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Simple Methods for Producing Tetraploids in Polyembryonic Citrus

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Abstract

Simple and easy methods for producing autotetraploids in polyembryonic citrus were developed. These methods require only colchicine treatment at the time of sowing and do not require special skills or equipment, such as *in vitro* culture. The ploidy of all growing plants was analyzed by flow cytometry. The percentage of tetraploid induction per seed in five accessions at optimal treatment was 11.0% - 42.5%. The optimum condition for tetraploid production was 0.005% colchicine for 24 h, excluding Kabuchii. The highest percentage of tetraploids was induced with 0.01% colchicine for 24 h in Kabuchii. In Kabuchii and 'Yoshida Ponkan,' the proportions of tetraploids were almost the same with 0.005% and 0.01% colchicine. In all accessions studied, 0.002% and 0.02% colchicine were not effective. The ploidy of some plants was confirmed by chromosome observation. The chromosome numbers of tetraploids determined by flow cytometry were 36 (2n = 4x = 36). In sequence-related amplified polymorphism (SRAP) analysis, no difference between tetraploids and original plants was detected in any of the five accessions. Hence, all analyzed tetraploids are considered autotetraploids (true-to-type tetraploids of a given accession).

Discipline: Horticulture
Additional key words: breeding, chromosome, colchicine, flow cytometry, polyploid

Introduction

Polyploid breeding is very important in fruit trees because tetraploid fruits tend to be larger because of their larger cell size and triploid fruits show seedlessness due to sterility caused by aberrations of meiosis (Sanford 1983). As seedlessness is one of the most important breeding objectives in citrus, triploid (2n = 3x = 27) cultivars have already been developed (Soost & Cameron 1980, 1985). Conversely, there is no tetraploid (2n = 4x = 36) commercial cultivar because fruits of citrus tetraploids are commonly smaller and have thicker rinds (Cameron & Frost 1967, Yamao et al. 1993, Nukaya et al. 2011). However, citrus tetraploids are valuable as parents for interploidy breeding (2x × 4x) to produce triploid seedlings (Soost & Cameron 1980, 1985).

Hence, the production and selection of new tetraploid accessions have been conducted. The simplest way to obtain autotetraploids (true-to-type tetraploids of a given accession) is to select tetraploids from nucellar seedlings in polyembryonic accessions (Cameron & Frost 1967). However, the proportion of spontaneous tetraploids was reportedly low (Hutchinson & Barrett 1981, Kawase et al. 2005) and autotetraploids cannot be obtained in monoembryonic accessions. Thus, several methods for producing tetraploids have been developed and various autotetraploids were produced (Oiyama & Okudai 1986, Yahata et al. 2004, Zhang et al. 2007, Kaneyoshi et al. 2008, Yasuda et al. 2022). These studies combined antimitotic reagents (such as colchicine) and *in vitro* tissue culture or micrografting. Although these methods are useful for producing tetraploids, they require skills and equipment. Therefore, the development of an easier and more efficient way to produce tetraploids is required.

Conversely, we have investigated local citrus grown on the Ryukyu Islands, Japan. Some accessions bear fruits with high phytonutrient values or unique scents (Teramoto et al. 2017, Yamamoto et al. 2019). Thus, they are considered promising parents for citrus breeding. However, many of those are seedy. Because the number of seeds is an inheritable trait (Yamamoto et al. 1992), seedy accessions are undesirable for seedless or few-seed breeding. Hence, the production of autotetraploids of these accessions is necessary for the advancement of

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triploid seedless breeding. In addition, in a few local citrus, such as Shiikuu (*Citrus* sp.) and Rokugatsumikan (*C. rokugatsu* hort. ex Y. Tanaka), processed products are made from rinds. Thicker rinds with larger and more prominent oil glands, which are a characteristic of tetraploids (Cameron & Frost 1967), may be desirable for processing. For these reasons, we attempted to produce autotetraploids in some accessions grown on the Ryukyu Islands.

In the present study, we aimed to produce autotetraploids in polyembryonic accessions using methods that do not require special skills and equipment. Tetraploids were produced in all five accessions subjected to these methods, and we report the results here.

Materials and methods

1. Plant materials

The following five accessions were used in the present study (Table 1): Shiikuu (*Citrus* sp.), Kuroshimamikan (*C.* sp.), Rokugatsumikan (*C. rokugatsu* hort. ex Y. Tanaka), Kabuchii (*C. keraji* hort. ex Tanaka), and 'Yoshida Ponkan' (*C. reticulata* Blanco). All accessions were local citrus grown on the Ryukyu Islands, except 'Yoshida Ponkan.' Shiikuu is cultivated on the Amami Islands, Japan, and the rind of its fruits has an aroma that is almost the same as that of 'Bergamot' (*C. bergamia* Risso et Piet.) (Teramoto et al. 2017). Kuroshimamikan is grown on islands in Kagoshima Prefecture (Yamamoto et al. 2021a). Rokugatsumikan and Kabuchii are distributed widely in the Ryukyu Islands (Yamamoto et al. 2021b). Ponkan is a major mid-maturing citrus in Japan.

Seeds were extracted from mature fruits harvested from preserved trees at the Toso Orchard of Experimental Farm, Faculty of Agriculture, Kagoshima University (Kagoshima, Japan, ca. 31°34′N, 130°32′E, and 65-m elevation), in each accession.

2. Colchicine treatment

The outer and inner coats of seeds were removed. Then, seeds were placed on colchicine-impregnated filter paper in Petri dishes. After colchicine treatment, the seeds were washed with distilled water and placed on water-impregnated filter paper in Petri dishes. The seeds were incubated at 25°C under dark conditions for approximately one week. After germination, seeds were transferred to Kanuma pumice (fine grain, Matsuzakien, Tochigi, Japan) in plastic pots (9 cm in diameter and 8 cm in depth). The plants were cultivated at 25°C ± 2°C under a 14-h photoperiod with a photon flux of 57 μmol m^{-2} s^{-1} provided by fluorescent lamps. Grown plants were transferred to pots (15 cm in diameter and 13 cm in depth) containing local soil in a greenhouse.

Shiikuu was treated with colchicine at concentrations of 0.002%, 0.005%, 0.01%, and 0.02% for 24 or 48 h at 25°C in the dark. One hundred seeds were used in each treatment.

In Kuroshimamikan, Rokugatsumikan, Kabuchii, and 'Yoshida Ponkan,' the concentrations of colchicine were the same as those in Shiikuu but only 24-h treatment was conducted. Seventy-five to one hundred seeds were used in each treatment.

3. Ploidy analysis by flow cytometry

Leaves (approximately 0.5 cm^2) were chopped using a sharp razor blade in nuclei isolation buffer (Partec, Münster, Germany). Crude samples were filtered and stained with a coloration solution (Partec) containing 4,6-diamidino-2-phenylindole (DAPI). DNA content was determined using a flow cytometer (PA-II Ploidy Analyzer; Partec).

4. Observation of chromosomes

Plants identified as tetraploids by flow cytometry in each accession were used as materials. Two plants were randomly selected in each accession. Young leaf samples (approximately 5 to 10 mm long) were immersed in 2 mM 8-hydroxyquinoline at 10°C for 4 h in the dark, fixed in methanol–acetic acid (3:1), and stored at −20°C.

For enzymatic maceration and air drying (EMA), young leaves were cut to a size of approximately 2 mm^2. EMA was performed as described by Yamamoto et al. (2004). The young leaves were washed in distilled water

Table 1. Citrus accessions used in the present study

Accession	Latin name	Distribution
Shiikuu	*C.* sp	Amami Islands
Kuroshimamikan	*C.* sp	Kagoshima Prefecture
Rokugatsumikan	*C. rokugatsu* hort. ex Y. Tanaka	Kyushu and Ryukyu Islands
Kabuchii	*C. keraji* hort. ex Tanaka	Ryukyu Islands
Yoshida Ponkan	*C. reticulata* Blanco	Commercial cultivar (Native to India)

to remove the fixative and macerated in an enzyme mixture containing 2% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult, Japan), 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd. , Japan), and 1 mM EDTA, pH 4.2, at 37°C for 45 min. After incubation, the macerated leaves were placed in distilled water for 20 min to remove the enzyme solution. The leaves were placed on glass slides, and water was removed using a piece of filter paper. The fixative was added and the leaves were tapped with forceps until the tissue was spread. Slides were air dried for at least 3 h.

Chromosomes were stained with 2% Giemsa solution (Merck Co., Germany) in 1/30 M phosphate buffer (pH 6.8) for 15 min, rinsed with distilled water, air dried, and then mounted with xylene. The chromosomes were observed under a microscope (Eclipse 80i, Nikon, Japan).

5. DNA analysis

Three tetraploids and one original plant in each accession and three diploid seedlings in Shiikuu were used as materials. Total DNA was extracted from the leaves using Isoplant II (Nippon Gene, Tokyo, Japan). Sequence-related amplified polymorphism (SRAP) analysis was conducted according to the study by Yamamoto et al. (2017). This was performed using 14 primer combinations useful for the cultivar identification of citrus (Tables 2, 3). The 12.5-μL PCR reaction mixture consisted of 10 ng of template DNA, 10 pmol of each primer, 0.5 units of Prime Taq DNA polymerase, and its 10× PCR buffer. PCR reactions were performed in a PC320 (Astec, Fukuoka, Japan) thermal cycler programmed as follows: initial heating at 95°C for 10 min, the first five cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, and extension at 72°C for 2 min, after which the annealing temperature was increased to 50°C for another 35 cycles, with a final extension of 10 min at 72°C. Amplified products were electrophoresed on 1.5% agarose gels (Seakem GTG Agarose; Takara Bio, Otsu, Japan) and stained with GelReD (Biotium, CA, USA). The bands were detected under UV light.

Results

In all experiments, the ploidy level of all 806 colchicine-treated plants was analyzed by flow cytometry (Fig. 1). The original diploid plants were used as the control. Diploids, tetraploids, and ploidy chimeras (2x + 4x) appeared in all accessions (Tables 4, 5).

Table 2. Forward and reverse SRAP primer information for the present study

Forward primer	Reverse primer
Me1: TGAGTCCAAACCGGATA	Em1:GACTGCGTACGAATTAAT
Me3: TGAGTCCAAACCGGAAT	Em2:GACTGCGTACGAATTTGC
Me4: TGAGTCCAAACCGGACC	Em3: GACTGCGTACGAATTGAC
Me5: TGAGTCCAAACCGGAAG	Em4: GACTGCGTACGAATTTGA
Me6: TGAGTCCAAACCGGACA	Em7: GACTGCGTACGAATTCAA
Me8: TGAGTCCAAACCGGACT	Em9: GACTGCGTACGAATTCAG
Me9: TGAGTCCAAACCGGAGG	Em10: GACTGCGTACGAATTCAT
Me10: TGAGTCCAAACCGGAAA	Em14: GACTGCGTACGAATTCTT
Me11: TGAGTCCAAACCGGAAC	Em15: GACTGCGTACGAATTGAT
Me12: TGAGTCCAAACCGGAGA	Em16: GACTGCGTACGAATTGTC

Table 3. SRAP primer combinations in the present study

Primer combination	
Me1/Em14	Me8/Em2
Me3/Em3	Me8/Em7
Me4/Em1	Me9/Em7
Me5/Em2	Me10/Em15
Me5/Em4	Me11/Em9
Me6/Em4	Me11/Em10
Me6/Em15	Me12/Em16

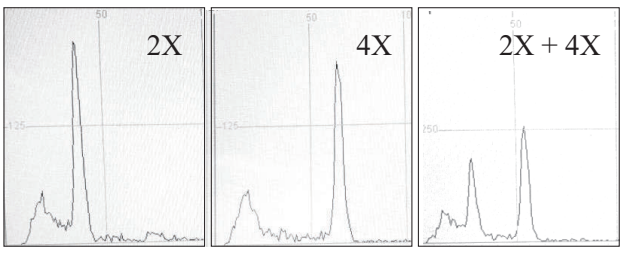

Fig. 1. Flow cytometer analysis of the plants induced by colchicine treatment of seeds in Shiikuu

Table 4. Effects of the colchicine concentration and treatment duration on the ploidy level of Shiikuu

Colchicine concentration (%)	Duration (hour)	No. of seeds treated	No. of plants grown (%)[a]	No. of each ploidy plant (%)[b]		
				2x	4x	2x + 4x
0.002	24	100	29 (29.0)	28 (28.0)	1 (1.0)	0 (0.0)
0.002	48	100	32 (32.0)	25 (25.0)	4 (4.0)	3 (3.0)
0.005	24	100	36 (36.0)	18 (18.0)	11 (11.0)	7 (7.0)
0.005	48	100	16 (16.0)	9 (9.0)	4 (4.0)	3 (3.0)
0.01	24	100	19 (19.0)	9 (9.0)	7 (7.0)	3 (3.0)
0.01	48	100	5 (5.0)	3 (3.0)	2 (2.0)	0 (0.0)
0.02	24	100	2 (2.0)	2 (2.0)	0 (0.0)	0 (0.0)
0.02	48	100	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] (No. of plants grown) / (No. of seeds treated) × 100
[b] (No. of each ploidy plant) / (No. of seeds treated) × 100

Table 5. Effects of the colchicine concentration on the ploidy level of four citrus accessions

Accession	Colchicine concentration (%)	No. of seeds treated	No. of plants grown (%)[a]	No. of each ploidy plant (%)[b]		
				2x	4x	2x + 4x
Kuroshimamikan	0.002	80	78 (97.5)	70 (87.5)	6 (7.5)	2 (2.5)
	0.005	80	58 (72.5)	18 (22.5)	34 (42.5)	6 (8.1)
	0.01	75	24 (32.0)	13 (17.3)	8 (10.7)	3 (4.0)
	0.02	75	5 (6.7)	4 (5.3)	1 (1.3)	0 (0.0)
Rokugatsumikan	0.002	100	81 (81.0)	72 (72.0)	5 (5.0)	4 (4.0)
	0.005	100	38 (38.0)	20 (20.0)	13 (13.0)	5 (5.0)
	0.01	100	14 (14.0)	9 (9.0)	3 (3.0)	2 (2.0)
	0.02	100	4 (4.0)	3 (3.0)	0 (0.0)	1 (1.0)
Kabuchii	0.002	100	83 (83.0)	71 (71.0)	6 (6.0)	6 (6.0)
	0.005	100	67 (67.0)	44 (44.0)	16 (16.0)	7 (7.0)
	0.01	100	45 (45.0)	23 (23.0)	19 (19.0)	3 (3.0)
	0.02	100	10 (10.0)	7 (7.0)	1 (1.0)	2 (2.0)
Yoshida Ponkan	0.002	80	58 (72.5)	49 (61.3)	3 (3.8)	6 (7.5)
	0.005	80	62 (77.5)	26 (32.5)	22 (27.5)	14 (17.5)
	0.01	80	32 (40.0)	12 (15.0)	20 (25.0)	0 (0.0)
	0.02	80	8 (10.0)	4 (5.0)	1 (1.3)	3 (3.8)

[a] (No. of plants grown) / (No. of seeds treated) × 100
[b] (No. of each ploidy plant) / (No. of seeds treated) × 100

In Shiikuu, the proportion of growing plants was low even with a low colchicine concentration (0.002%) compared with other accessions (Tables 4, 5). In general, the number of growing plants decreased with an increasing colchicine concentration or duration. The highest percentage of tetraploid induction per seed (11.0%) was observed in 24-h treatment using 0.005% colchicine. No tetraploid was obtained in 0.02% colchicine treatment. The occurrence of ploidy chimera was also the highest in 24-h treatment using 0.005% colchicine. As an effect of the treatment duration (24 or 48 h) was not detected, only 24-h treatment was conducted in the following experiments (Table 5).

The effects of the colchicine concentration on the ploidy level of Kuroshimamikan, Rokugatsumikan, Kabuchii, and 'Yoshida Ponkan' are shown in Table 5. The number of growing plants decreased with an increasing colchicine concentration, except for 'Yoshida Ponkan.' Only 4 to 10% of treated seeds grew in 0.02% treatment. In Kuroshimamikan and Rokugatsumikan, the highest percentage of tetraploid plants is obtained in 0.005% treatment. In particular, 42.5% of plants from

treated seeds under this condition were tetraploids in Kuroshimamikan. Conversely, both 0.005% and 0.01% were effective in producing tetraploid plants in Kabuchii and 'Yoshida Ponkan.' No or few tetraploids were observed in 0.02% treatment in all four accessions.

In all accessions, the chromosome numbers of tetraploids determined by flow cytometry were 36 (Fig. 2). These results indicate that the analyzed plants were tetraploids (2n = 4x = 36). We were able to confirm the ploidy of these plants at the chromosome level.

In SRAP analysis, using 14 primer combinations, no difference among tetraploids and original plants was detected in any of the five accessions. Figure 3 shows representative results (Me3 /Em3, Me9 /Em7, and Me11 / Em10).

In general, the tetraploid seedlings grew somewhat poorer than the diploid ones. However, some tetraploid seedlings grew well in Shiikuu (data not shown).

Discussion

In the present study, we were able to demonstrate straightforward methods for producing autotetraploids in polyembryonic citrus. These methods require only colchicine treatment at the time of sowing and do not require special skills or equipment, such as *in vitro* culture. The percentage of tetraploid induction per seed in each accession at optimal treatment was 11.0% - 42.5%. Hutchinson & Barrett (1981) obtained spontaneous tetraploids at a frequency of less than 3% in polyembryonic citrus hybrids. The proportion of tetraploids in the present study was much higher than that of Hutchinson & Barrett (1981). Conversely, Yahata et al. (2004) and Yasuda et al. (2022) produced tetraploids by combining colchicine treatment for seeds and *in vitro* culture. The proportions of tetraploids in their studies and that of the present study are similar. Thus, our methods are considered useful for the production of tetraploids in citrus.

The optimum condition for tetraploid production was 0.005% colchicine for 24 h, excluding Kabuchii. The highest percentage of tetraploids was induced in 0.01% colchicine for 24 h in Kabuchii. In Kabuchii and 'Yoshida Ponkan,' the proportions of tetraploids were almost the same in 0.005% and 0.01% colchicine. In all accessions studied, 0.002% and 0.02% colchicine were not effective. It has been considered that the former concentration was too low for the induction of tetraploids and the latter showed toxicity due to a high concentration and few plants grew. The number of grown plants decreased as the concentration of colchicine treatment increased. The effect of the treatment duration on tetraploid induction was experimented in Shiikuu. Treatment for 48 h was not better than that for 24 h. From the above, 0.005% colchicine for 24-h treatment may be considered a standard condition for the production of tetraploids in polyembryonic citrus. In previous studies (Yahata et al. 2004, Yasuda et al. 2022), tetraploids were produced by combining colchicine treatment of seeds and *in vitro* culture. The optimum colchicine concentration of their studies was 0.1%-0.2%. This concentration is 20-40 times

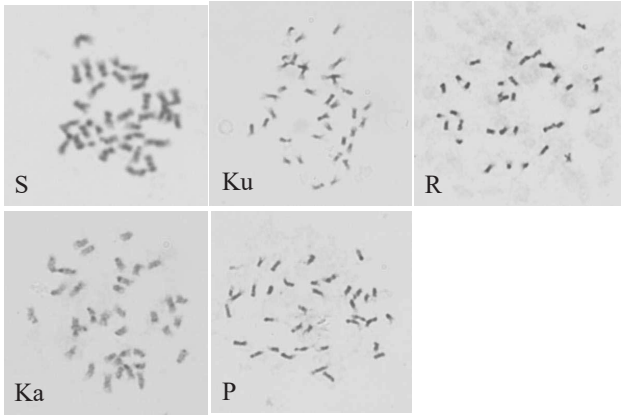

Fig. 2. Chromosomes of tetraploid plants (2n = 4x = 36)
S: Shiikuu, Ku: Kuroshimamikan, R: Rokugatsumikan, Ka: Kabuchii, and P: Yoshida Ponkan

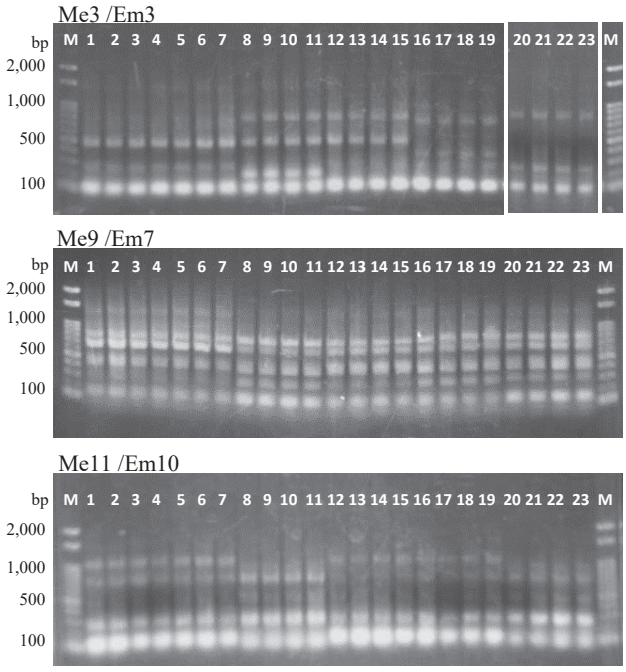

Fig. 3. SRAP analyses of tetraploid plants
M: DNA marker, 1-7: Shiikuu, 8-11: Kuroshimamikan, 12-15: Rokugatsumikan, 16-19: Kabuchii, and 20-23: Yoshida Ponkan. 1, 7, 8, 12, 16, and 20: original diploid plants. 2-4, 9-11, 13-15, 17-19, and 21-23: tetraploid plants. 5-7: diploid seedlings

more than our optimum concentration. The colchicine treatment methods in the previous and present studies are different: seeds were soaked in colchicine solution for 24 h or more in their treatment (Yahata et al. 2004, Yasuda et al. 2022). *In vitro* culture as an embryo rescue culture may have promoted the development of high-concentration-treated embryos. Conversely, the percentage of polyploid induction using *in vitro* culture was 20.0% in 0.05% colchicine for 24 h in sweet orange (Yasuda et al. 2022). They did not experiment with colchicine concentrations below 0.05%. By conducting experiments with colchicine treatment methods and concentrations that are in the same condition, it may be possible to clarify the difference in the optimal concentrations between their studies (Yahata et al. 2004, Yasuda et al. 2022) and the present study. Although the reason for the marked differences in optimum colchicine concentrations between their studies and the present study is unclear, tetraploids grew without *in vitro* culture because of low toxicity due to a low colchicine concentration. Our methods require only a small amount of colchicine. The methods in the present study are also excellent from this point.

Although the optimum condition for tetraploid production was almost the same in the five accessions tested, the percentage of tetraploid induction per seed was different among the accessions. Varietal differences in tetraploid induction by colchicine treatment were also reported by Yasuda et al. (2022). These results may be caused by differences in colchicine sensitivity among citrus accessions. However, the number of grown plants was low even with low colchicine concentrations in Shiikuu. In these accessions, the reason for the low percentage of tetraploids might involve the poor condition of treated seeds. The colchicine treatment of seeds was conducted approximately 80-90 days after extracting them from the fruits in Shiikuu. Although the seeds were kept in a refrigerator, storage conditions were considered not good.

In the present study, the ploidy of all 806 growing plants was analyzed by flow cytometry and the number of chromosomes of two tetraploid plants per accession for judged by flow cytometry was confirmed by chromosome observation. The ploidy levels based on flow cytometry and chromosome observation were identical. Recently, the ploidy of the candidates of artificial tetraploids was mainly examined by flow cytometry (Yahata et al. 2004, Kawase et al. 2005, Kaneyoshi et al. 2008, Yasuda et al. 2022). The high-level reliability of flow cytometry was also confirmed in the present study.

When producing autotetraploids from nucellar seedlings in polyembryonic accessions, the possibility of the emergence of zygotic seedlings must be considered. Because the proportion of emerging zygotic seedlings became high when the number of embryos per seed in the seed parent decreased (Yamamoto et al. 2023), tetraploids from zygotic embryo origin may appear in accessions with low numbers of embryos per seed. Hence, DNA analysis of produced tetraploids was conducted. All DNA-banding patterns between an original plant and tetraploids were identical in each accession. No extra band appeared in tetraploids. Therefore, all analyzed tetraploids are considered plants that arose from a nucellar embryo (autotetraploids). Although the numbers of embryos per seed of Kuroshimamikan were unknown, those of the other four accessions were reported (Yamamoto et al. 2023). Those of Rokugatsumikan, Shiikuu, and Kabuchii were approximately 10. As approximately 20% of seedlings were of zygotic embryo origin in accessions with approximately 10 embryos, DNA analysis was essential for the verification of the origin in those three accessions. By contrast, the number of embryos per seed was 35.7 in 'Yoshida Ponkan.' When it was 25 or more, zygotic seedlings hardly appeared. On the basis of these factors, DNA analysis may be necessary so that accessions with a few embryos are used as materials to produce tetraploids.

In conclusion, we successfully developed simple and easy methods to produce autotetraploids in polyembryonic citrus. Because this method requires only colchicine treatment at the time of sowing and not special skills or equipment, it is considered to contribute to the divergence of tetraploid accessions. As a matter of course, this technique can be applied to monoembryonic accessions. These methods may be useful for producing tetraploids in artificial hybrids derived from monoembryonic seed parents.

All tetraploids from the five accessions may be valuable parents for triploid breeding in citrus. In addition, because rind or essential oil extracted from fruit is used for processing in Shiikuu and Rokugatsumikan, tetraploids with thicker rinds with larger and more prominent oil glands (Cameran & Frost 1967) may be advantageous for this purpose. We are planning to evaluate the fruit traits of these tetraploids in comparison with those of diploids when they bear fruits.

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