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CRYPTICALLY FEMALE FLOWERS IN 'FUERTE': FACT OR FALLACY?

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ABSTRACT

A comparative study of flowers from high-producing and low-producing 'Fuerte' trees revealed that ca. 50% of 700 pollen sacs investigated, contained pollen grains of abnormal shape and diameter, low staining ability and weak resistance to rupturing. In anthers producing abnormal pollen grains the output and distribution pattern of the grains were irregular. As defective anthers occurred in all the stamens of a flower, looked normal and could not be identified macromorphologically, it was concluded that such flowers were cryptically female unisexual.

UITTREKSEL

'n Vergelykende studie van blomme van goeden swak-produserende 'Fuerte' borne hetgetoon datongeveerdie helfde van die 700 ondersoekte stuifmeelsakkies stuifmeelkorrels bevat wat abnormaal is in vorm en deursnee, swak kleur en maklik oopbars. In helmknoppe wat sulke stuifmeel vorm was die opbrengs en verspreidingspatroon van stuifmeel onreelmatig. Aangesien al die helmknoppe in 'n blom defektief was, maar normaal vertoon het en nie op grond van makromorfologiese eienskappe uitkenbaar was nie, is afgelei dat vroulike eenslagtigheid in hierdie blomme verskuil is.

INTRODUCTION

The extremely low fruit to flower ratio coupled with a huge variability in fruit production of individual avocado trees in clonal orchards are universal problems in avocado-producing countries. These phenomena raise serious doubts as to whether the avocado is indeed a hermaphroditic plant. The discovery of female sterile flowers on low-producing (E-type) 'Fuerte' trees in the orchards at Westfalia (Steyn *et al.,* 1993a), has recently raised the question as to whether some of the flowers on the high-producing (A-type) 'Fuerte' trees may not be cryptically female unisexual and male sterile (Steyn *et al.,* 1993b). Such flowers look normal, but the male organs (stamens) are defective. Pollen from such stamens may therefore be unsuitable for effecting normal seed development and, consequently, normal fruit set and growth in recipients of such pollen in the orchards. The main objective of the present study was to gain detailed and comparative information about the pollen-producing organs (stamens) of A-type and E-type trees and the pollen produced by these structures.

MATERIAL AND METHODS

Female-phase and male-phase flowers from three A-type trees, Nos. E2123, E2639, Q3B1 as well as from four E-type trees, Nos. W37281, W37283, Q4C238 and Q4A18 were collected in the morning (male-phase) and afternoon (female phase) and fixed in the orchards in a freshly prepared phosphate-buffered solution (pH 7.4) of 5% formaldehyde that contained 0.5% caffeine to improve the fixation of phenolic-containing cells (Mueller and Greenwood, 1977). The flowers of individual trees were kept in separate containers and stored in the fixative for investigation of the following aspects:

Comparative macromorphological floral data: Of each tree, at least 20 female and 20 male-phase flowers were examined by ascertaining, under the dissecting microscope, the number and state of the perigone, stamens, staminodes, nectaries and pistil of each flower. The length and width of each of the 9 anthers per flower were measured, as well as the length of each ovary and style. In male-phase flowers the number and position of dehisced locules per flower were assessed. The nine stamens and single ovary of each dissected flower were stored in the fixative in a labelled container (5ml Vac-u-test tube) for further investigation.

Staining of pollen: It has long been known that pollen grains change their shape and size very easily during different staining procedures (Kumazawa, 1937). In the Lauraceae, the delicate pollen grain has a thick inline layer that swells easily in aqueous media (Van Zinderen Bakker, 1956), causing rupturing of the thin exine covering. As the accurate assessment of pollen diameter and pollen number per locule depended to a large degree on the visibility and therefore the proper staining of the minute grains, a suitable stain as well as a technique that minimized rupturing of the fragile grains, had to be found and used throughout the investigation so that data could be compared. Various concentrations of several stains previously used for avocado pollen, e.g. toluidine blue (Sedgley, 1979), safranin and fast green (Schroeder, 1952) and gentian violet (Van Zinderen Bakker, 1956) were tested. Results indicated that 0.5 cc of a 0.25% aqueous solution of gentian violet, mixed with 10 gm pure glycerin produced the best results. Pure glycerine, as recommended by Praglowski (1970) for the pre-treatment of thin-walled pollen, was used instead of glycerin jelly. Specially prepared glass slides were devised so that the cover slip was slightly raised and exerted no pressure on the grains. As these slides could not be re-used after sealing the cover slips with nail polish, various alternative sealing agents were tested. Vulcanizing fluid (Tip Top tube patching solution) was considered most satisfactory as pollen preparations could be stored for weeks and the slides cleaned easily. With this technique normal pollen grains were adequately stained within minutes, less than 4% rupturing occurred and the re-usable slides save time.

Assessment of pollen production per anther and pollen/ovule ratio: For each of the seven trees, five of the abovementioned test tubes containing the dissected nine

stamens and single ovary of a female-phase flower were randomly selected. For each of five stamens per flower, the exact number of pollen grains in each of the four pollen sacs (locules) per stamen was assessed. In total, one hundred locules per tree were investigated as follows: The bilobed, 4-valvate anther was placed in a drop of staining solution on a glass slide and examined under the dissecting microscope to ensure that the flaplike valves covering the locules were intact, i.e. dehiscence had not yet occurred. By lifting one of the flaps, the pollen grains inside the locule could be exposed to the staining solution. Most grains floated out unaided into the solution. Grains that still remained inside the locule or were sticking onto the underside of the flap could be removed, because they had already absorbed some of the stain and were easily visible. The anther was then placed onto a clean slide and the process repeated. In this way the grains in each locule were separately obtained, covered and the edges of the cover slips sealed with vulcanizing fluid. A strip of graphic paper was attached to the underside of the slide to act as a grid. At this stage all the pollen grains under the cover slip were adequately stained and could be counted with the aid of a dissecting microscope. The total number of pollen grains per anther could therefore be assessed accurately. The pollen/ovule ratio of a flower was assessed as follows: The mean value of the pollen in the five anthers investigated was multiplied by the mean number of stamens per flower, as was determined by counting the number of stamens on 20 different flowers on each of the seven trees. The number of ovules per flower was determined in the same way. As in all flowers a solitary ovule occurred, the pollen/ovule ratio equaled the number of grains per flower. Pollen/ovule ratio per tree was obtained by determining the mean value of pollen produced by the five flowers studied.

Assessment of pollen size: For each anther dissected as described above, the diameter of 50 pollen grains in one proximal and one distal locule was measured by using a Kontron Mop-Videoplan analizer attached to a Zeiss stereomicroscope.

RESULTS AND DISCUSSION

Comparative macromorphological structure of reproductive organs

The flowers of high-producing trees (A-type flowers) as well as low-producing trees (E-type flowers) were actinomorphic and trimerous with nine functional stamens arranged in three alternating whorls (Fig. 1). Only in two flowers, both collected from tree No. C4A18, one of the nine stamens had not been formed. Each stamen had a 2-lobed, 4-valvate anther with a smaller distal and a larger proximal pollen sac (locule) in each anther lobe. During anther dehiscence the four flap-like valves covering the locules opened completely, except for a minute region on the distal side where the flaps remained attached to the anther (but see **Anther dehiscence** underneath). The fourth, innermost stamen whorl consisted of staminodes with basal parts filamentous and upper parts saggitate to heart-shaped, without locules. In most A and E-type flowers, only the stamens of the third whorl had paired, stalked nectaries attached to the bases of the filaments, but a variable number (1-7) of additional nectaries occurred in some of the flowers, collected from all trees. These extra nectaries were always associated with stamens of the two outer whorls, except for two flowers, collected from tree No. C4A18,

that had nectariferous structures on the perigone.

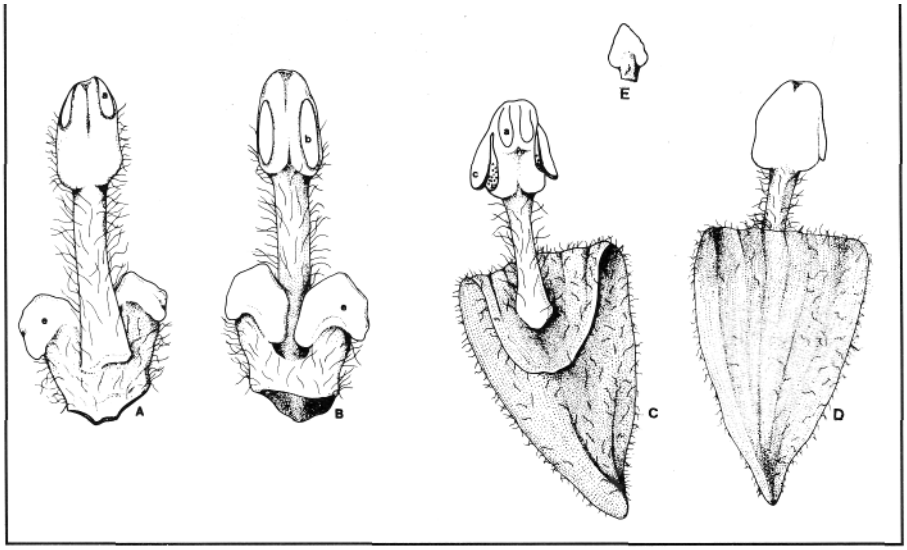


FIG. 1 A-E Macromorphological structure of avocado stamens. A - B. Stamen of the third whorl during the female phase, as seen from the back (A) and from the front (B). A pair of stalked nectaries (e) is attached to the base of the filament. Note position of the closed distal (a) and proximal (b) locules. C - D. Stamen of the first whorl during the male phase, as seen from the back (C) and front (D). Note position of the distal (a) and proximal locules and open valve (c). E. Staminode of the fourth whorl. a, distal locule; b, proximal locule; c, open valve of proximal locule; e, stalked nectary.

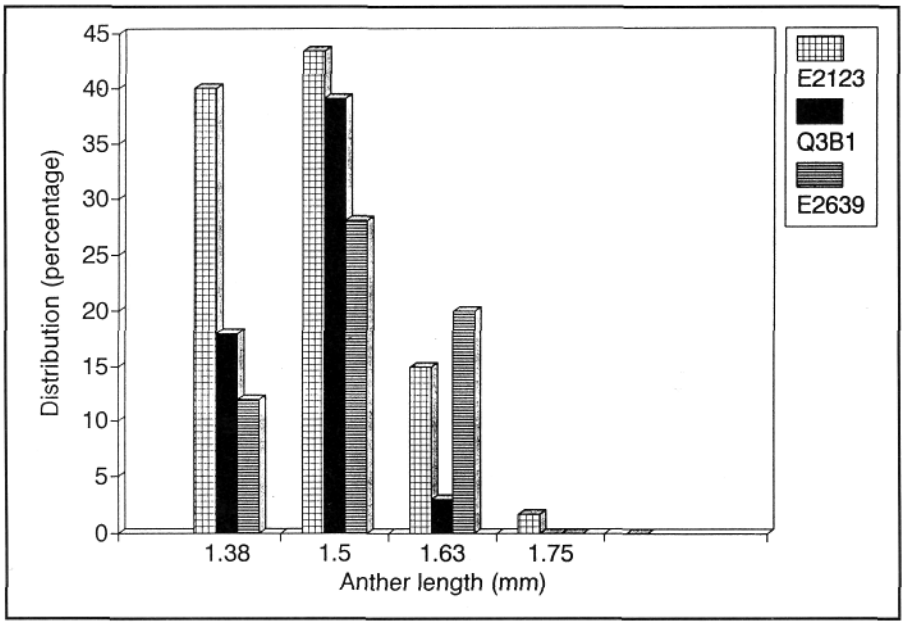


FIG. 2 Histogram depicting the percentage of short (1.38 mm.), medium length (1.50 mm.) and long (1.63-1.75 mm.) anthers in 20 flowers collected from each of three A-type trees, Nos. E2123, E2639 and Q3B1.

A Primitive, zygomorphic, 4.38 to 5.13 mm.-long pistils with unilocular, 2.00 to 2.5-mm.-long ovary with a single, anatropous and pendulous ovule were found in all A and E-type flowers examined.

Female-phase flowers had anthers that ranged from 1.38-1.75 mm. in length. In an individual flower the lengths of the nine anthers were relatively constant and usually did not vary more than 0.12 mm. in length so that flowers could be classified according to anther length. In A-trees anther length was negatively, and in E trees positively skewed (Figs. 2 & 3). However, individual A as well as E-trees varied in this respect, e.g. E-tree Nos. W37281 and Q4A18 seemed to have more flowers with anthers in the longer ranges (1.63-1.75 mm.) than did E-tree Nos. W37283 and Q4C238 (Fig. 3). Similarly, A-tree No. E2639 seemed to have less flowers with anthers in the short range (1.38 mm.) than did A-tree Nos. E2123 and Q3B1 (Fig. 2). An association between anther length and ovary length or anther length and number of nectaries could not be ascertained.

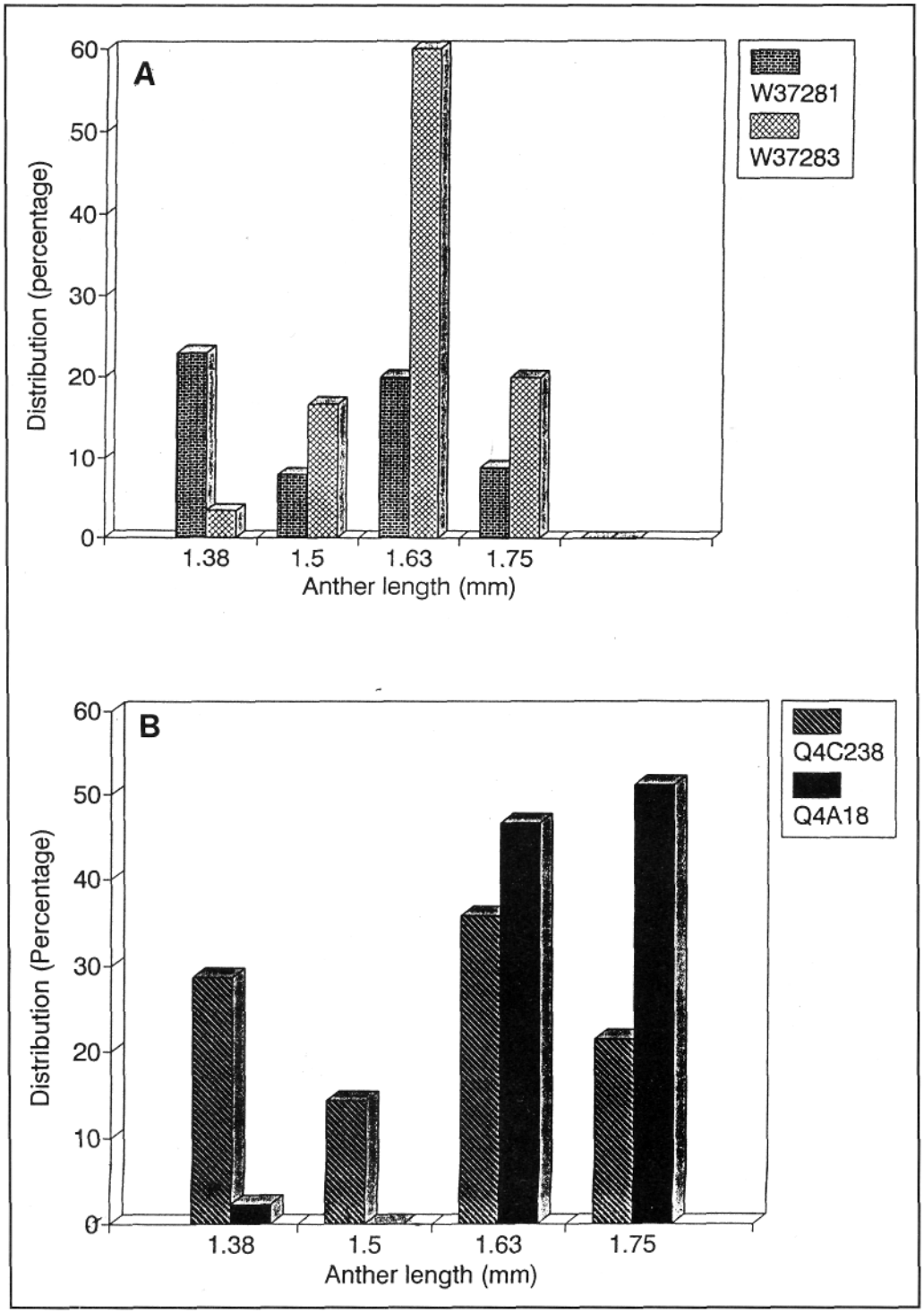


FIG. 3 Histograms depicting the percentage of short (1.38 mm.), medium length (1.50 mm.) and long (1.63-1.75 mm.) anthers in 20 flowers collected from each of four E-type trees. A. Trees Nos.W37281 and W37283 that tested positively for viroid infection and B. Trees Nos. Q4C238 and Q4A18 that tested negatively for viroid infection.

Pollen production per anther and pollen/ovule ratio

In both A-and E-type trees, the number of pollen grains per anther seemed directly related to anther size (Table 1). In anthers of medium length (1.50 mm.) the number of pollen grains varied between ca. 750 850 (see Anther Nos. 1-7 in Table 1), irrespective of whether the anthers occurred in the same flower (Anther Nos. 13), in different flowers on the same tree (compare Anther Nos. 1-3 with Anther Nos. 4 & 5), or in flowers on different trees (compare Anther Nos. 1-3 with Anther Nos. 6 & 7). Invariably, more grains were produced in the proximal locules. In medium length anthers a proximal locule yielded *ca.* 200-280 grains compared with ca. 130-180 grains per distal locule. Long anthers (1.75 mm.) contained approximately 1000 pollen grains, with ca. 200 220 grains in the distal and ca. 330 380 grains in the proximal locules (see Anther Nos. 8-10). In short anthers (1.38 mm.) the number of pollen grains totaled approximately 600 (see Anther Nos. 11 & 12 in Table 1).

On A as well as E-type trees, flowers occurred with anthers that deviated from this normal pattern (Table 2), the number of grains were extremely variable in both the distal and proximal locules, but usually much lower than in the locules of normal flowers with the same anther length. In deviating flowers, short anthers (see Anther Nos. 1, 6 & 7 in Table 2) usually contained ca. 350 grains in stead of ca. 600 grains, while long anthers (1.75 mm.) yielded 400-600 grains (see Anther Nos. 9 & 10 in Table 2), in stead of 1000. However, the flowers on A-tree No. E2123 seemed to be only slightly abnormal in this respect, as only one of the five flowers investigated had less than the expected number of pollen grains in the distal anther locules, while the proximal locules were unaffected (see Anther Nos. 3 & 4). Two of the five flowers of A-tree No. E2639 showed irregularities in pollen production per anther. The pollen distribution in two locules of one of the flowers is depicted in Table 2 (see Anther Nos. 1 & 2). None of the five flowers of A-tree Q3B1 had anthers that contained the expected number of pollen grains (e.g. Anther Nos. 5 & 6). The flowers of E-trees No. W37281 and W37283 showed no abnormalities with regard to pollen production. On the other hand, all the locules of the five flowers collected from E-tree Nos. Q4C238 and Q4A18 (totaling 100 locules per tree) were abnormal (see Anther Nos. 7-10).

The pollen/ovule ratio of the trees (i.e. mean number of pollen grains per flower) varied between 4000 and 7000. E-tree Nos. W37281 and W37283 had the highest ratios, *viz.* 7401 and 7120, respectively, compared with 6130 and 5157 for A-tree No. 2123 and A-tree E2639 respectively, while E-trees Nos. Q4C238 and Q4A18 had the lowest ratios, *viz.* 4420 and 4008.

TABLE 1 Association of flower type, pollen size and pollen grain production in proximal and distal locules of short (1.38 mm.), medium length (1.50 mm.) and long (1.75 mm.) anthers with a normal pollen distribution pattern.

Anther		Flower		Pollen production per anther					Pollen size (mm)	
				Distal locules		Proximal locules		Total		
No.	Length (mm)	No.	Type	No.1	No.2	No.1	No.2		Mean	S.T.D.
1	1.50	W37281/2	E	168	183	227	223	801	0.045	0.002
2	1.50	W37281/2	E	154	157	208	228	749	0.045	0.002
3	1.50	W37281/2	E	169	154	268	263	854	0.044	0.002
4	1.50	W37281/5	E	137	173	260	268	838	0.044	0.002
5	1.50	W37281/5	E	160	155	262	254	831	0.043	0.002
6	1.50	E2639/5	A	159	144	217	276	796	0.044	0.002
7	1.50	E2639/5	A	178	123	261	226	788	0.042	0.003
8	1.75	W37281/3	E	217	206	334	339	1096	0.044	0.002
9	1.75	W37281/3	E	193	206	384	327	1110	0.043	0.002
10	1.75	E2123/10	A	213	210	335	355	1113	-	-
11	1.38	E2123/7	A	156	115	177	163	611	0.042	0.002
12	1.38	E2123/9	A	118	123	169	191	601	0.040	0.002

TABLE 2 Association of flower type, pollen size and pollen grain production in proximal and distal locules of short (1.38 mm.) medium length (1.50 mm.) and long (1.75 mm.) anthers with an abnormal pollen distribution pattern.

Anther		Flower		Pollen production					Pollen size (mm)	
				Distal locules		Proximal locules		Total		
No.	Length (mm)	No.	Type	No.1	No.2	No.1	No.2		Mean	S.T.D.
1	1.38	E2639/2	A	65	122	93	84	364	0.053	0.006
2	1.50	E2639/2	A	97	91	129	160	477	0.051	0.010
3	1.50	E2123/9	A	42	16	259	288	5O6	0.043	0.005
4	1.38	E2123/9	A	29	85	224	260	598	0.049	0.004
5	1.50	Q3B1/5	A	176	164	133	95	568	0.037	0.011
6	1.38	Q3B1/5	A	64	115	69	84	332	0.033	0.003
7	1.38	Q4A18/11	E	68	72	104	119	363	0.056	0.007
8	1.50	Q4A18/11	E	0	10	91	88	189	0.060	0.005
9	1.75	Q4A18/11	E	110	0	21	273	404	0.057	0.004
10	1.75	Q4C238	E	144	142	167	170	623	0.043	0.006

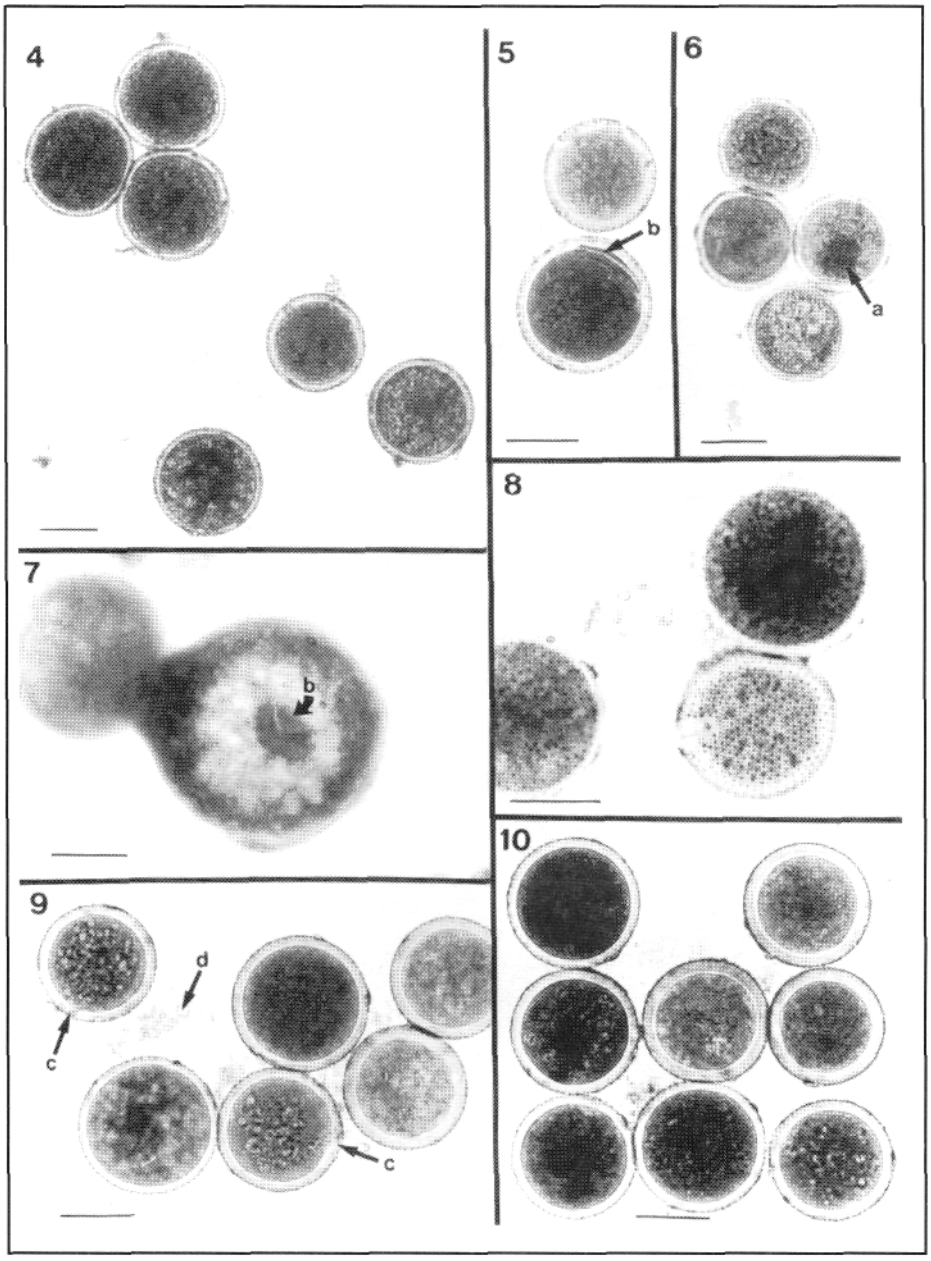
Assessment of pollen size

Pollen grains taken from A- and E-type flowers in the female phase were used to assess pollen size. Anthers that showed a normal production and distribution of pollen grains in the locules (Table 1) contained grains that usually varied in diameter from 0.037-0.046 mm. The mean diameter of the 50 pollen grains measured per locule varied between 0.040-0.045 mm. (S.T.D.= 0.002-0.003 mm, Table 1). In anthers with abnormal pollen production (Table 2), pollen diameter was extremely variable and ranged between 0.009-0.076 mm. so that the mean diameter of the 50 pollen grains measured per locule was very variable (0.033-0.60 mm in Table 2). This variation in size was reflected in high S.T.D. values (see Anther Nos. 2 & 5 in Table 2). In some locules most

of the pollen grains were either small (see Anther No. 6) or large (see Anther Nos. 8 & 9), so that the S.T.D. value was relatively low.

Comparative LM structure of pollen grains

Pollen grains taken from female-phase flowers with locules that showed a normal pattern of pollen production complied with the description and depiction of *Persea* pollen in the literature (Coetzer & Robbertse, 1986; Lieux, 1980; Van Zinderen Bakker, 1959). The grains were spherical, inaperturate and bi-cellular with an extremely thin exine layer that had minute, cone-shaped spinules evenly distributed over the entire surface (Figs. 48). The grains usually occurred in monads to tetrads or more seldomly, in loosely arranged, large groups (Figs. 4, 5, 6, 9 & 10). The intine layer varied in thickness and remained unstained. In all plants bi-cellular grains (Figs. 5 & 7) were observed, but the small generative cell was in most cases obscured by the large and lobed vegetative nucleus and extremely granular, intensely staining contents of the grains. The fact that both safranin and toluidine blue stained the contents, indicates that phenolic-containing substances may be the cause of the intense colouring. The extremely fragile pollen grains ruptured very easily and it seems possible that the spilled contents of the ruptured grains might have caused the grains to stick together (Figs. 9 & 10).

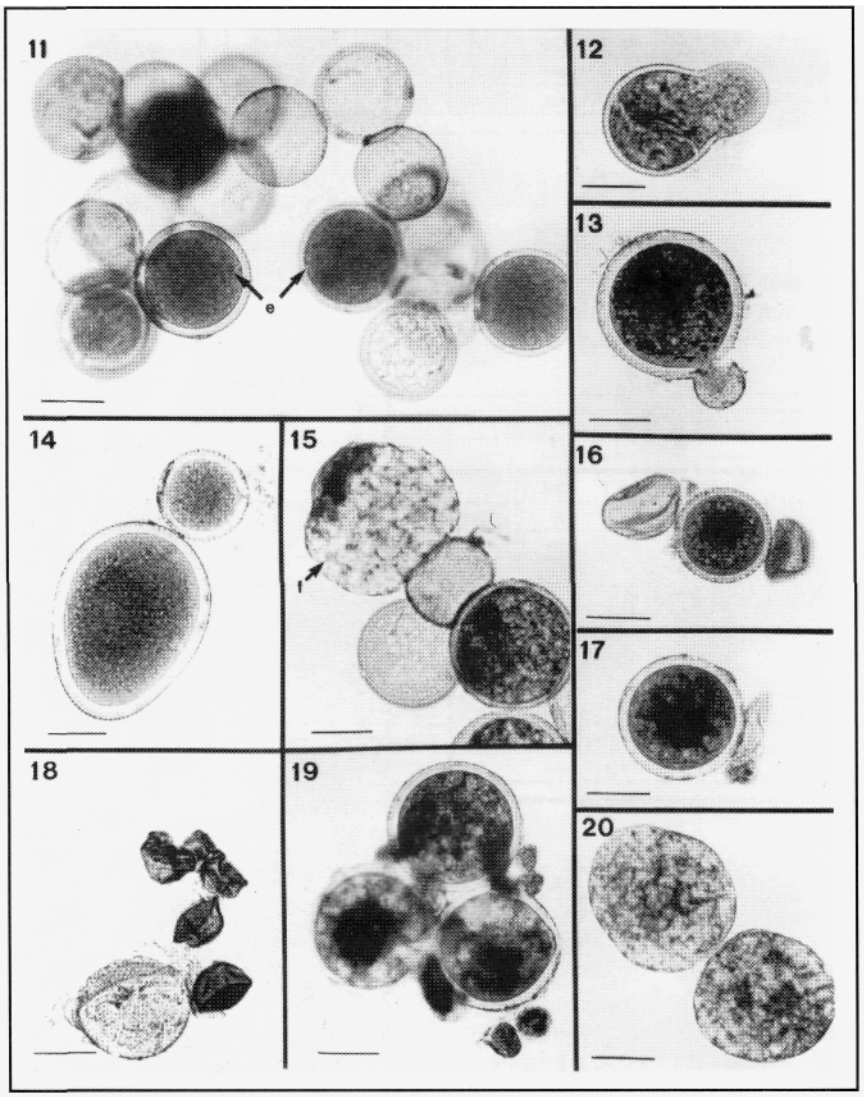


FIGS. 4-10 Microphotographs of pollen grains taken from anthers with locules showing a normal pollen distribution pattern. Figs. 4-6. Spherical, inaperturate, bicellular, densely-staining grains, arranged in monads to tetrads. a, large and lobed vegetative nucleus; b, generative cell. Fig. 7. Pollen grain at high magnification showing small generative cell. Scale bar 10μ. Fig. 8. Pollen grain in surface view showing spinules in exine. Figs. 9 & 10. Loosely-arranged large groups of grains illustrating similarity in pollen grain diameter, rupture (c) in pollen wall, spilled cell contents (d). All scale bars 25μ, except in Fig. 7.

On both E-and A-trees, abnormal pollen were found in the flowers with anthers that showed an abnormal pollen distribution (Figs. 11 20). When present, these grains

usually occurred in proximal as well as distal locules of the anthers and in all anthers of the flower. Many grains remained unstained or stained slightly after several hours in the staining solution (Fig. 11), were below average size or extremely large and varied in shape (Figs. 12, 13 & 14). Naked grains often occurred (Figs. 15 & 20), in others the exine pattern was irregular. The thickness of the intine layer was very variable. In some instances it seemed as if nakedness might have been caused by premature germination of the grain (Fig. 15); *Persea* pollen is different from all known pollen grains (Knox, 1984) in that the pollen tube at germination may be surrounded only by the plasma membrane, wall deposition does not occur until the pollen tubes have entered the stigma (Sedgley, 1979). Composite grains consisting of two lobes that varied in size were seen in the locules (Figs. 12 & 13). Numerous, very small, deformed and empty grains were very closely associated with seemingly normal or weakly-stained grains

(Figs. 16, 17, 18, & 19). These undersized grains were especially abundant in the anthers of flowers collected from A tree No. Q3B1 and E-tree Nos. Q4A18 and Q4C238. It is possible that the minute grains represent small lobes that have been cut off (compare Figs. 12 & 13 with Fig. 19) as a result of a defective meiotic devision. This would mean that the seemingly normal grains in association with the minute grains contain more than the usual number of chromosomes. Such grains might pass viability tests and still cause infertility. Under the dissecting microscope the small grains could not be counted. In addition, many grains were ruptured and large groups, comprising up to 70 grains, were formed so that some grains were obscured. These abnormalities could partly be responsible for low pollen counts in the locules. Rupturing and spilling of the contents often resulted in weak staining of grains, but in many cases there were no evidence of spilled contents, so that insufficient staining may have been caused by an absence of phenolic substances.



FIGS. 11-20 Micrographs of pollen grains taken from anthers with locules showing an abnormal pollen distribution pattern. All grains at the same magnification level. Fig. 11. Large group of pollen grains comprising normal grains (e) and numerous weakly-stained grains. Figs. 12-13. Lobed grains. Compare the size of lobed grain in Fig. 13 with the sizes of normal grains in Figs. 9, 10 & 11 (all grains at the same magnification level). Fig. 14. Large, ovate grain. Fig. 15. Large, naked grain (f). Fig. 16-19. Small, empty or deformed grains associated with seemingly normal or larger grains, but note the sizes of the grains in Fig. 19, as compared with normal grains (e) in Fig. 11. Fig. 20. Two naked grains. All scale bars 25μ.

Anther dehiscence

A comparative study of flowers collected from A and E-type trees in the male phase revealed that A-flowers have a very variable rate of anther dehiscence (5% 42%) compared with 79% 100% in E-flowers. The latter showed very obvious signs of nectary

activity. The staminodes were no longer shiny, but bore areas that seemed bruised and had turned black in the fixative. Such markings were totally absent on the nectar-secreting structures of A-flowers.

As these two floral functions (pollen release and nectar production) may have a large impact on the relative fruit set in A and E-trees, this part of the investigation will have to be repeated. The time of flower collection was not noted; it is possible that A-flowers were collected early in the morning while female flowers were closing. Deceived by the upright anthers of the closing flowers, we might erroneously have thought that the trees were in the male phase.

CONCLUSIONS

The results of this study suggest that many of the 'Fuerte' trees in the orchards are not suitable to act as pollen donors. A detailed investigation of 700 pollen locules showed that approximately 50% of these pollen-producing structures were defective. All A-type trees (Nos. E2123, E2639 and Q3B1) had some flowers that contained such locules. A high proportion of flowers with defective locules seemed to occur on E-tree Nos. Q4A18 and Q4C238, while on E-tree Nos. W37281 and W37283 no flowers with defective locules were found. However, since only a small number of the thousands of flowers produced per season were studied in detail, it can not be assumed that all pollen-producing structures on these two trees were normal.

The defective locules contained pollen grains of abnormal shape, poor staining abilities and a weak resistance to rupturing as compared with pollen grains from normal locules. Furthermore, the production and distribution pattern of the abnormal grains in the anthers were irregular. Some of these abnormalities indicate that the defects resulted from a disturbed meiotic division of the pollen mother cells. These cells start dividing when the flowers in the inflorescences are approximately 1 mm in length. The causal factors for the abnormalities therefore operate during the earliest flowering stages.

Pollen/ovule ratios of normal flowers indicated that flowers with long anthers (1.75 mm) produced ca. 1000 grains per anther and thus 9000 grains per flower, whereas flowers with short anthers (1.38 mm) yielded ca. 600 grains per anther or 5400 grains per flower. Anther size should therefore be one of the traits to be considered during the choice of a suitable pollinator. As E-type trees tended towards long-anther-flowers, pollen production should be higher on these trees than on A-type trees.

Defective locules occurred in anthers of short, long and medium length. These anthers cannot be distinguished by any macromorphological characters from normal anthers. The stamens were seemingly normal in number, size and structure and were not necessarily present in flowers with additional nectaries. The flowers that produce the abnormal pollen therefore seem to be cryptically female unisexual. Whether these grains are indeed sterile and incapable of effecting normal seed development and fruit growth can only be ascertained by experimental embryological procedures.

The results obtained during the present study undoubtedly indicate that A-type trees are not the only culprits in the orchards, as far as the production of abnormal pollen is concerned, since a seemingly high proportion of these grains was also produced by E-

type trees Nos. Q4A18 and Q4C238. The hypothesis that it is the high-producing trees in the orchards that are functionally female, i.e. have nonfunctional stamens, does therefore not seem to be valid.

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