

Biological characteristics of flowers and examination of pollen viability at different developmental stages of *Epimedium sagittatum*(Sieb. et Zucc.)Maxim

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Abstract

To gain a deeper understanding of the flowering pattern and reproductive characteristics of *Epimedium sagittatum*, to enrich the research on the flower development of *Epimedium sagittatum* and its reproductive regulation, and to screen the methods suitable for the rapid detection of pollen viability of *Epimedium sagittatum* and to promote its cross-breeding. The characteristics of its flower parts were observed, recorded and measured, and the pollen viability of *Epimedium sagittatum* was determined by five methods, including TTC staining, I₂-KI staining, red ink staining, peroxidase method and in vitro germination method. The flowering process of *Epimedium sagittatum* can be divided into five stages: calyx dehiscence, bract spathe, petal outgrowth, pollen dispersal, and pollination and withering. The results of I₂-KI staining and peroxidase method were significantly higher than those of other methods; the in vitro germination method was intuitive and accurate, but the operation was complicated and time-consuming; the red ink staining method was easy to operate and had obvious staining effect, and the results were the closest to those of the in vitro germination method; and it was found that the pollen of *Epimedium sagittatum* was not as effective as the in vitro germination method at the bud stamen stage, the flower stigma and the flower bud. It was also found that the pollen viability and germination rate of *Epimedium sagittatum* pollen were higher in the three periods of bud spitting, petal adductor and pollen dispersal. Comparing the five methods, the red ink staining method was found to be a better method for the rapid detection of pollen viability; the best pollination periods of *Epimedium sagittatum* were the bud stamen stage, petal adductor stage, and pollen dispersal stage of flowers at the peak of bloom. This study on the flowering and fruiting pattern of *Epimedium sagittatum*, and the related mechanism of sexual reproduction, can be used as a reference for the next step of research on the breeding of *Epimedium sagittatum*.

1. Introduction

Epimedium sagittatum (Sieb. et Zucc.) Maxim. (also known as “three branches and nine leaves grass”) is one of the most important traditional medicinal plants in China. It belongs to *Epimedium*, a perennial herbaceous plant in the Berberidaceae family (Wen et al.,2022). First recorded in Shennong's Classic of Materia Medica, it was listed as a top grade herb (Xu et al.,2020) with the effects of reinforcing kidney yang, strengthening muscles and bones, dispelling rheumatism, etc. It is often used to treat kidney yang deficiency, osteoporosis and other diseases (Wei et al.,2023; Wang et al.,2023). With the continuous in-depth research on *Epimedium* in modern science, the latest research has found that it also has anti-tumor effects, especially on patients with advanced liver cancer (Li et al.,2021). This has led to an increasing demand for *Epimedium*, and wild resources cannot meet the demand, resulting in rising prices. In order to obtain a large amount of *Epimedium*, artificial cultivation has begun. Currently, *Epimedium* seeds are expensive. In the cultivation process, it was discovered that although *Epimedium sagittatum* has abundant flowering, its reproductive ability is low, the seed setting rate is low, and the germination rate is extremely low. Therefore, there are few seedlings in natural populations (Cui et al.,2022).

It can be seen from the above that the weak reproductive ability and low seed germination rate of *Epimedium sagittatum* have always been a problem in *Epimedium* cultivation. Studies have found that the size of pollen viability directly affects the pollination and fertilization processes of plants, which is closely related to the yield and quality of plant seeds (Bai et al.,2023). Testing the viability of *Epimedium sagittatum* pollen at different flowering stages before pollination can understand the viability, development and physiological characteristics of *Epimedium sagittatum* pollen, and grasp the morphology of sterile pollen, providing a theoretical basis for artificial breeding (Li et al.,2023; Xu et al.,2012). Therefore, researching the viability and germination characteristics of *Epimedium sagittatum* pollen is very necessary to improve pollination breeding efficiency and reasonably carry out breeding to increase seed germination rate and obtain high-quality *Epimedium sagittatum* seeds. On this basis, it is of great value and significance to establish scientific and effective pollination methods for the future, accelerate the breeding process of excellent *Epimedium sagittatum* varieties, shorten the differentiation period between varieties, and thus enhance the competitiveness of *Epimedium sagittatum* germplasm. At the same time, it can provide a basis for the construction of the *Epimedium sagittatum* breeding system under artificial cultivation conditions and production application, thereby promoting the realization of maximized economic benefits in the large-scale production of *Epimedium sagittatum*. However, research and reports on pollen viability detection of this species have not been seen so far.

This paper systematically studies the flowering dynamics and floral characteristics of *Epimedium sagittatum*. It also determines the pollen viability at different developmental stages and screens suitable methods for rapid detection of pollen viability in *Epimedium sagittatum*. The aim is to deeply understand its flowering habits and reproductive characteristics, enrich data on flower development and reproductive regulation of *Epimedium sagittatum*, explore the limiting factors affecting its breeding process, discuss its breeding habits and pollination methods, and explore ways to improve the pollen viability of *Epimedium sagittatum*. This study aims to reveal the flowering mechanism of *Epimedium sagittatum*, clearly understand the physiological and biochemical characteristics of its pollen at different developmental stages, grasp the changes in pollen viability, thereby determine the optimal pollination time and pollen testing method. The purpose is to provide preliminary research basis for exploring artificial assisted fertilization technology, improving seed viability, guiding artificial cultivation management for high yield and efficiency, simplifying production procedures, and selecting new varieties in the future. This will provide preliminary research basis for renewing and reproductive biological studies as well as selecting new varieties of this species.

2. Experimental Materials and Methods

2.1 Experimental Materials

2.1.1 Sample Collection

The test site was located in the *Epimedium* plantation base in Pingyu County, Henan Province, at east longitude 114°61' and north latitude 32°96'. The test materials were *Epimedium sagittatum* seeded and

nursed in October 2021 with good growth after routine field management. Pollen was collected at the peak flowering period on sunny mornings from 9:00–10:00. Flowers at different blooming stages were collected and brought back to the lab for pollen extraction and testing.

2.2 Experimental Methods

2.2.1 Observation of Floral Organ Characteristics, Flowering Habits and Dynamics

From March to June 2023, 120 *Epimedium sagittatum* plants were randomly selected and tagged in the base. The date when the first flower bloomed was recorded as the initial flowering period, the period when over 50% of flowers bloomed was recorded as peak flowering period, and the date when the last flower bloomed was recorded as final flowering period. At peak flowering, 60 freshly bloomed single flowers were randomly selected every day and observed 6 times. Observations were made every 2 hours from initial to full bloom until withering. The collected fresh flowers were placed under a SZX2-ILLTQ stereo microscope to measure and record the dimensions of each floral part.

2.2.2 Pollen Viability Testing

2.2.2.1 Triphenyl Tetrazolium Chloride (TTC) Staining

TTC is an oxidation-reduction dye with a standard oxidation potential of 80mV. It dissolves into a colorless solution in water. After reduction, it forms the red insoluble triphenyl formazan. Its reduction amount can indicate dehydrogenase activity. This substance is relatively stable and not easily oxidized, so TTC is widely used in enzyme experiments to determine pollen viability. Referring to Liang Lu (Ling et al.,2022) et al.'s method with improvements, 1–2 drops of 0.5% TTC solution were dropped onto a concave slide. Pollen was sprinkled into the solution, mixed well, covered with a coverslip, and placed in a 35°C incubator for 1h. After taking out and microscopic examination, viable pollen appeared red, weakly viable pollen appeared light red, non-viable or sterile pollen was colorless.

2.2.2.2 I₂-KI Staining

The starch content in pollen can be used as a criterion to judge the level of pollen development. Normal mature pollen is mostly spherical with high starch content and can be stained blue by I₂-KI solution. Underdeveloped pollen is mostly deformed and generally contains no or little starch. It usually does not contain starch or contains relatively less starch and will not be stained or will be stained yellow or yellowish brown by I₂-KI solution. According to pollen staining, pollen viability can be identified. Referring to Song Jing (Song et al.,2022) et al.'s method with improvements, 1–2 drops of I₂-KI solution were dropped onto a concave slide. Pollen was sprinkled into the solution, mixed well, covered with a coverslip, and stained for 5 min before microscopic examination.

2.2.2.3 Red Ink Staining

The protoplasm of viable pollen cells has selective permeability and selective absorption capacity for external substances, such as red ink dyes cannot enter the cells and the pollen cannot be stained. However, the protoplasmic membrane of lifeless pollen cells will lose this activity, dyes will enter the cells and stain them. Therefore, pollen viability can be judged by whether pollen grains are stained. Referring to Wang Jinhua (Wang et al.,2022) et al.'s method with improvements, 5% red ink solution was prepared, 1–2 drops were dropped onto a concave slide, pollen was sprinkled into the solution, mixed well, covered with a coverslip, and examined under the microscope immediately. Viable pollen was not stained while non-viable pollen was stained red.

2.2.2.4 Peroxidase Method

Peroxidase is contained in pollen. Pollen with higher viability has stronger peroxidase activity. Peroxidase forms a complex with oxidants. The activated hydrogen peroxide in the complex can oxidize phenolic compounds. Pollen viability can be judged by the color change. Viable pollen is purple-red, while pollen with weaker viability or no viability is light red or colorless. Referring to Fu Qinchao (Fu et al.,2015) et al.'s method with improvements, 1 drop of aromatic amine coloring solution (0.5% aniline dye solution : 0.5% α -naphthol dye solution : aromatic buffer = 1:1:1) and 0.1% hydrogen peroxide each were dropped onto a concave slide. Pollen was sprinkled into the solution, mixed well, covered with a coverslip, and kept at 30°C for 15 min before microscopic examination.

2.2.2.5 In Vitro Germination

Normal mature pollen grains have strong viability. They can germinate and grow under suitable culture conditions. Germination number can be directly observed and counted under the microscope to calculate germination rate and determine pollen viability. Referring to Liu Xinyu (Liu et al.,2022) et al.'s method with improvements, prepared pollen germination culture medium was dropped onto concave slides. Pollen was sprinkled into the medium, mixed well, covered with coverslips, and placed in a germination box lined with wet filter paper. It was cultured in a 25°C incubator and examined under the microscope every 1 hour to count germinated pollen. Pollen with pollen tube length exceeding pollen diameter was considered as germinated.

Germination rate (%) = (number of germinated pollen grains / total number of pollen grains) $\times$ 100%

2.3 Data Statistics

Three slides were prepared for each treatment and observed under a BX53F2 optical microscope. 5 visual fields were selected on each slide, the total number of pollen grains counted was no less than 500. Pollen viability was calculated:

Pollen viability (%) = (number of viable pollen / total number of pollen) $\times$ 100%

2.4 Data Processing

The experimental data obtained were processed using Excel 2020 and SPSS 26 software.

3. Results and Analysis

3.1 Comprehensive Characteristics of *Epimedium sagittatum* Flowers

3.1.1 Biological Characteristics

Epimedium sagittatum is a long-day shade-loving plant(Figure 1). The flowering period is from March to June each year. The inflorescence is an indefinite panicle with numerous branches on the inflorescence axis. Each branch has three flowers and the terminal has one flower. The calyx has two whorls of four sepals each. The outer whorl is green with purple spots while the inner whorl is white. After blooming, the outer calyx falls off and the inner calyx opens up. The four petals are yellow in color. The four stamens grow out of the bud before the pistil. The style is green and the ovary is green with purple spots. After pollination, the ovary enlarges and the style gradually elongates, exceeding the stamens.

3.1.2 Floral Morphology

Epimedium sagittatum has a terminal panicle inflorescence, Flower organ parameter display (Table 1)10–35 cm long, with 20–50 flowers. The single flower is a complete flower without nectaries. The calyx has two whorls of four sepals each. The first whorl is green with purple spots, with 1 pair narrowly ovate and 1 pair oblong-ovate. The second whorl is white. After fully opening, the first whorl of sepals falls off. The lobes are triangular and reflexed. The inflorescence axis, pedicels and calyx surface all have purple spots. The petals are yellow or pale yellow, with 4 petals in a saccate, oblong or obovate shape. There are 4 stamens with yellow anthers in a cruciform position and purple-red filaments. There is 1 pistil with a purple-red cylindrical style and dark green stigma. When mature, the stigma surface secretes to adsorb pollen. The stigma position is about 8 mm higher than the anthers. The ovary is semi-inferior and spherical with 6–7 locules(Figure 2).

Table 1
Organ parameters of *Epimedium sagittatum* flowers

Floral Organ Parameters	Size/mm	Standard Deviation/mm
Petal length	2.24	0.14
Petal width	1.52	0.18
Filament length	3.69	0.19
Style length	2.05	0.21
Flower diameter	5.80	1.10
Calyx diameter	5.17	0.86
Ovary length	1.55	0.26

3.1.3 Flowering Dynamics Observation

The flowering period of *Epimedium sagittatum* is concentrated in March to June, with peak flowering in April. The overall flowering pattern shows concentrated mass flowering. It takes 2–4 hours from bud split to complete opening of the corolla. The average flowering period of the population is about 120 days, the flowering period of a single inflorescence is 10–20 days, and the duration of a single flower is 2–5 days. Through observation, it was found that the blooming process of a single *Epimedium sagittatum* flower can be divided into 5 stages (Fig. 3): (1) Sepal splitting stage - the bud swells and the sepals gradually split, exposing the internal tissues; (2) Stamen protrusion stage - the stamens break through the bud and are exposed outside; (3) Petal expansion stage - the petals push open the bud and unfold outwards, at this time the outer sepals fall off; (4) Peak pollen release stage - the flower is fully open, the pistil and stamens are completely exposed, and the anthers begin to release pollen; (5) Flower withering stage - the petal color gradually fades, the anthers begin to brown, and gradually wither and fall off, the ovary swells, and the style elongates.

It takes about 1 hour for the *Epimedium sagittatum* calyx to fully split. Then the stamens protrude through the bud with the stamen cluster spreading out. After about 1 hour, the yellow anthers begin to release pollen. At this time, the flower starts to bloom. After 1–2 hours, it fully blooms and enters the peak flowering period. At this time, pollen release decreases and the anthers begin to fall off. After blooming for 1–2 days, there is no significant change in the style, and the edges of the petals begin to wither. On the 2nd day after blooming, the filaments become dry and withered, on the 3rd day after blooming, the petals begin to fall off, the filaments become withered and curved shortened, the anthers gradually turn brown and begin to shrivel and fall off, the ovary begins to swell, and the style begins to elongate and gradually turns brown. On the 5th day after blooming, all petals have fallen off, the ovary sits on the fruit and gradually swells.

3.2 Comparison of Different Pollen Viability Testing Methods

3.2.1 TTC Staining

The TTC staining effect on *Epimedium sagittatum* pollen is shown in Fig. 4B. After TTC staining, all pollen turned yellowish brown, and no red pollen was observed. The pollen viability was 0, indicating that TTC staining does not easily stain *Epimedium sagittatum* pollen. Therefore, it is not suitable for determining the viability of *Epimedium sagittatum* pollen.

3.2.2 I₂-KI Staining

The I₂-KI staining effect on *Epimedium sagittatum* pollen viability is shown in Fig. 4C. Viable pollen with high starch content is stained red-brown, while non-viable pollen with low starch content is not stained and appears yellow-brown. The pollen viability measured by I₂-KI staining at 5 stages was 75.26%, 74.30%, 93.56%, 96.54% and 95.85%, respectively. The pollen viability was lowest at the stamen

protrusion stage at 74.30%; and highest at the peak pollen release stage at 96.53% (Table 2). The I₂-KI staining is fast, simple to operate, and the difference between viable and non-viable pollen is obvious. It is suitable for determining the viability of *Epimedium sagittatum* pollen.

Table 2

Statistics of pollen viability at different developmental stages of *Epimedium sagittatum* were examined by I₂-KI staining

Parameters	Sepal splitting stage	Stamen protrusion stage	Petal expansion stage	Peak pollen release stage	Flower withering stage
Average value(%)	75.26	74.30	93.56	96.54	95.85
Maximum value(%)	90.52	94.44	100.00	100.00	100.00
Minimum value(%)	43.55	50.00	90.59	88.89	93.38
Standard deviation	17.85	14.89	3.192	3.485	2.084

3.2.3 Red Ink Staining

The red ink staining effect on *Epimedium sagittatum* pollen viability is shown in Fig. 4D. Viable pollen has selective permeability of the cell membrane and cannot be stained by red ink, while non-viable pollen has complete permeability and is stained red. The pollen viability measured by red ink staining at the 5 developmental stages of *Epimedium sagittatum* was 84.73%, 44.99%, 24.30%, 31.76%, and 10.26%, respectively. The pollen viability was lowest at the flower withering stage, with the minimum value of 10.26%; and highest at the sepal splitting stage, with the maximum value of 84.73% (Table 3). Red ink staining is fast, simple to operate, and the difference between viable and non-viable pollen is obvious. It is suitable for determining the viability of *Epimedium sagittatum* pollen.

Table 3

Statistics of pollen viability at different developmental stages of *Epimedium sagittatum* were examined by red ink staining

Parameters	Sepal splitting stage	Stamen protrusion stage	Petal expansion stage	Peak pollen release stage	Flower withering stage
Average value(%)	84.73	44.99	24.30	31.76	10.26
Maximum value(%)	96.43	61.11	48.48	50.00	20.83
Minimum value(%)	66.67	29.45	11.76	0.00	0.00
Standard deviation	10.01	8.33	10.83	25.22	7.79

3.2.4 Peroxidase Method

The peroxidase staining effect on *Epimedium sagittatum* pollen viability is shown in Fig. 4E. Viable pollen has strong peroxidase activity and reacts with oxidants to appear purple-red, while pollen with weak or no activity appears light red or colorless. The pollen viability measured by the peroxidase method at the 5 developmental stages of *Epimedium sagittatum* was 99.23%, 81.51%, 99.30%, 19.93%, and 13.17%, respectively. The pollen viability was lowest at the flower withering stage, with the minimum value of 13.17%; and highest at the petal expansion stage, with the maximum value of 99.30% (Table 4). This method is slower, and the difference between viable and non-viable pollen is obvious, but it is easily interfered by other parts of the flower and is not suitable for determining the viability of *Epimedium sagittatum* pollen.

Table 4

Statistics of pollen viability of *Epimedium sagittatum* by peroxidase method

Parameters	Sepal splitting stage	Stamen protrusion stage	Petal expansion stage	Peak pollen release stage	Flower withering stage
Average value(%)	99.23	81.51	99.30	19.93	13.17
Maximum value(%)	100.00	88.52	100.00	41.18	50.00
Minimum value(%)	96.67	68.24	96.23	5.71	0.00
Standard deviation	1.438	6.075	1.398	15.08	14.69

3.2.5 In Vitro Pollen Germination Test

The germination of *Epimedium sagittatum* pollen on the germination medium is shown in Fig. 4F. The 1–8 hour germination rates of pollen at 5 developmental stages of *Epimedium sagittatum* are shown in Table 5. In vitro germination can directly observe pollen germination. When *Epimedium sagittatum* pollen at each stage was cultured by in vitro germination for 1–7 hours, due to the different pollen conditions, the time required for germination was also different and could not fully reflect the pollen germination. When the pollen was germinated for 7–8 hours, the basically fully germinated pollen with stronger activity, and the germination rate was relatively stable. The measured pollen viability at the 5 developmental stages of *Epimedium sagittatum* was 20.35%, 48.14%, 43.81%, 53.86% and 7.13%, respectively. The pollen viability was lowest at the flower withering stage, with the minimum value of 7.13%; and highest at the stamen protrusion stage, with the maximum value of 48.13% (Table 5). The in vitro pollen germination test results are relatively accurate, but this method requires higher cultivation temperature, nutrient composition, and pH value, and takes a longer time.

Table 5
In vitro germination rate of *Epimedium sagittatum* pollen from 1 to 8 h at different stages

Time	Sepal splitting stage	Stamen protrusion stage	Petal expansion stage	Peak pollen release stage	Flower withering stage
1h	0.00%	3.26%	0.93%	0.00%	0.00%
2h	1.21%	10.31%	4.13%	1.30%	0.00%
3h	7.33%	20.51%	15.11%	5.61%	0.13%
4h	10.51%	35.70%	17.53%	31.67%	6.57%
5h	15.67%	46.74%	35.79%	36.21%	6.77%
6h	20.31%	48.12%	43.74%	53.76%	6.98%
7h	20.33%	48.13%	43.76%	53.78%	7.04%
8h	20.35%	48.14%	43.81%	53.86%	7.13%
Standard deviation	9.15	4.73	6.83	6,75	3.15

3.2.6 Comparison of Different Pollen Viability Testing Methods

Pollen viability directly affects the results of fertilization and fruit setting rate after hybridization pollination. Varieties with higher pollen viability are preferred as male parents, which is more conducive to the successful breeding of new varieties (Yin et al.,2021). Currently, there are many methods for determining pollen viability, but most of the pollen testing methods obtain higher pollen viability than the actual viability. Due to the simple operation, less test time consumption, and ability to determine pollen viability in a short time, pollen staining methods are widely used in pollen viability detection of various

plants (Liang et al.,2022). Du Wenwen et al. concluded through the determination of pollen viability in Camellia that pollen staining was more suitable than I₂-KI staining and TTC staining (Du et al.,2021). Ren Feiyan et al. used TTC staining, MTT staining, Alexander staining, I₂-KI staining, red ink staining and in vitro germination to determine the pollen viability of Morus alba, and believed that Alexander staining and I₂-KI staining were more suitable (Ren et al.,2021). Liu Chengqin et al. used TTC staining, acridine red staining, I₂-KI staining, peroxidase staining and in vitro pollen germination to determine the pollen viability of Cuscuta chinensis, and believed that I₂-KI staining was the most suitable method for determining the viability of Cuscuta chinensis pollen (Liu et al.,2021). In addition, in vitro culture is another method for determining pollen viability. This method can directly observe pollen germination and gives more reliable results than pollen staining methods. It can be seen that different detection methods are suitable for different plants.

The results of this test found that in vitro germination can directly observe pollen germination. In this test, the pollen of *Epimedium sagittatum* at five developmental stages can germinate well after 8 hours of cultivation, and the germination rate is relatively stable. It is the preferred method for determining pollen viability, but this method has complex operation and long time consumption, and is not suitable for rapid detection of pollen viability. The staining method has the advantages of being fast and simple. It can reflect the viability of *Epimedium sagittatum* pollen to some extent, but is greatly affected by pollen characteristics. Different staining methods are suitable for different plants. TTC staining did not stain *Epimedium* pollen, and the measured pollen viability values were all 0, so pollen viability comparison analysis was not performed. As can be seen from Table 6, the pollen viability values measured by different methods vary greatly, with the order of peroxidase method > I₂-KI staining > pollen germination > red ink staining. Significance analysis showed extremely significant differences among the 5 methods. The results of I₂-KI staining and peroxidase method were significantly higher than red ink staining and in vitro germination. The results of red ink staining were closer to in vitro germination, and the staining effect was good and the operation was simple. It is the most suitable method for rapid detection of *Epimedium sagittatum* pollen viability.

Table 6
Comparison of results of different assays for pollen vitality in *Epimedium sagittatum*

Determination method	Sepal splitting stage	Stamen protrusion stage	Petal expansion stage	Peak pollen release stage	Flower withering stage
Pollen germination method	20.35	48.14	43.81	53.86	7.13
Peroxidase method	99.23	81.51	99.30	19.93	13.17
Red ink staining method	84.73	44.99	24.30	31.76	10.26
I ₂ -KI staining method	75.26	74.30	93.56	96.54	95.85
TTC staining method	0.00	0.00	0.00	0.00	0.00

3.3 Analysis of Pollen Viability at Different Developmental Stages

The results of this test found that the pollen viability of *Epimedium sagittatum* at different stages has extremely significant differences. From the results of the pollen germination test, it can be seen that the pollen germination rates of *Epimedium sagittatum* were higher during the stamen protrusion stage, petal expansion stage and peak pollen release stage. At this time, the pollen of *Epimedium sagittatum* has better pollination, but from the results of the pollen staining method, it can be seen that the pollen viability was also very high during the sepal splitting stage. Combined with microscopic observation, it was found that although the pollen viability was high at this time, the deformity rate was higher, up to more than 70%. If pollination succeeded at this time, it may lead to low germination rate of pollinated seeds. Therefore, the optimal pollination period of *Epimedium sagittatum* should be the stamen protrusion stage, petal expansion stage and peak pollen release stage. Through the pollen germination test, it was found that the pollen germination rate of *Epimedium sagittatum* was low, and the highest germination rate was only 53.86%. From the flowering process of *Epimedium sagittatum* (Fig. 3), it can be seen that the flowering of *Epimedium sagittatum* is protandrous with stamens protruding first and then pistil exposed for pollination. The pollen viability was stronger when the stamens protruded through the buds. Due to the different protruding times of stamens and pistils, the optimal pollination time may be missed. Combined with the investigation of local climate and ecology, due to temperature reasons, wild pollinating insects such as bees and butterflies in Pingyu, Henan Province were not active from March to April, making cross-pollination relatively difficult, resulting in poor fruit setting rate and seed germination rate of *Epimedium sagittatum*. Therefore, means such as artificial beekeeping can be adopted to increase the success rate of cross-pollination of *Epimedium sagittatum*, so as not to miss the optimal pollination time, thus enabling *Epimedium sagittatum* to breed high-viability, high-quality seeds.

4. Conclusion

In summary, the flowering period of *Epimedium sagittatum* is from March to June each year, with peak flowering in April. Its flowers are in panicles. Through observation, it was found that the blooming process of a single *Epimedium sagittatum* flower can be divided into 5 stages: sepal splitting stage, stamen protrusion stage, petal expansion stage, peak pollen release stage and flower withering stage. Experiments found that in vitro germination was the most accurate and effective method for detecting the pollen viability of *Epimedium sagittatum*. TTC staining, peroxidase method and I₂-KI staining were not suitable for determining pollen viability. Red ink staining had simple operation and obvious staining effect and was a good method for rapid detection of pollen viability. Among them, the detection results of red ink staining were closest to those of in vitro germination, making it the most suitable method for rapid detection of *Epimedium sagittatum* pollen viability. Combined with the flowering process and pollen viability at different developmental stages of *Epimedium sagittatum*, it was found that the optimal pollination period of *Epimedium sagittatum* should be the stamen protrusion stage, petal expansion stage and peak pollen release stage. It was also found that the pollen viability of *Epimedium sagittatum* was low, and the different protruding times of stamens and pistils may lead to missing the optimal pollination time. In addition, due to local climatic reasons, wild pollinating insects such as bees and butterflies were not active, resulting in lower cross-pollination rates and poorer fruit setting rate and seed germination rate of *Epimedium sagittatum*. Therefore, it is proposed to use artificial beekeeping to achieve cross-pollination and avoid missing the optimal pollination time, thereby increasing the success rate of cross-pollination of *Epimedium sagittatum*. The flowering results and related sexual reproduction mechanisms of *Epimedium sagittatum* in this study can provide reference for further research on the breeding of *Epimedium sagittatum*, in order to lay the foundation for hybrid breeding and cultivation promotion of *Epimedium sagittatum*.

Declarations

Author Contributions: B.J. and J.H. conceived and designed the experiments, J.H. and X.L. participated in the execution of the experiments, J.H. analysed the experiments, L.P. and J.H. supervised and completed the writing, P.L. and H.W. assisted in the design of the figures and tables, H.Z. reviewed the references, C.D. and S.C. reviewed the manuscript and provided valuable comments. All authors read and approved the published version of the manuscript

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is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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Figures



Figure 1

Flowering pictures of *Epimedium sagittatum*



Figure 2

Local plot of *Epimedium sagittatum* flowers



Figure 3

Blossom process of *Epimedium sagittatum*

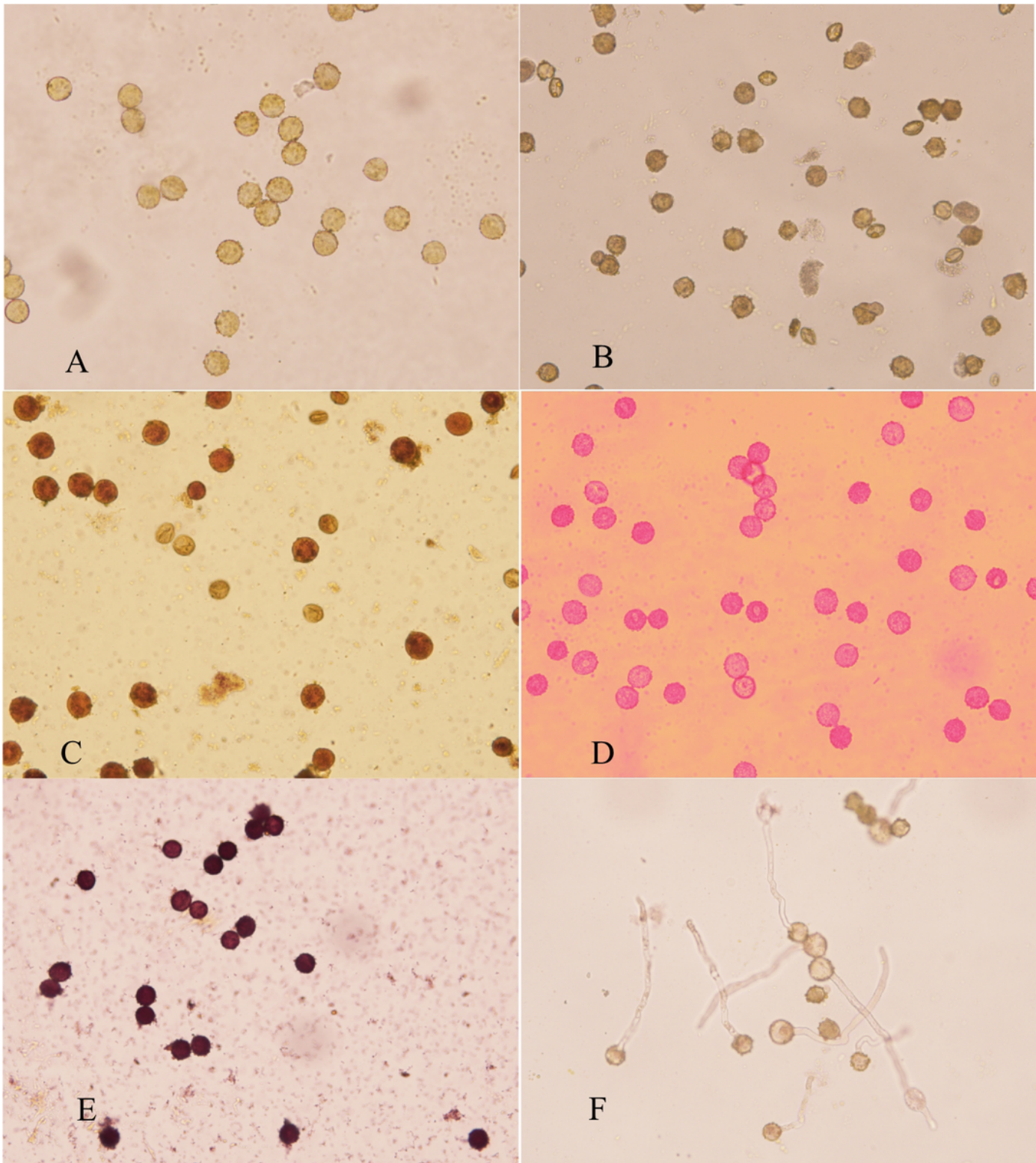


Figure 4

Pollen grains under the different activity assays