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Katokkon Pepper (*Capsicum chinense* Jacq.) Ploidy Determination by Morphological Characteristic and Flow Cytometry Analysis

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ABSTRACT

Katokkon pepper plant is originated from Toraja, South Sulawesi, Indonesia. This endemic pepper variety has unique hot and spicy characters with a distinctive bell pepper aroma, and aesthetic shape similar to paprika, but with smaller size. This research was conducted to identify ploidy level and plant morphology of colchicine induced Katokkon pepper at Laboratory of Plant Reproduction Bioscience and Biotechnology, Department of Agronomy, Faculty of Agriculture, Universitas Hasanuddin and experimental field of Agricultural Extension System Vocational High School Santo Paulus Tana Toraja (S 03°04'177" E 119°51'526"). Two weeks old seedlings were immersed for 4.5 hours in colchicine concentration (0.00%, 0.0125%, 0.025%, 0.05% and 0.10%). Flow cytometry analysis was carried out using Partec Cy-Flow Space™. Result showed that colchicine concentration (0.025%, 0.05% and 0.1%) produced mixoploid plants with two set of chromosomes (2n=24, 4n=48). This study also found morphological differences between mixoploids plants induced by 0.025%, 0.05% and 0.1% colchicine and diploid plants (0% and 0.0125% colchicine) during first two juvenile leaves phase. However, this difference did not occur further, and eventually morphology of adult mixoploid plant was not significantly different from control (diploid), which concurred to grading mixoploid grade 2. The mixoploid plants analyzed consist of higher diploids cells than tetraploid.

INTRODUCTION

Paprika or bell pepper (*Capsicum chinense* Jacq.) plant belongs to the *Solanaceae* family (Flowrenzhy & Harijati, 2017), which is classified as big chili. Another kind of *Capsicum* called "Katokkon" pepper is found growing endemically for generations in Toraja region at the northern part of South Sulawesi province, Indonesia (S 02°-03° E 119°-120°). The fruit shape like paprika but with much smaller size, shorter, blunt

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based fruit, and having a distinctive aroma (Amaliah, 2018) with a higher level of capsaicin spiciness than paprika (118,712.677 - 149,074.747 SHU) (Wätjen, Huyskens-Keil & Stöber, 2021).

Katokkon pepper, one of the leading commodities in Toraja is only loved by the Toraja people and becomes a local pride can only produce optimally at an altitude of 600 – 1200 m above sea level (Flowrenzhy & Harijati, 2017). Planting below an altitude of 600 m above sea level does not produce the same spiciness and aroma. Development of new variety for low altitude and quality improvement such as less spicy or sweeter fruit with the distinctive aroma and or larger fruit needs further serious attention for wider recognition and plant adaptable region and distribution (Marano et al., 2017).

There are many ways to improve the quality of Katokkon pepper, one of which is by polyploidization. Polyploidization is a mechanism to multiply set of chromosome (Stuessy & Weiss-Schneeweiss, 2019) Polyploidization occurs in nature, drive species evolution, and has taken an important role in the improvement of many plant species (Eng & Ho, 2019). Polyploidization research are closely related to plant diversification, innovations of gene functions, and improvement of plant important traits (Zhang, Wang, & Cheng, 2019). Polyploid plants show increased organ size, and improvement of heterozygosity and heterosis (Zeng et al., 2020). In ornamental plants breeding, polyploidization use to produce plant with lower growth rate, and longer flowering period of time, while in fruit crops, it use to produce seedless fruits (Sattler, Carvalho, & Clarindo, 2016). In *Canabis sativa*, for example, polyploid plants show a larger leaves, larger and less dense stomata, and enhanced in chemical compound of the leaves (Parsons et al., 2019).

Most of the *Capsicum* species are diploid (2n=24) i.e. *Capsicum annuum*, *C. chinense*, *C. frutencens* and *C. baccatum* (Sousa et al., 2015). The kind of *C. chinense* Jacq. also has the same number of chromosomes as in general *Capsicum*, which is 12 pairs of chromosomes (2n=24) (Souza et al., 2011). Recent research has been reported showing Katokkon pepper is a diploid plant (Tammu et al., 2021). To improve the quality of Katokkon pepper, polyploidization approach could be used.

Polyploidization can be achieved by the application of various chemical mutagens knows as antimitotic agents (AMA) (Salma, Kundu, & Mandal, 2017). Application of AMA in plants vary widely, ranging from seeds, seedlings, buds, plant shoots, both *in vitro*, *in vivo*, and *ex vitro* (Parsons et al., 2019). In *in vivo* systems, AMA is mainly applied by soaking, immersion, droplets, and mixed culture. There are various types of mutagen agent that can be used to induce polyploidy, such as colchicine, and antimitotic herbicides of oryzalin, trifluralin, flufenacet, mix of amiprophos-methyl and pronamide, and the use of gas N_2O (Chaikam et al., 2019).

Colchicine is known as the most potent mutagen used for polyploidy (Niazian & Nalousi, 2020). This mutagen prevents the formation of tubulin dimer, by binding to β-tubulin, and eventually prevents the formation of microtubules. The lack of microtubules during mitosis caused the chromosomes not being separated during the cell division proccess, so that the cell contains a doubled number of chromosome sets and a polyploid organism is formed (Chaikam et al., 2019).

Colchicine has been applied to various types of plants to produce polyploid plants such as *Chrysanthemum carinatum* L. (Kushwah et al., 2018), *Trachyspermum ammi* L. (Noori et al., 2017), *Gladiolus grandiflorus.* L. (Manzoor et al., 2018), *Trifolium alexandrinum* L. (El-Naby et al., 2012), and *Crocus sativus* L. (Kashtwari et al., 2021). In pepper, colchicine applications succeeded in obtaining polyploid plants, namely in cayenne pepper (Amanah et al., 2016), curly chilli (Murni, 2010), and *Capsicum annum* L. (Kulkarni & Borse, 2010).

Other than the squash method (Guo, 2012), analysis of plant polyploidy can be carried out quickly and accurately using flow cytometry (Maru et al., 2021). This method allows cell counting and sorting, identification of microorganism, biomarker detection, and protein engineering (Maru et al., 2021). Flow cytometry is a fast an easy technique to figure out plants level of ploidy (Némorin et al., 2013). This technique considered as the most accurate tools to determine plant ploidy level (Parsons et al., 2019). Ploidy from plants is determined by observing data in the form of peak curves or histogram (Fig. 1). Peaks shown on the monitor screen obtained is based on the luminescence captured by the detector on the flow cytometer (Dart et al., 2004).

Through polyploidyzation, diploid plants of katokkon pepper can still be developed for its quality improvement by making tetraploid or hexaploid plant having larger organs with better quality in taste, aroma, texture and aesthetic fruit. This study reported on attempt to produce polyploidy plants of katokkon pepper using colchicine mutagen and flow cytometry analysis.

MATERIALS AND METHODS

Germination and polyploidy induction with colchicine were carried out at the Laboratory of Plant Reproduction Bioscience and Biotechnology, Department of Agronomy, Faculty of Agriculture, Universitas Hasanuddin. The research took place from March 2019 to April 2020. Field planting was carried out in the Experimental Field of The Experimental Field of Agricultural Extension System Vocational High School Santo Paulus Tana Toraja (S 03°04'177" E 119°51526") at an altitude of 763 m above sea level. Katokkon pepper seeds materials used in this study were collected from Tana Toraja. Parental diploid plants were confirmed in advance by flow cytometry analysis. Other materials used were colchicine, $C_{22}H_{25}NO_6$ (Wako), hypochlorite 5.25% (Bayclin®), distilled water, Watman filter paper, fertilizer (NPK 16:16:16, SP36, KCl), pesticides (Decis, Furadan, Dithane M-45 and Score), and plastic mulch. Analytical digital scales (Mettler Toledo) were used for weighing. Analysis of ploidy level was carried out using the Flow Cytometry apparatus (Partec Cy-Flow Space™). Other instruments used were standard laboratory equipment and glasswares such as table top orbital shaker, petri dish, measuring cup, pipette, tweezers, caliper. Field machineries and equipment used were cultivator, hand tractor and sprayer.

Young seedlings of Katokkon pepper (approximately 25 mm long) were immersed in colchicine at concentration of 0%, 0.0125%, 0.025%, 0.05% and 0.1% (w/v) for 4.5 hours on orbital shaker (150 rpm). Preliminary data on immersion period of seedling in distilled water at 1.5, 3.0 and 4.5 hours has shown that 4.5 hours immersion time was applicable (data not shown). After treatment, seedlings were transferred to planting media in seedling trays (36 wells) for one month and then transplanted to polybags (Ø75 mm) for one month before transferred in the field.

Morphological parameters were observed every two weeks. Flow cytometry analysis for the level of ploidy was carried out on young leaves using Partec Cy-Flow Space™. Each sample of young leaves (0.5 x 0.5 cm) was placed in a petri dish. Cut leaves were given extracting buffer Cystain Pi as much as 250 µl and then chopped finely with a razor blade adequately for ±60 seconds. The extract was filtered on a sample tube filter to obtain about 0.2 µl of filtrate. A total of 800 µl of propidium iodide dye was added into the sample tube. The sample tube was placed under the suction needle of the Partec Cy-Flow Space™ machine and pushed upward manually so that the needle could draw up the filtrate into the system for analysis. Readings of the ploidy level could be obtained directly and is shown on a computer screen in graphic form of histogram.

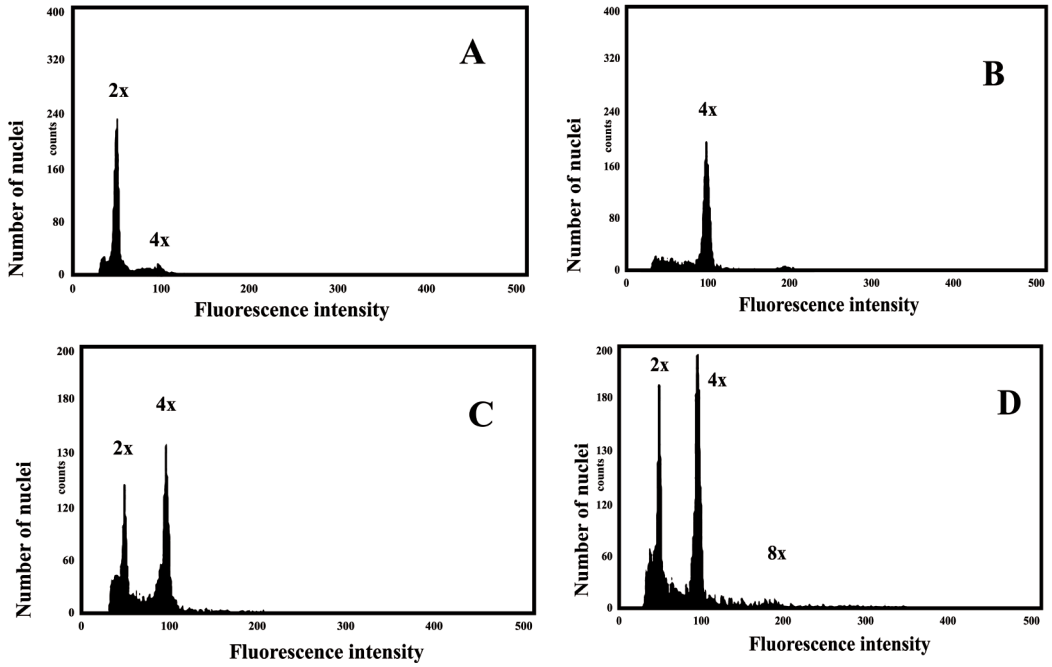

Fig. 1. Histogram result of flow cytometry analysis of *Zizyphus jujuba* Mill plant. cv. Zhanhua showing diploid (2n) (A), tetraploid (4n) (B), mixoploid (2n, 4n) (C) and mixoploid (2n, 4n, 8n) (D) (Gu et al., 2005)

RESULTS AND DISCUSSION

The results of flow cytometry analysis showed that polyploidy induction using immersion of colchicine for 4.5 hours at the levels of 0.025%, 0.05% and 0.10% succeeded in obtaining mixoploid plants (2n, 4n), while in the control (0.0000% colchicine) and 0.0125% colchicine treatment the ploidy level did not change. The results of the flow cytometry analysis were presented in Table 1.

This study did not obtain tetraploid Katokkon pepper plant, which is probably due to the lack time of immersion because at the same immersion time but with higher colchicine treatment (concentration of 0.025-0.100%) the effect of polyploidization was thought to be appropriate. The concentration and duration of colchicine treatment are interdependent and crucial for the success of polyploidization induction (Eng & Ho, 2019). Nevertheless, the increase of colchicine concentration decrease survival rate of the explant used (Zhou, Zeng, & Yan, 2017). There is an inverse relationship between AMA concentration and treatment duration. The desired result can be obtained by using high concentrations of AMA combined with short durations of treatment or with low concentrations of AMA and long durations of treatment (He et al., 2016). The reported preliminary research has shown also higher concentrations of 0.25% – 1% colchicine at 1.5 hours – 4.5 hours immersion did not give good results on Katokkon pepper (Kasmiati et al., 2020). This is because colchicine is highly toxic to plants, therefore, low doses with a long exposure period are considered reliable to reduce its toxic effect and increase polyploidy (Sajjad et al., 2013). Ranney (2006) also suggested the use of colchicine at the lowest possible concentration. The use of colchicine to induce several plants is relatively easy, but there are still limitations in the development of effective protocols for inducing polyploidy and basically every plant has a different response depending on the type and organ being treated (Azmi et al., 2016). The right concentration of colchicine and the duration of immersion are critical to induce polyploidy. If the duration of immersion is low it cannot induce the plant to become polyploid and vice versa. If the duration is too long it can cause the death of the plant (Limera et al., 2016). In this study it was recommended that the immersion time should be increased.

The ploidy level of the plant can be determined by observing the data from the formation of a peak curve or histogram of the luminescence captured by the detector on the flow cytometer (Dart et al., 2004). Fig. 2 (A) showed histogram peaks at channel 200 which indicated that the analyzed plants were diploid (2n), while Fig. 2 (B) showed histogram peaks at channels 200 and 400 which indicated that the analyzed plants were mixoploid (2n, 4n). Fig. 2 (B) showed that the histogram peak on channel 200 was higher than on channel 400 which indicated that there were more diploid (2n) cells than tetraploid (4n) cells.

Koutoulis, Roy & Price (2005) divide mixoploid plants into three classes, grade 1 for mixoploid plants with higher diploids than tetraploids, grade 2 for mixoploid plants where diploid and tetraploid are the same and grade 3 for mixoploid plants with higher tetraploids than diploids. The results of this study were in grade 1. The same results were also obtained by Tammu et al. (2021) in that Katokkon pepper seeds induced by colchicine at a concentration of 0.025% - 0.100% for 24 hours immersion resulted in grade 1 mixoploid (2n, 4n) plants.

Table 1. The induced ploidy level of katokkon pepper after colchicine treatments based on of flow cytometry analysis

Colchicine (%)	Index	Cell Count Mean	CV (%)	Chi-Square	Category
0.0000 (Control)	3.874	199.75	23.79	1.62	Diploid (2n)
0.0125	2.572	179.40	18.45	1.19	Diploid (2n)
0.0250	1.700	203.78	9.60	2.46	Mixoploid (2n, 4n)
	3.170	379.97	18.25	2.46	
0.0500	1.937	181.55	11.81	1.66	Mixoploid (2n, 4n)
	3.757	352.05	16.43	1.66	
0.1000	1.331	194.00	7.88	2.75	Mixoploid (2n, 4n)
	2.572	374.77	12.44	2.75	

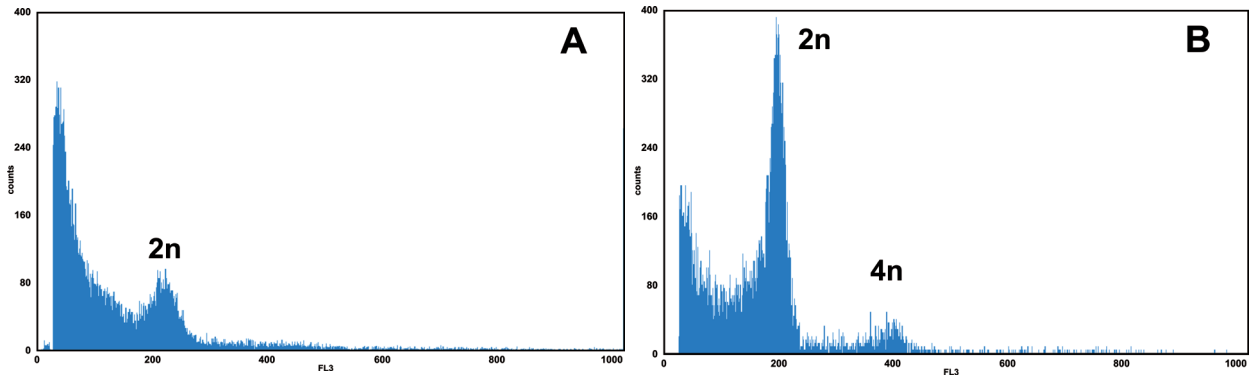

Fig. 2. Histogram of the results of the flow cytometry analysis of Katokkon pepper showing diploid (2n) (A) and mixoploid (2n, 4n) (B)

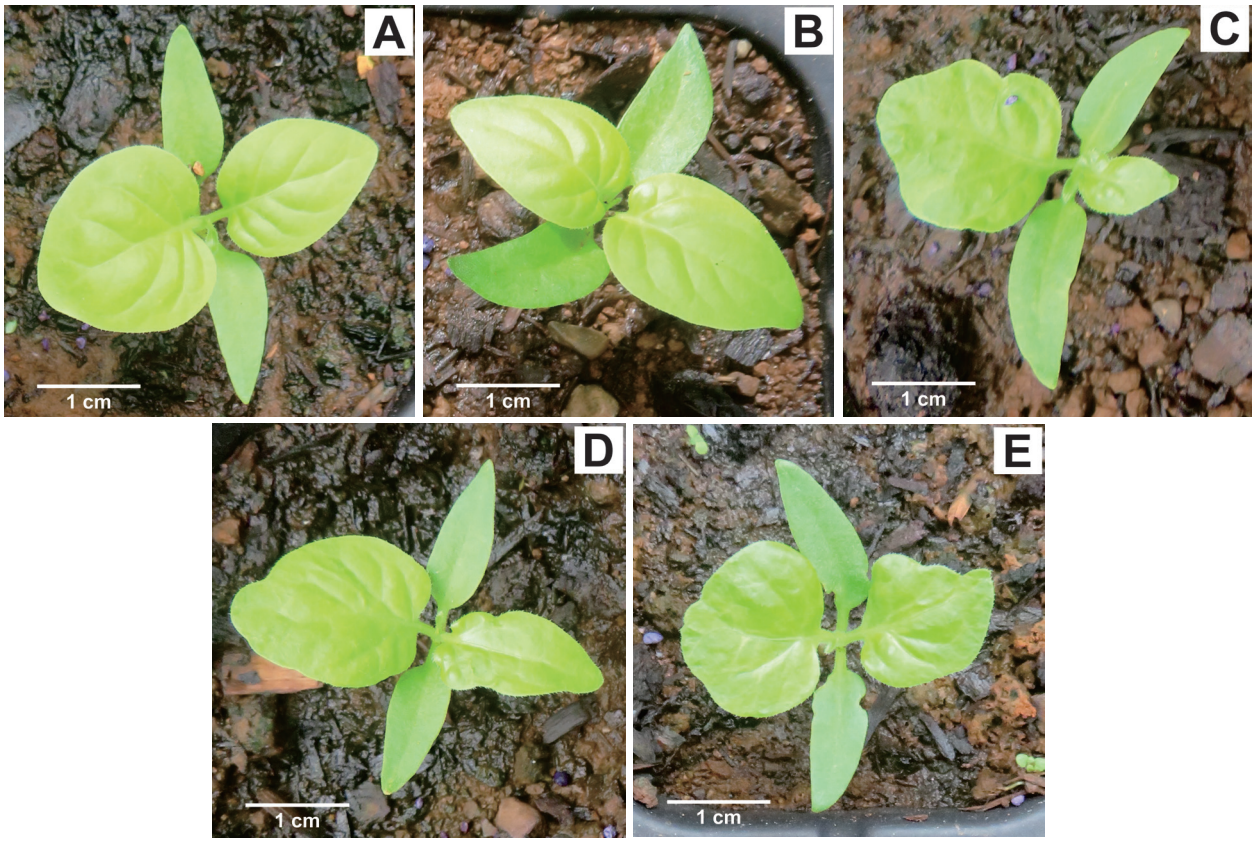

Fig. 3. The appearance of Katokkon pepper's first two leaves after colchicine treatments: A. 0%; B. 0.0125%; C. 0.025%; D. 0.050%; E. 0.100%. The first two leaves become wrinkled as shown in picture C, D and E

Based on the variance on the morphology of Katokkon pepper, it showed that the colchicine concentration treatment had no significant effect on plant height, stem diameter, leaf length, leaf width, leaf thickness, flowering age, fruit stalk length, fruit length, fruit diameter, fruit thickness, fruit weight and number of fruits per plant (data not shown). Morphologically, The mixoploid plants had no significant differences with diploid plants (Nagat, Kamla & Hoda, 2020). Several studies with longer immersion have succeeded in obtaining tetraploid plants with significant differences in morphology. Colchicine immersion for 3 days was able to attain tetraploid *Lychnis senno* Siebold et Zucc. plants with higher plant height than control plants (diploid) (Chen, Wang & Zhao, 2006). However, colchicine concentration at 0.0125% - 0.05% for 24 hours immersion and 48 hours can inhibit flowering in Jimsonweed (*Datura stramonium* L.) (Amiri et al., 2010).

This study found that the first two leaves of mixoploid plants katokkon pepper obtained from of 0.025%, 0.050% and 0.100% (C, D, E) colchicine concentration treatments had visual differences compared to diploid Katokkon pepper. The first two leaves of mixoploid plant are narrower and irregular in shape compared to control plants 0% and 0.0125% colchicine concentration treatments (A, B) which were diploid (Fig. 3). Mixoploid Katokkon pepper plant had narrowing of the leaves and an irregular shape like diploid Katokkon pepper (Sjahril et al., 2021). In this study there was an improvement in the morphology of Katokkon pepper plants, even though there was no significant difference in adult Katokkon plants. Roy, Leggett & Koutoulis (2001) corroborated our result where at first the leaves of mixoploid plants were narrower and shorter than later developed leaves.

CONCLUSION

Soaking diploid Katokkon pepper sprouts (2n=24) for 4.5 hours at colchicine concentrations of 0.025%, 0.05% and 0.10% induced the formation of mixoploid Katokkon pepper (2n=24, 4n=48) during the first juvenile phase with two young leaves, but the differences in leaf morphology did not continue until the plants matured. However, the immersion time was probably the critical point that have reduced the probability to obtain tetraploid plant (4n). Morphologically, the adult diploid Katokkon plants did not show significant differences from the adult mixoploid Katokkon plants. The resulting mixoploid Katokkon plant was a grade 1 mixoploid plant, which has higher diploid cells than its tetraploid cells.

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