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Induction of Polyploidy in *Cucumis melo* 'Chammel' and Evaluation of Morphological and Cytogenetic Changes

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Author Contribution Statement

Woo-Young Cho, Deen Mohammad Deepo, and Md Mazharul Islam wrote the manuscript. Si-Chun Nam, Jeung-Sul Han, Hong-Yul Kim, Chang-Kil kim, Mi-Young Chung and Ki-Byung Lim verified every step of experiment. Lim Ki-Byung supervised the work. All authors discussed the results and contributed to write a final manuscript.

Declaration of Competing Interest

This research is ready for publication. It was supported by Agriculture, Food and Rural Affairs. I hereby disclose all my conflicts of interest and other potentially conflicting interests, including specific financial interests and relationships and affiliations relevant to HORTICULTURAL SCIENCE AND TECHNOLOGY.

Abstract

This study explored the effects of oryzalin on the polyploidization of a new cultivar, *Cucumis melo* 'Chammel'. 58 diploid seedlings of 'Chammel' were treated with oryzalin. Their ploidy levels were checked by flow cytometry and number of 5S and 18S rDNA loci were examined by fluorescence in situ hybridization (FISH). Twelve among 58 plants were identified as tetraploids. The tetraploids were self-crossed, and their seeds were harvested. The seeds and seedlings of the tetraploids were compared to the corresponding diploids using morphology and cytogenetic analyses. The seed width, the cotyledon width, and the leaf thickness of the tetraploids increased significantly over those of the diploids. However, the length of the petiole of the tetraploids was shorter than that of the corresponding diploids. The stomata length and width of the guard cells in the tetraploids were longer than in diploids, but the number of stomata per area decreased in the tetraploids. The number of chromosomes was 24 and 48 for the diploids and tetraploids, respectively. Moreover, the chromosomal changes induced by oryzalin were confirmed; the diploids had one pair of 5S rDNA loci and two pairs of 18S rDNA loci, the corresponding numbers were doubled in the tetraploids. These results will help to generate new seedless oriental melon cultivars via polyploidization.

Additional key words: *5S rDNA*, *18S rDNA*, *FISH*, *oryzalin*, *tetraploid*

Introduction

Cucumis melo 'Chammel' is a new cultivar from a cross between oriental melon and melon. It has an orange pulp, similar to a melon, and a shape similar to an oriental melon. It has high sugar, citric acid, and beta-carotene contents, and a crisp taste. (Bae et al., 2019). Oriental melons are susceptible to powdery mildew, which frequently infects Cucurbitaceae plants (Lee et al., 2010) while, *Cucumis melo* 'Chammel' displays robust resistance to this disease (Bae et al., 2019).

Plant size, disease resistance, environmental stress tolerance, and seedlessness of fruits are all desirable traits in horticultural crops that are introduced by breeding and polyploidization (Ranney, 2006). Consumers prefer seedless fruits because of their higher sugar content (Marr and Gast, 1991), superior consistency, and higher yield (Henderson, 1977). As a result, one of the primary objectives of modern breeding is seedlessness. Seedless triploid watermelons are created by first generating tetraploids with a chromosome doubling reagent and then crossing the tetraploids with diploids (Suying et al., 1993; Jaskani et al., 2004).The most common chromosome doubling reagent is colchicine ($C_{22}H_{25}NO_6$) (Dermen, 1940). However, colchicine is carcinogenic and can cause infertility and chimerism (Wan et al., 1989). Therefore, the use of colchicine has been limited. Another chromosome doubling reagent, oryzalin (3, 5-dinitroN4, N4-dipropylsulfanilamide) has been successfully used to induce polyploidization in crops (Chauvin et al., 2005). Oryzalin is a synthetic herbicide used for weed control and inhibits microtubule assembly during cell division. Oryzalin has a higher affinity for plant tubulins (Doležel et al., 1994), and usually achieves the best results of polyploidization when young, actively growing meristems are treated (Ascough et al., 2008).

During polyploidization experiments, the change in nuclear DNA content needs to be monitored using various methods. For example, flow cytometry offers a quick, precise and simple way to determine nuclear DNA content (C-value) (Galbraith et al., 2009). In addition, fluorescence in situ hybridization (FISH) and karyotyping are commonly used techniques for cytogenetic studies (Anamthawat-Jónsson, 2004; Hwang et al., 2020; Islam et al., 2020). FISH analysis may provide basic details about each species' ploidy degree and chromosomal characteristics. Furthermore, the 5S, 18S, 25S, and 45S rDNA loci have been widely used to investigate the genetic relationships among plant species. (Pellerin et al., 2019; Sakhanokho et al., 2020). This study aimed to understand the effects of oryzalin on the polyploidization of *Cucumis melo* 'Chammel' with the goal of creating new seedless oriental melon cultivars.

Materials and Methods

Oryzalin Treatment of the Experimental Materials

Fifty-eight diploid seedlings of oriental melon (*Cucumis melo*) 'Chammel' with high-sugar content were used in this experiment. They were grown in a greenhouse (Spring Seed Co., Ltd, Korea) with day temperatures maintained between 25 to 30°C and night temperatures between 20 to 25°C. One week-old seedlings were treated with 50 $mg{\cdot}L^{-1}$ of oryzalin;10 μL was dropped at the shoot apex of young leaves immediately after cotyledons opened. The cotyledons were fixed with clips for 7 days to increase the absorption of oryzalin.

Flow Cytometry Analysis

The young leaves of treated plants were collected for flow cytometric analysis (Partec PA, Ploidy Analyzer, and Germany). The leaves were cut into (0.5 cm^2) pieces, put in a 5 × 12 mm Petri dish, and chopped with a blade in 500 μL of nuclei isolation buffer. Then, solution was filtered into a tube to remove the debris. Next, 2 ml of DAPI staining buffer (Partech, GmbH, Munster, Germany) was poured into the tube. The nuclei suspension was then introduced into a flow cytometry analyzer. After checking the ploidy level, the tetraploid plants were selected for self-crossing and seed collection for further cultivation.

Morphological Analysis

Seed Observation

The seed morphology of the tetraploid plants was compared to that of the diploid plants. The length, width, thickness, and weight of the seeds were measured.

Leaf Observation

The morphology of the cotyledon and the first true leaves diploid and tetraploid plants was observed. The cotyledon length and width; and the length, width, thickness of first leaves, and the length of petiole were measured.

Stomata Observation

The morphological features of the stomata of the diploids and tetraploids were compared. The epidermis was separated using forceps, placed on a glass slide, and observed under a microscope (BX61, Olympus, Japan) at 200X magnification.

Chromosome Analysis

Slide Preparation

Root tips of tetraploid and diploid plants were collected early in the morning, treated with 2mM 8-hydroxyquinoline at 20°C for 4 hours, and fixed overnight in a solution (ethanol: acetic acid=3:1). The root tips were then put in a refrigerator with 70% ethanol. They were washed with distilled water before being treated an enzyme solution of 0.3% pectolyase, 0.3% cellulase, and 0.3% cytohelicase in 150 mM citrate buffer for 40 minutes at 37°C. The root-tips were then squashed in 17 μL of 70% acetic acid on a glass slide and air-dried. Under a fluorescence microscope, the chromosomes were stained with 6-diamino-2-phynylindole (DAPI) in VECTASHIELD solution and counted (BX 61, Olympus, Japan).

FISH Treatment

FISH analysis was conducted according to Lim et al. (2001) with a minor modification. First, DAPI was removed from the slides using 2X SSC buffer for 10 min. Then, the slides were dehydrated by treating for 9 min each with 70%, 90%, and 100% ethanol. Briefly, slides were pre-treated with 100 $\mu g \cdot mL^{-1}$ RNase A at 37°C for 1 hour. After rinsing them with 2× SSC and fixing them with 4% paraformaldehyde for 10 min, we again rinsed the slides with 2× SSC and dehydrated them in an ethanol series (70%, 90%, and 100%), followed by air-drying.

The hybridization mixture contained formamide, 50% dextran sulfate, and 20 × SSC, 10% sodium dodecyl sulfate, herring sperm DNA, and rDNA probes. The mixture was placed in a water bath for DNA denaturation at 82°C for 2 min and then put on ice for 15 min for fixation. Forty microliters of the mixture were added to each slide, and a coverslip was placed on the slide, while ensuring there were no bubbles. The slides were put in a water bath for hybridization at 82°C for 2 min and then placed in a container with wet tissue paper and incubated in a humid chamber at 37°C for 18 − 20 hours. The slides were washed with 2× SSC buffer for 5 min followed by 0.1×SSC buffer for 30 min at 42°C with shaking and 2×SSC buffer for 5 min. Then, the slides were submerged into 1× detection buffer for 5 min. Streptavidin CY3 (Invitrogen, Carlsbad, CA, USA) and anti-digoxigenin fluorescein (Roche, Basel, Switzerland) were used to detect the labeled chromosomes. The slides were placed with coverslips in a humid chamber and incubated at 37°C for 1 hour. Three jars were

incubated containing a 1× detection buffer in the dark in a water bath at 37°C for 5 min, followed by dehydration in an ethanol series (70%, 90%, and 100%). Then, the slides were air-dried in the dark and counterstained with DAPI and Vectashield at a 2:100 ratio (Vector Laboratories, Burlingame, CA, USA).The prepared slides were examined with a model Nikon BX 61 fluorescence microscope (Tokyo, Japan). The probe signals were analyzed using ultraviolet excitation filters. Cytovision and imaging software were used to acquire the chromosomes' images with the 5S rDNA and 18S rDNA signals.

Statistical Analysis

Each of the characteristics was compared between the diploids and tetraploids using the SAS package (Statistical Analysis System, version 9.4, SAS Institute Inc.). The phenotypic similarity was analyzed using a 1% significance level in a t-test for a completely randomized design.

Results and Discussions

Flow Cytometry Analysis of the Ploidy Level

The DNA content of the 58 oryzalin-treated *Cucumis melo* 'Chammel' plants were analyzed by flow cytometry. The ploidy levels of were determined by the peak's channel position. An untreated diploid 'Chammel' plant was used as the control with a peak observed at channel 50 (Fig. 1A). The peak of a tetraploid line was observed at channel 100 (Fig. 1C), and the peaks of a mixoploid line were observed middle positions at channels 50 and 100 (Fig. 1B). Among the 58 plants diploid oryzalin treated 'Chammel' plants, 12 were detected as tetraploids, one was mixoploid, and the remaining were diploid (Table 1). In the case of watermelon treated by oryzalin, they found diploids, mixoploids, and desired tetraploids.

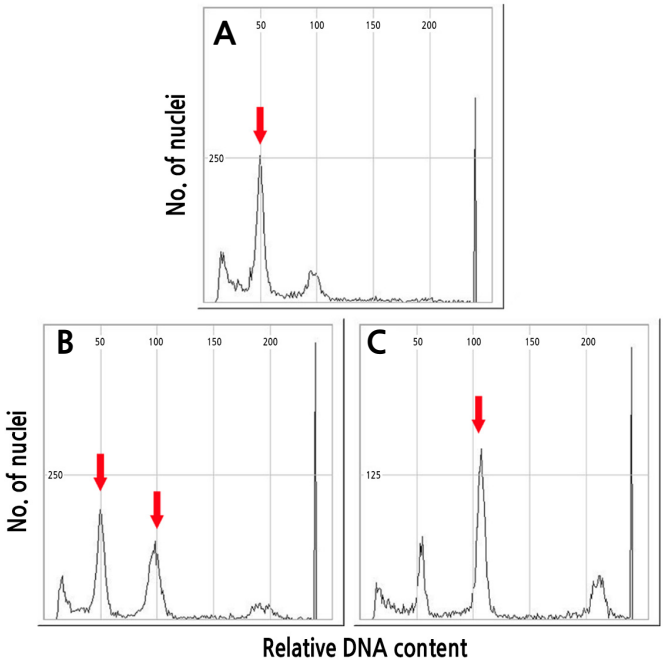

Fig. 1. Flow cytometric analysis of *Cucumis melo* 'Chammel' treated with 50 mg·L^{-1} oryzalin. A. Diploid. B. Mixoploid. C. Tetraploid.

(Kwack and Lee, 1999; Bae et al., 2020). In a study by Raza et al. (2003) using 0.01% colchicine in watermelon, the highest number of tetraploids was found. Tetraploid plants can be used to produce seedless nursery crops by creating triploids (Contreras et al., 2007). Therefore, these tetraploid oriental melons could be used for breeding with diploids and make new seedless oriental melon cultivars.

Table 1. The ploidy level of the 58 seedlings of *Cucumis melo* 'Chammel' treated with 50 mg·L^{-1} oryzalin

Plants	Mean	Ploidy	2C DNA content (Mbp)	pg (2C)^{z}
Control	52.30	2x	442.46	0.45
OM1	110.08	4x	931.28	0.95
OM2	57.60	2x	487.30	0.50
OM3	56.27	2x	476.04	0.49
OM4	109.59	4x	927.13	0.95
OM5	54.35	2x	459.80	0.47
OM6	55.64	2x	470.71	0.48
OM7	56.30	2x	476.30	0.49
OM8	57.25	2x	484.34	0.50
OM9	106.58	4x	901.67	0.92
OM10	107.24	4x	907.25	0.93
OM11	105.64	4x	893.70	0.91
OM12	55.47	2x	469.28	0.48
OM13	103.70	4x	877.30	0.90
OM14	100.46	MIX	849.89	0.87
OM15	105.02	4x	888.47	0.91
OM16	54.64	2x	462.25	0.47
OM17	54.59	2x	461.83	0.47
OM18	55.85	2x	472.49	0.48
OM19	54.34	2x	459.72	0.47
OM20	55.50	2x	469.53	0.48
OM21	105.13	4x	889.40	0.91
OM22	55.70	2x	471.22	0.48
OM23	53.93	2x	456.25	0.47
OM24	103.70	4x	877.30	0.90
OM25	51.74	2x	437.72	0.45
OM26	52.69	2x	445.76	0.46
OM27	53.90	2x	455.99	0.47
OM28	104.00	4x	879.84	0.90
OM29	52.12	2x	440.94	0.45
OM30	51.34	2x	434.34	0.44
OM31	54.03	2x	457.09	0.47
OM32	52.28	2x	442.29	0.45
OM33	54.66	2x	462.42	0.47
OM34	55.05	2x	465.72	0.48

Table 1. The ploidy level of the 58 seedlings of *Cucumis melo* 'Chammel' treated with 50 mg·L^{-1} oryzalin (Continued)

Plants	Mean	Ploidy	2C DNA content (Mbp)	pg (2C)[z]
OM35	52.55	2x	444.57	0.45
OM36	54.57	2x	461.66	0.47
OM37	54.36	2x	459.89	0.47
OM38	53.28	2x	450.75	0.46
OM39	53.66	2x	453.96	0.46
OM40	53.30	2x	450.92	0.46
OM41	54.72	2x	462.93	0.47
OM42	53.12	2x	449.40	0.46
OM43	53.76	2x	454.81	0.47
OM44	53.32	2x	451.09	0.46
OM45	54.51	2x	461.15	0.47
OM46	51.79	2x	438.14	0.45
OM47	53.22	2x	450.24	0.46
OM48	54.01	2x	456.92	0.47
OM49	52.14	2x	441.10	0.45
OM50	102.84	4x	870.03	0.89
OM51	54.16	2x	458.19	0.47
OM52	53.20	2x	450.07	0.46
OM53	55.47	2x	469.28	0.48
OM54	52.09	2x	440.68	0.45
OM55	51.39	2x	434.76	0.44
OM56	53.69	2x	454.22	0.46
OM57	53.82	2x	455.32	0.47
OM58	101.01	4x	854.54	0.87

[z]1 Mbp = 1/978 pg according to Doležel et al. (2007).

Morphological Analysis

Observation of Seeds

The tetraploid 'Chammel' seeds had average width of 4.65 mm and average weight of 31.82 mg, while the diploids had average width of 3.61 mm and average weight of 21.18 mg. (Table 2 and Fig. 2). However, the tetraploid and diploid seeds had similar lengths and thicknesses (Table 2). Previous studies found that polyploid seeds of Hylocerous (Cohen et al., 2013), Japanese dandelion (Hoya et al., 2007), and Dactylis glomerata (Maceira et al., 1993) were larger than the corresponding diploids.

Table 2. The comparison of seed morphology between *Cucumis melo* 'Chammel' diploids and tetraploids

Ploidy level	Length (mm)	Width (mm)	Thickness (mm)	Weight (mg)
Diploid	9.25 ± 0.13	3.61 ± 0.06	1.61 ± 0.02	21.18 ± 0.25
Tetraploid	9.39 ± 0.13	4.65 ± 0.05	1.62 ± 0.04	31.82 ± 0.18
Significance	NS	**	NS	**

**significant at $p < 0.01$. All the values are expressed as mean ± SE (n = 10).

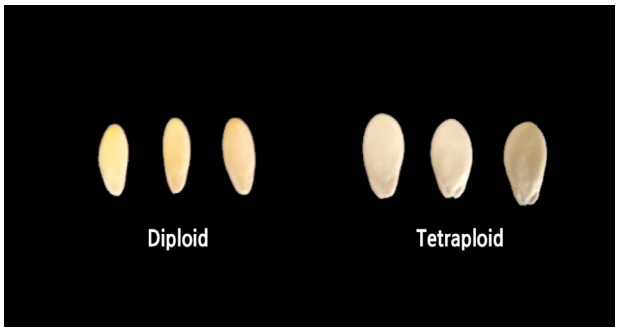

Fig. 2. The changes of seed morphology between *Cucumis melo* 'Chammel' diploids and tetraploids.

Observation of Leaves

Tetraploids had average cotyledon width of 2.05 cm and a first true leaf thickness of 0.64 mm, while diploids had average cotyledon width of 1.60 cm and a first true leaf thickness of 0.31 mm. (Table 3 and Fig. 3). A similar pattern was found in *Lycopersicon esculentum*, in which leaves of the tetraploids were broader than those of the diploids (Ishfaq et al., 2012). However, the petiole length of the tetraploid (3.42) cm was shorter than that of the diploid (4.87 cm), and significant difference were found in the length and width of the first true leaf between the diploids and tetraploids (Table 3). The difference at the leaf margin was noticeable. The leaf margin of the tetraploids was crenate, and the entire margin was smooth in the diploids (Fig. 3). The leaves of polyploid plants often show several distinct characteristics, such as thicker leaf lamina and shape changes (Eng and Ho, 2019).

Table 3. The comparison of leaf morphology between *Cucumis melo* 'Chammel' diploids and tetraploids

Ploidy level	Cotyledon		First true leaf			
	Length (cm)	Width (cm)	Length (cm)	Width (cm)	Thickness (mm)	Petiole length (cm)
Diploid	3.17 ± 0.03	1.60 ± 0.07	5.65 ± 0.04	6.35 ± 0.08	0.31 ± 0.01	4.87 ± 0.08
Tetraploid	3.17 ± 0.03	2.05 ± 0.04	5.03 ± 0.09	6.78 ± 0.05	0.64 ± 0.02	3.42 ± 0.06
Significance	NS	**	**	**	**	**

NS, ** Non Significant or significant at $p < 0.01$. All the values are expressed as mean ± SE (n = 6).

Fig. 3. The changes of leaf morphology between *Cucumis melo* 'Chammel' plants diploids ("2×") and tetraploids ("4×").

Table 4. The comparison of stomata distribution and size between *Cucumis melo* 'Chammel' diploids and tetraploids

Ploidy level	Stomata	Guard cell	
	Density (cell/mm^2)	Length (μm)	Width (μm)
Diploid	26.67 ± 0.88	32.04 ± 3.44	3.67 ± 0.26
Tetraploid	14.00 ± 0.58	48.67 ± 0.94	6.06 ± 0.25
Significance	**	**	**

$^{NS, **}$Non significant or significant at $p < 0.01$. All the values are expressed as mean ± SE (n = 5).

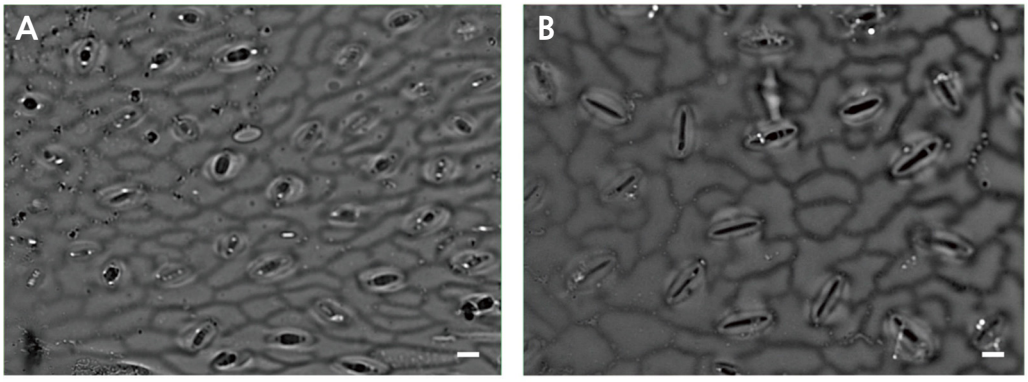

Fig. 4. The changes of stomata of the *Cucumis melo* 'Chammel' plants. A. Diploid (2n = 2x = 24). B. Tetraploid (2n = 4x = 48). Scale bar = 10 μm.

Observation of Stomata

The guard cell' length and width and the stomata' density of the diploids and tetraploids were measured (Table 4 and Fig. 4). The average length and width of the guard cells were 48.67 μm and 6.06 μm, respectively, for the tetraploids, and 32.04 μm and 3.67 μm, respectively, for the diploids. Polyploidy can be easily measured by visualizing the guard cells since tetraploid stomata become larger than diploid stomata (Yang et al., 2006). In this study, the tetraploid leaves had a stomatal density of 14 cell/mm^2, while diploid leaves had nearly twice the density at 26.67 cell/mm^2. This finding is similar to the results from watermelon (Bae et al., 2020) and coffee (Mishra, 1997). The polyploid plants may increase stress tolerance by reducing transpiration, such as the drought-tolerant autopolyploid in Arabidopsis (Del Pozo and Ramirez-Parra, 2015) and drought-tolerant allopolyploids in wheat (Xiong et al., 2006).

Chromosome Analysis

Chromosome Doubling

Determining chromosome numbers is a fundamental tool in cytogenetic studies (Mohammad et al., 2020). Korean melon is a diploid plant with a basic chromosome set (x) of 12 (2n = 2x = 24) (Kim et al., 2016). In this study, we found that the diploid Korean melon, also the control, had 24 (2n = 2x = 24) chromosomes, and the tetraploid oriental melon 'Chammel' plants had 48 chromosomes, (2n = 4x = 48) (Fig. 5). This result is consistent with the study of watermelon polyploidization in which diploid (2n = 2x = 22) plants treated with oryzalin became tetraploid (2n = 4x = 44) (Bae et al., 2020), and chromosome doubling of *Hibiscus* such as *H. syriacus* 'Oiseau blue' and 'Woodbrize' in which young leaves with colchicine generated polyploid cultivars (Van Laere et al., 2006).The effective induction of chromosome doubling

in diploid and triploid roses, by oryzalin has been documented (Kermani et al., 2003). In agriculture, several polyploid plants, e.g., wheat, cotton, coffee, oat, and canola, are widely cultivated, suggesting that polyploidy is very useful (Zeng et al., 2019).

FISH Analysis

FISH is a molecular cytogenetic technique that uses fluorescently labeled probes to determine the presences of a particular DNA sequence in the nuclei (Speicher and Carter, 2005; Hwang et al., 2020). FISH analysis was used for the accurate confirmation of ploidy in a previous study (Islam et al., 2020; Waminal and Kim, 2015). The 5S rDNA and 18S rDNA loci are usually located at different sites on different chromosomes, and their transcription is carried out by rRNA polymerases. These markers are widely used in FISH techniques. Frequently used 5S rDNA loci includes the 5S rRNA repeated units plus intergeneric spacer regions (IGS) and frequently used 45S rRNA loci include the 18S, 5.8S, and 25S rDNA repeated units, internal transcribed sequences (ITS1 and ITS2), and the IGS spacer region (Volkov et al., 2017).

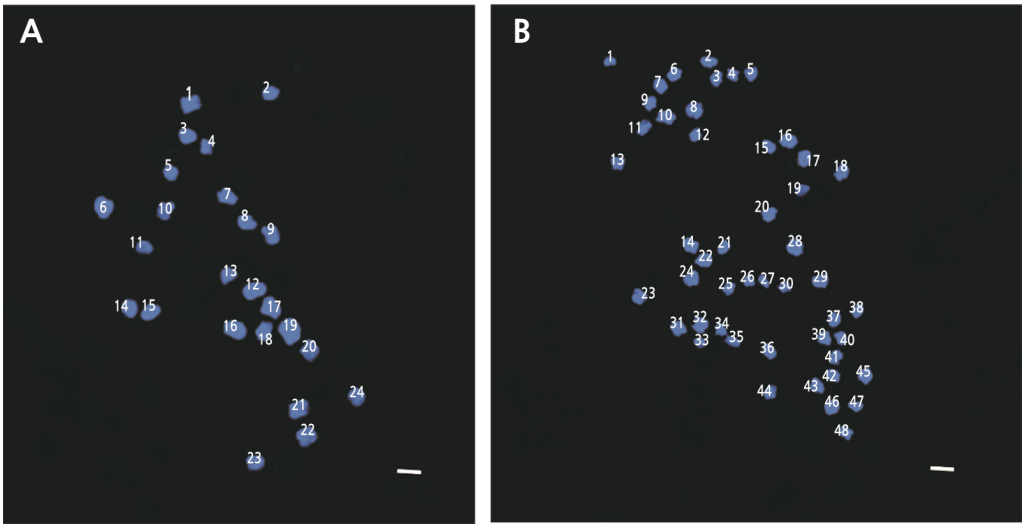

Fig. 5. Chromosome analyses of *Cucumis melo* 'Chammel' plants A. Diploids (2n = 2x = 24) and B. Tetraploids (2n = 4x = 48). Magnification: 400 ×. Scale bar = 10 μm.

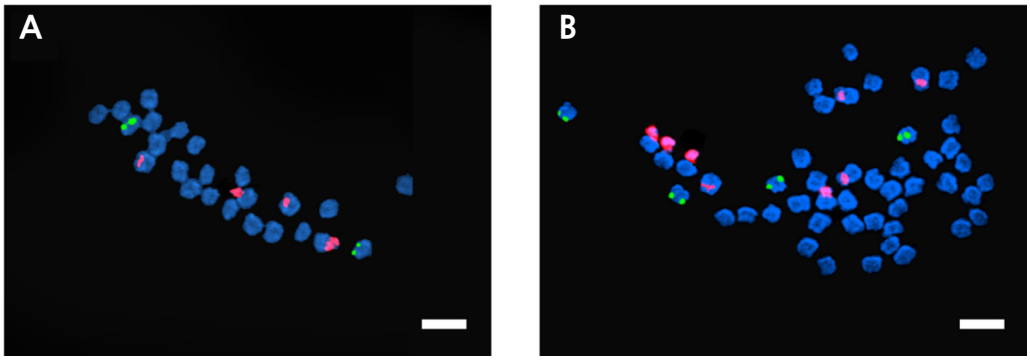

Fig. 6. Fluorescence in situ hybridization (FISH) images of 5S and 18S rDNA-labeled metaphase chromosomes of *Cucumis melo* 'Chammel' plants. A. Diploid (2n = 2x = 24).B. Tetraploid (2n = 4x = 48). The green and red fluorescence indicate the 5S and 18S rDNA loci, respectively. Magnification: 1000×. Scale bar = 10μm.

According to the FISH results, one pair of 5S rDNA loci and two pairs of 18S rDNA loci were detected in the diploid oriental melons, whereas two pairs of 5S rDNA and four pairs of 18S rDNA loci were detected in the tetraploid melons (Fig. 6). A study of cotton plants revealed that most diploids had one pair of 5S rDNA loci, and all the allotetraploid species had two pairs (Gan et al., 2013); the same result was found in the woody species of the genus *Rubus* (Wang et al., 2015). The 5S rDNA loci copy number, which was found in most FISH studies, is generally used to determine the ploidy level of plants (Younis et al., 2015; Mohammad et al., 2020).

Karyotyping is a technique for detecting chromosome variation and rDNA loci distributions, as well as creating a genetic map (Chung et al., 2018). It is the most used cytological technique in empirical and theoretical studies (Liang and Chen, 2015). In this study, 18S rDNA signals were found in chromosome 1 and 2 for both the diploids and tetraploids. In the case of 5S rDNA, its signals were detected near the centromere of chromosome 10 for both the diploids and tetraploids (Figs. 7 and 8). The FISH and karyotype data confirm the polyploidization of the 'Chammel' plants. This polyploidization method is expected to allow for more hybridizations with diploid oriental melons, allowing for the development of seedless cultivars.

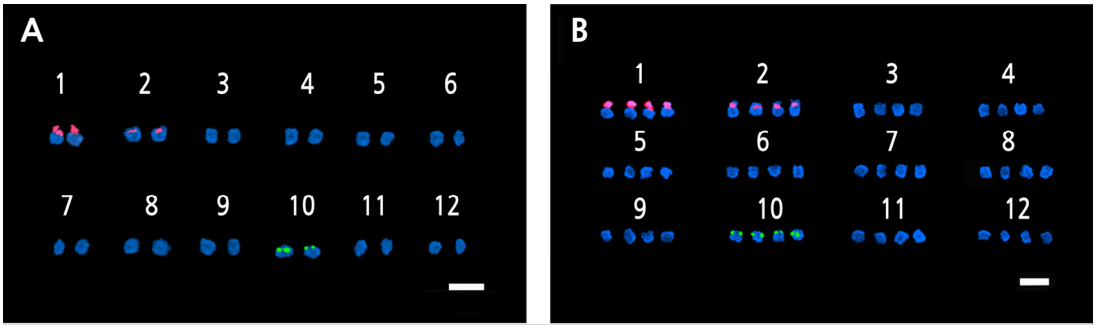

Fig. 7. FISH karyotype analysis of diploid (A) and tetraploid (B) *Cucumis melo* 'Chammel' plants with 5S rDNA (green) and 18S rDNA (red) probes. The chromosomes are arranged in the order of descending lengths of the short arm. Scale bar = 10 μm.

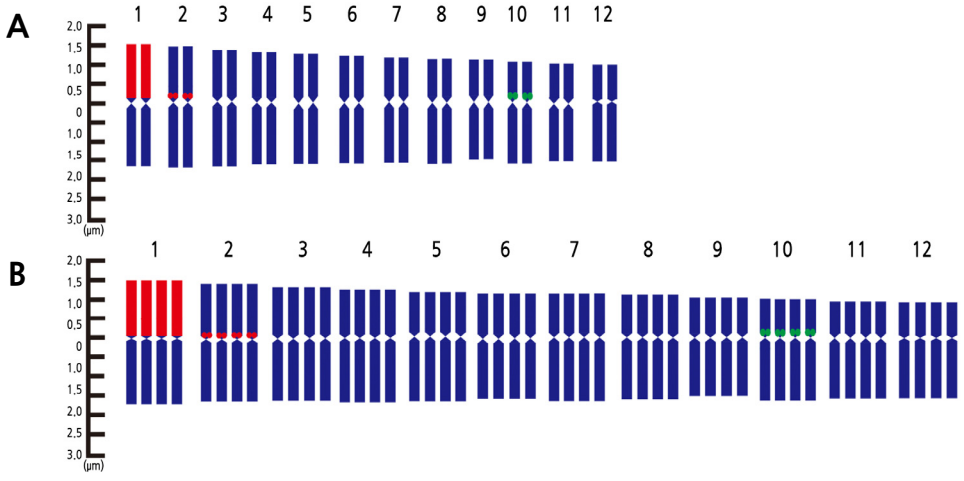

Fig. 8. FISH ideogram of 5S rDNA and 18S rDNA loci on chromosomes of diploid (A) and tetraploid (B) *Cucumis melo* 'Chammel' plants where green fluorescence indicates 5S rDNA and red fluorescence indicates 18S rDNA.

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