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Cultivable mycoflora on bleached, decaying and healthy *Posidonia oceanica* leaves in a warm-edge Mediterranean location

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ABSTRACT

Marine fungi are widely distributed in the ocean, playing an important role in the ecosystems, but only little information is available about their occurrence and activity. Seagrass bleaching is also a neglected phenomenon that seems to be linked to warm environments, even though the causes are still to be defined. In this study, the cultivable mycoflora associated to the leaf conditions (bleached, necrotic and live) and section (from the base to the tip) in the seagrass *Posidonia oceanica* was investigated in a Mediterranean warm-edge location (Cyprus). A total of 17 Ascomycota species/taxon were identified and results highlighted that mycoflora composition changed significantly in relation to both the leaf condition and section. A few known pathogens of terrestrial plants were detected only on bleached leaves, but it remains unknown whether they have any direct connections with *P. oceanica* bleaching phenomenon.

1. Introduction

Marine fungi are widely distributed in the ocean, being detected on sediment, wood, algae, seagrasses, sponges, corals and calcareous substrates (Amend et al., 2012; Rama et al., 2014; Rédou et al., 2015; Abdel-Wahab et al., 2019; Poli et al., 2022). They play an important role in the ecosystems, contributing to the food web functioning, by activating the nutrient cycling (Hyde et al., 1998; Amend et al., 2019; Grossart et al., 2019; Varrella et al., 2021) and mediating species interactions (Poli et al., 2020). Variations in marine fungi distribution due to climate change are expected worldwide, with promotion of decomposers and degradative succession of fungi, a shift in fungal community composition in the favor of saprotrophs (Asemaninejad et al., 2017; Venkatachalam et al., 2019; Kumar et al., 2021). Such changes could be exacerbated in the most threated areas by climate change, such as the Mediterranean Sea, among the fastest warming ocean regions (Marbà et al., 2015), as corroborated by sea surface temperature (SST) patterns of the last decades (Nykjaer, 2009; Pastor et al., 2020). Although the Mediterranean Sea is an important marine biodiversity hot spot (Coll et al., 2010), the mycobiota diversity is still poorly investigated and there is little evidence of the association of some mycoflora with certain algae (Gnavi et al., 2017; Garzoli et al., 2018) and seagrasses (Cuomo et al., 1988; Panno et al., 2013; Vohník et al., 2016, 2017, 2019; Ettinger and Eisen, 2019; Pasqualetti et al., 2020; Poli et al., 2020). The limited research available up to now suggests that there is an urgent need of further knowledge about fungi distribution and their role.

Seagrasses are flowering plants widely distributed that have key functions in ecosystems, providing goods and services such as nursery grounds, sediment stabilization, oxygen production, CO_2 removal and nutrient cycling (Hemminga and Duarte, 2000). In the Mediterranean Sea, the endemic *Posidonia oceanica* (L.) Delile forms extensive underwater coastal meadows, a European Union priority habitat (Habitat Directive 92/43/EEC) threatened by multiple local stressors, such as temperature and UV increase, that interact with climate changes (Micheli et al., 2013). Past *P. oceanica* die-offs were detected subsequent to long and intense marine heat waves (Marbà and Duarte, 2010); however, more recent studies provided promising evidence about its resilience (Stipcich et al., 2022a, 2023a; Bennett et al., 2022) and thermal adaptation in terms of canopy structure and shoot morphology to future conditions of higher temperature and marine heat waves occurrence (Pansini et al., 2021; Stipcich et al., 2022b). In warmer environments, especially after summer, leaf necrosis (Stipcich et al., 2022b) and leaf bleaching are quite common: leaves usually fall down already brown colored (necrotic hereafter) and then, once beached and

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exposed to the sun light (the surface layer of the beached cumulus), they bleach. Interestingly, in some warm edged locations of the Mediterranean basin, leaves bleach while still attached to the live shoots (Fig. 1A) and bleached leaf wreck accumulates on the seabed (Fig. 1B). Nevertheless, what exactly causes seagrass bleaching underwater, and whether this phenomenon is triggered by physical (high temperature and solar radiation) and/or biotic (e.g., mycoflora) factors, is still unknown. Solar radiation has been considered one of the major factors associated to the decomposition of the seagrass *Zostera marina*, by changing the biochemical structure of the leaves and therefore the bioavailability of organic matter and by producing the degradation of the absorbing components of the leaves and causing leaf bleaching (Vähätalo et al., 1998). However, in summer 2022, the presence of bleached meadows and the accumulation of bleached leaves on the seabed in Konnos Bay (Carpus) was a localized phenomenon since other meadows of the Island, with similar environmental conditions, were healthy, suggesting the importance of some local factors in triggering the bleaching. The leaves of *P. oceanica* are characterized by high concentration of tannic acids, that might change due to physical factors, activating certain microbial activities on specialized mycobiota (Pasqualetti et al., 2020). In this context, the contribution of fungi to this phenomenon should be considered, since on land, the variation in species composition of fungal assemblages on live, decomposed and bleached leaves has been described as the result of fungal succession related to the decomposition stages (Osono et al., 2008, 2009; Hirose et al., 2013), and, similarly, the presence of certain fungi might also be associated to the seagrass bleaching. Different mycobiota composition was associated to leaves, roots, matte and rhizomes of *P. oceanica* (Panno et al., 2013; Poli et al., 2020), but determining if the fungal composition is particularly associated to the different condition of leaves (e.g., live, necrotic and bleached) still remains unsettled. In addition, since leaf bleaching and browning begins from the tip, differences in mycobiota between the basal and distal sections of the leaf could help determine the condition of association with the plant. The aim of the study was to isolate and identify the mycoflora associated with the different conditions and different sections of leaves in the seagrass *P. oceanica*. The hypotheses were that i) the mycoflora was more abundant in bleached and necrotic leaves (both decaying) compared to green leaves (healthy), since more decomposers are expected in bleached and necrotic leaves, and also ii) more abundant in the distal (and older) sections of the leaves (20 and 30 cm from the base) compared to those closer to the base (0 and 10 cm), since the distal section is the oldest section of each leaf and it is more exposed. The results are needed to identify eventual associations of mycoflora species with each leaf condition and section, and based on their identity, to allow formulating hypotheses on their role on the seagrass health condition. In addition, results will provide assumptions about the fungal composition on *P. oceanica* leaves in a climate change future scenario, where the presence of necrotic and bleached leaves might be more frequent due to higher temperature (Stipcich et al., 2022b).

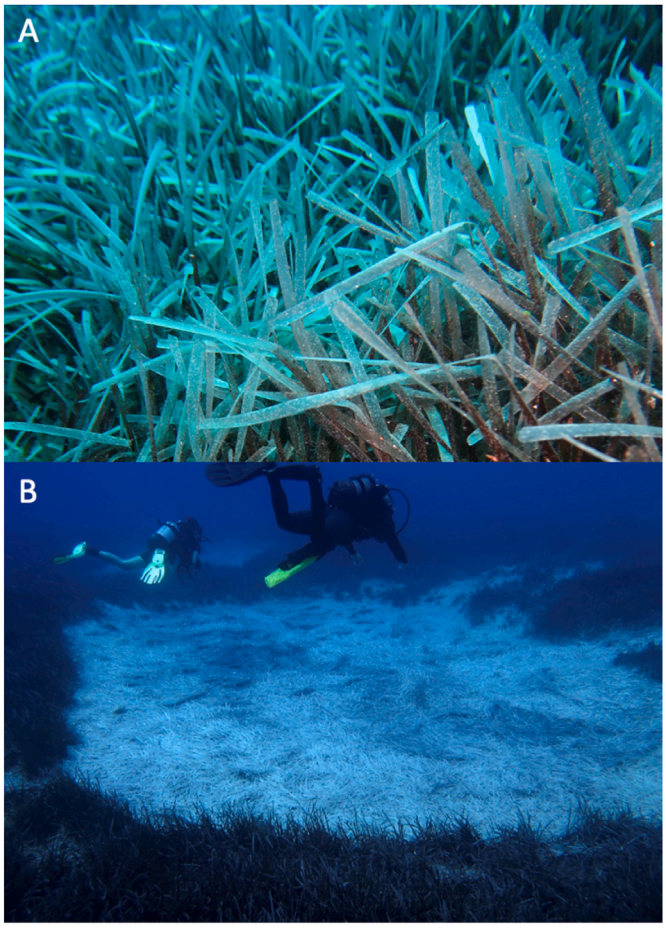

Fig. 1. A) Bleached leaves in the live meadow; B) bleached leaves accumulated on the seabed. Both pictures were taken in Konnos Bay, Cyprus, in July 2022.

2. Materials and methods

2.1. Study area and data collection

At the end of July 2022 shoots of *P. oceanica* were sampled in a warm-edge location (Bennett et al., 2022), Konnos Bay (N 34°59.174100, E 34°4.602300), South-East of Cyprus, where a meadow at 10 m of depth was selected. The mean seawater temperature in July 2022 (10 m depth) was 26.42 °C (measurements from data loggers HOBO Pendant Temp/Light 64 K Data logger, Onset Computer Corporation, USA) (Stipcich et al., 2023a). Green, white, and brownish leaves (each corresponding to the live, bleached, and necrotic leaf condition) were gently detached from a total of 21 shoots of the meadow (n = 7 for each leaf condition). In the laboratory, macro-epiphytes were removed by gently scraping the seagrass leaves with a razor blade and folded to be immediately placed in 50 ml tubes containing 15 ml of water agar (WA, Condalab, Madrid, Spain, prepared using seawater), in order to keep the material suitable during the transport to the laboratory (in Sardinia, Italy) for the fungal isolation. From each leaf, a 1 cm^2 piece of tissue was cut from 0, 10, 20, and 30 cm from the base, obtaining four segments per each leaf (84 segments in total). The leaves were placed on Potato Dextrose Agar (PDA, Condalab, Madrid, Spain, prepared using seawater) Petri dishes and incubated at 25 ± 1 °C for 12 days (depending on the growth rate) with 12 h photoperiod to facilitate the glorification of fungi. The emerging fungal colonies were transferred to fresh PDA plates supplemented with Streptomycin sulphate 100 mg/L and Tetracycline 100 mg/L, from which monosporium or monohyphal cultures were prepared. For each taxon two representative cultures were selected based on colony morphology (color and shape) as well as on the morphology of the reproductive structures observed under microscope.

Extraction of DNA was carried out following the protocol described by Aljanabi and Martinez (1997). The DNA extracted was amplified by PCR, using universal primers ITS1 and ITS4 which amplify a fragment of about 600 pb of the internal transcribed spacer regions 1 and 2 (ITS 1 and ITS 2) located before and after the 5.8 S rRNA gene (White et al., 1990). Briefly, a PCR reaction tube was composed as follows: 1xbuffer *Taq*polimerase, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer, 2 U *Taq*polymerase (all reagents were supplied by Invitrogen), 50–100 ng DNA, in 50 μl final volume.

The PCR conditions of amplification consisted of an initial denaturation step (94 °C for 3 min), followed by 33 cycles of denaturation (94 °C for 45 s), annealing (48 °C for 1 min), extension (72 °C for 90 s), and of a final extension (72 °C for 7 min). The PCR products were purified using a PureLinkTM Quick PCR Purification Kit, Invitrogen, following the

manufacturer's instructions and their concentration was then determined with the NanoDropOne (Thermo Scientific). About 20 ng of DNA template were collected and sent to BMR-Genomics, spin-off company of the University of Padova (Italy) for the sequencing service. For each isolate, both forward and reverse strands were sequenced, and the final sequences obtained by their combination, were compared to the sequences available in the NCBI database using the BLAST programme where the species identification was based on similarity >99.5%.

2.2. Data analyses

Differences in the presence/absence of different fungal taxon between leaf condition (live, bleached and necrotic) and leaf section (0, 10, 20 and 30 cm from the base of the leaf) were estimated by two different one-way PERmutational Multivariate Analyses Of Variance (PERMANOVA; Anderson, 2014) using a similarity matrix based on the Euclidean distance of untransformed data: one for the leaf condition (with three levels) and one for the leaf section (with four levels). *A posteriori* a pair-wise test was run to highlight the differences between levels of the same factor. The percentage contribution of the individual taxon to the diversity observed between groups was assessed by SIMilarity PERcentage (SIMPER) analysis. All the analyses were performed using PRIMER 7, PERMANOVA+ (Anderson, 2008).

3. Results

A total of 47 colonies (58.75% isolation success), belonging to 17 Ascomycota taxa, were isolated and identified from the different conditions of *P. oceanica* leaves (Table 1). Dothideomycetes and Sordariomycestes were the most represented classes with 76% of the organisms identified. *Penicillium*, *Fusarium* and *Cladosporium* were the most abundant genera, isolated from all the leaf conditions. In general, molecular analyses have not always given unambiguous evidence and in some cases two species were identified from the same sequence (Table 1). For the genus *Cladosporium,* the species were identified in two cases while in other three the sequence was not unequivocal (indicated as *Cladosporium* sp. 1, sp. 2 and sp. 3).

Nevertheless, seven species (*Alternaria infectoria*, *Apiospora arundinis/Periconia homothallica*, *Curvularia inaequalis, Fusarium oxysporum, Lasiodiplodia theobromae, Myriodontium keratinophilum* and *Penicillium citrinum*) were only recorded on bleached leaves (Fig. 2A and Table 1). Furthermore, bleached leaves displayed the highest number of fungal taxa (13), followed by both necrotic and live leaves (7) (Fig. 2A). Only two fungal taxa were associated with all the conditions of leaves (*Alternaria infectoria* and *Fusarium oxysporum*), two were in common between live and necrotic leaves (*Cladosporium* sp. 2 and *Cladosporium* sp. 3), two between necrotic and bleached leaves (*Aureobasidium melanogenum/A. pullulans* and *Penicillium citrinum*) and only one taxon was found in common between live and bleached leaves (*Cladosporium aggregatocicatricatum*) (Fig. 2A and Table 1).

There was no species found consistently among the leaf sections. Despite this variability, the highest number of taxa in common was found between the 0 and 10 (even though only two taxa: *Alternaria infectoria* and *Aureobasidium melanogenum/A. pullulans*) and between the 20 and 30 sections (two, *Apiospora arundinis/Periconia homothallica*; *Cladosporium* sp. 1) (Fig. 2B and Table 1).

A significant difference in terms of presence/absence of fungal taxa was found both between leaf conditions and leaf sections (PERMANOVA results, Table 2). The SIMPER analysis highlighted that the fungal taxa which contributed the most to the dissimilarities differed depending on the specific comparison (Table 3): 1) *Aureobasidium melanogenum/A. pullulans*, *Fusarium oxysporum* and *Lasiodiplodia theobromae* had a high contribution to the dissimilarities in the comparisons between necrotic leaves vs bleached leaves, 2) *Aureobasidium melanogenum/A. pullulans*, *Fusarium oxysporum* and *Cladosporium* sp. 3 between necrotic vs live leaves; 3) *Lasiodiplodia theobromae*, *Cladosporium* sp. 3 and sp. 1 and *Apiospora arundinis/Periconia homothallica* contributed most to the dissimilarities between the bleached and live leaves (Table 3).

4. Discussion

The mycobiota associated to the condition and sections of *P. oceanica* leaves was investigated in a warm-edge location in Cyprus. Overall, the results of this study were in accordance with previous findings, confirming that the fungi belonging to the phylum *Ascomycota* are well adapted to marine environments and represent the predominant marine mycoflora (Panno et al., 2013; Poli et al., 2020). Previous studies have identified the fungal taxa present in different parts of *P. oceanica* such as rhizome, roots, matte and leaves, highlighting a district specificity in terms of fungal load and number of species and determining the richness of the mycoflora associated to this seagrass (Panno et al., 2013; Gnavi et al., 2014; Poli et al., 2020). This study has evidenced a different kind of specificity since the mycobiota composition changed significantly in relation to both the leaf condition and leaf sections, even though for

Table 1
Fungal taxa isolated from *P. oceanica* leaves. Absence = 0; presence = 1.
**Cladoporium* sp.1 = *C. halotolerans; C. parahalotolerans; C. endophyticum; C. sphaerospermum*
× *Cladoporium* sp.2 = *C. allicinum; C. herbarum; C. sinuosum; C. floccosum; C. macrocarpum*
× *Cladoporium* sp.3 = *C. tenuissimum; C. cladosporioides; C. subuliforme; C. acalyphae; C. delicatulum.*

Accession code	Taxon	Leaf condition			Leaf section			
		live	bleached	necrotic	0	10	20	30
OR206523	*Alternaria alternata*	0	1	0	0	0	1	0
OR206526	*Alternaria infectoria*	1	1	1	1	1	0	0
OR206522	*Apiospora arundinis/Periconia homothallica*	0	1	0	0	0	1	1
	Aspergillus tabacinus/A. versicolor/A. sydowii	0	1	0	0	1	0	0
OR206514	*Aureobasidium melanogenum; A. pullulans*	0	1	1	1	1	0	0
OR206529	*Chaetomium elatum; C. rectangolare*	1	0	0	0	0	1	0
OR206517	*Cladosporium aggregatocicatricatum*	1	1	0	0	0	1	0
OR206520	*Cladosporium* sp. 1*	0	1	1	0	0	1	1
OR206524	*Cladosporium* sp. 2*	1	0	1	0	1	0	1
OR206527	*Cladosporium* sp. 3*	1	0	1	1	0	1	1
OR206518	*Curvularia inaequalis*	0	1	0	0	0	0	1
OR206519	*Fusarium fujikuroi species complex*	0	1	0	0	1	0	0
OR206525	*Fusarium oxysporum*	1	1	1	1	0	1	0
OR206515	*Lasiodiplodia theobromae*	0	1	0	0	1	1	1
OR206516	*Myriodontium keratinophilum*	0	1	0	0	1	0	0
OR206528	*Penicillium chrysogenum/P.rubens/P.commune*	1	0	0	1	0	0	0
OR206521	*Penicillium citrinum*	0	1	1	1	0	0	1

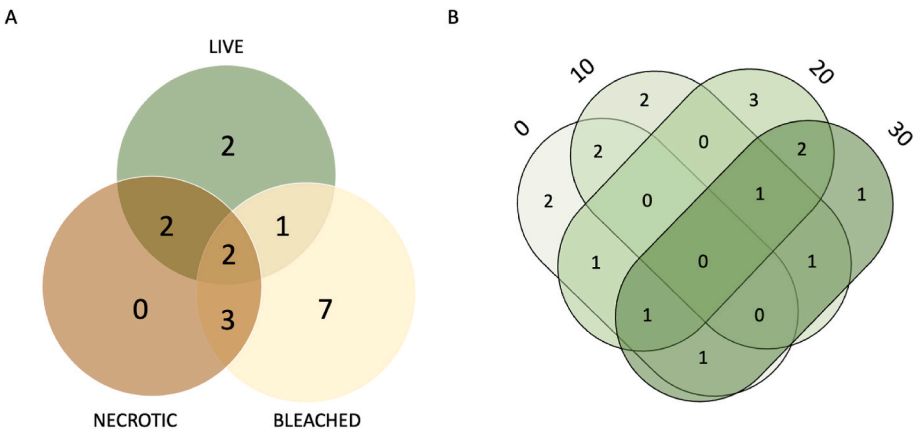

Fig. 2. Venn diagrams showing the total number of taxa and shared taxa among A) different condition of leaves and B) different leaf sections (0, 10, 20 and 30 cm from the base).

Table 2
Statistical analyses. One-way PERMANOVAs on the presence/absence of fungal taxa testing in leaf condition (three levels) and leaf section (four levels). * indicates significant results. Below, results of the pair-wise test. L = live leaf; B = bleached leaf; N = necrotic leaf.

PERMANOVA	Leaf condition	Leaf section
	Pseudo-$F_{2,157}$	*Pseudo-$F_{1,156}$*
Presence/absence of fungal taxa	2.55*	1.74*
Pair-wise test		
Leaf condition	L≠N≠B	
Leaf section	no alternative hypotheses	

Table 3
SIMPER analysis results based on the average abundance: the most contributing fungal taxa to dissimilarities between *P. oceanica* leaves condition (contribution in percentage, 50% cut off).

Species	Bleached av. Abund	Necrotic av. Abund	Contribution %
Aureobasidium melanogenum/A. pullulans	0.02	0.15	22.56
Fusarium oxysporum	0.02	0.08	13.43
Lasiodiplodia theobromae	0.09	0.00	12.13
Cladosporium sp. 1	0.04	0.02	8.33
Species	Bleached av. Abund	Live av. Abund	Contribution %
Lasiodiplodia theobromae	0.09	0.00	16.68
Cladosporium sp. 3	0.00	0.05	9.97
Cladosporium sp. 1	0.04	0.00	7.39
Apiospora arundinis/Periconia homothallica	0.04	0.00	7.39
Cladosporium aggregatocicatrum	0.02	0.02	6.95
Fusarium oxysporum	0.02	0.02	6.95
Species	Necrotic av. Abund	Live av. Abund	Contribution %
Aureobasidium melanogenum/A. pullulans	0.15	0.00	28.57
Fusarium oxysporum	0.08	0.02	18.49
Cladosporium sp. 3	0.02	0.05	14.31

these latter it was not possible identifying the fungal taxa mostly contributing to the differences. Regarding the leaf condition, only a few fungal taxa were found in common between bleached and necrotic leaves, between necrotic and live and between bleached and live leaves (3, 2 and 1, respectively), indicating most probably the result of the fungal succession related to the decomposition of the leaf (*i.e.* healthy leaf, necrotic and bleached). Successional aspects have already been highlighted in terrestrial plants, when the mycobiota was examined from live to dead leaves (Hirose et al., 2013). Additionally, the presence of specific fungal species on different leaf conditions confirms the ecological role played by these organisms on *P. oceanica* (Poli et al., 2020). An example can be represented by the presence of *P. chrysogenum*: this species was already found on live leaves of *P. oceanica* in the western Mediterranean basin rather than in matte (Poli et al., 2020), and its exclusive presence on live leaves in this study could support the hypothesis of a disease defense role in seagrasses as well, similar to patterns found on terrestrial plants (Chen et al., 2018). In fact, it has been reported that this species produces the Penicillium antifungal protein that inhibit the growth of several phytopathogenic fungi (Kaiserer et al., 2003). Further worth noticing is the presence of *A. pullulans* only in necrotic and bleached leaves: the species has been already found in marine environments and it has been thought to accelerate the decomposition of organic materials through the production of specific enzymes (Liu et al., 2009) that allow the fungus to degrade pectins (Pugh et al., 1963). On the other hand, this study has identified several fungal taxa that has never been associated to this seagrass: such apparent inconsistency could be due to the fact that the previous studies were focused on different parts of the plant, in different seasons and in the different areas of the Mediterranean Sea. To the best of our knowledge, this was the first time that mycoflora was investigated in different conditions and different sections of *P. oceanica* leaves, but moreover this was the first effort on the mycoflora isolated from a Mediterranean warm-edge population of *P. oceanica*. *P. oceanica* meadows can be found in warm, central and cool-edge regions of the Basin and evidence from the range of environmental conditions have highlighted that thermal regime affects plant morphology (Pansini et al., 2021; Stipcich et al., 2022b), biochemical composition and thus leaf nutritional value (Jiménez-Ramos et al., 2017; Stipcich et al., 2023b), and there is no reason for not hypothesizing an effect of the environmental conditions also on the mycoflora associated. In Australia elevated sea water temperature caused seagrass biomass loss and a change in bacterial community associated, enhancing the microbial decomposition of leaf detritus and also lignocellulose degradation (Trevathan-Tackett et al., 2017), so that a similar process could be expected in a Mediterranean warm-edge location. Lignin has a key role in improving the compactness and the resistance of terrestrial plants, but it was found also in seagrasses (Trevathan-Tackett et al., 2017), such as *P. oceanica* (Larkum et al., 2018), where contributes to the longevity of the tissues by protecting against microbial attacks (Klap et al., 2000). Only fungi with specific ligninolytic activity, such as those belonging to the Ascomycetes, (Klap et al., 2000; Panno et al., 2013) might be more present in an environment with fluctuating conditions. The eastern basin of the Mediterranean Sea is warming faster than the western basin (Nykjaer, 2009) and here *P. oceanica* leaf massive bleaching, and the subsequent piling of

bleached leaves in extensive areas at the study site, might be due to the increased temperature, since also in terrestrial plants bleached leaves are found especially in tropical sites (Osono et al., 2009). The role of solar radiation is presumed to be one of the major factors regulating the decomposition of seagrasses (Vähätalo et al., 1998), and high temperature environments are also characterized by high solar radiation, so that also climate change is expected to affect both of them (Li et al., 2012; Ohunakin et al., 2015), and disentangling their effects on seagrasses seems impossible only using correlative approaches. However, this phenomenon has not been described for *P. oceanica* before, and whether it is common or occurring only lastly in the eastern Mediterranean remains unclear. In this study, most of the fungal species isolated for the first time in *P. oceanica* were among those identified on bleached leaves. Most of them are plant pathogens (Logrieco et al., 2009; Moslemi et al., 2017; Salvatore et al., 2020a; Hopkins et al., 2023), and the pathogenic behavior seems to increase when the plant is under stress (Salvatore et al., 2020b). However, in this study none of the taxa detected on bleached leaves can be pointing as driver of the bleaching without any pathogenicity test; in fact, further inoculation experiment should be carried out to satisfy the Koch's postulates. Therefore, we could only speculate that the presence of these taxa may enhance the bleaching phenomenon concurrently with the environmental conditions at the study area and the stress level of the plants, even though it remains a hypothesis to be further tested. In a future warming scenario, the solar radiation or the increasing temperature might affect the biochemical composition of the leaves, and therefore their condition, which, consequently, might affect the mycobiota on *P. oceanica* associated. For instance, *L. theobromae*, that here was only found in bleached leaves, is a prevalently spread species in tropical and subtropical areas (Salvatore et al., 2020b) and past concerns about its propagation into temperate regions with the global warming may have already come true: this is now confirmed to be found associated to the seagrass *P. oceanica* in a warm-edge population, with higher temperature than in other areas of the Mediterranean basin. This concern can also be expanded into other fungal taxa that, until now, have been found only in warm-edge locations but in the future, due to high temperature or/and irradiance, may be present in other areas of the Mediterranean Sea.

This study, that certainly needs to be implemented by the use of further markers (to increase the taxonomic resolution of the investigation) and other cultivation approaches, represents an attempt to the understanding of the fungal composition on different condition of *P. oceanica* leaves emphasizing the bleaching phenomenon and how the mycoflora associated might change in a future climate warming scenario; this information will be useful not only to evaluate the good seagrass state, but also to estimate the ecological relevance that the mycoflora may play in the oceans and the potential use of fungal species as sources of bioactive compounds (Poli et al., 2020).

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CRediT author statement

Patrizia Stipcich: Conceptualization; Formal analysis; Investigation; Writing – original and revised draft, **Virgilio Balmas:** Methodology, Formal analysis, **Carlos E. Jimenez:** Conceptualization, **Safa Oufensou:** Methodology, Formal analysis, **Giulia Ceccherelli:** Conceptualization; writing – original and revised draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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