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Tetraploid Progenies from 2x X 4x Crosses of *Citrus* and Their Origin[1]

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Abstract. The analysis of ploidy levels in progenies from 2x X 4x crosses during embryogenesis and after germination of the seeds indicates that they are mixtures of triploids and tetraploids. The frequency of tetraploids varies from 6 to 94% depending on the pistillate parents used. Chromosome number determinations in the embryo and endosperm of sectioned young seeds provide conclusive evidence that the megagametophytes which produce tetraploids when fertilized contain diploid eggs and polar nuclei. The occurrence of diploid megagametophytes in diploids provides an additional approach for producing tetraploid stocks following 2x X 4x crosses.

The basic chromosome number in the genus *Citrus* and other members of the subfamily Aurantioideae is x=9 (9). Virtually all wild and cultivated forms of citrus are diploids (16). Polyploids, mostly triploids and tetraploids, are also known to occur either spontaneously or after certain crosses. Spontaneous tetraploids reported so far have appeared as nucellar seedlings in progenies from facultative apomict diploid pistillate parents. Apparently, they are autotetraploids which originate through doubling of the chromosome complement in a nucellar cell which later develops into an adventive embryo (2). Russo and Torrisi (21) reported an exceptional case where they found 3 hybrid tetraploids (allotetraploid) from 2x X 2x crosses. They considered the role of diploid gametes contributed by both parents, or doubling of the chromosome number in the first zygotic division to explain the origin of these allotetraploids.

Autotetraploids have been used in crosses with diploids to produce seedless (triploid) cultivars (3, 10, 24). It was found that progenies of 2x X 4x crosses contain unusually high frequencies of hybrid tetraploids in addition to normally expected triploids (3, 24). Tachikawa et al. (24) suggested "chromosomal aberration" to explain the occurrence of such tetraploids in frequencies as high as 70 to 80%. Cameron and Soost (3) proposed mechanisms such as doubling of the chromosome complement in the haploid egg cell due to delayed fertilization by diploid male gametes, or preferential fertilization of diploid egg cells by diploid male gametes in the presence of certain combinations of incompatibility alleles. They did not suggest that diploid female gametes were being produced by diploid pistillate parents because many of them had previously been used as pistillate parents in 2x X 2x crosses and produced only diploid progeny. Consequently, the origin of tetraploids from 2x X 4x crosses of *Citrus* had remained undetermined.

This paper reports the results obtained from progenies produced from 2x X 4x crosses made in 1969 and 1970 for the purpose of studying the origin of such tetraploids.

Material and Methods

Five diploid zygotic (monoembryonic) pistillate parents, 'Sukega' [*Citrus paradisi* Macf. X *C. sinensis* (L.) Osbeck[3]]; 'Temple' [*C. reticulata* Blanco X *C. sinensis* (L.) Osbeck[4]]; 'Clementine' [*C. reticulata* Blanco]; Pummelo CRC 2240 and 'Roeding's Pink' [*C. grandis* (L.) Osbeck] were hand-pollinated by tetraploid 'Paperrind' and an unnamed sweet orange (designated hereafter as sweet) [*C. sinensis* (L.) Osbeck]; 'Hall' and 'Seedy Marsh' [*C. paradisi* Macf.] and 'Lisbon' [*C. limon* (L.) Burm. f.].

Pollinations were carried out immediately after emasculation and in 1969 the twigs with pollinated flowers were bagged to exclude insects and stray pollen. In 1970, however, bagging was discontinued because chromosome counts in progenies obtained from unbagged twigs pollinated in 1969 indicated that there was no contamination by stray pollen. In addition, all of the pistillate parents except 'Temple' were self-incompatible.

Seeds were extracted 100 to 132 days after pollination and at fruit maturity. Fruits matured from 8 to 9 months after pollination depending on the cultivar. Seeds were classified as 1) fully developed, and 2) partially developed or empty. At maturity fully developed seeds contained well-developed and differentiated embryos that filled the entire seed cavity or when extracted at 100 to 132 days after pollination, they had well-developed endosperms which filled the entire seed cavity.

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[3]Staminate parent is not known definitely.
[4]Presumed parents; both pistillate and staminate parents are not known definitely.
[5]Esen, A. 1971. Unexpected polyploids in *Citrus* and their origin. PhD. Dissertation, University of California, Riverside.

At maturity most of the remaining seeds consisted only of empty seed coats. A few seeds were partially developed with undifferentiated and poorly differentiated embryos. Empty and partially developed young seeds had either no visible endosperm, or only a thin layer lining the seed cavity. Fully developed mature seeds from each cross were germinated for root tip chromosome counts. Chromosome counts were also made from young embryo squashes and sections of whole seeds extracted 100 to 132 days subsequent to pollination. For squash preparations, root tips were pretreated with 25 ppm. *o*-isopropyl-N-phenylcarbamate (IPC) (23), fixed in 2 1/2:1 (v:v) alcohol propionic acid, hydrolyzed in 1N HCl, stained in 1% lacto-propiono-orcein (6) and squashed. The same procedure was followed for young embryo squashes except that they were not pretreated with IPC before fixation. Additionally, whole young seeds were fixed in chromo-propiono-formalin (CRPF), dehydrated in an ethyl alcohol and tertiary butyl alcohol (TBA) series as described by Johansen (14), embedded in paraplast, sectioned at 12-13μ thickness and stained with 0.25% Heidenhein's hematoxylin.

Results

Seed set and development. The data in Table 1 show that 'Sukega' yielded the highest percentage of fully developed seeds (16.7 to 21.3) while 'Clementine' was extremely poor, producing the lowest percentages (1.1 to 4.9). Poor seed development was also evident in 'Roeding's Pink' pummelo (3.0%). 'Temple' and Pummelo CRC 2240 were somewhat intermediate with percentages of 12.0 to 18.7 and 11.9 to 16.5, respectively. Differences among pistillate parents were also evident in mean number of fully developed seed set per fruit (Table 1). Moreover seed set and development also appeared to be affected by the staminate parents as both percent of fully developed seeds and mean numbers of fully developed seeds per fruit were reduced when 'Hall' grapefruit and 'Lisbon' lemon were the staminate parents.

Analysis of progenies with respect to ploidy levels. Tables 2 and 3 show that the progenies consisted of triploids (Fig. 1, A) and tetraploids (Fig. 1, B) whose proportions fluctuated depending on the pistillate parents. There were also 3 cases of hexaploidy (Fig. 1, C). When comparisons were based on the surviving progeny (fully developed seeds on which chromosome counts were obtained), 'Sukega' produced the highest percentage of tetraploids (94.11%) followed by 'Temple' (51.85%), 'Clementine' (44.18%), 'Roeding's Pink' (6.67%) and Pummelo CRC 2240 (5.81%) (Table 2). Percentages based on potential survivors [seeds with well-developed endosperms when extracted 100 to 132 days after pollination minus those whose chromosome number could not be obtained because of the absence or scarcity of division figures (Table 3)] from 'Sukega' and Pummelo CRC 2240 pistillate parents were in reasonable agreement with those based on actual survivors (Table 2). The agreement was not good when 'Temple' was the pistillate parent, but only 6 seeds were available for counts made at 122 days after pollination. Differences among pistillate parents with respect to the percentages of tetraploids they produced remained similar when comparisons were based on fertilized ovules (cf. the last columns of Tables 2 and 3). However, the percentages were much lower when based on fertilized ovules because empty and partially developed seeds were included in calculations in addition to fully developed seeds. When expressing the percentages of tetraploids in terms of fertilized ovules, we assumed that all of the empty and partially developed seeds resulted from fertilized ovules and contained triploid embryos. This assumption was supported by the senior author's work on the mechanism of seed abortion following 2x X 4x crosses (Esen, 1971)[5]. He found that all empty and partially developed seeds as well as fully developed seeds arise from fertilized ovules. Also, all aborting seeds of 2x X 4x crosses, those that have defective, degenerating or completely degenerated endosperm 100 to 132 after pollination, contain triploid embryos which are destined to degenerate in the

Table 1. Seed set and development in crosses of 2x X 4x *Citrus* cultivars.[z]

♀ Parent 2x	♂ Parent 4x	Number of seeds obtained: No. of fruits obtained	Fully developed	Partially developed or empty	Total		% Fully developed	Fully developed per fruit
Sukega	Sweet	48	205	795	1000		20.5	4.27
	Paperrind	61	241	891	1132		21.3	3.95
	Hall	61	91	364	455		20.0	1.49
	Lisbon	13	13	65	78		16.7	1.00
	Total	183	550	2115	2665	Mean	20.6	3.01
Temple	Sweet	7	24	104	128		18.7	3.42
	Paperrind	2	6	44	50		12.0	3.00
	Hall	–	–	–	–		–	–
	Lisbon	1	–	16	16		–	–
	Total	10	30	164	194	Mean	15.5	3.00
Clementine	Sweet	37	24	515	539		4.5	0.65
	Paperrind	30	20	386	406		4.9	0.67
	Hall	29	8	300	308		2.6	0.28
	Lisbon	34	5	449	454		1.1	0.15
	Total	130	57	1650	1707	Mean	3.3	0.44
Pummelo CRC 2240	Sweet	5	62	461	523		11.9	12.40
	Paperrind	4	23	378	401		5.7	5.75
	Seedy Marsh	1	13	66	79		16.5	13.00
	Total	10	98	905	1003	Mean	9.77	9.80
Roeding's pink	Sweet	13	24	772	796		3.00	1.85

[z]Seeds extracted at maturity.

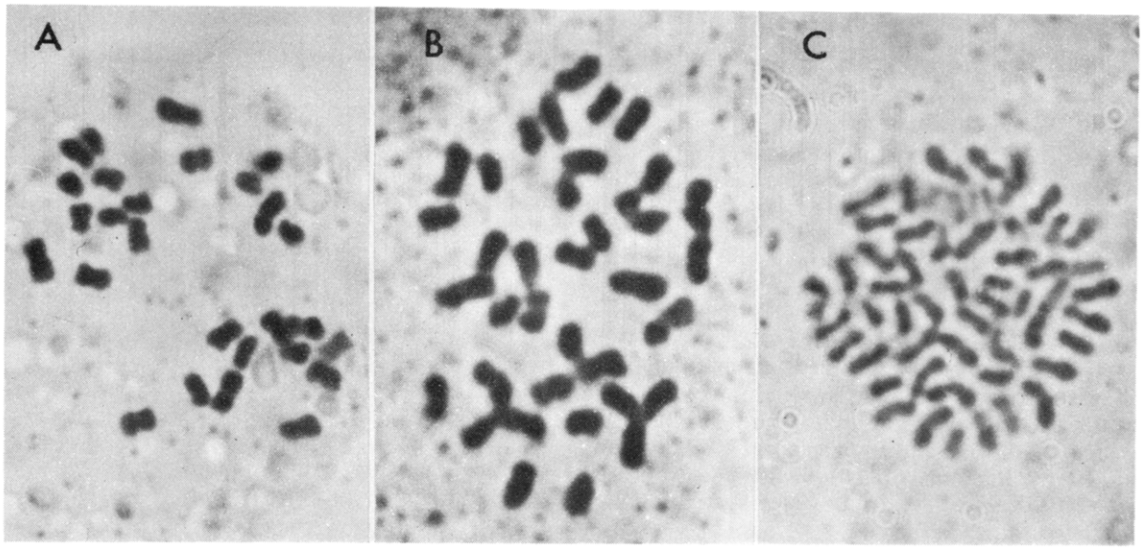

Fig. 1. Mitotic metaphase figures showing expected *(A)* and anomalous *(B, C)* ploidy levels in *Citrus* following 2x X 4x crosses: *A*, 3x Hybrid from Pummelo CRC 2240 X 4x sweet orange. *B*, 4x Hybrid from 2x 'Clementine' X 4x sweet orange. *C*, 6x Hybrid from 2x 'Sukega' X 4x 'Hall'. (*A*. X 4000; *B*. X 5800; *C*. X 3300)

absence of functional endosperm.

Relation of tetraploids to pistillate parents. Chromosome number data obtained from root tips (Table 2) demonstrated that the frequency and occurrence of tetraploids from 2x X 4x crosses were strictly dependent upon the pistillate parent. This relationship between pistillate parents and tetraploids was also verified through chromosome counts in the embryo and endosperm of sectioned seeds during embryogenesis (Table 3). Without exception, the endosperm was hexaploid and the embryo tetraploid in 187 seeds that had countable division figures available both in their embryos and endosperms. On the other hand, all of the sectioned seeds with tetraploid endosperm contained triploid embryo.

Discussion

Results of this study confirm the occurrence of tetraploids from 2x X 4x crosses of *Citrus* reported by Tachikawa et al. (24) and Cameron and Soost (3). In addition, we have demonstrated that production of diploid female gametes in varying frequencies depending on the pistillate parents is the

Table 2. Chromosome counts in the progeny of 2x X 4x *Citrus* crosses.[z]

♀ Parent 2x	♂ Parent 2x	No. of seeds		No. of hybrids with indicated degree of ploidy				Percent tetraploids among survivors	Percent tetraploids among fertilized ovules
		Fully developed	Total	Undetermined[y]	3x or near 3x	4x or near 4x	6x		
Sukega	Sweet	205	1000	13	13	179	..	93.22	18.13
	Paperrind	241	1132	11	13	216	1	93.91	19.26
	Hall	91	455	..	3	87	1	95.60	19.12
	Lisbon	13	78	..	..	13	..	100.00	16.67
	Total	550	2665	24	29	495	2	Mean 94.11	18.74
Temple	Sweet	24	128	3	11	10	..	47.61	8.00
	Paperrind	6	50	..	2	4	..	66.67	8.00
	Total	30	178	3	13	14	..	Mean 51.85	8.00
Clementine	Sweet	24	539	8	9	7	..	43.75	1.31
	Paperrind	20	406	6	8	6	..	42.85	1.50
	Hall	8	308	..	5	3	..	37.50	0.97
	Lisbon	5	454	..	2	3	..	60.00	0.66
	Total	57	1707	14	24	19	..	Mean 44.18	1.12
Pummelo CRC 2240	Sweet	62	523	8	52	2	..	3.70	0.38
	Paperrind	23	401	4	16	3	..	15.79	0.75
	Seedy Marsh	13	79	..	13	..	..	...	...
	Total	98	1003	12	81	5	..	Mean 5.81	0.50
Roeding's pink	Sweet	24	796	9	14	1	..	6.67	0.13

[z]Chromosome counts obtained from root tip squashes.
[y]Hybrids whose chromosome number could not be determined.

Table 3. Chromosome counts in developing seeds of 2x X 4x *Citrus* crosses[z].

♀ Parent 2x	♂ Parent 4x	No. of seeds		No. of seeds with indicated degree of ploidy				Percent tetraploids among potential survivors	Percent tetraploids among fertilized ovules
		Developing[y] normally	Total	Unde-termined[x]	3x or near 3x	4x or near 4x	6x		
Sukega	Sweet	331	1149	12	31	287	1	89.96	25.24
Temple	Sweet	6	142	..	0	6	..	100.00	4.22
Pummelo CRC 2240	Sweet	82	563	..	80	2	..	2.44	0.35

[z]Chromosome counts obtained from embryo squashes or sections of whole seeds.
[y]Seeds with well-developed endosperms and developing embryos at 100 to 132 days after pollination.
[x]Developing seeds whose chromosome number could not be determined.

mechanism leading to tetraploids. The following findings substantiate this conclusion: First, the frequency of tetraploids were primarily dependent upon the pistillate parents, for the frequency usually remained more or less constant when a given pistillate parent was pollinated by different staminate parents (Tables 2 and 3). Conversely, the frequency varied from one pistillate parent to another when pollinated by the same staminate parent (Tables 2 and 3). Second, the frequency of tetraploids from a 2x X 4x cross was approx equal to that of triploids produced by the same pistillate parent pollinated by diploids when comparisons were based on total number of ovules fertilized (Esen and Soost, 1971). Third, in 187 sectioned seeds with tetraploid embryos the endosperm was hexaploid. This indicates that the megagametophytes from which tetraploids arise were diploid. The occurrence of tetraploids in high frequencies from 2x X 4x crosses of other plant taxa suggests that this phenomenon is not limited to *Citrus* spp. Such unexpected tetraploids were reported from 2x X 4x crosses of *Dactylis* (4), *Sorghum* (7, 13), *Zea* (1, 19, 20), *Campanula* (12), *Medicago* (5, 17, 18), *Primula* (22) and *Solanum* (15, 25).

The mechanism leading to the production of the 3 hexaploids (Tables 2 and 3) in progenies from 2x X 4x crosses appears to be formation of double-unreduced (tetraploid) female gametes by diploid pistillate parents as 1 sectioned seed available with hexaploid embryo contained decaploid (10x) endosperm.

The differences in percentages of tetraploids among surviving progenies and fertilized ovules were found to be due to abortion of triploid embryos with tetraploid endosperm as opposed to full viability of tetraploid embryos with hexaploid endosperm. When all the empty and partially developed seeds from 2x X 4x crosses were considered as having triploid embryos with tetraploid endosperms, the percent survival of triploid embryos from 'Sukega', 'Clementine', 'Temple', Pummelo CRC 2240 and 'Roeding's Pink' all pollinated by tetraploids, was 1.35, 1.43, 8.07, 8.21 and 1.78, respectively. This indicates that about 92 to 99 percent of seeds with triploid embryo and tetraploid endosperm degenerate during embryogenesis, thus increasing about 4- to 50-fold the frequency of tetraploids among the surviving progeny of 2x X 4x crosses. It appears that this drastic reduction in the viability of triploid embryos with tetraploid endosperm results from unbalance of gene dosage between embryo and endosperm.

Although, we have established the origin of tetraploids from 2x X 4x crosses of *Citrus* as the diploid female gametes, the mechanism which leads to the production of such female gametes remains to be determined. It is very likely that some kind of irregularity in megasporogenesis and megagametogenesis is their origin. Gonial and somatic apospory can be ruled out as probable mechanisms, since all investigations on megasporogenesis in *Citrus* species have shown that megaspores are formed through meiosis by a well-defined megaspore mother cell (MMC) (11). The omission or failure of one of the meiotic divisions, or an extra chromosome replication in the functional megaspore may be a plausible explanation, but these possibilities need to be verified by a thorough study of megasporogenesis especially in 'Sukega', which produces about 25 percent diploid female gametes. The formation of diploid female gametes seems to be genetically determined. For example, 'Sukega' X 4x cultivars produced tetraploids in frequencies of 94.11% in 1969 and 89.41% in 1970 (Tables 2 and 3). We do not know if there is a similar mechanism operating to produce diploid microspores in the cultivars in question.

This study also suggests that one could determine the frequency of diploid female gametes produced by diploid cultivars by pollinating diploids with tetraploids and determining ploidy level in the surviving progenies. Since tetraploids from 2x X 4x crosses invariably survive and the number of surviving and aborted triploid seeds is known, calculations may indicate the frequency of diploid female gametes produced by diploid pistillate cultivars based on the frequency of tetraploids they produce following 2x X 4x crosses. Such information is valuable to judge the feasibility of producing seedless (triploid) cultivars by means of 2x X 2x crosses in which triploids could be distinguished from diploids in the seed stage on the basis of size (Esen and Soost, 1971). Moreover, one could obtain new tetraploid stocks at will, without relying on rare spontaneous tetraploids exclusively, by simply making crosses of 2x X 4x parents. Tetraploid stocks obtained in this manner can be used as pistillate parents in 4x X 2x crosses to produce triploids, as crosses in this direction are more successful than their reciprocals. Therefore, tetraploids from 2x X 4x crosses are not necessarily rogues that are undesirable.

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Nitrate Accumulation in Vegetable Crops As Affected by Photoperiod and Light Duration[1]

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Abstract. No NO_3 accumulated at any photoperiod in leaves or roots of table beets when N was not added to the soil. When N was applied at rates from 100 to 400 lb./acre less NO_3 accumulated in both plant parts as photoperiod was extended from 8 to 20 hr. Addition of N to the soil increased the total N content of leaves and roots. Larger total N concn were observed in plants grown under an 8-hr photoperiod than in plants grown under longer photoperiods.

Various radish, spinach, and snap bean cultivars were grown at different soil N rates and harvested 0, 6, and 12 hr after the initiation of the light period. Radish leaves and snap bean pods contained less NO_3-N as the plants were harvested further into the light period. Nitrate concn of radish roots and spinach leaves were not changed by harvesting at 6 AM, 12 noon or 6 PM. The addition of N fertilizer increased the NO_3-N concn of radish and spinach but decreased the NO_3-N concn of snap bean pods. Cultivars differed in their capacity to accumulate NO_3 in all 3 species. Nitrite accumulation was proportional to the quantity of NO_3 in the tissue.

With today's emphasis on production efficiency of vegetable crops, rates of N fertilizer are more liberal each year. Excessive quantities of soil N can lead to the accumulation of NO_3 by vegetable crops (2, 5, 6, 10, 14, 19, 20). Generally, vegetative portions of the plant accumulate more NO_3 than reproductive parts, (2, 5, 10, 14, 15, 18, 20, 24), yet a high quantity of NO_3 in any plant part may adversely affect quality of the final product (2, 14, 18, 19, 20, 24) or may even be detrimental to human health as it can cause methemoglobinemia.

Although NO_2 is the causal factor destroying the oxygen carrying power of the blood, high levels of NO_3 can be reduced to NO_2 by intestinal bacteria or by bacteria in the stored product and may cause NO_2 poisoning in humans (21). Another potential risk of high NO_3 levels is detinning of cans containing processed vegetables (14, 19, 21, 24).

Factors other than the soil N level, such as light, temp and moisture stress, may contribute to high NO_3 levels in plants (3, 6, 8, 9, 10, 17, 29). High light intensity increases nitrate reductase activity which leads to a decrease in NO_3 (4, 6, 8, 11, 16, 23). Similarily, nitrate reductase activity and NO_3 concn have been shown to be affected by photoperiod, diurnal variation and light quality. In wheat, long photoperiod increased nitrate reductase activity and decreased the NO_3 concn in the tissue compared to short photoperiod (13). Sampling corn at 1 PM instead of 5 AM led to an increase in nitrate reductase activity and a decrease in the NO_3 concn (12). Similar studies related to these factors and their affect on NO_3 accumulation in vegetable crops are scarce. Our objective was to determine the relationship of N fertilization, photoperiod, and duration of the light period to the accumulation of NO_3 by different vegetable crops.

Material and Methods

Effect of photoperiod on the NO_3 concn of table beets. Table beets (*Beta vulgaris* L. cv. Ruby Queen) were grown in 6-inch plastic pots in Ontario fine sandy loam. Nitrogen was added to the soil at rates of 0, 100, 200, or 400 lb. N/acre as NH_4NO_3. Seventy-five lb. P/acre as $Ca(H_2PO_4)_2$ and 150 lb. K/acre as K_2SO_4 were added to each N treatment.

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