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ORIGINAL PAPER

The formation of aegagropiles from the Mediterranean seagrass *Posidonia oceanica* (L.) Delile (1813): plant tissue sources and colonisation by melanised fungal mycelium

Laurence Lefebvre[1] · **Philippe Compère**[2] · **Sylvie Gobert**[1,3]

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Abstract
Aegagropiles are conglomerations of fibre debris from *Posidonia oceanica* meadows that are frequently found along Mediterranean beaches, but the plant organ from which these fibres arise remains unknown. In this study, a histological comparison of *P. oceanica* organs from 3 shoots with the structure of aegagropile fibres showed that most of them arise from leaf sheaths and rhizomes, suggesting that they are degradation products from the "matte" rather than from the leaf litter, which is mainly composed of detached leaf blades. Moreover, fungal hyphae, micro-sclerotia and typical degradation traces were found in the peripheral tissues of living *P. oceanica* organs, as well as in degrading aegagropiles. We assume, by comparing Vohník's observations and the observations made in this study, that these endophytic fungi and degradation traces might be attributed to a dark septate endophyte (DSE) in the Aigialaceae (Pleosporales), *Posidoniomyces atricolor*, which was recently described as an endosymbiont in *P. oceanica* roots. It constitutes one of the most important microorganisms by abundance that degrade *P. oceanica* tissues within the matte and give rise to the different fibre types in aegagropiles. This study shows that the proliferation of fungi causes organ degradation in *Posidonia,* starting early in living *P. oceanica* plants, continuing in the matte and, probably, in the leaf litter. The DSE plays a much more important role than that of a simple plant endosymbiont; its omnipresence within *P. oceanica* (and the degradation of the middle lamella and cell death during proliferation) causing the degradation of various *Posidonia* organs also contributes to the enrichment of the 'matte' compartment of this ecosystem, notably favouring nitrogen retention in its chitinous walls.

Keywords *Posidonia* · Aegagropiles · Marine plant histology · Marine fungus · Microbial degradation

Introduction

On Mediterranean beaches, it is common to find egg- or ball-shaped clumps of plant debris. These balls, called *Posidonia oceanica* aegagropiles, sometimes cover large areas of Mediterranean beaches, especially after storms. The aegagropiles are formed by hydrodynamic movements and are assemblages of *P. oceanica* seagrass debris and sand (Ganong 1905; Mathieson et al. 2000, 2015; Sanchez-Vidal et al. 2021; Lefebvre et al. 2021). The different steps of aegagropile formation were recently clarified (see in Lefebvre et al. 2021). Aegagropile formation begins with (1) the agglomeration of *P. oceanica* debris, mainly fibres, around a rhizomic nucleus, (2) the consolidation of the central part with mineral particles, and (3) the progressive erosion of the nucleus into fibres to give rise (4) to ball-shaped aegagropiles as they are found on Mediterranean beaches (see in Lefebvre et al. 2021). However, in Lefebvre et al. (2021), the different plant debris and fibrous elements found in aegagropiles were only classified on the basis of their shape and size without prejudice of the plant organs from which they originate. This approximation was mainly due to the lack of possible comparison

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✉ Laurence Lefebvre
l.lefebvre@uliege.be

[1] Oceanology, MARE Centre, UR-FOCUS, University of Liege, Sart-Tilman, B6c, 4000 Liège, Belgium

[2] Laboratory of Functional Morphology and Evolution, Center for Applied Research and Education in Microscopy (CAREM) and Biomaterials Interfaculty Center (CEIB), University of Liège, Liège, Belgium

[3] STARESO, Pointe de la Revellata, BP 33, 20260 Corse, France

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with *P. oceanica* fresh organs; this is one of the objectives of this study.

Like all Magnoliophyta, *P. oceanica* has four organ types: roots, rhizomes, leaves, and flowers (Larkum et al. 2006). These organs perform different functions (Boudouresque and Meinesz 1982; Boudouresque et al. 2012). Rhizomes have two growth axes; plagiotropic growth will result in creeping stems, whereas orthotropic growth produces upright stems (Ott 1980; Boudouresque and Meinesz 1982; Caye and Rossignol 1983; Molenaar et al. 2000; Boudouresque et al. 2012). At the rhizomes' apices, the set of 4–8 leaf blades is called the shoot. The leaf blades are 8–11 mm wide and 20–80 cm long. Each shoot consists of leaf blades that Giraud (1979) classified as (1) juvenile, or leaf blades less than 5 cm in length, (2) intermediate, or leaf blades of more than 5 cm, and (3) adult, or leaf blades that have completed their growth and are characterised by the presence of a basal sheath. When the adult leaf blades fall, their basal sheaths remain attached to the rhizomes and are called scales (Giraud 1979; Boudouresque and Meinesz 1982; Boudouresque et al. 1983; Pergent and Pergent-Martini 1990; Onoda et al. 2011; Nicastro et al. 2015).

The main changes in seagrasses compared to terrestrial monocotyledons (Kuo and den Hartog 2007) are (a) the absence of stomata, (b) a thinner cuticle (0.5 µm) that can be porous [in *Posidonia, Amphibolis* (C. Agardh.,1823) and *Thalassia* (Banks, ex K.D. Koenig., 1805)], (c) the reduction of vascular tissues, especially xylem, because the water supply is constant (Sculthorpe 1967; Tomlinson 1974), and (d) the implication of the epidermis of *P. oceanica* is made of a monolayer, made up of cells very rich in chloroplasts (Kuo and den Hartog 2007). This is an adaptation due to the loss of asymmetry between leaf sides. In addition, certain seagrasses have developed air lacunae in the mesophyll, called aerenchyma. The air lacunae are very large meatuses filled with air that ensure the leaves' buoyancy. The histological specificities of *P. oceanica* can be summarised as follows for each organ, from leaf blades to roots.

The histology of *Posidonia* plant organs has been described on the basis of Australian species such as *Posidonia australis, Posidonia sinuosa* and *Posidonia kirkmanii* (Kuo and Cambridge 1978) that show some ultrastructural differences. In the Mediterranean species *P. oceanica*, only the leaf histology was described as two epidermis one of this is an abaxial or inferior face and the other is adaxial or superior face with an filling tissue (the mesophyll) comprising vascular bundles (Kuo and McComb 1989; Olesen et al. 2002; Larkum et al. 2006). The epidermal cells are interconnected with the cells of the mesophyll. As in all marine Magnoliophyta, *P. oceanica* epidermal cells contain many chloroplasts, mitochondria, lipid micelles and starch grains. The general characteristics of the other *P. oceanica* organs are only known from studies of Australian *Posidonia* species.

Marine fungi growing in mangroves and salt marshes are well documented (Jones and Pang 2012; Kohlmeyer and Volkmann-Kohlmeyer 1991, 2001, 2002). However, fungi living in seagrass beds remain poorly understood (Alva et al. 2002; Gnavi et al. 2014). Seagrasses seem to be devoid of mycorrhiza, unlike terrestrial plants and aquatic plants (Nielsen et al. 1999). To date, only one root-fungus association has been reported for a marine magnoliophyte species (*P. oceanica*) (Vohník et al. 2015). 86% of angiosperms have mycorrhizae (Brundrett 2009), including freshwater aquatic plants (Sudova et al. 2011; Kohout et al. 2012) and haline plants (Radhika and Rodrigues 2007; Welsch et al. 2010; Eberl 2011; Sengupta and Chaudhuri 2002; Kothamasi et al.2006). However, some plant groups do not require mycorrhizal symbiosis for nutrient uptake. This is particularly true of plants in the families Poales, Brassicales, Lamiales and Caryophyllales and in the order Alismatales (an order that includes marine magnoliophytes (Larkum et al. 2018; Brundrett 2009). Mycorrhizae vary according to the morphology, distribution and physiology of their host plants. The taxonomic identification (genus and species) of mycorrhizae is only possible through their trophic function, especially via biochemical and genetic studies (Read 1991; Brundrett 2004; Vohník et al. 2015, 2016, 2019). Recently, Vohník and co-workers identified dark and yellow septate endophytic fungi in the peripheral root tissues of *P. oceanica* and interpreted them as slow-growing endophytic symbionts. The black morphotype of this species, the most abundant of the three morphotypes in and on the roots, is an ascomycete in Aigialaceae (Pleosporales) that was recently identified as *Posidoniomyces atricolor* (Vohník & Réblová), named for its host seagrass, *Posidonia oceanica*. It is a root mycobiont of *Posidonia oceanica*-dominated meadows (Vohník et al. 2019).

The yellow morphotype, which is the second most abundant, is *Lulworthiales* sp. Kohlm (2000) (Torta et al. 2015). Finally, the ochre morphotype is a basidiomycete of the Hymenochaetaceae family that was classified as *Fuscoporia torulosa* (Pers. T. Wagner & M. Fisch. 2001) (Vohník et al. 2016). Recently, Torta et al. (2022) presented some illustrations of hyphae under the light microscope colonising mainly the roots and, occasionally, the leaves and rhizomes of *P. oceanica*, but nothing is known neither about plant cell wall degradation as a result.

This study has two purposes. First, it seeks to image the tissues of fresh *P. oceanica* shoots to identify the degraded items found in aegagropiles. Different parts of *P. oceanica* shoots and selected aegagropile items were similarly fixed and embedded in resin for light microscopy (LM) and transmission electron microscopy (TEM) and fixed whole in resin to view polished block faces with scanning electron

microscopy (SEM) in addition to classical SEM imaging of fractured bulk samples. Second, the presence of fungal hyphae and micro-sclerotia and evidence of microbial degradation were investigated in the same samples of fresh *Posidonia* organs and aegagropile debris (Borovec and Vohník 2018).

Materials and methods

Biological material

Samples (1–2 cm in length) of fresh organs from *P. oceanica* shoots, i.e., leaf blades, leaf sheathes (the basal ligules of adult leaves), scales (the basal ligules of dead adult leaves attached to the rhizome), rhizomes, and roots, were collected from the meadows of the northwestern Mediterranean Sea in the Calvi Bay, Corsica (France), close to the STARESO research station (8.725° E 42.580° N).

Several aegagropiles that were harvested on the beaches of Calvi in 2015 and 2016 were selected for study. They were classified, according to Lefebvre et al. (2021), into two types: heterogeneous and homogenous, i.e., with or without rhizomic nuclei corresponding roughly with ellipsoidal or spheroidal shapes, respectively. The fibres from the four internal layers (L1, L2, L3 and the nucleus) of six freeze-fractured aegagropiles (Lefebvre et al. 2021) were used. The term 'fibre' includes all of the aegagropile plant components without prejudice regarding their origin (*P. oceanica* organs or not). The fibres were classified into three types by shape: (1) thin fibres, (2) flat fibres and (3) wide fibres—according to Lefebvre et al. (2021).

Preparation of the samples

To observe cross-sections of the aegagropile fibres and compare their structure with the tissues of fresh *P. oceanica* organs, samples were prepared in three ways: they were embedded in resin for thin sectioning and subsequent imaging with LM and TEM, used as bulk samples for surface imaging with SEM and embedded in resin as whole aegagropiles or plant tissues to view polished block faces in SEM with a backscattered electron detector (BSED).

Semi-thin and ultra-thin sections

The samples from fresh *P. oceanica* were immersed in a 4% formalin (CH_2O) fixative solution with 6 parts seawater, rinsed thrice for 10 min in seawater with 20 mM NaN_3, post-fixed in 1% OsO_4 for 1 h at room temperature, and finally rinsed thrice for 10 min in Milli-Q water. Isolated fibres from aegagropiles were fixed by immersion for 1 h in 2.5% glutaraldehyde in Na-cacodylate 0.1 M at a pH of 7.4, briefly rinsed in double-distilled water, then post-fixed for 1 h in 1% OsO_4 at 20 °C and rinsed thrice for 10 min in double-distilled water. The ethanol/propylene oxide-dehydration and the epoxy resin-embedding (SPI-PON 812, SPI-CHEM) procedures were classical, as described in Lefebvre et al. (2021) in the 'Fibre characterisation' part of the Materials and methods section (see also Hayat 1993; Kuo 2007). Transversal semi-thin sections were cut at 1 µm using a glass knife on an ultra-microtome (Porter-Blum MT2) and stained with toluidine blue (1%, pH 9.0) for LM observation (Hayat 1993; Kuo and den Hartog 2007). Ultra-thin sections (80 nm) were cut with a diamond knife on an ultra-microtome Reichert Ultracut E. They were placed on formvar-coated copper grids and classically stained with uranyl acetate and lead citrate.

Bulk specimens

Aegagropiles were frozen in liquid nitrogen and cut into 5-mm broad slices using a metal knife. For SEM surface observation, 15 fibres of each type of shape (thin, flat, and wide) from the 4 aegagropiles were picked-up and were glued on glass slides with double-sided carbon tape, then Pt-coated (BALZERS SCD 030 sputtering unit). The same fixing procedure was performed with slices of the frozen aegagropiles.

Polished block faces of entire aegagropiles

The 14 whole aegagropiles were gadolinium acetate-contrasted (or not), acetone-dehydrated, epoxy resin-embedded (EPOFIX, Struers), sliced with a metal saw in three planes, i.e. frontal, sagittal, and at least two transversal levels, on 14 aegagropiles) and mirror-polished following the same methodology as that described in Lefebvre et al. (2021) for 'Polished slices' (see Lepot et al. 2014; Hosogi et al. 2015).

Observation techniques

LM observations and images were conducted on the polished slices and semi-thin sections of *P. oceanica* fresh organs, as well as fibres from the aegagropiles. Two microscopes were used: an Olympus PROVIS AX70 equipped with a VisiCam 5.0 camera (VWR) and software for image capture and an Olympus CX21 with a Motic camera and Motic Image 3.0 software for image capture.

SEM imaging was conducted with an ESEM-FEG XL-30 (FEI/Philips, The Netherlands) and an ESEM Quanta 600 (FEI/Philips, The Netherlands). Several specimens, including isolated fibres (15 fibres per type), freeze-fractured aegagropiles and fresh *P. oceanica* organs were visualised in a high vacuum (10^{-7} Torr) with an E-T secondary electron detector (SE-SEM) at 20 kV accelerating voltage. The

polished surfaces of Gd-contrasted (or not) aegagropiles were visualised in a low vacuum (0.4 Torr) at 20 kV accelerating voltage using a BSED. To enhance the contrast of organic fibres, some of the polished slices were re-contrasted for 10 min in alcoholic uranylacetate ((UO)Ac) according to the classical staining method for electron microscopy (Kuo 2007).

TEM imaging was conducted on a TEM-STEM Tecnai G2 Twin, FEI, USA) at 80 kV accelerating voltage on the ultra-thin sections of isolated fibres.

Results

Histology and ultrastructure of *P. oceanica* fresh organs

P. oceanica leaf blades are long, flattened, and isobilateral (Fig. 1A, B and F). The general views of them show mesophyll cells sandwiched between two epidermises, adaxial and abaxial, covering the superior and inferior sides, respectively. Both epithelial and mesophyll cells contain cytoplasm with obvious chloroplasts and starch grains protruding in the vacuole and contain small meatuses (Fig. 1C–F). Chloroplasts appear as dense, ovoid structures that are visible on semi-thin sections (stained dark blue with toluidine blue) as well as on polished surfaces (bright, electron-dense areas when viewed in BSE-SEM). In contrast, starch grains remained clear on semi-thin sections and show low electron density in BSE-SEM or TEM (Fig. 1D). Each epidermis consists of a monolayer of living cells. The outer wall of epidermal cells is covered by a thin cuticle (0.5 µm) that is less electron-dense than the subjacent cell wall. The only differences between the adaxial and abaxial epidermises are that the epidermal cells of the adaxial side are prismatic and richer in chloroplasts than those of the abaxial side, which are rather cubic-shaped (Fig. 1A–F). Sclerenchyma fibre cells, which mechanically support the leaf blade structure, are present just below the epidermis. They form a discontinuous monolayer between the epidermal and mesophyll cells, often distributed separately or in small flat bundles of less than six cells. These sclerenchyma cells are identifiable by their very small diameter (5–10 µm), narrow, empty lumen and very thick walls that stain weakly (Fig. 1A–F). Three-to-four layers of broad mesophyll parenchyma cells occupy the space between the epidermis layers (80 µm). In these cells, a very thin cytoplasmic lining contains a few prominent chloroplasts and surrounds a large vacuole. The median mesophyll parenchyma cells appear larger than the subepidermal ones (Fig. 1A, B and F). Some of the median mesophyll cells are tannin cells (50–70 µm in diameter) characterised by spongy content and a brown-greenish colour in semi-thin sections (Fig. 1A and F) or osmiophilic (bright or electron-dense) areas on polished surfaces viewed with BSE-SEM (Fig. 1B). TEM views revealed that the cytoplasm forms the electron-dense spongy structure surrounding numerous electron-lucent granules. Vascular bundles (50–60 µm in diameter) are regularly spaced (each 80 µm of leaf blade width) in the median part of the mesophyll. The vascular bundles are surrounded by a row of thin-walled small mesophyll cells or bundle sheath cells with thin cell walls. The centres of the bundles are also composed of thin-walled cells, suggesting that phloem cells are dominant. These appear blue in semi-thin sections and are clustered towards the abaxial side of the leaf blades. Closer to the adaxial side, the xylem cells are difficult to discriminate; their thin, light-blue cell walls in semi-thin sections do not allow them to be distinguished from phloem cells (Fig. 1A, B and F).

The histological organisation of the basal sheaths of adult leaf blades, also called scales when the leaves die, is very similar to that of leaf blades (Fig. 2A, B and E) except that no vascular bundles were found. They also include epidermis layers on two sides (adaxial and abaxial), each supported by a monolayer of sclerenchyma cells and sandwiching a thick mesophyll of parenchyma cells (Fig. 2C, D and F). The epidermis looks similar on both sides, but that lining the abaxial side is very regular and contains small meatuses (Figs. 2C and F) made by aligned cubic-shaped cells that are about 12 µm thick and supported by a continuous monolayer of sclerenchyma cells. The basal sheaths are damaged in some places where the cuticle or the outer wall is lacking. On the adaxial side, the epidermal cells are slightly thicker (± 19 µm) and less aligned and the supporting subepidermal sclerenchyma is discontinuous (Fig. 2D). Sheaths also differ from leaf blades in having a thicker mesophyll parenchyma of about ten layers of cells (Fig. 2A, B and E). The biggest parenchyma cells are located close to the epidermis of both faces, while those in the median part look smaller. Some tannin cells were observed within this mesophyll (Fig. 2A and B), but no vascular bundles. At their bases, closer to the rhizome, basal sheaths persist as scales after the adult leaf blades they supported fall (Fig. 2G and H). From the outermost to the innermost layer, the different levels of the scales' bases appear in successive cross-sections close to their insertion into the rhizome. These sectioned scales contain regularly spaced fibre bundles of sclerenchyma cells within the mesophyll. From outside to inside (Fig. 2G and H), the subepidermal layer of fibres disappears at the adaxial side of the scales, while that of the abaxial side gradually sub-divides into separate flat bundles of about 40–50 cell columns that are several cell layers thick. These flat bundles become progressively rounder and form isolated cylindrical fibre bundles in the mesophyll, like those found in the rhizomes (see below).

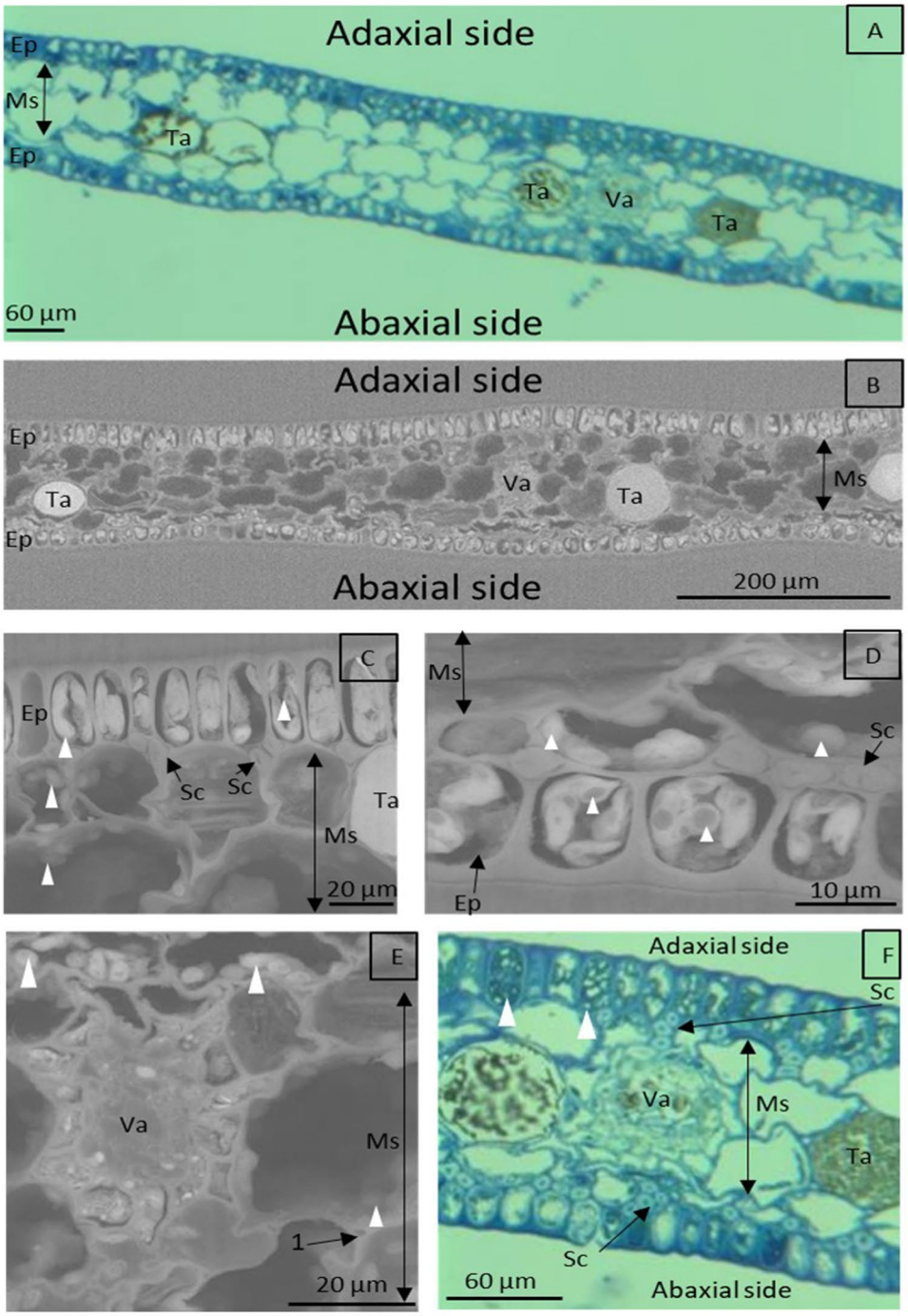
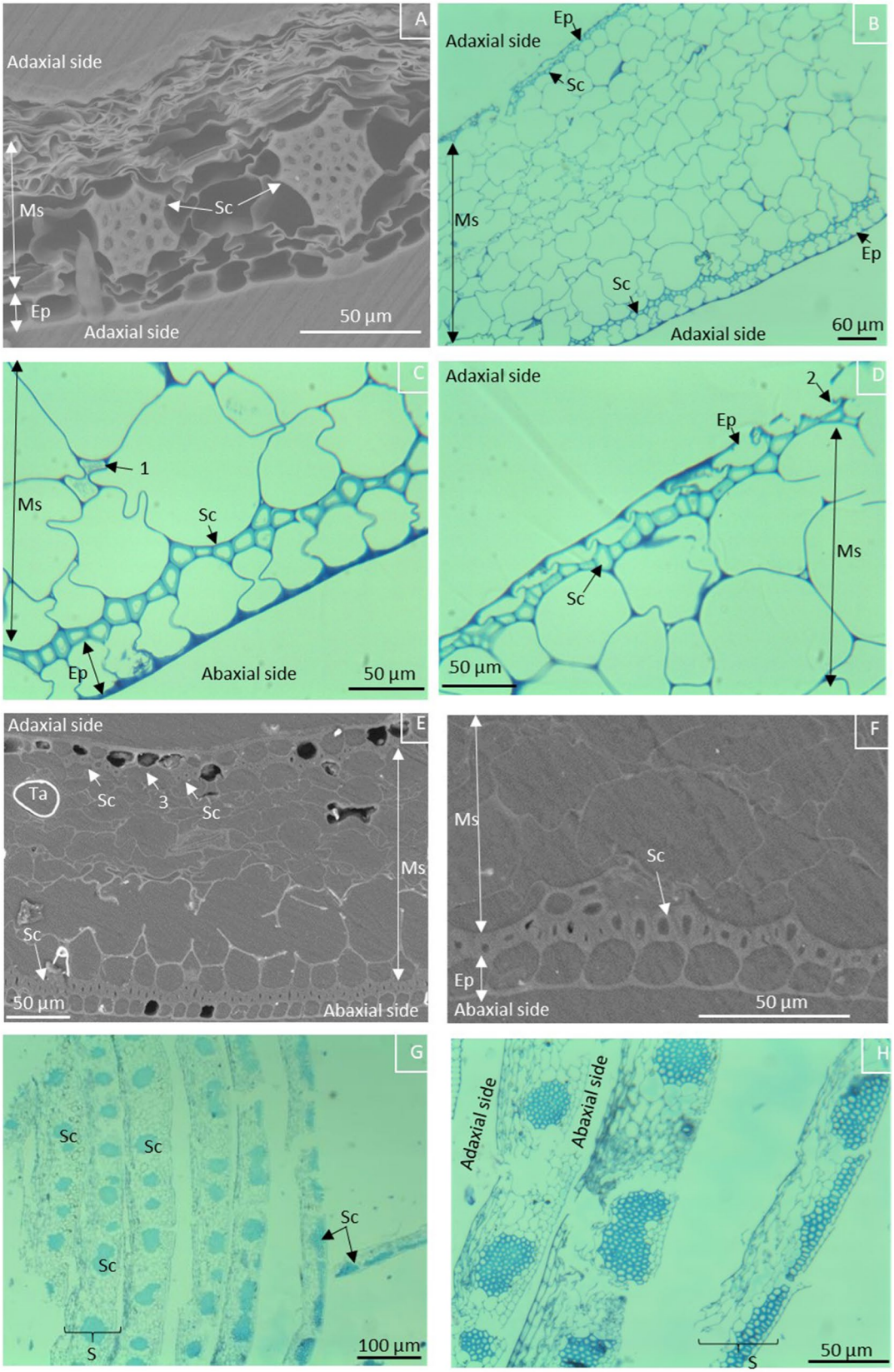


Fig. 1 LM (**A**, **F**) and BSE-SEM (**B**–**E**) views of transversal sections of *Posidonia oceanica* leaf blades. A and B: General views of a portion of a *P. oceanica* leaf showing both the adaxial and abaxial epidermis and the mesophyll (Ms) of large parenchyma cells with vascular bundles, tannin cells (brown-greenish in A and white in **B**). **A** Cytoplasmic content is visible in the lumen of the epidermal and parenchyma cells. **C** and **D** Detail of the adaxial and abaxial sides, respectively, showing epidermal cells of different shapes, isolated sclerenchyma cells (Sc), and chloroplast contents or starch grains, which appear dark in the chloroplast and clear or white (indicated by white triangles) in the cytoplasms of epidermal and parenchyma cells. **E** and **F** Magnifications of vascular bundles (Va) surrounded by small parenchyma cells and containing small thin-walled cells, likely phloem more than xylem. *Ep* epithelium; *Ms* mesophyll; *Sc* sclerenchyma; *Ta* Tannin cells; *Va* vascular bundle and *1* small meatus

The rhizomes, i.e., the stems of *P. oceanica,* are continuous and irregularly branched, roughly cylindrical in cross-section and surrounded by the remains of dead basal sheaths (or scales) (Fig. 3A and B). Histologically, the rhizomes consist of a central stele containing vascular tissue that is surrounded by a thick cortex and encased by the epidermis, a monolayer of cubic cells (10–20 µm), with an outer wall or cuticle that is sometimes damaged. The cortex is formed by an outer exodermis of thick-walled parenchyma cells and an inner thin-walled parenchyma (Fig. 3A, B and C). The exodermis consists of several layers of radially elongated cells (about 45 µm long and about 6 µm wide) with slightly thicker walls than the epidermal cells. It is rich in randomly distributed tannin cells (40–60 µm long and 5–10 µm wide) that appear bright on BSE-SEM images (Fig. 3C and D) and also contains cylindrical fibre bundles (40–60 µm in diameter) that are regularly distributed along a peripheral circle (Figs. 3A, B and D). Below the exodermis is the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◂**Fig. 2** BSE-SEM (**A**, **E** and **F**) and LM (**B**, **C**, **D**, **G** and **H**) views of transversal sections of *Posidonia oceanica* leaf sheaths. A and B: Low magnification views showing both adaxial (Ad) and abaxial (Ab) sides with flat discontinuous bundles of sclerenchyma fibre cells and a continuous monolayer of sclerenchyma cells under the epidermis, respectively, with a thick mesophyll of parenchyma (Ms) sandwiched between the epidermis layers. **C** and **D** Magnifications of the adaxial and abaxial sides, respectively. Damage to the epidermal small meatus (1) is visible at the corners of parenchyma cells (Ms), as well as an enlarged meatus (2). **E** BSE-SEM view of a leaf sheath in a transversal section with both sides and obvious interruptions in the adaxial sclerenchyma cell layer, (3) while that lining the abaxial side is continuous. **F** Magnification of the abaxial side showing cubic-shaped epidermal cells supported by a continuous monolayer of sclerenchyma cells. **G** and **H** LM of scales surrounding rhizomes, illustrating the progressive transformation of the abaxial sclerenchyma cell layer into cylindrical sclerenchyma fibre bundles. *Ep* epidermis; *Ms* mesophyll; *S* scale; *Sc* sclerenchyma and *Ta* Tannin cell

mesophyll, which exhibits some tannin cells and randomly distributed fibre bundles. The latter are enlarged towards the central stele of the rhizome, with diameters increasing up to 100 µm. Notably, in most of the semi-thin sections, the exodermis and parenchyma cells appear empty or dead, without any cytoplasmic content or exhibiting only some remains. Those observations were confirmed by TEM.

Root sections show a general organisation that is similar to that of rhizomes, with circular sections (Fig. 4A and B), an outer cortex with an exodermis and mesophyll, and a central stele. The cortical mesophyll cells increase in size towards the centre of the root. Unlike that in rhizomes, the exodermis (Fig. 4C and D) consists of thick-walled non-lignified collenchyma cells and lacks fibre bundles. The epidermal cells are rather large with an outer thick cuticle (Figs. 4E and F). As in rhizomes, the tissues regularly appear dead, with cytoplasm-free cells, damaged cuticles or walls and no stele, leaving a wide medullar hole. These observations are surprising, because roots and rhizomes were collected from living plants near growing ends.

Histology and ultrastructure of the fibres composing the aegagropiles

The fibres found in the aegagropiles were classified into three types according to their shape and size, i.e., thin, flat, and wide fibres (Lefebvre et al. 2021) without preconceived ideas about their tissue origin.

The fibres classified as 'thin' (Fig. 5A and B) always appeared cylindrical and were round in cross-section with diameters from 15 to 80 µm. These thin fibres have been identified as fibre bundles of *P. oceanica* composed of sclerenchyma cells (Fig. 5C and D). They can derive from stems (rhizomes) or scales at the very base of leaf sheaths that remain attached to the rhizomes (compare with Figs. 2 and 3).

The fibres classified as 'flat' (10 to 20 µm thick, Fig. 6A–C), are long, flat, ribbon-shaped fragments that are a few cell columns thick and include thick-walled sclerenchyma cells (with reinforced secondary walls) (Fig. 6A and B). These fragments are sometimes covered by epidermal cells (Figs. 6B and C). These fibres were mostly identified as fragments of *P. oceanica* leaf sheaths when they exhibit only one layer of sclerenchyma cells (compare with Fig. 2). Those with several layers of sclerenchyma cells forming flat fibre bundles (compare with Fig. 2) were likely from the base of leaf sheaths. However, these could also be fragments of the rhizome cortex with collenchyma cells (compare Fig. 6B with 3A). Fragments of leaf blades are rare or absent in aegagropiles. They would be recognisable by small clusters or isolated sclerenchyma cells below the epidermis.

The fibres classified as 'wide' (Fig. 7) appeared as large, irregular, but generally elongated fragments with a mean width of 0.57 ± 0.31 mm. These fibres are broader than the others and present a large range of sizes and shapes (Fig. 7A–C). They can be flat or round in cross-section. This variability is due to their size and composition, which includes several tissues. Most of these fibres are fragments of *Posidonia* organs that include numerous cell columns of several cell types (Fig. 7C–F). They regularly contain mesophyll associated with the epidermis and/or sclerenchyma fibres. The shape and relative disposition of cells compared with that in fresh *P. oceanica* organs allowed the identification of their origin. Most of the wide fibres have been interpreted as debris of *P. oceanica* rhizomes (Fig. 7C–F) or roots. Cortex fragments, with or without scales, and steles are found separately (Figs. 2G and H and 3A and B).

Microorganisms and microbial degradation traces

In fresh *P. oceanica* tissues

The examination of different sections of fresh *P. oceanica* organs with LM, BSE-SEM, and TEM revealed the presence of microorganisms or their traces within the tissues, suggesting that microbial colonisation and degradation already starts in living plants. They were mainly distributed in roots (Fig. 4C–F) and rhizomes (Fig. 3A) but also occurred in scales and leaf blades, to a much lesser extent (Fig. 8A). These microorganisms appeared mostly as filamentous fungal hyphae within the lumen of mesophyll or epidermal cells directly adjacent to living cells with cytoplasm and chloroplasts (Fig. 8D). In several images, the hyphae appeared septate and/or bearing micro-sclerotia (supposedly *P. atricolor*) (Figs. 3C, 4C–F and 8A–D and F). They are distinguished from epibionts (Fig. 9A) that occur on the surface of plant organs and were mostly identified as diatoms via SEM and some sections.

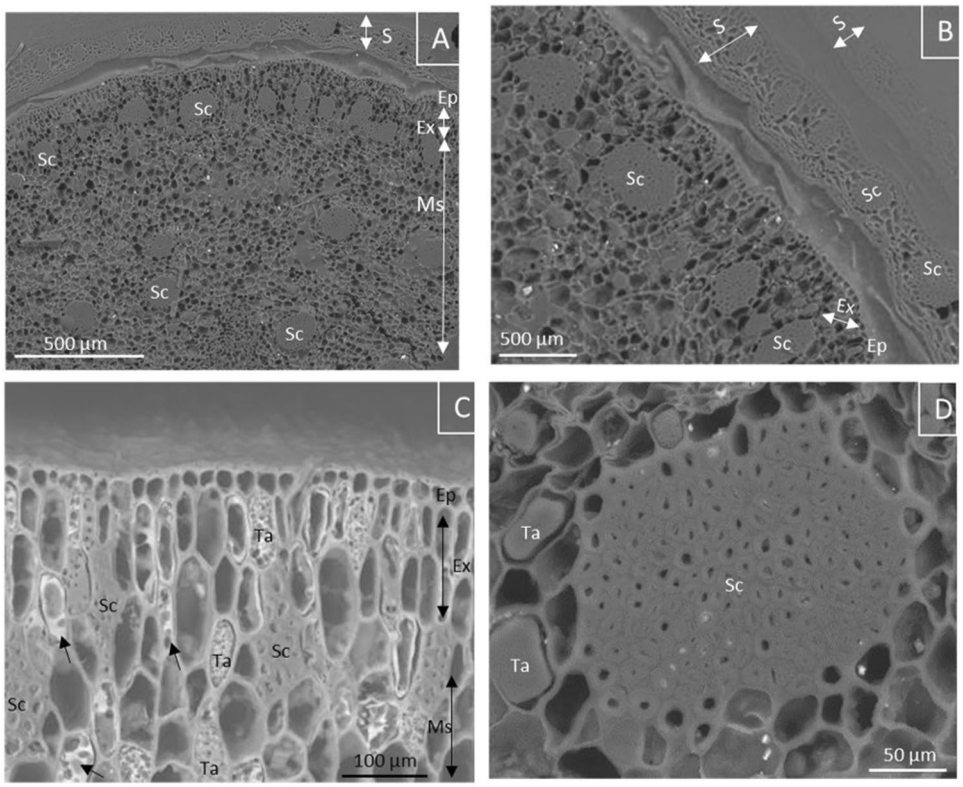

Fig. 3 BSE-SEM views of transversal sections of *Posidonia oceanica* rhizomes. **A** and **B** Low magnifications of the peripheral part of a rhizome showing two sectioned scales (S) attached and round bundles of sclerenchyma fibre cells (Sc). **C** Magnification of the rhizome epidermis and exoderm parenchyma with fibre bundles (Sc), tannin cells (Ta) and some parenchyma cells colonized by micro-sclerotia (indicated by black arrows). **D** Magnification of a broad fibre bundle composed of several sclerenchyma cells in a rhizome. *Ep* epidermis; *Ex* exodermis; *Ms* mesophyll; *S* scale; *Sc* sclerenchyma and *Ta* Tannin cells

Elongated fungal hyphae are 8–10 µm long and about 2 µm wide. They appear bright on BSE-SEM images (Figs. 8B and D) and sometimes form a network of filaments. In cross-section, round-shaped hyphae cross-sections filled the lumen of plant cells and walls contact to each other. In most of the cases, the colonised plant cells typically look dead and free of cytoplasm. Perforations of plant cell walls were also observed, especially in *P. oceanica* rhizomes (Fig. 8E and F).

Many observations (LM and BSE-SEM) indicated traces of fungal presence in the dead cells of the roots (Figs. 4C–F and 8B) and rhizomes (Figs. 3A and 8C–F) of *P. oceanica*. Micro-sclerotia (the reproductive organs of septate hyphae) were observed within the leaf epidermis (Fig. 8A), the rhizome exodermis and mesophyll (Figs. 3A and 8C–F) and the root epidermis, exodermis and mesophyll (Figs. 4C–F and 8B). They occasionally form rows of tubular sections (3 µm in diameter) on the inner side of the cell wall. In roots, cell wall alterations were visible in the epidermis and mesophyll as breaks and the disappearance of wall layers (Figs. 4C and D).

In the fibres composing *P. oceanica* aegagropiles

The examination of the different fibres composing aegagropiles also revealed the presence of microorganisms, their traces and degradation in *P. oceanica* cells and cell walls. These traces have been observed several times on the three types of fibres (thin, flat, and wide) found in aegagropiles (Figs. 5A and B, 6A, 7C, E and F, 9 and 10) regardless of the observation or preparation method. The observed alterations could be mechanical, but most of them are associated with microbial activity. First, epibionts (Fig. 9A) were observed on the surfaces of flat fibres derived from leaf blades and scales, as well as on the surfaces of thin fibres. SE-SEM views also reveal the total or partial degradation of the cuticle of the epidermis. Epiphytic organisms, such as diatoms (Fig. 9A) and bryozoans, can thus colonise the inner surface of epidermal cell walls. On fibre surfaces, fungal hyphae traces or 'dead' hyphae (because aegagropiles are dry) appeared in the form of long, thin 'ribbons' (3 µm in diameter) passing over several rows of parenchyma cells (Fig. 9A and B). In some cases, cavities or gutters and perforations were observed on inner wall surfaces (Figs. 8F and 9D), but the most frequent, obvious alteration is the cell wall separation that is likely caused by the degradation of the middle lamella (Fig. 9C). This point is detailed below.

Microorganism traces and degradation were also frequently seen inside the aegagropile fibres and tissue fragments (Figs. 5A and B, 6A, 7C, E and F, 9 A–D and 10 A–H), as within fresh plant organs. The presence of fungal hyphae (Figs. 7F, 9B and 10A and D) and micro-sclerotia (Figs. 7C and 10C) is often observed in wide fibres regardless of cell type. Cross-sections of hyphae are easily

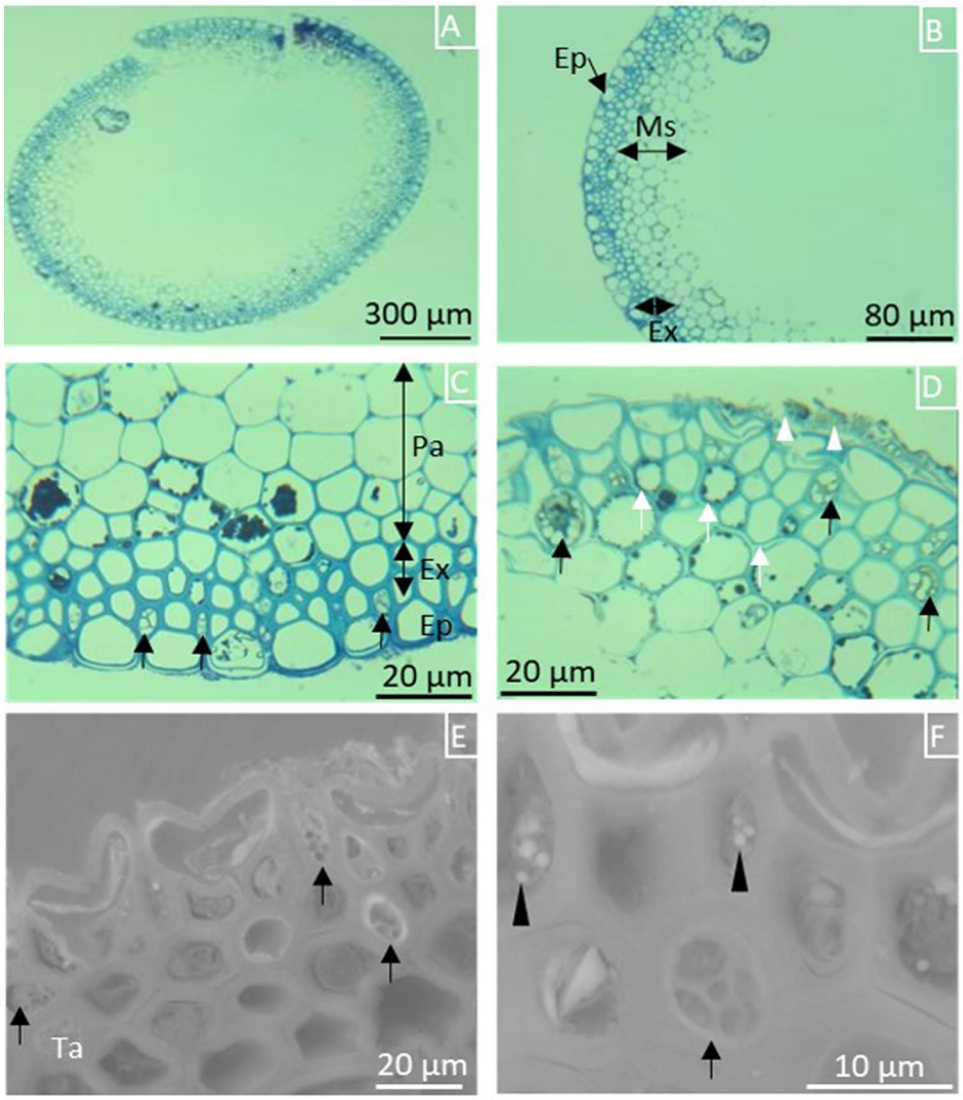

Fig. 4 LM (**A**–**D**) and BSE-SEM (**E**, **F**) views of transversal sections of *Posidonia oceanica* roots. **A** General view of a whole root cross-section with a medullar hole due to the loss of the central stele. **B**, **C** and **D** Magnifications of the external tissues (epidermis, exodermis, and parenchyma). Micro-sclerotia (indicated by black arrows) are obvious in the exodermal cells, as well as the degradation of cell walls, notably at the level of the middle lamella, causing cell separation and the presence of a hyphal mantel (indicated by white triangles). **E** and **F** Details of the exodermis showing the thick-walled collenchyma cells, some living cells with cytoplasm and chloroplasts (indicated by black triangles) and other dead cells containing micro-sclerotia. *Ms* mesophyll; *Ep* epidermis; *Ex* exodermis and *Ta* Tannin cells

recognisable in semi-thin sections (Fig. 7F), polished sections (Figs. 8D and 10A) and the fractured fibres seen in SE-SEM (Fig. 10D). They can also be identified in ultra-thin sections viewed with TEM (Figs. 10E–H). They can be accompanied by bacteria (Fig. 10H). In addition, the degradations observed in the cell walls of *P. oceanica* organs seem to be mostly due to fungi rather than bacteria. No characteristic striations from tunnelling bacteria (Nilsson and Singh 2014; Trevisan 2018) appear within the perforations of the middle lamella. Additionally, previous TEM images of plant fragments in the stomach content of gammarid amphipods associated with *P. oceanica* leaf litter in Calvi Bay reveal similar fungal traces (Trevisan 2018). The presence of micro-sclerotia traces within aegagropile fibres is also well illustrated in semi-thin sections and polished sections (Fig. 10). As in SE-SEM images (Figs. 9C and D), several features of cell wall degradation were frequently observed in semi-thin sections, polished sections, and TEM views. The most frequent features are the alteration or 'digestion' of the primary or secondary cell walls of epidermal and parenchyma (collenchyma) cells (Figs. 4C and D, 7C and 10B and C) causing the delamination of wall layers and breaks. The walls of sclerenchyma cells in fibre bundles (Figs. 5A, B and 10E, G) and the middle lamella are also affected, causing cell separation and the delamination of cell walls (Figs. 5B, 6A, 7D and E, 9C and 10E and G). Gutters resulting from the alteration of the secondary cell wall (Figs. 10G and H) and perforations of the whole cell wall close to hyphae (Fig. 10F) or only of the middle lamella (Fig. 10G) are apparent in ultra-thin sections and correspond to alterations seen on the inner face of opened cells in SE-SEM views (Fig. 9D). Sections thus show cavities or tunnels in the cell walls as well as perforations in the middle lamella, and these degradation features are likely to result in the large open spaces between cells (Figs. 4D, 5A and B, 7B–E and 9 C and D). This specific degradation

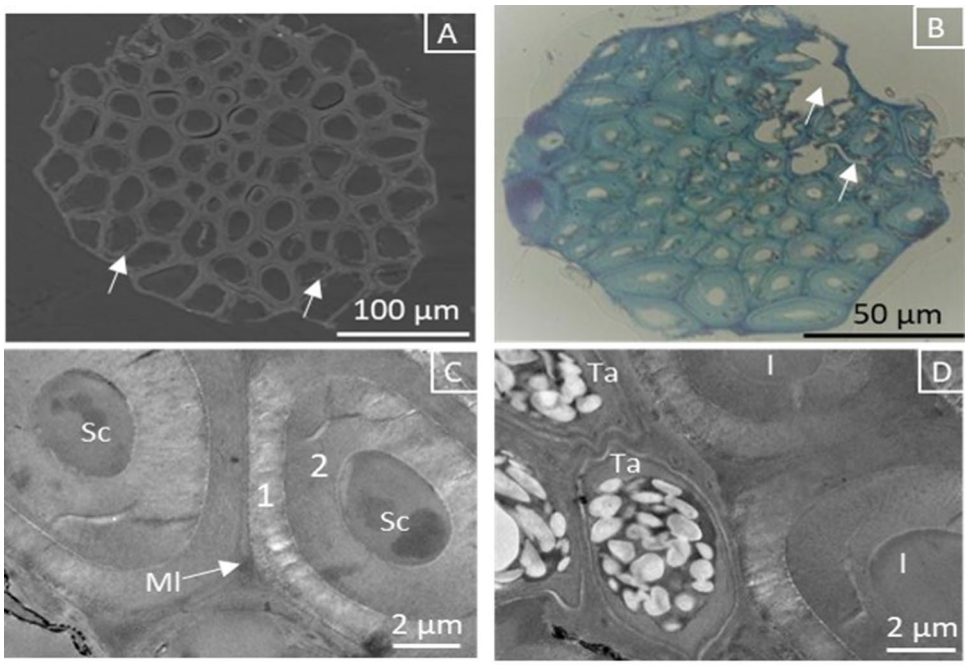

Fig. 5 BSE-SEM (**A**), LM (**B**, from Lefebvre et al. 2021), and TEM (**C**, **D**) views of thin fibres found in *Posidonia oceanica* aegagropiles. **A**, **B** Cross-sectioned fibre bundles showing sclerenchyma cells. The secondary walls are obvious in sclerenchyma cells, as well as perforations and breaks due to degradation (indicated with white arrows). **C** Sclerenchyma cells showing their thick primary (1) and secondary (2) walls, as well as the middle lamella (Ml). **D** Sclerenchyma cells (Sc) and tannin cells (Ta) with their contents; *l* sclerenchyma cell lumen; *Ml* middle lamella; *Sc* sclerenchyma cells and *Ta* tannin cells

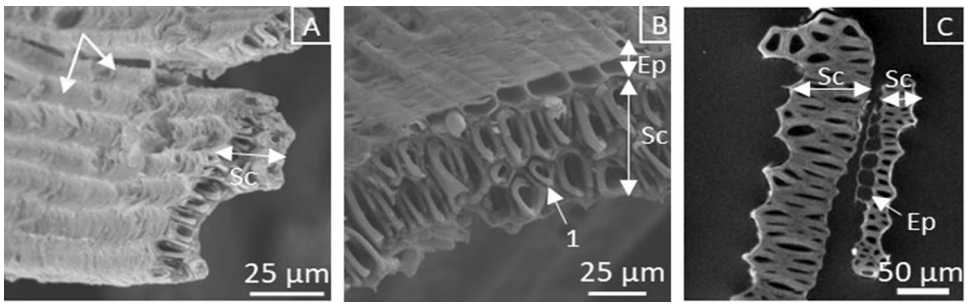

Fig. 6 SE-SEM (**A**, **B**, from Lefebvre et al. 2021) and BSE-SEM (**C**) views of flat fibres from *Posidonia oceanica* aegagropiles. **A** General view of a flat fibre made of one layer of sclerenchyma cells and showing fragmentation in two parts (indicated with arrows). **B** Magnification of a flat fibre showing two layers of sclerenchyma cells with degraded secondary cell walls (1) and covered by epidermal cells. **C** Sections of flat fibres showing multiple (left) and single layers of sclerenchyma cells (right), the latter with a few remaining epidermal cells. *Ep* epidermis and *Sc* sclerenchyma

contributes to the longitudinal fragmentation of cell columns at the level of the middle lamella and generates fibrous aegagropile constituents.

Discussion

To complement the existing histology of marine magnoliophytes, this study provides the first detailed description of *P. oceanica* organs (leaf blades, scales, rhizomes, and roots) with attention to particular features of the species. Moreover, the collected structural details were sufficiently precise to identify the cell types and tissues found in Mediterranean aegagropiles, i.e., fragments form rhizomes and fibres of various types. In addition, several traces of biotic degradation and microorganisms including fungal hyphae and bacteria, were found and characterised. The biotic degradation of tissues starts in living plants and may be primarily associated with fungal colonisation. The morphological characteristics of this fungus in the organs of *P. oceanica* are similar to those of the dark septate endophyte (DSE) previously described by Vohník et al. (2015, 2016, 2019) in the roots of the same species, and we assume that this could be the same taxon that they described. This dark septate fungus of the family Aigialaceae (Pleosporales), recently named *Posidoniomyces atricolor*, probably colonises several organs of living *P. oceanica*, because the presence of hyphae as well as fungal reproductive structures (micro-sclerotia) has been recorded not only in the roots but also in the rhizomes and leaf sheaths.

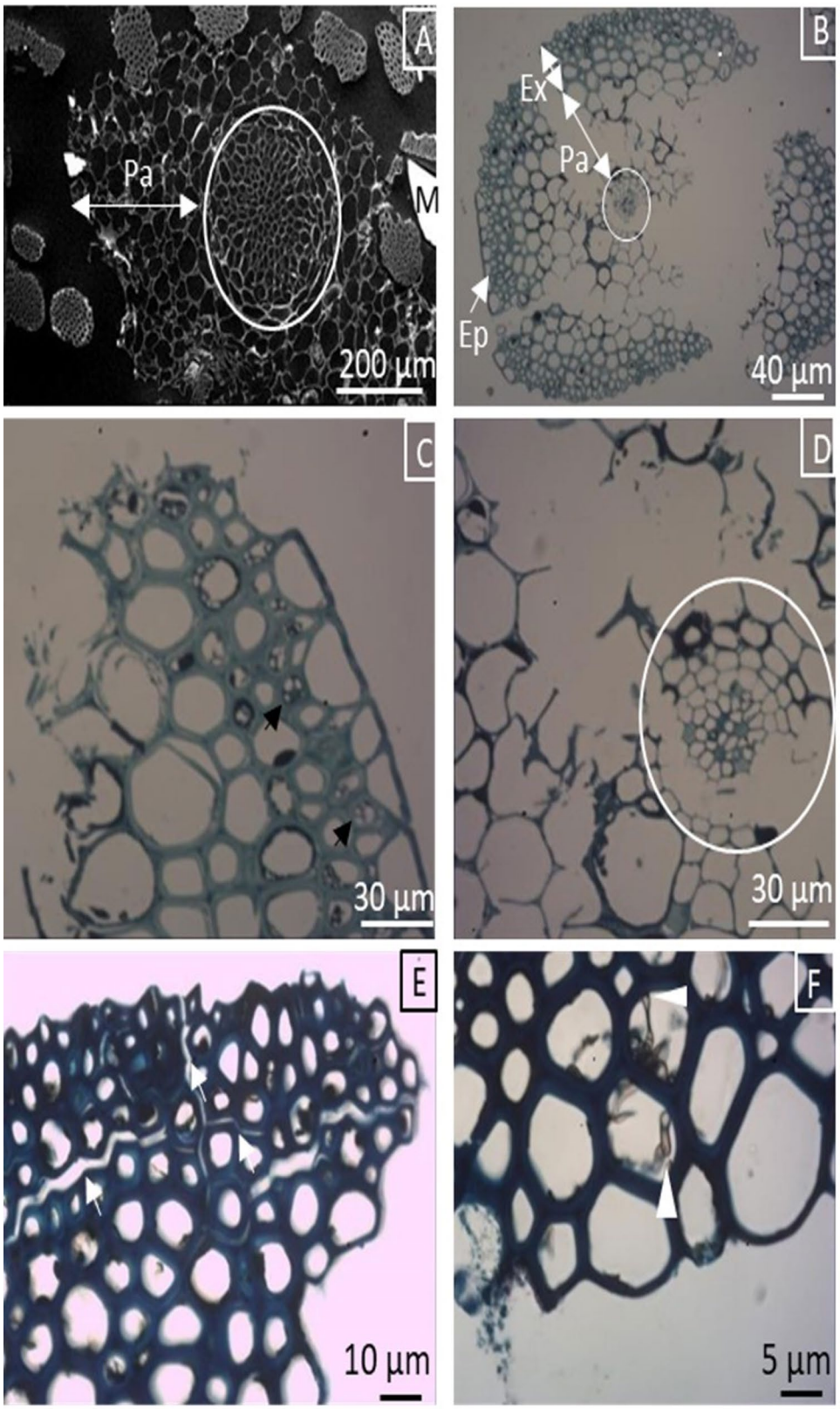

Fig. 7 BSE-SEM (**A**) and LM (**B**–**F**) views of cross-sectioned wide fibres from *Posidonia oceanica* aegagropiles. **A** Wide fibre containing the central stele (indicated with a white circle) of a rhizome surrounded by parenchyma remains and some sclerenchyma fibre bundles freed by parenchyma degradation. **B**, **C** and **D** Cross-sections of wide fibres identified as *P. oceanica* root fragments showing a general view of the root section (**B**) with the central stele containing vascular tissues (recognizable phloem), the peripheral part of the root with the epidermis (**C**) showing the exodermis and parenchyma, both containing micro-sclerotia (indicated with black arrows) and a magnification of the stele (**D**) surrounded by parenchyma. **E** and **F** Cross-sections of wide fibres identified as broad fibre bundles from scales close to the rhizomes. **E** (image from Lefebvre et al. 2021): The bundle of sclerenchyma cells is associated with parenchyma cells remaining at its periphery and shows different traces of alteration (indicated with white arrows) and degradation of the middle lamella, causing cracks in the bundle and cell separation. **F** Magnification showing fungal hyphae in the cell lumen (indicated with white arrowheads). *M* mineral; *Ep* epidermis and *Ex* exodermis

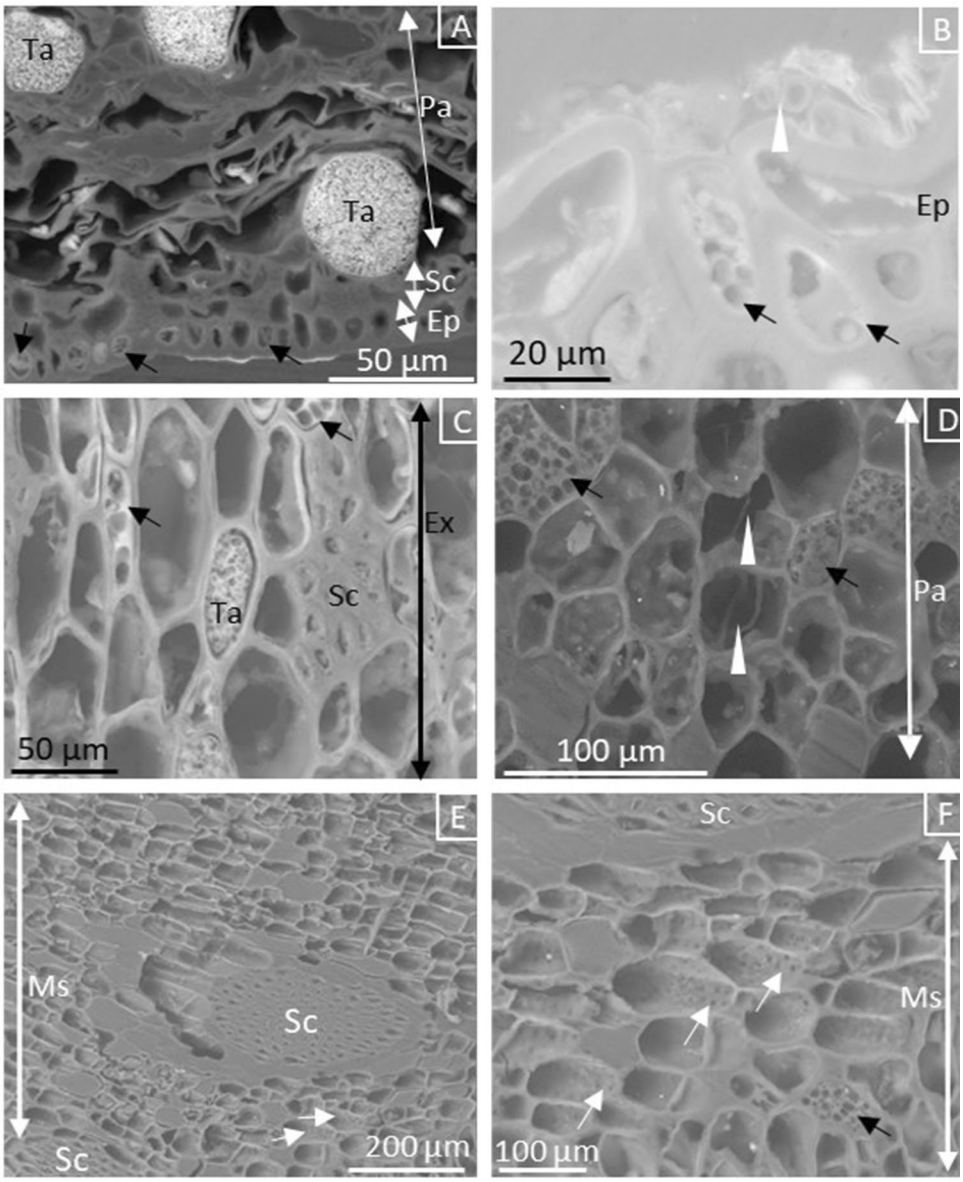

Fig. 8 BSE-SEM views of fungal hyphae and micro-sclerotia in the organs of living *Posidonia oceanica*. **A** *P. oceanica* leaf section showing micro-sclerotia in the epidermis (indicated with black arrows). **B** *P. oceanica* root section showing micro-sclerotia in the exodermis (indicated with black arrows) and cross-sections of fungal hyphae on the surface (indicated with white arrowheads). **C** and **D** Exodermis and parenchyma tissues of a *P. oceanica* rhizome, respectively, with micro-sclerotia (indicated with black arrows) and hyphae in the cell lumen (indicated with white arrowheads) alongside intact parenchyma cells (Ms) and tannin cells (Ta). **E** and **F** Perforations in the walls of the rhizome parenchyma cells (indicated with white arrows) and some micro-sclerotia (indicated with a black arrow). *Ms* mesophyll; *Ep* epidermis; *Ex* exodermis; *Sc* sclerenchyma and *Ta* Tannin cells

Histology and ultrastructure of *P. oceanica* organs

Our observations of fresh organs from *P. oceanica* agree with the literature dedicated to the general histology of various seagrasses, including the genus *Posidonia* (Tomlinson 1974; Kuo et al. 1981; Kuo and McComb 1989; Olesen et al. 2002; Kuo and den Hartog 2007; Larkum et al. 2006). Seagrasses have common characteristics with terrestrial grasses and all of their organs are established under the same histological basis. However, seagrasses have also acquired adaptations to their marine environments, so the histology, even of homologous structures, differs from that of the C3 and C4 terrestrial plants to which they are related.

- Leaf blades

The adaxial and abaxial sides of *P. oceanica* are narrow and, as in other seagrasses, less discernible than those of terrestrial monocotyledons (Kuo and den Hartog 2007). The only differences are that the cells of the adaxial epidermis are prismatic and richer in chloroplasts than those of the abaxial side, which are more cubic in shape. This distinguishes *P. oceanica* from *P. australis*, in which the leaf blade sides are identical with prismatic epidermal cells. Sub-epidermal fibre cells, also reported in *P. australis* and *Cymodocea* (Kuo and den Hartog 2007), are present either isolated or in small clusters. The major tissue of leaf blades is the mesophyll, which is composed of parenchyma cells, encloses vascular bundles, and contains specialised tannin cells with polyphenolic substances in their vacuoles (Kuo and den Hartog 2007). The latter were

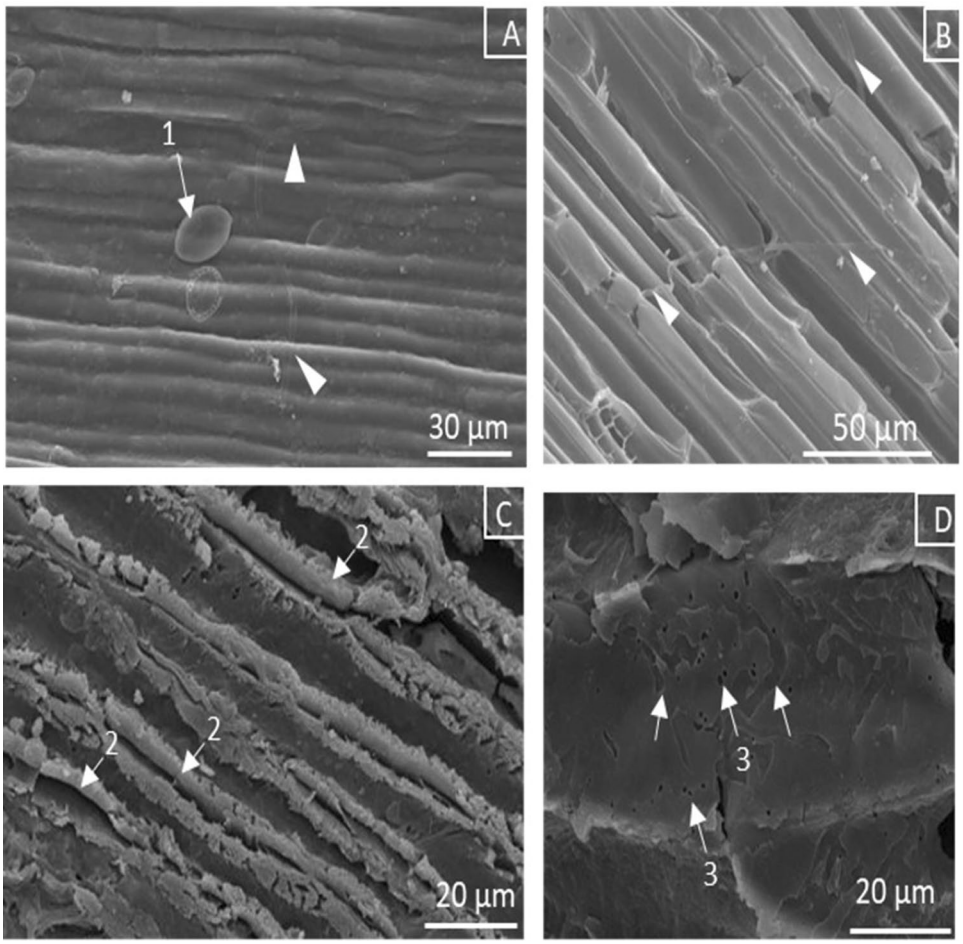

Fig. 9 SE-SEM surface views of fibres from aegagropiles of *Posidonia oceanica*. **A** Diatoms (1) and fungi hyphae traces (indicated with white arrowheads) on the surface of the epidermis of a flat fibre. **B** Hyphae crossing several cell rows (indicated with white arrowheads) on a flat fibre without an epidermis. **C** Longitudinal cell separation due to cell wall alteration and likely due to the degradation of the middle lamella (2). **D** Alterations of cell walls appearing as gutters (indicated with white arrows) and perforations (3) in the walls of parenchyma cells exposed at the surface of a wide fibre bundle

also found in different seagrasses, including *P. sinuosa* (Cambridge and Kuo 1979), and in species of the genera *Enhalus* (Rich., 1811), *Thalassia*, *Cymodocea* (K.D. Koenig., 1805), *Amphibolis* and *Thalassodendron* (Hartog, 1970). However, unlike those of many other seagrasses (*P. australis, Zostera marina, Zostera muelleri, Halophila ovalis* and *Halodule uninervis* (Kuo and den Hartog 2007), the mesophyll of *P. oceanica* is not an aerenchyma. No air lacunae, i.e., large air-filled spaces between parenchyma cells, were seen in the mesophyll. Air lacunae are not usually cells but rather enlarged meatuses. Unlike cells, they are not limited by their own walls but by the single walls of neighbouring parenchyma cells, presenting concave rather than convex walls. This disposition of discrete air lacunae as in *P. australis* was not observed in *P. oceanica.* Instead, regular triangle-shaped meatuses were observed. The absence of aerenchyma results in a low buoyancy of *P. oceanica* leaf blades that are not vertically positioned in the water column (except during strong hydrodynamic events) and sink to form litter after detachment. This contrasts with algae possessing air-filled vesicles (Hurka 1971) and other seagrasses with aerenchyma (Pedersen et al. 1998; Borum et al. 2006; Brodersen et al. 2018).

Generally, vascular bundles are abundant and mainly distributed near the margins of the leaf blades (Campbell and Reece 2004). This is true of *P. oceanica* and the vascular bundles show poorly distinct cell types. They are thoroughly surrounded by bundle leaf blade cells that are thin-walled and do not seem lignified as in *P. australis* and other seagrasses (e.g., *T. pachyrhizum* and *Halodule universis*). In the leaf blades of these species, a visible row of thick-walled bundle sheath cells encircles the vascular tissues. However, the vascular tissues are poorly distinguishable structurally; the xylem and phloem both exhibit only thin-walled cells. This agrees with the literature on seagrasses reporting a reduction of xylem, the absence of vessels and weak cell wall thickening and minimal lignification. These changes reflect the lower need for mechanical support and xylem transport in submerged species (Sculthorpe 1967; Tomlinson 1974).

- Leaf sheaths

The basal sheaths of leaves, or future scales, are the rigid, lignified structures at the base of the adult leaves of *Posidonia* bundles. According to Tomlinson (1974) and Kuo and

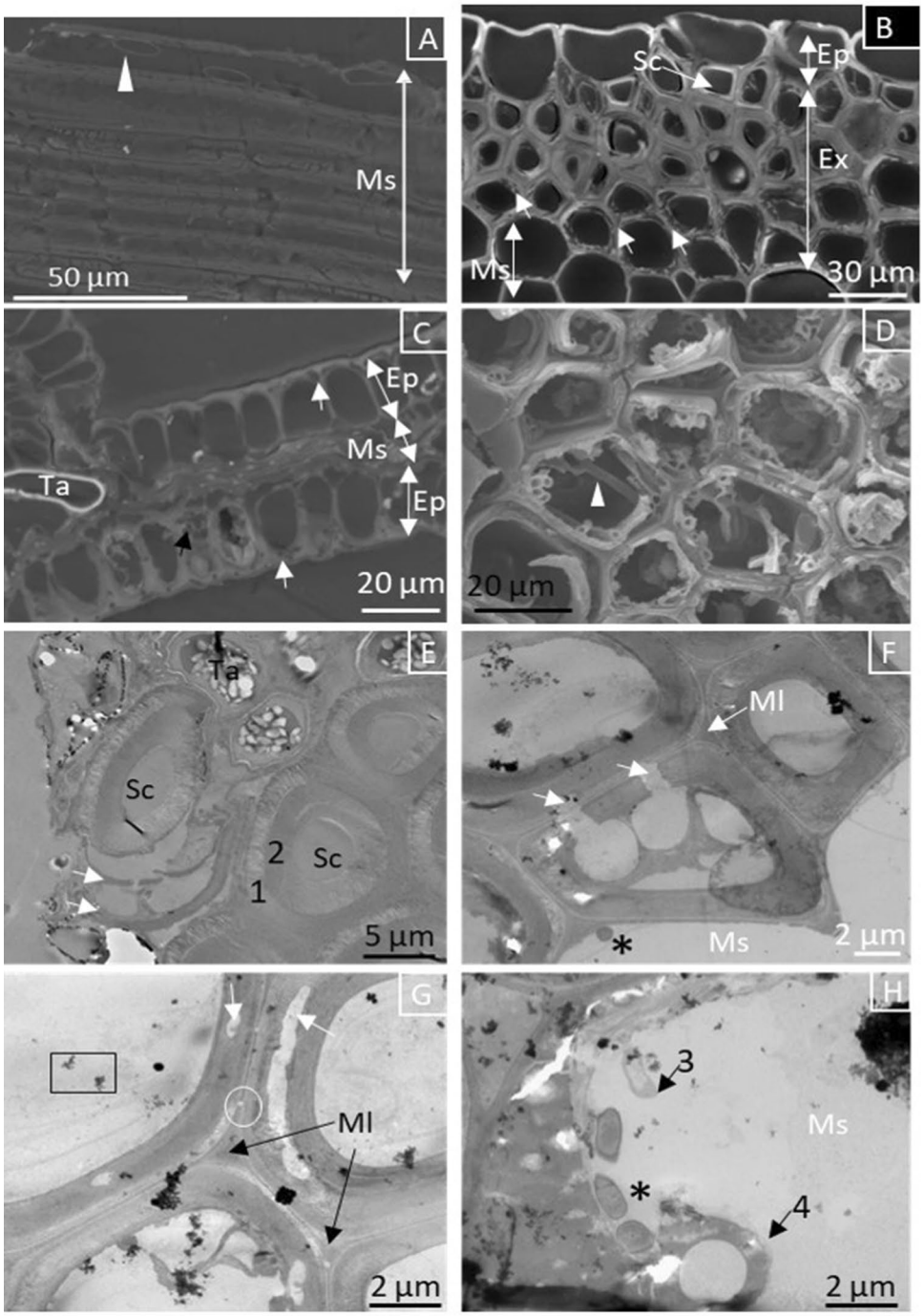

Fig. 10 BSE-SEM (**A**–**C**), SE-SEM (**D**), and TEM (**E**–**H**) images of fibres from aegagropiles of *Posidonia oceanica*. **A** and **B** Longitudinal and transversal sections of wide fibres showing hyphae in parenchyma cells (indicated with white arrowheads) and degradation traces (indicated with white arrows) in the cell walls of the exodermis (sclerenchyma and parenchyma cells). **C** View of a flat fibre showing cavities and tunnels in the walls of the epidermal cells (indicated with white arrows) and microsclerotia (indicated with a black arrow) in an epidermis cell lumen. **D** Evidence of fungal hyphae in the lumen of parenchyma cells (indicated with white arrowheads) in a flat fibre. **E** Delamination of the primary and secondary cell walls (indicated with white arrows) and alteration of the middle lamella in a thin fibre. **F** Perforations of the cell walls deep into the middle lamella (indicated with white arrows) and close to hyphae (asterisk). **G** Cavities and tunnels in cell walls (indicated with white arrows) and perforations in the middle lamella (indicated with a white circle) in a thin fibre with thick secondary (2) and primary walls (1) and electron-dense degradation products lining the cell lumen (indicated with a black square). **H** Evidence of the presence of hyphae (asterisk) and bacteria (3) with degradation products (4). *Ep* epidermis; *Ex* exodermis; *Ml* middle lamella; *Ms* mesophyll; *Sc* sclerenchyma and *Ta* Tannin cells

Cambridge (1978), these structures mainly play a mechanical role. They are often hidden in the sediment and probably perform little photosynthesis. The sheath then serves to maintain the adult leaf sheaths as well as the bundle of younger leaves sheltered in the centre of the shoot. When the leaf blades fall, a band of mechanical weakness leaves the scale around the rhizome. In this final stage, the leaf sheaths form scales that are dead and contain lignified fibre bundles that remain around the rhizome long after the tissues have rotted away (Kuo and Cambridge 1978; Kuo and den Hartog 2007).

Histologically, our observations confirm similarities with leaf blades including isobilateral symmetry, an important mesophyll with some tannin cells and distinct epidermal cell shapes on the adaxial and abaxial surfaces. This asymmetry in the epidermis was not reported in other *Posidonia* species (Kuo and den Hartog 2007). In *P. oceanica*, leaf sheaths differ from leaf blades in the presence of a continuous

subepidermal monolayer of fibre (sclerenchyma) cells on both sides, large parenchyma cells in the mesophyll and the absence of vascular bundles. The subepidermal monolayer of lignified fibre cells lines the majority of the sheath surface and is not present in other seagrasses, even in *P. australis*, where fibres in leaf sheaths were reported as fibre bundles in the mesophyll (see Fig. 5 in Kuo and den Hartog 2007). In *P. oceanica*, fibre bundles were only found at the bases of sheaths close to their attachment to the rhizomes. Towards the sheath base, the fibres disappear from the adaxial side, while those of the abaxial side progressively cluster into bundles. This was not reported by the previous studies and may be specific for *P. oceanica*. This difference in the fibre clustering between leaf sheaths and rhizome also explain from where the different fibre shapes in aegagropiles can come from. After the tissues have rotten, the sclerenchyma fibre cells persist because of their thick lignified cell wall that is hardly degraded (Trevisan 2018). The continuous monolayers of fibre cell of leaf sheaths give thus rise to the flat fibres with or without epidermis. Fibre bundles remain attached to rhizomes longer and give rise to thin fibres in aegagropiles (see Lefebvre et al. 2021) with round cross-sections, because these cells contain lignin, which impedes degradation.

In other seagrasses (Kuo and den Hartog 2007), the mesophyll of the leaf sheaths presents either very large air lacunae, as in *Z. marina*, or small, regularly distributed ones, as in *P. australis*. This is surprisingly not true of *P. oceanica* sheaths, where air lacunae seem to be absent as from leaf blades. Some enlarged meatuses were rarely found, but they cannot be interpreted as the air lacunae of an aerenchyma (Debeir et al. 2019). This absence of aerenchyma in the leaf sheaths of *P. oceanica* contrasts with the literature on other seagrasses, where aerenchyma is reputed to be more developed in leaf sheaths than in leaf blades. As a result, *P. oceanica* leaf sheaths do not float and remain attached to the rhizome.

Another point of contrast between *P. oceanica* and other seagrasses is the absence of vascular bundles in the mesophyll of leaf sheaths, although these were seen in leaf blades. This seems to be a peculiarity of *P. oceanica*; vascular bundles are common in *Z. marina* and *P. australis* leaf sheaths (see Fig. 5 in Kuo and den Hartog 2007) as well as in *P. sinuosa* and *Enhalus acoroides* (L.f., Royle., 1839) (Kuo 1978). This absence or reduction of vascular tissues in the leaf sheaths of seagrasses agrees with the idea that leaf sheaths have little role in the transport and absorption of solutes compared to leaf blades. Along with the presence of a continuous layer of lignified sclerenchyma (fibres) and that of a non-porous cuticle (Kuo and den Hartog 2007), the lack of vascular bundles likely limits the diffusion of nutrients. This excludes the possibility of photosynthesis in the mesophyll but not in the epidermis. In addition, the absence of vascular tissue in the leaf sheaths, at least of adult leaves, of *P. oceanica* suggests that sap transport is interrupted between rhizomes and their adult leaves. The latter must survive independently until they detach. Reciprocally, they are unlikely to sustain the rhizomes with photosynthesis products. These findings on nutrient transport should be considered in ecological models of *P. oceanica* meadows and the carbon–nitrogen–phosphorus (CNP) balance as there is no nutritive link between the adult leaves and the underground organs (roots and rhizomes).

One of the hypothetical consequences of the lack of vascular bundles within the leaf sheaths could be the development of scales. They are rich in lignified fibres and their other tissues rapidly die without photosynthetic production and supply from leaves. Scales thus form resistant protective structures around rhizomes, mainly contributing to the complex formation on which *P. oceanica* meadows develop: the matte (Pergent and Pergent-Martini 1990). *P. oceanica* is the only species of the genus *Posidonia* to form this kind of matte where anoxic zones and zones of intense decomposition are found (Kuo and den Hartog 2007).

- Rhizomes and roots (see Figs. 3 and 4)

The histology of *P. oceanica* rhizomes and roots roughly corresponds to that described for *P. australis* and *P. sinuosa* (Kuo and den Hartog 2007). Their histological organisation is very similar; however, some features related to differences in size and adventitious roots that branch and develop on the undersides of rhizomes at the level of nodes show variation. Rhizomes contrast with roots by the thickness cortical mesophyll (made of broad thin-walled cells) which contains tannin cells. However, the main difference is the presence of numerous fibre bundles in rhizomes just as described in *P. australis* (Kuo and den Hartog 2007). Moreover, *Posidonia* roots and rhizomes do not show the air lacuna or aerenchyma found in *Zostera* and *Cymodocea* seagrasses. This means that dead rhizomes remain and rot in place, forming the matte along with the leaf sheaths. However, fragments of live rhizome also (even alive) sunk in the meadow and, consequently, there is no possible dissemination in this fashion. This accumulation of dead plant fragments of rhizomes, roots, and scales is the primary cause of the matte that enriches the meadow environment with nutrients and fuels the ecosystem. This mode of decomposition seems more similar to what occurs in terrestrial environments, such as forests and humus. Continuous decomposition, therefore, makes reproduction by cuttings possible only locally and not over long distances. With global warming, *P. oceanica* meadows exhibit a greater frequency and intensity of sexual reproduction (Diaz-Almela et al. 2007). The nutrient-rich environment that *Posidonia* induce may also explain the scarcity of root hairs; *P. oceanica* seedlings do exhibit root

hairs, which are essential structures for the dissipation of water kinetic energy (Borovec and Vohník 2018).

The exodermis is composed of several cell layers with thick, non-lignified but suberised walls, as in *P. australis* and *Halophila* roots (du Petit-Thouars, 1806; Kuo and den Hartog 2007) and *Thalassodendron* and *Amphibolis* (Kuo and McComb 1989). Rhizomes differ from roots by the thickness of the cortical mesophyll, which is made of broad thin-walled cells and contains tannin cells. The main difference is the presence of numerous fibre bundles in the rhizomes, as in *P. australis* (Kuo and den Hartog 2007). The outermost fibre bundles are disposed in one or two rows just below the exodermis and then are randomly distributed in the cortical mesophyll and decrease in number towards the stele. They are slightly bigger than those seen in the leaf sheaths. The stele centre is surrounded by the endodermis and pericycle. Other genera, such as *Amphibolis* and *Thalassodendron*, have developed cells with thick lignified walls around the steles of their rhizomes (Kuo and den Hartog 2007).

The structure of the aegagropile fibres and determination of their origin (see Figs. 5, 6 and 7)

As in our previous work (Lefebvre et al. 2021) and confirmed by the results of this study, the fibres composing aegagropiles are categorised as follows: (a) thin, cylindrical fibres with small diameters (0.142 ± 0.051 mm) that are exclusively composed of sclerenchyma cells; (b) flat fibres that are fragments a few cells thick and many more wide (0.462 mm ± 0.095 mm) and are composed of several tissues, always including flat sclerenchyma fibres with mesophyll and/or epidermis, and (c) wide fibres that are larger, irregular in cross-section (2.295 ± 1.380 mm) and appear to be recognisable fragments of roots and rhizomes, the main organs of the *P. oceanica* ecosystem matte.

The comparison of the structure and tissue composition of the fibres found in aegagropiles with the tissues and their disposition in *P. oceanica* organs confirms that aegagropile fibres and elements come almost exclusively from *P. oceanica* organs. Moreover, it allows us to precisely identify the origin of the fibres of each type, a question that was not completely solved in our previous work (Lefebvre et al. 2021). These observations confirm and complement our hypotheses, supporting the idea that the degradation of plant fragments forms aegagropiles. Wide fibres are the remains of rhizomes and roots. These fragments are so big that they retain recognisable characteristics, such as the presence of a central stele. Even sections of peripheral fragments of rhizomes can be identified by the presence of large-fibre bundles in the cortical mesophyll as well as by cross-sections of scales close to their surface.

Their shape and exclusive composition of sclerenchyma fibre cells show that the thin fibres correspond to round fibre bundles. In *P. oceanica* organs, these are only found in the rhizome cortex and at the base of leaf sheaths. At this level, the sclerenchyma fibre cells of the subepidermal monolayer of the abaxial side of sheaths gradually adopt the same disposition as in the rhizomes, i.e., cylindrical bundles. This suggests that the fibre bundles of the outer rows in the rhizome are continuous with those of the sheaths at their attachment sites. This also explains why scales remain firmly attached to rhizomes, allowing them to assume their protective role.

The flat fibres, comprising flat sclerenchyma bundles together with epidermis and/or mesophyll, undoubtedly come from the surfaces of leaves. Leaf blades and leaf sheaths are the only *P. oceanica* organs with subepidermal monolayers of fibre cells. However, the size and aspect of flat fibres in aegagropiles are unlikely to derive from leaf blades. On both sides of the leaf blades, there are discontinuous layers of isolated fibre cells or small clusters of a few cells. In contrast, leaf sheaths are lined on both sides by continuous monolayers of fibre cells. Therefore, it is much more likely that the flat fibres in aegagropiles remain of the leaf sheaths, including their subepidermal sclerenchyma. This does not contradict previous conclusions on the contribution of *P. oceanica* leaves to aegagropiles, but the dominance of fibre from leaf sheaths suggests that aegagropiles are mainly formed by resistant tissues from the *P. oceanica* matte. This is consistent with the aegagropile formation and degradation processes on the seabed we proposed previously (Lefebvre et al. 2021). These processes were thought to involve the hydrodynamics of the water within the ripple marks of sandy areas between meadows and should occur in four steps as follows. The initial formation phase involves the aggregation of fibres around a nucleus (1), e.g., a fragment of rhizome retaining fibres between its scales. In the consolidation phase, the fibres are then cemented by sediment particles (2). Simultaneously, the growth phase continues through the aggregation of new fibres on the outside of the aegagropile (3) in sandy areas close to meadows and under wave influence at shallow depths close to the beach. The degradation phase (abiotic and biotic) of the fibres and the nucleus generates new thin fibres within the aegagropile until the nucleus completely disappears (4). This results in a progressive reduction of diameter (5), while the aegagropiles become spherical. The dominance of cylindrical fibre bundles and broad flat fibres from leaf sheaths, as well as rhizome fragments in the nucleus, suggest that aegagropiles mainly grow by the aggregation of *P. oceanica* debris present in the mat. Surprisingly, the contribution of fibres from leaf blades is minimal, although they dominate the degraded leaf litter. Rather than degradation-resistant litter elements (fibres) contributing to aegagropiles, as we first proposed (Lefebvre

et al. 2021), the results of this study suggest that aegagropiles are the degradation end products of the meadows via the mat. The degradation of detached leaf blades forming the leaf litter should be more rapid, occurring within 6 months (Remy 2016). Moreover, the leaf litter is sometimes transported far from the meadows and seems to offer carbon output from them. In contrast, the degradation of the remaining *P. oceanica* organs (leaf sheaths, roots, and rhizomes) occurs locally, within the matte on which the meadows develop. It constitutes a highly localised form of carbon recycling that may be comparable to terrestrial forest humus.

Fungal colonisation and plant tissue decay (see Figs. 8, 9 and 10)

Our observations showed that fungal hyphae or their remains were regularly observed together with micro-sclerotia and fungal decay traces in the cell walls of various fresh *P. oceanica* organs, as well as in the fibres and plant fragments found in aegagropiles. Micro-sclerotia contribute to the propagation of this mycobiont (Read 1999; Vohník et al. 2015, 2016). This means that fungi are already present in living plants.

Such traces of microbial degradation were also noted in aegagropile fibres in our previous work (Lefebvre et al. 2021). The morphology of the hyphae and micro-sclerotia, especially in the roots and rhizome tissues of fresh *P. oceanica*, is identical to those previously described by Vohník et al. (2015, 2016) in the living roots of the same *Posidonia* species. We, therefore, hypothesise that the fungal hyphae and micro-sclerotia that we observed belong to the same species of microorganism and should correspond to the dark septate endophyte (DSE) identified by Vohník et al. (2015, 2016, 2017).

Via microscopy, Vohník et al. demonstrated that the DSE association was specific to the host organism, *P. oceanica*. During pyrosequencing, they confirmed that the root mycobiome of *P. oceanica* is dominated by a single mycobiont belonging to Aigialaceae (Pleosporales). After a prolonged culture, they were able to deduce the phylogenetic relationships by sequencing large and small subunit nrDNA (LSU and SSU, respectively), as well as ITS nrDNA and DNA-directed RNA polymerase II (RPB2). The results suggested that this fungus belongs to a separate marine biotrophic lineage in the Aigialaceae (Pleosporales) and, therefore, they introduced a new name for this fungus: *Posidoniomyces atricolor* gen. and sp. nov. (Vohník et al. 2019). Several authors (Peterson and Massicotte 2004; Addy et al. 2005; Grünig et al. 2008) had previously observed fungal hyphae (DSE) on the root surface, but for the *P. oceanica* meadow, Vohník et al. (2015) were the first to show that the hyphae penetrate the tissues down to the exodermis. According to Schwarze (2007), ascomycetes can degrade the major components of plant cell walls (PCWs), such as lignin, hemicellulose, and cellulose. They are rare in marine habitats, however, and their degradation activity is lowered underwater due to the lack of oxygen (Singh 2012), which is necessary for the enzymatic digestion of lignin. Vohník et al. (2015, 2016) showed that DSE hyphae degrade PCWs and reduce their diameter to perforate the wall between adjacent cells.

Here, we hypothesise that the morphological features corresponding to the DSE are the black hyphae, their elongated shape and obvious septa, as well as the morphology of the black micro-sclerotia, but these morphological features are common to a broad range of fungi. Perforations of the PCW with thin hyphae and specific degradation of the middle lamella confirm the fungus's ability to degrade PCW components. As described by Vohník et al. (2015), colonisation should start from the surface. The fungus uses long, thin hyphae to fit through intercellular spaces and penetrate the exodermis. We also found fungal growth into the cortical mesophyll. This proliferation degrades the different PCW components, especially the middle lamella, and causes cell death as described by Vohník et al. (2015).

By analogy with terrestrial plants, Vohník et al. (2016) considered the DSE a symbiotic fungus that was associated with *P. oceanica* roots. Thus, as an organism of the rhizosphere, it was believed to be involved in nutrient uptake and nitrogen fixation in seagrasses. However, several arguments could contradict this hypothesis. In the literature, other fungi were found to have penetrated the epidermal cells of *P. australis* roots, but they were shown to lyse the thick PCW of the exodermal cells (Kuo et al. 1981). In this study, the distribution of melanised septate hyphae (hypothesised as the DSE described in Vohník et al.'s studies) is not restricted to roots, unlike that described by Vohník (Vohník et al. 2015, 2016; Vohník 2021). It is much larger, colonising the rhizomes, scales, and leaves (probably old) of living *P. oceanica* plants. This wide distribution of the fungus in *P. oceanica* organs, as well as the frequent fungal remains and degradation traces in aegagropile fibres, allows us to propose that the DSE is involved in the decay of *P. oceanica* organs in the meadow as well as in the matte or litter.

The role of microbial degradation in the formation of fibres in aegagropiles (see Fig. 11)

Fungal endophytes generally need oxygen and nitrogen to develop. Therefore, the living organs of *P. oceanica* represent ideal places for fungal growth, thanks to the cytoplasm in living cells and the presence of an oxygen-rich layer around the roots. Several studies have demonstrated the existence of a thin oxygen-rich layer surrounding the roots of certain marine magnoliophytes, including *P. oceanica*, *Cymodocea rotundata*, *Zostora marina* and *Halophila ovalis* (Pedersen et al. 1998; Connell et al. 1999; Holmer et al.

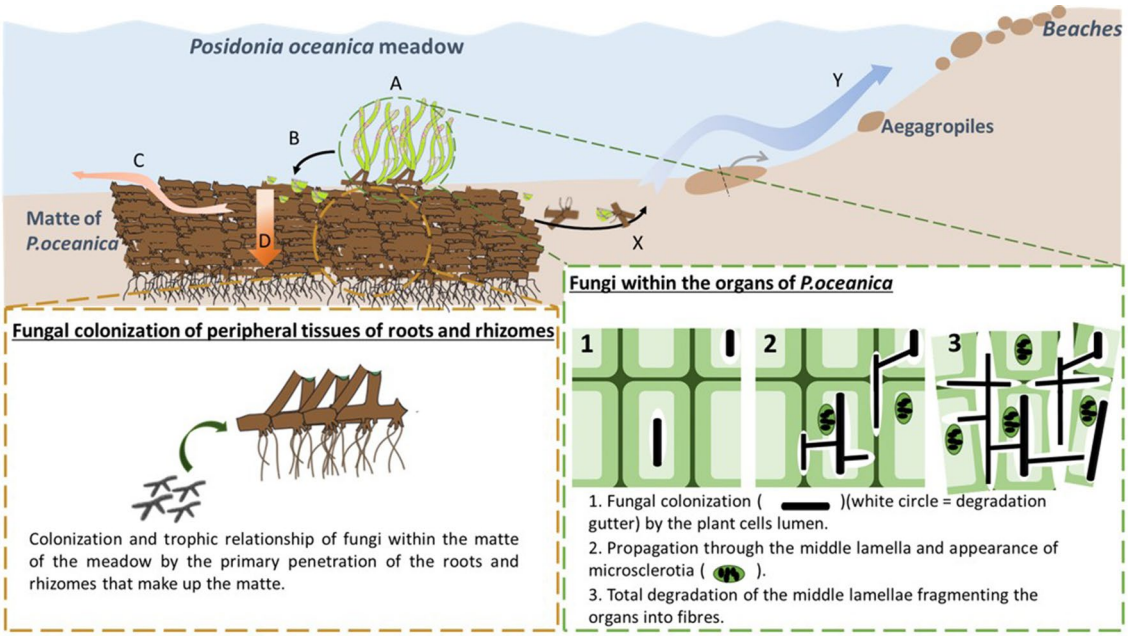

Fig. 11 Diagrammatic representation of two export pathways of organic matter from the *P. oceanica* meadow and the role of fungal degradation in living plants and in the fragmentation of tissues to form plant debris. **A** Living plants of *P. oceanica*; **B** periodic detachment of leaves (in autumn) and export to leaf litter; **C** degradation of the leaf litter away from the meadow (within 6 months); **D** degradation of the remaining leaf sheaths, old rhizomes with roots and accumulation in the mat; X: export of fibres and altered debris from the matte to form aegagropiles on the sandy bottom between meadows; Y: export of aegagropiles to beaches.

2003; Jensen et al. 2005; Abadie et al. 2017). Thus, fungal growth and degradation by fungal endophytes should start in roots and rhizomes, and then move into the leaf sheaths and blades. In different organs, the parenchyma cells are the first targets of fungal colonisation. Their thin walls and wide lumens are easily degraded due to their low lignin content. Our microscopic observations of fresh *Posidonia* organs show more widespread colonisation of parenchyma cells, with up to four hyphae in the lumen of a single parenchyma cell (Vohník et al. 2016; Schmidt et al. 2016). This preferential degradation of the parenchyma fragments the organs into their more-resistant fibres or isolates the stele in rhizomes. Tissue decay continues in the litter and the matte. In the litter, the fungal endophyte can periodically thrive during aerobic periods. The degradation of leaf blades rapidly destroys them through the disappearance of the mesophyll and non-lignified vascular bundles, leaving only isolated sclerenchyma fibres.

The environment of the matte in the *P. oceanica* ecosystem is conducive to the presence of fungi, particularly due to the prevalence of dead organs within this 3D structure (rhizomes and roots), thus leaving free access to the proliferation of fungi within dead parenchyma cells. However, the degradation of non-lignified tissues releases many more fibres than that in the leaf litter. These fibres are mainly the flat and large-fibre bundles of leaf sheaths and rhizomes. All of these continue to degrade in the mat. As the fungal endophyte has the ability to degrade lignin, hyphae were shown to perforate PCWs and to first specifically alter the middle lamella. This causes the separation of sclerenchyma cells and the splitting of large bundles into thinner fibres. As we suggested previously (Lefebvre et al. 2021), this process could result in the formation of thin fibres from the larger ones, producing the different fibre types found in aegagropiles. Biotic degradation, along with hydrodynamics, accelerates the fragmentation of the *P. oceanica* organs that comprise the foundation of the meadow. Furthermore, the fungi could continue to live and degrade the fibres within aegagropiles as long as they remain on the seabed and have an oxygen supply. Fungal hyphae die when they desiccate in aegagropiles on beaches. This allows us to assume that the matte of the *P. oceanica* seagrass ecosystem is essential for the activity of the fungal endophyte, which we believe is the major microorganism degrading *P. oceanica* tissues within the matte and, as a result, generating the different fibres that form into aegagropiles. The suggestion that it is the only microorganism responsible for *P. oceanica* tissue decay is much more speculative. However, this fungus is very dominant, as the number of hyphae and degradation traces found in fresh *P. oceanica* tissues and their remains in aegagropiles reveal. Comparatively, bacterial cells and bacterial degradation traces, although they are easy to identify, are very scarce; bacterial degradation leaves stripes within the perforations or degradation tunnels, which were

not observed in this study (Nilsson and Singh 2014, Singh 2012; Trevisan 2018).

The fungus does enrich the meadow with soluble nutrients. It may also favour nitrogen retention in its chitinous walls. This nutrient concentration and diffusion could explain the scarcity of root hairs on *P. oceanica* roots.

Conclusion

During this study (Fig. 11), histological observations of *Posidonia oceanica* organs confirmed their organisation and indicated some structural peculiarities that distinguish *P. oceanica* from most seagrasses, as well as from other species of the genus *Posidonia*. These are (1) the absence of aerenchyma within all of the plant organs, which reduces their buoyancy and confines them to the sea floor and (2) the absence of vascular bundles in the mesophyll tissue of the leaf sheaths, which become dead scales around the rhizomes after the leaf blades detach.

Posidonia oceanica can form a leaf litter of leaf blades and a matte that mixes degrading roots and rhizomes with leaf sheaths, all consolidated with sediment (Fig. 11). This matte is a specific area produced by *P. oceanica,* where plant organs decay slowly in place below the meadow. It provides a rich compost that recycles nutrients in the *P. oceanica* meadow. This is quite different from the litter that occurs on the seafloor away from the meadow and is composed mainly of detached leaf blades, which rot within 6 months. The matte also hosts another ecosystem and benefits other macrofauna invertebrates. Therefore, the meadow and its matte seem similar to terrestrial ecosystems in organisation and function. This may be a structural and adaptive consequence of the Messinian crisis that *P. oceanica* meadows faced when the Mediterranean Sea nearly dried up.

The results of this study also provide evidence that most of the fibres found in aegagropiles derive from *P. oceanica* organs degrading in the matte and strongly suggest that a dark septate endophytic fungus is the main degrading microorganism (Fig. 11A and D). This fungus begins to degrade plant cell walls in living *P. oceanica* organs, first targeting parenchyma cells and generating the various aegagropile fibre types by separating and fragmenting the sclerenchyma fibre bundles through the preferential alteration of the lignified middle lamella in leaf sheaths and rhizomes (Fig. 11A). These decay slowly, possibly over a period of years, and aggregate together with sediment to form aegagropiles in the ripple marks of sandy areas close to the meadow (Fig. 11X). As we previously suggested, aegagropiles grow, their components continue to degrade, and they evolve until they are brought to the beach by a storm (Fig. 11Y). Biotic degradation by microorganisms then ceases due to desiccation on beaches.

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