Establishment of Pollen Storage Method for Pointed Gourd (Trichosanthes dioica Roxb.) Originating in South Asia

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Original Paper

Establishment of Pollen Storage Method for Pointed Gourd (*Trichosanthes dioica* Roxb.) Originating in South Asia

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Abstract

As pointed gourd (*Trichosanthes dioica* Roxb.), which is dioecious and originates from South Asia, has ephemeral flowers (one day flowering), asynchrony of anthesis between male and female flowers is a major concern for fruits production. Properly stored pollen might therefore be helpful to overcome pollination problems. Pollen viability and pollen germination of this species was investigated at anthesis, and after short-term and long-term storage under different temperature conditions. Pollen stored at -20°C performed well with regard to pollen viability and germination for the maximum duration when compared to room temperature and 4°C. More than 20% of pollen grains induced germination after 3 days of storage at room temperature, after 1 week at 4°C and after 1 month at -20°C. Viable stored pollens after 1 month at 4°C and after 4 months at -20°C yielded fruit at 26.7% by hand pollination in an open field. However, a fruit set rate of over 80% was confirmed using pollens after 3 days stored at 4°C and after 2 weeks stored at -20°C.

Key words: dioecious, ephemeral flower, pointed gourd, pollen storage, *Trichosanthes dioica*

Introduction

Pointed gourd (*Trichosanthes dioica* Roxb.) is a dioecious plant, and is related to *T. cucumeroides* and *T. japonica*, which are distributed in Japan. Pointed gourd is cultivated mainly in India and Bangladesh, where it is one of the most important summer vegetables. Young fruits of this species are 10-12 cm length and 30-50 g, are edible at the green stage (Fig. 1), and are similar in appearance to *T. cucumeroides* and *T. japonica*. Fruits of pointed gourd are cooked in curry, fried, pickled and candied in confections (Paris and Maynard, 2008). The main cultivation season is February to September in South Asian countries, where temperatures often exceed 35°C.

Recently, the annual mean temperature in Japan has been increasing, and several crops are experiencing serious problems due to these high temperatures in the summer season. Pointed gourd may therefore become a new summer vegetable in Japan owing to its heat tolerance.

Pointed gourd has ephemeral flowers (one day flowering) that deflate 12-14 h after anthesis. The flowers come to bloom in the early evening after sunset and remained fully opened until the next morning or early afternoon (Kumar and Singh, 2012). Accordingly, when many of the female flowers bloom, and insufficient numbers of male flowers open, few fruits can be harvested because of unsuccessful pollination. As the natural crossing between female and male flowers leads to poor fruit setting, farmers use hand pollination to overcome this natural pollination barrier.

With regard to reproductive physiology, the shedding of pollen and the receptivity of the stigma of bisexual flowers may be simultaneous, distinct or overlapping (Pacini, 2000). However, flowering and pollen maturation are not often

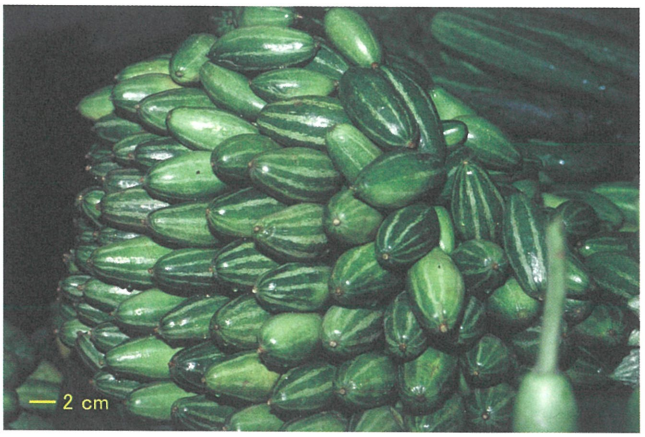

Fig. 1 Pointed gourd fruits in the market of Dhaka, Bangladesh.

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synchronized between two parental plants in dioecious species (Lim et al., 2014). Therefore, biologically active pollen should be stored without significant loss of viability until its counterpart plant is ready for pollination or to overcome any adverse pollination conditions.

Being a living organism, pollen preservation is very similar to seed preservation. Pollen viability is quickly lost if grains are left at room temperature, although low temperatures have been reported to be effective for long-term preservation of pollen with better viability (Giovannini et al., 2015). The variation in pollen longevity among plant species has been attributed to differences in desiccation tolerance of the pollen (Song and Tachibana, 2007). There is no universal test to precisely assess pollen viability, so the use of staining tests for pollen viability and *in vitro* germination tests are crucial for accurate prediction of germinability and fertilization ability (Dafni and Firmage, 2000).

Previous reports regarding pollen viability and *in vitro* germination of pointed gourd were mostly focused on the optimization of media composition (Zaman, 2006; Kumari et al., 2009). However, some aspects remain to be elucidated with regard to short-term and long-term pollen conservation techniques to overcome pollination problems in pointed gourd.

In the present study, pollen grains were stored at different storage conditions up to 12 months in order to provide information pollen viability and germination potential based on storage length.

Materials and Methods

Plant materials

Mature vine cuttings of 33 pointed gourd accessions collected from different locations of Bangladesh. They were introduced to the Institute of Tropical Agriculture, Kyushu University with consent of material transfer agreement between Kyushu University and Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh. In these accessions, the vines of one male (PGM01) and one female (PGF08) pointed gourd were planted in a plastic tray (35 x 50 cm) filling with soil (pamis sand : akadama soil = 1:1) November 2016. Sprouted vines of each genotype were transplanted into plastic pots (30 cm diameter) with pamis sand : akadama soil = 1:1 in an unheated glasshouse at Kyushu University, Hakozaki campus (lat. 33° 37′ N; long. 130° 25′ E), Japan, and were used for this experiment. The experiment had been conducted from April 2017 to July 2018 covering two flowering seasons in order to ensure required number of male and female flowers for pollen storage and filed fruit setting observation.

Pollen collection and storage

Male flowers from male plants (PGM01) were collected at the fully opened stage during the night (18:00-21:00). After collection, anthers were immediately placed in vials with silica gel and were stored under different temperature conditions; room temperature (25°C - 30°C), 4°C in a refrigerator and -20°C in a deep freezer.

Pollen viability at different storage temperatures and durations

Pollen viability of PGM01 was tested at anthesis, and after 1, 2, and 3 days, 1 and 2 weeks, and 1, 2, 4, 6, 8, and 12 months of storage. Pollen grains of PGM01 were collected with a needle on a glass slide and one or two drops of 1% acetocarmine were placed on these grains. Slides were covered with cover slips and were left for 10-15 minutes for proper staining of pollen grains. Slides were then observed under an optical microscope (Leica DM2500, Olympus DP70 model) at 10x magnification. Deeply stained and normal looking pollen grains were considered to be viable, whereas deformed, shriveled, slightly stained or colorless pollen grains were considered to be non-viable. The percentage of viable pollen was determined from three (3) randomly focused fields of a microscope, and 100 pollen grains were counted in each microscopic field. A total of 300 pollen grains per treatment were evaluated for viability assessment.

Pollen germination at different storage temperatures and durations

Pollen grains which were stored in a previous experiment were cultured in media containing 5% sucrose and 0.5% agar (Huyen, 2017). With a flat blade or needle, pollen was scraped onto smooth paper that was inclined and tapped to remove anthers and debris, leaving relatively pure pollen. The pollen was distributed on medium dispensed in 90-mm petri dishes. Petri dishes were covered with lids and the edges of these lids were wrapped with para-film paper to ensure uniform and high relative humidity. The covered petri dishes were incubated at 25°C under dark conditions for 24 h. Afterwards, observation slides were prepared by placing a small portion of germinating media with pollen grains and a cover slip was placed over the samples. Slides were then studied under an optical microscope (Leica DM2500, Olympus DP70 model) at 10x magnification. Pollen grains were considered to be germinated when pollen tube length was at least equal to or greater than the grain diameter (Leus, 2005). Germination of 100 pollen grains was counted for each microscopic observation field and this was performed

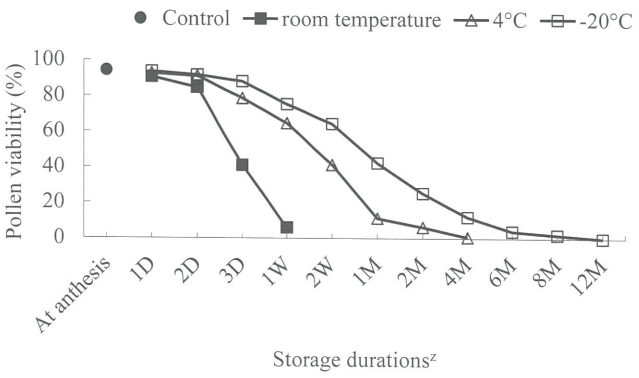

Fig. 2 Pollen viability (%) of pointed gourd at different storage durations under different temperatures.
[z] D: Day, W: Week, M: Month

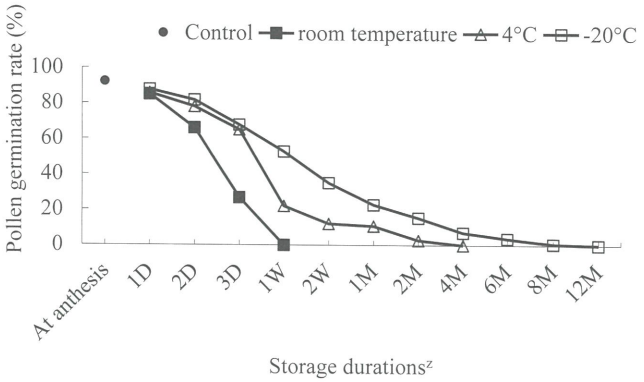

Fig. 3 Pollen germination rate (%) of pointed gourd at different storage durations under different temperatures.
[z] D: Day, W: Week, M: Month

for 3 different optical fields in each slide. The mean value of 3 observations was calculated and considered to be the germination rate. In total, the germinability of 300 pollen grains per treatment was assessed.

Effectiveness of fruit setting using stored pollen

The fertilization ability of stored pollen was assessed in terms of fruit set rate. In this study, a prehydration process was applied in order to improve the pollination process. For this purpose, pollen grains (fresh and stored) of PGM01 were soaked in 5% sucrose for 10 min at room temperature, after which they were used for pollination (Song and Tachibana, 2007). A few drops of Tween20 were also added in this solution to increase the adhesiveness of stigma and pollen grains. Five female flowers of PGF08 at anthesis were used for artificial crossings with fresh and stored pollen solution using a hand sprayer and this was repeated three times. The number of fruits was recorded for 15 days after pollination. The average value of three replications was recorded as the fruit set rate.

Results

Pollen viability at different storage temperatures and durations

The highest pollen viability (94.3%) was observed for fresh pollen collected at anthesis, and this was similar to that observed after one day of storage at room temperature (90.7%), 4°C (92.7%) and -20°C (93.6%) (Fig. 2). However, a significant decline in pollen viability from 90.7% to 41.0% was observed after 3 days of storage at room temperature. After the first week of storage at room temperature, very few (6.0%) pollen grains were viable. Meanwhile, pollen stored at 4°C and -20°C maintained 64.7% and 75.3% viability for 1 week. In the 2nd week, pollen viability decreased to 41.3% at 4°C, and this subsequently reduced to 11.3% and 6.3% after 1 and 2 months of storage, respectively. Only 0.7% of pollen grains were found to be viable at 4°C after 4 months of storage. Similarly, pollen stored at -20°C also showed a consistent reduction in viability where 12% and 2% pollen retained their viability after 4 and 8 months of storage, respectively.

Pollen germination at different storage temperatures and durations

The highest germination (92.3%) was observed in fresh pollen collected at anthesis and gradually decreased with different storage durations such as at room temperature, 4°C and -20°C (Fig. 3). At room temperature, 85% of pollen grains induced germination after 1 day of storage, and this sharply reduced to 26.6% beyond 3 days of storage. No pollen grains germinated after one week of storage, although some pollen grains were found to be viable at this stage in viability testing (Fig. 2). For pollen stored at 4°C, the maximum pollen germination (85.6%) was recorded after 1 day of storage and decreased slightly after 2 days of storage (78%). Moderate germination (65%) was observed after 3 days of storage. Subsequently, a marked reduction in pollen germination was noted after 1 week of storage (22%), and this finally reached 2.6% after 2 months of storage. None of the pollen was found to germinate beyond 4 months of storage. Meanwhile, at -20°C, pollen exhibited significantly higher germination with a minimal reduction up to 2 days of storage (87.7 to 82.7%). Moderate germination (52.6%) was observed after 1 week, and this decreased to 22.6% after 1 month of storage, which was statistically similar to the results for 2 months of storage (15.3%). The lowest percentage (0.6%) was counted after 8 months with the highest reduction from 87.7% to 0.6%.

Table 1. Percentage of fruits settings after pollination using fresh and stored pollen at different temperatures.

Pollen stored condition	Storage durations [z]											
	AA	1D	2D	3D	1W	2W	1M	2M	4M	6M	8M	12M
Room temperature	100.0 [y]	100.0	73.3	33.3	0.0	- [x]	-	-	-	-	-	-
4°C	100.0	100.0	100.0	86.7	73.3	46.7	26.7	6.7	0.0	-	-	-
-20°C	100.0	100.0	100.0	100.0	93.3	80.0	73.3	53.3	26.7	6.7	6.7	0.0

Five female flowers were crossed and repeats for three times.
[z] AA: At anthesis, D: day, W: week, M: month.
[y] Fruit settings were recorded after 15 days of pollination.
[x] - : Not examined.

Effectiveness of fruit setting using stored pollen

Pollen stored at room temperature beyond 1 week failed to produce any fruit, while that stored at 4°C and -20°C yielded fruit at 73.3% and 93.3%, respectively (Table 1). In addition, 26.7% and 73.3% fruit set rates were obtained using pollen stored for 1 month at 4°C and -20°C, respectively, and 26.7% fruit setting was observed using pollen stored for 4 months at -20°C, whereas no fruits were observed using pollen stored at 4°C. Finally, a 6.7% fruit setting rate was observed using pollen stored for 8 months at -20°C.

Discussion

A gradual decrease in viability and germination percentage was inferred from first day of storage until 12 months at all the storage temperatures in this study. Kumari et al. (2009) reported that 87.2% pollen grains from pointed gourd remain viable at three hours before anthesis, and that 22.1% remained viable at 30 hours after anthesis. However, the effects of different environmental factors on pollen germination and pollen tube growth are widely documented in different plant species (Dafni and Firmage, 2000; Taylor and Hepler, 1997). According to our study findings, more than 20% pollen grains maintained germinability for 3 days, 1 week and 1 month when stored at room temperature, 4°C and -20°C, respectively (Fig. 3). This illustrates that -20°C is more effective for long-term storage when compared to room temperature which agrees with previous findings (Kopp et al., 2002; Bomben et al., 1999; Anjum and Shaukat, 2008).

The fertilization ability of pollen stored at low temperature varied and has been reported in many crops with field level observations (Lyakh et al., 1998; Marks et al., 2014; El-Homosany and Sayed, 2015). Metz et al. (2000) reported that pollen from *Hylocereus* stored at 4°C for 3 to 9 months exhibited only 60-70% fruit set after pollination; however, 100% fruit set occurred when pollen was stored at subfreezing temperature for the same durations. According to our results, pollen grains were found to be effective for a considerable amount of fruit formation (26.7%) after 1 month of storage at 4°C and after 4 months of storage at -20°C. However, a fruit set rate of over 80% was confirmed using pollens after 3 days stored at 4°C and after 2 weeks stored at -20°C.

Among these pollen storage methods, pollen grain stored at 4°C (refrigerator) within 3 days was suggested as the most feasible to use for the farmers. Because, these stored pollen grains found effective to produce more than 80% fruit set at field level application.

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南アジア原産の食用カラスウリ（*Trichosanthes dioica* Roxb.）における花粉貯蔵法の確立

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摘　要

南アジア原産で雌雄異株性の食用カラスウリは一日花（一日開花性）であるため，雄花と雌花の開花の非同期性は果実の生産上大きな問題となる．適切に貯蔵された花粉は受粉における問題を克服できる．本研究では，本種の開花時および異なる温度条件下での短期貯蔵ならびに長期貯蔵での花粉活性と花粉発芽力を調査した．-20℃で貯蔵した花粉は，室温もしくは4℃で貯蔵した花粉と比較して最も長期間にわたって良好な花粉活性と花粉発芽力を維持していた．室温で3日間，4℃で1週間，および-20℃で1か月間貯蔵した花粉の発芽率はいずれも20%以上であった．4℃で1カ月間，および-20℃で4か月間貯蔵した花粉を用いて露地で人工授粉したところ，いずれも26.7%の着果がみられた．しかしながら，4℃で3日間，および-20℃で2週間貯蔵した花粉を用いると80%以上の着果率となることが確認された．

キーワード：一日花，花粉貯蔵，雌雄異株，食用カラスウリ，*Trichosanthes dioica*

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