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ORIGINAL ARTICLE

Insights on the fungal communities associated with needle reddening of the endangered *Abies nebrodensis*

Arcangela Frascella[1,2] · Sara Barberini[1] · Gianni Della Rocca[1] · Giovanni Emiliani[1] · Vincenzo Di Lonardo[1] · Stefano Secci[1] · Roberto Danti[1]

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Abstract

Abies nebrodensis is a species of fir endemic to Sicily, represented by only 30 trees in the natural population and is currently classified as critically endangered by IUCN. In such context, monitoring its health status is essential for the proper management and preservation of this species. Phytosanitary surveys of trees of the natural population of *A. nebrodensis* and on potted plants raised in the local forest nursery were carried out, and the phyllosphere fungal community was investigated. The health condition of trees in the natural population were fairly good, with needle reddening and blight as the most frequently observed symptoms on the foliage, while in the nursery similar disorders were registered on about the 1.3% of potted plants. Results on fungal isolations highlighted the presence of species belonging to *Valsa, Cytospora* (which includes anamorphs of *Valsa*) and *Rhizosphaera* genera as the most represented on both reddened and green needles; these results suggest that these fungi likely live as endophytes, resuming their growth when needles are affected by environmental stressors such as wind, hail, mechanical wounds and do not represent a biotic constraint for *A. nebrodensis*. The disorders observed appear mostly as a consequence of the harsh site in which the relic species lives. Together with the fungal community observed on symptomatic and healthy needles, they indicate that *A. nebrodensis* adapted and tolerates its altered habitat.

Keywords Sicilian fir · Mediterranean forests · Abies alba · Endophytic fungi · Threatened species

Introduction

Mediterranean forest ecosystems are exposed to pronounced climatic constraints such as wind, long drought but also heavy stormy rainfalls, high temperatures, which make them susceptible to biotic and non-biotic stress. Being in a transitional zone, affected by mid-latitude and tropical processes, they are vulnerable to climate changes (Giorgi and Lionello 2008; Palahi et al. 2008; Collevatti et al. 2011). Extreme weather events may impact plant health, reducing plant growth and vigour, altering phenology, impairing physiological processes, and creating wounds that promote the attacks of pathogens and pests (Panzavolta et al. 2017, 2021; Jactel et al. 2019). These effects may be even more detrimental for the endangered species living in altered and fragmented habitats (Garza et al. 2020).

The Sicilian fir, *Abies nebrodensis* (Lojac.) Mattei, is an endemic species of Sicily classified as critically endangered (A2cd) by IUCN (Thomas 2017) and can be considered as the rarest conifer in the European flora (Pasta et al. 2020). The residual population consists of only 30 mature trees and is restricted to an 84-ha area in the Madonie range in the north-central part of Sicily (Italy), between 1400 and 1700 m a.s.l. Its habitat is protected by the EU standards and is listed within the Annex I of the Habitat Directive (code 9220, 'Apennine beech forests with *Abies alba* and beech forests with *Abies nebrodensis*'). The natural population of the species is also included in the SCA Ita020004 and SPA Ita020050 of the Natura 2000 network.

✉ Sara Barberini
sara.barberini@ipsp.cnr.it

[1] Institute for Sustainable Plant Protection (IPSP), SS Sesto Fiorentino, National Research Council (CNR), Via Madonna del Piano 10, Sesto Fiorentino 50019, Italy

[2] Institute for BioEconomy (IBE), SS Sesto Fiorentino, National Research Council (CNR), Via Madonna del Piano 10, Sesto Fiorentino 50019, Italy

Some authors reported that *A. nebrodensis* occupied a wide territory in Sicily a few hundred years ago and was relatively frequent in the Madonie area in the 17th century. Extensive logging, grazing, fire and soil degradation would have led to a dramatic reduction of *A. nebrodensis* population (Morandini 1969; Venturella et al. 1997; Tinner et al. 2016; Pasta et al. 2020). According to IUCN, the main threats affecting *A. nebrodensis* relic population are i) the limited population size leading to genetic bottle-neck, ii) the poor health of the seedlings and saplings grown in the local forest nursery, iii) habitat degradation, grazing and trampling of seedlings by introduced herbivores such as wild boars and fallow deers, iv) the risk of genetic pollution exerted by non-native firs co-occurring in its natural range. Climate change, in terms of extreme events, including heat waves, may directly affect the species survival and indirectly increase the frequency of wildfires, altering the balance within the ecosystem (Pasta and Troia 2017).

Survival of *A. nebrodensis* therefore currently depends on the implementation of protective measures aimed at habitat improvement (Thomas 2017). In the last three decades, the Sicilian fir has been subject to specific conservation measures carried out by local authorities to tackle its main threats (Raimondo and Schicchi 2005). Currently, Life4fir (LIFE18 Nat/It/000164) project of the LIFE Nature and Biodiversity subprogram, is aimed at improving the conservation status of *A. nebrodensis* and averting the risk of extinction of this species through the implementation of *in situ* and *ex situ* protection measures (Frascella et al. 2022). To preserve endangered species, monitoring is essential to detect trends in species distribution through time, measure the impacts of threatening processes and evaluate the effectiveness of management responses (Lindenmayer et al. 2020). The phytosanitary inspection of *A. nebrodensis* population is one of the pivotal measures for *in situ* conservation of the species. Evaluating and monitoring the state of health of the natural population provides useful knowledge about occurring disorders and can assist in managing proper protection and conservation measures. Surveys were extended to the local 'Piano Noce' forest nursery, where a high rate of damaged and suffering plants was reported among the main threats to of the species by IUCN (Pasta and Troia 2017) and where genetically selected seedlings are being produced for reforestation purposes.

Some crown disorders, such as needle blight, have been observed to affect *A. nebrodensis* natural population for years, but generally they have not been described in detail (Raimondo and Schicchi 2005) and their real impact has remained unclear. Needle reddening and blight in *Abies* species have been reported since the beginning of the last century across Europe in natural populations, plantations and nurseries (Morelet 1975; Gourbière 1980; Martínez and Ramírez 1983; Kowalski and Andruch 2012). Various fungal species were reported to be associated with this symptomatology in *Abies* spp.: *Herpotrichia parasitica* (Kowalski and Andruch 2012), *Sydowia polyspora* (Talgø et al. 2010), *Lirula nervisequia* (Minter and Millar 1984; Barzanti et al. 1998), *Rhizosphaera* spp. (Diamandis and Minter 1980; Martínez and Ramírez 1983), *Phaecryptopus nudus, Delphinella abietis* (Solheim and Skage 2002; Talgø and Stensvand 2012), *Cytospora friesii* (Milijašević 1996; Lazarevic and Menkis 2022). In Italy, needle browning has been reported only locally and sporadically on *A. alba* (Barzanti et al. 1998; Capretti 2000).

The aims of this work are therefore to 1) report the occurrence and spread of needle blight on the foliage of *A. nebrodensis*, through surveys carried out both on the trees in the natural range and on the plants of the local nursery; 2) investigate the fungal communities associated with the needle disorders observed. Knowledge of the mycobiota associated with needle browning on *A. nebrodensis* is useful for the detection of potential pathogens and understand dynamics involved in the occurrence and evolution of symptoms. In addition, information about fungal species playing a key role within the microbial community could be raised, allowing the implementation of proper control measures to improve the health status of *A. nebrodensis*.

Materials and methods

Ecological features of the *A. nebrodensis* area

The Sicilian fir (*A. nebrodensis*) residual range is located in the municipality of Polizzi Generosa and is part of the Madonie Regional Park, covering an area of 40,000 hectares in Sicily (southern Italy) (Fig. 1).

Thirty adult trees (numbered from 1 to 32) constitute the relic population of *A. nebrodensis*. Only 24 out of the 30 trees are fertile and produce cones and seeds. They grow between the vegetation belt dominated by *Quercus petraea* (Matt.) Liebl. And *Ilex aquifolium* L. and that one dominated by *Fagus sylvatica* L., on a prevailing north, north-western slope. Beech forms wooded nuclei and groves, though they are fragmented. Soils are poor and shallow, on quartz-arenitic substrate, often extremely rocky and stony near ridges and screes. The range of Sicilian fir is extremely fragmented. Some trees are fully isolated, often growing on screes and bare soil, others grow near or inside beech groves or other broad-leaved formations.

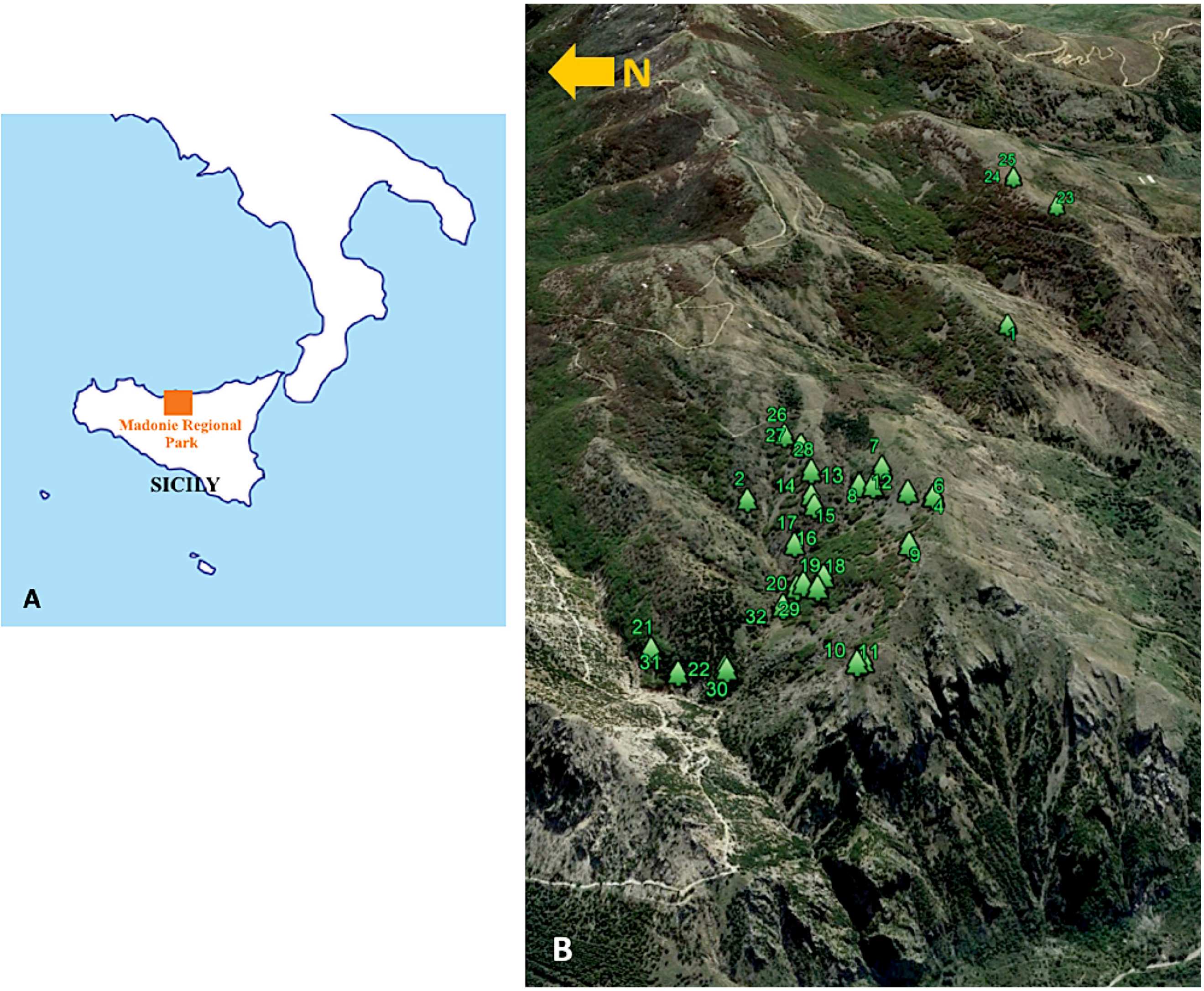

Fig. 1 **A** Position of the Madonie Regional Park within the Sicily region. **B** Position based on UTM coordinates of each of the 30 adult trees in the natural range of *Abies nebrodensis* in relation to the topography of the territory based on a Google Earth map

The Walter and Lieth diagram summarize average and seasonal climatic conditions for Polizzi Generosa over three decades, from 1991 to 2020 (Fig. 2).

Over this time period, the mean temperature was 15.0 °C, the maximum mean temperature was 31.6 °C, and the minimum mean was 2.5 °C. The mean annual precipitation was 418 mm, with a 5-month dry period extending from May to September.

The 'Piano Noce' nursery is located about 2.5 km in a straight line from the natural population of *A. nebrodensis*. Plants of *A. nebrodensis* raised in the 'Piano Noce' nursery were obtained from open pollinated seeds collected from 2008 to 2015 from the fertile trees of the natural population. Seeds were extracted from mature cones collected each year in October and sowed at the beginning of November, in pots containing a mixture of forest soil (collected in local sites), peat and tuff sand (3:1:1, v/v/v). One year after sowing, the seedlings obtained were transplanted into larger pots, containing forest soil mixed with sand. In the nursery, seedlings were divided by their own mother plant and sowing year, and then placed in three plots of the nursery.

Assessment of health conditions of trees and nursery plants

Surveys were carried out in October 2020 on the trees of the natural population and in November 2019 in the nursery. Trees in the natural stand were subjected to a careful visual examination to determine the occurrence and rate of foliage blight and needle reddening on the crowns of single trees. Impact (I) of disorders on the crowns was estimated

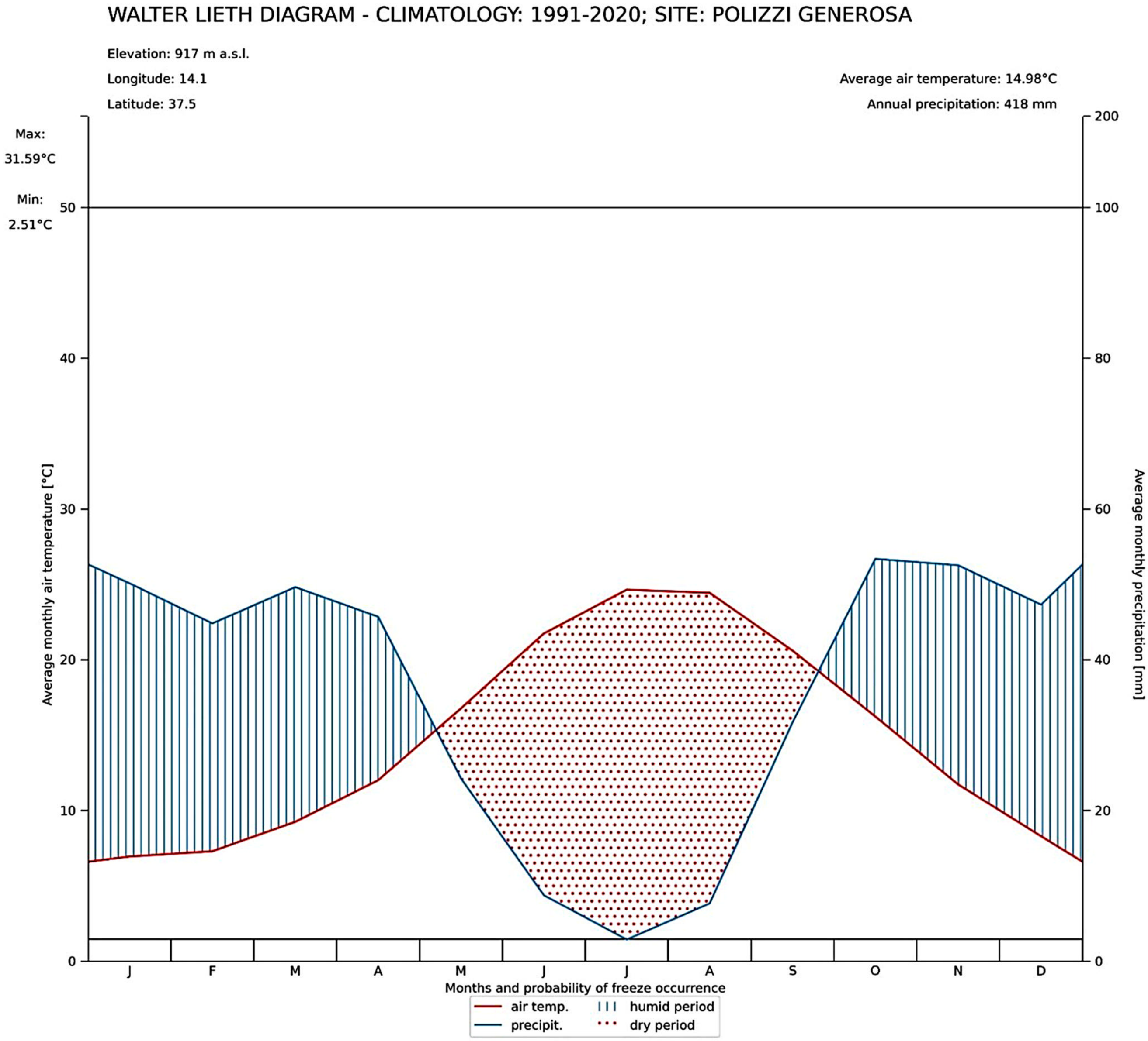

Fig. 2 The Walter and Lieth diagram for Polizzi Generosa

by dividing the number of symptomatic twigs counted throughout the crown (n) by the crown surface meant as a cone lateral surface area (L), with a height equal to that of the tree trunk and with a diameter at the base equal to the diameter of the crown at its bottom ($I = n/L$). The resulting value was the number of reddened twigs per unit of crown surface (m^2).

In the nursery, progenies were distinguished by mother plant and year of sowing. For each progeny, potted plants were counted and subjected to visual inspection to identify foliage disorders, with particular reference to needle reddening and blight, recording its incidence (in terms of percentage of damaged plants for each progeny). In total, more than 25,000 potted plants, coming from 12 mother trees were surveyed.

Sampling and fungal isolations

In the natural stand, 4 twigs showing needle reddening and blight were sampled from the 15 trees showing a higher rate of disorders (groups 3, 4 and 5 in Table 1). Healthy twigs showing no symptoms, adjacent to the symptomatic ones were collected alongside. Samples were separately inserted inside plastic bags, placed in a portable cooler, and taken to the laboratory in the same day of collection, where they were kept at 4 °C until being processed (within

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)

Tree n.	Trunk height (m)	Crown diameter (m)	L (m^2)	Elevation a.s.l. (m)	Position	I	Group of trees
20	9.2	2.9 × 3.2	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	7.4 × 6.7	117.4	1597	Isolated	0	
29	10.5	4.2 × 4.9	76.8	1468	Within a beech grove	0	
30	1.76	1.7 × 1.6	5.2	1400	Under oaks	0	
32	1.96	1.7 × 1.8	5.7	1449	Within a beech grove	0	
21	11.6	8.5 × 7.7	156.3	1433	Within oak grove	0.03	Group 2 0.01 < I > 0.1
8	11.0	7.7 × 6.9	132.9	1577	Near broadleaves	0.04	
17	10.7	8.7 × 7.3	143.5	1488	Isolated	0.04	
19	5.5	4.6 × 4.9	44.7	1487	Margin of a beech grove	0.05	
13	11.1	9.5 × 9.2	176.9	1567	Isolated	0.05	
2	14.4	9.2 × 9.1	217.2	1526	Isolated	0.06	
14	7.2	6.5 × 6.8	82.1	1556	Near oak trees	0.06	
26	6.0	3.8 × 3.6	36.5	1599	Within a beech grove	0.08	
7	5.7	5.3 × 5.5	53.5	1603	Near an oak tree	0.09	
10	7.5	7.5 × 7.3	97.2	1525	Isolated on a ridge	0.11	Group 3 0.11 < I > 0.5
18	7.6	5.2 × 4.5	60.1	1503	Near beech trees	0.13	
15	8.5	5.7 × 5.2	76.4	1539	Near oak trees	0.17	
16	5.3	7.7 × 4.8	59.8	1488	Isolated on shallow soil	0.18	
11	8.2	5.1 × 4.6	65.1	1520	Isolated on a ridge	0.20	
4	0.85	2.1 × 1.5	3.5	1639	Under a bigger *A. nebrodensis* tree	0.19	
6	7.8	7.2 × 6.3	90.1	1639	Isolated	0.26	
23	7.5	4.6 × 4.3	54.7	1673	Isolated on a ridge exposed to strong winds	0.33	
24	3.1	3.8 × 4.1	22.8	1705	Isolated on a ridge exposed to strong winds	0.53	Group 4 0.51 < I > 1.00
25	3.3	3.5 × 1.6	14.2	1705	Isolated on a ridge exposed to strong winds	0.71	
1	6.5	6.4 × 7.6	81.2	1651	Isolated and exposed to strong winds	0.74	
12	8.5	6.8 × 8.4	111.2	1604	Isolated on a scree exposed to strong winds	1.28	Group 5 I > 1.00
31	1.25	1.4 × 1.3	2.9		Small, damaged by wild herbivores	1.39	
9	1.8	3.6 × 3.6	14.4	1617	Isolated on rocks exposed to strong winds	2.43	
28	0.48	1.3 × 1.1	1.4	1586	Small, damaged by wild herbivores	2.96	

Grey shades indicate groups of trees showing a different I rate

L crown surface meant as a cone lateral surface area

2–3 days). Ten needles were detached from each sampled twig for fungal isolations. In a same tree, needles detached from twigs showing same symptoms (with the aid of stereomicroscope) were pooled. Overall, 250 reddened and 250 green (healthy) needles were used for isolations. In the nursery, 10–15 symptomatic twigs and shoots were collected from each of the 12 progenies raised in the nursery. Four needles per shoot were used for isolations. Samples were surface sterilized by immersion in a 70% ethanol solution for 1 min and then in a sodium hypochlorite solution (4–5% active chlorine) for 4 min, modifying the procedure described by Sieber-Canavesi and Sieber (1993). They were then rinsed with sterile water and dried on sterile filter paper. The needles were cut into 3–5 mm fragments and placed in Petri dishes containing PDA (Potato Dextrose Agar, Difco BD). Plates were incubated at 24 °C in the dark for 3 weeks. Each 2–3 days, plates were checked to assess the progressive development of the fungal colonies growing out from the fragments. Fungal colonies showing different morphology were counted, subcultured on PDA plates and incubated at 25 °C for 10 days in the dark.

The obtained colonies were grouped into morphotypes based on their morphological characteristics: color and shape of colony (front and reverse), mycelium texture and reproductive structures (when formed). The isolation frequency (IF) was calculated as percent using the following formula (Franceschini et al. 2005): $IF = Ni/Nt \times 100$, with Ni (number of fragments colonized by a fungus) and Nt (total number of plated fragments).

For DNA barcoding, 1–3 sample isolates of each morphotype were grown on a sterile cellulose membrane placed on PDA in Petri plates for DNA extraction. Plates were incubated in the dark at 25 °C for about 4 weeks and the mycelium was then scraped out and lyophilized. Samples were maintained at −20 °C in sterile tubes until genomic DNA extraction.

DNA extraction, PCR conditions and sequencing

DNA extraction was carried out with the 'NucleoSpin' Plant II kit (Macherey Nagel GmbH & Co. KG) on 20 mg of lyophilized mycelium following the manufacturer's instructions. Amplifications of the 5.8S rDNA (ITS) region of genomic DNA were carried out using the universal primers ITS1 and ITS4 (White et al. 1990). All PCR reactions utilized GoTaq Green master mix by Promega (Italy). Amplicons were then purified by "Wizard ® SV Genomic DNA purification system kit" and sequenced (ABI 3730xl DNA Analyzer; Eurofins genomics) using ITS1 as the sequencing primer. In case of low quality, amplicons sequenced with the reverse primer ITS4 were also used to achieve a greater accuracy in taxonomic classification.

The resulting sequences were matched for identification against available sequences from GenBank using the Blast online tool (Johnson et al. 2008). Sequences of the three most frequently isolated fungi were used for phylogenetic analyses with reference ITS sequences of the same genera identified in the Mediterranean area, available in GenBank (NCBI). The multiple sequence analysis and the construction of the neighbor-joining (NJ) phylogenetic tree were performed using MEGA 11 (Tamura et al. 2021), following the Kimura-two-parameters (K2P) method (Kimura 1980); 1000 bootstrap replicates were taken to examine the reliability of the interior branches and the validity of the trees obtained. The ambiguous nucleotide positions were removed for each sequence pair (pairwise deletion option).

Results

Tree health surveys in the natural population

Adult trees of the natural population were subdivided into five groups depending on the standardized number of reddened and blighted twigs per square meter of crown surface (I) (Table 1). Surveys showed that 7 out of 30 adult trees of the natural population reported no disorders (group 1); 9 trees showed an 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).

In the group 5 with the greatest impact of symptoms (I > 1.00), reddened twigs showed a thorough distribution along the tree canopy depth, while generally symptoms remained confined to the lower part of the crown in the other groups of trees (Fig. 3).

Some other symptoms were sporadically observed, though they were not included in this study. These were: defoliation of twigs and branches in the outer part of crowns (likely as natural evolution of the needle reddening and blight); presence of wounds due to herbivores on the distal part of branches and on the stem; defoliation in the lower crown parts due to excessive shading in *A. nebrodensis* trees located within beech or oak groves; chlorosis and atrophy of needles (Fig. 3).

From the trees of the natural population, 204 fungal colonies were obtained, 148 from reddened needles and 56 from healthy needles. No fungal colonies were obtained from 23.3% of reddened needles and 50% of green needles. PCR amplification of the 5.8S rDNA of fungal colonies representative of each morphotype allowed the identification of 21 different species belonging to 20 genera (Fig. 4). On reddened needles, *Valsa friesii* represented the most frequently isolated fungus (IF = 30%), followed by *Rhizosphaera* spp. (*R. oudemansii* and *R. pini* with IF = 7.3%), *Cytospora sophorae* (IF = 6.8%), *Thysanophora penicillioides* (IF = 4.4%) and *Lachnellula caliciformis* (IF = 4.1%). The same fungi, except *T. penicillioides,* were also isolated from green needles showing an IF of 13.9%, 2.0%, 2.7% and 9.7% respectively. *Phaeosphaeria* spp. was isolated only from green needles with a frequency of 2.7%. On the contrary, *Grovesiella abieticola, Tricharina* sp., *Phacidium* sp., *Epicoccum nigrum, Alternaria angustiovoidea, Crepatura ellipsospora, Dydimella* sp., *Apiognomonia errabunda* were isolated exclusively from reddened needles with frequencies ranging from 1.3% to 2.7%. *Phoma* sp. and *Penicillium murcianum* were instead isolated with similar frequencies in red needles and green needles (2.7% and 1.3%, respectively). In the list were also included fungal species displaying an IF less than 1% such as *Dendrophoma pleurospora, Lemonniera* sp., *Foliophoma* sp. and some unidentified fungi. *Crepatura ellipsospora* was the only belonging to the Basidiomycota, whereas all the other isolates belonged to Ascomycota.

Nursery plants survey

In the whole nursery, a mortality of 2.4% was recorded, and 1.3% of plants showed needle reddening and needle cast. Other symptoms such as small needles, chlorosis and stunted growth were also detected. In total, less than 8% of the potted plants in the nursery showed disorders (data not shown).

From the reddened needles sampled in the nursery, 277 colonies were obtained. No fungal colonies were isolated from the 19.6% of the plated needles. PCR amplification of the 5.8S rDNA allowed the identification of 20 different species belonging to 19 genera of Ascomycota (Fig. 5),

Fig. 3 **a** Example of needle reddening in tree no. 11 affecting the lower part of the crown. **b** Good crown condition of tree no. 7: the red circle shows a branch damaged by wild herbivores in the lower part of the crown. **c** Close-up picture showing the fair crown condition of tree no. 13 with cones developing on the distal portion of the branches. **d** Twig reddening and defoliation throughout the uphill crown portion of tree no. 12 exposed to strong winds

whereas 9 of these taxa were also isolated from the needles sampled in the natural population.

The fungal taxa most frequently isolated from reddened and blighted needles in the nursery were: *Valsa friesii* (IF = 28.0%), followed by *Rhizosphaera pini* (IF = 23.5%), *Strasseria geniculata* (IF = 8.1%), *Alternaria alternata* (IF = 7.0%), *Cytospora pubescentis* (IF = 6.0%) and *T. penicillioides* (IF = 4.6%). The remaining taxa showing a lower isolation frequency were *Didymella* sp. (3.7%), *Allanthomopsis lycopicodina* (3.7%), *Pestalotiopsis hollandica* (IF = 2.5%), *Ceuthospora phacidioides* (IF = 2.3%), *Phacidium faciforme* (IF = 1.8%), *Foliophoma* (1.5%) and some other fungi which were isolated from only one or few fragments.

Phylogenetic trees

Genetic distances among the *Valsa friesii, Cytospora* sp. and *Rhizosphaera* sp. isolates obtained from *A. nebrodensis* needles in this study and various Mediterranean species representing diversity of these genera are reported in Neighbor-Joining trees in Figs. 6 and 7.

Cytospora sophorae and *V. friesii* are grouped in a single cluster together with the reference isolate of *V. friesii*, identifying *C. hippophaes* as their phylogenetic nearest species (Fig. 6). *Cytospora pubescentis* clustered differently compared to *C. sophorae*. The obtained *Rhizosphaera* tree is split into two main clusters, the first containing *R. oudemansii, R. kalkhoffii, R. pinicola* and

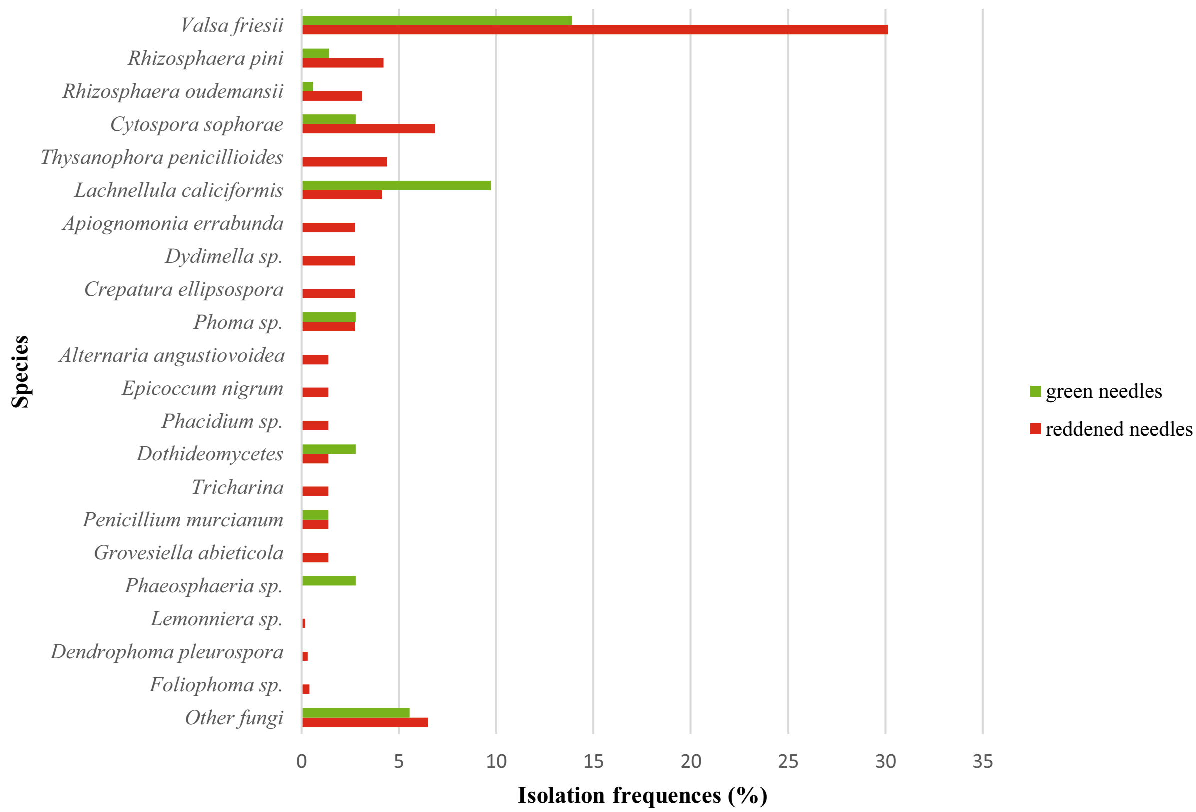

Fig. 4 Fungal isolation frequency (IF) from symptomatic and asymptomatic needles of *A. nebrodensis* trees of the natural population. The green and red bars represent isolations from green and reddened needle respectively

R. macrospora while *R. pini* grouped alone with the reference isolate (Fig. 7). GenBank accession numbers of ITS sequences generated in this study are shown in Table 2.

Discussion

In this work we surveyed for the first time the crown disorders of the critically endangered *A. nebrodensis* population providing insights to its phyllosphere fungal community. The crown assessment of trees in the natural population highlighted that the most common symptom is needle reddening and needle cast with subsequent defoliation of twigs and branches in the outer and lower part of the crowns. With few exceptions, disorders did not affect the upper two-thirds of the crowns. In general, the extent of symptoms observed is related to the environmental conditions that trees are facing at a microclimate and site level. Trees were separated into five groups based on the rate of reddened and blighted twigs observed per unit of crown surface. Trees belonging to group 1 (reporting no symptoms on the crown) are all located within beech groves where they benefit from better edaphic conditions due to a deeper soil layer and a greater amount of organic matter in the soil, and where they are also protected from strong winds. Cicşa et al. (2019) also reported an increased resistance to environmental stresses in mixed stands of silver fir and beech. The *A. nebrodensis* trees growing among beech trees generally showed defoliation of the lower branches due to shading, but their state of health is generally good. As far as the other groups are concerned, an increase in the number of symptomatic twigs was generally observed with the occurrence of more severe conditions which the trees are subjected to, depending on their topographic and site position. Trees of the fourth group (showing a needle reddening impact between 0.51 and 1.00) for example are isolated near ridges, such as trees no. 1, 24, and 25, where they are exposed to strong winds, storms, hail. On these trees symptoms were distributed throughout the canopy depth, though they had a greater impact in the lower portion. Tree no. 12 is growing in the middle of a large scree, very exposed to strong winds, where both atmospheric and edaphic conditions are certainly harsh. This tree is subjected to environmental stresses that probably favor the action of opportunistic fungi causing needle reddening and needle cast. Finally, trees no. 28 and 31, in group 5, showed a very stunted growth and advanced decline, due to repeated injuries caused by

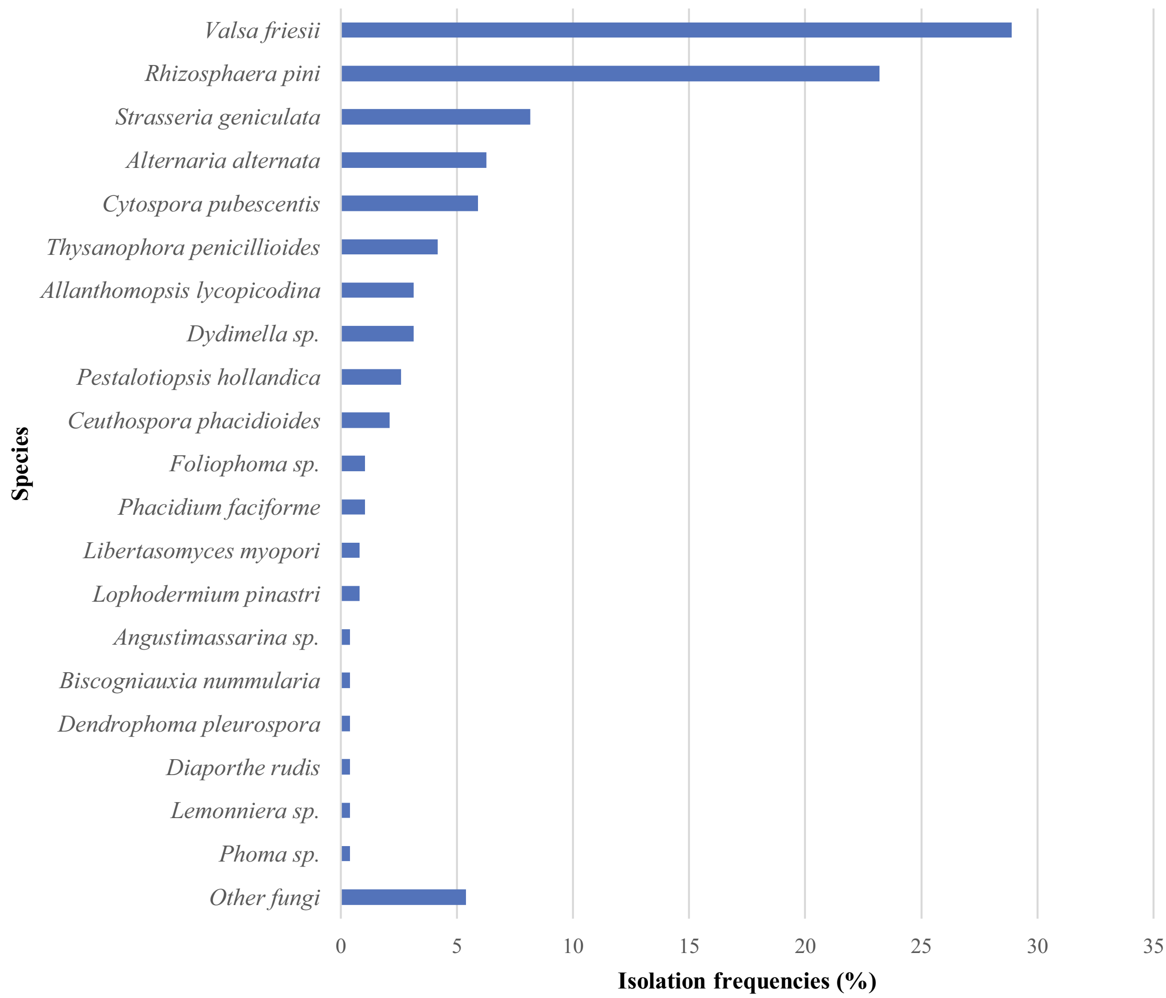

Fig. 5 Fungal isolation frequency (IF) from symptomatic plants of *A. nebrodensis* sampled in the nursery 'Piano Noce' nursery

wild herbivores leading to needle and twig blight. Attempt to improve their growth conditions requires the restoration of an adequate protective system of fences, to prevent wild herbivores from approaching. Generally, the symptoms observed in the crowns of the natural population indicate an equilibrium between the relic trees and the environmental conditions and do not appear to be caused by the introduction of aggressive and spreading pathogens. This situation is also supported by observations repeated in 2021 and 2023, which highlighted the occurrence of needle reddening in the crowns without substantial differences between the various years (data not shown).

Trees host a kind of endophytic fungi which are not able to kill healthy tissues but may switch to saprophytism, colonizing the needles weakened by environmental causes, favoring their fall (Sieber-Canavesi and Sieber 1993). In the case of *A. nebrodensis*, the environmental factors that mostly solicit plants are the strong winds which acts mechanically on needles and twigs, wild herbivores biting and rubbing their horns and hail. Results of fungal isolations from needles highlighted the presence of species belonging to *Valsa, Cytospora* (which includes anamorphs of *Valsa*) and *Rhizosphaera* genera as the most represented on both reddened and green needles. Species of the genus *Cytospora* are mainly opportunistic, being favored by trees weakened by drought, late frost or growing in bark damaged by other pathogens. They have also been reported as pathogenic fungi causing cankers on broadleaves and conifers, also known for their worldwide distribution and large host range (Adams et al. 2005, 2006; Fan et al. 2014a, b, 2015; Ariyawansa et al. 2015; Liu et al. 2015; Maharachchikumbura et al. 2016; Hyde et al. 2016; Li et al. 2016; Lawrence et al. 2017, 2018; Norphanphoun et al. 2017, 2018). *Valsa friesii* (teleomorph of *C. pinastri*) has been reported as an endophyte in senescent needles of *A. alba* in Switzerland (Sieber-Canavesi and Sieber 1993). In the same study, senescent needles were also colonized by *R. macrospora*. In Italy, *Cytospora* canker was exclusively reported on fruit trees and deciduous trees (Aiello et al. 2019;

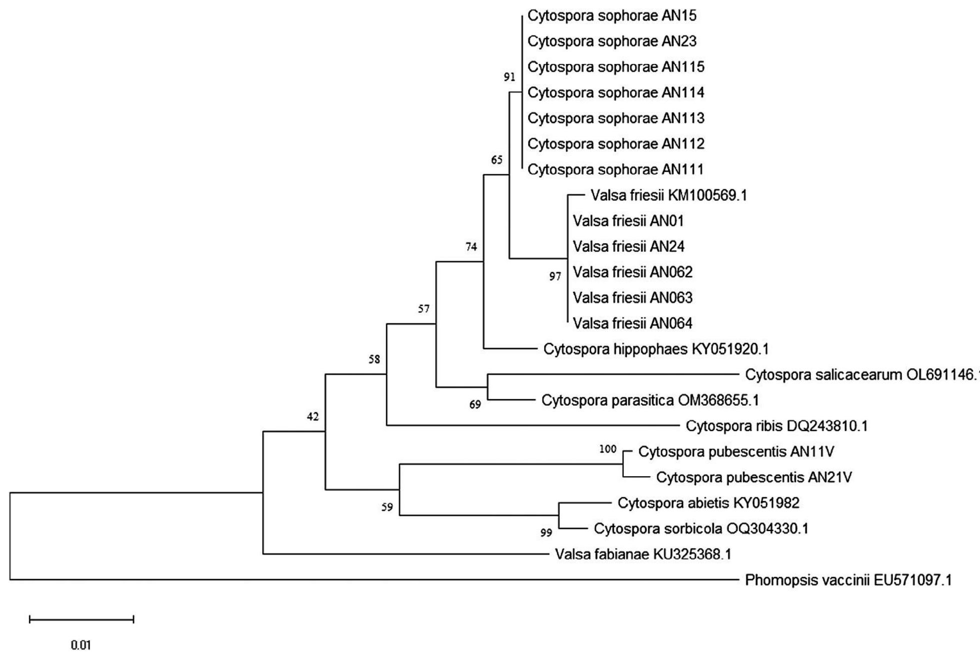

Fig. 6 Neighbor-joining (NJ) phylogenetic tree of *Cytospora* sp. and *Valsa* sp. isolates obtained from *A. nebrodensis* needles (marked with AN) based on 5.8S rDNA sequence. *Phomopsis vaccinii* was used as outgroup

Montuschi et al. 2006; Linaldeddu et al. 2016). In this work the presence of *Valsa friesii* and *C. sophorae* was observed on *A. nebrodensis*, representing the first example of such fungus-plant association on conifer in Italy.

Rhizosphaera oudemansii and *R. pini* were isolated from both reddened and green needles, although the frequency of isolation in the latter was lower. The genus *Rhizosphaera* has been considered as a little-known

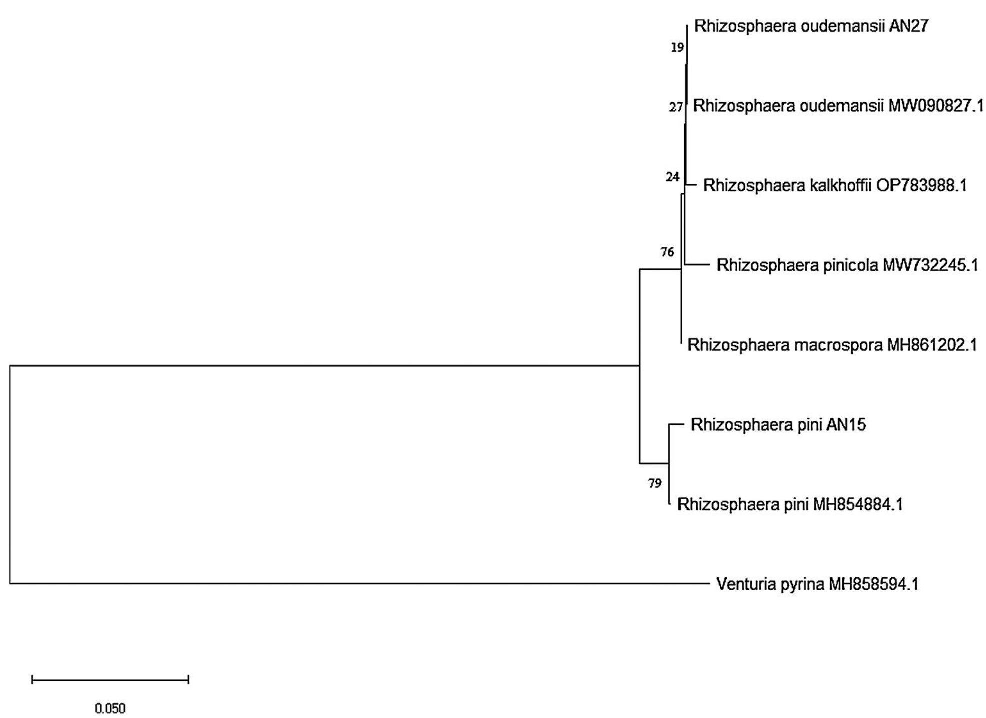

Fig. 7 Neighbor-joining (NJ) phylogenetic tree of *Rhizosphaera* sp. isolates based on 5.8S rDNA sequence. AN marks the isolates obtained from *A. nebrodensis. Venturia pyrina* was used as outgroup

Table 2 ITS sequences examined in this study

Species	Isolate	Host	Geographical origin	Genbank accession number
Cytospora sophorae	AN111	*Abies nebrodensis*	Italy	OQ626997
Cytospora sophorae	AN112	*Abies nebrodensis*	Italy	OQ626998
Cytospora sophorae	AN113	*Abies nebrodensis*	Italy	OQ626999
Cytospora sophorae	AN114	*Abies nebrodensis*	Italy	OQ627000
Cytospora sophorae	AN115	*Abies nebrodensis*	Italy	OQ627001
Cytospora sophorae	AN15	*Abies nebrodensis*	Italy	OQ641238
Cytospora sophorae	AN23	*Abies nebrodensis*	Italy	OQ641239
Cytospora pubescentis	AN11V	*Abies nebrodensis*	Italy	OQ678181
Cytospora pubescentis	AN21V	*Abies nebrodensis*	Italy	OQ678182
Valsa friesii	AN01	*Abies nebrodensis*	Italy	OQ627002
Valsa friesii	AN24	*Abies nebrodensis*	Italy	OQ627003
Valsa friesii	AN062	*Abies nebrodensis*	Italy	OQ627004
Valsa friesii	AN063	*Abies nebrodensis*	Italy	OQ627005
Valsa friesii	AN064	*Abies nebrodensis*	Italy	OQ627006
Rhizosphaera pini	AN15	*Abies nebrodensis*	Italy	OQ626995
Rhizosphaera oudemansii	AN27	*Abies nebrodensis*	Italy	OQ626996
Cytospora abietis	CPC 28394	*Abies alba*	Switzerland	KY051982
Cytospora ribis	CBS 128.36	*Ribes rubrum*	Netherlands	DQ243810.1
Cytospora salicacearum	AY41	*Vitis vinifera*	Turkey	OL691146.1
Cytospora parasitica	Shd194	*Malus domestica*	Iran	OM368655.1
Cytospora sorbicola	MEND-F-0113	*Prunus domestica*	Czech Republic	OQ304330.1
Cytospora hippophaes	CBS 259.88	*Hippophae rhamnoides*	Netherlands	KY051920.1
Valsa friesii	C2	*Abies alba*	Montenegro	KM100569.1
Valsa fabianae	P7_E3_1158	*Olea europeae*	Portugal	KU325368.1
Rhizosphaera pini	CBS:189.26	Not specified	Netherlands	MH854884.1
Rhizosphaera oudemansii	Ro29C-20GAa	*Abies alba*	Poland	MW090827.1
Rhizosphaera macrospora	CBS:208.79	Not specified	France	MH861202.1
Rhizosphaera kalkhoffii	AM8	*Picea abies*	Czech Republic	OP783988.1
Rhizosphaera pinicola	CAA1007	*Pinus nigra*	Spain	MW732245.1
Phomopsis vaccinii[a]	VAAT-PZ5	*Vaccinium macrocarpon*	Lithuania	EU571097.1
Venturia pyrina[a]	CBS:331.65	Not specified	Netherlands	MH858594.1

Sequences of the fungal species isolated from *A. nebrodensis* and indentified in this work are indicated with AN in the second column

[a]Represents the selected outgroup isolates

group of fungi associated with needle disease of conifers. *Rhizosphaera oudemansii* was found associated with a needle cast of *Abies pinsapo* in Spain (Martínez and Ramírez 1983). On *A. alba* needles, *R. oudemansii* was reported to behave as a specific primary saprophyte and occasionally as a weakness or opportunistic parasite (Gourbière 1986). However, the fungus may act as a pathogen collaborating with other biotic or abiotic factors. Gourbiere and Morelet (1979) clarified the taxonomy of the genus based on the cultural features of isolates and the ecology of *R. oudemansii* on *A. alba*. However, *Rhizosphera* sp. distribution is probably very little known since it has only been reported in The Netherlands, France, Germany and USA on needles of *A. alba, A. cephalonica* Loudon, *A. grandis* (Douglas ex D. Don) Lindl., *Picea glauca* (Moench) Voss, *P. omorika* (Panč.) Purk, *Pseudotsuga menziesii* (Mirb.) Franco and *Tsuga heterophylla* (Raf.) Sarg. (Butin and Kehr 2000; Kowalski and Andruch 2012; Hansen et al. 2018).

In this study of *Abies nebrodensis, Cytospora* and *Rhizosphaera* fungi were isolated from both symptomatic and green needles, suggesting an endophytic behavior for both. In the study of Sieber-Canavesi and Sieber (1993), *C. pinastri, R. oudemansii* and *R. macrospora* frequently isolated in senescent needles (partly brown or entirely brown) of *A. alba* Mill., were considered as 'transition fungi', i.e. endophytic fungi capable of changing their habit towards saprophytism. They display a different habit from purely endophytic fungi, capable of colonizing only green needles and from the purely saprophytic fungi isolated exclusively from litter needles. It is not clear whether these fungi play a role in needle necrosis in *A. nebrodensis* by interacting with various environmental stresses, or whether they become saprotrophs in dying needles. Transition fungi are not true pathogens, capable of killing healthy tissue, nor they are true saprophytes capable of decomposing dead tissue. It can be hypothesized that *C. pinastri, R. oudemansii* and *R. pini* already present in green needles, contribute to the necrotic process by interacting with various weakening factors, and favour senescence and fall of weakened needles that are no longer functional. The presence of fungi such as *Cytospora* and *Rhizoshaera* on *A. nebrodensis* in the southern Europe, combined with their reports on *A. alba* in Switzerland, on *A. pinsapo* Boiss. in Spain and on *A. concolor* in Serbia suggests a stable association of these fungi with European firs. *Cytospora* sp. have also been reported as causal agent of cankers on spruces and other conifers in USA (Kamiri and Laemmlen 1981; Jacobi 1994), but none of the *A. nebrodensis* trees examined showed the presence of cankers on branches or trunks.

Phylogenetic analysis of *Cytospora* isolates obtained in this study showed a clustering that was similar to that reported by Norphanphoun et al. (2017), where *C. sophorae* and *V. friesii* appeared genetically closer than *C. pubescentis* and *C. abietis*. On the contrary, the genetic distances obtained for *Rhizosphaera* species in this work are not fully coherent with the taxonomy reconstruction showed in Monteiro et al. (2021) where *R. oudemansii* and *R. pini* clustered in the same clade, resulting more distant from *R. kalkhoffii.* Such differences could be due to the diverse geographical origin (Korea) and host plant (*Pinus densiflora* Siebold & Zucc.) the isolates of Monteiro et al. (2021) come from. Further analysis need to be done by sequencing other genes to clarify this discrepancy in the results obtained.

Except for the Basidiomycete *Crepatura ellipsospora* which has been reported as a wood inhabiting fungus in China (Ma and Zhao 2019), all the isolated taxa are Ascomycetes (mostly in the asexual form), reported in the literature as opportunistic (weak) pathogens, saprotrophs or endophytes. *Thysanophora penicilliodies*, a fungus isolated from *A. nebrodensis* in the present study with a frequency of 4.4% on reddened needles, but not in green needles (see Fig. 4), was reported as a colonizer of litter needles of *A. alba*, as a typical saprotroph (Sieber-Canavesi and Sieber 1993). Despite *Lachnellula calyciformis* generally being regarded as a saprobe on conifers in USA, Canada, Asia, Europe and also associated with damaging cankers on *Pinus contorta* Douglas ex Loudon in Denmark (Minter 2005), in this work a higher frequency of isolation was obtained from green needles. This might suggest its endophytic phase on *A. nebrodensis,* inhabiting the healthy needles of trees before the appearance of needle-blight symptoms. Being generally reported on many broadleaved trees from north-eastern Europe to the Mediterranean, the presence of *Apiognomonia errabunda* in the needles of *A. nebrodensis* underlines the plasticity of this fungus and the ability to colonize hosts other than deciduous trees. Its presence on *A. nebrodensis* is probably favored by the proximity of *F. sylvatica* trees with which it is commonly associated (Viret and Petrini 1994; Sieber 2007) and by its spreading capacity (CABI Compendium 2023). The other isolated fungi were mostly found on reddened needles and are reported as ubiquitous fungi living in various habitats and hosts as saprobes or weak pathogens as the case for *Epicoccum nigrum,* and *Grovesiella abieticola, Phacidium* sp. and *Alternaria* sp. (Sieber-Canavesi and Sieber 1993; Vainio et al. 2017).

In the'Piano Noce' nursery, a similar scenario was observed for the symptomatic needles of *A. nebrodensis* potted plants where *Valsa friesii* and *Rhizosphaera pini* were the most frequently isolated fungi from reddened needles. In a recent work, *Rhizosphaera kalkhoffii* was found as the dominant fungus in *Pinus edulis* Engelm. seeds indicating its vertical transmission from the mother plant (Deckert et al. 2019). Likewise, the same behavior could be suggested for *R. pini* and *V. friesii* in this work. They were the most abundant fungi found in reddened needles sampled in the nursery. These plants are open pollinated seed progenies obtained from the fertile trees of *A. nebrodensis* in the natural population. Therefore, a vertical transmission of these fungi through seeds may have occurred. Direct transmission of the inoculum from the surrounding vegetation would be also possible, as around the nursery some planted *A. nebrodensis* trees are actively growing along with other conifer species.

Indeed, the assemblage of fungal taxa found in the 'Piano Noce' nursery seemed to be also related to the tree adult species growing inside and around the nursery area and in the ecosystem around represented by cypresses, pines, cedars, holm oaks, etc. and representing a significant source of inoculum. This is the case of *Strasseria geniculata, Alternaria alternata* and *Cytospora pubescentis* which are usually isolated from conifers and oak trees (James and Woo 1987; Pan et al. 2021; Tanney et al. 2023).

Unlike what is reported in the IUCN report on *A. nebrodensis* (Pasta and Troia 2017), the surveys in the 'Piano Noce' nursery have shown substantial good growth conditions of the plants and that the disorders observed have a relatively low and acceptable incidence among progenies (less than 8%). However, without a doubt, raising of seedlings is made difficult by the poor germinability due to the high rate of empty and not vital seeds (Jouini et al. 2023), the irregular fructification and the high mortality rate of seedlings in the early stages of growth

(Frascella et al. 2022). Although a good state of health has been found on the plants in the nursery, a regular and careful health surveillance in the nursery is required to prevent invasive pathogens from being introduced into the natural population with the planting of new reforestation nuclei (Chavez et al. 2016). Therefore, maintaining a high hygiene level in the nursery assigned to the propagation of *A. nebrodensis* is an essential measure to support the population of this species (Parke and Grunwald 2012). At the same time, it is necessary to carry on the monitoring of the health status of the natural population itself, keeping a constant eye on disorders and associated pathogens that could be exacerbated by climate change. This could assist in managing prompt mitigation and control measures.

Conclusions

To the best of our knowledge, this is the first detailed report on the health state of the trees in the *A. nebrodensis* relic population, except of a survey on scale insect fauna without evidence of pathogenic effects (Mazzeo et al. 2017).

Surveys on the health status of *A. nebrodensis* have brought to light new information about potential stress factors while providing knowledge for a better management strategy and conservation of this species. The needle reddening and blight symptoms reported in the present survey have been historically observed in the *A. nebrodensis* population but have never been associated with decline, whole crown defoliation or depletion of health state of plants. Indeed, the general good health state of *A. nebrodensis* trees previously reported by Raimondo and Schicchi (2005), encountered a positive vegetative growth during years and depicted an efficient example of adaptation of the species to the strict environmental condition it lives in.

This work shed light on Ascomycetes associated to needle reddening and blight symptoms in *A. nebrodensis*, underlining *Valsa* sp. (*Cytospora* sp.) and *Rhizosphaera* sp. as the prevailing fungi in both the natural population and the nursery. Their presence in green needles suggests that they can live as endophytes, resuming growth when needles are affected by environmental stressors, increasing their biomass while needles are still attached to the tree. As already proposed by Sieber (2021), their only goal seems to be in "pole position" when the leaf tissues die and are available for degradation.

This investigation allowed to exclude the involvement of aggressive pathogens as the cause of needle-disorders. Actually, all isolated fungi are classified as weak pathogens, endophytes or saprophytes, whose development on the symptomatic needles is associated with environmental disturbances to which the trees are subjected to in their natural habitat, such as wounds caused by herbivores, wind or hail. It would be likely suggested that the fungal species most frequently isolated from reddened needles are not dangerous for *A. nebrodensis* survival and neither represent a biotic constraint for the species.

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Data availability The data used in the current study are available on reasonable request from the Coordinator of the LIFE4FIR project Dr. Roberto Danti roberto.danti@ipsp.cnr.it.

Declarations

Ethical approval This article does not contain any studies requiring ethical approval.

Conflict of interest The authors confirm that they have no conflict of interest.

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