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**Isozyme Markers as a Tool in Avocado
Research**

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B. TABLE OF CONTENTS

	Page
A. Cover page	1
B. Table of contents	2
C. Abstract	4
D. Objectives of the research	5
E. Body of the report	6
 <u>I. Work done in Israel</u>	
I-1. Development of new enzyme systems and interpretation of their genetic control	6
I-2. Duplicated phosphoglucose isomerase genes in avocado	9
I-3. Determination of selfed and crossed proportions of avocado progenies produced by caged pairs of complementary cultivars	13
I-4. Isozyme analysis of mature avocado embryos to determine outcrossing rate in 'Hass' plot	19
I-5. Pollen parent effect on outcrossing rate in 'Hass' and 'Fuerte' avocado plots during fruit development	23

I-6	Genetic selection during the abscission of avocado fruitlets	51

II. Work done in U.S.A

II-1.	Segregation distortion and linkage analysis of hand pollinated avocados	53
II-2.	Total phenolics and high performance liquid chromatography of phenolic acids of avocado	59
II-3.	Production of protoplasts from avocado leaves	64
F.	Description of cooperation	66
G.	Main achievements of the research	67
H.	List of publications	69

C. ABSTRACT

Using three newly developed isozyme systems as well as three other known systems, we identified the pollen parent in avocado progenies obtained by enclosing 2 cultivars of complementary flower types in a screenhouse with a beehive. The observed percentage of outcrossing ranged from 2% to 100%. These results demonstrated the usefulness of isozyme analysis in screening avocado progenies of caged cultivars for pollen parentage. Such screening in the mature seed or young seedling stage increases greatly the efficiency of breeding programs.

'Ettinger' was found to excell as a pollen parent for various cultivars in cages. In the case of 'Hass', 'Ettinger' was found to be a highly effective pollen parent not only in cages but also in the orchard, where fruit yield decreased with increasing distance from 'Ettinger' and decreasing rate of hybrid formation. Contrary to previous reports, we found that cross-pollination was not limited to the nearest row adjacent to the pollen parent, but occurred also at much greater distances. We have shown that even a distance of 100 m cannot be considered as a safe distance for self-pollination for breeding purposes, as has been previously claimed.

We have also shown that the pollen parent has an important role in the selective abscission of avocado fruitlets. Furthermore, isozymic analysis of 'Ettinger' embryos at different stages of fruit development showed selective abscission of specific genotypes, indicating the importance of genetic selection in avocado fruitlet abscission.

Studies on genetic linkage groups for isozyme loci in population of Australian hand pollinated avocados have shown that only two genes were linked, Got-1 and Got-2. In another study on phenolic acids content in various avocado tissues, the content was found to be highest in cotyledons and least in mesocarp.

D. OBJECTIVES OF THE RESEARCH

- 1) To develop additional isozyme markers and to develop methods for testing additional tissues such as embryos and pollen. These advances could then be applied for identification of avocado cultivars and for parentage analyses of avocado progeny, in order to achieve objectives 2 and 3.

- 2) To develop the use of isozymic analysis for identification of hybrids as opposed to selfs in progeny produced by caging two cultivars of complementary flowering groups in a screenhouse. Parentage analysis at mature seed or young seedling stage should increase the efficiency of the breeding work conducted both in Israel and U.S.

- 3) To identify the male parent of avocado fruitlets and fruits in commercial plantings in order to assess the effectiveness of different avocado cultivars as pollen parents.

E. BODY OF THE REPORT

I. Work done in Israel

I-1. Development of new enzyme systems and interpretation of their genetic control.

Materials and Methods

The running system used for phosphoglucose isomerase (PGI) consisted of 0.03M tris-citrate (pH 8.0) gel buffer and 0.4M sodium borate (pH 8.7) tray buffer. For triosephosphate isomerase (TPI), the system consisted of 0.016M tris-citrate (pH 6.9) gel buffer and 0.049M tris-citrate (pH 6.9) tray buffer. The running system used for glutamate oxaloacetate transaminase (GOT) was Ashton (1). PGI, TPI and GOT were detected by staining procedures described in references 2, 3 and 4, respectively.

Results and Discussion

Methods were developed for resolving the isozyme systems of PGI, GOT and TPI in leaf, pollen and embryo tissues. Their genetic control was interpreted by comparing the isozyme patterns of the tissues leaf (diploid) versus pollen (haploid) and by segregation analysis of self-pollinated progeny.

Three PGI genes were identified: one monomorphic locus, Pgi-1, coding for the plastid isozyme and two independently assorting loci, Pgi-2 and Pgi-3, coding for the cytosolic isozymes. Evidence was obtained for PGI

gene duplication in avocado. The high isozymic variability of phosphoglucose isomerase rendered it a suitable 'fingerprint' for identification of avocado cultivars. The PGI study started during the period of BARD grant No. I-201-81 and the genetic analysis was completed during the period of the present BARD grant.

In GOT, we succeeded in identifying appropriate conditions for resolving the enzyme from leaves using Ashton buffer. Under the appropriate resolution conditions we have found that the cathodal set GOT-1 is a dimeric enzyme having fast (F) and slow (S) as the most common alleles.

In TPI, two sets of bands could be distinguished: the cathodal set TPI-1 and anodal set TPI-2. TPI-1 was found to be a dimeric enzyme originating from a single locus with two alleles, F and S. TPI-2 appears to be a plastid enzyme, yet not genetically analysed. (For further details see ref. 3.).

Tpi-I and Lap-2 were examined for evidence of genetic linkage in hybrid progeny of 'Hass' and 'Ettinger'. Allele segregation ratios were obtained by starch gel electrophoresis of the cotyledon tissue of 242 'Hass' fruits. Statistical analysis of these ratios indicated that these loci assort independently.

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I-3. Determination of selfed and crossed proportion of the avocado progenies produced by caged pairs of complementary cultivars.

Materials and Methods

Hybridization was attempted by caging 2 complementary cultivars in a screenhouse with a beehive during flowering. The following 3 different systems were used: (1) caging of 2 mature trees of complementary cultivars ('Ettinger' x 'Wurtz', 'Wurtz' x 'Ettinger', 'Ettinger' x 'Pinkerton' and 'Pinkerton' x 'Ettinger'); (2) caging of a single mature tree with a large central branch, comprising 10-20% of the tree canopy, grafted to a complementary cultivar 2 years earlier ('Tova' x 'Regina', 'Regina' x 'Tova', 'Tova' x 'Horshim', 'Horshim' x 'Tova', 'Ettinger' x 'Hass', 'Hass' x 'Ettinger', 'Ettinger' x 'Rosh-Hanikra' and 'Rosh-Hanikra' x 'Ettinger'); and (3) caging of a single mature tree with 2 grafted plants in large containers ('Ettinger' x 'Pinkerton' and 'Pinkerton' x 'Ettinger').

Samples of mature healthy leaves of authentic parents and progeny were prepared for horizontal starch gel electrophoresis as previously described (1). Isozymic analyses were carried out for MDH (1), TPI (2), LAP (3), PGM (3), PGI (9) and GOT (See "materials and methods" in section I-1).

Results and Discussion

Improvement of avocado fruit quality and productivity can be obtained by hybridization. The only economical way of producing avocado hybrids presently known is by using bees in a closed system to perform the millions of pollinations needed to obtain the thousands of seeds required for an

effective breeding program (5-7). Because of the synchronously dichogamous nature of the avocado flower, it was assumed that by caging two cultivars of complementary flowering groups, the opportunity for cross-pollination should be maximal and the majority of fruits produced would be hybrids (5-7). A previous study using malate dehydrogenase isozyme system, led us to the conclusion that, frequently, this assumption is not valid (1). This conclusion is strongly supported by the work done during the period of the present grant in which we have analysed 4000 seedlings for male parent, using the known isozyme systems (MDH, PGM and LAP) as well as the newly developed ones (PGI, TPI and GOT). Representative results are shown in Table 1, while Table 2 indicates the flower type of the corresponding caged cultivars. The observed percentage of outcrossing ranged from 2% to 100%. These results clearly confirm our conclusion that caging does not guarantee the formation of hybrids.

Our studies showed that different pollen parents differed markedly from each other in their effect on hybrid progeny produced in caged pairs (Table 1). A mature avocado tree has hundreds of thousands of flowers, while the number of mature fruits carried by the tree is usually only a few hundreds (8). The extent of fruitlet abscission may be enormous (9). Different pollen parents may differ widely in their effects on the survival of their progeny. The results (Table 1) suggested that a massive abscission of avocado fruitlets may give rise to a situation where very low percentage of pollination with a certain pollen will result in a high percentage of mature fruits originating from this pollen, if this pollen has advantage over other pollen during the stages of fruit-set and abscission

'Ettinger' was consistently found to excell as a pollen donor producing large percentage of hybrids when used as a pollen parent for 'Hass', 'Wurtz', 'Rosh-Hanikra' and 'Pinkerton' (95%, 100%, 74% and 100%, respectively). At the same time 'Ettinger' in the reciprocal combinations usually produced a large proportions of selfed progeny (Table 1). Apparently the 'Ettinger' pollen confers a better chance of survival to its progeny.

Table 1. Percentage of outcrossing in avocado progenies produced by caged pairs of complementary cultivars.

<u>Parents</u>		No	Outcrossing
<u>Female</u>	<u>Male</u>	<u>Seedlings</u>	<u>%</u>
'Tova'	'Regina'	110	50
'Regina'	'Tova'	100	53
'Tova'	'Horshim'	106	70
'Horshim'	'Tova'	400	100
'Ettinger'	'Hass'	86	2
'Hass'	'Ettinger'	107	95
'Ettinger'	'Wurtz'	136	2
'Wurtz'	'Ettinger'	69	100
'Ettinger'	'Rosh-Hanikra'	98	20
'Rosh-Hanikra'	'Ettinger'	76	74
'Ettinger'	'Pinkerton'	103	23
'Pinkerton'	'Ettinger'	218	100

Table 2. Flower type of caged avocado cultivars mentioned in Table 1.

<u>Cultivar</u>	<u>Flower type</u>
'Tova'	A
'Regina'	B
'Horshim'	B
'Ettinger'	B
'Hass'	A
'Wurtz	A
'Rosh Hanikra'	A
'Pinkerton'	A

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Pollen Parent Effect on Outcrossing Rate in 'Hass' and 'Fuerte'
Avocado Plots During Fruit Development

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Abstract

Outcrossing rates in 'Hass' and 'Fuerte' avocado blocks were determined during fruit development, using the isozyme systems of malate dehydrogenase (MDH) (EC 1.1.1.37), leucine aminopeptidase (LAP) (EC 3.4.11.1), and triosephosphate isomerase (TPI) (EC 5.3.1.1.) as genetic markers. Abscission of avocado fruitlets was selective and was greatly influenced by the pollen parent. Thus, in 'Hass' trees subjected to cross-pollination by 'Ettinger' and 'Fuerte', the population of 'Hass' fruitlets one month after fruit-set consisted mainly of 'Hass' selfs; however during fruit development the rate of 'Hass' selfs decreased and the rate of 'Hass' fruitlets produced by 'Ettinger' and 'Fuerte' increased, with a significant advantage to 'Ettinger' progeny. By the end of fruit abscission the surviving mature 'Hass' fruits were mostly 'Ettinger' hybrids.

The 'Hass' fruit yield was found to correlate significantly with the rate of outcrossing with 'Ettinger'

When 'Ettinger' served as a pollen parent for 'Fuerte' the outcrossing rate in trees adjacent to 'Ettinger' was about 40%, showing that cross-pollination among avocado cultivars of the same flowering group can be quite efficient at close proximity. The outcrossing rate in 'Fuerte' diminished with increasing distance from 'Ettinger', but no significant effect on yield was observed.

Avocado (Persea americana Mill.) is considered to be an outcrossing plant due to the synchronously dichogamous nature of its flowering behavior (2,9,14,22,23,24). Thus, in many orchards two complementary cultivars were usually planted in order to ensure adequate pollination and optimum yields (3,21,25). On the other hand it was noted that some cultivars were successfully self-pollinated, since their screened trees produced well (7,8,14), and large solid blocks of 'Hass' in California consistently set good crops (20).

The question of whether interplanting of cultivars is indeed necessary in commercial orchards was addressed by Bergh et al., who studied the effect of interplanting on avocado yield (4-6). They found that when different several avocado cultivars were planted adjacent to a complementary cultivar, significantly higher yields were obtained. The effect was limited however to the first row adjacent to the pollen donor. These authors suggested that cross-pollination was the primary cause of the yield increase and that close proximity or even overlapping of branches of the complementary cultivar is essential for pollen transfer by bees, which are considered to be the main avocado pollinators.

The development of isozymes as genetic markers in avocado (18,19,28,29) made it possible to distinguish hybrid offspring from selfs (10) and to determine rates of outcrossing between cultivars in the orchard (18,27,31). In the present work isozyme analysis was used to study the effect of pollen parent proximity on outcrossing rates during fruit development, with the objective of determining the

difference, if any, between the outcrossing rates at harvest time and the initial outcrossing rate at the beginning of fruit development.

Materials and Methods

The research was carried out in two avocado plots planted in 1979 in the commercial avocado orchard of Kibbutz Givat Brenner, near Rehovot.

1) The 'Hass' plot (Fig. 1, plot 3) has 28 rows (7.5 m apart), with 17 trees per row (4.5 m apart). The plot was terminated by two rows of 'Ettinger' at the western side and was in close proximity to one 'Ettinger' row across a narrow path at the eastern side. To the north, among 'Hass' trees in the adjacent plot 4, stands a group of 'Fuerte' trees, with another group of 'Fuerte' and 'Reed' trees nearby. Two trees in plot 3 were identified as 'Fuerte' and 'Reed' and are marked in Fig. 1.

2) The 'Fuerte' plot trees planted 7.5x6 m apart (Fig. 2, plot 7), was located adjacent to the 'Ettinger' plot (Fig. 2, plot 8).

During May-October 1984, fruitlets and fruits were sampled at different developmental stages. Their embryos were assayed in three isozyme systems: malate dehydrogenase (MDH), leucine aminopeptidase (LAP) and triosephosphate isomerase (TPI). In very young fruitlets (50-100 mg) the whole embryo with its endosperm were crushed in 100 μ l extraction buffer (10), and the extracts were absorbed on 4x6 mm

Whatman 3MM paper wicks. Extracts for electrophoresis were prepared according to Torres (26) from embryos of larger fruitlets.

The three isozyme systems were separated by horizontal starch gel electrophoresis (starch obtained from Sigma) and stained as described previously for LAP (11), MDH (10) and TPI (17,30).

Results

Outcrossing rate from fruit-set to maturity in 'Hass'

'Hass' fruitlets were sampled from the same trees on four dates, from the middle of May to the end of October. On each occasion usually five average-sized fruitlets per tree were picked from four pairs of trees in each row. The embryos obtained from the fruitlets were assayed in three different isozyme systems: TPI-1, LAP-2 and MDH-1. TPI-1 is a dimeric enzyme having fast (F) and slow (S) as the most common alleles (18,30). LAP-2 is a monomeric enzyme having F and S alleles (29). MDH-1 is a dimeric enzyme having F and S alleles (28). Table 1 presents the isozyme genotypes in these three systems for the four cultivars present in the area (Fig. 1).

'Hass' embryos heterozygous for Tpi-1 are the offspring of either 'Ettinger' or 'Fuerte'. Those heterozygous for Lap-2 are 'Ettinger' offspring, while those heterozygous for Mdh-1 could be the offspring of either 'Fuerte' or 'Reed'. Since only a few 'Reed' trees were present in the area (Fig.1), and since 'Reed' started to flower only

at the end of the 'Hass' bloom, we may assume that 'Hass' embryos heterozygous for Mdh-1 were 'Fuerte' offspring.

The crosses described above occur between a heterozygote ('Fuerte' for Mdh-1, 'Ettinger' for Lap-2, and both for Tpi-1) and a homozygote ('Hass' for all three). The hybrids may thus be expected to segregate in a homozygote to heterozygote ratio of 1:1 in each of the three isozyme systems. The overall rate of outcrossing should therefore correspond to the observed rate of heterozygotes multiplied by two.

The average rates of heterozygosity in 'Hass' fruitlets for each of the three isozyme systems determined on the four different sampling dates are presented in Table 2. The contribution of each of the two potential pollen donors, 'Ettinger' and 'Fuerte', was calculated. Judging by the percentage of heterozygotes for Tpi-1, the rate of hybrid formation increases consistently during fruit development and abscission up to a value of about 84% ($41.8\% \times 2$) at harvest time. The percentage of 'Fuerte' offspring (calculated from the percentage of heterozygotes for Mdh-1) increases during few weeks of early fruit development from May (0.8%) to June (36.4%) and then decreases to a value of 22.4% at maturity. The percentage of 'Ettinger' offspring (calculated from the percentage of heterozygotes for Lap-2) increases from about 44% in June to about 74% in October.

The distance-dependence of the effect of each pollen parent was then examined. The average percentage of heterozygotes for Lap-2, which identifies 'Ettinger' pollination, determined on different dates in the 'Hass' rows, is presented in Fig. 3. With increasing distance from

'Ettinger', i.e. towards the center of the plot, the rate of heterozygosity decreased, reaching its lowest value between rows 18 and 20 (135 m and 150 m from 'Ettinger' at the western side of the plot). It rose again on approaching the 'Ettinger' row to the east side of the plot (see Fig. 1). During the period between June and October, an increase in the rate of heterozygosity for Lap-2 was particularly noticeable in the middle of the plot. It should be noted that the rate in rows 3 and 4 was higher than 50%.

Figure 4 presents the percentage of 'Fuerte' progeny in all 'Hass' trees sampled, as a function of the distance between the 'Hass' tree and its nearest 'Fuerte' neighbor. As indicated by the three equations in Fig. 4 the percentage of 'Fuerte' offspring (i.e., the percentage of heterozygotes for Mdh-1) is a linear function of $\ln (\text{Dis F})$ (natural logarithm of the distance from the nearest 'Fuerte' tree). The time-related increase of the intercept and the decrease of $\ln (\text{Dis F})$ coefficient reflect the fact that mature 'Fuerte' offspring were found only very close to 'Fuerte' trees.

Statistical analysis of the relationship between heterozygotes for Mdh-1 ('Fuerte' offspring) and $\ln (\text{Dis F})$ and percentage of heterozygotes for Lap-2 ('Ettinger' offspring) showed significant correlations for September and October (equations 1 and 2) but not for June.

$$\text{eq. 1: } \% \text{ Mdh-1} = 40.5 - 6.4 \ln (\text{Dis F}) - 0.20\% \text{ Lap-2}$$

$$R^2 = 0.27 \quad p < 0.001$$

eq. 2: $\% \text{ Mdh-1} = 51.4 - 7.7 \ln (\text{Dis F}) - 0.027\% \text{ Lap-2}$

$$R^2 = 0.38 \quad p < 0.001$$

The conclusion to be drawn from these equations is that the survival rate of 'Fuerte' progeny depends on its distance from both 'Fuerte' and 'Ettinger' trees. Increasing the distance from 'Fuerte' and approaching 'Ettinger' (Fig. 1) results in a lower survival rate of 'Fuerte' offspring.

In February 1985 all 'Hass' fruits were harvested. Figure 5 presents the average yield per tree (16) together with the average rate of Lap-2 heterozygosity per tree as determined for each row in September and October. The data show a decrease in yield with increasing distance from 'Ettinger', and decreasing rate of heterozygosity ($r=0.85$).

Changes in percentage of 'Ettinger' offspring during 'Fuerte' fruit development

On nine dates, from the beginning of May to the end of October, fruitlets and fruits were sampled from eight 'Fuerte' rows at different distances from 'Ettinger' (Fig. 2). Because of the low fruit-set in the orchard, a small number of fruitlets was sampled from a relatively large number of trees. Fruitlets of average size were selected and then embryos were assayed for LAP-2. The only possible pollen donor responsible for 'Fuerte' embryos heterozygous for Lap-2 is 'Ettinger'.

In very young fruitlets no LAP-2 activity was detected. Table 3 presents the average rates of heterozygosity from consecutive periods of fruit development. No significant differences was found between these rates. Figure 6 presents the average rate of Lap-2 heterozygots in each row, and shows a decrease in the rate of 'Ettinger' offspring with increasing distance from 'Ettinger' trees. No correlation was found between rates of outcrossing and yields.

Discussion

A cross between heterozygous and homozygous plants is expected to yield a maximum of 50% of heterozygous offspring. Our results (Fig. 3) show that the percentage of 'Hass' fruits heterozygous for Lap-2 in the rows adjacent to 'Ettinger' to the west (i.e. rows 3-5) was higher than 50%, reaching a maximum value of 68%. These findings are consistent with the results of a preliminary study carried out one year earlier in the same plot, showing that the rate of heterozygosity for Lap-2 in the four rows adjacent to 'Ettinger' ranged between 60% and 74% (17). Furthermore, studies of outcrossing rates in another solid block of 'Hass' close to 'Ettinger' showed rates of heterozygosity for Lap-2 of 56% and 58% (18). The consistency of these results suggests that the unexpectedly high ratio of heterozygotes to homozygotes is unlikely to be due to chance. This phenomenon probably arises from genetic selection, similar to that found in self-pollinated 'Ettinger' progeny, and resulting in a high preference of heterozygotes over homozygotes (11). Obviously, under conditions of

genetic selection favoring heterozygotes, the overall rate of outcrossing is not a simple two-fold multiplication of the percentage of heterozygotes, but is a product of multiplication by a factor lower than 2. The precise magnitude of this factor remains to be determined.

Genetic selection, resulting in a better chance of survival of heterozygotes for Lap-2, is probably responsible for the fact that in 'Hass' fruits (Table 2) the observed rate of 'Ettinger' progeny (heterozygous for Lap-2) is higher than the difference between the total outcrossed progeny and 'Fuerte' progeny (heterozygous for Mdh-1) and not equal to this difference, as would be expected for an independent assortment of the loci.

The dependence of the outcrossing rate in 'Hass' on the distance from the 'Ettinger' pollen parent is shown in Fig. 3. 'Ettinger' (type B cultivar), previously shown to be a potent pollen parent (10,18), was again found to excel as a pollen parent for 'Hass', a complementary type A cultivar. On the verge of fruit maturity (September-October), the percentage of 'Hass' fruit identified as 'Ettinger' progeny (heterozygous for Lap-2) in the three rows adjacent to the adult 'Ettinger' trees (rows 3 to 5, Fig. 3), was in the range of 50% to 60%. Apparently, almost all fruits in these three rows were 'Ettinger' hybrids. A clear and consistent decline in the percentage of 'Ettinger' hybrids can be seen up to row 10, followed by a fairly constant value of approximately 20% (corresponding to approx. 40% 'Ettinger' progeny) up to row 22. On approaching the young 'Ettinger'

trees the rate of heterozygosity increased again to 40%. The relatively lower percentage of heterozygotes to the east of the 'Hass' block is probably attributable to the small size of the young 'Ettinger' trees and their relatively greater distance from 'Hass' trees (Fig. 1).

The proportion of identified 'Ettinger' hybrids in the 'Fuerte' rows adjacent to the 'Ettinger' block was about 20% (Fig. 6). As 'Fuerte' and 'Ettinger' belong to the same flowering group B, these results demonstrate that cross-pollination among cultivars of the same flowering group can be quite efficient at close proximity. Except for an unexplained drop in trees numbered 11 (3 trees away from the 'Ettinger' plot), there is a gradual and consistent decline in the rate of 'Ettinger' progeny down to a value of 2% in the trees numbered 1 (13 trees away from 'Ettinger' plot, i.e. a distance of approximately 80 m).

Our results (Figs 3 and 6) do not support the conclusion that cross-pollination is limited to the nearest row adjacent to the pollen parent or even restricted to overlapping branches (4-6). In addition, whereas others have claimed that a distance of 100 m can be considered as a safe distance for breeding purposes (2), the present results confirm our earlier finding (18) that a considerable rate of cross-pollination can occur at a distance of 100 m or more (Fig. 3) from a potential pollen parent.

Although the percentage of 'Hass' and 'Fuerte' heterozygotes show a consistent gradual decline with the distance from the pollen parent

(Figs 3 and 6), a significant percentage of cross-pollinated progeny occur even at a considerable distance. The fact that cross-pollination does not disappear completely over great distances from the pollen parent is probably the result of indirect pollen transfer by pollen exchange in the beehive (12). This might be responsible for the constant level of 'Ettinger' hybrids observed in rows 10-22 within the 'Hass' block (Fig. 3).

It should be noted that the rates of cross-pollination or cross-fertilization were not determined in this study. Hybrid tissues (embryo and endosperm) could be separated from maternal tissue (seed coat) only after the fruitlets had reached a minimum weight of 50 mg, by which time the fruitlets were already about 4 weeks old (13,15), and a massive abscission of flowers and fruitlets had occurred. The extent of this abscission may be affected by the pollen parent (1). Fruitlets originating from different pollen parents exhibit different rates of abscission; it therefore cannot be assumed that the percentage of hybrids in 1-month-old fruitlets reflects the rate of cross-pollination. In fact we found remarkable and significant changes in the percentage of 'Ettinger' and 'Fuerte' hybrids during 'Hass' fruit development (Table 2). One month after fruit-set the population of 'Hass' fruitlets consisted mainly of 'Hass' selfs, the rate of hybrid occurrence being 22% (11% identified). At the end of the abscission period the rate had increased to 84% (42% identified). This increase in percentage of hybrids resulted from selective abscission in which progeny of 'Ettinger' and 'Fuerte' displayed an advantage

over 'Hass' selfed progeny. A similar trend was found by Gazit and Gafni during the first three weeks of fruit development following hand pollination (15).

When 'Ettinger' served as a pollen parent for 'Fuerte' no selective abscission of fruitlets occurred (Table 3). It should be noted however, that we had to start the isozyme analysis with fruitlets weighing 100 mg, since LAP-2 activity could not be detected until that stage. It is therefore possible that in the early stages of fruit development selective abscission of 'Fuerte' selfs versus 'Ettinger' progeny took place. This is supported by the finding that a higher survival of 'Fuerte' x 'Ettinger' hybrid fruitlets than of 'Fuerte' selfs occurred after hand pollination (15).

The selective abscission of fruitlets may be affected by factors such as genetic selection, sensitivity to environmental conditions in the orchard, and ability to compete with vegetative growth and with neighboring fruitlets. The present study provides some insight into the nature of competition among neighboring fruitlets. The proportion of 'Hass' fruitlets which are 'Fuerte' progeny appears to have reached a maximum value of 36% (18% identified) in June and then decreased to 22% (11% identified). At the same time the rate of 'Ettinger' progeny showed a consistent increase (Table 2) until it reached a maximum value of approximately 80% (about 40% identified). It seems that in the first period, soon after fruit-set, the competition was between 'Hass' selfed fruitlets on the one hand and 'Fuerte' and 'Ettinger' progeny on the other. Later, when most of the surviving fruitlets were

the progeny of 'Fuerte' and 'Ettinger', there was competition between these fruitlets with a significant advantage to the 'Ettinger' progeny. This is in agreement with the finding that 'Hass' fruitlets originating from 'Ettinger' showed a significant advantage over 'Fuerte' progeny three weeks after hand-pollination of 'Hass' flowers had been accomplished (15).

The enormous number of flowers found in avocado and the massive abscission of fruitlets point to the importance of tolerance and competitive ability in the survival of fruitlets. It is possible to increase the survival chances of the fruitlets of a given cultivar by genetic improvement of the embryo and endosperm, using a pollen parent which confers traits of improved tolerance and greater competitive ability. Though 'Ettinger' was found to be a potent pollen parent for 'Hass' (Fig. 3 and (11)), it cannot be assumed that 'Ettinger' will always excel as a pollen donor. Its positive effect on yield is probably due mainly to its capability to bestow properties such as greater tolerance to environmental stress and greater ability to compete with vegetative growth. However, these effects will not be manifested under conditions where such stress or competition do not occur.

A high percentage of hybrid fruits does not necessarily ensure a high yield (18). Usually, however, a substantial increase in outcrossing rate may be assumed to be accompanied by a significant increase in fruit yield. A correlation between outcrossing rate and

yield in 'Hass' groves was demonstrated in the present study (Fig. 5), as well as by Vrecener-Gadus and Ellstrand (31).

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Table 1. Isozyme genotypes of four avocado cultivars present in the studied area ^z.

Cultivar	Tpi-1	Lap-2	Mdh-1
'Hass'	SS	FF	SS
'Ettinger'	FS	FS	SS
'Fuerte'	FS	FF	FS
'Reed'	SS	FF	FS

^z See Figure 1.

Table 2. Heterozygosity in 'Hass' fruitlets assayed by three isozyme systems at different stages of fruit development

Date	No. of tree pairs studied	No. of fruitlets assayed	Average fruit wt (g)	Percent heterozygotes		
				<u>Tpi-1</u>	<u>Mdh-1</u>	<u>Lap-2</u>
May 15	46	596	0.07	11.4c ^z	0.4c	- ^y
June 20	51	570	9.3	24.7b	18.2a	22.0c
Sept. 10	48	355	121	36.2a	10.7b	29.3b
Oct. 22	52	398	184	41.8a	11.2b	36.8a

^zResults (in vertical rows), followed by different letters are significantly different ($p < 0.001$), according to Duncan's.

^yLAP-2 was not detected in very young fruitlets.

Table 3. Percentage of 'Fuerte' fruitlets heterozygous for Lap-2,
which identifies 'Ettinger' pollination

Sampling period	No. of trees sampled	No. of fruitlets examined	Percent heterozygous for <u>Lap-2</u>
May-June ^z	67	264	11.5 $\pm$ 2.6 ^v
July ^y	87	234	11.7 $\pm$ 2.4
August ^x	75	237	11.9 $\pm$ 2.2
October ^w	61	246	11.8 $\pm$ 2.0

^zMay 10,24, June 6, ^yJuly7,17, ^xAugust 8,22, ^wOctober7,10.

^vMean $\pm$ SE.

Legends to Figures

- Fig. 1   Schematic map of 'Hass' plot 3 and the neighboring avocado plots in Givat Brenner.
- Fig. 2   Schematic map of 'Fuerte' plot 7 and the adjacent 'Ettinger' plot 8 in Givat Brenner.
- Fig. 3   Mean percentage of 'Hass' embryos heterozygous for Lap-2 ('Ettinger' offspring), in different rows of the 'Hass' plot on three different dates. 'Ettinger' trees were located near rows 3 and 26 (See Fig. 1).
- Fig. 4   Percentage of 'Hass' embryos heterozygous for Mdh-1 ('Fuerte' offspring) as a function of the distance of the 'Hass' tree from nearest 'Fuerte' tree.
- Fig. 5   Average yields and mean percentage of 'Hass' embryos heterozygous for Lap-2 ('Ettinger' offspring) at the verge of fruit maturity (September-October), in different rows of the 'Hass' plot.
- Fig. 6   Mean percentage of 'Fuerte' embryos heterozygous for Lap-2 in relation to the distance from the 'Ettinger' plot. Trees are numbered as in Fig. 2, i.e. trees numbered 13 are

closest to 'Ettinger'. The vertical lines represent the standard error of the mean value for data obtained on four dates for equidistant trees in rows 22 through 33.

Fig. 1

Fig. 3

Distance from nearest 'Fuerte' tree (m)

Fig. 4

Fig. 5

Fig. 6

II-3. Production of protoplasts from avocado leaves.

Materials and Methods.

Young tender avocado leaves were surface sterilized, abraded with carborundum and finely sliced to provide a protoplast source. The medium for digestion of the cell walls contained 0.5% Driselase and 0.75% Cellulysin, 12% Mannitol, 0.25mM MES, 2mM CaCl₂, 10mM Cysteamine, 5mM KCl, 0.5% BSA, 2mM MgCl₂, 0.4% PEG (3,3500MW), adjusted to pH 5.8. The addition of 0.5% bovine serum albumin (BSA) polyethylene glycol (PEG) at 0.4% and cysteamine increased the viability of the cells. To isolate the protoplasts from enzymes and debris filtration and density centrifugation a swinging bucket rotor were used. Protoplast culture was attempted in a standard tissue culture medium with 0.2ml drops x 5 per 35mm in sealed petri-dish (approx. 0.5 million protoplasts) kept dark at room temperature. After 2 days fresh culturing medium was added with 0.4% agarose 1:1. and every week 0.2% agarose in culturing medium added 1:1. The absence of a cell wall was verified by the visual appearance of the protoplast under the phase contrast microscope. Protoplasts are spherical without any cytoplasmic extrusions which indicate an incorrect osmoticum. An intact cell will not take up phenosafranine stain but will take up fluorescein diacetate which will fluoresce under U.V. fluorescence microscopy.

Cell wall regeneration was monitored with Calcaflour White which reacts with the B1,4 glucan linkages in the cell wall and fluoresces under fluorescence-UV microscopy. Protoplast density was measured with a haemocytometer.

Results and Discussion

Our goals for avocado protoplasts were to learn how to prepare them from leaf tissue, promote cell wall regeneration and callus formation, differentiation of tissues and eventually whole plant regeneration. This would have been followed by attempts at fusion, and so on. In brief we wanted to bring avocado technology on a par with what is being accomplished with herbaceous plants. We did not, and do not believe there was any reason this could not be accomplished. As the interest in this project developed late in the grant's life, there was not sufficient time to do a great deal. Avocado protoplasts were produced rather routinely and we believe walls reformed. In addition, some cells appeared to have divided. We did not achieve a callus a stage and did not get far enough to try fusion. A nice review of woody plant protoplast work and additional details of the avocado work was prepared by a graduate student for a non-thesis project. It is available to any interested parties from Torres.

F. DESCRIPTION OF COOPERATION

Throughout the period of work, information was exchanged between the two laboratores by mail and by visits. Dr. Torres visited Degani's laboratory at Bet-Dagan in 1985 and Dr. Degani visited Torres's laboratory at Lawrence in 1987. These visits provided the opportunity to exchange ideas and to formulate the broad outline of the cooperative effect.

G. MAIN ACHIEVEMENTS OF THE RESEARCH

- 1) Methods were developed for resolving the isozyme systems of phosphoglucose isomerase (PGI), glutamate oxaloacetate transaminase (GOT) and triosephosphate isomerase (TPI) from avocado leaf, pollen and embryo tissues. Genetic analyses of these isozyme systems have been carried out.
- 2) We have shown that the high isozymic variability of phosphoglucose isomerase renders it a suitable 'fingerprint' for identification of avocado cultivars.
- 3) The availability of the population of Australian hand pollinated avocados provided the opportunity to determine genetic linkage groups for isozyme loci. It was found that only two genes were linked, Got-1 and Got-2.
- 4) Our ability to identify the male parent at the mature seed stage, puts avocado breeding work on a more sound basis. Screening for the pollen parentage can now be done before planting out, thus saving considerable time, labor and other valuable resources and consequently increasing the efficiency of breeding programs.
- 5) Using isozymic analysis we can determine: (a) The effect of different pollen parents on outcrossing rates in the orchard. (b) The dependence of hybrids formation on the distance from the pollen parent, as well as the

correlation between yield and hybrids formation. This information should be very useful for choosing effective pollen parents and for designing future planting schemes of avocado orchards with increased fruit-set and production.

- 6) Contrary to previous reports, based on indirect evidence, claiming that cross-pollination was limited to the nearest row adjacent to the pollen parent and even restricted to overlapping branches, we showed that cross-pollination occurs at much greater distances. Our studies have shown that even a distance of 100 m cannot be considered a safe distance for self-pollination for breeding purposes, as has been previously claimed. A considerable rate of cross-pollination does occur at a distance of 100 m (or even more) from the pollen parent.
- 7) Our studies on avocado fruitlets during various stages of fruit development under orchard conditions have clearly indicated that the pollen parent plays an important role in the selective abscission of fruitlets. Parentage analysis during fruit development from the early stage of young fruitlets upto the stage of mature fruits clarified the advantage of pollen donors in enhancing the survival of their progeny.
- 8) Our studies on selfed 'Ettinger' embryos at different stages of fruit development showed selective abscission of 'Ettinger' fruitlets of specific genotypes. Specific selection as an important factor in avocado fruitlet abscission, is a phenomenon which has not been found in other fruit trees.

H. LIST OF PUBLICATIONS

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