PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 10(3): 203–210
https://doi.org/10.14719/pst.2257

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RESEARCH ARTICLE

Evaluation of grass pollen quality control for allergy test kit production using pollen morphological characters

Pakawat Jarupund[1], Chomdao Sinthuvanich [2], Prayoon Jantrakulroj [3] & Chalermpol Suwanphakdee[1*]

[1]Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[2]Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand
[3]Asoke Skin Hospital, Bangkok 10310, Thailand

***Email:** fscicps@ku.ac.th

OPEN ACCESS

ARTICLE HISTORY

Received: 30 November 2022
Accepted: 21 April 2023

Available online
Version 1.0 : 10 June 2023
Version 2.0 : 01 July 2023

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS,UGC Care etc. See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Jarupund P, Sinthuvanich C, Jantrakulroj P, Suwanphakdee C. Evaluation of grass pollen quality control for allergy test kit production using pollen morphological characters. Plant Science Today. 2023;10(3): 203–210. https://doi.org/10.14719/pst.2257

Abstract

Grass pollen is one of the leading causes of allergies in throughout the world. The symptom level depends on the personal immunity. Pollen allergy test kits are suitable for detecting allergic symptoms, which they are produced from pure pollen of specific species. The production of pollen allergy testing kits requires accurate botanical standards and identification for the process of quality control of raw materials basing on Thai Food and drug ministration (Thai FDA). To produce allergy test kit, we focused on the pollen of these 6 common grass species and also use pollen morphological characters for detection and identification. Therefore, this study focused on the method for qualification control and standardization for prototype product of grass pollen allergy testing kit. Six common grass species as *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L. Jacobs, *Oryza sativa* L., *Sorghum bicolor* (L.) Moench, *Urochloa eminii* (Mez) Davidse, *Urochloa mutica* (Forssk.) T.Q.Nguyen and *Zea mays* L. The criteria were: 1) common in Thailand and Southeast Asia; 2) high density in the atmosphere; and 3) severity of the allergic reaction. The modified acetolysis were applied for samples preparation. The pollen morphological (polar and equatorial axes, exine, porus, annulus and ornamentation) were observed and measurement under light and scanning electron microscope. The character matrix was analyzed based on a regression tree and Tukey's HSD test to assess support for the different pollen morphological characters. The distinguished character is ornamentation. The results were successfully described the pollen species and showed that the pollen morphologies were useful for contaminated detection. In addition, this method was suitable applied for qualification control of the raw materials for grass pollen allergy testing kits production.

Keywords

allergy testing kits, aeroallergens, palynology, regression tree

Introduction

The family Poaceae (Gramineae) is the fourth-largest flowering plant family, with over 12000 species in 800 genera and 12 subfamilies (Anomochlooideae, Aristidoideae, Arundinoideae, Bambusoideae, Chloridoideae, Danthonioideae, Micraioideae, Oryzoideae, Panicoideae, Pharoideae, Puelioideae and Pooideae), globally (1). Grasses are wind-pollinated (anemophilous), and most species have exserted stamens. The pollen is small, lightweight, and released in large quantities into the atmosphere. The

distance over which the pollen is distributed depends on the height at which the pollen is released, the weight and shape of the pollen, and the environmental conditions (2). In the Poaceae, about 4 times more pollen is released into the atmosphere than for other flowering plants. Consequently, there is a high density of grass pollen in the atmosphere, making it a major contributor to allergies and asthma in many countries (3).

Pollen allergy is common and associated with the pollen spreading in atmosphere. Pollen allergies are related to climatic factors, including wind, season and pollen density in atmosphere. These factors affect the flowering and release of pollen into the atmosphere. Allergens are substances that can trigger an immune reaction through IgE-mediated reactions, with most allergens being glycoproteins, weighing more than 10 kDa (4). Allergies are a major public health problem in America, Europe and Australia. In the United States, 20% of the population was found to have a pollen allergy and up to 15% of people was sensitive to grass pollen (5). In Australia, the prevalence of pollen allergy tends to increase every year. It has an allergy rate of 10% in adults and 30% in the child population (6). In Europe, grass pollen is still the leading cause of allergies. The countries where the grass is the main factor causing allergic reactions are Greece, Hungary, England, Germany, Denmark, Italy, France, and Turkey respectively. In Germany, the prevalence of allergies is 33.6% (7). In Poland, sensitization rates in adults are 10% (8). In Asian countries, 12% of the population in India is sensitive to grass pollen. As well as Malaysia and Taiwan, approximately 20% of the people are reported to be exposed to grass pollen (9). The grass family is distributed throughout the world, though different species have different distributions. Therefore, the grass allergens differ depending on the species of grass that are distributed in each area. Some grass species are a common source of allergens in Europe, with imported test kits often being the most common in Europe or America. Timothy grass is often used as a substitute for allergy testing, even though its distribution does not include in Thailand. Thailand, the frequency of allergy among Thai patients aged 10-59 years to Johnson grass was 21%, Bermuda grass was 17% and weed was 16%. The prevalence of pollen allergy was 32.6% in children 6-7 years, 43.4% in children 13-14 years, and 26% in adults (10). Reports confirmed that imported allergen test kits do not cover many types of allergens, with negative results often being produced even though the patients have allergy symptoms. Therefore, diagnosis and treatment have often been unsuccessful Thus, the allergy testing kits are not match for Thai and also Asian peoples. Therefore, imported allergy test kits are unsuitable for allergy testing in Thailand.

The pollen morphology of each species is different. Therefore, these properties can be useful to classify the species based on the occurrence, shape, aperture, ornamentation and exine thickeness (11). The pollen of the grasses is classified as stenopollinic pollen and is remarkably similar in morphology throughout the family (12-14). They are prolate to spheroidal, and monoporate surrounded by an annulus (15). Pollen grains in nature have robust annuli belonging to the cultivated species (16). The stenopollinic types taxa are classified based mainly on pollen morphological characters. Many studies have tried to use more detailed morphological characteristics based on both qualitative and quantitative factors. Although the pollen morphology of plants of this family has been widely studied but pollen morphological characters for species identification supporting is poorly reported (14, 17).

There is limited information on the allergens from pollen of the grass family in Asia (4, 5). Allergy testing kits from grass pollen species require to set the contaminated detection using pollen morphologies. To date, there has no the standard criteria of raw material quality for the production of test kits. Since grasses pollen are very similar, it is not possible to identified by pollen morphological character. Consequently, statistical analysis has been applied to determine the pollen morphological differentiation for the standard setting of raw materials. The current research aimed to establish standard criteria for raw materials using pollen morphological characters.

Materials and Methods

Specimen collection

Pollen of 6 grass species were collected: *Megathyrsus maximus* (Jacq.) B.K.Simon & S.W.L Jacobs. (green panic grass), *Oryza sativa* L. (rice), *Sorghum bicolor* (L.) Moench (sorghum), *Urochloa eminii* (Mez) Davidse (ruzi grass), *Urochloa mutica* (Forssk.) T.Q.Nguyen (para grass) and *Zea mays* L. (corn). The selected samples met the following criteria: the pollen types occur at a high density in the atmosphere, the pollen types are common in Thailand and the pollen types have been reported to cause allergic reactions.

Three pollen grass species (*M. maximus*, *S. bicolor* and *Z. mays*) were collected from the National Research Centre of Millet and Corn (Suwan farm), Pak Chong district, Nakhon Ratchasima province, Thailand. Two pollen grass species (*O. sativa* and *U. mutica*) were collected from the Nong Ya Sai district, Suphan Buri province, Thailand. *U. eminii* was collected from the Nakhon Ratchasima Animal Nutrition Research and Development Center, Pak Chong district, Nakhon Ratchasima province, Thailand (Table 1).

Pollen purification

The pollen was collected from fields and plantations. The samples were dried in a hot-air oven at 60 ˚C for 2 weeks. Next, the pollen grains were separated from the anthers using a blender. After that, a plankton net and filter papers were used to separate the pure pollen from anthers. Each type of pollen was at least 99% pure. The sample was stored at room temperature.

Pollen morphologies

Pollen grains of 6 grasses species were prepared using modified acetolysis techniques (18). Some samples were prepared for permanent slide and study under light microscope (LM) and also some were investigated under a

Table 1. Scientific name and common name of grasses pollen species.

Scientific name	Common name	Location
Oryza sativa L.	rice	Nong Ya Sai district, Suphan Buri province, Thailand
Zea mays L.	corn, maize	National Research Centre of Millet and Corn (Suwan farm), Pak Chong district, Nakhon Ratchasima province, Thailand.
Sorghum bicolor (L.) Moench	sorghum	National Research Centre of Millet and Corn (Suwan farm), Pak Chong district, Nakhon Ratchasima
Urochloa mutica (Forssk.) T.Q.Nguyen	para grass	Nong Ya Sai district, Suphan Buri province, Thailand
Urochloa eminii (Mez) Davidse	ruzi grass	Nakhon Ratchasima Animal Nutrition Research and Development Center, Pak Chong district, Nakhon Ratchasima province, Thailand
Megathyrsus maximus (Jacq.) B.K.Simon & S.W.L Jacobs.	green panic grass	National Research Centre of Millet and Corn (Suwan farm), Pak Chong district, Nakhon Ratchasima province, Thailand.

scanning electron microscope (SEM). The protocol is as follows: the pollen grains are separated from the anther. Put the pollen grains into 10% Potassium Hydroxide (KOH) and boiled for 2 mins. Pollen is transferred to a centrifuge tube and washed thoroughly with distilled water. The pollen is immersed in glacial acetic acid for a few minutes and the superfluous is decanted after centrifuged for 1 min at 3000 rpm. Then, the centrifuge tube containing pollen in the suspension of an acetolysis mixture (acetic anhydride and sulfuric acid, 9:1) is boiled for 1-2 min. The pollen suspension is centrifuged for 1 min at 3000 rpm and the supernatant liquid is decanted. Absolute ethanol is added and gently mixed by the vortex. Separated some pollen into 70 % ethyl alcohol for scanning electron microscopic observation. Put the benzene into another sample, gently mixed and centrifuged. Pour the benzene and add the 9000 stoke silicone oil and placed it in an oven at 50 ^{0}C for overnight. The pollen was mounted in the slide and the covered grass was sealed with paraffin. The pollen morphological characteristics were observed using a light microscope (LM, Zeiss-Axioskop2) and a scanning electron microscope (SEM, Quanta 450, Scientific Equipment Center, Faculty of Science, Kasetsart University, Bangkok, Thailand). The permanent slides were stored at the Palynology Special Research Unit, Department of Botany, Kasetsart University, Bangkok, Thailand. The study of pollen morphology was divided into 2 parts. First, qualitative characters were observed, consisting of the polar axis length, equatorial axis length, exine thickness, porus size and annulus size. Second, quantitative characteristics were observed consisting of pollen appearance, aperture and exine ornamentation.

Bioinformatics analysis

The statistical tests were carried out using the used the R Studio v.3.4.3 program (19). The Tukey's HSD (honestly significant difference) test at the 95% confidence interval was used to confirm significant differences for pollen morphological distances between species. The pollen species boundary of the grass family for the morphological characters of grass pollen were based on presence or absence information. The pollen morphological characters were developed into a character matrix of the six species that was subsequently used for regression tree analysis. Morphological distance was determined based on a regression tree using the "rpart.plot" package command in the rpart instruction set (20), because this distance analysis was designed to differentiate binary traits (0,1). The obtained values were analyzed based on a regression tree and graphed using the autoplot option with the "ggfortify" and "ggplot2" packages in the R software (21). This produced a graph showing the decision diagram to represent morphological character choices. The tree's nodes represented an event (yes, no) of a morphological character. The edge of the graph represented the decision condition.

Results

Pollen morphologies

The pollen grains were monad, heteropolar and had radial symmetry. The grains were small to large (20-100 µm). The aperture was monoporate. The ornamentation was microechinate, areolate, and perforate. The characters of each pollen species are described in the following sections (Table 2).

Megathyrsus maximus

Pollen grain is a monad. Polar axis is 24.2-31.8±2.32 µm long and equatorial axis is 25.1-33.4±2.63 µm long (P/E is 0.94). Exine thickness is 0.53-1.84±0.29 µm. Pollen grain is heteropolar, radially symmetrical and monoporate. Shape

Table 2. Pollen morphology of six grass species.

Species	Polar axis (µm)	Equatorial axis (µm)	Exine thickeness (µm)	Porus (µm)	Annulus (µm)	Size	P/E	Ornamentation
Oryza sativa	30.7±2.82 (25.8-34.6)	31.5±3.03 (26.8-36.9)	1.11±0.18 (0.72-1.50)	2.20±0.31 (1.73-2.85)	2.29±0.44 (1.55-2.99)	medium	0.95	areolate and perforate with microechinate
Zea mays	74.4±5.77 (63.5-84.0)	81.7±5.94 (71.2-93.3)	2.01±0.40 (1.21-3.07)	4.00±0.65 (2.47-4.95)	3.11±0.39 (2.44-3.65)	large	0.91	microechinate
Sorghum bicolor	34.4±1.77 (30.6-37.4)	36.6±1.53 (33.5-39.4)	1.44±0.21 (1.05-1.76)	2.13±0.22 (1.71-2.52)	2.03±0.31 (1.47-2.56)	medium	0.94	microechinate
Urochloa mutica	28.4±2.89 (23.6-34.7)	29.7±2.83 (25.9-36.1)	1.23±0.21 (0.99-1.58)	2.10±0.50 (1.39-3.25)	1.72±0.33 (1.11-2.60)	medium	0.96	areolate with microechinate
Urochloa eminii	24.7±2.12 (20.8-27.7)	27.0±2.40 (23.1-31.5)	1.16±0.21 (0.87-1.63)	1.75±0.27 (1.19-2.12)	1.78±0.28 (1.14-2.30)	small	0.92	areolate with microechinate
Megathyrsus maximus	28.4±2.32 (24.2-31.8)	30.1±2.63 (25.1-33.4)	1.19±0.29 (0.53-1.84)	2.18±0.45 (0.92-2.99)	2.10±0.45 (0.92-2.81)	medium	0.94	areolate with microechinate

is prolate to spheroidal. Ornamentation is areolate with microechinate (Fig. 1A-D).

Oryza sativa

Pollen grain is a monad. Polar axis is 25.8-34.6±2.82 µm long and equatorial axis is 26.8-36.9±3.03 µm long (P/E is 0.95). Exine thickness is 0.72-1.50±0.18. Pollen grain is heteropolar, radially symmetrical and monoporate. Shape is spheroidal. Ornamentation is combination of areolate and perforate with microechinate. (Fig. 1E–H).

Sorghum bicolor

Pollen grain is a monad. Polar axis is 30.6-37.4±1.77 µm long and equatorial axis is 33.53-9.4±1.53 µm long (P/E is 0.94). Exine thickness is 1.05-1.76±0.21 µm. Pollen grain is heteropolar, radially symmetrical and monoporate. Shape is prolate to spheroidal. Ornamentation is microechinate (Fig. 1I-L).

Urochloa eminii

Pollen grain is a monad. Polar axis is 20.8-27.7±2.12 µm long, equatorial axis is 23.1-31.5±2.83 µm long (P/E is 0.92). Exine thickness is 0.87-1.63±0.21 µm. Pollen grain is heteropolar, radially symmetrical and monoporate. Shape is prolate. Ornamentation is areolate with microechinate (Fig. 1M-P).

Urochloa mutica

Pollen grain is a monad. Polar axis is 23.6-34.7±2.89 µm long, equatorial axis is 25.9-36.2±2.83 µm long (P/E is 0.96). Exine thickness is 0.99-1.58±0.21 µm. Pollen grain is heteropolar, radially symmetrical and monoporate. Shape is prolate. Ornamentation is areolate with microechinate (Fig. 1Q-T).

Zea mays

Pollen grain is a monad. Polar axis is 63.5-84.0±5.77 µm long, equatorial axis is 71.2-93.3±5.94 µm long (P/E is 0.91). Exine thickness is 1.21-3.07±0.4 µm. Pollen grain is heteropolar, radially symmetrical and monoporate. Shape is spheroidal. Ornamentation is microechinate (Fig. 1U-X).

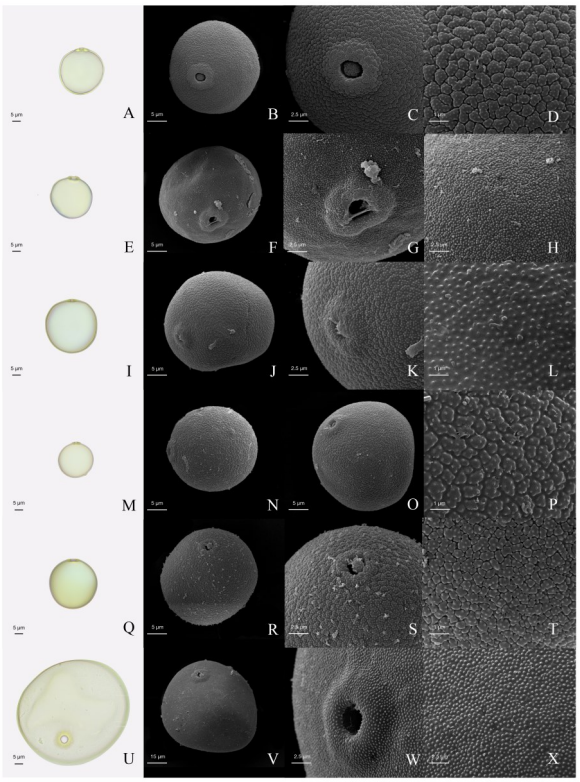

Fig. 1. Pollen morphology: *Megathyrsus maximus* **(A-D), A**: light microscope (LM), **B**: scanning electron microscope (SEM), **C**: aperture, **D**: areolate and perforate with microechinate ornamentation; *Oryza sativa* **(E-H)**, **E**: LM, **F**: SEM, **G**: aperture, **H**: microechinate ornamentation; *Urochloa eminii* **(I-L)**, **I**: LM, **J**: SEM, **K**: aperture, **L**: microechinate ornamentation; *Urochloa mutica* **(M -P)**, **M**: LM, **N**: SEM, **O**: aperture, **P**: areolate with microechinate ornamentation; *Sorghum bicolor* **(Q-T)**, **Q**: LM, **R**: SEM, **S**: aperture, **T**: areolate with microechinate ornamentation; *Zea mays* **(U-X)**, **U**: LM, **V**: SEM, **W**: aperture, **X**: areolate with microechinate ornamentation.

Bioinformatics analysis

The pollen morphological data of the 6 grass species consisted of quantitative characters (continuous data) and qualitative characters (discrete data). First, the continuous data characters were the polar axis, equatorial axis, exine thickness, annulus thickness and porus size. Second, the qualitative characters were from the exine ornamentation. The pollen morphologies of grass species are known to be very similar across the family. Therefore, data were analyzed using statistical tests to determine significant differences among the grass species. Tukey's HSD test was used to analyze the continuous data at the 95% confidence interval. Corn pollen's polar and equatorial axis lengths were significantly different from the other grasses. The pollen of sorghum with an average pollen grain size was significantly different from the pollen of the other grasses but the sorghum pollen grains were smaller than for corn pollen. The pollen of green panic grass, ruzi grass and rice had lengths of the equatorial axis that were not statistically different. For the pollen of ruzi grass, the equatorial axis was significantly different from the other grasses, except for the pollen of para grass. The polar axis length of corn pollen was significantly different from the other grasses. The pollen grains of ruzi grass and sorghum were not different in size when observed under a light microscope. However, the pollen of ruzi grass and sorghum were significantly different in polar axis and equatorial lengths from the other grasses. The axis lengths were not significantly different among the pollen grains of green panic grass, ruzi grass and rice. The annulus thickness for the corn pollen was significantly different from the other grasses, while green panic grass was significantly different from the pollen of para grass and rice but not different from the pollen of ruzi grass and sorghum. In addition, the corn pollen exine thickness was noticeably thicker than for the other pollen. The pollen of sorghum was similar to the para grass. The exine thickness of the sorghum pollen was significantly different from green panic grass, rice and ruzi grass. Finally, the results of porus size showed that there were no significant differences among the grass species, except for the corn (Fig. 2).

Regression tree analysis

The study of pollen morphologies using quantitative characteristics found that the pollen of corn was significantly different quantitatively from the other species in all characters (equatorial and polar axes, exine thickness, porus and annulus). The quantitative characters of pollen morphology were highly specific in their variation and could not be used to separate the six pollen species. The qualitative characters were targeted to support the differences between the six pollen grasses species. The pollen ornamentation of corn and sorghum was microechinate. Green panic grass, para grass and ruzi grass had a combination between areolate and microechinate, whereas rice has 3 combinations between areolate, microechinate and perforate. In general, pollen morphological characteristics are usually applied for species identification, with the quality data commonly used to support that information. The regression tree was applied for statistical analysis to support the different characters in stenoplynous families, such as the Poaceae. Combinations between qualitative and qualitative characters were analyzed and the tree showed difference for each species. The tree separated into 2 pollen sizes: medium and others. The medium-sized pollen species were green panic grass, para grass, rice and sorghum, while others were corn and ruzi grass. Corn was the largest and ruzi grass was the smallest. The medium group was separated using ornamentation as sorghum (microechinate) and rice (3 combinations between areolate, microechinate and perforate). Although, green panic grass and ruzi grass were quite similar in ornamentation, the annulus thickening was clearly different for separating the 2 closely related species (Fig. 3).

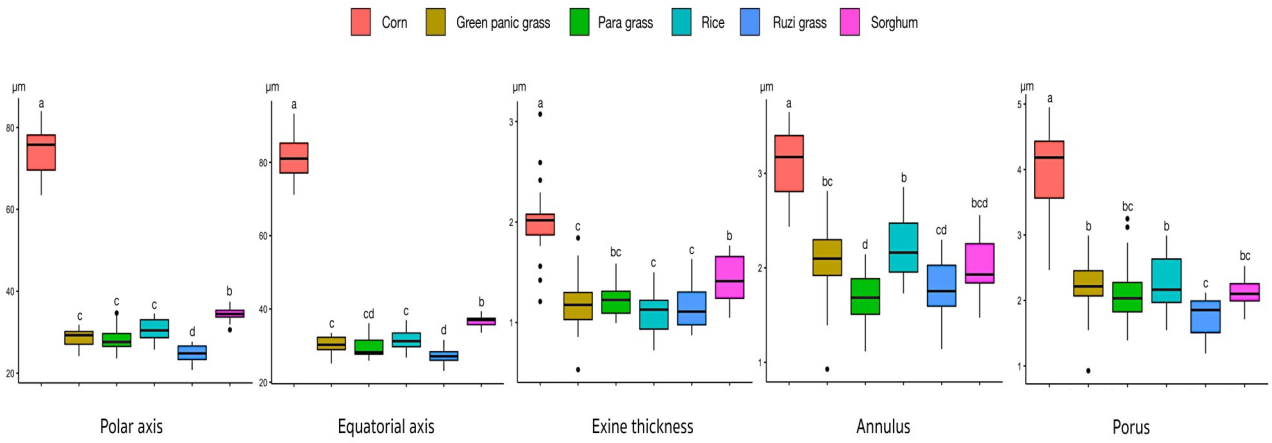

Fig. 2. Box plots of polar axis, equatorial axis, exine thickness, annulus and porus with Tukey's HSD test results. Treatments with the same lowercase letter are not significantly different (95% family-wise confidence level).

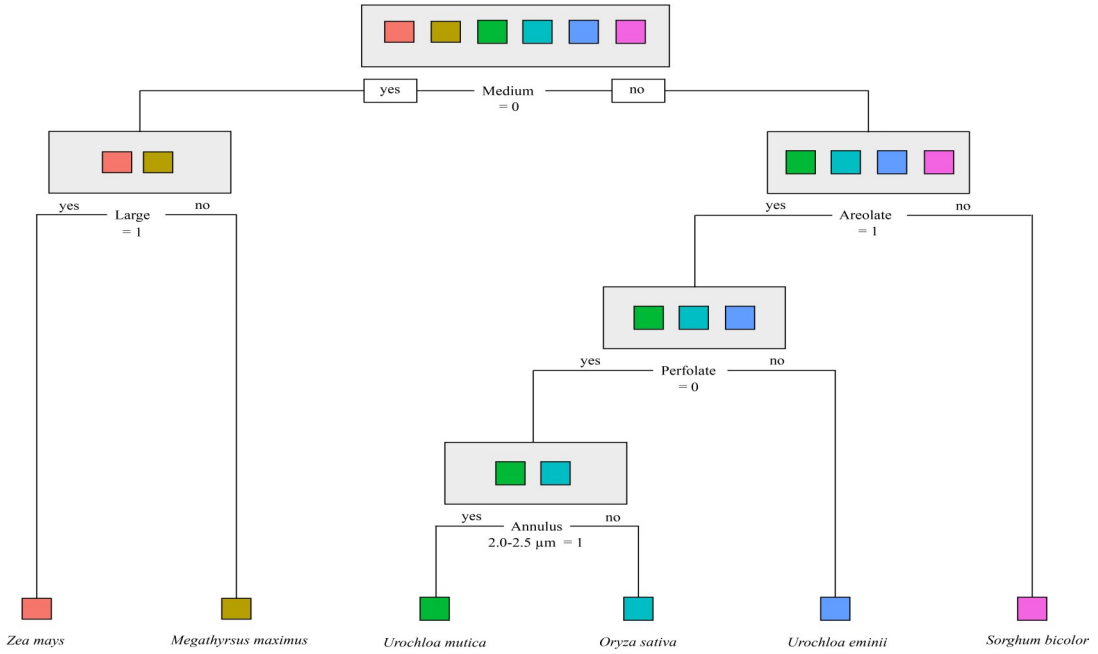

Fig. 3. Regression tree of pollen morphology.

Discussion

The purpose of this study was to evaluate suitable standardization of method for quality control of the raw materials for pollen allergy testing kit production. The pollen morphologies were observed using an LM and SEM for contamination detection. The pollen material was defined as not contaminated if there were less than 1% of non-pollen components. The results of the study were consistent with the earlier research (22) who reported on a standard method for extracting allergen proteins in *Lolium perenne* L. pollen and described a technique for detecting pollen contamination. The pollen samples were examined using an LM. The results were expressed as the number of non-pollen counts per low-power field containing approximately 200 pollen grains. The test was repeated at least 10 times. However, the report identified no exact figures for the extent of contamination of non-pollen components that did not affect the experiment. In the current study, non-contamination was defined as having less than 1% of non-pollen component contaminants and less than 1% of pollen of other plants. The amount of contamination in the current study corresponded (5), who studied pollen allergen extraction from *Cynodon dactylon* (L.) Pers., *Sorghum halepense* (L.) Pers and *Amaranthus hybridus* L. According to their report, the selection of the raw materials used in the manufacture of pollen allergy test kits was identified. In the process of line production, the purifying pollen for pollen extraction is very important. The pollen has to great efforts to avoid cross-contamination of raw pollen materials. Eventhough, the laboratory zones were separated. The purification of pollen needed to re-check. In the pollen storage process, it was suggested that pollen samples be kept in silica gel to avoid moisture changes (23). This advice was followed in the current study. The results showed that the pollen morphology of the 6 species studied was consistent with another study (15), indicating that grass is prolate-to-spheroidal monoporate surrounded by an annulus. In this current study, we tried to indicate the pollen grasses species using morphological characters. The criteria for samples selection were common, abundant and allergies causing tentative. Therefore, the morphological characters from LM and SEM were analyzed in the regression tree. The regression tree is widely used in machine learning and has given a new dimension to the concept of learning, which can be applied for the classification of pollen morphologies of six grass species. It also reduced the bias from person in analyzing the pollen morphological data (24). The quality control of pollen material in the manufacture of allergy assays has not previously been reported using tree regression statistical analysis. The current results were satisfactory and showed that grass pollen morphology could be useful for species identification. In addition, regression tree analysis has advantages in terms of computational processing and reducing the bias of human analysis. However, the current research was a prototype study using only 6 types of grass samples. The researchers also strongly suggest that regression tree analysis is one of methods that can be useful for detect the contamination for raw material quality control in allergy test kits production.

The raw materials of pollen quality control have to specify standardization for allergy testing kit production. However, the Thai Food and Drug Administration (FDA) have no specific criteria. Thus, the standardization must be specified by the producer.

Conclusion

To conclude that the pollen morphologies were remarkably observed as polar and equatorial axes, exine thickeness, porus, annulus and sculpturing respectively. The present study supports that the morphological characters of the 6 grasses pollen species are suitable for detected the contamination and also, useful for quality control of raw materials for a grass pollen allergy testing kits.

Acknowledgements

This research project is supported by National Research Council of Thailand (NRCT): NRCT5-RRI63002-M02. Authors thank David A. Simpson (Honorary Research Associate, Royal Botanic Gardens, Kew) for English proof reading and suggestions. Authors thank Dr. Ekaphan Kraichak for assistance with statistical analysis. Authors thank Deuanta Kongxaisavath for pollen purification process, Panyarat Laurchan for scientific advice. Authors thanks the Scientific Equipment Center, Faculty of Science, Kasetsart University for support on SEM (scanning electron microscope) studies. PJ also thanks the members of the Palynology Special Research Unit for their friendship, support and encouragement.

Authors contributions

PJ performed the experiments, data analysis and drafted the manuscript. CS contributed to provide important suggestions related to research. PYJ conceived the idea and participated in its design and coordination. CPS performed the research concept and approved of the article. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest : Authors do not have any conflict of interests to declare.

Ethical issues : None

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