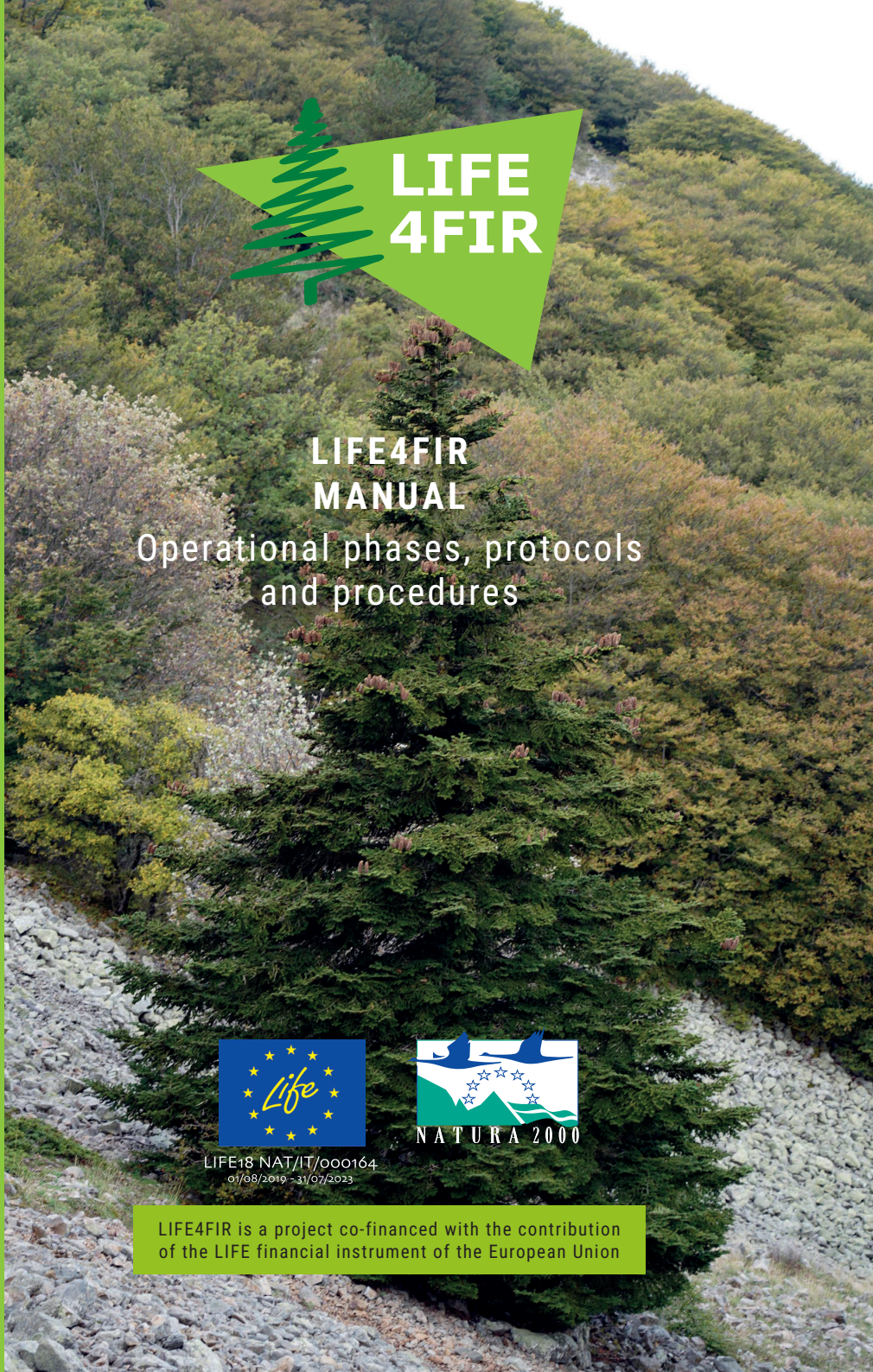

LIFE 4FIR

LIFE4FIR MANUAL

Operational phases, protocols and procedures

LIFE18 NAT/IT/000164
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1. GENETIC ANALYSES FOR CONSERVATION MEASURES

One the main activities of the LIFE4FIR project has been the evaluation of the genetic variability and the genetic relationships among the 30-adult trees and the 118 juvenile plants from the natural population of *Abies nebrodensis*. Knowledge of these aspects is pivotal for the conservation of the species through active management (genetic rescue) based on controlled crossings.
SNPs genotyping was used to perform the genetic characterization of both the adult trees and juveniles of the natural population. Then, paternity tests were carried out on the seedlings to determine the rate of outcrossing (cross between unrelated individuals), 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). Steps followed to achieve the aims of this actions are detailed herein.

1.1 SNPs selection.

High-quality and informative SNPs were identified using restriction site associated DNA sequencing (RAD-Seq). The PCR-based OpenArrays technology (Thermofisher Inc., United States) was used for SNP genotyping. A panel of 120 SNPs was developed for genotyping of *A. nebrodensis* individuals composed by information-rich SNPs in samples of *A. nebrodensis*, A. alba and A. cephalonica.
Among these, 20 SNPs were selected for their power to discriminate putative hybrids between *A. nebrodensis* and the other two Abies species through a PCoA (Principal coordinates analysis).
The remaining 100 SNPs were selected for analyzing the genetic structure of the population and for paternity tests.

1.2 *Abies nebrodensis* genotyping, analysis of the genetic diversity and structure of *A. nebrodensis*.

All the mature trees (n=30) and young individuals over 5-year old (n=118) in the natural population of *A. nebrodensis* were sampled, collecting a few needles. All these samples were preserved in silica gel

until the subsequent DNA extractions.

Population structure.
We used the Discriminant Analysis of Principal Components (DAPC), performed by using the "adegenet" package for the R software v.4.0.3, to study the population subdivision in *A. nebrodensis*.

Inbreeding level.
Based on the estimated pedigree, the inbreeding level of the population was assessed by estimating the inbreeding coefficient (Fis) of the population using the COLONY software. Furthermore, 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 estimated.

Genetic diversity.
The genetic diversity of adult trees was evaluated by calculating the following coefficients: PHt (proportion of heterozygous loci in an individual), Hs (Standardized heterozygosity based on the mean expected heterozygosity), IR (internal relatedness), HL (homozygosity by locus) and INBR (inbreeding coefficient). These coefficients mainly reflected the moderate homozygosity of *A. nebrodensis* plants due to inbreeding and selfing.

Genetic relatedness among trees.
The Ritland estimator (RIT) of pairwise co-ancestry between adult individuals was calculated to know the genetic relatedness of the 30-adult trees of *A. nebrodensis*. For genetic management of the population, outcrossing of individuals with more negative RIT values were detected and a list of the 30 most recommended crosses between mature trees of *A. nebrodensis* was reported. These indications were followed to increase genetic variability in the progenies through controlled crossings.

	PHt	Hs_exp	IR	HL	INBR
13M	0.49	1.13	-0.12	0.50	-0.20
22M	0.42	0.97	0.02	0.57	-0.02
29M	0.42	0.97	0.01	0.57	-0.01
12M	0.40	0.93	0.13	0.57	-0.01
14M	0.40	0.93	0.09	0.59	0.02
26M	0.40	0.93	0.08	0.58	0.04
10M	0.39	0.90	0.13	0.61	0.17
18M	0.39	0.90	0.11	0.61	0.12
11M	0.36	0.83	0.15	0.61	0.11
17M	0.36	0.83	0.17	0.63	0.10
20M	0.35	0.81	0.15	0.63	0.06
23M	0.34	0.79	0.16	0.63	0.07
8M	0.34	0.79	0.29	0.64	0.57
2M	0.33	0.76	0.23	0.65	0.15
9M	0.33	0.76	0.23	0.66	0.12
15M	0.32	0.74	0.22	0.67	0.08
24M	0.32	0.74	0.22	0.66	0.13
25M	0.32	0.74	0.23	0.66	0.18
4M	0.31	0.72	0.28	0.67	0.25
6M	0.30	0.69	0.27	0.68	0.20
7M	0.30	0.69	0.31	0.69	0.20
28M	0.28	0.65	0.34	0.71	0.17
1M	0.26	0.60	0.37	0.74	0.25
27M	0.26	0.60	0.39	0.73	0.31
21M	0.25	0.58	0.44	0.75	0.55
32M	0.25	0.58	0.45	0.74	0.36
30M	0.21	0.49	0.54	0.78	0.61
19M	0.18	0.42	0.63	0.83	0.79
16M	0.16	0.37	0.59	0.83	0.40
31M	0.14	0.32	0.68	0.86	0.65

Table 1-1. Genetic diversity estimations of adult trees. PHt (proportion of heterozygous loci in an individual), Hs (Standardized heterozygosity based on the mean expected heterozygosity), IR (internal relatedness), HL (homozygosity by locus) and INBR (inbreeding coefficient). Colors of coefficients range from lower (dark blue) to higher values (yellow)

1.3 Paternity test of natural regeneration plants of *A. nebrodensis*.

Paternity tests were carried out on natural regeneration seedlings/saplings to determine the rate of outcrossing, inbreeding and self-fertilization and to assess the eventual hybridization due to pollen coming from non-native Abies species. Using the COLONY software paternity tests allowed the origin of all young individuals to be inferred. For some seedlings, only one of the progenitors in the population was inferred and consequently they were be considered putative hybrids with other Abies species. To shed light on the possible hybrid origin of these individuals, a Principal Component Analysis (PCA) was carried out using 95 loci present in *A. nebrodensis*, A. cephalonica and A. alba. This allowed the potential hybrids to be genetically verified regarding their closeness to the three fir species.

1.4 Genetic characterization of pot plants raised in the local nursery.

Saplings raised in the local nursery before the start of the project represent the first selection step of plants to be used for reforestation, thus it is important to disentangle their genetic origin and to determine the rates of outcrossing (cross between unrelated individuals), 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 (namely, Abies alba and A. cephalonica). Following the SNPs genotyping, 2064 young individuals of *Abies nebrodensis* from the local nursery, derived from open pollination of 8 mother trees were genetically characterized. Paternity tests were carried out using the COLONY software.

The inbreeding level of the analyzed seedlings was estimated by calculating the inbreeding coefficient (Fis). The effective population size (Ne) was also calculated to estimate the number of individuals that effectively contributes offspring to the next generation. These findings were useful for selecting nursery seedlings to employ in the new reforestation plots.

1. Seedlings genetically characterized:	**1776 (86.05% of total seedlings)**
a) Seedlings whose parental origin was confidentially inferred:	1525
a.1) Both parents are *A. nebrodensis* adult-trees:	897
a.2) One of the parentals is *A. nebrodensis*, but the other one is unknown (i.e. another *Abies* species):	345
a.3) Both parents are unknown (i.e. another *Abies* species):	283
b) Seedlings whose parental origin could not be confidentially inferred:	251
2. Seedlings that could not be genetically characterized:	**288 (13.95% of total seedlings)**

Table 1-2. Results of the genetic characterization of the 2064 young plants of *Abies nebrodensis* sampled in the local nursery Piano Noce

2. INCREASING GENETIC VARIABILITY IN *A. NEBRODENSIS*

Small, isolated populations risk extinction due to inbreeding depression, random loss of beneficial variation, and reduced adaptability to environmental change. The decreased genetic variability can in fact lead to a reduced ability of populations to adapt to the new challenges proposed by their environment, such as infectious diseases or climate change.
Genetic rescue is the increase in population size through the movement of new genetic material from one population to another. This can happen through human-assisted intervention or natural migration. As a conservation tool, this strategy can increase the genetic diversity of small, isolated populations and help them recover from inbreeding.
In the case of *Abies nebrodensis*, the fragmentation of the population and the isolation of the plants favors self-fertilization with deleterious effects for the progeny and the evolutionary possibilities of the species. A controlled pollination plan has been developed in the LIFE4FIR project to promote outcrossing between isolated plants and encourage the new genetic combinations. Crossings should take into consideration factors such as increased reproductive fitness in terms of fruit set, number of seeds, germination rate and seedling survival as open pollination mainly leads to self-fertilisation.

2.1 Making controlled crosses.

Monitoring and detection of the beginning of full flowering.
In the natural population of *A. nebrodensis* full flowering generally occurs at the beginning of May. Therefore, from the second half of April, adult *A. nebrodensis* trees must be monitored by following the opening of the flower buds and the development of the reproductive structures until full flowering. Flowering occurs stepwise, also depending on elevation; furthermore, it can be extremely variable from one year to the next. Generally, abundant flowering (mast seeding) occurs every 4 years, interspersed with years in which the production of flowers is poor or completely lacking and years in which it is partial (not very abundant).

Isolation of female cones.

Before the opening of the male cones and of pollen release, female cones were isolated (covered) with terylene bags, a special hydrophobic paper material, which at the same time allows transpiration. Bags were closed at the base using soft rubber tape around the twigs.

Fig 2-1 Isolation of female cones with special terylene bags to perform controlled crosses

Collection of mature male cones.
Mature male cones (close to flowering) were collected separately from individual trees in the stand and stored in paper bags until used on female cones.

Pollen delivery.
At the beginning of the opening of the female cones, the isolating bags were opened for the insertion of the mature male cones inside them, separately according to the crossing combination. Typically, 10-15 male cones were inserted for each female cone. The natural movement of the bag due to the wind favored the dispersion of pollen from the cones inside the bag allowing the pollination of the female cones.

Crossbreeding combinations.
Pairings were carried out based on the results of the pairwise co-ancestry estimation between the plants of the natural population. With the aim of fostering crosses between plants with greater genetic distance. In May 2020 174 bags were used and 27 parental combinations were performed. In 2022, 23 crossing combinations were performed using 121 bags. The development of cones inside the bags was monitored during the summer at 15-day intervals.

Collection of cones and seeds.
Maturation of cones occurred regularly, and the bags were removed at the end of September to separately collect the cones before their disarticulation to extract seeds in October.

3. OPTIMIZED PRODUCTION OF SELECTED SEEDLINGS IN THE NURSERY

One of the pivotal actions of the LIFE4FIR project has been the production of seedlings from selected seeds obtained with controlled crosses and the raising of the seedlings in optimal conditions in the nursery to obtain a vigorous and improved stock to be used for reforestation. To attain this objective a series of diversified activities was implemented in the nursery.

3.1 Evaluation of health state of plants growing in the nursery.

Phytosanitary survey. At the beginning of the project, a phytosanitary survey was conducted on the pot plants already growing in the nursery before production of new selected seedlings was started. For each mother plant and year of sowing, the progenies were subjected to visual inspection, recording the mortality rate and the types of eventual symptoms observed, their frequency and impact (portion of damaged crown). This led to obtaining a picture of the size of each progeny and the frequency of the main symptoms observed.

	sector 1		sector 14		sector 15		Tot.	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
mortality	459	5,04	7	0,34	142	1	608	2,4
reddened needles	145	1,6	10	0,5	117	0,81	272	1,07
chlorosis	190	3,82	17	0,84	764	5,35	971	3,82
defoliation	9	0,1			35	0,24	44	0,17
small needles					6	0,04	6	0,02
blighted shoots	12	0,13	4	0,19	8	0,08	24	0,1
stunted growth			47	2,32	64	0,45	111	0,43

Table 3-1. Impact (as number and percentage) of the main symptoms observed on the aerial part of the saplings raised in the three nursery sectors

Sampling and analysis of the presence of pathogens.

In case of foliage disorders, samples of needles and twigs were taken from the symptomatic plants for further observations in the laboratory. Samples were carefully inspected to define in detail the signs of colonization by pathogens (tissue reaction, development of fungal fruiting bodies, etc.) and for the in vitro isolation of fungal microorganisms. Isolations were carried out from needles (reddened,

completely or partially necrotized) and from blighted shoots, after surface sterilization. Samples were then placed in PDA (Potato Dextrose Agar) plates, incubated at 25°C in the dark. The outgrown colonies were subcultured and then grouped into morphotypes based on their cultural characteristics. Morphotypes were identified through sequencing of specific regions of genomic DNA using the primers ITS1 and ITS4.

Fig 3-1 Symptoms of reddened and blighted needles observed in the young A. nebrodensis plants raised in the local nursery

Fig. 3-2 Fungal colonies grown on PDA plates, isolated from affected needles collected un the nursery

Evaluation of the presence of soil pathogens.

Soil of potted plants can host pathogenic microorganisms (oomycetes, fungi, bacteria, nematodes). Presence of very dangerous soil oomycetes, such as Phytophtora sp, cannot be overlooked in a nursery environment. This oomycete attacks the root system causing general deterioration of the whole plant (stunted growth, chlorosis, defoliation). Particular attention should be paid when symptoms of decline and death spread across an area or plot of the nursery, affecting many contiguous plants. For this reason, it is advisable that soil and root samples taken from the pots of symptomatic plants are analyzed.
The presence of Phytophtora sp. was determined through traditional methods that use bait for direct isolation from soil, organic debris and roots. Its identification was achieved with amplification and sequencing of the ITS locus.

Ecophysiological parameters and irrigation.
To optimize plant irrigation, the study of water relations (components of the xylem water potential of plants) allows us to establish the type of strategy that the plant adopts to respond the water deficit and, at the same time, identify simple and effective indicators to be used to the management of water resources. Pressure-volume (P-V) curves allow us to describe the relationship between the total water potential (Ψt) and its components as a function of the relative water content (R) of living organisms. These parameters help establish the correct quantity of water to be administered and the method of supply.
Soil analysis. The chemical-physical analyzes of soil samples allow us to verify the characteristics of the mixture used for germination and pot cultivation of plants and evaluate changes compared to the optimum of the species. The pH parameters; electrical conductivity (EC); organic matter and total carbonate content allow the properties of the soil to be defined. The soil samples taken from potted plants in the Piano Noce nursery were found to vary especially in salinity levels (EC) and total carbonate content. The need therefore emerged to use a standardized and optimized growth substrate for *A. nebrodensis*.

3.2 Measures to improve germination and seedling growth.

Selection of full seeds (with viable embryo). Through the use of an X-ray device (Gilardoni radiolight), a procedure was developed at IBE-CNR to select full seeds.

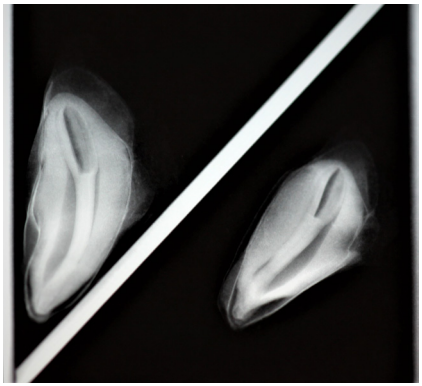


Fig 3-3 Healthy, full seeds (with embryo inside) of A. nebrodensis, photographed under Rx

In seeds obtained from open pollination collected in 2020 from 11 adult trees of the natural population of *A. nebrodensis*, the percentage of full seeds ranged from 0 (obtained for tree no. 19) to 54% (obtained for tree no. 7) with an estimated average value of 31.7%. The use of only full seeds allows better results from the sowings carried out.

Use of a standardized substrate.
A pot cultivation system for *A. nebrodensis* was developed that took into account standardized physical and chemical parameters capable of improving both seed germination and seedling growth. Among the different substrates tested, the best results were obtained with the complete Vigorplant soil, added with Agriperlite (in the ratio 70 lt + 10 lt), with which a germination percentage ranging from 20% to 80% was observed between seed lots of different mother plants, with an average of 31.4%.

Use of seed trays.
This type of container allows some benefits: easy handling in the nursery, good germination, high density of plants per unit area, reduced space requirement and, above all, less transplant stress for the seedlings. The latter point is of fundamental importance, as over 90% of losses in the nursery occurred immediately after transplanting. In fact, extraction may cause frequent breakages of the roots resulting in the death of the seedlings. Sowing in trays avoids this type of problem as the root ball (roots and soil) of the seedlings remains intact when extracted.

Mycorrhization.
The Basidiomycete Pisolithus tinctorius (Pers.), known as an ectomycorrhizal symbiont in A. alba and A. chephalonica, was used for mycorrhization of seedlings. One-year-old seedlings were inoculated in December at the time of transplanting, using 20 ml of a P. tinctorius spore suspension at a concentration of 107 spores/ml per plant. The inoculated seedlings were transferred to the greenhouse and regularly watered to accelerate fungal colonization.

Fig 3-4 After being transplanted, seedlings were treated with a suspension of the mycorrhizal fungus Pisolithus arhizus

At the end of the following summer, the evaluation of the effectiveness of mycorrhization was carried out. One-year-old seedlings were transplanted from the germination tray, and were transferred into 8 x 12 cm pots containing the same growth substrate (Vigorplant + agriperilte). The seedlings were kept in the new container for at least another year until they were planted out in the reforestation plots. At the end of the following summer, effectiveness of mycorrhization was evaluated. The inoculated seedlings showed intense green needles and better vegetative growth, with the production of the first lateral branches, compared to the non-inoculated seedlings. On average, inoculated seedlings were taller than non-inoculated ones (about 1.5 cm difference), had a thicker root collar (2 mm vs. 1 mm) and a higher Mycorrhization Index (MI) (7.84 vs. 6.25). The dry weight of the inoculated seedlings (both the epigeal part and the root system) was also 2-3 times greater than that of the non-inoculated controls.

4. MEASURES TO SUSTAIN THE NATURAL POPULATION

4.1 Monitoring and health survey.

Evaluating and monitoring the state of health of the natural population provides useful knowledge about occurring disorders and can assist in managing proper protection and conservation measures. The phytosanitary survey of *A. nebrodensis* population is one of the pivotal measures for assessing the health state of trees.

Crown inspections.

The 30 relic trees of *A. nebrodensis* were subjected yearly to a careful visual examination to evaluate their state of health crown shape and transparency, turning foliage, presence of dying, dried or damaged parts, occurrence of lesions. Disorders observed on the crown were described by recording the type of affected organ (trunk, branches, twigs, buds, needles), the portion of the crown involved, ideally divided into three parts along the longitudinal axis (lower, intermediate and upper third), the direction (north, south, east and west), the impact in terms of percentage of damaged crown and number of blighted or reddened twigs per unit area. The results of crown inspections were put in relation to the environmental conditions that trees are facing at a microclimate and site level.

Fig 4.1 Crown of A. nebrodensis tree showing reddened needles, needle cast and blighted shoots

Sampling and fungal isolations.
In the natural stand, 4 twigs showing needle reddening and blight were sampled from the trees showing crown disorders. Healthy twigs showing no symptoms, adjacent to the symptomatic ones were collected alongside. Ten needles were detached from each sampled twig for fungal isolations. Overall, 250 reddened and 250 green (healthy) needles were used for isolations. The obtained colonies were grouped into morphotypes based on their morphological characteristics. The isolation frequency (IF) was calculated as percent of the total number of plated fragments. For identification of fungal taxa (DNA barcoding), 1–3 isolates of each morphotype were used for DNA extraction. Primers ITS1 and ITS4 were used for sequencing. The resulting sequences were matched for identification against available sequences from GenBank using the Blast online tool.

4.2 Multispectral surveys.

Biotic or abiotic stresses act on the physiology and biochemistry, consequently changing the radiation absorbed or reflected by the foliage. Spectral reflectance is based on the absorption exerted by water and chlorophyll in the leaf. Various shaded areas within the vegetation were detected due to the type, health, leaf structure and moisture content of plants. Drone surveys were carried out on the natural population of *A. nebrodensis* at the beginning and at the end of the project. Two cameras were used in the survey: 1) conventional RGB camera to create an orthophoto of the terrain and a digital elevation model and find correlations between the topographic features and environmental stresses; 2) multispectral camera to obtain 4 simultaneous images for each band (red, red edge, green, near infrared). Using these images, reflectance maps were created from which different vegetation indices were obtained. The multispectral images were adequately analyzed for the production of a NDVI (Normalized Difference Vegetation Index) map, i.e. an indicator that describes the intensity and distribution of greenness, the relative density and health of vegetation for each element of the image, or pixels, in a drone image. Multispectral maps will allow monitoring the evolution over time of the

health status of the trees as a function of climate fluctuations and the conservation measures that will be implemented in the meantime.

4.3 Support to the natural regeneration.

Various factors limit the growth and settlement of the natural regeneration of *A. nebrodensis*: shallow and rocky soils, irregular flowering and fruiting over the years, high self-fertilization rate and high percentage of empty seeds, impact of wild herbivores damaging seedlings. Natural regeneration occurs mainly among cushion juniper and broom shrubs, under the cover of beech trees or in the presence of a humid layer of moss, where favorable microclimatic conditions and protection occur.

Census and mapping of the natural regeneration.

Surveys in situ were carried out to track and monitor the evolution of the natural regeneration. A survey protocol was developed based on measuring the distance in meters and the azimuth angle between each plant or seedling and the nearest mother tree, through the use of a professional compass.

Survey tables were prepared containing the parameters to be recorded: number of the mother tree (MP) and position with GPS, ID of the seedling, distance from MP, azimuth, height (cm), age, vegetative and health state, any notes. The data collected were used to implement a complete database and 15 maps, one for each mother plant. Pegs with labels were no longer used to mark young plants. In fact, those used in previous investigations seem to attract wild herbivores and were detached and chewed by deer shortly after their implantation.

Fig 4-2 Surveying and measuring a young plant of A. nebrodensis of the natural regeneration

Management measures.

Surveys on natural regeneration led to identify and record a total of 484 *A. nebrodensis* seedlings, divided among 15 mother plants.

The knowledge of the exact location of the natural regeneration and the mapping carried out were a reference for the placement of the new fences, aimed at maximizing the protection of the mother plants and the establishing young plants against fallow deer and wild boars.

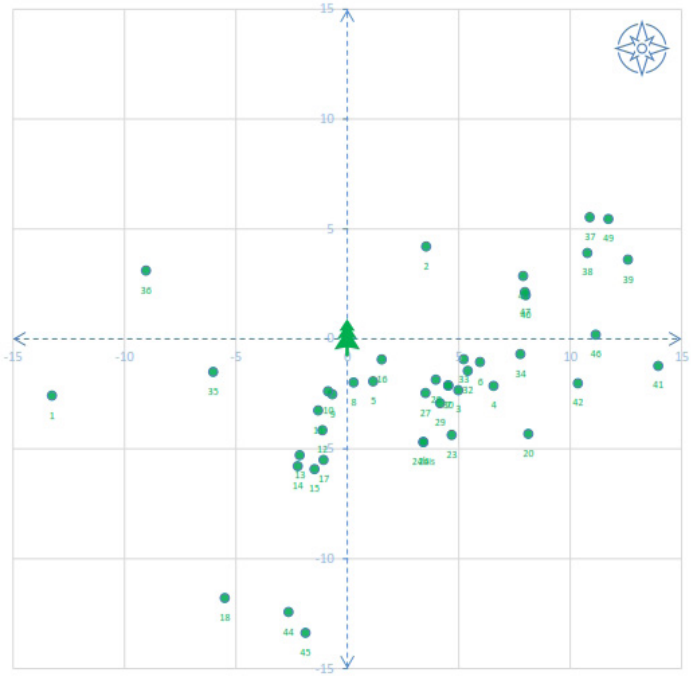


Fig 4-3 Map of the natural regeneration of the A. nebrodensis tree n. 18 (positioned at the center of the plot)

4.4 Installation of a new fencing system.

To encourage survival and development of the natural regeneration, fences set up around the adult *A. nebrodensis* trees are essential to avoid external disturbance (particularly from wild herbivores) to seed germination and seedling development.
The LIFE4FIR project planned the extension and strengthening of the fence system around the *A. nebrodensis* trees to meet three fundamental needs: 1) replace existing fences that were deteriorated and no longer functional; 2) support and protect the natural regeneration established outside the old fences; 3) strengthen protection to the adult trees with wider and higher fences. The new fence system was designed and installed on a larger surface area, increasing the protected surface around the *A. nebrodensis* trees to over 14,000 m2 in total. This measure will ensure the maintenance of optimal vegetative conditions, preserve the biocoenosis around each plant and consequently favor natural regeneration.

Implementation.

The new fences are made up of debarked chestnut poles with a diameter of no less than 7 cm at the top and a height of no less than 2.40 m, arranged at an average distance of 2 m and buried for 40 cm after caulking the bottom for 60 cm with cold tar. Fences are made of galvanized iron wire net with a degrading mesh, 1.60 m high, with a minimum weight of 0.70 kg per metre, fixed (by means of galvanized wire) on four rows of galvanized iron wire with a diameter of 2.70 mm anchored to the posts by means of staples and placed respectively at ground level, at 1.40 m, 1.60 m and 1.90 m. Each fence is equipped with an entrance via a 1.5 m wide gate built according to the scheme planned by the project using chestnut poles.
As trees are located in inaccessible places, mechanization of any activity was practically impossible. Initially it was necessary to restore and open small paths to reach the trees. Only later was it possible to transport the material necessary for the installation of the fences.

Fig.4-4 The new fences installed around the A. nebrodensis tree n. 12, as protection against wild herbivores

4.5 Video surveillance.

A video surveillance system has been installed as a deterrent and to control wildlife and abandoned livestock.

The system was installed by positioning 5 stations in the most visited sites of the *A. nebrodensis* range. For the installation, 5m long chestnut poles were used, with a section of 18-22 cm at the base and 12-16 cm at the top. The poles were firmly buried for 1m, avoiding the use of concrete plinths. The kit consisting of a tropicalized camera (water and dust resistant) with 2MP resolution and motion sensor and a 40 W photovoltaic panel, equipped with a 20 Ah battery, was installed at the top of the poles. Through an LTE/4G router, the acquired images can be transmitted to cell phones via SIM cards. The images can also be saved in local SD memories. The system was then upgraded to allow the real time transmission of the images acquired via satellite.

Fig 4-5 One of the five stations of the videosurveillance system

5. EX SITU CONSERVATION MEASURES

The LIFE4FIR project has implemented measures to ensure the ex situ conservation of *Abies nebrodensis* germplasm, through the creation of a clonal orchard and the launch of a seed bank and a cryobank. These measures will play a fundamental role in the conservation and management of the genetic heritage of *A. nebrodensis*.

5.1 Clonal orchard.

The clonal orchard is intended not only as a simple collection of germplasm, but also as a facility for the future production of seeds characterized by greater genetic variability, as cross-fertilization between the different genotypes is encouraged.
The clonal orchard will also allow constant monitoring of individual genotypes with regards to growth, habitus, phenology, etc., for scientific and educational purposes. In the future, when the plants reach maturity, the orchard will be used as a new source from which seeds or other propagation material can be collected, avoiding negative impacts on the natural population.
For the establishing the clonal orchard, each single genotype of the natural population of *A. nebrodensis* was propagated vegetatively with the grafting technique. Arrangement of the clones in the plot has been aimed at fostering cross-fertilization.

Grafting propagation.

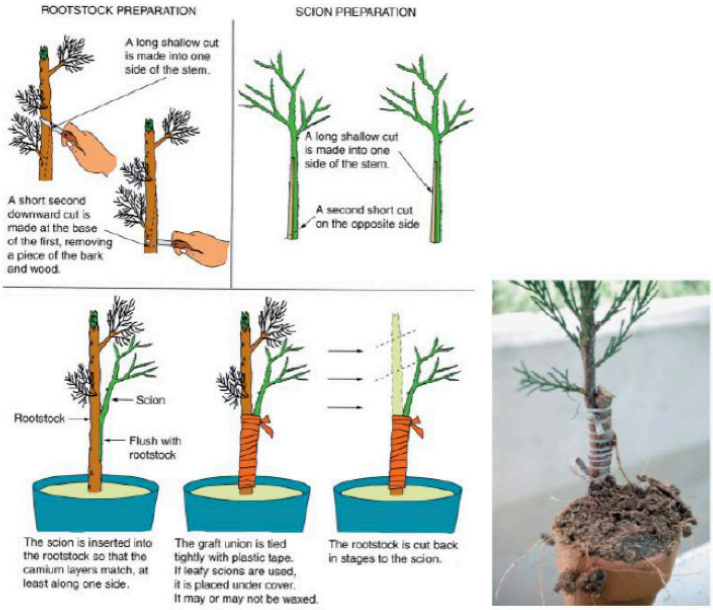
Propagation by cuttings and micropropagation are the most efficient techniques of vegetative reproduction. On the other hand, many species of conifers do not respond to these techniques, producing limited results, not sufficient to guarantee a suitable number of plants for reforestation purposes. In all these cases (including *Abies nebrodensis*) it is necessary to follow grafting propagation. The grafting technique that has been used in this Project is called "veneer-side grafting", by far the most used in the nurseries for the grafting propagation of conifers.

A protocol optimized for *A. nebrodensis*.

The specific procedure followed for the propagation by grafting of *Abies nebrodensis* is below summarized.

- Two weeks before grafting, the rootstocks (*A. nebrodensis* pot plants) were transferred to the greenhouse to force vegetative activity and root growth. The substrates were moistened, preventing them from being too wet. The lower 7-10 cm of the plant stems were kept clean by removing any branches, needles and soil eventually present.
- The scions were collected at the beginning of April, as soon as climatic conditions were suited depending on the presence of snow. However, at the time of harvesting the plants still had closed buds.
- Scions were kept at 4°C in a refrigerated room. Grafting was carried out within three days after they had been collected. Rootstocks, scions and equipment were assembled in a comfortable workstation in the nursery. A team of 6 people (experienced grafters, plus helpers) was organized. The grafting knives were prepared extremely sharp and clean.
- The collected scions were terminal shoots, mainly taken from the lower third of the tree. The scions for grafting (10-15 cm long) were prepared by removing any needles in the lower half.
- The first cut was made on a straight section, free of defects and wounds in the lower 4 inches of the rootstock stem. All cuts on the scion and rootstock were made in one smooth motion. This yielded the best surface for mating the scion with the rootstock. The first cut was made downwards to create a small flap on the rootstock stem. The width of this cut was as close as possible to the width of the scions, while still penetrating the bark of the rootstock.
- A downward cut on the scion was then made with a one angled cut at the end of the scion to create a flap. The length of the cut was equal to the length of the cut made on the rootstock;
- The scion was then inserted into the “pocket”, created in the basal cut part of the rootstock; the side of the scion aligned with the cut surface of the rootstock. When the grafting was done properly, the scion remained perfectly inserted in the rootstock pocket, with a

perfect alignment of the cut surfaces.
- The scions were then tied with a rubber strip to tighten the graft; the wrapped area of started and ended above and below the cuts. The grafted area was then covered with aluminium foil in order to prevent excessive drying;
- As conifers require high humidity while the scion is healing, the grafted plants were covered with a transparent plastic bag.



The "veneer-side graft", a typical grafting technique used in conifers

The grafted plants were moved back to the greenhouse, after cutting the upper part of the rootstock. The soil in the pots was periodically moistened, avoiding dripping. Particular attention was paid to avoiding dehydration of the soil, as this is a critical moment for the success of the grafts. Plants need light, but direct and intense solar radiation must be avoided. After 4-5 weeks, the plastic bag was removed and another third of the rootstock was cut, just above the insertion of the scion. Between mid and late summer, the elastic strip was removed to prevent it from excessively compressing the stem at the junction point. The grafted plants were then moved outside to a shaded area of the nursery. A survey carried out 4 months after grafting, showed that over 50% of

the grafts were successfull, a result of absolute excellence for a species like *A. nebrodensis*. The grafted plants must be supported with stakes for the first two years to favor a straight trunk.
Arrangement of genotypes in the clonal orchard was based on the need to foster cross-fertilization, also taking into account the data on the genetic distances between the ortets.

Fig 5 Grafting propagation

5.2 Seed bank.

Seed banks represent the most used ex situ conservation system for the conservation of plant biodiversity. The seed samples collected from the 30 adult *Abies nebrodensis* trees are stored in the Seed Bank, recently launched at the *Abies nebrodensis* Museum in the Municipality of Polizzi Generosa thanks to the LIFE4FIR project.
The maximum conservation time varies depending on the species, and can often reach many tens of years, during which germination and viability tests of the seeds are periodically repeated.

Seed collection.

Mature cones were collected from *A. nebrodensis* trees in October and then dried to equilibrium in a controlled environment. Mature seeds were cleaned of any remaining contaminant and their moisture content was measured on a sample of 3 seeds. The average moisture content of 6.3% was suited for their storage at +4ºC, until they were used for further experiments.

Seed selection.

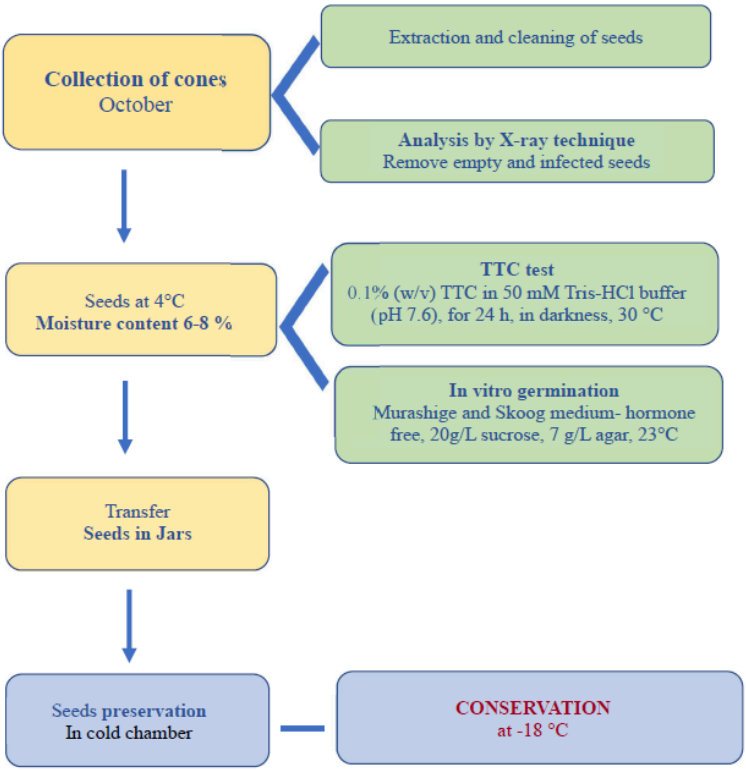
The massive presence of empty seeds, without embryos, represents a problem for conservation purposes. Therefore, in this project a procedure based on an X-ray scan of individual seed lots was applied to select full seeds.
The X-ray imaging allowed detection of empty seeds or seeds attacked by insects or diseased, which are removed.. Seeds were placed in the laboratory in 100-well square plastic plates (20x20 cm), then they were scanned and photographed with the Gilardoni radio light device, Lecco, Italy. The optimal procedure was developed using X-ray film, exposed to a radiation of 25 kV for 2 min., 3 mA (soft X-rays) at a distance of 45 cm from the X-ray source.. X-ray images were checked for the presence of endosperm and embryo.
Accordingly, the seed was considered abnormal if one or both structures were deformed. To validate this technique, a sample of seeds was opened after radiographic examination and checked under a stereoscope to confirm the presence of the embryo.

Tetrazolium Seed Viability test (TTC).
Mature seeds were kept at 4°C for 6 months. In vitro assays were applied on seeds and zygotic embryos to evaluate their viability and germination before starting conservation.
Seed viability was assessed with the tetrazolium test (2,3,5-triphenyltetrazolium chloride, TTC). The TTC test is based on the reduction of soluble and colorless tetrazolium salt to an insoluble red precipitate in the presence of dehydrogenase activity in live cells. The zygotic embryo stained red was the main indicator of seed viability.

In vitro germination test.
After surface sterilization with 70% ethanol and sodium hypochlorite, seeds were immersed in water for 48 h under sterile conditions before extraction of zygotic embryos.
The germination test was carried out using MS substrate (Murashige and Skoog 1962) without the addition of hormones. Every two weeks from the start of in vitro culture, the germination rate was calculated as the percentage of the number of germinated embryos compared to the number of cultured embryos. The germination rate of the sampled embryos was 66-100%.

Seed storage at low temperature (-18°C).
Following the international standard for long-term seed storage (FAO/IPGRI 1994), conservation was planned at −18°C or lower, with seed moisture content (MC) of 3–7%. A sample of seeds was used to test the effectiveness of this conservation procedure. After 6, 9, 12 months of storage at -18°C, germination and viability tests with TTC showed good results for each time frame evaluated. For conservation in the seedbank, seeds were placed in DAGKLAR 0.4 l transparent glass/stainless steel jars. Labels on the jars reported data of seed lots: location of the seed bank, species, ID number of the tree, year of collection, quantity (g), number of seeds, conservation start date.

4. Final Protocol for *A. nebrodensis* conservation in seed bank (-18°C)



5.3 Conservation of germplasm in cryobank.

Cryopreservation, or storage at ultra-low temperatures such as that of liquid nitrogen (-196°C), is the most innovative technique for the long-term conservation of plant genetic resources. The technique preserves organs and tissues obtained from in vitro culture and from the field, through an ultra-freezing process hinders almost all metabolic processes in the cell, preserving its structure and biological functionality.

Device for storing samples at -196°C.

The choice of liquid nitrogen dewar, where storing seeds, excised embryos, pollen, embryogenic callus of *Abies nebrodensis* was based on reliability, safety, guaranteed operation and low consumption of liquid nitrogen. This is the Locator 8 Plus, marketed by VWR International Srl of Milan, Italy. The salient features of the Locator 8 Plus climatic chamber are the following:

- container for storing samples (dewar) equipped with ultrasonic level monitor.
- capacity 121 litres
- 8 racks per unit, with a capacity of 10 boxes per rack and 25 2ml cryovials per box
- total capacity: 2000 cryovials
- static evaporation rate: 0.6 L/day
- neck diameter: 15.2 cm
- external diameter x height: 55.8 x 95.3 cm
- equipped with lockable lid.

Fig 5-1 Praparation of the cryoboxes containing A. nebrodensis germplasm (pollens, embryos, embryogenic lines) for conservation in the cryobank

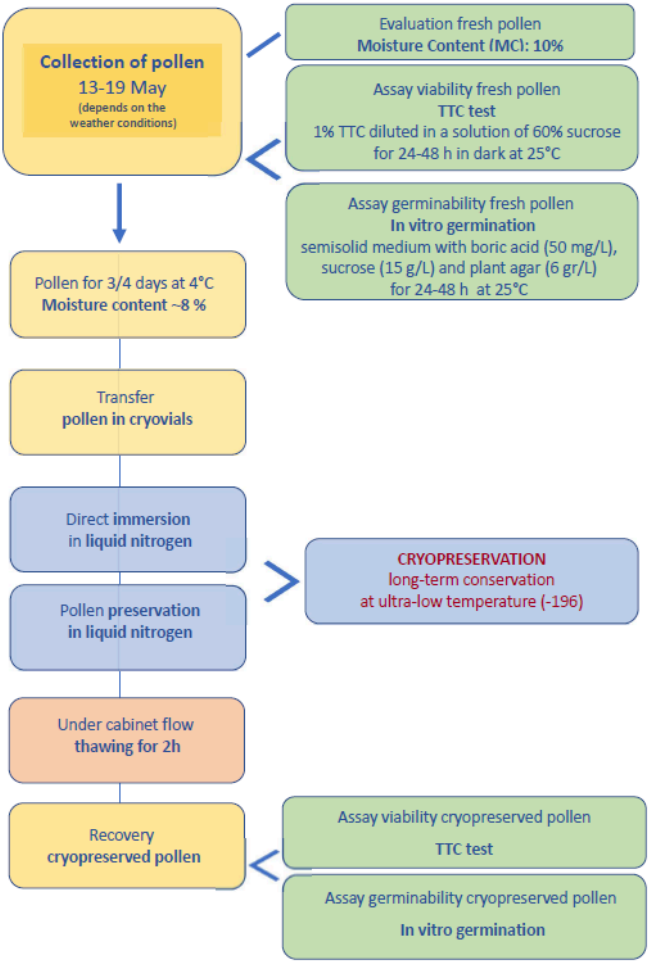
Storage of embryos at cryogenic temperature (-196°C).
For cryopreservation, zygotic embryos extracted from sterilized mature seeds were used, with a moisture content (MC) of 8.9% determined with a moisture analyzer.
To carry out the experiment, the zygotic embryos were divided into two groups, one treated with plant vitrification solution (+PVS2) and one untreated (-PVS2). After cryopreservation, the cryoboxes were removed from the liquid nitrogen and thawed in a water bath (40 °C) for 1 minute. A 90-95% germination rate of zygotic embryos was found both for embryos treated with the PVS2 solution and for untreated ones. Also, all TTC treated embryos were stained completely red after recovery from LN. Thus, the results highlight the potential of cryogenic technology for the preservation of *A. nebrodensis* zygotic embryos.

Storage of pollen at cryogenic temperature.
Pollen conservation is an important tool for maintaining plant genetic resources and can promote greater efficiency in breeding and germplasm conservation and exchange programs.
Pollen conservation should be understood as an additional means for the conservation of plant germplasm and not a substitute for the conservation of seeds or clonal materials.
Success of pollen preservation depends on environmental factors such as moisture content and storage temperature.
Pollen viability and germination rate vary over time in different species and must be assessed before and after cryopreservation to verify the success of conservation.
A pollen moisture content between 8 and 10% prevents the formation of ice crystals during the freezing process.

Collection, morphology and moisture content of *A. nebrodensis* pollen.
Field conditions and relative humidity at the time of collection influence the moisture content of pollen. Pollen was collected twice from various trees in May 2020.
After removal from the anthers, the collected pollen was sieved and

its morphology, moisture content (MC), viability and germinability were determined. The moisture content (MC) was determined with the Moisture Analyzer, on a sample of 0.2 g of pollen, resulting 10% on average. The pollen of *A. nebrodensis* was then observed using a stereomicroscope, optical microscope and ESEM.

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



Pollen viability and germination assays.
TTC tests were performed before and after pollen cryopreservation. Two drops of TTC mixture were placed on a microscope slide. *A. nebrodensis* pollen was then dusted over, covered with a coverslip and incubated in the dark for 24-48 hours at room temperature. After incubation, each sample was observed under a microscope. Pollen grains which stained bright orange or red were considered viable.

In vitro germination test.
Pollen germinates in vitro by placing pollen grains on a semisolid medium and measuring pollen tube elongation after a few hours. Pollen tubes that had reached a length equal to twice the diameter of the pollen grain were considered germinated. The medium used for *A. nebrodensis* pollen germination consisted of boric acid (50 mg/L), sucrose (15 g/L), and agar (6 g/L). Pollen was maintained at 25°C, the optimal temperature for germination testing.

Conservation in liquid nitrogen and evaluation of viability and germination.
After extraction from cones, the pollen was kept for three days at 4°C, to reduce the MC up to 8%. Pollen samples were then transferred into cryovials and placed in cryopreservation. After storage in liquid nitrogen, the cryovials were thawed by keeping them under a laminar flow hood for 2 hours at room temperature and then transferred to Petri dishes. By applying the TTC procedure described above, the percentage of viable pollen grains observed ranged between 88% and 96%, without significant deviations from fresh pollen. The same result was observed in the germination test, with a percentage of germinated granules ranging from 84% to 94%.

Conservation of embryogenic calli at cryogenic temperature.
Somatic embryogenesis, recognized as an advanced tool in forestry, has been applied for over three decades, and was initially developed for coniferous species. The pioneering application with spruce has demonstrated its potential, evolving into a beneficial method for

ecologically and economically significant species.
Somatic embryogenesis has been applied to various species of the Abies genus so far.
Combining this technique with cryopreservation allows for large-scale propagation and conservation of forest resources. Substantial success has been achieved in the production of somatic embryos of commercially important species. However, for *Abies nebrodensis*, obtaining the embryogenic callus has proven difficult.

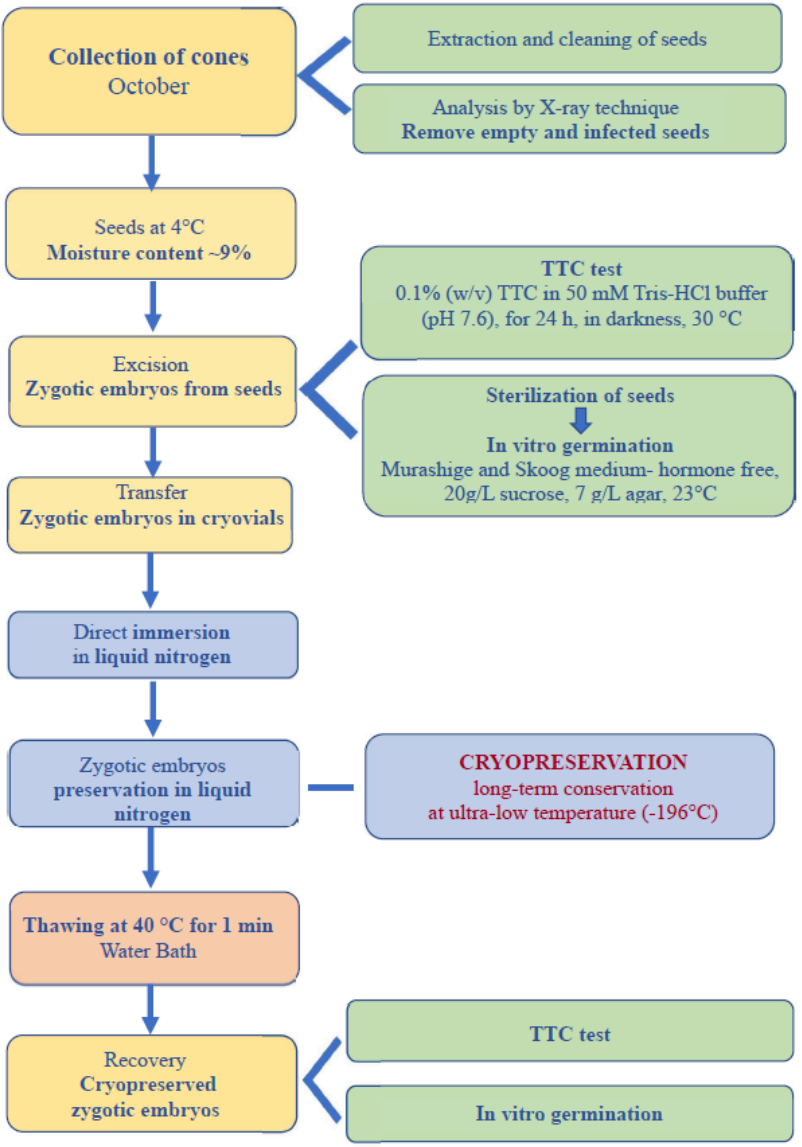
Somatic embryogenesis protocol for *A. nebrodensis*.
Unripe cones, containing seeds in an advanced stage of embryogenesis, were collected on two different dates, mid- and late July 2020, while mature cones were collected during the last week of September 2020.
Initially, seed selection was carried out using the X-ray procedure, observing the presence and morphology of the embryos.
Sterilization of seeds and extraction of embryos. Seeds were washed with detergent for approximately 30 minutes, then rinsed under running tap water for 4 hours. Immature seeds were then immersed in 70% ethanol for 1 min, and mature seeds for 5 min. Seeds were then rinsed five times with sterile distilled water.
Seeds were subsequently immersed for 20 minutes in a 20% (v/v) sodium hypochlorite solution to which a few drops of Tween 20 were added and rinsed 3 times with sterile water. Under a laminar flow hood, the seed coat was removed from the immature seeds and the megagametophyte was used as an explant.
The mature seeds were imbibed in sterile water for 48 hours, and then the zygotic embryos were carefully extracted using sterile tweezers.

Callus induction and proliferation from mature embryos.
Mature embryos were excised and cultured horizontally on different culture media for callus induction. Embryos were excised from mature seeds and placed horizontally in Petri dishes. For the first time in this species, embryogenic callus was obtained, using SH substrate (Schenk and Hildebrandt, 1972) with cytokinin (BAP, 1 mg/L). After 2 weeks incubation on induction substrate in the dark, the developed callus

was separated from the embryo and transferred to a fresh substrate for proliferation, treating each callus as an individual cell line. As a proliferation substrate, SH supplemented with 4.27 µM ABA, PEG-4000, 8% and 4% of maltose was used. Embryogenic tissue (ET) was kept in the dark at 25°C and subcultured onto fresh substrate every 15 days. After obtaining a sufficient amount of ET, the individual cell lines were transferred onto a maturation substrate (SH with 10 mg/L abscisic acid, 8% polyethylene glycol, and 40% maltose). Cultures were maintained at 25°C, in the dark, subculturing every 2 weeks. ET formation was continuously observed under the stereomicroscope, along with the development of somatic embryos.

Encapsulation and cryopreservation of embryogenic calli.
Small pieces (0.5 g) of embryogenic callus were encapsulated in alginate spheres according to the Micheli and Standardi (2016) protocol. Subsequently, they were washed with sterilized distilled water and then immersed in 3% (w/v) sodium alginate solution. Drops of encapsulating matrix containing ET were then transferred for 20 minutes into MS basal medium supplemented with 11.1 mg L-1 calcium chloride to obtain ET capsules. For the cryopreservation, after washing in sterile water, the beads were treated with the loading solution and transferred into a liquid solution containing MS medium supplemented with 34.2% sucrose. Subsequently, the ET capsules were treated with Plant Vitrification Solution 2 (PVS2) at 0 °C. All capsules with ET were then immersed in liquid nitrogen. After cryopreservation, ET capsules were thawed in a water bath (2 min at 40 °C) and transferred to SH medium supplemented with 4.43 µM BAP (the same medium used for proliferation) to test their viability.

5. Final Protocol for *A. nebrodensis* zygotic embryos conservation in cryobank (-196°C)



6. RE-POPULATION WITH SELECTED SEEDLINGS

One of the main objectives of the project is to create new reforestation nuclei using selected *Abies nebrodensis* seedlings obtained from cross-pollinations.
Selection of sites in which establishing the new plantations took into account to the results obtained in the plantations carried out in the previous projects. Inspections were carried out across the Madonie Park to identify the sites characterized by suitable ecological-environmental characteristics and which have given good results.
The selected sites are located in the Madonie Regional Park mainly between 1100 and 1600 m elevation a.s.l., in the municipalities of Polizzi Generosa, Isnello, Petralia Soprana, Petralia Sottana, Geraci Siculo and Gratteri. All the sites fall in areas managed by the Region's Agricultural and Territorial Development Department.
The sites located between 1100 and 1400 m elevation a.s.l. are part of the climax Ilici aquifolii-Quercetum austrotyrrhenicae, a relict forest association of notable geobotanical interest settled on the quartz-arenite substrates.
Sites located above 1400 m a.s.l. fall within the altimetric range of beech forests, linked to the climax Geranium versicoloris-Fagion association.
Some sites (Quacella, Piano Formaggio, Favarotti), despite being located on a calcareous substrate, have a deep and decalcified soil. They are potentially characterized by vegetation traits attributable to the mesophilous holm oak (Quercetum ilicis), an association characterized by the presence of Ilex aquifolium and some deciduous tree species.
The two sites selected at 750-850 m asl elevation are justified by the consideration that *A. nebrodensis* in the past grew at lower sites than the current residual population, restricted to poorly accessible areas between 1400 and 1600 m elevation asl.
Most of the sites have a N-NW exposure and are completely or partially covered by coniferous woodland plantations such as Pinus nigra, Cedrus atlantica, with the participation of broad-leaved trees such as Q. petraea, F.sylvatica, Q. ilex, Fraxinus ornus, Ilex aequifolium, Acer

campestre, or Q. ilex. The presence of a covering layer is essential in the juvenile growth of *A. nebrodensis*, to avoid direct sunlight.
The planting was carried out following the contour lines and the slope of the site. To allow the young plants to grow correctly, an adequate spacing was maintained, while avoiding a geometric layout. Where necessary, the herbaceous layer was removed, taking care not to damage any rare and/or endemic species present.
Based on the objectives of the LIFE4FIR project, the individual plots have a surface area from 3000 to 4000 m2 and the number of plants that have been planted was 400 each.
For the creation of the individual plots, interventions were carried out in the following phases: installation of the fences, opening of the holes, planting out of enhancing leguminous species, planting out of *A. nebrodensis* seedlings.

Fencing.
Fences were installed along the perimeter of each area, following the same procedure used for the fences set up to protect the trees of the natural population. Chestnut poles with a diameter of 8-10 cm and a length of 2.40 m were used, inserted into the ground for approximately 40 cm and placed at a distance of 2 m from each other.
The height of the fences is approximately 2 m above the ground.
Four layers of galvanized wire anchored to the poles were fixed to a progressive metal mesh with a height of 1.65 m and an overhead wire.
Access to the plots will be allowed by gates 1.5 m wide and 1.80 m high. Once installed, the gates will be held in place using iron gaiters anchored to the chestnut posts.

Digging holes.
The opening of the holes was carried out both using mechanical devices and with specific agricultural equipment. The holes will not have a well-defined spacing pattern but will be dug based on the characteristics of the surface and generally were 3-4 meters spaced. To ensure the young seedlings of *A. nebrodensis* a harmonious development of the root system and a greater water reserve, the holes had a truncated-cone or

Fig 6-1 A newly installed fence around one of the plots set up for A. nebrodensis repopulation

pyramid shape.
Both had a lower width of about 80 cm and an upper width of about 50 cm, with a depth between 50 and 60 cm.

Planting of enhancing Leguminosae.
The concurrent planting of shrubs belonging to the Fabaceae family, is aimed at improving soil fertility and ensuring adequate protection to the *A. nebrodensis* plantlet from both excessive sunlight, heat and summer drought after having been transplanted. The fast-growing nitrogen-fixing shrubs help to maintain suitable microclimate conditions for the development of *A. nebrodensis* plantlet in the spring-summer period and to protect them from strong winds. The shrubs that were used belong to the genera Genista, Spartium and Cytisus. They were 2-3 years old and were planted at a distance of about 50 cm from the *A. nebrodensis* plantlet. Generally, two shrubs will be planted for each single *A. nebrodensis* seedling.

Fig 6-2 A young A. nebrodensis plant is being planted out

Planting of *Abies nebrodensis* seedlings.

For each single plot, 400 seedlings raised in 9x9x20 cm containers in the nursery have been planted out. The seedlings were about 3 years old. In the nursery, they were transplanted after 1 year and were subjected to mycorrhization. Planting, as mentioned above, didn't follow a geometric arrangement but was performed according to the morphology of the soil and in compliance with the vegetation settled. Where a tree cover was present, the seedlings of *A. nebrodensis* were planted north of the trunk. In the reforested areas with a geometric spacing of the trees, the *A. nebrodensis* seedlings was arranged following a quincunx pattern. During planting, phosphorus-rich organic fertilizer was added to sustain the transplanted seedlings. After planting, a dip in the ground was created around each single plant to favor the accumulation of water. Furthermore, a mulching with plant material obtained from the herbaceous species present

in the immediate vicinity of the plots was made in spring to reduce evaporation.
Within the plots, about 50 holes of 40x40x40 cm were also dug for the direct sowing of *A. nebrodensis*, placing the seed at a depth of about 3 cm. Five seeds per hole have been sowed.

NOTES

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