Effect of drought stress on the anatomical structure of red flowering
Hoya coronaria Blume leaves

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ABSTRACT. *Hoya coronaria* is an epiphytic ornamental plant with non-succulent leaf types found in nutrient-poor, acidic, hot, and sandy forest areas (kerangas forest). Drought stress can inhibit growth affecting its morphology and anatomy. This study aims to determine changes in the characteristics and anatomical adaptations of *H. coronaria* plants to drought stress. This study uses a randomized block design (RBD) with one factor of five replications. The main factor is drought stress consisting of A0 (watering once every 1 day), A1 (watering once every 2 days), A2 (watering once every 3 days), A3 (watering every 4 days), and A4 (watering every 5 days). The incision uses the Whole mount method (paradermal), the freehand technique, and the paraffin method (transversal). Parameters observed, including stomata, leaf thickness, mesophyll thickness (palisade and sponge), upper epidermis thickness, lower epidermis thickness, and cuticle thickness, were then analyzed using the ANOVA test at a significance level of 95% and Duncan's Multiple Range Test (DMRT) of 5%. The results show that the A0 treatment experienced a significant difference in stomatal density (90.634 mm^2), stomatal index (8.8%), and epidermis width (21.51 μm). Treatment A1 showed no significant difference. The A2 treatment experienced a significant difference in guard cell length (27.73 μm) and epidermal width (23.78 μm). The A3 treatment showed a significant difference in the guard cell width (5.49 μm). The A4 treatment showed a significant difference in stomatal density (66.589 mm^2), stomatal index (7.1%), and guard cell length (29.40 μm). Increasing the watering interval in *H. coronaria* experienced changes in the anatomy of stomatal density, stomata size, and epidermal width.

Keywords: Drought stress; epidermal width; Hoya coronaria; leaf anatomy; stomata size;

INTRODUCTION

Hoya is a group of climbing epiphytes from the *Apocynaceae* family which has a variety of flower shapes and colors and is potentially used as ornamental plants with high selling value (Robika & Henri, 2020). Flowers in the *Hoya* genus are included in compound flowers which structurally have flowers consisting of five petals, crown, corona, and corolla (Muharrawati, 2015). The Hoya species found in Bangka Belitung is *Hoya coronaria* distributed in various Asian countries such as Southeast Asia, the Malay Peninsula, Thailand, Java, Kalimantan, Sumatra, and the Philippines (Rahayu & Fakhurrozi, 2020). *H. coronaria* has 6 varieties that can be distinguished by the main characteristic of flower color, namely pink corona with yellow corolla, white corona with yellow corolla, pink corona with pink honey-striped corolla, and white corona with pink corolla with honey stripes and white corolla (Deswanti *et al*., 2018).

Based on the leaf thickness, Hoya has three leaf types: succulent, semi-succulent, and non-succulent (Robika *et al*., 2015). The difference in Hoya leaf thickness is caused by the varying thickness of the cuticle, the thickness of the epidermal layer, and the thickness of the mesophyll tissue (Hakim *et al*., 2013). The level of leaf succulence is related to the path of photosynthesis (Robika *et al*., 2015). *H. coronaria* belongs to the non-succulent category with a C3 photosynthesis path. *H. coronaria* leaves have an epidermal layer which consists of a single layer of cells and is composed of dense epidermal cells like non-succulent leaf types in general. The anatomical structure of *H. coronaria* leaves is composed of cuticle tissue, palisade tissue which functions in absorbing photons for the process of photosynthesis, spongy tissue functioning for CO_2 fixation, has large and wide intercellular air cavities, and transport tissue with sheath cells (Robika *et al*., 2022). While

experiencing drought stress, plants with a C3 photosynthesis pathway will cause stomata to close and the rate of transpiration and photosynthesis to decrease (Joshi *et al*., 2022).

The habitat of *H. coronaria* in Bangka Belitung is found in a typical bush forest area (kerangas forest) and heath forest with soil characteristics of nutrient-poor, acidic (low pH), and hot with a dense layer of quartz sand (Rahayu & Fakhurrozi, 2020). Soil with lack nutrients can result in growing plant vegetation having specific characteristics as a result of adaptation to limited environmental conditions (Deswanti *et al.*, 2018). One of the conditions of nutrient deficiency in plants is a lack of water supply in the soil or an imbalance process between water demand and supply which is called drought (Rosawanti *et al*., 2015).

Drought stress can influence leaf anatomy observed leaf anatomical responses in several rice varieties (*Oryza sativa*) and found that drought stress resulted in smaller bulliform cell size, thicker epidermal cell walls, and smaller size and number of stomata (Zagoto & Violita, 2019). According to Mangena (2018) showed that drought stress had an effect on soybean plants (*Glycine max*), such as decrease in mesophyll thickness, decrease in stomata index, and decrease in the number of trichomes.

Moreover, Hidayati *et al*. (2017) stated that the number and density of stomata are closely related to plant adaptation to drought conditions. Therefore, this study aims to determine changes in the characteristics and anatomical adaptations of *H. coronaria* plants to drought stress. The research is expected to provide benefits, including as basic knowledge regarding the anatomical structural characteristics of the *H. coronaria* plant and become a reference in the process of cultivating plants as local ornamental plant candidates.

MATERIALS AND METHODS

The research was conducted in these locations: Laboratory of Biology and the Research and Experimental Garden, Faculty of Agriculture, Fisheries and Biology, Universitas Bangka Belitung.

Planting and maintenance of experimental plants. The *H. coronaria* cuttings that were used were 2-internode cuttings planted in polybags with black sand growing media. Black sand was used as a nursery medium because it allows for pore space that supports rooting and root growth during seeding until the cuttings produce roots (Putri *et al*., 2019). Then, after the plant cuttings have plant roots, they were transferred to a larger polybag with planting media. The planting media used a mixture of cockpit and chopped fern roots with a ratio of 1:5. The plants were treated under UV plastic shade and 70% paranet with once-every-day watering until the plants are 3 months old (totaling 20 leaves). In addition, the treatment applies NPK fertilizer with an element ratio of N:P:K, i.e., 1:1:1 and the dose was 2 g/L every week. Three months old of plants were treated with drought stress for 60 days. The experimental design used was a randomized block design (RBD) using 1 factor and 5 treatments, total of plant were 15 plants. The volume of water needed for watering is 300 mL/plant (Muharrawati, 2015).

Sampling and sample preparation. A sampling of *H. coronaria* leaves been treated with drought stress was carried out in the research and experimental garden at the Universitas Bangka Belitung. Purposive sampling technique was used in this study. Each drought stress treatment was carried out three individual repetitions, each individual had 3 leaves repetitions. The type of sample used has characteristics of medium leaf age, fresh and healthy, as well as still intact. The leaf sample used for the paradermal incision was cut and then put it into a plastic sample bottle that was filled with 70% alcohol; while for the petiole sample, the transverse incision was cut and then put it into a plastic sample bottle that was filled with 70% alcohol, then then each bottle was labeling (Johansen, 1940).

Making a paradermal incision. Paradermal incisions were made by the whole mount method (Sass, 1951). The steps for making semi-permanent paradermal preparations used the whole mount method that were as: *H. coronaria* leaves were cut into 3 x 4 cm sizes, then fixed, namely the cut leaves were soaked in 70% alcohol for at least 24 h. The next step was washing samples with water (aquades) for 5 min. The samples were then sliced paradermally using a razor by removing the upper

epidermis of the leaf. After the slicing process, the samples were soaked in bleach for 5 minutes to remove chlorophyll, then the samples were washed again using distilled water for 10 minutes. The next process was staining where the incision was placed on the glass preparation, and the incision was dripped with 0.5% safranin solution Then, the solution was absorbed using a tissue and dripped with 25% glycerin solution. After dripping with a 25% glycerin solution, the glass slide was covered with a cover glass and labeled with the treatment name. Observation of the incision was carried out under a light microscope with a magnification of 400 times (Sass, 1951).

Making a Transverse Incision. Transverse leaf incisions were made using the paraffin method with Johansen series solutions for dehydrating and clearing. The working stage in the paraffin method was that the leaves that have been fixed in 70% alcohol were immersed in a solution of FAA (formaldehyde, glacial acetic acid, and 70% alcohol in a ratio of 5:5:90 ml) for 3 days. Then, they were washed using 70% alcohol for 1 h and 50% ethanol 4 times per 1 h. Dehydration and clarification were carried out in stages and immersed in Johansen series I-VII solution. Then, paraffin was infiltrated with a melting point temperature of 60 °C gradually in an oven. Samples were embedded in blocks made of cardboard in the shape of cubes filled with pure paraffin and coded. After the paraffin block solidified, it was immersed in Gifford softener solution (glacial acetic acid, absolute glycerin, and 60% alcohol in a ratio of 20:5:80 ml) for 4 weeks. The paraffin block was trimmed and then affixed to the holder and sliced using a microtome with a thickness of 10µm. The results of the incisions were placed in an object glass smeared with albumin-glycerin (1:1 ratio) and dripped with distilled water then heated using a hotplate at 30 °C for ± 12 hours. Staining of samples using 1% safranin dye and 0.5% fast green was then given Entellan closed using a cover glass and labeled. The transverse incision preparations were put into the oven at 40 °C for ±12 hours and observed under a microscope with magnifications of 10, 100, 400, 1000 times (Johansen, 1940).

Microscopic observation of preparations with a light microscope. Observation of paradermal leaf incisions there were include the number of stomata and epidermal cells and the length and width of guard cells The observation of leaf transverse incisions there were the thickness of the upper and lower cuticle, upper and lower epidermis, and mesophyll tissue. While, observations in the petiole was include the diameters of the stalk leaves and xylem. Observations were carried out under a light microscope with a magnification of 400 times. Each leaf paradermal section was observed in 5 areas, while the transverse section of the stem was observed in 3 areas. Measurements were made with a micrometer calibrated with a light microscope. Paradermal observations on stomatal density were carried out through manual calculations using the formula (Wilmer, 1983; Dorly *et al*., 2016). Stomatal density per field of view is obtained using the following formula:

$$\text{Stomata density} = \frac{\text{Number of stomata per field of view}}{\text{Area of stomata's field per view (mm}^2)}$$

Stomata index per field of view is obtained using the following formula:

$$\text{Stomata index} = \frac{\text{Number of stomata per field of view}}{(\text{Number of stomata} + \text{Number of epidermis})\text{per field of view}} X\ 100$$

Data analysis. Data were analyzed descriptively to determine and compare the effect of drought stress on the anatomical structure of *H. coronaria* leaves. Paradermal leaf anatomy variables were including stomata density, stomata index, and stomata size. Analysis of leaf transverse data used a variable value which was the average value of the measurement results of the three areas of the field of view. Anatomical analysis data of *H. coronaria* for each treatment uses SPSS 25.0 software and then was tested using the ANOVA with a significance level of 95%. In addition, if there was a significant difference, the Duncan Multiple Range Test (DMRT) was then administered.

RESULTS AND DISCUSSION
Paradermal anatomical structure of the *Hoya coronaria* **leaves.** Fig. 1 shows that the paradermal anatomical structures include stomata, epidermis, neighboring cells, trichomes, and guard cells. *H. coronaria* plants have anisocytictomata with kidney-shaped guard cells. The anisocytic

stomata is characterized by guard cells surrounded by 3 neighboring cells that have different sizes. *H. coronaria* has a hypostomatic stomata, which is indicated by the location of the stomata on the underside of the leaf (abaxial).
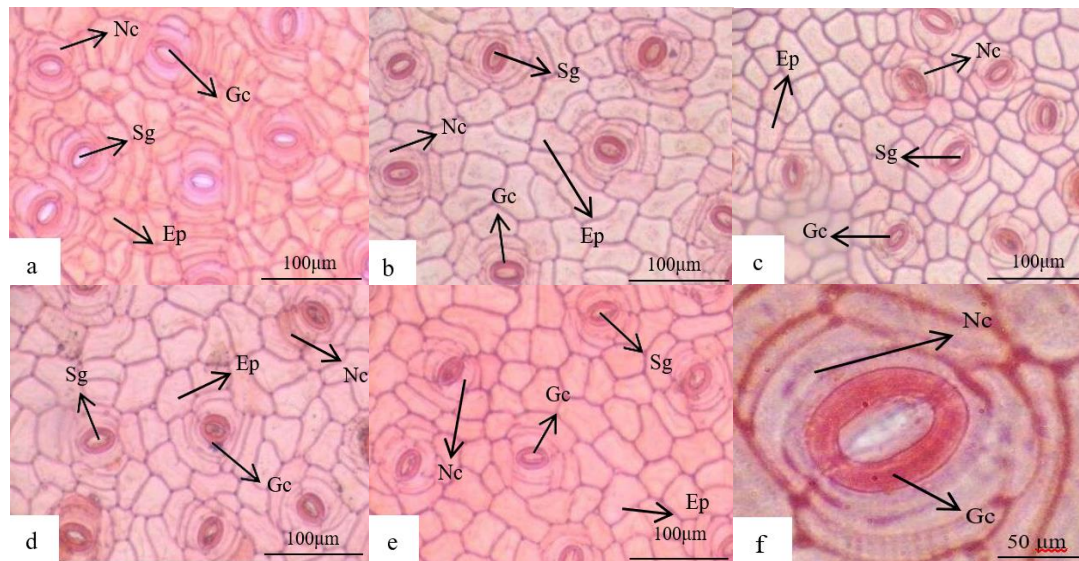

Fig. 1. Paradermal section: a. A0; b. A1; c. A2; d. A3; e. A4; f. Stomata. Nc= Neighbor cells; Gc= Guard cells; Ep= Epidermis; Sg= Stomata gap

The results of paradermal anatomy analysis of *H. coronaria* leaves show that drought stress treatment on paradermal anatomy affected the parameters of stomatal density, stomatal index, stomata size, and epidermis width. The control treatment shows a significant difference in stomatal density, stomatal index, and epidermis width. Treatment A1 shows no significant difference. In the A2 treatment, significant differences were found in guard cells length and epidermis width. Treatment A3 shows a significant difference in guard cell width, whereas treatment A4 shows a significant difference in stomatal density, stomata index, and guard cell length (Table 1).

Table 1. Measurement of paradermal anatomical parameters of *Hoya coronaria*

Treatment	SD (mm^{-2})	SI (%)	SW (μm)	GCW (μm)	GCL (μm)	EW (μm)	EL (μm)
A0	90.634 [b]	8.8 [b]	21.51 [a]	6.00 [b]	28.44 [ab]	21.51 [a]	40.14 [a]
A1	93.244 [b]	8.6 [b]	20.60 [a]	5.93 [b]	28.46 [ab]	22.71 [ab]	38.56 [a]
A2	86.533 [ab]	7.9 [ab]	20.66 [a]	5.90 [b]	27.73 [a]	23.78 [b]	38.95 [a]
A3	77.400 [ab]	7.4 [a]	20.53 [a]	5.49 [a]	27.92 [ab]	22.34 [ab]	37.89 [a]
A4	66.589 [a]	7.1 [a]	21.48 [a]	5.89 [b]	29.40 [b]	22.46 [ab]	40.22 [a]

Note: Numbers followed by the same letter in the column represent no significant difference based on Duncan's test with a 95% confidence level; SD = Stomata Density; SI = Stomata Index; SW = Stomata Width; GCW = Guard Cell Width; GCL = Guard Cell Length; EW = Epidermis Width; EL = Epidermis Length; A0 = Watering once a day; A1 = Watering every 2 days; A2 = Watering every 3 days; A3 = Watering every 4 days; A4 = Watering every 5 days

Stomata density is an indicator of the level of transpiration, metabolism, absorption of water and minerals (Ilahi *et al*., 2018). The results of statistical analysis show that drought stress affects stomatal density parameters. The stomata density in the treated *H. coronaria* plants showed significant differences in treatment A4, whereas those for A0, A1, A2, and A3 did not show significant differences (Table 1). However, the data display shows a decrease in stomata density for the treatment given and stomata density in *H. coronaria* plants is relatively low. This can be seen based on the Marantika category (2021), namely low stomata density (<300/mm^2), medium density (300-500)/mm^2) and high density (>500/mm^2), A0 (watering once a day) (90.634 mm^2), A1 (watering every 2 days) (93.244 mm^2), A2 (watering once every 3 days) (86.533 mm^2), A3 (watering once every 4 days) (77.400 mm^2), and A4 (Watering once every 5 days) (66.589 mm^2).

This is because the long interval of watering causes stress on *H. coronaria*. This is in line with Widianti *et al*., (2017), stating that the *Jatropha curcas* plant experienced a decrease in stomatal density from 70% to 40% water stress, which was thought to reduce the rate of excess transpiration in the plant. The stomatal density in plants affects drought, as a form of plant adaptation that experiences a lack of water will cause low stomata density in plants (Rindyastuti & Hapsari, 2017).

Stomatal density affects two important processes in plants, namely transpiration and photosynthesis. Plants with a higher stomatal density have a higher transpiration rate than plants with a low density (Hasanuzzaman *et al*., 2023). A higher density of stomata is an indicator of a high rate of transpiration, metabolism, and absorption of minerals and water. A high rate of transpiration causes a high rate of water loss resulting in plants experiencing drought stress (Arzani *et al*., 2013).

Reducing a large number of epidermal cells compared to reducing the number of stomata per leaf area will affect the stomata index in a plant, where the stomata index will increase *(Windarsih et al*., 2022)*. The results of the statistical analysis show that drought stress treatment had significant difference in treatments A0 (8.8%) and A4 (7.1%), compared to treatments A1, A2, A3 which were not significantly different and there was a decrease in stomata index of each treatment given (Table 1). This is in line with Widianti *et al*., (2017) stating that there was a decrease in the stomata index in Cisokan plants when watering every 6 days and every 12 days, it is suspected that Cisokan plants are more resistant to drought stress. Furthermore, Basu *et al*., (2016) stated that the stomata index decreases with increasing drought stress as a form of plant adaptation to drought stress conditions to prevent excessive transpiration.

The index of stomata is more resistant to drought because it can reduce the transpiration rate. In limited water conditions, plants will close their stomata to regulate water loss and return of CO_2 which is essential for the availability of CO_2 fixation during photosynthesis (Guerrieri *et al*., 2019). Furthermore, Pirasteh-Anosheh *et al*., (2016) found a higher stomatal index in stressed conditions causes plants to wilt easily because the transpiration rate increases due to the increased number of stomata. Stomata size and density are related to drought resistance. In plants experiencing drought stress, the number of stomata decreases to reduce water loss during transpiration (Hidayati *et al*., 2017).

The stomata length and width are closely related to the size of the stomatal pores; the larger the size of the stomata, the larger the stomata pores will be. This results in a high rate of transpiration because more water comes out which will increase the absorption of nutrients in the soil *(Dama et al.*, 2020). Moreover, Juairiah (2014) stated that stomata length is classified into 3: less long (<20 μm), long (20-25 μm), and very long (> 25 μm); while for stomata width, they are classified into three parts, namely less wide (<19.42 μm), wide (19.42-38.84 μm), and very wide (>38.84 μm). The results of stomatal measurements show that the stomata length ranges from (27.73-29.40 μm) which is classified as very long; while for stomata width (20.53–21.51 μm), it is categorized as wide. Moreover, the results of the data analysis show the stomata width on the treatment of long watering intervals was no significant difference, whereas the stomata length showed a significant difference in treatments A2 (27.73 μm) and A4 (29.40 μm).

Transverse anatomical structure of the *Hoya coronaria* leaves. The results of image observation on the transverse anatomy of *H. coronaria* leaves show there were upper cuticle, upper epidermis, mesophyll which is composed of palisade, spongy and intracellular spaces, lower epidermis, and lower cuticle. Epidermal tissue in the *H. coronaria* plant is composed of dense epidermal cells and only consists of a single layer of cells (uniserat), which is found on the adaxial and abaxial sides and is covered by a layer of cuticle. In the mesophyll tissue of *H. coronaria* leaves, it differentiates into palisade parenchyma and spongy parenchyma. The palisade on *H. coronaria* leaves was found only on one side of the adaxial part of the leaf; while on the other side, there was a sponge so that the leaves are called dorsiventral or bifacial leaves. Observations of the transverse anatomy of the leaves can be seen in (Fig. 2).
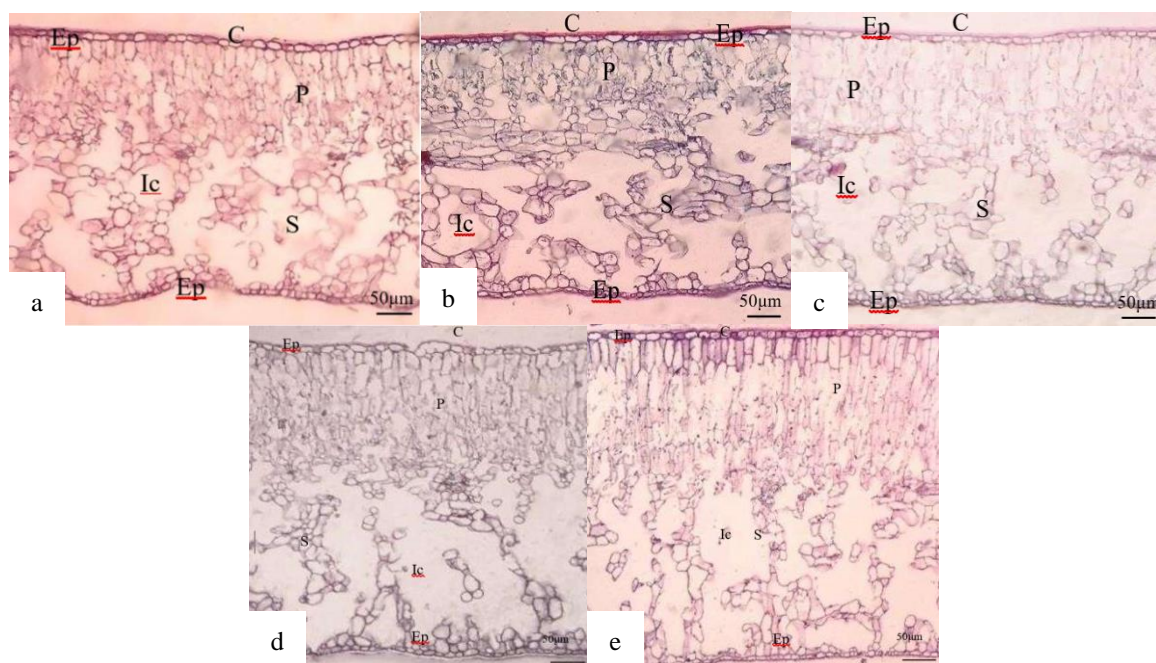
Fig. 2. Transverse section: a. A0; b. A1; c. A2; d. A3; e. A4. C= Cuticle; Ep= Epidermis; P= Palisade; S= Sponge; Ic= Intracellular cavity

The results of the statistical analysis show that drought stress had no significant effect on the transverse anatomy as evidenced by no significant difference found in each treatment. However, the results of the image observations show that the damage to the palisade tissue occurred along with the increase in different watering intervals (Table 2).

Table 2. Measurement of the transverse anatomical parameters of *H. coronaria* leaves

Treatment	LT (μm)	UET (μm)	PT (μm)	ST (μm)	MT (μm)	LET (μm)	BCT (μm)	UCT (μm)
A0	751.08 [a]	19.68 [a]	247.65 [a]	462.22 [a]	713.58 [a]	16.00 [a]	4.48 [a]	6.91 [a]
A1	832.56 [a]	18.97 [a]	279.69 [a]	512.72 [a]	792.13 [a]	15.48 [a]	4.88 [a]	7.47 [a]
A2	832.25 [a]	22.10 [a]	293.15 [a]	496.54 [a]	794.29 [a]	14.37 [a]	5.65 [a]	7.73 [a]
A3	867.44 [a]	20.35 [a]	307.96 [a]	517.47 [a]	823.92 [a]	16.30 [a]	5.05 [a]	7.31 [a]
A4	913.73 [a]	21.57 [a]	306.79 [a]	571.98 [a]	871.30 [a]	17.27 [a]	4.77 [a]	7.89 [a]

Note: Numbers followed by the same letter in the column represent no significant difference based on Duncan's test with a 95% confidence level; LT = Leaf Thickness; UET = Upper Epidermis Thickness; PT = Palisade Thickness; ST = Sponge Thickness; MT = Mesophyll Thickness; LET = Lower Epidermis Thickness; BCT = Bottom Cuticle Thickness; UCT = Upper Cuticle Thickness; A0 = Watering once a day; A1 = Watering every 2 days; A2 = Watering every 3 days; A3 = Watering every 4 days; A4 = Watering every 5 days

The transverse anatomy of *H coronaria* leaves is composed of the upper cuticle, upper epidermis, palisade, spongy, lower epidermis, and lower cuticle. The thick leaves of the *H. coronaria* plant are mostly composed of mesophyll tissue. The results of data analysis on leaf thickness show that there was no significant difference in the treatment, but there was an increase in leaf thickness with the treatment given (Table 2) at A0 (751.08 μm), A1 (832.56 μm), A2 (832.25 μm), A3 (867.44 μm), A4 (913.73 μm). According to Basu *et al*., (2016), found one form of plant adaptation to drought stress is the addition of leaf thickness, because the cuticle layer will be thicker which can inhibit water loss. An increase in leaf thickness can be an indicator of plant tolerance to drought stress *(Savira et al.*, 2023).

The increase in leaf thickness is accompanied by an increase in the thickness of the epidermal cells. Epidermal tissue in *H. coronaria* plants is composed of dense epidermal cells and consists of only one layer of cells (uniserat). The thickness of the epidermal cells in the treatment of this study showed no significant difference between the treatments (Table 2) but showed an increase in the size of the epidermal cells; the longer the watering interval, the thicker the epidermal layer. Moreover, Paluvi *et al.*, (2015) stated that thickening of epidermal cells indicates a reduction in the rate of

transpiration. This certainly provides distinct advantages for plants in maintaining the amount of water in their tissues. This is in line with Zagoto & Violita (2019) stating that the anatomical response of *Oryza sativa* leaves to drought stress causes an increase in the thickness of the epidermal cell walls. In addition to the thickening of the epidermal cells that protect the cells and tissues beneath them, the epidermal cells are covered by a cuticle.

The cuticle is the outermost layer of epidermal cells which is divided into two layers, namely the outermost layer which consists of the cutin layer (true cuticle) and the inner layer (cuticular layer) which contains cutin (Alponsin *et al*., 2017). The results of statistical analysis show there was no significant difference in cuticle thickness in the upper and lower cuticles, but there was an increase in cuticle thickness in each treatment given (Table 2). The increase in cuticle thickness is thought to be an adaptation to reduce excessive water expenditure on leaves due to a stressed environment (Bi *et al*., 2017). Thicker cuticles are an adaptation to water stress (Le Provost *et al*., 2013).

H. coronaria leaves have mesophyll tissue which is differentiated into palisade and spongy tissue which is located under the epidermal cells. The palisade on *H. coronaria* leaves was found only on one side of the adaxial part of the leaf; while on the other side, there was a sponge so the leaves are called dorsiventral or bifacial leaves. The shape of the palisade tissue of *H. coronaria* leaves resembles spongy cells, tightly arranged without intercellular cavities, and consisting of several layers; whereas sponge tissue has cells that are not tightly arranged so that there are wide and large intercellular cavities. Based on observations of the palisade tissue in treatments A0 and A1, the arrangement was denser when compared to treatments A2, A3 and A4, and the intracellular cavity in the sponges was relatively larger as the watering intervals increased. The palisade tissue functions in absorbing photons for the process of photosynthesis, while spongy tissue functions for CO_2 fixation. The presence of intercellular cavities in sponges can facilitate the CO_2 gas diffusion process and can increase the efficiency of photosynthesis by contact between the intracellular space and mesophyll cells containing chloroplasts (Robika *et al*., 2022).

The function of the cavity found between the sponge cells is as a place for CO_2 (for photosynthesis) and O_2 (results of photosynthesis) to enter (Oguchi *et al*., 2018). Based on the results of statistical analysis on mesophyll thickness, palisade thickness, and sponge thickness, no significant difference was found, but there was an increase in thickness with the length of the watering interval given (Table 2). The measurement results also show that spongy tissue was thicker than palisade tissue. According to Alponsin *et al*., (2017) stated that the increase in mesophyll thickness (palisade and spongy) is related to the fixation of CO_2 in the air. Moreover, Tihurua *et al*., (2020) stated that the mesophyll of mangrove leaves is thicker to maximize CO_2 binding in the process of photosynthesis, especially in drought stress environments. This is thought to be the cause of the difference in the thickness of the palisade and the sponge.

CONCLUSION

Drought stress showed a significant effect on the parameters of stomata density, stomata index and stomata size of *H. coronaria* plants. The longer the watering interval given shows a decrease in stomata density, a decrease in stomatal index, and damage to the palisade tissue. There was a tendency to increase the thickness of leaves, cuticle, epidermis, and mesophyll tissue although it was not significantly different. The increase and decrease in cell size in *H. coronaria* indicates a form of plant adaptation to drought stress. So it can be concluded that *H. coronaria* plants are vulnerable as seen from changes in anatomical structure and tissue damage that occurs to drought stress.

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