Effect of low storage temperature on pollen viability of fifteen herbaceous peonies

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1. Introduction

Herbaceous peony (\textit{Paeania lactiflora} Pall.), which has a long history of cultivation, is one of the traditionally famous flowers of China. Given its elegant and vibrantly colourful flowers and diverse range of plants, herbaceous peony flowers have high potential in national market, as well as the international market, as fresh cut flowers. As the global demand for these flowers increases, it is necessary to breed new cultivars with desirable characteristics.

Most often, herbaceous peonies are propagated by seed or asexually (Qin, 2004), but new varieties are generally bred by cross breeding. The flowering period for herbaceous peonies in China lasts from the end of April to mid-May depending on climatic conditions. Pollen vitality and non-synchronised flowering are key factors influencing breeding. Some herbaceous peony cultivars are restricted from being parents in breeding programmes due to the problem of asynchronised flowering, so it is necessary to store the pollen, which is collected from male donor parents for later hand pollination. Normally pollen is sensitive to temperature and easily loses viability under natural conditions; consequently, pollen preservation is problematic. However, different storage temperatures are suitable for different species and varieties and optimum temperature for pollen germination and tube growth depends on species and varies between cultivars [1]. Some research suggests that the viability of Pinyin pollen rose decrease to zero absolutely after one year of storage at about –5°C, as tested by in vitro germination [2]. Mango pollen can be stored for 8 weeks at room temperature, while the trend continues for up to 24 weeks at –4°C and –20°C by in vitro germination. Viability of fresh pollens was confirmed by fluorescein diacetate (FDA) and acetocarmine tests to be higher in pollinator mango cultivars [3]. Pollen from bromeliads could be stored in liquid nitrogen (–196°C) without a significant loss of viability after in vitro germination [4]. Cherimoya pollen can be efficiently stored at –20°C [5]. However, studies of the long-term storage viability of herbaceous peony pollen are seldom conducted in inland China.

 Aim of the present study was to evaluate the effect of temperature on pollen viability and tube growth. Furthermore, we used two methods to measure the pollen viability of fifteen herbaceous peony cultivars under different storage conditions to evaluate the optimal method for short- and long-term pollen storage.
2. Materials and methods

2.1. Plant material

According to the standard for cut flowers, 20 herbaceous peony cultivars were selected at the horticulture testing station of Shandong Agricultural University in 2015. Five cultivars underwent stamen abortion according to ex-episode inquiry activity. We retained 15 cultivars: ‘Hongfeng’, ‘Fenchijinyu’, ‘Qingtianlan’, ‘Guifeichacui’, ‘Bingshan’, ‘Tianshanhongxing’, ‘Dafugui’, ‘Hongxuqi’, ‘Xuefeng’, ‘Xueyuanhonghua’, ‘Chifen’, ‘Dongjingnvlang’, ‘Gaoganhong’, ‘Hongfushi’, and ‘Yangfeichuyu’. These parent plants were healthy enough to provide pollen. The 15 herbaceous peony cultivars were divided into three groups according to blooming sequence: early-blooming cultivars (‘Dafugui’, ‘Gaoganhong’, ‘Hongxuqi’, ‘Hongfeng’, and ‘Fenchijinyu’), mid-season blooming cultivars (‘Xuefeng’, ‘Hongfushi’, ‘Qingtianlan’, ‘Xueyuanhonghua’, and ‘Guifeichacui’) and late-blooming cultivars (‘Bingshan’, ‘Chifen’, ‘Tianshanhongxing’, ‘Dongjingnvlang’, and ‘Yangfeichuyu’).

2.2. Pollen collection and storage

Pollen was collected between 8:30 and 10:30 a.m. on a sunny morning and placed in the laboratory in the shade. The next morning, anthers and pollen were packed in parchment paper and put in hermetic bags with silica gel after anthers were desiccated and pollen was washed out. Every cultivar pollen sample was divided into four storage conditions (4 °C, –4 °C, –20 °C, and –76 °C) and stored for ten different time intervals, viz. 0 d, 3 d, 7 d, 1 month, 3 months, 6 months, 10 months, 11 months, 12 months, and 13 months.

2.3. Pollen germination and viability tests

For the in vitro pollen germination test, the germination medium contained 10 g/100 mL sucrose + 2 g/100 mL agarophyte + 5 mg/100 mL boric acid [6]. Pollen germination and pollen tube growth were evaluated after 3 h. If the length of the pollen tube was equal to or exceeded the pollen diameter after being cultured for three hours at 20 °C, pollen was classified as having germinated (Fig. 1).

In the I2-KI staining method [6], after staining was observed for several minutes, the stained pollen grains were scored as “viable,” while abnormally sized and unstained pollen were scored as “non-viable” (Fig. 2).

For both methods, at least 50 pollen grains per visual field were counted randomly under a light microscope at 100× magnification.

2.4. Verification by hybridisation

For ‘Hongfeng’, a male parent, fresh pollen and the pollen stored at –20 °C for about 12 months were selected to hybridise with female parent ‘Qihualushuang’ during the flowering season. Next, we investigated the percentage fruit set in the fruit maturity period.

2.5. Statistical analysis

Data were expressed as means ± standard errors. Differences were tested with one-way ANOVA and the least significant difference using SPSS22.0. P-values of <0.05 were considered to be significant.

3. Results

3.1. Viability of fresh pollen

Regardless of the cultivar, different viability tests showed differential response. When confirmed by in vitro germination and the I2-KI staining method, fresh pollen viability across all fifteen herbaceous peony cultivars showed significant differences (p
Comparatively higher pollen viability was observed using the I2-KI staining method than in the in vitro germination test. There were 11 cultivars where the viability exceeded 60.00% using the I2-KI staining method and three cultivars that were confirmed by in vitro germination (Table 1). The maximum germination of fresh pollen was found in ‘Bingshan’ (67.80%) and the minimal viability was ‘Dongjingnvlang’ (49.19%) as confirmed by in vitro germination (Table 1). However, fresh pollen observed using the I2-KI staining method showed that ‘Bingshan’ (83.56%) had the highest pollen viability (Table 1).

3.2. The effect of different storage temperatures

Pollen viability declined the fastest at 4°C, which was confirmed by both the in vitro pollen germination test and the I2-KI staining method. The germination percentage of most cultivars declined to zero at later stages of storage (Fig. 3). Viability of all cultivars decreased to 0.00% at 13 months, except the ‘Yangfeichyu’ cultivar still had a low pollen viability (1.83%) using in vitro germination (Fig. 5Q). The minimum pollen viability was found in ‘Hongfeng’ (10.10%) at 13 months, as measured by the I2-KI staining method (Fig. 3E).

Pollen was still viable in ‘GaoganHong’, (2.88%) ‘Hongxiuqiu’, (0.91%) (Fig. 3B) ‘Hongfushi’, (2.52%) (Fig. 4) ‘Dongjingnvlang’, (1.92%) and ‘Yangfeichyu’ (6.73%) (Fig. 5E) after 13 months of storage at –4°C while pollen viability of other cultivars declined to zero (in vitro germination). Pollen viability of ‘Dafugui’, (Fig. 3B) ‘Xuefeng’, ‘xueyuanhonghua’, (Fig. 4), was reduced to 0.00% by (in vitro germination) after 13 months of storage at –4°C, but pollen remained highly viable with the I2-KI staining method at 22.20%, 32.17%, and 20.00% respectively (Fig. 3F) (Fig. 4N). Pollen of ‘Bingshan’ showed a significantly higher viability percentage than other cultivars at all storage conditions (I2-KI staining method) (Fig. 5R).

Pollen stored at –20°C showed significantly higher viability percentages compared to pollen stored at 4°C and –4°C, as confirmed both by in vitro germination and by the I2-KI staining method of pollen viability. With in vitro germination of ‘Hongfeng’, (Fig. 3C) ‘Qingtianlan’, and ‘Guifeichacui’, (Fig. 4K), pollen viability was reduced to 0.00% after 13 months of storage, and the others still germinated at –20°C. However, pollen viability was significantly lower for up to 10 months. Pollen of ‘Bingshan’ also showed the significantly highest viability at all storage stages, just like at –4°C (Fig. 5S). The lowest viability was obtained from the I2-KI staining method in ‘Qingtianlan’ (Fig. 5O).

The pollen viability in all peony cultivars was the maximum at storage conditions of –76°C compared to other storage temperatures across all stages. Pollen viability at –76°C showed similar trends compared with pollen viability at –20°C among fifteen herbaceous peony cultivars on all observation dates, as confirmed by in vitro germination and the I2-KI staining method. Pollen viability was significantly lower for up to 10 months just as storage at –20°C and the descending rate of germination from 11 to 13 month of storage occurred slowly when tested by the in vitro germination method (Fig. 3D), (Fig. 4L), (Fig. 5T). All of the fifteen herbaceous peony cultivars were still highly viable after 13 months of storage at –76°C (Fig. 3D and H), (Fig. 4L and P), (Fig. 5T and X).

In early-blooming cultivars, the rate of reduction in pollen viability of ‘Hongfeng’ was relatively fast when tested by in vitro germination and by the I2-KI staining method (Fig. 3). In five mid-season blooming cultivars, the rate of reduction in ‘Guifeichacui’ was relatively fast when tested by the I2-KI staining method.
(Fig. 4). In other cultivars at later storage times, especially at –20 °C and –76 °C when tested by the in vitro germination test, viability of ‘Yangfeichuyu’ was obviously high and ‘Bingshan’ was relatively low, even though its fresh pollen viability was highest (Fig. 5). Overall, the reduction rates of pollen viability at four storage temperature conditions were very similar, with few exceptions, using I2-KI staining method, and the slowest rate of decline occurred at –76 °C. Cultivars stored at four different temperature conditions each had vastly different decline rates when confirmed by the in vitro germination method.

3.3. The effect of different storage temperatures on pollen tube

The pollen tube length was different of different cultivars and the tube length of fresh pollen has no direct relation with storage pollen. Higher pollen tube lengths were measured at lower storage temperature levels for all of the studied cultivars (Fig. 6). The maximum pollen tube length was 101.9 μm for ‘Fenchijinyu’ recorded after 13 months storage (Fig. 6a). The minimum pollen tube length was 28.4 μm for ‘Xuefeng’ after recorded 13 months storage (Fig. 6b).

3.4. Verification by hybridisation

The results showed that the seed-setting of ‘Qhualushuang’ was extremely low and the development of seeds showed poor growth under natural conditions without manual pollination. At the same time, the female parent ‘Qhualushuang’ still possessed a very high percentage of fruit-set hybridising with ‘Hongfeng’ fresh pollen or pollen stored for one year. Through investigation in the fruit maturity period, we found that even the viability of ‘Hongfeng’ pollen had fallen to zero (in vitro germination) and 17.24% (I2-KI staining method) which was lower than other fourteen cultivars (Figs. 6 and 7).

Fig. 4. Pollen viability of five medium blooming-seasoned cultivars, ‘Xuefeng’ ‘Hongfushi’ ‘Qingtianlan’ ‘Xueyuanhonghua’ ‘Guifeichacui’, stored at 4 °C, −4 °C, −20 °C and −76 °C as tested by two methods. In vitro germination test (I–L), I2-KI staining method (M–P).

Fig. 5. Pollen viability of five later flower cultivars, ‘Bingshan’ ‘Chifen’ ‘Tianshanhongxing’ ‘Dongjingsnvlang’ ‘Yangfeichuyu’, stored at 4 °C, −4 °C, −20 °C and −76 °C as tested by two methods. In vitro germination test (Q–T), I2-KI staining method (U–X).
In this study, the differential rates of reduction in pollen viability stored at different temperature regimes may be the result of temperature-dependent rates of metabolic activities in pollen. Pollen germination increased after in vitro culture at 20 °C, similarity, tube length increased. Regarding shelf-life during storage and the pollen tube growth, the most important factors are temperature and humidity (Qiao et al., 2010). Another important factor affecting pollen shelf-life is the moisture content [7,8]. Different temperature and relative humidity incidents, even for a short period, effect pollen germination and growth capacity [9]. In addition, pollen with high initial water content has been reported to be sensitive to stress and is typically short-lived during storage [10]. Drying is the most frequently used method for pollen preservation as it can prevent the growth and reproduction of microorganisms and minimize many of the moisture-mediated degradation reactions to enhance shelf-life [11]. In addition, longevity and storage stability of pollen may be connected to the presence of substances such as proteins and starches [12].

At room temperature, the high temperature and humidity, strong activity of respiration and metabolism, severity of water loss and rapid decline of vitality all lead to poor storage characteristics. Cryo-drying, however, inhibits metabolism and reduces enzyme activity and respiration, which slow the rate at which pollen viability declines. Yamin and Liangbi (1998) also note that low temperature, low humidity, and low oxygen can prolong storage life. When pollen was stored under –76 °C especially, viability was lower at 10 months than at 11 months. Most likely the storage temperature in March after 10 months was too low, which would have a negative effect on the pollen performance, though this result was in disagreement with previous studies in olive [9]. Under natural conditions, pollen viability increases as the temperature rises within a certain range. This coincides with previous scholars’ conclusions [13]. Pollen germination and tube growth are essential steps for plant reproduction. This process is affected by multiple biotic and abiotic stresses, including low and high temperatures [14,15]. Low temperature (LT) has generally negative effects on pollen germination and tube growth in multiple species [16,17]. Pollen germination decreased after an in vitro culture at 30 °C; in contrast, however, tube length further increased [9]. An increasing temperature also reduced pollen germination and accelerated pollen tube growth in sweet cherry [18] (Fig. 8).

The results showed that significantly different viability results were obtained with different methods, which is consistent with previous research on other crops where viability was estimated at the end of each period by staining and in vitro germination [19], though the I2-KI staining method is not suitable for pepper and senecio cruentus (Wang, 1998). According to Smith-Huerta and Vasek, [20] it is more accurate to test the fertilised availability of pollen to seed set ratio. In the study, I2-KI staining method was more reliable for testing pollen viability. It is possible that the in vitro germination method is usually subjected to various constraints, which lead to germination rates that cannot actually reflect pollen viability. Therefore, ‘Qihualushuang’, the female parent, still possessed a very high percentage fruit-set, even if viability of ‘Hongfeng’ pollen had fallen to zero after one year of storage when tested by the in vitro germination method.

According to the results of the present study, we speculate that ‘Dafugui’, ‘Gaoganhong’, ‘Hongxiuqiu’, ‘Xiaomei’, ‘Hongfushi’, ‘Xueyuanhonghua’, ‘Guifeichacui’, ‘Bingshan’, ‘Chifen’, ‘Tianshanhongxing’, and ‘Dongjingnvlang’ still could be used to pollinate after one year of storage at 4 °C. In addition, ‘Fenchijinyu’ and ‘Yangfeichuyu’ also could be used to pollinate after one year of

4. Discussion
storage at -4 °C, while all fifteen cultivars could be used as hybrid parents after one year of storage at both -20 °C and -76 °C.

5. Conclusion

From this study, we conclude that the I2-KI staining method may be more convenient and reliable when compared with in vitro germination tests in herbaceous peonies. In production, we suggest that herbaceous peony pollen could be sufficiently stored at 4 °C for hand-pollination among cultivars having non-synchronized flowering in a season. According to the results of the present study, we speculate that ‘Dafuliu’, ‘Gaoganhong’, ‘Hongxiuqiu’, ‘Xuefeng’, ‘Hongfushu’, ‘Xueyuanhonghua’, ‘Guifeichacui’, ‘Bingshan’, ‘Chifeng’, ‘Tianshanhongxing’, and ‘Dongjingnvlaang’ still could be used to pollinate after one year of storage at 4 °C. In addition, ‘Fenchijin’ and ‘Yangfeichuyu’ also could be used to pollinate after one year of storage at -4 °C, while all fifteen cultivars could be used as hybrid parents after one year of storage at both -20 °C and -76 °C. Pollen stored at -76 °C showed significantly higher viability when compared to all the other storage conditions. However, further experimentation is needed to study how long herbaceous peony pollen could be stored at -76 °C. Obstacles such as spatial and temporal isolation of the parents and resource absence, aroused by unsuccessfully collecting pollen, could be solved by long-term storage of pollen.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, “Effect of low storage temperature on pollen viability of fifteen herbaceous peonies”.

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