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Characterization of Pollen Grain and Pollen Extract from Common Allergenic Plants in the Philippines

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Allergic rhinitis and asthma are prevalent in tropical countries like the Philippines. Pollen grains from anemophilous plant species may trigger these respiratory allergies. Pollen extracts from common allergenic plants are currently produced locally in the Philippines and are utilized for the diagnosis and immunotherapy of patients with respiratory allergies. Further improvement of their quality is necessary to avoid false negative or false positive diagnoses, as well as to increase the effectiveness of immunotherapy and avoid adverse reactions. This study aimed to characterize the morphology of the collected pollen grains and assess the pollen purity, protein concentration, and electrophoretic profile of the allergenic pollen extracts. Pollen identification and percent purity of the pollen collections were assessed *via* microscopy and hemocytometer. Lowry assay was performed to identify the total protein concentration of the pollen extracts before subjecting them to SDS-PAGE and silver staining. The pollen samples from varying plant families have distinct features that will aid in their proper identification. Collected aggregates of pollen samples with pollen purity of $<$ 90%, like *T. procumbens*, *A. saman*, *L. leucocephala*, and *M. indica*, manifest darker colors compared to their actual color. On the other hand, the color of collected pollen samples with pollen purity $\geq$ 90% are commonly yellow or off-white, which is their natural color. The total protein concentration of the crude pollen extracts ranges from 2.87–9.63 mg/mL. The pollen extracts revealed protein bands at molecular weights ranging from 10–250 kD on SDS-PAGE. Proper characterization of a particular pollen may help in assessing the purity of the collected pollen. Higher pollen purity may contribute to the assurance that the positive diagnosis was due to the target pollen of a plant and not due to contaminations from other components. The result of this study may be considered for the improvement of the crude pollen allergen extract production for clinical use. It is also recommended to assess the effect of the environmental condition where the pollen was collected in terms of the actual proteins present after allergen extraction.

Keywords: allergen, pollen extract

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INTRODUCTION

Tropical countries like the Philippines are inhabited by a wide variety of plant species producing pollen. An aeropalynological survey conducted by Sabit *et al.* (2016) showed that Urticaceae, Cannabaceae, Poaceae, and Moraceae were the most prevalent among the 18 pollen types, and 87% of the total airborne pollen was obtained during the dry season (November–May). In a review of aeroallergen skin tests done between 2006–2011 among patients at the allergy clinic of the University of the Philippines–Philippine General Hospital (UP-PGH) Out-Patient Department, the predominant outdoor allergenic pollen are Johnson grass (*Sorghum halepense*), pigweed (*Amaranthus spinosus*), and mango tree pollen (*Mangifera indica*) (Santos-Estrella *et al.* 2013).

Airborne pollen grains are known to induce type I hypersensitivity or an immediate reaction involving the release of immunoglobulin E (IgE) antibodies. Pollen grains contain proteins and enzymes described as allergens to which the immune system reacts. In allergic patients, the immune system recognizes a pollen allergen as foreign to the body, causing the T cells to secrete Th2-cytokines that enhance IgE production by plasma cells. These IgE antibodies then bind to the high-affinity receptors on the surface of mast cells and basophils. Subsequent exposure to the pollen allergen will now induce an IgE-mediated allergic reaction through mast cell degranulation. In an allergy skin test, a small amount of the pollen allergen is introduced into the skin of the patient, thereby causing mast cell degranulation that is manifested as a wheal and flare reaction 15–20 min after the skin test. On the other hand, specific allergen immunotherapy is the only disease-modifying treatment for respiratory allergies. Increasing amounts of the allergen to which the patient is allergic is gradually introduced to the patient. Allergen immunotherapy results in decreased basophil sensitivity and eosinophil infiltration (Drazdauskaitė *et al.* 2021).

Currently, local pollen grains are processed to produce crude pollen extracts used for allergy skin prick tests and for subcutaneous specific allergen immunotherapy of patients with respiratory allergies. However, these extracts are not fully characterized yet. The collection of pollen grains from certain plants with high purity and distinct protein bands remains to be challenging. Characterization of extracts is needed since the amount of the allergenic molecule varies depending on the source materials, as well as the production processes; and for the allergen immunotherapy to be effective, the extract should contain a specific amount of the allergen component (Heinzerling *et al.* 2013). Crude allergen extracts contain numerous undefined components aside from the allergens with varying potencies and ratios. Thus, the data on the presence and quantity of allergenic molecules in each extract can be used as a basis for the quality check of manufactured extracts used in allergy skin prick tests and immunotherapy.

This study aimed to characterize the morphology of the collected pollen grains utilized for the diagnosis of patients with respiratory allergies in the Philippines, to assess the purity of the collected pollen samples, and to identify the protein concentration and electrophoretic profile of the allergenic pollen extracts. The results of the study may help in further standardization and quality improvement of the allergenic pollen extracts produced in the country for clinical purposes such as skin tests and immunotherapy.

MATERIALS AND METHODS

Pollen Collection

Mature flowers of *Albizia saman* ("acacia"), *Amaranthus spinosus* (pigweed), *Cocos nucifera* (coconut), *Imperata cylindrica* ("cogon"), *Leucaena leucocephala* ("ipil-ipil"), *Mangifera indica* (mango), *Mimosa pudica* ("makahiya"), *Oryza sativa* (rice), *Pennisetum polystachion* (foxtail grass), *Sorghum halepense* (Johnson grass), and *Tridax procumbens* (wild daisy) were collected from various locations such as Bulacan, Cavite, Laguna, Manila, Nueva Vizcaya, and Zamboanga del Sur during their respective anthesis stage or the period during which the flower is fully open. The collection was done around 09:00 AM–12:00 PM during sunny days from September–May from the years 2020–2022. Pollen-containing anthers, except for *M. indica*, were separated from the flowers through gentle brushing directly into the test sieve. Pollen collections were passed through test sieves according to the mesh size of 25, 38, 45, or 150 μm depending on the pollen size. *C. nucifera* pollen grains were provided by the Philippine Coconut Authority Zamboanga Research Center. As for *M. indica*, whole anthers with the presence of pollen attached to them were plucked using tweezers. Pollen grains were stored at –20 °C prior to use. Pollen grain samples and their purity were visualized and measured using the OPTIKA Microscope and PROview Software under LPO (40X) and HPO (400X). Ten milligrams (10 mg) of pollen were randomly picked from a thoroughly mixed collection of each pollen sample, and 990 μL of distilled water was added to make a pollen mixture. This mixture was processed within 1 h to avoid pollen bursting. Pollen and debris or contaminants present in a sample were counted in a hemocytometer using two quadrants opposite each other. Pollen and debris or contaminants overlapping within the top and left side of each square from the quadrants are counted, whereas those overlapping the bottom and right side of each square are not considered. Viewing and counting were done under HPO (400X)

magnification thrice per sample (n = 3) for a total of six quadrants (two quadrants per trial). The percent purity of samples was calculated by performing the formula:

$$\% \text{ purity} = \frac{\text{Total no. of intact target pollen sample}}{\text{Total no. of target pollen and impurities}} \times 100$$

The final percent purity of each pollen sample was calculated using the average purity of all three trials.

Extraction of Pollen Allergen

Pollen samples were defatted first with a 1:5 (w/v) ratio of pollen (g) to diethyl ether (mL) at room temperature thrice for 50 min each with shaking at 155 rpm. The pollen grains were made to settle down for 10 min before removing the excess diethyl ether and air-dried for 24 h.

Allergens from dried defatted pollen were extracted using a 1:10 (w/v) ratio of pollen (g) to 1X phosphate-buffered saline (PBS, pH 7.3) (mL). For those pollen extracts with degraded protein profiles (*A. saman* and *M. indica*), even when the collected pollen grains were stored at –20 °C before extraction, an additional Promega G6521 Protease Inhibitor was included in 1X PBS (0.3% final concentration). This is to prevent the protease degradation of the protein and improve its electrophoretic profile. The pollen solutions were agitated at 160 rpm overnight under 4 °C. Samples were centrifuged afterward for 30 min at 12,000 rpm under 4 °C. The solutions were then differentially filtered using 0.80, 0.45, and 0.20 µm Whatman syringe filters accordingly. Extracted pollen solutions were then stored at –20 °C for future use.

Protein Content Determination

Lowry assay was performed to determine the protein content of each pollen extract. Pollen samples were diluted to a 1:10 (v/v) ratio of pollen samples with 1X PBS. One hundred microliter (100 µL) of the standard or sample solution was mixed with 25 µL of 5X copper-tartrate solution in the microplate wells and incubated for 10 min at room temperature in the dark. After incubation, 10 µL of 1:1 ratio of Folin-Ciocalteu and 0.1M NaOH was mixed in. Further incubation was done for 20 min at room temperature in the dark. The absorbance value was measured at 630 nm using SPECTROstar Nano Microplate Reader.

Physicochemical Characterization of Pollen Extract Proteins

Protein profiles of the pollen extracts were carried out using 10–15% gradient SDS-PAGE gel using Major Science Mini SDS-PAGE Apparatus. The gel was subjected to Pierce™ Silver Stain kit based on the manufacturer's recommendation to visualize the protein band. A Vivantis PR0623 Whole Blue Range Prestained Protein Ladder was used to assess the molecular weight of the protein bands.

Statistical Analysis

The normality of data gathered from the study was analyzed using the Shapiro-Wilk test. The non-parametric Kruskal-Wallis test for independent samples and its *post hoc* Dunn's test were performed to analyze if there were significant differences between the pollen purity of the collected pollen samples and between protein concentrations of the pollen extracts. All statistical analyses were performed through IBM SPSS version 22, and the alpha values were set to $\alpha = 0.05$.

RESULTS

Pollen Characterization

From Table 1, it was evident that pollen samples from varying plant families (Amaranthaceae, Anarcardiaceae, Arecaceae, Asteraceae, and Fabaceae) manifest distinct features that will help in their proper identification. However, pollen samples coming from grasses (Poaceae family) have very similar characteristics, including their morphology (monad, spheroidal, with single pore surrounded by annulus) and color (yellow) with little differences in terms of their sizes, as shown in Table 1.

Pollen collections from plant samples with sticky pollen that adheres to the anthers like *T. procumbens*, *A. saman*, *L. leucocephala*, and *M. indica* pollen were < 90% pure. It is challenging to completely separate the pollen from its anther and other structures of the flower or inflorescence through sieving, thus affecting the purity of the pollen samples. Dried-up anthers and other floral parts turned black or brown, resulting in a darker color of the actual pollen collection than the expected off-white color, as listed in Table 1.

Protein Concentration of Pollen Extracts

The total protein concentration of the crude pollen extracts ranged from 2.87–9.63 mg/mL, with the lowest concentration coming from *M. pudica* and the highest concentration from *A. spinosus* (Figure 1). These protein concentrations represent both the allergenic and non-allergenic components of the pollen extracts.

Protein Profile of the Crude Pollen Extracts

The pollen extracts resolved from the gradient SDS-PAGE revealed protein bands at molecular weights ranging from 10–250 kD (Figure 2).

Fifteen (15) distinct protein bands were observed from *A. spinosus*. Nine distinct protein bands were observed from

Table 1. Comparative characteristics of 11 pollen collections as observed from actual samples.

Pollen Sample	Family	Pollen Sample under Microscope	Pollen Size (µm) Mean ± SE	Actual Collected Pollen Sample	Pollen Purity (%)	Color of Pollen Aggregate		Pollen Morphology in Accordance with Literature	Collection Area
						Before Collection	After Collection		
A. spinosus	Amaranthaceae		29.17 ± 2.64		96.06[a,d]	Yellow	Yellow	Monad Spheroidal Poly-pantoporate (Siriwattanakul *et al.* 2015; Talebi *et al.* 2016)	Bulacan, Nueva Vizcaya
M. indica	Anarcardiaceae		21.25 ± 0.49		69.07[b]	Off-white	Brown	Colporate Prolate Striato-reticulated (Shubharani *et al.* 2013)	Manila, Bulacan
C. nucifera	Arecacea/Palmae		41.67 ± 4.99		90.86[a,c,d]	Pale yellow	Pale yellow	Monad Monocolpate (Rimna *et al.* 2017)	Zamboanga del Sur
T. procumbens	Asteraceae		23.75 ± 0.56		73.45[b,c]	Off-white	Brown	Monad Spheroidal Surface surrounded by spines (Salamah *et al.* 2019)	Pangasinan
A. saman	Fabaceae		122.31± 3.92 x 149.22± 8.52		80.05[b,c,d]	Orange	Brown	Triporate Oblate Polyad (Ebigwai and Egbe 2017)	Bulacan
L. leucocephala	Fabaceae		48.27 ± 2.73		66.79[b,c]	Off-white	Brown	Prolate Tricolporate Non-angular (Aftab and Perveen 2006)	Pangasinan
M. pudica	Fabaceae		9.58 ± 0.72		94.85[a,d]	Off-white	Off-white	Spheroidal Tetrad (Ibrahim *et al.* 2012)	Nueva Vizcaya
I. cylindrica	Poaceae		29.58 ± 1.00		91.23[a,b,c,d]	Yellow	Yellow	Monad Spheroidal With single pore surrounded by annulus (Mander *et al.* 2013)	Bulacan, Las Piñas
O. sativa	Poaceae		34.17 ± 3.40		93.19[a,c,d]	Yellow	Yellow	Monad Spheroidal With single pore surrounded by annulus (Mander *et al.* 2013)	Bulacan

Table 1. Cont.

Pollen Sample	Family	Pollen Sample under Microscope	Pollen Size (μm) Mean ± SE	Actual Collected Pollen Sample	Pollen Purity (%)	Color of Pollen Aggregate		Pollen Morphology in Accordance with Literature	Collection Area
						Before Collection	After Collection		
P. polystachion	Poaceae		40.33 ± 1.67		83.33[a,c,d]	Yellow	Yellow	Monad Spheroidal With single pore surrounded by annulus (Mander *et al.* 2013)	Bulacan, Laguna
S. halepense	Poaceae		41.22 ± 2.41		90.01[a,b,c,d]	Yellow	Yellow	Monad Spheroidal With single pore surrounded by annulus (Mander *et al.* 2013)	Bulacan, Laguna

*Scale bar = 20μm
**Same letters indicate no significant differences (α =0.05)

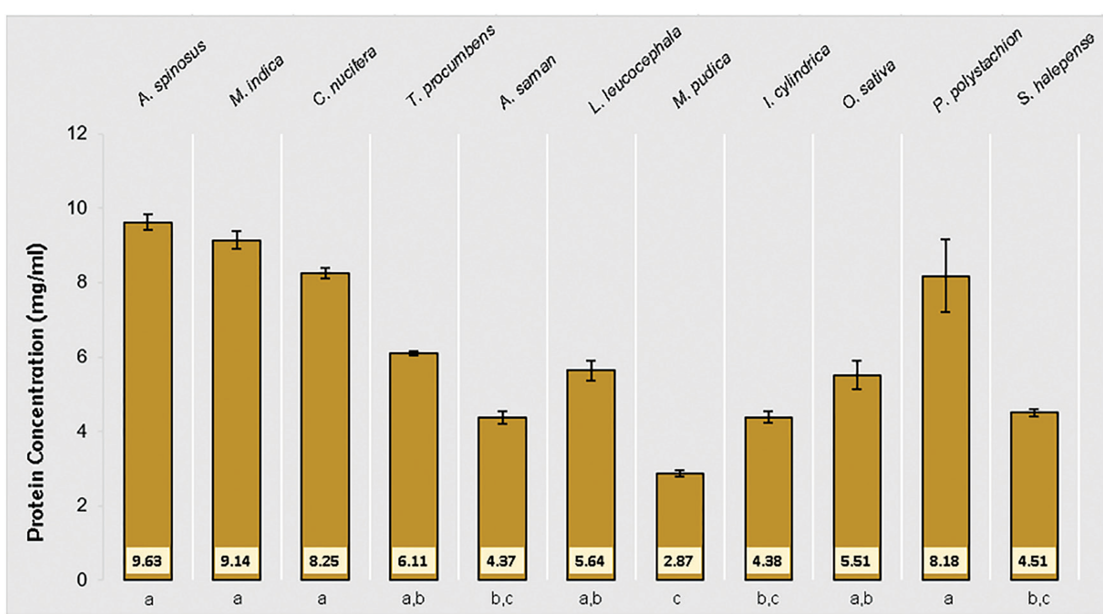

Figure 1. Determined total protein concentration of pollen extracts using Lowry assay.

M. pudica, whereas seven distinct protein bands were observed from *T. procumbens*. The pollen extracts from these plants showed electrophoretic profiles different from each other with unique bands at ~ 17 kD (*M. pudica*), 20 kD (*A. spinosus*), and > 100 kD (*T. procumbens*).

At least 12 distinct protein bands were observed for the grass pollen extracts. Notably, *I. cylindrica*, *O. sativa*, *P. polystachion*, and *S. halepense* showed very distinct bands at ~ 17, ~ 27, ~ 30, and ~ 60 kD.

Different electrophoretic profiles were also observed among the trees, with *A. saman* showing the greatest number of protein bands at a molecular weight range of 10–72 kD. *C. nucifera* showed 13 distinct protein bands, whereas there were six distinct protein bands for *M. indica*.

As seen in Figure 2, crude pollen extracts from *L.*

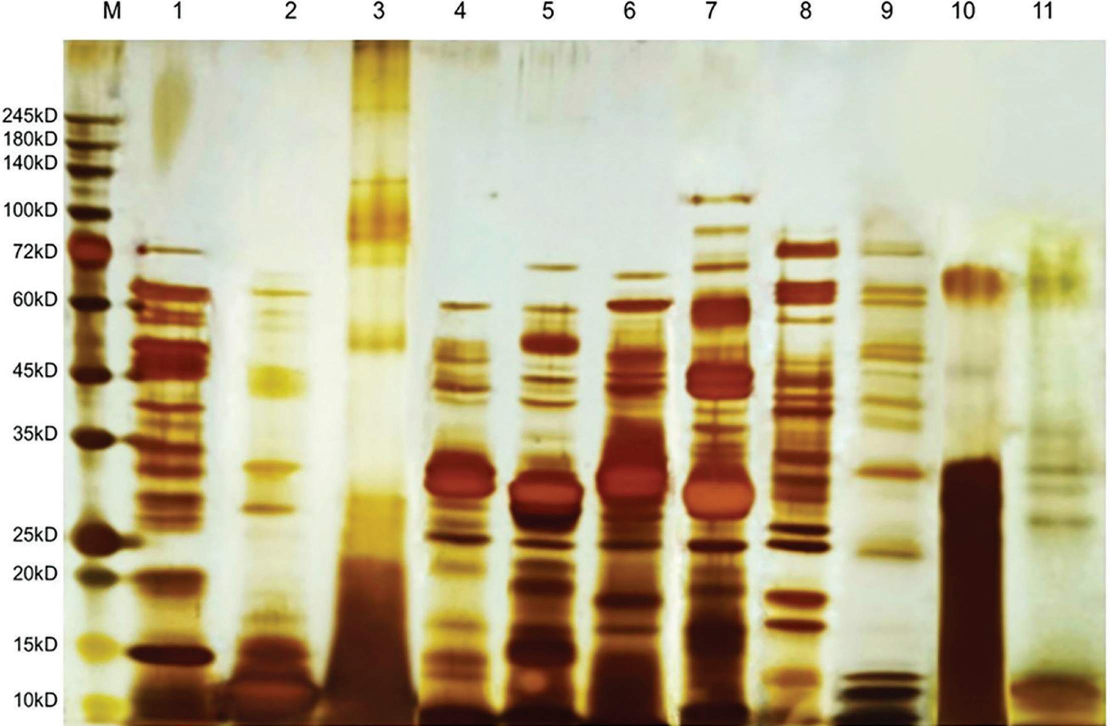

Figure 2. Electropherogram profile of pollen allergen extracts from silver stained gradient SDS-PAGE gel. Lanes: [M] protein marker, [1] *A. spinosus*, [2] *M. pudica*, [3] *T. procumbens*, [4] *I. cylindrica*, [5] *O. sativa*, [6] *P. polystachion*, [7] *S. halepense*, [8] *A. saman*, [9] *C. nucifera*, [10] *L. leucocephala*, and [11] *M. indica*.

leucocephala, *M. indica*, and *T. procumbens* had fewer protein bands as compared with the other pollen extracts. Collected pollen samples from these three had the lowest purity among all the pollen samples from the study. Their pollen purity ranged from 66.79, 69.07, and 73.45%, respectively (Table 1). However, despite *M. pudica* having 94.85% pure pollen collection, its crude pollen extract still displayed few protein bands. *M. pudica* pollen purity was detected to be significantly different ($p < 0.05$) from the purity of *L. leucocephala*, *M. indica*, and *T. procumbens* pollen samples. On the other hand, crude pollen extracts from the remaining seven pollen samples (*A. spinosus*, *I. cylindrica*, *O. sativa*, *P. polystachion*, *S. halepense*, *A. saman*, and *C. nucifera*) displayed more protein bands (Figure 2) than *M. pudica* despite having no significant differences ($p > 0.05$) between their pollen purity (Table 1).

Moreover, among those pollen extracts with fewer protein bands (Figure 2), *M. pudica* had the lowest protein concentration (2.87 µg/mL) and showed significant differences ($p < 0.05$) against the protein concentrations of *L. leucocephala* (5.64 µg/mL), *M. indica* (9.14 µg/mL), and *T. procumbens* (6.11 µg/mL). Also, *M. pudica* still manifested the lowest protein concentration among the seven remaining pollen extracts (Figure 1) with multiple distinct protein bands (Figure 2). Among these seven, the protein concentrations of *I. cylindrica*, *S. halepense*, and *A. saman* were not significantly different ($p > 0.05$) from the protein concentration of *M. pudica.*

DISCUSSION

Knowledge regarding the actual morphology of specific pollen grains from certain plants will help in properly identifying the target allergenic pollen to be extracted. It is essential that these allergenic extracts are of high purity to ensure their quality if they will be utilized for clinical purposes. In this case, there is an assurance that the positive allergenicity results were due to the target pollen of a plant and not because of contaminations from other components. This helps the physicians to provide accurate diagnoses and administer effective immunotherapy to their patients.

There were studies that suggest the inclusion of multiple grass pollen in an extract for diagnosis and immunotherapy, especially if they are from the same subfamily (Chabre *et al*. 2009; Gangl *et al*. 2013). This can be done since most grasses (Poaceae family) usually manifest similar allergens because of their taxonomic and biochemical relationships. Thus, grass allergens were grouped according to their shared amino acid sequences and the reason why most of them are cross-reactive

(Garcia-Mozo 2017; WHO/IUIS Allergen Nomenclature Database). For instance, *I. cylindrica*, *P. polystachion*, and *S. halepense* all belong to the subfamily Panicoideae, whereas *O. sativa* belongs to the subfamily Oryzoideae. However, *O. sativa* was likely to be of high purity, especially if the pollen grains were gathered from a rice field away from the pavements prone to contamination from dust and other plant species.

Powdery pollen from Amaranthaceae, Arecaceae, and Poaceae family are anemophilous or wind-pollinated, and their presence in the air usually triggers symptoms of allergy (Songnuan 2013; Siriwattanakul *et al.* 2015; Talebi *et al*. 2016). On the other hand, sticky pollen grains from the Asteraceae, Fabaceae, and Anarcardiaceae families are entomophilous or insect-pollinated (Gangl *et al.* 2013). Entomophilous pollen can still be considered aeroallergens if they are producing sufficient pollen to make them airborne (amphiphilous) and cause sensitization but not as threatening as the anemophilous pollen (Vieira *et al*. 1998; Weber 2014).

The protein concentrations of the pollen extracts from this study were different from the results of Cabauatan and Ramos (2012), wherein grass pollen extracts have protein concentrations ranging from 11–22 mg/mL, as well as with the study of Castor *et al.* (2016) with protein yield of 0.28–0.97 mg/mL. Both studies used the Bradford assay, whereas the study of Baer *et al.* (1986) used the Lowry assay on pollen extract of Bermuda grass, which yielded around 2.8–4.3 mg/mL. Gavrović *et al.* (1997) utilized the Lowry assay to determine the protein content of the pollen extract from Timothy grass (*Phleum pratense*), which yielded a protein content of 1.04–3.30 mg/mL. These results from Baer *et al.* (1986) and Gavrović *et al.* (1997) are closer to the results obtained by this study, which also used the Lowry assay.

Although it was not included in this study, there were researchers that addressed the significant effect of pollen exposure to air pollution, air humidity, and atmospheric precipitation on the protein concentrations of the pollen (Shahali *et al*. 2009; Sénéchal *et al*. 2015; Ščevková *et al*. 2020). This depends on the species and the thickness of their exines which is the outer layer of the pollen. Some pollen exines may be very fragile when exposed to pollutants, humidity, and precipitation. This causes the pollen to burst and release their protein contents in the air such as allergens and other components involved in viability and germination (Shahali *et al*. 2009; Sénéchal *et al*. 2015). It was also observed that pollen enclosed inside the anther, like anemophilous pollen, was less susceptible to pollution and other atmospheric conditions (Sénéchal *et al*. 2015). Thus, pollen protein concentration does not usually have a significant correlation with protein or allergen concentration (Sénéchal *et al*. 2015; Ščevková *et al*. 2020).

The low protein concentration of *M. pudica* pollen extract, despite high pollen purity, (> 90%) could be attributed to its pollen being entomophilous. Their pollen adheres outside the surface of the anther and is more susceptible to the above-mentioned meteorological factors. This is in contrast to *A. spinosus* and other grass pollen collections, which were highly pure but anemophilous in nature, making them protected inside their anther. Crude pollen extracts from less pure, entomophilous pollen collections such as *T. procumbens*, *A. saman*, *L. leucocephala*, and *M. indica* could have higher protein concentrations than *M. pudica* due to the presence of non-pollen proteins from the contaminants.

A local study from Cabauatan and Ramos (2012) showed degraded protein profiles from the pollen extracts of *O. sativa* and *I. cylindrica* compared to the results of this study. Cabauatan and Ramos (2012) did not utilize protease inhibitor during pollen allergen extraction, and the purity of the pollen collections was not disclosed. On the other hand, Castor *et al.* (2016) mentioned the use of a 0.3% protease inhibitor for extracting *I. cylindrica* pollen allergen, but no pollen purity was disclosed. The resulting protein profile was better than that of Cabauatan and Ramos (2012) but with little smearing; their protein bands were similar to this study (~ 15, ~ 25, ~ 30, ~ 35, ~ 45, ~ 55, and ~ 60 kD).

Air pollutants could be another factor that may also influence the protein profile of the entomophilous pollen (*M. pudica*, *T. procumbens*, *A. saman*, *L. leucocephala*, and *M. indica*). As plants respond to these stressors, they tend to produce more stress-induced proteins, and such proteins are usually not expressed by plants in a less polluted area (Sénéchal *et al*. 2015). There were reports that certain allergens emerged, whereas the others disappeared when exposed to pollution. In a study by Shahali *et al.* (2009), a significant decrease in the expression of the *Cupressus arizonica* major pollen allergen Cup a 1, a 45-kD pectate lyase protein, was observed when collected in a polluted area. Meanwhile, a 35-kD thaumatin-like protein Cup a 3 allergen was expressed in higher concentration in the pollen if the plants were exposed to pollution (Cortegano *et al*. 2004; Suárez-Cervera *et al*. 2008). For *Cupressus sempervirens*, a 34-kD Cup s 3 allergen thaumatin-like protein was not detected from pollen grains collected in a less polluted site (Shahali *et al*. 2012).

CONCLUSION

Recognizing the morphological features of the collected pollen is a step necessary to gather pollen grains with high purity and effectively use them in the production of good quality extracts for clinical purposes. This will ensure that

no other components from contaminants are included in the extracts themselves. The results of this study showed that pollen collections with ≥ 80% purity have distinct resolved protein bands rather than in the form of a smear. It does not necessarily mean that impurities were the source of additional protein bands in a sample. Moreover, the use of a protease inhibitor may also improve the quality of the electrophoretic profile of the pollen extracts. It is also recommended to assess the effect of the environmental condition where the pollen was collected in terms of the actual proteins present after allergen extraction. The results of this study may be considered in further studies regarding local pollen allergens in the Philippines and in the improvement of the crude pollen allergen extract production for clinical use.

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