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EFFECTS OF STORAGE TEMPERATURE AND DURATION ON POLLEN GRAIN VIABILITY AND POLLEN-TUBE ELONGATION IN CHINESE CHINQAPIN (*CASTANEA HENRYI* SKAN)

HUAN XIONG, DEYI YUAN, ZHI-YU DENG, GENHUA NIU AND FENG ZOU*

The Key Lab of Non-wood Forest Products of Forestry Ministry, Central South University of Forestry and Technology, Changsha 410004, Hunan, P. R. China

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Abstract

Chinese chinquapin [*Castanea henryi* (Skan) Rehder & E.H. Wilson] is used as a food and timber crop in southern China. Most chinquapin cultivars are self-incompatible and bloom at different times; consequently, artificial pollination is used to ensure fruit set and nut yield. Effective pollen storage that enables producers and breeders to use stored pollen for cross-pollination at a later date is important. In this study, the cultivar Changmangzi was used to estimate the viability and pollen tube length of pollen stored at room temperature, and at 4, −20, and −80°C using *in vitro* germination tests. It was observed that pollen grain germination significantly decreased at all four storage temperatures. Pollen viability was 14.4% after only 24 days of storage at room temperature. The germination rate was 13.3% after 90 days of storage at 4°C, and 14.5% after 180 days at −20°C. The initial germination rate of pollen stored at −80°C was 56.3% at the beginning of the test and decreased to 15.4% after 240 days. Pollen-tube length decreased with increased storage duration; mean pollen-tube lengths ranged from 109.44 to 257.51 μm. Based on these results, it is suggested that a storage temperature of −80°C for Changmangzi pollen is good.

Introduction

Chinese chinquapin (*Castanea henryi*) is grown in warm temperate subtropical areas of China. The tree grows in the Yangtze River Valley and southern regions, including Fujian, Zhejiang, Hunan, Sichuan, and Guizhou provinces (Xiang *et al*. 2016). The nuts of Chinese chinquapin are rich in nutrients, with a high starch content ranging from 47.58 to 56.94% (Zheng *et al*. 2003) and high mineral nutrition content. Nuts also contain 18 types of amino acids, eight of which have been reported to be good for health (Fan *et al*. 2015). The nuts are eaten as a traditional food in many areas of China, where they are consumed fresh, cooked, candied, and as a source of flour for pastries. The demand for the nuts is growing and the cultivation area of this species in China has reached 1,000,000 ha (Xiong *et al*. 2018). Chinquapin production is the main economic activity for many Chinese farmers in rural regions.

Chinquapin a monoecious species is mostly wind-pollinated, bearing both staminate (male) and pistillate (female) flowers on the same tree (Fan *et al*. 2017), and is self-incompatible; thus, a different cultivar is required for pollination (Zhang *et al*. 2016). However, most chinquapin cultivars do not flower at the same time, even if the flowering period is very long, and do not always overlap (Tang *et al*. 2013a). For this reason, artificial pollination is used to ensure adequate pollination in various compatible chinquapin cultivars and to improve yields (Zhang *et al*. 2016). In artificial pollination and breeding experiments, awareness of pollen-storage problems is critical (Bryhan and Serdar 2008). Thus, an understanding of the optimal pollen-storage and preservation conditions is important when using conserved pollen grain at the appropriate time for artificial pollination and hybridization (Youmbi *et al*. 2012). Pollen requires adequate storage conditions to avoid losing viability (Alburquerque *et al*. 2007). The present investigation was aimed to determine how long chinquapin pollen remains viable under various storage conditions.

*Author for correspondence: <zoufeng06@126.com>.

Recently, workers have demonstrated the importance of storage temperature in pollen grain preservation (Cruzatty *et al*. 2015). Zheng *et al.* (1997) reported that the maximum germinating percentage (22.4) of chinquapin cultivar Bailuzi pollen grains was achieved after storage for 5 days at room temperature. Earlier Zheng *et al*. (2004) reported that a low temperature (8°C) for 15 days was favorable for pollen storage. However, Tian *et al.* (2013) indicated that the germination rate was higher at −20 than at −70 or 0°C. After 30 days of storage at −20°C, chinquapin pollen germination rates remained over 69% (Tian *et al.* 2013). Optimal pollen storage conditions also vary among species or cultivars (Naik *et al.* 2013). Fernando *et al.* (2006) showed that storage of American chestnut (*C. dentata*) pollen at 4°C for 2 weeks significantly increased the percentage germination (48) compared to storage at −20 or −80°C. Lai *et al.* (2017) suggested that low temperatures of 4 - 7°C could prolong pollen viability in different chinquapin cultivars.

Although the pollen viability of *Castanea* species has been reported previously (Fernando *et al*. 2006, Beyhan and Serdar 2008, 2009, Liu *et al.* 2013, Zou 2016), there have been only a few studies on pollen storage in chinquapin cultivars for short or long periods at different temperatures (Zheng *et al*. 1997, 2004, Tian *et al.* 2013a, Lai *et al.* 2017). Pollen viability and longevity have been found to differ among genotypes (Fernando *et al*. 2006, Bryhan and Serdar 2009, Lai *et al.* 2017). Pollen viability and longevity has largely been recognized in pollination biology as a priority in understanding the reproductive performance of *Castanea* species and for successful implementation of breeding programs. However, conserving pollen viability depends on storage conditions. Conservation of germination capacity also depends on pollen tube length. There is little information available regarding pollen viability and longevity in the cultivar *C. henryi* Changmangzi. Thus, the main aim of this study was to determine the optimal storage temperature conditions for Chinese chinquapin pollen.

Materials and Methods

Castanea henryi cv. Changmangzi was collected from a chestnut orchard at the Central South University of Forestry & Technology Center of Ruchen (southeast Hunan Province, China). Ruchen is located at 25°33′43″ N latitude and 113°45′08″ E longitude. The average annual temperature, rainfall, and sunshine hours are 18.2°C, 1547.1 mm, and 1713 hrs, respectively. The chinquapin cultivar Changmangzi originated in Fujian province and is a late-ripening local Chinese cultivar (Fan *et al*. 2015). Trees were grown in red soil under normal management practices for commercial fruit production. Mature catkins were randomly collected from seven-year-old trees in full bloom on 10 May, 2015. Catkins were brought to the laboratory and laid on clean black paper sheets at room temperature until the anthers dehisced (Bryhan and Serdar 2009). Pollen grains were harvested in 1.5 ml clean microcentrifuge tubes and divided into several aliquots to reduce the stress related to thawing. To evaluate the ideal long-term storage temperature for the maintenance of long-term high pollen viability, four different storage temperatures (Room temperature, 4, −20, and −80°C) were tested.

Pollen samples were evaluated after 0, 3, 6, 9, 12, 15, 18, 21 and 24 days of storage at room temperature; 0, 5, 10, 15, 20, 30, 40, 60, and 90 days of storage at 4°C; 0, 10, 20, 30, 60, 90, 120, 150, and 180 days of storage at −20°C; and after 0, 30, 60, 90, 120, 150, 180, 210, and 240 days of storage at −80°C. The frozen pollen grains were kept at room temperature for 10 min and were not rehydrated before shedding on the germination medium (Youmbi *et al*. 2012). After thawing, pollen was collected to determine the germination rate using the *in vitro* culture method (Alburquerque *et al*. 2007). Standard germination medium was supplemented with 10% sucrose, 1% agarose, and 0.005% boric acid, and was used to evaluate pollen germination. Pollen was dusted onto a glass slide and the plates were covered and incubated at 30°C for 30 hrs in the dark

(Tang *et al*. 2013b). The germination capacity of stored pollen was counted until viability decreased to less than 15%. The experiment was set up using a completely randomized design. Three repetitions were performed for each treatment (storage temperature).

After the glass slide plates had been stored for 30 hrs in the dark, germinated pollen grains were counted using an optical microscope with 20× ocular magnification. Pollen grains were considered germinated when the length of the pollen tube exceeded the grain diameter (Alburquerque *et al*. 2007) (Fig. 1). Randomly selected at least three optical fields that contained approximately 100 pollen grains were selected for each count. The germination rate was calculated according to Xiong *et al*. (2016). For each treatment combination (temperature and storage time), the lengths of at least 30 germinated pollen tubes were measured using ImageJ software (National Institutes of Health, USA).

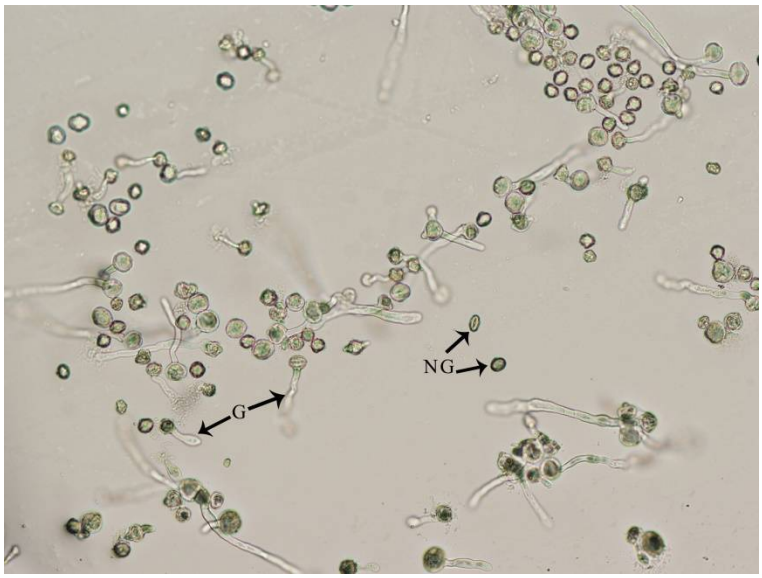

Fig. 1. *Castanea henryi* Changmangzi pollen grains; germinated (G), showing the pollen tube, and not germinated (NG), without pollen tube.

One-way analysis of variance (ANOVA) was used to evaluate the effects of storage temperature on pollen grain germination ability and pollen-tube elongation. Analysis was performed using SPSS 19.0 statistical software (IBM, USA). Significant differences among means were assessed using Duncan's multiple comparison at $p \leq 0.05$. Regression analyses of pollen germination rate and storage days, and of pollen-tube length and storage days, were conducted using Microsoft Excel 2010 software (Youmbi *et al*. 2012). Figures were prepared using Origin Pro 8.5 software (Origin Laboratory, USA).

Results and Discussion

The germination of pollen grains decreased significantly under the four storage conditions (Fig. 2A-D). A significant positive correlation was observed between pollen germination rate and storage time at room temperature ($R^2 = 0.9712$, $p < 0.01$) (Fig. 3A), 4°C ($R^2 = 0.8791$, $p < 0.01$) (Fig. 3B), −20°C ($R^2 = 0.9499$, $p < 0.01$) (Fig. 3C), and −80°C ($R^2 = 0.9815$, $p < 0.01$) (Fig. 3D). In this case, the average germination rate of fresh Changmangzi pollen grains was 56.3%. After 15

days at room temperature, the pollen germination rate was 25.6%; however, it decreased to 14.4% after 24 days. The results obtained in this study indicate that chinquapin cultivars have a short period of viability at room temperature. Similar results were also reported in different chinquapin cultivars by Zheng *et al.* (2004) and Lai *et al.* (2017). Zheng *et al.* (2004) reported that the germination rate of chinquapin cultivar Baniluzi was 1.5% after 4 days at room temperature. Lai *et al*. (2017) observed that the germination rate of chinquapin cultivar YLZ01 was 2.12% after 10 days at room temperature. The reduction in germination capacity of pollen grains stored at room temperature may be due to the inactivation of essential germination enzymes and substrates (Youmbi *et al.* 2012). The enzymes will readily diffuse into the surrounding medium. Enzyme activity, which decreases respiratory substrates, causes pollen viability to decline during storage (Gandadikusumah *et al.* 2017).

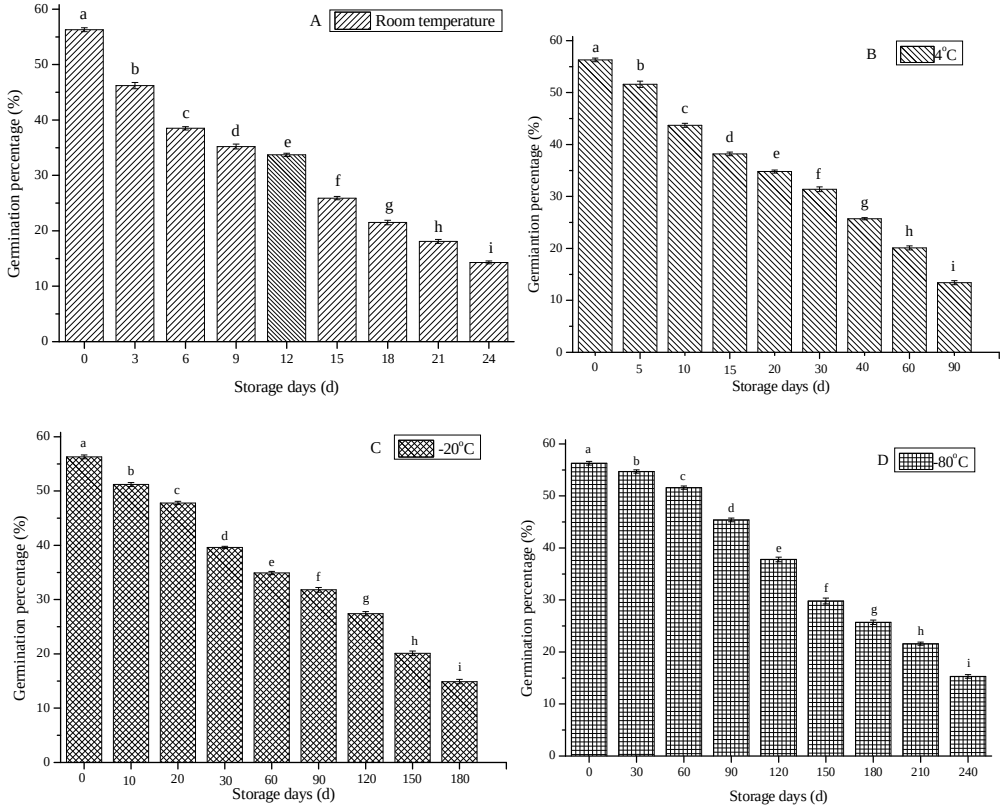

Fig. 2. *In vitro* germination of pollen grains of *Castanea henryi* Changmangzi after storage at room temperature (A), 4°C (B), −20°C (C) or −80°C (D) for different periods.

Changmangzi pollen had greater longevity when stored at −80°C than under all other storage conditions. After 30 days of storage at 4°C, the germination rate was 31.3%, but this declined to 13.3% after 90 days. After 180 days of storage at −20°C, the germination rate was 14.5%. However, after 240 days of storage at −80°C, Changmangzi pollen grains still germinated at a rate of 15.4%. These findings corroborate the results obtained by other researchers, who demonstrated that pollen viability was higher at lower temperatures (Fernando *et al*. 2006, Tian *et al*. 2013, Novara *et al*. 2017). Fernando *et al.* (2006) observed a germination rate of 19% in American

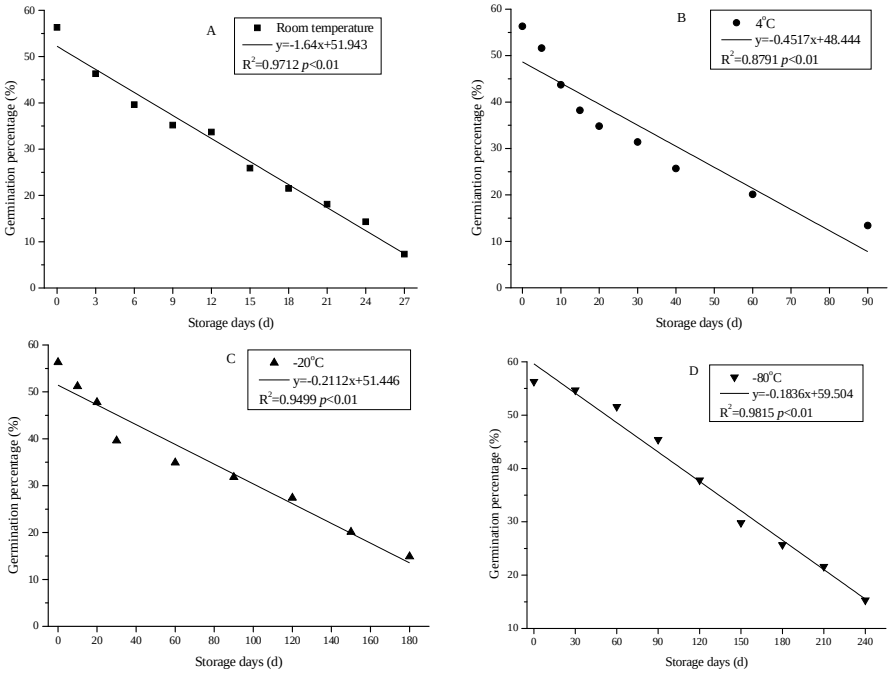

Fig. 3. Linear regression between pollen germination percentage and storage days in *Castanea henryi* Changmangzi. A. Room temperature; B. 4°C; C. -20 °C; D. −80°C.

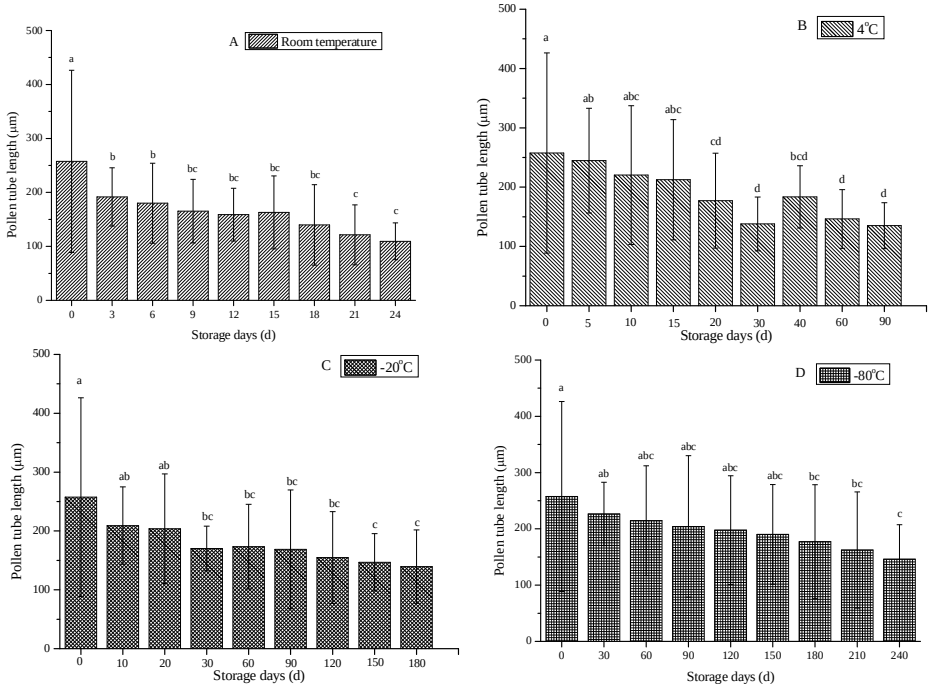

Fig. 4. *In vitro* germination of pollen-tube length of *Castanea henryi* Changmangzi after storage at room temperature (A), 4°C (B), −20°C (C) or −80°C (D) for different periods.

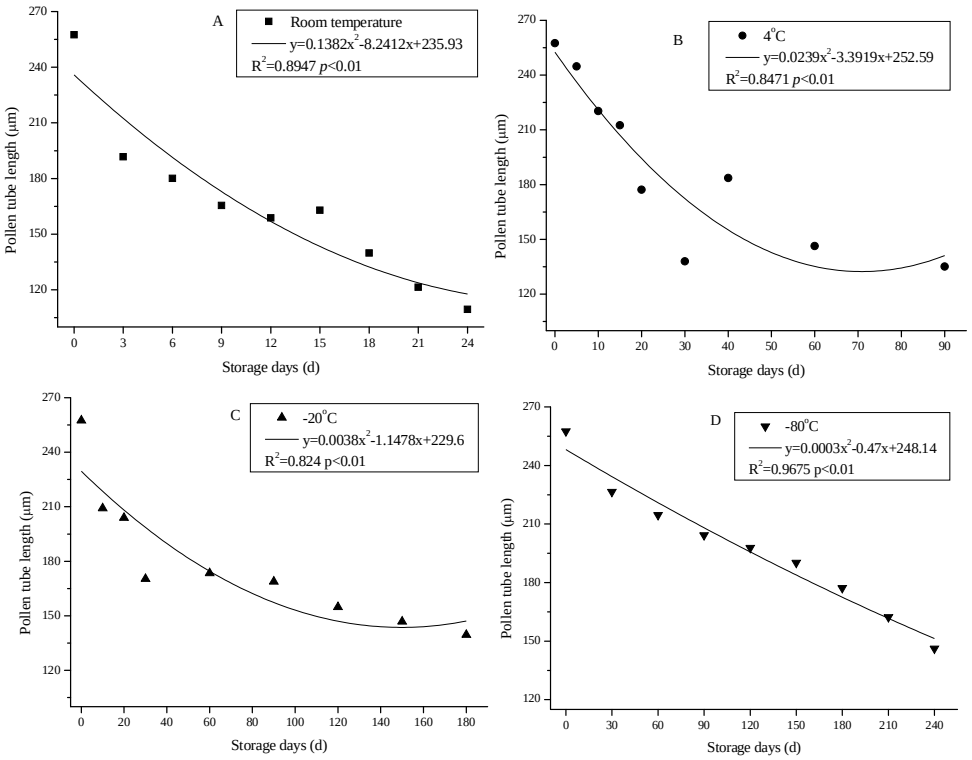

Fig. 5. Polynomial regression between pollen tube length and storage days in *Castanea henryi* Changmangzi. A. Room temperature; B. 4°C; C. −20°C; D. −80°C.

chestnut pollen stored for 1 year at −80°C. However, Tian *et al*. (2013) reported that the germination rate of *C. henryi* pollen was only 0.9% after 360 days of storage at −70°C. In addition to inherent differences among species or varieties, pollen quality is also affected by storage temperature. It is suggested that the loss of pollen structural integrity may be one of the principal causes of the decrease in pollen viability at lower temperatures. Similar results were also reported by Youmbi *et al*. (2012) for the four *Cola* species.

Pollen-tube length decreased with storage duration at the various temperatures (Fig. 4A-D). The maximum pollen-tube length was observed at 0 days (before storage). After 15 days at room temperature, the pollen-tube length was 162.92 μm. However, pollen-tube length decreased to 109.44 μm after 24 days. After 30 days of storage at 4°C, the pollen-tube length was 137.96 μm, which was not significantly different from measurements taken at 90 days (135.07 μm). At a storage temperature of −20°C, the pollen-tube length was 139.50 μm after 180 days. The pollen-tube length reached 146.24 μm after 240 days of storage at −80°C.

Polynomial regression analyses indicated a high correlation between pollen-tube elongation and storage time at room temperature ($R^2 = 0.8947$, $p < 0.01$)(Fig. 5A), 4°C ($R^2 = 0.8471$, $p < 0.01$) (Fig. 5B), −20°C ($R^2 = 0.824$, $p < 0.01$) (Fig. 5C) and −80°C ($R^2 = 0.9675$, $p < 0.01$) (Fig. 5D). The regression equation of the pollen-tube length for Changmangzi pollen stored at room temperature had a very high amplitude (1.35), indicating a strong reduction in pollen-tube length with storage duration. However, the smallest amplitude (0.76) was observed for the regression equation of pollen-tube length for pollen stored at −80°C, indicating a minimal reduction in

pollen-tube length with storage time. These results imply that storage may reduce the quantity of nutritive compounds in pollen. Loguercio *et al*. (2002) reported that the variation in pollen-tube elongation may reflect a characteristic genotypic constitution of gametophytic populations. The results presented here imply that elongation is more dependent on the age of the pollen than on the pollen species.

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