The effect of shading on density and size of glandular trichomes in artemisia cina tetraploid, the source of anti-cancer artemisinin

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Abstract. Artemisinin is a sesquiterpene lactone found in Artemisia cina having many medicinal properties. Artemisinin produced and stored in Glandular trichomes The purpose of this study was to determine the effect of shade 0%, 50% and 75% on the density and size of glandular trichomes and the content of artemisinin in A. cina tetraploid plants. The shade treatment significantly affected the density and size of glandular trichomes and the content of artemisinin in Artemisia cina tetraploid. 50% shade treatment showed the highest density of Glandular trichome, the width of glandular trichomes, and Artemisinin content. The results of the correlation analysis show that there is a very significant relationship between the density of glandular trichomes and the content of artemisinin.

1. Introduction
Artemisia cina (Asteraceae) is a plant of a family with Artemisia annua, in Indonesia, there are many weeds in the highlands. Artemisia cina can grow in Indonesia but does not flower because of day length and temperature that is not appropriate [1]. Artemisinin content in A. cina is still low around 0.00148 - 0.16% so that efforts are needed to increase the content of artemisinin in A. cina (Herawati et al. 2014). One of the efforts that have made in increasing the content of artemisinin through artificial polyplody or induction of artificial polyplody. The content of artemisinin A. cina polyplody induced by growth regulators was higher that is 0.066% - 0.162% compared to diploid plants which were 0.047% - 0.061% [2].

Efforts to increase the content of artemisinin in addition to genetic engineering can also be made through technical culture. One way that can take in increasing the content of secondary metabolites in technical culture is to provide stress both biotically and abiotically. Plant growth will greatly influence by environmental factors where the plant grows. Light is one of the environmental factors that influence plant growth. The sun is a source of light energy needed by plants for photosynthesis, plays a role in regulating temperature, respiration and, water loss. In addition to physiological processes in the plant body, the production of secondary metabolites influenced the light. Treatment of light stress can be in the form of giving low or high light intensity, the period or duration of plants exposed to light and exposure to plants at specific wavelengths. The intensity of sunlight also affects the formation of tissues or organs that play a role in protecting plants against the stress of solar radiation.

Trichomes are the hairs that are on the surface of plants, namely the surfaces of leaves, stems, flowers, or fruit. Trichomes originate from epidermal cells and perform various functions. Some secrets
of sticky exudates to trap insects, others produce essential oils that attract beneficial insects or repel pests [3]. Their presence can also modulate airflow over the leaf surface, which affects the rate of respiration and transpiration. Trichomes act as reflectors of light under conditions of high light intensity so that plants do not experience stress. Trichomes also have chemical defense functions as they accumulate large amounts of bioactive chemicals in many plants [4][5].

There are two main types of trichomes: glandular and nonglandular trichomes. Glandular trichomes excrete a mixture of chemicals that have been found to have a wide variety of uses in pesticides, pharmaceuticals and industrial flavors [6];[7]. Glandular trichomes (GT) on Artemisia annua produce also, store the anti-malarial and anti-cancer compound artemisinin (AN).

Artemisinin is a sesquiterpene lactone endoperoxide produced in glandular trichomes (GLT) in Artemisia annua L.[8];[9]. Currently, artemisinin is not only used effectively as a malaria treatment but also effective against other human diseases such as schistosomiasis, hepatitis B, and several other cancers [10]. The potential of Artemisinin as an anticancer lies in endoperoxide which is bound to sesquiterpene lactone. Endoperoxide will form free radicals when reacting with iron. Iron is needed for cell division and proliferation. Compared to normal cells, cancer cells have aggressive tumors. Cancer cell aggressiveness becomes higher depending on the number of surface surfaces of transferrin receptors that take up iron through interactions with plasma iron carrying protein transferring. Thus, cancer cells will be more susceptible to the cytotoxicity of artemisinin because of the high iron absorption rate [11]. The purpose of this study was to determine the effect of shade on the density and size of glandular trichomes and the content of artemisinin in A. cina tetraploid plants

2. Materials and methods

The study was conducted at the Laboratory of Genetics of the Faculty of Agriculture, Gadjah Mada University, Laboratory of Tissue Culture and the experimental gardens of the Faculty of Agriculture and Business, SatyaWacana Christian University, Salatiga.

2.1 Plant material

The plant material of this research is Artemisia cina tetraploids (2n = 4x) resulting from polyploidy induction with plant growth regulator agents. The microscopic determination of chromosomes has confirmed the number of chromosomes.

2.2 Leaf area analysis

Leaf area measurements were done with a Leaf Area Meter Mark 2 type, Delta T, Burwell Cambridge, England. The leaves used in the measurement of leaf area were the ones that had been completely unfolded and were located in sections 3, 5, 8 and 10.

2.3 Glandular trichomes observation

Glandular trichomes characteristics were observed in the 6th segment. The epidermal surfaces of abaxial leaves were brushed with non-colored nail polish and covered with transparent plastic tapes. The tapes were taken off and placed on object-glass. The glandular trichomes length, width and density were observed using an Olympus microscope with photomicroscope Optilab.

2.4 Artemisinin analysis

Making a standard curve with pure Artemisinin as a standard. Making a standard curve with pure Artemisinin as a standard is the first step that must take in the measurement of an active ingredient using HPLC. Making a standard curve using pure Artemisinin as a standard to get the regression value so that the results of measuring samples using HPLC can calculate levels of Artemisinin-based on the results of the regression of the standard curve.

2.5 Extraction
Samples were dried by curing at 30 °C until they reach a constant weight. A dry sample was mashed with mortar. 100 mg of the dry weight of the sample was then extracted with 2 mL of toluene, and filtered using filter paper and then put into the flacons.

2.6 Quantitative analysis
Quantitative artemisinin analysis was performed using HPLC. At first, Artemisinin hydrolyzed in an alkaline solution. The hydrolysis product called Q260 measured at a wavelength of 260 nm [12]. The procedure of the quantitative analysis is as follows: the extraction results with toluene taken 500 μL then evaporated/dried, the residual residue is dissolved again in 200 μL methanol. The next 800 μL NaOH solution (20 mgL⁻¹ w / v) added and the mixture formed was agitated with vortex and heated into a water bath for 30 minutes at 50 °C. After chilling added 200 μL methanol and 800 μL acetic acid 0.2 M, then measurements of Artemisinin by HPLC, at a wavelength of 260 nm, using a Licrospher RP-18 column 15 cm long. The mobile phase used is Methanol: Potassium dihydrogen phosphate 0.05 mM (55: 45), the flow rate of 0.5 mL.minute⁻¹. Retention time ranges from 14.

2.7 Data analysis
The data were analyzed using analysis of variance (ANOVA). The parametric data analysis using the Tukey-test at a 5% level was conducted to determine the significance between the two treatments on the observed data of leaf area, glandular trichomes density, glandular trichomes size, and using T-test at 5% level for artemisinin content analysis. The program used for data analysis is SAS 9.1.3.

3. Result and discussion

3.1 Effect of shading on leaf area
The 50% to 75% shade treatment has a significant effect on increasing leaf area compared to without shade. The leaf area significantly increased with increasing shade. It suspected that under low light intensity conditions, the plant would carry out self-defense mechanisms against stress conditions. One mechanism that can be done by plants is to increase the efficiency of light conversion per unit of plant tissue by increasing the proportion of leaf area. Shaded plants have thin leaves and low dry weight [13].

| Shading | Leaf Area (cm²) | Glandular Trichomes | Artemisinin Content (%) |
|---------|----------------|---------------------|------------------------|
|         |                | Density (μm²)       | Size (μm)              |                        |
|         |                |                     | Length | Width | |
| 0%      | 21.615 b       | 123.283 b           | 27.52  a   | 8.65  b | 0.042  b |
| 50%     | 30.623 a       | 155.610 a           | 30.10  a   | 10.46 a  | 0.265  a |
| 75%     | 29.983 a       | 120.467 b           | 29.67  a   | 9.46  b  | 0.027  b |

The mean value in each column, followed by the same letter was not significantly different in the Tukey test (α= 5%).

3.2 Effect of shading on glandular trichomes density
The shade treatments of 0%, 50%, and 75% have a significant influence on the density of glandular trichomes. The 50% shade treatment gives significantly different results to the glandular trichomes density than the 0% shade treatment and 75% shade treatment. The density of glandular trichomes shows the number of glandular trichomes per unit area. At 50% shade gives higher glandular trichome density compared to 0% shade treatment while 75% shade gives lower glandular trichome density than 0% and 50% shade. Glandular trichomes and trichomes present in the leaf have a role as light reflectors. In the shade treatment, 50% of the plants have not experienced a light deficit so that the development of
glandular trichomes as a reflection of light is still proper. Besides that, the higher leaf area also influences the number of glandular trichomes that formed. At 75% dense shade, plants will experience a light deficit caused by dense shade. Plants experience a light deficit caused by dense shade characterized by the lack of light reflecting on the surface of the leaf caused by low cuticle development, trichomes and the absence of a waxy layer [13].

3.3 Effect of shading on the size of glandular trichomes
The length and width of the glandular trichomes are the sizes of the glandular trichomes. Giving 50% to 75% shade gives a gland trichome length not significantly different than without shade. Polyploid plants are characterized by cell size and a more significant number of cells as a result of chromosome multiplication. Glandular trichome length is the result of an increase in size and number of cells. Increase in the number of chromosomes will cause an increase in the DNA content per cell. An increase in enzyme activity per cell and an increase in cell volume, which, as a whole, affects the increase in plant size [14].

The provision of 50% shade shows that the width of glandular trichomes is significantly more extensive than the treatment without shade and 75% shade. Glandular trichome width is the result of cell elongation and expansion. Spread and elongation of cells in the trichome gland that treated with shade are thought to be related to auxin activity in shaded plants.

3.4 Effect of shading on artemisinin content
Table 1 shows that the 50% shade treatment results in a significantly higher content of artemisinin compared to the 0% and 75% shade treatments that are 0.262%. Artemisinin is the result of secondary metabolism produced by the Artemisia sp. Abiotic and biotic factors influence the production of secondary metabolites. Abiotic factors such as environmental stress often affect the accumulation of plant secondary metabolites [15].

Light is one of the abiotic factors that can affect the production of metabolites. Light can stimulate the production of secondary metabolites such as gingerol and zingiberene in Zingiber officinale [15]. Shading is an environmental modification that aims to reduce the intensity of light received by plants so that plants experience a stress condition that will stimulate the formation of secondary metabolites. Besides that, the shade is also a modification of the microclimate, especially the temperature in the plant's growing environment.

The effect of shade on the artemisinin compound is thought to be related to the intensity of light received by plants. That influences the process of biosynthesis of artemisinin. HMGR is an enzyme that acts as a regulatory enzyme in the biosynthesis of artemisinin. The HMGR plays a role in isoprenoid biosynthesis, including artemisinin [16]. HMGR activity in plants regulated by light. The intensity of light received by plants at 50% shade treatment is thought to be the appropriate light intensity for HMGR activity. Shaded conditions also affect the ambient temperature to be lower due to the intensity of the received light is also lower than without shade. Under low-temperature conditions, the artemisinin level is higher [17]. The provision of shade, to affecting the intensity of light received, becomes lower, which affects the ambient temperature, also affects the presence of glandular trichomes in plants. A glandular trichome is a place of synthesis and storage of artemisinin in Artemisia cina. The density of glandular trichomes will affect the levels of artemisinin.

3.5 Relation between leaf area, density, and size of glandular trichomes and artemisinin content
Pearson's Correlation Coefficient between leaf area, length, and width of glandular trichomes and artemisinin content in Artemisia cina shown in table 2. Based on the sig. (2-tailed) A value between the density of glandular trichomes and Artemisinin content of 0.002 <0.05, which means there is a significant correlation between the variable density of glandular trichomes with artemisinin content. Furthermore, the relationship between the glandular trichomes width and artemisinin has a Sig. (2-tailed) value of 0.011 <0.05, which means there is a significant correlation between the width of the glandular trichomes variable and the content of artemisinin.
Based on the r count (Pearson Correlation) known r count value for the relationship between glandular trichomes density and artemisinin content of 0.867 > r table 0.666 which means there is a strong correlation between glandular trichomes density and artemisinin content. Furthermore r count for the relationship between glandular trichomes width and artemisinin content of 0.793 > r table of 0.666, which means there is a correlation between glandular trichomes width and artemisinin content. Biosynthesis and storage of artemisinin can be found in glandular trichomes. Glandular trichome density correlates with artemisinin content. Table 2 shows the correlation between the levels of artemisinin and the morphological and anatomical characters of A. cina. The level of artemisinin has a positive correlation and is very significant until it is evident by the width of the glandular trichomes and the density of the glandular trichomes. Thus the higher the density of the glandular trichome, the artemisinin content will increase. This is in line with the results of research Dangash et al. which states that glandular trichome density correlates with artemisinin content [18].

**Table 2.** Correlation between leaf area, density, and size of glandular trichomes and artemisinin content

| Correlations | KGT | PGT | LGT | LD | AN |
|--------------|-----|-----|-----|----|----|
| KGT Pearson Correlation | 1 | .541 | .678* | .359 | .867** |
| Sig. (2-tailed) | | .132 | .045 | .343 | .002 |
| N | 9 | 9 | 9 | 9 | 9 |
| PGT Pearson Correlation | | .541 | 1 | .666 | .654 | .427 |
| Sig. (2-tailed) | .132 | 1 | .050 | .056 | .251 |
| N | 9 | 9 | 9 | 9 | 9 |
| LGT Pearson Correlation | .678* | .666 | 1 | .719* | .793* |
| Sig. (2-tailed) | .045 | .050 | .029 | .011 |
| N | 9 | 9 | 9 | 9 | 9 |
| LD Pearson Correlation | .359 | .654 | .719* | 1 | .490 |
| Sig. (2-tailed) | .343 | .056 | .029 | .180 |
| N | 9 | 9 | 9 | 9 | 9 |
| AN Pearson Correlation | .867** | .427 | .793* | .490 | 1 |
| Sig. (2-tailed) | .002 | .251 | .011 | .180 |
| N | 9 | 9 | 9 | 9 | 9 |

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

4. Conclusion
The shade treatment significantly affected the density and size of glandular trichomes and the content of artemisinin in Artemisia cina tetraploid. 50% shade treatment showed the highest density of Glandular trichome, the width of glandular trichomes, and Artemisinin content. The results of the correlation analysis show that there is a very significant relationship between the density of glandular trichomes and the content of artemisinin.

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