HARVESTING TIME AND VIABILITY OF *Ixora coccinea* 'DWARF RED COCCINEA' POLLEN

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ABSTRACT

The cultivated variety of the non-native *I. coccinea*, Dwarf Red Coccinea (DRC), is most popular and widely spread all over Thailand. However, knowledge about its pollen morphology and fertility for plant breeding purposes, is limited. This study aimed to investigate the quantity, viability and germinability of pollen grains collected from the flowers of DRC at different times on a summer day, particularly from 8 AM to 4 PM. Pollen quantity was determined using a haemacytometer while its viability and germinability were examined after staining with 1% acetocarmine and allowing the pollen to germinate on a modified agar-gelled germination medium. The pollen collected at 10 AM had the highest pollen density (53.3×10^4 pollen/mL) and viability percentage (72.05%). When these pollen were allowed to germinate on an artificial medium supplemented with various sucrose concentrations, the highest *in vitro* pollen germinability was found at the medium containing 10% sucrose. Hence, the best time to collect the *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen was at 10 AM. However, further investigations are recommended on the effects of daily or hourly environmental changes particularly, ambient temperature and humidity, on the quantity and quality of harvestable pollen as well as on the pistil phenology, to develop a more complete breeding strategy for the *Ixora* species.

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* or Dwarf Red Coccinea (DRC) is the most popular and widely grown 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).

DRC 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 the quantity and quality of pollen collected from flowers are important in plant breeding (Ashman *et al.* 2004; Colling *et al.* 2004). Both the abiotic (humidity and temperature) and biotic (pollinator) components of the environment also influence the pollen amount and viability of the flowering plant (Aronne 1999; De Luca *et al.* 2013). Hence, it is of fundamental interest to study pollen. Few studies have focused on pollen morphology of *Ixora* spp. (De Block & Robbrecht 1998; Sreekala *et al.* 2003). However, the pollen quantity and quality of *I. coccinea*, cv. Dwarf Red Coccinea, have never been described. Thus, the objective of this study was to determine the morphology, and changes in viability, density, and germinability of DRC pollen collected at different times of the day when the flowers were in full bloom. Under a light microscope, the pollen of this species has a generally prolate shape which is different from that of *I. congesta* and *I. arborea.*

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MATERIALS AND METHODS

Plant Material

Inflorescences were collected from the blooming *Ixora coccinea,* cv. Dwarf Red Coccinea (DRC) plants at the garden of 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

The DRC flowers were randomly selected at different times of the day (8 AM, 10 AM, 12 midday, 2 PM and 4 PM). The pollen grains were released by holding each flower upside down over a glass slide and by tapping (Fig. 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. The pollen viability was then investigated by staining with 1% (w/v) acetocarmine. The unstained pollen grains were non-viable while the red stained grains were considered viable. Before and after pollen staining, its shape was clarified by using the P/E ratio (Punt *et al.* 2007; Hesse *et al.* 2009). All pollen grains were randomly selected with 50 and 30 replications to determine pollen size and viability, respectively.

Pollen Density

The density (or quantity) of DRC pollen grains was investigated at different times (8 AM, 10 AM, 12 AM, 2 PM and 4 PM) using a modified method based on Bunderson *et al.* (2012). Pollen grains from ten flowers were placed in a microcentrifuge tube. Some 60 µL glycerol and 40 µL distilled water were then added into the tube and were mixed for 30 s using a vortex mixer. Then, 8 µL of the pollen suspension was placed in a haemacytometer (Improved Neubauer rulings, BOECO, Germany) using a micropipette. The pollen grains were then covered with a glass and 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 (Fig. 2). Those pollen grains that touched the line on the bottom and right of the grid were omitted from counting.

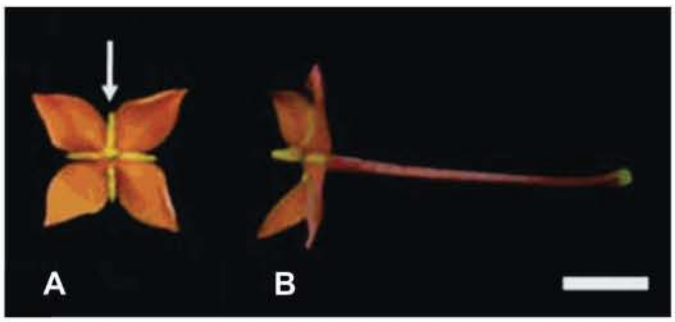

Figure 1 *Ixora coccinea,* cv. 'Dwarf Red Coccinea' flower
Notes: A = Top view (an arrow pointed at an anther); B = Side view (Bar = 1 cm).

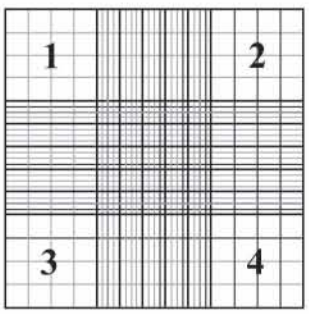

Figure 2 Grid layout of haemacytometer illustrating the position of number 1, 2, 3 and 4 pollen grains that were counted (modified from LeGresley and McDermott, 2012)
Notes: The formula for pollen density calculation (LeGresley and McDermott, 2012) is as follows:
The average number of pollen per mL = [(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 the 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 grains 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 and 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 (DRC), most pollen grains were probably shed from the anther before 4 PM as there was insufficient number (less than 10 grains per flower) by that time. Therefore, pollen at this time was excluded in the experiments. Under the light microscope, the DRC pollen had polar axis longer than equatorial diameter and the P/E ratio was around 1.80 to 1.94 before staining (Table 1). Pollen with a polar axis longer than equatorial diameter or P/E ratio of 1.33-2.00 was described as prolate (Punt *et al.* 2007; Hesse *et al.* 2009). Moreover, 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, the DRC pollen from 8 AM to 2 PM was generally prolate in shape and had a medium size before staining (Fig. 3; Table 1). When compared with other *Ixora* species observed under the light microscope, the shape of DRC pollen was dissimilar to those of *Ixora congesta* (suboblate) and *I. arborea* (oblate spheroidal) (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		
8 AM	39.4 ± 0.210b	21.8 ± 0.238a	1.8 ± 0.012b	prolate
10 AM	39.6 ± 0.261b	22.0 ± 0.178a	1.8 ± 0.008b	prolate
12 AM	39.1 ± 0.169b	20.2 ± 0.130c	1.9 ± 0.011a	prolate
2 PM	40.2 ± 0.135a	21.0 ± 0.176b	1.9 ± 0.014a	prolate

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

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

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

Pollen staining with acetocarmine is one of the most widely used technique in estimating pollen viability (Malayeri *et al.* 2012). After the DRC pollen grains were hydrated with 1% (w/v) acetocarmine, the shape of the dry pollen changed from prolate to spheroidal (Fig. 5) and the diameter was around 32.6 - 36.7 μm (Table 2). Moreover, the viable pollens, as manifested by those exhibiting acetocarmine staining, were most numerous at 10 AM (72.05%) (Table 2). In another study, (Sreekala *et al.* 2003) the anther dehiscence in *Ixora agasthyamalayana* occurred at 7 AM to 1 PM and the peak anther dehiscence was also at 10 AM. In the current research, the experiment was done until 4 PM when there was a noticeable decline in pollen quantity and quality.

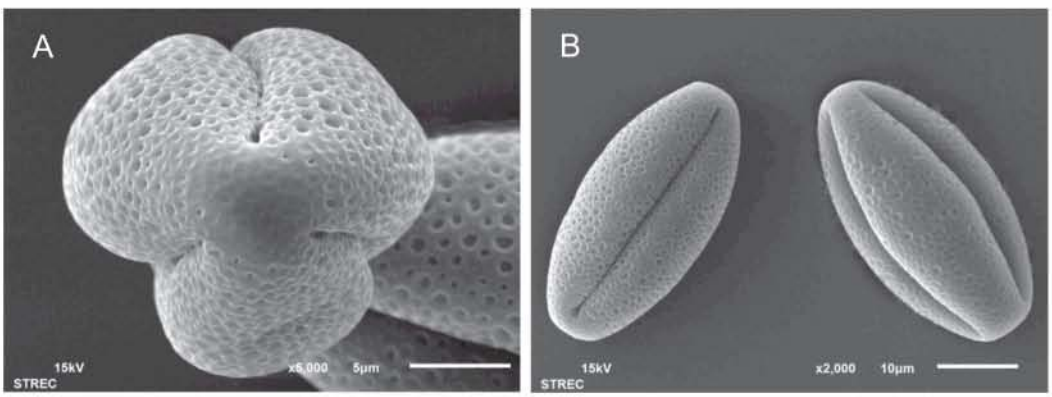

Figure 4 Polar (A) and equatorial (B) views of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' pollen under a scanning electron microscope

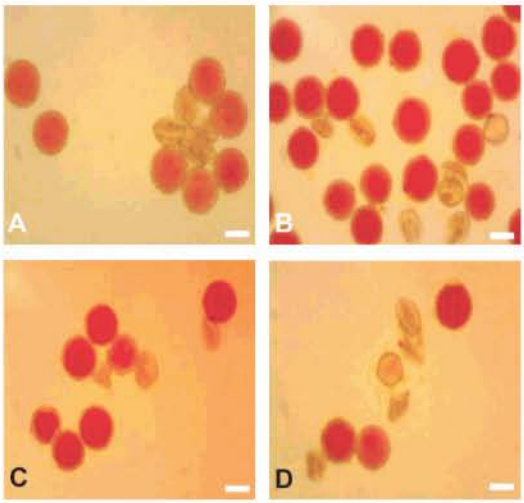

Figure 5 Pollens of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' after staining with 1% (w/v) acetocarmine
Notes: Pollens were collected at different times: A) 8 AM; B) 10 AM; C) 12 noon; and D) 2 PM. Bar = 20 μm.

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

Time	Diameter[1] (μm)	Viability[2] (%)	Density[3] (pollen per mL)
8 AM	36.4 ± 0.5a	61.8 ± 1.7b	$8.9\times10^4 \pm 4.6\times10^3$c
10 AM	36.7 ± 0.3a	72.0 ± 1.0a	$53.3\times10^4 \pm 0.2\times10^3$a
12 AM	36.6 ± 0.3a	60.7 ± 0.6b	$18.7\times10^4 \pm 3.8\times10^3$b
2 PM	32.6 ± 0.1b	36.0 ± 1.0c	$7.7\times10^4 \pm 1.9\times10^3$c

Notes: [1]Values are means of 50 replications ± SE; [2]Values are means of 30 replications ± SE; [3]Values are means of 6 replications ± SE. Data marked by the same letter in a column are not significantly different ($P < 0.05$).

Although the Thai Rubiaceae plants are mainly dependent on animal-assisted pollination (Puff *et al.* 2005), the best time to collect highly viable pollen of *I. coccinea,* cv. 'Dwarf Red Coccinea' for artificial breeding would be at 10 AM. This was also the time of the day that the highest number of pollen per mL (53.292×10^4) was collected (Fig. 6; Table 2). Possibly, the pollen density was reduced after 10 AM because of the continuous shedding of pollen from the anther to the external environment. Pollen numbers are effectively counted using a haemacytometer (Godini 1981; Kelly *et al.* 2002; Bunderson *et al.* 2012), and in the current study, a Neubauer improved haemacytometer was used to successfully determine the pollen density. Density of pollen collected at different times throughout the day was rarely investigated. Considering the findings of the present study, it is possible that not only different amounts of pollen are produced at different flowering seasons (Piotrowska 2012; Peel *et al.* 2014), but also different quantities of pollen may be obtained at different times of the day. Hence, further studies are recommended to investigate the effects of environmental conditions particularly, ambient temperature and humidity of pollen shedding/collection at different times of the day.

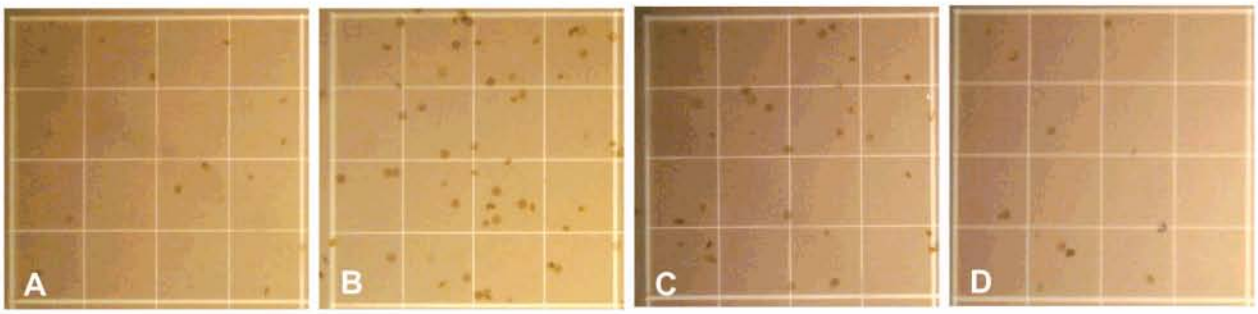

Figure 6 Pollen density of *Ixora coccinea,* cv. 'Dwarf Red Coccinea' on a Neubauer improved haemacytometer
Notes: Pollens were collected at different times: A) 8 AM; B) 10 AM; C) 12 noon; and D) 2 PM; All figures from grid A.

Since little information is available on the germination potential of *I. coccinea,* cv. 'Dwarf Red Coccinea' pollen, the pollen collected at 10 AM was used in this study to determine 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 at the medium containing 10% sucrose (Table 3). At higher sucrose concentrations (20% sucrose) fewer than 10% of pollen germinated. This is consistent with other studies showing that sucrose concentration is an important factor for *in vitro* pollen germination (Fig. 7; 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 possibility 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 the future. The optimal sucrose concentration for *in vitro* pollen germination appears to depend on the plant species. 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 germination percentage of DRC pollen was sharply reduced at sucrose concentrations that were lower and higher than 10%.

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

Notes: Values are means of 20 replications ± SE. Data marked by the same letter in a row do not significantly differ ($P < 0.05$).

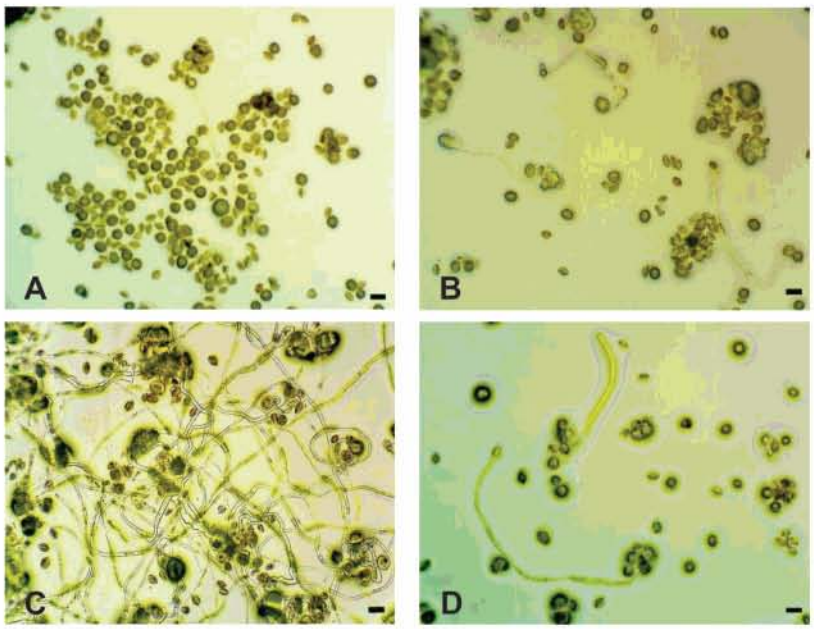

Figure 7 *Ixora coccinea,* cv. 'Dwarf Red Coccinea' pollen germination on modified Mercado *et al.* (1994) medium
Notes: A = 0%; B = 5%; C = 10%; D) 20% (w/v) sucrose. Bar = 50 μm.

CONCLUSION

The quantity of *I. coccinea,* cv. 'Dwarf Red Coccinea' (DRC) pollen produced was related to the different production times during the day. The number of DRC pollen grain peaked at 10 AM which also exhibited the highest estimated viability based on 1% (w/v) acetocarmine staining and density measurement with a Neubauer improved haemacytometer. This is probably related to the varying environmental conditions like ambient temperature and humidity at different times of the day of pollen collection. Moreover, the optimal sucrose concentration of 10% (w/v) was essential for the DRC pollen germination. 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 the 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. To develop a more complete breeding strategy, further study is recommended on the pistil phenology of this species and other related species, and on the effect of temperature and humidity on *Ixora.*

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