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Flavonoid pattern inheritance in the allopolyploid *Spartina anglica* – comparison with the parental species *S. maritima* and *S. alterniflora*

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Chemical compounds studied in this article:

Schaftoside (CID 442658)

Isoschaftoside (CID 3084995)

Isoorientin 2''-*O*-(β-glucosyl) (CID 9986192)

Isoorientin (CID 114776)

Isovitexin 2''-*O*-(β-glucosyl) (CID 25202838)

Isovitexin (CID 161672)

Isoscoparin 2''-*O*-(β-glucosyl) (CID 72193659)

Isoscoparin (CID 442611)

Tricin 7-diglucuronide (RN 380468-519)

Isoorientin 2''-*O*-(6-(*E*)-*p*- caffeoylglucoside) (RN 1637757-97-1)

Isoorientin 2''-*O*-(6-(*E*)-*p*-feruloylglucoside) (RN 220948-75-4)

Isoscoparin 2''-*O*-(6-(*E*)-*p*-caffeoylglucoside)

Isoscoparin 2''-*O*-(6-(*E*)-*p*-feruloylglucoside) (RN 372113-55-8)

Isovitexin 2''-*O*-(6-(*E*)-*p*-feruloylglucoside) (RN 220948-77-6)

Tricin 7-*O*-glucoside (RN 32769-01-0)

Tricin 4'-*O*-(*erythro*-β-guaiacylglyceryl) ether 7-*O*-β -glucoside (CID 101193641)

Tricin 4'-*O*-(*threo*-β-guaiacylglyceryl) ether 7-*O*-β-glucoside (RN 462100-42-2)

Tricin 4'-*O*-guaiacylglyceryl ether *erythro* (RN 369390-51-2)

Tricin 4'-*O*-guaiacylglyceryl ether *threo* (RN 369390-52-3)

Tricin (CID 5281702)

Abbreviations used in Figures and Tables

BDL: below detection limits

Sam: *Spartina maritima*

Sal: *Spartina alterniflora*

San: *Spartina anglica*

Abstract

The invasive species *Spartina anglica* arose in Europe by a cross between the Afro-European species *S. maritima* (native, paternal ancestor) and the introduced North American *S. alterniflora* (invasive, maternal ancestor). Aqueous methanolic extracts were prepared from plant tissue for chemotaxonomical comparison between the three species and determination of the phenolic pattern inheritance in *S. anglica*. A total of 20 phenolic compounds were detected in the aerial tissues of *S. anglica* and *S. alterniflora,* but only seven in *S. maritima*. They were isolated from their respective crude extracts, and their structures were determined according to spectroscopic data analysis and chemical evidence. They all belong to the flavonoid class, with 13 of them identified as *C*-glycoflavonoid and seven as *O*-glycoflavonoid. All these products were detected for the first time from *S. anglica,* fourteen of them for the first time from *S. alterniflora*, and three of them for the first time from *S. maritima*. The individual concentrations in the three species were determined by quantitative HPLC. The two parental species were found to differ markedly in their foliar phenolic fingerprint, whereas that of *S. anglica* showed a clear maternal dominance. Eight of the fourteen major compounds identified were of maternal origin among which, six were over-expressed, only three were from paternal origin but under-regulated, while two originated from the two parents. As far as we know, this work represents the first exhaustive report of the phenolic fingerprints of *S. alterniflora* and *S. anglica* and of the phenolic pattern inheritance in *S. anglica.* The similarity in the phenolic chemistry of the introduced and invasive *S. alterniflora* to its progeny could play a role in the physiological vigour and invasion success of *S. anglica*. This work provide a foundation for further studies, considering the reported biological activities of *C*-glycosidic flavonoids and tricin derivatives, and the lack of knowledge of the ecological chemistry of the genus *Spartina*.

Keywords: Spartina anglica; S. maritima; S. alterniflora; flavonoid inheritance; quantitative HPLC; LC/MS; NMR.

Introduction

Spartina Schreb. (Poaceae, subfamily Chloridoideae Kunth ex Beilschm., tribe *Zoyseae* Benth) is a small genus consisting of approximately 15 species, most of which are native to the New World (Chevalier 1953; Saarela, 2012). Until the beginning of 19th century, *S. maritima* (Curtis) Fernald was the only species found in Europe. During the 19th century, various members of the genus spread outside of their native boundaries and become invasive in their new settlement. In Europe, this is the case of *S. alterniflora* Loisel., which native range is the Atlantic and Gulf Coasts of the United States. The introduction of *S. alterniflora* in England and its subsequent crossing with the European native species *S. maritima* resulted in the sterile F1 hybrid *Spartina* × *townsendii.* After it doubled in chromosome number, it became able to sexually reproduce as a new much more vigorous allotetraploid species *S. anglica* C.E. Hubbard (see as examples: Ayres and Strong, 2001; Baumel et al., 2002). *S. anglica* was first recorded in France in 1906 and has now largely ousted *S. maritima* from much of its native range in Western Europe.

At first, *S. anglica* was considered a useful plant because of its ability to trap large amounts of sediment, its rapid clonal growth, strong competitive and reproductive capability, and high tolerance for tideland habitats, including low or high salt marsh, mudflats and cobble beaches. As a result, *S. anglica* has been intentionally introduced to coastal and estuarine mudflats throughout the world to aid in foreshore protection, reclamation of land and stabilisation of marshes. Only the introductions into South America and South Africa were unsuccessful. However, some of the very traits that make *S. anglica* valued are also the greatest causes for concern. Through a combination of intentional planting and natural or accidental dispersion, the species has become a notorious invasive species throughout the world. This very aggressive invader significantly alters both the physical structure and biological composition of tidal marshes, mudflats and creeks (Dethier and Hacker, 2004; Nehring and Hesse, 2008). In many cases, *S. anglica* has dramatically changed tidal marsh characteristics, excluding native species, which results in significant harmful ecological impacts and its classification in the list of the "100 of the World's Worst invaders" (Invasive Species Specialist Group, 2019). The maternal ancestor *S. alterniflora* has also become an urgent topic due to its invasiveness in many places where it was introduced (Campos et al., 2004; Stiger-Pouvreau et al., 2015; Xie and Han, 2018).

The problem of invasive species and their control is one of the most pressing applied issues in ecology today. The main causes of invasion success have been reviewed by Catford et al. (2009). Among them, the "Novel Weapons Hypothesis" attributes exotic plant success to the fact that upon introduction many exotics are liberated from their specialist herbivores and pathogens. As a result,

invasive species may gain disproportionate competitive advantages through the effects of specialized metabolites to which species in invaded areas have not had the opportunity to adapt (Callaway and Ridenour, 2004). In addition, hybridization may influence the expression and evolution of resistance to natural enemies. Consequences of hybridization for the generation of qualitative and quantitative variations in specialized chemistry and the possible effects on hybrids fitness in relation to herbivore resistance have been reported (Cheng et al., 2011). However, although plant chemical defences are thought to play an important role in the success of invasive plants, often little is known about natural variation in their allelochemicals and much remain unknown about the inheritance of many of their defensive chemical compounds. This is especially the case of most of the members of *Spartina* Schreb., whose specialized metabolism has been poorly documented.

Phenolic compounds constitute one of the largest groups of plant specialized metabolites. They take part in the regulation of seed germination, cooperate in regulating the growth of plants, and play an important role in plant defence against pathogens, herbivores, and environmental stress (Kulbat, 2016; Treutter, 2005). The capacity to produce phenolics as defensive compounds against herbivores or against competitors is often involved in the competition between alien and native plant species. Highest concentrations of total phenolic compounds and differences in their effects were found in a comparative study of invasive versus native plants (Kim and Lee, 2011). We have recently characterized the phenolic fingerprint of the endangered species *S. maritima* (Grignon-Dubois and Rezzonico, 2019). This prompts us to do the same with *S. alterniflora* and *S. anglica.* The aims of the present study were: (1) to investigate the phenolic expression variation between the two parental species and the allopolyploid of their F1 hybrid; (2) to determine the inheritance pattern of phenolic compounds in *S. anglica*; (3) to obtain some clues regarding factors contributing to the reproductive success of this species.

Although *S. anglica* is not *sensu stricto* a F1 hybrid of *S. maritima* and *S. alterniflora* but its allopolyploid derivative, terms such as parent, paternal, maternal, daughter … have been used throughout this publication. It is noteworthy that *Spartina* species are now found in the International Plant Index Name as *Sporobolus* (IPNI, 2019). However, the former name *Spartina* remains still used in The World Register of Marine Species (WORMS 2019), The Plant List and in the recent literature, which led us to do the same in this study (see paragraph 4.2.1 for details).

2. Results

Plant samples were collected on the same date at two locations on the French coasts of the southern Bay of Biscay *i.e*. Arcachon Bay for *S. maritima* and *S. anglica* and Hossegor Lake for *S. alterniflora* (Fig. 1). For each species, three independent plant samples (S1-S3) were collected along a transect perpendicular to the shoreline. In addition, samples consisting of 70 individuals were randomly collected from the whole meadow of each species (samples G). Each of the twelve resulting collections was separately extracted with aqueous methanol, and then the phenolic content of the resulting extracts was analysed.

2.1. Determination of the foliage phenolic fingerprint of Spartina species by HPLC-DAD and LC/MS

HPLC-DAD stand alone and coupled with LC/MS were used to determine the phenolic profile of the three species. The crude extracts were subjected to two different columns (Polartec and HTec), which provided a check for co-elution problems. While HTec is a classical C18 hydrophobic phase, the Polartec column is a C18 phase with embedded amido group leading to pronounced hydrophilic properties and separation mechanisms based both on hydrophobic (van der Waals) and polar interactions. The chromatograms obtained with the two columns are depicted in Figure 2. Examination of the HPLC profiles revealed important qualitative and quantitative differences in the phenolic composition of the two parental species *S. maritima* and *S. alterniflora*, while *S. anglica* indicated substantial similarities with *S. alterniflora.* A total of sixteen peaks were detected for *S. alterniflora* and *S. anglica* and only seven peaks for *S. maritima* (Fig. 2). The hydrophilic properties of the Polartec column allowed the separation of peaks **1**, **2**, **3**, which were in part co-eluted on HTec, and also of peak **9**. The HTec column allowed a perfect separation of peaks **7** and **8**, which were co-eluted on Polartec, and of peaks **10** and **11**, which were poorly separated on Polartec. Compounds were identified from retention times, mass spectra, on-line UV spectra and comparison to standard when available.

The three phenolic profiles were largely dominated by flavonoids. Comparison of retention time and UV spectra with those of reference standards indicated the absence of flavonols and flavones aglycones, except traces of tricin identified in the three species by comparison with an authentic standard (full list of standards used is given in Table S1, and on-line UV spectra in Fig S1; supplementary materials). Structural assignments were achieved by direct comparison with standards (compounds **4** and **10**), or with previously positively identified compounds from *S. maritima* (compounds **7**-**8, 11**; Grignon-Dubois and Rezzonico, 2019). Repeated analysis of the samples following acid hydrolysis permitted positive identification of compounds **1**-**8** and **10**-**14** as flavone *C*-glycosides and compounds **9** and **16** as flavone *O*-glycosides. Peaks **4**, **7**, **8**, **10**, **11** were

respectively identified as homoorientin, isovitexin 2"-*O*-glucoside, isoscoparin 2"-*O*-glucoside, isovitexin and isoscoparin (Fig. 3, Tables S2-S3).

2.2. Purification of the extracts and structure identification of compounds **1–20**

Compounds **1**-**20** (Fig. 3) were isolated from the respective crude extracts by successive chromatography on C-18 silica gel, and then analysed by spectroscopic methods.

Peaks **1** and **2** differed by their retention time (respectively 12.8 and 13.3 min), but showed both identical parent ion with a mass of *m/z* 565 (M^+) and fragmentation patterns and UV spectra (Fig. S1). They were found with about the same 55/45 ratio in both *S. anglica* and *S. alterniflora,* but were not detected in *S. maritima*. Prolonged acid or alkaline hydrolysis failed to liberate glycoside moieties or to alter the products suggesting that they were *C*-glycosyl flavonoids. They were isolated as a mixture, but small amount of peak **1** was obtained as a pure compound. The UV (λ_{max} 336 nm) and NMR data clearly indicate that the two compounds are 6, 8-di-*C*-glycosylapigenin (absence of signals for H-6 and H-8, two doublets (each 2 H, *J* ~ 8.8 Hz) and two methines (each 2 C) typical of a 1, 4-disubstituted benzene moiety). The ^{13}C chemical shifts of the two isomers were very close, making assignment of each signal difficult. The ^{1}H-NMR spectrum of the carbohydrate portion was complex due to the overlap of the four sugar moieties. In contrast, 2D NMR spectra and ^{1}H chemical shifts and coupling constant of the aromatic rings allowed identification of compound **1** as apigenin 6-*C*-β-glucopyranosyl-8-*C*-α **-**arabinopyranoside (schaftoside) and compound **2** as apigenin 6-*C*-α -arabinopyranosyl-8-*C*-β-glucopyranoside (isoschaftoside) (Asenstorfer et al., 2006; Du et al., 2011; Hopper et al., 2010; Xie et al., 2003; Zhang et al., 2015) (see supplemental materials for details). Their order of elution on C18 column is in agreement with data from the literature (Chimichi et al., 1998; Rehwald et al., 1994).

Peak **3** (M 610) was isolated as a pure sample. ^{1}H and ^{13}C spectra clearly showed the presence of two glucose moieties. Only two singlet protons were observed in the aromatic regions, which confirm that sugars are attached to a carbon. The presence of a *C*-bound β-glucopyranosyl unit was evident from the coupling values between H-1" and H-2" (9.9 Hz). No change was observed upon alkaline treatment of compound **3**, while acid hydrolysis led to glucose and compound **4** identified as isoorientin by comparison with an authentic standard (Table 2). Downfield shift of the proton H-2" and carbon C-2" signals (respectively at 4.37 and 81.1 ppm compared to its monoglucoside (**4**: 4.03 and 70.1 ppm) suggest that the second sugar is attached to the C-2" position of the first sugar. The 2"→ 1'" interglycosidic connectivity was confirmed by the HMBC correlation between H-2" and C-1'". The second sugar anomeric carbon signal at δ 105.9 ppm suggested the second sugar to be an *O*-glycoside. Comparison with literature data confirmed

the structure of **3** as isoorientin 2''-*O*-glucoside (Besson et al., 1985; Markham et al., 1998; Xie et al., 2016).

Isolation and characterization of peaks **7**, **8**, **10** and **11** from *S. alterniflora* and *S. anglica* confirmed their identity with those isolated from *S. maritima* and were respectively assigned as isovitexin 2''-*O*-glucoside (**7**), isoscoparin 2''-*O*-glucoside (**8**), isovitexin (**10**), and isoscoparin (**11**) (Grignon-Dubois and Rezzonico, 2019).

Peak **9** was isolated as a pure sample from the three species. Its mass spectrum indicated an $[M]^+$ ion at *m/z* 683. Acid hydrolysis of **9** led to compound **20**, which was unambiguously identified as 4′, 5, 7-trihydroxy-3′, 5′-dimethoxyflavone (tricin) by comparison to standard and spectroscopic data (UV, MS and NMR). The presence of two glucuronic units in compound **9** was confirmed by eight characteristic carbon signals observed in the region 81 to 71 ppm, two carbonyl carbons at δ 172.2 and 172.9 ppm, and two anomeric protons at δ 5.15 ppm (d) and 4.57 (d) in the ^{1}H NMR spectrum, which correlated respectively with carbon at δ 98.5 and 103.4 ppm in HSQC. The β-configuration of the glucuronic units was proved by the coupling constant (J = 7.2 and 7.8 Hz) for the anomeric proton. The 2''→ 1''' interglycosidic connectivity was confirmed by the HMBC correlations between H-1''' (4.57 ppm) and C-2'' (81.0 ppm), and between H-2'' (3.58 ppm) and C-1''' (103.0 ppm). The position of attachment of the diglucuronic unit was deduced from the ^{1}H and ^{13}C NMR data and confirmed by HMBC correlations. The downfield shifts of the H-6 and H-8 signals observed in compound **9** (respectively 6.56 and 7.14 ppm) compared to the unsubstituted tricin (6.21 and 6.56 ppm) are in agreement with *O*-7 glucuronylation. A similar effect was observed for the C-6 and C-8 signals (respectively 100.2 and 96.6 ppm with compound **9** versus 98.5 and 94.1 ppm for tricin). This assignment was confirmed by the HMBC correlation observed between the H-1'' anomeric proton signal (δ 5.15 ppm) with the aromatic carbons assigned to C-7 (163.1 ppm). Comparison with literature data confirmed the structure of **9** as tricin 7-*O*-glucuronopyranosyl-(1→2)-*O*- glucuronopyranoside (Harborne and Hall, 1964; Kim et al., 2008; Li et al., 2016).

The on-line UV spectra of peaks **5, 6**, **12**-**14** (Fig. S1) showed a dissymmetric and more intense long wavelength band than compounds **1**-**4**, **7**-**11** and **15**, which is characteristic for flavonoid glycosides acylated by hydroxycinnamic acid (Markham, 1982). The hydroxycinnamates **5** (M 772), **6** (M 786), and **12** (M 786) were not in sufficient concentration in the crude extract to be isolated as pure compound and fully identified by NMR. However, on alkaline hydrolysis **5** yielded isoorientin 2''-*O*-glucoside (**3**) and caffeic acid, **6** yielded isoorientin 2''*O*-glucoside (**3**) and ferulic acid, and **12** yielded isovitexin 2''-*O*-glucoside (**7**) and caffeic acid. On acid hydrolysis, **5** and **6** yielded isoorientin (**4**), while **12** yielded isovitexin (**10**) (Table 2). These chemical behaviors associated with the UV and MS data allowed identification of **5** as isoorientin 2''-*O*-

caffeoylglucoside (Kowalska et al., 2014), **6** as isoorientin 2''-*O*-feruloylglucoside, and **12** as isoscoparin 2''-*O*-caffeoylglucoside (Markham et al., 1998). Compounds **13** and **14** were isolated as a mixture. Alkaline hydrolysis led to ferulic acid along with isovitexin 2''-*O*-glucoside (**7**) and isoscoparin 2''-*O*-glucoside (**8**), while acid hydrolysis afforded ferulic acid along with isoscoparin (**11**), isovitexin (**10**), and vitexin resulting from a Wessely-Moser rearrangement (Walace and Mabry, 1970) (Table 2). The NMR spectra of the mixture **13**-**14** confirmed both the presence of two *(E)*-feruloyl moieties and two 2''-*O*-linked glucopyranosyl residues. Analysis of the sugar signals with reference to those of isovitexin 2''-*O*-glucoside (**7**) and isoscoparin 2''-*O*-glucoside (**8**) revealed the downfield shift of the protons H-6''' (+0.63 ppm) and of the C-6'''carbon (+1.9 ppm). Such shifts typically indicate acylation at the C-6''' (Besson et al., 1985; Markham, 1982). All together, these results allowed identification of **13** as isoscoparin 2''-*O*-(6-(*E*)-feruloylglucoside) and **14** as isovitexin 2''-*O*-(6-(*E*)-feruloylglucoside) (Abou-Zaid et al., 2001, Besson et al., 1985; Markham et al., 1998).

Six minor compounds (**15-20**) were also found in *S. anglica* and *S. alterniflora*, and three (**18**-**20**) in *S. maritima*, but their respective concentration in the crude extract were below the limit of detection, except for compound **16**. However, they were concentrated in the less polar sub-fractions during the purification process, and small amounts were isolated in mixture, of which compound **20** was unambiguously confirmed as tricin (NMR, LC/MS, UV and comparison to standard). The molecular weight of compound **15** was determined to be 492, and its UV spectrum was found identical to those of tricin (λ_{max} 353 nm). Compounds **16** and **17** differed in their retention time (respectively 17.7 and 18.5 min on HTec column), but showed identical on-line UV (λ_{max} 340 nm) and mass spectra (*m/z* 689 in positive mode), which was compatible with the molecular formula $C_{33}H_{36}O_{16}$. Compounds **18** and **19** also differed in their retention time (respectively 26.8 and 27.1 min on HTec column), but showed identical on-line UV (λ_{max} 340 nm) and mass spectra (*m/z* 527 in positive mode compatible with the molecular formula $C_{27}H_{27}O_{11}$). Upon acid treatment, **15** led to **20**, while **16-17** led to **18-19**, which is in agreement with the observed difference in molecular weight (162), due to loss of a glucose group (Table 2). These data suggested that compounds **16** and **17** are *O*-glucoside of compounds **18** and **19,** while **15** was assigned to tricin 7-*O*-glucoside (Kwon et al., 2002).

Sub-fractions containing mixture of these products were isolated and analysed by NMR. The ^{1}H NMR spectra (DMSO-d_6) were very complex due to overlapping signals, especially between 5.5 and 3.0 ppm. However, two sets of four signals (intensity 2:1:1:1) and two sets of three multiplets (intensity 1:1:1) were observed in the aromatic region, which suggested for **16**-**19** a tricin-type structure linked to a phenolic ring. Additionally, the spectra of fractions dominated by **16-17** (F2) and **18-19** (F4) showed the presence of two sets of methoxy group which differed in their intensity

and chemical shift with the most intense at about 3.82-3.85 ppm and the less at about 3.73-3.74 ppm. This suggests that both flavone fragment and aromatic ring were substituted by methoxy groups.

Fraction F2 contains a mixture of **16** and **17** (ratio 36:64) along with smaller amount of **15.** A symmetrical tri-substituted B-ring was evident from the two-proton singlets for H-2' and H-6' respectively at 7.35 (compound **16**) and 7.37 ppm (compound **17**) in combination with methoxyl resonances (s, 6 H) at 3.88 (**16**) and 3.85 ppm (**17**). Two sets of coupled doublets (J = 2 Hz) at respectively 6.48 and 6.94 ppm (compound **16**) and 6.46 and 6.93 ppm (compound **17**) were typical for meta coupled proton H-8 and H-6, while the one-proton singlets at 7.16 (compound **16**) and 7.03 (compound **17**) were assignable to H-3. Two sets of three multiplets overlapped between 6.69 and 6.98 ppm and singlets at 3.73 (**16**) and 3.75 ppm (**17**) were in agreement with a guaiacyl ring. The aliphatic regions showed the presence of glucose- and glycerol-type moieties. These data suggested that **16** and **17** were composed of tricin linked at *O*-4' by an identical guaiacylglycerol fragment and differed only by the stereochemistry of the aliphatic part of the molecules. The downfield shifts of the H-6 and H-8 signals in compound **16** (respectively 6.48 and 6.94 ppm) and compound **17** (6.46 and 6.93 ppm) suggest that the glucosyl fragment was attached to *O*-7. It was confirmed by the presence of resonances at 12.87 and 8.78 ppm for compound **16**, and 12.96 ppm and 8.75 for compound **17**, which eliminate the possibilities of glycosylation at C-5 (flavone fragment) or at C-4'' (guaiacyl fragment). In addition, since the ring B has to be symmetrically substituted, the ether between tricin and the guaiacylglyceryl fragment can only be at C (4')-*O*.

By comparing these data with literature reports, the diastereoisomeric pair **16/17** was 'identified as the two epimers of tricin 4'-*O*-(*β*-guaiacylglyceryl) ether 7-*O*-*β*-glucopyranoside (Bouaziz et al., 2002; Bukhari et al., 2016; Colombo et al., 2005; Lee et al., 2015a), and consequently **18**-**19** as the two epimers of tricin 4'-*O*-(*β*-guaiacylglyceryl) ether. Compounds **18**-**19** were obtained in mixture with compounds **17** and **20**. Insufficient sample quantitites did not allow further purification and the identity of the isomeric pair **18/19** could not be fully confirmed by NMR. However, it was possible to analyze the aromatic and methoxy regions in the ^{1}H NMR spectra, and the results confirmed assignment to the tricin 4'-*O*-(*β*-guaiacylglyceryl) ether pair of isomers (Bouaziz et al., 2002; Huang et al., 2010; Lee et al., 2015b; Nakajima et al., 2003; Syrchina et al., 1992; Wenzig et al., 2005) (Tables S3 in supplement materials).

Due to overlap of signals in the 4-5 ppm region, it has been impossible to determine the ^{3}J (H-7'', H-8'') coupling values to assign the *erythro*/*threo* stereochemistry. However, compounds **16**-**19** were respectively eluted at 17.7, 18.5, 24.6 and 24.9 min. Comparison of these retention values with those reported by Bouziz et al. (2002) in similar analytical conditions (Rt for M 688: *erythro* 15.1 min, *threo* 15.6 min; Rt for M 526: *erythro* 19.3 min, *threo* 19.8 min) suggest that compounds

16 and **18** might be more likely the *erythro* forms and compounds **17/19** the *threo* forms. NMR data are available in Table S4 (Supplementary materials). According to the most accepted hypothesis, flavonolignans are likely biosynthesized by oxidative radicalization of their flavonoid precursors and coniferyl alcohol, followed by coupling of the two radicals.

2.3. Quantification of individual flavonoid compounds within the three species

The individual phenolics were quantified in the four crude extracts (S1-S3 and G) obtained from each species. Comparison of the results obtained with samples S1-S3 and G within a species showed the same HPLC profile and a very weak variability in the phenolic concentrations. In contrast, HPLC profiles, phenolic content and concentrations varied substantially between the species (Fig. 4 and Table S5). Considering the low variability between samples S1-S3 and G of a given species, the comparison between daughter and parental specimens has been discussed only for samples G, which are consisting of 70 individuals and should ensure the best representation of their respective meadow. However, the full set of data is available in supplementary material (Table S5). Results obtained from samples G are summarized in Table 1 (peaks given in order of elution on the HTec column).

2.3.1. Comparison of the parental species

The two parental species were found to differ dramatically in their foliar phenolic chemistry. Visual inspection of the HPLC profiles revealed clear qualitative and quantitative differences in composition for the two parent lines (Fig. 2, Table 1). A total of 15 individual flavonoids (peaks **1-14** and **16**) were quantified in the crude extract of *S. alterniflora,* whereas only seven (peaks **3-4** and **7-11**) were found in *S. maritima.* Six of the seven common products are flavone 6-*C*-mono and diglucosides of luteolin (**3**, **4**), apigenin (**7**, **10**) and chrysoeriol (**8**, **11**), and one flavone *O*-glycoside of tricin (**9**), but their concentrations differ considerably between the two parents. A large predominance of compounds **7-8** and **10-11** was observed in the paternal parent *S. maritima* (respectively 2.265, 1.279, 1.363 and 0.695 $mg.g^{-1}$), while compound **9** was only present in low amount (0.104 $mg.g^{-1}$). In contrast, compounds **7-8** and **10-11** were found in lower amounts in the maternal *S. alterniflora* (respectively 0.170, 0.540, 0.145, and 0.109 $mg.g^{-1}$), while compound **3** was about four time more abundant in *S. alterniflora* (0.695 $mg.g^{-1}$) than in *S. maritima* (0.157 $mg.g^{-1}$). However, whatever the parental species, flavone 6-*C*-monoglucosides (**4**, **10**, and **11**) were found in lower concentrations than their respective 2''-*O*-glucosides (**3**, **7**, and **8**). Tricin 7-*O*-diglucuronic acid (**9**) was also common to the parental species, but while it is the most abundant phenolic in *S.*

alterniflora (3.942 mg.g^{-1}, 49% of the total), it is also the least abundant in *S. maritima* (0.104 mg.g^{-1}, 2% of the total).

Eight flavonoids (**1**-**2**, **5**-**6**, **12**-**14** and **16**) were only found in the maternal parent *S. alterniflora.* They were identified as schaftoside (**1**: 0.293 mg.g^{-1}), isoschaftoside (**2**: 0.240 mg.g^{-1}), isoorientin 2''-*O*- caffeoylglucoside (**5**: 0.371 mg.g^{-1}), isoorientin 2''-*O*- feruloylglucoside (**6**: 0.431 mg.g^{-1}), isoscoparin 2''-*O*-caffeoylglucoside (**12**: 0.253 mg.g^{-1}), isoscoparin 2''-*O*-feruloylglucoside (**13**: 0.533 mg.g^{-1}), isovitexin 2''-*O*-feruloylglucoside (**14**: 0.087 mg.g^{-1}), and tricin 4'-*O*-guaiacylglyceryl ether 7-*O*-glucoside (**16**: 0.084 mg.g^{-1}). The greater flavonoid chemical diversity observed in *S. alterniflora* is mainly due to the presence of the acylated forms of the flavone *C*-glucosides by caffeic and/or ferulic acid (**5**- **6** and **12**-**14**). All together, they represent 21% of the total flavonoid. Acylation of flavone is known to modulate the physiological activity of the resulting flavonoid ester by changing solubility, stability, reactivity and interaction with cellular targets (Viskupicova et al., 2012 and references therein). It has been reported to play a role in UV-B protection mechanisms (Kowalska et al., 2007).

In addition, five tricin derivatives (**15**, **17-20**), were below the detection level in the crude extracts, and their concentrations were not determined in this study. Compounds **18-20** were found in both *S. maritima* and *S. alterniflora*, whereas compounds **15-17** were only found in *S. alterniflora.*

2.3.2. Comparison of the phenolic fingerprint of S anglica to its two progenitors

Examination of Table 1 and Figure 2 shows that all the phenolic traits of the maternal parent *S. alterniflora* have been transmitted to *S. anglica*. Indeed, all of the phenolics **1-20** are kept in *S. anglica*, and most of them are over-expressed (**1-3**, **6**, **13-14**, **16**). Compound **12** was found in similar amount in *S alterniflora* and *S. anglica*. Compound **9** which was the dominant phenolic in *S. alterniflora* (3.942 mg.g^{-1}) is under-expressed in *S. anglica* (2.385 mg.g^{-1}). However, although less abundant than in the maternal parent, compound **9** remains the dominant flavonoid of *S. anglica* and represent 20.3% of the total.

Transmission of paternal phenolic traits appeared globally less efficient. Compounds **4** and **8,** which were present in both parents, but more abundant in *S. maritima,* were additively transmitted. Compounds **7** and **10**, which were the major phenolics in the paternal parent (respectively 2.265 and 1.363 mg.g^{-1}) were under-expressed in *S. anglica* (respectively 0.944 and 0.244 mg.g^{-1}), as was compound **11** (respectively 0.695 and 0.286 mg.g^{-1}). Noteworthy, the concentration of compound **3** in *S. anglica* (1.817 mg.g^{-1}) was about twice greater than the sum of the amount in the parental

species (0.162 mg.g^{-1} in *S. maritima* and 0.717 in *S. alterniflora*). The highest total concentration of individually measured phenolic compounds was found in *S. anglica* (11.71 mg.g^{-1}dw), then *S. alterniflora* (8.03 mg.g^{-1}), followed by *S. maritima* (6.01 mg.g^{-1}). Considering the effect of acylation on the biological properties of flavones (Viskupicova et al., 2012), the presence in *S. alterniflora* and *S. anglica* of five hydroxycinnamates of 6-*C* flavones diglucosides (compounds **5**, **6, 12**-**14),** while only the non-acylated forms were present in *S. maritima* is noticeable.

The relative flavonoid composition within the three species is summarized at the bottom of Table 1, in which results are given as proportion between aglycone type (lines 1-4), or between substituent type (lines 5-9). The percentage values clearly show:

- the similarity and highest phenolic diversity of *S. alterniflora* and *S. anglica,*

- the preponderance of apigenin- and chrysoeriol 6-*C*- mono and diglucosides, the quasi absence of tricin derivatives, and the absence of hydroxycinnamates in *S. maritima.*

The flavonoids concentrations in the samples of the three species (Table S5) were subjected to a principal component analysis (PCA) and to a hierarchical cluster analysis (HCA). The PCA showed a cumulative variance of 98.7% up to the second principal component with values of PC1 and PC2 equal to 73.3% and 25.4%, respectively (Fig. 5-A). Inspection of the loadings indicates that PC1 (horizontal axis) is composed primarily of the contributions from concentration of compounds **1**-**3**, **5**-**7** and **9**-**16**, and PC2 (vertical axis) of the contributions from compounds **4** and **8**. The PCA biplot graphic PC1 x PC2 (Fig. 5-A shows that samples of *S. maritima* cluster along the negative PC1, while samples of *S. anglica* and *S. alterniflora* cluster along the positive PC1 and are separated by PC2. The dendrogram obtained from the cluster analysis of the samples in relation to the concentrations of chemical constituents confirms that *S. maritima* samples form a separate group, while the second branch of the tree is subdivided into two distinct subclusters, one being *Spartina anglica* and the other *S. alterniflora* (Fig. 5-B). These results confirm that their flavonoid contents are more similar to each other than those of *S. maritima*. The dendrogram obtained from the cluster analysis of the variables identifies five clusters at the 2% dissimilarity level (Fig. 5-C). There is one individual cluster (compound **9**, representing 49% of the total flavonoids in *S. alterniflora*)**,** a large cluster that groups together **4** with all the compounds only detected in *S. alterniflora* and *S. anglica* (**1**, **2**, **5**, **6**, **12**, **14**, **16**), and three clusters each grouping two compounds (**3** and **13**, overexpressed in *S. anglica*; **7** and **8**, abundant in both *S. maritima* and *S. anglica*; **10** and **11,** characteristic of *S. maritima*).

3. Discussion

Phenolics constitute an extremely diverse class of highly bioactive, and in many cases, highly toxic substances. They are the most abundant specialized metabolites of plants, which need them for pigmentation, growth, reproduction, resistance to pathogens and predators, and many other functions in plant ecology and physiology (see as examples Cheynier et al., 2013; Kulbat, 2016; Lattanzio et al., 2006; Treutter, 2005). The unpalatability of salt-marsh plants is often associated with chemical defence systems, and numerous studies have provided evidence for the role of phenolics in the interactions with their natural herbivore enemies (Arnold and Targett, 2002; Sieg and Kubanek, 2013).

3.1. Previous reports for Spartina species

Little consideration had been given to the specialized chemistry of the genus *Spartina.* We have recently characterized the phenolic content of *S. maritima* (Grignon-Dubois and Rezzonico, 2019). Apart from this, no attempt had been made to fingerprint and quantify phenolics in the other species and only a few compounds have been well characterized. On the basis of their resistance to acid hydrolysis, the presence of flavone *C*-glycosides was detected in the species *S. arundinacea*, *S. pectinata*, and *S. townsendii*, but their structures were not identified (Harborne and Williams, 1976). Over the last decades, American specimens of *S. alterniflora* have been shown to express chemical defences that deter a variety of herbivores (Valiela et al., 1979; Valiela and Rietsma, 1984; Rietsma et al., 1988; Baerlocher and Newell, 1994). Similar findings were observed with *S. patens* (Siska et al., 2002). In all these studies, the feeding deterrent effects were attributed to free phenolic acids, especially ferulic acid detected in *S. alterniflora* leaves. More recently, a mixture of phenolic compounds isolated from *S. alterniflora* was found to deter grazing by the snail *Littoraria irrorata,* of which orientin was fully characterized (Sieg et al., 2013). Two flavonol *O*- glycosides were isolated from a Chinese specimen of *S. anglica* and identified as 5,4'-dihydroxy-3'-methoxy-flavonol-3-*O*-glucosyl-(1→6)-glucosyl-7-*O*-rhamnoside and 5-hydroxy-3'-methoxy-flavonol-3-*O*-glucosyl-(1→6)-glucosyl-7-*O*-rhamnoside (Sun et al., 2013). Their molecular weights (respectively 786 and 770) are identical to those of products **6**, **12** and **13** found in our samples of *S. alterniflora* and *S. anglica*, but it is coincidental. Indeed, if present in our samples, they would have led to flavonols on acid hydrolysis, and their absence was confirmed by comparison to standard. Two algicidal compounds isolated from another Chinese specimen of *S. anglica* were identified as isorhamnetin-3-*O*-sophoroside-7-*O*-rhamnoside and syringetin-3-*O*-galactoside (Xu et al., 2009; publication in Chinese-language). Neither these products, nor their corresponding aglycone (isorhamnetin and syringetin) were detected in our crude extracts or in their acid hydrolysis mixtures, and there was no data available upon which to base an examination of the results of these publications. A mixture of flavonoids consisting of schaftoside, isoorientin, vitexin, scoparin, tricin,

salcolins A and B have been identified by LC/MS in a Chinese specimen of *S. alterniflora*, but the amounts were not determined (Xu et al., 2019, patent in Chinese-language).

Part of these results differs from ours, especially in the regiochemistry and/or the identity of flavonoids, but it was impossible to draw conclusions from these discrepancies in the absence of spectral data. Nevertheless, plant interactions with environmental factors can cause changes in the metabolism and regulation of biochemical and physiological processes, and pronounced geographical variation may show significant disparity in plant specialized metabolites patterns. The divergence between results obtained with Chinese specimen and ours could indicate geographical variations in the phenolic chemistry of *S. anglica* and *S. alterniflora.*

3.2. Biological potential of the Spartina flavonoid content

Compared to other classes of flavonoid, little is available in the literature about the biological role of *C*-glycosidic flavonoids in plants. However, activities ascribed to *C*-glycosidic flavones include their functioning as antioxidants (Ramarathnam et al., 1989; Kitta et al., 1992), efficient glucosidase and pectinase inhibitors (Ravisé and Chopin, 1981), insect feeding attractants (Kim et al., 1985), larvae feeding deterrents (Haribal and Renwick, 1998), antimicrobial agents (Li et al., 2013), promoters of mycorrhizal symbioses (Akiyama et al., 2002), and UV-protective pigments (Les and Sheridan, 1990). *C*-glycosidic flavones function as antibiotic agents through their subsequent conversion to the more toxic quinones (Wiseman and Carpentier, 1995). In oats, they have been identified as a defence against plant-parasitic nematodes (Soriano et al., 2004) and as crop protectant against weeds (de Bertoldi et al., 2009; Kong et al., 2004). Isoschaftoside has been shown to act as a seed germination inhibitor (Hooper et al., 2010).

From a biosynthetic point of view, natural flavonoid glycosides are mainly synthesized by *O*-glycosyltransferase, but a few by *C*-glycosyltransferase (Yang et al., 2018). Compared to the large accumulation of tricin-7-*O*-diglucuronide in *S. alterniflora* and *S. anglica* (respectively 49 and 20% of the total flavonoid, the quasi absence of *O*-glycosidic flavonoids in *S. maritima* is noticeable (1.7% of the total flavonoid).

The occurrence of tricin and tricin derivatives in plants has been reviewed (see as example Li et al., 2016). They are widely distributed in herbaceous and cereals plants, in which they exist as free tricin, tricin-glycosides, tricin-lignans, and tricin-lignan-glycosides. Tricin and its derivatives were reported to function as efficient and strong antioxidants, anti-weeds, anti-herbicide and insect deterrents (Li et al., 2016). Tricin has also been found to possess antibacterial, antifungal, insecticidal activity, and to be an effective protectant against biotic and abiotic stress (Li et al., 2016). Strong phytotoxic activities have been reported for flavonolignans derived from tricin (Liu et al., 2017). Tricin 7-*O*-glucuronopyranosyl-(2"→ 1"')-*O* glucuronopyranoside (compound **9**) has

been reported to play a role in the antifeedant activity of Indian Barnyard millet extracts against brown planthopper (Kim et al., 2008).

The reaction steps leading to tricin biosynthesis has been reconstructed as naringenin→apigenin→luteolin → chrysoeriol → selgin → tricin, and a unique flavonoid B-ring hydroxylase (CYP75B4) indispensable for tricin formation has been identified in rice (Lam et al., 2015). This enzyme showed 5'-hydroxylase activity that was restricted to chrysoeriol, and CYP75B4 knockout mutant were found tricin deficient, with unusual accumulation of chrysoeriol (Lam et al., 2015). Comparison of these data with our results suggests that the flavonoid 5'-hydroxylase, if present in *Spartina* species, should be efficient in *S. alterniflora* (tricin derivatives 49%; chrysoeriol derivatives 18%), but poorly expressed in *S. maritima* (tricin derivatives 2%; chrysoeriol derivatives 33%). From this point of view, *S. anglica* appears intermediate between the parental species (tricin derivatives 21%; chrysoeriol derivatives 32%) (Table 1).

All the phenolics detected in *S. anglica* were found to be of parental origin, none were missing and no substitute compound was present. Of the 15 phenolics quantified, ten were inherited from *S. alterniflora,* three from *S. maritima,* and two from the two parents. The absence of substitute compounds suggests low changes of the phenolic biosynthetic pathways during the hybridization. Structural relationship between the 20 flavonoids identified in this work is presented in Figure 6.

In contrast to the lack of phytochemical study, the *Spartina* genus has been fairly well investigated at the genetic level. *S. anglica* results from hybridization of the genomes of *S. alterniflora* (maternal donor) and *S. maritima* (paternal donor) (Ferris et al., 1997; Baumel et al., 2003). Hybridization is often associated with changes in biochemistry. Qualitatively, hybrids may express all of the specialized chemicals of the parental taxa, may fail to express certain parental chemicals, or may express chemicals that are absent in each parent (Orians, 2000). The presence of all the parental phenolics in *S. anglica* is consistent with the parental genome additivity reported for this species (Baumel et al., 2001, 2002). The gene expression variation between *S. anlica* and the two parental species has been recently reported (Ferreira de Carvalho et al., 2017). Interestingly, a significant maternal dominance was found in *S. anglica* for the cinnamoyl-CoA reductase, which catalyzes the first specific step in the phenylpropanoids metabolic pathway. Its level of expression in *S. anglica* was found to be about the same as in *S. alterniflora*, and twice as high as in *S. maritima* (Ferreira de Carvalho, 2017). These data are in good agreement with the trend observed here with the respective phenolic contents.

Exotic plants may become invasive when they produce chemical defences that are not found in native plants. It has been shown that successful exotic plant species had a higher diversity of metabolites compared with congeneric native species (Macel et al., 2014). Our results show that the

introduced *S. alterniflora* has a higher diversity of phenolic than the European native *S. maritima,* and that the phenolic pattern of *S. anglica* is mostly inherited from *S. alterniflora*. This suggests that the phenolic chemistry of *S. anglica* might play a role in its invasive character. Noteworthy, *S. anglica* and *S. maritima* having been collected at the same time from two adjacent meadows only separated by a few meters, no environmental effect may be invoked to explain the difference in the phenolic content observed in this study.

Concluding remarks

Numerous reports of phenolic-mediated interactions suffer from the lack of reliable phytochemical data in the Literature. The specialized metabolites produced by the plant may participate in the defence against other plants and pathogens. In particular, an important step to understand how plant hybrids are affected by herbivory is to study how characters important to herbivores are inherited from the parental species to the hybrids (Orians, 2000). From this point of view, the phenolic content variability between *S. anlica* and its two parents is of interest. This study constitutes the first detailed profiling of phenolic metabolites in three members of the genus *Spartina,* the first report of quantitative data on the individual flavonoids and, to the best of our knowledge, the first report of compounds **1**-**20** in *S. anglica* and of compounds **3** and **5**-**14** in *S. alterniflora.*

Considering the lack of knowledge about the phenolic chemistry of *Spartina* species and the biological activities reported in the literature for the *C*-glycosidic flavonoid- and tricin derivatives, this work provides a foundation for further studies. Understanding the flavonoid chemistry in *Spartina* species and its possible link with ecological factors might help to elucidate the functioning of *Spartina* communities.

4. Experimental

4.1. General methods

Analytical-grade water was from Sodipro Company (Echirolles, France). Trifluoroacetic acid (TFA), glucuronic acid and all the solvents used (HPLC-grade) were purchased from Aldrich Chemical Company (Saint-Louis, Missouri, USA). The other standards were purchased from Extrasynthèse (Genay, France). C18 reverse-phase silica gel and HPLC columns were from Macherey-Nagel (Düren, Germany). TLC analyses were carried out using Si 60 HPTLC plates from Merck (Darmstadt, Germany) in PrOH - EtOAc - H_2O (4:0.5:0.5 v/v/v). After development, plates were sprayed with a butanolic solution of orthophosphoric acid and aniline, then heated at 130°C until sugar spots were seen.

Ultrasound-assisted extraction experiments were performed with a Sonorex Digitec H 512 sonicator, 35 kHz (Bandelin, Berlin, Germany), and centrifugation with a Jouan B4i centrifuge (Thermo electron, Waltham, MA, USA).

^{1}H, ^{13}C NMR and 2D NMR spectra were recorded on AVANCE 300 and 600 MHz instruments (Bruker) in DMSO d_6 /D_2O (Euriso-Top, Gif-Sur-Yvette, France). Ultraviolet (UV) spectra were recorded on a V-630 UV-VIS Jasco spectrophotometer (Easton, USA) in HPLC-grade water. High performance liquid chromatography (HPLC) combined with diode array detection (DAD) was performed on a Thermo Electron liquid chromatography system. LC/MS was performed using a HP1100 (Hewlett-Packard) equipped with an Agilent MSD 1946B simple quad mass spectrometer and a HP Chemstation software.

4.2. Plant materials

4.2.1. Taxonomy of the species

Until recently, the species *Spartina maritima* (Curtis) Fernald (common name small cordgrass), S. *alterniflora* Loisel, and *S. anglica* C.E. Hubbard were considered members of the genus *Spartina* Schreb. (Poaceae, subfamily Chloridoideae Kunth ex Beilschm., tribe Zoyseae Benth). On 2014, Peterson et al. proposed inclusion of *Spartina* in a large, monophyletic genus *Sporobolus*, with conservation of the name *Sporobolus* over the older generic name *Spartina*. However, if these species are now found in the International plant index name (IPNI, 2019) as *Sporobolus*, other databases still use the former name *Spartina*. This is the case of the World Register of Marine Species (WORMS 2019), The Plant List, and all the recent literature.

4.2.2. Study sites and collection of the samples

The Bay of Arcachon is a semi-enclosed mesotidal system located on the South-West coast of France. The surface area of this coastal lagoon is approximately 185 km^2, with an exposed intertidal area of 65%. The upper tidal levels are occupied by coastal salt-marshes formed by several halophytic plant communities. First recorded in the Bay in 1985, *S. anglica* has rapidly expended displacing native species like *S. maritima*, *Zostera* and *Salicornia* species. Stands of *S. maritima* and *S. anglica* are mainly located in the inner part of the Bay, where they form high-density monospecific meadows. Whole plant individuals were sampled on September 2016 at low tide along the seashore of Taussat (1°07'95" W, 44°72' 26" N; Fig. 1), where *S. maritima* and *S. anglica* meadows cohabit at the same location in the intertidal zone.

Hossegor Lake is located 130 km south of Arcachon. This small (0.9 km^2) shallow marine water body is connected to the Atlantic Ocean by a short and narrow channel. Intertidal flats

represent 0.28 km², and *Zostera noltei* covers 0.012 km². Whole plant individuals were sampled at low tide on September 2016 (-1°42'71" W, 43°67'51" N; Fig. 1).

The three species of *Spartina* colonize low marsh zones and are exposed to cyclical tidal inundations. Plant samples were collected on the same date at two locations on the French coasts of the southern Bay of Biscay *i.e*. Arcachon Bay for *S. maritima* and *S. anglica* and Hossegor Lake for *S. alterniflora* (Fig. 1). For each species, three independent plant samples (S1-S3) were collected along a line transect perpendicular to the shoreline. In addition, to ensure each of the three monospecific *Spartina* beds were identically represented, 70 specimens were randomly collected from the whole meadow of each species (samples G).

After collection, samples were washed in seawater to remove sediment, and then with freshwater for 1 to 2 min (twice repeated) to remove salt. Associated debris and algae were removed when present. Samples were referenced as Sa-BA-sept 2016 (*S. anglica*), Sm-BA-sept 2016 (*S. maritima*), and Sal-HL-sept 2016 (*S. alterniflora*). Identification of the species was based on morphology, plant anatomy and on comparison with identification keys reported in literature (Bissot, 2016; CBNSA 2014; Géhu, 2008; Kern et al., 2008; Saarela, 2012).

Plant material was separated into aerial parts and rhizomes. Leaves were air-dried until constant weight, and then cut in small pieces. Samples G were mixed manually to obtain a homogenous sample reflecting the biological diversity of their respective meadows.

The drying method (lyophilisation or air-dried at room temperature) had no effect on the results.

4.3. Extractions

The same typical procedure was applied for the three species. Plant material (10 g) was thoroughly mixed with 150 ml of a 50:50 (v/v) mixture of de-ionised water and methanol, ultra-sonicated for 30 min, and then macerated at room temperature (24 h). The extraction process was repeated once. The two resulting extracts were pooled together, and centrifuged (5°C, 30 min, 6000g). The supernatant was evaporated under *vacuo* until complete elimination of the methanol, and then the remaining aqueous solution was freeze-dried.

Phenolic compounds were identified based on HPLC retention time (Rt), on-line DAD spectra, and MS molecular ions in comparison with authentic standards (Tables 1 and S1, Fig. 2-3).

4.4. Purification of compounds for structural elucidation

Extracts were obtained from 70 g of each *Spartina* species. The individual phenolics were isolated from the crude extracts by successive rounds of gravity open column chromatography

using C18 silica gel. Gradient elution was done with H_2O-MeOH as the mobile phase starting from 99:1 to 20:80. Fractionation was monitored by HPLC. Isolated compounds were concentrated to dryness and the structure determined by UV, MS and NMR spectroscopies. Comparison with literature data confirmed the structure of compounds **1-14** and **16** (Table 1). Five minor compounds (**15** and **17-20**) were below the limit of detection in the crude extracts, but were isolated in mixture during the purification process. Their structures were tentatively assigned by UV, MS and NMR spectroscopies. Spectral data are reported in Tables S2-S4 (Supplementary materials).

4.5. Acid hydrolysis of the crude extracts

A 100-mg sample of crude extract was dissolved in 100 ml of methanol and heated with 1M HCl at 100 °C for 4 h (monitored by HPLC). The hydrolysate was controlled for sugars against a standard mixture (HPTLC; toluene-n-BuOH-pyridine-H_20, 2:5:3:3). The flavonoid content of the reaction mixture was analysed by HPLC, LC/MS, UV, and comparison with authentic samples of vitexin, isovitexin, orientin, isoorientin, luteolin, apigenin, chrysoeriol and tricin**.** Results of acid hydrolysis are summarized in Table 2. The eventual presence of other flavone- and flavonol-*O*-glycosides, whose molecular weight matched with the LC/MS results or data from the literature, was also checked, but none of which were present. The full list of standards used is given in Table S1 (Supplementary material).

4.6. Qualitative and quantitative HPLC analysis

Separation and quantification of phenolics in *Spartina* crude extracts were performed using a liquid chromatography system (Thermo electron) equipped with a SCM 1000 solvent degasser, a thermostatically controlled column compartment, an AS 3000 autosampler with a 100 µl loop, a PDA UV6000LP detector and a Chromquest Chromatography Workstation. Reverse phase C8 and C18 columns were tested using the same or similar gradients of methanol-water with 0.1% TFA to separate the complex mixture of flavonoids in *S. anglica* and *S. alterniflora*. The Nucleodur C18 HTec column (110 Å pore size, 3 µm particle size, 150 × 4.6 mm i.d.) was found to offer the best separations, but it was not optimum for all regions of the chromatograms. For the best results over the entire range of polarities of the extracted compounds, the use of a C18 phase with embedded polar amido group provided a check for co-elution problems (Nucleodur Polartec: 110 Å pore size, 5 µm particle size, 250 × 4.6 mm i.d). In both case, separations were carried out at 40 °C and the analytes were eluted at a flow rate of 1 ml min^{-1} using the binary gradient 0.1% (v/v) TFA in water (A) and methanol (B). The following linear gradient was used: 0 min, 80% B; 60 min, 99% B. Run time, stop time and post time were respectively 60 min, 60 min, and 6 min with the Polartec

column, and 30 min, 30 min and 6 min with the HTec column. UV spectra were collected over the range of 220-400 nm. The injection volume was 20 µl. The data were integrated using Chromquest software. In addition, they were processed to create a chromatogram, in which each peak represents the absorbance of the eluting substance at its λ_{max} (max-plot chromatogram). Stock solutions of the dried crude extracts were prepared in HPLC-grade water at a concentration of 3 mg ml^{-1}. All solutions were filtered prior to analysis through a 0.20-µm syringe filter and injected three times into the HPLC.

Chromatograms were systematically recorded at 270, 328 and 350 nm, which allowed a clear distinction between benzoate-, hydroxycinnamate-, and flavonoid derivatives. Chromatographic peaks of the crude extracts were checked for peak purity and identification was achieved by matching the Rt and on-line UV spectra of standards.

Quantitative determinations of flavonoïds were carried out using a calibration curve of the structurally related aglycone *i.e.*: apigenin at 335 nm (R^2 = 0.9996, seven points) for compounds **1-2**, **7, 10,** and **14**, luteolin at 350 nm (R^2 = 0.9993, six points) for compounds **3-6,** chrysoeriol at 348 nm (R^2 = 0.9994, six points) for compounds **8** and **11-13**, tricin at 350 nm (R^2 = 0.9991, six points) for compounds **9** and **16**. A molecular weight correction factor was applied to convert individual flavonoids calculated as the external standard equivalents to their respective forms as described for anthocyanins by Chandra et al. (2001). The correction factors were determined by dividing the molecular weight of the product to be quantified by that of the standard flavonoid used. This led to the following values: compounds **1-2,** 2.09; **3**, 2.13; **4**, 1.57; **5**, 2.70; **6**, 2.75; **7**, 2.20; 8, 2.08; **9**, 2.07; **10**, 1.60; **11**, 1.54; **12**, 2.62; **13**, 2.67; **14**, 2.85. Data are expressed in mg per gram of dry matter ($mg.g^{-1}$ dw) of plant tissue. They are presented as mean ± standard deviation (SD) of three determinations (Table 1). The HPLC analysis of standards was performed using exactly the same conditions as for the extracts.

4.7. LC/MS analysis

LC/MS was performed using an HP1100 (Hewlett-Packard) equipped with an Agilent MSD 1946B single quad mass spectrometer and HP Chemstation software. Positive and negative mode ESI spectra of the column eluate were recorded in the range of *m/z* 120-1000 a.m.u. Absorbance was measured at 280 and 350 nm. Compounds were separated using a Zorbax poroshell C18 column (Agilent): 100 mm × 3 mm i.d, 2.7 µm particle sizes. The analytes were eluted at a flow rate of 0.2 $ml.min^{-1}$ using the binary gradient (v/v) formic acid in water (pH = 2.55, A) and methanol (B). The following linear gradient was used: 5 % B to 100 % B (15 min). Separation of the analytes was carried out at 50 °C. The injection volume was 2 µL. For mass

spectrometric analysis, compounds were detected using the following conditions: nebulising gas pressure, 60 psi; drying gas flow rate, 12 l.min^{-1}; drying gas, temperature, 350 °C; capillary voltage, 4000 V; temperature source, 350 °C. Data were acquired in full scan mode (*m/z* 100-1000) at a fragmentor voltage of 70 V.

4.8. Statistical treatments

For each individual compound, differences between species were statistically analyzed using Statistica software, v 7.1. After log transformation of the data, homogeneity of variance was achieved (Cochran test) and a one way-ANOVA was performed (with plant identity as fixed factor), followed by a Tukey test in the case of multiple treatment (*i.e.* more than 2 plant species). Bonferroni correction was applied. Results are summarized in Table 3.

Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed with XLSTAT software (Addinsoft) to analyze the differences and similarities between the three species. Results are presented in Figure 5.

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Captions for Figures and Tables

Table 1: Peak number, HPLC retention time (Rt), molecular weight, phenolic concentration ($mg.g^{-1}$ dw, mean values ±SD) of flavonoid compounds from *S. maritima, S. alterniflora* and *S. anglica* (samples G).

Table 2. Hydrolysis products obtained from compounds **1-20**.

Table 3. Results from one-way ANOVA and, when applicable, from Tukey test. df: degree of freedom; Sma : *S. maritima*; Sal: *S. alterniflora*; San: *S. anglica*).

Table S1. List of standards used.

Table S2. ^{1}H NMR ($DMSO/D_2O$) data for compounds **3**, **4**, **7-11**.

Table S3. ^{13}C NMR ($DMSO/D_2O$) data for compounds **3**, **4**, **7-11**.

Table S4. NMR data ($DMSO/D_2O$) for compounds **16-17.**

Table S5. Comparison of the phenolic concentration ($mg.g^{-1}$ dw, mean values ±SD) of flavonoid compounds **1**-**14** and **16** in samples S1, S2, S3 and G for *S. maritima, S. alterniflora* and *S. anglica.*

Figure 1. Study sites on the Southern French Atlantic coast. Symbol **+** indicate position of the sampling sites.

Figure 2. Comparison of the HPLC chromatograms obtained on HTec (top) and Polartec (bottom) column: (a) *S. maritima;* (b) *S. alterniflora;* (c) *S. anglica.*

Figure 3. Formulae of compounds **1-20**.

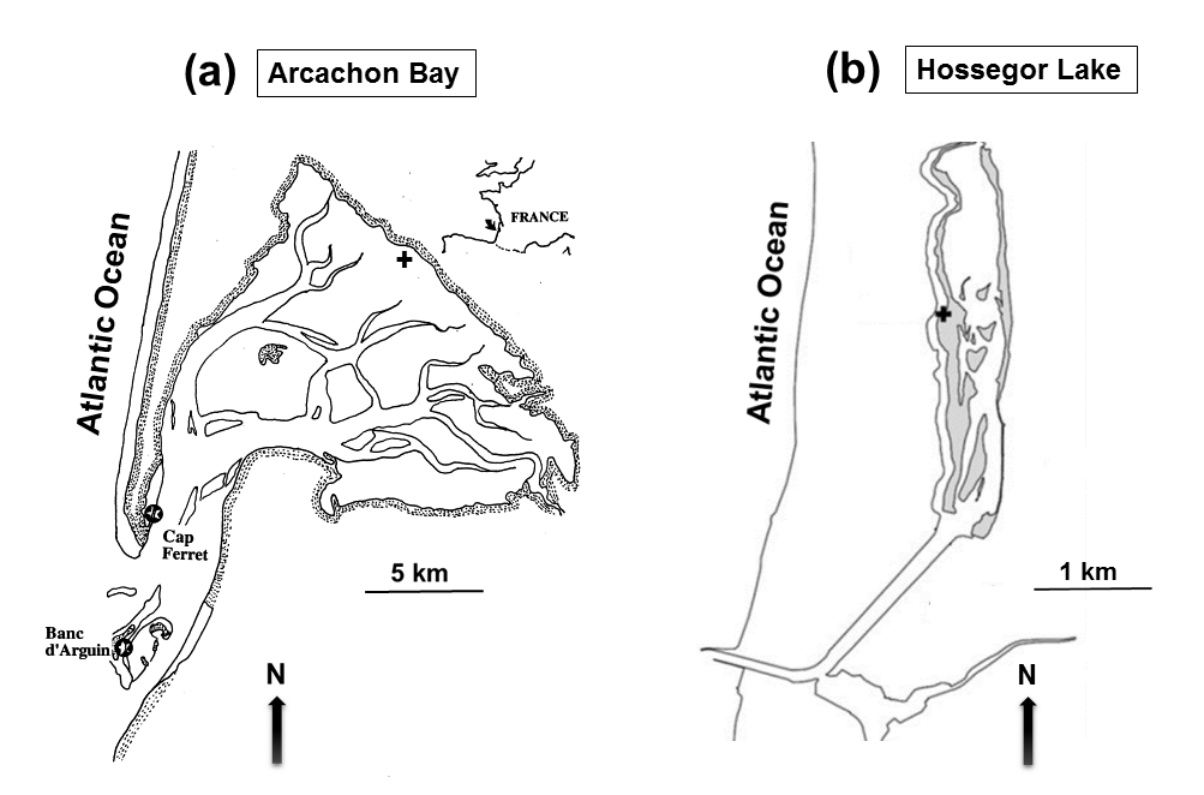
Figure 4. Comparison of the phenolic content of samples S1-S3 and G for each *Spartina* species: *S. maritima* (top), *S. alterniflora* (middle) and *S. anglica* (bottom). Black bars, samples G; white bars, samples S1; light gray bars, samples S2; dark gray bars, samples S3. Compound numbers on

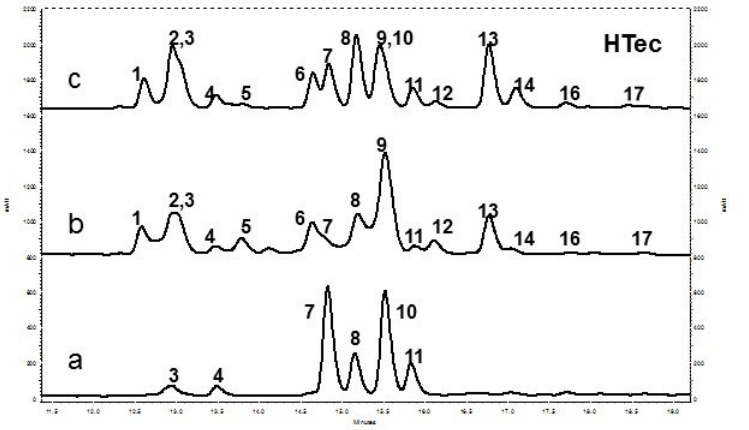
abscissa are the same as in Table 1 and Fig. 3. Concentrations values on ordinate are given as mg.g^{-1} dw, mean values (n = 3; see Table S5 for full data).

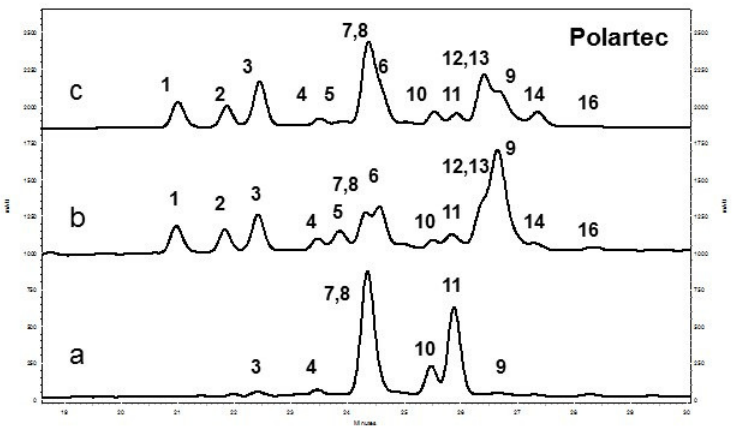
Figure 5. A - PCA biplot graph based on PC1 and PC2 scores; B - dendrogram of the three species based of their flavonoid contents; C - dendrogram obtained from the cluster analysis of the variables. Sma: *S. maritima*; San: *S. anglica*; Sal: *S. alterniflora.* Compound numbers are the same as in Table 1 and Fig. 3.

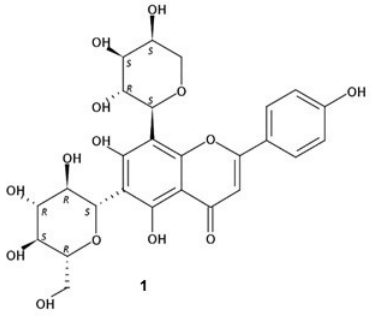
Figure 6. Structural relationship between flavonoids **1**-**20** identified in the three *Spartina* species (MW: molecular weight).

Figure S1. On-line UV spectra for peaks **1-16**.

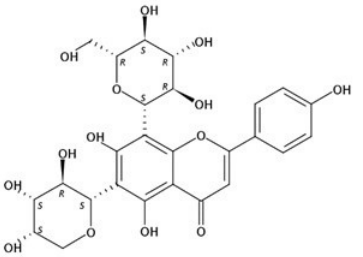

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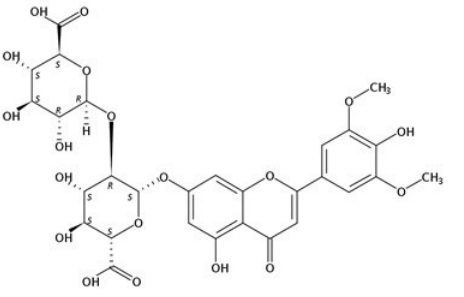

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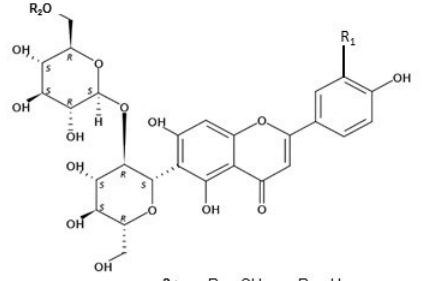
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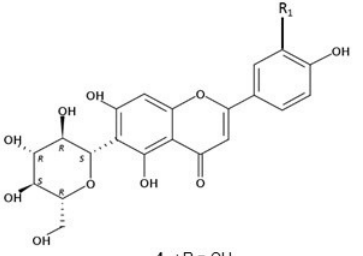
2



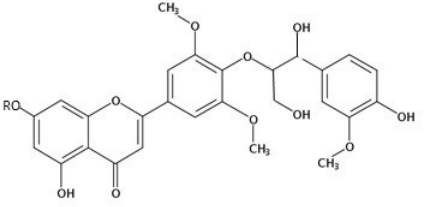
9



3 : R_1 = OH R_2 = H
5 : R_1 = OH R_2 = caffeoyl
6 : R_1 = OH R_2 = feruloyl
7 : R_1 = H R_2 = H
8 : R_1 = OMe R_2 = H
12 : R_1 = OMe R_2 = caffeoyl
13 : R_1 = OMe R_2 = feruloyl
14 : R_1 = H R_2 = feruloyl



4 : R = OH
10 : R = H
11 : R = OMe



16 : R = Glucosyl (erythro)
17 : R = Glucosyl (threo)
18 : R = H (erythro)
19 : R = H (threo)

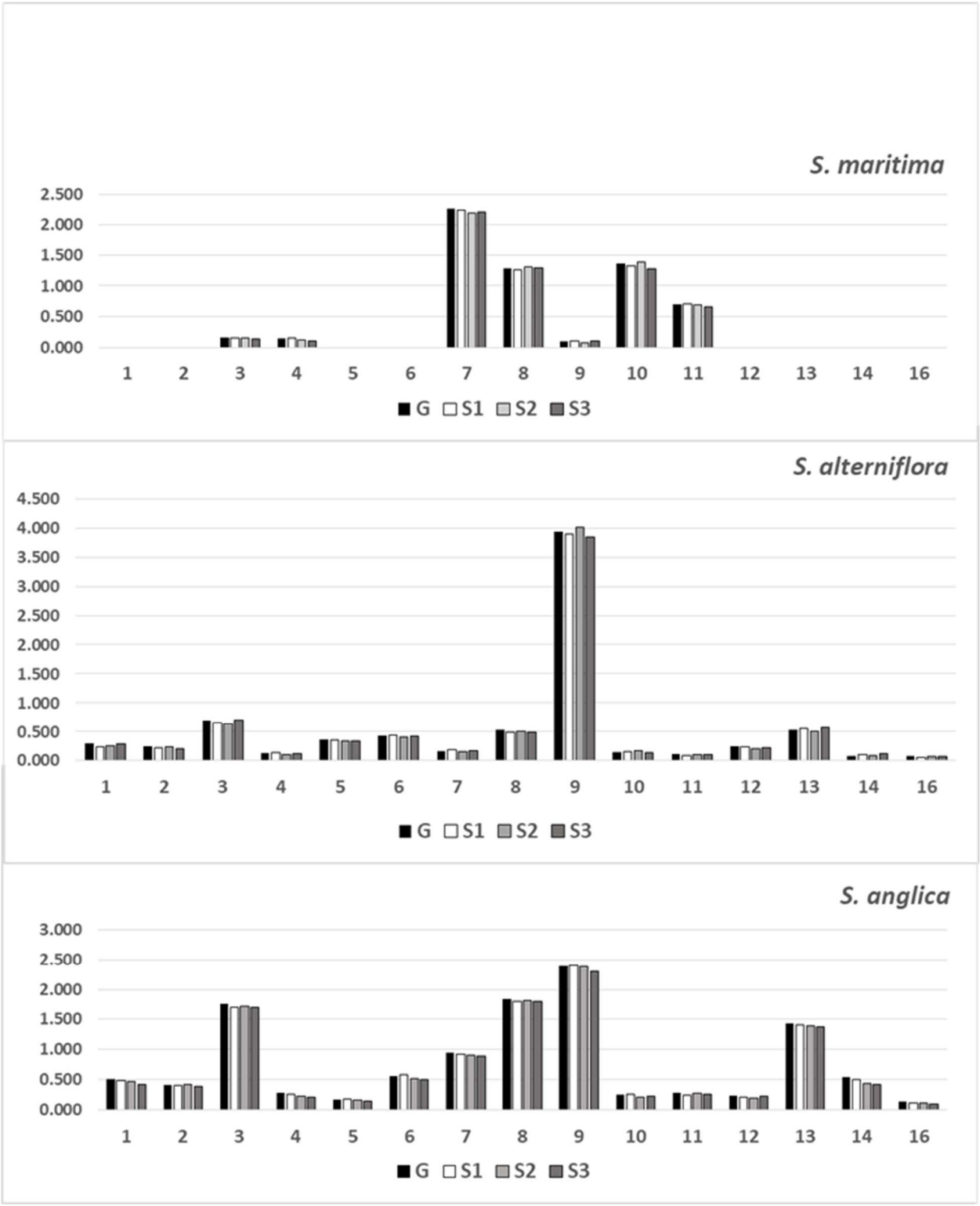


Figure 4. Comparison of the phenolic content of samples S1-S3 and G for each *Spartina* species: *S. maritima* (top), *S. alterniflora* (middle) and *S. anglica* (bottom). Black bars, samples G; white bars, samples S1; light gray bars, samples S2; dark gray bars, samples S3. Compound numbers on abscissa are the same as in Table 1 and Fig. 3. Concentrations values on ordinate are given as $mg.g^{-1}$ dw, mean values (n = 3; see Table S5 for full data).

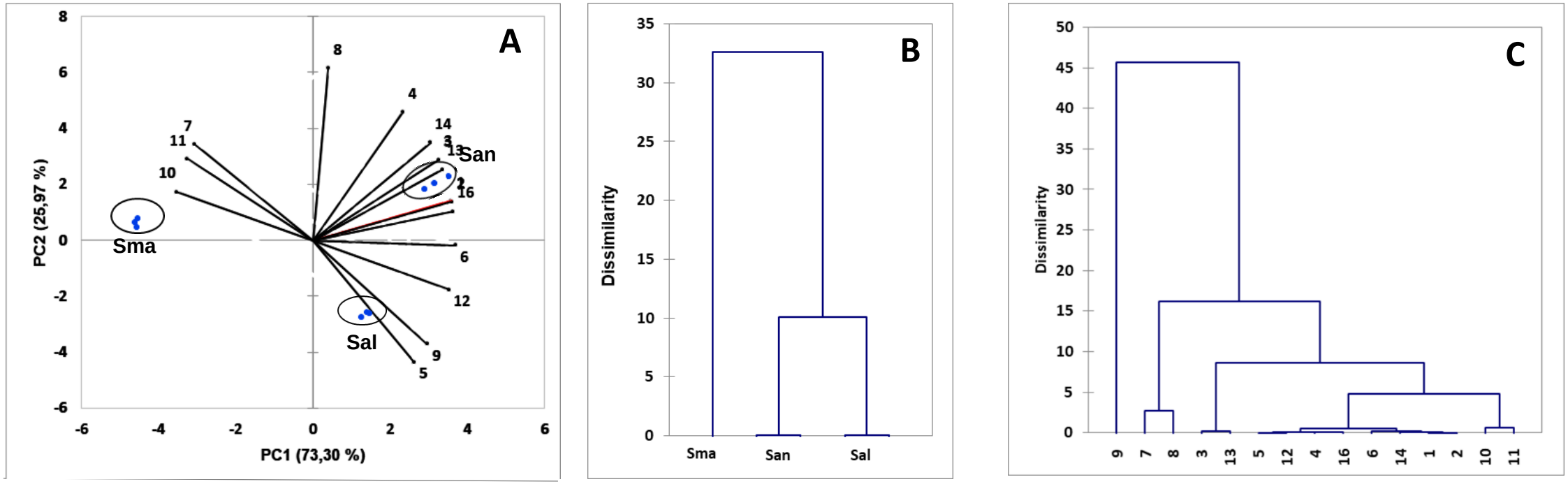
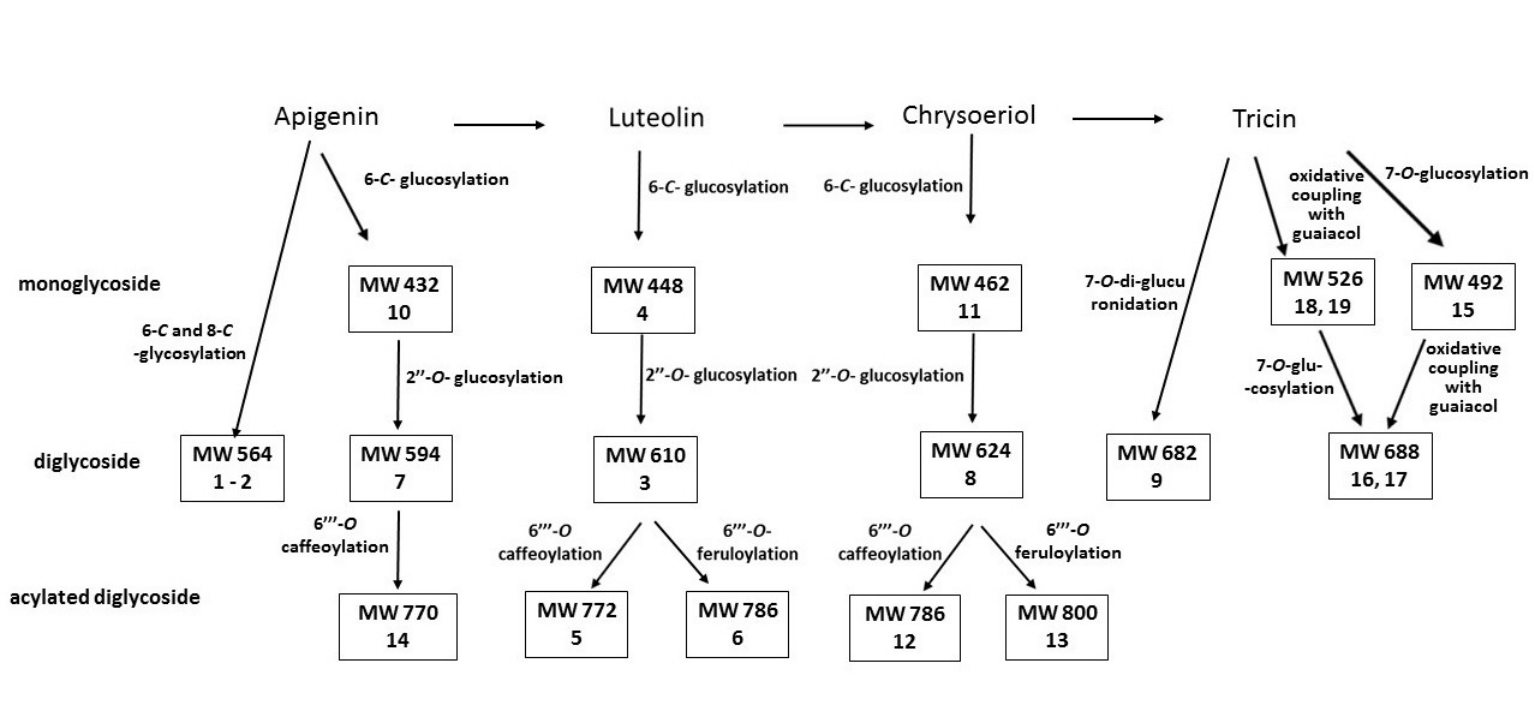


Figure 5. A - PCA biplot graph based on PC1 and PC2 scores; B - dendrogram of the three species based of their flavonoid contents; C - dendrogram obtained from the cluster analysis of the variables. Sma: *S. maritima*; San: *S. anglica*; Sal: *S. alterniflora*. Compound numbers are the same as in Table 2 and Fig. 3.


Apigenin
Luteolin
Chrysoeriol
Tricin
6-C- glucosylation
6-C- glucosylation
6-C- glucosylation
oxidative coupling with guaiacol
7-O-glucosylation
monoglycoside
MW 432
10
MW 448
4
MW 462
11
7-O-di-glucu ronidation
MW 526
18, 19
MW 492
15
6-C and 8-C -glycosylation
2''-O- glucosylation
2''-O- glucosylation
2''-O- glucosylation
7-O-glu--cosylation
oxidative coupling with guaiacol
diglycoside
MW 564
1 - 2
MW 594
7
MW 610
3
MW 624
8
MW 682
9
MW 688
16, 17
6'''-O caffeoylation
6'''-O caffeoylation
6'''-O-feruloylation
6'''-O caffeoylation
6'''-O feruloylation
acylated diglycoside
MW 770
14
MW 772
5
MW 786
6
MW 786
12
MW 800
13

Peak number	Rt (min)	MW (g)	Aglycon	Structure assignment*	Phenolic concentration			Pattern of inheritance	
					S. maritima	*S. alterniflora*	*S.anglica*	donnor	level of expression
1	12.8	564	Apigenin	Schaftoside	*BDL*	0.293±0.005	0.505±0.003	maternal	over
2	13.3	564	Apigenin	Isoschaftoside	*BDL*	0.240±0.002	0.413±0.005	maternal	over
3	13.3	610	Luteolin	Isoorientin 2''-*O*-glucoside	0.157±0.002	0.695±0.005	1.753±0.019	both	over
4	13.8	448	Luteolin	Isoorientin	0.148±0.003	0.132±0.001	0.279±0.004	both	additive
5	14.1	772	Luteolin	Isoorientin 2''-*O*- caffeoylglucoside	*BDL*	0.371±0.003	0.168±0.003	maternal	under
6	14.9	786	Luteolin	Isoorientin 2''-*O*-feruloylglucoside	*BDL*	0.431±0.003	0.558±0.004	maternal	over
7	15	594	Apigenin	Isovitexin 2''-*O*-glucoside	2.265±0.037	0.170±0.002	0.944±0.009	paternal	under
8	15.4	624	Chrysoeriol	Isoscoparin 2''-*O*-glucoside	1.279±0.009	0.540±0.005	1.839±0.009	both	additive
9	15.8	682	Tricin	Tricin 7-*O*-diglucuronide	0.104±0.004	3.942±0.025	2.385±0.020	maternal	under
10	16	432	Apigenin	Isovitexin	1.363±0.014	0.145±0.002	0.244±0.003	paternal	under
11	16.1	462	Chrysoeriol	Isoscoparin	0.695±0.009	0.109±0.001	0.286±0.003	paternal	under
12	16.3	786	Chrysoeriol	Isoscoparin 2''-*O*-caffeoylglucoside	*BDL*	0.253±0.002	0.233±0.002	maternal	total
13	16.9	800	Chrysoeriol	Isoscoparin 2''-*O*-(6-(*E*)-feruloylglucoside)	*BDL*	0.533±0.003	1.439±0.007	maternal	over
14	17.2	770	Apigenin	Isovitexin 2''-*O*-(6-(*E*)-feruloylglucoside)	*BDL*	0.087±0.002	0.533±0.005	maternal	over
15	17.4	492	Tricin	Tricin 7-*O*-glucoside	*BDL*	*BDL*	*BDL*		
16	17.7	688	Tricin	Tricin 4'-*O*-guaiacylglyceryl ether 7-*O*-glucoside	*BDL*	0.084±0.002	0.132±0.002	maternal	over
17	18.5	688	Tricin	Tricin 4'-*O*-guaiacylglyceryl ether 7-*O*-glucoside	*BDL*	*BDL*	*BDL*	maternal	
18	24.6	526	Tricin	Tricin 4'-*O*-guaiacylglyceryl ether	*BDL*	*BDL*	*BDL*	both	
19	24.9	526	Tricin	Tricin 4'-*O*-guaiacylglyceryl ether	*BDL*	*BDL*	*BDL*	both	
20	25.1	330	Tricin	Tricin	*BDL*	*BDL*	*BDL*	both	
(a) Total flavonoids **1**-**16**					6.011	8.025	11.711		
(b) Total apigenin derivatives**					3.628 (60%)	0.935 (12%)	2.639 (23%)		
(c) Total luteolin derivatives**					0.305 (5%)	1.629 (20%)	2.758 (24%)		
(d) Total chrysoeriol derivatives**					1.974 (33%)	1.435 (18%)	3.797 (32%)		

(e)Total tricin derivatives**	0.104 (2%)	4.026 (49%)	2.517 (21%)
(f) Total 6-C 8-*C* diglycosides**	*BDL*	0.533 (7%)	0.918 (8%)
(g) Total 6-*C* glucosides**	2.206 (37%)	0.386 (5%)	0.809 (7%)
(h) Total 6-*C* diglucosides**	3.701 (62%)	1.405 (18%)	4.536 (39%)
(i) Total 6-*C* diglucoside caffeates**	*BDL*	0.624 (8%)	0.402 (3%)
(j) Total 6-*C* diglucoside ferulates**	*BDL*	1.051 (13%)	2.530 (22%)

*See Fig.3 for formulae

**Values between brackets (%) indicate the percentage of abundance of compound type compared to the total of flavonoids detected (aglycone identity : lines b-e; substituent identity: lines f-j).

BDL : below detection level

peak	Acid hydrolysis products	Alkaline hydrolysis products	Assignment
1	no change	no change	Schaftoside
2	no change	no change	Isoschaftoside
3	Isoorientin (**4**)	no change	Isoorientin 2''-*O*-glucoside
4	no change	no change	Isoorientin
5	Isoorientin (**4**)	Isoorientin (**4**) and caffeic acid	Isoorientin 2''-*O*-caffeoylglucoside
6	Isoorientin (**4**)	Isoorientin (**4**) and ferulic acid	Isoorientin 2''-*O*-feruloylglucoside
7	Isovitexin (**10**)	no change	Isovitexin 2''-*O*-glucoside
8	Isoscoparin (**11**)	no change	Isoscoparin 2''-*O*-glucoside
9	Tricin	-	Tricin 7-diglucuronide
10	no change	no change	Isovitexin
11	no change	no change	Isoscoparin
12	Isoscoparin (**11**)	Isoscoparin (**11**) and caffeic acid	Isoscoparin 2''-*O*-caffeoylglucoside
13	Isoscoparin (**11**)	Isoscoparin (**11**) and ferulic acid	Isoscoparin 2''-*O*-(6-(*E*)-p-feruloylglucoside)
14	Isovitexin (**10**)	Isovitexin (**10**) and ferulic acid	Isovitexin 2''-*O*-(6-(*E*)-p-feruloylglucoside)
15	Tricin (**20**)	-	Tricin 7-*O*-glucoside
16	Tricin 4'-*O*-guaiacylglyceryl ether (**18**)	-	Tricin 4'-*O*-guaiacylglyceryl ether 7-*O*-glucoside
17	Tricin 4'-*O*-guaiacylglyceryl ether (**19**)	-	Tricin 4'-*O*-guaiacylglyceryl ether 7-*O*-glucoside
18	no change	-	Tricin 4'-*O*-guaiacylglyceryl ether
19	no change	-	Tricin 4'-*O*-guaiacylglyceryl ether

Table 3. Results from one-way Anova and, when applicable, fromTukey test (df: degree of freedom; Sma: *S. maritima*; Sal: *S. alterniflora*; San: *S. anglica*).

Flavonoid number	df	p-value	Multiple comparison
1	1	0.001	Sal<San
2	1	<0.001	Sal<San
3	2	<0.001	Sma<Sal<San
4	2	<0.001	[Sal=Sma]<San
5	1	<0.001	San<Sal
6	1	0.013	Sal=San
7	2	<0.001	Sal<San<Sma
8	2	<0.001	Sal<Sma<San
9	2	<0.001	Sma<San<Sal
10	2	<0.001	[Sal=San]<Sma
11	2	<0.001	Sal<San<Sma
12	1	0.126	Sal=San
13	1	<0.001	Sal<San
14	1	<0.001	Sal<San
16	1	0.0026	Sal<San