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METAPHASE CHROMOSOME SPREAD PREPARATION OF CITRUS SPECIES

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ABSTRACT

Citrus is one of the popular fruit crops in Vietnam with many commercial varieties, however, its cytogenetic characteristics are currently limited. This study was conducted to find out a procedure for metaphase chromosome preparation in some citrus species. The results showed that after a 4-hour treatment with 0.002 M 8-hydroxyquinoline solution, the mitose indices of the root tip samples differed by species. Specifically, the tangerine and orange samples yielded the highest results at 11:00 am, the lemon root sample at 10:00 am, and the kumquat sample at noon. The explants were fixed in carnoy solution for at least 24 h; in what, the cell wall could be digested with 5 M HCl in 7−10 min (orange) or 10−15 min (tangerine) or with 2% pectinase and cellulase mixture in 30−35 min for kumquat and lemon. Then, their stained in aceto-orcein for 7−10 min and observed under the microscope. As with previous research, the chromosomal set of the assessed samples revealed that they all possessed a chromosome set of 2n = 18. The study's findings identified the factors influencing the quality and chromosomal dispersion of several citrus samples, as well as the sample fixing time. Therefore, this procedure for preparing specimens can be applied to other citrus-family plants for further cytoogenetics research.

Keywords: Cell wall digestion, cell division inhibitor, cell cycle, metaphase arrest.

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INTRODUCTION

Citrus is one of the most significant fruit crops in the world and is grown in over 114 nations. The northern region produces more than 70% of the world's citrus, mostly in China, Brazil, India, the United States, and nations around the Mediterranean. Vietnam produces citrus with a wide range of shapes, cultivars, and regional names that are unique to the country such as Cam Sen Yen Bai, Cam Sen Dinh Ca - Bac Son, Cam Bu Ha Tinh, Cam Sanh Ham Yen, etc. Citrus is a high-quality fresh fruit with high nutritional and usage values. Citrus fruits are used for fresh eating, making jams, making soft drinks and for medicinal purposes. Essential oils distilled from the peels, leaves and flowers are widely used in the food and cosmetic industries (Bora et al., 2020).

Along with morphological and molecular biological factors, chromosome number is one of the most important information for plant classification. The physiological state and productivity of plant species are determined by differences in the number, size and shape of chromosomes, so the study of chromosome status is a fundamental part of plant cytogenetics and breeding. Chromosomes are isolated from cells of living tissues and analyzed at the metaphase of mitosis, when they are most condensed and therefore easier to observed. The number of chromosomes in each species and their variations (polyploidy, aneuploidy) are revealed by chromosomal studies. According to published data, different citrus species have varying levels of chromosome numbers. For example, six citrus species including *Citrus maxima* (Burm.) Merr. var. Sha-tian-you pummelo, *Citrus reticulata* (Blanco) var. Red Tangerine, *Citrus medica* (L.), *Microcitrus australasica* (F. Muell.) var. Swingle, *Citrus mangshanensis* (S.W. He & G.F. Liu) var. Wild Mangshan Mandarin and *Poncirus trifoliata* (L.) Raf., all have 2n = 18 (He et al., 2020). While, *Citrus sinensis* Osbeck (Sweet orange cv. mosambi), *C. reticulata* Blanco (Nagpur mandarin) and *Citrus jambhiri* Lush (Roughlemon) with diploid (2n = 2x = 18), triploid (2n = 3x = 27), tetraploid (2n = 4x = 36), hexaploid (2n = 6x = 54) (Narukulla, 2022). Therefore, identify the genetic variation of the genus *Citrus* with cytogenetic is required. In addition, the chromosome counting of commercial citrus varieties in Vietnam and the factors influencing the quality of metaphase chromosome spread, have not been conducted. Thus, the study was carried out to assess the impact of several factors on the chromosome quality of some commercial citrus varieties in Vietnam, including seed germination conditions, sample fixation time, mitotic inhibitors, and manipulations at the steps of mitotic metaphase preparation.

MATERIALS AND METHODS

Plant samples

We planted the seeds of orange, lemon, kumquat, and tangerine that we had bought from the fruit kiosk at Da Lat Market to collect young root tips-growing tips with cells that are constantly dividing. The seeds were collected from fruits and then planted in both *in vitro* and *ex vitro* conditions to produce plantlets. The plantlets' root tips were used as explants for fixation and mitotic metaphase preparation.

Seed germination conditions

Seeds were collected and cultured under *ex vitro* and *in vitro* conditions with minor modification (Domingues et al., 2024) that (1) *Ex vitro*: seeds were washed under running water for 15 min, then the seed coat was removed and sown on coconut fiber (Eco Source Co. Ltd., Vietnam) or (2) *In vitro*: seeds were washed under running water, shaken in 0.1% soap solution (Sunlight, Electrical Co., Ltd., Binh Duong, Vietnam) for 5 min, then washed with tap water for at least 30 min. The explant surface was sterilized with 2% Javel solution for 15 min and washed with sterile water at least three times in a laminar hood. The seed coat was removed and cultured in MS (Murashighe & Skoog, 1962; Merck KGaA, Darmstadt, Germany) medium supplemented with 30 g/L sucrose and 7 g/L agar (Viet Xo Vegetable & Fruit J.S.C., Hai Phong, Vietnam).

Fixation time

The 30-day root tips were collected at 3 different time points: 10:00 am, 11:00 am and 12:00 am (Maravilla et al., 2023; Planchais et al., 2000), then cut about 1 cm long and fixed in carnoy solution (3 acetic acid: 1 ethanol) for at least 24 hours, then proceed to hydrolyze the cell wall (Maravilla et al., 2023). To calculate the mitotic index, the number of cells in the mitotic stages were counted after stained and observed under an optical microscope.

Cell division inhibitors and the preparation steps

In order to improve the quality of chromosome spread and increase the number of dividing cells, samples must be pretreated prior to fixation. Pretreatment solvents were selected basing on treatment concentration, chromosome size, and cell structure include distilled water at 4 ^{o}C, 8-hydroxyquinoline, colchicine, or α-bromonaphthalene (Planchais et al., 2000). Investigating the conditions of appropriate treatment chemicals for each plant species is therefore essential. The root tips were treated either with 0.002 M 8-hydroxyquinoline or 0.1% colchicine for 4 h at room temperature (25 ± 2 ^{o}C) to evaluate the effectiveness of these two chemicals on the inhibition of cell division in the investigated samples. Cell walls were treated in the sample with HCl or enzymes.

In this study, the root tips of investigated species were treated by 8-hydroxyquinoline, cut about 1cm long and fixed in carnoy solution (3 acetic acid: 1 ethanol) for at least 24 hours. Afterwards, samples were treated with (1) 5M HCl for 7, 10 and 15 minutes (HCl_7, 10, 15) at room temperature then rinsed with 45% acetic acid or (2) an enzyme mixture of 2% PC (2% pectinase and cellulase) at 37 ^{o}C for 25, 30, and 35 minutes (PC_25, 30, 35) with washing 2 times × 10 min by 0.01M sodium citrate buffer before and after enzyme treatment. Depending on the softness of the sample after treatment, the sample can be crushed in 45% acetic acid solution or left intact on the glass slide then stained with 2% aceto-orcein, covered with a coverslip, and gently pressed before being observed under an optical microscope.

Chromosome staining

The sample (one root tip per slide) was placed on a glass slide, a small amount of 45% acetic acid was added, and it can either be crushed or left intact. The sample was then stained with 2% aceto-orcein (Tonzetich, 2004), covered with a coverslip, and gently pressed before being observed under an optical microscope.

Culture conditions

Ex vitro: seeds were sown on coconut fiber substrate and grown in greenhouses with an average temperature of 18 ± 2 ºC, and were exposed to 50% natural sunlight with a sunshade net.

In vitro: seeds were de-coated and cultured in MS medium which provided water and nutrients, the temperature maintained at 25 ± 2 ºC as well as stable light for 16 hours/day (Domingues et al., 2024).

Observe images and data analysis

After staining, nuclei and chromosomes were captured by microscope (Olympus CX23) at × 400 and × 1,000 magnification. The images were then processed using Image J software to convert them to black and white image mode, chromosomes were marked and counted the number in the investigated samples.

RESULTS AND DISCUSSION

Seed germination conditions on root growth

After sowing seeds under two different conditions, root length was measured after 10, 15, and 30 days to evaluate the effect of sowing conditions on root system development. The results showed that the seed germination and root growth depended on the seed germination conditions and the species. In the *ex vitro* condition, the seeds germinated after 10−12 days of sowing with a survival rate of about 80% while in the *in vitro* condition, the seeds germinated earlier (5−7 days of culturing) with a survival rate of

100%. In addition, the root growth under *in vitro* conditions was better than that under *ex vitro* conditions in all four tested species after 30 days. The *in vitro* condition was very favorable for seed germination and growth (Barpete et al., 2015; Haj Sghaier et al., 2022). Tagarines gave the longest root length with 48.7 mm under *in vitro* condition, and opposites under *ex vitro* condition, root growth was the slowest with only 18.6 mm. In both conditions, kumquats roots (41.18 and 24.68 mm, respectively) grew faster than lemons (31.76 mm and 20.18 mm) and oranges (34.33 mm and 19.67 mm). This indicated that whereas root development was generally slower in *ex vitro* settings, and there was no significant difference between the study samples after 30 days (Fig. 1). The standard error (SE) value demonstrated the possible impact of external conditions on root growth and physiological properties of each plant.

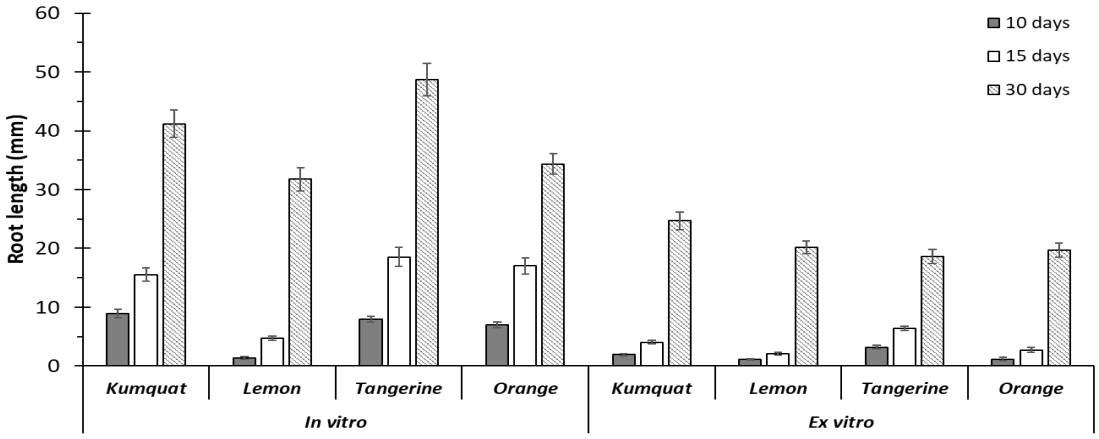

Figure 1. The effect of *in vitro* and *ex vitro* conditions on root growth of *Citrus* explants

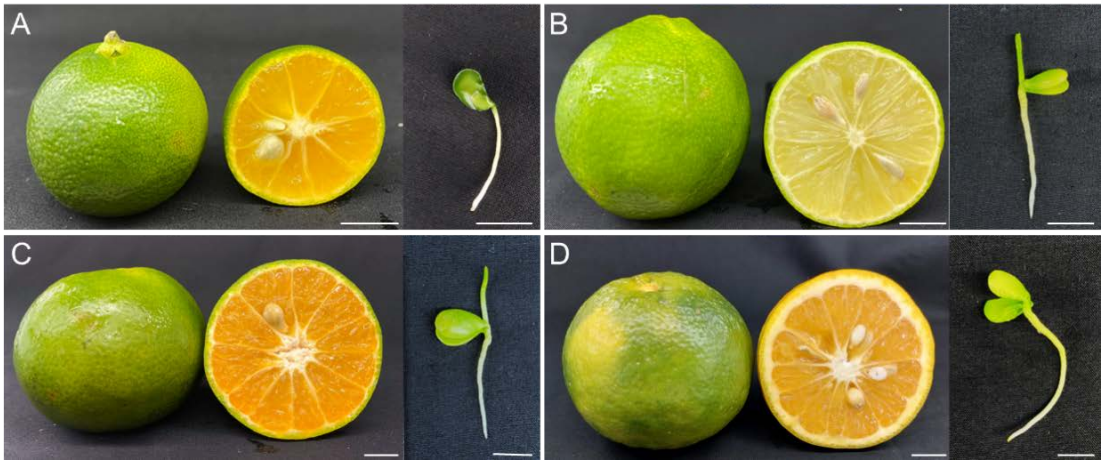

Figure 2. The fruit (left) and *in vitro* germinated plantlet (right) with 30-day root tips used as samples for mitotic chromosome spread. A: Kumquat; B: Lemon, C: Tangerine; D: Orange. Scale bar: 1 cm for A, B, C, D

In addition to the survival rate and fast root growth rate under *in vitro* condition, the seeds were cultivated in sterile circumstances on an artificial nutrient medium, which was advantageous for evaluating the morphology and development of the roots. From the results

obtained, the *in vitro* roots were the most suitable for collecting root tips to prepare metaphase chromosome spread (Fig. 2).

Fixation time on mitotic index

Each species has a unique cell cycle, some reports have shown that sample fixation time significantly affects the observation of cells in the metaphase of mitosis (Fukui & Iijima, 1991; Georgi et al., 2002; Ma et al., 1996). The shape of the chromosomes varied depending on the stage of cell division (Fig. 3).

To assess the variation in the proportion of cells in the stages of mitosis, the root tips of kumquat, lemon, tangerine, and orange were collected and fixed at different time including 10:00 am, 11:0 am, and 12:00 am (Table 2). In the kumquat, the mitose index peaked at 12:00 am with 48.80% of dividing cells and fell to 41.44% by 10:00 am. With 43.60% of dividing cells, the greatest mitose index for lemon samples was reported at 10:00 am. This index progressively decreased at subsequent treatment time points, reaching 38.34% at 11:00 am and 35.7% at 12:00 am. The greatest mitose index values for the orange and tangerine samples were recorded at 11:00 am, at 40.08% and 50.22%, respectively, while the lowest values were obtained at 10:00 am, at 42.01% and 34.31%. The mitotic index, which measures the rate of cell division, can peak at different periods in different plant species, as well as within the same species, due to differences in biological clocks and developmental timing. Each plant species has an internal clock that controls its developmental activities, including mitosis. For instance, some plants may peak in the summer, while others may have a higher mitotic index in the spring when growth is more active (Westin et al., 1999). Other factors that can affect mitotic rates include temperature, light, pH, and the availability of nutrients.

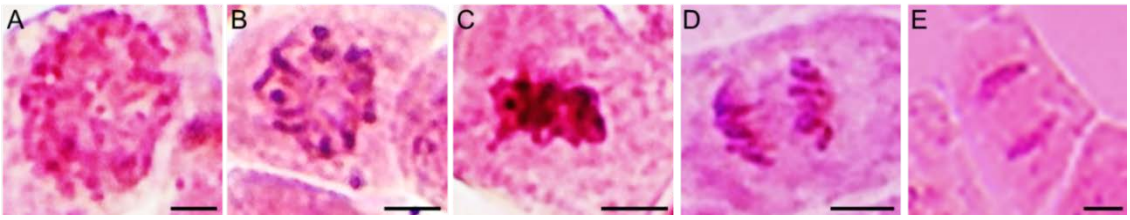

Figure 3. Cell division stages of the lemon chromosome. A: Interphase; B: Prophase; C: Metaphase, D: Anaphase; E: Telophase. Scale bar: 5 µm for A, B, C, D, E

Table 2. Effect of fixation time n mitose index of 4 *Citrus* explants

Sample	Fixation time	% cell in mitosis phases					Mitose index (%)
		Interphase	Prophase	Metaphase	Telophase	Anaphase	
Kumquat	10:00	58.56	32.56	1.69	1.06	6.13	41.44
	11:00	55.26	32.19	3.24	2.02	7.29	44.74
	12:00	51.20	40.91	2.15	0.96	4.78	48.80
Lemon	10:00	56.40	38.86	3.32	0.24	1.18	43.60
	11:00	61.66	32.06	1.79	2.02	2.47	38.34
	12:00	64.34	27.92	0.57	1.32	5.85	35.66
Tangerine	10:00	58.00	34.46	1.88	2.26	3.39	42.00
	11:00	49.78	45.98	2.46	1.12	0.67	50.22
	12:00	56.92	36.55	2.87	1.57	2.09	43.08
Orange	10:00	65.69	27.21	1.23	1.23	4.66	34.31
	11:00	59.92	31.02	0.77	1.35	6.94	40.08
	12:00	62.52	26.16	1.67	0.93	8.72	37.48

Mitotic chromosome spread

Cell division inhibitors

According to Singh et al. (2018), high concentrations of colchicine were proven to cause polyploidy, and low concentrations (0.1 to 0.5% for 1 to 2 h, at room temperature) were recommended in the pretreatment step. A treatment time of 1.5 h gives the best results for soybean chromosomes. This study examined the differences in treatment effectiveness between root tip samples treated with 0.002 M 8-hydroxyquinoline and 0.1% colchicine for 4 h at room temperature (Fig. 4).

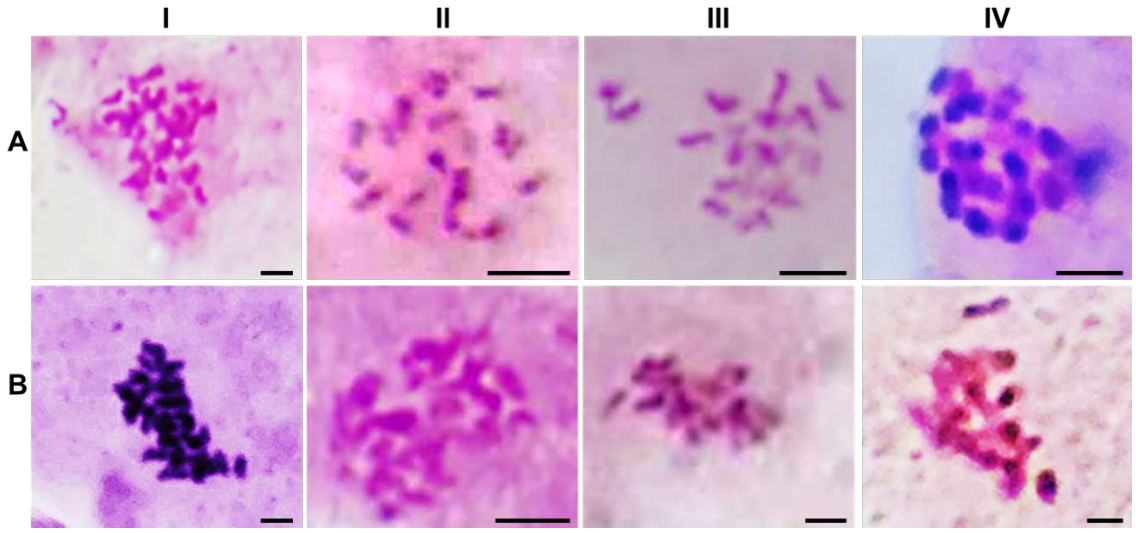

Figure 4. The effects of 8-hydroxyquinoline (A) and colchicine (B) on mitotic chromosome spreads of kumquat (I), lemon (II), tangerine (III) and orange (IV). Scale bar: 5 µm for A, B, I–IV

In general, compounds generally prevented cell division, producing many cells in the metaphase stage with condensed chromosomes, but the dispersion of chromosomes was not good (because it also depended on the preparation step). With the preliminary results obtained in this experiment, 0.002 M 8-hydroxyquinoline and 0.1% colchicine can both be utilized to stop cell division, however, 8-hydroxyquinoline is less hazardous to the environment and human health than colchicine. Therefore, in order to examine the impact of the preparation steps on well-dispersed metaphase slides, 8-hydroxyquinoline was used in the sample processing step of the subsequent experiment.

The preparation steps

According to Freschet et al. (2021), using HCl to soak root samples with appropriate concentration and time helps hydrolyze cell walls, which facilitates sample compression and uniform cell distribution on glass slides. The sample will be still hard, difficult to spread, and difficult to observe if it is processed in a short time or in low concentration because the cell walls won't have enough time to break down by HCl or enzymes; if it is processed too long, the cells will be deformed because of the loss of cell walls and dehydration from protoplasm shrinkage.

In order to clean the cytoplasm, and allow chromosomes to spread out during the compression process, the sample must be soaked in 45% acid during the staining process. The cytoplasm will be lysed by treating the sample in an acetic acid solution, which will facilitate the observation of the chromosomes and nuclei.

The results obtained under experimental conditions showed significant differences between the investigated species. In general,

the HCl treatment worked better than the enzyme mixture when applied to root samples of investigated citrus species. Most HCl treatment produced protoplasts, the cytoplasm was lysed, the dispersion of nuclei was generally good, chromosomes were well-stained and -observed (Table 3 & Fig. 5).

From the results obtained in the above experiments, the preparation process of chromosome specimens of the studied species was slightly adjusted (Table 4) and cells in metaphase with coiled and dispersed chromosomes were obtained (Fig. 6).

The most crucial step to prepare mitotic chromosome slides is to adjust the staining time, acetic acid treatment, or grinding and pressing of the sample based on its hardness or softness following the cell membrane resolution step. This ensures that the cells are evenly distributed and do not overlap. Therefore, the quality of the mitotic spread depends on the skill and experience of the technician/researcher.

Although polyploid citrus species have been discovered such as *C. sinensis* Osbeck (Sweet orange cv. mosambi), *C. reticulata* Blanco (Nagpur mandarin) and *C. jambhiri* Lush (Roughlemon) with diploidy (2n = 2x = 18), triploidy (2n = 3x = 27), tetraploid (2n = 4x = 36), hexaploid (2n = 6x = 54) (Narukulla, 2022). For these research samples, seeds were used as original material, so the chromosome set could only be 2n or 4n because 3n plants are sterile (cannot produce seeds). The result obtained in this study showed that the chromosome set of the investigated samples were all 2n = 18 chromosomes (Fig. 6), The data obtained was also similar to previous studies on some citrus species (Stace et al., 1993; Guerra et al., 2000; Bhuvaneswari et al., 2020). However, the size and shape of chromosomes will vary from species to species. Deng et al. (2019) have also reported citrus species with tiny chromosomal sizes (2–4 um) and comparable chromosome morphologies (Deng et al., 2019).

Table 3. The effects of treatment with 5M HCl for 7, 10 min, 15 min and 2% PC for 25, 30, 35 on chromosome spreads preparation of investigated samples

<table>
<tr><th>Treatments</th><th>Kumquat</th><th>Lemon</th><th>Tangerine</th><th>Orange</th></tr>
<tr><td>HCl_7</td><td>Cell walls not degraded, nucleus not observed</td><td colspan="2">The cell wall had been degraded, formed single cells with dye-stained nuclei.</td><td rowspan="2">Sample was moderately soft; the cells and chromosomes were well observed.</td></tr>
<tr><td>HCl_10</td><td>The cell wall had been degraded, formed single cells with dye-stained nuclei.</td><td>Both nucleus and cytoplasm were stained, difficult to observe.</td><td rowspan="2">The sample was moderately soft, the cells and chromosomes were well observed.</td></tr>
<tr><td>HCl_15</td><td colspan="2">Cells were over-digestion, both nucleus and cytoplasm were stained, difficult to observe.</td><td>Both nucleus and cytoplasm were stained, difficult to observe.</td></tr>
<tr><td>PC_25</td><td>Cell wall not degraded, nucleus not observed.</td><td>Both nucleus and cytoplasm were stained, difficult to observe.</td><td rowspan="3">Cells were over-digestion, both nucleus and cytoplasm were stained, difficult to observe.</td><td>The nucleus was clearly visible.</td></tr>
<tr><td>PC_30</td><td colspan="2" rowspan="2">The sample was moderately soft, the cells and chromosomes were well observed.</td><td rowspan="2">Cells were over-digestion, both nucleus and cytoplasm were stained, difficult to observe.</td></tr>
<tr><td>PC_35</td></tr>
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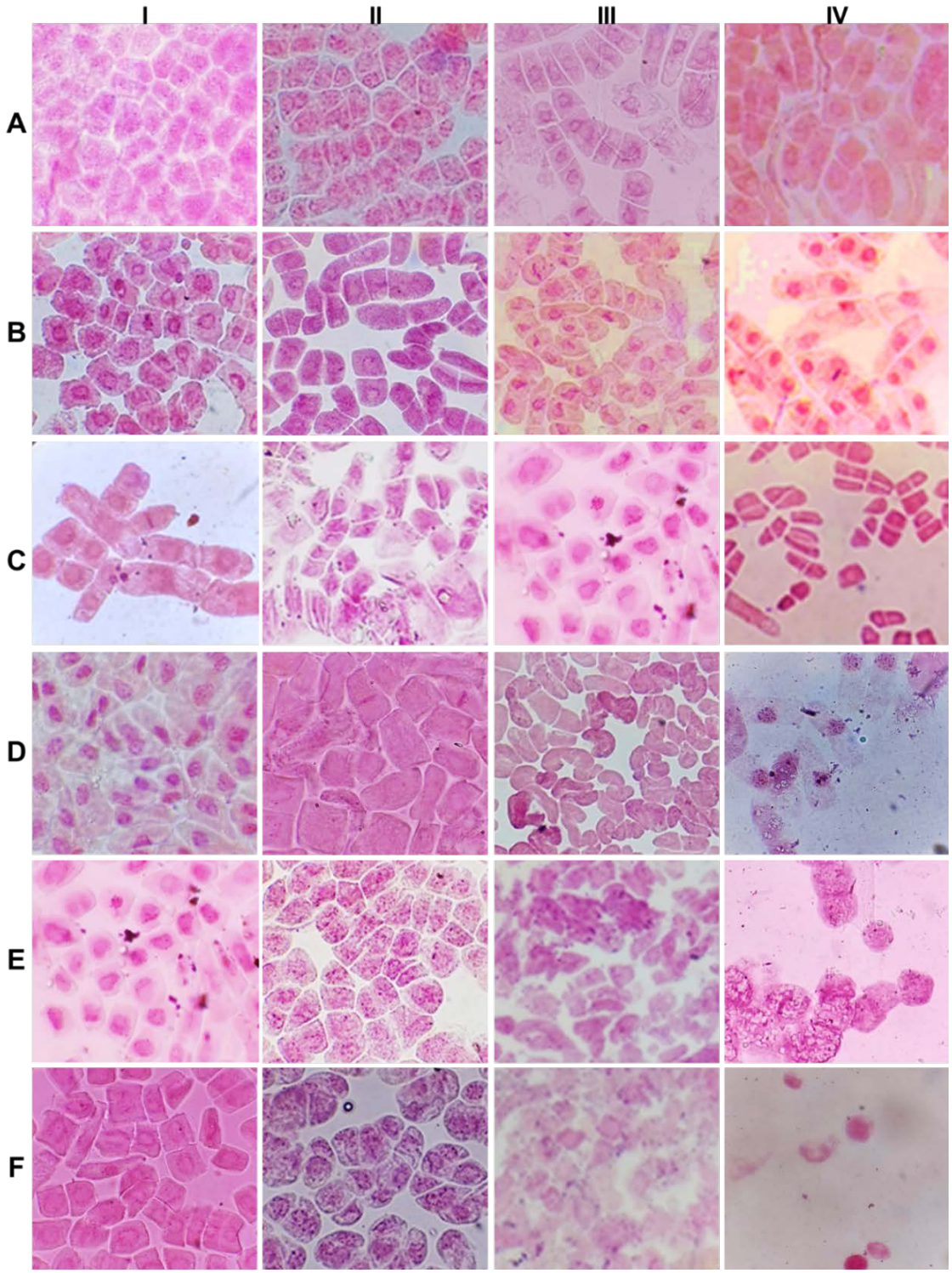

Figure 5. The effects of treatment with 5M HCl for 7 min (A), 10 min (B), 15 min (C) and 2% PC for 25 min (D), 30 min (E), 35 min (F) on chromosome spreads of kumquat (I), lemon (II), tangerine (III) and orange (IV)

Table 4. Some noticeable points when performing chromosome spreads preparation of investigated samples

Sample	Slide preparation modifications
Kumquat	Crush the root tip in 45% acid acetic, heat gently for 3 min, stain by aceto-orcein, heat again for 7 min, and press the coverslip.
Lemon	Stain by aceto-orcein stain for 10 min, wash with 45% acid acetic 45% for 3 min and stain again for 3 min.
Tangerine	
Orange	

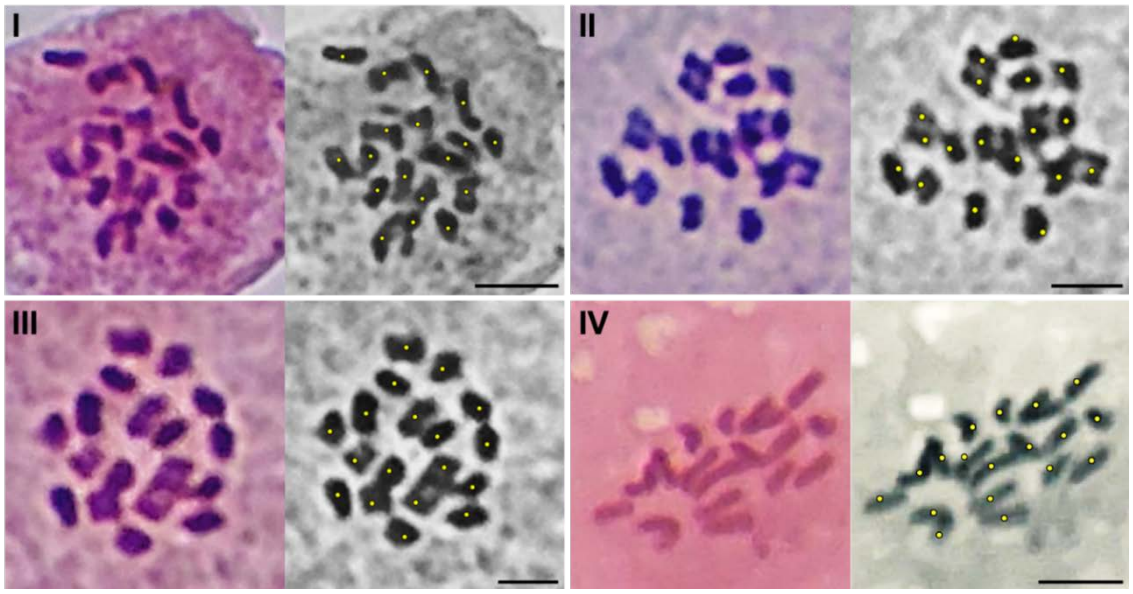

Figure 6. The metaphase spreads of kumquat (I), lemon (II), tangerine (III) and orange (IV) with stained chromosomes (left) and numbered by yellow dot (right). Scale bar: 5 µm for I–IV

CONCLUSION

The results showed that *in vitro* seed germination promoted the samples to grow faster, making it easier to collect root tip samples for chromosome preparation. Both mitotic inhibitors can be used to arrest metaphase in the investigated samples, however, using 0.002 M 8-hydroxyquinoline solution for 4 h was more suitable. The time of sample fixation also differs by species, specifically lemon at 10:00 am, tangerine and orange at 11:00 am and kumquat at 12:00 am. The cell wall digestion also differs between the samples by 5 M HCl for 7–10 min (orange) or 10–15 min (tangerine) or by a mixture of pectinase and 2% cellulase for 30–35 min for kumquat and lemon. The chromosome set of the samples studied in this study is all 2n = 18. The study's findings revealed that the quality and chromosomal dispersion of the investigated citrus samples were impacted by procedural manipulation and sample fixing time. Therefore, this sample preparation method can be used for many cytogenetic studies on other citrus plants.

REFERENCES

Barpete S., Oguz M., Ozcan S., Anayol E., Ahmed H. A., Khawar K. M., Ozcan S., 2015. Effect of temperature on germination, seed vigor index and seedling growth of five Turkish Cotton (*Gossypium hirsutum* L) cultivars, *Fresenius Environmental Bulletin*, 24: 2561.

Bhuvaneswari G., Thirugnanasampandan R., Gogulramnath M., 2020. Effect of

colchicine induced tetraploidy on morphology, cytology, essential oil composition, gene expression and antioxidant activity of *Citrus limon* (L.) Osbeck, *Physiology and molecular biology of plants: an international journal of functional plant biology*, 26: 271. https://doi.org/10.1007/s12298-019-00718-9

Bora H., Kamle M., Mahato D. K., Tiwari P., Kumar P., 2020. Citrus Essential Oils (CEOs) and Their Applications in Food: An Overview, *Plants* (Basel, Switzerland), https://doi.org/910.3390/plants9030357

Deng H., Cai Z., Xiang S., Guo Q., Huang W., Liang G., 2019. Karyotype Analysis of Diploid and Spontaneously Occurring Tetraploid Blood Orange [*Citrus sinensis* (L.) Osbeck] Using Multicolor FISH With Repetitive DNA Sequences as Probes, *Frontiers in Plant Science*: 10, https://doi.org/1010.3389/fpls.2019.00331

Domingues N. F. J., Carneiro D. C. d. S., Putti F. F., Rodrigues J. D., Tecchio M. A., Leonel S., Silva M. d. S., 2024. Physiological Indexes in Seed Germination and Seedling Growth of Rangpur Lime (*Citrus limonia* L. Osbeck) under Plant Growth Regulators, *Agronomy*, 14: 2066. https://doi.org/10.3390/agronomy14092066

Fukui K., Iijima K., 1991. Somatic chromosome map of rice by imaging methods, *Theoretical and Applied Genetics*, 81: 589. https://doi.org/10.1007/BF00226723

Georgi A. B., Stukenberg P. T., Kirschner M. W., 2002. Timing of Events in Mitosis, *Current Biology*, 12: 105. https://doi.org/10.1016/S0960-9822(01)00662-5

Guerra M., Dos Santos K. G., Barros E. S. A. E., Ehrendorfer F., 2000. Heterochromatin banding patterns in Rutaceae-Aurantioideae--a case of parallel chromosomal evolution, *American journal of botany*, 87: 735.

Haj S. A., Tarnawa Á., Khaeim H., Kovács G. P., Gyuricza C., Kende Z., 2022. The Effects of Temperature and Water on the Seed Germination and Seedling Development of Rapeseed (Brassica napus L.), *Plants* (Basel, Switzerland), https://doi.org/1110.3390/plants11212819

He L., Zhao H., He J., Yang Z., Guan B., Chen K., Hong Q., Wang J., Liu J., Jiang J., 2020. Extraordinarily conserved chromosomal synteny of *Citrus* species revealed by chromosome-specific painting, *The Plant Journal*, https://doi.org/10310.1111/tpj.14894

Ma Y., Nurul M., Faridi I., Crane C., Stelly D., Price H., Byrne D., 1996. A New Procedure to Prepare Slides of Metaphase Chromosomes of Roses, *HortScience*, https://doi.org/3110.21273/HORTSCI.31.5.855

Maravilla A. J., Rosato M., Rosselló J. A., 2023. Preparation of Mitotic Chromosomes with the Squash Technique, *Methods in molecular biology* (Clifton, N.J.), 2672: 141. https://doi.org/10.1007/978-1-0716-3226-0_7

Narukulla V., 2022. Ploidy analysis among *Citrus mutants* using leaf meristematic tissue, *Journal of Horticultural Sciences*, 17: 34. https://doi.org/10.24154/jhs.v17i1.1186

Planchais S., Glab N., Inzé D., Bergounioux C., 2000. Chemical inhibitors: a tool for plant cell cycle studies, *FEBS Letters*, 476: 78. https://doi.org/10.1016/S0014-5793(00)01675-6

Stace H. M., Armstrong J. A., James S. H., 1993. Cytoevolutionary patterns in Rutaceae, *Plant Systematics and Evolution*, 187: 1. https://doi.org/10.1007/BF00994089

Tonzetich J., 2004. Orcein staining and the identification of polytene chromosomes, *Methods in molecular biology* (Clifton, N.J.), 247: 249. https://doi.org/10.1385/1-59259-665-7:249

Westin J., Sundblad L., Strand M., Hällgren J., 1999. Apical mitotic activity and growth in clones of Norway spruce in relation to cold hardiness, *Canadian Journal of Forest Research*, 29: 40.