Changing of Morphological, Anatomical, Cytological Characteristic and Artemisinin Content in *Artemisia cina* by Colchicine Treatment

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Abstract. The induction of artificial polyploidy has been considered a valuable tool that can improve many plants' genetics and change their morphological, anatomical, and physiological characteristics. *Artemisia cina* plants' polyploidy is induced through shoot culture to increase artemisinin content. This research consisted of stages of explant propagation, polyploid induction, and acclimatization. The colchicine concentrations of 100 mg/L, 150 mg/L, 200 mg/L and application times of 24 h, 48 h, 72 h, and 96 h were the treatments given. Descriptive analysis was used to analyze the chromosome determination. The number of alive explants after induction was analyzed at a 5% level using analysis of variant (ANOVA). The paired T-test was also conducted at a 5% level to determine the significance of the plant height, leaf area, stomata, chlorophyll content, glandular trichomes density, and glandular trichomes size and content of artemisinin between the two treatments on the observed data. In Colchicines, treatment with concentrations of 100, 150, and 200 mg/L and time application of 24, 48, 72, and 96 h can induce polyploidy on *A. cina.* Colchicines' improved concentration and application time are of lower percentage in living explants. Eight plants are the highest number of polyploidy crops on colchicines treatment of 100 mg/L for 72 h. The resulting ploidy level varies on $2n = 2x = 16$, $2n = 3x = 24$, $2n = 4x = 36$, $2n = 5x = 40$, and $2n = 6x = 48$. The highest level of polyploidy $2n = 4x = 32$ is 20% in the treatment Colchicines 100 mg/L for 72 h. The modal number of chromosome x obtained in this study was = 8. The ploidy level's highest plant height, leaf area, chlorophyll content, and stomata sizes were $2n = 4x = 32$. The number of stomata and glandular trichomes on polyploidy plants was smaller than the diploid plants. Polyploidy plants generally have larger leaves, stomata, and glandular trichome sizes than diploid plants. However, the number of stomata and glandular trichomes is less than in diploid plants. Artemisinin content on polyploidy plants is higher than on diploid plants.

Keywords: *Artemisia cina* · Colchicine · Chromosome · Polyploidy · Ploidy level

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1 Introduction

Artemisia cina is one of the species of genus Artemisia, which contains artemisinin, a bioactive antimalarial substance. Artemisinin is a sesquiterpene lactones with internal peroxides, compounds that are active as antimalarials [1]. In Indonesia, *Artemisia cina* is often abundantly found as weeds in the mountain. The content of artemisinin in *Artemisia cina is* lower than in *Artemisia annua*, ranging from 0.0075%-0.066% [2, 3].

Polyploidy induction is widely recognized as one of the active breeding techniques among various breeding devices. It broadens the genetic base, develops breeding pathways quickly, improves the fertility of interspecific hybrids, and makes viable crosses between genotypes with different levels of ploidy [4]. Improved vigor and better performance are the features that make polyploid plants preferable to their diploid relatives [5]. Polyploidy induction can also increase the production of secondary metabolites in plants.

Developing a suitable method for increasing the secondary metabolites found in these plants, and enhancing their resistance against environmental stress can help to conserve these precious species. The induction of artificial polyploidy has been considered as a valuable tool that can improve many plants' genetics and change the morphological, anatomical, and physiological characteristics [6, 7]. Polyploidy can be induced through sexual polyploidization or somatic doubling (or mitotic) [8]. Chromosome doubling can be induced artificially by using colchicines that inhibit the segregation of chromosomes during cell division and growth-regulating substances. Colchicine changes the number of chromosomes and induces gene mutations in seeds and vegetatively propagated plants [9].

The ability of polyploid plants to live in various habitats and survive in adverse environments, makes them better than diploids. It is because of the addition of alleles that increase their heterozygosity [10]. Polyploid plants increase in cell size due to the addition of additional gene copies. For example, the plant height, leaf length, and stem diameter increase in polyploidy-induced Ajowan plants (*Trachyspermum ammi* L) [11]. This effect on polyploidy is known as the "gigas effect" [5].

The increase of secondary metabolite productions via induced polyploidy has been carried out, such as on like *A. annua* [12, 13] and *Mentha longifolia* [14]. Chromosome doubling is accompanied by striking changes in secondary metabolism and the primary metabolism on various types of plants [15]. Some of these changes include an increase in the overall enzymatic activity, isozyme diversity, and changes in chemical constituents, which can lead into the increasing of production and specific qualitative changes in the biosynthesis of secondary metabolites [13, 15].

Polyploidy plants are known to have larger photosynthetic potentiality than diploid plants. It is shown by the dark green colored of the leaves because they have more giant enormous leaf cells and a higher amount of chlorophyll [16]. The examples were show on *Sophora flavescens* Aiton [17], *Mentha longifolia* [14], *Gentiana triflora* [18] and *Plox subulata* [19], Petunia "Mithcell" [20], the *Bacopa manieri* [21] and *Echinacea purpurea* [22]. Based on the results of previous studies, colchicine treatment was able to induce polyploidy which had an impact on changes in morphological characteristics, anatomy, cytology and the content of active ingredients in plants. The treatment of Colchicine concentration and the period of treatment are expected to have an effect on polyploidy.

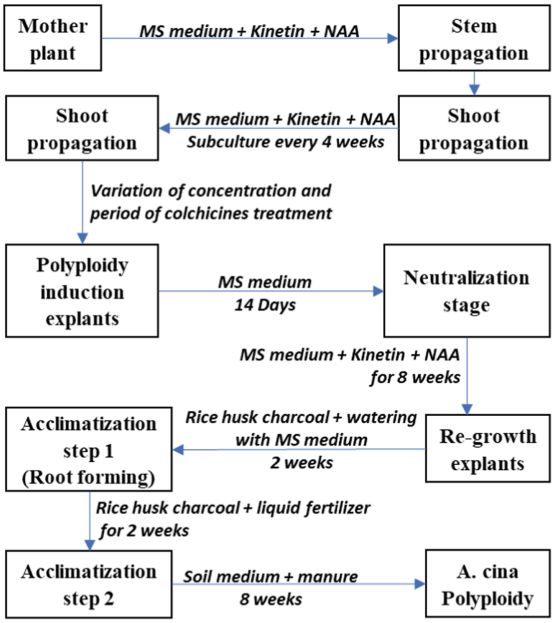

Fig. 1. *A. cina* polyploidy induction process.

2 Materials and methods

This study is a multi-year study conducted from 2014 to 2020. Polyploidy induction research was carried out in the tissue culture laboratory of the Faculty of Agriculture and Business, Satya Wacana Christian University, Salatiga, Indonesia (Fig. 1).

2.1 Explants Propagation

The source of explants in this study was taken from the Center of Medicinal Plant Research Tawangmangu Central Java. The Explants were maintained and propagated in vitro on Murashige - Skoog medium containing salts and vitamins, 20 $g.L^{-1}$ sucrose, 8 $g.L^{-1}$ agar, and plant growth regulator 10 mgL^{-1} kinetin and 1 $mg.L^{-1}$ NAA. The Cultures were maintained at 25 °C with 16 h daylight of 260 $\mu mol^{-1}.m^{-2}$ cool white fluorescent illumination. Stem segments as explants consisting of three segments transplanted in vitro. Planted segments derived from S ect. 2, 3, 4, 5 and 6. Propagation of shoots was done by cutting each segment containing 3 nodes with 2–3 leaves and then were planted in the shoot propagation medium. Subculture was done every 4 weeks by cutting along the three segments and then planting plant it back to the fresh medium. Propagation was done until the required number of explants had been fulfilled for the induced polyploidy actions.

Table 1. Number of explants alive after induction

Time aplication (hour)	Colchicines concentration			Average of time aplication
	100	150	200	
24	17.00	13.67	11.33	14.00 a
48	12.00	8.33	7.67	9.33 b
72	6.67	5.67	0.33	4.22 c
96	4.67	4.33	0.0	3.00 c
Average of colchicines concentration	10.08 p	8.00 q	4.83 r	(-)

Remarks: Mean followed by different letters behind the numbers showed a significant.

2.2 Polyploidy Induction

Induction of polyploidy was done by soaking *Artemisia cina* shoots in a sterile distilled water containing colchicines. Colchicines treatment concentration 100 mg.L^{-1}; 150 mg.L^{-1}; 200 mgL^{-1} and the period of treatment were 24; 48; 72; and 96 h. Explants used were tops in S ect. 2, 3 and 4, with apical buds removed. The Soaking was done using a sterilized Petri dish, then filled with a colchicine solution of appropriate treatment. It was sealed with plastic wrapping, and placed inside a laminar air flow according to the treatment period. After the subsequent immersion period, the explants were transferred to MS medium without growth regulator or neutralization stage. The neutralization stage has been done for 14 days after the explants are transferred to the growth medium. The growth medium used was MS medium supplemented with 10 mg.L^{-1} kinetin and 1 mgL^{-1} NAA. Then, the explants were grown in the growth medium for eight weeks. The Explants of *A. cina* were also cultured in MS medium without plant growth regulator as sources of explants without polyploidy induction.

2.3 Acclimatization

In this study, the explants of *Artemisia cina* have not taken from a root induction stage, but eight weeks after being in the growth medium. Then, the acclimatization was conducted afterward. This action was done because it took a long time on a preliminary test to bring up the explants' roots, besides despite the high mortality rate at the root induction phase. The Acclimatization was conducted for 12 weeks with the following stages: the plantlets were removed from the media to the rice husk charcoal then placed it on a plastic cup. Watering was done with 0 MS medium and placed in the laboratory for two weeks. At this stage, the roots were already formed. The next stage was placing the plantlets in the greenhouse were when it still grew on rice husk charcoal - and fertilized with a liquid fertilizer with a concentration of 2 mL.L^{-1} for two weeks. At this stage, the chromosome determination has been done to determine the ploidy level of the induction process. Two weeks later, the plantlets were transferred to soil mixed with manure at 1:1 ratio and put in the polybag with a diameter of 30 cm for eight weeks. NPK fertilization has also been given at this stage. Finally, an observation involving leaf area, plant height, stomata

density, length and width of stomata, analysis of chlorophyll content, and artemisinin content was conducted.

2.4 Chromosome Determination

After two weeks of growth in the greenhouse, the chromosome quantities of the plantlet were observed. One cm length of the root tip was cut. The root tips were treated with iced water at 4 °C for 24 h. Then, the plant's root tips were fixed into Carnoy's solution/fixation solution (3 ethanol: 1 glacial acetic acid) and stored in the refrigerator at 4 °C for at least 1 h. The root tips were rinsed with distilled water and hydrolyzed using HCl 1 N for 15 min at 60 °C in a water bath. After that, the root tips were stained with Fuchsin for 20 min and replaced in an objective glass. The chromosomes were observed using an Olympus Microscope connected to Optilab photo-microscope.

2.5 Analysis of Chlorophyll Content of Leaves

The chlorophyll content of leaves was extracted using DMSO as solute [23] that has been modified. Leaf analysis has been done to the completely unfolded ones in the third section. First, measure 0.04 g sample of leaves, slice them into small pieces, add 5 ml of DMSO, and incubate in the dark at room temperature for 48 h. Filter it with filter paper, then measure the absorbance value with Spectrophotometer (UV mini-1240, UV VIS Spectrophotometer, Shimadzu) at a wavelength of 480, 649, and 665 nm [24]. Chlorophyll content expressed in $mg.g^{-1}$ fresh weight.

$$\text{Chlorophyll a} = ((12/19) \times A_{665}) - ((3/45) \times A_{649}) \quad (1)$$

$$\text{Chlorophyll b} = (21.99 \times A_{649}) - ((5/32) \times A_{665}) \quad (2)$$

$$\text{Total Chlorophyll} = ((18/54) \times A_{649}) + (6.87 \times A_{665}) \quad (3)$$

2.6 Leaf Area Measurement

Leaf area is one measure of plant growth measurement of leaf area to determine the difference in growth between induced polyploid and diploid plants. The leaf area measurement has done with a Leaf Area Meter Mark 2 type, Delta T, Burwell Cambridge, England. The leaves used in the leaf area measurement were wholly unfolded and located in S ect. 3, 5, 8 and 10.

2.7 Stomata Observation

Observation of stomata has been done in 60 days after planting. The leaves used for the observation must have opened correctly on the fourth segment. The epidermal surfaces of abaxial leaves were smeared with non-coloured nail polish and covered with transparent plastic tapes. The tapes were taken off and placed on an object-glass. The stomata length, width, and density were observed using an Olympus microscope with photomicroscope Optilab at 10- and 40-times magnification.

Table 2. Effect of colchicines concentration on polyploid induction of *Artemesia cina*

Colchicine treatment	Time application	Number of plants continuing to grow in green house	Number of diploid plants	Number of polyploid plants	Ratio of poliploid plants (%)
100 mg/l	24 h	21	18	3	14.3
	48 h	14	8	6	42.9
	72 h	20	12	8	40.0
	96 h	13	11	2	15.4
150 mg/l	24 h	7	4	3	42.9
	48 h	3	3	0	0.0
	72 h	7	4	3	42.9
	96 h	10	8	2	20.0
200 mg/l	24 h	6	3	3	50
	48 h	16	13	3	18.8
	72 h	1	0	1	100.0
	96 h	0	0	0	0.0

2.8 Glandular Trichome Observation

Observation of the glandular trichome has been done using the epidermis surface of leaves smeared with non-coloured nail polish. It was taped on tape, then peeled and placed on a microscope object, then calculated the size (length and width) and density or number of trichomes in the area. The trichome gland's size (the length and width) has been measured with the Optilab camera USB and Image raster program. The unit for glandular trichome size is μm, and the unit for glandular trichome density is μm2.

2.9 Percentage of Polyploidy Obtained

The percentage of polyploidy obtained was counted by counting the polyploidy obtained in every treatment divided by the total number of polyploidy occurrences multiplied by 100%.

2.10 Artemisinin Content Analysis by High-Performance Liquid Chromatography

An Analysis of artemisinin content has performed 12 weeks after plantlets were transferred to the soil. The Shoots were cut then dried in an oven at a temperature of 40 °C until dry. Furthermore, artemisinin content was analysed using HPLC [25], 100 mg dry weight of the sample was then extracted with 2 ml of Toluene and filtered using filter

Table 3. Percentage (%) of polyploidy level on *Artemesia cina*

Colchicine treatment	Time aplication	Triploid (2n = 24)	Tetraploid (2n = 32)	Pentaploid (2n = 40)	Heksaploid (2n = 48)
100 mg/l	24 h	2.9	2.9	2.9	0.0
	48 h	8.6	8.6	0.0	0.0
	72 h	2.9	20.0	0.0	0.0
	96 h	0.0	5.7	0.0	0.0
150 mg/l	24 h	2.9	5.7	0.0	0.0
	48 h	0.0	0.0	0.0	0.0
	72 h	2.9	5.7	0.0	0.0
	96 h	8.6	2.9	0.0	0.0
200 mg/l	24 h	0.0	0.0	2.9	2.9
	48 h	2.9	2.9	0.0	2.9
	72 h	0.0	2.9	0.0	0.0
	96 h	0.0	0.0	0.0	0.0

paper and then inserted into the flacon. Analysis of artemisinin was quantitatively done using HPLC [25]. First, artemisinin is hydrolyzed in the alkaline solution. The hydrolysis product called Q260 was measured at the wavelength of 260 nm [26]. The quantitative analysis procedure was as follows: 500µl of them with toluene extract taken then evaporated/dried, the residue was dissolved in 200 mL of methanol again. Next, 800 mL of NaOH solution (0.2% w/v) was added, and the mixture was agitated with a vortex and heated in a water bath for 30 min at 50 °C. After it got cold, 200µl of methanol and 800 mL 0.2 M acetic acid were added, then artemisinin was measured by HPLC, at a wavelength of 260 nm, using an RP-18 column Licrospher length of 10 cm. The mobile phase used was methanol: 0:05 Potassium dihydrogen phosphate mM (55: 45), a flow rate of 0.5 mL.min^{-1}. The retention time was 14 min.

2.11 Data Analysis

The descriptive analysis was used to analyse the chromosome determination. The number of alive explants after induction was analysed at 5% level using analysis of variant (ANOVA). The T-test was also conducted at 5% level to determine the significance the plant height, leaf area, stomata, chlorophyll content, glandular trichomes density and glandular trichomes size and content of artemisinin, between the two treatments on the observed data. The analysis of data used program of SPSS 22.0.

3 Results and discussion

3.1 Effect of Colchicines Concentration on Explants Alive After Induction

The survival rate is an essential factor when evaluating the efficiency of polyploid induction after colchicine treatment. The treatment between the concentration and the application time of colchicines have no interaction on at the number of explants alive after the induction. Still, there are differences in the influence of both concentration and application time.

The concentration of 100 mg.L^{-1} Colchicines causes the number of explants life more than the other level. The increase of 100 mg.L^{-1} colchicine to 200 mg.L^{-1} decreases the number of live explants (Table 1).

Twenty-four application time had significantly cause resulted in more explants compared to other applications time. However, when the application time was extended from 24 h to 96 h the number of alive explants after induction was decreased. The toxicity of the colchicines may have caused this effect. The results of the survival rate were in line with the work of Heo [27]. Jeloudar et al. reported the negative effect of high colchicine concentration on survivability in *L. leichtlinii* plantlets [28].

Colchicines are a phytoalkaloid that binds to tubulin and prevents its polymerization into microtubules-blocking the mitotic spindle formation and arresting nuclear division at metaphase [29]. It will decrease cell division, and it will affect plant growth. Colchicines are not only had an effect on cell division, but they also spread throughout the cells. It disrupts cellular mechanisms and causes toxicity at high concentrations. Singh and Roy (1988) also reported that high colchicine concentration could cause the death of plantlets by damaging several parts of cells. Cook and Loudon (1952) stated that Colchicines seem to affect the viscosity of the cytoplasm so that the cells cannot function normally. The action of colchicines on the meristems may be cumulative and have a physiological disturbance resulting in a reduced rate of cell division or death of explants [30].

3.2 Polyploid Induction and Confirmation of Polyploidy by Cytological Analysis

Colchicines has been used experimentally to visualize the metaphase chromosome in cytogenetic studies. It includes inducing polyploidy in plants [31]. The result of chromosome determination from the root tip showed that colchicines treatment 100 mg.L^{-1}, 150 mg.L^{-1}, 200 mg.L^{-1} for 24, 48, 72, and 96 h induced polyploidy efficiency on *A. cina*. Table 3 shows that increased colchicine concentration and application time decrease polyploid plants. This result is in line with the statement of Maneerattanarungroj et al. [32]. The treatment of colchicines is widely used in plant improvement. The right concentration and incubation time need to be done because colchicines are toxic to plants. Colchicines' toxicity increased when incubation time increased.

The highest number of polyploidy plants on colchicine treatment of 100 mg.L^{-1} for 72 h was 8 (Table 2). Ploidy level was obtained by varying the $2n = 2x = 16$, $2n = 3x = 24$, $2n = 4x = 32$, $2n = 5x = 40$, and $2n = 6x = 48$ (Fig. 2). The highest level of polyploidy was $2n = 4x = 32$ at 20% in the treatment Colchicines 100 mg.L^{-1} for 72 h (Table 3). Colchicines interfered with the process of mitosis by binding to the tubulin wall inhibiting the formation of microtubules. Furthermore, the chromosomes'

Table 4. Plant height, leaf area, stomata density, stomata size and chlorophyll content on diploid and polyploid plant of *Artemesia cina*

Ploidy level	Plant height (cm)	Leaf area (cm^2)	Stomata density (μm^2)	Stomata Size (μm)		Chlorophyll content ($mg.g^{-1}$)
				Length	Width	
Diploid	13.00 ± 3.9	24.90 ± 5.61	106.13 ± 8.53	18.02 ± 0.87	13.12 ± 1.2	1.96 ± 0.23
Triploid	23.27 ± 2.83	41.76 ± 12.90*	72.38 ± 5.14*	23.40 ± 2.91*	13.47 ± 3.40	2.10 ± 0.18
Tetraploid	26.80 ± 4.51*	48.53 ± 16.01*	70.62 ± 7.45*	70.62 ± 7.45*	17.73 ± 2.63*	2.14 ± 0.13*
Pentaploid	21.50 ± 6.36	40.71 ± 15.98	51.21 ± 8.89*	51.21 ± 8.89*	15.18 ± 2.63	2.01 ± 0.07
Heksaploid	21.50 ± 4.95	38.15 ± 13.87	68.27 ± 11.84*	68.27 ± 11.84*	14.62 ± 1.63	1.95 ± 0.09

Remarks: An asterik behind the number within the column indicate significant difference of mean (± SD) between diploid and polyploid tested by T-test.

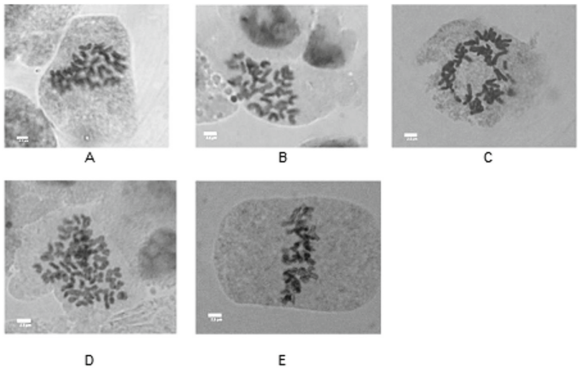

Fig. 2. The effect of colchicines treatment on level polyploidy *A. cina* A. chromosome 2n = 3x = 24; B. chromosome 2n = 4x = 32; C. chromosome 2n = 5x = 40; D. chromosome 2n = 6x = 48; E. chromosome 2n = 2x = 16.

movement toward the poles produces a cell with double the number of chromosomes [33]. The speed of chromosome doubling of each different cell results in varying levels of polyploidy. In this study, the obtained modal number of chromosome x was 8. *A. cina* has unique chromosome numbers with modal numbers of x = 8 and x = 9. The normal plants of these species have chromosome numbers of diploid 2n = 2x = 18 (from modal x = 9) or polyploidy 2n = 4x = 32 (from modal x = 8) [33, 34].

3.3 Leaf Morphological and Anatomical Characteristic

It is known that polyploids generally show morphological changes in another diploid. In this study, there were differences in morphology and anatomy, which were significant at the induction result of *Artemisia cina.*

Observation of morphological and anatomical characteristics of polyploidy plants obtained from the induction of colchicines by observing plant height, leaf area, stomatal

Table 5. Glandular trichome density, glandular trichome size and artemisinin content on diploid and polyploid plant of *Artemesia cina*

Ploidy level	Glandular Trichome density/ μm^2	Glandular trichome size (μm)		Artemisinin content (%)
		Length	Width	
Diploid	104.463 ± 7.996	24.636 ± 1.524	8.910 ± 0.974	0.047
Triploid	93.764 ± 10.765	26.857 ± 1.774	10.539 ± 1.361*	0.056
Tetraploid	74.993 ± 13.104*	28.995 ± 2.772*	10.705 ± 0.793*	0.056
Pentaploid	75.590 ± 7.212*	30.650 ± 2.517*	11.135 ± 0.375*	0.058
Heksaploid	78.915 ± 17.557*	34.535 ± 0.940*	10.770 ± 0.707*	0.051

Remarks: An asterik behind the number within the column indicate significant difference of mean (± SD) between diploid and polyploid tested by T-test.

density and stomata size (length and width of the stomata), chlorophyll content (Table 4) and glandular trichome density and glandular trichome size (Table 5).

The polyploidy of *Artemisia cina* plants showed significant changes in plant height, leaf area, stomatal density, stomatal size, and chlorophyll content. In polyploidy plants, the plant height and leaf area were higher than diploid plants, especially in tetraploid. Polyploid plants showed lower stomata density than diploid plants but larger stomata size. *Artemisia cina* tetraploid plants showed lowest stomata density but had the most significant size (length and width) (Table 4). Stomata size is one indicator that is widely used in the identification of polyploidy. Table 4 shows a negative relationship between the ploidy levels and stomatal density. There is a decrease in stomata per unit area at an increasing ploidy level. This result of the research is in line with the results of the study by Lin et al. [35] on *Artemisia annua* plants and Moghbel et al. [36] on *Glycyrrhiza glabra* plants. Glandular trichome density and glandular trichome size show the same results as stomata.

Glandular trichome density shows a negative relationship with ploidy levels. Glandular trichome size in polyploid plants is higher than diploid. Polyploidy plants had more chromosomes than diploid plants, causing the cell size and nucleus to become more significant. The larger cell size would result in a larger plant size in general. Chlorophyll content in polyploid plants was higher than that in diploid plants, especially in tetraploid, which significantly increased compared to diploid (Table 4). Polyploid plants had more giant leaf cells and a higher amount of chlorophyll.

3.4 The Effect of Polyploidy Induction on Artemisinin Content in Artemisia Cina

The content of artemisinin in polyploid plants is higher than the diploid, although not statistically significant (Table 5). Artemisinin is stored in the leaves, especially in the glandular trichome. *A. cina* polyploid plants show a larger glandular trichome size than diploid plants. Larger leaf size and glandular trichome size in polyploid plants indicate the possibility of polyploid plants producing higher artemisinin. The study by Dangash et al. [37] concluded that artemisinin secreted in glandular trichomes, the trichome

density, and trichome size are directly proportional to the percent artemisinin content. So, the higher the trichome, the amount and size, the higher will be artemisinin content.

The number of chromosomes is most influential in the number of genes that express enzymes involved in the biosynthesis of artemisinin. The results of the study Lin et al. [35] concluded that the average Artemisinin in *Artemisia annua* tetraploid increased through modulation of the expression of genes involved in the biosynthesis artemisinin.

In conclusion, colchicines treatment at various concentrations and application time can induce polyploidy. The increased concentration and application time of colchicines caused the viability of explants reduction after treatment. The results of induction on *A. cina* produced varying ploidy level, that was, 2n = 2x = 16, 2n = 3x = 24, 2n = 4x = 32, 2n = 5x = 40, 2n = 6x = 48 and modal number of chromosomes was x = 8. Changes in morphology, anatomy, cytology and Artemisinin content in *A. cina* induced polyploid. Plant height, leaf area, chlorophyll content, artemisinin content, glandular trichome size, and stomata size in polyploidy plants were bigger than the diploid. In contrast, stomata density and glandular trichome density per unit area were smaller than diploid. The results of this study are expected to provide information for further research and plant breeding.

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