2018

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10.1016/j.orggeochem.2018.07.017
This is an Author's Accepted Manuscript of:
Kaal, J., Serrano, O., José, C., & Rencoret, J. (2018). Radically different lignin composition in Posidonia species may link to differences in organic carbon sequestration capacity. *Organic Geochemistry*, *124*, 247-256.
https://doi.org/10.1016/j.orggeochem.2018.07.017

Radically different lignin composition in *Posidonia* species may link to differences in organic carbon sequestration capacity

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Abstract

There is considerable variability in the ability of seagrass ecosystems to sequester organic carbon (C_{org}) in their sediments, which act as natural carbon sinks contributing to climate change mitigation. In this work, we studied the chemistry of two *Posidonia* seagrass species aiming to elucidate whether differences in chemical composition might explain differences in their C_{org} sequestration capacity. Pyrolysis-GC-MS and Thermally assisted Hydrolysis and Methylation (THM-GC-MS) showed a remarkable difference in phenolic compound patterns between *P. oceanica* and *P. australis* bulk plants and individual organs (leaves, sheaths, roots and rhizomes). The lignin of *P. australis* generates a series of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) products that are typical of herbaceous plants, whereas *P. oceanica* is particularly rich in *p*-hydroxybenzoic acid (*p*BA) derivatives. The structural characteristics of the lignins were further investigated by two-dimensional Nuclear Magnetic Resonance (2D-NMR) and Derivatization Followed by Reductive Cleavage (DFRC), focusing on sheath tissues. The analyses confirmed important differences in the lignin content (19.8% in *P. australis* and 29.5% in *P. oceanica*) and composition between the two species; intriguingly, the cell-walls of *P. oceanica* sheaths were highly enriched in *p*BA, a component that was completely absent in *P. australis*. 2D-NMR and DFRC further revealed that *p*BA was esterified to the lignin, acylating the γ-OH of the lignin side-chain. Interestingly, *P. oceanica* lignin presented an extremely high degree of *p*-hydroxybenzoylation in both guaiacyl (73%) and syringyl (61%) lignin units, the highest *p*-hydroxybenzoylation degree reported in plant lignins to date. It is tempting to conclude that the higher soil C_{org} storage capacity of *P. oceanica* ecosystems might be related to the higher abundance of *p*BA-rich lignin and its recalcitrant nature.

Keywords: *Posidonia australis*, *Posidonia oceanica*, lignin, *p*-hydroxybenzoates, analytical pyrolysis, 2D-NMR, DFRC, blue carbon

1. Introduction

Coastal vegetated ecosystems – blue carbon ecosystems (tidal marshes, mangroves and seagrasses) – occupy 0.2% of the ocean surface, but contribute 50% of carbon burial in marine sediments (Duarte et al., 2005; Nellemann et al., 2009). Seagrass meadows of the genus *Posidonia* rank amongst the most effective blue carbon sinks. More specifically, the organic carbon (C_{org}) storage in *P. oceanica* and *P. australis* (ranging from 80 to 880 Mg C_{org} ha^{-1}; Serrano et al., 2016a) is exceptional compared to other seagrass species (ranging from 6 to 120 Mg C_{org} ha^{-1}; Lavery et al., 2013; Campbell et al., 2015; Miyajima et al., 2015). Furthermore, *P. oceanica* (750 Mg C_{org} ha^{-1} and 84 g m^{-2} yr^{-1}) has up to three-fold higher C_{org} stocks and up to seven-fold higher C_{org} sequestration rates compared to *P. australis* (250 Mg C_{org} ha^{-1} and 12 g m^{-2} yr^{-1}) (Serrano et al., 2016a). These differences have been attributed, among other reasons, to the vertical rhizomal growth (i.e., orthotropic) of *P. oceanica*, producing a deep mat of plant debris embedded within the sediment that can persist for millennia (Boudouresque et al., 1980; Gobert et al., 2006; Mateo et al., 1997; Lo Iacono et al., 2008; Serrano et al., 2014).

Differences in the chemical composition of seagrass tissues can play a key role in the accumulation and preservation of C_{org} in seagrass sediments (Klap et al., 2000; Torbatinejad et al., 2007; Trevathan-Tackett et al., 2015, 2017). Nevertheless, molecular-scale studies on seagrass composition have focused on free low molecular weight organic matter (OM), and mostly in *P. oceanica* (Ncibi et al., 2009; Zapata and McMillan, 1979; Cuny et al., 1995). Little attention has been given to the composition of the structural biopolymers, such as lignin, even though degradation/preservation dynamics ("recalcitrance") largely depends on that OM fraction and the capacities of the microbial community to decompose such debris (Trevathan-Tackett et al., 2017). It is well known that plant recalcitrance is caused, to a large extent, by the presence of lignin, a highly complex aromatic cell-wall polymer typical of vascular plants that plays crucial roles for plant growth and development, as well as for pathogen protection. The lignin is formed by radical coupling of mainly three *p*-hydroxycinnamyl alcohols, namely, *p*-

coumaryl, coniferyl, and sinapyl alcohols, differing in their degree of methoxylation. These monolignols give rise to the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively, producing a variety of structures and linkages within the lignin polymer (Vanholme et al., 2010). The lignin content as well as the monomer composition widely varies with taxon, cell type, and tissue (Harris, 2005). In general terms, gymnosperm lignins are composed of G- units with minor amounts of H-units, woody and non-woody angiosperm lignins are G-S lignins with variable S:G ratios, whereas grasses (monocots) contain all the three units (Boerjan et al., 2003). Additionally, abundant research has indicated that lignins are also produced from γ-acylated monolignol conjugates, with acetates, *p*-hydroxybenzoates, *p*-coumarates, and ferulates (del Río et al., 2008; Martínez et al., 2008; Lu et al., 2015; Karlen et al., 2016). Lignin is present in considerable amounts in both *Posidonia* species, especially in the fiber-rich leaf sheaths (Kuo, 1978; Ncibi et al., 2009). A recent study using analytical pyrolysis techniques (Kaal et al., 2016) showed that the roots, rhizomes and sheaths (not leaves) of *P. oceanica* contain large amounts of phenolic material with a significance abundance of *p*-hydroxybenzoic acids (*p*BA), but the lignin of *P. australis* has not been studied on the molecular scale.

In the present study we describe the molecular composition of several *P. australis* and *P. oceanica* plant organs, by conventional analytical pyrolysis (Py-GC-MS) and thermally assisted hydrolysis and methylation (THM-GC-MS). Furthermore, we study the lignin composition of sheaths in both *Posidonia* species, by state-of-the-art bidimensional Nuclear Magnetic Resonance (2D-NMR) and Derivatization Followed by Reductive Cleavage (DFRC). The ultimate objective is to provide the necessary framework for assessing differences in C_{org} storage capacity based on differences in the recalcitrance of *Posidonia* tissues.

2. Material and Methods

2.1 Sample materials and pre-treatment

Plants of *P. australis* were sampled in Shark Bay (Indian Ocean, Western Australia), while plants of *P. oceanica* were sampled in Port Lligat (Spain). All materials were rinsed with distilled water, assuring that epiphytes attached to the leaves and other allochthonous tissues were removed, and subsequently oven-dried at 60 ºC until constant weight. Subsamples of *P. australis* were kept as "whole plants", whereas others were subdivided into "leaves", "leaf sheaths" and "roots+rhizomes". Subsamples of *P. oceanica* were obtained for "leaf sheaths" only, which complements existing data for leaves, roots, rhizomes and three subfractions of sheaths published by Kaal et al. (2016). This enabled comparisons between *P. australis* and *P. oceanica*. All samples were ball milled to powder and then split for subsequent analyses.

For Py-GC-MS and THM-GC-MS, powder samples of whole plants of *P. australis*, the dissected organs of *P. australis* and sheaths of *P. oceanica* were analyzed without any further treatment. For 2D-NMR and DFRC, subsamples of *P. australis* and *P. oceanica* sheaths were previously extracted with water (3 x 40 mL), 80% ethanol (3 x 40 mL), and finally with acetone (2 x 40 mL), by sonicating in an ultrasonic bath for 30 min, centrifuging (8000 rpm, 25 min) and eliminating the supernatant. The extractive-free samples were dried and then ball-milled for 2 h using a Retsch PM 100 planetary ball mill at 600 rpm. The lignin content of *P. australis* and *P. oceanica* samples were determined as Klason lignin (Tappi, 2004).

For detailed NMR analyses, lignin-enriched cell wall preparations (cellulolytic enzyme lignin; CEL) were obtained by enzymatically hydrolyzing the polysaccharides fraction with Cellulysin (Calbiochem, San Diego, CA), a crude cellulase preparation from *Trichoderma viride* that also contains hemicellulase activities. 200 mg of extracted ball-milled material were suspended in 20 mM NaOAc buffer (30 mL, pH 5.0), in a 50 mL centrifuge tube, together with 8 mg of Cellulysin, and the reaction slurry was incubated at 30 ºC for 48 h. The solids were recovered by centrifugation (8000 rpm, 4 ºC, 20 min), and the process was repeated twice with fresh buffer and enzyme. The lignin-enriched preparation was finally recovered by filtration, washed with distillated water and then lyophilized.

2.2 Analytical pyrolysis

Pyrolysis-GC-MS and THM-GC-MS were performed as described by Kaal et al. (2016). Briefly, approximately 0.5 mg of plant materials were introduced into quartz tubes and embedded in quartz wool, which were both pre-combusted at 900 ºC using a muffle furnace. The sample-containing quartz tubes were pyrolyzed using a Pyroprobe 5000 (CDS Analytical) at 650 ºC (setpoint temperature) for 10 s (heating rate 10 ºC ms^{-1}). The pyrolysis interface, isothermal at 325 ºC, was connected to a 6890 gas chromatograph (Agilent Technologies) equipped with a HP-5MS non-polar column (length 30 m; internal diameter 0.25 mm; film thickness 0.25 µm). The oven temperature program was from 50 to 325 ºC at 20 ºC min^{-1}. The mass selective detector (Agilent 5975B) operated in electron impact (EI) mode at 70 eV, with the ion source at 230 ºC and the quadrupole detector at 150 ºC, and measuring fragments in the *m*/*z* 50–500 range. The interfaces between the pyrolyzer, gas chromatograph and the mass selective detector were at 325 ºC. The same analytical conditions were used for THM-GC-MS, but prior to the analysis a droplet of 25%(aq) tetramethyl ammonium hydroxide (TMAH, Sigma-Aldrich) was added to the sample-containing quartz tubes and allowed to impregnate for one hour (at room temperature).

The sheath materials of *P. oceanica* and *P. australis* were analyzed by both methods in triplicate. For the sheaths, relative proportions of the products were calculated as the percentage of the total quantified peak area (% TQPA), using the main fragment ions (*m*/*z*) of each product, creating separate datasets for Py-GC–MS and THM-GC–MS, focusing on the phenolic compounds (phenols, guaiacols, syringols). A one-way analysis of variance (ANOVA) was applied to identify statistically significant differences in relative proportions of compounds, groups of compounds and compound ratios, between the two *Posidonia* species. Replicates were not obtained for the other samples (whole plant and different organs of *P. australis*) and the interpretation of the chromatograms is based on visual inspection.

2.3 Two-dimensional Nuclear Magnetic Resonance (2D-NMR)

For the 2D-NMR study, whole cell walls of *P. oceanica* and *P. australis* sheaths were analyzed in two different ways: i) "*in situ*" (at the gel state) according to the method previously described (Kim and Ralph, 2010), and ii) after acetylation, which provides NMR spectra with a better resolution (Lu and Ralph, 2003). In the case of the samples without derivatization, ~85 mg of extracted and finely milled sheaths were swelled in 1 mL DMSO-d_6:pyridine-d_5 (4:1, v/v), until forming a gel inside the NMR tube. For the NMR analysis of acetylated samples, 50 mg of ball-milled extractive-free samples were previously acetylated with *N*-methylimidazole and acetic anhydride (Lu and Ralph, 2003), and were subsequently dissolved in chloroform-*d*. In addition, *P. oceanica* lignin enriched preparation (CEL) was also acetylated and dissolved in DMSO-d_6:pyridine-d_5 (4:1). Heteronuclear single quantum coherence experiments (2D-HSQC) were acquired at 300 K on a Bruker AVANCE III 500 MHz spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5 mm TCI cryoprobe. The spectra were acquired using an adiabatic pulse sequence (hsqcetgpsisp.2) and the following NMR parameters were set: spectral width from 10 to 0 ppm in F2 (^{1}H) using 748 data points for an acquisition time (AQ) of 74.8 ms, an interscan delay (D1) of 1 s, and from 200 to 0 ppm in F1 (^{13}C) using 256 increments of 32 scans, for a total acquisition time of 2 h 34 min. The $^1J_{CH}$ used was 145 Hz. Spectra were processed using Bruker's Topspin 3.5 software and typical matched Gaussian apodization in F2 dimension (LB=-0.1 and GB=0.001) and squared cosine-bell apodization in F1 dimension (LB=0.3 and GB=0.1) were applied. The residual peaks of DMSO (δ_C/δ_H 39.5/2.49) and $CHCl_3$ (δ_C/δ_H 77.0/7.26) were used as internal references. 2D-HSQC correlation signals were assigned by literature comparison (Rencoret et al., 2013; Lu et al., 2015). The lignin compositions of *P. oceanica* and *P. australis*, in terms of H-, G- and S-lignin units, and *p*BA were estimated based on the volume integrals of the ^{1}H$-^{13}$C correlation signals. The signals C_2–H_2 (from G units) and $C_{2,6}$–$H_{2,6}$ (from H and S lignin units, and from *p*BA) were used to estimate their abundances. It is important to note that *p*BA quantitation relative to the lignin might be overestimated because of the longer relaxation times of these end-units compared to the rapidly relaxing polymer.

178 *2.4 Derivatization Followed by Reductive Cleavage (DFRC)*

179 The DFRC degradation method was performed according to the protocol by Lu et al.
180 (2015). Fifty mg of extractive-free *P. australis* and *P. oceanica* whole plant samples were
181 treated with 4 mL of an acetyl bromide/acetic acid solution (1/4 v:v), and stirred at 50 ºC for 3
182 h. The solvents were removed by rotary evaporation at reduced pressure. The products were
183 then suspended in 5 mL of dioxane/acetic acid/water, 5:4:1 (v/v/v) and 200 mg of powdered Zn
184 was added, and the mixture was stirred at room temperature for 24 h. After completion, the
185 reaction mixture was transferred into a separation funnel with dichloromethane (10 mL) and
186 saturated ammonium chloride (10 mL). The aqueous phase was adjusted to pH < 3 by adding
187 3% HCl(aq), the mixture vigorously mixed, and the organic layer separated. The water phase
188 was extracted twice more with 10 mL of dichloromethane. The combined dichloromethane
189 fractions were dried over anhydrous Na_2SO_4, and were evaporated on a rotary evaporator. The
190 residue was then acetylated with 5 mL of pyridine:acetic anhydride solution, 1:1 (v:v) for 24 h.
191 The acetylated lignin degradation products were collected after rotary evaporation of the
192 solvents and analyzed by GC/MS on a Saturn 4000 (Varian, Walnut Creek, CA) instrument
193 fitted with a medium-length high-temperature capillary column (DB5-HT, 15 m × 0.25 mm i.d.,
194 0.1 μm film thickness; from J&W Scientific). Helium was used as carrier gas at a rate of 2 mL
195 min^{-1}. The samples were injected with an autoinjector (Varian 8200), which was programmed
196 from 120 °C (0.1 min) to 340 °C at a rate of 200 °C min^{-1} and held at the maximum temperature
197 until the end of the analysis. The oven temperature was programmed from 120 °C (1 min) to
198 340 °C (10 min) at a rate of 10 °C min^{-1} and the transfer line temperature was set at 300 °C
199 during the analysis.

200

201

202 **3. Results and discussion**

203

204 *3.1 Analytical pyrolysis of Posidonia australis organs*

205 The Py-GC-MS chromatograms of the whole plant sample of *P. australis* contain large
206 peaks for products of polysaccharides (acetic acid, 3/2-furaldehyde, 4-hydroxy-5,6-dihydro-
207 (2*H*)-pyran-2-one, 5-hydroxymethyl-2-dihydrofuraldehyde-3-one, 1,4:3,6-dianhydro-α-D-
208 glucose and levoglucosan), guaiacyl lignin (4-vinylguaiacol, $C_{3:1}$-guaiacols), syringyl lignin (4-
209 methylsyringol, 4-vinylsyringol, $C_{3:1}$-syringols), chlorophyll (phytadienes) and C_{16}-fatty acid, a
210 combination that is typical of a predominantly lignocellulose material (Fig. 1A). Among the
211 phenolic products, 4-vinylphenol and G and S products are dominant, typical of herbaceous
212 species in general (Hedges and Mann, 1979). The chromatograms of individual *P. australis*
213 organs show that the leaves (Fig. 1C) release, besides G and S moieties (from lignin) and
214 polysaccharides, relatively large amounts of fatty acids (probably occurring freely) and
215 phytadienes (from phytol in chlorophyll). Sheaths (Fig. 1E) have relatively large peaks of G and
216 S moieties, indicative of high lignin content. Roots and rhizomes (Fig. 1G) produce
217 predominantly G-type phenols and conspicuous peaks for catechol and methylcatechols, which
218 reflect the relatively large tannin content. The results obtained for *P. australis* whole plant and
219 individual organs are in concert with those of *P. oceanica* (Kaal et al., 2016), with the key
220 exception of the distribution of phenolic compounds.
221 These results are corroborated by the THM-GC-MS fingerprints of the same materials
222 (Fig. 1B-H). All samples were prolific in C_{16}-fatty acid methyl ester (FAME) and a
223 carbohydrate product with *m*/*z* 101, 111, 187 and 219 (Yeloff et al., 2008). None of the samples
224 from *P. australis* produced a significant peak for *p*BA methyl ester. The roots and rhizomes of
225 *P. australis* produce clear peaks for 1,3,5-trimethoxybenzene and 2,4,6-trimethoxytoluene,
226 which is in agreement with their interpretation as tannin markers (Nierop et al., 2005). The leaf
227 sample of *P. australis* produced a peak for 9,10-dimethoxy-C_{16}-FAME from cutin in leaf
228 cuticles (del Río and Hatcher, 1998) and all samples showed peaks for typical carbohydrate
229 products (e.g. methylated C_6-metasaccharinic acids) and G and S type phenols.
230
231 *3.2 Analytical pyrolysis of P. australis and P. oceanica sheaths*

The sheaths showed the "cleanest" fingerprint of lignin-derived phenolic compounds (least signal from tannin, polysaccharides, chlorophyll, cutin and fatty acids) for both *P. australis* and *P. oceanica* (Fig. 1). Therefore, leaf sheaths were used for a further comparison of lignin in *P. oceanica* and *P. australis* (triplicate analysis). The Py-GC-MS chromatograms show that only *P. oceanica* (Fig. 1I) produces a dominant peak for phenol, whereas *P. australis* has a more heterogeneous fingerprint of G phenols (4-vinylguaiacol, $C_{3:1}$-guaiacols) and S phenols (4-vinylsyringol, $C_{3:1}$-syringols) (Fig. 1E). Markers of non-decarboxylated intact *p*BA (*m*/*z* 121 base ion) were found only in the chromatograms of *P. oceanica*. The ANOVA analysis of the relative abundance of the phenolic products show that phenol and *p*BA compounds from Py-GC-MS are significantly enriched in *P. oceanica* and all other compounds (including C_1- and C_2-alkylphenols and most G and S compounds) are enriched in *P. australis* ($P < 0.001$; Table 1). Moreover, ratios that describe the phenolic composition such as *p*BA/total phenols (*p*BA/Ph_t) –calculated as the sum of phenol and *p*-hydroxybenzoic acids divided by the sum of the all phenols and methoxyphenols (Table 1)–, G/Ph_t and S/Ph_t, shows that these parameters are significantly different between the two species (ANOVA, $P < 0.001$ for all ratios). This can also be inferred for the THM-GC-MS fingerprints (compare Fig. 1F for *P. australis* and Fig. 1J for *P. oceanica*; Table 2) albeit with lower degrees of significance ($P < 0.05$). These results provide unambiguous evidence of a major difference in lignin composition of the sheaths of the *Posidonia* species analyzed. Furthermore, the abundance of phenol (Py-GC-MS) and *p*BA (THM-GC-MS) of *P. oceanica* is unrivalled by any other plant material. These compounds also dominate millennial soil materials underneath *P. oceanica* meadows (Gadel and Bruchet, 1987; Kaal et al., 2016), which is indicative of the good preservation of *p*BA-rich lignin. Hence, ratios such as *p*BA/total phenols (*p*BA/Ph_t) can be used to estimate the contribution of *P. oceanica* in blue carbon soils and sediments, likely enhancing the capability to discern between autochthonous and allochthonous sources using stable carbon isotopes alone (Kennedy et al., 2010)

3.3 2D-NMR analyses of sheaths of P. oceanica and P. australis

The Klason lignin contents of the extractive-free sheath materials were 19.8% and 29.5% (of dry weight sample) for *P. australis* and *P. oceanica*, respectively. This remarkably high lignin content of sheath materials of *P. oceanica* was also reported by Ncibi et al. (2009). A detailed analysis of the lignin composition in both species was performed by 2D-NMR.

The aromatic regions of the HSQC NMR spectra of the acetylated whole cell-wall from *P. oceanica* and *P. australis* revealed notable differences between them (Fig. 2). The HSQC spectrum of *P. australis* (Fig. 2A) shows correlation signals corresponding to the aromatic rings of typical H-, G- and S-lignin units. The signal of syringyl units ($S_{2,6}$) is readily observed at δ_C/δ_H 103.8/6.59, whereas the signals of guaiacyl units are detected at δ_C/δ_H 110.8/6.93, 118.6/6.86 and 122.1/6.99, and from H-lignin units at δ_C/δ_H 128.2/7.38, corresponding to $C_{2,6}H_{2,6}$ ($C_{3,5}H_{3,5}$ is overlapped with $G_{5/6}$ signals). A semi-quantitative analysis, based on the signals volume integrals, shows that the lignin from *P. australis* is enriched in G-lignin units with an H:G:S composition of 7:57:36 (molar ratio). The S/G ratio obtained upon NMR (S/G of 0.6) is in good agreement with that obtained from Py-GC-MS (S/G of 0.5) (Table 1).

On the other hand, the aromatic region of the HSQC spectrum of *P. oceanica* (Fig. 2B) shows the typical signals of H-, G- and S-lignin units, and two prominent signals at δ_C/δ_H 121.5/7.12 and 131.0/7.91, which correspond unequivocally to the $C_{3,5}H_{3,5}$ ($pBA_{3,5}$) and $C_{2,6}H_{2,6}$ ($pBA_{2,6}$) correlation signals of *p*BA (Leplé et al., 2007; Lu et al., 2015; Rencoret et al., 2013, 2018). The semi-quantitative analysis gave a H:G:S lignin composition of 3:81:16, but accompanied with a *p*BA content of 102% (relative to total lignin defined as H+G+S = 100%). 2D-NMR data corroborated the pyrolysis results, and indicated that the lignin in *P. oceanica* is more enriched in G-units than the lignin in *P. australis*, and that *p*BAs are the main components in *P. oceanica*, whereas *p*BA is completely absent in *P. australis*. It is noted that the high abundance of *p*BA might be exacerbated by the fact that *p*BA could be found as terminal group, which are overestimated by 2D-HSQC (Lu et al., 2015).

In order to determine whether the *p*BA moieties in *P. oceanica* are linked to lignin or to carbohydrates, a lignin-enriched cell wall preparation obtained after enzymatic hydrolysis of carbohydrates (CEL) was also analyzed by 2D-HSQC and its spectrum was compared with that of the whole cell walls (Fig. 3). The HSQC spectrum of the whole cell walls (Fig. 3A) displays correlation signals of carbohydrates, especially from glucose (Gl_1-Gl_6), together with signals of *p*BA. After the enzymatic hydrolysis and removal of the carbohydrates, the above mentioned glucose signals strongly decreased, whereas signals from *p*BA remained unchanged (Fig. 3B). This implies that the vast majority of the *p*BA units are not connected to the polysaccharides in the cell-wall of *P. oceanica* sheath. Indeed, a comparison between the aromatic regions of the spectra, after increasing their intensity (Fig. 3C and D), shows that the *p*BA/(H+G+S) ratio remains essentially unaffected by the enzymatic hydrolysis of carbohydrates, which suggests that *p*BA units in *P. oceanica* sheaths are bound to the lignin moiety. This is an important result because if the *p*BA would have been located primarily in glycosidic linkages, as was hypothesized by Kaal et al. (2016), degradation of the carbohydrates would release the *p*BA units. The incorporation of the *p*BA in lignin structures implies a stronger (theoretical) inherent chemical recalcitrance, especially under anoxic conditions (Arnosti et al., 1994; Traoré et al., 2017).

Taking into account that the *p*BA can only be connected to lignin via ester (acting as acylating group) or via ether (whose formation involves the 4-OH group) linkages, an acetylation assay was performed to determine whether the 4-OH of *p*BA was free or etherified. The 2D-HSQC of the non-acetylated and acetylated CEL preparation of *P. oceanica* clearly shows the shift in the *p*BA signals (especially $pBA_{3,5}$ that moves from δ_C/δ_H 115.0/6.84 to 121.8/7.21) after acetylation (Fig. 4), which indicates that the 4-OH groups of *p*BA in *P. oceanica* occur freely and are not etherified. Consequently, the *p*BA moieties in *P. oceanica* sheath are bound to lignin through ester linkages, acylating the γ-OH of the lignin side-chain, and bearing a free phenolic (4-OH) group, as also occurs in the lignins of other monocotyledonous plants (Rencoret et al., 2013, 2018; Lu et al., 2015; del Río et al., 2017).

312 Note that the positioning of *p*BA as terminal and pending substructures to G-dominated lignin is
313 similar to that of neolignans isolated from the rhizomes of *P. oceanica* (Bitam et al., 2012).
314
315 *3.4 DFRC analysis*
316 The incorporation of *p*BA into the lignin polymer of *P. oceanica* was further
317 conclusively demonstrated by using DFRC, a degradation method that cleaves β-ether linkages
318 (the most abundant linkages in the lignin polymer) but leaves esters unaltered (Lu et al., 2015).
319 This method has been recently employed to confirm that *p*BA are also found acylating the lignin
320 of some palm fruit endocarps (del Río et al., 2017; Rencoret et al., 2018). The chromatograms
321 of the DFRC degradation products of *P. oceanica* and *P. australis* sheaths are presented in Fig.
322 5. Both seagrasses released the *cis*- and *trans*-isomers of *p*-hydroxyphenyl (*c*H, *t*H), guaiacyl
323 (*c*G, tG), and syringyl (*c*S, *t*S) lignin monomers (as their acetylated derivatives) arising from
324 normal (γ-OH) units in lignin. The H:G:S lignin compositions obtained upon DFRC were of
325 6:54:40 for *P. australis* and 1:68:31 for *P. oceanica*, confirming the results obtained by Py-GC-
326 MS and 2D-NMR. More importantly, the chromatogram of *P. oceanica* was dominated by
327 prominent peaks of the *cis*- and *trans*- isomers of γ-*p*-hydroxybenzoylated G- (*c*G-*p*BA, *t*G-
328 *p*BA) and S-lignin units (*c*S-*p*BA, *t*S-*p*BA), which were identified by their mass spectra (Fig.
329 5C), and which were completely absent in *P. australis*. DFRC also indicated that *p*BA in *P.*
330 *oceanica* lignin extensively acylates both guaiacyl (73%) and syringyl (61%) lignin units. This
331 observation was crucial to confirm that *p*BAs are linked to a lignin polymeric network of G and
332 S moieties, and that the vast majority of G and S units carry covalently bound *p*BA.
333 Lignin acylation with *p*BA has been reported in other monocotyledonous plants, such as
334 in palms (Rencoret et al., 2013, 2018; Lu et al., 2015; del Río et al., 2017; Karlen et al., 2017),
335 as well as in some eudicotyledonous plants like poplar, willow and aspen (Venverloo, 1971;
336 Landucci et al., 1992; Morreel et al., 2004). Interestingly, the pattern of *p*-hydroxybenzoylation
337 of *P. oceanica* widely differs from those observed in the lignins of other plants where *p*BA are
338 preferentially acylating the S-lignin units whereas G-units are barely *p*-hydroxybenzoylated (Lu

et al., 2015; del Río et al., 2017; Rencoret et al., 2018). On the other hand, the monolignol-*p*BA ester conjugates in *P. oceanica* are responsible not only for the high extent of *p*-hydroxybenzoylation of the lignin polymer but also for the production of γ-*p*-hydroxybenzoylated neolignans (Bitam et al., 2012). Knowledge on the microbial or abiotic decay of either lignin with an extraordinary *p*BA load or enigmatical neolignan is not available, which implies that we can not elaborate on the link between the peculiar structure of polymeric phenolic compounds in *P. oceanica* and its capacity as a C_{org} sink. Molecular assessment of seagrass tissues before and after laboratory or field incubation assays might contribute to establishing this link.

3.5 Implications on C_{org} preservation dynamics

Gobert et al. (2006) and Waycott and Les (2000) reported that there is a large genetic difference between the Mediterranean-endemic *P. oceanica* and the Australian counterparts, such as *P. australis*. Their habitat separation may have taken place as early as the Late Eocene, associated with the disappearance of the Tethys Sea. Considering that the Australian seagrasses have a phenolic composition that is similar to that of most angiosperms and in particular herbaceous species, it remains to be assessed how (and why) the molecular skeleton of *P. oceanica* had undergone a big evolutionary shift towards a lignin (or neolignan) complex dominated by *p*BA-acylated G units.

The tissues of both *Posidonia* species studied are rich in lignin. Especially the leaf sheaths are heavily lignified, which has been linked to the persistence of sheath materials after plant death (Kuo, 1978; Torbatinejad et al., 2007). It is well-known that lignin has a large preservation potential under anoxic conditions such as those of mat deposits in such seagrass ecosystems (Burdige and Lerman, 2006), which might be related to the capacities of both *Posidonia* species as C_{org} sinks. However, this effect of lignin composition on the preservation of dead OM from *Posidonia*, and the relative importance of other biogeochemical factors (e.g. Serrano et al., 2016b), is yet to be determined. The main chemical differences between *P.*

366 *oceanica* and *P. australis* are the higher lignin content and the high proportion of *p*BA in the
367 former and H, G and S moieties in the latter. It was conclusively demonstrated that the abundant
368 *p*BA units in *P. oceanica* form part of the lignin macromolecule, possibly transferring an
369 enhanced chemical recalcitrance to the cell-wall materials. These results are in agreement with
370 the description of *P. oceanica* as an outlier among seagrasses in terms of their biology, ecology
371 and C_{org} sink capacity (Walker et al., 1999; Fourqurean et al., 2012).
372 Even though the biogeochemical behavior of lignin in *Posidonia* seagrasses remains
373 largely unknown, detailed molecular characterization of macromolecular tissues seems to be a
374 promising approach to unravel the processes and pathways of C_{org} preservation, including
375 evaluating the magnitude and rate of greenhouse gas emissions with disturbance (Lovelock et
376 al., 2017).
377
378 **4. Conclusions**
379 Detailed analyses of the composition of *P. oceanica* and *P. australis* tissues, and
380 especially their lignin composition, showed that they are very different. The lignin of *P.*
381 *australis* presents a moderate enrichment of G-units (H:G:S molar ratio of 7:57:36) whereas the
382 lignin of *P. oceanica* is largely enriched in G-units (3:81:16) and is extensively acylated at the
383 γ-OH with *p*BA. *P. oceanica* lignin presented the highest *p*-hydroxybenzoylation degree (73%
384 of G- and 61% of S-lignin units are acylated with *p*BA) reported in plant lignins to date. It
385 seems likely that differences in C_{org} storage capacity between *P. oceanica* and *P. australis* are
386 partly explained by differences in the abundance of lignin and its molecular composition, which
387 may enhance the chemical recalcitrance of cell walls. Molecular characterization of more types
388 of seagrasses ecosystems (e.g. living plants and detritus embedded within soils) together with
389 incubation studies to assess the lability of different molecular structures in oxic and anoxic
390 conditions, are necessary to unravel the importance of phenolic composition for C_{org}
391 accumulation-storage-release dynamics.
392

Acknowledgments

This study has been funded by the Spanish projects CTQ2014-60764-JIN and AGL2017-83036-R (co-financed by FEDER funds). OS was supported by an ARC DECRA DE170101524. We thank Dr. Manuel Angulo for running the NMR experiments that were acquired on a Bruker Avance III 500 MHz instrument from the NMR facilities of the General Research Services of the University of Seville (SGI CITIUS). We thank the anonymous reviewers for valuable suggestions.

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FIGURE CAPTIONS

Figure 1. Pyrolysis-GC-MS (left) and THM-GC-MS (right) total ion current chromatograms of whole plant *P. australis* (**A** and **B**) and its plant organs: leaf (**C** and **D**), leaf sheath (**E** and **F**) and root+rhizome (**G** and **H**). The chromatograms of the sheath material of *P. oceanica* (**I** and **J**) are also displayed. Symbols for Py-GC-MS: CAT= catechol, F16= C_{16}-fatty acid, G= guaiacol, 4MG= 4-methylguaiacol, 4VG= 4-vinylguaiacol, C3G= *trans*-4-propenylguaiacol, C3S= *trans*-4-propenylsyringol, P= phenol, Ps1= acetic acid, Ps2= 3/2-furaldehyde, Ps3= 5-hydroxymethyl-2-dihydrofuraldehyde-3-one, Ps4= levoglucosan, Ph1= pristene, Ph2= phytadiene 1, Ph3= phytadiene 2. Symbols for THM-GC-MS (for *p*BA, G and S derivatives, see Table 1): F16= C_{16}-fatty acid methyl ester, Ps5-Ps8= unidentified carbohydrate products (Ps8= *m/z* 101,111,187,219 compound, *see text*), T1= 1,3,5-trimethoxybenzene, T2= 2,4,6-trimethoxytoluene.

Figure 2. 2D-HSQC NMR spectra of acetylated *P. australis* (**A**) and *P. oceanica* (**B**) sheath whole cell walls. The main lignin structures identified are depicted at the bottom. *p*BAs are the most abundant aromatic substructures in *P. oceanica*, whereas they are completely absent in *P. australis*. The abundance of the different lignin units (H, G and S) are calculated from contour volume-integrals, and *p*BA is estimated based on H+G+S=100%. It is important to note that *p*BA are found as endgroup on the lignin polymer, presenting a higher mobility and therefore their signal integration is over-represented in 2D-HSQC NMR outputs.

Figure 3. 2D HSQC spectra of acetylated *P. oceanica* sheath whole cell walls (**A**) and its corresponding lignin enriched preparation, CEL (**B**), in DMSO-d_6:pyridine-d_5 (4:1,v:v). Note that the intensities of the spectra **A** and **B** have been selected to clearly show the relative proportion carbohydrates/*p*BA. If the intensity of the spectra are augmented, the correlation signals of H-, G- and S-lignin units can also be observed (**C** and **D**).

Figure 4. 2D-HSQC NMR spectra of *P. oceanica* EL preparation, before (**A**) and after (**B**) acetylation, in DMSO-d_6:pyridine-d_5 (4:1,v:v). The shifts in the *p*BA correlation signals (principally $pBA_{3,5}$) after acetylation demonstrate that *p*-hydroxybenzoate on lignin occurs in its free phenolic form, susceptible to being acetylated, and are not etherified (in this case, the acetylation of *p*BA group cannot take place and the *p*BA signals should therefore remain in essentially the same place within the HSQC spectrum).

Figure 5. Reconstructed ion chromatograms (*m/z* 192+222+252+121, characteristic for the H, G, S and *p*BA compounds) of the DFRC degradation products released from (**A**) *P. australis* and (**B**) *P. oceanica*. *c*H, *t*H, *c*G, *t*G, *c*S, and *t*S represent the normal *cis*- and *trans*-*p*-coumaryl, guaiacyl and syringyl monomers, respectively (as their acetyl derivatives). G-*p*BA and S-*p*BA represent the coniferyl and sinapyl alcohol γ-acylated with *p*BA. (**C**) The structures of these compounds and the mass spectra of G-*p*BA and S-*p*BA.

Table 1. Py-GC-MS products of the sheaths of *P. australis* and *P. oceanica*, and average relative proportions (%) with standard deviation (SD) of three replicates. ANOVA differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS $P > 0.05$) and the *Posidonia* species with higher concentration is indicated. *p*BA = *p*-hydroxybenzoic acid, Ph_t = total phenolic compounds, G = guaiacyl, S = syringyl, H = *p*-hydroxyphenyl.

	Main source	*P. australis*		*P. oceanica*		ANOVA
		(%)	SD	(%)	SD	(*P* value)
phenol		26.2	5.7	87.8	0.7	*** *P. oceanica*
4-hydroxybenzoic acid (*p*BA) methyl ester	*p*BA	0.0	0.0	1.7	0.2	*** *P. oceanica*
4-hydroxybenzoic acid (*p*BA)	*p*BA	0.0	0.0	1.0	0.1	*** *P. oceanica*
4-methoxybenzoic acid methyl ester	*p*BA	0.0	0.0	0.2	0.0	*** *P. oceanica*
2/3-methylphenol		4.2	0.2	1.6	0.2	*** *P. australis*
4-methylphenol		14.7	0.3	1.5	0.3	*** *P. australis*
C_2-alkylphenol		2.5	0.1	0.3	0.1	*** *P. australis*
C_2-alkylphenol		3.7	0.1	0.4	0.1	*** *P. australis*
C_2-alkylphenol		0.9	0.1	0.1	0.0	*** *P. australis*
4-vinylphenol	H	4.3	0.4	0.4	0.1	*** *P. australis*
guaiacol	G	5.9	0.7	0.8	0.1	*** *P. australis*
4-methylguaiacol	G	5.4	0.6	0.7	0.1	*** *P. australis*
4-ethylguaiacol	G	1.9	0.4	0.2	0.0	** *P. australis*
4-vinylguaiacol	G	10.6	1.4	1.4	0.3	*** *P. australis*
4-allylguaiacol	G	1.0	0.2	0.1	0.0	*** *P. australis*
cis-4-propenylguaiacol	G	0.8	0.1	0.1	0.0	*** *P. australis*
trans-4-propenylguaiacol	G	3.4	0.5	0.5	0.1	*** *P. australis*
syringol	S	1.8	0.4	0.2	0.0	** *P. australis*
4-methylsyringol	S	1.2	0.2	0.1	0.0	*** *P. australis*
4-ethylsyringol	S	0.6	0.1	0.0	0.0	*** *P. australis*
4-vinylsyringol	S	4.9	0.8	0.4	0.2	*** *P. australis*
4-allylsyringol	S	1.0	0.1	0.1	0.0	*** *P. australis*
cis-4-propenylsyringol	S	0.8	0.1	0.1	0.0	*** *P. australis*
trans-4-propenylsyringol	S	4.4	0.5	0.2	0.1	*** *P. australis*
*p*BA/Ph_t		0.4	0.1	0.9	0.0	*** *P. oceanica*
H/Ph_t		0.1	0.0	0.0	0.0	*** *P. australis*
G/Ph_t		0.4	0.0	0.0	0.0	*** *P. australis*
S/Ph_t		0.2	0.0	0.0	0.0	*** *P. australis*
S/G		0.5	0.0	0.3	0.1	** *P. australis*

Table 2. THM-GC-MS products of the sheaths of *P. australis* and *P. oceanica*, and average relative proportions (%) with standard deviation (SD) of three replicates. ANOVA differences are indicated with asterisks (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS $P > 0.05$) and the *Posidonia* species with higher concentration is indicated. *p*BA = *p*-hydroxybenzoic acid, Ph_t = total phenolic compounds, G = guaiacyl, S = syringyl, H = *p*-hydroxyphenyl.

	Main source	*P. australis* (%)	SD	*P. oceanica* (%)	SD	ANOVA (*P* value)
4-methoxybenzaldehyde (P4)		2.0	1.0	0.0	0.0	* *P. australis*
x-methoxybenzoic acid methyl ester (P6 isomer)		0.0	0.0	20.6	14.6	NS
4-methoxybenzoic acid methyl ester (P6)	*p*BA	3.2	1.5	71.8	16.9	** *P. oceanica*
3,4-dimethoxybenzaldehyde (G4)	G	7.0	3.7	0.4	0.2	* *P. australis*
3,4-dimethoxybenzoic acid methyl ester (G6)	G	6.5	3.4	0.8	0.6	* *P. australis*
3,4,5-trimethoxybenzaldehyde (S4)	S	4.1	1.1	0.1	0.1	** *P. australis*
2-methoxyethenyl-3,4-dimethoxybenzene (G7,G8)	G	18.8	6.9	3.2	1.5	* *P. australis*
3,4,5-trimethoxybenzoic acid methyl ester (S6)	S	0.6	0.2	0.1	0.0	* *P. australis*
cis/trans-1-(3,4,5-trimethoxybenzene)-2-methoxyethylene (S7,S8)	S	35.6	14.3	2.1	0,7	* *P. australis*
threo/erythro-1-(3,4-dimethoxybenzene)-1,2,3-trimethoxypropane (G14)	G	5.4	3.2	0.0	0,0	* *P. australis*
threo/erythro-1-(3,4-dimethoxybenzene)-1,2,3-trimethoxypropane (G15)	G	3.3	2.5	0.1	0,0	NS
trans-3-(3,4-dimethoxyphenyl)-3-propenoic acid methyl ester (G18)	G	7.1	2.5	0.7	0,7	* *P. australis*
threo/erythro-1-(3,4,5-trimethoxybenzene)-1,2,3-trimethoxypropane- (S14)	S	4.4	2.8	0.0	0,0	NS
threo/erythro-1-(3,4,5-trimethoxybenzene)-1,2,3-trimethoxypropane- (S15)	S	1.8	1.0	0.0	0,0	* *P. australis*
*p*BA/Ph_t		0.0	0.0	0.9	0,0	*** *P. oceanica*
H/Ph_t		0.0	0.0	0.0	0,0	* *P. australis*
G/Ph_t		0.5	0.1	0.1	0,0	*** *P. australis*
S/Ph_t		0.5	0.1	0.0	0,0	** *P. australis*
S/G		1.0	0.4	0.4	0,0	NS