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Pollen Morphology and Pollen Elemental Composition of Selected Philippine Native Gingers in Tribe Alpinieae (Alpinioideae: Zingiberaceae)

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ABSTRACT: The pollen morphology and pollen elemental composition of the selected Philippine native gingers in tribe Alpinieae (Alpinioideae: Zingiberaceae) *viz.*, *Amomum muricarpum* Elm., *Etlingera dalican* (Elmer) A.D.Poulsen, *E. philippinensis* (Ridl.) R.M.Sm. and *Hornstedtia conoidea* Ridl. are not completely determined as well as their impacts in the pollen germination and pollen tube growth. In this study, the analyses were performed by light microscopy (LM), scanning electron microscopy (SEM) and energy dispersive x-ray (EDX) spectrometry to better understand their pollen surfaces and pollen elemental composition. Data revealed that the pollen sizes of *A. muricarpum* measured 45-80µm, *E. dalican* measured 65-75µm, *E. philippinensis* measured 60-65µm while *H. conoidea* measured 50-90µm. The four native species possess spheroidal shape and inaperturate pollen. However, pollen color of *A. muricarpum* and *H. conoidea* were yellowish-brown, while green to greenish-yellow for *E. dalican* and greenish for *E. philippinensis*. Ornamentation or exine sculpture of *A. muricarpum* is echinate, *E. dalican* is gemmate while *E. philippinensis* and *H. conoidea* is psilate. A greater proportion of potassium (K^+) and sulfur (S^{2-}) were observed in the pollen of the four native gingers amongst other detected elements by EDX. Hence, studies on pollen characterization are important to perceive and reveal their morphological features, elemental composition and are useful for future studies on *in vitro* germination of the selected species.

Keywords: *Amomum muricarpum, Etlingera dalican, Etlingera philippinensis, Hornstedtia conoidea,* Philippine endemic

INTRODUCTION

Pollen morphological characteristics are utilized for taxonomic classification purposes (Erdtman and Roger, 2007). Further, its taxonomic significance has long been recognized and used to assess evolutionary relationships (Welsh *et al.*, 2009). Pollen are also useful in studies of plant taxonomy because many pollen traits are influenced by the strong selective forces evolved in various reproductive processes including pollination, dispersal and germination (Oswald *et al.*, 2011; Ghosh and Karmakar, 2012). In plant systematics, pollen are especially used to determine the pollen size, pollen shape, pollen type, structure of the pollen wall, pollen architecture, number of aperture, aperture position and aperture shape (Amlin, 2013).

Zingiberaceae is among the poorly collected groups and its members are said to be poorly taxonomically known with its comprehensive monograph written only in 1904 (Campbell and Hammond, 1989; Acma, 2014). This family comprises perennial herbs which are mostly creeping horizontal or tuberous rhizomes with about 47 genera and 1,000 species (Carr and Carr, 2004). Zingiberaceae with its subfamily Alpinioideae have 16 genera including the three genera in tribe Alpinieae *viz.*, *Amomum* Roxb., *Etlingera* Giseke and *Hornstedtia* Retz.

Amomum are generally evergreen herbs inhabiting wet forests, especially in light gaps and at forest margins (Sakai and Nagamasu, 1998). *Amomum muricarpum* Elm. is a Philippine native ginger which is locally known as “tugis” and are distributed in South East of China to Indo-China and in the Philippines particularly in Mindanao (Govaerts, 2005).

On the other hand, *Etlingera* is a large genus of about 100 species distributed in the Indo-Pacific region representing 10 species in the Philippines including *Etlingera elatior* (Jack) R.M.Sm. which is known only from cultivation (Poulsen, 2006; Pelser *et al.*, 2011 onwards). Two of which are *E. dalican* (Elmer) A.D. Poulsen which is known for its local names "dalikan" in Bukidnon and "tagbak" in Bisaya and *E. philippinensis* (Ridl.) R.M. Sm. which is also locally known as "tagbak" in Bisaya (Elmer, 1915; Acma, 2010). Meanwhile, to the best of the authors' knowledge, this present paper is the first report on the palynology of the genus *Hornstedtia*. *H. conoidea* Ridl. is known as "panaon", "pinoon" and "panon" in Bisaya, "tagbak" in Mandaya and "puso-puso" in Tagalog (Barbosa *et al.*, 2016). *H. conoidea* was first collected in the Philippines at Cuernos Mountains, Dumaguete, Negros Oriental by Ridley on 1909. Generally, seeds from the ripe fruits of *A. muricarpum* and *H. conoidea* are considered edible and eaten by the local people in the province of Bukidnon and claimed to cure stomach disorders (Acma, 2010).

Studies on Zingiberaceae in the Philippines are few, outdated and wanting. So far, only the laboratory studies of Acma (2010) on flavonoid analysis of the four species, Barbosa *et al.* (2016) on antioxidant activities and phytochemical screening of *A. muricarpum, E. philippinensis* and *H. conoidea* and Mendez *et al.* (2017) on comparative pollen viability and pollen tube growth of *E. dalican* and *E. philippinensis* are the available literature for these studied species. This present paper reports additional information on the pollen morphological characterization and elemental differences in the composition of the selected Philippine native gingers by light microscopy (LM), scanning electron microscopy (SEM) and energy dispersive x-ray (EDX) spectrometry that could serve as baseline data for future evaluation and taxonomic characterization of these species. The four species were selected based on the phenology and availability of the pollen samples.

MATERIALS AND METHODS

A. Inflorescence, Flower and Pollen Collection

Three inflorescences per species were collected and brought to the laboratory for dissection and measurement of parts. On the other hand, fresh pollen samples were collected from matured stamens of 10 plants in the same population during anthesis (flowers fully open). The collection was in accordance with the natural flower opening time for each species. Flowers and fruits were preserved in 70% ethanol for the spirit/pickled collection, while dried vegetative parts and inflorescences were pressed for herbarium specimens. The spirit/picked and herbarium collections were deposited at the Botany Section of Central Mindanao University Herbarium (CMUH).

All pollen samples used in the study were fresh from plants growing in the living ginger collections at the Mt. Musuan Zoological and Botanical Garden (MMZBG) and Acma's residence at the Market Site of Central Mindanao University, University Town, Musuan, Bukidnon, Philippines on November 2016 to March 2017. Both populations were found in shady places. 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 minutes from collection and were maintained at a room temperature of 20°C.

B. Plant Measurement and Description

Five plants per species were morphologically measured and described. Lengths of the live specimens were measured using a meter stick. Quantitative data were obtained namely a. plant height b. length and width of leaf c. length of ligule d. length and diameter of petiole e. distances of the node and f. length and diameter of rhizome.

C. Pollen Morphology and Micromorphological Studies

For light microscopy examinations, fresh pollen samples 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. Pollen color was classified based on hydrated pollen samples which were examined under LM. For the other pollen morphological features such as shape, aperture and ornamentation, they were classified following the criteria of Theilade *et al.* (1993), Saensouk *et al*. (2009) and Chen & Xia (2011).

D. SEM and EDX Analysis

The pollen samples for SEM examination were processed in Mindanao State University–Iligan Institute of Technology (MSU-IIT), Iligan City, Philippines. The pollen samples were mounted on the sample holder using carbon tape. Then, the mounted samples 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 EDX analysis were performed for determination of morphology and elemental composition of the four Philippine native gingers.

Three images of each sample were taken at a magnification of 200X, 500X and 1,000X. SEM EDX was used since it is a non-destructive analytical method for high resolution surface imaging and quantitative identification of elements present in the samples (Singh *et al.,* 2014).

RESULTS AND DISCUSSION

A. Plant Description

A. muricarpum plants reach a height of 1-1.5 m, long. Leaves found at upper 2/3 portion, 1/3 lower portion has reduced leaves. Leaves are lanceolate, generally 23-28 × 6-7 cm, base rounded, apex acuminate, margins entire. Petiole subsessile. Ligules wavy to truncate, 1.5 to 2.0 mm and smooth. Inflorescence obconic, 7–6 × 3.5–4 cm. Bracts, membranous, triangular and slightly pubescent, pinkish. Bracteoles tubular, apex irregularly toothed, pinkish measuring 6–7 mm long. Calyx elongated, fused, tubular, pinkish in color but tips are light green and hairy. Dorsal lobe oblong while lateral lobe narrow oblong, yellow with pink lines measuring 20 × 8 mm for dorsal lobe and 20 × 5 mm for lateral lobes. Labellum obovate, about 3 × 2 cm, white at sides, yellow with red marks at center. Fruits rambutan-like, with spines, fruits reddish purplish when mature (Fig. 1A).

E. dalican plants reach a height of 2.5–3 m tall. Inflorescence is obconic, measuring 8 × 4 cm, 20-26 flowers in one inflorescence, 7-10 flowers opened at the same time during its anthesis or when an inflorescence is in full bloom forming a truncate top. Bracts oblong, tips notched and hairy, pink towards top and white below, larger ones measuring 4 × 1 cm.

Fig. 1. Floral dissection of *A. muricarpum* (A), *E. dalican* (B), *E. philippinensis* (C) and *H. conoidea* (D). Inflorescence (inf), flower (fl), bracts (b), bracteoles (bt), calyx (cx), corolla lobes (cl), labellum (lm), stigma (sg), style (s), epigynous gland (eg), anther sac (as).

Bracteoles tubular, tips hairy, pink towards upper portion while white at base, 3 cm long. Calyx elongated, fused, tubular, 3-tipped, pink, measuring 3.5 × 0.3 mm. Corolla lobes oblanceolate, measuring 3 × 1 cm, yellow in color. Corolla tube white in color (Mendez *et al.*, 2017) (Fig. 1B).

E. philippinensis plants reach a height of 2–2.5 m tall. Inflorescence is cone-shaped, measuring 9 × 2 cm, 5-7 flowers in one inflorescence with 1-2 flowers opened at the same time during its anthesis. Bracts elliptic-lanceolate, reddish but white at base, 3.5 × 2 cm. Bracteoles tubular, about 3.5 cm long. Calyx elongated, fused, tubular, 3-tipped, red in color except basal part which is white, 5 cm long. Corolla oblong, red but base is white about 3 × 0.4 cm. Labellum very bright red or scarlet, ovate, 2.5 × 1 cm (Mendez *et al.*, 2017) (Fig. 1C).

H. conoidea plants reach a height of 3 m tall. Rhizome green to brownish. Leaves broad lanceolate, coriaceous, base cuneate or obtuse. Petiole sessile. Ligule oblong, pubescent. Inflorescence spindle-shaped, 9 × 3 cm. Involucre present. Bracts triangular, 6 × 2 cm, red except at base which is whitish. Bracteole tubular, membranous, pink at upper part white at base, 3 cm long. Calyx elongated, membranous and light pink 40 × 5cm. Corolla oblong, red, 1.5 × 0.5cm. Labellum oblong, without staminodes, red, 1.5 × 0.8cm. The spindle–shaped inflorescence, presence of an involucre, and absence of staminode qualifies this species to belong to the genus *Hornstedtia* (Fig. 1D).

B. Pollen Morphology

Pollen of *A. muricarpum* measured 45-80 µm which appeared yellowish-brown in color having spheroidal shape, inaperturate with echinate ornamentation (Fig. 2A). *E. dalican* pollen measured 65-75 µm which appeared greenish-yellow in color having spheroidal shape, inaperturate with gemmate ornamentation (Fig. 2B). *E. philippinensis* pollen measured 60-65 µm which appeared greenish in color having spheroidal shape, inaperturate and with psilate ornamentation (Fig. 2C). *H. coniodea* pollen measured 50-90µm which appeared yellowish brown in color having spheroidal shape, inaperturate and with psilate ornamentation (Fig. 2D).

These findings on the pollen size, shape, aperture and ornamentation of *A. muricarpum* coincided and agreed to the report of Mangaly and Nayar (1990) on the studied pollen of *A. hypoleucum* Thwaites and *A. pterocarpum* Thwaites which are sub-spheroidal to ovoid to spherical in shape with diameter 30-90 µm and 35-75 µm, respectively. Additionally, it also supported Kaewsri and Paisooksantivatana (2007) which reported and concluded that Thai *Amomum* are spherical to subspherical in shape, 30-70 µm in diameter with the intine layer 1-7 µm thick, inaperturate and the exine ornamentation is either echinate or psilate.

On the other hand, the work of Mendez *et al.* (2017) was the first report and documentation on the pollen morphology of the genus *Etlingera* with tests on its viability and germination and measurement of tube growth. There is scanty of literature in the palynology of genus *Etlingera*. It was reported that the pollen sizes of *E. dalican* (65-75µm) are bigger compared to that of *E. philippinensis* (60-65µm). Reported herein also are the observations that those populations of *H. conoidea* found in the provinces of Mindanao Island *viz.*, Bukidnon, Davao Del Norte and Surigao del Sur often bore fruits. *A. muricarpum* was also found to bear fruits "especially those populations found in the city of Malaybalay and municipality of Kibawe in Bukidnon province; however, it mainly depends on its fruiting season. For the period of 2010 to 2017, the authors observed the *ex situ* conservation of *E. dalican* in Bukidnon which always bore fruits and the *E. philippinensis* bore fruits only once. This study is the first report regarding the fruiting of *E. philippinensis* which was failed to observe by Elmer (1915). The fruiting of *E. philippinensis* is hard to observe in both wild and cultivated populations, since the flowers last 1-2 days only and a new flower will emerge. Then, a week after the first flowering, the inflorescence become dried. Thus, it was observed that for the duration of the study, the species with bigger pollen – *H. conoidea* often bore fruits, followed by *A. muricarpum* and *E. dalican* which bears fruit every year. While *E. philippinensis* rarely bore fruits.

The inaperturate pollen of the four examined species also supported the findings of Furness & Rudall (1999) regarding the widespread occurrence of inaperturate pollen among monocotyledonae.

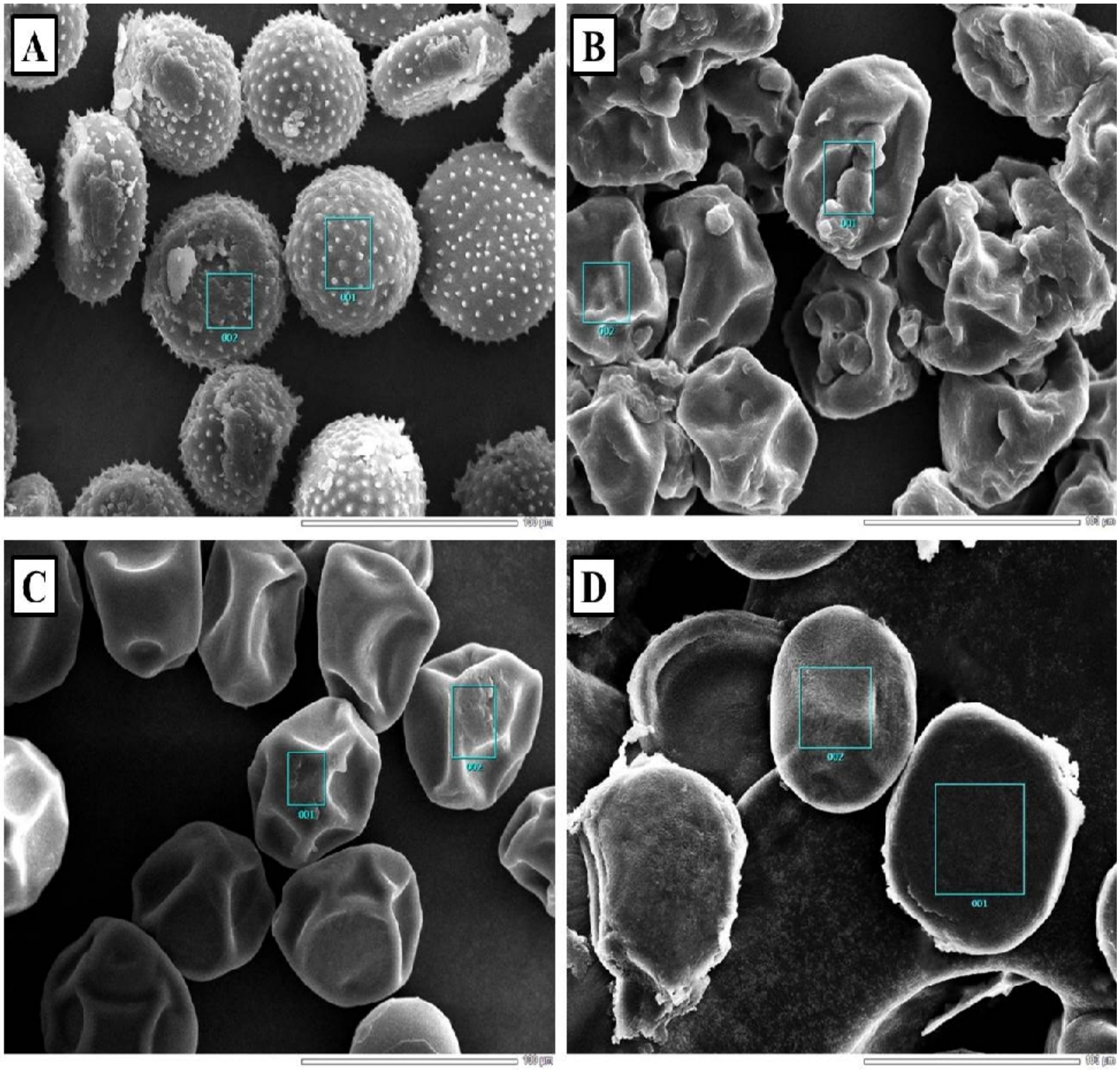

Fig. 2. SEM images of pollen in *A. muricarpum* (A), *E. dalican* (B), *E. philippinensis* (C) and *H. conoidea* (D) under SEM (500x).

These data agrees with report of Chen and Xia (2011) which stated that *Curcuma kwangsiensis* S.G.Lee & C.F.Liang and *Boesenbergia longiflora* (Wall.) Kuntze possess inaperturate pollen which are ovoid to spherical and varied from 51.9 ± 7.2µm in *C. kwangsiensis* and 109.4 ± 12.5µm in *B. longiflora*. Theilade *et al.* (1993) studied the *Zingiber* pollen and reported that the species of said genus possess spherical shape (with a cerebroid or reticulate sculpturing) or ellipsoidal pollen with diameter ranging 55-85 µm. Saensouk *et al*. (2015) also reported that the majority of the genus *Curcuma* have large sized pollen in the range of 50.5–86.9 µm and Chen (1989) further reported that the exine sculpturing of the pollen of *Curcuma* are psilate which is similar to our examined pollen of *E. dalican, E. philippinensis* and *H. conoidea.*

Generally, it appears that the ranges of the pollen sizes of the studied species fall between the pollen sizes of related genera under Zingiberaceae *viz.*, *Boesenbergia*, *Cornukaempferia*, *Curcuma* and *Zingiber* (e.g. Liang, 1988; Liang, 1990; Mangaly and Nayar, 1990; Theilade *et al*., 1993; Theilade and Theildae, 1996; Kaewsri and Paisooksantivatana, 2007; Saensouk *et al.*, 2009; Chen and Xia, 2011; Saensouk *et al.*, 2015). Although Kaewsri and Paisooksantivatana (2007) reported that pollen morphology in *Amomum* is less useful for subgeneric classification, this study will still provide information which will be useful in future related researches in species delineation of ginger species, since pollen features can often be used to identify a particular taxon (Uno *et al.*, 2001).

C. Elemental Composition

The elements detected in the four native ginger species were potassium (K^{+}), sulfur (S^{2-}), aluminum (Al^{3+}) and magnesium (Mg^{2+}). The predominant minerals in the pollen samples of *A. muricarpum* were K^{+} (2.07%), followed by S^{2-} (0.32%), Mg^{2+} (0.11%) and Al^{3+} (0.11%) (Fig. 3A). *E. dalican* were S^{2-} (0.35%), followed by Al^{3+} (0.09%), K^{+} (0.8) and Mg^{2+} (0.07) (Fig. 3B).

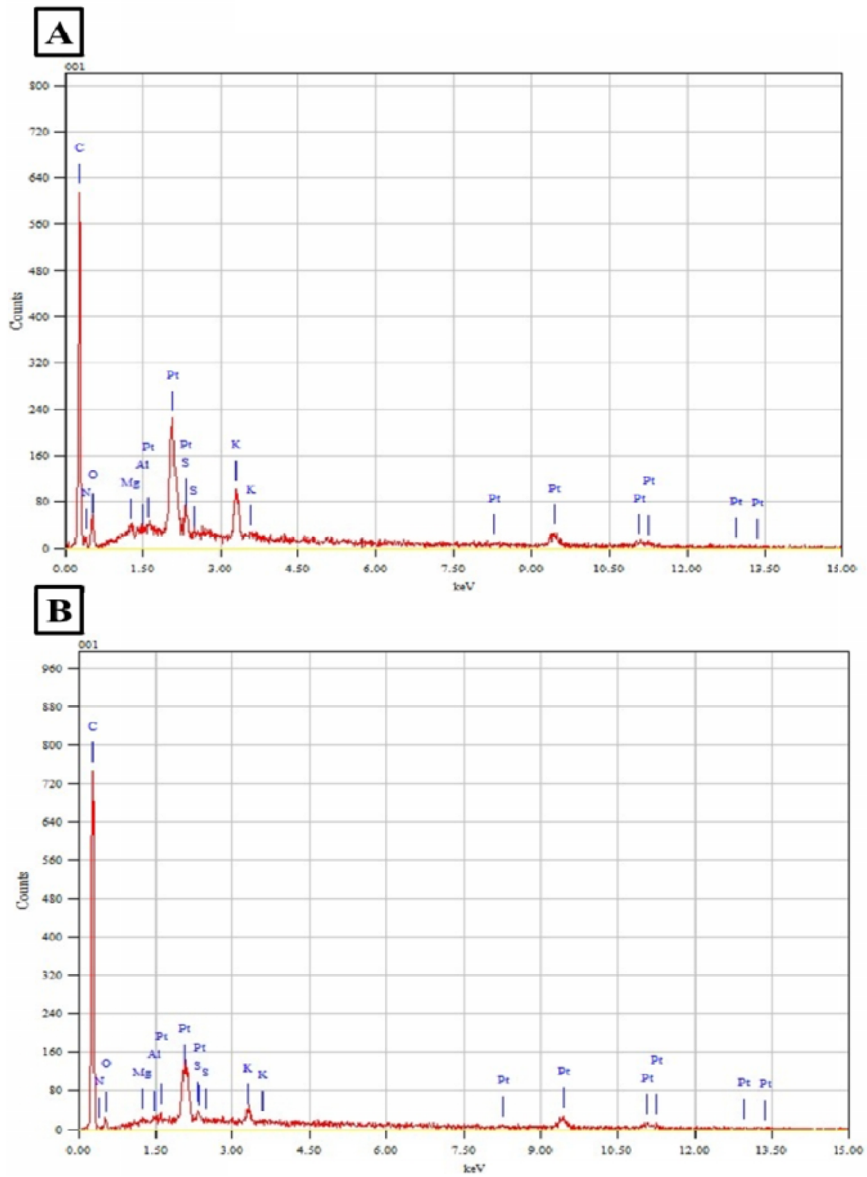

Fig. 3. EDX analysis showing the elemental composition of the areas (blue boxes) shown in Figure 2. A) *A. muricarpum*, (B) *E. dalican*.

Similar to that of *A. muricarpum*, *E. philippinensis* were K^{+} (2.02), followed by S^{2-} (0.76%), Mg^{2+} (0.25%) and Al^{3+} (0.03) (Fig. 3C). *H. conoidea* were K^{+} (6.13%), followed by S^{2-} (1.61%), Al^{3+} (0.19%) and Mg^{2+} (0.08) (Fig. 3D). These findings supported the study of Carpes *et al*. (2009) which have predominant materials of K^{+}, Calcium (Ca^{2+}) and Mg^{2+}.

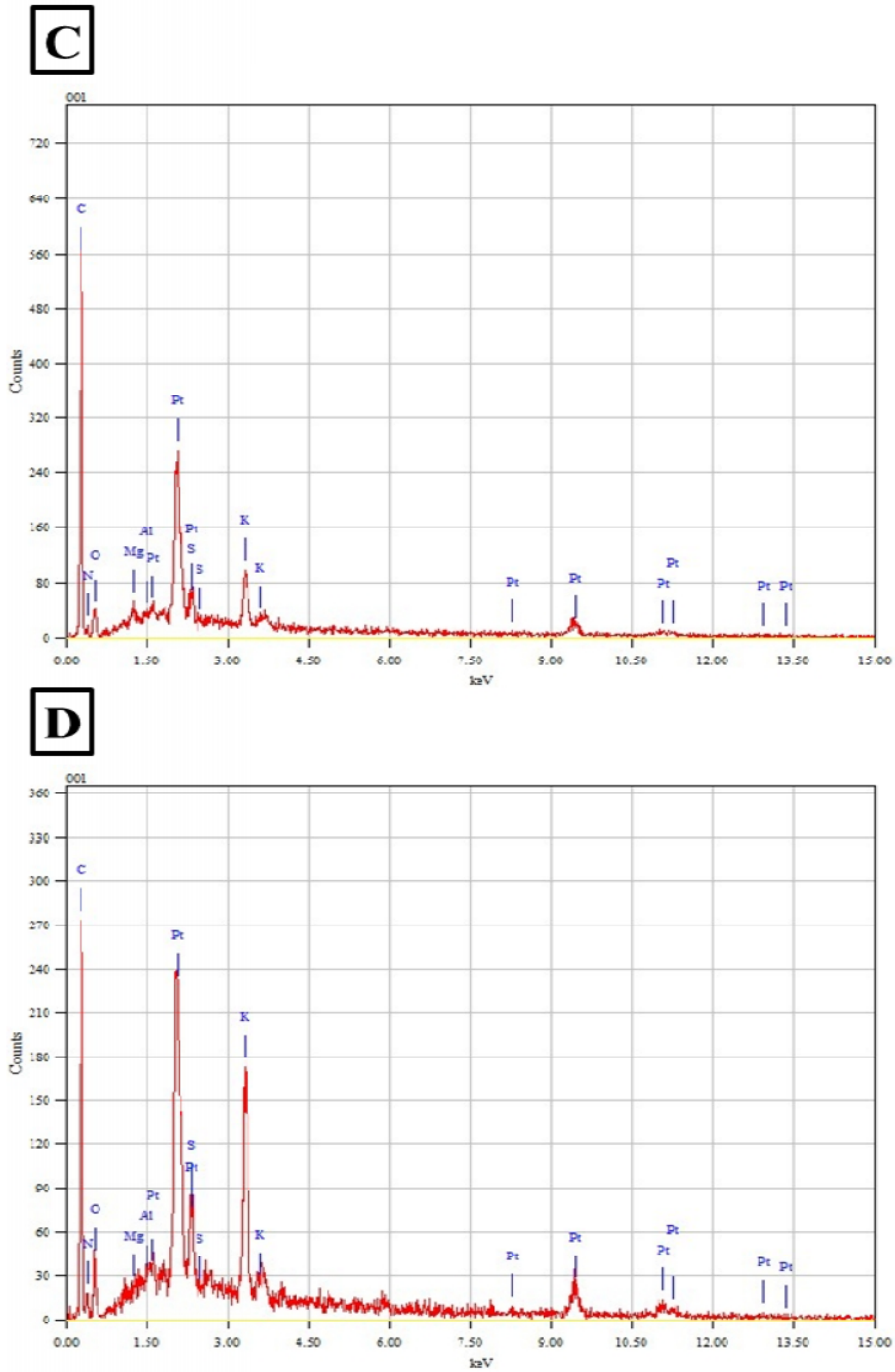

Fig. 3. EDX analysis showing the elemental composition of the areas (blue boxes) shown in Figure 2. (C) *E. philippinensis* and (D) *H. conoidea*.

Additionally, Nilsson and Berggren (1991) on the analysis of a larger pollen surface area showed that the dominant elements were silicon (Si^{4+}), phosphorous (P^{3}), S^{2-} and K^{+}. The EDX analysis in this study also revealed the presence of carbon (C^{4-}), oxygen (O^{2-}) and nitrogen (N^{3-}) in the pollen of the four species which are the principal elements of proteins. These elements are essential to the pollen germination and tube growth. Germination and tube growth of pollen are sensitive to Al^{3+} (Konish *et al.*, 1988; Searcy and Mulcahy, 1990; Sawidis and Reiss, 1995). One likely cause of inhibition of pollen tube growth by Al^{3+} is disturbance of the tip-to-base cytoplasmic Ca^{2+} gradient by blocking plasma membrane Ca^{2+}-permeable channels (cf. Piñeros and Tester, 1995, 1997; Rengel *et al.*, 1995). In other species, Al^{3+} inhibits pollen tube growth in tomato (Searcy and Mulcahy, 1990), lily (Sawidis and Reiss, 1995) and tea (Konish *et al.*, 1988). Furthermore, K^{+} together with Ca^{2+} are interdependent with each other because the inward K^{+} channels are greatly regulated by Ca^{2+} as in case of *Arabidopsis* pollen (Fan *et al.*, 2001) as well as stomatal guard cells (Schroeder and Hagiwara, 1989; Blatt *et al.*, 1990; Fairley-Grenof and Assmann 1992a, b; Lemtri-Chlieh and MacRobbie, 1994; Kelly *et al.*, 1995; Grabov and Blatt, 1997). According to Fan *et al*. (2001), external supply of K^{+} ion enhanced the rate of pollen germination as well as pollen tube growth in *Arabidopsis*. Moore and Jung (1974) pointed out that Mg^{2+} enhanced the tube growth in the case of *in vitro* pollen germination of sugarcane. Prajapati and Jain (2010) indicated that Mg^{2+} is significant in pollen tube growth of *Luffa aegyptiaca* Mill. The detected elements in our pollen samples may also play key roles in the germination and tube growth of the four native species.

CONCLUSIONS AND RECOMMENDATIONS

This study revealed that among the examined pollen, *H. conoidea* showed to have the biggest pollen, followed by *A. muricarpum, E. dalican* and *E. philippinensis*, respectively. The selected Philippine native gingers possess spheroidal shape pollen and are inaperturate. However, pollen color of *A. muricarpum* and *H. conoidea* appeared yellowish-brown, green to greenish-yellow for *E. dalican* and greenish for *E. philippinensis*. Ornamentation or exine sculpture of *A. muricarpum* is echinate, *E. dalican* is gemmate while *E. philippinensis* and *H. conoidea* is psilate. A greater proportion of K^{+} and S^{2-} were observed in the pollen of the four native gingers amongst other detected elements by EDX which can enhance the rate of germination and tube growth of pollen.

It is recommended, however, that further studies be conducted on other species of Zingiberaceae specifically in subfamily Alpinioideae. There is also a need to test the germination rate and measure the tube growth of the pollen of four species to better understand the roles of each detected element. With the utilization of the SEM, the inner structure of the exine and the intine of the Zingiberaceae pollen should also be done so as to acquire more information for future taxonomic evaluation and *in vitro* propagation.

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