Comparative Pollen Viability and Pollen Tube Growth of Two Endemic Philippine *Etlingera* (Zingiberaceae, Alpinioideae)

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**ABSTRACT**

The pollen viability and pollen tube growth of *Etlingera dalican* and *Etlingera philippinensis* (Zingiberaceae) were examined from fresh samples under the light microscope (LM) and scanning electron microscope (SEM). Pollen measurements were 68-75µm for *E. dalican* and 60-65µm for *E. philippinensis*, having a spheroidal shape for hydrated pollen and an irregular shape for dry pollen for both species. *E. dalican* pollen has greenish-yellow color while that of *E. philippinensis* is greenish. Both species have inaperturate pollen but differ in their ornamentation, which is gemmate in *E. dalican* and psilate in *E. philippinensis*. *E. dalican* had 88.56% pollen viability while *E. philippinensis* had only 40.69%. The rate of pollen tube growth was faster in *E. dalican* (17.75 µm per day) than *E. philippinensis* (8.17 µm per day). The possible pollinators observed for the two species were butterflies of the genus *Catopsilia*, ants and flies. Additional information on the inflorescence and flower description of the two species are herein presented.

**KEY WORDS:** *Etlingera dalican*  
*Etlingera philippinensis*  
Philippine Native Gingers  
Pollen germination  
Pollen morphology

**INTRODUCTION**

Zingiberaceae, also called “gingers”, are herbaceous plants that produce essential oils (Larsen et al., 1999; Larsen & Larsen, 2006). These plants are widely used in traditional medicine to relieve colds, inflammations, diarrhea, stomach disorders, fever and cramps. They are also valuable as food flavoring and spice agents, and are generously included in postpartum diets (Larsen et al., 1999; Leong-Skornickova & Gallick, 2010; Boonmee et al., 2011). According to Pelser et al. (2011), the Philippine Zingiberaceae includes 14 genera with 107 species.

*Etlingera*, a large genus of about 100 species which is distributed in the Indo-Pacific region has 9 species in the Philippines (Poulsen, 2006; Pelser et al., 2011) of which two species are considered in this study. *Etlingera dalican* (Elmer) A.D. Poulsen is a Philippine endemic species which was renamed in 2003 of its former name *Amomum dalican* (Elmer) Merr. and known for its local names “dalikan” in Bukidnon and “tagbak” in the Visayas (Acma, 2010). *Etlingera philippinensis* (Ridl.) R.M. Sm. is another Philippine endemic species also locally known as “tagbak” in the Visayas which holds a new record of reported occurrence in the eastern Mindanao corridor (Acma, 2010).

Reconnaissance observations of these two species by the authors indicate the rarity of fruits in *E. philippinensis* compared to *E. dalican* populations. Although many physical, nutritional and biological factors in general could influence fruit setting in plants, the aspect on pollen viability, pollen tube growth and pollen morphology are given specific attention because of their taxonomic and phylogenetic values. Hence, this study presents empirical information on pollen characteristics of two *Etlingera* species that could be scrutinized in future evaluation and taxonomic characterization of these species.

**MATERIALS AND METHODS**

**Collection of Samples.** Fresh pollen samples were collected from matured stamens of 10 plants in the same population during anthesis (flowers fully open) between the months of November 2015 and January 2016 from 5:00 AM to 6:00 AM. Pollen collection was in accordance with the natural flower opening time for each species. Herbarium specimens and spirit collections were deposited at the Botany Section of Central Mindanao University Herbarium (CMUH) with the accession number 00010859 for *E. dalican* and 00010860 for *E. philippinensis*. All pollen people (Acma, 2010). *Etlingera philippinensis* (Ridl.) R.M. Sm. is another Philippine endemic species also locally known as “tagbak” in the Visayas which holds a new record of reported occurrence in the eastern Mindanao corridor (Acma, 2010).

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samples used in the study were freshly collected from plants growing in the living ginger collections at the Mt. Musuan Zoological and Botanical Garden (MMZBG) of Central Mindanao University (CMU) and Acma’s residence at the Market Site of CMU, University Town, Musuan, Bukidnon. The flower samples were placed separately in zip-lock cellophane bags to prevent drying and were transported to the laboratory for further processing within 15-20 min from collection and were maintained at room temperature of 20°C.

Five plants per species were morphologically measured and described. Lengths of the live specimens were measured using a meter stick. Three (3) inflorescences per species were also collected and brought to the laboratory for dissection and measurement of parts.

**Pollen Morphology and Micromorphological Studies.** Fresh pollen samples for light microscopy examination were removed from the anther sacs using forceps, mounted with water in a glass slide, examined under microscope and described as to: a. size b. shape c. color d. aperture and e. ornamentation. Measurable features like the size of the pollen were determined from scanning electron microscope (SEM) photographs. Pollen color was visually classified based on hydrated pollen which were examined under light microscope (LM). Samples were not acetolyzed based on the reports of Liang (1988) and Saensouk et al. (2009) that members of the Zingiberaceae are mostly not resistant to acetolysis due to their very thin exine and thick intine wall. For the other pollen morphological features such as shape, aperture and ornamentation, they were classified following the criteria of Liang (1988), Liang (1990), Theilade et al. (1993), Saensouk et al. (2009), and Chen & Xia (2011).

The pollen samples for SEM examination were sent to Mindanao State University–Iligan Institute of Technology (MSU-IIT), Iligan City. The possibility of dehydration of the pollen samples was considered due to the 5-6 hrs travel between CMU and MSU-IIT. The pollen samples were mounted on the sample holder using carbon tape. Then the mounted pollen were coated with platinum using the JEOL JFC-1600 Autofine Coater to make the surface of samples suitable for SEM characterization. The SEM images of the samples were taken using the JEOL JSM-6510LA Analytical SEM coupled with an energy dispersive x-ray spectrometer at 200x, 500x and 1000x magnifications.

**Testing for Starch and Pollen Viability.** A common method to assess pollen viability is by staining and direct counting (Barrett, 1985; Dudash, 1991; Willis, 1999). Parfitt and Ganeshan (1989) have determined that the pollen stain tests (acetocarmine, Alexander Stain’s, TTC, MTT, and NBT) are not reliable or consistent and are not positively correlated with *in vitro* germination tests (as cited by Bolat and Pirlak, 1999). Hence, this study used IKI (iodine + potassium iodide) solution for the stain test. Fresh pollen were mounted on glass slides and tested with a drop of IKI solution to reveal the presence of starch. Another set of pollen samples for the determination of the pollen viability was prepared separately with pollen added with a drop of IKI solution. These were covered with a cover slip and subjected to light microscopy using low power objective (100x) and counted for the percentage viability. In getting the percentage viability, a modified formula was used as shown below:

\[(\text{Viable pollen / Total # of pollen}) \times 100 = \text{Percentage Viability}\]

**Determination of Pollen Germination**

**Pollen germination assays.** Pollen samples from another set of 5 plants for each species from the same population were collected for germination test. These samples were processed right after the collection. Pollen collection time was limited to 15-20 min to avoid exposure to contaminants. The germination medium consists of 100 g sucrose (C\textsubscript{12}H\textsubscript{22}O\textsubscript{11}), 500 mg calcium nitrate [Ca(NO\textsubscript{3})\textsubscript{2}], 4H\textsubscript{2}O], 120 mg magnesium sulfate (MgSO\textsubscript{4}), 100 mg potassium nitrate (KNO\textsubscript{3}) and 120 mg boric acid (H\textsubscript{3}BO\textsubscript{3}) were dissolved in 1000 ml deionized water following Karni & Aloni (2002). The pH of the germinating medium was 6.37 at 55°C. An estimated 10 ml of the medium was poured/dispeased into three Petri plates for each species in the treatment and allowed to cool for about 15 min to allow the agar to solidify. The plates were covered and incubated and maintained at a room temperature of 20°C. No staining of the pollen was needed (Ozler et al., 2009) and each plate was considered a replicate. The petri plates with the germinating medium were exposed under the ultraviolet light for 15 min. The pollen samples were inoculated aseptically to the petri plates containing the germinating medium. Lids of petri plates with pollen samples were sealed with a tape, labeled accordingly and brought to the growth room with the same maintained temperature.

**A. Evaluation of the onset of pollen germination.** After 24-h of incubation, the pollen were counted for pollen germination. Pollen samples inoculated on the Petri plates were mounted on glass slide and viewed under the light microscope. Those found within the microscopic field (100x magnifications) were considered for scoring. A pollen was classified as germinated if at least the beginning of a developing pollen tube could be seen emerging from one of the pores or reach the pollen diameter (Steiner & Gregorius, 1998; Tosun & Koyuncu, 2007). Percentage pollen germination was calculated following the formula of ISTA (1999) as shown below:

\[(\text{Pollen germinated / Total # of pollen}) \times 100 = \%\text{Germination}\]

**B. Pollen tube lengths.** Pollen were considered...
germinated when its tube length equaled the grain diameter (Luza et al., 1987). The pollen tube lengths were measured within 14 days at 2 days interval after initial plating. Observations were done with an ocular micrometer fitted to the ocular/eyepiece of the microscope. The determination of the percentage pollen tube lengths depended upon the pollen tubes that emerged outside the pollen wall.

RESULTS AND DISCUSSION

Plant Description. *E. dalican* plants reach a height of 2.5–3 m and the inflorescence (with 10-18 flowers) is obconic, measuring 8 x 4 cm with a truncated top when flowers open. Bracts are oblong, tips notched and hairy, pink towards the top and white below. Bracteoles are tubular, tips hairy, pink towards upper portion while white at base. Calyx elongated, fused, tubular, three tipped and pink. Corolla lobes are oblongolate, yellow in color, and corolla tube white in color; stigma reddish color. Anther position is lower than stigma and the pollen are located between corolla and anther sacs (Fig. 1). Populations of *E. dalican* are seen near streams and/or forest habitat where there is no direct sunlight. The flowers of the plant do not open at the same time. *E. philippinensis* on the other hand, reaches a height of 2–2.5 m and the inflorescence (with 5-8 flowers) is cone-shaped, 9 x 2 cm. Bracts are elliptic-lanceolate, reddish but white at base; bracteoles tubular. Calyx elongated, fused, tubular, three tipped, red in color except the basal part which is white. The corolla is oblong, red but base is white. Labellum is very bright red or scarlet with ovate shape. All parts are lighter in color at the bottom and darker towards the tip. Pollen samples are also located in the anther sacs (Fig. 2). Rhizomes are long; creeping along the soil, inflorescences either grow distantly from the main rhizome or are partially buried in the soil. This species is also found in shady places.

*E. philippinensis* have dominantly reddish color in its entire inflorescences. Like in *E. dalican*, individual flowers of *E. philippinensis* do not open at the same time. In the report of Melati (2015) on *Zingiber officinale* Rosc. temperature and relative humidity could influence the time of anthesis and increase in temperature and relative humidity accelerates the opening of flowers. However, as to whether the same factors are behind the anthesis in these two *Etlingera* species needs further investigation. Likewise, Darjanto & Satifah (1990) also stated that changes in these factors change plants’ response to flowering.

Throughout the study, it was observed that the two *Etlingera* species were often frequented by two species of butterflies belonging to genus *Catopsilia*. This observations support the earlier report of Larsen et al. (1999) and Larsen & Larsen (2006) that *Etlingera* species are pollinated by butterflies. *E. dalican* was further observed hosting black-colored ants in its inflorescences (Fig. 3). *E. philippinensis*, on the other hand, harbored fruit flies and red-colored ants. These insects were distributed on the entire plant particularly on the leaves and inflorescences (Fig. 4). The probability that they could be possible pollinators is considered. Pollinators (butterflies and ants) are known to frequently visit plant species due to nectar secretion. The amount of reward offered to a flower visitor also depends on the concentration of sugar in the nectar. However, humidity, temperature and soil moisture strongly influence high rate of nectar secretion (Brown, 1959; Real & Rathcke, 1991; Aswani et al., 2013).

Pollen Morphology. The characters of pollen studied using LM and SEM are summarized in Table 1. The results on the morphological features of the pollen showed resemblances as well as differences between the species in their features. According to McGrath (1999), pollen of many plants can be used to classify the genus and sometimes the species on the basis of such characteristics like the size, shape, aperture,
and ornamentation.

The present study revealed that *E. dalican* has bigger pollen compared to *E. philippinensis* in which pollen size range for *E. dalican* was 68 - 75 µm while for *E. philippinensis* was 60 - 65 µm. *E. dalican* pollen appeared greenish-yellow in color having spheroidal shape (hydrated pollen) while *E. philippinensis* pollen appeared greenish in color having spheroidal shape (hydrated pollen). These findings on the pollen size ranges and shapes agree with the reports of Theilade et al. (1993), Chen & Xia (2011) and Saensouk et al. (2015). On the other hand, the outline of dry pollen in the two species as seen in polar view are irregular specifically its infoldings, and their pollen are inaperturate. The main difference for the two species is in its ornamentation in which *E. dalican* is gemmate while *E. philippinensis* is psilate (Figs. 5-8).

In this observation, it is clear that *E. dalican* have bigger pollen compared to *E. philippinensis*. The inaperturate pollen of the two species supports the findings of Furness & Rudall (1999) regarding widespread occurrence of inaperturate pollen among monocotyledonae. These data closely resembled the inaperturate pollen in *Curcuma kwangsiensis* and *Boesenbergia longiflora* of Chen and Xia (2011) which range from ovoid to spherical and varied from 51.9 ± 7.2µm in *C. kwangsiensis* and 109.4 ± 12.5µm in *B. longiflora*. Furthermore, it was also reported that *B. albomaculata* and *B. longiflora* pollen are spherical, 83.0 ± 7.8µm in diameter and appeared nonaperturate. It was likewise observed that the most inner layer was developed only in certain regions and appeared to have excrescences similar to *B. tiliiflora* (Mangaly and Nayar, 1990) with a circular area around the distal pole where the intine is thinner.

Theilade et al. (1993) studied the *Zingiber* pollen and reported that the species of said genus possess a spherical shape (with a cerebroid or reticulate sculpturing) or ellipsoidal pollen with a diameter ranging 55-85 µm. Pollen were also reported that no structures indicating the presence

<table>
<thead>
<tr>
<th>Species</th>
<th>Pollen size</th>
<th>Pollen shape (hydrated pollen)</th>
<th>Pollen shape (dry pollen) and infolding</th>
<th>Pollen color</th>
<th>Pollen aperture</th>
<th>Pollen ornamentation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. dalican</em></td>
<td>68–75 µm</td>
<td>Spheroidal</td>
<td>Irregular</td>
<td>Greenish–yellow</td>
<td>Inaperturate</td>
<td>Gemmate</td>
</tr>
<tr>
<td><em>E. philippinensis</em></td>
<td>60–65 µm</td>
<td>Spheroidal</td>
<td>Irregular</td>
<td>Greenish</td>
<td>Inaperturate</td>
<td>Psilate</td>
</tr>
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</table>
Saensouk et al. (2015) on the other hand reported that the majority of the genus *Curcuma* has large sized pollen in the range of 50.5–86.9 µm. Chen (1989) further reported that the exine sculpturing of the pollen of *Curcuma* are psilate which is similar to *E. dalican* and *E. philippinensis*.

It appears that the ranges of our pollen size fall between the pollen sizes of related genera viz., *Boesenbergia*, *Cornukaempferia*, *Curcuma* and *Zingiber* (e.g. Liang, 1988; Liang, 1990; Mangaly and Nayar, 1990; Theilade et al., 1993; Theilade and Theildae, 1996; Saensouk et al., 2009, Chen and Xia, 2011; Saensouk et al., 2015). These reports on pollen morphology are significant, since pollen function as agents of reproduction and their features can often be used to identify a particular taxon (Uno et al., 2001).

**Pollen Viability.** Previous studies on pollen samples indicate that pollen of *E. dalican* turn dark brown in the iodine test, while *E. philippinensis* pollen turn yellowish as indications of the presence of starch following Simpson (2006). Firmage & Dafni (2001) also reported that if the pollen has starch it means that they are viable. Using the test, the current study shows that the percentage viability of *E. dalican* pollen (88.56%) is higher than that of *E. philippinensis* (40.69%).

There are a number of nongenetic causes of pollen inviability including pollen age and physical factors such as temperature and humidity (Kelly et al., 2002). Viability also mainly depends on relative atmospheric humidity at shedding and during pollen transport (Frankel & Galun, 1977; Pacini, 1990; Paoletti, 1992). It has been considered that pollen remain viable longer at low relative humidity levels (Stanley & Linskens, 1974). However, recent studies
Table 2. Two (2) weeks observation for Pollen Tube Growth of *E. dalican* and *E. philippinensis* under the light microscope (400x).

<table>
<thead>
<tr>
<th>Species</th>
<th>2nd day</th>
<th>4th day</th>
<th>6th day</th>
<th>8th day</th>
<th>10th day</th>
<th>12th day</th>
<th>14th day</th>
<th>Average microns (per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. dalican</em></td>
<td>3.6–4.4 µm</td>
<td>7.8–9.2 µm</td>
<td>13.6–21.6 µm</td>
<td>28.6–33.3 µm</td>
<td>43.4–49.6 µm</td>
<td>63.4–66.9 µm</td>
<td>72.3–79.4 µm</td>
<td>17.75 µm</td>
</tr>
<tr>
<td><em>E. philippinensis</em></td>
<td>0.8–2.4 µm</td>
<td>2.4–4.4 µm</td>
<td>4.8–6 µm</td>
<td>13.6–15.3 µm</td>
<td>22.9–24.1 µm</td>
<td>28.8–31.4 µm</td>
<td>33.6–38.3 µm</td>
<td>8.17 µm</td>
</tr>
</tbody>
</table>

pointed out that pollen of different species need a high level of relative humidity to germinate as well (Corbet & Plumridge, 1985; Digonnet-Kerhoas & Gay, 1990; Mulugeta et al., 1994; Loupassaki & Vasilakakis, 1995). The link between pollen viability and fruit and seed set has been earlier evidenced (Stone et al., 1995). As to whether this connectivity is applied to the two Etlingera species studied still requires further investigation.

**Pollen Germination and Pollen Tube Growth.** Culture medium containing a gelling agent (agar or gelatin) has been used successfully in many species (Jayaprakash & Sarla, 2001; Kakani et al., 2002; Kozai et al., 2008). In this study, agar was used instead of gelatin due to the report of Stanley & Lisksens (1974) that there are several advantages of using agar germination tests such as the ease of taking carbohydrate, creating stable relative humidity and providing aerobic conditions. The germination medium used for this study followed the formulation of Karmi & Aloni (2002). This is in accordance with the suggested reports of Khan and Perveen (2006) and Imani et al. (2011) that culture medium aside from having carbohydrates (particularly sucrose) should also contain germination-stimulating substances such as boric acid, calcium nitrate, potassium nitrate and magnesium sulfate wherein all aforesaid substances are the major components.

*E. dalican* and *E. philippinensis* as observed in this study have similar percentage germination of 0% after 1 day of incubation. However, for pollen tube growth, the observation after 2 days of incubation indicates that the first pollen tube length in *E. dalican* measures 3.6–4.4 µm on the (2nd day observation) and 72.3–79.4 µm in the 14th day observation. On the other hand, pollen tube length in *E. philippinensis* on the 2nd day measures 0.8–2.4 µm and 33.6–38.3 µm in the 14th day observation. As shown in Table 2, average increase in pollen tube length of *E. dalican* is higher compared to that of *E. philippinensis*.

Germination has already been used to indicate viability of pollen (Schoenike & Stewart, 1963) and a linear relationship is seen between pollen viability and germination capability in many fruit species (Stanley & Linskens, 1974). Moreover, Einhardt et al. (2006) concluded that if pollen seems non-viable, and if vigorous pollen tubes are present, it is still good enough to ensure at least a moderate efficient fruit set, despite the low germination rate. Pollen germination and tube growth thus emerge as highly dynamic and coordinated processes, integrating many different signals from the local environment to regulate growth and development (Rodriguez-Enriquez et al., 2013). The present data on pollen viability and pollen tube growth in *E. dalican* and *E. philippinensis* collected in Central Mindanao University appear to provide additional support to these earlier observations.

**Conclusions and Recommendations**

The distinguishing features of pollen in *E. dalican* and *E. philippinensis* are presented. The pollen of *E. dalican* are distinguished from that of *E. philippinensis* by their sizes and exine ornamentation in which *E. dalican* measured 68-75 µm and has gemmate ornamentation while *E. philippinensis* measured 60-65 µm and has psilate ornamentation. Furthermore, *E. dalican* pollen viability and pollen tube growth is higher than that of *E. philippinensis*.

In view of our results, we recommend further studies on pollen of other ginger species to be examined/characterized using SEM in order to delineate species. Implications on the different stages of germination should also be noted and described. Moreover, these species should be germinated/propagated using other germination media and recording the tube growth as well. More research using molecular and population genetic studies are needed to investigate the relationships of the genus *Etlingera*. Further studies to establish relationship between pollen viability and germination with fruit setting in the two species must be done.

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