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DETERMINING APPROPRIATE METHODS FOR ESTIMATING POLLEN VIABILITY AND GERMINATION RATES IN LISIANTHUS

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ABSTRACT

Crossbreeding is a multi-stage process with inherent challenges and risks in developing new varieties. Success hinges on selecting highly fertile parents. In species like lisianthus, uncertainty persists regarding the optimal methods for assessing pollen quality, which is crucial for evaluating pollen parent fertility. This study seeks to identify the most reliable techniques for this purpose. Fresh and dead pollen from four lisianthus (*Eustoma grandiflorum*) varieties was used. The dead pollen was obtained by thermal inactivation. Five chemical staining methods (iodine-potassium iodide, 2,3,5-triphenyltetrazolium chloride – TTC, lactophenol cotton blue, safranin, acetocarmine) were employed to assess pollen viability, and two biological methods (Petri dishes, hanging drops) were used to determine the germination rate. Four solid medium cultures were employed in Petri dishes, while the hanging drop utilised four liquid medium cultures. Thirteen tests were conducted for each variety, evaluating fresh and dead pollen. The study found significant variations in pollen quality among lisianthus varieties and methods. Fresh pollen showed viability rates ranging from 56.87% to 99.41% and germination rates from 0.20% to 45.11%. TTC exhibited the lowest viability rate across all varieties, while the highest germination rate was observed in the liquid culture medium with only boric acid and PEG1500. Notably, TTC was the sole viability method that did not stain dead pollen, and no germination occurred in any method for dead pollen. TTC is the most reliable staining method, and a liquid culture medium with boric acid and PEG1500 effectively determines lisianthus pollen quality. Varying boric acid and PEG1500 concentrations are advisable.

Keywords: antioxidant, antimicrobial, chemical composition, *Diospyros lotus*, macro and micronutrient

INTRODUCTION

Developing new varieties is essential to enhance agricultural productivity, adapt to environmental conditions, and meet market demands. One of the most widely used methods in developing varieties is crossbreeding, which consists of various stages, including parental selection, pollination, fertilisation, fruit set, seed formation, fruit harvesting, seed maturation, seed germination, and progeny selection [Zlesak 2007, Nadeem et al. 2013]. Although each stage carries specific difficulties and risks, parental selection is critical. When low-fertility parents are involved in pollination, it reduces fruit and seed set rates, thereby decreasing the likelihood of selecting a potential candidate variety during the selection stage. In order to

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obtain a large number of seeds, it is necessary to create a gene pool by selecting parents with high fertility to ensure successful pollination and fertilisation and then to identify combinations with high compatibility [Farooq et al. 2013].

Fertility in paternal parent selection is directly related to pollen quality. Sufficient pollen and high pollen viability and germination rates are desired [Farooq et al. 2016]. Methods that determine pollen viability and germination rates are used to select genotypes with high pollen quality. These methods are divided into *in vivo* and *in vitro* [Soares et al. 2013]. In *in vivo* conditions, examining pollen development in the style after pollination and determining the number of seeds formed after fertilisation can provide information about pollen quality [Huang et al. 2013, Jiang et al. 2017]. However, since many other factors affect pollen development in the style or seed formation under *in vivo* conditions, it is imperative to perform pollen viability and germination tests under *in vitro* conditions as a comparison criterion [Paydaş et al. 1996].

Pollen quality tests under *in vitro* conditions are divided into two groups, including chemical and biological methods. There are many chemical and biological methods, and the success of these methods varies according to species and varieties. Moreover, significant variations in results can arise among methods, even for the same species or variety. In some methods, pollen can be stained before it matures, while in other methods, pollen that has lost its viability can also be stained. The success of pollen germination rates depends on the efficacy of in vitro nutrient media designed to simulate species-specific stigma fluid [Tushabe and Rosbakh 2021]. Therefore, it is crucial to determine the most optimised and reliable species -specific methods for determining pollen viability and germination rates. Identifying the most optimal and reliable methods can enhance hybridisation success, eliminating significant factors in breeding studies like time loss, cost, and labour force that may arise during the selection of a method for paternal parent selection. The literature determined that there are almost no studies on pollen viability and germination rate of lisianthus. This study aimed to determine the most appropriate and reliable chemical and biological methods for lisianthus species. The study also compared chemical methods on both fresh and dead pollen, revealing the optimal staining method for all plant species.

MATERIAL AND METHODS

Studies to determine the pollen quality of lisianthus pollen under *in vitro* conditions were carried out in the cytology laboratory of Ankara University, Faculty of Agriculture, Department of Horticulture, between September and January 2022.

Plant material

Pollen of four different varieties of lisianthus (*Eustoma grandiflorum*), which ranks 5th among the top 20 cut flowers in the world-cut flower trade (Royal Flora Holland 2023), was used as plant material. The pollen of Alissa 1 White, Sorrie Pink Picote, Abc 2 Purple, and Abc 2 Rose varieties were obtained from Focus Flowers, a commercial cut lisianthus producer located in Antalya, Turkey (36°56'35.88'' N, 30°45'32.8674'' E).

Flowers of all varieties were harvested at the floral opening stage (stage 4) just before the full blooming stage (stage 5) with a stem length of 30 cm in the cool evening hours [Velasco-Ramírez et al. 2022]. The harvested flower stems were placed in buckets with tap water and transported from Antalya to Ankara, where the study was conducted. Pollen was collected at the full blooming stage of the flowers, which is the pollen's maturation period, and used immediately to determine pollen viability and germination rates [Wang et al. 2011]

Methods

The iodine-potassium iodide (IKI), 2,3,5-triphenyltetrazolium chloride (TTC), lactophenol cotton blue (LCB), safranin (SAF), and acetocarmine tests (AC), which are most commonly encountered methods in the literature and relatively easy to perform even under minimal conditions, were used to determine pollen viability rates. Petri dish and hanging drop methods were utilised to assess pollen germination rates. In the Petri dish method, four solid culture mediums commonly preferred across various species were used, while the hanging drop method involved four different liquid culture mediums. In total, 13 dif-

ferent methods were tested for each variety. Each method was applied to both fresh and dead pollen. After collection, the pollen was divided into two groups. Fresh pollen was used immediately, whereas dead pollen was used after undergoing thermal inactivation through incubation at 80°C for 24 hours [Parfitt and Ganeshan 1989, Wang et al. 2004].

In all methods, a Leica DM1000 model microscope and imaging system with ×20, ×40, and ×100 magnification objectives were used for counting pollen grains.

Pollen viability tests

IKI. One drop of IKI solution, prepared by adding 1 g of potassium iodide and 0.5 g of iodine to 100 mL of distilled water, was placed on the slide. Pollen was sprinkled onto each drop using a brush, covered with a coverslip, and observed under a microscope after 5 minutes. Pollen grains stained black or dark brown were categorised as "absolutely viable", those stained light brown, orange, or red were categorised as "semi -viable", and those stained yellow or colourless were categorised as "non-viable". The count of semi-viable pollen, theoretically assumed to be 50% viable, was added to the count of absolutely viable pollen [Eti 1990].

TTC. 100 mg of TTC was dissolved in 1 mL, and 5.4 g of sucrose was dissolved in 9 mL of distilled water. These solutions were mixed, and a drop was placed on a slide. Pollen was scattered using a brush on the drops, and the slide was covered with a coverslip. The preparation was kept in a dark and humid environment (it should not be below 50%) for 2.5 hours (determined through preliminary studies) before counting. Pollen grains stained dark red and red were considered "absolutely viable", light red or pink were "semi-viable", and unstained were "non-viable" pollen. The count of semi-viable pollen, 50% theoretically viable, was added to the absolute viable pollen count [Eti 1991].

LCB. A drop of the LCB solution, prepared from 20 mL each of crystal phenol, lactic acid, glycerol, distilled water, and 5 mL of a 1% aniline blue mixture, was placed on a slide. Pollen was sprinkled and covered with a coverslip, and the preparation was left in a dark and humid environment for 45 minutes. Pollen grains-stained dark blue were "viable", while blue, light blue, yellow, or colourless were "non-viable" [Abdelgadir et al. 2012, Saarela 2012].

SAF. 1 g of safranin was dissolved in 40 mL of ethyl alcohol and completed to 100 mL with distilled water, forming a stock solution. A safranin solution was then prepared by mixing the stock solution with glycerol and distilled water (1:2:1 v/v). The preparation was left in a humid environment for 45 minutes, and pollen grains stained dark red were "viable", while light red, pink, or unstained were "non-viable" [Eti 1990, Bolat and Pırlak 1999].

AC. 55 mL of distilled water and 45 ml of glacial acetic acid were mixed to make a 45% acetic acid solution. The mixture was heated for 10 minutes until it reached the boiling temperature, after which 0.5 g of carmine powder was added while stirring. After cooling and separating from the sediment, the solution stood for 12 hours and was filtered. A drop of 1% acetocarmine solution was dropped on a slide. After being covered with a coverslip, the slide was left in a dark, humid environment for 45 minutes before counting. Pollen grains stained dark or red were "viable", while light yellow or unstained were "non-viable" [Tingting 2009].

Pollen germination tests

Petri dish method. Four different solid media were used, as shown in Table 1. After preparing the media, they were poured into Petri dishes with an approximate thickness of 2 mm and left to cool. Once the medium had cooled down but before it fully solidified, it was divided into four separate areas and pollen inoculation was performed on each area using a brush. The petri dishes with the inoculations were sealed with lids containing two layers of filter paper moistened with distilled water. Pollen grains, which had been inoculated into the germination media, were incubated at a temperature of 22°C and a relative humidity of 70% in a growth chamber for 12 hours (determined based on preliminary studies), taking into account the specific optimal growth temperature and humidity for the lisianthus. Pollen grains were considered germinated when the pollen tube length reached at least 1.5 times the diameter of the pollen [İmrak 2010, Giovannini et al. 2013].

Hanging drop method. Four different liquid media were used, as shown in Table 1. After the media were prepared, a drop of these media was placed on the coverslips with a pipette. Pollen was sprinkled on these drops with the brush. Then, the coverslip with

Table 1. Solid and liquid medium cultures and their contents used for determining pollen germination rates

Petri dish method / solid medium culture	*Brewbaker and Kwack culture (K-1)	10% sucrose + 100 mg/L boric acid + 300 mg/L calcium nitrate + 200 mg/L magnesium sulfate + 100 mg/L potassium nitrate + 3.5 g agar
	**modified Monnier culture + agar (K-2)	370 mg/L magnesium sulfate + 950 mg/L potassium nitrate + 85 mg/L potassium phosphate + 880 mg/L calcium chloride + 412.5 mg/L ammonium nitrate + 175 mg/L potassium chloride + 7.45 mg/L disodium EDTA + 5.55 mg/L iron II sulfate + 50 mg/L boric acid + 16.50 mg/L manganese II sulfate + 10.50 mg/L zinc sulfate + 0.83 mg/L potassium iodide + 0.25 mg/L sodium molybdate + 0.025 mg/L copper II sulfate + 0.025 mg/L cobalt II chloride + 1.0 mg/L thiamine + 1.0 mg/L pyridoxine + 100 g/L PEG4000 + 3.5 g agar
	agar culture-1 (K-3)	3.5 g agar + 20% sucrose + 10 ppm boric acid
	agar culture-2 (K-4)	3.5 g agar + 20% sucrose + 10 ppm boric acid + 300 mg/L calcium nitrate
Hanging drop method / liquid medium culture	modified Monnier culture* + PEG4000 (S-1)	370 mg/L magnesium sulfate + 950 mg/L potassium nitrate + 85 mg/L potassium phosphate + 880 mg/L calcium chloride + 412.5 mg/L ammonium nitrate + 175 mg/L potassium chloride + 7.45 mg/L disodium EDTA + 5.55 mg/L iron II sulfate + 50 mg/L boric acid + 16.50 mg/L manganese II sulfate + 10.50 mg/L zinc sulfate + 0.83 mg/L potassium iodide + 0.25 mg/L sodium molybdate + 0.025 mg/L copper II sulfate + 0.025 mg/L cobalt II chloride + 1.0 mg/L thiamine + 1.0 mg/L pyridoxine + 200 g/L PEG4000
	modified Monnier culture* + PEG1500 (S-2)	370 mg/L magnesium sulfate + 950 mg/L potassium nitrate + 85 mg/L potassium phosphate + 880 mg/L calcium chloride + 412.5 mg/L ammonium nitrate + 175 mg/L potassium chloride + 7.45 mg/L disodium EDTA + 5.55 mg/L iron II sulfate + 50 mg/L boric acid + 16.50 mg/L manganese II sulfate + 10.50 mg/L zinc sulfate + 0.83 mg/L potassium iodide + 0.25 mg/L sodium molybdate + 0.025 mg/L copper II sulfate + 0.025 mg/L cobalt II chloride + 1.0 mg/L thiamine + 1.0 mg/L pyridoxine + 300 g/L PEG1500
	liquid culture (S-3)	20% sucrose + 10 ppm boric acid
	liquid culture (S-4)	200 gr/L PEG1500 + 10 ppm boric acid

* Jayaprakash 2018
** Zhao et al. 2005, Zhao et al. 2008

the drop was placed on the hollow slides with a ring of petroleum jelly around the edge, with the drop in the centre of the petroleum jelly. They were placed in Petri dishes containing two layers of filter paper moistened with distilled water and covered with filter paper lids [Macovei et al. 2016]. The incubation and germinated pollen grain count procedures were performed as described in the petri dish method.

Data analysis

The randomised complete block design established studies to determine pollen viability and germination rates under in vitro conditions in three replicates. Two coverslips and two areas of each coverslip were counted for each genotype. Averages of 500 pollen grains were counted in each area. The data obtained were expressed as %, and after applying angle transformation, variance analysis was performed using IBM SPSS 22 statistical package programs subjected to variance analysis (one-way ANOVA). The Duncan Multiple Comparison Test revealed the difference between the averages. A hierarchical heat map was created using XLSTAT to understand patterns and groupings within the dataset, bring together data points with similar characteristics, and identify and visualise different groups.

RESULTS

Different pollen viability and germination methods were compared in lisianthus varieties in this study, and the results of the analysis of variance revealed that pollen viability and germination rates of fresh and dead pollen showed statistically significant differences according to both varieties and methods.

Pollen viability tests

In all viability tests for fresh pollen, lisianthus varieties' viable pollen rate was above 50%. The lowest viable pollen rate in all varieties was obtained from the TTC test, while the highest viable pollen rate was determined in the SAF test. When an assessment was made regardless of the varieties, the TTC test showed the lowest viable pollen rate among the tests. When evaluating the overall average of all tests, Alissa 1 White and Sorrie Pink Picote varieties exhibited higher viability rates than Abc 2 Purple and Abc 2 Rose varieties (Table 2).

In order to determine the reliability of staining methods in determining viable pollen rates, the stains made with thermally inactivated pollen were examined. All the staining tests except TTC stained dead pollen grains in all varieties. In other words, IKI, acetocarmine, safranin, and LCB methods stained non -viable pollen, which were unreliable (Table 3).

Pollen germination tests

The germination rate was 25% or less in all mediums except the S-4 liquid medium. For all varieties, the highest germination rate was observed in the S-4 medium. The lowest germination rates varied depending on the methods used. Following the S-4 medium,

Table 2. Viable pollen rates of lisianthus varieties in different staining methods

Variety	Pollen viability tests for fresh pollen (viable pollen rate, %)					
	IKI	LCB	SAF	AC	TTC	Mean
L1	94.30 b	96.43 ab	99.41 a	74.51 c	66.89 d	86.31 A
L2	85.52 b	89.41 b	99.14 a	80.93 c	74.15 d	85.83 A
L3	90.50 b	79.61 c	98.10 a	82.61 c	56.87 d	81.54 B
L4	86.81 b	77.75 d	98.31 a	81.16 c	65.21 e	81.85 B
Mean	89.28 B	85.80 C	98.74 A	79.80 D	65.78 E	–

$p \leq 0.05$, the difference between the averages of different treatments in the same variety is shown with lowercase letters in the same row. Differences between varieties are shown with capital letters in the same column, and differences between treatments are shown with capital letters in the same row. L1: Alissa 1 White, L2: Sorrie Pink Picote, L3: Abc 2 Purple, L4: Abc 2 Rose, IKI: iodine-potassium iodide, LCB: lactophenol cotton blue, SAF: safranin, AC: acetocarmine, TTC: 2,3,5-triphenyltetrazolium chloride

Table 3. Viable pollen rates of lisianthus varieties in different staining methods

Variety	Pollen viability tests for dead pollen (viable pollen rate, %)				
	IKI	LCB	SAF	AC	TTC
L1	89.42	78.25	77.85	90.00	0
L2	93.15	86.21	74.21	98.00	0
L3	91.26	91.25	85.63	95.31	0
L4	88.62	89.00	86.00	94.21	0

$p \leq 0.05$, the difference between the averages of different treatments in the same variety is shown with lowercase letters in the same row. Differences between varieties are shown with capital letters in the same column, and differences between treatments are shown with capital letters in the same row. L1: Alissa 1 White, L2: Sorrie Pink Picote, L3: Abc 2 Purple, L4: Abc 2 Rose, IKI: iodine-potassium iodide, LCB: lactophenol cotton blue, SAF: safranin, AC: acetocarmine, TTC: 2,3,5-triphenyltetrazolium chloride

Table 4. Germinated pollen rates of lisianthus varieties in different culture mediums

Variety	Pollen germination tests for fresh pollen (germinated pollen rate, %)								
	S-1	S-2	S-3	S-4	K-1	K-2	K-3	K-4	Mean
L1	10.36 c	3.34 e	0.20 f	45.11 a	8.29 cd	25.06 b	10.12 c	6.01 de	13.5 A
L2	4.53 c	6.11 c	0.92 d	30.10 a	1.24 d	16.09 b	6.31 c	0.33 d	8.20 C
L3	11.63 c	5.12 d	5.50 d	39.05 a	4.09 d	16.28 b	6.33 d	0.92 e	11.1 B
L4	5.64 c	5.45 c	2.74 c	29.01 a	5.83 c	10.74 b	5.89 c	2.22 c	8.44 C
Mean	8.04 C	5.00 D	2.34 E	35.82 A	4.86 D	17.04 B	7.16 C	2.37 E	–

$p \leq 0.05$, the difference between the averages of different treatments in the same variety is shown with lowercase letters in the same row. Differences between varieties are shown with capital letters in the same column, and differences between treatments are shown with capital letters in the same row. L1: Alissa 1 White, L2: Sorrie Pink Picote, L3: Abc 2 Purple, L4: Abc 2 Rose; S: liquid culture medium, K: solid culture medium

the highest germination rates were noted in the K-2 solid medium. The solid K-4 and liquid S-3 media recorded the lowest germination rates. Similar results were found across treatments, regardless of the variety. Irrespective of the treatments, the highest germination rate was recorded in Alissa 1 White (Table 4). Conversely, the lowest germination rate was determined in Abc 2 Rose.

None of the mediums showed any germination in the inoculations aimed at determining the germination rates of the heat-treated pollen.

Hierarchical heat map analysis of the tests

According to the hierarchical heat map, the tests have been categorised into two main clusters. The first cluster comprises chemical methods, while the second encompasses biological methods. The first cluster is divided into two sub-clusters. The first subset includes the TTC method, distinct from the other chemical methods. Likewise, the second cluster is divided into two sub-clusters, with the S-4 liquid culture medium in the first sub-cluster standing apart from the other biological methods. The hierarchical heat map highlights that

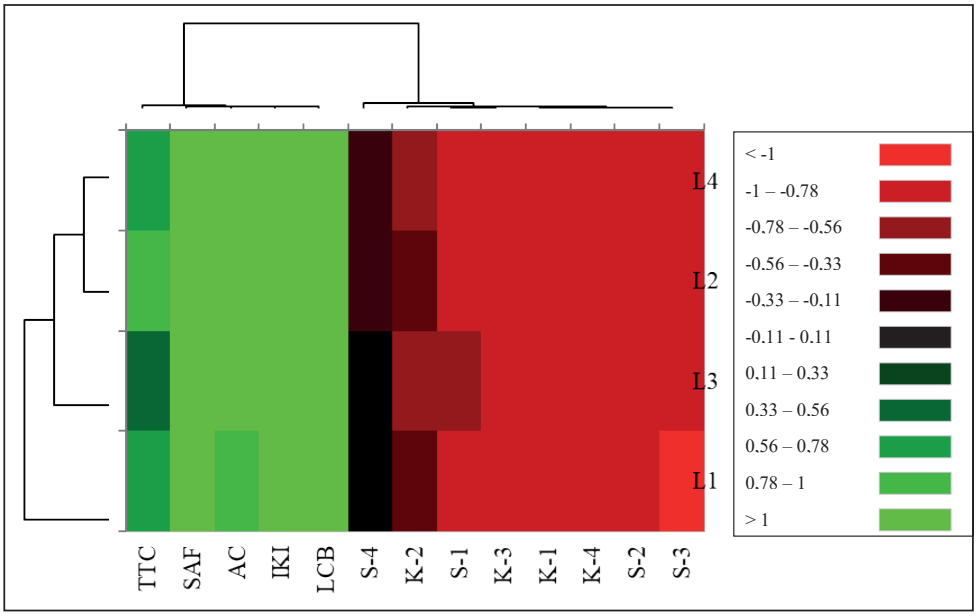

Fig. 1. Hierarchical heat map of pollen germination and viability methods in different lisianthus varieties for fresh pollen. The colour-coded scale indicates an increase from red to green through black, L1: Alissa 1 White, L2: Sorrie Pink Picote, L3: Abc 2 Purple, L4: Abc 2 Rose, TTC: 2,3,5-triphenyltetrazolium chloride, SAF: safranin, AC: acetocarmine, IKI: iodine-potassium iodide, LCB: lactophenol cotton blue

within the same central cluster, the darker-shaded TTC method and S-4 liquid media significantly differ from the other tests and media. It suggests that the TTC test and S-4 culture media may hold the potential to be more reliable or effective than others (Fig. 1).

DISCUSSION

In this study, aimed at optimising pollen viability and germination rates in cut *Lisianthus* varieties, the findings revealed significant differences both within the same variety and among different varieties regarding pollen viability and germination methods. It has been widely acknowledged that pollen viability and germination methods vary depending on genotypes [Eti 1990, Kalyoncu et al. 2013, Macovei et al. 2016], and the effectiveness of these methods can vary depending on the species and cultivar used [Bolat and Güleryüz 1994, Kalyoncu et al. 2013].

Besides genetic differences, another reason for variation among pollen viability methods may be differences in the way the staining method used stains the pollen. The IKI test can stain starch in pollen [Melloni et al. 2013]; the TTC test is a staining method based on the reduction of a red insoluble substance called formaza with a colourless soluble tetrazolium salt in the presence of dehydrogenase enzyme [Şensoy et al. 2003]; and the AC, LCB, and SAF tests can stain pollen components such as cytoplasm and cellulose [Stanley and Linskens 1974, Melloni et al. 2013]. The variation observed in pollen germination methods is thought to be because the culture medium is different from or similar to the stigma fluid specific to the species or variety concerning the amount of components used in the culture medium, along with genetic differences. Fragallah et al. [2019] reported that the components and amounts of pollen germination media may differ according to species; each pollen grain has specific requirements, and different media may be needed. Culture media preparations in pollen germination methods may vary according to plant species and many internal and external factors. Rodriguez-Enriquez et al. [2013] stated that in vitro approaches are based on a culture medium that simulates the conditions of the style stigma.

The IKI, AC, SAF and LCB tests had higher pollen viability rates than the TTC test. It is estimated that the TTC test showed lower viability values due to the

staining ability of methods other than TTC, which can stain fresh and dead pollen. Indeed, in various studies conducted on different species and varieties, it was reported that the TTC test showed lower viability rates compared to other viability tests [Kalyoncu et al. 2013, Sanşili 2014, Erbaş et al. 2015].

Similarly, viability rates obtained from methods other than the TTC method were considerably higher than those from all germination tests. However, closer ratios to certain biological tests were obtained in the TTC method, albeit varying according to the varieties. A study by Parfitt and Ganeshan [1989] found that chemical methods did not show similarity to biological methods. Although a linear relationship between live pollen and germination rate might be expected [Martins et al. 2016], higher viability values can be observed in chemical methods compared to results obtained from biological methods due to the capability of chemical methods to stain immature pollen [Şensoy et al. 2003]. However, experiments conducted on pollen that had lost viability after being held at 80°C for 24 hours revealed that, except for the TTC method, the other staining methods also stained non-viable pollen. The inability of pollen grains to germinate in the culture mediums supports this finding. Wani et al. [2015] also reported that some staining methods can stain non -viable pollen. Therefore, it was determined that the difference between pollen viability and germination methods may arise from the TTC test staining only live pollen, while the other viability tests can stain non -viable pollen.

Among pollen germination methods, it was observed that liquid culture mediums had higher germination rates compared to solid culture mediums. This difference might be attributed to variations in dehydration tolerance levels among varieties. Pacini and Dolferus [2019] stated that pollen tolerance to dehydration may vary depending on the species and varieties. Additionally, while varying according to the varieties, it was generally observed that pollen germination rates obtained from mediums containing sugar were lower than those obtained from mediums containing PEG. This may be because PEG more effectively regulates osmotic pressure than sucrose. Alternatively, the concentration of sucrose in the study might have been too high for the *Lisianthus* species, hindering germination. Research results indicate that PEG is a good osmoregulator; it may be more successful in regulating osmotic potential in pollen tube growth, and high concentrations of sugar can inhibit germination [Hong-Qi and Croes 1982, Shivanna and Sawhney 1995, Lin et al. 2017, Li et al. 2023].

In conclusion, among easily applicable pollen viability methods, the TTC test was found to be the most reliable, while among pollen germination methods, the S-4 liquid culture medium was determined to be the ideal medium for the *Lisianthus* species. It is recommended that different concentrations of this culture medium containing boric acid and PEG1500 be tested.

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