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Full Length Article

Germination Characteristics and Dynamic Changes of Antioxidant Enzymes during Storage of *Viola dissecta* Pollen

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Abstract

Knowledge about pollen ultra-morphology, storage characteristics and germination rate are essential for directional plant breeding and plant improvement. The objective of this study was to determine a suitable medium for pollen germination *in vitro* of *Viola dissecta* and to evaluate the effect of different storage temperatures on its pollen longevity. The pollen ultra-morphology of *V. dissecta* was observed using scanning electron microscopy (SEM), the suitable medium for pollen germination *in vitro* was determined by orthogonal test. Dried pollen of *V. dissecta* was stored at different temperatures (room temperature, 4, -20 and -80°C) and different storage times (24, 40, 72, 120, 184, 264 and 365 d), the germination rate of the stored pollen and the activities of SOD, POD and CAT were investigated. Pollen grains of *V. dissecta* were medium-sized with three germination ditches. The surface ornamentation was smooth with small grains set on the surface, which was different from *Viola* spp. pollen. The most suitable medium for *V. dissecta* was composed of 285 g•L^{-1} sucrose, 6 g•L^{-1} agar, 50 mg•L^{-1} GA_3, 250 mg•L^{-1} boric acid, and 200 mg•L^{-1} $Ca(NO_3)_2$, The best storage temperature of pollen was -80°C, after 365 d of storage, the germination rate was still 57.86%. During storage, the pollen germination rate decreased significantly after the peak of the activities of the three antioxidant enzymes. Correlation analysis showed that SOD was major factor affecting the germination rate of *V. dissecta* pollen, and it has a significant positive correlation with pollen germination rate, followed by CAT and POD. SOD was a sensitive antioxidant enzyme at room temperature, 4 and -80°C, whereas at -20°C, both SOD and CAT were sensitive antioxidant enzymes.

Keywords: *Viola dissecta*; Ultra-morphology; Pollen germination; Antioxidant enzyme activity; Storage

Introduction

Viola dissecta Ledeb is a perennial herb native to China, which belongs to the family Violaceae with palmately lobed leaf and heat resistance in China. Although *V. tricolor* and *V. cornut* has a long flowering period, but with a simple leaf shape, lower heat resistance and cold resistance, which seriously restricted their application and popularization. Due to its strong resistance to stress and aesthetic qualities, *V. dissecta* is considered to be one of the most promising plants for genetic improvement of *V. tricolo*r and *V. cornut*.

Hybrid breeding technology is one of the most common means for breeding new varieties in family Violaceae (Horisaki and Niikura 2004; Guo *et al.* 2017). Hybrid breeding requires a large amount of high-quality pollen for pollination and fertilization (Abdullah *et al.* 2000; Chen *et al.* 2011). Therefore, the evaluation of the germination ability of the pollen after storage is the key to the success in *Viola* breeding (Dane *et al.* 2004; Kaur and Singhal 2019). *In vitro* germination is a commonly used technique to identify pollen viability. Pollen from *V. dissecta* is short-lived after releasing from anthers and thus pollen preservation is essential for overcoming the seasonal and geographical limitations of hybridization, and it is also an effective method to preserve plant germplasm resources. Pollen germination rate is the main indicator to evaluate the success of the storage method (An *et al.* 2011). The evaluation of pollen germination *in vitro* can validate germination *in vivo* (Jia *et al.* 2015).

Studies have shown that under different temperature stresses, the physiological balance of pollen is disrupted, resulting in excessive accumulation of reactive oxygen species (ROS) in pollen and oxidative damage to pollen cells (Cao 1986; Liu *et al.* 2013). Therefore, the pollen germination rate gradually decreases with the extension of storage time (Tan 2011; Qi *et al.* 2014; Zhang *et al.* 2018). The antioxidant enzymes can eliminate ROS and protect pollen to reduce damage (Guan *et al.* 2012; Qi *et al.* 2014) and the level of antioxidant enzyme activity can be used as an indicator of pollen germination to a certain extent.

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There is a correlation between pollen germination rate and antioxidant enzyme activity (Tan 2011; Zhao *et al.* 2004; Zhang *et al.* 2018; Jia *et al.* 2020). At present, studies on pollen of Violaceae family is mainly focused on pollen preservation of Violaceae, optimizing the medium for pollen germination (Mu *et al.* 2013) and optimizing temperature for pollen storage (Guo *et al.* 2017). Systematic studies on germination and storage in Violaceae pollen have not been reported and the physiological mechanism of pollen cell senescence is still elusive. The pollen viability and storage characteristics of *V. dissecta* pollen have not been reported. Therefore, in this study, pollen ultra-morphology, pollen germination rate and storage characteristics of *V. dissecta* were studied. This study aimed to determine an accurate medium for evaluating the germination of *V. dissecta* pollen, the physiological and biochemical reactions of pollen during storage were explored in terms of the antioxidant enzyme activity of pollen to provide experimental basis for selecting viable pollens for *Viola* interspecific hybridization.

Materials and Methods

Pollen collection

Viola dissecta flowers were collected from Taihang Mountain in Henan province, China. Flowers were collected at flowering stage before the anthers released pollen. The collected flowers were placed in an ice box, and taken back to the laboratory; the unspattered anthers were taken out of 5000 flowers, put in petri dishes and placed in a cabinet at 25°C for 24 h. The pollen grains released from anthers were collected and divided into two parts: one part was used to determine the pollen germination rate and the ultra-morphology, the other part was dried for 24 h in a blast drying oven at 30°C and the dried pollen was filled and sealed in a centrifuge tube for later use.

SEM observation on pollen morphology

The pollen grains were dried in a blast dryer at 50°C for 6 h, and sprayed gold with a sputter coater, then pasted the pollen grains on the sample stage with black double-sided conductive adhesive and placed under a scanning electron microscope (SEM) for observation. The shape and ornamentation of pollen grains were observed; the photos of pollen grains were taken at 500–3500 times magnification. The description of pollen morphology was mainly based on the terminology and definition of "Introduction to Palynology"(Wang and Wang 1983).

Optimization of culture medium for *in vitro* germination of pollen

An orthogonal experimental ($L_9[3]^4$) design was used to optimize the germination medium for *V. dissecta* pollen. The number '4' means 4 factors ([1] sucrose, [2] H_3BO_3, [3] GA_3 and [4] $Ca(NO_3)_2$), '3' means 3 levels (1) sucrose (265, 285, and 305 g•L^{-1}), [2] H_3BO_3 (150, 200, and 250 mg•L^{-1}), [3]GA_3 (50, 100, and 200 mg•L^{-1}) and [4] $Ca(NO_3)_2$ (100, 200, and 300 mg•L^{-1})), and '9' means 9 trials. Each treatment was repeated three times, CK was the control medium (only 6 mg•L^{-1} agar added). The heated and melted medium components were poured into petri dishes with a diameter of 50 mm. After cooling and solidification, fresh pollen grains were scattered on the surface with a sterile paintbrush. The petri dishes were then placed in an incubation chamber at 24°C for 15 h. Fresh pollen grain germination was assessed, and the germinated pollen grains were observed and counted by the Olympus Fluorescent Microscope BX53 at 10X magnification. A pollen grain was considered germinated if the length of the pollen tube was longer than the diameter of the pollen grain.

Germination rate and antioxidant enzyme activity of stored pollen

The dried pollen grains were divided into 4 parts and stored them at room temperature, 4, -20 and -80°C, respectively. Twenty centrifuge tubes with 1.5 g pollens were placed at each temperature. A few pollen grains were taken out from each centrifuge tube after 24, 40, 72, 120, 184, 264 and 365 days and the germination rate was determined using the medium optimized in the experiment.

The germination rate, SOD activity, POD activity, and CAT activity of pollen grains stored at room temperature, 4, -20 and -80°C were evaluated *in vitro* after 24, 40, 72, 120, 184, 264 and 365 d. The medium was prepared with [sucrose (285 g•L^{-1}), boric acid (250 mg•L^{-1}), GA_3(50 mg•L^{-1}) + $Ca(NO_3)_2$ (200 mg•L^{-1})]. Stored pollen grains at -20 and -80°C were thawed in a 45°C water bath for 3–5 min until the ice melted. After thawing, stored pollen grains were cultured in petri plates containing pollen germination medium in incubators. These petri plates were incubated at 25°C for 24 h, and the germination of stored pollen grains were evaluated.

Antioxidant enzyme assay experiments were performed at 4°C. A 1.0 g pollen was putted in a pre-cooled mortar, added 0.5 mol·L^{-1} phosphate buffer (pH 7.0), 50 mg polyvinylpyrrolidone (PVP) and 0.1g quartz, and homogenized for 30 s. The homogenate was centrifuged at 4°C at 15 000×g for 20 min and the supernatant was used to determine the enzyme activity.

The activities of SOD, POD, and CAT were determined by the nitroblue tetrazolium (NBT) reduction method (Zhang *et al.* 2007), the Guaiacol method and the potassium permanganate titration method (Pan 2001), respectively. All enzyme assay experiments were performed 3 times.

Statistical analysis

SPSS 19.0 was used to perform statistical analysis and

analyze the orthogonal test for pollen germination. Means grouping was done with Duncan's multiple test (P<0.05 or P<0.01). In addition, Microsoft Excel 2016 software (USA) was used to generate figures and tables.

Results

Plant and pollen morphological characteristics

V. dissecta leaves were pinnate and deep-lobed, the flower was papilionaceous corolla in rose red color, with high ornamental value (Fig. 1A–C). The average length of the polar axis of *V. dissecta* pollen was 45.12 μm, the average length of equatorial diameter was 22.51μm, and the ratio of the length of the polar axis (P) to the equatorial diameter (E) in *V. dissecta* pollen ≈2, so the shape of *V. dissecta* pollen was spheroidal. Pollen was nearly circular in polar view, oblong in equatorial view. The pollen has three germination ditch, each with a width of approximately 3 μm, which dehisced from one pole to the other along the longitudinal axis (Fig. 1D–G), The ornamentation of pollen grains was smooth with small grains set on the surface, and the small cave-like carved lines had fine and curved net ridges that were more uniform but irregular in shape and size (Fig. 1H). Malformed and underdeveloped pollen accounted for 14.56% of the total pollen grains.

Pollen germination in different media

Table 1 shows that the pollen germination rate of *V. dissecta* were significantly different among 10 medium treatments (P<0.05). The average pollen germination rate of No. 6 medium was 86.64% (Table 1; Fig. 2B), which was better than the control medium (CK) with only 16.11% pollen germination rate (Table 1; Fig. 2A). Boric acid was the most important factor affecting pollen germination, followed by sucrose, GA_3 and $Ca(NO_3)_2$ (Table 1). Due to the interaction of the 4 factors, the optimal medium for pollen germination of *V. dissecta* pollen was 285 g•L^{-1} sucrose+50 mg•L^{-1} GA_3 +200 mg•L^{-1} $Ca(NO_3)_2$ +250 mg•L^{-1} boric acid (Fig. 2B), which was significantly higher than other media (Fig. 2C).

Effects of storage temperatures and storage times on pollen germination

Pollen germination rate was significantly influenced by storage temperature and time (Fig. 3). Pollen germination rate decreased gradually with increase in storage duration. The pollen rate decreased rapidly with storage time duration at room temperature, the pollen germination rate dropped to 0, and the pollen lost vitality when stored for 120 d. The pollen germination rate fell to 0 when stored at 4°C after 184 d of pollen storage. Under the storage conditions of -20 and -80°C, the germination rate of pollen decreased rapidly from 0~120 d of pollen storage, pollen activity showed a slow downward trend from 120~365 d. The pollen germination rate was the highest (45.43%) at -80°C after 365 d of pollen storage, which was 52.92% of the germination rate of fresh pollen, followed (24.50%) by -20°C. The findings of this study indicated the pollen longevity at -80°C is longer than 365 d.

Table 1: Effects of different media on pollen germination of *V. dissecta*

Treatment	Sucrose (g•L^{-1})	Boric acid (mg•L^{-1})	GA_3 (mg•L^{-1})	$Ca(NO_3)_2$ (mg•L^{-1})	Germination rate (%)
1	265	150	50	100	41.3±1.32de
2	265	200	100	200	31.83±1.40g
3	265	250	200	300	45.40±1.82bc
4	285	150	100	300	46.00 ±0.11b
5	285	200	200	100	42.00±0.10cd
6	285	250	50	200	86.64±1.30a
7	305	150	200	200	38.42 ±0.10e
8	305	200	50	300	34.48±1.20f
9	305	250	100	100	41.64±1.87d
CK	0	0	0	0	16.11±1.13h
K1	39.51	41.91	54.14	41.65	
K2	58.21	36.10	21.49	52.30	
K3	30.74	57.89	39.83	41.96	
R	8.87	9.72	5.60	4.08	

Note: The data with different capital letters indicate significant differences at the 0.05 level

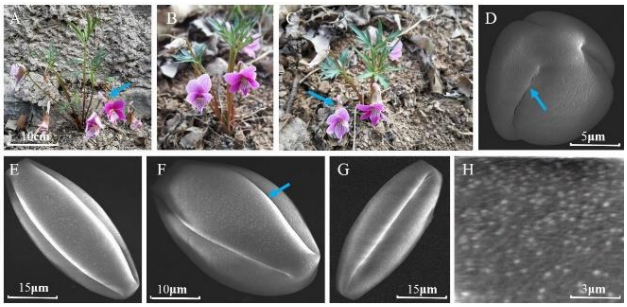

Fig. 1: Ultra-morphology of pollen grains of *V. dissecta* under the SEM. A-C: *V. dissecta*; D-E: Polar view; E-G: equatorial view; H: Exine sculptures

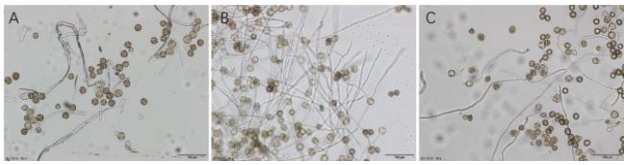

Fig. 2: Germination rate of *V. dissecta* pollen in different culture media. A: the control; B:286 g•L^{-1} sucrose, 250 mg•L^{-1} boric acid 50 mg•L^{-1}GA_3 200mg•L^{-1}Ca $(NO_3)_2$; C:305 g•L^{-1} Sucrose, 250 mg•L^{-1} boric acid, 100 mg•L^{-1} GA_3 100 mg•L^{-1} Ca $(NO_3)_2$)

Pre- and post-storage changes of antioxidant activities in pollen

SOD activity: As shown in Fig. 4, storage temperature and time had a much greater impact on SOD activity (P<0.05). With the extension of storage time, the SOD activity in *V.*

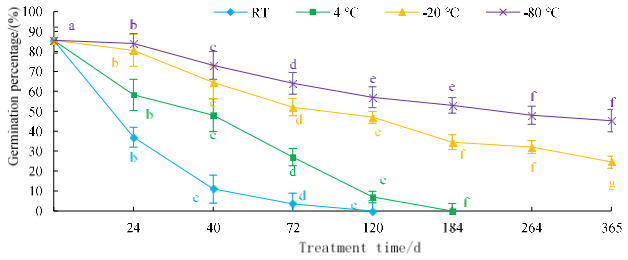

Fig. 3: Effects of different storage methods on pollen germination

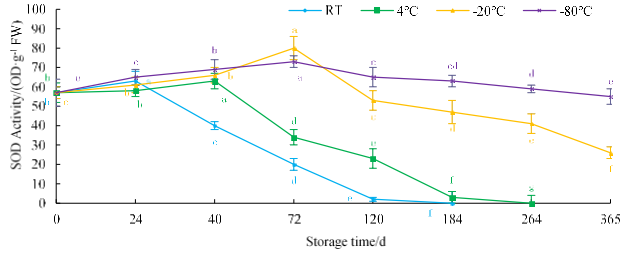

Fig. 4: Effects of different storage times and temperatures on pollen SOD activity

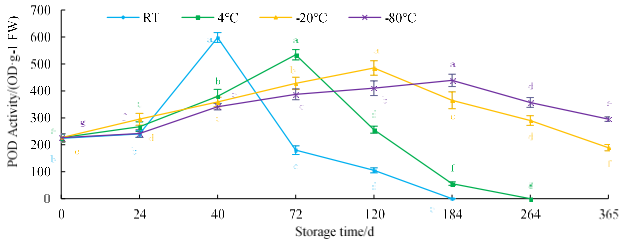

Fig. 5: Effects of different storage times and temperatures on pollen POD activity

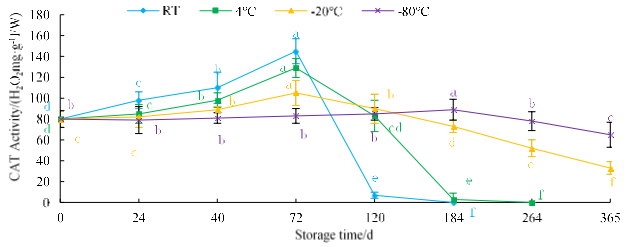

Fig. 6: Effects of different storage times and temperatures on pollen CAT activity

dissecta pollen rapidly increased, and peaked at 24 d at room temperature and at 40 d at 4°C, and then decreased, and the SOD activity in pollen dropped to 3 after storage of 184 d at 4°C, which was only 5.26% of its peak, and then declined rapidly with storage time duration, dropped to 0 at 184 d and at 264 d, respectively. At -20°C, SOD activity increased, and reached a peak at 72 d of storage and then gradually declined. At-80°C, SOD activity went up slowly, reached a peak with 73 OD•g^{-1} at 72 d, decreasing to 60 from 72 to 120 d of storage, and then showed a slow decrease from 120 d to 365 d, SOD activity decreased to 55 after 365 d of storage, which was 96.49% of that before preservation, this suggest that pollen has a strong ability to remove active oxygen.

POD activity: Under different storage time and temperature, the POD activity of *V. dissecta* pollen changed significantly (Fig. 5; P<0.05). Under 4°C and room temperature, POD activity showed a curve of first rapid increase and then rapid decline with the extension of storage time. After 40 d of storage at room temperature, POD activity reaches a peak with 598 OD·g^{-1} FW, and then rapidly decreases, and dropped to 0 at 184 d. At 4°C, POD activity reached a peak at 72 days and then rapidly dropped to 0 at 264 d. At -20 and -80°C, the change in POD activity was similar, POD activity showed a slow increase and then a slow declining trend. Peak activity was observed with 485 OD·g^{-1} FW at 120 d at -20°C, and with 439 OD·g^{-1} FW at -80°C at 184 d, respectively. POD activity at 365 d decreased to 189 OD·g^{-1} FW and 295 OD·g^{-1} FW respectively, which accounted for 83.62% and 130.53% of the POD activity of pollen before storage. This indicated that pollen can eliminate H_2O_2, phenols, aldehydes, etc. to get protected from damage to maintain high viability.

CAT activity: There were significant differences in CAT activity at different storage temperatures (Fig. 6; P<0.05). The CAT activity of *V. dissecta* pollen showed a rapid increase and then a rapid decline with storage time duration at room temperature and 4°C. After storage for 184 d and 264 d, the CAT activity dropped to 0. The CAT activity in *V. dissecta* pollen increased first and then decreased with storage time duration at-20 and -80°C. Peak of CAT activity was found with 105 H_2O_2 mg·g^{-1} FW at 72 d and with 89 H_2O_2 mg·g^{-1} FW at 184 d, respectively. At 365 d, CAT activity decreased to 65 and 33 H_2O_2 mg·g^{-1} FW, which accounted for 81.25 and 41.25% of the CAT activity in pollen before storage, respectively. This indicated that pollen can scavenge H_2O_2 in time at a low temperature and protect pollen from damage.

Correlation of pollen germination percentage with enzyme activities

Correlation analysis between pollen germination percentage and the three antioxidant enzymes was performed and is shown in Table 2. The pollen germination percentage in *V. dissecta* had a positive correlation with CAT and POD activities, which was significantly positively associated with SOD activity (Table 2; P <0.05). The SOD activity played a dominant role in the pollen germination percentage during storage. The effect of SOD activity on the pollen germination was higher than that of CAT and POD activities. SOD activity exhibited a significantly positive association with POD activity or CAT activity (P<0.05).

Table 2: Correlation between pollen's germination and antioxidant enzymes activity

Antioxidants	Germination rate	SOD	POD
SOD	0.862^{**}		
POD	0.459^{ns}	0.759^{**}	
CAT	0.518^{*}	0.726^{**}	0.784^{**}

Note: "**" indicates that the correlation\ analysis appeared a very significant level; "ns" non-significant (P>0.05)

Discussion

In this study, average size of *V. dissecta* pollen was "medium", which significantly differed from *V. variegate*, which has small pollen (Guo *et al.* 2017), but was in line with *V. tricolor* (Lu *et al.* 2005). Furthermore, compared with the pollen of other species of *Viola* (Jiang *et al.* 2000; Guo *et al.* 2017), the *V. dissecta* pollen has unique characteristics that specifically include the following: (1) the germination ditch was narrow, while this was wider in other species; and (2) the pollen surface was more smooth, which was different from that of Violaceae, the ornamentations of pollens surface are generally striated, wrinkled corrugated and verrucous. These differences suggest that *V. dissecta* may have a special taxonomic status in the genus *Viola*. According to the study of Walker (1974), the evolution of the pollen surface decoration was smooth-mesh-stripe surface wart thorn-like; indicating that *V. dissecta* was more primitive.

It is very essential to determine the requirement of pollen germination for cross-pollination (Abdelgadir *et al.* 2012), since different plants have different requirements for pollen germination (Huang and Wu 2011). It has been reported that sucrose (Kremer and Jemri´c 2006; Salles *et al.* 2006), boric acid (Báez *et al.* 2002; Zhang *et al.* 2018), GA_3 and $CaCl_2$ (Hirose *et al.* 2014) can satisfy the requirements for pollen germination and play important roles in pollen germination. Our study showed that the pollen grains of *V. dissecta* germinated even without sucrose, boric acid, GA_3 and $Ca(NO_3)_2$, but the germination rate was only 16.11%, by comparison, the germination rate of *V. dissecta* pollen ballooned with the addition of sucrose, boric acid, Ca^{2+} and GA_3. This finding explained the germination of most pollen requires the supply of exogenous nutrients. Our study showed that the optimal medium for pollen germination of *V. dissecta* had 285 g/L sucrose, 250 mg/L H_3BO_3, 200 mg/L $Ca(NO_3)_2$ and 50 mg/L GA_3 (Table 1). Excessive concentrations of sucrose, $Ca(NO_3)_2$, H_3BO_3 and GA_3 inhibited pollen germination, which was in accordance with previous experiments on the pollen, including *Chaenomeles sinensis* (Guan *et al.* 2012), *Camellia japonica* (Jia *et al.* 2015). Multiple comparisons showed that boric acid was the major factor influencing the germination of *V. dissecta* pollen. Furthermore, pollen germination rate (86.64%) correlated with pollen deformity rate (14.56%) of *V. dissecta* (Table 1), this demonstrated that pollen deformity was the main reason why a few pollens cannot germinate.

The pollen preservation is of great importance for breeding new varieties through hybridization (Keller *et al.* 1996; Báez *et al.* 2002), low moisture content and low temperature can effectively reduce the physiological activity and nutrient consumption during pollen storage, and extend pollen longevity (Tan 2011; Liu *et al.* 2020). The storage pollen at -20°C in sweet cherry had greater pollen viability rather than pollen those at -80 or 4°C (Özcan 2020). Contrasting results have been reported by Liu *et al.* (2020) and Jia *et al*. (2015) that pollen germination in *Keteleeria fortunei* and *Paeonia qiui* was superior at -80°C than other temperatures. We found that pollen stored at room temperature, 4 or -20°C only had a low germination rate compared with those stored at -80°C in *V. dissecta*. Pollen germination rate decreased slowly at 4°C, the pollen germination rate dropped to 0 after 184 d at 4°C (Fig. 3). This suggested that the 4°C was only suitable for short-term preservation of pollen. At -80°C, the pollen germination rate still reached 45.3% after 365 d of pollen storage, which was 53.17% before storage. This indicated that the longevity of *V. dissecta* pollen may be more than 12 months at -80°C.

SOD, POD and CAT activities are the main physiological indicators of the level of damage to plants (Miltiadis and Porlingis 1985; Cakmak and Horst 1991; Kanazawa *et al.* 2000). The level of antioxidant enzyme activity can reflect pollen germination ability to a certain extent (Mao *et al.* 2016; Guo *et al.* 2017; Ren *et al.* 2021). During pollen storage, the rapid decrease of antioxidant enzyme activity after reaching the peak often indicates a rapid decline in pollen germination rate (Akhond *et al.* 2000; Abdelgadir *et al.* 2012; Dong *et al.* 2017; Liu *et al.* 2020). Our study showed that at 4°C, the time when the pollen germination rate drops to 0% was delayed by 80 d compared with that at room temperature. The activities of SOD, POD and CAT all showed a trend of first increasing and then decreasing. Compared with at room temperature, the peak time of POD and SOD activity was delayed by 16–32 d at 4°C storage. This showed that the accumulation of ROS in pollen at 4°C was slower than at room temperature, pollens produced not only reactive oxygen species but also possibly toxic substances, such as amines.

At -20 and -80°C, the germination rate of *V. dissecta* pollen was 45.3% after 365 d storage at -80°C, which was 53.17% of that before storage, the activities of SOD, POD and CAT were more than 80% before storage, the finding was in good agreement with that reported for *Keteleeria fortune*i pollen (Liu *et al.* 2020). This indicated that pollen has the ability to scavenge active oxygen and free radicals, and can delay pollen senescence (Xu *et al.* 2015). Tan (2011) reported that pollen germination rate correlated strongly with antioxidant enzymes during pollen storage. This study showed that the pollen germination rate was positively correlated with SOD, CAT and POD activities. This showed that the activity level of antioxidant enzymes could be used as indicators of pollen viability. The antioxidant enzymes played different roles at different

storage temperatures, under room temperature, 4 and -80°C, the peak of SOD activity appeared earlier than POD and CAT activity. This indicated that SOD played a crucial role for protecting pollen at 3 temperatures. The peak of SOD and CAT activity first appeared at 72 d of storage at -20°C, followed by that of POD, which showed that SOD and CAT played a sensitive role at -20°C. In brief, SOD acted as a sensitively antioxidant role at room temperature, 4 and -80°C, whereas SOD and CAT acted as sensitively antioxidant roles at -20°C.

Conclusion

Pollen surface ornamentation and size for *V. dissecta* were significantly different from *Viola spp.* An optimal medium composition for *V. dissecta* was 285 g•L^{-1} sucrose+200 mg•L^{-1} Ca $(NO_3)_2$+50 mg•L^{-1} GA_3+250 mg•L^{-1} boric acid, resulting in a germination rate of 86.64%. Boric acid was the main factor affecting the pollen germination. Storage of *V. dissecta* pollen at −80°C offered a more reliable way of pollen viability preservation than storage at room temperature, 4 and -20°C, the pollen longevity at -80°C was longer than 365 days. SOD was the main factor affecting the germination rate of *V. dissecta* pollen, followed by CAT and POD. SOD acted as a sensitively protective role at room temperature, -4 and -80°C, while at -20°C both SOD and CAT showed sensitivity. Further studies are needed to find the possible cytological mechanism(s) of pollen programmed death in the present study.

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Author Contributions

WJ SH designed the experiments; WJ, YG, YW and DK performed the experiments and analyzed the data. YG and YW prepared figures and tables.

Conflict of Interest

We, the authors, declare no conflict of interest of any kind among ourselves of the institutions where the work was done

Data Availability Declaration

All data reported in this article are available with the corresponding author and will be produced on demand

Ethics Approval

Not applicable

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