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EFFECT OF DIFFERENT POLLEN HARVESTING TIMES ON QUANTITY, VIABILITY AND IN VITRO GERMINABILITY OF *Ixora coccinea* 'Dwarf Red Coccinea' POLLEN

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ABSTRACT

Knowledge about pollen of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' to be collected for basic investigations or plant breeding purposes is limited. Under a light microscope, *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen was generally prolate in shape which was different from that of *I. congesta* and *I. arborea*. In addition, the quantity, viability and germinability of pollen collected at different times from 8 am to 4 pm in a summer day from the flowers of *I. coccinea,* cv. 'Dwarf Red Coccinea' were investigated. Pollen quantity was determined using a haemacytometer while the viable and germinable pollen was examined after staining with 1% acetocarmine and germinating on a modified agar-gelled germination medium, respectively. The *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen collected at 10 am exhibited the highest pollen density and germination percentage of 53.3×104 pollen/ml and 72.05%, respectively. When these pollen were germinated on the artificial medium supplemented with various sucrose concentrations, the highest *in vitro* 'Dwarf Red Coccinea' pollen germinability was found on a medium containing 10% sucrose. It was concluded that the best time to collect *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen was at 10 am. Further studies would be worthwhile to investigate more closely the effect of changes in the environmental factors (for example, ambient temperature and humidity) even within the same day on harvestable pollen quantity and quality as well as pistil phenology to develop a more complete breeding strategy for *Ixora*.

Keywords: plant breeding, pollen fertility, Rubiaceae

INTRODUCTION

Ixora is one of the pantropical genera in the Rubiaceae family comprising at least 500 species (Mouly *et al*. 2009). Among the 28 cultivated varieties, the non-native *I. coccinea* 'Dwarf Red Coccinea' is most popular and grown widespread all over Thailand. It is used as flower bed border, living fence, pot plant or individual shrub (Puff *et al.* 2005; Mouly *et al*. 2009; Chamchumroon 2014). There may be some interest in breeding of *I. coccinea* which has a superior character as a year-round and non-stop bloomer. Its weak characters include a requirement of full sunlight to partial shade and excellent drainage in pots.

Both pollen quantity and quality collected from flowers of a plant are some of the important factors in plant breeding (Ashman *et al*. 2004; Colling *et al*. 2004). Besides, both abiotic (for example, humidity and temperature) and biotic (for example, pollinator) components in the environment also

influence pollen amount and viability of the flowering plant (Aronne 1999; De Luca *et al*. 2013). It is also of fundamental interest to study pollen. So far, there were some reports on pollen morphology of *Ixora* spp. (De Block & Robbrecht 1998; Sreekala *et al*. 2003). However, pollen quantity and quality of *I. coccinea,* cv. 'Dwarf Red Coccinea', have never been described. Thus, the objective of this study was to determine morphology, and changes in viability, density, and germinability of *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen collected at different times of a day when the flowers were in full bloom.

MATERIALS AND METHODS

Plant material

Inflorescences were collected from the blooming *Ixora coccinea,* cv. 'Dwarf Red Coccinea' plants in the garden of the King Mongkut's University of Technology North Bangkok at 2 hour intervals from 8 am to 4 pm on a sunny day in three consecutive weeks of March, the summer season in Thailand.

Pollen size, shape and viability

After inflorescence collection at different times of the day (8 am, 10 am, 12 midday, 2 pm and 4 pm), of *I. coccinea,* cv. 'Dwarf Red Coccinea', flowers were randomly selected and pollen grains were released by holding each flower upside down over a glass slide and by tapping (Figure 1). Overall, pollen from 20 anthers from 5 different plants of different populations were placed on a glass slide for observation of pollen size and shape under a light microscope. Then, pollen viability was investigated by staining with 1% (w/v) acetocarmine. The unstained pollen grains were non-viable while the red stained pollen were considered to be viable. Before and after pollen staining, its shape was clarified by using P/E ratio (Punt *et al*. 2007; Hesse *et al*. 2009). All pollen grains were randomly selected for 50 and 30 replications to determine pollen size and viability, respectively.

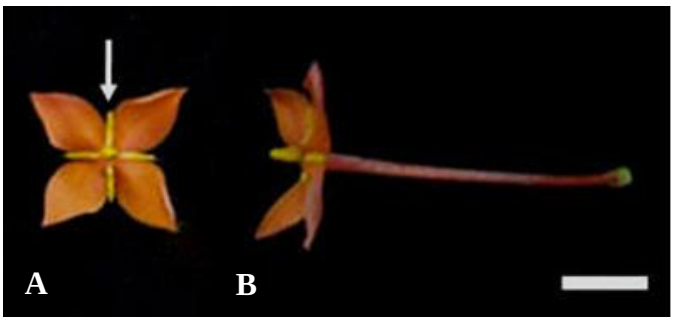

Figure 1 *Ixora coccinea,* cv. 'Dwarf Red Coccinea' flower: A – Top view (an arrow pointed at an anther); B – Side view (Bar = 1 cm)

Pollen density

The density (or quantity) of *I. coccinea*, cv. 'Dwarf Red Coccinea' pollen grains at different times (8 am, 10 am, 12 am, 2 pm and 4 pm) was investigated using a modified method based on Bunderson *et al*. (2012). Pollen grains from ten flowers were placed in a microcentrifuge tube. Afterwards, 60 µl glycerol and 40 µl distilled water were added into the tube and were mixed for 30 s using a vortex mixer. Then, 8 µl of the pollen suspension was filled in a haemacytometer (Improved Neubauer rulings, BOECO, Germany) with a micropipette. After placing a cover glass, pollen grains were spread over the grid which was divided into nine large squares. Only the pollen grains in the grid number 1, 2, 3 and 4 were counted (Figure 2). Those pollen grains that touched the line on the bottom and right of the grid were omitted from counting.

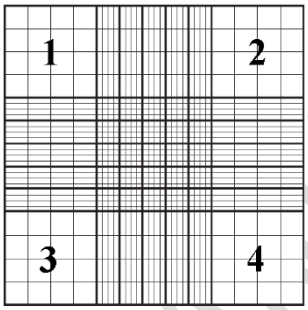

Figure 2 Grid layout of haemacytometer illustrating the position of number 1, 2, 3 and 4 that pollen grains were counted (modified from LeGresley and McDermott, 2012)

The formula for pollen density calculation (LeGresley and McDermott, 2012) as follows:

The average number of pollen per ml = [(pollen number in chamber 1+2+3+4)/4] × 10^4

= average count per large square × 10^4

Data from this experiment were collected from 6 replications.

Pollen germination

In vitro pollen germination test was carried out using modified Mercado *et al*. (1994) medium which consisted of 0.1 mM boric acid, 1 mM calcium chloride and various sucrose concentrations [0, 5, 10 and 20% (w/v)]. The medium was adjusted to pH 5.7, gelled with 0.9% (w/v) agar and sterilized at 121 °C, 15 psi for 20 min. Later, pollen grains from ten flowers were brushed over the surface of the germination medium. The treatments were incubated at 25±5 °C for 24 h in a dark room. Pollen grains were considered to have germinated when pollen tube length was twice longer than the diameter of pollen grain. Percentages of germination data were averaged from 20 replications.

Scanning electron microscope analysis

Pollen samples were collected at 10 am and sent to the Scientific and Technological Research Equipment Centre, Chulalongkorn University for morphological analysis using a scanning electron microscope (SEM-EDS, model JSM-6610LV, JEOL Ltd., Tokyo, Japan).

Data analysis

ANOVA of all data were carried out. If appropriate, the mean values in pollen size, viability, density and germination were compared using the Duncan Test at $P<0.05$.

RESULTS AND DISCUSSION

One of the characters used to describe and identify a pollen grain of a flowering plant is its size and shape. For this purpose, measurements of the polar axis (P) and equatorial axis (E) have been broadly used (Groppo *et al*., 2010; Chwil 2015). In the case of *I. coccinea,* cv. 'Dwarf Red Coccinea', most pollen grains were probably shed from anther before 4 pm as there was insufficient number (less than 10 grains per flower). Therefore, pollen at this time was excluded in further experiments. Under a light microscope, *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen before staining had polar axis longer than equatorial diameter and the P/E ratio was around 1.80 to 1.94 (Table 1). According to Punt *et al*. (2007) and Hesse *et al*. (2009), pollen with a polar axis longer than equatorial diameter or P/E ratio of 1.33-2.00 was described as prolate. Besides, the largest diameter was generally used for specifying the size of pollen. Pollen diameter between 26 and 50 μm was categorized as a medium size (Hesse *et al*. 2009). Thus, *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen from 8 am to 2 pm before staining was generally prolate in shape and had a medium size (Figure 3 and Table 1). When compared with other *Ixora* species observed under a light microscope, the shape of *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen was dissimilar to *Ixora congesta* and *I. arborea* which were suboblate and oblate spheroidal pollen, respectively (Ibrahim *et al*. 2012; Prabhakar & Ramakrishna 2014).

Table 1 Pollen size and shape of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' (under a light microscope) at different times before staining

Time	Length of the axis (μm)		P/E ratio	Shape
	P	E		
8am	39.4±0.210b	21.8±0.238a	1.8±0.012b	prolate
10am	39.6±0.261b	22.0±0.178a	1.8±0.008b	prolate
12am	39.1±0.169b	20.2±0.130c	1.9±0.011a	prolate
2pm	40.2±0.135a	21.0±0.176b	1.9±0.014a	prolate

Note: Values are means of 50 replications ±SE. Data marked by the same letter in a column are not statistically significant different ($P<0.05$).

Figure 3 Pollen morphology of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' before staining (Bar = 20 µm)

To examine more closely, *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen were also investigated under a scanning electron microscope. It was found that the polar and equatorial shape of this monad pollen was tri-lobulate and prolate, respectively. The grain had tricolpate aperture and psilate-perforate sculpturing (Figure 4). This was different from *I. congesta* as the pollen of this species had suboblate shape, microreticulate sexine ornamentation, pericolpate aperture and quadrangular outline (Ibrahim *et al*. 2012). These results suggest that pollen from the genus *Ixora* may have divergent forms in different species.

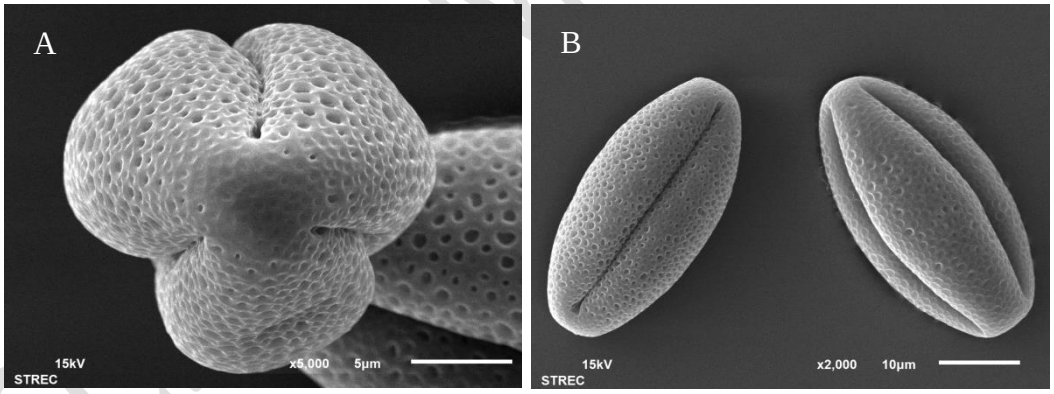

Figure 4 Scanning electron micrographs of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' pollen at polar (A) and equatorial (B) views

Staining pollen with acetocarmine is one of the most widely used technique to assist with estimating pollen viability (Malayeri *et al*. 2012). After 'Dwarf Red Coccinea' pollen was hydrated with 1% (w/v) acetocarmine, the shape of the dry pollen changed from prolate to spheroidal (Figure 5) and the diameter was around 32.6-36.7 µm (Table 2). Moreover, viable pollen as revealed from those exhibiting acetocarmine staining was mostly found at 10 am (72.05%) (Table 2). Sreekala *et al*. (2003) observed that anther dehiscence in *Ixora agasthyamalayana* occurred at between 7 to 1 pm

and peak anther dehiscence was at 10 am. In the present research, the experiment was done until 4 pm when there was a noticeable decline in pollen quantity and quality.

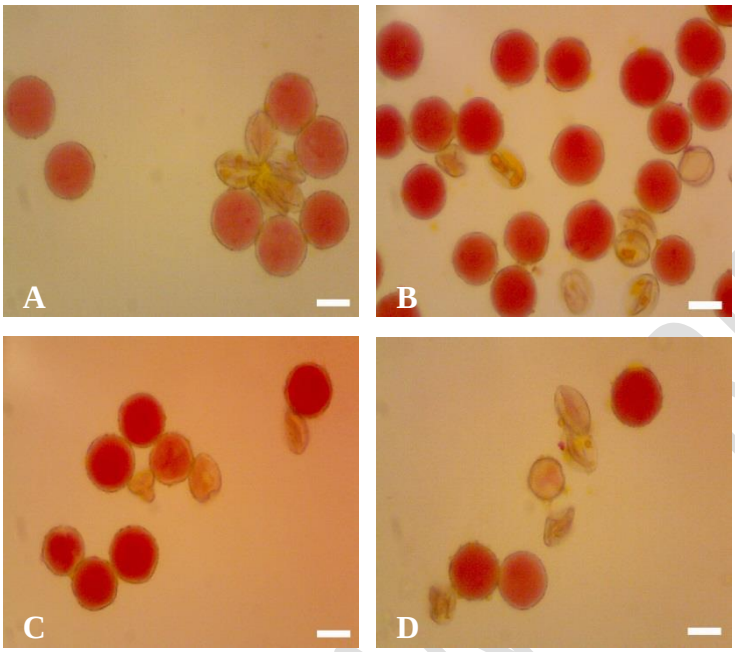

Figure 5 Pollen of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' after staining with 1% (w/v) acetocarmine. Pollen collected at different times: A) 8 am, B) 10 am, C) 12 noon, and D) 2 pm (Bar = 20 µm)

Table 2 Pollen diameter and viability after staining and pollen density of *Ixora. coccinea,* cv. 'Dwarf Red Coccinea' at different times

Time	Diameter[a] (µm)	Viability[b] (%)	Density[c] (pollen per ml)
8am	36.4±0.5a	61.8±1.7b	$8.9\times10^4\pm4.6\times10^3$c
10am	36.7±0.3a	72.0±1.0a	$53.3\times10^4\pm0.2\times10^3$a
12am	36.6±0.3a	60.7±0.6b	$18.7\times10^4\pm3.8\times10^3$b
2pm	32.6±0.1b	36.0±1.0c	$7.7\times10^4\pm1.9\times10^3$c

Note: [a]Values are means of 50 replications ±SE.
[b]Values are means of 30 replications ±SE.
[c]Values are means of 6 replications ±SE.
Data marked by the same letter in a column are not statistically significant different ($P<0.05$).

Although Thai Rubiaceae plants are mainly dependent on animal-assisted pollination (Puff *et al*. 2005), for artificial breeding of *I. coccinea,* cv. 'Dwarf Red Coccinea', the best time to collect pollen that are highly viable would be in the morning particularly at 10 am. Another advantage to collect pollen at this time of the day was that the highest number of pollen per ml (53.292×10^4) was found (Figure 6 and Table 2). It was possible that pollen density was reduced after 10 am because continuously shedding of pollen from the anther to the external environment took place. The number of pollen in many plant species was effectively counted using a haemocytometer (Godini 1981; Kelly

et al. 2002; Bunderson *et al*. 2012). In this research, a Neubauer improved haemacytometer was successfully used to determine pollen density as well. Pollen density from different times throughout the day was rarely investigated. Taking the findings of the present study into consideration, it would seem that not only different amounts of pollen may be produced at different times during a flowering season (Piotrowska 2012; Peel *et al*. 2014), but also different quantities of pollen may be obtained at different times of a day. This could be related to the varying environmental conditions (for example, ambient temperature and humidity) at different times of the day affecting pollen shedding/collection. Further studies are recommended to investigate this possibility.

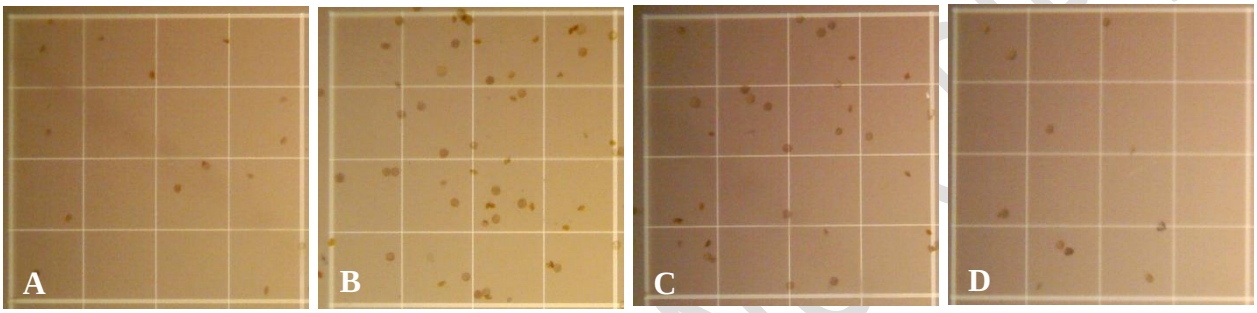

Figure 6 Pollen density of *Ixora coccinea*, cv. 'Dwarf Red Coccinea' on a Neubauer improved haemacytometer. Pollen collected at different times: A) 8 am, B) 10 am, C) 12 noon, and D) 2 pm (all figures from grid A)

As there is a lack of information on the germination potential of *I. coccinea*, cv. 'Dwarf Red Coccinea' pollen, pollen collected at 10 am was used for a study of the effect of different sucrose concentrations on pollen germination on an agar medium (modified based on Mercado *et al.* 1994). The highest percentage of pollen germination (about 34%) occurred on medium containing 10% sucrose (Table 3). At lower concentrations or 20% sucrose fewer than 10% pollen germinated. This is consistent with other prior studies showing that sucrose concentration is an important factor for *in vitro* pollen germination (Figure 7 and Table 3). The *Ixora* pollen may lose their viability quickly after collection. The fresh pollen exhibited 72% viability as revealed by acetocarmine staining. However, during *in vitro* germination, more pollen could have lost viability. Another possilibity is that sucrose may not be the only factor to enhance *Ixora* pollen germination. To increase the germinability, other factors (for example, boric acid concentrations, Fragallah *et al*. 2019) could also be studied in future studies. The optimal sucrose concentration for *in vitro* pollen germination appears to depend on the plant species under study. For example, cannonball tree pollen needed a high level of sucrose (20% w/v) while two forms of day-blooming native Thai waterlily responded very well on a low sucrose concentration (5% w/v) (Bodhipadma *et al*. 2013; 2016). In contrast, the percentage of

I. coccinea, cv. 'Dwarf Red Coccinea' pollen germination was sharply reduced at these sucrose concentrations.

Table 3 Percentage of *Ixora coccinea*, cv. 'Dwarf Red Coccinea' pollen germination at 10 am on modified Mercado et al. (1994) medium supplemented with different sucrose concentration

	Sucrose concentrations			
	0%	5%	10%	20%
Germination (%)	0.0±0.0d	3.2±0.5c	33.8±1.7a	6.6±0.56b

Note: Values are means of 20 replications ±SE. Data marked by the same letter in a row are not statistically significant different (P<0.05).

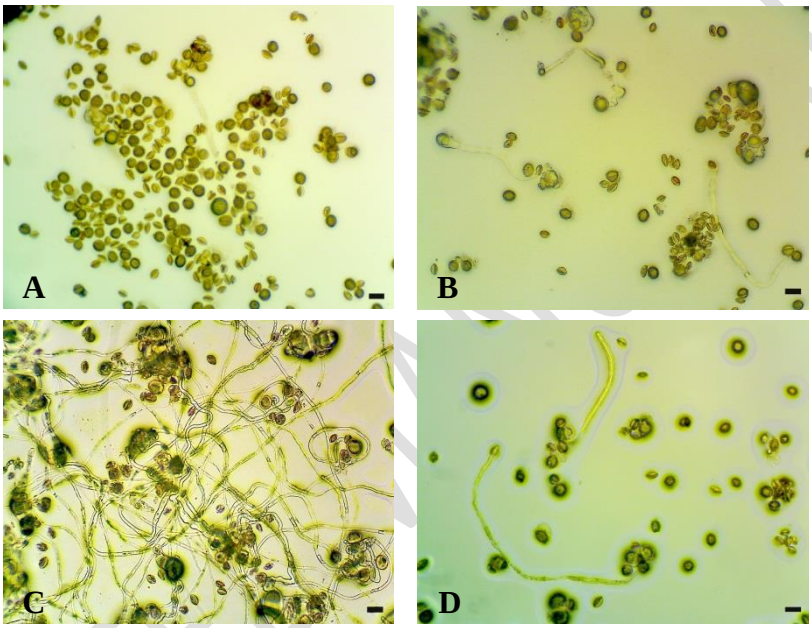

Figure 7 *Ixora coccinea*, cv. 'Dwarf Red Coccinea' pollen germination on modified Mercado et al. (1994) medium with A) 0%, B) 5%, C) 10%, and D) 20% (w/v) sucrose (Bar= 50 µm)

CONCLUSION

It has been known that different amounts of pollen may be produced at different times during a flowering season (Piotrowska 2012; Peel *et al*. 2014). In this study, the quantity of *I. coccinea*, cv. 'Dwarf Red Coccinea' pollen produced was related to the different times during the day. This, therefore, has implications in studies of pollen from other plants. The number of *I. coccinea*, cv. 'Dwarf Red Coccinea' pollen grain peaked at 10 am which also exhibited the highest viability estimated based on 1% (w/v) acetocarmine staining and density measurement with a Neubauer improved haemacytometer. This could be related to the varying environmental conditions (for example, ambient temperature and humidity) at different times of the day affecting pollen collection. Further studies are recommended to investigate this possibility. Additionally, sucrose concentration was essential for *I. coccinea*, cv. 'Dwarf Red Coccinea' pollen germination and the optimal sucrose

concentration was 10% (w/v). Since other studies on *Ixora* pollen were mainly focused on pollen morphology, it would be of interest to study the effect of sucrose level on germination of pollen from other *Ixora* spp.. Another implication of the present study is that for the breeding of *Ixora coccinea* 'Dwarf Red Coccinea', the critical time to collect pollen would be 10 am. In further study, it would also be necessary to study pistil phenology of this species and other related species to develop a more complete breeding strategy.

REFERENCES

Aronne G. 1999. Effects of relative humidity and temperature stress on pollen viability of *Cistus incanus* and *Myrtus communis*. Grana 38:364-367.

Ashman TL, Knight ML, Steets JA *et al*. 2004. Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology 85:2408-2421.

Bodhipadma K, Noichinda S, Thaiyanto P, Leung DWM. 2013. Morphology, viability, and germinability of pollen from two forms of *Nymphaea nouchali* var. *versicolor*, A day-blooming waterlily. ScienceAsia 39:214-218.

Bodhipadma K, Noichinda S, Permchalad K, Changbandist S, Phanomchai S, Punnakanta L, Leung DWM. 2016. A study of cannonball trees in Thailand: Hood staminodes are larger than ring stamens but only germination of staminal ring pollen can be stimulated by exogenous sucrose. KMUTNB: IJAST 9:167-173.

Bunderson DL, Water VP, Wells H, Levetin E. 2012. Predicting and quantifying pollen production in *Juniperus Ashei* forests. Phytologia 94:417-438.

Chamchumroon V. 2014. Two new species of *Ixora* (Rubiaceae) from Thailand. TFB (Botany) 42:85-90.

Chwil M. 2015. Micromorphology of pollen grains of fruit trees of the genus *Prunus*. Acta Sci Pol-Hortoru 14:115-129.

Colling G, Reckinger C, Matthies D. 2004. Effects of pollen quantity and quality on reproduction and offspring vigor in the rare plant *Scorzonera humilis* (Asteraceae). Am J Bot 91:1774-1782.

De Block P, Robbrecht E. 1998. Pollen morphology of the Pavetteae (Rubiaceae, Ixoroideae) and its taxonomic significance. Grana 37:260-275.

De Luca PA, Bussie`re LF, Souto-Vilaros D, Goulson D, Mason AC, Vallejo-Marı´n M. 2013. Variability in bumblebee pollination buzzes affects the quantity of pollen released from flowers. Oecologia 172:805-816.

Fragallah SADA, Lin S, Li N, Ligate EJ, Chen y. 2019. Effects of sucrose, boric acid, pH, and incubation time on *in vitro* germination of pollen and tube growth of Chinese fir (*Cunnighamial lanceolata* L.). Forests 10: 102.

Godini A. 1981. Counting pollen grains of some almond cultivars by means of an haemocytometer. In : GREMPA Colloque 1980, CIHEAM-Options Mediterranéennes Série Etudes 1981-I, Institut Agronomique Méditerranéen de Zaragoza (IAMZ), Paris. p. 83-86.

Groppo M, da Cruz-Barros MAV, da Silva Correa AM. 2010. Pollen morphology of species of *Hortia* (Rutaceae). Rev Bras Bot 33:13-20.

Hesse M, Halbritter H, Weber M *et al*. 2009. Pollen terminology: An illustrated handbook. New York: SpringerWein.

Ibrahim IF, Balasundram SK, Abdullah NAP, Alias MS, Mardan M. 2012. Morphological characterization of pollen collected by *Apis dorsata* from a tropical rainforest. Int J Botany 8:96-106.

Kelly JK, Rasch A, Kalisz S. 2002. A method to estimate pollen viability from pollen size variation. Am J Bot 89:1021-1023.

LeGresley M, McDermott G. 2012. Counting chamber methods for quantitative phytoplankton analysis - haemocytometer, Palmer-Maloney cell and Sedgewick-Rafter cell. In: Karlson B, Cusack C, Bresnan E, editors. Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis (IOC manuals and guides, no. 55). Paris: UNESCO. p. 25-30.

Malayeri BE, Noori M, Jafari M. 2012. Using the pollen viability and morphology for fluoride pollution biomonitoring. Biol Trace Elem Res 147:315-319.

Mercado JA, Fernandez-Muzor R, Quesada MA. 1994. In vitro germination of pepper pollen in liquid medium. Scientia Horticulture 57:273-281.

Mouly A, Razafimandimbison GS, Khodabandeh A, Bremer B. 2009. Phylogeny and classification of the species-rich pantropical showy genus *Ixora* (Rubiacrae-Ixoreae) with indications of geographical monophyletic units and hybrids. Am J Bot 96:686-706.

Peel RG, Ørby PV, SkjØth CA, Kennedy R, Schlünssen V, Smith M, Sommer J, Hertel O. 2014. Seasonal variation in diurnal atmospheric grass pollen concentration profiles. Biogeosciences 11:821-832.

Piotrowska K. 2012. Meteorological factors and airborne *Rumex* L. pollen concentration in Lublin. Acta Agrobot 65:45-52.

Prabhakar R, Ramakrishna H. 2014. Palynodiversity in Boath mandal forest division of Adilabad district, Telangana State, India. Int J Pharm life Sci 5:3685-3693.

Puff C, Chayamarit K, Chamchumroon V. 2005. Rubiaceae of Thailand: A Pictorial Guide to Indigenous and Cultivated Genera. Bangkok: The Forest Herbarium, Department of National Parks, Wildlife and Conservation.

Punt W, Hoen PP, Blackmore S, Nilsson S, Le Thomas A. 2007. Glossary of pollen and spore terminology. Rev Palaeobot Palyno 143:1-81.

Sreekala AK, Rajkumar G, Pandurangan AG. 2003. Studies on floral biology of *Ixora agasthyamalayana* Sivadasan & Mohanan - a rare southern Western Ghats endemic. Zoos Print J 18:1041-1042.