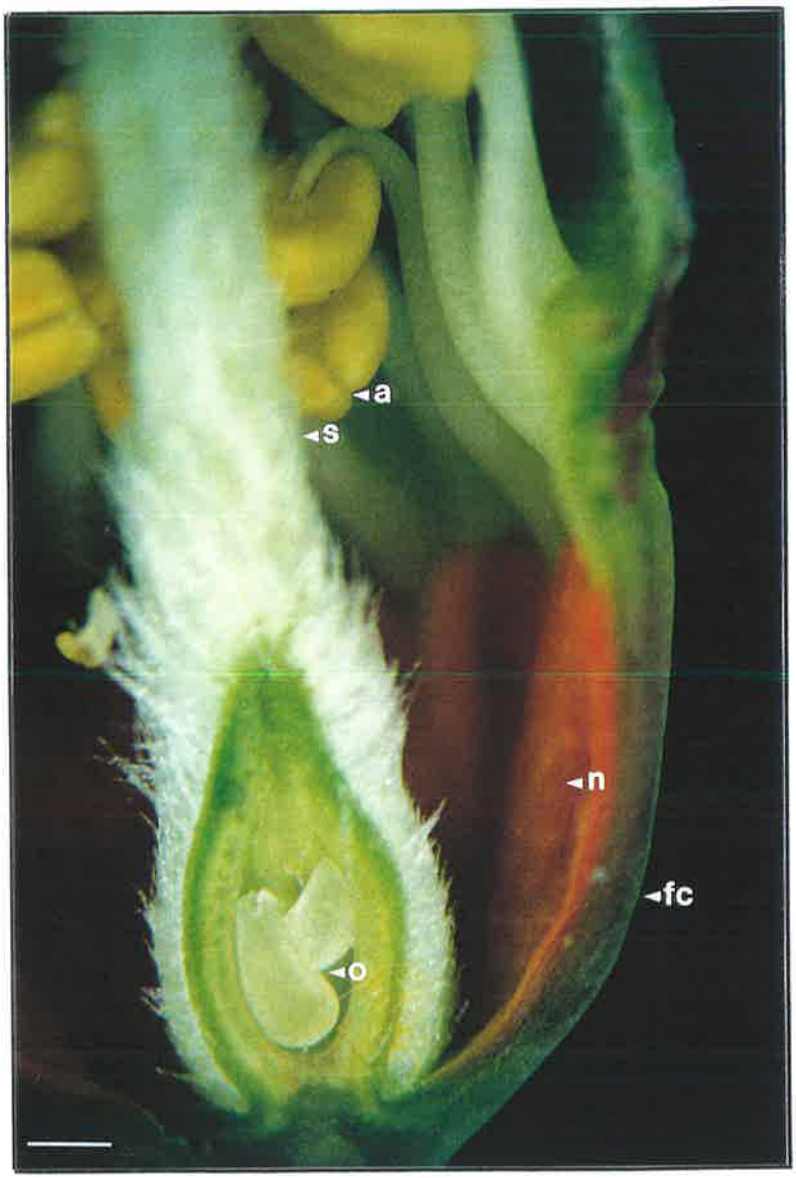
1.2.2 Characteristics of the almond

The cultivated almond is a strong, vigorous tree of medium size, 6-9 m high (Micke and Kester, 1978a). The wood is relatively hard, branches are glabrous and, when they are one year old, are pale green to reddish brown in colour. In most Mediterranean varieties the leaves are petiolated, lanceolate or ovate with an acute apex (Micke and Kester, 1978b). The flower primordia appear in the buds at the beginning of autumn (Weinbaum and Spiegel-Roy, 1985). The flower buds are primarily produced laterally on shoot spurs 2-9 cm long and in some cultivars on lateral or long shoots (Kester, 1981). Flowers are hermaphrodite, with 5 sepals, and 5 white or pink petals. The pistil is single containing two ovules and there are 20-40 stamens (Fig. 1.2.2.1). Flowering which is extremely precocious, takes place from January to March in the northern hemisphere and from July to August in the southern hemisphere. The fruit consist of an egg-shaped drupe with a sigmoid growth pattern (Kester, 1981). The mesocarp is generally thin (5-15 mm) and dehisces at maturity. The endocarp, may vary in shape, surface appearance and consistency. The endocarp is a fundamental factor used to identify different cultivars, e.g. as hard shell, semi-hard shell, soft shell and paper shell. The endocarps contain one or two embryos (kernels) which vary in size and weight from about 0.5-2 g (Woodroof, 1978; Kester, 1981).

1.2.3 Almond nut production

The highest almond production is from the U.S.A. (335,700 tonnes). Europe produces 459,000 tonnes (Spain 250,700 tonnes, Italy 127,300 tonnes), Asia 323,700 tonnes (China 125,000 tonnes, Iran 56,000 tonnes) and Australia 7,000 tonnes (FAO, 1991; Summary of Crops Australia, 1990-91). Almonds normally grow in regions with a Mediterranean-type climate and due to this production is restricted to a small part of Australia. A large proportion of Australian's almonds are produced in South Australia, mainly in the Adelaide plains and the Riverland area. In 1975 South Australia produced 99% of the total Australian production. In 1992 almond production in the South Australian and Victorian Riverland ereas expanded greatly. The total area under nut production compared with that under other fruit crops in South Australia is shown in Figure 1.2.3.1 (Fruit South Australia, 1990-91). In 1992 total area under almond production was

Fig. 1.2.2.1 Longitudinal section of almond flower (Nonpareil cultivar); **a** (anther), **fc** (floral cup), **n** (nectary), **o** (ovules) and **s** (style).



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approximately 5,000 hectares for bearing and non-bearing almond trees; of this area 2,795 hectares was in South Australia (410,000 trees 6 years and over and 149,000 trees under 6 years). In the State of Victoria 2,060 hectares was under cultivation (245,000 trees 6 years and over and 167,000 under 6 years). In the State of New South Wales 65 hectares only was under cultivation (1,000 trees 6 years and over and 12,000 trees under 6 years). (Summary of Crops Australia, 1991-1992) (Fig. 1.2.3.2). The increase in production from 1986 to 1992 for Australia is shown in Figure 1.2.3.3.

We have reason to believe that the almond production figure stated by the FAO (7,000 tonnes) is too high for Australia (Almond Cooperative). The Cooperative reports that for 1991 Australia overall had approximately 3,500 hectares under almond cultivation, yielding approximately 4,000 tonnes of kernels (5.8 kg kernel/tree). The average yield of almond production in Australia increased from 2.7 kg kernel/tree in 1956-57, to 3.9 kg kernel/tree in 1970-71, to 5.5 kg kernel/tree in 1975 (Baker and Gathercole, 1977). In South Australia the average yield per tree is now more than 7 kg. However in the Riverland area near the Victoria border almond production is more than 10 kg kernel/tree (Summary of Crops Australia 1991-92). In the Riverland area there is a high potential for almond production due, we believe, to the better conditions and environment for pollination and fruit set.

1.2.4 Climate and almond production

Almond production is virtually restricted to areas with Mediterranean climates. Less than two percent of the earths land surface has a Mediterranean climate spread within five regions between 30° and 40° of latitude in both hemispheres (Leeuwrike, 1974). Mediterranean-type environments are found in the Mediterranean Basin of southern Europe, central and southern California, central Chile, south-west Cape province of South Africa and in southern Australia (south west of Western Australia and in South Australia) (Gibbon, 1981).

The state of South Australia has the Southern Ocean to the south and to the north is bordered by land. The state covers a total area of 984,377 square kilometres (one eight of the area of the Australian continent). One third of this area has no significant agricultural use and over

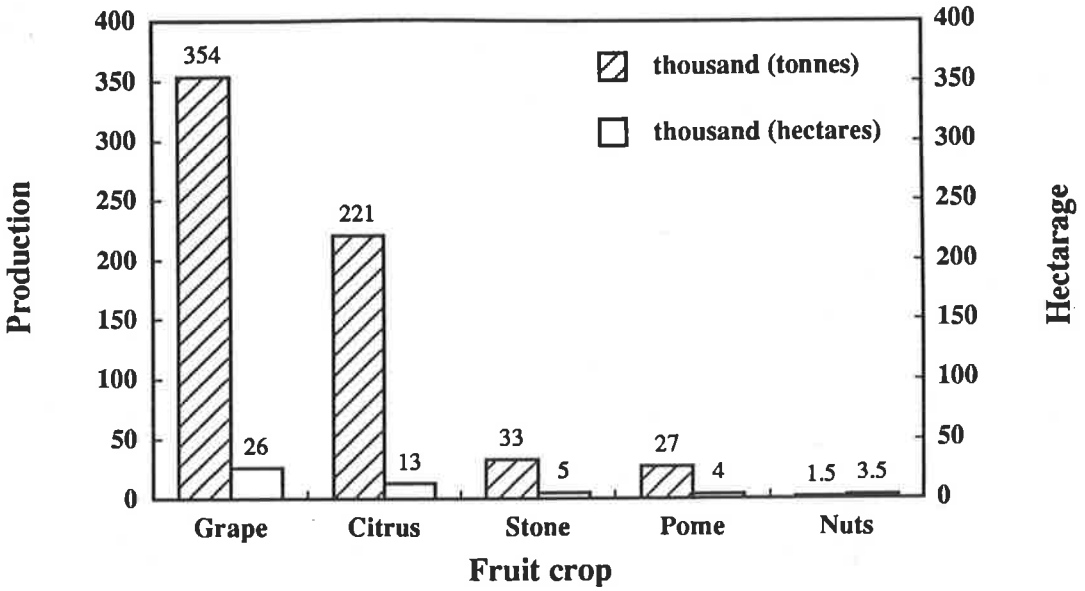


Fig. 1.2.3.1 Major fruit crop production in South Australia (1990-1991)

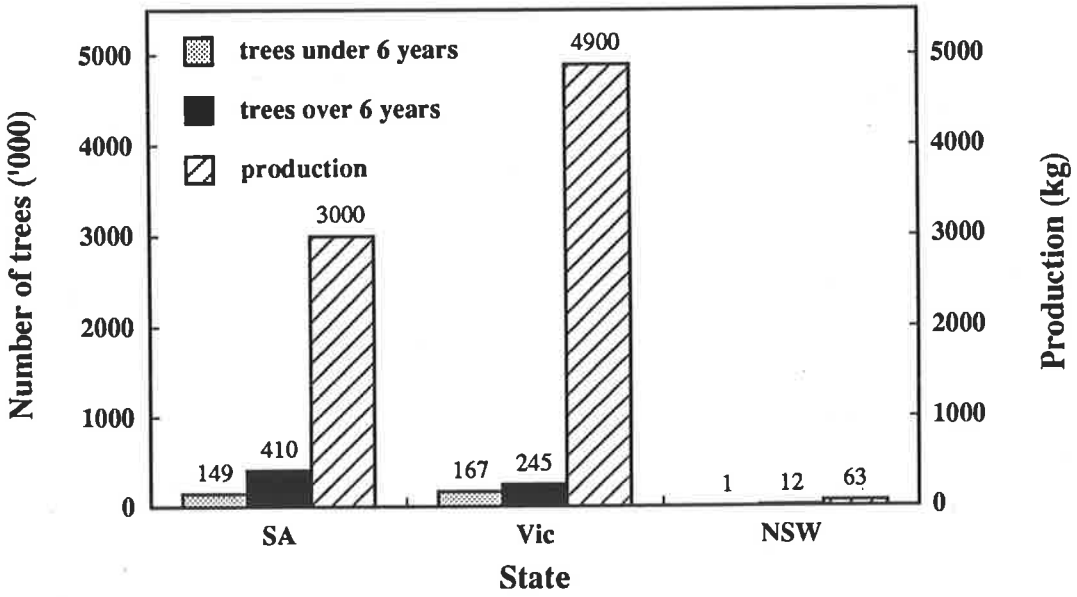


Fig. 1.2.3.2 Number of almond trees and in-shell production in Australia (1992)

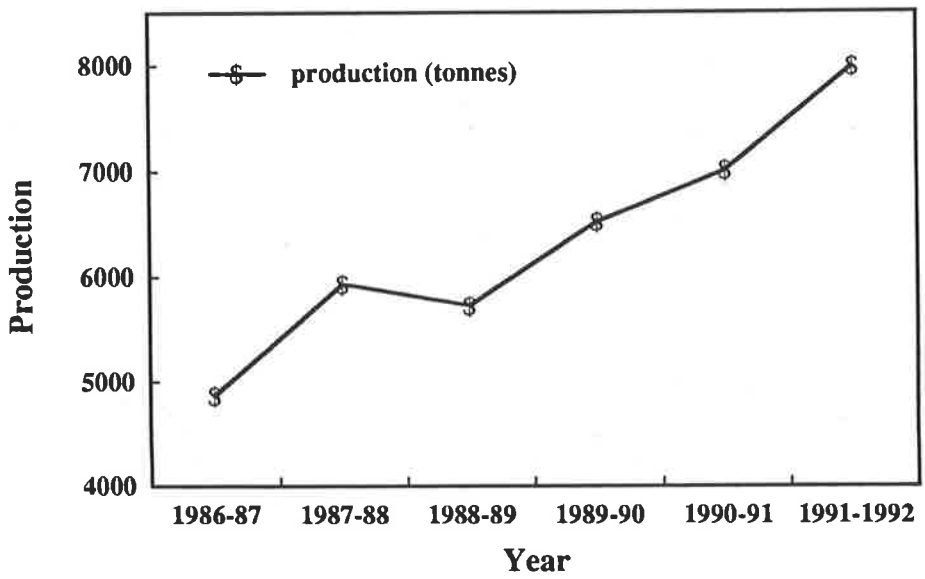


Fig. 1.2.3.3 In-shell almond production in Australia (1987-1992)

half is devoted to extensive pastoral pursuits. Almonds are cultivated in the Adelaide plains and in the Riverland area which traverses the South Australia/Victoria border (Murray River). Adelaide is the driest capital city in Australia, having less rainfall and a lower relative humidity than any other capital. Temperature in the summer frequently exceeds 30° C, July is usually the coldest winter month when the mean maximum is 15° C. However the lowest minimum ever recorded is -0.4° C. August is only slightly warmer than July, but after August, the temperature rises slightly. Adelaide's summer rainfall is light and unreliable, June in winter is the wettest month (Fig. 1.2.4.1a, b) (South Australian Yearbook, 1993).

It will be seen from the data (Fig. 1.2.4.1a) that Adelaide is normally mild in winter months and warm in summer. Extreme cold is never experienced because the Southern Ocean effectively protects the state from the bitter cold polar air mass. Occasionally, however, during the summer months, the very hot weather (hot continental air mass) drifts down from the north and several days of unpleasantly hot weather can result. All of the above suggests that the Adelaide plains portion of South Australia is similar for almond production to the other four regions of the world with a Mediterranean climate. In Figure 1.2.4.1a, b flowering and harvest times are shown relative to the local climatic changes through the year. It is possible that the temperature during flowering is a little low and research reported below suggests that a move to higher temperature regions in South Australia could well be beneficial.

1.2.5 Pollen ultrastructure in the *Rosaceae* family

1.2.5.1 Pollen wall structure

There are two domains of pollen walls; the exine which is the outer patterned layer is made of sporopollenin and the intine is the inner smooth polysaccharide layer. The layers are distinct genetically, morphologically and developmentally (Heslop-Harrison, 1975). The sporopollenin wall polymer is remarkable for its resistance to biological degradation and is considered to be formed by the oxidative polymerization of carotenoids and carotenoid esters (Show, 1971; Brooks and Show, 1978). In morphological terms there are two types of exine, tecta type (pattern surface layer covered by a roof or tectum supported by baculum)

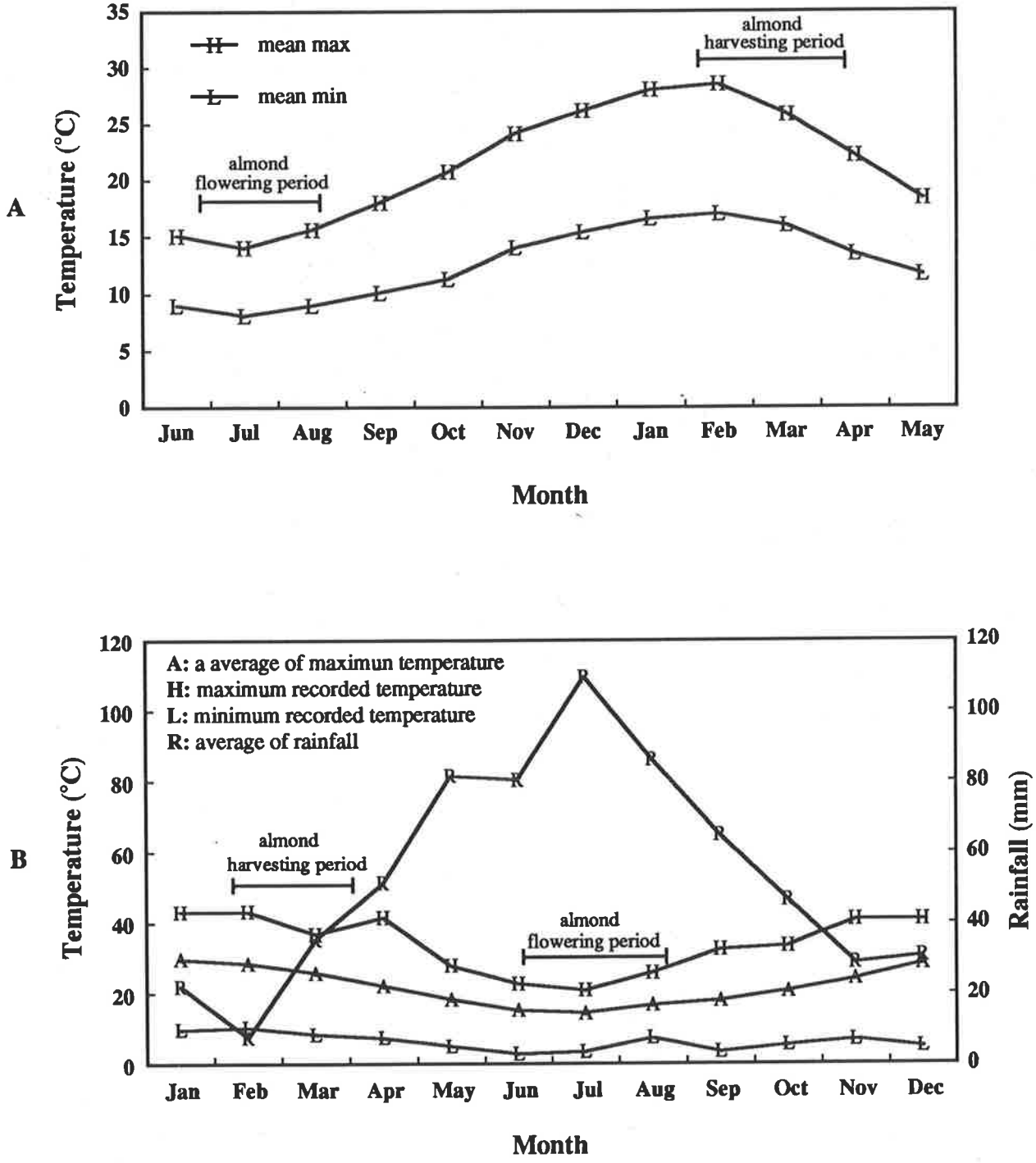


Fig. 1.2.4.1 (A) Maximum and minimum temperature for Adelide maximum (highest recorded) and minimum (lowest recorded), (B) average rainfall and average maximum temperature during (1983-1993).

and pilate (where the patterned surface layer is open at the tips) (Knox, 1984).

1.2.5.2 Pollen finger print, sculpture and pores

Pollen grain sculpture is not easy to describe accurately in words, and consequently somewhat unsatisfactory for analytical description. The major typical patterns of exine structure are: knobby, needle, net, finger print, and combinations of needle with net or knobby (Iwanami *et al.*, 1988). Most pollen grains possess apertures (pores). These thin exine areas are the location for the pollen tube emergence during germination and allow the volume of the grain to alter during humidity changes (Walker, 1974). There are two types of pollen grain aperture, porate (which has apertures in the exine) and colpate (which has long furrows in the exine surface) (Knox, 1984).

In most cases pollen grains in the sub-family of *Prunoidae* and *Pomoidae* of *Rosaceae* family are elliptical-truncate isopolar and triculpate. In these sub-families pollen grain exine has a 'finger print' sculpture. Maas (1977) described pollen ultrastructure of the strawberry (*Fragaria*), and showed the pollen grain in this case to be elliptical, tricolporate and moderately to prominently ridged. The size of pollen and prominance of exine ridges appeared to correspond with the ploidy level. Pollen grain ultrastructure of 31 cultivars of apple was studied by Marcucci *et al.* (1984). They found the size of pollen grains was quite uniform within a given cultivar. The exine pattern may help for the identification of some cultivars or group of genetically close cultivars. In crab-apple Martens and Fretz (1980) showed that all pollen grains were approximately equivalent in size, tricolpate elliptical in shape with tectate-perforate exine type. Exine ridging was superficially similar among the cultivars, but close examination revealed sufficient detail to distinguish between cultivars. Fogle (1977a) compared the pollen grain ultrastructure in apple, sweet cherry, European plum, nectarine and peach. Exine patterns were different for apple and cherry which were, however, similar in size. Patterns enabled a distinction to be established between clones within each of the species. Peach and nectarine clones varied within the same ranges of pollen sizes and exine characteristics. Plum exine patterns were distinct from those of other tree fruits, but ridging and pore characteristics of the exine separated plum clones into distinct categories. In another investigation Fogle (1977b) showed differences in the size

and surface of pollen grain ultrastructure which were useful for distinguishing between species of fruit trees such as, peach, plum, apricot, cherry, apple and pear. Westwood and Challice (1978) used the anther and pollen grain ultrastructure to distinguish between pear species. He found that the combination of pollen grain ultrastructure and topography of anthers were useful to separate the species examined. Mulas *et al.* (1988) described pollen ultrastructure differences between twenty almond cultivars originating from Italy, France, Russia, and the U.S.A. (California). Mulas *et al.* (1989) have also shown that the ultrastructural morphology of Nonpareil almond does not vary according to rootstock, irrigation or geographical location.

When the pollen germinates, the pollen tube emerges through one of the pores. Pollen grains that have one long furrow are called mono-colpate, this kind of pollen is common in monocotyledons and primitive dicotyledons. A large number of dicotyledons including *prunus* have these long furrows that are equidistant from each other. The majority of dicotyledons have three apertures 'tricolpate pollen' (Knox, 1984).

1.2.6 Pollination with particular reference to fruit and nut crops

1.2.6.1 Pollination in fruit and nut crops

When pollen is mature, the anthers open and pollen grains escape. This stage is called 'anthesis' and often known as 'full bloom' (Jackson, 1986). Pollination is defined as the movement of pollen grains from anther to stigma, but not in every instance does pollination leads to the production of a fertilized fruit or nut, even when pollination is followed by the most favourable set of circumstances possible.(Kester, 1981; Ryugo, 1988).

Self-pollination is the transfer of pollen between flowers of the same cultivar; cross-pollination is the transfer of pollen between flowers of two different cultivars of the same or related species (Kester 1981). Pollination is a requirement for the production of most fruit crops and subsequent fertilization of egg (s) and fruit set. Horticultural crops rely greatly upon honeybee (*Apis mellifera L.*) colonies to meet their pollination needs (McGregor, 1976).

Many fruit tree crops, with the exception of those that are anemophilous or produce parthenocarpic fruit, require insect-pollination to produce seed and develop fruit (McGregor, 1976; Free, 1970a; Crane and Walker, 1984). The honeybee is the most commercially significant vector for pollination (McGregor, 1976). Many members of the *Rosaceae* family such as almond, apple, plum and sweet cherry are genetically self-incompatible and require cross-pollination. Golden Delicious apple, tart cherry and most peach cultivars, are at least partially self-compatible.

1.2.6.2 Pollination by insects

Insects are the only animals that feed in sufficient numbers on the flowers of fruit trees to achieve an acceptable incidence of effective pollination, and many species of the insect orders Hymenoptera, Lepidoptera, Diptera, Coleoptera and Hemiptera visit flowers in orchards (Free, 1970a; Boyle and Philogene, 1983, 1985). Not only self-incompatible trees can benefit from insect visits but also self-fertile trees because insects can transfer pollen within a flower from the anthers to the stigma (Free, 1970a; Lane, 1979; Sedgely and Griffin, 1989). Bees in general, and social bees in particular, are the most specialized pollen vectors for cultivated plants (DeGrandi-Hoffman, 1987). The major role of insects, especially honeybees, in pollination of pome and stone fruit has long been recognized. The effect of bee-pollination on quality and quantity of some horticultural crops has been studied in cherries (Lane, 1979), peaches (Langridge and Goodman, 1979) and apricots (Langridge and Goodman, 1981).

Pollination and fruit-set due to wind-pollination is usually insignificant and the pollen of fruit trees (*Rosaceae* family) can only be transported by very strong winds (Wood, 1937; Langridge and Goodman, 1979, 1981). The pollen of this family is sticky and hence difficult for wind to dislodge unless the pollen is dried by high temperatures and low humidity (Crane, 1985). Pear pollen is different from the pollen of *Prunus* and *Malus* in that it is lighter and less sticky and so it is more likely to be transported by wind (Stephen, 1958).

1.2.6.3 Floral attractiveness and foraging behaviour

The three major components of flower recognition for insects are through odour, colour, and the nutritional value of the nectar and pollen (Harborne, 1982). Plants release odours at times when temperatures are high and their pollinators are more active. Odours are composed of mono-or sesquiterpenes, simple aliphatic alcohols, ketones, or esters and can originate from petals, leaves, or flowers. Normally, a mixture of these compounds provides a flower's scent (Harborne, 1982; Williams, 1983). Honeybees can distinguish between flowers of different colours in the ultraviolet-blue green-yellow spectral range (Kevan and Baker, 1983). Nectar-guides, also known as honey-guides, which occur on the petals of flowers, are patterns of lines that converge on the nectaries. They supposedly enhance the attractiveness of flowers and are more pronounced in flowers in which the nectaries are harder to find (Free, 1970a). Bees are attracted to flowers that appear blue or yellow to the human eye (Kevan and Baker, 1983). Using artificial patches of blue and yellow flowers it was shown that some honeybees are constant to colour and others to odour (Wells and Wells, 1985). Flowers with a broken outline as well as olfactory nectar guides on the petals are highly attractive (Free, 1970a; Jones and Buchmann, 1974; Kevan and Baker, 1983).

Some bees forage only on flowers of particular shapes and sizes, but other bees also visit tall and stunted plants of the same species, and flowers at different stages of opening (Free, 1970a, 1970b). The shape and size of almond flowers does not vary greatly between cultivars but there is enough difference to make it possible for bees to distinguish between the cultivars. Apetalous flowers were often visited for nectar, while newly opened flowers without dehisced anthers were not visited by honey bee (Williams and Brain, 1985).

1.2.6.3.1 Nectar

The primary reason honeybees and many other pollinators visit flowers is to collect nectar and pollen for the colony's nutritional needs. The three main sugars most commonly found in nectar are glucose, fructose, and sucrose, although other sugars occur in small amounts such as maltose, raffinose, melibiose, trehalose and melezitose (Baker and Baker, 1983). Nectar is largely comprised of water and several sugars, but small amounts of other substances, such as organic acids, volatile oils, polysaccharides, proteins, enzymes and

alkaloids contribute to its aroma and the characteristics of the honey prepared from it (Shuel, 1955; Baker and Baker, 1975, 1983).

Not all the sugars that occur in nectar are equally attractive to bees, and the proportion of the various sugars in the nectar differ greatly between species and cultivars within *Prunus*, although the proportion of different sugars is usually consistent amongst flowers within cultivars (Wykes, 1952a, 1952b; Baker and Baker, 1983). There are three groups of nectaries: 1) sucrose dominant, 2) those containing primarily glucose and fructose, and 3) those with approximately equal amounts of glucose, fructose, and sucrose (Weinbaum and Spiegle-Roy, 1985). Honeybees prefer nectars containing sucrose to those with either glucose or fructose. Fructose is preferred over glucose (Wykes, 1952a, 1952b) and sucrose dominant solutions are also preferred over nectar containing equal parts of sucrose, glucose, and fructose. Amino-acids and protein are present in nectar in trace amounts only (Baker and Baker, 1973a, 1973b). The amount of nectar in a flower and its sugar concentration are strongly related to humidity (Corbet *et al.*, 1979a, 1979b). More nectar is secreted on sunny than cloudy days, indicating that nectar sugars are a direct product of photosynthesis, which is influenced by sunlight (Shuel, 1955).

Nectar provides a carbohydrate source for honeybees, while pollen supplies the remainder of their nutritional needs (Haydak, 1970) Foragers that find species with higher sugar concentrations dance with more vigour, and more successfully recruit new foragers than those finding nectar with lower sugar concentrations (Lindauer, 1948). Individual almond flower may exude between 1.1 to 4.4 mg of nectar during a 24 h period (Simidchiev, 1973).

Honeybees prefer nectars that contain 20 to 50% sugar, and rarely collect nectar of lower concentrations, perhaps because dilute nectar needs an excessive amount of energy to remove the water and produce honey (Gary, 1979).

Sugars constitute between 29-35% of almond nectar, glucose and fructose are the major sugar constituents, but sucrose is also present (Battaglini and Battaglini, 1974, 1976; Vezvaei and Jackson unpublished data). Almond nectar absorbs UV light strongly and fluorescences brilliantly in the visible portion of the spectrum (Thorp *et al.*, 1975). The ranges of average sugar concentration recorded are; almond 20-40% , apple 25-55%, apricot

5-25%, nectarine 20-25%, peach 20-38%, pear 2-37%, plum 10-40%, sour cherry 15-40%, and sweet cherry 21-60% (Free, 1970a). These ranges suggest that almond nectar is probably always attractive to honeybees; therefore the importance of the concentration of almond nectar probably only depends on whether or not the almond nectar is the most concentrated nectar available and hence the most attractive to honeybees. Nectar from *Prunus* flowers including almond, are low in sucrose and rich in glucose and fructose (Battaglini and Battaglini, 1974), so almond nectar may not always be as attractive as the nectar of other plant species.

Nectar foragers have been found to be effective pollinators in certain cases in almond orchards (Ester *et al.*, 1983). Foragers that visit flowers after their effective pollination period (EPP) has expired are useless for pollination. Pollen-collectors predominantly visit flower within 1 or 2 days of opening when pollen has started to dehisce, whereas nectar-gatherers favour older flowers (Langridge and Goodman, 1981). This tendency was also observed in almond, nectar collectors tend to visit older flowers and so are less likely than pollen collectors to effectively pollinate flowers that have a very short EPP of '1-3 days' (Griggs and Iwakiri, 1964).

1.2.6.3.2 Pollen

Pollen is a rich source of food, and in particular of protein (Stanley and Linskens, 1974; Baker, 1977). Pollen provides bees with proteins, fats, carbohydrates, vitamins and trace amounts of inorganic salts and is essential for brood rearing in honeybee colonies (Stanley and Linskens, 1974; Klungness and Peng, 1984). Pollen collectors have a greater chance of contacting the stigma than nectar collectors in almonds and apples (Robinson, 1979; Thorp, 1979). Todd and Bretherick (1942) found that the percentage of protein in peach and almond pollen was well above the mean value for 126 flower species examined. Some varieties of almond (Tufts, 1919; Hill, 1985) and apple (Webster *et al.*, 1949) produce more pollen than others. Almond pollen is a highly nutritious and attractive pollen (Standifer *et al.*, 1980), so that the attractiveness of pollen to honeybees is probably not a problem for almond pollination.

The optimum temperature range for anther dehiscence in almond is 18° C to 27° C, and dehiscence is retarded at temperatures below 15° C (Micke and Kester, 1978b). Generally, low relative humidity and high temperature are thought to favour anther dehiscence, and rain is thought to reduce the rate of dehiscence (Free 1970a; Langridge and Goodman, 1979, 1981). One hundred almond flowers may produce enough pollen for between 2 and 8 fully laden honeybee pollen loads; i.e. a tree with 10,000 flowers may produce a total of between 200 and 800 pollen loads. A tree with a peak of 2,000 flowers at full bloom may produce 20 to 80 pollen loads per day during the peak of flowering.

1.2.6.4 Climatic effect on honeybee activity and foraging

Weather conditions during the almond blooming period is critical for successful cross-pollination. Climatic variables such as wind, rain, air humidity, temperature, light intensity and spectral quality, are the triggers for the timing of flowering in plants and for pollen vector activity. The major factors influencing honeybee flight initiation are temperature and solar radiation (Burrill and Dietz, 1981). Temperature seems to be a particularly important factor (Wafa and Ibrahim, 1957, 1958). Flight and temperature are positively and linearly related at least between 14° C-22° C (Szabo, 1980; Burril and Dietz, 1981). Honeybees will not fly if the temperature is below 9° C. Even if a suitable temperatures exists, flight will not occur without sufficient light. Some bees fly on cloudy days, but they tend to stay close to the hive (Philips, 1930 a, 1930b). Honeybee prefer to fly in sunlight and visit flowers in sunlight, and many foragers return home when clouds form overhead (Free and Spencer-Booth, 1964a).

Temperature is not the only weather variable that affects foraging activity. Honeybees do not fly extensively during rainy, cool weather, or collect pollen from wet flowers. They may, however, continue to forage for nectar in drizzling rain (Erickson *et al.*, 1975). Rain has also been held responsible for removing large amounts of pollen from anthers (Micke and Kester, 1978b). Rain can burst pollen, but apparently that is not a significant problem in almond (Griggs, 1958; Micke and Kester, 1978b).

Winds below 8 kph have little effect on foraging activity, but foraging activity is low during cool periods with winds of 10 to 20 kph, and few honeybees forage when the wind is over 40 kph, regardless of the temperature (Thorp *et al.*, 1973).

In the morning and afternoon, flight activity is positively related to solar radiation. During the solar noon period when the sun is at its zenith flight activity appears to be negatively related to solar radiation (Burrill and Dietz, 1981). Flight activity is negatively related to humidity, as would be expected since temperature and humidity are inversely related (Szabo, 1980). During unfavourable weather, a lower percentage of the foraging population of larger colonies leaves the hive compared to that of smaller colonies (Free and Preece, 1969).

1.2.6.5 Almond pollination requirement

Almond trees require cross-pollination between cross-compatible cultivars to produce an economic crop (Griggs, 1953; McGregor, 1976; Baker and Gathercole, 1977). All major commercial varieties of almond cultivars in Australia and the U.S.A. are essentially self-incompatible (Baker and Gathercole, 1977; Kester, 1969, 1981; Jackson and Clarke, 1991). A profitable almond crop depends upon the cross-pollination of practically all flowers. In order to obtain this, the bee population should be large enough so that repeated visits are made to every flower. The bees must not only visit many flowers on one tree but also visit between cultivars to obtain their loads of nectar and pollen. In this way, the pollen is spread from one tree to another to the maximum extent (Kester, 1958). The grower wants the heaviest possible set of almonds, because there is no fruit thinning problem (Griggs, 1953; Kester and Griggs, 1959b). The failure of any almond flowers to be cross-pollinated reduces yield by just that much. By comparison, 5% fruit set is economic for apple trees.

Fruit trees that require cross-pollination due to self-incompatibility must have at least one other cultivar planted nearby to serve as a compatible pollen source. The pollinizer must bloom annually and at about the same time as the main cultivar (Free, 1960b). Generally it is thought that the pollinizer should bloom 1-2 days before the main cultivars, although this may vary from one location to another (Dennis, 1979). Bloom phenology is influenced by temperature; cool weather increases the blooming time, while warm weather decreases it

(Morris, 1921). The behaviour of honeybees on fruit trees has been the subject of considerable study (Free, 1960b, 1970a; McGregor, 1976). Bee visitation rates are positively related to bloom density. Honeybees can in some cases discriminate between cultivars and tend to show cultivar-specific fidelity (Free and Spencer-Booth, 1964a; Free, 1966a; Robinson, 1981). Stephen (1958) found that cross-pollination was greater in an intermixture of varieties throughout an orchard. The percentage of fruit set can decrease as the number of rows between the main crop trees and the row of pollinizers increases. Free (1962b) and Free and Spencer-Booth (1964b) demonstrated this effect of increased distance between the main cultivar and pollinizer varieties on the initial and final fruit sets obtained in plum, apple, pear, and sweet cherry orchard. Brown (1951) found that only 2.8% of the flowers in the central part of a well-trained plum tree set fruit, whereas flowers of the two outer parts, each adjacent to a pollinizer, had sets of 6 and 9% . Free (1962b) found that plum trees adjacent to pollinizers has greater sets on their sides facing pollinizers than on their far sides (10.8 and 4.3%).

In Australia almond growers usually plant one 'main' cultivar which is pollinated by one or more pollinizer cultivars whose nuts may be less valuable than that of the 'main' cultivar. Having only two cultivars per orchard is efficient for orchard management, but the inclusion of more cultivars is aimed at reducing the risk of insufficient cross-pollination when there are few flowers on the trees of one cultivar or when the overlap of the flowering periods is poor (Baker and Gathercole, 1977; Hill *et al.*, 1985).

1.2.6.6 The effective pollination period (EPP)

Every flower has a limited life beyond which pollination cannot lead to the fertilization of an ovule. Williams (1965, 1966, 1970) referred to this time limit as the effective pollination period (EPP) defined as the period determined by the longevity of the ovules minus the time necessary for the pollen-tubes to penetrate to the ovules. Occasionally, however, the receptive period of the stigma is less than the calculated EPP, and in this case the actual EPP is then equal to the receptive period of the stigma (Griggs *et al.*, 1965; Weinbaum *et al.*, 1980; Stosser and Anvari, 1983). Pollen cannot move to a stigma unless the flowers are open, and almond stigmas are receptive to pollen at anthesis

(Free, 1970a; Micke and Kester, 1978a). Therefore the time during which effective pollination can occur in almond is the time from anthesis to the expiration of the EPP. The mean EPP is usually delimited by the time when nut-set becomes significantly less than the nut-set of newly-opened flowers, so some flowers can be effectively pollinated after the EPP for the tree has expired (Williams, 1965; Stosser and Anvari, 1983). Another factor that should be taken into account is that the experiments of Griggs and Iwakiri (1964) were performed during warm weather, so the EPPs for many pairs of almond cultivars may often be less than 4 days during cooler weather. On the other hand, Weinbaum *et al.* (1980) obtained 32% and 36% set after hand-pollinating 6-day-old almond flowers, and so EPPs may be more than 4 days in some circumstances.

Ovule longevity and pollen-tube growth, and hence EPP, vary greatly depending not only on the particular cultivar but also the age of the flower. Generally, ovule longevity decreases, and the rate of pollen-tube growth increases, with increasing temperature (Mellenthine *et al.*, 1972; Jefferies and Brain, 1984a, 1984b; Vasilakakis and Porlingis, 1984; Postweiler *et al.*, 1985), but there may be an optimum temperature above and below which the EPP, and nut-set following open-pollination, decreases (Marcellos and Perryman, 1987). Flower position may also be important; ovules of Delicious apple tend to degenerate later in the terminal flowers than in the lateral flowers of clusters. This may be why the fruit-set of terminal flowers is often superior (Hartman and Howlett, 1954). Apple cultivars with a tendency to biennial bearing often have longer EPPs in years of higher yields, but the significance of this coincidence is unknown (Williams, 1970). Griggs and Iwakiri (1964) found that almond flowers must be pollinated by compatible pollen within four days of first opening and, therefore, flowering periods of compatible cultivars should coincide with these four days. Ovule degeneration in Italian Prune commences at the chalazal end of the nucellus so that even if the egg is fertilized this will not result in fruit set (Thompson and Liu, 1973). Almond ovules do not mature until anthesis, so the stage of development of almond ovules at anthesis may depend on several critical factors just prior to anthesis (Pimienta and Polito, 1983).

1.2.6.7 Pollen tube growth and fertilization

In almond the stigma is usually receptive as the blossom opens, or shortly thereafter, and remains receptive for about 3 to 4 days under favourable weather conditions or longer in cool, cloudy and wet weather (Kester, 1981). A positive correlation has been demonstrated in plum between the number of pollen grains deposited on the stigma and the rate of pollen tube growth in the style (Lee, 1980). In many crops pollen tube growth to the base of the style is relatively more rapid than the tube growth between the base of the style and the ovule. In almond the pollen tube reaches the base of the style after four days, but three or four more days are needed to reach to the embryo sac, despite the former distance being far greater than the latter (Pimienta *et al.*, 1983). This phenomenon has also been reported for cherry (Stosser and Anvari, 1981), hazelnut (Thompson, 1979), and apple (Anvari and Stosser, 1981). The ovary of hazelnut and the embryo sac of almond are not capable to reaching maturity in the absence of compatible pollination (Thompson, 1979; Pimienta and Polito, 1983). In almond the abortion of the second ovule is due to blockage of the vascular tissue, and the case degenerates prior to fertilization (Thompson, 1979; Pimienta and Polito, 1983).

Generally, the rate of pollen-tube growth is increased by increasing temperatures, so if incompatibility can be overcome, it is more likely to occur at higher temperatures (Lewis, 1942; Griggs and Iwakiri, 1975; Socias i company *et al.*, 1976). The speed at which pollen-tubes grow in a stigma differs between cultivar-pairs, and the differences appear to be unrelated to any of the incompatibility reactions. Generally, the fastest growth of pollen tubes in almond occur when the temperature is between 18° C and 30° C. Below 15° C the pollen-tubes often fail to reach the ovary, regardless of the cultivars involved (Griggs, 1958; Socias i company *et al.*, 1976; Weinbaum *et al.*, 1984). This information has been used to support the suggestion that orchard temperatures generally should be kept above 13° C by supplementary heating and the use of windbreaks to reduce the wind-chill factor. The growth of pollen-tubes is probably an important factor in nut-set in Australian almond orchards because daily maximum air temperatures in most districts are usually below 13° C during at least the first half of the almond flowering season. It is important that more research be carried out in this area.

Griggs and Iwakiri (1964) found that almond flowers were most receptive to pollination on the day immediately after they opened; set was significantly reduced when pollination was delayed until the third day after opening, and practically no set occurred when pollination did not occur until five or more days after opening. In most species, there is little or no growth of the ovary during anthesis (Crane, 1964; Coombe, 1976). Many authors (Kester and Griggs, 1959a; Griggs and Iwakiri, 1964; Griggs, 1970; Gary *et al.*, 1976; Baker and Gathercole, 1977; Micke and kester, 1978a) assumed that usually about 30% of flowers in commercial orchards produce mature nuts. Micke and Kester (1978a) stated further that the percentage can be 'from below 20% to over 40%, depending on season, weather variables, the number of flowers on the tree and other factors'. However, those percentages appear to have been based on the nut-set that the more productive orchardists obtain, and not on the mean nut-set that is theoretically possible.

1.2.6.8 Other factors affecting pollination

The pollen in the first flowers of the almond flowering season is not as viable as pollen in the flowers that open later (Hill, 1985). Female-sterility is also a significant factor in nut-set, microscopic examination of almond flowers showed that some flowers thought to be female-fertile are actually female-sterile (Pimienta and Polito, 1983). Many almond flowers are incapable of producing nuts because they lack mature ovules. Such flowers may be referred to as 'female sterile flowers'. Generally the occurrence of significant numbers of female-sterile flowers is not unusual amongst the flowers of fruit trees (Howlett, 1926; Jones, 1968; Socias i Company *et al.*, 1976; Postweiler *et al.*, 1985; Rallo and Fernandez-Escobar, 1985).

Honeybees prefer some varieties of fruit crop to others (Karmo, 1958), so when cross-compatible varieties are selected for planting in the same orchard, their relative attractiveness to bee should be considered, as should that of varieties in surrounding orchards. The preference of bees for particular varieties seems to be related to differences in nectar secretion, and correlations have been found between the numbers of honeybees visiting different varieties and the amount and/or concentration of nectar present in apple and plums (Brown, 1951).

By using pollen traps and analysis the pollen loads it was shown that soursobs (*Oxalis pes-caprae L.*), and wild mustard (charlock) (*Sinapis arvensis L.*) and salvation Jane (*Echium plantagineum L.*) are the main species responsible for the reduction of almond pollen collection (Jackson and Vezvaei, unpublished data). It is recommended that almond orchards be kept clear of weeds and that area around the orchard by sprayed to control broadleaf weeds (Hill, 1985).

Information on the foraging areas of individual honeybees is important when trying to solve many pollination problems, because bees that keep to one fruit tree or one cultivar during a trip are valueless as cross-pollinators. Minderhoud (1931) found that during a single trip the foraging area of a honeybee was not greater than 100 m^2 and suggested that large fruit trees, standing well apart from others, might not be sufficiently cross-pollinated. MacDaniels (1931) observed that individual honeybees tend to keep to a single tree, or to two trees. Roberts (1956) reported that, when trees are large with plenty of flowers, a bee is likely to keep to one tree per trip. Free (1960a) found that, in orchards where trees within rows were closer together than trees in adjacent rows, bees changed trees more frequently within rows than between rows. Singh (1950) supposed that a honeybee's foraging area can extend to several fruit trees when forage is scanty, and Free (1960a) found that, during unfavourable weather, bees visited fewer flowers per tree than in good weather.

In almonds foragers usually visit 100 flowers per foraging trip and make 10 trips per day during good weather. However, adverse weather decreases the forager density, and the remaining foragers make fewer trips per day through increasing the time required to collect a load and not through decreasing the time each forager spends foraging (Free, 1960b, 1970a). A positive correlation was found between the number of colonies and cherry and pear production (Stephen, 1958).

Commercial almond production depends on cross-pollination facilitated by honeybees. The nut-set of the average orchard is commonly 10 to 30% but over 50% is possible (Hill, 1987). In Australia almond growers favour two kinds of patterns in orchard design; two rows main cultivar and one row of pollinizer, and/or one row of the main cultivar to each row of the pollinizer. In a hypothetical orchard where each tree is of a single cultivar, the trees are planted to a square grid pattern, and the trees canopies are spherical and

do not touch each other, a forager can move in a straight line from one tree to any of the eight surrounding trees (Hill, 1989). Information available to Australian almond growers states that 'at least 3 hives per hectare are recommended and 6 to 8 hives per hectare for better results' (Baker and Gathercole, 1977). Generally, less than 10% of the pollen carried into hives is as mixed species loads (Free, 1963) but many mixed loads may go unnoticed because pollen from some plant species, especially within Prunus, are difficult to distinguish without using a scanning electron microscope (Fogle, 1977a, 1977b; Thorp, 1979; Iezzoni and Hancock, 1984; Marcucci *et al.* 1984; DeGrandi-Hoffman *et al.*, 1984).

1.2.6.9 Pollination related phenomena

Apomixis, polyembryony, parthenocarpy, xenia and metaxenia, all affect horticultural production. Apomixis and polyembryony are not important in pome and stone fruits but parthenocarpy, xenia and metaxenia (effect of pollen parent on seed and fruit) are important in these two sub families.

Parthenocarpy is the formation of fruit without pollination (Sedgley and Griffin, 1989). In nut crops such as almond and pistachio, where the economic section is the kernel parthenocarpy is a disadvantaging phenomenon (Crane, 1973). Griggs and Iwakiri (1954) indicated that temperature was the determining factor in the parthenocarpic fruit set of 'Bartlett' pear. In most fruit the seed is the source of giberellic acid (GA), but in the certain varieties the pericarp is also able to produce GA, and the GA produced by the pericarp is sufficient to induce and maintain fruit growth. The GA production is sensitive to temperature. Thompson and Liu (1973) noticed that cool weather during flowering time can cause parthenocarpic function in apple.

Parthenocarpy in almonds refers to the development of a fruit that appears normal but which does not have a seed (kernel). The ultimate aim of almond growers is to produce almond kernels, so parthenocarpic almonds are useless for almond production. In almonds, ovule abortion is the result of ovule degeneration following callose deposition in the chalazal region (Thompson and Liu, 1973); embryo abortion can also be correlated with a high temperature at the time of pollination (Thomson and Liu, 1973). Large numbers of parthenocarpic nuts are sometimes produced by the trees of some almond cultivars, perhaps

from flowers that opened early in the flowering seasons. Little is known about parthenocarpy in almond and so the significance of parthenocarpic fruit as a factor of nut-set in almond is unknown. Parthenocarpy has been studied in other crops, and larger numbers of parthenocarpic fruit can be produced by spraying trees with giberellic acid. Naturally occurring parthenocarpic fruit are also common on the trees of some peach and nectarine cultivars (Weinbaum and Erez, 1983).

McKay and Crane (1938) considered the effect of different pollen on various female recipients in chestnut. The fruit resulting from crosses between *Castanea mollissima Blume.* as the pollen source and a variety of Japanese chestnut called 'Male Sterile' yielded smaller nuts than crosses between this variety and *Castanea crenata Sibold & Zuii.* In this investigation it was shown that the pollen source has a direct effect on the size of the seed. For example, fertilization by pollen from varieties bearing small nuts on varieties normally bearing large nuts produces nuts smaller in size than those of the self-pollinated female tree. It was demonstrated by Cedo *et al.* (1984) that pollen has a direct effect on the type of endosperm in the Makapuno coconut (*Cocos nusifera L.*).

Romberg and Smith (1946) reported statistical evidence that cross pollination produced pecan nuts significantly larger in volume and with heavier kernels than those produced by self-pollination. Any changes to the embryo of pecan were considered to be effects of heterosis and any increase in the maternal section was attributed to metaxenia. Crane and Iwakiri (1980) reported that there was no significant effect on percentage of fruit set from hand pollination of *Pistachia vera L.* with different pollen sources such as Peters, Ask, Aegine B, Hybrid cultivars, and *Pistachia atlantica Desf.* All of the cultivars used as a male parent had an equal compatibility with the Kerman variety. Fruit resulting from these cross-pollinations matured at the same time as fruits produced with *Pistachia vera L.* pollen. The lengths and diameter of whole fruits produced by the various pollen sources were not significantly different, but dry weight and kernel length were significantly different in inter and intra specific-hybridization. Time of maturity was not altered by different kind of pollens. These authors have shown shell splitting is related directly to kernel development rather than to type of pollen source. In another experiment, Nevo *et al.* (1974) found that

the source of pollen does not influence the structure of the endocarp nor its dehiscence, but it does affect the size of the kernels.

Whitehouse *et al.* (1963) showed that the pollen source has an effect on the time of ripening of fruits. Pollen of pistachio other than *Pistachia vera L.* tended to retard nut development. The greatest delay in ripening of nuts occurred when *Pistachia chinensis Bunge.* and *Pistachia integerima Stewait ex Brandis.* were used. In general larger kernels and shell splitting resulted from applying *Pistachia vera L.* pollen. Whitehouse also stated that kernel size and shell splitting are varietal characteristics and that the pollen has only a small influence.

1.2.7 Self-incompatibility

Self-incompatibility is one of the important outcrossing mechanisms and is genetically controlled. Self-incompatibility is best defined as the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination (Nettancourt, 1977). It is a mechanism that usually ensures obligate outbreeding, but it may be reproductively inefficient. Self-incompatibility results from the failure of selfed pollen grains to adhere to or germinate on, the stigma; or for the pollen tube to grow normally the full length of the style, with one possible exception *Borago officinalis L.* (Crowe, 1971). In the latter, if the pollen tube is formed at all, it grows so slowly that it may never reach the ovule; or if it does it will be so late that the ovule either will have been pollinated by compatible pollen or will have withered. This definition thus rules out failure of sexual fertilization due to sterility, embryonic lethality or breeding barriers. Sedgley and Griffin (1989) treat self-incompatibility in the broadest sense, to include both pre- and post-zygotic mechanisms, and suggest that the distinction between the two in terms of genetic control may be more apparent than real. Because self-incompatibility is a form of plant growth regulator acting on pollen and pollen tubes it is amenable to study by bioassey (Jackson and Linskens, 1990).

1.2.7.1 Gametophytic incompatibility response

Two kinds of styles can be distinguished; the solid style and the hollow style. Solid styles have a central core of transmitting tissue, and hollow styles usually have a canal which

is sometimes divided into branches. The hollow style is very common in monocotyledons. The pollen tube passes through the canal to reach the ovary, in self-incompatible species the pollen tube usually is arrested in the tissue of the style (Ascher and Drewlow, 1971). In *Trifolium* the hollow style is surrounded by secretory cells, and the canal and stigma exudates are the same composition (Heslop-Hrrison, 1982). In citrus (Kahn and Demason, 1985) and gooseberry (Arasu, 1985) the pollen tube is arrested in the canal. Brewbaker (1967) reported that self-incompatibility in the ovary may also be correlated with hollow styles.

Solid styles have transmitting tissues with the files of cells separated by intercellular substances. Acid phosphate, peroxidase and carbohydrates are found in the intercellular material (Herrero and Dickinson, 1979). Pandy (1962) observed that most incompatible pollen tubes in self-and cross-incompatible crosses were arrested about one-third of the length down the style from the stigma of *Solanum spp*. In the neck of the style of *Petunia* hybrids are large spherical cells, where the pollen tubes are arrested (Kenrick and Knox, 1985).

Small molecular weight glycoproteins have been correlated with S genotype in *Petunia hybrida Hort.* (Kamboj and Jackson, 1986). The upper part of the style is also the inhibitory site in *Prunus serotina Ehrh.* (Yamashita *et al.*, 1990), and *Berassica* (Moore and Nasrallah, 1990; Kandasomy *et al.*, 1990). Sedgley *et al.* (1985) observed that the pollen tubes of *Macademia* were arrested in the upper part of the style. Ellis *et al.* (1991) reported that upper part of the style in *Eucalyptus* was the site of inhibition in inter specific crosses. In *Nicotiana elata Link lotto*. the highest concentration of S-glycoproteins was present in the upper part of the style at flowering time. Anderson *et al.* (1986) reported that using the stigmatic secretion from a compatible mature flower with mature pollen on immature stigmas from the same plant resulted in selfed seed. Thus, incompatibility substances (S-glycoproteins) are not present in immature flowers, Koltunow (1990) supported this finding in tobacco.

1.2.7.2 Gametophytic self-incompatibility in the *Prunus* genus

Most members of the *Prunus* genus are self-incompatible and will not produce commercial crops if planted in solid blocks of one cultivar; also certain groups of cultivars are cross-incompatible. For effective cross-pollination, at least 2 to 3 cross-compatible cultivars must be planted in an orchard and bees provided as pollinators. However in self-incompatible cultivars, even in a good pollination season, fruit production can be erratic and is dependent upon weather conditions before and during the bloom period. The amount of pollen production may vary from year to year and the natural bee population and their foraging patterns may also vary (Free,1960b). Most of these problem can be eliminated if self-fertile cultivars are used.

In *Prunus* genus there is a homomorphic monofactorial, multiallelic gametophytic incompatibility system (Crane and Brown, 1937; Tehrani and Brown, 1992). In this system the pollen / pistil interaction is genetically controlled by the haploid genome of each pollen grain and the diploid genome of the pistil tissue. One S locus with a large number of alleles S1, S2 ...Sn, controls the compatibility relationships. The major recognition gene, S, used to be referred to as a sterility factor, but since both pollen and embryo sacs are functional and not sterile, it is now called the incompatibility gene.

1.2.7.3 Compounds involved in gametophytic self-incompatibility

The biochemical and radiogenic studies of Lewis (1949, 1960) showed that the S-locus in monofactorial gametophytic systems has a tripartite structure. According to this hypothesis, the incompatibility gene consists of three linked segments which determine the incompatibility phenotype of the pollen and style. The first segment is the S locus specificity segment, common to both pollen and pistil. It individualizes the allele and contains specific genetic information that either prevents or fails to stimulate pollen tube growth if one of the two specificity segments present in the diploid style is also present in the haploid pollen grain. The second segment controls activation of S activity in the pollen (pollen activity), and the third segments controls S activity in the pistil (stylar activity). In cherry X-irradiation has led to the recognition that each of the two activity segments can be mutated independently, and linkage relationships between the mutated activity parts and the

specificity segments have been demonstrated (Lewis 1949, 1960). All three part of the locus in cherry are very closely linked according to this hypothesis (Lewis,1960).

In *Prunus*, especially in cherry, some research has been carried out for compounds in the secretions that are involved in the incompatibility reaction (Nettancourt, 1977). Stylar antigens of *Prunus avium (L.) L.* have been studied by Raff and Clarke (1981) and Raff *et al.* (1981). Two major antigenic components were detected in stylar extracts from mature flowers, one was uniquely associated with stylar tissues but not related to the S-genotype, and the other was restricted to the style, and was absent from immature styles in which the incompatibility response is not found. These compounds met the criteria for involvement in the self-incompatibility reaction. The amounts of these components increased gradually during floral development, and were correlated to the progressively increased capacity to reject incompatible tubes. Their concentration is highest in the transmitting tissue of the style where the rejection occurs. Mau *et al.* (1982) isolated the series of style components corresponding to different S-allele groups of *P.avium (L.) L.* They isolated and partially characterized components of *P.avium (L.) L.* styles. A glycoprotein was the major component of the isolated material, but a uronic acid containing component and an arabinogalactan were also present. An antigenic glycoprotein associated with the S_3S_4 self-incompatibility genotype (antigen S), and a component found in the style of all *Prunus* species (antigen P) were two additional minor components of the style. Williams *et al.* (1982) found that antigen S was a potent inhibitor of *in vitro* pollen tube growth, and the other isolated components were not effective in inhibiting pollen tube growth. So the finding that the antigen S is a powerful inhibitor of pollen tube growth is consistent with the idea that it may be a product of, or related to, the S gene.

In cherry and pear it appears to function via glycoproteins which correlate with the incompatibility alleles (Raff *et al.*, 1981; Hiratsuka *et al.*, 1985a, 1985b, 1986). The glycoproteins have a molecular weight of 37000-39000 in cherry (Mau *et al.*,1982) and 52000-58000 in pear (Hiratsuka *et al.*, 1986), and appear to have the biological activity of Antigen S as demonstrated by the inhibition of *in vitro* germination of self-pollen (Williams *et al.*, 1982; Hiratsuka *et al.* 1987) Messenger ribonucleases (mRNAse) and proteins play a role in the regulation of pollen tube growth (Lin *et al.*,1987), and S-glycoproteins in the

Solanaceae are reported to be ribonucleases (Ai *et al.*,1990; Singh *et al.*, 1991; Lee *et al.*, 1994; Murfett *et al.*, 1994).

Two cultivars will be cross-incompatible when the specific S-allele present in the haploid pollen grains of one is the same as one of the two S-alleles in the diploid tissue of the pistil of the other. Most of the research into self-incompatibility in the *Prunus* species has been carried out in sweet cherry. A large number of alleles at the S-locus controls cross-incompatibility among sweet cherry cultivars. Crane and Lawrence (1931) hypothesized that at least six alleles controlled the system. Crane and Brown (1937) later extended this estimate to at least nine alleles. (Tehrani and Brown, 1992).

1.2.7.4 Self-incompatibility in almond

All commercially important almond cultivars in Australia and California exhibit gametophytic self-incompatibility (Socias i Company *et al.*, 1976). Thus, fertilization and fruit set by a plant of given genotype requires pollen of an intercompatible genotype (Griggs, 1953). Incompatible pollinizers are one of the barriers to maximum almond productivity (Kester and Griggs, 1959a). Commercial cultivars, even if self-fertile, when planted with other cross-compatible cultivars should exhibit fruit set percentages of approximately 25-30% or higher (Kester and Griggs, 1959a).

Self-incompatibility in almond is homomorphic and controlled by a single multi-allelic gene, which is gametophytically controlled. Self-fertilization is prevented when pollen carrying an S-allele which is identical to that carried by the stigma on which it lands (incompatible) and is discriminated from pollen carrying an S-allele which is not carried by the stigma (compatible). Three type of pollination may be observed: fully compatible, half-compatible and incompatible (Fig. 1.2.7.4.1). The S-gene is, by its nature, multi-allelic. The minimum number of S-alleles required to maintain the polymorphism is three, since individuals are heterozygous, and the number of S-alleles which could be maintained in a population is theoretically very large (Kester and Asay, 1975; Frankel and Galun, 1977; Tehrani and Brown, 1992).

Only a few of the numerous almond cultivars grown worldwide are self-compatible. The majority of these come from the Italian region of Apulia (Reina *et al.* , 1986). These

cultivars where shown to be capable of transmitting their self-compatibility to their offspring (Socias i Company and Felipe, 1977). The incompatibility systems in the genus *Prunus* appear to be controlled by a single, multi-allelic gene which is expressed in the pollen which is gametophytic (Crane and Brown, 1937; Crane and Lawrence, 1947; Lewis and Crowe, 1954). This system seems to occur in almond species (Socias i Company, 1990; Socias i Company and Feilip, 1992a). Assuming that the inheritance of self-compatibility in almond is the same as that in other *Prunus* species, Dicentia and Garcia (1993) suggested that self-compatibility is determined by an allele of the self-incompatibility gene which is dominant over all the other alleles at this locus; this is supported by other authors (Socias i Company, 1984; Socias i Company and Felipe, 1988). The gene concerned with self-compatibility is monofactorial and is dominant over all self-incompatibility alleles of the S-gene (Dicentia and Garcia 1993). The gametophytic self incompatibility system in almond, is controlled by the S locus. Like any other gene, this locus can undergo mutation, naturally or artificially induced. A mutation can establish a self-compatible allele Sf (East, 1929). The transfer of self-compatibility genes from peach and other species of *Punus* has been an objective of almond breeding programs in many countries (Kester and Asay, 1975, Kester, 1978).

Numerous methods have been used to overcome the self-incompatibility barrier on the stigmatic surface including physiological, physical, mechanical and genetic methods (Wright, 1989). There are several physical factors related to this study including bud pollination; there is one of the oldest methods for overcoming self-incompatibility (Linskens 1964, Shivanna *et al.* 1978). The method has been applied successfully to various gametophytic systems, e.g. in *Petunia*, *Prunus*, and *Trifolium*. Delayed pollination can occur; Kakizaki (1930) observed that in some incompatible combinations seed set was enhanced by putting fresh pollen on aged stigmas. End of season compatibility is another way of overcoming self-incompatibility, it has been observed repeatedly that the incompatibility reaction of a plant weakens towards the end of its life cycle (Nettancourt, 1977; Litzow and Ascher, 1983).

1.2.8 Cyanogenic glycosidase in plants

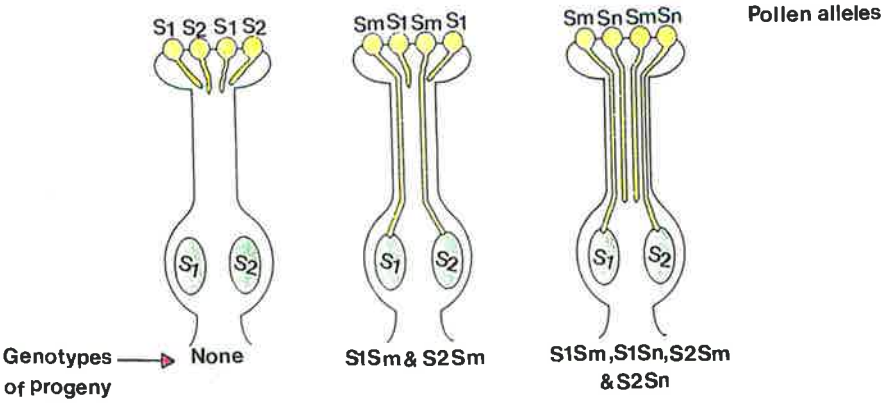
Glycosides are organic compounds in which there is usually a semiacetal linkage between the reducing group of a sugar and an alcoholic or phenolic hydroxyl group of a nonsugar compound called an aglycone. The semiacetal link takes place through oxygen and so the compounds are known as O-glycosides. These glycosides are easily hydrolyzed to the sugar and aglycone by either enzymes or acids (Conn, 1980).

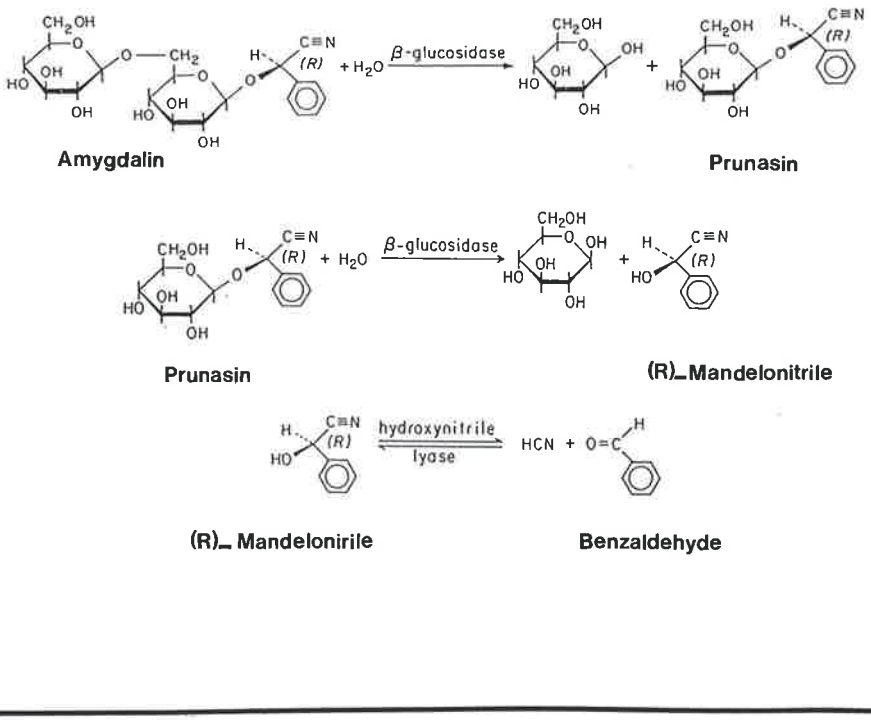
The production of hydrogen cyanide (prussic acid) by living organisms is known as cyanogenesis. Higher plants which exhibit this phenomenon contain one or more compounds which liberate the cyanide on hydrolysis. The cyanogenic substances in higher plants are of two types: cyanogenic glycosides and cyanogenic lipids. Both are derivatives of hydroxynitriles (cyanohydrins), and both liberate a carbonyl compound and HCN when the sugar of the respective fatty acid moiety is removed (Conn, 1980). Glycosides forming HCN on hydrolysis are widely distributed in the plant kingdom (Hegnauer, 1977). Because of their toxicity to man and other animals, the existence of these glycosides has been known for a long time. In higher plants, HCN is tied up in the form of a cyanogenic glycoside. The structure of the cyanogenic glycosides may be illustrated by the formula for amygdalin (see Fig. 1.2.8.1).

The ability to make cyanogenic glycosides which liberate hydrogen cyanide on hydrolysis is widespread among plant. At least 2050 species from 110 plant families have Cyanogenic glycosidic compounds (Hegnauer, 1977). Examination of chemotaxonomic treatises by Gibbs (1974) suggested that as many as 2000 species of higher plants are cyanogenic. The list of species includes ferns, gymnosperms, and angiosperms, distributed among approximately 110 families. According to Gibbs (1974) the cyanogenic families are *Rosaceae* (150 species), *Fabaceae* (125), *Poaceae* (100), *Araceae* (50), *Asteraceae* (50), *Euphorbiaceae* (50), and *Passifloraceae* (30). While the number of cyanogenic species is large, the number of cyanogenic glycosides found in these species is small, numbering only 23. Only four cyanogenic lipids are known and these occur in a single family, the *Sapindaceae*.

Fig. 1.2.7.4.1 Compatible and incompatible pollination in almond (after Tehrani and Brown, 1992).

Fig. 1.2.8.1 Possible pathway for cyanogenesis compounds in almond (from Conn, 1979).


S1 S2 S1 S2
Sm S1 Sm S1
Sm Sn Sm Sn
Pollen alleles
S1
S2
Genotypes of Progeny
None
S1Sm & S2Sm
S1Sm, S1Sn, S2Sm & S2Sn


β-glucosidase
Amygdalin
Prunasin
Prunasin
β-glucosidase
(R)-Mandelonitrile
hydroxynitrile lyase
HCN + O=C
(R)-Mandelonirile
Benzaldehyde

1.2.8.1 Amygdalin in the *Rosaceae* family

In the family *Rosaceae*, the compounds commonly found are amygdalin from almond, peach and apricot, and prunasin from almond and *Prunus macrophylla*. Amygdalin is distributed extremely widely in rosaceous species. This compound is found in the seeds of the *Rosaceae*, such as those of the bitter almond, apricot, plum, peach, apple, quince, Japanese medlar, and others. It is also found in the leaves and bark of the peach, and in the leaves, flowers, and bark of the bird cherry (*Prunus padus L.*). The glycoside has been obtained in 1.8% yield from the seed of bitter almond (Wester, 1913) and in 2% yield from the bark of peach (Rabate, 1933).

Prunasin is the beta-D-glucoside of mandelonitrile, whereas amygdalin is the corresponding gentiobioside. Prunasin is widely distributed among many families in contrast to amygdalin which is limited to the *Rosaceae*. Prunasin has been reported in the leaves and branches of *Prunus padus L.*, the bark and leaves of *Prunus serotina Ehrh.*, the leaves of *Prunus laurocerasus L.*, and *Prunus microphylla*, and the green leaves of *Photinia serrulata Lindl.*, all members of Rosaceae (Nahrstedt, 1973).

In *Prunus avium (L.) L.*, in all aerial parts D-mandelonitrile-beta-glucoside (prunasin) has been identified in the vegetative parts, while prunasin and D-mandelonitrile-beta-gentiobioside (amygdalin) are found in the generative parts (Nahrstedt, 1972). The cyanogenic glycoside prunasin occurs in leaves and fruit of Cotoneaster species, whereas amygdalin was found in the ripe fruits.(Nahrstedt, 1973).

1.2.8.2 Bitterness in almond

Bitterness in almond kernels is due to a glycoside, amygdalin, which accumulates within the seed of those almond cultivars which have the bitter genotype (McCarty *et al.*, 1952; Woodroof, 1967; Frehner *et al.*, 1990). The amygdalin precursor (prunasin) is not produced in the kernel, but is inherited from the mother plant, which has the bitter genotype (Frehner *et al.*, 1990). Heppner (1923) suggested that the original almond in an evolutionary sense was bitter. If true, it is possible that a mutation occurred in the bitter almond tree with the sweet almond as the result. Bitterness is primarily a problem when seeds from a bitter tree are inadvertently mixed with kernels from a sweet tree. Bitter kernels

historically have been a problem in older orchard planted out with trees generated from seeds rather than clones, because up to 25% of the trees may be the bitter genotype in these trees (Spiegel-Roy and Kochba, 1974; Kester and Asey, 1975). Bitterness cannot appear when pollen from a bitter tree fertilizes a blossom on a 'sweet' trees (Kester and Asay, 1975). A 'slightly bitter' flavour can be detected from some cultivars that sometimes is considered pleasing. Slightly bitter forms could correspond to heterozygous trees (Ss) and occasionally the presence of the recessive allele may produce a certain degree of bitterness, due to an alteration in the total dominance of the sweet flavour.(Dicentia and Gercia, 1993).

Prunasin is the sole glycoside in almond fruit until 100 days after flowering. Thereafter the relative abundance of the corresponding diglucoside amygdalin increases significantly and after 160 days amygdalin makes up composed more than 90% of the total cyanogenic glycoside present. This pattern of accumulation is consistent with the fact that bitter almond seeds contain mainly amygdalin as their cyanogenic glycoside (Nahrstedt, 1972). A similar pattern of accumulation and relative abundance of prunasin and amygdalin has been observed in *Prunus avium (L.) L.* and in *Cotoneaster bullatus Boiss.* (Nahrstedt, 1973). In almond prunasin glucosyl-transferase, the enzyme thought to be involved in amygdalin syntheses, has not yet been identified. Between 80 and 130 days after flowering, the amount of cyanogenic compounds in the almond fruit increases rapidly. From 130 until maturation at 180 days the cyanide compounds increase only slightly (Frehner *et al.*, 1990).

1.2.8.3 Genetics and inheritance of bitterness

Inheritance of kernel flavour in almond is determined by just one gene with two alleles, the dominant S which is responsible for the sweet flavour, and the recessive s which is responsible for the bitter flavour (Heppner, 1923, 1926; Spiegel-Roy and Kochba, 1981). A bitter almond therefore has a genotype of ss. Bitterness may represents a chemical defence. It is brought about by the presence of the glycoside amygdalin, which becomes transformed into the poisonous compound prussic acid (hydrogen cyanide), after crushing chewing, or any other injury to the seed. Sweet cultivars have been identified through breeding tests as the homozygous (SS) or heterozygous (Ss).

The fact that almond kernels are the offspring generation and results from genes of the both maternal and the pollen parents, suggests that expression of bitterness might vary within a tree depending on the genotype of individual embryos. Crane and Lawrence (1947) reported that when the almond cultivar 'Marie Dupuy' was pollinated by pollen from a bitter almond, the resulting seeds were decidedly bitter, but Kester and Asay (1975) concluded that when bitter almond was used as a pollen sources no bitter seeds were produced. Most commercial cultivars are thought to be heterozygous for bitterness (Ss). Although segregation of the genes must occur to give some ss genotype, bitterness is not expressed in these ss embryos, but does show up in nuts produced from trees grown from these embryos. Heppner (1923, 1926) claimed that inheritance is monofactorial, and inherited in the Mendelian monohybrid ratio. Almond cultivars may be homozygous or heterogynous for sweetness for example Nonpareil, Mission, Peerless and Ne Plus Ultra have the heterozygous character (Spiegel-Roy and Kochba, 1981).

1.2.9 Gene flow in crops pollination by insect

Pollen-mediated gene dispersal in flowering plants is effected primarily by animals and air currents (Levin and Kerster, 1969). Among gene flow studies of vascular plants, a large number of experiments have been conducted on the population genetics of angiosperms, gymnosperms and pteridophytes (Haufler, 1987). Gene flow can be mediated by either seed or pollen dispersal. Pollen movement generally shows restricted patterns that vary with the type of dispersal agent and the morphology of the pollen grain (Handel, 1982). Much work on estimating pollen flow is based on detailed observation of pollinator movements, which assumes a close correlation between these movements and the pollen transferred among flowers.

Actual gene flow has been measured in several agronomic studies where interest has been in the minimum distance needed to isolate cultivars. In these studies, crop cultivars with genetic markers were used, and then the F_1 progeny examined for the phenotypes of these markers (Bateman, 1947; Jackson and Clarke, 1991). Few such studies on actual gene flow have been carried out in natural populations because the existence of large monomorphic patches within a population of contrasting phenotypes is rarely found in

nature. For better understanding of gene flow in orchards it is necessary to understand the patterns of pollen flow; for such estimates to be made it is essential that pollen be marked, or be otherwise identifiable in some way. Many studies of pollen travel are based largely or entirely on observation of the foraging animal, which can be a very inaccurate and misleading technique. Furthermore, no information can be gathered for wind-pollinated plants in this way.

However, the travel of anemophilous pollen can be estimated by the use of pollen traps (Stanley and Linskens, 1974). Studies of inter-populational gene flow have been conducted on only a few species of horticultural crops. Consequently, studies in experimental orchards are of particular interest in understanding micro-evolutionary processes in plant populations (Jackson and Clarke, 1991). It is to be emphasized that gene flow by pollen is potentially quite different from the pollen flow pattern alone, as gene flow analysed by progeny phenotype includes interactions additional to merely bee foraging behaviour: gamete competition, viability of the pollen grains, pollen-pistil interaction and differences in viability among the progeny before the phenotypes are scored for gene flow estimates (Sari-Gorla *et al.* 1975; Ochendon and Currah, 1978; Handel, 1982). Pollen flow in entomophilous plants is a function of pollinator flight distance, pollen dispersal schedule, and directionality of successive pollinator flights. If all the pollen is deposited on the first recipient so that there is no carry-over, the pollen dispersal distance will be equivalent to the pollinator flight distance (Levin *et al.*, 1971).

1.2.9.1 Flight behaviour to gene flow response

Levin and Kerster (1969) have shown that plant density and spacing control the feeding-flight behaviour of bees and the movement of the pollen they bear. Bees are highly responsive to plant spacing, therefore bee-mediated pollen dispersal and associated gene dispersal is strongly correlated with plant spacing, the plant species and floral mechanism notwith-standing (Levin and Kerster, 1969). The authors have discussed two models (short and long range) for the amount of distortion which pollen carry-over effects have on the relationships between flight distance and dispersal distance. The short-range model assumes that 80% of pollen from a plant is deposited on the next one visited, 15% is dropped on the

second, and 5% is dropped on the third. The second model assumes that 50% of pollen from a plant is deposited upon the next plant, 25% on the second plant, 12.5% on the third plant, 6.5% on the fourth plant, and 6% on the fifth plant.

Bee flight distance means and pollen dispersal distance means would be greater in sparsely populated areas and smaller in the densely populated areas (Levin and Kerster, 1969). Levins (1964) proposes that gene flow among populations is part of the adaptive system of a species, and that optimum gene flow rates depend on the statistical structure of the environment. Bees learn to recognize a food source, tend to remain loyal to it, forage over short distances, and often return to the same area within a colony after flying to the hive (Free, 1966a). Kirkpatrick and Wilson (1988) measured a range of 0% to 15% gene flow from experimental plots of cultivars of *Cucurbita pepo L.* to several single wild plants of *Cucurbita texan Ecan.*, the two species were isolated from each other by a distance of over 450 m. Foraging behaviour of bees major importance for patterns of gene dispersal in plant populations (Levin, 1979). Foraging behaviour may be affected by the quality and distribution of the nectar sugar rewards offered by flowers (Heinrich and Raven, 1972; Heinrich, 1975). According to these authors most bees visited only a few flowers on a plant, then moved to a neighbouring plant. Bees visited only 1-2 flowers on 48.8% of plant visits, and 3-4 flowers on 22.7% of plant visits. The frequency of more flowers visited per plant was low, although some bees visited up to 30 flowers on a plant (Handel, 1982).

1.2.9.2 Pollen travel within and between plants

Generally, to understand the micro evolution, niche width, population subdivision, and evolutionary potential of plant populations it is necessary to investigate the genetic structure of those populations. This genetic structure is rigidly controlled by the breeding system of the plants, and a major factor in the nature of the breeding system is pollen travel (Richards, 1986). Levin and Berube (1972) have studied some features of the efficiency of the pollination system in *Phlox pilosa L.* and *P. glaberrima L.* visited by the sulphur butterfly (*Colias eurytheme*); they estimated that between 10% and 17% of pollen was deposited on the next flower visited, and thus a single visit between flowers only transported about 1% of the available pollen. Gerwitz and Faulkner (1972) used a source plant marked

with P^{32} as a radioactive marker for their work with *Brassica*, and showed that the proportion of radioactive pollen was decreased by about 30% for each subsequent non-radioactive flower visited. Typically, up to 50% of pollen was deposited on the first flower visited after collection, and deposition on subsequent flowers decrease rapidly in a leptokurtic fashion; less than 1% of pollen usually survives on the pollinator after eight visits. Gene flow by pollen in *Lupinus texensis Hook.* was longer than estimates based on pollinator movement alone (Schaal, 1980).

1.2.9.3 Methods for estimating gene flow by pollen in plants

Several methods have been used for gene flow by pollen experiments, all using markers. They fall into four distinct categories: 1) external marking of pollen grains, using dyes, stains, or fine powders; these methods measure pollen movement (pollen flow) onto stigmas or flowers 2) dominant genetic markers on a known pollen source, which can be detected readily in offspring of potential recipients either readily (e.g flower colour, leaf colour, taste) 3) molecular markers from isozyme analysis can be used to characterize particular genes in the F_1 progeny 4) pollinator marking (capture, tagging with ferrous metal and capture again with magnet in the inflorescence. Molecular markers from isozyme analysis were used to characterize the level and patterns of genetic diversity in fern (Ranker, 1992). Ellstrand *et al.* (1989) used genetic markers in experimental and natural stands of wild radish to measure the patterns of variation of gene flow by pollen into small populations. In both cases, they found considerable heterogeneity among populations in their gene flow by pollen studies. Colwell (1951) examined pollen labelled with P^{32} to understand better the pattern of microspore distribution of ponderosa pine and Douglas fir; he found that the number of radioactive pollen grains from the liberated area had a negative correlation with distance between (150-400 m). Distance of gene flow was estimated before capture of honeybees and tagging with ferrous metal disks that were recovered on subsequent foraging trips by a magnet attached to an inflorescence (Gary, *et al.*, 1977).

Handel (1982) used a marker gene which is expressed in the F_1 generation as a yellow cotyledon. In this experiment there was a negative correlation between frequency of green heterozygote seedling and distance from the gene source in the centre of the field. In

plants gene flow can occur via seed or pollen dispersal. The level of gene flow due to pollen dispersal has usually been inferred, either by marking pollen with a chemical or radioactive label for wind pollination species or by determining the distribution of pollinator foraging flight distances for insect-pollinated species, or using gene markers (Schaal, 1980).

1.2.9.4 Leptokurtic distribution

The outstanding feature of both anemophilous and anemophilous pollen travel is its leptokurtic distribution, a pattern that is common to many biotic movements (Handel, 1983b). The leptokurtosis typical of pollen and gene travel in most zoophilous flowers, certainly when visited by bees, butterflies and most birds, is found with respect to both within-plant and between plant travel, and results from an interaction of several behavioural and physical features. Pollen carry-over itself tends to be leptokurtic in distribution, thus most pollen is carried to the next flower visited. For most flower visitors, length of flight between flowers is dependent on their behavioural strategy at that moment; for a foraging bee, between 90% and 99% of flights are short distance foraging flights. The remainder are escape flights over much greater distances, for the purposes of visiting new patches, escaping predators or returning to the hive or nest. Richards (1986) claimed that, for bee pollinated plants, it is usual to find that at least 80% of flights are less than 1 m and 99% are less than 5 m. Occasionally pollen grains may, however travel much further, such is the nature of pollinator behaviour and the resulting leptokurtic dispersal distribution.

Also, the mean distance of pollen dispersal is dependent not only on pollinator behaviour, but also on plant density. Travel will be much shorter in areas of high plant density, as originally emphasised by Levin and Kerster (1968). The authors found pollen dispersal in *Primula veris L.*, highly dependent on plant density. Jackson and Clarke (1991) suggest that each bee visits only one almond cultivar in almond orchard by flying along the rows of that cultivar before it visits another cultivar in the next row, perhaps accidentally visiting an alternative cultivar, which only then may result in fertilization and nut set. Nicholas and Price (1983) have indicated that in *Delphinium nelsoni Greene* and *Ipomopsis aggregata (Pursh) Grant.* optimal out-crossing distance for these species is indeed short, between one and 100 m. These authors estimated pollen carryover, using dye powders as

pollen analogues. Some dye particles travel as far as the eighteenth successive flower after being picked up by bumblebees. The specific pattern of pollen flow changes with weather, taxa in the pollinator pool, and the floral biology of the plants (Free, 1966b; Schmitt, 1980).

1.2.9.5 Distance of pollen flow

Very local patterns of pollen flow have been observed in several plant populations, often with a leptokurtic distribution of pollen flow or pollinator visits (Levin and Kerster, 1974). Measurements of restricted actual and potential gene flow lead to estimates of small neighbourhood size in plant populations (Jain and Bradshaw, 1966; Levin and Kerster, 1974). Honeybees will often visit only small areas of uniform planting's that are at extremely close quarters (Free, 1970a; Waddington, 1980). Handel (1982) found that bitter pollen was deposited within 2-3 m of the central block with bitter *Cucumis* genes, and a small percentage of the fruits with the bitter gene were found at the end of the rows. This leptokurtic pattern is consistent with several other studies on the distribution of pollen by bee-pollinated plants. Gary *et al.* (1977) showed that in an onion field most of the honey bees tended to forage near the initial capture locations, which are related to large and homogeneous flower distribution. They found that cross-pollination of onion by honeybee is high between the male sterile and the male-fertile lines during foraging.

Levin *et al.* (1971) produced a carry-over model to estimate directionality on the pollen dispersal. This model assume that 40% of the pollen was deposited on the first plant, 20% on the second, 10% on the third, 5% on the 4th through 8th and 2.5% on each of the 9th and 10th plants. In *Diervilla onicera* most pollen grains were deposited on the first few flowers, but some grains were deposited much further away, the maximum carry-over being 54 flowers. In *Diervlla lonicera*, bees deposited significantly more grains on flowers which contained large amounts of nectar than on drained flowers (Thomson and Plowright, 1980).

1.2.10 Genetic markers used in fruit crop identification

Morphological variation in fruit trees cannot reflect the real extent of their genetic variability, and many morphological traits can arise as sports and become cultivars by means of asexual propagation. Protein electrophoresis and isozyme polymorphism has proven

useful for cultivar identification in several crops. Proteins, as the first products coded by genetic material, are ideal markers because they may reflect even small genetic changes, and they are least affected by environment (Bailey, 1983). Isozyme analysis has proved useful to detect differences in gene expression in several organs of the same plant, or to distinguish between closely related cultivars (Ben-Hayyim *et al.*, 1982). Isozymes are closely related to gene products, and their electrophoretic mobility's are due to different allele combinations. Isozymes have been successfully used for the identification of cultivars of several crops, including some fruit tree. Polymerise chain reaction (PCR) is a more recent technique which is now being utilized in a similar way through the random amplification polymorphic DNA (RAPD) approach.

1.2.10.1 Isozymes

Enzymes can be separated into different molecular forms, called isozymes. Isozymes are ideal markers because they are genes products, commonly codominant in effect and relatively unaffected by the environment. The codominance property makes these markers particularly useful for identification purposes (Richardson *et al.*, 1986; Pasteur *et al.*, 1988).

Isozymes are readily separated and detected by standard biochemical techniques, e.g. gel electrophoresis. Thus isozymes are valuable biochemical markers, which are particularly useful for plant breeders concerned with genetic improvement of long-lived woody perennial fruit trees. In almond breeding, isozyme analysis has been used for identification of cultivars, characterization and relationships, hybrid identification, inheritance and linkage studies (Jackson and Clarke, 1991; Jackson, 1992; Granger *et al.*, 1993; Vezvaei *et al.* 1994).

1.2.10.2 Determination of isozyme marker techniques for identification of *Prunus* species

Isozyme electrophoresis has been applied to cultivar identification in many crop species, including fruit trees such as the *Prunus* genus (Arulsekar *et al.*, 1986a; Chaparro *et al.*, 1987; Durham *et al.*, 1987; Hauagge *et al.*, 1987a; Messeguer *et al.*, 1987).

Almond is a perennial outbreeder which displays wide genetic variation. This heterozygosity is stabilized by vegetative propagation for horticultural purpose such that each named cultivar, new hybrid seedling or sport represents a unique gene combination which can be maintained indefinitely. In peach, modern cultivars are vegetatively reproduced, but existing information suggests that the level of isozyme variation in the species is low. Parfitt *et al.* (1985) reported that two enzyme systems PGM and PGI were monomorphic in the peach cultivars. Durham *et al.* (1985) found three enzyme systems MDH, PRX and GAL after an analysis of 13 well-resolved enzyme systems. Arulsekar *et al.* (1986a) found only one polymorphic enzyme MDH from 10 enzymes examined. Messeguer *et al.* (1987) examined 14 enzyme systems for identification of 81 peach cultivars. Variation among cultivars was observed in 4 enzymes EST, IDH, MDH and APS. Because of the lack of polymorphism in peach cultivars with the usage of morphological characteristics in addition to the isozymes, the number of identified cultivars can be increased.

The greatest degree of variability was seen in almond and plum, with apricot being intermediate. The variability found in the *Prunus* genus is associated with their breeding behaviour and the broadness of the germ plasm sampled. The highest mean heterozygosity per locus corresponds to plum and almond, which have self-incompatibility systems (Layne and Sherman, 1986; Weinberger, 1975; Byrne, 1990). Hauagge *et al.* (1987a, 1987b) studied the variation and inheritance of seven enzyme systems in almond leaves; three of these systems showed no variation. The variability of these four enzymes in 76 almond cultivars allowed their separation into 40 classes for identification. Cerezo *et al.* (1989) studied nine enzyme systems namely PGM, AAT, GPI, LAP, 6GPD, ACP, CAT, IDH and ADH. Most polymorphisms were found in PGM, LAP, CAT and ACP, the variability enabled them to distinguish between most of the cultivars. Isozyme variation in various species in almond such as AAT, GPI, IDH, LAP and PGM had more polymorphisms than PER, PGD and SKDH. Some isozymes like ADH, ALD, GDH and ME were monomorphic in all almond clones examined (Mowery *et al.*, 1990).

In a comparative study of isozymic variability in two *Prunus* species, peach and almond, peach was polymorphic in only one out of 12 loci, while almond had ten polymorphic loci for the same isozyme systems. This difference in variation was attributed

in part to the pollination system, which in almond is outbreeding (Arulsekar *et al.*, 1986a). Byrne (1990) found the lowest variability in peach among diploid stone fruit. Only three out of 12 isozymes systems in peach showed variations in a study carried out by Durham *et al.* (1987). Phosphoglucose isomerase and phosphoglucomutase seemed to be monomorphic (Parfitt *et al.*, 1985), Messeguer *et al.* (1987) studied 13 isozyme systems from pollen and found polymorphisms only for EST, IDH, MDH and ACP.

Ibanez *et al.* (1993) showed that isozyme analysis of 26 peach cultivars had a low variability level, as compared with morphological variation in this species. Isozyme diversity was lower between freestone, clingstone and nectarine groups than within each group showing that the artificial selection which originated the current cultivars had no effect on the genes controlling isozymes.

Hauagge *et al.* (1987b) used four enzyme systems AAT-1, GPI-2, LAP-1, PGM-1 and PGM-2 for separation and relationship between almond cultivars. They suggest that Nonpareil and Mission are the dominant gene pool source for other Californian cultivars. Messeguer *et al.* (1987) studying isozymes in peach pollen concluded that isozymes were only effective for peach cultivar identification when employed as a complement to other characteristics. Arulsekar *et al.*, (1986a) who compared the isozyme variability in the peach and almond found almond cultivars had much more variability at the isozyme loci studied as compared to peach varieties and attributed this to the outcrossing nature of almonds. However differences in banding patterns among cultivars were observed for four enzymes, namely EST, IDH, ACP and MDH used to classify 13 of the 81 cultivars collected. Ten cultivars were uniquely classified on morphological characters alone. Combination of the two classification systems enabled 48 of the 81 cultivars to be individually distinguished. The lack of variability in peach cultivars for the enzyme systems is due to the narrow genetic base of domesticated peaches depending on the self pollinating characteristic of peaches. Chaparro *et al.* (1987) used starch gel electrophoresis of two enzyme systems PGM and 6PGD to confirm increased variability in isozymes phenotypes in peach brought about by hybridization with plum or almond.

Granger *et al.* (1993) used ten isozymes systems including 6GPD, G6PD, GPI, IDH, PGM, FDP, SKDH, PER, MDH and AAT for identification of 78 different sweet

cherry cultivars. They showed that these ten enzymes, each having variation at only one locus, provided 70 unique genotype profiles from each extraction.

1.2.10.3 Linkage studies

These studies assign the relationship of allozyme coding loci close to one another along chromosomes. While isozymes have been used as genetic markers for inheritance studies, they may also have value as biochemical markers for other genes. For example if an enzyme allele of one parent was closely linked to the gene controlling an important character, than the progeny could be screened for this allele to identify hybrids possessing the gene sought; in tomato, an acid phosphatase allozyme is tightly linked to a gene for nematode resistance. This relationship has been used to assist in transfer of resistance (Rick and Fobes, 1974). Also it may be possible to tag monogenic traits of fruit crops with enzymes.

It may be possible to find association between isozyme genotypes and certain quantitative traits. The chosen combination can be screened for in progeny to select individuals coding for the desired quantitative trait. Isozymes can also be used as biochemical markers to screen for maximum heterozygosity which may be desirable, especially to reduce the effects of genetic load. In apple, Manganaris and Alston (1992) found that there was a close linkage between LAP-2 and resistance of rootstock to mildew. In raspberry five enzyme systems were studied including IDH, MDH, PGI, PGM and TPI. These five enzyme systems were enough to distinguish between cultivars. Also linkage groups were found between PGM-1/TPI-2 , PGM-1/MDH-2 , MDH-2/TPI-1 and 2, PGI-2/IDH-1 and IDH-1/TPI-1 (Causineau and Donelly, 1992). In olives a significant positive association was found between vigour and heterozygosity at the EST-1 locus in crossed progeny, and at EST-1 and LAP-1 loci for trees from natural population (Oiazzani *et al.*, 1993).

Inter specific hybridization between peach and other *Prunus* can be used for linkage studies, through hybridization of peach clones to produce more isozyme markers for linkage studies (Mowery *et al.*, 1990). crosses between *Citrus* and *Poncirus* species 37 pairs of genes studied, GOT-1 / MDH-1, MDH-2 / MEO-1, MDH-2 / MEO-2 and ME / MEO-2 were found to be linked (Torres *et al.*, 1985). A linkage analysis in hand pollinated avocado

showed a very tight linkage between GOT-1 and GOT-2 (Torres *et al.* 1986). Progenies from 45 apple cultivars showed close linkage between PRX-2 and PRX-3, and PRX-1 appeared to be in a same group; in addition there was linkage between PRX-4 and PRX-5. (Manganaris and Alston, 1992). In *Asparagus officinalis L.* analysis of linkage relationship by Maestri *et al.* (1991) showed linkage groups for loci encoding IDH-2/CPI/GOT-2, MDH/CAT/GOT-3, 6PGD/GPI, and GPI/ACP-1. In cherimoya (*Annona cherimola Mill.*) inheritance analyzed at 18 isozyme loci from crosses suggested fourteen possible loci with non-Mendelian inheritance for the potative heterozygotes. Tests of joint segregation for 46 of the 78 combinations of these loci identified two linkage groups involving GOT-2/MDH-1/ADH/TPI-1/ACO-2 and TPI-2/PGI-1/GOT-1/ACO-1 (Lee and Ellstrand, 1987).

In apple Manganaris and Alston (1987) demonstrated a linkage between GOT-1 and the incompatibility S locus, and also a close link between GOT-1 and IDH-1. Hauagge *et al.* (1987a) tested the independent assortment of paired enzymes in almond using a chi-square analysis for linked genes. Independence of genes is shown in all combination except in LAP-1/PGM-2, AAT-1/GPI-2 and LAP-1/PGM-2. They concluded however that due to insufficient data the results were not completely convincing. In blueberry forty-seven pairs of loci were tested for independent assortment. Two of these linkage groups PGI-2/LAP-1 and PGM-2/6PGD, which appeared independent of each other were investigated (Heemstra *et al.*, 1991). Polymorphism in 10 isozymes in wild cherry (*Prunus avium L.*) has been analysed; joint segregation of 13 locus pairs showed linkage between LAP-1 and GOT-1 and between LAP-1 and ME-1 (Santi and Lemoine, 1990). Moran and Bell (1983) reported linkage of GPI and LAP in *Eucalyptus*. The GOT enzyme system has been used to established the S genotypes of apple cultivars (Manganaris and Alston, 1987). GOT was found to be closely linked to the incompatibility S locus and to the IDH locus. Both GOT and IDH were therefore established as markers for the S incompatibility in apple.

Weeden and Wendel (1990) have reported the use of isozymes for identifying synteny. Conservation of proteins of chromosomal linkage groups was suspected in several other cases. Comparison of the *Pisum* and *Lens* linkage maps has revealed that at least 5 of the 14 chromosomal arms exhibit partial synteny (Weeden *et al.*, 1987). Linkage groups have been reported in *Citrullus* and *Cucurbita*, at least two appear to be conserved between

the genera. Navot and Zamir (1986) found linkage between the loci coding the plastid and cytosolic isozymes of GPI in *Citrullus*. Studies with monosomic alien addition lines in *Cucurbita* placed a homologus pair of loci on a single *Cucurbita Palmata* chromosome (Wendel *et al* , 1986)

1.2.10.4 Isozyme separation technique

The separation of the allozymes (isozyme) and choice of supporting medium takes in place in an electric field. A solid medium such as starch or acrylamide is necessary to mediate the separation after the electric field is turned off. The medium serves as a sieve, separating molecules by size and shape. In gels with a single pH the proteins move constantly through the gel (Richardson *et al.*, 1986).

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

A variety of different supporting matrices in which the electrophoretic separation of enzymes can be carried out is now available. Among them are starch gel, acrylamide gel, agarose gel and cellogel. The supporting matrices function as molecular filters. Hence, starch gel electrophoresis separates enzymes on both charge and molecular weight (Shields *et al.*, 1983). To some extent, the choice of methods is simply a matter of inclination and convenience, but for some enzymes clear separation is only obtained using particular support matrices. In this present work, cellogel has been used because it has the capacity to carry a large number of samples (50 samples in a gel) and is remarkably uniform in texture, making easier to 'score' each run and to compare the results of each run with other runs. When the enzymes have been resolved by means of electrophoresis in a solid matrix, they are then

stained with the appropriate reactant to locate their positions as an array of bands (Shields *et al.*, 1983). The substrates and other solutes of the reactant diffuse into the gel. The product of enzymatic reaction then reacts with a reagent such as a diazonium salt, and forms a coloured precipitate.

1.2.10.5 Interpretation of isozyme electrophoresis

While allozyme electrophoresis refers to the electrophoretic separation and histochemical recognition of differences in banding patterns between individuals proteins the true power of this methodology lies in the ability to assign a genetic basis to the observed phenotype (the banding pattern). Once the genetic basis of differences in banding pattern is understood, it becomes possible to make and confirm predictions about the inheritance of that genetic variation in the population (Jackson, 1992).

Usually each enzyme has a constant number of subunits. The result of a single locus with two alleles for the monomeric enzymes is two types of single-bands homozygous individuals may be observed along with a two-banded heterozygous type. The two bands are reflective of the two different protein products coded by the two alleles. The gene product of a loci that code for a specific multimeric enzyme usually combine at random to produce the active form of the enzyme. For example, an individual that is heterozygous for a dimmer enzyme encoded by a single locus with two alleles 'a' and 'b' produces a three-banded phenotype on the gel. The allelic products combine to give rise to three forms of the active enzyme represented by bands 'aa', 'ab', 'bb'. These three bands appear in relative concentration of 1:2:1 since random combination of subunits follows the binomial theorem. This heterozygous genotype can be contrasted with the single-banded phenotypes produced by the two possible homozygous genotypes 'aa' and 'bb'. The 'ab' band of the heterozygote's phenotype is often termed a 'hybrid band', but it is more aptly termed a heteromeric band. A heteromeric band cannot be formed in heterozygotes for monomeric enzymes since the active protein is only a single polypeptide (Jackson, 1992).

1.2.10.6 Null alleles

Some structural genes have alleles which have no product that can be detected by electrophoresis. These alleles are known as 'silent' or 'null' alleles, and normally they are recessive. Heterozygotes have a null allele 'n' and an active allele 'a' (an individuals) and show the same electrophoretic phenotype as 'aa' homozygotes, 'nn' heterozygous, without banding after electrophoresis. There are two ways in which the presence of a null allele can be determined. Firstly, by crosses between individuals, the offspring of heterozygotes can be predicted and verified. Secondly, by comparison with isozymes from other loci in the same system, if the bands are of equal intensity, this shows that the intensity differences observed at the problem locus are due to differences in allele dosage (Weeden and Wendel, 1990).

Alleles that are no longer transcribed or that code for defective polypeptides lacking enzymatic activity are generally referred to as 'null alleles'. Null allele were predicted in polyploids and other duplicated gene systems on theoretical grounds (Weeden and Wendel, 1990; Jackson, 1992).

1.2.10.7 Polymerase chain reaction (PCR) and species identification

The principle of the polymerase chain reaction (PCR) is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target DNA sequence. The oligonucleotide primers are directed with their 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase and its substrates result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of the DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, perhaps up to several million fold in a few hours.

By using the thermostable *Taq* DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* instead of the *E. coli* Klenow fragment of DNA polymerase I

utilized in the first attempts at the PCR, it has been possible to avoid inactivation of the polymerise which necessitated the addition of enzyme after each heat denaturation step. The theoretical basis of PCR is presented schematically in Figure 1.2.10.7.1.

1.2.10.8 Comparison of PCR with other methods for identification of species and cultivars

A number of techniques that are currently used to identify individual plants (Tingey *et al.*, 1992; Shrock *et al.*, 1992). Include:

(i). Comparison of morphological and agronomic characters.

(ii). Cytogenetic analysis

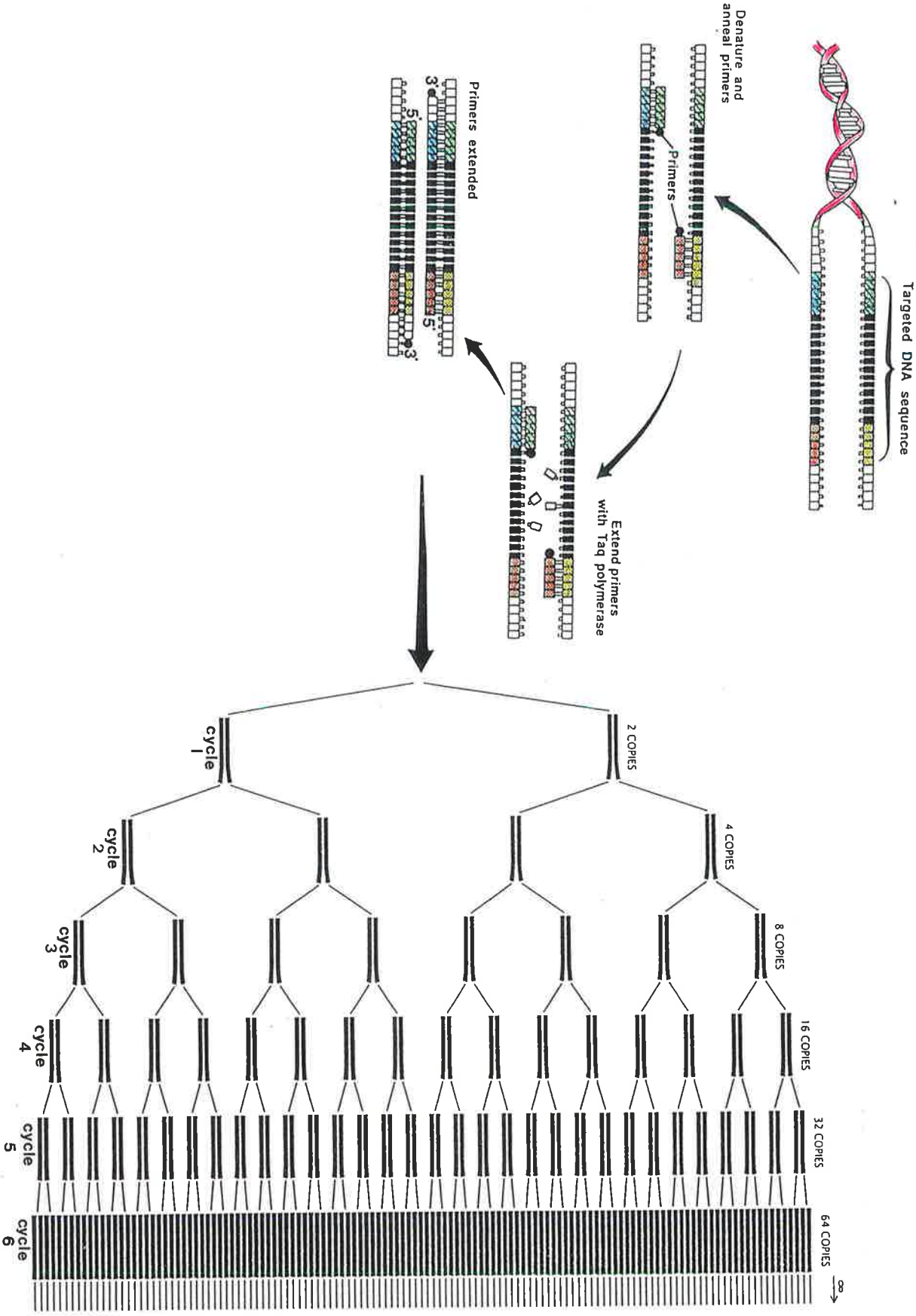
(iii). Isozyme analysis (Tanksley and Orton, 1983)

(iv). DNA profiling techniques, including RFLP and RAPD.

DNA based markers offer a number of advantages over isozymes and other biochemical methods for identifying distinctness. Firstly, the conditions and management practices secondly, the presence of the same DNA in every living cell of the plant allows tests to be conducted on any tissue at any stage of growth (provided that DNA of sufficient purity can be isolated). Thirdly, new DNA profiling techniques enable us, for the first time, to quickly and easily scan large sections of the genome in search of polymorphisms that can be used to demonstrate distinctness. The recent development of the polymerase chain reaction (PCR) has enabled promising new DNA profiling techniques that are simpler and faster to perform, and often require less development time than RFLP analysis.

In 1990, Williams *et al.* (1990) and Welsh and McClelland (1990) developed the second approach to PCR, randomly amplified polymorphic DNA (RAPD). In this approach, short DNA primers, of known sequence, but chosen on an arbitrary basis, are used to amplify those regions of the genome where the primer (s) bind sufficiently close on opposite strands to allow amplification of the intervening DNA (Tingey and Del Tufo, 1993; Newbury and Ford-Lioyd, 1993). The advantage of this approach is that no prior knowledge of the DNA sequence is required.

Fig. 1.2.10.7.1 Amplification of target DNA by arbitrary primer during PCR (after Kirby, 1990 and Mullis, 1990).


Targeted DNA sequence
Denature and anneal primers
Primers
Extend primers with Taq polymerase
Primers extended
5'
3'
3'
5'
cycle 1
cycle 2
cycle 3
cycle 4
cycle 5
cycle 6
2 COPIES
4 COPIES
8 COPIES
16 COPIES
32 COPIES
64 COPIES
∞

1.2.10.9 Applications of the RAPD assay

Development of genetic maps

One of the first practical uses of RAPD markers was in the creation of high-density genetic maps. By using a more efficient assay, Reiter *et al.* (1992) were able to place over 250 new genetic markers on a recombinant inbred population of *Arabidopsis haliana* in only 4 person-months, clearly demonstrating the utility of RAPD markers for quickly saturating both a global and local genetic map (Tingey and Del Tufo, 1993).

Because RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region (Williams *et al.*, 1990; Parks *et al.*, 1991), polymorphisms are usually noted by the presence or absence of amplification product from a single locus. This also means that the RAPD technique tends to provide only dominant markers. Individuals containing two copies of an allele are not distinguished quantitatively from those containing only one copy of the allele.

Targeting Genetic Markers

Several groups have used the RAPD assay as an efficient tool to identify molecular markers that lie within regions of a genome introgressed during the development of near isogenic lines (Klein-Lankhorst *et al.*, 1991; Martin *et al.*, 1991; Paran *et al.*, 1991). By definition, any region of the genome that is polymorphic between two near-isogenic plants is potentially linked to the introgressed trait. Thus, Klein-Lankhorst *et al.* (1991) were able to identify RAPD markers specific to chromosome 6 of tomato by screening a *Lycopersicon esculentum* substitution line, and Martin *et al.* (1991) were able to confirm linkage of RAPD markers to the *Pto* locus in tomato after screening two near-isogenic lines (Tingey and Del Tufo, 1993).

Pooling Strategies

Recently, another technology has been developed that is designed to identify genetic markers linked to very specific regions of the genome (Arnheim *et al.*, 1985). Markers linked to that locus are identified by their linkage dis-equilibrium with respect to the rest of the population. The limitation of this approach is that it relies on RFLP technology, called bulk segregant analysis, which two bulked DNA samples gathered from individuals

segregating in a single population. Each bulk is composed of individuals that differ for a specific phenotype or genotype, or individuals at either extreme of a segregating population. For simple genetic traits, all loci in the genome should appear to be in linkage equilibrium except in the region of the genome linked to the selected locus. Markers linked to this locus should appear polymorphic between the pools for alternate parental alleles (Tingey and Del Tufo, 1993).

Population Genetics

Several groups have reported on the utility of RAPD markers as a source of phylogenetic information. Arnold *et al.* (1991) were successful in using RAPD markers to test for inter-specific nuclear gene flow between *Iris fulva* and *I. hexagona*, and to study the presumed hybrid origin of *I. nelsonii*. Hu and Quiros (1991) were able to show that the amplification products from only four random primers were sufficient to discriminate between 14 different broccoli and 12 different cauliflower cultivars (*Brassica oleracea L.*) .

1.2.10.10 Advantage of using PCR for DNA - genotype determination

PCR requires only small amounts of DNA and often crude mini prep procedures yield DNA of sufficient quantity and quality. The PCR process involves fewer steps than RFLPs and is therefore faster to perform. The PCR is technically straight forward once conditions have been established; it does not require the use of radioactivity to visualize polymorphisms. The PCR can be readily automated at all stages from DNA extraction to data collection and analysis. The vast range of potential primer sequences that can be used gives the technique great diagnostic power (Williams *et al.*, 1991).

There are a number of advantages to using a PCR assay for determining the genotype of a DNA sample. The assay takes only a few hours compared to Southern blotting which can take days or weeks. Secondly, PCR is capable of being automated, allowing large numbers of samples to be rapidly typed with little labour required (Tingey *et al.*, 1992). Finally, it is economical with DNA; only picograms or nanograms are required, whereas nanograms or micrograms are needed for Southern blotting of genomic DNA. In one PCR reaction, many distinct genetic loci can be typed at the same time by multiplex amplification using multiple primer pairs. As a result of these economies, a number of laboratories have

converted known RFLP markers into a PCR format and many searches for new polymorphisms have focussed on those that can be assayed by PCR (Wagner, 1992).

Although DNA of woody plants is usually isolated with polysaccharides, tannins or phenolic and can have portions of it methylated, improvement of the DNA isolation technique to produce less contaminated DNA does not seem to increase the efficiency of the RAPD protocol significantly (Smith and Chin, 1992; Gogorcena and parfitt, 1992). However, Weeden *et al.* (1992) suggest otherwise, blaming impurities in DNA for blurred or faint bands found in the gel profiles.

RAPD polymorphisms can be detected in all organisms looked at so far even in highly inbred lines where other types of polymorphisms may not have been detected. Large genome size is not a problem as PCR based methods are more sensitive in detecting polymorphisms than are Southern hybridization based methods (Welsh *et al.*, 1991). However the number of segments amplified from bacterial samples with small genomes suggest that some of these may have originated from mismatches between the primer and DNA template (Williams *et al.*, 1991).

Most RAPD markers segregate in a Mendelian fashion. They can segregate in a 1:1 or 1:3 fashion depending upon whether the parents are homozygous or heterozygous for the particular fragment. Though some fragments do not segregate in a Mendelian fashion this is no different than that found in RFLP and isozymes (Ohm and McKenzie, 1992; Reiter *et al.*, 1992; Devos and Gale, 1992; Tulsieram *et al.*, 1992; Woodward *et al.*, 1992). Possible reasons for distorted segregation include deleterious translocated segments containing the particular gene or gamete/ zygote selection against the particular RAPD marker (Uphoff and Wrickle, 1992; Echt *et al.*, 1992).

If genome pooling strategy or bulk segregant analysis are used (Kesseli *et al.*, 1992) the efficiency of using RAPD's in genetic mapping is increased. These data suggest that, for genetic mapping, gene tagging and varietal identification RAPD technology is very useful and reliable (Weeden *et al.* , 1992).

Chapter 2

General materials and methods

Chapter 2

General materials and methods

2.1 Chemical analysis

2.1.1 Determination of oil content and fatty acid in almond kernels

In almond kernels, oil is the major component of the fruit, being 50-65 % of dry weight (Mehran and Filsoof, 1974; Filsoof *et al.*, 1976; Nassar *et al.*, 1977). The main fatty acids reported are oleic (59-78%) and linoleic (19-30%) together with small amounts of palmitic, palmitoleic, stearic, and linolenic acid (Nassar *et al.*, 1977). Synthesis of the lipids begins from 50 days after fruit set and increases to the maximum rate at 70 days after fruit set (Munshi *et al.*, 1982). This phenomenon begins when the jelly-like interior of the nuts hardens to a solid material.

During kernel development the oil content displays a double sigmoid pattern. Oleic and linoleic acids accumulate to similar levels, but the linolenic acid content decreases during development (Munshi and Sukhija, 1984). The oil fraction during ripening varies from 35% to 61% at harvest time according to the cultivar examined. In almond, as well as in other oil seeds, phospholipids and glycolipids are first synthesized. Later, at an intermediate stage the accumulation of the acylglycerides begins, a fraction that represents about 95% of the oil in mature fruits (Shing and Privett, 1970; Munshi *et al.*, 1982).

2.1.1.1 Total oil determination (ether extraction)

Oil determination can be measured directly or indirectly (Fichney and White, 1985). A Soxhlet apparatus was used for oil extraction. For the direct method one g of crushed almond kernel was weighed into the clean and numbered paper thimble (Whatman 30 x10 cm). The thimble with the sample was placed in a Soxhlet extraction unit. A dried and tared 250 ml round-bottomed flask (with several glass beads) was placed under the extraction unit. Solvent Shell x 55 (150 ml) was added to the flask and the flask heated by an electric plate. The volatilized solvent was condensed by means of a water condenser during extraction. After extraction the Soxhlet unit was removed and the solvent remaining in the flask collected by distillation. To avoid oxidation of the oil a small amount of solvent was

added before distillation. The flask, beads, and oil were carefully dried in an oven at 105° C overnight, cooled in a desiccator and weighed. The difference between the weight of the flask before and after the extraction gives the amount of the oil in the sample.

The indirect method also begins with one g of crushed almond which was folded into filter paper (Whatman No. 541) carefully to contain the sample and secured with two paper clips. The packets were numbered with pencil and samples placed into the oven at 105° C overnight, weighed direct from the oven and placed in Soxhlet apparatus and extracted for 8-16 hours (Several packets might be placed in each unit). After extraction the packets are removed from Soxhlet apparatus and allowed to dry in an oven at 105° C for at least 6 hours. Samples are weighed direct from the oven after 20 minutes cooling in the desiccator. Crude fat is the loss of weight and is expressed as a proportion of the sample dry matter determined simultaneously. The amount of oil generally found in almond is between 45 and 55 percent.

2.1.2 Determination of total nitrogen (crude) with Kjeldahl method

The principle involved with this method is that the nitrogen of the protein and other compounds are transferred into ammonium sulphate by acid digestion with boiling sulphuric acid. The acid digest is cooled, diluted with water, and made strongly basic with sodium hydroxide. The ammonia is released and distilled into the boric acid solution. The ammonia in the boric acid solution is titrated with standardized sulphuric acid solution (Scales and Harrison, 1920).

After crushing the almond kernels, one g was weighed on a filter paper, the paper folded and twisted around the sample and introduced into the Kjeldahl test tube. Two reagent blanks were run (with filter paper) through all stages of procedure and the blank titration subtracted from the sample titration. A special Kjeldahl catalyst tablet was added to each tube (each tablet contains: 1.0 g sodium sulphate, anhydrous and the equivalent of 0.01 g of selenium). The rake of the tube was positioned inside the fume hood and add 15 ml of concentrated sulphuric acid added with an automatic dispenser. Samples were heated at 400° C in the 1015 Tecator digester under the fume hood. Samples were cooled during the first 10 minutes of digestion by tap water turned full on, after that time the water was turned

down for a further 40 minutes. The test-tube rack was removed from the digester. The solution should be clear and all the carbon oxidised. Fifty ml of distilled water was added per tube. For total nitrogen content an Auto Kjeltec 1030 Analyser (Tecator) was used, which automatically measures ammonia released after alkali addition. The prepared tubes were placed in pre-adjusted Kjeltec Auto 1030 Analyser for 3 minutes and nitrogen content of the samples determined. Crude protein content can be estimated from this value. For determination of moisture content another one g of sample is weighed in a glass dish and put into an oven at 100° C for 24 hours. The sample was cooled in a desiccator and weighed again.

2.1.3 Determination of ethanol soluble and other non-structural carbohydrate in almond kernels

This method has been applied by Dubois *et al.* (1956) for the determination of simple sugars, oligo-saccharides, poly-saccharides, and methyl ethers with free or potentially free reducing groups. These compounds give an orange-yellow colour when treated with phenol and concentrated sulphuric acid. The reaction is sensitive and the colour stable.

Almond kernels were ground finely using a mortar and pestle, one g of sample was added to boiling 80 % ethanol and allowed to boil for a further 15 minutes. The slurry was decanted into a 10 or 50 ml centrifuge tube, and the mortar and pestle washed with 5 ml of 80 % ethanol and added to decanted slurry. Samples were centrifuged for 10 minutes at 2000 r.p.m to separate solids and supernatant. The supernatant was transfer with a pipette into a 25 ml volumetric flask. Five ml of 80 % ethanol was added to the pellet and heated the slurry to boiling in the water bath for 5 minutes (prevent frothing of the samples with a glass rod). The supernatant was transferred with the pipette to the 25 ml volumetric flask and made up to volume with 80 % ethanol.

Reagents used to develop the colour reaction include concentration sulphuric acid in an automatic dispenser (5 ml volume), 5 % phenol solution and standard sucrose solution (10 mg/100 ml). A series of 15x18 mm glass test tubes were selected, which were clean and free from dust or cellulose fibres. In the first step 0.1 ml of the 80 % ethanol extract was taken and to it added 0.9 ml of water, simultaneously adding 1 ml of 5 % phenol. A jet of 5 ml of concentrated sulphuric acid was pumped to the central part of the sample and mixed

(this step is potentially dangerous, and solution comes to the boil). A face mask must be worn, and acid added behind the screen in the fume hood. The optical density was measured at 490 nm when the tubes have cooled, The procedure was standardized with standard of sucrose (5-70 μg per tube).

2.1.4 Mineral elements measurement in almond kernels

With inductively coupled plasma spectrometery (ICPS) it is possible to determine P, K, S, Ca, Mg, Na, Al, Zn, Mn, Fe, Co, Mo, and B in almond nuts. ICPS has the potential to determine all the nutritional elements (except nitrogen) with a polychromator sequentially scanning monochromator in a single digestion (Zarcinas *et al.*, 1987). The advantage of this method is that is simple, quick and precise, plant material is made ready for this method by digestion using nitric acid to allow the simultaneous determination of the 13 elements.

Almond samples were dried overnight at 65-70° C before digestion. The almond kernel was ground finely with a mortar and pestle. One g of sample was weighed and transferred into a tube with a mark to indicate 25 ml. Ten ml of nitric acid was added (Univar A.R.70% w/w) with the aid of a dispenser, under the fume hood.(lab coat, plastic gloves and face mask must be worn for safety reasons at this stage). After adding nitric acid, the sample was covered loosely with plastic film and left overnight or longer in the fume hood. The tubes were then heated for 4 hours at 120° C and the temperature was increased to 140° C. Digestion was continued at this temperature until about 1 ml of acid remained (pre-digest vortex and a cold start prevents combined from frothing over). After cooling, the digest was diluted to 20 ml with 1% v/v nitric acid and decanted into a polystyrene tube (avoiding solids being transferred). The digest stood overnight before ICPS analysis to allow precipitation of any suspended solids. The ICP instrument for used was a 3580B ICP Spectrometer Sim/Seq. with 21 simultaneous channels manufactured by Applied Research Laboratories (Switzerland).

2.1.5 Qualitative and quantitative determination of cyanogenic compounds in almond

2.1.5.1 Qualitative method

Sodium picrate soaked paper was used for qualitative cyanogenic compounds determination in almond kernels. It is an easy, quick method for assay of a large number of samples. Sodium picrate paper was used as explained by (Guignard, 1906; Kingsbury, 1964; Horwitz, 1980; Brinker and Seigler, 1992). The almond kernel was ground in a cold mortar and pestle with liquid nitrogen. One g of this sample was placed into a 10 ml plastic test tube, to which was added 0.5 ml of phosphate buffer pH 6.8 including 20 µl b-glucosidase (100 µg b-glucosidase in 2 ml of phosphate buffer), and then a wet strip of sodium picrate paper (3 x 1 cm) was suspended over the test tube, the lid clamped shut and the sample incubated for at least 4 hours at 37° C. Where cyanogenic compounds were present in the kernels, the sodium picrate paper changed from yellow to orange, or to deep orange at higher 'cyanide' concentrations.

2.1.5.2 Quantitative method

In principle quantitative analysis of cyanogenic compounds in plant tissues involves hydrolysis of the cyanogenic glucoside and liberation of HCN which is trapped in a basic solution and determined colorimetrically (Nahrstedt *et al.*, 1981; Brinker and Seigler, 1989).

The approximate amount of cyanogenic compound in the kernel can be estimated by a preliminary qualitative test (sodium picrate paper see above). Each kernel was ground separately in a cold mortar and pestle with liquid nitrogen. To a 100 ml flask, inside which a glass vial forms the central part of the bottom of the flask, one g of ground sample was added outside the central vial or well. Then 0.5-0.6 ml of a solution of sodium phosphate pH 6.8 including 20 µl b-glucosidase (100 µg b-glucosidase in 2 ml of phosphate buffer) was added to the ground sample in that part of the flask outside the central vial in the bottom of the flask. Inside the central vial was placed 1 ml of freshly made up 1 M sodium hydroxide. The flask was sealed and incubated for 4 hours at 37° C. During incubation the HCN released from the sample is trapped by sodium hydroxide as NaCN inside the vial. Cyanide was determined according the Lambert *et al.* method (1975). In this method 0.1

aliquots of the sodium hydroxide solution were removed from samples and put in a set of 150 x18 mm test tubes and diluted to 1 ml. To each tube 0.5 ml 1M acetic acid, 5 ml of succinimide /N-chlorosuccinimide (2.5 g succinimide and 0.25 g N-chlorosuccinimidein 1 litre of water) and 1 ml of barbituric acid/pyridine reagent was added. The latter reagent was made up by mixing 12 g barbituric acid with a small amount of water to make a paste, then 120 ml pyridine was added with enough water to give a final volume of 400 ml. The tubes were shaken or vortexed vigorously and allowed to sit it for 10 minutes. In the presence of the cyanide a purple colour appears and the optical density measured at 580 nm. The colour is stable for at least for 30 minutes. For preparation of a standard curve, aliquots of a standard NaCN solution (Brinker and Seigler, 1989) were made up to 1 ml with 0.1 sodium hydroxide.

A standardized solution of NaCN was prepared as follow: a 0.02 M solution of NaCN in 0.1 N NaOH was prepared (0.980 g NaCN/l) and the exact amount of CN^- in the solution was determined by a modified Liebig titration method (Kolthoff and Sandell, 1952; Ayres, 1958; Vogel, 1961), as follows: 1) A standard 0.1 M $AgNO_3$ solution was prepared: 16.987 g of dried $AgNO_3$ was weighed and diluted to 1 litre with water in a volumetric flask. This solution was stored in the dark. 2) Twenty five ml of the NaCN solution was pipetted to a 50 ml flask equipped with a stirring blade, then 1.5 ml 6 N NH_4OH and 0.5 ml 10% KI was added and titrated with the standard $AgNO_3$ solution until the solution just remained faintly opalescent or turbid; this endpoint was easier to see against a black background (about 2.4 ml $AgNO_3$ solution was usually required.

2.2 Isozyme analysis

2.2.1 Extraction of various almond tissues

Leaf samples must be obtained from fresh new growth due to the lower concentration of phenolic compounds in rapidly growing tissues. Pollen was obtained from the flowers just before opening (balloon stage), anthers were excised, and allowed to reach anthesis in the laboratory, contained in petri dishes. Extraneous anther tissue was removed with a sieve, as described by Jackson (1989a). Pollen was stored in a small vial or used

immediately for isozyme analysis. Nuts used were fully mature, and before extraction the embryos excised (Hawker and Buttrose, 1980) for isozyme analysis. Nuts were stored as free kernels at room temperature for at least 1 year before analysis without any obvious effect on isozyme patterns of the contained embryo (Jackson, 1992).

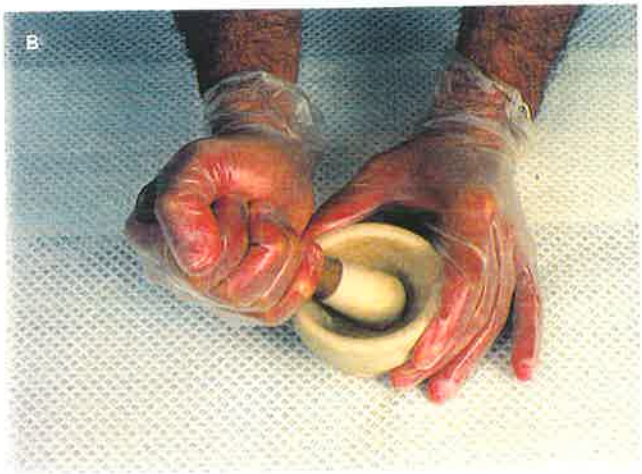
Leaf material was extracted by grinding 300 mg leaf with 150 mg polyvinylpyrrolidone and 2 ml of extraction buffer containing 0.05 M Tris (650 mg), 0.15% citric acid (150 mg), 0.12% cysteine HCL (120 mg) and 0.1% ascorbic acid (100 mg), pH 8. Grinding was carried out in a pestle and mortar, the mixture was centrifuged at 3000 x g in an Eppendorf 5414S centrifuge, and the supernatant used for gel electrophoresis. Pollen was extracted in a similar way, except that 200 µg was extracted with 250 µl of extraction buffer, no polyvinylpyrrolidone being used due to the lack of phenolic compounds in the pollen. Each pollen 'pellet' obtained from a pollen trap in a bee hive was extracted by 200 µl of extraction buffer. Embryos were extracted as described above for leaf material except that 400 mg of embryo was used. In all cases, gel electrophoresis of the supernatant for isozyme analysis was carried out within 1 hour of extraction (Fig. 2.2.1.1)

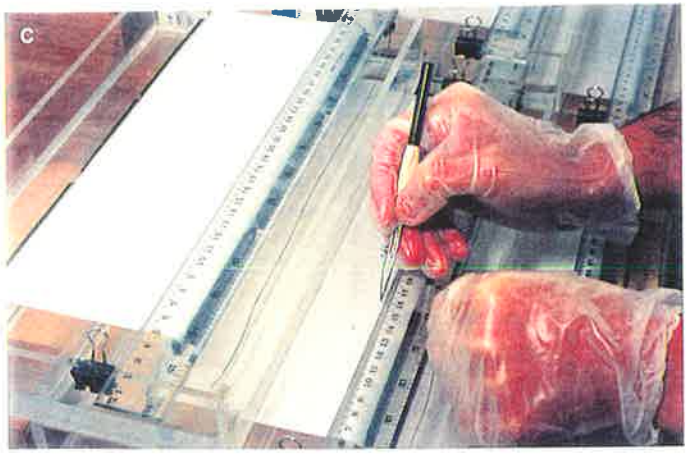
2.2.2 Gel electrophoresis

Cellogel (Cellulose acetate gel) was used in this study, and is manufactured by Chemtron (Milan, Italy). Cellogel was supplied in airtight plastic bags containing aqueous methanol, and was stored at 4° C. Several sheets were transferred to a covered tank containing 30% methanol shortly before use, cut to the size needed with a scalpel and ruler. Dry gels were not exposed to the air for more than a few seconds. Since one side of the gel is porous and appears dull and the other side has a plastic-coated non-porous surface, care should be taken to apply the sample to the porous side of the gel. After cutting to size the gels were soaked in buffer (see below for details of buffer which varies according to the isozyme being detected) for a least 10 minutes, blotted, and positioned in the electrophoresis tank so as to take a position between the anode and cathode compartments containing buffer. Three long bar magnets were placed at the inner wall of each buffer compartment (above the level of liquid), in order to hold the cellogel in a horizontal plane between the anode and cathode compartment. The magnets were held in this position by attraction to metal strip on

Fig. 2.2.1.1 Procedures used during isozyme analysis. **A** (equipment), **B** (extraction), **C** (loading), **D** (electrophoresis) and **E** (staining).


A
Normal
extraction


B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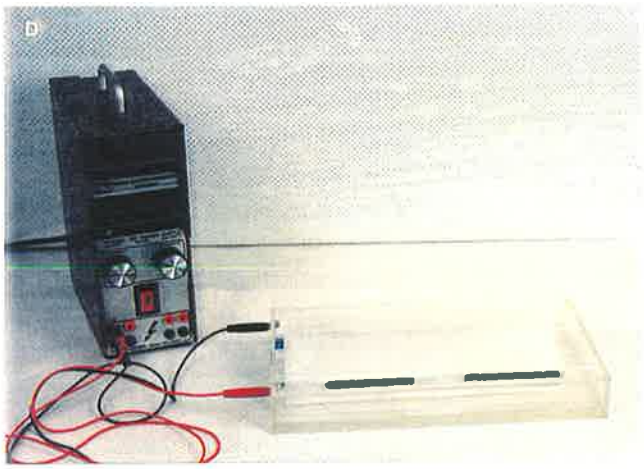
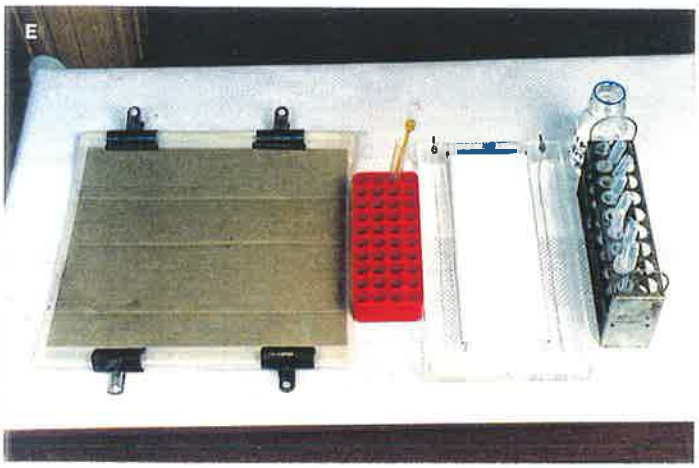

D


E


F

the other side of the plastic wall. Buffer was placed in the electrophoresis chamber, the buffer used depending on the isozymes to be detected on the gel. For GPI, LAP, AAT, IDH, and SKDH, 0.05 M Tris-maleate, pH 7.8 was used; for PGM, 0.025 M Tris-glycine, pH 8.5; for G6PD, 0.02 M sodium phosphate, pH 7. Fifty samples were extracted at a time, and 50 extract supernatants applied to each of seven gels in seven different electrophoresis tanks set up in a 4° C cold room. Seven gels were needed so that the supernatants could be used to analyse for all the isozymes). To facilitate loading of approximately 1 µl of supernatants, a bank of wooden blocks was set up so as to place each of the seven tanks containing seven gels for easy access for loading. A plastic ruler was held in place over each gel to act as a guide for the loading of the samples, a draftsman pen (architect's type) being used to apply the supernatant. When loading, sufficient pressure had to be exerted to lightly mark the surface of the gel, the length of each mark being monitored on the ruler to allow for 50 different samples in line across the gel. For almond isozymes in the buffer systems indicated, the samples were applied in a line (called origin) 0.5 to 1.5 cm from the cathode edge of the flat porous side of the gel. Each tank was then connected to the power pack, so that all tanks received a constant 200V, and the electrophoresis 'run' was carried out at 4° C for 1.5-2.0 hours. At the end of the run, gels which so far had been treated the same way, apart from variations in the buffer used, were ready for enzymes-specific staining.

2.2.3 Staining for isozymes

Staining solutions for each enzyme were made up immediately before use by mixing stock solutions and spreading the final mix on a disposable plastic sheet which was stretched over cardboard with the aid of clips. After turning off the electric current, the gel was then taken out of the electrophoresis tank, holding it by the portions that were in contact with cathodal and anodal buffer liquid. The gel was dipped into the stain smeared on the plastic sheet and rocked gently back and forth. The stain and gel were left in contact with occasional rocking motion for 30 to 60 seconds. The gel was then blotted to remove excess stain, and placed between two plastic sheets. The gel was then incubated at 37° C to allow isozyme reaction, some took a few seconds, others up to 30 minutes, to show visible bands

reflecting positions of isozymes on the gel. A record of band positions was done by photocopying wrapped gels. Staining ingredients for each of the isozymes and notes on the enzyme-specific staining are given below.

2.2.3.1 Isocitrate dehydrogenase (IDH)

The principle of reaction is as follows. Where isocitrate dehydrogenase is located on the gel, it catalyzes the conversion of isocitrate to a-Ketoglutarate in the presence of $MgCl_2$ and at the same time the reduction of NADP to NADPH. The NADPH produced enzymatically reacts chemically with the dye MTT, using PMS as intermediate, yielding insoluble purple formazan. Purple areas therefore correspond to the location of isocitrate dehydrogenase. The stain solution is composed of 10 mg DL-isocitric acid, 2 ml 0.1 M Tric-HCl pH 8, 0.1 ml 25 mM NADP, 0.1 ml 0.2 mM $MgCl_2$, 0.1 ml 14.5 mM methyl thiazolyl blue (MTT), 0.1 ml 6.5 mM phenazine methosulphate (PMS) (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.2 Glucose-6-Phosphate dehydrogenase (G6PD)

The principle of reaction is that where G6PD lies on the gel, it catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconate in the presence of $MgCl_2$, and at the same time reduces NADP to NADPH. As for IDH above, the NADPH so produced reacts chemically with the dye MTT (in the presence of PMS) to give purple formazan. The staining solution is composed of 6 mg glucose-6-phosphate, 2 ml 0.1 M Tris-HCl pH 8, 0.1 ml 25 mM NADP, 0.1 ml 1 M $MgCl_2$ 0.1 ml 14.5 mM MTT, 0.1 ml 6.5 mM MTT, 0.1 ml 6.5 mM PMS (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.3 Shikimate dehydrogenase (SKDH)

The principle of the reaction is as follows. Where SKDH is on the gel, shikimate is oxidized to 3-dehydroshikimate and NADP is reduced to NADPH. As for IDH and G6PD above, purple formazan is precipitated where bands of SKDH are located. The staining solution contains, 6 mg shikimic acid, 0.1 ml 25 mM NADP, 0.1 ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS, 2ml 0.1 M Tris-HCl pH 8.5 (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.4 Glucose Phosphate isomerase (GPI)

The principle of the reaction: wherever GPI appears on the gel, fructose is converted to glucose-6-phosphate then to 6-phosphogluconate while also reducing NADP to NADPH in the presence of Mg Cl_2. As for the other enzymes above, the NADPH reacts chemically with MTT (+PMS) to give purple formazan which is precipitated on to bands where GPI is located on the gel. The stain solution contains, 5 mg fructose-6-phosphate, 2 ml 0.1 M Tris-HCl pH 8, 0.1 mM 25 mM NADP, 0.1 ml 1 M $MgCl_2$, 0.1 ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS, 2 international units of glucose-6-phosphate dehydrogenase (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.5 Leucine aminopeptidase (LAP)

The principle of reaction is that where LAP is located on the gel, it catalyzes the hydrolysis of leucyl-b-naphthlamide to leucine and naphthol. The naphthol so formed reacts chemically with Black K to give a violet precipitate overlaying areas of peptidase activity. Staining solution contains 5 mg L-leucyl-b- naphthylamide, 1.5 mg Black K salt, 0.25 M Mg Cl_2, 2ml 0.2 M , Tris-maleate pH 5.5 (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.6 Asparatate aminotransferase (AAT)

Principle of reaction is, where AAT lies on the gel, it catalyzes the transferase reaction between a-Ketoglutarate and L-asparatate to yield glutamate and oxaloacetate. The oxaloacetate so formed reacts chemically with the diazonium salt Fast Garnet GBC to give a coloured precipitate which become occluded in the gel where AAt is located. AAT is also known as glutamate-oxaloacetate transaminase (GOT).

The stain solution contains 6 mg Fast Garnet GBC salt, 0.2 ml 50 mg/ml L-asparatate pH 8, 0.2 ml 50 mg/ml a-Ketoglutarate pH 8, 2 ml 0.1 M Tris HCl pH 8 (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.7 Phosphoglucomutase (PGM)

The principal of reaction is as follows. Where PGM is located on the gel , glucose-1-phosphate is enzymatically converted to glucose -6-phosphate in the presence of glucose-

1, 6-diphosphate. The glucose-6-phosphate so formed is in turn converted to 6-phosphogluconate in the presence of glucose-6-phosphate dehydrogenase and $MgCl_2$, at the same time NADP is reduced to NADPH. As for the above staining reactions yielding NADPH, this compound reacts chemically with MTT in the presence of PMS to give an insoluble purple formazan which thus becomes occluded in the areas of the gel containing active PGM. The stain solution is contains 10 mg glucose-1-phosphate containing 1% gluucose-1, 6 diphosphate, 0.1 ml 25 mM NADP, 0.1 ml 1 M $MgCl_2$, 0.1 ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS, 2 international units of glucose-6-phosphate dehydrogenase (also used in the GPI stain mixture above) (Richardson *et al.*, 1986; Jackson, 1992).

2.2.3.8 Interpretation of stained gels

First, it is essential to know how many polypeptides make up the molecular structure of the active enzyme involved. For almond, only LAP and PGM of the enzymes studied have a monomer structure, the interpretation of gel is the simplest. Thus, in homozygotes (i.e., in homozygote diploid embryo or leaf) only one kind of polypeptide is synthesized and there is only one band for each isozyme. In another embryo or leaf, which is homozygous for a second allele, there is also one band, but it may migrate a different distance from the origin compared to the first mentioned. The genotype of each diploid embryo or leaf could be designated 'aa' and 'bb'. In the heterozygote, genotype 'ab', the cells of the embryo or leaf will produce both 'a' polypeptide and 'b' polypeptide, and so there will be two bands. Two alleles in almond for LAP-1 and PGM-1 and PGM-2 were observed. The numbers appearing after the isozyme name refer to the particular locus (or gene) involved. Usually, the bands for each locus are well apart and there is no difficulty in telling one from the other. In the case of PGM-1 and PGM-2, where both are heterozygous (as in Nonpareil almond cultivar, for example), the genotype is 'ab' for PGM-1 and 'ab' for PGM-2 and four clear enzyme bands are seen on the gel, stained for PGM. For the IDH enzyme which is a dimer and has three alleles in almond, the use of pollen which is haploid simplifies the interpretation since it cannot yield hybrid dimers in stained gels. An almond cultivar which is heterozygous 'ab' for IDH yields three bands for diploid cells composed of dimer ('a' protein + 'a' protein), dimer ('b' protein + 'b' protein), and hybrid dimer ('a' protein + 'b'

protein). For haploid pollen two bands are seen for the heterozygote 'ab', dimer ('a' protein + 'a' protein) which comes from 'a' genotype pollen and dimer ('b' protein + 'b' protein) which comes from 'b' genotype pollen and no hybrid dimer as there is only one gene per cell in pollen from these heterozygous plants (Jackson, 1992).

2.3 Other materials and methods

Other materials and methods are described within the following chapter as follows:

DNA isolation and PCR amplification section 7.3.2.

DNA amplification conditions section 7.3.3.

Pollen germination tests section 8.3.2.

Chapter 3

Characterization of Australian almond cultivars and comparison with Californian cultivars by isozyme polymorphism

Chapter 3

Characterization of Australian almond cultivars and comparison with Californian cultivars by isozyme polymorphism

3.1 Summary

Australian-derived almond cultivars Baxendale, Bruce, Chellaston, IXL Seedling, Johnson's Prolific, Keane's Seedling, Pethick's Wonder, Somerton and White Brandis were identified by means of isozyme polymorphism at 8 genetic loci: AAT-1, GPI-2, LAP-1, PGM-1, PGM-2, IDH, G6PD and SKDH. The same genetic markers were used to compare the Australian almond cultivars with commonly grown Californian cultivars Carmel, Davey, Drake, Fritz, Mission, Ne Plus Ultra, Nonpareil, Peerless, Price and Thompson.

The assignment of alleles at the various loci was aided by examination of the inheritance of these markers by crossing Nonpareil with pollen from 17 Australian and Californian cultivars. This demonstrated for the first time, null genes in Price at LAP-1 and in Drake at AAT-1. The Australian cultivars differed from the Californian at several loci. These differences arise from the separate development in Australia of almond cultivars from what is believed to be largely Spanish 'Jordan' types, before a shift to larger irrigated orchards using Californian methods and Californian-Australian cultivars.

3.2 Introduction

Australian cultivars of almond [*Prunus dulcis (Mill.) D.A. Webb syn P. amygdalus Batsch.*], developed for commercial orchards in South Australia, have not been subjected to detailed genetic characterization. Over the years, Australian growers selected desirable chance seedlings which were often planted to the exclusion of the original parents. One such selected seedling, Chellaston, from the Edwardstown area of Adelaide, made up half of the Australian commercial plantings as recently as 1970.

In view of the recent interest in almond characterization and improvement (Socias i Company and Felipe, 1992a), it is timely to begin such studies of Australian almond

cultivars, as was recently reported for cherry (Granger *et al.*, 1993). Some Australian varieties are known to differ by at least 1 isozyme locus (Jackson, 1992). Research into gene flow via pollen in almond plantings (Jackson and Clarke, 1991) requires a more extensive set of genetic markers, such as isozyme markers than is presently available. We therefore investigated Australian and Californian almond cultivars for isozyme loci and carried out inheritance studies.

3.3 Materials and methods

3.3.1 Plant materials

Healthy young leaves are suitable for isozyme analysis (Jackson, 1992) and were obtained for each cultivar from a commercial orchard at Angle Vale (35 kilometres north-east of Adelaide, South Australia). They were held in crushed ice during transport to the laboratories at the Waite Agricultural Research Institute. Sampling was continued during vegetative growth. Cultivars for this study included Carmel, Davey, Drake, Fritz, Mission, Ne Plus Ultra, Nonpareil, Peerless, Price and Thompson (Californian), and Baxendale, Bruce, Chellaston, IXL Seedling, Johnson's Prolific, Keane's Seedling, Pethick's Wonder, Somerton and White Brandis (Australian). The commercial orchard that was the source of all almond cultivars in this study is also maintained as a nursery and is the source of a significant proportion of Australia's almond seedlings for commercial plantings.

3.3.2 Isozyme analysis of almond leaf, pollen and embryo

Seventy isozyme systems were surveyed (see e.g. Richardson *et al.*, 1986), before choosing the following 8 systems that showed polymorphism in almond: AAT-1 (aspartate amino transferase at locus 1), LAP-1 (leucine aminopeptidase), GPI-2 (glucose phosphate isomerase), G6PD (glucose-6-phosphate dehydrogenase), IDH (isocitrate dehydrogenase), SKDH (shikimate dehydrogenase) PGM-1 and PGM-2 (phosphoglucomutase). Characteristics for IDH in almond are described by Cerezo *et al.* (1989), Jackson and Clarke (1991) and Jackson (1992), polymorphism for G6PD and SKDH in some Californian varieties is reported by Jackson (1992). Isozyme analysis was carried out as described in section 2.2 in this thesis. Cellogel was used as the medium for electrophoresis.

3.3.3 Inheritance Studies

Controlled crosses were carried out on the Californian cultivar Nonpareil as sole mother tree, using pollen from the 17 Australian and Californian cultivars. Viability of pollen was tested on agar plates (1% agar, 0.01% boric acid, 0.03% calcium nitrate, 0.02% magnesium sulphate, 0.01% potassium nitrate and 15% sucrose) based on Brewbaker and Kwack (1963). In all cases, viability was scored more than 80%.

3.4 Results

3.4.1 Australian and Californian Cultivars

Inferred genotypes are shown in Table 3.1 for both Australian and Californian cultivars. Typical banding patterns for the 8 polymorphic isozyme loci in almond and distance from loading point are depicted in Figures 3.1a and b. At least 1 of the Californian cultivars was homozygous for 'b' allele at the AAT-1 locus, homozygous for the 'a' allele at PGM-1, carried the 'b' allele at GPI-2 or carried a null genes at AAT-1 and LAP-1. No Australian cultivars showed any of these characteristics. On the other hand only Australian cultivars possessed the 'a' allele at SKDH, and all were heterozygous 'ab' at PGM-2, except for Pethick's Wonder, which was 'bb' (Table 3.1). The Australian variety Bruce was found to possess a fast component at GPI-2, not seen in any other cultivar.

3.4.2 Inheritance studies

All embryos resulting from crosses between Nonpareil and the 17 pollen donors were examined at the 8 polymorphic loci. Of the 136 sets of outcomes only 15 showed significant deviation from Mendelian ratios (Tables 3.2, 3.3, 3.4, 3.5, 3.6). The fast band exhibited only by Bruce at GPI-2 did show up in the progeny, but in a smaller number than expected (34:16 and $p = 0.01$). The segregation ratio for G6PD and SKDH was not significantly different from Mendelian (data is not shown).

Inheritance studies were crucial to the designation of null genes; a heterozygous diploid plant carrying a null allele might otherwise have been scored as homozygous for its other allele at that locus (e.g. na can be scored as aa). We demonstrated that the Californian cultivar Price, like Mission and Fritz (Hauagge *et al.*, 1987a, 1987b) has a null gene at LAP-

1, as does Carmel. Inheritance studies also suggest a null gene at AAT-1 in Drake and Fritz, and that Peerless is 'nn' at AAT-1.

4.4 Discussion

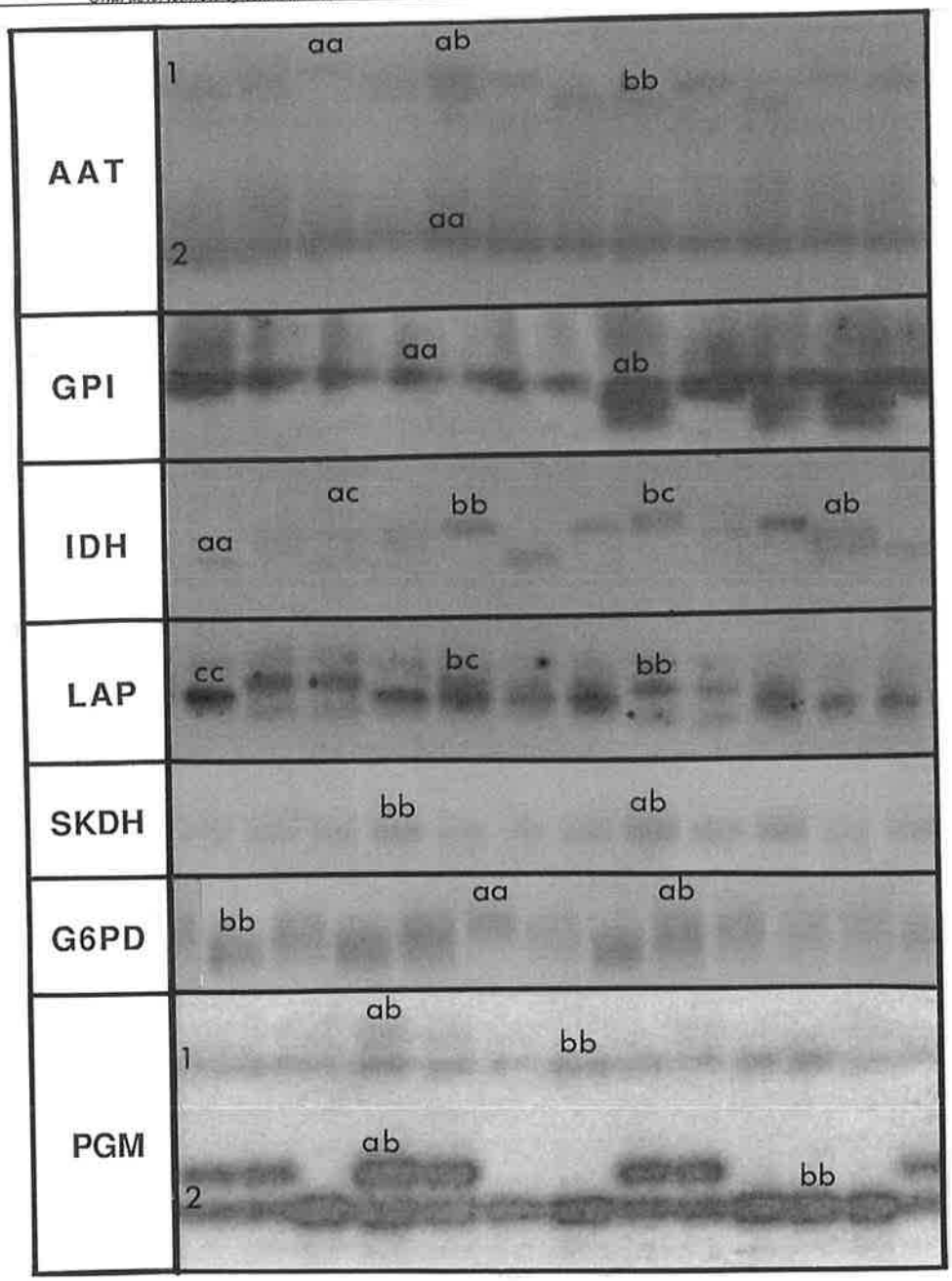
The Californian almond cultivars were characterized by Hauagge *et al.* (1987a, 1987b) using leaf isozyme polymorphism at 5 loci AAT-1, GPI-2, LAP-1, PGM-1 and PGM-2. A wider range of almond cultivars was characterized by Cerezo *et al.* (1989) using isozyme analysis of pollen. That group investigated almond cultivars from the different geographic zones of Spain, and from France, Italy, Russia and California. Their results did not always coincide with those of Hauagge *et al.* (1987 a, 1987b), perhaps due to the more reliable interpretation possible with haploid pollen tissue. It has shown several differences between the Californian and Australian cultivars. The Californian cultivars show null genes at LAP-1 and AAT-1 which are lacking in Australian cultivars. This characteristic of the Californian cultivars is a property of a group of almond genotype thought to have originated from Mission parents. This group also shows 'ab' alleles at the GPI-2 locus, a characteristic not seen in any of the Australian cultivars. Similarly, the homozygous 'aa' genotype at PGM-1 and homozygous 'bb' at AAT-1 are shown by some of the 'Mission group', and not seen in any of the Australian almond types, only Pethick's Wonder is homozygous 'bb' at PGM-2, whereas several of the Mission group of Californian cultivars exhibit this genotype.

We conclude that, unlike the situation in California (Hauagge *et al.*, 1987a, 1987b) Mission (or the Mission group) was not used to generate selections that gave rise to the recognized Australian cultivars. It is likely, however, that Nonpareil, the other cultivar that dominates in Californian selections as a parents, has played a role in Australian selections. Evidence that the Australian cultivars have been developed independent of those in California is found at the SKDH locus, where the 'a' allele occurred only in the Australian cultivars; we did not find in any Californian material. Quinn (1928) suggests that the Jordan almond of Spain was used in early selections in South Australia (Kester and Asay, 1975); it is likely that Nonpareil was also used. We therefore conclude that the Australian almond cultivars

represent a pool of genotypes derived quite separately from the Californian cultivars, and are of interest therefore in their own right as a source of variation for the development of improved varieties.

It was noted by Hauagge *et al.* (1987b) that their results with isozyme markers suggest that Carmel, which was alleged to have arisen as a bud mutation of Nonpareil in the U.S.A., could not have been derived in such a manner. Our results support this conclusion, as inspection of Table 1 reveals several genotype differences between Carmel and Nonpareil. The significant departures from Mendelian inheritance evident in the present studies are important when studies of gene flow via pollen are carried out in almond orchards.

A


AAT
1
aa
ab
bb
2
aa
GPI
aa
ab
IDH
aa
ac
bb
bc
ab
LAP
cc
bc
bb
SKDH
bb
ab
G6PD
bb
aa
ab
PGM
1
ab
bb
2
ab
bb

B

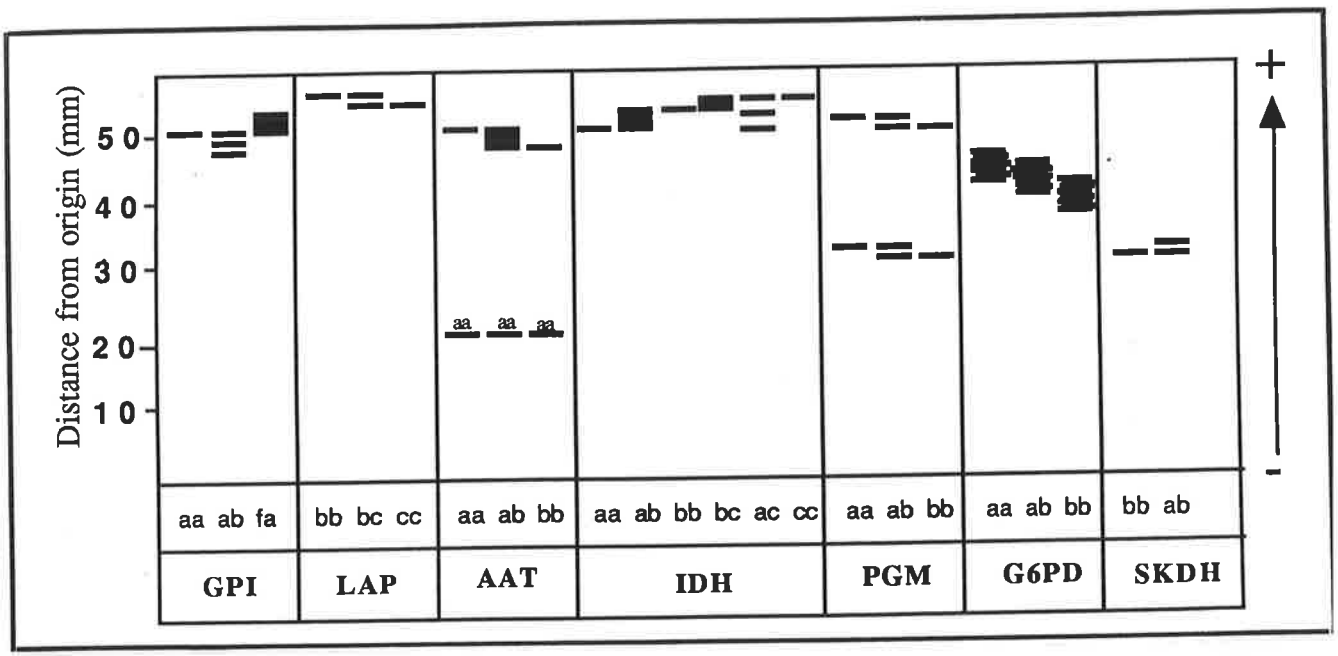

Distance from origin (mm)
50
40
30
20
10
aa
aa
aa
+
-
aa ab fa
bb bc cc
aa ab bb
aa ab bb bc ac cc
aa ab bb
aa ab bb
bb ab
GPI
LAP
AAT
IDH
PGM
G6PD
SKDH

Table 3.1 Inferred genotypes of Australian and Californian Cultivars at eight loci

Cultivar	Individual locus							
Australian cultivars	**AAT-1**	**GPI-2**	**LAP-1**	**PGM-1**	**PGM-2**	**IDH**	**G6PD**	**SKDH**
Baxendale	ab	aa	bc	ab	ab	ab	ab	ab
Bruce	aa	fa	bb	bb	ab	bb	bb	bb
Chellaston	aa	aa	bb	ab	ab	ab	bb	bb
IXL Seedling	ab	aa	bb	ab	ab	bc	bb	ab
Johnson's Prolific	aa	aa	cc	bb	ab	ab	bb	ab
Keane's Seedling	ab	aa	cc	ab	ab	ac	bb	ab
Pethick's Wonder	aa	aa	bc	bb	bb	cc	bb	ab
Somerton	aa	aa	bc	ab	ab	ab	bb	bb
White Brandis	aa	aa	bb	bb	ab	bb	ab	bb
Californian cultivars								
Carmel	ab	aa	nc	ab	bb	ab	aa	bb
Davey	ab	aa	bc	ab	ab	ab	ab	bb
Drake	na	aa	bb	bb	ab	bc	bb	bb
Fritz	na	ab	nb	ab	bb	ab	ab	bb
Mission	ab	ab	nc	aa	bb	aa	bb	bb
Ne Plus Ultra	ab	aa	bc	bb	ab	ac	ab	bb
Nonpareil	ab	aa	bc	ab	ab	ab	ab	bb
Peerless	nn	aa	bb	ab	ab	bb	ab	bb
Price	aa	ab	nc	aa	bb	ab	ab	bb
Thompson	bb	aa	bc	ab	ab	aa	ab	bb

Table 3.2. Summary of goodness of fit test in Nonpareil cultivar cross with 17 different pollen sources at AAT-1 locus

Crosses	Parental genotype	observed ratio			Expected ratio	df	X^2	P
		aa	ab	bb				
Nonpareil x Baxendale	ab x ab	27	57	18	1 : 2 : 1	2	3.11	ns
Nonpareil x Bruce	ab x aa	29	21	-	1 : 1	1	1.29	ns
Nonpareil x Carmel	ab x ab	22	55	25	1 : 2 : 1	2	0.44	ns
Nonpareil x Chellaston	ab x aa	129	118	-	1 : 1	1	0.49	ns
Nonpareil x Devey	ab x ab	22	38	15	1 : 2 : 1	2	1.35	ns
Nonpareil x Drake	ab x na	35	31	34	2 : 1 : 1	2	9.27	0.097
Nonpareil x Fritz	ab x na	67	45	38	2 : 1 : 1	2	2.30	ns
Nonpareil x Johnson	ab x aa	53	45	-	1 : 1	1	0.65	ns
Nonpareil x Keane's Seedling	ab x ab	23	35	16	1 : 2 : 1	2	1.48	ns
Nonpareil x Mission	ab x ab	45	105	56	1 : 2 : 1	2	1.24	ns
Nonpareil x Ne Plus Ultra	ab x ab	24	34	6	1 : 2 : 1	2	9.02	0.011
Nonpareil x Peerless	ab x nn	60	-	38	1 : 1	1	4.98	0.026
Nonpareil x Pethic's Wonder	ab x aa	47	33	-	1 : 1	1	2.46	ns
Nonpareil x Price	ab x aa	48	52	-	1 : 1	1	0.16	ns
Nonpareil x Somerton	ab x aa	29	21	-	1 : 1	1	1.29	ns
Nonpareil x Thompson	ab x bb	-	110	90	1 : 1	1	2.00	ns
Nonpareil x White Brandis	ab x aa	35	40	-	1 : 1	1	0.33	ns

Table 3.3. Summary of goodness of fit test in Nonpareil cultivar cross with 17 different pollen sources at IDH locus

Crosses	Parental genotype	observed ratio					Expected ratio	df	X^2	P
		aa	ab	bb	ac	bc				
Nonpareil x Baxendale	ab x ab	20	51	29	-	-	1 : 2 : 1	2	1.70	ns
Nonpareil x Bruce	ab x bb	-	22	28	-	-	1 : 1	1	0.72	ns
Nonpareil x Carmel	ab x ab	27	50	22	-	-	1 : 2 : 1	2	0.50	ns
Nonpareil x Chellaston	ab x ab	70	107	70	-	-	1 : 2 : 1	2	4.42	ns
Nonpareil x Devey	ab x ab	18	34	23	-	-	1 : 2 : 1	2	1.27	ns
Nonpareil x Drake	ab x bc	-	31	21	24	16	1 : 1 : 1 : 1	3	11.12	0.011
Nonpareil x Fritz	ab x ab	33	73	44	-	-	1 : 2 : 1	2	1.68	ns
Nonpareil x Johnson	ab x ab	30	48	20	-	-	1 : 2 : 1	2	2.05	ns
Nonpareil x Keane's Seedling	ab x ac	11	19	-	21	23	1 : 1 : 1 : 1	3	4.91	ns
Nonpareil x Mission	ab x aa	111	94	-	-	-	1 : 1	1	1.41	ns
Nonpareil x Ne Plus Ultra	ab x ac	10	10	-	24	16	1 : 1 : 1 : 1	3	8.41	0.038
Nonpareil x Peerless	ab x bb	-	40	58	-	-	1 : 1	1	3.32	ns
Nonpareil x Pethic's Wonder	ab x cc	-	-	-	34	46	1 : 1	1	1.81	ns
Nonpareil x Price	ab x ab	24	54	23	-	-	1 : 2 : 1	2	0.64	ns
Nonpareil x Somerton	ab x ab	11	31	8	-	-	1 : 2 : 1	2	3.38	ns
Nonpareil x Thompson	ab x aa	96	104	-	-	-	1 : 1	1	0.32	ns
Nonpareil x White Brandis	ab x bb	-	42	33	-	-	1 : 1	1	1.08	ns

Table 3.4. Summary of goodness of fit test in Nonpareil cultivar cross with 17 different pollen sources at LAP-1 locus

Crosses	Parental genotype	observed ratio			Expected ratio	df	X^2	P
		bb	bc	cc				
Nonpareil x Baxendale	bc x bc	33	41	22	1 : 2 : 1	2	7.11	0.028
Nonpareil x Bruce	bc x bb	28	22	-	1 : 1	1	0.72	ns
Nonpareil x Carmel	bc x bc	26	22	51	1 : 2 : 1	2	0.42	ns
Nonpareil x Chellaston	bc x bb	124	123	-	1 : 1	1	0.004	ns
Nonpareil x Devey	bc x bc	37	25	13	1 : 2 : 1	2	20.5	0.001
Nonpareil x Drake	bc x bb	37	63	-	1 : 1	1	6.84	0.043
Nonpareil x Fritz	bc x nb	75	44	31	2 : 1 : 1	2	2.26	ns
Nonpareil x Johnson	bc x cc	22	28	-	1 : 1	1	0.04	ns
Nonpareil x Keane's Seedling	bc x cc	-	50	48	1 : 1	1	4.42	0.035
Nonpareil x Mission	bc x nc	-	46	28	1 : 2 : 1	2	0.83	ns
Nonpareil x Ne Plus Ultra	bc x bc	55	54	96	1 : 2 : 1	2	3.09	ns
Nonpareil x Peerless	bc x bb	10	36	14	1 : 1	1	1.02	ns
Nonpareil x Pethic's Wonder	bc x bc	44	54	-	1 : 2 :1	2	1.51	ns
Nonpareil x Price	bc x nc	24	27	46	1 : 2 : 1	2	0.22	ns
Nonpareil x Somerton	bc x bc	10	22	18	1 : 2 : 1	2	3.04	ns
Nonpareil x Thompson	bc x bc	61	91	48	1 : 2 : 1	2	3.18	ns
Nonpareil x White Brandis	bc x bb	37	38	-	1 : 1	1	0.01	ns

Table 3.5. Summary of goodness of fit test in Nonpareil cultivar cross with 17 different pollen sources at PGM-1 locus

Crosses	Parental genotype	observed ratio			Expected ratio	df	X^2	P
		aa	ab	bb				
Nonpareil x Baxendale	abxab	20	37	43	1 : 2 : 1	2	15.43	0.0004
Nonpareil x Bruce	abxbb	-	25	25	1 : 1	1	0.00	ns
Nonpareil x Carmel	abxab	26	51	22	1 : 2 : 1	2	0.42	ns
Nonpareil x Chellaston	abxab	58	129	60	1 : 2 : 1	2	0.52	ns
Nonpareil x Devey	abxab	12	39	24	1 : 2 : 1	2	4.20	ns
Nonpareil x Drake	abxbb	-	49	51	1 : 1	1	0.04	ns
Nonpareil x Fritz	abxab	14	119	17	1 : 2 : 1	2	55.38	< 0.001
Nonpareil x Johnson	abxbb	-	41	57	1 : 1	1	2.60	ns
Nonpareil x Keane's Seedling	abxab	21	36	17	1 : 2 : 1	2	0.48	ns
Nonpareil x Mission	abxaa	118	87	-	1 : 1	1	4.71	0.03
Nonpareil x Ne Plus Ultra	abxbb	-	28	32	1 : 1	1	0.27	ns
Nonpareil x Peerless	abxab	23	54	21	1 : 2 : 1	2	1.11	ns
Nonpareil x Pethic's Wonder	abxbb	-	51	29	1 : 1	1	6.13	0.0113
Nonpareil x Price	abxaa	42	58	-	1 : 1	1	2.28	ns
Nonpareil x Somerton	abxab	22	20	8	1 : 2 : 1	2	8.80	ns
Nonpareil x Thompson	abxab	47	99	54	1 : 2 !	2	0.51	ns
Nonpareil x White Brandis	abxbb	-	31	44	1 : 1	1	2.26	ns

Table 3.6. Summary of goodness of fit test in Nonpareil cultivar cross with 17 different pollen sources at PGM-2 locus

Crosses	Parental genotype	observed ratio			Expected ratio	df	X^2	P
		aa	ab	bb				
Nonpareil x Baxendale	ab x ab	22	62	16	1 : 2 : 1	2	6.77	0.034
Nonpareil x Bruce	ab x ab	19	22	9	1 : 2 : 1	2	4.37	ns
Nonpareil x Carmel	ab x bb	-	55	25	1 : 1	1	0.25	ns
Nonpareil x Chellaston	ab x ab	66	112	69	1 : 2 : 1	2	2.21	ns
Nonpareil x Devey	ab x ab	27	37	11	1 : 2 : 1	2	6.96	ns
Nonpareil x Drake	ab x ab	18	48	34	1 : 2 : 1	2	5.20	ns
Nonpareil x Fritz	ab x bb	-	76	74	1 : 1	1	0.03	ns
Nonpareil x Johnson	ab x ab	19	52	27	1 : 2 : 1	2	1.77	ns
Nonpareil x Keane's Seedling	ab x bb	-	44	30	1 : 2 : 1	2	2.66	ns
Nonpareil x Mission	ab x bb	-	108	97	1 : 1	1	0.59	ns
Nonpareil x Ne Plus Ultra	ab x ab	16	30	14	1 : 2 : 1	2	0.13	ns
Nonpareil x Peerless	ab x ab	17	48	33	1 : 2 : 1	2	5.32	ns
Nonpareil x Pethic's Wonder	ab x bb	-	44	36	1 : 1	1	0.80	ns
Nonpareil x Price	ab x bb	-	51	49	1 : 1	1	0.04	ns
Nonpareil x Somerton	ab x ab	10	27	13	1 : 2 : 1	2	0.71	ns
Nonpareil x Thompson	ab x ab	63	57	50	1 : 2 : 1	2	4.89	ns
Nonpareil x White Brandis	ab x ab	14	40	23	1 : 2 : 1	2	3.85	ns

Chapter 4

Pollen ultrastructure of ten Australian and Californian almond cultivars

Chapter 4

Pollen ultrastructure of ten Australian and Californian almond cultivars

4.1 Summary

Pollen grains of ten almond cultivars [*Prunus dulcis (Mill) D.A. Webb, syn P. amygdalus Batsch.*] including three Australian and seven Californian cultivars were examined using scanning electron microscopy (SEM). The following parameters were examined: pollen length, width, and L/W ratios, distance between germinal furrows (valves), the number of pits, the number of ridges and stria, and their respective widths, all within a standard area of the equatorial region of the exine. These parameters were found to be useful in distinguishing cultivars tested and indicated significant differences between cultivars at the $P < 0.01$ level. Six of these cultivars can be identified with one or two characters, while for the remaining cultivars a combination of parameters is necessary for identification. In almond, pollen grain morphology and exine characteristics are most useful if combined with other morphological and molecular genetics markers for distinguishing cultivars.

4.2 Introduction

Pollen grain shape and ornamentation has often been used for identification of species, cultivars and clones of horticultural crops. Fogle (1977a, 1977b) has used pollen ultrastructure to distinguish between species of peach, plum, apricot, cherry, apple and pear and their cultivars. Westwood and Challice (1978) also were able to distinguish species of pear. Pollen ultrastructure has been used to distinguish the cultivars of pecan (Haulik and Holtzhausen, 1988), apple (Marcucci *et al.*, 1984; Martens and Fretz, 1980) and grape (Ahmedullah, 1983). Mulas *et al.* (1988) described pollen ultrastructure differences among twenty almond cultivars originating from Italy, France, Russia, and the U.S.A. (California). Mulas *et al.* (1989) have also shown that the ultrastructural morphology of Nonpareil almond does not vary according to rootstock used, irrigation or geographical location.

The aim of this study is to characterize pollen morphology so as to be able to use this to trace the origins of pollen foraged by honeybees in commercial orchards. This would add a further dimension to our studies on gene flow by pollen as determined by molecular genetic markers (Jackson and Clarke, 1991).

4.3 Materials and methods

4.3.1 Pollen collection

Pollen samples were obtained from selected trees in a commercial almond orchard near Angle Vale (34° 39 S, 138° 40 E), 35 km NW of Adelaide, South Australia. The climate is of the Mediterranean type with a hot, dry summer and wet winter, rainfall 460 mm. The almond cultivars sampled for this study were Carmel, Fritz, Mission, Nonpareil, Ne Plus Ultra, Peerless, and Price (Californian cultivars) and Chellaston, Johnson's Prolific and Pethic's Wonder (South Australian cultivars) as described morphologically by Baker (1973) and genetically by Vezvaei *et al.* (1994).

4.3.2 Preparation of pollen for electron microscopy

Five trees for each of the ten cultivars were selected, and a completely randomized design was used. Fifty flower buds ready to open were collected from each tree at random from the whole canopy and placed into paper bags. The anthers were separated from the flowers of each tree and placed in petri dishes to dry at room temperature for 24 hours. Two samples were taken from each petri dish. The anthers were removed from the samples and air-dried pollen was sifted onto double-sided adhesive tape attached to an aluminium stab. At each stage contamination of samples by pollen from different cultivars was carefully avoided.

The samples were coated with about 200 A° gold alloy using a J.E.O.L. vacuum evaporator unit and observed with a Cambridge S 250 MK3 scanning electron microscope at 20 KV. At least 50 pollen grains from each cultivar were viewed before selecting representative mature pollen. Measurements made were: pollen size (length, width and L/W ratio), distance between two valves (germinal furrows), the number of pits in the equatorial

region, and number of ridges and stria crossing one side of the equatorial region; 10 pollen grains examined for each cultivar.

4.3.3 Data analysis

Analysis of variance was performed on the various measurements, to test for differences between cultivars. Mean values for the cultivars were separated by Tukey's wholly significant difference. The statistical package used was Genstat.

4.4 Results

4.4.1 Pollen grains sculpture

Pollen grains of all cultivars examined were truncate, isopolar and triaperturate with the germinal furrows extending the length of the grain (Figs. 4.1 and 4.2), except for pollen of Pethic's Wonder which was elliptical in pollen shape for most of the samples (Fig. 4.2.c). Some characteristics of the pollen grain and exine are shown in Tables 4.1 and 4.2.

4.4.2 Pollen grain diameter

Pollen grain lengths varied from a minimum of 55.6 µm for Pethic's Wonder to 62.1 µm for Peerless. Pollen grain widths ranged from 28.7 µm for Fritz to 31.5 µm for Ne Plus Ultra. Length/width ratios varied from 1.76 for Pethic's Wonder to 2.15 for Carmel and Peerless. The distance between valves was lowest at 18.8 µm for Peerless and highest for Pethic's Wonder at 21.7 µm. The number of pits in 50 μm^2 of exine surface was found to be very variable among the cultivars, mean ranging from a value of 5.0 for Pethic's Wonder to 72.0 for Peerless. The number of ridges crossing 7 µm of exine equatorial region varied only slightly, the lowest value was found for Pethic's Wonder (11.4) and the highest for Fritz (17.6). Ridge mean widths were from 0.32 µm for Chellaston to 0.43 µm for Fritz. The number of stria crossing 7 µm of exine was very close to the number of ridges and complementary to it, the lowest being Pethic's Wonder (10.5) and the highest Fritz (19.9). Mean stria widths were found to be close to the mean of ridge width, the lowest recorded being for Price (0.25 µm) and the highest for Peerless (0.33 µm).

4.5 Discussion

Analysis of results indicated that in almond, there is less pollen morphological variability than in other species such as apple (Marcucci *et al.*, 1984). One of the reasons may be the lower ploidy level (8n for almonds). In apple, plum, peach, strawberry and raspberry species there appears to be a direct relationship between pollen size and level of ploidy (Fogle, 1977a; Scott, 1951; Maas, 1977). Comparison of the values given in Tables 4.1 and 4.2 and inspection of pollen electron micrographs shown in Figures. 4.1 and 4.2, suggest that differences are significant enough to enable us to separate and identify six cultivars relatively easily. Thus Chellaston pollen has very narrow ridges; Peerless pollen has the smallest distance between the two valves and is pitted over the entire surface of pollen grains; While Fritz had widest interwoven ridges and width of ridge; Pethic's Wonder has elliptical pollen grains in most samples and very low pit numbers and wide distance between the two valves; Price has very narrow stria and high numbers of stria in the equatorial section; and Ne Plus Ultra has the greatest width. While pollen from the four remaining cultivars can share similar characteristics in one or more parameters, it is clear that each cultivar identified, when considered over all parameters, has a unique set of parameters.

While it was shown by Mulas *et al.* (1988) that pollen grain size by itself is too variable to be used for almond cultivar identification, nevertheless the length/width ratio does seem to be a useful parameter for identification. Thus, comparing this ratio for the three Californian cultivars as found by Mulas *et al.* (1988) in the Northern hemisphere with the ratio found in the present study in the Southern hemisphere (Table 4.1), it can be seen that this parameter is similar in the two studies and does not vary according to the geographical location; e.g. for Ne Plus Ultra (1.92±0.07) as compared with our result (1.96±0.04); for Nonpareil (2.20±0.07) as compared with the present (2.05±0.02) and for Mission (2.06±0.07) as against the present (2.04±0.03).

Other pollen characteristics can be used as an aid to identification, notably the number of ridges and stria which are moderately constant for each cultivar, and the exine pattern resulting e.g. parallel or interwoven which certainly is an identifiable trait. So too is the width of stria and ridges, as can be seen in Table 4.2. Such properties as the number of pits in the exine can be rather too variable and should only be used for widely different

cultivars (Table 4.2). We have thus confirmed the ultrastructural characteristics of pollen for the three Californian cultivars Ne Plus Ultra, Nonpareil and Mission studied by Mulas *et al.* (1988) as giving similar results under very different growing conditions (Italy vs South Australia).

In conclusion, it is found that several morphological parameters obtained from an examination of electron micrographs can be used to distinguish between pollen samples from ten almond cultivars. However they are best used in combination with other cultivar characteristics (e.g. molecular genetic markers) to be most useful for gene flow by pollen studies.

Table 4.1 Characteristics of pollen grains from ten almond cultivars

Data in the same column having different letters (a, b, c) are significantly different using Tukey's WSD ($P < 0.01$).

Values are mean ± standard error of ten samples.

* Australian cultivar.

Cultivar	Length (μm)	Width (μm)	L/W ratio	Distance between two valves (mm)
Carmel	62.0±0.8 b	29.8±0.6 ab	2.15±0.06 b	20.1±0.5 ab
* Chellaston	60.6±0.6 b	30.0±0.4 ab	2.02±0.02 ab	19.8±0.4 ab
Fritz	58.2±0.2 ab	28.7±0.8 a	2.04±0.07 ab	19.9±0.5 ab
* Johnson's Prolific	60.8±0.8 b	31.2±0.3 b	1.94±0.04 ab	21.6±0.7 b
Mission	60.0±0.8 b	29.4±0.2 ab	2.04±0.03 ab	21.0±0.7 ab
Ne Plus Ultra	61.8±0.91 b	31.5±0.6 b	1.96±0.04 ab	19.2±0.3 ab
Nonpareil	59.8±0.2 b	29.3±0.3 ab	2.05±0.02 ab	20.3±0.5 ab
Peerless	62.1±0.8 b	29.6±0.3 ab	2.10±0.02 b	18.8±0.7 a
* Pethic's Wonder	55.6±0.9 a	31.1±0.4 b	1.76±0.04 a	21.7±0.4 b
Price	58.9±0.2 ab	29.6±0.3 ab	1.98±0.02 ab	19.7±0.4 ab

Table 4.2 Exine characteristics of pollen grains from ten almond cultivars

Data in the same column having different letters (a, b, c) are significantly different using Tukey's WSD ($P \leq 0.01$).

Values are mean ± standard error of ten samples.

* Australian cultivars.

Cultivar	No. of pit in 50 μm² of exine	No. of ridge in 7 μm of exine	Mean ridge width (μm)	No. of stria in 7 μm of exine	Mean stria width (μm)
Carmel	55.0±4.0 cd	14.3±0.4 ab	0.35±0.01 ab	14.0±0.8 ab	0.32±0.01 b
*Chellaston	48.0±3.0 c	15.8±0.1 b	0.32±0.01 a	15.5±0.7 bc	0.30±0.02 ab
Fritz	61.0±4.0 cd	17.6±0.5 c	0.43±0.02 b	19.9±1.1 c	0.25±0.01 ab
*Johnson's Prolific	20.0±3.0 ab	14.9±0.5 b	0.36±0.01 ab	15.8±0.8 bc	0.28±0.01 ab
Mission	38.0±4.0 bc	14.8±0.5 b	0.41±0.01 b	14.6±0.9 ab	0.30±0.01 ab
Ne Plus Ultra	14.0±2.0 ab	13.7±0.6 ab	0.34±0.01 ab	13.6±0.8 ab	0.28±0.01 ab
Nonpareil	41.0±2.0 bc	14.4±0.6 b	0.36±0.01 ab	13.7±0.4 ab	0.30±0.02 ab
Peerless	72.0±7.0 d	15.7±0.6 b	0.39±0.01 b	14.8±1.0 b	0.33±0.02 b
* Pethic's Wonder	5.0±2.0 a	11.4±0.4 a	0.41±0.02 b	10.5±0.4 a	0.25±0.01 ab
Price	25.0±1.0 b	16.5±0.5 b	0.36±0.01 ab	15.9±0.6 bc	0.24±0.01 a

Fig. 4.1 Electron micrographs of pollen grains (x 1500) and showing exine characteristics in equatorial region (x 10,000) **A** (Nonpareil), **B** (Carmel), **C** (Fritz), **D** (Mission), **E** (Ne Plus Ultra).

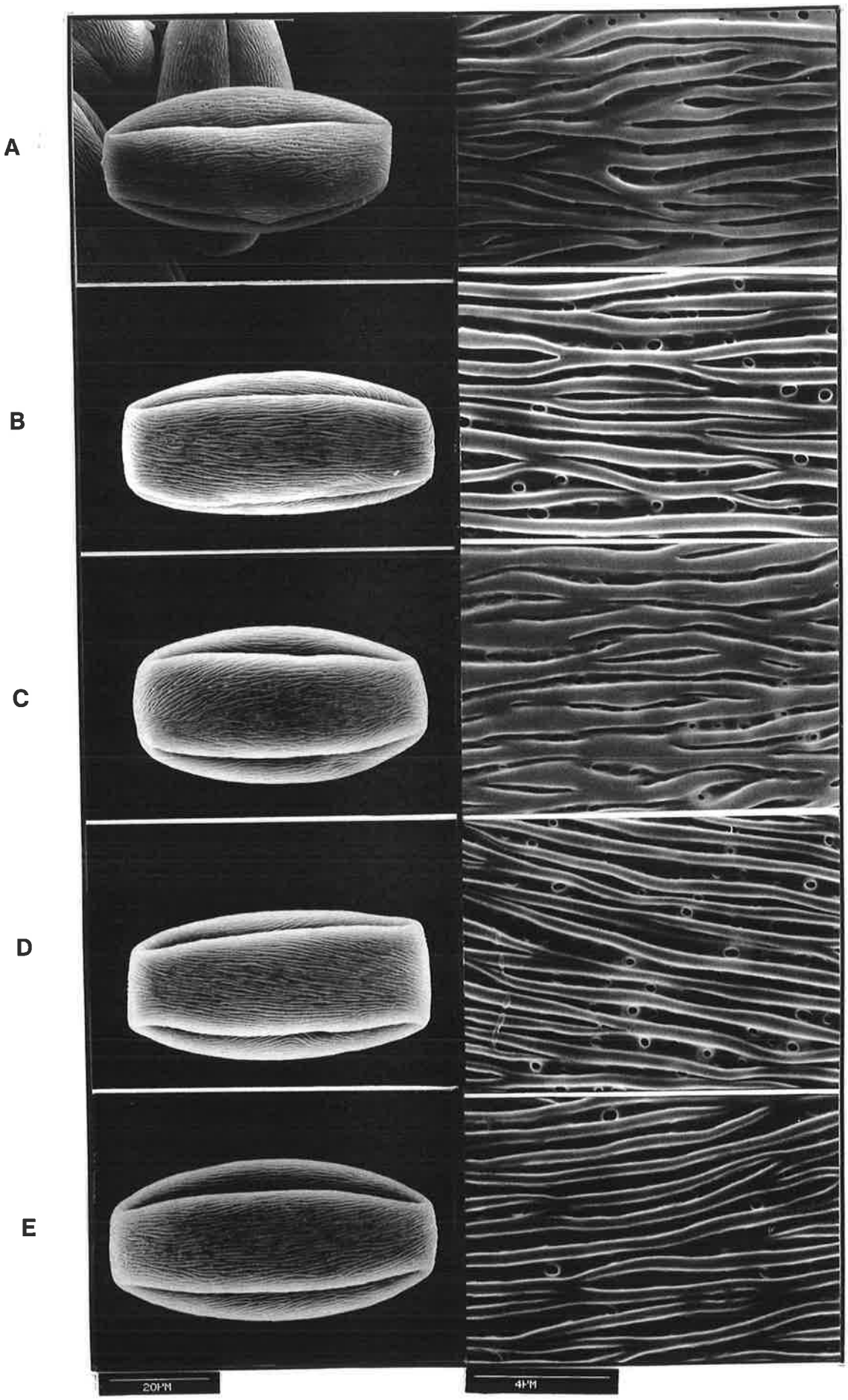
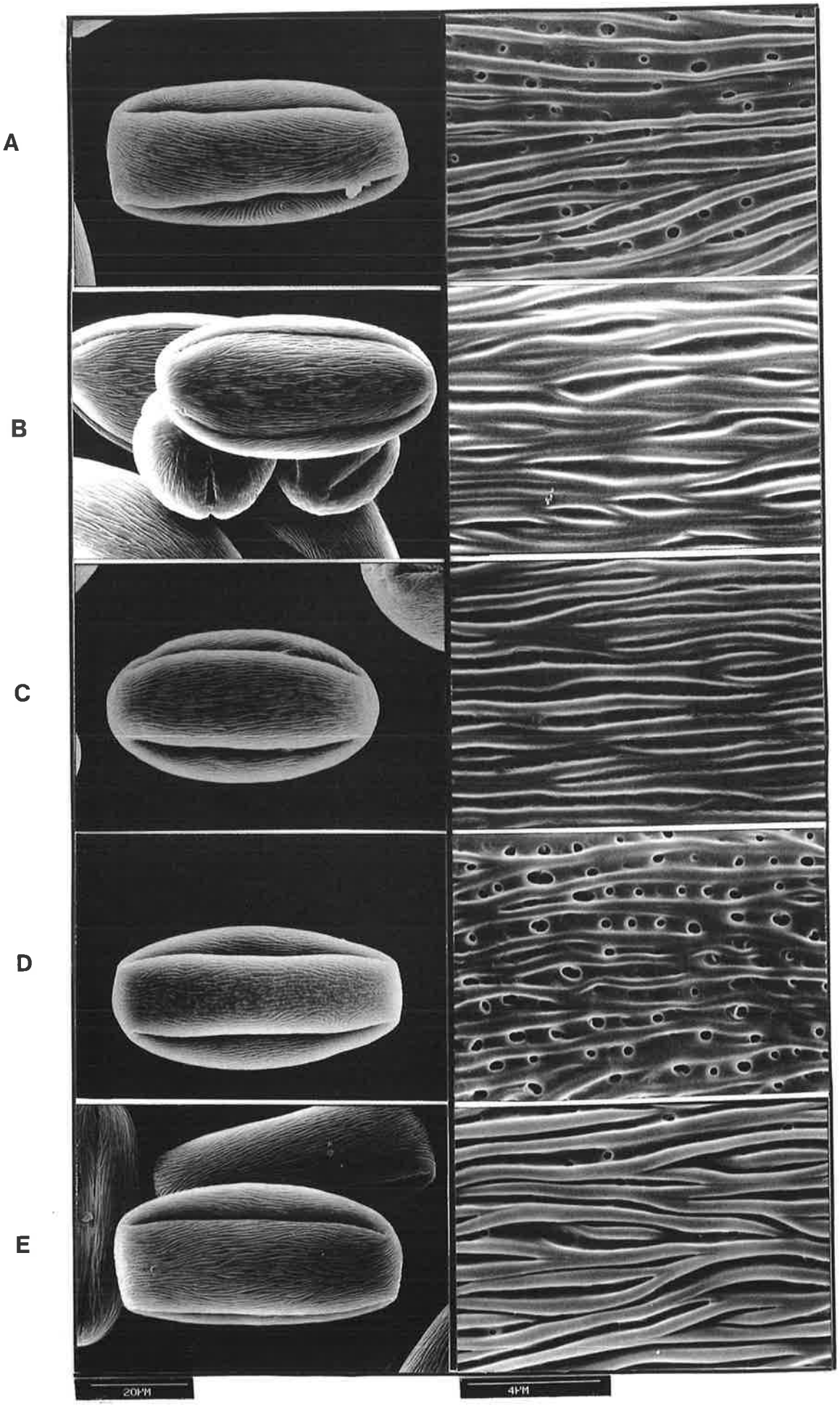

A
B
C
D
E
20µM
4µM

Fig. 4.2 Electron micrographs of pollen grains (x 1500) and showing exine characteristics in equatorial region (x 10,000) **A** (Chellaston), **B** (Johnson's Prolific), **C** (Pethic's Wonder), **D** (Peerless), **E** (Price).


A
B
C
D
E
20µM
4µM

Chapter 5

Inheritance and linkage of isozyme loci in almond

Chapter 5

Inheritance and linkage of isozyme loci in almond

5.1 Summary

The segregation of seven isozyme marker genes was investigated using 8 controlled crosses in almond. In all crosses the cultivar Nonpareil was the maternal parent. Pollination was achieved using 8 different cultivars and a total of 3200 individual kernels assessed. For each isozyme the goodness of fit test was used to test for departure from the expected frequencies assuming Mendelian inheritance. Given a higher than expected number of significant results for individual isozymes, independent segregation between pairs of isozymes was tested using the chi-square statistic on the resulting two-way contingency tables. In all crosses a highly significant association (P-value < 0.001) was observed between (i) the AAT-1 and IDH isozymes and (ii) the LAP-1 and PGM-2 isozymes which leads to the conclusion that the respective isozyme pairs are linked.

In addition, a significant association (P-value < 0.001) was observed between LAP-1 and GPI-2 when pollen sources were Fritz, Mission or Price, but could not be tested for the remaining five pollen sources because they are homozygous at these loci. If LAP-1 is linked with GPI-2 and PGM-2, it might be expected that we should find evidence of linkage between GPI-2 and PGM-2. The lack of a significant association between these two isozymes suggests that LAP-1 is located centrally on the chromosome. These three pairs of linked loci are the first to be established in almond.

5.2 Introduction

Isozyme analysis has been used for genetic mapping through linkage studies (Torres *et al.*, 1985). However application of such linkage studies are rare for woody plants for several reasons *viz.* space and other required resources, long generation times, and in many cases, technical difficulties in obtaining progeny from controlled crosses (Torres, 1983). Also as woody plants are frequently heterozygous at individual loci, this complicates such studies. Recently linkage in some species of gymnosperms and angiosperms have been investigated (Eckert *et al.*, 1981; Lee and Ellstrand, 1987; Wehling, 1991; Fuong *et al.*,

1993). The knowledge of the linkage relationships among isozyme loci would be useful for further genetic work and provide information on linkage conservation (Heemstra *et al.*, 1991). Linkage between pairs of isozyme loci has been reported in a number of horticultural genera as follow: in citrus two related linkage groups were found between seven isozyme loci investigated; one of these linkage groups is between AAT-1 and MDH-1 (Torres *et al.*, 1985). (In this communication we use the term AAT where others quoted refer to GOT; they refer to the same isozyme). In avocado a close linkage has been found in AAT-1 and AAT-2 (Torres *et al.*, 1986). In apple, Manganaris and Alston (1987) reported linkage between AAT-1 and a self-incompatibility loci, and a close relationship was demonstrated between AAT-1 and IDH. In blueberry, Heemstra *et al.* (1991) found two independent linkage groups between (i) GPI-2 and LAP-1 and (ii) PGM-2 and 6GPD-2. In fig a sex determining gene is linked to the peroxidase gene, and AAT and esterase genes are linked (Valizadeh, 1973). Santi and Lemoine (1990) identified two linkage groups in sweet cherry (i) LAP-1 and AAT-1 and (ii) LAP-1 and ME-1. Linkage was reported in grape between GPI-c and LAP-1 by Weeden *et al.* (1988).

We are now in a position to study linkage more thoroughly in almond (*Prunus dulcis [Mill] D.A. Webb.*), since in addition to the five loci identified by Hauagge *et al.* (1987b) (*viz* AAT-1, GPI-2, LAP-1, PGM-1 and PGM-2), polymorphism has been demonstrated for IDH (Cerezo *et al.*, 1989; Jackson and Clarke, 1991) and SKDH (Jackson, 1992).

5.3 Materials and methods

5.3.1 Plant materials

Healthy young leaves are suitable for isozyme analysis in almond (Jackson, 1992). Leaves were sampled from each of nine cultivars (Carmel, Fritz, Grant, Keane, Mission, Ne Plus Ultra, Nonpareil, Peerless and Price) from a commercial orchard at Angle Vale (35 kilometres NE of Adelaide, South Australia). All samples were packed in crushed ice during transport to the laboratory at the Waite Agricultural Research Institute.

5.3.2 Isozyme analysis of almond leaves

The seven polymorphic isozyme loci examined were: (i) asparatate amino transferase at locus one (AAT-1), (ii) glucose phospho isomerase (GPI-2), (iii) isocitrate dehydrogenase (IDH), (iv) leucine amino peptidase (LAP-1), (v) phosphoglucomutase at locus one (PGM-1), (vi) phosphoglucomutase at locus two (PGM-2) and (vii) shikimate dehydrogenase (SKDH). Cellogel was used as the medium for electrophoresis, which was carried out as described for almond in chapter 2.2.

5.3.3 Inheritance studies

Controlled crosses were carried out using pollen from the eight pollen sources on the Nonpareil cultivar as sole maternal source as described by Kester and Asay (1975). Inheritance studies were carried out in this way for practical reasons and because Nonpareil is the most desirable commercial cultivar and we want to use results to aid a study of gene flow by pollen to Nonpareil in commercial orchards. Two Nonpareil trees were isolated in the orchard by nylon mesh cages for this purpose. For each cross four hundred embryos (kernels) were analysed as described by Jackson (1992).

5.4 Results

All observed isozyme patterns for the various genotypes in the seven isozyme loci studied are represented diagrammatically in Figure 5.1. Inheritance studies were crucial to the designation of null genes, which, when present in heterozygous diploid plant cells, can be scored as homozygous at a locus where it appears (e.g. 'na' can be scored as 'aa'). We have demonstrated that the cultivar Price has a null gene at LAP-1 (Vezvaei *et al.*, 1994), as do Fritz and Mission (Hauagge *et al.*, 1987a and 1987b). In addition Peerless is 'nn' at the AAT-1 locus (Jackson and Clarke 1991).

5.4.1 Testing for Mendelian segregation at each enzyme locus

Mendelian inheritance of isozyme banding patterns was tested statistically with the goodness of fit test of expected frequencies. For each cross the expected ratio of phenotypes was calculated for each isozyme locus. Table 5.1 shows details for the seven isozyme loci

for each of the eight crosses. It should be noted that for crosses where there is no segregation occurring because both parents are homozygous, the goodness of fit test cannot be applied. Such cases are indicated by a "-" in Table 5.1 and occur for 12 of the 56 combinations considered. Of the 44 tests, 28 support Mendelian inheritance, but the remaining 16 tests showed significant departure from the expected ratio. This high proportion of departure needs to be carefully investigated, but we will assume that inheritance is Mendelian and proceed to test for evidence of linkage between the seven loci.

5.4.2 Testing for linkage between isozyme loci

If two loci are unlinked, then the segregation pattern at one locus should be independent (or unrelated) to the segregation pattern at the other locus. Given the data recorded, the potential exists to test the 7x(7-1)/2 =21 pairwise combinations of the 7 isozyme loci for each of the eight crosses investigated. However as segregation is not occurring at all loci in all crosses, it will not be possible to perform a test in all instances. The observed phenotypes were tabulated for each pair of isozyme loci for each cross. Given the high proportion of departure from the expected ratios when individual isozyme loci were tested, it seemed unwise to test the two-way contingency tables against their corresponding expected ratios with the goodness of fit test. Instead, for each pair of isozyme loci the null hypothesis that the two isozyme loci independently segregate was tested by carrying out a chi-square test of independence on the appropriate contingency table.

The results of the chi-square test for independence are summarised in Table 5.2. Table 5.3 parts i-iii show the observed and expected frequencies for each significant association.

For every cross a highly significant association (P-value < 0.001) was observed between the following pairs (i) AAT-1 and IDH and (ii) LAP-1 and PGM-2. Additionally, a highly significant association (P-value < 0.001) found between LAP-1 and GPI-2 when the pollen source was Fritz, Mission or Price, but could not be tested for the remaining five pollen sources, as there was no segregation. An isolated association of PGM-1 and IDH was found for the cross involving Carmel (P-value < 0.05), but in the absence of supporting evidence for the other parents, this appears to be a chance result and not proof of linkage.

Given the large number of pairwise combinations tested (100), it is not surprising that this seemingly anomalous result was found.

5.4.3 Identifying parental type when isozyme loci are linked

By considering the genotype of the parent and the observed frequency of the phenotypes in the embryo, it is possible to identify the pairing of the alternative alleles at the linked isozyme loci. For example, consider the Nonpareil x Ne Plus Ultra cross for the isozyme loci LAP-1 and PGM-2. Consider both parents are bc for LAP-1 and a'b' for PGM-2. The two way contingency table, showing observed frequency and expected frequency for these crosses is given in Table 5.3 (i). The placement of the alleles on the chromosomes could be either of the following two alternatives for both Nonpareil and Ne Plus Ultra $\frac{b\ \ a'}{c\ \ b'}$ or $\frac{b\ \ b'}{c\ \ a'}$ for both parents. By considering the expected frequency of parental and comparing these to the observed numbers in the contingency table, it is possible to conclude the most likely linkage arrangement for both Nonpareil and Ne Plus Ultra is $\frac{b\ \ a}{c\ \ b'}$.

Thus the b allele of the LAP-1 loci is linked with the a' allele of the PGM-2 loci and similarly c is linked with b' for both Nonpareil and Ne Plus Ultra. In Table 5.4 the relationship among the alleles for the linked loci is indicated for all eight crosses considered.

5.5 Discussion

In this, the first linkage study of almond with eight crosses tested for seven isozyme loci, a significant association (P-value < 0.001) is found between AAT-1 and IDH and also between LAP-1 and PGM-2 which leads us to conclude that these isozyme pairs are linked. Additionally a highly significant association is found between LAP-1 and GPI-2. However, as LAP-1 appears linked to two isozyme loci, evidence of linkage might be expected between PGM-2 and GPI-2. The lack of a significant association between these two isozymes suggests that LAP-1 is located more centrally on the chromosome with the other two isozymes at opposite ends of the same chromosome.

Two of these linked isozyme loci are also found to be linked in other studies. Linkage between LAP-1 and PGM-2 is tentatively suggested in almond by Hauagge *et al.*, (1987a and 1987b), and linkage is indicated between AAT-1 and IDH in apple where both in turn appear linked to the to self-incompatibility gene (Manganaris and Alston, 1987). It can be suggested that as the mechanism of self-incompatibility is gametophytic in both related genera, it could be that the linkage group AAT-1 and IDH is itself linked to self-incompatibility in almond; this idea can be investigated in the future. In blueberry Heemstra *et al.* (1991) found a similar linkage group between LAP-1 and GPI-2. Moran and Bell (1983) reported linkage of LAP-1 and GPI-2 in *Eucalyptus*. In *Asparagus officinalis L.* Maestri *et al.* (1991) found linkage between IDH-2 and AAT-2 and GPI-1. Conservation of linked loci among different species can have important implications for the evolution of the various species (Weeden and Wendel, 1990). It must be recognised that for the results presented here, where there is significant departure from the expected ratio using a goodness of fit test as in Table 5.1, it could be misleading to estimate linkage distance.

An isolated association of PGM-1 and IDH was found for the cross involving Carmel (P-value < 0.05), but we concluded that this appears to be a chance result and not proof of linkage. Finally, we consider there is significant evidence in almond of linkage between two pairs of loci, namely (i) AAT-1 and IDH and (ii) LAP-1 and PGM-2. Additionally LAP-1 and GPI-2 were observed to be linked when the pollen source was heterozygous for GPI-2. However as PGM-2 and GPI-2 are not significantly associated, we propose that LAP-1 is located in a more central position on the chromosome with PGM-2 and GPI-2 at opposite ends.

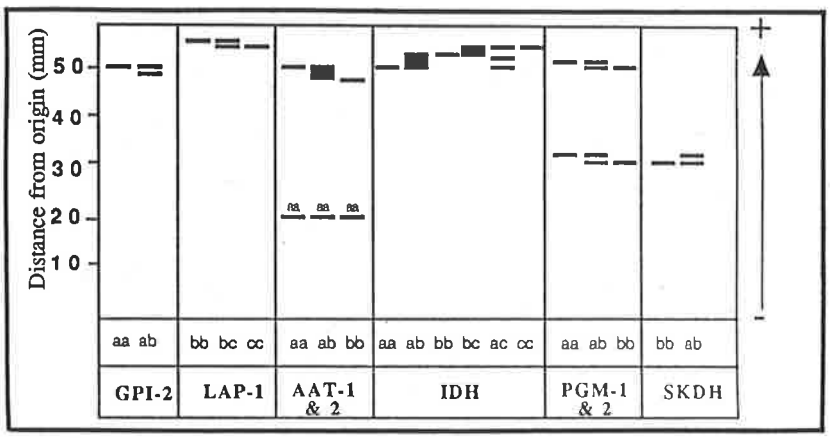


Fig. 5.1 Diagram of banding patterns of seven isozyme loci in progeny of the crosses.

Table 5-1: Summary of observed frequencies, expected ratios and goodness of fit test for each of the eight crosses by seven isozyme loci

Cultivar / Nonpareil	AAT-1 ab	GPI-2 aa	IDH ab	LAP-1 bc	PGM-1 ab	PGM-2 ab	SKDH bb
Carmel	ab	aa	ab	nc	ab	bb	bb
Expected	aa:ab:bb=1:2:1	aa=1	aa:ab:bb=1:2:1	bb:bc:cc+nc=1:1:2	aa:ab:bb=1:2:1	ab:bb=1:1	bb=1
Observed	135:187:78	400	89:207:104	94:92:214	89:217:94	188:212	400
Goodness of fit	17.13 on 2 df	-	1.65 on 2 df	1.98 on 2 df	3.03 on 2 df	1.44 on 1 df	-
Significance level	* * *		ns	ns	ns	ns	
Fritz	na	ab	ab	nb	ab	bb	bb
Expected	aa+na:ab:bb=2:1:1	aa:ab=1:1	aa:ab:bb=1:2:1	bb+nb:bc:cc=2:1:1	aa:ab:bb=1:2:1	ab:bb=1:1	bb=1
Observed	219:102:79	174:226	86:209:105	171:111:118	71:196:133	194:206	400
Goodness of fit	6.54 on 2 df	6.72 on 1 df	2.7 on 2 df	8.65 on 2 df	19.3 on 2 df	0.36 on 1 df	-
Significance level	*	* *	ns	*	* * *	ns	
Grant	aa	aa	bc	bb	ab	ab	bb
Expected	aa:ab=1:1	aa=1	bb:ab:ac:bc=1:1:1:1	bb:bc=1:1	aa:ab:bb=1:2:1	aa:ab:bb=1:2:1	bb=1
Observed	212:188	400	101:119:82:98	199:201	50:203:147	116:208:76	400
Goodness of fit	1.44 on 1 df	-	6.9 on 3 df	0.01 on 1 df	49.99 on 2 df	9.03 on 2 df	-
Significance level	ns	-	ns	ns	* * *	*	
Keane	ab	aa	ac	cc	ab	bb	ab
Expected	aa:ab:bb=1:2:1	aa=1	aa:ab:ac:bc=1:1:1:1	bc:cc=1:1	aa:ab:bb=1:2:1	ab:bb=1:1	ab:bb=1:1
Observed	98:207:95	400	123:102:94:81	171:229	69:191:140	195:205	193-207
Goodness of fit	0.45 on 2 df	-	9.19 on 3 df	8.44 on 1df	45.42 on 2 df	0.25 on 1 df	0.49 on 1df
Significance level	ns	-	*	* *	* * *	ns	ns

Table 5.1: (Continue)

	AAT-1	GPI-2	IDH	LAP-1	PGM-1	PGM-2	SKDH
Nonpareil	**ab**	**aa**	**ab**	**bc**	**ab**	**ab**	**bb**
Mission	ab	ab	aa	nc	aa	bb	bb
Expected	aa:ab:bb=1:2:1	aa:ab=1:1	aa:ab=1:1	bb:bc:cc+nc=1:1:2	aa:ab=1:1	ab:bb=1:1	bb=1
Observed	104:216:80	199:201	199:201	102:82:216	152:248	178:222	400
Goodness of fit	5.7 on 2 df	0.01 on 1 df	0.01 on 1 df	4.47 on 2 df	23.26 on 1 df	4.84 on 1 df	-
Significance level	ns	ns	ns	ns	* * *	*	-
Ne Plus Ultra	ab	aa	ac	bc	bb	ab	bb
Expected	aa:ab:bb=1:2:1	aa=1	aa:ab:ac:bc=1:1:1:1	bb:bc:cc=1:2:1	ab:bb=1:1	aa:ab:bb=1:2:1	bb=1
Observed	125:198:77	400	115:97:79:109	129:141:130	202:198	99:194:107	400
Goodness of fit	11.55 on 2 df	-	7.77 on 3 df	35.33 on 2 df	0.04 on 1 df	0.67 on 2 df	-
Significance level	* *	-	ns	* * *	ns	ns	-
Peerless	nn	aa	bb	bb	ab	ab	bb
Expected	an:bn=1:1	aa=1	ab:bb=1:1	bb:bc=1:1	aa:ab:bb=1:2:1	aa:ab:bb=1:2:1	bb=1
Observed	198:202	400	218:182	190:210	85:228:87	94:193:113	400
Goodness of fit	0.04 on 1 df	-	3.24 on 1 df	1 on 1 df	7.89 on 2 df	2.24 on 2 df	-
Significance level	ns	-	ns	ns	*	ns	-
Price	aa	ab	ab	nc	aa	bb	bb
Expected	aa:ab=1:1	aa:ab=1:1	aa:ab:bb=1:2:1	bb:bc:cc+nc=1:1:2	aa:ab=1:1	ab:bb=1:1	bb=1
Observed	225:175	198:202	99:205:96	109:92:198	186:214	202:198	400
Goodness of fit	6.26 on 1 df	0.04 on 1 df	0.29 on 2 df	1.3 on 2 df	1.96 on 1 df	0.04 on 1 df	-
Significance level	*	ns	ns	ns	ns	ns	-

Table 5.2 Summary of all possible chi-square tests of association between isozyme loci

The symbols in the table mean:

***	association significant at P<0.001.
*	association significant at P<0.05.
ns	association non-significant.
-	two-way table cannot be formed (at least one loci homozygous).

		AAT-1	GPI-2	IDH	LAP-1	PGM-1	PGM-2
GPI-2	Carmel	-					
	Fritz	ns					
	Grant	-					
	Keane	-					
	Ne Plus	-					
	Mission	ns					
	Peerless	-					
	Price	ns					
IDH	Carmel	***	-				
	Fritz	***	ns				
	Grant	***	-				
	Keane	***	-				
	Ne Plus Ultra	***	-				
	Mission	***	ns				
	Peerless	***	-				
	Price	***	ns				
LAP-1	Carmel	ns	-	ns			
	Fritz	ns	***	ns			
	Grant	ns	-	ns			
	Keane	ns	-	ns			
	Ne Plus Ultra	ns	-	ns			
	Mission	ns	***	ns			
	Peerless	ns	-	ns			
	Price	ns	***	ns			
PGM-1	Carmel	ns	-	*	ns		
	Fritz	ns	ns	ns	ns		
	Grant	ns	-	ns	ns		
	Keane	ns	-	ns	ns		
	Ne Plus Ultra	ns	-	ns	ns		
	Mission	ns	ns	ns	ns		
	Peerless	ns	-	ns	ns		
	Price	ns	ns	ns	ns		
PGM-2	Carmel	ns	-	ns	***	ns	
	Fritz	ns	ns	ns	***	ns	
	Grant	ns	-	ns	***	ns	
	Keane	ns	-	ns	***	ns	
	Ne Plus Ultra	ns	-	ns	***	ns	
	Mission	ns	ns	ns	***	ns	
	Peerless	ns	-	ns	***	ns	
	Price	ns	ns	ns	***	ns	
SKDH	Carmel	-	-	-	-	-	-
	Fritz	-	-	-	-	-	-
	Grant	-	-	-	-	-	-
	Keane	ns	-	ns	ns	ns	ns
	Ne Plus Ultra	-	-	-	-	-	-
	Mission	-	-	-	-	-	-
	Peerless	-	-	-	-	-	-
	Price	-	-	-	-	-	-

Table 5.3 Summary of observed (obs) and expected (exp) frequencies for Nonpareil crossed with 8 different pollen sources

(i) AAT-1 & IDH

Pollen source		obs	exp	obs	exp	obs	exp	obs	exp
		IDH							
Carmel	AAT-1	aa		ab		bb			
	aa	**10**	30.04	**61**	69.53	**64**	35.44		
	ab	**35**	41.61	**117**	96.31	**35**	49.09		
	bb	**44**	17.35	**28**	40.17	**6**	20.48		
Fritz		aa		ab		bb			
	aa+an	**28**	47.08	**104**	114.43	**87**	57.49		
	ab	**49**	21.93	**45**	53.29	**8**	26.77		
	bb	**9**	16.99	**60**	41.28	**10**	20.74		
Grant		ab		bb		ac		bc	
	aa	**41**	63.07	**78**	53.53	**22**	43.46	**71**	51.94
	ab	**78**	55.93	**23**	47.47	**60**	38.54	**27**	46.06
Keane		aa		ab		ac		bc	
	aa	**10**	30.14	**20**	25.14	**16**	22.30	**52**	20.33
	ab	**53**	63.35	**66**	53.05	**60**	46.87	**27**	42.74
	bb	**60**	29.52	**17**	24.72	**15**	21.84	**4**	19.92
Mission		aa		ab					
	aa	**32**	51.24	**71**	51.76				
	ab	**112**	107.96	**105**	109.04				
	bb	**55**	39.80	**25**	40.20				
Ne Plus Ultra		aa		ab		ac		bc	
	aa	**21**	35.94	**18**	30.31	**20**	24.69	**66**	34.06
	ab	**45**	37.21	**66**	48.26	**49**	39.30	**39**	54.23
	bb	**49**	21.85	**13**	18.43	**10**	15.01	**4**	20.71
Peerless		ab		bb					
	an	**53**	107.91	**145**	90.09				
	bn	**165**	110.09	**37**	91.91				
Price		aa		ab		bb			
	aa	**27**	55.69	**116**	114.75	**82**	54.56		
	ab	**72**	43.31	**88**	89.25	**15**	42.44		

Table 5.3 Summary of observed (obs) and expected (exp) frequencies values for Nonpareil crossed with 8 different pollen sources

(ii) LAP-1 & PGM-2

Pollen Source		obs	exp	obs	exp	obs	exp
		PGM-2					
Carmel	LAP-1	ab		bb			
	bb	**80**	44.18	**14**	49.82		
	bc	**72**	43.24	**20**	48.76		
	cc+cn	**36**	100.58	**178**	113.42		
Fritz		ab		bb			
	bb+bn	**136**	82.94	**35**	88.06		
	bc	**25**	53.83	**86**	57.17		
	cc	**33**	57.23	**85**	60.77		
Grant		aa		ab		bb	
	bb	**97**	57.71	**83**	102.98	**19**	38.31
	bc	**19**	58.29	**124**	104.02	**58**	38.69
Keane		ab		bb			
	bc	**123**	83.36	**54**	100.26		
	cc	**67**	111.64	**144**	97.74		
Mission		ab		bb			
	bb	**87**	56.61	**15**	45.39		
	bc	**70**	45.51	**12**	36.49		
	cc+cn	**65**	119.88	**151**	96.12		
Ne Plus Ultra	LAP-1	aa		ab		bb	
	bb	**74**	31.94	**46**	62.56	**9**	34.51
	bc	**12**	34.65	**93**	67.90	**35**	37.45
	cc	**13**	32.42	**55**	63.53	**63**	35.04
Peerless		aa		ab		bb	
	bb	**69**	44.65	**90**	91.20	**31**	54.15
	bc	**25**	49.35	**102**	100.8	**83**	59.85
Price		ab		bb			
	bb	**86**	54.77	**23**	54.23		
	bc	**68**	46.23	**24**	45.77		
	cc+cn	**47**	100.00	**152**	99.00		

Table 5.3 Summary of observed (obs) and expected (exp) frequencies for Nonpareil crossed with 3 different pollen sources

(iii) LAP-1 & GPI-2

Pollen Source Fritz	LAP-1	obs GPI-2 aa	exp	obs ab	exp
	bb+bn	**82**	96.61	**89**	74.38
	bc	**101**	62.72	**10**	48.28
	cc	**43**	66.67	**75**	51.33
Mission		aa		ab	
	bb	**16**	50.49	**86**	51.51
	bc	**65**	40.59	**17**	41.41
	cc+cn	**117**	106.92	**99**	109.08
Price		aa		ab	
	bb	**11**	53.96	**98**	55.04
	bc	**77**	45.54	**15**	46.46
	cc+cn	**110**	98.50	**89**	100.49

Table 5.4 Deduced parental type showing linkage of alleles for (i) AAT-1 and IDH; (ii) LAP-1 and PGM-2; and (iii) LAP-1 and GPI-2

Cultivar	Isozyme linked groups		
Cultivar	**AAT-1 & IDH**	**LAP-1 & PGM-2**	**LAP-1 & GPI-2**
Nonpareil	a b' / b a'	b a' / c b'	b a' / c a'
Carmel	a b' / b a'	c b' / n b'	
Fritz	a a' / n b'	b b' / n b'	b a' / n b'
Grant	a b' / a c'	b a' / b b'	
Keane	a c' / b a'	c b' / c b'	
Mission	a a' / b a'	c b' / n b'	c a' / n b'
Ne Plus Ultra	a c' / b a'	b a' / c b'	
Peerless	n b' / n b'	b a' / b b'	
Price	a a' / a b'	c b' / n b'	c a' / n b'

Chapter 6

Isozyme diversity in Iranian almond populations

Chapter 6

Isozyme diversity in Iranian almond populations

6.1 Summary

Isozymes have been used as genetic markers to evaluate gene diversity in several cultivated genotypes and seven wild species. Six isozyme systems namely, AAT, GPI, LAP, MDH, PGM and SKDH were used. Isozyme analysis of embryos showed that there is a high level of gene diversity between genotypes and between individuals collected from different parts of Iran. These six isozyme systems showed some new alleles, which are reported for the first time in the almond.

Gene diversity in cultivated genotypes collected from the northern parts of Iran was greater than that in cultivated genotypes from southern provinces. Gene diversity in cultivated almond was greater than in the wild species; among wild species *P. lysioides* and *P. scoparia* had the highest polymorphism possibly due to the wide distribution of these species.

6.2 Introduction

Almond *(Prunus dulcis D.A. Webb. Syn P. amygdalus Batsch.)* is a self-incompatible species with considerable genetic variability. Isozyme markers have been used as a tool for identification of species and cultivars. The study of isozymes for cultivar identification has been carried out in many horticultural crops such as grape (Stavrakakis and Loukas, 1983), apple (Manganaris and Alston, 1987 and 1992), avocado (Torres *et al.*, 1986), citrus (Torres *et al.*, 1985), Macademia (Vitanage and Winks, 1992), peach (Arulsekar *et al.*, 1986b) and cherry (Santi and Lemoine, 1990; Granger *et al.*, 1993). In almond Arulsekar *et al.* (1986b) reported polymorphism in AAT-1, PGM, MDH and 6PGD. Hauagge *et al.*, (1987b) reported the polymorphisim of four enzyme sytstems LAP, GPI, PGM and AAT in almond leaves, and determined the inheritance of these enzymes in almond and almond x peach hybrids. Chaparro *et al.* (1987) used two enzyme systems PGM and 6PGD for identification of peach x almond hybrids. Mowery *et al.* (1990) described

seventeen isozyme systems in clones of almond, and three wild species sub members of the sub genus *Amygdalus*. Jackson and Clarke (1991) used seven enzyme systems to study gene flow in an almond orchard. Australian almond cultivars have been characterized genetically and compared with Californian varieties by (Vezvaei *et al.*, 1994).

Iranian almond genotypes and wild species have not been characterized genetically, although Khatamsaz (1992) used morphological characters to identify wild species of almond in Iran. The aim of this work is to evaluate isozyme diversity in some Iranian cultivated genotypes and species collected from different parts of Iran.

6.3 Materials and methods

6.3.1 Geography and climate of Iran

Iran is situated in south western Asia with an area of 1,647,063 square kilometres. It is bordered on the north by the Soviet Union and separated into two sections by the Caspian sea. On the east, Iran is bordered by Afghanistan and Pakistan. On the west by Iraq and Turkey.

Iran has three types of terrain. The centre of the country is a vast, high plain, or plateau. Surrounding this plateau is a ring of mountains along the shore of the Caspian sea in the north and along the southern coast. The central plateau of Iran is one of the driest, most desolate regions on earth. It is not, however, flat and featureless. Its average height is about 1,200 meters above sea level and the plateau is covered with low, rounded hills with wide, sloping sides. In the east and south of Tehran (the capital) is a large desert called the Dasht-e-Kavir (Great Salt Desert). Unlike other deserts around the world, the Dasht-e-Kavir consists of neither sand nor gravel. Instead, it is a crust, or in some spots a 'paste', of salt. The soil in this region is extremely salty, and in winter occasional rains or flood waters from the mountains create a swampland and dissolve the salt in the soil. The southern and eastern parts of the plateau are the driest and emptiest. Southeast of the Dasht-e-Kavir lies another large desert, The Dasht-e-Lut, in the centre of a vast depression. The Dasht-e-Lut is a sand desert, bare of vegetation, covered with rank after rank of peaked dunes, and torn by winds and sandstorms. The rim of mountains that surrounds the central plateau covers about half

of Iran's total area. It consists of three major ranges: The Zagros Mountains in the west, the Elburz Mountains in the north, and the Khorasan mountains in the east. In the south and southeast, smaller mountain ranges and cliffs form the edge of the plateau.

Probably the single most important fact about Iran's climate is that it is dry. Although the country has many lakes, streams, and rivers, most of them are shallow and dry up during summer. Most of Iran receives little rainfall. The deserts and the southern coast average about 13 centimetres a year. But although rain seldom falls along the southern coast, parts of this area, especially along the Persian Gulf, are extremely humid. The rest of the central plateau, including Tehran, gets a little more rain an average of about 30 centimetres a year. Summer is the driest time; at least half of all the rainfall in Iran occurs during the winter months. At the highest altitudes, precipitation takes the form of snow. Water from the melted snow is a major part of Iran's water supply during the hot, dry summer. Azerbaijan, in the northwest, is the country's coldest region. In winter, temperatures can drop to as low as -37° C. Khuzistan, in the southwest, is the hottest region, with summer temperature reaching 55° C. Tehran's temperatures range from -3° C to 7° C in January, the coldest month, and from 22° C to 37° C in July, the hottest month. One other important aspect of Iran's weather is wind. The high humidity at the coast of the Persian Gulf is caused by warm, damp winds that blow across the Gulf from Arabia. The low temperature of Azerbaijan and Khorasan in winter are caused by fiercely cold, dry winds blowing down across central Asia from Siberia.

6.3.2 Distribution of cultivated almond and wild species in Iran

Almonds are grown normally in regions with a Mediterranean climate; the major almond production areas in Iran are located in north west 'Azarbaijan', north east 'Khorassan', Central province; 'Ghom', 'Kashan', 'Tafresh', 'Saveh' and in the south 'Esfhan', 'Yazd', 'Kerman' and 'Shiraz'. Almond production is restricted in the north to regions near the Caspian sea, in the central desert and near the Persian Gulf. More than 20 wild species normally grow in these areas as well as in the Zagros, Alborz and Khorasan mountains.

6.3.3 Collection of almonds nuts and isozyme analysis

Nut samples were collected in different parts of Iran and passed through Australian quarantine to check for pests and diseases. From the 2941 samples, the first fifty embryos were sown in a quarantine growth cabinet in 1990 and checked for viral diseases. Twenty five plants showed symptoms of *Prunus ring spot virus* and were discarded. The plants remaining were used for leaf analysis. Embryos were analysed for six isozyme markers namely, AAT-1, GPI-2, IDH, PGM 1 & PGM-2 SKDH and MDH. Because of the lack of polymorphism in MDH isozyme the data is not shown for this enzyme system. Isozyme analysis was carried out according to the methods described in chapter 2.2.

6.4 Results

6.4.1 Isozyme diversity in cultivated genotypes in different parts of Iran

The inferred genotype at seven isozyme loci for 25 almonds collected in Iran is shown in Table 6.1. Analysis of embryos collected from different parts of Iran showed that there is a high level of isozyme diversity between the different locations and genotypes. Gene diversity in the cultivated genotypes of northern Iran was greater than gene diversity of those from the southern provinces. Gene diversity in cultivated genotypes was greater than that for wild species. The banding patterns for the different enzyme systems and diversity of isozymes for cultivated and wild species are shown in Figure 6.1 and Tables 6.2 to 6.6. The wild species *P. lycioides* and *P. scoparia* showed the greatest polymorphisms possibly due to the wide distribution of these two species.

6.4.2 Isocitrate dehydrogenase (IDH)

Variability of isozyme banding patterns was found in IDH for haploid materials as reported by Cerezo *et al.* (1989). Jackson and Clarke (1991) used haploid and diploid materials for isozyme analysis, and found five combinations of alleles in Australian and Californian almonds (aa, ab, bb, ac, bc). Some new alleles are reported for Iranian almond here for the first time (cc and cd). These alleles were observed in samples collected from the Azarbaijan and Khorasan provinces only. Wild species are recognised by a large number of homozygous 'aa' or 'bb' alleles; only a few samples showed 'ac' and 'bc' alleles in *P.*

Lycioides and *P. scoparia* respectively. IDH showed the highest degree of polymorphism, having seven different combinations of alleles with two zones of activity. A faint anodal and a strong cathodal band were also seen, the migration distance being between 40 and 50 mm from the loading point (Fig. 6.1).

6.4.3 Leucine amino peptidase (LAP)

In cultivated almonds three genotypes (bb, bc and cc) have been described previously for this locus (Arulsekar *et al.*, 1986b; Hauagge *et al.*, 1987b; Jackson and Clarke, 1991; Vezvaei *et al.*, 1994). In the Iranian cultivated almond populations for the northern provinces 'ab' alleles are now reported for the first time, although in wild species only 'bb', 'bc' and 'cc' allele combinations were observed and only 'ab' alleles was observed in *P. lycioides* (Fig. 6.4). The migration distance was between 40 and 60 mm from the loading point.

6.4.4 Asparatate amino transferase (AAT)

AAT has a dimer structure in almond and was detected at two loci (Hauagge *et al.*, 1987b). In Australian and Californian almond cultivars at AAT-2 locus the band near the loading point was found to be monomorphic, but in the Iranian almond population this locus was polymorphic. AAT-1 is normally polymorphic in almond. Three different allele conbinations were observed in Californian and Australian cultivars, namely 'aa', 'ab' and 'bb'. In the Iranian almond population four new allele combinations (ff, fa, bd and dd) were recorded for the first time in almond, these new alleles were found in samples collected from the Azarbaijan, Khorasan and central provinces. In wild species further new alleles were observed (Figs. 6.4 and 6.5), but the variability of these alleles in *P. lysioides* and *P. scoparia* were greater than for other wild species in the populations studied.

6.4.5 Glucose phospho isomerase (GPI)

This enzyme showed only one zone of activity. Australian cultivars showed three different allele combinations 'fa', 'aa' and 'ab' Iranian cultivars showed three more alleles (ff, ac and bb) all of them reported now for the first time (Figs. 6.2 and 6.3). Only 'bb' is

found in the wild species (Fig. 6.4). In this enzyme the migration distance of the zone of activity from the loading point was between 40 and 60 mm.

6.4.6 Phosphoglucomutase (PGM)

Two zones of activity (loci) are present in almond for PGM (Arulsekar *et al.*, 1986b; Chaparro *et al.*, 1987 and Hauagee *et al.*, 1987b). PGM is a monomeric enzyme with two loci in almond. PGM-1 and PGM-2 are both polymorphic, PGM-1 shows three allele combinations 'aa', 'ab' and 'bb' in cultivated and wild species, always the frequency of 'aa' alleles is lower than that of other alleles in cultivars and wild species. In PGM-2 three combinations, 'aa', 'ab' and 'bb' were detected in Australian cultivars but in Iranian cultivars and species studied four more allelic combinations were found namely 'fa', 'ac', 'bc', 'fb' and 'fc' (Figs. 6.2 to 6.5).

6.4.7 Shikimate dehydrogenase (SKDH)

SKDH is a monomeric enzyme encoded by a single locus in almond (Mowery *et al.*, 1990). Most of the Australian and Californian cultivars are 'bb' homozygous and a few 'ab' heterozygous. Iranian almonds showed a high percentage of homozygous 'bb' in comparison with *ab* heterozygous, while a few samples of the Azarbaijan population and *P.reticulata* showed 'aa' for SKDH.

6.5 Discussion

Isozyme variability in almond is generally higher than that for other *Prunus* species (Byrne, 1990). Byrne suggests that this is due primarily to its allogamous nature. Among the various world almond populations, the European population had a higher level of isozyme variability than did the Californian. In the Iranian almond population, it is not surprising that more alleles were seen since the prevalent sexual propagation of almond for horticultural purposes in Iran should result in a higher level of diversity. Natural hybridization of cultivated almonds with the relatively large population of wild species (20) might be another reason for high diversity of alleles in Iranian almond populations. Thus the variability found in almond is associated with its breeding behaviour and the broadness of

the germ plasm sampled. The higher heterozygosity per locus in almond is consistent with its self-incompatibility (Layne and Sherman, 1986). The variability of banding patterns showed that IDH, AAT-1, LAP and PGM-2 are more variable in almond than in other isozymes studied.

Often the level of variability in cultivated angiosperms is higher than that in uncultivated angiosperms (Byrne, 1990). The present studies are consistent with Byrne's generalization, except for *P. lysioides* and *P. scopria*. In the latter we found more variability, especially at the PGM-2, LAP-1 and AAT-1 loci.

In other studies of cultivated and wild plants of the same or closely related species, in comparison with cultivated populations, there have been natural populations having more variability (*e.g.Raphanus*), less variability (*e.g.Cucurbita*), and the same isozymic variability (*e.g.Camelia* and *maize*) (Brown, 1978; Decker and Wilson, 1987; Ellstrand and Marshall, 1985; Wendel and Parks, 1983). More work needs to be carried out to clarify the reasons for these contrasting situations.

It is concluded that isozyme markers can be used as a powerful tool for identification of almond species and cultivars and also for population studies. This is the first approach to the study of diversity of isozyme markers in Iranian almond populations, further work is needed.

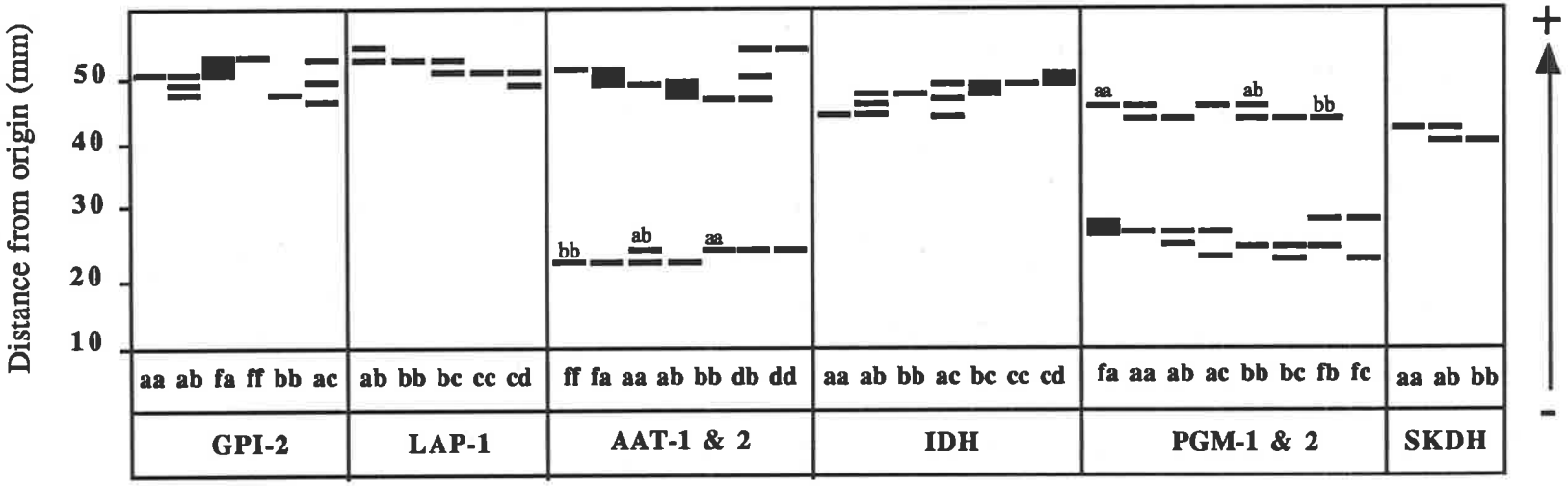


Fig. 6.1 Diagrammatic illustration of GPI, LAP, AAT, IDH, PGM and SKDH banding patterns accompanied by the allelic and phenotypic designation of Iranian almond populations.

Fig. 6.2 Zymogram of banding patterns for almond collected from Shahrood province, Iran.

A-1 (Shahrood 1)

A-2 (Shahrood 13)

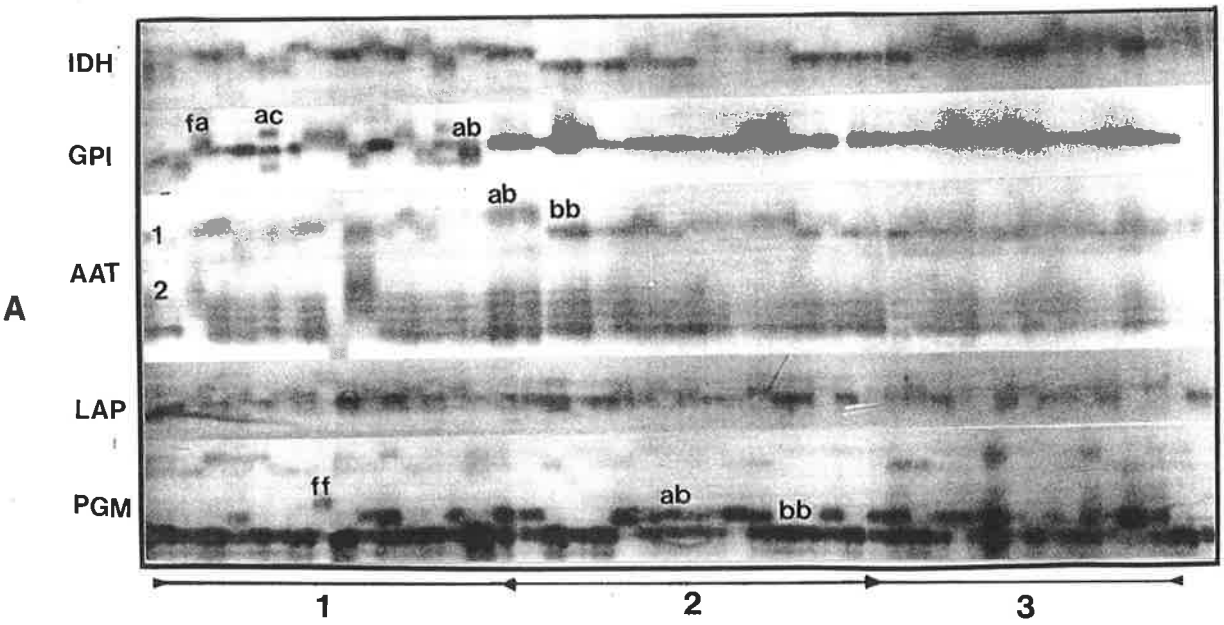
A-3 (Shahrood 11)

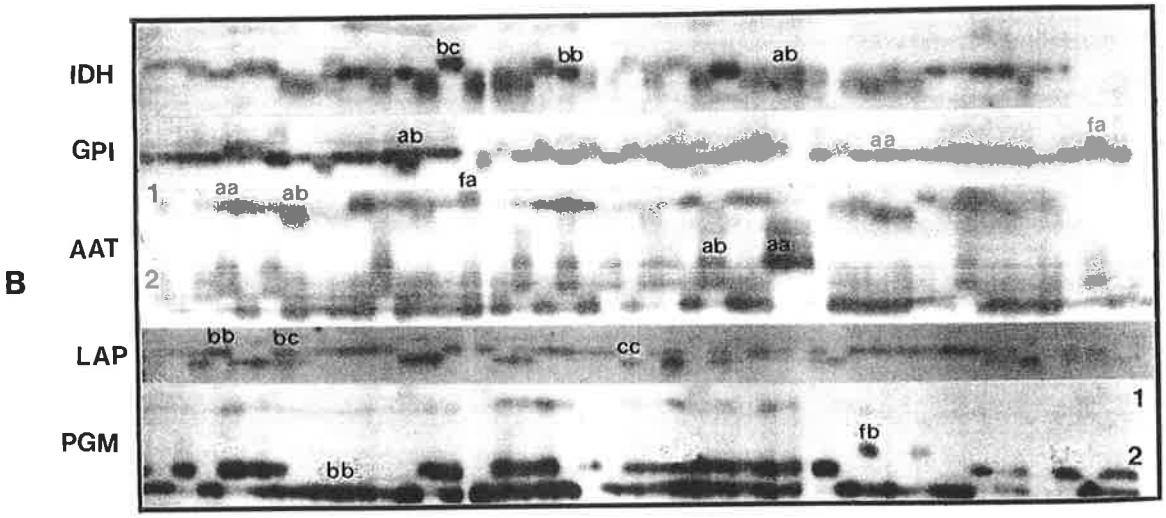
B (Shahrood Province)

C-1 (Adli 11)

C-2 (Bitter 25)

C-3 (No.3)


A
IDH
GPI
AAT
LAP
PGM
fa
ac
ab
ab
bb
1
2
ff
ab
bb
1
2
3


B
IDH
GPI
AAT
LAP
PGM
bc
bb
ab
ab
aa
fa
1
aa
ab
fa
2
ab
aa
bb
bc
cc
1
fb
bb
2

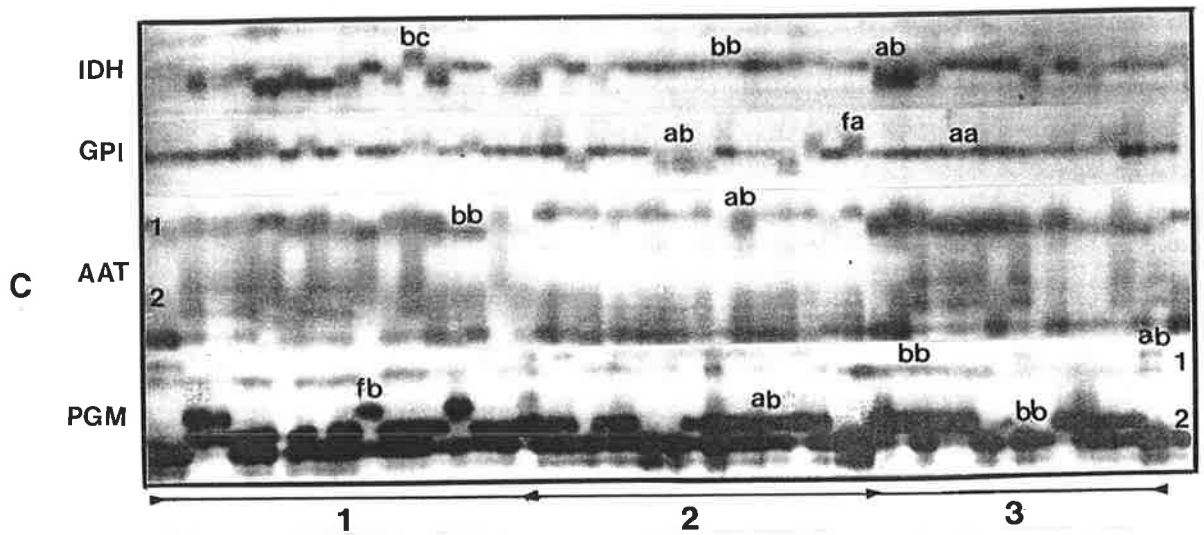

C
IDH
GPI
AAT
PGM
bc
bb
ab
ab
fa
aa
1
bb
ab
2
bb
ab
1
fb
ab
bb
2
1
2
3

Fig. 6.3 Zymogram of banding patterns in almond populations in 3 different provinces of Iran.

A (Azarbijan)

A-1 (AH 3)

A-2 (AH 25)

A-3 (AH 50)

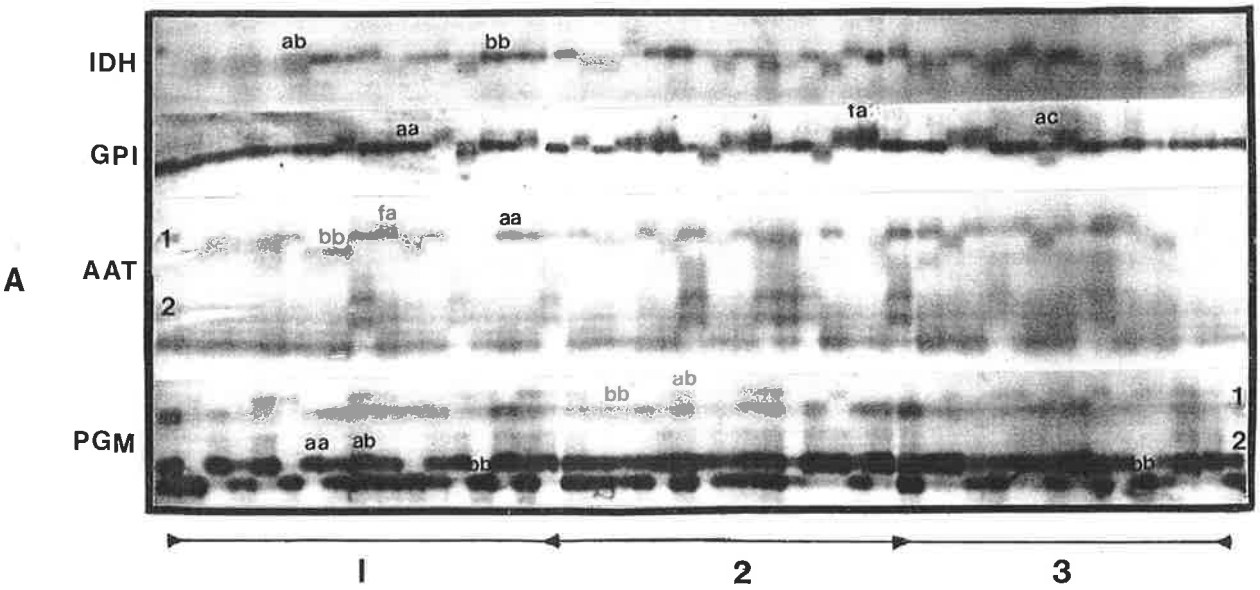
B (Shahrood and Ghom provinces)

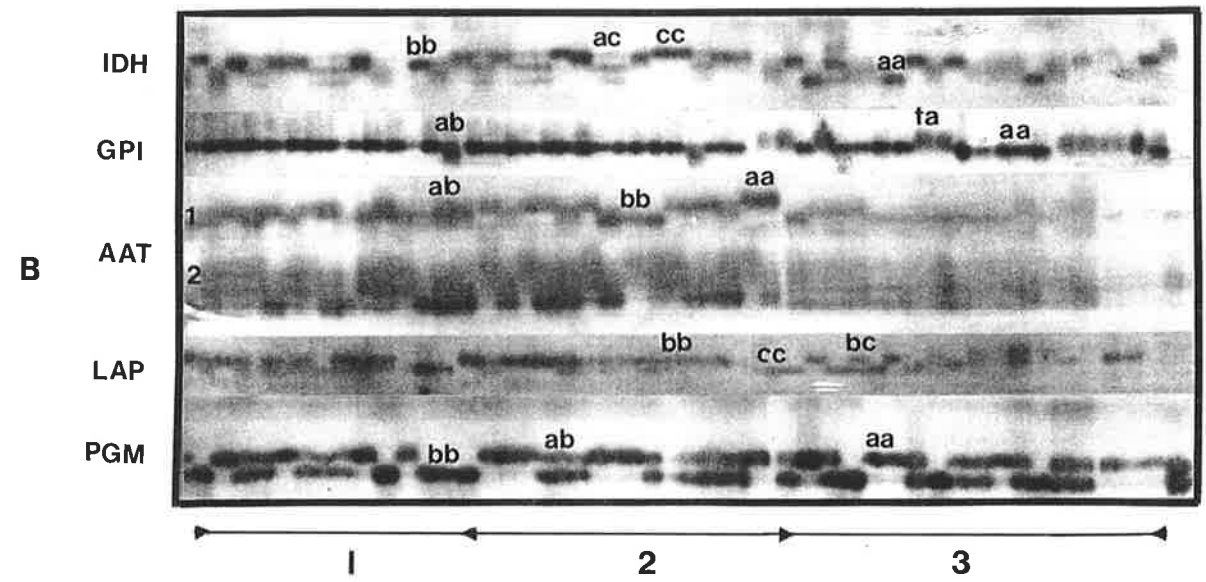
B-1 (Shahrood 12)

B-2 (Shahrood 8)

B-3 (Ghom 24)

C (Shemiran Population)


A
IDH
GPI
AAT
PGM
ab
bb
aa
fa
ac
1
2
1
2
3


B
IDH
GPI
AAT
LAP
PGM
bb
ac
cc
aa
ab
fa
1
2
bc
1
2
3

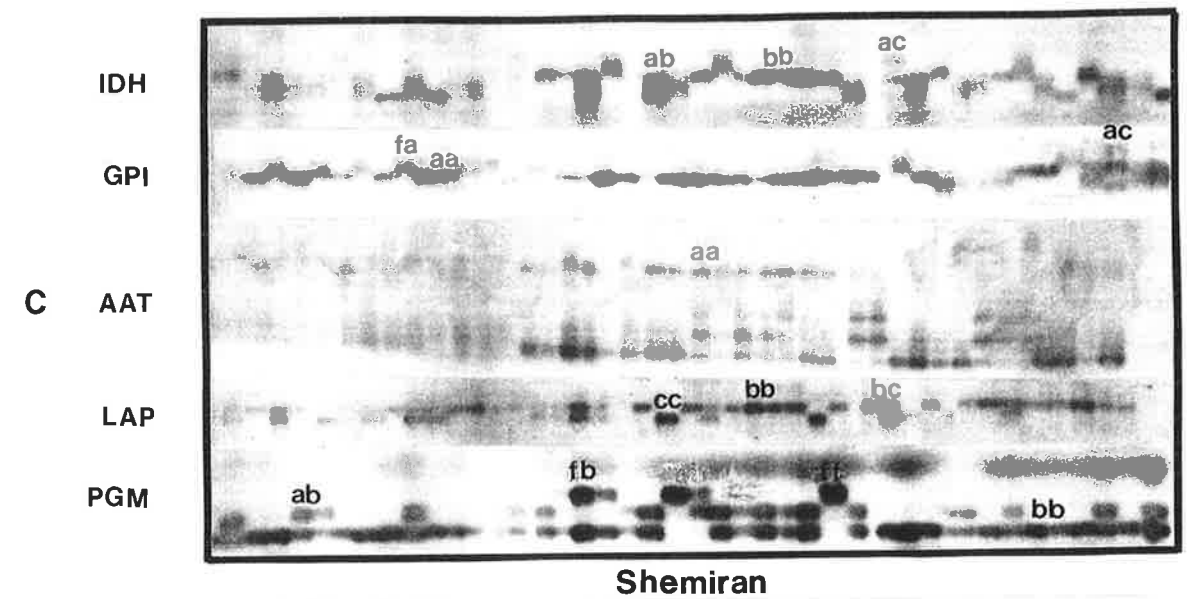

C
IDH
GPI
AAT
LAP
PGM
ab
bb
ac
fa
aa
cc
fb
Shemiran

Fig. 6.4 Zymogram of banding patterns of 4 wild almond species from Iran and Waite collection.

A-1 *(P. lycioides)*

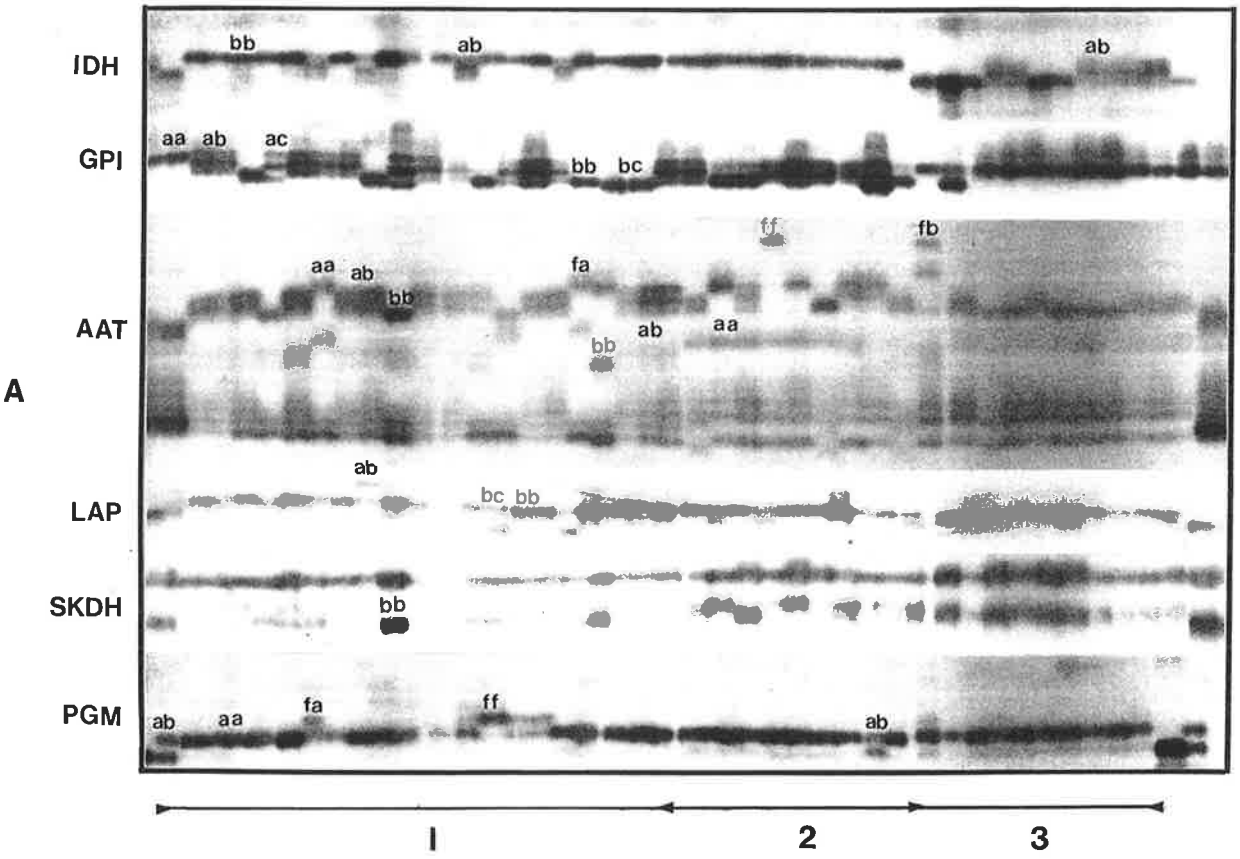
A-2 *(P. scoparia)*

A-3 *(P.eleagnifolia)*

B-1 *(P.webbii [leaf])*, collected from Waite arboretum, Adelaide.

B-2 *(P. webbii [embryo])*, collected from Waite arboretum, Adelaide.

B-3 *(P. eleagnifolia)*


A
IDH
GPI
AAT
LAP
SKDH
PGM
bb
ab
aa ab ac
bb bc
ff
fb
aa ab
bb
fa
ab
aa
bb
ab
bc bb
bb
ab aa
fa
ff
ab
1
2
3

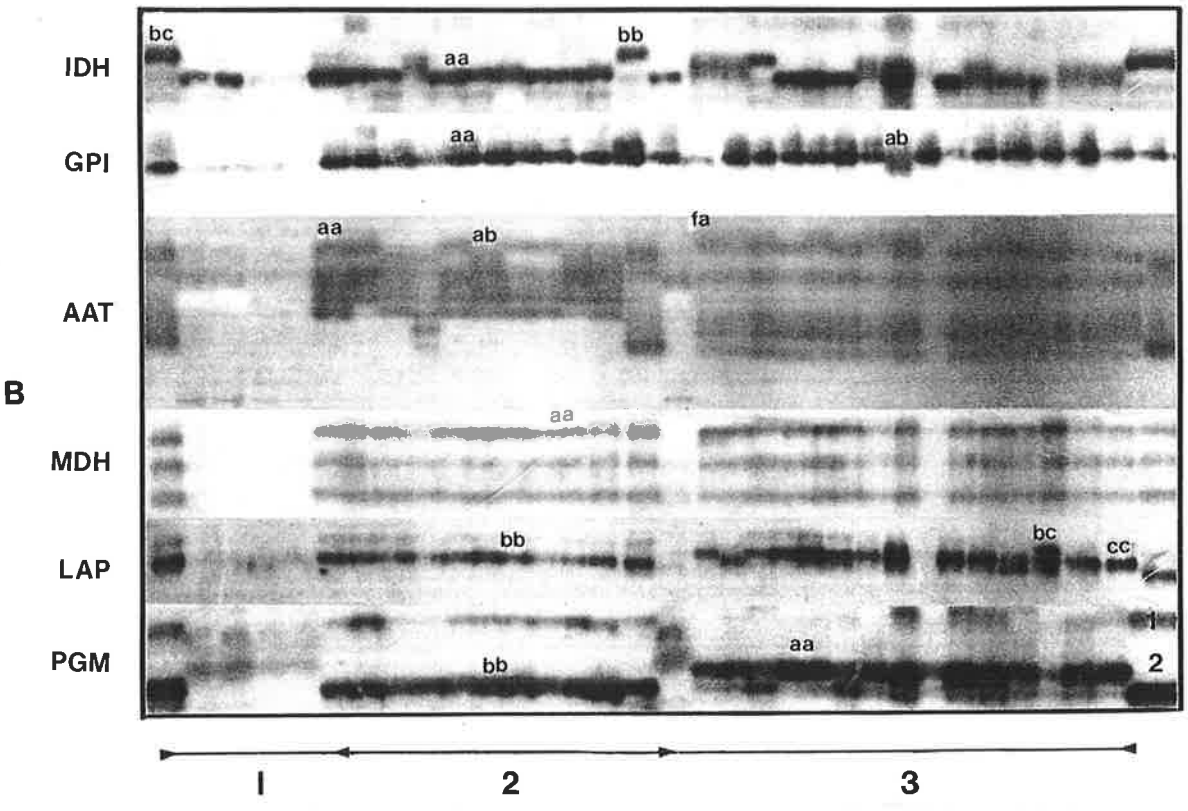

B
IDH
GPI
AAT
MDH
LAP
PGM
bc
aa
bb
aa
ab
aa
ab
fa
aa
bb
bc
cc
aa
bb
1
2
1
2
3

Fig. 6.5 Zymogram of 6 isozyme systems in 4 wild almond species.

A (*P. lycioides*)

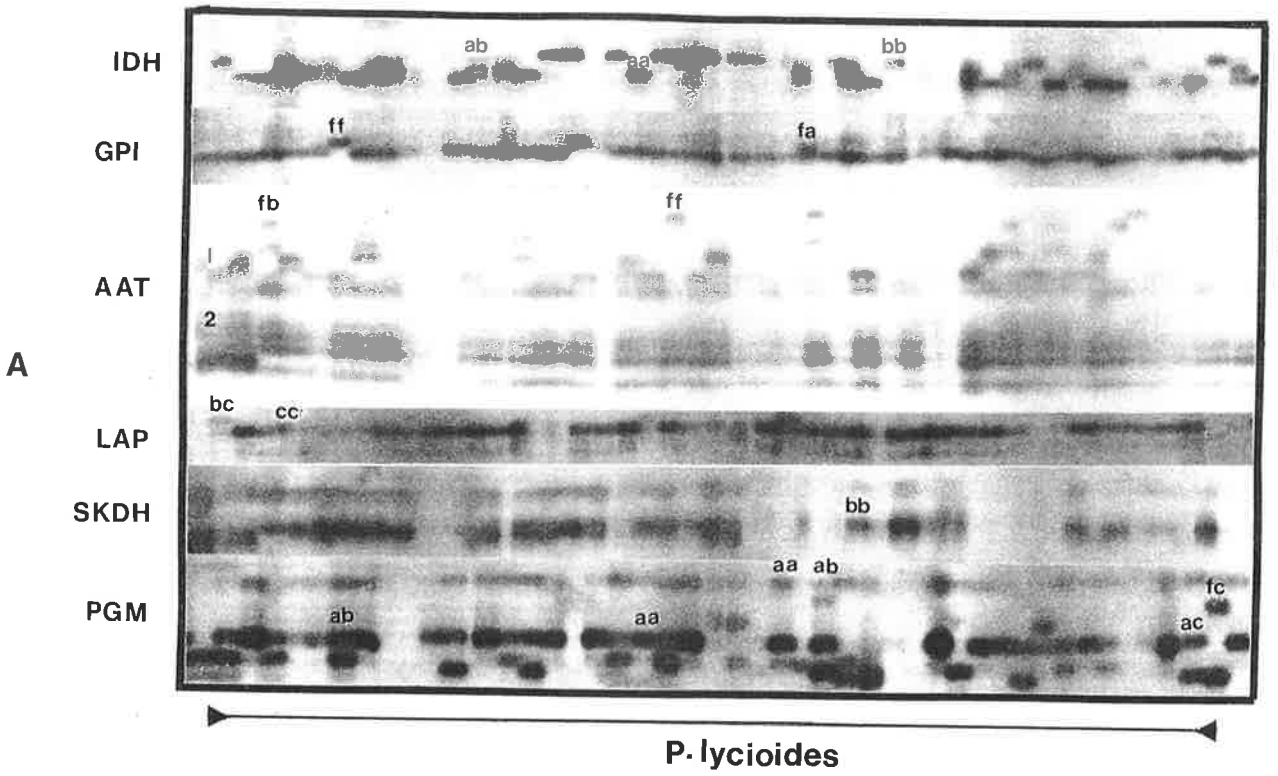
B-1 (*P. scoparia*)

B-2 (*P. eleagnifolia*)

B-3 (*P. lycioides*)

B-4 (*P. reticulata*)

B-5 (*P. scoparia*)


A
IDH
GPI
AAT
LAP
SKDH
PGM
ab
aa
bb
ff
fa
fb
ff
1
2
bc
cc
bb
aa ab
fc
ab
aa
ac
P. lycioides

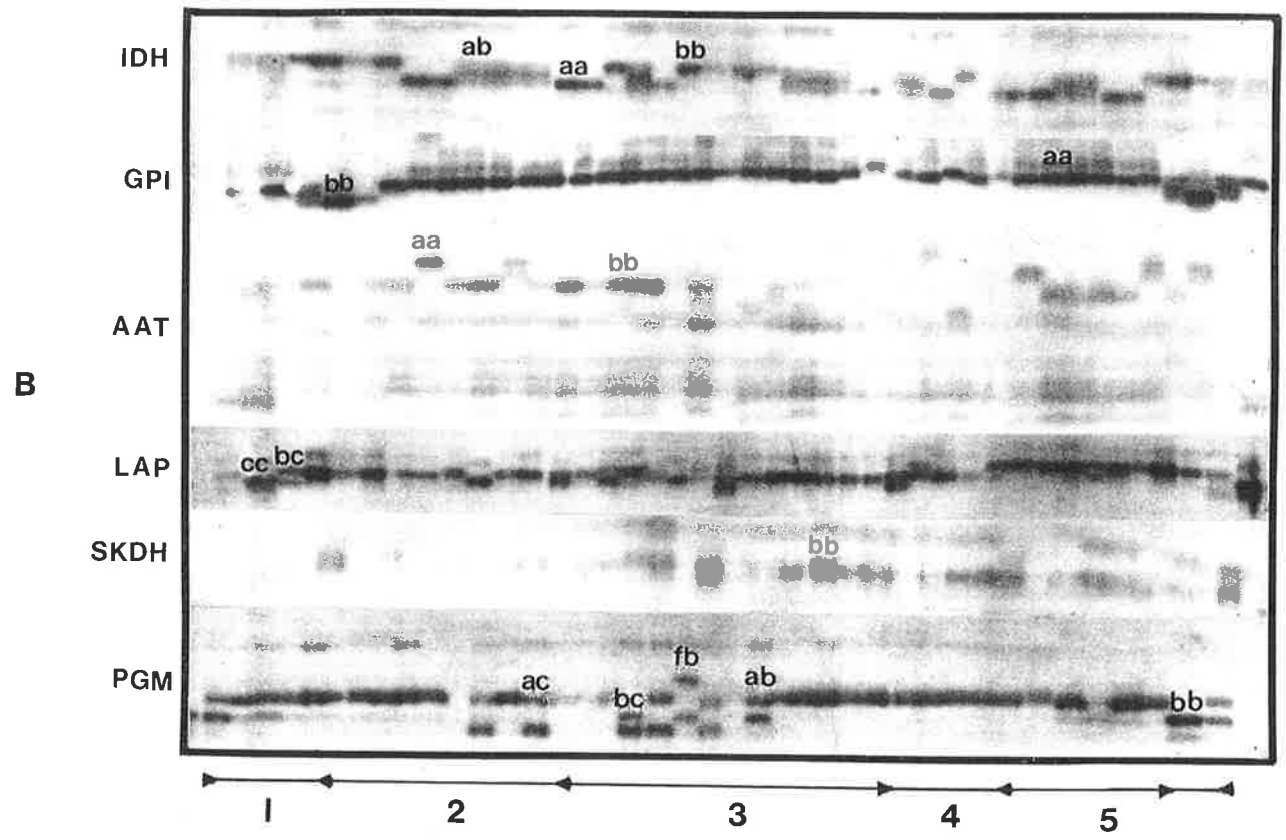

B
IDH
GPI
AAT
LAP
SKDH
PGM
ab
aa
bb
bb
aa
aa
bb
cc bc
bb
fb
ac
bc
ab
bb
1
2
3
4
5

Table 6.1 Genotype designation at 7 isozyme loci for 25 Iranian almonds

Genotype	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
Shemiran 20	aa	bb	bb	bb	bb	bb	bb
Shemiran 20	aa	bc	bb	ab	bb	bb	bb
Shemiran 20	fa	bc	ab	bb	bb	ab	bb
Shemiran 20	aa	bc	aa	bb	bb	bb	bb
Shemiran 20	aa	bc	ab	ab	bb	bb	bb
Shemiran 20	aa	bb	ab	bb	bb	ab	bb
Shemiran 20	aa	bb	bb	bb	bb	ab	bb
Shemiran 20	fa	bc	bb	bb	bb	ab	bb
Kashan 20	aa	bb	nn	nn	nn	nn	bb
Shemiran 85	aa	bc	bb	bb	bb	bb	bb
Shemiran 85	aa	bb	bb	bb	bb	bb	bb
Shemiran 85	aa	bb	bb	bb	bb	ab	bb
Shemiran 25	aa	bb	bb	aa	bb	ab	bb
Shemiran 25	aa	bb	bb	aa	bb	bb	bb
Shemiran 25	aa	bb	ab	aa	bb	ab	bb
Shemiran 25	aa	bb	bb	aa	bb	bb	bb
Shemiran 40	fa	cc	bb	bb	bb	aa	bb
Shemiran 40	aa	bb	ab	ab	bb	aa	bb
Shemiran 20	aa	bb	bb	aa	ab	ab	bb
Shemiran 6	aa	bb	ab	bb	bb	ab	bb
Shemiran 6	aa	bb	ab	aa	bb	bb	bb
Shemiran 6	aa	bb	ab	aa	bb	bb	bb
Shemiran 20	aa	bb	bb	aa	ab	bb	aa
Shemiran 20	aa	bb	bb	ab	ab	bb	bb
Shemiran 20	fa	bb	ab	aa	bb	bb	bb

Table 6.2 Frequency of isozyme alleles in Iranian almond populations (Azarbijan province)

*. New alleles for almond

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
50	AH 3	fa : 9 aa : 38 ab : 3	bb : 12 bc : 26 cc : 12	fa* : 3 aa : 26 ab : 15 bb : 6	ab : 21 bb : 23 bc : 6	aa : 6 ab : 25 bb : 19	aa : 19 ab : 21 bb : 10	bb : 50
35	AH 25	fa : 7 aa : 26 ab : 2	bb : 2 bc : 14 cc : 17 - : 2	fa* : 8 aa : 15 ab : 4 bb : 4 db* : 2	ab : 19 ac : 1 ab : 19 bb : 4 bc : 6	aa : 5 ab : 20 bb : 10	aa : 18 ab : 12 bb : 5	bb : 25 ab : 10
20	AH 46	aa : 20	bb : 4 bc : 16	bb : 20	bb : 20	bb : 20	bb : 10 ab : 10	bb : 20
35	AH 50	fa : 17 aa : 14 ab : 4	bb : 7 bc : 16 cc : 12	fa* : 2 aa : 13 ab : 20	ab : 15 bb : 15 bc : 7	aa : 8 ab : 12 bb : 15	aa : 10 ab : 25	aa : 2 ab : 7 bb : 26
25	Monago	fa : 5 aa : 20	bb : 5 bc : 15 cc : 5	aa : 3 ab : 21 bb : 1	aa : 3 ab : 9 ac : 1 bb : 4	bb : 19 ab : 6	aa : 7 ab : 18	bb : 25
50	Falagh	fa : 20 aa : 30	bb : 32 bc : 16 cc : 2	fa* : 4 aa : 14 ab : 30 bb : 2	aa : 8 ab : 14 ac : 10 bc : 8 bb : 10	aa : 2 ab : 4 bb : 44	aa : 14 ab : 30 bb : 6	bb : 50
50	Kaghasi doposteh	fa : 20 aa : 30	bb : 26 bc : 8 cc : 14	fa* : 2 aa : 22 ab : 17 bb : 9	aa : 4 ab : 26 ac: 4 bb : 16	aa : 2 ab : 21 bb : 27	aa : 12 ab : 28 bb : 6	bb : 50
20	Badam neime sangi	aa : 20	bb : 8 bc : 8 cc : 2	aa : 2 ab : 18	ab : 12 ac : 6 bb: 2	bb : 4 ab : 16	aa : 4 ab : 8 bb : 8	bb : 20
20	No. 62	fa : 6 aa : 14	bc : 6 cc : 14	ab : 10 bb : 10	ac : 6 bc : 14	bb : 12 ab : 8	aa : 16 ab : 4	aa : 4 ab : 4 bb : 12

Table 6.2 (Continue)

*. New alleles for almond

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
40	Badam dastmaly	fa : 3 aa : 34 ab : 3	bb : 16 bc : 18 cc : 6	aa : 16 ab : 6 db* : 12 bb : 6	aa : 4 ab : 12 bb: 18 bc : 6	aa : 4 ab : 14 bb : 20	aa : 14 ab : 24 bb : 2	ab : 4 bb : 36
20	Badam sangy draz	fa : 2 aa : 12 ab : 6	bb : 4 bc : 12 cc : 3 - : 1	ab : 16 bb : 4	bb : 18 ab : 2	bb : 18 ab : 2	aa : 5 ab : 8 bb : 6 - : 1	ab : 8 bb : 12
20	Badam sangy gerd	fa : 2 aa : 11 ab : 7	bc : 8 cc : 12	ab : 4 bb : 3 db* : 13	ab : 4 bb : 6 bc : 6 cc : 4	aa : 4 ab : 8 bb : 8	bb : 15 ab : 5	bb : 20
20	Badam kaghazi	aa : 20	bb : 2 bc : 15 cc : 3	ab : 7 bb : 13	aa : 10 ab : 10	aa : 10 ab : 10	aa : 8 ab : 12	ab : 4 bb : 16
344	Azarbaijan population	ff* : 3 fa : 57 aa :279 ab : 5	ab* : 6 bb :60 bc :135 cc :143	fa* : 6 aa : 55 ab :142 db* : 20 bb :113 - : 8	aa : 57 ab :106 ac : 32 bb : 82 bc : 43 cc* :10 cd* :14	aa : 31 ab : 162 bb: 148 - : 3	aa : 68 ab : 177 bb : 99	ab : 10 bb :334

Table 6.3 Frequency of isozyme alleles in Iranian almond populations (Khorassan province)

*. New alleles for almond

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
30	Shahrood A (10)	aa : 28 ab : 2	bb : 6 bc : 8 cc : 14 - : 2	aa : 6 ab : 22 bc : 2	aa : 6 ab : 22 bc : 2	aa : 8 ab : 25 bb : 12	aa : 2 ab : 10 bb : 18	bb : 30
30	Shahrood B (11)	aa : 24 ab : 6	bc : 14 cc : 16	aa : 20 ab : 10	ab : 14 bb : 12 bc : 4	aa : 8 ab : 12 bb : 10	aa : 4 ab : 14 bb : 12	bb : 30
30	Shahrood C (9)	aa : 30	bb : 4 bc : 6 cc : 18 - : 2	aa : 12 ab : 8 bb : 2 db* : 2 - : 6	aa : 12 bb : 16 bc : 2	aa : 6 ab : 14 bb : 10	ab : 14 bb : 18	bb : 30
20	Shahrood D (1)	fa : 4 aa : 20 ab : 6	bc : 14 cc : 16	fa* :12 ab : 12 db* : 2	ab : 4 ac : 2 bb : 10 cc* : 2	aa : 8 ab : 8 bb : 14	*fa : 1 ab : 11 bb : 18	bb : 20 ab : 10
30	Shahrood E (13)	aa : 17 ab : 13	bb : 9 bc : 4 cc : 17	ab : 15 bb : 15	aa : 20 ab :5 ac : 5	aa : 5 ab : 12 bb : 13	aa : 4 ab : 14 bb : 12	ab : 6 bb : 24
30	Shahrood F (16)	aa : 24 ab : 6	bb : 11 bc : 16 cc : 3	ab : 20 bb : 10	aa : 10 ab : 12 ac : 8	aa : 14 bb : 6 ab : 10	aa : 5 ab : 14 bb : 6	ab : 6 bb : 24
30	Shahrood G (4)	fa* : 8 aa : 10 ab : 8 ac* : 4	bb : 25 bc : 5	aa : 25 ab : 5	aa : 10 ab : 4 ac : 4 bc : 4 - : 2	ab : 6 bb : 24	ab : 15 bb : 15	bb : 30
30	Shahrood H (12)	aa : 29 ab : 1	bb : 8 bc : 18 cc : 4	aa : 9 ab : 21	ab : 10 ac : 8 bb: 2 bc : 8 *cc : 2	aa : 2 ab : 10 bb : 18	aa : 8 ab : 12 bb : 10	bb : 30

Table 6.3 (Continue)

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
30	Shahrood I (18)	aa : 28 ab : 2	bb : 13 bc : 6	aa : 10 ab : 14	aa : 6 ab : 13	aa : 2 ab : 12	aa : 10 ab : 8	bb : 30
30	No. 1	aa : 30	bb : 14 bc : 10 cc : 4 - : 2	aa : 30	ab : 8 bb : 18 bc : 4	aa : 10 ab : 10 bb : 10	aa : 4 ab : 2 fb* : 6 bc* : 18	aa : 4 ab : 4 bb : 20 ab : 10
30	No. 2	aa : 14 ab : 16	bb : 4 bc : 18 cc : 8	aa : 9 ab : 16 bb : 5	ab : 14 bb : 12 bc : 4	aa : 2 ab : 15 bb : 13	aa : 4 ab : 12 bb : 14	bb : 20 ab : 6
30	No. 3	fa* : 5 aa : 25	bb : 17 bc : 13	ab : 14 bb : 16	aa : 5 ab : 10 bb : 13 bc : 2	ab : 3 bb : 27	aa : 7 ab : 15 bb : 8	bb : 30
30	No. 5a	aa : 27 ab :3	bb : 23 bc : 6 cc : 1	aa : 8 ab : 14 bb : 8	ab : 7 bb : 19 bc: 3 - : 2	aa : 4 ab : 21 bb : 5	aa : 6 ab : 4 bb : 14 bc : 4 - : 2	bb : 30
30	No. 5b	fa* : 4 aa : 10 ab : 4 ac* : 2	bb : 20 bc : 4 cc : 6	aa : 4 ab : 10 db* : 6 bb : 10	aa : 6 ab : 12 bb : 4 bc : 8	aa : 6 bb : 13 ab : 11	ab : 12 bb : 18	ab : 6 bb : 24
30	No. 6	fa : 12 aa : 16 ab : 1 ac* : 2	bb : 15 bc : 6 cc : 7 - : 2	aa : 30	ab : 21 bb : 3 bc : 6	ab : 10 bb : 20	ab : 2 bb : 28	bb : 30
30	No. 7	fa : 5 aa : 20 ab : 5	bb : 15 bc : 6 cc : 9	aa : 25 ab : 5	ab : 10 ac : 2 bb : 6 bc : 8 cc* : 4	ab : 6 bb : 24	aa : 7 ab : 7 bb : 16	bb : 30
30	No. 8	fa :14 aa : 16	bb : 6 bc : 20 cc : 4	ab : 10 bb : 18 - : 2	ab : 19 bb : 11 bc : 4	ab : 14 bb : 16	ab : 4 bb : 26 bb : 14	bb : 30

Table 6.3 (Continue)

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
30	No. 11	fa :14 aa : 16	bb : 13 bc : 11 cc : 6	aa : 2 ab : 20 db* : 2 bb : 6	aa : 4 ab : 16 bb: 6 bc : 4	ab : 6 bb : 24	ab : 20 ac : 4 bb : 2 bc* : 4	ab : 2 bb : 28
30	No 13	aa : 23 ab : 7	bb : 10 bc : 16 cc : 4	aa : 15 ab : 15	ab : 7 bb : 19 ab : 21 ac : 1 ab : 21	aa : 4 ab : 21 aa : 4 ab : 12 bb : 14	aa : 6 ab : 4 ab : 12 bb : 16 - : 2	bb : 30 bb : 30
30	No. 14	aa : 15 ab : 15	bb : 1 bc : 19 cc : 10	aa : 12 ab : 16 bb : 2	aa : 6 ab : 16 ac : 2 bc : 6	aa : 2 ab : 18 bb : 10	ab : 16 ac* : 1 bb : 13	bb : 30
30	No. 15	aa : 25 ab : 5	bb : 19 bc : 7 cc : 4	ab : 18 bb : 12	aa : 20 ab : 4 ac : 6	aa : 5 ab : 15 bb : 10	ab : 11 bb : 19	bb : 27 ab : 3
30	No. 17	aa : 24 aa : 6	bb : 19 bc : 7 cc : 4	aa : 13 ab : 17	aa: 2 ab : 10 ac : 6 bb : 6 bc : 4	aa : 6 ab : 20 bb : 4	aa : 4 ab : 16 bb : 10 bb : 14	bb : 30
30	No. 19	aa :19 ab : 11	bb : 10 bc : 10 cc : 10	aa : 13 ab : 12 bb : 5	aa : 3 ab : 6 bb : 21	aa : 6 ab : 17 bb : 7	aa : 1 ab : 24 bb : 5	bb : 30
30	No. 21	aa : 15 ab :15	ab* : 2 bb : 2 bc : 18 cc : 8	aa : 19 ab : 11	bb : 30	aa : 6 ab : 12 bb : 12	aa : 8 ab : 10 ab : 12	bb : 30
30	No. 22	aa : 22 ab : 8	bb : 13 bc : 17 cc : 10	aa : 14 ab : 16 bb : 2	ab : 4 ac : 9 bb : 6 bc : 9 - : 2	aa : 4 ab : 20 bb : 6	aa : 1 ab : 13 bb : 16	bb : 30
30	Late bloom No. 7	aa : 30	bb : 12 bc : 18	aa : 30	aa : 12 ab : 18	ab : 9 bb : 21	aa : 24 ab : 6	ab : 2 bb : 28

Table 6.4 Frequency of isozyme alleles in Iranian almond populations (Central province)

*. New alleles for almond

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
30	Shemiran 85	ff* : 3 fa : 10 aa : 16 - : 1	bb : 16 bc : 11 cc : 3	aa : 3 ab : 22 bc : 5	aa : 5 ab : 20 ac : 2 bb : 3	aa : 2 ab : 12 bb : 6	ab : 2 bb : 28	bb : 30
160	Shemiran population	fa : 12 aa :148	bb : 66 bc : 84 cc : 10	ab : 80 bb : 76 ac : 4	ab : 66 bb :44 ac : 18 bb : 20 bc : 12	aa : 32 ab : 128	aa : 44 ab : 76 bb : 40	bb :160
30	Ghom 24	fa : 10 aa : 20	bb : 15 bc : 12 cc : 3	ab : 11 bb : 19	aa : 6 ab : 16 bb : 6 cd* : 2	aa : 5 ab : 17 bb : 8	aa : 9 ab : 15 bb : 6	bb : 30
20	Kashan 8	fa : 8 aa : 12	bb : 16 bc : 4	ab :10 bb : 6 db* : 4	aa : 8 ab : 4 ac : 4 bc : 4	bb : 20	aa : 8 ab : 10 bb : 2	bb : 20
200	Central province population	ff* : 2 fa : 26 aa :172	bb : 92 bc : 86 cc : 20	*fa :10 aa : 8 ab : 83 bb : 84 - : 15	aa : 51 ac : 21 ab : 63 bb : 22 bc : 43	aa : 16 ab : 61 bb : 123	aa : 50 ab : 84 bb : 66	ab : 2 bb :198

Table 6.5 Frequency of isozyme alleles in Iranian almond populations (Southern province)

*. New alleles for almond

No. of samples	Genotypes	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
86	Esfehan population	aa : 72 ab : 14	bb : 26 bc : 44 cc : 16	fa*: 2 aa : 14 ab : 37 bb : 33	ab : 30 bb : 56	aa : 6 ab : 10 bb : 70	aa : 30 ab : 40 bb : 16	bb : 86
80	Kerman population	aa :80	bb : 70 bc : 10	aa : 56 ab : 16 bb : 8	aa : 54 ab : 6 bb : 20	ab : 8 bb : 72	aa : 2 ab : 33 bb : 45	bb : 80
170	Shiraz population	ff* : 3 fa : 48 aa :119	bb : 42 bc : 91 cc : 37	aa : 34 ab : 85 bb: 41 - : 10	aa : 68 ab : 61 ac: 10 bb : 20 bc : 11	aa : 11 ab : 115 bb : 44	aa : 35 ab : 81 bb : 54	bb : 170

Table 6.6 Frequency of isozyme alleles in 7 Iranian wild almond species

*. New alleles for almond

No. of samples	Species	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
62	*Prunus eleagnifolia*	aa : 62	ab* : 6 bb : 46 bc : 8 cc : 2	ff* : 8 fa* : 16 aa : 24 ab : 10 bb : 4	aa : 19 ab : 33 bb : 10	aa : 30 ab : 18 bb : 14 bb : 6	aa : 58 bb : 4	bb : 62
142	*Prunus lysioides*	ff* : 4 fa :2 aa :106 ab : 2 bb*:28	ab* : 2 bb : 31 bc : 8 cc : 97 cd* : 4	aa : 15 ab : 80 db*: 14 bb : 23 db* : 5 ff* : 5	aa : 35 ab : 84 bb : 19 bc : 4	aa : 32 ab : 128	aa : 34 ab : 43 ac* : 10 bb : 30 fb* : 8 bc* : 6 fc* : 11	bb : 142
20	*Prunus nairaica*	aa : 18 bc : 2	bb : 12 bc : 6 - : 2	fa* : 2 aa : 2 bb : 16	aa : 18 ab : 2	aa : 18 ab : 2	aa : 2 ab : 10 bb : 8	bb : 20

Table 6.6 (Continue)

No. of samples	Species	GPI-2	LAP-1	AAT-1	IDH	PGM-1	PGM-2	SKDH
140	*Prunus reticulata*	aa :140	ac* : 2 bb :109 bc : 15 cc : 14	ff* : 2 fa* : 6 aa : 82 db* : 4	aa : 66 ab : 60 bb : 34	aa : 34 ab : 64 bb : 42	aa : 134 ab : 6	aa : 5 ab : 6 bb :129
242	*Prunus* scoparia	aa :162 ab : 36 bb*: 42	aa* : 8 ab*: 40 ac* : 8 bb :106 bc : 28 cc* :53	aa : 99 ab : 53 db* : 28 bb : 42 ff*: 12 - : 8	aa :118 ab : 30 ac : 2 bb: 92	aa : 242	aa : 122 ab : 38 bb : 82	bb : 242
80	*Prunus* spinosissima	aa : 50 ab : 10 bb*: 20	bb : 18 bc : 8 cc : 54	fa* : 2 aa : 40 ab : 23 bb : 15	aa : 80	aa : 6 ab : 74	aa : 52 ab : 4 bb : 26	bb :80
20	*Prunus* webbii	aa : 20	cc : 20	aa : 2 ab : 16 db* : 2	aa : 20	bb : 20	bb : 20	bb :80
80	*Prunus* reticulata x eleagnifolia	aa : 32 ab : 40 bb : 8	bb : 2 bc : 8 cc : 70	aa : 80	aa : 68 bb : 12	aa : 22 ab : 46 bb : 12	aa : 80	bb :80

Chapter 7

Identification of wild species and cultivated almond by RAPD markers

Chapter 7

Identification of wild species and cultivated almond by RAPD markers

7.1 Summary

RAPD analysis was applied to 12 cultivated and wild species, by using seven primers. All cultivars and species were distinguished by comparisons of differences in DNA banding patterns. The dendogram showed that among the three wild species, *P.lysioides* is more closely related to *P.scoparia* than to *P.reticulata*. In cultivated almond, with one exception, Australian cultivars were clearly separated from Iranian genotypes. Comparison of PCR banding patterns between cultivated almonds and wild species shows that the variability of bands was lower for wild than cultivated almonds.

7.2 Introduction

Traditional markers based on morphological differences between individuals have been employed for identification of cultivars and species (e.g. pollen ultrastructure). The subsequent development of isozymes and other biochemical markers represented a significant improvement since they offered greater diversity (Tanksley and Orton, 1983). Restriction fragment length polymorphism (RFLP) is depend on the use of probes to identify single or lower copy sequences in DNA. This method is very powerful and has been used to construct detailed linkage maps of several crop species including tomato and potato (Tanksley *et al.*, 1989). However they are expensive and time consuming and technically difficult to use in some species with large and complex genomes. It has recently been proposed that random amplified polymorphic (RAPD) can be exploited as a source of genetic markers (Williams *et al.*, 1990).

All the above methods have met with success, but each one has different advantages and disadvantages. Pollen ultrastructure is useful only when combined with other morphological characters and requires generative phases of plant growth. Isozyme analysis is highly reproducible but has relatively little polymorphism and cannot always be applied to the discrimination of closely related genotypes due to insufficient polymorphism among closely related cultivars. Because protein is a product of gene expression, they may vary in

different tissues, developmental stages and environments (Beckmann and Soller, 1983). On the other hand, DNA markers such as RLFPs give a much higher degree of polymorphism and stability, but disadvantages include the laborious procedures involved, the relatively high cost and use of radioisotopes. As an alternative the random amplified DNA polymorphism assay was developed by Williams *et al.* (1990) to provide greater levels of polymorphisms. However in some material it can be less reproducible. The method is based on amplification by the polymerase chain reactions (PCR) of random DNA segments using single primers of arbitrary nucleotide sequences. Unlike the other PCR-based strategies proposed, this new assay does not require any specific sequence information on the target genome. The amplified DNA fragments referred to as RAPD markers which typically utilize decamer oligonucleotide primers of arbitrary sequence but with a GC content of 50% or higher, are used to amplify segments of genomic DNA. They have been shown to be highly useful in the construction of genetic maps (Klein-Lankhorst *et al.*, 1991).

In the last few years RAPD has been used extensively for identification of many plants, especially horticultural crops. Mulcahy *et al.* (1993) have characterized twenty five accessions of apple by RAPD markers; they can distinguished between cultivars by obtaining a distinctive finger print for each cultivar. Five rose cultivars were identified by using eight primers. All cultivars were distinguished by comparing differences in DNA banding patterns (Torres et al, 1993). Relationships between 16 cultivars of mume (*Prunus mume Sieb. et Zucc*) were examined by Takehiko *et al.* (1993). They concluded that the RAPD assay was useful for parental identification and classification in mume. The object of the present study is to assess the species and cultivar identification and the relationships between them by RAPD markers.

7.3 Materials and methods

7.3.1 Plant materials

Seeds of three wild almond species (*Prunus lycioides*, *Prunus reticulata* and *Prunus scoparia*), five controlled crossed cultivars (Fritz, Keane, Missiom, Nonpareil and Peerless) and four Iranian genotype (AH3, Shemiran 20, Shemiran, 26, and Shemiran85) were planted at 25° C in a growth room.

7.3.2 DNA isolation and PCR amplification

Leafy plant material was used for total nucleic acid extraction, and DNA was isolated using a CTAB extraction technique (Doyle and Doyle, 1987) as follows: 5 ml of CTAB solution buffer (100 mM Tris-HCl, pH 8.0- 1.4 M NaCl- 20mM EDTA- 2% CTAB- 0.4 % β mercaptoethanol) in a 10 ml plastic centrifuge tube was preheated in a water bath to 60° C. Fresh leaves (0.5-1g) were ground to a powder in liquid nitrogen using a mortar and pestle (ground samples were rinsed into the tube, contents mixed to a slurry and incubated at 60° C for 30 minutes with agitation approximately every 5 minutes). After cooling the mixture to 50° C, tubes were filled with an equal volume of chloroform / isoamyl alcohol (24:1 , V:V) and mixed gently for 5 minutes. Tubes were centrifuged at 4000 r.p.m for 4 minutes in a swinging bucket rotor centrifuge. The upper (aqueous) phase was transferred using a wide bore pipette to a clean 10 ml tube where 1/10 volume of 7.5 M ammonium acetate was added to lower the pH and allow better precipitation of DNA. Then 1.5 volumes of cold (-20° C) isopropanol was added gently and mixed slowly.

Nucleic acids were precipitated and recovered in one of two ways: a) If DNA strands were visible they were spooled, spun onto a glass rod and transferred directly into a tube of wash solution. b) If DNA appeared flocculent the solution was centrifuged 2000 r.p.m for 20 minutes. The supernatant was then poured off gently and the precipitate collected. The pellet was washed with 70% ethanol for resuspension of DNA. Samples were centrifuged at 2000 r.p.m for 20 minutes and once again the supernatant was poured off. The pellet was then resuspended in 0.4 ml TE [10mM Tris-HCl (pH 7.4)- 1M EDTA] to dissolve the nucleic acids. Samples were let stand at 4° C over night. One µl of RNAase A (10 mg/ml) was added to 100 µl of DNA solution and incubated at room temperature for 30 min. The DNA concentration for each sample was estimated by comparing band intensity on electrophoresis in agarose gel with standard DNA controls of known concentrations. After preparing a 1 percent agarose gel in TBA buffer (0.089 M Tris- 0.089 M boric acid- 0.002 M EDTA), 8 µl of DNA dissolved in TE buffer was mixed with 2 µl of ficoll loading dye and wells were loaded with 8 µl of mixed solution. The DNA bands after treatment with RNAase are shown in Figure 7.1.1

7.3.3 DNA amplifications conditions

Amplifications by PCR reactions were performed in volumes of 30 microliters containing 10Xbuffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0 at 25° C), 1% Triton X-100 buffer] 3 µl, 25 mM $MgCl_2$ 4 µl, 1.25 mM deoxyribonucleotide triphosphates (dNTPs) 4.8 µl, primer 1.5 µl, 5 mg/ml T4 Gene 32 protein 0.2 µl (Boehringer Mannheim) and 5 units/µl *taq* polymerase 0.18 µl (Promega), H_2O and 25 ng/µl template DNA 1µl. The mix was overlaid with 30 µl of mineral oil.

Amplification was conducted in a Perkin Elmer Cetus Thermocycler. The samples were incubated at 94° C for 2 minutes then amplified for 40 cycles consisting of 1 min at 95° C, 10 sec at 50° C, 15 sec at 45° C, 20 sec at 40° C, 1 min at 35° C, 30 sec at 45° C, and 1min 45sec at 72° C and a final extension step for 5 min at 72° C. Amplification products were separated by electrophoresis on 1.6% agarose gel and detected by staining with ethidium bromide.

A total of 7 primers (Operon Technologies, Almeda, Calf.) were used. Their sequences were as follows from the 5' end: OPA-03 = AGTCAGCCAC, OPA-07 = GAAACGGGTG, OPA-10 = GTGATCGCAG, OPA-11 = CAATCGCCGT, OPB-06 = TGCTCTGCCC, OPB-12 = CCTTGACGCA, OPB-18 = CCACAGCAGT.

7.3.4 Data analysis

For each individual primer, the PCR products were scored for computer analysis on the basis of the presence or absence of the amplified products. If a product was present in a particular genotype it was designated '1'; if absent, it was designated '0'. Pair-wise comparison of genotypes, based on the presence or absence of unique and shared polymorphic products, was used to generate similarity coefficients (McCullagh and Nelder, 1989). The similarity coefficients were then used to construct a dendogram by UPGMA (un-weighted pair-group method with arithmetical averages) using a Genestat computer program.

7.4 Results

7.4.1 Cultivar and species identification

A survey with seven arbitrary primers revealed a great amount of polymorphism in amplified DNA fragments ranging in length from 435 bp to 1904 bp. The number of bands produced by each primer varied from as a few as 2 (Fig. 7.1.8) to as many as 20 or more (Fig. 7.1.2). The seven primers resulted in a large number of amplifications, the electrophoresis separations of RAPD from sample 1-12 with the seven primers are shown in Figure 7.1 (2-8). A total of three wild species and nine cultivars of almonds were successfully separated by the RAPD fingerprints. The RAPD method for association of a different cultivar was quite consistent. Replication of electrophoresis runs showed that the banding patterns were constant with the same primers.

Since an almost limitless number of primers can be analysed and several DNA bands can be differentiated for each one, the number of possible combinations is large. In the present work, differences among cultivars were obvious and expressed consistently with most primers. In the almond species and cultivars examined each primers can identify cultivars and species. With all primers, DNA banding patterns for each cultivar was consistent. Occasional differences in the intensity of some non-discriminatory bands, between replications of the samples were observed. This was probably due to differences in the quality of DNA isolated from respective samples.

7.4.2 Cultivars and species relationship

A dendogram tree generated by UPGMA is shown in Figure 7.2. Among the three wild species *P.lysioides* is more closely related to *P. scoparia* than to *P. reticulata*. In cultivated almond, with one exception, Australian cultivars were separated from Iranian genotypes. Among the 12 cultivars and species, two clusters were resolved. The larger cluster, which was assigned as group A consisted of cultivated almonds while group B contained two wild species (*P. lysioides* and *P. scoparia*). Coefficients of similarity showed that *P. reticulata* is more closely related to cultivated almonds than to wild species.

7.5 Discussion

The polymorphism obtained by amplification of arbitrary primers is very extensive in almonds. The RAPD technique, therefore provides an easy and reliable system for cultivar identification in almond. Alternative systems for cultivar identification such as isozyme analysis appear to show less polymorphism than does RAPD. Weeden and Lamb (1985) and Samimy and Cummins (1992) found that a survey of 6 isozyme systems was required to distinguish apple cultivars. In almonds, we have shown that 4 isozyme systems are necessary to distinguished almond cultivars, the number of isozyme systems needed for wild almond species is greater due to the low diversity of alleles in these species. A comparison of PCR banding patterns between cultivated almond and wild species shows that the variability of bands was less for wild than for cultivated almonds.

Although additional work is needed to confirm the results obtained in this initial study, it seems that the RAPD technique can be used to characterize almond cultivars. It could be particularly useful in protecting protect plant variety rights.

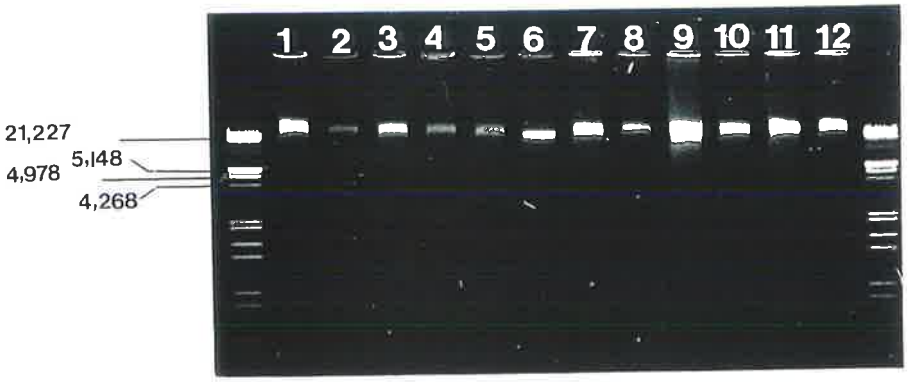
Fig. 7.1.1 DNA banding patterns (after treatment with RNAase) for 12 cultivated and wild almond species, run on a 1% agarose gel.

Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

Fig.7.1.2 RAPD patterns for 12 cultivated and wild almond species generated with primer number **A-3** (numbering from left to right after marker line).

Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

1



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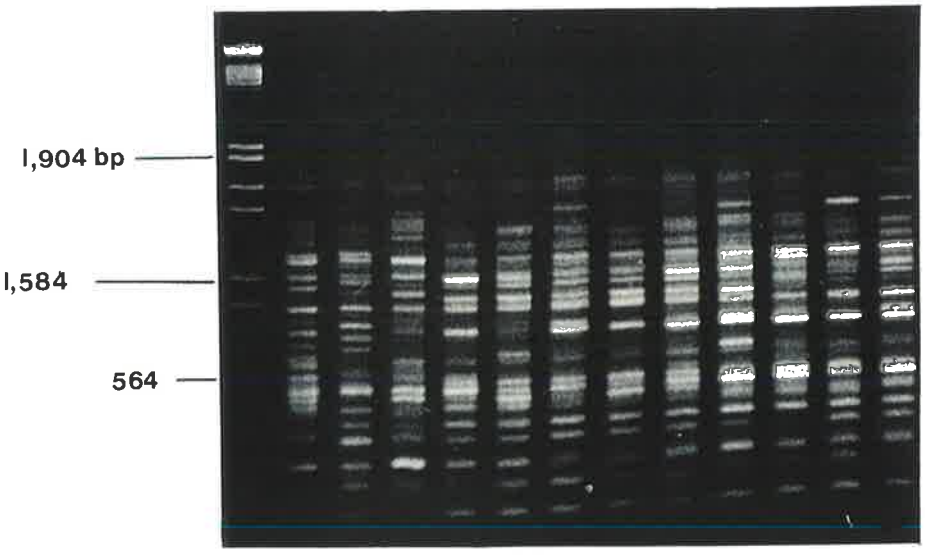


OPA–03

Fig.7.1.3 RAPD patterns for 12 cultivated and wild almond species generated with primer number **A-7** (numbering from left to right after marker line).

Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

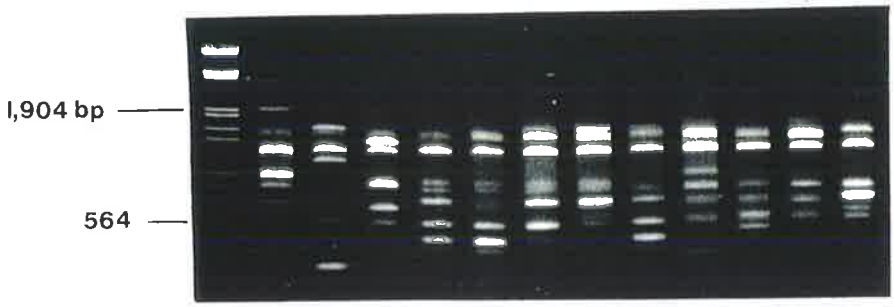
Fig.7.1.4 RAPD patterns for 12 cultivated and wild almond species generated with primer number **A-10** (numbering from left to right after marker line).

Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

Fig.7.1.5 RAPD patterns for 12 cultivated and wild almond species generated with primer number **A-11** (numbering from left to right after marker line).

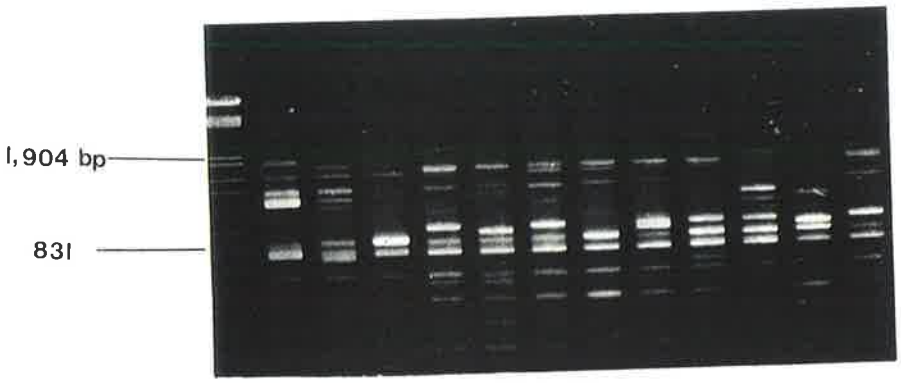
Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

3



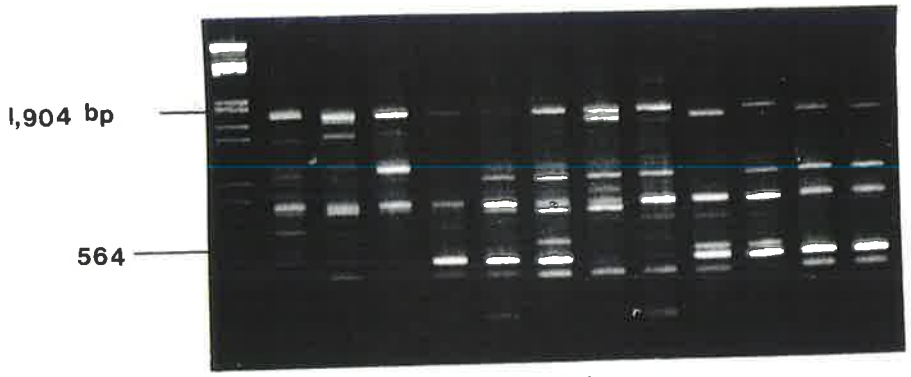
OPA–07

4



OPA–10

5



OPA–11

Fig.7.1.6 RAPD patterns for 12 cultivated and wild almond species generated with primer number **B-6** (numbering from left to right after marker line).

Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

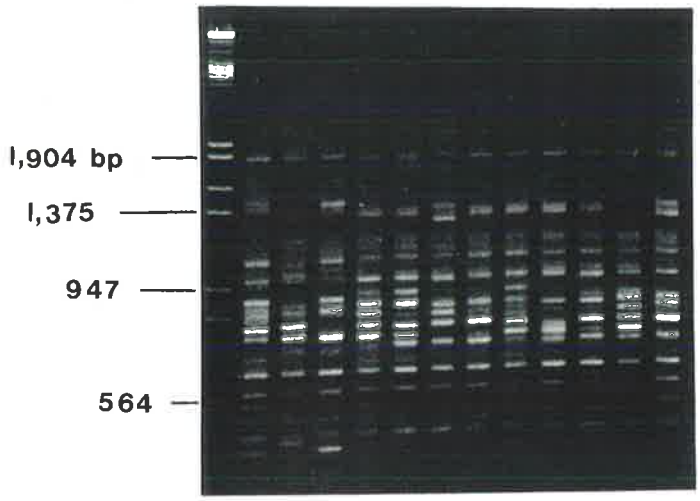
Fig.7.1.7 RAPD patterns for 12 cultivated and wild almond species generated with primer number **B-12** (numbering from left to right after marker line).

Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

Fig.7.1.8 RAPD patterns for 12 cultivated and wild almond species generated with primer number **B-18** (numbering from left to right after marker line).

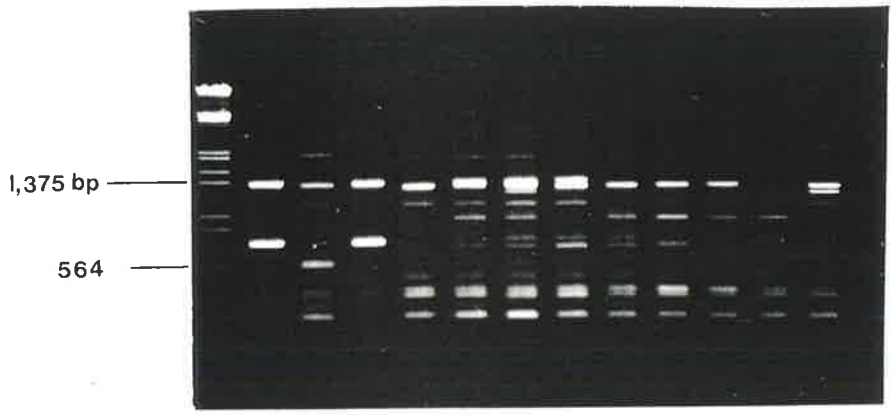
Sample 1 - *Prunus lycioides*
Sample 2 - *Prunus reticulata*
Sample 3 - *Prunus scoparia*
Sample 4 - Nonpareil
Sample 5 - Keane
Sample 6 - Fritz
Sample 7 - Mission
Sample 8 - Peerless
Sample 9 - Shemiran 26
Sample 10 - Shemiran 58
Sample 11 - Shemiran 20
Sample 12 - AH3

6



OPB–06

7



OPB–12

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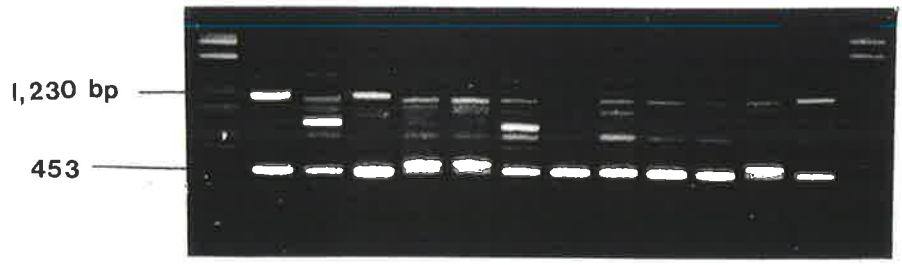


OPB–18

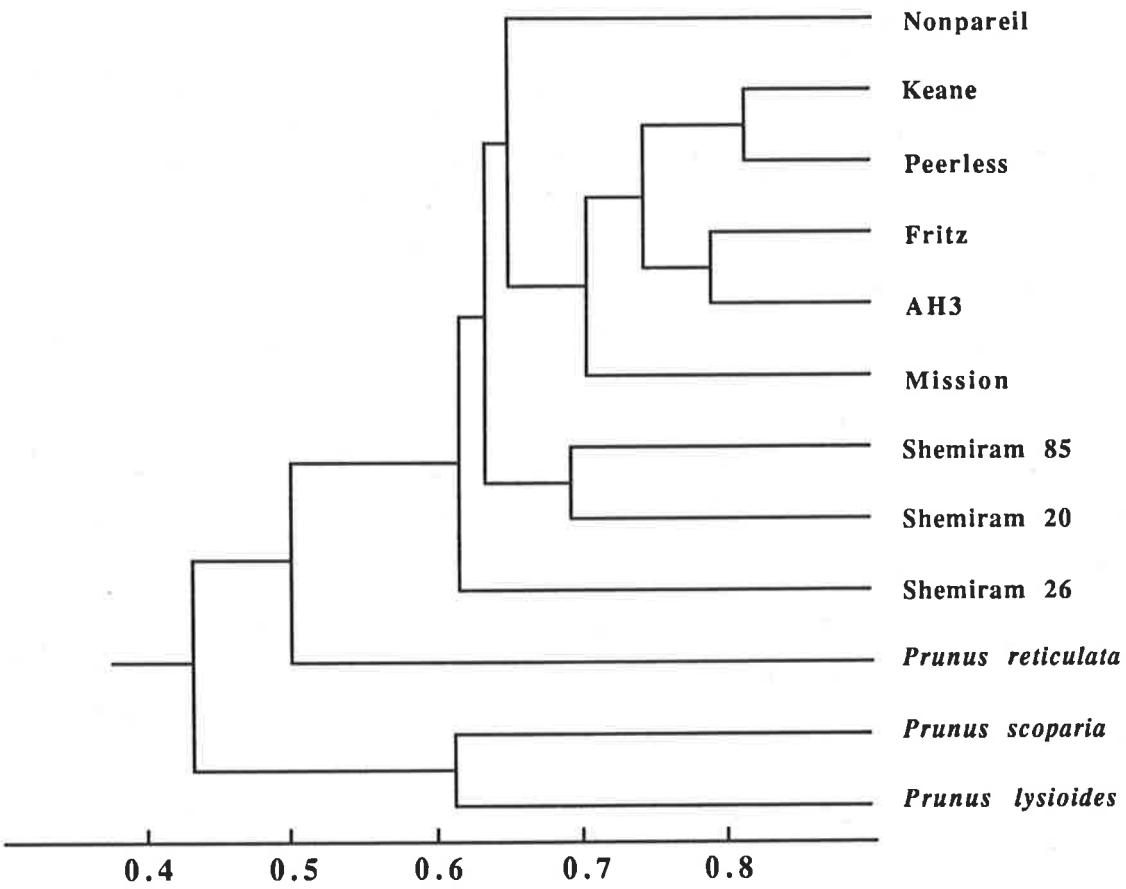


Fig. 7.2 Dendrogram of almond genotype, constructed using UPGMA based on Jaccard's similarity coefficients. The scale is Jaccard's coefficient of similarity.

Chapter 8

The effect of pollen parent and stage of flower development on almond nut production

Chapter 8

The effect of pollen parent and stage of flower development on almond nut production

8.1 Summary

Pollination of the Nonpareil almond cultivars at 6 succeeding stages (days) of flower development showed that newly opened flowers are more fertile than at the 5 other stages of development. This was true when pollen from 18 different almond cultivars was used, except for that from IXL seedling, which was incompatible with Nonpareil cultivar.

Pollen from 8 of these cultivars was examined for differences in proportion of pollinated Nonpareil flowers which set nuts and for quality of nuts produced. While there were no significant differences in physical and chemical traits of nuts produced, there were significant differences in proportion of flowers setting nuts. Thus pollen from cultivars Peerless, Fritz, Keane and Price set highest numbers of nuts, Grant, Mission and Ne Plus Ultra intermediate numbers, and Carmel set the lowest numbers on Nonpareil.

When 5 different pollen donors were used on Price and Keane cultivars as female recipients, only pollen from Keane cultivar produced nuts on Price which were significantly different from the others (they were heavier than expected), while pollen donors did not influence kernel weight on nuts set on Keane cultivar. Ne Plus Ultra pollen was found to be incompatible with Price cultivar.

8.2 Introduction

Almond (*Prunus dulcis [Mill.] D.A. Webb syn. Prunus amygdalus Batsch.*) is a member of the *Rosaceae* family grown commercially for kernels in several countries with a Mediterranean-type climate (Kester and Asey, 1975). Most of the commercial almond cultivars are self-incompatible (Bowman, 1939; Kester and Griggs, 1959a; Socias i Company *et al.*, 1976). Since the edible portion of the almond is the embryo (kernel), fertilization of the egg is essential (Griggs, 1953). Currently, almond orchards must be inter-planted with at least 2 cross-compatible and simultaneously blooming cultivars, and pollinating insects are required to carry out the inter-cultivar transfer of pollen (Tufts, 1919;

Griggs, 1953). However in commercial orchards percentage of fruit set is commonly only 5-30% of that possible (Tufts, 1919; Griggs, 1949; Kester and Griggs, 1959a; Gary *et al.*, 1976). For Australian conditions, Hill (1985) stated that an average nut set of 50 % should be achieved by manipulating the factors affecting pollination and fruit set.

Several investigators have reported an effect of different pollen sources on quality and quantity of fruits produced on some plant species (e.g. see Denny, 1992). In pecan, pollen effects are observed following selfing as compared with cross pollination (Romberg and Smith, 1946; Marquard, 1988). In pistachio and chestnut, pollen from a small-seeded cultivar or species produces a reduction in nut size when applied to a cultivar or species which normally produces large seeds (McKay and Crane, 1938; Crane and Iwakiri, 1980; Sedgley and Griffin, 1989). Pollen effects were found in the embryo and pericarp of pistachio (Peebles and Hope, 1937), but Crane and Iwakiri (1980) showed that in this case pollen only affected the embryo. In coconut, controlled crosses showed that pollen directly influences the type of endosperm produced (Ninan *et al.*, 1936; Cedo *et al.*, 1984). In the only such work reported for almond, Godini *et al.* (1991) showed that it is no difference between 12 different pollen donors on the proportion of pollinated flowers which set nuts on Ne Plus Ultra and Ferragnes cultivars. The effect of pollen sources on nut set on Nonpareil, the most important commercial cultivar in Australia hes not previously been investigated.

The object of this study is to investigate the percentage fruit set obtained on Nonpareil almond at different stages of flower development with a large range of almond pollen sources. The effect of different pollen sources on kernel quality was also examined, as was cross compatibility between the various cultivars.

8.3 Materials and methods

8.3.1 Experimental site

The investigation was carried out over 3 seasons in the lmond collection block at the Waite Agricultural Research Institute (1990-1992) and during 1992-1993 in a commercial almond orchard at Angle Vale 35 km north-west of Adelaide (South Australia) Figures 8.1a and b.

8.3.2 Pollen collection and germinability

Pollen was freshly gathered from flowers at the balloon stage (1 day prior to opening) from each cultivar in a commercial almond orchard at Angle Vale. Anthers were removed and stored at 20° C for 24 h and prepared for pollination according to methods described by Jackson (1989a). *In vitro* germinability of freshly collected pollen was tested on a germination medium containing agar (1%), sucrose (15%) and a mixture of salts (Brewbaker and Kwack, 1963). All pollen tested was found to be more than 80% viable. Pollination treatment was conducted as described by Kester and Asey (1975). The flowers were pollinated with small soft brushes. To avoid contamination of pollen, separate brushes were used and washed with 70% ethanol after each treatment.

8.3.3 Hand cross-pollination

In June 1990 a 9 year-old Nonpareil tree was selected and completely covered with nylon mesh (80 % light transmission) before flowering. Hand cross-pollination was conducted at 6 different stages of flower development [2 days before opening (-2), 1 day before opening at balloon stage (-1), newly opened flower (0), 1 day after opened flower (+1), 2 days after flower opened (+2), and 3 days after opening (+3)]. Pollination was carried out with pollen from 18 pollen sources, namely Baxendale, Bruce, Carmel, Chellaston, Davey, Drake, Fritz, Grant, IXL Seedling, Johnson's Prolific, Keane, Mission, Ne Plus Ultra, Peerless, Pethic's Wonder, Price, Somerton, Thompson, and White Brandis. Forty flowers in each stage were pollinated with each of these 18 pollen donors. Flowers were emasculated at stage (-2) and stage (-1) only.

In June 1991, two 10 years-old Nonpareil trees were covered with nylon mesh separately, and 32 branches were selected in each tree (4 replicates by 8 pollen sources). Hand cross-pollination was carried out with pollen from 8 different cultivars (Carmel, Fritz, Grant, Keane, Ne Plus Ultra, Mission, Peerless and Price). In each branch 250 flowers were pollinated at stage (+1).

In 1992, crosses were carried out on Keane and Price trees with 5 different pollen sources. Two trees were covered separately and 20 branches were selected in each tree (cultivar). Five hundred flowers were pollinated using flowers at stage (+1) on each branch.

Keane was pollinated with pollen of Fritz, Ne Plus Ultra, Nonpareil, Price and Peerless and Price was pollinated with pollen of Fritz, Keane, Ne Plus Ultra, Nonpareil and Peerless.

Percentage fruit set was calculated after 3 months and at the time of maturity in each year. Fruit was harvested in February (6 months later) when mesocarp had started to dry and had dehisced. Mesocarps were then removed and nuts dried in-shell at room temperature. Fifty in-shell nuts were selected at random from each replication for further analysis in each year.

8.3.4 Physical and chemical analysis

Fifty nuts from each replication were examined for physical traits (weight, length, width, and thickness of in-shell nut and kernel) in 1991, 1992 and 1993. Chemical analyses were carried out on almond kernels for total crude protein with the Kjeldahl method (section 2.1.2), total oil was extracted by the Soxhlet method (section 2.1.1), mineral elements were analysed with inductively coupled plasma spectrometery (ICPS) method (section 2.1.4) and ethanol soluble sugar was measured by a colorimetric method (section 2.1.3). Cyanide measurement as described by Brinker and Seigler (1989) was modified for almond (section 2.1.5). Further details of cyanide analysis are described by Vezvaei and Jackson (1994).

8.3.5 Statistical analysis

Analysis of variance was performed on each of the measured traits, to test for differences between the effects of pollen sources. Mean values for the cultivars were separated by Tukey's honestly significant difference (HDS). The Genstat statistical package was used in all calculations (Genstat 5 Committee 1987).

8.4 Results

8.4.1 Changes in receptivity of Nonpareil flowers

Nonpareil flowers were able to produce nuts during all stages of flower development tested. For each of the 18 different pollen sources percentage of flowers pollinated which set fruit was highest for the newly opened flower (stage 0) and was significantly lower at earlier and later stages. mean of the percentage of fruit set for 18 different pollen sources was shown in Figure 8.2.

8.4.2 Physical, chemical and mineral element analysis of Nonpareil nuts

There were no difference ($P < 0.01$) between the quality traits examined (physical, chemical and mineral elements) for nuts obtained by crossing Nonpareil with any of the 8 different pollen donors (Tables 8.1, 8.2 and 8-3).

8.4.3 Percentage of fruit set on Nonpareil , Price and Keane

Carmel pollen gave a significantly lower proportion of pollinated flowers which subsequently set nuts on Nonpareil cultivar than did pollen from several other cultivars (Fig. 8.3a). Pollination of Price with pollen from 5 different cultivars had no significant effect on the percentage of flowers settings nuts (except when the cross-incompatible Ne Plus Ultra was used) (Fig. 8.4a). Pollination of Keane with the different pollen sources on the other hand did have a significant effect on the percentage of flowers which set nuts by the different pollen sources (Fig. 8.5a). When the Price cultivar was fertilized with pollen from different sources, significant differences in the weight of resulting individual kernels were found, but this did not occur with Keane (Figs. 8.4b and 8.5b).

8.4.4 Cross-incompatible cultivars

Results from all combination of crosses showed that all the almond cultivars used were cross-compatible except for that between Nonpareil x IXL Seedling (from 240 flowers is pollinated no fruit resulted) and the Price x Ne Plus Ultra was unsuccessful (Fig. 8.4a).

8.4 Discussion

In general, the development of receptivity of the stigmatic surface may vary widely between species from several days before anthesis to several days after the flower opening (Palser *et al.*, 1989). Previous studies from California on the effective pollination period (EPP) for Nonpareil almond flowers showed an EPP varying from 3 to 7 days for each flower (Griggs and Iwakiri, 1964; Kester, 1981). A similar EPP is shown here for Australian conditions. Under South Australian (Adelaide) conditions it is found that the highest percentage of fruit set occurred on flowers during the first day of opening (stage 0), whereas Griggs and Iwakiri (1964) reported under Californian conditions the highest nut set

on Nonpareil flowers was one day after flower opening, at stage (+1). This difference possibly could be accounted for by the lower winter temperatures experienced in California. According to Sedgley and Buttrose (1978), low temperature may be cause slow maturation of the ovule in watermelon. Despite the fact that pollen used from all cultivars was better than 80% viable, the maximum percentge of flowers setting nuts was no better than 45%. The question arises as to whether this is the absolute maximum obtainable. Since for practical reasons, flowers were pollinated only once during the day, repeated applications every hour after opening could give a higher result. However under orchard conditions we cannot be certain that flowers are frequently pollinated during stage 0, as Hill (1985) notes for the same orchard in the present study, pollen dehiscence does not begin until after the first day of opening (*viz* at stage +1). According to Williams and Brain (1985) and Goodwin (1986) pollen gathering bees are reluctant to visit flowers not yet dehiscing pollen from the anther and it is the pollen-gatherer that is said to be responsible for the major part of cross-pollination in almond (Thorp, 1973). This reduces the maximum possible set to more the like 38% found for stage (+1), and moreover for Adelaide conditions, Hill *et al.* (1985) suggests that the number of bee visits to each flower after opening varies from none to 12. Perhaps the only way to increase the percentage of fruit set as determined by the above factors is to set up orchards in warmer areas (as is happening with the move of many orchards to the Riverland areas of South Australia), where dehiscence of anthers may be more likely to occur earlier (Jackson, 1989b), perhaps during the first day of flower opening (stage 0) and where bee activity is enhanced by the temperature increase.

The concept that the various pollen donors can have different effects on yield and fruit quality in addition to merely providing pollen for cross-pollination purposes, is a relatively new area of interest in horticulture (Crane and Iwakiri, 1980; Degani *et al.*, 1990). When the most desirable commercial variety of almond Nonpareil was looked at, we found no significant effect of pollen donors on physical and chemical traits of nuts produced. However, when nuts produced on the cultivar Price (planted to act as a pollinizer for Nonpareil) were investigated there was a significant effect of pollen donor on the weight of individual nuts obtained. As described by Sedgley and Griffin (1989), the effect of pollen from a cultivar which normally produces large kernels on a cultivar producing small kernels

is greater than for the reciprocal crosses. For almond then it is not surprising that we find pollen from Keane (which itself produces large nuts), significantly increases the weight of individual nuts produced on Price (normally producing smaller nuts). There was no significant effect of the pollen donor on either the physical or chemical properties of nuts set on Keane, the other pollinizer studied. While a large part of the almond orchardists' income is derived from the sale of Nonpareil nuts, sales by weight of nuts produced by the Nonpareil pollinizers is significant and so pollen donor effects on these should be considered when planning an orchard . From the results shown here, the planting of IXL Seedling with Nonpareil and Ne Plus Ultra with Price, should be avoided since these pairs are incompatible. These two cross-incompatible pairs of almond cultivars have also been reported to be cross-incompatible very recently by Kester *et al.* (1994).

Although ultimately experimental plantings should be observed for best approaches to orchard design, it is pertinent to consider the results found hear for pollen donor effects on proportion of flowers setting nuts on whole branches of Nonpareil. As reported here Peerless, Fritz, Keane and Price as pollen donors all gave high nut set on Nonpareil; Grant, Mission and Ne Plus Ultra gave lower nut set, and Carmel the lowest. These figures do not take into account overlap of flowering times between these pollinizers and Nonpareil, this property should also be allowed for in orchard design. While numbers of nuts set on Price did not seem to be significantly affected by pollen donors, as reported by Godini *et al.* (1991) for Ne Plus Ultra, it is shown in the present studies that there is an effect of pollen donor on the percentage of nuts set on Keane.

Table 8.1 Physical characteristics of Nonpareil nuts obtained by crosses with each of 8 different pollen sources

Pollen sources	In-shell weight (g)	In-shell length (mm)	In-shell width (mm)	In-shell thickness (mm)	Kernel weight (g)	Kernel length (mm)	Kernel width (mm)	Kernel thickness (mm)	Shell weight (g)
Carmel	2.69 ± 0.014	3.60 ± 0.10	23.8 ± 0.10	14.1 ± 0.05	1.54 ± 0.009	24.6 ± 0.07	14.6 ± 0.05	7.3 ± 0.03	1.14± 0.009
Fritz	2.52 ± 0.016	34.8 ± 0.11	23.5 ± 0.08	14.8 ± 0.06	1.42 ± 0.012	23.8 ± 0.08	14.0 ± 0.05	7.4 ± 0.03	1 10 ± 0.011
Grant	2.63 ± 0.015	35 2 ± 0.10	23.7 ± 0.06	14.4 ± 0.05	1.47 ± 0.012	24.0 ± 0.09	14.2 ± 0.05	7.5 ± 0.03	1.15 ± 0.011
Keane	2.76 ± 0.017	36.0 ± 0.14	24.1 ± 0.08	14.8 ± 0.05	1.57 ± 0.014	24.6 ± 0.01	14.6 ± 0.06	7.5 ± 0.03	1.18 ± 0.009
Mission	2.80 ± 0.016	36.1 ± 0.12	24.0 ± 0.08	14.3 ± 0.05	1.56 ± 0.010	24.4 ± 0.08	14.6 ± 0.06	7.3 ± 0.03	1.22 ± 0.011
Ne Plus Ultra	2.51 ± 0.015	34.4 ± 0.10	23.2 ± 0.07	14.3 ± 0.05	1.45 ± 0.011	24.2 ± 0.08	14.1 ± 0.05	7.3 ± 0.03	1.06 ± 0.008
Peerless	2.50 ± 0.015	34.1 ± 0.11	22.8 ± 0.07	14.1 ± 0.05	1.42 ± 0.010	23.6 ± 0.08	13.9 ± 0.05	7.4 ± 0.03	1.07 ± 0.008
Price	2.55 ± 0.016	34.7 ± 0.11	23.2 ± 0.06	14.1 ± 0.04	1.48 ± 0.011	23.9 ± 0.08	14.2 ± 0.05	7.4 ± 0.02	1.07 ±0.008
Significance level	NS	NS	NS	NS	NS	NS	NS	NS	NS

Values are mean ± standard error of 400 samples, shell water content 6.3%, kernel water content 4.8 %.

Table 8.2 Total protein, oil, ethanol soluble sugar and cyanid analyses in Nonpareil almond kernels obtained by crosses with each of 8 different pollen sources

Pollen sources	Protein content %	Oil content %	sugar (Ethanol soluble) (mg/g)	Cyanogenic compound as NaCN (µg/g)
Carmel	23.0 ± 0.38	42.5 ± 0.50	3.4 ± 0.39	0.014 ± 0.001
Fritz	23.7 ± 0.34	46.8 ± 1.12	3.4 ± 0.03	0.015 ± 0.001
Grant	22.6 ± 0.43	42.6 ± 1.03	2.8 ± 0.14	0.016 ± 0.001
Keane	25.0± 0.38	45.8 ± 0.73	3.6 ± 0.22	0.016± 0.001
Mission	23.7 ± 0.46	45.0 ± 0.48	3.0 ± 0.14	0.017 ± 0.002
Ne Plus Ultra	21.5 ± 0.30	44.3 ± 0.42	3.6 ± 0.28	0.014 ± 0.003
Peerless	23.4 ± 0.37	23.4 ± 0.37	3.2 ± 0.23	0.016 ± 0.001
Price	24.1± 0.41	45.2 ± 1.50	3.1 ± 0.26	0.016 ± 0.001
Significance level	NS	NS	NS	NS

Column 1. values are mean ± standard error of 98 samples, dry weight.

Column 2, 3, 4. values are mean ± standard error of 24 samples, dry weight.

Table 8.3 Mineral element analyses of Nonpareil almond kernels obtained by crosses with 8 different pollen sources

Pollen sources	Ca (Ug/g)	K (µg/g)	Mg (µg/g)	Mn (µg/g)	P (µg/g)	S (µg/g)	Zn (µg/g)
Carmel	3598	11813	3928	26.2	7465	1952	33.4
	± 921	± 1084	± 249	± 2.5	± 445	± 259	± 4.0
Fritz	2914	11627	3574	23.1	7138	1748	33.3
	± 554	± 1416	± 344	± 2.2	± 523	± 248	± 4.0
Grant	3726	11980	4023	27.5	7505	1752	33.3
	± 531	± 1358	± 327	± 2.5	± 588	± 319	± 4.3
Keane	3267	11921	3649	25.2	7308	1899	35.9
	± 677	± 1582	± 335	± 3.1	± 487	± 396	± 6.2
Mission	3334	11141	3741	26.3	7942	2040	38.0
	± 560	± 1192	± 318	± 3.1	± 570	± 321	± 6.0
Ne Plus Ultra	3674	11980	3967	27.2	7653	1765	36.5
	± 353	± 862	± 377	± 3.6	± 469	± 207	±3.8
Peerless	3794	11369	3811	26.7	7269	1726	32.6
	± 746	± 1412	± 316	± 3.9	± 473	± 228	± 4.0
Price	3799	11974	3897	27.2	7647	1916	35.1
	± 776	± 2246	± 427	± 2.5	± 425	± 344	± 4.3
Significance level	NS	NS	NS	NS	NS	NS	NS

Values are mean ± standard error of 32 samples, dry weight.

Fig. 8.1A View of almond orchard at Angle Vale.

Fig. 8.1B Construction of cage supports around Nonpareil tree at Waite Institute.

A

B

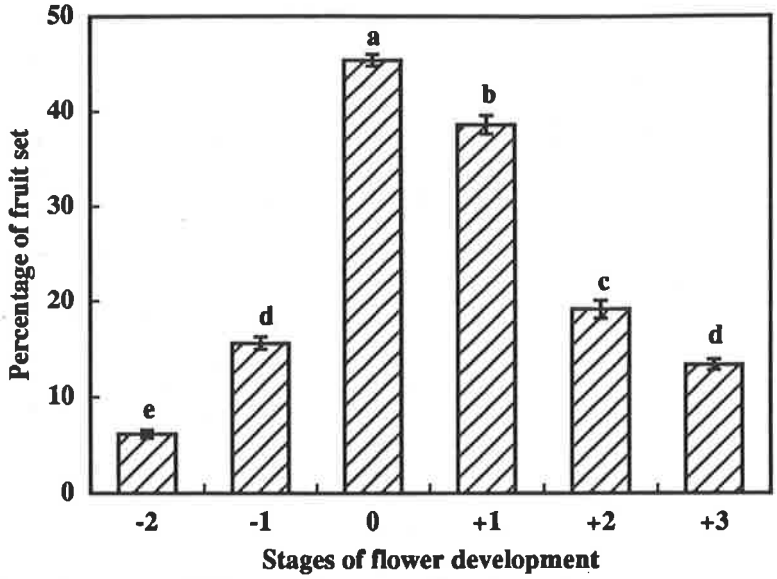


Fig. 8.2 Effect of stages of Nonpareil flower development on percentage of flowers pollinated which set nuts.
Values marked with the same letter are not significantly different based on Tukey's HSD ($P < 0.01$).
Bars indicate standard error of the mean.

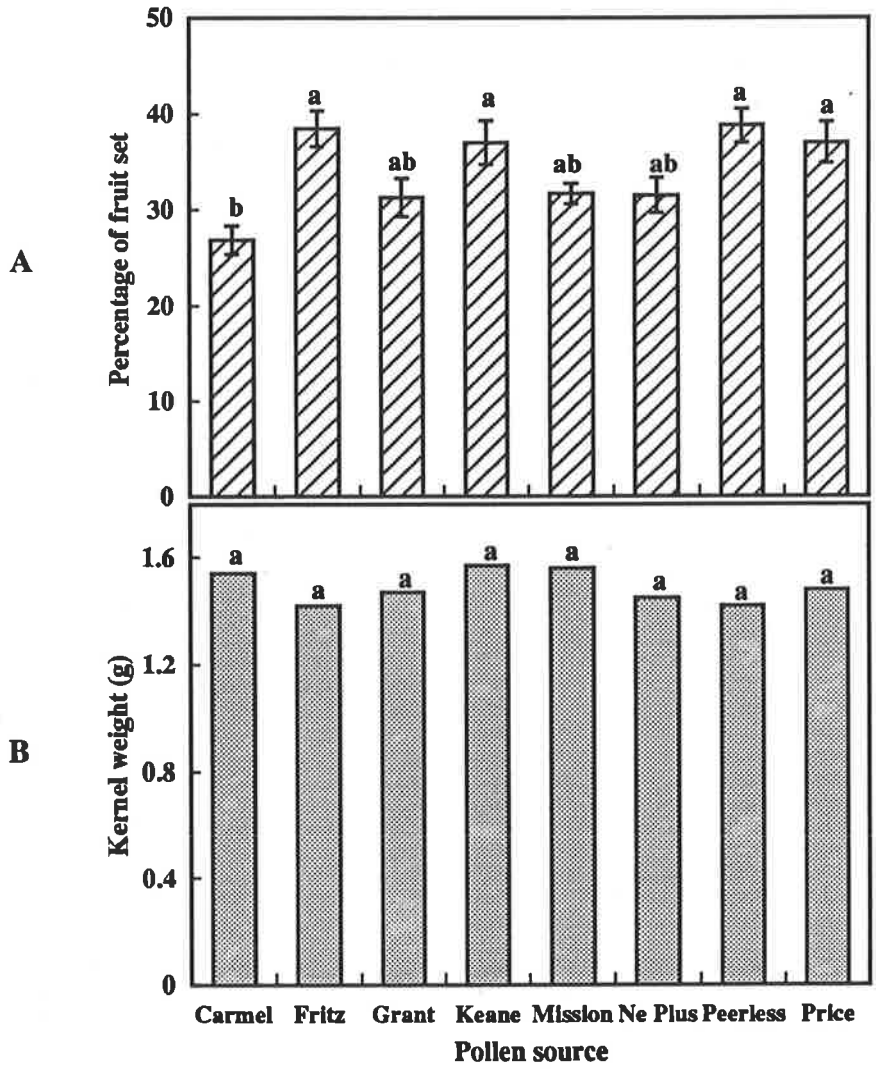


Fig. 8. 3 Effect of pollen parent on percentage of Nonpareil flowers pollinated which set nuts (A) and kernels weight * (B).
Values marked with the same letter are not significantly different based on Tukeys HSD ($P < 0.01$).
* Bars for standard error of mean very small and therefore not shown.

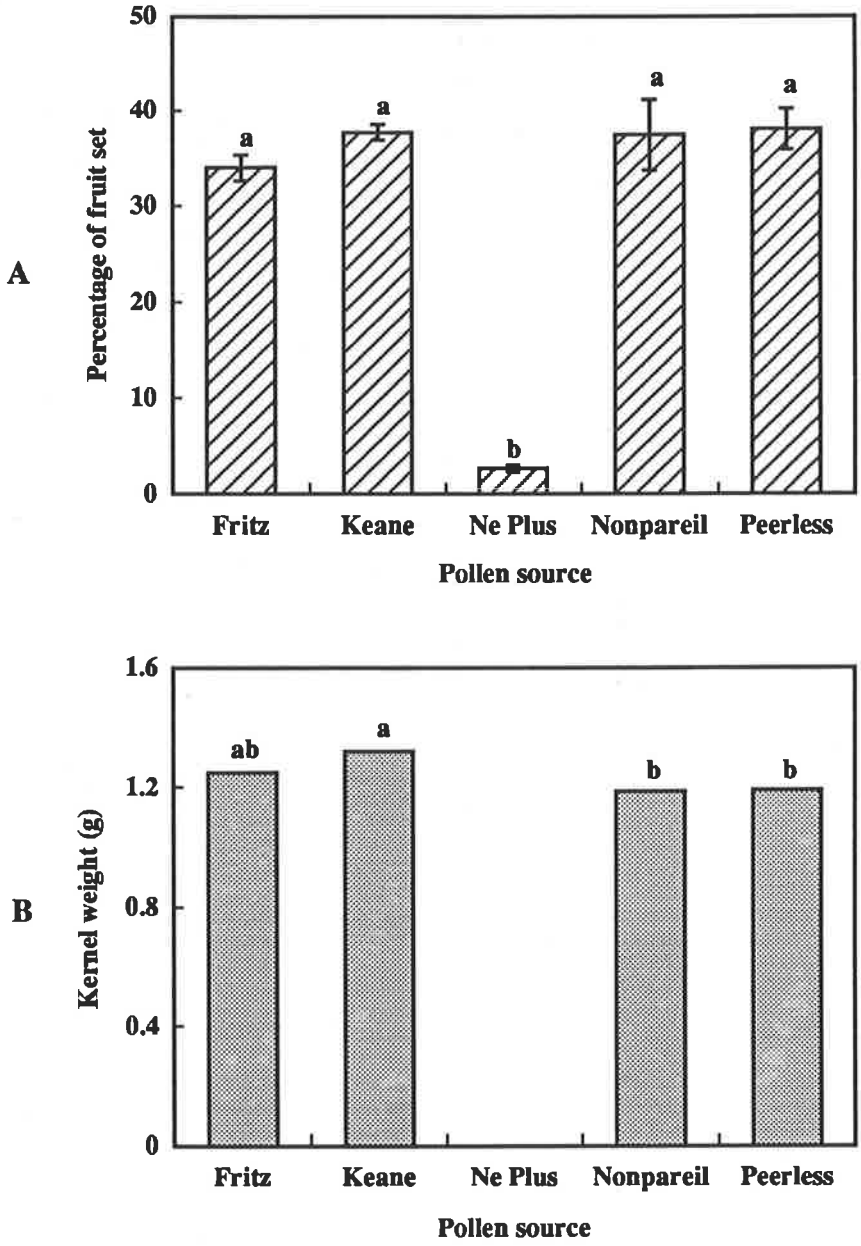


Fig. 8.4 Percentage of flowers pollinated which set nuts (A) and kernel weight * (B), on Price cultivar crossed with 5 different pollen sources.

Values marked with the same letter are not significantly different, based on Tukey HSD ($P < 0.01$).
* Bars for standard error of mean very small and therefore not shown.

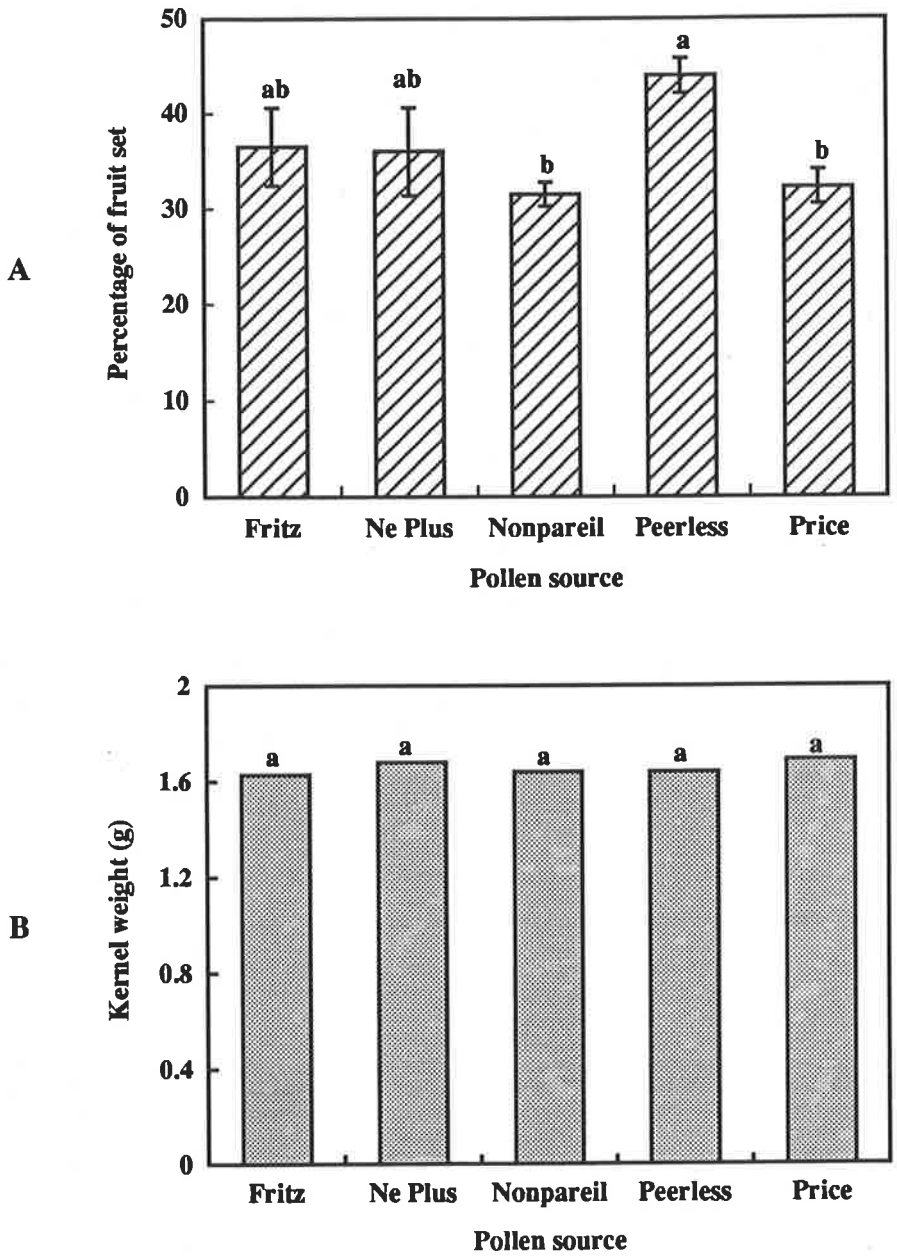


Fig. 8.5 Percentage of flowers pollinated which set nuts (A) and kernel weight * (B), on Keane cultivar crossed with 5 different pollen sources.

Values marked with the same letter are not significantly different, based on Tukey's HSD ($P < 0.01$).
* Bars for standard error of mean very small and therefore not shown.

Chapter 9

Pollen tube growth in Nonpareil almond in relation to pollen genotype, temperature and competition among mixed pollen

Chapter 9

Pollen tube growth in Nonpareil almond in relation to pollen genotype, temperature and competition among mixed pollen

9.1 Summary

Pollen tube growth in Nonpareil almond cultivar was studied in three different temperature regimes (22° C, 15° C and field). Pollen tube growth was examined by light microscopy after 6, 12, 24, 48, 72, 96, and 144 hours after hand cross pollination with two cross-compatible cultivars, namely Keane and Peerless. Additionally, embryos resulting from fertilization by a mixture of pollen from the two above pollen sources were examined using isozyme analysis to estimate the frequency of particular pollen genes in the embryos.

Pollen germination on the stigma surface was found to be related to temperature, and germination began 6 hours after pollination. Pollen tube penetration in upper part of the style began for Peerless pollen after 12 hours at 22° C only, and after 24 hours for the other temperatures. There was no significant difference between the number of pollen tubes observed for all temperatures at days 2, 3, 4 and 6. Pollen tubes were observed at the base of the style after three days for Peerless at 22° C, and after four days for other temperatures. The pollen tube in the micropyle was observed after 4 days in all treatments, except for Keane at 15° C which did not enter the micropyle until after 6 days. The percentage of pollen tubes in the ovule in the field conditions was always higher than that in the controlled growth room experiments at 22° C and 15° C respectively.

Competition between Keane and Peerless pollen when applied together on Nonpareil stigmas was investigated by observations on pollen genes in the resultant embryos. Comparison of 5 different isozyme loci showed that the frequency of pollen genes in the embryo was higher for Peerless, being twice that for Keane genes. Self-pollination of Nonpareil resulted in the pollen tube being rejected on the stigma as well as in the style; swelling at the tip of the tube and also branching of the pollen tube was only rarely observed. Deposition of callose on the tip without swelling was found to be a common indication of incompatible pollen.

9.2 Introduction

Commercial almond cultivars are self-incompatible (Socias i Company *et al.*, 1976; Godini, 1981; Vezvaei *et al.*, 1994) and for a commercial fruit set of 30% or higher, pollinizers are required (Kester and Griggs, 1959a). Almond pollination is a complex phenomenon related to the effective pollination period (EPP) among many other factors. EPP is the period of ovule viability minus the time taken for the pollen tube to grow to the ovule. It represents the period during which pollen transfer must occur for fruit production. The effect of self and cross pollination on pollen tube growth and embryo sac development has been studied by Pimienta and Polito (1983). The rejection of incompatible male gametophytes in almond was found by these workers to occur on the stigma, as well as in the style. In general, self-pollination is characterized by lower pollen grain retention on the stigma, reduced and delayed pollen germination and also by a low frequency of pollen tube growth through the style (Pimienta *et al.*, 1983). In self-compatible cultivars, Godini (1981) showed that self-compatible pollen tubes grow more slowly than cross-compatible pollen tubes. However, Socias i Company and Felipe (1992b) observed that self-pollen tubes in the self-compatible almond cultivar under the study grew as fast as non-self and there was no significant difference between self and non-self pollen tubes grown at 15° C or 22° C. Socias i Company (1976) demonstrated that the rate of pollen tube growth varied after selfing and crossing in both self-incompatible and self-compatible cultivars. Vasilakakis and Porlingis (1984) reported that pollen tube growth in self-pollination studies was faster than that observed for cross-pollination in the self-compatible almond cultivar 'Truoito'. In this cultivar pollen tubes grew faster in self-pollinated than in cross-pollinated flowers at 10° C and 15° C, but at similar rates at 20° C and 25° C, and slower at 30° C. At the present time there are no published comprehensive studies regarding pollen tube growth rates in different cross-compatible cultivars or studies on competition of two mixing pollen sources in almond cultivars.

The objectives of this work were to evaluate the effect of two different pollen genotypes on pollen tube growth in Nonpareil almond at different temperatures, as well as to study competition within a mixture of these two pollen sources in order to determined the frequency of fertilization of ovule by the different male gametes.

9.3 Materials and methods

9.3.1 Pre experimental treatment

Six Nonpareil almond trees (four years-old) were used in this experiment. The trees were taken out of the experimental orchard at the Waite Agricultural Research Institute in June 1993. Trees were placed in large galvanized 150 litre pots and the roots were treated with indole-3-butyric acid (IBA) at 1250 ppm concentration for recovery of the roots and left in the field until blooming time. In July 1993, two trees were moved to a growth room with temperatures of 15° C during the day and 9° C at night and two trees were placed in a growth room with temperatures of 22° C during the day and 12° C at night (Fig. 9.1a). In both growth rooms, the photoperiod was 12 hours (from 7 am to 7 pm) with light intensity of 330 $\mu M / cm^2 / SEC^{-1}$. The remaining two trees were left in the field and covered completely with insect proof nylon mesh, allowing 80 % light transmission.

9.3.2 Pollination treatment

Hand cross-pollination was conducted with pollen from two different pollen sources, Peerless and Keane cultivars. Pollen was collected from branches which had been removed from trees in a commercial orchard before opening and held at 25° C in a growth room, standing in 5% sucrose solution to force the flowers to open and the anthers to dehisce. After collecting the pollen, pollen viability was tested on an agar germinating medium Brewbaker and Kwack (1963). The flowers on each tree in each treatment were emasculated at the balloon stage, and pollinated at random 2 days later (Fig. 9.1b). The pollination treatment was carried out daily over a two week period between 7-10 am. Flower samples from each tree were taken at seven different times, namely 6, 12, 24, 48, 72, 96, and 144 hours after pollination. The pistils were fixed immediately after collection in Carnoy's fluid fixative containing absolute alcohol, chloroform and acetic acid (6:3:1).

For microscopic examination, the style and ovules were dissected from each pistil and both were hydrated in a series of different alcohol/water mixtures (70% and 30%) for 30 minutes, then washed twice with distillated water for half an hour. Samples were autoclaved at 60° C in a solution of 0.8 N sodium hydroxide for an hour. This procedure softens the tissues, which make it easier to stain afterwards with aniline blue at 0.1% in 0.1N potassium

phosphate, and to prepare them for fluorescent microscopic observation of the germination of pollen on the stigma and of pollen tube growth in the style (Linskens and Esser, 1957; Martin, 1959; Sedgley, 1977). Pistils were prepared according to the method of Socias i Company *et al.* (1976), the outer part of the pistils were removed and the transmitting tissues through which the pollen tube grows were assessed by observation under the UV microscope. Fluorescence of the callose deposits in the pollen tubes were induced by aniline blue staining after squashing with one drop of glycerine.

Pollen grains and tubes were observed and counted in three different parts of the style (upper, middle and base) as well as in the ovules, and this was used to estimate pollen tube growth. The data was obtained from 28 pistils in each treatment. In the field conditions temperature and rainfall were recorded during the flowering period (Figs. 9.2a and 9.2b).

9.3.3 Statistical analysis

A generalized linear model (McCullagh, and Nelder, 1989) with poisson error was fitted to the number of pollen grains on the stigma. The number of pollen tubes in the upper style is a function of the number of pollen grains on the stigma, and accordingly a generalized linear model with binomial error structure was fitted. Similar models were fitted to the counts of pollen tubes in the mid and base style, ovary, and ovule, regarding each as a count out of the number of pollen tubes in the immediately preceding pistil section. For example, the number of pollen tube in the lower style was modelled as counts out of the number of pollen tubes in the mid style. Separate models were fitted for each time under style.

The resulting analysis of deviance Tables were used to test for effects of pollen source and temperature.

9.3.4 Pollen ultrastructure of exine surface

The pollen grain ultra structure of the exine surface of Keane and Peerless pollen was studied after preparation for electron microscope at a magnification of x1500 and x10,000.

9.3.5 Pollen mixture treatment

The pollen mixtures were prepared from an equal volume of pollen obtained from Peerless and Keane cultivars, and thoroughly mixed with a vortex mixer; then used in the field conditions. Progeny (153) kernels resulting from hand cross pollination of mixed pollens were analysed individually with four enzyme, systems namely asparatate amino transferase at locus one (AAT-1), isocitrate dehydrogenase (IDH), leucine amino peptidase at locus one (LAP-1), and phosphoglucomutase at loci one and two (PGM-1 and PGM-2) in order to determine which male gamete gene was in the embryo. Electrophoresis conditions for isozyme analysis were as described in section 2.2 of this thesis.

9.4 Results

9.4.1 Average of pollen grains retained on the stigma

The number of pollen grains retained per stigma varied with temperature and pollen source (Fig. 9.3). Pollen germination on the stigma surface was observed from 6 hours after pollination and increased with time. Pollen germination has a positive relationship with increasing the temperature and depends on the genotype. Pollen germination on the stigma at 22° C was observed to be more than that measured at 15° C and in the field conditions.

9.4.2 Proportion of pollen tubes in upper part of style, base of the style and ovule

Pollen tube growth in the style varies significantly with time and temperature ($P < 0.01$). The percentage of pollen tubes present in the upper part of the style increases with time; pollen tube penetration in the upper part of the style started after 12 hours at 22° C only for Peerless, and after one day for genotype or temperature (Table 9.1). There were no significant difference between the number of pollen tubes present at days 2, 3, 4 and 6 for the three different temperature. Pollen tubes were observed at the base of the style after 3 days only for Peerless at 22° C and 4 days for all other genotype, treatment combinations (Table 9.2). Pollen tubes were observed in the micropyle after 4 days in all treatments except for Keane at 15° C, where they were first observed started after 6 days (Table 9.2). The percentage of pollen tubes which had reached the ovule in the field conditions was higher than that observed for the 22° C and 15° C temperatures respectively (Table 9.3). In

almond as for other *Prunus* species, two anatropous ovules are present in the flower bud, one of these ovules in Nonpareil almond is typically aborted. In this experiment, four of the samples at 22° C showed the two presented ovules had pollen tube in the micropyle.

9.4.3 Frequency and growth of pollen tubes in the style

The number of pollen tubes that growing in the upper part of the style was always more than continued to grow observed in the middle style and the base of the style (Figs. 9.4c and 9.4.g). In this particular experiment no more than one tube was observed in the micropyle of the ovules (Fig. 9.4d).

9.4.4 Effect of pollen genotype on germination and pollen tube growth

Germination and the number of tubes in the upper portion and base of the style and in the ovules differed significant for the different pollen genotypes at $P < 0.05$.

9.4.5 Frequency of fertilization from a mixture of two pollen sources

Comparison of four different isozyme systems showed that the frequency of genes in the Nonpareil embryo which can detected was twice as much for Peerless as for Keane (Table 9.4).

9.4.6 Self pollination treatment

In self-pollination treatments on Nonpareil flowers, swelling of the pollen tube tip and branching of the pollen tube, although present, is only rarely observed (Figs. 9.4e and 9.4f). Rejection of the pollen tube takes place on the stigma as well as in the style. Callose deposition at the tip of the tube without swelling was a more common indication of the incompatible tube.

9.4.7 Pollen grain ultrastructure

Pollen grain ultrastructure and exine pattern finger prints are shown in Figurs 9.4a and 9.4b. The pollen grains of Keane cultivar are smaller than Peerless and have fewer pores in the exine and higher depth of ridges.

9.5 Discussion

In temperate tree crops the rate of pollen tube growth to the base of the style is quite low (Sedgley, 1989). Pollen tube growth in the style of almond is not as rapid as in other fruit trees such as avocado (Sedgley, 1977). This experiment showed that in Nonpareil almond, Peerless pollen tubes take 4 days to reach the base of the style both at 22° C and under field conditions, but at 15° C only a small proportion of tubes were observed at the base of the style by this time. These results are similar to those of Pimienta *et al.* (1983). They found 4 days was needed for the pollen tube to reach the base of the style and 7-8 days to reach the embryo sac. Pollen tube behaviour in almond is therefore similar to that described by Lewis (1942) for cherries.

Observation of pollen tube growth in self pollinated Nonpareil showed that inhibition occurred in the stigma as described by Pimienta *et al.* (1983), as well as in the middle part of the style; this result is typical of the gametophytic incompatibility systems found for the *Rosaceae* (Crowe, 1964). Swelling of the tip of self pollen tubes in the almond was reported by Socias i Company *et al.* (1976). We observed that inhibition of tubes in the style was accompanied by swelling and callose deposition at the tips. This has been considered to be a classical expression of the incompatibility response of almond, but swelling at the tip was rarely observed by Pimiento *et al.* (1983). In almond, pollen tube retrogression could be due to the destruction of the incompatible pollen tube as has been observed in cherry and *Lycopersicon peruvianum* (Nettancourt *et al.*, 1973). Typically, studies which compare pollen tube growth of compatible and incompatible pollinated pistils showed that incompatible pollen tube grow more slowly than compatible pollen tubes. Our observations indicate that in almond differences between two compatible pollen sources in reaching the micropyle might be due to faster germination and penetration of the stigma and style rather than the rate of pollen tube growth in the style. This phenomenon is supported by the study of a comparison of self and cross pollinated Nonpareil almond by Pimienta *et al.* (1983). These authors found that the time required for compatible and incompatible tubes to reach the base of the style in almond pistils is different. This difference, as shown here, almost entirely due to delayed pollen germination in self pollinated pistils, rather than to a slower rate of growth in the style. Pollen on the stigma of Nonpareil almond started to

penetrate the style after 12 hours at 22° C for Peerless and at 24 hours after pollination in other temperature treatments. In most cases there is faster penetration of the Peerless pollen on the stigma of the Nonpareil almond compared to that by the Keane cultivar. We believe that faster germination of the pollen tube during the first day after pollination is an important factor in reaching the embryo sac more rapidly and fertilizing the egg (before the end of the EPP). The rate of the pollen tube growth is not greatly different in compatible pollen tubes. In this experiment rate of the pollen tube growth at 15° C is much slower than at 22° C and in the field experiment, slower in the 4 days after pollination but greater at 6 days after pollination.

Delay in pollen germination might be related to a genetic response, perhaps due to the pollen size and exine sculpture of the pollen grain. As Sedgley (1989) reports, pollen germination and tube growth is promoted by temperatures above 10° C, thus there appears to be a balance between female and male fertility which has led to the concept of (EPP).

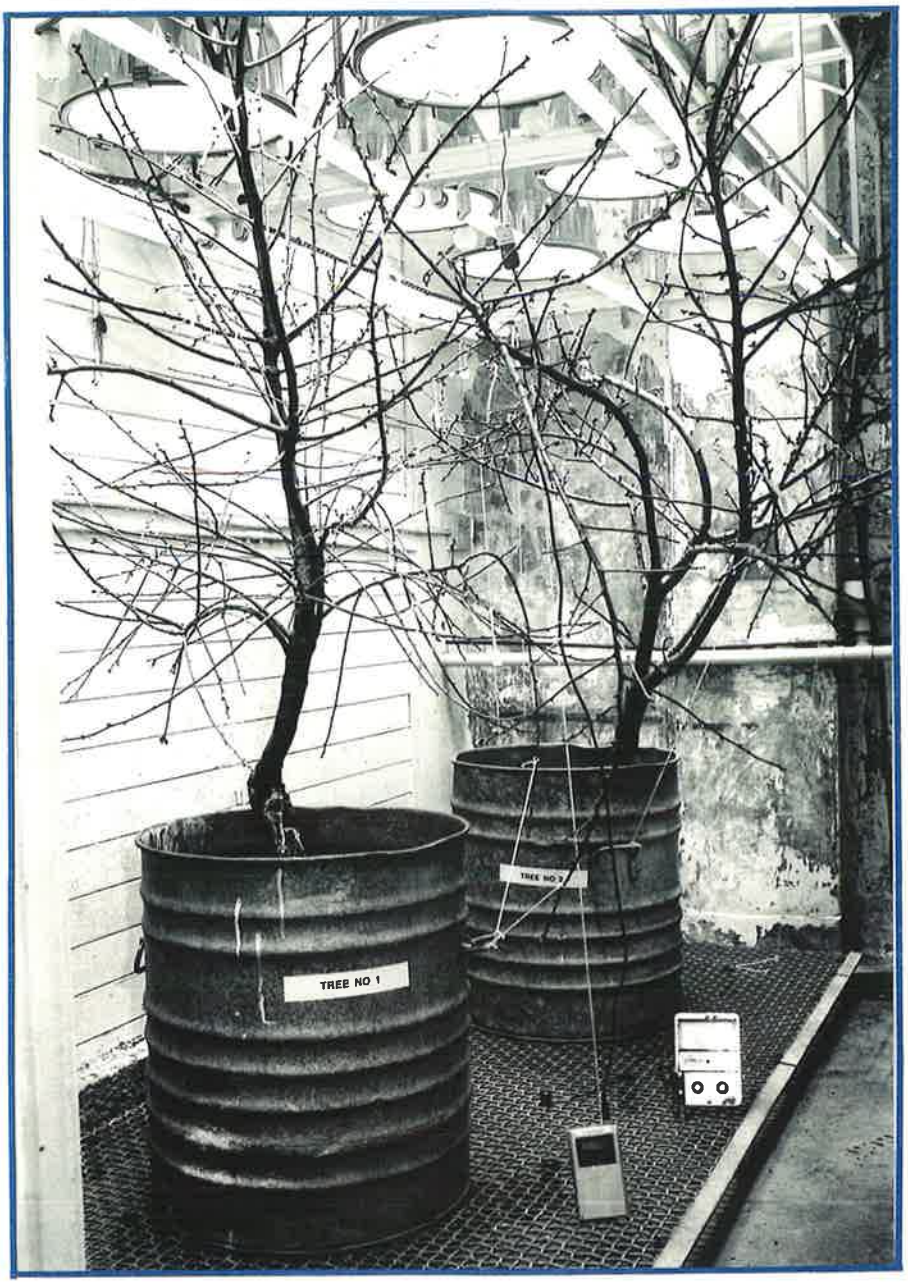
In almond, the embryo sac is not quite mature when the tubes reach to ovule the base of the style and the tube is not immediately attracted as was described in avocado by Sedgley (1989). In cross pollinated Nonpareil almond, pollen tube growth to the base of the style and the upper portion of ovary stops or slows greatly within 4 days until the final stage of embryo sac development differentiation is completed. Three to four days later the embryo sac is fully developed (Pimienta *et al.*, 1983). In this study we found that embryo sac development was delayed at 15° C and promoted at 22° C. Maturation of ovules in almond is similar to that of peach as described by Herrero and Arbeloa (1989).
Isozyme analysis of embryos obtained from the mixture of pollen showed that there was a significant difference between fertilization of ovules by pollen from Peerless and Keane cultivars.

We concluded that the effects of observed for pollen genotype, time, and temperature are significant. It is clear that significantly more ovules are successfully penetrated on day 6 than day 4. Pollen from Peerless results in higher ovule penetration than that from Keane. The temperature effects were significantly different, but repeating the experiments would be advantageous. Nonetheless, the temperature effects were clear, with 22° C / 12° C giving significantly higher pollen tube growth than for field conditions and for 15° C / 9° C regime.

Fig. 9.1A Almond trees in growth room for pollen tube study.

Fig.9.1B Emasculation of almond flowers for hand-cross pollination.

A



B

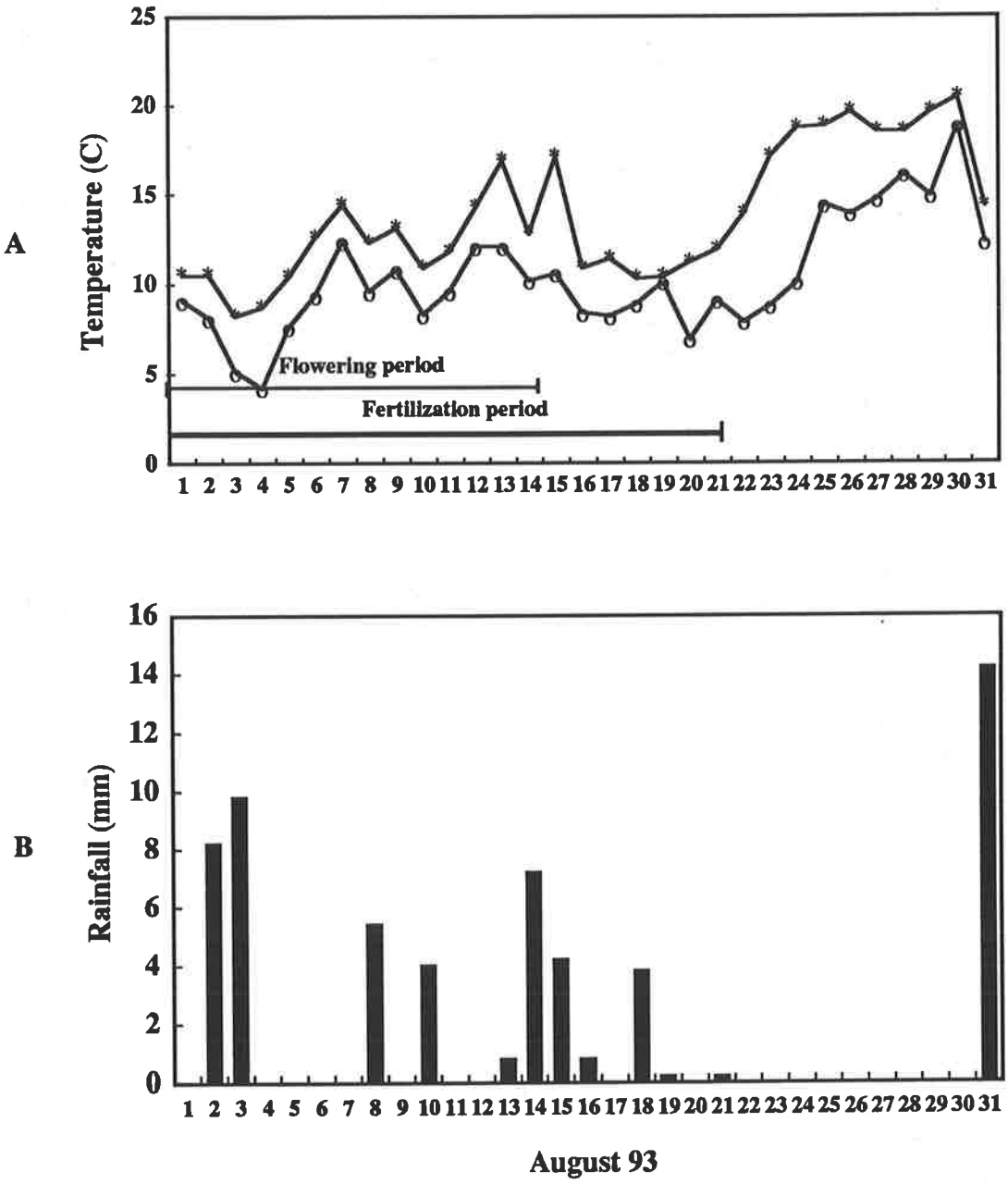


Fig. 9. 2 Daily values for Maximum and Minimum temperature (A) and rainfall (B) during flowering period in August 1993.

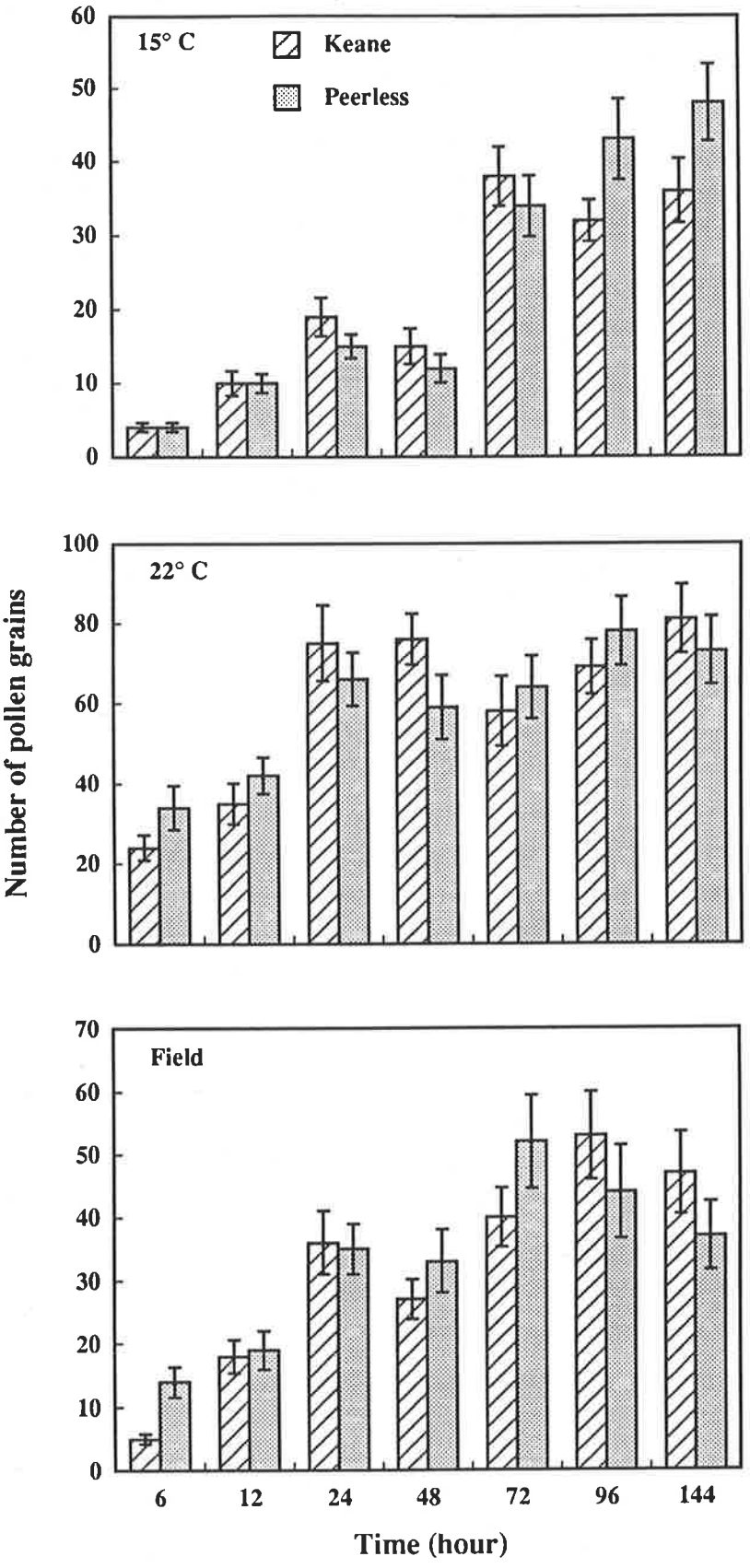


Fig. 9.3 Mean number of pollen grains on the stigma surface at three different temperatures.

Table 9.1 Percentage of pollen tubes penetrating different parts of the pistil at 22° C

Pollen Source	Time (hour)	22° C		
		Up style [1]	Base style [2]	Ovule [3]
Keane	6	0.0	0.0	0.0
Peerless	6	0.0	0.0	0.0
Keane	12	0.0	0.0	0.0
Peerless	12	17.0± 3.3	0.0	0.0
Keane	24	39.0± 2.8	0.0	0.0
Peerless	24	55.0± 2.7	0.0	0.0
Keane	48	64.0± 2.6	0.0	0.0
Peerless	48	67.0± 3.8	0.0	0.0
Keane	72	58.0± 2.4	0.0	0.0
Peerless	72	69.0± 2.6	4.0± 1.1	0.0
Keane	96	57.0± 2.7	10.0± 0.86	21.0± 6.0
Peerless	96	63.0± 3.0	12.0± 2.2	19.0± 5.2
Keane	144	55.0± 2.9	10.0± 1.1	27.0± 5.2
Peerless	144	63.0± 3.4	13.0± 2.2	40.0± 6.8

1. % pollen tubes penetrating the upper style given the number of pollen grains applied to stigma.
2. % pollen tubes penetrating to base of style given the number of pollen tubes in the upper style.
3. % pollen tubes penetrating the ovule given the number of pollen tubes in the base of style.

Mean ± standard errors of 28 samples.

Table 9.2 Percentage of pollen tubes penetrating different parts of the pistil at 15° C

Pollen Source	Time (hour)	15° C		
		Up style [1]	Base style [2]	Ovule [3]
Keane	6	0.0	0.0	0.0
Peerless	6	0.0	0.0	0.0
Keane	12	0.0	0.0	0.0
Peerless	12	0.0	0.0	0.0
Keane	24	35.0± 3.8	0.0	0.0
Peerless	24	40.0± 3.9	0.0	0.0
Keane	48	60.0± 5.0	0.0	0.0
Peerless	48	67.0± 4.5	0.0	0.0
Keane	72	60.0± 3.3	0.0	0.0
Peerless	72	58.0± 3.0	0.0	0.0
Keane	96	51.0± 3.0	0.4± 0.7	0.0
Peerless	96	56.0± 3.1	7.0± 1.3	2.0±.2.0
Keane	144	54.0± 3.7	12.0± 3.5	14.0± 6.4
Peerless	144	67.0± 3.3	11.0± 1.5	18.0± 6.6

1. % pollen tubes penetrating the upper style given the number of pollen grains applied to stigma.
2. % pollen tubes penetrating the base of style given the number of pollen tubes in the upper style.
3. % pollen tubes penetrating the ovule given the number of pollen tubes in the base of style.

Mean ± standard errors of 28 samples.

Table 9.3 Percentage of pollen tube penetrating the different parts of the pistil under field conditions

Pollen Source	Time (hour)	Field conditions		
		Up style[1]	Base style[2]	Ovule[3]
Keane	6	0.0	0.0	0.0
Peerless	6	0.0	0.0	0.0
Keane	12	0.0	0.0	0.0
Peerless	12	0.0	0.0	0.0
Keane	24	36.03.5	0.0	0.0
Peerless	24	49.0± 3.4	0.0	0.0
Keane	48	59.0± 4.6	0.0	0.0
Peerless	48	71.0± 5.0	0.0	0.0
Keane	72	66.0± 4.6	0.0	0.0
Peerless	72	71.0± 2.5	2.0± 0.5	0.0
Keane	96	45.0± 3.6	13.0± 2.3	17.0± 6.6
Peerless	96	53.0± 3.3	14.0± 3.8	28.0± 7.8
Keane	144	52.0± 4.1	15.0± 4.8	41.0±.7.6
Peerless	144	53.0± 4.4	20.0± 4.7	49.0± 9.3

1. % pollen tube penetrating the upper style given the number of pollen grains applied to stigma
2. % pollen tubes penetrating the base of style given the number of pollen tubes in the upper style.
3. % pollen tubes penetrating the ovule given the number of pollen tubes in the base of style.

Mean ± standard errors of 28 samples.

Table 9.4 Genotype of Nonpareil almond used (as a mother source), Keane and Peerless used as the (two mixed pollen donors) and possible isozymes genotype of resulting embryo for distinguishing male genes in the individual embryo

Cultivar	individual isozyme loci				
	AAT	IDH	LAP	PGM-1	PGM-2
Nonpareil	ab	ab	bc	ab	ab
Keane	ab	ac	cc	ab	bb
Peerless	nn	bb	bb	ab	ab
Possible genotypes in progeny after mixed pollen application	aa:either combination bb:either combination ab: Keane	aa: Keane bb: Peerless ac: Keane bc: Keane ab: either combination	bb: Peerless cc: Keane bc: either combination	aa: either combination bb: either combination ab: either combination	aa: Peerless bb: either combination ab: either combination

Number of male genes positively identified at each of the locus studied

	AAT	IDH	LAP	PGM-1	PGM-2
Keane	8	21	11	-	-
Peerless	-	49	28	-	12

Overall Percentage of male genes detected in embryos

Keane	26.1
Peerless	58.2
Undecided	15.7

Fig. 9.4 Pollen ultrastructure, exine surface and pollen tubes growing in the style of almond.

A - Pollen ultrastructure (x1500) and exine surface (x10,000) for Peerless.

B - Pollen ultrastructure (x1500) and exine surface (x10,000) for Keane.

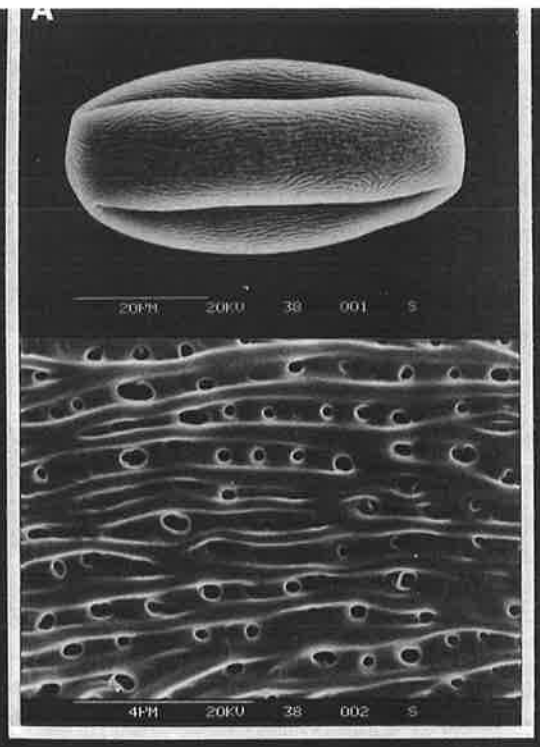
C - Pollen tubes growing on stigma of Nonpareil cultivar at 22° C.(x 75)

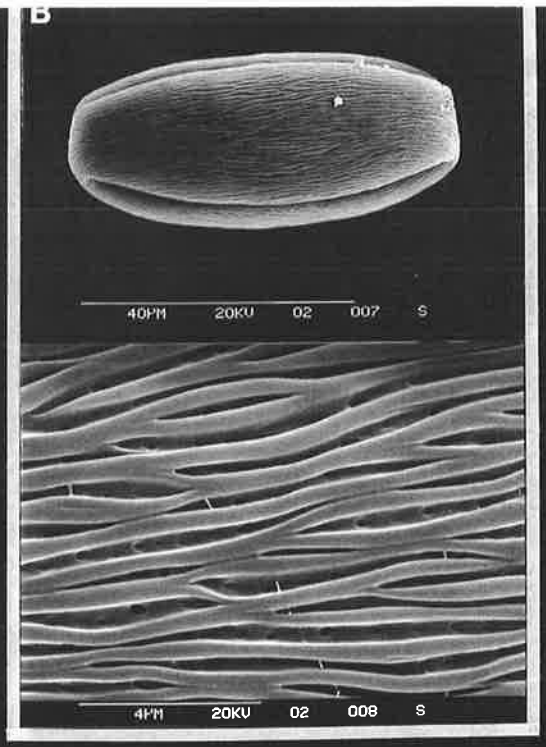
D - Pollen tube in the base of the style (**pt**) and embryo sac (**e**) (x 75) of Nonpareil.

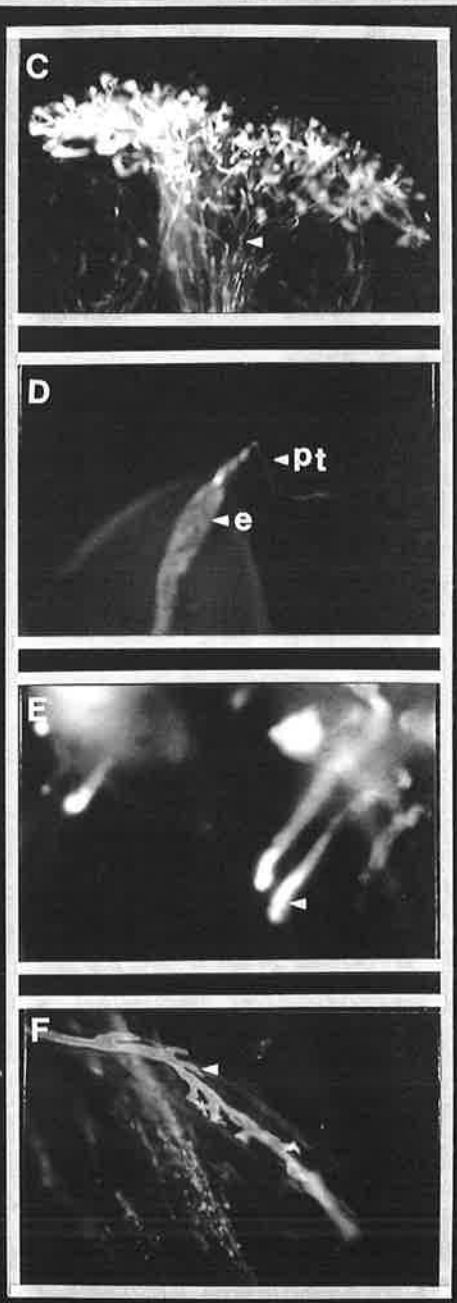
E - Pollen tubes arrested in self-incompatible Nonpareil cultivar stigma. Arrow shows callose deposition in tube tip (x 150).

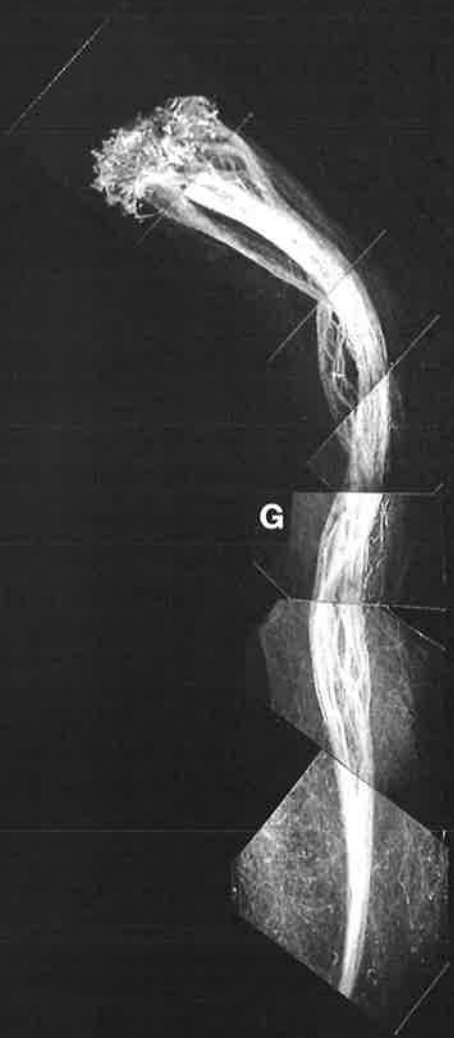
F - Branching of self-incompatible pollen tube in the style of Nonpareil almond (x 150).

G - Peerless pollen tube growing in Nonpareil almond style (x 37.5).


A
20PM 20KV 38 001 S
4PM 20KV 38 002 S


B
40PM 20KV 02 007 S
4PM 20KV 02 008 S


C
D
pt
e
E
F


G

Chapter 10

Gene flow by pollen in an almond orchard as determined by isozyme analysis of individual kernels and honeybee pollen loads

Chapter 10

Gene flow by pollen in an almond orchard as determined by isozyme analysis of individual kernels and honeybee pollen loads

10.1 Summary

The pattern of gene flow by pollen in a line of 18 trees in a commercial orchard was investigated. The orchard planning followed a 1:1 design of a row of main 'Nonpareil' cultivar alternating with rows of pollinizers including Carmel, Fritz, Keane, Peerless and Price rows, the rows being at right angles to the line of 18 tree studied. Results showed that in most cases gene flow by pollen is highly leptokurtic, with by far the highest flow coming from the nearest neighbour tree. An exception was the pollen gene flow between Fritz and Keane trees, which was higher than expected for trees not nearest neighbours. Examination of pollen brought back to the hive suggested that 80 to 90 % of pollen loads consisted pollen from only one cultivar (not mixed), suggesting a high degree of fidelities for honeybees collecting from one cultivar only.

10.2 Introduction

Pollen dispersal and pollen mediated gene dispersal in flowering plants is affected primarily by animals (insects, birds and mammals) and air currents (Levin and Kerester, 1969). Among gene flow studies of vascular plants, a considerable number of experiments have been conducted on the population genetics of angiosperms, gymnosperms and pteridophytes (Haufler, 1987). Much work on estimating pollen flow is based on detailed observation of pollinator movements, which assumes a close correlation between these movements and pollen transferred among flowers (Free, 1970a). Actual gene flow has been measured in several agronomic studies where interest has been in the minimum distance needed to isolate a particular cultivar. In the present studies, cultivars with known genetic markers were used, and the F_1 progeny is examined for the marker phenotypes (Bateman, 1947; Jackson and Clarke, 1991).

Studies of inter-population gene flow have been conducted in only a few species of horticultural crops (Gary *et al.*, 1977; Handel, 1982 and 1983a; Jackson and Clarke, 1991).

Pollen flow in entomophilous plants is a function of pollination flights made by the insect; if all pollen is deposited on the first recipient plant so that there is no carry-over, the pollen dispersal distance will be equivalent to the pollinator flight distance (Levin *et al.*, 1971). Molecular markers were used to characterize the level and pattern of genetic diversity in fern and its implications for gene flow by spore (Ranker, 1992). Ellstrand *et al.* (1989) used genetic markers in experimental and natural stands of wild radish to measure the pattern of variation of gene flow by pollen into small populations. Handel (1982) used a gene marker in melon (*Cucumis melo*) which is expressed in the F_1 generation as a yellow cotyledon. He found that there is a negative correlation between the occurrence of genetically heterozygote seedlings and distance from the gene source in the centre of the field. In other studies Handel (1983a) used cucumber (*Cucumis sativa*) which were homozygous for the dominant bitter allele which gives the cotyledons and leaves of the plants a distinctive distasteful flavour. He found that the pattern of gene flow by pollen was very restricted, most of the bitter pollen was deposited 2-3 m from the central bitter block, the results were consistent with his first experiments.

Gene flow by pollen is important in entomophilous plants such as almond with gametophytic self-incompatibility and in which fertilization of the egg is essential and directly affected by transfer of pollen by the honeybee. The aim of the present study is to elucidate the pattern of gene flow by pollen in an entire line of 18 almond trees including several different cultivars in a commercial orchard at Angle Vale, South Australia. From these results we can estimate which cultivar are the best pollinizers for Nonpareil and work out a better planting design for orchards in this area.

10.3 Materials and methods

10.3.1 Orchard design

The almond orchard used for this study is at Angle Vale South, Australia. It is sited 35 km NW Adelaide, it has a Mediterranean climate with average rainfall of 460 mm. Trees are irrigated with a low sprinkler system. Bees were brought into the orchard in June, 1992 and 1993 just before flowering (10 hive/h), arranged in groups of 4 to 10 hives at several points through the orchard. The orchard is made up of rows of almond trees, each row

being composed of one particular cultivar so that a row of Nonpareil cultivar is always next to a 'pollinizer' cultivar row, followed by another row of Nonpareil and so on (i.e.a 1:1 planting). Gene flow by pollen was studied in a line of 18 trees at right angles to the single cultivar rows. In other words in a line across 18 rows (Fig. 10.1). Distance between the rows is 7.3 m and between the trees in the rows 5.5 m. The main cultivar is Nonpareil (8 rows) and pollinizers (10 rows) included Carmel, Fritz, Keane, Peerless and Price.

10.3.2 Plant materials

In 1992 and 1993 at harvest time, 200 nuts were collected at random from two sections of each tree in the study. These sections corresponded to the geographical east and west halves of the canopy of each tree. Each row in the orchard runs N to S. The nuts were then used for isozyme analysis, in order to determine which pollen genes were in the embryo of each nut. In 1993 pollen 'pellets' were collected from one of the hives fitted with a pollen trap, with the aim of analysing the pollen brought back by honeybees to the hive, for its cultivar of origin. From this we can determine which cultivars the bees are foraging from at various times. Flowering times for each of the cultivar was recorded and the total number of fruits and individual kernel weights recorded in the selected rows during the two years of study.

10.3.3 Isozyme analysis

Seven isozyme systems were utilized: glucose phosphate isomerase (GPI-2), leucine amino peptidase (LAP-1), asparatate amino transferase (AAT), phosphoglucomutase at the first and second loci (PGM), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6PD) and shikimate dehydrogenase. Paternity analysis of each nut collected was examined by the methods described in section (2.2) of this thesis.

10.4 Results

10.4.1 Flowering time and total yield

Flowering times of the cultivars recorded during the two years of study are shown in Table 10.1. Fritz and Keane are early blooming cultivars, Nonpareil and Peerless

intermediate and Price and Carmel later than the others. Total yield and kernel weight of each tree in the selected rows are shown in Table 10.2. Yield as kernels varies between the rows and in some parts the Nonpareil is higher than others especially in the Nonpareil row between the two pollinizer Keane and Price. Because of the variation between the rows we could not calculated the analysis of variance. The highest kernel yield were recorded for Nonpareil and Keane trees and lowest for Peerless. Nut characteristics of cultivars under study are shown in Table 10.3.

10.4.2 Isozyme paternity and gene flow distribution

Isozyme paternity using 7 isozymes is shown in Table 3.1 (chapter 3) for each cultivar studied. Gene flow distribution by pollen in the experimental section is detected by comparison with the existing maternal alleles for each cultivar. Histograms showed that in most cases gene flow distribution is leptokurtic with the highest percentage of the gene flow by pollen from the nearest neighbour to each cultivar (Fig. 10.2). The percentage of pollen gene from a particular source decreased sharply in the second tree away from the pollen source and become very small in the third tree away, and so on. For example gene flow distribution for particular alleles (genes) showed that for Keane cultivar pollen, the percentage of 'Keane' genes detected in the nuts of the cultivar Price in the first row away from the pollen source is 30% of total genomic content in the east (nearest) canopy section and 17% in the west canopy section and in the second row away (Nonpareil cultivar) 9% east and 3% west.

However the leptokurtic distribution 'rule' breaks done in the third row away from the Keane pollen source. The Fritz cultivar is found in this row, its nuts showed 21% Keane pollen gene in both its east and west canopy sections. In the row of Nonpareil (fourth row) percentage of Keane pollen gene detected is only 2% in the east and 1% in the west. Comparison of the first five tree in the line and most other results from the study demonstrated that the maximum gene flow is normally just two trees away (15 m). However the increasing percentage of pollen genes from Keane in trees 4 and 12 (both Fritz trees) showed that bees must fly with increased frequency between these two cultivars to explain the abnormally high amount of Keane genes in Fritz nuts. The reason for this may

be due to overlapping of flowering time at the beginning of blooming period in the orchard when no other cultivars are flowering, or according to the 'scent' of these two cultivars. The percentage of genes detected from the Keane cultivar in Fritz nuts is greater than from Fritz to Keane (although both are high). Gene flow study in four Nonpareil trees in the line of gene flow study over two years (1992 and 1993) showed that in most cases gene flow distribution and percentage of gene detected was similar for both years (Fig. 10.3).

10.4.3 Isozyme analysis of honeybee pollen load

Isozyme analysis of pollen pellets showed that the fidelity of the honeybee is high and for more than 80 % of the time the bees visited only one cultivar during any particular flight (Fig. 10.4).

10.4.4 Competition between almond pollen and other species

Inspection of pollen loads which were collected from bee hive in 'pollen traps' showed that honeybee collected pollen from other species when the almond pollen available is low (at the beginning and at the end of flowering period). Percentage of fidelity at the beginning and at the end of flowering is lower than at full bloom, it is possible that cross-pollination between cultivars would be greatest at beginning and end of flowering period (Table 10.4).

10.5 Discussion

Results from gene flow experiments showed that as found by others for anemophilous pollen movement (Handle, 1983b; Richards, 1986), distribution of almond pollen genes is highly leptokurtic. As pollen carry-over itself tends to be leptokurtic in distribution, thus most pollen is carried to the next flower visited. For most flower visitors, length of flight between flowers is dependent on their behavioural strategy at that moment for a foraging bee. As described by Levin and Kerster (1968) for bee pollinated plants between 90% and 99% of flight are short distance foraging flights. The percentage of Keane genes detected in nuts from Fritz cultivar were more than from other cultivars, including the tree next to it, the reason for this may depend on the lack of blooming of other cultivars at the time both are flowering (flower density) or may be due to other flower characteristics

such as odour or nectar sugar encouraging more visits by bees (Heinrich and Raven, 1972; Heinrich, 1975). Similar results were found in another almond orchard with different design in the same area (Jackson and Clarke, 1991). The authors suggest that each bee visits only one cultivar by flying alone the rows cultivar before it visits another cultivar in the next row. They also suggest that accidental visiting an alternative cultivar may result in most fertilization and fruit set resulting in the comparatively low yield of nuts compared with what is theoretically possible.

Maximum distance of gene flow from Keane cultivar with particular and distinctive allele 'c' for IDH was highest in the first row next to it and lower in the second row away and lowest in row number 5 (30 m) with the exception of Fritz which has the high percent of pollen genes from the Keane cultivar. For the most part gene flow distribution in the orchard is similar to the second model of Levin and Kerster (1969) possible reasons for this are discussed above; this model assumes that 50% of pollen from a plant is deposited upon the next plant, 25% on the second plant, 12.5% on the third plant, 6.5 % on the fourth plant, and 6% on the fifth plant. Also similar to Levin *et al.* (1971) they produced a carry-over model to estimate directionality on the pollen dispersal, this model assume that 40 % of the pollen was deposited on the first plant, 20% on the second, 10% on the third 5% through 8th and plant, at least 80% of flights are less than 1 m in the distance and 99% are less than 5 m. 2.5% on each of the 9th and 10th plants. Gene flow studies shown here suggest that the combination of the two pollinizers Keane and Carmel for Nonpareil is not recommended because these two cultivar as pollinizer do not overlap well in flowering time, but the combination of Keane and Price or Keane with Fritz is recommended on purely pollination results. Results obtained in 1992 was similar to that found in 1993.

Although Fritz pollen genes do appear in certain parts of the orchard in higher than expected amounts, nevertheless we are not recommending extensive use of this cultivar in the Angle Vale area of South Australia due to a small nut size, and its sensitivity to salt levels especially with the rootstock (Nemaguard) used at the present. Carmel blooming appears to be later than Nonpareil and is not such a vigorous tree at Angle Vale. We are therefore not recommending Carmel either. However Price, Peerless and Keane are effective pollinizers and flowering overlaps well with Nonpareil. The fact that in the most cases pollen genes

predominantly from the nearest tree, implies that pollinizer trees, should be next to Nonpareil, even to the extent that they are alternated in the Nonpareil rows. This may cause problems at harvest time but our results suggest improved yields would result. Four possible improved planting designs for orchards are shown in Figure 10.5.

At the end we believe that the almond yield would increase from planting design A to D respectively, but in design C and D the income might be decreased due to possible mixing of nuts at harvest.

Fig. 10.1 Plan of part of the commercial almond orchard selected for gene flow at Angle Vale. Centrally located lines indicate the line of trees used to study gene flow by pollen.

★ Carmel cultivar.

Table 10.1 Flowering data during 1992-1993 for almond cultivars in Keane orchard at Angle Vale

Cultivar		Year 1992					year 1993				
Carmel	a	8/8	16/8	19/8	24/8	5/9	13/8	16/8	19/8	24/8	29/8
	b	1%	40%	60%	75%	100%	3%	50%	70%	80%	100%
Fritz	a	31/8	7/8	13/8	20/8	_	7/8	13/8	15/8	18/8	-
	b	3%	40%	80%	100%		1%	60%	80%	100%	
Keane	a	31/7	7/8	13/8	16/8	-	7/8	13/8	16/8	-	-
	b	2%	30%	70%	100%		1%	80%	100%		
Nonpareil	a	31/7	7/8	13/8	16/8	19/8	7/8	12/8	16/8	19/8	24/8
	b	4%	20%	40%	80%	100%	5%	10%	50%	95%	100%
Peerless	a	31/7	7/8	13/8	18/8	20/8	7/8	13/8	16/8	19/8	24/8
	b	5%	10%	50%	80%	100%	5%	10%	60%	90%	95%
Price	a	8/8	13/8	16/8	18/8	-	7/8	13/8	16/8	19/8	-
	b	5%	20%	60%	100%		2%	5%	50%	100%	

(a) date

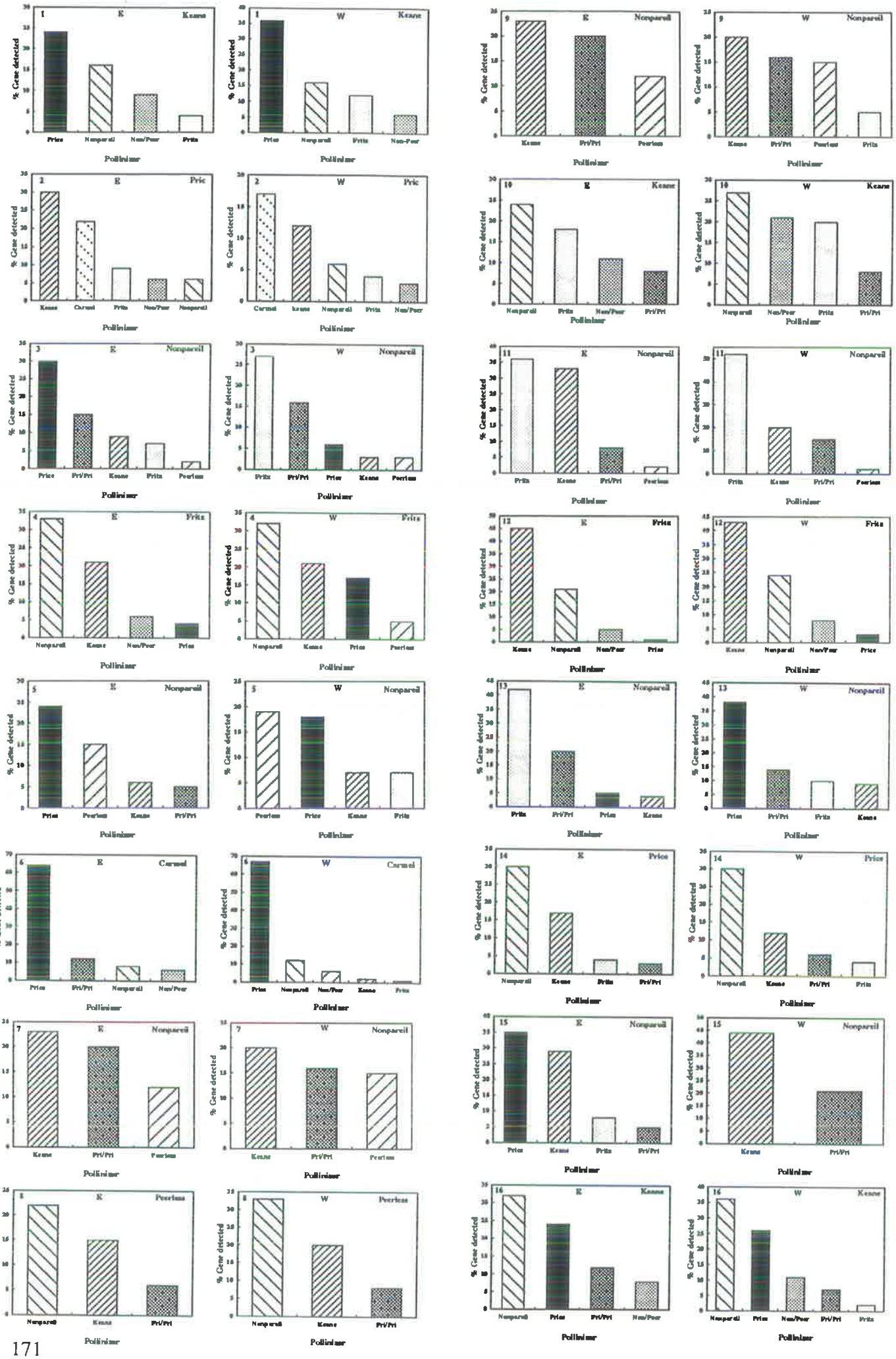
(b) % of of flowers opened to that date.

Table 10.2 Number of fruit and kernel weight in two selected lines for gene flow study

Tree No.	Cultivar line 1	No. of fruit	Kernel weight (g)	Cultivar line 2	No. of fruit	Kernel weight (g)
1	Keanes	5715	7715	Keane	4143	5665
2	Price	8490	9609	Price	5035	5264
3	Nonpareil	3879	5139	Nonpareil	7555	5925
4	Fritz	5670	6166	Fritz	7000	7469
5	Nonpareil	4798	6423	Nonpareil	3481	4430
6	Carmel	6285	7689	Price	6454	7338
7	Nonpareil	6562	7840	Nonpareil	4931	5576
8	Peerless	3471	4927	Peerless	3002	4274
9	Nonpareil	3702	4639	Nonpareil	6620	8846
10	Keane	6432	8508	Keane	6208	7950
11	Nonpareil	5730	6453	Nonpareil	7364	9039
12	Fritz	5670	6305	Fritz	6371	6667
13	Nonpareil	7595	9112	Nonpareil	6220	7373
14	Price	6665	7363	Carmel	4065	4838
15	Nonpareil	7548	9218	Nonpareil	8680	10881
16	Keane	9015	11428	Keane	6101	7984
17	Nonpareil	8088	9898	Nonpareil	5312	6886
18	Price	8290	8881	Price	5213	5926

Table 10.3 Nut characteristics of cultivars under gene flow study

Cultivar	Shell weight (g)	Kernel weight (g)	Percentage of double kernel	Percentage of empty kernel
Nonpareil	0.61	1.20	6	2
Keane	1.22	1.32	0	-
Price	0.65	1.11	6	2
Carmel	0.76	1.20	3	1
Fritz	0.95	1.06	1	2
Peerless	2.07	1.42	6	2


1 E Keane
1 W Keane
2 E Pric
2 W Pric
3 E Nonpareil
3 W Nonpareil
4 E Price
4 W Price
5 E Nonpareil
5 W Nonpareil
6 E Carmel
6 W Carmel
7 E Nonpareil
7 W Nonpareil
8 E Peerless
8 W Peerless
9 E Nonpareil
9 W Nonpareil
10 E Keane
10 W Keane
11 E Nonpareil
11 W Nonpareil
12 E Price
12 W Price
13 E Nonpareil
13 W Nonpareil
14 E Price
14 W Price
15 E Nonpareil
15 W Nonpareil
16 E Keane
16 W Keane
% Gene detected
Pollinizer

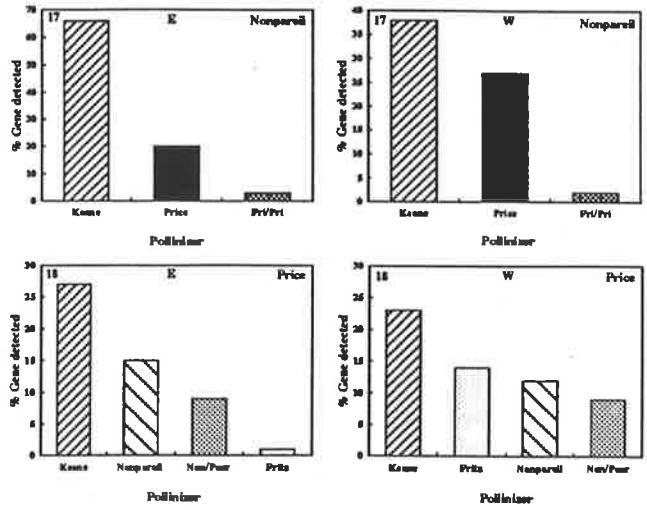


Fig. 10.2 Gene flow by pollen. percentage of pollen genes detected in nuts from each tree in a line of 18 trees in an almond orchard at Angle Vale [East (E) and west (W) parts of each tree are shown].

For convenience the full Fig. 10-2 is shown on pages 171 (opposite) and 172.

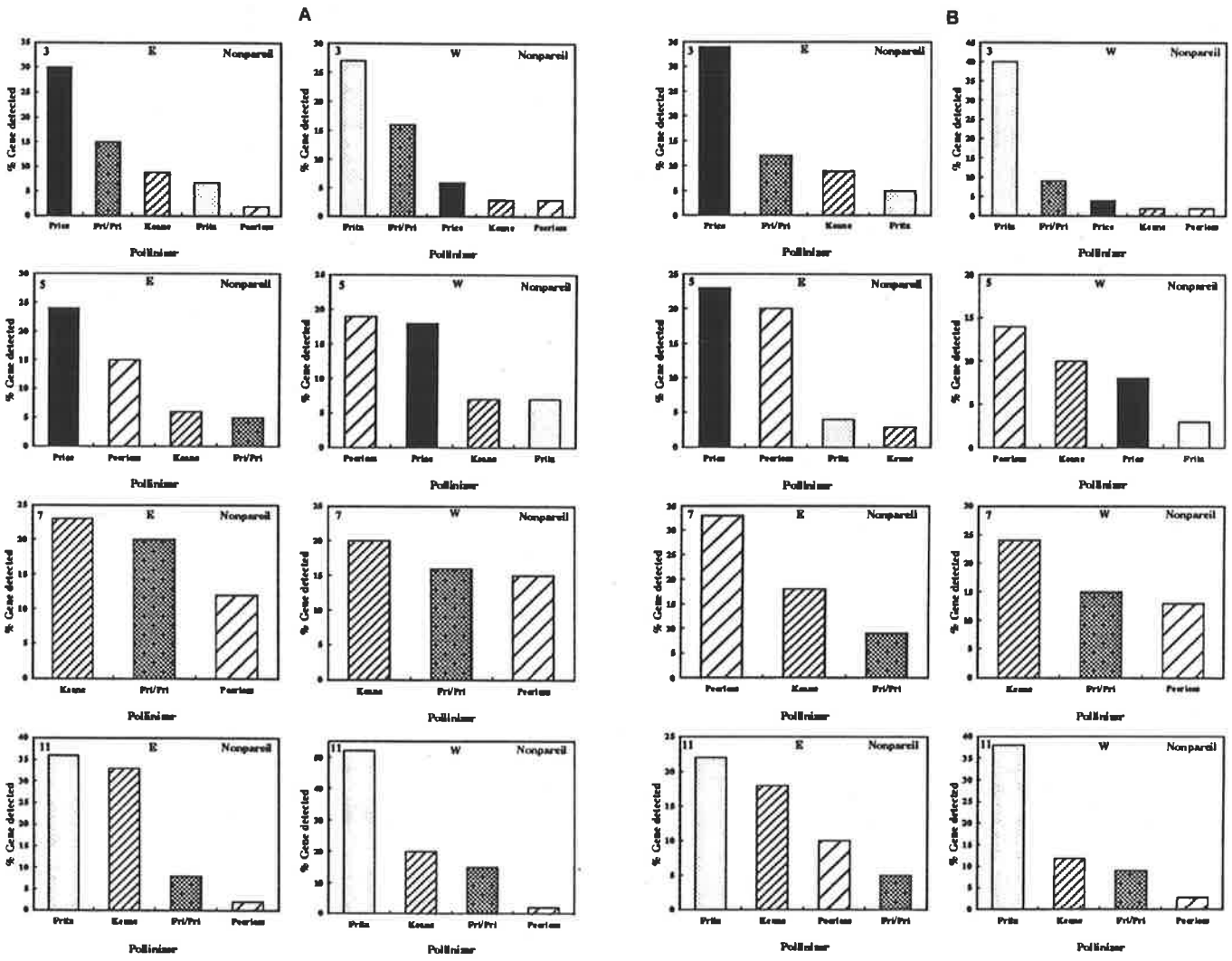


Fig. 10.3 Comparison of gene flow by pollen to Nonpareil for the two years 1992 (**A**) and 1993 (**B**).

Fig. 10.2 Zymogram of 4 enzyme systems from honeybee pollen loads collected with a trap from the honeybee hive. The red arow shows the presence of a non-almond pollen load. All other patterns show pollen loads each comprised of only one almond cultivar, blue arrows show some of the less common pollen loads from different almond cultivars.

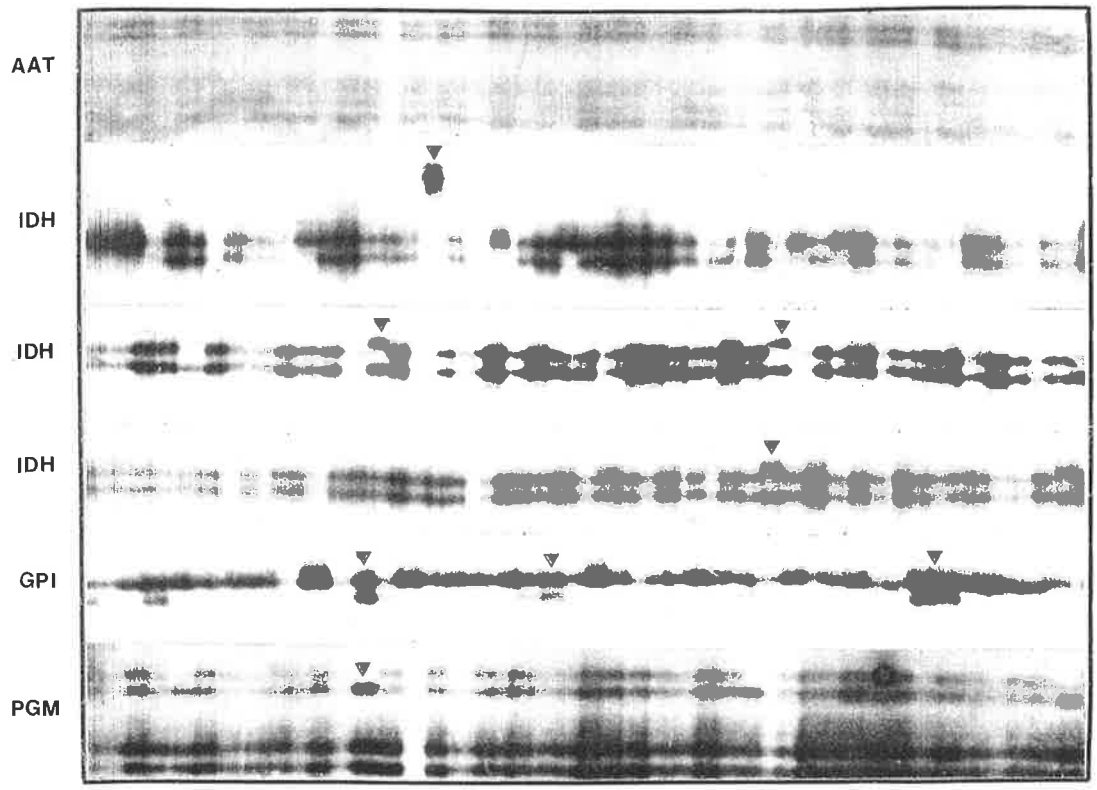

AAT
IDH
IDH
IDH
GPI
PGM

Table 10.4 Honeybee pollen load weight and % fidelity in a section of almond orchard at Angle Vale

Date	Pollen pellet weight (g)		Percentage of fidelity
	almond	Others	
12/8/93	7.6	5.2	77%
19/8/93	56.4	7.3	91%
22/8/93	24.6	10.8	88%
25/8/93	9.7	9.1	76%
27/8/93	5.3	6.3	78%

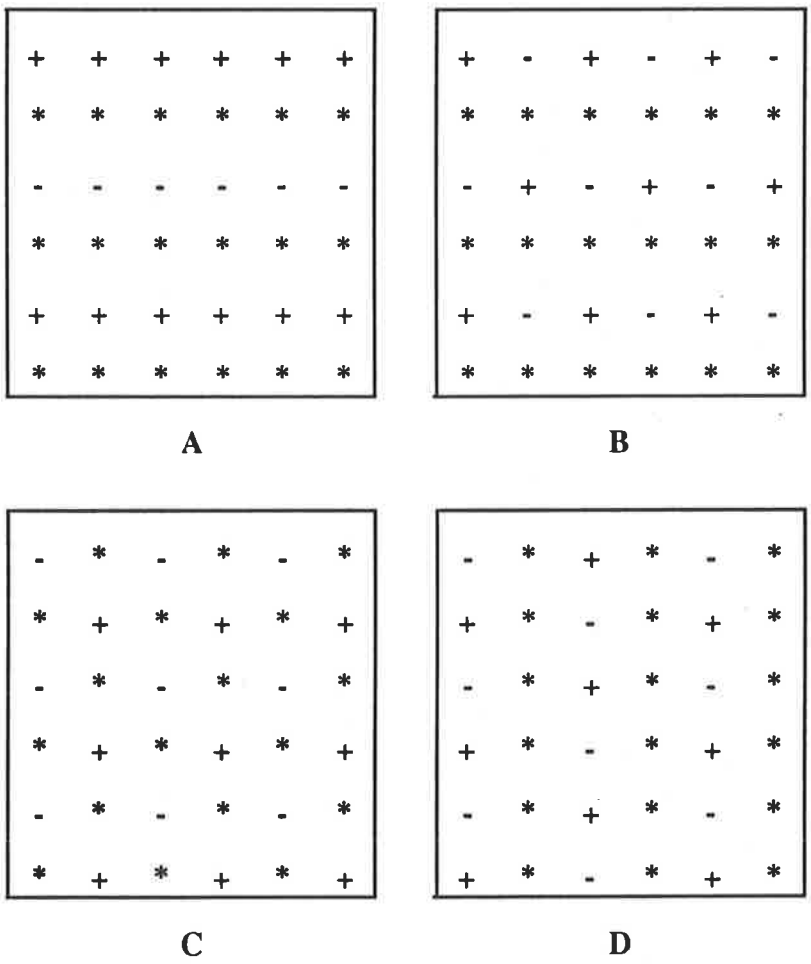


Fig. 10.5 Orchard design for higher almond production.
*. Main cultivar
-. Pollinizer
+. Pollinizer

Chapter 11

Pollination of almond in isolated cage by honeybees

Chapter 11

Pollination of almond in isolated cage by honeybees

11.1 Summay

Percentage of fruit set in bee self and cross pollination as carried out by bees was investigated for Nonpareil almond cultivar in South Australia. Percentage of self pollination leading to nut set in Nonpareil almond was found to be very low at 0.18% of flowers produced on tree. Therefore cross pollination can be done for all practical purposes without emasculation of flowers. The results suggest that the proportion of flowers settings nuts can be higher by increasing honeybee populations. The number of nuts set decreased with increasing distance from the bee hive in the cage. Finally comparison of linkage of isozyme groups in bee mediated self-pollination versus bee mediated cross-pollination (reciprocal crosses) showed that in both studies a highly significant association was found between the same two pairs of enzyme loci, namely IDH with AAT-1 and LAP-1 with PGM-2.

11.2 Introduction

Most almond cultivars are self-incompatible and require the pollen of another cultivar for successful fertilization (Socias i Compny *et al.*, 1976; Godini, 1981; Vezvaei *et al.*, 1994) Growers cannot produce a commercial crop if they plant solid blocks of one cultivar. Self-incompatibility in almond varies from 99.9% to a slight preference for foreign pollen, it can also vary from one environmental condition to another (Bowman, 1939; Weinbaum, 1985). With current commercial practices no thinning of nuts is ever necessary in almond, the growers objective is to obtain a maximum set of nut (Kester and Griggs, 1959a). In order to obtain this, adequate numbers of pollinizer trees and honeybee colonies must be properly distributed throughout the orchard.

The object of this study was to evaluate percentage of self-pollination of Nonpareil almond cultivar in Adelaide conditions and the maximum almond production in trees isolated in cages. Comparison of linkage of enzyme groups in bee mediated self-pollination versus bee mediated cross-pollination has also been evaluated.

11.3 Materials and methods

11.3.1 Caged Nonpareil tree with a hive of bees

The study was conducted over two years in the experimental orchard at the Waite Institute and in a commercial almond orchard at Angle Vale, 35 km North the Adelaide.
A Nonpareil almond tree was covered with a nylon mesh in June 1990. Soon before flowering a strong hiveof bees was introduced into the cage for pollination purposes, honeybees were fed with 50% sucrose solution until flowering. Nuts were harvested after six months and kernels were analysed for six isozyme systems (AAT-1, GPI-2, IDH, LAP-1, PGM-1 & PGM-2 and SKDH) as described in section (2.2) of this thesis.

11.3.2 Caged Nonpareil and Keane trees together with a hive of bees

In 1992 two adjacent trees (Nonpareil and Keane cultivars) were covered with an insect proof mesh at Angle Vale and a hive of bees placed in between the two trees before flowering. The amounts of total yield and percentage of nuts set was recorded for different parts of each tree.

11.3.3 Isozyme analysis

All of the nuts from bee mediated self-pollination and 400 nuts from each tree of bee mediated cross-pollination were examined for isozyme analysis and evidence of linkage between isozyme loci.

11.4 Results

11.4.1 Percentage of self-pollination in Nonpareil almond and linkage study

Percentage of self pollination in Nonpareil almond was found to be a very low 0.18% of flowers present; from 35,700 flowers produced on the tree in 1990 only 65 nuts were produced. Isozyme analysis showed that all the kernels produced were the result of self fertilization from self pollen, except for 3 kernels which must have resulted from fertilization of pollen from outside the cage (probably Fritz pollen) (Table 11.1). This is deduced from the fact that Nonpareil genotype for GPI-2 locus is 'aa' and only the Fritz cultivar in the orchard is heterozygous 'ab' for GPI-2. Chi-square test two way contingency

table showed a highly significant evidence for linkage between two pairs of loci namely IDH with AAT-1 (P value < 0.001) and LAP-1 with PGM-2 (Table 11.2).

11.4.2 Nut production in cage with two trees and linkage study

When Keane and Nonpareil trees were caged together with a hive of bees, many nuts resulted, the number of nuts set decreased with increasing distance from the hive (Table 11.3). The number of nuts produced on both of these trees in the year before and after the caging (isolation) is given for comparison in Table 11.4. The yield of the Nonpareil and Keane trees was higher than for other trees in the orchard due to the palnting of these two trees in the same row. A proportion of increasing yield must be related to yearly natural growth of trees. Again (chi-square test with two contingency tables showed that, there is a high association (P value < 0.001) between to pairs of enzyme loci, LAP-1 with PGM and AAT-1 with IDH, suggestion linkage between these two pairs as before (Table 11.5).

11.5 Discussion

Almond pollen is highly nutritious and attractive to the honeybee. So that attractiveness of pollen to honeybees is probably not a problem for almond pollination. Honey bees collect either pollen, or both nectar and pollen. Nectar secretion begins slowly at first at about the same time as the flower opens. Pollen collectors predominantly visit newly opened flowers after pollen dehiscence begins, whereas nectar-gatherers bees favour older flowers (Langridge and Goodman, 1981). This tendency to visit older flowers makes it less likely that nectar gatherers are as effective in pollination as are the pollen gatherers. The percentage of fruit set in the Nonpareil almond tree isolated with a beehive was less than 0.18 % of flowers produced on the tree and is similar to the result found for a similar experiment carried out in California. The result support the suggestion that emasculation of flowers is not necessary for hand cross pollination experiments carried out on the Nonpareil almond cultivar in South Australia, as reported for Nonpareil in California by Kester and Asey, 1975). Almond pollen can only be transported by very strong wind (Wood, 1937; Longridge and Goodman, 1981). Almond pollen is sticky and hence difficult for wind to dislodge unless the pollen is dried by high temperature and low humidity (Crane, 1985).

Wild bees and other insects could well have brought about the setting of almond nuts, crops said to have been set through wind pollination (Wood, 1937). In the experiment with a Nonpareil tree isolated with a hive of bees, we found that just 3 of 65 nuts were pollinated with foreign pollen. It is possible that these three resulted from cross-pollination of the Nonpareil by a very small insect able to get through the mesh of the cage (eg. a small 'hover' fly) or the passing of pollen from bees outside the cage to those inside by deposition (accidental) of pollen on the cage network by bees outside the cage.

As to the experiment with two trees in the same cage, nut production on Keane was lower in comparison with the previous year when uncaged, probably because the tree starts to flower one week earlier than Nonpareil and so there is no pollen source for pollination of Keane when isolated in the cage at these early stages. Nut production varied according to the distance from bee hive and decreased with increasing distance from the hive. The nut production could be increased if two pollinizers and a main cultivar were to be covered with a the nylon mesh.

We conclude that under South Australian conditions hand cross-pollination can be effectively examined without prior emasculation of flowers, and also that for higher production at least two overlapping pollinizers with main cultivar is necessary. We also suggested that by increasing the bee population, nut set can be increased. Finally the result of linkage studies showed that the linkage groups were the same in both sets of pollination experiments carried out by bees in the cages, and the same as that found in the hand ceoss-pollination experiments described in Chapter 5.

Table 11.1 Isozyme analysis of seven polymorphic isozymes in embryos obtained from bee pollinated Nonpareil cultivar in the cage and chi-square test of progeny

*. Represents nuts probably set from foreign Fritz pollen.

No.	AAT-1	GPI-2	IDH	LAP-1	PGM-1	PGM-2	SKDH
1	ab	aa	bb	bb	bb	ab	bb
2	ab	aa	bb	bc	ab	ab	bb
3	bb	*ab	bb	bb	ab	ab	bb
4	ab	aa	ab	cc	bb	ab	bb
5	aa	*ab	aa	bb	ab	ab	bb
6	bb	aa	ab	bc	ab	ab	bb
7	ab	aa	ab	bb	ab	ab	bb
8	ab	aa	ab	bb	ab	aa	bb
9	aa	aa	ab	cc	ab	bb	bb
10	ab	aa	bb	bc	bb	ab	bb
11	bb	aa	ab	bc	bb	bb	bb
12	aa	aa	ab	cc	bb	ab	bb
13	ab	aa	ab	cc	ab	ab	bb
14	aa	aa	bb	bc	bb	ab	bb
15	aa	aa	bb	cc	ab	bb	bb
16	ab	aa	ab	bc	ab	ab	bb
17	aa	aa	ab	bc	ab	ab	bb
18	ab	aa	ab	bc	ab	ab	bb
19	ab	aa	ab	bc	ab	ab	bb
20	bb	aa	aa	bc	ab	ab	bb
21	bb	aa	ab	cc	ab	bb	bb
22	ab	aa	aa	bc	ab	aa	bb
23	ab	aa	ab	bc	ab	ab	bb
24	aa	aa	ab	bb	ab	ab	bb
25	ab	aa	aa	bc	bb	bb	bb
26	bb	aa	aa	bc	ab	ab	bb
27	ab	aa	ab	bc	bb	ab	bb
28	aa	aa	ab	cc	bb	bb	bb
29	bb	aa	ab	cc	ab	bb	bb
30	ab	aa	aa	bc	ab	ab	bb
31	aa	aa	ab	cc	bb	bb	bb
32	ab	aa	ab	bc	bb	ab	bb
33	ab	aa	ab	bc	ab	bb	bb
34	ab	aa	ab	bc	bb	ab	bb
35	aa	aa	bb	cc	bb	ab	bb

36	aa	aa	bb	bc	ab	ab	bb
37	aa	*ab	aa	bc	ab	ab	bb
38	ab	aa	aa	bc	bb	ab	bb
39	bb	aa	ab	cc	bb	bb	bb
40	ab	aa	ab	cc	bb	ab	bb
41	aa	aa	bb	bc	ab	ab	bb
42	bb	aa	ab	bc	ab	ab	bb
43	ab	aa	ab	cc	bb	bb	bb
44	bb	aa	aa	bc	ab	ab	bb
45	bb	aa	aa	bc	bb	ab	bb
46	ab	aa	aa	bb	ab	ab	bb
47	bb	aa	ab	bc	ab	aa	bb
48	bb	aa	aa	bc	ab	aa	bb
49	ab	aa	ab	bc	ab	ab	bb
50	bb	aa	ab	bc	ab	bb	bb
51	ab	aa	ab	bb	ab	aa	bb
52	ab	aa	ab	bc	ab	ab	bb
53	ab	aa	ab	bc	ab	ab	bb
54	ab	aa	ab	bc	ab	ab	bb
55	aa	aa	bb	bc	ab	bb	bb
56	ab	aa	ab	bc	ab	ab	bb
57	bb	aa	aa	bc	ab	ab	bb
58	aa	aa	bb	bc	ab	ab	bb
59	ab	aa	aa	cc	ab	bb	bb
60	ab	aa	ab	cc	ab	bb	bb
61	aa	aa	ab	cc	bb	bb	bb
62	ab	aa	aa	bb	ab	ab	bb
63	aa	aa	bb	bc	bb	ab	bb
64	ab	aa	ab	bc	ab	ab	bb
65	aa	aa	ab	bb	ab	ab	bb

Nonpareil	**AAT-1**	**GPI-2**	**IDH**	**LAP-1**	**PGM-1**	**PGM-2**	**SKDH**
genotype	**ab**	**aa**	**ab**	**bc**	**ab**	**ab**	**bb**
Progeny	**abxab**	**aaxaa**	**abxab**	**bcxbc**	**abxab**	**abxab**	**bbxbb**
	aa: 18	aa: 62	aa: 15	bb: 10	aa: 0	aa: 5	bb: 65
	ab: 32	*ab: 3	ab: 38	bc: 39	ab: 45	ab: 44	
	bb: 15		bb: 12	cc: 16	bb: 20	bb: 16	
Significant	**0.26**	-	**2.18**	**4.00**	**37.60**	**14.36**	-
level	**NS**		**NS**	**NS**	***	***	

Table 11.2 Linkage groups between enzyme loci in bee vectored self-pollination of a caged Nonpareil tree

Nonpareil x Nonpareil					
Linkage groups	**Value of X^2**	**Significant level**	**Linkage groups**	**Value of X^2**	**Significant level**
IDH x LAP-1	4.34	ns	LAP-1 x PGM-1	6.25	ns
IDH x PGM-1	1.38	ns	LAP-1 x AAT-1	6.86	ns
IDH x PGM-2	3.65	ns	LAP-1 x PGM-2	23.97	***
AAT-1 x IDH	20.35	***	PGm-1 x PGM-2	3.35	ns
AAT-1 x PGM-2	5.65	ns			

Table 11.3. Percentage of fruit set in different parts of the caged Keane and Nonpareil trees (m represents distance in meters from the centrally located bee hive)

Keane					Nonpareil				
5m	4m	3m	2m	1m	1m	2m	3m	4m	5m
4.1	15.4	17.5	32.4	30.5	27.0	26.5	16.0	17.0	13

Table 11.4 Number of nuts and kernels weight obtained from caged Keane and Nonpareil trees in 1992-93 and comparison with the 1991-92 and 1993-94 when trees were not caged

Keane				Nonpareil			
Year	1991-92	1992-93 (caged)	1993-94	**year**	1991-92	1992-93 (caged)	1993-94
Kernels number	7234	6382	7659	**Kernels number**	9230	10512	11051
Kernels weight (Kg/tree)	9.400	8.300	10.00	**Kernels weight (Kg/tree)**	10.500	12.600	13.261

Table 11.5 Linkage groups found for isozyme loci in nuts produced after pollination of Keane and Nonpareil by bees in a cage

Keane x Nonpareil			Nonpareil x Keane		
Linkage groups	Value of X^2	Significant level	Linkage groups	Value of X^2	Significant level
LAP-1 x AAT-1	3.04	ns	LAP-1 x AAT-1	0.28	ns
LAP-1 x PGM-1	0.05	ns	LAP-1 x PGM-1	3.24	ns
LAP-1 x PGM-2	103.04	***	LAP-1 x PGM-2	87.14	***
LAP-1 x IDH	1.03	ns	LAP-1 x IDH	0.64	ns
AAT-1 x PGM-1	3.12	ns	AAT-1 x PGM-1	6.65	ns
AAT-1 x PGM-2	2.67	ns	AAT-1 x PGM-2	1.86	ns
AAT-1 x IDH	153.78	***	AAT-1 x IDH	192.18	***
PGM-1 x PGM-2	0.40	ns	PGM-1 x PGM-2	5.88	ns
PGM-1 x IDH	6.52	ns	PGM-1 x IDH	7.42	ns
PGM-2 x IDH	2.10	ns	PGM-2 x IDH	1.00	ns

Chapter 12

General discussion

Almond is a self-incompatible species, needing the honeybee for cross-pollination to produce commercial crops. Cross-pollination and fertilization is a dynamic phenomenon related to climate, honeybee behaviour, flower conditions, the cultivars present and the distance between them. This project has emphasized the effect of these factors in trying to bring about on increase in almond production under South Australian conditions. Discussion on the more specialized issues onvolved is given in each chapter above as it arises, only the more general aspects are included here.

Several traits were investigated for potential use as markers for almond cultivar identification, including pollen ultrastructure, RAPD markers and isozyme markers. The decision to use isozyme to identify the various almond cultivars for most of the investigations described here was made because isozyme analysis can be carried out at a fraction of the cost of the RAPD method and more quickly . Many thousands of embryos were subject to analysis for presence of pollen genes and other contributions to the overall embryo genetic make-up. These investigations included genetic linkage, gene flow by pollen and studies on the genetic relationships between the various cultivars and species. Linkage between several genetic markers has now been established in almond for the first time as a result.

An interesting finding was that under South Australian conditions (Adelaide plains) Nonpareil flowers set highest numbers of nuts when hand-pollinated on the first day of opening, in contrast to Californian findings that the second day gave the highest nut set. It seems that bee visits are lower on the first day, and increase on the second day when pollen begins to dehisce under Adelaide plains conditions. It is possible that the South Australian Riverland climatic conditions are closer to those in California, and the timing of flower receptivity and bee visits more suitable as a result. Further investigation are needed to resolve this.

A study of the various Australian and Iranian cultivars showed that both gene pools are significantly different to that in California, and thus of interest to future breeding

programs for improved almond cultivars. Establishment of clear isozyme genetic markers for Australian cultivars and the addition of several more to the already useful ones known for the Californian cultivars, has been useful in confirming that gene flow by pollen in Australian commercial orchards is quite restricted. In most cases, the predominant source of pollen for nut set on a particular tree is from the nearest tree, and often from the nearest portion of the canopy of nearest trees, if potential pollinizers are equidistant. This has implications for best design of almond orchards. It would seem that designs incorporating rows of trees of one cultivar only are not going to be fully efficient in cross-pollination. Thus the best practice according to growers now is that orchards should comprise a row of Nonpareil, pollinated by a row of pollinizer, then a row of Nonpareil and followed by a row of a second pollinizer cultivar and so on (1:1 design).

For better cross pollination the present studies would suggest that each row be composed of alternating cultivar trees e.g. pollinizer 1 : Nonpareil : pollinizer 2 : Nonpareil : Pollinizer 1, etc. So that each tree has cross pollinizing nearest neighbour trees on all sides. Not so clean for harvest purposes, but if highest yields are needed then much a plan is essential. The choice of cultivars for pollinizer 1 and pollinizer 2 in such a design will depend on many factors, including those making harvest easier (e.g. late or early maturing nuts). Good overlap of flowering time with Nonpareil, heavy annual flowering and value of nuts produced by pollinizers are also important. Overall for the Adelaide plains Peerless and Price or Keane would be a reasonable choice, in combination with Nonpareil. Breeding progrmes set up with the with the long aim of producing self-compatible cultivars and also better rootstock would be areas for future study for higher almond production in Australia and elsewhere.

Studies with Iranian almond samples show that there is a very high degree of diversity in cultivated almonds in Iran, no doubt reflecting that many (but not all) orchards are begun from seed and not cloned trees of particular cultivars. Almond orchards in Iran established from the seed have advantage and disadvantage; these orchards are a great gene pool for genetic study in the future, e.g. selection for horticultural traits and asexual reproduction as a new cultivar for established new orchards. Natural inter specific hibridization can be selected and examined as rootstock for almond and or other related

species. The nuts produced from these orchard have different shape size, colour and taste and some of trees produce bitter almond, sometimes bitter nuts are mixed with sweet nuts a factor that reduces the price in the market. Orchard established from the seed have increased percentage of bitter genotype due to recessiveness of this gene.

Gene flow in Iranian almond orchards is not a problem due to variability of genotype (each tree could have a different genotype) and so cross-incompatibility may not be such a serious problem. The low crop is some Iranian orchards is due to the small size and weight of the kernels produced and also to some agrotechniqual problems and irrigation. In the more recently estblished orchards cross-incompatibility and gene flow must be considered. Selection from local genotyps and hybridization between local cultivars and some cultivars from overseas such as Nonpareil should improve quality and quantity of nuts in Iran.

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