

Research Article



## The Effect of Colony Addition of Bees (*Apis cerana* and *Tetragonula laeviceps*) in Pollination and Fruit Set of Pummelo (*Citrus maxima* (Burm.) Merr.)

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### ABSTRACT

Pummelo (*Citrus maxima* (Burm.) Merr.) (Rutaceae) is a cultivated species with a high economic value and several cultivars are found in Indonesia. Bageng Taji is a seedless pummelo cultivar found in Bageng village, Pati regency, Central Java, Indonesia. This research aims to measure the effect of colony addition of two bee species, *Apis cerana* and *Tetragonula laeviceps* in pollination and fruit set of pummelo. We set-up four treatments of pollination, i.e., bagged flowers (control), open flowers, colony addition of *A. cerana* (honey bee), and colony addition of *T. laeviceps* (stingless bee). Visiting activity of bees were observed by using focal sampling method in the sunny days. Pollen load was measured using the acetolysis method. The effectiveness pummelo pollination was measured based on fruit formations. Results showed that the longest visitation and the number of flowers visited of *A. cerana* (15.2 seconds/flower and 37.3 flowers/5 minutes) and *T. laeviceps* (45.5 seconds/flower and 17.1 flowers/5 minutes) occurred at 09.30 am. The average number of pollens carried by *A. cerana* (19,908 pollen grains) was higher than *T. laeviceps* (4,308 pollen grains). Addition of *A. cerana* and *T. laeviceps* colonies increased 34% and 32% of fruit formation compared to control plants.

## 1. Introduction

Pollinator insects are one of the most important ecosystem services and about 35% of crops depend on pollinator (Klein *et al.* 2007). Roubik (1995) estimated 1,330 plants or about 70% in the Asian region are pollinated by insects. The honey bee (*Apis cerana*) is a social bee that has superior characteristics, such as able to adapt to the tropical climate, resistant to parasitic mites, aggressive and easy to cultivate (Morse and Hooper 1985). Social bees are known as potential pollinating agents that increase agricultural production (Thomas *et al.* 2009; Erniwati and Kahono 2011). Atmowidi *et al.* (2007) reported *A. cerana* was the highest abundance of pollinating insect of mustard (*Brassica rapa*) in

agricultural ecosystems in West Java and the highest visits occurred in the morning (08.30-10.30). In the north slope of Mount Slamet, Central Java, Widhiono and Eming (2015) reported *A. cerana* was a pollinating insect with a high abundance (25.61%) in agricultural crops. In Magetan, East Java, Cholis *et al.* (2020) stated *A. cerana* and *Tetragonula laeviceps* increase the pummelo fruits by 18.71% and 15.66%, respectively. Stingless bee is a highly social insect with diverse species (Camargo and Roubik 1991). This species is a major visitor of various agricultural crops (Slaa *et al.* 2006). *Tetragonula laeviceps* (stingless bee) has a small body size (3.44-4.88 mm in length) (Sakagami 1978). The role of stingless bees in crops pollinating has been reported previously. Wulandari *et al.* (2017) reported pollination by *T. laeviceps* increased 231% of pods, 48% of seeds per pod, 204% of seed weight, and 24% of seed germination.

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Pummelo (*Citrus maxima* (Burm.) Merr.) (Rutaceae) is a cultivated species with a high economic value and several cultivars are found in Indonesia. Pummelo has a perfect flower (Susanto *et al.* 2013) and self-pollination occur. However, most pummelo varieties have a self-incompatibility (SI). SI inhibit pollination mechanism due to a genetic barrier of pollen and pistils of the same tree. Cross-pollination by insects reduce the negative impact of SI (Cholis *et al.* 2020).

In Indonesia, the peak flowering of pummelo usually occurs in October-November in the early of rainy season (Cholis *et al.* 2020). Bageng village is the largest producer of "Bageng Taji" variety or seedless pummelo in Pati Regency, Central Java. The variety of "Bageng Taji" has been patented at the Center for the Protection of Plant varieties of the Ministry of Agriculture of the Republic of Indonesia with Certificate Number: 01/PVL/2008. The productivity of pummelo is significantly affected by pollinating insects and until now, no available data on pummelo production related to colony addition of *A. cerana* and *T. laeviceps*. This study aims to measure the effect of colony addition of bees (*A. cerana* and *T. laeviceps*) on pummelo pollination and fruit set, as well as the flower visiting activity and pollen load.

## 2. Materials and Methods

### 2.1. Study Area and Pummelo Cultivar

The study was conducted at the pummelo "Bageng Taji" garden at Bageng village, Gembong sub-district in Pati Regency, Central Java. Gembong sub-district is the largest producer of seedless variety of pummelo in Pati. Geographically, Bageng village has an area of 645.04 ha in the highlands of Muria mountain ranging from 40 to 220 meters above sea level. This study was conducted in the "Pamelo Agri" garden with an area of 7,000 m<sup>2</sup>. This garden is also planted with eggplant, long beans, chilies, dragon fruit, and banana.

### 2.2. Observation of Pummelo Flowers

The morphology of the pummelo flower, consist of color, number of petals, petal width and stalk length, number of stamens, stamen position, and number of flowers per inflorescence were observed (Dewi *et al.* 2015). The nectar volumes were measured in the morning (06.00 am) by using a micropipette and concentration of nectar sugar were measured by refractometer. Pollen morphology, including the pollen shape and

size, aperture, and ornamentation, also were observed (Setiawati *et al.* 2015).

### 2.3. Measurement of Pollination Effectiveness

Measurement of pummelo pollination effectiveness was carried out in September 2021 to April 2022. In a total, twelve pummelo plants were selected, and each treatment used three plants. Four pollination treatments were used, i.e., five flowers were bagged by screen with 10 replication per plant (total 30 replication for three plants (control plants), five flowers were opened, (total 30 replication for three plants), addition of one colony of *A. cerana*, and addition of three colonies of *T. laeviceps* in the pummelo plantation. Each treatment was conducted for 7 days and a total of 28 days for all treatments.

### 2.4. Measurement of Bee Visiting Activities

Visiting activity of bees (*A. cerana* and *T. laeviceps*) were observed in opened flowers using the focal sampling method (Martin and Bateson 2007). The visiting activities observed were the number of flowers visited per minute and the duration of visits per flower in 07.30-17.30 in sunny days. The environmental parameters also were measured consist of relative humidity and temperature, wind velocity, and light intensity.

### 2.5. Measurement of Bee Pollen Load

A total of 20 individuals of each species of bee were used to measure pollen load. One individual of bee was caught using insect net and was put into a micro tube containing 0.1 mL of 70% ethanol, then was rotatored for 5 minutes. After that, the sample was centrifuged at 3,500 rpm for 5 minutes, and then bee was removed. The solution was centrifuged at 2,000 rpm for 3 minutes, then the supernatant was discarded. The precipitated pollen was added with 0.1 mL of acetolysis solution (acetic dihydride and H<sub>2</sub>SO<sub>4</sub> in ratio 9:1). Then, the solution was heated using a water bath in 80°C and the supernatant was discarded. The 1 mL of aqua dest was added in the solution and was centrifuged at 2,000 rpm for 5 minutes. A 0.1 mL of the solution was taken using a pipette and placed on the hemocytometer, then the pollens was observed under a light microscope embedded by camera. The number of pollen load of one individual of bee was calculated by the following formula:

$$\frac{V1}{N1} = \frac{V2}{N2}$$

Where:

- V1 : volume of four quadrants  
V2 : total volume of solution  
N1 : the number of pollen count  
N2 : total pollen load

## 2.6. Measurement of Fruit Formation

The fruit formation measured were the number fruit produced, the number of normal and abnormal fruits, and fruit weigh, diameter, and shape. The fruit measurements were carried out after 6 months of pollination. The proximate analysis (carbohydrates, fats, protein, water and ash contents, and vitamin C) was conducted at Center for Agro Industry, Bogor, West Java, Indonesia.

## 2.7. Data Analysis

The visiting activity of *A. cerana* and *T. laeviceps* were shown in line graphic. The relationship between bees visiting activity and environmental parameters was analyzed using Pearson correlation in the Paleontological Statistics (PAST) software. The average number of fruits, percentage of normal and abnormal fruits, fruit diameter and weight among treatments were analysed using Analysis of Variance (ANOVA) followed by Tukey's test.

## 3. Results

### 3.1. Morphology of Pummelo Flower

One inflorescence of pummelo consists of 4-10 individual of flowers. Each flower has 4-5 petals, 6 sepals, 26-35 anthers, and 1 pistil. The petals (or crown) are white in shape with a pointed tip. The sepals are green with a bowl-like shaped. The pedicel is green with a size >1 cm. The "Bageng Taji" variety has a compound flower with the flower position in the leaf axis. The nectar volume taken in the morning (06.00 am) using a micropipette ranging from 0.02-0.03 mL and the sugars content in the nectar ranges from 17-23%.

### 3.2. Visiting Activity of *A. cerana* and *T. laeviceps*

In general, *A. cerana* visited more pummelo flowers than *T. laeviceps*. In *A. cerana*, the longest visit (15.2 seconds/flower) and the number of flowers visited (37.3 flowers/5 minutes) occurred at 10.30 am, while the lowest visit (8.8 seconds/flower) and the number of flowers visited (36.7 flowers/5 minutes) occurred at 5.30 pm. While in *T. laeviceps*, the longest visits duration (45.5 seconds/flower) and the number of

flowers visited (17.1 flowers/5 minutes) occurred at 10.30 am, while the lowest visit duration (28.1 seconds/flower) and the number of flowers visited (18 flowers/5 minutes) occurred at 5.30 pm (Figure 1). Visiting activity of both species, *A. cerana* and *T. laeviceps*, on pummelo flowers showed the same pattern. These activities increase at 07.30 until 09.30, decreased at 11.30-12.30 am, and low activities in the afternoon (Figure 1). Based on Pearson correlation analysis, the activity of *A. cerana* and *T. laeviceps* were positively correlated with temperature ( $r = 0.246$ ,  $p = 0.03$  and  $r = 0.276$ ,  $p = 0.014$ ), light intensity ( $r = 0.255$ ,  $p = 0.024$  and  $r = 0.335$ ,  $p = 0.002$ ), and negative correlated with wind velocity ( $r = -0.286$ ,  $p = 0.012$  and  $r = -0.505$ ;  $p = 0.000$ ) and humidity ( $r = -0.108$ ,  $p = 0.346$  and  $r = 0.018$ ,  $p = 0.87$ ) (Table 1).

### 3.3. Pollen Load

The average number of pollens carried by *A. cerana* (19,908 pollen grains) was higher than *T. laeviceps* (4,308 pollen grains) (Figure 2A). Pummelo flowers have a large number of pollens. The pollen of pummelo has a spherical shape with 2.11  $\mu\text{m}$  in length (Figure 2B) and the pollen ornamentation is psilate with diporate aperture.

### 3.4. Fruit Formation

The number of fruits formation in plants added by colony of *A. cerana* and *T. laeviceps* were higher (4.50 fruits and 4.10 fruits) than in opened plants (3.03 fruits) and control plants (0.8 fruit). Open pollination, colony addition of *A. cerana* and *T. laeviceps* produced more normal fruits than control plants. Bee pollination increased fruit size and weight of pummelo (Table 2). Addition of *A. cerana* and *T. laeviceps* colonies increased 34% and 32% of fruit formation compared to control plants. Addition of *A. cerana* and *T. laeviceps* colonies decreased abnormal fruits (16% and 8.67%), respectively compared to open pollination and control (42.67% and 90%). The normal fruit has a spheroid shape - pyrifoam (larger diameter in the middle and smaller in the end), red flesh and thick fruit skin. The skin thickness of ripe fruit reach 1-2 cm with green-yellow color. The abnormal fruit has irregular shape with light-green color. Normal fruit of pummelo has red flesh with a sweet taste, while the abnormal fruit has a pale color with a bitter taste and has no seeds. Based on proximate analysis, addition colony of *A. cerana* and *T. laeviceps* increased vitamin C and fruit nutritional

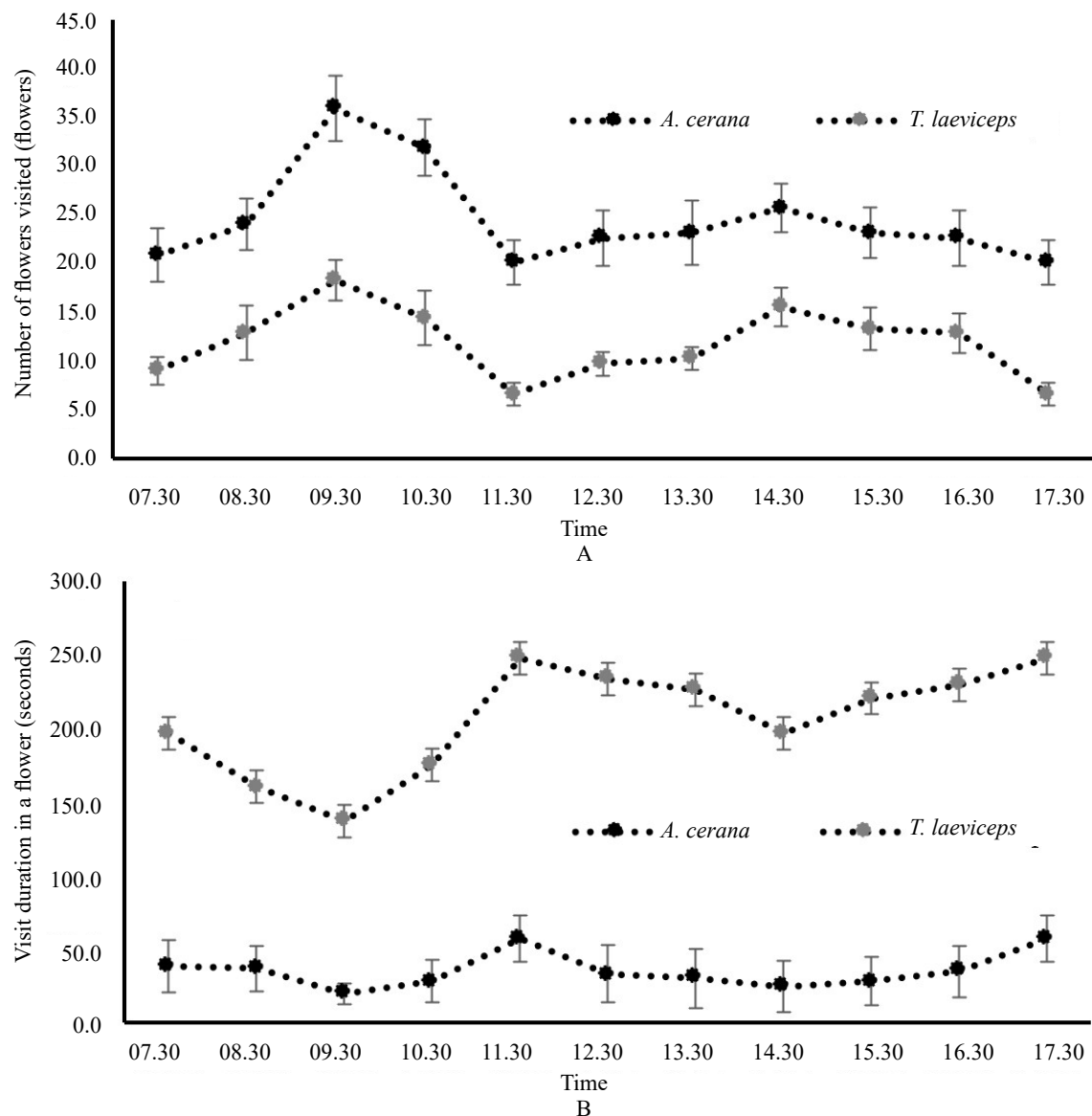


Figure 1. Visiting activities *Apis cerana* and *Tetragonula laeviceps* on pummelo flowers: (A) number of flowers visited per 5 minutes, (B) duration of visits per flower

Table 1. Pearson correlation analysis between visiting activity of *Apis cerana*, *Tetragonula laeviceps* and environmental parameters

Pearson correlation	Bee species	Temperature (°C)	Humidity (%)	Light intensity (lux)	Wind velocity (m/s)
Coefisien r	<i>A. cerana</i>	0.246243	-0.10876	0.25543	-0.28581
Sig. (p value)		0.030593	0.34642	0.024955	0.011744
Coefisien r	<i>T. laeviceps</i>	0.27643	-0.018827	0.33506	-0.50503
Sig. (p value)		0.014949	0.8709	0.0028949	2.81E-06

content of carbohydrates, fats, and protein compared to opened and control plants. The fruit water and ash contents in the bagged flowers (control) had a higher than other treatments (Table 3).

#### 4. Discussion

The morphological characters of pummelo flower are a white color, stamen length <1 cm with flower

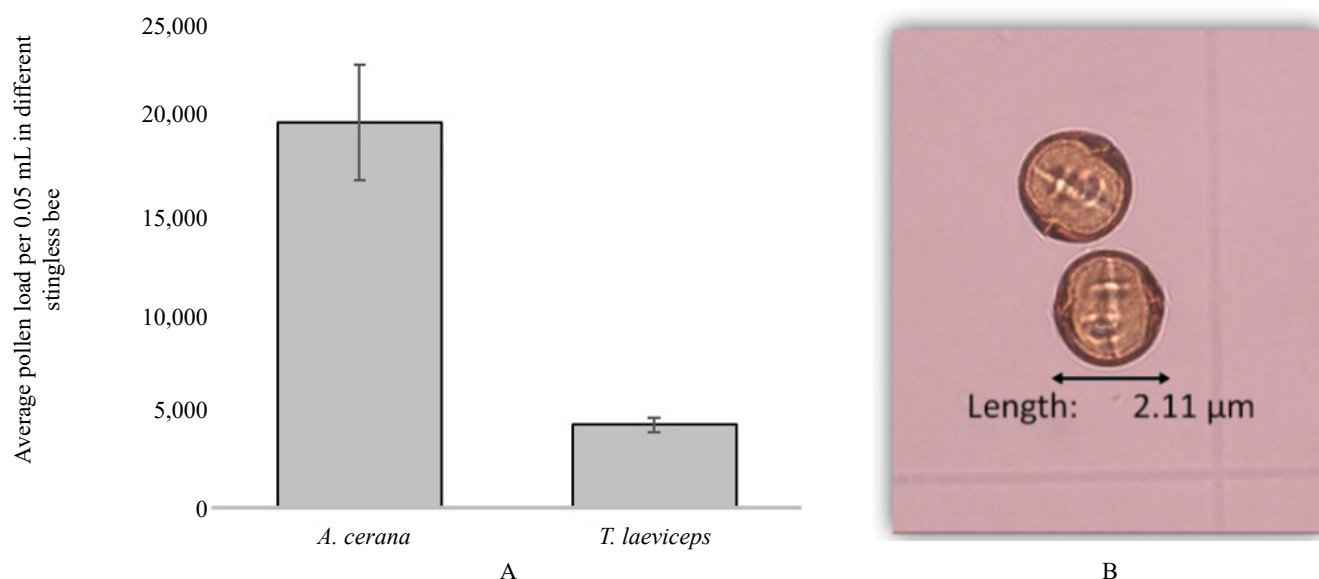


Figure 2. (A) The average pollen carried by *Apis cerana* and *Tetragonula laeviceps*, (B) morphology of pummelo pollen. The standard error is shown on each graph

Table 2. Fruit formation of pummelo by addition of *Apis cerana* and *Tetragonula laeviceps* colonies

Fruit set	Pollination treatment			
	Bagged flowers (control)	Opened flowers	Addition colony of <i>T. laeviceps</i>	Addition colony of <i>A. cerana</i>
Number of treatments flower	30	30	30	30
Average number of fruits formed (fruit)	0.8 <sup>c</sup>	3.03 <sup>b</sup>	4.10 <sup>a</sup>	4.10 <sup>a</sup>
Percentage of normal fruit (%)	10 <sup>c</sup>	57.3 <sup>b</sup>	84 <sup>a</sup>	84 <sup>a</sup>
Percentage of abnormal fruit (%)	90 <sup>a</sup>	42.67 <sup>b</sup>	16 <sup>c</sup>	16 <sup>c</sup>
Transverse diameter of fruit (cm)	23.52 <sup>c</sup>	24.57 <sup>b</sup>	30.5 <sup>a</sup>	30.5 <sup>a</sup>
Longitudinal diameter of fruit (cm)	22.46 <sup>c</sup>	23.56 <sup>b</sup>	28.73 <sup>a</sup>	28.73 <sup>a</sup>
Fruit weight (kg)	0.99 <sup>b</sup>	1.7 <sup>b</sup>	2.2 <sup>s</sup>	2.2 <sup>s</sup>
Increase of fruit formation (%)	0	5	32	34

The letters in the same row showed significantly different based on ANOVA and Tukey test

Table 3. Proximate analysis of pummelo fruits produced by four treatments

Component	Pollination treatment			
	Bagged flowers (control)	Opened flowers	Addition colony of <i>T. laeviceps</i>	Addition colony of <i>A. cerana</i>
Vitamin C (mg/kg)	461	490	615	669
Carbohydrates (%)	8.64	9.41	9.63	9.25
Fat (%)	0.06	0.1	0.04	0.08
Protein (%)	0.71	0.38	0.38	0.73
Ash content (%)	0.29	0.21	0.25	0.24
Water content (%)	90.3	89.9	89.7	89.7

stalk length 0.8-1.2 cm and a greenish-white color (Zufahmi and Nurlaila 2018; Adlini and Umaroh 2020; Cholis *et al.* 2020) and consist of 4-5 petals (Orwa *et al.* 2009). The position of pistil to the anther affects the pollination process. Position of anthers of pummelo flower are higher than the stigma, so it could self-

pollinate. However, the pollinator insects facilitate cross-pollination on pummelo. Crossing plants using high viability pollen result superior quality of pummelo fruits (Dewi *et al.* 2015).

Results showed that the visiting activity of bees increased in the morning and decreased in noon (11.30



am) (Figure 1). Visiting activity of *A. cerana* was higher than *T. laeviceps*. The higher activity of *A. cerana* may related to a higher level of colony requirement than *T. laeviceps* which has a smaller body and colony size (Leksikowati *et al.* 2018). The longer visit duration in a flower of *T. laeviceps* aim to maximize resource acquisition (Wulandari *et al.* 2017) and may be related to the shorter proboscis compared to honey bees. The difference in visiting activity of bees also related to body size and the large bees visited more flowers with shorter visits in a flower (Hoehn *et al.* 2010; Oronje *et al.* 2012). In addition, the number of pollens carried also are influenced by the visiting time to flowers. Bees that earlier visit will carry more pollens (Vidal *et al.* 2010). A'yunin *et al.* (2019) also reported that more flowers visited make more efficient of the insect as pollinator. The visit duration of bees also was influenced by nectar sugar-content (Ruslan *et al.* 2015), food sources (Tan *et al.* 2015), and colony needed (Hendriksma *et al.* 2019). During foraging, bees carry nectar and resin (Wicaksono *et al.* 2020). Hills *et al.* (1997) reported honeybees showed flower constancy, i.e., during foraging, individual of bee visit flowers of one plant species and flower constancy increase pollination efficiency (A'yunin *et al.* 2019). Flower constancy is influenced by flower color and shape, volume and nectar concentration (Engel and Bakels 1980; Gruter and Ratnieks 2011; Pangestika *et al.* 2017). The activities of *A. cerana* and *T. laeviceps* were positively correlated with temperature and light intensity (Table 1). In pummelo plantation, the day temperature is relatively high and affected the number of flower-visiting insects. In high temperatures, foraging activity require more energy (Basari *et al.* 2018) and bees tend to stay in the hive (Bruijn and Sommeijer 1997).

Body size of bee affects the number of pollens carried (Pearce *et al.* 2012). Large body size carried more pollen than smaller bees (Huda *et al.* 2015). The food sources of flowers consist of nectar and pollen. By bees, collected nectar then is processed into honey as an energy source. Pollen is a source of protein, while nectar is a source of carbohydrate (Kuntadi 2008; Riendriasari *et al.* 2022). Results showed *A. cerana* carried more pollens than *T. laeviceps*. The similar result also was reported by Alpionita *et al.* (2021) on strawberry. Pummelo has a yellow, large, and sticky

pollen. During visit the flowers, pollens attach to the bee's body (A'yunin *et al.* 2019).

Bee, *Apis cerana* is the main pollinating insects in some crops, such as apples, melons, watermelons, and oranges. Some agricultural crops cannot produce seeds or fruit without cross-pollination by insects (Hoopingarner and Waller 1992; Shresta 2008). The highest fruit formation was by addition of *A. cerana* (91.3%), followed by *T. laeviceps* (84%), open flowers (57.3%), and bagged flowers (10%). The increase of fruit formation was higher in addition of *A. cerana* (34%), and *T. laeviceps* (32%) than control plants. Addition of bee colonies in pummelo plantation also produced a large size of fruits. In addition, Bahlis *et al.* (2021) also reported pollination by *T. laeviceps* increase fruit size, fruit weight, number of seeds per fruit, seed germination, and sugar content. These data showed the pummelo requires pollinator insects for pollination.

Fruit of pummelo has a spheroid shape (Susanto *et al.* 2013; Dewi *et al.* 2015). The fruit of “Bageng Taji” variety has a peel color when ripe with a larger diameter than other varieties (Dewi *et al.* 2015). This variety belong to the accession group of seedless fruit. Fatima *et al.* (2010) reported the morphological character of seedless fruit in sweet orange and mandarin cultivars are influenced by genotype. The “Bageng Taji” variety have morphological characters, such as a medium leaf-size, a thick epicarp, green-yellowish peel color in ripe fruit, and pyriform-spheroid shape (Dewi *et al.* 2015). Pollination by bees increased vitamin C of pummelo fruit (Table 3). This result similar in fruit of strawberry pollinated by bees (*A. cerana* and *T. laeviceps*) that increased vitamin C, as well as fruit formation, fruit size and weight (Alpionita *et al.* 2021).

This study concluded that honey bee (*A. cerana*) and stingless bee (*T. laeviceps*) contributed to the pollination process of pummelo. The peak visiting activity of bees occurred in the morning and these activities decreased in the afternoon. *Apis cerana* carried more pollens (262,750 pollen grains) than *T. laeviceps* (95,344 pollen grains). The addition of *A. cerana* and *T. laeviceps* colonies in pummelo plantation produced high normal fruits (91.3% and 84%) and decreased the abnormal fruits (8.67% and 16%, respectively). The addition of

the bee colonies in plantation also increased the fruit vitamin C content.

## Conflict of Interest

The authors declare no conflict of interest.

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