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Isozyme Analysis of Mature Avocado Embryos to Determine Outcrossing Rate in a 'Hass' Plot

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Abstract. **Outcrossing rate in a solid block of 'Hass' avocado *(Persea americana* Mill) was determined 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), for which evidence concerning the genetic control of the latter is presented. Almost all 'Hass' mature fruits were found to have resulted from cross-pollination. Among the potential pollen donors—'Hass', 'Ettinger', and 'Reed'—'Ettinger' excelled, producing almost all of the hybrid fruits.**

Several studies have attempted to determine the effect of different avocado cultivars on each other's productivity (1, 4, 5, 9, 16). These efforts mainly measured the influence that one cultivar has on the fruitfulness of adjacent trees of another cultivar. The reason for this work was the daily synchronous dichogamic flowering behavior of avocado (3, 16), which tends to favor cross-pollination between complementary cultivars. Bergh (3) found the effect of pollen parents on yield to be limited to the first row adjacent to the pollen parent, especially with overlapping branches. He suggested that this was due to the foraging pattern of the main pollinator of avocado, the honey bee.

The limited effect of pollen parents on the yield of the first row only convinced Bergh that appreciable cross-pollination can occur only when the pollen donor tree is adjacent to the pollinated tree. He also concluded that a distance of 100 m may rule out the possibility of undesirable cross-pollination for breeding purposes (2). These conclusions were based on indirect evidence, such as yield differences. The development of isozymes as genetic markers in avocado (17-19) provided the necessary tool for addressing this problem directly. In this study we identified the pollen parents of mature 'Hass' fruits and thus determined directly the extent of cross-pollination in the orchard.

Materials and Methods

The present study was undertaken in the Moranim orchard, located in the coastal plain of Israel near Rehovot. There were no other avocado trees within 2 km of the orchard. The trees were planted 6 x 6 m apart in 1976. The orchard was crowded, with branches of adjacent trees overlapping. The planting pattern of the four cultivars at this location

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('Ettinger', 'Hass', 'Nabal', and 'Reed') is illustrated in Fig. 1. The identity of all 'Hass' trees was carefully verified; no rootstock sprouting was found.

Three different isozyme systems were used in this work. Two of them, malate dehydrogenase (MDH) and leucine aminopeptidase (LAP), have been described by Torres and Bergh (19, 20). MDH-1 is a dimeric enzyme having fast *(F)* and slow *(S)* as the most common alleles, and LAP-2 is a monomeric enzyme having *F* and *S* as alleles. The 3rd system, triosephosphate isomerase (TPI), was described by Vrecenar-Gadus and Ellstrand (20), who showed *TPI-1* to be a dimeric enzyme, more evidence concerning TPI genetic control will be presented here.

The following methods were used for the analysis of TPI genetic control. Leaves were prepared for analysis as described by Degani and Gazit (6). Pollen grain extracts were prepared as follows: 10 stamens, with their anthers open, were shaken into 100 µl of an extraction buffer (6). They were then crushed and the extracts absorbed on 4 x 6 mm Whatman 3MM paper wicks. The running system used for TPI consisted of a 16 mM Tris-citrate (pH 6.9) gel buffer, and an electrode buffer of 49 mM Tris·citrate (pH 6.9). Gels were run at 1.1 $mA \cdot cm^{-2}$ gel cross-section. TPI was stained by the slightly modified procedure of Soltis et al. (14). The reaction mixture consisted of 4 ml 0.1 M Tris-HCl (pH 8.0), 150 mg sodium arsenate, 10 mg EDTA, 75 units glyceraldehyde 3-phosphate dehydrogenase, 1.5 mg dihydroxyacetone-phosphate, 15, mg nicotinamide-adenine dinucleotide (NAD), 5 mg 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), 1 mg phenazine methosulfate (PMS), and 6 ml 1% agarose.

Mature 'Hass' fruits were sampled for analysis in Oct. 1984 from five alternate rows in the orchard (Fig. 1), five from each tree, 10 trees per row. Cotyledons were assayed with the three isozyme systems [MDH (6), LAP (18), and TPI] using the extraction procedure described by Torres (17).

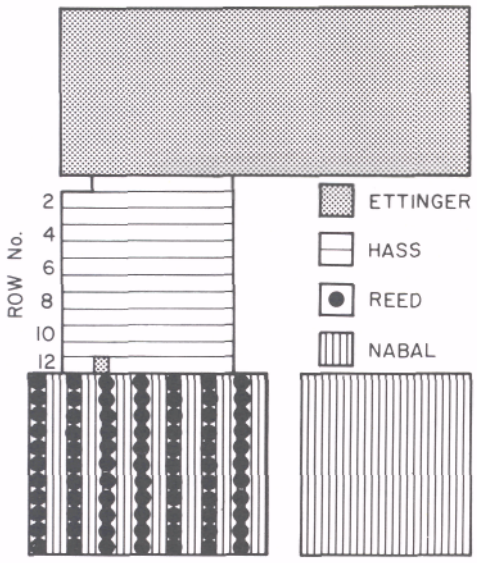

Fig. 1. Schematic map of Moranim orchard.

Table 1. *Tpi-1* genotypes of 15 avocado cultivars.

Cultivar	*Tpi-1* genotype
Anaheim	*SS*
Benik	*SS*
Ettinger	*FS*
Fuerte	*FS*
Hass	*SS*
Irving	*FS*
Nabal	*SS*
Pinkerton	*SS*
Reed	*SS*
Rincon	*FS*
Sharwill	*SS*
Stewart	*FF*
Teague	*SS*
Topa-Topa	*SS*
Wurtz	*SS*

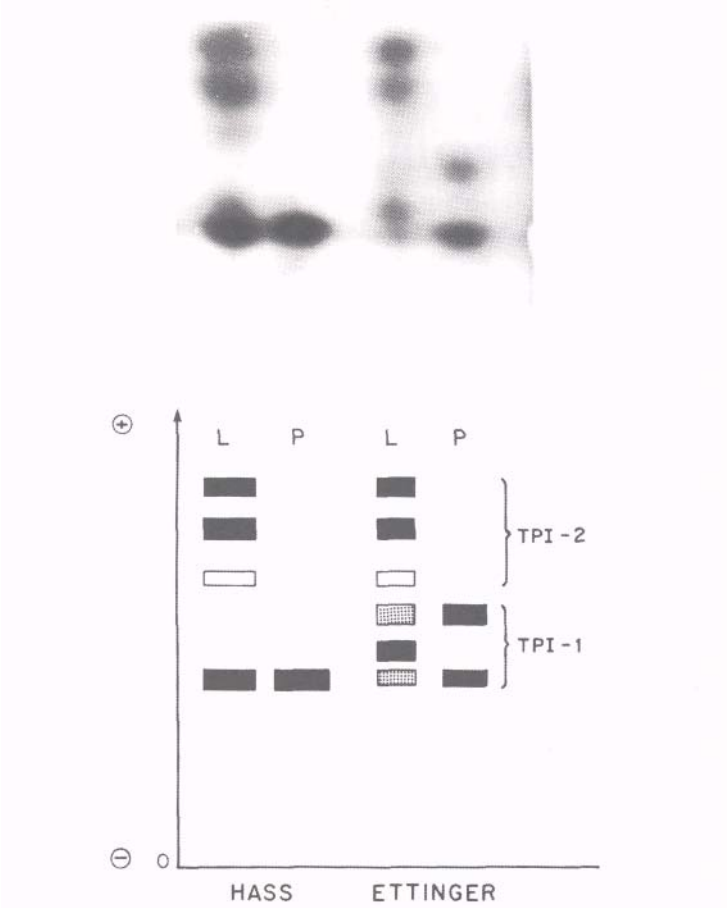

Fig. 2. Electrophoretic patterns of TPI isozymes on starch gel. Comparison of leaf (L) and soaked pollen (P) extracts from 'Hass' (**left**) and 'Ettinger' (**right**).

Results

TPI isozyme system. Fifteen avocado cultivars were assayed, and their isozyme

genotypes for *TPI-1* are presented in Table 1.

To interpret the genetic control of TPI, the isozyme patterns of leaves and pollen in 'Hass' and 'Ettinger' were compared, as shown in Fig. 2. Two sets of bands could be distinguished: The cathodal set, *TPI-1,* appeared either as a single-banded pattern, suggesting a homozygous condition *(SS* or *FF);* and as a three-banded pattern, suggesting the heterozygous condition*(FS),* in agreement with Vrecenar-Gadus and Ellstrand (20). The anodal set *TPI-2* appeared invariably as a triplet.

The TPI patterns in Fig. 2 are distinguished by the low activity of the more anodal bands in the pollen as compared with the leaf, suggesting that *TPI-2* is the plastid isozyme (11, 22). A 2nd difference is the disappearance of the allozyme heterodimer of *TPI-1* in the pollen pattern of 'Ettinger'. An intralocus hybrid enzyme cannot be produced in the pollen, thus its absence identifies the corresponding band in leaf extract as the intralocus enzyme (11, 22).

Hence, *TPI-1* is 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 analyzed.

Cross pollination rate in a solid 'Hass' plot. Three isozyme systems *(LAP-2, MDH-1,* and *TPI-1*) were used to detect the pollen parents of mature 'Hass' fruits from Moranim orchard (Fig. 1). Table 2 presents the isozyme genotypes of the four cultivars in the orchard.

'Hass' embryos, heterozygous *at LAP-2* or *TPI-1,* are the offspring of 'Ettinger', whereas heterozygotes for *MDH-1* are from 'Reed'. 'Nabal's' offspring cannot be distinguished from those of self-pollinated 'Hass'. The isozyme analysis of 'Hass' embryos from Moranim is presented in Table 3.

About 49% of the embryos were heterozygous at *LAP-2* and about 47% were heterozygous at *TPI-1.* Since a cross between a heterozygous plant ('Ettinger') and a homozygote ('Hass') is expected to yield heterozygous and homozygous offspring in a ratio of 1:1, we can conclude that almost all the fruits were the result of cross-pollination by 'Ettinger'. The contribution of 'Reed' as a pollen parent was very limited; among the fruits assayed only three embryos (2.1%) were heterozygous at *MDH-1.*

These isozyme analysis data also were used for linkage studies of *TPI-1* and *LAP-2.* We named the alleles coding for *F* and *S* isozymes *LAP-F,* and *LAP-S* and *TPI-F,* and *TPI-S,* respectively. Table 4 presents the distribution of the four genotypes in the 'Hass' embryo population. The results showed that the two genes assort independently in avocado.

A similar analysis was carried out by Vrecenar-Gadus and Ellstrand with 'Bacon' (20). However, they used a different electrophoretic system for LAP-2 separation, and from their results it appears that the isozyme they refer to as *LAP-2* is not the same as our *LAP-2.* 'Bacon', which was found to be heterozygous for *LAP-2* under their conditions, was homozygous for *LAP-2* under the conditions used by Torres et al. (18, 19) and by us.

'Hass' fruits were harvested in Mar. 1985. The fruits of each tree were weighed and the yields (kg/tree) for each row are summarized in Table 5. It should be noted that the yield was very low. A noticeable positive effect of 'Ettinger' was evident only in the first 'Hass'

row. Nevertheless a significant correlation was found between yield and distance from Ettinger *(r* = -0.708, *P* = 0.01).

Table 2. Isozyme genotypes of four avocado cultivars in Moranim orchard.

Cultivar	*Mdh-1*	*Lap-2*	*Tpi-1*
Hass	*SS*	*FF*	*SS*
Ettinger	*SS*	*FS*	*FS*
Reed	*FS*	*FF*	*SS*
Nabal	*SS*	*FF*	*SS*

Table 3. Rate of heterozygote 'Hass' embryos from Moranim orchard in three isozyme systems.

Row no.	No. embryos assayed	Heterozygotes (%)		
		Lap-2	*Tpi-2*	*Mdh-1*
2	50	56.0	50.0	0.0
4	49	58.0	48.5	0.0
6	48	35.5	35.2	2.1
8	50	38.3	51.5	4.0
10	46	58.8	49.1	0.0
Mean		48.9	46.7	1.2

Table 4. Linkage analysis of *Lap-2* and *Tpi-1* in avocado (number of embryos analyzed: 242).

Genotype	Observed ratio (%)	Expected ratio for unlinked loci (%)	Contribution to χ^2
Lap-FS; Tpi-FS	24.4	25	0.037
Lap-FF; Tpi-SS	27.7	25	0.202
Lap-FS; Tpi-SS	24.8	25	0.004
Lap-FF; Tpi-FS	23.1	25	0.334
			$\chi^2 = 0.577$
			df = 3, $P = 0.90$

Table 5. 'Hass' yield at Moranim orchard (Mar. 1985).

Row	Yield (kg/tree)
1	33.6 ± 6.9
2	23.4 ± 5.9
3	19.4 ± 6.1
4	26.0 ± 10.6
5	21.9 ± 6.5
6	18.7 ± 5.0
7	22.7 ± 7.0
8	24.9 ± 6.5
9	21.6 ± 6.4
10	8.4 ± 3.3
11	9.6 ± 5.2
12	17.8 ± 7.1

Discussion

The finding that almost all mature 'Hass' fruits in a solid block of 10 rows (Fig. 1) resulted from cross-pollination (Table 3) was unexpected. Bergh and co-workers

concluded that cross pollination occurs only when trees are in close proximity (2, 3, 5). However, we have found that even six rows away, almost all 'Hass' fruits were the result of cross-pollination by 'Ettinger'. Similar results were reached by Vrecenar-Gadus and Ellstrand (21) in a study done with 'Hass' where 'Bacon' was the pollen parent. However, in contrast to their results, our findings (Table 3) showed that there was no consistent decline in the rate of cross-pollinated fruits with increasing distance from the pollen parent.

Evaluation of our results should consider the flowering behavior of the cultivars involved. There is about 3 weeks of overlap between the flowering periods of 'Hass' and 'Ettinger'. 'Reed' flowers later, at the end of 'Hass' flowering. 'Nabal' is the last to flower, and its flowering does not coincide with that of 'Hass' (13, 15). 'Hass' and 'Reed' both belong to flowering group A, while the other two cultivars belong to group B (16).

The observed high rate of cross-pollination is consistent with the fact that 'Hass' and 'Ettinger' belong to complementary flowering groups and their flowering periods coincide. On the other hand, a certain amount of self-pollinated 'Hass' fruits could also be expected, especially with increasing distance from the 'Ettinger', in view of the fact that pure 'Hass' orchards, in the absence of any pollen donor cultivar, may bear large crops (2, 3, 5). The observed negligible rate of self-pollinated 'Hass' fruits may be due to a) a possible defect in 'Hass' pollen or b) a high abscission rate of selfed 'Hass' fruitlets. In the first case, almost no 'Hass' selfed fruitlets were set and the only efficient fertilizations were those with 'Ettinger' as a pollen parent. In the 2nd case, the initial set included both selfed and cross- pollinated fruitlets, but only 'Ettinger' offspring survived the long months of massive abscission (12).

We favor the 2nd possibility. Recent studies showed that during the first 3 weeks the abscission rate of self hand-pollinated 'Hass' fruitlets was much higher than that of hand-pollinated 'Hass' X 'Ettinger' hybrids when present on the same inflorescence (8, 10). Similar preferential abscission of selfed 'Hass' fruitlets is likely to have occured in the Moranim orchard. Selfed 'Hass' fruitlets, if formed, probably abscised gradually during development, either due to competition with offspring of 'Ettinger' or independently due to greater susceptibility to climatic stresses. Apparently, when the possible pollen donors were 'Hass', 'Ettinger', and 'Reed' on occasion, fruitlets resulting from 'Ettinger' pollen had the greatest chance to survive.

We do not know the initial rates of cross and self-pollinations at present. The massive abscission of fruitlets in avocado (12) may transform a negligible initial rate of cross-pollination into a high rate of cross-pollinated mature fruits. Our results show that with the potent pollen parent 'Ettinger' (6, 8-10), most of the surviving 'Hass' fruits were hybrids, even six rows (36 m) away from any 'Ettinger' tree (Fig. 1). Thus, even a distance of 100 m should not be considered as a safe barrier for breeding purposes (2); on the contrary, with the above combination of 'Hass' and 'Ettinger', a considerable rate of cross-pollination might occur even at a 100-m distance.

There is no consistent decline in the production rate of 'Ettinger' offspring with increasing distance from 'Ettinger' trees (Table 3). This lack of decline might be the result of initial set of 'Ettinger' fruitlets, which was higher than the bearing capacity of the trees. Thus, even with an initial gradient in cross- pollination, it could not be detected at

harvest time. On the other hand, it is possible that although bees working on 'Ettinger' flowers visited only adjacent 'Hass' flowers (2), pollen exchange that may have occurred in the beehive (7) might be responsible for widespread, low-intensity, cross-pollination throughout the orchard. Under such conditions, no gradient in cross-pollination should be expected, since the pollen was not transferred directly from 'Ettinger' to 'Hass'.

Our findings (Table 3) do not warrant the conclusion that 'Ettinger' will always be an effective pollen parent for 'Hass', or that 'Hass' will produce only when cross-pollinated. Different environmental conditions, especially climatic, may yield different results in different places, and these differences may be reflected in cross-pollination rate, fruitlet survival, and yield.

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