Growth, stomata and trichome characteristics of diploid and tetraploid Artemisia annua L. plants

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Abstract. Tetraploid A. annua have been reported to have higher artemisinin level compared to diploid plants. This research aimed to evaluate growth, stomata, and trichome characteristics of diploid compared to tetraploid plants. Two accessions of diploids (K.1.B and K1.2.2) and two accessions of tetraploid plants (K2.2.9 and K3.2.1) VOM1 were grown in the field for 11 weeks. Growth of plants was investigated every week by measuring plant height, number of branches per plant, fresh and dry weights. Density and size of stomata were observed using light microscopy; the number of trichomes was observed by scanning electron microscopy. The artemisinin level was analyzed by HPLC. The results showed that the plant height of both diploid and tetraploid plants was comparable. From week-1 to week-11, all plants still increased in their plant height. Similarly, numbers of branches also increased except for diploid K.1.B plants. The highest growth rate of numbers of branches was found in tetraploid clone K3.2.1. However, the highest biomass production was found in diploid clone K1.2.2. The artemisinin level of tetraploid plants was higher than that of diploid plants. The level of ploidy was confirmed by flow cytometry analysis.

1. Introduction
Annual wormwood (Artemisia annua L.) (family Asteraceae), is also referred to as Artemisia, is the only species of genus Artemisia to synthesize artemisinin. Artemisinin-derived drugs shown against a variety of parasites that affect the health of humans and animals. They have also successfully been used for the treatment of malaria and cancer [1]. Since 2001, artemisinin with other antimalarial drugs (artemisinin-based combination therapy, A.C.T.) has been recommended by World Health Organization (WHO) to fight malaria in the countries where highly resistant strains of the protozoan have been certified [2]. The critical feature of artemisinin as a widely used antimalarial compound is that artemisinin has shown no
significant side effects [3].

Artemisia annua is native to China and naturalized in a region including the United States, Europe, and South America. This plant is naturally grown in the subtropical and highland areas, but nowadays, it is successfully cultivated in the tropics such as Indonesia [4]. Artemisinin constituent depends on their geographical distribution [5]. Some cultivar of A. annua were cultivated in Indonesia has low artemisinin content from 0.1 to 0.3%, it has become one of the problems of cultivation in Indonesia [6].

Commercial artemisinin is mainly extracted from the leaves of cultivated and wild-grown plants, which produce artemisinin in glandular trichomes [7]. A concentration of artemisinin is dependent on genotype, plant tissue, and time of harvesting and influenced by soil and climatological conditions [2]. A breeding program to develop a plant that produces a high level of secondary metabolite production has been done with recent efforts, including producing polyploid plants by using somatic cell manipulation. Polyplody of A. annua using colchicine and oryzalin treatment to obtain in vitro polyploid plantlets [8,9]. In vitro growth enhancement and leaf regeneration of tetraploid plants were also conducted on Murashige and Skoog (MS) [10] medium containing cytokinins in combination with auxins [11-13]. Evaluation of the artemisinin level on both diploid and polyploid in vitro plantlets were also evaluated [8,14]. The objective of the research was to investigate growth, stomata, and trichome characteristics of diploid and tetraploid accession of A. annua grown in the field. This research is useful to evaluate the growth characteristics of polyploid A. annua resulted from in vitro regeneration plant.

2. Materials and Methods

2.1. Plant material and site of experiments
Four accessions of A. annua, namely, K.I.B, K.1.2.2 (diploids), K.2.2.9, and K.3.2.1 (tetraploids) were used in this study. Stomata and trichome measurements and ploidy confirmation were conducted at Research Center for Biotechnology LIPI, and Scanning Electron Microscopy (SEM) analysis was conducted at PT Fajar Mas Murni. Artemisia annua cultivation and plant growth measurements were conducted at Cibodas Botanic Gardens. Furthermore, artemisinin extraction conducted at Research Center for Chemistry LIPI, and determination of artemisinin levels was conducted at the Center of Biomedical and Applied Health Technology, National Institute of Health Research and Development, Ministry of Health Republic Indonesia.

2.2. Confirmation of ploidy level
Ploidy level confirmation was carried out on plants growing in the field using a flow cytometer (BD Accuri C6+, U.S.A.) analysis following a modified method [8]. Pieces of leaves at about 0.5 x 0.5cm were placed on a petri dish, added with 250 µl Nuclei Extraction Buffer, then chopped into very tiny pieces with a razor blade. The leaves were then filtered with a 30 µm millipore filter. The filtrate was placed into a cuvette tube to add with staining solution, propidium iodide, RNAse. Diploid plant leaves were used as standard. The mean DNA content and coefficient of variation (CV) of each sample at each peak were observed and compared with the control plants sample (diploid). Ploidy levels of plants were determined according to the average amount of DNA, which was read based on the histogram that appeared after the flow cytometer analysis [8].

2.3. Stomata and trichome measurement
Artemisia annua leaves from the field were taken using scissors and put in a jam bottle containing 100 ml of water, then stored in the refrigerator until ready for analysis. Stomata observations were carried out on the upper and lower leaf epidermis following the method [15], using the LEICA DFC310 FX microscopy with 400x magnification and SEM Hitachi SU3500 with 200x, 500x and 1000x magnification. Calculation of length, width, and number of stomata per field of view was focused on a bright, clean, and undamaged field of view. The calculation was done in 10 different fields of view. The
stomata density was recorded by counting the number of stomata per mm$^2$. Data was averaged for each individual plant observed.

2.4. Artemisia annua cultivation and plant growth measurement

Propagation of selected clones is carried out using seeds produced from generation 1 (M1). These clones are namely K.I.B; K.1.2.2; K.2.2.9; and K.3.2.1. Seeds were sown on topsoil and roasted husks using trays. After the seedling height reached 10-15 cm, then they were moved to the field. Planting in the field was carried out in stages on different beds to prohibits cross-pollination between clones. The plant spaces used in the field were 50x50 cm, while the distance between the beds was 50 cm. Fertilization using NPK with a dose of 40:40:40 kg/ha. The first fertilization was P fertilizer 40 kg/ha, N and K fertilizer 20 kg/ha were given one week after planting and added for 20 kg/ha N and K fertilizer one month after planting. Irrigation of the plants conducted during the dry season and weed controlled routinely until the plants were two months old. The parameters observed were plant height and number of branches observed every week with ten plants for replicates. Biomass weight was measured from 3 plant samples obtained after harvesting three months after planting. Dry weights were obtained by drying biomass in an oven at 60°C for two days. From the data obtained, the growth rate of plant height and the rate of growth of the number of branches were calculated. The data obtained were then tabulated for analysis, and all parameters were statistically tested with ANOVA.

2.5. Artemisinin extraction

Determination of artemisinin levels in A. annua using High-Performance Liquid Chromatography (HPLC) modification method [16]. Simplisia leaves of A. annua (200 mg) was placed in a 10 mL volumetric flask, added 5 mL acetonitrile as a solvent. Samples were homogenized using a sonicator for 10 min. Then acetonitrile was added up to 10 mL. Samples were then filtered using 0.45 µm sieve and transferred to vials for analysis. The comparative standard for artemisinin (Sigma Aldrich) at 12.5 mg were placed in a 25 mL volumetric flask dissolved with 5 mL acetonitrile, then homogenized using a sonicator for 10 min. Furthermore, acetonitrile is added up to 25 mL (500 ppm). Comparison standard was made in the series level 50; 100; 200; 300; 400; 500 ppm and transferred into vials. Comparative standards and A. annua leaf samples were analyzed using KCKT Waters, column X-Cartridge C18 4.6 × 150 mm, with a flow rate of 0.60 mL/min, injection volume of 10 µL at a wavelength of 214 nm. The mobile phase used is acetonitrile: methanol: distilled water (40:20:40). Artemisinin concentration were calculated by a dry mass basis.

3. Results and Discussion

Ploidy level of both diploid and tetraploid A. annua plants was confirmed by flow cytometry analysis. Table 1 shows the ploidy level of four accessions tested. The results showed that K.I.B. and K1.2.2 were diploids, K2.2.9 and K3.2.1 were tetraploids, as they were also confirmed by histograms presented in figure 1. Table 1 and figure 1 show the peak sizes of diploid plants (K.I.B and K1.2.2) was at channels 137.12 and 154.54, tetraploids (K2.2.9 and K3.2.1) on channel 300.78 and 343.31 with a coefficient of variation (CV%) ranged from 4.13 to 8.19%. Tetraploid plants had a propidium iodide (P.I) value twice than that of diploid plants. Ploidy level confirmation using flow cytometer has been widely used in various plants such as Artemisia annua [8], pamelo [17], Centella asiatica [18], taro [19,20], banana [21], guava [22], and water spinach [23]. The flow cytometer is widely used for determining ploidy levels of plants because it can analyze faster and easier.
Table 1. Mean P.I. and CV of *A. annua* from flow cytometry analysis.

| No. | Accession | Mean P.I | CV % | Ploidy   |
|-----|-----------|----------|------|----------|
| 1   | K.I.B     | 154.54   | 5.43 | Diploid  |
| 2   | K1.2.2    | 137.12   | 8.19 | Diploid  |
| 3   | K2.2.9    | 343.31   | 4.85 | Tetraploid |
| 4   | K3.2.1    | 300.78   | 4.13 | Tetraploid |

![K.I.B (diploid) K1.2.2 (diploid) K2.2.9 (tetraploid) K3.2.1 (tetraploid)]

**Figure 1.** Histogram of four accessions *A. annua* from flow cytometry analysis.

Stomata characteristics of diploid and tetraploid *A. annua* plants also varied (table 2). The results showed that abaxial epidermis cells had higher stomata density than that of adaxial cells; this is common to all plant species. Tetraploid K2.2.9 had the largest size of stomata so that the stomata density was the lowest. The highest stomata density of adaxial cells was found at tetraploid K2.2.9. However, the highest of that abaxial cells was found at diploid K1.2.2 plants. Figure 2 shows an anatomical structure of diploid and tetraploid *A. annua* plants analyzed with SEM and light microscopy. No abnormal structure was found between diploid and tetraploid plants.

Based on the measurement of the size and density of stomata, it is showed that the plants, generally, have a larger stomata size but lower stomata density than diploid plants. However, for tetraploid K2.2.9 clones, the size of stomata was smaller than diploid K1.2.2 plants, which were also the result of colchicine induction; this shows that the induction of polyploid with colchicine even though not doubling the number of chromosomes, it affected the size of stomata. In pamelo, stomata density decreases with increasing ploidy levels [17]. Tetraploid *Dioscorea rotundata* plants produced by colchicine also have a larger stomata size but lower stomata density than their diploid plants [24].

Table 2. Stomata density and size of adaxial and abaxial epidermal tissue of *A. annua* V0M1 generation (measured on 400 x magnifications).

| Accession | Epidermis | Stomata Density (no/mm²) | Stomata Length (µM) | Stomata Width (µM) |
|-----------|-----------|--------------------------|---------------------|-------------------|
| K.I.B     | Adaxial   | 85.55±33.37              | 14.38±1.51          | 9.20±1.08         |
|           | Abaxial   | 442.48±90.99             | 13.21±0.84          | 9.31±0.88         |
| K1.2.2    | Adaxial   | 153.39±80.25             | 15.77±1.77          | 10.93±1.57        |
|           | Abaxial   | 398.23±157.16            | 15.38±0.88          | 11.24±1.46        |
| K2.2.9    | Adaxial   | 191.74±100.50            | 13.74±1.51          | 9.62±1.12         |
|           | Abaxial   | 259.59±67.91             | 14.33±1.52          | 9.86±0.88         |
| K3.2.1    | Adaxial   | 67.85±33.34              | 23.63±2.07          | 12.91±1.86        |
|           | Abaxial   | 227.14±57.68             | 22.29±1.48          | 12.05±1.22        |
Figure 2. Stomata of *A. annua*. A. Photographs were taken by scanning electron microscopy, (500x left), (1000x middle), (2000x right). B. Photographs were taken by light microscopy (400x magnifications).

Trichome of *A. annua* is a place for artemisinin and some other metabolite products [25,26]. Table 3 indicates that most abaxial cells had higher numbers of trichomes. Only diploid K.I.B plants have lower trichome numbers on their abaxial cells. However, diploid K1.2.2 and tetraploid K3.2.1 had less number of trichomes compared to that of diploid K.I.B and tetraploid K2.2.9 (figure 3).

Table 3. Trichome numbers of adaxial and abaxial epidermal tissue of *A. annua* V0M1 generation (measured on 400 x magnifications).

| Accession | Epidermis | Number of trichome |
|-----------|-----------|--------------------|
| K.I.B     | Adaxial   | 10.67±1.14         |
|           | Abaxial   | 8.67±1.52          |
| K1.2.2    | Adaxial   | 2.00±0.71          |
|           | Abaxial   | 2.25±0.83          |
| K2.2.9    | Adaxial   | 9.00±1.22          |
|           | Abaxial   | 10.00±1.22         |
| K3.2.1    | Adaxial   | 3.00±1.58          |
|           | Abaxial   | 3.50±1.34          |

Figure 3. *Artemisia annua* trichomes under scanning electron microscopy. A. 200x; B. 500x; C. 1000x magnifications.
Growth of diploid and tetraploid A. annua plants were still increasing from week-1 to week-11 with a similar pattern of growth of plant height for all accessions (figure 4A). At the beginning of growth, K2.2.9 had higher growth; however, after eight weeks of planting, the growth was similar with K3.2.1 plants. Diploids plants had lower height compared to that of tetraploid plants. In contrast with the height, numbers of branches varied among the accessions. After 11 weeks of planting, tetraploid plants remained to have higher numbers of branches compared to that of diploid plants. Tetraploid K3.2.1 had the highest, and diploid K.I.B had the lowest branch numbers (figure 4B).

Although no significant differences were seen, tetraploid plants showed higher growth of stems and leaves than diploid plants. Morphological comparisons between diploid and tetraploid plants could be seen in the vegetative part and flower character. The differences that arise include plant height, number and size of leaves, and flowering period. Tetraploid plants are not significantly different in stem diameter, but have darker leaf color characteristics compared to diploid [27].

The relative growth rate of A. annua plant height was not significantly different (figure 5A). The relative growth rate of the number of branches K3.2.1 was highest significantly different from K.I.B, which had the lowest value (figure 5B). Diploid K1.2.2. had a higher relative growth rate of branches numbers with diploid K.I.B, but it had similar to that of tetraploid K2.2.9.

**Figure 4.** Growth of A. annua V0M1 generation from week-1 to week-11 after planting. A. Plant height. B. Numbers of branches. Accession K.I.B and K1.2.2 are diploids, Accession K2.2.9 and K3.2.1 are tetraploids.

**Figure 5.** The relative growth rate of A. annua V0M1 generation. A. The relative growth rate of plant height. B. The relative growth rate of the number of branches. Accession K.I.B and K1.2.2 are diploids, Accession K2.2.9 and K3.2.1 are tetraploids.
The biomass of K.3.2.1 tetraploid plant was highest, but not significantly different from the accession K.2.2.9 tetraploid, and K.1.2.2. diploid. On the other hand, K.1.B diploid accession has the lowest biomass. The allocation of biomass is associated with the mobilization of assimilation between sink and source physiologically. The tetraploid plants have higher biomass than the diploid because of the source values more significant than the diploids [28].

Figure 6. Fresh and dry weights of A. annua V0M1 generation. Accession K.1.B and K1.2.2 are diploids, Accession K2.2.9 and K3.2.1 are tetraploids.

Analysis of artemisinin of A. annua by HPLC is presented in figure 7. The results indicated that tetraploid plants had higher artemisinin levels compared to diploid plants. The highest level of artemisinin was found on tetraploid K2.2.9 (0.52 mg/g D.W.); the lowest of the artemisinin level was on diploid K1.2.2. (0.33 mg/g DW). Artemisinin content of diploid and polyploid in vitro shoots have also been evaluated [14]. The treatment of colchicine concentration and soaking period resulted in shoots of in vitro A. annua with varying levels of artemisinin. All tetraploid shoots had higher levels of artemisinin compared to diploid and mixoploid shoots with the same concentration of colchicine treatment. The range of artemisinin levels in tetraploid shoots was 0.139 to 0.387%, with the highest levels in shoots treated with 0.25 with a soaking time of 2 days. Increased levels of artemisinin on tetraploid shoots ranged from very low to more than six times higher compared to that of the diploid shoots. A similar finding was reported in A. annua tetraploid hair roots, which is six times higher than diploid roots [29]. Increased levels of artemisinin in tetraploid plants resulting from the treatment of 0.1% colchicine also occurred with 1.5 times higher compared to diploid plants [30].

Figure 7. Artemisinin level of A. annua V0M1 generation. Accession K.1.B. and K1.2.2 are diploids, Accession K2.2.9 and K3.2.1 are tetraploids.

4. Conclusion

In conclusion, growth, artemisinin content, and stomata, as well as trichome characteristics of A. annua M0V0, was affected by the genotype of the plants. Growth of diploid and tetraploid plants were comparable, but the size of stomata and artemisinin content of tetraploid plants were more significant than that of diploid plants. Ploidy level was stable for both diploid and tetraploid plants.
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