LIFE4FIR – Project LIFE18 NAT/IT/000164

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

"Impact in *Abies nebrodensis* conservation report" - Action D2.

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1. Introduction

The LIFE4FIR project had the general objective of developing and implementing innovative strategies for the in situ and ex situ conservation of *Abies nebrodensis*, a species of fir endemic to the Madonie Park, located in the central-northern part of Sicily. The Madonie Fir is classified as critically enangered and is included in the IUCN red list. The residual natural population is currently made up of only 30 adult trees, distributed in an area of about 84 hectares within the Madonie Park.

Abies nebrodensis is actually very vulnerable due to a series of threats such as: genetic erosion, fragmentation (and consequent autogamy), slow development of the natural regeneration, rocky and superficial soils, overpopulation of wild herbivores (fallow deer and wild boar) and possible hybridization with non-native firs, reduced germination and survival of seedlings in nurseries. In light of these threats, the LIFE4FIR project has been focused on a set of objectives that could jointly improve the conservation status of the species.

- The first objective was to support and protect the population of the relic adult trees and the young plants of the natural regeneration of *Abies nebrodensis*, helping them to counter the main threats, intervening directly in their natural habitat.
- At the same time, efforts were directed to increase the genetic diversity of the species by promoting cross-pollination through a controlled cross-breeding program.
- After a phase of growth in the nursery, these seedlings were used in a reforestation plan in selected areas with the aim of creating new repopulation and re-diffusion nuclei and restoring the dynamic structure of the population.
- Lifefir wa salso aimed at ensuring the conservation of the species also through ex-situ measures such as the establishment of a clonal orchard and the launch of a seed bank and a cryobank.
- In addition to these technical objectives, the consortium has pursued the objective of promoting and spreading the protection measures implemented, also ensuring their sustainability and replication in the years to come. Increasing awareness on the importance of biodiversity and the protection of endangered species, as well as specific training and education measures for the replication of the LIFE4FIR models, are fundamental tools to ensure a lasting and effective impact of the project.

All these activities have produced a series of results and have allowed the development and fine-tuning of procedures and tools for monitoring, propagating, managing of the genetic resources, raising of seedlings in nurseries, for the ex situ conservation of germplasm, that will

certainly allow the species to be supported and will produce positive impact on its conservation in the years to come.

2. Impact of measures implemented by Life4fir in the *Abies nebrodensis* conservation

2.1 A new system of fences for protecting *Abies nebrodensis*

The Life4fir Project carried out the extension and strengthening of the fences around the *Abies nebrodensis* trees (C1.1) to meet two basic needs: 1) most of the current fences (installed more than 20 years ago) 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) seedlings of the natural regeneration were found to grow outsides the perimeters of the extant fences and required adequate protection against bite and soil compaction by herbivores; 3) strengthening protection to the adult trees with wider and higher fences. So, a new fence system has been installed to better protect the natural regeneration on a broader surface and to enlarge the 'protected area´ for the relic trees of the population. The new fences were meant to protect the *Abies nebrodensis* population also from anthropogenic pressure and from the numerous visitors traveling along the paths of the park (and the surrounding space) reaching the trees.

New fences

Fenced area	14050 sqm
Wire mesh length	3750 m
Number chestnut poles used (2,50m long)	1800
Galvanized Iron wire	5 t

The surface of the protected area has been extended to over 14,000 sqm. This will ensure the maintenance of optimal vegetative conditions, will preserve the biocenosis around each tree and will consequently favor the development of the natural regeneration.

2.2 Increasing natural regeneration

According to the census carried out at the beginning of the project in 2020, the number of young plants of natural regeneration has increased significantly compared to the previous census of 2014, from 274 to 484 (+ 75%). This occurred despite the old fences, built 20 years earlier, were worn and inefficient. In the years following the installation of the new fences (2021), the presence of new

seedlings, not previously counted, was observed. In the last monitoring carried out in December 2024, shortly before the end of the project, it was possible to observe a further increase in the number of seedlings as well as in the number of trees contributing to natural regeneration. In particular, the number of trees regenerating increased from 15 to 17, while the number of growing seedlings was 522. Therefore, a further development and increase of natural regeneration is expected in the next years, as the new fences are providing a more effective protection. A new census will allow this effect to be quantified.

2.3 Video surveillance system

A video surveillance system has been installed throughout the natural range of *Abies nebrodensis* as a deterrent and to control wildlife, abandoned livestock and visitors.

The system was made by 5 stations installed in the most visited sites of the *Abies nebrodensis* range. Through an LTE/4G router, the acquired images can be transmitted to cell phones via SIM cards. The images can also be saved in local SD memories. The system was then upgraded to allow the real time transmission of the images acquired via satellite.

Video surveillance will help control the flow of visitors along the path crossing the *Abies nebrodensis* population and will allow monitoring the use of the site, as well as providing useful indications for the control of wild herbivores. The cameras are able to shoot images based on motion sensors. Since the installation (May 2024), an average of 2 videos per week have been viewed for each of the 5 cameras installed, for a total of about 320 videos, in which the passage of people and animals was visible.

2.4 Improved practices for raising healthy and vigorous plants in the nursery

The surveys conducted on the young plants of *Abies nebrodensis* present in the nursery 'Piano Noce' at the beginning of the project allowed to evaluate growth and health conditions and to identify the cultivation practices to be improved for assuring optimal growth to plants. This point was considered essential to produce quality and vigorous nursery stock to be used in the reforestation.

Surveys involved approximately 25,000 open-pollinated plants from 12 families. The observations allowed us to define the incidence of disorders (<8%), the most frequent symptoms (chlorosis and reddening of the needles, 3.8% and 1.0 %, respectively) and to exclude the development of

pathogens which may be harmful in the nursery set (e.g. Phytophtora). Growth conditions of the seedlings were generally good.

However, some cultivation practices have been identified that needed to be improved to ensure that the young plants have the most suitable growth conditions: 1) use of a standardized substrate; 2) regular transplants every two years; 3) use of seedling trays to reduce the risk of damaging the root system during transplant; mycorrhization even with commercial formulations of ectomycorrhizal fungi that have demonstrated efficacy in promoting the growth of the root systems of seedlings. The availability of healthy and vigorous seedlings, genetically selected, is important to support reforestation activity and therefore for the conservation of *Abies nebrodensis*. At the end of the project 4000 seedlings have been raised following the improved procedure described. They were used for reforestation purposes and 3500 of them were planted out in the new plots created by Life4fir.

2.5 Effective protocols for genetic analysis, monitoring, propagating (grafting and crossing) and ex situ conservation of germplasm.

2.5.1 Genetic characterization

The PCR-based OpenArrays technology (Thermofisher Inc., United States) was used for SNP genotyping. A panel of 120 SNPs was developed for genotyping of *Abies nebrodensis* individuals composed by information-rich SNPs in samples of *Abies nebrodensis*, *A. alba* and *A. cephalonica*. The developed procedure represents a modern and reliable tool for population genetics studies through the evaluation of the following parameters: population structure, inbreeding level, genetic diversity, genetic relatedness among trees, rate of self-fertilization and eventual hybridization with non-native Abies species in the natural regeneration and in the progenies raised in the local nursery. The results obtained are very useful for conservation management. The analyses highlighted a high level of inbreeding and self-fertilization in the natural regeneration, suggesting the need for assisted management to promote gene flow through controlled crosses. Cross combinations were then defined and carried out based on the pairwise co-ancestry estimation between the plants of the natural population, with the aim of fostering crosses between plants with greater genetic distance. From the crosses carried out in various years 4000 outbred seedlings were obtained for reforestation.

2.5.2 Health state monitoring

Health state monitoring of the trees in the natural population was carried out through morphometric surveys, crown inspections, sampling and laboratory analyses for the isolation of fungal microorganisms. These observations were integrated with multispectral analyses using drones to measure the physiological state of the trees based on the radiation reflected by the crowns.

The crown disorders of the critically endangered *Abies nebrodensis* population were surveyed for the first time providing insights on the phyllosphere fungal community.

The health condition of trees in the natural population were found to be fairly good. The most frequent symptoms were needle reddening and needle cast with subsequent defoliation of twigs and branches in the outer part of the crowns. But these symptoms were sporadic and sparse. In general, the extent of symptoms observed were found to be related to the environmental conditions that trees are facing at a microclimate and site level, as reported in the table below.

Tree n.	Trunk height (m)	Crown diameter (m)	L (m^2)	Elevation a.s.l. (m)	Position	I	Group of trees
20	9.2	2.9x3.2	44,7	1480	Within a beech grove	0	Group 1 No symptoms
22	12.0	5.90	56,0	1400	Within a beech grove	0	
27	10.0	7.4x6.7	117,4	1597	Isolated	0	
29	10.5	4.2x4.9	76,8	1468	Within a beech grove	0	
30	1.76	1.7x1.6	5,2	1400	Under oaks	0	
32	1.96	1.7x1.8	5,7	1449	Within a beech grove	0	
21	11.6	8.5x7.7	156,3	1433	Within oak grove	0.03	Group 2 0.01 < I > 0.1
8	11.0	7.7x6.9	132,9	1577	Near broadleaves	0.04	
17	10.7	8.7x7.3	143,5	1488	Isolated	0.04	
19	5.5	4.6x4.9	44,7	1487	Margin of a beech grove	0.05	
13	11.1	9.5x9.2	176,9	1567	Isolated	0.05	
2	14.4	9.2x9.1	217,2	1526	Isolated	0.06	
14	7.2	6.5x6.8	82,1	1556	Near oak trees	0.06	
26	6.0	3.8x3.6	36,5	1599	Within a beech grove	0.08	
7	5.7	5.3x5.5	53,5	1603	Near an oak tree	0.09	

10	7.5	7.5x7.3	97,2	1525	Isolated on a ridge	0.11	Group 3 0.11 < I > 0.5
18	7.6	5.2x4.5	60,1	1503	Near beech trees	0.13	
15	8.5	5.7x5.2	76,4	1539	Near oak trees	0.17	
16	5.3	7.7x4.8	59,8	1488	Isolated on superficial soil	0.18	
11	8.2	5.1x4.6	65,1	1520	Isolated on a ridge	0.20	
4	0.85	2.1x1.5	3.5	1639	Under a bigger A. nebrodensis tree	0.19	
6	7.8	7.2x6.3	90,1	1639	Isolated	0.26	
23	7.5	4.6x4.3	54,7	1673	Isolated on a ridge exposed to strong winds	0.33	
24	3.1	3.8x4.1	22,8	1705	Isolated on a ridge exposed to strong winds	0.53	Group 4 0.51 < I > 1.00
25	3.3	3.5x1.6	14,2	1705	Isolated on a ridge exposed to strong winds	0.71	
1	6.5	6.4x7.6	81,2	1651	Isolated and exposed to strong winds	0.74	
12	8.5	6.8x8.4	111,2	1604	Isolated on a scree exposed to strong winds	1.28	Group 5 I > 1.00
31	1.25	1.4x1.3	2,9		Small, damaged by wild herbivores	1.39	
9	1.8	3.6x3.6	14,4	1617	Isolated on rocks exposed to strong winds	2.43	
28	0.48	1.3x1.1	1,4	1586	Small, damaged by wild herbivores	2.96	

Table 1 Adult trees of *A. nebrodensis* were subdivided in five groups based on the number of reddened and blighted twigs per unit of crown surface (Impact, I). L: crown surface meant as a cone lateral surface area. Grey shades indicate groups of trees showing a different I rate (from Frascella et al., 2024. Insights on the fungal communities associated with needle reddening of the endangered *Abies nebrodensis*, Journal of Plant Pathology)

Surveys showed that 7 out of 30 adult trees of the natural population reported no disorders (group 1); 9 trees showed an impact (I) ranging from 0.01 to 0.1 (group 2); 7 trees from 0.11 to 0.50 (group 3); 4 trees from 0.51 to 1.00 (group 4); 4 trees showed an I > 1.00 (group 5).

Isolations were carried out from 250 reddened needles and 250 green needles, plating 2000 leaf fragments, leading to 204 fungal colonies and 21 identified taxa. Isolations excluded the involvement of aggressive pathogens, revealing the participation of opportunistic fungi, endophytes and saprophytes on needles affected by environmental stressors such as wind, hail, and mechanical wounds. These fungi do not represent a biotic constraint for *Abies nebrodensis*. The plants were found to be in good or fair health state and seem able to tolerate the environmental stresses of their habitat (detailed information in Frascella et al., 2024 https://doi.org/10.1007/s42161-024-01639-7).

The multispectral surveys revealed a correspondence between the visual inspections and the NDVI index obtained for the individual crowns, which was well above the threshold indicating a state of suffering (0.6). The median NDVI obtained for the crowns of the A. nebrodensis trees ranged from 0.75 to 0.85.

The data collected represent a reference line for evaluating the evolution of the growth and health status of plants in the years to come, also in relation to the protection interventions carried out and to the climate change.

2.5.3 Grafting propagation and setting up of the clonal orchard

To set up the clonal orchard, vegetative propagation of the trees in the *Abies nebrodensis* population was required for their replication. The grafting technique that has been used in this Project is called "veneer-side grafting", by far the most used in the nurseries for the grafting propagation of conifers.

ID Mother tree	Nr. living grafts	No. of grafts planted out in the orchard
1	9	8
2	15	12
4	9	8
6	22	14
7	21	14
8	17	15
9	18	14
10	11	9
11	13	11
12	24	15
13	23	14
14	27	14
15	15	12
16	18	15
17	26	17
18	14	11
19	23	14
20	12	12
21	33	15
22	17	12
23	5	4
24	9	8
25	13	8
26	16	18
27	15	14
28	4	4
29	14	12
30	4	2
32	9	7
tot	456	333

Table 2. Number of grafts performed for each A. nebrodensis mother tree and success in terms of living grafts and percentage.

However, for some species, including *Abies nebrodensis*, the success of the grafts is very low for reasons related to the biology of the species. Hence, this technique has been refined, and some steps have been fine-tuned. This lead to a rate of success never achieved before, allowing us to obtain 456 grafted plants and to replicate all the plants of the population which are all represented in the clonal orchard which included 333 grafted plants.

The clonal orchard is intended not only as a simple collection of germplasm, but also as a facility for the future production of seeds characterized by greater genetic variability, as cross-fertilization between the different genotypes is encouraged, eluding the distance between the trees in the natural population.

The clonal orchard will also allow constant monitoring of individual genotypes with regards to growth, habitus, phenology, etc., for scientific and educational purposes. In the future, when the plants reach maturity, the orchard will be used as a new source of seeds or other propagation material, avoiding negative impacts on the trees natural population.

2.5.4 Ex-situ conservation of *Abies nebrodensis* germplasm: samples conserved in the seedbank

Seed banks represent the most used ex situ conservation system for the conservation of plant biodiversity. The seed samples collected from adult *Abies nebrodensis* trees are stored in the Seed Bank, recently launched at the *Abies nebrodensis* Museum in the Municipality of Polizzi Generosa thanks to the Life4fir project.

To create the seed bank, procedures were developed for the conservation at -18°C of seed samples from fertile *Abies nebrodensis* plants by performing assays on: seed collection, seed selection, seed vitality and germination tests before and after low-temperature conservation.

Currently samples of seeds collected from 12 trees are conserved. Only full and viable seeds were selected for storage. They have been carefully selected following the X-ray screening procedure. The collection of seeds was affected by the irregular and variable flowering that characterizes this species, so that not every year cones were available. Furthermore, not all trees are fertile (only 24 out of 30). One of the tasks that will be carried out in the after-Life phase will be to integrate the seed bank by inserting samples of the missing plants.

Tree (id)	2020		2022	
	Nr of conserved seeds	weight (gr)	Nr. of conserved seeds	weight (gr)
6	334	19.5	134	8.22
7	458	20.6	-	-
8	784	36.2	181	13.02
10	1000	50	193	13.85
12	531	28.2	118	7.13
13	344	17.01	471	22.02
17	42	3.3	-	-
21	686	44.78	323	18.69
22	598	25.4	285	11.13
23	-	-	99	4.53
25	-	-	3	0.19
27	200	9.2	156	8.05

Table 3. For each mother tree, the number and weight of seeds conserved at -18°C in the A. nebrodensis seedbank are reported.

2.5.5 Ex-situ conservation of A. nebrodensis germplasm: samples conserved in the cryobank

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

Within Life4fir, procedures have been developed for the cryopreservation of pollen, zygotic embryos, and embryogenic callus lines through viability, germination, and growth assays before and after liquid nitrogen preservation.

Pollen samples		
Trees Id.	No. cryovials (year2020)	No. cryovials (year 2022)
1	1	5
2	1	2
6	1	10
7	1	2
8	1	14
9	1	20
10	1	7
11	1	10
12	1	10
13	1	14
14	1	12
15	1	15
16	1	3
17	1	6
18	-	4
19	1	3
21	1	1
22	1	18
23	1	2
24	1	5
25	1	0
27	1	9
29		1

Table 4. Pollen samples of A. nebrodensis conserved in the cryobank

Zygotic embryos		
Tree Id.	No. cryovials (year 2020)	Total No. of embryos
6	20	100
8	21	105
10	18	90
13	15	75
21	18	90
22	18	90
27	16	80

Table 5. Number of zygotic embryos conserved in the cryobank per each A. nebrodensis mother tree

Embryogenic callus lines (encapsulated)		
Tree Id.	No. cryovials 2020	No. encapsulated calli
7	11*	44
8	11	44
10	11	44
11	x	x
15	x	x
16	x	x
21	11	44
22	10	40

Table 6. Number of embryogenic calli conserved in the cryobank per each mother tree.

* 4 capsules are contained in each cryovial. x: callus lines obtained but still to be inserted into the cryobank.

One of the tasks of the after-Life activity will be to widen the germplasm collection in the cryobank by adding the samples of the trees still missing.

2.6 Launch of the seed bank and the cryobank

The seed bank and cryobank have been set up in the Museum of *Abies nebrodensis* at the Municip29ality of Polizzi Generosa. Their functioning at full capacity represents a safe way to assure the ex situ conservation of the *Abies nebrodensis* germplasm in the face of natural threats that could jeopardize the survival of the plants in the natural population (e.g. fires). Their management and the possibility of being visited allow the involvement of local communities and students on issues related to biodiversity and its conservation.

2.7 Repopulation with selected seedlings

One of the main objectives of the project is to create new reforestation nuclei using selected *Abies nebrodensis* seedlings obtained from cross-pollinations.

The new plots were set up in 10 areas of the park, using genetically selected seedlings obtained with controlled crosses. The areas were chosen based on the ecological requirements of *Abies nebrodensis*, preferring sites with a north, north-east exposure, under the cover of existing tree formations. Broom plants were placed side by side to ensure adequate protection for the young fir seedlings. The new plots were previously fenced to protect them from wild herbivores. Shelters

were applied to the 3500 seedlings in the plots as an additional form of protection against wild fauna and to favor a water supply deriving from the condensation of water vapor in the air.
In the Mandarini and Favarotti plots, set up in May 2023, 22 and 17 dead plants were respectively detected in the surveys carried out at the end of the project. The fails will be replaced with the 500 extra plants obtained from the controlled crosses.
The plantations created are an important investment for the future of the species. They significantly increase the population size and improve its genetic pool through the use of selected outbred progeny to overcome the high rate of self-fertilization found in natural regeneration. This will lead to an increase in genetic diversity, addressing genetic erosion and reducing homozygosity. This potentially will lead to a better adaptation capacity of the species to environmental stresses and to climate change. Furthermore, the re-diffusion cores will be properly realized in areas where the *Abies nebrodensis* can eventually have the chance to migrate upward to counter global warming. The success of the new plots will therefore have a decisive impact on defining the conservation status of *Abies nebrodensis* in the coming years. Great care of the created nuclei and the post-planting interventions will be important activities during the after-life phase of the project.

3. Conclusions

3.1 Main points emerged

Extension of the area in strictly protected status (fenced)	14,000 sqm
Perimeter of the fenced area	3750 m
Videosurveillance: images downloaded and checked	320
Increase of the natural regeneration	522 plantlets (8% increase compared to 2020)
Outbred seedlings obtained	4000
Plants used for reforestation	3,500
Living plants at project end	3,463
Area interested by repopulation measures	40,000 sqm
Grafted plants obtained	456
Grafted plants in the clonal orchard	333
Number of plants raised with nursery improved procedures	25,000
Trees stored in the seedbank	12
Cryobank Trees with stored pollen Trees with stored embryos Trees with stored embryogenic callus lines	 23 7 5 (+3)
Health state Median NDVI of the crowns	0.75-0.85

From a strictly numerical point of view, some indicators can usefully represent the effect of some measures implemented by the Life4fir project:

Among the implemented measures, only some are able to produce an immediate effect on the conservation of *Abies nebrodensis*, such as the new fences, the seed bank and the cryobank.

Effects of most activities on the conservation of *Abies nebrodensis* will be assessable in the years to come, such as the new repopulation nuclei and the clonal orchard. The success of the new plots will be assessable when the plants are settled and are able to grow without the need of cares, showing good growth rates and good health. The plots set up with Life4fir will be added to those created in previous projects since 2000, some of which show excellent results with plants that have reached or are reaching fertility and become generators of progeny themselves.

Among the parameters that can be used as indicators for the conservation of *Abies nebrodensis* in the years to come, the following can be considered:

- Temporal change in the number of plantlets of natural regeneration;
- Number of outbred seedlings obtained though the controlled crosses campaigns;
- Survival, growth and health state of the genetically selected plantlets in the repopulation sites;
- Number, genetic origin and health state of plantlets raised in the local nursery;
- Growth and health condition of trees of the natural population;
- Reduction of the fallow deer and wild boar populations;
- Survival, growth and state of health of plants in the clonal orchard;
- Regular operation of the cryobank and seed bank and amount of samples conserved;
- Absence of non-native firs in the surroundings of *Abies nebrodensis* population.
- Increase awareness of local people and schools on the issues of *Abies nebrodensis* conservation and on biodiversity, number of visitors.
- Frequency and spread of disorders in the crowns of the A. nebrodensis trees.
- Health state of crowns expressed as NDVI.

Life4fir has produced a joint strategy to respond the main threats affecting the critically endangered *Abies nebrodensis*. Although the concrete effects on the conservation of the species will be visible and assessable in the years to come, the large amount of work carried out (made up of various phases: discussion and debate, laboratory assays, inspections and interventions in in situ) has

allowed establishment of solid basis and good practices to be carried forward even after the end of the project to improve the conservation status of the species and reduce its risk of extinction.

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