DIPLOID-TETRAPLOID PERICLINAL CHIMERAS AS BUD VARIANTS IN CITRUS[1]*

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INTRODUCTION

NUMEROUS genetic variations affecting portions of Citrus trees have occurred under cultivation. "Bud-variation" or "bud-mutation" forms have been studied extensively from the horticultural viewpoint in various commercial varieties, such as the Eureka and Lisbon lemons (SHAMEL et al. 1936), but little is known about the causation of the changes concerned.

Certain conspicuously mixed bud-variation forms, such as lemon "strains" with white-margined leaves (SHAMEL 1932) and orange strains with two kinds of fruit rind (SHAMEL et al. 1925), appear to be essentially periclinal chimeras which include the parent type as one component. In other cases, as with the horticulturally important Wase satsuma (TANAKA 1932), it seems very doubtful whether frequent somatic reversion of a bud-variant form to the parent type is due primarily to an originally chimeral condition or to genetic instability of the new type.

The extreme diversity of Citrus forms and the very abundant heterozygosis which seems to be general in this genus (WHITE 1914, FROST 1926) suggest that gene mutation occurs comparatively often. This process may therefore be considered as one probable cause of bud variation in Citrus, although the conditions are unfavorable for its discrimination from somatic crossing over or structural aberrations in chromosomes.

"Twin" variations in rind characters, which change adjacent sectors in opposite directions from the parental condition, are occasionally observed. These presumably indicate the occurrence of abnormal mitosis. When, as occasionally happens, both rind thickness and rind color are changed in opposite directions in two adjacent sectors, some shift involving more than one gene locus seems clearly indicated. Aside from such cases (and germ-layer shifts in chimeras, of course), the general nature of bud variation in Citrus seems likely to be determinable only when a cytologically visible alteration is involved.

Of the possible kinds of cytological evidence on the causation of bud

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variations in Citrus, evidently only changes in number of chromosomes are likely to be detected at all readily. Microsporocytes and root tips of stem cuttings presumably show the chromosome number only of two different germ layers from which they are derived. Their combined evidence, with bud-variation forms, therefore needs to be supplemented, especially by direct or indirect determination of the number of chromosomes in the epidermis derived from the first germ layer. These detailed investigations are necessary in order to distinguish between entirely modified variants and chimeras.

In recent years many investigations have been carried out to determine the number of germ layers in the primordia of plants. SCHMIDT (1924) proposed the "tunica-corpus" theory, visualizing two main germ regions, and FOSTER (1936, 1939) has shown that the number of germ layers participating in the development of the various tissues varies considerably in different species. BLAKESLEE et al. (1939) and SATINA et al. (1940, 1941), working with various colchicine-induced chimeras in Datura, have clearly demonstrated that three germ layers take part in the development of this plant. Apparently nothing is known about this subject with regard to Citrus.

Few determinations of chromosome number are available for Citrus forms of known bud-variation origin. NAKAMURA (1929) examined microsporocytes of two variant branches of satsuma classed as Wase. He found the same chromosome number ($2n = 18$) in these branches as in the "normal" part of the same trees and observed no difference in chromosome behavior. The Shamouti orange of Palestine, which probably originated from the Belladi orange by bud variation and which frequently produces branches of Belladi (OPPENHEIM 1929), has been found to have the normal number of chromosomes in microsporocytes (OPPENHEIM and FRANKEL 1929).

Under at least some climatic conditions tetraploidy is common among Citrus seedlings produced asexually (through nucellar embryony) by diploid parents of many varieties (FROST 1925, 1938; LAPIN 1937). Since triploids, but no tetraploids, have been found among seedlings of known gametic origin (F_1 interspecific hybrids) in the same cultures with nucellar tetraploids, it seems that the doubling of chromosome number which produces tetraploidy in Citrus seedlings must usually occur prior to the beginning of the embryonic divisions. Frequent cases of production of both tetraploid and diploid seedlings by the same seed strongly suggest that the doubling usually occurs in the pistil, and probably in the nucellus.

Tetraploid branches on otherwise diploid Citrus trees, indicating the probable origin of tetraploidy in shoot meristems, do not seem to have been reported prior to the present study. On the basis of the tree charac-

teristics of the bud-variation forms which have been studied in California, it is probable that tetraploid bud variations are rare in Citrus. In the case here reported, a small portion of a seedling tree was partially tetraploid, and the change in chromosome number probably occurred after the germination of the seed.

MATERIAL AND METHODS

From a hybrid mandarin produced by pollination of King mandarin by Dancy tangerine at the UNIVERSITY OF CALIFORNIA CITRUS EXPERIMENT STATION, 128 seedlings were grown from 111 seeds resulting from open pollination. On examination in January, 1932, at the age of about four and one-half years, all these plants seemed identical with the seed parent as to genetic type, and therefore all presumably had originated asexually by nucellar embryony. On one of these trees, however, one small branch (later designated "b"), which arose from the trunk at the height of about one and one-half meters, bore four lateral shoots, of which the largest was observed to have broad, apparently thick leaves, suggestive of tetraploidy. No description was recorded for the other three laterals. After the new shoots and leaves of the following spring had developed, branch b was examined again. Among a total of 13 shoots bearing two or more leaves each, only one then seemed to be diploid, ten appearing tetraploid, and two possibly intermediate. The entire variant branch died after the tree was transplanted.

The remainder of the seedling tree (designated "a") seemed to be entirely diploid. The progeny trees a-1 to a-3 of the following discussion were propagated from two shoots distant from branch b. Two pairs of trees (b-1 and b-2, b-3 and b-4) were propagated from two nonadjacent shoots on branch b. Trees a-1 to b-4 were all budded on trifoliate-orange rootstocks and were grown in consecutive order in the nursery. Tree b-5 was budded on sweet-orange rootstock from one of the bud sticks mentioned. In 1936 these trees were set in the orchard, in the order a-1 to b-5.

In August, 1940, leafy cuttings were taken from scattered terminal shoots of four trees, a-1, a-2, b-1, and b-4, and placed in sand in a closed hotbed with electric bottom heat. Rooted cuttings were potted in October and kept in a greenhouse.

In January, 1941, root tips from some of these cuttings were fixed in the Craf fixing solution (RANDOLPH 1935) for 24 hours, dehydrated by the tertiary butyl alcohol method (JOHANSEN 1940), and embedded in paraffin; sections 6 microns thick were stained with Heidenhain's haematoxylin. All drawings were made with camera lucida at table level at a magnification of approximately 3900×.

In April of the same year flower buds of different sizes were collected

from trees a-1, b-1, and b-4 (a-2 had no buds, probably because of overproduction of fruits in the preceding season) and fixed for 12 hours in three parts alcohol:one part acetic acid and then stored in 70 percent alcohol; smears were made with acetocarmine (concentrated solution in 45 percent acetic acid) and heated nearly to boiling before sealing the cover glass. Chromosome counts were made in dividing pollen mother cells with approximately the same magnification as used for counts in root tips.

At the same period the tips of fast growing shoots and small central portions of mature leaves were also fixed in Craf, dehydrated as were the

TABLE 1

Thickness of leaves on trees from which cuttings were taken.

TREE	LEAF THICKNESS (MM×100)		
	MEAN	DIFFERENCE	DIFF./S.E.
a-1	27.25±.26		
a-2	27.70±.44		
b-1	36.32±.34	8.62	15.4*
b-4	37.45±.31	9.75	18.1†

* Difference between a-2 and b-1 highly significant (t=2.71 for n=40 at P=0.01. GOULDEN, 1939, table 94).

† Difference between a-2 and b-4 also highly significant.

Difference between b-1 and b-4 hardly significant—Diff./S.E.$=\frac{1.13}{0.46}=2.46$ (t=2.42 for n=40 at P=0.02).

root tips, and cross-sectioned at 8 to 10 microns; they were also stained with Heidenhain's haematoxylin.

MORPHOLOGY OF BUDDED PROGENIES

General characters of trees and leaves; fruiting habit

In February, 1941, trees a-1 to a-3 had a considerable crop of fruit (very heavy on two trees, light on one) and appeared to be typical specimens of the seed-parent form in all respects (fig. 1). Of the five trees budded from branch b, trees b-1 and b-5, especially the former, were relatively short and broad, and they had not more than four fruits each (suggesting the relative unfruitfulness characteristic of tetraploids); b-1, at least, had foliage which seemed clearly indicative of tetraploidy and was especially broadened, as a result of very low branching (fig. 2). Trees b-2 to b-4, representing both bud sticks from branch b, were tall and erect, about like trees a-1 to a-3 in shape, but on the whole were intermediate

in yield of fruit (b-2 and b-3 about medium, b-4 very light) (fig. 3). However, much if not all of their foliage seemed to indicate tetraploidy, as did that of b-5.

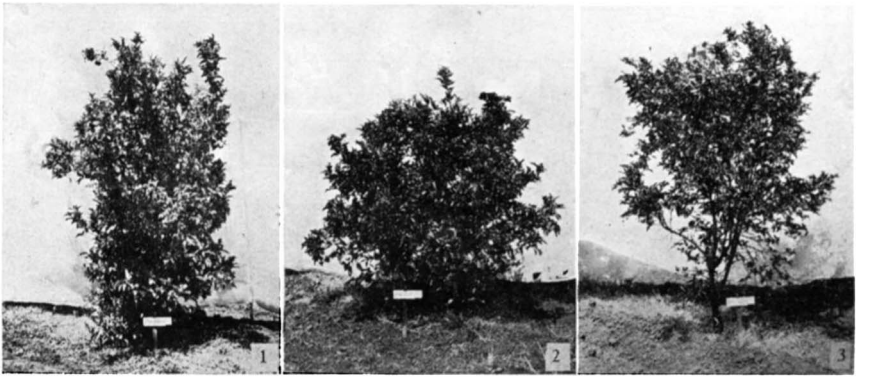

FIGURE 1.—Progeny tree a-1. Erect and typical of seed-parent form.

FIGURE 2.—Progeny tree b-1. Foliage is indicative of tetraploidy. Broadening of tree shape is probably exaggerated by very low branching after budding.

FIGURE 3.—Progeny tree b-4. Similar in shape to a-1 but with most of the foliage indicative of tetraploidy.

Leaf thickness

Twenty mature leaves, similarly located and of similar size, were collected in April, 1941, from each of the trees from which cuttings had been taken. Two measurements of thickness were made near the middle of each leaf, with a coverglass gauge reading in hundredths of a millimeter. The means and their standard errors are given in table 1.

The means for trees b-1 and b-4 are compared with that for tree a-2, which has higher mean and variability than a-1, for determination of the significance of the differences. The leaves from both of the trees propa-

TABLE 2

Relative breadth of leaves of cuttings.

TREE	NUMBER OF LEAVES	LEAF-SHAPE INDEX		
		MEAN	DIFFERENCE	DIFF./S.E.
a-1 and a-2	23	2.035 ± .025		
b-1	19	1.916 ± .030	0.119	3.05*
b-4	12	1.880 ± .032	0.155	3.78†

* Difference between general average of (a-1 and a-2) and b-1 is significant ($t = 2.71$ for $n = 40$ at $P = 0.01$).

† Difference between the same average and b-4 is also significant ($t = 2.72$ for $n = 35$ at $P = 0.01$).

Difference between b-1 and b-4 is not significant: Diff./S.E. = 0.78 ($t = 2.76$ for $n = 29$ at $P = 0.01$).

gated from the variant branch (b) were significantly thicker than those of the trees propagated from other parts (a) of the parent seedling. On the other hand, the difference between the two "b" trees was hardly significant, and that between the two "a" trees was not significant.

Leaf index

To secure an unbiased sample for determination of the shape index (length/width) of the leaf blade, all available original leaves (omitting seriously distorted ones) on the cuttings were measured. The means and standard errors are given in table 2.

Results for 50-leaf samples of other varieties, previously studied at the Citrus Experiment Station, indicate that the differences here found, in spite of their statistical significance, are not highly significant in relation to the actual chances of sampling from replicate trees. The differences, however, indicate greater relative width of leaves on the trees budded from the variant branch. Eleven tetraploid Citrus forms grown at the Citrus Experiment Station all showed greater leaf breadth than the corresponding diploids, the differences usually being much greater than those here presented.

CYTOLOGY AND HISTOLOGY

The study of the above-mentioned morphological characters of the progeny trees of the variant branch b suggests very strongly that it originated through chromosome doubling. To test this hypothesis, chromosome counts were made in root tips of cuttings, at meiosis, and later in young leaves and stems; the size of stomata was determined; and the general histology of leaf primordia, mature leaves, and young shoots was studied.

Chromosome number in root tips of cuttings

As mentioned above, chromosome counts were made in root tips of cuttings which were taken from trees a-1, a-2, b-1, and b-4. Special atten-

TABLE 3

TREE	NUMBER OF CUTTINGS EXAMINED	NUMBER OF ROOTS EXAMINED	CHROMOSOME NUMBERS
a-1	1	3	$2n=18$
a-2	1	3	$2n=18$
b-1	4	15	$4n=36$
b-4	4	21	$2n=18$

tion was paid to secure counts in several cell layers of the meristematic tissue. With the exception of two cases, the same number was found in all

layers of a particular root tip. In one of the exceptions, apparently two diploid nuclei, one of which appeared as a metaphase polar view with approximately 18 chromosomes, were dividing in one single cell surrounded by tetraploid ($4n=36$)[4] tissue. In another instance one single tetraploid cell was found in otherwise entirely diploid tissue.

Excluding the above exceptions the following results were obtained:

As expected, trees a-1 and a-2 produced normal diploid roots on their cuttings (fig. 4); b-1 originated tetraploid roots (fig. 5), and b-4, rather unexpectedly, had diploid roots on its cuttings.

Chromosome number at meiosis

In the spring of 1941, trees a-1, b-1, and b-4 produced a small number of flower buds, which were examined for their chromosome numbers at meiosis. The counts were made at MI, AI, and especially at MII and AII. Furthermore, observations were made on the presence of laggards, the number of microspores derived from each microsporocyte, and the variability of pollen size. The results were as follows:

Tree a-1: No chromosome counts could be made, but the number of microspores derived from each microsporocyte was, with rare exceptions, four, and the pollen was noted to be normal and uniform in size.

Tree b-1: The exact number of chromosomes at the different phases of the division of the microsporocytes could be determined only in a limited number of cases, since clear distinctions were often not possible between univalent, bivalent, and multivalent chromosomes. At MI usually from 14 to 18 apparent bivalents could be observed, including possible multivalents in instances of less than 18; at AI various sets (two in each cell) of 16 to 17 apparent univalents were counted; at this stage and at TI occasional laggards were encountered; in one MII configuration two plates of approximately 14 univalents were counted, several laggards being distributed in the cytoplasm; at various second anaphases groups of 17 and 18 chromosomes were observed; at two of these phases, however, groups of only nine chromosomes were found. Only 70 percent of 200 examined microsporocytes gave rise to four apparently normal microspores, 0.5 percent giving three, 21.5 percent five, and 8 percent six microspores. Mature pollen grains were variable in size.

Tree b-4: Various counts showed nine apparent bivalents at MI, two groups of nine suspected univalents at AI, and nine univalents at MII phase and AII. At two first metaphases, however, approximately 18 apparent bivalents were counted. Of the examined microsporocytes 87 percent developed into four microspores, 13 percent into five; the pollen

[4] In this paper "n" (here = x) is used exclusively to designate one genome, not to distinguish between reduced (n) and somatic (2n) conditions.

grains were noted to be uniform in size, perhaps a little more variable than the ones from tree a-1.

These results indicate that tree a-1 has normal diploid microsporocytes and b-1 apparently tetraploid ones. The data concerning tree b-4 are too scanty for a definite conclusion regarding the cytological constitution of its microsporocytes. However, since tetraploids vary with respect to their chromosome behavior at meiosis (FROST 1938), and since it is not always possible to make definite distinctions between uni- and multi-valent chromosomes at meiosis of Citrus, tree b-4 may also have tetraploid microsporocytes. The data above mentioned which disagree with this assumption may be due to errors regarding the number of chromosomes intimately associated at meiosis, or to a shift in germ-layer composition within tree b-1 or b-4.

Size of stomata

Stomata measurements were made under low magnification by means of a micrometer ocular; for that purpose freehand sections of lower epidermis were prepared, two from each side of the midrib in the central portion of each of ten leaves collected at random on the south side of the three budded progeny trees, and fixed in formalin-acetic-alcohol (5–5–90; 50 percent alcohol). The sections were examined without staining; from each leaf 20 stomata (about five from each section) were measured, totaling 200 stomata for each progeny tree. The area of each stoma was calculated by multiplying the product length×width of the area occupied by the two guard cells, by $\pi/4$, assuming that this area has the shape of an ellipse. The variance "between" and "within" leaves was calculated for each tree, and BRIEGER'S (1937) table of ζ (giving the probabilities of ratios of standard deviations) was used for evaluation of the results. This study indicated that the variances mentioned were not significantly different. Consequently, the total variability in stomatal area for each tree is used in estimating the standard error of that tree's mean stomatal area. The mean areas and their standard errors are presented in table 4.

In spite of the fact that the above statistical analysis reveals significant differences in mean stomatal area between trees a-1 and b-1 and also between trees b-1 and b-4, it is concluded that these differences must be due to some environmental factor and not to chromosome doubling, since they did not agree with actual chromosome counts in the epidermis of leaf primordia, as presented later, which revealed that this cell layer is for all three trees diploid; they also disagreed with observations of the width of the epidermal cells of mature leaves, which was found to be equal for all three trees. Unpublished investigations under way at the INSTITUTO AGRONÔMICO revealed recently that the differences of mean stomata areas in diploid and tetraploid epidermis of otherwise genetically identical Citrus

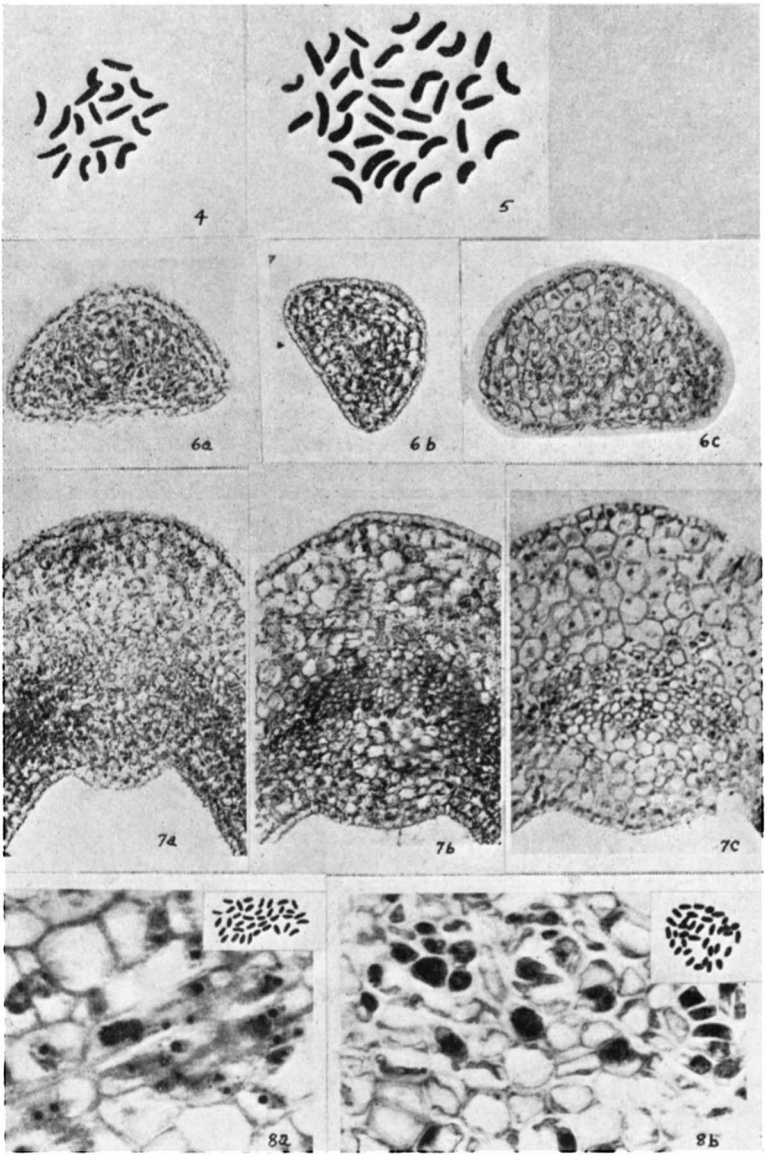

FIGURE 4.—Metaphase chromosomes in root tip of cutting from progeny tree a-1 (2n = 18). ×2600.

FIGURE 5.—Metaphase chromosomes in root tip of cutting from progeny tree b-1 (4n = 36). ×2600.

FIGURE 6.—Cross sections of leaf primordia (×133): a—from progeny tree a-1; b—from progeny tree b-1; c—from progeny tree b-4.

FIGURE 7.—Cross sections through midrib region of young leaves (×133): a—from progeny tree a-1; b—from progeny tree b-1; c—from progeny tree b-4.

FIGURE 8.—Procambium cells in young leaves of progeny tree b-4 (×667): a—4n = 36 in procambium of leaf blade; b—4n = 36 in procambium of midrib region.

forms are about four times as large as those mentioned for trees a-1 and b-1 in table 4.

Leaf primordia

The sectioned material permitted the study of the histology and the determination of the chromosome number in different cell layers of very young leaf primordia and in later stages when the leaf blade was already partially developed. Very young leaf primordia appear in cross sections as disk-like structures of a few more or less concentric cell layers. A little later two protuberances, one on each side, begin to project outward, forming the blade of the leaf. At this stage the vascular tissue of the mid-

TABLE 4

*Mean area of stomata in micrometer units.**

TREE	NUMBER OF STOMATA	AREA IN SQUARE UNITS		
		MEAN	DIFFERENCE	DIFF./S.E.
a-1	200	94.42±1.09		
b-1	200	106.38±0.97	11.96	8.19†
b-4	200	93.76±0.87	0.66	0.47‡

* The actual area of stomata in square microns is obtained by multiplying these means by 2.89, as each unit of the micrometer ocular corresponded to 1.7 micron.

† Difference between means of a-1 and b-1 is highly significant.

‡ Difference between means of a-1 and b-4 is not significant.
Difference between means of b-1 and b-4 is also highly significant (Diff./S.E. = 9.71) (t = 2.60 for n = 200 at P = 0.01).

rib also starts to develop, and in some cross sections procambial regions are easily detected in the blade, which are connected with the vascular tissue of the midrib. The differentiation into spongy and palisade parenchyma occurs much later.

The following differences were found among the sectioned material of the three trees:

A-1 (*Fig. 6a and 7a*). In round-shaped cross sections the cells of the epidermis are approximately of the same size as the internal cells. In later stages, due to the fact that the epidermis develops into a more organized layer, its cells usually become a little longer in the radial direction, appearing somewhat smaller than the others. The cells of the parenchyma tissue on the dorsal or abaxial side of the midrib become much larger than the epidermal cells. The cells of the procambium in cross sections of the blade are narrow and elongated, containing densely stained cytoplasm. In material of a-1, as expected, $2n = 18$ was determined in most cell layers.

B-1 (*Fig. 6b and 7b*). The development of the leaf primordia is, of

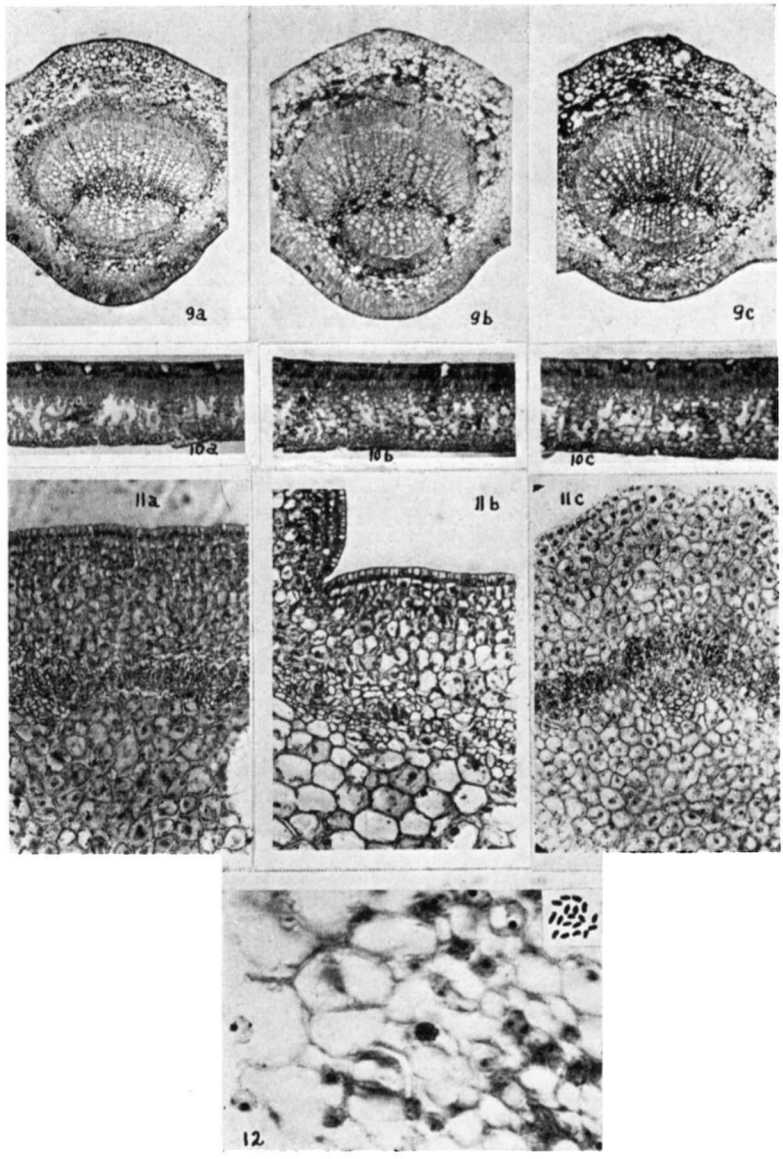

FIGURE 9.—Cross sections of midrib regions of mature leaves (×37): a—from progeny tree a-1; b—from progeny tree b-1; c—from progeny tree b-4.

FIGURE 10.—Cross sections of blades of mature leaves (×37): a—from progeny tree a-1; b—from progeny tree b-1; c—from progeny tree b-4.

FIGURE 11.—Cross sections of portions of young stems (×133): a—of progeny tree a-1; b—of progeny tree b-1; c—of progeny tree b-4.

FIGURE 12.—Portion of the pith and procambium of a young stem of progeny tree b-4; $2n = 18$. ×1427.

course, identical with that in a-1; the differences in cell size, however, are as follows: the subepidermis and adjacent layers are formed by cells which, from the beginning, are much larger than the epidermal cells; the difference is further evident upon comparison of the epidermis and the adjacent parenchymatous tissue of the dorsal side of the midrib of somewhat older leaves; the size difference is here more conspicuous than in tree a-1. The procambium cells, on the contrary, do not seem to differ in size from those observed in tree a-1. Extensive search to determine the chromosome number in the various layers of these cross sections revealed that the epidermis is diploid ($2n=18$), the subepidermis and adjacent layers tetraploid ($4n=36$), and the procambium cells also tetraploid ($4n=36$).

B-4 (*Fig. 6c, 7c, and 8a, b*). No appreciable differences in cell size and structure could be detected between leaf primordia of tree b-4 and those of tree b-1; the chromosomal constitution of the cell layers was the same as for b-1—that is, the epidermis was diploid ($2n=18$), while the subepidermis, the adjacent layers, and the procambium were tetraploid ($4n=36$).

Mature leaves (*fig. 9a, b, c and 10a, b, c*)

The study of cross sections through the central portion of mature leaves, including the midrib and parts of the blade, furnished the following information: the epidermis is formed by cells of approximately the same size in all three plants, a-1, b-1, and b-4; the palisade parenchyma is definitely wider in b-1 and b-4 than in a-1, the cells being, in average, longer and broader in the former two; the spongy parenchyma occupies more space and its cells are larger in b-1 and b-4 than in a-1. At the midrib region the palisade and spongy parenchyma are much more developed in b-1 and b-4 than in a-1; in the vascular tissue the vessels, tracheids, etc., apparently have a larger diameter in b-1 and b-4 than in a-1. No appreciable differences in size of cells could be detected between the material of b-1 and that of b-4.

Young stems (*fig. 11a, b, c and 12*)

Cross sections of the terminal portions of young shoots were examined. The cells of their epidermis were found to be of similar size in all three plants, a-1, b-1, and b-4; the cortex is made of larger cells in b-1 and b-4 than in a-1. In these young shoots no differences in cell size could be detected in the procambium and vascular tissue which begins to be formed at this region. In material examined from trees a-1 and b-1 a considerable difference in size was found between the cells of the pith and of the cortex, the former having cells about twice as large as the latter; in tree b-4, however, this difference in cell size of these two regions was insignificant, all the cells being, on the average, of the same size. The following chromosome counts were made: the epidermis, as expected, was diploid for all

three trees; the parenchymatous cells of the cortex were tetraploid for b-1 and b-4, diploid only for a-1; the procambium and pith cells of trees a-1 and b-4 were diploid, these tissues being tetraploid only for b-1.

DISCUSSION AND CONCLUSIONS

The results of the above investigations indicate that the original variant branch, found on one of the progeny trees of a hybrid mandarin (King ×Dancy), owed its peculiar characters to the occurrence of somatic chromosome duplication and has produced buds of at least two types of cytological constitution. Both are periclinal chimeras, and assuming that, as in Datura (BLAKESLEE et al. 1939, 1941; SATINA et al. 1940, 1941), three germ layers participate in the development of the vegetative shoot of Citrus, these chimeras are of the following nature: one is 2n − 4n − 4n, respectively, for its first, second, and third germ layers, and gave origin to progeny tree b-1; the other, originating progeny tree b-4, is 2n − 4n − 2n for the same layers. It cannot be determined when the duplication occurred, whether in a bud of the original parent tree or further back during its ontogeny, nor can it be determined how the differentiation into these two chimeras took place.

Based on the cytological constitution of these two chimeras, the following considerations may be presented:

The first germ layer takes part only in the formation of the epidermis; differences in size of stomata between two individuals, in spite of being of statistical significance, are not always an indication of differences in chromosome number of the epidermis. Caution should be exercised therefore in making deductions regarding the chromosomal constitution of this layer, based on the size of its stomata.

The second germ layer is responsible for the formation of the internal leaf tissue, including the procambium and the vascular tissue, as was clearly demonstrated by chromosome counts in the procambium of leaf primordia for plant b-4, whose second layer, and only this one, is tetraploid.

The similarity of leaf thickness and of leaf shape, as indicated by the leaf-shape index of trees b-1 and b-4 (tables 1 and 2) constitutes further proof that the cytological constitution of the leaves of these trees is identical. This fact is not in accordance with the findings of SATINA and BLAKESLEE (1941), since in Datura the vascular tissue of the leaves is derived from the third layer.

The fact that the leaves of the chimeras are indistinguishable and that they resemble very closely the leaves of true tetraploid plants, in spite of having a 2n epidermis, is an indication of the outstanding role which the second germ layer plays in the formation of Citrus leaves.

The cell layers underneath the epidermis in the young stem (cortex)

are also derived from the second layer. As in other plants, this layer is responsible for the formation of the microsporocytes. Since it is tetraploid in both chimeras, it produces in them the relative unfruitfulness characteristic of tetraploid Citrus forms.

All tissues derived from the procambium and later the cambium of the stem and its pith originate from the third germ layer. The fact that this layer is diploid in one of the chimeras (b-4) and tetraploid in the other (b-1) probably explains why these two progeny trees differ in growth habit, b-4 being tall and erect and about like a-1 in shape, and b-1 short and broad. If this explanation is valid, it may be concluded that the third germ layer plays an important role in the determination of the growth habit of the trees. Chromosome counts in root tips of cuttings of the two chimeras have proven that these roots, which develop from a callus, are always derived from the third layer. It has been observed that callus at the base of a stem cutting forms beneath the bark and appears to arise from the fascicular cambium alone.

The breadth of the leaves was found to be smaller for the two chimeras than usually in 11 Citrus forms, presumably tetraploid throughout, which have been studied at the CITRUS EXPERIMENT STATION; this difference may be due to the diploid nature of the epidermis of the chimeras.

In progeny tree b-4, as stated above, the vascular tissue of the leaves is tetraploid, the corresponding tissue in the stem, however, being diploid. Since, according to several investigators, the procambial cells in leaf primordia differentiate in two directions—namely, upward toward the leaf apex and downward toward the shoot apex—to form the vertical leaf traces, it may be concluded that in tree b-4 the tetraploid leaf vessels probably unite with the diploid stem vessels at the insertion region of the petiole.

Since both chimeras owe their origin to somatic chromosome doubling which occurred in nature and since they cannot easily be distinguished by their morphological characters from true tetraploids, it is obvious that great care must be taken in selecting tetraploid Citrus seedlings for study or breeding. The evidence, however, indicates that partially tetraploid trees are probably rare.

Further germ-layer studies on Citrus are needed, particularly with reference to the generality of the two-layer pattern of leaf ontogeny which we have found in one form, and with reference to the germ-layer relations of those morphologically complex structures, the pistil and the fruit. It is not evident how the two-layer pattern could prevail in the leaves of such forms as SHAMEL'S (1932) variegated lemon, since these leaves are mainly white-margined, with variegated interior, and therefore evidently must have two genetic types within the epidermis (FROST in press).

SUMMARY

The present investigation deals with the cytological nature of a bud variant which originated on a progeny tree of a hybrid mandarin (King × Dancy). From the variant branch several progeny trees were grown in comparison with others derived from the normal part of the tree. The results of extensive investigation indicate that the budded progeny of the variant branch includes at least two types of chimeral constitution: one is 2n–4n–4n respectively for its first, second, and third germ layers, and the other is 2n–4n–2n for the same layers.

On the assumption that three germ layers exist in Citrus, it was demonstrated that the first germ layer forms the epidermis, the second one all leaf tissues (with the exception of the epidermis), the microsporocytes and at least part of the cortex of young vegetative shoots, and the third one forms the procambium, cambium, and the pith of the stem. Determination of chromosome number in root tips of cuttings of these two chimeras, demonstrated that these roots, at least when they derive from a callus, have their origin in the third germ layer. Comparison of stomata of mature leaves of different plants indicates that size differences, even if they are of statistical significance, are not always due to differences in chromosome number in the epidermis. The progeny trees of these chimeras differ considerably in growth habit, the one with a diploid third germ layer being approximately normal (erect), the other with a tetraploid third layer being low and broad. This fact suggests that the third layer plays a considerable role in determining the growth habit of the trees. Both chimeras are rather unfruitful, which is typical for Citrus tetraploids.

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