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Effect of induced polyploidy on plant growth, chlorophyll and flavonoid content of *Artemisia cina*

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Abstract. *Artemisia cina* is one of a member of genus *Artemisia* that has potential as a medicinal plant. However, the levels of *Artemisia* medicinal bioactive compounds are very low. Polyploidization is an alternative method that can enhance of growth and secondary metabolite productions of plants. The aims of this research were to determine the effect of polyploid induction using colchicine and plant growth regulator toward plant growth, the chlorophyll, kaemferol and quercetin contents of *A. cina*. Four different *A. cina* used in this research consisted of two diploid genotypes (TWN and KJT) and two polyploid genotypes (J and M). Induction of mutant polyploid was conducted using colchicine and combination of plant growth regulator benziladenyl (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The measured plant growth parameters were dry weight, leaf area, and plant height. The chlorophyll content of leaves was determined spectrophotometrically, and flavonoid content determined using HPLC. The result showed that the polyploid genotype (M) significantly decreased leaf and root growth compared to the KJT and TWN. In *A. cina* plants, polyploidization using colchicine is more effective in increasing the biomass than using combination plant growth regulator BA and 2,4-D. The flavonoid content of KJT was the lowest, and significantly different compared to the other plants. This study provides new information about the effect of polyploid on growth and flavonoid content in *A. cina*. This can be useful information to develop *A. cina* to become a medicinal plant.

Key words: *Artemisia cina*, polyploid, colchicine, shoot culture, plant growth regulator

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INTRODUCTION

Artemisia cina is a medicinal plant belongs to Compositae family that producing artemisinin as a major secondary metabolite compound. Artemisinin is used as antimalaria and effective against drug-resistant malaria. Artemisinin and its derivatives also have the potential for the therapy of several infectious diseases such as leishmaniasis, hepatitis-B, and against cancer cell lines (Romero et al. 2005; Sen et al. 2007; Li et al. 2016). In addition to artemisinin, flavonoid such as quercetin and kaempferol compounds contained in *A. cina* is a secondary metabolite that is also potential for the development of herbal medicines. Kaempferol and quercetin have been reported to have several pharmacological properties as antimicrobial, anti-inflammatory, antioxidant, antitumor, cardioprotection, neuroprotection, antidiabetic, anti-hepatitis and also are being applied in cancer chemotherapy (Kelly, 2011; Cheng et al. 2015; Krishnadhas et al. 2016; Imran et al. 2019).

In general, plants naturally produce bioactive compounds in low concentrations. Biosynthesis of bioactive compounds is restricted to a single organ, such as leaves, roots, flowers or fruits, but accumulation and processing of the corresponding product can be detected in several other plant tissues (Giri & Lakshmi, 2000). The growth of plant organ and biosynthesis of many bioactive compounds is easily affected by environmental factors such as nutrient availability, stress factors, light, growth regulators and alteration ploidy (De Jesus, 2003). Artificial polyploidy is an alternative method that can enhance the vegetative and reproductive growth of plant organs and also plant biomass. The gene doses in the polyploid mutant were duplicated and will affect not only plant growth and biomass, but also plant activity enzymes, isozyme diversity, and responsible for improving secondary metabolite production (De Jesus, 2003; Kim et al. 2004). In the case of medicinal plant, polyploid is more helpful and affordable due to the enhanced biomass and biosynthesis of bioactive compounds (Salma et al. 2017). The enhancement of plant biomass and the secondary metabolite productions via induced polyploidy has been reported by several researchers. Corneillie et al. (2019) reported that in *Arabidopsis* polyploid mutants, the dry weight of the stem was doubled and saccharification yield was significantly increased. The polyploid adventitious roots play a role in enhancing the production of ginsenoside and biomass in *Panax ginseng* (Kim et al. 2004). The induced polyploid medicinal orchid (*Anoectochilus formosanus*) was reported significantly enhance on

various growth parameters including dry weight, fresh weight, shoot length, root length, leaf width, the size of the stoma, and the number of chloroplasts per stoma. The tetraploid medicinal orchid plants also produced significantly higher contents of total flavonoid and gastrodin than the diploid plants (Chung et al. 2017).

The genetic improvement of *Artemisia* through artificial polyploidization has been done on the *A. annua* plant by several researchers. De Jesus (2003) reported that tetraploid clones of *A. annua* resulting from colchicine-treated hairy root culture showed major differences in growth and development and yield artemisinin 2-5 times higher than diploid clones. Banyai et al (2010) reported that *Artemisia annua* were successfully induced to be tetraploid plants by treating leaves tissue culture with 0.1% colchicine. The tetraploid of *A. annua* plants showed larger sizes of the root system, stomata, and glandular secretory trichomes and yield artemisinin 1.5 times, whereas leaf size in tetraploid was smaller but thicker than diploid plant. Polyploid plantlet of *A. annua* was also reported to have a bigger size of stomata and produced more artemisinin than diploid plantlet (Rahman et al. 2016). *A. cina* is another species member of genus *Artemisia* that also has potential as a medicinal plant and source of bioactive compound (anticancer, antibacterial and antifungal). Moreover, the effect of polyploidization on shoot culture of *A. cina* using plant growth regulator treatment toward plant growth has also been reported by Herawati et al. (2015).

The aims of this research were to determine the effect of induced polyploidization using colchicine and plant growth regulator toward plant growth, the chlorophyll and content of quercetin and kaempferol on *A. cina*. This research was expected to provide additional information that reinforces the importance of polyploidization in enhancing the growth and production of bioactive compounds of medicinal plants, especially *A. cina*.

METHODS

The research was conducted using a Completely Randomized Design. The four genotypes of *A. cina* were used in this research. The two diploid genotypes of *A. cina* namely TWN and KJT were obtained from The Center for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT), Tawangmangu, Central Java, Indonesia. KJT genotypes were the diploid plants of *A. cina* that were obtained through shoot culture. While TWN genotype was harvested directly from the plantation land The plants were identified at the Herbarium Bogoriense, Biology Research Center, Bogor, Indonesia. Polyploid genotypes J and M were considered as mutant polyploid putative 1 (P1).

Propagation of Explants

The medium for shoot culture was MS medium containing 10 mg L-1 of kinetin and 1 mg L-1 of NAA (1-Napthalaeneacetic acid). The shoot explants used were from *in vitro* culture. Each shoot explants had consisted of three stem segments. The segments from two until six were planted in MS medium containing 10 mg L-1 of kinetin and 1 mg L-1 of NAA. Every four weeks, the explants were sub-cultured by cutting off three stem segments and replanting them in the new MS medium like before.

Induction of Polyploidy

Colchicine at the concentration of 100 mg/l was used to induce J mutant polyploid and combination of 2 mg/ml of BA (Benziladenyl) and 3 mg/ml of 2,4 D was to induce M mutant polyploid of *A. cina*. In the first step, the identical explants were selected. The transfer of explants to polyploid induction media was done two weeks after the final propagation subculture stage followed by incubation until the plantlet formed.

Acclimatization of Plantlet

The plantlets of *A. cina* were transferred into plastic pots containing husk charcoal as a medium, then they were incubated at room temperature for 14 days. The plantlets were fertilized using 2 ml of NPK liquid fertilizer. At the end of the incubation period, the plantlets were transferred to the glasshouse for 14 days. After that process, the plantlet was transferred into a 20 cm diameter polybag with a mixture of garden soil medium and manure (1:1).

Determination of Shoot and Root Dry Weight

The plant growth were determined at the end of the acclimatization period through its shoot and root dry weights. The shoots were harvested at the soil surface level, and the roots were dried without direct sunlight until they wither. Drying the shoots and roots was conducted by heating in a 40^{o}C oven until it reached a constant weight.

Determination of Leaf Area

The leaf area was measured using Leaf Area Meter Mark 2 type (Delta T, Burwell Cambridge, England). The leaves measured were the completely unfolded leaf and in the 3, 5, 8 and 10 sections.

Determination of Chlorophyll Content

The chlorophyll content of leaves was determined using Dimethyl sulfoxide (DMSO) as a solvent. The sample used was the third segment with completely

unfolded leaves. As much as 0.04 grams of leaves were sliced into small pieces and added with 5 mL DMSO and incubated in the dark at room temperature for 48 hours. At the end of incubation, the mixtures were filtered with filter paper. The absorbance of the mixture was measured using a spectrophotometer (UV mini-1240, UV VIS Spectrophotometer, Shimadzu) at a wavelength of 649 and 665 nm. The chlorophyll content was calculated using the following equation:

Chlorophyll a µg/mL = (12:19 x A665) - (3:45 x A649)

Chlorophyll b µg/mL = (21.99 x A649) - (5:32 x A665)

Total Chlorophyll µg/mL = (18:54 x A649) + (6.87 x A665)

Determination of Flavonoid Content

The flavonoid content (kaempferol and quercetin) was analyzed using High-Performance Liquid Chromatography (HPLC) (Tokusoglu et al. 2003). For separation of quercetin and kaempferol, the HPLC conditions were using the Chromosorb Column RP C18 (150 x 5 mm id - KNAUER), H_3PO_4 0.1%: acetonitrile (60:40) as the mobile phase, the flow rate 1 ml/min, the injection volume of 20 µl, ambient temperature, and using UV 370 nm detector. The quantitation of the amounts of the quercetin and kaempferol in *A. cina* sample extracts were determined by using a standard curve of quercetin and kaempferol pure compounds calibration .

Data analysis

The analysis data were conducted statistically using a one-way analysis of variance (ANOVA) to evaluate differences among all genotypes in plant growth and content of chlorophyll and flavonoid. Mean separation was performed using the Tukey test with a P≤0.05 probability level.

RESULTS AND DISCUSSION

The organ growth (root dry weight, shoot dry weight and leaf area) of the four tested *A. cina* genotypes, did not show totally significant difference between diploid (KJT and TWN genotypes) and polyploid (J genotype), except for mutant polyploid genotype (M) (Table 1). The root and shoot dry weight of J was higher than the KJT and TWN. The leaf and root growth of M genotype (polyploid) experienced a significant decrease, shown by having the lowest growth in terms of root dry weight, shoot dry weight and leaf area compared to three others (KJT, TWN and J genotypes). The highest dry weight of roots and shoots was found in J, while the highest leaf area was found in KJT.

Table 1. The growth of the diploid (KJT and TWN) and polyploid mutant (J and M) of *A. cina*

Genotype	Level of ploidy	Root dry weight (g)	Shoot dry weight (g)	Leaf area (cm2)
KJT	2x=2n	12.63± 1.19 ab	9.21± 0.83 b	33.55 ± 0.71 a
TWN	2x=2n	14.48± 2.64 a	10.49± 0.12 b	32.87 ± 0.84 a
J	2x=4n	16.84± 3.97 a	13.07± 0.51 a	33.27 ± 1.39 a
M	2x=4n	6.94± 1.30 b	7.22± 0.72 c	29.93 ± 0.66 b

Results are the mean ± standard deviation (SD) of three replications. Values with the same letter in a column are not significantly different (p > 0.05).

Table 1 also shows that colchicine mutagen was more able to increase leaf and root growth compared to the combination of the plant growth regulator of BA and 2,4-D. Artificial polyploid induction in *A. cina* plants using different mutagens shows different growth responses, as seen in leaf growth (Figure 1). Ade and Rai (2010) reported that colchicine has the ability to induce polyploidy and can be used to convert sterile hybrids into fertile. The colchicine mutagen has been shown to induce polyploidy in various plants, including *Scutellaria baicalensis* (Gao et al. 2002), *Panax ginseng* (Kim et al. 2004), *Artemisia annua* (De Jesus, 2003; Banyai et al. 2010; Rahman et al. 2010) 2017), *Codonopsis lanceolata* (Kwon et al. 2016), *Glycyrrhiza glabra* var. *glandulifera* (Bernard et al. 2012), and *Artemisia cina* (Herawati et al. 2015).

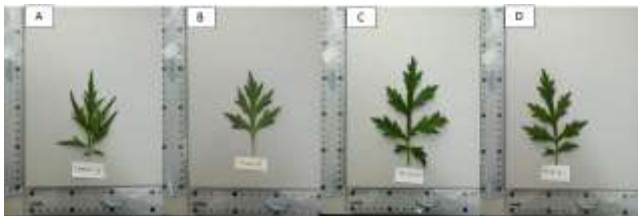


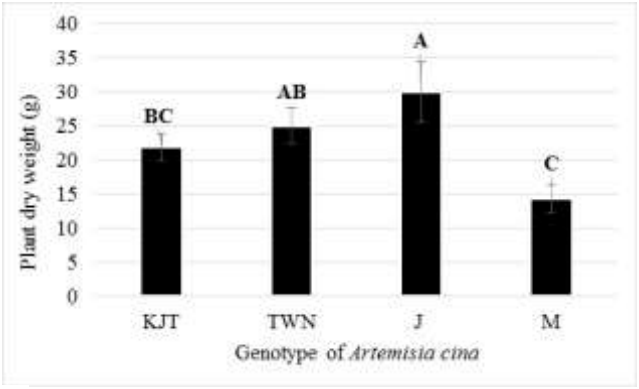
Figure 1. The different leaves growth of the four genotypes of *A. cina* plants. (A) KJT, (B) TWN, (C) J and (D) M.

Although colchicine treatment in this study can induce polyploidy in *A. cina* and also increase leaf and root growth, the results are not significant. This can be caused by several factors. The effect of colchicine in polyploidization is different regarding its concentration, method, duration of treatment, and also genetic factors of the treated plants (Aina et al. 2012; Dhooghe et al. 2011; Moghbel et al. 2015). When compared to colchicine, the combination of BA and

2,4-D had no significant effect on the formation of *A. cin*a polyploid mutants. In general, the combination of BA and 2,4-D plays a role in stimulating the formation of callus and plantlets in some plants including soybeans (Kristanti et al., 2013), *gendarussa* (Wahyuni et al., 2017), and pineapple (Zulkarnain et al., 2018).

The growth of diploid and polyploid of *A. cina* is also determined based on plant dry weight (Figure 2A) and plant height (Figure 2B). The plant dry weight of the two diploid plants was not significantly different either the plant that was directly harvested from the land (TWN) or the one obtained from shoot culture (KJT). Differ from the diploid plants, the plant dry weight of the two polyploidy plants were significantly different from each other. The plant weight of J was higher (29.91 ± 4.46 g) than the M (14.16 ± 2.02 g). The plant height was significantly different among four tested *A. cina* genotypes. In this study, artificial polyploidization using mutagen both in the form of colchicine and a combination of plant growth regulators (BA and 2,4D) on *A. cina* reduced plant height compared to diploid plants (wild type). The lowest plant height was found in genotype M polyploid mutants at 33.13 ± 1.00 cm, and the highest plant height was found in wild type plant (TWN genotype) at 58.67 ± 0.61 cm.

A.



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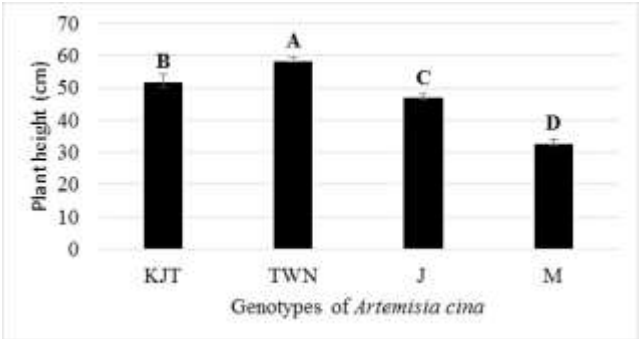


Figure 2. The plant dry weight (A) and plant height (B) of the diploid plant (KJT and TWN) and polyploid mutant of *A. cina* (J and M).

The increased plant growth due to polyploidization has been also reported by many researchers. A two-fold increase in stem dry weight in *Arabidopsis* polyploid mutants compared to the wild type was reported by Corneillie et al. (2019). The increased ginseng plant biomass due to polyploid induction was also reported by Kim et al. (2004). The induced polyploid medicinal orchid (*Anoectochilus formosanus*) was also reported significantly enhanced on various growth parameters including dry weight, fresh weight, shoot length, root length, and leaf width (Chung et al. 2017). However, the decrease in plant height shown on polyploid *A. cina* plants in this study was related to duplicated gene doses in polyploid mutants that would affect not only growth but also enzyme activity. The slow and stunted growth of *A. cina* polyploid mutant was one of the successful indicators of polyploidy induction. The decrease in plant height of *A. cina* polyploid may be due to physio-chemical disturbance of cell and induction of polyploidy (Mazoor et al. 2018).

Artificial polyploid induction significantly affects the total chlorophyll content in *A. cina* (Figure 3). The total chlorophyll content of polyploid plants (J and M) is lower than diploid plants (KJT and TWN). However, among of the diploid plants and mutant polyploid plants, the chlorophyll content were not significantly different from each other. The chlorophyll content of KJT, TWN, J and M genotypes of *A. cina* respectively are as follow, 3.00 ± 0.10, 2.85 ± 0.17, 2.16 ± 0.08, and 2.25 ± 0.06 µg/ml. The reduction in total chlorophyll content of the *A. cina* polyploid mutant in this study is not in line with the results of studies reported by several previous researchers. Polyploid induction is reported to increase chlorophyll content in some plants including *Acacia mearnsii* (Mathura et al. 2006), *Pogostemon cablin* (Wu & Li, 2013), *Stevia reboudiana* (Zhang et al. 2018) and *Artemisia annua* (Yunus et al. 2018). However, Manzoor et al. (2018) reported that colchicine-induced polyploid in *Gladiolus grandiflorus* was decreased the chlorophyll content, this result supports the result of this study. According to Xu et al. (2010), the reducing of total chlorophyll content in induced tetraploid plants occurred due to structural modifications such as disintegration in the lamellar or thylakoid membrane of chloroplast which affect the synthesis of chlorophyll.

Polyploidy is considered a method of increasing bioactive compound potential in plants. In this study, the effect of induced polyploidization using colchicine and a combination of plant growth regulators (BA and 2,4-D) on the content of flavonoids in the form of quercetin and kaemferol compounds in *A. cina* was also determined.

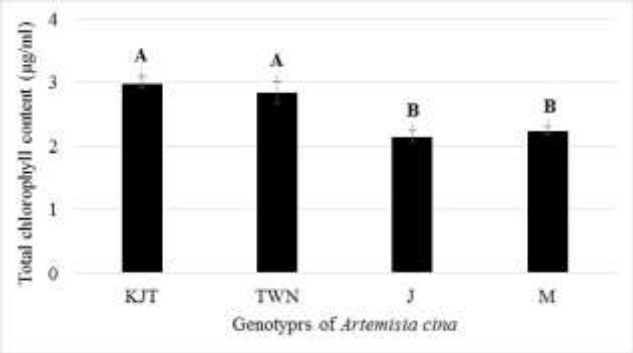
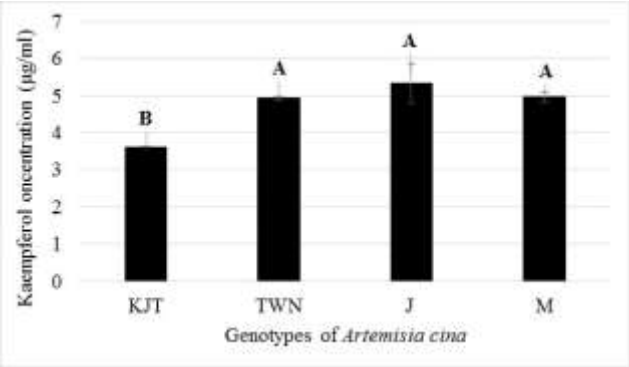


Figure 3. The total chlorophyll content of the diploid plant (KJT and TWN) and polyploid mutant (J and M) of *A. cina*

The results showed that induced polyploid in *A. cina* significantly increased quercetin and kaemferol contents. The quercetin and kaempferol content of KJT shows the lowest value, and significantly different from another diploid plant (TWN) and polyploid plants (J and M) (Figure 4). The content of quercetin among the TWN, J and M was not significantly different. The kaempferol content of KJT, TWN, J and M were 3.61 ± 0.01, 4.92 ± 0.05, 5.33 ± 0.54, and 4.97 ± 0.13 µg/ml, respectively (Figure 4A). The lowest quercetin content was found in KJT by 39.81 ± 0.41 µg/ml, and the highest was found in M by 52.92 ± 1.09 µg/ml (Figure 4B).

A.



B.

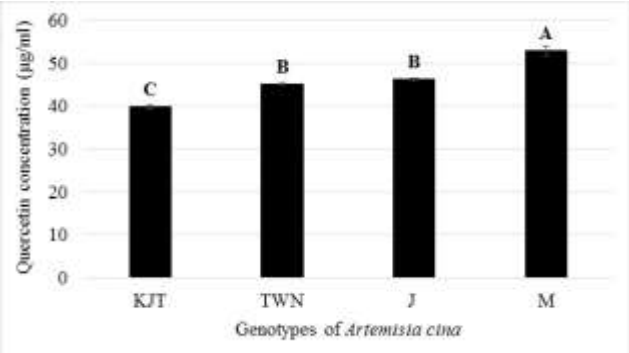


Figure 4. The kaempferol (A) and quercetin (B) contents of the diploid plants (KJT and TWN genotypes) and polyploid mutants (J and M genotypes) of *A. cina*

The increase in flavonoid content (quercetin and kaemferol) in induced polyploid *A. cina* in this study is supported by Chung et al. (2017) who also reported an increase in flavonoid content in the medicinal orchid plant (*Anoectochilus formosanus*). According to Parida and Mishra (2015), polyploidization can induce changes in the quality and quantity of secondary metabolites such as phenols, terpenoid, anthocyanin, and flavonoid, and higher ploidy levels are clearly reflected in higher accumulation of flavonoids in plants. Some induced polyploidy plants are reported to produce more flavonoids or terpenoids than diploid wild types, including in *Artemisia annua* (De Jesus, 2003) and *Scutellaria baicalensis* (Gao et al. 2002). According to Salma et al. (2017), polyploid plays a major role and is more prospective than diploid in the development of medicinal plants because the increase in the number of alleles in polyploid can mask lethal recessive mutations, the formation of heterosis (allopolyploid and autopolyploid) which can confer transgressive performance and hybrid vigor, the doubling of alleles might be the cause of various new functions to increase resistance to various diseases and environmental changes.

The research on artificial polyploidization to increase the biomass and content of artemisinin and other secondary metabolites has been carried out in member of *Artemisia*, but it has not been much studied on *A. cina*. The finding of appropriate and effective methods to induce polyploid and increase flavonoid content in *A. cina* through this research is important to support the development of *A. cina* as one of the potential medicinal plants.

CONCLUSION

The growth of organ, biomass and chlorophyll content of *A. cina* not totally significantly different between polyploid and diploid plants. The polyploid genotype (M) significantly decreased leaf and root growth compared to the KJT and TWN. The induced polyploid in *A. cina* significantly increased quercetin and kaemferol contents compared to diploid plants. Colchicine is the most effective to induce polyploidy in *A. cina*. Colchicine mutagen shows higher leaf and root growth compared to the combination of the plant growth regulator of BA and 2,4-D.

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