LIFE4FIR – Project LIFE18 NAT/IT/000164

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

"Recommended actions for conservation programs".
Action C2.

Istituto per la Protezione Sostenibile delle Piante
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REPUBBLICA ITALIANA
REGIONE SICILIANA
ASSESSORATO REGIONALE
DELL'AGRICOLTURA, DELLO SVILUPPO RURALE
E DELLA PESCA MEDITERRANEA

Parco delle Madonie

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1. Introduction. Species with small population size: main threats and conservation efforts

Populations characterized by reduced size or isolation resulting from habitat loss and fragmentation generally exhibit a reduced genetic diversity caused by restricted gene flow, genetic drift and inbreeding. Genetic erosion is the loss of genetic diversity within a species. Because variability is inherently related to evolvability, genetic erosion in small, recently fragmented populations may contribute to their endangerment. Influences that could contribute to genetic erosion in native plant species include major losses of habitat and the resident plant populations; fragmentation of habitat; management activities such as thinning, harvesting, or nursery selections that target certain features of plants; and planting material from a narrow genetic collection in revegetation efforts. It can also happen as a consequence of a catastrophic event that removes large numbers of individuals and their habitat. Genetic erosion can represent the loss of entire populations genetically differentiated from others, the loss or change in frequency of specific alleles within populations or over the species as a whole, or the loss of allele combinations. Genetic erosion can result from habitat loss and fragmentation and seed collections that are too restricted.

Genetic diversity is important to a species' fitness, long-term viability, and ability to adapt to changing environmental conditions. Also, plant populations that are less genetically diverse may be more susceptible to pathogens or other environmental stresses and may be less competitive with introduced invasive species.

Genetic erosion is dramatically a burden on the *Abies nebrodensis* population, reduced to only 30 adult trees. For the purposes of protecting this species, the Life4fir project has implemented measures aimed at increasing the genetic variability in the *A. nebrodensis* offspring and at using selected outcrossed seedlings to create new repopulation nuclei. This activity required a series of steps to first understand the structure and genetic diversity of the population as well as the genetic distances between plants, and then undertake a program of controlled crosses between the genetically most distant plants. Other measures have been undertaken in situ to protect adult plants and the natural regeneration in their habitat and ex situ for the long-term conservation of germplasm, protecting it from adverse events.

2. Genetic analysis of the *Abies nebrodensis* population

The general aim of this action is the evaluation of the genetic variability and the genetic relationships among the 30-adult trees and the juvenile plants from the natural regeneration of *A. nebrodensis*. Genotyping with 120 SNPs was used for genetic characterization of these individuals.

The genetic diversity and structure of the natural population of *A. nebrodensis* was studied. Then, paternity tests were carried out on the natural regeneration to determine the rate of outcrossing, inbreeding and self-fertilization and to assess the rate of introgression (eventual hybridization) due to fertilization of female cones with pollen coming from alien firs (*Abies alba* and *Abies cephalonica*). Finally, we calculated the genetic relatedness of the 30-adult trees of *A. nebrodensis* based on the pairwise co-ancestry.

Despite genetic analyses evidenced a significant genetic diversity among the *A. nebrodensis* adult trees, the genetic diversity of the resulting natural regeneration dropped as consequence of the high rate of selfing. The estimated pedigree evidenced a remarkable level of inbreeding for *A. nebrodensis* individuals, and the effective population size (*Ne*) (which is a key parameter in population genetics that estimate the number of individuals that effectively contributes offspring to the next generation) was found to be as low as *Ne* = 6, reflecting the strong impact of genetic drift and inbreeding on the evolutionary dynamics of this population. Results of analysis also reflected the moderate homozygosity of *A. nebrodensis* plants due to inbreeding and selfing. Genetic characterization of open pollinated plants raised in the nursery evidenced again a very high selfing rate and significant introgression with non native fir species (*A. alba* and *A. cephalonica*).

Findings showed that active management of this population could help in mitigating genetic loss. Based on pairwise co-ancestry, a list of the 30 most recommended crosses between mature trees of *A. nebrodensis* was produced based on co-ancestry. The possibility of genetic rescue should be considered as a way to maintain evolutionary potential. However, to mitigate vulnerability of the species to environmental changes and alleviate the extinction risk, assessing the effect of selfing on inbreeding depression and the potential use of hybrids for future adaptation in their natural habitat is crucial for effective conservation strategies.

2.1 Controlled crosses for assisted genetic flow

A plan of controlled crosses has been aimed at promoting outcrossing between trees, given the high rate of inbreeding and genetic erosion. In the Life4fir project the crossings were carried out in 2020 and 2022, based on flowering which was extremely variable between one year and the next (Fig. 1). In 2019, 2021 and 2023 it was very scarce or completely absent. In 2020 the flowering was very abundant and the seed crop was large (mast year), allowing 27 crossing combinations to be carried out, obtaining 488 cones and approximately 60,000 seeds. In 2022 flowering was intermediate and 23 crossing combinations were carried out resulting in 389 cones and approximately 45,000 seeds. When carrying out the crosses, the genetic distance (co-ancestry) between the parent trees was considered, with the aim of favouring combinations between unrelated or less related plants.

The germination rate of the seeds was significantly lower in 2022 compared to 2020. The latter was a mast year that, in addition to favoring the production of a greater number of flowers, also seems to have favored the development of seeds and their germination. Therefore, the irregular flowering of *A. nebrodensis*, in addition to complicating the planning of crossings, also affects the germination capacity of the seeds.

Fig. 1. Controlled crosses among *A. nebrodensis* trees performed by a skilled technician.

3. Production of genetically selected plants in the nursery

For the conservation of threatened species affected by genetic erosion, it is important to increase the genetic diversity of the species. Selection of seedlings and their optimal growth in the nursery is crucial to obtain healthy, vigorous and improved stocks to be used for setting up the new repopulation nuclei.

Assays were carried out in the nursery to verify the germination rate of the seeds, incidence of disorders and mortality, and identify the biotic and/or abiotic factors eventually affecting plants, with the aim of implementing proper corrective measures.

Selection of full seeds. Based on the use of a X-ray device a procedure has been developed to assess the percentage of full seeds and to select viable seeds. In open-pollinated seeds collected in 2021 from 11 adult trees of the *A. nebrodensis* natural population the percentage of full seeds ranged between 0 (obtained for the tree no. 19) to 54% (obtained for the tree no. 7) with a mean value estimated as 31.7%. The use of full seeds allows an increase of the germination rate and of productivity.

Use of a standardized soil mixture. Chemical-physical analyses of soil samples suggested some adjustments to standardize the preparation of the substrate to make it perfectly suitable to *A.*

nebrodensis, as: a pH of 5.5-6.0, an adequate water and air retention capacity, high porosity, and a good concentration of organic matter. Using a standard substrate, a germination rate ranging from 20 to 80% was observed among the seed lots of the different mother trees.

Mycorrhization. Mycorrhiza is a beneficial, mutualistic relationship between a fungus and the roots of its host plant. This relationship is a natural infection in which the plant supplies the fungus with sugars and carbon and receives water and/or mineral nutrients in return. The hyphae of the fungus increase the root surface area of absorption from soil, benefiting the plants by providing access to large amount of water and nutrients (particularly nitrogen, phosphorus, zinc, manganese and copper), and increasing pathogen resistance, drought and salinity stress tolerance, water and

Fig.2. Inoculation of *A. nebrodensis* seedlings with a spore suspension of *Pisolithus arhizus* (left); greater development of an inoculated seedling (center) compared to an uninoculated one (right).

nutrient uptake, transplanting success and growth.

The Life4fir project has planned and implemented mycorrhization of seedlings obtained from artificial cross-pollination and raised in the nursery with a formulation prepared with the ectomycorrhizal Basidiomycete *Pisolithus arhizus* (Fig. 2a). The beneficial effect of mycorrhization on inoculated seedlings was visible as: longer root system (as the length of the primary root); longer seedlings, larger collar diameter, higher number of total root tips, higher percentage of active tips, higher mycorrhization index (ratio total tips/root length) (Fig. 2b).

Use of seedling trays for sowing. This type of container provides a series of benefits: easy handling in the nursery, good germination, high density of seedlings per square meter, low space requirement, and above all, less transplant stress (Fig. 3). The latter takes on considerable importance, as over 90% losses were previously recorded after seedlings were transplanted from pots. Actually, extraction causes frequently break of taproots with consequent death of seedlings.

Sowing in trays avoid this kind of problem as the root ball (roots and soil) remains intact and roots are not affected.

3.1 Plant health surveys in the nursery

The nursery activity plays a fundamental role to produce healthy, vigorous, and genetically selected plants, for use in reforestation plots. Assessment of incidence of disorders and mortality and identification of the biotic and/or abiotic constraints is required to set up proper corrective measures.

The health status of the plants in the local 'Piano Noce' forest nursery was surveyed at the beginning of the project. Incidence of disorders was recorded to control the impact of factors that can disturb the growth and vigor of the plants. Fungal isolations from affected twigs and needles were also performed. The presence of Oomycetes in the nursery was investigated through isolations and gene sequencing from soil and roots samples collected from a pool of plants showing related symptoms of the pathogen such as chlorosis and defoliation.

Surveys allowed the recording of the most frequently observed disorders: chlorosis; reddened

Fig. 3 *A. nebrodensis* seedlings growing in trays (left); healthy *A. nebrodensis* pot plants raised in the Piano Noce forest nursery (right)

needles; stunted growth; defoliation; blighted shoots; and small needles. Mortality rate was 2.4% in average. Overall, less than 8% of the surveyed plants showed symptoms. The disorders observed were scattered within the nursery. Isolations from soil samples and from the roots of the chlorotic and defoliated plants were negative for *Phytophthora* sp. and other pathogenic Oomycetes. The fungal microflora isolated from reddened or yellowed needles and from blighted shoots was found to be represented by saprophytes, endophytes and weak pathogens. Damages to needles and shoots seem to be due to predisposing external factors such as delayed transplants or direct sun light exposure that favor the action of weak pathogens (opportunistic), often already present within asymptomatic tissues as endophytes. The regular execution of transplants should be considered as a useful measure to reduce occurrence of chlorosis, stunted growth and mortality.

4. Monitoring and control of biotic and abiotic stressors

Monitoring the health state of natural forest areas provides basic knowledge about occurring threats, as well as proper protection and conservation measures, providing clues to understand the influence of disturbances, such as climate change and invasive species on forest ecosystems. As consequence of climate change there is concern that tree mortality will increase due to physiological stress, insect outbreaks and wildfires driven by future climate change, particularly affecting Mediterranean forest ecosystems.

For conservation of threatened species, monitoring is essential to detect trends in abundance and distribution through time, measure the impacts of threatening processes and evaluate the effectiveness of management responses.

In the framework of the Life4fir project it has been useful to describe the impact of disorders, investigating on their causes and monitoring their evolution in relation to the environmental conditions and climate change. The project has planned to carry out health state surveys surveys on the natural population of the Madonie fir to detect and monitor the occurrence and extent of biotic or abiotic disturbances and to eventually implement proper control measures. Surveys on plant health were based on the visual inspections on single trees alongside multispectral analysis to monitor eventual physiological disorders at the whole crown level.

Tree inspections and samplings. Careful visual examination was aimed at evaluating tree disorders based on evaluation of crown shape and transparency, turning foliage, presence of damaged crown portions due to blight, diebacks, necrosis (cankers), and occurrence of lesions on trunk and branches (Fig. 4). Disorders observed on the crowns were separately described, recording the type of affected organ (trunk, branches, twigs, shoots, needles), the extent and position of the crown involved, the impact in terms of percentage of damaged crown.

Isolation and characterization of fungi. Laboratory analyses were conducted on samples of twigs and needles through observations under stereomicroscope, isolation and culture of fungal colonies and their genetic characterization through PCR and sequencing of target loci. This allowed identification of the fungal microflora associated with the observed disorders and eventually detect the occurrence of harmful pathogens. The surveys carried out allowed the plants of the natural population to be grouped based on the degree of the damaged foliage, the position of the symptoms in the crown, and the main types of symptoms observed (reddened shoots and twigs, defoliation, wounds due to herbivores, little leaf and chlorosis, desiccation of twigs and small branches). Fungal isolations allowed insights into the fungal community in relation to the observed disorders. Presence of aggressive pathogens was excluded, as all isolated fungi were weak pathogens, endophytes or saprophytes, whose development in the trees was associated with environmental constraints to which the trees are subjected in their natural habitat, as: strong winds, summer drought, late frosts, high temperatures during summer, in addition to the damage caused by wild herbivores, in particular fallow deers that have massively reproduced in the Park territory in the last years.

The fair health state of *A. nebrodensis* trees that emerged from the last surveys in the natural population, as well as from previous surveys, depicted a sort of tolerance of the species to the strict environmental condition it lives in.

Fig. 4. Sporadic necrotic branches were observed, especially on the trees growing in harsh sites. Necrotic tissues were found to be colonized by weak pathogenic and saprophytic fungi.

4.1. UAV Multispectral surveys

The loss of the forest is encouraging the use of new technologies to detect areas where different sources of stress are making an impact. Drone technology can be used to evaluate the health of the forest using different high-resolution images, enabling a more cost-effective approach. Biotic or abiotic stresses on trees involve their physiological and biochemical disorder which in turn modifies the radiation absorbed or reflected by the crown. Multispectral cameras will measure the visible and near-infrared radiation reflected by plants. A survey of the *A. nebrodensis* trees and the surrounding vegetation by remote sensing technology based on the use of a drone equipped with both a RGB digital camera and a multispectral camera was carried out in the project. Drone flights were conducted in October 2020 and August 2023. Using the captured images, reflectance maps were created and different vegetation indices have been obtained. The 3D reshaped DTM (Digital Terrain Model) of the *A. nebrodensis* natural range allowed to report the distribution of trees in relation to the morphology of the territory. The high reflectance potential of the leaves in the NIR allowed the evaluation of the defoliation of the forest through these sensors. The infrared images showed in red the reflectance of the cover canopy of the trees based on water and chlorophyll absorption in the leaf. Various shades of red were related to type, health, leaf structure and moisture content of trees. Multispectral images were analyzed for the production of a NDVI (Normalized Difference Vegetation Index) map (Fig. 5). NDVI is an indicator that describes the greenness, the relative

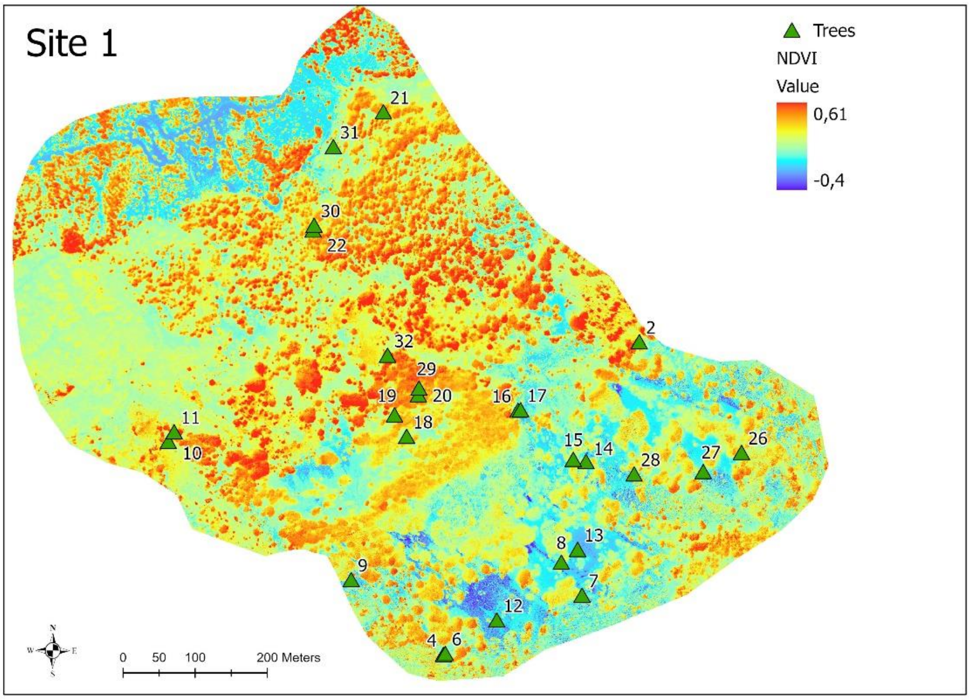

Fig. 5. NDVI map of the area in the Vallone Madonna degli Angeli, hosting the main nucleus of the *A. nebredensis* population.

density and health of vegetation for each picture element, or pixel, in a drone image. The maps obtained are useful for conducting a comparative investigation of the health state of the single trees in relation to environmental conditions through a spatial autocorrelation analysis. By carrying out a second drone survey after 3 years from the previous one, multispectral maps were also useful to monitor the evolution over time of the health state of trees as a function of climatic fluctuations and of the protection measures implemented meanwhile.

6. Protection measures in situ

6.1 Support to the natural regeneration: mapping and fencing

Natural regeneration is a biological process that can be assisted and managed to increase forest cover and achieve the recovery of the native ecosystem or some of its functions. Various factors limit the growth and establishment of natural regeneration of the *A. nebrodensis* population: the superficial and rocky soils, the irregular flowering and fruiting over the years, the high rate of self-fertilization and the high percentage of empty seeds, the impact of wild herbivores. For the conservation of the species, it is therefore necessary to protect and support the natural regeneration of *A. nebrodensis* which, based on the surveys carried out, was found to be slowly spreading (484 seedlings registered in 2020, compared to 274 in 2014 and 80 in 2000). Census and mapping of the young plants of the natural regeneration were carried out to trace them and monitor their evolution as well as to optimize the protective function of the new fences (Fig. 6). To carry out exhaustive survey a series of parameters were recorded for each seedling/plantlet: distance in meters and the azimuth angle from its respective mother tree, GPS position, height (cm), age, growth and health state. The collected data were used for the implementation of a comprehensive database and 15 maps, one for each mother tree. The data acquired, with the exact position of the seedlings around the mother trees, were of great help for the placement of the new protective fences around each mother tree.

Fig.6. Mapping the natural regeneration.

6.2 Protection of the relic trees with a system of fences

The installation of adequate fences is a necessary measure to protect the trees of the *A. nebrodensis* population from the impact of wild herbivores and anthropogenic pressure (Fig. 7). The first system of fences for the protection of the *A. nebrodensis* trees dated back to the 1950s and due to its deterioration over time, it was replaced with new fences built in the following years. The Life4fir Project planned the extension and strengthening of the fences around the *A. nebrodensis* trees to meet three basic needs: 1) most of the extant fences (which dated back to 2004) showed again signs of deterioration and were damaged by the increased populations of fallow deers and wild boars, having lost much of their functionality; 2) the extant fences were often overrun by fallow deers and therefore it was necessary to replace them with taller fences; 3) many seedlings of the natural regeneration were found to grow outsides the perimeters of the extant fences and adequate. protection measures were needed.

Fig 7. The new fence built around the *A. nebrodensis* tree n. 6

So, a new fence system has been planned and installed to strengthen and enlarge the 'protected area´ for the relic trees of the population and the natural regeneration. The new fences are meant to protect the *A. nebrodensis* population also from anthropogenic pressure and from the numerous visitors who walk the paths of the Park reaching the trees and their surroundings. The area protected by the new fences has been increased to 14.000 sq.m. This will ensure the maintenance of the microhabitat, will preserve the biocenosis around each tree and will consequently favor the development of the natural regeneration also preventing wild herbivores to damage trees with their bites and horns.

7. Repopulation with selected seedlings

One of the core actions LIFE4FIR project is aimed at the creation of *A. nebrodensis* repopulation nuclei using outbred seedlings obtained from controlled crosses, raised in the 'Piano Noce' nursery in Polizzi Generosa. Objective of this action is to improve the gene pool and promote the future dynamism of the species in relation to different biotic and abiotic pressures. Ten sites have been selected for reforestation within the Madonie Park based on ecological, pedological and topographic characteristics suitable for the species. The choice also took into consideration the outcomes of the reforestation plots of *A. nebrodensis* set up in previous projects. The selected sites are mainly included between 1100 and 1600 m a.s.l. elevation, characterized by mesophilic deciduous formations of the following climax associations: *Quercion ilicis*, *Quercion roboris* and *Geranium versicoloris - Fagion*. Only two plots are located at a lower elevation, between 750 and 850 m a.s.l. In these areas, however, the microclimatic conditions appear however potentially suitable to the development of *A. nebrodensis*. The sites located between 1100 and 1400 m a.s.l. elevation are part of the climax association of the *Ilici aquifolii-Quercetum austrotyrrhenicae,* a relict forest association of considerable geobotanical interest, established on quartz arenitic substrate. The plots located above 1400 m a.s.l. elevation fall within the woods of beech, related to *Geranium versicoloris-Fagion* climax association. The reforestation sites were also chosen based on the possibility for *A. nebrodensis* to move uphill under the pressure of climate change.

Based on the ecology of the species, most of the selected sites have a North or North-West slope and are characterized by continuous or partial tree cover under which the young *A. nebrodensis* plants will be adequately protected. For the success of the new plantings, a few measures have been taken to provide seedlings with the necessary support. Each area was previously delimited with 2 m high fences, buried 50 cm, to prevent the entry of wild boars. The plantation was carried out following a series of precautions. The holes have a truncated cone or pyramid shape (with the base wider than the upper opening) to encourage root development and increase the water reserve. Spacing, where possible, was quincunx with a distance of 3-4 m between the plants, while in the rocky points, holes had not a regular spacing.

The holes will be made north of the pre-existing tree vegetation to guarantee greater protection for the seedlings (Fig.8). An organic fertilizer rich in phosphorus was administered at the time of planting. Leguminous plants of the genus *Genista* were planted near the *A. nebrodensis* seedlings to improve soil fertility and ensure further protection to the young firs from both excessive sunlight, heat and summer drought after having been transplanted. The fast-growing shrubs help to maintain suitable microclimate conditions for the development of seedlings in the spring-summer period and to protect them from strong winds.

Fig. 8 Reforestation nucleus carried out in Favarotti. Position of holes where seedlings were planted out (left); a small newly planted seedling (right).

8. Ex situ germplasm conservation

The ex-situ conservation of the *A. nebrodensis* germplasm has been planned by the Life4fir project to achieve different purposes: the creation of a clonal orchard, the seed bank and the cryobank. These measures play a fundamental role in preserving the genetic heritage of *A. nebrodensis*.

8.1 Clonal orchard

The setting up of a clonal orchard has been planned by the Life4fir project. Main objective of the clonal orchard is gene conservation, ensuring the long-term enhancement of the genetic diversity presently available to meet future needs. For *A. nebrodensis*, the clonal orchard is meant not only as a simple germplasm collection, but also as a source of seed with increased genetic variability in the future, since crossing between the different genotypes can eventually occur. This will lead to produce seed with a broadened genetic base. The clonal orchard will also allow a constant monitoring of the single tree genotypes (as concerning growth, phenology ecc.), for scientific aims and teaching. In the future, when the plants will reach their maturity, the orchard will be used as a new source of seed on behalf of the original trees, avoiding the negative impacts due to the repeated harvesting from the natural population. To establish this collection of clones, each individual genotype of the *A. nebrodensis* natural population had to be vegetatively propagated.

Grafting propagation

In conifers, vegetative propagation for the reproduction of 'plus trees', selected or endangered trees is carried out both by cutting or grafting. Cutting propagation is not always effective with conifers. Tests conducted in the past had already highlighted the low rhizogenic potential of *A. nebrodensis* cuttings. A sound alternative is grafting propagation, where a portion of the shoot is grafted on a seed rootstock, suitably prepared. For the propagation of *A. nebrodensis* a 'veneer-side graft' procedure has been fine tuned. In this type of graft, a 10 cm long apical portion of a branch is prepared with a 'pen' cut of the base and this is inserted into a 'pocket' produced on the rootstock, in a lateral position; the graft is then suitably tightened with an elastic to facilitate contact of the regenerating parts (cambium of both rootstock and scion) and the grafted point protected from dehydration with aluminium foil. During the first weeks, grafts are maintained under plastic bags until graft healing occurs. This technique was successfully used for the vegetative propagation of the *A. nebrodensis* trees of the natural population to be used for establishing the clonal orchard. A survival rate over 50% after 1 year from grafting had never been achieved before (Fig. 9).

Fig. 9. Looking after the *A. nebrodensis* grafts in the greenhouse.

8.2 Seed bank

Seed Banks represent the most used ex situ conservation system for the conservation of plant biodiversity. To date, there are about 1750 seed banks in the world, managed by public and private institutions. Seed banks operate at a temperature of -18° C, keeping the seeds inside glass or plastic containers. The maximum storage time strictly depends on the species, but it frequently reaches tens of years, during which germination and viability tests are periodically repeated.

Seeds of *A. nebrodensis* are being preserved in the Seed Bank, established at the MAN (Museum of Abies nebrodensis) in the Polizzi Generosa municipality building.

Fig. 10. The *A. nebrodensis* seedbank established at Polizzi Generosa for the conservation of seed germplasm at -18°C.

For *A. nebrodensis*, the large rate of empty seeds, represent a real problem for preservation. The selection of full and viable seeds represents a fundamental step for their conservation. Therefore, we applied an X-ray test procedure to detect and remove empty seeds. This allowed to implement the seed bank with only full and viable seeds.

The ripe cones were collected in October. Seed were then extracted and conserved at 4°C for a short time. After X-ray analysis, only full and healthy seed were selected for storage. Before being stored, the selected seeds were subjected to viability test and in vitro germination assays on solid medium. Since the seed moisture content is critical factor to determine the success of seed bank storage, it was evaluated (6-8%) by Moisture Analyzer (Mettler-Toledo). The seeds were then weighted and placed in labelled jars reporting all the data of the stored seeds: location of seed bank; species; tree id number; collection year; quantity (gr); number of seeds; starting date of conservation. Jars were stored in a freezer chamber (-18°C) with the following specifics: two transparent glass doors for easily inspection of the content; temperature up to -20° C for storage of seeds; wheels and safety lock with keys; internal equipment: nr. 6 shelves; internal lighting with led tubes, with automatic activation both at each door opening and by means of a special button on the control panel placed in the upper part of the structure. The seed bank was established in a room provided with all the due safety devices.

8.3 Cryobank

Cryopreservation, i.e. the storage at ultra-low temperatures such as that of liquid nitrogen (-196°C), is the most innovative method which enables long-term conservation of plant genetic resources. This technique preserves organs and tissues by means of an ultra-fast cooling process that, if properly developed and well adapted to the specific plant specimen, arrests almost all metabolic processes in the cell, while preserving its structure and biological functionality. Arrest of cellular metabolism produced by cryogenic temperatures make cryopreservation a safe method in terms of the genetic stability of the stored material. For the above, cryopreservation represents an important additional option for the conservation of *A. nebrodensis* genetic resources. Various procedures have been developed for the preservation of excised embryos, pollen and embryogenic callus samples, the latter included in synthetic seeds, from as many as possible of the 30 trees of *A. nebrodensis*. A Cryobank has been established at the MAN (Museum of Abies nebrodensis) in Polizzi Generosa. Staff with adequate skills is employed for management of the cryo-bank.

Pollen. Mature anthers were collected from *A. nebrodensis* trees during May. The collected pollen grains were sieved and morphologically characterized at stereomicroscope, optical microscope and ESEM. The pollen moisture content was measured by Moisture Analyzer instrument using 0.2 g of pollen. For cryopreservation, no desiccation process was needed since a water content of 8-10% was reached after a storage of three days at 4°C. The pollen samples were then transferred into cryovials and immersed directly into liquid nitrogen (-196 ^{0}C). The cryovials containing pollen were thawed under a laminar flow cabinet for 2 hours at room temperature. To validate the protocol, viability of cryopreserved pollen was assessed with TTC test and in vitro germinability assessment on solid substrate, before and after cryopreservation.

Zygotic embryos. Ripen female cones were harvested in October and then dried under a controlled environment. Mature seeds were cleaned and maintained at 4° C for a short time. The mature seeds were washed with tap water, disinfected with ethanol (70%, 1 min), sodium hypochlorite (2%, 20 min) containing a few drops of Tween 20%, and rinsed in sterile distilled water. Seeds were then imbibed in water for 48h under sterile condition and opened to extract the zygotic embryos (Fig. 30). The moisture content of excised embryos was determined by a Moisture Analyzer, considering <10% as an optimal level of humidity for conservation in liquid nitrogen. The excised embryos were inserted in cryovials, treated with Plant Vitrification Solution 2 or not, and transferred inside cryoboxes before the immersion in liquid nitrogen at -196 ^{0}C.

All the cryovials after thawing in a water bath (1 min at 40 °C), were placed under the laminar flow and PVS2 solution was removed and replaced with washing solution (liquid MS medium with 1.2 M sucrose) for 20 min. To validate the protocol, TTC and in vitro germination tests were applied on cryopreserved zygotic embryos to estimate the viability and in vitro germination rate on Murashige and Skoog (MS) hormone-free medium.

Fig.11. samples of pollen, zygotic embryos and embryogenic callus lines conserved in the *A. nebrodensis* cryobank (left); the launch of the cryobank in the presence of mayor of Polizzi Generosa (right).

Embryogenic callus lines. For *A. nebrodensis*, embryogenic callus lines were obtained with the induction from mature zygotic embryos. Embryos were excised from mature seeds harvested in October 2020, from the following adult trees: 6, 7, 8, 10, 12, 13, 19, 21, 22, 27 and placed horizontally in Petri dishes. Embryogenic callus was obtained, for the first time in this species, only in SH medium supplemented with a cytokinin (1 mg/L BAP). After 8–12 weeks on initiation media in the dark, callus was separated from the embryo and transferred to a fresh medium as individual cell lines for proliferation. Embryogenic tissue (ET) was cultured in the dark at 25 °C and subcultured onto fresh medium every 15 days. When sufficient amount of ET developed, individual cell lines were transferred onto a maturation medium (SH with abscisic acid 10 mg L-1, polyethylene glycol 8% and maltose 40%). Cultures were transferred to fresh media every 2 weeks and kept at 25° C in darkness. ET formation was continuously monitored under microscope, along with the development of somatic embryos.

The encapsulation/vitrification technique was applied for the cryopreservation of the embryogenic callus lines. This procedure is based on encapsulation in sodium alginate of the callus portions and in the subsequent treatment with the ‘Plant Vitrification Solution 2’. The callus propagules coated with this procedure showed the ability to proliferate after the encapsulation process.

9. Conclusions

The measures implemented in situ for the protection of *A. nebrodensis* within the Life4fir project have a multidisciplinary character to help the species cope with its vulnerability factors: the production of genetically selected seedlings and their use in new reforestation sites, the protection of the natural population with new fences, the video surveillance system, the phytosanitary monitoring with visual and multispectral analyses. To these are added the forms of protection implemented by ex situ measures, such as the seed bank and the cryobank. While the latter are able to produce their effect from the moment they are put into operation, the ex situ measures are destined to produce effects in the medium to long term and their results will be appreciable in the years to come. However, these measures represent a solid basis of good practices that it is necessary to continue, manage and develop after the end of the project through various forms of financing, starting from initiatives of the regional administration and the Park Authority. The protection of the Madonie fir requires a continuity of interventions that ensure the improvement of the habitat and reduce the vulnerability of the species.

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