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: 'Seed-bank and cryobank constitution'
Action C.5

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REPUBBLICA ITALIANA
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1. Introduction

The LIFE4FIR project includes, among its aims, the constitution of a seed-bank and a cryobank, hereinafter referred as Bank of Germplasm (BG), for the storage of tissue and organs from *Abies nebrodensis*, with the goal to create a safe long-term *ex situ* repository for this endangered species. Originally, the BG was supposed to be established inside a building already existing at the "Museo Naturalistico Francesco Minà Palumbo" in Castelbuono. Subsequently, the Municipality of Polizzi Generosa, during a meeting held on October 13, 2020 (*see 2.1.*) offered to host the BG, inside the already existing MUSEUM ABIES NEBRODENSIS (MAN; Fig. 1) and this was immediately considered the best suitable location for the seed-bank and the cryobank, within a prestigious context.

Fig. 1. Polizzi Generosa, the city of *Abies nebrodensis*, and its MAN (MUSEO ABIES NEBRODENSIS).

Indeed, successful conservation and effective use of plant biodiversity are very important to ensure sustainable increases of production and contribute greatly to agricultural ecosystems. The erosion of plant genetic variability is attributable to many important factors, including partial degradation of the natural habitat, changes in land use, replacement of traditional varieties with modern cultivars, intensification of agriculture, as well as the increase in population, poverty, land degradation and climate change. Over time, there has been a dramatic depletion of the genetic heritage and an increase in the number of threatened species

which has alarmed and sensitized the entire planet. *Abies nebrodensis* is one of these highly endangered species under the risk of extinction. The conservation of plant germplasm can be carried out *in situ* and *ex situ*. In the first case, the species are kept in their natural environment through conservation practices of habitats and ecosystems, or conservation is pursued by promoting on farming for landraces and ancient varieties of crops. However, inevitable damage and transformation of natural environments may cause huge reductions or decline of species, populations and ecosystems, with consequent loss of the biodiversity, which demonstrates that *in situ* strategies alone are rarely sufficient to guarantee the conservation of the plant genetic heritage. Therefore, it is essential to integrate plant biodiversity conservation programs through additional approaches using *ex situ* strategies which keep the biological materials in artificial environments, with the possibility of reintroducing them to their natural habitats at any time. In addition to the traditional approaches of seed-banking and clonal orchards for the *ex situ* conservation of tree genetic resources, there is today a recently developed and rapidly evolving technique, i.e. cryopreservation, that can be validly considered as integrated and complementary in the plant biodiversity conservation programs, providing a further guarantee against accidental loss of genetic resources.

1.1. Conservation of germplasm in seed bank

The conservation of plant biodiversity in seed banks is the classic approach for maintaining the germplasm of species characterized by gamic propagation. Around the world, about 1750 seed banks are currently registered, spread over all Continents, under the aegis of important international organizations, including FAO and Bioversity International. In 2010, the 2nd FAO "World Plant Genetic Resources Report" indicated in about 7.4 million accessions maintained the various conservation centers around the world; as many as 90% of these are kept in seed banks, largely comprising cereals, but to an important extent also forest species.

The storage at low temperature of seeds is a strategic approach for genetic resource conservation. In seed banks, a large amount of genetic diversity can be conserved in a single room, hence they are considered convenient and recognized globally. In seed banks, important plant germplasm is stored. This germplasm is a valuable source of genes for breeding. Seed banks also provide database systems which enable recording easily the eco-geographic details of each seed population in collection.

For conifers, the seed represents an important and valid germplasm conservation system. Storage temperature and seed moisture content are the two critical factors, determining the success of the storage in seed-banks. Depending on the species, seeds are dried to an appropriate

moisture content. The international standards state the reduction of seed moisture content to 3-7%, by means of air drying at 10-25°C up to 10-15% relative humidity. It should be underlined that conifer seeds are generally characterized by embryos with a natural low level of water content (less than 10%), hence it is often not required any moisture content reduction for the storage at a temperature of -18°C, i.e. the one generally applied in seed-banks.

Based on all the above remarks, this form of conservation for the *Abies nebrodensis* germplasm was considered important in the LIFE4FIR project, also in consideration of the high risk of genetic erosion (even extinction) that the species is presently facing.

1.2. Conservation of germplasm in cryobank

Cryopreservation, i.e., the storage at ultra-low temperatures such as that of liquid nitrogen (-196°C), is the most innovative technique which enables long-term conservation of plant genetic resources. The technique preserves organs and tissues, from in vitro culture and from the field, by means of an ultra-freezing process that, if properly developed and well-adapted to the specific plant specimen, hampers almost all metabolic processes in the cell, while preserving its structure and biological functionality. In fact, at a cryogenic temperature very few biological reactions and significant variations of the physiochemical properties remain active. Liquid nitrogen, a cryogenic gas easily available and of limited cost, is used universally in cryobanks and ensures the maintenance of temperatures ranging from approximately -165°C (for samples stored in the space of the container filled with the gas vapors) to -196°C (for samples immersed in the liquid phase of nitrogen).

Among the main advantages of cryopreservation in woody plants, in comparison to traditional conservation in the field or in in vitro banks, there are (Fig. 2):

- the minimum space requirements. For instance, the use of shoot tips or excised embryos as conservation units makes possible the storage from 7.000 to over 30.000 samples in small- and medium-sized dewars (i.e., from 35 to 200 liters), with very low conservation costs (in practice, only those necessary for the control of the cryobank and maintenance of the appropriate level of liquid nitrogen);
- the low long-term costs. A study has shown that the introduction and maintenance of one accession in cryopreservation is more expensive than traditional conservation in seed-banks and clonal collections. On the other hand, in the long term (over 20 years), the maintenance of genetic resources in cryobank becomes significantly cheaper than all these forms of conservation, especially when operating with a high number of accessions;

- high genetic stability. Numerous experimental works, particularly in the last 30 years of experiences of plant cryopreservation, have evaluated the phenotypic, cytological, biochemical and molecular stability aspects of the material subjected to conservation in liquid nitrogen, never showing significant stable alterations of material after long-term storage;
- the long-term storage. Theoretically, the storage time can be unlimited, and for sure over 100 year.

Among the main drawbacks of cryopreservation, the availability and accessibility to liquid nitrogen has been carefully considered for the establishment of the cryobank of *Abies nebrodensis*, with satisfactorily solutions (*see 3.3.*).

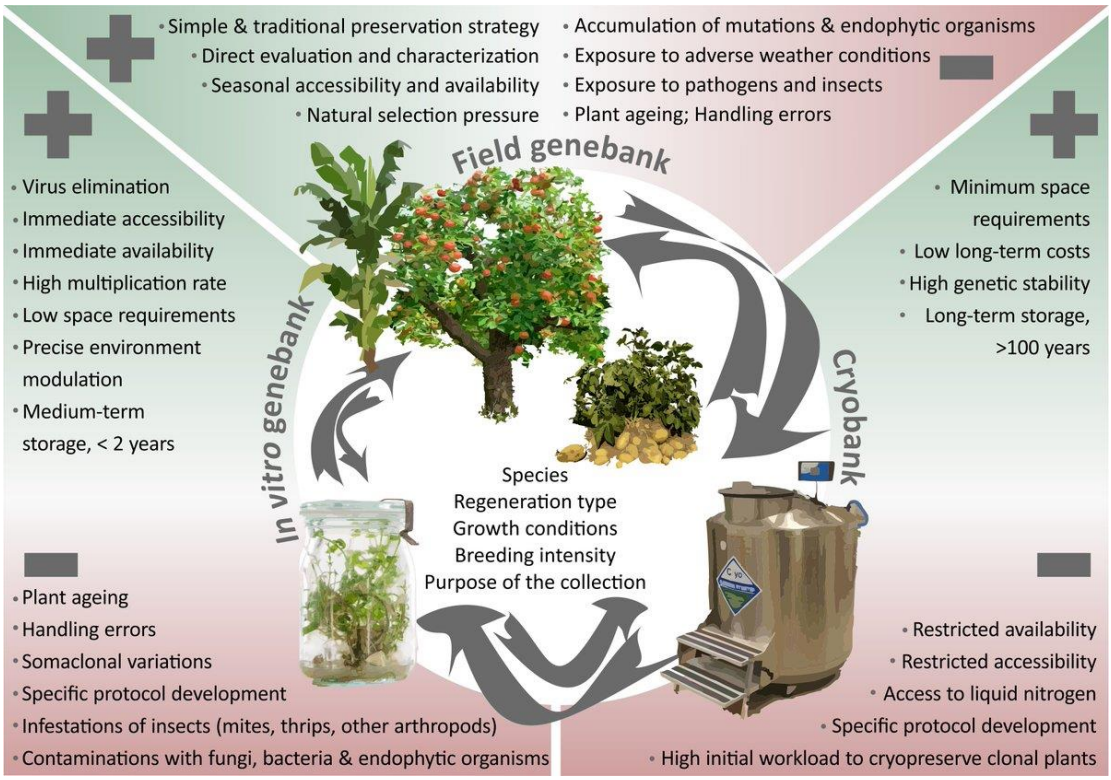

Fig. 2. Comparison among field genebank, in vitro genebank and cryopreservation of trees, in terms of advantages and drawbacks (from Panis et al. Plants, online November 2020).

2. Roadmap followed to establish the *Abies nebrodensis* Bank of Germplasm, implemented with a seed-bank and a cryobank

The decision to establish the BG at the MAN and the related implementation agreements were taken during two meetings held at the Municipality of Polizzi in the days October 13, 2020, and May 25, 2021.

2.1. Meetings in Polizzi Generosa municipality on October 03, 2019

The first meeting was held in the Municipality of Polizzi Generosa, with the partecipation, among others, of the Major, Sig. Giuseppe Lo Verde, the Director of the Parco delle Madonie, Dr. Peppuccio Bonomo, and a delegation of the LIFE4FIR project (Dr. Roberto Danti, Coordinator of the project, Prof. Rosario Schicchi, Dr. Maurizio Lambardi, Dr. Gianni Della Rocca and Dr.ssa Carla Benelli; Fig. 3). During the meeting, the Mayor expressed great interest and full availability to host the BG within the MAN. The MAN is an educational museum of *Abies nebrodensis*, inside which visitors can become fully aware of the importance of the species and the need to safeguard the 30 relict plants, through the vision of images, films and plant material. For these reasons it was decided to accept the offer of the Municipality of Polizzi Generosa, identifying the MAN as the best location for the BG (seed-bank and cryobank). The MAN of Polizzi Generosa is the subject of frequent visits by interested people, scientists, school and study groups: in this context, the BG will also be able to perform an important function of disseminating knowledge on the innovative strategies available today for the conservation of threatened genetic resources, strategically important and complementary to the classic *in situ* conservation approach.

The meeting ended with agreement to go on with the constitution of the BG in one available room inside the MAN. At the same time, the interventions to be made for the preparation and compliance of the room were listed (*see 4.*).

Fig. 3. Meeting at the Municipality of Polizzi Generosa and visit at the MAN, 03.10.2019.

2.2. Meeting in Polizzi Generosa Municipality on May 25, 2021

The second meeting was held in the Municipality of Polizzi Generosa on 25.5.2021, with the partecipation, among others, of the new Major, Sig. Gandolfo Librizzi, the Director of the Parco

delle Madonie, Dr. Peppuccio Bonomo, and a delegation of the LIFE4FIR project (Dr. Roberto Danti, Coordinator of the project, Prof.sa Maria Antonietta Germanà and Dr. Maurizio Lambardi; Fig. 4). The Mayor confirmed the willingness of the Municipality of Polizzi Generosa to host the BG at the MAN. The Polizzi Generosa administration has also made a commitment to guarantee the financial support, necessary to maintain the BG after the end of the project. In this sense, the annual cost of supplying liquid nitrogen was quantified at 2,000 Euros per year. The administration will meet this cost and also the other minor costs of managing the structures contained therein. The CNR and the University of Palermo have also made a commitment to guarantee the necessary consultancy for the management and implementation of the seed-bank and cryobank in the years to come, after the end of the LIFE4FIR project. An inspection was then carried out on the room intended for the BG (Fig. 5).

Fig. 4. The persons attending the meeting at the Municipality of Polizzi Generosa on May 25, 2021. From left to right: Prof.ssa Maria Antonietta Germanà, Dr. Maurizio Lambardi, Dr. Gandolfo Librizzi (Major), Dr. Roberto Danti (Coordinator of LIFE4FIR) and Dr. Peppuccio Bonomo.

Fig. 5. The room selected to host the Bank of Germplasm of *Abies nebrodensis*. Some of the equipments, purchased with the project funds, were already present at the meeting time of 25.5.2021.

3. Acquisition and description of the equipment for the BG (seed-bank and cryobank)

The pandemic Covid-19 has unfortunately slowed down the complete implementation of the BG of *Abies nebrodensis*, as it was stated in the LIFE4FIR program. However, at the time of drafting this report, the previously requested room masonry works were already carried out, as well as some of the interventions related to safety standards required in an ambience containing liquid nitrogen. In addition, all the necessary equipment for the future activity of the BG was acquired (*see 3.1., 3.2*).

3.1. Climatic chamber for the seed conservation at -18°C

After a careful market survey, the choice of the climatic chamber that will house the seeds of the relict plants from *Abies nebrodensis*, in storage at -18°C, fell on a product of the company EVERmed Srl of Montaggiana (Mantova, Italy), *www.evermed.it*, the LFG 1160S (Fig. 6).

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E-mail: evermed@evermed.it - Http: www.evermed.it

ISO 9001:2008 EN ISO 13485:2012

Fig. 6. The climatic chamber selected as seed-bank of *Abies nebrodensis*. *Left*, the image from the brochure of EVERmed SRL; *right*, the climatic chamber, just arrived at the BG of the MAN of Polizzi Generosa.

The salient features of the climatic chamber are the following:

- two transparent doors for easily inspection of the content
- temperature up to -20 ° C for storage of seeds
- wheels and safety lock with keys
- glass doors: Nr. 2 hinged, side by side, made with an anodized aluminum perimeter frame, triple-crystal heat-insulating tempered glass equipped with heating elements to prevent frost and condensation, vacuum cavities to increase the insulation coefficient.
- internal equipment: Nr. 6 shelves (3 per door), made of non-toxic plasticized steel wire, supported by anti-tipping guides in 18/10 AISI 304 stainless steel, adjustable in height
- shelf dimensions (W x D cm): 53 x 55
- internal lighting: with LED tubes, positioned in the side walls of the storage cell, with automatic activation both at each door opening and by means of a special button on the control panel
- control panel: positioned in the upper part of the structure
- refrigerant gas: R404a without CFC
- defrost: completely automatic, with pre-programmed interventions from the remote thermostat.

- temperature: adjustable in the range -5°C /-20°C
- gross capacity (liters): 1295 - net capacity (liters): 1160
- dimensions (L x W x H cm): 150 x 70 x 210.

From the above, it is believed that this climatic chamber will respond perfectly to the need for space, operating characteristics and alarm systems, necessary for the maintenance in perfect safety of the precious seed of the relict plants of *Abies nebrodensis* that will be stored inside. Moreover, the glass doors will allow to show the content of the climatic chamber to the future visitors of the BG of *Abies nebrodensis*.

3.2. Device for the conservation of samples at -196°C

As for the dewar of liquid nitrogen, where various material of *Abies nebrodensis* (seeds, excised embryos, pollen, embryogenic callus) will be stored for unlimited conservation at -196°C, it was decided to select the same model functioning at the cryobank of the CNR-IBE in Sesto Fiorentino (Firenze), as already well known for its reliability, safety, guarantee of operation and low consumption of liquid nitrogen. It is the Locator 8 Plus, marketed by the VWR International Srl of Milano, Italy (Fig. 7).

Fig. 7. *Left*, the Locator 8 Plus, dewar for the conservation of samples in liquid nitrogen, where the cryobank of *Abies nebrodensis* germplasm will be established; *right*, rack and box where cryovials are allocated.

The salient features of the Locator 8 Plus the climatic chamber are the following:

- container for storing samples (dewar) in liquid nitrogen at -196°C, equipped with ultrasonic level monitor
- capacity 121 liters
- unit racks 8, with a capacity of 10 boxes for rack and 25 2-mL cryovials per box
- total capacity: 2000 cryovials
- static evaporation rate: 0.6 L/day
- neck diameter: cm 15.2
- external dimensions diameter x height: cm 55.8 x 95.3
- lid with lock.

3.3. Provision of liquid nitrogen

For the periodic supply of liquid nitrogen, when the cryobank will come into operation with the implementation of plant material, various supplier companies have been contacted, located in Palermo (about 1.5 hour from Polizzi Generosa). A final decision has not yet been taken, but the trend is to select the company Air Liquide which boasts an excellent organization in the supply of technical gases, and is the main producer and supplier of liquid nitrogen in Europe.

4. Establishment of the Bank of Germplsm at the MAN of *Abies nebrodensis*

Fig. 8 illustrates the location of the BG, on the 2nd basement floor, under the MAN of the Municipality of Polizzi Generosa. A 4 x 2 m room with a window overlooking a side street of the Municipality has been selected (Fig. 9, a and b).

The room has been completely sanitized before the arrival of the equipment, while some work on the electrical system is still in progress. The room will contain the seed-bank, the cryobank and a support shelving. In addition, it will be furnished with posters illustrating the LIFE4FIR project, and the innovative conservation techniques applied to save the endangered species. The supply of liquid nitrogen will take place through an access door to the basement that overlooks a side street of the Municipality (Fig. 9, c).

Regarding safety standards, the room will be equipped with (i) two oxygen detectors, placed in diametrical points of the room, (ii) a window on the access door that allows a full view of the interior when the door is closed (Fig. 9, d), and (iii) protective corset, mask and gloves (Fig. 10) to be used during the operations of filling the dewar with liquid nitrogen and handling the samples in storage.

Fig. 8. Planimetry of the 2nd basement floor, under the MAN. In red, the room for the Bank of Gemoplasm.

Fig. 9. The Bank of Germplasm of *Abies nebrodensis*. a, the room with the dewar for sample conservation in liquid nitrogen; b, the side with the window opening to the street outside; c, the access door to the basement that overlooks a side street of the Municipality; d, the window on the entrance door for inside inspection.

Fig. 9. Corset (*left*), mask (*centre*) and gloves (*right*) for protection during the operations with liquid nitrogen.

The management of the BG will be entrusted to an employee of the Municipality of Polizzi Generosa who will be previously trained by Dr. Maurizio Lambardi and Dr. Carla Benelli.

5. Implementation of the seed-bank and cryobank

The travelling difficulties that arose in the period April 2020-May 2021, due to the Covid-19 pandemic, led to significant delays in the acquisition of *Abies nebrodensis* plant material and, as a consequence, in the development of the related conservation protocols, whose deadline was in fact moved to 31.12.2021. Therefore, the onset of the implementation of the seed-bank and the cryobank with pollen, seeds, excised embryos and embryogenic callus was also postponed. As reported in previous reports, currently the progress in the development of protocols is as follows:

- pollen - the pollen germination tests have been carried out (Fig. 10, a) and the development of the storage protocol in liquid nitrogen is underway;
- seeds - the operations of extracting the seeds from the cones, their cleaning, sanitization and evaluation of germination have been perfected (Fig. 10, b). Subsequently, an X-ray investigation protocol was developed to separate the full seeds from the empty seeds (Fig. 10, c); this will soon allow to start implementing the seed-bank with only or largely full and germinable seeds;
- embryos - the procedure for the excision of embryos from the outer coatings and from the endosperm was perfected (Fig. 10, d), as well as the evaluation of germinability with TTC

and direct germination tests. The development of the conservation protocol in cryobank is underway;

- embryogenic callus - it has not yet been possible to obtain efficient lines of embryogenic callus, also due to the almost total absence of seeds in trees in the 2021 season. Hence, actions to obtain embryogenic callus will resume in the 2022 season.

Fig. 10. *Abies nebrodensis*. a, a grain pollen; b, seeds after extraction and cleaning; c, seeds at the X ray image: *left*, a full seed; *right*, an empty seed; d, an excised embryo.

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