LIFE4FIR – Project LIFE18 NAT/IT/000164

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

Report: 'Set up of protocols to investigate biotic and abiotic stresses of seedlings in the nursery'

Action A1.3

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REPUBBLICA ITALIANA
REGIONE SICILIANA
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DELL'AGRICOLTURA, DELLO SVILUPPO RURALE
E DELLA PESCA MEDITERRANEA

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1. Introduction

This deliverable reports the activities carried out and the results obtained in the A1 preparatory action, and in particular in the sub-action A1.3 'Set up of protocols to investigate biotic and abiotic stresses of seedlings in the nursery'.

The 'Piano Noce´ forest nursery is located 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. Here, production of plants to be used for reforestation purposes in the publicly-owned areas of Sicily is carried out.

Propagation by seed and raising of the *Abies nebrodensis* seedlings is an activity that has been carried out for years by the nursery 'Piano Noce'. This activity has also been supported by previous projects aimed at the conservation of this species, through afforestation measures and plantings carried out by public and private subjects inside the Park territory.

Within the Life4fir project, the nursery activity plays a fundamental role for the production of healthy, vigorous and genetically selected saplings, to be used in reforestation plots meant as re-diffusion cores of *A. nebrodensis*. Production of 4500 seedlings is expeted for this purpose by the Life4fir project.

In 2018, when the Life4fir project was being compiled, mortality and disorders were reported to affect seedlings and young plants growing in the nursery. For this reason, the Project has planned a preparatory action aimed at verifying the actual incidence of disorders and mortality, to identify the biotic and/or abiotic factors associated with observed losses and damages and to eventually implement proper control measures. The primary objective is to identify and reduce the impact of factors that can constrain the growth and vigor of seedlings and pot plants. Actually, one of the primary requirements of the Project, is the production of healthy and vigorous seedlings, with increased genetic variability.

A procedure was then followed by IPSP-CNR to monitor the state of health of the plants growing in the nursery, to detect emergencies and evaluate the incidence of the observed disorders.

2. Nursery surveys

Investigations in the Piano Noce nursery were conducted by IPSP-CNR in November 2019 and were repeated in October 2020.

The plants of *A. nebrodensis* currently raised in the nursery derive from the open pollinated seeds collected in the past years (from 2008 to 2015) from the mature (fertile) trees of the natural population. For seed exctraction, mature cones were collected in October and appropriately stored in dry and well aired rooms at the Piano Noce nursery to promote their disarticulation.

After one month, the seeds were separated from the wings, scales and other impurities. Sowing of seeds was carried out at the beginning of November, in pots containing a substrate made of vegetable soil (collected in local sites), peat and tuff sand. In each pot, from 5 to 20 seeds were generally sown. The emergency occurred stepwise about four months later, from mid-March on. At the beginning of May, the above ground part of the seedlings was 3 to 6 cm tall. One year after sowing, the seedlings obtained were transplanted into larger pots, using a suitable forest soil mixed with sand.

Inside the nursery, plants of *A. nebrodensis* are divided by mother plant, sowing year, and lot (within the same sowing year) and are placed in three plots of the nursery: plot 1, plot 14 and plot 15.

2.1 Frequency of symptoms

For each plot, mother plant, year of sowing and possibly lot, the plants were counted and subjected to visual inspection in order to identify 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). This led to obtain a picture of the size of each progeny and of the distribution of the different types of symptoms among the progenies.

2.2. Samplings for fungal isolations

Samples of affected needles and twigs were taken from the diseased saplings for further observations in the laboratory. With the aid of a stereoscope, samples were observed to define in detail the signs of colonization by pathogens (reaction tissues, fruiting bodies of fungi, etc.) and used for the in vitro isolation of fungal microorganisms. Isolations were performed by needles (reddened, entirely or partially necrotized) and from blighted shoots. For surface sterilization, needles and shoots were first immersed in a 70% ethanol solution for 1 min and then in a sodium hypochlorite solution (4-5% active chlorine) for 4 min. Then they were rinsed with sterile water and dried with sterile filter paper. The needles were cut into 3-5 mm fragments and placed in Petri dishes containing PDA (Potato Dextrose Agar). Plates were incubated at 24° C in the dark for 3 weeks. Each week the plates were checked to assess the progressive development of the fungal colonies growing out from the fragments and to arrange for their subculturing.

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).

To this end, fungal isolates colonies of each morphotype were grown on a sterile cellulose membrane placed on Potato Dextrose Agar medium (BD Difco) 2% in Petri plates. Plates were incubated in the dark at 25°C for about 10 weeks and the mycelium was scraped out and lyophilized. Samples were maintained at -20°C in sterile tubes up to genomic DNA extraction. Extraction was carried out with the 'NucleoSpin' Plant II kit (Macherey Nagel GmbH & Co. KG) using on 20 mg of lyophilized mycelium. Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using the primers ITS1 and ITS4. Amplicons were then purified and sequenced. The resulting sequences were 'blasted' against available sequences from GenBank database for identification.

A table of fungal taxa isolated from the different types of samples and their respective isolation frequencies was then obtained.

2.3 Assessment of *Phytophtora* inoculum

Among those observed, chlorosis and defoliation of plants are generic symptoms that can be due to various causes, both biotic and abiotic (environmental stress). Among the former, the action of very dangerous soil oomycetes, such as *Phytophtora* sp, cannot be neglected in the nursery setting, as it can be really dangerous. This oomycete attacks the root system of plants causing generalized deterioration of the crown (stunted growth, chlorosis, defoliation). For this reason, isolations were performed in the laboratory from soil and root samples collected from the pots of the symptomatic plants, following the protocol reported here below.

Phytophthora isolation

Soil and root samples were collected from the pots of 10 symptomatic saplings (showing significant chlorosis and defoliation), in a part of the nursery where symptoms were more frequent (PM 11, lot 1, plot 15). The samples were placed separately in plastic bags, transferred to the IPSP-CNR laboratory within 48 hours inside a refrigerated box at 5°C.

Three different procedures were followed for the isolation of *Phytophtora*: soil bating, direct plating and apple technique.

Soil baiting. The principle of soil baiting is that by flooding soil with water, zoospores will be released from propagules in the soil, swim upward because they are negatively geotropic, and infect the leaf pieces used as baits. Infected baits then can be plated on a medium selective for *Phytophthora* species. Baiting has several advantages over direct plating. First, a larger quantity of soil can be tested, which increases the likelihood of detecting a pathogen present at a low population density. Secondly, homothallic species that survive as dormant oospores are more likely to be detected by baiting than by direct plating.

Soil and fine roots were cut into 2-3 mm fragments with sterile scissors and placed in trays with sterile water (1/3 soil, 2/3 sterile water by volume). After few hours, young healthy leaves of *Edera helix*, *Quercus ilex*, *Rosa canina*, *Arbutus unedo* were placed on the surface of the water as bait. Tissues of the baits becoming necrotic within 3-4 days were then plated the selective substrate NARPH (containing the antibiotics Nystatin, Ampicillin, Rifampicin) in Petri plates, which were then incubated at 20°C in the dark for 20 days.

Direct plating. Root samples 0.5-1.5 mm thick taken from the soil, were carefully washed with tap water and dried with sterile filter paper. The roots were cut into 3-5 mm long fragments placed directly on NARPH in Petri dishes and incubated as described above.

Apple technique. One apple (Granny Smith var. due to their higher acidity) for each soil sample was used.

1. Preparation of apples: apples were externally sterilized with 70% ethyl alcohol and a hole of about 13 mm diameter and 25 mm depth was realized using a cork borer (fig.1).
2. Preparation of samples: a portion of soil containing roots and/or infected tissues was added of distilled water to obtain a fairly dense consistence (mud).
3. The sample was introduced inside the hole in the apple until it was filled and the hole covered with transparent Scotch tape. Apples were kept in laboratory for 5-6 days at 20-22°C.
4. Once the typical Phytophthora lesion was developed (superficial, hard and dark brown), some little pieces at the margin of the lesion were cut with sterile scalpel and placed on Petri dishes (90 mm) containing NARPH (selective media for Oomycetes). Plates were incubated at 20°C in the dark for 10 days.
5. Phytophthora colonies were then transplanted on PDA and incubated at 20°C in the dark for 5 days.

Identification

Fungal isolates resembling *Phytophtora* were grown on a sterile cellulose membrane placed on Potato Dextrose Agar medium (BD Difco) 2% in Petri plates. Plates were incubated in the dark at 22°C for about 10 weeks and the mycelium was scraped out and lyophilized. Samples were maintained at -20°C in sterile tubes up to genomic DNA extraction. Extraction was carried out following the procedure previously described for the other fungal colonies. Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using the primers ITS4 and ITS6 as described by and Cooke et al., 2000. Amplicons were then purified and sequenced. The resulting sequences were blasted against available sequences from GenBank data base for identification.

2.4 Ecophysiology measurements

Some ecophysiological measurements were carried out on two groups of pot plants of *Abies nebrodensis*.The two groups consisted of plants of different age: 10-12 years-old and 4 years-old, each comprising 5 pot plants. The aim was to study and compare the water relations between the two age groups of plants. Measurements to obtain the pressure-volume (PV) curves were carried out in July and between the last ten days of November and the beginning of December, using the same procedure. In ecology, pressure-volume curves describe the relation between total water potential (Ψt) and the relative water content (R) of living organisms. These values are widely used in research on plant-water relations, and provide valuable information on the turgor, osmotic and elastic properties of plant tissues.

The pressure-volume curves were obtained following the bench dehydration method, using twigs of the same year and of comparable size between the different samples. Four PV curves were created for each age group.

2.5. Soil analysis

Chemical-physical analyses of soil samples were carried out to verify the characteristics of the mixture used for the cultivation in pots of the *Abies nebrodensis* plants in the Piano Noce nursery. Any excess, shortage or imbalance of the various soil parameters may interfere with growth of seedlings and pot plants and their evaluation will allow to make adjustments to restore optimal conditions, based on the requirements of the species.

The intrinsic variability of the soil is an aspect that must be taken into due consideration for the purpose of soil analysis and requires careful execution of samplings. In the nursery, where the soil mixture can be prepared following a determined procedure and using the same raw materials, the variability to be sampled should be quite small.

Six different soil samples were sampled from pot plants: 1) last transplant carried out in December 2020; 2) sowed in 2013, transplant 2016; 3) sowed in December 2020; 4) sowed in 2008, transplanting 2012; 5) sowed in 2008, transplanting in 2014 and 2017; 6) sowed in March 2020. This kind of sampling was aimed at analyzing the growth substrate of pot plants that had been transplanted at different intervals of time and of seedlings derived from recent sowing still not transplanted. The following traits of soil samples were analyzed: pH; electric conductivity (EC) based on saturated paste extract; organic matter and total carbonate content. Soil pH is an indicator of the level of acidity or alkalinity of the soil. A reading of 7 is neutral; crops typically grow best when pH is between 6 (slightly acidic) and 7.5 (slightly alkaline). Nutrient availability may be hindered if soil pH is not within the optimum range. The pH of the soil greatly influences the microbiological activity, the availability of mineral elements and the adaptability of the various plant species.

Soluble salts in the soil, whether derived from the soil itself, from groundwater or irrigation water or from fertilizers, are essential for plant nutrition; but their concentration must be contained within certain values. High soluble salt content (or salinity) can cause water stress, nutrient imbalances in plants, and affect nutrient uptake. Seedlings are more sensitive to higher than normal soluble salts content compared to older plants. High soluble salt levels above 4 dS/m can potentially damage plants. Salts can accumulate from excessive fertilizer applications and poor quality irrigation water, and where rainfall is limited.

Organic matter. Generally speaking, the higher the organic matter, the healthier the soil. This is reported as a percent, and it measures the the ability of the soil to supply nutrients, water and other physical wellbeing to growing plants. Row crops should be at around 2.5% of organic matter or higher, though it is not uncommon for sandy soils to be lower. The organic carbon present in the sample is closely related to the quantity of organic matter in the soil itself. The analysis of these two components is fundamental for soil analysis as they influence many properties of the soil including: structure, ability to retain mineral and nutritional elements such as: nitrogen, phosphorus, potassium and sulfur; water retention capacity.

Total carbonate content is meant as the mineral component of the soil which is mainly composed by calcium, magnesium and sodium carbonates. Since the former is predominant over the others and the analytical method does not allow the distinction between the various forms, conventionally the limestone of the soil is expressed as calcium carbonate ($CaCO_3$). The presence of limestone in the soil, within certain limits, is to be considered positive for the nutritional function of calcium in relation to plants and for its favorable effects on the structure and mineralization of organic substances. However, when it is contained in excessive amount, and above all in very active mineralogical forms, the typical drawbacks of alkaline soils can occur.

3. Results

3.1 Nursery surveys

Table 1 shows the list and the amount of the open pollinated progenies raised in the Piano Noce nursery in November 2019. Twelve mother plants are represented. The total number of plants counted is conspicuous and exceeds 25000, growing in three nursery plots: 1, 14 and 15. The number of plants per each mother plant is very variable: from a minimum of 158 for PM 12 to a maximum of 6127 for PM 22. Age of the pot plants varies from 4 years (sowing 2015: PM 1, PM 7, PM 8, PM 13 , PM 16, PM 21, PM 22, PM 27) to 11 years (sowing 2008: PM 11, PM 13, PM 21). Some mother trees had offspring of different years of age (PM 8, PM 11; PM 13, PM 21, PM 22, PM 27) others of a same year of age (PM 1, PM 7, PM 10, PM 15, PM 16).

	S 1	S 14	S 15	tot.
PM 1			2340	2340
PM 7			780	780
PM 8	118		687	805
PM 10			284	284
PM 11		798	4077	4875
PM 12			158	158
PM 13	736	1224	2804	4764
PM 15			172	172
PM 16			268	268
PM 21	3705		629	4334
PM 22	4542		1585	6127
PM 27			491	491
tot.	9101	2022	14275	25398

Table 1. Extent (as number of potted plants) of the open-pollinated *A. nebrodensis* progenies of each mother tree (PM) represented in the Piano Noce nursery, in November 2019. Progenies are located in three plots: S 1, S 14 and S 15.

	mortality		diseased	
	No.	%	No.	%
Plot 1	459	5,04	320	3,51
Plot 14	7	0,34	73	3,61
Plot 15	140	0,98	978	6,85
tot	606	2,38	1371	5,39

Table 2. Damages in terms of dead and diseased plants
are here reported for each of the three nursery plots.

The lack of the progeny of many trees (there are 24 trees in the population recognized as fertile) and the great variability found among the progenies of the mother trees in the number of plants growing in the nursery, reflects the differences in terms of cone production among the fertile trees of the population. Some, such as PM 21, PM 22, PM 13 and PM 11, generally produce a greater amount of cones than others. Although the fruiting of trees in the natural population is subjected to marked annual variations, production remains relatively low and very uncertain for many of them. Investigations carried out in the past also revealed marked differences between the trees in the percentage of empty seeds and therefore in the percentage of germinated seeds. To preserve and increase the genetic basis of *A. nebrodensis* and favor its variability, it would be advisable to obtain and grow seedlings from the trees that are not currently represented in the nursery. In the Lif4fir project we will try to address this point, exploiting the years of abundant fruiting (as 2020 has been).
In the whole nursery, 606 dead plants (2.4%) and 1371 plants with symptoms to the crown (5.4%) were detected, as indicated in Table 2. In total, less than 8% of the pot plants in the nursery were damaged. The frequency of dead plants was higher in plot 1 (5%) than in plots 14 and 15 (0.34 % and 0.98 %, respectively). The frequency of diseased plants was instead higher in plot 15 (6.85%) than in the other two plots (3.5 %, plot 1; 3.6%, plot 14). In the plot 1 and plot 15 the incidence of damage observed (dead and symptomatic plants) was higher (8.5 % and 7.9 %, respectively) compared to plot 14 (4 %).

	plot 1		plot 14		plot 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

Tabella 3. Frequence (as number and percentage) of the main symptoms observed on the aerial part of the saplings raised in the three nursery plots.

The most frequently observed symptoms are listed in table 3. Only chlorosis (3.8 %) and reddened needles have an incidence greater than 1% of the total number of saplings. The other symptoms: defoliation, small needles, blighted shoots and stunted growth were sporadic. The percentage of plants with chlorosis was higher in plot 15 (5.35%) and in plot 1 (3.82%) compared to plot 14 (0.84%). The frequency of plants with reddened needles was higher in plot 1 (1.6%) than in the other two plots. The frequency of dead plants was higher in plot 1 (5%) compared to plot 15 (1%) and plot 14 (0.3%).

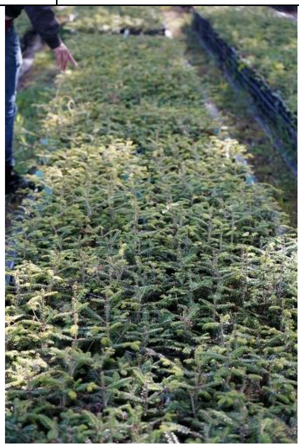
 1	 2	 3
 4	 5	 6
 7	 8	 9
 10	 11	 12

Table 4. Symptoms observed in the nursery survey: reddened needles (1, 2, 3, 10); chlorosis (5, 6, 11); blighted shoots (1, 4, 10); stunted growth (7); mortality (7, 12). Phases of the inspection (8, 9)

The percentage of damaged saplings (dead and symptomatic) was very variable among the progenies of the different mother trees (Fig. 1). The lowest percentages were found on the progeny of PM 1, PM 7, PM 13, with a value less than or equal to 5%. The highest percentages were reported for PM 10 (19%), PM 15 (15%), PM 27 (12%). An overall damage of 5 to 10% was recorded for the remaining progeny. The progenies with the highest percentage of damaged plants (PM 10, PM 15 and PM 27) had a relatively low weight on the whole plants, due to their reduced numerical size.

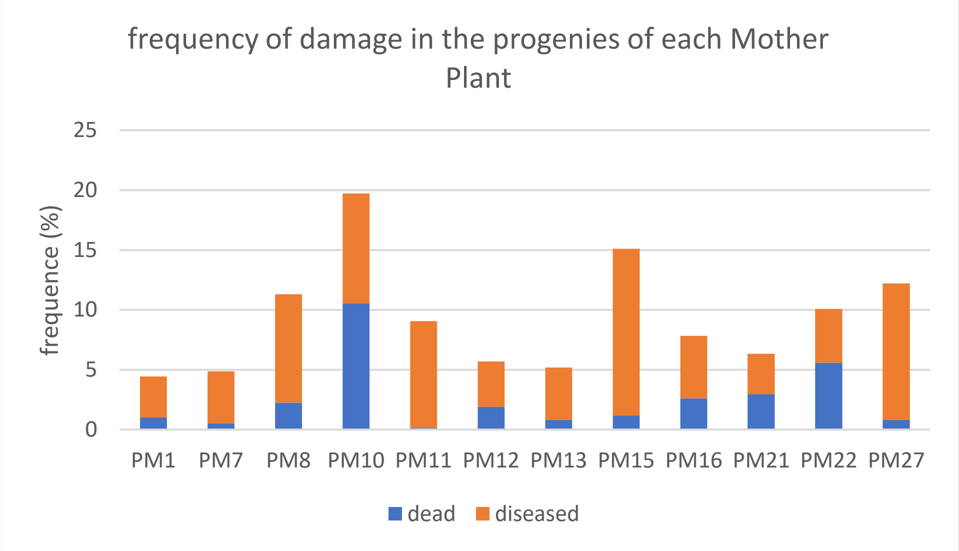


Fig. 1. Frequency of damaged saplings (dead + diseased) recorded in the progenies of the mother trees grown in the nursery.

Even within a same mother tree, the frequency of damaged or dead plants was different between progenies derived from seed collected in different years and between lots of a same year.
A Correspondence analysis (MCA) was performed with the frequency of the three symptoms most commonly recorded (chlorosis, CL; reddened needles, AR; and mortality, M) on the progenies of the twelve mother tree grown in the Piano Noce nursery (Fig. 2). In the graph, the position of the mother trees (PM) is due to the relative frequency of the three symptoms observed (AR, reddened needles; CL, chlorosis, M, mortality). Thus PM 11, PM 13, PM 21 and PM 22 are placed lower than the other PMs because they have reported a relatively higher frequency of plants with reddened needles and are closer to the symbol representing this symptom (AR). PM 10 and PM 12 are instead those closest to the symbol representing mortality (M) whose frequency lead to locate them in the right part of the graph. Then there are the PMs located at the top left near the 'chlorosis' symbol (CL), which is the symptom most recorded in the progenies of these mother trees. Finally, the mortality symbol M placed

in the opposite position with respect to the symbols of the other two symptoms (AR and CL) in relation to the main axis 1, indicates that in the progenies where the percentage of dead plants is greater, the percentage of the other two symptoms is reduced and vice versa.

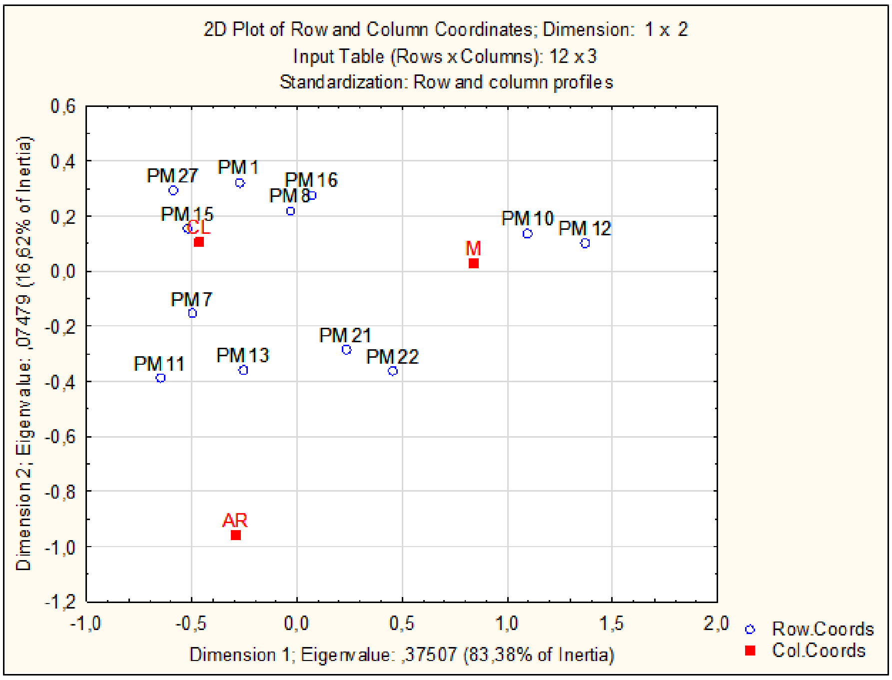


Fig. 2. Correspondence analysis performed using the frequencies of the three main symptoms recorded on the progenies growing in the nursery of the 12 represented mother trees. M: mortality; AR: reddened needles; CL: chlorosis.

3.2 Fungal isolations from affected samples

From the 270 samples plated, 115 colonies were obtained. Sequencing of the ITS1-ITS4 DNA region lead to the identification of 14 fungal taxa. (Fig. 3 and 5). Frequency of the 14 fungal taxa isolated from the affected needles (yellowed, reddened, partially or entirely necrotized) (Fig. 4) are reported in Tables 5 and 6, as percent of the obtained colonies and as frequency of isolation, respectively. The 77% of all colonies obtained are represented by 5 taxa: *Alternaria alternata*, *Cytospora* sp. (anam. of *Valsa abietis*), *Allanthomopsis lycopicodina*, *Alternaria* sp., *Coniothyrium aleuritis*. All these fungi are Ascomycetes (mostly in the asexual form), reported in bibliography as saprophytes, opportunistic pathogens or endophytes. Endophytic fungi often become pathogenic when their host has been weakened by various types of stress factors (biotic or abiotic) and many of the cited fungi may have this kind of behaviour. *Alternaria alternata* is a ubiquitous fungus reported in numerous habitats and hosts, known for its saprophytic behavior, although some species of *Alternaria* are weak pathogens.

Cytospora sp. is the asexual form of *Valsa abietis*, reported as associated with blighted shoots and cankers of some conifers damaged by other biotic or abiotic stressors. *Allanthomopsis lycopicodina* was isolated from pine needles blighted by *Phytophtora*, while *Coniothyrium* sp. (asexual form of *Dothideomycetes*) is reported as a leaf endophyte of conifers and as a pathogen on juniper.

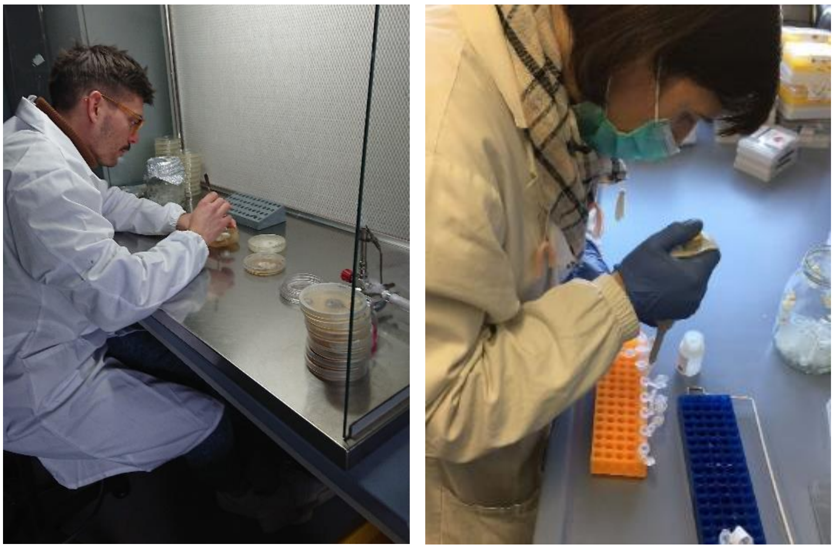


Fig. 3. Phases of subculturing of fungal colonies and processing for DNA amplification and sequencing

Fig. 4: Needles affected by partial or total necrosis, observed at a Stereoscope

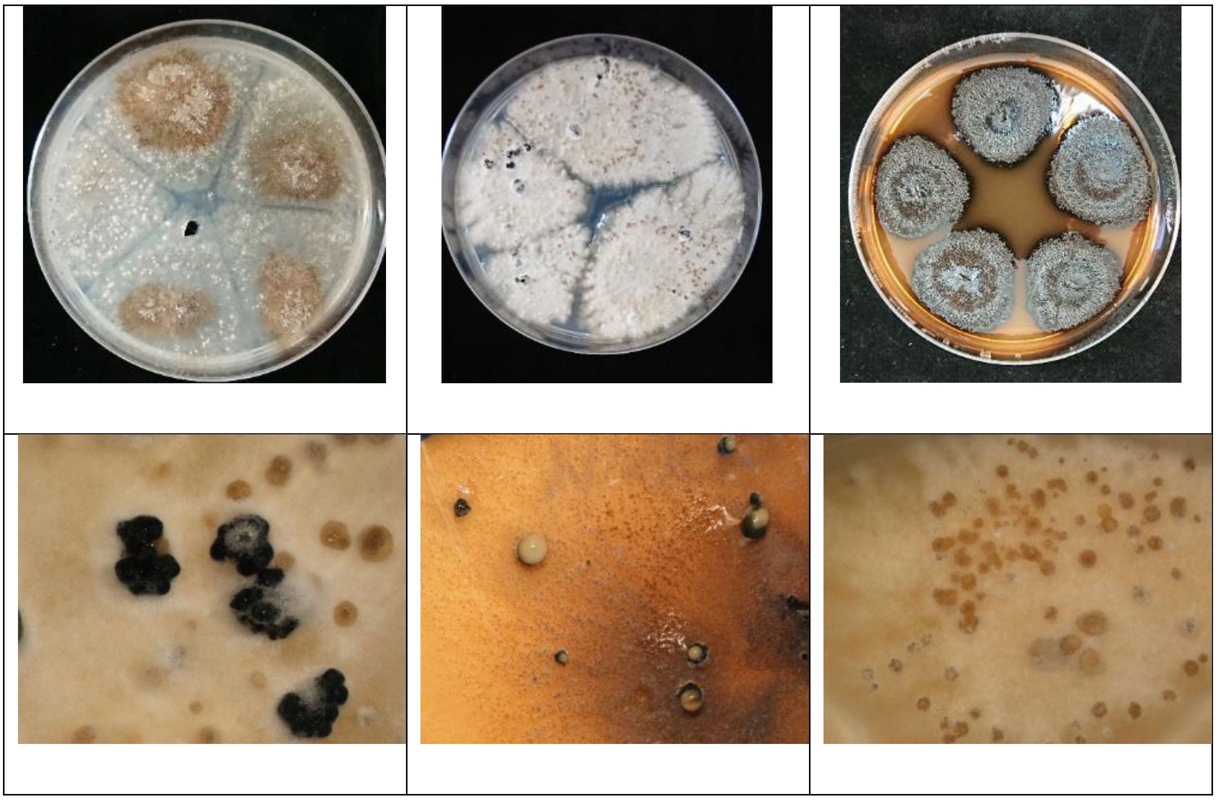


Fig. 5: different types of fungal colonies growin out from the plated needle samples, and fruitbodies developed in vitro (bottom).

Fungal taxa	**Percent of all obtained isolates**
Alternaria alternata	30
Cytospora sp. Anam. of Valsa	15
Allantophomopsis lycopicodina	11
Alternaria sp.	10
Coniothyrium aleuritis	9
Ceuthospora phacidioides	6
Pestalotiopsis funerea	5
Libertasomyces myopori	3
Dothydeomycetes sp.	3
Elytroderma deformans	3
Phacidium sp.	1
Foliophoma sp.	1
Diaporthe rudis	1
Angustimassarina sp.	1

Table 5. Relative frequence of fungal taxa obtained isolated from reddened, blighted and chlorotic needles collected from the A. nebrodensis saplings raised in the Piano Noce nursery

Fungal taxa	Yellow needles	reddened needles	Needles partially dried	Blighted shoots
Alternaria alternata	41,6			
Allantophomopsis lycopicodina		9		
Libertasomyces myopori	8	4		
Pestalotiopsis funerea				4,4
Cytospora sp. anam. of Valsa				5,8
Ceuthospora phacidioides		3		2
Alternaria sp.	8			7,4
Phacidium sp.				1,5
Coniothyrium aleuritis				5,8
Foliophoma sp.				1
Dothydeomycetes sp.		3		1
Diaporthe rudis			1	
Elytroderma deformans				3
Angustimassarina sp.				1

Table 6. Number of needles (as percent of the number of plated needles) colonized by a given fungal taxon for each type of symptom.

The remaining taxa, isolated less frequently, are however represented by ubiquitous fungi, such as *Pestalotiopsis funerea*, and by fungi reported as endophytes or associated with disorders due to other causes, eg. *Foliophoma* sp., isolated from olive leaves attacked by Venturia and Pseudocercospora leaf spot, and *Lybertasomyces myopori* (a *Phoma*-like fungus), reported as saprophyte, endophyte or pathogen (facultative pathogen) on different species.

The assemblage of fungal taxa found in the symptomatic needles of *A. nebrodensis* plants in the Piano Noce nursery is also related to the tree species growing inside the nursery area and in the ecosystem around: cypresses, pines, cedars, holm oaks, etc. which represent a significant source of inoculum *Pestalotiopsis funerea*, for example, is a fungus frequently isolated from Cupressaceae, both as an opportunistic pathogen and saprophyte, although it has sometimes been reported as responsible for major cankers and diebacks on cypress. In general, the assembly of endophytes of a species, in a certain site, depends on the plant species that are part of the surrounding ecosystem. Only a few endophytes have specificity for a particular host.

Regarding the frequency of needle colonization, most of the isolated fungi was obtained from blighted shoots, although the highest percentage of colonization was found for *Alternaria alternata* on yellow needles. Only three taxa have been isolated from needles with different symptoms.

3.3 Isolations aimed at *Phytophtora* sp. detection

Following the three different isolation techniques described, no isolates of *Phytophtora* species were obtained from the soil and root samples examined. The colonies found, both from the soil and from the roots, were isolates of *Mortierella* sp., a Zygomycete very widespread as a saprophyte in the soil, on declining leaves and other organic material. Together with *Penicillium*, *Trichoderma* and *Mucor*, *Mortierella* belongs to an ecological group of microorganisms that are the first in colonizing the roots and growing on them. Some *Mortierella* species have a dual endophyte-saprophytic behavior with their host. *Phytophthora* sp. was not recovered from our samples. It may be due to the simple fact that *Phytophthora* was absent in the examined samples, but also to the sampling itself. Isolation of *Phytophtora* is not always an easy task. *Phytophthora* species attack only healthy plant material, including roots. Thus, the pathogen can be present when no symptoms are obvious. *Phytophthora* species are difficult to isolate from necrotic tissue because the tissue often harbors many secondary pathogens, among which, *Mortierella* sp. is very frequent. Successful isolation of *Phytophthora* species from diseased tissue involves careful selection of freshly infected tissue. *Pythium* spp. are also invariably present on both healthy and diseased roots, crowns, and lower stems of plants.

Fig. 6. Soil baiting for Phytophtora detection and isolation. Left: soil and fine roots were placed in a tray; center: sterile water was poured over the soil layer (1/3 soil, 2/3 water); right: leaves of *Edera helix*, *Quercus ilex*, *Rosa canina*, *Arbutus unedo* placed on the surface of the water as baits.

3.4 Ecophysiology measurements

Four pressure-volume curves were obtained for each of the two age class of potted plants. Table 7 reports the values of water potential at the turgor loss point (Ψ_{tlp}) and of osmotic potential at full turgor (π_0). For both parameters, there were no significant differences either between the two age groups or between the summer (Jul) and autumn (Nov) within a same group. This indicates that younger (4 years old) potted plants are 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.

Age class	Ψ_{tlp} (Mpa) Jul	π_0 (Mpa) Jul	Ψ_{tlp} (Mpa) Nov	π_0 (Mpa) Nov
4 years	-2.08 ± 0.28	1.65 ± 0.33	-2.07 ± 0.40	1.39 ± 0.35
10-12 years	-2.11 ± 0.34	1.56 ± 0.31	-2.20 ± 0.25	1.45 ± 0.13

Table 7. Average values of water potential at turgor loss point (Ψtlp) and of osmotic pressure at full turgor (π_0), derived from the analysis of the pressure-volume curves

3.5 Soil analysis

The results of the analysis of soil samples collected from pots where A. nebrodensis plants were growing are shown in Tables 7 and 8. In addition to soil analysis, some parameters of the water used the Piano Noce nursery for irrigation were measured. The electrical conductivity (EC) of irrigation water was found to be 0.369 dS / m, the pH 6.5 (Table 9)

Soil samples	pH	EC (conductivity) (1:5) (μS/cm)	EC saturated paste extract (dS/m)	Organic matter (g/Kg)	carbonati totali (g/Kg)
1 - T 2020	6,59	831	1,962	35,34	85,7
2 - S 2013, T 2016	7,16	160,6	0,379	50,93	188,5
3 – S Dec. 2020	6,8	1527	3,605	64,61	282,8
4 – S 2008, T 2012	7,36	322	0,760	54,45	85,7
5 – S 2008, T 2014, 2017	7,46	276	0,652	41,05	137,1
6 – S March 2020	7,41	185,7	0,438	71,84	64,2

Table 7. Soil analysis of six samples collected from pots in the Piano Noc Nursery. The year of sowing and transplant is reported. T: transplant; S: sowing

Soil samples (g/kg)	Argilla	Limo	Sabbia
1 - T 2020	113,5	119	767,5
2 - S 2013, T 2016	28,5	155	816,5
3 – S Dec. 2020	22,5	139,5	838
4 S 2008, T 2012	44,5	284	671,5
5 – S 2008, T 2014, 2017	186,5	187,5	626
6 -S March 2020	39,5	88	872,5

Table 8. Soil texture, proportion of sand, silt and clay.

	EC	pH
Irrigation water	0.369 dS / m	6.5

Table 9. Main traits of the irrigation water

The pH varied in a range between 6.6 and 7.4 and is substantially neutral for all the samples, though the two extreme values are slightly acidic and alkaline.

The electrical conductivity (EC) is a measure of the amount of salts in the soil (salinity) and was found to be quite variable between the samples examined. The highest value, referred to the saturated paste extract, was 3,605 dS / m found for sample no. 3. This is quite high value and could interfere with the good growth of plants. The values found for the other samples did not represent limiting salinity conditions, including the 1.9 dS / m measured for sample no. 1 which can be considered an average value.

The variability in salinity found between the examined samples requires a deeper evaluation of its causes. It could depend on the characteristics of the raw substrates used for the preparation of the mixture, which is made of soil, sand and peat. The soil and sand used in the mixture are collected locally by the nursery staff, while peat is purchased.

The irrigation water, on the other hand, is quite balanced in terms of conductivity and pH and it doesn't seem to represent a disturbing factor for the potplants.

The organic matter content was high for all the samples examined, although it was rather variable, ranging between 6% and 12%. It is believed that the organic matter in the soil must not drop below 2% to ensure good growth of plants. Below this threshold, the soil shows signs of a decline in the ability to fully perform its physical, chemical or biological functions.

The total carbonate content was also very variable, ranging from the 6.4% to 28.2% found for samples no. 6 and n. 3, respectively, with a nearly 5-fold difference between the two. The former corresponds to a moderately calcareous soil, while the latter corresponds to a strongly calcareous soil. Above 10% of total carbonate, the soils are considered very calcareous, and 3 out of 6 of the examined samples showed to exceed this threshold. The latter value was recorded in the sample n. 3 that also reported the highest level of EC (salinity). A high amount of limestone in the soil, especially if active, does not have the positive effect of a greater availability of fertilizing elements. On the contrary it cause a negative effect, as it cause some nutrients essential for normal plant development become insoluble.

As far as texture is concerned, al the examined soil samples showed a high sand content: above 75% of sand they are loamy sand, below 70% they are sandy loam. The clay content was rather low for all the samples, ranging between 2.2% and 18.6%. The sandy soils are very permeable and mobility of nutrients is promoted. The considerable aeration favours the rapid mineralization of the organic matter, with a marked releases of concentrated nutrients in a relatively short time and not always coinciding with the cultivation needs of the plants.

Analysis of soil samples in the Piano Noce nursery showed a significant variability for the total carbonate content and salinity, which may affect the good growth of plants. The range of variation of the pH, the organic matter content and the texture seemed not to represent a disturb for the growth of the *A. nebrodensis* plants.

4. Conclusions

Main points emerged.

- A large number of open-pollinated progenies of *A. nebrodensis* has been found in the Piano Noce nursery: more than 25000 potted plants were counted.
- The potted plants currently present vary in age from 5 to 12 years. The most suitable for use in reforestation are the young ones, because they have a well-developed and conformed root system. The older ones have problems of spiraling root due to their long stay in the pots after the last transplant. Their root system is deformed, not very branched, and is therefore more vulnerable to pathogens and stresses and may compromise the mechanical stability of plants.
- The propagation activity of *A. nebrodensis* that has been conducted for years in the Piano Noce nursery is aimed at producing plants for reforestation or to be planted throughout the territory through their assignment to public and private entities. Therefore, the material currently grown in the nursery should be considered as inherited from previous projects and initiatives. The Life4fir project is evaluating the possibility of using the plants already raised in the nursery for reforestation interventions, by means of a careful genetic selection. Furthermore, the evaluation of the growth conditions of the plants growing in the nursery allows to identify any detrimental aspects of production and to intervene promptly to ensure the most appropriate care to the new progenies that will arise from the action C2.1 'Enhancement of the genetic diversity of the natural population: promoting the outbreeding through manual cross-pollination '. These will be used to set up new reforestation plots with selected material. The ongoing genetic analyzes will provide indications for selecting outbred plants. The selected plants can be adequately prepared for planting in the reforestation plots provided for in the project.
- Among the observed symptoms, chlorosis is the most frequently detected, having been found on 970 plants (3.8%), albeit with differences between the three plots. The incidence of chlorosis was particularly high in lot 1 of PM 11 in the plot 15, where as many as 316 out of 791 plants (40%) showed this symptom. In most cases, plants showed partial chlorosis, limited to the shoots of the last year or the previous year. Cases of chlorosis spread to the entire crown were relatively few and were often accompanied by reduced growth and defoliation. Generally, chlorosis is a symptom of a generic etiology and may be due to various causes, either infectious, or to environmental stress and nutritional imbalances. Based on our surveys, the latter seems the most likely cause, because

otherwise widespread symptoms would be observed in the nursery, while instead the symptoms are uneven.

- Surveys on the health conditions of the *A. nebrodensis* plants raised in the Piano Noce nursery showed that the damage observed is substantially scattered, in the different plots and in the different lots of a same plot. Surveys ruled out the presence of generalized or patchy damage around a diffusion center or uniform damage affecting particular portions of the nursery, which may indicate the presence of harmful pathogens. Also, isolations from the soil and from the roots of the chlorotic and defoliated plants were negative for *Phytophtora* sp. This seems to rule out the presence of this dangerous pathogen which frequently damages nursery production due to the favorable conditions encountered in nurseries, such as frequent irrigation, the close position of the plants, etc.).
- In the Piano Noce nursery, both the sowing and the raising of the seedlings are carried out in pots. The growing medium used 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. Among the fungal pathogens that reside in the soil of forest nurseries the most frequent are *Cylindrocarpon* spp., *Fusarium* spp., *Phoma* spp., *Phytophtora* spp., *Pythium* spp., *Rosellinia* spp, *Rhyzoctonia* spp., *Verticillium* spp.

  The investigations carried out seem to rule out this type of threat. No generalized death was observed in any of the three plots of the nursery. Dead plants were reported in almost all the surveyed lots, but the mortality rate was generally quite low (mean 2.4 %). Only in the six lots of PM 22 of plot 1 the mortality rate was higher than 5% (average 7.3%). These plants are from sowing carried out in 2009 and benefited from a single transplant in 2012. So, it is possible that they have suffered from the long stay in the same pot, with problems of spiraling roots and soil compactness. To reduce mortality and decline, seedlings and pot plants should be transplanted regularly every 2 years into larger pots to keep the root system well developed and conformed and to ensure good growing conditions.
- The fungal microflora isolated from reddened or yellowed needles and blighted shoots was found to be represented by taxa that have a weak pathogenic activity and by saprophytes or endophytes. Damages to needles and shoots seem to be due to predisposing external factors such as delayed transplants or direct sun light exposure that favor the action of weak pathogens (opportunistic), often already present within asymptomatic tissues as endophytes. Assays did not show the presence of inoculum of fungal pathogens reported with a certain recurrence in the nursery in the needles and shoots of fir, like those of the

genera *Lirula*, *Herpotrichia*, *Kabatina*. At present, disease control measures are not required. Therefore no particular measures must be followed for the control of pathogens in the nursery.

- Assays of the water-relations, even if referred to a period of a few months (July-November 2020), showed that the pot plants in the nursery havenot experienced adaptation to conditions of water stress, and are therefore subjected to an adequate irrigation regime.
- The analysis of soil samples carried out in the Piano Noce nursery showed a certain variability in the various parameters examined. The values that showed the greatest variation between samples are those related to salinity (EC) and total carbonate content. Both reached limiting levels in some of the samples tested. It is important to shed light on the causes that originate this marked variability, which probably reside in the preparation of the soil mixture used in the pots. In order to standardize the preparation of substrate used for sowing and transplanting and to make it perfectly suitable for *A. nebrodensis* plants requirements, it is necessary to draw up and follow a protocol that reports the proper methods of collecting the raw material and of preparing the soil mixture.

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