Biosaintifika 9 (3) (2017) 423-429

Biosaintifika
Journal of Biology & Biology Education

http://journal.unnes.ac.id/nju/index.php/biosaintifika

Size and Density of *Artemisia annua* Stomata Soaked in Water Extract of *Gloriosa superba* seeds

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DOI: 10.15294/biosaintifika.v9i3.11234

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History Article

Received 26 August 2017
Approved 9 November 2017
Published 31 December 2017

Keywords

Size; Density; Stomata; *Artemisia annua*; *Gloriosa superba*

Abstract

Artemisia annua is a herbaceous plant that produces artemisinin as a malaria drug, haemorrhoids therapy, aromatherapy, antiviral, anticancer and antibacterial. *Gloriosa superba* is a plant that contains high colchicine compounds, especially on the seeds. *Gloriosa superba* extracts of tubers, stems, seeds, and leaves were used as biomutagen for many plants. Colchicine contains of these plants as antimitotic have been studied and proven by the mitotic index plants. Water extracts of *Gloriosa superba* seeds was used as a mutagen for *Artemisia annua*. The aim of this study was to determine the size and density of *Artemisia annua* stomata soaked in water extract of *Gloriosa superba* seeds as a mutagen. Extraction of *Gloriosa superba* seeds obtained naturally on Krakal Beach, Gunung Kidul by using a maceration method with water solvent (1:1). *Artemisia annua* sprouts were obtained from B2P2TOOT Tawangmangu. Variables treatment on sprouts using water extract concentration of *Gloriosa superba* seeds and soaking time of *Artemisia annua* sprouts. Measurements of stomatal length, width and density were conducted in epidermis of *Artemisia annua* leaf. Observation and measurements of the stomata were conducted by using a light microscope. The results showed that the length and width of stomata were 0.025 mm and 0.017 mm respectively. The stomatal density of the control leaf (174.69 amount/mm2) was lower than the other treated plants. Stomatal size and density has increased with the increasing concentration extracts on treated plants. Water extracts of *Gloriosa superba* seeds proved the effects on stomatal size and density of treated plants.

How to Cite

Rahmawati, S. I., Yunus, A., & Susilowati, A. (2017). Size and Density of *Artemisia annua* Stomata Soaked in Water Extract of *Gloriosa superba* seeds. *Biosaintifika: Journal of Biology & Biology Education*, 9(3), 423-429.

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p-ISSN 2085-191X
e-ISSN 2338-7610

INTRODUCTION

Artemisia annua is a potential Chinese medicinal plant to be cultivated in Indonesia (Figure 1). This medicinal plant produces the active ingredient artemisinin as a malaria drug to replace quinine that has been resistant to *P. Falciparum*. Liu, *et al.* (2011) has proven that the production of drugs from artemisinin derivate works as high efficient chemotherapy for low plasmodial and toxic antibodies. Issue of *Artemisia annua* plant development in Indonesia according to Lestari, *et al.* (2010) is that the available genotype has a very low artemisinin content. Improving the yield and the artemisinin content is the main objective for breeding this herb. Graham *et al.* (2010) reported that the production of artemisinin is challenging because *Artemisia annua* remains relatively undeveloped as a crop. Increased ploidi in medicinal plants is needed to increase secondary metabolite levels so that the price of affordable medicinal materials (Rantau *et al.,* 2014). Artificial polyploid is a technique to increase the chromosome quantity in plant. Polyploidy induction of *Artemisia annua* is able to increase artemisinin production (Banyai *et al.,* 2010; Huang *et al.,* 2010). Plant induction of ploidy can increase the content of artemisinin as has been proven in the research by Lin, *et al.* (2011).

Figure 1. *Artemisia annua*

Gloriosa superba is a herbaceous plant that grows naturally around Krakal Beach, Gunung Kidul, Yogyakarta. Ghosh, *et al.* (2002) explained that this plant is a semi-wooden herb that grows propagate (Figure 2). *Gloriosa superba* is one of the important species in Asia and Africa which produce two important alkaloids colchicine and gloriosine that present in seeds and tubers (0.7% to 0.9%). The other alkaloids that have been isolated from the plant are lumi colchicine, 3-demethylcolchicine, 3-demethyl-N-deformyl-N-deacetylcolchicine, N-formyl deacetylcolchicine (Chulabhorn *et al.* 1998). The content of colchicine in *Gloriosa superba* reported by Arambewela (1991) is 0.15% to 0.25% in the seeds. *Gloriosa superba* is a rich source of colchicine used to treat gout, cirrhosis and is commonly used in plant breeding studies to produce polyploidy (Pandey and Banik, 2012). High colchicine content accompanied by prospects of good availability from both wild and cultivated sources make the seeds of *Gloriosa superba* a potential commercial source of colchicine (Sivakumar & Krishnamurthy, 2002). Ernawiati (2008) also explained that the content of alkaloid colchicine compounds in *Gloriosa superba* plant can be used as a potential mutagen (polyploid).

Figure 2. Flower and Seeds of *Gloriosa superba*

Haryanti, *et al.* (2009) stated that polyploid can be made artificially with chemicals such as colchicine because most of these substances are easily soluble in water and effectively induce polyploid. A study by Ade and Mahendra (2010) showed that colchicine is also used for inducing polyploidy in plant cells during cellular division by inhibiting chromosome segregation during meiosis. Half the resulting gametes therefore contain no chromosomes, while the other half contain double the usual number of chromosomes (i.e., diploid instead of haploid as gametes usually are) and lead to embryos with double the usual number of chromosomes (i.e. tetraploid instead of diploid).

Polyploid plants treated by colchicine are characterized by larger chromosomal, morphological and stomatal size differences compared to diploid plants (Rantau *et al.,* 2014). Larger leaf size in polyploidy plants may indicate a potentially higher biomass indicating that the higher quantity of desired compound existing in the leaves could be obtained (Kun-Hua *et al.,* 2010). This research expected water extract of *Gloriosa superba* seeds influence the cytological of treated plants and gives effect to stomata leaf in length, width, and density. The aims of this study was to determine the size and density of *Artemisia annua*

stomata soaked in aquadest extracts of *Gloriosa superba* seeds that containing colchicine as polyploid agent.

METHODS

The method used in this research was a laboratory experimental method that aimed to determine the presence or absence of causal relationships and how much the relationship by way of giving a particular treatment on the variables studied (Nazir, 1988). This research's method includes extraction of the *Gloriosa superba* seeds, analysis of colchicine content, soaking the *Artemisia annua* sprouts, stomata preparation and stomata observation by using the microscope.

Extraction of *Gloriosa superba* Seeds and Soaking Process of *Artemisia annua* Sprouts

Extraction of *Gloriosa superba* seeds was by using maceration method with water solvent (1:1). The water that used in this extraction was a distilled water (aqua dest). Analysis of colchicine content of the extracts was conducted by TLC-Densitometry method. Colchicine detection in water extract of *Gloriosa superba* seeds using TLC method and determination of colchicine content in water extract using densitometry method. Water extracts of seeds was used as a polyploid agent of *Artemisia annua* soaking sprouts. *Artemisia annua* sprouts used were about 1-2 weeks old. The treatment variables in this study were the concentration of water extracts of *Gloriosa superba* seeds (0%, 25%, 50%, 75% and 100%) and the soaking time of *Artemisia annua* sprouts (0 minutes, 30 minutes, 60 minutes and 90 minutes). Sprouts used were initially maintained on the polybags. Design of treatment in this research is presented in Table 1 as follow.

Table 1. Research Design

Times / Concentration	A (0 minutes)	B (30 minutes)	C (60 minutes)	D (90 minutes)
A (0%)	AA	AB	AC	AD
B (25%)	BA	BB	BC	BD
C (50%)	CA	CB	CC	CD
D (75%)	DA	DB	DC	DD
E (100%)	EA	EB	EC	ED

Stomata Observation

Stomata observations in this study were conducted on 20 treated plants with five times repetition. Leaves of *Artemisia annua* that used in stomata observation was 1-2 months old. Parts of leaf used on observation is the lower epidermis. The lower epidermis has more stomata and easier to observe under the microscope. Stomata sample preparation was done in the morning when the plants are photosynthesizing, this is the condition where the stomata of lower epidermis is open. Leaf picking was done randomly at the center of the plant stem by selecting the fresh and fairly old leaves. First preparations of stomata samples using nail polish was smeared on lower epidermis of leaf. After drying of the nail polish approximately 1-2 minutes, picked the leaf then affixed to the tape. Next step leaf that affixed on tape pulled from the tape slowly and carefully. Leaf epidermis mold on tape affixed to the object glass. It must be clean to observe the stomatal length and width easier. Stomata preparation such as a glass preparation can last for ±6 months to be observed.

Observation of stomatal size and density was conducted using a microscope with magnification of 400 times. The calculation of the length, width and number of stomatal per field of view is focused on a clear, clean and undamaged field of view. Number of stomata recorded in the field of view (mm^2). Number of wide field of view calculated from number of length field of view and number of width field of view. Observation data were then averaged for each treated plant. The stomatal density of each treatment was calculated using the following formula :
Stomatal Density= (Number of stomata)/(Wide field of view (mm^2))

RESULT AND DISCUSSION

Water extract of *Gloriosa superba* seeds that contains colchicine in this research used to soaked *Artemisia annua* sprouts. Detecting and determination colchicine content on extracts used TLC-Densitometry method was resulted same compounds colour, Rf value and wavelength with standard colchicine. Colchicine content on water extract of *Gloriosa superba* seeds is 12.84 µg/µl (± 2.88). Stomatal size and density observed at 20 treated plants leaf include control leaf plant with each treatment was performed five replications. Observation data based on this research obtained stomata length, width and density presented in Table 2. Size and Density of Stomata *Artemisia annua*.

The Size of *Artemisia annua* Stomatal Soaked in Water Extract of *Gloriosa superba* seeds

Lower epidermis has more stomata to observe and calculate. AA leaf (0%, 0 minutes) is

a control leaf sample that has a stomatal length of 0.025 mm and stomata width of 0.017 mm, while in BA (0.027 mm; 0.018 mm), CA (0.033 mm; 0.020 mm), DA (0.036 mm; 0.021 mm) and EA (0.036 mm; 0.021 mm) the length and width larger than control.The result indicated that the increase of stomatal length and width is in line with the increase of the concentration. However, sample that has soaking sprouts time longer than 0 minutes shows a lower number of length and width, example CA (0.033 mm; 0.020 mm), CB (0.033 mm; 0.019 mm), CC (0.032 mm; 0.018 mm) and CD (0.031 mm; 0.017 mm). There are variation data of stomatal length and width in all leaves samples.

Stomata width in AD (0.016 mm) and BD (0.016 mm) is lower than in AA (0.017 mm) as a control. Leaf sample treated with variable concentration 100% water extract of *Gloriosa superba* seeds and 90 minutes soaking sprout times has lower stomata width (0.019 mm) compared to the other leaf treatments. Stomatal length in EB (100%, 30 minutes) treated leaf shows an increase size in length (0.037 mm) than other treated leaves. While the widest stomata in EC (100%, 60 minutes) leaf treatment is 0.024 mm. This condition may be occurred due to the environment factors (temperature and light) during the growth phase in the field. Therefore, there are any variation of stomatal size between treatments and environment factors.

Artemisia annua plant that is soaked in water extracts of *Gloriosa superba* seed showed the characteristics of ploidy plant evidenced by the different size and length of stomata in the control plants compared to the treated plants. In this research increased concentration with same soaked sprout times on treated plants showed an increase in stomatal size as presented in Figure 3. The results of this study indicate the similarity with research by Banyai *et al.* (2010) showing that plants in *Artemisia annua* L. tetraploid have larger stomatal size than diploid plants. Suryo (1995), ploidy plants have a larger size in epidermal cells of the leaves, stomata, cells at the growing point, flowers and fruit.

The Density of *Artemisia annua* Stomatal Soaked in Water Extract of *Gloriosa superba* seeds

Stomatal density calculate from number of stomata divided field of view. Number field of view was calculated from length field of view (0.250 mm) multiplied by width of field of view (0.187 mm) was 0.004675 mm^2. The number of stomata of each treatment is different so that if it divided by number field of view will produce the variation of stomatal density value. Density variations can be clearly observed using graph analysis comparing the increasing of concentrations aquadest extract of *Gloriosa superba* seeds compared to the time of soaking sprouts *Artemisia annua*. There are four garphs for compare the stomatal density that present in Figure 4. Densi-

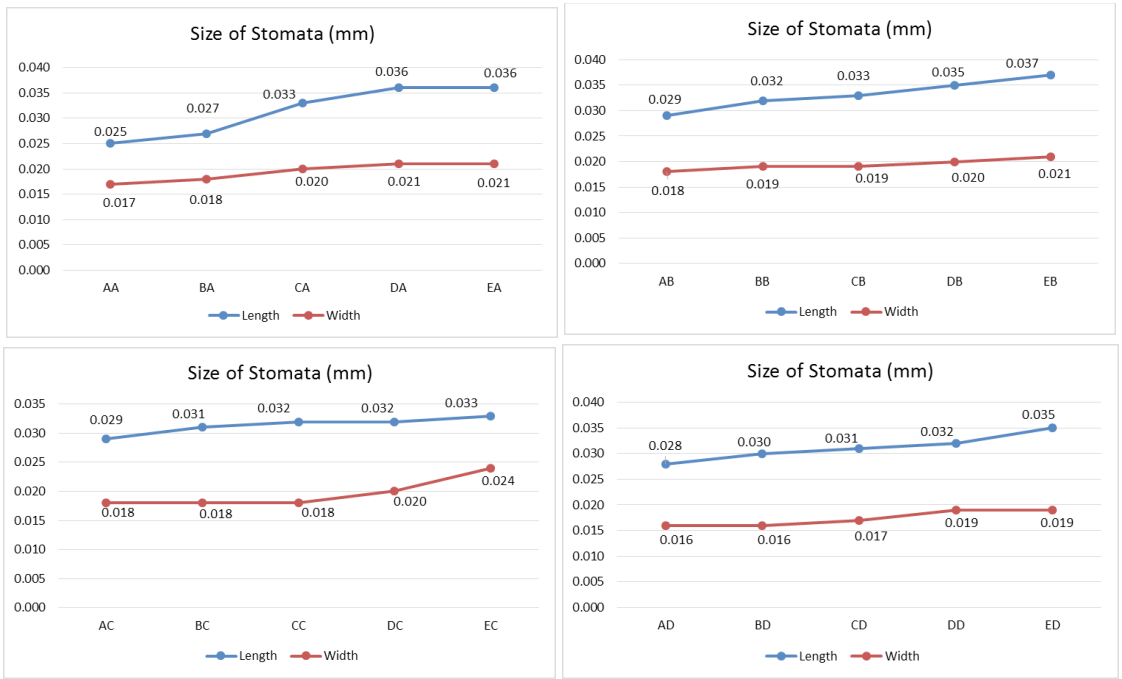

Figure 3. Size Graph of Stomata *Artemisia annua* Soaked in Water Extract of *Gloriosa superba* seed in concentration increased and same soaking times

ty Graph of Stomata *Artemisia annua* Soaked in Water Extract *Gloriosa superba* seeds in concentration increased and same soaking times. Density stomata data based on observed also present in Table 2. that shows all the numbers of stomatal density in each treatments (concentrations and times).

Table 2. Size and Density of Stomata *Artemisia annua*

Leaves Sample	Stomata		
	Length (mm)	Width (mm)	Density (amount/mm^2)
AA	0.025	0.017	174.7
BA	0.027	0.018	146.2
CA	0.033	0.020	128.3
DA	0.036	0.021	124.8
EA	0.036	0.021	104.3
AB	0.029	0.018	153.3
BB	0.032	0.019	149.7
CB	0.033	0.019	142.6
DB	0.035	0.020	135.5
EB	0.037	0.021	121.2
AC	0.029	0.018	168.1
BC	0.031	0.018	162.6
CC	0.032	0.018	146.2
DC	0.032	0.020	128.3
EC	0.032	0.024	124.8
AD	0.028	0.016	224.6
BD	0.030	0.016	206.8
CD	0.031	0.017	174.7
DD	0.032	0.019	158.3
ED	0.035	0.019	135.5

Number of stomatal density of control sample/AA (0%, 0 min) was 174.69 amount/mm^2. It is higher than other leaves sample in same soaking sprouts times and increasing concentration of *Artemisia annua* BA (146.2 amount/mm^2), CA (128.3 amount/mm^2), DA (124.8 amount/mm^2) and EA (104.3 amount/mm^2). The data showed that the control sample AA have lower stomatal density than the others. Number of stomatal density present the stomatal density in leaves samples. When the number calculate result of stomatal density high, it present that have lower stomatal density. While in number calculate result of stomatal density lower, it present that have higher stomatal density in leaves samples.

Increasing concentration of water extract of *Gloriosa superba* seeds and same soaking sprouts times of *Artemisia annua* in Figure 4**.** shows the increasing of stomatal density in each treatment. Number of stomatal density in control sample/AA (174.7 amount/mm^2) stomatal lower than the other, except two leaves samples AD (0%, 90 min) with 224.59 amount/mm2 and BD (24%, 90 min) with 206.77 amount/mm^2. Condition of both AD leaf sample and BD leaf sample may be occurred due to the ploidy levels of it and environment factors. In other results, there are some treated leaf samples that have a lower density values compared to the control plants. Treated sample of EA (100%, 0 minutes) was higher (104.3 amount/mm^2) in stomatal density than the other samples because it has lower number of stomatal density. While the sample of AD (0%, 90 minutes) has lower (224.6 amount/mm^2) stomatal density because it has higher number of stomatal density.

Variation of stomatal density in this study related to length and width or size of stomata. Stomata that longer and wider than others have higher stomatal density and lower number of stomatal density. Therefore the difference number of stomata lenght and stomata width in same wide field of view influence the number stomatal density. Based on the data that present in Table 2 proved in treatment with same soaking sprouts time 90 minutes. Leaf sample AD (0%, 90 minutes) and BD (25%, 90 minutes) have the same and lower number of width (0.016 mm) than other leaf samples. In number of stomatal length both AD (0.028 mm) and BD (0.030 mm) have low number. These data prove that both samples have higher number of stomatal density and lower stomatal density than others treated leaves sample.

The research has been done to show that the plant by increased concentration treatment water extracts of *Gloriosa superba* seeds is increasing in the stomatal density. It is possible that the wider stomatal diameter of the plants is the indication of the ploidy that is greater than the normal diploid plant. Therefore, the number of length and width directly proportional with number of stomatal density. Research conducted by Rantau, *et al.* (2014) proved that by the treatment of colchicine on the in vitro of *Artemisia Annua* L. germination resulted in the variation in size and density of stomata depending on the concentration and duration of soaking in *Artemisia annua* L sprouts. (Rantau *et al.,* 2014).

Herawati, *et al.* (2015) stated that leaf stomatal density was strongly influenced by ploidy level. The polyploid plants had low stomatal density. A low density of stomata on polyploidy

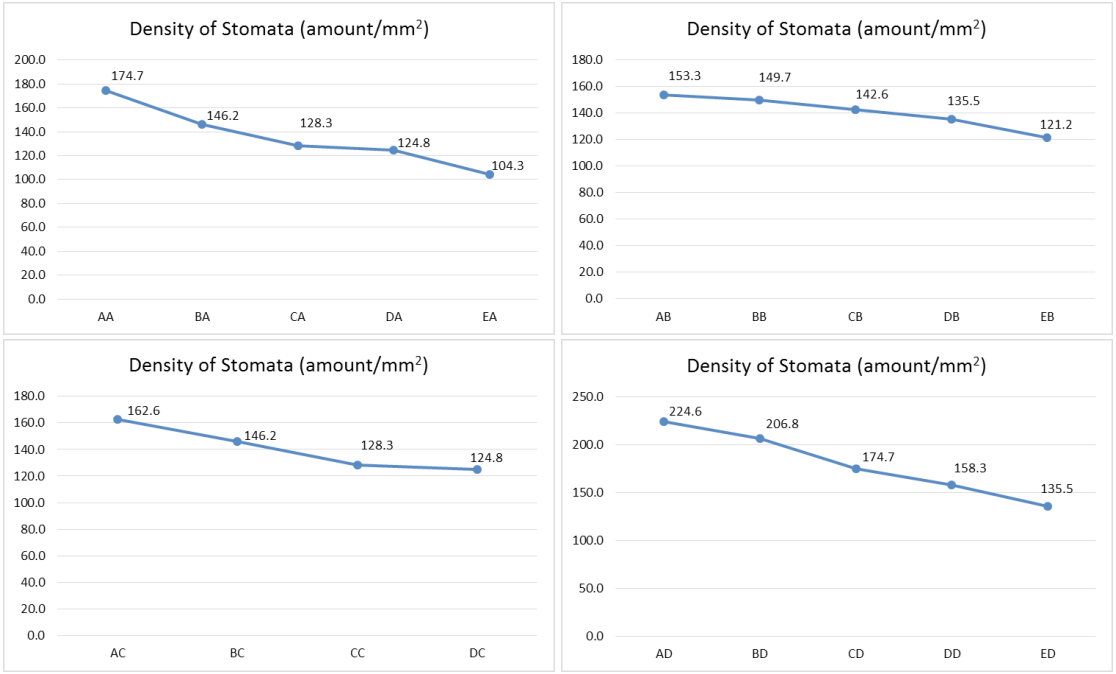

Figure 4. Density Graph of Stomata *Artemisia annua* Soaked in Water Extract of *Gloriosa superba* seeds in concentration increased and same soaking times

plants was influenced by sizes of the stomata. The stomatal size on polyploidy plants was greater than that of diploid plants. Greater stomatal sizes may be used as consideration factors for polyploidy cytological determination.

This study shows that water extract of *Gloriosa superba* seeds influence the size and density of *Artemisia annua* stomata. Polyploid plants had a larger stomatal size and density than diploid plants. The results of this study is possible to appear *Artemisia annua* polyploid indicated by stomatal size and density. Bio mutagen in *Gloriosa superba* can be utilized for plants induction to produce ploidy plants and increasing the metabolism of plants. In this study is also possible that treated *Artemisia annua* produce a higher artemisinin than diploid plant of *Artemisia annua*.

CONCLUSION

Based on the results of the research, there are variation in size and density of *Artemisia annua* stomata soaked in water extract of *Gloriosa superba* seeds. Analysis result from the data observation of stomatal size and density shows that there are notably different between treated plants. Stomatal size and density has increased with the increasing concentration extracts. Different individual plant responses result in different stomatal sizes and densities. The stomata observation results indicated level of *Artemisia annua* ploidy different each other.

ACKNOWLEDGEMENT

This research was financially supported by Prof. Dr. Ir. Ahmad Yunus, M.S. Thanks to Dr. Ir. Yuli Wiyastuti as field supervisor from B2P-2TOOT. *Artemisia annua* seeds in this research were obtained from B2P2TOOT, Tawangmangu, Karanganyar.

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