LIFE4FIR – Project LIFE18 NAT/IT/000164

"Decisive in situ and ex situ conservation strategies to secure the critically endangered Sicilian fir, *Abies nebrodensis*"

Report on: Protocol for long-term conservation of *A.nebrodensis* pollen at ultra-low temperature
Action A.1

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DELL'AGRICOLTURA, DELLO SVILUPPO RURALE
E DELLA PESCA MEDITERRANEA

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1. Introduction

The LIFE4FIR project includes, among its aims, the constitution of a cryobank for the storage of tissue and organs (seeds, pollen, isolated embryos and embryogenic callus lines) from *Abies nebrodensis* (Action C.5), with the goal to create a safe long-term ex situ repository for this endangered species at the Municipality of Polizzi Generosa inside the already existing Museum Abies Nebrodensis (MAN).

The project provides for preparatory activity to achieve the above-mentioned objective. In particular in the Action 1.4 includes the development of a protocol for long-term storage of *A. nebrodensis* pollen in liquid nitrogen (cryopreservation).

Plant cryopreservation, is a process of cooling and storing vegetal structure as plant cells, tissues, or organs in liquid nitrogen (LN; -196°C) or LN vapour (-160°C). This methodology ensures the maintenance of samples viability after thawing with the possibility of an indefinite storage. A clear indication on cryopreservation as a useful and necessary tool for conservation of plant species has been underlined in the Plant Conservation Report 2020 which reports the cryopreservation among alternative conservation methods (www.cbd.int/doc/publications/cbd-ts-95-en-lr.pdf)

2. Need and Importance of Pollen Conservation

Pollen conservation is an important tool for the maintenance of plant genetic resources and can promote improved efficiency in breeding programs and germplasm conservation and exchange. Pollen has great significance in other research areas, such as climate changes, medicine, history and archaeology.

The creation of pollen banks and new methodologies aimed at maintaining the long-term viability of pollen are of interest for the scientific community.

The importance of pollen preservation has been always considered very seriously, many research already had dealt with topic in the 70-80's, preserving pollen in different condition of temperature (4°C, -20°C and -80°C). Collection and storage of pollen grains can be a tool to obtain a wide range of genetic diversity in a population and it represents an effective propagule for genebanks.

Advantages of the pollen preservation are:

- Collections pollen can be made available to breeders upon request, indeed, with adequate pollen available, it is possible to load additional pollen onto stigmas to increase pollination and yield.

- Pollen serves as a source of genetic diversity in collections where it is hard to maintain diversity with seeds (species of low fecundity, large seeds, or seeds that require an investment of labour to store).
- Pollen is available for research programs. Storage of pollen within genebanks also ensures its availability year-round for basic biology and allergy research programs.
- Pollen can also be shipped internationally, often without threat of disease transfer, like seeds, pollen international exchange is considered safe, as harmful pests and diseases are rarely transferred through pollen.

Collected pollen serves to maintain and preserve the alleles of an individual or population. Sampling strategies have often recommended collecting a set number of individuals per population to ensure that the common alleles are captured. Pollen can also be collected from individual trees within a genebank both to conserve alleles specific to each individual and to provide male gametes for breeding purposes.

For these reasons, pollen conservation should be integrated into the conservation programs of germplasm banks, to avoid loss of the genetic material of male parents. Pollen conservation should be an additional means of conserving plant germplasm and not a substitute for the storage of seeds or clonal materials.

Pollen storage success depends on environmental factors of humidity and storage temperature . Low temperatures and humidity are usually linked to pollen metabolism decrease, which allows greater longevity. Assuming relatively ideal storage temperature and pollen moisture, viability is independent of the storage period.

3. Cryopreservation of Pollen and Cryobanks

Cryopreservation is a method enabling the long-term preservation of genetic diversity of the population sampled on a relatively small space. The possibility to store pollen for long-term and prolong its longevity increases the opportunity for researchers in biotechnological and basic studies particulary in breeding programs overcoming seasonal and geographical restrictions. Pollen is manageable for cryopreservation and this preservation method has been applied in different fruit species (e.g. actinidia, *Prunus* sp, *Olea europaea*, *Psidium* sp., *Vitis* sp., Citrus) and forest species (Pinus, oak, beech, horse-chestnut, Picea). A pollen cryobank can maintain genetically diverse stocks of pollen collected from such plants and provide the required male parent in a viable and fertile form for primary and supplementary pollination needs, to improve seed set for species amplification, thereby increasing the chance of species recovery for eco-restoration programs.

Pollen, is an interesting material for genetic resource conservation of various species and it stored in LN by several Countries. In India, the National Bureau of Plant Genetics Resources (NBPGR) conserves cryopreserved pollen of 65 accessions belonging to different species and the Indian Institute for Horticultural Research conserves pollen of 650 accessions belonging to 40 species from 15 different families, some of which have been stored for over 15 year. In the USA, the National Centre of Genetic Resources Preservation (NCGRP) preserves in liquid nitrogen the pollen of 13 pear cultivars and 24 Pyrus species.

To maintain the pollen viability as high as the fresh pollen during long-term storage, it is necessary to follow protocols of collection, moisture content, storage temperature and viability pollen tests.

Viability of pollen varies with time in different species and needs to be assessed before and after cryopreservation.

Moisture content plays a major role during cryopreservation, excess moisture in pollen forms ice crystals damaging pollen membranes irreversibly affecting viability.

According to Sprague and Johnson, a pollen moisture content from 8 to 10% avoids the formation of ice crystals during the freezing process, regardless of the final cold storage method but in particular when using -80 to -196°C temperatures. Moreover the pollen viability and germinability evaluation is the essential step to verify the successful of storage.

4. Steps to Achieve the Protocol for Cryopreservation of *A. nebrodensis* Pollen

4.1 Collection, morphology and moisture content of A. nebrodensis pollen

Field conditions and relative humidity at the time of harvest affect the pollen moisture content. During May 2020, after preliminary inspections of *A. nebrodensis* trees in the Madonie park, anther harvests were made twice from various plants.

Table 1 reports the trees from which pollen was collected (the number refers to the plant marked in the Park), classified in groups on the basis of the amount of pollen collected in the first harvest on 13 May, and in the second harvest, on 19 May 2020. Several trees showed a low amount of pollen in the first collection, but it was possible to reach an adequate amount for conservation in the next one.

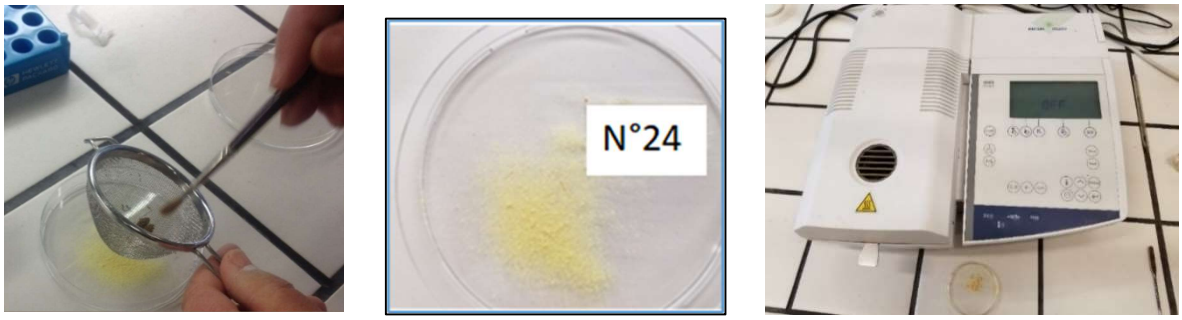
Table 1 - Abies Nebrodensis trees and amount of sampled pollen collected in 2020

Harvest time pollen	**TREES (n°)**		
	Scarce	*Moderate*	*Abundant*
13 May	9-11-12-15-17-25	10-14	1-2-7-8-13-24-27
19 May	19-21		6-9-11-12-17-22-23

In 2021, the pollen production has been very limited, consequently nullifying the cones and seeds production; the pollen samples collected in May were minimal (only 12 and 24 trees).

After removal from anthers, the pollen collected was sieved (Fig. 1A and 1B) and the morphology characterization, moisture content, viability and germinability of pollen test were promptly performed.

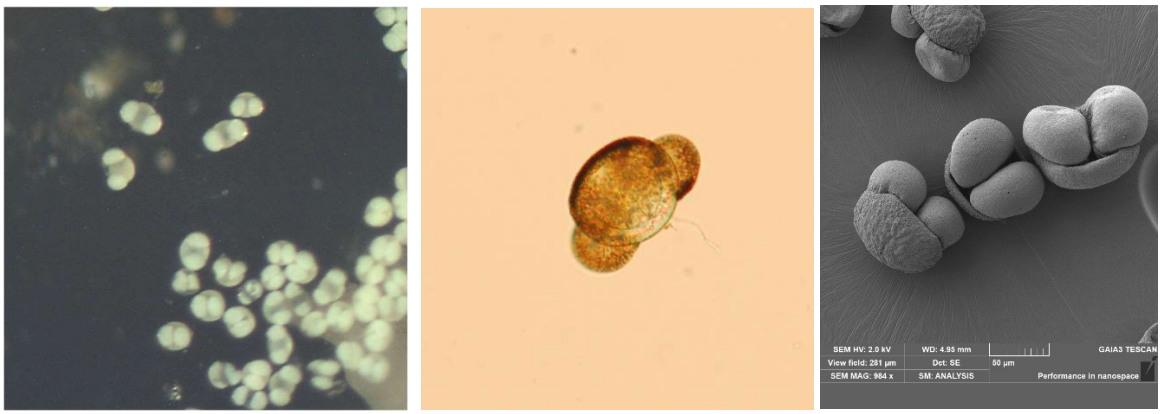
The pollen moisture content (MC) was determined by Moisture Analyzer instrument weighing 0.2 gr of pollen. In the fresh pollen the MC was on average 10% (Fig. 1C)



(A) **(B)** **(C)**

Fig. 1. Pollen sieving (A); Fresh pollen (B); Evaluation of the moisture content (C)

The pollen of *A. nebrodensis* was observed by Leika steromicroscope (Fig. 2 A), Leika Optical Miscroscope (Fig. 2B) and Environmental Scanning Electron Microscope (ESEM -Fei Quanta 200; Fig. 2C). Pollen grains are isodiametric with an elliptical central body with two lateral air sacs (bisaccate) and one aperture leptoma. The air sacs are clearly protruding from the body (Fig. 2).



(A) **(B)** **(C)**

Fig. 2. Pollen grains morphology A. nebrodensis observed under steromicroscope (A), optical microscope (B) and Environmental Scanning Electron Microscope (ESEM;C)

4.2 Viability and germinability assays

TTC test and in vitro germination were carried out to evaluate the viability and the germinabilty of fresh pollen, respectively.

TTC test

Tetrazolium (2, 3, 5 triphenyl tetrazolium chloride) test is also known as the TTC test. All living tissues, which respire, possess the ability of reducing a colour less chemical 2, 3, 5 triphenyl tetrazolium chloride into a red coloured compound formazan by H transfer reactions catalysed by the enzyme dehydrogenases. This test is based on the reduction of soluble colorless tetrazolium salt to reddish insoluble precipitate in the presence of dehydrogenase activity in the pollen grain cells. The concentration of tetrazolium salt, temperature and period of incubation needs to be standardized to get optimal and precise results in various pollen samples.

1% TTC was prepared by adding 200 mg of 2, 3, 5 triphenyl tetrazolium chloride and 12 g of sucrose in 20 ml distilled water. Two drops of this mixture were dropped on a microscope slide and the *A. nebrodensis* pollen was dusted over it and covered with a coverslip in dark at room temperature for 24-48 h. Following incubation, each sample was observed under the microscope and pollen grains stained orange or bright red colour were scored as viable.

In vitro germination

Two germinabilty assays were tested: 1) In vitro germination on appropriate semisolid-medium and 2) 'Hanging Drop' method (Fig.3).

1) Pollen germinated *in vitro* by placing pollen grains onto a semisolid medium and measuring the elongation of the pollen tube after a few hours. Pollen tubes that elongate to a length at least two times the diameter of the pollen grain were considered germinated (Fig. 4). The medium

used for *A. nebrodensis* pollen was as follows: boric acid (50 mg/L), sucrose (15 g/L) and plant agar (6 g/L). The pollen was maintained at 25°C, the optimal temperature for *in vitro* germination assays of most species.

2) The hanging-drop method: pollen was put on micro drops of a solution composed of boric acid, calcium nitrate and sucrose. Micro drops were placed on the inside of the lid of a petri dish in which 3 mL of water were added in the base to create a humid chamber. The petri dish with pollen was maintained at room temperature.

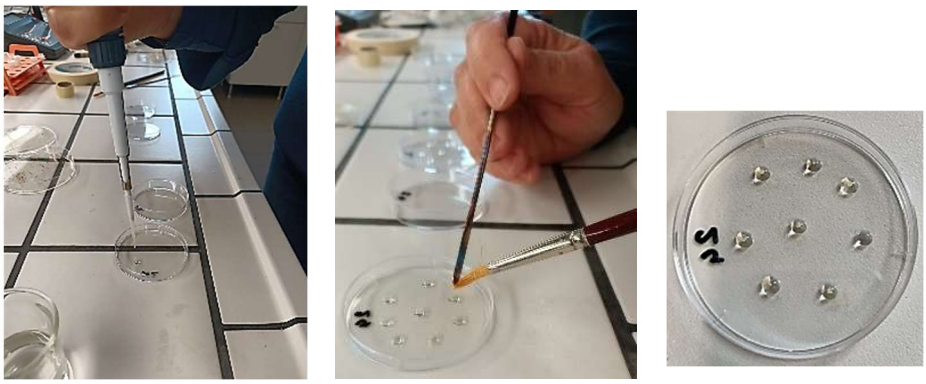


Fig. 3. 'Hanging Drop' method for in vitro germination

For observation, petri dishes with germinated pollen on the medium or into the micro drops were positioned under a microscope.

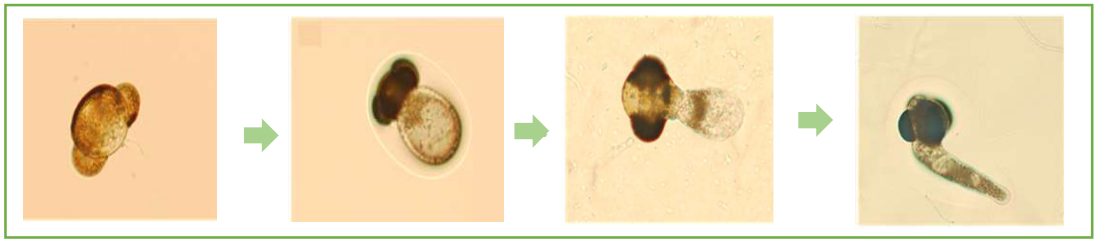


Fig. 4. A. nebrodensis pollen grain: in vitro germination and pollen tube growth during 48 h

4.3. Immersion, storage in liquid nitrogen and recovery of pollen

After the collection, the pollen was maintained three days at 4°C, thereby the MC was reduced until to 8%, and no desiccation pollen was needed before the immersion in liquid nitrogen. Samples of pollen was transferred in the cryovials and immersed directly into liquid nitrogen (Fig. 5).

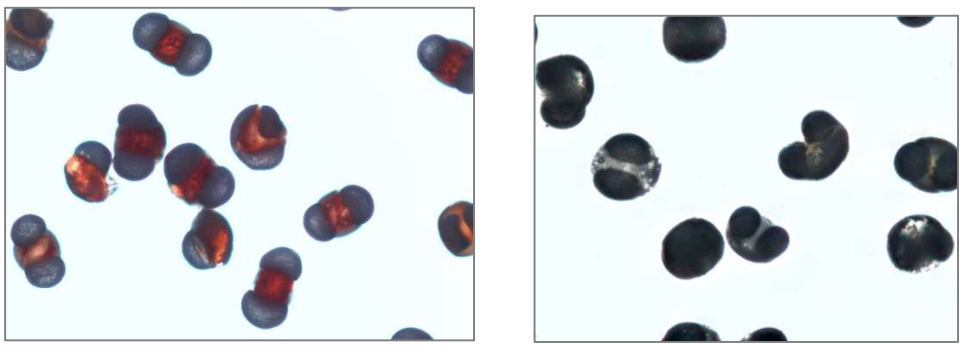
Fig. 5. Pollen in cryovials, immersion and storage in liquid nitrogen (cryopreservation)

After storage in liquid nitrogen, the cryovials containing the pollen were thawed by placing them under a laminar flow cabinet for 2 hours at room temperature and then transferred in petri dishes.

4.4 Assays on cryopreserved pollen.

Viability and in vitro germination were assessed on cryopreservd pollen samples.

The TTC test was used to evaluate the pollen viability after cryopreservation as described above (Fig. 6). For in vitro germination assay on cryopreserved pollen, the test with solid medium was used (Fig.7), this method, indeed, has proven more effective for *A. nebrodensis* pollen .



(A) **(B)**

Fig. 6. TTC test in A. nebrodeinsis pollen: *viable pollen grains (A), not viable pollen grains (B)*

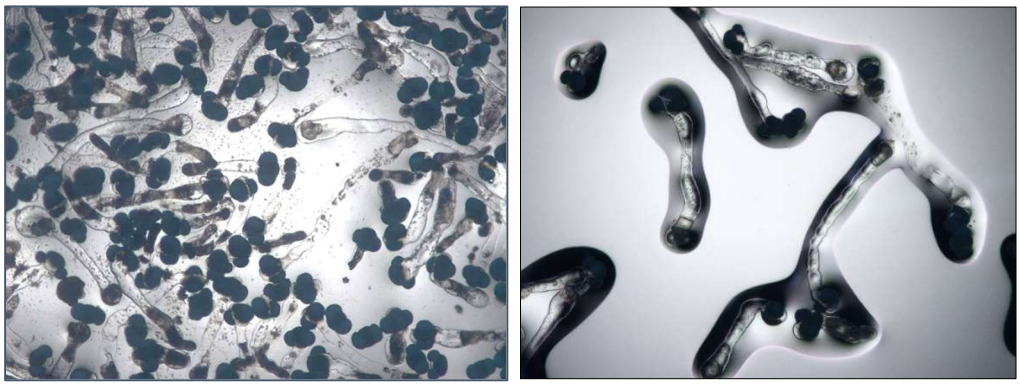


Fig. 7. Pollen in vitro germination of A. nebrodensis on semisolid media after 48 hours

4.5 Results after cryopreservation of A. nebrodensis pollen

By application of this protocol the percentage of viable pollen grains observed was ranging between 88 % up to 96% in *A. nebrodiens* trees evaluated, without significant deviations from the fresh pollen. The same trend is recorded in the germination test, the percentage of germinated pollen grains ranged from 84% to 94%.

For difficulties due to the Covid-19 pandemic, the implementation the cryobank with pollen, and other explants (seeds, excised embryos and embryogenic callus) was postponed until the next year (2022), but the above described protocol will allow a fast and effective storage for the long-term conservation of *A. nebrodensis* pollen.

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5. FINAL PROTOCOL FOR *A. NEBRODENSIS* POLLEN CRYOPRESERVATION