LIFE4FIR – Project LIFE18 NAT/IT/000164

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

Report on the effective production of mycorrhizal Abies nebrodensis seedlings and on the improved health status of the seedlings of the local nursery of 'Vivaio Piano Noce´.

Action C4.2

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1. Introduction

Abies nebrodensis (Lojac.) Mattei, is a Sicilian endemism considered, according to IUCN criteria, as a "Critically Endangered" (CR) species. The relative level of extinction risk is determined by a process called Red-Listing, a species-based conservation assessment, which considers individual species either globally, nationally, or within a particular geographic region. The status of Critically Endangered species of *A. nebrodensis* is due to several factors: the high number of empty seeds, the low survival rate of the seedlings grown in tree nurseries, the low sexual performance of adult individuals, the genetic pollution due to frequent hybridization with other firs occurring in its habitat (*Abies alba* Mill., *Abies cephalonica* Loudon, and the relatively low presence of ectomycorrhizal associations. Monitoring and assessment programs are needed for providing broad overviews to help strategic and tactic planning development. Although over the years several conservation and restoration programs have been initiated, the natural population currently includes 30 mature trees located in Vallone Madonna degli Angeli, Monte Scalone, Monte dei Pini and, Monte Cavallo in the Madonie Natural Park (N. Sicily) (Mirabile et al., 2022). The reforestation activities are carried out by the "Piano Noce" forest nursery (Polizzi Generosa, PA), where thanks to the Life4fir project several reproduction and propagation measures have been developed. In particular, in this deliverable are reported the results of the activities carried out in the Action C4 of Life4Fir project "*Inoculation of Abies nebrodensis seedlings with ectomycorrhizal fungi for the in situ and ex situ conservation of the natural population of Madonie fir.*"

2. Procedures

2.1. Inoculation of *A. nebrodensis seedlings*

At the end of November 2021 artificial mycorrhization assays were initiated at the Piano Noce Nursery on *Abies nebrodensis* seedlings (1 year old). For this purpose, the Basidiomycete *Pisolithus arhizus* (Scop.) Rauschert 1959 (Figure 1), an epigeal gasteromycete known as an ectomycorrhizal symbiont in *Abies alba*, *Abies cephalonica* and *Pinus pinea* (Castellano and Trappe, 1991; Rincòn et al., 2001; Krajnˇa´kova´ et al., 2012), was used as source of propagules. This fungus sporulates abundantly almost all year round and produces a very high amount of basidiospores that germinate readily once in contact with seedling roots. These traits make it an ideal mutualistic symbiont that can also be used in artificial inoculation assays (Garbaye et al., 2006) and commercial ectomycorrhizal formulations.

Figure 1 – Basidiome of *Pisolithus arhizus*

Basidiomes of *P. arhizus* growing under *Eucalyptus camaldulensis* and *Pinus* spp. trees were collected and brought to the lab of the Department of Agricultural, Food and Forest Sciences (SAAF) of the University of Palermo. The basidiomes were dried in a universal dryer at 475 Watt at room temperature for 48 hours, in order to preserve spores for long storage periods. Spore suspensions (10^7 spores/ml) were prepared in sterilized distilled water 24 hours before seedlings inoculation and maintained at 4°C for 12-18 hours. Inoculation activities were carried out in the "Piano Noce" nursery applying the spore suspension at the root systems during the transplant (Figure 2). Control plants were treated with sterilized distilled water. Soon before the transplant, root length, height and collar diameter of seedlings was measured (Figure 3). After the inoculations, the seedlings were transferred in greenhouse and were daily irrigated to favour fungal colonization.

Figure 2 – Inoculation of *Abies nebrodensis* seedlings with spore suspension of *Pisolithus arhizus.*

Figure 3 – Seedlings (1 year old) of *Abies nebrodensis* while being transplanted.

Observations to monitor the growth and to detect differences between inoculated and non-inoculated seedlings were carried out at 6 months and 1 year after inoculation. The measured parameters were:

- length of the root system (as the length of the primary root);
- seedling height (as the distance from the collar to the apical bud);
- collar diameter;
- number of total root tips;
- percentage of active tips;
- mycorrhization index (IM, total tips number/root length).

For each survey (6 month and 1 year after inoculation), 40 seedlings (20 inoculated and 20 non-inoculated) were randomly collected, taken out of the pot. The root systems were washed under running water to remove soil and debris. For tips observations, three root fragments were taken from each root system and observed at stereoscopic microscope.~~:~~ F~~f~~or each fragment the total length was measured and the following traits were registered: total number of mycorrhizal tips; active tips, recognizable by their colour and enlarged felt-like shape; non-vital, necrotic and/or desiccated tips..

3. Results

The results of the measurements conducted on seedlings inoculated with the *P. arhizus* spore suspension and on non-inoculated seedlings are shown in Tables 1 and 2. Analysis of the growth parameters of the seedlings showed, in both surveys, that the inoculated ones had a more developed root system, and collar diameter and seedling height were higher than in the non-inoculated ones as well (Table 1; Figure 4 A-D). In most of the seedlings, moreover, the aerial part showed a darker green colour of the foliage and lateral primary branching, which were absent in the non-inoculated seedlings (Figure 5).

Observation of root tips showed an IM of 4.79 and 5.28, at the first and second surveys, respectively, for inoculated seedlings, and 4.47 and 4.58 for non-inoculated ones. Notable differences were found in the percentages of active apices (Figure 6A), which reached more than 80% in both surveys, in inoculated seedlings and about 50% in non-inoculated ones (Table 2). In the latter, in fact, a higher presence of inactive and necrotic apices was observed (Figure 6B).

	Inoculated seedlings		**Non inoculated seedlings**	
Parameters[a]	**6 months**	**1 year**	**6 months**	**1 year**
Root system lenght (cm)	22.9 ± 3.8	27.3 ± 4.6	18.9 ± 1.98	20.78 ± 2.48
Seedling height (cm)	8.9 ± 1.33	9.3 ± 1.93	7.65 ± 0.87	7.67 ± 1.03
Collar diameter (mm)	1.97 ± 0.33	2.4 ± 0.19	1.41 ± 0.39	1.73 ± 0.32

[a]parameters were measured on 20 seedlings for each survey.

Table 1 – Growth parameters detected on *Abies nebrodensis* seedlings at 6 month (T1) and 1 year (T2) after the inoculation.

	T1						T2					
Seedlings	**Total tips[a]**	**Active tips [a]**	**Non-active tips [a]**	**active tips (%) [a]**	**Root lenght (cm) [a]**	**IM**	**Total tips[a]**	**Active tips [a]**	**Non-active tips [a]**	**active tips (%) [a]**	**Root lenght (cm) [a]**	**IM**
Inoculated	90,75	80	10,75	88,2	19,1	4,2	109,9	95,3	14,7	86,7	20,7	4,7
Non-inoculated	70,5	35,3	35,2	50,1	15,8	2,2	77,2	36,7	40,6	47,5	17,1	2,2

[a] mean of 20 samples.

Table 2 – Analysis of root tips observed in inoculated and non-inoculated seedlings at 6 months (T1) e 1 year (T2) after the inoculation

Figure 4 – Measurement on inoculated and non-inoculated seedlings: A) inoculated seedlings 6 month after inoculation, C) inoculated seedlings 1 year after inoculation; B) non-inoculated seedlings 6 month after inoculation, D) non-inoculated seedlings 1 year after inoculation.

Figure 5 – Differences in vegetative development of the aerial part of inoculated seedlings (bottom row) and non-inoculated ones (top row).

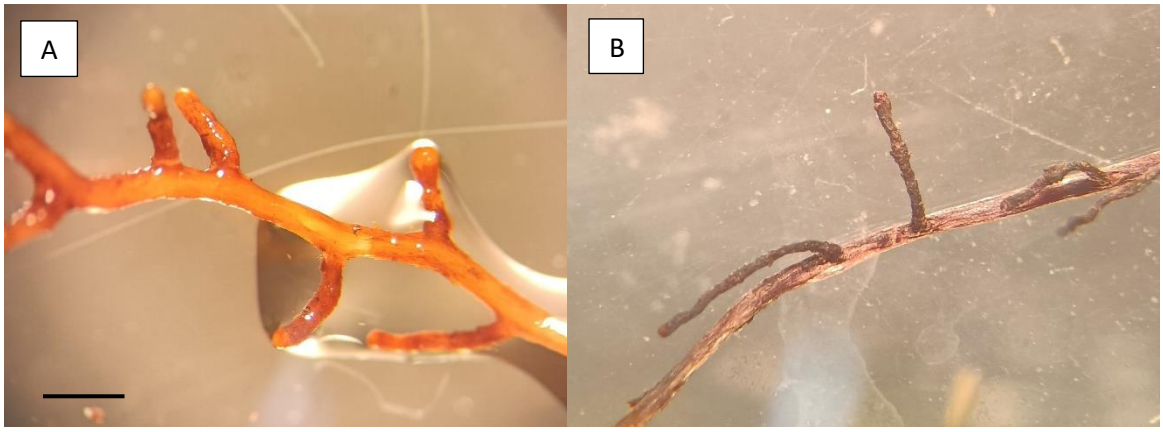

Figure 6 – Root tips observed at the stereoscopic microscope: A) active tips in inoculated seedlings; B) dead and necrotic tips in non-inoculated seedlings. Bar = 500 µm.

4. Concluding remarks

Mycorrhization trials conducted with *Pisolithus arhizus* spore suspension showed promising results on seedlings of *Abies nebrodensis*. Surveys conducted at 6 months and 1 year after inoculation, in fact, showed increased vigour in both the root system and the aerial vegetative part of inoculated seedlings compared with the non-inoculated ones. In addition, the greater presence of active tips in inoculated seedlings compared to non-inoculated ones confirms the success of mycorrhization. Considering these results, it can be stated that mycorrhization of *A. nebrodensis* seedlings can be used to produce more vigorous seedlings useful for reforestation programs. Faster growth and a more robust root system, in fact, could result in greater adaptation and survival of the seedlings in the transplanting phase, which represent one of the most delicate phases in *Abies nebrodensis* conservation.

5. References

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