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<https://doi.org/10.5109/4625>

出版情報：九州大学大学院農学研究院紀要. 50 (1), pp.93-102, 2005-02-01. Faculty of Agriculture, Kyushu University

バージョン：

権利関係：

Production of Tetraploid Forms of Acid Citrus Cultivars by Top Grafting of Shoots with Sprouting Axially Buds Treated with Colchicine

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(Received November 5, 2004 and accepted November 11, 2004)

To induce the tetraploid forms of three diploid acid Citrus cultivars ($2n=2x=18$), 'Yuzu' (*C. junos*), 'Kizu' (*C. kizu*) and 'Hanayu' (*C. hanaju*), sections of one-year-old spring shoots with sprouting axillary buds were treated with various concentrations of colchicine and immediately top-grafted on 30-year-old satsuma mandarin trees at the beginning of April. Characteristics of the plants developed from the top grafted scions were scored five years after the treatments. Tetraploid forms of the cultivars, which were certified by tetraploid chromosome number in their seedlings, were derived from the shoot sections treated with 0.05–0.4% colchicine solutions for 12 hours for 'Yuzu', 0.05–0.4% for 6 hours for 'Kizu' and 0.1 and 0.2% for 6 hours for 'Hanayu'. As compared with the original plants, these colchicine-induced tetraploid plants produced large flowers, fruits with thick rind, large oil gland and large seeds, and showed high leaf shape index (leaf width / leaf length). The large seeds from open-pollinated tetraploid plants showed the same degree of polyembryony as seeds from their original diploid cultivars, and their seedlings were tetraploid. These results suggested not only that the tetraploid forms of the three cultivars can be directly used for breeding of triploid seedless acid citrus cultivars as seed or pollen parents, but also that the present method is superior to the other methods to produce citrus tetraploid forms directly applicable for triploid breeding at two to four years after colchicine treatments.

INTRODUCTION

Triploid *Citrus* plants with high parthenocarpic ability are of great interest for establishing seedless cultivars, because of their horticulturally favorable characteristics and high productivity. Soost and Cameron (1980, 1985) firstly bred two seedless cultivars 'Oroblanco' and 'Melogold' that are triploid pummelo-grapefruit hybrids from $2x \times 4x$ crosses. However, introduction of triploid *Citrus* cultivars has so far been very limited.

For the breeding of triploid *Citrus* cultivars, literatures suggest two methods, i.e., usage of diploid megagametophytes of tetraploids and diploids, and usage of diploid

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pollen of tetraploids. The percentage of diploid megagametophytes or unreduced eggs varied from less than 1% to about 25% in diploid *Citrus* cultivars tested (Esen and Soost, 1971; Geraci *et al.*, 1975; Wakana *et al.*, 1982). In Japanese acid citrus cultivars tested, however, the rates were so low that we might not use them as triploid breeding materials (Wakana, unpublished data). Thus, the usage of tetraploids seems to be the best method for the production of triploid acid citrus plants.

Most tetraploid *Citrus* plants that have been established were obtained as variant nucellar seedlings in seedling populations grown for other purposes (Soost and Cameron, 1975). Generally, tetraploid frequency varied from less than 1% to 3% in the seedling populations (Soost, 1987). In addition to the low frequencies, the tetraploid nucellar seedlings have very long juvenile periods from five to more than ten years as is observed in diploid nucellar ones. Hence, induction of tetraploid sports on shoots of target diploid cultivars is considered to be the most efficient method for shortening the periods.

After the work of Tachikawa (1971), tetraploids were derived from diploid cultivars treated with colchicine. Barrett (1974) successfully induced tetraploids from diploid *Citrus* cultivars as well as periclinal ploidy chimeras by colchicine treatment on the sprouting axillary buds. In polyembryonic diploid cultivars, Gmitter and Ling (1991) recovered non-chimerical tetraploids from the undeveloped ovules cultured on the medium supplemented with 0.01 and 0.1% colchicine. Tetraploid plants were also produced by somatic hybridization between polyembryonic cultivars and between polyembryonic and monoembryonic cultivars using embryogenic callus from polyembryonic cultivars (Kobayashi and Ohgawara, 1988; Grosser and Gmitter, 1990). Presence of barrier of nucellar embryony in these tetraploids, however, led them to the use as pollen parents, since production of triploids is very difficult or at extremely low frequencies (Soost, 1987).

Although most acid citrus cultivars are moderately or highly polyembryonic, some acid citrus cultivars such as 'Yuzu', 'Hanayu' and 'Lisbon' lemon show very low degree of polyembryony and produce monoembryonic seeds and zygotic seedlings with high frequencies. Therefore, they are useful not only as pollen parents but also as seed parents for breeding triploid *Citrus* cultivars.

The aim of this study is to develop most convenient and efficient method of colchicine treatment to scions and subsequent top working, by which we produce tetraploid forms of diploid acid citrus cultivars that will be directly used for triploid breeding as seed and/or pollen parents as early as possible.

MATERIALS AND METHODS

Plant materials

Three diploid acid citrus cultivars ($2n=2x=18$), 'Yuzu' (*C. junos* Sibb. *ex* Tanaka), 'Kizu' (*C. kizu* hort. *ex* Y. Tanaka) and 'Hanayu' (*C. hanaju* hort. *ex* Shirai) were used as plant materials to produce their tetraploid forms. These cultivars were about 30-year-old trees grown at Sasaguri orchard of the University Farm, Kyushu University, Fukuoka. 'Yuzu' and 'Hanayu' showing low degree of polyembryony produce 2.3 and 1.2 embryos per seed in average, whereas highly polyembryonic 'Kizu' produces >10.0 embryos per seed in average. 'Yuzu' is a leading cultivar of acid citrus cultivated in Japan, while 'Kizu'

and 'Hanayu' are minor ones. Tetraploid 'Kizu', 1-No.5, was also prepared to demonstrate the characteristics of tetraploid plants

Colchicine treatment

When axillary bud break occurred in shoots of these cultivars at early to middle in April 1996, sections (5 cm in length) were excised from one-year-old spring shoots and trimmed so that at least three axillary buds remained on the shoot sections. The trimmed sections were immersed in colchicine solutions supplemented with a few drops of Tween 20 per liter. The immersion treatment was made in combination with various concentrations of colchicine and different immersion times, i.e., 0.05, 0.1, 0.2, 0.4 and 0.8% for 'Kizu' and 'Hanayu', 0.05, 0.1, 0.2 and 0.4% for 'Yuzu'; 4, 8, 12 and 16 hours for 'Yuzu' and 6 hours for 'Kizu' and 'Hanayu'. During the immersion treatment, vessels with 15 sections each were shaken 60 times per minute. The colchicine-treated shoot sections (scions) were rinsed twice with water, and immediately grafted on blanches of about 30-year-old 'Okitsu Wase Unshiu' (*C. unshiu* Marc.), covered with TX film and allowed to growth.

Morphology of shoots

After the first flower initiated on the shoots developing from the scions, the morphology of shoots was compared with that of the original plants. The characteristics of flower buds and flowers were also observed and photographed. Five mature fruits from open pollination were collected from each of the colchicine-induced tetraploid-like shoots at October. Then, the weight of fruits and seeds, number of perfect seeds and rind thickness were scored.

For the examination of leaf characters, ten typical leaves were collected from each of the colchicine-induced tetraploid-like plants and the original diploid cultivars. Then, their wing leaves were removed, and width and length of these leaves were measured and leaf shape index (width / length) was calculated.

Chromosome observation

The perfect seeds were divided into two categories, large and small. For chromosome observation, only large seeds were randomly chosen in each of the original cultivars and plants with tetraploid-like morphology. Seed coat of the large perfect seeds was removed with forceps. The embryos extracted from each seed with forceps were placed on wet filter paper and incubated at 25 °C. Root tips of the germinated embryos were collected and the chromosome number in the root tip cells was counted under a microscope according to the procedure reported by Park *et al.* (1999). One embryo extracted from three large perfect seed each was used for chromosome number observation.

RESULTS

Some of the shoots developing from colchicine-treated scions showed sectorial-chimera-like or mixoploid-like morphology up to one or two years after the grafting. However, plants with this morphology disappeared by five year after grafting.

Of 340 scions treated with various concentrations of colchicine and grafted on satsuma mandarin trees, 123 produced plants five years after the grafting or five-year-old

branches. In the plants, flower formation and fruit set started from two years after grafting and were observed at almost all of the plants by five years after the grafting.

Rate of plants with tetraploid-like morphology

Of 240 colchicine-treated scions of 'Yuzu', five scions immersed for 12 hours produced plants with typical morphology of tetraploid (Table 1). Each of the five scions produced one tetraploid-like plant, and one of the five also produced one additional plant without any morphological variation. The colchicine concentrations that resulted in tetraploid-like plant formation were 0.05, 0.10, 0.20 and 0.40% (Table 1).

Of 50 'Kizu' scions, six scions treated with 0.05–0.40% colchicine solutions for six hours produced tetraploid-like plants (Table 2). One scion immersed in 0.05% and one scion immersed in 0.10% colchicine solution produced two shoots each. Only one of the two shoots developed into plants with tetraploid-like morphology.

Of 50 'Hanayu' scions, one scion immersed in 0.05% colchicine solution for 6 hours and four scions immersed in 0.10% colchicine solution for 6 hours developed into plants with the typical morphology of tetraploid (Table 2). In 'Hanayu' and 'Kizu', shoots successfully developed from about half of the scions treated with low concentration of colchicine (0.05–0.20%). However, no shoots developed from scions treated with 0.80% of colchicine in the both cultivars and only two shoots developed from only two scions

Table 1. Number of diploid and tetraploid plants successfully developing from 'Yuzu' scions treated with different concentration of colchicine solutions in combination with different treatment time. The results were scored five years after the grafting.

Exposure time to colchicine (hr)	Concentration of colchicine (%)	No. of scions treated	No. of scions producing shoots with indicated ploidy (No. of shoots) ^a			
			2x	4x	2x and 4x	Total
4	0.05	15	9	0	0	9
	0.10	15	6	0	0	6
	0.20	15	3	0	0	3
	0.40	15	7	0	0	7
8	0.05	15	3	0	0	3
	0.10	15	3	0	0	3
	0.20	15	2	0	0	2
	0.40	15	1	0	0	1
12	0.05	15	6	1	0	7
	0.10	15	7	1	0	8
	0.20	15	8	1	1(1/2) ^b	10
	0.40	15	4	1	0	5
16	0.05	15	11	0	0	11
	0.10	15	3	0	0	3
	0.20	15	2	0	0	2
	0.40	15	3	0	0	3
Total	–	240	78	4	1	83

^aPloidy was estimated by their morphological characteristics. ^b Two shoots, one 2x and one 4x, developed from one axillary bud on the scion.

Table 2. Number of diploid and tetraploid plants successfully developing from grafted scions treated with different concentration of colchicine solutions for six hours. The results were scored five years after the grafting.

Cultivar	Concentration of colchicine (%)	No. of scions treated	No. of scions forming shoots with indicated ploidy ^a			
			2x	4x	2x & 4x (No. ^b)	Total
Kizu	0.05	10	5	0	1(1/2)	6
	0.10	10	4	0	1(1/2)	5
	0.20	10	4	3	0 (-)	7
	0.40	10	1	1	0 (-)	2
	0.80	10	0	0	0 (-)	0
	Total	50	14	4	2(2/4)	20
Hanayu	0.05	10	5	0	0 (-)	5
	0.10	10	3	1	0 (-)	4
	0.20	10	5	2	2(2/5)	9
	0.40	10	2	0	0 (-)	2
	0.80	10	0	0	0 (-)	0
	Total	50	15	3	2(2/5)	20

^aPloidy was estimated by their morphological characteristics. ^b A total number of 4x shoots / a total number of 2x shoots.

treated with 0.40% colchicine in each of the cultivars.

Ploidy of seedlings

Chromosome numbers in the root tip cells of seedlings from the tetraploid-like plants were 38 in all seedlings examined. Hence, the tetraploid-like plants were certified to be tetraploid plants in which at least the second germ layer is tetraploid useful for triploid citrus breeding.

4x Plant morphology

Compared with the original diploid cultivars, the colchicine-induced tetraploid plants had (1) thick and wide dark-green leaves (Fig. 1), (2) very large flower buds, anthers and pistils (Fig. 2), and (3) thick and large petals and calyxes (Fig. 2). The plants produced larger fruits with larger seeds, thicker rind and larger oil grand than the original diploid cultivars (Table 3, Fig. 3). The induced tetraploid plants of each cultivar were morphologically uniform and, except those of 'Hanayu', showed the same extent of tree vigor as the original diploid. 'Hanayu' tetraploids showed horizontally oriented growth and a slower growth rate than diploid.

There was relatively large variation in leaf length between and among the original and colchicine-induced tetraploid plants, probably due to the extent of tree vigor. However, significant difference was observed in leaf width between them, i.e., the leaf width in tetraploids was 1.13–1.50 times as wide as that in the original diploid cultivars. The difference was more clearly detected by the usage of leaf shape index (width / length): the indices in each of the induced tetraploids of 'Yuzu', 'Hanayu' and 'Kizu' were almost simi-

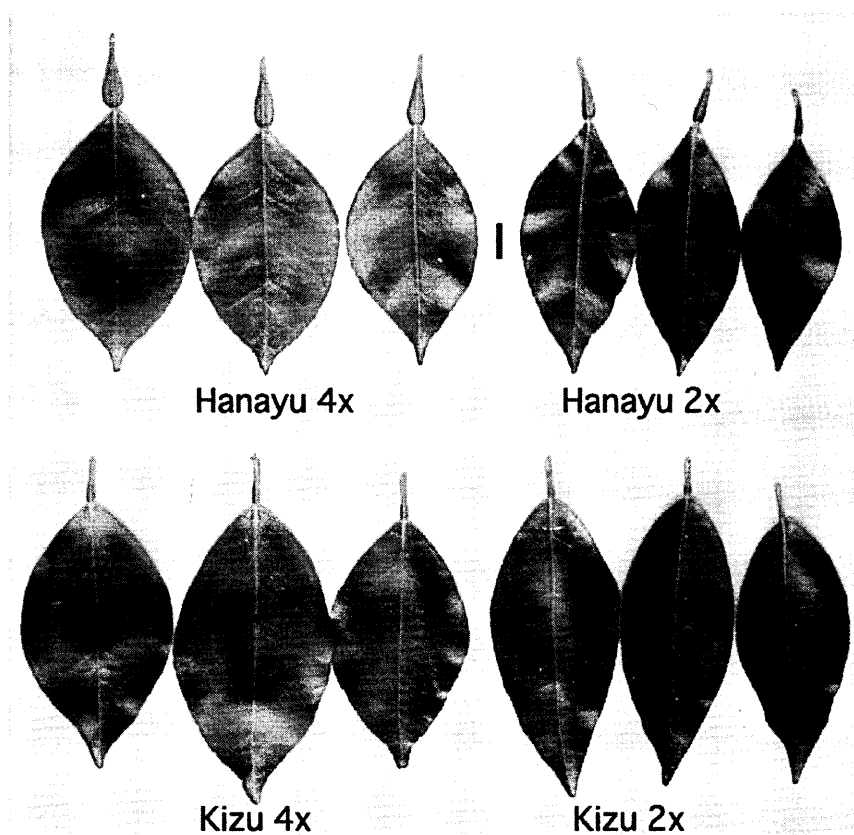


Fig. 1. Leaf morphology in colchicine-induced tetraploids (left) and the original diploid (right) 'Hanayu' and 'Kizu'. The vertical bar indicates 1 cm.

lar and were about 1.3 times higher than those in the original diploid cultivars (Table 3).

Fruits of the induced tetraploid plants were larger than those of the original diploid in any cultivars and had very thicker rind, which was 1.3, 1.4 and 1.7 times thicker than that of the original diploid 'Yuzu', 'Hanayu' and 'Kizu' respectively (Table 3). Compared with the original cultivars, average weight of large seeds of the tetraploid plants was about 1.1 times heavy for 'Yuzu' and 'Hanayu', and was about 1.3 times heavy for 'Kizu' (Table 3). The seeds from the induced tetraploid plants contained almost the same number of embryos as those from the original diploid cultivars, i.e., 1.11 for 'Hanayu', 2.35 for 'Yuzu' and >10 for 'Kizu'. The induced tetraploid plants contained small plump seeds with relatively high frequencies as compared with the original diploids (Table 3). This phenomenon seemed to be related to pollination of the tetraploid plants with diploid plants locating adjacent to them.

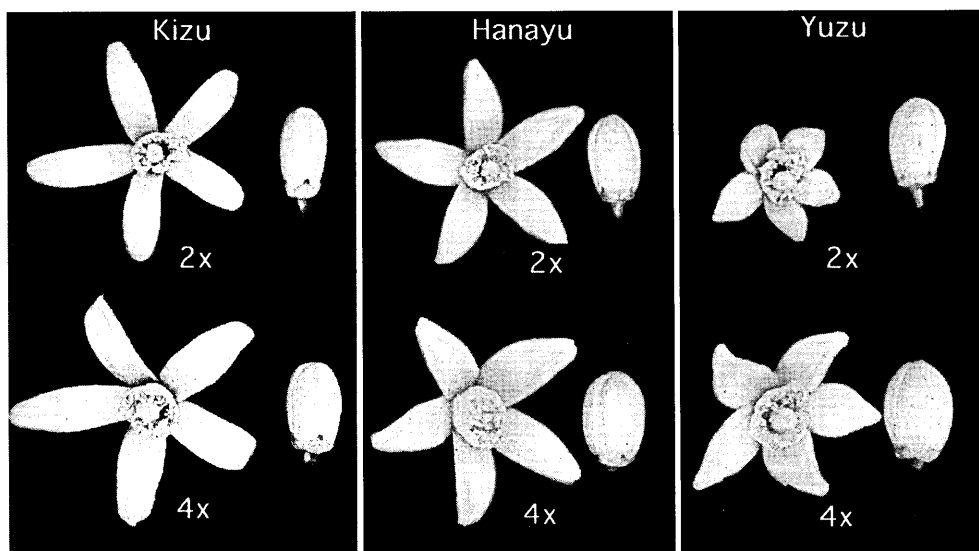


Fig. 2. Morphology of just-opened flowers and flower buds just before anthesis in colchicine-induced tetraploids (lower) and the original diploid (upper) 'Kizu', 'Hanayu' and 'Yuzu'.

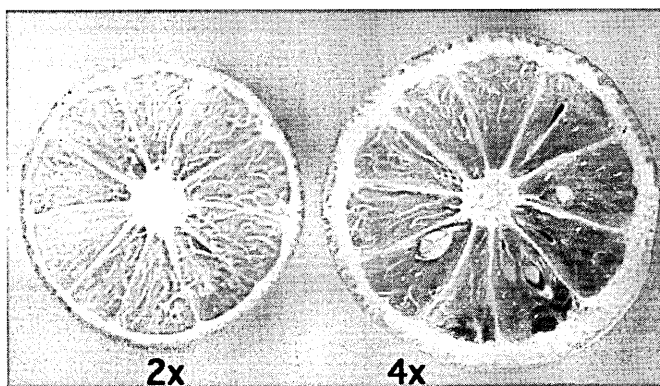


Fig. 3. Bisected mature fruits derived from diploid (left) and colchicine-induced tetraploid (right) 'Kizu'. Note the difference of rind thickness and fruit size between 2x and 4x fruits.

Table 3. Leaf and fruit characteristics in colchicine-induced tetraploid plants and their original diploid acid citrus cultivars.

Plant No. ^a	Weight of fruit (g)	Rind thickness (mm)	Leaf width (mm)	Leaf length (mm)	Leaf shape index ^b	Number of seeds (No. ^c)	Weight of seed (mg)	Ploidy of seedling ^d
Yuzu								
Original 2x	114	5.0	3.3	6.8	0.49	24.3(0.9)	290	2x
0.1-12-No.1	159	6.5	4.2	6.9	0.61	11.3(3.0)	330	4x
Hanayu								
Original 2x	40	3.9	4.0	9.2	0.44	6.8(0.2)	371	2x
0.1-6-No.1	87	5.6	4.8	8.5	0.56	10.8(2.6)	423	4x
0.2-6-No.4	73	5.5	5.4	9.6	0.57	17.5(1.0)	406	4x
0.2-6-No.5	64	5.8	4.8	8.4	0.57	10.0(1.0)	390	4x
Kizu								
Original 2x	80	2.7	3.8	7.9	0.48	4.3(0.0)	380	2x
0.4-6-No.2	141	4.8	4.8	8.1	0.60	5.4(0.6)	500	4x
0.2-6-No.3	84	4.3	5.3	8.2	0.65	2.6(1.6)	500	4x
0.1-6-No.5	120	4.4	4.9	7.7	0.63	3.5(1.0)	505	4x
0.2-6-No.7	115	3.9	5.7	8.7	0.62	3.6(0.0)	478	4x
0.1-No.1 ^e	148	5.3	4.3	6.8	0.62	6.0(2.0)	500	4x

^a Number indicates colchicine concentration (%) followed by exposure time to colchicine (hr) and individual seedling number. ^b Leaf width / leaf length. ^c Number of small plumped seeds supposed to be triploid (Wakana *et al.*, 1982). The number of perfect seeds includes the number of small seeds.

^d The seedlings were derived from sowing of large perfect seeds. ^e Tetraploid plant 0.1-No.5 was directly induced as a bud sport by the treatment of a solution of 0.1% colchicine on an axial bud of intact shoot without immersion treatment and subsequent grafting.

DISCUSSION

The present study demonstrates that immersion of one-year-old shoots with sprouting axillary buds in colchicine solution and subsequent top grafting of the immersed shoots on adult trees is a very useful and efficient technique for the production of tetraploid forms of desired diploid *Citrus* cultivars, since the induced tetraploid plants set fruits two to four years after the top grafting and is directly used for crossings to produce triploid hybrids. The diploid *Citrus* cultivars used in this study are polyembryonic, and their tetraploid forms induced by colchicine also showed the same degree of polyembryony in the seeds. The induced tetraploid forms of 'Yuzu' and 'Hanayu' produced seeds of which about half were monoembryonic, suggesting the tetraploids are useful not only for pollen parents but also for seed parents. However, tetraploid form of 'Kizu' is available only for a pollen parent, since it exhibited high polyembryony as the original diploid did.

Tetraploid forms of diploid *Citrus* cultivars have been derived from nucellar seedlings (Soost and Cameron, 1975; Soost, 1987), through *in vitro* culture of undeveloped ovules of polyembryonic cultivars (Gmitter and Ling, 1991) and through protoplast fusion *in vitro* with embryogenic callus induced from polyembryonic citrus ovules (Kobayashi and Ohgawara, 1988; Grosser and Gmitter, 1990). These tetraploid seedlings require long

years of vegetative growth to reach their adult phase because of their long juvenile periods. Furthermore, these tetraploids, which are either nucellar or nucellar with fused nuclei, are available only for a pollen parents because of their high degree of polyembryonic ability. Oiyama and Okudai (1986) induced tetraploid plants through micrografting of colchicine-treated shoot apex of axillary buds in monoembryonic citrus cultivars. However, this method also requires long time up to flowering of the grafted plants on seedlings. Hence, the present method of colchicine treatment and top working is superior to these reported ones to develop triploid breeding in acid citrus.

The high colchicine concentration (0.8%) depressed shoot development from the treated axillary buds and resulted in failure of top working in two cultivars used in this study, whereas the low concentrations (0.05–0.20%) are considered to have no effect on the shoot development and top working. Differential sensitivity to the 0.4% colchicine treatment was observed among cultivars, i.e., it was suppressive for shoot meristem development in axillary buds of 'Hanayu' and 'Kizu'. Thus, this concentration is not widely applicable to produce tetraploid forms of diploid *Citrus* cultivars, although one tetraploid plant was recovered from one axillary bud treated with this concentration in 'Kizu' and 'Yuzu'. The appropriate exposure time to colchicine was 12 hours for 'Yuzu', while in 'Hanayu' and 'Kizu' many tetraploid plants were recovered by the exposure time of six hours with a maximum recovery rate of 40%. Hence, it is referred from these results that the appropriate exposure time of trimmed shoots with sprouting axillary buds to colchicine solution is 6–12 hours and that the concentration is 0.1–0.2%.

The axillary buds forming on shoots of 'Hanayu', 'Yuzu' and 'Kizu' are the compound buds composed of a thorn (a main bud for 'Kizu') and an accessory bud (a first bract leaf axillary bud initiating on the base of first bract leaf of the main bud or thorn) with two secondary accessory buds developing at the first and second bract leaf axils of the accessory bud (Wakana, 2002). Because of the development of more than two shoots from one compound axillary bud in these acid citrus cultivars, the 2x and 4x shoots are considered to originate from two buds within one compound axillary bud treated with colchicine.

Disappearance of sectorial-chimera-like or mixoploid-like morphology in some shoots by five year after grafting suggests that these shoots are unstable to maintain their structure. Conversely, it is concluded that the successfully induced tetraploid plants have stable shoot structure. Although ploidy level in each of the three germ layers of the shoots was not determined in this study, the morphological and cytological data indicate that the colchipooids are stable tetraploid plants, at least, whose LII layer is tetraploid. Since the reproductive cells originate from the LII layer (Frost and Krug, 1942; Tilney-Vassett, 1982), these colchipooids or tetraploid forms of diploid cultivars will be effectively and directly utilized for triploid breeding programmes to develop triploid seedless acid citrus cultivars.

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