Journal of Horticultural Science & Biotechnology (2007) **82** (1) 29–34

Effect of moisture content of tomato pollen stored cryogenically on *in vitro* germination, fecundity and respiration during pollen tube growth

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(Accepted 1 October 2006)

SUMMARY

The moisture content (MC) of tomato pollen was conditioned to a range between 3.8% and 53% of fresh weight by equilibration in air with a relative humidity (RH) ranging from 0 – 100%. After storage in liquid N (–196°C) for 5 d, followed by thawing and rehydration, germination was measured *in vitro* on a semi-solid substrate. Pollen with a MC of 6.8% or 9.3%, which was achieved by exposure to 50% or 60% RH, respectively, gave the highest percentages of germination (56.8% and 57.4%, respectively). Further studies involving three MC levels (6.8%, 9.3% and 11.2%, equilibrated at 50%, 60% and 65% RH, respectively) and cryogenic storage for periods of up to 22 months, indicated that both the germination ability and the length of the pollen tubes formed after incubation for 6 h did not depend on the duration of cryopreservation, provided the pollen was rehydrated after thawing. The ability of pollen to set fruit and to produce seeds was not affected by storage in liquid N_2 for up to 22 months, when the MC was 6.8% or 9.3%. However, when the MC was 11.2% prior to storage for 14 or 22 months in liquid N_2, the percentage of successful pollinations was lower and fewer seeds were formed per fruit. Cumulative O_2 consumption, due to the respiration of pollen during germination, increased linearly with incubation time and the rate of increase was similar for both fresh pollen and pollen that had been stored cryogenically for 6 or 13 months. It is concluded that tomato pollen may be stored in liquid N_2 for at least 22 months without loss of viability, provided its MC is adjusted to between 6.5% and 9.5% prior to cryopreservation and that, after thawing, the pollen is rehydrated.

Many applications related to plant breeding, such as cross-pollination of plants that flower at different times or locations, conservation of germplasm in a minimum volume, and/or the production of haploids, may require long-term storage of viable pollen (Towill, 1985; Hanna and Towill, 1995). Storage under cryogenic conditions (i.e, temperatures below –70°C) seems to be the most effective means to maintain pollen viability for long periods, while temperatures of approx. –20°C enable the maintenance of pollen viability for 1 year (i.e., season to season) or even longer (Towill, 1981; Bajaj, 1987; Towill and Walters, 2000). However, the optimum temperature for long-term storage of pollen varies between species, or even between different cultivars of the same species. For example, a temperature of –20°C was more favourable for the storage of kiwi (*Actinidia delisiosa*) and almond (*Prunus dulcis*) pollen than –80°C (Abreu and Oliveira, 2004; Martinez-Gómez *et al.*, 2002).

A crucial factor for successful storage of pollen under cryogenic conditions is its moisture content (MC; Johri and Vasil, 1961; Towill, 1985; Connor and Towill, 1993; Towill and Walters, 2000). In most cases, partial desiccation is required before storage, to ensure maintenance of viability (Hecker *et al.*, 1986; Towill, 1987; Luza and Polito, 1988; Bowes, 1990; Parton *et al.*, 2002), because this reduces the risk of intracellular ice formation (Ching and Ching, 1964; Ching and Slabaugh, 1966). However, critical moisture levels for cryogenic storage of pollen vary among species (Towill, 1985; Connor and Towill, 1993). In some cases, pollen may be exposed to cryogenic temperatures immediately after collection, indicating that some desiccation has already occurred after release from the anthers (Visser, 1955; Crisp and Grout, 1984; Polito and Luza, 1988; Rajasekharan *et al.*, 1994); but, in most cases desiccation to a certain MC is needed (Luza and Polito, 1988; Parton *et al.*, 2002). On the other hand, excessive desiccation may be harmful to pollen viability during storage at cryogenic temperatures (Bajaj, 1987; Marchant *et al.*, 1993). Hence, for satisfactory storage of pollen of a particular species under cryogenic conditions, the optimum MC of the pollen grains must be determined.

Tomato is an important greenhouse species which is propagated commercially from hybrid seed. Although storage of tomato pollen may be relevant for both the commercial production of hybrid seed and the development of new hybrids, little information is available regarding its storage under cryogenic conditions. The information that is currently available refers mainly to the effects of cryogenic storage on initial and final germination rates *in vitro* (Visser, 1955) and pollen fecundity (Sacks and St. Clair, 1996). Alexander and Ganeshan (1989) reported a very low number of seeds per fruit when the pollen used for fertilisation had been stored at –196°C. However, the optimum temperature and MC for cryogenic storage of tomato pollen have not been defined to date.

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This study was therefore designed to assess: (i) the effects of the MC of tomato pollen on its germination ability after storage in liquid nitrogen (–196°C) and on the rate of pollen tube elongation; (ii) changes in pollen fecundity during storage; and (iii) the respiration rate of cryogenically-stored tomato pollen during *in vitro* germination. The latter may reveal the timing of metabolic events (Dickinson, 1965), thereby providing a physiological basis for the interpretation of possible differences in germination ability between fresh pollen and pollen stored under cryogenic temperatures for a particular time.

MATERIALS AND METHODS

Pollen grains of tomato (*Lycopersicon esculentum* Mill.) cv. 'Paxoi', an inbred line characterised by profuse production of pollen, were collected from a greenhouse crop in May. Pollen was collected between 0900 – 1100 h by vibrating open flowers bearing mature pollen on the anther cone using an electric vibrator connected to a small vial which served as the pollen collector. During collection, the minimum and maximum air temperatures were 18°C and 28°C, respectively, and the relative humidity ranged from 60% to 70%.

Determination of moisture content (MC) equilibrium

Immediately after collection, pollen grains were transferred to the laboratory and separated into portions, each of 3 – 4 mg. Each portion was spread over the surface of a shell vial which was inserted into a sealed beaker containing a second vial with H_2SO_4 at a concentration that enabled the establishment of a target relative humidity (RH) regime (Solomon, 1951). Using this technique, seven different RH levels ranging from 20 – 80% were established. The air temperature in the beakers was maintained at 25°C. In addition, to attain a RH regime of 100%, one of the beakers was filled with distilled water, whereas a RH regime of 0% was obtained using anhydrous $CaCl_2$. The beakers were held at 25°C for 24 h to permit equilibration of the pollen MC and the RH regime. Regular measurements of pollen weight during storage in the beakers indicated that constant weight, and hence humidity equilibrium, was established within 3 h of exposure to each RH regime, which agrees with the findings of Connor and Towill (1993). After 24 h, the pollen samples were weighed to determine their fresh weight (FW) under each RH regime, followed by oven-drying at 90°C to constant weight (generally achieved within 5 h) to determine the dry weight (DW), as suggested by Connor and Towill (1993). The difference between the FW and DW of each sample was used to calculate the MC (%) of the pollen under each RH regime. Measurements of pollen MC were repeated four times for each RH regime.

Cryogenic storage of pollen

After establishing the relationship between RH and the MC of tomato pollen, additional pollen samples were collected, separated into portions of 2 – 3 mg and stored at RH levels of 0, 20, 30, 40, 50, 60, 70, 80 and 100% using H_2SO_4 solutions as described above. Following equilibration, each pollen sample was placed in a 5 ml glass tube, which was sealed and immersed immediately in liquid N_2, as suggested by Nath and Anderson (1975). The cooling rate was approx. 100°C min^{-1} (Towill, 1985). After 5 d of cryogenic storage, the glass tubes were removed from the liquid N_2 and placed in a water bath at 30°C for 15 s. The pollen samples were allowed to equilibrate to 100% RH in air, then used for *in vitro* germination measurements. The data obtained were used to determine the range of pollen MC values (%) during cryogenic storage that did not inhibit germination.

Following this, fresh pollen was collected on three subsequent dates, separated into portions, equilibrated under RH regimes of 50%, 60% and 65% and stored at –196°C, as described above. At regular intervals, pollen samples were removed from storage, brought to room temperature, divided into two sub-samples and tested for *in vitro* germination. One sub-sample was tested at the actual pollen moisture status, while the second was tested after pollen rehydration (establishment of moisture equilibrium at 100% RH). Furthermore, some pollen from each sampling date was reserved for pollination tests to assess its fecundity.

In vitro *germination of pollen*

The *in vitro* germination of pollen was performed in Petri dishes containing a semi-solid culture medium consisting of 1% (w/w) agar, 15% (w/w) sucrose, and 50 mg l^{-1} boric acid, according to Maisonneuve and Den Nijs (1984). Pollen grains were scattered over the surface of the substrate with the aid of a brush and the Petri dishes were then sealed and incubated at 16°C for 6 h. At the end of this time, germination of pollen was stopped by spraying with acetocarmine. Subsequently, 300 pollen grains per dish were selected at random and germination was recorded with the aid of an optical microscope (10 × 10). Six dishes (replicates) were used for each germination test. Germination was defined as pollen grains in which the pollen tube was equal to, or longer than the diameter of the grain, as suggested by Towill (1987). Germination data were subjected to arcsin transformation prior to statistical analysis. In addition, pollen tube length, after 6 h of incubation, was measured in 20 randomly selected pollen grains per replicate with the aid of the optical microscope.

Pollination procedures

The fecundity of pollen that had been conditioned to three MC levels and stored cryogenically for 10, 14, or 22 months, was tested by pollinating open flowers of the same inbred line ('Paxoi'). The pollen was rehydrated prior to pollination. The percentage of successful pollinations and the number of seeds per fruit were used as evaluation criteria. The number of seeds per fruit may be influenced by the sequence of inflorescence induction on the plant as well as by the sequence of flowers within the inflorescence (Rylski, 1979). To exclude these sources of variation, flowers located at the same position on each plant were used for each pollination test. Thus, pollen from the basal flowers of the first inflorescence was collected for storage in liquid N_2 for 10 or 22 months, and pollen from the fourth inflorescence was collected for storage in liquid N_2 for 14 months. The number of pollinations per treatment varied from three to ten, depending on the availability of cryogenically-stored pollen. Each plant was used for only one pollination.

Flowers were emasculated at the stage of partial flower opening, pollen was then applied to the stigma with the aid of a brush, and the pollinated flowers enclosed in paper bags to prevent unintended pollination from neighbouring plants. After visible fruit set, the bags were removed and the fruits were allowed to grow until fully ripe. A comparable number of fruit set by means of natural pollination was also allowed to develop, as controls, to compare the numbers of seeds per fruit. To achieve a continual supply of flowers at the first-to-fourth truss stage, tomato seedlings were planted successively throughout the experiment.

Respiration of germinating pollen

The respiration of fresh and cryogenically-stored pollen was measured manometrically using a Warburg apparatus (Umbreit *et al.*, 1964). Each flask contained 1.7 ml medium [12% (w/w) sucrose and 50 mg l^{-1} H_3BO_3] plus 0.5 ml 0.1 M KOH in the well to bind CO_2. Samples (10 mg) of pollen, that had previously been equilibrated at 100% RH at 15°C, were added to the medium and incubated *in vitro* at 16°C for 400 min. Respiration was expressed in terms of total O_2 consumption in µl. Measurements of respiration were made in both fresh and cryogenically-preserved pollen (–196°C for 180 d or 390 d). Prior to cryogenic storage, the tomato pollen had been equilibrated in a 60% RH atmosphere at 25°C, resulting in a MC of 9.5% (on a FW basis). Means were calculated on the basis of four pollen samples (replicates).

RESULTS

Non-linear regression revealed an exponential relationship between the RH of the air and the MC of the tomato pollen, following equilibration of pollen and air (Figure 1). This relationship was highly significant (R^2 = 0.996).

The percentage of *in vitro* germination of tomato pollen stored at –196°C for 5 d was markedly affected by the MC of the grains (Figure 2). Germination was highest (56.8 – 57.4%) in pollen with an MC level of 6.8 – 9.3%. Higher and lower pollen MC levels resulted in reduced germination after storage for 5 d in liquid N_2. Pollen grains with a high MC (53%), following equilibration in a saturated atmosphere (RH 100%), showed a pronounced decrease in germination, which fell to 10.7%. Fresh, unstored pollen, which had a MC of 10.6%, exhibited a germination rate of 52.7%, which was significantly lower than that of slightly desiccated, stored pollen (MC 6.8 – 9.3%).

Tomato pollen was capable of maintaining its initial germination ability for at least 660 d of storage in liquid N_2, when its MC prior to cryopreservation had been conditioned to between 6.8 – 11.2%, provided that it was rehydrated after thawing (Figure 3). However, the *in vitro* germination ability of pollen that had not been rehydrated after thawing exhibited a progressive decline with an increasing duration of cryopreservation, regardless of its MC prior to being placed in liquid N_2. Furthermore, the relative length of the pollen tubes (expressed as the ratio of the length of the tube to the diameter of the pollen grain), was not affected by MC during storage in liquid N_2, or by the duration of cryopreservation (Figure 4).

Cryogenically-stored tomato pollen, with a MC of 6.8% or 9.3% prior to storage, retained the ability to pollinate tomato flowers in the greenhouse and to produce fruit for a period of 22 months (Table I). The resulting fruits were comparable in seed number to those derived from natural pollination with fresh pollen. However, pollen conditioned to a MC level of 11.2% prior to cryopreservation exhibited a reduced ability to successfully pollinate fruits, and formed significantly fewer seeds per fruit compared to pollen with MC levels of 6.8% and 9.3%. Seeds originating from pollination with cryogenically-stored pollen exhibited germination percentages exceeding 99% when subjected to germination tests (data not shown).

The cumulative oxygen uptake by fresh pollen, or pollen that had been cryogenically stored for 6 or 13 months, increased linearly with time, over 400 min of incubation (Figure 5). The linear relationships corresponding to the three different pollen treatments were almost identical.

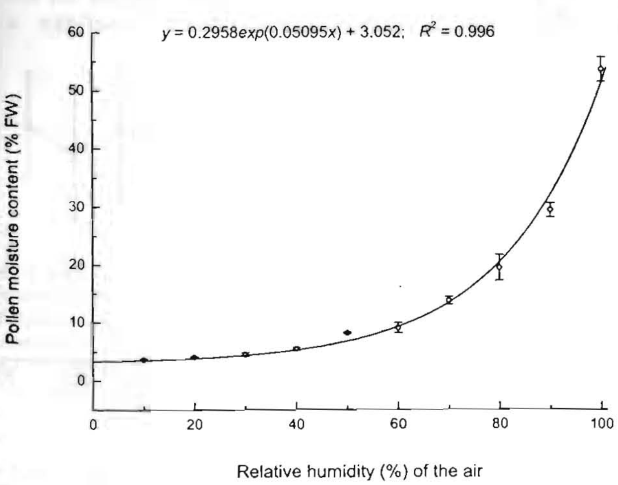

FIG. 1
Relationship between relative humidity of the air and moisture content of tomato pollen at moisture equilibrium. The points indicate means of four measurements, while the vertical bars depict ± standard errors. The solid line represents the best fit regression curve.

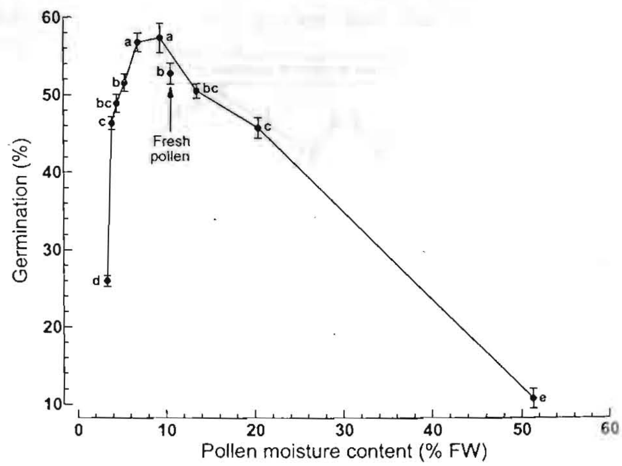

FIG. 2
In vitro germination of fresh tomato pollen (single marked value) and pollen stored at –196°C for 5 d (values connected by a solid line) as influenced by moisture content. Vertical bars indicate ± standard errors of means (n = 6). Values followed by the same lower-case letter do not differ significantly at P = 0.001 according to the Student-Newman-Keuls' Multiple Range Test.

TABLE I

Percentage of successful pollinations and number of seeds per fruit in tomato as influenced by moisture content and duration of cryogenic storage (CS) of the pollen

Moisture content of pollen (% w/w)	CS for 10 months		CS for 14 months		CS for 22 months	
	Successful pollinations (%)	Seeds per fruit	Successful pollinations (%)	Seeds per fruit	Successful pollinations (%)	Seeds per fruit
Fresh pollen (10.6%)	–	197.0 a	–	152.0 a	–	222.2 a
6.8	100	286.4 a	100	157.0 a	–	–
9.3	100	180.0 a	100	132.0 a	100	236.6 a
11.2	100	281.0 a	80	37.0 b	90	81.3 b

In each column, values followed by the same lower-case letter do not differ significantly at $P = 0.05$ according to the Student-Newman-Keuls' Multiple Range Test.

This response indicates that the rate of respiration of germinating pollen that had been stored in liquid N_2 for at least 13 months was similar to that of fresh pollen.

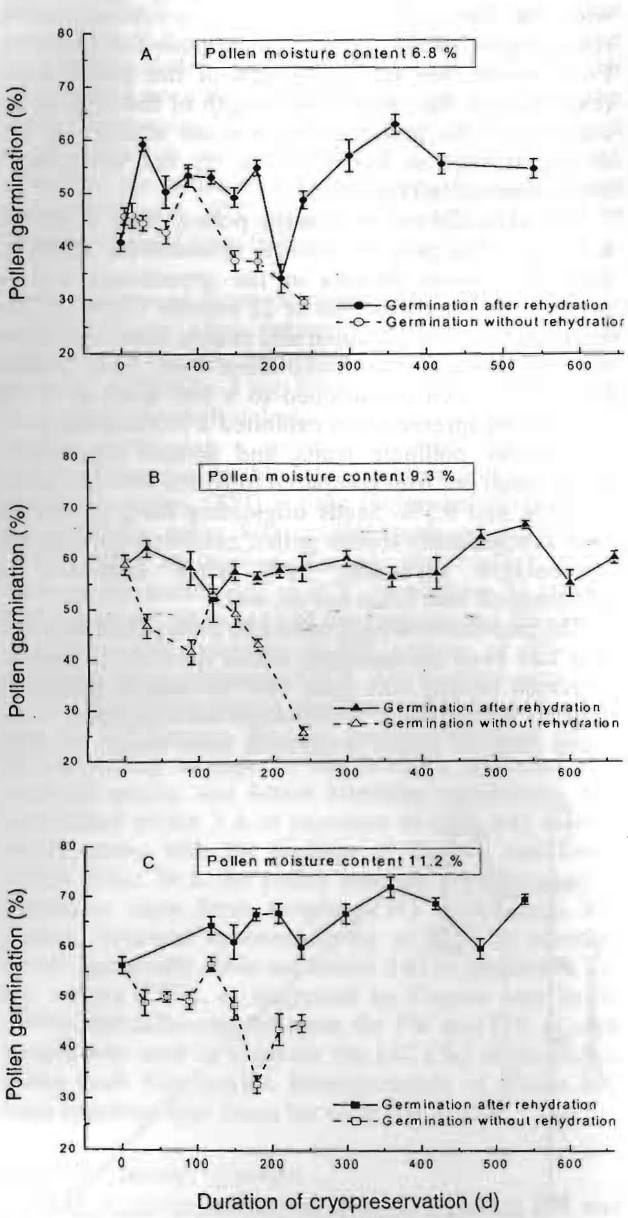

FIG. 3

Changes in germination ability (% germination) of tomato pollen during storage in liquid nitrogen (–196°C) for 660 d as influenced by rehydration after thawing. The moisture content (FW basis) of the pollen had been equilibrated to 6.8% (Panel A), 9.3% (Panel B) or 11.2% (Panel C) in air at 50%, 60%, and 65% relative humidity, respectively. Vertical bars indicate ± standard errors of means (n = 6).

DISCUSSION

The results of the present study clearly indicate that tomato pollen may be stored at cryogenic temperatures without adverse effects on its ability to germinate. These results are consistent with the observations of Sacks and St Clair (1996), who reported successful storage of tomato pollen at –80°C for 5 weeks. The ability of pollen to survive after storage at cryogenic temperatures has been confirmed for many plant species (Hanna and Towill, 1995). However, the MC of the pollen during storage is crucial for its germinability after thawing. Our results revealed that the MC of tomato pollen during cryopreservation should be between 6.8 – 9.3% to obtain maximum germination, whereas pollen with lower or higher MC levels showed a reduction in germination ability during cryogenic storage. Adjustment of the MC of tomato pollen to 6.8 – 9.3%, prior to cryogenic storage, enhanced its germination ability, compared even to untreated, fresh pollen with a moisture content of 10.6%. The importance of partial desiccation of pollen prior to cryopreservation was also emphasised by Luza and Polito (1988), who found that pollen of English walnut (*Junglans regia* L.) was unable to survive storage in liquid N_2 if its MC was >7.5%. On the other hand, the pollen of *Glycine* spp. could be stored successfully at –196°C without prior desiccation (Tyagi and Hymowitz, 2003); while, the pollen of some species (e.g., *Rosa* sp., *Dioscorea* spp.) was susceptible to excessive dehydration prior to cryopreservation (Marchant *et al.*, 1993; Daniel *et al.*, 2002).

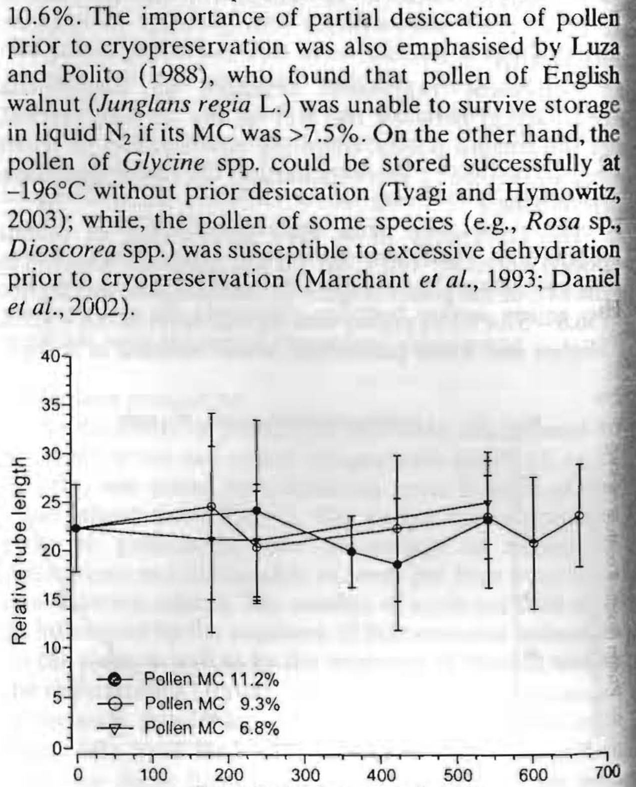

FIG. 4

Relative length (pollen tube length:pollen grain diameter ratio) of pollen tubes formed from tomato pollen after *in vitro* germination for 6 h as influenced by the duration of cryogenic storage and the moisture content (MC) of the pollen prior to placement in liquid nitrogen. Vertical bars indicate ± standard errors of means (n = 6).

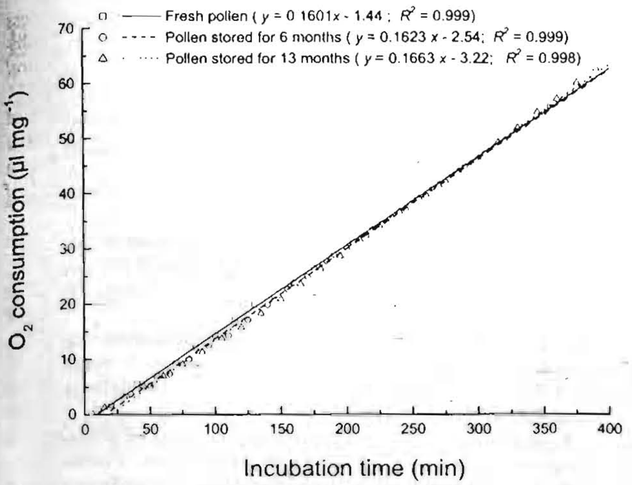

FIG. 5
Cumulative O_2 consumption (in µl mg^{-1} fresh pollen) by germinating tomato pollen as influenced by storage in liquid nitrogen for 0, 6 or 13 months. Symbols depict measured values, while lines indicate the linear relationships between incubation time and the total amount of O_2 consumed.

The optimal MC for cryogenically-stored tomato pollen (6.8 – 9.3%) may be achieved by placing the pollen grains in air with a RH of 50 – 60% for 3 h. Nevertheless, as pointed out by Towill (1985), Van der Walt and Littlejohn (1996), Metz *et al.* (2000), Parton *et al.* (2002), and Martinez-Gómez *et al.* (2002), a prerequisite for slightly dehydrated pollen to exhibit a high germination ability after cryogenic storage was to rehydrate the grains after thawing. Our results support this statement (Figure 3). In contrast, Visser (1955) did not rehydrate tomato pollen after storage in liquid N_2, and observed a reduction in germination from 47% to 35%.

The biological value of cryogenically-stored pollen depends not only on its germinability, but also on its ability to set viable seeds. Parton *et al.* (2002) reported reduced seed set after pollination of *Vriesea* 'Christiane' with *Vriesea* 'Leen' pollen from anthers stored at –80°C, compared to pollination with fresh pollen of the same species. Furthermore, broccoli pollen stored in liquid N_2 retained its viability, but the seeds obtained from such pollen exhibited reduced vigour and rapidly lost germinability (Crisp and Grout, 1984). Hence, when testing the ability of pollen from a particular plant species to be stored at cryogenic temperatures, it is also essential to assess the capability of the cryopreserved pollen to set fruit and to produce viable seeds.

In the case of tomato, our results indicate that the ability of tomato pollen to set fruit and viable seeds was not adversely affected by storage in liquid N_2, if the MC of the pollen is 6.8 – 9.3% during cryopreservation. This result agrees with those of a previous report (Sacks and St Clair, 1996). However, cryogenic storage of tomato pollen with a higher MC may reduce its viability, as indicated by the lower seed number in fruits obtained using pollen with a MC of 11.2% that had been stored cryogenically for more than 6 months. The number of seeds per fruit correlates with fruit size in tomato (Rylski, 1979). Thus a reduced seed number should normally be accompanied by a lower mean fruit weight. Nevertheless, since cryogenically-stored pollen is used for breeding rather than for fruit production, the impact of cryopreservation of tomato pollen on fruit size is relatively immaterial.

The similar respiration rate of germinating fresh pollen and that of pollen stored for 6 or 13 months under cryogenic temperatures (Figure 5), and the similar lengths of pollen tubes observed after germination for 6 h, indicate that storage at –196°C does not affect the germination process of tomato pollen. This is due to the fact that placing the pollen at cryogenic temperatures immediately and completely blocks cellular metabolism (Mazur, 1984; Towill, 1985; Bajaj, 1987). Exposure of pollen to such low temperatures inhibits enzymatic processes so rapidly that the intermediates of enzyme reactions may be detected in cells (Douzou, 1977; Fink, 1977; Jaenicke, 1981).

In conclusion, tomato pollen can be stored in liquid N_2 (–196°C) for at least 22 months without any significant loss in germinability or viability, provided its MC is adjusted to 6.5 – 9.3% prior to exposure to the cryogenic temperature, and the pollen is rehydrated after thawing. The desired MC level may be attained by placing the pollen in a RH regime of 50 – 60% until equilibrium is established between the pollen and the air, which normally occurs within 3 h.

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