

# Prolonged Cold Storage Affects Pollen Viability and Germination along with Hydrogen Peroxide and Nitric Oxide Content in *Rosa hybrida*

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## Abstract

Roses (*Rosa hybrida*) are the most important ornamental cut-flowers and breeders' main focus is to develop new desirable modern cultivars. Rose breeding programs center on the introduction of new flower colors, thornless stems, higher production and good post-harvest performance. The study of the main pollen traits, such as pollen quantity and quality, viability, longevity, morphological homogeneity, germination and tube growth, is important for building suitable breeding programs. Recently, a number of studies have shown that reactive oxygen species, like hydrogen peroxide, and nitric oxide, are involved in a wide range of signaling processes including pollen tube growth and pollen-pistil recognition. Pollen viability after anther dehiscence is crucial for successful crossbreeding. In the present work, pollen grains from 5 hybrid tea rose cultivars were stored at -20 °C up to 12 months. Pollen viability and germination rate was monitored in order to provide useful information about pollen storage length. Additionally, pollen grains were tested for their content in hydrogen peroxide and nitric oxide by using a novel approach where the fluorescence is read in a quantitative RealTime PCR (qRT-PCR) machine. Pollen viability and *in vitro* pollen germination capacity varied among the rose genotypes, while a progressive decrease was evidenced during 12 months of storage at low temperature. Both hydrogen peroxide and nitric oxide production were found to be genotype-dependent, whilst accumulation of the two molecules was observed during the storage period. A putative detrimental effect of these molecules during pollen conservation is hypothesized.

**Keywords:** hybrid tea rose, pollen conservation, pollen grains, reactive nitrogen species (RNS), reactive oxygen species (ROS)

## Introduction

Rose breeding programs have a long history throughout centuries (Scariot *et al.*, 2006; De Cock *et al.*, 2007). Nowadays, the breeding programs are mainly focused on new desirable cultivars with novel flower colors, thornless stems, higher production, and prolonged post-harvest performance, but some major problems related to poor seed production and germination are frequently encountered (Pipino *et al.*, 2011a; Pipino *et al.*, 2012; Caser *et al.*, 2014; Bosco *et al.*, 2015). Cultivated roses often show reduced pollen viability resulting from male meiotic or post-meiotic aberrations (Jacob and Ferrero, 2003). To avoid the risk of very low seed production, breeders commonly use high pollen quantities from a limited number of male parents chosen for their known fertility and

often need to overcome asynchrony in flowering among crossing parents by storing pollen (Zlesak 2006; Pipino *et al.*, 2011b).

Pollen viability is quickly lost if grains are left unprotected at room temperature; though, when stored at cold temperatures, pollen viability is kept for a longer period of time which varies among cultivars (Giovannini *et al.*, 2015). Hence, a key aspect in breeding programs is linked to pollen conservation along with the identification of parameters related to pollen fertility and viability. This is of utmost importance and can aid to increase the overall efficiency of breeding programs.

Pollen preservation is dependent on aging and, consequently, some common features can be envisaged with the seed system, in which most of the damage associated with aging results from reactive oxygen (ROS) and reactive nitrogen species (RNS) production during seed desiccation and prolonged storage

(Donà *et al.*, 2013a). Besides, ROS and RNS are key components in seed signal transduction pathways, and it has been hypothesized that seed germination can be completed only when the ROS content is maintained under a critical threshold that allows the induction of ROS-mediated signaling mechanisms (Balestrazzi *et al.*, 2011; Ventura *et al.*, 2012). Therefore, several tools tested on seeds could be expanded also for pollen investigations. Recently, it has been shown that nitric oxide (NO), a main constituent of RNS, is involved in plant reproductive processes (Zafra *et al.*, 2010). Moreover, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), a key player among ROS, has a role to play in pollen tube growth and pollen-pistil interactions (Speranza *et al.*, 2012). However, the elucidation of the roles played by these molecules within the plant gametes is still incipient.

In the present study, pollen grains collected from five hybrid tea rose cultivars were stored at -20 °C up to 12 months. Pollen viability and germination rate were monitored in order to provide breeders with useful information about pollen storage length. In addition, H<sub>2</sub>O<sub>2</sub> and NO content were measured in fresh and stored pollen grains at indicated time points to evaluate their eventual role as possible viability markers. The acquired information can assist breeders in setting up more effective breeding strategies for hybrid tea rose varieties.

## Materials and Methods

### Plant material

Pollen grains from five commercial *Rosa hybrida* cultivars ('Dolphin', 'Golden Fashion', 'New Fashion', 'Swan' and 'Touch of Class') were provided by NIRP International (Bevera, Imperia, Italy). Anthers were placed at room temperature in open petri dishes to favor pollen release and drying. After two days, fresh pollen was used either for further analysis or stored at -20 °C for a total of 12 months. Intermediate analyses were performed at 3, 6 and 12 months of conservation.

### Pollen viability and germination rate

Pollen viability was assessed by TTC (Tryphenil Tetrazolium Chloride) staining as previously reported (Eti, 1991). *In vitro* pollen germination was assessed on PGM medium (40 mg L<sup>-1</sup> H<sub>3</sub>BO<sub>3</sub>, 152 mg L<sup>-1</sup> CaCl<sub>2</sub>(H<sub>2</sub>O), 150 g L<sup>-1</sup> sucrose, 7 g L<sup>-1</sup> agar, pH 5.6) (Leus, 2005). Samples were evaluated after 24 h incubation at 22±2 °C under dark conditions. Pollen grains were considered as germinated when the pollen tube reached a length of at least 1.5 times its diameter (Leus, 2005). The observations were performed using a Reichert Biovar microscope (Reichert, Germany) (100×), with incorporated Olympus C-5000 photocamera. All analyses were performed in triplicate (100 pollen grains scored for each slide).

### Detection of hydrogen peroxide and nitric oxide

H<sub>2</sub>O<sub>2</sub> and NO levels were measured by staining the pollen grains with dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Milan, Italy) and diamino fluorescein diacetate (DAF<sub>2</sub>-DA, Sigma-Aldrich), respectively (Pasqualini *et al.*, 2011). The pollen (1 mg) was incubated for 15 min with 10 μM DCFH-DA, and for 30 min with 10 μM DAF<sub>2</sub>-DA. Relative fluorescence was determined at λ = 517 nm in a Rotor-Gene 6000 PCR apparatus (Corbett Robotics, Brisbane, Australia), setting the program for one cycle of 30s at 25 °C. As a positive control, pollen grains were subjected to heat shock (10 min,

65 °C). The relative fluorescence (represented as RFU) was calculated by normalizing the samples to controls. Photos were taken using a ZEISS Axioplan microscope equipped with a CCD (Computer Coupled Device) videocamera (Photometrics, Arizona, USA). All analyses were performed in triplicate.

### Statistical analysis

Arcsine transformation was performed on all percent incidence data before statistical analysis in order to improve homogeneity of variance. Effects of genotype and storage duration on the analysed traits were evaluated by one-way ANOVA using Ryan-Einot-Gabriel-Welsch's multiple stepdown F (REGW-F) test (p ≤ 0.05). All analyses were performed with SPSS 21.0 Inc. software (Chicago, Illinois, USA).

## Results and Discussion

### Changes in pollen viability and germination rate during storage at -20 °C

For this study, five cultivars ('Golden Fashion', 'Dolphin', 'Swan', 'Touch of Class', 'New Fashion') with high fertility pollen, based on their diameter larger than 30 μm (Pipino *et al.* 2011b), were selected; these cultivars were also suggested by NIRP International, provider of the used material, as cultivars with high potential for breeding programs. The pollen aspect during TTC coloration and pollen grain germination is shown in Fig. 1A and B, respectively. The changes in pollen viability and germination rate during one year of storage at -20 °C are presented in Fig. 1C and D. In fresh pollen (month 0), the pollen viability ranged from a minimum of 72.3% in 'Golden Fashion' to a maximum of 83.3% in 'Dolphin', without significant differences. After 3 months of conservation only a slight decrease (from 81.2% to 71.0%) in pollen viability belonging to 'Swan' cultivar was observed. In the other cultivars, the pollen viability strongly decreased between 3 and 6 months of conservation, reaching the lowest values at 12 months, with the lowest reduction (~ 65%) in 'Dolphin' and 'Touch of Class' and the highest (~ 86%) in 'Swan' (Fig. 1C). When the fresh pollen germination rate was investigated, none of the tested cultivars presented a germination percentage above 50% (Fig. 1D). The pollen germination rate ranged from 26.8-27.1% in 'New Fashion' and 'Touch of Class' to 46.4-47.0-49.0% in 'Swan', 'Golden Fashion' and 'Dolphin', respectively. After 3 months only a slight decrease in 'New Fashion' (from 26.8% to 21.0%) was observed. Similarly to pollen viability, the conservation at low temperature affected the germination rate between the months 3 and 6. The lowest percentages were counted after 12 months with the lowest reduction (~ 75%) in 'Touch of Class' (from 27.1% to 6.7%, respectively) and the highest (~ 93%) in 'Golden Fashion' and 'Swan' (from 47.0% to 3.3% and from 46.4% to 3.3%, respectively). Similar findings were recorded in *R. dumalis* and *R. villosa* (Ercisili, 2007), as well as in other six *R. hybrida* commercial cultivars (Giovannini *et al.*, 2015). The decrease in pollen viability during storage condition may be due to dehydration, resulting in loss of pollen colloidal properties (Pacini, 2000; Franchi *et al.*, 2011).

### Hydrogen peroxide and nitric oxide production during storage at -20 °C

The levels of H<sub>2</sub>O<sub>2</sub> and NO in hybrid tea rose pollen grains were measured using the standard technique of DCFH-DA and

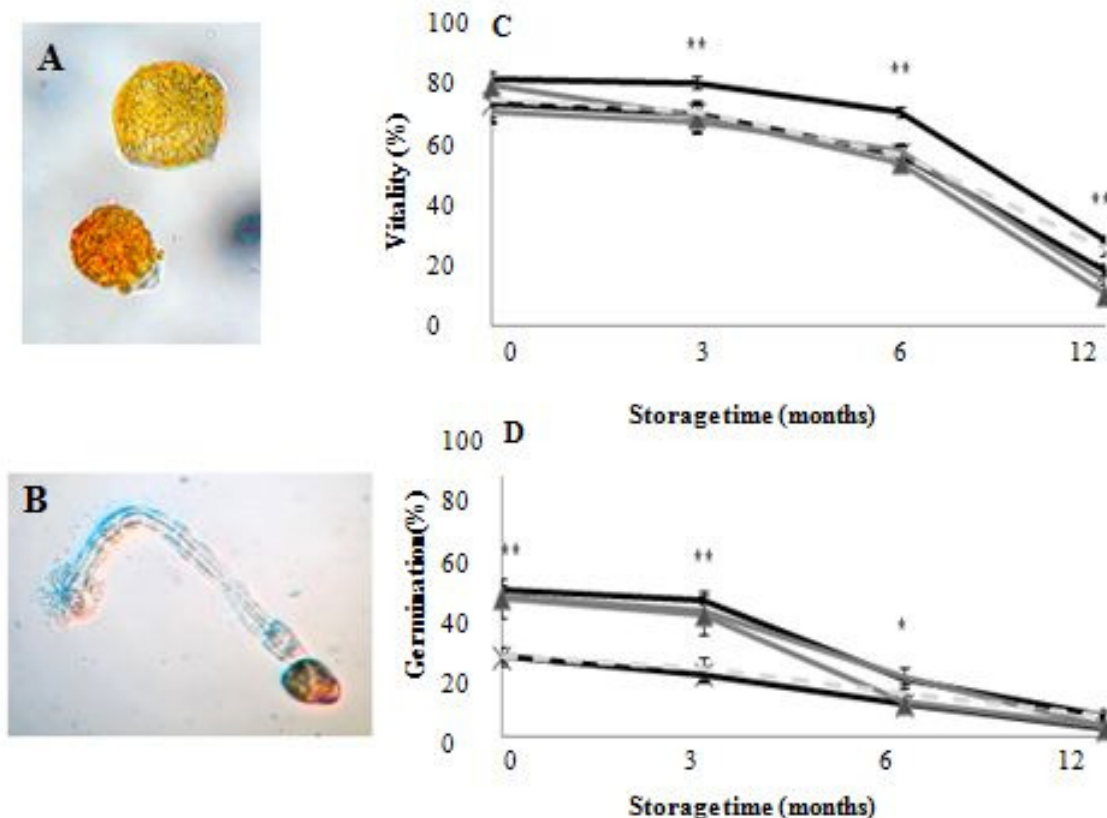


Fig. 1. Evaluation of hybrid tea rose pollen viability and germination during cold storage. (A) Pollen grains stained with TTC. (B) Germinated pollen grain. (C) Dynamics of pollen viability (C) and germination rate (D) in 5 selected hybrid tea rose cultivars ('Dolphin', solid black line; 'New Fashion', solid black line with X; 'Golden Fashion', solid dark grey line; 'Swan', solid dark grey line with ▲; 'Touch of Class', dotted light grey line). Asterisks indicate the significance of differences between genotypes as determined by one-way ANOVA and REGW-F comparison tests (\* =  $P < 0.05$ , \*\* =  $P < 0.001$ ). Vertical bars indicate  $\pm$  standard error,  $n = 5$ .

DAF2-DA staining, but with improvements regarding the detection of fluorescence by using a qRT-PCR machine (Donà *et al.*, 2013b). Additionally, images of stained pollen grain with DCFH-DA and DAF2-DA are shown in Fig. 2A and B, respectively. The results showed that the  $H_2O_2$  and NO content varied among the tested cultivars (Fig. 2C and D). In fresh pollen (month 0),  $H_2O_2$  varied significantly among cultivars (Fig. 2C). After 3 months of conservation a significant increase in  $H_2O_2$  and NO levels were observed in 'New Fashion' and 'Dolphin'. Later, at 6 and 12 months, the highest values appeared in 'Touch of Class' and 'Dolphin' (7.4- and 10.6-fold, 4.6- and 5.5-fold, respectively, as compared with the fresh pollen content). In the other cultivars,  $H_2O_2$  content kept constant during the experiment without significant variations. Concerning the NO content, in fresh pollen (month 0) significant differences were observed among cultivars, while no variations were found after the first 3 months of storage in all the studied cultivars (Fig. 2D). On the other hand, at 6 and 12 months a significant increase, between 2- to 4-fold, was observed in 'Touch of Class', 'Dolphin', and 'Swan'. The other cultivars kept constant content during all the experiment without significant differences.

In this study the  $H_2O_2$  and NO production resulted to be cultivar dependent, with high levels of accumulation after 6 months of storage, mostly in 'Touch of Class' and 'Dolphin'. In

addition, their levels appear to have an interrelation with the loss of pollen viability and germination during cold storage conditions. Similarly, when the pollen of *Paeonia suffruticosa*, *Paulownia tomentosa* and *Ambrosia artemisiifolia* were exposed to ultraviolet radiations (UV-B) or ozone ( $O_3$ ) fumigation,  $H_2O_2$  and NO generation was detected and correlated with a reduction of in vitro pollen germination and tube growth (He *et al.*, 2006; Pasqualini *et al.*, 2011).

### Conclusion

Our study indicates that hybrid tea rose pollen can be efficiently stored at  $-20\text{ }^\circ\text{C}$  up to 3 months. Longer storage periods affected pollen viability and germination, with the highest impact after 12 months of storing. Thus, our study provides key information for rose breeders regarding rose pollen storing time. Moreover, the  $H_2O_2$  and NO content could represent a marker to assess the loss of pollen viability and germination during cold storage conditions; however, further studies which include more cultivars are needed to validate this result. Future investigations pointed to elucidate the crosstalk between ROS and RNS and define improved storage methods suitable for prolonged pollen longevity are still required.

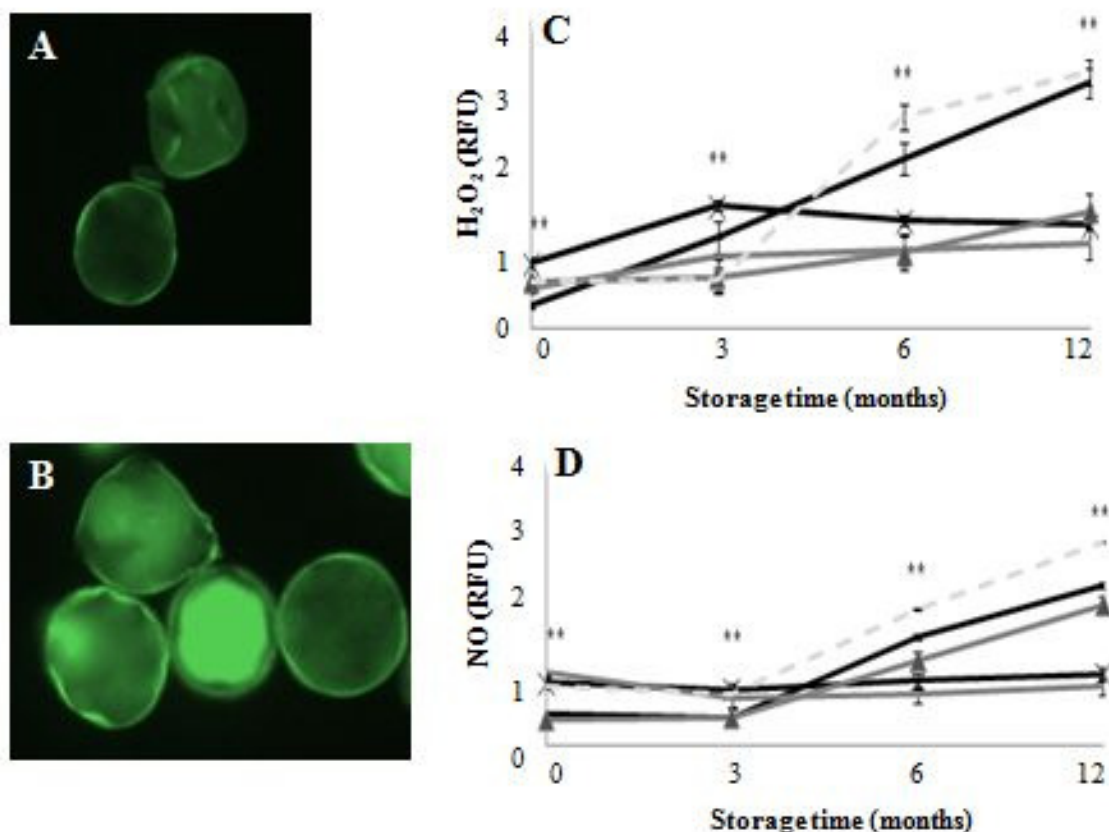


Fig. 2. Evaluation of hybrid tea rose pollen viability and germination during cold storage. (A) Pollen grains stained with TTC. (B) Germinated pollen grain. (C) Dynamics of pollen viability (C) and germination rate (D) in 5 selected hybrid tea rose cultivars ('Dolphin', solid black line; 'New Fashion', solid black line with X; 'Golden Fashion', solid dark grey line; 'Swan', solid dark grey line with ▲; 'Touch of Class', dotted light grey line). Asterisks indicate the significance of differences between genotypes as determined by one-way ANOVA and REGW-F comparison tests (\* =  $P < 0.05$ , \*\* =  $P < 0.001$ ). Vertical bars indicate  $\pm$  standard error,  $n = 5$ .

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