

Anther Dehiscence, Pollen Viability and Stigma Receptivity Study on Cultivars of Black Pepper (*Piper nigrum* L.)

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ABSTRACT

A study on floral biology of black pepper cultivars that cover anther dehiscence, pollen viability and stigma receptivity was carried out with the aim to improve the efficiency of intervarietal hybridisation in black pepper breeding work. In this study, 10 black pepper cultivars were used, namely ‘Semongok Aman’, ‘Kuching’, ‘Semongok Emas’, ‘Semongok Perak’, ‘Semongok 1’, ‘Nyerigai’, ‘India’, ‘Lampung Daun Lebar’, ‘Sarikei’ and ‘Yong Petai’. The results show that anthesis in the 10 black pepper cultivars occurred between 10.25 pm and 10.50 pm. In the pollen viability study, results suggest that pollen are more viable between five and 10 hours after anther dehisced. However, there are variations among the cultivars for the optimum viable stage. For stigma receptivity, the results show that stigmas at Stage 2 (elongation and spreading of stigmata) and Stage 3 (complete emergence and wide spreading of stigmata) had better receptivity than Stage 1 (first appearance of stigmata). There was no difference in stigma receptivity stages among the 10 cultivars. This study thus, shows the most suitable time for intervarietal hybridisation via artificial pollination.

Keywords: Anther dehiscence, black pepper cultivars, pollen viability, stigma receptivity

ARTICLE INFO

Article history:

Received: 20 October 2017

Accepted: 4 January 2018

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INTRODUCTION

Black pepper, scientifically called *Piper nigrum* L., is a spice plant from the family of Piperaceae. The plant is known as the King of Spices, and is the world's most widely used spice due to its unique aroma and pungency. In Malaysia, the

plant plays a pivotal role as a cash crop for smallholders and has become a major agricultural commodity, particularly in the state of Sarawak, since it was introduced in 1856 (Sim, 1993).

Hybridisation ensures a wide variety exists among the cultivars of black pepper ensuring quality attributes and yield. High yielding black pepper varieties are needed to compensate high production cost constraints in Malaysia (Sim, 1993). Intervarietal hybridisation has become the main thrust in the black pepper breeding programme since it was initiated in Agriculture Research Centre, Semongok of Sarawak in 1957. However, the breeding work is limited in achievement so far, with only one promising hybrid developed from crosses between Balankotta and Kuching. The hybrid, named Semongok Emas was released to farmers in 1991 (Sim, 1993). In India, two achievements through conventional breeding have been reported so far, that is, Panniyur 1 and Panniyur 3, both from crosses between Uthiaranotta and Cheriyanakaniyakan (Ravindran, Nair, & Matthew, 1981). Limited achievement of conventional black pepper breeding is possibly due to lack of fundamental information on the nature of the reproductive biology, particularly on pollen viability and stigma receptivity.

Ravindran et al. (1981), Sim (1985) and Chen (2011) have reported similar artificial pollination procedures for black pepper. In general, the artificial hybridisation was carried out by inseminating the pollen suspension of the male plant onto the

stigma of female plant. However, these reports had no specification concerning the viable stage of pollen for collection and the receptive stage of the stigma. So, there is some doubt regarding the reliability of the procedure and the technique of artificial pollination. In addition, intricacy in artificial pollination of black pepper is perhaps due to the catkin type of inflorescent, minute sized flower and lack of uniformity in emergence of anther and time of anthesis. Hence, a fundamental study in floral biology of black pepper is needed to ensure efficiency of conventional breeding.

The objectives of this study are to determine the time of anthesis, the nature of pollen viability and the stigma receptivity of black pepper, while in tandem, the aim is also to improve the efficiency of conventional breeding.

MATERIALS AND METHODS

Materials

The experiment was carried out from March to July 2016. The plant materials used in this study were obtained from a plant house at the Malaysian Pepper Board and a black pepper germplasm collection plot located at the Agriculture Research Center, Semongok. A total of 10 black pepper cultivars were used, namely Semongok Aman (SA), Kuching (KCH), Semongok Emas (SE), Semongok Perak (SP), Semongok 1 (S1), Nyerigai (NYE), India (IND), Lampung Daun Lebar (LDL), Sarikei (SAR) and Yong Petai (YP). For

each cultivar, 10 plants were planted in pots sized 10 inches in diameter, with a potting mixture combination of topsoil, sand and peat moss at 1:1:1 ratio.

Methods

Pollen Morphology Study. Palynology study is crucial for identification of pollen. For this study, only cultivar SA was used. Previous research has proven the unlikeliness of morphological differences among black pepper cultivars' pollen (Ravindran et al., 2000). Anthers were collected from the mature flowers and stored in a plastic centrifuge tube filled with 70% alcohol. These anthers were crushed with a glass rod and the solution was filtered through fine meshes with a 0.32mm aperture to collect pollen grains. The pollen grains were then prepared for light and scanning electron microscopy (SEM) by the standard method as described by Arora and Modi (2008). For the SEM study, pollen grains were suspended in a drop of ethanol and directly transpired with a fine pipette to a metallic stub using double sided cello tape and coated with gold palladium in a sputtering chamber. The SEM examination was carried out on a LEO electron microscope (Model LEO 430). The terminology of the pollen characteristics and analysis used is in accordance with Bhattacharya, Mujumdar and Bhattacharya (2006) and Agashe (2006).

Anther Dehiscence. Anther dehiscences on all cultivars were carried out in the first five months of 2016 at the plant

house, Malaysian Pepper Board. In each month, 10 days were randomly selected for carrying out the observation, regardless of weather conditions. The anthers selected for the study were at the pre-dehiscenced stage. Observation, with one hour intervals each time, was carried out starting from early morning until anther dehiscence was noticed. The preliminary judgment on anther dehiscence was made on the basis that whitish powder-like pollens were seen by the naked eye. Then, the released pollens were confirmed through stereomicroscope observation.

Pollen Viability Study. A total of 10 cultivars were evaluated in this study. Based on the finding of the anther dehiscence study, the pollen was classified into five stages for verification of the viability:

- Stage 1: Right after anther dehiscenced
- Stage 2: Five hours after anther dehiscenced
- Stage 3: 10 hours after anther dehiscenced
- Stage 4: 15 hours after anther dehiscenced
- Stage 5: 20 hours after anther dehiscenced

The test was carried out in vitro. A moist condition for pollen germination was created by placing a glass slide on top of bent glass tubing, which was placed inside a petri dish with small amount of water added. This created a moist environment to maintain humidity of the medium to promote germination. Chen (2011) published the germination medium protocol for pollen viability test on *P. colubrinum*, which was then adapted to *P. nigrum* pollen

germination. The optimised liquid medium consisted of 10% sucrose, 100 mg/L boric acid, and 300 mg/L calcium nitrate.

To initiate the observation, pollen grains at the desired stage were carefully transferred onto the liquid medium. The pollen of *P. nigrum* takes at least six hours to initiate germination (Chen, 2011). After six hours of treatment, the glass slide was taken out of the petri dish, and the germinated pollen was then fixed with Carnoy's fixative and stained with Safranin. Pollen grains were observable under the compound microscope with 400x magnification. Percent germinated pollen was counted via microscope and the length of the pollen tube was measured via a reticule (built-in scale on eyepiece). For all five stages of anther dehiscence, 100 grains of pollen for three replicated treatments were counted to obtain the percentage of pollen germination. For pollen tube length evaluation, 30 germinated pollen grains from three replications were calculated. Both the germination percentage and the pollen tube length were evaluated to indicate the level of pollen viability. The data were analysed with one-way ANOVA to identify the most viable stage of pollen.

Stigma Receptivity Study. This study was performed on the 10 cultivars to check receptivity difference. The receptivity is indicated by production of peroxidase on stigmatic surfaces. The production of peroxidase is indicated by a formation of blue dot on stigmatic surfaces (Chen, 2011). The dissolved Peroxtesmo KO

paper is needed because the stigma of black pepper is so minute (about one millimeter in size) and fragile that the test cannot be done with direct contact of the stigma with Peroxtesmo KO paper. The Peroxtesmo KO solution was prepared with a concentration of 5 paper/1 mL of distilled water. Observation must be done immediately within five minutes, after the treatment using the Fluorescent Stereo Microscope Leica M165 FC. The stigma at three distinctive stages was sampled for observation, that is:

- Stage 1: First appearance of stigma (Day 1 of emergence)
- Stage 2: Elongation and spreading of stigma (Day 2-3 of emergence)
- Stage 3: Complete emergence and wide spreading of stigma (Day 4-6 of emergence)

The description on stages of stigma was based on description by Sim (1979). For each cultivar and each stage of stigma, 30 stigma/inflorescence was collected for the test. One-way ANOVA statistical analysis was carried out to determine the most receptive stage of the stigma.

RESULTS AND DISCUSSION

Pollen Morphology Study

Pollen morphology of the black pepper cultivar 'SA' was studied via SEM observation with the aim to precisely collect the right pollen for anther dehiscence and viability study. SEM observation showed pollen grain size is about <10 µm

in diameter, categorised under myosotis, spherical shaped, radially symmetrical and with irregular pinulose sculpturing. *Piper nigrum* exhibits much uniformity in pollen morphology (Ravindran et al., 2000). This

is supported by Federico et al. (2017) in their study on *Jatropha* cultivars and also Sanja et al. (2013) on sweet cherry cultivars study. Thus, only one cultivar, Semongok Aman was selected for this study.

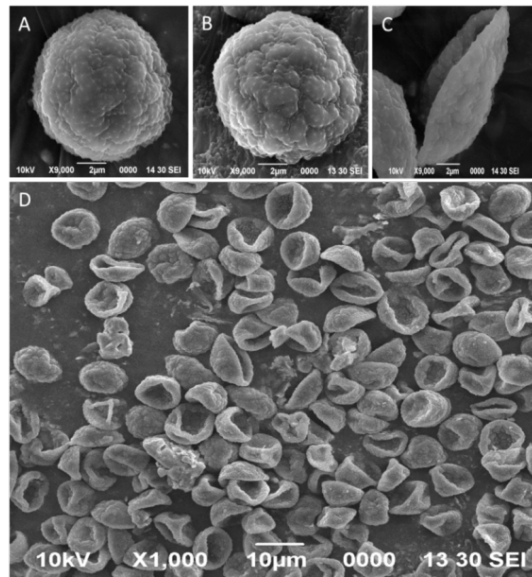


Figure 1. SEM micrographs showing size, shape and sculpture of black pepper cultivar ‘SA’ pollen grains

A and B are viable pollen with turgid appearance, C relates to unviable pollen with flattened appearance and D is the mixture of viable and unviable pollens.

Anther Dehiscence

The objective of this observation is to reveal the estimated time, to ease tediousness in checking the anthesis time. However, observation showed there is no significant difference in time of anther dehiscence among cultivars. Normally,

pollen dispersal under field conditions can be erratic over time and dependent on relative humidity (RH) and temperature (Yates & Darrel, 1993).

In this paper, only cultivar ‘SA’ was reported. Investigation on anther dehiscence of cultivar ‘SA’ showed the median time of anther dehiscence was between 11.00 pm to 12.00 pm. Observation study revealed that the months of March and April showed high variation in the median time of anther dehiscence, ranging from 10.30 pm (March 2016) to 10.25 pm (April 2016)

(Figure 2), while in May, June, and July 2016, the median time recorded were 11.15 pm, 11.45 pm, and 11.50 pm, respectively. There were small variations in the time of anther dehiscence for the months of May, June, and July, compared to the months of March and April (Figure 2). However, statistical analysis proved the variation was a non-significant difference at ($p \leq 0.05$).

Based on the rainfall data in 2016 (Malaysian Meteorological Department), the months of March and April had higher rainfall compared to the subsequent months of May, June, and July. Reduced rainfall between May and July may

introduce higher temperatures and lower RH to the environment. Thus, the delay in anther dehiscence for the three months is most likely due to higher temperatures and lower RH factors. This finding is supported by Sato et al. (2000), who suggested that high temperature exposure could inhibit the anther dehiscence process. Other researchers (Sharp & Chisman, 1961; Jovanovic & Tucovis, 1975; Yates & Darrel, 1993; Ellis et al., 1998; Marcela et al., 2017) also reported the adverse effects of elevated temperatures and low RH influences on anther dehiscence in several crops that eventually affect the fruit set.

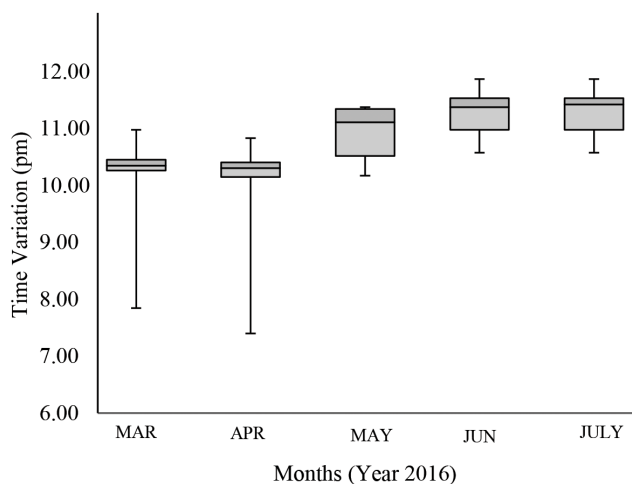


Figure 2. Time of anther dehiscence in cultivar Semongok Aman between March and July 2016

Pollen Viability Study

This study on pollen viability aimed to identify the optimum time for pollen collection for each of the 10 cultivars. Assessment of pollen germination

percentage and pollen tube length measurement via in vitro induced germination (Figure 3) was carried out to indicate the viability of pollen at various stages of collection.

Pollen germination percentage results (Figure 4) proved that there were variable differences in the viability of pollen collected at the five stages of the anther dehiscence, and the trend of viability among the cultivars also showed some variations. In most of the cultivars, the percentage of germination showed significant differences in Stage 5 compared to Stages 2, 3, and 4. The germination of pollen in Stage 1 was not significantly different compared to Stage 5. This was observed on cultivars KCH, SE, SP, NYE, and SAR. Cultivars LDL and YP also showed similar viability trends; however, insignificant variation was observed at Stage 3 on cultivar LDL, and Stage 3 and Stage 4 for cultivar YP. The viability trend of cultivar SA only showed significant differences at Stage 5 pollen, while from Stage 1 to Stage 4, the pollen germination percentages showed no significant difference. Cultivar S1 also exhibited slightly different viability trends, where Stage 1 and Stage 5 showed no significant difference in germination percentage but was significantly different compared to Stage 2, Stage 3, and Stage 4. Cultivar IND showed no substantial difference in pollen germination percentage for all five distinct stages of the anther dehiscence.

Pollen tube length of in vitro germinated pollen at various stages was also investigated (Figure 5). The tube length was measured using stereomicroscope with a built-in reticule. Results show cultivars SA, KCH, SE, SP, LDL, SAR and YP exhibited similar trends of viability in all the pollen stages. All cultivars only showed significant differences

in pollen tube length measurements at Stage 5, pollen collected after 20 hours of anther dehiscence. Pollen collected at Stages 1, 2, 3, and 4 showed no significant difference in tube length assessment. The pollen tube length ranged from 9.2 μm (cultivar SAR, Stage 5 pollen) to 22 μm (cultivar KCH, Stage 4 pollen). Cultivars S1, NYE, and IND demonstrated different trends of pollen tube growth. Cultivar S1 exhibited three significantly different mean groupings among five stages of pollen, with Stage 1 (mean group 1) significantly greater in length compared to mean group 2 (Stages 2, 3, & 4) and mean group 3 (Stage 5). Stage 5 pollen in mean group 3 had significantly shorter length compared to pollen at mean group 2, thus, it is among the less viable stages of pollen, based on the tube length indicator. Cultivar NYE also has three distinctively different mean groups: mean group 1 (Stage 1 to Stage 3), mean group 2 (Stage 4), and mean group 3 (Stage 5). Among the three mean groups, mean group 1 showed the greatest length, ranging from 23.70 μm to 24.80 μm , while mean group 3 with Stage 5 pollen only achieved an average length of 11.20 μm . Cultivar IND showed a similar trend to NYE, where the greatest length recorded was 20.20 μm in the first distinct group, followed by 19.80 μm (mean group 2) and 9.70 μm (mean group 3). Pollen collected from Stages 2, 3, and 4 showed better pollen tube growth performance compared to pollen at Stage 1 and Stage 5. In Stage 5, the length of the pollen tube was among the shortest due to retarded growth.

ANOVA tests proved that the pollen collected between 5 and 15 hours of anther dehiscence is generally more viable based on the percentage of germination and pollen tube elongation. Pollen collected at Stage 1 showed low viability of about 67.90%, as recorded for cultivar YP. However, the pollen tube elongation performance for Stage 1 pollen is comparable to Stages 2, 3, and 4 pollens. This may be due to delayed germination for pollen collected right after anther dehisced. In most cases, pollen grains are metabolically dormant and highly desiccated when released from the anthers (Buitink et al., 2000; Heslop-Harrison, 1979). Thus, slightly poorer performance of Stage 1 pollen in germination studies may not be due

to the poor viability of pollen collected at this stage. Kearns and Inouye (1993) reported that pollen collected immediately after dehiscence was generally the most viable.

Another study found that pollen collected after 20 hours showed relatively low germination percentage and short pollen tube elongation. The pollen collected from this stage may be non-viable, even though the pollen is able to achieve satisfactory high germination percentage and pollen tube elongation. According to Heslop-Harrison (1979), non-viable pollen grains may hydrate to the same extent as living pollen grains, swell, and even develop short tubes before the tubes eventually rupture.

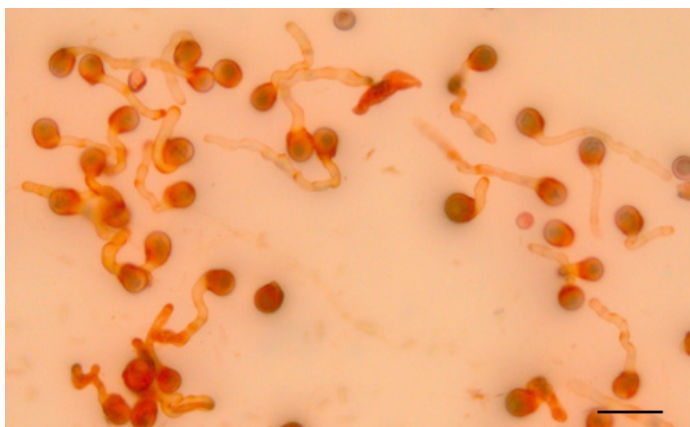


Figure 3. *In-vitro* germinated pollen at viable stage
The scale bar for the image above is 10 μ m.

Pollen germination percentage

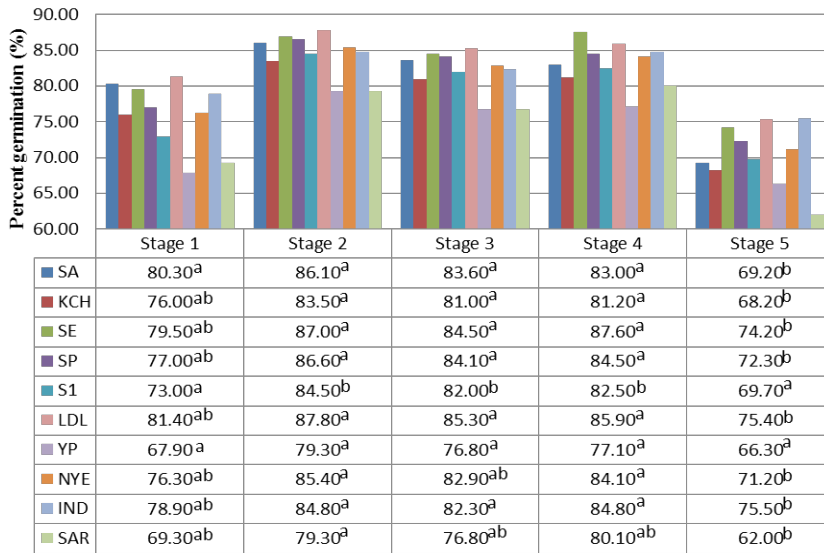


Figure 4. Percentage of pollen germination at various stages of pollen for the 10 cultivars

SA - Semongok Aman; KCH - Kuching; SE - Semongok Emas; SP - Semongok Perak; S1 - Semongok 1; NYE - Nyerigai; IND - India; LDL - Lampung Daun Lebar; SAR - Sarikei; YP - Yong Petai. The mean scores followed by the different superscript letter within the same column are significantly different at $p \leq 0.05$.

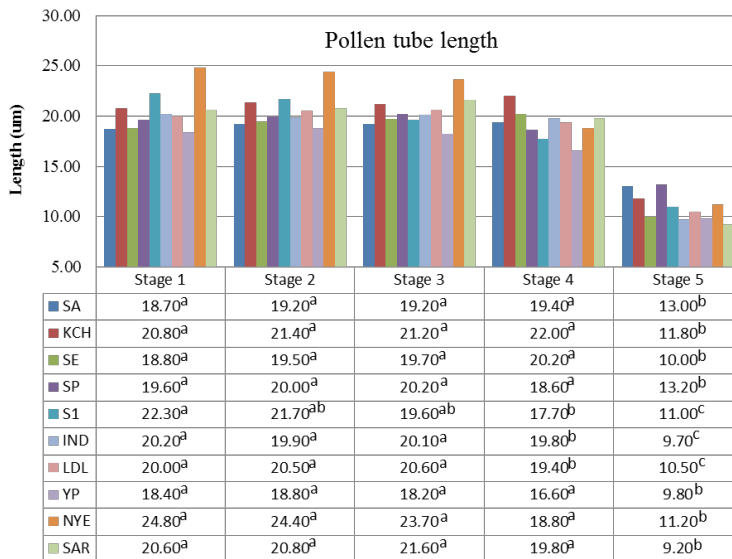


Figure 5. Pollen Tube length at five various stages of the anther dehiscence for the 10 cultivars

SA - Semongok Aman; KCH - Kuching; SE - Semongok Emas; SP - Semongok Perak; S1 - Semongok 1; NYE - Nyerigai; IND - India; LDL - Lampung Daun Lebar; SAR - Sarikei; YP - Yong Petai. The mean scores followed by the different superscript letter within the same column are significantly different at $p \leq 0.05$.

Stigma Receptivity Study

In this study, three stages of stigma (Figure 6) were sampled to examine their receptivity level. The stages are as follows:

- Stage 1: First appearance of stigma (Day 1 of emergence)
- Stage 2: Elongation and spreading of stigma (Day 2-3 of emergence)
- Stage 3: Complete emergence and wide spreading of stigma (Day 4-6 of emergence)

Development of a blue dot on the stigma after the treatment was recorded as a positive result, while no colour change was recorded as a negative result. The percentage of receptive stigma was calculated for each stage, and the results are tabulated in Table 1. The results show nine out of 10 cultivars that have been tested showed comparable results on the receptivity trend. Statistical analysis showed only Stage 1 stigma has a significantly lower receptive level, compared to stigma at Stages 2 and 3, for all nine cultivars except cultivar SA. Cultivar LDL recorded 52.60% (Stage 1) as the lowest, while cultivar IND achieved 97.70% at Stage 2, the highest overall

percentage. Cultivar SA showed three stages of stigma which were significantly different on a receptive level, with Stage 2 recording the highest percentage at 97.70%, followed by Stage 3 at 90.10%, and Stage 1 at 80.30%.

Peroxtesmo KO test paper is a tool for quick and easy detection of peroxidase (Dafni & Maue's, 1998). Galen and Plowright (1987) and Dafni (1992) proved the reliability of Peroxtesmo KO test paper for identification of stigma receptivity. Stigma receptivity could be evaluated by the arrival of peroxidase on stigmatic surfaces of the black pepper flower while the presence of peroxidase is indicated by the appearance of a blue or greenish colour (Dafni & Maue's, 1998). This method has been adopted in reproductive biology study of other plants, including *Macleania bullata* (Luis, 2000), *Origanum syriacum* (Rodriguez-Riano & Dafni, 2007), and *Manekia naranjoana* (Tatiana & Joseph, 2008).

In most plants, the stigma is receptive to pollination over a wide range of floral developmental stages (Amy & Rosanna, 2010; Chen et al., 2013). However, the results obtained from this study via ANOVA test showed that the stigma of black pepper at Stage 1 has significantly lower receptivity compared to Stages 2 and 3. Purseglove (1968) reported that peak receptivity occurred at three to five days of emergence. He added that stigma may remain receptive for up to 10 days. This is also supported by Kalinganire et al. (2000) and Sedgley, Blesing, and Vithanage

(1985) in their studies on silky oak and macadamia, respectively. They recorded that lower receptivity occurred at early stigma emergence stage. However, Chen (2011) revealed a study on receptivity via a hydrogen peroxide test, showing no significant difference at any stages of stigma, including the early emergence stage. Thus, the variation in this study, even though significant, at Stage 1 may become an indicator as a reasonably receptive stage of stigma for the 10 cultivars studied.

Helen and Lauren (2002) reported that stigmatic age is uncorrelated with receptivity. The stigma normally remains receptive at any stage before receiving pollen. After the stigma receives pollen, the stigmatic cells collapse and eventually dry up, once pollen hydration and germination occur (Wetzstein & Sparks, 1989). Without pollination, stigmatic surfaces may remain receptive for a longer period (Wetzstein & Sparks, 1989).

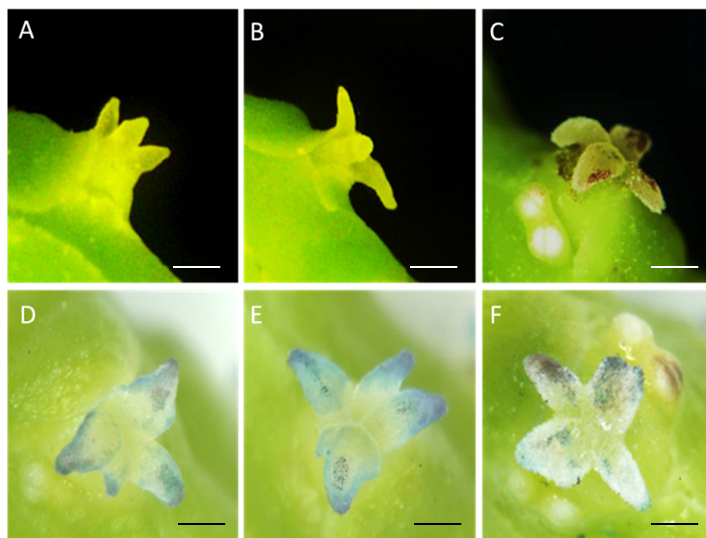


Figure 6. Stages of stigma

A refers to Stage 1, which is the first appearance of stigma. B is Stage 2, where elongation and spreading of stigma occurs. During C (Stage 3), there is complete

emergence and wide spreading of stigma. D, E and F are images of stigma after Peroxtesmo KO treatment. The scale bar for all the images above is 0.5 mm.

Table 1
Receptivity difference at three stages of stigma for 10 cultivars

SA		KCH		SE		SP	
Stage	Mean	Stage	Mean	Stage	Mean	Stage	Mean
1	80.30 ^a	1	64.20 ^a	1	82.60 ^a	1	69.00 ^a
2	97.70 ^b	2	96.50 ^b	2	97.40 ^b	2	96.50 ^b
3	90.10 ^c	3	95.90 ^b	3	96.80 ^b	3	97.30 ^b
S1		NYE		IND		LDL	
Stage	Mean	Stage	Mean	Stage	Mean	Stage	Mean
1	69.00 ^a	1	62.60 ^a	1	63.60 ^a	1	52.60 ^a
2	97.30 ^b	2	97.80 ^b	2	97.70 ^b	2	96.90 ^b
3	97.00 ^b	3	93.80 ^b	3	96.10 ^b	3	95.40 ^b
SAR		YP					
Stage	Mean	Stage	Mean				
1	70.60 ^a	1	74.30 ^a				
2	96.90 ^b	2	96.90 ^b				
3	96.70 ^b	3	95.50 ^b				

Note: SA - Semongok Aman; KCH - Kuching; SE - Semongok Emas; SP - Semongok Perak; S1 - Semongok 1; NYE - Nyerigai; IND - India; LDL - Lampung Daun Lebar; SAR - Sarikei; YP - Yong Petai

Stage 1 is the first appearance of stigmata, while Stage 2 is the elongation and spreading of stigmata and Stage 3 is the complete emergence and wide spreading of stigmata. Means followed by the different superscript letter in the same column are significantly different at $p \leq 0.05$.

CONCLUSION

Floral biology study provides invaluable information to improve the efficiency of artificial pollination in black pepper breeding. Based on the results, it is proposed that pollen collection for all 10 cultivars be done between 4.00 am and 2.00 pm, since their anther dehiscence has been found to occur between 10.00 pm and 12.00 pm. The stigma was proven

more receptive at Stages 2 and 3 for all the cultivars studied, cultivar 'SA' showed that it was most receptive with stigma of Stage 3. The information generated from this study may assist breeders with dispersal of the pollen onto the stigma at the right stage and time to increase pollination efficiency and enhance success in black pepper breeding programmes.

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