

## A METHOD FOR MID-TERM STORAGE OF *EPIMEDIUM PUBESCENS* (BERBERIDACEAE) POLLEN

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### Abstract

Due to the different species of *Epimedium* separated by seasonal isolation and geography, developing a method for mid-term storage of *E. pubescens* is necessary to facilitate germplasm conservation, interspecific hybridization and identification of hybrid species. In order to optimize the process of hand-pollination, in this work we have studied the conservation potential of *E. pubescens* pollen stored for approximately 2 months at -20°C, -70°C and -196°C. The optimal desiccation time of *E. pubescens* pollen was found to be 210 min. *In vitro* pollen germination of fresh pollen was 56.0%, and it was progressively reduced after conservation at -20°C, -70°C and -196°C reaching a minimum of 4.2%, 6.0% and 4.4%, respectively after 2 months of storage. The germination capacity of pollen stored at -20°C and -196°C was similar to that observed at -70°C, therefore for reasons of convenience and economy, pollen can be efficiently stored at -20°C. There were no fruit set differences between the field pollinations performed with stored pollen and pollinations performed with fresh pollen. These results indicate that *E. pubescens* pollen can effectively stored at subzero temperatures for several weeks to months.

### Introduction

*Epimedium*, a genus of the Berberidaceae, is a plant group from which a botanical supplement is widely used as a tonic, aphrodisiac and antirheumatic in China, Japan and Korea. *Epimedium* extract can strengthen immunity and has been proven effective against osteoporosis and cardiovascular diseases (Wang & Huang, 2005; Meng, 2005). Five species are currently recorded as medicinal plants in the Chinese Pharmacopoeia, including *E. pubescens* Maxim, *E. sagittatum* (Sieb. et Zucc), *E. pubescens* Maxim, *E. wushanense* T. S. Ying and *E. koreanum* Nakai (Anon., 2005). The wild resources of medicinal *Epimedium* species have been dramatically reduced due to years of over-harvesting and habitat destruction since the 1990s, and some species have even become endangered (Ward 2004; Xu *et al.*, 2007, 2008). Pollen storage is useful for breeding programmes, genetic conservation and artificial pollination (Perveen & Khan, 2009), and Pollen stored at low temperature presented germination capacity better than high temperature (Stanley & Linskens 1974; Khan & Perveen, 2008). No previous studies have reported procedures for conserving pollen of *E. pubescens*. Developing a method for midterm storage of *E. pubescens* is necessary to facilitate germplasm conservation, interspecific hybridization and identification of hybrid species by artificial pollination using stored pollen. Cryopreservation is commonly used for long-term storage, as pollen can be kept viable for long periods using this method (Omura & Akihama, 1980; Lee *et al.*, 1985; Polito & Luza, 1988; Sparka & Yates, 2002; Khan & Perveen, 2010). *Epimedium* pollen stored for one to two months is sufficient for most purposes, including breeding studies. Therefore we hypothesized that subzero conservation would also be suitable for mid-term storage of *E. pubescens* pollen. In this report, we compared storage conditions at different subzero temperatures in order to optimize the current process of hand-pollination among *Epimedium* species.

### Materials and Methods

Inflorescences of *E. pubescens* were collected in the protected area of Jinchengshan Forest Park (106°28'E 30°45'N, at altitudes of 568-790 m) on 15th March 2007, and stored on ice until reaching our laboratory. Immediately after anther dehiscence in cultivar inflorescences, pollen was subjected to different treatments. Due to the very small size of *E. pubescens* pollen (13-16µm), samples were collected when pollen was adhering to the walls of dehisced anthers (Fig. 1A-B).

The control experiment consisted in evaluating pollen longevity of fresh pollen stored at 4°C in a refrigerator. Pollen viability was evaluated daily as described below. Other experiments involved desiccation of pollen prior to storage.

In order to optimize desiccation time, the effects of different pollen moisture contents (obtained by extending the desiccation time) on pollen longevity were investigated at a fixed storage temperature of -20°C. Pollen grains collected from dehisced anthers of 215 flowers were continuously dehydrated on filter paper in silica gel desiccators. Partially dehydrated pollen grains were taken out after 90 min. Drying and then every 60 min for observation.

After the desiccation step, pollen was placed in 1.5ml cryovials and stored at -20°C, -70°C and 196°C in the refrigerator, freezer and liquid nitrogen, respectively. All the pollen viability tests were carried out after 5min of thawing at room temperature.

The optimal liquid media of *E. pubescens* pollen consisted of 150 mg L<sup>-1</sup> sucrose, 40 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 40 mg L<sup>-1</sup> Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, pH 5 (Quan *et al.*, 2007). The liquid media were dropped into the bottom of concave glass, pollen granules were scattered into the medium and mixed with a small brush. Glass slides were put into culture dishes containing wet filter paper on the bottom. After storage for in an artificial climate chamber (temperature, light intensity were 25°C, 600LX respectively) (Quan *et al.*, 2007), pollen germination was observed and scored under a light microscope (Olympus CX21). Pollen was considered as germinated when the length of the tube was longer than the grain diameter.



Fig. 1. Flowers of *E. pubescens*. A. The open flowers of *E. pubescens*. B. *E. pubescens* pollen adheres to the thecal walls of dehisced anthers.

Field pollinations using fresh pollen or pollen stored at  $-20^{\circ}\text{C}$  up to one month were performed by applying pollen with a paintbrush onto the stigmas just before flower opening. Artificial xenogamy with fresh pollen: 70 flowers. Flowers of 5 panicles were emasculated before anthers dehisced, and the stigmas of these flowers were pollinated with fresh pollen collected from another population. Artificial xenogamy with stored pollen: 56 flowers. Flowers of 5 panicles were emasculated before anthers dehisced, and the stigmas of these flowers were pollinated with stored pollen. All the panicles were bagged after pollination (the untreated flowers were removed). The fruit set of these two treatments were observed and recorded.

All data were analyzed using one way ANOVA with Duncan analysis as a posterior test and Nonparametric test using SPSS11.0 software.

## Results and Discussion

The germination frequency of freshly collected pollen was  $55.95\pm 4.36\%$  (Fig. 2), which was significantly higher than that of the pollen stored at  $4^{\circ}\text{C}$  (One-Way ANOVA with Duncan analysis,  $F_{7,34}=59.60$ ,  $p<0.01$ ). This germination frequency is similar to that observed in other species such as *Brassica campestris* (Mulcahy and Mulcahy, 1988), *Zea mays* (Inagaki, 2000) or *Annona cherimola* (Lora, 2006). As the storage time increased, the pollen germination frequency decreased. These results indicated that fresh *E. pubescens* pollen stored at  $4^{\circ}\text{C}$  exhibited reduced germination capacity and no *In vitro* pollen germination was obtained after 6 days of storage at  $4^{\circ}\text{C}$ .

Figure 3 illustrates the changes in pollen viability after desiccation for different lengths of time and a storage temperature of  $-20^{\circ}\text{C}$ . In this experiment, pollen germination was scored *In vitro* after 1, 6, 12, 25 and 40 days of storage. As storage time increased, the germination frequency of desiccated pollen grains decreased. Pollen germination frequency after desiccation for 210 min was significantly higher than other treatments except for the extended storage period of 40 days. Pollen germination frequencies for grains desiccated for 210 min

were  $80.10\pm 5.01\%$ ,  $58.61\pm 3.82\%$ ,  $42.10\pm 3.93\%$ ,  $33.11\pm 3.02\%$  after 1, 6, 12 and 25 days of pollen storage, respectively. When the storage time reaches 40 days, there were almost no differences between the desiccation treatments. Therefore, the optimal desiccation time of *E. pubescens* pollen was determined to be 210min, when the best pollen germination is obtained.

Pollen germination after 1 day of storage at  $-20^{\circ}\text{C}$ ,  $-70^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$  demonstrated significant differences ( $p<0.01$ ) of  $43.67\pm 2.91\%$ ,  $32.71\pm 2.05\%$  and  $55.81\pm 4.01\%$ , respectively (Fig. 4). Differences in germination frequency were reduced with extending storage time. Pollen stored for 6 days at  $-196^{\circ}\text{C}$  demonstrated a high germination frequency of  $49.09\pm 2.10\%$ , and no significant difference was observed between pollen stored at  $-20^{\circ}\text{C}$  and  $-70^{\circ}\text{C}$ , with  $37.09\pm 2.02\%$  and  $29.86\pm 3.24\%$  germination frequencies respectively ( $p>0.05$ ). Significant differences of  $23.41\pm 3.03\%$ ,  $17.71\pm 3.06\%$ ,  $32.62\pm 3.63\%$  in pollen germination capacity were also observed after 51 days of storage at  $-20^{\circ}\text{C}$ ,  $-70^{\circ}\text{C}$  and  $-196^{\circ}\text{C}$ , respectively ( $p<0.01$ ). No significant differences in germination ability were observed among the three different treatments at 71 days ( $p>0.05$ ). Consequently, although pollen conservation at  $-196^{\circ}\text{C}$  may be useful for long-term storage such as conservation of genetic resources, pollen can be cheaply and efficiently stored at  $-20^{\circ}\text{C}$  for purposes such as hand pollination.

Although a decrease in pollen germination was observed after extended storage, artificial pollination using pollen stored for up to 1 month at  $-20^{\circ}\text{C}$  showed no fruit set difference compared to pollinations performed with fresh pollen (Nonparametric test,  $P=0.173$ ) (Fig. 5).

## Conclusions

The present study is the first report of a method for midterm storage of *E. pubescens* pollen at subzero temperatures. Except for *Amorphaphallus konjac cv* (Sacks & Clair, 1996) and *Lycopersicon esculentum* (Zhang *et al.*, 2000) pollen, which can be directly stored in liquid nitrogen, water content is the key factor for pollen longevity during storage. For other species it is

necessary to do lyophilize pollen before storage (Baenabas 1976, 1988; Luza & Polito, 1988). Our research on *E. pubescens* pollen indicated that a lyophilization treatment of 210 min was optimal for these species because longer and shorter treatments resulted in reduced germination frequency.

Fresh *E. pubescens* pollen exhibited short longevity compared to the pollen stored at subzero temperatures. Similar observations have been recorded for pollen from such species as *Vitis vinifera*, *Simmondsia chinensis*, *Pistocia vera*, *Carya illinoensis*, *Annona cherimola*, *Abelmoschus esculentus*, *Morus alba*, *Citrullus Lanatus*, *Praecitrullus fistulosus* (Omura & Akihama, 1980; Lee *et al.*, 1985; Polito & Luza, 1988; Sparka & Yates, 2002; Lora, 2006; Khan & Perveen, 2006; 2008; 2010; Perveen & Ali, 2011). Interestingly, the germination capacity of pollen stored at -20°C and -196°C was higher than that

stored at -70°C. Although pollen conservation at -196°C was optimal, pollen can be cheaply and efficiently stored at -20°C for purposes such as hand pollination. Although a decrease in pollen germination was observed concurrent with increased conservation time, field pollinations with pollen stored for up to 1 month at -20°C demonstrated no yield differences compared to pollinations performed with fresh pollen. Similar results were also obtained by Crisp & Grout (1984) and Lora *et al.*, (2006). This study indicates that *E. pubescens* pollen can be effectively stored during the flowering-season of *Epimedium* and could thus enable easy transport of pollen across species of *Epimedium*. Consequently, pollen collected and stored at sub-zero temperatures can be used for field pollinations, and could solve the problem concerning different species of *Epimedium* separated by geography or limited by flowering time.

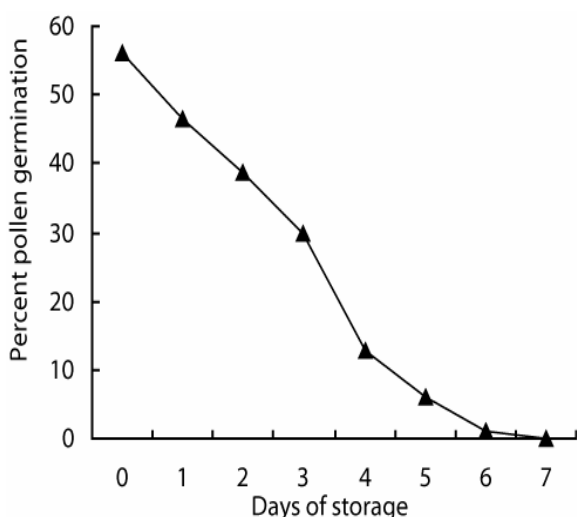


Fig. 2. *E. pubescens* pollen germination after storage at 4°C.

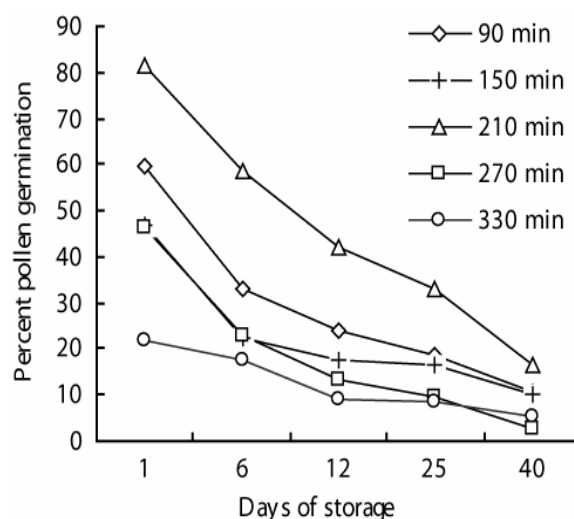


Fig. 3. Germination of *E. pubescens* pollen containing different moisture contents and stored at -20°C.

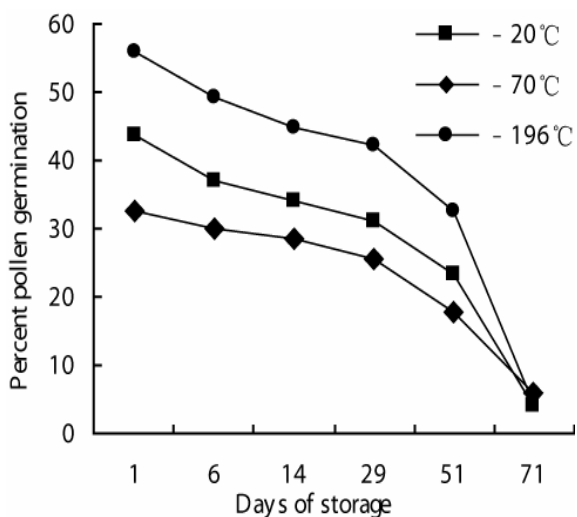


Fig. 4. *E. pubescens* pollen germination after storage at -20°C, -70°C and -196°C.

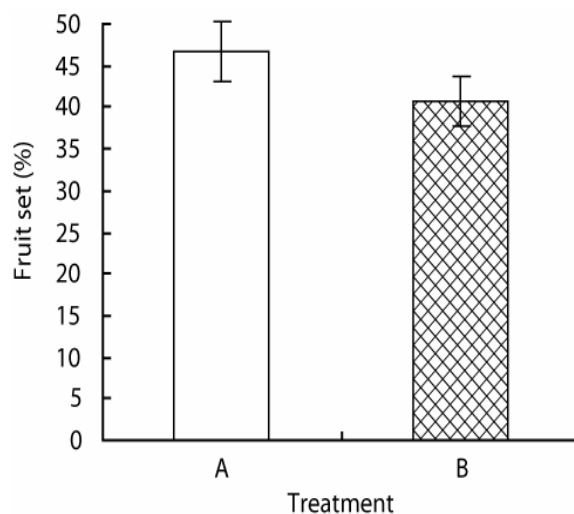


Fig. 5. Fruit set after artificial pollination using fresh pollen and stored pollen. Data is presented as the mean of measurements and error bars represent [standard deviation] A Treatment with fresh pollen, B Treatment with stored pollen.

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(Received for publication 5 March 2009)