Euphytica (2017) 213:231
DOI 10.1007/s10681-017-2019-9

Pollen viability and stigma receptivity in *Lilium* during anthesis

Guoren He · Fengrong Hu · Jun Ming · Chun Liu · Suxia Yuan

Received: 16 January 2017 / Accepted: 31 August 2017 / Published online: 9 September 2017
© Springer Science+Business Media B.V. 2017

Abstract Pollen viability and stigma receptivity are prerequisites for successful pollination and seed set in flowering plants. In this study, the pollen viabilities and stigma receptivities of nine *Lilium* genotypes (six cultivars and three species native to China) were assayed by in vitro pollen germination and the benzidine-H_2O_2 method, respectively. Embryo sac development during anthesis was observed to further ensure the timing of controlled pollination. In addition, the relationship between stigma secretion and stigma receptivity was studied to estimate the pollination time based on phenotype. Anthers cracked on the day of flowering in all genotypes, but pollen germination during anthesis was not observed in Asiatic hybrids excepted for 'Tiny pudhye', which exhibited low pollen viability for a short period of time (from 0 to 1 day after anthesis). In the other genotypes, pollen germination rates were highest on anthesis (five of seven genotypes), 0–1 day after anthesis (*L. sulphureum*), or 0–2 days after anthesis (one Longiflorum hybrid), and then gradually decreased with days after anthesis. While, stigma receptivity first increased and then decreased during anthesis. For most genotypes, stigmas began to be receptive 1 day after anthesis, and all genotypes exhibited stigma receptivity at 2 days after anthesis. The durations of stigma receptivity and strongest stigma receptivity, were genotype dependent, and were 5–8 days and 1–4 days, respectively. Moreover, on the first flowering day, 6 of 7 genotypes had mature embryo sacs, and at the time at which stigmas began to be receptive, all tested genotypes had mature embryo sacs. Some *Lilium* genotypes showed stigma secretion, which can be a sign of stigma receptivity. Stigmas became receptive and reached highest receptivity within 1 day of the first appearance of secretion on the surface of the stigma and at peaking, respectively. The results of this study are valuable for the implementation of successful *Lilium* breeding programs.

Keywords *Lilium* · Pollen viability · Stigma receptivity · Embryo sac development · Stigma secretion

Guoren He and Fengrong Hu have contributed equally to this work.

G. He · J. Ming · C. Liu · S. Yuan (✉)
Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing 100081, China
e-mail: yuansuxia@caas.cn

G. He · F. Hu
College of Landscape Architecture, Nanjing Forestry University, Nanjing 210037, China

Introduction

The genus *Lilium* includes approximately 100 species and an abundant number of commercially important cultivars (McRae 1998; Van Tuyl et al. 2011). Lilies

are widely used in medicine, food industry, and the flower market. China is the center of the distribution of *Lilium*, with approximately 55 species (nearly 50% of the global total) (Liang and Tamura 2000), and most wild species have excellent commercial properties. For example, *Lilium regale* Wilson, *Lilium sulphureum* Baker and *Lilium henryi* Baker have been extensively used in breeding (McRae 1998; Prosevičius and Strikulyte 2004). *Lilium lancifolium* Thunberg (3x, 2n = 36) is a natural triploid in the genus (Noda 1986) and is native to eastern temperate Asia, in China, Japan and Korea. It is widely planted as an ornamental in subtropical and temperate areas, and its bulbs and flowers (without stamens) are edible (McRae 1998; Lim 2014). The cultivation of *L. lancifolium* in China has a long history, as it is one of the edible lilies and a legal medical resource, recorded in The Pharmacopeia of People's Republic of China and other historical Chinese herbal records (Lim 2014; Liu 2015).

Lily species are taxonomically classified into seven different sections (Lilium, Martagon, Pseudolirium, Archelirion, Sinomartagon, Leucolirion and Oxypetalum) based on various morphological and physiological characteristics (Comber 1949; Khan 2009). According to the description of McRae (1998), hybrid lilies are presently categorized into ten divisions (Asiatic hybrids, Martagon hybrids, Candidum hybrids, American hybrids, Longiflorum hybrids, Chinese trumpet hybrids, Oriental hybrids, Orienpet hybrids, Species and Miscellaneous hybrids) under the horticultural classification. At present, Most of popular cultivars are interspecific hybrids between the sections (e.g., Oriental × Trumpet hybrids, Longiflorum × Oriental hybrids, Longiflorum × Asiatic hybrids, etc.), and some are interspecific hybrids (Longiflorum hybrids, Asiatic hybrids and Oriental hybrids) within the sections (especially Leucolirion, Archelirion and Sinomartagon). *Lilium* species exhibit great variation in DNA content, ranging from 13.7 to 47.9 pg/1C with an average of 36.0 pg/1C (Bennett and Leitch 2012). Accordingly, the genetic backgrounds of modern hybrids with different ploidies are complicated, and most genetic loci have a high degree of heterozygosity (Zhou 2007; Xie 2012). Furthermore, some hybrids are sterile, especially triploids (Asano 1982). In our previous research, we found that the abnormal meiotic frequency of pollen mother cells during meiosis was closely related with heterozygosity. (For example, the wild species *L. regale* had the lowest observed abnormal meiotic rate of 7.65% at the tetrad phase, and two Oriental hybrids had lower abnormal meiotic rates than the Oriental × Trumpet hybrid 'Robina', which had a high abnormal meiotic rate of 71.57%; Li et al. 2012.) Therefore, pollen viability is related to heterozygosity in lilies.

In *Lilium* breeding, crossing is the most important tool to combine agronomic traits in new hybrids. Within a section it can be made with relative ease, but intersectional hybridization is very difficult (Barba-Gonzalez 2005). To address this challenge, studies of the factors affecting pollen growth in intra- and interspecific pollinations, pre- and post-fertilization barriers in interspecific crosses and the interaction between the pollen tube and pistil have been conducted (Ascher and Peloquin 1966, 1968; Ascher 1975; Amaki and Yamamoto 1988; Van Tuyl et al. 1982, 1991; Janson 1992; Janson et al. 1993), and many different techniques have been developed to overcome pre- and post-fertilization barriers in lily breeding (Asano and Myodo 1977; Asano 1978; Van Creij et al. 1990; Van Tuyl et al. 2011). Whether intra- or inter-sectional crosses are involved, both pollen viability and embryo development are important for ensuring successful controlled pollination. In addition, when performing crosses of compatible lilies within sections or hybrid divisions, stigma receptivity is an important factor affecting seed set.

Stigma receptivity and pollen viability are critical for the effective initiation of the pollen-pistil interaction (Shivanna 2003). A stigma is receptive when it has the ability to support pollen germination, and the onset of stigmatic receptivity is accompanied by a number of changes that occur upon stigma maturation (Sanzol and Herrero 2001). The duration of receptivity can vary from a few hours to up 10 days (Dafni 1992; Shivanna 2003). According to Dafni (1992), stigma receptivity can be investigated to identify the optimum flower age for artificial pollination procedures and to increase pollination efficiency. In addition, a sticky secretion that indicates receptivity usually accompanies the female stage of the flower (Heslop-Harrison and Shivanna 1977). In *Eucalyptus woodwardii*, the amount of stigma secretion and the ability to support pollen germination and tube growth were found to increase with time, reaching a peak at 7 days after anthesis (Sedgley and Smith 1989). In *Lilium*

longiflorum, exudate production was studied from approximately 8 days before anthesis to 2 days after anthesis, and Janson et al. (1994) observed that the exudate production on the stigma and in the style started before anthesis, although it was unclear whether any of the exudate fluid originated from the micropyle. At approximately 8 days before anthesis, the stigma remained largely dry. At three to 4 days before anthesis, some papillae at the stigma were collectively covered by a layer of exudate, and at 2 days after anthesis, a thick layer of exudate was visible on the stigma (Janson et al. 1994). Moreover, the stigmatic sap and the stylar sap accelerated the growth of pollen tube in *L. longiflorum* (Amaki et al. 1989). Both the speed and the guidance of the pollen tube appeared to be determined by the properties of the exudate (Janson et al. 1994; Willemse et al. 1995). The production of stigma secretions appears to indicate receptivity for pollination and does not appear to be triggered by pollination. The pollen viability phase is the time during which pollen is able to germinate on the appropriate (receptive and compatible) stigma (Dafni 1992; Dafni and Firmage 2000). This phase varies among species and ranges from minutes to months after shedding (Shivanna and Johri 1985). Pollen viability studies are recognized as essential for understanding the reproductive performance of species and the successful implementation of breeding programs (Dafni and Firmage 2000).

Studies of pollen viability and stigma receptivity support the successful controlled pollination of *Lilium* because the application of viable pollen to the receptive stigmas increases seed set. However, systematic information for many *Lilium* species or hybrid series is lacking. Therefore, the aims of the present research were to determine the timing of the pollen viability and stigma receptivity of 6 *Lilium* cultivars and 3 species native to China and to elucidate the relationships between stigma receptivity and both embryo sac development and stigma secretion.

Materials and methods

Plant materials

A total of 6 cultivars and 3 species native to China in the *Lilium* genus were studied (Table 1). Four cultivars were introduced from the Netherlands and included three Oriental hybrids (OO) ('Sorbonne', 'Siberia', 'Starfighter'), and one Asiatic hybrid (AA) ('Tiny pudhye'). The remaining two cultivars, a *Longiflorum* hybrid (LL) ('Baiguang No. 3') was from Japan, and a AA hybrid ('Jinghe') were bred by the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China (Yuan et al. 2016). The three *Lilium* species native to China were *L. henryi*, *L. sulphureum* and *L. regale*, and were provided by the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences, Beijing, China. For each genotype, 30–50 plants were planted in experimental fields in Beijing and Kunming, Yunan Province, that belong to the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences, Beijing, China. Each bud was protected with a sulfuric acid paper bag before anthesis to avoid cross-pollination. Samples were collected from healthy plants from June to September, 2016.

Pollen germination

Pollen viability at different flowering times during anthesis was determined based on germination. For each genotype, pollen grains of dehisced anthers were collected from 3 or 6 flowers with the same flowering time between 10:00 and 11:00 am in the field, and their viability was assayed using in vitro liquid medium techniques (Griffin et al. 1982). The same number of flowers was sampled at different flowering times for each genotype. The pollen incubating solution consisted of 5% sucrose, 150 mg/L boric acid, and 20 mg/L $CaCl_2$. Approximately 5 μL of the solution was dropped on a glass slide, and then a small amount of pollen that had been collected using a toothpick was added to it. After incubation at 25 °C for 4 h, pollen germination was recorded by observation under a microscope (Olympus CX31) at ×400 magnification. A pollen grain was considered to have germinated if the pollen tube was twice as long as the diameter of the pollen (Rodriguez and Dafni 2000).

Each replicate was composed of at least 300 pollen grains from 1 or 2 flowers, and every treatment was replicated three times. Pollen germination data were analyzed by ANOVA (SAS 8.1 software and Microsoft Excel 2007), and means were compared by Duncan's multiple-range test at $P = 0.05$.

Table 1 Characteristics and locations of plant materials and the sampling times of floral organs

Cultivar/species	Series	Ploidy	Plant location	Sampling time
'Sorbonne'	OO	Diploid	Beijing	July, 2016
'Siberia'	OO	Diploid	Beijing	July, 2016
'Starfighter'	OO	Diploid	Beijing	August, 2016
'Tiny pudhye'	AA	Diploid	Beijing	September, 2016
'Jinghe'	AA	Diploid	Beijing	June, 2016
'Baiguang No. 3'	LL	Diploid	Beijing	July, 2016
L. henryi	Species endemic to China	Diploid	Kunming,Yunnan province	July, 2016
L. sulphureum	Species endemic to China	Diploid	Kunming,Yunnan province	July, 2016
L. regale	Species endemic to China	Diploid	Beijing	June, 2016

Stigma receptivity

Peroxidase activity was examined on non-pollinated stigma surfaces. Three to six fresh flowers at the same flowering time during anthesis were collected between 8:00 and 9:00 am in the field and maintained under hydration until analysis. Stigmas were then excised from the flowers using scalpels and placed on a glass slide in a drop of benzidine-H_2O_2 solution (1% benzidine:3% H_2O_2 hydrogen peroxide:water = 4:11:22, v/v; Dafni 1992). Stigma receptivity was recorded after 10–15 s by observation under a dissecting microscope. Receptivity was determined based on a reaction to the solution, in which the stigma turns blue and bubbles appear on its surface. A deeper blue with more bubbles indicated stronger stigma receptivity.

Embryo sac development

Based on the results from the stigma receptivity experiments, flowers at the first day of flowering and with initial stigma receptivity were collected, and their ovaries were removed to observe the developmental stage of the embryo sac. Five ovaries were selected for each flower development stage, yielding a total of ten ovaries over both development stages for each genotype. The middle part of each ovary was fixed in FAA (50% ethanol:glacial acetic acid:38% formaldehyde = 18:1:1) for at least 2 days at 4 °C. The samples were then dehydrated through a graded ethanol series, infiltrated with xylene and embedded in paraffin wax by conventional methods (Hu 2005). The ovaries were then sectioned on a microtome (10 µm thick sections) and stained with safranine and fast green.

Stigma secretion

The color and quantity of the stigma secretions were observed with the naked eye during anthesis.

Results

Pollen germination

Anthers began to crack on the first day of flowering in all genotypes. For the three OO hybrids and two wild species (*L. henryi* and *L. regale*), the pollen germination percentage was highest on the first day of anthesis and then decreased over time (Table 2). The highest pollen germination rate was longest in 'Baiguang No. 3' (LL) (from 0 to 2 days after anthesis), followed by *L. sulphureum* (from 0 to 1 day after anthesis). The AA hybrid 'Jinghe' exhibited no pollen germination and 'Tiny pudhye' (AA) only exhibited low pollen viability for a short period of time (from 0 to 1 day after anthesis).

Stigma receptivity

According to the results of pollen germination, seven genotypes having better fertility were selected in this experiment (Table 3). All tested genotypes showed stigma receptivity at 1 or 2 days after anthesis, and had 5–8 days of stigma receptivity. (Table 3; Fig. 1a). Stigma receptivity first increased and then decreased over time after anthesis (Table 3; Fig. 1b). The time and duration of the period of highest receptivity were genotype dependent. The period of highest stigma

Table 2 Pollen germination percentage during the course of anthesis in different lily genotypes

Genotype	In vitro germination at different days after anthesis (%, mean ± SD)							
	0	1	2	3	4	5	6	7
'Sorbonne'	80.0 ± 2.7a*	73.4 ± 2.1ab	70.9 ± 2.4b	67.4 ± 1.6b	57.0 ± 3.2c	57.0 ± 3.8c	55.0 ± 1.5c	–
'Siberia'	79.1 ± 1.0a	73.7 ± 1.8ab	72.4 ± 1.8b	63.5 ± 2.4 cd	60.5 ± 2.5 cd	54.9 ± 2.8d	54.0 ± 1.6d	44.9 ± 2.2e
'Starfighter'	28.4 ± 0.5a	26.6 ± 2.2ab	23.6 ± 1.1abc	23.0 ± 3.7abc	19.9 ± 2.2bc	17.4 ± 1.6c	11.0 ± 1.7d	–
'Tiny pudhye'	5.1 ± 0.7b	17.8 ± 1.5a	0	0	0	0	–	–
'Jinghe'	0	0	0	0	0	0	0	–
'Baiguang No. 3'	77.1 ± 1.9a	77.1 ± 1.6a	75.3 ± 2.8a	71.4 ± 1.0ab	70.9 ± 0.7ab	67.3 ± 2.3bc	61.6 ± 0.9c	54.0 ± 2.9d
Lilium henryi	78.6 ± 3.6a	75.6 ± 2.0ab	62.3 ± 4.7bc	64.8 ± 2.0c	59.6 ± 1.6 cd	56.3 ± 6.7 cd	47.6 ± 4.0d	–
Lilium sulphureum	81.8 ± 2.4a	81.0 ± 1.7a	75.6 ± 1.8b	73.2 ± 1.1bc	67.1 ± 1.8 cd	69.2 ± 2.0d	64.3 ± 1.3de	60.7 ± 0.9e
Lilium regale	84.9 ± 1.3a	74.3 ± 0.6b	73.2 ± 3.3b	73.8 ± 3.1b	69.2 ± 2.1b	68.3 ± 0.8b	52.0 ± 0.7c	–

SD standard deviation, – values missing due to the natural shedding of anthers

* Values within a genotype followed by the same lowercase letter are not significantly different at the 0.05 level of probability according to Duncan's multiple-range test

Table 3 Stigma receptivity during the course of anthesis in different lily genotypes

Genotype	Stigma receptivity at different days after anthesis								
	0	1	2	3	4	5	6	7	8
'Sorbonne'	–	–	+	+	++	++	++	+	+/–
'Siberia'	–	+	++	++	+++	+++	++	+	–
'Starfighter'	–	++	+++	+++	++	++	+/–	–	
'Baiguang No. 3'	–	+	++	++	+	+			
Lilium henryi	–	–	+/–	+	+	++	+		
Lilium sulphureum	–	+/–	++	+++	+++	+++	+++	++	+
Lilium regale	–	+	++	++	+	+/–			

– no reaction, +/– partly positive reaction, + weak positive reaction, ++ strong positive reaction, +++ very strong positive reaction

Blank space: values missing due to the natural shedding of styles

receptivity lasted for only 1 day in *L. henryi* and for 4 days in *L. sulphureum*.

Relationship between embryo sac development and stigma receptivity

Similarly, during anthesis, embryo sac development was observed in those seven genotypes (Table 4). On the first day of flowering, six genotypes had mature embryo sacs (Fig. 2), whereas development lagged in the other one genotype ('Sorbonne'). Embryo sacs in 'Sorbonne' matured 2 days after anthesis, when the stigmas began to show receptivity. In summary, all the tested genotypes had mature embryo sacs at the beginning of stigma receptivity.

Relationship between stigma secretion and stigma receptivity

No stigma secretion was observed with naked eye in the two AA hybrids. In the remaining genotypes, the secretions were colorless, shiny, wet and sticky (Fig. 1a). The results indicated that when the secretions began to appear on the surfaces of the stigmas, receptivity had begun or would begin 1 day later (Table 5). In most genotypes stigmas were most

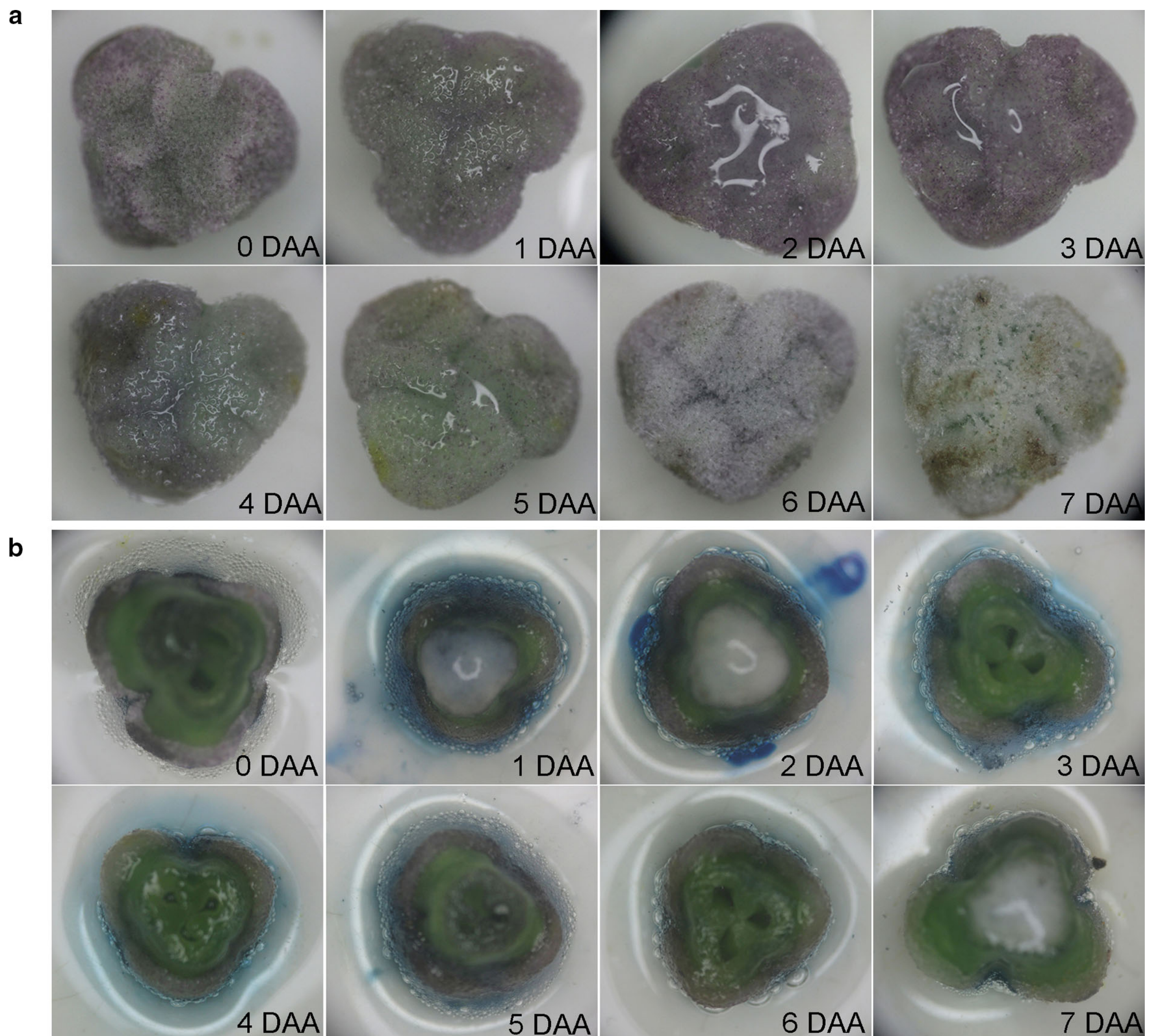

Fig. 1 'Starfighter' stigmas from 0 to 7 days after anthesis (DAA). **a** Stigmas showing no reaction but showing wet secretions (from 0 to 6 days after anthesis; Table 3). **b** Stigmas after treatment with benzidine-H_2O_2 solution

receptive when the secretions peaked, or reached highest receptivity 1 day later as in the cases of *L. henryi* and *L. sulphureum.*

Discussion

During the last few decades, polyploidy cultivars originated from hybridization of cultivars that belong to different taxonomic sections have been replacing the diploid forms due to their superior characteristics (Barba-Gonzalez et al. 2005; Zhou 2007; Zhou et al. 2008; Khan et al. 2009). Moreover, allotriploids are predominantly polyploids. These allotriploids are difficult to use as parents in breeding because the triploids are mostly sterile due to abnormal meiosis (Asano 1982, 1983). According to the hypothesis "Five same genomes of endosperm are essential for its development in *Lilium*", male-sterile triploid lilies can be used as the female parent to cross with appropriate diploid and tetraploid males to produce aneuploid progenies, but the breeding efficiency is very low (Zhou et al. 2012, 2014). Therefore, in this research, diploid genotypes representing three important groups

Table 4 Development of embryo sacs at different flowering times

Genotype	Development of embryo sac on the first day of flowering		Development of embryo sac at the initiation of stigma receptivity	
	Days after anthesis	Embryo sac development stage	Days after anthesis	Embryo sac development stage
'Sorbonne'	0	Immature	2	Mature
'Siberia'	0	Mature	1	Mature
'Starfighter'	0	Mature	1	Mature
'Baiguang No. 3'	0	Mature	1	Mature
Lilium henryi	0	Mature	2	Mature
Lilium sulphureum	0	Mature	1	Mature
Lilium regale	0	Mature	1	Mature

of hybrids (Longiflorum, Asiatic and Oriental) were tested, including some cultivars (e.g., the three OO hybrids 'Sorbonne', 'Siberia' and 'Starfighter') that are popular lilies with good characters for cut flowers in China. Another 3 species in the *Lilium* genus that are native to China were selected: *L. henryi*, *L. regale* and *L. sulphureum*, which are important parents of trumpet hybrids in lily breeding (McRae 1998). All the materials investigated in the study appear to have great potential as donor materials in breeding.

Successful seed set generally depends on the viability of pollen grains, which can be assessed by different methods, including staining with non-vital dyes, in vitro germination tests (Heslop-Harrison et al. 1984; Shivanna and Johri 1985) and in vivo tests, such as analyses of final seed set (Shivanna and Johri 1985; Razora and Zsuffa 1986). Analyzing final seed set is the most reliable method (Shivanna and Johri 1985; Bhattacharya and Mandal 2004) but has the disadvantage of a high time investment, whereas non-vital stains are useful for rapidly determining pollen viability (Kearns and Inouye 1993). The in vitro germination method has been found to be reliable for determining pollen viability in *Shorea robusta* (Bhattacharya 2011) and was used to determine pollen viability in the present study. In this research, 'Jinghe' (AA) did not exhibited pollen germination and 'Tiny pudhye' (AA) only exhibited low pollen viability for a short period of time (from 0 to 1 day after anthesis (Table 2). Furthermore, in practice, to cross with diploid AA hybrids, no seeds were got when 'Jinghe' was used as the female parent and only very few seeds can be obtained sometimes when 'Tiny pudhye' as the female parent (no data shown). So the two AA hybrids were mostly sterile. This maybe relate to AA's genetic background. These cultivars in the group of Asiatic hybrids are derived from interspecific hybridization among approximately 12 species of the Sinomartagon section, while OO hybrids are bred from interspecific hybridization between 6 species in section Archelirion and LL hybrids originate from section Leucolirion containing 4 species (Woodcock and Stearn 1950; McRae 1998). In addition, in most of those genotypes showing pollen germination, the rates were highest on the first flowering day and then slowly decreased in the days following anthesis, remaining at approximately 50% until the natural shedding of the anthers. In this experiment, the pollen germination rate of 'Starfighter' (OO) was very low throughout the flowering period; it is possible that the liquid medium used for pollen germination was not suitable for this genotype. In future research, we will attempt to formulate a

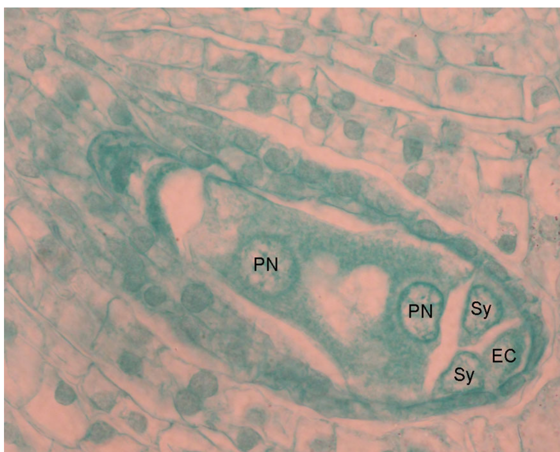

Fig. 2 Mature 'Dandie' embryo sac. *PN* polar nuclei, *Sy* synergid, *EC* egg cell

Table 5 Relationship between stigma secretion and stigma receptivity

Genotype	The time at which a secretion began to appear on the stigma (days after anthesis)	The time at which a secretion began to peak (days after anthesis)	The time at which the stigma began to be receptive (days after anthesis)	The time at which stigma receptivity began to peak (days after anthesis)
'Sorbonne'	1	4	2	4
'Siberia'	1	4	1	4
'Starfighter'	1	2	1	2
'Baiguang No. 3'	0	2	1	2
Lilium henryi	2	4	2	5
Lilium sulphureum	0	2	1	3
Lilium regale	0	3	1	2

liquid medium that is suitable for pollen germination in a wider variety of genotypes.

Stigma receptivity has been studied in only a limited number of ornamental plants (Shivanna 2003; Baskorowati 2009; Kalinganire et al. 2000; Sliva et al. 2013; Souza et al. 2016). It can be determined based on the stigma's morphological changes, pollen germination on its surface, and staining or testing for enzymatic activity; however, none of these methods provide precise estimates. According to Shivanna (2003), the only definitive method to determine stigma receptivity is through controlled pollination and subsequent analysis of effective pollen germination until fruit and seed set. However, this method is not widely applicable in *Lilium* because the genetic backgrounds of modern hybrids of different ploidies are complicated, and many crosses are not compatible. Receptive stigmas invariably present several enzymes such as esterases, peroxidases, and acid phosphatases, and the activities of these enzymes on the stigma surface have often been considered to indicate receptivity (Dafni 1992; Dafni and Maués 1998). In this study, peroxidase activity was examined on non-pollinated stigma surfaces with a benzidine-H_2O_2 solution (Dafni 1992). This solution provides a fast, simple, and easily performed method for determining stigma receptivity. In *Shorea robusta*, the duration of stigma receptivity was shown to be long, initially increasing and then decreasing during anthesis (Bhattacharya 2011). Our results in *Lilium* are similar. Stigma receptivity duration was 5–8 days in the 7 tested genotypes. For most genotypes, the stigmas began to be receptive 1 day after anthesis, and all genotypes had receptive stigmas at 2 days after anthesis. The duration of stigma receptivity was long, and at the late stage of flowering, it was not suitable for pollination. This latter finding reflects the facts that the stigma is more prone to falling off after long flowering times and that there might not be sufficient time available for pollen germination on the stigma.

The clarified ovaries of *Lilium* showed the presence of fritillaria-type embryo sac development (Maheshwari 1950) with eight cells: three antipodals, two synergids, one egg cell, and two polar nuclei (one small (n) and one large (3n)) (Fig. 2). We found the antipodal lifespan to be very short, and the cells easily disintegrated in the mature embryo sac. Furthermore, the haploid and triploid polar nuclei fused into a central cell in the mature embryo sacs only after pollination. Janson and Willemse (1995) also found that the polar nuclei did not fuse until fertilization in *L. longiflorum*. In the present research, six of 7 genotypes had mature embryo sacs on the first flowering day, and all tested genotypes had mature embryo sacs at the time at which the stigmas first became receptive. The timing of stigma receptivity initiation, the time of highest receptivity, and the duration of stigma receptivity were genotype dependent (Table 3). Therefore, the benzidine-H_2O_2 method was useful for determining the time of stigma receptivity and can thus facilitate successful controlled pollination. However, the solution is toxic, the procedure is time intensive, and stigma receptivity may be affected by external environmental conditions.

Therefore, we attempted to find a morphological index related to stigma receptivity. In practice, a wet

secretion was observed on the stigma surface during anthesis in the OO, OT and LL hybrids and some of the wild species; this secretion was colorless, shiny and sticky. In *Melaleuca alternifolia*, the secretion is yellow in color (Baskorowati 2009). Therefore, it is possible that the color of the secretion depends on the species. In *L. longiflorum*, a thick layer of exudate was observed on the top of stigma at 2 days after anthesis (Janson et al. 1994). Similarly, in our study, the secretion began to peak on the stigma surface at the same flowering time in 'Baiguang No. 3' (a *L. longiflorum* hybrid). In *Melaleuca alternifolia*, the appearance of the secretion indicated that the stigma was receptive (Baskorowati 2009). In our study, its appearance was also a sign of stigma receptivity. When the secretion began to appear on the surface of the stigma, it indicated that the stigma was receptive or would soon be receptive, and when the secretion peaked, the stigma was or would soon be at its most receptive. Therefore, for genotypes with stigma secretion in *Lilium*, the optimal pollination time can be determined based on the amount of secretion.

Acknowledgements This work was supported by the National Natural Science Foundation of China (31301804), the Special Fund for Agro-scientific Research in the Public Interest (201203071), the Chinese National Key Technology R&D Program (2012BAD01B0701), the National Center for Flower Improvement, and the Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Ministry of Agriculture, P. R. China. We also acknowledge partial funding from the Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-2016-IVFCAAS).

References

Amaki W, Yamamoto Y (1988) Pollen tube growth in the pistils grafted with style of different cultivar or with cross or self pre-pollinated styles of *Lilium longiflorum*. J Jpn Soc Hortic Sci 57(2):269–272

Amaki W, Tezuka T, Yamamoto Y (1989) Effect of pistillary sap on the in vivo pollen tube growth and changes in components of stylar sap by pollination in *Lilim longiflorum* thumb. J Jpn Soc Hortic Sci 75(4):648–654

Asano Y (1978) Studies on crosses between distantly related species of lilies. III. New hybrids obtained through embryo culture. J Jpn Soc Hortic Sci 47(3):401–414

Asano Y (1982) Chromosome association and pollen fertility in some interspecific hybrids of *Lilum*. Euphytica 31:121–128

Asano Y (1983) Random distribution of the number of chromosome pairings in interspecific hybrids of *Llilum*. Cytologia 48:803–809

Asano Y, Myodo H (1977) Studies on crosses between distantly related species of lilies. I. For the intrastylar pollination technique. J Jpn Soc Hortic Sci 46(1):59–65

Ascher PD (1975) Special stylar property required for compatible pollen-tube growth in *Lilium longiflorum* Thunb. Int J Plant Sci 136(3):317–321

Ascher PD, Peloquin SJ (1966) Effect of floral aging on the growth of compatible and incompatible pollen tubes in *Lilium longiflorum*. Am J Bot 53(1):99–102

Ascher PD, Peloquin SJ (1968) Pollen tube growth and incompatibility following intra- and interspecific pollinations in *Lilium longiflorum*. Am J Bot 55(10):1230–1234

Barba-Gonzalez R (2005) The use of *2n* gametes for introgression breeding in Oriental × Asiatic lilies. PhD-thesis, University Wageningen

Barba-Gonzalez R, Ramanna MS, Visser RGF, Van Tuyl JM (2005) Intergenomic recombination in F1 lily hybrids (*Lilium*) and its significance for genetic variation in the BC1 progenies as revealed by GISH and FISH. Genome 48:884–894

Baskorowati L (2009) Stigma receptivity and pollen viability of *Melaleuca alternifolia*. J For Res Jpn 6(1):26–36

Bennett MD, Leitch IJ (2012) Plant DNA C-values database (release 6.0, December 2012). http://data.kew.org/cvalues/

Bhattacharya A (2011) Does canopy height determine the pollen viability and stigma receptivity? A cross-population observation on *Shorea robusta* Gaertn. f. Our Nat 9:41–48

Bhattacharya A, Mandal S (2004) Pollination, pollen germination and stigma receptivity in *Moringa oleifera* Lamk. Grana 43(1):48–56

Comber HF (1949) A new classification of the *Lilium*. Lily Yearb R Hortic Soc 13:86–105

Dafni A (1992) Pollination ecology: a practical approach. Oxford University Press, Oxford

Dafni A, Firmage D (2000) Pollen viability and longevity: practical, ecological and evolutionary implications. Plant Syst Evol 222:113–132

Dafni A, Maués MM (1998) A rapid and simple procedure to determine stigma receptivity. Sex Plant Reprod 11:177–180

Griffin AR, Ching KK, Johnson KW, Hand FC, Burgess IP (1982) Processing *Eucalyptus* pollen for use in controlled pollination. Silvae Genet 31:198–203

Heslop-Harrison Y, Shivanna KR (1977) The receptive surface of the angiosperms stigma. Ann Bot 41:1233–1258

Heslop-Harrison J, Heslop-Harrison Y, Shivanna KR (1984) The evaluation of pollen quality and a further appraisal of the fluorochromatic (FCR) test procedure. Theor Appl Genet 67:367–375

Hu SY (2005) Reproductive biology of angiosperm. Higher Education Press, Beijing

Janson J (1992) Pollen tube - pistil interaction and fertilization in *Lilium Iongiflorum.* PhD-thesis, University Wageningen

Janson J, Willemse MTM (1995) Pollen tube penetration and fertilization in *Lilium longiflorum* (Liliaceae). Am J Bot 82(2):186–196

Janson J, Reinders MC, van Tuyl JM, Keijzer CJ (1993) Pollen tube growth in *Lilium longiflorum* following different pollination techniques and flower manipulations. Acta Bot Neerl 42(4):461–472

Janson J, Reinders MC, Valkering AGM, van Tuyl JM, Keijzer CJ (1994) Pistil exudate production and pollen tube growth in *Lilium longiflorum* Thunb. Ann Bot 73:437–446

Kalinganire A, Harwood CE, Slee MU, Simons AJ (2000) Floral structure, stigma receptivity and pollen viability in relation to protandry and self-incompatibility in silky oak (*Grevillea robusta* A. Cunn). Ann Bot 86:133–148

Kearns CA, Inouye DW (1993) Techniques for pollination biologists. Colorado University Press, Colorado, p 583

Khan MN (2009) A molecular cytogenetic study of intergenomic recombination and introgression of chromosomal segments in lilies (*Lilium*). PhD-thesis, University Wageningen

Khan MN, Zhou S, Ramanna MS, Arens P, Herrera J, Visser RGF, Van Tuyl JM (2009) Relevance of diploid BC progenies for intergenomic recombination and introgression breeding in Longiflorum × Asiatic hybrids of lilies (*Lilium* L.). Euphytica 166:399–406

Li J, Yuan S, Liu C, Tian M, Yu N (2012) Actors affecting viability and development of isolated microspore in *Lilium*. North Hortic 03:59–63

Liang SY, Tamura M (2000) *Lilium*. In: Wu ZY, Raven PH (eds) Flora of China, vol 24. Science Press, Beijing, pp 118–152

Lim TK (2014) *Lilium lancifolium*. In: Lim TK (ed) Edible medicinal and non medicinal plants, vol 8. Flowers. Springer, Dordrecht, pp 215–220

Liu Y (2015) *Lilium lancifolium* (Baihe, Tiger Lily). In: Liu Y, Wang ZM, Zhang J (eds) Dietary Chinese herbs. Springer, Vienna, pp 147–148

Maheshwari P (1950) An introduction to the embryology of angiosperms. McGraw-Hill, London, pp 84–152

McRae EA (1998) Lilies: a guide for growers and collectors. Timber Press, Portland

Noda S (1986) Cytogenetic behavior, chromosomal differentiations, and geographic distribution in *Lilium lancifolium* (Liliaceae). Plant Species Biol 1:69–78

Prosevičius J, Strikulyte L (2004) Interspecific hybridization and embryo rescue in breeding of lilies. Acta Universitatis Latviensis Biology 676:213–217

Razora OP, Zsuffa L (1986) Pollen viability of some Populus species as indicated by in vitro pollen germination and tetrazolium chloride staining. Can J Bot 64:1086–1088

Rodriguez RT, Dafni A (2000) A new procedure to asses pollen viability. Sex Plant Reprod 12:241–244

Sanzol J, Herrero M (2001) Review: the effective pollination period in fruit trees. Sci Hortic 90:1–17

Sedgley M, Smith RM (1989) Pistil receptivity and pollen tube growth in relation to the breeding system of *Eucalyptus woodwardii* (Symphyomyrtus: Myrtaceae). Ann Bot 64:21–31

Shivanna KR (2003) Pollen biology and technology. Science Publishers, Enfield

Shivanna KR, Johri BM (1985) The angiosperm pollen: structure and function. Wiley Eastern Ltd., New Delhi

Sliva LAC, Pagliarini MS, Santos SA, Valle CB (2013) Stigma receptivity, mode of reproduction, and mating system in *Mesosetum chaseae* Luces (Poaceae), a native grass of the Brazilian Pantanal. Genet Mol Res 12(4):5038–5045

Souza EH, Carmello-Guerreiro SM, Souza FVD, Rossi ML, Martinelli AP (2016) Stigma structure and receptivity in Bromeliaceae. Sci Hortic 203:118–125

Van Creij MGM, van Raamsdonk LWD, Van Tuyl JM (1990) Wide interspecific hybridization of *Lilium*: preliminary results of the application of pollination and embryo-rescue methods. Lily Yearb North Am Lily Soc 43:28–37

Van Tuyl JM, Marcucci MC, Visser T (1982) Pollen and pollination experiments. VII. The effect of pollen treatment and application method on incompatibility and incongruity in *Lilium*. Euphytica 31:613–619

Van Tuyl JM, Van Diën MP, Van Creij MGM, Van Kleinwee TCM, Franken J, Bino RJ (1991) Application of in vitro pollination, ovary culture, ovule culture and embryo rescue for overcoming incongruity barriers in interspecific *Lilium* crosses. Plant Sci 74:115–126

Van Tuyl JM, Arens P, Ramanna MS, Shahin A, Khan N, Xie S, Marasek-Ciolakowska A, Lim KB, Barba-Gonzalez R (2011) *Lilium*. In: Kole C (ed) Wild crop relatives: genomic and breeding resources, plantation and ornamental crops. Springer, Berlin, pp 161–183

Willemse MTM, Plyushch TA, Reinders MC (1995) In vitro micropylar penetration of the pollen tube in the ovule of *Gasteria verrucosa* (Mill.) H. Duval and *Lilium longiflorum* Thunb.: conditions, attraction and application. Plant Sci 108:201–208

Woodcock HBD, Stearn WT (1950) Lilies of the world: their cultivation & classification. Country Life, London

Xie S (2012) A molecular cytogenetic analysis of chromosome behavior in *Lilium* hybrids. PhD-thesis, University Wageningen

Yuan S, Liu C, Ming J (2016) A new lily cultivar 'Jinghe'. Acta Hortic Sin 43(9):1845–1846

Zhou S (2007) Intergenomic recombination and introgression breeding in Longiflorum × Asiatic lilies, PhD-thesis, University Wageningen

Zhou S, Ramanna MS, Visser RGF, Van Tuyl JM (2008) Genome composition of triploid lily cultivars derived from sexual polyploidization of Longiflorum × Asiatic hybrids (*Lilium*). Euphytica 160:207–215

Zhou S, Li K, Zhou G (2012) Analysis of endosperm development of allotriploid × diploid/tetraploid crosses in *Lilium*. Euphytica 184:401–412

Zhou S, Yuan G, Xu P, Gong H (2014) Study on lily introgression breeding using allotriploids as maternal parents in interploid hybridizations. Breed Sci 64:97–102