

# Pollen germination and hand pollination in pitaya (*Hylocereus undatus*)

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## Original article

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# Abstract

Hand pollination is a necessary aiding method for pitaya (*Hylocereus undatus*) production to achieve a high yield. With the cultivated area of pitaya going up exponentially in the recent years, a systematic study was carried out to understand the mechanism behind the high yield. In this study, we first developed an optimal medium for the in vitro germination of pitaya pollen. Upon testing the activity of the pollen collected from or stored for different time, we observed that the relative high pollen germination rates (27.2–65.1%) were those collected at between 2 h before blooming and 6 h after blooming, the highest activity was at 2 h after blooming, and that storing them for 24 h at 4°C reduces their germination rate from 65.2% to 35.5% and their production to about 82%. Therefore, it is not appropriate to pollinate plants with pollen that have been stored for more than 24 h, without bringing a breakthrough in pollen storage. We also observed that stigma receptivity and pollen activity are synchronous, which together determine fruit setting rate and fruit size. Pollination within 6 h after blooming provides the best fruit setting percentage and fruit size, the other favorable option being pollination at 6:00 pm, that is, 2 h before blooming; however, pollination at 6:00 am the next morning is expected to lead to 23% reduction in the production. These results will be useful for reproductive biology studies in this species. Moreover, this work set an important foundation for collecting pollen and selecting the right time of hand pollination to improve the yield and breeding efficiency in pitaya.

# Introduction

Pitaya (*Hylocereus undatus*) is one of the most important fruit trees in the tropical and subtropical regions of the world native from Latin America which has received increased attention during the last decade for their potential as new exotic fruit crops and been planted throughout the world (Lichtenzweig et al., 2000). The planting area of which has increased exponentially in China in the last 5 years due to its high market demand. Pitaya belongs to the Cactaceae family, flowers at night, and is pollinated by nocturnal pollinators, which chiefly include bats and moths (Tran et al., 2015). However, since many of the pitaya varieties is self-incompatibility, therefore, nocturnal pollinators is far less than enough in production, hand pollination is now the most important supplement for production. An extensive knowledge on pollen and pollination is, therefore, necessary.

The florescence of pitaya is about 5 days for one turn, and the bloom period of pollen source variety and mainly-planted variety may vary by 1 or 2 days, making the supply of fresh pollen not seasonable, so pollen storage is inevitable. Pollen can remain viable from several minutes to tens of years varies from species (Shivanna, 2019), for pitaya, pollen sealed and stored below 4 °C can still be used for pollination for the next day or even the day after next day in production, but the fruit setting percentage and fruit size were found to drop. However, there are no data till date to show the exact loss of fruit setting percentage and fruit size because of long pollen storage, more effective storage method have no reported yet for now.

Pitaya blooms at about 8:00 pm and withers at about 8:00 am the next morning. The time-varying activity of pollen during this period and the time-varying stigma receptivity have not yet been studied. Therefore, a systematic study should be undertaken to explore the time-dependent pollen activity and stigma receptivity in pitaya.

In vitro germination is one of the important methods to understand the characteristics of pollen, and studies have revealed different kinds of media for pollen germination and pollen tube extension. A conventional pollen germination medium consists of sucrose, boric acid, calcium nitrate, magnesium sulfate, and potassium nitrate (Brewbaker and Kwack, 1963)(Roberts et al., 1983), media consist of those component with species differ concentration have been widely used. Apart from the nutrients, pH and temperature are also important environmental factors influencing in vitro pollen germination. Most of the media, for example, those of *Prunus laurocerasus* (Sulusoglu and Cavusoglu, 2014), *Garcinia mangostana* (Sutthinon et al., 2018), *Triticum aestivum* (Turan et al., 2018), and *Hydrangea macrophylla*, *Dichroa febrifuga* and their hybrids (Alexander, 2019) have been developed based on the Brewbaker and Kwack medium, which comprises 10% sucrose, 100 ppm boric acid, 300 ppm calcium nitrate, 200 ppm magnesium sulfate, and 100 ppm potassium nitrate (Brewbaker and Kwack, 1963). In fact, the very essential ones may contain only sucrose or/and boric acid (Sarkar et al., 2018); however, the presence of iron will give a better result (Brewbaker and Kwack, 1963). An optimal medium for pitaya pollen germination is essential for an in vitro pollen study.

In order to understand the activity of pollen and stigma receptivity, we are trying to develop an optimal pollen germination media to observe the activity of pollen using in vitro germination and stigma receptivity by studying the fruit setting percentage and fruit size. The ultimate goal is to achieve the time point or stage at which the activity of pollen is the highest, collect the pollen, and use it to pollinate on the stigma at the best state to enhance production.

## **Materials And Methods**

### **Plant material**

“Red Crystal” and “Big Red” pitaya varieties were used in this study. “Big Red” was used as the pollen source for “Red Crystal”, and the plants were grown in a greenhouse at the Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou, China (23°09'14.9"N 113°22'22.0"E). All data were collected between June and October 2019, during which the average low and high temperatures in the region were 24.9 and 32.1°C, respectively, and the average daytime was about 13 h.

### **Methods**

#### **Medium for pollen germination**

The Brewbaker and Kwack medium (pH 7.0) was used as the control. We first investigated the time-varying germination rate in the control medium to estimate the time required to reach the highest

germination rate at four different temperatures, 25, 28, 30, and 32°C (from ambient average low to high temperatures). We then investigated the time-varying germination rate at the optimum temperature for seven different concentrations (0, 50, 100, 200, 300, 500, and 800 ppm) of sucrose, boric acid, calcium nitrate, magnesium sulfate, potassium nitrate and varying pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) values. After the single-factor experiment, combine those factors to test their interaction to get a best combination of concentration, temperature, and pH. To avoid the interference of the population effect of pollen, all the germination experiments were adjusted to about 10,000 pollen grains per milliliter medium. The germination rates were estimated using a microscope with at least 5 visual fields for one sample.

### **Germination rates of pollen collected at different time points**

Pollen from the “Big Red” plants were collected every 2 h, starting from 8:00 am on the first day (12 h before flower blooming) to 8:00 pm on the next day (12 h after flower withering), and germinated immediately using the optimal medium, and the germination rate was recorded for all the time points. The pollen collected from three plants was mixed as one sample.

### **Fruit setting percentage for pollination at different time points**

The “Red Crystal” plants were pollinated every 2 h starting from 8:00 am on the first day (12 h before flower blooming) to 8:00 pm on the next day (12 h after flower withering) with pollen collected at 8:00 pm before the first day from “Big Red” when the flowers just start blooming, and the fruit setting percentage was recorded for all the time points. The uniformity of pollination is controlled using a sprinkling can, with 1 g pollen being dispersed in 50 ml germination medium and one punch per flower at 5 cm away from the stigma (0.7 ml per punch).

### **Pollen storage and activity**

The fresh pollen collected from “Big Red” at 8:00 pm were divided into four parts. The “Red Crystal” plants were pollinated using the first part immediately. The other three parts were stored under 4°C and used to pollinate the “Red Crystal” plants at 24, 48, and 72 h (the second day, third day, and fourth day), respectively. For this experiment, both pollen collection and pollination were done at 10:00 pm, about 2 h after blooming. Each time, pollen were collected and mixed from at least 10 flowers, and 30 flowers were pollinated.

### **Fruit size and seed number description**

Fruits from the treatment groups (at least 30 fruits from each treatment group) were weighed individually to determine their fruit sizes. The seed number per unit area was calculated from the transection of the fruits; the number of seeds at the center of the section, that is, in an area of 2 cm\*2 cm, were counted to obtain the seed intensity.

## **Results**

## Pollen germination and pollen tube length in different temperatures and incubation times

For all treatment groups, the germination rate kept increasing from the 1<sup>st</sup> to the 10<sup>th</sup> h, after which it was almost constant till the 24<sup>th</sup> h (Figure 1a). Although the germination rate at the 10<sup>th</sup> h was good enough as a representative final result for one of the treatment groups, we used the data of the 12<sup>th</sup> h as the representative result in order to be sure. On temperature experiment, 28°C is optimal for germination rate (Figure 1a), and the measurement of pollen tube length at 12<sup>th</sup> h point to the same result (Figure 1b), so all the subsequent *in vitro* germination experiment will under 28°C.

## Optimal *in vitro* pollen germination medium

By change one factor of the control medium under 28°C and estimated the germination rate after 12 h, it was seen that while the pollen were very sensitive to the change in the concentrations of sucrose and boric acid, and the pH (Figure 2a), they were highly tolerant to the change in the concentrations of calcium nitrate, magnesium sulfate, and potassium nitrate (Figure 2b). It was observed that, under conditions of pH below 6.5 or above 7.5, boric acid concentration below 100 ppm or above 500 ppm, and sucrose concentration below 10% or above 25%, the germination rates dropped rapidly. The optimal concentrations of sucrose and boric acid are 20% and 100 ppm, respectively, and the optimal pH is 7.5. Through the lack of calcium nitrate, magnesium sulfate, and potassium nitrate results in a drop in the germination rate, the change in their concentration from 50 to 800 ppm has very little influence on the germination rate. Therefore, we fix calcium nitrate, magnesium sulfate, and potassium nitrate at the optimal concentrations of 100, 300, and 300 ppm, respectively, and then explore the germination rates with different combinations of boric acid (100, 200, 300, and 500 ppm) and sucrose (10%, 15%, 20%, and 25%), and pH (6.5, 7.0, and 7.5; Figure 2c). The optimal germination medium for pitaya pollen is 20% sucrose + 300 mg/L H<sub>3</sub>BO<sub>3</sub> + 100mg /L Ca (NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O + 300mg /L KNO<sub>3</sub> + 300 mg /L MgSO<sub>4</sub>·7H<sub>2</sub>O under 28°C, pH 7.0 (Figure 2c).

## Pollen storage and activity

The activity of pollen stored at 4°C or room temperature were estimated by *in vitro* germination every 2 h from 0 h (freshly collected pollen) to 48 h. At room temperature, the germination rate dropped to almost 0% in 18 h (Figure 3a). For pollen stored at 4°C, the situation was much better because the germination rate was 29.5% at 24 h, and the pollen displayed some activity even at 48 h (Figure 3b). When pollen stored at 4°C for 24, 48, and 72 h were used for hand pollination, the fruit setting rates were 92.5%, 16.7%, and 2.5% (Figure 3c) and the fruit sizes were 283.6, 120.1, and 73.3 g (Figures 3d and 4), respectively, which were significantly lower than those when pollinated using fresh pollen (the fruit setting percentage was 99.2% and the average fruit size was 319.5 g). Therefore, it may be concluded that it is better to use fresh pollen for pollination as compared to pollen stored below 4°C for 24 h pollination because the latter leads to a 18% reduction (according to the reduction of fruit setting percentage and fruit size) in production.

The seed intensity was not different for fruits from different treatment groups (Figure 4). In other words, it may be concluded that fruit size is positively related to the seed number and the pollen activity.

### **Time-varying pollen activity and stigma receptivity**

To make the time point easier to understand, we define 8:00 pm, that is, the time flowers begin to bloom as 0 h, 2 to 12 h before blooming as -2h to -12h, 2 to 12 h after blooming as 2 h to 12 h, 2 to 12 h after the flowers withered as +2 h to +12 h. The pollen collected at -12 h or -10 h have almost no activity; the activity keeps increasing after -10 h, with the germination rate reaching the highest value of 65.1% at 2h. The germination rate starts dropping after 2 h, reaching 0% at

+8 h (Figure 5a). Similar to pollen activity, the stigma pollen acceptance capacity also reaches a peak and then goes down, the capacity being determined by the fruit setting percentage. The pollen used in this experiment was freshly collected or stored at 4°C for less than 24 h. The germination rate (37.6%–65.1%) for each time point is shown as a line chart and the corresponding fruit setting percentage is shown as a boxplot in Figure 5b. At -2 h, it can be observed that the stigma already has the capacity to accept the pollen, with a fruit setting percentage of 93.3%, while pollinate at -4 h or earlier, the highest fruit setting percentage is only 71.7%; between 0 h and 8 h, the fruit setting percentage is almost 100%; at 10 h, that is, at 6:00 am the next morning, the fruit setting percentage is 92.5%, after which it drops to less than 89.2% and finally to 2.5% at +12 h (Figure 5b). The fruits were weighed after maturation (Figure 5c); the fruit size at -2, 0, 2, 4, and 6 h was the same level and that of fruits at -4, 8, 10, 12, and +2h was significantly than the fruits of 0 h ( $p$ -value<0.01), size of other time points were didn't measured due to inadequate numbers of fruit.

The data mean that pollination taking place in 8 h after blooming will result in about 100% of fruit setting and significantly bigger fruits than those occurring at other time points. Pollination at -2 h will result in a reduced fruit setting percentage but will not affect the fruit size. Pollination at 8 h, when the flowers are still open, the fruit setting percentage is as high as 99.2%, but the size of fruits is already became significantly smaller (10%) than those of 0 h, the size reduction became more and more serious for fruit pollination at 6 h or later. Pollination in 4 hours after blooming is the best choice; however, considering the fact that it is much easier carry out the process during daytime, pollination before dark or the next morning is usually carried out. Our data show that pollination at -2 h is much better than that carried out the next morning with respect to both fruit setting percentage and fruit size.

## **Discussion**

In vitro pollen germination has been described by hundreds of studies, with a variety of media being used. Based on such studies, we observe that while sucrose serves as the most common carbon source (Nygaard, 1977; O'Kelley, 1955), boric and/or calcium are essential for pollen tube growth (Brewbaker and Kwack, 1963; Potts and Marsden-Smedley, 1989). Based on these predecessors, we developed a relatively optimal medium (20% sucrose + 300 mg/L  $H_3BO_3$  + 100 mg /L  $Ca(NO_3)_2 \cdot 4H_2O$  + 300 mg /L  $KNO_3$  +

300 mg /L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  under 28 °C, pH 7.0) for the pollination of pitaya. However, there is no best medium because in natural, pollen germinate and grow in the pistil, which is a complex, time-changing environment involving different phases of pollen growth and different requirements (Holdaway Clarke et al., 2003). The highest pollen germination rate with this optimal medium is 65.1%, which is reached in 10 h, increasing the time does not result in a higher germination rate or a longer pollen tube, that may because 65.1% being very close to that obtained by natural pollen activity or the bound defect of the medium.

Pollen storage is an established technique in many species, it could be cryopreservation at -20 °C, -80 °C or -196 °C, organic solvent preservation, sealed dry preservation and so on (Iwanami and Nakamura, 1972; Harrington, 1970; Martínez-Gómez et al., 2002; Pinney and Polito, 1989; Shivanna et al., 1991; Towil, 2010). In our study, we also refer to those methods, but no activity was detected after one week of storage (data not show). At present, there is no effective method for long-term pollen storage. Further study need be carrying out on pitaya pollen storage.

With the optimal medium for germination, our results suggest that pollen collected at 22:00 pm (about 2 h after blooming) have the highest activity, which means that pollen collected at 22:00 pm are the most suitable to be stored for hand pollination later. However, a higher pollen activity does not mean a higher fruit setting percentage or fruit size since there seems to be a saturation value for pollen activity. The germination rate changes from 56.3–65.1% (from 8:00 pm to 2:00 am the next day) result the same fruit setting percentage (about 100%) and same level of fruit size (from 306 to 313 g with no significant difference). Even pollination using pollen with a germination rate of 37.6% (pollination at 6:00 pm with pollen collected at 10:00 pm the day before) does not affect too much of the yielding. Although pollination during daytime is much easier, it is better to carry out the pollination not earlier than 2 h before blooming to minimize production losses. Pollination the next morning will lead to more production losses. The best choice for hand pollination is to pollinate within 6 h after blooming (from 8:00 pm to 2:00 am next morning). For pollination at 4:00 am, the fruit setting percentage can reach up to 99.2% but the fruit size is significantly 10.5% smaller. In pollinations carried out after 4:00 am, both the fruit setting percentage and fruit size drop rapidly.

Both pollination at earlier than 2 h before blooming or later than 8 h after blooming will lead to production losses, but the pattern is different. The production loss of pollination at morning is caused by the decrease in the fruit size, although the fruit setting percentage is still at a relatively high level. We speculate that it is because in the morning, the temperature becomes higher and higher, with water loss from the stigma inhibiting pollen germination, thus reducing the fruit size. The production loss of pollination before blooming is mainly caused by low fruit setting percentage, while the fruit size has not been affected too much. It seems is because that the pistil is not totally ready yet before blooming, but the pollen on the stigma can wait for the mature of pistil with a tiny reduction of activity thus lower the fruit setting percentage. Pollen stored at 4 °C for 24 h will show a reduction in their activity, thus decreasing the fruit setting percentage and fruit size. However, it can still be considered as an alternative option with an 18% reduction in production.

In pitaya production, the process of hand pollination is usually by virtue of experience. But experience won't always work. In some complex scenario, like a short-term rainstorm, the work of pollination will be ruined. So, will hand pollination be successful before or after rainstorm, i.e. before blooming or several hours after blooming? Our study confirmed that the pollen had a high germination rate (> 35.5%) and the stigma had a high receptivity (> 92.5%) from 2 h before blooming to 10 h after blooming ( $\geq 92.5\%$ ), during which the pollen collection and pollination can ensure normal fruit setting and yield. Our research on pollen activity and stigma receptivity can be a technical guidance to hand pollination, and thus, avoid losses or improve production and breeding efficiency.

## **Declarations**

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### **Declarations**

#### **Ethics approval and consent to participate**

Not applicable

#### **availability of data and materials**

All the materials are stored in our lab, all the data are show in the manuscript as graph, original data is available by contact the aauthor.

#### **Consent for publication**

Not applicable

#### **Conflict Interest:**

Juncheng Li declares that he has no conflict of interest.

Honghui Shi declares that he has no conflict of interest.

Xiaoling Huang declares that she has no conflict of interest.

Yulin Wang declares that he has no conflict of interest.

Junsheng Zhao declares that he has no conflict of interest.

Hongfen Dai declares that he has no conflict of interest.

Qingming Sun declares that he has no conflict of interest.

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## Author's contribution

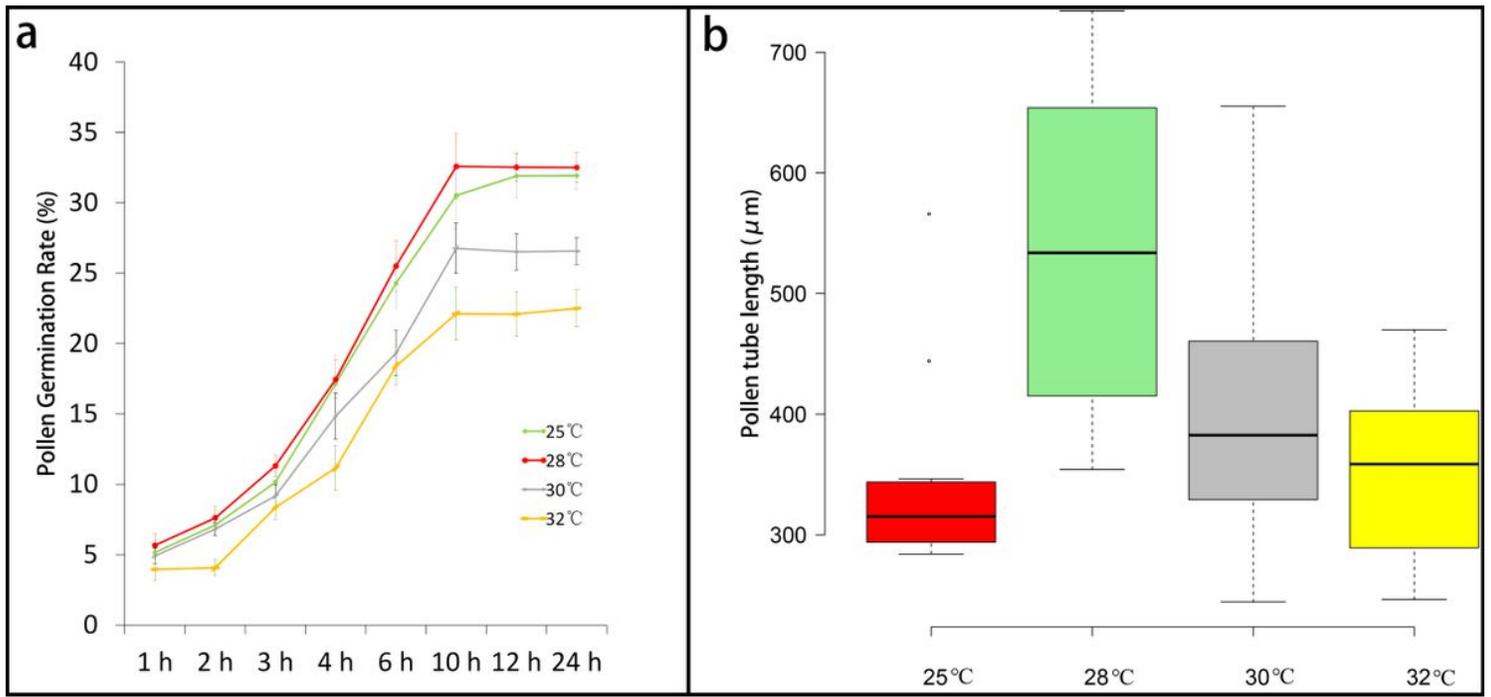
Qingming Sun designed experiments; Juncheng Li, Honghui Shi, Xiaoling Huang, Yulin Wang carried out experiments; Juncheng Li, Hongfen Dai, Junsheng Zhao analyzed experimental results; Juncheng Li, Hongfen Dai analyzed sequencing data; Juncheng Li wrote the manuscript.

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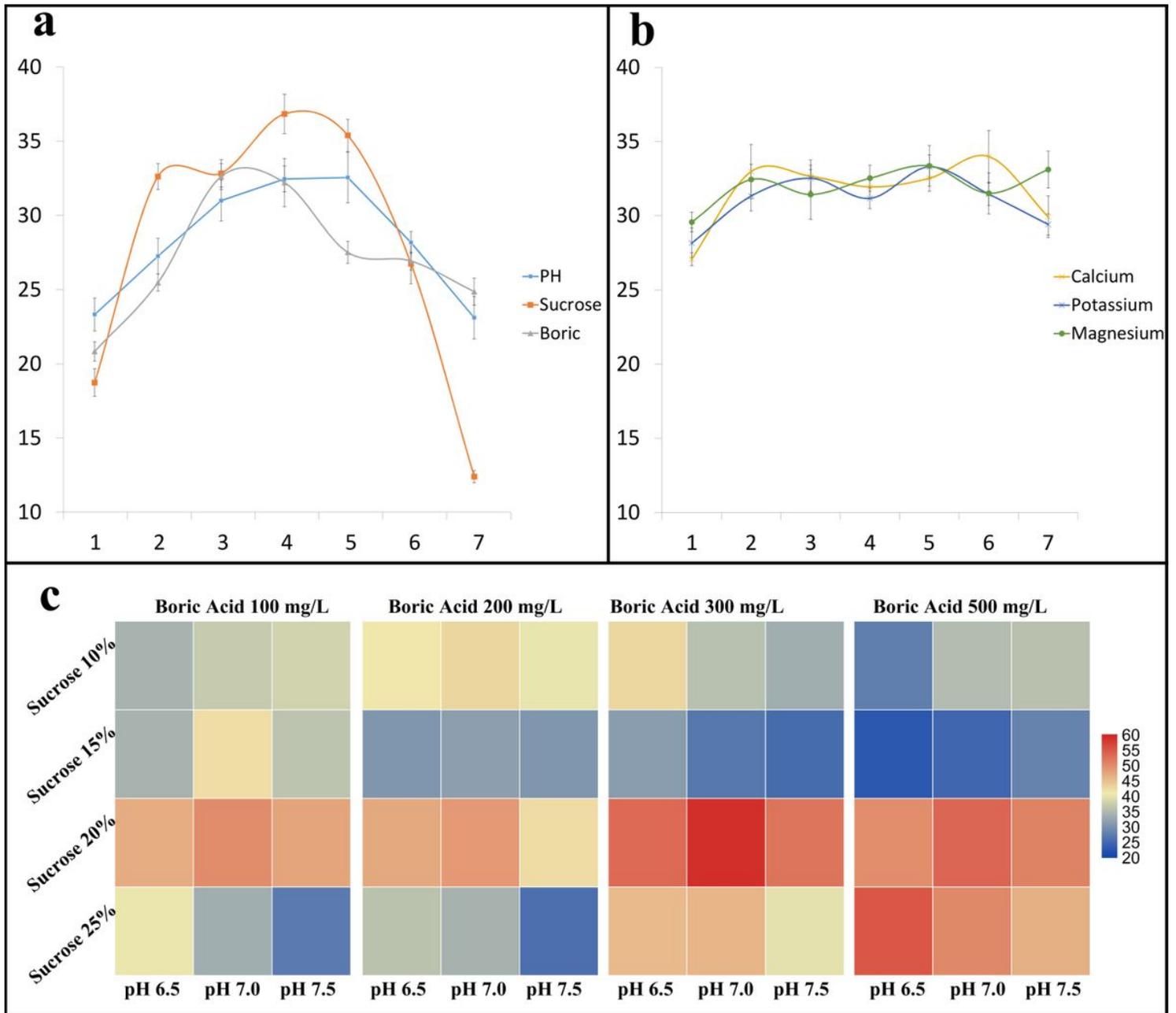
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## Figures



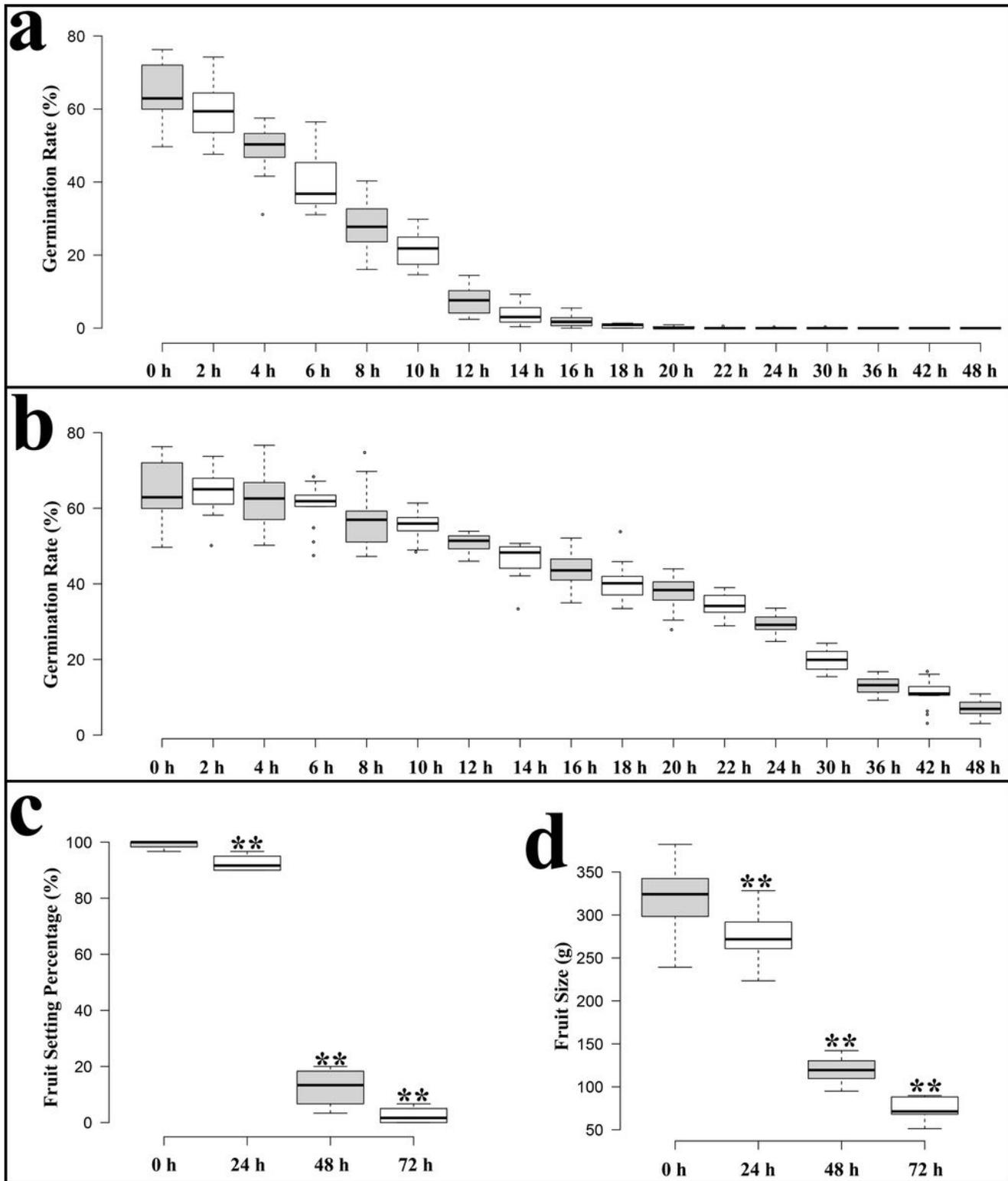
**Figure 1**

In vitro pollen germination rate and pollen tube length under different temperatures and incubation times. (a) Time-varying germination rate under different temperatures. (b) Pollen tube length statistics at the 12th h.



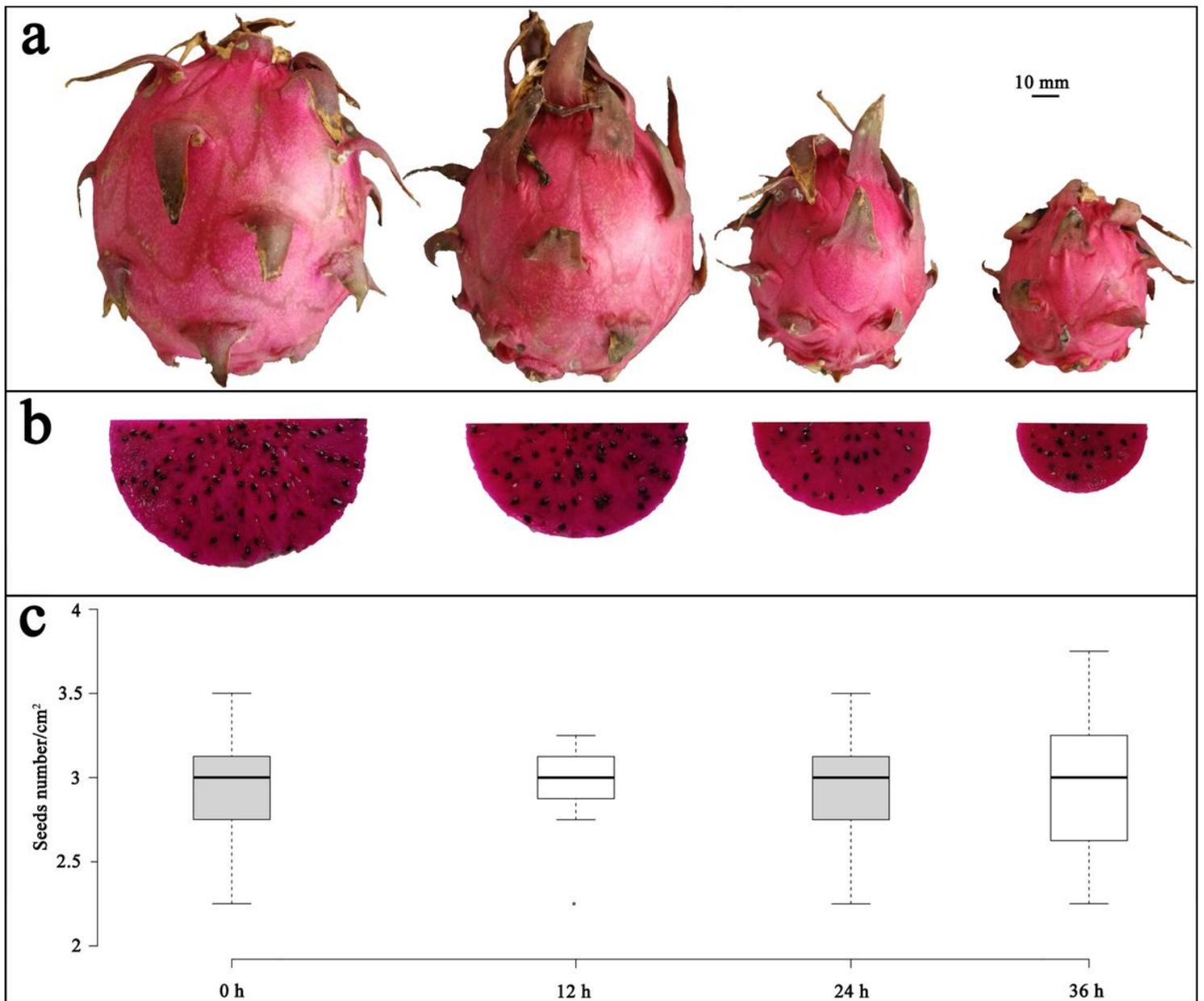
**Figure 2**

Germination rates under different media. (a) Germination rates under different concentrations of sucrose and boric acid, and varying pH. (b) Germination rate under different concentrations of iron, calcium, potassium, and magnesium. (c) Germination rates under different combinations of pH, sucrose, and boric acid. The number on the x-axis 1-7 means concentration 0, 50, 100, 200, 300, 500, 800 ppm or pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5.



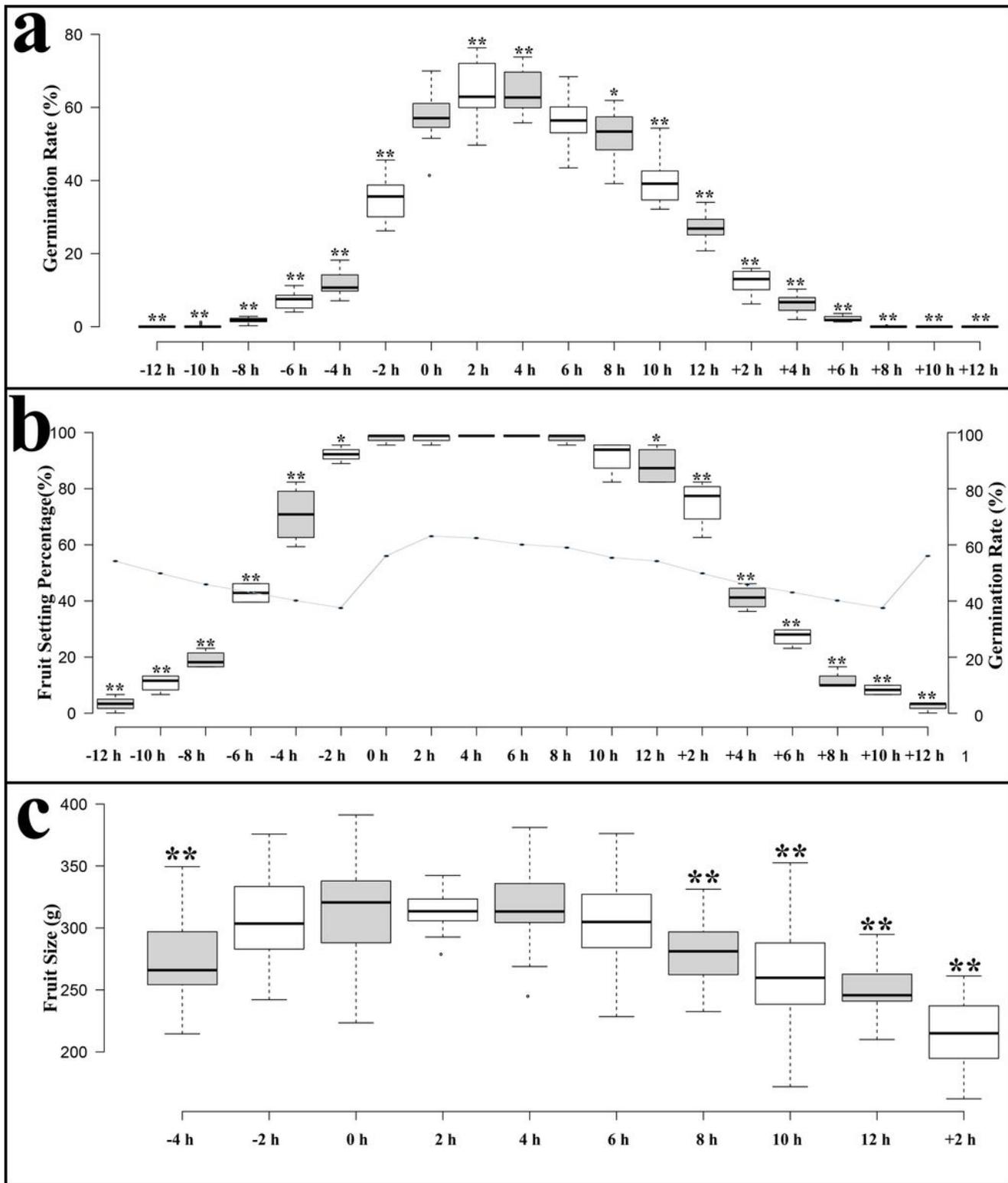
**Figure 3**

(a) Time-varying activity of pollen stored at room temperature. (b) Time-varying activity of pollen stored at 4°C. (c) Fruit setting percentage of pollinations with pollen stored for different time periods. (d) Fruit size of pollinations with pollen stored for different time periods.



**Figure 4**

(a) Fruit size of fruits from different treatment groups, bar=10 mm. (b) Fruit transection images of fruits from different treatment groups, bar=10 mm. (c) Seed intensity of fruits from different treatment groups.



**Figure 5**

(a) Activity of pollen collected from 12 h before blooming to 12 h after withering; (b) Fruit setting percentages for pollination from 12 h before blooming to 12 h after withering are shown as boxplots, and the corresponding pollen activities are shown as line charts; (c) Fruit size of fruits from pollination at different time points. All the comparisons, fruit size, germination rate, fruit setting rate are compare with the data at 0 h, \*\* and \* means extremely significant different and significant different.