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A novel *in vitro* germination method revealed the influence of environmental variance on the pecan pollen viability

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ABSTRACT

Pecan is one of the most important horticultural nut crops in the world. Germination tests are vital to the safe application of artificial pollination and cross breeding of pecan. Although many *in vitro* germination methods have been proposed, the realization of robust and reproducible methods to induce morphologically normal growth pollen tubes *in vitro* for detailed dynamic observation has often proved challenging in pecan. We employed a mixing and spreading method to realize the uniform distribution and germination on agarose medium, which resulted in the unprecedented level of germinational repeatability and observability. With this method, we investigated the influence of environmental variance on the *in vitro* germination of pecan pollen. The data from germination rate, burst rate, pollen tube length and germination speed indicated the pecan pollen had an optimal germination temperature at 25 °C but optimal rehydration temperature at 16 °C which was lower than minimum germination temperature. And this optimal rehydration temperature was harmful to pollen germination if pollen was ready to germinate after rehydration. It helped researchers understand the mechanism of probable promotion of high day/low night temperatures to pollen germination in nature. Results further revealed that pecan pollen didn't lose their viability rapidly in first several days at ambient conditions but changed to a different rehydration and germination condition. In addition, we proposed here a new germination speed parameter GT50 to indicate the early change of pollen viability.

1. Introduction

Pecan (*Carya illinoinensis*), belonging to the *Juglandaceae* family, originally grows in south-central North America and is one of the most important horticultural nut crops in the world (Wetzstein et al., 1996). It is a wind-pollinated monoecious plant with staminate flowers organized into an ament or catkin and female flowers borne on a spike (Wetzstein and Sparks, 1986). Because of the heterodichogamy and short flowering period, breeding programs often have to use stored pollen to perform particular pecan crossing. Efficient and reproducible viability testing before crossing is essential to avoid the use of nonviable stored pollen, or it will result in the loss of the cross for the year (Sparks and Madden, 1985). Germination tests have generally been considered to be the best *in vitro* indicator of pollen usefulness, which assess the viability of pollen samples by germinating a sample of pollen grains in an artificial media (Galleta, 1983). Previous studies have proved that the *in vitro* germination of most pollens are mainly affected by the osmotic potential regulator (such as sucrose), concentration of borate and so on in medium although the effects of these ingredients in medium are not completely understood (Holdaway-Clarke et al., 2003). In pecan, trials have proved that fresh pollen can germinate in an artificial medium (Wetzstein and Sparks, 1985; Yates et al., 1986) and controlled rehydration of dry pollen before placing in the germination media is vital to determining accurate germination rate (Yates and Sparks, 1989). The rehydration experiment of dry pecan pollen helps prove that pollen stored for nearly 2 years at −80 °C or −196 °C, but not −10 °C, can retain germination capacity equal to freshly collected pollen by both *in vitro* germination test and fruit set analysis (Yates and Sparks, 1989). Further studies reveal that pecan pollen maintained at −12 °C for 2 years is as viable as freshly collected pollen if it is oven dried at 35 °C to a constant weight and stored in moisture-proof bags (Yates et al., 1991). Some experiments even reveal that the viability of pecan pollen does not decline in frozen storage up to 13 years (Sparks and Yates, 2002). On the other hand, researchers also find that pecan pollen loses their viability rapidly in first several days although it can retained the low germinational capacity for 59 days at ambient laboratory conditions (Yates and Sparks, 1989). It is surprising that pecan pollen will

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lose its viability rapidly in short time since this type of desiccation-tolerant pollen can survive for a long period of time after anthesis. In addition, when it is introduced from its original region into other climate zone, pecan may encounter some problems in pollination and germination although the seedlings still grow well (Peng et al., 2012). To deeply explore the detailed influence of environmental variance on pollen viability, we need a high robust and reproductive method to induce *in vitro* pollen germination and morphologically normal growth of pollen tube. But the designing of such method in pecan is always a challenging.

In recent years, a new cellophane based method is proposed to improve the germination rate and repeatability of pecan pollen, which has considerable utility compared to the traditional drop, tube, multi-well liquid and multi-well agar methods (Conner, 2011). However, there still exist some obvious defects in current method. First, non-replicable results still exist due to the use of 'dusting' method to spread pollen on medium surface. Pollen density can influence the germination of plant pollen no exception of pecan pollen whose germination proves decreasing below or above the optimal concentration (Yates et al., 1986). Second, the current method can't be used to observe the germination process directly and dynamically. The advancing of novel method in model plant *Arabidopsis thaliana* using agarose and cellophane as support greatly facilitates efficient, reproducible studies of the post-hydration development of pollen from *A. thaliana* and its relatives (Rodriguez-Enriquez et al., 2013). But, for *in vitro* germination of pollen from pecan and its relatives, a robust and reliable germination method, through which germination process of differently treated pollen can be compared in detail, has remained elusive.

In this paper, we proposed a novel method for efficient *in vitro* germination with very high repeatability which can not only promote germination and tube growth, but also realize dynamic observation of germination process. We used this system to evaluate the influence of environmental variance on *in vitro* germination of pecan pollen.

2. Materials and methods

2.1. Collection and use of pollen

Studies on the pollen germination were conducted using 'Pawnee' pollen collected in early May of 2013. Pollens were collected, dried and stored according to the reference (Conner, 2011). They were aliquoted into 50-ml polypropylene centrifuge tubes and frozen at −80 °C until use. Germination tests were conducted using newly thawed and RT-stored pollen samples. For a newly thawed pollen sample, a 50-ml sample tube was taken out of the freezer and immersed in ice to thaw for 2 h and then transferred to dessicator at room temperature for 2 h. For RT-stored pollen sample, the above-mentioned sample tube was finally stored at dessicator at room temperature for 3d or 7d before germination tests.

2.2. Pollen culture medium and apparatus

Germination medium was mainly made on the base of previous studies with some necessary optimization for the method in this paper (Conner, 2011). It consisted of 20% sucrose, 4 mM Boric acid, 3 mM $CaCl_2$, 0.67 mM KCl, 10 mM MES-Tris (pH5.8) and 1% agarose (Biowest, Spain). The liquid part of the germination medium was made by dissolving sucrose first and then adding the other materials except agarose. The liquid part was used here to mix rehydrated pollen for spreading. For the final germination medium, the appropriate agarose was added to the above solution and briefly heated in a microwave. Each 5 ml medium was then poured into 5 cm petri dish before its solidification and blown openly in clean bench for 10 min. The plate can be stored at 4 °C temporarily for a couple of days before use.

2.3. Pollen germination method

Germination tests here included several necessary steps (Fig. 1A). First, the thawed pollen was rehydrated for hours before transferring to germination medium. In this step, pollen samples were dusted into petri dishes and suspended above water in a sealed container at required rehydration temperature. Second, the rehydrated pollen was weighed into 1.5 ml centrifuge tube and then fully mixed with liquid germination medium in proportion by tip. Here 10 μl suspension was dropped into the germination medium plate for a single test right after the mixing. Third, the pollen suspension was spread evenly on plate using cell spreader (1 cm side length) by a unidirectional spreading. Each germination plate was immediately covered after spreading to prevent drying. Finally, the plate, suspended above water in a sealed box, was incubated in the dark at certain temperature to germinate for at least 24 h. When taken for examination every 3 h, the plate was covered and observed directly by inverted microscope. Each treatment of every sample was repeated three times.

2.4. Microscopy and data analysis

During the germinational process, the germinational phenomena in all tests were observed and recorded by Olympus inverted fluorescence IX71 microscope with DP71 camera at 100× magnification. A pollen grain was considered to have germinated if the pollen tube was equal to or greater in length than the width of the pollen grain. And a pollen grain was considered to be burst if the inclusion of pollen diffuse. The grain both have germination and burst (usually burst from the pollen tube) will be considered as germination instead of burst. Germination images were further measured including count and pollen tube measurement by Image Pro Plus 6.0. In each replication, three vision fields were examined with each field containing 200 or so pollen grains. The data were exported to Excel software for further analysis including arithmetic mean and error analysis. The arithmetic mean of data from three vision fields was taken as one experimental repeat. For length measurement of pollen tube, 50 pollen tube or all (if the quantity was not enough) was measured from each of three vision fields and their arithmetic mean was taken as one experimental repeat. Experimental errors were estimated through data from three repetition. Paired *t*-test was performed in Excel for group data comparison and all indicated *P* values were two tailed.

3. Results

3.1. The selection strategy of rehydration and germination condition

We used ultra cold stored pollen samples instead of freshly collected pollen samples in our experiments, which is on one hand because of better repeatability and convenience, on the other hand because of equal germination capacity to freshly collected pollen by both *in vitro* germination test and fruit set analysis (Sparks and Yates, 2002; Yates et al., 1991; Yates and Sparks, 1989). In our preliminary experiments, we used the mixing and spreading method to make sure the optimal pollen concentration for high germination rate, which was about 0.02 g rehydrated pollen per 100 μl liquid germination medium for the sample we used. Our studies also indicated that the pollen could hardly germinate below 22 °C which was then set as the temperature boundary between rehydration and germination. We also noticed from the climate record in pecan's naive region that the day mean minimum temperature