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SHORT COMMUNICATION

Effects of cryopreservation on germinability of olive (*Olea europaea* L.) pollen

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Abstract Long term storage of viable pollen is important for bank germplasm constitution to preserve resources that can be used in breeding programs, biotechnologies and genetic engineering. Pollen from 12 olive (*Olea europaea* L.) cultivars was stored for 1 year in liquid nitrogen at −196°C. The morphology of pollen grains and germination rates on fresh and long term stored pollen were observed. Results on in vitro pollen germinability, both before and after cryopreservation, showed highly significant responses among the 12 cultivars. The relationship between germinability and pollen grain size did not reveal any significant relationships in both treatments. Our findings will contribute to the improvement of olive pollen preservation and lead to find a more efficient method for its long term storage, while retaining its germinability.

Keywords Cryopreservation · *In vitro* pollen germinability · *Olea europaea* L. · Pollen grain size

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Introduction

Long term storage of viable pollen is important for bank germplasm constitution so that it is possible to preserve resources that can be used in breeding programs, biotechnologies and genetic engineering (Vergano et al. 1994; Alba et al. 2000; Piotto et al. 2010). As reported by Engelmann (2004) cryopreservation, the storage of biological material at ultra low temperature, usually that of liquid nitrogen (−196°C), is the only technique currently available to ensure the safe and cost-efficient long-term conservation of different types of germplasm. At this temperature, all cellular divisions and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time. Moreover, it is stored in a small volume, protected from contamination and requires a very limited maintenance. Pollen, which is an interesting material for genetic resource conservation of various species, is stored by several Institutes around the world. In particular the NBPGR

(India) conserves cryopreserved pollen of 65 accessions belonging to different species, the Indian Institute for Horticultural Research (IIHR, Bangalore) conserves pollen of 600 accessions belonging to 40 species from 15 different families and in the USA, the NCGRP conserves pollen of 13 pear cultivars and 24 *Pyrus* species.

In Italy, a large-scale utilization of cryopreservation for pollen storage is still limited due to the poor knowledge on the physiological, structural, functional aspects of pollen grains and in particular the timing and methods of sampling that characterize different plant species.

There are some disadvantages in pollen grains storage as the small amount produced by many species, the lack of transmission of organelle genomes via pollen, the loss of sex-linked genes in dioecious species and the general inability to regenerate into plants (Hoekstra 1995). On the contrary an advantage is that pests and diseases are rarely transferred by pollen grains. This allows safe movement and exchange of germplasm as pollen. However, few papers have been published to assist genebank curators in the establishment and management of long term pollen storage. Because of its high potential, it is expected that pollen cryopreservation will become more frequently employed for long term conservation of plant genetic resources. For this purpose it needs more studies to get information on *in vitro* pollen grain germinability in different species which are influenced by storage length, storage temperatures and genotypes (Ferri et al. 2008).

That being stated a study on morphology and size of olive (*Olea europaea* L.) pollen grains in different Italian cultivars and a comparison between in vitro germination rates on fresh pollen and after a long term storage in liquid nitrogen was carried out.

Materials and methods

In vitro germination rates were assessed on pollen grains of 12 olive cultivars: 'Carolea', 'Cima di Melfi', 'Coratina', 'Faresana', 'Fasolino', 'Gremignolo Bolgheri', 'Leccino', 'Manzanilla', 'Nocellara Belice', 'Nociara', 'Palmarola', 'Rotondella'. Samples of olive flowering branches of the selected cultivars were collected in the experimental fields of "Incoronata" farm, Melfi di Potenza (Basilicata region), at the end of May and early June 2007. The single flowers picked from inflorescences were stored for dehydration 24 h at room temperature in Petri dishes with Silica Gel. For each cultivar two samples of pollen grains, extracted from dehiscent anthers, were placed in polypropylene vials and stored for cryopreservation at −196°C in liquid nitrogen (LN_2) for 365 days after full bloom. According to Pinney and Polito (1990) olive pollen grains were rehydrated about 3 h in a controlled environment at +20°C and 95% relative humidity (RH) prior to being assayed for germination; *in vitro* growth of pollen tube was adopted to evaluate pollen grain germinability. Pollen was germinated in 15 × 60 mm Petri dishes containing 10 ml of an agar-solidified growing medium: agar 1%, sucrose 10%, boric acid (H_3BO_3) 100 ppm and calcium chloride ($CaCl_2$) 1 mM. Calcium has an essential role in pollen germination and pollen tube growth (Brewbacker and Kwack 1963). Pollen grains were sprinkled in a fine layer over the surface of the growing medium and incubated for 24 h in dark at 21°C; according to Reale et al. (2006) they were considered germinated when the pollen tubes were longer than the diameter of the pollen grains. The pollen was observed by a light microscope and the germination rates were determined on 100 pollen grains. Similarly pollen grains of each genotype, stored 1 year in liquid nitrogen at −196°C, were again tested again under the same conditions to assess *in vitro* germination rates after the treatment. Polar axis (P), equatorial axis (E) and areas of pollen grains were determined by a He/Ne laser scanning microscope at 543 nm with a scanning time of 64 s and a morphometric image analysis program 1.38. No pretreatment was made on pollen grains; they were only dipped overnight in distilled water, causing a strong hydration, and then examined under the laser scanning microscope. For each cultivar 70 randomly selected pollen grains were measured. Data were subjected to analysis of variance by the MSTAT-C software and the Duncan's multiple range test was made.

Results and discussion

The scanning laser microscopy was used to study the morphology and size of pollen grains of selected

olive cultivars. In all genotypes were found elliptical trizonocolpate pollen grains; Fig. 1a shows the elliptical shaped grains with three distinct furrows along the polar axis. For each cultivar 70 randomly selected pollen grains were measured and results indicated that the mean value of polar axis (P) was 59.66 μm ranging between 54.64 and 64.14 μm, while the mean equatorial axis (E) was 32.05 μm ranging between 30.1 and 35.87 μm. Through morphometric analysis the pollen grains areas were measured for each genotype giving a mean value of 1,498 $\times$ 10^{-12} m^2 and a range from 1,278.73 $\times$ 10^{-12} m^2 in the smallest grains ('Manzanilla') to 1,720.56 $\times$ 10^{-12} m^2 in the biggest ones ('Faresana'); while the remaining olive cultivars showed intermediate values. The relationship between germinability and pollen grain size did not reveal any significant relationships in both treatments: before and after cryopreservation. P/E ratio varied from 1.79 to 1.94 with a mean value of 1.86; then the pollen grains of all studied olive cultivars were classified medium in size and were placed in prolate (elliptical) shape group based on P/E ratio. A previous work by Javady and Arzani (2001) on pollen morphology of five Iranian olive cultivars, using Scanning Electron Microscopy (SEM), reported measures of polar axis (P) ranging between 22.76 and 28.58 μm and mean values of equatorial diameter (E) between 15.95 and 19.14 μm. Isocrono and Vallania (2009) reported mean values between 20 and 30 μm in the polar diameter (P) and between 17 and 20 μm in the equatorial diameter of different Italian olive cultivars. The values of polar and equatorial axes and consequently of areas of pollen grains resulted greater than those reported in previous studies. This could be explained as follows: measurements of pollen grains by Scanning Laser Microscopy don't require any pretreatment except their immersion for 12 h in distilled water. Consequently, the hydration increases pollen grain sizes respect to other techniques which generally cause their coartaction with a strong decrease of polar and equatorial axes and generally of their areas.

The results on *in vitro* pollen grains germinability observed before and after long term storage in liquid nitrogen (Fig. 1b, c) showed highly significant responses among the 12 obseved cultivars of *Olea europaea* L. as showed in Fig. 2. In particular, fresh pollen grains of the 'Carolea' showed the highest in vitro pollen germination rate (84.5%) that decreased

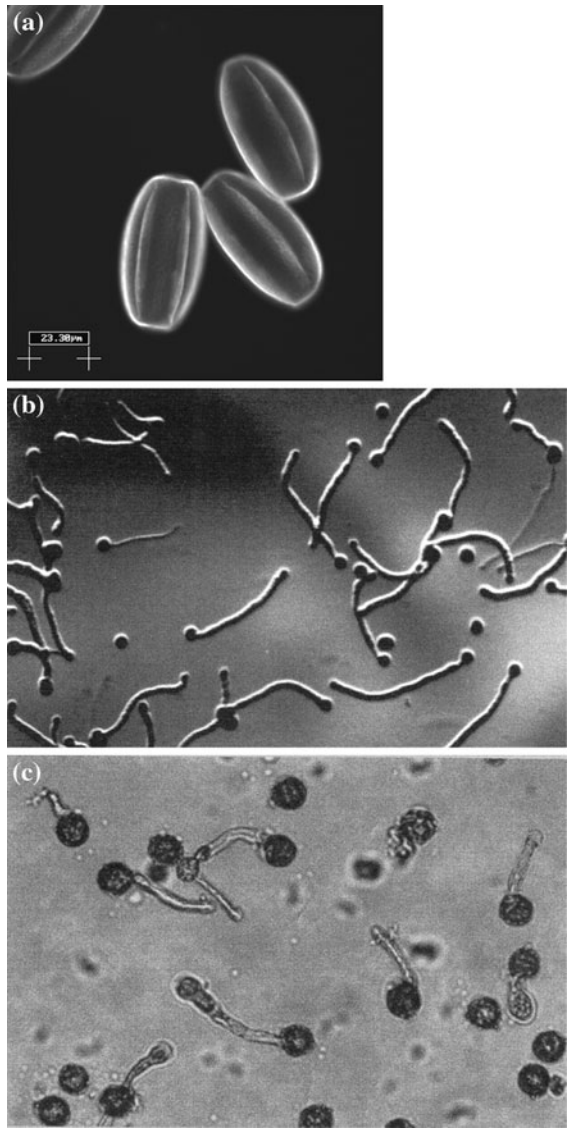

Fig. 1 **a** Olive pollen grains observed under He/Ne laser scanning microscope; **b** germinated pollen grains at flowering time, cv. 'Manzanilla' (5×); **c** germinated pollen grains after cryopreservation, cv. 'Palmarola' (32×)

to 30% after cryopreservation; a similar trend was observed in 'Cima di Melfi' (78.1%), 'Manzanilla' (75.4%), 'Fasolino' (71.2%) and 'Palmarola' (70%) while a strong decrease in these genotypes to 22.3, 33.1, 23.1 and 32.7%, respectively was obtained after the long term storage of pollen in liquid nitrogen. Lower germination rates, ranging between 40 and 60%, not significantly different from each other were observed on fresh pollen grains of the following

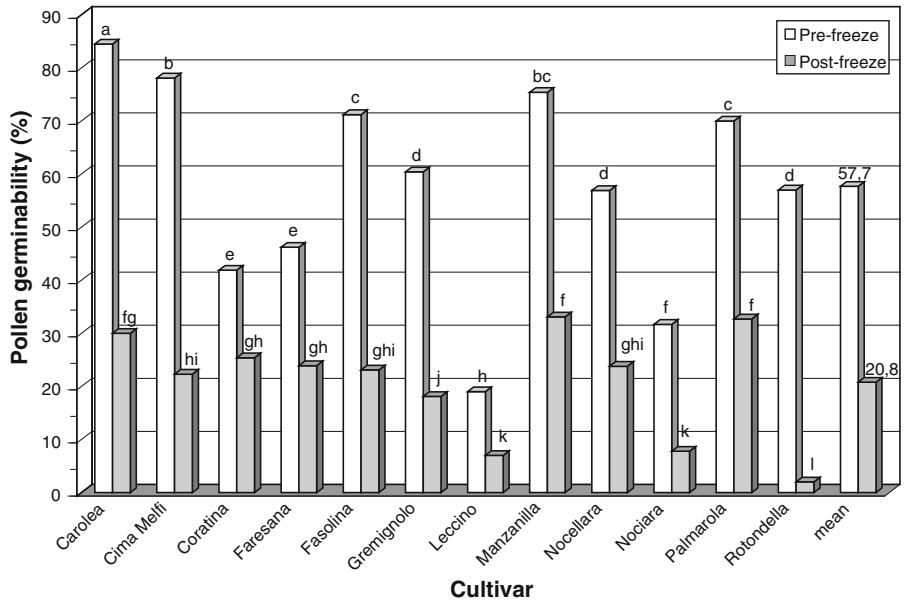

Fig. 2 Pollen germinability (%) at flowering time and after cryopreservation in 12 olive (*Olea europaea* L.) cultivars. Significant differences at $P \leq 0.01$ are indicated by different alphabetic letters (Duncan test)

genotypes: 41.9% in 'Coratina', 46.2% in 'Faresana', 60.4% 'in 'Gremignolo', 56.9% in 'Nocellara' and 'Rotondella', which, after 1 year at −196°C, showed even lower mean values: 25.4, 23.9, 18.1, 23.8 and 2.3%, respectively. So, after the treatment a strong reduction in pollen germinability (about 50%) respect to the fresh pollen was observed. Furthermore the mean value of 2.3% of the genotype 'Rotondella' resulted a very negligible value resulting the lowest pollen germinating rate. Finally pollen grains of cultivars 'Nociara' and 'Leccino' showed a very low *in vitro* germinability: 32.3 and 19.2% on fresh pollen decreased to 8.1 and 6.9% after cryopreservation, respectively.

Over all cultivars the observed average germinability on fresh and stored pollen grains was 57.7 and 20.8%, respectively. A similar trend was observed by Vergano et al. (1994) after 4 years of cryopreserved pollen at liquid nitrogen temperature in different fruit trees and, more recently, by Ferri et al. (2008) which reported *in vitro* germination rates of olive pollen grains stored 35 and 200 days at a temperature of −20°C.

As currently known, several factors affect the *in vitro* germinability of pollen grains: genotype, temperature and time of conservation, composition of growing media (Pinney and Polito 1990; Barnabas and Kovács 1997; Ferri et al. 2008). Furthermore, it is important to underline that pollen viability and germinability, both before and after cryopreservation, depend from the initial conditions of pollen grain in relation to: external temperature and air humidity, grain water content, reserve substances, gamete maturity and their interactions (Franchi et al. 2002; Nepi et al. 2001; Pacini 1996; Pacini and Hesse 2004; Pacini et al. 2006; Speranza et al. 1997; Vesprini et al. 2002).

According to Ganeshan et al. (2008) which reviewed the current status of pollen cryopreservation in most species the genotype effect, stage of pollen beyond complete maturity, physiological status of plant, cold treatment and the methodology followed are the major factors influencing post cryopreservation survival. In most tropical species, dry-mature pollen grains freshly dehisced from anthers are in ideal physiological conditions to be processed for cryopreservation. In addition, the problem of the water content has been studied by Franchi et al. (2002) which classified pollen grains of several species between "partially hydrated pollen", with a water content greater than 30%, like recalcitrant seeds, and "partially dehydrated pollen", with a water content of less than 30%, like orthodox seeds (Kermade and Finch-Savage 2002). Most of pollen of

Gymnosperms and Angiosperms has been found partially dehydrated. In this case pollen resists hostile environments (high temperatures and low relative humidities) better because it is able to maintain its water content in a certain range by mobilizing carbohydrate reserves (Guarnieri et al. 2006). Partially hydrated pollen, especially that with low sucrose and polysaccharide content, is not able to control water content because of its reduced possibility to interconvert carbohydrates and to increase turgor pressure; it quickly loses water and dies (Nepi et al. 2001; Franchi et al. 2002). Unfortunately in literature there are scarce evidences regarding olive pollen germinability in relation to water content and its cryopreservation. In light of the evidences previously cited and according to morphological similarities with pollen grains of other Angiosperms species classified by Franchi et al. (2002) it could be possible to consider olive pollen grains as partially dehydrated pollen; so, it could be less difficult to store it at low temperatures as requested by long term storage in liquid nitrogen.

According to Ferri et al. (2008) we must consider that, differently from other species, olive pollen is very sensitive to the *in vitro* growing conditions, so we found a significative decreasing of germinability respect to natural conditions. Experimental evidences from literature seem to suggest the opportunity to define a most efficient prefreezing treatments on olive pollen grains to optimize its water content that has a major influence on the ability to store pollen at ultra-low temperatures (Barnabas 1983).

Conclusions

Among olive cultivars investigated our results have showed significative variation for both traits: *in vitro* germinability, morphology and size of pollen grains. The results of this research have highlighted the operational effectiveness of olive germplasm by pollen cryopreservation, whose technique, however, should be improved further in order to optimize several parameters. Particular attention should be devoted to the prefreezing treatments especially in relation to pollen water content. Care must be taken to avoid ice crystallisation during the freezing process, which otherwise would cause physical damage to the tissues. The existing cryogenic strategies rely on air-drying, freeze dehydration, osmotic dehydration, addition of penetrating cryoprotective substances and adaptive metabolism (hardening) or combinations of these processes. As previous reported few papers have been published to assist genebank curators in the establishment and management of long term pollen storage. There is still a limited number of cases where cryopreservation is used routinely for pollen storage, mainly because the techniques need to be adapted for each species as in the case of *Olea europaea* L. Further experiments are needed to investigate olive pollen for long term storage period at ultra low temperature standardizing moisture content of pollen prior to freezing. Because of its high potential, it is expected that pollen cryopreservation in liquid nitrogen will become more frequently employed for long term storage of plant genetic resources. Our preliminary findings will contribute to the improvement of olive pollen preservation and lead to find a more efficient method for its long term storage, while retaining its germinability.

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