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Full Length Article

Pollen Ultra-Morphology and Pollen Viability Test of *Lilium* Oriental Hybrids

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Abstract

Ultra-morphology and pollen viability assessment are essential to understand evolutionary ecology and sterility problem, and to design hybridization program in cross-breeding. The pollen ultra-morphology of two *Lilium* Oriental hybrid cultivars, 'Sorbonne' and 'Siberia' were observed using scanning electron microscope. And the pollen germination condition *in vitro* and four dye tests methods for pollen viability were determined. The results showed that pollens of two *Lilium* Oriental hybrid cultivars were much alike in ultra-morphology with ellipsoidal shape, reticulate surface decoration and single germination ditch, indicating a close genetic relationship between two cultivars. The suitable medium for pollen germination of two cultivars composed of 10% sucrose, 0.1% boric acid and 0.5% agar. The pollen germination percent reached the highest at 25°C. Although I_2-KI stain, TTC stain and peroxidase precipitation methods were not suitable for lily pollen viability test, the pollen viability tested by acetocarmine method were close to that by *in vitro* pollen germination at suitable condition, which suggested acetocarmine stain can be as a quick test method for pollen viability of *Lilium* Oriental hybrids. © 2018 Friends Science Publishers

Keywords: Lily; *In vitro* germination; Medium composition; Acetocarmine

Introduction

Genus Lilium comprises more than 80 species (Asano, 1989) and 9,400 cultivars (International Lily register, http://www.lilyregister.com/). These are classified mainly into three groups, Longiflorum, Asiatic, and Oriental hybrids. Different lily species have a wide variety of valuable characters such as flower size, color, flowering time, and resistance to different pathogens. Oriental hybrid lilies (*Lilium* spp.) had larger and attractive flowers with wide range of white, pink, and yellow colors, strong fragrance and resistance to *Botrytis elliptica*.

Oriental hybrids 'Siberia' and 'Sorbonne' have been the most popular lily cultivars as cutting flowers around the world. 'Siberia' has straight and hardness stem, large and white flowers and strong fragrance, whereas 'Sorbonne' with pure rose-pink flowers and white margins, as well as its fragrant and beautiful shapes, have been loved by people (Du *et al*., 2005). Many studies were carried out on two cultivars to accelerate its extensive planting and utilization, including cultivation techniques research (Li *et al*., 2011; Zhang *et al*., 2012), chilling treatment on bulb (Kang *et al*., 2009) and rapid propagation *in vitro* (Chen, 2007; Zhai *et al*., 2010), together with crossing as parents to obtain new cultivars (Xia *et al*., 2010).

Up to now, the cross breeding was the only way to combine these vital horticultural traits into one cultivar, since genetic transformation approaches have not been well developed for lily yet (Arwa, 2012). It was essential to assess pollen viability for understanding sterility problem and designing hybridization program in artificial pollination and cross-breeding, as well as evolutionary ecology (Gupta and Murty, 1985; Thomson *et al*., 1994). A variety of dyes has been used to test pollen viability, such as Alexander's procedure, acetocarmine, aniline blue in lactophenol, TTC, MTT and X-Gal. But it is not clear which dye is suitable to Oriental hybrids. Simultaneously suitable medium composition and pollen culture temperature for pollen germination *in vitro* are also need to be studied. Characters of pollen morphology can be used as markers in analysis of phylogenetic relationship because they are relatively stable (Li and Qin, 1993). Up to now, ultra-morphology characteristics of lily has rarely been exploited.

In this work, two *Lilium* Oriental hybrid cultivars, 'Sorbonne' and 'Siberia', were used as experimental materials to study pollen ultra-morphology of lily by SEM and efficient method to test pollen viability *in vitro*.

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Materials and Methods

Materials

Two Oriental hybrid lily (*Lilium* spp.) cultivars, 'Sorbonne' and 'Siberia' were used as experimental materials. Upcoming flower opening, anthers were collected from two cultivars separately and put into a closet placed with dry silica gel at room temperature. Then fresh pollens were collected from naturally cracking anthers.

Observation of Pollen Ultra-morphology

The pollens were naturally dried and coated with gold, and then pasted on the sample platform with the double-sided adhesive tape and observed by scanning electron microscope (SEM, Model S-450, Japan). The morphology of the pollens including the equatorial plane, polar surface and ornamentation were surveyed under 1200 and 6000 magnification, respectively. These morphological characters were described according to the terminology of G. Erdtman (Wang and Wang, 1983).

Pollen Germinating Tests

A two-variable-two-level design was used to produce nine experimental combinations involving two key factors, sucrose concentration (5, 10 and 15%) and boric acid H_3BO_3 concentration i.e., 0.1, 0.2 and 0.3% (Table 1). Solid medium with 0.5% agar were prepared as given in Table 1 and dipped into cavity of slide and cooled to become semisolid, respectively. Then a small amount of pollen was scattered on medium with a dissecting needle. After that, the slides were placed on a piece of moist filter paper in a Petri dish and incubated for 14 h at 25°C without light. The germination of pollens was observed under a microscope (10×10 magnification). More than 150 pollens were observed to calculate the germination percentage. Pollen was scored as germination only when the pollen tube elongated longer than pollen diameter. Each medium combination had three replicates. To find the optimum temperature for pollen germination, pollens were cultured on the screened optimal germination medium incubated for 14 h at 15, 20, 25 and 30°C in the dark, respectively. The germination percentage was calculated. Each treatment had three replicates.

Pollen Viability Test by Staining Method

I_2-KI: It can detect the presence of starch. The solution consisted of 0.1% I_2-KI. The pollen grain was considered viable if it turned blue (Zhang and Geng, 2012).

Acetocarmine: It can detect the presence of nucleus or chromosome. The test solution consisted of saturation carmine in 5% acetic acid with a trace of iron ion. The pollen grain was considered viable if it turned red.

TTC: It can detect the presence of dehydrogenase. The test solution consisted of 0.5% 2, 3, 5- Triphenyl-chlorotetrazolium chloride. The pollen grain was considered viable if it turned red after being immersed in fresh test solution at 25°C for 15 min.

Peroxidase precipitation: This test detected the presence of myeloperoxidase. The test solution consisted of solution I which included 0.5% benzidine, 0.5% α-naphthol and 0.25% sodium carbonate, and solution II which included 0.3% hydrogen peroxide. The two solutions were blended just before use. The pollen grain was considered viable if it turned red after being immersed in test solution at 30°C for 10 min.

Statistical Analysis

An ANOVA (Analysis of Variance) was executed on DPS7.55 software, applying a Tuckey test, to provide the significant differences of pollen germination percentage between medium compositions, and differences of pollen viability detected between staining methods.

Result

SEM Surface Ultrastructure of Pollen

The ultra-morphology of pollens of *Lilium* Oriental hybrid cultivars, 'Sorbonne' and 'Siberia', was observed by SEM. The results showed that the pollens of two cultivars were alike with all elliptical or spindle-shaped, with single germination ditch, which dehisced from one pole to the other along the longitudinal axis (Fig. 1A, B, C and D). The ornamentations of pollens surface were latticed and had single row of columnar glyphs which consisted of obvious or no obvious disc beads-like particles (Fig. 1E and F). The difference was that the mesh size of the latticed ornamentations of 'Sorbonne' was larger than 'Siberia', in which the average size was 76.4 μm×31.0 μm in the former and 75.8 μm × 29.7 μm in the latter. In addition, the mesh of pollen ornamentations in the 'Sorbonne' had spikes or strip-like protuberances and the mesh ridge had some breakpoints and sparse tumor-like particles. However, in the 'Siberia', the mesh had rods or strip-like protuberances with the dense verrucous granule-like ridge (Fig. 1E and F). In brief, the morphology of the pollens in two cultivars was very similar except that the mesh of pollen ornamentation had some slight differences.

Optimization for *In vitro* Pollen Germination Condition

The pollen germination percentages were different significantly among various medium compositions, from 2.7 to 44.7% in Sorbonne, and from 3.1 to 26.3% in Siberia (Table 2). The effects of two ingredients of sucrose and H_3BO_3 on pollen germination were separately analyzed.

Table 1: The mediums for pollen germination *in vitro* with various sucrose and H_3BO_3 contents

No.	Content of sucrose (%)	Content of H_3BO_3 (%)
1	5	0.1
2	5	0.2
3	5	0.3
4	10	0.1
5	10	0.2
6	10	0.3
7	15	0.1
8	15	0.2
9	15	0.3

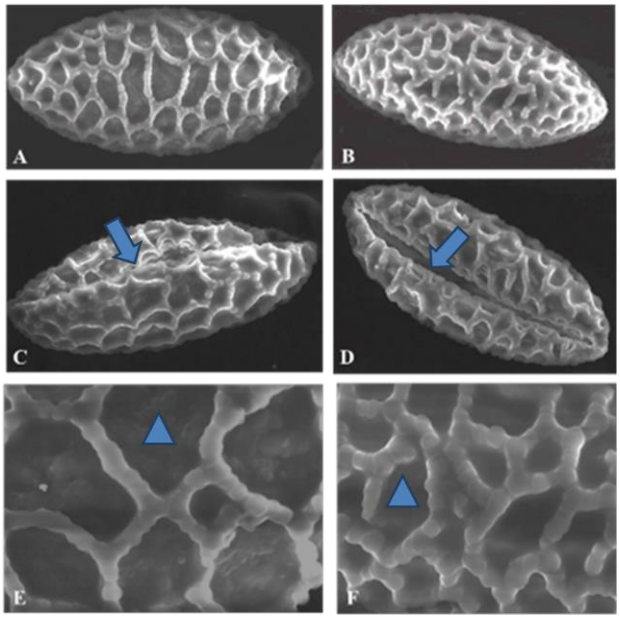

Fig. 1: Ultra-morphology of pollen grains of two Oriental hybrid lily cultivars under the SEM**.** A, C and E showed the pollen morphology of the 'Sorbonne'. B, D and F showed the pollen morphology of the 'Siberia'. A-B: pollen shape and ornamentation under 1200 magnification; C-D: germination ditch (arrows) under 1200 magnification; E-F: the mesh of pollen ornamentation (arrowheads) under 6000 magnification

The results showed that the optimum sucrose content for both pollens germination was 10%. Because the pollen germination percentages were reduced when the sucrose concentration was higher or lower than 10%, whether the H_3BO_3 content was low or high (Table 2). The optimum H_3BO_3 content for pollen germination of both cultivars was 0.2% in low concentration (5%) of sucrose, but was 0.1%under higher concentration (10%) of sucrose (Table 2). The germination medium with the highest of pollen germination percentage was 10% sucrose with 0.1% H_3BO_3 for both Sorbonne and Siberia.

On the basis of these data, the germination medium including 10% sucrose and 0.1% H_3BO_3 was employed and both pollens were separately incubated at 15, 20, 25 and 30°C to find the optimum temperature for pollen germination. The results showed that the pollen germination percentage of two cultivars reached the highest at 25°C, that was, 43.67% in 'Sorbonne' and 29.33% in 'Siberia'. However, the pollen germination percentage decreased significantly when the temperature increased to 30°C. Hence, the idea temperature for pollen germination of *Lilium* Oriental hybrids was 25°C (Fig. 2).

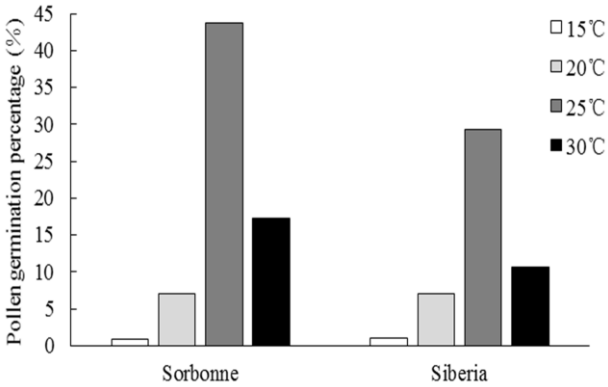

Fig. 2: *In vitro* germination percentage of fresh pollens from two cultivars' Sorbonne' and 'Siberia' under different temperatures

Comparison of Four Dye Methods to Test Pollen Viability

The results of pollen viability using the four dyes have been shown in Table 3. Two dyes including I_2-KI and peroxidase precipitation didn't stain pollens of two Oriental hybrid lily cultivars. 10.33% pollen of 'Siberia' was stained by TTC, but no pollen of 'Sorbonne' was stained. However, acetocarmine could stain pollen of both cultivars, and staining percentage arrived at 42.67% for 'Sorbonne' and 25.67% for 'Siberia', which was consistent with the optimum pollen germination percentage shown as Fig. 2. Thus, acetocarmine staining of pollen was considered to be a possible measure for two lily cultivars.

Discussion

According to the pollen characteristics of ultra-morphology, the two cultivars of *Lilium* Oriental hybrids showed consistency in the shape of pollen, the number and shape of germination aperture. The outer shape of pollen was ellipsoid. The germination aperture was single ditch which dehisced from one pole to the other along the longitudinal axis. Decoration of pollen wall was all textured. The results were consistent with previous study results in other plant species that the characters of pollen morphology were relatively stable within one species, but distinct among different species (Li and Qin, 1993).

Table 2: *In vitro* germination percentage of fresh pollens from two cultivars 'Sorbonne' and 'Siberia' on nine different medium compositions

Composition	0.1% H_3BO_3		0.2% H_3BO_3		0.3% H_3BO_3	
	Sorbone	Siberia	Sorbone	Siberia	Sorbone	Siberia
5% sucrose	10.0±5.0 cd	5.0±4.24 b	16.3±3.2 bc	9.7±3.5 b	11.0±1.0 cd	3.3±1.5 b
10% sucrose	44.7±7.6 a	26.3±1.9 a	14.3±1.53 c	12.3±4.9 b	24.0±9.5 b	4±4.4 b
15% sucrose	16.7±0.6 bc	11.7±2.5 b	16.3±3.51 bc	6.7±1.2 b	2.7±2.1 d	3.0±2.6 b

Mean ± standard deviation. Values sharing same letters differ non-significantly (*P*>0.05)

Table 3: Viability percentage of fresh pollen from two cultivars 'Sorbonne' and 'Siberia' tested by four vital dyes

Methods	Viability percentage (**%**) of fresh pollen in two lily cultivars	
	Sorbonne	Siberia
I_2-KI	0 b[x]	0 c
TTC	0 b	10.33 b
Peroxidase precipitation	0 b	0 c
Acetocarmine	42.67 a	25.67 a

Mean ± standard deviation. Values sharing same letters differ non-significantly (P>0.05)

The ultra-morphology characteristics of pollen could be employed as identification indicators for *Lilium* Oriental hybrids, which would be helpful in analysis of phylogenetic relationship of lily.

In vitro pollen germination was one of the most reliable methods to determine pollen viability. For this method, the composition of medium play important role on pollen germination. Sucrose was an indispensable element in the medium, not only as a nutrient and energy source for pollen germination, but also maintaining the osmotic potential balance between pollen and medium. The pollen germination in different species needs different concentration of sucrose. Zhao and Liu (2001) reported that sucrose concentration of 5% was suitable to Laiyang dwarf cherry and plum for pollen germination, but 10% sucrose was appropriate for the hawthorn, kiwi fruit and pomegranate. In addition, the pollen of apple and strawberry germinated well on the medium with 10-15% sucrose. Our study showed the sucrose concentration of 10% was suitable to pollen germination of *Lilium* Orient hybrids, as same as hawthorn, kiwi fruit and pomegranate. Boric acid was another important element in the medium. It could promote the absorption and metabolism of sugar, increase the absorption of oxygen and participate in pectin synthesis, which was conducive to the construction of the pollen tube in the growth wall (Hunan Agricultural University, 1992). Around 80 and 20-70 mg/L boric acid were optimal to promote the pollen germination of kumquat and litchi, respectively (Liu, 1988; Lü *et al*., 1995). Our results showed that 0.1 mg/L boric acid was optimal for the pollen germination of *Lilium* Orient hybrids, which were consistent with the proposal that the medium containing boric acid and sucrose had good effects on germination of many dinuclei pollen (such as soybean, peach and lily), but poor on the trinuclear pollen (such as Cruciferae and Asteraceae) (Leduc and Monnier, 1990; Shivanna and Linskens, 1991; Li and Qin, 1993; Wang and Chen, 1993). Temperature was also one factor, which could influence pollen germination. In this experiment, the pollen germination percentage was significantly different at different temperatures but 25°C was optimum. In short, this study showed that the medium with 10% sucrose and 0.1% boric acid was suitable for pollen germination of *Lilium* Orient hybrids under 25°C condition.

Although *in vitro* pollen germination was one of the most reliable methods, it needs to prepare an appropriate germination medium and took a long time to get results. The quick and easy test methods for pollen viability had been developed using a large variety of dyes, such as acetocarmine, TTC and X-Gal, based on different principles. However, the appropriate dyes to stain viable pollen were different for different species. For example, I_2-KI test detected the presence of starch in pollen, and TTC test detected the presence of dehydrogenase. The results of this study showed that I_2-KI, peroxidase precipitation and TTC test were not suitable for the determination of pollen viability of *Lilium* Oriental hybrids, since these dye almost stained none of pollens. The reason maybe pollen wall of *Lilium* Oriental hybrids was too thick to allow permeation of these dyes (Li and Qin, 1993), or the content of starch and activity of myeloperoxidase and dehydrogenase were too low to detect. These speculation need to be further confirmed. Fortunately, the test results of pollen viability by acetocarmine were very close to the highest percentage by *in vitro* germination. Therefore, acetocarmine test was feasible to pollen viability test of *Lilium* Oriental hybrids.

Conclusion

The morphology of the pollens of two Oriental hybrid lily cultivars was very similar except that the mesh of pollen ornamentation had some slight differences, showing a close genetic relationship between them. *In vitro* pollen germination tests revealed that the medium

with 10% sucrose and 0.1% boric acid was suitable for pollen germination of two cultivars under 25°C condition, and the pollen germination percentage of 'Sorbonne' (44.67%) was higher than 'Siberia' (24.67%). For quick and easy test methods for pollen viability, acetocarmine test was the suitable method to determine pollen viability.

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