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THE FORAGING ACTIVITY AND POLLINATION SERVICES OF THREE STINGLESS BEE SPECIES TO ENHANCE FRUIT QUALITY AND QUANTITY OF OKRA (*ABELMOSCHUS ESCULENTUS* L.)

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Abstract

Insect pollination has been reported to have a better yield quality and quantity than artificial pollination. Wild bees, including stingless bees contribute to effective and efficient pollination on okra (*Abelmoschus esculentus* L.: Malvaceae). However, previously no report about okra pollination by native Indonesian stingless bees, which can be potential as pollinators. Therefore, this research aims to compare foraging activities of three species of stingless bees (*Heterotrigona itama*, *Tetragonula laeviceps,* and *T. clypearis*) on okra plants and fruits quality and quantity parameters among four pollination treatments (three stingless bee species and without pollinator). Four cages with 50 okra plants were used for pollination treatments. The foraging observation of different stingless bee species on okra was conducted in the cages, and the okra yield from each treatment was compared. Our results showed the foraging activities of three stingless bee species were higher in the morning than those in the afternoon. In the morning, the number of visits of *T. clypearis* and *T. laeviceps* were negatively correlated with visit duration. The current study also showed that size and weight of pod, the number of seeds, and the percentage of fruit set of okra were enhanced by pollination of three stingless bee species. Unfortunately, the pollination of stingless bee species did not affect the seed germination and flavonoid content of fruits. The native of Indonesian stingless bees are good pollinators to improve pod quality and quantity.

Keywords: flavonoid, fruits, *Heterotrigona itama*, pollinator, *Tetragonula laeviceps*, *Tetragonula clypearis*

INTRODUCTION

Bees were reported to pollinate the most dominant flowering plants and have increased fruit production, weight and seeds of various plants compared to artificial pollination (Atmowidi *et al.,* 2007; Bommarco *et al.,* 2012; Garratt *et al.,* 2014; Mudri-Stojnić *et al.,* 2012; Navatha and Sreedevi, 2012; Sáez *et al.,* 2019). Pollination by honey bees increased the size, the number of seeds, and seed weight of pumpkin (Walters and Taylor, 2006). Bee pollination decreased of malformation fruits of strawberries (Nye and Anderson, 1974; Klatt *et al.,* 2014). The fruit set and the number of seeds of tomatoes pollinated by bees were also higher than those by self-pollination (Santos *et al.,* 2014). In melon, pollination by honey bees increased the fructose content of fruits by 9% and 13% compared to control plants (Shin *et al.,* 2007).

Recent studies showed stingless bees tend to collect more pollens and the effectiveness of stingless bee for pollination are as well as honey bees (Kakutani *et al.*, 1993; Heard, 1994; Santos *et al.*, 2014). Stingless bees have more contact with stigma than honey bees during foraging and show more nectar collecting activities (Heard, 1994). Stingless bees were the most effective and efficient pollinators due to their small size and wide distribution (Kwapong *et al.,* 2010). Stingless bees have been widely used as the primary pollinator in various crops, such as *Tetragonula laeviceps* in chili pepper (Putra *et al.*, 2016). High profit and convenience in meliponiculture have spread stingless bees extensively in Indonesia (Kahono *et al.,* 2018; Priawandiputra *et al.*, 2020).

Currently, India dominates the world's okra production (around 6 million tons in 2019). In Indonesia, the use and consumption of okra has been extensive. Unfortunately, the high demand for okra in Indonesia has not been followed by productivity in the market. Okra plays an essential role in human nutrition provides sufficient fat, protein, carbohydrates, minerals, and vitamins (Benchasri, 2012). Okra fruits contain antioxidant compounds which prevent inflammatory responses (Liu *et al.,* 2021). Okra pollination by insects increased the number of matured seeds and decreased fruits malformation (Azo'O *et al.,* 2011). Previous studies showed that *Tetragonula iridipennis* and *Lithurgus atratus* increased pod size, the number of seeds per pod, and the seed germination of okra (Perera and Karunaratne, 2019). However, the species of Indonesian stingless bees have never been reported to pollinate of okra. Therefore, this study aims to compare: 1) foraging activities of three species of Indonesian stingless bees (*Heterotrigona itama*, *Tetragonula laeviceps*, and *T. clypearis*) on okra flowers, 2) seven fruits quality and quantity parameters among pollination services of three Indonesian stingless bee species compared to self-pollination.

MATERIALS AND METHODS

Study Area

This study was conducted from November 2020 to April 2021. The observation of foraging activity of bees, yield measurement, and seed germination were conducted at the greenhouse of IPB University. The okra flavonoid concentration was measured at the Laboratory of the Center for Biopharmaceutical Studies, IPB University.

Preparation of Plants and Stingless Bees

This study was conducted in the greenhouse of IPB University, sized 20 m × 7.5 m × 5.5 m. We set up four cages using gauze in the greenhouse, and the size of each cage was 4 m × 4 m × 3 m. Each cage was used for each treatment. Four treatments used were: (1) control plants (without pollinator), (2) pollination by *H. itama,* (3) pollination by *T. laeviceps,* and (4) pollination by *T. clypearis.*

In each cage, seeds of okra were planted in trays, and after one week, each plant was transferred into a polybag. After two weeks, this plant was fertilized with one tablespoon of NPK (16 : 16 : 16). In each cage consist of 50 okra plants. All stingless bees used for this study were a great and healthy colony and free from pests and diseases. When the plants start flowering, one colony of each bee species, i.e., *H. itama*, *T. laeviceps*, and *T. clypearis* was introduced in each cage. Meanwhile, in control plants, there was no pollinators. Based on observation, there are 5–10 okra flowers bloom in each cage.

Observation of Stingless Bee Visiting Activity

The observation of visiting activity of stingless bees on okra flowers in each cage was carried out by using the focal sampling method (Martin and Bateson, 1993) in the morning (8:00–10:00 am) and afternoon (12:00–02:00 pm). Environmental parameters, i.e., air temperature and humidity and light intensity, were measured using a 4-in-1 digital instrument during the observation of bee visiting activity. The visiting activities measured were the number of flowers visited per 5 minutes and visit duration in one flower for seven sunny days.

Pollen Load Measurement

Ten individuals of each stingless bee species who returned to its hive was taken, and then each individual was put in a tube containing 1 mL of 70% ethanol: glycerol (4 : 1). Then, attached pollen on bee bodies was separated using acetolysis method (Erdtman, 1960). The sample was centrifuged in 3500 rpm for 5 minute and then the individual of stingless bee was removed. After that, the solution was centrifuged at 2000 rpm for 3 minutes, and the supernatant was removed and by leaving pollen deposits. The pollen deposit was added by 1 mL acetolysis solution (9 acetic anhydrides: 1 acetic acid). The tube was heated in 80 °C in the water bath for 5 minutes, and the supernatant was removed. The remaining deposit was rinsed using distilled water and then was centrifuged in 2000 rpm for 3 minutes. Finally, the supernatant was removed and by leaving 0.1 mL of solution. Remained solution was homogenized and dropped into a Neubauer-type hemocytometer. The number of pollens were counted under compound microscope with 400 × 10 magnification. The procedure was replicated as many as 10 individuals of each species. To observe and measure of the pollens, *Image Raster* 3 software was used. The pollen load was measured based on formulae:

$$\frac{v_1}{n_1} = \frac{v_2}{n_2}. \tag{1}$$

Where

v_1.... volume of four quadrants,
v_2.... volume of the total solution,
n_1.... number of pollens count,
n_2.... total pollen load.

Measurement of Fruit Set and Seed Germination

All blooming flowers were labeled to analyze the percentage of fruit set (pods) (Fig. 1). Seven days after flowering, the pods were harvested and measured the length, width, and weight of pod and the number of seeds per pod. Seed germinations were measured from pods of 1.5–2 months after flowering. The mature seeds were characterized by brown, dry, and open pods to reveal the seeds. The seeds of okra were collected from ten pods of each treatment. Afterward, 100 seeds were put into a Petri dish with wet tissue and stored in a dark place. The germinated seeds were counted after three days. The percentages of seeds germination were counted. The germination measurement was repeated five times of each treatment. Okra seeds germination was measured and compared among treatments.

Measurement of Flavonoid Concentration

In each treatment, 10 pods were collected from 5 different trees. The pods were baked at 35 °C for one week. The dried okra pods were mashed using a blender and weighed 5 grams. Samples of dried powder were sent to Biopharmaca Research Center, IPB University to analyze the flavonoid concentration using spectrophotometry. In each treatment, measuring the flavonoid concentration was repeated five times.

Data Analysis

The relationship between environmental parameters and visiting activities of bees was analyzed using the *Spearman* correlation test. The seed germination data among treatment was analyzed using One-Way ANOVA. The fruit and seed parameters among treatments was compared using the Kruskal-Wallis and Mann-Whitney Pairwise test. All data analysis was performed using R (R Core Team, 2014).

RESULTS

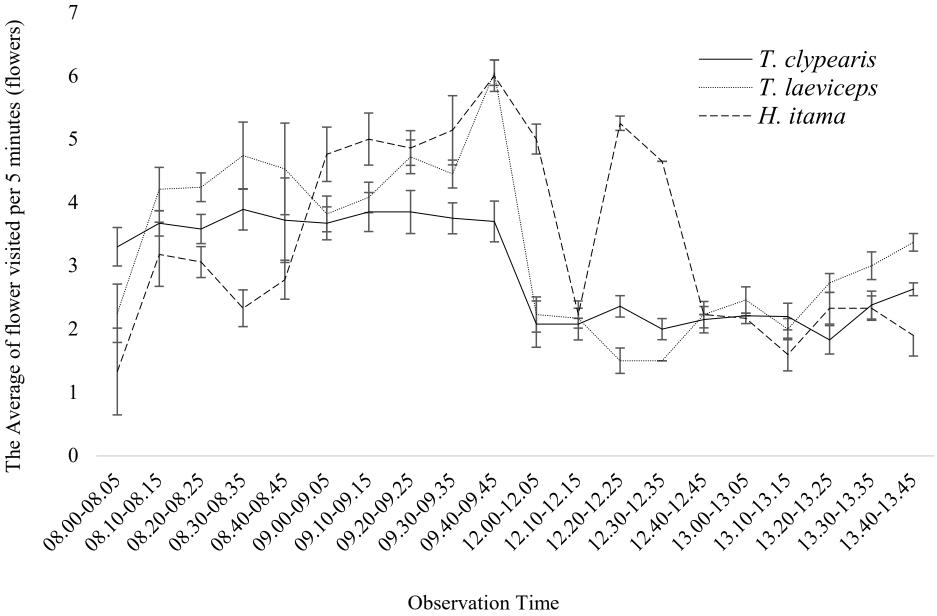
Visiting Activity of Stingless Bees

The visiting activities of stingless bees varied. Generally, stingless bees were more active in the morning than those in the afternoon (Fig. 2). *Tetragonula clypearis* showed a constant visiting activity with visiting three and two flowers in the morning and afternoon, respectively. Meanwhile, *T. laeviceps* and *H. itama* showed more fluctuated in flower visiting. The peak of visiting activity of *T. laeviceps* and *H. itama* occurred at 09:40–09:45 (6 flowers per 5 minutes). While in *H. itama*, the peak of visiting activity occurred at 08:35–09:45 (4 flowers/5 minutes). Contrary to flower visitation, the visit duration of three stingless bee species was lower in the morning than in the afternoon (Fig. 3). The lowest (40 seconds/flower) and the highest (90 seconds/flower) visits duration of *T. clypearis* occurred in the morning. In the afternoon, the visit duration of *T. laeviceps* is constantly about 100 seconds/flower. In the afternoon, a similar pattern was also showed in *T. laeviceps* and *H. itama* that visit duration ranged 100–130 seconds/flower.

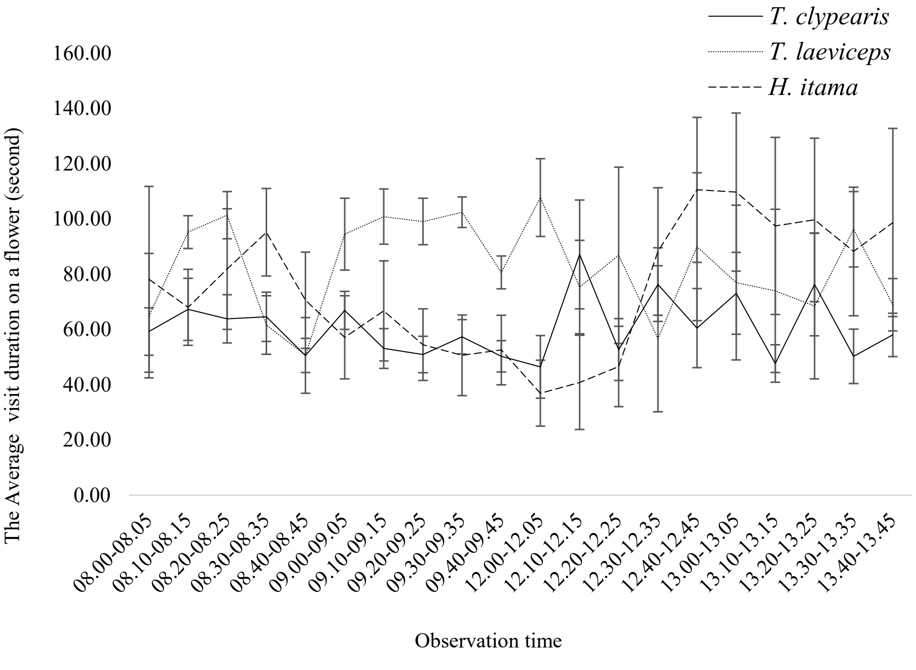
The number of flowers visited per 5 minutes of *H. itama* was positively correlated with light intensity ($p = 0.00$). The number of flower visited of *T. clypearis* was positively correlated with humidity ($p = 0.00$) and temperature ($p = 0.00$), but negatively correlated with light intensity ($p = 0.00$). Meanwhile, the number of flower visited of *T. laeviceps* was negatively correlated with humidity ($p = 0.00$) and positively correlated with light intensity ($p = 0.00$) (Tab. I). The visit duration of *H. itama* was negatively correlated with temperature and light intensity ($p = 0.06$ and $p = 0.00$). The visit duration of *T. clypearis* was positively correlated with humidity ($p = 0.00$), but negatively correlated with temperature ($p = 0.02$) and light intensity ($p = 0.06$). Meanwhile, the visit duration of *T. laeviceps* was not significantly correlated with three environmental factors measured ($p > 0.05$) (Tab. II). Results showed in the morning, the number of flower visited


(1a) (1b) (1c)

1: *Okra plants: 1a the formed pods; 1b the formless pods; 1c bee activities on okra flowers*



2: *The average of the number of flowers visited per 5 minutes by the three stingless bee species on okra flowers. Standard errors were shown in the graphic.*



3: *The average of visit duration on a flowers of stingless bees. Standard errors were shown in the graphic.*

was negatively correlated with visit duration in *T. clypearis* ($p = 0.003$) and *T. laeviceps* ($p = 0.00$), while in the noon this correlation was positive in *T. laeviceps* ($p = 0.00$) (Fig. 4).

Pollen Load

Pollen of okra had a large size (diameter 155.75 µm × 105.36 µm) and spinose pollen grain attached to bee bodies (Fig. 5). Based on the pollen load measurement, *H. itama* carried the highest number of pollens (131.25 pollen grains), followed *T. laeviceps* 56.3 pollen grains, and *T. clypearis* (85.6 pollen grains, respectively. The pollen load among stingless bee species was significantly different ($p < 0.05$).

Pods and Seeds Set

Pollination by three species of stingless bees enhanced fruit formation of okra measured by length, width, the weight of pods and the number

I: *Correlation between the environmental factors and the number of bee visits per 5 minutes of observation*

Spearman Correlation	Visit Activity	Relative Humidity (%)	Temperature (°C)	Light Intensity (lux)
Coefficient (r)	*H. itama*	-0.02	-0.02	0.23
Sig		0.64	0.68	0.00
N		344	344	344
Coefficient (r)	*T. clypearis*	0.17	1	-0.21
Sig		0.00	0.00	0.00
N		377	377	377
Coefficient (r)	*T. laeviceps*	-0.16	0.07	0.19
Sig		0.00	0.15	0.00
N		1104	1104	1104

II: *Correlation between the environmental factors and the duration of bee visits per 5 minutes of observation.*

Spearman Correlation	Visit Activity	Relative Humidity (%)	Temperature (°C)	Light Intensity (lux)
Coefficient (r)	H. itama	0.02	-0.09	-0.20
Sig		0.62	0.06	0.00
N		344	344	344
Coefficient (r)	T. clypearis	0.18	-0.11	-0.09
Sig		0.00	0.02	0.06
N		377	377	377
Coefficient (r)	T. laeviceps	0.04	-0.03	-0.04
Sig		0.4	0.55	0.39
N		1104	1104	1104

of seeds and pods set. There are significant differences in length (p = 0.00), width (p = 0.00), weight (p = 0.00), the number of seeds (p = 0.00), and percentage of fruit set (p = 0.00) of pods among treatments. The fruit size of okra without pollinating insects had significantly different with plants pollination by stingless bees (Fig. 6). The pollination services by *H. itama* enhanced the highest fruit production, followed by *T. clypearis*, and *T. laeviceps*, respectively. *Heterotrigona itama* increased pod length by 3.51% (p = 0.00), width by 2.6% (p = 0.00), weight by 25.89% (p = 0.00), the number of seeds by 26.95% (p = 0.00), and the number of pods by 16.02% (p = 0.00) compared to control. The okra pollinated by *T. clypearis* increased in pod length by 2.38% (p = 0.03), pod width by 1.35% (p = 0.1799), pod weight by 15.93% (p = 0.00), the number of seeds by 26.87% (p = 0.00), and the number of pods by 16.76% (p = 0.00). Similarly, *Tetragonula laeviceps* increased pod weight by 14.61% (p = 0.00), the number of seeds by 19.48% (p = 0.00), and the number of pods by 13.55% (p = 0.00), while increased of pod length by 0.27% (p = 0.4659) and pod width by 0.6% (p = 0.8825), statistically was not different with control.

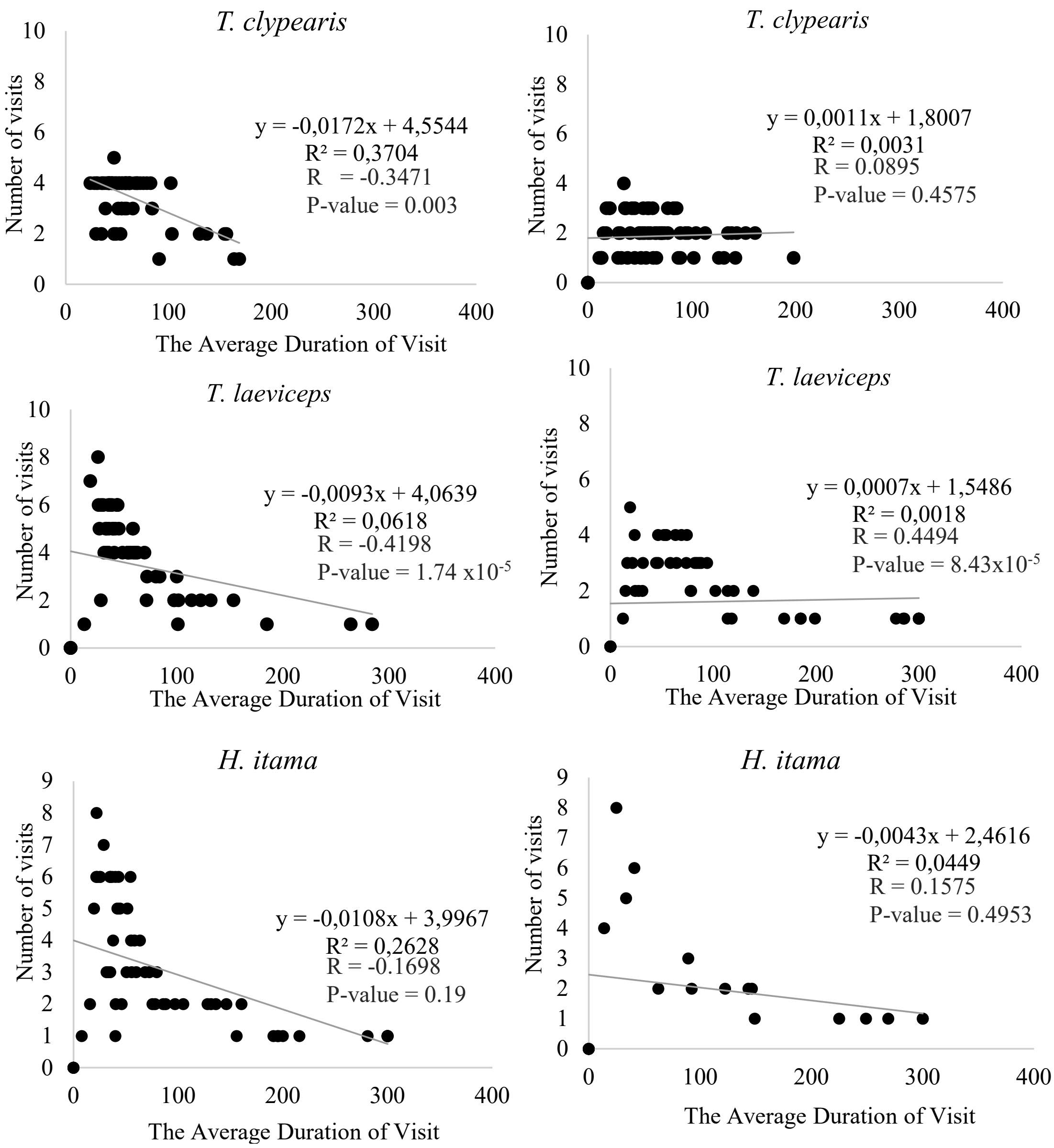
Flavonoid Concentration and Seed Germination

The flavonoid concentration of okra pods varied among treatments. The flavonoid of okra pods pollinated by *H. itama* showed the highest concentration (0.166%), followed by *T. clypearis* (0.072%), and *T. laeviceps* (0.056) (Fig. 7a). However, with control plants, flavonoid concentrations of okra pollination by *H. itama* were not significantly different (p = 0.057). The seeds germination of plants assisted by stingless bee pollination was higher than that of the control treatment (Fig. 7b), although no significant difference among treatments (p = 0.349).

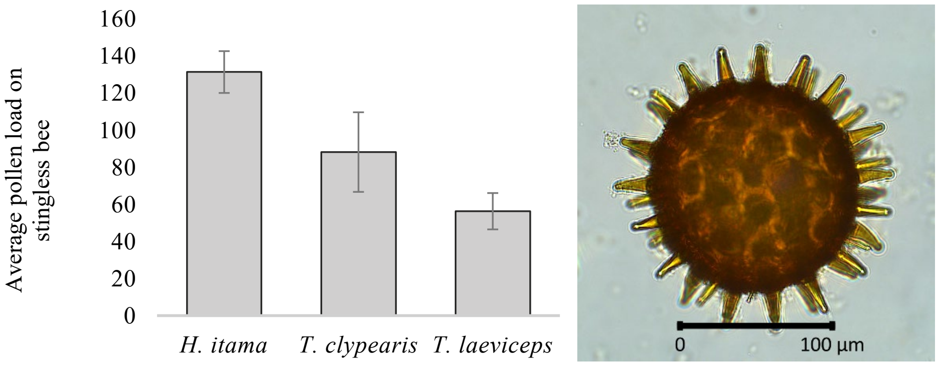
DISCUSSION

Visiting Activity of Stingless Bee

Stingless bees forage throughout the day, but their foraging is most significant in the morning (Kajobe and Echazarreta, 2005). The current study showed that the peak flower visitation of three stingless bee species (*T. clypearis*, *T. laeviceps*, *H. itama*) was at 09.00–09.45. This data was also supported by foraging activities of the other stingless bee species, *Lepidoptrigona terminata*, where the highest activities was at 09.00–10.00 (Azo'O *et al.,* 2011;

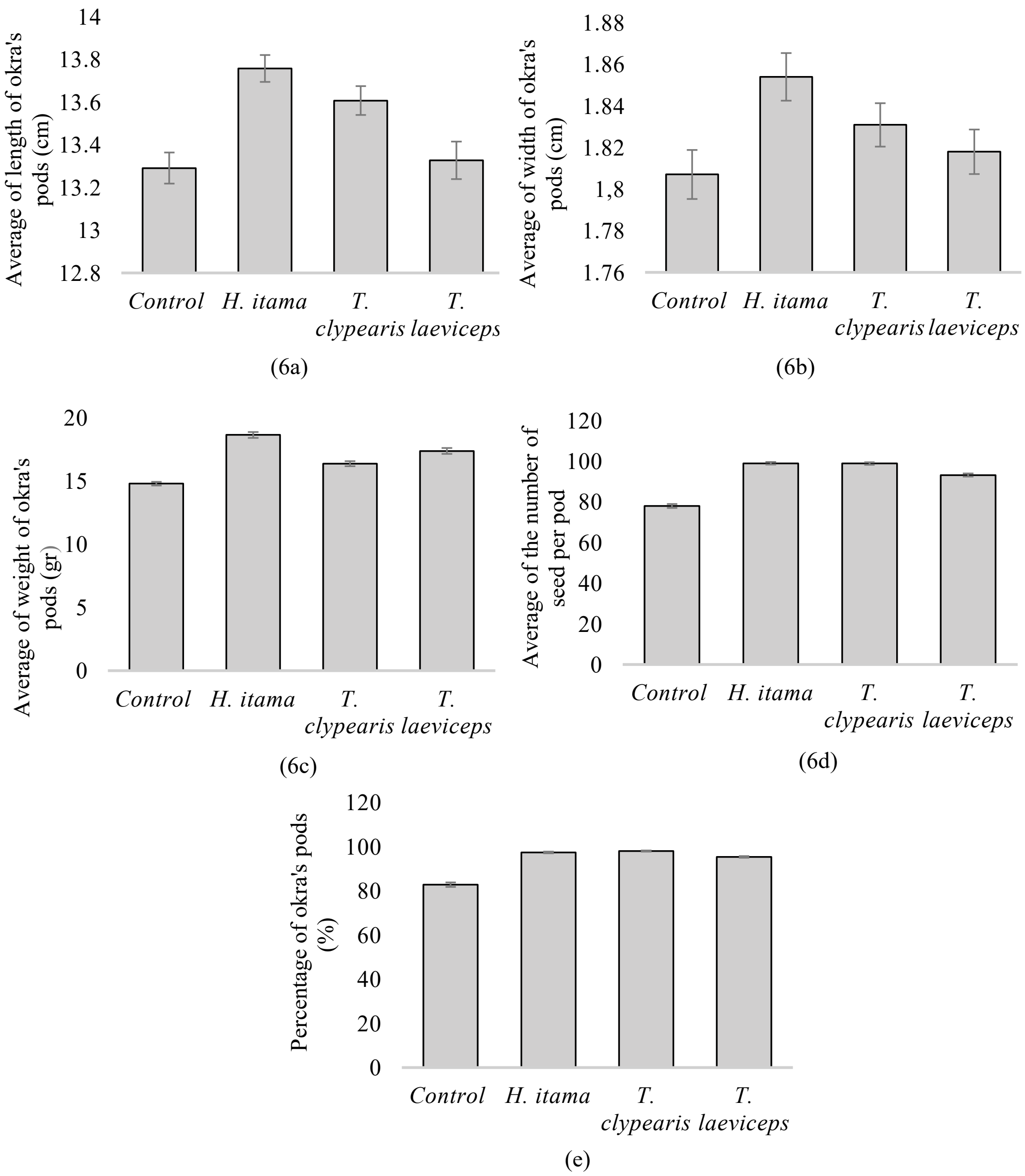


4: *The correlation between the number of flowers visited and visit duration of stingless bees in the morning (left) and in noon (right)*

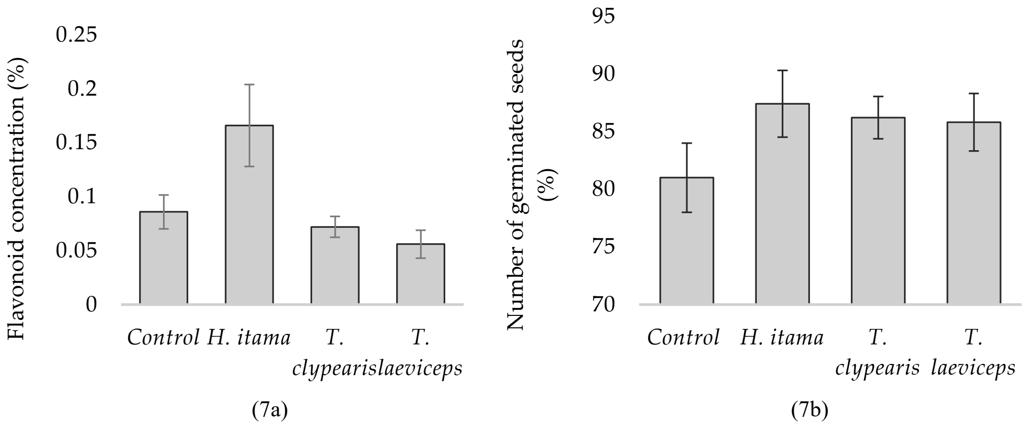


(5a) (5b)

5: *5a Pollen load on stingless bees. Standard errors were shown in each bar; 5b Morphology of okra pollen.*



6: *Yield of okra fruit: 6a pod length; 6b pod width; 6c pod weight; 6d the number of seeds per pod; 6e the percentage of fruit set. Standard errors were shown in each bar.*



7: *The Average number of: 7a seeds concentration of okra's pod in four different treatments; 7b the percentage of seed germination. Standard errors were shown in each bar.*

A'yunin *et al.*, 2019). The flower visitation of three stingless bee species drastically decreased at noon. A high ambient temperature may influence this low visiting activity during the day. In high temperature, stingless bees tend to stay in the hive (De Bruijn and Sommeijer, 1997).

Our result showed at 08.00–09.45, *H. itama, T. clypearis* and *T. laeviceps* visited 2–6 flowers per 5 minutes. These data contrast with previous study of *Trigona minangkabau* that the visitation rate at 08.00–10.00 ranged 1–1.5 flowers per 5 minutes (Kakutani *et al.,* 1993), and *Tetragonisca angustula* visited three flowers per minute (Sánchez *et al.,* 2001). This difference in visitation rate may occurs due to differences in pollen and nectar availability (Kishan *et al.,* 2017).

The variation in flower handling time may be caused by many factors, such as variation of species, body size, colony size of stingless bee, and plant species composition. We found *T. clypearis*, *T. laeviceps*, and *H. itama* spent 40–90 seconds/flower, 100 seconds/flower, and 100–130 seconds/flower, respectively. Previous study showed *T. laeviceps* spent 9.47 to 18.34 seconds per flower of musk melon (Gadhiya and Pastagia, 2019), while *H. itama* spent 13.6 seconds per flower of chayote (A'yunin *et al.,* 2019). The visit duration of both species, *T. laeviceps* and *H. itama* on okra flowers, were longer than on musk melon and chayote flowers. The other study reported that *T. iridipennis* spent 235.8–256.3 seconds/capitulum on sunflower (Kishan *et al.,* 2017) and 3.9–7.8 seconds/flower on cucumber (Kumar *et al.,* 2020). The difference in visit duration of three stingless bees may be caused by differences in nectar and pollen availability on okra's flower during day and noon. The flower's pollen and nectar availability are generally high in the morning and low in the afternoon and evening (Murrell and Nash, 1981; Herrera, 1990; Gottlieb *et al.,* 2005). In high concentrations of nectar during the day, stingless bees difficult to absorb (Pleasants, 1983; Thomson, 1986; De Bruijn and Sommeijer, 1997). Results showed the visitation rate of stingless bees negatively correlated with visit duration. The higher visiting rate caused the less time used to handle the flowers. The morning is the primary time for foraging for stingless bees because high food resources of flowers (Nicodemo *et al.,* 2013; Nunes-Silva *et al.,* 2013; Azmi *et al.,* 2015), so bees will visit many flowers shortly.

Pod and Seed Yields

Okra are self-pollinating plants which can produce pods without pollinators. However, the current study showed the percentage of okra pods without insect pollination was only 85% of the total fruit produced. The control plants (no stingless bee) produced the lowest percentage of pods. Several okra plants did not have pods in the control plants due to the ovule failure to develop in the pods (Chautá-Mellizo *et al.,* 2012). This may not be enough pollen on the stigma that make not all ovules are fertilized (Pangestika *et al.,* 2017).

The ability to carry pollens by stingless bees could be the main factor in okra's successful pollination. Results showed that *H. itama* carried more pollen than *T. clypearis* and *T. laeviceps*. The body size of stingless bee influenced the pollen load. *Heterotrigona itama* has the largest body size (5.34 mm) (Priawandiputra *et al.,* 2020) followed by *T. clypearis* (4.00–4.17 mm) (Suriawanto *et al.,* 2017), and *T. laeviceps* (3.7–4.5 mm) (Atmowidi *et al.,* 2018), respectively. There is a positive correlation between a bee's body size and pollen load. The larger body size of bee also had a larger corbicula that carried more pollens during their foraging (Veiga *et al.,* 2013; Pangestika *et al.,* 2017). Pangestika *et al.* (2017) also reported *H. itama* carried more pollens (69,802 pollen grains) than *T. laeviceps* (40,802 pollen grains). *Heterotrigona itama* carried pollens of Arecaceae and Solanaceae, while *T. laeviceps* carried pollen of Rutaceae and Poaceae (Pangestika *et al.,* 2017). The small pollen load on three stingless bees studied may be related to the large size of okra pollen (diameter 155.75 µm × 105.36 µm). The pollen size determines the number of pollen carried by stingless bees (Wicaksono *et al.,* 2020; Cholis *et al.,* 2020). The small number of pollens in the three stingless bee species was also due to the large size and spinose pollen grains. Lunau *et al.* (2015) reported that bumblebees did not collect *Alcea rosea* (Malvaceae) pollen characterized by large, sticky, and spinose grains as mechanical protection of pollen.

Stingless bees increase pollination effectiveness by increasing the auxin hormone concentration that accelerates fruit development (Wietzke *et al.*, 2018). Stingless bee pollination increases fruit set, such as length, width, pods' weight, and the number of seeds per pod. This study supported a previous study of okra pollination using some species of insects (Azo'O *et al.,* 2011; Woodcock *et al.,* 2014; Perera and Karunaratne, 2019). Visitation of entomofauna on okra flowers increased the fruit set by 1.4–3% and 5.5–6.1% compared to self-pollination (Azo'O *et al.,* 2011). Honey bee, *Apis mellifera*, which dominantly pollinated okra increased 0.03% of yield and 1.26% of seed number (Angbanyere and Baidoo, 2014). Similarly, pollination using two species of stingless bees *T. iridipennis* and *Lithurgus attratus* in Sri Lanka also enhanced about 50% of the number of seeds and seed germination, 17% of pod diameter, and 4% of pod length (Perera and Karunaratne, 2019).

Insect pollination also increases nutrition in some plants. Levels of fat and vitamin E in almonds are strongly influenced by insect pollination (Brittain *et al.* 2014). However, not all plants showed an increase in nutrient levels after insect pollination. Pollination has an overwhelming effect on yield quantity of pear and apple (Hünicken *et al.,* 2020).

The current study showed no significant difference in the flavonoid concentration between the control plants and pollination by *H. itama*. However, both treatments (control and *H. itama*) were significantly higher in flavonoid concentration than other treatments (*T. laeviceps* and *T. clypearis*). It indicated stingless bee pollination might not affect the flavonoid concentration in the pods. Another study also stated that the cross-pollination of citrus does not affect the fruits' flavonoid levels (Wang *et al.,* 2019).

Our result indicates that seed germination of self-pollination of okra tends lower than other treatments. Pollination by pollinating agents increases the potential for cross-breeding in plants. Cross-breeding increase heterozygosity, genetic exchange, genetic variation, and prevent inbreeding depression in plants (Wang *et al.,* 2019). In some plants, inbreeding depression due to self-pollination results in decreased seed germination (Bellusci *et al.,* 2009). The current study showed no significant difference of seed germination of okra among treatments. Results of our research also supported several previous studies on *Eugenia uniflora* (Fidalgo *et al.,* 2019). Similarly, the pollination of okra by stingless bees was not significantly different from the control (Sihag, 1986).

The three stingless bee species used in the study have the potential as effective and efficient pollinator agents in some crops. However, their utilization has not been optimally applied in Indonesia. This study showed how important the species of stingless bees improve fruit performances (Asma *et al.,* 2019; Putra *et al.,* 2020; Putra *et al.,* 2014; Travero *et al.,* 2012), including to enhanced okra productivity in Indonesia. However, the ability of stingless bees adaptation on agricultural landscapes should be considered before applying on a large scale. *Tetragonula laeviceps* that adapt to any kind of habitat can be selected as the most potential pollinator agent in agricultural habitats (Buchori *et al.,* 2020). In the future, the two other stingless bee species also should be assessed to perform in large-scale of agricultural habitats.

CONCLUSION

The activity of three species of stingless bees was higher in the morning, and the peak of visiting activity occurred at 08:00–10:00. Contrary, the visits duration of three stingless bee species was higher in the afternoon. Pollination by three species of stingless bees increased the length, width, weight of pods and the number of seeds per pod. The pollination of stingless bee species did not affect the flavonoid content and seed germination. The three species of stingless bees are good pollinators to improve pods and seed set of okra.

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