LIFE4FIR is a project co-financed with the contribution of the LIFE financial instrument of the European Union

LIFE 4FIR

MANUAL OF GOOD PRACTICES FOR THE IN SITU AND EX SITU CONSERVATION OF THE THREATENED MEDITERRANEAN ABIES SPECIES

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PROLOGUE

Natural ecosystems are a multifunctional source of goods, resources and services for the benefit of mankind, such as: food, clean air, pure water, energy and raw materials, health, recreational and social benefits. The biodiversity of healthy ecosystems favours the delivery of their multiple services and helps them respond to the impacts of climate change. However natural ecosystems are increasingly stressed due to climate change, loss and fragmentation of habitats, pollution and introduction of alien species, as consequence of excessive anthropogenic pressure.

The LIFE projects are tools for implementing the EU environmental policy. In particular, the LIFE Nature and Biodiversity sub-program has funded since 1992 good practice, pilot and demonstration projects for the conservation of biodiversity, habitats and species. The LIFE4FIR project (LIFE18 Nat / It / 000164) "Decisive in situ and ex situ conservation strategies to secure the critically endangered the Madonie fir (Abies nebrodensis) in Sicily" has been funded by this sub-program.

The Madonie fir is the most representative and well-known endemism of Sicily. Due to its critically endangered status, acknowledged by IUCN, this species plays a symbolic role, because it fully represents the effects of habitat degradation and the loss of biodiversity caused by the overexploitation of natural resources. For the Madonie fir, the decline began in the 1800s, when this species was still reported to be widespread in the mountains of the northern part of Sicily. The massive utilization of mature trees, as source of valuable construction timber and uncontrolled grazing have drastically reduced the population of this species, up to the 30 relict plants that are counted today.

Over the past two decades, a series of projects have been devoted to the safeguarding of this species and the conservation of biodiversity in the Madonie Park territory. Among these, a previous LIFE2000NAT/IT/7228 project, followed by an APQ Project (CIPE 2004). The LIFE4FIR project has taken the results and knowledge acquired by previous projects to implement a thorough, multidisciplinary and integrated strategy, based on the use of innovative techniques, to respond to the main vulnerability factors affecting Abies nebrodensis and improve its conservation status. The joint work of the institutions involved (Istituto per la Protezione Sostenibile delle Piante e l'Istituto per la BioEconomia del CNR; UNIPA-SAAF; l'Ente Parco delle Madonie; Departamento de Biología Vegetal y Ecología dell'Università di Siviglia e il Dipartimento per lo Sviluppo Rurale e Territoriale delle Regione Sicilia) has led to significant progresses in the five key actions of the project: 1. Sustain to the residual trees of Abies nebrodensis in their natural habitat (in situ conservation). 2. Maintaining the purity of the population and increasing biodiversity in the progenies. 3. Nursery production and selection of pure, outbred and vigorous seedlings. 4. Replanting interventions in suitable areas of the Madonie park using genetically selected seedlings to promote the rediffusion of the species. 5. Ex situ conservation with traditional and innovative methods through implementation of a seed bank and a cryobank.

These thorough and synergic pool of actions provides a model of procedures for the long-term conservation of this species, that could be effectively replicated. This handbook reports the effective and sustainable guidelines that are producing good results on the conservation of Abies nebrodensis that can represent a useful reference for people committed to safeguarding the biodiversity of threatened and endangered firs (and conifers) in the Mediterranean area.

CHAPTER 1
Assessment of the genetic diversity and structure of the *Abies nebrodensis* relic population

Biodiversity provides the foundation of ecosystem stability, functioning, and productivity, as well as provisions of ecosystem services. There is also evidence that a standing genetic diversity benefits ecosystem functioning, and resilience providing the raw material for individuals, populations, and species to evolve and adapt especially under new environmental conditions (abiotic and biotic stress) and changing climate. As such, individuals and populations that have high genetic diversity should have better chances of persistence and adaptation. This may be particularly true for genetic diversity in climate-responsive genes. In summary, genetic diversity provides the basis for conservation of genetic resources within and across species, and its maintenance is essential for the survival, adaptation, and evolution of individuals, populations, and species for a functional ecosystem management.
Because forest trees are normally the keystone species of the forest ecosystems, their genetic diversity has special significance and has been identified as the foundation of forest sustainability. The forest harvesting, the renewal practices and natural disturbances can impact population demography and several evolutionary processes, such as genetic drift, gene flow, inbreeding, and selection, by affecting local tree density. This led to creating bottleneck, fragmentation, and spatial genetic structure, which can adversely affect genetic diversity and differentiation in post-harvest tree populations.

1.1 Assessment of genetic diversity of *Abies nebrodensis*
Due to the past overexploitation, the habitat of A. nebrodensis has become increasingly fragmented, with the natural population size and individual numbers of plants decreasing dramatically, resulting in a significant loss of genetic resources. Currently, population is small, fragmented, and scattered, increasing the probability of inbreeding and selfing and the potential for genetic drift (Fig. 1, 2).

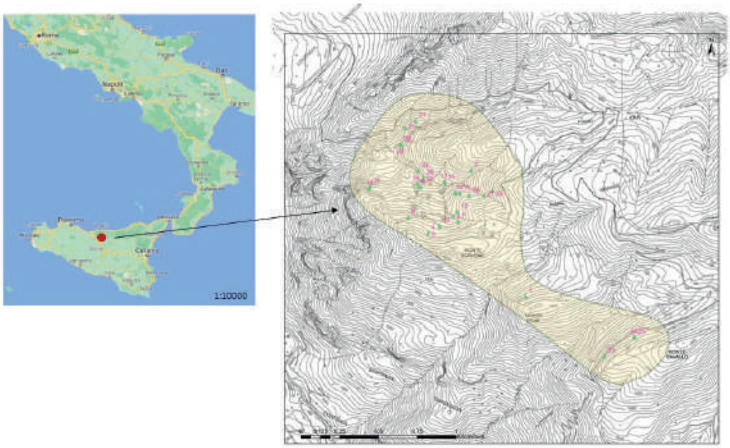


Fig. 1. Localization of Madonie park in the Sicily region (left) and map of the A. nebrodensis natural population (right). Position of each 30 adult trees is reported in a 1:10000 Sicily regional map.

Fig. 2. The scattered distribution of A. nebrodensis in its natural habitat.

Also, irregular seed production, difficult seedling renewal, and the lack of a specific mechanism for long-distance seed dispersal have resulted in poor population regeneration. Conservation of the species is therefore critically important. Genetic resource conservation and plant breeding programs require an evaluation of the genetic diversity and structure of the endangered species.

One of the load-bearing action of the Life4fir project is aimed at the evaluation of the genetic variability and the genetic relationships among the adult trees and the natural regeneration of the threatened A. nebrodensis forest population. For the A. nebrodensis, SNPs genotyping was used to assess the genotype individual trees and to study the genetic diversity and structure 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).

1.2 SNPs genotyping

For SNP genotyping the PCR-based OpenArrays technology (Thermofisher Inc., United States) was used. This technology provides a robust and flexible platform achieving superior data quality and high sample throughput at low per-sample costs, making it ideally suited for studies involving large volumes of samples.

The adoption of this approach is very useful for the population genotyping and the observation of mutations occurring throughout the genome. The International Union for the Protection of New Varieties of Plants recommends SNPs and microsatellites as preferred markers for DNA fingerprinting and varietal characterization. A single nucleotide polymorphism (SNP) is a variation of the genetic material on a single nucleotide present in the population in a proportion higher than 1%. Nucleotide differences allow to distinguish between varieties and ecotypes, or even individuals within the same species. The technology allows the generations of millions gene sequences and information simultaneously, in a short time and at low cost. The data produced by the sequencing, compared with the information in the public databases relating to the genomes of the already sequenced species (reference), lead to the identification of the nucleotide differences. This generates a very detailed genetic profile, characterized by thousands of SNPs each of which is well identified for its position in the genome of the species under analysis (Fig. 3).

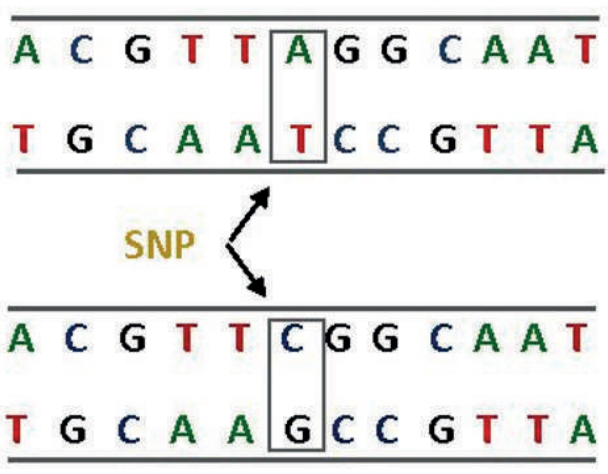


Fig. 3. Schematic representation of Single Nucleotide Polymorfism

1.3 Paternity test

Paternity testing is conducted to determine the biological linkage between offspring and an alleged father, to generate a probability of paternity. On the basis of genetic similarity results obtained by SNPs genotyping, a specific software inferred the origin of each individuals, assigning their putative parents, 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. The presence of a high rate of inbreeding or self-fertilization is often accompanied by a genetic depletion (homozygosis) leading to a population more susceptible to biotic and abiotic stresses. The paternity test allows to determine the best crossings for a species genetic improvement increasing the percentage of heterozygosis.

Findings obtained with *A. nebrodensis* population

1. The accurate and reliable results obtained with Open-array using 100 SNPs clearly indicate that most adult trees are genetically close. Effective population size, a key parameter in population genetics to estimate the number of individuals that effectively contributes offspring to the next generation, is very low. These findings are in contrast with results of previous studies based on other molecular markers, which showed high rates of genetic diversity in the natural A. nebrodensis population.

2. Paternity test showed that more than 90% of the seedlings of the natural regeneration were originated by self-pollination. Genetic drift and inbreeding are expected to decrease the ability of seedlings to cope with a wide variety of environmental stressors, which probably would explain the low survival rate of seedlings reported in previous studies.

3. Similarly, pure A. nebrodensis open pollinated offspring raised in the local nursery showed high rates of self-fertilization (more than 95%). A significant rate of hybrids coming from crosses with other firs was also found at the nursery.

4. For regeneration activities in the population, a hand-pollination program using genetically distant individuals has been designed to improve genetic diversity of A. nebrodensis population and seedling survival. A list of the 30 most recommended crosses between mature trees of A. nebrodensis was obtained accordingly (see chapter 2).

1.4 Conclusions

The results on the genetic uniformity of the population and the high rate of self-fertilization found on natural regeneration were largely expected and the use of controlled crosses between plants had already been planned. Outputs of the genetic analyses carried out are of great help for conducting the next controlled crosses because the combinations between plants with greater genetic distance were defined and the new crosses will be carried out accordingly to increase the genetic variability of the progenies as much as possible. Less expected was the identification of suspected hybrids among the open pollinated progenies raised in the local nursery. This finding needs to be considered with great caution for future population management and will have an impact on other actions of the project.

CHAPTER 2
Performing controlled crosses to increase genetic variability of the offspring

The main issue in the conservation of threatened species is that they have undergone a gradual or sudden decrease in numbers, increasing the risk of extinction. The resulting genetic structure of such a bottlenecked population may also have important consequences to its long-term viability and therefore should be of concern to conservation managers. One consequence of small population sizes is that the frequency of inbreeding can increase, which can lead to the immediate loss of fitness (i.e. decreased survival or reproductive success, termed 'inbreeding depression'). A second but entirely different consequence is the loss of genetic variation, which can reduce the potential of populations to adapt to new challenges in their environment such as infectious diseases or climate change. Although eliminating the agent of decline should always take priority, both inbreeding depression and loss of genetic variation can also lead to an increased risk of extinction.

A potentially negative consequence of habitat fragmentation is restricted gene flow between remaining small populations which can lead to genetic depletion and reduced individual fitness (Charlesworth & Charlesworth 1999; Leimu et al. 2010; Angeloni et al. 2011). Small populations are also affected by genetic drift which can cause the fixation of recessive deleterious alleles leading to the loss of population genetic diversity and further reduction of fitness. A large body of empirical research has demonstrated causal links between reduction in genetic diversity, elevated inbreeding and reduced fitness in wild plant populations (reviewed by Leimu et al. 2006 and in tropical trees specifically by Lowe et al. 2005).

Genetic rescue is the supplementation of genetically impoverished populations with new individuals (or genotypes) with the purpose of alleviating genetic erosion (Thrall et al. 1998) and enhancing population viability (Tallmon et al. 2004; Pimm et al. 2006). Studies on annual and short-lived perennial plants have demonstrated that pollen-mediated gene flow between populations, and introductions of new individuals into a population, can also improve various fitness components and increase population viability, an effect that appears more pronounced in small populations.

Controlled pollination allows the crossing between plants that are isolated and favors the creation of new genetic makeup. Cross trials should evaluate the effect on increment of reproductive fitness in terms of fruit set, seed set, germination rate and seedling survival compared to open pollination consisting mainly in selfing.

2.1 Execution of controlled crosses in *A. nebrodensis*

In April 2020, A. nebrodensis adult trees were carefully monitored with particular attention to the opening of the buds, development of the reproductive structures and the full flowering. In 2020 the flowering of A. nebrodensis occurred at the beginning of May and was very abundant, unlike the previous year. All the 25 mature trees of the natural population produced male and female cones, some in large amount. Manual cross pollination among trees were performed by skilled technicians working for Unipa.

Mature (and close to full flowering) male cones (Fig. 4a) were collected separately from individual trees slightly earlier than their opening and put inside paper bags and stored for 2-3 days in a controlled room with a dehumidifier in plastic trays, to avoid the development of molds until they were used on female cones (Fig. 4b). These were isolated from external pollen before their opening when they reached 2-3 cm in length by using terylene bags, a special material that is hydrophobic, but at the same time allows transpiration. Bags were placed in a vertical position to let the cone grow after pollination (Fig. 4c). Since flowering occurs stepwise, it was necessary to monitor the development of flowers on individual trees to proceed properly based on their development. Plants at lower elevations typically flower earlier than plants at higher elevations.

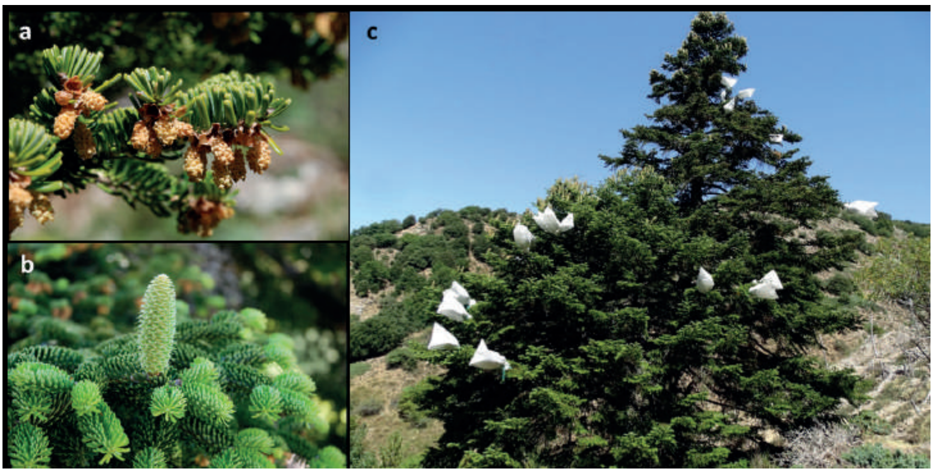


Fig. 4. Male and female cones (a and b) of A. nebrodensis. c) Terylene bags isolating female cones for execution of controlled crosses in the mother trees no. 16 and 17.

When the female cones were opening, 10-15 mature male cones were inserted inside the bags based on the crossing combination. The natural movement of the bag due to the wind favors the release and dispersion of the pollen from the cones inside the bag, allowing the pollination of female cones.

For each pollinated tree, the number of bags and the number of cones for each bag were recorded, indicating the plant that provided the pollen. A total of 174 sacs were used on 24 trees and 488 female cones were protected from external pollen. Crosses were performed according to 27 different combinations of parents, based on the geographic distance between the parental trees (as in 2020 results on genetic distance between trees were not available yet). Development of cones inside bags was monitored during the summer at 15 days intervals. Maturation of cones occurred regularly and bags were removed at the end of September to collect cones before they disarticulate and extract seeds in October (Fig. 4d).

Fig. 4d. Mature cones of A. nebrodensis

Seeds were separately extracted from cones and sown at the Piano Noce forest nursery starting from December.

As in 2021 flowering didn't occur at all in the A. nebrodensis population, hand pollinations were not performed. In May 2022 flowering was fair (not as abundant as in 2020) and controlled crosses were again feasible. This time, the parental combinations were based on the genetic distance between plants, as resulted in the study on the population genetics based on SNIPs genotyping. In all 23 cross-combinations were carried out, using 121 bags to include 389 female cones (Tab. 1).

TYPE OF CROSS	PARENT 1	PARENT 2
Cross 1	19M	26M
Cross 2	11M	19M
Cross 3	17M	31M
Cross 4	9M	30M
Cross 5	31M	32M
Cross 6	21M	30M
Cross 7	7M	32M
Cross 8	8M	9M
Cross 9	8M	19M
Cross 10	16M	30M
Cross 11	10M	27M
Cross 12	30M	31M
Cross 13	22M	7M
Cross 14	7M	30M
Cross 15	25M	28M
Cross 16	21M	22M
Cross 17	1M	22M
Cross 18	18M	9M
Cross 19	27M	25M
Cross 20	1M	29M
Cross 21	8M	12M
Cross 22	24M	28M
Cross 23	4M	25M
Cross 24	25M	26M
Cross 25	2M	14M
Cross 26	21M	32M
Cross 27	13M	15M
Cross 28	27M	24M
Cross 29	10M	30M
Cross 30	19M	28M

Table 1. List of 30 recommended crosses between mature adult trees of A. nebrodensis ordered by more distant co-ancestry and, therefore, more convenient crosses to increase genetic diversity. Among the recommended outcrossing non reproductive individuals are also included.

Seeds obtained from the hand-pollination carried out in 2020 were sowed in the second and third decade of December of the same year. Sowing was carried out in seedling trays, each containing 35 (5x5x12 cm) cells. Five seeds were sown in each cell. The number of trays used in December was 140, for a total number of 24,500 sowed seeds. Germination began in the first days of March 2021. In May 2021 the rate of germinated seeds ranged between 10% and 60% across the various mother trees and was higher compared to the germination rate recorded for open pollinated seeds collected from the same mother trees (Fig. 5).

Fig. 5. Germination phases of A nebrodensis seed in seedling trays located at "Piano Noce" nursery (Polizzi Generosa).

Size of cones also resulted different between the mother trees, ranging from 9 to 16 cm in length and from 13.5 and 16 cm in circumference. Weight of the collected seeds was also markedly different among the various parental combinations: from 13 g to 873 g. Genotyping of a sample of seeds obtained from the controlled crosses is useful to evaluate the success of the hand pollinations.

In April 2021 a new sowing was carried out using 184 seedling trays and 32200 seeds. So, a total of 56000 seeds derived from controlled crossed were sowed in the nursery. Currently, due to the low rate of full seeds, the low germination rate, to physiological losses and to damage caused by rodents, the number of seedlings derived from hand pollinated cones growing in the nursery is about 5000.

These selected seedlings represent new genetic pools and will be used in the reforestation plots planned in the project, meant as re-diffusion cores of the species. Conservation of A. nebrodensis can be improved if a proper strategy is established to improve the gene pool and promote the future dynamism of the species in relation to biotic and abiotic pressures.

CHAPTER 3
Production of genetically selected plants in the nursery

One of the main objectives of the Life4fir project is to select outbred seedlings, to raise the selected seedlings in the nursery in optimal conditions so as to obtain healthy, vigorous and improved material to be used for the construction of the new reforestation nuclei planned in the project. To achieve this goal, the implementation in the nursery of a series of diversified activities and measures is required. Within the Life4fir project, production of plants to be used for reforestation purposes in the publicly owned areas of Sicily is carried out by the 'Piano Noce' forest nursery. The nursery is in Polizzi Generosa, within the Madonie Regional Park, few kilometres away from the A. nebrodensis natural population. The nursery is conducted by the DRSRT (Regional Department of the Rural and Territory Development) of Sicily, which is a beneficiary of the Life4fir project.
Propagation and raising of the Abies nebrodensis seedlings is an activity that has been carried out for years by the nursery 'Piano Noce', supported by previous projects aimed at the conservation of this species. At the beginning of the project, more than 25000 A. nebrodensis plants were growing in the nursery.
One of the primary requirements of Life4fir, is the production of healthy and vigorous seedlings, with increased genetic variability. For this reason, the Project has planned a preparatory action aimed at verifying the germination rate of the seeds, the incidence of disorders and mortality, to identify the biotic and/or abiotic factors associated with observed losses and damages and to eventually implement proper corrective measures. Primary objective is to define and reduce the impact of factors that reduce seeds germination and constrain the growth and vigor of seedlings and pot plants.
A series of surveys allowed the evaluation of the germination and growth of the seedlings in the nursery, to characterize any biotic and abiotic stresse and disorder and implement adequate mitigation and control measures.

3.1 Assessment of germination and survival rate after the transplant

At the Piano Noce forest nursery numerous pot plants (over 25,000) of Abies nebrodensis were surveyed at the beginning of the project. The plants came from sowings carried out since 2003.. The seed (open pollinated), which derived from the mature mother plants, was collected during the first ten days of October, before cones disarticulated. Cones were separately collected and stored in dry and airy conditions. Sowing was carried out in the next spring in March - April by placing 10 - 15 seeds inside 9.5 x 16.5 cm film nursery pots, on a substrate formed by agricultural soil, mixed with sand. The raw material used in the mixture comes from unknown sites and the substrate is prepared without a standardized procedure. Transplant was carried out in the following spring with removal of the seedlings and repotting always on agricultural soil in 14.5 x 24.5 cm film nursery pots (Fig. 6).

The germination rate varied according to the reproductive potential of each single mother plant, but was generally low, while after being transplanted mortality rate of seedlings was approximately 90%. So, the need of developing a pot cultivation system of A. nebrodensis that took into account standardized physical and chemical parameters and that can improve both the seed germination and the growth of seedlings arose.

Fig. 6. Transplanted A. nebrodensis seedlings in film nursery pots at Piano Noce nursery.

Percentage of full seeds

A procedure based on the use of a X-ray device (Gilardoni radiolight) has been developed at IBE-CNR 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%.

Aanalysis of the substrate

Chemical-physical analyses of soil samples were carried out to verify the characteristics of the mixture used for the germination and cultivation in pots of the Abies nebrodensis plants in the Piano Noce nursery. The following traits of soil samples were analyzed: pH; electric conductivity (EC) based on saturated paste extract; organic matter and total carbonate content. Soil samples collected from potted plants were found to vary in salinity levels (EC) and total carbonate content. So, the need of standardizing the preparation of substrate used for sowing and transplanting emerged, to make it perfectly suitable for A. nebrodensis plants requirements: a pH of 5,5-6, an adequate water and air retention capacity, high porosity, and a good concentration of organic matter. Among the 4 tested substrates, the highest percentage of germination was observed with the complete Vigorplant soil, additioned with Agriperlite (in the ratio 70 lt + 10 lt) (Fig. 7), for which the reported percentage of seed germination ranged from 20 to 80% between the different seed lots, with an average of 31.4%.

Fig. 7. Complete Vigorplant soil, additioned with Agriperlit

Use of seedling trays for sowing

The choice of this type of container relied on some important factors such as: easy handling in the nursery, good germination, high density of seedlings per square meter, low space requirement, and above all, less transplant stress. The latter takes on considerable importance, as over 90% losses have been recorded at transplant (Raimondo, F., & Schicchi, R. 2005). Actually, extraction of seedlings causes the taproot frequently breaks with consequent death of the seedlings. Sowing in trays avoid these kind of problem as, at the root ball (roots and soil) remains intact and roots are not affected. In each single cells, 5 seeds were placed for germination (Fig. 8 and Tab. 2).

Fig. 8. Sowing of *A. nebrodensis seeds* in trays at the "Piano Noce" nursery

CHARACTERISTICS		**TIPO 350**
Size of trays	cm	30,0 x 48,5
Number of cells	n.	35
Size of cells	c.c.	350
Upper size of cells	cm	6,1 x 5,8
Lower size of cells	cm	3,4 x 3,8
Depth	cm	16
Thick	mm	1,6
Colour		black
Number of seedlings / m^2	n.	240

Table 2. Characteristcs of seedling trays at "Piano Noce" nursery

3.2 Occurrence and frequency of disorders

For each plot, mother plant, year of sowing ecc., the plants were counted and subjected to visual inspection to identify the damaged organs, recording the number of dead plants and the different types of symptoms observed, their frequency and impact (in terms of percentage of damaged crown) (Tab. 3). This led to obtain a picture of the size of each progeny and of the frequency of the observed symptoms among and within the progenies.

Symptoms	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. Frequency (as percentage) of the main symptoms observed in the aerial parts of the seedlings that develop in three plots of the nursery.

3.3 Samplings for fungal isolations

Samples of affected needles and twigs were taken from the diseased saplings for further observations in the laboratory. Samples were carefully inspected to define in detail the signs of colonization by pathogens (reaction tissues, fruiting bodies of fungi, etc.) and for in vitro isolation of fungal microorganisms. Isolations were performed from needles (reddened, entirely or partially necrotized) and from blighted shoots, after surface sterilization. The colonies obtained were grouped into morphotypes based on their cultural characteristics. Morphotypes were distinguished based on their morphology (mycelium and reproductive structures) and by the sequencing of specific regions of genomic DNA used for their diagnostic value (ITS1-ITS4).

3.4 Assessment of soil pathogens

Chlorosis and defoliation of plants are generic symptoms that can be due to various causes, both biotic and abiotic (environmental stress). The growing medium of pot plants is a mixture of topsoil, peat and sand. This substrate can host pathogenic microorganisms (fungi, bacteria, nematodes) or, if the pots are placed on the ground, harmful microorganisms can pass inside the pots mixture and damage the plants. Trade of ornamental plants is recognized as a principal pathway for the introduction of alien plant pathogens.

The action of very dangerous soil oomycetes, such as Phytophtora sp, cannot be neglected in the nursery setting. This oomycete attacks the root system of plants causing generalized deterioration of the crown (stunted growth, chlorosis, defoliation). For this reason, isolations should be performed in the laboratory from soil and root samples collected from the pots of the plants affected by chlorosis, defoliation, wilting and general decline. Particular attention should be pay when symptoms of decline and death are spread to an area or plot of the nursery, affecting many contiguous plants.
Presence of Phytophtora can be determined using traditional baiting and isolation from soil, organic debris and roots. PCR amplification using the primers ITS4 and ITS6 and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA gene is useful for Phytophtora detection.

Among the fungal pathogens that reside in the soil of forest nurseries, frequent are also *Cylindrocarpon spp., Fusarium spp., Phoma spp., Pythium spp., Rosellinia spp, Rhyzoctonia spp., Verticillium spp.*

3.5 Ecophysiology measurements

The study of water relations (components of the xylem water potential of plants) is aimed at establishing in detail the type of strategy that the plant adopts to tolerate water deficit and, at the same time, identify simple and effective indicators to be used to outline the operational lines for the management of the water resource, or the irrigation of potted plants in the nursery in the specific case. In ecology, the technique of the pressure-volume curves (P-V) describe the relation between total water potential (Ψt) and its components as function of the relative water content (R) of living organisms.
For Abies nebrodensis two groups of pot plants of different age, 10-12 years-old and 4 years-old, each comprising 5 pot plants were sampled in July and November 2020. The P-V curves were obtained following the bench dehydration method, using twigs of the same year and of comparable size between the different samples. Values of water potential at the turgor loss point (Ψtlp) and of osmotic potential at full turgor ($\pi 0$) were recorded. Four P-V curves were obtained for each age group. Results indicated that younger (4-years-old) potted plants were no more susceptible to dehydration than 10–12-year-old potted plants. Moreover, between July and November, potted plants of both age groups had not implemented osmoregulation mechanisms to adapt to a water shortage. So, over the considered time lapse, the plants sampled in the nursery were not affected by water shortages and the irrigation regime can be considered suitable.

3.6 Mycorrhization

Mycorrhiza, which means “fungus-root,” is defined as a beneficial, or mutualistic relationship between a fungus and the roots of its host plant. This relationship is a natural infection of a plant's root system which occurs naturally in about 90 % of the vegetal taxa, in which the plant supplies the fungus with sugars and carbon and receives water and/or mineral nutrients in return. This type of relationship has been around since plants began growing on land about 400 to 500 million years ago. There are several thousand different species of mycorrhiza fungi.

Types of Mycorrhizal Fungi

Mycorrhizae are classified into three types, based on the location of the fungal hyphae in relation to the root tissues. In the endomycorrhiza the fungi produce hyphae inside the roots in intra- and intercellular spaces; in the ectomycorrhiza the fungal infection is spread between the cells of the external layers of roots; in the ectoendomycorrhizae fungi invade the roots colonizing the external layers of cortical cells in inter- and intracellular way. Trophic exchanges take place at the contact sites between cells and hyphae. In the arbuscular mycorrhizae (AM), the most common type of endomycorrhizal (EM) fungi affecting most of the plant species of agricultural interest, hyphae invade the cells of the root cortex, without damaging the cell membrane, branching dichotomously and producing a structure similar to small tree, called arbuscules. These structures are considered the major site of exchange between the fungus and host.

Benefits

Endomycorrhizal fungi benefit a majority of the plants in the world. Ectomycorrhizal fungi, which account for about 3 percent of mycorrhizhae, are more advanced and benefit mainly woody and tree species. In total, mycorrhizal fungi benefit 80 to 90 percent of all plant species.
Plants that do not respond to mycorrhizae include some Brassicaceae, Cariophyllaceae and a few other species.
The main benefit mycorrhizal fungi provide is access to large amount of water and nutrients (particularly nitrogen, phosphorus, zinc, manganese and copper). This is because the hyphae increase the root surface area of absorption from soil. The mycorrhizal hyphae are smaller in diameter compared to plant roots and can reach areas unavailable to the roots. Other reported benefits of the mycorrhiza include: increased pathogen resistance, increased drought and salinity stress tolerance, higher transplanting success, increased crop yield with enhanced flowering, increased water and nutrient uptake, improved soil structure.

Use, Products and Cost

Mycorrhizae are designed for many uses, including vineyards/orchards, nurseries, commercial growers, landscapes, homeowners or for land reclamation projects. The use of mycorrhizal fungi is also popular in organic production. In few cases mycorrhizal fungi could be artificially inoculated in host plant to obtain the fruiting bodies (truffle, Boletus spp., Amanita caesarea, etc.). It is important to note that mycorrhizae can be found in most soils naturally, so it might not be necessary to purchase mycorrhizae. Most soilless mediadonot contain mycorrhizae, so they could be incorporated if growing in containers. Mycorrhizal fungi can be found as granular, powder or in concentrated solution.

Applications

Application of mycorrhizal fungi in production can be conducted as direct infection of cuttings or plugs during transplanting, incorporating into the media or the soil or applied through the irrigation. The inoculant can reproduce with ideal circumstances, such as adding mulch and compost. Avoid overwatering and excessive fertilization applications. However, irrigation, harvesting and Crop rotation may influence the root-fungi combination. Some fungi can colonize new roots within a week, while others may take as long as a month.

One of the objective of the Life4fir project is the mycorrhization of seedlings obtained from artificial cross-pollination and grown up in pots.
In absence of scientific data about Abies nebrodensis mycorrhization, the Basidiomycete Pisolithus tinctorius (Pers.), known as ectomycorrhizal symbiont in A. nebrodensis and related species such as A. alba and A. cephalonica (Castellano and Trappe, 1991; Rincòn et al., 2001; Krajnˇa´kova´ et al., 2012), was selected. This epigeal gasteromycete is characterized by the high production of fruiting bodies during the whole year and the high amount of basidiospores that easily germinate in presence of seedlings roots. These characteristics make it an ideal mutualistic symbiont also in artificial inculation assays and in commercial ectomycorrhizal formulations. Moreover, for long-term storage of fungal inoculum the fruiting bodies of *P. tinctorius* can be dehydrated under air flow at room temperature for 48 hours and stored until inoculation assays. In this case, spores need to be reactivated in sterilized distilled water and maintained at 4°C for 12-18 hours before seedlings inoculation. To facilitate the interaction between the two symbionts, inoculation with spore suspension during transplant is recommended.
Inoculation of one-year old seedlings of A. nebrodensis was carried out during the transplant in winter (December 2021). In detail, 20 ml of *P. tinctorius* spore suspensions at the concentration of 107 spore per plants were directly injected thereabout the seedlings root systems (Rincòn et al., 2001) (Fig. 9).

Fig. 9. Inoculation of spore suspension of *P. tinctorius* in transplanted *A. nebrodensis* seedlings.

Seedlings were transferred in greenhouse and constantly irrigated to speed up fungal colonization. At the end of Summer 2022 (early September) the evaluation of mycorrhization efficacy was carried out. Several parameters were measured to check mycorrhization effects: general growth state; seedlings height (=distance from root collar to apical bud), root collar diameter, index of mycorrhization (IM= number of total tips/lengths of root fragments), dry weight of root system and of the aerial part. The inoculated seedlings showed an intense green color of the needles and a better vegetative growth, with the production of the first lateral branches, compared to uninoculated seedlings. In average, inoculated seedlings resulted higher than non-inoculated ones (about 1.5 cm of height difference), with a thicker root collar (2 mm vs. 1 mm) and a higher IM (7.84 vs 6.25). Differences were consequently detected in the dry weight of the inoculated seedlings and the controls. In fact, the aerial part and the root system of the inoculated seedlings were twice and three times heavier than the non-inoculated ones, respectively.

CHAPTER 4

Plant health survey of the *Abies nebrodensis* trees and control of the biotic and abiotic stresses.

Monitoring the health state of natural forest areas provides basic knowledge about occurring threats, as well as proper protection and conservation measures. Moreover, the collected information may provide clues to understand the influence of disturbances, such as climate change and invasive species on forest ecosystems. The impacts of climate change on forests have already been observed in Europe and there is concern that tree mortality will increase due to physiological stress, insect outbreaks and wildfires driven by future climate change. The finding that Europe's most vulnerable forests are located in the Mediterranean indicated that, due to warmer temperatures caused by climate change, Mediterranean forests will be at risk of forest fires, pest outbreaks and desertification.
In the context of threatened species conservation, 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. It is also important for informing legislative protection and securing investment in management and is a powerful communication tool that allows for meaningful engagement with a broad range of stakeholders. Effective monitoring can inform decision making and management to enhance threatened species conservation. The fragile existence of threatened species further demands that monitoring has adequate precision and sensitivity to detect subtle changes in populations to inform important decisions and future management without delay. Monitoring-management frameworks should begin by defining and scoping the problem (or problems) affecting a species. These initial steps focus on developing compatible conservation, monitoring, and management aims and on outlining management actions and strategies.
In designing a fit-for-purpose program, innovative approaches could be investigated that enable more cost-effective and or data-specific methods. For example, advancements in drone technology can facilitate greater precision in data capture (Hodgson et al. 2016) and eDNA has proven to be an effective tool in monitoring some endangered species or threats; both techniques promise benefits in cost-effectiveness.

4.1 Monitoring of the *A. nebrodensis* trees within LIFE4FIR

The action C1 of the Life4fir project 'Support and preserve Abies nebrodensis in its natural habitat' relies on a series of activities aimed at the preservation of the relic trees of this unique natural population by monitoring and controlling their threats (in situ conservation). These include the outcomes and information obtained by the surveys conducted within the sub-action C1.5 'Spatial and health analysis of A. nebrodensis natural population using drone technology'.
In addition to the threat posed to plants by wild herbivores (expecially fallow deers and

wild boars), disorders have been sporadically observed over the years on the crowns of A. nebrodensis trees (Fig. 10).

Fig. 10. Examples of needle reddening and blight, twigs and branch desiccation and injuries due to wild herbivores of A. nebrodensis in the natural population.

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 phytopathological 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. Monitoring the health conditions through regular surveys has been also aimed at evaluating the effect of the protection measures implemented in the course of the project to the relic trees.
Surveys on plant health were based on the visual inspections the single trees alongside multispectral analysis to monitor eventual physiological disorders at the whole crown level.

4.2 Tree inspections and samplings

Trees were yearly subjected to a careful visual examination to evaluate their state of health based on observations of the crown shape and transparency, turning foliage, presence of declining or desiccated parts, and occurrence of lesions. Disorders observed on the crowns were separately described, recording the type of affected organ (trunk, branches, twigs, shoots, 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.
Laboratory analyses were conducted on the collected samples through: observations under stereomicroscope, isolation and culture of fungal colonies and their genetic characterization through PCR amplification and sequencing of target loci (Fig. 11).

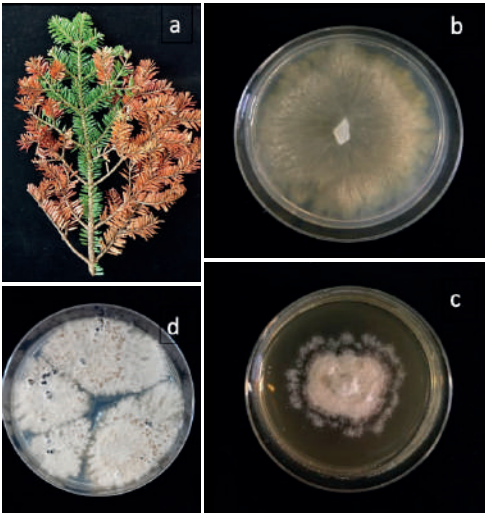


Fig. 11.
a) A sampled twig showing reddened needles and defoliation. Fungal isolates most frequently sampled from reddened needles of A. nebrodensis.
b) Cytospora abietis on PDA plate;
c) Rhizosphaera macrospora on PDA plate;
d) Pestalotiopsis funerea on PDA plate

This allowed to identify 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 on the basis of 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 the involvement of aggressive pathogens to be excluded as the cause of the observed disorders. All isolated fungi were classified as weak pathogens, endophytes or saprophytes, whose development in the plant is associated with environmental disturbances to which the trees are subjected in their natural habitat, as: summer drought, late frosts, high temperatures during summer and intense solar radiation, 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.

4.3 Surveys on the fungal microbiome

The plant microbiome (phytobiome), is defined as a full set of microbial genomes associated with a host plant. The importance of plant-associated microbes for host plant fitness, health and nutrition is universally accepted. In particular, certain fungi and bacteria promote plant growth and increase their stress resistance. Multiple studies showed also that herbivore damage in forest ecosystems is clearly influenced by tripartite interactions between trees, insects and their microbiomes. However, not all symbiotic organisms provide benefits to the host, as plant-microbial interactions can also be neutral (commonly referred to as commensalism) or even deleterious for the host (parasitism or pathogenicity).
In the Life4fir project, the characterization of the fungal microbiome in healthy and disordered shoots and twigs of A. nebrodensis is being carried out to unveil the biodiversity of epiphytic and endophytic fungi residing in the canopy at an ecological level, in relation with different physiological conditions of the tissues and different site conditions.

4.4 UAV surveys and multispectral analysis

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 (UAV - Unmanned Aerial Vehycle) equipped with a digital camera and a hyperspectral images, has been carried out in the initial phase of the project. A second drone survey has been planned by the end of the project to compare the health state of the habitat after the implementation of the planned protection measures.
Two cameras were used: 1) RGB conventional camera. The images taken were geolocated. An orthophoto of the terrain and Digital Elevation Model was made to find correlations between the topographic traits and the development of stresses. 2) Multispectral Camera to obtain 4 simultaneous images, one for each band: Red, Red Edge, Green, Near Infrared. Using these captured images, reflectance maps were created. Different vegetation indices can be obtained from the combination of these reflectance maps. These spectral indices will be specifically designed to produce a value that indicates the amount or vigour of the vegetation.
The frames (in RGB and in the spectrum of green, red, red edge and near infrared) were aligned and, through the Structure from Motion (SfM) procedure, the cloud of points was created. This was used to obtain the orthophoto and the production of Digital Terrain Model (DTM) and surfaces (DSM), through the procedure of triangulation.

The 3D reshaped DTM (Digital Terrain Model) of the area where the natural population of A. nebrodensis allowed to report the distribution of trees in relation to the morphology of the territory (Fig. 12).

Fig. 12. 3D reshaped Digital Terrain Model of the A. nebrodensis population. distributed along the Vallone Madonna degli Angeli. Monte dei Pini e Monte Cavallo.

Orthophotos of the A. nebrodensis natural range can be used as maps, to measure true distances and in Geographic Information System; for combining the image or the results of its processing with other maps or comparing the image or the results of its processing with points representing the 'ground truth'.
For the multispectral images only, detected in the RED (Red), REG (Red Edge), GREEN (Green), NIR (Near Infrared), the reflectance maps were produced after appropriate calibration of the camera made by means of the light sensor mounted on the drone and using the known reflectance values obtained from the calibration panel.
The high reflectance potential of the leaves in the NIR allowed the evaluation of the defoliation of the forest through these sensors. The 50% of the radiation is reflected by the forest canopy if the forest is healthy. The defoliation of forest decreases the reflectance of the NIR.
The infrared images showed in red the reflectance of the cover canopy of the trees. The spectral reflectance is based on water and chlorophyll absorption in the leaf. There are various shades of vegetation due to type, health, leaf structure and moisture content of plants (Fig. 13).

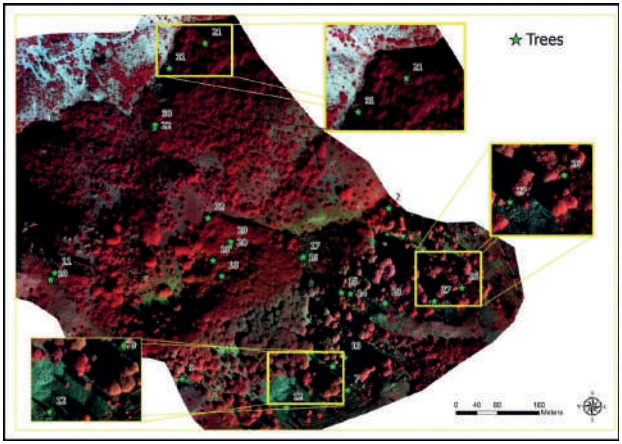


Fig. 13. Infrared map of the area 1. including the main nucleus of the A. nebrodensis population, with some zoomed sites where A. nebrodensis trees can be distinguished.

Multispectral images were suitably analyzed for the production of a NDVI map. NDVI is an indicator that describes the greenness, the relative density and health of vegetation for each picture element, or pixel, in a drone image (Fig. 14).

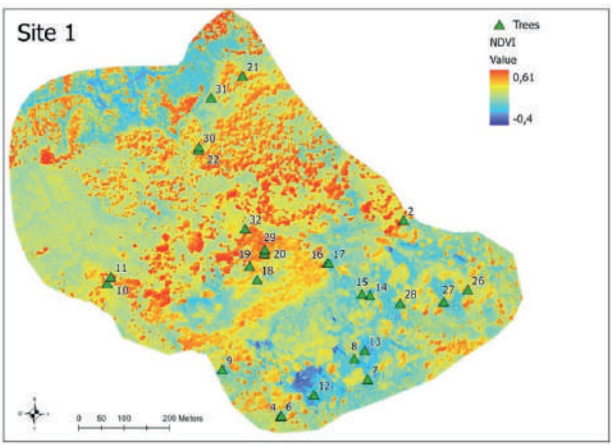


Fig. 14. NDVI map of the area 01 including the main nucleus of the A. nebrodensis population.

NDVI values may range from +1.0 to -1.0. The NDVI of the A. nebrodensis habitat ranged between -0.4 and +0.61 and the areas where the index was higher than 0.5 (red and orange areas) that corresponded to the presence of dense vegetation are rather limited.
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 before the end of the project, multispectral maps can be also useful to monitor the evolution over time of the health state of trees as a function of climatic fluctuations and of the measures that will be implemented meanwhile.

Chapter 5
Sustain to the natural regeneration

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. Ecological restoration relies on natural regeneration processes for achieving forest ecosystem recovery. In addition to enhancing resilience and supplying multiple ecosystem goods and services, natural regeneration can be highly effective for recovering local biodiversity and species interactions.
During natural regeneration local biodiversity is enriched by: 1) natural propagation of trees and shrubs by seeds, root sprouts, stumps, or coppices; 2) regeneration of local genetic sources adapted to local soil and climate conditions; 3) associated pollinators, herbivores, and seed dispersal agents of colonizing trees. Many of these benefits can also be achieved using direct seeding and tree planting approaches, but at significantly higher effort and costs.
A number of social, regulatory, and ecological conditions need to be met for natural regeneration to occur. Natural regeneration may occur when the following ecological factors are met: low levels of soil disturbance and retention of topsoil; proximity to forest remnants or reserves that allows colonization of vegetation from dispersed seeds, root sprouts, or stem sprouts; protection from fires, grazing, and extensive harvesting; minimal presence of fire-prone grasses, ferns, woody vines and invasive species that can impede tree establishment. Where some of these conditions are not present, enrichment planting, weeding, or intensive planting may be required to restore forest cover and qualities. Favorable ecological conditions for natural regeneration are associated with particular social and regulatory contexts for land use (da Feri Policy brief).

5.1 The natural regeneration of *Abies nebrodensis*
Various factors limit the growth and establishment of natural regeneration of the Abies 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 that eat the seedlings (Fig. 15).

Fig. 15 Mature cones of A. nebrodensis for seeds collection

Often the action of localized disturbance (digging action of wild boars, grazing of fallow deers and livestock etc.) has hindered the success of seedlings in the recent past. In 2005, a survey conducted at the end of the previous Life Natura project (LIFE2000NAT/IT/7228) "Conservation in situ and ex situ of Abies nebrodensis (Lojac.) Mattei", reported that the natural regeneration of A. nebrodensis in its natural area consisted of 80 seedlings aged between two and over 20 years, found in the vicinity of eight of the twenty-four plants of the natural population capable of producing seeds. In the survey conducted in 2014, eleven mother trees with 274 accessions were recorded. The increase in the number of seedlings detected indicates a reversal in the dynamics of the Abies nebrodensis population. The young plants of the natural regeneration mainly grow among the cushion shaped plants of Juniperus hemisphaerica and Genista cupanii where they find favorable microclimatic conditions and protection from the biting of herbivores. Furthermore, the presence of a moss layer increases the water content in the soil, which is essential for the survival of the seedlings (Fig. 16).

Fig. 16 Young plants of the natural regeneration growing under Fagus sylvatica and on a moss layer.

5.2 Census and mapping of the juveniles of the natural regeneration

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. Surveys were conducted within the Life4fir project in summer and fall 2020 for the detection and identification of the natural regeneration of Abies nebrodensis in the relic population.

To carry out exhaustive inspections in the field, a survey protocol was developed. It was based on measuring the distance in meters and the azimuth angle of each plantlet or seedling to its respective mother tree, through the use of a professional compass. Survey tables were prepared containing the parameters to be recorded: no. of the mother tree (MP) and GPS position, seedling Id, distance from MP, azimuth, height (cm), age, vegetative and health state, any notes. The collected data were used for the implementation of a comprehensive database, and 15 maps, one for each mother tree, were produced (Fig. 17).

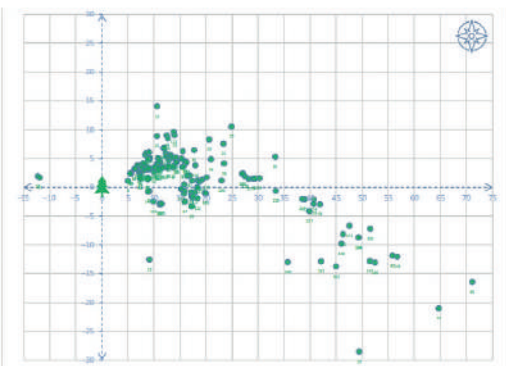


Fig. 17 Distribution map of natural regeneration of mother plant n. 10

Based on the experiences of previous surveys, among which the last dated back to 2014, pegs with labels were not used as signals of young plants. Infact, those used in the previous surveys detached and were chewed by deers after a few weeks as they entice these animals to approach.

5.3 Amount and localization of the natural regeneration

The survey of natural regeneration allowed to detect and record a total of 484 plantlets and seedlings of Abies nebrodensis, subdivided among 15 mother trees. This data is extremely significant considering that in the previous survey conducted in 2014, eleven mother trees with 274 accessions were recorded. New reports concerned the mother tree no. 2, with 3 accessions; the mother tree no. 13 with 4 accessions; the mother tree no. 20, with 31 accessions and the mother tree no. 11, with 31 accessions. The mother trees with the highest number of recorded seedlings were the no.10 (169 seedlings), the no. 22 with 87 seedlings, the no. 21 with 58 seedlings and the no. 18 with 43 seedlings. The young plants ranged between 1 and 24 years old (Tab. 4).

Mother tree (no.)	No. plants of the natural regeneration	No. of seedlings (age < 2 years)	No. tot natural regeneration	Plants already surveyed in 2014	Plants newly reported in the 2020 survey
1	24	5	29	13	16
2	3		3		3
6	2		2	2	
8	14	13	27	2	25
10	143	26	169	21	148
11	26	5	31		31
13	3	1	4		4
17	4		4	4	
18	40	3	43	35	8
20	4		4		4
21	3	55	58	1	57
22	73	14	87	38	49
23	1		1	1	
27	3		3	3	
29	8	11	19	7	12
Tot.	351	133	484	127	357

Table 4. Summary table of the amount of the natural regeneration for each mother tree.

The analysis of the distribution maps showed that a slow growing natural regeneration of Abies nebrodensis has been established around most mature mother trees, preferring protected sites with greater ease of rooting, such as shady and humid sites near beech groves, or oak or holm oak groves, or pillow-shaped juniper (J. hemisphaerica) and broom (G. cupani) shrubs, moss layers and ledges of cliffs facing north. Natural regeneration was found up to distances as far as seventy meters from a mother tree. For example, the 10/82 seedling was found 71 meters southeast of the mother tree no. 10 and the seedling 8/23 was found at

65 meters near a beech stump, along the path located upstream the mother tree no. 8.
A comparison with the data collected in the previous survey of 2014, highlight that the seedlings of A. nebrodensis were subjected to a relevant mortality rate. For example, in the case of the mother tree no. 22, fourty-seven one-year-old seedlings were reported in 2014, whereas only 21 plants with a compatible age (5/7 years) were found in 2020, showing a mortality rate of 55%.
For the mother tree no. 29, 28 seedlings of about one year of age (scattered between 5 and 34 m apart) were detected in 2014, in addition to 8 well established plants. Today 7 out of the 8 established plants were found, but none of the seedlings recorded in 2014. Instead, 12 new seedlings less than two years old were found. Therefore, in this case a 100% mortality of seedlings occurred.

5.4 The new system of fences

For the establishing of the natural regeneration, the fences set up around the adult A. nebrodensis trees have had considerable importance, avoiding external interference in the processes of germination of seeds and development of seedlings. The first system of fences for the protection of the relic A. nebrodensis trees dates back to the 1950s and due to its deterioration over time, it was replaced with new fences built in the following years. The last fences, were installed by the previous LIFE-Natura 2000 project "Conservation in situ and ex situ of Abies nebrodensis (Lojac.) Mattei" carried out between 2000 and 2005.

The Life4fir Project planned the extension and strengthening of the fences around the A. nebrodensis trees to meet two basic needs: 1) most of the extant fences showed again signs of deterioration and were damaged by the massive population of fallow deers and wild boars, having lost much of their functionality; 2) many seedlings of the natural regeneration were found to grow outsides the perimeters of the extant fences and adequate protection measures against herbivores were needed; 3) the extant fences were often overrun by fallow deers and therefore it was necessary to replace them with taller fences. So, a new fence system has been planned and installed to better protect the natural regeneration on a broader surface and to enlarge the 'protected area´ around the relic trees of the population. 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 (and the surrounding space) reaching the relic trees.
To support the natural regeneration and the conservation of the microhabitats around the thirty relict trees, the area protected by the new fences from wildlife and human pressure has been increased to 14,000 m2. The new system of fences has been established to increase the buffer zone around trees and hence to protect the natural regeneration over a larger surface. This will preserve the biocenosis around each tree and will consequently favor the development of the natural regeneration (Fig. 18).

Fig. 18. Fences installed to protect the natural population of A. nebrodensis.

The new fences are made of chestnut poles with a top diameter of no less than 7 cm and a length of no less than 2.40 m. Before installation, they were caulked for the lower 60 cm portion with cold tar and were then put in place 2 m away one another and inserted for 40 cm in the ground.
The metal net is made of 1.60 m height galvanized iron wire with a degrading mesh, with a minimum weight of 0.70 kg per linear meter. It was fixed (by means of galvanized wire) on four orders of galvanized iron wires with a diameter 2.70 mm, which were anchored to the poles by means of staples and placed respectively at ground level, at 1.40 m, at 1.60 m and at 1.90 m from the ground. Each fence is equipped with a 1.5 m wide gate entrance, built according to the scheme planned by the project as shown in Fig. 19.

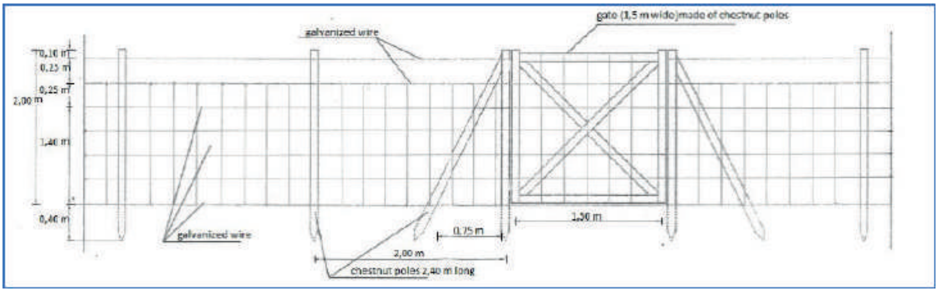


Fig. 19. Design of the fences as reported in the project.

For fences installation, 1800 chestnut poles, 5000 kg of galvanized iron wire, 3750 m of wire mesh and 24 wrought iron joints for the wooden gates were used. Other hardware consisting of hinges and side and ground locks for gates was also used.

5.5 Measures for managing the natural regeneration

Despite the high mortality rate of seedlings, the overall increase of the natural regeneration showed that a gradual expansion of A. nebrodensis in its habitat has occurred in the last years, despite the low effectiveness of the old, worn fences in protecting the mother trees and the young plants growing in their surroundings. The actual origin of natural regeneration, especially the plantlets and seedlings found at a greater distance from the closest mother tree, can be assessed only with the genetic analyses. Nevertheless, the correct census of the natural regeneration as well as the updating the consistency of the population of A. nebrodensis allowed to address the management choices for the conservation of this species. As part of this project, detection and signalling of the actual position of each single accession has been of considerable importance for managing the natural regeneration. The removal of the old fences and their replacement with new fences was in fact carried out in June 2021 without causing any damage or disturb to plantlets and seedlings, which are not easily visible to people not used to looking for them. The installation of the new fences will reinforce the protection to A. nebrodensis seedlings and mother trees and a further increase in the number of naturally regenerated plants is expected in the next years.

Chapter 6
Ex situ conservation of the *A. nebrodensis* germplasm

The ex-situ conservation of the Abies 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

6.1 Establishment of a clonal orchard
The setting up of a clonal orchard has been planned by the Life4fir project. It is meant not only as a simple germplasm collection, but also to produce seed with increased genetic variability in the future, since crossing between the different genotypes is favoured. This will lead to produce seed with a broadened genetic base. The clonal orchard will also allow a constant monitoring of the individual 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 population, 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 must be vegetatively propagated.

6.1.1 Grafting propagation
In conifers, vegetative propagation for the reproduction of 'plus trees,' selected or endangered trees is carried out both by cutting propagation and grafting. In cutting propagation, portions of shoots of about 10-15 cm are treated at the base with talc preparations, containing an auxin (generally, IBA), and placed to root in a misted bench. This is a simple and often effective technique, but not with all conifers. Tests conducted in the past had already highlighted the low rhizogenic potential of Abies nebrodensis cuttings. A sound alternative is grafting propagation, where a portion of the shoot is grafted on a seed rootstock, suitably prepared. The most widely used graft with conifers is the 'veneer-side graft.' In this type of graft, an apical portion of the branch of about 10 cm is prepared with a 'pen' cut of the base and this is inserted into a 'pocket' produced on the rootstock, in a lateral position (Fig. 20a); the graft is then suitably tightened with an elastic to facilitate contact of the regenerating parts (cambium of rootstock and scion) and the grafted point protected from dehydration with aluminium foil (Fig. 20b). During the first period after grafting, the grafted plants are maintained under plastic bags (Fig. 20c) until graft healing occurs (Fig. 20d). This technique was used with material from Abies nebrodensis trees for the vegetative propagation of the A. nebrodensis trees of the natural population to be used for the constitution of the clonal orchard.

(a) (b) (c) (d)

Fig. 20 The grafting of Abies nebrodensis.

6.2 Seed-bank

6.2.1 Purposes of Seed-banks

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, often under the aegis of the most important conservation organizations (such as CGIAR, Bioversity International, CIAT and others). The 'Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture' (SoWPGR-2) of the FAO reported over 7,500,000 accessions, mainly cereals and other species reproduced by vegetative propagation, preserved with this technique. The 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 many tens of years, during which germination and viability tests are periodically repeated. For the above, seeds of the 30 trees of Abies nebrodensis will be preserved in the Seed Bank, established at the MAN (Museo dell'Abies nebrodensis) in the Municipality of Polizzi Generosa.
For conifers, and so for A. nebrodensis, the storage of seeds at low temperature is an important and valid germplasm conservation system, but the large presence of empty seeds, together with normal seeds, can be a problem for preservation. Therefore, we applied X-ray analysis to detect and eliminate empty seeds.

6.2.2 Selection of full seeds

A X-Ray device was used to assay Abies nebrodensis seeds. Seeds were cleaned and placed in plastic square well plates (20x20 cm; Fig. 21).
100 seeds per treatment were individually exposure to X-ray analysis, with appropriate and selected parameters (25 kV, 3 mA at a distance of 45 cm from the X-ray source for 2 min).
Full and empty seeds were identified; in the full seed the embryo was well evident (Fig.22).
To validate this technique, a sample of seeds after X-ray was opened and checked under the stereomicroscope to confirm the presence of an embryo; in a full seed, the embryo is well evident with the presence of endosperm.
To ensure the survival of seeds after X-ray, in vitro germination tests were performed for their viability and germination capacity; the results showed that 100 % survived. This confirmed that X-ray exposition is a non-destructive method when an appropriate (non-lethal) dose of X-ray is used.
Based on those X-ray images (Fig.23), empty seeds or those infested by insects or diseases were removed and considered not valid for preservation. This allowed to maintain and implement the seed-bank (at -18°C) with only full and germinable seeds.

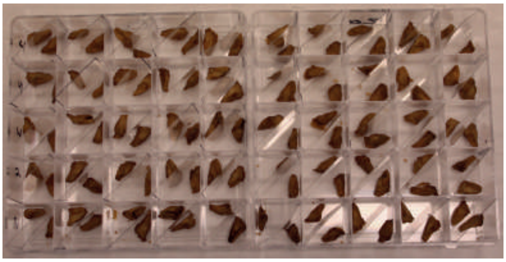


Fig. 21 100 seeds in square-well plates

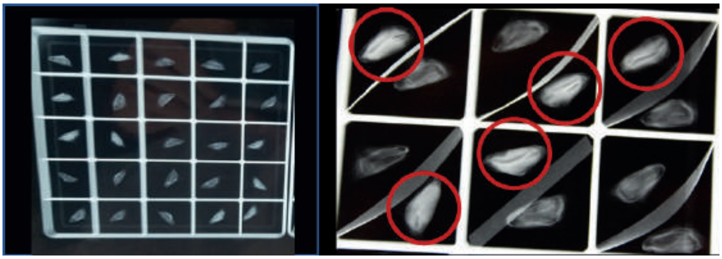
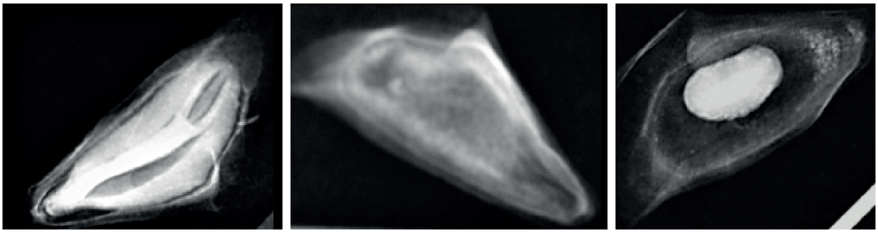


Fig. 22. Seeds on the X-Ray film (left);
full seeds (red circle) and empty seeds (right).



(a) (b) (c)

Fig. 23. Full seed with embryo (a); empty seed (b); seed with larva (c)

6.2.3 Protocol for Seed Conservation at Low Temperature (-18°C)

The cones were collected in October and then the seeds were removed and conserved at 4°C for a short time. After X-ray analysis, only full and healthy seed were selected to storage.
Before being stored, the selected seeds were subjected to viability test (using the TTC/ Triphenyl Tetrazolium Chloride test) and in vitro germination on solid medium (MS hormone-free, 20 g/L sucrose and 7 g/l agar)
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 weighted and placed in labelled jars containing all the associated information (Fig. 24): location of seed bank; species; plant number; collection year; quantity (gr); seeds number; starting date of conservation. Jars were stored in freezer chamber (-18°C).

Fig. 24. Jar labelled with seed sample information
and transferred to Seed-bank

Fig. 25. Seed-bank of *A. nebrodensis*

6.2.4 Constitution of Seed-bank for *Abies nebrodensis*

For the seed-bank, it was fundamental to identify an adequate room. After an inspection to check accessibility for the visitors, the chosen room was placed inside the Municipality of Polizzi Generosa. The room has been completely cleaned before the arrival of the equipment and the interventions related to safety standards required were done.

In selected room, the freezer chamber (Fig. 25) for storage at -18°C of seed from plants of A. nebrodensis was located and it had the following main specifications:

- 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
- control panel: positioned in the upper part of the structure

Inside the freezer chamber (-18°C), labelled jars with full seeds of A. nebrodensis were placed.

6.3 Cryo-bank

6.3.1 Purposes of Cryo-banks

Cryopreservation, i.e., the storage at ultra-low temperatures such as the temperature of liquid nitrogen (LN; -196°C), is the most innovative method which enables long-term conservation of plant genetic resources. The method preserves organs and tissues, from in vitro culture and the field, 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. In fact, at a cryogenic temperature very few biological reactions and significant variations of the physiochemical properties remain active. Nitrogen is a cryogenic gas that, at the liquid phase, is easily available, has a limited cost, and is used universally in cryobanks where it ensures the maintenance of temperatures ranging from approximately -150°C (for samples stored in the space of the container filled with the gas vapors) to -196°C (for samples immersed in the liquid phase of LN). The total absence of subcultures and the arrest of cellular metabolism produced by cryogenic temperatures make cryopreservation a safe method in terms of the genetic stability of the

stored material. Proof of this is found in the numerous experimental works that, particularly in the last 30 years have evaluated the phenotypic, cytological, biochemical and molecular stability aspects of the material subjected to conservation in LN, never showing significant stable alterations. For the above, cryopreservation represents an important additional option for the conservation of Abies nebrodensis genetic resources, and in time various procedures have been developed that allow the conservation of a pool of organs and tissues from in vitro culture or directly from the field. A Cryobank has been established at the MAN (Museo dell'Abies nebrodensis) in the Municipality of Polizzi Generosa and it will preserve excised embryos, pollen and embryogenic callus samples, the latter included in synthetic seeds, from as many as possible of the 30 trees of Abies nebrodensis.

6.3.2 Constitution of Cryo-bank for *Abies nebrodensis*

The room of cryo-bank has been completely cleaned before the arrival of the equipment and the interventions related to safety standards required in an ambience containing liquid nitrogen was done.

Regarding safety standards, the room was equipped with (i) two oxygen detectors, placed in diametrical points of the room, (ii) a window on the access door that allows a full view of the interior when the door is closed and (iii) protective corset, mask and gloves (Fig. 26) to be used during the operations of filling the dewar with liquid nitrogen and handling the samples in storage.

The salient features of dewar used for cryopreservation of A. nebrodensis (Locator 8 Plus; Fig. 27) are the following:

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

- lid with lock.

For the periodic supply of liquid nitrogen (every two months), supplier companies have been contacted, located in Palermo.
Staff with adequate skills will be employed for management of the cryo-bank.
The room will be furnished with posters illustrating the LIFE4FIR project, and the innovative conservation techniques applied to save the endangered species.

Fig. 26. Corset (left), mask (centre) and gloves (right) for protection during the operations with liquid nitrogen.

Fig. 27. Locator 8 Plus, dewar for the conservation of samples in liquid nitrogen, for cryobank of Abies nebrodensis germplasm (left); rack and box where cryovials are allocated (right).

6.3.3 Cryopreservation protocols for *Abies nebrodensis*

Protocol for cryopreservation of pollen

Mature anthers were collected from A. nebrodensis trees during May. After the removal from anthers, the collected pollen grains were sieved and a morphology characterization was also carried out by stereomicroscope, optical microscope, and Environmental Scanning Electron Microscope (ESEM); this showed that the pollen grains of A. nebrodiensis are isodiametric with an elliptical central body with two lateral air sacs (bisaccate) and one aperture leptoma. The air sacs are clearly protruding from the body.

The pollen moisture content was measured by Moisture Analyzer instrument weighing 0.2 gr of pollen. For the pollen cryopreservation, no desiccation process was needed since their water content of 8-10% is reached after three days of store at 4°C. The pollen samples were transferred into cryovials and immersed directly into liquid nitrogen (-196^{o}C). The cryovials containing pollen were thawed under a laminar flow cabinet for 2 hours at room temperature. To validate the protocol, the cryopreserved pollen was subjected to tests of viability by 2, 3, 5 triphenyl tetrazolium chloride (TTC) and germinability in vitro on solid substrate, before and after cryopreservation.

For TTC test, two drops of TTC solution (1%, 200 mg of 2, 3, 5 triphenyl tetrazolium chloride and 12 g of sucrose in 20 ml distilled water) were dropped on a microscope slide with pollen grains and covered with a coverslip. After 24-48 h of incubation in darkness, an observation under the microscope was done and pollen grains stained orange or bright red colour were considered viable (Fig. 28). In vitro germination of cryopreserved pollen was tested onto a semisolid medium composed of boric acid (50 mg/L), sucrose (15 g/L) and plant agar (6 g/L) at 25°C and after incubation of 24-48 h it was checked under the microscope. Pollen grain was considered germinated when the length of pollen tube was 3-fold the grain diameter (Fig. 29).

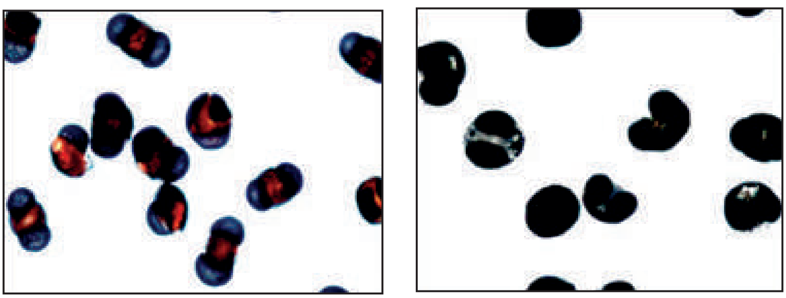


Fig. 28. TTC test -Pollen grain viable (left), no viable (right)

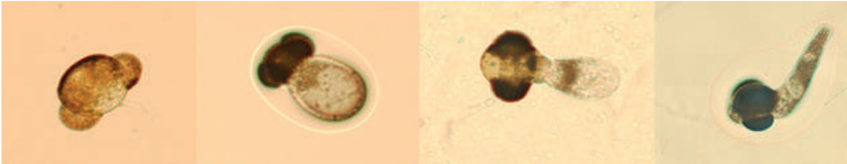


Fig. 29. In vitro germination pollen on solid medium: development of pollen tube

6.3.4 Protocol for cryopreservation of zygotic embryos

The cones from the Abies trees 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 then followed by rinsing in sterile distilled water. Later, the seeds were 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 Moisture Analyzer reporting <10% as an optimal level of humidity before the immersion in liquid nitrogen

The excised embryos were inserted in cryovials, treated with Plant Vitrification Solution 2 (PVS2; 30 % glycerol, 15% ethylene glycol, 15% dimethyl sulfoxide (DMSO) in MS, 0.4 M Sucrose) or not (-PVS2), and transferred inside the cryobox before the immersion in liquid nitrogen at -196^{o}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 germination. The zygotic embryos were completely immersed in TTC (0.1% w/v) for 24 h in darkness at 30°C. Later, they were washed with distilled water and the development of red colour was the main indicator if the zygotic embryo was alive or not. The in vitro germination was carried out by culturing the cryopreserved embryos in vitro on Murashige and Skoog (MS) hormone-free medium.

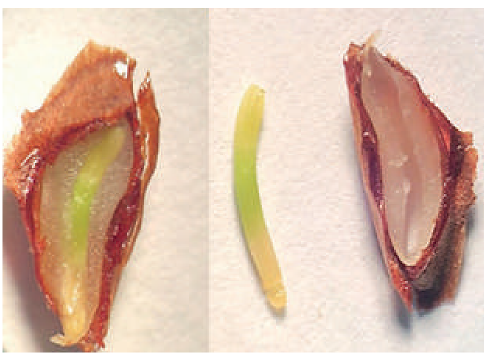


Fig. 30. Sterilized mature seed with embryo (left), excided embryo (right)

6.3.5 Protocol for cryopreservation of embryogenesis callus lines

For Abies nebrodensis, embryogenic callus lines were obtained with the induction from mature zygotic embryos. Embryos were excised from mature seeds harvested in October 2020, from adult trees with the following identification number (ID): 6, 7, 8, 10, 12, 13, 19, 21, 22, 27 and placed horizontally in Petri dishes. Three media were tested: SH Medium (Schenk and Hildebrandt, 1972) supplemented with different concentrations of auxin and cytokinin. Embryogenic callus (Fig. 31) 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. The proliferation medium was the same for the initiation stage. 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 observed under microscope, along with the development of somatic embryos.

The encapsulation/vitrification technique (Fig. 32) was applied for the cryopreservation of the embryogenic callus lines. The protocol consists in the 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.

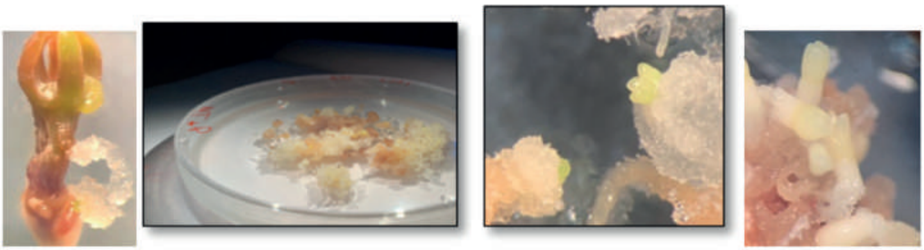


Fig. 31 Induction and development of embryogenic callus lines

Fig. 32 Encapsulation/vitrification technique for the criopreservation of the embryogenic callus lines.

Chapter 7
Repopulation in re-diffusion nuclei with selected seedlings

Through the action C6, 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.
Inspections carried out in the territory of the Madonie Park, allowed the identification the of sites showing adequate ecological-environmental conditions, also considering the indications provided by the results of the reforestation plots of A. nebrodensis set up in previous projects. The results obtained with the experimental plots created between 2001 and 2005 with the LIFE2000NAT/IT/7228 Project "Conservation in situ and ex situ of Abies nebrodensis (Lojac.) Mattei " were especially taken into account. In all, ten sites were identified in the Madonie area, distributed within the municipalities of Polizzi Generosa, Isnello, Petralia Soprana, Petralia Sottana, Geraci Siculo and Gratteri (Tab. 5 and Fig. 33). All sites fall within the perimeter of the Madonie Park, in areas managed by the Department of Agricultural and Territorial Development of the Regional Government.

sito	Comune	latitudine	longitudine	Altitudine (m slm)
Casa Prato	Polizzi Generosa	37°50'49.79"N	14°1'58.23"E	1610
Sanguisughe (Portella Fatuzza)	Polizzi Generosa	37°48'55.52N	14°1'53.78"E	1140
Quacella	Polizzi Generosa	37°50'51.71"N	14°0'45.07"E	1240
Piano Formaggio	Isnello	37°53'36.80"N	14°0'15.21"E	1220
Favarotta	Isnello	37°54'21.87"N	13°59'11.92"E	820
Savochella	Petralia Soprana	37°50'20.15"N	14°6'39.78"E	1450
Mandarini	Petralia Sottana	37°51'39.69"N	14°5'38.07"E	1290
Fegotti	Geraci Siculo	37°50'26.18"N	14°8'43.18"E	1270
Pantano	Geraci Siculo	37°50'24.47"N	14°9'25.03"E	1110
Serra Arcia	Gratteri	37°57'26.97"N	13°58'6.06"E	750

Table 5 - Localization of the ten reforestation sites of A. nebrodensis within the Madonie Park.

Fig. 33 Map reporting the position of the ten reforestation plots in the Madonie Park territory.

7.1 Vegetation traits in the selected areas of repopulation

The ten 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 potentially suitable to the development of A. nebrodensis seedlings.
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 the quartz arenites of the Numidian Flysch. These woods are characterized by Quercus petraea subsp. austrotyrrhenica as main species, associated with Acer obtusatum, A. campestre and, to a lesser extent, A. monspessulanum, Sorbus torminalis and Ulmus glabra. In the cooler sites and at higher elevations, species of the upper belt such as Fagus sylvatica and Acer pseudoplatanus can be found. The understorey consists mainly of Ilex aquifolium which finds here optimal growth conditions, forming a dense layer of vegetation interrupted only by Malus sylvestris, Crataegus orientalis subsp. orientalis, Prunus spinosa, Euonymus europaeus, Daphne laureola, Rhamnus catharticus and Ruscus aculeatus. Among the herbaceous plants, in addition to Aquilegia vulgaris typical of this association, there are some species of phytogeographic interest such as, Anemone apennina, Cyclamen repandum, C. hederifolium, Dactylorhiza romana, Hieracium racemosum subsp. pignattianum, Drymochloa drymeia, Lathyrus venetus, Primula acaulis, Symphytum gussonei, Viola reichenbachiana.
The plots located above 1400 m a.s.l. elevation fall within the woods of beech, related to Geranium versicoloris-Fagion climax association. These woods are dominated by Fagus sylvatica which is often associated with aged trees of Acer pseudoplatanus. The understorey

is characterized by the presence of Sorbus graeca, Orthilia secunda subsp. secunda, Euphorbia meuselii and, sporadically, Rhamnus catharticus and Ilex aquifolium. In the more open and less favorable sites, Astragalus nebrodensis, Crataegus laciniata, Prunus mahaleb subsp cupaniana and Rosa sicula are also found. The herbaceous layer is made of a few species, among which the most frequent are: Allium pendulinum, Anemone apennina, Galium odoratum, Cardamine chelidonia, Cyclamen hederifolium subsp. hederifolium, Corydalis solida, Doronicum orientale, Galium odoratum, Geranium versicolor, Lamium flexuosum var. pubescens, Luzula sylvatica subsp. sicula, Monotropa hypopitys, Lactuca muralis, Neottia nidus-avis, Scilla bifolia and Hieracium racemosum subsp. pignattianum.
Some sites located between 800 and 1200 m a.s.l. elevation (Quacella, Piano Formaggio, Favarotta), though located on a calcareous substrate, have a deep, decalcified soil. They are affected by vegetational traits related to the mesophilic holm oak wood (Aceri campestris-Quercetum ilicis), an association characterized by the presence of Ilex aquifolium and some deciduous tree species such as Acer campestre, A. monspessulanum, Sorbus greca, Malus sylvestris and oaks related to Quercus pubescens. In the understorey in addition to the species already cited, many taxa are can be found, as Euphorbia characias, Fraxinus ornus, Lonicera etrusca, Pyrus amygdaliformis, Rubia peregrina, Rosa sempervirens, Ruscus aculeatus, Clematis vitalba, Daphne laureola e Tamus communis. The herbaceous layer is represented by Brachypodium sylvaticum, Cyclamen hederifolium, C. repandum, Lamium flexuosum, Asplenium onopteris, Thalictrum calabricum, Trifolium pratense, Viola dehnhardtii, Paeonia mascula subsp. mascula.
A site located at about 750 m a.s.l. elevation (Serra Arcia) is justified by the consideration that A. nebrodensis in the past grew at lower elevations compared to the residual population which, due to the anthropogenic action, is now restricted to less accessible sites located between 1400 and 1600 m a.s.l. elevation.

7.2 Planting procedures

As regards the planting of the A. nebrodensis seedlings in the individual plots, the procedure will take into due account the positive results obtained with the plantations carried out in the previous LIFE project.
Planting should be done following the contour lines and the exposure of the site. Taking into account the objectives of the Life4fir project, the individual plots, will have an area between 3000 and 4000 m2 and the number of plants that will be planted is 400. Spacing must allow the plants to grow without disturbance and will be defined to avoid damage to any species rare and/or endemic present.
For each single plot, the following measures will be implemented stepwise: fencing, opening of the holes, planting of enhancing legumimous shrubs, planting of A. nebrodensis seedlings.

7.3 Fencing

The fences will be installed along the perimeter of each plot following the same procedure used for the protecting fences set up around the relic trees of the natural population.
Chestnut poles with a diameter of 8-10 cm and a length of 2.40 m will be used. They will be inserted into the ground for about 40 cm and placed at a distance of 2 m each other (Fig. 34). Their height above ground will be about 2 m. Entry to the plots will be allowed by gates 1.5 m wide and 1.80 m high (Fig. 35).

Fig 34. Installation of a fence

Fig. 35. Placement of a gate

7.4 Digging holes for planting

The opening of the holes will be carried out in the soil both with mechanical devices and with specific agricultural equipment. The holes spaced about 3-4 meters apart from each other will not have a well-defined spacing pattern but will be positioned based on the characteristics of the site. To ensure the young seedlings of A. nebrodensis a harmonious development of the root system and a greater water reserve, the holes will have a particular shape. Truncated-cone holes and pyramidal holes will be preferred.
The former will have a lower diameter of about 80 cm and an upper diameter of about 50 cm, with a depth between 50 and 60 cm (Fig. 36). The pyramidal holes will have the lower side of 80 cm, the upper one of 50 cm, while depth will be 60 cm (Fig. 36).

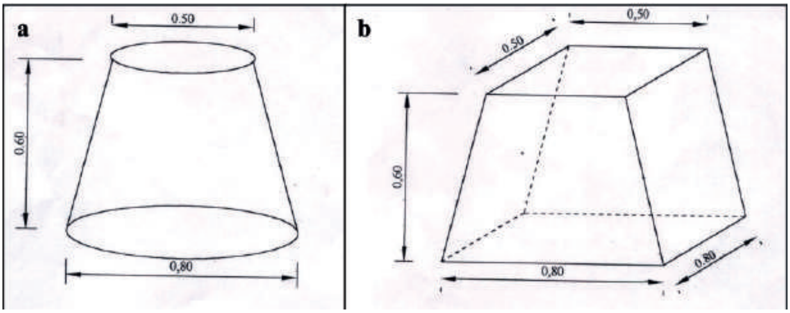


Fig. 36 Scheme of a truncated-cone shaped hole (a) and of truncated pyramid shaped hole (b).

7.5 Planting of enhancing *Leguminosae* species

The planting of shrubs belonging to the Fabaceae familiy, is aimed at improving soil fertility and ensure adequate protection to the A. nebrodensis seedlings from both excessive sunlight, heat and summer drought after the transplant in the field.
The shrubs that will be used belong to the Genista, Spartium and Cytisus genera and will be 2-3 years old. These will be planted near the A. nebrodensis seedlings at a distance of about 50 cm, helping to maintain a suitable microclimate for the development of the seedlings in the spring-summer period and to protect them from strong winds. Generally, two shrubs will be planted around each single A. nebrodensis seedlings.

7.6 Planting of *A. nebrodensis* seedlings

For each single plot, 400 seedlings raised in 9x9x20 cm containers in the nursery will be planted out. The seedlings will be about 3 years old. In the nursery, they were transplanted after 1 year and were subjected to mycorrhization. As mentioned above, planting will not follow a geometric pattern but will be performed according to the morphology of the soil and in compliance with the vegetation settled. During planting, phosphorus-rich organic fertilizer will be added to the hole to help growth of the transplanted seedlings. After planting, a dip in the ground will be created around each single plant to favor the accumulation of water. Furthermore, to reduce evaporation from the ground around the hole, a mulching with plant material obtained from the herbaceous species present in the immediate vicinity of the plots will be made in spring.
Within the plots, about 50 holes of 40x40x40 cm will also be created for the direct sowing of A. nebrodensis, placing the seed at a depth of about 3 cm. Five seeds will be sowed per hole.

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