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SEM based study for palynological and pollen germination of *Lilium longiflorum cv.* Pavia

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Abstract

Pollen is the first choice of germplasm curator, geneticist, breeder and physiologist for conservation and crop improvement programs. A pollen palynological attributes and pollen germination study of *Lilium longiflorum cv.* 'Pavia' was conducted using scanning electron microscope (SEM). Various morphological characteristics were studied. The results showed that pollens were much alike in ultra-morphology with elliptical to ellipsoidal shape, reticulate exine and single germination ditch. The pollen viability was tested by the wet room method with 3 dyes: Acetocarmine, TTC, Lugol's iodine. 49.66% pollen viability was recorded with Acetocarmine dye, which suggested acetocarmine stain can be used as a quick test method for pollen viability. The pollen germination ability was examined at set intervals, thereby establishing the dynamics of this process for 72 hours. Pollens collected just 1 h after anthesis were recorded with the highest germination (85%) compared to pollen harvested before or one day after. The suitable medium for pollen germination was 3% sucrose and 15% PEG, which was recorded with the highest pollen germination (95%) after 72 h of inoculation. Understanding evolutionary ecology and the sterility problem and designing hybridization programmes in cross-breeding necessitates a thorough understanding of pollen morphology and viability. Pollen ultra-morphology traits could be useful in determining the evolutionary relationship of lilies.

Key words: Lilium, SEM, pollen viability, pollen germination, pollen staining

Introduction

Floriculture, a vital agri-business, generates jobs and entrepreneurship, with a growing focus on cut flower exports. The industry seeks novel species or cultivars for commercial viability (Kumar and Singh, 2019).

The *Lilium* genus (Liliaceae family) has approximately 110 recognized species unique to the Northern Hemisphere (GRIN 2016). All the species are diploid (2n = 2x = 24) except *L. lancefolium*, which has either diploid or triploid. Lilium *cv.* "Pavia" is classified as LA hybrids (*Lilium asiatica x L longiflorum*), and is triploid with 2n = 3x = 36 chromosomes (Zhou and Zhou, 2012). Lilies ranked fourth among cut flowers in Flora-Holland (www.floraholland.com, 2018-19) while being the second flower bulb crop on the commercial market.

By scanning multiple surface morphologies with an electron beam, scanning electron microscopy (SEM) can reveal valuable information (Goldstein *et al.,* 2003). Pollen germination is an important phase in seed plant sexual reproduction. Furthermore, germinating pollen is frequently utilized as a model for research into the mechanisms that drive polarisation and polar development (Rounds and Bezanilla, 2013). Palynological research revealed that the genus Lilium has numerous characteristics (Pupuleku *et al.,* 2010) and pollen carbohydrate content (Clement and Audran, 1995). Comprehending the sterility problem and designing hybridization programmes for cross-breeding and artificial pollination require knowledge of pollen viability (Thomson *et al.,* 1994). To assess pollen viability, a variety of pollen stainers have been employed, including Acetocarmine, Triphenyl-chlorotetrazolium chloride (TTC), Alexande's method, Lugol's iodine, MTT, and X-Gal. However, it is unclear which coloring is best for Lilium cultivars. Simultaneously, the proper media composition for pollen germination must be investigated. Pollen Palynological characters can be utilized as phylogenetic connection markers because they are relatively stable (Li and Qin, 1993). In India, the ultra-morphology properties of the lily have rarely been harnessed. Thus, we studied the pollen morphological characteristics, viability and germination of *L. longiflorum* cv Pavia in this investigation.

Material and methods

The palynological characterization and pollen germination study of *Lilium cv.* Pavia was conducted at the Division of Plant Genetic Resources of ICAR-Indian Institute of Horticultural Research (IIHR), Bengaluru. *Lilium* bulbs were procured from Florance Flora Company, Bangalore, India. Bulbs were planted in the experimental greenhouse of the Division of Floriculture and Medicinal Crops. The fresh pollens of Pavia were used as experimental material to study the palynological characterization, pollen viability and germination process.

SEM observations: Pollen morphological characterization was performed using SEM. After 1h of anthesis, the pollens were collected from the naturally cracking anthers. The harvested pollens were placed on petri dishes and kept at room temperature for 4 h. Shape of pollen grains, polar surface and exine ornamentations were observed by SEM (Hitachi-TM-3030 Plus

SEM) under 1200 and 6000 magnifications, respectively. Each sample was observed by SEM, microphotographs at different magnifications were taken. These morphological characteristics were defined using G. Erdtman's nomenclature (Wang and Wang, 1983).

Pollen viability test by staining method

Lugol's iodine: It can detect the presence of starch in pollen tissue. The 0.1% of Lugol's iodine working solution was used. Only blue-stained pollen grain was considered viable (Zhang and Geng, 2012).

TTC dye: It can detect the presence of dehydrogenase in pollen tissue. The 2, 3, 5- triphenyl-chlorotetrazolium chloride (0.5%) test solution was used for staining. The pollen grain was considered viable if it turned red.

Acetocarmine dye: It can aid in confirming the presence of nucleus or chromosome in pollen tissue. Saturation of carmine in 5% acetic acid with a trace of iron ion was used as the test solution. If the pollen grain turned crimson, it denotes a viable or positive test.

Pollen germinating tests: Different concentrations of polyethylene Glycol (15% PEG) and sucrose were prepared in double distilled water (concentrations: 0 (control), 1, 3, 5, 7, and 10%). Pollens were collected from 6.00 am to 12.00 pm and germinated in "wet rooms" at room temperature 25±1°C. Observations were taken at intervals of 1, 6, 24, 48, and 72 hours to observe the dynamics of pollen germination capacity. The germination capacity was calculated as a percentage by dividing the total number of pollen grains by the number of germinated grains. Pollen germination was observed under a microscope at a magnification of 1010x. A total of 100 pollen grains were observed to calculate the germination percentage.

Statistical analysis: Duncan's multiple-range test (DMRT) analyzed significant variations in pollen germination (%) due to medium compositions and staining procedures for pollen viability via analysis of variance. Viability was assessed by observing 100 pollen grains per treatment in triplicate.

Results

Palynological characterization: The ultra-morphology of pollens revealed that pollen of *Lilium cv.* "Pavia" were elliptical with the reticulate exine, monosulcate, boat-shaped, heteropolar, 1-3-porate, almost spherical with a single germination ditch, which dehisced from one pole to the other along the longitudinal axis (Fig. 2C and D). The diameter measured for the polar axis

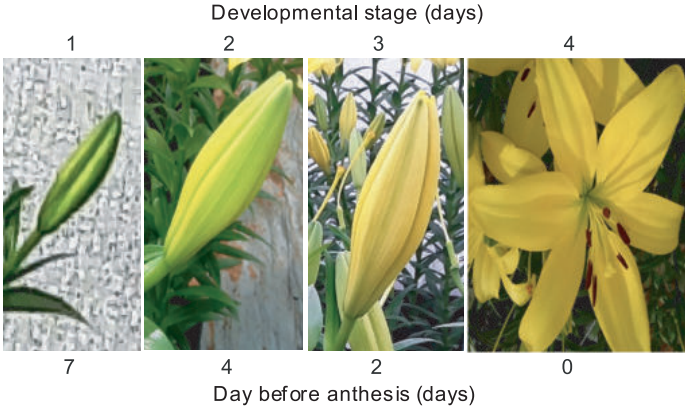

Fig. 1. Developmental stages of flower buds in Lilium *cv.* Pavia

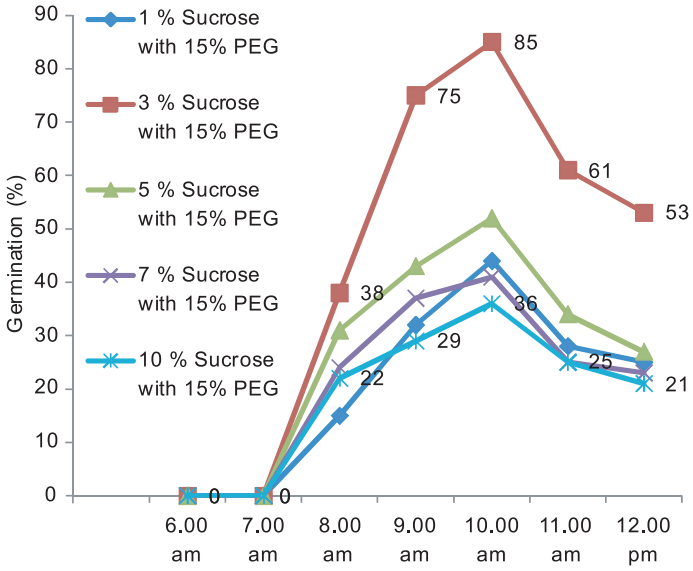

Fig. 2. Interaction effect of pollen collection time and sucrose concentration on pollen germination

was 122 µm and for the equatorial axis was 45 µm. The average between polar and equatorial axis was around 2.71. Sulcus was narrow in the center, broad and deep with rounded ends (Fig. 2A and B). Pollen surface ornamentations were latticed, with a single row of columnar glyphs consisting of obvious or non-obvious disc beads-like particles. The mesh size of the latticed ornamentations ranged from 76.4 m 31.0 m to 75.8 m 29.7 m. Furthermore, the pollen ornamentation mesh included spikes or strip-like protuberances, and the mesh ridge contained some breakpoints and sparse tumor-like particles (Fig. 2E and F).

Pollen viability test: The pollen viability results utilizing the three colours are reported in Table 1. Lugol's iodine dye did not discolour pollen from lily cultivars. 17% of pollens were stained by TTC solution (3%) after 6 hours of staining. However, acetocarmine stained maximum pollens (49.66 %), consistent with the ideal pollen germination percentage (Fig. 4). As a result, acetocarmine was chosen as the best feasible staining agent for pollen.

Table 1. Viability percentage of fresh pollen of Lilium *cv.* Pavia tested by three vital dyes

Dye used	Percentage (%) solution used			
	0.5	1.0	2.0	3.0
Acetocarmine	27.33^{c}	35.00^{c}	42.99^{c}	49.66^{c}
TTC	5.66^{b}	7.33^{b}	12.00^{b}	17.00^{b}
Lugol's iodine	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}

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

Optimization of pollen germination condition: Pollen viability was determined based on germination at different flowering times during anthesis. The pollen germination percentages significantly differed among various developmental stages (Fig. 1) and medium compositions. The interaction effect of pollen collection time and sucrose concentration on pollen germination was studied to find out the best time of pollen collection with maximum germination percentage. The results showed that the best time of pollen collection was just after anthesis when natural cracking appeared on anthers. When pollen was collected at this time, 85% of pollen germination was recorded (Fig. 2). The effects of sucrose and polyethylene glycol (PEG) on pollen germination were analyzed to optimize the best medium concentration for maximum pollen

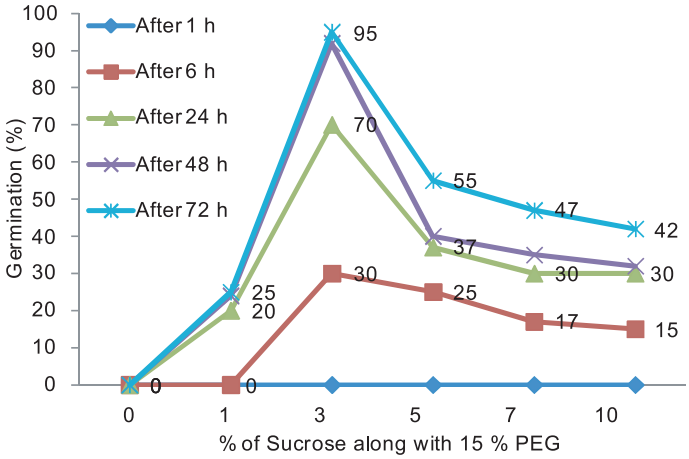

Fig. 3. Effect of different sucrose concentration with 15% PEG on pollen germination

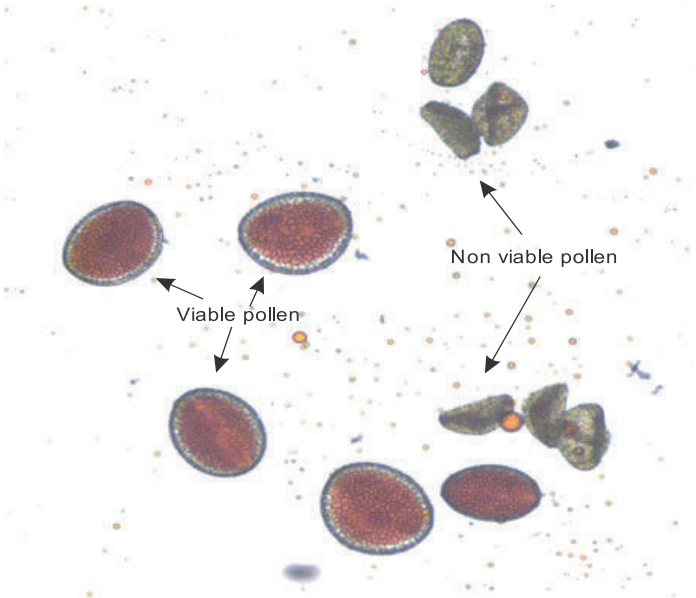

Fig.. 4. Lilium *cv.* Pavia pollen staning by Acetocarmine dye to check viablility under microscope. 1. Viable pollen stained red. 2. Non-viable pollen remain dark green.

germination. Maximum pollen germination (95%) was observed in a medium of 3% sucrose and 15% of PEG (Fig. 3). The pollen germination percentage reached 95 percent when inoculated on sucrose (3%) and PEG (15%) for 72 h at room temperature. It was observed that when the sucrose concentration was increased to 10%, the percentage of pollen germination decreased significantly. The pollen showed a very meager response to germination under control treatment compared to other treatments, which can clearly be observed in Fig. 6. According to a dynamic analysis of pollen germination, the pollen germination process is highly impacted by sucrose concentration in culture media. The extraordinarily high pollen germination rate (over 90% of pollen) suggests that the hybrid genotype's meiosis is normal.

Discussion

Lilium pollen matures shortly after anthesis, followed by desiccation to create a low-moisture environment conducive to germination. Germination entails diverse biochemical and morphological changes. Each pollen grain displays a distinct pattern on its surface during germination, observable via SEM. SEM-based palynological studies establish that pollen grains are monosulcate, a fundamental trait among seed plants and common in monocotyledons (Furness and Rudall, 2001). Similar findings were reported by Dhyani *et al.* (2009) and Muratović *et al.* (2010) in the Lilium genus. Ultra-morphological analysis reveals uniformity in pollen shape, quantity, and germination aperture shape in Lilium cv. Pavia, with ellipsoid outer form. The germination aperture, a single groove, splits longitudinally from one pole to another, with textured wall decorations.

Li and Qin (1993) noted stable intraspecies pollen morphological traits but inter-species variations. Various dyes like acetocarmine, TTC, and X-Gal were used for pollen viability testing based on different concepts. However, the best dyes varied per species. For instance, the I2-KI test indicated starch presence through deep blue color, while the TTC test detected dehydrogenase activity. This study found I2-KI, peroxidase, and TTC tests ineffective for determining pollen viability in Lilium Oriental hybrids due to limited staining. This could be due to thick pollen

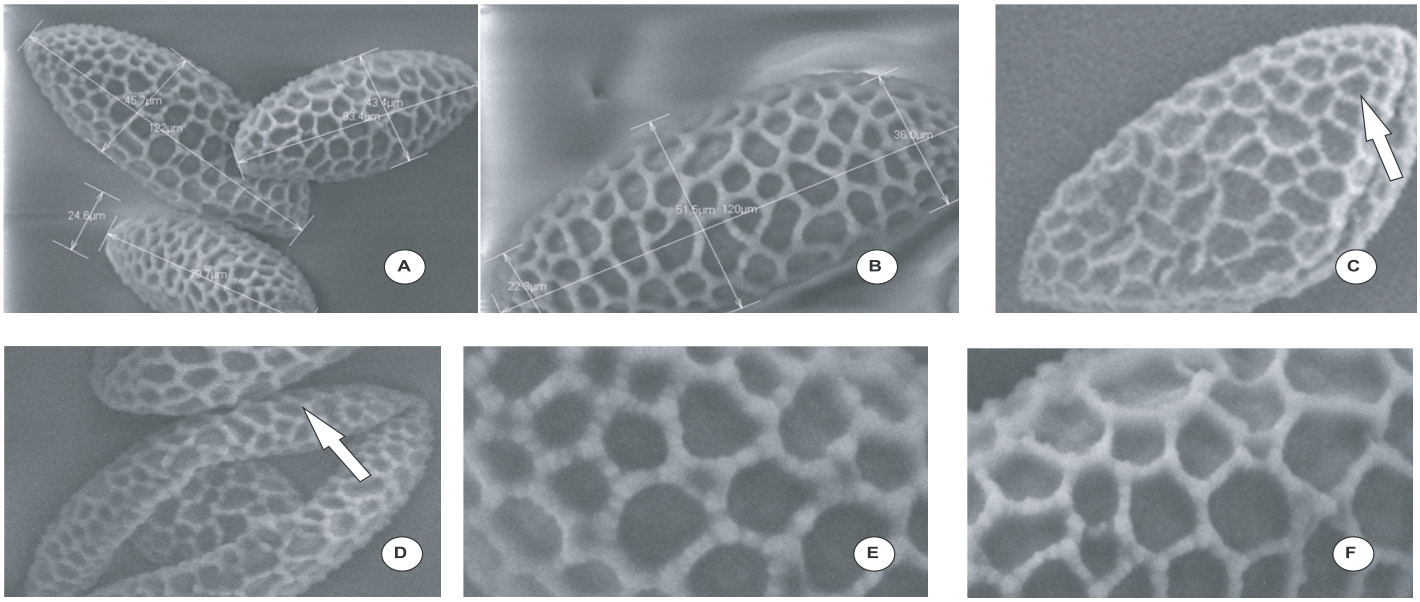

Fig. 5. Ultra-morphology of pollen grains Lilium *cv.* Pavia under the Microscope. A-B: Pollen shape and ornamentation under 1200 magnification; C-D: Germination ditch (arrows) under 1200 magnification; E-F: The mesh of pollen ornamentation (arrowheads) under 6000 magnification

walls hindering dye penetration or low starch, myeloperoxidase, and dehydrogenase activities (Li and Qin, 1993), requiring further verification. Fortunately, acetocarmine viability testing closely matched highest *in vitro* germination percentages.

Sucrose in culture mediums affects germination based on concentration, with different species requiring specific sucrose levels for pollen germination. Sugar sources, *e.g.*, maltose and sucrose, were inadequate for *Lilium oriental* and *Lilium davidii* (Quiet *et al.,* 2012). Lilium pollen could germinate in PEG-only media. Lilium pollen displayed higher amylase activity than Gladiolus pollen in PEG and boric acid solution, with reducing glucose content during pollen tube growth. Lilium pollen's PEG-mediated germination rate and independence from sugar sources could relate to natural carbohydrate types, contents, and enzyme activities (Geng *et al.,* 2013). These findings support Shukla *et al.* (2022) emphasis on phenotypic characterization's role in enhancing *Meconopsis aculeata* crop improvement."

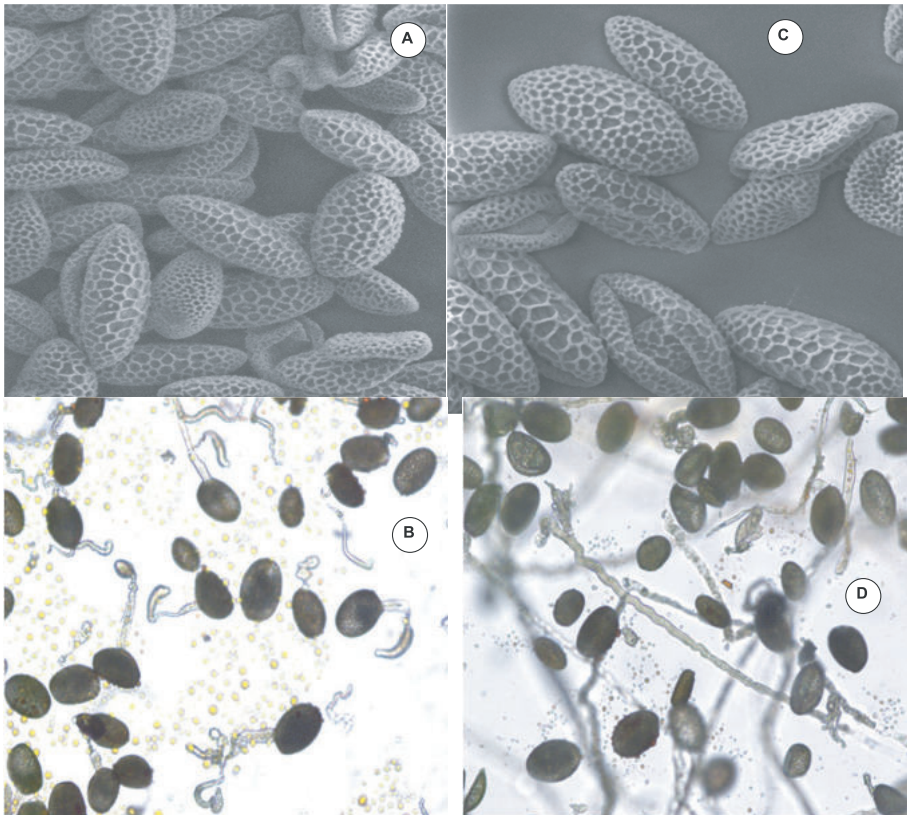

Fig. 6. A-B. Pollen grain germination of Lilium *cv.* Pavia under control; C-D: Pollen grain germination of Lilium *cv.* Pavia treated with 15% PEG and 3% sucrose

Analysis of Lilium cv. 'Pavia' pollen showed unique elliptical grains with reticulate exine and distinct ornamentations. Optimal viability testing employed acetocarmine dye, correlating well with germination outcomes. Timing and medium composition were crucial for germination rates, with peak results post-anthesis using 3% sucrose and 15% PEG in the medium. These insights enhance knowledge of pollen behavior, viability, and hold significance for plant breeding.

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