LIFE4FIR – Project LIFE18 NAT/IT/000164

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

"Report of actions related to control and prevention of native and invasive pests and pathogens".
Action C1.4.

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REPUBBLICA ITALIANA
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DELL'AGRICOLTURA, DELLO SVILUPPO RURALE
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1. Introduction – concepts on plant disease

A plant disease is defined as a malfunction of the plant in response to continuous stress factors. Diseases can affect the plant's ability to produce, reproduce or grow properly and can cause very different symptoms.

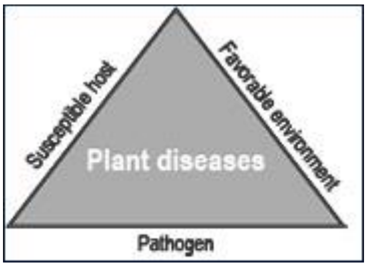
Diagnosing a disease can sometimes be difficult and distinguishing between changes due to a pathogen and an abiotic disorder is critical to developing an effective management plan. The causative agents of plant diseases are biotic, or living, and are called pathogens. Abiotic disorders are caused by non-living factors and are represented by environmental stresses. Understanding the difference between the two is critical to diagnosing the cause of plant damage.

Plant diseases often provide useful clues about the factors that made a plant susceptible to pathogens. These factors could include an unsuitable site, nutrient imbalance, water stress etc. In many cases, addressing the underlying cause of the plant's problems helps control the disease process and allows the plant to regain health and vigor to withstand such problems in the future.

Many pathogens have an opportunistic behaviour, and settle on stressed and debilitated plants, taking advantage of the weakened condition of the host. Some opportunistic pathogens are characterized by an endophytic (latent) phase, during which they live in the plant tissues in a condition of equilibrium, without causing apparent symptoms, and then begin colonization when the host weakens.

When control measures need to be taken, it is necessary to decide which management measures are most appropriate. An integrated pest management, or IPM, strategy is more prudent and effective because it involves using a combination of management techniques.

Plant disease occurrence triangle



A disease that has a biotic cause (due to a pathogem) is likely to occur only when three conditions are present.

A triangle is often used to illustrate how plant diseases occur. A disease will only occur when three conditions are present, as represented by the three sides of the triangle (Figure 1). 1) A pathogen, such as a bacterium or fungus, encounters a… 2) susceptible host plant during… 3) environmental conditions favorable to the development of the disease. A disease will develop only if all three conditions are present. The presence of the pathogen is the first condition, but it is not sufficient for the development of the disease. The probability of development of a disease on a resistant plant is minimized; therefore, plant selection may be a key factor in disease management. Finally, environmental conditions must be favorable for the development of the disease. These conditions allow the growth and reproduction of the pathogen, reducing plant vigor and predisposing the plant to infection. The best management approach is to exclude any of the three conditions that form the sides of the triangle. Keeping these conditions in mind will help you gain insight into plant diseases and their control.

2. Invasive Pests and Pathogens

IPPs are responsible for tree and/or production losses in forests, urban areas, and commercial forests (Moore 2005). In Europe, the total annual costs of invasive species have been roughly estimated at around 10,000 million euros (Kettunen et al. 2009), but little data is available on forestry PPIs (Kenis and Branco 2010). This is a small percentage of the €109 billion annual production value of the forestry sector (Forests Europe 2011), but purely financial analyzes of the effect of PPIs overlook the potentially larger costs of damage to ecosystem services, amenities and other ecological values (Kenis et al. 2009; Lambertini et al., 2011). Therefore, protection from invasion risks is important both ecologically and economically (Parker et al. 1999; Aukema et al. 2011).
PPI control strategies can be divided into three categories: prevention and interception, early detection and surveillance, and reporting and management (e.g. Blackburn et al. 2011). Within the EU, these strategies must be supported by individual member states to be successful. Greater public involvement and understanding of the threats posed by forestry IPPs can increase willingness to legislate or take action (e.g. Hulme et al. 2009b; Simberloff et al. 2013).

3. Surveys on Abies nebrodensis for monitoring

In the case of *Abies nebrodensis*, subaction C1.4 was dedicated to monitoring the health status of the relic population and mitigating biotic and abiotic stresses, with particular attention to invasive species. Phytosanitary monitoring of such a small population can have significant relevance for its protection. The project therefore carefully assessed the onset of disorders affecting the trees of the A. nebrodensis population, determining their spread and impact and understanding their cause, in order to prepare and implement adequate prevention and control measures.
Some crown disorders, such as needle blight, defoliation have been observed to affect A. nebrodensis natural population for years (even in the previous Life project implemented in 2000-

2004), but generally their spread have not been described in detail (Raimondo and Schicchi, 2005) and their real impact remained unclear. The precise assessment of spread and impact of disorders on individual trees was pursued in the Life4fir project.

As part of the project, surveys were carried out to evaluate the health status of the trees of the natural population of *A. nebrodensis*. The surveys made it possible to record a series of parameters capable of defining growth and morphology of trees and to obtain information on the balance of the plants in relation to the surrounding vegetation and environment. This also allowed to define a starting baseline, to which refer in subsequent surveys to evaluate the effect of the measures implemented for the protection of the relic trees and monitor the occurrence of new symptoms.

3.1 Tree health surveys

Particular attention was given to recording the disorders on the crowns of single trees. The parameters used to have a clear picture of the state of health of the plants are the following: transparency of the foliage, turning foliage, presence of disorders (diebacks, shoot and needle blights, bark lesions, defoliation, etc.), the type of damaged organ (foliage, shoots, branches, trunk), their extension on the canopy, their impact based on the percentage of damaged canopy.

The comparison between measurements carried out at different times made it possible to evaluate the evolution of the symptoms observed and to record an improvement or worsening of the health state of a tree.

The surveys on the trees of the natural population carried out in November 2019 and May 2023 showed a situation of substantial balance between the trees and the natural environment, despite the microclimate and the unfavorable soil conditions in many cases. Table 1 shows in detail the results of the observations made. The most frequent disorders found are needle reddening and shoot blight, defoliation due to shading exerted by adjacent broad-leaved trees, lesions to the woody organs caused by wild herbivores. The symptoms observed are mainly due to the effect environmental stresses due to strong winds, temperature excursion, hail. The information collected allows plants to be classified based on the spread of the observed disorders, compared to the surface of the foliage, and to relate it to the microclimatic and soil conditions. In Table 2 the impact of needle reddening and blight was related to site conditions of each tree based on topography, soil and surrounding vegetation. Through sampling and isolation in the laboratory, the fungal microflora associated with the observed disorders was investigated (see paragraph 3.4).

	Phytosanitary survey November 2019 - May 2023							
Abies nebrodensis **natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
1	Nov. 2019	0	-	70 small shoots	Lower and intemediate thirds	N prevailing	10	Shoots with reddened needles
			-	branches	Lower third	All	5	Branches showing mechanical injuries and desiccated due to wild herbivores
	May 2023	0	-	50 small shoots branches	Lower third Lower third	All S-E	5 2	Shoots with reddened needles Mechanical injuries with defoliation
2	Nov. 2019	0-20	-	5 twigs 1° order	Lower third	S-O	10-15	Dried out and defoliated, no due to shading
				Twigs 1° order	Lower third	All		Chlorosis and small needles
				Branch	Lower third	NE	2	October 2020: distal portion (50 cm) defoliated
	May 2023	0-20	-					stable
4	Nov. 2019	0-20	-	Twig 1° order Terminal shoots	Thorough Lower third	All S	5 2	Desiccated due to shading Reddened needles
	May 2023	0-20	-	Twig 1st order shoots	Thorough thorough	All S	5 1	Former defoliation Reddened needles
6	Nov. 2019	0-20	-	Twig 1° order	Thorough	All	4	branches distal portion (100 cm) dried out (old symptom)
				Twigs 2 order	Lower third	N	3	October 2020: distal shoots reddened
				Branch	Lower third	N	2	October 2020: 50 cm distal defoliated + reddened needles

	Phytosanitary survey November 2019 - May 2023							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
	May 2023	0	-	Small twigs, shoots	Lower third	N	1	Reddened needles, dotted
				branch	Lower third		1	Dried up branch with injuries
7	Nov. 2019	0-20	-	Twig 1° order	Lower third	W		Branch dried out distal portion (100cm)
			-	N° 2 twigs 2° order	Lower third	N	< 1	Distal shoots with reddened needles
			-	Twig 1° order	Lower third	W		Twigs dried out, distal part 20-30 cm
			-	Branch 1° order	Lower third	N	1	Wound due to friction with the soil
			-	Branch 1° order	Lower third	S	1	Wounds due to herbivores
	May 2023	0-20	-	twigs 2nd order branch	Lower third Lower third	W S	1 <1	Reddened needles Wounds due to herbivores
8		0-20	-	N° 2 twigs 1° order	Lower third	S	1	Branches with wounds due to herbivores
				Terminal shoots	Lower third	N	<1	Reddened shoots
	May 2023	0-20	-	Small twigs, shoots branches	Lower third Lower third	N S	<1 20%	Reddened needles Internal branches defoliated due to shading
9	Nov. 2019	0-20	-	Twigs 1° e 2° order	thorough	All, N prevailing	10	Distal portions dried out
	May 2023	0-20	-	Branch Small twigs, shoots	Upper part Lower third	W N prevailing	5 1	Branch just below the cut of trunk is dried up Reddened needles, dotted

	Phytosanitary survey November 2019 - May 2023							
Abies nebrodensis **natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
10	Nov. 2019	0-20	-	Twig 2° order	Lower third	All	1	Distal part with reddened needles
				Branch 1° order	Lower third	S-O	< 1	Branch broken dried out in its distal part (40 cm)
				Branch 1° order	Lower third.	N-E	1	Branch drying and defoliating, also wounds by herbivores
	May 2023	0-20	-	Branch 1st order Small twigs and shoots	Lower third Lower third	N-E N, E and NE prevailing	4 3	Branch defoliated with wounds by herbivores. Reddened needles
11	Nov. 2019	0	-	Twigs 2° order (60 -70)	Lower and intermediate thirds	All, N prevailing	1	Distal part with reddened needles
				N° 3 twigs 1° order	Lower third	N	1	Branches dried out at 1-1,5 height from the ground.
	May 2023	0	-	Small twigs and shoots	Lower third	N, E and NE	5	Reddened needles
12	Nov. 2019	10 mainly in the upper part	About 30%	Twigs 2° order	thorough	All	10	Distal part with reddened needles, 2-10 cm in length
				Twigs 1° order	thorough	All	10	Distal portion (100 cm) dried out
				Twigs 1° e 2° order	thorough	All	10	Yellowed distal portion up to 30-40 cm
	May 2023	10 upper portion	20%	Twigs 1st order	Thorough	All	25S, 20N	Reddened needles

	Phytosanitary survey November 2019 - May 2023							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
				Branches	Lower third	N		Internal branches defoliated due to shading
13	Nov. 2019	0-20	-	Twigs 2nd order	Lower third	SE (monte)	< 1	inner twig reddened (20 cm)
				Twigs 2 e 3 order	Lower third	SE (monte)	<1	5 twigs reddened and defoliated close to the ground
	May 2023	0-20	-	5-6 shoots branches	Lower third Lower third	N All	<1	Reddened needles Internal branches defoliated due to shading
14	Nov. 2019	0-20	-	7 branches 1° order	Lower third	NE-N-O	5	Branches with wounds in the distal 1-2 m due to herbivores
		0-20	-	11 twigs 2° order	Lower third	All, N prevailing	< 1	Reddened needles in the distal 2-10 cm part
	May 2023	10 (S shaded by oak)	-	2 branches 2 shoots	Lower third Lower third	N N	3 <1	Defoliated due to former injuries Reddened needles
15	Nov. 2019	0-20	-	7 twigs 2° order	Lower third	All	< 1	Outer crown
		0-20	-	Twig 1° order	Lower third	S	1	Twig dried out in the distal 30 cm, close to the ground
		0-20	-	Twig 1° order	Lower third	O	1	Distal part (20 cm) with reddeded needles
	May 2023	0-20	-	Branch	Lower third	N	<1	50 cm distal portion dried out due to rubbing with the protective wall.

	Phytosanitary survey November 2019 - May 2023							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
				1 shoot	Lower third	N	<1	Reddened needles
16	Nov. 2019	0-20		2 twigs 2° order	Lower third	S-O	< 1	Reddened needles
		0-20		Twigs 2° order	Lower third	All		Small xeromorph needles
		0-20		15 twigs 2° order	Lower third	All	< 1	Outer twigs with reddened needles, expecially S-O direction
	May 2023	0-10	-	Twigs 2nd order	Lower third	S, O	<1	Formerly defoliated no reddened needles
17	Nov. 2019	0-20	-	N° 4 twigs 2° order	Lower third	All	< 1	Distal part with reddened needles
				Branch	Lower third	W	1	Twigs dried out are inserted in a branch damaged by herbivores
	May 2023	0-10	-	Twigs 2nd order and shoots branch	Lower third Lower third	All W	<1	Reddened needles The wound caused by herbivores is healing
18	Nov. 2019	0-20 (a S-E 20-40 e 40-60)	-	Branches and twigs	Lower and intermediate thirds	S-E	20	Three branches and twigs dried out due to shading by beech trees
		0-20	-	N° 3 twigs 1° order		N-O	2	Branches dried out at 1 m height from the ground
	May 2023	0-20	-	branches	Lower third	E, S	35	stable

	Phytosanitary survey **November 2019 - May 2023**							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
19	Nov. 2019			N° 2 branches 1° order	Lower third	E	2	Twigs dried out due to injuries by herbivores
				Twigs 2 order	Lower third	SE	1	Two twigs with reddened needles
	May 2023			Branches Small twigs	Lower third Lower third	SE SE	5 <1	Branches dried up due to oak shading 5 twigs with reddened needles
20	Nov. 2019	25	-	Crown	Lower and intermediate thirds	S	40	Tree with half of the crown defoliated
		25	-	Branches 1° order	Lower third E 1/3 Int.	N-E and N-O	10	Branches dried out
	May 2023							stable
21	Nov. 2019	0-20	-	Branches 2° order	Lower third	All, SE prevailing	4	Distal half of branches is defoliated also those not shaded
				Twigs 2 order	Upper third		<1	October 2020: two reddened twigs
	May 2023							Stable, no reddened twigs
22	Nov. 2019	0-20		Branches and twigs	Lower third	N-S-O-E	20	Twigs long-dried out due to shading
	May 2023							stable
23	Nov. 2019	0-20	-	Twigs 2nd order	Thorough	All	5-7	Distal portions with reddened needles, up to 20 cm in length, small needles

	Phytosanitary survey November 2019 - May 2023							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
	May 2023	0-20	-					stable
24	Nov. 2019	0-20	-	Twigs 1st order	Lower third E 1/3 Int.	S (uphill)	5	Twigs broken due to herbivores
		0-20	-	Twigs 2st order	Lower third	S (uphill)	3-4	Distal portions (2-10 cm) with reddened needles, wounds due to herbivores
	May 2023	0-20	-					stable
25	Nov. 2019			Twig 1st order	Thorough	N	2	Twigs 10-20 cm long dried out and defoliated, slight damage by herbivores
	May 2023							stable
26	Nov. 2019	20		Branch, twigs	Lower third	All	5	Branches and wigs dried out up to 2 m height from the ground due to shading
				Twigs 3 order	Lower third	S (monte)	1	Three reddened twigs due to rubbing
	May 2023							Stable, shading by beech trees
27	Nov. 2019	0-20	-	Twigs 1° and 2° order	Lower and intermediate thirds	All	2	Inner twigs dried out due to shading
	May 2023	0-20	-					Stable, inner twigs defoliated due to shading

	Phytosanitary survey November 2019 - May 2023							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
28	Nov. 2019	50	0-20	Crown	Thorough	S (uphill)	50	Thorough damage due to herbivores
				Twigs 1st and 2nd order	1/3 upper	All	20	Defoliated branches and twigs, reddened needles
	May 2023	50	15-20	Twigs branch	 Lower third	All	10 10	Reddened needles, Branch dried up with browning needles. Substantially stable
29	Nov. 2019	25-30	-	crown	Lower third	all	40	Crown dried out up in the lower half due to shading by the adjacent beech
	May 2023	25-30					50	Foliage lacking in the lower half, stable
30	Nov. 2019	-	-	Twig 2° order	Intermediate third.	E	1	Small healing wound on the trunk, daughter of the PM 22, first cone produced in 2019 (empty)
				Twig 2° order	Lower third	E	1	
				Twig 1° order	Lower third	N-E	0	Healing wound close to trunk
				Trunk	Lower third	E	0	Wound due to friction, broken twig
	May 2023	-	-	crown		uphill		Defoliation due to mechanical causes, stable 3 female cones
31	Nov. 2019		60-80 e >80	Branche di 1° e 2° order	Thorough	All	60	Branches dried out, declining

	Phytosanitary survey November 2019 - May 2023							
***Abies nebrodensis* natural population**								
Tree no.	**Survey year**	**Crown transparency**	**Turning foliage (%)**	**Affected organ**	**Position in the crown**	**Direction (N, S, E, W)**	**Percentage of damaged crown**	**Description**
	May 2023			Branch		W	50	1 branch defoliated Stable, no new reddenings, crown shaded by a holm oak uphill
32	Nov. 2019			Trunk	Half height		15	Four branches dried out, with wounds due to herbivores
	May 2023							Leader dried out (formerly), replaced by a lateral twig. Stable, no new disorders

Table 1. Disorders observed on A. nebrodensis the natural population are reported separately for each tree. Comparing data of the first survey of Nov. 2019 and the last survey of May 2023 (grey rows), no significant differences can be observed in the course of the years.

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 2 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.

3.2 Dendro-auxometric parameters

The project planned to record the dendro-auxometric parameters of each tree to monitor their growth over time. This will allow to have a baseline to refer to in the future to assess the evolution and growth of the trees in quantitative terms, also in relation to the protection measures that will be carried out during the project. For each tree, the main dendro-auxometric parameters were recorded, such as: trunk height in meters, diameter of the stem at the base, diameter of the stem at breast height (bh: 1.3 m from the ground), diameter of the crown and its morphology.

3.3 Surveys in the nursery

In the nursery, the surveys on the health conditions of the plants were aimed at evaluating whether the raising procedure was adequate or it was necessary to make changes. The nursery activity plays a fundamental role to produce healthy, vigorous and genetically selected plants for use in the new repopulation nuclei.

In the nursery, progenies were distinguished by mother tree, year of sowing and plot. For each progeny, potted plants were counted and subjected to visual inspection to identify foliage disorders, also recording their incidence in terms of percentage of damaged plants for each progeny.

In the Piano Noce forest nursery, progenies of 12 mother trees (PM) were represented. The total number of plants growing in three nursery plots exceeded 25000. The number of pot plants of each mother tree varied from a minimum of 158 for PM 12 to a maximum of 6127 for PM 22.
In the whole nursery, a mortality of 2.4% was recorded, and 4.6% of plants showing needle reddening, blight and needle cast symptoms (4.6%). Other symptoms such small needles, chlorosis and stunted growth were sporadic. Totally, less than 8% of the seedlings in the nursery was damaged. The impact of the disorders on the plants in the nursery was relatively low.
Fungal sampling and isolation were carried out on symptomatic plants in the nursery in order to determine the microflora associated with the disorders observed and exclude the action of pathogens capable of spreading rapidly and causing serious damage. Particular attention was given to very harmful oomycetes in nursery environments. The presence of *Phytophthora* sp. in the nursery was investigated in soil and roots samples collected from a pool of plants showing related symptoms of the pathogen such as chlorosis and defoliation through ITS sequencing (DNA barcoding).

Symptoms	No.	%
mortality	608	2,4
reddened needles	272	1,07
chlorosis	971	3,82
defoliation	44	0,17
small needles	6	0,02
blighted shoots	24	0,1
stunted growth	111	0,43

Table 2. Frequence (as number and percentage) of the main symptoms observed on the aerial part of the pot plants raised in the Piano Noce forest nursery.

3.4 Sampling, fungal isolations and DNA barcoding identification

Samples of blighted twigs, shoots and needles were taken both from trees of the natural population and from pot plants in the nursery for further observations and analysis 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. On the trees of the natural population, isolations were performed also from healthy (green) needles.
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. 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 twice 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 (mycelium and reproductive structures). Morphotypes were then characterized by sequencing specific regions of genomic DNA, known for their diagnostic value (ITS1-ITS4). The resulting sequences were Blast matched against available sequences from GenBank for identification.

From the twigs sampled from the trees of the natural population, 204 fungal colonies were obtained, 148 from reddened needles and 56 from healthy needles. Based on ITS sequencing they belonged to 20 different taxa (Fig. 1). From the shoots sampled in the nursery 277 colonies were obtained and 20 different species belonging to 19 genera were identified (Fig. 1). Nine of these species were isolated also from the needles sampled in the natural population.

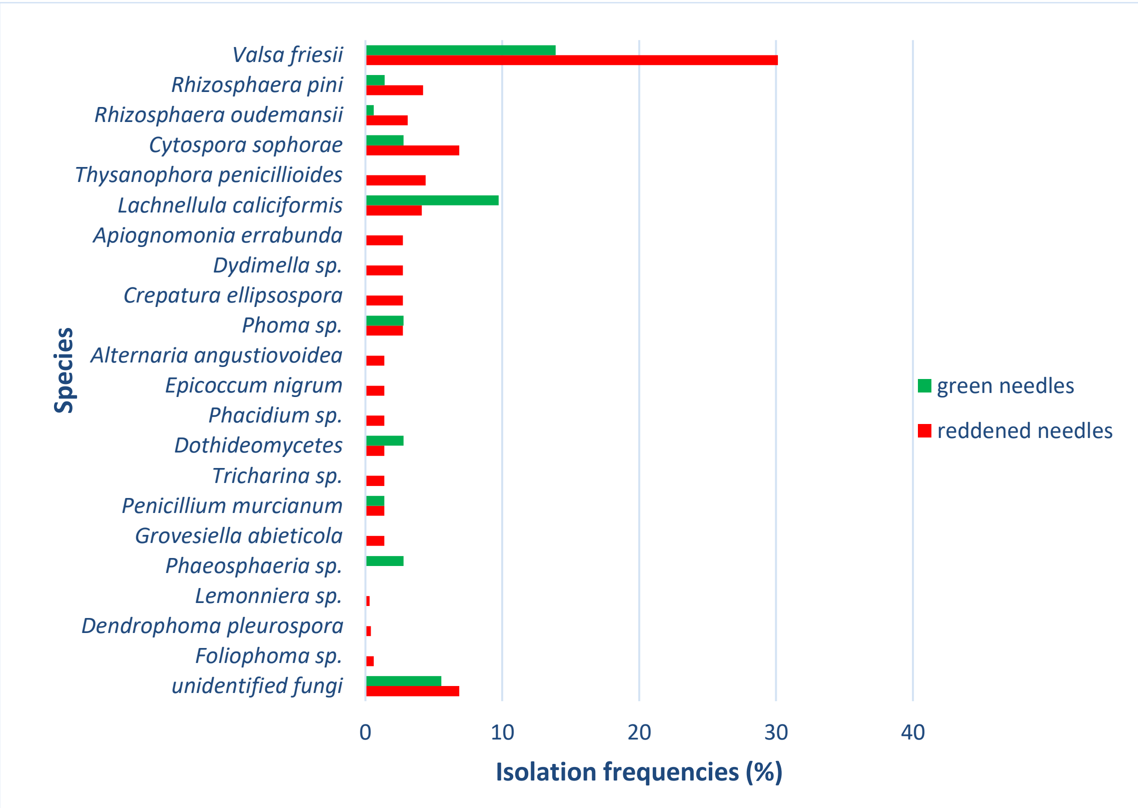


Figure 1: Fungal isolation frequency (IF) from symptomatic and asymptomatic needles of *A. nebrodensis* trees of the natural population. The green and red bars represent isolations from green and reddened needle respectively.

Results on fungal identification performed in plants of the natural population highlighted the presence of species belonging to *Valsa* (and its anamorph *Cytospora*) and *Rhizosphaera* genus as the most represented on both reddened and green needles. All these fungi are reported as mainly opportunistic, being favored by weakening factors of trees such as drought, late frost or growing in the bark damaged by other pathogens. Isolation of these fungi from green needles suggests their presence as endophytes and their ability to become saprophytic in needles weakened and stressed by environmental factors. These fungi are known as 'transition fungi', stably associated with senescent needles of *Abies* species and have an intermediate behavior between true pathogens and true saprophytes. Being already present in green needles, they contribute to the necrotic process by interacting with various weakening factors, favoring the senescence of weakened needles that are no longer functional.

Among the remaining fungal taxa, the investigation carried out excluded the participation of true pathogens in the necrotic process of the needles observed on branches of *A. nebrodensis* in the

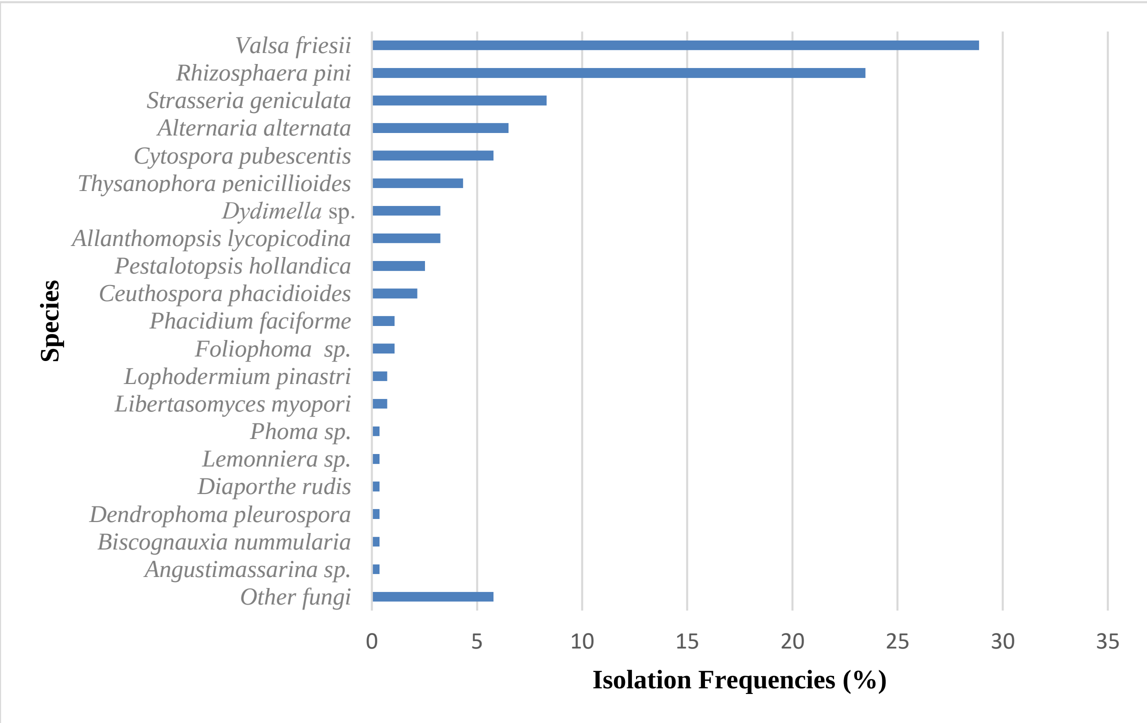


Figure. 2 Fungal isolation frequency (IF) from needles and shoots of *A. nebrodensis* sampled in the 'Piano Noce' nursery.

natural population.

Even on the plants in the nursery, the microbial mycoflora associated with the disorders observed was not very different compared to the plants in the natural population. *Valsa friesii* and

Rhizosphaera pini were the most prevalent taxa isolated from the symptomatic samples examined too. All other fungi are reported as endophytes, disease pathogens and saprophytes. These do not include any invasive alien species.

Both the observed symptoms and the fungal taxa isolated from the samples of needles and shoots allowed the involvement of aggressive pathogens and newly introduced pathogens to be excluded. Damages to needles and shoots seem to be due to predisposing external factors such as delayed transplants or direct sun light exposure that favor the action of weak pathogens (opportunistic), often already present within asymptomatic tissues as endophytes.

The investigations conducted have made it possible to identify some nursery practices to improve, such as the standardized preparation of the substrate for potted plants, the carrying out of transplants more frequently, watering every 5 days when the temperature exceeds 25°C, adequate shading.

4. Key points

- Monitoring the growth conditions and the state of health of A. nebrodensis trees is essential for the in situ protection of the natural population.
- In the Life4fir project, an action was dedicated to monitoring the health state of A. nebrodensis through repeated surveys aimed at evaluating the disorders of the foliage and determining their spread and impact on individual trees. This allowed obtaining a detailed picture of the symptoms observed.
- Control and prevention of pathogens has been based on monitoring of the state of health of trees through regular surveys of crown disorders, sampling of symptomatic organs and fungal isolations, identification of fungal taxa by DNA barcoding.
- In addition to the surveys of the disorders, dendro-auxometric measurements were carried out to record the growth parameters of the trees: height and diameter of the trunk, width of the crown, its symmetry.
- The data collected in the two previous points allowed us to establish a baseline, to which we can refer in subsequent surveys to monitor the evolution of tree growth and crown disorders.
- The symptoms most frequently observed on trees in the natural population were: reddened shoots and needles, desiccation of twigs, wounds to lower branches due to wild herbivores, small leaves and chlorosis.
- No substantial differences emerged regarding the spread and impact of the alterations between November 2019 and May 2023.

- Isolations from symptomatic and asymptomatic needles and twigs were carried out to examine the fungal microflora associated with the observed alterations.
- The identification of the fungi obtained from the isolations was made with DNA barcoding (ITS sequencing).
- Both the symptoms and the associated fungal microflora led to exclude aggressive pathogens as cause of the disorders observed. The isolated fungal community was composed of endophytes, weak pathogens and saprophytes. The action of these fungi is related to the occurrence of environmental stress to which trees are subjected: strong winds, hail, shallow and rocky soil, damage from herbivores. The trees located near the ridges or isolated among stony ground showed needle reddening to a greater extent compared to trees located in better conditions, such as those near or within beech nuclei, which are more protected and in more favorable edaphic conditions.
- Disorders can therefore be attributed to the hard site conditions to which the residual trees of the A. nebrodensis population are subjected. Overall, the plants show good tolerance to the environment in which they live, except for the pressure exerted by populations of wild herbivores such as fallow deers and wild boars.
- The surveys in the nursery were aimed at verifying the health conditions of the plants grown, assessing the presence of any pathogens, and eventually evaluating the need for corrective nursery measures.
- Isolations from the soil excluded the presence of dangerous pathogens which can cause severe losses in the nursery set. (e.g. *Phytophtora*). Isolation from reddened and dried needles and twigs highlighted the presence of weak pathogens and saprophytes, similar to what was observed in the natural population and excluded the effect of aggressive pathogens.
- In the Piano Noce nursery, the incidence of mortality and disorders did not exceed 8% and overall the over 25,000 potted plants examined showed substantial good growth conditions. Unlike what is reported by the IUCN, the health of the seedlings and saplings grown in the local forest nursery does not currently appear to represent a threat to *A. nebrodensis*.

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