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Anatomical, morphological and physiological leaf characters of black betel (*Piper betle* L. var. *nigra*) in varying natural and man-made habitats

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Abstract. *Kuswandi PC, Ariyanti NA, Yunus MF, Amri CNAC. 2023. Anatomical, morphological and physiological leaf characters of black betel (*Piper betle *L. var.* nigra*) in varying natural and man-made habitats. Biodiversitas 24: 3236-3244. Piper betle* L. var. *nigra* or black betel (known as *Sirih hitam* in Indonesia) contains valuable secondary metabolites, such as alkaloids, flavonoids, saponins, tannins, phenols, carotenoids, steroids and terpenoids. Black betel leaf extract has been shown to have antimicrobial activity thus there is a prospect to be developed as a promising herbal plant. Nevertheless, little information is available about its development as a medicinal plant. This research studies the influence of different habitats on several characters of black betel leaves with the aim to understand the suitable environmental conditions for the optimum growth of black betel plants. We used a survey method and random sampling of black betel leaves in four locations in Java Island, Indonesia, namely Banyuwangi which represents natural habitat, and Karanganyar, Ngaglik and Pakem which represent man-made habitats. Measurements of temperature, humidity, soil moisture and light intensity were carried out at each location. Analyses of leaf area, leaf water content, total leaf chlorophyll content and flavonoid content were undertaken and statistically analyzed using SPSS software. Leaf transverse sections were also observed. The results showed that the environmental parameters differed in the four locations. Leaves samples from the natural habitat in Banyuwangi were significantly different ($P<0.05$) from the three man-made habitats for chlorophyll and flavonoid content. For water content, significant difference was only for Banyuwangi samples with those from Karanganyar and Pakem. For leaf area, significant difference was only found between the Banyuwangi samples and Karanganyar. Observations on the transverse cross section of midrib of black betel leaves from the four locations showed structures that are generally found in *Piper betle* species, namely the presence of an epidermal layer, trichomes in the abaxial part of the leaf, several layers of the hypodermis, visible vascular tissue and the presence of secretion cells. There were several differences in the leaf anatomy such as greater number of trichomes on the leaves from Karanganyar, the secretory cells that were more visible in the leaves from Ngaglik and Banyuwangi and the sclerenchymal tissue that was more visible in the leaves from Banyuwangi. Such differences are likely influenced by variations in environmental parameters thus showing that the man-made habitat in the Karanganyar location can affect leaves characters similar to black betel plant grown in its natural habitat in Banyuwangi.

Keywords: Black betel, chlorophyll, flavonoid, habitats, leaf area, transverse section, water content

INTRODUCTION

In terms of plant diversity, Indonesia is the second largest country in the world after Brazil. Many plant species have been used for medicinal purposes by the people in Indonesia. Medicinal plants are plants that can be used as medicinal materials. The parts of the plant that can be used for medicinal uses include leaves, stems, fruits, tubers, rhizomes and roots (Nugroho 2017; Nugroho and Ningsih 2017). One plant group often used for medicinal purposes is from the *Piper* genus. This genus is widely distributed throughout tropical and subtropical regions, such as Indonesia, India, Sri Lanka, Malaysia, Thailand, Taiwan, and other Southeast Asian countries.

One important species from the genus is *Piper betle* L. (commonly known as betel) and it is widely used as a traditional medicine in Southeast Asia. There are several types of betel in Indonesia that can be differentiated based on the color of the leaves. These include green betel, red betel and black betel, yet the green betel is the most used variety (Sari et al. 2020). Traditionally, the betel leaves have been considered as herbal remedy. Chewing the raw leaves can stimulate the central nervous system activity and treat several diseases, such as high blood pressure, wounds, asthma, cough and bronchitis (Junairiah et al. 2017). The content of saponins and tannins in betel leaves is known as antiseptic for wounds and anti-inflammatory agent (Novita 2016).

Previous studies have shown that black betel in Indonesia is rich in phytochemical compounds and has the potential to be developed as supplement to maintain health (Junairiah et al. 2017). Suriani et al. (2020) stated that the black betel has its own unique volatile oil, commonly known as betel oil. A broad range of phytochemical compounds contained in the black betel leaves includes alkaloids, terpenoids/steroid, flavonoids, polyphenols, saponins, tannins and eugenol (Junairiah et al. 2018a; Junairiah et al. 2019), amides derivatives of piperenamide A and piperenamide B (Prasetya et al. 2021a). Moreover, phytochemical compounds like 1-pentene, 3-butenoic acid, and furan, acetic acid, methylamine, and pyridine have

been traced through GC-MS analysis (Junairiah et al. 2018b). In addition, Prasetya et al. (2021b) mentioned that 2-octenoic acid and 2-hexenoic acid, both fatty acid derivatives, were successfully extracted from the betel leaves. Indonesia's black betel anti-bacterial assays show that there is antimicrobial activity against pathogenic microbes such as *Staphylococcus aureus, Escherichia coli, Candida albicans, Streptococcus mutans* and *Streptococcus sanguinis* (Junairiah et al. 2017; Prasetya et al. 2012). Black betel can also be used as an eco-friendly biocontrol agent in organic rice cultivation. In Indonesia, a mixture of black betel leaves extract, *Piper caninum* Blume (locally known as *sirih hutan*) and plant growth-promoting rhizobacteria can inhibit the growth of *Pyricularia oryzae*, a fungus that causes rice blast disease, and improve the overall morphological characteristics such as height, leaves number and panicles, resulting in the increase in grain yields (Suriani et al. 2020).

In the development of medicinal plants industry, a sufficient number of raw materials is needed to obtain adequate extracts. This can be achieved by the improvement in the cultivation practices of the targeted plant so that the yield can meet the demand for the medicinal industry. However, there are several problems that usually occur in medicinal plant propagation efforts, including the absence of optimal propagation technology, the lack of farmer's knowledge regarding the quality of plant materials needed by the industry, and lack of funding in the development of medicinal plant agribusiness (Nugroho and Ningsih 2017).

Piper leaves contain flavonoids, a secondary metabolite that is produced by plants as a response to external factors, such as temperature, light, water availability or pathogen attacks. The effects of environmental factors, such as light intensity, vary in different species and can cause an increase in plant flavonoid content (Idris et al. 2018). In the context of black betel cultivation, previous study showed that propagation by cuttings in artificial habitat produced only two leaves with diameter of 3 cm after three months of planting while in its original habitat the leaves can grow up to 5-10 cm in diameter (Harjunowibowo and Rinanto 2020). Such study implies that there is a knowledge gap on how to improve the morphological characters of black betel to meet the needs of the farmers and medicinal industry. Therefore, it is necessary to conduct research to obtain a cultivation environment that can produce high quality betel plants. This study aims to investigate the influence of environmental conditions of two different growing sites: (i) natural habitat; and (ii) man-made habitat (i.e., cultivation site), on several morphological, physiological and anatomical characteristics of black betel. We expected the results of this study might provide preliminary understanding of leaf anatomical, morphological and physiological adaptation in various habitats and help black betel cultivation practices.

MATERIALS AND METHODS

Samples origin

This is an observational study with samples of black betel leaves obtained from four locations (Figure 1): (i) a natural habitat (a forest in Mount Raung, Banyuwangi, East Java); and (ii) man-made habitats (farm and gardens) in Karanganyar (Central Java), Ngaglik and Pakem (located in Sleman, Special Region of Yogyakarta). The location of black betel in Banyuwangi was in its natural habitat, where the black betel plant grew naturally in the forest supported by different tree species. The samples of betel leaves from Mount Raung Banyuwangi were considered as control and were compared with betel leaves from man-made habitats where the betel plant growing environment was manipulated to meet the growth needs of betel plants.

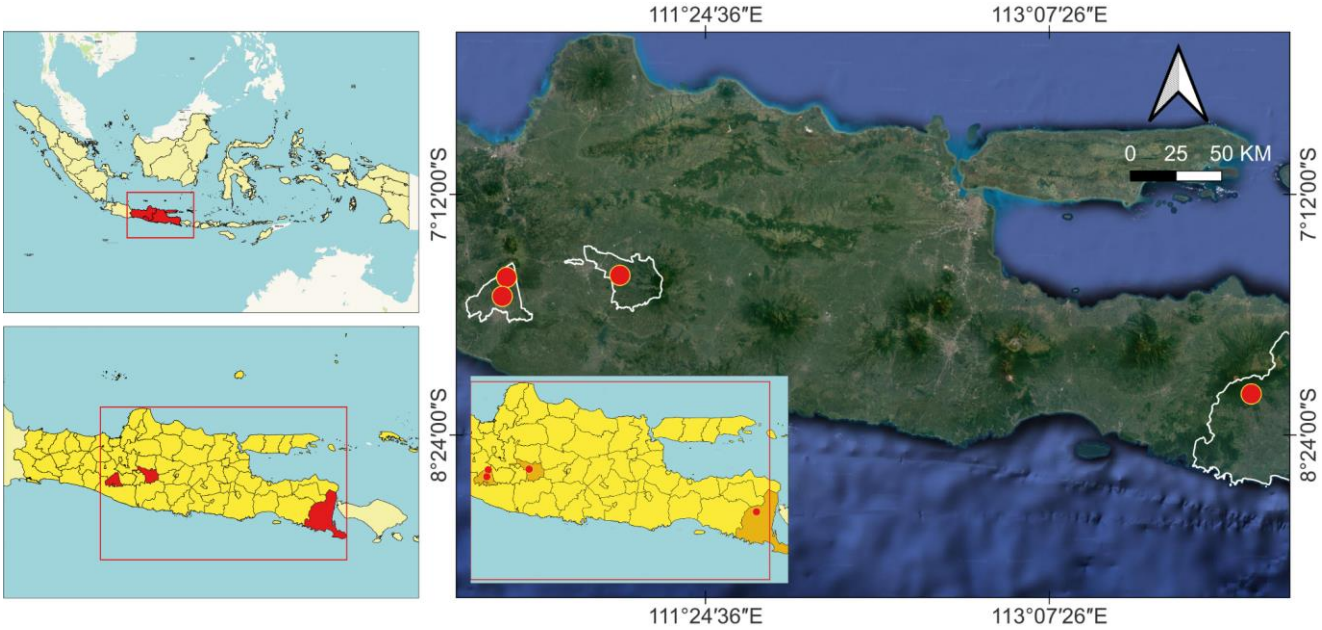

Figure 1. Map of location where the samples of black betel leaves were collected: A. Pakem (top), Ngaglik (bottom) in Sleman (Yogyakarta), B. Karanganyar (Central Java), C. Banyuwangi (East Java), Indonesia

From each site, the leaf samples taken were second to sixth leaves from the tip of ten random plants. The independent variables for this study were environmental factors measured in each location where the black betel grew, namely air temperature and humidity, soil moisture and light intensity which were measured in 5 positions for each location to find the average value. The environmental data of four locations were measured in June 2022 and during 10.00-11.00 AM. The dependent variables were leaf water content, leaf area, chlorophyll content, and flavonoid content of the betel leaves. The anatomy of the leaves was also observed using a transverse cross section of the midrib.

Measurement of leaf water content (% fresh weight)

Leaves were weighed to obtain its fresh weight then dried in the oven at 60°C for 36 hours and weighed to obtain its dry weight. The water content of leaves as a percentage of its fresh weight was determined by the following equation:

$$m = 100\% = \frac{Wm}{Wm + Wd} \times \frac{Wm}{Wt} \times 100\%$$

Where:
m : Water content (% fresh weight)
Wm : Fresh weight (g)
Wd : Dry weight
Wt : Total weight

Measurement of leaf area

Leaf length was measured from the base to the tip of the leaf and leaf width was measured at the widest part of the leaves. Leaf area was calculated using the formula:

$$A = L \times W \times c$$

Where:
A : Leaf area (cm^2)
L : Leaf length (cm)
W : Leaf width (cm)
C : Constant

The constant used was 0.672 which is usually used for *Piper betle* leaf shape (Susilo 2015).

Measurement of chlorophyll content

Chlorophyll content was measured by grinding one gram of leaves with 85% acetone. The mixture was then centrifuged for 1200 rpm and filtered. The filtrate was transferred into a 100 mL measuring flask and added with 85% acetone solvent to a final volume of 100 mL. Chlorophyll solution was poured into the cuvette to the limit line and absorbance levels were recorded for each wavelength. The amount of chlorophyll a, b, and total chlorophyll were calculated according to Arnon (1949) formula:

Chlorophyll a = 12.7 D-663 - 2.69 D-645 (mg/L)
Chlorophyll b = 22.9 D-645 - 4.68 D-663 (mg/ L)
Total Chlorophyll = 20.2 D-645 + 8.02 D-663 (mg/L)

Transverse section of leaf midrib

Preparation of the slices was based on the Sass's Safranin-Fast green coloring method (Sass 1958). The leaf anatomical study involved the processes of fixation, dehydration, de-alcoholization, paraffin infiltration and embedding, cutting, and staining. For the fixation stage, the sample was cut, then soaked into a fixative solution and allowed to stand for 24 hours. The fixative solution consisted of 95% ethanol : glacial acetic acid : formalin : aquadest with ratio of 50:5:10:35. The next step was dehydration where samples from the fixative solution were fed into 50% ethanol twice for 30 minutes each, ethanol 70% twice for 30 minutes each, ethanol 80% twice for 30 minutes each, ethanol 90% twice for 30 minutes each, and ethanol 96% three times for 30 minutes each.

Then, the leaves were placed into solutions of ethanol : xylol 1 (3:1) for 20 minutes, ethanol : xylol 2 (1:1) for 20 minutes, ethanol: xylol 3 (1:3) for 20 minutes and pure xylol twice until the sample appeared transparent. The parafinization and embedding stages were carried out in xylol : paraffin (1:1) for 15 minutes, then into pure paraffin 1, pure paraffin 2, and pure paraffin three times each for 15 minutes. After that, the samples were put into a mold containing liquid pure paraffin and allowed to stand for 24 hours at room temperature. Samples in paraffin were glued together with wood blocks and allowed to stand for 24 hours. The samples were then cut using a rotary microtome with a thickness of 12 micrometers, then placed on a glass of objects that have been smeared with glycerin albumin and aquadest. The cutting was placed on a hot plate at a temperature of 30°C then allowed to stand until dry and stretched.

The staining of the sample was carried out by inserting the sample on the object glass into a solution of pure xylol for 20 minutes, then dipped in the solution of xylol : ethanol with different ratio of (3:1, 1:1, 1:3), ethanol (96% 45%, 0%) each for 1 minute. Then, the samples were transferred into 1% solution of Safranin (aq) for 30 minutes, washed with aquadest, dehydrated with a 45%, 96% serial alcohol solution, followed by staining with a 0.1% fast green solution for 2 minutes. After that, the samples were quickly soaked in absolute alcohol, then dealcoholized with a solution of xylol: ethanol with different ratio of (1:3, 1:1 and 3:1) for 30 seconds, placed in pure xylol, then mounted using Entelan®. Lastly, the samples were dried on the hotplate at a temperature of 30°C.

Flavonoid analysis

For the flavonoid analysis, leaves were dried using an oven at 60°C for 48 hours then ground to obtain a fine powder. Five milligrams of the powder were used for analysis of total flavonoid content.

Procedure for making standard curves

Ten milligrams of Quercetin were added to 0.3 mL of 5% sodium nitrite for 5 minutes. The next step was adding 0.6 mL of 10% aluminum nitrate, waited for 5 minutes, added 2 mL of 1 M sodium hydroxide, then adjusted the volume to 10 mL. The solution was diluted according to the standard curve concentration and the absorbance was read at λ 510 nm.

Procedure for determination of total flavonoid equivalent quercetin using spectrophotometric method

One hundred milligrams of samples were weighed then added with 0.3 mL of 5% sodium nitrite for 5 minutes. The next step was adding 0.6 mL of 10% aluminum nitrate, waited 5 minutes and then added with 2 mL of 1 M sodium hydroxide. The volume of the solution was adjusted to a volume of 10 mL, diluted 5 times and the absorbance was read at λ 510 nm.

Data analysis

Data were obtained from ten plants from each location with three leaves used for analysis from each plant. Statistical analysis was undertaken for the leaf area, leaf water content and chlorophyll content using Analysis of Variance (ANOVA) in the SPSS program.

RESULTS AND DISCUSSION

Environmental factors

The results show that there were differences in several environmental parameters at four locations (Table 1). The natural environment of black betel in the forest of Mount Raung Banyuwangi is an area with low light intensity (250-1000 lux) and high air humidity (85-88%) (Harjunowibowo and Rinanto 2020).

The results show that the air temperature across the four sample locations ranged from 24-30°C. The lowest temperature was measured in Banyuwangi and the highest temperature was in Karanganyar. The natural forest where black betel plants grew in Banyuwangi was overgrown with tall trees, making the light intensity low. In Karanganyar, a two-layer paranet was used to lower the light intensity, still it was not as low as in Banyuwangi. In Ngaglik, paranet was not used but the black betel plants were grown with other plants that provide some shading, while in Pakem the light intensity was quite low even though no paranet used. Air humidity ranged from 65-88% across the four locations. Measurement of soil moisture showed large variation ranging from 13-86% which could be due to different watering times at Pakem and Karanganyar sites, so it was likely that during the time of measurement, the planting medium had not been watered. At the Ngaglik location, regular watering was carried out and at the natural habitat in Banyuwangi, soil moisture was maintained due to the presence of a canopy and the accumulation of leaves falling from trees around the place where black betel plants grow. In addition, the black betel plants also grew on different types of support. In the natural habitat it grew on different tree species while in the man-made habitats they were supported by bamboo cuttings or climbing on walls (Figure 2).

Leaf water content

Water is important for plant growth and can be an indicator of how suitable a certain habitat is for the growth of a plant species. Insufficient amount of water for plant growth is reflected in the plant's organs such as leaves. Leaf water content also affects other leaf characters such as leaf photosynthetic rate and leaf area. Statistical analysis showed that there were significant differences between leaf water content from four different locations (Figure 3). Black betel leaves from Karanganyar and Pakem showed significant difference compared to leaves from the natural habitat in Banyuwangi even though the humidity and soil moisture was lower in those two locations (Table 1). Interestingly, the leaves from Banyuwangi contained lower water content (Figure 3) compared to leaves from other locations, thus suggesting a non-linear relationship between soil moisture and humidity in each location and water content of black betel leaves. This can be explained by a study conducted by Wang et al. (2022), where a theoretical model was used to predict the relationship between leaf area and photosynthetic rate and leaf water content and temperature. The results showed that photosynthetic capacity and leaf area did not increase in linear with leaf water content. The authors also proposed that leaf water mass is a better predictor of whole-leaf photosynthesis and leaf area than whole-leaf nitrogen and phosphorus masses.

Leaf water content also reflects whether the black betel plant experienced drought stress in the sampling locations. Drought stress is an environmental factor that can affect the formation of secondary metabolite compounds which is important for plants that have medicinal properties. Secondary metabolites are compounds produced by plants usually in stressed conditions, in contrast to the primary metabolite compounds necessary for plant growth (Perangin-angin et al. 2019). An optimal condition is needed so that enough water can be obtained by the plants but can also promote the plant to produce secondary metabolites that are used for medicinal purposes.

Leaf area

Leaf area was measured in this study because it is one of plant traits that determines light perception capacity thus affecting plant biomass. However, the relationship between leaf area and plant biomass can be non-linear and vary depending on carbon partitioning (Weraduwage et al. 2015). The results of this study showed significant difference between samples from Banyuwangi with those from Karanganyar (Figure 4). No significant difference was found between samples from Banyuwangi to samples from Ngaglik and Pakem. The man-made habitats in Karanganyar, Ngaglik and Pakem had higher light intensity than in Banyuwangi and showed leaves with a lower leaf area. Low light intensity can cause the size of the plant leaves to become wider because the plant adapts to obtain enough light for photosynthesis.

Other factors such as nutrient is also an important factor affecting plant growth. A study by Fender et al. (2011) showed that soil moisture and air humidity did not have a significant influence on the leaf area of plant seeds of the genus of Fagus. However, water and nitrogen still have an important role in leaf growth even though their direct role is not yet known. Thus, further investigation is needed to find correlation between nutrients in the soil or planting media of black betel plants.

Chlorophyll content

Chlorophyll was measured in this study due to its important role in photosynthesis which is needed as an energy source for plants. Plants are known to adapt to various habitats to obtain the optimum photosynthesis (Li et al. 2018). In this study, Duncan's post hoc test analysis showed the total chlorophyll content of the leaves from Banyuwangi was significantly lower ($p<0.05$) than that from the other locations (Figure 5). This suggests that light intensity that is at similar level does not directly affect the chlorophyll content of black betel leaves. An environment with low light intensity can adversely affect the total content of chlorophyll.

Low light intensity is known to cause an increase in total chlorophyll (Zhang et al. 2016) thereby increasing the effectiveness of photosynthesis. The low light intensity in Banyuwangi did not result in the same total chlorophyll content as the leaves from Pakem where the light intensity was also quite low. The influence of other factors, such as nutrition, diseases and water availability, can also affect the chlorophyll content of the leaves in the different habitats. These results imply that providing shade, such as paranet, does not always result in the increase of growth of black betel plants by affecting their photosynthesis inferred from the chlorophyll content of the leaves.

Piper betel plants prefer to grow in lower light intensity. However another factor such as nutrients is also important in the plant's growth and development especially in producing secondary metabolites. Studies in green *Piper betel* grown in different shades (Muttaleb et al. 2018) showed that chlorophyll content was higher in plants grown under 30% and 50% shades and with addition of 100 kg/ ha of N in the soil. Full sunlight with no addition of nitrogen fertilizer was unsuitable for the plant as the high light intensity caused withering leaves and stunted growth, however it increased accumulation of secondary metabolites and antioxidant activates.

Leaf flavonoid content

Flavonoid is one of the secondary metabolites found in black betel leaves. It is known that the production of secondary metabolites is affected by the habitat conditions of a plant. Previous study showed that a higher light intensity caused the production of more flavonoids in the leaves of green betel (Muttaleb et al. 2018). Our research shows that the leaves samples from Banyuwangi had significantly lower total flavonoid content compared to the leaves from the other three locations (Figure 6). Banyuwangi showed a lower light intensity (Table 1) which might be caused the presence of many trees which served as the media for the black betel plant to grow. This finding is in accordance to other studies where a higher light intensity can cause a response against oxidative damage from UV radiation. A higher photosynthetic capacity can also increase flavonoid content in plants due to more precursor available for the production of secondary metabolites (Li et al. 2016). Thus, the result of our study which showed a higher flavonoid level in leaves from locations with higher light intensity is in accordance with the findings of other studies. Although flavonoid is only one type of secondary metabolites, this is a promising finding that suitable artificial habitat can be conditioned for the production of secondary metabolites.

Leaf anatomy

Leaf anatomy is also important parameters to observe in this study because of its structural plasticity. Leaf structure can vary in different environment and is important for plants as a form of adaptation (Hu et al. 2022). The anatomical structure of black betel leaves from the different locations showed some differences in the midrib cross sections. The thickness of the black betel leaves from Karanganyar differed from the samples from the other locations (Figure 7B). This may be due to the different growth of the leaves according to the area and other factors that can affect plant growth. The effect of temperature on leaf thickness has been observed in various plant species, such as *Arabidopsis thaliana* (L.) Heynh. (Jin et al. 2011), *Brassica oleracea* L. (Rodríguez et al. 2015) and *Glycine max* (L.) Merr. (Jumrani et al. 2017), where temperature had a significant effect on mesophyll thickness, cell size and cell number in those species. The higher temperature was found to cause significantly thicker mesophyll due to the increased permeability of plasma membrane (Purnama et al. 2018).

Observation on the leaves trichomes showed that the samples from Karanganyar recorded more trichomes compared to the other samples (Figure 7B). Simple multicellular trichome with short, pointed-end (trichomes that have more than two cells, short with sharp-pointed on the apex) was observed in the samples from all locations. The presence of trichomes can influence the water content of the plants through temperature regulation, by either reducing the absorption of radiant energy or by enhancing its dissipation once it has been absorbed and as the site of essential oil biosynthesis (Queiroz 2018; Chen et al. 2022). Trichomes also have structures that can protect leaves from abiotic and biotic disturbances (Kim et al. 2012). The type of trichomes can also be useful in identification of certain species such as in Acanthaceae species (Amri et al. 2018).

Findings on leaf epidermal characteristics showed the presence of hypodermal cells under the epidermis layer of species studied. Raman et al. (2012) revealed 1-4 layers of hypodermis cells that can be found in *Piper sarmentosum* Roxb. and *Piper betle* leaves. Research by Dewi et al. (2020) also recorded the presence of hypodermal layers in green *Piper betle*. Yuanyue et al. (2009) explained the importance of eco-physiological characters in selected mangrove species from China with the presence of hypodermis cells in *Acanthus ilicifolius* L. that functioned as water storage tissue. This unique anatomical feature in plants can help withstand extreme environmental habitat such as in coastal and mangrove area.

The arrangement of vascular bundles in the midribs can be used in the classification and identification process of selected plants species (Rudall 2020). The vascular bundle of midrib consisted of main vascular bundle (opened system with continuous rings of vascular bundle) with U-shaped were recorded in all the black betel leaf samples. The presence of sclerenchyma cells around the vascular

bundle can be used to differentiate the samples from different locations. Sclerenchyma cells are important for mechanical functions and can provide strength for the plant (Jarvis 2012). Layers of collenchyma cells are also present in all species studied. Collenchyma also functions to strengthen and protect plant structure (Chen et al. 2019).

Secretory cells are also found in the black betel leaves samples. Secretory cells are also found in green betel leaves (Raman et al. 2012). Secretion cells function in storing and secreting certain compounds. Such compounds can be secondary metabolites that can have the potential to be natural ingredients of a drug. The development of secretory cells is influenced by genetic and environmental factors. It is necessary to conduct further research on the anatomy of black betel in several environmental conditions to see the correlation between environmental factors and the ability of black betel plants to produce secondary metabolite compounds.

Table 1. Environmental parameters at four sampling sites

Location	Altitude (m asl.)	Temperatures (°C)	Air humidity (%)	Light intensity (lux)	Soil moisture (%)
Banyuwangi	701	24	88	1000	86
Karanganyar	573	30.34	65.4	1561.8	37
Ngaglik	238	26.62	79.4	1538.4	68.2
Pakem	418	24.6	69.2	1394	13

Figure 2. The growing condition of black betel plants at four different locations: A. Banyuwangi, B. Pakem, C. Ngaglik, D. Karanganyar, Indonesia

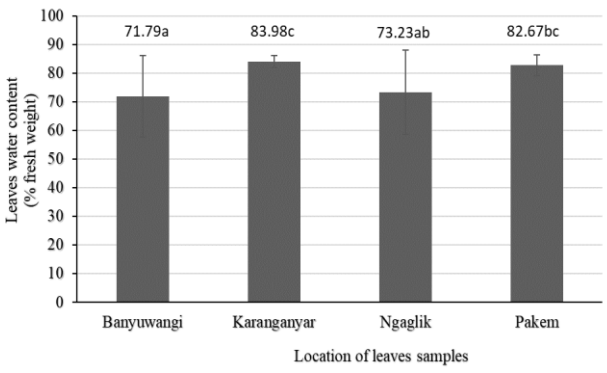

Figure 3. Water content of black betel leaves from four different locations. Letters a, b and c indicate values to be significantly different at $p<0.05$

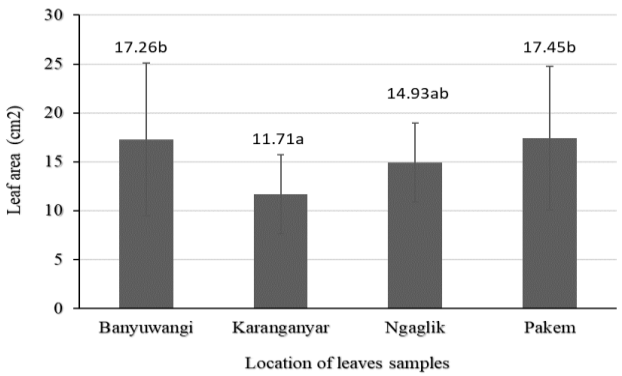

Figure 4. Black betel leaf area from four locations. Letters a, b and c indicate values to be significantly different at $p<0.05$

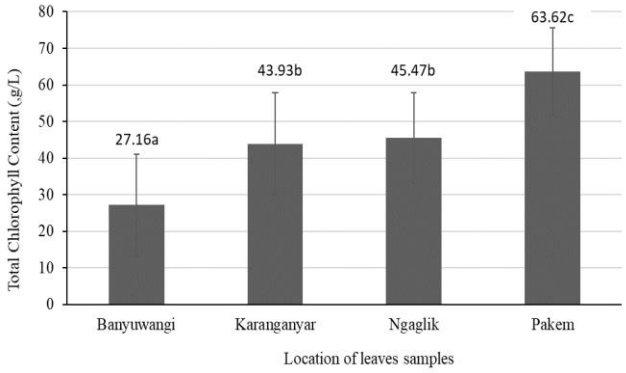

Figure 5. Total chlorophyll content of black betel leaves from four sampling sites. Letters a, b and c indicate values to be significantly different at p<0.05

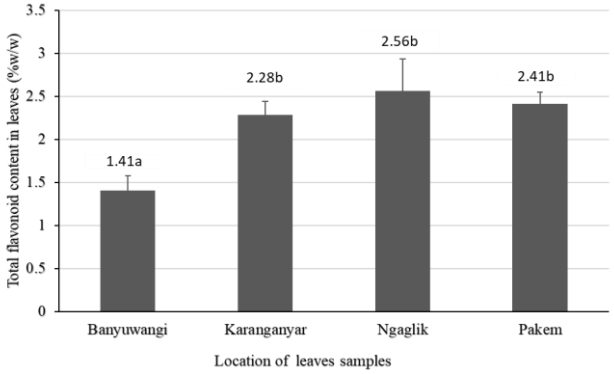

Figure 6. Total flavonoid content of black betel leaves from four sampling sites. Letters a, b and c indicate values to be significantly different at p<0.05

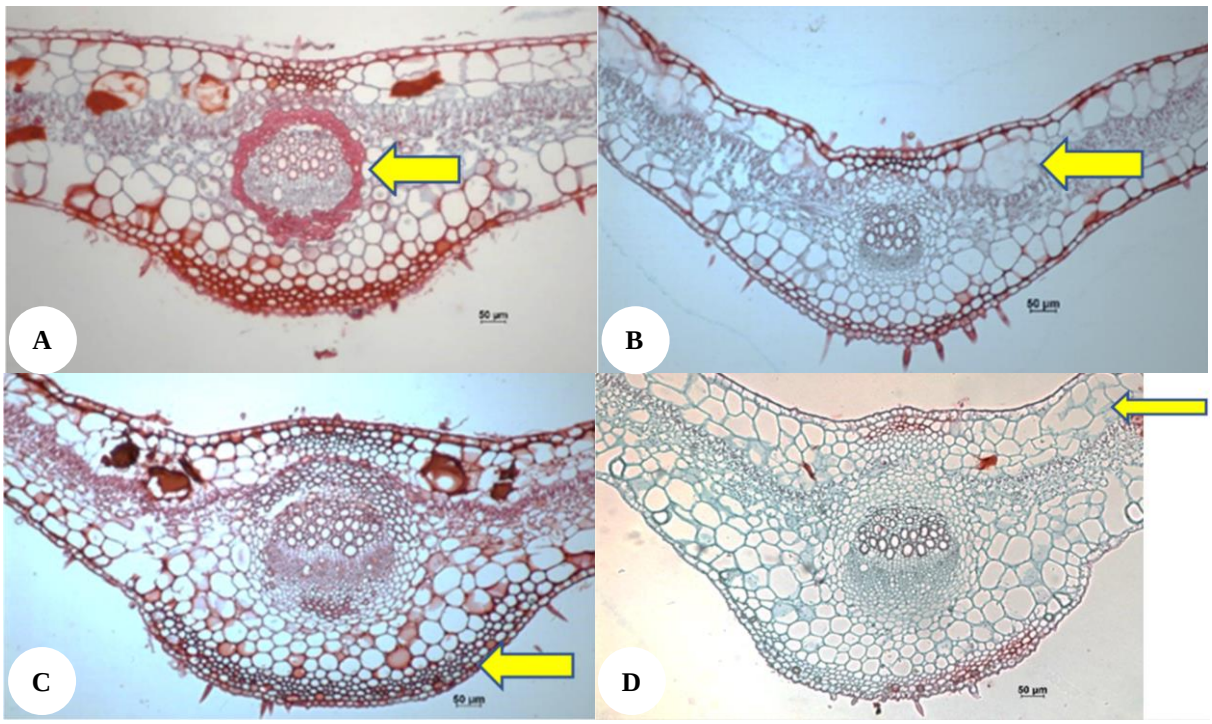

Figure 7. Cross section of midrib of black betel leaves from four locations: A. Banyuwangi: Sclerenchyma cells around vascular bundle (yellow arrow), B. Karanganyar: Mucilage cell present in adaxial epidermis cells (yellow arrow), C. Ngaglik: Collenchyma cells present under the abaxial epidermis cell (yellow arrow), and D. Pakem: Hypodermis cells present under the adaxial epidermis cell (yellow arrow)

In conclusion, leaves samples of black betel from the natural habitat in Banyuwangi was significantly different (P<0.05) from the three man-made habitats in term of chlorophyll and flavonoid content. For water content, significant difference was found between the Banyuwangi samples with those from Karanganyar and Pakem while for the leaf area, significant difference was only found between the Banyuwangi samples and Karanganyar. Observations on the transverse cross section of midrib of black betel leaves from the four locations showed structures that are generally found in *Piper betle* species, namely the presence of an epidermal layer, trichomes in the abaxial part of the leaf, several layers of the hypodermis, visible vascular tissue and the presence of secretion cells. However, there were several differences, namely the greater number of trichomes on the leaves from Karanganyar, the secretory cells that were more visible in the leaves from Ngaglik and Banyuwangi and the sclerenchymal tissue was more visible in the leaves from Banyuwangi. The man-made habitat in the Karanganyar location showed that it can affect leaves characters similar to black betel plant grown in its natural habitat. However, it is still necessary to conduct further research on the differences in the content of secondary metabolite compounds and leaf anatomy in more diverse locations or habitats to determine the factors that most influence the formation of secondary metabolites in black betel leaves.

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REFERENCES

Amri CNAC, Tajudin NS, Shahari R, Azmi FM, Talip N, Mohamad AL. 2018. Comparative leaf anatomy of selected medicinal plants in Acanthaceae. Intl Med J Malays 17 (2): 17-24. DOI: 10.31436/imjm.v17i2.944.

Arnon DI. 1949. Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24 (1): 1-15. DOI: 10.1104/pp.24.1.1.

Chen D, Melton LD, Zujovic Z, Harris PJ. 2019. Developmental changes in collenchyma cell-wall polysaccharides in celery (*Apium graveolens* L.) petioles. BMC Plant Biol 19 (1): 81. DOI: 10.1186/s12870-019-1648-7.

Chen JJ, Sun Y, Kopp K, Oki L, Jones SB, Hipps L. 2022. Effects of water availability on leaf trichome density and plant growth and development of *Shepherdia x utahensis*. Front Plant Sci 13: 855858. DOI: 10.3389/fpls.2022.855858.

Dewi GP, Kuntorini EM, Pujawati ED. 2020. Struktur anatomi dan uji histokimia terpenoid dan fenol dua varietas sirih hijau (*Piper betle* L.). Bioscientiae 17 (2): 1-14. DOI: 10.20527/b.v17i2.3448. [Indonesian]

Fender AC, Mantilla-Contreras J, Leuschner C. 2011. Multiple environmental control of leaf area and its significance for productivity in beech saplings. Trees 25: 847-857. DOI: 10.1007/s00468-011-0560-z.

Harjunowibowo D, Yudi R. 2020. Penerapan Parameter Habitat Hutan Raung pada Pembiakan Sirih Hitam (Black *Piper betle* L.). Laporan Akhir Pengabdian Kepada Masyarakat (PKM) Kerjasama dengan UMKM Nyawiji Agriraya. LPPM UNS, Surakarta. [Indonesian]

Hu Y, Yang L, Gao C, Liao D, Long L, Qiu J, Wei H, Deng Q, Zhou Y. 2022. A comparative study on the leaf anatomical structure of *Camellia oleifera* in a low-hot valley area in Guizhou Province, China. PLoS ONE 17 (1): e0262509. DOI: 10.1371/journal.pone.0262509.

Idris A, Linatoc AC, Abu Bakar MF, Ibrahim ZT, Audu Y. 2018. Effect of light quality and quantity on the accumulation of flavonoid in plant species. J Sci Technol 10: 32-45. DOI: 10.30880/jst.2018.10.03.006.

Jarvis MC. 2012. Sclerenchyma. In: Encyclopedia of Life Sciences. John Wiley & Sons Ltd, Chichester. DOI: 10.1002/9780470015902.a0002082.pub2.

Jin B, Wang L, Wang J, Jiang KZ, Wang Y, Jiang XX, Ni CY, Wang YL, Teng NJ. 2011. The effect of experimental warming on leaf functional traits, leaf structure and leaf biochemistry in *Arabidopsis thaliana*. BMC Plant Biol 11: 35. DOI: 10.1186/1471-2229-11-35.

Jumrani K, Bhatia VS, Pandey GP. 2017. Impact of elevated temperatures on specific leaf weight, stomatal density, photosynthesis and chlorophyll fluorescence in soybean. Photosynth Res 131 (3): 333-350. DOI: 10.1007/s11120-016-0326-y.

Junairiah, Zuraidassanaaz NI, Izdihar FN, Manuhara YSW. 2017. Callus induction of leaf explant *Piper betle* L. var *nigra* with combination of plant growth regulators Indole-3-Acetic Acid (IAA), Benzyl Amino Purin (BAP) and kinetin. AIP Conference Proceedings 1888, 020028. 6th International Conference and Workshops on Basic and Applied Sciences, ICOWOBAS 2017. Erbil, Kurdistan, Iraq, 18-19 Mar 2017. DOI: 10.1063/1.5004305.

Junairiah, Ni'matuzahroh, Zuraidassanaaz NI, Sulistyorini L. 2018a. Isolation and identification of secondary metabolites of black betel (*Piper betle* L. var *nigra*). Jurnal Kimia Riset 3 (2): 131-138. DOI: 10.20473/jkr.v3i2.12064.

Junairiah, Purnomo, Utami ESW, Ni'matuzahroh, Sulistyorini L. 2018b. Callus induction of *Piper betle* var *nigra* using 2,4-dichlorofenoxyacetic acid and 6-benzil aminopurin. Biosaintifika 10 (3): 588-596. DOI: 10.15294/biosaintifika.v10i3.15962.

Junairiah, Mahmuda A, Manuhara YSW, Ni'matuzahroh, Sulistyorini L. 2019. Callus induction and bioactive compounds from *Piper betle* L. var *nigra*. IOP Conf Ser Earth Environ Sci 217: 012026. DOI: 10.1088/1755-1315/217/1/012026.

Kim HJ, Seo EY, Kim JH, Cheong HJ, Kang BC, Choi DI. 2012. Morphological classification of trichomes associated with possible biotic stress resistance in the genus *Capsicum*. Plant Pathol J 28 (1): 107-113. DOI: 10.5423/PPJ.NT.12.2011.0245.

Li AM, Li SH, Wu XJ, Zhang J, He AN, Zhao G, Yang X. 2016. Effect of light intensity on leaf photosynthetic characteristics and accumulation of flavonoids in *Lithocarpus litseifolius* (Hance) Chun. (Fagaceae). Open J For 6 (5): 445-459. DOI: 10.4236/ojf.2016.65034.

Li Y, He N, Hou J, Xu L, Liu C, Zhang J, Wang Q, Zhang X, Wu X. 2018. Factors influencing leaf chlorophyll content in natural forests at the biome scale. Front Ecol Evol 6: 64. DOI: 10.3389/fevo.2018.00064.

Muttaleb QA, Abdullah TL, Hassan SA, Rashid AA, Taheri S, Ahmed OA, Abdulameer DA. 2018. The role of shade and nitrogen on physiological traits and secondary metabolites of *Piper betle* L. J Hortic 5 (2): 1000230. DOI: 10.4172/2376-0354.1000230.

Novita W. 2016. Uji aktivitas antibakteri fraksi daun sirih (*Piper betle* L.) terhadap pertumbuhan bakteri *Streptococcus mutans* secara in vitro. Jambi Medical Journal: Jurnal Kedokteran dan Kesehatan 4 (2): 140-155. DOI: 10.22437/jmj.v4i2.3579. [Indonesian]

Nugroho AW. 2017. Review: Konservasi keanekaragaman hayati melalui tanaman obat dalam hutan di Indonesia dengan teknologi farmasi: Potensi sains dan tantangan. Jurnal Sains dan Kesehatan 1 (7): 377-383. DOI: 10.25026/jsk.v1i7.71. [Indonesian]

Nugroho RA, Ningsih EA. 2017. Produksi Tanaman Obat. In: Salim Z, Munadi E (eds). Info Komoditi Tanaman Obat. Badan Pengkajian dan Pengembangan Perdagangan Kementrian Perdagangan Republik Indonesia, Jakarta. [Indonesian]

Perangin-Angin Y, Purwaningrum Y, Asbur Y, Rahayu MS, Nurhayati. 2019. Pemanfaatan kandungan metabolit sekunder yang dihasilkan tanaman pada cekaman biotik. Agriland 7 (1): 39-47. DOI: 10.30743/agr.v7i1.3471. [Indonesian]

Prasetya F. 2012. Formulasi pasta gigi berbahan aktif ekstrak daun sirih hitam sebagai antimikroba penyebab radang gusi (gingivitis) dan gigi berlubang (caries). J Trop Pharm Chem 2: 19-25. DOI: 10.25026/jtpc.v2i1.44.

Prasetya F, Salam S, Rahmadani A, Haikal K, Febrina L, Anshory H, Arifuddin M, Siregar VO, Narsa AC, Herman H, Ahmad I, Indriyanti N, Ibrahim A, Rusli R, Rijai L, Kuncoro H. 2021a. Novel amides derivative with antimicrobial activity of *Piper betle* var. *nigra* leaves from Indonesia. Molecules 26 (2): 335. DOI: 10.3390/molecules26020335.

Prasetya F, Salam S, Rahmadani A, Kuncoro H, Rusli R, Ruchaemi A, Sukartiningsih. 2021b. Chemical constituents from *Piper betle* L. var. *nigra* (Piperaceae). J Trop Pharm Chem 5 (3): 229-232. DOI: 10.25026/jtpc.v5i3.322.

Queiroz GA, Silva PHL, de Oliveira RA, Sodré GA, do Bomfim Costa LC. 2018. Drying temperature changes trichome integrity, chemical content and composition of the essential oil of pepper-rosmarin. Ciênc Rural 48 (11): e20180283. DOI: 10.1590/0103-8478cr20180283.

Raman V, Galal AM, Khan IA. 2012. An investigation of the vegetative anatomy of *Piper sarmentosum*, and a comparison with the anatomy of *Piper betle* (Piperaceae). Am J Plant Sci 3 (8): 1135-1144. DOI: 10.4236/ajps.2012.38137.

Rodríguez VM, Soengas P, Alonso-Villaverde V, Sotelo T, Cartea ME, Velasco P. 2015. Effect of temperature stress on the early vegetative development of *Brassica oleracea* L. BMC Plant Biol 15: 145. DOI: 10.1186/s12870-015-0535-0.

Rudall JP. 2020. Anatomy of Flowering Plant. An Introduction to Plant Structure and Development. (4th Ed.). Cambridge University Press, Cambridge.

Sari EF, Prayogo GP, Loo YT, Zhang P, McCullough MJ, Cirillo N. 2020. Distinct phenolic, alkaloid and antioxidant profile in betel quids from four regions of Indonesia. Sci Rep 10: 16254. DOI: 10.1038/s41598-020-73337-0.

Sass JE. 1958. Botanical Microtechnique. Iowa State University Press, Ames

Suriani NL, Suprapta DN, Nazir N, Parwanayoni NMS, Darmadi AAK, Dewi DA, Sudatri NW, Fudholi A, Sayyed RZ, Syed A, Elgorban AM, Bahkali AH, Enshasy HA, Dailin DJ. 2020. A mixture of *Piper* leaves extracts and Rhizobacteria for sustainable plant growth promotion and bio-control of blast pathogen of organic Bali rice. Sustainability 12 (20): 8490. DOI: 10.3390/su12208490.

Susilo DEH. 2015. Identification of constanta value of leaf shape for leaf area measurement using length cross width of leaf of horticulture plant in peat soil. Anterior Jurnal 14 (2): 139-146. DOI: 10.33084/anterior.v14i2.178. [Indonesian]

Wang Z, Huang H, Wang H, Peñuelas J, Sardans J, Niinemets Ü, Niklas KJ, Li Y, Xie J, Wright IJ. 2022. Leaf water content contributes to

global leaf trait relationships. Nat Commun 13: 5525. DOI: 10.1038/s41467-022-32784-1.

Weraduwage SM, Chen J, Anozie FC, Morales A, Weise SE, Sharkey TD. 2015. The relationship between leaf area growth and biomass accumulation in *Arabidopsis thaliana*. Front Plant Sci 6: 167. DOI: 10.3389/fpls.2015.00167.

Yuanyue L, Zhongbao L, Peng L. 2009. The study on the leaf anatomy of some mangrove species of China. In: 2009 International Conference on Environmental Science and Information Application Technology. Wuhan, China. DOI: 10.1109/ESIAT.2009.397.

Zhang H, Zhong H, Wang Ji, Sui X, Xu N. 2016. Adaptive changes in chlorophyll content and photosynthetic features to low light in *Physocarpus amurensis* Maxim and *Physocarpus opulifolius* "Diabolo". PeerJ 4: e2125. DOI: 10.7717/peerj.2125.