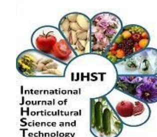
International Journal of Horticultural Science and Technology
Journal homepage: http://ijhst.ut.ac.ir



Comprehensive Analysis of Pollen Viability, *in vitro* Germination, and Self-incompatibility in *Hamelia patens* Jacq. Pollen

Kanneri Anukrishna, Mohankumar Saraladevi Resmi, Ramseena Vadakkethil Rasack, Padmanabhan Jayanthikumari Vivek*

Post-Graduate and Research Department of Botany, Sree Neelakanta Government Sanskrit College, Pattambi, Palakkad, Kerala state, India

ARTICLE INFO

Article history:

Received: 20 February 2024,
Received in revised form: 6 May 2024,
Accepted: 7 June 2024

Article type:

Research paper

Keywords:

Aniline blue Fluorescence assay,
Gametophytic self-incompatibility,
Hamelia patens,
Pollen germination,
Pollen viability

COPYRIGHT
© 2025 The author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other medium is permitted, provided the original author(s) and source are cited, in accordance with accepted academic practice. No permission is required from the authors or the publishers.

ABSTRACT

Pollen, or the male gametophyte, plays a crucial role in genetic exchange, establishment, and survival of higher plants. Experiments were conducted to assess pollen viability, *in vitro* pollen germination, and *in vivo* pollen tube growth in *Hamelia patens* Jacq., a notable species from the Rubiaceae family. Among the various staining techniques applied, the modified Alexander staining method proved to be the most effective for evaluating pollen viability in this species. The viability results obtained using this method (81.80%) closely aligned with the highest pollen germination rate observed *in vitro* (81.33%). *In vitro* analyses indicated that incorporating an optimal sucrose concentration (10%) along with lower concentrations of boron (0.01%) and calcium (0.01%) in the artificial medium significantly enhanced both pollen germination percentage and pollen tube growth rate. The isolated application of these factors was less effective in improving germination rates compared to their combined use, which substantially boosted pollen germination and tube elongation. Additionally, *in vivo* pollen germination studies using aniline blue fluorescence microscopy support previous findings of a self-incompatibility system in *Hamelia patens*, confirming that it does not involve a late-acting self-incompatibility (LSI) system or ovarian sterility (OS).

Introduction

The germination of pollen and the synchronized growth of pollen tubes through floral tissues are critical processes in the life cycle of angiosperms, ensuring the delivery of sperm cells to the egg for fertilization (Scheible and McCubbin, 2019). Pollen plays a vital role in the sexual reproduction of seed plants as the male gametophyte carrying genetic information (Liu et al., 2023). The vitality of pollen is crucial for plant crossbreeding and genetic improvement research. Assessing pollen viability involves examining its stainability, germination potential, and fertilization ability (Dafni and Firmage, 2000). Oloumi and Rezanejhad (2009) highlight that evaluating pollen viability is essential to ensure the effectiveness of hand pollination in breeding experiments.

*Corresponding author's email: vivekpj@gmail.com

Pollen remains a fascinating subject in plant research (Tushabe and Rosbakh, 2021). A comprehensive understanding of pollen biology, particularly pollen viability, germination, and tube growth, is indispensable for developing informed strategies to enhance productivity (Pirlak and Bolat, 1999). Assessing the functional quality of pollen is crucial for various applications, including pollen storage, genetic research, investigations into pollen-stigma interactions, crop enhancement, breeding programs, and studies on incompatibility and fertility (Dafni, 1992). Techniques such as staining, *in vitro* and *in vivo* germination, and assessing the final seed set are employed to evaluate pollen viability (Dafni and Firmage, 2000).

Pollen grains are commonly treated with non-vital stains such as acetocarmine, iodine potassium iodide, and aniline blue in lactophenol, which provide rapid methods for studying pollen viability. These stains can color both fresh and dead pollen grains (Shivanna and Rangaswamy, 1992). *In vitro* germination is frequently used as a standard viability test in genetic enhancement programs, although each species requires tailored protocols and specific culture media (Machado et al., 2014; Zambon et al., 2014). Selecting appropriate *in vitro* pollen germination media is essential for both basic and applied pollen research (Tushabe and Rosbakh, 2021).

Controlled pollinations, followed by germination of pollen grains on the stigmatic surface and subsequent growth of pollen tubes in the style, constitute an *in vivo* pollen viability test (Shivanna and Rangaswamy, 1992). This approach helps to understand pollen-pistil interactions, a critical post-pollination phase determining reproductive success in seed plants. *In vivo* pollen germination studies also reveal whether a species is self-incompatible or self-compatible. Aniline blue fluorescence assays can track the growth of pollen tubes in the pistil following 'self' and 'cross' pollinations (Shivanna and Tandon, 2014).

Self-incompatibility (SI) is a genetically controlled mechanism in flowering plants that rejects self or genetically related pollen grains. SI is classified into gametophytic SI (GSI) and sporophytic SI (SSI) based on genetic studies (Zheng et al., 2018). Inhibition of incompatible pollen or pollen tubes can occur at any point from the stigma surface to the micropyle (Williams et al., 1982). Inhibition of incompatible pollen tubes in the style is particularly characteristic of GSI (Dowd et al., 2000). In GSI, the SI phenotype of the pollen is determined by its own S-genotype. Almost all GSI systems exhibit rejection of self-pollen tubes during their growth in the style, even though they can successfully germinate on the stigma of another plant (Kao and Tsukamoto, 2004; Oloumi and Rezanejhad, 2009). *In vivo* studies of pollen germination and tube growth provide optimal results for understanding pollen germination as they reveal the actual mechanisms on the plant (Karabıyık, 2022).

Hamelia patens Jacq., commonly known as the 'fire bush,' is a potential plant from the Rubiaceae family with various ethnomedicinal uses (Bano et al., 2015). *Hamelia patens* consistently produces a significant number of self-incompatible (SI) flowers throughout the year, which are naturally pollinated by hummingbirds (Cunningham, 1994; Bawa et al., 1985). It features triaperturate pollen grains, an ancestral characteristic of the Rubiaceae family. These characteristics make *H. patens* an important subject for developmental and molecular studies within the Rubiaceae family. This investigation provides a comprehensive understanding of pollen viability and the GSI system in *H. patens* using the aniline blue staining technique combined with fluorescence microscopy.

Material and Methods

Plant materials

We selected two accessions of *Hamelia patens* Jacq. for our study from the Botanical Garden of Sree Neelakanta Government Sanskrit College in Pattambi, Palakkad District, Kerala. Pollen grains were collected from freshly dehisced anthers between 08:00 and 08:30 a.m. for pollen viability and germination studies (Galetto, 2009; Newstrom et al., 1994).

Pollen viability tests

Several staining methods were evaluated to distinguish between viable and non-viable pollen. Five staining techniques were employed in our pollen viability experiment: Acetocarmine, Modified Alexander stain, Iodine potassium iodide (IKI), Tetrazolium (TTC), and Aniline blue. These staining solutions were freshly prepared following the procedure outlined by Rathod et al. (2018).

Acetocarmine test (2%): This stain detects the presence of chromatin. Viable pollen grains exhibit shades of pink to deep red, while non-viable pollen grains remain colorless (Marutani et al., 1993).

Modified Alexander staining method: Alexander's stain is a complex blend of chemicals, including malachite green, acid fuchsin, orange G, and

others, that selectively stain cellular components based on their chemical properties and the cell's physiological condition. In viable pollen grains, the protoplasm absorbs acid fuchsin, resulting in a magenta-red color due to the dye's interaction with cellular content. In contrast, non-viable pollen grains, which lack active protoplasm and consist mainly of cell walls, are stained by malachite green, producing a blue-green coloration. This differential absorption allows for the clear identification of viable versus non-viable pollen grains (Alexander, 1969).

Iodine potassium iodide (IKI) test: This stain measures the starch content of pollen grains. It reacts with the starch in viable pollen grains, resulting in a distinctive blue-black coloration that indicates fertility (Sunnichan et al., 2005).

Tetrazolium (TTC) test: This method relies on the reduction of a colorless, soluble tetrazolium salt, specifically 2,3,5-triphenyl tetrazolium chloride (TTC), to a reddish, insoluble substance known as formazan, facilitated by dehydrogenases, a group of enzymes crucial to the respiratory cycle (Shivanna and Rangaswamy, 1992).

Aniline blue test: This test is used to detect the presence of callose in viable pollen grains (Fragallah et al., 2019). Fresh pollen grains stain dark blue, while dead ones remain colorless.

In vitro pollen germination studies

The sitting drop culture method was employed to investigate the effects of sucrose, boron, and calcium on *H. patens* pollen germination and tube growth. Pollen cultures were prepared by evenly distributing freshly collected pollen grains across various liquid media and incubating them in a humidity chamber at room temperature for 4 hours. Distilled water was used as the control. The eleven liquid media prepared for this study are listed in Table 1.

Table 1. Artificial liquid media used for *in vitro* pollen germination and tube elongation of *H. patens*.

Media code	Sucrose (%)	Boric acid (%)	Calcium nitrate (%)	Distilled water
A	-	-	-	
B	5	-	-	
C	10	-	-	
D	15	-	-	
E	-	0.01	-	100ml
F	-	0.1	-	
G	-	0.2	-	
H	-	-	0.01	
I	-	-	0.1	
J	-	-	0.2	
K	10	0.01	0.01	

A (control), B (sucrose 5%), C (sucrose 10%), D (sucrose 15%), E (boric acid 0.01%), F (boric acid 0.1%), G (boric acid 0.2%), H (calcium nitrate 0.01%), I (calcium nitrate 0.1%), J (calcium nitrate 0.2%), K (Sucrose 10% + boric acid 0.01% + calcium nitrate 0.01%).

To calculate the germination percentage, we divided the number of germinated pollen grains in each field of view by the total number of pollen grains in that field of view, expressing the result

as a percentage. The mean pollen tube length was determined by averaging the lengths of ten pollen tubes measured from each slide. For statistical analysis, we applied one-way ANOVA and Tukey's test ($P < 0.05$) to assess the significance of differences among treatments.

In vivo pollen germination and assessment of self-incompatibility

Hand pollinations were conducted followed by an aniline blue fluorescence assay to analyze *in vivo* pollen germination and pollen tube growth in the pistil. Sample preparation was carried out according to the protocol of Chen et al. (2012). Artificially pollinated flowers were collected at 1, 2, 3, 4, 6, 8, and 24 hours after pollination. To prepare for the aniline blue fluorescence assay, pistils were fixed in a solution of formalin, acetic acid, and alcohol (F.A.A; 1:1:8 v/v) for at least 24 hours. After fixation, the pistils were soaked in a 2 M NaOH solution for 3–4 hours to soften and clarify the tissues, following rinsing with demineralized water. The pistils were then stained with a 0.1% aniline blue solution dissolved in 0.15 M K_2HPO_4. Each pistil was gently squashed and mounted on a clean microscopic slide using glycerine to spread out the material. A Leica DM6B fluorescence microscope was used to monitor the germination and growth of pollen tubes through the style.

Statistical data analysis

Data analysis was performed using IBM SPSS® version 22 software (SPSS Inc., Chicago, USA). The staining percentage was determined by dividing the number of stained pollen grains by the total number of pollen grains per field of view and expressing it as a percentage. Statistical analysis involved one-way ANOVA and Tukey's tests ($P<0.05$) to assess variations among treatments. Comparisons of viable and non-viable pollen grains in each treatment were made using a Student's t-test.

Results

Pollen viability test

Among the five staining methods employed, the highest average pollen viability percentage was observed with the modified Alexander staining technique (81.80%), while the lowest was noted with the iodine potassium iodide (IKI) method (51.20%) (Fig. 1). The F-statistic ($F = 41.39$) indicates that the variability in the mean percentage of pollen viability among the different staining methods is statistically significant ($P<0.05$) (Table 2). The Student's t-test revealed that, with the exception of IKI, there is a statistically significant difference between the means of viable and non-viable pollen grains ($P<0.05$) (Table 3). Consequently, the IKI test was unable to distinguish between the two types of pollen and is therefore unsuitable for detecting pollen viability in *H. patens*. Notably, the pollen viability determined using the modified Alexander staining method (81.80%) also aligns closely with the highest observed percentage of pollen germination obtained through *in vitro* experiments (81.33%). Thus, the modified Alexander staining method proves to be a more reliable and suitable approach for assessing pollen viability in this species.

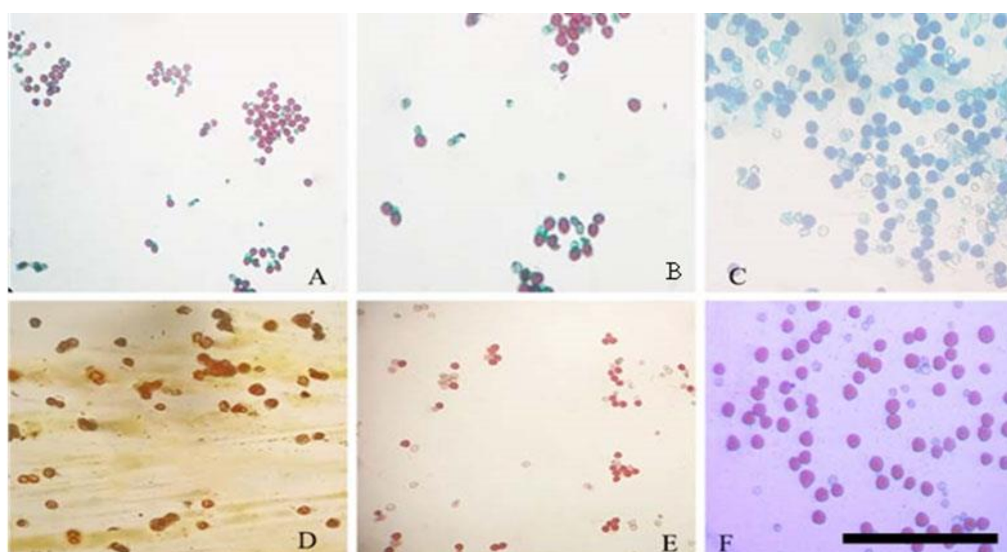


Fig. 1. Different staining methods for pollen viability assessment in *H. patens*. (A and B) Modified Alexander stain, (C) Aniline blue, (D) Iodine Potassium iodide, (E) Tetrazolium (TTC), (F) Acetocarmine. Scale Bar: 100µm.

Table 2. One way ANOVA analysis to compare the mean percent of pollen viability based on various staining techniques.

Stain	Mean (%)	SD	N	F	Sig.
Acetocarmine	79.34	1.56	5	41.39	0.000*
Modified Alexander method	81.80	0.55	5		
Iodine potassium iodide	51.20	1.22	5		
TTC	61.32	1.20	5		
Aniline blue	70.13	3.56	5		

*Indicates significant differences ($P<0.05$).

Table 3. Mean number of fresh and dead pollen grains within each treatment were compared using t-test.

Test	Fresh pollen (viable)	Dead pollen (non- viable)	N (No. of fields)	t value	P value
Acetocarmine	72.60 ± 5.61	18.80 ± 1.24	5	9.351	0.000*
Modified Alexander method	86.20 ± 5.97	19.20 ± 1.46	5	10.886	0.000*
Iodine potassium iodide (IKI)	62.80 ± 2.81	60.00 ± 3.47	5	0.625	0.549
TTC	59.00 ± 3.74	37.60 ± 4.67	5	3.574	0.007*
Aniline blue	66.80 ± 7.30	28.20 ± 3.05	5	4.876	0.001*

* Statistically significant difference between the mean values of viable and non-viable pollen grains ($P < 0.05$). Data are mean values (± SE) for five fields.

In vitro pollen germination and pollen tube growth

Eleven distinct compositions of pollen germination media were utilized to examine the effects of sucrose, boron, and calcium on the germination and tube growth of *H. patens* pollen. Pollen grains were capable of germinating even in the sole presence of water, although at a relatively low rate (12.47%). The addition of sucrose, H_3BO_3 (boric acid), and Ca^{2+} significantly enhanced the germination rate of pollen, underscoring the importance of these components in supporting and stimulating pollen germination and tube elongation.

Effect of sucrose concentration on pollen germination and tube growth

In the pollen germination experiment, three different sucrose concentrations (5%, 10%, and 15%) were used, designated as B, C, and D, respectively. The effect of sucrose was most pronounced and beneficial at a 10% concentration, yielding a germination rate of 44.27%. However, increasing the sucrose concentration to 15% led to a significant decrease in pollen germination, dropping to 23.40%. ANOVA analysis revealed that variations in pollen germination percentage and pollen tube growth across the different media were statistically significant ($P<0.05$). The average percentage of pollen germination was highest in medium C (44.27%) and lowest in medium B (25.61%). The F-test also indicated that sucrose concentrations significantly affect pollen tube length, with the

longest average pollen tube length found in medium C (197.2 μm) and the shortest in medium B (108.4 μm).

Effect of boric acid on pollen germination and tube growth

Pollen growth media with boron concentrations of 0.01%, 0.1%, and 0.2% were prepared and labeled as E, F, and G, respectively. ANOVA analysis did not reveal significant differences among treatments at the 5% level. The results demonstrate that a lower concentration of boric acid significantly enhances both pollen germination and tube growth rates. Conversely, higher boric acid concentrations lead to a reduction in both pollen germination and tube growth rates. The highest average rate of pollen germination and tube length was observed in medium E (23.88%, 61.40 μm), while the lowest germination rate was noted in medium G (19.40%, 36 μm).

Effect of calcium nitrate on pollen germination and tube growth

Three distinct pollen germination media containing 0.01%, 0.1%, and 0.2% calcium nitrate were prepared and designated as H, I, and J, respectively. ANOVA analysis revealed significant differences among all treatments at the 5% level. The H-type medium, containing 0.01% calcium nitrate, exhibited the highest rate of pollen germination and tube length (73.80%, 1489 μm). The lowest germination rate was observed in the I-type medium (27.08%), while the shortest tube length was recorded in the J-type medium (104.20 μm), which contained 0.2% calcium nitrate.

Overall effect of media types on pollen germination and tube elongation

When pollen grains were cultured in an artificial medium supplemented with various concentrations of sucrose, boron, and calcium (Fig. 2), the highest *in vitro* pollen germinability and tube growth were achieved with a medium containing optimal levels of all three nutrients, specifically 10% sucrose, 0.1% boric acid, and 0.01% calcium nitrate (Fig. 3A, B). The optimum concentrations of these nutrients were determined through separate additions to the artificial media. When administered individually, these factors did not significantly increase germinability. However, when combined, a substantial improvement in pollen germination (81.33%) and tube growth (1565.13 μm) was observed. Thus, for optimal *in vitro* pollen germination of this species, the recommended conditions were a sucrose concentration of 10% and lower concentrations of boron (0.01%) and calcium (0.01%).

In vivo pollen germination and assessment of self-incompatibility

Fluorescence microscopy is a widely used technique for detecting self-incompatibility in various plant species. In our study, we observed that pollen from both self-pollination and cross-pollination germinated within 1-hour post-pollination, with no discernible differences in the initial growth of pollen tubes. However, pollen tubes resulting from self-pollination exhibited a markedly slower growth rate, advancing only to the midpoint of the style (Fig. 4). In contrast, within 4 hours after pollination, pollen tubes from cross-pollination had successfully reached the ovary (Figs. 5 and 6). Further observations at 8 and 24 hours after pollination revealed that most pollen tubes from cross-pollination had reached the ovary. Conversely, even 24 hours after self-pollination, no pollen tubes were observed at the ovary. This indicates that in self-pollinated pistils of *H. patens*, pollen tubes are unable to reach the ovary, and their growth is arrested approximately midway through the style.

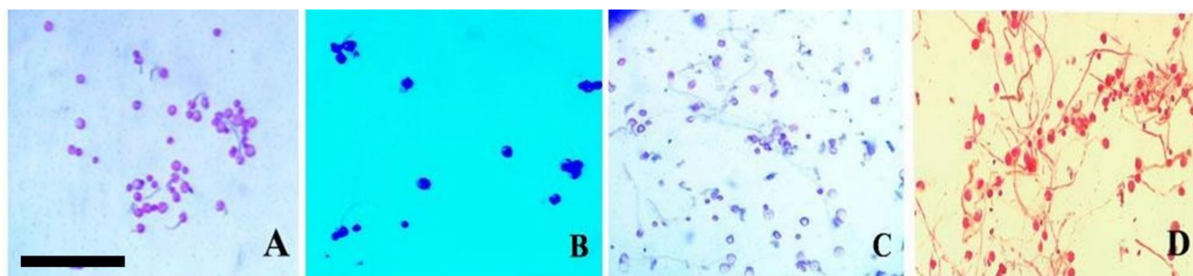


Fig. 2. *In vitro* pollen germination of *H. patens* across various media. (A) sucrose 10%, (B) boric acid 0.01%, (C) calcium nitrate 0.01%, (D) Sucrose 10% + boric acid 0.01% + calcium nitrate 0.01%. Scale Bar: 100μm.

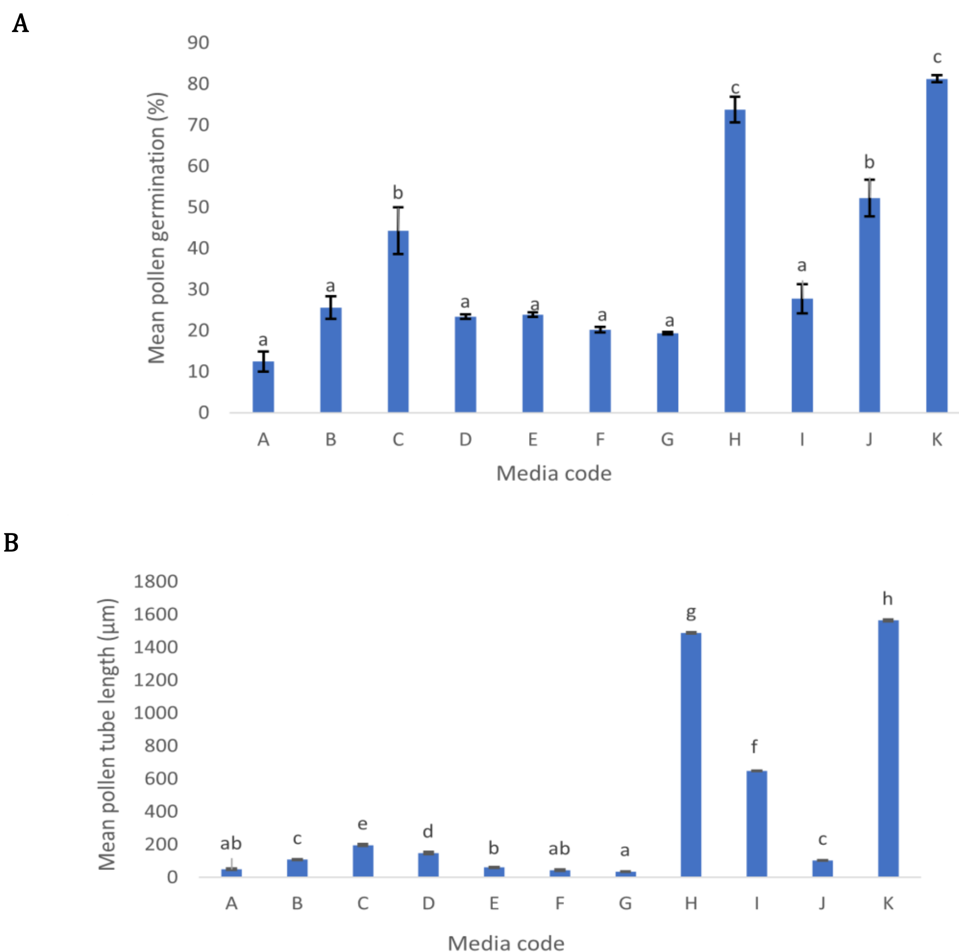


Fig. 3. Effect of media types on *in vitro* pollen germination and pollen tube elongation of *H. patens*. Media code A (control), B (sucrose 5%), C (sucrose 10%), D (sucrose15%), E (boric acid 0.01%), F (boric acid 0.1%), G (boric acid 0.2%), H (calcium nitrate 0.01%), I (calcium nitrate 0.1%), J (calcium nitrate 0.2%), K (Sucrose 10% + boric acid 0.01% + calcium nitrate 0.01%). Means with different letters are significantly different (Tukey's HSD $P < 0.05$).

On the other hand, in cross-pollinated flowers, the ability of pollen tubes to reach the ovary within 4 hours after pollination suggests the presence of a self-incompatibility mechanism in *H. patens*. The inhibition of self-pollen tube penetration around the midpoint of the style in this species indicates the absence of late-acting self-incompatibility (LSI) or ovarian sterility (OS). In an LSI system, blockage occurs within the ovary rather than at the style level.

Discussion

Pollen viability test

In flowering plants, the success of pollination relies on the vigor and viability of pollen grains or the male gametophyte (Khan et al., 2024). Pollen, representing plant germplasm, plays an indispensable role in plant genetic breeding and germplasm resource research. The vitality of pollen in artificial pollinations directly influences the outcome of plant hybridization, determining its success or failure (Shi et al., 2023).

Chemical staining is a widely utilized technique for assessing pollen viability, favored for its simplicity and rapid results (Jia et al., 2022). Certain staining techniques can selectively color viable pollen (Khatun and Flowers, 1995). Of the five staining methods evaluated in this study, acetocarmine, the modified Alexander method, TTC, and the aniline blue test could distinguish viable pollen from non-viable pollen. The IKI test failed to differentiate between fresh and dead pollen in this species. The highest average pollen viability percentage was observed with the modified Alexander staining technique.

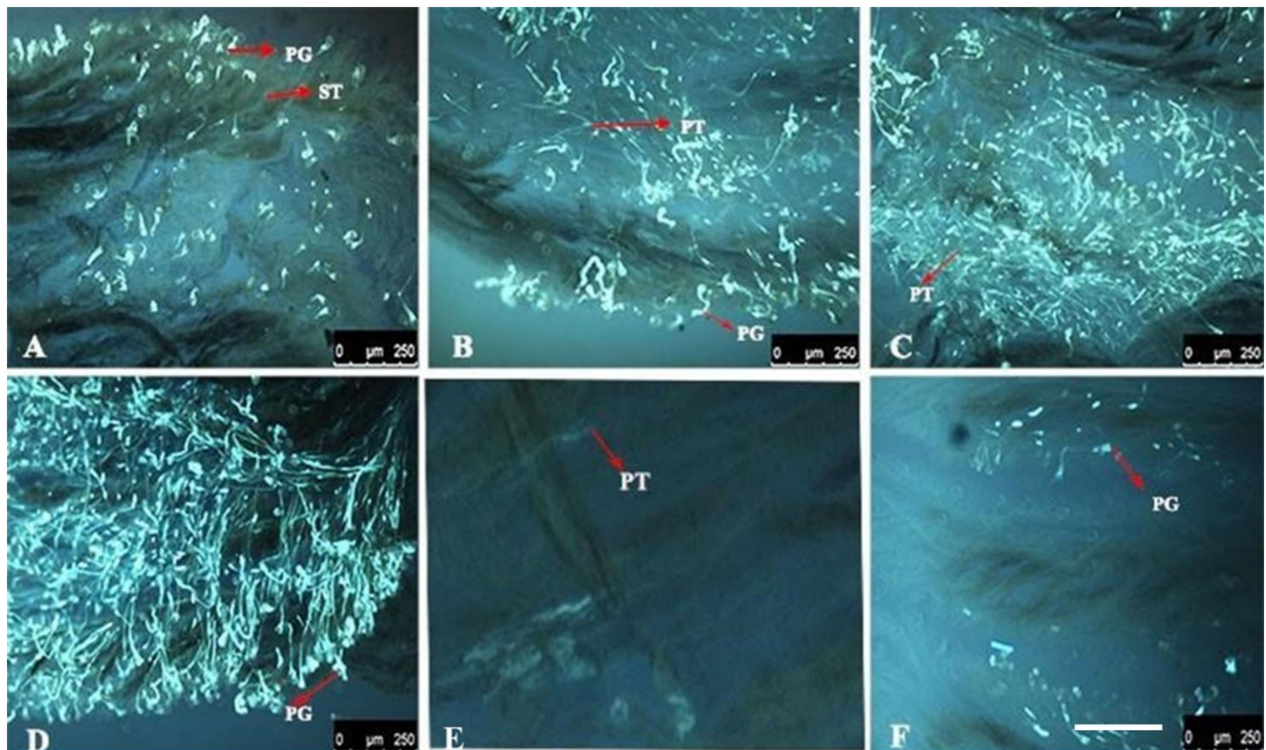


Fig. 4. *In vivo* pollen germination and tube growth in *H. patens* after self-pollination. (A-D) Pollen germination on the stigma at 1 h (A), 2 h (B), 3 h (C), and 8 h (D) post-pollination, (E) Pollen tubes reach a quarter of the style length with arrested growth, (F) Reduced pollen viability 24 h after pollination, PG = Pollen Grain, PT = Pollen Tube, ST = Stigma, SY = Style. Scale Bar: 250 µm.

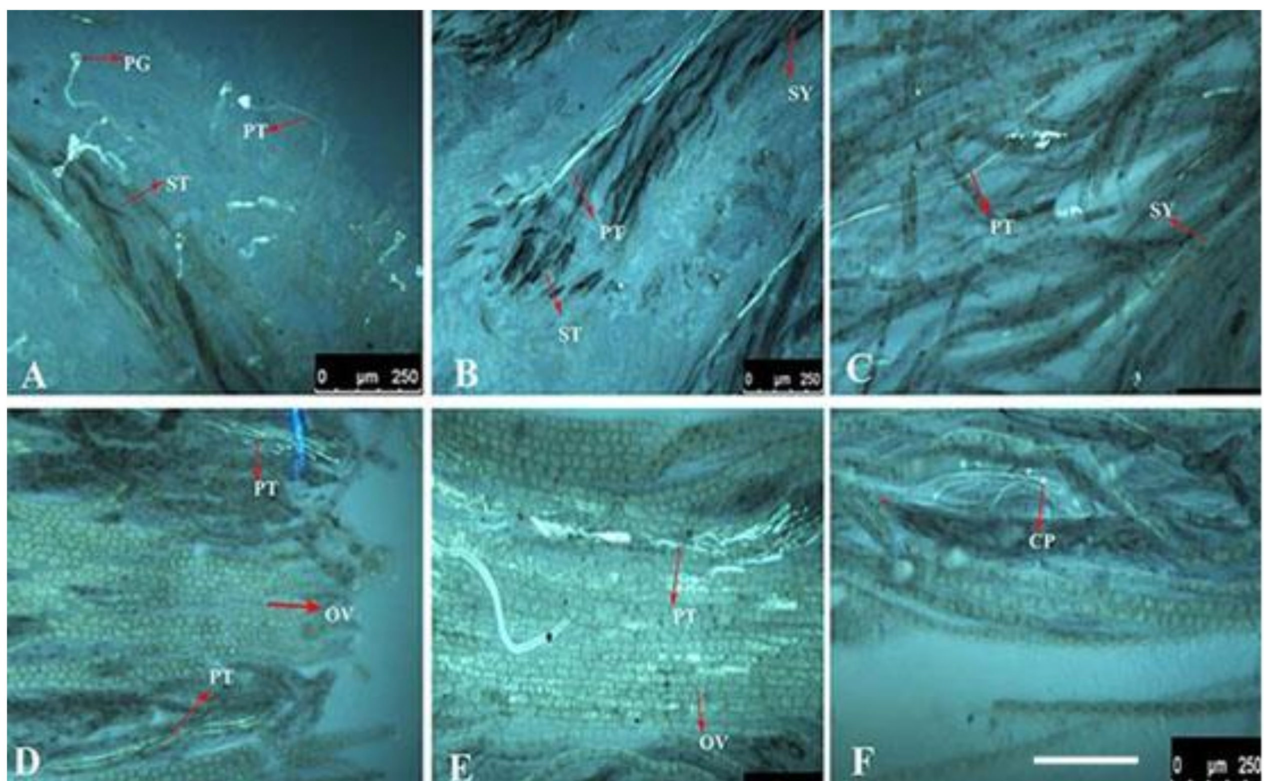


Fig. 5. *In vivo* pollen germination and tube growth in *H. patens* after AXB cross-pollination. (A) Pollen grains germinate on the stigma, 1 h post-pollination, (B) Pollen tubes penetrate stigmatic tissue into the style, (C) Pollen tubes advance through the style, 3 h post-pollination, (D) Pollen tubes reach the ovary at 4 h, (E) Increased pollen tubes at the ovary by 6 h, (F) Callose plug forms in a tube, PG = Pollen Grain, PT = Pollen Tube, ST = Stigma, SY = Style, CP = Callose Plug. Scale Bar: 250 µm.

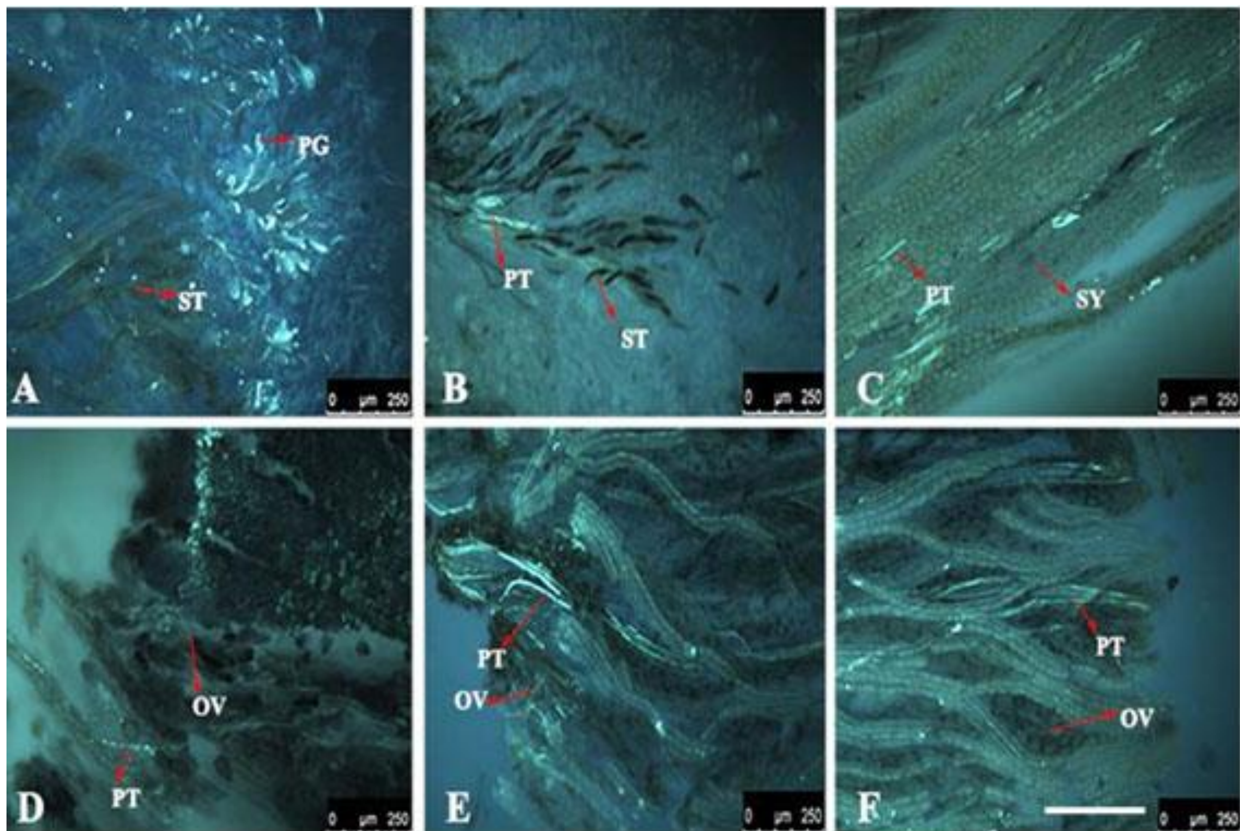


Fig. 6. *In vivo* pollen germination and tube growth in *H. patens* after BXA cross-pollination. (A) Pollen grains germinating on the stigma, (B) Pollen tubes enter the style, 1 h post-pollination, (C) Pollen tubes move through the style, 3 h post-pollination, (D) Pollen tubes reach the ovary at 4 h, (E) Increased pollen tubes at the ovary by 8 h (F) Dense accumulation of pollen tubes in the ovary at 24 h. PG = pollen grain, PT = pollen tube, ST = stigma, SY = style. Scale Bar: 250 μm.

This result suggests that pollen viability estimation through staining techniques is not universally applicable (Skrzypkowski et al., 2023) and can vary across species. Negative results have been documented for various staining techniques in different plant species. For instance, in *Jatropha curcas*, tetrazolium salt (TTC) was the only stain that effectively distinguished between viable and non-viable pollen (Abdelgadir et al., 2012). Huang et al. (2004) reported that in *Leymus chinensis* (Poaceae), TTC did not stain dead pollen, whereas IKI stained dead pollen similarly to fresh pollen. In *Festuca arundinacea* (Poaceae), Zeng-Yu et al. (2004) found that TTC, MTT, FCR, and aniline blue could not differentiate between fresh and dead pollen. Similarly, in some *Prunus* spp. (Rosaceae), MTT intensely stained heat-killed pollen (Parfitt and Ganeshan, 1989). Abdelgadir et al. (2012) also noted that non-differentiation of viable and non-viable pollen with different staining techniques is common. Sulusoglu and Cavusoglu (2014) observed that the TTC test provided a more accurate estimation of pollen viability compared to the IKI stain in *Prunus laurocerasus*. The high pollen viability detection using the Alexander stain can be attributed to its ability to differentially stain pollen grains based on viability, providing a clear color distinction and facilitating the assessment of pollen quality (Peterson et al., 2010). Additionally, the mean percentage of pollen viability indicated by the modified Alexander method showed a high correlation with fresh pollen germination *in vitro*.

In vitro pollen germination studies

Experiments involving *in vitro* pollen germination and tube growth are crucial for both fundamental and applied research in pollen biology (Sakhanokho and Rajasekaran, 2010). The formulation of an effective pollen germination medium varies among species (Sunnichan et al., 2005). Our findings revealed that pollen grains could germinate solely with water, albeit at a reduced rate of 12.47%. This suggests that an ergastic substance within the pollen may provide necessary nutrients for pollen tube growth. However, when sucrose, H_3BO_3, and Ca^{2+} were introduced, the germination rate of *H. patens* pollen increased significantly, indicating that the internal nutrients of most *H. patens*

pollen grains are insufficient for supporting germination and that there is a reliance on external nutrients (Jia et al., 2022).
Our study confirms the essential roles of sucrose, boron, and calcium in enhancing pollen germination and tube elongation. Sucrose serves a dual purpose in pollen germination by supplying nutrients and carbon sources, and by regulating environmental osmotic pressure (Jia et al., 2022). Lin et al. (2017) reported that sucrose at an appropriate concentration can promote both pollen germination and tube development, whereas high concentrations inhibit these processes.

Boric acid plays a crucial role in pollen germination, pollen tube growth, and the guidance of pollen tubes (Shi et al., 2023). An increase in boric acid concentration decreases pollen germination and tube elongation. Fang et al. (2016) found that high levels of boron hinder pollen germination and cause morphological abnormalities in pollen tubes in *Malus domestica*. Brewbaker and Kwack (1963) explored the role of calcium in pollen germination and tube growth across 86 species from 39 plant families, suggesting that calcium in the pollen culture medium at an appropriate concentration is essential for pollen germination. Suboptimal concentrations of calcium ions can adversely affect the pollen tube cytoskeleton, leading to abnormal pollen tube morphology (Wasag et al., 2022).

In vivo pollen germination and assessment of self-incompatibility

In vivo pollen germination experiments, followed by aniline blue fluorescence microscopy, reveal the pattern of pollen tube growth in the pistil after self- and cross-pollinations. The response of pollen tube growth to controlled pollinations is strongly related to self-incompatibility. Staining with aniline blue fluorochrome specifically colors the glucan layer in the inner callosic wall of pollen tubes, giving them a ladder-like appearance (Oloumi and Rezanejhad, 2009). In species with Gametophytic Self-Incompatibility (GSI), inhibition of incompatible pollen tubes typically occurs within the style following successful pollen grain germination (Bedinger et al., 2017). For example, in *Cornus florida*, fluorescence microscopy indicated a GSI system where self-pollinated pollen germinated freely on the stigma, but pollen tubes penetrated only the upper third of the style, while cross-pollen tubes reached the base of the style by 48 hours (Reed, 2004). Milatović and Nikolić (2007) analyzed self-incompatibility in 36 apricot cultivars using fluorescence microscopy and reported that in self-incompatible cultivars, pollen tube growth ceased in the style with characteristic swellings.
In *Camellia sinensis*, a GSI plant, self-pollinated flowers failed to set seeds even though pollen tubes could grow to the ovary, a phenomenon known as ovarian SI or Late-acting SI (LSI) (Chen et al., 2012). Our study confirms that in *H. patens*, the self-incompatibility barrier occurs at the style level of the pistil, indicating a conventional GSI system rather than an LSI system. These findings support the report by Louis et al. (2012), which described an RNase-based gametophytic self-incompatibility system in *H. patens*.

Conclusion

This research on *Hamelia patens* Jacq., a member of the Rubiaceae family, elucidates its reproductive biology and highlights the Modified Alexander staining method as highly effective for assessing pollen viability. The viability results of 81.80% obtained with the Modified Alexander staining technique closely match the highest observed *in vitro* pollen germination rate of 81.33%. Optimal *in vitro* germination and pollen tube growth were achieved in a medium containing 10% sucrose and low concentrations of boron (0.01%) and calcium (0.01%), underscoring the importance of a balanced nutrient environment. Notably, the research determined that the self-incompatibility mechanism in *H. patens* does not involve late-acting self-incompatibility (LSI) or ovarian sterility (OS). The insights gained regarding optimal pollen germination conditions and the plant's self-incompatibility system provide valuable information for horticulturists and conservation biologists working to ensure the survival and propagation of this species.

Acknowledgements
Kanneri Anu Krishna acknowledges CSIR (Council of Scientific and Industrial Research) for the Junior Research Fellowship (File no-08/592(0004)/2019-EMR-1, dated: 21-10-2019).

Author contributions
KAK investigation, original draft. MSR review, and editing. RVR investigation. PJV conceptualization, supervision, resources, review, and editing.

Conflict of Interest
The authors indicate no conflict of interest in this work.

References

Abdelgadir HA, Johnson SD, Van Staden J. 2012. Pollen viability, pollen germination and pollen tube growth in the biofuel seed crop *Jatropha curcas* (Euphorbiaceae). South African Journal of Botany 79, 132-139.

Alexander MP. 1969. Differential staining of aborted and non-aborted pollen. Stain Technology 44, 117-122.

Bano J, Santra S, Menghani E. 2015. *Hamelia patens* a potential plant from *Rubiaceae* family: A Review. International Journal of Scientific and Engineering Research 6, 960-973.

Bawa KS, Perry DR, Beach JH. 1985. Reproductive biology of tropical lowland rain forest trees. I. Sexual systems and incompatibility mechanisms. American Journal of Botany 72(3), 331-345.

Bedinger PA, Broz AK, Tovar-Mendez A, McClure B. 2017. Pollen-pistil interactions and their role in mate selection. Plant Physiology 173, 79-90.

Brewbaker JL, Kwack BH. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. American Journal of Botany 50, 859–865.

Chen X, Hao S, Wang L, Fang W, Wang Y, Li X. 2012. Late-acting self-incompatibility in tea plant (*Camellia sinensis*). Biologia 67, 347-351.

Cunningham SA. 1994. Measuring the relationship between floral duration and fruit set for *Hamelia patens* (*Rubiaceae*). Biotropica 26, 227-229.

Dafni A, Firmage D. 2000. Pollen viability and longevity: practical, ecological and evolutionary implications. Plant Systematics and Evolution 222, 113-132.

Dafni A. 1992. Pollination Ecology: A Practical Approach. Oxford: Oxford University Press. 250 pp. ISBN: 0-19-963298-7.

Dowd PE, McCubbin AG, Wang X, Verica JA, Tsukamoto T, Ando T, Kao TH. 2000. Use of *Petunia inflata* as a Model for the Study of Solanaceous Type Self-incompatibility. Annals of Botany 85, 87-93.

Fang K, Zhang W, Xing Y, Zhang Q, Yang L, Cao Q. 2016. Boron toxicity causes multiple effects on *Malus domestica* pollen tube growth. Frontiers in Plant Science 7, 1–12.

Fragallah SADA, Lin S, Li N, Ligate EJ, Chen Y. 2019. Effects of sucrose, boric acid, pH, and incubation time on *in vitro* germination of pollen and tube growth of Chinese fir (*Cunnighamial lanceolata* L.). Forests 10, 102.

Galetto L. 2009. Reproductive biology of *Hamelia patens* Jacq. (*Rubiaceae*) in northern India. The International Journal of Plant Reproductive Biology 1, 63-71.

Huang Z, Zhu J, Mu X, Lin J. 2004. Pollen dispersion, pollen viability and pistil receptivity in *Leymus chinensis.* Annals of Botany 93, 295–301.

Jia W, Wang Y, Mi Z, Wang Z, He S, Kong D. 2022. Optimization of culture medium for *in vitro* germination and storage conditions of *Exochorda racemosa* pollen. Frontiers in Plant Science 13, 994214. https://doi.org/10.3389/fpls.2022.994214.

Kao T, Tsukamoto T. 2004. The Molecular and Genetic Bases of S-RNase-Based self-incompatibility. Plant Cell 16, 72–83. https://doi.org/10.1105/tpc.016154.

Karabıyık Ş. 2022. Effects of temperature on pollen viability and *in vivo* pollen tube growth in *Citrus sinensis*. Journal of Applied Botany and Food Quality 95.

Khan I, Naeem MK, Shahzad A, Zhang Z, Chen J, Sajjad M. 2024. Optimizing wheat pollen preservation for enhanced viability and *in vitro* germination. Agronomy 14, 201.https://doi.org/10.3390/agronomy14010201.

Khatun S, Flowers TJ. 1995. The estimation of pollen viability in rice. Journal of Experimental Botany 46, 151–154.

Lin Y, Wang Y, Iqbal A, et al. 2017. Optimization of culture medium and temperature for the *in vitro* germination of oil palm pollen. Scientia Horticulturae 220, 134–138.

Liu Q, Yang J, Wang X, Zhao Y. 2023. Studies on pollen morphology, pollen vitality and preservation methods of *Gleditsia sinensis* Lam. (Fabaceae). Forests 14, 243. https://doi.org/10.3390/f14020243.

Louis CF, Raj NRS, Radhamany PM. 2012. Identification and characterization of incompatibility factors in *Hamelia patens* Jacq. (*Rubiaceae*). Journal of Horticultural Science and Biotechnology 87, 531–538.

Machado CA, Moura CRF, Lemos EEP, Ramos SRR, Ribeiro FE, Lédo AS. 2014. Pollen grain viability of coconut accessions at low temperatures. Acta Scientiarum. Agronomy 36, 227–232.

Marutani M, Sheffer RD, Kamemoto H. 1993. Cytological analysis of *Anthurium andraeanum* (Araceae), its related taxa and their hybrids. American Journal of Botany 80, 93–103.

Milatović D, Nikolić D. 2007. Analysis of self-(in)compatibility in apricot cultivars using fluorescence microscopy. Journal of Horticultural Science & Biotechnology 82, 170–174.

Newstrom LE, Frankie GW, Baker HG. 1994. A new classification for plant phenology based on flowering patterns in low land tropical rain forest trees at La selva, Costa Rica. Biotropica 266, 141-159.

Oloumi H, Rezanejhad F. 2009. Response of pollen tube growth and seed set to controlled pollination and their relation to self-incompatibility in different cultivars of *Petunia hybrida*. Grana 48, 102–108.

Parfitt DE, Ganeshan S. 1989. Comparison of procedures for estimating viability of *Prunus* pollen. Horticultural Science 24, 354–356.

Peterson R, Slovin JP, Chen C. 2010. A simplified method for differential staining of aborted and non-aborted pollen grains. International Journal of Plant Biology 1(2), e13. https://doi.org/10.4081/pb.2010.e13.

Pirlak L, Bolat I. 1999. An investigation on pollen viability, germination and tube growth in some stone fruits. Turkish Journal of Agriculture and Forestry 23, 383-388.

Rathod V, Behera TK, Munshi AD, et al. 2018. Pollen viability and *in vitro* pollen germination studies in *Momordica* species and their intra and interspecific hybrids. International Journal of Chemical Studies 6, 32–40.

Reed SM. 2004. Self-incompatibility in *Cornus florida*. Horticultural Science 39, 335–338.

Sakhanokho HF, Rajasekaran K. 2010. Pollen biology of ornamental ginger (*Hedychium* spp. J. Koenig). Scientia Horticulturae (Amsterdam) 125, 129–135. https://doi.org/10.1016/j.scienta.2009.12.037.

Scheible N, McCubbin A. 2019. Signaling in pollen tube growth: beyond the tip of the polarity iceberg. Plants 8, 156. https://doi.org/10.3390/plants8060156.

Shi R, Shi F, Wang S. 2023. Influence of sucrose, boric acid and calcium chloride on *in vitro* pollen germination and tube elongation of alfalfa. Legume Research 46(9), 1192-1198. https://doi.org/10.18805/LRF-74.

Shivanna KR, Rangaswamy N. 1992. Pollen Biology: A Laboratory Manual. 1st ed. Springer-Verlag Berlin Heidelberg.

Shivanna KR, Tandon R. 2014. Reproductive Ecology of Flowering Plants: A Manual. Springer, India. https://doi.org/10.1007/978-81-322-2003-9.

Skrzypkowski W, Galek R, Adamus A, Kiełkowska A. 2023. Pollen development and stainability in *Vicia faba* L. and *Lupinus angustifolius* L. Agriculture 13, 2065. https://doi.org/10.3390/agriculture13112065.

Sulusoglu M, Cavusoglu A. 2014. *In vitro* pollen viability and pollen germination in cherry laurel (*Prunus laurocerasus* L.). The Scientific World Journal 1–7. https://doi.org/10.1155/2014/657123.

Sunnichan VG, Mohan Ram HY, Shivanna KR. 2005. Reproductive biology of *Boswellia serrata*, the source of salai guggul, an important gum-resin. Botanical Journal of the Linnean Society 147, 73–82.

Tushabe D, Rosbakh S. 2021. A compendium of *in vitro* germination media for pollen research. Frontiers in Plant Science 12, 709945.

Wasag P, Suwinska A, Lenartowska M, Lenartowski R. 2022. RNAi-Mediated knockdown of calreticulin3a impairs pollen tube growth in petunia. International Journal of Molecular Sciences 23(9), 4987. https://doi.org/10.3390/ijms23094987.

Williams EG, Knox RB, Rouse JL. 1982. Pollination sub-systems distinguished by pollen tube arrest after incompatible interspecific crosses in Rhododendron (Ericaceae). Journal of Cell Science 53, 255–277.

Zambon CR, Silva LFO, Pio R, Figueiredo MA, Silva KN. 2014. Estabelecimento de meio de cultura e quantificação da germinação de grãos de pólen de cultivares de marmeleiros. Revista Brasileira de Fruticultura 36, 400–407.

Zeng-Yu W, Yaxin G, Scott M, Spangenberg G. 2004. Viability and longevity of pollen from transgenic and non-transgenic tall fescue (*Festuca arundinacea*) (Poaceae) plants. American Journal of Botany 4, 523–530.

Zheng YY, Lin XJ, Liang HM, Wang FF, Chen LY. 2018. The long journey of pollen tube in the pistil. International Journal of Molecular Sciences 19, 1–15. https://doi.org/10.3390/ijms19113529