원예과학기술지 17(3):337-340. 1999

# Convenient Evaluation of Stored Apple Pollen Viability by Fluorochromatic Reaction

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**ABSTRACT** In order to evaluate stored apple pollen viability, *in vitro* germination test was performed on a microscope slide coated with the culture medium containing fluorescein diacetate (FDA). However, the inclusion of FDA to the culture medium declined pollen germination. Alternatively, the fluorochromatic reaction procedure was tested. The procedure involved dusting pollen grains onto drops of 10% sucrose solution containing 0.002% FDA and allowing them to accumulate fluorescein. Within 30 min after the fluorochromatic reaction, viable pollen grains clearly fluoresced under ultraviolet light. Both the *in vitro* germination test and the fluorochromatic reaction procedure revealed that stored apple pollen viability was not considerably decreased over storage up to at least 39 months. Of the cultivars examined by both methods, 'Fuji' and 'Senshu' pollen viability was highest, 'Tsugaru' was intermediate, and 'Jonagold' was lowest. The fluorescing percentages appeared approximately comparable to the germination percentages except for the 'Senshu' pollens stored for 3 months, although the fluorescing percentages was slightly higher than the germination percentages. Strong and highly significant correlations were found between the two methods. It can thus be concluded that the fluorochromatic reaction procedure provides a convenient and reliable evaluation of stored apple pollen viability.

***Additional key words***: fluorescein diacetate, fluorescence, pollen germination

## Introduction

Various procedures have been used for estimation of pollen viability (Abdul-Baki, 1992; Heslop-Harrison et al., 1984; Huang and Johnson, 1996; Jefferies, 1977; Parfitt and Ganeshan, 1989; Sahar and Spiegel-Roy, 1984; Werner and Chang, 1981). Among these, *in vitro* germination and staining with tetrazolium salts have commonly been used to evaluate pollen viability. The *in vitro* germination involves germinating the pollens on an artificial culture medium and determining germinability and pollen tube growth (Abdul-Baki, 1992; Sahar and Spiegel-Roy, 1984). However, the *in vitro* pollen germination depends on optimization of the medium and temperature and on the availability of an adequate amount of pollen in the tested sample (Heslop-Harrison et al., 1984; Sahar and Spiegel-Roy, 1984). Staining pollens with tetrazolium salts for determining pollen viability requires relatively less time than the *in vitro* germination, thus making it most suitable for routine screening of many samples (Werner and Chang, 1981). Since tetrazolium salts can damage pollen membranes and inhibit germination at the concentrations recommended for staining (Abdul-Baki, 1992), however, it has often resulted in inaccurate estimation of pollen viability (Oberle and Waston, 1953; Parfitt and Ganeshan, 1989). Other staining reagents such as aniline blue lactophenol, safranin, and acetocarmine can also damage pollens (Parfitt and Ganeshan, 1989; Werner and Chang, 1981) and thus cannot be used in evaluating pollen viability.

Fluorochromatic reaction using fluorescein diacetate (FDA) might be an alternate method to evaluate pollen viability. The fluorochromatic reaction is based on the hydrolysis of FDA by esterase to yield fluorescein which fluoresces under ultraviolet light (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984; Rotman and Papermaster, 1966).

In this study, the fluorochromatic reaction procedure was examined to compare with the *in vitro* germination test with or without FDA and to determine if it could be used as a rapid and reliable method for evaluating stored apple pollen viability.

※ This work was supported in part by Wonkwang University in 1998.

## Materials and Methods

Mature 'Fuji', 'Senshu', 'Tsugaru', and 'Jonagold' apple trees grown at commercial orchard in Iksan, Chonbuk province, Korea were used as pollen source in this study.

Flowers at the balloon stage were harvested randomly from various shoots within and among 20 trees of each cultivar. Pollens from 30 flowers of each cultivar were collected in a 35-mm petri dish by using an anther dehiscence picker. The pollens were not sieved, since coarse preparation of pollens are better for long-term storage. After the coarse pollen preparations were pooled, they were divided into portions, each for one determination, and stored in sealed greasy paperbags containing silica gel at -15℃ until used. For comparison purpose, some preparations were stored in the same manner as above, but without using silica gel. Pollens of many horticultural crops have generally been stored at freezing temperature for long-term storage (Akihama and Omura, 1986; Galletta, 1983).

The viability of the stored pollens was determined as pollen germination on a culture medium. The medium consisted of 1% agar, 10% sucrose, 20 ppm $H_3BO_3$, and 300 ppm $Ca(NO_3)_2 \cdot 4H_2O$ with or without 0.01% FDA as reported (Abdul-Baki, 1992; Huang and Johnson, 1996). A thin layer of the medium was developed onto a microscope slide by dipping the slide into the hot liquid medium, withdrawing it quickly, and placing it on a glass plate horizontally. Pollens were dusted uniformly, with a camel hair brush, onto a slide containing the medium. The slide was kept in a petri dish with moist paper towels to maintain high humidity and incubated at 25℃. Percentage of germinated pollens was determined after 2, 4, and 6 h incubation under a light microscope. Each determination was made by randomly counting the germinated pollen grains at five locations in a single slide. At least 200 pollen grains were evaluated in each determination. A pollen grain was considered to have germinated when the length of pollen tube exceeded the diameter of pollen grain.

Alternatively, pollen viability was evaluated as fluorescing percentages following the fluorochromatic reaction using FDA (Heslop-Harrison et al., 1984; Jefferies, 1977). Following pollens were shaken onto

a microscope slide, a few drops of 10% sucrose solution containing 0.002% FDA were pipetted and the pollens were covered with a cover glass. After 30 min, brightly fluorescing pollens were determined as viable under a light microscope with ultraviolet light provided by a mercury lamp through excitation and barrier filters at 485 and 520 nm, respectively. For comparison purpose, the representative slide was photographed under ultraviolet light and under visible light provided by a halogen lamp.

The experiment was completely randomized with at least five replications for each determination. Simple linear regressions were made between the fluorescing percentages and the *in vitro* germination percentages of the pollens. Data were analyzed using the paired-comparison *t* test.

## Results and Discussion

Germination percentage of 'Fuji' apple pollens stored at -15℃ for 27 months was determined on a culture medium with or without 0.01% FDA over 6 h. On the medium without FDA, 66.3, 70.5, and 74.7% germinated after 2, 4, and 6 h incubation, respectively, at 25℃ (Fig. 1). On the medium with FDA, however, the germination percentage was only 43.8% after 6 h incubation (Fig. 1). This result indicates the adverse effect of FDA on apple pollen germination. In contrast to our results, Abdul-Baki (1992) and Huang and Johnson (1996) have reported that pollen viability is not reduced by FDA when included in a culture medium. This discrepancy might be resulted from different sources of pollens and/or FDA concentrations used. The FDA concentrations used for evaluating pollen viability have greatly varied with the plant species, ranging from 0.001 to 0.04% (Abdul-Baki, 1992; Huang and Johnson, 1996; Heslop-Harrison et al., 1984). From our preliminary experiments, it was found that with 0.001% FDA some of the nonfluorescing grains appeared almost as dark as the background (data not shown). Abdul-Baki (1992) reported the similar observations. Since FDA is slowly hydrolyzed to fluorescein in aqueous solutions, thereby reducing the sensitivity of the assay (Heslop-Harrison and Heslop-Harrison, 1970), the 0.001% FDA may be too low. If FDA concentration is too high, on the contrary, pollen viability may be overestimated as Heslop-Harrison et al. (1984) pointed out. Thus, we have chosen the FDA concentration at 0.01% as Huang and Johnson (1996) utilized in their culture medium. In our experiments, however, FDA at this concentration inhibited apple pollen germination and thus there was no merit of including FDA in the culture medium.

Instead of determining the exact FDA concentration in a culture medium for evaluating pollen germination, we have decided to evaluate pollen viability on a microscope slide directly in a way of dusting pollen grains onto drops of 10% sucrose solution containing 0.002% FDA and allowing them to accumulate fluorescein. Within 30 min after the fluorochromatic reaction with FDA, fluorescing pollen grains clearly appeared as shown in Fig. 2. Under ultraviolet light, viable pollen grains could easily be recognized by their fluorescence (Fig. 2A), since only viable pollens can uptake and subsequently hydrolyze nonfluorescing FDA by esterase to yield fluorescein (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984; Rotman and Papermaster, 1966). In contrast, nonviable pollens are not capable of hydrolyzing FDA and do not fluoresce, since the semipermeability nature of their plasma membranes have been lost (Jeffries, 1977). Under visible light, however, viable pollen grains were not distinguished from nonviable ones (Fig. 2B).

The viability of 'Senshu' apple pollens determined either by the fluorochromatic reaction or by the *in vitro* germination was not considerably decreased over storage duration up to at least 39 months (Table 1), suggesting that apple pollens with the absorbent can be stored at freezing temperature for a long time without significantly losing viability. However, the pollens stored for 15 months without using silica gel lost their viability significantly (Table 1), indicating that relative humidity during the storage is an important factor influencing pollen viability even at freezing temperature. The *in vitro* germination test represented that the apple pollens stored for 3 months exhibited lower germination percentage than those for 27 months (Table 1).

Considerable differences in pollen via-

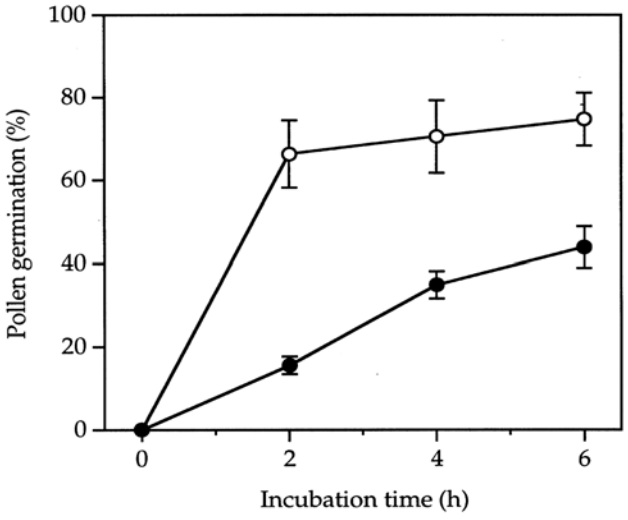


**Fig. 1.** Germination percentage of 'Fuji' apple pollens in a medium with (●) and without (○) 0.01% FDA. The pollens stored for 27 months were used. Error bars are ± SE of the means from five replications.

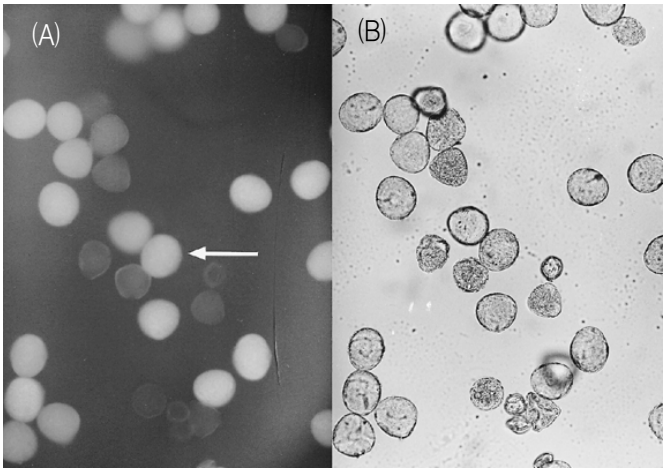


**Fig. 2.** Pollen viability as fluorescing pollen in 10% sucrose solution containing 0.002% FDA. The same sample was photographed under a light microscope at 100 magnification under ultraviolet light provided by a mercury lamp (A) or under visible light provided by a halogen lamp (B). Arrow indicates one of the fluorescing pollens.

**Table 1.** Comparison of fluorescing percentages with germination percentages of 'Senshu' apple pollens stored for 3, 15, 27, and 39 months.

| Storage duration (month) | Fluorescing (X) (%) | Germination (Y) (%) |
|---|---|---|
| 3[z] | 85.4 | 65.2 |
| 15 | 25.2 | 27.3 |
| 27 | 74.4 | 71.8 |
| 39 | 71.7 | 63.3 |
| | Y = 10.797 + 0.718X ($r^2$ = 0.910**) | |

[z]The apple pollens have been stored at -15℃ without using silica gel.
**Significant at P ≤ 0.01 using the paired-comparison *t* test.

**Table 2.** Comparison of fluorescing percentages with germination percentages of pollens from four apple cultivars stored for 27 months. Some data are quoted from Table 1.

| Cultivar | Fluorescing (X) (%) | Germination (Y) (%) |
|---|---|---|
| Fuji | 87.8 | 74.7 |
| Senshu | 74.4 | 71.8 |
| Tsugaru | 49.9 | 43.8 |
| Jonagold | 23.9 | 21.3 |
| | Y = 0.603 + 0.886X ($r^2$ = 0.978**) | |

**Significant at P ≤ 0.01 using the paired-comparison *t* test.

bility determined either by the *in vitro* germination or by the fluorochromatic reaction were observed among apple cultivars (Table 2). 'Fuji' and 'Senshu' pollen viability was highest, 'Tsugaru' was intermediate, and 'Jonagold' was lowest. Considering that 'Jonagold' is a triploid cultivar (Kim and Kim, 1998; Weeden and Lamb, 1985), it is not surprising that 'Jonagold' pollens have less viability.

The fluorescing percentages appeared approximately comparable to the germination percentages on the culture medium except for the 'Senshu' pollens stored for 3 months (Tables 1 and 2). However, the fluorescing percentages was slightly higher than the germination percentages. These results are consistent with observations on avocado (Sahar and Spiegel-Roy, 1984).

The regression equations of the germination percentages (Y-axis) versus the fluorescing percentages (X-axis) were Y = 10.797 + 0.718X for pollens stored for different storage durations (Table 1) and Y = 0.603 + 0.886X for pollens from different cultivas (Table 2). The slope values indicate that the fluorochromatic reaction procedure slightly overestimates the *in vitro* pollen germination. However, the positive intercept values imply that a few of germinable pollens are not responsive to the fluorochromatic reaction. The correlations between the fluorescing percentages and the germination percentages were strong ($r^2$ = 0.910 and 0.978) and highly significant (P ≤ 0.01). This results indicate that the fluorochromatic reaction procedure is a convenient and reliable evaluation of stored apple pollen viability. Similar observations have been reported on blueberry (Huang and Johnson, 1996), olive (Pinney and Polito, 1990) and tomato (Abdul-Baki, 1992).

In conclusion, the fluorochromatic reaction procedure we tested provides a satisfactory evaluation of stored apple pollen viability. The fluorochromatic reaction procedure, in conjunction with the storage of pollen, will be helpful to improve breeding techniques and to facilitate commercial mass pollination for apple trees. The method may also be useful in determining pollinizer requirements and in evaluating the viability of pollens which develop under environmental and cultural stresses.

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## 형광염색반응에 의한 장기 저장 사과 화분의 활력 측정


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### 초 록


장기 저장한 사과 화분의 활력을 측정하기 위하여 기본배지와 fluorescein diacetate(FDA)를 첨가한 배지를 slide glass 위에 얇게 도포한 후 기내발아율을 조사한 결과, FDA를 첨가한 배지에서는 화분 발아가 저하되었다. 따라서 FDA 용액을 이용한 형광염색반응법으로 사과 화분의 활력을 측정할 수 있는지 구명하기 위하

여 10%의 sucrose 용액을 slide glass 위에 한 방울 떨어뜨린 후 0.002%의 FDA를 첨가한 곳에 화분을 치상한 결과, 형광반응이 일어나는 치상 후 30분 경에 활력이 있는 화분은 자외선 하에서 명확히 형광을 나타냈다. 기내발아율과 형광반응률을 측정한 결과, 39개월 장기 저장한 사과의 화분도 활력이 감소하지 않는 것으로 나타났다. 같은 방법으로 조사한 품종별 화분의 활력은 '후지' 와 '천추'가 가장 높게 나타났으며 '쓰가루'는 중간 정도, '조나골드'는 낮게 나타났다. 형광염색반응법에 의한 형광반응률은 대체로 기본배지에서의 발아율과 비슷하게 나타났으나 3개월간 저장한 '천추'의 화분은 형광반응률이 발아율보다 다소 높게 나타났다. 형광반응률과 발아율은 높은 정의 상관을 보여 형광염색반응법에 의해 장기 저장 사과 화분의 활력을 편리하고 정확하게 측정할 수 있었다.

추가 주요어 : fluorescein diacetate, 형광, 화분 발아