Pollination, Fruit Set and Identification
Studies in Pear

by

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A view from the Magarey family's orchard located in Coromandel valley a region of South Australia, where the majority of experiments were conducted

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1-Sharifani, M.M., J.F. Jackson.(1995). Pollination efficiency in a pear orchard. New Zealand Society for Horticulture Science (4-6 Sept.1995). PP; 28

2-Sharifani, M.M. and J.F. Jackson. (1996). Isozyme inheritance in controlled crosses with Packham Triumph pear. Acta Horticulturae. 437:169-174

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Pollination, Fruit Set and Identification Studies in Pear

Summary

Cross pollination is necessary for seed production amongst incompatible fruit tree crops such as almond, cherry, pear, etc.. Insufficient pollination may lead to reduction in yield and/or an increase in misshapen fruits. The research reported here was carried out to evaluate pollination efficiency, gene flow by pollen and fruit set in Packham Triumph cultivar pear trees located in a mixed planting of pear cultivars in a commercial orchard in the Coromandel Valley region of South Australia.

In order to reconstruct gene flow by pollen and fruit set in Packham Triumph it was first necessary to identify molecular markers for the various cultivars for use in such work. Isozyme markers and randomly amplified polymorphic DNA (RAPD) finger printing were examined for this purpose. DNA extraction and RAPD fingerprinting methods were optimized for pear and identification of pear species and cultivars carried out by the RAPD-PCR technique. Several oligonucleotide 10-mer primer produced band markers characteristic for the various cultivars grown in the Coromandel Valley orchard, with the oligonucleotide of sequence GTCCCGACGA giving the best results. In order to see how extensively useful the method could be, wild pears from Iran were also successfully identified with the GTCCCGACGA primer through RAPD-PCR DNA fingerprinting. However, when this primer was tested on progenies of the crosses of the Coromandel valley cultivars Josephine X Packham Triumph and Lemon Bergomot X Packham Triumph, the expected parent band markers in the progenies were not reproduced. It seems then that these RAPD markers while eminently suitable for identifying pear cultivars are not suitable for identifying progeny and for gene flow by pollen studies because of the unpredictable nature of the inheritance of RAPD markers.

Isozyme systems were however found to be more useful. Pollen rather than leaf tissue was found to be convenient for observing polymorphic banding patterns in GPI, PGM, ADH, IDH and GDH isozyme systems. Further work using seed obtained from controlled crosses showed clear banding patterns for GPI and ADH isozymes in these seeds and produced polymorphic bands specific for the crosses. PGM and IDH isozyme systems did not show good resolution of bands on the gel. Banding patterns

for 26 pear species and cultivars were applied to a similarity matrix software program to obtain relatedness. These results showed smaller differences in isozyme banding patterns between the wild pear species than between the commercial pear cultivars using the GPI isozyme system. Inheritance of isozyme banding patterns resulting from crosses of different pollen parents from trees in the commercial orchard with the cultivar Packham Triumph showed an inheritance ratio of 2:1:1 for the banding patterns in the GPI isozyme system for three of these crosses, i.e. Josephine, Lemon Bergomot and Duchess crossed with Packham Triumph.

To assist in the interpretation of gene flow by pollen studies, flower number, flowering time, pollen production and nectar sugar and amino acid analysis was recorded for two pear cultivars often used as pollinizers, Josephine and Lemon Bergomot, and was also recorded for the main receptive cultivar, Packham Triumph. This work showed there was a decided lack of synchrony in flowering time between Lemon Bergomot and Packham Triumph. Josephine was found to flower more in synchrony with Packham, but produced much lower flower numbers. Measurement of flower number and pollen production among the three cultivars showed that Packham Triumph had greater numbers of flowers and a higher pollen production. Packham Triumph nectar analysis showed the presence of sucrose which is an attractant for honeybees, while it was found that Josephine and Lemon Bergomot had little or no sucrose in the flower nectar and may therefore be less attractive to honey bees.

Caging of Packham Triumph trees gave abundant fruit set, the fruit having no seed. This showed that Packham Triumph is fully self-incompatible but able to set fruit parthenocarpically. Comparison with nearby uncaged trees showed no significant difference in number of fruit set between caged and uncaged trees, but there was a significant increase in average weight of fruit in the caged (parthenocarpic) situation (about 25% more). There was also a significant increase in number of misshapen fruit in cages (about 30% more). When another pear cultivar, Lemon Bergomot, was caged and compared with non-caged trees, there was no increase in number of misshapen fruits due to the lack of seeds as there was found with Packham Triumph. The effect of number of seeds on the shape of fruit in Packham Triumph showed that the more seeds the better the shape, no misshapen fruit was found where there was more than 4

seeds per fruit (10 is theoretical maximum). With Lemon Bergomot in contrast, there was no effect of number of seeds per fruit on shape, however fruit abscised early had less seeds than retained fruit. It can be readily seen then that very good pollination to yield better than 4 seeds per fruit is necessary to give well shaped Packham Triumph fruit. To test these observations and to examine gene flow by pollen in the open orchard situation, fruits from Packham Triumph trees were examined from an area in the orchard where there was a large stand of Packham Triumph trees. On the edge of this stand there was one row of Josephine cultivar and no other cultivar compatible with Packham Triumph for at least 10 rows. It was found that fruit on the first row of Packham Triumph nearest the Josephine had most seeds (average 3.7 per fruit) and the number of seeds per fruit decreased with increasing distance from the Josephine row. By row five, an average of only one seed per fruit was seen. Use of GPI and IDH isozyme markers showed that all seeds in these Packham Triumph fruits had Josephine pollen genes and no pollen genes from other cultivars in other parts of the orchard were found. As expected from the caged tree experiments, the average weight per fruit and number of misshapen fruit increased with increasing distance from the Josephine row, consistent with a decrease in seed number. In this experiment, the placement of the honey bee hives showed no significant relationship with any of these parameters, which only depended on the distance from the Josephine pollinizer trees. It is worth noting that even when Packham Triumph trees are right next to Josephine trees, seed set averages below 4 seeds per fruit and we know that we need to have more than 4 seeds per fruit to have no misshapen fruits. Clearly, pollination is inadequate in this orchard even when pollinizers and recipient trees are next to one another. In mixed planting is another part of the orchard, Josephine was found to be a more efficient pollinizer of Packham Triumph than Lemon Bergomot. Lemon Bergomot on the other hand is pollinated very effectively by the more numerous Packham Triumph, Josephine making limited contributions to Lemon Bergomot pollination. Preliminary work on gene flow by pollen among several cultivars along a Tatura trellis system suggested, that pollen genes flow further along such an open trellis system compared to unrestrained rows of pear trees.

The pollen ultrastructure of 26 pear species and cultivars was studied because this knowledge may assist in future pollination identification work. Pollen morphometric

characteristics such as pit surface area (%) was measured by an image analysis computer program. Pollen length and width were measured, as well as ridge diameter. The results showed that pollen pit surface area and ridge diameter and orientation were useful in characterizing pollen grain origin. The effect of pollen parent on fruit produced as a result of controlled crosses was to change the percentage of seed and fruit set. The effect was small but significant. Analysis of total nitrogen in the fruit showed no changes in total nitrogen with changes in pollen parent. Further work showed that increasing number of seeds increased total free amino acids resulting from protein hydrolysis of the fruit.

Overall, the present studies have provided several molecular markers and other means for pear identification. Some of these were used to show that pollination in pear is very local within the orchard, taking place most efficiently from one tree to its nearest neighbour. The presence of seeds in the fruit of Packham Triumph cultivar improved the shape, but, in the orchard studied, the number of seeds per fruit did not exceed four, the minimum needed to ensure good shape. Methods for improving pollination in pear orchards were discussed.

Declaration

I hereby declare that this work presented here has been 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 reference is made in the text.

I am willing to have this copy of my thesis being made available for loan and photocopying when deposited in the University of Adelaide library.

Mohamad Mehdi Sharifani

Acknowledgements

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1-Sharifani, M.M., J.F. Jackson.(1995). Pollination efficiency in a pear orchard. New Zealand Society for Horticulture Science (4-6 Sept.1995). PP; 28

2-Sharifani, M.M. and J.F. Jackson. (1996). Isozyme inheritance in controlled crosses with Packham Triumph pear. Acta Horticulturae. 437:169-174

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Chapter 1

General Introduction and Literature Review

1.1   General Introduction

Pollination is one of the major events in the flowering plant. Pollination is the transfer of pollen from the pollinizer, the source of the pollen, to the receptive stigma by the agent of transfer, which is the pollinator in entomophilous flowering plants. Pollination is important because it is the first step preceding other steps leading to the double fertilization. Usually, pollen germinates on the receptive stigma and a pollen tube enters the stigma tissue and passes through the style via apoplast, finally reaching the ovary. Then the pollen tube enters the ovule and embryo sac respectively. Finally the two generative nuclei in pollen tube fuse with the egg nuclei and polar nuclei in embryo sac. The result is a plant embryo and endosperm, which are the starting points of fruit set and growth. Environmental factors such as temperature, humidity, radiation, foraging behaviour of pollinators and genetic structure of pollen and pistil and their interactions can affect the result of fertilization. Climatic conditions have an effect on the behaviour of pollinators like the honey bee, and on anther dehiscence time and nectar composition. The juxtaposition of pollinizer cultivars and receptive cultivars in the orchard may also affect the efficiency of pollination. Objectives of this study are to evaluate pollination efficiency and gene flow by pollen in the commercial pear orchard. This will enable us to decide which cultivars are effective pollinizers and to decide optimal juxtaposition of pollinizer and main producer cultivars in the orchard and the importance of the honeybee in this process. Parthenocarpy which is the ability to form the fruit without fertilization is another mechanism leading to fruit set and development. Parthenocarpy in the pear is quite important, because under poor pollination conditions it replaces fruit set by fertilization in the orchard. The effect of parthenocarpy processes on fruit set and shape and weight of fruit was therefore also studied. Parthenocarpy in relation to fruit set efficiency in the orchard is an area that has hitherto received little consideration.

Coming out of this study then will be pattern of gene flow by pollen amongest the various cultivars in the orchard obtained by application of isozyme or DNA fingerprinting technique. Identification of cultivars and species of pear by pollen isozymes permit reliable results for gene flow study. Identification both by isozyme and RAPD -PCR DNA fingerprinting provide a detailed view of cultivars and species differences. As the amounts of sugar in the nectar and pollen in the flower play a role in honeybee attraction, these were also measured. Further, the effect of seed number varied by pollination of various numbers of styles on the shape of the fruit in these pear cultivars, Packham Triumph and Lemon Bergomot were investigated. The effect of pollen source on fruit set, seed set and fruit shape was also investigated.

1.2   Literature Review

1.2.1   Pear

1.2.1.1 History

Considering fossils recorded from the mountainous regions of western China, the first appearance of the pear goes back geologically to the tertiary period. Historically the pear dates back at least to 1100 B. C (Challice and Westwood, 1973; Hedric *et al.*, 1921) and it was at this time that wild and edible pear began to differentiate. About 300 B.C., pear culture was well established in Greece. Distinct cultivars were propagated via cutting and grafting. Ancient Rome contributed greatly to the knowledge of pear growing around 235-150 BC.

1.2.1.2   Botanical Description, Speciation and Evolution

Pear belongs to the family Rosaceae, subfamily Pomidea and genus *Pyrus*. This genus has been classified into 22 primary species, grouped by geographical distribution and taxonomic relationships. The Pomidea are distinguished by a basic haploid chromosome number of 17 other subfamilies of Rosacea have 7 to 9. The origin

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of the Pomidea has been described as an amphidiploid of two primitive forms of Rosaceae. One form or species having eight chromosomes and the other having nine (Sax, 1931; Rubtsov, 1944). According to Challice and Westwood (1973), the geographical isolation of the *Pyrus* population by mountain ranges and the adaptation to different environmental conditions led to the speciation and monophyletic origin of the pear. Pear flowers are hermaphrodite, white, and possess five petals and sepals. They have among 20 to 30 stamens with red anthers. The number of styles varies from two to five. The styles are free and constricted at the base. The ovary normally in domesticated pear has five locules with two ovules per locule which means the maximal seed set is ten seeds per fruit. As pear is genetically heterozygous and allopolide in origin, different species and cultivars show an extended range of variability in their chemical and botanical characteristics. Meanwhile, the effect of environment on the creation of ecotype should be noted in the classification. The appearance of cork on the leaf surface of some African species may be the result of the effect of a warm climate. The leaf shape in pear is very variable, In *Pyrus calleryana*, it is crenate, while the shape in *Pyrus betuleafolia* is a coarse serrate shape. In *Pyrus nivalis*, it is entire or crenulare and in *P. pyrifolia* it is serrate to setose (Challice and Westwood ,1973). The midrib of the leaf is either free from glands or it is covered with small glands, throughout its entire length. The leaf colour varies from pale to dark green and within the darker shades of green there is considerable variation (Crane and Lewis 1940). The number of carpels also varies among *Pyrus* species. Some species have two, others' three to four and some up to as many as five or six carpels. Domesticated varieties generally posses 5 carpels. The shape of the fruits varies greatly from oblate to elongate. The skin surface is usually either smooth as in *Pyrus pyraster* or russet as in the Winter Nelis cultivar. Other variations exist in seed weight and length and in tree size for different species of pear (Westwood and Challice, 1973). It has been indicated that different species of pear require a different period of time for cold hardiness and breaking of dormancy for seed germination (Westwood and Bjornstad, 1968; Rajashekar *et al.*, 1982). Differences in flowering time for pear species have also been stressed (Faust *et al.*, 1976). Differences in phenolic content were considered by some workers as a new means of pear species identification and for phylogenic studies (Challice and Westwood 1973;

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Challice 1981). Interspecific hybridization, introgression, mutation and polyploid induction have all contributed to the creation of new cultivars and subspecies in pear. This creative process began to take place shortly after the pear migration occurred from its origin site to the "New World". Migration occurred from a few centres of origin in Asia (Vavilov, 1951;) These were 1) the Chinese centre; 2) South of Caspian sea; 3) Northwest India; Afghanistan, Tadjikistan and Uzbekistan. Different interspecific hybrizidation led to the creation of new cultivars, however the ancestor of common pear is believed to be P. *communis* X *P.pyraster* and *P. communis* X *P. Cacuasica* (Bell and Hough, 1986). Pear species have been subdivided into five groups based on botanical and chemotaxonomic characteristics (Challice and Westwood, 1973) as outlined below. As this subdivision was achieved by various botanical and chemotaxonomic methods, there was no way to understand a complete picture of genetic relationships between the species. Because of the highly heterozygous nature of pear, many new genotypes are expected to occur in their progeny due to segregation of the gene. Thus it may be difficult to recognize primitive species from intra- and interspecies hybrizidation progenies.

Asian species
P.betulaefolia
P.dimorphophylla
P.fauriei Schneid
P.calleryana
P.koehnei schneider

West Asian species
P.regelii   (syn.*P.heterophylla*)
P.amygdaliformis
P.elaeagrifolia
P.salicifolia
P syriaca
P.glabr

Medium large fruited Asian species
P.ussuriensis maxim
(*syn. P.cossoni Rehder*
P.pyrifolia
P.hondoensis Kikuchi &Nakai
P.pashia
Kansu Pear

North African species
P.longipes
P.gharbiana Trab
P.mamorensis Trab.

European species
P.communis sensu lato
P.nivalis
P.cordata

1.2.1.3 Polyploidy

Polyploidy has been achieved by doubling with colchicine or natural doubling as mentioned by Crane and Lewis (1940) whereby pear has arisen from a double haploid with an alloploid origin with basic chromosome number of $n = 8 + 9$ to be $2n = 34$. Most pear species are diploid, but tetraploid cultivars in Pyrus do exist e.g, Beurre Diel, Duchess and Pitmaston and, the latter can be recognized by their larger than normal shoots and fruits and increased leaf thickness. Triploids have been derived by crossing a diploid cultivar with a tetraploid an example is Merton pride which was derived from the cross of Glou Morceau [2n} x Bartlett [4n] (Janick and Moore, 1975). Dermen (1947) obtained a triploid by crossing a colchicine produced tetraploid with several diploid cultivars.

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Polyploid forms of pears are known of 4n=68 and hexaploid 6n=102. The benefits of polyploidy include larger fruit size and higher self compatibility in the case of tetrapolids. However they have the disadvantage of a greater generative sterility than seen in diploids.

1.2.1.4 Blooming Date Differences of Pear Species and Cultivars

The time of blooming among the various cultivars of pear is important, if one is to maximize cross pollination in the self incompatible cultivars. Another aspect is that early flowering pears may face frost injury in early spring in some cultivation areas. The time of flowering is influenced by two main factors; A chilling requirement for breaking the rest period and a heat requirement to develop flower buds to bloom (Landsberg, 1974; Faust *et al.*, 1976). The rate of these two quantities is different with variations in the genetic structure of pears. Inadequate chilling in some years results in late and weak blooming. The amount of chilling requirement should be considered for species and cultivars selection in orchard design. In those areas where may not be satistied chilling requirement, those cultivars should be replaced with cultivars with relatively low chilling requirement.

Elevated temperature requirements for flower development is different with variation in pear genotypes (Overcash, 1965). For breeding purposes, a late flowering cultivar can be obtained by making a cross and then backcross with a late flowering cultivar (Faust *et al.*, 1976).

The time of flowering in pear species is, in general, very extended. It was reported (Faust *et al*, 1976) for Westwood,s "collection"(at Corvallis Oregon), the range of pear bloom extends to 50 days, starting with *P.kochnei* and wild type *P. Longipes w, P.regelii* and *P.nivalis* blooming 10 to 21 days after Bartlett. The genetic reasons for differences of flowering at different temperatures still are not known.

For comparison in South Australia at the Waite Arboretum, Glen Osmond, the time of flowering of different species has been recorded to extend from as early as 15 July to 10 October. The following is from records of flowering times in different years which obtained by Mr, D.Symon and adjusted by the author :

Pear species	Start of flowering	End of Flowering
P.syrica	10 September	30 September
P. serotina	1 October	10 October
P.tadijshikestan	10 October	20 October
P.salcifolia	20 Sptember	10 October
P.pyrifolia	10 September	30 September
P.pashia	10 September	30 September
P.pyraster	10 September	10 October
P.mamorensis	10 July	30 July
P.kawakanski	15 August	20 September
P.koroshinsky	15 August	10 September
P.lindley	20 September	10 October
P.longipes	1 October	20 October
P.cossoni	1 October	20 October
P.communis	20 September	10 October
P.amygdaliformis	15 July	15 September
P.boissierana	late September	early October
P.betulaeufolia	1 September	25 September
P.calleryana	24 August	20 Septmeber

1.2.2 Pollination and Pollinators

Most species and cultivars of fruit trees, require pollination to induce fruit set. Although apomixis and vegetative parthenocarpic fruit crops are exceptions to the necessity of pollination, efficient pollination can lead to higher fruit set in terms of flowers per tree. Many fruits and flowers that are not pollinated and fertilized soon abort. Newly set fruits with larger numbers of seeds are more stable than fruits with fewer seed numbers. It is possible that shape of the fruits especially in the pome fruits can be influenced by the seed numbers. Low number of seeds and an uneven distribution of the seeds in the fruit that

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may be the result of too little pollination may lead to misshapen fruits. In this way, pollination not only affects fruit set and yield of tree, but also can influence fruit quality. Records of these effects are not common, however. The seeds in the centre of fruit may be the source of gibberellin and auxin hormone that prevent dropping of the fruitlets. Seeds can only develop if double fertilization has taken place, the result of double fertilization will be a nucleus, and endosperm and embryo development. Double fertilization will take place if pollen from a compatible cultivar is deposited on the stigma, followed by pollen tube growth, etc.

Efficient pollination in an orchard then depends greatly on several relevant factors. These include the microclimate effects, foraging behaviour of insect pollinators, ecosystem conditions, attraction of flowers, orchard design, distance between pollinizer cultivars and main cultivars, number of hives and flowering time of pollinizers compared with main cultivars, particularly pear trees which are mostly self-incompatible (DeGrandi-Hoffman *et al.*, 1989) such as the cultivar Packham Triumph.

1.2.2.1 Foraging Behavior of Pollinators

Research into the foraging behaviour of pollinators has led to improved pollination results in the orchard (Thomson, 1982; Waddington, 1983; Free and William, 1983). Studying foraging behaviour of pollinators is important because of its effect on pollination efficiency and patterns of gene flow by pollen (Jackson and Clarke 1991a,b). It is particularly important for almond, apple, pear, and cherry orchards where cross pollination is necessary. Efficient behaviour of pollinators in regard to pollinizers could possibly increase fruit set and quality of fruit, including shape. The foraging behaviour of bees is influenced by ecological conditions such as microclimate of pollination, space and orientation of plants in the population. The optimum temperature for foraging varies between different species of bees (Free, 1993). Honeybee behaviour is influenced by many

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dynamic and interacting factors in the orchard. Levine and Kester (1969) showed that different densities of plants in the population affect flight distance parameters. Furthermore, they indicated that interspecific differences in plant stature, floral mechanism, pigmentation and odour have a major influences on the flight behaviour of bees. The colour of flower can act as an attraction for the honey bee. Mayer *et al.*, (1989) indicated that the honey bee has more interest in the blue range to white and non-white flowers than pink coloured flowers in crabapple. Other important factors are flower structure, odour, nectar, sugar, pollen amount and number of flowers. On the other hand, the conditions in the hive can change the behaviour of honey bees for foraging. Eckert *et al.* (1994) found that the size of a colony and population size of honeybees affects the overall activity. These workers indicated that the individual nectar forager from large colonies tended to work harder than individuals from small colonies. Furthermore, the value of nectar and pollen resources to colony changes depending on the state of the colony and individual foragers that modify their behaviour accordingly. Honeybees appear to be able to estimate the energy which they gain from the source. Seely (1994) stated that bees behave as though they register the probability of a nectar source without sensing the energy gain per foraging trip or the rate of energy gain per trip, but rather by sensing the energetic efficiency of their foraging. Honey bees are sensitive to the volatiles of plants and pheromones emitted by other kinds of honey bees like the queens of the hive. Currie *et al.* (1992) applied three concentrations of honeybee queen mandibular pheromone to 0.4 ha blocks of apple (*Malus domestica*) and pear (*Pyrus communis*) by an air blast sprayer. Concentrations of 1000 queen equivalents (a.i) /ha increased the number of forager honeybees on both apple and pear. They could obtain the same result in the wild over a range of environmental and geographical conditions. A study of the attractiveness to bees of five volatile compounds in alfalfa, showed a high degree of attractiveness to linalool (Henning *et al.*, 1992). One conclusion of this study was that plant breeders should select for increased level of more attractive volatiles in alfalfa to enhance honeybee foraging on this crop.

1.2.2.1.1 The effect of Flower Structure and Flower Allochemicals on Pollination

Free (1993) observed that the structure of the flower affects the foraging method of the honey bee. When the flower has stamens which are very close together the bee stands on the petals of the flower and pushes its tongue between the stamens and collects nectar. In this case, the bee cannot touch the stigma so pollination does not occur. Conversely, where the stamens are separate the honeybee tries to stand among the anthers pushing its tongue through the stamens to collect the nectar, in this method the body of the honeybee often touches the stigma. So the degree of spread of the anthers largely determines the proportion of the honeybees that work the flower from the top or the side (Robinson and Fell, 1981). When the stamens are short and more flexible, bees preferred to work on top of the flower, among the stamens, rather than from the side.

Robinson (1979) found that 86% of the bees foraging on Delicious apple flowers stood on the petal and collected the nectar from the side through the basal gaps. For five other cultivars of apple, however, 94% per cent of foragers worked on top of the flower among the stamens for the nectar collection and only 6% of the honeybees were sideworkers. Robinson and Fell (1981) reported only 7 % and 8% of flowers which were pollinated by sideworker foragers set fruit, while top worker foragers could set 22 to 50 % of the flowers to fruits. Some pollinators cannot pollinate certain cultivars whose stigmas are too long where they may be far above the stamens (Roberts, 1945).

Pham (1991) suggested that allochemicals in flowers mediate the foraging behaviour of honeybees. These include: 1)-total concentration of sugar in nectar, 2)-amount of nectar which is secreted, 3)- volatile constituents of floral aroma. Pham (1991) showed that nectar is the key factor influencing honeybee's choice while distant plant recognition is based on aroma differences.

Differences among the various cultivars of a particular species in flower structure and allochemical content on attraction and behaviour of the honey bee are an area where much research is needed, particularly for fruit crops. Different cultivars of species may have different attractiveness to honeybees (Boyle and Philogene, 1983). The relative attractiveness of weeds and other plants growing in and near fruit orchards is also important.

Thus Stephen (1958) found that pear orchards with large number of *Stellaria* spp and *Stellaria.alba* growing in them, had poor crops and he observed that honeybees only visited pear flower abundantly when the ground flora was overpopulated by bees. The sugar concentration of *Stellaria.alba* and Stellaria spp. were 48-64% and 51-58%, respectively, and sugar concentration for pear was only 7-34%.

Differences in the amount of nectar, pollen production, colour and scent of different cultivars probably lead to different attraction rates for honeybees. It was indicated by DeGrandi-Hoffman *et al.* (1991), that those cultivars of almond with higher density of flowers attracted the highest number of honeybees per flower. This can show up as differences in attraction between cultivars and activity efficiency of honeybee per tree and per flower. Furthermore Wells and Smith (1981) indicated that the number of flowers visited per trip was negatively related to the mean nectar yield per flower. Kevan and Barker (1983) indicated that flowers with highly broken designs are more attractive to honey bees than flowers that have a smooth outline.

1.2.2.1.2 The Effect of Volatile Compounds in Flowers on Pollination

Volatile compounds or fragrances are materials that can be interesting for pollinators as an attractant (Henning *et al.*, 1992). In recent years, some effort has been focused on understanding the chemical structure of these volatiles and the relation between these compounds and the foraging behaviour of pollinators (Pham *et al.*, 1992). Dobson *et al.*

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(1991) found differences in the composition of volatile compound between various species of Composites. They noticed volatiles were more attractive than pollen for honeybees. Jackobson and Oleson (1994) surveyed the effects of temperature light and humidity on the composition and secretion rate of volatiles in *Trifolium repens*. Temperature can affect the secretion quantity of volatiles. They found that a high rate emission of volatiles occurred at high irradiance. Further work showed that humidity had effects on the emission of fragrance as well.

Dobson *et al*. (1990) observed differences in fragrance compounds among the various flower parts of the *Rosa rugosa* flowers. This was conducted by analysing volatiles from different parts of *Rosa rugosa*. The compounds found included benzoid alchohols and terpenoids in petal volatiles and sesquiterpenes and fatty acid derivatives, 2 tridecanol, tetradecanol and tetradecyl acetate in sepal.

Analysis of volatiles in kiwifruit flowers showed the presence of 32 carbonyl, 24 alcohol, 6 ester 32 hydrocarbon, 6 ester and 2 miscellaneous compound (Tatsuka *et al.*, 1990). The relative importance of these compounds as bee attractants is not known.
Pham *et al*. (1994) suggested that the honeybee attraction to plants is based on an associative conditioning between food, provided by nectar and nectar pollen, and pollen volatiles. The honeybee recognizes those volatiles are different between male and female sunflowers. Bees can distinguish between plant species by recognition of fragrances from whole flower and pollen coat odour (Dobson., 1985). Bees can distinguish pollen odours among the different species of *Rosa* (Dobson *et al.*, 1987). It was found that differences in the chemical profiles of the pollen of different species can work as a signal for the honeybee (Dobson *et al.*, 1987).

As can bee seen from the above examples, it is imperative that studies be made of the attractant volatiles in fruit crops in order to better understand honey bee behaviour and therefore the efficiency of pollination in the orchard.

1.2.2.1.3 The Foraging Range of the Honeybee

The foraging range of the honeybee depends to a large extent on the availability of the nectar and pollen in the orchard. When there is not enough nectar and pollen the bee will range further to find more. But when there is an adequate supply and approximately equal distribution of pollen and nectar then honeybees remain foraging along the row of orchard trees. Kurennoi *et al.* (1984) reported that 89% of the honeybees foraged along the row in densely planted apple orchard and only 11% cross from row to row. It was reported that when food for the honey bee is scanty the foraging area of the honeybee can be extended to several sites (Singh, 1950).

It was reported (Levin and Kester, 1969) that the foraging area is inversely proportional to the colony density and directly proportional to spacing distance between trees in orchard. Consequently, changes in colony density are reflected in bee behaviour and gene flow by pollen rate, unfavourable weather conditions and disturbance by some other insects can lead to an extension of the foraging area (Free, 1960). In this case, it is possible that more cross pollination takes place. The distribution of bees on the various cultivars in an orchard depends on the unique attraction and amount of flowers and nectar and overlapping of flowering time among the cultivars. The foraging behaviour and area foraged by bee also depends on the species of bees; for example the honeybee appears to fly more methodically whilst other bees such as the bumble bee and solitary bee fly more freely and from one tree to another (Kurennoi *et al.*, 1984). In this regard, the bumble bee and solitary bee could be more efficient than honey bees for cross pollination (Paarmann, 1977). Research into the possibility of establishing artificial colonies of bumblebees for cross pollination has merit.

1.2.3 Pollination and the Weather

Weather is a key factor for activation of pollinators. The accumulative effects of temperature, humidity, radiation and wind can have effects on the flight activity of honeybees. It has been reported that in low temperatures' pollinators do not forage for pollen and nectar in the orchard (Szabo, 1980). Flight and temperatures are positively related to each other, honeybees generally do not fly at temperature below 9°C (Szabo, 1980). There is a difference between honeybee and bumblebee for adaptation to low temperature. In a field experiment carried out to compare the effect of temperature on the activity of the bumblebee and honeybee, it was found that the bumblebees, *Bombs terrestris lucorum B.pascourum* and *B.hortorum*, all began foraging at a lower temperature threshold than the honeybee (Corbet *et al.*, 1993). Adequate light is necessary for flight activity of honeybees. On a cloudy day, there is a reduction in number of honeybees in flight activity (Free, 1993). Wind has a decreasing effect on the activity of honeybee. Szabo (1980) stated that wind speeds of more than 14 miles per hour can block honeybee activity. Humidity also has a reverse effect on the foraging activity of the honeybee. Honeybees may stop activity before rain.

1.2.3.1 Effects of Temperature and Humidity on Pollen and Stigma

Temperature has been considered an important factor during pollination because of its effects on pollen humidity, hydration, pollen size and shape, germination of the pollen, tube growth, ovule longevity and the effective period of pollination (EPP) (Gilissen,1977; Corbet and Plumbridge, 1985; Heslop-Harrison, 1987).
As the humidity influence the viability of pollen grains, temperature can affect humidity and hence viability (Struik *et al.*, 1986).

In wheat it has been reported that high temperature and low relative humidity could reduce pollen viability (Struik *et al.*, 1986). Humidity affects the size and shape of pollen grains. Thus the size can affect the buoyancy. Jones and Newell (1946) indicated that

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weather conditions could affect the timing of pollen release on several species of grass and concluded that low temperature or high humidity leads to delayed release of pollen.

It has been stated that relative humidity could have effects which affect the movement of pollen on insect bodies (Corbet *et al*, 1982; Erickson and Buchmann, 1983).

Low temperature, especially post bloom temperature can delay pollen germination, pollen tube growth and pollen tube penetration into the style (Thompson and Liu, 1973). Cerovic and Ruzic (1992) concluded that the highest average number of pollen tubes in the style and ovary of cherry, occurred at 15°C.

The receptivity of the stigma in some species depends on the stigmatic exudate (Heslop-Harrison, 1987; Knox, 1984) which usually is an aqueous solution. Air humidity rate can affect the rate of this exudate and hence receptivity of the stigma. Reversibly, it was reported that high temperature and a low rate of humidity reduced the duration of stigmatic receptivity (Struik *et al.*, 1986; Souza, 1972).

1.2.3.2 Effects of Temperature and Humidity on Nectar

As the amount of sugar in the nectar is important for attraction of pollinators, the concentration of sugar can be changed by humidity and temperature. In humid climates nectar can also be diluted by prolonged exposure to high humidity. (Corbet *et al.*, 1979) Conversely, in dry weather with high temperatures, rapid evaporation may concentrate sugars in the nectar.

It was shown by Corbet (1978) that changes in nectar amount and sugar concentration of *Echium* at different times of the day was affected by shading and sunshine. They found that nectar volume and sugar content went up in the early morning between 6-7 am when the temperature had gone from 10 to 15° C, and that the amount of nectar went up in the middle of the day when the temperature was higher, but the volume had decreased by 8

pm.   William and Brouchu (1969) showed that vapour pressure deficit that is in equilibrium with temperature and humidity, affects the activity of nectaries and nectar concentration in the flower.   Reabsorption of nectar by nectaries can occur by raising of the temperature and decreasing humidity rate. Increasing the sugar content of nectar is accompanied by a secretion of nectar. The length of flowering and the secretion of nectar is dependent on temperature and relative humidity (Prabucki *et al.*, 1989). It has also been stated that, in cool, wet periods the nectar of *Vicia faba* contained 24.5% sugar, whilst in warm, dry periods it contained as much as 35.5% sugars.

1.2.4 Gene Flow and Pollination

The term, gene flow by pollen, refers to the dissemination of genes by pollen from different paternal sources (monoecious or dioecious or hermaphrodite) to maternal parents within a plant population.

Gene flow by pollen in plants is a dynamic system which is controlled by ecological and genetic variables, such as pollinator behaviour, abundance of weed plants and flowering patterns, all of which may affect the frequency and rate of gene flow by pollen in plants. The importance of this area is not only related to pollination of horticultural crops but also has links to other branches of biology such as population genetics, ecology, genetics, botany, etc.   Gene flow by pollen relates to the pollination efficiency and effectiveness of pollinizers and pollinators in the orchard. Entomologists have contributed to the study by observing the foraging behaviour of insect pollinators and their effectiveness in pollination.

The movement of pollen genes can be caused by two transfer factors, namely wind or insects. Pollen movement brought about by insect transfer is called entomophilous and by wind, anemophilous.   The mechanisms controlling the flow of entemophilous and anemophilious pollens are different, in the former wind direction and speed define the pattern of flow, but in the former one, the foraging behaviour of pollinators strongly affect the pollen distribution.   It should be mentioned that the pattern of pollen flow alone and

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gene flow by pollen are two different terms, for the former only the behaviour of pollinator is an important factor, but, for the latter genetic, consideration such as compatibility or incompatibility effects are also of utmost importance. General factors such as microclimate, behaviour of pollinators, population size, density, shape and effect of neighbourhood size control the pattern of gene flow by pollen (Levin and Willson, 1978; Ellastrand et al., 1978).

1.2.4.1 Estimation of Pollen Flow in Population and Effective Factors

Four general approaches are used to estimate gene flow by pollen in a population: 1) measurement of pollen dispersal from point sources, 2) measuring gene dispersal from point and block sources, 3) deducing gene flow from natural population genetic structure and 4) paternity analysis of progeny in sink population. The first two methods are applied frequently for gene flow by pollen estimation. The first method involves measuring pollen either indirectly from pollinator foraging distance or directly with marked or naturally polymorphic pollen (Ellastrand, 1992; Campbell and Waser; 1989; Thomson and Thomson, 1989). The second method involves finding markers by different possible crosses and progeny testing of seed harvested at varying distance from the source of pollen (Marquard, 1988; Smyth and Hamrick, 1987). Those species of plants that have high rates of selfing, generally have very low rate of gene flow by pollen in their population from other sources (Hamrick,1987; Hamrick et al.1991). All studies of gene flow by pollen showed that the genotype of the plant and the type of vector and plant density affected the pattern and rate of gene flow in the population (Schmitt, 1980; Tonsor, 1985; Levin and Kerster, 1969; Campbell and Waser, 1989).

The movement of pollen grain from a specific source is difficult to determine directly because the stigma may receive pollen from many different directions. Some workers have applied different materials such as methylene blue dye or a fluorescent dye to follow pollen distribution patterns in the plant population (Price and Waser, 1979). Usually such dyes have shown (Waser and Price, 1983) that positioning of a heterozygote plant in the

centre of a group of homozygote plants could be useful in understanding the distance and direction of pollen flow. The application of dye alone in this type of work does not enable one to recognize the pattern of gene flow with any certainty, although pollen flow is measured. Reinke and Bloome (1979) applied radiochemicals to flower buds before anthesis. After anthesis, they collected stigma at a set distance from the inoculated plant. By finding the distribution of radiochemical grain on the stigmas, they could precisely draw the picture of pollen flow. Some other workers have followed the pattern of pollen flow through a plant population by recognition of pollen surface morphology (DeGrandi Hoffman *et al.*, 1984; DeGrandi-Hoffman, *et al.*, 1992). Some workers (Free, 1962) have show that the distribution of cross pollen on adjacent cultivars is more than on other more distant cultivars. Free (1962) explained that when a bee leave one tree, it usually moves to the nearest one and the greatest set on the part of a tree nearest to a pollinizer, or to a bouquet, suggest that a bee arriving on a main variety tree from nearby pollinizer flowers effectively pollinates only the first few flowers it visits. Dilution of pollen from a pollinizer with the pollen from the main cultivar can decrease the dispersion strength of pollen with increasing distance from pollinizer. Levin and Kester (1967) showed the amount of pollen movement between taxa was strongly affected by the distance between the populations and increasing distance led to lower pollen transfer.

In contrast to the work of Free (1962), Degrandi-Hoffman *et al.* (1992) found that the distribution of cross pollen is homogeneous on a tree and was not biased by the adjacent pollinizer. With respects to the difference between the work of Free (1962) and that of Degrandi Hoffman *et al.* (1992), it can be understood that orchard design, flowering season, microclimate and types of plant may change the results of pollen flow in the orchard. Jackson and Clarke (1991b) showed a pattern of gene flow by pollen in almond orchards similar to that suggested by Free (1962). The pattern of pollen flow may depend on the structure and characteristics of the plant population.

Murawaski *et al.*, (1990) in a low density population of Bombacaceous trees showed that, there was a positive correlation between the flowering tree density and the outcrossing

rate. As the amount of pollinizer's flowers decreased relative to the main cultivar, the rate of outcrossing decreased. Differences in nectar amount or quality in different parts of a population caused by different cultivars can result in more foraging by pollinators in that part of the population and so a higher pollen flow results (Weaver, 1979). Levin and Kester (1974), demonstrated that plant spacing and flight parameters are strongly linked when considering pollen flow. Ellestrand *et al.*, (1978) investigated the effect of different densities of *Hellianthus annus* on the rate of crossing in the population. They concluded that an increasing density of a main cultivar led to a reduction in the rate of outcrossing within a population.

1.2.4.2 Estimation of Pollen Gene Flow by Detection of Marker Genes in the Seed

Estimation of gene flow by pollen by examination of the seeds can be conducted in different ways. Examination of different crosses by noting the morphological characteristics of seedlings resulting from these crosses and comparing these characteristics to possible parent plants are an obvious way to proceed. Counting the number of ovules set in a population of two groups of self incompatible and cross compatible plants can be another way of estimating gene flow by pollen. Molecular markers such as isozyme polymorphism, restriction fragment length polymorphism (RFLP) or polymerase chain reaction (PCR) DNA sequence markers are used to identify pollen genes in the seed. In this case the first step is identification of parents by these methods followed by controlled crosses to see how these markers can also be inherited. Knowledge of the segregation ratio can help in determining the allele and gene frequency and linkage analysis in the population.

Kendall and Soloimon (1973) showed that the arrangement of pollinizers in an apple orchard has an effect on the rate of cross pollen flow in the orchard. He recorded both the percentage of flowers setting fruit and the percentage setting ovules for several orchards

with different arrangement of pollinizer and main cultivar, Where a pollinizer was included in a dwarf tree orchard one for every eight main cultivar in all rows, 40% of flowers set fruit and 23% ovules. In an orchard with an interplanted mixture of large trees, 32% and 25% was recorded; where the orchard had alternate rows of pollinizer and main cultivar 18% and 7% was obtained, and for one row of pollinizer for every four rows of main cultivar 5% and 5%. Gene flow by pollen has been studied in sunflower planting by following isozyme markers in the seed set (Arias and Riesberg., 1994). They found that the gene flow pattern was inversely proportional to the distance from the pollen source. Hybrid frequency decreased with increasing distance from the sunflower standard as pollen source. Gene flow from the pollen source continued even over 1 km distance. Gene frequency decreased from 0.27 at a distance of 3m to 0.02 at a distance of 1 km from the pollinizers.

Marquard (1988) investigated the rate of outcrossing in a pecan orchard by recording diagnostic isozyme markers between self and cross plants. His finding showed that increasing distance from the row of the pollinizers resulted in a reduction in the rate of outcrossing. Pecan is wind pollinated, self or cross pollinated species with no self-incompatibility. In many cultivars pollen shedding takes place before the stigma becomes receptive and in some cultivars stigma maturity precedes the pollen dehiscence, thus the cross pollination is inevitable.

Comparison of strawberry fruit weight in two plots at different distances from groups of hives 25-50 m and 150-200 m away showed that the mean berry weight was 33% higher for the plot nearer to the hive (Severson, 1993). Since the fruit weight in berries is in direct relationship with number of seeds, the increased number of seeds in berries in the plot near the hive indicated a higher gene flow by pollen to the plot closer to the hives.

Levin and Wilson (1978) showed that gene frequency in plant populations is affected by both plot size and pollen dispersal. Gene flow in an almond orchard was studied by Jackson and Clarke (1991). They found that the gene flow was greatest to the side of the main cultivar row next to the row of pollinizers, and that the rate of gene flow was

reduced in the side away from the pollinizer. It was concluded that the behaviour of pollinators by focusing on one cultivar can play the determining role of gene flow pattern in the orchard. It has been shown in an avocado orchard, that there is a decrease of cross pollination with increasing distance from pollinizers (Degani et al., 1989). By application of three specific markers in three isozyme systems including MDH, TPI, LPA, it was found that the rate of abscission in the avocado for Hass cultivars depends on the pollen parents. Hybrid progenies of Hass cultivar with Ettinger cultivar could survive more than self progenies of Ettinger cultivars. In this work, they found that when Ettinger was used as a pollen parents for Fuerte cultivar, the outcrossing rate in the tree adjacent to Ettinger was about 40%. This showed the rate of crossing and selfing at close distance to the Ethinger was approximately equal despite the fact that the outcrossing rate in Fueret decreased with increasing distance from Ethinger, there was no effect of yield of fruit from Fuerte tree (Goldring et al., 1987).

1.2.5 The use of Isozyme Markers in Cultivar Identification

1.2.5.1 History

The first research hinting at the existence of isozymes was started by Mallettle and Dawson (1949) who obtained five different purified tyrosinase preparations from the common mushroom. Other workers such as Gillespie et al. (1952) found there were multiple forms of esterase, amylase, β-glucosidase, sucrose, cellulase, protease and alkaline phosphatase in Aspergillus spp. Roberts (1956) found that wheat acid phosphatase activity in leaves is due to several distinct enzymes with narrow substrate specificity. The birth of the isozyme technique began with the development of starch gel electrophoresis by Smithies (1955).

The second major event was the demonstration that these enzymes could be visualized directly on a starch gel when stained with a specific histochemical stain. (Hunter and Markert 1957). Gradually the term isozyme was used as first coined by Markert and Moller (1959) to show different forms of an enzyme with similar substrate specificity.

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1.2.5.2 Applications

The isozyme technique has been applied extensively to different areas of plant biology such as breeding, genetic identification, population genetics, genetic linkage studies and mapping of chromosomes (Chyi and Weeden, 1984, Fobes, 1980). Identifying variability in plants by isozyme techniques is an important approach to establish new genetic lines by sexual and somatic hybrids. Furthermore confirmation of hybrids and parental identification has been carried out by isozyme technique (Gogorcena *et al.*, 1990).

Linkage studies have also utilized isozyme techniques. Mangarian and Alston (1992) found a close linkage between LAP2 and resistant genes to mildew in apple. Torres *et al.*, (1986) indicated a linkage between two loci of GOT1 and GOT2. Meanwhile Mangarian and Alstone (1992) found a linkage between GOT1 and the S locus for incompatibility in apple.

When the protoplasts of *Soybean spp* and *Nicotina gluca* were fused together, the resulting somatic hybrids were identified by the isozymes of alcohol dehyrogenase (ADH) and asparate aminotransferase (AAT). The bands screened from both parents as they appeared on zymograms, allowed linkage studies to be carried out on the F1 generation (Tanksley and Rick, 1980). As the isozyme bands are codominant for most alleles, this sort of study can easily be achieved. In linkage studies, a proposed genes which linked to an isozyme or a group of isozymes may be discovered by expanding a linkage map through using the isozyme markers. Linking to these isozyme markers can be applied in finding desirable genes in segregating populations. For example the linkage relationship is now being used extensively in breeding programs to transfer resistant genes from one cultivar to another (Rick and Fobes, 1974).

The saving of time for the plant breeder is another aspect of the application of isozyme techniques. For example, when genes are to be introgressed from one genotype into

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another, a number of backcrosses may be necessary after the initial cross in order to re-obtain the recurrent phenotype. The amount of time which must be invested in this procedure makes the backcrossing a costly technique for the plant breeder. Isozyme techniques have been employed to reduce the number of backcross generations necessary for the return to the recurrent parent genotype (Tanksley and Rick, 1980). Differences in isozymic alleles between the donor and the recurrent parent have been exploited for this purpose. Isozymes have been employed for breeding self and cross pollinated progeny. To do this the two parents and F1 can be identified by their isozyme profiles (Tanksley and Rick 1980). Furthermore in cross- pollinated crops allozyme frequencies could be applied to estimate the amount of heterozygosity present in cross-pollinated crops as distinct from selfed ones (Nijenhuis, 1971). Isozymes were used for the estimation of cross pollination in 26 populations of wild barley at 22 polymorphic loci. The result of this experiment showed that the rate of cross pollination was 1.6% (Brown, 1978). This work showed that *Hordeum vulgaris* was as the major source of allozyme diversity in wild barley populations. This result led breeders to carry out crosses with *Hordeum vulgaris* in order to change of the genetic characteristics of barley (Shaw and Allard, 1982; Arulsekar and Bringhurst, 1981). Muller (1977) used a marker tree with a rare isozyme allele to estimate the amount of cross pollination which occurred in trees at various distances from the marker tree.

1.2.5.3 Application of Isozymes in Pome Fruits

In general the genetic aspects of fruit trees are more complex than for annual plants. Thus identification of fruit trees by some morphological characters which are controlled by multiple genes is difficult. By using systems which are controlled by a single gene can make it easier to identify fruit tree cultivars. During the past decades many efforts have been focused on the identification of fruit crops (Weeden and Lamb, 1987; Parfitt *et al.*, 1985) by isozyme techniques.

In some plant tissue Phenolic compounds which block the activity of isozymes and, in some cases, lower the activity of some isozymes have been a problem for resolution of the

zymogram by staining.   In this case, some workers have used pollen samples to increase the activity where no phenols are present during staining.

1.2.5.3.1 Apple

Menendez *et al.* (1986) tested 33 clones of apple rootstocks for 4 isozyme systems. The large number of different banding patterns obtained for each isozyme system, indicated the possibility of considerable genetic diversity among these apple rootstocks. Acid phosphatase (ACP) and peroxidase (PX) showed great diversity of bands with these clones.

Weeden and Lamb (1985) characterised 45 apple cultivars using 6 isozyme systems, the most useful enzyme systems were found to be 6-phosphogluconate dehydrogenase (GP6D) and aspartate amino transferase (AAT).   The authors could identify possible parents for different hybrids. These workers have recently elucidated two areas of activities for GPI.   They used young leaf tissue after leaf bud flushing. GPI-2 showed polymorphism for these 54 apple cultivars (Weeden and Lamb,1985). Meanwhile IDH presented triple banding patterns, which were interpretted as *aa* for the most anadol band, the middle being *ab* and the most cathadol one as *bb*. These researchers found that diaphorase isozyme system showed polymorphism at locus 2, triosphosphate (TPA) showed activity but no polymorphism in their work. For MDH Weeden and Lamb (1985) reported five possible loci. Marquard and Chan (1995) reported polymorphism banding patterns for SKDH ,6PGD, AAT, GPI and ADH using leaf and dormant bud tissues of crab-apple.   By this work, it was concluded that ADH was regulated by two loci, one of which is polymorphic.   Samimy and Cummins (1992) tested 13 apple rootstocks in 6 isozyme systems two of them PGM and 6PGD were found to be the most useful. By using the ADH isozyme system, three zones of activity were found. Differences in Zone 2 of ADH led to a method of recognition among these rootstocks. Already, Chevreau *et al*. (1985) had reported that ADH is a dimeric enzyme and was coded for by two loci in apple.   Furthermore these worker showed 4 main band positions by PGM for apple

cultivar. Meanwhile it was concluded that 6PGD showed more variability than the other 5 enzyme systems. By using this enzyme pattern the rootstock could be divided into 10 distinct sets. Chyi and Weeden (1984) could identify different intensity and number of bands in 6PGD for Johnathon, Jongold and Spigold apple cultivars. These authors stated that differences in the intensity of heterodimer bands of *ab* and bb with *aa* could be a reason for variability in dosage effect of this gene. Presence of different copies of alleles in the gene can be a reason for these differences.

1.2.5.3.2 Pear

Santsmoure and Demuth (1980), were the first workers to use isozymes for pear identification. The aim of their work was the recognition of two cultivars; Redspire and Whitehouse cultivar, which were supposed seedlings of Bradford.

Parental recognition of some seedlings resulting from open pollination of Chanticleer and Bradford was another goal for their work. They obtained polymorphisms on peroxidase banding patterns, with 10 cathodal and 5 anodal bands. They used both leaf and dormant cambium tissue of pears for this work. Interestingly the banding patterns were not quite the same for both tissues. The authors identified seedlings which resulted from Chanticle open pollinated from Bradford, but they also found markers enabling the recognition of selfed progeny. They showed a similarity between William and its budburst Max Red Bartlet. Meanwhile another similarity was found between Crab-apple Favourite and its budburst Starkimson. Menendez and Daley (1986) used PX, ACP and EST isozyme systems for identification of some pears. They employed double isoelectric polyacralymide gel for their work. ACP and PX showed more polymorphism banding patterns than EST. Crezo *et al.* (1989) looked at pollen from 18 pear cultivars, performed 6 isozyme systems. These isozymes included AAT, ACP, ADH, GPI, LAP and PGM. The highest numbers of polymorphisms were detected for GPI, PGM, AAT, ACP and ADH in that order. As the dimer nature of some enzymes appeared to be heterodimer, it reinforced the hypothesis on the allopolyoid origin of the cultivars.

1.2.6 Identification, using Polymerase Chain Reaction(PCR)

DNA Fingerprinting

1.2.6.1 Technical Explanation

The polymerase chain reaction (PCR) is a technique which can be used to amplify specific regions of DNA by DNA polymerase and application of short nucleotide primers (McPherson *et al*, 1991; Newton and Graham, 1995).

The PCR technique was devised by Mullis and Faloona in 1988 as an *in vitro* enzymatic method, different temperatures replacing the enzymatic function for DNA denaturation and annealing of primer to target DNA. PCR consists of 3 main steps (Fig 1.1) including A) denaturation, B) annealing C) extension (Hill *et al.* 1992).

Denature DNA sample
to separate DNA strands
(94°C, 5 min)

Primers bind to
DNA strands
(30–65°C, 30 s)

Denature to separate
DNA strands
(94°C, 30 s)

Polymerase synthesizes
new DNA strands
(65–75°C, 2–5 min)
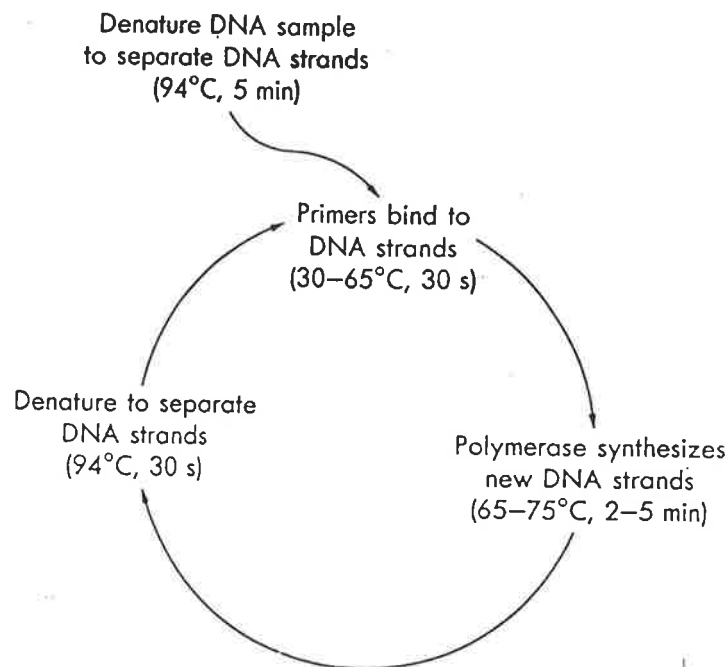
Fig.1.1 The PCR cycle, consisting of denaturation, annealing and extension, with different temperatures applied at each step (from Watson *et al.*,1992).

A) Denaturation Two strands of DNA separate by heating at temperature just below boiling point 94°C and convert to single strands. As the DNA polymerase cannot tolerate this temperature thermostable Tag DNA polymerase is used. This Tag Polymerase is isolated from the thermophilic bacterium *Thermus aquaticus.*

B) Annealing Refers to the lowering the temperature to between 37-65° C in order to anneal primers to the target DNA on the site flanking the region of DNA to be amplified Both DNA strands work as template for annealed primers for the synthesis of DNA. (Hill *et al.* 1992).

C) Extension At this stage the temperature is raised to 65- 74°C which is the optimal temperature for the activity of Tag (*Thermus aquaticus*) polymerase. This stage takes about 2-5 minutes. Synthesis of DNA starts from 3' to 5' direction (Watson *et al*, 1992). And so the cycle continues denaturation, annealing and extension allowing amplification of DNA in an arithmetical format. The number of newly synthesised DNA strands are dependent on the number of cycles; $N = (2)^{n-1}$ where N is the number of DNA strands product and n number of PCR cycles (Innis *et al.*, 1990).
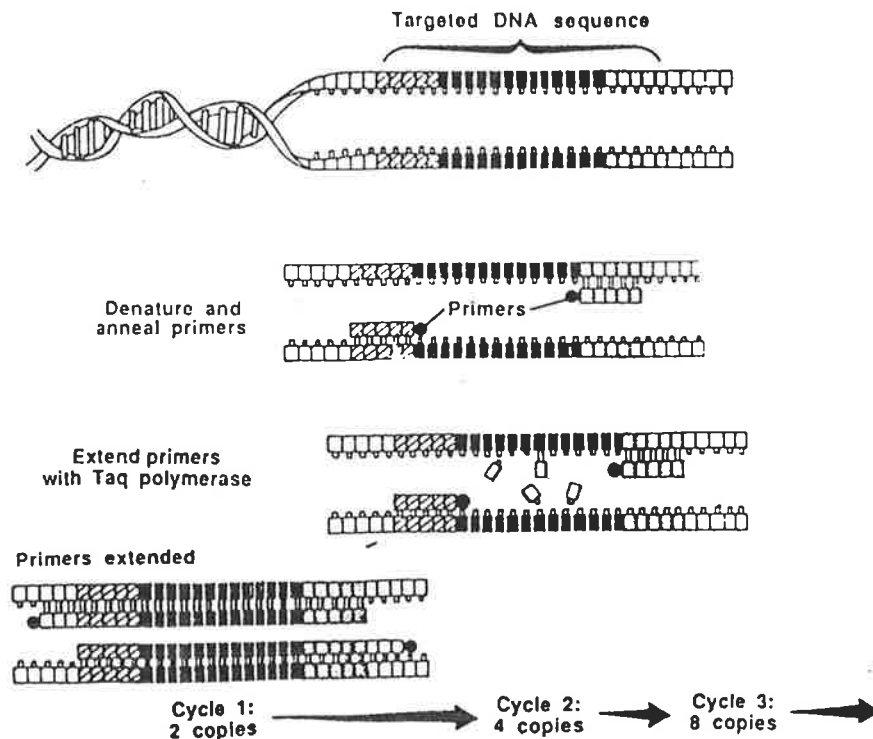
Fig.1.2. Newly synthesised DNA as product separates in the first step of another cycle

(From; Kirby,1990)

1.2.6.2 Application of DNA PCR fingerprinting for tree fruit crops
Random Amplification Polymorphism DNA (RAPD)

RAPD and microsatellite PCR DNA fingerprinting can be useful methods for identification of close genetic phylogeny of plants (Teramoto *et al.*, 1993). Mulcahy *et al.*(1993) applied two 10-mer primers in order to understand genetic variations in eight cultivars in 25 accession of apples. A total of eight cultivars were successfully separated by these primers. Koller *et al.* (1993) used 10-mer sequence 5'ACGAGGGACT and also the sequence of 5'AAGACCCCTC with DNA extracted from 11 apple cultivars for PCR identification. The amplified segements with these primers divided these cultivars into only two groups. Tancred *et al.*(1994) differentiated a new Queensland apple cultivar from 3 others with similar phenotypes. The primer OPM04 was used in this work. OPM04 produced specific similar marker bands of size 700bp for the 3 cultivars which made a distinction for recognition of the Queensland cultivar.

Polito and Hormaza (1994) found a sex determinator marker for pistachio by application of decamer oligonucleotide primer CCTCCAGTGT. This primer produced a 945bp amplified band which was presently in the bulked female samples. The absence of this band in male bulked enables it to be used as sex detector in pistachio. Teramoto *et al.* (1993) attempted the on identification of Japanese pears by employing human minisatellite DNA probes. They were able to show limited differences in Japanese pears. On the other hand they did obtain an analysis of parental relationships for some of these pears. This work indicates that many other fruit trees could well also utilize DNA fingerprinting with human minisatellite probes. RAPD and other PCR DNA fingerprinting methods show great promise in the future for fruit tree identification.

1.2.7 Fruit Set

The control of fruit set is based on both hormonal and nutritional factors (Faust,1989). Fruit set has been defined as the persistence and development of an ovary with adjacent tissue following anthesis (Westwood, 1978). As the mechanism of fruit set is different between parthenocarpic fruit and normal fruit the definition of fruit set should be more precisely defined for each. In this way, we can define fruit set from an hormonal aspect which is the growth of the ovary wall caused by some hormone originating from the seed (Luckwill, 1948). The hormone may appear in the locule or ovary wall by some stimulator such as the pollen tube and lead to fruit growth. The mechanism, of vegetative parthenocarpy still is not clear. Stephenson (1981) defined fruit set as the deposition of pollen, which is a rich source of hormones, on the stigmatic surface providing the stimulus for the renewed growth of the ovary. However, there is little evidence for this.

In general, factors which affect fruit set can be divided into two sections. Firstly, environmental factors such as temperature during bloom and early post bloom, wind, bee activity, rainfall, light during pollination and pollinizers. Cultural practices such as orchard design and pollinizers, pruning, thinning, nutrition, scoring and application of growth regulators have promoting effects on fruit set. In fact, fruit set is influenced by vegetative growth, optimal growth of tree leads to a balance between the vegetative and generative parts of tree. If the plant could send enough sap to reproductive organs during stages of flower initiation and anthesis and post bloom perhaps more fruits would be constituted. A second group of factors affecting fruit set are internal factors within the plant. These are ovule longevity, compatibility of pollen, the rootstock, leaf to fruit ratio, leaf area index, ovule normality and flower structure.

1.2.7.1 Fruit Set and Temperature

Temperature has been considered to be an important factor during pollination because it affects pollen viability, germination, pollen tube growth, ovule longevity and the effective period of pollination (EPP) (Sedgley and Griffin, 1989). Jackson *et al.* (1983) reported temperature in the pre blossom period is negatively associated with fruit set. They indicated that fruit set on Cox's Orange Pippin apple was increased when they were cooled during February, March and April by placing them in a cold store at 5°C or by misting in the orchard whenever temperature rose up 4.5°C.

Mellenthin *et al.*(1972) found that temperature drastically affected the rate of pollen tube growth. For example, Vasilakakis and Porlinigis (1985) demonstrated that the EPP of Tsakoniki pear varies between five days at 20°C and 13 days at 8°C. Also they indicated that pollen tube growth to the style base of Tsakoniki pear; for both self and cross pollination took 6 days at 15°C, compared to 3 days for Anjou and Williams at the same temperature. Thus different cultivars showed different speeds of pollen tube growth.

The EPP is modified by temperature and genetic composition of the plant, and therefore is an important factor in fruit set. Vasilakakis and Porlinigis (1985) determined that pear ovules were viable for 11 days at 5°C. Thus, at 5°C, the EPP was zero, because at this temperature it took 12 days for pollen tube to reach to the base of the style while at 15°C it was 6 days. Accordingly, slow growth of tubes at low temperature is thought to be the principal reason for low set of fruit during cool spring seasons. It was reported by Sedgley and Griffin (1989) the effective period pollination (EPP) in tree fruit crops is very temperature dependent. Different cultivars in pome fruits have different EPP. Temperature affects on both rate of pollen tube growth and the rate of ovule degeneration Also, the rate of pollen tube growth for different pollen and pistils is variable at different temperatures (Vasilakakis and Porlinigis, 1985).

Temperatures could provide good conditions for efficient pollination and fruit set with regard to other factors. As a result the activity of bees is an important factor for pollination and fruit set, suitable temperatures can promote activity of bees, hot or cold weather can inhibit their activity. Warm temperatures during bloom are important as honey bees do not fly at temperatures below 10°C. Brittain (1933) found a rise in activity of honey bees from 10°C to 18°C followed by a gradual decrease to a low level at temperatures of 30C and above.

As a result, different species of pome fruits show different adaptation to temperature, but in general, average maximum and minimum temperature between 20°C and 10°C respectively is reported to be the most suitable temperature for obtaining greatest fruit set during pollination period among the species of pome fruits (Mitra *et al.*, 1991).

1.2.7.2 Ovule Longevity and Fruit Set

Fruit set is also influenced by ovule longevity and fertility, greater longevity and fertility being accompanied by greater fruit set. In addition to temperature, other factors such as hormones and presence of pollen can extend or shorten the longevity, and thus affect fruit set. It was reported by Herrero and Gascon (1987) that pollination or GA3 treatment of *Pyrus communis* does not alter embryo sac development, but both prolong embryo sac viability. It was demonstrated that in untreated unpollinated flowers, the ovule degenerated between 12 and 21 days after anthesis while in cross pollinated and GA3 treated flowers, this degeneration is postponed by about 10 days. In a cross pollinated species this extends the period over which successful fertilization can take place. This extension of the period of embryo sac viability observed in cross-pollinated species occurs before fertilization takes place. It was shown by Herrero and Gascon (1987) that embryo sacs initiate rapid elongation, two weeks before fertilization and thus this extends embryo sac viability. The hypothesis that greater ovule longevity depends on the pollen cultivar may be true because on pollination a wave of cytoplasmic and biochemical activity precedes the pollen tube along the length of the pistil, and this is capable of inducing

activity in various tissue of this organ. This is evidenced by an increase in polysome number (Herrero and Dickinson, 1979), RNA changes and alteration in the level of protein synthesis (Herrero and Gascon,1987). Different types of pollen create different patterns in RNA and protein synthesis which leads to changes in the activity of pistil tissue (Herrero and Gascon,1987).

It was reported for Agua De Aranjuez pear (Herrero, 1983), maturation of the ovules does not proceed for all ovules in a single flower for the same way, 38% of ovule being functional after anthesis, while most of them are functional 3 days after anthesis. Meanwhile Lombard et al., (1972) noticed differences in stigma receptivity between different cultivar of pears. Earlier they reported 'Comice pear; had less receptivity of stigma than other pears.

1.2.7.3 Effects of Pollinizers, Cross Pollination, Orchard Design and Flower Structure on Fruit Set

For maximum fruit set, all of the factors increasing both pollination and fertilization should be of the optimum. These factors could be a combination of main variety or varieties with their best compatible pollinizers. Furthermore, pollinizers cultivars should have sufficiently overlapping bloom period with the receptive cultivar. Viability of pollen is another important factor affecting pollination. It was indicated cultivars Waite and Magness are completely male sterile and obviously can not function as pollinizers (Mitra et al.,1991). It was reported (Hiratsuka et al, 1985) cross pollination of self-incompatible Japanese pear cultivars produced 90-100% more fruit set in comparison with 20-26% obtained from self-pollination. Fruits from self-pollination were more malformed and slightly smaller in size. Also, good fruit set depends on the distance between pollinizer and main variety, and this distance may have a marked effect on fruit set. Other factors are flower structure and amount of nectar for each cultivar to attracted honey bees which leads to greater pollination of flowers. Soltesz and Nyeki (1982) demonstrated that optimal placement of pollinizers varieties can lead to greater fruit set. In another study on four varieties of

apple it was found that, distance from a beehive had an effect on the fruit set. The distance varied between 4 and 440 m from the nearest beehive and fruit set, number of seeds per fruit and yield were all adversely affected by greater distance from the hives, while the effect on average fruit weight was the opposite. Also it was shown that the sensitivity of cultivars to distance from the hive could be different, for example Jonathan was less sensitive than Golden Delicious and Starking (Mijacika, 1986). Different male cultivars as pollen donor have different setting potential (Fig.1.4). With some of the differences being related to self-compatibility. Self fruitful cultivars such as Golden Delicious might be expected to set well in comparison with self-unfruitful ones such as Delicious. Also some varieties such as Granny Smith seems to have poor set in comparison with other cultivars such as Jonathan and McIntosh. Reasons for this may be include ovule longevity, flower structure and response to temperature and carbohydrate reserves.

Key to signs :

1 Hardy vojkörte ♀ × Téli esperes ♂
2 Téli esperes ♀ × Hardy vojkörte ♂
3 Hardenpont téli vojkörte ♀ × Hardy vojkör
4 Hardy vojkörte ♀ × Hardenpont téli vojk
5 Hardenpont téli vojkörte ♀ × Téli espere

Yield in row

(kg tree)

Distance from pollinizer(m)
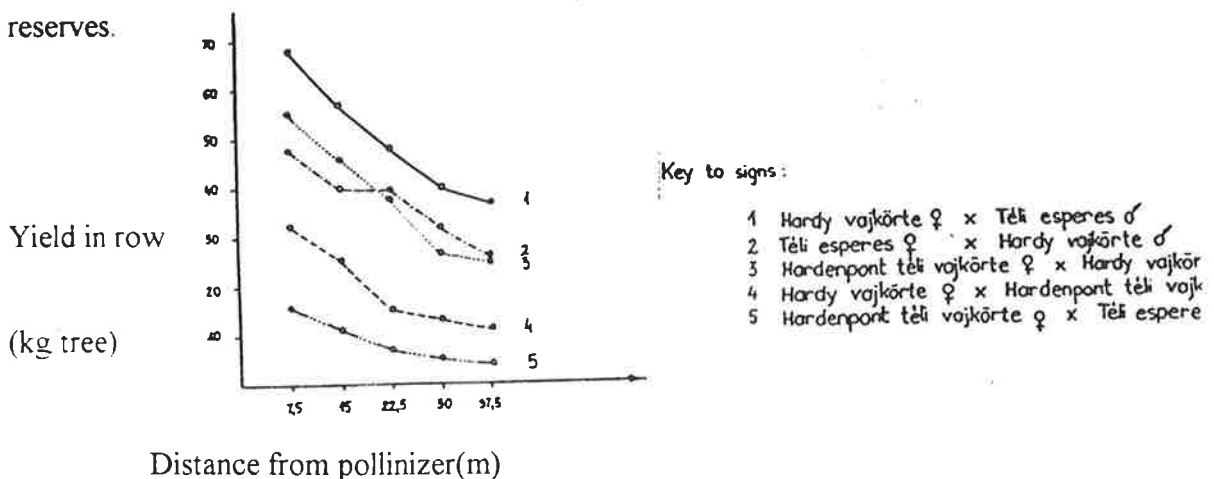
Fig. 1.3 The influence of the pollinizer distance on the yield and placement of varieties in the row (Soltes and Nyeki, 1982).

Flower structure can play a role for attraction of honey bee (Robinson,1979; Dennis,1986) studied the foraging of bees on flowers of a series of apple cultivars in New York, Pennsylvania and Nova Scotia. He observed differences in basal gaps between stamen filaments of different cultivars. These gaps were much larger in delicious than in 11 other cultivars. The size of these gaps for Golden Delicious was between 273 to 546

uM and the maximum for the other cultivars was 137uM (for Rhode Island). As the honey bee glossa (tongue) is about 180u in breadth, nectar collection through the basal gaps would be difficult in cultivars other than Golden Delicious. Based on this, the bees could extract nectar without crawling over the top of the blossom. As a result, types of pollinizers, number of pollinizers, their placement, flower structure and rate of nectar could have a significant effect on pollination and fruit set. Other factors in addition to basal gaps, such as strength of flower as a sink, and ovule longevity in different strains, could change the results of fruit set (Dennis, 1979).

1.2.8. Parthenocarpic Fruit Set

Parthenocarpy refers to the setting of fruit without seed formation in absence of functional pollination, Noll (1902) was the first to define this term. Parthenocarpy has been reported for many fruits and vegetables such as citrus, date, pear, grape, fig and cucumber, tomato, tobacco (Gustafson, 1942).

1.2.8.1 Genetic and Enviromental Aspect of Parthenocarpy

Gustafson has mentioned two types of parthenocarpy facultative and obligative. Obligative results from genetic sterility, while facultative parthenocarpy refers to the situation where fruit sets with seeds in favourable environments and to the inducement of seedless fruit in unfavourable weather conditions. There has been some research into finding the genetic basis of parthenocarpy. Lin et al. (1984) found a single recessive gene Pat 2 in Severianian tomato cultivar. They showed that expression of this gene is strong in unfavourable environment condition , e.g. high night and day temperatures. These authors stated that another cultivar of tomato Oregon T5-4 sets parthenocarpic fruit when temperature is below 18C. Furthermore they added, the environment that favours expression of parthenocarpy acts on sporophytic rather than gametophytic tissue . Johnson and Hall (1954) found a parthenocarpy of tomato under high temperature (31-37° C). However high temperature as well as low temperature plus short days and high

humidity also favours parthenocarpy in tomato. Furthermore Lin *et al.*, (1983) added lack of pollen plus rapid growth of ovary are responsible for the development of parthonocarpic fruits under green house conditions. There is considerable evidence to suggest that there are differences in hormonal balance between ovules of parthenocapic and seeded fruit. Many researchers have concluded that for parthenocarpy the auxin content of ovaries at the time of blossoming were high enough to induce ovary growth without fertilization of ovules. For pear (Grigges *et al.,* 1970) and for grape (Iwahori *et al.,* 1968), auxin contents were greater than that already found in seeded fruits. Some pear cultivars such as Bartlett and Anjou are self sterile, but they behave as self-fruitful under certain conditions because of the production of parthenocarpic fruits. The extent of parthenocarpic fruits is determined by the location, season and tree vigour. Furthermore, parthenocarpic fruits are more prone to pre-harvest drop than the seeded fruits.

1.2.9 Fruit Shape

Fruit shape is influenced by a combination of endogenous and exogenous factors. Endogenous factors include such things as hormones (concentration, localisation), genes, cell structure, seeds, rootstock, ovary position (Herrero, 1989). Exogenous factors may be growth regulators applied to the plant, pollen which may affect the gene expression during fruit set and development and change the hormonal balance in fruit, thinning, pruning, and the temperature before and after bloom. Temperature indirectly affects the shape of fruit by its effect on the number of flowers to be set and seed set (Tromp, 1990). Changes in shape can be caused by local changes in growth rate or changes in direction of growth or a combination of these. Changes in growth rate of cells can be caused by changes in the plane of cell division and enlargement. Some hormones as gibberellin and cytokinin can promote division and some other factors such as gamma irradiation, inhibit cell division. With this respect that the pattern of cell division and extension affect on fruit shape, there is still a lack of knowledge about factors which affect cell shape such as the exact role and mechanism of cytoskelton in the determining of cell shape and cell division (Lyndon, 1990).

1.2.9.1 Influence of Seeds on Fruit Shape

The formation of seeds within the pericarp has a profound effect on fruit shape and development. Pome fruits that develop a full complement of seeds are more symmetrical than those with fewer seeds (Ryugo,1988). It has been suggested (Nakagawa et al. 1968) that seed development leads to greater extension of locules and ovary, affects the cell division and extension of the outer sections of locules. The reason for this is probably due to hormones such as gibberellin and cytokinin. Nakagawa et al., (1968) explained that localization of hormone can define the shape of the fruit, but there is no definitive proof of this as yet, because the pattern for vegetative parthenocarpy is still not clear. Marcucci and Visser (1983) explained the shape of the fruit by its core and locule form. They explained that in Bonne Louise and Conference pear fruits, locules with normal seed are much larger than locules with only aborted seeds. Parthenocarpic fruits have a much

smaller core than normal fruits without abortive seeds and the core of the latter are smaller than in fruit with normal seeds. Generally the locules of the large fruits are only slightly wider than those of smaller fruit thus it is clear that the size of locule can affect the size of core and that both can affect the shape of fruit. This is demonstrated by Conference fruit which had a smaller diameter, and a smaller core in comparison with the fruit which had seeds, and a much larger core than those without or with aborted seeds. Nakagawa *et al.*, (1968) showed that core diameter of parthenocarpic fruits in Japanese pear induced by gibberellin was greater in length and smaller in width than seeded fruits. Nakagawa *et al.*, (1968) also concluded that the seeds play an important role in the shaping of the fruits. Unilateral position of seeds in *Pyrus nivalis* may cause local enlargement of the core. Since the layer of the flesh outside of the core is uniformly thick, a rather asymmetric fruit is formed. Marcucci and Visser (1983) noticed that seeds do, in fact, shape the fruit indirectly by shaping the core and showed that the cortex diameter is largely independent of the presence of the seeds (Nakagawa *et al.*, 1968). It may be concluded that with respect to cortex development, the seeds play a minor role. Meanwhile, with respect to different pattern of shape, correlation of locule and core shape in pears cannot be a reasonable and full illustration of the shape of the fruits. A highly significant correlation was found in tomato between ovary size at anthesis and final fruit diameter. Ovary diameter gave the same results in relation with fruit diameter (Owen and Aung, 1990). Proctor and Schechter (1992) pointed out, that the fresh weight and deformity of fruit is correlated with seed number. They conclude that ovule damage increased fruit deformity and reduced fruit weight, but had no effect on fruit length. The precise mechanism controlling fruit shape is still not clear, but the factors affecting pattern of cell divsion and extension during fruit development may define the shape.

1.2.9.2 Effects of Hormones and Growth Regulators on Fruit Shape

Spraying of cytokinin, gibberellin, and 2,4,D at different stages of fruit development could lead to different shape of the fruits. It was reported that Delicious apples which were grown in a hot climate were oblate, and became more elongated by using the spray three days after petal fall (Westwood, 1978). Herrero (1989) showed that treatment with GA3

at different times with different concentrations could lead to different shape of pear fruit. Early treatment at flowering or 2 consecutive treatments of 5 ppm, one at flowering and the other at petal fall produced malformed fruit. But late treatment (10 ppm) either at the onset or during petal fall produced good shaped fruits. Herrero (1989) concluded from this work, that early treatment at flowering induced mainly parthenocarpic setting, but following the late treatment during petal fall, most of the fruit were seeded. A possible reason for this is that gibberellin could prolong embryo sac viability. It was reported by Stembridge and Morrell (1972), that cytokinin, 6-benzyladenine (BA) and gibberellin GA4+A7 (but not GA3) increased the ratio of fruit length to diameter (L/D) ratio of Delicious apple. The prominence of points near the calyx was increased more by BA than GA4+GA7 but the increase was greater when the two were combined. Sprays during bloom were more effective than post bloom. Nakagawa *et al.*, (1968) found that unequal localization of hormone applications could be a reason for forming asymmetric fruit in Japanese pear and apple. Application of GA3 to one side of the fruit 2 weeks after bloom led to both a cell number and size increase in the treated side as compared to the non treated side in both types of fruit. Greater responses were observed in parthenocarpic than seeded fruit. It was also observed that there was a striking increase in cortex thickness due to a greater cell size and number on the treated side in comparison with the non treated side following treatment with GA7, 4, 6 and 8 weeks after bloom. Based on this experiment GA3 failed to induce asymmetric fruit growth. Also recent work (Nakagawa *et al.*, 1968) showed that kinetin and IAA have little influence on fruit shape, but 2,4,D causes small size oblate fruit.

1.2.9.3 Influence of Rootstock on Fruit Shape

Rootstock type can also influence fruit shape. It has been demonstrated (Westwood and Blaney, 1963) that fruit from trees on non-dwarfing rootstocks are relatively more elongated than those from dwarfing rootstock. Different rootstocks therefore (Westwood and Blaney, 1963) could change the fruit shape of Red Delicious. Rootstocks can affect

fruit size which is associated with weight of fruit. In Russia (Larsen, 1982) compared the fruit quality of 28 cultivars on rootstocks of quince A and wild pear seedling. Quince rootstock increased fruit uniformity and size. In Yugoslavia (Stancevic *et al.*, 1972) fruits of Bartlett, and Clapp which were developed on quince were larger than on seedling rootstocks.

1.2.9.4 Effects of Climate and District Location on Fruit Shape

It was shown that geographical situation including climate, altitude and latitude affects the fruit shape (Greenhalge and Godley, 1976). The reason may be that in different districts, different radiation, climate and humidity could be found. All of these could affect the pattern of fruit development. Greenhalge and Godley, (1976) reported a relation between apple fruit shape and the altitude at which the trees were grown. Shaw (1914) observed that apples with the highest L/D ratio came from the district with coolest climate during flowering and fruitlet stage and oblate fruit comes from regions with high temperature within 16 days after bloom. Greenhalge *et al.*, (1977) pointed out that the temperature during the seven days period following full bloom is critical in determining final fruit shape. Greenhalge *et al.* (1977) found a correlation (r = -0.86) between soil temperature and fruit L/D ratio of Red Delicious apple. Also, it was shown that, in cooler districts, apples develop a shape with straight sides, concave near the crown of the fruit with a tendency to a pentagonal rather than circular outline in the equatorial plane, with a pronounced calyx lobe and fruits are longer than wide (high L/D ratio). In districts with mild to warm climates, however fruits appear with a low L/D ratio. Westwood *et al.*, (1968) reported that Delicious grown in a climate with warm clear days and cool nights are more conicelongate than those grown in hot days and warm nights. Westwood and Burkhart (1968) reported also that changes in shape occur for 60 to 90 days after bloom. Fruit length also was found to be negatively correlated with the number of heat units above 5°C during the entire season and not just those immediately after bloom. Frost

could change shape of fruit to malformed shape. The reason is that the placenta could be killed by frost, consequently preventing ovule development. If only a portion of the placenta and ovules is killed the fruit may survive (Strang *et al.,* 1980). Strang *et al.* (1980) reported that simulated frost injury to ovaries at intervals after full bloom significantly increased fruit malformation and reduced fruit weight. Finally, the effects of temperature radiation and humidity on cell division and expansion of fruit is an area for further study.

1.2.10 Fruit Weight

Fruit weight is a combination of different elements after fruit development such, as cell number, cell density, air space between cells, interacellular space between cells, lignification of cells, water content and soluble solid. Fruit weight can be affected by different factors which change these elements. These factors are rootstock, thinning, pruning, application of hormones, climate, irrigation and nutrition (Westwood,1978). It was also reported that flower position could effect fruit weight. Fruit coming from the centre blossom had larger fruits than side blossoms (Westwood *et al.*, 1960). As high fruit weight is associated with large fruit, the factors which affect fruit size also should be considered. In this way, it should be considered that cell size and number combine differently to give various size of fruit. Westwood *et al.,* (1960) reported that the factors affecting cell size of the fruit could be many cell per fruit, heavy bloom and set, low soil moisture, weak fruiting, low N, low leaf-fruit ratio, early thinning (increase cell number) chlorotic or diseased leaves, moderate chemical thinning.

1.2.10.1 Effects of Pruning and Thinning on Fruit Weight

Pruning and thinning are both cultural practices which increase the nutritional level of the fruit and thus lead to more fruit weight. Guignebault and Trilliot (1989) indicated that increasing the level of pruning decreases the number of pear fruits (Conference) but

increases individual fruit weight. In their work it was showed that the proportion of fruits > 55 mm in diameter increased as the degree of pruning increased. Marini and Sowers ((1991) found that spur pruning of Golden Delicious apples increased yield, but did not increase fruit weight. George *et al.*, (1986) found that fruit weight of Custard apple was negatively related to fruit number per tree (r = -0.69), but the author confirmed that the potential growth rate of the fruit is controlled endogenously. Burge *et al.* (1991) reported that heavy flower thinning of Hosui (*Pyrus pyrifolia*) reduced initial fruit set and resulted in a significant increase in fruit size. Time of thinning is a factor which could affect on fruit size and weight. Bergh (1991) showed that thinning during the first 2 weeks after full bloom could increase the cell number in the cortical region of apple cultivars of Starkrimson and Starkspur. But late thinning (6 weeks after full bloom) reduced fruit size.

1.2.10. 2 Effect of Shade on Fruit Weight

The amount of sunlight penetrating fruit trees has an important effect on the maximum yield of high quality fruits including fruit weight, fruit soluble solid concentration (Kapple,1989). It was reported by Webster and Crowe (1971) that shading also changed the shape of McIntosh apple fruits. Kapple (1989) reported that, when branches of "Bartlett" pear were shaded 50 days after full bloom, there was a linear reduction in fruit weight at harvest time for different percentages of shading (Fig.1.4).
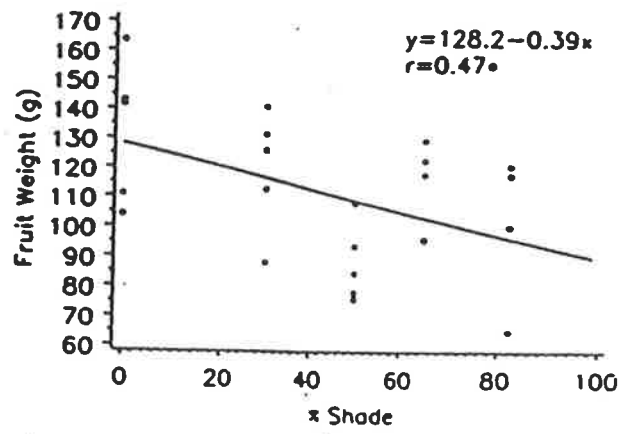
Fig.1.4 Response of mean fruit weight of 'Bartlett'pear to various shade treatments

(Kapple,1989)

Chapter 2

General Materials and Methods

2.1 Determination of Total Nitrogen Using Kjeldahl Method

Twenty grams of pulped pears were dried out using an oven at 100°C for 36 h. One gram of the dried samples was put in nitrogen free paper, folded and introduced into the Kjeldahl test tubes. Two blank tubes and two standard samples were run together with other samples of pears. A recipe of Kjeldahl catalyst tablet was added to each tube containing 1 g anhydrous sodium sulphate (SO_4Na_2) and the equivalent of 0.01 g of selenium. The the tube rake was placed inside the fume hood and 15 ml of concentrated sulphuric acid (SO_4H_2) was added by an automatic dispenser. Samples were heated at 400°C in the Tecator digester under the fume hood. Samples were cooled within 10 minutes of digestion using tap water. After that time, the water was reduced volume for a 40 minutes and the test tubes were removed from the digester. All the carbon was oxidized. An Auto Kejltec 1030 Analyser (Tecator) was employed which auotmatically measures ammonia released after alkali addition. The prepared tubes were placed in pre-adjusted Kjeltec Auto analyser for 3 minutes and the nitrogen content of the sample determined. Crude protein content can be estimated from this value. The percentage of moisture was obtained after drying the samples.

2-2 DNA Extraction, Purification and Analysis

2-2. 1 Examination of Different Methods for DNA Extraction from Pear Leaf

In order to find the best method for DNA extraction from pear leaf, different methods were tested. Some methods were not effective for DNA extraction from leaves of "middle age", because there was no powerful detergent to lyse cell layers easily. The pear leaves had high amounts of phenol oxidase and carbohydrate, and cell layers had a firm structure not easy to break. Using strong detergent and chemical agents to lyse the cell was inevitable. Furthermore application of anti-polyphenol materials such as

polyvinylpyrrolidone which adsorb tannins was beneficial. Sodium bisulphite, 2-mercaptoethanol or dithiothreitol functioned as an anti- polyphenol oxidase and assisted the DNA extraction. Evaluation of different methods are presented in Table 2.1.

Table 2.1 Comparison of DNA Extraction Methods from Pear Leaf.

Methods	Detergent	Anti oxidant	Effective
1-Doyle and Doyle (1988)	CTAB	0.2 % Mercaptoethanol	NO
2-Thomas *et al.* (1993)	Sarkosyl	Mercaptoethanol + PVP	Yes
3-Guillemaut and (1992) Marechal-Drouard	SDS	Cystein	Yes
4-Mulcahy (1993)	CTAB	0.4% Mercaptoethanol	No
5-Collins and Symons (1992)	Sarkosyl	Sodium Bisulphite ($Na_2 SO_3$)	Yes
6-Torres *et al.* (1993)	CTAB	Sodium Bisulphite ($Na_2 SO_3$)	No
7-Zhu *et al.*(1993)	Benzyl Chloride +SDS		Yes

The results obtained using these methods showed that Sarkosyl functions better than other detergents. SDS was more efficient than CTAB, especially when cheesecloth was used as a filter in the Guillemaut and Marechal-Drouard (1992) method after mixing the ground tissue with DNA extraction buffer. Among these methods, method No 2 and 5 worked most efficiently. The most effective method which was used for most of the work described here was a modification of the method No 5. The modification was to add Nacl to adsorb carbohydrate and also PVP to adsorb tannin. Since the residues of SDS could inhibit TAG polymerase activity during DNA amplification (Hill *et al.*,1992), then those methods using SDS were not used.

2.2.2 DNA Purification Using Gene Clean Kit

The method finally adopted for DNA extraction is given in detail in chapter 3. The purification of DNA after extraction was made to remove remaining secondary metabolites or residues of materials used during DNA extraction. 5 µl of silica matrix ('glass milk", geneclean bio 101, inc) was added to a concentration of 500 ng of DNA dissolved in TE buffer. The mixture in an Eppendorff tube was centrifuged for approximately 5 seconds. The mixture was held on ice and vortexed every 1-2 minutes. Thus the DNA adhered to 'Glass milk".200µl of wash buffer was added to the mixture and was left 5 minutes on ice. After another centrifugation, the supernatant was discarded. Washing with 'wash buffer" was repeated two more times. The pellet in bottom of the tube was resuspended in TE buffer and heated to between 45-55°C for 2-3 minutes. The suspension was centrifuged for 30 seconds, and the supernatant which contained DNA transferred to a new tube.

2.2.3 DNA Concentration

DNA dissolved in TE buffer was measured using a TK105 glass fluorimeter. This instrument employs fluorescence of dye bound to the minor groove of double stranded DNA. 365 nm light (long uv) excites this bound dye, enabling us to estimate the concentration of double stranded DNA. DNA fluorescence is related to the AT content of DNA sample, so it was important to use a standard DNA similar to the sample under investigation. A dye solution which were consisted of TNE buffer, 10 ml (Tris base, EDTA, NaCl), 90 ml distilled filtered water with DNA standard (calf thymus DNA, Hoefer Scientific) concentration of 100ng/µl, applied for reading of absorbed fluorescence to DNAs. TNE buffer and distilled water was filtered before using to remove any suspension in the solution. Calibration of the instrument was performed between 0 -100 ng/µl DNA concentration. Two ml of dye solution was pipetted into a cuvette plus 2 µl of standard DNA solution. After calibration, pear DNA samples were measured.

2.2.4 Quality of DNA Samples

The UV spectrum of DNA samples was examined using a Shimzadzu UV-160 A spectrophotometer. The absorbency value at 260 nm and 280 nm of each DNA sample were determined. The best quality DNA had maximum absorbency at 260nm and lower absorbency at 280 nm (Fig. 2.1). The result of ratio A260/A280 nm for good quality DNA is 2. The A260/A280 ratio of the DNA samples extracted from pear leaf were consistently in the range of 1.6-2. This quality of DNA obtained after using Gene Clean Kit. As the absorbency curve for RNA and DNA for 260 and 280 are the same, DNA quality measurement were conducted after removing RNA using RNase 40 $\mu g/.\mu l$. High 280 nm reading indicate contamination materials such as protein, etc .
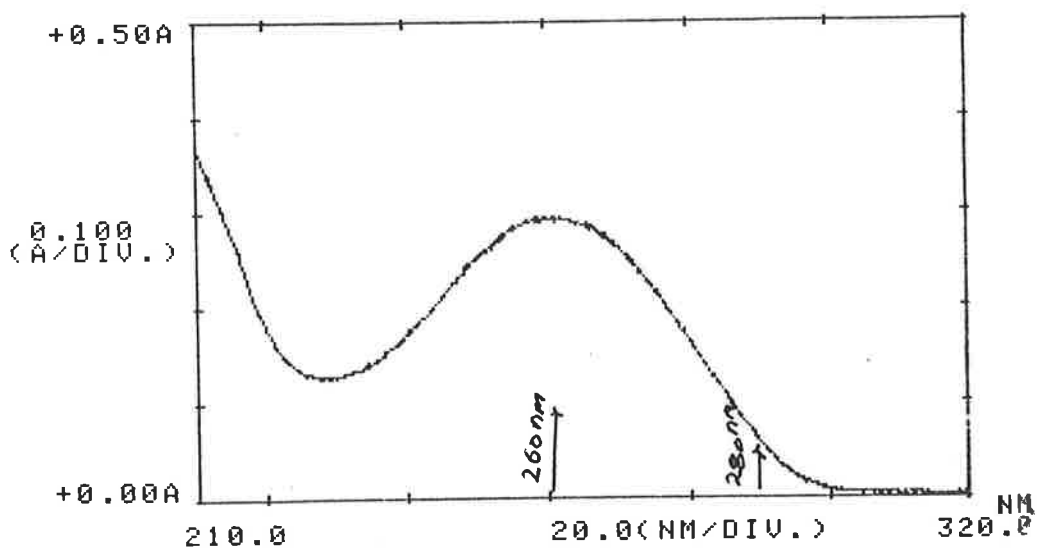
Fig. 2.1 Absorption spectrum of DNA in 260 and 280 nm.

2.3. Extraction of Pollen and Seed for Isozyme Analysis

Pollen grains were obtained from the flowers before opening. Anthers were excised and allowed to dehiscence at normal room temperature. Pollen was collected by removing extraneous anther tissue with a sieve as described by Jackson (1989). Pollen was stored in a small Eppendorff tube at -20°C or used immediately for isozyme analysis after collection. Seeds of pears were taken from fruits and let to dry for 24 h at room temperature. After the drying period, seeds were stored at 4° C . The relative amount of pollen and buffer for isozyme analysis was crucial. In practice 18 mg of pollen was used with 110- 120 µl extraction buffer. The extraction buffer included 0.05M Tris HCl, 0.15 % citric acid , 0.12 % Cysteine-HCl and 0.1 % ascorbic acid, pH 8. Polyvinylpolypyrrolidine (PVPP) was used adding it at half the seed weight. The coat of the seed was removed before grinding. Pear seed weight depends on the cultivar used. Packham seeds without a seedcoat were around 45-50 mg each to which 250 µl buffer was used for extraction of individual Packham seeds and 200µl for with Lemon Bergomot seed. The weight of individual seeds of Lemon Bergomot without a seedcoat was 25-30 mg. Grinding was carried out in a mortar and pestle. No PVPP was necessary for the case of isozyme extraction from the pollen, due to the lack of phenolic compounds in pollen. The mixture was centrifuged at 3000 x g in an Eppendorff 5414S centrifuge (Fig.2.3).

2.4 Gel Electrophoresis

Cellogel (cellulose acetate gel) was used in this work ,it was prepared ready for use by the Chemitron company (Milan, Italy). Cellogel was supplied in airtight plastic bags contained in methanol solution. The Cellogel came as 30 X 30 cm which were cut to 30 X 15 cm for these experiments. The Cellogel was kept in 30 % methanol solution and stored at 4°C. Before installation of cellogel in the electrophoresis box, cellogel was soaked in the specific buffer for the isozyme system to be separated (see Fig 2.2).

In this work two buffers were used, the first 0.05 M tris-malate pH 7.8 which was used for isozymes; GPI, IDH , MDH and GDH. The second buffer system utilized was 0.02 M sodium phosphate buffer, pH 7.0 for PGM and ADH isozyme systems.

Since one side of the cellogel is porous and appears dull and the other side has a plastic coated non porous surface, care was taken to use the porous side of the gel for loading the samples. The gels were soaked in the buffers before installation and loading the samples. After soaking excess moisture was removed from the gel and it was positioned between the cathode and anode compartments containing the buffers. A few long bar magnets were placed at the inner wall of each buffer compartment above the liquid buffer, in order to hold the cellogel in an horizontal plane between cathode and anode compartments. (Fig.2.3E). Supernatant from an extract was used for loading on the gel, using a draftsman's pen. A plastic ruler was held in place over each gel to act as a guide for loading of the samples. The samples were loaded in a line 2 cm distance from the cathode edge of the gel. Each electrophoresis box was connected to a power pack delivering a constant voltage of 200V and electrophoresis continued at 4° C for 1.5-2 hours. After ending the run period, the electrophoresis box was transferred to the lab where the stain material was prepared

2.5 Staining of the Gel for Different Isozymes

Staining was performed for each isozyme system by placing a small amount of staining solution on a disposable plastic sheet which was stretched over cardboard with the aids of clips. The gel was taken out from the electrophoresis box holding it by the portions that were in contact with cathodal and anodal buffer liquid. The gel was dipped into the stain solution smeared on the plastic sheet and rocked gently back and forth (Fig. 2.4F). This occasional rocking motion continued for 30-60 seconds. The gel was blotted to remove excess stain and was positioned between two plastic sheets.

The gel was then incubated at 37°C (see Fig.2.4G). The time of incubation was different for different isozyme systems, ADH took between 5-10 minutes and GPI and IDH between 15-20 minutes. PGM took between 25-30 minutes. Photocopying was conducted from each gel to record band position for interpretation of gels.

2.5.1 Alchohol Dehydrogenase (ADH)

The principle of reaction is as follows, where ADH appears on the gel, alcohol (ethanol) is converted to an aldehyde, while also reducing NAD to NADH in presence of ADH. Then the NADH reacts chemically with MTT (+ PMS) to give a purple formazan which is precipitated in bands where ADH is located on the gel. The stain solution consisted of 0.2 ml ethanol, 2 ml 0.1 M tris HCl pH 8.0, 0.1 ml 40 mM NAD, 0.1ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS. (Richardson *et al.*, 1986; Jackson, 1992).

2.5.2 Glucose Phosphate Isomerase (GPI)

Where GPI appears on the gel, fructose is converted to glucose-6-phosphate and then in presence of glucose 6 phosphatase dehydrogenase is converted to 6-phosphogluconate. During this reaction, NAD is reduced to NADH which reacts chemically with MTT +PMS to give a purple formazane 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 ml, 25 mM NADP, 0.1 ml 1 M MgCl2, 0.1 ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS and 2 international units of glucose-6-phosphate dehydrogenase (Richardson, *et al.*, 1986; Jackson, 1992).

2.5.3 Phosphoglucomutase (PGM)

The principle of reaction is as follows, where PGM is located on the gel, the glucose 1-phosphate is enzymatically converted to glucose-6-phosphate in the presence of glucose 1, 6 diphosphate. Then the glucose-6-phosphate dehydrogenase in the presence of glucose-6-phosphate dehydrogenase enzyme and $MgCl_2$ is converted to 6-phosphogluconate. At the same time NADP is reduced to NADPH, this compound react chemically with MTT in the presence of PMS to give an insoluble purple formazan which thus becomes obstructed in the areas of the gel containing active PGM The stain solution is composed of 10 mg glucose 1 phosphate, 2 ml 0.1 M Tris HCl pH 8, 0.1ml, 25 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).

2.5.4 Isocitrate Dehydrogenase (IDH)

The principle of this isozyme staining reaction is that where isocitrate dehydrogenase is located on the gel it catalyses 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 position of isocitrate dehydrogenase. The stain solution contained 10 mg DL-isocitric acid, 2 ml 0.1 M Tris HCl pH 8, 0.1ml, 25 mM NADP, 0.1 ml 1 M Mgcl2, 0.1 ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS, and 2 international units of glucose -6-phosphate dehydrogenase (Richardson, *et al.*, 1986; Jackson, 1992).

2.5.5 Glutamate Dehydrogenase (GDH)

The principle reaction for this isozyme is as follows, where glutamate dehydrogenase is located on the gel, it converts glutamic acid to ketoglutrate and, during this reaction NADP reduces to NADP. Then the NADH reacts chemically with MTT (PMS) to give purple formazan which is precipitated as bands where GDH is located on the gel. The stain solution contains , 20 mg of glutamic acid, 2 ml 0.1 M tris HCl pH 8.0, 0.1 ml 40 mM NAD, 0.1ml 14.5 mM MTT, 0.1 ml 6.5 mM PMS. (Richardson *et al.*, 1986; Jackson, 1992).

2.6 Interpretation of the Stained Gel

Interpretation of isozyme bands depend on how many polypeptides are contained in the molecular structure of the active enzymes. For PGM the quaternary structure of the enzyme is as a monomer while ADH, GPI and IDH are dimers.

Homozygosity or heterozygosity is another criteria assisting interpretation of bands.

In homozygous genotypes, only one kind of polypeptide is synthesised which gives only one band on electrophoresis but in heterozygous genotypes two bands may be shown. For example, a heterozygous structure for PGM is *ab* and homozygous *aa* or *bb*.

The number of loci is defined by obvious distance between bands on gels and independent phenotype segregation for example numbering is as follows PGM-1 for locus 1 and PGM-4 for locus 4.

There is a difference between the appearance of bands for haploid and diploid cells. A pear cultivar which is a heterozygous *ab* diploid, shows 3 bands equivalent an *aa, bb* and a hybrid intermediate bands *ab* in between the aa and *bb* bands. But for haploid cells of the same plant only two *aa* and *bb* equivalent bands can be seen for a heterozygous haploid genotype (Jackson, 1992).

2.7 Other Materials and Methods

Further methods are described in other chapters:

Anthocyanin measurement in chapter 6

Amino acid determination in nectars in chapter 6

Pollen collection and germination tests in chapter 8

Fig.2.2: A; Taking out the gel from 30% methanol and after drying was soaked in specific buffer, **B**; grinding the seed by mortar and pestle using extraction buffer and PVPP.

A

B

Fig.2.3, **C**; Centrifugation, **D**; Loading the supernatant extract on the gel, **E**; Running the gel using power pack.

C

D

E
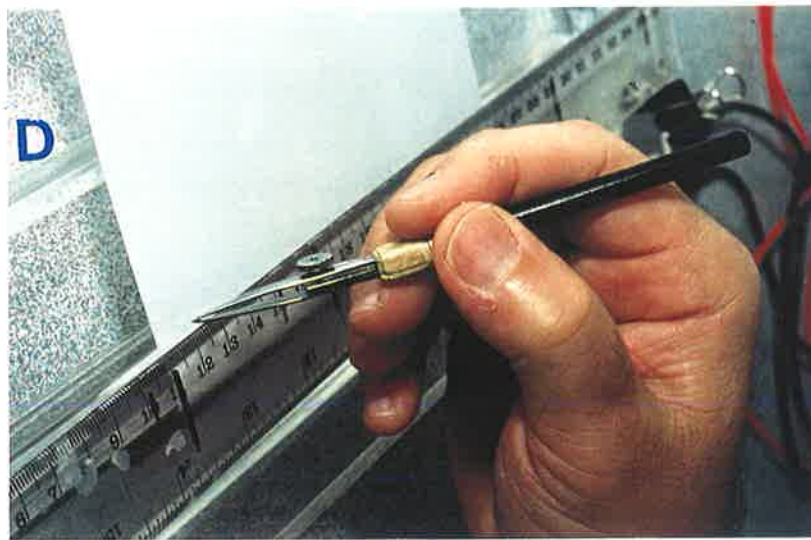
Fig.2.4. F, Staining the gel, **G**; After incubation.

F

G

Chapter 3

Randomly Amplified Polymorphic DNA (RAPD)

Characterization of Pears

3.1 Introduction

Randomly amplified polymorphic DNA (RAPD) which has been proposed by William *et al.*, (1990) as a source of genetic markers is an extension of polymerase chain reaction (PCR) techniques described by Mullis and Faloona (1987). RAPD techniques have been used extensively in horticulture to remove ambiguous identity from plant materials and differentiate between them (Gogorcena and Parfitt, 1994; Polito and Hormoz,1994; Tancred *et al.*, 1994). The influence of environment on botanical characters of plants, makes this molecular biology technique more valuable since it uses basic genetic structure directly (Morell *et al.*, 1995). Genetic investigation of inheritance and linkage identification and genetic maps are other applications of this technique (Ronning and Schnell., 1995; Omura *et al.*, 1993). The preference of RAPD over other molecular biology techniques lies in the large number of primers available which makes for more opportunities to find more polymorphic bands within treatments. Many primers such as synthetic oligonuclotides, microsatellites, synthesized primers based on specific probes, randomly amplified microsatellite (RAMP), sequence tagged sites (STS) or sequence tagged microsatllites (STM) are alternatives used to find polymorphic patterns which would be a reflection of different template DNA sequences. Teramoto *et al.*, (1993) used minisatellite primers obtained from human myoglobin DNA as a probe after application of the *Hinf*I restriction enzyme. Recent research has shown hybridized polymorphism in banding patterns within Japanese pears using RAPD techniques, and in *Malus* M13 bacteriophage repeat probe has been utilized to distinguish between different apple cultivars (Rogstad *et al.*, 1988, Nybom *et al.*, 1990). Harada *et al.*, (1993) made specific arbitrary primers of 19 nucleotides in sequence which were able to be used in

paternity analysis in some apple cultivars. Mulcahy *et al.*, (1993) used Operon primers A05 and A011 to identify 25 accessions of apple. Tancred *et al.*, (1994) differentiated an apple cultivar from 3 other similar cultivars using a decamer oligonucleotide primer with the sequence 5' GGCGGTTGTC 3'. Because of questions concerning the efficiency of oligonucleotide (10 mer) primers in distinguishing different pear cultivars and their cross progenies the present study was undertaken.

The primary aim of the investigation described in this chapter was to attempt to find RAPD markers using 10-mer oligonucleotide primers which could distinguish among Packham Triumph, Josephine and Lemon Bergamot pear cultivars and to trace the pattern of such markers in progeny of the crosses between the pear cultivars. A subsidiary aim was to look for RAPD markers to discriminate different pear species and cultivars used in this study. Finally, we used Iranian wild pears seedlings to detect differences.

3.2 Materials and Methods

3.2.1 Plant Materials

Plant materials consisted of 17 pear species and cultivars (see explanations in Table 3.1). Leaves from pear cultivars were collected from a commercial orchard located in Coromandel Valley a region in Adelaide Hills (South Australia). The Pear cultivars were of the equal ages. Leaves from pear species were obtained from the Waite Agricultural Research Institute Arboretum. The controlled crosses of Josephine, Lemon Bergomot and Nashi pear cultivars (as males) with Packham Triumph cultivar (female) were conducted to obtain F_1 seeds. These seeds were stratified for 2 months at 4°C and then planted in pots. Seedlings of these crosses with Packham Triumph were then used to prepare progeny DNA. The seedling DNAs amplified with primer OPC07 in 4 replications for each cross were all subjected to PCR analysis. In order the Iranian wild pear seeds to be germinated a period of stratification period was undertaken then the pear seedlings were obtained.

Table 3.1 List of pear species and cultivars and their origins used for RAPD DNA fingerprinting.

	Species	Cultivars	Origin
1	*P.amygdaliformis*		Mediterranean Sea, South Europe
2	*P.betuleafolia*		North and Central China
3	*P.calleryana*		Central and Southern China
4	*P.communis*		West, Southeast Europe
		Josephine	
		Lemon Bergomot	
		Packham Triumph	
		Duchess	
5	*P.gharbiana*		Moroco
6	*P.pyraster*		South Europe
7	*P.pyrifolia*	Chojru	Japan, China , Korea
		Kosui	
		Shinsui	
		Twntieth Century	
8	*P.ussuriensis maxim*		Northern China, Korea, Siberia
9	*P.syriaca*		Northeast Africa, Lebanon, Iran

3.2.2 DNA Extraction

Pear leaves contain high amounts of polyphenol compounds which can disturb DNA extraction. To overcome this limitation a different number of DNA extraction protocols were used. Two of these protocols (Thomas *et al.*, 1993; Collins and Symons, 1993) were found more productive and efficient for DNA extraction from pear leaves. We have introduced several modifications and have found that the addition of PVPP (polyvinylpolypyrollidone) and Na_2SO_3 to the extraction buffer can greatly improve the quality of the extracted DNA. Washing DNA with 70 % and pure ethanol also improved the DNA quality for the polymerase chain reaction.

Leaves from individual plants (0.6 g) were frozen in liquid nitrogen and ground to a fine powder and suspended in 5 ml of isolation buffer. The buffer was composed of sarkosyl 30%, 0.1 M Tris HCl, 0.1 M Na_2SO_3, 10mM EDTA, 1% PVPP (MW 40,000) and 500mM NaCl with final of pH 8.0 The suspension was incubated at 65°C for 30 minutes, with occasional shaking.

An equal volume of phenol /chloroform / isoamyl alcohol (25:24:1) was added to the suspension and mixed. The phases were separated by centrifugation at 9000 X g for 12 min. The supernatant was collected and an equal volume of chloroform/isoamyl alcohol (24:1) was added and the mixture was shaken and subjected to centrifugation as above. The aqueous layer was collected and 1/10 volume 3 M Na acetate at pH 4.6 was added, shaken gently and stored on ice for 10 minutes. The mixture was centrifuged and the supernatant transferred to a new tube where the DNA was precipitated with two volumes cold pure ethanol. After washing with 70% ethanol the DNA was dissolved in TE buffer to a concentration 40 ng/μl. RNA was removed by incubation with 7μl of 40mg/ul RNAse.

3.2.3 Amplification Conditions

The PCR reaction mixture in a volume of 30 μl was composed of 10X buffer 500mM KCl, 100mM Tris -HCl (pH 9 at 25° C), 200μM dNTPs, 3.3 mM $MgCl_2$, 0.04 U/μl tag polymerase, 0.25 μM primer, 40ng/μl genomic DNA, 5mg/ml T4 gene 32 protein, sterile distilled water was added to the reaction mixture to make up to 30μl. Each sample was overlaid with two drops of mineral oil to prevent evaporation during heating period in the thermocycler. The samples were exposed to the following temperature profiles using a Perkin Elmer DNA Thermal Cycler: Firstly the samples were incubated at 94°C for two minutes, then immediately amplification was performed for 40 cycles. Each cycle consisted 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° and 1 min 45 sec at 72°C and a final extension step for 5 min at 72°C. Finally, the mixture was kept at 4° C until electrophoresis. The amplification products were mixed with 5 μl formamide dye solution and separated by electrophoresis on 1.8 % agarose gel at 75V for 3 h. The amplified DNA bands were detected by staining in ethidium bromide 0.001 mg/ml (Maniatis, 1982).

The primers used were 10-mer oligonuclotides purchased from Operon Technology (Alameda, CA.). A large number of primers were used to distinguish those that produced abundant polymorphisms. These were included OPA08, OPA10, OPA12, OPA20, OPB06, OPB10, OPB11, OPB12, OPC04, OPC06, OPC07, OPC08 OPC09, OPC10, OPC15, OPC16, OPD02, OPD03, OPD04, OPD06. We looked for the

primer or primers capable of providing a multiple banding pattern and then used it to perform random amplification of DNA extracted from pear cultivars and species. Amplification was repeated with each primer several times to make sure of the reproducibility of the bands.

3.2.4 RAPD Analysis

To understand efficiency of each primer, the number of total bands produced by the primers were calculated for Josephine, Lemon Bergomat and Packham Triumph cultivars. The number of polymorphic bands produced by each primer divided to total bands to obtain percent of polymorphic bands.

3.3 Results.

3.3.1 Notes on Preparation of DNA from Pear Tissue

Optimization of the method in this work showed that DNA for amplification should be very clean, and free of any carbohydrate or phenolic compounds which could lead to inhibition of Tag DNA polymerase activity during extension of the DNA. The optimal concentration of DNA was identified as 40-50 ng/μl. Using Na_2SO_3 and PVPP is essential in order to avoid oxidation of phenolic compounds which then interfer with the reaction. It was found that very young tissue of pear is easy to extract but it gave only limited amounts of DNA. The best leaf tissue to use for DNA extraction is from trees 3 to 8 weeks after flowering because the leaf tissue at this stage is soft and contains less phenolic compounds and the DNA content per dry weight is maximal. After adding cold ethanol to extract, dissolved DNA and being allowed to stand overnight, DNA was suspended in a tube. It was wound out onto a glass rod rather than pelleting by centrifugation, as this gave a better result for PCR work. Washing the DNA a few times with 100 % and 70 % ethanol is important to remove any remaining chloroform and sodium acetate. It was also found that using 'gene clean kit" materials is beneficial for obtaining clean DNA for PCR work. DNA preparations from pear leaf had high amounts of RNA. 7μl of 40mg/ml RNAse was necessary to remove RNA from 50μl DNA solutions.

3.3.2 RAPD Analysis of Pear Species and Cultivars

From the initial group of 20 primers, tried 9 were found to be useful in distinguishing pear cultivars and other *pyrus* species (see Table 3.2). Of the other primers used very little or none showed polymorphism, and were thus considered as inadequate for the intended objectives.

Table 3.2 Sequence of the nine oligonucleotide primers suitable for identification.

Primer	sequence 5'to 3'	M. W
OPA08	GTGACGTAGG	3099
OPA10	GTGATCGCAG	3059
OPA12	TCGGCGATAG	3059
OPB12	CCTTGACGCA	2979
OPC06	GAACGGACTC	3028
OPC07	GTCCCGACGA	3004
OPD01	ACCGCGAAGG	3053
OPD02	GGACCCAACC	2973
OPD10	GGTCTACACC	2979

Each primer yielded a wide array of strong and weak bands. Only reproducible fragments with distinct bands were scored in our comparison. The number of total fragments and number of polymorphic bands with percent of polymorphism are shown in Table 3.3. As can be observed the percent of polymorphism ranged from 12.5% (OPD10) to 50 %(OPD02) (Table 3.3). The size of amplified fragments ranged from 150 bp (OPC06) to 2200 bp (OPC06) (Fig. 3.5) Primer OPD02 produced a banding pattern which enabled us to discriminate between all three cultivars Lemon Bergamot, Packham Triumph and Josephine (Fig. 3.1). The latter primer produced band markers located between 1766-1033bp (Fig. 3.1). Using primer OPD10 gave a greater number of bands over that obtained with OPD02 on the agarose gel in the area of 2176-1033 bp(Fig. 3.2). Two marker bands at 1230 bp were obtained for Lemon Bergamot and Packham Triumph which were not produced for Josephine (Fig. 3.3) using primer

OPC07. Application of the latter primer demonstrated clear differences among Chojru (Japanes pear), *P.betulaefolia* and Winter Nelis (Fig. 3.4 ,lane 4, 5, 6, and 7). Primer OPC06 revealed a strong and unique marker band for Josephine which was located at slightly more than 653 bp (Fig. 3.5). The results obtained using primer OPC06 with DNA extracted from four wild pear species and several *P.coummunis* cultivars and Japanese pear cultivars are shown in Fig 3.6 . Using primer OPC07 showed the presence of a sequence in the area of 453-298 bp for Shinsui, Duchess and *P.ussuriensis maxim* which was absent for Chojru and Twentieth Century.

DNA amplification for *P. amygdaliformis* with primer OPC07 showed a marker band at 1100 bp which was not produced in four other pears as illustrated in Fig. 3.7. *P. gharbiana* (Fig. 3.7 lane 5) as an African pear produced a pattern similar to *Pyrus pyrifolia*, this result suggesting that probably these African pears did not originate in Africa, but rather migrated there from other parts of the world.

The close relationship between European pears, as these originated mainly from *P.communis* made identification among those difficult by using the random primers. Application of the primers in series A from Operon technology OPA08 and OPA12 and series B only B12 showed a range of polymorphism between Josephine, Lemon Bergomot and Packham Triumph. In series C of Operon technology primers OPC06 and OPC07 were effective. Primers OPD01, OPD02 and OPD010 showed a range of polymorphism. Primer OPD02 was more effective than others in series D (Table 3.3).

Table 3.3: Primers used and number of different DNA amplified fragments and percent of polymorphism observed between 3 cultivar of pears

Primer	Total	No. Fragments observed	
		Polymorphic	Percent polymorphic
OPA08	9	2	22
OPA10	7	1	14
OPA12	6	2	33
OPB12	7	2	28.5
OPC06	6	1	16
OPC07	6	1	16
OPD01	5	1	20
OPD02	6	3	50
OPD10	8	1	12.5

3.3.3 Progeny Testing

As illustrated in Fig. 3.3, two good markers bands were found for Lemon Bergomot and Josephine using primer OPC07. The result are shown in Fig. 3.8, DNA amplification for two cross progenies of Josephine (male) X Packham Triumph showed traces of the two marker bands at 1230 and 1100bp (Fig. 3.8 lanes 2 and 5). DNA profiles (Fig. 3.8, lanes 3, 4) obtained from the latter cross progenies showed marker band just at 1100bp which apparently was the band specific to Packham Triumph female parent. In cross progenies of Lemon Bergomot (male) X Packham Triumph, the only band marker was revealed at 1230 bp which apparently originated from Lemon Bergamot as pollen donor (Fig. 3.8 , lanes 6, 7, 8, 9). Similar DNA profiles were observed between two cross progenies of Twentieth Century (Nashi) X Packham Triumph (Fig. 3.8, lanes 10, 11, 12, 13) and Josephine X Packham Truimph. This result made identification between these two cross progenies difficult. Using primer OPC08 as a testing primer for these progenies did not show a distinct difference between progenies (Fig. 3.9). Already testing of parents using primer OPC08 did not produce polymorphic bands.

3.3.4 Iranian Wild Pear Species

Testing of DNA extracted from seven Iranian wild pear species showed quite different polymorphic patterns from other pear species treated here using primers OPA011 and OPA12 (Fig. 3.11 and Fig. 3.12). Using primer OPC08 did not produce valuable polymorphic bands (Fig. 3.10). Pear species in lane 3 and 4 for primers OPA12 (Fig. 3.12) and OPA 11 (Fig. 3.11) did show distinct differences. Furthermore lane 2 indicated a distinct difference with lane 1 and 3 using primer OPA12.

3.4 Discussion

Good resolution and production of bands with the RAPD technique depends on many factors such as the good quality of DNA, sequence of DNA, annealing temperature which may be different between the primers and other factors. The polymorphic bands were stable and repeatable because the band markers were consistent among several DNA extractions. In our experiments the plant samples were of the similar growing conditions for pear species and cultivars respectively. Also, the DNA profiles were obtained from one specific thermocycler. But the constancy of the results for pear species grown at different locations have not been proved. The results obtained from application of random 10 mer primers showed that the sequences of these primers when used individually are not adequate to analyse completely genetic differences between pear cultivars especially those which originated from one species. But on the other hand application of these primers can be advantageous to recognize genetic differences among pear species or cultivars with wider genetic distance, and several oligonuclotides 10 mer primers produced bands characteristics for the various local cultivars. Using specific primers which could be made from a polymorphic probe could be more helpful in obtaining better polymorphism. While the RAPD technique was found to be a good way of distinguishing between pear cultivars and species, unlike the isozyme techniques described before it was not found to be practical in paternity testing. Thus the OPCO7 10 mer oligonucleotide of sequence GTCCCGACGA was especially useful in distinguishing certain commercial cultivars. The progeny of crosses between these cultivars did not inherit "amplified polymorphic DNA bands" in any predictable way which could be useful in paternity testing .

Some researchers have been successful in using RAPD fingerprinting in deciding paternity in certain crops-e.g. Hashizume *et al* (1993) for watermelon and tomato. Luro *et al* (1995) used a minisatellite probe as primer in citrus and was able to recognize nucellar from zygotic seedling. Recognition of parental band markers in F1 progenies has been discussed by Newton and Graham (1995). They concluded that PCR methods like RAPD may not be the method of choice in deciding paternity in cases of close kinship relationship with common origin. Further work is recommended for the identification of pear species and *P. communis* cultivars using STM, STS, RAMP and synthesized specific primer marker probes.

Fig. 3.1 RAPD profiles obtained from 3 cultivars using primer OPDO2 (1-DNA molecular weight marker VI, 2-Josephine, 3-Lemon Bergomot, 4-Packham T.).

Fig. 3.2. RAPD profiles obtained from 3 cultivars using primer OPD10 (1-DNA molecular weight marker VI, 2-Josephine, 3-Lemon Bergomot, 4-Packham Triumph).

Fig. 3.3 RAPD profiles obtained from 3 cultivars using primer OPC07 (1-DNA molecular weight marker VI, 2-Josephine, 3-Lemon Bergomot, 4-Packham Triumph).

Fig 3.1

bp

1766 →
1033 →

1 2 3 4

Fig. 3.2

2176 →
1766 →
1033 →
653 →

1 2 3 4

Fig 3.3

2176 →
1033 →

1 2 3 4
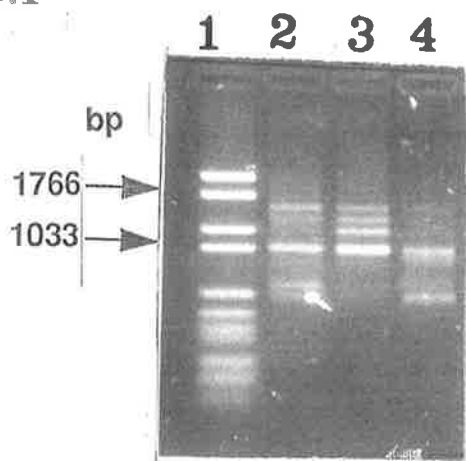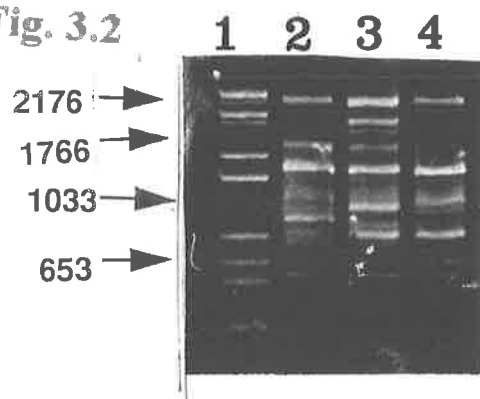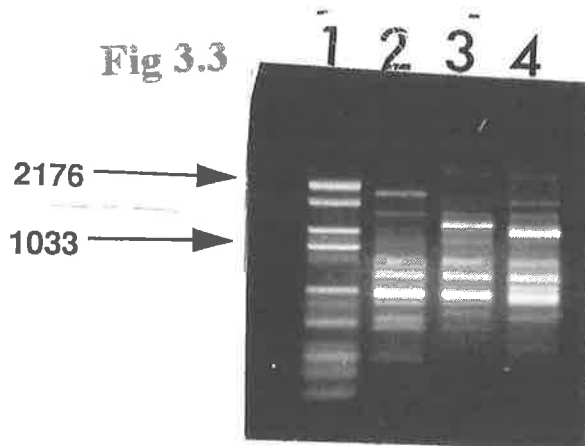
Fig. 3.4. RAPD profiles generated by primer OPC07 obtained from pear species and cultivars; (1-DNA molecular weight marker VI, 2-Josephine, 3-Lemon Bergamot, 4-Packham Triumph, 5-Chojru, 6-*P.betulaefolia*, 7-Winter Nelis, 8-*P.ussriensis maxim* 9-Shinsui, 10-Duchess, 11-*P.calleryana*,12-Twentieth Century, 13-DNA molecular weight marker VI).

Fig. 3.5. RAPD profiles obtained from 3 cultivars using primer primer OPC06 (1-DNA molecular weight marker VI, 2-Josephine ,3-Lemon Bergomot , 4-Packham Triumph).

Fig. 3.6. RAPF profiles generated by primer OPC06 obtained from pear species and cultivars; (1-DNA molecular weight marker VI, 2-*P.betulaefolia*, 3-*P.calleryana*, 4-Duchess, *P.ussriensis maxim*(misnamed), 6-*P.syriaca*, 7-blank, 8-Chojru, 9- Shinsui, 10-Twentieth Century, 11 blank, 12 DNA marker VI).

Fig. 3.7 RAPD profiles obtained from 5 cultivars and species using primer OPC07 (1-DNA molecular weight marker VI, 2-*P.amygdaliformis* 3-Blank, 4-Kosui, 5-*P. gharbiana*, 6-*P.pyraster*, 7-*P.pyrifolia*).

Fig. 3.4

1 2 3 4 5 6 7 8 9 10 11 12 13

1230 →
653 →

Fig. 3.5

1 2 3 4

653 →

Fig. 3.6

1 2 3 4 5 6 7 8 9 10 11 12

bp

2176 —
1033 —
298 —
150 —

Fig. 3.7

1 2 3 4 5 6 7

2176 —
1766 —
1230 —
1033 —
653 —
298 —

bp
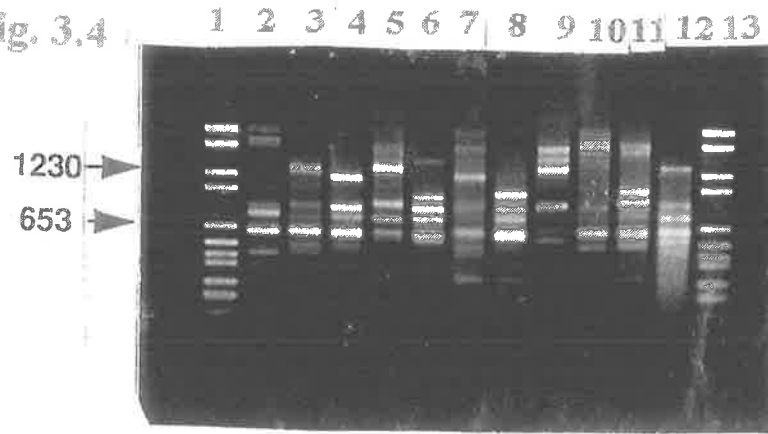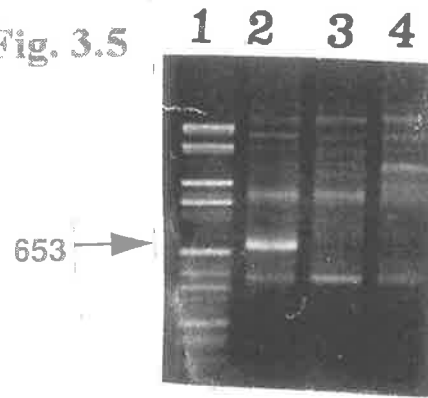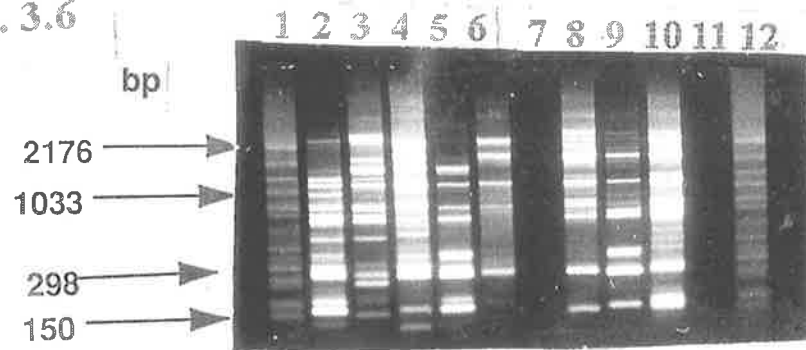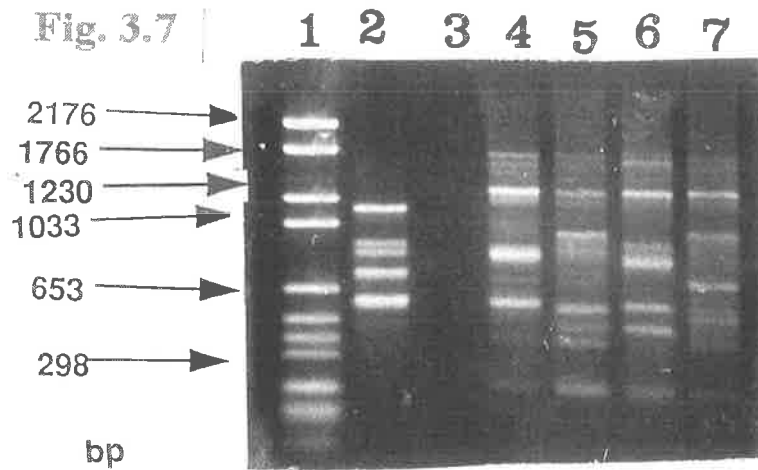
Fig. 3.8 Amplification of cross progenies of pear DNAs using primer OPC07 (Lane1, DNA molecular weight marker VI, lane 2, 3, 4, 5 cross progenies Josephine (male) x Packham Triumph, lane 6, 7, 8, 9 cross progenies Lemon Bergomot (male) x Packham Triumph, lane, 10, 11, 12, 13, Nashi (male) x Packham Triumph.

Fig. 3.9 Amplification of cross progenies of pear DNAs using primer OPC08 (Lane1, DNA molecular weight marker VI, lane 2, 3, 4, 5 cross progenies Josephine (male) x Packham Triumph, lane 6, 7, 8, 9 cross progenies Lemon Bergomot (male) x Packham Triumph, lane, 10, 11, 12, 13, Nashi (male) x Packham Triumph.

Fig. 3.10 RAPD profiles obtained from 8 wild pears using primer OPC08
1- DNA molecular weight marker VI, 2-Bane2, 3-Khoy1,4-Azar,
5-Mame Sarme, 6-Maryvan ,7-Kavyle, 8-Kavyle2, -Khoy2.

Fig. 3.8
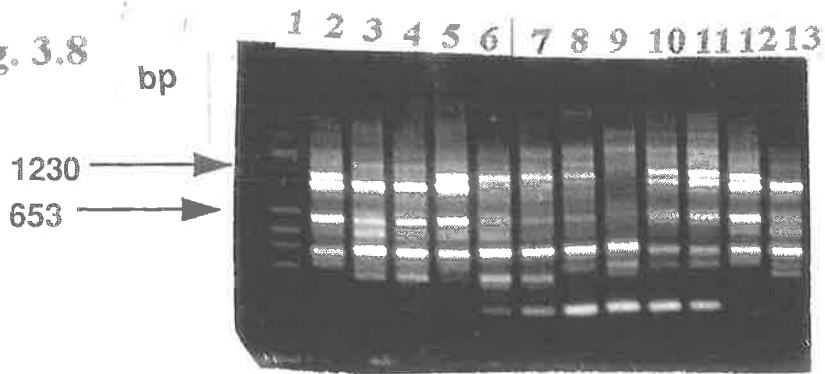
Fig. 3.9

Fig 3.10
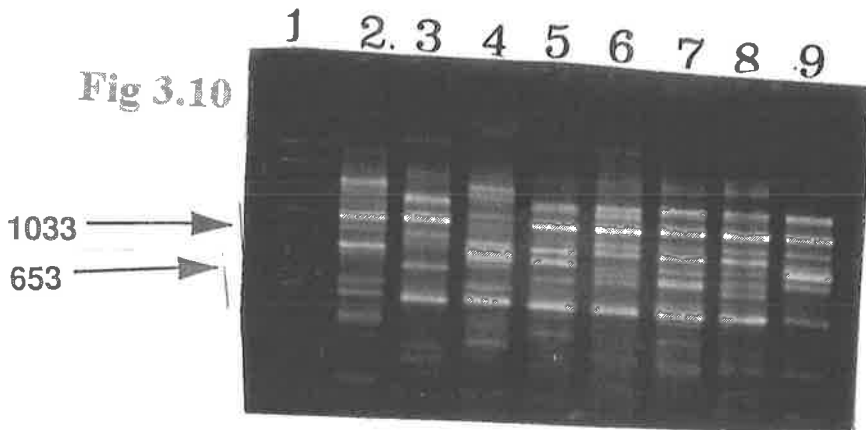
Fig. 3.11 RAPD profiles obtained from 8 wild pears using primer OPA11;
1-DNA molecular weight marker VI, 2-Bane2, 3-Khoy1, 4-Azar,
5-Mam Sarme, 6-Maryvan 7-Kavyle, 8-Kavyle2, 9-Khoy2 .

Fig. 3.12 RAPD profiles obtained from 8 wild pears using primer
OPA12; 1-DNA molecular weight marker VI 2-Bane2, 3-Khoy1,
4-Azar, 5-Mam Sarme, 6-Maryvan 7-Kavyle, 8-Kavyle2, 9-Khoy2,
10-DNA molecular weight marker VI.

Fig. 3.11
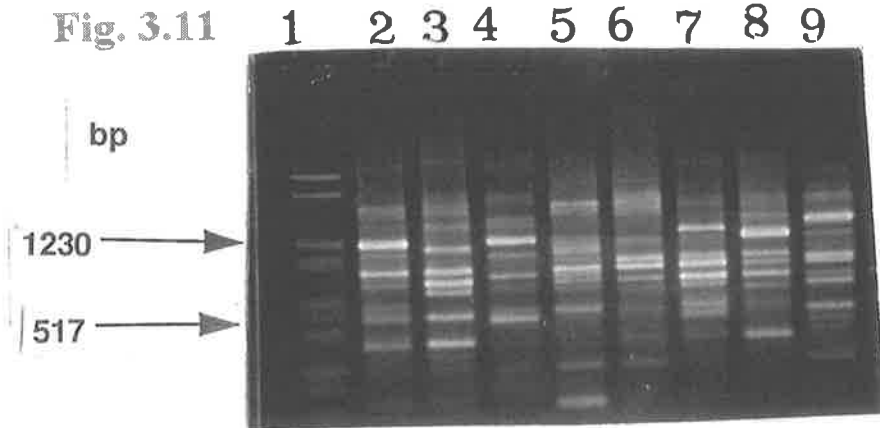
bp

1230

517

1 2 3 4 5 6 7 8 9

Fig 3.12

bp

1766

1230

653

1 2 3 4 5 6 7 8 9
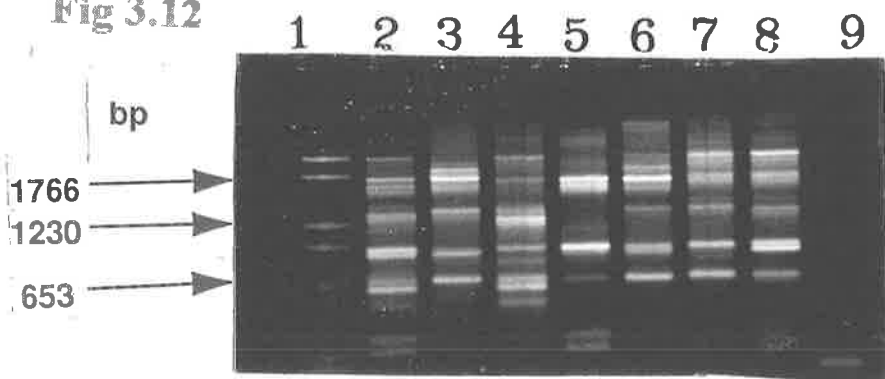
Chapter 4

Characterization of Pear Species and Cultivars Using Isozyme Polymorphism

4.1 Introduction

Self-incompatibility and heterozygosity in the genus *Pyrus* has led to high genetic diversity in this genus (Bell and Hough, 1986; Chevreau and Skirvin, 1992). This same high genetic diversity can be useful for application of isozyme techniques to characterize pear species and cultivars. In two earlier decades, identification and evaluation of pear species was based on botanical and chemotaxonomical characters (Challice and Westwood, 1973; Challice,1981). Since then the use of isozymes for pear recognition has come into use (Santmour and Demut, 1980). Menendez and Daley (1986) used a gradient polyacrylamide gel electrophoresis system to identify some cultivars and species of pears with acid phosphatase (ACP), esterase (EST) and proxidase (Prox) isozyme systems. As there is a large amount of phenolic compounds in pear leaf, working with leaf as sample materials was a problem for isozyme extraction and activity. Pollen has several benefits in comparison with leaf, such as higher enzymatic activity, easier extraction and a simpler isozyme banding pattern since pollen cells are hapioid and pollen extracts are relatively free from phenolic compounds Crezo *et al* (1989) have investigated pear identification using pollen extracts Jang *et al*., (1991) identified species and cultivars of pears by using peroxidase isozyme system. The objective of the present work was the identification of pear cultivars and species using isozyme techniques on pollen extracts. Recognition of isozyme genetic variation between cultivars can be useful for hybrid identification, pollination studies and paternity analysis (Jackson and Clarke, 1991 a,b).

4.2. Materials and Methods

4.2.1 Leaf Protein Enzyme Extraction

Four different extraction buffers were tested to select the best one for of isozyme extraction and electrophoresis. These four were based on; A); Samimy and Cummins(1992), B); Jackson (1992) C); Arulsekar and Parfitt (1986), D);Valizade (1977) It was found that B and C were satisfactory, in total cases, leaf material (300 mg) was ground with 150 mg PVPP (polyvinylpolyporyldone) and 2.0 ml extraction buffers. For young, normal and aged leaves from pear cultivars Packham Triumph, Lemon Bergomot and Josephine, both methods yielded 0.5-0.6 µg/g leaf tissue of sample protein as determined by the method of Bradford (1976).

4.2.2. Collection of Pollen

The experiments were conducted in 1994 and 1995. Flowers from several pear species and cultivars were collected from two different sites. Flowers were gathered from a commercial orchard located in Coromandel Valley a region in the Adelaide Hills (Australia). The pear cultivars included, Chojru, Housi Kosui, Shinsui and Twentieth Century all examples of *Pyrus pyrifolia* and Josephine, Anjou, Lemon Bergamot, Packham Triumph, Howell ,Winter Nelis. examples of *Pyrus communis* and Hwa Hong,Yali and Tsu Li examples of *P. bretschneideri.*

Flowers from other pear species were collected from the germplasm collection located at the Waite Agricultural Research Institute Arboretum. These included; A) West Asian pears; *P.amygdaliformis*, *P. syriaca* and *P.lindley,* B) Asian pears; *P. calleryana*, *Pyrus betulaefolia*, *Pyrus ussuriensis maxim*, *Pyrus kawakami*, *Pyrus boissierana* C) North African pears included, *P. gharbiana* and *P. mamorensis*, and D) European pears; *P. pyraster.* Pollen was collected before anthesis and stored at -20°C.

4.2.3.Pollen Enzyme Extraction

Pollen samples were ground in a mortar and pestle with an equal amount of sand and a ratio of one pollen sample (mg) to 6 times buffer (μl) . Extraction buffer contained 0.1 M Tris-HCl, 0.15% citric acid, 0.12% cystein HCl and 0.1% ascorbic acid, pH 8. The mixture was centrifuged at 3000 x g for 10 minutes. Supernatant was used for isozyme separation (Jackson,1992). Eleven isozyme systems including MDH, ACP, ADH, SKDH, PGM, GPI, IDH, GPI, GDH, G6PD and 6GPD were examined. Those isozyme systems which showed polymorphism and better resolution were selected. Those included; GPI (glucosephosphate isomerase), PGM (phosphoglucomutase), IDH (isocitrate dehydrogenase), ADH (alcohol dehydrogenase) and GDH (glutamate dehydrogenase).

4.2.4 Gel Electrophoresis

Two running buffer systems were used, including A); 0.02 M sodium phosphate pH 7 for ADH and PGM and B); 0.05M Tris-malate pH 7.8 for GPI, GDH and IDH (see Richardson *et al.* 1986). Cellogel acetate sheets of 200 μm thick and dimension 15 X 30 cm were used in this study. Cellogel was supplied in airtight plastic bags containing aqueous methanol and was stored at 4°C. Methanol was washed out before use. Electrophoresis was carried out at a constant voltage of 200 V(D.C) for 2 hours.

4.2.5. Staining

Staining was achieved based on Richardson *et al.*, (1986). The pH of the stain buffer for ADH Isozyme systems was optimized and pH 7.75 was found best for staining. This isozyme system is very sensitive to the pH of stain buffer.

Data was based on presence of alleles in each locus collected in four isozyme systems. These systems included GPI, ADH, PGM, IDH. GDH banding patterns was not used in similarity matrix calculation due to the lack of data for some pears. Presence of a band is scored as 1 and its absence as 0. GPI showed nine different bands at increasing distance from loading zone. PGM, IDH and ADH showed different number of bands 4, 4, and 3 respectively. The similarity of matrix was calculated by a Macro-comparison program using an Excel Macintosh software (see Table 4.3).

Zymogram showing GPI isozyme banding patterns in extracts
from leaves for 9 cultivars of pears:
1) Chojuro, 2) Howell, 3) Italian, 4) Josephine 5) Lemon,
6) Nashi, 7) Packham, 8) Winter Nelis 9) Yali, 10) Almond
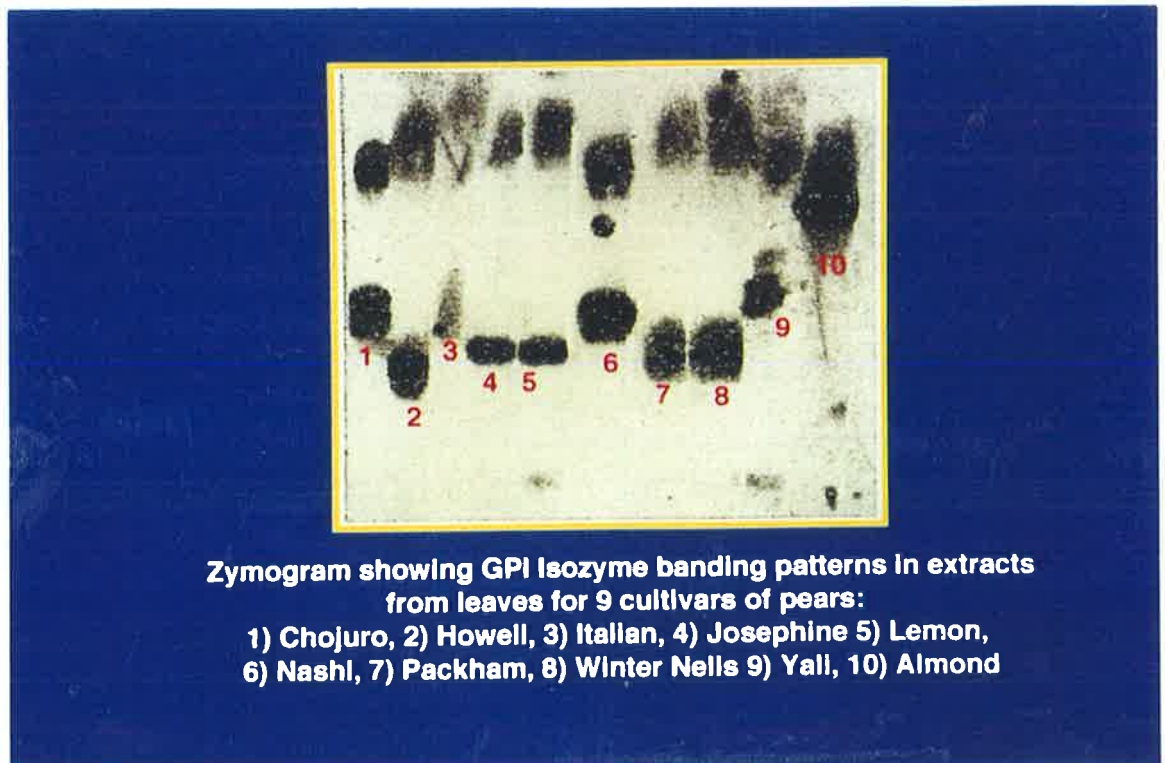
Fig.4.1 Zymogram showing GPI izosyme banding pattern in extracts
from leaves of nine cultivars of commercial pear.
1-Chojru, 2-Howell, 3-Italian, 4-Josephine, 5-Lemon Bergomot
6-Nashi, 7-Packham Triumph, 8-Winter Nelis, 9-Yali,
10- Control (almond nut- from Nonpareil cultivar).
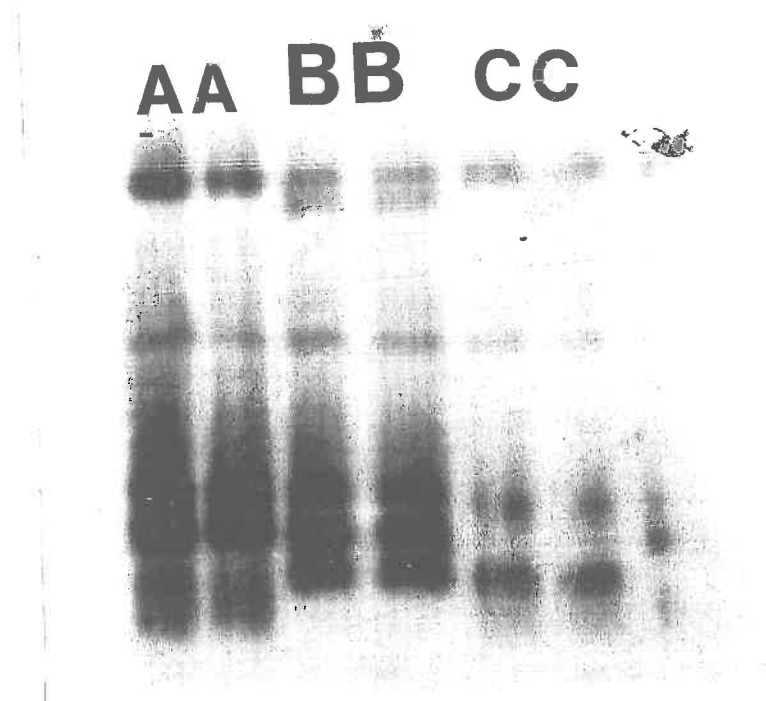
Fig.4.2. Zymogram showing PGM isozyme banding patterns in extract from pollen for 3 cultivars of pear; A; Josephine, B; Lemon Bergomot, C; Packham Triumph.

4.3. Results

4.3.1 Leaf Isozyme

The results of several experiments involving leaf isozyme for different pears showed that while polymorphism was evident there was a difficulty in this tissue, many bands overlapping and not separating adequately (Fig.4.1).

Further works with pollen extracts showed a good appearance and clear separation of bands on the cellogel (Fig.4.2). It was decided therefore to use pollen extracts to monitor isozyme polymorphism in the different pear species and cultivars.

4.3.2. Phosphoglucomutase (PGM)

PGM showed four distinct zones of activity representing four separate loci. In general PGM-3 and PGM-4 showed better resolution than the two other loci. In the earlier work we obtained a good resolution of all four separate loci using pollen isozyme electrophoresis. Categorizing of pears based on structures of the two PGM-3 and PGM-4 divides pears into 9 groups (Table 4 1 and Fig 4 3). Each of the loci PGM-3 and PGM-4 were contained to 3 alleles: aa, ab, bb (Fig 4.2) and (Fig. 4.3) and see the Figs 4.13h, 4.14I, j, 4.15k, l

4.3.3. Glucose Phosphate Isomerase (GPI)

GPI as a dimer, showed higher range of variations within pear cultivars than other isozyme systems. A large number of pear species showed patterns A and B while a few gave patterns C to I (Table 4.1 and 4.2, Fig.4.4). See also Fig 4.10a, b, 4.11c, d, 4.12e, f and 4.13g. Pear cultivars Josephine, Lemon Bergomot, Winter Nelis showed

patterns similar to *P.gharbiana, P.pyraster, P.calleryana, P.betulaefolia, P.amygdaliformis, P.ussuriensis* and *P.kawakami*.
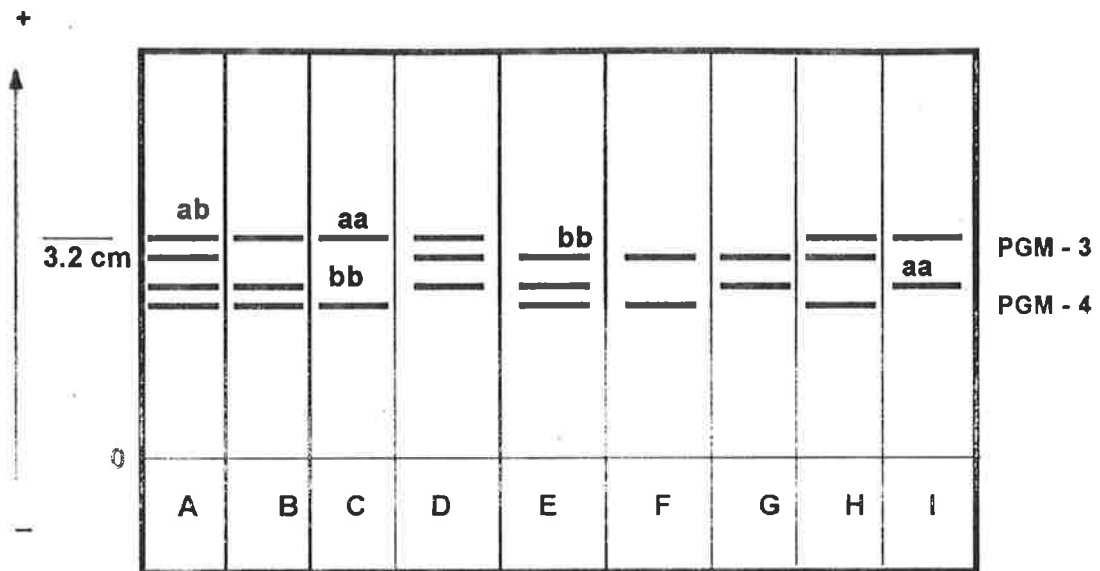
Fig. 4.3 Schematic diagram showing PGM-3 and PGM- 4 banding patterns for different pears. See Table.4.1 and 4.2 for species and cultivars in groups A through to I.
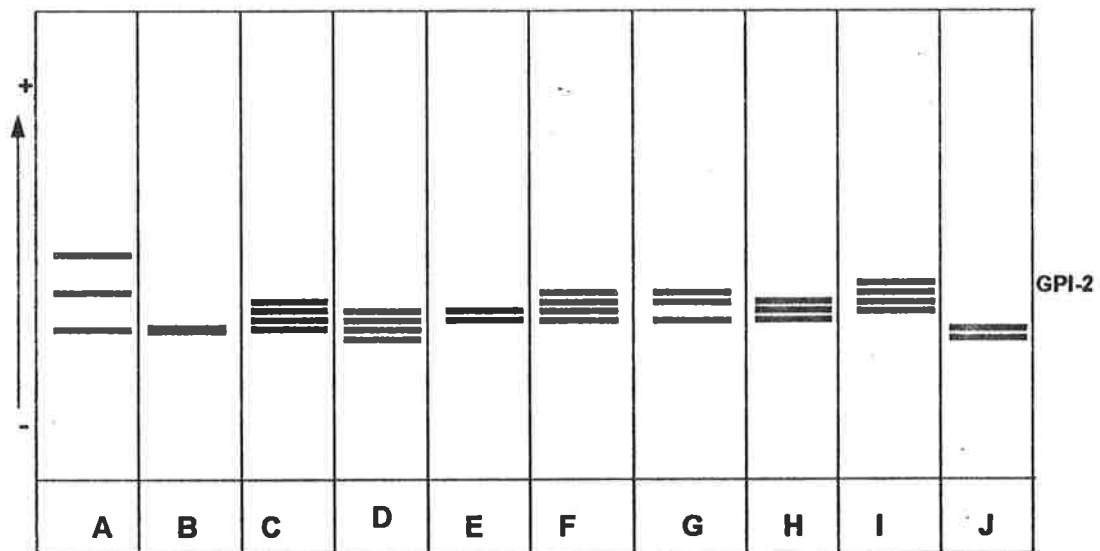
Fig. 4.4 Showing schematic illustration of GPI-2 for different pear species and cultivars (for details refer to Table 4.1).

Table. 4.1 Isozyme phenotype patterns in pear species and cultivars.

Pyrus species	Cultivar	PGM	GPI	ADH	IDH	GDH
P.amygdaliformis		F	B	A	B	A
P. betulaefolia		C	B	A	D	A
P.boissierana		D	A	C	C	A
P.bretschneideri	Hwa Hong	A	C	A	D	A
P.bretschneideri	Tsu Li	A	H	A	C	A
P.bretschneideri	Yali	A	I	A	C	A
P.calleryana		B	B	A	A	A
P.communis	Anjou	D	J	A	D	A
P.communis	Howell	D	H	C	B	—
P.communis	Josephine	H	B	A	C	A
P.communis	Lemon Bergomot	D	B	C	C	B
P.communis	Packham Triumph	E	D	C	B	A
P.communis	Winter Nelis	E	B	A	C	A
P.gharbiana		C	B	C	C	B
P. kawakami		C	B	A	A	A
P. lindley		D	A	C	B	A
P. mamorensis		A	A	A	D	B
P. pyraster		G	B	C	C	A
P. pyrifolia	Chojru	D	F	B	C	B
P. pyrifolia	Housi	A	G	B	C	B
P. pyrifolia	Kosui	I	F	A	F	B
P. pyrifolia	Shinsui	D	H	A	E	B
P. pyrifolia	Twentieth Century	A	E	A	C	B
P.syriaca		E	J	A	D	B
P.ussuriensis maxim		B	B	A	D	A

4.3.4. Alcohol Dehydrogenase (ADH)

Three groups of bands for ADH-2 including A, B, C (Fig. 4.5) were found in the pears. Three zones of activity were observed for *Pyrus calleryana*. These zones could be the expression of three loci for this enzyme. Wild species of pears were located in group A and C. Some pears such as Anjou and *P. syriaca* showed lower dosage activity of allele 1 in group A (Fig .4.5). A few Japanese and Chinese pears including Shinsui, Twentieth Century Yali, Tu Li and Hwa Hong revealed an A phenotype. Pear species *P.gharbiana, P.pyraster, P.boissierana* and *P.lindley* showed a C phenotype.

4.3.5. Isocitrate Dehydrogenase (IDH)

The IDH isozyme system produced 4 different zones of activity indicating four possible loci. Zones 2 and 3 showed higher activity. This could reflect higher gene dosage of loci 2 and 3. Locus 2 was used for comparing the various pear species and cultivars (Fig 4.6). This isozyme system produced six different banding patterns A, B, C, D, E and F [having alleles: bd, ac, ab, cc, cd and bc respectively (see Fig. 4.6)]. The A and B banding patterns are composed of 3 separate bands when looking at extracts from pollen. This condition may emphasis two separate but close loci for these A and B banding patterns (Fig. 4.6). Most pears are located in group C which is ab. *P.kawakami* and *P. calleryana* showed great similarity with pears of group B including *P.lindley* and *P amygdaliformis* Two pears of the African group *P.mamorensis* and *P.gharbiana* did not show close similarity to each other in this isozyme system (see Table 4.1). *P.gharbiana* as African pear showed a similar pattern to *P.pyraster and* Winter Nelis as European pears (see Table 4.1)
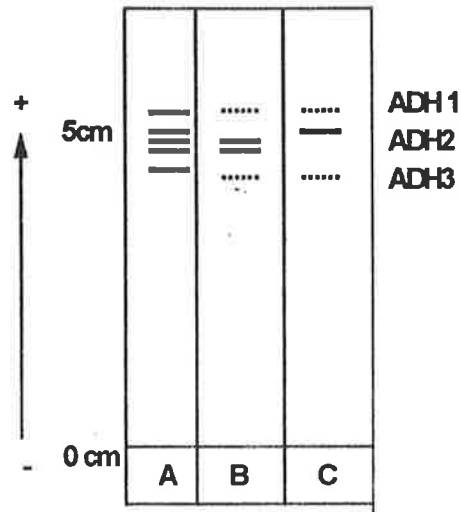
Fig. 4.5 Schematic diagram showing ADH banding patterns for different pears (for details refer to Table 4.1).
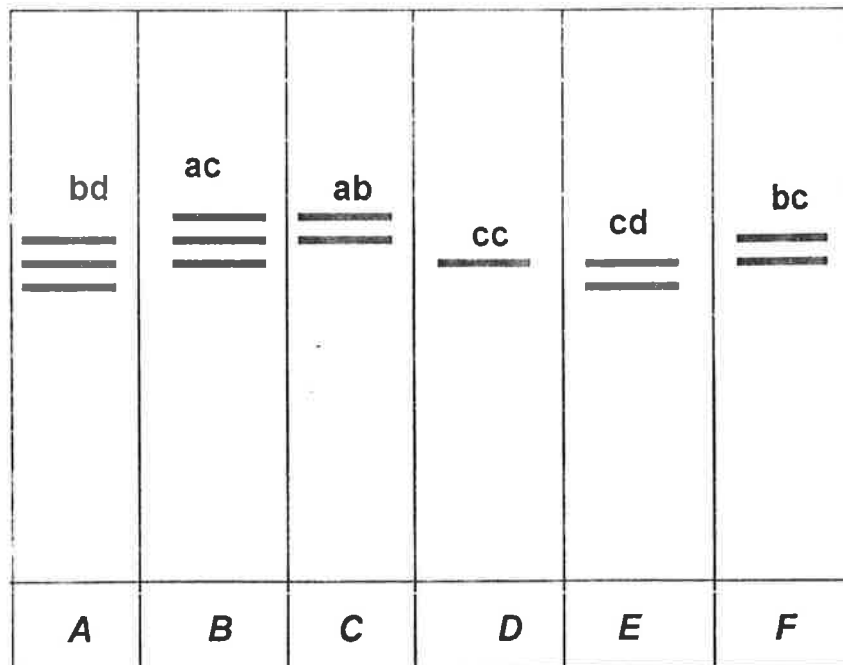
Fig. 4.6 Showing schematic illustration of IDH for different pear species and cultivars.

4.3.6. Glutamate Dehydrogenase (GDH)

Glutamate dehydrogenase is a large complex of subunits. This isozyme usually has one locus and a number of alleles. Results of work with GDH enabled us to divide pears into two groups of A and B (Fig. 4.7). Group A consisted of at least 4 bands.. *P. calleryana*, *P. amygdaliformis*, *P.kawakami*, *P.ussuriensis maxim*, Packham Truimph, Josephine, *P.pyraster*. *P. betulaefolia*, Anjou and Hwa Hong were categorized in group A. Group B consisted of two bands. Group B pattern shown by *P.gharbiana*, Shinsui, Kosui, Twentieth Century, *P.mamorensis*, Housi and Lemon Bergamot.
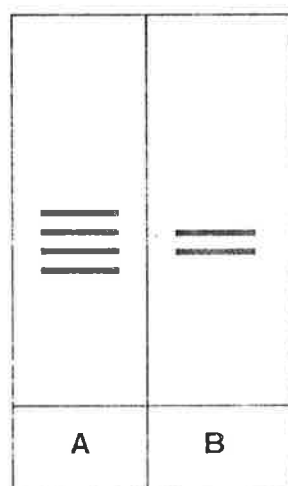
Fig. 4.7 Schematic illustration of Glutamate dehydrogenase (GDH)
(banding patterns for pears which grouped into two forms of **A** and **B**
(see explanation at Table 4.1).

4.3.7. Malate Dehydrogenase

MDH in pears showed four separate loci. MDH did not show enough polymorphism to be of value in showing relationships between pear species and cultivars.

Table 4.2 Frequency of isozyme phenotypes observed among 25 pear species and cultivars.

Phenotype	ADH		GPI		IDH		PGM	
	species	cultivar	species	cultivar	species	cultivar	species	cultivar
A	6	8	3	0	2	0	1	4
B	0	2	6	3	2	2	2	0
C	4	3	0	1	3	7	3	0
D		-	0	1	3	3	1	5
E			0	1	0	1	1	0
F			0	2	0	1	1	2
G			0	1			1	0
H			0	3			0	0
I			0	1			1	1
J			1	0				0

4.3.8. Phylogeny Study of Pears

By application of a Macro-comparison program and Excel Macintosh software (Rolf, 1993). we obtained a similarity matrix between pear species and cultivars based on results obtained from GPI, ADH. PGM and IDH isozymes (Table 4.3). In a comparison we found that there was a relatedness between *P.kawakami* with *P.calleryana and P.betulaefolia* at a 90 % similarity (see Fig 4.10, 4.11). *P.amygdaliformis* showed smilarity with Winter Nelis, Josephine and *P.syriaca* at similarity between 80-90 %. On the other hand *P.syriaca* as a West Asian pear showed a good relatedness with Winter Nelis, Hwa Hong, Tu li and *P.ussriensis maxim* at 85% similarity. Howell, *P.pyraster* and Lemon Bergamot showed a multilateral similarity at 80-85%. Between Lemon Bergamot and Howell a similarity at 90%. was observed. The recent result emphasizes that Lemon Bergamot, Howell and *P.pyraster* have a close kinship to óne another. Chojru, Housi and Twentieth Century showed a similarity of 80% with Yali. Packham Truimph an ilarity at 90%. Both African pear species did not show a high similarity to each other. A 95% similarity (Table 4.3) between *P.lindley* and *P.boissierana* in isozymes banding patterns was observed and closer inspection showed a remarkable similarity in

botanical characteristics as well. It is possible to conclude that d Howell showed a sim *P.lindley* and *P.boissierana* are actually one species under two names. Such a similarity was found also for *P.ussuriensis maxim* with *P.calleryana* (see Table 4.3), a point taken up in the next section.

4.3.9. *P. ussuriensis maxim* a Misnamed Species in Australia

Recently it has been reported (Kellow and Will, 1996) that *Pyrus ussuriensis maxim* has been misnamed in various parts of Australia. These authors matched the botanical characters of the *P.ussuriensis maxim* grown in Australia with the standard description from Rehder (1940) and Krussman (1985). In this comparison, *P.ussuriensi maxim* did not match in fruit size, calyx form and leaf teeth shape with the currently named *P.ussuriensis .maxim* in Australia, it did, however, match the characters of *P.calleryana*. Furthermore, results of isozyme banding patterns in the present laboratory work using the five isozyme systems also confirmed this mistake. The comparison of banding patterns showed 90% similarity between *P.calleryana* and *P. ussuriensis. maxim* from the Waite arboretum (Table 4.3 and Fig 4.8). A study of pollen ultrastructure gave further confirmation of the similarity (see section 12.3). The present author has few a specimen growing in the Mount Lofty Botanic Garden, south Australia which matches the description of *P. ussuriensis.maxim* and which is labelled correctly. Botanical characteristics are shown in Fig 4.8, it can bee seen that the specimen *P. ussuriensis.maxim* is indeed remarkably similar to that labelled *P.calleryana* and very different to the specimen in the Mount Lofty Botanic Garden and now known to be genuine *P.usssriensis.r...xim*.

4.3.10 Similarity of *P.lindley* to *P.boissierana*

A close similarity between P.*lindley* and P.*boissierana* in the Waite Arboretum was observed in this work. Inspection of a similarity matrix between these two species showed a 95% similarity in isozyme banding patterns and with respect to botanical characteristics of fruit and leaf, there was also a close similarity (Fig. 4.9).

It is possible that *P.lindley* and *P.boissierana* are one species but under two names. Further botanical studies of these trees in the Waite Arboretum is recommended.

(Table.4.3) Showing similarity matrix for pear species and cultivars : 1; *P. Amygdaliformis*, 2; *P.betulaefolia*, 3; *P.boissierana*, 4; *P.gharbiana*,5, *P.kawakami* 6; *P.lindley* 7, *P.mamorensis*; 8; *P.pyraster*;9; *P.syriaca*.10; *P.ussuriensis maxim*, 11; *P.calleryana*12; Anjou, 13; Chojru,14; Housi, 15;Howell, 16; Hwa Hnog, 17; Josephine, 18; Kosui, 19; Lemon Bergomot, 20; Packham Triumph, 21; Shinsui, 22; Twentieth century, 23; Tsu Li, 24; Winter Nelis, 25;Yali.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1																								
2	75%																							
3	60%	45%																						
4	75%	70%	75%																					
5	75%	90%	45%	70%																				
6	65%	50%	95%	70%	50%																			
7	55%	70%	45%	40%	60%	50%																		
8	65%	50%	75%	70%	50%	70%	50%																	
9	80%	75%	50.%	55%	65%	55%	75%	65%																
10	75%	90%	55%	55%	80%	60%	70%	50%	85%															
11	75%	80%	55%	55%	90%	60%	60%	50%	75%	90%														
12	70%	75%	60%	55%	65%	65%	75%	55%	80%	85%	75%													
13	50%	45%	60%	45%	45%	55%	45%	65%	50%	45%	45%	50%												
14	60%	55%	60%	55%	55%	55%	55%	55%	65%	60%	55%	55%	50%											
15	60%	45%	80%	75%	45%	75%	55%	85%	60%	55%	55%	50%	90%											
16	65%	70%	45%	50%	60%	505	70%	50%	85%	80%	70%	75%	65%	60%	60%									
17	90%	75%	70%	85%	75%	65%	55%	65%	70%	75%	75%	70%	60%	65%	55%	55%								
18	50%	65%	50%	45%	65%	55%	55%	55%	60%	65%	65%	60%	70%	70%	65%	70%	65%							
19	70%	55%	90%	85%	55%	85%	45%	85%	60%	65%	65%	60%	80%	70%	50%	75%	50%	50%						
20	70%	45%	60%	65%	45%	65%	55%	75%	70%	55%	55%	70%	60%	60%	90%	55%	80%	50%	50%					
21	50%	65%	40%	35%	65%	45%	65%	55%	70%	65%	65%	60%	50%	50%	80%	65%	60%	50%	70%	70%				
22	70%	65%	60%	65%	65%	55%	65%	75%	70%	65%	65%	60%	80%	60%	50%	85%	50%	80%	50%	50%	50%			
23	60%	75%	40%	45%	65%	45%	755	55%	80%	75%	65%	60%	80%	80%	70%	75%	80%	70%	70%	70%	70%	70%		
24	90%	65%	70%	75%	65%	65%	55%	75%	75%	65%	70%	70%	70%	50%	95%	60%	80%	50%	60%	90%	80%	90%	80%	
25	60%	55%	60%	55%	55%	55%	55%	55%	50%	55%	55%	50%	80%	70%	65%	90%	50%	80%	70%	50%	80%	60%	80%	70%

Fig. 4.8 A comparison of leaf and fruit for *P.callerana*
and *P.ussuriensis maxim* from two different sites
Waite Arboretum and Mount Lofty Botanic garden.

Fig. 4.9 A comparison of leaf and fruit for *P.lindley*
and *P.boissierana* collected from Waite Arboretum.

Pyrus ussuriensis maxim

Location: Mount Lofty Botanic Garden

Pyrus ussuriensis maxim

Location: Tree 780F, Arboretum. Waite Institute

Pyrus calleryana

Location: Tree 777B, Arboretum. Waite Institute

Fig. 4.8

Pyrus lindley

Location : Arboretum. Waite Institue

Pyrus boissierana

Location: Arboretum. Waite Institute
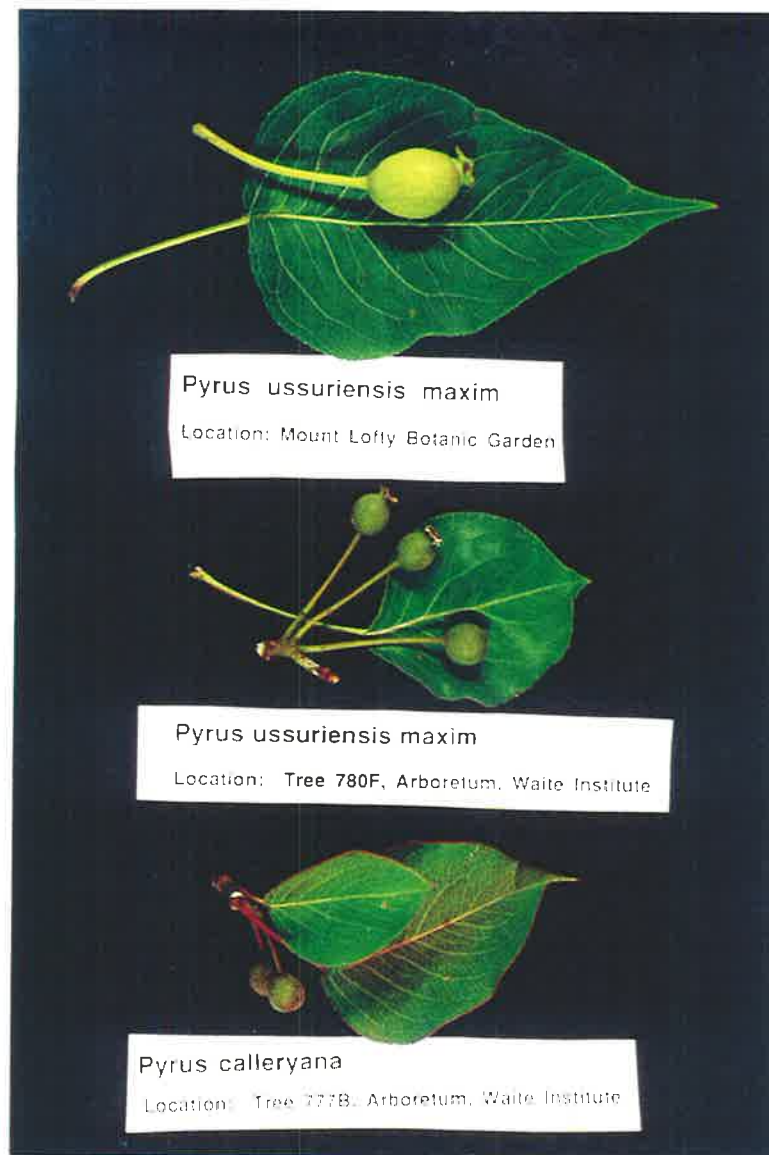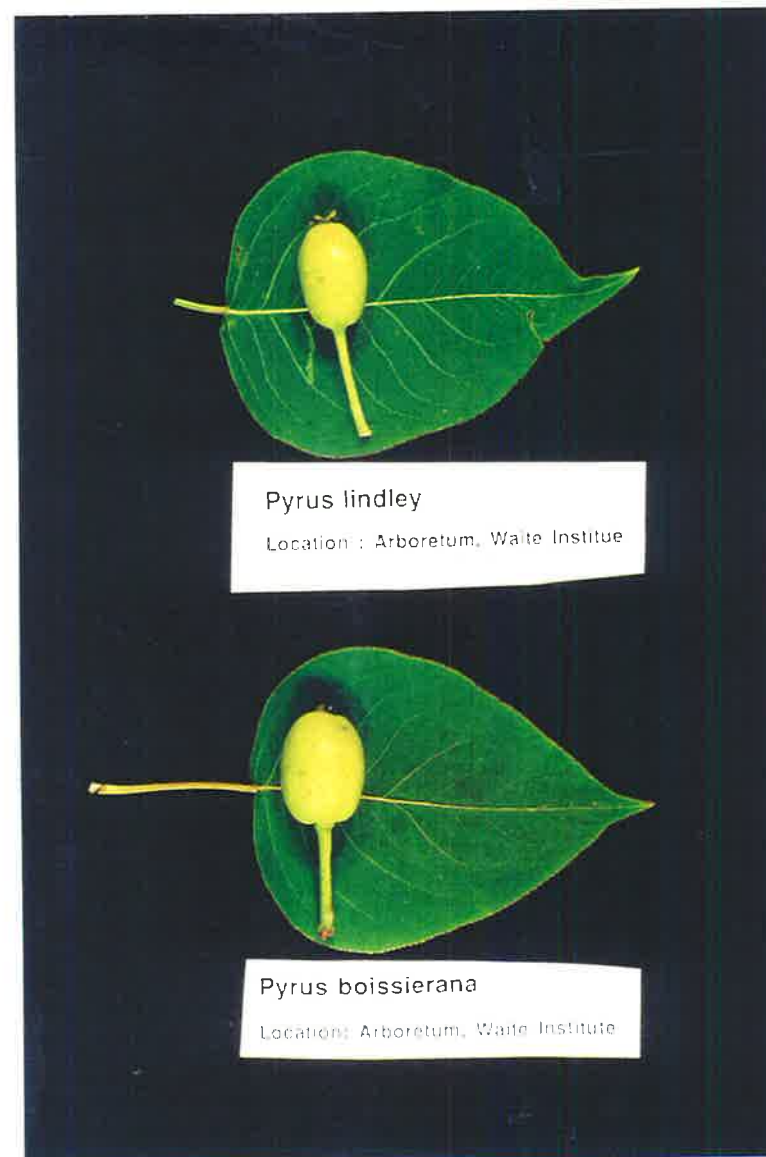
Fig. 4.9

4.4 Discussion

Crezo *et al.* (1989) found most variability in banding patterns for pear pollen isozymes occurred for AAT, GPI, PGM, ACP, ADH. The banding patterns for GPI-2 described in their work were the same as shown here. Meanwhile the similarity patterns of GPI for some pears are similar to those described by Marquard and Chan (1995) and Weeden and Lamb (1985) for apple. Crezo *et al.*,(1989) obtained similar patterns in GPI-2 for almond, B and E and J (Fig 4.4) banding patterns occurring in both almond and pear. The number of bands with respect to the dimeric character of GPI-2 support the presence of more than one locus in this zone or can be a sign of an alloploid origin for pear (Crezo *et al.*, 1989). However scoring the complicated banding patterns was difficult. Difficulty in scoring of apple for GPI has been reported, (Chevreau *et al.*,1985). Further work gave expression of a duplicated gene for GPI as an interpretation for a pattern which was not a dimer (Chevreau and Laurens, 1987).The low diversity of species located in groups A and B showed a low level of variations for the pear ancestors (wild species of pears). Occurrence of hybridization with respect to the self-incompatible nature of pear led to greater diversity. Although it is probable that primary pears were in few forms in one area, Crezo *et al.* ,(1989) could not obtain a complete set of bands for PGM-3 and PGM-4 loci for pear. However they showed a better resolution for PGM-1 and PGM-2. In this study, we observed a greater difference in banding patterns of PGM for pear species in comparison with the pear cultivars grown in the Coromandel valley orchard Weeden and Lamb (1987) have shown five loci for apple PGM isozyme. They stated that PGM-4 and PGM-5 were cytosolic in apple. They showed a monomer and heterozygous structure for apple PGM which is similar to pears in this regard. Kenner *et al* (1995) give evidence for the presence of four loci for MDH in Cucumber. They pointed out that MDH-2 and MDH-4 (counted from loading zone) code for cytosolic isozymes and MDH-3 codes for a mitochonodrial isozymes. ADH banding patterns found here for pollen from pear coincide with the work of Crezo *et al.*, (1989) and we extent it to characterise more species. Two anadoic bands revealed for Chojru and Housi were different to other

pears.   Crezo *et al.*, (1989) detected 3 loci for ADH which was in agreement with the present pear ADH banding patterns.   Marquard and Chan (1995) indicated a greater range of ADH banding patterns for Crabapples.

The close similarity of *P.gharbiana* with *P.pyraster* suggests the probability that *P.gharbiana* had migrated from Europe to Africa. GPI, PGM and IDH revealed more polymorphism than other isozyme systems used in this study.   Discriminatino identification of pears in GPI and PGM isozyme systems demonstrated the usefulness of isozyme technique to identify different pears.   Interestingly those cultivars with narrow genetic differences can be identified using PGM GPI, ADH and IDH.

Results obtained from the similarity matrix of pears showed that among some pear species and cultivars there was significant relatedness. Even though these similarities were not the outcome of a whole picture of characters of pear species and cultivars, they do help to provide clearer comparisons among pears. Due to the geographical restriction with pear species distribution, similarity among pear species may be the results of intraspecies hybridization or inter hybridization among early pear species. The relatedness of *P.calleryana* and *P.betulaefolia* with *P. kawakami* supports the suggestion that the origin of the above mentioned species was China (Challice and Westwood, 1973).   The ancestor of early European pears can be species other than *Pyrus communis*.   It is shown here that *P.amygdaliformis* which has a Southern Europe origin has a similarity at 80-90% with Winter Nelis and Josephine, which are both European pears. We did not measure the similarity between *P.communis* and the European pear cultivars grown in the orchard. The similarity of *P.syriaca* (West Asian pear) with *P.ussriensis maxim*, Winter Nelis, Hwa Hong and Tsu Li can suggest that European pear cultivars arose from several species and not only from *P.communis* (Catlin and Olsson, 1966) .   African pears *P.gharbiana* and *P.mamorensis* did not show high similarity to each other which was in accordance with Challice and Westwood (1973).   Similarity of Japanese pears including Chojru, Hosui, Twentieth Century with Yali which has a Chinese origin support this hypothesis that Chinese and Japanese pears have a common ancestor.

Figs. **4.10a**, **b** GPI isozyme phenotypes for different pear species and cultivars.

a

GPI Isozyme banding patterns in extracts from pollen for pear cultivars:
A) Josephine, B) Hwa Hong, C) Lemon Bergomot, D) Packam Triumph,
E) Twenty Century, F) Winter Nelis

b

Zymogram showing glucosephosphate isomerase bands
in extracts from pollen for pear species and cultivars
A) P.betlufolia, B) Josephine, C) P.boissierana, D) Packham, E) Yali
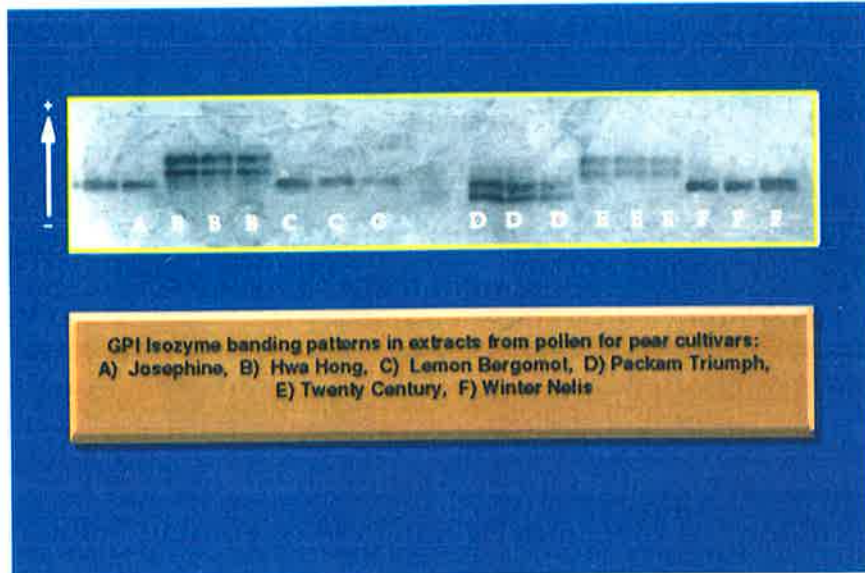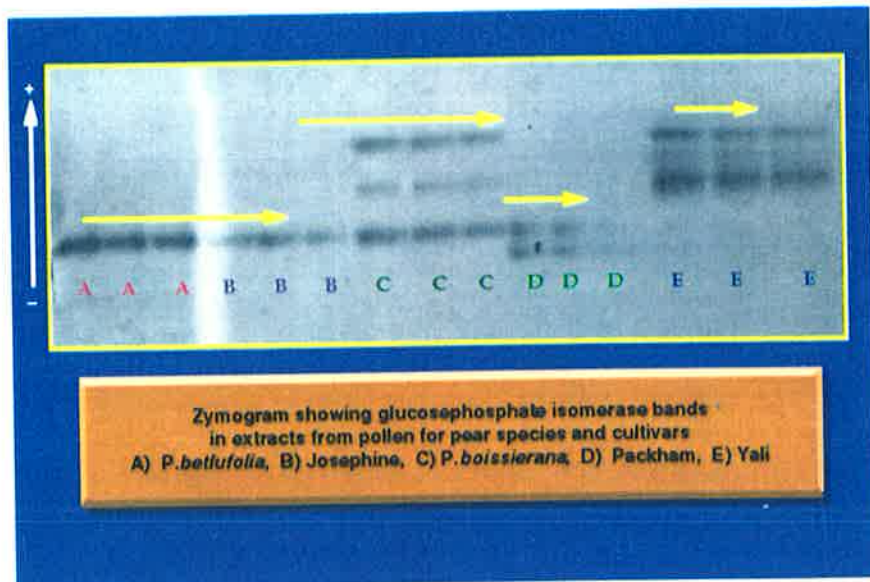
Figs.4.11c, **d** GPI isozyme phenotypes for different pear species and cultivars.

c

GPI 2

A A A A B B B B
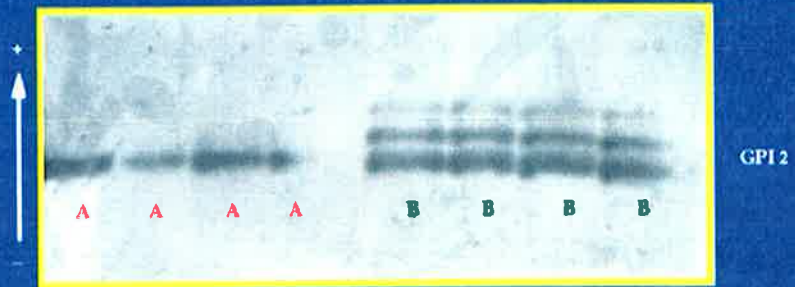
Glucosephosphate isomerase banding patterns
in extracts from pollen for two species of pears:
A) P. mamorensis, B) P. amygdaliformis

d

GPI 2

A A A A B B B B C C C D D D

Glucose phosphate isomerase (GPI 2), banding patterns
in extracts from pollen for japanese pear cultivars as follow:
A) Chojuro, B) Housi, C) Kosui, D) Shinsui

Figs.4.12e, f GPI isozyme phenotypes for different pear species and cultivars.

e

Glucosephosphate isomerase banding patterns observed
in extracts from pollen for 4 species and Cultivars of pears:
(A) P.*amygdaliformis*, (B) Packham, (C) P.*pyraster*, (D) Twenty century

f

GPI 2

Glucosephosphate isomerase banding patterns
in extracts from pollen for 4 pear cultivars:
A) Anjou, B) Howell, C) Packham, D) Tsu li
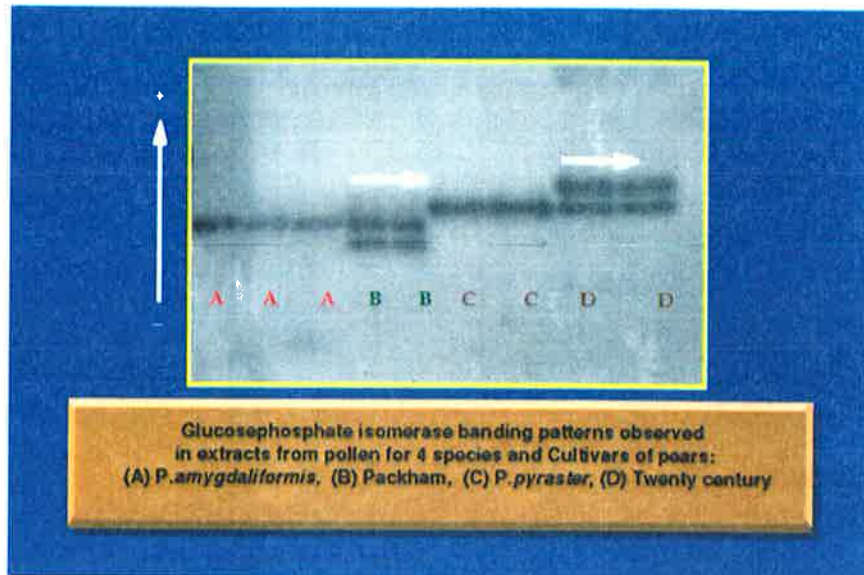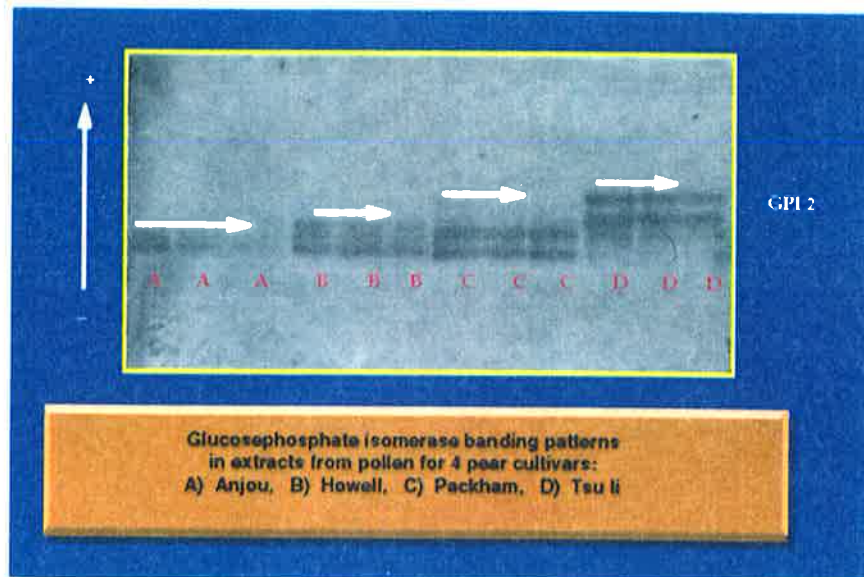
Fig. **4.13g**. GPI isozyme phenotypes for three pear species.

Fig. **4.13h**. PGM Isozyme phenotypes for different pear cultivars and species.

g

Zymogram showing gloucosephoshate isomerase (GPI 2) banding patterns
in extracts from pollen for 3 pear species:
A) P. *boissierana* , B) P.*lindley* , C); P. *syriaca*

h

Pgm3
Pgm 4

A   B   D   C
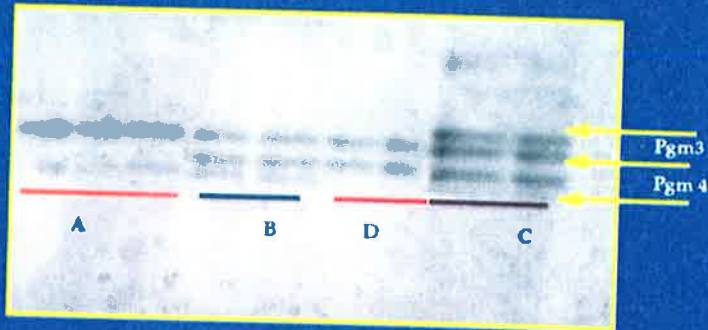
Zymogram showing phosphoglucomutase (PGM) banding patterns
in extracts from pollen for 4 Cultivars and Species of pears:
A) P.*amygdali formis*, B) Packham, C) P.*pyraster*, D) TW.C

Figs. **4.14i**, **j**. PGM Isozyme phenotypes for different pear cultivars and species.

i
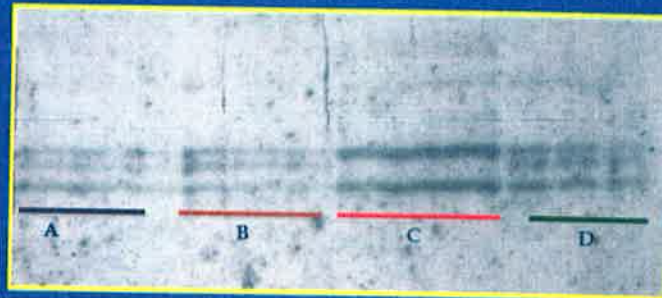
Zymogram showing PGM isozyme banding patterns
in extracts from pollen for pears:
A) Chojuro, B) Housi, C) Kosui, D) Shinsui

j
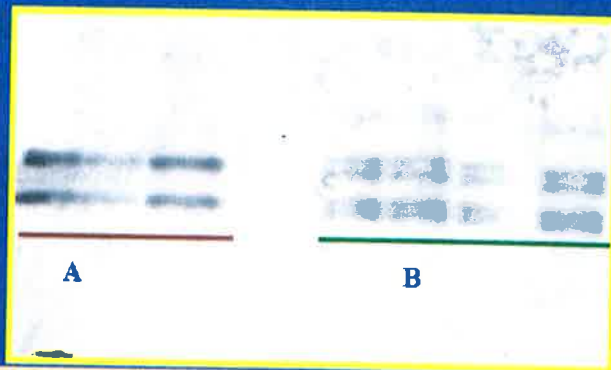
Zymogram showing PGM isozyme banding patterns
in extracts from pollen for pears:
A) P. *amygdaliformis*, B) P. *mamorensis*

Figs.4.15k, l. PGM Isozyme phenotypes for different pear cultivars and species.

k

Zymogram showing PGM izosyme banding patterns
in extracts from pollen for pears:
A) P. *betulofolia*, B) Josephine, C) P. *boissierana*, D) Packham, E) Yali

l

Zymogram showing PGM banding patterns
in extracts from pollen for pear species and cultivars:
A) P. *kawakani*, B) Kosui, C) Lemon Bergomot D) P.*lindley*,
E) P.*pyraster*, F) 20 century, G) Winter Nelis
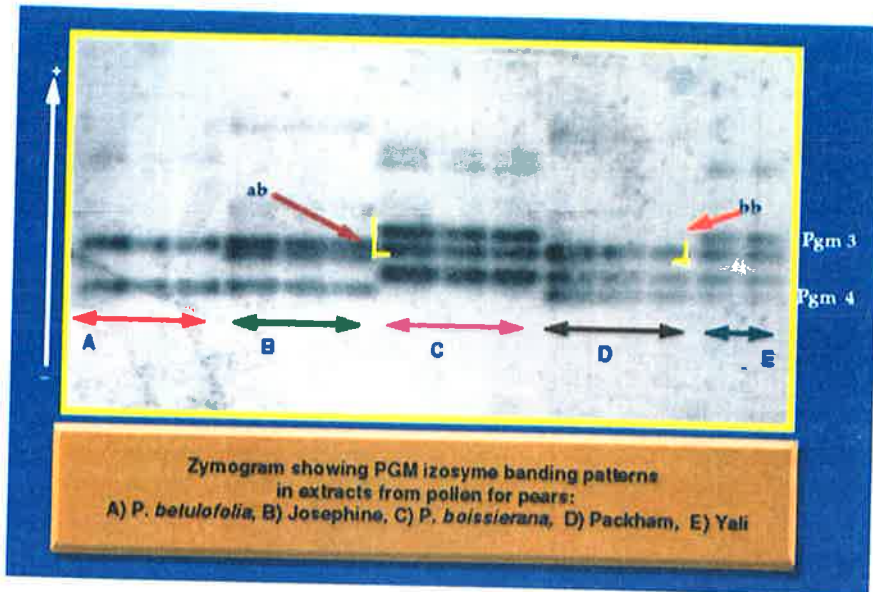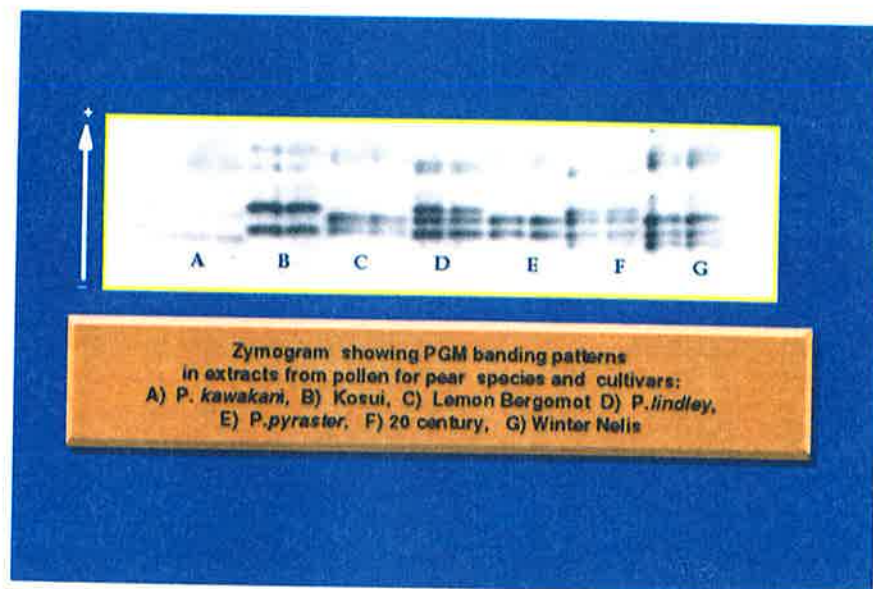
Fig.4.16m.IDH Isozyme phenotypes for three pear species.

Fig.4.16n. ADH Isozyme phenotypes for different pear cultivars and species.

m
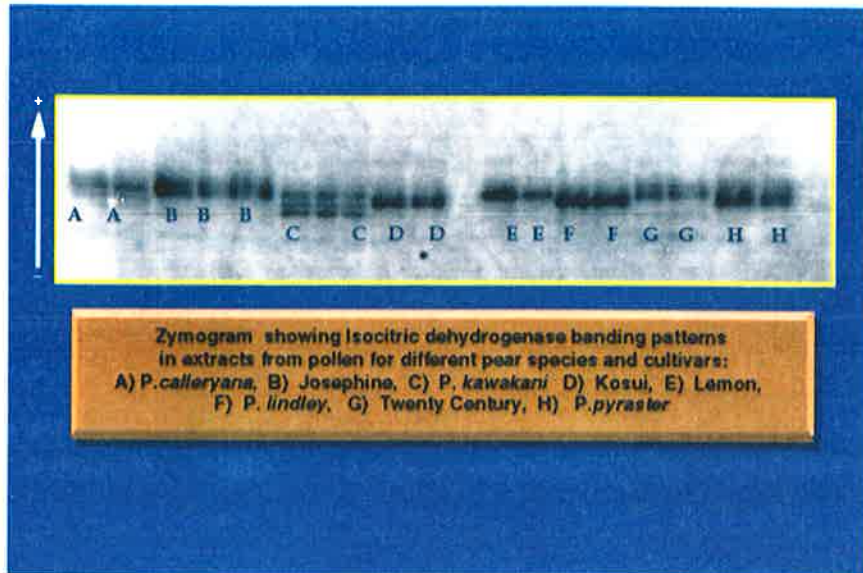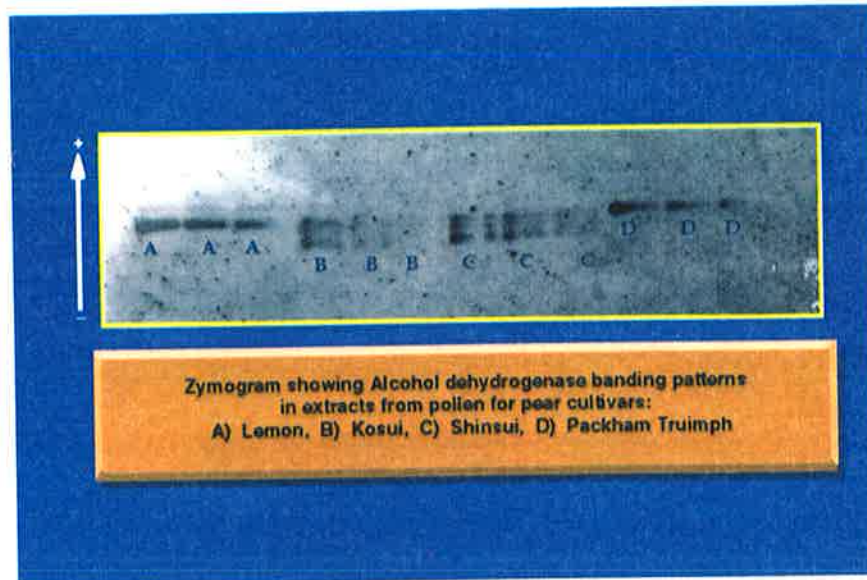
Zymogram showing Isocitric dehydrogenase banding patterns
in extracts from pollen for different pear species and cultivars:
A) *P. calleryana*, B) Josephine, C) *P. kawakani* D) Kosui, E) Lemon,
F) *P. lindley*, G) Twenty Century, H) *P. pyraster*

n

Zymogram showing Alcohol dehydrogenase banding patterns
in extracts from pollen for pear cultivars:
A) Lemon, B) Kosui, C) Shinsui, D) Packham Truimph

Chapter 5

Inheritance of Isozyme Phenotypes in Controlled Crosses with Packham Triumph Pear

5.1 Introduction

Controlled crosses to find patterns of inheritance of molecular markers can be applied to follow gene flow by pollen in plant populations (Arias and Rieseberg, 1994; Jackson, 1992; Marquard, 1988; Tanksley, 1984). Genetic linkage of these markers can be studied through this work also (Chevreau and Laurens, 1987; Beaver and Iezzoni, 1993; Arus *et al.*, 1994; Vezvaei *et al.*, 1995). Rare alleles or allelic polymorphism within cultivars or species can be helpful (Muller, 1977). Homozygous isozyme banding patterns or production of just one isozyme phenotype in a cross with a cultivar or species may show the homozygotic parental structure of that gene within that cultivar or species.. The main purpose of the work described in this chapter was to describe isozyme phenotype inheritance in crosses with Packham Triumph cultivar which can then be used to assist in studying gene flow by pollen within the pear orchard.

5.2   Materials and Methods

5.2.1 Pollen Collection and Cross Pollination

Flowers of Packham Triumph pear were selected as the female parent. Pollen was collected from other pear cultivars and species. The male parents used included Josephine, Lemon Bergomot, Duchess and Winter Nelis. *Pyrus boissierana* and *Pyrus lindley* were selected as wild species male parents to observe the inheritance of banding patterns of these species in comparison with commercial cultivars to assist in better interpretation. Pollen was separated from flowers before anthesis. All the brushes which were used for cross pollination were washed and sterilised with 70% ethanol before and after use. Anthers were dried and dehiscence took place after 48 hours at a room temperature of 22°C. Pollen was sieved with a mesh number 100 sieve (opening .0060 inch to separate pollen

from dried anthers). Pollen grains so obtained was placed in an Eppendorf tube and kept at 4°C.

Although Packham Triumph is fully self-incompatible nevertheless it was emasculated before hand pollination with a brush. The flowers were then bagged and labelled after pollination avoid contamination with other pollen source.

5.2.2 Isozyme Analysis

Seeds from different crosses were collected, and after 24 hours drying at room temperature were kept in a cold room at 4°C. Five isozyme systems including ADH, PGM, GPI, IDH and MDH were utilized. Testa was removed from seeds before extraction. The seed was ground with a mixture of 20mg PVPP and 250 µl normal extraction buffer (see Chapter 4). The mixture was centrifuged at 14000 rpm for 15 minutes. Supernatant was loaded on to cellogel acetate sheets. Two buffer systems were used. These included 0.02 M sodium phosphate buffer pH 7.0 for ADH and PGM and 0.05 M Tris-malate pH. 7.8 for GPI and IDH and MDH. Electrophoresis was performed at a constant voltage of 200 VDC for 2 hours in a cold room at 4°C. Staining was achieved as described by Richardson *et al.,* (1986).

5.3 Results

5.3.1 PGM, IDH and GPI

Examination of seeds with the PGM isozyme system did not reveal any detectable bands, suggesting low or no activity of this isozyme in seed.
This is in contrast to high activity in pollen extracts. Testing of seed extracts for IDH isozyme activity revealed weak bands, suggesting low activity of IDH in seeds. PGM and IDH were not used therefore in these gene flow by pollen studies.
As seen before the GPI isozyme system showed a high number of variational phenotypes in extracts from pollen, so it was expected that many new combinations would be seen in seeds. As shown in Fig.5.1, Fig 5.5 and Fig 5.6, three different phenotypes were observed in three different crosses. Isozyme

phenotypes in all crosses indicated both parental phenotypes in their progenies as can be seen by comparison in Fig 5.2.

| A1 | B1 | C1 | A2 | B2 | C2 | A3 | B3 | C3 |
| Packham X P.*boissierana* | | | Packham X Duchess | | | Packham X Josephine | | |

Fig. 5.1 Schematic zymogram GPI isozyme phenotypes in three different crosses with Packham Triumph (female recipient).

A1. B1. C1 are schematic GPI isozyme phenotypes obtained in progenies of the cross *P.boissierana* (as male donor) with Packham Triumph.

A2, B2, C2 are schematic GPI isozyme phenotypes obtained in progenies of the cross Duchess (as male donor) with Packham Triumph.

A3, B3, C3 are schematic GPI isozyme phenotypes obtained in progenies of the cross Josephine (as male donor) with Packham Triumph.
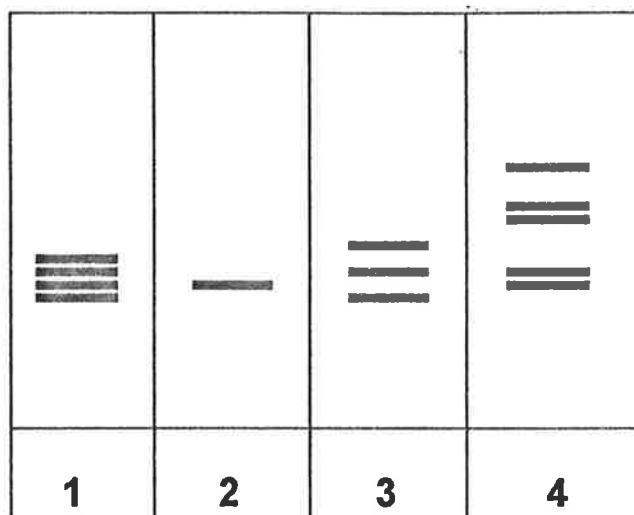
Fig.5.2. GPI isozyme phenotypes in extracts from pollen for 1-Packham Triumph, 2-Lemon Bergomot, Winter Nelis and Josephine, 3-Duchess, 4-*Pyrus boissierana*.

Segregation of phenotypes in crosses of Josephine, Lemon Bergomot and Winter Nelis as male donor with Packham Truimph showed a similar pattern. In earlier work, these male donor cultivars showed a similar pattern for GPI which was bb (Fig.5.2).

In practice the above segregation was applicable for crosses of Packham with Winter Nelis and Lemon Bergomot, because the parental phenotypes were equal and segregation would be the same as above.

Due to difficulty in assigning alleles for pollen and seed isoenzyme phenotypes for GPI F1 progeny from the crosses with Packham the progeny types were labelled as A, B, C. Segregation of GPI isozyme phenotypes in the cross of Duchess with Packham showed a ratio of 2:1:1 for phenotypes, A2: B2: C2 respectively (Table 5.2).

Table.5.1 Segregation analysis for cross between Packham Triumph X Duchess and Packham Triumph X *P. boissierana* in GPI Isozyme system.

Crosses segregation	Phenotypes frequency			X^2	Expected
Packham X Duchess					
	A2	**B2**	**C2**		**2:1:1**
Observed	21	10	9	0.15 * P< 0.05	
Expected	20	10	10		
Packham X P.*boissierana*					
	A1	**B1**	**C1**		**1:1:1**
Observed	19	18	17	0.1 * P<0.05	
Expected	18	18	18		
Packham X Josephine	**A3**	**B3**	**C3**		
Observed	39	27	19	2.1 * P<0.05	**2: 1: 1**
Expected	42	21	21		

5.3.2 Phenotypic Segregation For Alcohol Dehydrogenase (ADH)

The ADH isozyme system produced two main phenotypes in different crosses. The first group which included Lemon Bergomot, Packham Triumph and *P.boissierana* showed as parents one single band in extracts from pollen for locus 2. Another group such as Winter Nelis, Duchess and Josephine as parents give 3 different bands (see chapter 4, Table 4.1)). It was found that when Josephine or Winter Nelis was used as male donor with Packham Triumph, progeny showed two banding patterns (A and B) which resulted in the progeny, and *P.boissierana*, *P.lindley*, Duchess as male donor gave only B banding pattern in the progeny when crossed with Packham Triumph (Fig.5.3). This result was reproducible and clear to detect and so can then be used for gene flow by pollen work also.
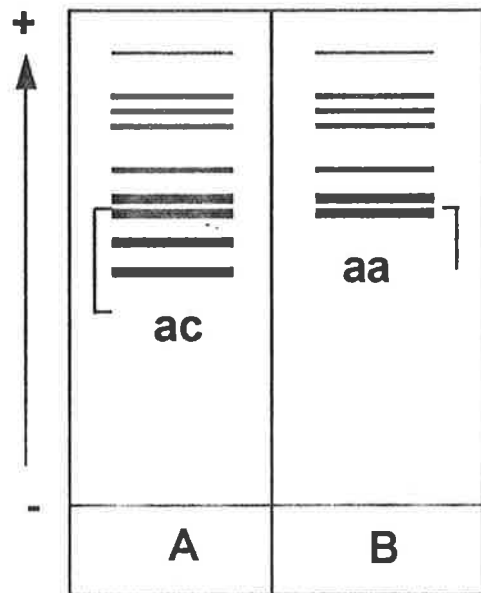
Fig 5.3 Showing two ADH isozyme phenotypes in progenies of controlled crosses of group A and B phenotypes which were obtained by male donors (Josephine or Winter Nelis) with Packham Triumph. Group B phenotype which was obtained by males (*P.boissierana*, *P.lindley*, Duchess or Lemon Bergomot) with Packham Triumph.

Table 5.2 Showing segregation analysis for two **A** and **B** phenotypes of ADH in extracts from seeds.

	Phenotypes				
Crosses	**A**	**B**	X^2	Expected segregation	
Packham X Josephine	39	35	0.2	P<0.05	**1:1**
Packham X Lemon	0	68	—	—	**0:1**
Packham X Winter	26	28	0.1	P<0.05	**1:1**
Packham X Duchess	0	40	—	—	**0:1**

5.3..3. Malate Dehydrogenase

While MDH gave clear bands (**Fig.5.4**), it did not show polymorphism and was not therefore used further in this work.

5.4 Discussion

The segregation ratio in controlled crosses for GPI indicated two types of inheritance. Detection of a 2.1.1 ratio was significant for crosses of Josephine and Duchess with Packham Triumph (Table 5.2). But for the cross between the wild species *P.boissierana* and Packham Triumph the ratio changed to 1:1:1. Chevreau and Laurens (1987) has already reported that different parental peroxidase phenotypes give different segregation ratios. Other researchers indicated a different format of segregation including 1:1 and 2:1:1 and 3:1 for F1 progenies in different crosses (Hauagge *et al*, 1987; Chevreau and Laurens, 1987).

Similar banding pattern was the outcome of two male parents (Josephine and Lemon Bergomot) crossing with Packham Triumph (as female recipient). Thus there was no isozyme marker could be used in discrimination between gene flow by pollen from Josephine and Lemon Bergomot to Packham Triumph.. Also a similar segregation ratio of GPI phenotypes in the progenies obtained from the crosses of Duchess and Josephine (male parents) with Packham Triumph was observed (Table 5.1). This similarity representing that Duchess and Josephine more probably had similar GPI isozyme phenotype. Further study, is needed to complete understanding of alleles and loci structures in GPI isozyme for different

pears. Achiving the controlled test crosses on specific locus or loci can be helpful for understanding of more genetic inheritance and probable linkages in pear. However ADH and GPI can be used for this purpose.

The segregation analysis for cross between Josephine (as male donor) with Packham Triumph showed a ratio of 1:1 for A and B phenotypes of ADH (Table 5.3), while the cross between Lemon Bergomot (male) with Packham Triumph only produced B phenotype (Table 5.3). Winter Nelis (as male) behaved in a similar manner to Josephine for ADH isozyme system (Table 5.3).
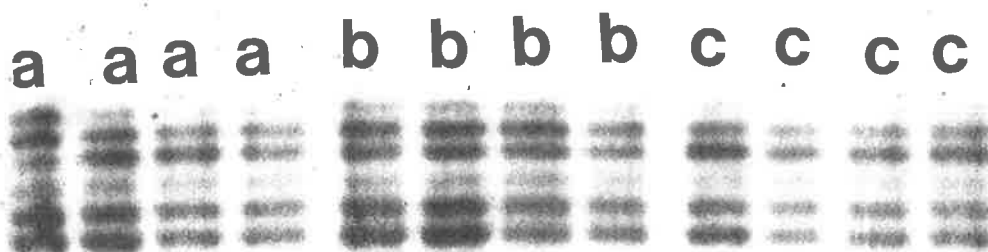
Fig.5.4. Zymogram showing MDH isozyme banding pattern in extracts from seeds for different crosses, a; Packham Triumph X *P.boissierana*, **b;** Packham Triumph X Duchess, **c;** Packham TriumphX Lemon Bergomot.
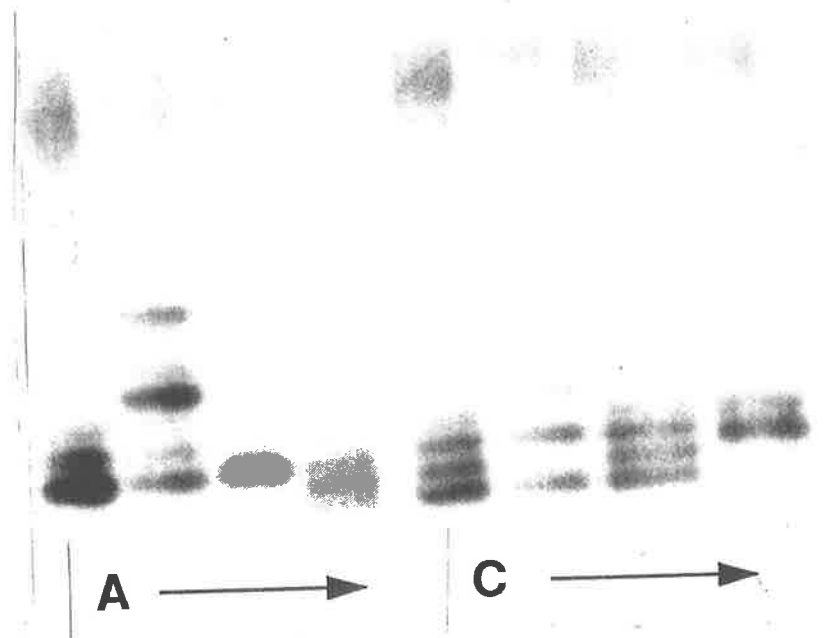
Fig.5.5 Zymogram showing GPI isozyme phenotypes in cross of male donors; *P.boissierana* X Packham Triumph (A) and Duchess (male) X Packham Triumph (B).
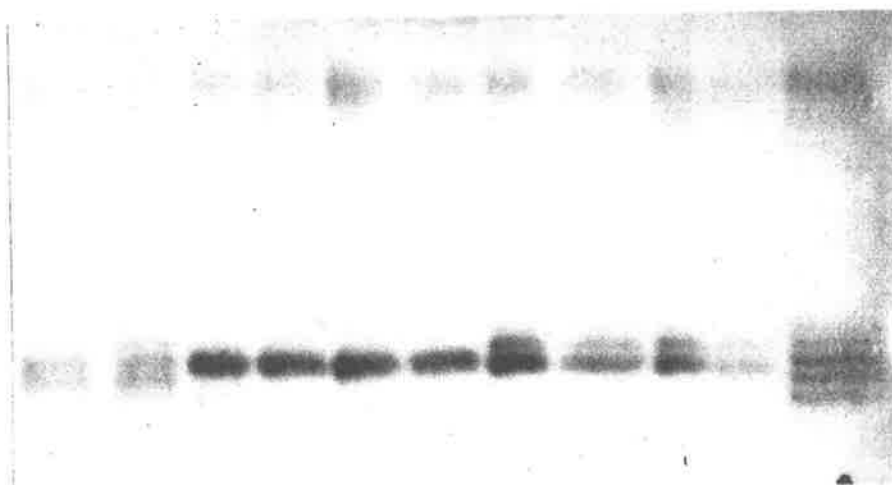
Fig.5.6 Zymogram showing GPI isozyme phenotypes in cross
of male donor Lemon Bergomot with Packham Triumph.

Chapter 6

Nectar Analysis, Anther Anthocyanins and Pollen Production Measurement for Pears

6.1 Introduction

The quality and quantity of nectar and pollen are a part of important elements which can affect ine foraging behavior of pollinators within plant populations (Goodman and Fisher,1991). It was reported that there are many different levels of attractiveness of tree fruit flowers to pollinators (Free, 1993). This was attributed mostly to differences in amounts and composition of amino acids, volatiles and sugars between different flower nectars (Dobson, 1985). It was Zauralov (1983) who pointed out that it was nectar sucrose level rather than fructose and glucose which enhanced bee activity in populations of apple and sour cherries. De Grandi-Hoffman *et al.*, (1992) concluded that those almond cultivars which had the greatest number of flowers per cubic meters had the most nectar and pollen per cubic meter, so attracted the greatest number of honeybees. Alm *et al.* (1990) supported the hypothesis that amino acids of nectar contributed to honey bee attraction and feeding on cabbage. Results of different works showed that within the cultivars of various fruit trees there are variations in concentration of different amino acids and sugars in flower nectar (Meheriuk and Lane, 1987). The present investigation was undertaken firstly to analyse amino acids and sugars in nectars of different pear cultivars and species. The second aim was to measure pollen production in certain pear cultivars. Measurement of anthocyanin on the surface of anthers was also carried out for pear cultivars and species in case the colour of anthers changed attractiveness to bees. The importance of a study of pollen production study was related to the need to understand the adequacy of pollen supply and dispersion from certain cultivars for pollination and fertilization purposes.

6.2 Materials and Methods

6.2.1 Preparation of Nectar Extract

Six species of pears including *P.betulaefolia*, *P.longipes*, *P.koroshinsky*, *P.brestchneideri*, *P.boissierana*, *P.cossoni* and five pear cultivars of *P.communis* Josephine, Lemon Bergomot, Sensation, Nashi (Twentieth Century), and Packham Triumph, were selected. The plant species were located at the Waite Agricultural Institute Arboretum in South Australia. As pear flowers in general had low amounts of nectar, direct collection of nectar was not possible. The method for collection followed that of Grunfeld *et al.*, (1989) and Jolls *et al.*(1994). One hundred opened flowers per cultivar *P.communis* and other species were picked. Petal., styles and stamens were removed immediately. The calyx from one hundred flowers which contained the nectar was soaked for 20 minutes in 100 ml distilled water. Then, the mixture was filtered through Whitman filter paper No 42. Twenty five ml of this solution was freeze dried and 5 ml distilled water was added to the remaining solid to increase the concentration 5 fold.

6.2.2 HPLC Analysis of Amino Acids

A sample of 200μl from the lyophilised solution was concentrated again . Lyophilised samples were redissolved in 100μl of HCl (0.01N) containing 0.5 mM norvaline and sarcosine as internal standards. Redissolved samples were centrifuged for 5 minutes at 14000 r.p.m. in an Eppendorf 5415 centrifuge. Analysis was achieved with one μl of the supernatant. Amino acid analysis was carried out on a Hewlett-Packard Amino Quantitative Amino Acid Analyser, consisting of an HP 1090 series II liquid chromatography controlled by HP Chem station software. An autosampler was used to derivatize the amino acids which were then separated by reversed -phase HPLC on an amino quant C18 column. Derivatization was performed with ortho-phthaldhyde and FMOC (9-fluorenylmethyl chloroformate) and quantified by U.V absorbance. Amino acid

calibration standard, derivatizing regents and the amino acid analysis column were obtained from Hewlett -Packard.

6.2.3 Sugar Analysis of Nectar

Sucrose , glucose and fructose was measured with an enzymatic kit using methods from Boehringer Mannheim where NAD reduction is measured at a wavelength of 340 nm using a Varian /DMS 200 UV-Visible spectrophotometer. A mixture solution of 0.5 g/l for each of glucose, fructose and sucrose was used as standard sugar. The D-glucose deflection was read b _ore and after enzymatic hydrolysis of sucrose. The D-fructose deflection was read after D-glucose.

6.2.4 Anthocyanin Measurement

Anthers from different *P.communis* cultivars and other species were collected and 0.1 gram of anthers was homogenised in 2 ml of 50% ethanol and centrifuged at 2500 g for 10 minutes. 2.5 ml of 1 M HCl was added to 0.5 ml of clear supernatant of anther extract and left to stand overnight. This homogenised solution was used for spectrophotometry measurement using a varian /DMS 200 UV Visible spectrophotometer at 520 nm wavelength.

6.2.4 Pollen Production Measurement

Fifty newly opened flowers from each cultivars were picked . Anthers were detached and placed in dishes. Anthers were kept in air temperature for 48 h to dry and after drying, they were weighed to obtain dried anthers with pollen. After sieving to collect pollen from anthers, anthers without pollen were weighed and the difference taken as pollen weight.
Anther dry weight and fresh weight were measured for further studies in pollen production.

6.3 Results

6.3.1 Amino Acids

Analysis of nectars for 20 free amino acids showed a high degree of variability within this collection of pears (Table 6.1). Asparagine was found to be present in higher concentration in all nectars, followed by glutamine, asparatic acid, glutamic acid, serine, alanine, arginine and proline. The overall concentration of amino acids varied markedly in nectars from the various sources. Some other amino acids such as cysteine, tryptophan, lysine and leucine showed very low amounts in different nectars (Table 6.1).

Sensation and *P.cossoni* were found to have higher concentration of amino acids while. *P.koroshinsky*, *P.boissiereana*, *P.brestchneideri* and *P.betulaefolia* showed the lowest amount of amino acids in nectars.

From the cultivars in the Coromandel valley orchard, Packham Triumph nectar had the highest concentration of amino acids, closely followed by Lemon Bergomot, while Josephine and Nashi had the lowest.

6.3.2 Sugar Analysis of Nectars

Results of sugar analysis showed a higher level of total sugar in nectar from *P.cossoni* and *P.comunis* 'Sensation. These two also had higher concentrations of amino acids. The lowest concentration of total sugars was found inn *P.boissierana* with 200 mg/l (Fig. 6.1). Josephine, Lemon Bergomot and Packham Triumph had 280, 270 and 230 mg/l of total sugars respectively. For fructose, *P.cossoni* and *P. communis* Josephine had the highest concentration at 240 and 180 mg/l, respectively. Lowest fructose level were found in *P.koroshinsky* and *P.betulaefolia*, 20 and 70 mg/l, respectively (Fig. 6.2).

P.communis cultivar Sensation and *P.cossoni* showed 540 and 400 mg/l glucose in nectar (Fig. 6.3), while *P.betulaefolia* and Packham Triumph had the lowest glucose level with

60 and 80 mg/l respectively. Analysis of nectars for sucrose showed a different pattern in comparison with other sugars (Fig. 6.4).

Table 6.1 Free amino acids which were found in necatrs for 11 species and *P.communis* cultivar of pears including No 1) *P.betulaefolia*, 2) *P.longipes*, 3) *P.koroshinsky*, 4) Lemon Bergomot, 5) Sensation, 6) Packham Triumph, 7) Josephine, 8) *P.brestchneideri*,9) *P.boissierana*, 10) *P.cossoni*, 11) Nashi (Twentieth Century). Each vlaue is in ug/ml.

Amino Acids	No.1	No. 2	No. 3	No. 4	No. 5	No. 6	No.7	No. 8	No. 9	No. 10	No.11
Asp.	5.3	31.8	5.5	14.2	32.8	19.1	5.2	1.8	4.6	43.7	7.7
Glu.	3.4	23.7	6.1	15.6	26.7	12.5	5.9	1.2	2.5	35.0	3.1
Asn.	27.1	163.9	51.2	120.5	275.6	162.5	42.1	23.5	20.9	202.3	45.0
Ser.	10.2	31	6.8	21.1	36.0	29.4	11.1	3.7	4.1	35.5	10.8
Gln.	5.6	34.9	11.3	26.7	106.7	51.1	16.5	3.9	20.9	33.9	11.1
His.	1.7	6.3	1.1	2.9	4.9	3.7	1.5	0.8	nd	6.6	1.3
Gly.	3.7	7.1	2.4	3.6	7.5	5.3	3.9	1.0	1.1	7.0	3.3
Thr.	2.6	11.8	2.8	7.3	13.1	8.0	2.5	0.9	1.5	15.2	2.8
Ala.	4	23.8	6.4	23.4	32.7	25.5	8.3	1.9	3.7	28.7	6.1
Arg.	4.6	28	8	14.8	37.0	22.5	6	2.4	5.6	29.8	7.8
Tyr.	<0.1	0.5	nd	0.2	0.7	0.7	0.1	nd	nd	0.8	nd
Cys.	1.6	3	1.0	1.3	3.2	1.8	1.4	nd	nd	3.8	1.3
Val.	1.9	14.7	3.0	11.2	17.2	10.6	3.3	1.2	2.0	20.8	4.1
Met.	1.1	7.8	2.2	4.1	8.4	5.1	1.2	0.3	0.2	10.3	1.2
Trp.	0.6	2.4	0.6	2.4	2.4	1.5	nd	nd	nd	3.5	nd
Phe.	1.1	14.3	1.8	7.9	12.1	5.5	2.6	0.2	1.7	19.6	2.7
Ile.	1.1	13.2	2.8	10.6	18.5	11.4	4.4	1.4	1.3	17.7	4.7
Leu.	0.5	9.1	0.4	1.8	8.2	3.2	1.0	0.1	nd	12.6	2.0
Lys.	0.4	1.6	nd	0.8	1.7	1.3	0.8	nd	nd	1.9	0.8
Pro.	6.1	37.6	9.2	6.3	28.6	9.0	3.1	1.7	2.0	33.7	6.5

Asp. = Asparatic acid, Glu. = Glutamic acid, Asn. = Asparagine, Gln. = Glutamine, His. = Histidine, Glyc. = Glycine, Thr. = Threonine, Ala. = Alanine, Arg. = Arginine, Tyr. = Tyrosine, Cys. = Cysteine Val. = Valine, Met. = Methionine, Trp. = Tryphtophan, Phe. = Phenylalanine, Ile = Isoleucine, Leu. = Leucine, Pro. = Proline, Ser. = Serine nd= not detected
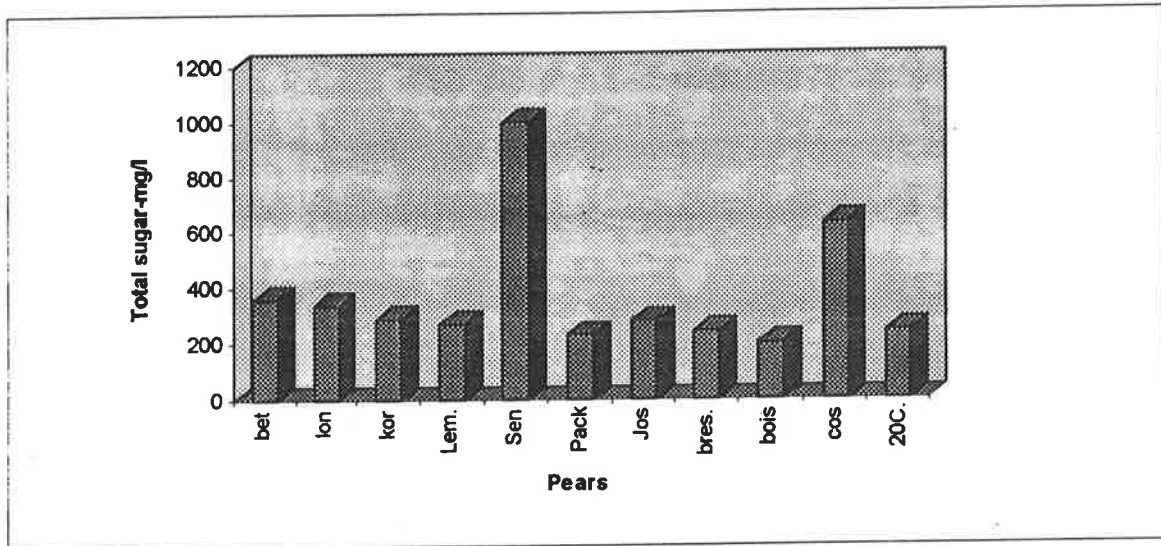
Fig. 6.1 Total nectar sugar concentration for some pear species and *P.communis* cultivas The explanation for abbreviation of the species and cultivars are as follow; bet.=*P.betuleafolia*, lon.=*P.longipes*, kor.=*P.koroshinsky*, Lem.=Lemon, Sen.= Sensation, Pack.=Packham Triumph, bres=*P.breschendri*, bois=*P.boissierana*,.coss =*P. cossoni*, 20c = Twentieth Century
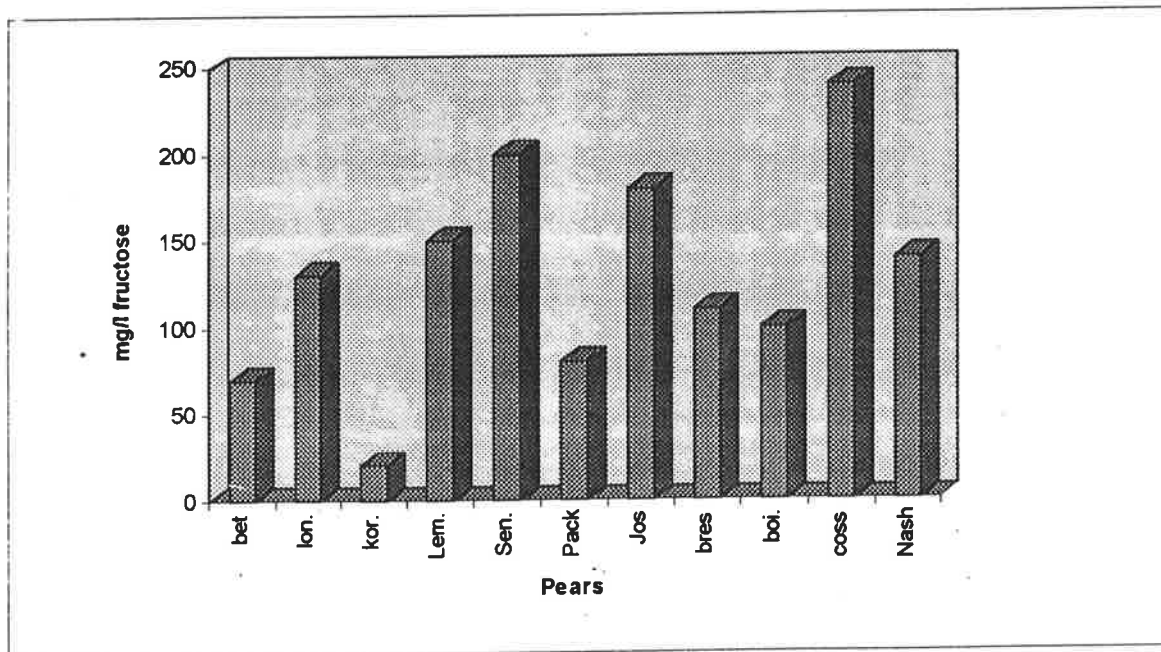
Fig. 6.2 Nectar fructose concentration for some pear species and *P.communis* cultivars. The explanation for abbreviation of the species and cultivars are as follow; bet=*P.betuleafolia*,lon=*P.longipes*, kor=*P.koroshinsky*, Lem=Lemon, Sen=Sensation, Pack=Packham Triumph, bres=*P.breschendri*, bois=*P.boissierana*, coss = *P.cossoni*, Nashi.

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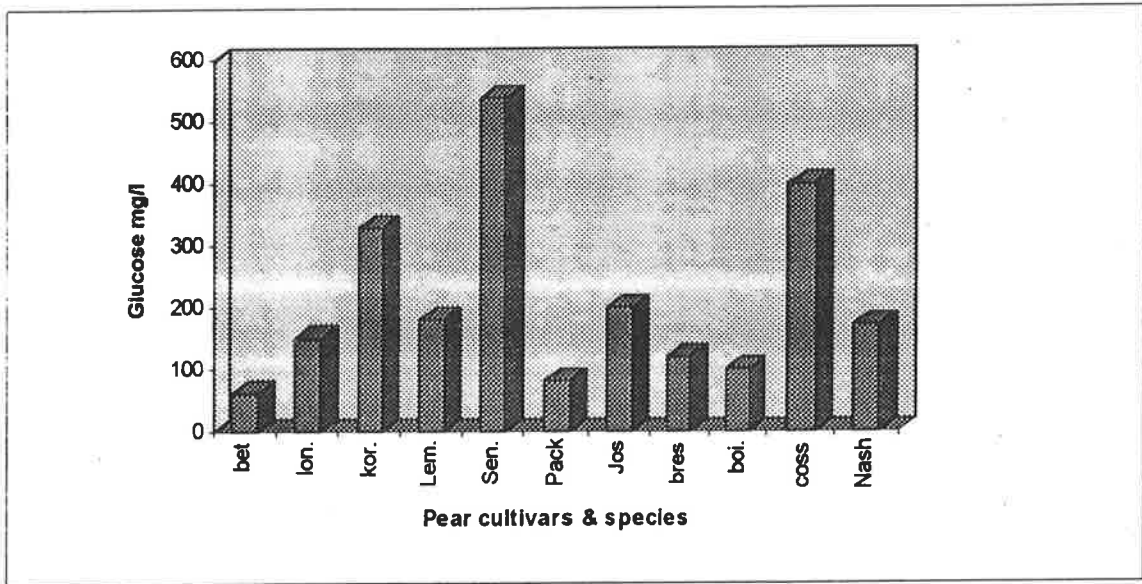
Fig. 6.3 Nectar glucose concentrations for some *pyrus* species and cultivars.
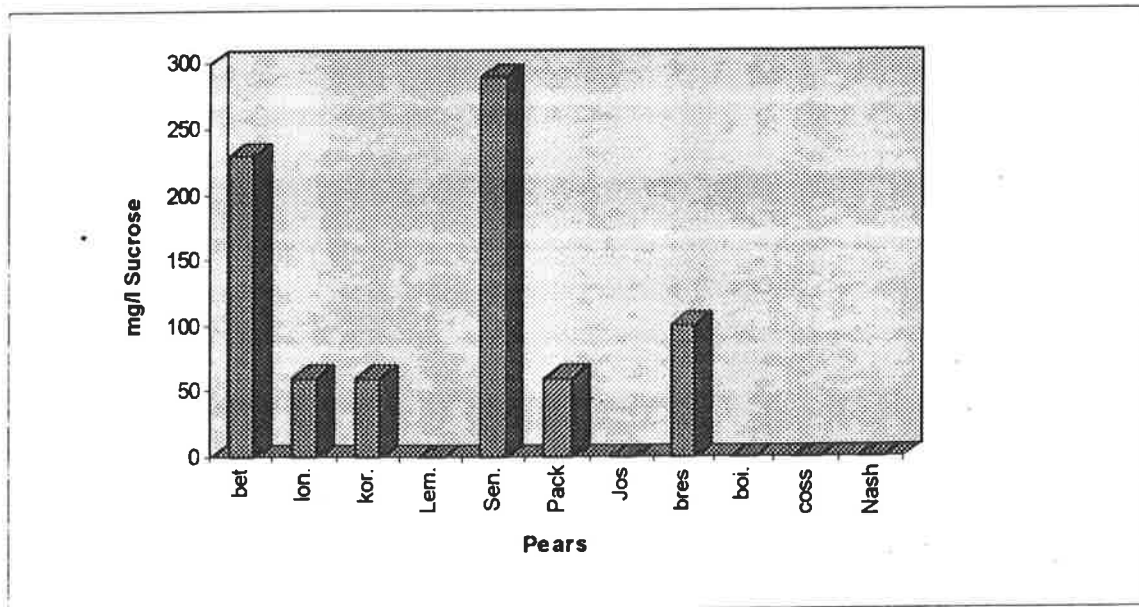
Fig. 6.4. Nectar sucrose concentration for some *pyrus* species and cultivars.

Some *P.communis* cultivars and other species showed no or very low sucrose amounts, these included Lemon Bergomot, Josephine, *P.boissierana*, *P.cossoni* and Nashi. Sucrose in nectar for some species and cultivars including; *P. communis* Sensation, and *P.betulaefolia* and *P.brescheneideri,* was the highest amount, 290, 230, 100 mg/l respectively. But *P.communis* Packham Triumph, *P.koroshinsky* and *P.longipes* had the lower amount of 60 mg/l (Fig. 6.4).

6.3.3 Anthocyanin in Anthers

The results of anther anthocyanin measurement showed pears could be divided into three groups based on anthocyanin concentration. *P.betulaefolia* easily had the largest amount.
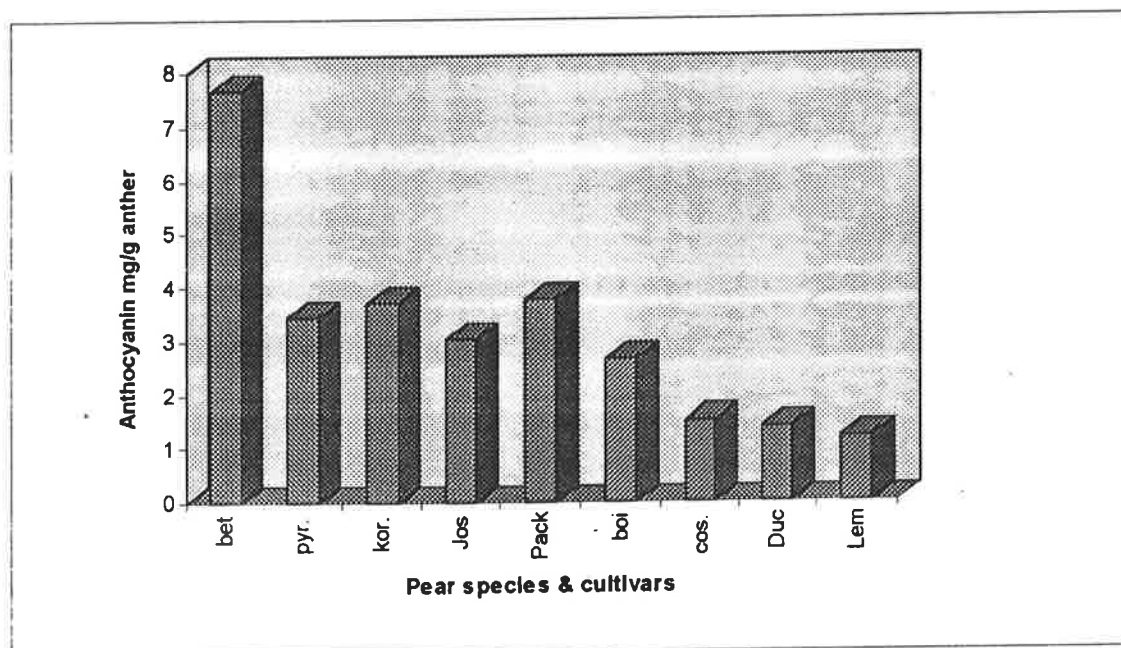
Fig. 6.5 Variations in the amount of anthocyanin mg/g anther for pear species and cultivars.

Packham Triumph, Josephine, *P.pyraster* and *P.koroshinsky* formed a second group with moderate amounts, Lemon Bergomot and Duchess as *P.communis* cultivars and *P.cossoni* had lowest concentrations of anther anthocyanin.

6.3.4 Pollen Production

Measurement of pollen production per 50 flowers indicated that *P.communis* cultivars and other pear species varied consideably in pollen production (Fig. 6.6). The obtained data (Table 6.6) was applied to a regression statistical program to find out probable correlation of pollen weight to anther dry weight and its moisture for 50 flowers. There was not found to be any significant relationship.

Packham Triumph produced the highest amount of pollen for all pears studied. Packham Triumph produced 74 mg/50 flowers while Josephine 60 /50 flowers and Howell 54 mg/50 flowers and Lemon Bergomot 48 mg/50 flowers. The lowest amount of pollen production produced was found in Nashi (Twentieth century) with 25 mg/50 flowers. Generally the wild species of pears produced lower amount of pollen for 50 flowers than the *P.communis* cultivars., but *P.amygdaliformi and P. longipes* gave as much as 56 and 40 mg/50 flowers, respectively.
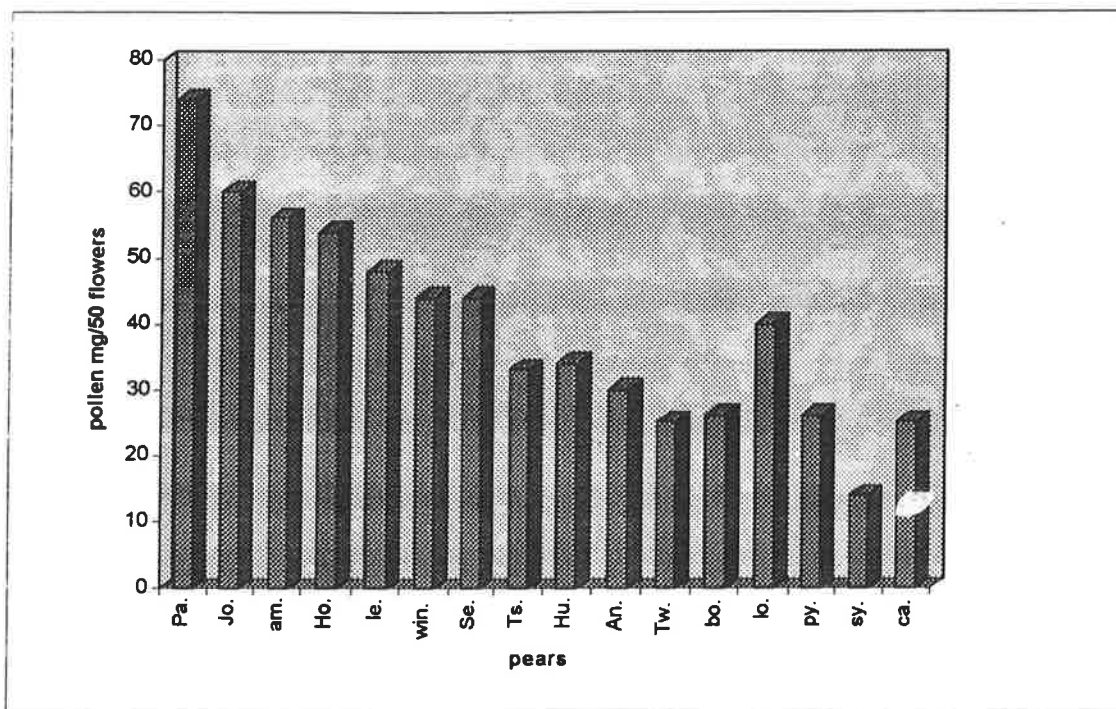
Fig. 6.6. Variations in pollen production for pear species and *P.communis* cultivars.

Table 6.2. Pollen production for pear species and *P.communis* cultivars. In addition to the amount of pollen(mg) produced from 50 flowers, fresh and dry weight of anthers and moisture content of 50 flowers are also shown.

Pears	Dry anthers (g)	Fresh anthers (g)	Moisture (g)	Pollen (mg)
Packham Triumph	0.260	1.288	1.028	74
Josephine	0.296	1.156	0.860	60
P.amygdaliformis	0.223	0.819	0.595	56
Howell	0.302	0.680	0.378	54
Lemon Bergomot.	0.185	0.957	0.772	48
Winter Nelis	0.350	1.0	0.630	44
Sensation	0.334	0.917	0.583	44
Tsu li	0.266	0.823	0.557	33
Hu- Hong	0.344	1.0	0.657	34
Anjou	0.175	0.718	0.416	30
Nashi (Tw.C.)	0.186	0.718	0.532	25
P.boissierana	0.382	0.996	0.595	26
P.longipes	0.226	0.641	0.226	40
P.pyraster	0.250	0.607	0.357	26
P.syriaca	0.266	0.781	0.621	14
P.calleryana	0.115	0.494	0.379	25

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6.4 Discussion

The concentration of amino acids in the flower nectar is a variable element which depends on flower age , temperature, humidity, and flower damage (Gottsberger *et al.*, 1990; Corbet, 1978; Jackobsen and Kristjansson, 1994). In the present work all of these variables were kept as close to one another as possible for all pear species. The concentration of asparagine was high in all flowers nectars investigated, which was in accordance with the results of Gottsberger *et al.*, (1990). They also found a high level of the same amino acid in the nectar of *Hibscus rosa-sinensis*. The importance of amino acids for feeding and attraction of pollinators has already been reported (Alm *et al.*, 1990). The preference of amino acid for pollinators is a question which has recently been raised. Potter and Bertin (1988) found a preference of *Sarcophaga bullata* for histidine in an artificial nectar but these flies refused it in combination with lysine. Glycine was preferred by those flies much more than histidine (Potter and Bertin, 1988).

Anthocyanin seems to accumulate in the epidermal cells of anthers. The physiological role of anthocyanin in pear flower anthers is not known. However the possible importance of anthocyanin as a colour signal for pollinators in the interior of the flower can not be discounted (Goodman and Fisher,1991). The marked differences between the amount of anthocyanin may suggest differences in attraction of pollinators to different pear flowers. Packham Triumph again scores well here. Further study is needed to investigate the effect of varying concentration of anther anthocyanin on pollinators attraction to pear flowers.

Sugar analysis of nectars showed that glucose in general occurs at higher concentration than fructose and sucrose. However *P.betulaefolia* showed sucrose as a dominant sugar in its nectar. This result was in accordance with Meheriuk and Lane (1987) for pear sugar analysis of nectars.

The results of a pollen production survey indicated differences in pollen production between the various pear cultivars and species. Church and William (1983a) examined pollen production for different desert apple and ornamental *Malus* cultivars, and found differences in both pollen production and flower number. Pollen production for 50

flowers was a mean of pollen production calculation for each cultivar or species. Apparently, pollen production and flower number is affected by, rootstock, pruning and tree age and microclimate (Church and William., 1983a). Recently Dumanoglu and Celik (1994) found that pear cultivars with equal number of anther produced different numbers of pollen grains per flower. Furthermore they also found that individual anthers within each pear cultivar produced significantly different numbers of pollen grains. This work showed that the genetic diversity of pears might cause such differences in pollen grain production(Dumanoglu and Celik, 1994). Certainly, Packham Triumph in the orchards under study produces an abundance of pollen. Further research is needed to investigate factors which define physiological and morphological factors affecting pollen production in different pear species.

Chapter 7

A Comparison of the Effects of Caging Packham Triumph and Lemon Bergomot Cultivars

7.1 Introduction

Packham Triumph pear cultivar *(Pyrus communis)* was developed in Australia in the early 1900s, from a cross between Bell x William Bon Chertien cultivars. This self-incompatible cultivar, produces more flowers than most other cultivars, including Nashi, Winter Nelis, Lemon Bergomot, and Josephine. As reported here, the amount of pollen per flower in Packham Triumph is also higher (see chapter 6). The problem of the occurrence of some misshapen fruits on Packham Triumph may be due to the lack of sufficient pollination in the orchard, a possibility to be explored in the present work. This caging experiment was conducted in part to evaluate the possibility that wind pollination could set some seed in Packham Triumph as suggested by Westwood *et al.* (1966). Westwood and Grim (1962) reported that pears which were located around the border of an orchard bore more fruits than trees inside the block. Westwood *et al.* (1966) observed that when branches of flowers were bagged by screens or cheesecloth some seeds were produced in fruits, but when branches were covered tightly with muslin, no seeds were obtained. It has been mentioned that parthenocarpic fruits may be set in unfavourable conditions (Lin *et al.*, 1983), e.g, where there is not enough pollen or cross pollen when the receptor cultivar is a self-incompatible. On the other hand, it has been claimed that when Packham Triumph has not sufficient pollinizers present it sets less fruit (Wauchope, 1968). Furthermore Selimi (1971) reported that fruits obtained on bagged limbs of unpollinated Packham Triumph trees were completely seedless and mostly misshapen. In order to test the ideas of Westwood *et al.*, (1966) and Selimi (1971), under the condition of South Australia, two Packham Triumph trees were each caged with a mesh which was impenetrable to honey bees, but which would admit any possible wind borne pollen. Initial Fruit set(%) seed set (%), number of misshapen fruits and fruit weight (%) were measured on these two trees and compared with two uncaged Packham Triumph trees

nearby. Two Lemon Bergomot trees were caged also for comparisons with the uncaged tree.

7.2 Materials and Methods

7.2.1 Caged Packham Triumph

This experiment was conducted in the Coromandel Valley region of South Australia. Four trees of Packham Triumph numbered 3A1, 2B19, 2B21 and 8B15, approximately equal in size and age were selected next to rows of pollinizers in two sites of the orchard. Trees 3A1 and 2B19 were selected as cage treatment and ie two others as open pollinated controls. The cage was of nylon mesh, beeproof and transmitted 80% light, it was put over the trees before blossoming (Fig.7.1). One hundred flowers were labelled on each of four sides of the trees (West, East, North, South) to measure initial fruit set and retained fruit set percentage. Ten random fruits were selected from each side of all the treatments to compare fruit weight between cage and none-cage treatments. Seed set efficiency was taken as the number of seeds per ten fruits. For 100 % seed set it is expected there would be 100 seeds per 10 fruits for Packham Triumph fruits. Initial fruit set was measured one month after flowering and retained fruit set two weeks before the crop was harvested. Misshapen was defined as uneven growth of fruit. Although it was difficult finding a botanical definition for misshapen in this experiment a fruit was scored as misshapen when it showed uneven shape in one side only. Data obtained by cage and non-cage treatments were analysed in a randomised complete block design using a Super ANOVA software program. Means of variables for the cage and non cage treatments were separated according to Tukey,s test.

7.2.2 Caged Lemon Bergomot

Two Lemon Bergomot trees of similar size numbered J10 and K18, were selected for cage treatments. The cages were installed on the trees before flowering time (Fig. 7.2). Trees J11 and K17 were selected as non-cage control treatments. These trees were located adjacent the Packham Triumph rows. Means of initial fruit set, fruit weight and seed set were for cage and non-cage treatment in a RCBD design according to Tukey's test were compared.

Fig. 7.1. Installation of cage on Packham Triumph before flower anthesis.

Fig.7.2. A cage had been installed on Lemon Bergomot before flower opening.

7.3 Results

7.3.1 Packham Triumph,

7.3.1.1 Fruit Weight

Significant differences were observed between cage and non-cage treatments for this variable. The average weight of fruits under the cage treatment were significantly higher than non cage treatments (Table 7.1) and (Fig. 7.3).

7. 1 Effects of cage and non-cage treatments on fruit weight (g) seed set, fruit set, fruit retained (%) and misshapen fruits (%) of Packham Triumph trees.

Treatments	Fruit Wt	Seed Set	Fruit Set	Fruit Ret.	Missh.fruits
Cage	190 a	0 a	15.95 a	9.06 a	7.1 a
Non-Cage	135 b	38.37 b	15.94 a	8.62 a	3.6 b

Different subscript letters within column groups represent a significant difference, according to Tukey's test.
Significant at $p < 0.01$. For misshapen fruits significant at $p < 0.05$
Fruit Wt = Fruit weight, Fruit Ret= Fruit retained
The percentage of misshapen fruit calculated based on 160 fruits (100%) per tree.

7.3.1.2 Seed Number

No seeds were present in fruits obtained from the cage treatments. Only traces of seeds much smaller than fully developed seeds and which had no endosperm and embryo were found in these fruits (Fig. 7.4). Seeds were obtained in non-cage treatments. The cage prevented cross entomophilous pollination of Packham Triumph flowers.

7.3.1.3 Initial Fruit Set and Retained Fruit Set

There was no significant difference for initial fruit set and retained fruit set percentage between cage and non-cage treatments (Table 7.1) and (Fig.7.5).
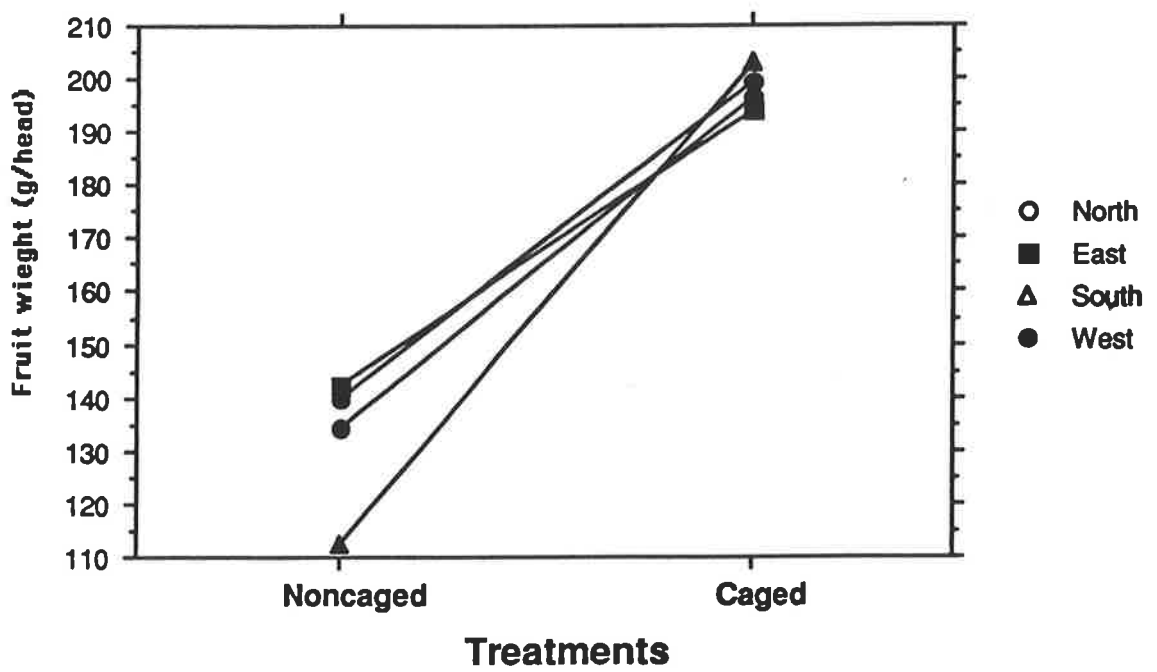
Fig. 7.3 Comparison of average fruit weight for cage and non-cage treatment obtained from different parts of the Packham Triumph trees.

Fig. 7.4. Traces of seeds resulting from Parthenocarpic Packham Triumph fruits. The fruits were obtained from tree 2B19 under the cage.
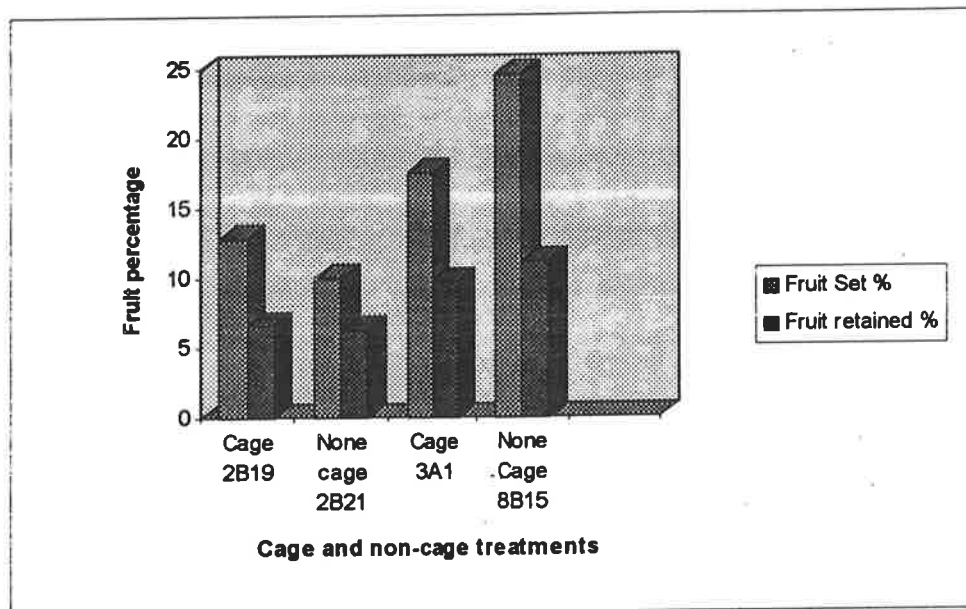
Fig. 7. 5 Comparison of initial fruit set and retained fruit set percentage(%) for the two treatments of cage and non-cage on Packham Triumph.

7.3.1.4 Misshapen Fruit

Some misshapen pears were obtained in both cage and non cage treatments. The mean number of misshapen pears in the cage treatment was more than observed in non-cage treatments however (Table 7.1, Fig. 7.6).

7.3.2 Lemon Bergomot

7.3.2.1 Fruit Set

Results of initial fruit set (%) did not show significant differences between the two caged trees and one non-cage control treatments (Table 7.2).

7.3.2.2 Fruit Weight

There was no observed significant difference between cages and non-cage treatment for average fruit weight.

Table 7. 2 A comparison of caging and non-caging treatments for fruit set (%), fruit weight (g) and seed set efficiency (%) of Lemon Bergomot

Treatments	Fruit Set	Fruit	Seed Set
Cage	33 a	174 a	0 a
Non-Cage	34 a	169 a	52 b

Different subscript letters within column groups represent a significant difference, according to Tukey's tests. Significant at P< 0.05.
Fruit Wt = Fruit weight

7.3.2.3 Seed Set Percentage

No seed were observed in fruit set on Lemon Bergomot caged trees. For non-caged control, Lemon Bergomot fruit showed an average of 5.2 seeds per fruit.

7.3.2.4 Misshapen Fruits

Under the cage treatments no unevenly misshapen pears such as was found for Packham Triumph fruits were observed. However a small percentage of fruit with a changed fruit evenly shape format was observed. These fruits were more elongated in comparison with normal shaped fruits (Fig 7.7).

7.4 Discussion

The absence of seeds in fruits of both Packham and Lemon Bergomot showed that wind did not carry pollen for seed set. Westwood (1968) suggested that pear pollen could be carried by wind, however Free (1993) stated that pear pollen is characteristically moist or sticky, suggesting that it is not anemophilous and distributed by wind but rather is

entomophilous and carried by insects. Also it was mentioned by the former author that pear varieties in this aspect may not be uniform. The weather condition such as relative humidity and moisture content of pollen can affect pollen flow by wind.
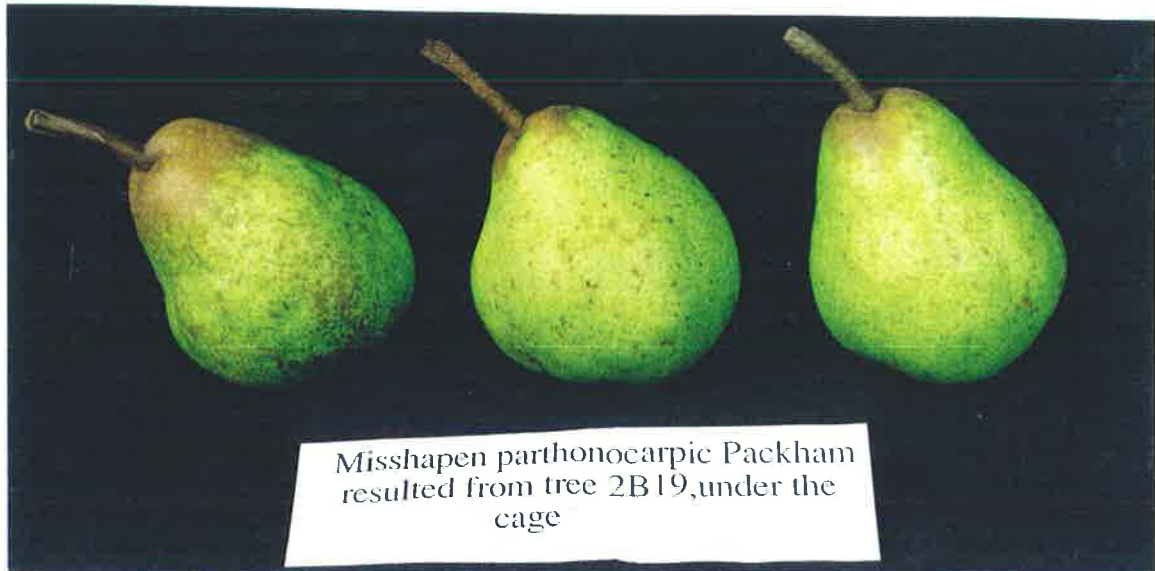
Fig. 7.6. Misshapen Packham Triumph fruits obtained from the cage treatment.

Fig. 7.7. Lemon Bergomot fruits which changed shape as a result of lack of pollination by cage installation on the tree before anthesis.

Furthermore, overcrowding of the stigma by self-incompatible pollen grains might reduce seed set through inhibition of germination or tube growth by probable air mediated cross compatible pollen grains. This condition which has been defined as 'clogging' of stigmas may account for no seed formation within the fruits under cage treatment. Even if airborne pear pollen was present but, due to the clogging of stigma it might have little importance in pollination of pear. This is in agreement with Langridge and Jenkins (1972).

Results of this study showed that fruit set (%) in Packham Triumph and Lemon Bergomot was not affected by cage treatment, which is in coincide with the result of Langridge and Jenkins (1972). They reported nearly the same rate of fruit set (%) as was reported here for Packham Triumph. This result was not in agreement with Nyeki et al., (1994) because already they had stated a four percentage of fruit set (%) for Packham Triumph in the condition of Hungary. More probably the different geographical situations caused this difference. Based on Nyeki et al., (1994) pear varieties were assigned to 6 groups on the basis of measurement of natural parthenocarpy. Based on this category, Packham Triumph and Lemon Bergomot cultivars had high strength of producing fruits through vegetative parthenocarpy in Coromandel Valley. Further, during the recent experiments the stamens from a few bunches of Packham Triumph flowers were removed and the flower bunches were labelled and bagged. After few weeks vegetative parthenocarpic fruits were obtained which had no seeds inside.

In contrast to the results of some researchers (Marcucci and Visser, 1983) parthenocarpic Packham Triumph pears were not elongated and had no reduction in diameter in comparison with seeded fruits. Parthenocarpic Packham Triumph fruits in comparison with seeded fruits, significantly had higher weight which this was a new result. Also these over size parthenocarpic fruits of Packham Triumph were not marketable. This result was in contrast with those parthenocarpic fruits of cylinder shape which were smaller in size and harvested from other self-fruitful cultivars in other parts of the world (Mitra et al., 1991). It is clear that, under normal conditions in the Coromandel Valley orchard there is not enough "cross" pollen for full

fertilization of flowers on Packham Triumph, especially two trees or more away from a pollinizer tree. Under these conditions some parthenocarpy is expected and in addition the partly-seeded fruit will often be unevenly misshapened. Packham Triumph appears to be very sensitive to this lack of pollen leading to some misshapen fruits, as experiments with Lemon Bergomot show that while parthenocarpy in this cultivar did lead to a minor but even change of shape from a round-oblate form to something more ovate-pyriform, nevertheless the fruit could not be said to be unevenly misshapen.

Lemon Bergomot fruits with few seeds (say 1 or 2) or no seeds were not unevenly misshapen either. Thus Packham Triumph shape could be said to be very susceptible to changes in seed content in the fruit, a genetically controlled trait which can lead to misshapen fruit. This trait may not be so obvious in other pear cultivars. Parthenocarpic fruit on Packham Triumph was heavier than seeded fruit, but of similar average weight on Lemon Bergomot. Lemon Bergomot trees cultivars showed higher fruit set (%) than Packham Triumph trees in this work.

7.4.1 Conclusion

To conclude, it seems that the strength of parthenocarpy in these two cultivars is quite high. For both Packham Triumph and Lemon Bergomot lack of pollination did not dramatically change the numbers of fruit set, but did affect the seed set enormously. It was proved that wind had no effect on cross pollination. The shape of the fruits was more affected in Packham Triumph than Lemon Bergomot by changes in seed numbers.

The significant increase of average fruit weight in Packham Triumph observed in the cage treatment showed a distinct physiological differences between these two cultivars. Fruit set cannot be used as a criteria for pollination efficiency measurement in orchards with these cultivars because there was no significant difference in fruit set between cage and non-cage treatments. However it does appear that pollination is a major requirement for Packham Triumph cultivar to obtain normal fruits with normal fruit shape and fruit weight. Further research is needed to learn more about the physiological and genetic explanation of parthenocarpy in fruit crops.

Chapter 8

Effects of Seed Number on Pear Fruit Set and Shape

8.1 Introduction

During fruit development seeds are a rich source of hormones including gibberellin, cytokinin and auxin needed for fruit growth (Crane, 1969). These hormones may function to mobilize metabolites from sap towards the fruit. Thus the seeds have an important role for fruit set and development especially for non parthenocarpic fruits. A different hormonal pattern has been illustrated for parthenocarpic and nonparthenocarpic fruits (Talon and Primo-Millo, 1990; Takeno and Ise, 1992).

In pome fruits seed formation not only has a drastic effect on fruit set, but also can influence the shape of the fruit (Rohitha and Klinac, 1990). The economical value of the standard fruit shape makes the relevant research valuable. Packham Triumph is a productive pear cultivar grown extensively in South eastern Australia. The large number of unevenly misshapen Packham fruits obtained in commercial orchards has been associated with inadequate pollination and the occurrence of parthenocarpic fruits. This investigation was undertaken to determine the effect of seed number on fruit shape and fruit set in Packham Triumph and Lemon Bergomot cultivars. Lemon Bergomot was selected for this study because the shape of its fruit in commercial orchard is round-oblate and therefore it is very different from Packham. No unevenly misshapen fruits have been observed on Lemon Bergomot trees within the orchard. On the other hand, Packham Triumph pear fruit is oblong ovate-pyriform and usually many unevenly misshapen fruits have been observed. The effect of different number of pollinated styles on fruit weight of Packham Triumph pear was also examined in this work.

8.2 Materials and Methods

8.2.1 Pollination with Different Number of Stigmas

The flowers of pear cultivars usually have five styles and five stigmas and are mainly self incompatible. Each style leads to one locule with two ovules. A successful pollination of each stigma and fertilization in embryo sacs can produce two seeds per stigma. By removing all or some of the styles and stigmas before pollination, different numbers of seeds can be expected. Two experiments were designed for Packham Triumph. In each experiment, 5 treatments included the removal of 1, 2, 3, 4, or all 5 styles and stigmas with replication of 80 flowers per treatment selected. Experimental flower bunches had between 3-9 flowers. Bunches of flowers for experiments were selected from different sides of Packham Triumph and Lemon Bergomot trees. Josephine flowers were collected as a pollen source for hand pollination of self incompatible Packham Triumph cultivars. For hand pollination of self incompatible Lemon Bergomot cultivars, Packham Triumph pollen was used. Each treatment was conducted by removal of the appropriate number of styles and stigmas and a small soft pen brush used to place cross pollen on each stigmatic surface (Kester and Asey, 1975). Flowers before hand pollination were emasculated. To avoid foreign pollen contamination, polyethylene bags were placed over flowers after hand pollination. Flowers for hand pollination were at the stage of one day before opening. Means of variables obtained from different treatments were separated according to Dunacn and LSD,s test.

8.2.2 Pollen Germinability Test

In vitro pollen germination tests were carried out for pollen collected from Josephine and Packham Triumph cultivars before application. Germination medium contained agar (1%), sucrose (15%) and a mixture of salts (Brewbaker and Kwack, 1963). The results of this test showed more than 80% viability for both Josephine and Packham Triumph pollen.

8.2.3 Fruit Set and Shape Calculation

Fruit set number was calculated as percentage of total flowers setting fruit and remaining attached of those treated one month after hand pollination. Data for fruit set percentage was obtained from 8 bunches of flowers randomly selected as replications per treatment. These data were compared in a Randomized Complete Block Design (RCBD) using a SuperAnova software program. Misshapen fruits were separated from each treatment (see Fig. 8.5 and Fig. 8.6) and the percentage calculated, based on total number of fruits per individual treatment. Misshapen fruits were those that visually differ from round (Lemon Bergomot) or oblate (Packham Triumph) shape. A comparison of fruit weights was made for these treatments.

8.3 Results

8.3.1 Fruit Set and Number of Seeds per Fruit

Fruit set (%) was influenced by pollination of different number of stigmas. This difference in pollination of number of stigmas, produced different number of seeds for each treatments as shown in Table 8.1.

Table 8.1. Ranges in seed number which were obtained by pollination of different number of stigmas in Packham Triumph and Lemon Bergomot.

Stigma Number Pollinated					
	1	2	3	4	5
Seed Number	(0-2)	(2-4)	(3-6)	(4-7)	(6-9)

Fruit set percentage for Packham Triumph increased with the number of stigmas pollinated. As shown in Fig 8.1 pollination of 5 and 4 stigmas produced higher fruit set(%) in comparison to one stigma treatment. A significant difference was found between pollination of 4 and 5 stigmas with 1 stigma. In this respect a significant P value equal to .01 was obtained.

The results obtained for Lemon Bergomot showed the same pattern except that pollination of a single stigma showed an exceptionally high significant fruit set. In this experiment pollination of 4 stigmas indicated significantly increasing   of fruit set

in comparison with the pollination of 2 and 3 stigmas. A P value equal to .05 was obtained for the recent difference (Fig. 8.2. and Table 8.2).

Table 8.2 Average (mean ± standard error) fruit set (%) after pollination of different number of stigmas for Packham Triumph and Lemon Bergomot cultivars.

Cultivar	1	2	3	4	5
Packham	42.7± 7.8 a	57.6± 8.1ab	55± 4.3 ab	67.3± 5.8 b	65.68±4.7b
Lemon Bergomot	69.37± 10 b	35.2± 3.8 a	42± 5.1 a	54.4 ± 3.8ab	51.4 ± 6.4 ab

Different subscript letters within column groups represent a significant difference, according to Duncan,s test. Significant at p≤ 0.05.

8.3.2 Fruit Shape

For Packham Triumph a significantly higher percentage of unevenly misshapen fruits was obtained where a low number of stigmas were pollinated. Misshapen fruits (%) showed values of 62.5 % for 1 stigma pollinated 54.5 % for two stigmas, 12.7 % for three stigmas and 0 % for 4 and 5 stigmas pollinated (Fig. 8.3) The percentage of misshapen fruits obtained in relation to seed number for open pollination in the orchard for Packham Triumph was investigated for one tree only in order to compare with the results obtained for hand pollinated flowers with one through to 5 stigmas pollinated see Table 8.4.

Table 8.3 Misshapen fruits and seed set values (%) for a Packham Triumph tree located in an area of orchard known to have poor pollination. Percentages for misshapen fruits was based on a total 80 fruits collected including good and misshapen fruits. There was no evidence for fruit with 10 seeds).

Seed Number	0	1	2	3	4	5	6	7	8	9
Misshapen fruits (%)	14	8	9	6	3	0	0	0	0	0
Total fruits (%)	41	14	15	11	6	5	4	2	2	0

For Lemon Bergomot, in contrast to Packham Triumph, no unevenly misshapen fruits were observed with any of treatments (one through to 5 stigmas pollinated). Nor were there any misshapen Lemon Bergomot fruits on the open pollinated trees.

8.3.3 Fruit Weight

Data was obtained by weighing 8 randomly selected fruits for each treatments for Packham Triumph. Data was compared as explained before. The result showed a significant difference for fruit weights between 1 and 5 stigmas pollinated with a P value equal to 0.01. Fruits produced by pollination of 5 stigmas showed a considerably higher weight compared to one, 2 and 3 stigmas pollinated treatments particularly when compared to 1 stigma pollinated. ·· One stigma pollinated treatment produced significantly fruits with lower weight The effect of number of stigmas pollinated on fruit weight is shown in Fig. 8.4.
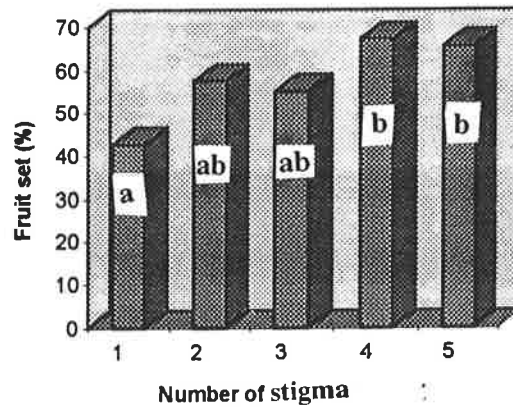
Fig 8.1 The effects of number of stigma: pollinated on fruit set (%) for Packham Triumph.
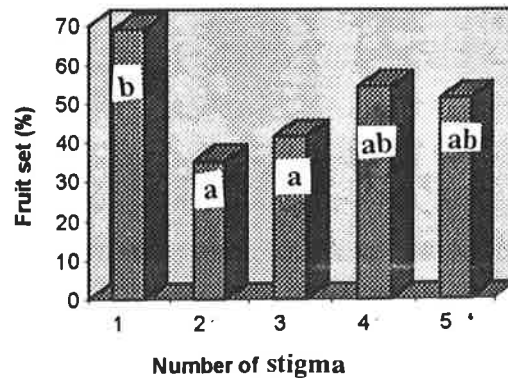
Fig. 8.2 The effect of number of stigma pollinated on fruit set (%) for Lemon Bergomot.
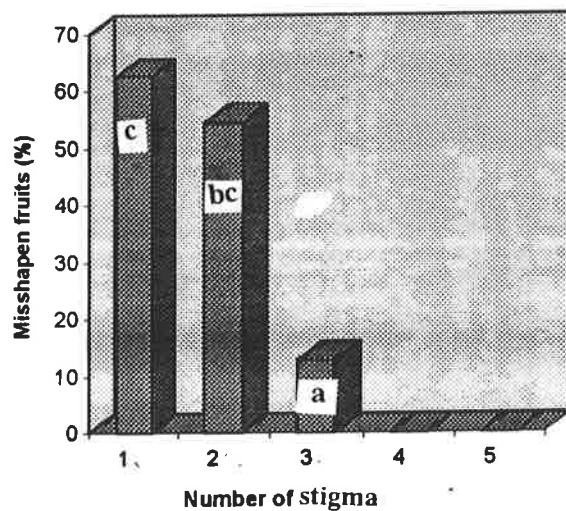
Fig. 8.3. Effect of number of stigma pollinated on the percentage of misshapen fruits obtained for Packham Triumph.
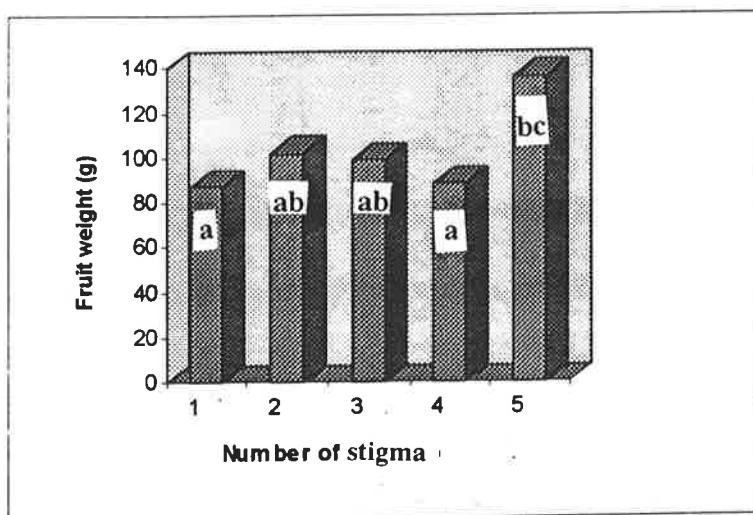
Fig. 8.4. Effect of number of stigma pollinated on fruit weight for Packham Triumph.

8.4 Discussion

The influence of seed number on fruit set, shape and weight has already been reported for certain apple and pear cultivars not investigated here (Garcia-Papi and Garcia-Martinez ,1984; Bukovac and Nakagawa, 1968; Rohitha and Klinac, 1990). The results of the present work were in general agreement with the work of Rohitha and Klinac (1990). In this study it was understood that seed number has a marked effect on fruit set and weight of the two cultivars. The production of auxin during fertilization and seed development may well contribute to these effects.

Packham Triumph fruit with greater number of seeds had more symmetric shape than those fruits with fewer seeds. Probably this effect is related to seed number influences on hormonal balance within the fruit cells. The effect of seed number on fruit shape and weight in this study was in accordance with the results of Proctor and Scheter (1992). As mentioned above the seed number had no marked effect on the fruit shape of Lemon Bergomot fruit, which might be due to a lower fruit cell sensitivity of Lemon Bergomot to seed number.

Other factors are involved in fruit quality, but improving pollination to obtain more seeds may be the easiest way to improve quality and quantity of fruits.

It was shown that pollination of one stigma, still produced fruit even if no seed was present. If the role of seed is a major element for fruit set, what are the other factors which control fruit set other than seeds? Already it has been reported (Lin *et al.*, 1982; 1984) that the occurrence of parthenocarpy happens in unfavourable conditions, including where there is no "cross" pollen for self incompatible cultivars, unviable pollen or adverse weather conditions. So it is probable that there is a switching signal to begin expression of a parthenocarpy gene, due to non pollination of a higher number of stigmas in treatments of one stigma pollination. It is possible that this expression can produce an hormonal balance that leads to fruit growth and not to fruit abscission. Hormonal balance is different in ovaries of parthenocarpic fruits at the time of anthesis in comparison with seeded fruits. In parthenocarpic fruits it has been reported there is a greater concentration of auxin at the time of anthesis (Iwahori *et al.*, 1968; Griggs *et al.*,1970; Wang *et al.*, 1993).

Meanwhile it has also been reported that there is a lower concentration of cytokinin in the ovary of parthenocarpic tomato (Mapelli, 1981). Talon and Primo-Millo (1990)

reported a considerable difference in concentrations certain gibberellins, GA53, GA44, GA19, GA20, GA1, GA4 and GA9 in ovaries of Satsuma parthenocarpic mandarin at 7 days before and after anthesis. However, the concentration of these hormones in pome fruits with different numbers of seeds and any relationship to shape and weight is a matter for further research. Lemon Bergomot was observed to always produce a good set of fruits, where only one style was pollinated which could be a parthenocarpic gene activity replacing seed processing. The number of misshapen fruits obtained in relation to seed number in the experiments for Packham Triumph reported here was in accordance with distribution of misshapen fruits observed in the orchard where there was open pollination. It can be concluded that the effect of seed number on the shape of pear fruit is different for the two pear cultivars investigated. Lemon Bergomot with a round shape showed no uneven shape sensitivity to seed number in contrast to Packham Triumph.

It is recommended therefore that Packham Triumph be sufficiently pollinated to give at least 5 or 6 seeds in order to attain a high proportion of nicely shaped fruit in commercial orchards.
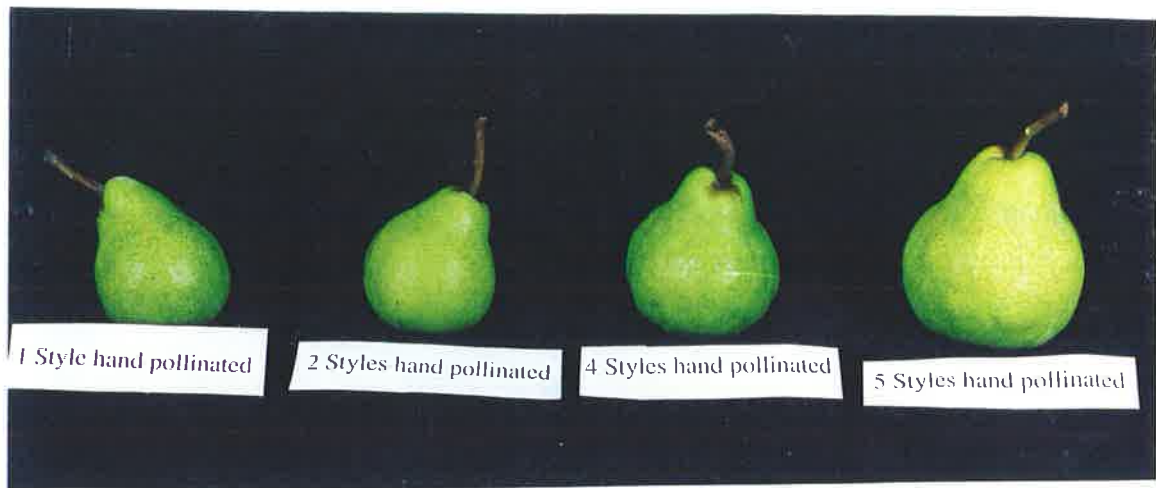
Fig. 8.5 Packham Triumph pears obtained for different numbers of styles and stigmas pollinated.

Fig 8.6 Lemon Bergomot pears obtained for different numbers of styles and stigmas pollinated.

Chapter 9

Pollination Efficiency in a Block of Packham Triumph Trees

9.1 Introduction

Pollination is a dynamic process involving environmental, genetic and physiological factors (Kevan and Barker, 1983; DeGrandi-Hoffman et al., 1986; DeGrandi-Hoffman, 1987: McCall and Primack, 1992)

The efficiency of pollination is a complex matter which goes back to the effectiveness of each factor involved (DeGrandi-Hoffman et al. 1989). Suitable weather conditions is a factor influencing anther dehiscence, pollen dispersion, insect pollinator activity and the physiological processes of fertilization (Corbet, 1990). Just as wind is important for anemophilous plants, insect pollinators play a central role for pollination of entomophilous plants. Foraging activity of the honeybee not only depends on suitable weather conditions (Langridge and Goodman, 1981) but also on internal and external hive conditions (Eckert et al., 1994). As the honeybee usually looks for the best reward during foraging (Weaver, 1979), the presence of better sources of pollen and nectar in a neighbouring orchard for instance means that the honeybees will forage there preferably (Larrey et al., 1979). Free (1993) suggests that, depending on food resources and weather conditions the number of bees decrease with increasing distance from the hive, so that the distribution of hives in the orchard is also an important factor. Orchard design is another major complex element which can affect the pattern of gene flow by pollen (Marquard, 1988; Wood and Marquard,1992).

Packham Triumph pear is considered to be an outcrossing cultivar due to its self-incompatibility characteristics. Poor pollination in pear leads to a reduction in seed set efficiency and thus a loss of fruit setting, especially for nonparthenocarpic pear fruits. Another major impact of poor cross pollination is a loss in seed number which can lead to misshapen and parthenocarpic pear fruits (Rohitha and Klinac, 1990)

In order to understand and evaluate pollination efficiency for Packham Triumph trees in an open orchard situation, fruits were examined in an area of the orchard where there was a large block of Packham Triumph trees. On one edge of this stand there was one row of Josephine cultivar and no other cultivar compatible with Packham

Triumph for at least ten rows. A group of beehives was placed to one side of the block of Packham Triumph trees . The object was to observe the effect of distance from pollinizers to bee hives on seed set, fruit set and fruit weight on the Packham Triumph trees. As Packham Triumph is completely self-incompatible, then the seed numbers in fruits reflect the degree of cross pollination. Counting the seeds within the fruits on trees in the different rows with increasing distance from pollinizers was selected therefore as a way of determining the pattern of gene flow by pollen within the stand of Packham Triumph trees.

9.2 Materials and Methods

9.2.1 Relative Flowering Times of Pollinizer Cultivars and Packham Triumph

The experiment was conducted in a commercial orchard in the Coromandel Valley region of South Australia in the Adelaide Hills. Josephine and Lemon Bergamot were the main potential pollinizers. There was a significant difference between the pollinizers and the main cultivar Packham Triumph in flowering times (Fig 9.1) there is only a partial overlap between them.
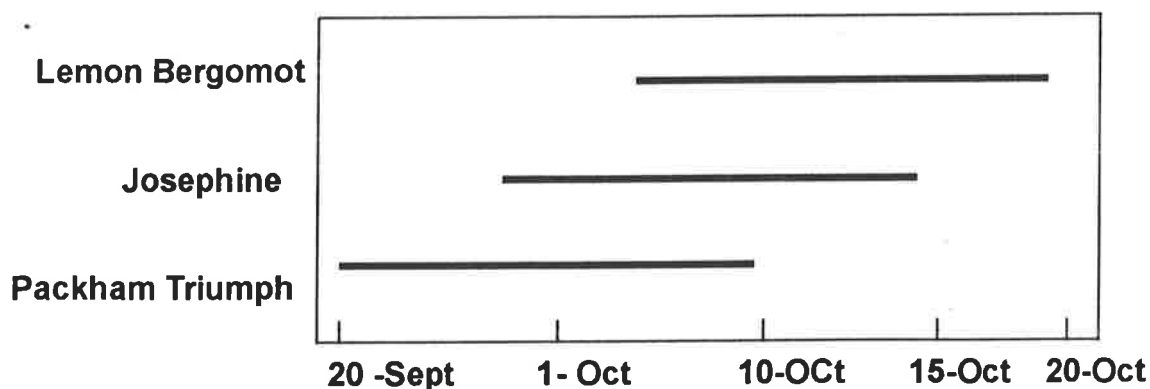
Fig. 9.1 Flowering dates of two pollinizers Lemon Bergamot and Josephine and main cultivar Packham Triumph.

As can be deduced from Fig. 9.1, when two thirds of Packham Triumph flowers are on full bloom, Lemon Bergamot starts its flowering flush. To make matters worse, there was no a balance for flower number between pollinizers and Packham Triumph. Josephine trees were shown to have range of flower numbers between 2,500- 4,000

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and Lemon Bergomot trees a number of 3,000-4,000 flowers, while Packham Triumph trees had a range of flower number between 15,000-20,000 flowers for each tree.

9.2.2 Collecting Data on Fruit and Seed Set

The presents experiment were conducted in a particular area of the pear orchard. This part contained one row of Josephine pollinizers trees on the outside of a solid stand of many rows of Packham Triumph (Fig. 9.2). Hives of honeybees were placed near the Josephine row during flowering time, to one side of the trees being investigated (see Fig. 9.2)
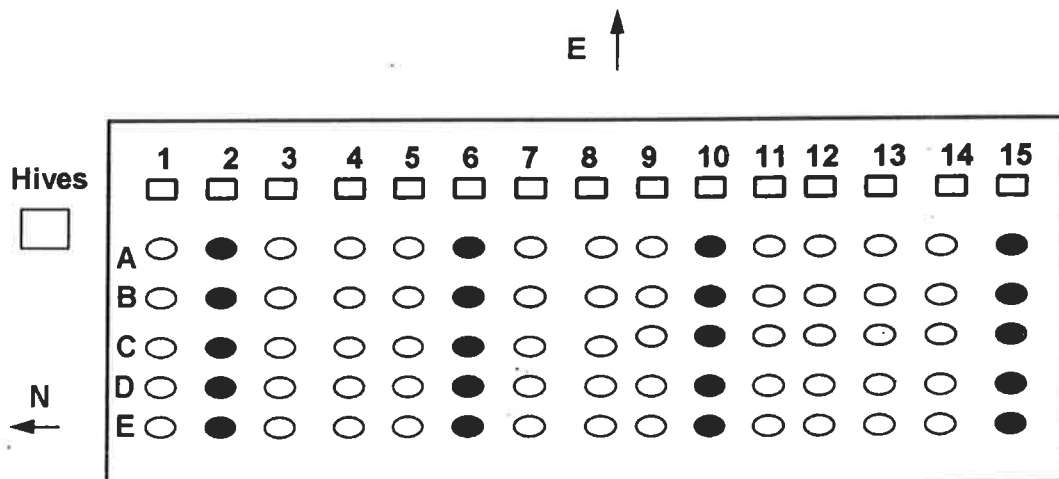
A stand of Packham Triumph trees in the commercial orchard located alongside a single row of Josephine pollinizers.

○ Packham Triumph trees, A, B, C, D, E are individual rows of Packham Triumph the numbers refer to tree number in rows.

● Fruit examined in these Packham Triumph trees.

□ Josephine trees.

Fig. 9.2 A schematic design of stand of Packham Triumph located next to a row of Josephine pears.

Five consecutive Packham Triumph trees at increasing distances from the single Josephine pollinizer row were selected for investigation (Fig. 9.2). For each distance

from the pollinizer, four replications were used. For example, Packham Triumph tree No2, row A was the first of the trees to be examined at 5.5 meters from pollinizers and Packham trees 6A, 10A , 15A were replications for the same distance from the pollinizer row (5.5 metres). Trees in rows A,B, C, D and E where located at a distances of 5.5, 11, 16.5, 22 and 27.5 metres from the Josephine pollinizers. A total of 20 trees were thus used for the experiment (Fig. 9.2).

120 flowers were counted and labelled at the time of anthesis on the eastern side of each of these trees for fruit set observation and investigation. The percentage of fruit set from the selected flowers was measured one month after anthesis. Ten fruits from the eastern side of every tree were picked at random and the number of seeds recorded for each fruit. As the greatest possible number of seeds set in each fruit is ten, assuming 100% fertilization, then the number of seeds per 10 fruits was taken as a seed set percentage efficiency for that tree. Fruit weight was also recorded. A regression in a Super Anova software program was employed to establish equation relation between distance from pollinizer row and fruit set, seed set efficiency and fruit weight.

9.2.3 Paternity Test- Isozyme Analysis of Seed Set

As a control, seeds of pears from two specific crosses carried out by hand of Packham Triumph X Josephine and Packham Triumph X Lemon Bergamot were subjected to isozyme analysis using the ADH, GPI and MDH systems. Seeds from fruits on the Packham Triumph trees being investigated were taken randomly from fruits and isozyme analysis performed on these to also find the pollen source leading to successful fertilization producing those seeds. Twelve seeds from each tree were used for isozyme analysis. Each seed was ground with 20 mg polyvinylpyrrolidone and 260 æl normal extraction buffer containing 0.05 M Tris HCl, 0.15% citric acid, 0.12% cystein HCl and 0.1% ascorbic acid, pH 8. Grinding was carried out in a mortar with pestle, the mixture was centrifuged at 3000 X g in an Eppendorf 5414s centrifuge and the supernatant used for gel electrophoresis. Cellulose acetate gel (cellogel) was used with two running buffers; 0.05M Tris -malate pH 7.8 for GPI and MDH and 0.02M sodium phosphate pH 7.0 for ADH isozyme systems. Staining was carried out as described by Richardson *et al.,* (1986).

9.3 Results

9.3.1 Effect of Distance from Pollinizer Trees and from Bee-Hives on Fruit Set, Seed Set and Fruit Weight on Packham Triumph Trees

Statistical analysis of data for initial fruit set, seed set and fruit weight showed clear relationships with distance from the pollen source. Thus, initial fruit set declined significantly in a linear manner with distance from the pollinizer (Fig. 9.3), with a correlation coefficient r = 0.58 (Table 9.1). Seed set was also affected by distance from pollinizer, increasing distance from pollinizers led to a linear reduction in seed set (Fig. 9.4 and Table 9.1). Average fruit weight actually increased with increasing distance from the pollinizer (Fig. 9.5). As the p value for the linear relationship was less than 0.05 and the correlation coefficient, R = 0.75, then the increase in weight with distance was quite significant.

Analysis of data obtained from the Packham Triumph trees located next to the row of pollinizers (row A, see Fig.9.2) showed no significant changes of initial fruit set, seed set and fruit weight with increasing distance from the bee hive (Table 9.2).

Table 9.1 The effect of distance from row of pollinizers on initial fruit set (%), seed set (%) and average fruit weight(g) of Packham Triumph fruit. Each value for initial fruit set (%) is an average of 4 replications each for 120 flowers, fruit weight and seed set (%) are average of 10 fruits per tree, for each row, four trees were investigated as shown in Fig. 9.2 .

Row	Initial Fruit set (%)	Seed set (%)	Average fruit weight (g)
A	20 ab	36.5 b	131.8 a
B	21 b	24.6 a	143.37 ab
C	19.5 ab	13.7 a	158 b
D	14 a	15.5 a	166 b
E	13 a	11 a	167.7 b

Different subscript letters within column groups represent a significant difference, according to LSD Fisher at P= .05
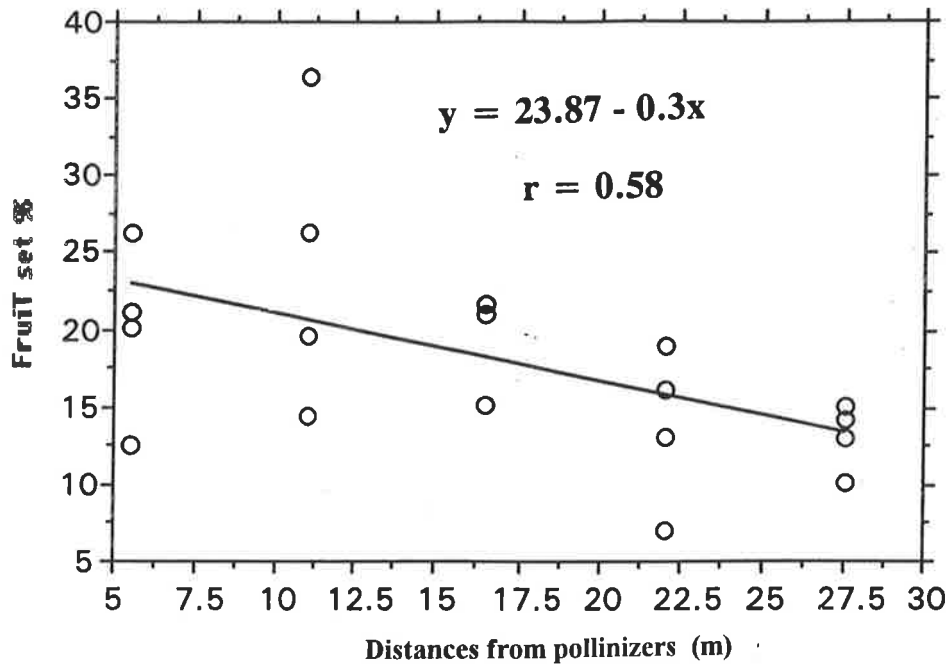
Fig.9.3. Effect of distance from pollinizers on initial fruit set (%) of Packham Triumph.
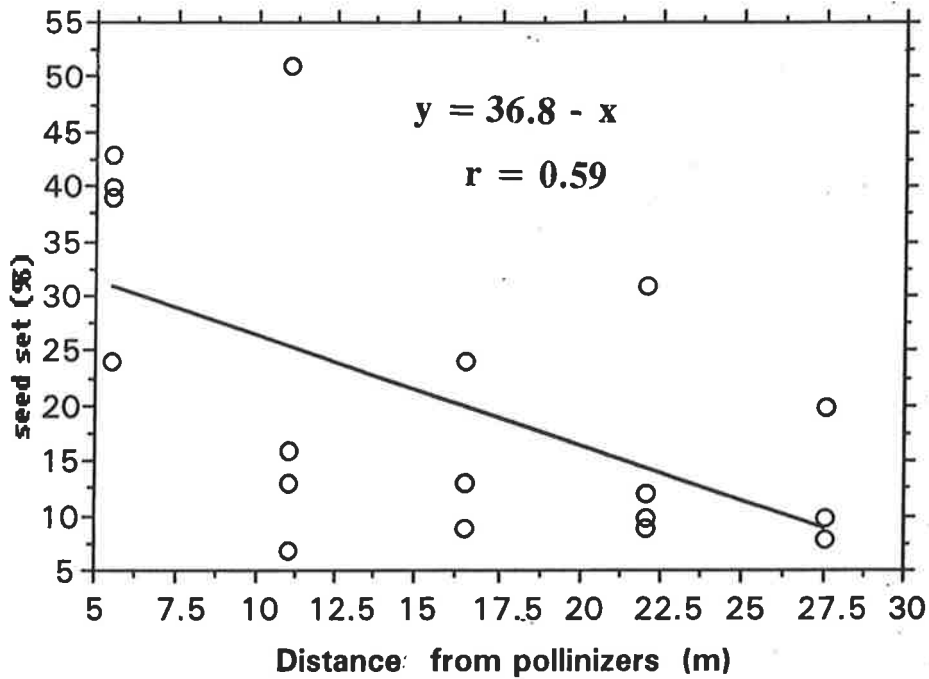
Fig. 9.4 Seed set efficiency (%) as a function of distance from pollinizer.
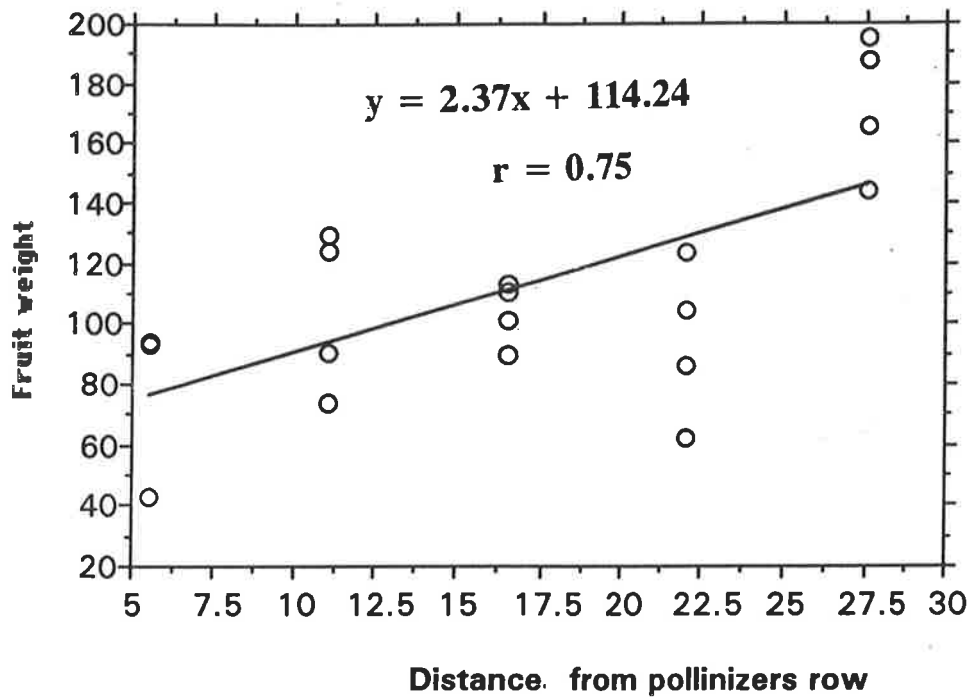
Fig. 9.5 The effect of distance from pollinizer on average fruit weight on Packham Triumph tees.

Table 9.2. The effect of distance from bee-hive on initial fruit set(%), seed set (%) and average fruit weight (g) for Packham Truimph.

Distance from hive(m)	Fruit set (%)	Seed set (%)	Average fruit weight(g)
11	16.8 a	43 bc	139 c
22	15.6 a	26 ab	104 a
33	26.2 a	24 a	127.6 bc
44	22.4 a	45 c	124 b
55	21.5 a	40 bc	131 bc
66	24 a	42 bc	121 b
77	27 a	35 ab	116.8 ab
88	24.5 a	32 ab	105 a
99	19.5 a	46 c	125 bc

Different subscript letters within column groups-represent a significant difference, according to LSD Fisher at P= .05, Each value of fruit set was a measurment from120 flowers, each value for seed set and average fruit weight(g) was obtained from 10 fruits from each of the experimental trees.

9.3.2 Isozyme Analysis to Determine Pollen Parents

Alcohol dehydrogenase (ADH) produced a different banding pattern for Josephine in comparison with Lemon Bergomot and Packham Triumph. These patterns showed an *ac* genotype band pattern for Josephine and *aa* genotype for Lemon Bergomot and Packham Triumph (see Chapter 4).

Results of controlled crosses for Packham Triumph X Josephine showed a Mendalian segregation with ratio of 1:1 for *ac* and *aa* (see also Chapter; 5). Segregation of progenies for crosses of Packham Triumph X Lemon Bergomot produced only *aa* bands (Fig. 9.6).
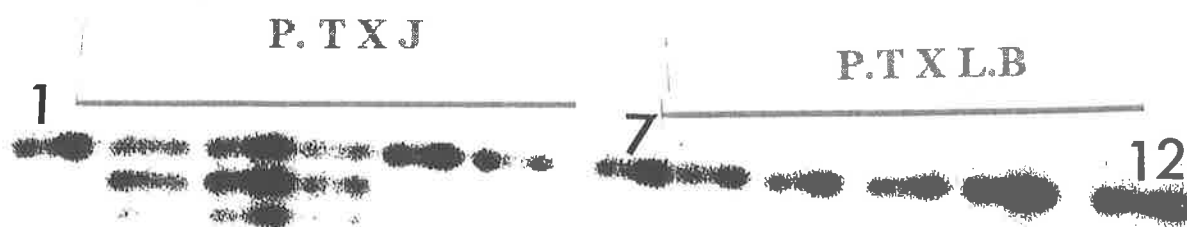
Fig. 9.6 Alcohol dehydrogenase banding pattern in extracts from seeds for two crosses of Packham (female) X Josephine (1-6) and Packham X Lemon Bergomot (7-12).

Results of isozyme analysis of randomly selected seeds from the stand of Packham Triumph trees showed approximately half were ac and half aa (Fig. 9.7), suggesting that Josephine was the major pollen source for the seed set presumably from the Josephine cultivar row to the east of the block of Packham Triumph trees (Fig. 9.2).

Fig. 9.7 ADH isozyme banding pattern for randomly selected seeds from row D located 22 meters from pollinizers. Patterns for seeds from other rows were similar (not shown).

9.4 Discussion

As expected, we found a decreased seed set with increasing distance from Josephine pollen donors. Reduction of flow of compatible pollen and consequently seed set efficiency and initial fruit set with increasing distance from pollinizer is in accordance with observations on other tree crops (Free, 1962; Marquard, 1988; Jackson and Clarke 1991a, b). The vegetative parthenocarpy characteristic of Packham Triumph compensates to some extent for the lack of pollen in setting fruit. Thus average fruit weight showed a positive correlation with increasing distance from the pollinizer. In work described before (Chapter 7) with two caged Packham Triumph trees heavie fruit was produced by parthenocarpy. Initial fruit set, average fruit weight and seed set showed no simple relationship to distance of trees from the hives of bees, in contrast to the effect of distance from pollinizer trees. It is likely then that the placement of hives is not as critical as placement of pollinizers. It is worth noting that even for the Packham Triumph trees next to Josephine pollinizer trees, seed set was less than 4 per fruit, considerably less than the possible 10 per fruit and less than the number of seeds needed to give good shaped fruit. Pollination is therefore inadequate in this orchard, a result no doubt of several factors. Efficient pollination depends on good combination of pollinizers with the main cultivar. Good pollinizers which have tight simultaneous flowering with the receptor variety and have reciprocal fertilization with the receptor can be selected to improve pear pollination. Also pollinizers should have high fertility and should no be too closely related genetically to the pollinated variety .(Neyki *et al*, 1994).

We noted that Josephine trees bear considerably less flowers than Packham Triumph (about one quarter, in fact), and flowering time of the two cultivars does not completely overlap. It is worth considering at this stage what could be done to improve in this type of the orchard for Packham Triumph pears. From the present results, pollinizer cultivars should virtually surround each Packham Triumph tree to give better than the 5 seeds per fruit needed for good shape. Two pollinizer cultivars should be selected to give better overlap of flowering with Packham Triumph.

One of these flowering earlier to cover the first 50 % flowering of the main cultivar and the other pollinizer to cover the second half of the flowering period. Such an orchard design is shown in Fig.9.8.
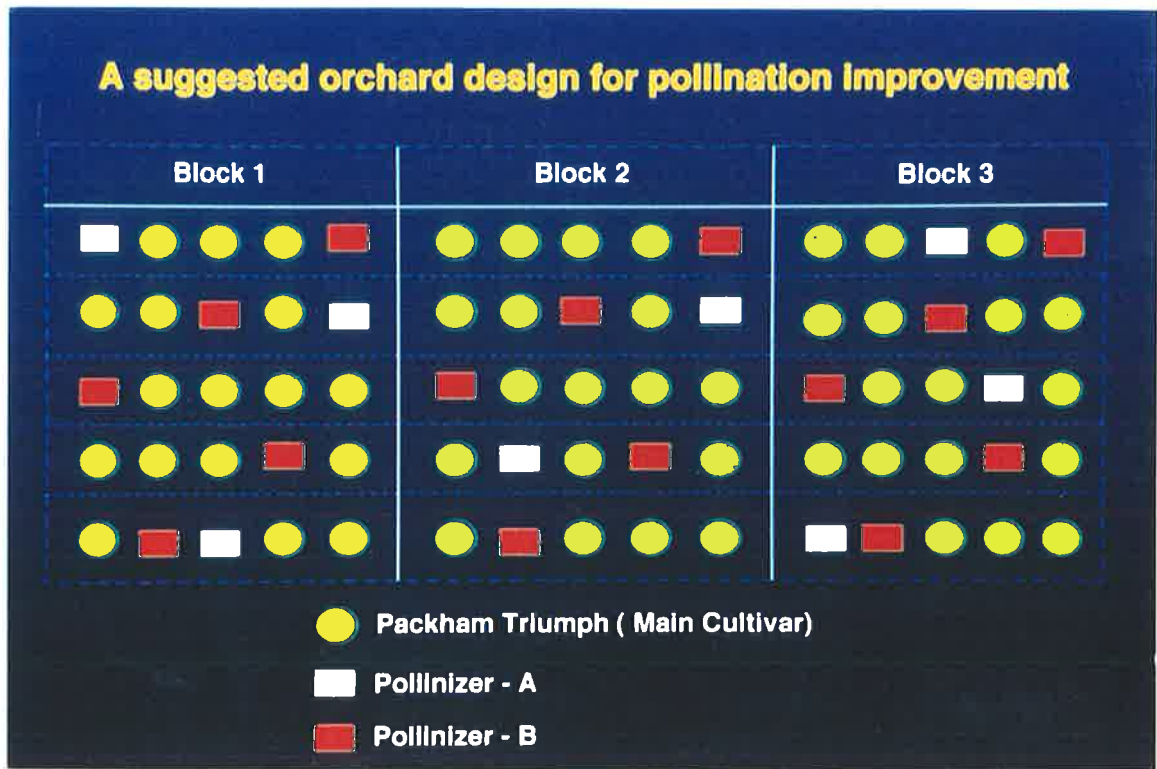
Fig. 9.8. A suggested design for improvement of pollination using two cultivars of pollinizers within the rows.

Chapter 10

Gene Flow by Pollen to Packham Triumph Pear Trees Interplanted with Other Cultivars

10.1 Introduction

Gene flow by pollen is a complex substantial matter of study that measures pollen dispersal and consequent successful fertilization events from point or block sources (Ellastrand, 1992). Estimation of gene flow basically is based on population genetic structure and paternity analysis of progeny in sink populations (Loveless and Hamrick, 1984; Handel, 1983; Hamrick, 1987).

The genetic nature of plant populations with respect to synchronous anthesis and self incompatibility or compatibility of plants within the population can affect gene flow by pollen (Hamrick, 1987). The spatial distribution and density of plants, number of pollinizers and orchard design and frequency of mating groups in the population are other major elements which affect gene flow in the orchard. (Levin and Kester, 1969). The quantity of cross compatible pollen which is available and its dispersion in the plant population is an important issue of consideration. The amount of cross compatible pollen may be restricted by an higher quantity of self incompatible pollen. Microclimate humidity and moisture of pollen have profound synergistic influences on the slow stream of pollen dispersal in the population by insect vector or by wind in anemophilous plants (Corbet, 1990; Gilbert and Punter, 1990; Yates and Sparks, 1993). It has been reported that microclimate and the pollen vector's behaviour play a major role on dispersal of pollens (Waddington, 1983; Thomson and Plowright, 1980). The foraging behaviour of pollinators is a function of resource quality including nectar, aroma, flower colour and plant spatial arrangement and density. The foraging behaviour of the pollen vector is therefore another factor effective on gene flow (Jackson and Clarke, 1991a). The following study was conducted using isozyme techniques to compare the relative effectiveness of two pollinizers in fertilizing the Packham Triumph cultivar in a commercial pear orchard. In addition, nectar analysis and pollen production was measured for both pollinizers and the Packham Triumph cultivar.

10.2 Materials and Methods

10.2.1 Frequency of Seed Number

The relative distribution of the various cultivars in the portion of the commercial orchard in Cormandel Valley used for this experiment is shown in Fig. 10.1.

Twenty fruits from each of Packham Triumph trees K10, L10 and M9 and M8 were picked at random and frequency of seed number within these were counted. The data was used to gain a knowledge of compatible pollen dispersal to each tree at the time of Packham Triumph pollination. Some seeds were also collected randomly from Packham Triumph fruits on trellis trees in another part of this orchard to test gene flow by pollen as a preliminary work. This latter work was designed to investigate gene flow on trellis systems. This trellis row consisted of Winter Nelis, Twentieth Century, Shinsui, Duchess as pollinizers and of course Packham Triumph. In three other trellis rows, Josephine, Twentieth Century, Winter Nelis and Kosui were also presented as pollinizers.

10.2.2 Isozyme Analysis of Seeds

The isozyme patterns resulting from controlled crosses of Packham (female) X Josephine and Lemon Bergomot (males) were at first determined. For the ADH isozyme system, segregation showed equal numbers ac and aa genotypes for the cross Packham Triumph X Josephine while only homozygous aa genotype resulted from Packham Triumph X Lemon Bergomot crosses. Considering the above results, seeds from trees K10, L10, M9 and M8 were examined to determine what proportion of each pollinizer is in the pollen gene flow stream during the period of Packham Triumph pollination. For this purpose, seeds were tested from within fruits gathered on both the East and West sides of each tree. The method for isozyme extraction, electrophoresis and staining was based on Jackson (1992).

The number of ac bands multiplied by 2 was taken to reflect the number of successful fertilization events by Josephine pollen on Packham Triumph trees. The remaining aa bands numbers not assigned as 1:1 with ac were taken as being fertilized by Lemon Bergomot pollen.
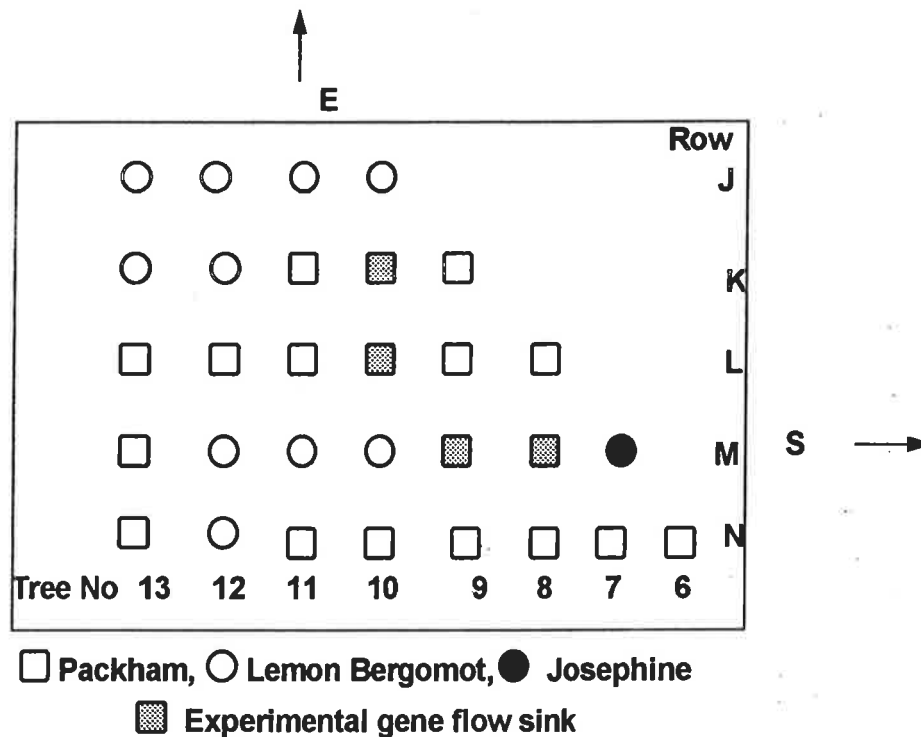
Fig. 10.1 Schematic design of a part of the orchard used to evaluate two pollinizers (Josephine and Lemon Bergomot) for pollination of Packham Triumph cultivars investigated as gene flow sinks. Trees K10, L10, M8 and M9 were the Packham Triumph trees investigate.

10.3 Results

10.3.1 Seed Number Distribution

Previous work indicated that 4-5 seeds per fruit, implied reasonable cross pollination while 0-1 seeds per fruit indicated grossly insufficient cross pollination. The maximum number possible is of course 10 seeds per fruit. For fruits obtained in this particular part of the orchard we found 0 to 4 seeds per fruit (Table 10.1, Fig. 10.2, Fig. 10 3). It would seem that there is inadequate cross pollination in this part of the orchard. It is possible that insufficient overlap of flowering times of the various cross pollinizers with Packham Triumph would be one of the reasons for the poor number of seeds per fruit, among many possibilities.

Table 10.1 Seed number distributions for Packham Triumph fruits.

Tree No	Fruits (%) with different seed number										
	Seed Number										
	0	1	2	3	4	5	6	7	8	9	10
K10	20	30	30	10	10	0	0	0	0	0	0
L10	20	25	15	10	20	10	0	0	0	0	0
M8	20	50	5	5	10	10	0	0	0	0	0
M9	30	5	20	20	20	5	0	0	0	0	0
Average	25	28.3	16.5	11.2	15	7.5	1.2	0	0	0	0
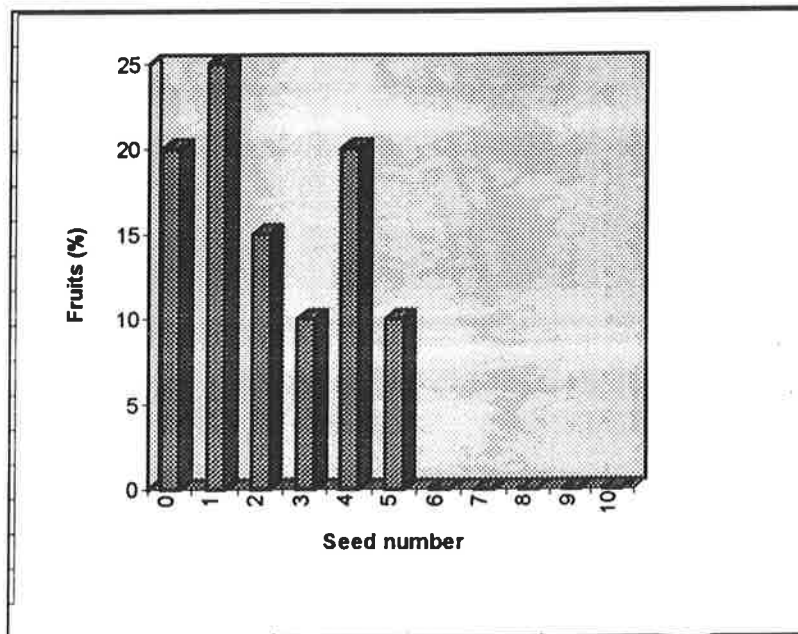
Fig.10.2. Showing relation of seed number distribution with fruit (%) for Packham Triumph tree L10.
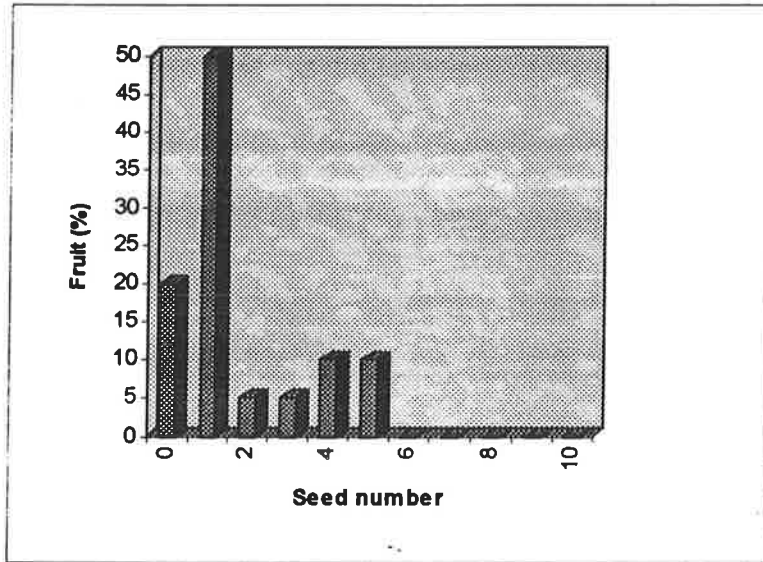
Fig. 10.3. Showing seed number distribution in relation with fruit (%) for Packham Triumph tree M8.

Flowering calender for 3 cultivars
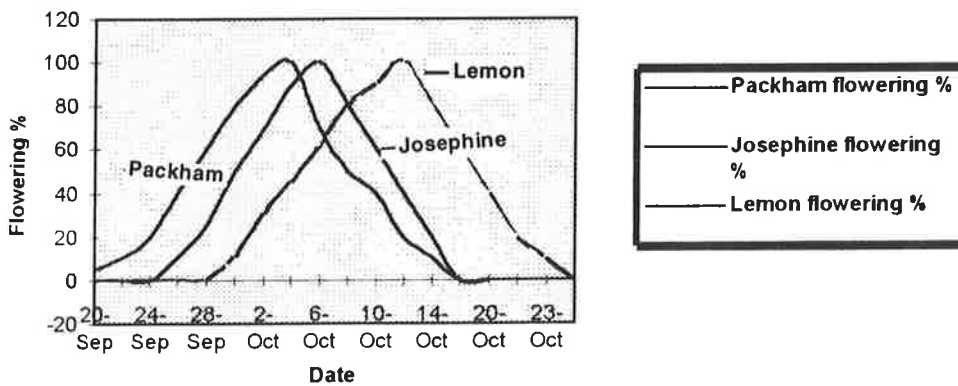
Fig.10.4 Showing flowering flush, full bloom and ending flowering calendar for 3 Pear cultivars in 1994.

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10.3.2 Gene Flow by Pollen to Packham Triumph

The results obtained (Table 10.2) using the ADH isozyme system to identify pollen genes in the seed set in Packham fruit showed approximately an equal distribution of Josephine and Lemon Bergomot genes in the experimental sinks, L10 and M9. Gene flow to K10 showed a higher flow of Lemon Bergomot pollen most likely from the Lemon Bergomot trees in the adjacent row J (Fig. 10.1). Gene flow to Packham M8 however was highest from Josephine (Table 10.2). Significantly, tree M8 had a Josephine cultivar right next to it. What is surprising is that Josephine pollen genes are found in all Packham Triumph fruit investigated, even though only one Josephine tree M7 is planted in that part of the orchard amongst many Packham Triumph and Lemon Bergomot trees. Tree K10 three rows from the Josephine pollinizer still showed evidence of Josephine pollen genes in seeds, although naturally less than found in Packham Triumph seeds from the trees closer (Table 10.2) to the Josephine tree. This suggests that Josephine is a more effective pollinizer than Lemon Bergomot for Packham Triumph under the conditions of the experiment in the orchard.
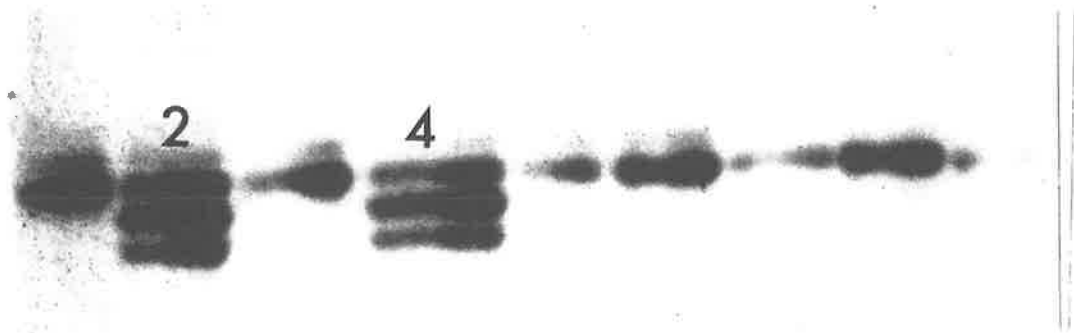
Fig. 10 5. Showing ADH isozyme zymogram for nine seeds selected at random from tree K10 (Lane two and four show an *ac* pattern, the other seven lanes show *aa*).

With respect to the flowering calendar of Josephine and Lemon Bergomot relative to Packham Triumph (Fig. 10.4), there would be appear to be a less than perfect overlap of Lemon Bergomot with Packham Triumph helping to explain the above result. In addition, remarkable differences were observed in the flowers number on Packham Triumph trees in comparison with Josephine and Lemon Bergomot. Results of flowers accounting for these cultivars showed that Packham Triumph had an average range of 16,000-18,000, Lemon Bergomot 4,000-4,500 and Josephine 3,000-4,000 flowers per

tree in the orchard condition. Since there is considerably more Packham Triumph flowers, per tree, then this reduces the probability of successful fertilization for every Packham flower and explaining the overall poor seed set in Packham. Moreover it is possible that a surplus of Packham pollen could tend to block fertilization by smaller amount of Lemon Bergomot and Josephine pollen, but we have no evidence for this. Lack of adequate pollination would also produce more parthenocarpic fruits. Measurement of the percentage of fruits with different seed numbers per fruit showed that highest proportion of fruits in these trees were either parthenocarpic fruits or fruits with one seed per fruits (Fig.10.2, Fig. 10.3, Table 10.1).

Table. 10.2 Percentage of source genes which were found in Packham experimental sinks.

Tree	Seed No	Lemon(%)	Josephine (%)
K10	47	66	34
L10	50	50	50
M8	47	30.5	69.5
M9	49	50.1	46.9

10.3.3 Gene Flow by Pollen in a Trellis System

Analysis of seeds in fruits sampled from trees in a Tatura trellis system using the GPI isozyme system showed indications of several different pollen genes in the seeds. As shown in Fig. 10.6 bands typical of Duchess, Winter Nelis and Twentieth century (Nijisseiki) can be seen in extract of the seeds. It would seem from this preliminary study that an open form of trellis helps to give gene flow by pollen over a greater distance in comparison with normal plantings.

Fig. 10.6   The GPI banding patterns given by extracts from seeds collected from Packham Triumph fruits in trees in a trellis system. The pollen parents assigned for each lane from left to right is as follow; 1- Duchess, 2- Winter Nelis, 3-Winter Nelis, 4-Winter Nelis, 5-Twentieth Century, 6- Twentieth century, 7-Winter Nelis,8-Twentieth Century, 9-Twentieth Century, 10-Duchess, 11-Twentieth Century.

10.4 Discussion

Lombard *et al.* (1971) reported that EPP (effective period for pollination) for Packham Triumph ovule is 1-2 days at normal temperatures, so inadequate pollination in the early flowering times of Packham Triumph flowers could lead to embryo sac collapse and lower seed production. Unequal amounts of pollen production among the cultivars presented in the orchard (see chapter 6) makes for different availability of pollen. The cultivar which produces higher amounts of pollen but is otherwise of equal attractiveness to honeybees visiting flowers, probably has a higher chance for pollen dispersal than others. Important factors also include the amount of pollen production, rate of anther dehiscence (related to pollen moisture) and attractiveness of flowers to pollinators (Kendall and Smith, 1976). Sucrose in flower nectar has been reported to play a more important role than glucose and fructose for honeybee attraction (Wykes, 1952; Kevan and Barker, 1983). The analysis of sugar in flowers for these pear cultivars indicated that Packham Triumph nectar had considerable amount of sucrose while Josephine and Lemon Bergomot had none (Fig. 10.8). This was backed up by my own personal observation that honey bees frequented Packham Triumph cultivars and were observed in lower numbers on Josephine and Lemon Bergomot trees. The reduction of cross pollen flow on Packham Triumph (K10) with increasing distance from Josephine source (M7) was in agreement with other work (Jackson and Clarke, 1991b; Free 1962).

10.5 Conclusions

It is suggested that Lemon Bergomot is not an efficient pollinizer for Packham Triumph, probably due to inadequate flowering time overlap between the two cultivars (Fig 10.4) and a lack of attractiveness of Lemon Bergomot flowers to honeybees. The Packham Triumph cultivars investigated in this part of the orchard in general showed inadequate pollination, although Josephine did perform better as a pollinizer. Josephine like Lemon Bergomot also has a low sucrose in the nectar and low flower
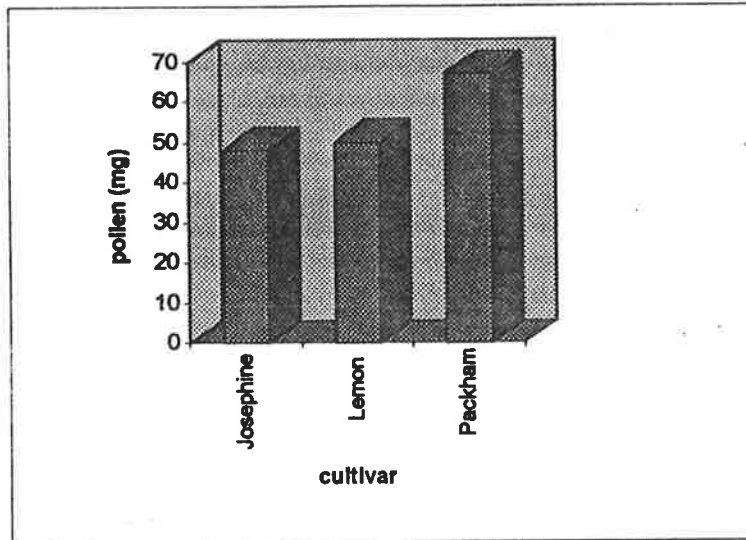
Fig. 10.7 Pollen production (mg per 50 flowers) for three pear cultivars.
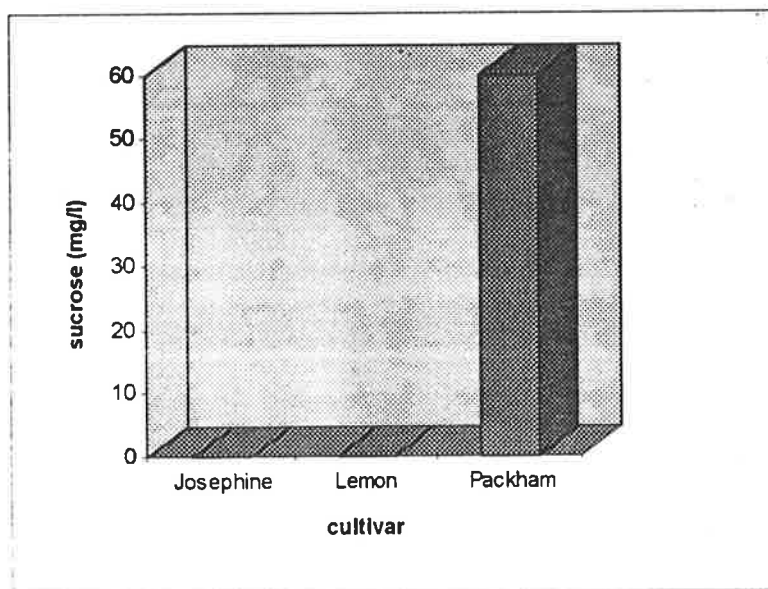
Fig.10.8 Sucrose concentrations in nectar samples from Josephine, Lemon Bergomot and Packham Triumph cultivars.

numbers, but nevertheless does appear to be a better pollinizer for Packham Triumph trees. In that part of the orchard under study here, the plentiful supply of Packham Triumph pollen means a good seed set in fruits on Josephine and Lemon Bergomot trees (Chapters 6 and 11), and conversely poor seed set in Packham Triumph due to its larger number of flowers and not enough cross compatible pollen.

Chapter 11

Gene flow by Pollen to Lemon Bergomot Pear Trees Interplanted with Other Cultivars

11.1 Introduction

Isozyme genetic markers have been used to identify different fruit crop genotypes and their self and outcrosses (Parfitt *et al.*, 1985, Durham *et al.*, 1987).

Estimation of outcrossing rates has been one use of these markers in pollination studies (Tanksley, 1984; Degani *et al.*, 1989). In addition isozyme markers have been used in gene flow studies to describe pollen movement patterns from point or block sources into sinks (Jackson and Clarke, 1991a, b). An understanding of pollen source efficiency relies on the results of such gene flow studies.

Effect of pollen source on abscission has already been studied (Degani *et al.*, 1989) as has genetic selection of avocado during abscission(Goldring *et al*, 1987).

This investigation was aimed at understanding the gene flow by pollen from Packham Triumph and Josephine sources to Lemon Bergomt cultivar in a commercial orchard, In addition a study was made of genetic selection, if any, during abscission of Lemon Bergomot pear fruitlets.

11.2 Materials and Methods

11.2.1 Placement of Trees in the Orchard

A Lemon Bergomot tree (tree number 15 in row I, see Fig.11.1) was selected as a gene flow sink tree (the experimental tree) in a predominantly Packham Triumph pear section of the orchard. This tree was located between several rows of Packham Triumph. Some other Lemon Bergomot trees were located nearby and one Josephine at some distance three rows away. During the Lemon Bergomot flowering, it was estimated that there was enough pollen from Packham and Josephine to give some flow of pollen to the Lemon Bergomot (Chapter 10). Because of the high number of the Packham Triumph flowers and large number of Packham Triumph trees in the

153

vicinity, it was expected that there would be little or no Josephine pollen flow to the Lemon Bergomot tree under study. Bear in mind however, that a single Josephine was found to be a relatively effective pollinizer of Packham Triumph flowers from a similar distance (Chapter 10), and so its effectiveness must be tested here.
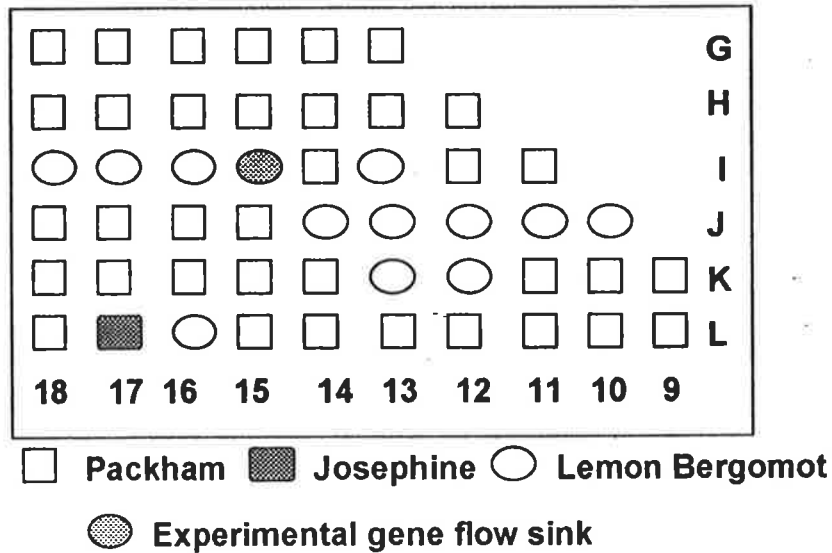
Fig. 11.1 A schematic design for a part of orchard, where the Lemon Bergomot pear tree (tree no 15, row I) as a gene flow sink to survey gene flow by pollen to this tree.

11.2.2 Frequency of Seed Number

Forty Lemon Bergomot fruits from 4 different sides of the experimental Lemon Bergomot pear tree were picked at the time of maturity. Another forty fruits which had fallen naturally from the tree, were also collected from under the same tree, to study factors associated with fruit abscission. The number of seeds per fruit was recorded. Abscised fruit was collected between the first and second drop time.

11.2.3 Isozyme Analysis

Twelve randomly seeds were selected for both the fruits retained on the tree and abscised fruits for each of the four sides of the Lemon Bergomot tree. Isozyme analysis was carried out on these using GPI and ADH. GPI isozyme banding patterns for controlled crosses Lemon Bergomot X Josephine and Lemon Bergomt X Packham Triumph are illustrated in (Fig. 11.4). The GPI banding pattern in progenies of crosses obtained from Lemon X Josephine indicated a simple *bb* band (see Fig. 11.5) Isozyme phenotypes for the cross Lemon Bergomot X Packham Triumph showed three different phenotypes *ac*, *bb*, *bc* equally as obtained for the cross Josephine X Packham Triumph (Fig.11.4) with a segregation ratio 2:1:1. The method for performing seed isozyme analysis was as described by Jackson (1992) and Richardson *et al.*, (1986).

11.3 Results

11.3.1 Seed Number Analysis

Distribution of seed numbers within the Lemon Bergomot pear fruits showed considerable differences between the abscised and fruits held on the tree (Fig. 11.2 and Fig. 11.3). Approximately sixty percentage of total abscised fruits had 4 or less seeds per fruit which more than 70 % of retained fruits averaged 4 to 7 seeds per fruit. It is possible then that a lower number of seeds may lead to lower fruit retention in Lemon Bergomot. These results are in accordance with results obtained in Chapter 8.

11.3.2 Paternity by GPI

In practice, it was difficult to judge the difference between Packham Triumph and Josephine pollen contributions to the GPI patterns seen in extracts of seeds from Lemon Bergomot trees. However the presence of Josephine pollen genes should show the normal 1:2:1 segregation of *ac:cc:cd* seen with Packham Triumph X Lemon

Bergomot crosses and that was not detected here (Table 11.1). This is in agreement with the observation from paternity testing with ADH (as shown).
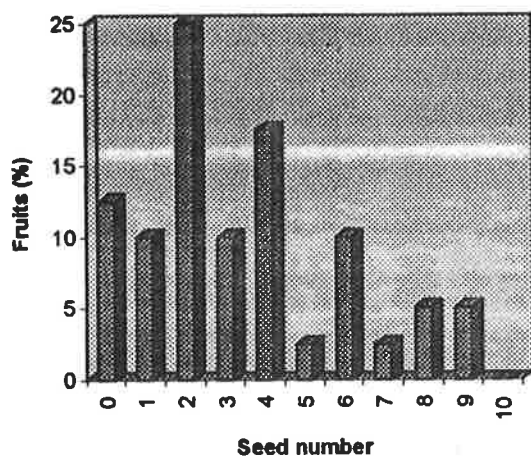
Fig. 11.2 Distribution of seed number per fruit in fruits naturally abscised early from the experimental Lemon Bergomot tree.
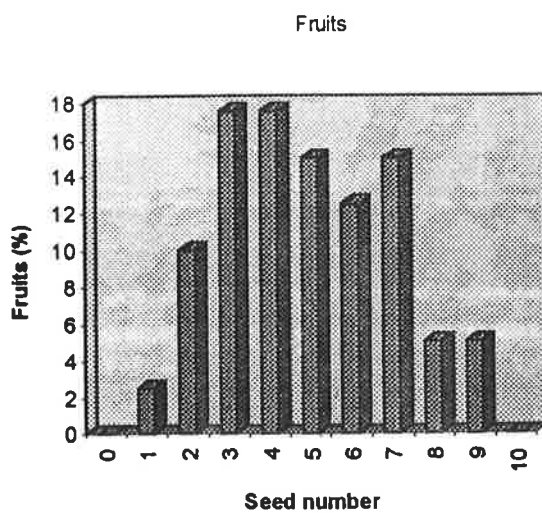
Fig. 11. 3 Distribution of seed number per fruit in

fruits picked from the experimental Lemon Bergomot tree.
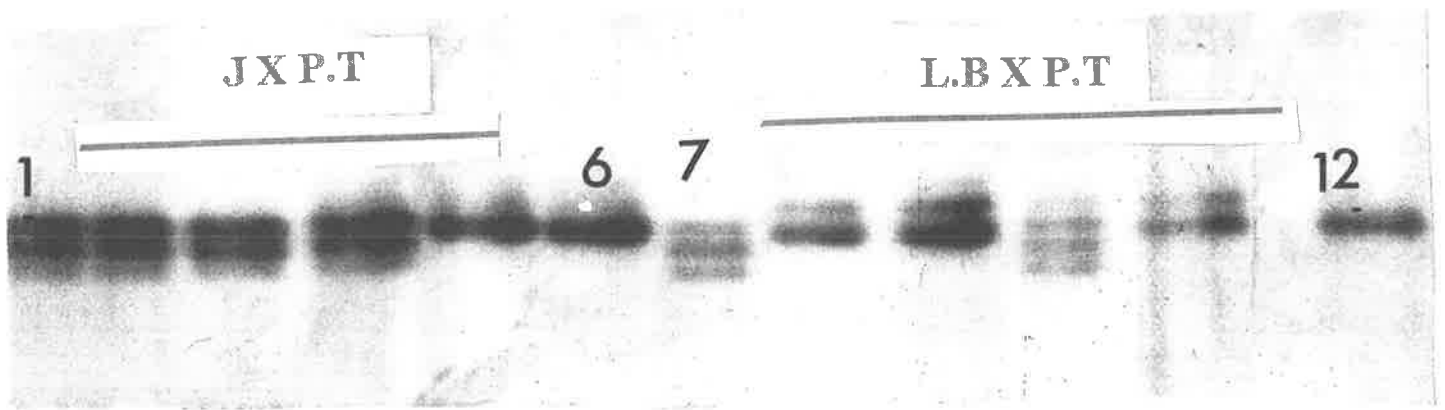
Fig.11.4 GPI isozyme banding patterns for crosses between Josephine X Packham Triumph (1-6) Lemon Bergomot X Packham Triumph (7-12).
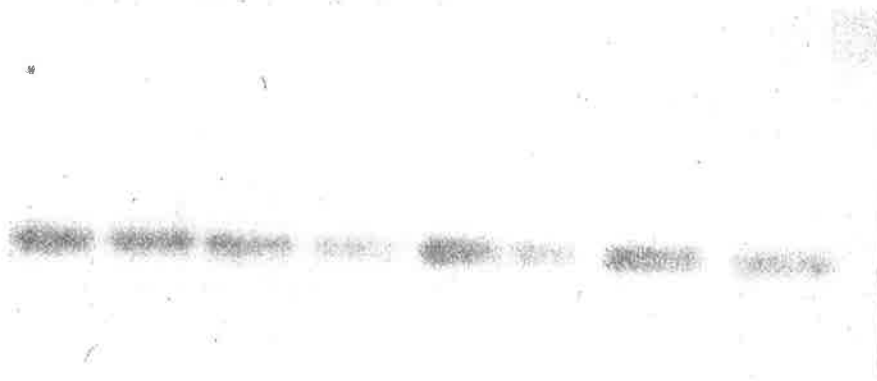
Fig.11.5 GPI isozyme banding patterns for the crosses of Lemon Bergomot X Josephine

Table 11.1 Genotype distribution and X^2 test for the two groups of seeds

using the GPI isosyme system.

Treatments	seeds tested	ac	bb	cd	x^2	P
Picked fruits	48	12	11	25	.016	< 0.05
Abscissed fruits	45	9	9	27	1.5	< 0.05

11.3.3 Paternity by ADH

With the ADH isozyme system an *ac* band can only arise from Josephine (male) X Lemon Bergomot which crosses while *aa* bands result from Packham Triumph (male) X Lemon Bergomot (see section 5.3.2 p.96). As can be seen in Fig. 11.6, only *aa* bands were observed in seeds from Lemon Bergomot fruits. It is deduced that there is no seed set from Josephine pollen on the Lemon Bergomot.

11.4 Discussion

Results of isozyme analysis showed that the Josephine cultivar did not act as a pollinizer for Lemon Bergomot in the part of the orchard tested. The reason for this is probably due to the high density of Packham Triumph cultivar with its greater amount of pollen production and higher numbers of flowers in comparison with Josephine (see chapter 10). The result was pollination of Lemon Bergomot by Packham Triumph only.

Isozyme analysis of seeds from abscised fruits indicated a phenotypical isozyme pattern quite similar to seeds collected from fruits picked from the experimental Lemon Bergomot tree. In this case, it was concluded that there was no pollen source except Packham Triumph which pollinated the Lemon Bergomot. The segregation of alleles in abscised fruits followed the same pattern as that for seeds collected from the picked fruits.
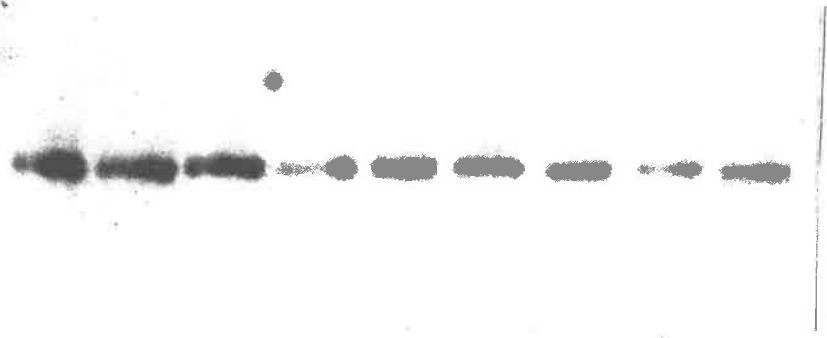
Fig. 11.6 ADH isozyme banding patterns for random seeds obtained from abscised fruits, showing no trace of Josephine pollen genotypes.
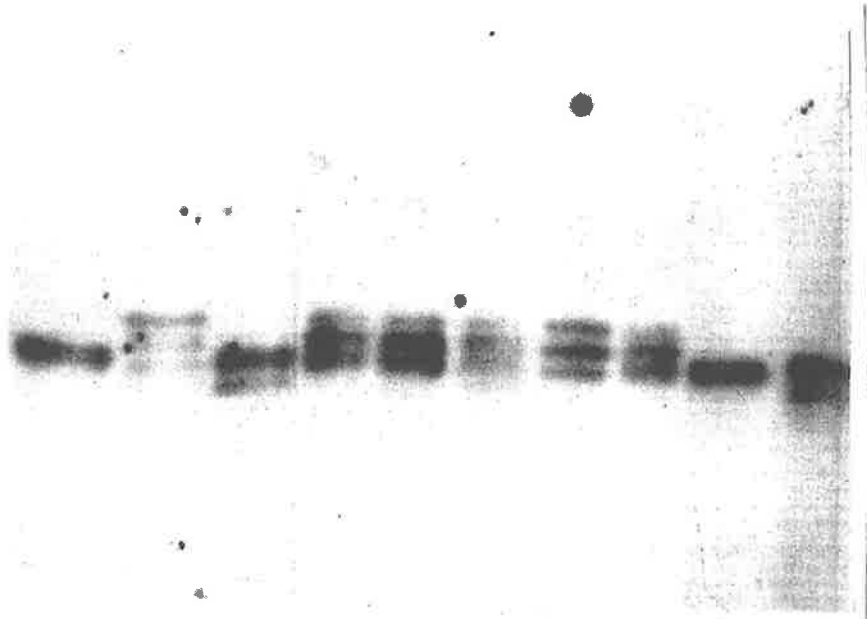
Fig. 11.7 GPI isozyme banding patterns in extracts from seeds from picked fruits on random seeds obtained from the experimental Lemon Bergomot.

These results were in accordance with that obtained for avocado by Degani *et al.*, (1986). These authors obtained a similar genotype segregation pattern for LAP-2 between picked fruitlets and abscised ones in the first fruit drop. However Degani *et al* (1986) showed a higher frequency of FS LAP-2 rather than an FF pattern for fruits in the second drop in July-June in comparison with April-May final drop, suggesting some sort of genotypic selection in this drop for avocado.

The results of this research showed that there is no genetic selection during abscission. The only factor which was observed to differ between abscised and retained fruits was the number of seeds, with a lower seed number associated with early fruit drop.

Chapter 12

Pollen Ultrastructure of Pear Species and Certain Cultivars

12.1 Introduction

Pollen morphology has been used as a key for identification and phylogenic study of plant species (Kitaura *et al.*, 1986). Pollen ultrastructure has also been used as a means of studying plant populations (DeGrandi-Hoffman *et al.*, 1992) and for following patterns of pollen flow throught a population (DeGrandi-Hoffman *et al*, 1984; DeGrandi- Hoffman *et al*, 1986). Differences in exine patterns on pollen surface has enabled researchers to distinguish between certain fruit cultivars and even certain varieties of fruits (Marcucci *et al.*, 1984, Fogle, 1979). It has been understood that some components of the pollen surface are more variable within a genus or species than others. These components are number and surface area of perforations and diameter and numbers of ridges on the pollen surface (Ueda and Tomita, 1989). Erdtman (1952) was one of the first researchers who used pollen orientation components for the purpose of taxonomy. Diploidy and polyploidy of strawberries were distinguishable by prominent differences between pollen grain size and ridges of corresponding genetic structures. Westwood and Challice (1978) used morphometric analysis of pollen for different pear species. Mulas *et al.*, (1989) showed that environmental effects did not change the pollen ultrastructure of twenty almond cultivars. The present investigation was proposed to identify morphometric characteristics of pollen species and certain pear cultivars which could have potential for identification purposes.

12.2 Materials and Methods

12.2.1 Pollen Collection

Pollen from different pear species and certain cultivars were collected in South Australia. These species included *P.longipes, P.koroshinsky, P.betulaefolia,*

P.calleryana, *P.gharbiana*, *P.kawakami*, *P.lindley*, *P.pyraster*, *P.syriaca*, *P.mamorensis*, *P.boissierana* collected from the Arboretum of the Waite Agricultural Research Institute. The *P.communis* and *P.pyrifolia* cultivars were Anjou, Chjoru, Hosui, Shinsui, Kosui, Lemon Bergomt, Josephine, Packham Triumph, Yali, Twenty Century, Hwa Hong , Howell, Italian, Winter Nelis and Tsu Li, which were collected from a commercial orchard located in Corommandel Valley, a region of South Australia. The anthers were separated from the flowers of each species and certain cultivars and placed in a Petri dish and kept at room temperature for 48 h to allow the anthers to dehisce. Pollen was collected on an aluminium paper using a sieve with mesh no. 100 and opening of 0.0060 inches.

12.2.2 Electron Microscopy

The air-dried pollen was placed onto a double-sided adhesive tape attached to an aluminium stub. Attention was taken not to mix samples and stabs carefully labelled. These samples were kept in a desiccator containing silica gel to dry.

The samples were coated with about 200 A° gold alloy using a Benton vacuum evaporator unit and observed with a SEM (scanning electron microscope, Philips XL 30 Feg) at 12 KV. The magnification was performed at 2061 times for a general view of pollen and 10823 times for more precise examination of pollen ultrastructure. Six representative pollen were selected from each sample for examination. Variables including polar length, equatorial width and germinal furrow length were measured using a SIS software program to measure dimensions of pollen directly on the screen. The ratio of L/W for the different pollen was obtained from the data. The surface area covered by perforations (%) was measured on the pollen grain using a COMOS (Bio Rad Labroatries Pty. Ltd.) image analysis software program. All data which was obtained for pollen length and width was compared in a statistical randomized complete block design using a Superanova software program. Means of variables were separated according to Duncn,s test significant at .05% level.

12.3 Results

Substantial differences in pollen dimension including length, length/width ratio, and length of germinal furrow were observed between the pear species and certain cultivars (Table 12.1). There was no significant differences among pears used in this study for the character of pollen width. Size and number of the pits (surface area of pits) made the separation of some cultivars from the other possible while slight differences in the ridge diameter were observed. Separation of the means for significant variables, including polar length, length/width ratio and length of furrow divided pears into 3 groups. These were maximum, moderate and maximum groups. Shinsui, Twentieth Century, a Chinese cultivar Yali and African species *P.mamorensis*, and *P.lindley* were located in the maximum group. From pear species *P. syrica and P. kawakami* were also grouped in minimum one and for the moderate group Packham Triumph and Italian cultivars were examples. Josephine had a longer polar axis length in comparison with Packham Triumph and Lemon Bergomot, but had lower equatorial length than the two others. *P.mamorensis* from the African group significantly was much larger than another member of its group *P.gharbiana.*

Italian 19.69% (Fig.12.10), Winter Nelis 5.15% (Fig.12.24), and Anjou 4.89 % (Fig12.1). As a result pit surface area (%) which was a reflection of size and number of pits was accounted here as a significant variable for pear identification. Using this character also could divide the pears into 3 different groups of maximum moderate and minimum. The shape, orientation of ridges were different among the pear species and cultivars studied here but, diameter of ridge was slightly different among the pears studied here. Anjou showed thicker ridges and Kosui had thinner one (Figs.12.1, 12.13). Some samples including Howell and Packham Triumph (Fig.12.7 and Fig.12.17), showed a smooth surface with no or less visible ridges. Pit surface area (%) can be used to discriminate between the cultivars. Italian, Winter Nelis,and Josephine, *P.koroshinsky, P.pyraster,* Packham Triumph, Housi and *P.gharbiana* had the highest surface area of pits respectively which cover the exine of pollen near the area of the germinal furrow. Some other species and cultivars had a moderate area of pits on the exine such as Shinsui, Tsu li, Lemon Bergomot, Yali and *P.longipes.*

Table 12.1. Measurements for different pollen characteristics of pear species and cultivars. Each value is a mean of 6 replications for pollen length and width, germinal furrow length, ridge diameter and pit surface area .

No	Species cultivars	Pollen polar axis (μm)	Equator axis (μ)	Furrow length (μm)	L/W ratio	Surface area of pits (%)	2 ridges diameter (nm)
1	Anjou	39.8 d-f	20.6 a	33 de	1.9 b-e	2.35	322 a
2	*P.betulaefoli.*	40.4 c-e	20.7 a	36.6 b-d	1.95 a-e	2.59	256 bc
3	*P.calleryana*	37 ef	19.7 a	32.6 e	1.87 cf	0.68	199
4	Chojru	40.2 c-e	18.8 a	35 c-e	2.1 a	2	238 cd
5	*P.gharbiana*	40.5 c-e	25.8 a	34.2 de	1.56 h	3.1	215 de
6	Hosui	42.3 d	20.4 a	38.8 a-c	2.07 ad	3.1	230 cd
7	Howell	38 ef	19.5 a	33.5 de	1.94 a-e	2.91	---
8	Hwa Hong	43.9 ab	22.5 a	39.1 ab	1.95 a-e	0.95	281 cd
9	*P.boissieran*	43.4 bd	21 a	39.1ab	2.06 ad	0.77	227 cd
10	Italian	40.9 de	20.5 a	36.5 be	1.95 a-e	13.61'	266 bs
11	Josephine	44 ab	20.6 a	41 a	2.1 a	5.1	239 cd
12	*P.kawakami*	36.6 ef	20.5 a	33 de	1.78 ef	1.1	222 c-e
13	Kosui	44.9 ab	22 a	39.2 ab	2.04 ad	1.18	180 e
14	Lemon	40.08 de	21.6 a	36.6 bd	1.85 df	1.71	249 bd
15	*P.lindley*	44.3 a	20.7 a	38.5 a-c	2.1 a	0.57	230 cd
16	*P.mamorens.*	45.2 a	22.3 a	40.7 a	2.02 a-d	---	242 cd
17	Packham	40.7 de	21.2 a	36.3 b-e	1.9 b-e	3.36	251cd
18	*P.pyraster*	39.9 de	20.8 a	33.9 de	1.91b-e	3.59	256 bc
19	Shinsui	44.0 a	22.2 a	39.9 ab	1.98 a-c	1.45	219 de
20	*P.syriaca*	38.8 ef	22.1 a	33.3 de	1.75 f	2.9	236 cd
21	Tsu li	40.0 de	21.7 a	35.2 de	1.84 d-f	1.15	225 c-e
22	Twenty Cen.	46.6 a	22 a	41.7 a	2.1 a	0.99	237 cd
23	Yali	45 a	21.2 a	41 a	2.1	2.1 a	235 cd
24	Winter Nelis	43.8 bc	21 a	37.9 ac	2.08 ab	6.2	219 de
25	*P.longipes*	42.5 a-d	20 a	37.5 a-c	2.1 a	2	214 de
26	*P.koroshinsk.*	40.6 c-e	20.7 a	37.1 a-c	1.96 a-c	3.59	208 de
27	*P.u.maxim*	39.2 d-f	20.1 a	36.4 b-e	1.95 a-e	0.81	205 de

The minimum and maximum standard errors for samples of pollen length were ± 0.485and ±1.931.
The minimum and maximum standard errors of samples of pollen width were ± 0.341 and ±1.137
The minimum and maximum standard errors for samples of ridges were ± 6.145 and ± 22.2 and for
pit surface area (%) were ± 0.068 and± 0.770.
Different subscript letters within column groups represent a significant difference according to
Duncn's test.

Other sample such as *P.lindley*, *P.calleryana*, *P.boissierana* had a relatively low area of pits on the exine (Figs.12.3, 12.9, 12.15)

12.4 Discussion

Westwood and Challice (1978) obtained morphometric characteristics of pollen for some of the *Pyrus* species. In their work three species *P.longipes*, *P.betulaefolia* and *P.calleryana* were common with the present study. They reported the *P.longipes* pollen length and width respectively as 48.8 and 26.0 μm, but here the length and width was shown to be 42.5 and 20 um (Fig.12.25). Mulas *et al.*, (1989) indicated that the season, geographical situation and kind of irrigation and cultivation can affect the pollen grain dimension. Thus, it is probable that different localities could lead to different pollen dimension as recorded. With respect to the P value which was lower than .05 and low rate of standard error for pollen length and germinal furrow length, length/width ratio found here, these characters could therefore be used efficiently for mean of identification. Calculation of pits surface area using image analysis software program on the pollen exine for the first time showed that this measurement could also be used for successful discrimination between these samples. Marcucci *et al.*, (1984) already could showed that size and number of pits were useful characters for distinguishing of apple clones.

P.calleryana and *P.ussuriensis maxim* showed similarity for pollen characteristics (see section 4.3.9). Comparison of six above characters for these two species confirmed the *P.ussuriensis maxim* should be the same species *P.calleryana*.

Results of this study show that, polar length, germinal furrow length, pollen length/width ratio, pits surface area and orientation of ridges are the most suitable criteria to apply for pollen recognition in the *Pyrus* species.

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig.12.1 Anjou

Fig.12.2 *P.betuleafolia*

Fig.12.3 *P.calleryana*

12.1

Acc V   Spot Magn   Det   WD ⊢─────── 2 µm
12.0 kV 3.0 8367x   SE   34.3 2Anjou

Acc V   Spot Magn   Det   WD ⊢─────── 10 µm
2.0 kV 3.0 1593x   SE   34.3 2Anjou

12.2

Acc V   Spot Magn   Det   WD ⊢─────── 2 µm
12.0 kV 3.0 8367x   SE   34.2 P.betufolia

cc V   Spot Magn   Det   WD ⊢─────── 10 µm
0 kV 3.0 1593x   SE   34.1 P.betufolia

12.3

Acc V   Spot Magn   Det   WD ⊢─────── 2 µm
12.0 kV 3.0 8367x   SE   16.8 P.calleryana

Acc V   Spot Magn   Det   WD ⊢─────── 10 µm
12.0 kV 3.0 1593x   SE   16.8 P.calleryana

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.4 Chojoru

Fig. 12.5 *P.gharbiana*

Fig. 12.6 Housi
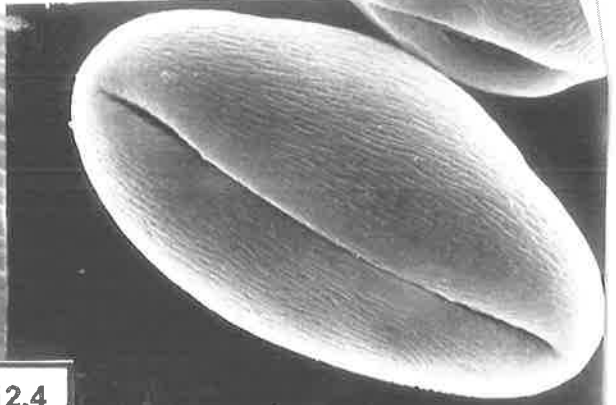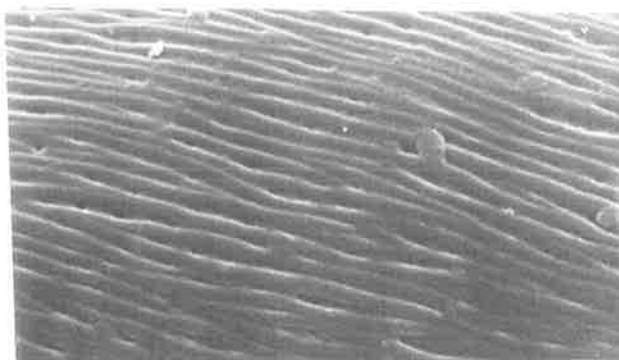
12.4

Acc V Spot Magn Det WD |———————| 2 µm
12 0 kV 3.0 8367x SE 25.9 chojoru

Acc V Spot Magn Det WD Exp |———————| 10 µm
15 0 kV 3.0 2061x SE 34.2 1 14 Chojoru
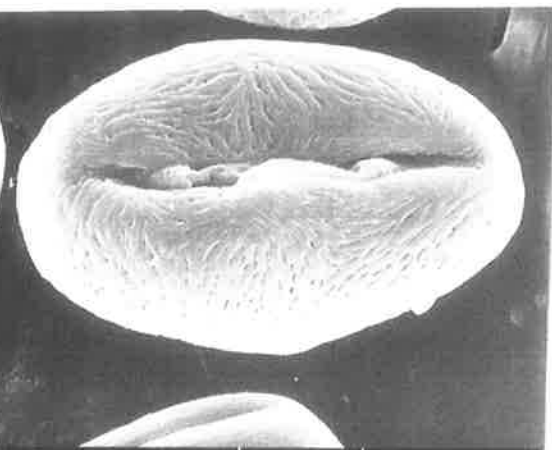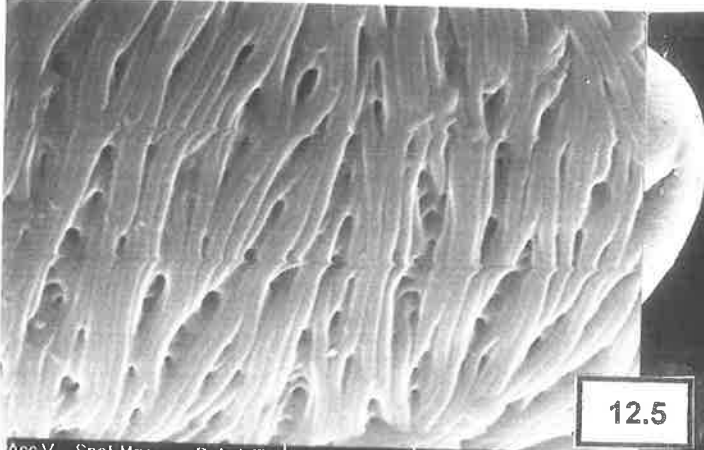
12.5

Acc V Spot Magn Det WD |———————| 2 µm
15 0 kV 3.0 8367x SE 25.9 P.Gharbiana

Acc V Spot Magn Det WD |———————| 10 µm
15 0 kV 3.0 1593x SE 25.9 P.Gharbiana
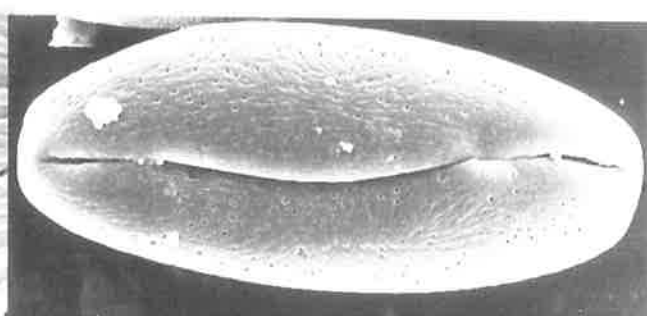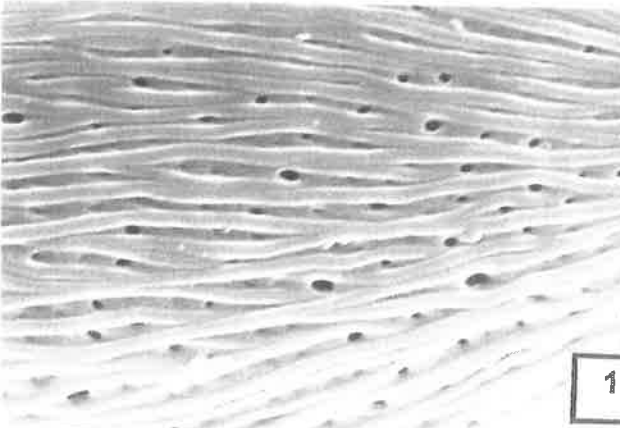
12.6

Acc V Spot Magn Det WD |———————| 2 µm
12.0 kV 3.0 8367x SE 33.8 Housi

Acc V Spot Magn Det WD |———————| 10 µm
12.0 kV 3.0 1593x SE 33.8 Housi

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.7 Howell

Fig. 12.8 Hwa Hong

Fig. 12.9 *P.boissierana*

12.7
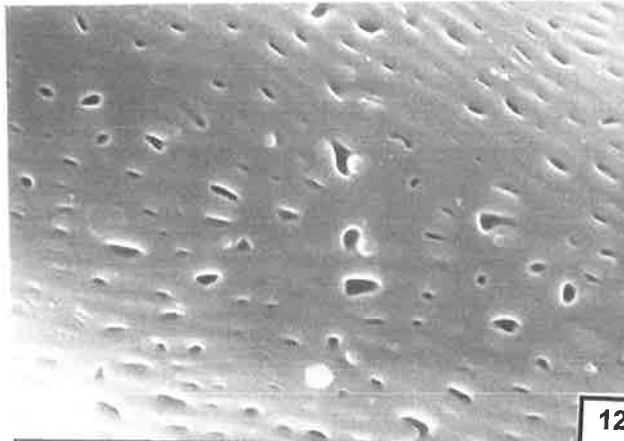
Acc V Spot Magn Det WD 2 µm
12 0 kV 3 0 8367x SE 30 4 Howell
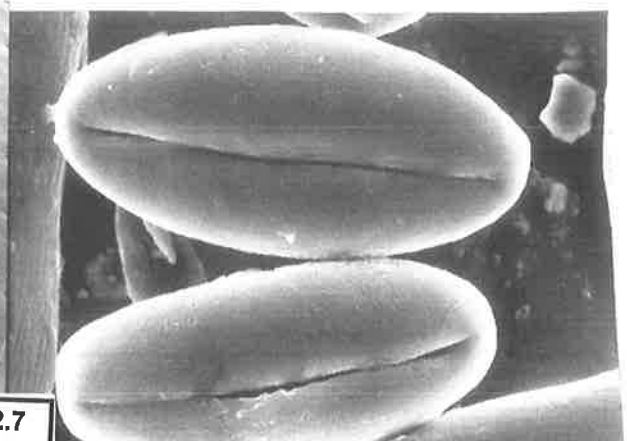
c V Spot Magn Det WD 10 µm
0 kV 3 0 1593x SE 30 4 Howell

12.8
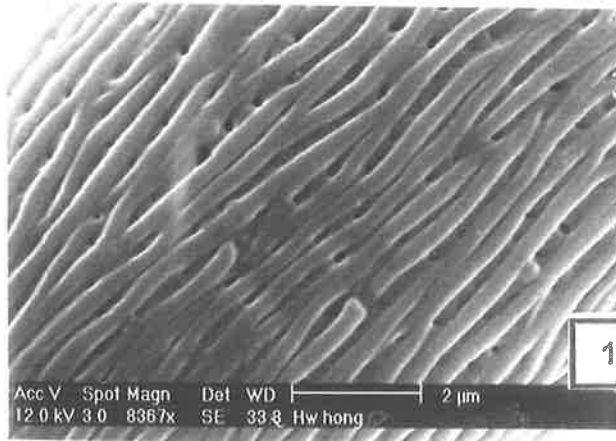
Acc V Spot Magn Det WD 2 µm
12 0 kV 3 0 8367x SE 33 8 Hw hong
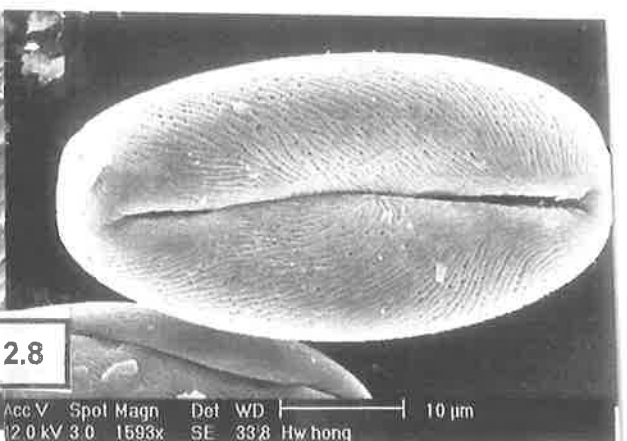
Acc V Spot Magn Det WD 10 µm
12 0 kV 3 0 1593x SE 33 8 Hw hong

12.9
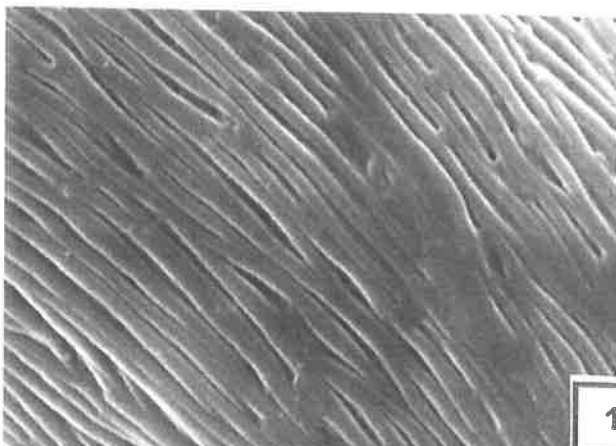
Acc V Spot Magn Det WD 2 µm
12 0 kV 3 0 8367x SE 33.9 P biossierena
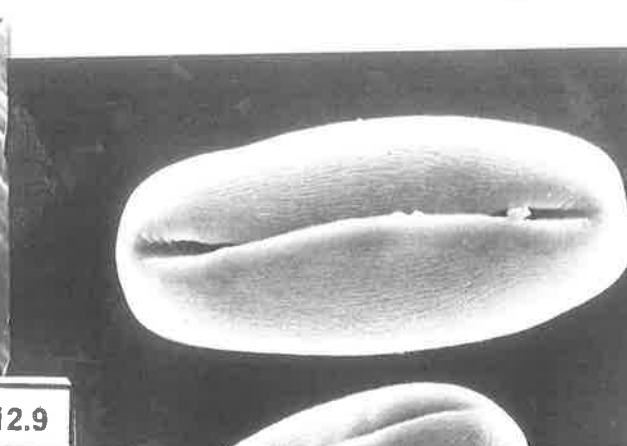
Acc V Spot Magn Det WD 10 µm
12 0 kV 3 0 1593x SE 33.9 P biossierena

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.10 Italian

Fig. 12.11 Josephine

Fig. 12.12 *P.kawakami*
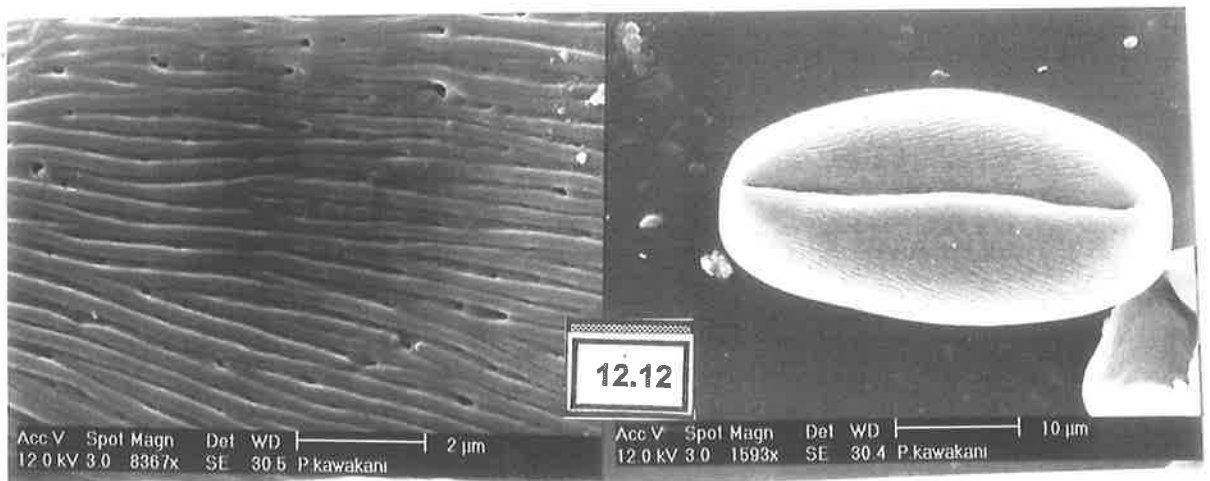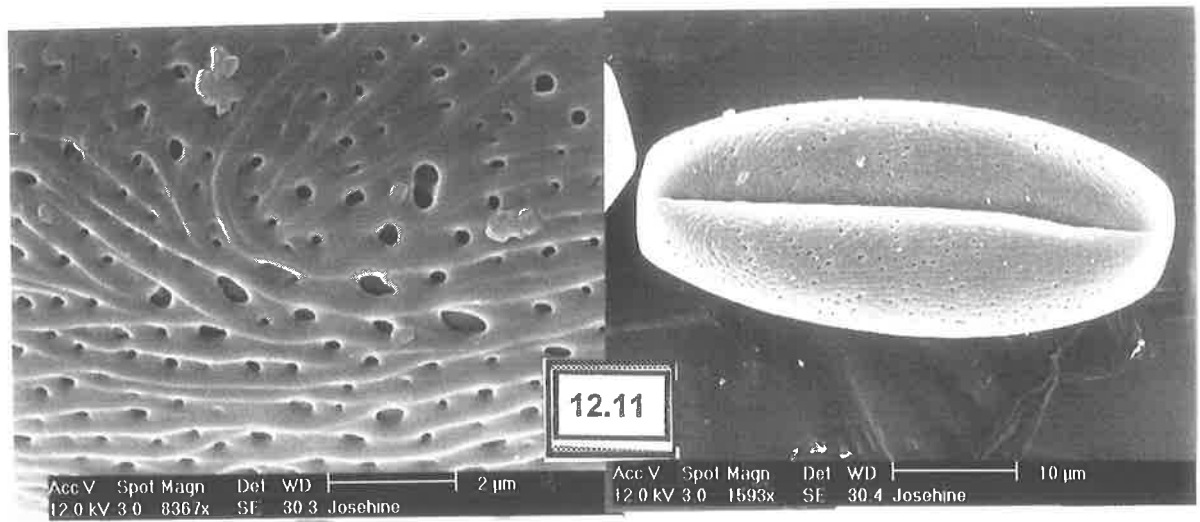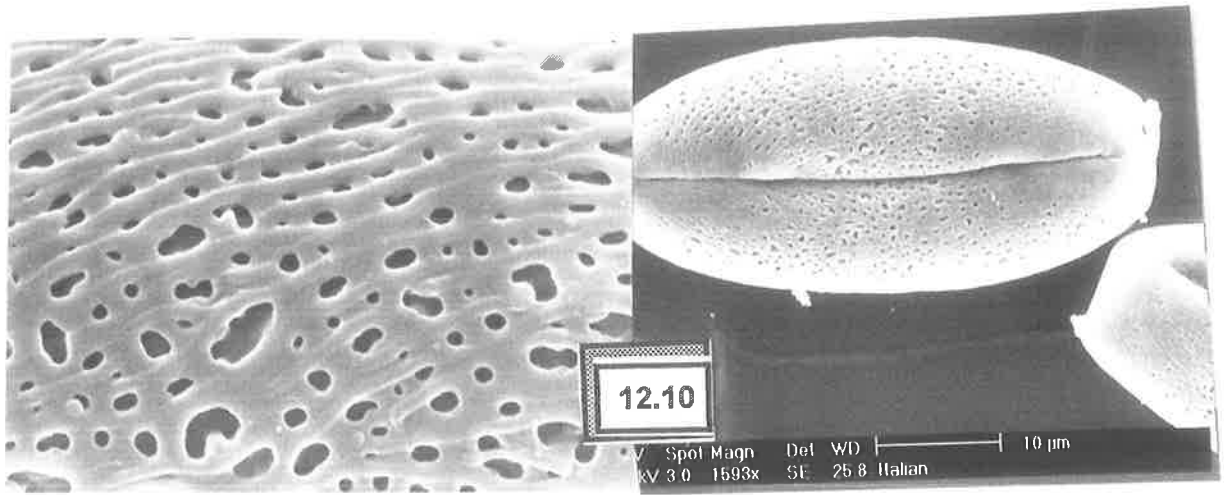
12.10

12.11

Acc V Spot Magn Det WD ⊢———————⊣ 2 μm
12.0 kV 3.0 8367x SE 30.3 Josehine

Acc V Spot Magn Det WD ⊢———————⊣ 10 μm
12.0 kV 3.0 1593x SE 30.4 Josehine

12.12

Acc V Spot Magn Det WD ⊢———————⊣ 2 μm
12.0 kV 3.0 8367x SE 30.5 P.kawakani

Acc V Spot Magn Det WD ⊢———————⊣ 10 μm
12.0 kV 3.0 1593x SE 30.4 P.kawakani

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.13 Kosui

Fig. 12.14 Lemon Bergomot

Fig. 12.15 *P.lindley*
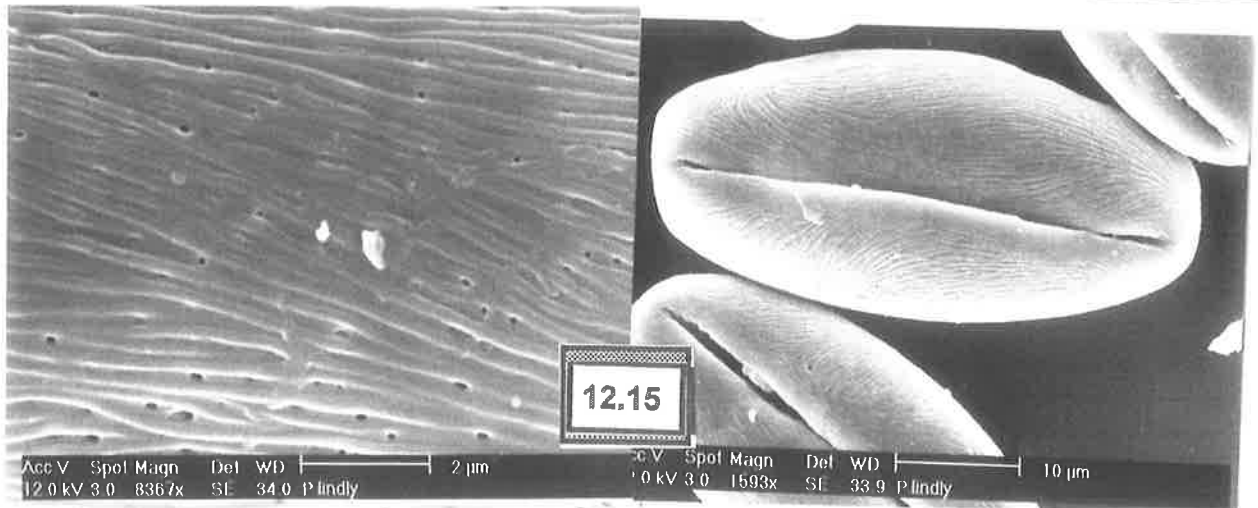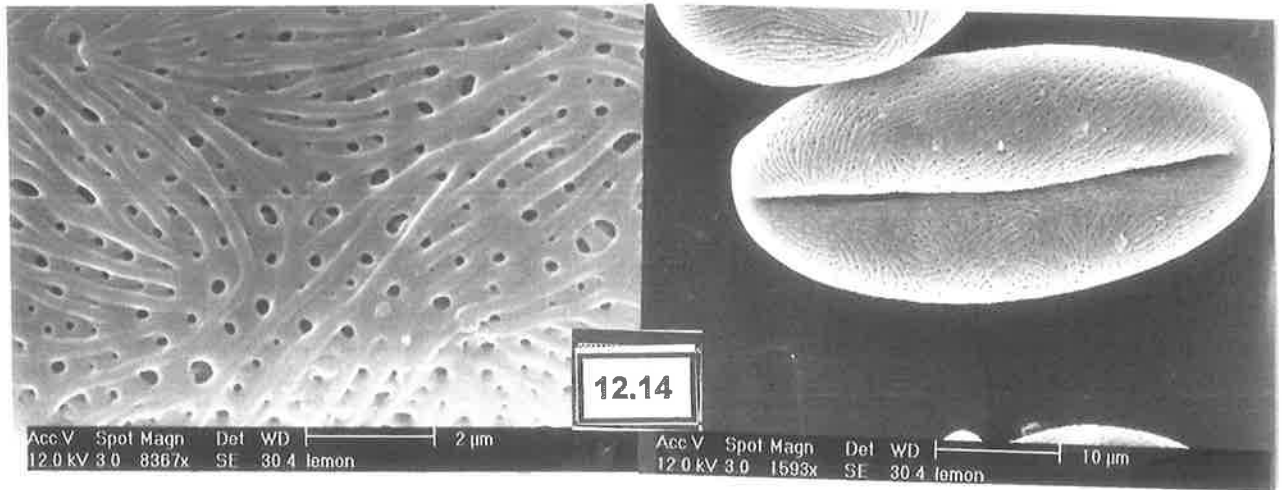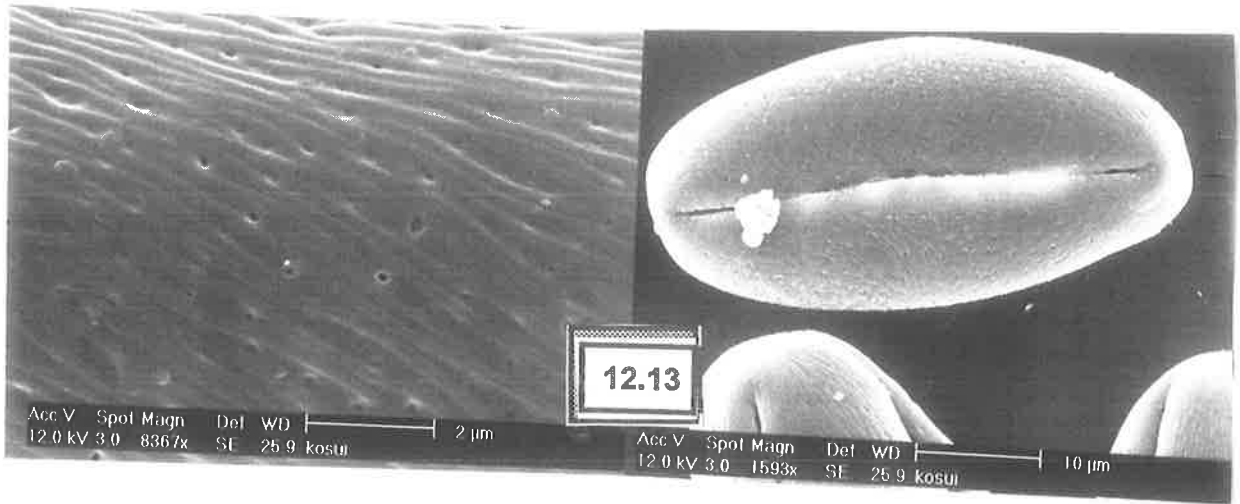
12.13

12.14

12.15

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.16 *P.mamorensis*

Fig. 12.17 Packham Triumph

Fig. 12.18 *P.pyraster*
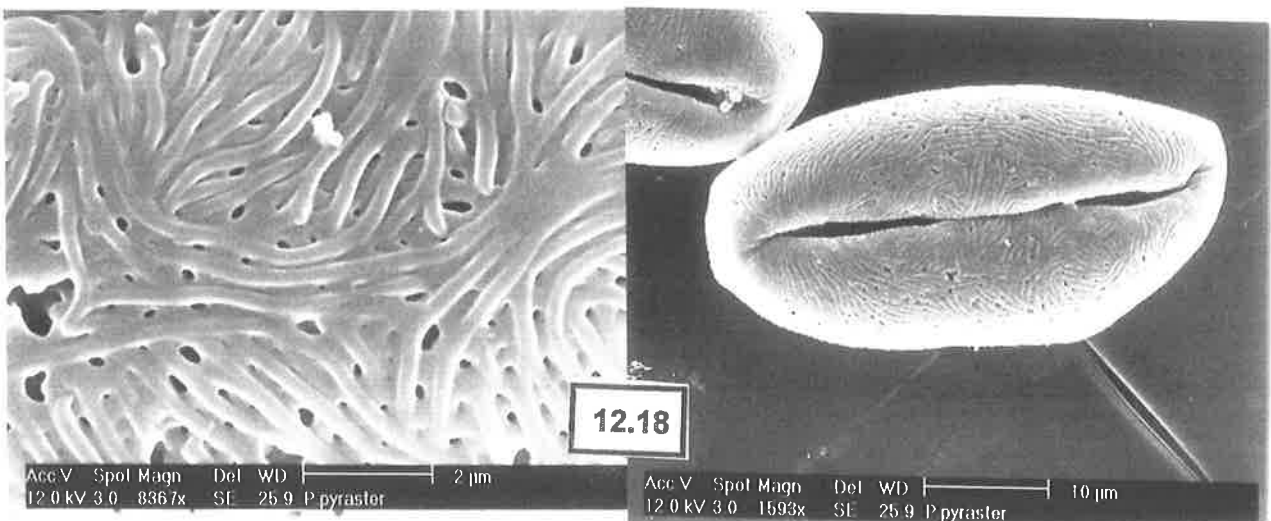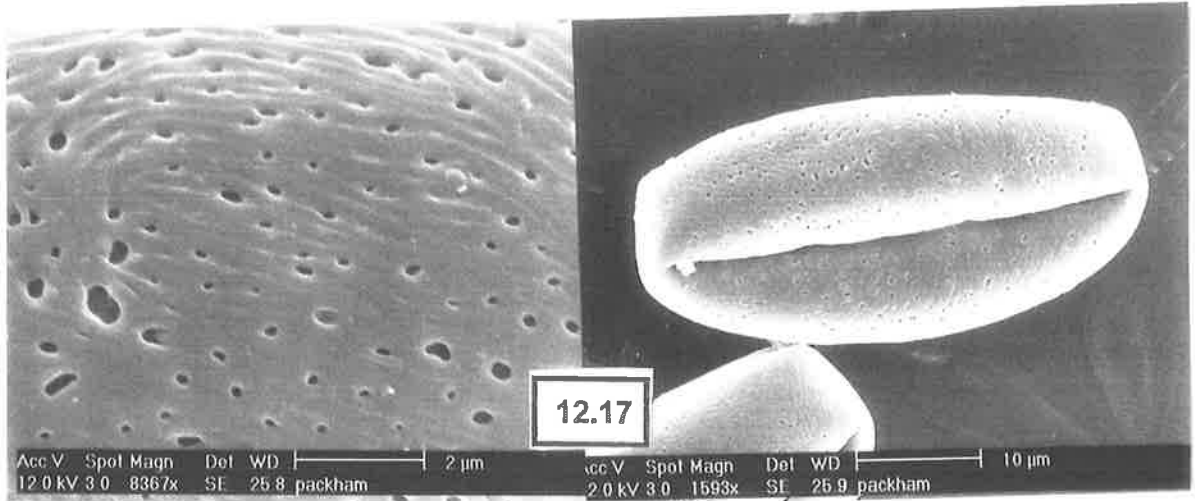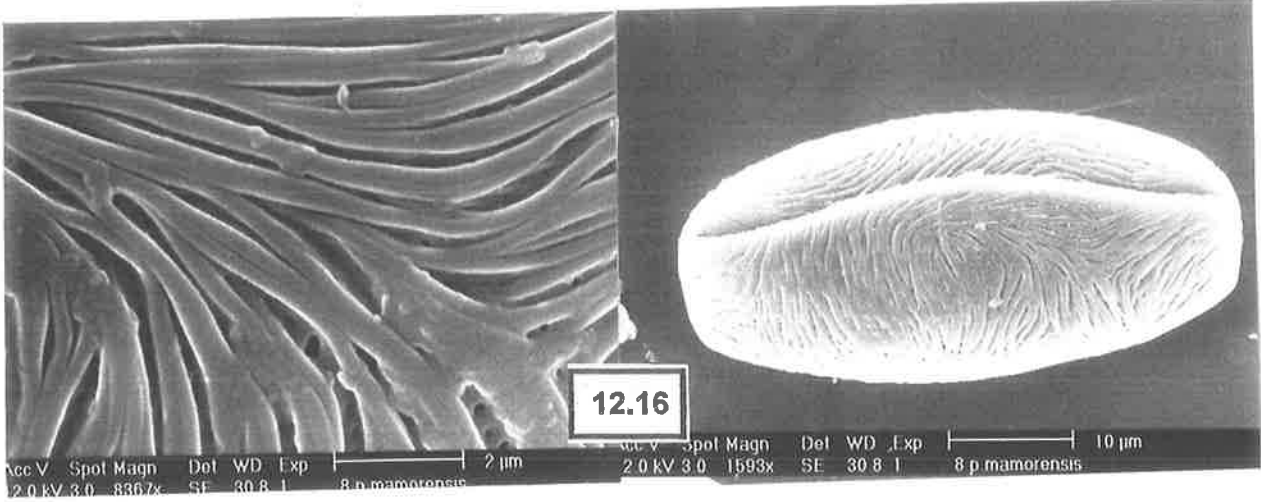
12.16

Acc V Spot Magn Det WD Exp   2 µm
2 0 kV 3 0 8367x SE 30 8 1   8 p mamorensis

Acc V Spot Magn Det WD Exp   10 µm
2 0 kV 3 0 1593x SE 30 8 1   8 p mamorensis

12.17

Acc V Spot Magn Det WD   2 µm
12 0 kV 3 0 8367x SE 25 8 packham

Acc V Spot Magn Det WD   10 µm
2 0 kV 3 0 1593x SE 25 9 packham

12.18

Acc V Spot Magn Det WD   2 µm
12 0 kV 3 0 8367x SE 25 9 P pyraster

Acc V Spot Magn Det WD   10 µm
12 0 kV 3 0 1593x SE 25 9 P pyraster

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.19 Shinsui

Fig. 12.20 *P. syriaca*

Fig. 12.21 Tsu li
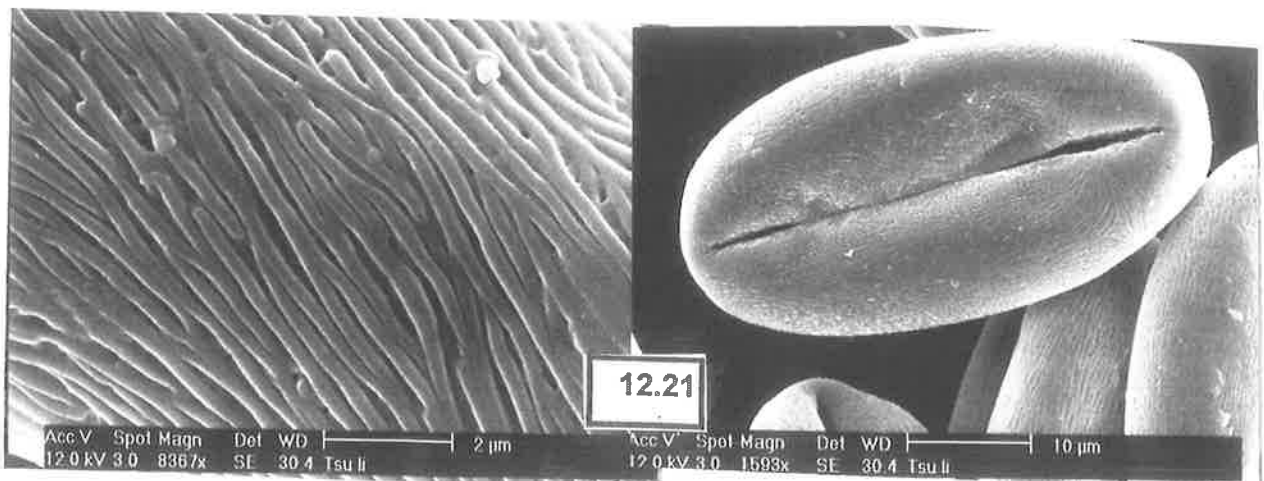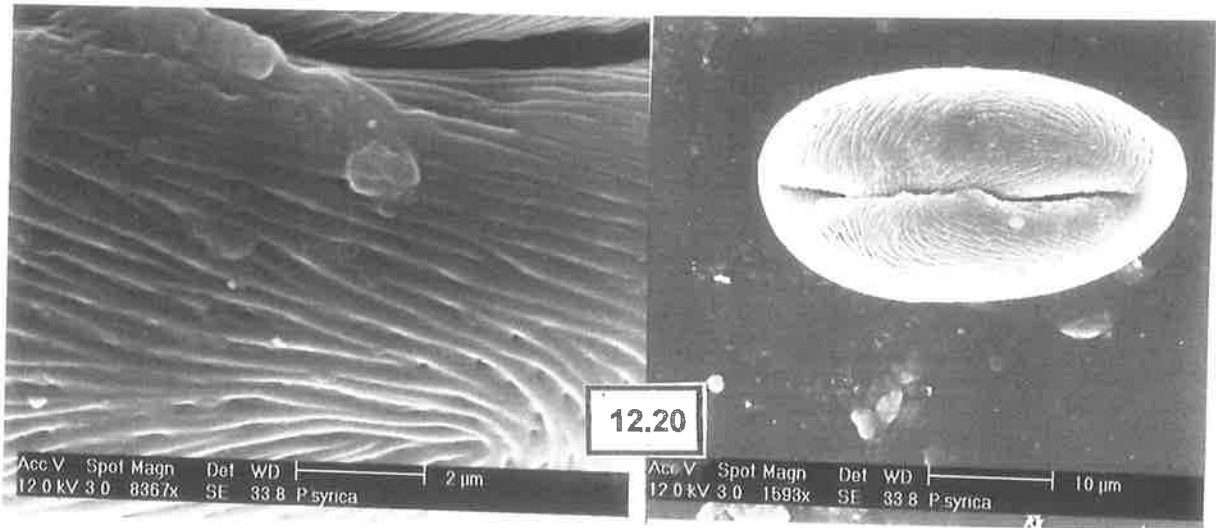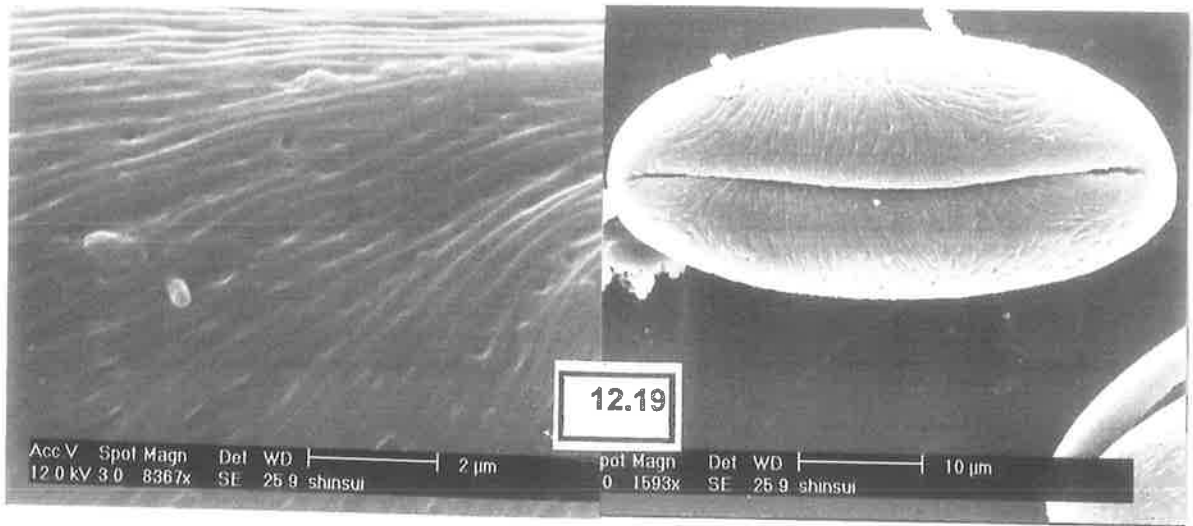
12.19

Acc V   Spot Magn   Det   WD ┣━━━━━━━━━┫ 2 µm   pot Magn   Det   WD ┣━━━━━━━━━┫ 10 µm
12 0 kV 3 0   8367x   SE   25 9 shinsui   0   1593x   SE   25 9 shinsui

12.20

Acc V   Spot Magn   Det   WD ┣━━━━━━━━━┫ 2 µm   Acc V   Spot Magn   Det   WD ┣━━━━━━━━━┫ 10 µm
12 0 kV 3 0   8367x   SE   33 8 P.syrica   12 0 kV 3 0   1593x   SE   33 8 P syrica

12.21

Acc V   Spot Magn   Det   WD ┣━━━━━━━━━┫ 2 µm   Acc V' Spot Magn   Det   WD ┣━━━━━━━━━┫ 10 µm
12 0 kV 3 0   8367x   SE   30 4 Tsu li   12 0 kV 3 0   1593x   SE   30 4 Tsu li

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.22 Twentieth Century

Fig. 12.23 Yali

Fig. 12.24 Winter Nelis
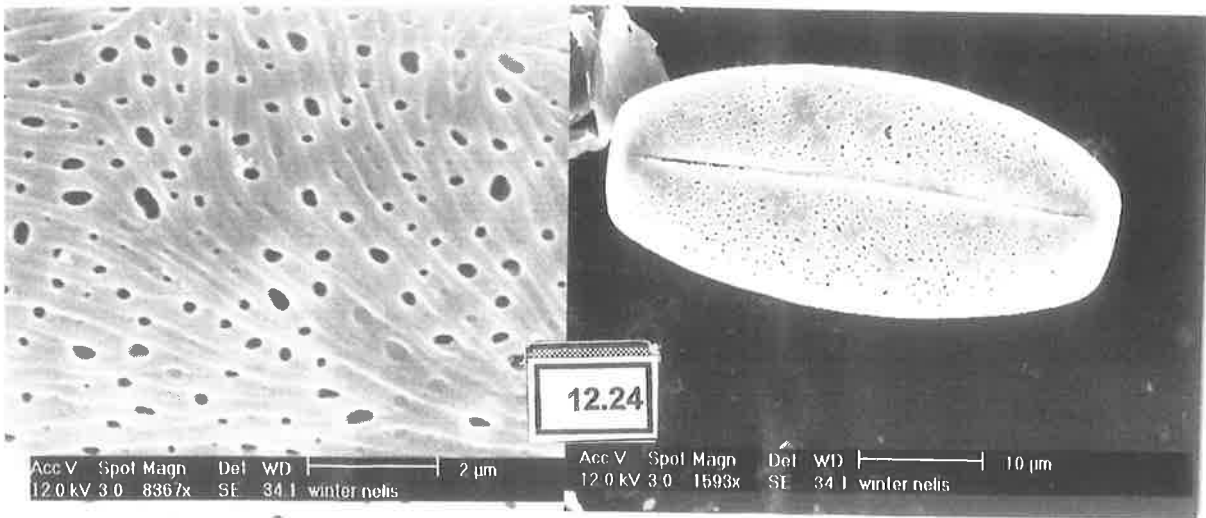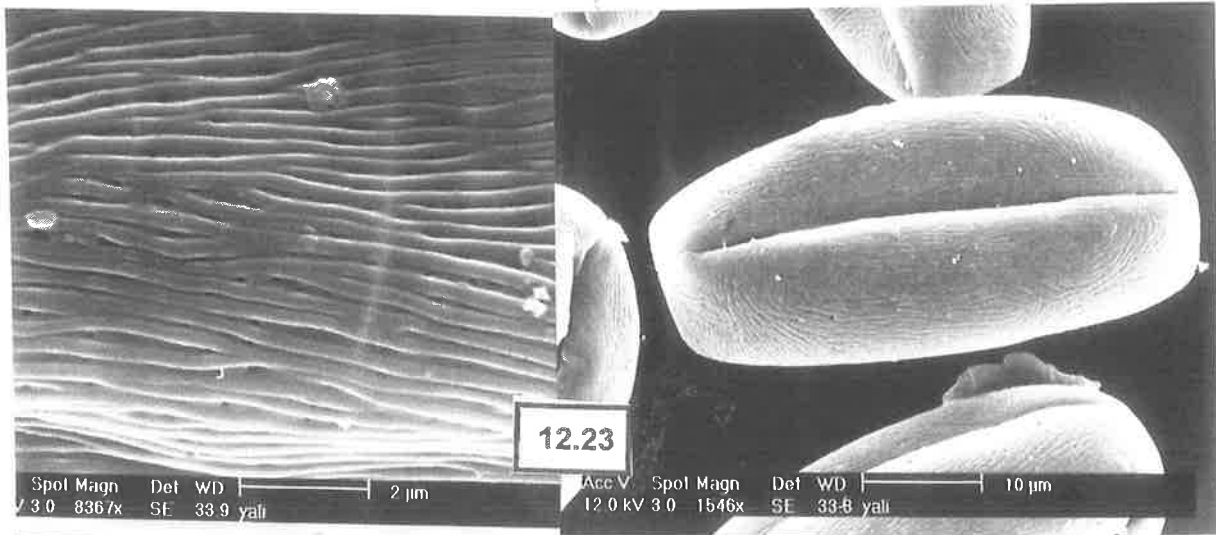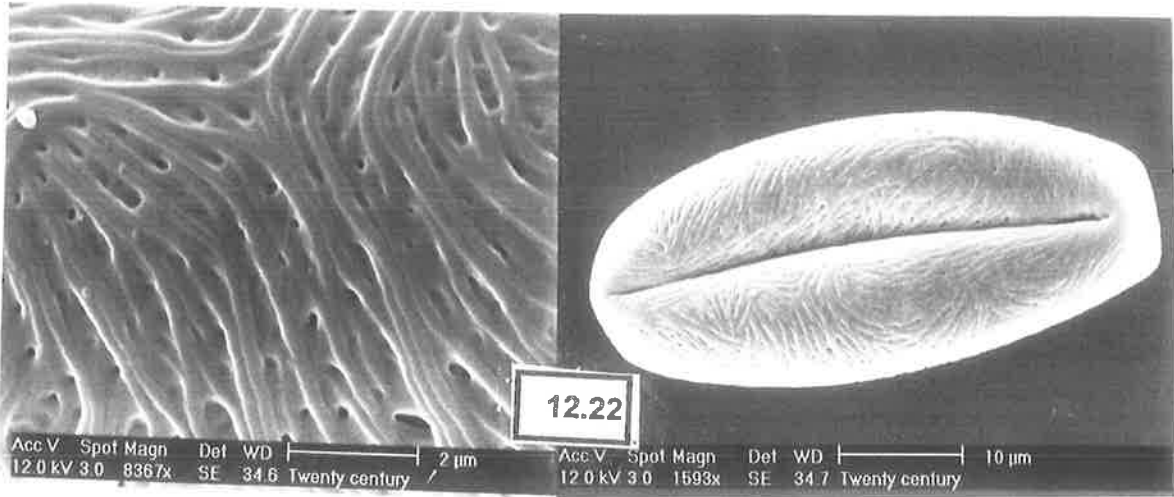
12.22

Acc V   Spot Magn   Det   WD |————————| 2 μm
12.0 kV 3.0 8367x   SE   34.6 Twenty century

Acc V   Spot Magn   Det   WD |————————| 10 μm
12.0 kV 3.0 1593x   SE   34.7 Twenty century

12.23

Spot Magn   Det   WD |————————| 2 μm
3.0 8367x   SE   33.9 yali

Acc V   Spot Magn   Det   WD |————————| 10 μm
12.0 kV 3.0 1546x   SE   33.8 yali

12.24

Acc V   Spot Magn   Det   WD |————————| 2 μm
12.0 kV 3.0 8367x   SE   34.1 winter nelis

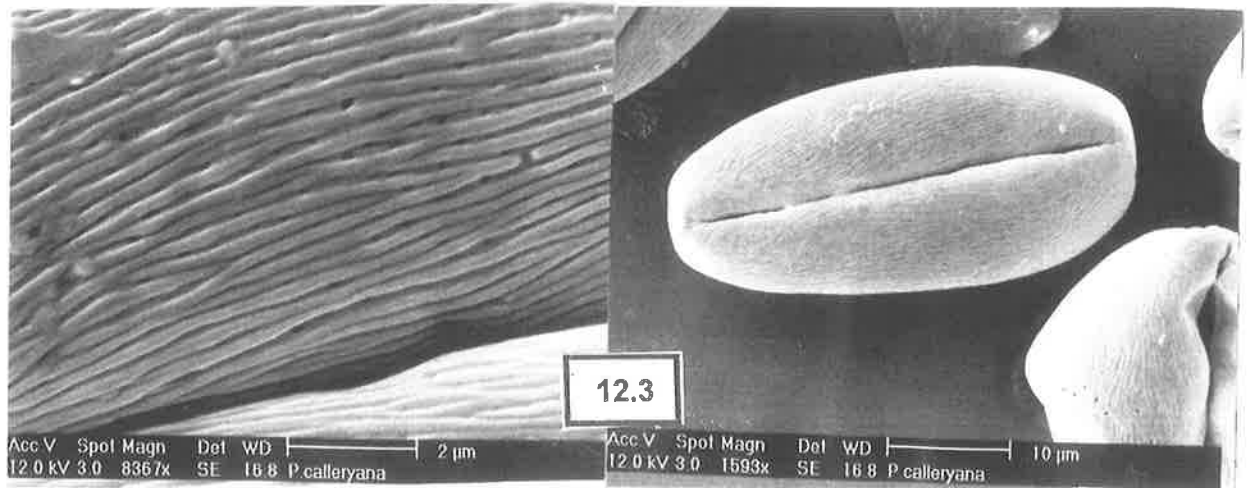Acc V   Spot Magn   Det   WD |————————| 10 μm
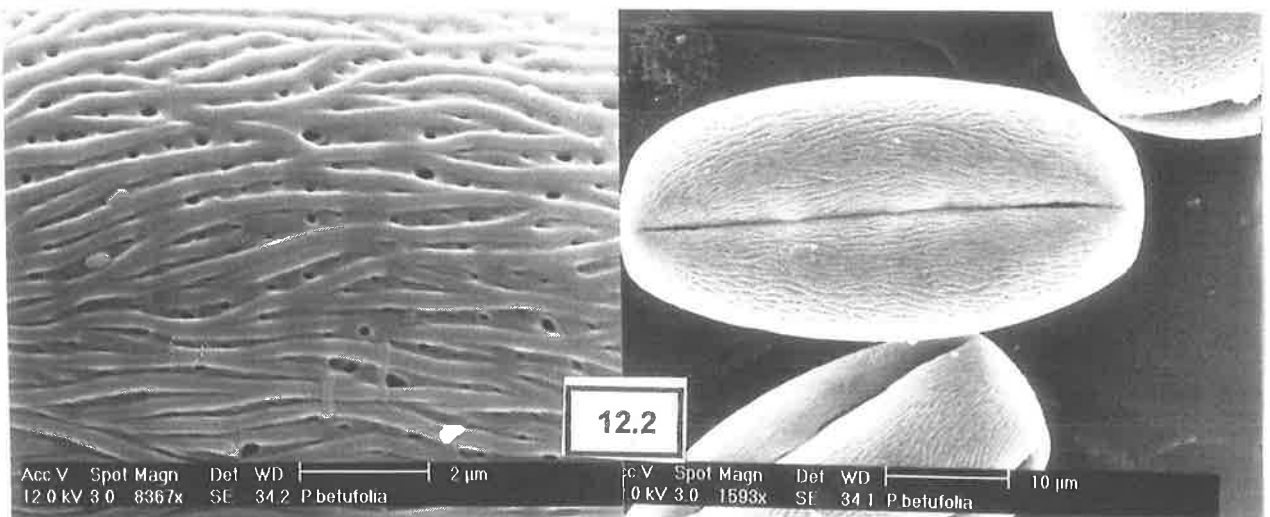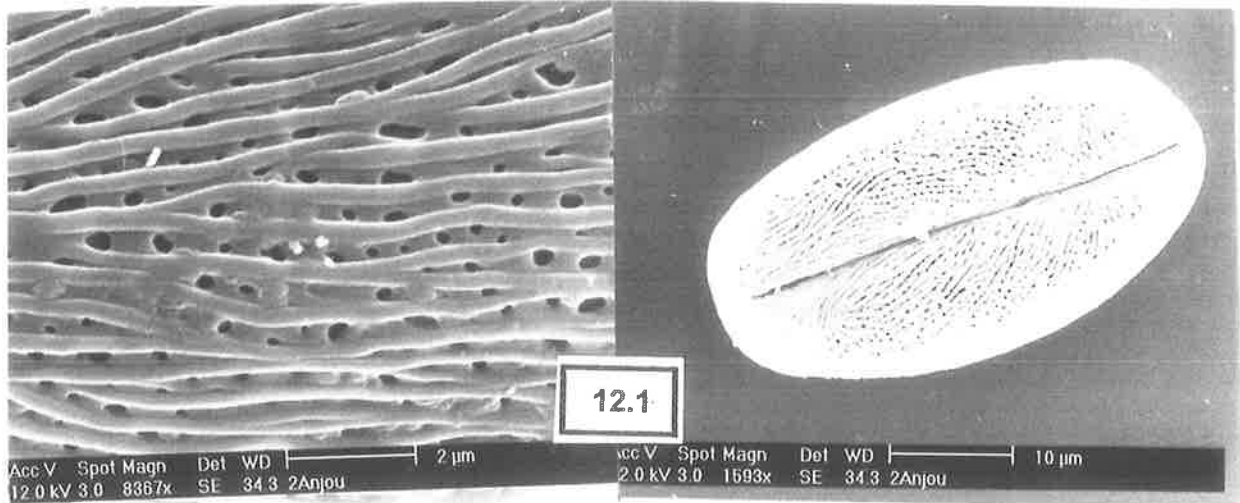12.0 kV 3.0 1593x   SE   34.1 winter nelis

Electron micrograph of pollen grains, showing exine charateristics for cultivars and species of pear listed below.

Fig. 12.25 *P.longipes*

Fig. 12.26 *P.koroshinsky*

Fig. 12.27 *P.ussuriensis maxim*

12.25

Acc V Spot Magn Det WD   2 μm
12.0 kV 3.0 7731x SE 33.9 p.logipes

Acc V Spot Magn Det WD   10 μm
2.0 kV 3.0 1593x SE 33.9 p.logipes

12.26

Acc V Spot Magn Det WD   10 μm
2.0 kV 3.0 1593x SE 33.9 p.koroshinsky

12.27

Acc V Spot Magn Det WD   10 μm
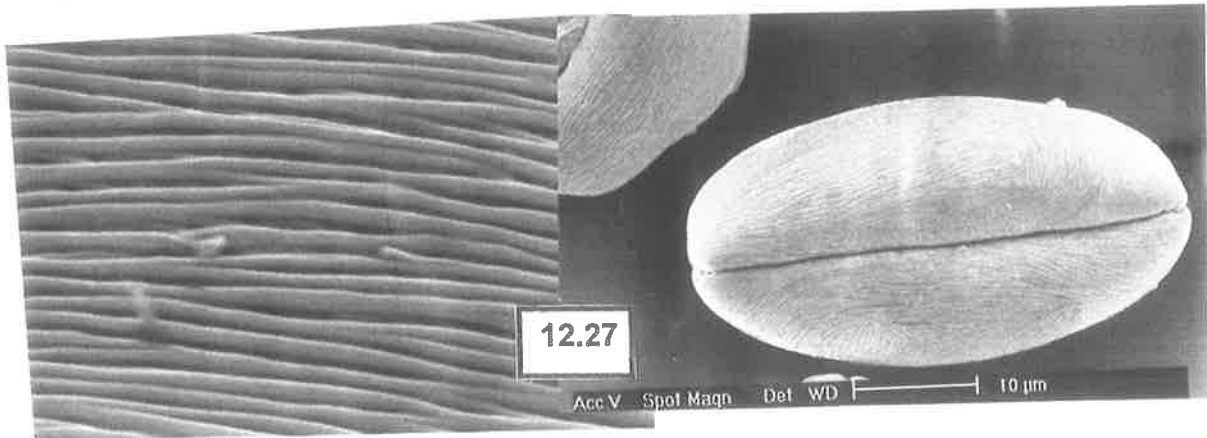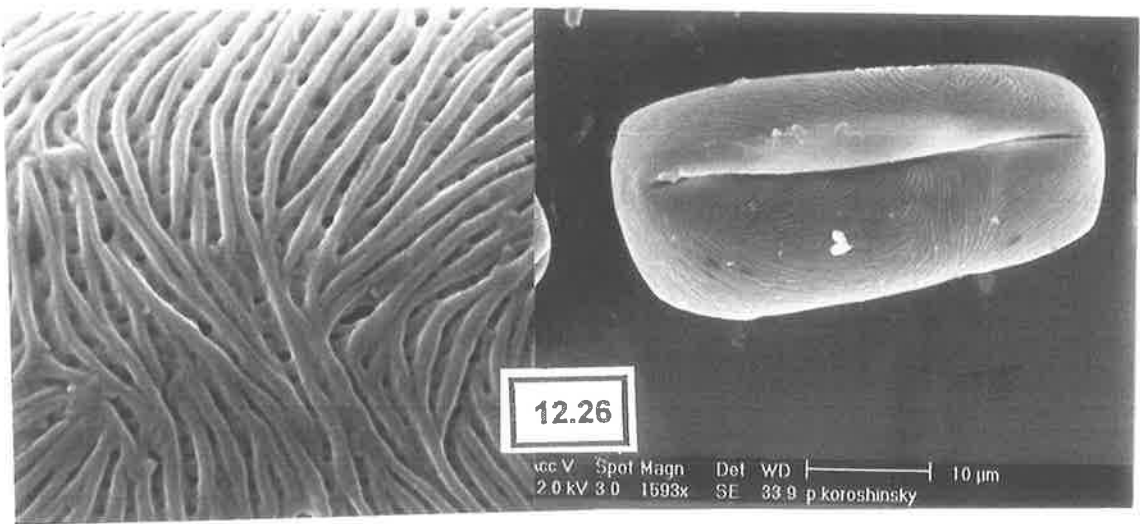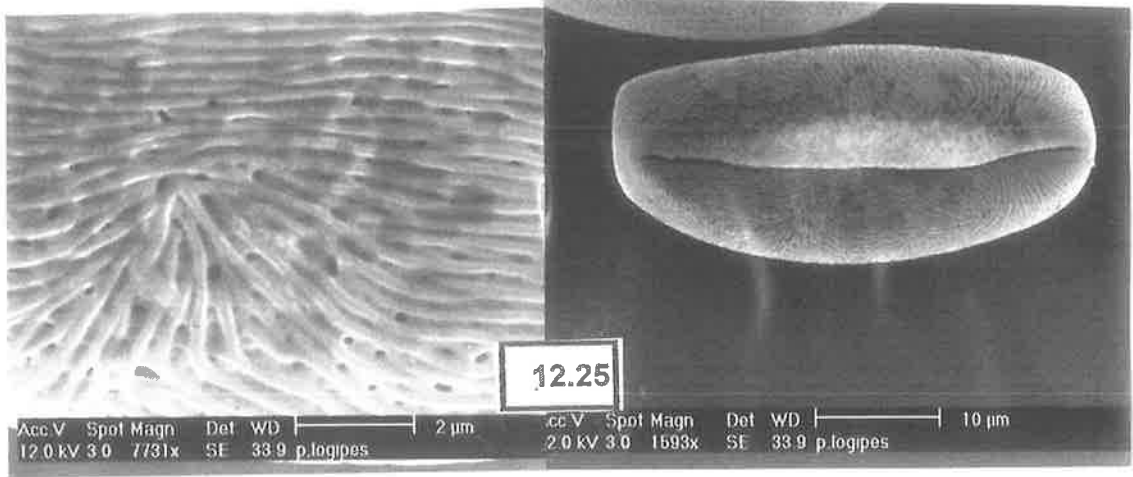
Chapter 13

Pollen Parent Effects on Fruit Characteristics of Pear Cultivars

13.1 Introduction

Fruit set and seed set rates in the various fruit tree cultivars are different. This is due to a combined effects of environment and genetic content on these plants. In self-incompatible plants, pollen parents and maternal tissue can both affect fruit set and seed set. The effects of pollen parent on fruit characteristics have already been reviewed (Khan *et al.*, 1994; Denney 1992). Difference in pollen dispersion "strength" and pollen pistil interaction between cultivars are important aspects of pollen parent effects. The term metaxenia which was coined by Swingle (1928) and extended by Denney (1992) and clarified as pollen parent effects include effects on both embryo and maternal tissue such as pericarp. For date palm, it has been shown that pollen source effects pericarp (Swingle, 1928). Marquard (1988) found that cross pollination increased the nut weight of pecan in comparison with self-pollination. Vezvaei and Jackson (1995) found pollen from Kean cultivar of almond produce heavier nuts on the Price cultivar female recipient. The present study was conducted to examine the effects of pollen sources on seed weight, nitrogen content of fruit, fruit shape, weight and free amino acid contents in different crosses with Packham Triumph and Lemon Bergomot. Free amino acids content in fruit pulp was tested for different crosses.

13.2 Materials and Methods

13.2.1 Pollen Collection and Germination Test

Flowers were collected from each cultivar at the balloon stage (1 day before flower opening). Anthers were excised and stored at room temperature for 48 h. Pollen was collected from dehisced anthers according to methods described by Jackson (1989).

Germination tests for pollen from all parents were carried out using an *in vitro* germination medium containing agar (1%), sucrose (15%) and a mixture of salts (Brewbaker and Kwack 1963). All pollen tested had viability higher than 78% .

13.2.2 Hand Cross-Pollination

Two Packham Triumph trees approximately equal in size diameter of girth and age was selected as female recipients. Josephine, Lemon Bergomot, Howell, Nashi, (Twentith Century) were selected as pollen parent donors. Cross pollinations were achieved based on the methods of Kester and Asey (1975). Before pollination emasculation of the flowers was carried out. Hand pollination was conducted on fifty flowers at the stage of 15 h before flower opening.

13.2.3 Fruit Weight, Fruit Set, Seed Weight and Seed Set (%)

Twenty fruits from each of several crosses were selected and fruit weight, seed set percentage and seed weight measured. Twenty open pollinated fruits on the same trees were picked at random from the four sides of the tree as control treatment. Seed weight for each fruit was obtained by averaging weight of all seeds in the fruit. The data was analysed in a Randomized Complete Block Design program by a Super Anova software. Initial fruit set(%) was measured for 50 flowers one month after pollination and final retained set two weeks before harvesting time.

13.2.4 Nitrogen Measurement

The nitrogen content of the fruit pulp from fruits resulting from different crosses with Lemon Bergomot female recipient was measured to examine possible effects of pollen parents on protein content. Total nitrogen was determined as described in Chapter 2. Three crosses were used for this experiment, included Lemon Bergomot X Nashi, Lemon

Bergomot X Josephine, and Lemon Bergomot X Packham Triumph. Ten samples of fruits for each treatment was obtained at random.

13.2.5 Amino Acid Analysis

Amino acid analysis was carried out on fruits resulting from different crosses with Packham Triumph pears. The effect of the presence and absence of seed was also surveyed in this work. Ten gram from a peeled pear fruit was ground with 5 ml 80% ethanol. The mixture was passed through the filter paper and centrifuged for 10 minutes at 13000 rpm to precipitate all suspended particles. The supernatant was passed through the filter again and was kept at -18°C. The sample solution was analysed for amino acids by HPLC as described in Chapter 2.

13.2.6 Fruit Shape

The two crosses Howell X Packham Triumph (female) and *P.lindley* X Lemon Bergomot were selected for fruit shape study. The ratio of fruit length to diameter was selected as a criteria for changes in fruit shape. The data for each cross and its control was compared in a randomized complete block design. Replication for control treatment for each cross were randomly taken from the same tree but from open pollinated fruits. Other data leads us to believe that open pollination on these trees would most likely have been by Josephine and Packham Triumph respectively, Howell and *P.lindley* pollen were selected because they come from trees with very different fruit shape.

13 . Results

13.3.1 Fruit Set

Results of initial and final retained set on Packham Triumph by hand cross pollination showed that Winter Nelis and Josephine pollen produced higher percentage of fruit set in comparison to other two pollen sources (see Table 13.1).

177

Table 13.1 Fruit set (%) in different crosses with Packham Triumph

Crosses (pollen sources)	Initial set (%)	Final set (%)
Josephine	66.6	44.7
Lemon Bergomot	50	34.3
Nashi	43	21
Winter Nelis	85	50.2

13.3.2 Seed and Fruit Weight

The pollen parent did have a significant effect on fruit weight and seed set percentage (Table 13.2 and Table 13.3). The cross Packham Triumph (female) X Josephine produced a significantly higher seed set (%) than control and the cross Howell X Packham Triumph gave the lowest seed set of the hand pollinated tests (Table 13.2).

Table 13.2: Seed set (%) in different crosses with Packham Triumph.

Crosses	Seed set (%)	Std Error ± (%)
Control (open pollination)	33 a	4.7
Packham X Howel	56 b	6.8
Packham X Josephine	69 bc	6.3
Packham X Lemon	71 bc	4.8
Packham X Nashi	76 c	8.3
Packham X Winter Nelis	58 b	5.8

a, b and c denotes values which are significantly different
from one another. P value <.05.

Table 13.3 Effect of pollen parents on fruit weight in different crosses with Packham Triumph.

Crosses	Fruit weight (g)	Std Error ±(gr)
Control	199.2 ab	4.7
Packham X Howel	196.3 a	13.9
Packham X Josephine	193.7 a	11.57
Packham X Lemon	229.1 b	14
Packham X Nashi	208 ab	10.6

P value < .05 .

Due to the effects of seed number on fruit weight (see Chapter 8), a replication of 10 fruits for three crosses of Josephine, Lemon Bergomot and Nashi with Packham Triumph were used for a comparison for fruit weight. The result also showed that Lemon Bergomot pollen produced significantly heavier fruit.
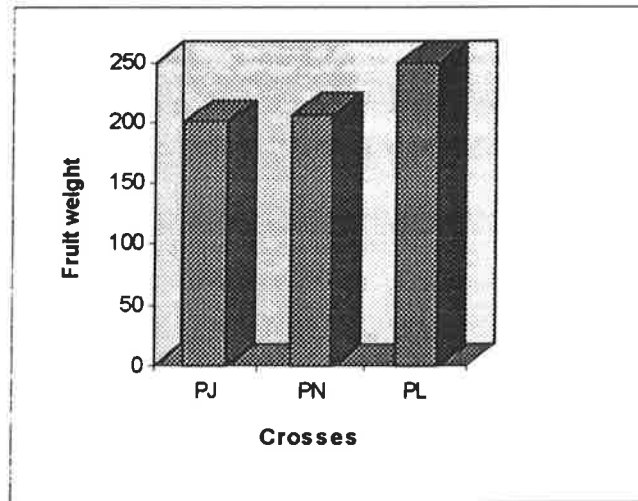
Fig.13.1. Fruit weight comparisons in different crosses with equal number of seeds, (PJ= Packham X Josephine PN= Packham X Nashi, PL= Packham X Lemon).

13.3.3 Dry Weight and Nitrogen Content

Results of drying pear samples showed that between 11.5% and 13.8% of weight of fresh fruit is dry matter. There were significant differences in dry weight of fruits resulting fror different crosses (Table 13.4). Nashi pollen gave rise to a slightly higher dry weight in fruit on Lemon Bergomot in comparison with Josephine and Packham Triumph pollen parents. The nitrogen content of the fruits produced by the various crosses were not significantly different (Table 13.5).

Table 13 .4. A comparison of dry weight of fruits after drying out 20 (g) pulp in oven for the different crosses. Each value is mean of 10 replications.

Crossesx pollen source	Dry weight (g)	Std Error ±(gr)
Lemon B. X Josephine	2.43 a	.049
Lemon B. XPackham	2.34 ab	.029
Lemon B.X Nashi	2.55 b	.046

a,b,c denote values which are significantly different from one another.

Table 13.5. A comparison for nitrogen content in different crosses with Lemon Bergomot as a female parent.

Crosses x pollen source	Nitrogen (g)	Std Error ±(gr)
Control	.011 ns	.010
Lemon B. X Josephine	0.12 ns	.010
Lemon B. XPackham	0.09 ns	.012
Lemon B.X Nashi	0.12 ns	.010

ns denote no significant differences from one another. P> .05

13.3.4 Fruit Shape

The comparisons for each individual cross with its control treatment did not show significant changes in shape of the fruits on Lemon Bergomot and Packham Triumph trees as influenced by pollen from *P.lindley* and Howell. (Table 13.6).

Table 13.6 Two comparisons for effects of ɩ _ɹlen parents on the ratio of L/D in Lemon Bergomot and Packham Triumph fruits.

Crosses	L/D	Std Error ±
Lemon B. X *P.lindley*	0.94 ns	..017
Control	0.96 ns	.025
. PackhamX Howell	1.15 ns	.025
Control	1.13 ns	.025

. Each value is a mean of 10 replications. Ns denote
no significant differences. P >.05

13.3.5 Effect of Pollen Parents on Amino Acid Concentrations

Results of analysis of free amino acid in fruits from different crosses showed variations in four free amino acids including glutamic acid, asparagine, serine and proline (Table 13.7). Lower amounts of free amino acid was observed in parthenocarpic fruit (control) in comparison with other fruits (crosses) which had seeds.

Table 13.7. Analysis of four free amino acids(μg/ml)in different crosses obtained from the fruit pulps.

Treatments	Glutamic acid	Asparagine	Serine	Proline
Control (parthenocarpic fruit)	14	21	4.3	22.2
Packham X *P.boissierana*	46.8	563	8.5	22.9
Packham X Winter Nelis	13.6	16.6	3.5	16.6
Packham X Josephine	36.8	152.8	6.5	16.2
Packham X *P.betulaefolia*	27.7	215.3	6.7	72.2

Hydrolysis of Protein of pulp from fruit in two crosses showed significant differences in the level of valine and lucine (Table 13.8).

Analysis of free amino acids in Lemon Bergomot fruits with different seed numbers showed strikingly different levels of asparagine. Increasing numbers of seeds led to greater accumulation of this free amino acid (Table 13.9).

Table 13.8 Valine and leucine levels in hydrolysates of protein from pulp of fruit from two crosses.(nmol/ml).

Crosses	Valine	leucine
Packham X Winter Nelis	346	500
Packham X Josephine	484	627

Table 13.9 Concentration of the free amino acid asparagine (nmol/ml) in Lemon Bergomot fruits with different number of seeds.

Seed Number	2 seeds	3 seeds	4 seeds	8 seeds
Asparagine content	560	441	3432	7635

Hydrolysis of protein from Lemon Bergomot fruits with 2 and 8 seeds showed a higher level of several amino acids in 8 seeded fruit compared with 2 seeded. This higher level was shown for glutamic acid, serine, valine, methionine, phenylalanine, isoleucine and lysine.

13.4 Discussion

This work has shown that pollen from different cultivars had different effects on fruit set. Fruit shape and seed weight did not show significant differences however. Dry matter (%) and total nitrogen (%) was reduced on Lemon Bergomot female tissue by Packham Triumph pollen. On the other, hand fruit weight on Packham Triumph maternal tissue was increased by Lemon Bergomot pollen as compared to Josephine pollen. As the fruit weight is a result of multiple factors such as fruit number on the branch, seed number in the fruit and fruit position on the branch, it is difficult to interpret these results. Probably, pollen parent affects fruit weight indirectly. Some factors such as pollen pistil interaction, level of the pollen compatibility with pistil, pollen and pistil age may affect on fruit weight. It is also difficult to come to a full understanding of the relationship between fruit weight, dry matter percentage, nitrogen and other major elements and compounds in fruits. Effects of pollen parents on fruit set and seed set has already been confirmed by Church and William (1983b) for apples and Khan *et al.*, (1994) for Cherimoya (*Annona cherimola*).The effect of pollen parents on the chemical make-up of fruits characters has rarely been considered in pome fruits. The degree of fruit ripening in this experiment could possibly influence these results. Hydrolysis of protein showed remarkable differences for valine and leucine in the two crosses of Josephine and Winter Nelis pollen

with Packham Triumph.   Hydrolysis of protein from the pulp of fruits confirmed the effect of seeds in bringing about a considerable increase in total amino acids .

At this stage it is difficult to   make practical suggestions as to preferred pollen sources inferred from the present work.   It would seem however that nutritionally, speaking seeded fruit is better than parthenocarpic fruit due to a higher amino acid and protein content in the former.

Chapter 14

General Discussion

Pyrus fruit tree species and the cultivars are mainly self-incompatible. In these trees cross pollination by agents of pollen transfer such as the honey bee is important to obtain a satisfactory yield of fruit. As found here insufficient pollination lowers the number of seeds within the fruit and leads to uneven growth of fruits resulting in misshapen pears in the case of Packham Triumph cultivar. Efficient pollination relies on many factors including climate, presence of adequate " cross" pollen and pollen dispersal within the orchard, honeybee activity, synchronous flowering between receptive cultivar and other pollinizers, planting system and orchard design. The present project has emphasized some of these factors and the relation between them to better understand pollination efficiency in the orchard.

In chapter 8, the effects of seed number on pear fruit set and shape were evaluated by pollination of different numbers of styles. In this experiment two cultivars, Packham Triumph and Lemon Bergomot were used. The shape of the fruit on Packham Triumph was more affected by seeds or lack of seeds than was fruit on Lemon Bergomot. A greater number of misshapen pears was in accordance with lower number of seeds found in Packham Triumph fruits in the orchard where there was open pollination. Increasing the number of seeds in the fruit had a positive effect on final fruit set (percentage) in both Lemon Bergamot and Packham Triumph. These results are consistent with a hormone released by the seed influencing the relative growth of different parts of the fruit as well as fruit drop or lack of it. The effects of seeds on fruit shape and fruit weight reported here agree quite well with the work of Nakagaw *et al.,* (1968) and Rohitha and Klinac (1990). Pollination of flowers with only one style gave fruits which had no seeds. This results suggests a switching on of a signal to begin expression of a parthenocarpy gene due to non pollination of a higher number of stigmas.

In order to study gene flow by pollen in the orchard, different isozyme systems were employed to find suitable genetic markers by means of controlled crosses. Pollen isozyme analysis was the first step used to characterize pear and species and cultivars.

In chapter 4 GPI, PGM ADH and IDH were found to be suitable isozyme systems for the recognition of the different pears. The banding patterns in the GPI-2 isozyme system for different pears were in accordance with the work of Crezo *et al.*, (1989) and Marquard *et al.*, (1995). With respect to dimeric structure of GPI, interpretation of GPI-2 pattern poses some difficulty (Crezo *et al.*, 1989). PGM showed four distinct zones of activity suggesting four probable loci responsible for this isozyme. The results for ADH were similar to that reported by Crezo *et al.*, (1989) and Marquard *et al.*, (1995). A comparison of similarity matrix using isozymes banding patterns between *P.calleryana* and what has been named *P.ussuriensis maxim* in Australia showed a similarity of 90% between these two. Interestingly, comparison of pollen ultrastructure characteristics between *P.calleryana* and *P.ussuriensis maxim* confirmed this conclusion (Chapter 12). Application of primers GTCCCGACGA and GAACGGACTC on DNA extracts from both *P.calleryana* and so called the *P.ussuriensis maxim* did not show any differences in bands obtained, even though these primers did produce different bands for many other species. All the recent results for comparison of these two species were in agreement with Kellow and Will (1995) who suggested that *P.ussuriensis maxim* in Australia is the same as *P. calleryana.*

In controlled crosses (Chapter 5), there was observed two kinds of segregation ratios for GPI isozyme banding patterns. The ratio 2:1:1 was observed for the cross of Josephine and Lemon Bergomot and Duchess with Packham Triumph as female recipient, while the ratio 1:1:1 was obtained in cross of *Pyrus boissierana* with Packham Triumph. This latter segregation ratio only occurred between crosses involving domesticated with wild pear. Chevreau and Laurens (1987) had already shown that different parental peroxidase phenotypes gave different segregation ratios.

In chapter 10, GPI and ADH isozyme markers are described that allow discrimination between Josephine and Lemon Bergomot pollen genes in seeds borne on Packham Triumph trees. In this same chapter, we used these facts to study gene flow by pollen within the orchard. The outcome was that Josephine was shown to be a better pollinizer than Lemon Bergomot for Packham Triumph under the conditions. It was perhaps due in part to the better synchrony of flowering between Josephine & Packham Triumph. In another part of the orchard, it was shown that increasing distance from a row of Josephine pollinizers showed a reduction in pollination which was reflected in a reduction of seed set (%) per fruit. A decreasing gene flow by

pollen pattern was shown for the number of the seeds within the fruit in relation to the increasing distance from the pollinizers row (as seen also in almond by Jackson and Clarke, 1991b). These results are in general agreement with those of Free (1962) and Marquard (1988), and suggest that closer interplanting of pollinizer with Packham Triumph would be beneficial. A balance between flower number, pollen production and flowering time between main cultivar and pollinizers can assist to create better conditions for increasing pollination efficiency in the orchard. The gene flow by pollen study also showed that there was no trace of Josephine pollen genes in fruits on Lemon Bergomot cultivars, all pollen genes were from Packham Triumph. This may well be because Packham Triumph trees have more flowers and a higher production of pollen per flower in comparison with Josephine leading the bees to favour foraging from Packham Triumph. Furthermore, there were lower numbers of Josephine pollinizer trees in the orchard, compared to Packham Triumph. Another interesting result of gene flow by pollen to Lemon Bergomot was a similar segregation ration in GPI isozyme banding patterns in seeds of fruits that abscised early and fruits that remained on the Lemon Bergomot tree. This result is in accordance with that of Degani et al., (1989) for Avocado. This showed that Packham Triumph trees were probably the only pollen source for pollen deposited on the Lemon Bergomot sink.

The results obtained from the cage experiments (Chapter 7) showed that wind-assisted pollination did not take place in this pear orchard. This result was not in agreement with the experiments of Westwood et al. (1966). It is possible that geographical condition and microclimate characteristics such as wind speed and moisture content of pollen may affect mediation and deposition of pollen.

A comparison of fruit set on both Packham Triumph and Lemon Bergomot trees between cage and non-cage treatment showed no significant difference between them. The cage treatment did not show greater abscission of flowers than non cage treatments. The mechanism for switching on to production of parthenocarpic fruits is obscure. Some fruits developing parthenocarpically in the cage lost their normal shape, this evidence was observed specifically for Lemon Bergomot fruits, which while they could not said to be " misshapen" were often of a different shape (pyriform rather than round oblate) to seeded fruit. It was observed that pollen production for different pear species and cultivars was quite different. The reasons for this difference is still not understood, but is obviously under genetic control. Using different random

oligonucleotide 10-mer primers with the RAPD technique did not produce enough polymorphism to be able to tell the difference between all pear cultivars and species grown in the orchards (Chapter 3).

The primers which produced a few characteristic marker bands for some of the pear species and cultivars were OPA12, OPC07, OPC06, OPD02 and OPD10 (Operon Technology) with sequences of TCGGCGATAG, GTCCCGACGA, GAACGGACTC, GGACCCAACC and GGTCTACACC respectively. Of these primers, OPC07 or GTCCCGACGA was the most effective in giving marker bands. However, using the OPC07 primers with RAPD technique on the progenies of controlled crosses showed that inheritance of marker bands was not predictable enough to allow it to be used for paternity testing of seeds in fruits. RAPD methods were therefore not used to follow gene flow by pollen. Application of specific primers, such as RAMP (Random amplified microsattelite), STS (sequence tagged site) or STM (sequence tagged microsattlite) or an M13 DNA probe could well give greater success in this area ᐧ (Nybom *et al.*, 1990).

As described in chapter 12, pollen ultrastructure can be quite useful to characterize differences between pear species and cultivars. Application of an Image Analysis Computer Program for pit surface area percentage was advantageous for understanding differences between cultivars. The present work builds extensively on that of Westwood and Challice (1978) and confirms some observation made in that earlier work. A difference between the pollen length and width of *P. longipes* found in the present investigation when compared with that of Westwood and Challice (1978) could well be simply due to the climatic differences between that here and that of the USA for the work of Westwood and Challice (Mulas *et al.*, 1989).

Pollen parents had distinct effect on fruit set and seed set on recipient pear trees. This could be due to differences in pollen pistil interaction (Khan *et al*, 1994, Vesvaei and Jackson, 1995). Pollen parents had no significant effect on pear fruit shape, emphasizing influence of female recipient on fruit shape. Other characters such as nitrogen content was not influenced by pollen parents. Dry matter in fruit showed an increase when Nashi pollen was used to set fruit. Further experiments are needed to confirm this result only tested once so far. A preliminary study showed differences in few free amino acids in fruits resulting from four controlled crosses.

Significantly, it was shown that parthenocarpic fruit had much lower levels of amino acid (protein) than seeded fruit. The rate of ripening for different fruits can make for difficulties in measuring differences in amino acid and other compounds in fruit from different treatments, however.

14.1 General Conclusion

The work described here shows that, setting seeds in Packham Triumph pear is very important as affects the shape and weight of the fruit. Increasing seed number production in the fruit depends on the improvement of pollination efficiency in the orchard.

It was concluded that pollination efficiency of the Packham Triumph pear relies on many factors, including orchard design, attractiveness of pollinizers to pollinators, flowering synchrony of its pollinizers and pollen production of the pollinizers. Planting of pollinizers within the row of the main cultivars in comparison with planting out of the row may assist in giving better pollen flow and better pollination of Packham Triumph.

Nonsynchronous flowering between pollinizers and Packham Triumph pear trees resulted in a reduction of Packham Triumph pollination. In addition, an imbalance in pollen production and flower number between the pollinizers and the main cultivar assisted to creating poorer conditions for Packham Triumph pollination. Packham Triumph pear readily sets fruit parthenocarpically and so lower pollination did not affect fruit set, but it did influence shape and weight of the Packham Triumph pears.

Parthenocarpic fruits obtained from the cage treatment of the Packham Triumph had a higher weight of fruit compared with pollinated fruits. However more misshapen fruits were obtained from the cage treatment and so there is a clear preference for fruits with a complete set of seeds with a good shape. It was found that insect-mediated pollination were only means of pear pollination and wind independently had no effect on pear pollen flow and pollination. Of the two pollinizers, Josephine showed better efficiency for pollination of Packham Triumph than did Lemon Bergomot. The results from this study suggested that breeding pollinizers with the high attractiveness to honeybee shown by Packham Triumph would be of value for increasing pollination of Packham Triumph in commercial orchards. Equal attractiveness for the two pollinizers

and the main cultivar can be attained by an increase in the number of flowers, nectar production and similar composition in nectar.

Diversity in isozymes banding patterns, flowering and pollen ultrastructure of pear cultivars and species in this work showed available resources in pear for the breeding of more effective pollinizers. Application of different pollen parents for the pear pollination would not affect the many different aspects of pear quality and quantity but it could influence the fruit set, seed set, and probably the dry matter and some free amino-acids. The different pollen parents did not influence the shape of the fruit and abscission after initial fruit set.

14.2 Future Research Perspectives

Improvement in pollination because of the very dynamic nature of pollination depends on many factors. Some of these factors such as orchard design, pollinizer selections, orchard site, hives number, location and condition can all be controlled by the orchardists and researchers now, in the light of the studies on gene flow by pollen recorded here.

Researchers could improve aspects of pear pollination by :

1-Breeding of improved pollinizers for Packham Triumph so as to increase number of flowers, pollen production, flowering synchrony and to create better nectar quality for honeybee attraction.

2-Finding the relationship of pollen moisture and pollen dispersion by wind, in view of the present finding that wind pollination does not occur in the Adelaide Hills.

3-Relationship study of climate and pollen mediation and dispersion.

4-Finding biochemical reasons for misshapen fruits in Packham Triumph, a result of inadequate pollination leadings to too few seeds in the fruit.

5-Studying the possibility of using enpollination on pears. Enpollination is the artificial addition of cross pollen to honeybees as they depart from the hive for food gathering.

6- Investigation of relationship between vegetative growth and parthenocarpy and growth of fruit when seeds are present.

7- Investigation of relationship between chemical characters of pear nectar and honey bee attraction.

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