Journal of Oil Palm Research Vol. 29 (3) September 2017 p. 311 – 317

ESTIMATING NUMBERS OF OIL PALM (*Elaeis guineensis*) POLLEN GRAINS USING IMAGE ANALYSIS AND PROCESSING

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ABSTRACT

Elaeidobius kamerunicus *is the most important oil palm pollinator in Indonesia and Malaysia. However, the mechanism and efficiency of pollen transfer by this weevil are clearly not understood. The lack of study on pollination process in oil palm* (Elaeis guineensis) *is mostly caused by difficulties in pollen counting due to their small size. Most of the counting was conducted manually which is prone to mistakes, required extensive training, and time-consuming. The aim of this study is to provide a novel technique for counting pollen that is rapid, consistent, and efficient with a comparable accuracy to manual counting. Male and female of* E. kamerunicus *were collected from male and female oil palm inflorescences (N=60). Extracted pollen were placed and distributed in a flat microscope slide separated by designated observation chambers. Images of each chamber were captured as a JPEG format and analysed by ImageJ. Multiple macros were constructed for image processing steps to obtain the pollen numbers. Comparison with manual counting using paired T-test, Pearson's correlation and linear regression showed a high similarity between both methods.*

Keywords: *Elaeis guineensis, Elaeidobius kamerunicus*, pollen counting, image processing, ImageJ.

Date received: 18 February 2015; **Sent for revision:** 19 June 2015; **Received in final form:** 3 August 2017; **Accepted:** 4 August 2017.

INTRODUCTION

One of the major concerns in oil palm (*Elaeis guineensis* Jacq.) plantation is poor fruit set due to limited pollination. Therefore, in 1981, the oil palm weevil (*Elaeidobius kamerunicus*) was introduced to address this problem. Subsequently, there has been many reports corroborated that the insect significantly improved fruit set (Syed *et al*., 1982; Harun and Noor, 2002). This success story has stimulated many studies on the field of pollination biology of oil palm (Sipayung and Lubis, 1987; Dhileepan, 1994; Poinar *et al*., 2002; Eardley *et al*., 2006; Purba *et al*., 2012). Nevertheless, most of these studies only focused on the correlation between the presence of *E. kamerunicus* and oil palm yield, while the mechanism and the main role of this weevil on pollen transfer remain unclear. For this, we need such a method which can provide rapid, yet precise estimation of the numbers of pollen produced by the plant and how it is distributed by *E. kamerunicus*. Quantification of pollen would provide critical information to understand of the male reproductive function (Kearns and Inouye, 1993), fitness of different plant morphs (Harder and Barrett, 1993), and crucial to understand the number of pollen grains that is removed and carried by pollinators (Thomson and Goodell, 2001; Adler and Irwin, 2006).

Study on pollen deposition rate by pollinators requires two crucial steps: (1) pollen extraction from pollinator's body and (2) pollen counting from the obtained samples. Pollen separation techniques have been developed extensively by applying several types of solvent, gel, and detergent

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solution (Bernhardt, 2005; Costa and Yang, 2009). Though, these techniques mostly suitable for large insects, rather than small insects like oil palm weevil. Furthermore, pollen counting was usually performed using a haemocytometer (Chinchilla-López and Richardson, 1991; Dhileepan, 1992; Prada *et al*., 1998). This method could estimate the total number of pollen grains by extracting data from sub-sample counting. However, application of this method requires special training, time-consuming and prone to disproportionate pollen settling on grids, especially for the large pollen grains are large, leading to erroneous estimates (Kannely, 2005). Alternatively, pollen numbers are counted manually based on photographed pollen. However, this method is labour intensive and consequently prone to human error. Electronic counters, which utilise electric currents to determine the presence of particles offers a faster counting process. Unfortunately, this type of counter could not discriminate debris particles from pollens (Kearns and Inouye, 1993). This problem later was solved by further development and application of laser-based counter for pollen counting (Kawashima *et al*., 2007). However, high operational cost impedes its operation by frequent users.

Therefore, it is necessary to develop a simple method that could generate rapid and accurate estimation of pollen numbers from the oil palm. One possibility is to use an image processing tool and analysis program which offer a more accurate, user friendly, and consistent estimation (Bechar *et al*., 1997; Aronne *et al*., 2001; Fonseca *et al*., 2002; Costa and Yang, 2009; Geissman *et al*., 2013; Mudd and Arathi, 2012). This tool is equipped with a particular computer software to scan pollen images, which previously captured as raw images by a camera. The scanned pollen objects are then counted as separated units (Bechar *et al*., 1997; Arone *et al*., 2001).

In this study, we used ImageJ, a free-Java based image analysis software provided by the US National Institute of Health (http://rsbweb.nih.gov/ij/; Rasband, 1997-2000; Schneider *et al*., 2012). ImageJ has already applied as a method to estimate numbers of small biological objects such as insect eggs, insect adults, and yeast cells (Mains *et al*., 2008; Kesavaraju and Dickson, 2012; Choudhry, 2016). Using the similar approach, we also developed a specific image preparation procedure and macro to provide an accurate estimation on the pollen numbers of oil palm.

MATERIAL AND METHODS

Pollen Preparation for Image Analysis

In this study, image analysis and processing method were applied to count pollen obtained from the body of *E. kamerunicus*. Both male and female weevils which carried pollen grains were randomly collected from male and female inflorescences of 7 years old oil palm. Unlike airborne pollen, animal-dispersed pollen are normally sticky, which tend to form pollen clumps. Thus, a specific washing method is required to prevent this clump formation prior image analysis. The collected insects were placed into a small sample glass bottle, then washed with 100 μl KOH 10% (w/v). Washing method was performed by a 'pump-suck' technique using a small pipette to dissociate pollen clumps from all parts of the weevil's body.

In order to reduce pollen density, 100-200 μl KOH was added. The 100-300 μl pollen solution was pipetted and dropped onto microscope slide. The slide was divided into smaller sub-sample chambers (0.5 x 0.5 x 0.1 cm^3) by black Nachi Tape® to maintain accuracy of software analysis (Costa and Yang, 2009). Pollen samples from each *E. kamerunicus* were distributed into five to thirty chambers to generate images with ideal pollen density.

Image Analysis

Individual slide was mounted on a stereo microscope for image capturing. A Nikon SMZ 1500 stereomicroscope and Nikon Digital Sight DS-FI 1 camera was used to capture pollen images for each chamber. The light source was supplied from the bottom and adjusted to allow an optimal light condition for image analysis. All pictures were processed by NIS Element D 3.1® software and saved as JPEG format.

Prior to analysis, captured images were cropped to remove the edges of each viewing chambers. All debris were eliminated using any photo editor software (in our case we used Photoshop®) then analysed by Image (*Figure 1*). The accuracy of pollen analyser macro was determined according to Costa and Yang (2009).

Accuracy and Statistical Analysis

Data of number of pollens obtained by image analysis were compared with those from manual counting. Data of manual counting obtained using cell counter plugin from ImageJ Plugins-Analyze menu. Results from both counting were compared using paired T-test. Correlation between both data was analysed by Pearson's correlation and linear regression. All statistical analysis was carried out by SPSS 13.

RESULTS AND DISCUSSION

Compatible Multiple Macro for Oil Palm Pollen Grains Counting

Every flowering plant has different pollen characters, so that the image processing should be

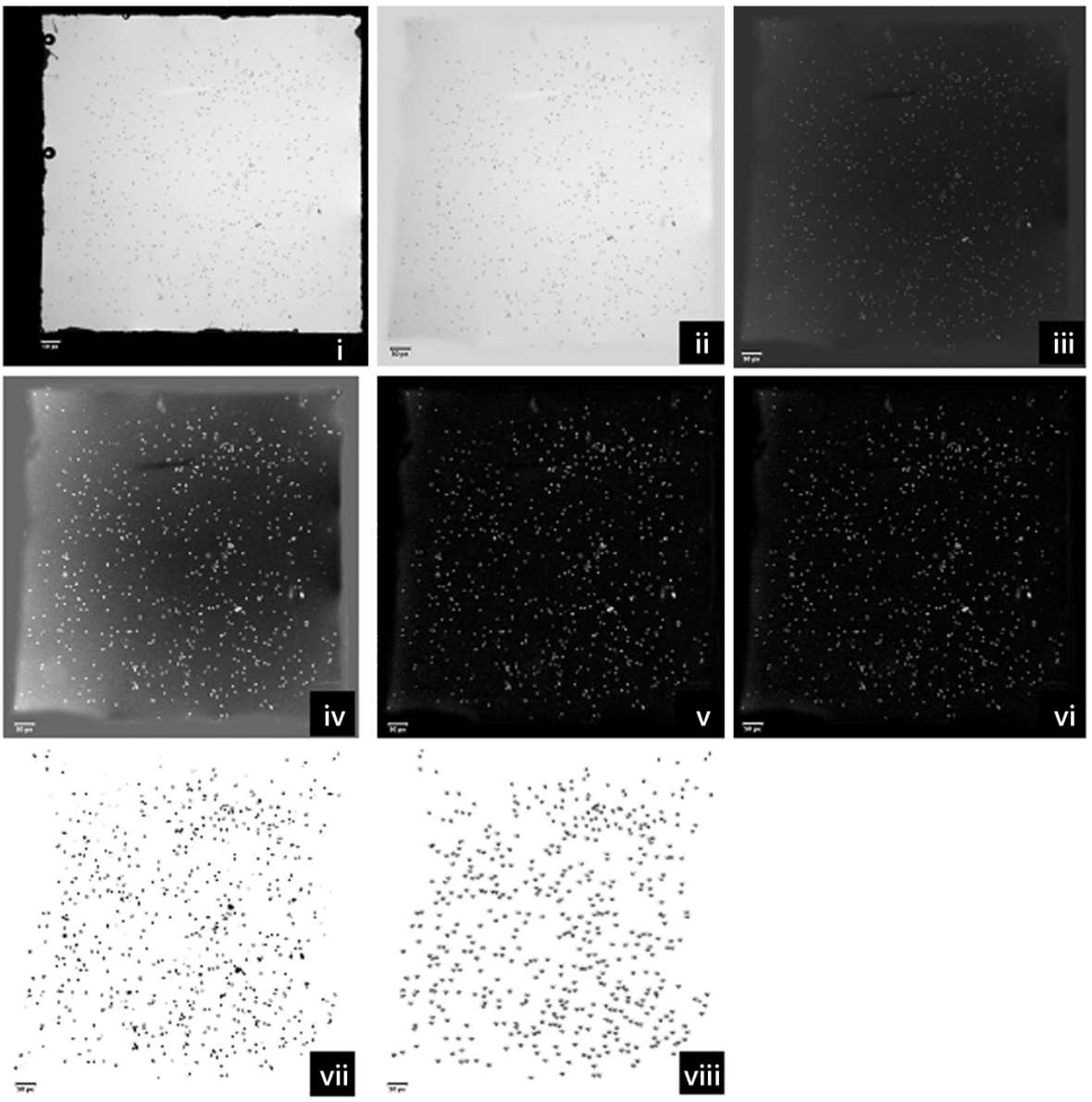

Figure 1. Various images resulting from step-by-step of image processing and analysis using multiple macro on ImageJ program. Serial command (macro) was (i) unprocessed image, (ii) prepared image after cropping and clearing from the debris, (iii) inverted image, (iv) image adjusted for brightness and contrast, (v) image with subtracted background, (vi) 8-bit image, (vii) image adjusted for threshold, (viii) image for particle counting and analysis.

adjusted accordingly. This prompted us to construct procedures for image preparation and macro prior to analysis with ImageJ. The preparation for oil palm pollen processing consisted of six sequential steps: (i) inverting the image to create contrast between dark background and pollen as a light pixels, (ii) setting up brightness and contrast (saturated at 0.15) - to increase image contrast, (iii) background substraction (rolling value at 50 px) to remove the background image, (iv) converting the image to an 8-bit and adjusting the threshold adjustment at 0, 55 to transforming the image to binary pixels and improving the proportion of pollen pixels, (v) re-inverting to reverse pollen image from light into dark pixels. This step is critical since the software only recognises black pixels as objects to be counted. The final step was employing a particle analyser (vi), which counted pollens based on its size and circularity *(Figure 2*).

In order to improve the estimation of pollen number by ImageJ, it is necessary to find correct brightness and contrast, threshold and particle analyser setting. Brightness and contrast should be set be fine-tuned accordingly (*Figure 2b*) as low intensity hinders many dark pixels and leads to underestimation (*Figure 2b*). High contrast might increased the noise resulting in overestimation (*Figure 2c*). Threshold macro is a command to convert 8-bit image pixels to binary pixels (black and white). The process is based on threshold value that was adjusted previously. A higher value will produce low image conversion while lower value

might cause too many pixels conversion. Correct value is a necessity as the number of pixels is a basic parameter for pixel identification and pollen object classification.

The final and the most important step is defining the range value for pollen size and its circularity. This value should be adjusted between the minimum and the maximum size also circularity of pollen pixels to reduce overestimation. Any objects with shape range outside this range should be omitted. Therefore, separation process of pollen, by KOH or detergents, should be considered as the greatest concern as clumped pollens tend to underestimate pollen count. Our study found that the best setting for size and circularity for oil palm pollen ranged from 8 to 40 pixels^2 and 0.3 to 1.0, respectively.

In order to improve flexibility and speed of image counting, multiple macros, including close command, should be added to batch macro. The constructed batch macro is provided as follow:

```
macro "Counting of E. guineensis pollen grains (2011)"
{requires ("1.33s") ;
inputFolder = getDirectory ("Batch Counting!!: Choose your
input folder!") ;
images = getFileList (inputFolder) ;
for (i=0; i<images. length; i++) {
input Path = inputFolder + images [i] ;
      open (inputPath) ;
      run ("XXX") ; (multiple command)
run ("Close") ;
close() ; }
```

Based on analysis from 925 images, ImageJ was able to detect and count pollen grains with high similarity with manual counting (Pearson's r=0.97,

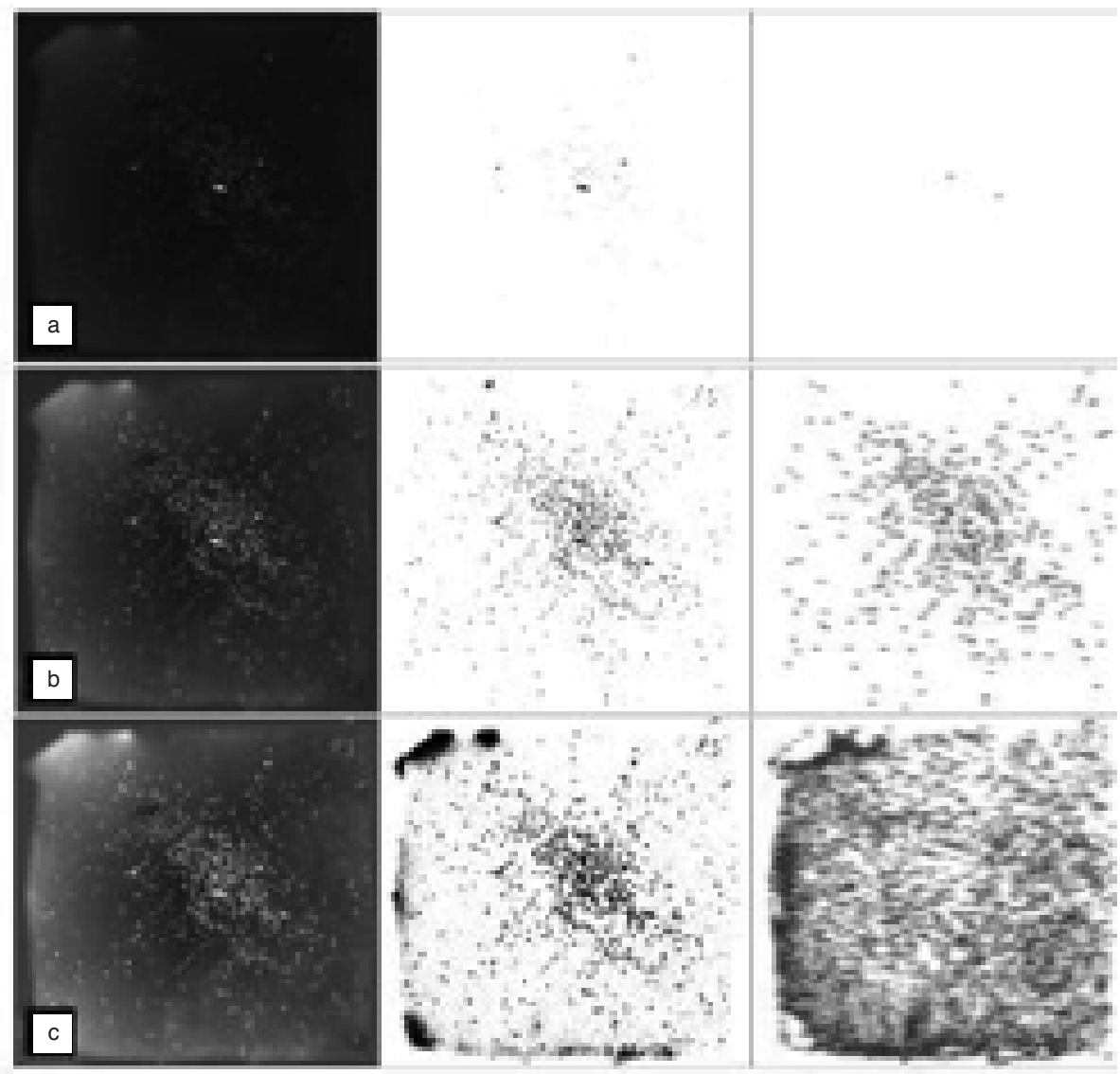

Figure 2. Three sample images representing poor and best macro setting. (a) Low brightness and contrast (0), high threshold (65), narrow range of size (8-30) and circularity (0.3-0.9) lead to under estimation. (b) Middle macro setting (0.15; 55; 8-40; 0.3-1.00) resulted in good prediction. (c) Over brightness and contrast (1), low threshold (20), and wide range of size (0-50) and circularity (0-1) lead to overestimation.

P<0.0001). Linear regression (*Figure 3*) also showed that image analysis was successful to predict pollen grain, in accordance with Equation y = 1.556x – 9.9925 (r^2=0.952). We also found that only 84 images (9.08%) produced estimates less than 75% of actual number.

The high accuracy of pollen number estimation, in this study was also reported in other studies with different plant families that have different pollen size and shape (Mudd and Arathi, 2012; MacInnis and Forrest, 2017). This flexibility may be beneficial for further application on various oil palm cultivars.

The comparison of pollen prediction by image analysis and manual counting (N=15) is depicted in *Table 1*. P-value of paired T-test shows that pollen prediction for each group is not significantly different at α=0.05 (two tailed), except for MM group. We believe the difference between manual counting and image analysis found in MM group was caused by high pollen and debris densities on the body of male weevils. Pleura setae present in males weevils provide additional body surface which allowed the smaller debris to contaminate pollen samples (Dhileepan, 1992). Debris might form clusters with pollen which make it difficult for software to delineate individual pollen to give a correct count (Mudd and Arathi, 2012). However, for many biological studies involving counting of small biological particle, a moderate deviation (lower than 20%) is often negligible compared to noise generated by other factors (Geissman, 2013). Yet, further studies are needed to: (1) improve method for separation of pollen from debris, (2) improve sensitivity of the pollen detection program, allowing for a better understanding of pollination process in oil palm, and (3) further application of this software to counting small circular objects in oil palm research (*i.e*. bacterial colony counting, debris counting, *etc*.) can also be pursued.

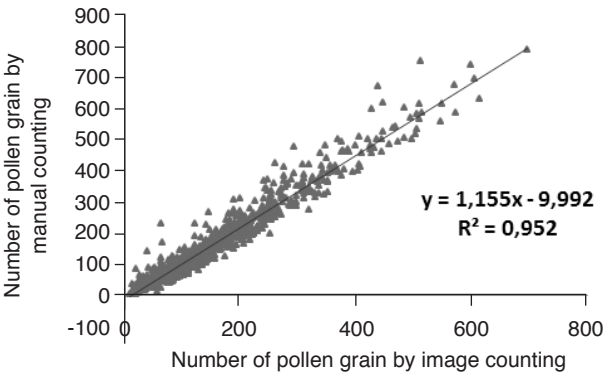

Figure 3. Linear regression between manual counting and image analysis. Manual counting was performed by cell counter plug-in on ImageJ and counting by image analysis was performed by ImageJ with multiple macro (N=925).

CONCLUSION

In conclusion, image analysis of pollen by ImageJ could be applied as a reliable method for pollen counting. The cost for entire process is considerably low as all processes could be carried out by equipments which are available in any standard biology laboratory and the program is provided as an open source program. Furthermore, image processing using ImageJ reduces the chance of false estimation, ensures a greater consistency on counting and reducing labour requirements for reliable pollen counting.

ACKNOWLEDGEMENT

We thank Palgunadi and Satyoso for allowing us to use the laboratory and photographic equipment; Agus Widodo, Bargowo Addianto for allowing us to use their personnel; Ali Imran and Jari for helping with manual counting; Prima for her assistance in the field; Erwin for helping with statistical analysis; Ageng Mufti SH for support and critical suggestion in developing macro; Damiran and Hani for helping with research material preparation; Firman, Yusuf, Solihul Amal, Ramadhani Eka, M Kamal Muzzaki, Adeline Prapaisie, Yuvika, Ni Putu Indah, Wahyu Santika for valuable suggestion during research design. This research was partly funded by PT Astra Agro Lestari Tbk and PUPT Grant from Ministry

TABLE 1. COMPARISON OF POLLEN COUNTING BY MANUAL AND IMAGE ANALYSIS METHODS

Sex (N=15)	Group	Pollen count (mean ± S.E.)		Pearson's correlation (r)	P-value (paired T-test)
		Manual counting using ImageJ cell counter plugin	Image analysis counting by ImageJ multiple macro		
Male	♂(MM)	5 721 (± 376)	5 075 (± 317)	0.984	0.000
	♀(MF)	698 (± 97)	727 (± 97)	0.949	0.360
Female	♂(FM)	3 357 (± 368)	3 172 (± 287)	0.971	0.123
	♀ (FF)	293 (± 31)	277 (± 27)	0.96	0.112

Note: * MM - male weevil at male inflorescences, MF- male at female inflorescences, FM - female at male inflorescences, FF- Female at female inflorescences.

of Research, Technology and Higher Education granted to the first author.

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