Causes and Consequences of Stigma Closure in *Oroxylum indicum* Vent. (Bignoniaceae)

Tuanjit Sritongchuay

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Ecology (International Program)
Prince of Songkla University
2009

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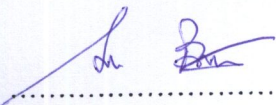Prince of Songkla University
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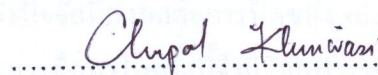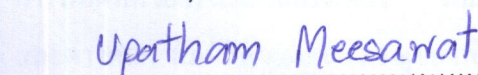**Major Advisor:**   **Examining Committee:**

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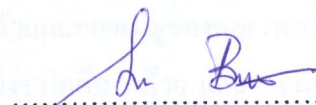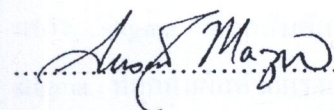
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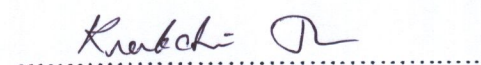
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ชื่อวิทยานิพนธ์   สาเหตุและผลดีของลักษณะการปิดของ stigma ในดอกเพกา (*Oroxylum indicum*, Vent.

ผู้เขียน   นางสาว เตือนจิต ศรีทองช่วย

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บทคัดย่อ

ลักษณะการปิดของ stigma เป็นลักษณะที่พบได้ทั่วไปในพืชวงศ์ Bignoniaceae รวมทั้งใน ดอกเพกา (*Oroxylum indicum*) แต่การศึกษาถึงปัจจัยที่ส่งผลต่อการปิดของ stigma และ ผลที่เกิดจาก การปิดของ stigma นั้นมีค่อนข้างน้อย ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาให้เข้าใจ ถึงสาเหตุและผลของลักษณะการปิดของ stigma ในพืชชนิดนี้ โดยศึกษาปัจจัยที่ส่งผลต่อการปิด คือ การสัมผัสด้วยแรงเพียงอย่างเดียว สัมผัสด้วยละอองเรณูของดอกเพกาทั้งจากภายในดอกเดียวกัน และต่างดอก สัมผัสด้วยละอองเรณูของพืชต่างชนิดคือ กล้วย และ สะตอ สัมผัสด้วยละอองเรณูใน จำนวนที่ต่างๆกัน จากนั้นสังเกตระยะเวลาในการปิดและเปิดของ stigma จากการศึกษาพบว่า stigma ที่ถูกสัมผัสด้วยแรงเพียงอย่างเดียวปิดเร็วกว่า stigma ที่ถูกสัมผัสโดยละอองเรณูของดอก เพกา stigma ที่ถูกสัมผัสโดยละอองเรณูของเพกาไม่มีการเปิดหลังจากปิดไปแล้วรอบแรกขณะที่ stigma ที่ถูกสัมผัสด้วยแรงเพียงอย่างเดียวและ สัมผัสด้วยละอองเรณูของพืชต่างชนิดนั้นมีการเปิด การศึกษาถึงผลของลักษณะการปิดของ stigma นั้นทดสอบสมมติที่ว่า ลักษณะการปิดของ stigma ช่วยป้องกันละอองเรณูจากดอกเดียวกัน ช่วยกักเก็บละอองเรณูจากต่างดอก เพิ่มการกระจายของ ละอองเรณู ป้องกันการอุดตันของ stigma ซึ่งเกิดจากจำนวนละอองเรณูมาตกที่ stigma มากเกินไป จากการศึกษาพบว่า ลักษณะการปิดของ stigma ช่วยลดการแข่งขันของหลอดละอองเรณู ดังนั้นการ ปิดของ stigma ในดอกเพกาสามารถส่งผลดีและผลเสียต่อพืชชนิดนี้ซึ่งจะมีการอภิปรายในการศึกษา ในครั้งนี้

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ABSTRACT

Stigma closure following pollination is common in the Bignoniaceae, but the factors influencing this behavior and its consequences are not well understood. I investigated several factors influencing the speed of stigma closure and reopening in *Oroxylum indicum*, and its beneficial consequences to this plant. The effects of pressure alone, conspecific pollen, heterospecific pollen (of *Musa* and *Parkia*), and pollen load size on stigma behavior were examined. Pressure alone resulted in faster closing than the application of conspecific pollen, and faster reopening than the application of heterospecific pollen. Stigmas never reopened following the deposition of conspecific pollen. Stigmas discriminate between conspecific and foreign pollen; by reopening after pollination with the latter. Stigma closure did not affect the number of pollen grains received either self pollen or outcross pollen, number of pollen exported. I also counted pollen tubes in the styles of flowers that were either hand-supplemented with outcross *O. indicum* pollen or open-pollinated. Pollen tube numbers were highest following light pollination (~900 grains), revealing evidence for stigma clogging following pollination with very heavy loads (>6000 grains). The potential advantages and disadvantages of stigma closure and reopening in *O. indicum* were discussed.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Bignoniaceae, Scrophulariaceae, Martyniaceae, and Lentibulariaceae produce two tongue-shaped stigma lobes, which in some species move in response to pressure and to pollination (Newcombe, 1922; Stephenson and Thomas, 1977; Bertin, 1982; Fetscher and Kohn, 1999). Following the application of pressure or pollination, the lobes fold together such that the receptive surface of each lobe is pressed against the other. Pattern of stigma closure varies among species. For example, in *Mimulus cardinalis* (Newcombe, 1922) and *M. aurantiacus* (Fetscher and Kohn, 1999), the stigma reopens after the primary closure. In contrast, in *Campsis radicans*, stigmas do not reopen (Newcombe, 1922). Understanding both the causes of this behavior and its effect on pollen receipt and pollen performance is a first step towards evaluating its adaptive significance.

Several studies have detected proximate causes for stigma closure and re-opening in these taxa. For example, the application of light pressure alone can cause stigma closure in some Bignoniaceae species (Newcombe, 1922; Bertin, 1982). However, the stigmas of *Spathodea campanulata* do not close completely following pressure; complete closure requires a combination of both pressure and pollen (Newcombe, 1924). Stigmas of several species have been found to reopen following the application of pressure without pollen, but longer-duration and permanent closure requires pressure and pollen combined (Newcombe, 1924; Stephenson and Thomas, 1977; Fetscher and Kohn, 1999; Richardson, 2004; Yang *et al.*, 2004). In some cases, heterospecific pollen deposition has also been found to cause stigma closure (Waser and Fugate, 1986).

Stigma closure can result in a fitness cost if it occurs following the deposition of insufficient conspecific pollen to achieve full seed set (Fetscher and

Kohn, 1999; Somanathan and Borges, 2001; Richardson, 2004; Yang *et al.*, 2004). In addition, seed set may be reduced if stigmas close following the deposition of heterospecific pollen because they will be unavailable for pollination until they reopen (Waser and Fugate, 1986). The costs of such stigma closure are likely to be highest in taxa in which fruit or seed set are chronically pollen-limited.

Stigma closure may also confer fitness benefits. For example, rapid stigma closure may prevent interference among pollen grains and tubes if closure prevents multiple visits that result in very high pollen loads. Stigma closure increases pollen receipt by preventing pollen removal from stigma surface (Fetscher and Kohn, 1999). Stigma closure may also increase pollen disperse (and increase male reproductive success) and decrease the self pollination by reducing interference between anthers and stigmas (Fetscher, 2001).

Oroxylum indicum is self-incompatible plant in family Bignoniaceae (Srithongchuay *et al.*, 2008). In *Oroxylum indicum*, stigma closure may incur fitness costs. Trees of *O. indicum* exhibit lower fruit set in open-pollinated flowers (mean fruit set is 31.2%; range among trees: 14-40%; N = 7 trees) than in hand-supplemented cross-pollinated flowers (mean fruit set = 47.7%; range among trees: 16-80%), which may be due to pollination failure. In addition, the minimum number of out-cross pollen grain needed to initiate fruit set is at least 900 pollen grains (Srithongchuay *et al.*, 2008). Insufficient pollinator visitation is unlikely to be the reason for pollen-limited fruit set in this species because the nectar bat, *Eonycteris spelaea*, is a reliable and frequent visitor to *O. indicum* (Start, 1974; Srithongchuay *et al.*, 2008). Even where fruit set is very low, individual flowers are fed upon an average of 65 times per night (Srithongchuay *et al.*, 2008). A more likely reason for the low fruit set observed in *O. indicum* is that stigma closure results in insufficient conspecific pollen deposition, resulting in pollen-limited fruit set. To test this , the causes and consequences of stigma closure need to be fully understood.

1.2 Project Objectives

The objects of this study are to determine:

1) What are the causes of stigma closure in *O. indicum?*

2) What are the benefits of stigma closure to the plant?

1.3 Hypotheses

My hypotheses for the second question are:

1) If stigma closure reduces intrafloral pollens, deposition of intrafloral pollens on the closed stigma will be lesser than on forced to remain open stigma.

2) If stigma closure fixes cross pollen, deposition of cross pollen in the closed stigma will be higher than that in the forced to remain open stigma.

3) If the stigma closure increases pollen exported, less pollen will remain in flowers with closed stigmas than in flowers in which the stigmas are artificially forced to remain open.

4) If stigma closure helps to reduce pollen interference, the we can detect evidence pollen tube success following lightly hand-pollination will be higher than in nature or heavily hand-pollination.

CHAPTER 2

LITERATURE REVIEW

2.1 *Oroxylum indicum*

Oroxylum indicum is self-incompatible plant in family Bignoniaceae. In study of Srithongchuay *et al.* (2008), there were five pollination treatments to test the breeding system of *O. indicum.* Following hand-crossed pollination, 47.7% of flowers set fruit while 31.2% of the flowers in open pollination set fruit. In contrast, spontaneous selfing and insect pollination set no fruit. Only a single flower from one tree in self-induced pollination treatment set fruit. Srithongchuay *et al.* (2008) found that at least 900 out-cross pollen grains were needed to initiate fruit set when three amount of pollen grain treatments (300,600, 900 pollen grains) were hand-pollinated in 12 flowers in each treatment. Only 7 flowers hand-pollinated with 900 pollen grains set fruit, while other treatment set no fruit.

The number of flowers per inflorescence varied between 30 and 62 flowers. Only one or two flowers per inflorescence were open in each night, and each inflorescence generally flowered for 10 weeks. The flowers opened from 1900h to 2030h. Flower opening was quite rapid, taking about 5 minutes. The corolla droped at about 0200h on the night of opening. The number of ovules and pollen grains in each flower was 306 ± 20.8 and 269600 ± 750, respectively. The pollen:ovule ratio was 884:1 (n = 25). The stigma was receptive during the period of flower opening. Anthesis occurs between 1900h-2040h which is time of flower opening or slightly thereafter. More than half of pollen grains remain germinating in 15% sucrose solution throughout the night and decrease to about 30% 24 h after anthesis (Figure 1). Flowering occurs throughout the year in southern Thailand; individual trees flower continuously. The stigma of *O.indicum* takes a few seconds to totally close after following pressure, and close permanently if stigma recieves conspecific pollen (Figure 2).

From the study of Srithongchuay *et al.* (2008), nectarivorous bat, *Eonycteris spelaea* was the main visitors to *O. indicum*. The first bat arrived to the flower at 1930h, the peak of visiting time was 2200h – 2300h. On average, bats visited each flower 65 ± 3.5 times per night. Bats usually landed on the flowers using their feet and claws to grab the corolla tube, and then inserted snout into the corolla tube to feed on the nectar and consequently, dusted the forehead with pollen. Each
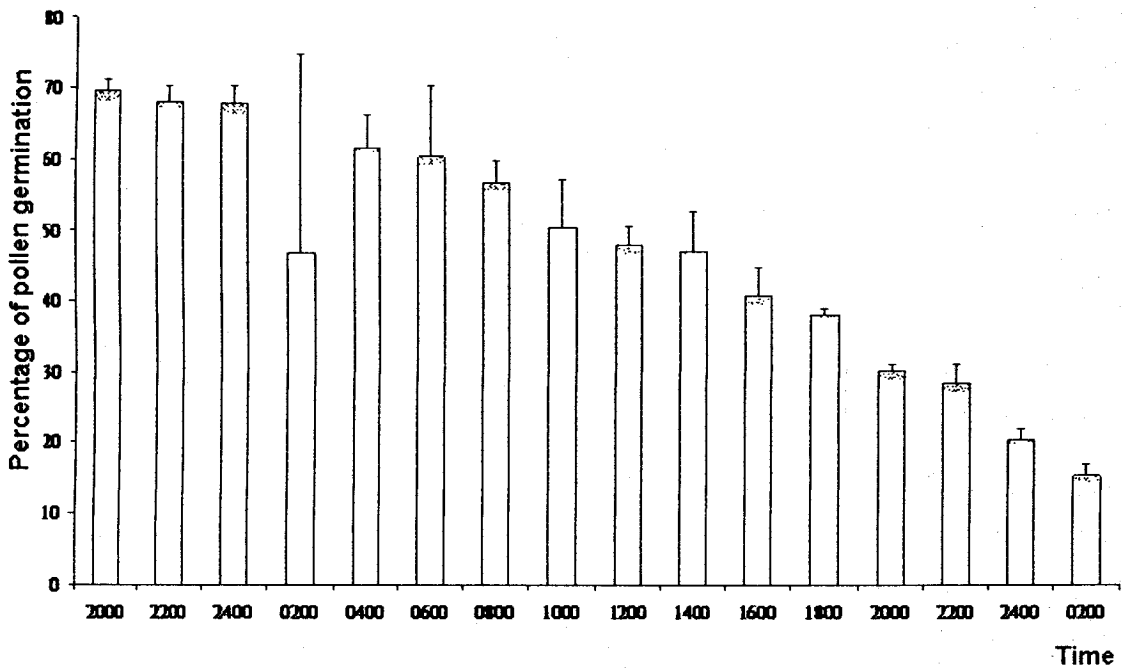
visit lasted for only 1-2 s. A single sphingid moth was observed to visit a flower, but it did not come into contact with the stigma or stamens.

Figure 1. Average germination percentage (+ SD) of pollen of *O. indicum* in 15 percent sucrose solution (n = 3 flowers sampled per time interval). In each time interval, a mean of 460.13 ± 229.17 pollen grains germinated (range 180-937). Standard deviation is shown (Srithongchuay *et al.*, 2008).
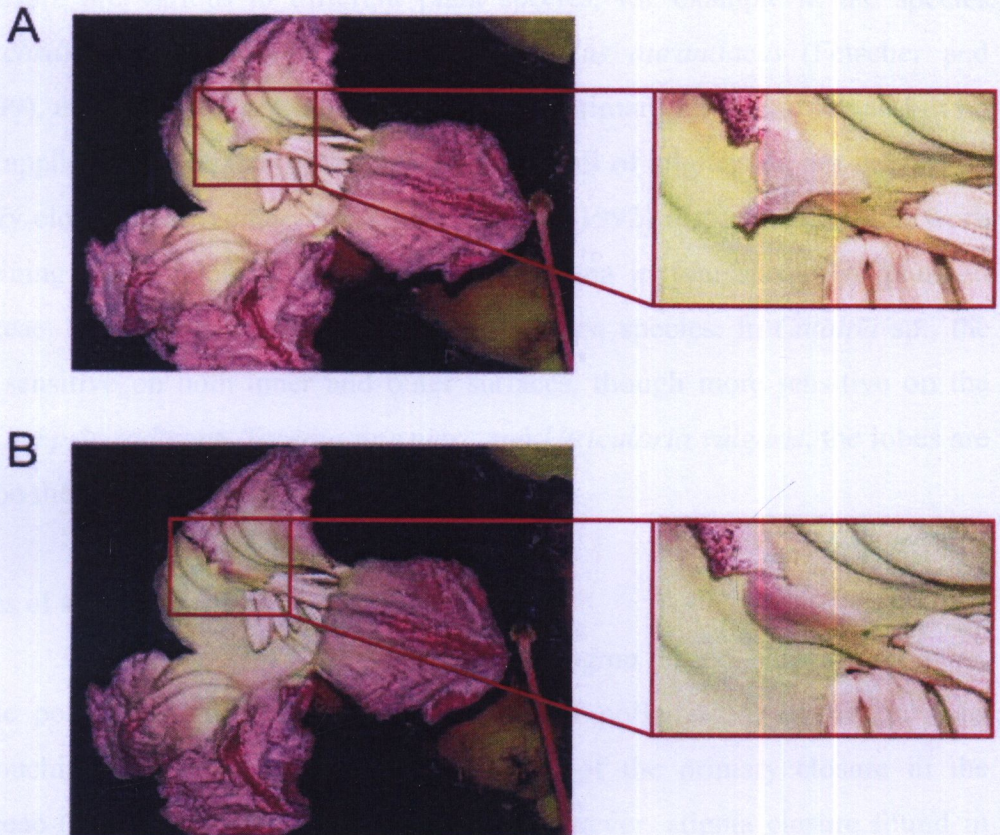
Figure 2. *Oroxylum indicum* stigmas (A) In the unstimulated flower, stigma lobes are spread apart. (B) After being touched, the stigma lobes close such that their inner surfaces are pressed together

2.2 Stigma closure

Stigma of plants in some families have two tongue-shape lobes (Newcombe, 1922; Fetscher and Kohn, 1999; Bertin, 1982). In nature two lobes of stigma are closed together by the pressure of visitors' body. The primary stigma closure occur when two divergent stigma lobes close together in the first time after

flower opening. In some plants, the stigmas remain permanently closed when pollen grains were deposited on stigma surface. However in other plants, the stigma may be opened following the first closing, and then it close without a second stimulation. The later closing are called secondary closure (Newcombe, 1922; 1924). The patterns of stigma closure are various in different plant species, for example in the species *Mimulus cardinalis* (Newcombe, 1922) and *Mimulus aurantiacus* (Fetscher and Kohn, 1999), the stigma reopen in a period after the primary closure, if touch, but no pollen, is applied. In contrast, in *Campsis radicans*, all of stigmas do not open after the primary closure (Newcombe, 1922). Newcombe (1992) also conducted the work in determining the location of sensitive area of stigma in four species of plant in Bignoniaceae. The sensitive area are different between species. In *Catalpa* sp., the lobes are sensitive on both inner and outer surfaces, though more sensitive on the inner. In *Campsis radicans, Torenia fournieri*, and *Utricularia vulgaris*, the lobes are sensitive on their inner surface only.

2.3 Causes of stigma closure

There are various factors that cause stigma closed, such as pressure, conspecific pollen, heterospecific pollen, amount of pollen load. In the previous studies, touching by normal pressure is the cause of the primary closure in the Bignoniaceae (Newcombe, 1922; Bertin, 1982). However, stigma closure found in *Spathodea campanulata* differ from all other members of the family Bignoniaceae (Newcombe, 1924). The stigmas of this plant do not close completely by pressure stimulation, but a complete closure of stigma is due to pressure and pollen combined. In addition, Bertin (1982) also found that stigma closure in *Campsis radicans* only occurs if effective pollination occurs. The study of Fetscher and Kohn (1999) exhibits that time to reopening is much longer, and the frequency of reopening much lower, when stigma was pollinated than when they were touched but no pollen transfered. In addition, interspecific pollens can also cause the stigma closure. For example, pollen of *Delphinum nelsonii* causes the reflexed lobes of *Ipomopsis agregata* stigma to close together, and the response of stigma increaseed with size of *D. nelsonii* pollen load (Waser and Fugate, 1986). Indeed, the primary closing may be induced by other factors including an electric current through the stigma (Newcombe, 1922),

hydrocyanic, ammonia vapor, crushing a portion of the style (Newcombe, 1922). These evidences indicated that different species show interspecific variation in proximate causes of stigma closure.

The causes of the secondary stigma closure are: 1) effect of pollen germination, the stigma remains closed after the primary closing or secondary closing because the pollen tubes make the way into, and take up water from cell in the stigma and style (Newcombe, 1922; Bertin, 1982; Fetscher and Kohn, 1999). 2) effect of dehydration from the stigmatic cells, the stigmata open and remain open after the primary closing if the air around them is very moist. The secondary closure is occurs and stigma remains closed if the air around them is dry. The air moisture is the important factor in *Catapa speciosa, Campsis radicans, Torenia fournieri, Mimulus cadinalis, Diplacus glutinosus* (Newcombe, 1924). In addition the stigma closure is also caused by the dry and moist pollen grains (Newcombe, 1922). The evidence indicated that the secondary closing and continued closure are due to the hydrostatic pressure of the vacuole contents pressing on the rigid cell wall of stigmatic tissue.

2.4 Benefit of stigma closure

One of suggestion on benefit of stigma closure is capturing pollen receipt. The pollen grains that come to the stigma surface with the first pollinator visiting will be removed by the next pollinator if the stigmas are still open (Fetscher and Kohn, 1999). Fetcher and Kohn (1999) compared the number of pollen grains deposition on the natural stigmas and forced to remain open by glue, and found that the mean number of pollen grains deposited by pollinators on natural stigmata is significantly lower than the mean for manipulated stigmata. However, the number of seed produced is not different between both treatments.

The stigma closure has the potential to reduce stigma and anthers interference within the same flower so avoids intrafloral self pollen to deposit on the stigma of the self incompatible plant was suggested as the benefit of stigma closure (Newcombe, 1922; 1924; Fetscher, 2001). Fetscher (2001) compared the self pollen deposition on the stigma of natural flowers and forced to remain open stigma flowers in *Mimulus* sp., and the results in this study do not show any significant difference of pollen deposition in both treatments.

Consequence of stigma closure reducing the stigma and anthers interference is that stigma closure provides more pollen dispersal and anthers behind open stigma may be protected from pollinator. Fetscher (2001) found that stigma closure in Humming bird pollinated plant; *Mimulus* sp., is highly effective in reducing the stigma interference with pollen export. The pollen export from the natural stigma flowers is 2.4 time of the number of pollen export from the flower that stigmas were forced to remain open.

Covering the stigma surface by the large number of pollen grains may reduce the seed set in the plants because the high number of pollen load have more competition or interference between each pollen. Consequently, the pollen germination of pollen from in the dense area is not well and has short level of pollen tube (Scribailo and Barrett, 1991). The former study about stigma closure help to reduce the pollen interference is not shown.

2.5 Costs of stigma closure

The stigma closure can be a cost to the plant when a few out-cross pollen grains deposit on stigma, since the stigma closure prevents later pollen deposition, In *Chilopsis linearis*, stigma closure by the legitimate visitors cost the plant fitness because these visitors carry insufficient pollen grains to stigma at the first visitation (Richardson, 2004). Moreover, permanent stigma closure by inadequate pollen grains is potentially costly to plan fitness. Richardson (2004) found several visitors of *C. linearis* carrying not enough pollen grains such as *Apis* sp., *Megachile* sp., *Anthophora* sp. In addition, interspecific pollen deposition can also cost to plant . In the study of Waser and Fugate (1986), they compared the seed set of *Ipomopsis aggregata* flowers between competition treatment which flowers were received pollen grains of *Delphinium nelsonii* for 6 hours before following the pollen of *I. aggregata*, and the control treatment in which the stigmas received only conspecific pollen. They found that heterospecific pollen decreased mean seed set by almost 50% because foreign pollen often caused stigma to close together within 1.5-6 hour, reducing stigma receptivity.

CHAPTER 3

MATERIAL AND METHODS

3.1 Study species

Oroxylum indicum Vent. (Bignoniaceae) is a self-incompatible, evergreen or partly deciduous small tree growing up to 20 m in height. Fruit bat, *Eonycteris spelaea* (Figure 3) is the main pollinator of *O. indicum* (Srithongchuay *et al.*, 2008). Although *E. spelaea*, is a frequent visitor and pollinator of this species (Srithongchuay *et al.*, 2008), fruit set is low, ranging from 3.3% to ~31% (Gould, 1978; Srithongchuay *et al.*, 2008). The flowers are nocturnal, hermaphroditic, and bell-shaped (Corner, 1988), with a cream-colored corolla tube and light purple petal lobes (Figure 4).

Oroxylum indicum is common in disturbed habitats such as open forest, roadside, villages, rice fields and along streams (Ridley, 1930; Corner, 1988; Zhiyun and Santisuk, 1998). Pods are long flat, and seeds are large flat discs (1.5 cm diam) surrounded by a large papery wing (Figure 4) that assists in wind or water dispersal (Ridley, 1930; Corner, 1988). This species is used locally for multiple purposes, including as a green vegetable, as goat feed, as a hedge row, and as lightning rods ; the seeds and bark are used medically for alleviating pain and as an anti-inflammatory agent to treat arthritis. (Corner, 1988; Ali *et al.*, 1998; Zhiyun and Santisuk, 1998; Laupattarakasem *et al.*, 2003; Lambertini *et al.*, 2004). Extracts of *O. indicum* have also been investigated for their anti-cancer properties; it has anti-proliferative properties against human breast cancer cell lines (Lambertini *et al.*, 2004; Costa Lotufo *et al.*, 2005, Palasuwan *et al.*, 2005).

Figure 3. The main pollinator of *O. indicum, Eonycteris spelaea* : A) *E. spelaea* has short chocolate brown hair, long slender snout and neck hair that are often tinged yellow. B) *E. spelaea* is visiting and inserting its long snout into the corolar tube to reach nectar at the base.

Figure 4. The productive part of *Oroxylum indicum*: (A). the funnel shape opening flower (B). Long flat pod. (C). Flat seeds surrounded by large papyrus wing

3.2 Study site

The experiments in this study were conducted on seven trees located in Songkhla Province (6° 7' N- 7° 56' N, 100° 01' E -101° 06' E, (Figure 5)). Experimental trees were at least 1 km apart (Figure 5). Flowers of these trees were

reached using permanently installed towers ca. 7 meters high. Two trees (Figure 6) were within the boundaries of the Prince of Songkla University (PSU), Hat Yai campus (07° 00.4' N, 100° 30.7' E, near Kor Hong hill), which is about 6 km long and 1 km wide with an elevation ranging from 30-140 m asl. The vegetation of the surrounding area is characterized by old growth mixed with secondary forest and a rubber plantation (Bumrungsri *et al.*, 2006). The mean annual precipitation in 2007 in this region is 2100 mm (Data from Khohong Agromet station). Another two trees were in the suburban area ~2 km south of the campus; they were surrounded by lawn, flower gardens, small trees, and single or two-story buildings. The other three trees were in the rural area ~10 km southeast of the PSU campus, surrounded by rubber plantations and orchards, with a small stream flowing adjacent to one of the trees.

Figure 5. Map of Thailand shows the study area in Hat Yai, Songkhla province, Southern Thailand.
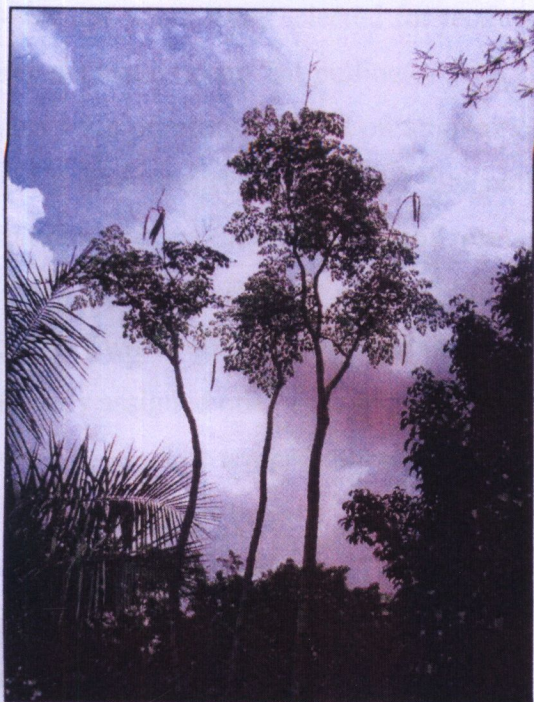
Figure 6. The experimental trees are in the boundary of Prince of Songkla University, Hat Yai campus

3.3 Experimental design

3.3.1 Causes of stigma closure and re-opening

The experiments were conducted from May 05 to August 11, 2007, over 98 nights. Nine experimental treatments were applied to detect the factors affecting stigma closure, including: (1) application of pressure only; the inner surfaces of the stigma lobes were gently touched by cotton wool covered with transparent plastic (Figure 7). The application of pressure were conducted on the inner surface because this area is the sensitive location of stigma (Sritongchuay per. Obs.); (2-3) the effect of pressure combined with *O. indicum* self pollen (low vs. high pollen loads: 300 vs. 900 grains); (4-5) the effect of pressure combined with outcross conspecific pollen (300 vs. 900 grains (initiative number to set fruit)); (6-7) the application of pressure with pollen from *Musa acuminata* (20 vs. 200 grains); (8-9)

the application of pressure with pollen from *Parkia speciosa* (20 vs. 200 polyads). *Parkia* and *Musa* were selected for testing the effects of foreign pollen because they share the same nectar bat pollinator with *O. indicum* and because the pollen of these plants were previously found on stigmas of *O. indicum* (Srithongchuay *et al.,* 2008). From former observation, 20 and 200 pollen grains of heterospecific pollen are the minimum and maximum number of foreign pollen grains found on the stigma of *O. indicum,* so we used these numbers to test the effect of pollen load on the stigma closure. Two to three flowers per tree were tested per night, and each flower was randomly assigned one of the nine treatments above.

The temperature of the air 10 cm away from each flower was recorded at the time of pollination using a digital hygrothermometer (Oregon Scientific, Portland, Oregon; model no. RTGR328n). In each treatment, a total of 30 stigmas were hand-pollinated using a cotton swab wrapped in a layer of transparent plastic film on which was placed the appropriate type and amount of pollen. The wrapped cotton swab can transfer all of pollen to stigma surface.The quantity of pollen used in the high vs. low pollen load treatments was estimated using a magnifying glass, as follows. The pollen to be applied were spread on a 5-cm diam concave glass dish. The pollen grains were examined under magnification, and a needle was used to count and group individual grains (20 at a time) until the number of pollen grains reached the desired number (e.g., 200, 300 or 900 grains), all of which were then used for pollination. The out-cross and foreign anthers were transfered from the tree, and put in to vial less than 2 hours before counting and hand pollinating.

Following each pollination, the stigma was observed to record the time to closing and to reopening after each treatment. The duration before stigma closure and the duration of closure (i.e, the time before reopening) were recorded. Stigma closure was defined to be complete when the interior surfaces of the two lobes were no longer visible; a stigma was defined to be reopened when the two lobes had opened to a 45-degree angle. After stigmas reopened, they were observed further to determine whether there was any secondary closure. The experiments were conducted each night from 2000h - 0600h, and then stigmas were observed every five hours for two days. The observation was carried out on the tower about 2 meters far from the flower that can prevent the flowers from naturaly pollinator visiting.

Figure 7. The inner surface of two lobed stigma of *O. indicum* flower was touched by the cotton wool covered with transparent plastic.

3.3.2 Benefit of stigma closure

Stigma closure fixing outcross pollen and reducing the autogamous self pollen

To test the hypothesis that stigma closure fixes outcross pollen and reduces the autogamous self pollen, the flowers were divided into two treatments. One is "opened stigma" treatment that stigmas were held open by adhering a small piece of paper to the back of each lobe using a thin layer of superglue. The other one is "natural stigma" treatment which stigmas were permitted to close naturally (Figure 8).

The anthers from 60 in both sets of flowers, opened stigma treatment and natural treatment from six trees, were sprinkled with aniline blue powder, which distinguished the pollen transferred from a flower's anthers to its own stigma from and outcross pollen; within-flower pollinations were identified by pollen grains stained with aniline blue. From preliminary study, the number of dyed pollen grain in

each flower were counted. On everage, 87.56% (SE = 4.37, N = 25) of the pollen grains in each flower were dyed by aniline blue (Sritongchuay unpubl data).

On successive nights for 45 days, one or two flowers per night were observed for ~5 hours, or until they each received 60 nectar bat visits. After 60 visits, the stigmas in the natural treatment would always have closed naturally. After the observation period, the flowers in both treatments were bagged for 24 hr to exclude further visitation, after which the stigmas were collected and the number of pollen grains recorded. The numbers of pollen grains on stigmas that were the result of within-flower transfers were determined using a fluorescence microscope and then the number of outcross pollen was recorded using a transmission light microscope.
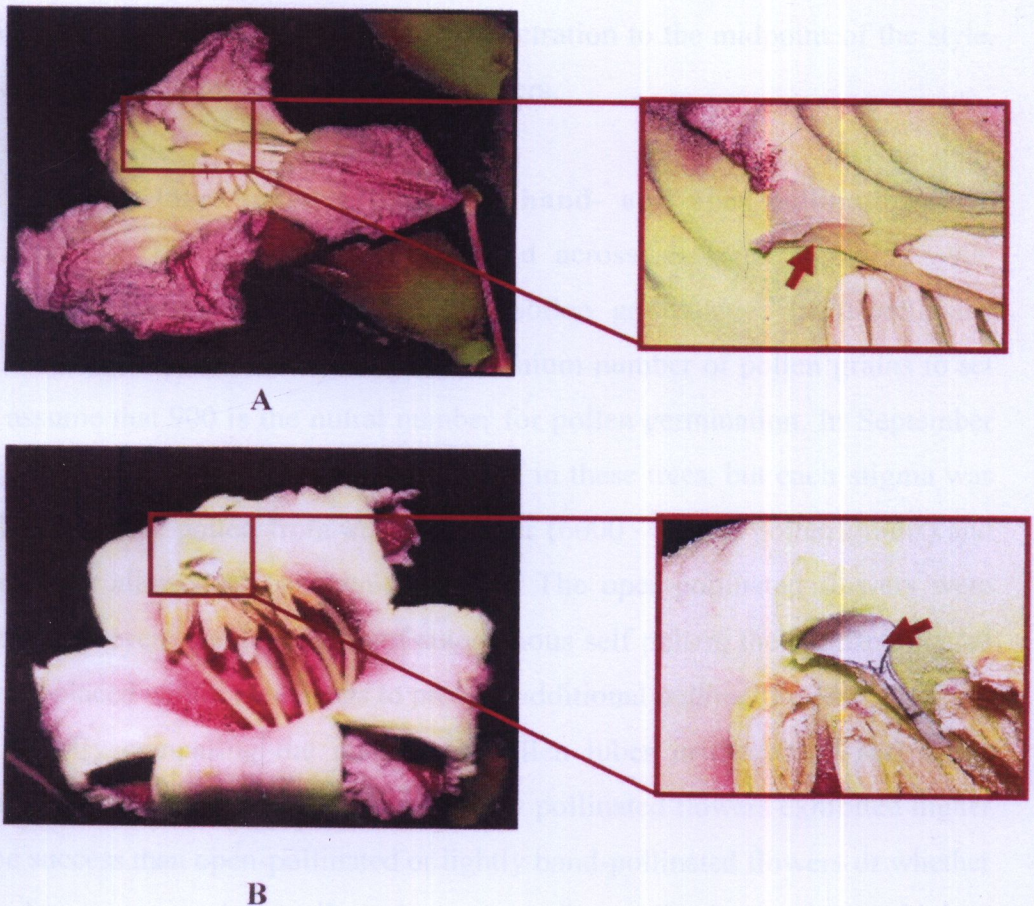
Figure 8. (A) The stigma in natural treatment. (B) The stigma in opened stigma treatment was forced to open adhered the small piece of paper with super glue on the back of the stigma lobes.

The stigma closure increase pollen exported

From June to September 2009, 30 flowers in natural flowers and 30 flowers in forced to remain open stigma from six trees were allowed to be visited naturaly by bats for single time. All of stigma in natural flower were closed after bat visiting. All of remained pollen grains were removed from five anthers and preserved in 75 % EtOH. The remained pollen grains in anthers of each flower were counted and compared between both treatments.

Stigma closure avoiding pollen interference

Detecting evidence of pollen interference: To determine whether interactions between pollen grains on stigmas may impede or interfere with pollen tube growth, we examined pollen receipt, and pollen tube penetration to the midpoint of the style, in a total of 95 naturally- or hand-pollinated flowers.

Number of pollen tubes in style (following hand- and open-pollination): In October 2007, 30 flowers were hand-pollinated across six trees, applying ~900 outcross pollen grains per stigma. The 900 pollen grains were hand-pollinated because ,in *O. indicum*, this number is the minimum number of pollen grains to set seed. We assume that 900 is the nutral number for pollen germination. In September 2007, 30 additional flowers were hand-pollinated in these trees, but each stigma was pollinated with all the pollen from a single anther (6000 – 10000 pollen grains) and 30 flowers were allowed to be open-pollinated. The open-pollinated flowers were emasculated to prevent the deposition of autogamous self pollen; the hand-pollinated flowers were placed within mesh bags to prevent additional pollination.

By comparing the number of pollen tubes in the hand- and open-pollinated styles, we could assess whether heavily pollinated flowers exhibited higher pollen tube success than open-pollinated or lightly hand-pollinated flowers or whether pollen interference may reduce pollen tube success when pollen loads are very high.

Forty-eight hours after the initiation of each treatment, the stigmas and styles were collected and preserved in FAA. Forty-eight hours was sufficient for pollen tubes to reach the base of the style (data not shown), so I assumed that counting the number of pollen tubes reaching the midpoint of the style's length after

48 hr would provide a reasonable estimate of the number of pollen tubes that could ultimately gain access to the ovary. The fixed styles were transferred to 8 N NaOH for 24 h in order to soften and clear the pistil tissue. The styles were then rinsed carefully with distilled water in a Petri dish for 12 h and then soaked for 24 h in a 0.005% aniline blue solution (prepared first as a 0.05% aniline blue solution, and then diluted to 0.005% with 0.07 N Sodium Phosphate buffer). The styles were then mounted on a slide in a drop of glycerin and gentle pressure was applied to a coverslip in order to flatten the tissue. The number of pollen tubes visible half-way down the style was recorded using a fluorescent microscope. To count the number of pollen grains deposited on the naturally-pollinated flowers, each stigma was separated from its style and placed on a slide with fuschin stain, pressed flat with a cover slip, and then viewed under a light microscope.

3.4 Statistical analyses

Analyses of covariance (ANCOVA) were conducted to detect the effects of individual tree, treatment, temperature of the flower at pollination, and all two-way interactions on the time to closure. For the time to stigma closure, all pollination treatments (including pressure only) were included; for the time to stigma reopening, pollinations using *Oroxylum* pollen were excluded because stigmas did not reopen. In these analyses, tree was considered to be a random effect. No significant differences among trees (or significant interactions that included trees) were detected (results not shown), so tree was dropped from the model.

Analyses of covariance (ANCOVA) were then conducted to examine the effects of pollination treatment, temperature, and the interaction between them on the time to stigma closure and the time to reopen. When pollen load was found to have no effect on the time to stigma closure, high- and low-pollen load flowers in each taxon were pooled and an ANCOVA was conducted to determine the effects of taxon (or pressure alone), temperature, and the interaction between them on the time to stigma closure. The statistical analyses were carried out using the JMP statistical program (version 6.0).

The effects of stigma closure on pollen receipt: Two-way mixed ANOVAs were conducted to detect significant differences among trees (random), treatments (forced-open vs. naturally closing stigmas) (fixed), and the interaction between them with respect to the amount of pollen deposited through autogamous selfing and with respect to the amount deposited due to both geitonogamy and outcrossing. There was no significant effect of tree or its interaction with treatment, so tree was excluded from the model and conducted one-way fixed ANOVAs to detect effects of stigma closure on pollen receipt.

The effects of stigma closure on pollen exported: One-way ANOVA were conducted to detect significant difference among treatment (forced-open vs. naturally closing stigma). This analysis was carried out using the R statistical program (version 2.8.1).

Factors affecting pollen tube penetration to detect evidence of pollen interference: Two-way mixed ANOVAs were conducted to detect significant differences among trees (random), treatments (heavily pollinated, lightly pollinated, and naturally pollinated flowers) (fixed), and the interaction between them with respect to the number of pollen tubes observed ½-way down the style after 48 hours.

CHAPTER 4

RESULTS

4.1 Causes of stigma closure

The ambient moisture and temperature around the flowers were 90% to 95% and 25 degrees C to 29 degrees C respectively.

Table 1 and Figures. 9 - 10 show the mean time to stigma closure and the mean time to re-open (and the sample sizes) for each treatment.

4.1.1 The effect of the pressure only.

The gentle touch of cotton wool on the stigma surface caused the stigma to close immediately. The angle between the two lobes changed from 45 deg to 0 deg within 3 - 6 sec (mean $\pm$ SE = 5.07 $\pm$ 0.17, N = 30). The stigma reopened 20 - 30 min after touching (mean $\pm$ SE = 25.6 $\pm$ 0.48, N = 30), and these stigmas did not close again unless they were touched.

4.1.2 The effect of pressure vs. pollen.

Stigmata closed significantly faster after the application of pressure alone than after pollination with conspecific pollen (Table 2; Figs. 9). Following pollination with *Musa* or *Parkia* pollen, however, the stigmas closed as rapidly as with pressure alone (Fig. 10). Stigmas with heterospecific pollen reopened significantly more slowly than stigmas to which pressure only was applied.

Table 1. Mean number of stigma closure and re-opening time in all treatments.

Treatment (N = 30 in all cases)	Mean (+ SD) time to stigma closure (sec)	SE of time to stigma closure (sec)	Mean (+ SD) time to stigma reopening (min)	SE of time to stigma reopening (min)
Pressure Only	5.07 ± 0.95	0.17	25.60 ± 2.67	0.48
Self (300 pollen grains)	5.70 ± 1.21	0.22	Never re-opened	-
Self (900 pollen grains)	6.20 ± 1.28	0.23	Never re-opened	-
Outcross (300 pollen grains)	5.53 ± 1.22	0.22	Never re-opened	-
Outcross (900 pollen grains)	5.70 ± 0.92	0.17	Never re-opened	-
Musa (20 pollen grains)	5.43 ± 0.90	0.16	66.50 ± 5.59	1.02
Musa (200 pollen grains)	5.43 ± 1.07	0.20	67.83 ± 6.14	1.12
Parkia (20 polyads)	5.00 ± 0.98	0.18	67.00 ± 6.90	1.26
Parkia (200 polyads)	5.43 ± 0.90	0.16	66.67 ± 5.66	1.03

Table 2. Summary of ANOVA to detect significant differences among treatments (Pressure Only, *Oroxylum* pollen [self vs. outcross and high vs. low pollen loads] *Musa* [high vs. low pollen loads], and *Parkia* [high vs. low pollen loads]) with respect to the mean rates of stigma closure and reopening. *Oroxylum* was excluded from the analysis of the time to stigma reopening because stigmas do not reopen following pollination with conspecific pollen.

A. Time to Stigma Closure

Source	Df	Sum of Squares	F-ratio	P-value
Treatment	8	33.794	3.90	**0.0002**
Temperature	1	3.332	3.08	0.0805
Treatment x Temperature	8	15.059	1.74	0.0897
Model	17	48.883	2.66	**0.0005**
Error	252	272.617		
Total	269	321.500		

B. Time to Stigma Reopen

Source	Df	Sum of Squares	F-ratio	P-value
Treatment	4	39842.608	313.88	**<.0001**
Temperature	1	4.454	0.14	0.7085
Treatment x Temperature	4	43.735	0.34	0.8475
Model	9	41215.434	144.31	<.0001
Error	140	4442.806		
Total	149	45658.240		

4.1.3 The effect of pollen load using conspecific pollen.

The two-way ANOVA detected no significant differences in the mean speed of stigma closure between selfed or outcrossed flowers receiving 300 vs. 900 grains (Figure 9). Following pollination with conspecific pollen, the stigmas did not reopen.
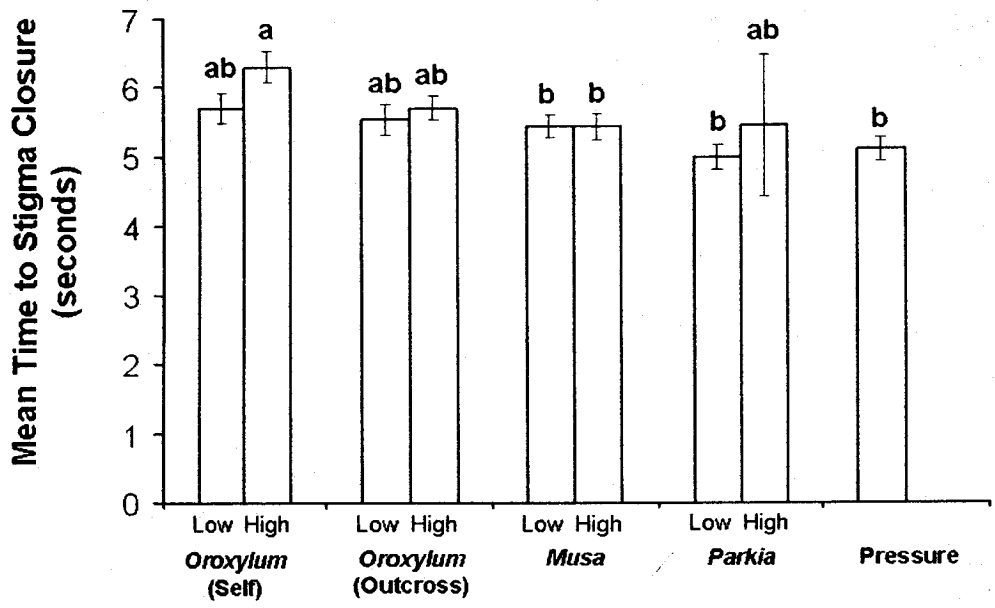
Figure 9. Mean time to closure (+ std. errors) of stigmas following each treatment. According to label above the bars, there are three significantly different groups in time to stigma closure; (a) the slowest group , (ab) intermediate group, which time to stigma close is not significantly difference from group (a) and (b), and (b) the fastest group. Group (a) and (b) are significant difference in time to stigma closure.

4.1.4 The effect of self vs. cross conspecific pollen.

The source of conspecific pollen did not affect the rate of stigma closure. Following pollination with self pollen, stigmas closed within 5 - 10 sec (mean $\pm$ SE = 5.95 $\pm$ 0.15, N = 60); following pollination with outcross pollen, they closed within 4 - 10 sec (mean $\pm$ SE = 5.62 $\pm$ 0.15, N = 60).

4.1.5 The effect of conspecific vs. heterospecific pollen.

Significant differences were detected among the three species with respect to the rate of stigma closure (Species effect: $F_{3,270}$ = 5.83, p < 0.0007; Full model: F = 3.46; p < 0.0015). Following pollination with conspecific (*O. indicum*) pollen, stigmas closed significantly more slowly than following the application of *Parkia* pollen or pressure alone (Figure 9). There was no significant difference, however, between conspecific pollen and *Musa* pollen with respect to the speed of stigma closure.

4.1.6 The effect of different foreign pollen grains.

Pollination with either *Musa* or *Parkia* pollen resulted in stigma closure within seven sec, followed by re-opening within 60-90 min. The rates at which stigmas closed and opened did not differ significantly between these species (Figue 10). No secondary closing was observed in either the pressure only treatment or in the pollination treatments.
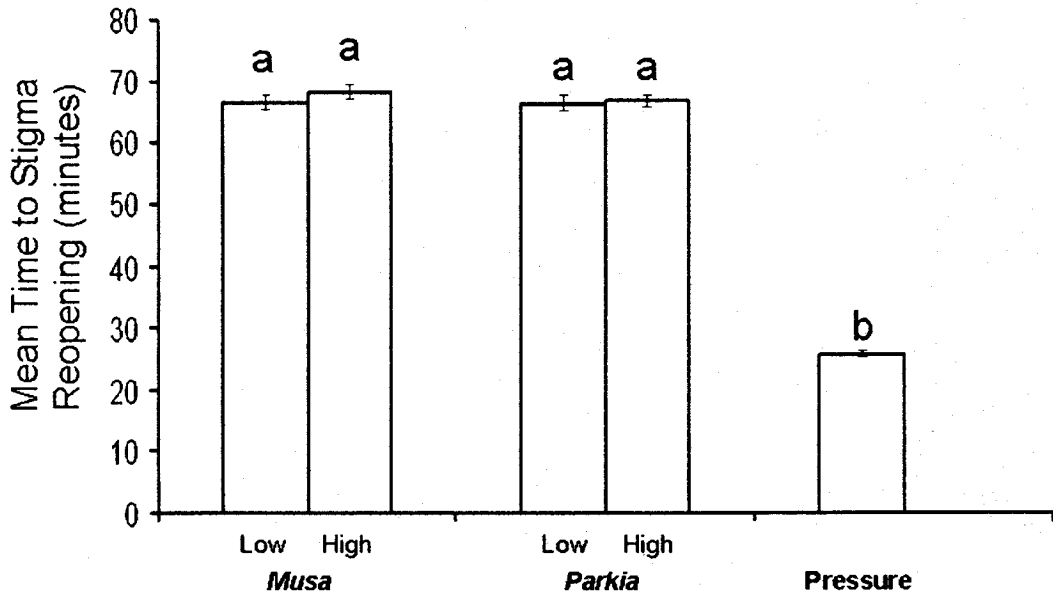
Figure 10. Mean time to reopen (+ std. errors) of stigmas pollinated with *Musa* or *Parkia* pollen or touched with pressure only. Within each taxon, stigmas with high and low pollen loads were pooled.

4.2 Benefits of stigma closure

4.2.1 The effects of stigma closure on pollen receipt

Effects of stigma closure on amount of self pollen received through autogamous pollination (comparing forced-open vs. closed stigmas). A one-way ANOVA detected no significant effect of stigma closure (vs. forcing the stigma to remain open) on the number of pollen grains received by autogamous selfing (Tables 3 and 4).

Effects of stigma closure on amount of pollen received through both geitonogamous pollination and outcrossing. There was no significant effect of stigma closure (vs. forcing the stigma to remain open) on the number of pollen grains received (Tables 3 and 4)

Table 3. Mean number of *Oroxylum* pollen grains received on stigmas that were forced to remain open vs. permitted to close naturally. Stigmas permitted to close naturally probably closed after the first pollinator visit. N = 35 stigmas in each treatment.

Stigma treatment	Mean $\pm$ SD of number of pollen grains deposited by autogamous pollination	SE of number of pollen grains deposited by autogamous pollination	Mean $\pm$ SD of number of geitonogamous pollination and outcrossing pollen grains receipt	SE of number of geitonogamous pollination and outcrossing pollen grains receipt
Forced Open	160.2 $\pm$155.01	26.20	1566.0 $\pm$ 1038.56	175.55
Permitted to Close	185.9 $\pm$ 187.73	31.73	1530.0 $\pm$ 961.51	162.52

Table 4. Two-way mixed ANOVA to detect significant differences among treatments (forced-open vs. naturally closing stigmas) with respect to the number of pollen grains deposited on stigmas due to autogamous selfing or due to the combination of geitonogamy and outcrossing.

A. Amount of autogamous self pollen received

Source	Df	Mean Square	F-ratio	P-value
Stigma treatment	1	0.0712	0.6917	0.4085
Error	68	0.1030		

B. Amount of outcross + geitonogamous pollen received

Source	Df	Mean Square	F-ratio	P-value
Stigma treatment	1	0.0001	0.0021	0.9640
Error	68	0.0634		

4.2.2 Stigma closure support pollen export.

The mean of pollen grains that remained at the flowers after bats visiting in natural stigma flowers was 247.93 pollen grains (SD = 4.173, N = 30), while the mean of pollen grains in the flowers that stigmas were forced to remain open was 298.17 pollen grains (SD = 4.384, N = 30). There was no significant effect of stigma closure (vs. forcing the stigma to remain open) on the number of remained pollen grains (Table 5)

Table 5. One-way ANOVA to detect significant differences among treatments (forced-open vs. naturally closing stigmas) with respect to the number of pollen grains remaining after bats visiting.

Source	Df	Mean Square	F-ratio	P-value
Stigma treatment	1	37851	2.2958	0.135
Error	58	16487		

4.2.3 Detecting evidence of pollen interference

Number of pollen tubes in style (given full hand- or natural-pollination): The three pollination treatments (lightly hand-pollinated, heavily hand-pollinated, and open-pollinated) yielded different levels of pollen tube growth (Table 6 and figure 11). A two-way mixed ANOVA indicated that there were no significant differences among trees (random effect) and no significant tree x treatment interaction term, but that the number of pollen tubes observed in the lightly pollinated flowers (~900 grains deposited) was significantly higher than in either the open-pollinated or the heavily hand-pollinated treatments ($F_{2,10}$ = 76.97; p < 0.0001).

Table 6. Summary of the number of pollen tubes observed following hand- and open-pollination. The heaviest pollination resulted in lower pollen tube growth or access to the style. Means with distinct superscripts differed significantly based on a two-way mixed ANOVA to detect the effects of Tree (random) and Treatment on Logt10-transformed number of pollen tubes observed.

| Pollination Treatment	Mean ($\pm$ SD) number of pollen grains received	Type of pollen	Mean number ($\pm$ SD) of pollen tubes ½-way down the style	N	Date
Lightly hand-pollinated	~900	Outcross	$460.0^a \pm 142.30$	30	October 2007
Heavily hand-pollinated	6000 – 10000	Outcross	$201.1^b \pm 40.86$	30	September 2007
Open-pollinated	1424 ± 540.13	Outcross and geitonogamous self	$191.2^b \pm 34.83$	30	September 2007
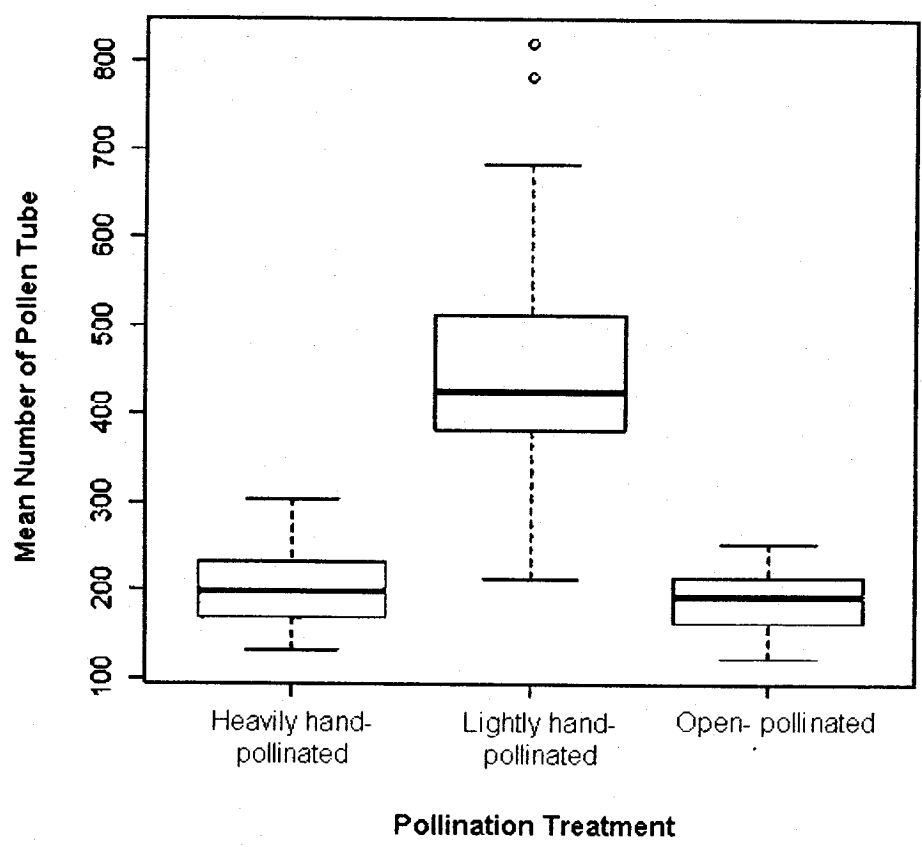
Figure 11. Number of pollen tube were observed at half way down the style in three treatments. The box represents lower quartile, median, and upper quartile.

CHAPTER 5

DISCUSSION

5.1 Factors affecting the speed of stigma closure and reopening

In the experiments reported here, all stigmas closed within a few seconds following the application of mechanical pressure with or without pollen, although pressure alone resulted in slightly faster closure than pollination with conspecific pollen. Such rapid and consistent stigma-closing behavior has been proposed to be due to a reduction in the turgor pressure of cells located in the "hinge" of the stigmas (Newcombe, 1922; 1924) in response to mechanical pressure. We found some evidence that *Oroxylum indicum* responds differently to conspecific vs. heterospecific pollen, as pollination with *Parkia* pollen resulted in slightly faster stigma closure than *Oroxylum* pollen. The biochemical or mechanical reasons for this sensitivity are unknown, although *Parkia* polyads (120 – 150 um diam) are more than twice the diameter of *Oroxylum* (60 um) or *Musa* (53 um) pollen (Stroo, 2000).

Stigmas pollinated with conspecific pollen do not reopen, unlike those that receive mechanical pressure alone or heterospecific pollen. Reopening after heterospecific pollination is clearly beneficial in that it allows the opportunity for subsequent successful conspecific pollination. Interestingly, *Oroxylum* responds differently to heterospecific pollen than to pressure alone: stigmas receiving heterospecific pollen take longer to reopen than those receiving pressure alone. This slow reopening of stigmas following heterospecific pollination reduces their potential exposure to both heterospecific and conspecific pollination. The net benefit or cost of this slow reopening depends on the likelihood and amount of pollen deposition with each pollen type.

Most previous studies of stigma behavior have found that conspecific pollen causes the most rapid, and the most permanent, stigma closure. For example, stigmas pollinated with conspecific pollen remain closed longer (and are less likely to reopen) than stigmas receiving pressure alone in *Mimulus aurantiacus* and *Campsis*

radicans (Fetscher and Kohn, 1999; Yang *et al.*, 2004). In *Campsis radicans*, pollinated stigmas also close more rapidly than those receiving mechanical pressure alone (in contrast to our observations of *O. indicum*). Similarly, in *Ipomopsis aggregata*, stigmas close slowly and reliably (in 1.5 – 6.0 hours) in response to conspecific or heterospecific pollen, but rarely in response to mechanical pressure alone (Waser and Fugate, 1986).

The failure of pollinated stigmas to reopen has been proposed to be due to the hydration and germination of pollen (particularly conspecific pollen) after adhesion; as water, nutrients, and other small molecules from stigmatic tissue are transported rapidly into pollen grains and emerging pollen tubes, the stigma becomes increasing dehydrated, and the continued low turgor pressure prevents the lobes from reopening (Newcombe, 1924; Edlund *et al.*, 2004). In at least one species, however, (*Heterophragma quadriloculare*: Bignoniaceae), the stigma does not reopen following the application of either mechanical pressure alone or of conspecific pollen (Somanathan and Borges, 2001), so the mechanisms affecting reopening may differ among species.

The closed stigma of *O. indicum* does not reopen following self-pollination, even though this species is self-incompatible. Similar to other Bignoniaceae, *O. indicum* is probably characterized by late-acting self-incompatibility, in which self pollen can germinate and pollen tubes can reach the ovary and even fertilize the ovules (Bawa, 1974; Sage *et al.*, 1999). Consequently, the affect of self pollen on stigma behavior appears to be maladaptive, at least with respect to its effects on female reproductive success.

Investigators of other species have also reported that stigmas do not discriminate between self and outcross pollination. In *Campsis radicans*, Yang *et al.* (2004) found no difference between self and outcross pollen with respect to their effect on the timing of stigma closure or reopening. Unlike *O. indicum*, however, *C. radicans* stigmas reopen within 72 – 146 min following pollination with conspecific pollen (the time to reopening increases with the pollen load). In *Ipomopsis aggregata* (Polemoniaceae), Waser and Fugate (1986) found no difference between the effects of self and outcross pollen on the probability of stigma closure. By contrast, Richardson (2004) reported that, in *Chilopsis linearis* (also in the Bignoniaceae),

stigma reopening was more likely following the application of self relative to outcross pollen.

The observation that, in *O. indicum*, heterospecific pollen results in a longer period of stigma closure (i.e., delayed reopening) than mechanical pressure alone may be due to the hydration process after pollen adhering at stigma surface. Although heterospecific incompatibility systems usually take place in higher plants, foreign pollen may absorb water from the stigmatic tissue of some plants (Hülskamp *et al.*, 1995). The resulting dehydration of the stigma may be the cause of its closure in *O. indicum* (and its delayed opening relative to pressure alone) following pollination with *Musa* or *Parkia* pollen.

5.2 Fitness consequences of stigma closure

5.2.2 Stigma closure fixes outcross pollen and reduces the autogamous self pollen

We predicted that stigma closure may affect the receipt of either self or outcross pollen, but we detected no significant difference between stigmas that were forced to remain open and those permitted to close naturally with respect to the amount of either autogamously transferred pollen or the amount of geitonogamous and outcross pollen (combined). Given that *Oroxylum* stigmas close immediately following the receipt of conspecific pollen and do not reopen, if the proportions of geitonogamous vs. outcross pollen do not differ between the forced-open and the naturally-closed stigmas, then stigma closure does not confer an immediate fitness "cost" due to reduced outcross pollen receipt. We cannot foresee a mechanism that would result in stigma closure affecting the proportions of geitonogamous and outcross pollen deposited on individual stigmas.

Our results for pollen receipt contrasts with those of *Mimulus aurantiacus*, in which stigmas forced to remain open received more outcross pollen than those permitted to close (Fetscher and Kohn, 1999). Fetscher (2001) reported that stigma closure is unlikely to reduce the amount of self pollen deposited on *M. aurantiacus* stigmas, and ultimately concludes that the benefits of stigma closure in that species derive from its effects on the rate of pollen export.

Two general results suggest that in *O. indicum*, stigma closure may incur a fitness cost, at least with respect to female reproductive success. First, as described above, heterospecific pollination results in rapid stigma closure and delayed reopening relative to pressure alone, reducing the opportunities for conspecific pollination during the period of stigma closure. The cost of stigma closure following the deposition of heterospecific pollen will be most severe when pollinators are rare. Surprisingly, we found that the duration of stigma closure was similar in response to both high and low heterospecific pollen loads; we had expected that stigmas with high pollen loads would reopen more slowly due to increased stigma dehydration.

Second, although we found that stigmas that were forced to remain open received the same amount of non-autogamous pollen as those permitted to close, our hand-pollinations found that stigmas close rapidly even when the number of conspecific pollen grains deposited is as low as 300 pollen grains, which is more than the number of ovules per flower but less than the amount necessary to set fruit (e.g. 900 outcross pollen grains in *O. indicum*, Srithongchuay *et al.*, 2008). This high sensitivity can therefore prevent the receipt of the amount of pollen necessary for fruit set. Moreover, a previous study found that 40% of the stigmas collected after a single visit by its principal pollinator, *Eonycteris spelaea*, received fewer than 900 grains (Srithongchuay *et al.*, 2008). Given the low fruit set in *O. indicum*, stigma closure following pollination with low pollen loads would seem to be selectively disadvantageous. The potential for stigma closure to be responsible for low fruit set was suggested by Somanthan and Borges (2001) to apply to *Heterophragma quadriloculare* (Bignoniaceae). In contrast to *O. indicum*, permanent stigma closure occurs in *Campsis radicans* only if enough pollen is received to ensure fruit set (Yang *et al.*, 2004).

Other studies have reported that stigma behavior can be highly sensitive to pollen loads in patterns that appear to be adaptive. For example, in *Chilopsis linearis*, the stigmas that reopen following conspecific pollination have fewer pollen grains than those that remain closed, implying that larger pollen loads are more likely to result in permanent closure (Richardson, 2004). In *Campsis radicans*, following pollination with high pollen loads (>350 grains), stigmas closed more rapidly, opened more slowly (if they did reopen), and were less likely to reopen

(10% vs. 95%) than stigmas pollinated with low pollen loads (<150 grains) (Yang *et al.*, 2004). When pollinated with interspecific pollen (*Delphinium nelsonii*), the stigmas of *Ipomopsis aggregata* generally close within 1.5 hours (very slowly relative to *O. indicum*), and this response is most frequent following the deposition of heavier pollen loads (Waser and Fugate, 1986). In our study, pollen load did not affect any component of stigma behavior following either conspecific or heterospecific pollination; Fetscher and Kohn (1999) similarly found no effect of pollen load on the probability of stigma reopening in *Mimulus aurantiacus*.

In spite of these apparent costs of stigma closure, this behavior has been proposed to provide benefits such as enhancing pollen receipt, avoiding or reducing intrafloral self-fertilization, improving germination conditions, increasing pollen export, and preventing stigma clogging (Newcombe, 1922; 1924; Fetscher amd Kohn, 1999; Fetscher, 2001; Scribailo and Barrett, 1991). Additional experiments to determine the advantages of stigma closure in this plant are required to investigate these possibilities.

5.3.3 The stigma closure increase pollen exported

According to, the number of pollen grain that remained at flower allowing bat visiting was not significantly different between naturally closed stigma treatment and forced to remain opened stigma treatment, thus we can infer that stigma closure does not provide the benefit to *O. indicum* in term of increasing pollen exported. The results in this study are different from the previous study in *Mimulus* sp. (Fetscher, 2001). Fetscher (2001) found that flowers with closed stigma export more pollen than in which the stigma is forced to open, thus the stigma closure play the important role reducing female interference with male function.

5.3.4 Detecting evidence of pollen interference

Two results suggest that pollen grains may interfere with each other following pollination of *O. indicum* stigmas, although further research is necessary. First, heavily hand-pollinated flowers exhibited fewer pollen tubes than lightly hand-

pollinated flowers (201.1 vs. 460). These observations were made in different months (October vs. September 2007), however, so we cannot rule out environmental differences as a contributing factor. Second, the heavily hand-pollinated flowers exhibited the same number of pollen tubes as the open-pollinated flowers, even though the former received 5 – 10 times as many outcross pollen grains. These results suggest that stigma closure may improve pollen tube access to the style by preventing excess pollination. On the other hand, open-pollinated flowers (which may receive a considerable amount of incompatible pollen transferred from other flowers on the same tree) exhibited fewer pollen tubes than the lightly hand-pollinated treatment (which received only outcross pollen). Perhaps stigma closure limits pollen tube success by reducing the number of outcross pollen grains received. In addition, our emasculation of the open-pollinated flowers prevented autogamous self pollination, reducing to some extent the potential for interference among pollen grains. This may have increased the success of pollen tubes in this experimental treatment compared to open-pollinated flowers that had not been emasculated. In nature, open-pollinated flowers may experience even lower pollen tube success than what we observed here.

CHAPTER 6

CONCLUSION

The stigmas touched by pressure only and pressure combined with foreign pollens reopened after closed for 20-30 and 60-90 minutes respectively. In the contrast, the stigmas never reopened after touched by conspecific pollens either self or cross, and either many (900 pollen grains) or a few (300 pollen grains). The stigma closure provide disadvantage to *O. indicum* if the stigma is closed by the small number of pollen and self pollen.

Stigma closure did not affect the number of pollen grains received; stigmas permitted to close naturally received the same number of autogamously deposited self pollen and the same total number of conspecific pollen grains as stigmas that were mechanically forced to remain open. Stigma closure did not affect the number of pollen export. In the detecting evidences of pollen interference experiment, pollen tube numbers were highest following light pollination (~900 grains), revealing evidence for pollen interference following pollination with very heavy loads (>6000 grains). Consequencely, stigma closure may improve pollen tube access to the style by preventing excess pollination.

A more comprehensive study would include estimating the effects of stigma closure on measures of female reproductive success such as self vs. outcross pollen receipt, pollen tube growth, and ovule fertilization rates.

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