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# Cryogenic preservation of grape (*Vitis vinifera* L.) pollen

by

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**Gefrierkonservierung von Pollen der Rebe *(Vitis vinifera* L.)**

Zusammenfassung: Pollen von fünf Rebsorten, der in flüssigem Stickstoff (−196 °C) bis zu 64 Wochen lang gelagert worden war, zeigte nach dieser Zeit keine signifikante Verminderung seiner Keimfähigkeit *in vitro*. Dagegen verlor der bei Zimmertemperatur aufbewahrte Pollen seine Lebensfähigkeit schon innerhalb von 4 Wochen. Zwischen den fünf Sorten wurden nach 64 Wochen dauernder Gefrierlagerung signifikante Unterschiede der Keimfähigkeit beobachtet. Die beschriebene Technik der Gefrierkonservierung von Pollen kann sowohl für Rebenzüchter als auch für Genbanken von Nutzen sein.

Key words: pollen, variety of vine, cold, storage, germination.

## Introduction

Pollen of several grape cultivars lose viability and fertilizing capacity after 2 weeks of collection and storage under ambient conditions (NAGARAJAN *et al.* 1965). Maximum longevity for pollen of Thompson Seedless, Muscat of Alexandria and Monukka was reported as 4 years, when stored at −12 °C and 28 % relative humidity (OLMO 1942), resulting in a reduced germination profile. Pollen storage for a period of 4 months to over 1 year at low temperatures (−12 to 8 °C) within a humidity range of 0—50 % was also found suitable (NEBEL and RUTTLE 1937; NAGARAJAN *et al.* 1965; BAMZAI and RANDHAWA 1967), but not without a progressive reduction in viability after storage. Studies on the effects of varying humidity for preserving viability (GOLLMICK 1942; RANDHAWA *et al.* 1982) and storage in organic solvents (AGARWAL 1983) resulted in reduced germination *in vitro* with increasing storage durations. Fluctuations in storage conditions alter the microclimate in which pollen is preserved, leading to an accelerated loss of viability (STANLEY and LINSKENS 1974).

Although successful pollinations were achieved with 4 years' stored pollen producing germination *in vitro* as low as 6 % (OLMO 1942), preserving pollen viability with negligible loss in terms of its germination potential and fertility would be most desirable. A 'pollen bank' containing stocks preserved with high viability profiles can be of prolific use to grape breeders. Thus, pollination between early and late flowering cultivars could be carried out, irrespective of their geographic location and time of flowering. Recently, long-term pollen preservation for genetic resources conservation has been given a serious consideration (HARRINGTON 1970; ROBERTS 1975; OMURA and AKIHAMA 1980; OMURA *et al.* 1982). The possibility of using liquid nitrogen storage conditions for preserving viability of grape pollen was demonstrated (PARFITT and ALMEHDI 1983). Pollen samples rapidly frozen and thawed after holding for 1 h at −196 °C retained viability *in vitro* equivalent to fresh pollen.

Application of cryogenic techniques to preserve pollen viability for prolonged durations has been found suitable in grape (YAKIMOV 1977, 1979) and pollen of other economically important crops (FARMER and BARNET 1974; NATH and ANDERSON 1975; BARNA-

BAS and RAJKI 1976, 1981; KOBAYASHI *et al.* 1978; WEATHERHEAD *et al.* 1978; AKIHAMA *et al.* 1979; FISCHER and WALTHER 1981; TOWILL 1981; FRANK *et al.* 1982; TISSERAT *et al.* 1983; PARFITT and ALMEHDI 1984 a, b). The present paper establishes the viability state of cryopreserved grape pollen in terms of germinability *in vitro* and provides a simple method for prolonging viability under cryogenic conditions.

## Materials and methods

### Pollen collection and processing for cryopreservation

Pollen was collected from plants in well established vineyards of Bangalore Blue, Bangalore Purple, Black Champa, Queen of Vineyards and Anab-e-Shahi using the method described by OLMO (1942). Pure pollen was transferred into gelatin capsules, which were individually packed in 5 ml Corning culture tubes with screw caps, containing activated silica gel. Culture tubes were bound with adhesive tape and wrapped with aluminium foil after encasing them in small 5 cm long PVC tubes. Storage at −196 °C was accomplished by direct immersion in liquid nitrogen after precooling to −20 °C. Culture tubes with pollen samples were stacked in canisters of a Mach-SM-43 cryobiological system (MVE, USA) and immersed in liquid nitrogen. Complete immersion of samples throughout the storage duration was ensured by frequent refilling of the cryoflask with liquid nitrogen and capping the canisters with perforated lids, in order to prevent the stored samples from floating out into the Dewar. The cryoflask was maintained in the laboratory at 22 ± 2 °C.

### Viability tests

Pollen viability was tested by of its ability to germinate in an artificial medium. Fresh and cryopreserved pollen samples were cultured *in vitro* following hanging drop technique, after 0 (fresh), 4, 26 and 64 weeks. Samples were kept for thawing at ambient temperature for 30 min before culture, after which the remaining pollen was refrozen to −196 °C via −20 °C. The germination medium consisted of 20 % sucrose solution in which pollen germinated profusely. Hanging drop cultures were incubated for 5 h at 25 ± 2 °C and stained with a drop of ALEXANDER's stain (1980). Slides with germinated pollen were scanned and scored using a Leitz Neo-Promar projection microscope. Pollen whose tube lengths measuring more than the grain diameter were considered viable. More than 500 grains were scored for each replication. Three replicates, each of control and frozen pollen were assayed for viability after each storage duration. Data on germination were analysed for variance by completely randomised (factorial) design.

## Results and discussion

Table 1 shows *in vitro* pollen germination percentages recorded after different durations of cryopreservation, as compared with fresh pollen. All five cultivars recorded germination rates equivalent to fresh pollen, after 4, 26 and 64 weeks of storage in liquid nitrogen. However, pollen samples from different cultivars showed significant variability in germination profiles after cryopreservation. Pollen could withstand direct freezing to −196 °C via −20 °C, rapid uncontrolled thawing to room temperature and refreezing to cryogenic temperatures (via −20 °C) during viability assessment after each storage duration. On the contrary, pollen samples stored at room tempera-

Table 1

Pollen germination in different grape cultivars after storage in liquid nitrogen (mean values as per cent)

Pollenkeimung bei verschiedenen Rebsorten nach Lagerung in flüssigem Stickstoff (Mittelwerte in %)

| Cultivar | Duration of storage (weeks) | | | | Mean |
|---|---|---|---|---|---|
| | 0 (fresh) | 4 | 26 | 64 | |
| Bangalore Blue | 54.3 | 54.0 | 50.8 | 59.5 | 54.7 |
| Bangalore Purple | 61.8 | 58.1 | 58.2 | 81.9 | 64.9 |
| Black Champa | 80.5 | 66.1 | 71.3 | 84.4 | 75.6 |
| Queen of Vineyards | 74.8 | 75.3 | 79.0 | 80.2 | 77.3 |
| Anab-e-Shahi | 64.0 | 62.8 | 73.2 | 71.7 | 67.9 |
| Mean | 67.1 | 63.3 | 66.5 | 75.6 | |

| | Cultivar | Duration | C × D Interaction |
|---|---|---|---|
| Standard error mean | 3.612 | 3.230 | 7.224 |
| LSD (P 0.05) | 10.322 | 9.232 | 20.643 |
| LSD (P 0.01) | 13.810 | 12.352 | 27.620 |

ture (control) continuously decreased in their capacity to germinate *in vitro*, approaching less than 5 % by the end of 2 weeks, after which viability was completely lost within 4 weeks. Control pollen samples, cultured along with cryopreserved samples after 26 and 64 weeks, did not germinate. Pollen stored in liquid nitrogen continued to germinate as successfully as fresh pollen. Analysis of variance between cultivars, storage durations and their interaction is given in Table 2.

Differential staining with ALEXANDER's stain was observed for fresh and cryopreserved germinated pollen, until the 64th week of viability assay. Germinated grains stained in two colours, the light pink grains becoming more greenish as the tubes elongated. The non-germinated grains stained dark pink.

Pollen samples of all cultivars stored in liquid nitrogen maintained total viability after 64 weeks, indicating that a rapid cooling rate (from −20 to −196 °C) had no significant effect on the biological integrity of pollen under cryogenic conditions. Pollen preserved under these conditions could remain viable for 12 years (YAKIMOV 1979) with negligible loss of its potential to germinate *in vitro*, retaining high fertility rates by producing normal fruit and seed set when used in field pollinations. This method of pollen preservation is considered superior to storage under low temperature and humidity, where viability was gradually reduced after prolonged durations of storage.

Field pollinations with stored pollen germinating as low as 6 % *in vitro* was reported to be sufficient for effecting a normal berry set (OLMO 1942). In the present investigation, pollen of all five cultivars recorded very high germination profiles after 64 weeks of cryopreservation, which is 10—14 times more than the stipulated requirement for a normal berry set. Therefore, the major implication of this study from a breeding view point is that storage in liquid nitrogen over a prolonged duration allows grape breeders to pollinate early flowering cultivars with pollen from late flowering cultivars taken during previous years.

Table 2

Analysis of variance of pollen germination percentages

Varianzanalyse der prozentualen Pollenkeimung

| Source | df | MS | F | Significance |
|---|---|---|---|---|
| Cultivar | 4 | 995.16 | 6.357 | ** |
| Duration | 3 | 413.04 | 2.638 | NS |
| C × D Interaction | 12 | 83.28 | 0.532 | NS |
| Error | 40 | 156.55 | | |

As a simple technique, storing fresh grape pollen (without the need for desiccation or freeze-drying) for long-term preservation of plant germplasm is possible by cryopreservation in liquid nitrogen at – 196 °C. This can be used as a routine method by grape breeders and gene banks involved in conservation *in vitro.*

## Summary

Pollen of five grape cultivars stored in liquid nitrogen ( – 196 °C) showed no significant decrease in percentage germination after 64 weeks, whereas pollen stored under laboratory conditions lost viability within 4 weeks. Significant differences in pollen viability were recorded among the cultivars after the storage duration of 64 weeks. This technique of pollen cryopreservation will be advantageous for grape breeders and gene banks involved in pollen preservation for conservation of grape genetic resources.

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