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Leaf and Stomata Morphometrics of Gayam *Inocarpus fagifer* (Fabaceae) at Different Altitudes

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ABSTRACT

Gayam (*Inocarpus fagifer*) is one of the members of the angiosperm flora in Ambon City, Indonesia, that grows and develops at various altitudes. This research aimed to analyze the leaf and stomata morphometrics of these plants in the Aer Louw and Ema Village areas. Leaf samples were taken from the upper, middle, and lower strata and considered as replicates. The morphometric characteristics were measured manually using millimeter block paper and the formula for calculating leaf ratio. Furthermore, the stomata were stained using the direct incision method and safranin. The incision results were analyzed using an Olympus CX23 microscope at 400x magnification. The measurement and observation were analyzed descriptively and correlatively. The results showed that the average leaf width and length, as well as the midrib length were greater in Aer Louw Village than in Ema Village; while the leaf tip and stalk length were greater in Ema Village than in Aer Louw Village. The characteristics of stomata length and width in Ema Village were greater than in Aer Louw Village; otherwise, the number, index, and density of stomata in Aer Louw Village were greater than in Ema Village. Meanwhile, the correlational analysis showed that the environment influenced the variations of leaves and stomata. Therefore, the variations of leaves and stomata in the areas could predict plant adaptations to different environments.

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INTRODUCTION

Inocarpus fagifer (Parkinson ex Zollinger) Fosberg is a woody, leguminous plant with a tree habitus distributed in tropical and subtropical areas. It has a shallow taproot, while the lateral roots appear on the soil surface. The tree bark is rough and brown or gray, while the leaves are oval, arranged alternately, dark green, and has a rough surface. The flowers are arranged in clusters on branches, stems, and twigs with five petals and shows white or yellowish color variations. The fruit is oval, irregular, and slightly flattened, while the young fruit is green and turns orange-brown when ripe. Moreover, the seeds are white, fibrous, and thin (Setyowati & Wawo, 2015; Wawo et al., 2011).

I. fagifer is known in different countries under its local names, thus *aila* in Papua New Guinea, *chataignier de Tahiti* in French Polynesia, *ivi* in Fiji, and *Tahitian chestnut* or *Polynesian chestnut* in England (Pauku, 2006). Pauku et al. (2010) also stated that most farmers use *I. fagifer* as an agricultural crop. Meanwhile, *I. fagifer* in Ambon City is grown by the community but not cultivated as a crop, such that the seeds that fall on the ground will grow naturally. The city is one of the areas with a large distribution of these plants, namely in Ema and Air Louw Villages. The distribution in the two villages represents the highlands and lowlands.

According to Hamidah & Fitriani (2018), some plants can grow well in the lowlands to the highlands; hence, they have a wide distribution. This explanation shows that *I. fagifer* is a plant with a wide distribution in these two villages. The topography and slope of the place and environmental factors such as light intensity, wind speed, temperature, and CO_2 pressure vary greatly in high and low areas (Gao et al., 2019; Kofidis & Bosabalidis, 2008).

The varying conditions of environmental factors certainly affect the modification and adaptation of plants in the two villages. One of the characteristics that is easy to observe is the morphology of plant organs such as roots, stems, leaves, flowers, fruits, and seeds. However, the tree has a large trunk diameter and a height of up to 3 m. This makes it difficult to observe and measure some morphological characteristics. Meanwhile, flowers, fruits, and seeds are classified as seasonal organs, making morphological observation difficult. Leaves are one of the vegetative organs obtained to observe and measure morphological characteristics. Leaf morphometric measurement is conducted to determine leaf area, length, and ratio, which are also very useful for determining physiological processes. Observation of stomata characteristics can easily be performed through the leaf organ. Liu et al. (2020) also stated that plant adaptation to changes in environmental factors is carried out by reducing leaf area and increasing the thickness, mesophyll tissue thickness, and stomata density.

According to Ruszala et al. (2011) and He & Liang (2018), stomata are tissues very sensitive to the environment. Therefore, it is very important to observe and measure the stomata of the *I. fagifer* leaves to determine their shape, length, width, aperture, density, and index. Paembonan et al. (2021) stated that highlands affect the number of stomata but reduce the size and index of the Makassar ebony (*Diospyros celebica* Bakh.). Tumpa et al. (2022) also noted that geographical location affects the leaf size of *Salix triandra* L., a process of morphological adaptation to environmental changes. According to Muradoglu & Gundogdu (2011), leaf surface area relates to the stomata frequency in walnut plants. Based on these results, the leaf and stomata morphometric measurement of *I. fagifer* plants was conducted based on the difference in altitude in two areas, namely Ema and Aer Louw Villages. Morphometric analysis of leaf and stomata at different altitudes can be a prediction for *I. fagifer* plants to cope with climate change in the future. Therefore, this research analyzed leaf and stomata morphometrics based on different altitudes.

MATERIALS AND METHODS

Sampling Location

This research was conducted in Aer Louw Village with an altitude 200 m above sea level (asl) and Ema Village with an altitude of 600 m above sea level (asl) (Figure 1).

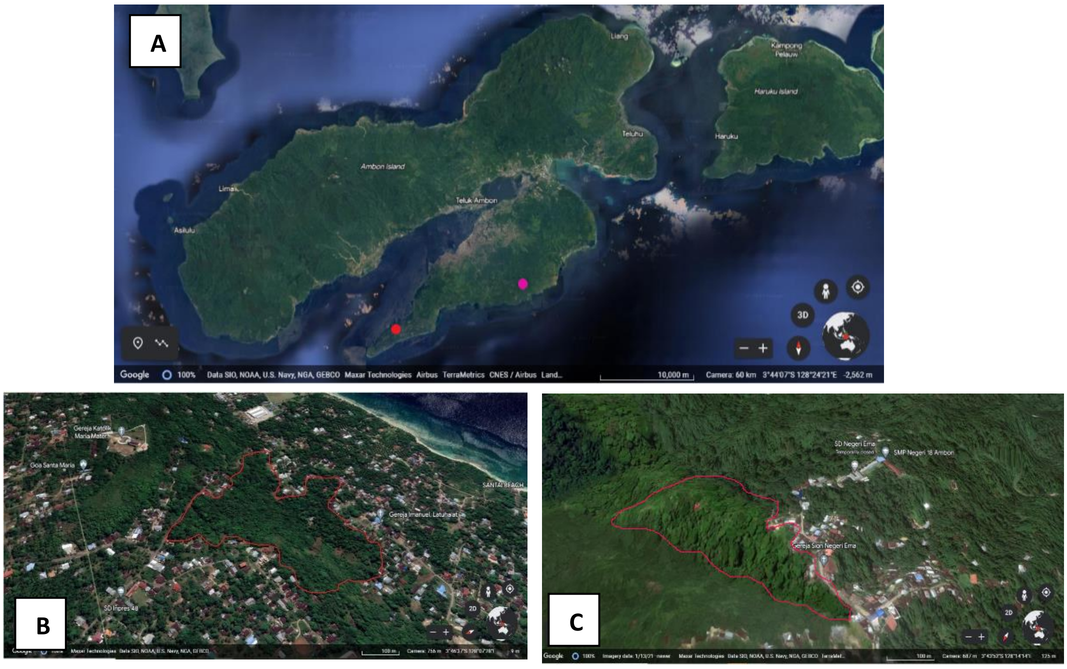

Figure 1. A. Map of Ambon Island; B. Research Locations in Aer Louw Village; C. Research Locations in Ema Village. Source: https://earth.google.com/web/search/

Sampling

Leaf samples for stomata observation and morphometric measurement were taken separately. The samples for observing stomata were taken from one of the largest and tallest *I. fagifer* trees at the two sampling locations. Meanwhile, morphometric measurements were taken from 10 trees in the two locations by considering the upper, middle, and lower strata. Sampling for stomata measurement was carried out on the left and right branches and focused only on green leaves. Leaf samplings were repeated five times with a total was 30 dark green leaves.

Research Procedure

Environmental factors such as temperature, light, and altitude were measured. The morphometric measurement of *I. fagifer* leaves was conducted in the following stages: (1) leaf samples were cleaned of dirt and dust using a tissue, (2) the samples were placed on millimeter block paper and marked using a pen, (3) the results of the markers were measured using a ruler as shown in Figure 2. After obtaining the values for the length, width, tip, stalk, and leaf midrib length, the next step was to calculate the formula for the ratio of leaf length and width (Shi et al., 2020).

The *I. fagifer* leaf stomata morphometric measurement was conducted in the following stages: (1) the leaf samples taken were cleaned of dirt and dust; (2) the samples were sliced crosswise at the bottom using a razor blade; (3) the leaf slices were soaked in commercial bleach (bayclin) for ± 5 min until they turn white; (4) the leaf slices were washed using distilled water and soaked in 1% safranin for 1 min; (5) the slices were washed again using distilled water; (6) the slices were observed using an Olympus CX23 microscope with 400x magnification; (7) the observation results were photographed using a digital camera connected to a computer; (8) the observed photos were inserted into the image master to measure length, width and opening size of stomata, count the number, and observe the location and type of stomata.

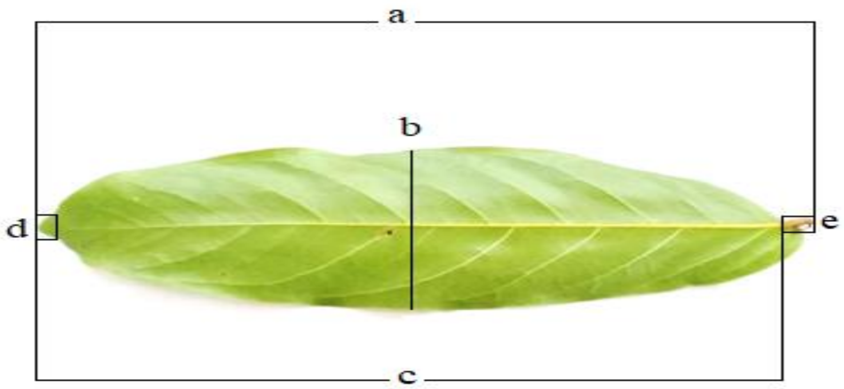

Figure 2. Morphometric characteristics of *I. fagifer* leaf measurement. (a) leaf length; (b) leaf width; (c) leaf midrib length; (d) leaf tip length; (e) leaf stalk length

$$\text{Ratio of leaf length and width} = \frac{\text{leaf length}}{\text{leaf width}}$$

$$\text{Ratio of leaf length and leaf midrib length} = \frac{\text{leaf length}}{\text{leaf midrib length}}$$

$$\text{Ratio of leaf length and leaf stalk length} = \frac{\text{leaf length}}{\text{leaf stalk length}}$$

$$\text{Ratio of leaf length and leaf tip length} = \frac{\text{leaf length}}{\text{leaf tip length}}$$

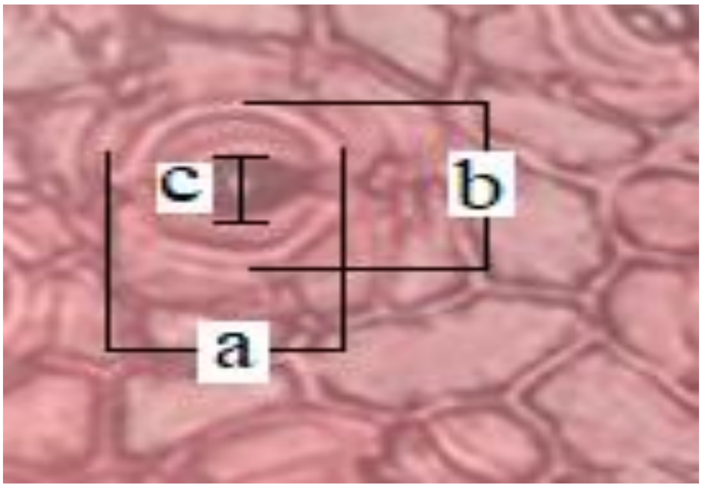

Figure 3. Morphometric characteristics of *I. fagifer* leaf stomata measurement. (a) stomata length; (b) stomata width; (c) stomata opening size

Data Analysis

Data from *I. fagifer* leaf and stomata morphometric calculation were collected and analyzed descriptively to determine the average value and standard deviation. Furthermore, the data were analyzed to determine correlation value of environmental factors and effective contribution using multiple linear regression inferential statistics (Wang et al., 2019; Sun et al., 2021). An analysis of correlation value and effective contribution is used based on the following formula:

Formula of product moment correlation

$$r_{xy} = \frac{n\sum XY - (\sum X)(\sum Y)}{\sqrt{\{n\sum X^2 - (\sum X)^2\}}}$$

(Chawla et al., 2016; Kumari & Yadav, 2018).

Then analyze the effective contribution (EC):

$$EC\%X_n = BX_n \; x \; r_{xy} \; x \; 100\%$$

(Turkheimer & Waldron 2000).

Note:
EC: effective contribution; BX_n: B coefficient of the predictor; X_n: predictors such as temperature, light, and altitude, r_{xy}: correlation coefficient.

The calculation for the stomata index and density was conducted based on the Fetter et al. (2019) formula as follows:

$$\text{Stomata Density} = \frac{\text{Number of Stomata}}{\text{Field of View Unit}}$$

$$\text{Index Stomata} = \frac{\text{Number of stomata}}{\text{Number of stomata + epidermal cells}}$$

The data was analyzed using Excel and SPSS for Windows 18.

RESULTS AND DISCUSSION

Inocarpus fagifer Leaf and Stomata Morphology

Morphologically, the color of *I. fagifer* leaves in Ema and Aer Louw Villages was the same, dark green on the upper surface and light green on the lower surface. The upper surface of the leaves is smooth and greasy, while the lower surface is rough (Figure 4). Meanwhile, the stomata morphology between the two areas has the same shape but differs in size and number of stomata in one field of view (Figure 4).

Stomata are a type of differentiation from leaf epidermal tissue (Peterson et al., 2013; Torii, 2021; Zuch et al., 2022). *I. fagifer* stomata are found on the lower surface of the leaves. The stomata in these two areas have an actinostic type, with guard cells surrounded by neighboring cells in a radius. The number of neighboring cells is 4 or more, while the stomata's guard cells are kidney-shaped with thin side walls and thicker top and bottom walls (Prabhakar, 2004; Ahmad et al., 2009; Song et al., 2020).

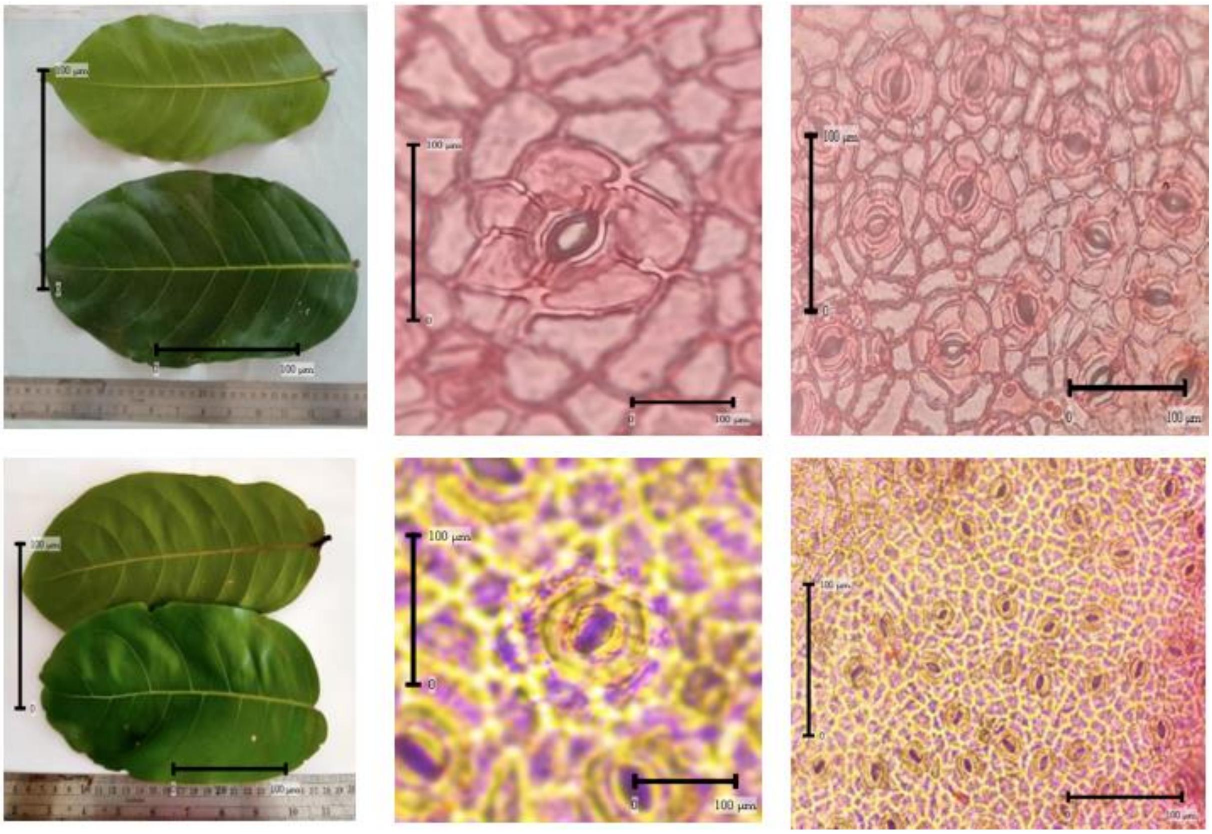

Figure 4. *I. fagifer* leaf morphology. (Top row) Samples from Ema Village (Altitude of 600 m asl). (Bottom row) Samples from Aer Louw Village (Altitude of 200 m asl).

Leaf and Stomata Morphometrics of Inocarpus fagifer

The morphometric measurement in the two areas with different altitudes varies greatly, as summarized in Table 1. The same result was reported by Paridari et al. (2013) wherein *Carpinus betulus* L. growing at high altitudes had a small leaf lamina compared to those growing at low altitudes. According to Liu et al. (2020), adapting plants in the highlands reduces leaf area. This shows that *I. fagifer* plants that grow at different altitudes have adapted to have an average leaf width and length at high altitudes (Ema Village) of 119.35 mm and 259.77 mm, while those growing at low altitudes (Aer Louw Village) have an average leaf width and length of 135.43 mm and 312.52 mm. The long and wide leaves of the *I. fagifer* plants in Aer Louw Village also have a midrib average length of 268.47 mm, while in Ema Village the midrib average length is 245.8 mm. This result agrees with that of Madeline et al. (2014), where broad leaves have high vein density accompanied by stomata density. Meanwhile, the broad leaves of *I. fagifer* in Aer Louw Village had shorter tips and petioles on average 3.69 mm and 5.45 mm, while the average tips and petioles in Ema Village were 6.07 and 8.7. According to Serdar & Kurt (2011), leaf parameters can be used as a variable to detect the level of phenotypic variability among plant species in a population. The stomata morphometric measurement of *I. fagifer* leaves in the two areas with different altitudes varied greatly, as recapitulated in Table 2. The high altitude in Ema Village (600 m asl) resulted a lower stomata density than Aer Louw in the lower area (200 m asl). Fustier et al. (2019) reported that stomata density decreased with increasing altitude. According to Li et al. (2021), plants with large stomata have low densities, but large size affects plant adaptation. Furthermore, Idris et al. (2019) stated that high intensity affects stomata density to support high assimilation processes in plants.

Table 1. Morphometric characteristics of *I. fagifer* leaves at two places with different altitudes

Morphometric Characteristics of *I. fagifer* Leaves	Location	Upper strata (mm)	Middle strata (mm)	Lower strata (mm)
Leaf length	Ema	265.7±3.37	258.2±4.03	255.4±4.17
	Aer Louw	302.26±2.12	293.22±1.03	342.08±18.4
Leaf width	Ema	116±2.93	111.8±2.63	111.7±2.83
	Aer Louw	140.14±2.12	135.88±1.03	130.26±0.82
Leaf midrib length	Ema	251 ±3.22	244.7±3.83	241.7±3.92
	Aer Louw	278.26±4.23	269.22±2.89	257.94±2.37
Leaf tip length	Ema	9.7±0.17	4.1±0.12	4.4±0.14
	Aer Louw	3.66±0.66	3.76±0.04	3.66±0.05
Leaf stalk length	Ema	8.8±0.26	8.7±0.26	8.6±0.3
	Aer Louw	5.40±0.1	5.56±0.07	5.40±0.1
Ratio of leaf length and width	Ema	23.2±0.37	23.09±21.56	22.8±0.29
	Aer Louw	21.64±0.19	21.56±0.14	25.56±1.16
Ratio of leaf length and leaf midrib length	Ema	10±0	10±0	10±0
	Aer Louw	10.99±0.12	10.89±0.05	13.09±0.64
Ratio of leaf and leaf stalk length	Ema	303±7.93	294.5±6.83	314.4±6.28
	Aer Louw	487.9±27.9	500.12±18	535.68±54.2
Ratio of leaf length and leaf tip length	Ema	535.1±32.3	59.04±12.3	531.7±15.6
	Aer Louw	856.5±24.3	785.01±9.19	966.59±68

Table 2. Stomata morphometric characteristics of *I. fagifer* leaves at two areas with different altitudes

Stomata Morphometric Characteristics	Loc.	Upper Strata (µm)		Middle Strata (µm)		Lower Strata (µm)		Description
		Right Branch	Left Branch	Right Branch	Left Branch	Right Branch	Left Branch	
Stomata Length (Mean±SD)	Ema	47.54± 0.40	47.50± 0.33	44.07± 0.26	44.21± 0.45	41.88± 0.39	42.39± 0.46	Very long
	Aer Louw	16.68± 0.14	16.63± 0.12	14.89± 0.22	14.96± 0.21	12.75± 0.995	12.79± 0.75	Less long
Stomata Width (Mean±SD)	Ema	46.86± 0.56	47.09± 0.39	44.4± 0.26	44.02± 0.37	42± 0.46	41.99± 0.63	Very wide
	Aer Louw	16.93± 0.30	17.09± 0.29	14.96± 1.06	15.26± 0.54	14± 1.07	14.3± 1.64	Less wide
Stomata Opening Size (Mean±SD)	Ema	16.03± 0.27	16± 0.32	14.11± 0.34	14.06± 0.25	12± 0.24	12.02± 0.24	Wide
	Aer Louw	6.058± 0.11	6.2± 0.13	5.01± 0.1	5.16± 0.24	4.8± 0.39	4.92± 0.46	Less wide
Number of Stomata (Mean±SD)	Ema	13± 1.52	14.4± 2.30	10± 0.71	9.8± 0.84	7.2± 0.84	6.4± 1.14	Few
	Aer Low	43± 7.01	40.2± 9.15	24.4± 4.62	27.4± 8.05	24± 3.27	21.4± 1.52	Many
Stomata Index (Mean±SD)	Ema	0.18± 0.01	0.18± 0.01	0.15± 0.01	0.15± 0.01	0.1± 0.02	0.1± 0.01	Low
	Aer Louw	0.58± 0.02	0.57± 0.03	0.43± 0.02	0.44± 0.01	0.37± 0.01	0.36± 0.01	High
Stomata Density (Mean±SD)	Ema	152.3± 17.24	163.6± 26.16	113.6± 8.04	111.4± 9.51	81.81± 9.51	72.72± 12.96	Low
	Aer Louw	670.8± 108.9	624.2± 142.1	379.9± 70.23	425.5± 124.9	370.6± 48.9	333.3± 21.61	High

Description: Loc: Location

The difference in altitude is an environmental factor affecting the plant microclimate. According to Lamprecht et al. (2018), ecosystems at high altitudes have low temperatures. Meanwhile, Idris et al. (2019) reported that the stomata density increased when exposed to high sunlight. Environmental characteristics at different altitudes also affect the stomata morphometric features. Tiwari et al. (2013), also stated that altitude was positively correlated with stomata density, index, and guard cell length. According to Akbarinia et al. (2011), variations in shape, size, index, area, and stomata can vary within one species. The stomata length characteristic of *I. fagifer* leaves is directly proportional to its width. Muradoglu & Gundogdu (2011) also stated a positive relationship between stomata length and width. According to Li et al. (2011), the stomata index of *Quercus aquifolioides* Rehder & E.H. Wilson decreased at high altitudes and increased at low altitudes. Meanwhile, the morphological characteristics of stomata related to its density are inversely proportional to the length and width of it, as well as to the size of the stomatal opening, which is inversely proportional to stomata density (Hong et al., 2018; Haworth et al., 2023).

Some of these findings have supported this research that the length of the *I. fagifer* stomata leaves are also directly proportional to the width of the stomata and the size of the opening of the stomata is directly proportional to the number, index, and density of stomata.

The variation of stomata in the two areas with different altitudes shows that altitude plays a role in morphometric characteristics. According to Alonso-Amelot (2008), highland plants have high adaptability to extreme environments. It was stated by Ahmad et al. (2020) that the ability of plants to adapt in the highlands is by adjusting their morphological and physiological characteristics. Halbritter et al. (2018) and Montesinos-Navarro et al. (2011) also confirmed that the elevation gradient greatly affects abiotic factors, such as humidity, temperature, and light intensity in an area.

Variations in leaf and stomata morphometrics of *I. fagifer* as affected by environmental parameters

Environmental characteristics in the two areas with different altitudes are shown in Table 3. The condition of the two areas showed that light intensity influences temperature, while altitude is related to light intensity as indicated in Table 3. The condition of the two areas showed that light intensity influences temperature, while altitude is related to light intensity as indicated in Table 3. Altitude is an environmental factor that greatly determines the relationship between leaf and stomata morphometrics in *I. fagifer* plants. The relationship of environmental factors to the leaf morphometric characteristics of *I. fagifer* plants is shown in Table 4.

Table 3. Environmental characteristics

Environmental Characteristics	Ema Village	Air Louw Village
Light intensity	17,000 Lux	20,000 Lux
Temperature (°C)	25°C	28°C
Altitude	600 m asl	200 m asl

Table 4. Correlation of environmental factors with leaf morphometric characteristics

Leaf Characteristics	R	R^2	Sum of Square		Mean Square		F	F sig (p)
			Reg.	Res.	Reg.	Res.		
Leaf length	0.31	0.099	417.4371	3798.868	417.43713	65.5	6.373308	0.014(*)
Leaf width	0.46	0.2136	74.32614	273.564	74.32614	4.717	15.75835	0.00(*)
Leaf midrib length	0.32	0.1025	77.11201	675.319	77.112007	11.64	6.622793	0.012(*)
Leaf tip length	0.32	0.1003	0.074907	0.671787	0.074907	0.012	6.467212	0.014(*)
Leaf stalk length	0.65	0.418	1.581127	2.201347	1.581127	0.038	41.65875	0.00(*)
Ratio of leaf length and width	0.01	0.0001	0.001815	16.26631	0.001815	0.28	0.006472	0.936

Leaf Characteristics	R	R^2	Sum of Square		Mean Square		F	F sig (p)
			Reg.	Res.	Reg.	Res.		
Ratio of leaf length and leaf midrib length	0.3	0.09	0.411682	4.163537	0.411682	0.072	5.7349 17	0.019(*)
Ratio of leaf length and leaf stalk length	0.42	0.1727	7390.602	35402.1997 6	7390.6021	610.4	12.108 14	0.001(*)
Ratio of leaf length and leaf tip length	0.47	0.2163	16625.69	60222.5031 4	16625.692	1038	16.012 12	0.00(*)

Description: Reg: Regression; Res: Residual; (*): significant

Table 5. Effective contribution of environmental factors to leaf morphometric characteristics

Leaf characteristics	Effective Contribution of Environmental Factors			Total (%)
	Temperature	Light	Altitude	
Leaf length	0.00	0.00	9.901	9.901
Leaf width	0.00	0.00	21.36	21.36
Leaf midrib length	0.00	0.00	10.25	10.25
Leaf tip length	0.00	0.00	10.03	10.03
Leaf stalk length	0.00	0.00	41.8	41.8
Ratio of leaf length and width	0.00	0.00	-	-
Ratio of leaf length and leaf midrib length	0.00	0.00	8.998	8.998
Ratio of leaf length and leaf stalk length	0.00	0.00	17.27	17.27
Ratio of leaf length and leaf tip length	0.00	0.00	21.63	21.63

Table 6. Correlation of environmental factors with stomata morphometric characteristics

Stomata Characteristics	R	R^2	Sum of square		Mean square		F	F sig (p)
			Reg.	Res.	Reg.	Res.		
Stomata length	0.992	0.983	2667.697	44.952	2667.697	4.495	593.452	0.00(*)
Stomata width	0.992	0.985	2493.795	39.027	2493.795	3.903	638.997	0.00(*)
Stomata opening size	0.964	0.93	229.338	17.248	229.338	1.725	132.961	0.00(*)
Number of stomata	0.843	0.711	1180.083	480.833	1180.083	48.08	24.542	0.00(*)
Stomata index	0.915	0.838	0.288	0.056	0.288	0.006	51.575	0.00(*)
Stomata density	0.879	0.772	370572.395	109402.096	370572.4	10940	33.873	0.00(*)

Description: Reg: Regression; Res: Residual; (*): significant

Table 7. Effective contribution of environmental factors to stomata morphometric characteristics

Stomata characteristics	Effective Contribution of Environmental Factors			Total (%)
	Temperature	Light	Altitude	
Stomata length	0.00	0.00	98.3	98.3
Stomata width	0.00	0.00	98.5	98.5
Stomata opening size	0.00	0.00	93	93
Number of stomata	0.00	0.00	71.1	71.1
Stomata index	0.00	0.00	83.8	83.8
Stomata density	0.00	0.00	77.2	77.2

The relationship of environmental factors to the stomata morphometric characteristics of *I. fagifer* leaves is shown in Table 6. Environmental factors of light, temperature, and altitude have a significant relationship with all morphometric characteristics of *I. fagifer* leaves (p=<0.05). Previous research confirmed that environmental factors greatly affect stomata opening size (Casson & Gray, 2008). Qi & Torii (2018), reported that environmental factors stimulate stomata density. Harrison et al. (2020) also stated that environmental factors correlated with stomata size and density.

The effective contribution of environmental factors was calculated to determine which stomatal morphometric characteristics were more dominant. The altitude effectively contributed to these stomatal morphometric characteristics, as indicated in Table 7. Aslantaş & Karakurt (2009) stated that high areas have high rainfall while temperature, O_2 and CO_2 levels decreased. This shows that the environmental factors of temperature, light, O_2, CO_2, and humidity depend on altitude.

The low and high altitudes are related to temperature, light, and humidity. These environmental factors affect stomata length, width, opening size, number, index, and density simultaneously. Specifically, stomata opening is influenced by light (Elhaddad et al., 2014) and high temperature (Lawson & Blatt, 2014). Driesen et al. (2020) stated that stomata opening is influenced simultaneously by light, CO_2, temperature, and humidity. Altitude greatly influences plant physiology, such as stomata density (Qiang et al., 2003). Richardson et al. (2017) confirmed that stomata are adaptive tissues that modify their stomatal density, size, and form in response to environmental changes.

CONCLUSION

The results showed that different environmental conditions can provide variations in the morphology of the leaves and stomata of *I. fagifer* plants. Altitude is related to other environmental factors, such as temperature and light intensity, which can directly influence variations of leaves and stomata. This research can predict *I. fagifer* plants' survival and adaptation to environment changes.

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