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Evaluation of *Stevia rebaudiana* Leaf-Axillary Shoot Formation, Cultured in MS Medium Supplemented with IAA-BAP and MS Medium Supplemented with Kinetin

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Abstract

Stevia rebaudiana leaves can be used as a sweetener alternatives because they contain steviol glycoside derivative compounds, including steviosides and rebaudioside-A. Propagation of *Stevia* is more optimally carried out using *in vitro* culture when compared to conventional propagation through seeds or cuttings. This study aimed to evaluate the formation and growth of *Stevia* shoots and leaves in MS medium containing a mixture of IAA and BAP with MS medium containing kinetin only, as well as evaluating the use of a liquid medium containing kinetin. *Stevia* was initiated from apical shoot then grown in MS medium containing a mixture of IAA and BAP with MS medium containing kinetin only. *Stevia* was subcultured every 4 weeks. Several parameters measured were number of axillary shoots and number of leaves. It was transferred into a liquid medium for 7 days. The results showed that the formation and growth of axillary buds and leaves at the initiation stage were better in medium containing IAA and BAP compared to medium containing single hormone kinetin. At the stage of shoot multiplication and maintenance, cultivation in semi-solid medium containing kinetin showed more leaves and axillary shoots compared to that cultivated in semi-solid medium with the addition of IAA and BAP. Plants acclimatized in liquid medium supplemented with 1 ppm kinetin showed fast plant growth but were not accompanied by sturdy stem growth. The presence of brownish color on certain parts of the plant such as in some leaves and stems was also observed.

Keywords: *axillary shoot, BAP, IAA, kinetin,* Stevia rebaudiana

1. Introduction

Diabetes is one of the biggest health problems in the world. In 2017, it was estimated that around 4 million people died from diabetes and its complications [1]. The main cause of diabetes is the unhealthy lifestyle of modern society, including consuming too much high-energy diet [1]. *Stevia rebaudiana* is one of the plants that has a great potential as a producer of alternative sweetener compounds. The leaves of this plant produce steviol glycoside compounds which are known to be about 250 to 300 times sweeter than sucrose [2]. In additionn, *Stevia* extract can replace the sugar and be marketed as a non-caloric sweetener. The most abundant steviol glycosides are stevioside and rebaudioside-A. Since 1995, many food industries used Stevia extracts to sweeten their food products, such as in Japan, Brazil, and other countries [3].

One of the problems in the regeneration of *Stevia* is the non-uniform quality of seed. Regeneration of *Stevia* by *in vivo* production is not an ideal propagation method because the germination rate is low and the variety of offspring is high [4]. Therefore, to overcome that problem, in this study, Stevia propagation was developed using tissue culture technique.

Tissue culture or micropropagation is a method of plant propagation using an aseptic culture of cells, tissues, organs, and their components under *in vitro* aseptic conditions with predetermined chemical and physical conditions [5]. The principle of tissue culture is the totipotency of plants, where plant cells can grow into new individuals identical to their parents. Tissue culture has many advantages over conventional plant propagation methods. With this method, large numbers of new plants can be produced in a short time and without requiring a lot of space. The properties of the tillers produced by the

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tissue culture method will also resemble the properties of the parents. Plants resulting from micropropagation can also be given improved characteristics through manipulation of physical and chemical conditions, such as diseases-free plant and more secondary metabolites production [6].

In vitro culture of *Stevia* has been carried out by several researchers [7, 8]. Previous studies used a different composition of plant growth regulators (PGRs) to produce optimal growth, including 1.13 ppm BAP and 0.35 ppm IAA in the study by Sumaryono and Sinta [8] and 1 ppm kinetin in the study by Melviana et al. [7]. The purposes of this study were to evaluate the formation and growth of shoots and leaves of *Stevia* in semi solid medium containing a mixture of IAA and BAP or kinetin solely, and to evaluate the use of a liquid medium containing kinetin for shoot maturation.

2. Methodology

2.1 Stevia Explant

Explants were obtained from the Indonesian Center for Biotechnology and Bioindustry Research, Bogor, West Java. Apical shoot containing three nodes from 4 weeks-old of *S. rebaudiana* plants, with a height about 30-50 cm, were used as an explant.

2.2 Shoot Initiation

Stevia shoots (Figure 1) were washed in running tap water for 3 minutes, soaked in a solution containing 0.5 % AntracolTM fungicide, then rinsed twice with distilled water and dried using filter paper. Explants were then sterilized using 70% alcohol, followed by 0,27% (v/v) NaClO of commercial bleach solution added with 2 drops of Tween-20 for 5 minutes. Subsequently, the explants were rinsed three times with sterile distilled water and dried using sterilized filter paper. The explants were then transferred into a petri dish lined with sterile filter paper.

Explants were then cultured in semi-solid Murashige-Skoog (MS) medium [9] containing 30 g/L sucrose and 8 g/L agar, supplemented with 0.35 ppm IAA and 1.13 ppm BAP or 1 ppm kinetin solely. The pH of the medium was adjusted to 5.6 to 5.8. The cultures were then incubated in room temperature under 12/12 hours photoperiod and light and light intensity 1000 lux using 36-watt TLD lamps. Subculture was carried out three times in every 4 weeks using similar initiation medium. The new regenerated shoots were separated and transferred into new medium during subculture.

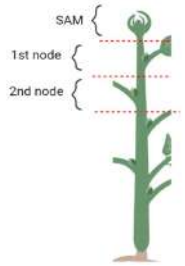

Figure 1. Explant used in this research

2.3 Shoot culture in liquid medium

Regenerated shoot from semi-solid medium were transferred into liquid medium, which consisted of half-strength MS medium, supplemented with 20 g/L sucrose and 1 ppm kinetin. The pH of the medium was adjusted to 5.6 to 5.8. The culture was then incubated on 40-rpm shaker in room temperature with 12/12 photoperiod.

3. Result and Discussion

3.1 Stevia Initiation

At the initiation stage, the medium containing IAA and BAP produced leaves and more axillary shoots, which were developed from the explant's nodes, compared to kinetin (Figures 2 A and B). Explants growing in IAA and BAP has regenerated leaves at 2 weeks after initiation (Figure 3A), meanwhile explants in medium containing kinetin did not show any growth, and some explants indicated yellowing (Figure 3B).

Shoot initiation stage usually need combination of auxin and cytokinin to regenerate new axillary buds. Auxins and cytokinins influence organ regeneration by controlling cell differentiation [7]. Auxins will trigger cell elongation and cell growth, while cytokinins stimulate cell division and shoot formation in culture. At the initiation stage, therefore, concentration of cytokinin in growth medium was usually higher than auxin in order for the explant can grow more shoots [10, 11].

Two weeks after subculture, the formation of axillary shoots and leaves in the medium containing IAA and BAP was slower and less than the number of axillary shoots and leaves regenerated in medium containing kinetin (Figure 4A). During the rest of the subculturing period, faster axillary shoot regeneration occurred in medium containing IAA and BAP. At 4 weeks after subculture, therefore, the number of axillary shoots in both media were same. Leaf formation in medium containing IAA and BAP was slower than in medium containing kinetin. Number of leaves in those medium, therefore, were fewer compared to the leaves in medium containing kinetin. Longer internodes were observed in medium containing kinetin (Figures 5A and B).

The addition of kinetin to Stevia results in higher number of leaves and internodes compared to other PGRs such as IAA and BAP. The higher number of internodes can produce more explant when it is sub-cultured. The higher number of leaves can produce more steviol glycosides in *Stevia*. Kinetin is a cytokinin-type growth regulator, which can increase shoot propagation and cell division or plant biomass and plant cell differentiation [12]. Giving PGR kinetin as much as 1 ppm was the best concentration for the growth of Stevia shoot. Melviana et al. [7] showed that the addition of 1 ppm kinetin to the semi-solid culture of *Stevia* was able to produce a relatively high number of shoot and leaf multiplication compared to other PGRs, and the resulting plantlets were

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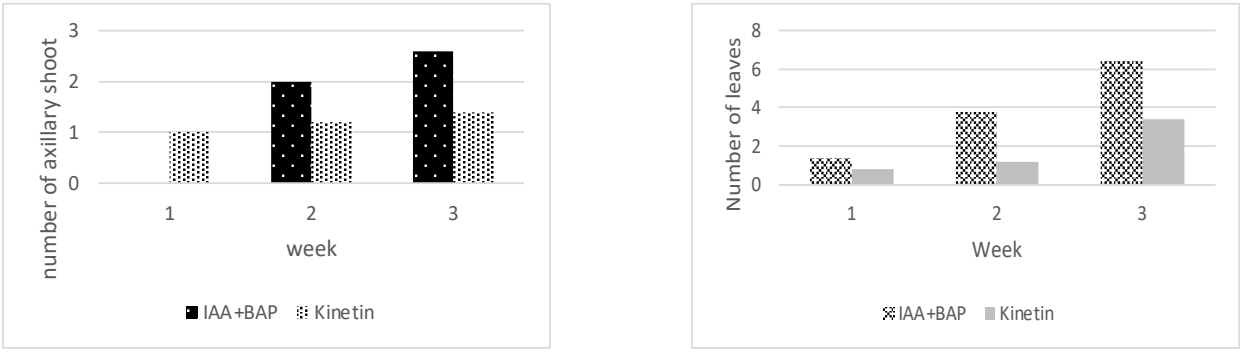
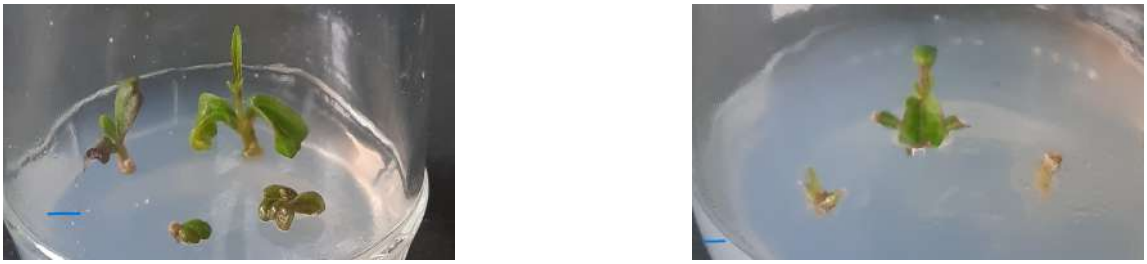
also green, indicating healthy plant growth. Similar result was shown in *Hypericum spectabile* where the highest number of shoot buds was produced in medium containing 1 ppm kinetin. Nevertheless, medium containing BAP was faster to produce shoot bud and leaves than kinetin. Based on Muhammad et al. [16], multiplication rate of shoot bud development was dependent upon cytokinin type, its concentration, and medium used.



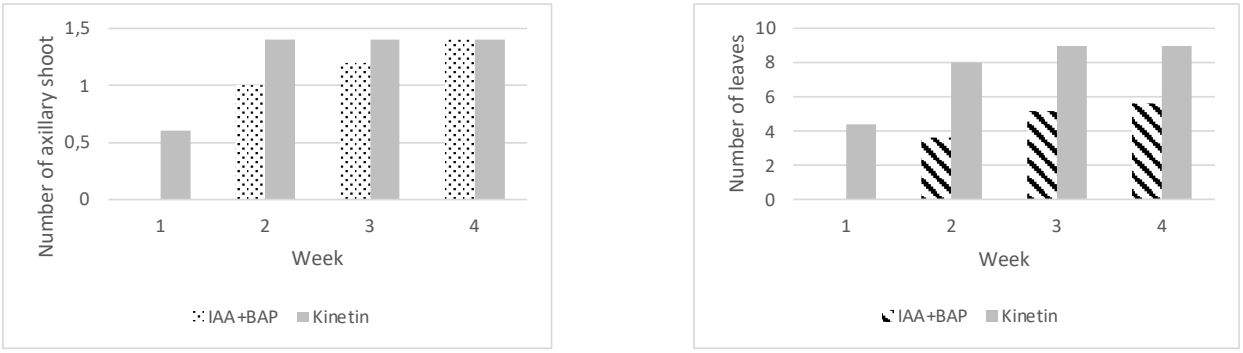
(A) (B)

Figure 2. (A) Number of axillary shoots and (B) Number of leaves at 3 weeks after initiation stage



(A) (B)

Figure 3. (A) *Stevia* culture on IAA and BAP medium and (B) *Stevia* culture on kinetin medium at 2 weeks after initiation. Scale bar indicated 1 cm.



(A) (B)

Figure 4. Number of axillary shoots and (B) number of leaves at 4 weeks after multiplication stage

3.2. Culture on Liquid Medium

Shoot maturation was conducted to strengthen the shoot for further research which included plantlet regeneration and multiplication in bioreactor as well as for increasing secondary metabolite content in plantlets. After the explants were propagated in sufficient quantities, the culture was transferred to MS half-strength liquid medium with 1 ppm kinetin PGR for 7 days [7]. In Figure 6, it can be observed that the cultured plants were brown in some parts. The browning can be caused by stress on plants such as changes in temperature, osmotic pressure, or other changes [13]. These changes can lead to enzymatic oxidation reactions of phenolic compounds that can produce quinones that are brownish-yellow in colour. Quinone compounds can damage plant tissues causing stunted plant growth [11,14]. In this study, stress occurred in plants due to continuous immersion in plants previously cultivated in solid media which was causing the browning and yellowing of tissues.

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(A) (B)

Figure 5. (A) Shoot culture in IAA and BAP medium; (B) Shoot culture in kinetin medium. Scale bar indicated 1 cm.

Figure 6. *Stevia* after being cultured in liquid medium. Circle = brown leaf

4. Conclusion

At the initiation stage, the shoot culture of *Stevia* cultivated in a semi-solid medium containing IAA and BAP showed good growth such as higher leaf number and axillary shoots, while at the shoot multiplication and maintenance stage, cultivation on semi-solid medium with the addition of kinetin showed good growth such as higher leaf number and axillary shoots compared to cultivation in semi-solid medium with the addition of IAA and BAP. Acclimatized plants cultured in a liquid medium with the addition of 1 ppm kinetin showed fairly fast plant growth but was not accompanied by strong stem growth and the presence of brown colour in certain plant parts such as some leaves and stems.

Acknowledgement

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The Behavioral Response of Komodo Dragons (*Varanus komodoensis* OUWENS, 1912) During Mating and Nesting Periods towards Tourist Presence in Loh Buaya, Komodo National Park

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Abstract

It has been recognized in many studies that wildlife tourism practices might generate a negative impact on wildlife, particularly during the reproductive period. Some wildlife may lower their sensitivity towards tourist presence, for instance in Komodo. Understanding to what extent habituation occurs in Komodo would be necessary for tourism management in Komodo National Park (KNP). Therefore, this study aimed to identify the response of Komodo to tourist presence during mating and nesting activities. The observation was conducted in Loh Buaya, which is one of the tourism sites in KNP. Komodo's responses were divided into (1) avoidance; (2) neutral; and (3) aggressive under categorized stimulus: tourist number (i.e., < 5 persons; 5-10 persons; and > 10 persons) and distance (i.e., < 5 m; 5-10 m; and > 10 m). Correlation analysis was performed to identify any influences on mating and nesting activities. Our results revealed that Komodo inhabiting tourism facilities have been habituated to tourist presence. Different tourist frequencies did not influence Komodo mating activities ($r(20)= 0.036$, $p=0.873$), the nest preparing activity (i.e., digging proportion; $r(22)= 0.054$, $p=0.803$) and the guarding activity (i.e., nesting proportion; $r(22)= 0.314$, $p=0.135$). Nevertheless, our results indicated possible impacts due to tourism activities and its supporting facilities, such as dominated mating pairs, threats to female reproductive success, and human-Komodo conflicts. Therefore, habituation evidence must be carefully considered in order to develop more corresponding strategies and achieve sustainable tourism practices.

Keywords: *wildlife tourism, Komodo, reproductive behavior, habituation*

1. Introduction

Interaction between humans and wildlife has occurred for a long time. It could come up with consumptive activities, e.g., hunting, or non-consumptive activities, such as tourism [1]. Tourist motivation for visiting nature could be varied and not limited to observing wildlife, for instance, visitors' activities in Bako National Park were mostly related to trekking, enjoying nature scenery, observing wildlife, experiencing a relaxing environment, and taking nature photographs [2]. Nevertheless, in wildlife tourism, which makes wildlife encounters the main product, tourist satisfaction might be greatly influenced by the quality of their experience generated from the interaction with wildlife [1]. Many managers would come up with some strategies to improve the outcomes by building trekking tracks [3], watch towers, artificial water holes [4], and wildlife feeding [4–6].

The impacts of tourism activities on wildlife have been reported in many studies, particularly during the breeding seasons. Animal reproductive activities become a highly interesting attraction for tourists, yet wildlife may experience great pressure [7]. Human visits during the early incubation period resulted in a lower nesting success rate in Common Eiders compared to late period visits [8]. Furthermore, human presence may force wildlife to exhibit antipredator behavior, which may reduce their body conditions [9,10] and cause behavioral changes [11]. In the study of the Iberian rock lizard, antipredator behavior caused the male individuals to spend more time in refuge and ultimately lose their mating opportunities [12].

In order to offset the cost due to antipredator behavior, some wildlife could adjust their response through habituation.

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Muting the antipredator behavior by lowering their sensitivity may provide benefits for wildlife. Habituated individuals can relatively improve their body conditions [13] and maintain their reproductive success [11]. Nevertheless, habituation does not always come with positive consequences, such as human-wildlife conflict [14,15]. Regardless of the benefit and cost to wildlife, habituation sometimes becomes a desirable result in wildlife tourism practices [1,13,16].

In general, wildlife might be forced to decrease their sensitivity level due to the absence of alternative habitats [17] or because the benefit provided exceeds the cost [7]. Habituation may differ between species or individuals based on genetic and learning abilities to specific stimuli or environmental conditions [14,18]. Individual characteristics, such as gender, may influence the rate of habituation [19]. Moreover, the degree of stimulus will also influence the wildlife sensitivity level [11]. Therefore, with these varying factors and results in the habituation process, more comprehensive information would be necessary to draw the conclusions in the wildlife response to human presence.

The world's largest living lizard, the Komodo (*Varanus komodoensis* OUWENS, 1912), has a very limited distribution in five islands in the East Nusa Tenggara region, four of which are within the Komodo National Park [20–22]. Komodo National Park (KNP) is one of the conservation areas intended to protect the well-known lizard population and their natural habitat. The tourism practice with Komodo as the main attraction has grown steadily since the establishment of KNP in the 1980s [4]. According to the Bureau of Statistics [23], the total number of visitors in KNP increased from 68,000 (2015) to 178,683 (2018) individuals/year. Furthermore, the peak season for visitors was July-August, which also happens to be Komodo dragon reproduction season [24]. The mating and nesting activities might be highly attractive for visitors to observe, yet it was also the most sensitive period for the Komodo population.

Komodo habituation to visitors has been discussed in a previous study. It was reported that the Komodo inhabiting the high human activity areas were less sensitive (or habituated) [25]. Despite an extensive examination of human activity's impact on the population, the discussion of Komodo's habituation was limited to direct responses, with no extension to other behaviors such as mating and nesting. According to Auffenberg [24], Komodo will exhibit aggressive behavior during mating and nesting activities. Therefore, understand- ing the Komodo response to tourist presence during those times may provide more information about the extent of habituation.

This study aimed to identify the Komodo's response to tourist presence during mating and nesting activities. The response would be assessed under different tourist numbers (i.e., low, moderate, and high) and distances (i.e., near, moderate, and far). In particular, we would like to identify whether there were certain numbers or distances that could possibly terminate mating and nesting activities. Furthermore, the relationship between the stimulus and reproductive behavior would be assessed to determine the influence of tourist presence. Ultimately, it may provide extensive information regarding the habituation of Komodo.

2. Methodology

2.1 Description of Study Area

This study was conducted at a tourism area in Loh Buaya, Rinca Island, Komodo National Park (Figure 1). The ecosystems in that area were dominated by deciduous monsoon forest and savanna grassland. The climate consists of a long-dry season in March-November and a short-wet season in December-February [21,24]. Six nests had been observed in this study, but one of those was different from the last study reported by Jessop et al. [26].

Mating observation was conducted around the Tourism Supporting Facility Area (TSFA) such as the barrack, kitchen, café, guest house, and office. Meanwhile, a nesting observation was performed on a potentially active nest. According to a previous study, the active nests of Komodo are usually located under ≤ 25% of vegetation canopy coverage [27]. In order to carry out our research design, the observed nest should be located under 10 meters with the tourism track. It was determined by komodo's ability to clearly distinguish a person from another object at six meters [24]. Therefore, there were only two possible nests that meet the criteria, which were LBM1 (i.e., Loh Buaya Mount-Nest) and LBM2 (Figure 2).

Figure 1. Map of study area in Loh Buaya that shows TSFA (Tourism Supporting Facility Area that consists of barac, cafetaria, kitchen, guest house, office, and other facilities), nest distribution (red: active nest and dark: not active nest) and its relative distance to tourism track.

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Figure 2. Observed Komodo's Nest (A) LBM1 and (B) LBM2.

LBM1 and LBM2 are mount-type nests and located in a deciduous monsoon forest. The average solar radiation intensities were 90.97 (SE) 1.52 Lux (LBM1) and 90.20 (SE) 1.30 Lux (LBM2). Due to the direct exposure to an open area (> 75% of total surrounding cover), LBM1 is likely to experience higher pressure than LBM2.

2.2 Behavior Observation

This study was conducted at a tourism area in Loh Buaya, Rinca Island, Komodo National Park (Figure 1). The ecosystems in that area were dominated by deciduous monsoon forest and savanna grassland. The climate consists of a long-dry season in March-November and a short-wet season in December-February [21,24]. Six nests had been observed in this study, but one of those was different from the last study reported by Jessop et al. [26].

Mating activities were observed using the Focal sampling method and limited to the pairs that were observed mating around TSFA. Those pairs would be categorized into resident and visitor pairs. The resident pairs (i.e., both male and female individuals) were distinguished by their regular presence around TSFA. All individuals were firstly identified by their natural marks, such scars [28]. Copulation duration and interval (i.e., pre-copulation and post-copulation) were recorded during observation. When one of the two individuals left the TSFA, the observation would come to an end.

A similar method was also performed for nesting observation. The observations were limited from pre-egg laying phase to the first week of the post-egg laying phase. This period was thought to be the most vulnerable to disturbances [8]. A single female individual from either LBM1 or LBM2 would be continuously observed for 12 h (i.e., 06.00-18.00) based on Komodo's daily active period [24]. The observation will be focused on single female individual that was firstly recorded to starting her nesting activity. Duration of other nesting activity, including resting would be recorded during this observation.

2.3 Assessment of Response Toward Tourist Presence

The direct responses were divided into three categories: (1) Avoidance, i.e., Komodo stop the activities and run away from tourists, which results in a broken mating pair or nest abandonment; (2) Neutral, i.e., Komodo continue the mating or nesting activities; and (3) Aggressive, i.e., Komodo stop the activities and run toward tourists in an aggressive course.

The tourism stimuli were categorized by the number of tourists in one group and observation distance. The management of Komodo National Park has decided that one guide will accommodate a maximum of five visitors in a single group. It is also possible that a single group will have more than 5 participants and will be led by more than one guide. Therefore, this threshold was used to define the number of tourist categories: low (i.e., < 5 persons in a group); moderate (i.e., 5-10 persons in a group); and high (i.e., > 10 persons in a group). The observation distance was also classified as close (5 m), moderate (5-10 m), and far (> 10 m). That was based on Komodo's ability to clearly recognize an object at 6 meters [24]. Under the conditions where multiple groups occurred, the total number of visitors, which was then calculated into tourist frequency (i.e., visitors/minute), and the nearest distance would be recorded.

2.4 Statistical Analysis

Differences in mating activity (i.e., pre-copulation, copulation, post-copulation, and total mating) between resident and visitor pairs were analyzed using a t-test. If the data could not meet the parametric test assumptions, then the Mann Whitney U test would be performed [28]. Similar methods were used to analyze differences in nesting activity (i.e., digging activity, other nesting behavior, total nesting, and foraging) between the pre-egg laying and post-egg laying phase. Furthermore, the Chi-square test will be used to examine the difference in Komodo's response to all stimulus categories. Furthermore, the Pearson's correlation test will be used to determine any impact of tourist presence on mating behavior. It aimed to see the relationship between visitor frequency and mating proportion (i.e., copulation behavior/mating interval). Similar methods were used to investigate the effect of visitor frequency on nesting behavior. We tried to identify whether the visitor frequency influences nest-preparing behaviors (i.e., digging/other nesting behaviors) as well as nest-guarding behaviors (i.e., nesting/foraging). The R program (R GUI version 4.0.4, assisted by R Studio version 1.4.1106) was used for all statistical analysis.

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3. Results and Discussion

3.1 Mating Behavior

Two pairs, which are resident (i.e., ♂1 and ♀1; Figure 3 A and B) and visitor (i.e., ♂5 and ♀4; Figure 3 C and D), were observed mating around TSFA during this study (Table 1 & Figure 4). The resident pair was observed mating 13 times in two consecutive days and visitor only mating nine times in a single day. The resident pair exhibited a longer duration of total mating (U= 84.5, p= 0.044), pre-copulation (U= 88, p= 0.022) and post-mating (U= 94, p= 0.019). Nevertheless, cop- ulation duration was not significantly different between those pairs (U= 45.5, p= 0.396).

Table 1. Mating and nesting behavior of Komodo

No	Behavior	df	Duration (minute)			
			Min	Max	Avg	± SD
Mating Behavior						
1	Pre-Copulation	21	1	46	12.82	13.84
2	Copulation	21	2	10	4.36	2.14
3	Post-Copulation	19	0	65	18.63	15.59
4	Total Mating	21	6	99	33.27	25.04
Nesting Behavior						
1	Digging	23	0	642	98.04	146.76
2	Other nesting behaviors	23	0	684	293.42	241.62
3	Total Nesting	23	0	716	391.46	236.74
4	Foraging	23	0	725	290.50	234.72

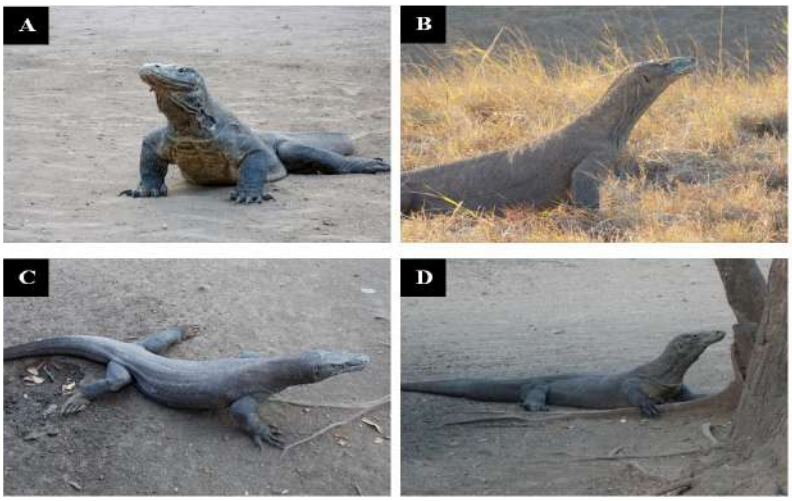

Figure 3. Focal Individuals (Resident pair: (A) ♂1 (Johnson) and (B) ♀1 (Jessica). Visitor pair: (C) ♂5 (Jeremy) and (D) ♀4 (Jane)).

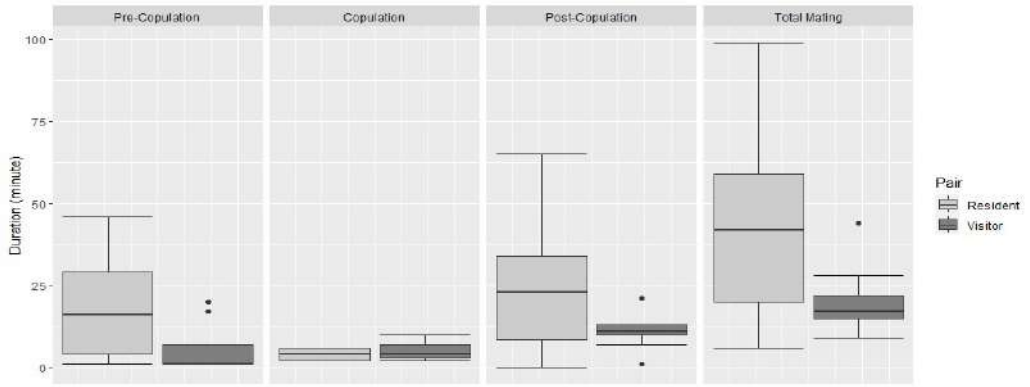

Figure 4. Komodo mating behavior.

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3.2 Nesting Behavior

During our fieldwork, we recorded that three of six Komodo nests were active, which were LBM1, LBM3, and LBM 6 (Figure 1). The active nest was distinguished by the presence of female individual during the nesting period. Nevertheless, the observation was carried out at LBM1, since it met with the given criteria. Individual ♀1 was observed nesting at LBM1 in 21 days after the last mating with ♂1. Nevertheless, she was also spotted mating with another male individual the day before it, but the data was not included in the analysis. Nesting activity became more intensive on day 6th and carried on for another 12 days until eggs were laid. There was a different in nesting activity between preparation and guarding phase (Table 1 & Figure 5). Total nesting (U= 30, p= 0.032) and other nesting behaviors (U= 11, p= 0.001) were significantly higher during the post-egg laying period. On the other hand, digging (U= 86.5, p= 0.044) and foraging (U= 94.5, p= 0.028) were recorded significantly lower in that period.

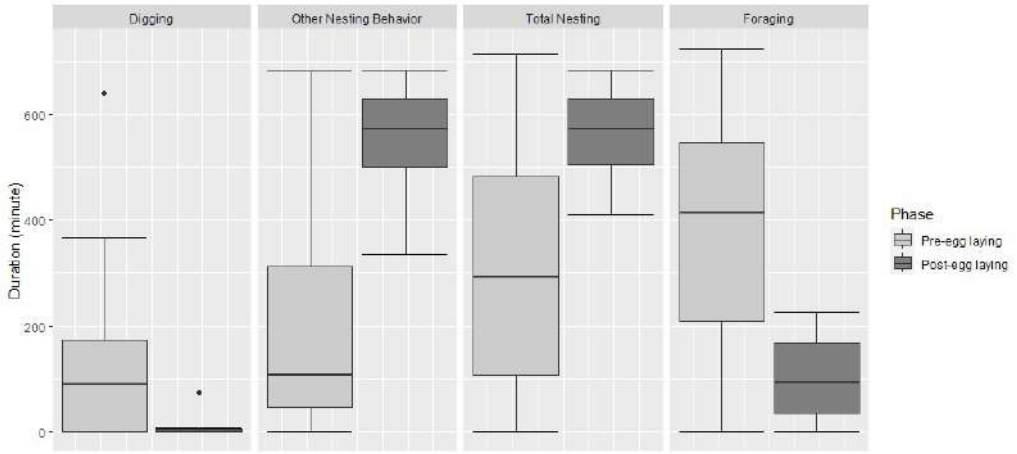

Figure 5. Komodo nesting behavior.

3.2 Komodo Response Towards Tourists

Both resident and visitor pairs exhibited neutral responses in all stimulus categories (Table 2). The highest number of visitors was 40 persons with the closest distance was 3 meters. The average tourist frequency was found to be significantly higher in the residents (6.08 ± (SE) 0.98 tourists/minute) than visitor pairs (1.76 ± (SE) 0.61 tourists/minute; U=1895, p=0.001). The resident pair was observed mating more frequently than the visitor pair around the study area. It increased the possibility of multiple tourist groups occurring and affected the average tourist frequency. In addition, different tourist frequencies did not influence Komodo mating activities ($r_{(20)}$= 0.036, p=0.873; Figure 6). All the stimulus categories and pairs were not analyzed separately due to the limited data available.

Table 1. Komodo response towards tourist presence during mating and nesting activities

Parameters	Mating Response				Nesting Response			
	N	1	2	3	N	1	2	3
Less than 5 persons								
< 5 m	32	0	32	0	30	0	30	0
5-10 m	28	0	28	0	204	0	204	0
>5 m	1	0	1	0	11	0	11	0
5 – 10 persons								
< 5 m	15	0	15	0	9	0	9	0
5-10 m	22	0	22	0	119	0	119	0
>5 m	0	0	0	0	14	0	14	0
More than 10 persons								
< 5 m	6	0	6	0	3	0	3	0
5-10 m	6	0	6	0	45	0	45	0
>5 m	1	0	1	0	4	0	4	0

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Figure 6. Relationship between Komodo mating behavior (i.e., copulation proportion) and tourist frequency

Individual ♀1 exhibited a neutral response in all stimulus categories during pre-egg laying and post-egg laying phase (Table 2). During the nesting observation, the highest number of tourists was 35 persons with the closest distance was 5 meters. The average tourist frequency for the pre-egg laying (4.13 ± (SE) 0.29 tourists/minute) was significantly lower than the post-egg laying (5.83 ± (SE) 0.11 tourists/minute; U=1476, p=0.003). Individual ♀1 was recorded to spend more time around the nest during post-egg laying phase. Therefore, multiple groups were more likely to occur, which increased the average tourist frequency. Furthermore, different tourist frequencies had no influence on nest preparation activity (i.e. digging proportion; $r_{(22)}$= 0.054, p=0.803; Figure 7) and guarding activity (i.e. nesting proportion; $r_{(22)}$= 0.314, p=0.135; Figure 7). All the stimulus categories and nesting phases were not analyzed separately due to the limited data available.

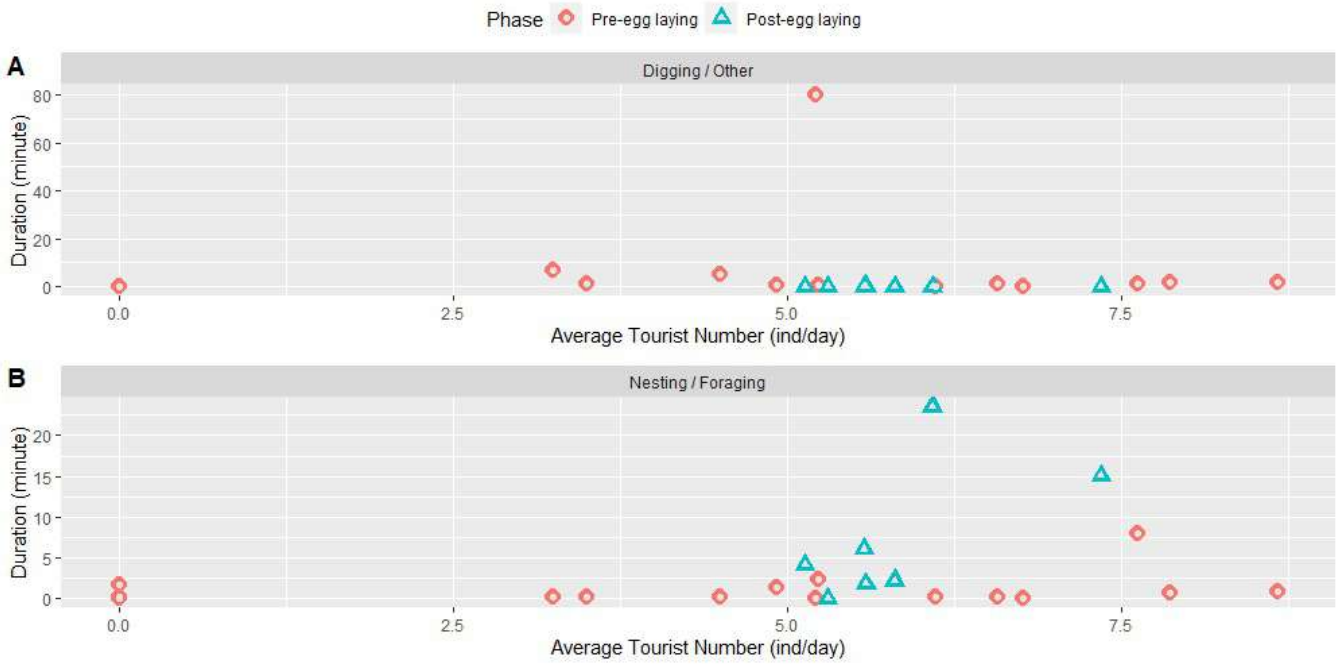

Figure 7. Relationship between Komodo nesting behavior and tourist frequency. (A) Digging proportion and (B) Nesting proportion

Both resident and visitor pairs appeared to be already habituated to tourist presence. All the mating events were not influenced by the tourist's presence (i.e., there was no evidence in broken mating pair). Moreover, different tourist stimulus categories did not seem to cause the mating duration shorter or longer. Nevertheless, according to our observations, mating occurred more frequently under the low tourist frequency. Unfortunately, we could not make any clear judgements regarding the relationship between mating frequency and tourist numbers due to the limited data available.

Furthermore, we were unable to determine whether the lower mating occurrence on visitor pairs was due to their lower habituation level when compared to resident pairs. We recorded that the visitor pair performed mating only on a single day around the TSFA. According to the data, the pair exhibited no response toward tourist presence during mating. We

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assumed that the mating occurred when the male individuals extended their home range during the reproductive period and overlapped with the female [29]. It has been reported in a previous study that mating in Komodo often take place near carcass [24]. Therefore, human activities in the TSFA may attract Komodo to aggregate [25]. Without the presence of the dominant resident male, other individuals, particularly the subordinate male or visitors, could have an opportunity to mate around the TSFA [30].

The resident exhibited a longer duration in mating activities (i.e., pre-copulation and post-copulation) than the visitors. Mating duration in Komodo could be influenced by the male's experience. A younger male individuals take longer mating time than a more experienced older male individuals [24]. The longer mating duration in the resident pair might be influenced by a high individual density around the TSFA which could increase the competition among male individuals. It has been reported in the previous study that Komodo were attracted to tourism facilities, particularly the kitchen [25]. With a male:female ratio that is skewed toward male (i.e., 3.3:1), the competition will be on the male individuals to find a mating partner [24,30]. Our observations supported this suggestion. All the resident pair mating activities were occurred in the middle of individual aggregation around the TFSA. There were almost 15 occurrences of mating disturbances recorded during the mating observation.

Moreover, a high frequency of interaction among resident individuals due to aggregation may also influence female receptivity. According to Auffenberg [24], female individuals would tend to exhibit aggressive behavior toward male individuals. Bigger male individuals ofter attack smaller individuals, including females, while eating a carcass in aggregation. Mating rejection by females may also be affected by their receptive period, which could be different among individuals [30,31]. Nevertheless, female individuals will finally accept mating to avoid a bigger cost generated from male coercion [32]. Unfortunately, our observations could not distinguish the receptive period of the female resident during mating activities.

Human disturbance during the nesting period often leads to some unfavorable consequences, such as nest abandonment [8]. Nevertheless, such evidence was not detected on our focal female. Individual ♀1 did not abandon her nest during tourist visits in both phases. During preparation phase, high tourist frequency did not interfere digging activity. Moreover, it also did not seem to have any influence on nesting behavior during the guarding phase. Unfortunately, less habituated females were not covered by this study, which might prefer to avoid nesting in disturbed areas [17].

Low availability of suitable nest might turn the habituation as a preferable way for individual ♀1 in order to depress the cost, otherwise more energy should be spent for exploring other potential nests outside the disturbed area [7,8,17,33]. Nevertheless, adverse consequences might remain to emerge. Our camera trap results indicated that the LBM1 had experienced the highest pressure from egg predators due to its surrounding openness compared to the other more isolated active nests (e.g., LBM3 and LBM6). Opening habitat is often implemented in tourism practice, and it has been reported that this could potentially increase nest predation [11,34]. Furthermore, it might threaten the body condition of individual ♀1. However, the limited nest available and food attraction (i.e., attracted by food smells around kitchen and feeding attraction) seemed to overcome the cost. Nonetheless, long-term effects may occur, jeopardizing the reproductive success of Komodo dragons.

Human-wildlife interaction frequently results in conflict, such as an attack on humans, and the likelihood is higher in habituated wildlife [15,35,36]. Several cases of Komodo attack have been reported around the study site, but they have not been properly documented. During our observation, individual ♀1 was once spotted foraging into the ranger's facilities (i.e., barac). Therefore, it could be assumed that the human-Komodo encounter could take place in an undesired location. Without any proper facilities to treat the bite-wounds, it could have fatal consequences.

Even though our results showed that komodo exhibited a neutral response toward tourist presence, they might potentially take an aggressive course when the tourist was within closer range. As has been reported by Auffenberg [24], Komodo was often observed exhibiting ignorance behavior toward tourists up to 1-2 meters while they were around carcasses. During the observation, an aggressive response just occurred when the disturber (i.e., other Komodo individuals) came within a certain distance (i.e., 1 meter during mating and 2 meters during nesting). According to our data, the closest distance of the tourist while observing Komodo was still higher compared to the recorded proximity of disturber individuals. We believe that the unwanted interaction could possibly occur if the threshold was exceeded [37].

Misinterpreting the evidence of habituation may lead to an unwanted conflict between Komodo and tourists. Appropriate management should be implemented by the manager in order to achieve sustainable tourism practices. Therefore, several plans were proposed as follows:

1. Food attraction and feeding practices should be strictly regulated by the manager, as has been proposed in the previous study [25]. The rules of Who, When, and Where must be considered as a baseline in constructing the plan. Who: Feeding practice needs to be limited only for a certain type of visitor (i.e., those who has a special interest, such as researchers or documentary filmmakers) and was held under tight surveillance by the KNP manager. When: The practices could only be carried out for a certain period of time considering some adverse impacts that may follow, such as lowering body condition and decreasing the

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komodo's natural hunting ability. Where: Individual aggregation due to food attraction should be relocated out of TSFA for the purpose of avoiding unwanted interaction in an undesirable place. The baiting method was encouraged to be used in a preferable location, such as a forest. We believed that tourists would gain a greater experience by encountering Komodo in the wild rather than around TSFA.

2. Habitat modification was only conducted in insensitive areas, such as trekking tracks, baiting areas, or nest observation spots [1]. Clearing path along trekking track should be necessary due to the safety purposes towards unintentionally ambushing attack by komodo. Clearing habitat around the nest would not be encouraged due to the potential impact, as has been discussed above.
3. Tourist behavior should also be regulated to avoid unintentional conduct that could provoke the komodo to take an aggressive course [1,37]. A minimum distance between komodo and tourist should be established during their encounter. In addition, any sudden movement should also be prevented. Auffenberg [24] mentioned that komodo was not only sensitive to a chemical signal, but also a sudden motion. Moreover, the tourist number should also be regulated carefully. According to the study of the Nubian ibex in southern Israel, the species' tolerance increased with the number of humans present. Nevertheless, it would be a desirable outcome for a short period yet could lead to maladaptation in a long period [38].

Finally, tourist perception and knowledge of wildlife would be important and may become a key factor in establishing sustainable tourism practices [1]. Therefore, it should be taken into account by the KNP manager. Furthermore, multidisciplinary research will be encouraged in order to provide a more comprehensive picture.

4. Conclusion

Komodo, particularly those inhabiting the tourism area, have become habituated to the tourist presence during mating and nesting activities. Nevertheless, tourism activity should be managed carefully in order to avoid any negative consequences for both humans and Komodo. Our study suggested that the intensity of interaction between humans and Komodo should be limited. There must be a threshold for tourist numbers in order to avoid any further decrease in Komodo's tolerance level, which might lead to maladaptation. Direct interaction should be restricted to minimize any conflicts, such as an unintentional attack, that could possibly occur. Furthermore, habitat modification should avoid any sensitive areas, such as nests. The open access to the nest due to altered habitat could decrease a female Komodo's body condition and reproductive success. In the long term, it might jeopardize the species population viability. Finally, with a comprehensive plan by the manager, tourism activity in Loh Buaya could generate a positive impact for both the socio-economic aspect and the conservation of the Komodo population.

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Primer Design and Optimization of Annealing Temperature for Analysis of Glutathione Reductase Gene Expression in Rice (*Oryza sativa* L.)

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Abstract

Glutathione Reductase (GR) belongs to the NADPH-dependent flavoprotein oxidoreductase family and is found in both prokaryotes and eukaryotes. The GR gene is considered to play a key role in the elimination of oxidative reaction products by looking at the level of gene expression of GR rice in dealing with drought stress using qPCR. One of the important steps to develop a specific, effective and efficient qPCR is the primer design. Several studies analyzing GR gene expression in rice have also designed primers. However, the primer still lacks an ideal characteristic of primer, as it still has a secondary structure. This studies aims to design rice GR specific primers and optimize the annealing temperature for GR gene expression analysis on rice. Primers were designed using the Primer3 and Geneious Prime and checked for specificity using the Primer-BLAST tool. The selected primer pairs were then optimized for annealing temperature using gradient PCR. The best primer design results were GR-Forward 5'-ACGATTGCAGCCAGTGAAGA-3' and GR-Reverse 5'-TGCGGCAATACTATCAACATCC-3', with an amplicon length of 204 bp, primer base lengths of 20 and 22 nucleotides, Tm values of 60°C and 58.9°C, %GC of 50% and 45.5%, respectively. This primer pair had no secondary structure, both hairpin and self dimer. Gradient PCR showed the optimum annealing temperature for this primer pair was 52.2°C so that the primer can be used as a specific primer to analyze the GR gene expression in rice using qPCR.

Keywords: *annealing temperature, glutathione reductase, primer design, rice*

1. Introduction

Glutathione reductase, also known as GSR or GR belongs to the NADPH-dependent flavoprotein oxidoreductase family and is found in both prokaryotes and eukaryotes. GR contains different domains such as NADPH binding domain, FAD binding domain and interface domain for joining two GR subunits [1]. Although GR is localized to chloroplasts, cytosol, and mitochondria, more than 80% of its activity in photosynthetic tissues is in chloroplast isoforms [2]. GR is known to be involved in protecting photosynthesis against oxidative stress [1]. One of which occurs due to drought stress [3].

Glutathione reductase plays an important role in cellular defense against reactive oxygen metabolites efficiently maintaining the cellular pool of reduced glutathione (GSH) by catalyzing the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) with a concomitant increase in NADPH oxidation. GR converts GSSG to GSH, thereby assisting in maintaining a high GSH/GSSG ratio under various abiotic stresses [2].

Glutathione reductase is one of the antioxidant enzymes produced by plants as a defense mechanism against drought stress. Based on the results of Violita (2007), GR enzyme activity increased with the length time of drought stress treatment in soybean plants. The highest increase in GR activity occurred on the 10th day of the drought stress treatment. This increase in GR activity is related to the role of GR in protecting plants from oxidative stress due to a decrease in the relative moisture content of the leaves. GR enzymes protect cells from oxidative damage through the regulation of cyclic ascorbate glutathione (ASH-GSH) [2]. GR enzyme activity in the ASH-GSH cycle is controlled by genes GR differ depending

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on the type of group. This is in line with the results of Refli & Purwestri, (2016) research, that transcription patterns of GR gene change in different ways in response to drought stress and salinity in rice plants. GR gene transcription regulates glutathione reductase activity in plants. Therefore, GR enzymes may have less activity in drought treated rice seedlings compared to those treated with salinity. This may be due to more GR genes which involves setting strategies for adapting rice seedlings to salinity com- pared to drought strategies [4].

To find out how the mechanism of the GR enzyme in dealing with drought stress, further research on the expression of the GR gene in dealing with drought stress is required. This can be accomplished by examining differences in the activity levels and transcription of genes encoding the GR enzyme, which is expected to play a vital role in reduced glutathione defense (GSH) and the removal of oxidative reaction products [4].

Gene expression is compromised of two stage such as transcription and translation. To express its genetic information, cells carry out various processes, including copying genetic information from DNA to mRNA (messenger RNA), this process is called transcription, and then followed by the translation of the genetic information contained in the mRNA molecule into protein, this process is called translation [5].

The technique most frequently used to analyze differential mRNA expression is quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR is an effective method for observing changes in gene expression during processes such as cellular differentiation because it is highly sensitive and specific [6]. The qRT-PCR was used to see the quality of gene expression through formation of complementary DNA (cDNA) formed from RNA and calculate the quantity of cDNA amplification produced [7]. In the cDNA amplification stage with PCR, a pair of primers is needed (forward and reverse), to limit the area to be amplified [8].

PCR success depends on the primer used. Primers are single stranded oligonucleotide molecules consisting of approximately 18-30 bases. A good primer is determined by several primer criteria. These criteria include: primer length between 18-22 mer, %GC ranging from 40% - 60%, Tm (melting temperature) 58-60ºC and not more than 65ºC, no primer interactions (dimers and hairpins), primer stability, repeats, runs and false priming [9].

The key to PCR success is inseparable from bioinformatics studies when designing specific primers for target genes [10]. The success of gene amplification by PCR using specially designed primers is largely determined by the accuracy of the primer attachment temperature (annealing) with a DNA template. If the temperature is too low it can cause the primer to stick to the genomic DNA or attach to a non-specific place, while the temperature is too high it will prevent the amplification process from occurring [11]. For this reason, it is necessary to optimize the PCR process so that the PCR results obtained are optimal [12]. Several studies analyzing GR gene expression in rice have also designed primers [13], [14]. However, the primer still lacks an ideal characteristic of primer, as it still has a secondary structure, both hairpin and dimer. This secondary structure can reduce PCR efficiency. Based on this, the purpose of this study were to design rice GR specific primers and optimize the annealing temperature for GR gene expression analysis on rice.

2. Methodology

This study was research based on molecular biology and computational testing (bioinformatics). Gene nucleotide sequence of GR (NCBI accession number: XM_015757647.2) which will be modified referring to research [13], [14]. Primers were designed using the program primer3 on the site Primer3web (https://primer3.ut.ee/). Generated primer candidates were analyzed further using Geneious Prime software [15], then target gene specificity was analyzed using primer-BLAST on the NCBI website [10]. The primers obtained were tested for their annealing temperature using gradient PCR, electrophoresis using 1.5% agarose gel and visualization using Gel Doc.

2.1 Primer Design

GR accession number of (XM_015757647.2) was entered on column search on the NCBI site. The symbol for this gene is LOC4348623 with the locus tag OSNPB_100415300 found in chloroplasts [16]. Then the primer generated using 'pick primer' tools. The length of the PCR product was adjusted for qPCR analysis in the "PCR product size" column, which was a minimum of 150 and a maximum of 250 bp.

2.2 Primer Analysis Using Geneious Prime and Primer-BLAST

The FASTA file of GR sequence (XM_015757647.2) was downloaded from the NCBI website, then imported to the Geneious Prime software. All candidate primer sequences that have been designed using 'pick primer' on the NCBI website were copied and saved as primer sequences in Geneious Prime. The primer criteria were shown on primer annotation and also the DNA fold displayed for each primer. Then a primer pair that fits the criteria for a good primer was chosen. After obtaining the best primer, then the specificity of the primer was checked using the primer-BLAST tool on the NCBI website. Selected primer pair was synthesized in IDT, Singapore.

2.3 Rice Root RNA Extraction

TRNA extraction from rice root was using GENEzol™ Reagent (Cat. GZR100). All stages of extraction followed the GENEzol procedure™. Samples were weighed 50–100 mg and then crushed using liquid nitrogen with the help of a micropestle. To the samples 500 µl GENEzol was added and

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incubated for 10 minutes at room temperature. After that, 200 µl of chloroform was added to the sample tube as much as 500 µl by shaking gently for 10 seconds. Sample mixture was centrifuged at 16,000 rpm for 15 minutes at 4^{O}C. The aqueous phase containing RNA was transferred to a new tube. Isopropanol alcohol was added according to the volume of RNA into the tube and then incubated at room temperature for 10 minutes. Sample was centrifuged at 16,000 rpm for 10 minutes at 4^{O}C then the supernatant was discarded without disturbing the pellet. RNA pellets were washed using 70% ethanol and centrifuged at 16,000rpm, 5 minutes at 4^{o}C. The supernatant was discarded again without disturbing the pellet then air dried the RNA pellet for 10 minutes at room temperature. To resuspend the RNA pellet 50 µl of nuclease-free water was added then incubated at 55^{o}C to dissolve the pellets.

2.4 cDNA Synthesis

cDNA synthesis was performed using reverse transcriptase (RT) enzymes in combination with qPCR with the Sensifast cDNA Synthesis Kit. qRT-PCR was carried out by preparing a cDNA synthesis reaction mixture. The sample was briefly homogenized with a vortex, then put into the thermocycler and run for 30 minutes, with annealing settings at 25^{o}C for 10 minutes, reverse transcription stage at 42^{o}C for 15 minutes, and inactivated at 85^{o}C for 5 minutes and the cDNA can be directly used as a template for qPCR and stored at -20^{o}C.

2.5 Gradient PCR

Optimization of annealing temperature was conducted using gradient PCR . PCR reaction in 10 µl consisting of 0.5 µl rice root cDNA, 0.4 µl of 10 µM forward and reverse primers, 5 µl of 2x GoTaq PCR master mix (Promega) and 3.7 µl nuclease-free water. PCR temperature cycle, i.e. initial step 95°C for 3 minutes, followed by 35 cycles of denaturation 95˚C for 30 seconds, annealing temperature was set gradient 50-60°C for 30 seconds, elongation 72°C for 30 seconds, and final elongation 72°C for 5 minutes. The PCR products were separated using electrophoresis using 1.5% agarose gel with 1x TAE buffer. Electrophoresis was carried out at 100 V for 35 minutes. Furthermore, the electrophoresis result was visualized using Gel Doc.

3. Results and Discussion

3.1 Primer Design

Based on the design results on the program 'pick primer', 10 forward primers and 10 reverse primers (10 primer sets) were obtained Table 1. Forward primer is a primer located at the front end of the DNA target and serves to mark the front end of the DNA strand to be duplicated; in other words, forward primer will go from the 5' end to the 3' end. Meanwhile, the reverse primer is located at the back end of the DNA target [9].

Of the 10 sets of primer candidates generated, each has a different amplicon length and nucleotide arrangement. Amplicons are target DNA strands that have been successfully duplicated during the PCR process [17]. The length of the PCR amplification amplicons depends on the purpose of the research to be carried out. Standard PCR amplicon lengths are 100-500 bp. Products with a length of 1000 bp require 1 minute for the extension process at the PCR stage [18]. Based on the data of the primer candidates in Table 1, each primer has a length of <2000 bp so that the required extension time for PCR is also less than 1 minute.

3.2 Primer Analysis Using Geneious Prime and Primer-BLAST

Analysis of primer candidates was conducted based on good primer criteria using Geneious Prime. In general, the optimal base length for qPCR primer was 18-22 nucleotides [19]. Based on the primer candidate data (Table 1), all primer candidate sets 1 to 10 are good primers forward and reverse which have primer length of about 20-22 nucleotides, this meets the criteria for optimal base length.

Melting temperature (Tm) is the temperature at which 50% of the DNA double strands separate. The optimal primer Tm for qPCR is 58^{o}C – 60^{o}C [20]. In this study, 9 sets of primer pair candidates (forward and reverse) have a Tm between 58-60^{o}C. The already meets the optimal primer Tm for qPCR. While primer set 6 has reverse primer Tm of 54.2^{o}C so that it does not meet the criteria for a good Tm (Table 1). If the Tm primer is too low, the primer tends to anneal elsewhere and produce non-specific products. While if Tm is too high (> 65oC), it will reduce the effectiveness of annealing which can lead to failure of the DNA amplification process. Primer pair must have a difference in the Tm value of not more than 5 because it can cause a decrease in the amplification process or even no amplification process occurs. Tm can be calculated manually using the formula Tm = 2(A+T) + 4(G+C). The Tm of a primer must be chosen carefully because it has a significant impact on temperature annealing used in the PCR process [19].

Percentage (%) GC is the number of percentages of guanine and cytosine in a primer. %GC should be in the range of 40-60% [9]. In this study, all sets of primers forward and reverse already met the criteria for a good %GC (41.7% - 55%), except primer set 8 because the reverse primer only has 39.1% (Table 1). Primers with %GC below 50% require a base length of more than 18bp to keep Tm above the recommended minimum. The 9 sets of primer pair candidates have fulfilled the primer criteria of %GC because they are in the range of 40-60% [17].

The ideal PCR product size or primer amplicon length for qPCR is between 100-250 bp to increase amplification efficiency [20]. In this study, only 2 primer sets (forward and reverse) which met the ideal amplicon length criteria, namely set 2 with an amplicon length of 196 bp and set 10 with an am-

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plicon length of 204 bp. Meanwhile, the other primer candidates do not meet the ideal amplicon length criteria (Table 1).

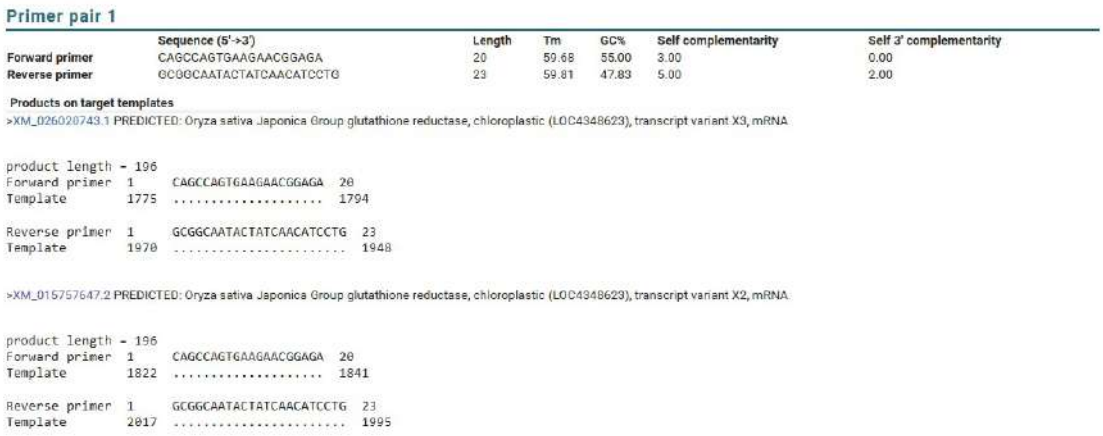
Table 1. Specification of GR primer candidates

Set	Primer	Sequence (5' > 3')	Tm	% GC	Amplicon Size
1	Forward	TGTTGCAGTCTGACCAGTCG	60.2	55.0	289 bp
	Reverse	TGCGGCAATACTATCAACATCC	58.9	45.5	
2	Forward	CAGCCAGTGAAGAACGGAGA	59.7	55.0	196 bp
	Reverse	GCGGCAATACTATCAACATCCTG	59.8	47.8	
3	Forward	AACCGCGGAAACAACAGACA	60.7	50.0	262 bp
	Reverse	GCGGCAATACTATCAACATCCTG	59.8	47.8	
4	Forward	CCGCGGAAACAACAGACAGA	60.9	55.0	260 bp
	Reverse	GCGGCAATACTATCAACATCCT	58.6	45.5	
5	Forward	TTGCAGTCTGACCAGTCGAT	59.0	50.0	288 bp
	Reverse	ATGCGGCAATACTATCAACATCC	59.2	43.5	
6	Forward	TTGTGTTGCAGTCTGACCAGT	60.1	47.6	291 bp
	Reverse	GCGGCAATACTATCAACATCC	54.2	47.6	
7	Forward	TCTAACCGCGGAAACAACAGA	59.9	47.6	268 bp
	Reverse	CATGCGGCAATACTATCAACATCC	60.4	45.8	
8	Forward	CGCGGAAACAACAGACAGAT	58.9	50.0	263 bp
	Reverse	TCATGCGGCAATACTATCAACAT	58.6	39.1	
9	Forward	ACCGCGGAAACAACAGACA	60.2	52.6	265 bp
	Reverse	TCATGCGGCAATACTATCAACATC	59.5	41.7	
10	Forward	ACGATTGCAGCCAGTGAAGA	60.0	50.0	204 bp
	Reverse	TGCGGCAATACTATCAACATCC	58.9	45.0	

A good primer can't have a secondary structure like a hairpin and dimer. Stability secondary structure determined by the free energy (ΔG) and the melting temperature. This causes the primer to not anneal to the DNA template. A hairpin is a structure formed by polynucleic acid base pairing between complementary single-stranded sequences of either DNA or RNA. The formation of hairpin structures in primers should be avoided, but it is very difficult to obtain primers without hairpin structures. Primer also may not bond with their partner primer which is called pair dimer nor self-dimer which is formed by intermolecular interactions between the two (same sense) primers, where the primer is homologous to itself. Of the primer candidates that have been designed, only 2 primer sets meet these criteria, namely primer set 2 and primer set 10 (Table 1).

Based on the analysis of primer above, the selected primers are primer sets of 10 that have met the criteria for good primers, namely with base lengths of 20 and 22 nucleotides, Tm 58.9 and 60^{O}C, %GC 50% and 45.5 %, amplicon length 204 bp and has no secondary structure (hairpin and self-dimer). The specifications for the selected primer candidates can be seen in Table 2. This primer set 10 is then named by GR primer.

The specificity of the designed primers was checked using the Primer BLAST tool on the NCBI website (Achyar et al., 2021). NCBI Primer BLAST results (Figure 1) indicate that primer pairs can amplify GR mRNA sequence with amplicon length GR of 204 bp (Figure 2).



Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CAGCCAGTGAAGAACGGAGA	20	59.68	55.00	3.00	0.00
Reverse primer	GCGGCAATACTATCAACATCCTG	23	59.81	47.83	5.00	2.00

Products on target templates

>XM_026020743.1 PREDICTED: Oryza sativa Japonica Group glutathione reductase, chloroplastic (LOC4348623), transcript variant X3, mRNA

```
product length = 196
Forward primer  1     CAGCCAGTGAAGAACGGAGA  20
Template        1775  ....................  1794

Reverse primer  1     GCGGCAATACTATCAACATCCTG  23
Template        1970  .......................  1948
```

>XM_015757647.2 PREDICTED: Oryza sativa Japonica Group glutathione reductase, chloroplastic (LOC4348623), transcript variant X2, mRNA

```
product length = 196
Forward primer  1     CAGCCAGTGAAGAACGGAGA  20
Template        1822  ....................  1841

Reverse primer  1     GCGGCAATACTATCAACATCCTG  23
Template        2017  .......................  1995
```

Figure 1. NCBI primer BLAST results from primer set 10

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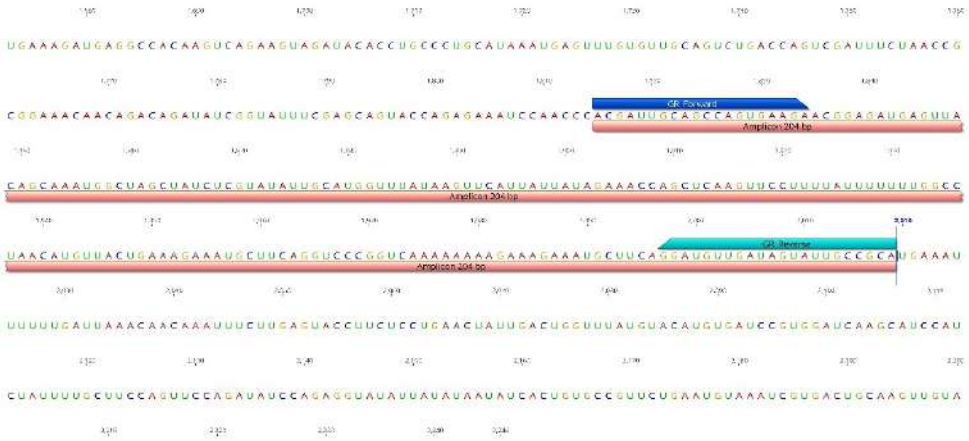
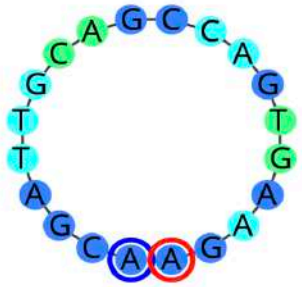
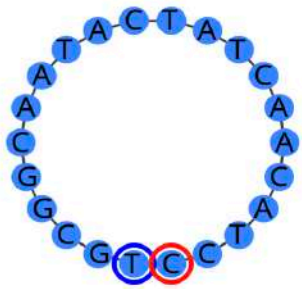


Figure 2. Amplicon length of primer set 10

Specificity checks were carried out to determine the number and type of genes GR that can be detected using the primer as well as other organisms that can also be detected by the primer [21].

Table 2. Selected primers (primer set 10) characteristics analyzed using Geneious Prime

No	Primary characteristics	DNA Fold
1	Name: GR_Forward Type: Primer Bind (primer_bind) (Created by primer3) Length: 20 Interval: 1,815 -> 1,834 %GC: 50.0 Hairpin Tm: None Self Dimer Tm: None Tm: 60.0 Sequence: ACGATTGCAGCCAGTGAAGA Product size: 204 bp Mismatches: 0 # Local Off-target Sites: 0 Local Off-target: XM_015757647	
2	Name: GR_Reverse Type: Primer Bind (primer_bind) (Created by primer3) Length: 22 Interval: 2,018 -> 1,997 %GC: 45.5 Hairpin Tm: None Self Dimer Tm: None Tm: 58.9 Sequence: TGCGGCAATACTATCAACATCC Product size: 204 bp Mismatches: 0 # Local Off-target Sites: 0 Local Off-target: XM_015757647	

3.2 Primer annealing temperature (Ta) optimization

Optimization of annealing temperature (Ta) using gradient PCR aims to test primer pairs in order to obtain the optimum Ta in amplifying target genes [22]. The optimal Ta is typically 5ºC lower than the Tm of the primer-ssDNA template. The Ta is determined by the number and order of nucleotides in the primer [23].

Based on the results of agarose gel electrophoresis of primer set 10 (Figure 3), the optimum annealing temperature is at 52.2^{O}C because the resulting DNA bands are brighter and thicker than the other DNA bands. According to Iqbal et al., (2016), bands that are clear, brilliant, unbroken or not smeared are considered to meet the requirements for good DNA bands. In this study all samples had bands that were clearly visible, brilliant, thick and no unspecific band nor dimer. The size of the amplicon is also in accordance with the results of the design. Mubarak et al. (2020) stated that an annealing temperature that is too high prevents optimal primer-template binding, whereas an annealing temperature that is too low can result in non-specific binding and, consequently, non-specific PCR products. After the primers hybridize to the templates, extension occurs [23].

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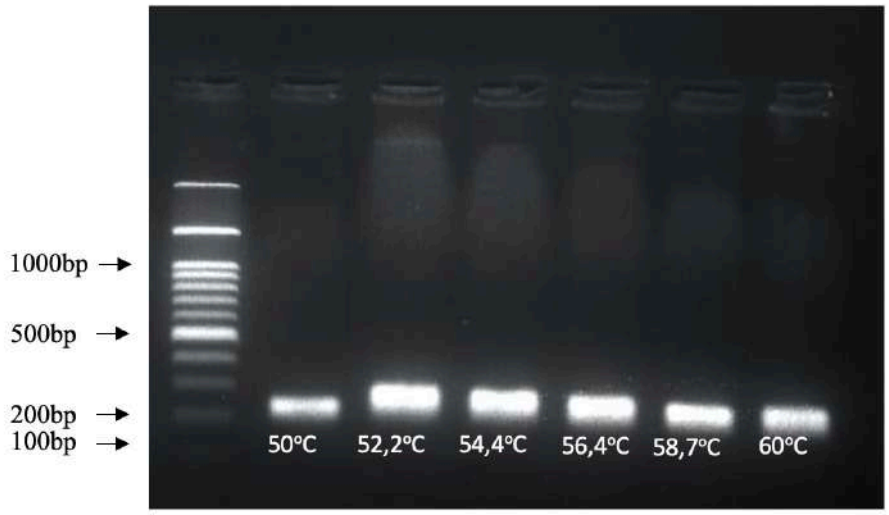

Figure 6. Visualization of electrophoresis results of PCR products with primer set 10 on cDNA of rice roots stressed by drought at a temperature range of 50-60°C using a 100 bp marker

4. Conclusion

The best primer design results were primer set 10 which is named GR primer. The characteristic of GR primer are GR-forward 5'-ACGATTGCAGCCAGTGAAGA-3' and GR-reverse 5'-TGCGGCAATACTATCAACATCC-3', amplicon length 204 bp, base lengths 20 and 22 nucleotides, Tm values 60 °C and 58.9 °C, %GC 50% and 45.5%, respectively. Both primers had no secondary structure (hairpin and self dimer) with 52,2°C as optimum annealing temperature so that these primers can be used as a specific primer to amplify the GR gene in rice using qPCR.

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Stand Structure and Composition and Model for Estimating Stand Volume Potential at the Citragaluh Sustainable Community Forest Management Unit, Subang Regency, West Java

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Abstract

In general, community forest management is still limited to the management of individual farmers so that it affects diversity, especially in the form of stands. This study aimed to explore the stand structure and composition characteristics and develop a model for estimating the potential stand volume at the Citragaluh community forest management unit with 55 observation plots based on a combination of slope and density classes. The data taken includes slope; plant species; plant coordinates; planting pattern and spacing; tree diameter, tree height, and canopy density. The results of plot observations showed that the cropping pattern of Citragaluh Community Forest Management Unit (CFMU) consisted of monoculture (10.9%), mixed stands (20%), agroforestry (29%), dry fields (27.27%), wet fields (9%), bamboo stands (1.8%) and built-up area (1.8%). The trees species found were Jeungjing, Mahogany, Teak, Tisuk, Sobsi, Akasia, and Puspa. Based on the results of stratification, diameter distribution, and stand volume, mixed gardens were the best cropping pattern. This research proves the role of community forest as a transition between plantation forest and natural forest based on the stand form and composition. The stand volume potential estimator model chosen was linear with the equation $Y = 0.074X_1 + 2.924\ X_2 - 1.679$ where X_1 = slope and X_2 = Normalized Difference Vegetation Index (NDVI). The values of R_2 models are 51.3%. The average potential for the Citragaluh is 119,835 m^3/ha, which tends to be higher than other community forest studies.

Keywords: *community forest, agroforestry, stand structure, stand composition, stand volume potential*

1. Introduction

The community forest is a forest that is outside the state forest area and the land is owned by the general public so that the form of management is unique compared to other forest types. Community forest management is generally still on a traditional individual per family-scale with limited knowledge and experience of farmers [1]. This form of management affects diversity, especially in the form of land stands such as patterns and spacing. The planting pattern of the community forest is influenced by the needs and habits of the landowner, as well as the biophysical condition of the land [2][3]. The needs and habits of the landowner can be seen when viewed from the land owner's profession. Landowners who work as farmers tend to have agroforestry planting patterns, while landowners with other professions have monoculture or polyculture cropping patterns [2].

Land biophysical factors in the form of land contours, climate, season, and soil conditions and types are also one of the considerations for farmers in choosing cropping patterns. Land with steep slopes will have a high potential for erosion and runoff so that it can experience critical conditions. Therefore, land with steep slopes will generally be dominated by perennial/forestry species with denser spacing than those with low slopes. The closer the spacing, the higher the canopy cover density, where the denser the canopy cover, the higher the vegetation index value [5]. Consideration of these two factors is important for farmers in determining the shape of the pattern and spacing to avoid failure [4].

In addition to affecting the shape of the stand, the planting pattern and spacing of community forests also affect the amount and type of product produced. The main product of

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community forest is wood, but other products can also be produced, especially on land with agroforestry patterns. Community forests can be an alternative source in increasing national log production. Based on data from the Ministry of Environment and Forestry, the total production of logs in 2019 from natural forests and plantations was 6.77 million m^3 and 36.23 million m^3, respectively. This number has decreased from 2018 which was 8.60 million m^3 of 40.14 million m^3 for natural forests and industrial forests [6]. The level of wood production in community forests can be determined by estimating the potential stand volume. Until now, it is still difficult to get the right form for estimating the potential stand volume in community forests, so involving geographic information systems (GIS) and remote sensing is expected to produce a good model.

The Citragaluh Sustainable Community Forest Management Unit (CFMU) is one of the certified community forests, located in four villages namely Cimeuhmal, Rancamanggung, Gandasoli, and Cibuluh. The Citragaluh CFMU has a total area of 3906 land units with a total land area of 516.38 hectares. Although it has been in the form of a management unit, the management of the Citragaluh CFMU is still traditional in which each member is individual, so it does not have a clear plan and does not fully guarantee sustainability in terms of farmers' income and the existence of community forests [1]. This study aims to explore the structural characteristics of the stand composition on the land and determine a model for estimating the potential for stand volume on the Citragaluh CFMU land.

2. Methodology

The research was carried out in March – April 2021, which was located at Citragaluh CFMU, Tanjungsiang District, Subang Regency. The boundary of the research coordinates is between 107° 47' – 107° 50' east longitude and 6° 41' – 6° 45' south latitude. The area of the CFMU Citragaluh based on the certification is 516.38 ha, but in this study, the area used is 1724,797 ha as shown in Figure 1 below.

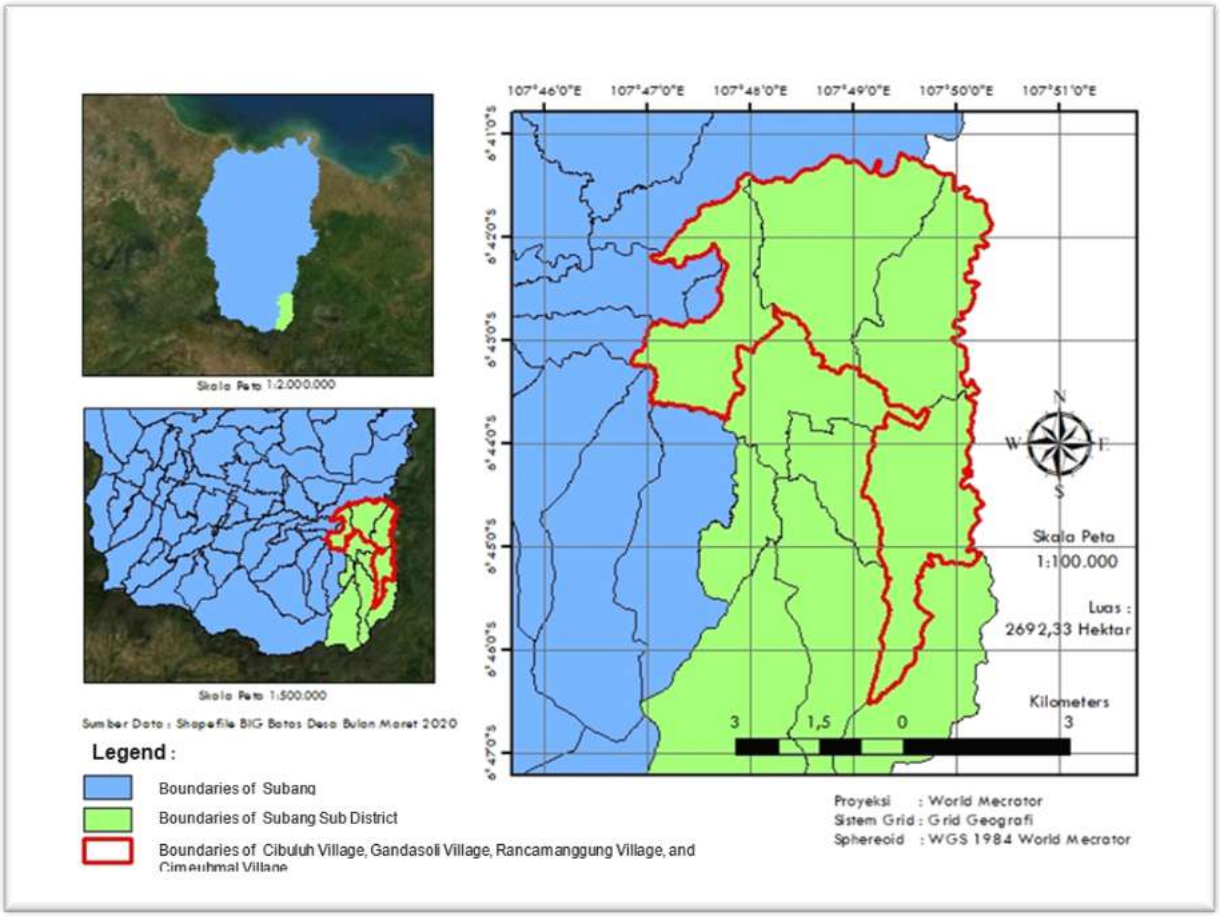

Figure 1. Map of Research Locations (Boundary of the four villages)

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The data used in this study were the Citragaluh CFMU certification documents in the form of images of land cover maps of the four villages in 2016; shapefile map of the boundaries of the four villages sourced from the Geospatial Information Agency (BIG) web-based on data in March 2020; Digital Elevation Model (DEM) data for the Subang Regency area from the National DEM/DEMNAS; and a panchromatic SPOT 6 satellite image dated June 1, 2020, sourced from the Lembaga Antariksa dan Penerbangan Nasional (LAPAN). The data retrieval stages were carried out entirely with the ArcGIS 10.8 application. The creation of the Area of Interest (AOI) is carried out by georeferencing and digitizing the certification document of the mixed plantation land cover map image. The slope map is made from DEM data based on the Regulation of the Minister of Forestry of the Republic of Indonesia No. P.32/ Menhut-II/2009 concerning Procedures for Compiling Technical Plans for Forest and Watershed Land Rehabilitation [7] with classifications as shown in Table 1 below.

Table 1. Slope class classification

Class	Slope percentage range	Explanations
1	0 – 8%	Flat
2	8 – 15%	Sloping
3	15 – 25%	A bit steep
4	25 – 40%	Steep
5	> 40%	Very steep

The vegetation density class map is made based on the Normalized Difference Vegetation Index (NDVI) value, with the following formula.

$$NDVI = \frac{Near\ Infra\ Red\ Band - Red\ Band}{Near\ Infra\ Red\ Band + Red\ Band}$$

The results of the calculation of the NDVI value will vary in the range of values from -1 to 1. This study uses a range of NDVI values between 0.3-0.7 or more to distinguish tree-vegetated areas in community forests [8]. The interval of the density class NDVI value is determined based on the desired number of classes using the following formula [9].

$$Interval\ Class = \frac{highest\ NDVI\ value - lowest\ NDVI\ value}{total\ desired\ class}$$

This study has divided the vegetation density class into five classes as shown in Table 2 below.

Table 2. Vegetation density class classification

Class	NDVI value range	Explanations
1	(-0,187 - 0,3)	No vegetation
2	(0,3 - 0,402)	Very sparse vegetation
3	(0,402 - 0,504)	Sparse vegetation
4	(0,504 - 0,606)	Medium/enough vegetation
5	(0,606 - 0,708)	Dense vegetation
6	(0,708 - 0,810)	Very dense vegetation

The results of overlaying the slope map and vegetation density map are then intersected to produce 27 classes of overlay of slope and density. This study used stratified random sampling based on the combination of slope and density classes which had 55 samples of square plots measuring 20x20 meters square (0.04 ha). The number of plot allocations per combination of slope and density is determined based on the results of the calculation of the proportion of the area. Data were taken during field observations in the form of the plant species (either forestry or agriculture); the number of plants; planting pattern and spacing; tree diameter at breast height/ DBH; total tree height; branch-free tree height; canopy width radius; tree coordinates; and the slope of the land.

2.1 Analysis of Structural Characteristics and Stand Composition

The existing/actual condition of the stand characteristics composition structure was analyzed using diameter distribution diagrams and tables. The type of stand structure can be identified using a diameter distribution diagram. If the shape of the diagram resembles an inverted 'J', it describes a forest that is not the same age/similar to a natural forest; Otherwise, if the shape resembles the form of a bell/normal distribution, it describes a forest of the same age/similar to a plantation forest [10]. The form of the stand structure can also affect the sustainability of its management, where the stand structure of a good community forest is similar to the concept of a normal forest [1]. In addition, the shape of the stand structure will also be visualized with a vertical profile diagram (3D model) and a horizontal stand (tree canopy diagram) using the SExi-FS. The tree canopy diagram was analyzed using the Tree Cramming method, which is measuring the percentage of canopy cover by moving and closing the positions of the tree

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canopy sizes so that they become one group [11]. The results of the 3D model of the stand will be able to know the stratification, while the results of the canopy profile diagram can know the percentage of the canopy cover area. The stratum division is grouped into five based on natural forest, namely stratum A (>30m), stratum B (20-30m), stratum C (4-20m), stratum D (1-4m), and stratum E (0-1m) [12]. To determine the composition of stands, they are divided into monoculture forests, mixed forests, natural forests and non-forests such as gardens, agroforestry and others.

2.2 Statistical Analysis of Stand Volume Potential Estimator Model

The stages of building a stand volume potential model consist of calculating the volume of each plot, testing correlations between variables, testing the validity of the combined vegetation density and slope class mapping, determining the basic shape of the model, testing classical assumptions, testing verification and model validation, selecting the best model, and volume prediction map creation based on the model. The calculation of the total volume in each plot with the following formula.

$$V_i = \frac{1}{4} x\, \pi\, x\, DBH^2\, x\, Tbc\, x\, f \qquad V_{teg} = \sum_{i=1}^{n} V_i$$

Explanations :

V_i = tree volume (m^3)

V_{teg} = total volume in each plot (m^3/plot)

π = 3,14

DBH = diameter at breast height (m)

Tbc = branch-free tree height (m)

f = tree form factor (f = 0,7)

The correlation testing between variables is carried out to determine the relationship between two or more variables, with the Pearson correlation formula as follows [13].

$$r = \frac{n\sum X_i Y_i - (\sum X_i)(\sum Y_i)}{\sqrt{\{n\sum X_i^2 - (\sum X_i)^2\}\{n\sum Y_i^2 - (\sum Y_i)^2\}}}$$

Explanations :

r = coefficient correlation

n = number of sample

X_i = first variable value

Y_i = second variable value

The results of the correlation coefficient test will produce a value between -1 to 1 with the confidence level used in the study is 95%. The form of interpretation of the value of the relationship between variables can be seen in full in Table 3 below [14].

Table 3. Interpretation of correlation level

Coefficient range	Correlations
0,00 – 0,199	Very low
0,20 – 0,399	Low
0,40 – 0,599	Medium/enough
0,60 – 0,799	Strong
0,80 – 1,00	Very strong

Testing the validity of the combined density and slope class mapping is carried out by checking the field conditions (ground check) from the observations based on the combined slope and density map. Testing the accuracy of the two variables was carried out using the confusion matrix, Overall Accuracy (OA) calculations, Kappa value and its interpretation in Table 4 as follows.

$$Kappa = \frac{N\sum_i^r X_{ii} - \sum_i^r X_{i+} X_{+1}}{N^2 - \sum_i^r X_{i+} X_{+1}}\, x\, 100\%$$

$$OA = \frac{\sum_i^r X_{ii}}{N}\, x\, 100\%$$

Explanations:

X_{ii} = diagonal value from row i and column i

X_{i+} = total column pixel i

X_{+i} = total row pixel i

N = total sample plot

Table 4. Interpretation of correlation level

Kappa value	Correlations	Reliability percentage
0 – 0,2	None	0 – 4%
0,20 – 0,39	Minimum	4 – 15%
0,40 – 0,59	Low	15 – 35%
0,60 – 0,80	Medium/enough	35 – 63%
0,80 – 0,90	Strong	64 – 81%
> 0,90	Very strong	82 – 100%

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The modeling uses two scenarios, namely a model with one estimator variable, namely the NDVI (X) value; and a model with two estimating variables, namely slope (X1) and NDVI value (X_2) to estimate the potential stand volume (Y). The form of the basic equation used is as follows.

1) Linear regression formula ($Y = \alpha + \beta_1 X$)
2) Logarithm formula ($Y = \alpha + \beta_1 LnX$)
3) Invers formula ($Y = \alpha + \frac{\beta_1}{X}$)
4) Quadratic regression formula ($Y = \alpha + \beta_1 X + \beta_2 X^2$)
5) Cubic regression formula ($Y = \alpha + \beta_1 X + \beta_2 X^2 + \beta_3 X^3$)

Explanations:
Y = Stand volume potential
α = intercept
βn = n regression coefficient
X_1 = Slope variable
X_2 = NDVI variable

Before making the model, the data must meet the classical assumptions, namely normality, multicollinearity, heteroscedasticity, and autocorrelation tests [16]. This study was not conducted autocorrelation test because the data is not in the form of time series. All classical assumption tests were performed with the IBM SPSS 25.

The model verification and validation testing is carried out using several criteria such as coefficient of determination, adjusted coefficient of determination, Root Mean Square Error (RMSE), error (e), aggregate deviation (AD), mean deviation (MD), and Chi square test. The calculation of the coefficient of determination (R2) aims to determine the effect of the level of accuracy and the closeness of the relationship between variables in the regression model, while the corrected coefficient of determination (adjusted R2) is a modification of the corrected coefficient of determination which shows the effect of the number of estimators/predictors on model predictions [17]. The formula for calculating R2 and adjusted R2 is as follows.

$$R^2 = \frac{SSR}{SST} = \frac{SST - SSE}{SST} = 1 - \frac{SSE}{SST} = 1 - \left\{\frac{\sum(y_i - \hat{y}_i)^2}{\sum(y_i - \bar{y})^2}\right\}$$

$$\bar{R}^2 = \frac{(1 - R^2)(n-1)}{n-k-1}$$

Explanations:
R_2 = coefficient of determination
SSR = Sum of Squared Regression
SST = Sum of Squared Total
SSE = Sum of Squared Error
y_i = observed value
$\bar{y}_i$ = predicted value
$\bar{y}$ = mean value
$\bar{R}^2$ = adjusted coefficient of determination
n = number of observations
k = total parameter used in model

The calculation of RMSE (Root Mean Square Error) is carried out to determine the level of error / error that occurs in the calculation results of the model when compared to the actual value [18]. The formula for calculating RMSE is as follows.

$$RMSE = \sqrt{\frac{\sum\left(\frac{E-O}{O}\right)^2}{n}}\ x\ 100\%$$

Explanations:
RMSE = Root Mean Square Error
E = expected value
O = observed value
n = total sample plot

The calculation of error (e) is carried out to determine the level of error in the study that can be caused by errors in measurement or technical errors/human error [18]. The formula for calculating the error is as follows.

$$e = \sum\left\{\frac{\left(\frac{E-O}{O}\right)}{n}\right\} x\ 100$$

Explanations:
e = error
E = expected value
O = observed value
n = total sample plot

The calculation of the aggregate deviation (AD) is the ratio of the difference between the number of actual values and the total estimated values of the model with the total estimated values of the model, with a range of values between -1 to +1[18]. The formula for calculating SA is as follows.

$$AD = \left(\frac{\sum E - \sum O}{\sum E}\right)$$

Explanations:
AD = aggregate deviation
E = expected value
O = observed value

The calculation of the mean deviation (MD) is the absolute sum of the ratio of the difference between the estimated value and the actual value with the estimated value, which is divided by the total estimated value [18]. The formula for calculating SR is as follows.

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$$MD = \left(\frac{\sum \left| \frac{E - O}{E} \right|}{n} \right) x\ 100\%$$

Explanations:
MD = mean deviation
E = expected value
O = observed value

The calculation of the mean deviation (MD) is the absolute sum of the ratio of the difference between the estimated value and the actual value with the estimated value, which is divided by the total estimated value [18]. The formula for calculating SR is as follows.

$$X^2{}_{cal} = \sum \frac{(O - E)^2}{E}$$

Explanations:
$X^2{}_{cal}$ = calculated X value Chi Square
O = observed (actual volume value/Va)
E = expected (model volume value/Vm)

Hypotheses in the significant test:
H0: Vm = Va; the model volume value is similar to the actual volume value
H1: Vm ≠ Va; the model volume value is not similar to the actual volume value.

The best model is selected based on the ranking score of each criterion based on the test results of each model. The higher the score value, the lower the ranking. The criteria that will be used in selecting the best model are as follows.

1) Has a high R2 value and a high R2 adjustment.
2) Has a low RMSE value.
3) Meet the real difference test / Chi Square.
4) Has a bias value closest to zero.
5) Has a MD value below 10%.
6) Has an AD value in the range of -1 to +1.

The distribution map of the potential standing volume of Citragaluh CFMU was made based on the selected model equation with ArcGIS 10.7.

3. Results and Discussion

3.1 Characteristics of Structure and Composition of Stands

The characteristics of the structure and composition of stands at Citragaluh CFMU are quite diverse, including monoculture, polyculture, and agroforestry. Monoculture is a homogeneous cropping pattern that only grows one type of forest plant species; while polyculture or mixed stands is a heterogeneous cropping pattern that grows more than one species with forestry plant types. Agroforestry is the most homogeneous cropping pattern among the three because there is a combination of two or more types of crops between forestry, plantation, and agriculture in one area. The agroforestry found on the Citragaluh CFMU land is in the form of complex agroforestry with a combination of the three types of forestry, agricultural, and plantation crops that resemble natural forest ecosystems. The agroforestry complex is divided into two based on the location, namely the yard and the agroforest.

The type of yard is located near a residential area with three phases of form, the first form is garden, then the second form is a mixed garden, and the last form is talun garden. In the first phase, the dominant annual crops are planted by farmers after land clearing or the beginning of planting; in the mixed garden phase, farmers begin to plant various types of trees side by side with annual crops; the final phase of the talun garden has tree species that have dominated and growth large in size so that there are almost no annual plants. The second type is agroforest, located far from the owner's house and does not produce seasonal food crops. Seasonal food crops are only planted at the beginning of land clearing and will be replaced directly by planting various types of trees. Another form of cropping pattern found is a field of annual crops, with a few trees planted in the form of a line as a land boundary fence.

Based on the results of field observations, the stand compostion at Citragaluh CFMU can be identified into four types, namely monoculture patterns, polyculture patterns (mixed stands), agroforestry patterns, and other patterns. In the monoculture cropping pattern, 6 plots (10.9%) were found with two types of plants; mixed stands pattern was found in 11 plots (20%) with four dominant plant species; two forms of agroforestry patterns were found in 16 plots (29%) based on their location; other patterns were found as many as 22 plots (40%) which were dominated by dry fields. The overall stand composition of Citragaluh CFMU.can be seen in Table 5 below.

Each cropping pattern will be represented by one sample plot for visualization of the 3D stand model and tree canopy profile diagram as shown in Table 6. In the monoculture pattern, the spacing used is 2x2 m2 so that the stands are very close together; the mixed stand pattern varies quite a bit between 2x2 m and 3x3 m; the agroforestry pattern (mixed garden, talun garden, and agroforest) do not have regular spacing; and the form of dry fields of 2x2 m or 3x3 m between fence trees. The pattern and spacing also affect the stand volume. The denser the stand, the volume will also increase. Based on the results of data processing, the highest volume value was obtained with an average of 3,959 m3/plots, followed by talun gardens of 3,453 m3/plots, then mixed stands of 1,743 m3/plots, mixed gardens of 1,262 m3/

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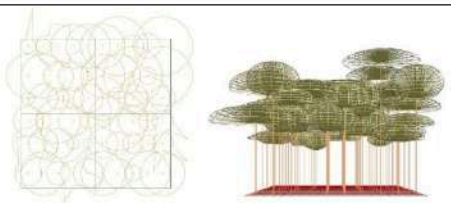
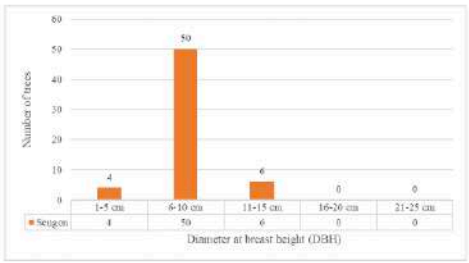
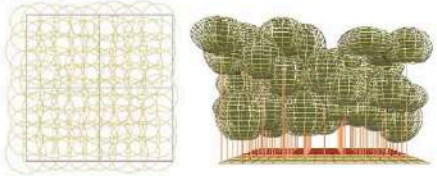
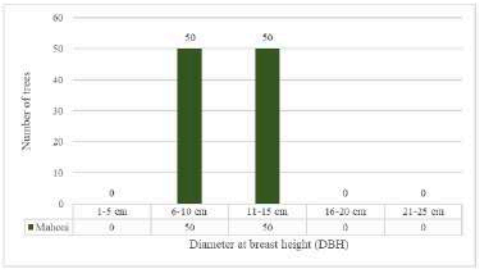
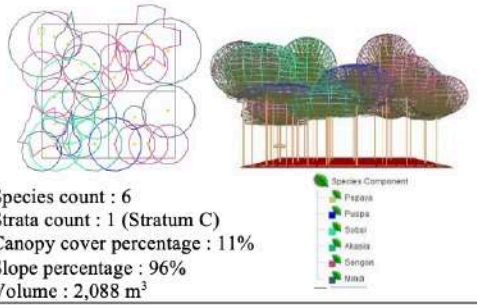
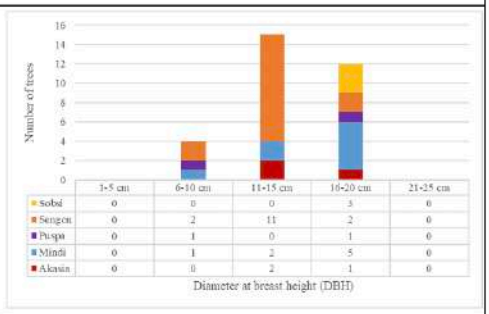
plots, dry fields of 0.347 m3/plots, and agroforests of 0.087 m3/plots. The volume in the agroforest is the lowest because there is only one plot. The distribution of volume values for each cropping pattern can be seen in Table 7 below.

Table 5. Stand Composition of CFMU Citragaluh's cropping pattern

Stand characteristics		Plot number	∑ Plot	Rasio (%)
Monoculture	Sengon	6	2	3.63
	Mahoni	54	4	7.27
Mixed Tree Stand	Sengon Dominant	29	3	5.45
	Mahoni Dominant	48	5	9.09
	Tisuk Dominant	25	1	1.81
	Sobsi Dominant	40	2	3.63
Agroforestry	Mixed garden	20	11	20
	Talun garden	10	4	7.27
	Agroforest	12	1	1.81
Others	Dry fields	32	15	27.27
	Rice fields	-	5	9.09
	Bamboo stand	-	1	1.81
	Building area	-	1	1.81

Table 6. The form of visualization of the structure and composition of stands per cropping pattern

Stand characteristics	Canopy diagram/ Canopy Stratification / Explanations/ Species components	Diameter distribution graph
Sengon Monoculture	 Species count : 1 Strata count : 1 (Stratum C) Canopy cover percentage: 100% Slope percentage : 7% Volume : 1,853 m^3	
Mahoni Monoculture	 Species count : 1 Strata count : 1 (Stratum C) Canopy cover percentage : 100% Slope percentage : 28% Volume : 5,203 m^3	
Mixed Tree Stand Sengon Dominant	 Species count : 6 Strata count : 1 (Stratum C) Canopy cover percentage : 11% Slope percentage : 96% Volume : 2,088 m^3	

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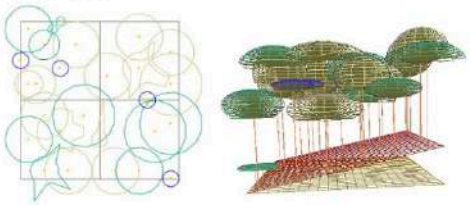
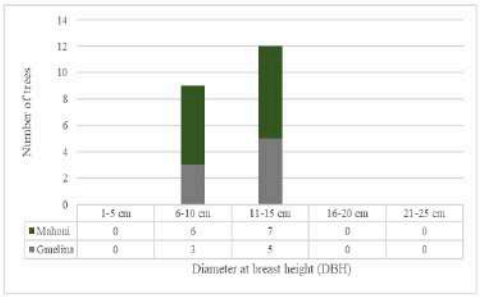
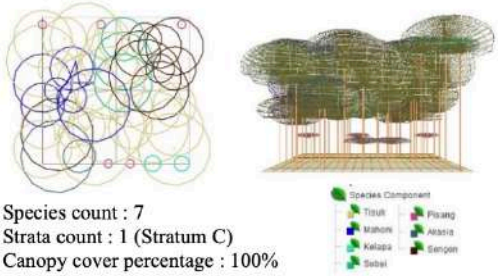
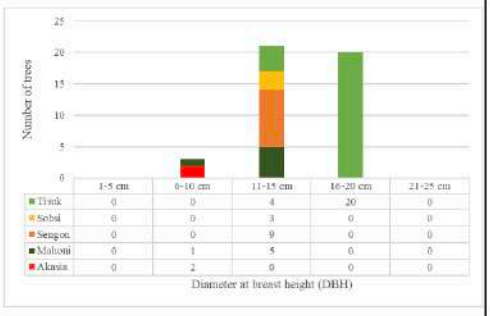
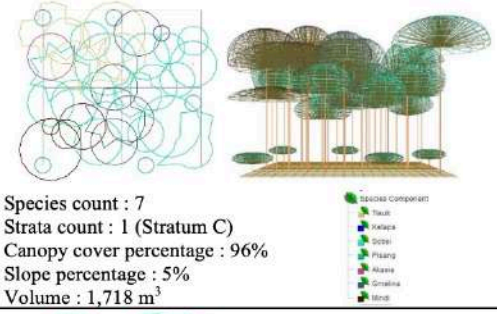
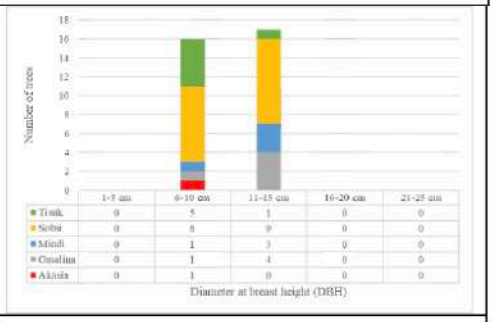
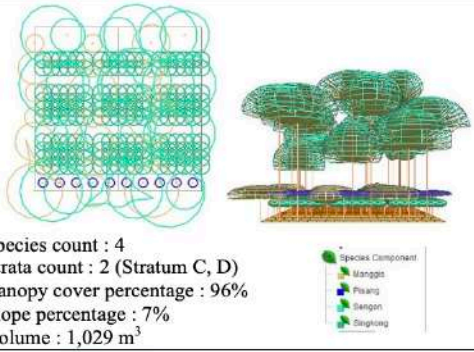
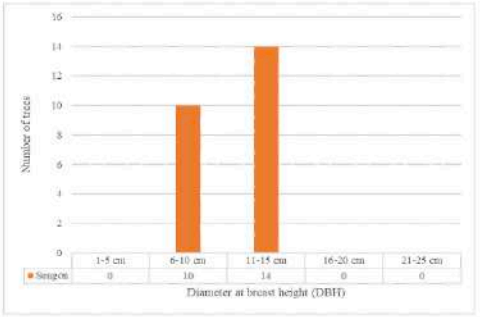
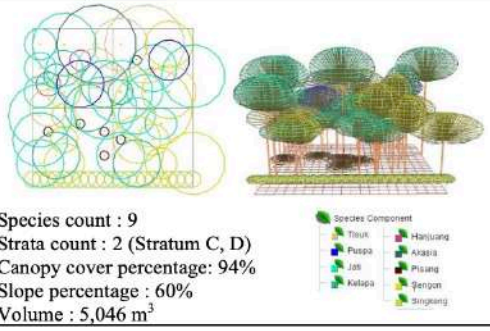
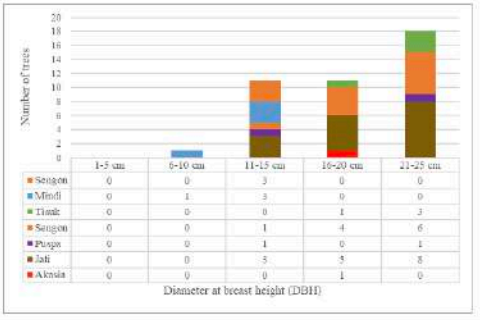
Mixed Tree Stand Mahoni Dominant	 Species count : 4 Strata count : 1 (Stratum C) Canopy cover percentage : 20% Slope percentage : 96% Volume : 1,099 m^3	
Mixed Tree Stand Tisuk Dominant	 Species count : 7 Strata count : 1 (Stratum C) Canopy cover percentage : 100% Slope percentage : 5% Volume : 3,239 m^3	
Mixed Tree Stand Sobsi Dominant	 Species count : 7 Strata count : 1 (Stratum C) Canopy cover percentage : 96% Slope percentage : 5% Volume : 1,718 m^3	
Mixed Garden	 Species count : 4 Strata count : 2 (Stratum C, D) Canopy cover percentage : 96% Slope percentage : 7% Volume : 1,029 m^3	
Talun Garden	 Species count : 9 Strata count : 2 (Stratum C, D) Canopy cover percentage: 94% Slope percentage : 60% Volume : 5,046 m^3	

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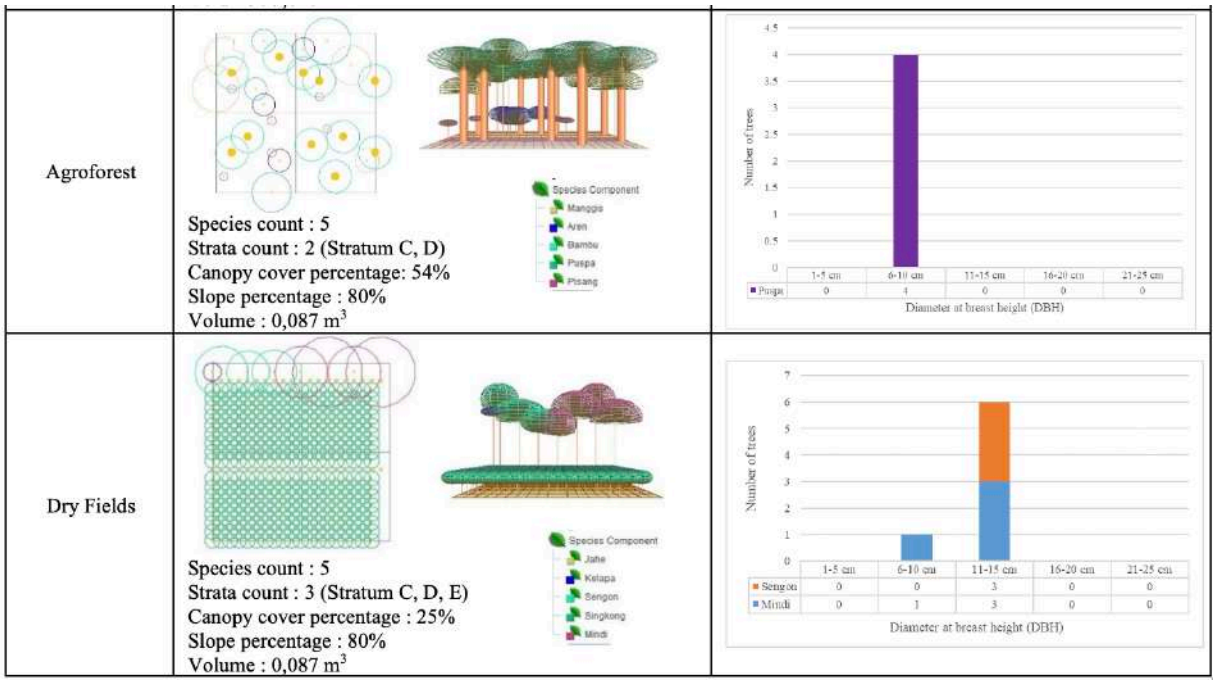

Table 7. The form of visualization of the structure and composition of stands per cropping pattern

Cropping pattern	Volume (m^3)		
	Min	Max	Mean
Monoculture	1.854	5.203	3.959
Mixed tree stand	0.769	4.039	1.743
Mixed garden	0.075	3.404	1.262
Talun garden	1.682	5.046	3.453
Dry fields	0.000	1.002	0.347
Agroforest	-	0.087	-

The number of plant species depends on the type of cropping pattern, which is a commercial type. The types of forestry plants found were jeunjing/sengon (*Falcataria moluccana*), mahoni (*Swietenia mahagoni*), tisuk (*Hibiscus macrophyllus*), sobsi (*Maesopsis eminii*), ki maung (*Bischofia javanica*), mindi (*Melia azedarach*), white teak (*Gmelina arborea*), and Teak (*Tectona grandis*). Types of plantation crops found were mangosteen (*Garcinia mangostana*), coffee (*Cofea arabica*), sugar palm (*Arenga pinnata*), banana (*Musa paradisiaca*), coconut (*Cocos nucifera*), and hanjuang (*Cordyline fruticosa*). The dominant types of crops are cassava (*Manihot esculenta*), aromatic ginger (*Kaempferia galanga*), ginger (*Zingiber officinale*), taro (*Colocasia esculenta*), and long beans (*Vigna unguiculata*). The more types of plants that make up a stand, the more the number of strata.

Monoculture patterns and mixed stands, only had one stratum, namely stratum C; agroforestry pattern has two strata, namely stratum C and D; and the form of the field has three strata, namely stratum C, D, and E. The field has small annual plants so that it can have more strata. In addition to stratification, Citragaluh CFMU stands shape can also be identified through the distribution of tree diameters. Overall, the shape of the diameter distribution graph for each cropping pattern is similar to a bell/normal distribution curve. This shows that the shape of the stands at Citragaluh CFMU tends to resemble a plantation forest where the planting is carried out simultaneously (for a lifetime), but with quite varied types of plants. Based on the results of data processing, the average tree height was 10,455 m and the dominant diameter was 11-15 cm. The diameter size tends to be small and based on the tree's survival level, the size is still at the pole level. The shape of the diameter distribution graph for each cropping pattern can be seen in Table 6.

3.2 Statistical Analysis for Estimating Stand Volume Potential Model

The results of the calculation of the plot volume for each form of cropping pattern can be seen in full in Table 7 and the sampling error value is 0.013. Based on the results of the correlation test of all variables, a moderate relationship (r = 0.636) was obtained between the percentage of canopy cover and NDVI so that it could be used in the ground check (ground-truth). The relationship between NDVI and volume is also moderate (r = 0.438), but the relationship between

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slope and volume is low (r = 0.388) so two scenarios were carried out in constructing the model. Based on the results of the Ground check, the Kappa and OA values for the slope are 0.502 and 0.672, respectively; while the vegetation density is 0.454 and 0.654. Kappa values in vegetation density tend to be low because many plots have high NDVI values but in actual conditions do not have appropriate canopy cover. Based on the Kappa value, the reliability level of slope and NDVI data is 15 – 35%. The slope, NDVI, and volume data were then tested for classical assumptions and divided into training data and validation data with a proportion of 80:20. The form of the model that was successfully raised can be seen in full in Table 8 below.

Models number one to six are the first scenario models (using only the NDVI variable), while models number seven and number eight (using the NDVI variable and slope variable). The results of the validation and verification tests of the model can be seen in Table 9, Table 10, Table 11, and the best model selection can be seen in Table 12.

Table 8. Model form and estimator equation

Model form	No.	Model equations
Linear	1	$Y = 3{,}106\,X - 1{,}5173$
Logarithmic	2	$Y = 1{,}863\,\mathrm{Ln}\,(X) + 1{,}403$
Invers	3	$Y = 2{,}281 - 1{,}025/X$
Quadratic	4	$Y = 7{,}444\,X^2 - 6{,}731\,X + 1{,}504$
Cubic	5	$Y = 16{,}405\,X^3 - 24{,}622\,X^2 + 13{,}328\,X - 2{,}455$
	6	$Y = 5{,}817\,X^3 - 3{,}642\,X^2 + 0{,}219$
Multi-linear	7	$Y = 0{,}074X_1 + 2{,}924\,X_2 - 1.679$
Multi-Quadratic	8	$Y = -0{,}042{X_1}^2 + 6{,}641{X_2}^2 + 0{,}388X_1X_2 + 0{,}145X_1 - 7{,}45X_2 + 1{,}515$

Table 9. R^2, Adjusted R^2, and RMSE value from training model

No. Model	R^2	*Adjusted* R^2	RMSE
1	0,299	0,282	0,763
2	0,273	0,255	0,748
3	0,240	0,222	0,733
4	0,333	0,317	0,794
5	0,337	0,321	0,795
6	0,335	0,319	0,794
7	0,323	0,290	0,841
8	0,406	0,377	0,790

Table 10. R^2, Adjusted R^2, and RMSE value from testing model

No. Model	R^2	*Adjusted* R^2	RMSE
1	0,320	0,245	0,696
2	0,300	0,222	0,676
3	0,266	0,184	0,657
4	0,327	0,252	0,752
5	0,317	0,241	0,762
6	0,323	0,248	0,756
7	0,513	0,391	0,644
8	0,285	0,106	0,731

Table 11. error, AD, MD, dan Chi-Square value

No. Model	e	AD	MD (%)	*Chi Square*
1	0,199	-0,067	45,9	13,609
2	0,190	-0,073	47,6	14,042
3	0,179	-0,076	49,2	14,836
4	0,206	-0,056	46,9	14,646
5	0,208	-0,042	46,8	14,827
6	0,205	-0,052	47,1	14,825
7	0,199	-0,037	40,9	13,277
8	0.182	-0.080	51,2	14,093

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Table 12. Best model selection based on rankings

No. Model		1	2	3	4	5	6	7	8
Training phase	R^2	6	7	8	4	2	3	5	1
	Adjusted R^2	6	7	8	4	2	3	5	1
	RMSE	3	2	1	5	7	6	8	4
Testing phase	R^2	4	6	7	2	5	3	1	8
	Adjusted R^2	4	6	7	2	5	3	1	8
	RMSE	4	3	2	6	7	8	1	5
Model Validation	e	4	3	1	7	8	6	5	2
	SA	5	6	7	4	2	3	1	8
	SR	2	6	7	4	3	5	1	8
Total		38	46	48	38	41	40	28	45
Ranking		3	7	8	2	5	4	1	6

All models have met the requirements of the Chi-Squared test criteria where the value of Xcount < Xtable (18.307) at a 95% confidence level. Based on Table 12, the best model has the lowest total score, namely linear two variables with the equation $Y = 0.074X_1 + 2.924\ X2 - 1.679$. This model describes a positive relationship between volume and slope and NDVI, where the effect of NDVI is greater than slope. The values of R_2, Adjusted R_2, RMSE, models are 51.3%, 39.1%, and 0.644, respectively. This study also shows that variations in the level of land slope and variations in NDVI values can explain the volume variation of 51.3% and the remaining 48.7% can be explained by other factors. The graphic form of the two-variable linear model equation can be seen in Figure 2 below.

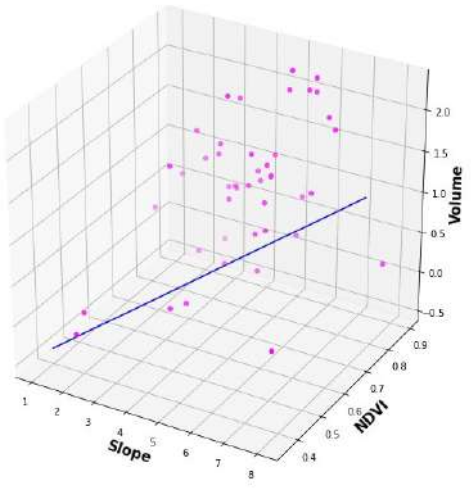

Figure 2. Graph of a 2-variable linear model (multi-linear)

The R_2 value of the linear model of these two variables has a considerable difference between the training and validation stages when compared to the R_2 value of other models. This can be caused by the lack of data making up the model so that the R_2 value of the model that appears is not optimal. However, when compared with the results of other community forest stand volume studies, the R_2 value of the linear model of the two variables is still lower and no one has used the slope of the land variable in constructing the model [19]. The results of the selected model mapping resulted in a volume value range of 1,983 x 10-5 m^3/ha to 2192,825 m^3/ha, with an average stand volume potential of 119,835 m^3/ha. Due to the shape of the volume graph being indicated to be positively skewed, the mapping of the potential volume distribution of the stands is divided into four classes based on quartiles so that the proportion of potential volumes could be known. Quartile 1 has a value range of 1,983 x 10-5 m^3/ha to 25,976 m^3/ha with an area of 302,409 ha (20.61%); Quartile 2 has a range of values from 25.976 m^3/ha to 68.795 m3/ha with an area of 422.784 ha (28.82%); Quartile 3 has a range of values from 68.795 m^3/ha to 168.388 m^3/ha with an area percentage of 394.989 ha (26.92%); Quartile 4 has a value range of 168,388 m^3/ha to 2192,848 m^3/ha with a percentage of 346,578 ha (23.62%) of area.

Based on this division, it can be seen that the second quartile has the largest proportion with a volume potential range of 25.976 m^3/ha to 68.795 m^3/ha. If these results are compared with other community forest studies, the quartile range and the

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average potential volume of this study have similar results to the average potential volume of around 40-80 m^3/ha [19][20]. When compared with natural forest and plantation forest, the average yield potential of this study is close to plantation forest and much higher than natural forest [21][22]. Based on the results of this study, it can be seen that the stand structure of Citragaluh CFMU resembles a plantation forest of the same age through the graph of its diameter distribution; with a stand composition resembling a natural forest through its mixed cropping pattern of varying plant types with more than one canopy stratification. The more strata of the canopy of a stand, the better the performance of the plant in increasing water and soil conservation. Strata A to D can slow down the kinetic energy of rainwater by interception and stem flow, while strata E can reduce the surface runoff [23]. In addition, the shape of the structure and composition of the stand also greatly affects the volume of the stand, where the observed volume value tends to be small due to the cropping pattern being dominated by the form of fields that only have a few trees. This shows that community forests with mixed cropping patterns can act as a transition between plantation forests and natural forests.

4. Conclusion

The stand structure at Citragaluh CFMU has the characteristics of an age-old plantation based on its diameter distribution and the characteristics of a natural forest based on its stratification and composition of plant species. Planting pattern affects the amount of stratification and composition of plant species, while spacing affects tree size. The average tree size at Citragaluh CFMU is 11 m for tree height and 11-15 cm for diameter. The best model for estimating the potential stand volume at Citragaluh CFMU is a linear model of two estimating variables, namely slope (X_1) and NDVI (X_2), having the equation $Y = 0.074X_1 + 2.924\ X_2 - 1.679$ with a value of R_2 is 51.3%.

Acknowledgement

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Short Note on Asteraceae as Traditional Food and Medicinal Plants in Cihanjawar Village, Purwakarta Regency, West Java

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Abstract

Asteraceae is known as the largest family of flowering plants. Despite some species members being invasive plants, these species are often adopted and utilized by local community groups for food, traditional medicine, and other uses. In our ethnobotanical study of Asteraceae, we identified ways a local Sundanese community group in West Java utilizes a diverse range of species in the family for different purposes. Our study focuses on a Sundanese village called Cihanjawar, located in the regency of Purwakarta, using ethnobotany and ethnomedicine approaches. People of Cihanjawar utilize some species of Asteraceae for food as '*lalapan*' and traditional medicinal purposes. In-depth, semi-structured interviews with the people of Cihanjawar were conducted to collect primary data regarding the utilization of Asteraceae species as food and traditional medicine. A total of eight species of Asteraceae were found during the field-guided exploration in Cihanjawar Village, which include *Acmella paniculata, Ageratum conyzoides, Calyptocarpus vialis, Crassocephalum crepidioides, Dichrocepala integrifolia, Emilia sonchifolia, Erechtites valerianifolia, Sphagneticola trilobata*, some of which are considered invasive alien species. The species of *A. paniculata, C. crepidioides, E. sonchifolia, Er. valerianifoli*a is eaten as a raw food (lalapan, Ind.). Then *Ag. Conyzoides* and *C. crepidioides* are utilized in traditional medicine. *C. vialis, D. integrifolia*, and *Sphagneticola trilobata* are not used by the people of Cihanjawar as food or as traditional medicine.

Keywords: *Asteraceae, ethnobotany, ethnomedicine, lalapan, Sundanese*

1. Introduction

Asteraceae Bercht. & J.Presl (1820) or the sunflower family is known as the largest plant family in Angiospermae with 1,700 genera and 24,000 species that are well distribued worldwide except Antarctica [1]. Due to their distribution, species of Asteraceae are considered invasive alien species, which grow well outside their native range. According to Setyawati et al. [2] and Trjitrosoedirjo et al. [3], some species of Asteraceae have been recorded as important alien species e.g., *Ageratum conyzoides*, *Austroeupatorium inulifolium, Bidens pilosa,* and others. Invasive alien species raises a certain issue on its impacts, including a threat to local biodiversity, human health, and economic interest [4]. Apart from being invasive, local people still utilize Asteraceae daily for food and traditional medicine. This is particularly true for Sundanese people who have unique habits of consuming fresh plants as vegetables known as lalapan [5]. Septiani et al. [6] recorded that Sundanese people in Naga Traditional Village, Tasikmalaya Regency, consume various species of Asteraceae, including *Conyza sumatrensis, Lactuca sativa,* and other species. For medicinal purposes, Tahnia [7] also discovered that the Sundanese people of Cireundeu Traditional Village, Cimahi City, utilize *Blumea balsamifera* to treat diarrhea.

One of the Sundanese villages in West Java that are particularly interesting due to their seclusion and traditional practices is Cihanjawar Village, located in the Purwakarta Regency, West Java. The people of Cihanjawar are mainly farmers. However, they also utilize natural resources for their daily needs due to their proximity to forests, rice fields, and farmlands. People of Cihanjawar use a wide range of plants for different purposes. For example, they grow paddy (*Oryza sa-*

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tiva), cassava (*Manihot esculenta*), lima bean (*Phaseolus lunatus*), common bean (P. *vulgaris)*, winged bean (*Psophocarpus tetranogonolobus*), banana (*Musa* spp.), tomato (*Solanum lycopersicum*), scallion (*Allium fistulosum*), and garlic chives (A. *tuberosum*) for food and market crops. Various species of Asteraceae are also found in Cihanjawar Traditional Village and are utilized extensively despite being considered weeds in their farm. This study therefore aims to explore the relationship between the species of Asteraceae and the people of Cihanjawar through their use as food and traditional medicine.

2. Methodology

The study was conducted in Cihanjawar Village, Purwakarta Regency, West Java in May 2017. In-depth, semi-structured interviews with two key informants which are also farmers in Cihanjawar were conducted to collect primary data regarding the utilization of Asteraceae species as food and traditional medicine. In addition to this, an exploration was also carried out using the field guide method, during which we observed the surrounding paddy fields and farmlands with of a local guide from the Cihanjawar Villagers. The species of Asteraceae which have been collected during the field-guided exploration were then identified using the 'A Guidebook of Invasive Plant Species in Indonesia' [2], '75 Important Invasive Plant Species in Indonesia' [3], and 'Weeds of Rice in Indonesia' [8]. The maps and environment of Cihanjawar is shown in figure 1 and 2, respectively.

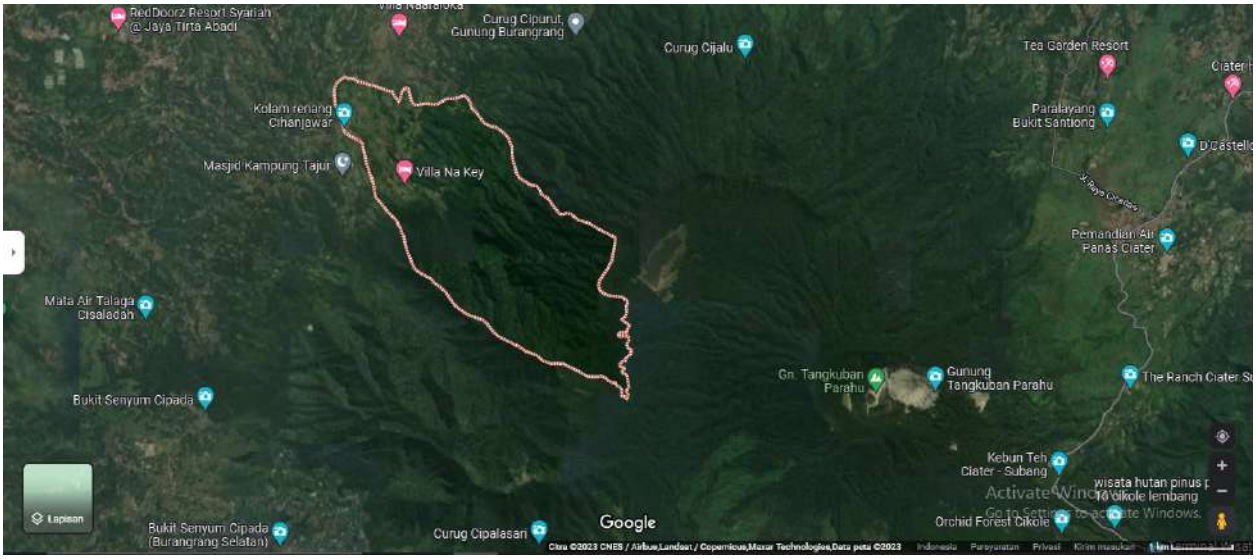

Figure 1. Maps of Cihanjawar Village, Purwakarta Regency, West Java (Google Maps, 2023)

Figure 2. Cihanjawar Village, Purwakarta Regency, West Java

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3. Results and Discussion

3.1 Diversity and Traditional Uses of Asteraceae in Cihanjawar Village

A total of eight species of Asteraceae were found during the field-guided exploration, mainly in the paddy fields and farmlands in Cihanjawar Village, Purwakarta Regency, West Java, of which only five species are utilized as food or medicinal plants. The five species are *Acmella paniculata, Ageratum conyzoides, Crassocephalum crepidioides, Emilia sonchifolia,* and *Erechtites valerianifolia*. Three species, i.e., *A. paniculata, E. sonchifolia*, and *Er. valerianifolia* are only used as food. In addition, *Ag. conyzoides* is the only species used for medicinal purposes, while *C. crepidioides* is the only species used for food and medicinal plants. The people of Cihanjawar do not utilize the other three species. This includes *Calyptocarpus vialis, Dichrocephala integrifolia,* and Sphagneticola trilobata. The complete result of Asteraceae species including traditional uses in Cihanjawar Village is shown in Table 1.

Table 1. List of species and utilization of Asteraceae in Cihanjawar Village, Purwakarta Regency, West Java

Species	Local Names	Traditional Uses		Plant Parts use
		Food	Medicine	
Acmella paniculata	Jotang	Sautéed	-	L, I
Ageratum conyzoides	Babandotan	-	External wound	L
Calyptocarpus vialis	-	-	-	-
Crassocephalum crepidioides	Sintrong	Eaten raw as *lalapan*, sauteed	Headache and hypertension	L
Dichrocepala integrifolia		-	-	-
Emilia sonchifolia	Jonge	Eaten raw as *lalapan*, sautéed	-	L
Erechtites valerianifolia	Jonggol	Sautéed	-	L
Sphagneticola trilobata	Wedelia	-	-	-

Note: L = Leaves; I = Inflorescence

In the context of traditional food, the way people of Cihanjawar consume *A. paniculata, C. crepidioides* and *E. sonchifolia* are by eating them raw or known as lalapan as the Sundanese habit and then sautéed as vegetables. *A. paniculata* is the only species which both the leaves and inflorescence are consumed. For *C. crepidioides* and *E. sonchifolia*, leaves are the only part eaten. For the traditional medicinal aspect, the people of Cihanjawar believe that *Ag. conyzoides* can heal an external wound. The crushed leaves of *Ag. conyzoides* are rubbed onto the wounded skin surface. Another plant used for traditional medicine, *C. crepidioides*, is believed to treat headaches and hypertension by consumption of the leaves, although further implementation was not fully explained. Some species of Asteraceae are shown in Figure 3.

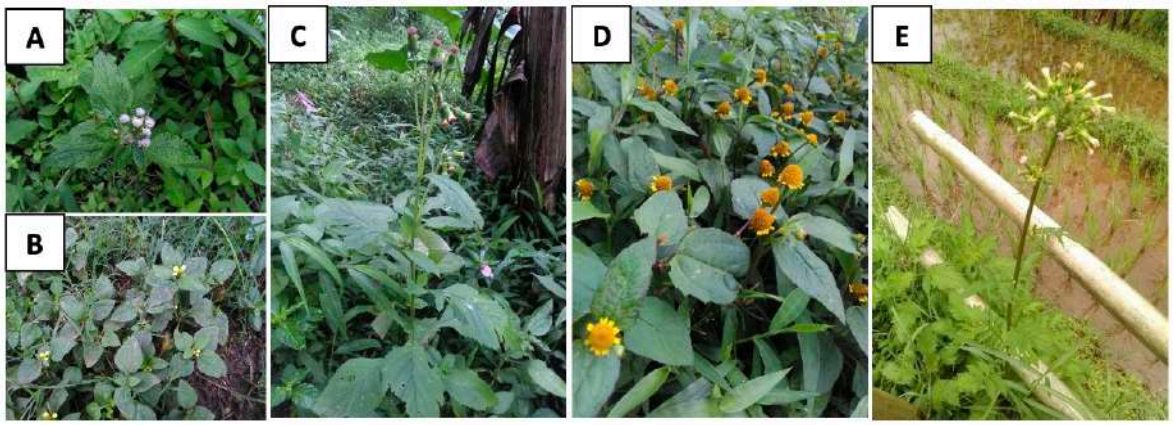

Figure 3. (A) *Ageratum conyzoides*, (B) *Calyptocarpus vialis*, (C) *Crassocephalum crepidioides,* (D). *Acmella paniculata,* and (E) *Erechtites valerianifolia*

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3.2 The Validation on Traditional Food Uses

Some species of Asteraceae are found and utilized by people of Cihanjawar and other Sundanese people in Naga Traditional Village, Tasikmalaya Regency. According to Septiani et al. [6] people of Naga also consume *E. sonchifolia* and A. *paniculata* as '*lalapan*', which are eaten directly without any process. People of Cihanjawar utilize the leaves, the same plant part as Naga's. However, the people of Naga only consume *A. paniculata* leaves, unlike those of Cihanjawar who consume both leaves and flowers.

Besides in West Java, the Sundanese in Banten or well known as Baduy Tribe, utilized Asteraceae as food plants. The study from Iskandar & Iskandar [9] discovered that Sundanese Baduy Tribe consume *Er. Valerianifolia* as vegetables. Other than the Sundanese people in West Java & Banten, research conducted by Kurniawan et al. [10] in Dieng Plateau, Central Java, found 18 species of Asteraceae, most of which are also used as food and medicinal plants. Some of the species in the Dieng Plateau can also be found and consumed by people in Cihanjawar Village, such as *A. paniculata* and *C. crepidioides*. In Dieng Plateau, the leaf is also the plant part which is consumed. Besides, study from Fauziana & Susandarini [11] in Tawangmangu, Karanganyar Regency, Central Java, showed that other than being utilized as medicinal plants, *E. sonchifolia* is also consumed as vegetables.

Leaves are the most utilized plant part, particularly in food uses. It is mainly because leaves are the part that has the highest regeneration rate, in a sense that it can sprout repeatedly and therefore will not impact that much the growth of the plant regardless of the photosynthesis taking place inside the leaves [12]. Leaves possess a plenty of metabolites from photosynthesis [13,14]. In addition, leaves are rich in vitamin B9, vitamin K, and carotenoids [6, 15]. Leaves are the most accessible plant part and can grow faster than others [6].

3.3 The Validation on Traditional Medicinal Uses

In an ethnobotanical study, the utilization of plant for traditional medicine by local people can be validated by conducting research regarding the chemical compounds or secondary metabolites which possesses bioactivity of the plant. A study carried out by Dash & Pn [16] showed that methanol and aqueous extracts of *Ag. conyzoides* leaves showed a faster rate of wound healing in wounded rats. According to Fitriani [17], leaves of *Ag. conyzoides* possess secondary metabolites, such as alkaloid compounds. This secondary metabolite could be the reason why treatment using its leaves will recover faster.

According to Adjatin et al. [18], *C. crepidioides* is utilized for blood pressure regulation by local people of Benin (in Africa region). From this, people of Cihanjawar also use *C. crepidioides* as a treatment for hypertension and headache. In terms of the chemical compounds and secondary metabolites, *C. crepidioides* contains tannin, flavonoid, and phenols also possesses the potential as anti-inflammatory, antioxidant, immunomodulatory, antimicrobial, anti-tumour, and anti-diabetic [19].

Another Sundanese people in West Java, particularly from Banceuy Traditional Village, Subang Regency, utilize some species of Asteraceae as medicinal plants, according a study from Weking et al. [20]. For example, they utilize, *Ag. conyzoides* and *C. crepidioide*s which are also found and applied in Cihanjawar Village are used to treat external wounds and hypertension, respectively.

3.4 A Note on The Invasiveness of The Eight Species

Out of the eight species of Asteraceae that we have found during our field exploration, *A. paniculata, Ag. conyzoides, C. crepidioides, E. sonchifolia, Er. valerianifolia*, and *S. trilobata* are considered invasive alien species [2,3], while *D. integrifolia* is the only native species commonly found in their natural habitat in West Java. In addition, *C. crepidioides, Er. valerianifolia*, and *S. trilobata* were recorded as 75 Important Invasive Plant Species in Indonesia [3]. Since most the Cihanjawar people have livelihood as farmers and live next to the ricefield, some of the Asteraceae species which found were also recorded as a weed of ricefield by Soerjani et al. [8], such as *Ag conyzoides, C. crepidioides*, and *E. sonchifolia*.

These invasive species have been known to cause problems in different places with a few control has been implemented. In conservation sites, some Asteraceae species are commonly found such as in Cibodas Botanical Park [21], Masigit-Kareumbi Hunting Park [22, 23], and in plantation site like corn plantation [24], ricefield [25], and pine & sweet potato plantation [26], and sugarcane [27]. The invasive species we found in Cihanjawar, on the other hand, is not particularly controlled, but due to active weeding and their use as both food and medicine, their population can be controlled. We still do not have sufficient data to show how intensive the use of these species is for consumption to certainly state that there is a balance between the invasiveness of the plant and their utilization. However, a changing dietary pattern and shift to modern medicine will certainly disrupt this mode of traditional control and potentially the ecosystem. Further study is needed to explore this phenomenon.

4. Conclusion

In Cihanjawar Village, Purwakarta Regency, West Java, we found eight species of Asteraceae during the field guided exploration in Cihanjawar Village, including *Acmella paniculata, Ageratum conyzoides, Calyptocarpus vialis, Crassocephalum crepidioides, Dichrocepala integrifolia, Emilia sonchifolia, Erechtites valerianifolia, Sphagneticola trilobata. A. paniculata, E. sonchifolia,* and *Er. valerianifolia* are only used as food. *Ag. conyzoides* is the only species used for medicinal purposes, while *C. crepidioides* is the only species that uses food and medicinal plants. Leaves are the most plant part used as food and traditional medicine, while the flower of *A.*

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paniculata is used as food. *C. vialis, D. integrifolia*, and *S. trilobata* are not used by the people of Cihanjawar as food and traditional medicine. From this, we understand that the practice of local knowledge in Cihanjawar Village regarding plant utilization traditionally is still maintained. The fact that some of these Asteraceae are invasive species shows that the traditional community has adapted to new species by integrating them into their daily lives. This also hints on local ways to control the population of invasive species through sustainable utilization. A shift to a different dietary pattern might disrupt this balance, which requires further research to be carried out.

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