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Asexual Embryogenesis in the Mango
(Mangifera Indica L.)

S. Gazit, R. J. Knight, Jr.

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C. ABSTRACT

The main objectives of the research were: a. A survey of polyembryony in mango; b. to study the effect of self and cross pollination on fruit set and embryo development in polyembryonic mango cvs.

The occurrence of polyembryony was examined in 38 different cvs. In contrast with Horn's results we found a clear-cut difference between monoembryonic and polyembryonic cvs; the emergence of multiple seedlings from one seed was prevalent in polyembryonic cvs, and was absolutely absent in almost all monoembryonic cvs. In all polyembryonic cvs the occurrence of one seedling emerging from a seed was noted. In most cases it occurred in damaged polyembryonic seeds. However, genuine monoembryonic seeds were found in 5 out of 6 polyembryonic cvs which were examined. 7 Ovules from 2 months old fruits were successfully cultured in modified solid Murashige and Skoog media. Precocious germination occurred from ovules of 5 cvs, 2-3 weeks later. No nucellus could be isolated from these ovules. Somatic embryogenesis occurred in nucellus excised from ovules of 5 cvs. Later, embryogenesis was induced in callus that was developed from nucellus taken from 2 polyembryonic cvs.

In almost all hand pollination experiments, initial fruit set (2-4 weeks after pollination) was minuscule. The results from a successful study indicated strongly that 'Turpentine' is self-incompatible, suggested that '13-1' may also be self-incompatible and that there may be a cross-incompatible reaction between '13-1' (female) and 'Turpentine' (male).

'Turpentine' and '13-1' trees were caged (with screen) individually and in pairs, during the flowering season. Results tend to support the conclusions that '13-1' is self-incompatible and cross-incompatible, as a female, with 'Turpentine'.

D. OBJECTIVES OF THE RESEARCH

1. To gather and document reliable data about the phenomenon of polyembryony in a wide range of monoembryonic and polyembryonic mango cultivars.
2. To study the effect of self and cross pollination (with monoembryonic and polyembryonic pollen) on fruit set, and embryo development and survival in polyembryonic cultivars.

E. SCIENTIFIC REPORT

E.1. Polyembryony Rate in Mango Seeds

Introduction

The phenomenon of polyembryony was found in mango more than 100 years ago (2, 15, 16). The occurrence of polyembryony in many mango cvs is well known to professionals working with mango. It has been used as an important descriptor of mango (11, 12, 13, 14, 15, 18). In many tropical countries polyembryonic cvs have been propagated cheaply from seeds. As a rule the seedlings produced come true to type (9, 11, 12, 13, 17). This uniformity of progenies is very advantageous in rootstock production (3, 9, 11, 12, 13). In Israel, polyembryonic rootstocks like 'Sabre', '4-9' and '13-1' are used almost exclusively in commercial propagation of mango (4, 12).

Though the phenomenon of polyembryony is of great importance in breeding and propagation, there is very little documented, detailed information about it. (1, 5, 6, 7, 8, 10, 17). The most extensive study was reported by Horn (5). He examined 7,780 germinating seeds from 200 cvs and reported that many monoembryonic cvs such as 'Alphonse', 'Mulgoa' and 'Sandersha' had a small percentage (1 to 13) of polyembryonic seeds. At the same time a high incidence of monoembryonic seeds was reported in known polyembryonic cvs. The rate was as high as 84.1% for 'Pico' and 56.1% for 'Cambodiana'. Horn's results are at variance with other findings (6, 7, 8, 15), and with data reported by nursery personnel. This scanty, conflicting information convinced us that a thorough study of these phenomena is warranted.

Material and Methods

Mature fruits were harvested, the fibrous bony endocarp separated from the flesh, and the seed then extracted. The papery translucent outer seed covering was removed. Most of the seeds were planted the same day they were extracted. Planting was sometimes delayed up to 3 days. In such a case, the seeds were kept moist in polyethylene bags. The seeds were planted in a well aerated, porous medium. In Israel a mixture of peat moss and "Kalkar" (granulated polystyrene) was used. The seeds were planted in small elongated containers (0.7 l) in a cooled hothouse (temp. fluctuated between 20 and 30 degrees C). Most seeds (95-99%) germinated. In Florida sphagnum moss was used as a medium, and seeds were sown in shallow plastic boxes (flats) prepared for the purpose.

Six weeks after planting the seeds were taken out of the medium. The number of individual seedlings (with one or more branches, and one taproot) was determined (1, 5). Though optimal conditions were provided for germination, we found that not all embryos had germinated. Some became brown and died, while others remained alive. We did not count ungerminated embryos, either dead or alive. Thus, our results fall short of the true number of embryos per seed. We discarded, and did not include in the results, seeds that did not sprout at all.

Results

The number of seedlings sprouting from one seed was determined for 41 different cultivars and selections (19 in Israel and 22 in Florida). Overall 5,245 germinating seeds were examined (4,742 in Israel and 503 in Florida). The results of the work carried out in Israel are presented in Table 1.1.

Table 1.1. Rate of polyembryony in germinating seeds from 6 monoembryonic and 13 polyembryonic mango cultivars in Bet Dagan, Israel*

Cultivar	No. seeds examined		Ave. no. plants per seed		% seeds with one plant	
	1981	1982	1981	1982	1981	1982
Alphonso	200	--	1.00	--	100.0	--
Dasher1	207	--	1.00	--	100.0	--
Haden	207	--	1.00	--	100.0	--
Irwin	221	--	1.00	--	100.0	--
Maya	195	--	1.00	--	100.0	--
Sandersha	203	--	1.00	--	100.0	--
Carabao	201	--	2.05	--	32.0	--
Colombo Kidney	--	19	--	2.20	--	37.0
Gedong	201	--	4.00	--	14.0	--
Mistikau1	180	201	3.99	4.87	12.0	2.0
Peach	200	162	4.83	3.93	3.0	3.0
Rupp1n	207	42	1.01	1.00	99.0	100.0
Sabre	204	388	3.54	3.94	14.0	13.0
Turpentine	--	182	--	3.92	--	9.0
Warburg	192	162	2.25	2.39	32.0	25.0
4-9	--	118	--	4.27	--	3.0
8-16	157	--	2.93	--	15.0	--
13-1	148	341	3.28	2.87	7.0	13.0
14-12	204	--	2.81	--	8.0	--

*Seedlings were examined 6 weeks after sowing.

Not one of the 1,233 monoembryonic seeds (from 6 cvs) gave rise to more than one seedling per seed (Table 1.1). 'Ruppin', which had been considered as polyembryonic, was found to be almost 100% monoembryonic: out of 251 seeds tested only one sprouted more than one seedling. In contrast, the remaining 12 polyembryonic cvs performed as expected, producing more than one seedling per seed in the case of most seeds (63% - 98%). The average number of seedlings per seed ranged from 2.05 to 4.87. 'Carabao', 'Colombo Kidney' and 'Warburg' produced less seedlings per seed, compared to the other 9 cvs. The highest number of seedlings per seed encountered was 12 plants, from a single 'Peach' seed. Seeds from 6 cvs were tested during 2 consecutive years. The variation found between years was not pronounced.

The results of the work carried out in Florida are presented in Table 1.2. Only one seedling each emerged from seeds of known monoembryonic selections (HC3S-31, -33, -40, -41). The selection HC3S-56 showed a very weak tendency to produce more than one seedling per seed (Table 1.2). For the 17 polyembryonic cvs the average number of seedlings per seed ranged from 1.1 to 5.7. The highest number of sprouts (13) emerged from a 'Turpentine' seed. Anthracnose destroyed some emerging seedlings; this disease was especially prevalent during the second, more rainy season (Table 1.2). The lower number of seedlings per seed in the second season may be the result of this problem.

Certainly factors other than genotype exert a profound influence on the number of plants that germinate from a single seed, as data for 'Carabao' and M-20222 indicate (Table 1.2). In 1981, seeds of both these cvs gave rise to an average of 3.3 young plants whereas the next year both cvs averaged only 2.3 plants from a single seed. Furthermore there was a

great difference by years in the number of seeds that gave rise to only one plant: in 1981, only 4.3% of the 'Carabao' and 5.5% of the M-20222 seeds yielded a single seedling, whereas in 1982 21.4% of the 'Carabao' and 25.9% of the M-20222 seeds produced only one seedling. On the other hand, 2 different trees of 'Ono' gave fairly similar results in the year 1982, averaging 2.1 and 2.6 plants per seed, respectively, with 21.4 and 23.8% of the seed populations giving rise to one single plant (Table 1.2). Furthermore, the same clone in two different environments performed differently. Note that 'Carabao' in Israel in 1981 (Table 1.1) produced 2.05 plants per seed (vs. 3.3 in Florida) and 32% of its seeds produced only 1 plant (vs. 4.3% in Florida) whereas 'Sabre' in Israel (1983) averaged 3.94 plants per seed, vs. only 2.1 in Florida. 'Turpentine' in Israel in 1982 also averaged more plants per seed (3.92) than the highest reading for Florida that year, 3.8 (Tables 1.1 and 1.2).

The HC3S population, seedlings of HC3 open-pollinated, show segregation for the polyembryonic trait. Anecdotally, HC3 is derived from a cross of 'Haden' (monoembryonic) by 'Carabao' (polyembryonic) made by Edward Simmonds, an early director of the Miami station. 'Haden' traditionally is believed to result from a spontaneous cross of 'Mulgoba', its known seed parent, by 'Turpentine', the common seedling race widely grown in Florida in the late 19th century.

In Florida seedlings were observed in regard to their position from proximal (upper end of the seed) to distal (lower end) locations, and rated for vigor. A few generally were observed to be relatively large (rating 1), many of medium size (rating 2), and some of relatively small size (rating 3). The size of a particular plant appeared directly related to the amount of cotyledonal material that had broken off to form the segment

that fed that plant during the course of its development. In general (for most polyembryonic cvs), the larger, more vigorous seedlings appeared near the upper end of the seed, but results were not conclusive. In most cases seedlings tended to cluster at the upper end of the seed; this apparently was the result of where the nucellar buds appeared during the seed's early development (See E.5, below).

Table 1.2 Rate of polyembryony in germinating seeds from 4 monoembryonic and 17 polyembryonic mangos in Miami, Florida.

Cultivar	No. seeds examined	Ave. no. plants per seed	% seeds with one plant
<u>1981:</u>			
Carabao	23	3.3	4.3
M-20222	18	3.3	5.5
Turpentine	23	5.7	0.0
<u>1982:</u>			
HC3S-31	16	1.0	100.0
HC3S-33	17	1.0	100.0
HC3S-40	24	1.0	100.0
HC3S-41	15	1.0	100.0
Arumanis	27	1.7	44.4
Cambodiana	8	1.1	87.5
Carabao	14	2.3	21.4
Chino	9	2.8	11.1
Golek	6	1.5	50.0
HC3 (Parent)	11	1.5	63.6
HC3S-47	14	2.0	42.8
HC3S-50	20	1.3	80.0
HC3S-51	10	2.0	50.0
HC3S-56	21	1.05	95.2
HC3S-58	19	2.2	21.0
Heart	24	1.9	54.2
M-20222	27	2.3	25.9
Madoe	10	3.0	20.0
Ono (posn 1-1)	21	2.6	23.8
Ono (posn 2-7)	14	2.1	21.4
Sabina	8	2.0	25.0
Sabre	26	2.1	42.3
<u>Turpentines:</u>			
N2-1-7-2	28	2.6	25.0
N3-1-2-6	24	2.7	33.3
N4-1-1-10	26	3.8	11.5



Fig. 2.1. A 13-1 embryo (seedcoat removed).

2. Visual Estimate of Embryo Number

When the seedcoat is removed and the embryo or embryos are exposed it is possible to identify polyembryony (Fig. 2.1). We removed the seedcoat (a tedious chore) from ca. 750 seeds of 6 cvs used for the prior survey. We estimated the number of embryos per seed and then planted these seeds in labelled containers. Results are presented in Table 1.3.

We were surprised to find monoembryonic-like seeds in 5 of the 6 cvs tested. Seeds of 'Peach' only were consistently polyembryonic. In contrast, about 20% of the 'Carabao' seeds appeared monoembryonic (Table 1.3). After germination, we found in most cvs a high positive correlation between the visual estimate of embryo number per seed and the actual number of emerging seedlings. However, the results also show that no absolute accuracy can be achieved by a visual estimate. The clearest discrepancy can be seen in the seeds that we estimated to have only one embryo; of the 52 seeds in that category, 11 were eventually found to be polyembryonic. We checked carefully the 5 'Peach' seeds which were evaluated to be polyembryonic, but produced only one seedling per seed (Table 1.3). In every case, we found dead embryos in these seeds.

Discussion

Planting the seeds in light, well-aerated media provided optimal conditions for germination. The media enabled easy uprooting of the seedlings, thus facilitating reliable (1, 5) determination of their correct number.

Our results (Tables 1.1, 1.2, 1.3) show that, as a rule, there is a clear-cut difference between monoembryonic and polyembryonic mangos: seeds of monoembryonic mangos produced only one seedling plant per seed. This conclusion contradicts Horn's finding (5), but it is supported by the fact that no true multiple seedlings were found when thousands of monoembryonic seeds were planted for breeding purposes, both in Israel and Florida. Seeds of polyembryonic mangos usually produced more than one seedling per seed. However, in all cvs of this type tested, some seeds produced only one seedling. This phenomenon also was found by Horn (5). Our observations indicate that often this is the result of partial damage to a polyembryonic seed. In one of our studies (Table 1.3), 62% of 109 seeds with only one seedling, were identified earlier as polyembryonic. However, the occurrence of some genuine monoembryonic seeds is supported by our results (Table 1.3). This phenomenon was especially common in 'Carabao', where 17% of the seed were found, both visually and actually, to have only one embryo. In 4 other cvs this phenomenon also occurred, but was quite rare (in about 3% of the seeds). Only 'Peach' was found to produce 100% polyembryonic seeds.

The only cv that does not fit into this scheme is 'Ruppin' (HC3S-56 may be similar). 'Ruppin' had been identified as polyembryonic (11, 12). We found only one seed with more than one seedling out of 251 planted.

Table 1.3. The relation between visual estimate of embryos per seed and the actual number of seedlings emerged for 6 polyembryonic mango cultivars. Experiment performed in 1982 at Bet Dagan.

Cultivar	No. of seeds tested	Seeds grouped by visual estimate		No. of seeds with N seedlings								Correlation coef. est.: actual	
		No. embryos per seed	No. of seeds	N=1	2	3	4	5	6	7	8		
Carabao	168	1	33	29	4								0.99
		2	93	25	52	9	6	1					
		3	40	3	20	12	3	2					
		4	2			1	1						
14-12	94	1	7	3	2	2							0.81
		2	29	2	15	11	1						
		3	34	2	12	12	7	1					
		4	23	1	6	11	5						
		5	1					1					
8-16	61	1	4	2		1		1					0.27
		2	14	6	4	2	1	1					
		3	14	1	2	6	4	1					
		4	18		5	3	5	5					
		5	11	1	1	9							
Gedong	112	1	4	3	1								0.74
		2	36	6	12	11	7						
		3	48	1	5	14	10	10	3	3	2		
		4	20			5	4	5	4	2			
		5	4			1	2	1					
Mistikawi	147	1	4	4									0.98
		2	5	4	1								
		3	17	4	8	2	3						
		4	24	2	9	8	5						
		5	35	5	1	8	11	6	1	3			
		6	23		2	7	3	5	4	2			
		7	22		1	5	2	7	2	4	1		
		8	17				3	6	6	1	1		
Peach	149	3	12	1	5	4	2						0.90
		4	44	3	2	6	17	10	2	4			
		5	39	1	3	4	8	8	10	4	1		
		6	28			2	2	4	11	6	3		
		7	16				4	5	3	2	2		
		8	10				1		2	3	4		

Oppenheimer, who observed a lot of 'Ruppin' seedling trees, found that they do not come true to type. It seems that 'Ruppin' is an exception to the rule, a monoembryonic cv that may rarely produce a polyembryonic seed. This conclusion means that 'Ruppin' should not be included anymore in the list of recommended polyembryonic mango rootstocks in Israel.

We found a great variation in the number of embryos and seedlings per seed, in seeds of the same cv (Table 1.3). This variation was noticed also in other studies (7, 15). It is believed that environmental conditions can influence the realization of the polyembryonic potential in Citrus. The same belief appears well conceived in the case of Mangifera.

Monoembryonic seeds of polyembryonic mango cvs are not necessarily zygotic. 'Warburg' can be safely propagated by seeds (11, 12), as almost all seedlings will be true to type. This indicates that the high percentage of single seedlings per seed (Table 1.1) are nucellar. The same argument can be applied to 'Carabao'. Though 32% of its seeds gave rise to only one seedling in Israel (Table 1.1), and 17% of its seeds were probably monoembryonic (Table 1.3), 'Carabao' is known to come true to type (9).

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E.2. Anatomical Study of Early Polyembryony in '13-1' and 'Turpentine'

Introduction

A number of anatomical studies of polyembryony have been published (1, 2, 3, 4, 5, 6, 7). Before the present study, it had already been found that at anthesis dense nucellar cells already can be discerned at the micropylar end of the embryo sac. After the formation of endosperm, these cells start to divide rapidly, developing into adventitious embryos that protrude and enter into the embryo sac. We followed the polyembryonic developmental process in the 2 cvs ('13-1' and 'Turpentine') that we used for most of our experimental work in Israel.

Material and Methods

'13-1' and 'Turpentine' flowers and fruitlets were sampled near Bet Dagan, Israel and were fixed in FAA. Pistils, or ovaries, were excised, embedded in paraffin and cut serially in lengthwise sections of 12 μ thickness. Sections were stained with safranin and fast green.

Results

The development of the nucellar embryo was followed up to the age of 6 weeks. No significant differences were found between '13-1' and 'Turpentine'. Results can be seen in Figs. 2.1, 2.2, 2.3, 2.4 and 2.5. A large number of nucellar embryos could be already seen at the age of 2-3 weeks (Fig. 2.2). At the age of 3-4 weeks the nucellar embryos began to be incorporated into the embryo sac. This was the last stage when the zygotic embryo could still be identified with certainty; at this stage it was surrounded completely by the endosperm, while the nucellar embryos were still located close to the micropylar nucellus wall. At the age of 4-6 weeks it was no longer possible to identify the zygotic embryo (Figs. 2.3, 2.4, 2.5).



Fig. 2.3. Nucellar embryos (NE) in 3-4 weeks old '13-1' fruitlet (x 125).

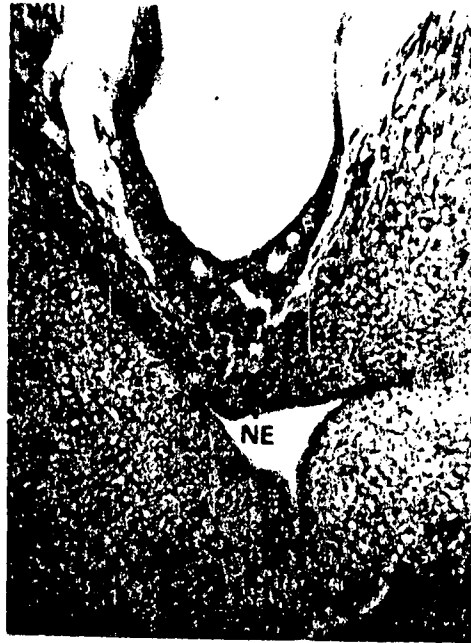


Fig. 2.2. Early stage of nucellar embryos (NE) development. '13-1' fruitlets, 2-3 weeks old (x 125).



Fig. 2.1 Nucellar cells with pronounced large nuclei (NU) in '13-1' ovule at anthesis (x 125). (x



Fig. 2.5 Nucellar embryos in 5-6 weeks old '13-1' fruitlet (x 40).



Fig. 2.4. Nucellar embryos (NE) in 4-5 weeks old '13-1' fruitlet (x 125).

Discussion

The early development of nucellar embryos in '13-1' and 'Turpentine' mangos did not differ in any significant detail from prior reports (1, 2, 3, 4, 5, 6). The information obtained in this study was used in studying the effect of controlled hand pollination on subsequent development of young embryos (E.6).

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F. Description of Cooperation

Information about research techniques, difficulties encountered, results and ideas were freely exchanged between the two cooperating scientists. Dr. Gazit went to Miami in 1984 and Dr. Knight's visit to Bet Dagan and Rehovot in 1985, provided ample opportunity for a wide range of discussions on the research. About 100 fruitlets, set after hand pollination in Miami, were processed and anatomically examined in Rehovot.

G. Evaluation of Research Achievements

1. Reliable data on the phenomenon of polyembryony in a large number of monoembryonic and polyembryonic mango cvs were amassed. This information corrects some erroneous information found in the literature. All monoembryonic cultivars produced 100% monoembryonic seeds. Thus, no nucellar seedlings should be expected from this group. 'Ruppin' previously was recommended as a polyembryonic rootstock. We found this cv to be practically monoembryonic. Some genuinely monoembryonic seeds were observed in several polyembryonic cvs. They should be checked to determine whether they are of nucellar or zygotic origin.

2. Mango ovules were successfully cultured on solid modified Murashige and Skoog media. When the ovules were at a stage in which no nucellus could be identified, precocious germination occurred. This technique may be used to maximize the rate of embryo germination and survival.

3. Somatic embryogenesis was achieved from cultured nucellus of polyembryonic cvs. This may lead to wide-scale vegetative propagation by tissue culture.

Tissue culture work had not been included in the original research

plan. However, when faced in 1982 with a very scanty fruit set in Miami we decided to use the small number of fruitlets in the most effective way. Dr. Litz from the University of Florida, at Homestead, was ready to collaborate and went to work with the plant material that Dr. Knight supplied.

4. Isozyme analysis was used successfully to identify the zygotic seedlings from tagged '13-1' seed "families". The location of the emerging zygotic seedling was found not to differ from that of its emerging nucellar siblings. We found almost all seedlings, zygotic as well as nucellar, to emerge from the proximal part of the seed. Thus, Tammes' suggestion to cut off the proximal part of the seed, in order to get rid of the sexual embryo, will result in destroying all nucellar embryos as well, in most '13-1' seeds.

5. Dismal results from hand pollination led to a study of factors affecting successful mango pollination in Israel. We were surprised to find that in the coastal plain of Israel, successful pollination (and fruit set) occur only at the end of the flowering season. This dismal situation emphasizes the urgent need to develop methods to delay the flowering season.

6. Our studies (hand pollination with anatomical examination of the resulting young fruitlets, and of fruitlets set on caged trees) indicate that both 'Turpentine' and '13-1' are self-incompatible and that though '13-1' is apparently a good pollenizer for 'Turpentine', 'Turpentine' is apparently a poor pollenizer for '13-1'. These conclusions have great importance for breeding new rootstocks, and for seed production of these leading rootstocks in Israel and Florida.

H. List of Publications

1. Gazit, S. and Roizman, Y. 1989. Factors responsible for inadequate successful pollination under subtropical climatic conditions. (A paper to be delivered at the 3rd Int. Mango Symposium and published in Acta Hort.)
2. Litz, R. E., Knight, R. and Gazit, S. 1982. Somatic embryos from cultured ovules of polyembryonic Mangifera indica L. Plant Cell Rep. 1:264-266.
3. Litz, R. E., Knight, R. and Gazit, S. 1984. In vitro somatic embryogenesis from Mangifera indica L. callus. Scientia Hortic. 22:233-240.