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Seed vitality and fungal contamination in *Abies nebrodensis*

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ABSTRACT

Seeds of *Abies nebrodensis* were subjected to laboratory tests aimed to detect fungal contaminants and to obtain xenobiotic-free seedlings, by the use of different surface sterilising agents. Moreover, hot water at 60 °C was used to suppress any fungal microorganisms colonizing the inner tissues. *Alternaria alternata, Aspergillus flavus* and *Stemphylium vesicarium* were the most frequent fungal contaminants. Non-contaminated seeds showed germination values ranging from 0 to 36.4% depending on the applied sterilization protocol. Further analyses will be carried out to establish the influence of these fungi on the seed germination process and their relationship with seedlings of *A. nebrodensis*.

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Introduction

Abies nebrodensis (Lojac.) Mattei, is a Sicilian endemism considered, according to IUCN criteria, as a "Critically Endangered" (CR) species (Pasta and Troia 2017). 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 (Capotorti et al. 2020; Wagensommer et al. 2021). The status of Critically Endangered species of *A. nebrodensis* is due to several factors: the high number of empty seeds (Scialabba 2019), the poor health and 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, *Abies nordmanniana* (Stefen) Spach) (Pasta and Troia 2017), and the relatively low presence of ectomycorrhizal associations (Iotti et al. 2016). Monitoring and assessment programs are indispensable for providing broad overviews to help strategic and tactic planning development (Corona et al. 2010; Burrascano et al. 2011; Biondi et al. 2014). Although over the years several conservation and restoration programs have been initiated (Venturella et al. 1997), 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). Only 24 trees in this stand are capable of producing cones (Pasta et al. 2020). Moreover, fungi naturally infecting the embryos, depending on their symbiotic nature (pathogenic or mutualistic), could influence the vitality of seeds (Amza 2018; Shuwen et al. 2021).

In order to acquire more information on the seed vitality, on the level of seedborne fungal contamination and on the possibility to obtain xenobiotic-free seedlings, samples of seeds of *A. nebrodensis* were collected and subjected to the appropriate protocols.

Materials and methods

Seed sampling

Seed samples were collected from mature cones of three specimens of *Abies nebrodensis* (ID number 10, 13, and 21) in the nursery Vivaio Piano Noce, Polizzi Generosa (Palermo). In the laboratory, the seeds were first subjected to separation from mature cones and rachis and, after, wings were manually removed. For each specimen, the percentage of the weight of seeds on the total weight of cone structures was determined.

Seeds observation

To detect the presence of injuries or alteration of tissues, 100 seeds for each specimen were individually evaluated. For this purpose, the seeds, both intact and longitudinally dissected, were analyzed under a stereomicroscope (Zeiss, Oberkochen, Germany) to detect both brownings, rotting, or other symptoms and the status of the embryo.

Fungal isolation

Fungal isolation was carried out according to the "direct plating technique," which consists in placing the whole seeds directly on the growth substrate, after surface sterilization (Czaban and Wróblewska 2006; Tournas et al. 2006). In detail, 100 seeds for each specimen were subjected to two surface sterilization techniques and plated in Petri dishes (90 mm Ø)

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containing Potato Dextrose Agar (PDA, Condalab). Seeds were treated by: 1) immersion in 30% NaClO for 30 min (NaClO30); 2) immersion in 75% EtOH for 3 min (EtOH3) (Kolotelo et al. 2001). After the treatments, seeds were washed three times in sterile distilled water.

A third treatment was carried out on an equal number of seeds to suppress any fungal microorganisms colonizing the inner tissues. In particular, according to Kolotelo et al. (2001), seeds were immersed in sterile distilled water at 60° C for 55 min (heat treatment, HSW55); such treatment should not limit the vitality of the embryos.

All treated seeds were left to dry in an underflow chamber and five seeds per plate were put in Petri dishes and incubated at 25 ± 1 °C in the dark for one week. All the experiments were carried out in duplicate.

After the incubation period, the developing colonies were subcultured on new PDA plates and pure colonies were used for morphological and molecular analysis. The fungal isolation frequency (FIF) was calculated as follows: $FIF = \Sigma(Ni/Nt) \times 100$, where Ni was the number of seeds from which each fungal taxon was isolated and Nt was the total number of plated seeds (Boncaldo et al. 2010).

Seed germination test

For the three different treatments, non-contaminated seeds (seeds from which no fungal colonies have developed) were put in a sterile glass tube containing 10 ml of Water Agar (15 g of Agar - Oxoid, Milano, Italy - in 1 L of distilled water, WA) and incubated at room temperature under natural photoperiod to obtain seedlings under axenic conditions. Seeds were considered germinated when they had ca. 5-mm long radicles (Boncaldo et al. 2010).

Morphological identification of fungal colonies

Small portions of mycelial mass grown in PDA plates were mounted whit a drop of lactophenol solution (25 ml distilled water, 25 ml glycerin, 25 ml lactic acid, 25 g phenol crystals), added 0.01% of methylene blue. Microscopic observations were conducted using a light microscope (Axioskop; Zeiss, Oberkochen, Germany) coupled to an AxioCam MRc5 (Zeiss, Oberkochen, Germany) digital camera. Images were captured using the software AxioVision 4.6 (Zeiss, Oberkochen, Germany). All obtained fungal colonies, according to their macroscopic and microscopic features, were grouped in morphotypes (Ragazzi et al. 2001).

Molecular identification

One isolate for each morphotype was used for fungal DNA extraction from 7-day old pure colonies using the Extract-N-Amp™ kit (Sigma-Aldrich, St. Louis, USA) following the manufacturer's instructions. The internal transcribed species (ITS) region of the rDNA was amplified by polymerase chain reaction (PCR) on 4 µl of the freshly extracted DNA, mixed with 10 µl of the Extract-N-Amp PCR reaction mix (Sigma-Aldrich, St. Louis, USA), 1 µl of each primer at 10 µM and 4 µl of sterilized distilled water. The reaction was performed using the primer pair ITS1F/ITS4 (White et al. 1990; Gardes and Bruns 1993). PCR products were separated on 1.5% agarose gel and when positive were sent to Eurofins Genomics (Ebersberg, Germany) for sequencing with the ITS1F primer used in PCR. The obtained sequences were compared with sequences deposited in GenBank through BLASTn tool. Phylogenetic analyses were performed as described by Giambra et al. (2016). Alignments were made using ClustalW software (Thompson et al. 1997) and phylogenetic trees were automatically generated with the Neighbor-joining method using MEGA-X v 10.1.8. Confidence values for individual branches were determined by bootstrap test (1000 replicates).

Statistical analysis

Data on Fungal Isolation Frequency (FIF) were tested for differences using the one-way analysis of variance (ANOVA; general linear model) followed by post hoc Tukey's multiple range test applied for pairwise comparison. The statistical analysis was performed using XLStat® add-in ver. 2014.5.03 (Addinsoft, Paris, France) for Microsoft Excel®.

Results

Seeds observation

The weight of seeds on the total weight of cone structures was, in average, about 23% for each specimen. The analysis of the seeds of the three trees of *A. nebrodensis* showed that 47% of seeds collected from specimen ID 10, 45% from ID 21, and 24% from ID 13 had healthy embryos (Figure 1a), while the remaining had aborted embryos or completely browned endosperm (Figure 1b).

Fungal isolation

After one week of incubation the surface-sterilized seeds of *A. nebrodensis* showed fungal colonization with different values of FIF. In particular, 556 fungal colonies were obtained from all the analyzed seeds and each FIF is reported in Table 1. Among the three sterilization methods the highest decontamination was reached by the HSW55 treatment, that showed statistically significant differences compared to the other two. In particular, fungal growth was inhibited by 91–95%, 11–15% and 7–11% of seeds respectively for HSW55, NaClO30 and EtOH3.

Seed germination test

Only in a few cases axenic seedlings developed from non-infected seeds placed on WA since a very small percentage of them completed the germination process, as reported in Table 2.

Morphological identification of fungal colonies

The morphological observations on the isolated fungal colonies permitted us to identify them as belonging to the taxa

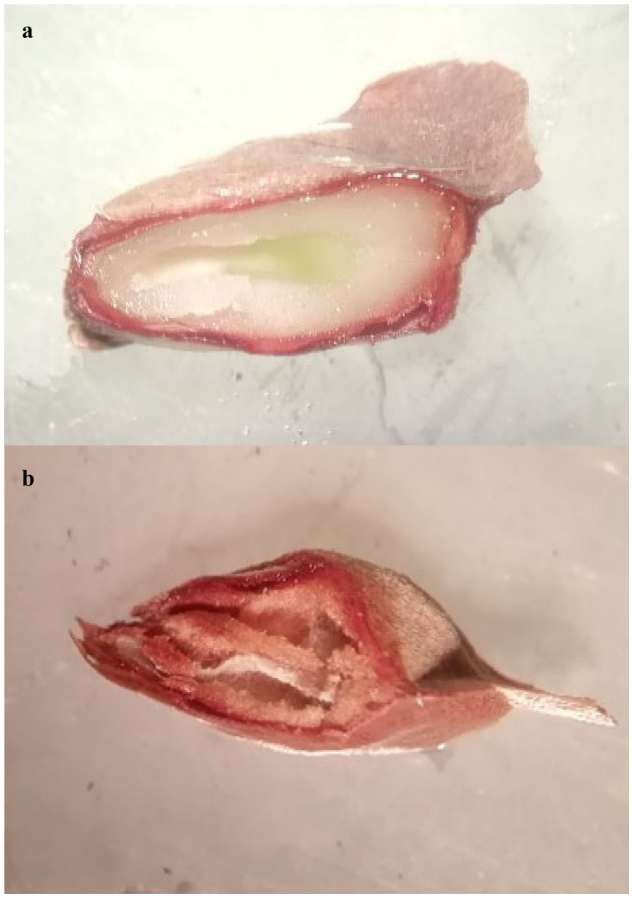

Figure 1. Longitudinal section of *Abies nebrodensis* seeds with healthy embryo and endosperm (a) and with abortive ones (b).

Table 1. Fungal isolation frequency (FIF) of *Abies nebrodensis* seeds after decontamination processes. Values are mean of two replication ± standard deviations.

	Seeds treatments		
	NaClO30	EtOH3	HSW55
Specimen ID		FIF (%)	
21	$89^{a} \pm 0.05$	$91^{a} \pm 1$	$8^{b} \pm 2$
10	$87^{b} \pm 1.5$	$93^{a} \pm 2$	$5^{c} \pm 2$
13	$85^{b} \pm 2$	$89^{a} \pm 1$	$9^{c} \pm 1.5$

FIF = Fungal isolation frequency. Data within a line followed by the same letter are not significantly different according to Tukey's multiple range test. $P \leq 0.05$. Lowercase letters indicate statistically significant differences for the three seeds treatments within the same specimen ID.

Table 2. In vitro germination of *Abies nebrodensis* seeds.

	Seeds treatment					
	NaClO30		EtOH3		HSW55	
ID	NC	GS	NC	GS	NC	GS
21	11	2 (18.2%)	9	3 (33.3%)	92	6 (6.25%)
10	13	1 (7.7%)	7	1 (14.3%)	95	10 (10.5%)
13	15	0 (0%)	11	4 (36.4%)	91	8 (8.8%)

NC = non-contaminated seeds; GS = germinated seeds; number in parenthesis indicates the percentage of germination.

Alternaria Nees, Syst. Pilze, Suppl., *Stemphylium* Wallr. and *Aspergillus* Michieli ex Haller section *Flavi*. In particular, *Alternaria* colonies, the most frequent amongst all the isolated colonies (60%), were characterized by a white-grayish mycelium at the margins and dark green mycelium in the inner zones. After nine days of incubation in PDA, colonies covered the entire plate surface. The color of mycelium varied from dark green to dark brown (Figure 2a). Microscopically, colonies showed the typical *Alternaria* asexual structures. The conidia appeared brown in color, ovoid in shape with a short conical beak at the tip, with 4–6 transverse septa, one or two longitudinal ones and ranging from 30 to 40 µm in length and from 10 to 13 µm in width (Figure 2b). Other fungal colonies (30%) after nine days of incubation on PDA were characterized by a slower growth (70 mm in diameter), yellowish to olive floccose and rough mycelium (Figure 2c). For these colonies, microscopically, the typical conidiophore of genus *Aspergillus* was recognized. The conidiophores were biseriate or uniseriate with globose vesicles ranging from 18 to 36 µm in diameter and smooth or finely rough conidia of 3.2–5.8 µm in diameter (Figure 2d). Based on these characteristics the colonies were identified as *Aspergillus* section *Flavi*. *Stemphylium*-like colonies (10% of the total isolated colonies), after nine days on PDA, reached 80 mm in diameter and looked with light brown mycelium (Figure 2e). Conidiophore appeared brown, oblong, or oval conidia, 21–35 × 12–15 µm in size with 1–6 transverse septa and 1–3 longitudinal septa (Figure 2f). Three isolates named AN1, AN2 and AN4, selected from each morphotype, were subsequently used for molecular and phylogenetic analysis.

Molecular identification of fungal colonies and phylogenetic analysis

Blast analysis of the three isolates AN1, AN2 and AN4 pointed out 99% of similarity respectively with *Aspergillus flavus* Link, *Alternaria alternata* (Fr.) Keissl. and *Stemphylium vesicarium* (Cooke) Wint. sequences present in GenBank database. The obtained ITS sequences were deposited (Table 3) and aligned with other selected sequences available from GenBank. Neighbour-joining trees based on phylogenetic analysis of the ITS region were obtained using three different datasets. The analysis showed that our isolates clustered with *A. flavus* (Figure 3a), *A. alternata* (Figure 3b), and *S. vesicarium* (Figure 3c) strains isolated in other studies (Woudenberg et al. 2015; Vu et al. 2019; Vaghefi et al. 2020).

Discussion

The presence of fungal contaminants in coniferous seeds was detected in the early 1900s and it was known that these infections could be correlated to damping-off and decay of seedlings (Rathbun-Gravatt 1931). Fungi are usually found on seedcoat or/and in the embryo and the colonization begins from seed lesions during its development in cones or during harvest (Boncaldo et al. 2010). In this study, we found that *A. alternata* (the most recurrent fungal species), *A. flavus* and *S. vesicarium* are frequently associated with *A. nebrodensis* seeds. In general, these three fungal species are known for their pathogenicity both towards agricultural plants and towards foodstuffs (Logrieco et al. 2009; Leach et al. 2020; Li et al. 2021), but nothing is known about their presence on *A. nebrodensis* seeds. In particular, *A. alternata* is one of the most important seed-borne pathogenic fungi, causing also leaf spot in over 100 host species of plant and

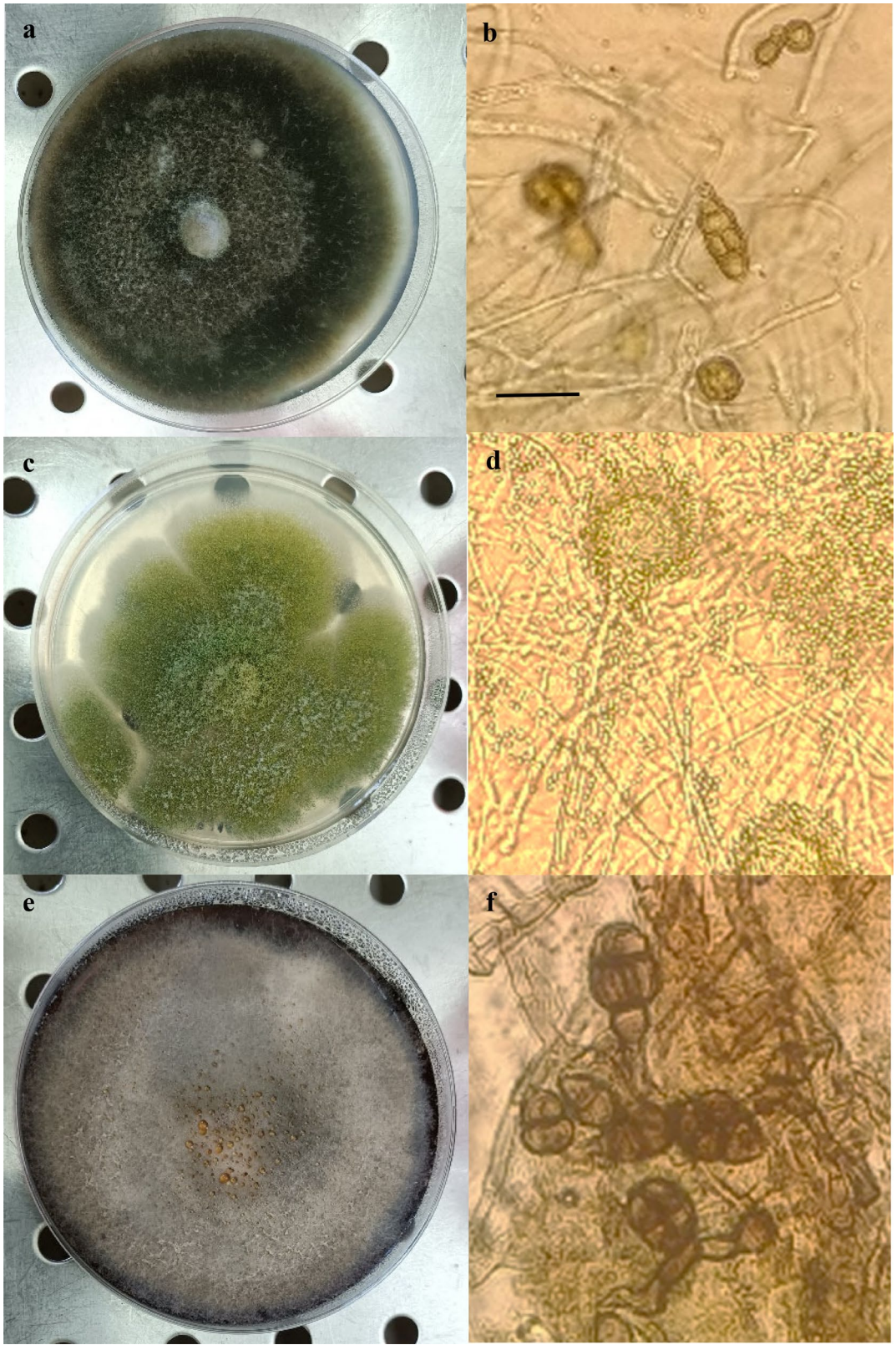

Figure 2. Morphological characteristics of fungal isolates: colonies of *Alternaria alternata* (a), *Aspergillus flavus* (c) and *Stemphylium vesicarium* (e) after nine days of incubation on PDA; *Alternaria alternata* conidia (b); conidiophore and conidia of *Aspergillus flavus* (d); *Stemphylium vesicarium* conidia (f). Bar= 30 µm.

post-harvest infections in various crops (Thomma 2003; Kustrzeba-Wójcicka et al. 2014). Previous studies highlighted the presence *A. alternata* on *Abies* sp. pl. seeds (Talgø et al. 2010). *A. flavus* is a well-known foodstuffs contaminant that causes infections and rots mostly in cereals, legumes and

Table 3. Fungal isolates obtained in this study.

Isolate	Species	GenBank Acc. number
AN1	*Aspergillus flavus*	OL823125
AN2	*Alternaria alternata*	OL823126
AN4	*Stemphylium vesicarium*	OL823128

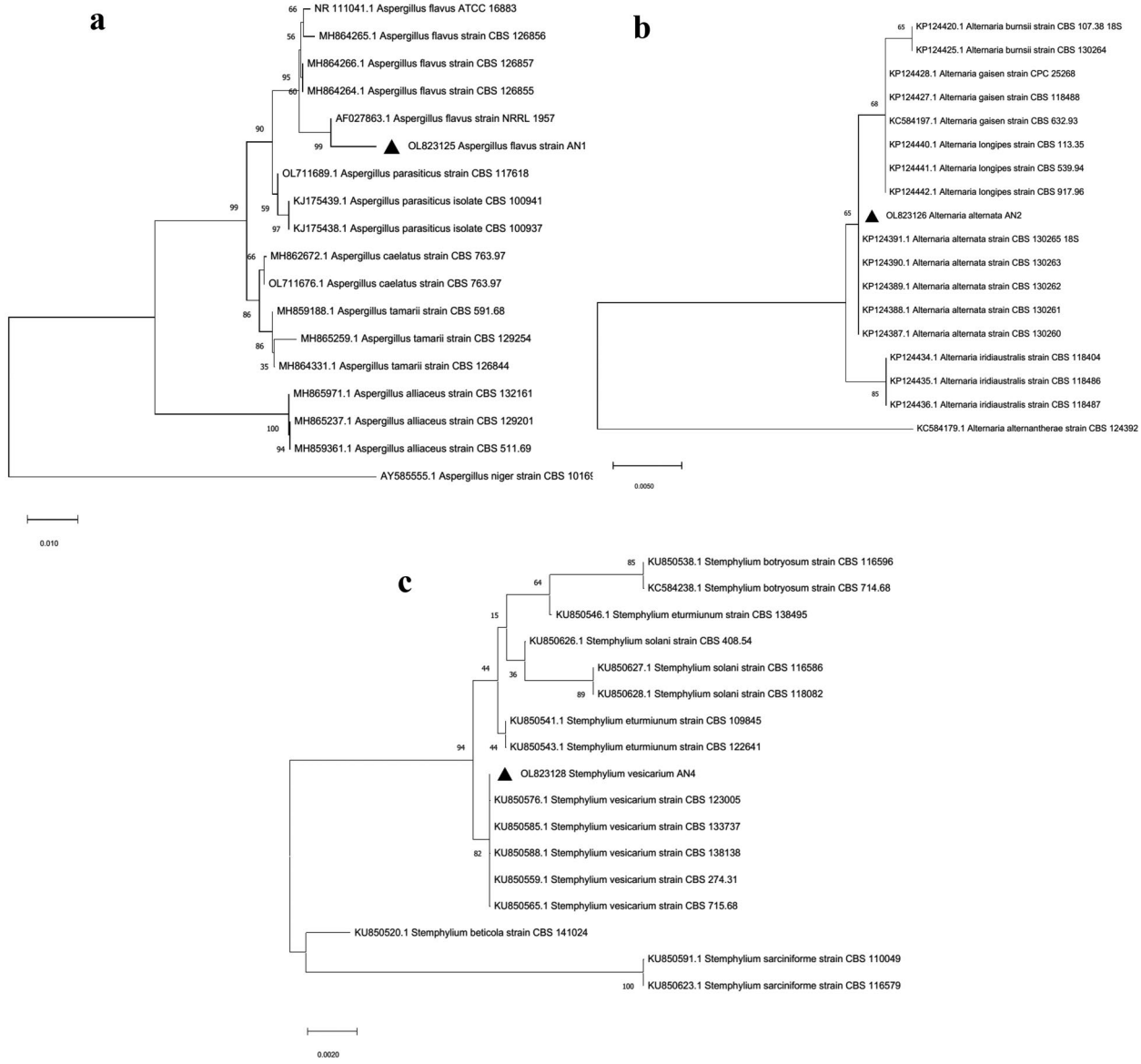

Figure 3. Neighbour-joining tree based on phylogenetic analysis of the ITS1-5.8S rDNA-ITS2 sequences of *Aspergillus* sec. *Flavi* (a), *Alternaria* (b) and *Stemphylium* (c) species. Bootstrap percentages calculated from 1000 resampling are indicated at nodes. GenBank numbers with black triangles represent the sequences obtained in this study and deposited at the GenBank Database.

nuts (Mirabile et al. 2021). It also infects products during post-harvest management and in particular environmental conditions can produce toxic substances known as mycotoxins dangerous both for human and animals (Arapcheska et al. 2015). In peanut (*Arachis hypogaea* L.) *A. flavus* is considered a virulent seed and seedling pathogen, greatly reducing seed germination (Ali et al. 2021). Furthermore, concerning conifers, Tournas et al. (2006) reported both *A. flavus* contamination in pine nuts and the risk of transmission of this pathogen through infected materials. Also, *S. vesicarium*, agent of the brown spot of pear (*Pyrus communis* L.), a disease of economic importance in fruit-growing areas of southern Europe (Alberoni et al. 2005; Rossi et al. 2005), was detected as a seed-borne pathogen on radish (*Raphanus sativus* L.) (Belisario et al. 2008) and onion (*Allium cepa* L.) (Aveling et al. 1993). *A. alternata, A. flavus* and *S. vesicarium* are reported for the first time as contaminating fungi in seeds of *A. nebrodensis*. Moreover, to our knowledge, this is the first report of *S. vesicarium* associated with coniferous seeds.

Among the two surface sterilization treatments, NaClO30 showed higher activity against fungal contaminants than EtOH3 (FIF= 85–89% and 89–91%, respectively), but the thermic treatment (HSW55) permitted to obtain the highest values of decontamination (FIF= 5–9%). These results suggest that the majority of fungal contaminants were located inside the seeds (endosperm and embryo tissues). Regarding the vitality of seeds in nature, as reported by Scialabba (2019), the highest germination capability of randomly selected seeds of *A. nebrodensis* is 25%, increasing to 61% in selected seeds with healthy embryos. In this study, the highest germination rate for all specimens (14.3–36.4%) was observed after EtOH3 treatment, followed by NaClO30 (0–18%), while HSW55 showed the lowest values (6–10%). Hence, NaClO30 and HSW55 treatments seem to confirm the negative effects on the germination of seeds, as reported in previous works

(Kasten Dumroese et al. 1988; Boncaldo et al. 2010). Therefore, to obtain xenobiotic-free seedlings new strategies of treatments must be employed; in particular, although the HSW55 method is the most effective in reducing fungal contamination, time and temperature of treatment needs to be reconsidered so as not to negatively affect the germination capacity of the seeds.

Further investigations are needed to establish the role of these fungi on the germination process of *A. nebrodensis* seeds and to define their symbiotic relationship (parasitism, neutral or mutualism, (Redman et al. 2001)) with the host during the early stages of growth.

Disclosure statement

No potential conflict of interest was reported by the authors.

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