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# Fertilizing ability of cryopreserved grape (*Vitis vinifera* L.) pollen[1)]

by

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**Befruchtungsfähigkeit von gefrierkonserviertem Pollen der Rebe (*Vitis vinifera* L.)**

Zusammenfassung: Durch kontrollierte Bestäubung wurde die Befruchtungsfähigkeit gefrierkonservierter Pollen von Black Champa (BC) und Queen of Vineyards (QVY) nach bis zu 5jähriger Lagerung geprüft. Verschiedene Elternkombinationen unter Einschluß von Selbstungen zeigten keine signifikanten Unterschiede des prozentualen Beerenansatzes; im Vergleich zu den jeweiligen Kontrollen mit Frischpollen war jedoch eine signifikante Abnahme der Befruchtungsfähigkeit zu verzeichnen. Bei allen Kreuzungen unter Verwendung von gefrierkonserviertem Pollen ging die Anzahl der Kerne je Beere zurück; dies weist auf eine mögliche Abnahme der Fertilität hin. Übertragung von gefrierkonserviertem Pollen (QVY) auf die Narben einer abweichenden Sorte (BC) ergab den höchsten prozentualen Beerenansatz bei schwacher Kernausbeute. Bei der *in-vitro*-Keimung des Pollens nach unterschiedlicher Dauer der Gefrierkonservierung wurde eine hohe Vitalitätsrate — frischem Pollen vergleichbar — registriert; signifikante Unterschiede zwischen den beiden Sorten, der Dauer der Gefrierlagerung oder deren Interaktionen wurden jedoch nicht festgestellt. Die vorliegende Untersuchung beweist die Lebensfähigkeit von gefrierkonserviertem Pollen auch nach langer Lagerungsdauer, wobei jedoch im Freiland mit niedriger Fertilität, geringem Fruchtansatz und schwacher Kernausbeute gerechnet werden muß.

Key words: pollen, variety of vine, cold, storage, germination, pollination, fecundation, fruit set, shattering, seed number.

## Introduction

Long-term pollen preservation enables secure maintenance of fruit tree genetic resources (AKIHAMA and OMURA 1986). A large amount of genetic material could be conserved in a 'pollen cryobank'. Such a facility for temperate fruit trees was first established in Japan (AKIHAMA *et al.* 1979). Recently, three centres in the U.S.A. have initiated work on pollen cryopreservation (STANWOOD *et al.* 1986; ANONYMOUS 1987; PARFITT 1988).

Establishment of pollen cryobanks primarily need definite protocols optimized for maintaining pollen viability and fertility over extended periods. PARFITT and ALMEHDI (1983) demonstrated the possibility of using cryogenic conditions for preserving viability of grape pollen. GANESHAN (1985) established the viability of cryopreserved grape pollen in terms of its potential to germinate *in vitro* after a period of 64 weeks. The present report relates to fertilizing ability of cryofrozen grape pollen after 5 years of cryostorage in liquid nitrogen, substantiated with *in vitro* germination and field pollination data.

## Materials and methods

The methodologies involved in pollen collection, processing for cryopreservation and viability tests of fresh and cryofrozen material have been described earlier

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(GANESHAN 1985). Pollen samples of Black Champa and Queen of Vineyards collected in the 1981—1982 season were 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 pre-cooling 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 was ensured by frequent refilling of the cryoflask with liquid nitrogen and capping the canisters with perforated lids. The cryoflask was maintained in the laboratory at 22 ± 2 °C.

Pollen samples were thawed from a frozen state to ambient temperature after various intervals of storage, by initially holding the canister over liquid nitrogen fumes for 15 min, after which the culture tubes were removed from the canister and allowed to thaw at ambient temperature for 30 min before culture. Laboratory assays were carried out to test pollen viability of cryostored samples. The germination medium consisted of 20 % sucrose solution, used for hanging drop cultures. Pollen samples after culture were incubated for 5 h at 25 ± 2 °C, stained (ALEXANDER 1980) and scored following procedures described by GANESHAN (1985). Data on *in vitro* pollen germination (3 replicates) recorded periodically (after 0, 1, 6, 15, 42 and 60 months) along with controls were statistically analysed by completely randomised factorial design.

Field pollinations were carried out with 5-year-old cryofrozen Black Champa and Queen of Vineyards pollen. More than 150 flowers (2 inflorescences) per cross combination from both these cultivars were emasculated and cryostored pollen was applied after thawing to ambient temperature, using a sterilized '0' size painting brush. Emasculated flowers with exposed receptive stigma were touched with cryostored pollen, taken on the brush. For controls, fresh pollen was similarly applied on emasculated flowers. The following parental combinations were used in the crossing schedule:

| Pollen parent | Seed parent |
|---|---|
| 1. Black Champa (cryofrozen) | Black Champa |
| 2. Black Champa (fresh) | Black Champa |
| 3. Queen of Vineyards (cryofrozen) | Queen of Vineyards |
| 4. Queen of Vineyards (fresh) | Queen of Vineyards |
| 5. Queen of Vineyards (cryofrozen) | Black Champa |

Data recorded on fruit and seed set for different crosses were statistically compared using a test for differences in proportions by normal deviate test (FISCHER and YATES 1979). The seed set data were averaged to number of seeds per berry for each cross, after deleting the dropped berries.

## Results and discussion

The pollen viability profiles as tested by *in vitro* germination using cryopreserved Black Champa and Queen of Vineyards pollen is presented in Table 1 a. Pollen from both cultivars have recorded germination rates equivalent to fresh pollen, after 5 years of cryostorage ($P = 0.05$). There was no significant difference between these cultivars, with regard to their viability after prolonged cryogenic freezing. On the contrary, pollen samples stored at ambient temperature (control) lost viability within 4 weeks, which has also been reported earlier (GANESHAN 1985). Analysis of variance between cultivars, storage durations and their interaction is given in Table 1 b.

Table 1a

Pollen germination in Black Champa and Queen of Vineyards after storage in liquid nitrogen (mean values as per cent)

Pollenkeimung bei Black Champa und Queen of Vineyards nach Lagerung in flüssigem Stickstoff (Mittelwerte in %)

| Cultivar | Duration of storage (months) | | | | | | Mean |
|---|---|---|---|---|---|---|---|
| | 0 (fresh) | 1 | 6 | 15 | 42 | 60 | |
| Black Champa | 74.0 | 66.1 | 71.2 | 84.4 | 84.0 | 77.0 | 76.1 |
| Queen of Vineyards | 72.1 | 75.3 | 78.9 | 80.2 | 88.6 | 90.0 | 86.8 |
| Mean | 73.0 | 70.7 | 75.0 | 82.3 | 86.3 | 83.5 | |

| | Cultivar | Duration | C × D Interaction |
|---|---|---|---|
| Standard error mean | 2.649 | 4.589 | 6.489 |
| LSD (P = 0.05) | 7.736 | 13.400 | 18.950 |

Table 1 b

Analysis of variance of pollen germination percentages

Varianzanalyse der prozentualen Pollenkeimung

| Source | DF | MS | F | Significance |
|---|---|---|---|---|
| Cultivar | 1 | 199.95 | 1.582 | NS |
| Duration | 5 | 244.24 | 1.932 | NS |
| C × D Interaction | 5 | 65.95 | 0.521 | NS |
| Error | 24 | 126.35 | | |

Tables 2 a and b present results of field pollinations with cryofrozen Black Champa and Queen of Vineyards pollen. The differences in proportions test for the parental cross combinations recorded significant variation for the berry set percentage values, for crosses involving fresh versus cryofrozen pollen on the same as well as different female parents. Crosses involving cryofrozen pollen recorded almost similar berry set on their own female parents, which are significantly low, compared to fresh pollen (controls). The highest berry set percentage, however, was recorded for the cross involving cryofrozen Queen of Vineyards pollen with Black Champa (68.8 %). A high rate of berry drop was associated with crosses involving cryofrozen Queen of Vineyards pollen, which was correspondingly low with that of cryofrozen Black Champa pollen compared to its fresh pollen cross, which was associated with a high berry drop frequency. The average seed number per fruit was low among crosses involving cryofrozen Queen of Vineyards pollen, while with that of Black Champa the reduction was only marginal when compared with respective controls.

Cryofrozen pollen of Black Champa and Queen of Vineyards maintained total viability after prolonged storage (5 years) as tested by *in vitro* germination. However, a variation in fertility profiles could be perceived, when these samples were used in field pollinations on their own female counterparts or on a different female parent (cryofrozen Queen of Vineyards pollen on Black Champa females). The average number of

seeds set per fruit was much less in crosses involving cryofrozen pollen, compared with controls. Although this difference was only marginal for crosses involving cryofrozen Black Champa pollen, a considerable reduction in seed set was recorded for crosses involving cryofrozen Queen of Vineyards pollen.

A low seed set may result with cryofrozen pollen if the initial moisture content in pollen is high. Seed set was increased in potato, when the initial moisture content in pollen was reduced prior to cryogenic storage (TOWILL 1984). Therefore, a suitable treatment of pollen before and after cryogenic exposure could increase seed set.

The success of field pollinations with stored pollen depends on its fertility status and the probability of a viable pollen grain inducing seed set. This is quite high for grapes even when the pollen had a low viability profile *in vitro*. Pollen germination rates as low as 6 % (*in vitro*) was reported to be sufficient for effecting normal berry set

Table 2 a

Field pollinations with Black Champa (BC) and Queen of Vineyards (QVY) pollen after 60 months of storage in liquid nitrogen

Freilandbestäubung mit Pollen von Black Champa (BC) und Queen of Vineyards (QVY) nach 60monatiger Lagerung in flüssigem Stickstoff

| No. | Cross | | % berry set (PBS) | % berry drop (PBD) | Av. seed number/ fruit (SNF) |
|---|---|---|---|---|---|
| | Female | Pollen | | | |
| 1. | BC | BC (LN) | 34.4 | 11.3 | 1.2 |
| 2. | BC | BC (F) | 59.2 | 36.2 | 1.4 |
| 3. | QVY | QVY (LN) | 38.7 | 35.0 | 0.8 |
| 4. | QVY | QVY (F) | 51.7 | 1.6 | 2.3 |
| 5. | BC | QVY (LN) | 68.9 | 29.0 | 1.3 |

LN = Liquid nitrogen.
F = Fresh.

Table 2 b

Comparison of PBS obtained from different crosses using test for difference in proportions by normal deviate test (FISCHER and YATES 1979)

Vergleich der prozentualen Verrieselung mit Hilfe des „test for difference in proportions by normal deviate test" (FISCHER und YATES 1979)

| Crosses | BC × BC (F) | QVY × QVY (LN) | QVY × QVY (F) | BC × QVY (LN) |
|---|---|---|---|---|
| BC × BC (LN) | −0.24** | −0.04 | −0.17** | −0.34** |
| | (−4.38) | (−0.80) | (−2.96) | (−5.35) |
| BC × BC (F) | | 0.20** | 0.07 | −0.09 |
| | | (−3.49) | (1.21) | (−1.46) |
| QVY × QVY (LN) | | | −0.12* | −0.30** |
| | | | (−2.14) | (−4.55) |
| QVY × QVY (F) | | | | −0.17* |
| | | | | (−2.51) |

Figures in parentheses indicate corresponding 'Z' values.
P = 0.01 when Z = > 2.56.
P = 0.05 when Z = > 1.98 but < 2.56.
Not significant when Z < 1.98

(OLMO 1942). In the present studies, cryostored grape pollen recorded viability profiles equivalent to fresh pollen after 5 years of cryostorage. A good seed formation could result with stored pollen despite a poor viability recorded *in vitro* (STANLEY and LINSKENS 1974) and, conversely, pollen having a good viability after storage could induce a poor fruit and seed set (KING 1965). Field pollinations with 5-year-old cryofrozen grape pollen showed reduction in fertility rates, associated with reduced berry number and seed yield, although the viability status *in vitro* remained unaffected. The present investigation, therefore, demonstrates that long-term cryostored grape pollen can remain viable but with a reduced fertility, inducing fruit and seed set when used in field pollinations.

The primary objective of a frozen pollen program should be to save almost all alleles in some unknown combination with alleles at other loci (NAMKOONG 1981). Therefore, the pollen sampling strategy must insure that even rare alleles will be included in a given gene pool, although their probability of occurrence is minimum. It would be virtually impossible to preserve unique allelic combinations which exist in germ cells, but an attempt could be made to save the alleles in a gene pool that can be recombined in future breeding generations, as long as they are present in the conserved pool. This could be accomplished by broadening the gene pool over time (SCHOENIKE and BEY 1981). Intensive pollen sampling from diverse populations such as seed-grown grape plants, clonal material, test plantings, etc., could contribute to maintain allelic frequencies. Besides this, samples from different grape growing regions held in a pollen cryobank could enrich the gene pool and allelic combinations desired by the breeder.

## Summary

Cryopreserved grape pollen of Black Champa (BC) and Queen of Vineyards (QVY) were used in controlled pollinations to test their fertilizing ability after 5 years of storage. Parental combinations involving cryofrozen pollen on their own female parents showed no significant difference in percent berry set, whereas in comparison with the respective controls (fresh pollen) recorded a significant decrease. There was a decrease in seed yield per berry in all crosses involving cryofrozen pollen, indicating a possible fertility decline. Crosses involving cryofrozen pollen (QVY) on a different female parent (BC) produced the highest berry set percentage, but with a poor seed yield. *In vitro* pollen germination recorded high viability profiles (equivalent to fresh pollen) after different durations of cryopreservation, with no significant differences among the two cultivars, durations and their interactions.

The present investigation establishes the viability status of long-term cryostored grape pollen, but with low fertility rates, reducing fruit and seed set when used in field pollinations.

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