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MACROMOLECULAR COMPOSITION OF THE PROPAGULE WALL OF *NELUMBO NUCIFERA*

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Key Word Index—*Nelumbo nucifera*; Nelumbonaceae; fruit wall; pyrolysis; ^{13}C NMR; lignin-cellulose; tannin–polysaccharide complex.

Abstract—The macromolecular constituents of the sclerotic propagule wall of *Nelumbo nucifera* and seed coat of *Nymphaea caerulea* were studied using scanning electron- and light microscopy in combination with Curie-point pyrolysis-gas chromatography-mass spectrometry. In addition, the *Nelumbo* material was analyzed using solid state ^{13}C nuclear magnetic resonance and in-source pyrolysis-mass spectrometry. The sclerotic seed coat of the *Nymphaea caerulea* revealed the presence of angiosperm lignin-cellulose similar to that found in most sclerotic plant remains. In sharp contrast, the fruit wall plus seed coat of *Nelumbo* is believed to be composed of a complex of polysaccharides, based on primarily galactose and mannose units, and insoluble tannins, which are suggested to play the same structural role as the lignin-cellulose in the sclerotic seed coat. The distinctive nature of the chemical constituents present in the propagule wall of *Nelumbo*, supports the systematic distinction of this genus in the separate family Nelumbonaceae. The characteristic chemical composition of the propagule walls of *Nelumbo* could be an additional factor in favour of a prolonged longevity of these fruits. However, the distinctive composition of polysaccharides and tannins without the presence of lignin is considered to be the main reason for the absence of these propagules in the fossil record, despite their physical resistance. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Water lilies (Nymphaeaceae *sensu lato*; Nymphaeales) are distributed worldwide [1–4] and often occur abundantly in modern fresh-water environments, such as lakes, swamps and marshes. Hence, it is not surprising that their remains are microscopically recognized as substantial contributors to the sedimentary organic matter in peat deposits [5–7]. Water lily remains also contribute to sediments, as witnessed by the frequent recognition of leaves, rhizomes and seeds in the fossil record throughout the Quaternary and Tertiary and as far back as the late Cretaceous [2–4, 8–10].

Based on, primarily, morphological evidence, water lily genera are now subdivided into three families [2–4]: (1) Nymphaeaceae *sensu strico* (*Nymphaea*, *Nuphar*, *Victoria*, *Euryale*, *Ondinea*, *Barclaya*); (2) Cabombaceae (*Cabomba*, *Brasenia*); and (3) Nelumbonaceae (*Nelumbo*). However, it should be mentioned that the systematic position of this latter family is ambiguous and new morphological evidence implies that *Nelumbo* is distinct from the Nymphaeales and only distantly related to them [11]. This is further corroborated by recent data on the chemical composition of the epicuticular waxes [12] and rbcL-analyses [13], indicating that the Nelumbonaceae may not belong to the Nymphaeales. Remains of all three families are recognized in the fossil record, however, there is an intriguing phenomenon concerning the presence of the different propagules. Seeds of several modern genera of Nymphaeaceae *s.s.* and *Brasenia* (Cabombaceae) are represented in the fossil record [8–10], whereas the fruits of the genus *Nelumbo*, often termed seeds [3, 14], are absent prior to the Holocene (*ca* 0.01 Ma). This observation is rather surprising considering the fact that *Nelumbo*-like leaves are known from the Cretaceous onwards [3, 9, 10]. Moreover, a high preservation potential could be predicted for *Nelumbo*

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fruits based on the fact that they possess a physically tough, water- and air-tight wall and are famous for their extreme longevity, being capable of germinating after many centuries [14–17]. Their absence in the fossil record is therefore rather puzzling.

In the present work, the sclerotic (i.e. hardened, thickened) fruit wall of *Nelumbo nucifera* is examined using scanning electron- and light microscopy, in addition to Curie-point pyrolysis-gas chromatography(-mass spectrometry), in-source pyrolysis-mass spectrometry and solid-state ^{13}C NMR, in order to characterize the insoluble constituents. The Curie-point pyrolysis results are compared with those of the sclerotic outer seed coat (testa) of *Nymphaea caerulea*, the chemistry of which is considered to be representative of that of the sclerotic propagule walls of Nymphaeaceae *s.s.* and Cabombaceae [18]. Comparison between the macromolecular constituents present in these two samples will be discussed with reference to the chemosystematic position of *Nelumbo* and the absence of its propagules in the fossil record.

RESULTS

Microscopy

Nymphaea caerulea propagules are seeds that are small, *ca* 1–5 mm long and ovoid. The outer wall of these propagules is the seed coat, which consists of a sclerotic outer testa and a thin inner tegmen [cf. 8, 19].

The propagule of *Nelumbo* is a single-seeded fruit which is rather similar in overall appearance to the seed despite of its utterly distinct botanical nature. It is larger than the seed, up to 15 mm long, but also ovoid and with terminal features [Fig. 1(a)] which resemble those on the operculum of the seeds. The resemblance is purely superficial as the larger of these structures [Fig. 1(a)] is the remains of the stigma and the smaller is an oval scar the function of which is unknown [11].

As expected from its botanical nature the *Nelumbo* propagule also has very distinct anatomy. The external surface [Fig. 1(b)] of the fruit wall is covered by fine tubercles and, in the area which protrudes from the receptacle, also contains stomata [Fig. 1(b)] which are connected to the internal parenchyma by channels [o; Fig. 1(c) and (d)]. In section, the propagule wall, which is a combined fruit wall and seed coat, has four layers [Fig. 1(c)]. We consider that the innermost layer [Fig. 1(c)] is the unspecialized seed coat (sc), but this is almost identical in anatomy to the very thick inner layer of the fruit wall (i). This tissue is thick-walled parenchyma. Another distinctive layer in the fruit wall consists of closely-packed, narrow elongate cells [e; Fig. 1(c) and (d)], which are white under light microscopy. These are not columnar sclereids, but are somewhat flexible and thin-walled. We do not know their exact anatomical nature. The outermost fruit wall layer consists of cuboid thick-walled cells [c; Fig. 1(d)].

Chemistry

In order to characterize the macromolecular composition of the sclerotic propagule walls, the *Nymphaea* sclerotesta and the whole propagule wall (fruit wall plus seed coat) of *Nelumbo* were subjected to CuPy-GC(-MS). In addition, the *Nelumbo* sample was analyzed using Py-MS and solid-state ^{13}C NMR. Peak

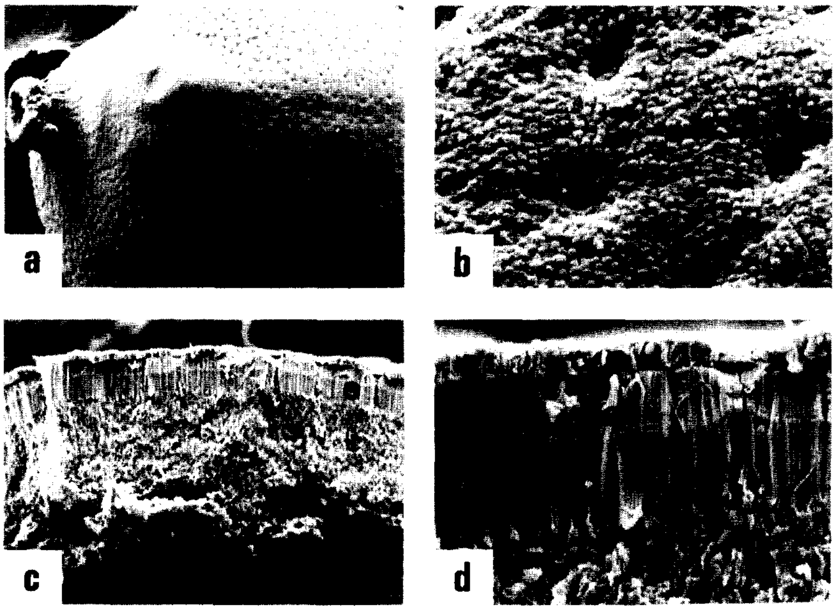

Fig. 1. Scanning electron micrographs of modern *Nelumbo nucifera* single-seeded fruit. (a) apical portion showing stigma on the far left and scar left of centre; × 10; (b) surface detail showing tubercules and three stomata; × 160; (c) transverse section of complete wall showing seedcoat (sc), inner layer of fruitwall (i) and elongated cells (e); × 42; (d) transverse section of outer portion of wall showing detail of elongate cell layer (e), cuboid thick-walled cells (c) and a channel (o); × 160.

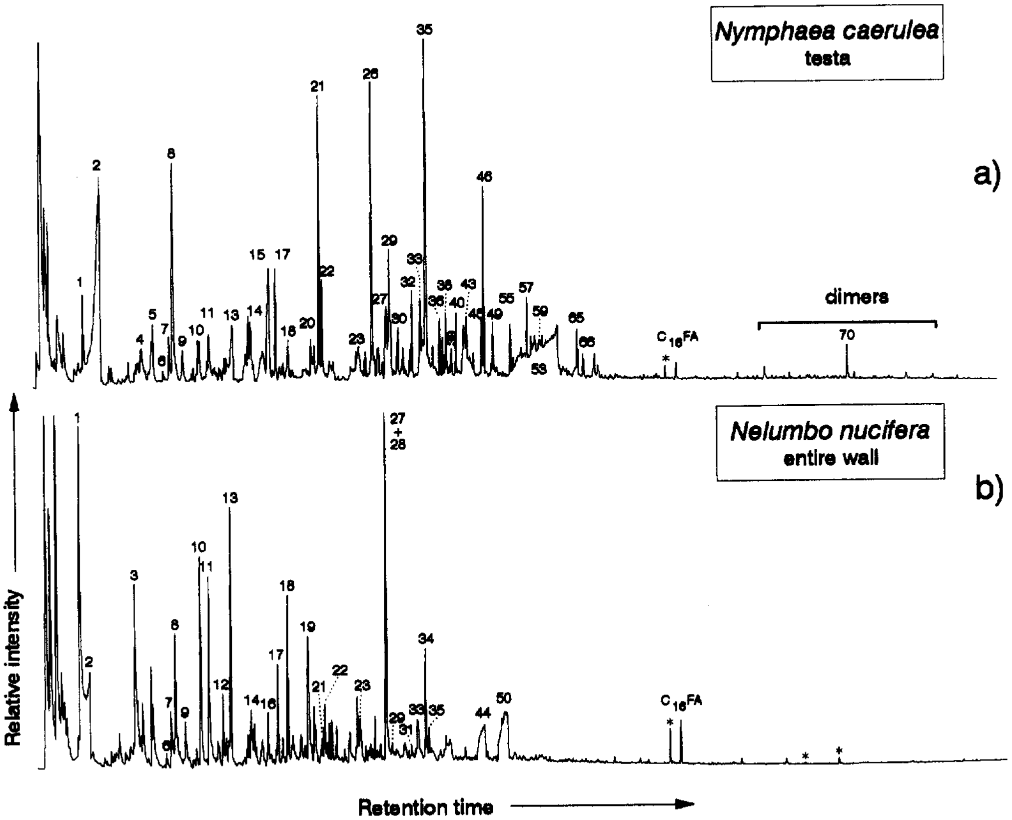

Fig. 2. CuPy-GC traces (Curie-temperature 610°) of (a) testa of modern *Nymphaea caerulea* and (b) whole propagule wall of modern *Nelumbo nucifera*. Key: * = contaminants; $C_{16}FA$ refers to hexadecanoic acid. Numbers refer to compounds listed in Table 1.

numbers in Fig. 2 refer to compounds listed in Table 1.

Curie-point pyrolysis-gas chromatography-mass spectrometry

The pyrolysates of both samples are dominated by phenolic and polysaccharide pyrolysis products. However, the contribution of and distribution within these two classes varies substantially between the pyrolysates [Figs 2(a) and (b)]. The pyrolysate of the testa sample of *Nymphaea* is dominated by methoxyphenols with 2-methoxyphenol (**21**), 4-methyl-2-methoxyphenol (**26**), 4-ethenyl-2-methoxyphenol (**35**) and 4-(2-(*E*)-propenyl)-2-methoxyphenol (**46**) as the most important contributors [Table 1; Fig. 2(a)]. In addition, substantial amounts of acetic acid (**2**), furan- (e.g., 2-furaldehyde; **8**) and pyran derivatives (e.g. 4-hydroxy-5,6-dihydro-(2*H*)-pyran-2-one; **15**), as well as anhydrosugars [e.g. 1,6-anhydro-β-D-glucopyranose (levoglucosan); **53**] are present. 2,6-Dimethoxyphenols (e.g. **36**, **45**, **57** and **65**), alkylphenols (**17**, **20** and **22**), 1,2-benzenediol (**27**) and methoxyphenol-dimers (e.g. **70**) are found in relatively small amounts.

In contrast, the pyrolysate of the *Nelumbo* sample [Fig. 2(b)] is dominated by polysaccharide pyrolysis products (e.g. **3**, **8**, **10**, **11**, **13**, **18**, **19**, **28**, **44** and **50**), in addition to 1,2-benzenediols (**27** and **34**). It should be noted that the peak annotated **27**+**28** [Fig. 2(b)] represents *ca* equal amounts of 1,2-benzenediol and 5-hydroxymethyl-2-furaldehyde. Methoxyphenols and (alkyl)phenols are present in minute amounts. The distribution pattern of the polysaccharide pyrolysis products is very different from that in the pyrolysate of the testa [cf. Fig. 2(a)]. In particular, the relatively low amount of 2-furaldehyde (**8**), the high amounts of 2-hydroxymethylfuran (**10**), 5(*H*)-furan-2-one (**11**), 2,3-dihydro-5-methylfuran-2-one (**13**), 2-hydroxy-3-methyl-2-cyclopenten-1-one (**18**), 5-hydroxymethyl-2-furaldehyde (**28**), the virtual absence of 4-hydroxy-5,6-dihydro-(2*H*)-pyran-2-one (**15**) and the absence of 1,6-anhydro-β-D-glucopyranose (= levoglucosan; **53**) should be noted. Moreover, two relatively important pyrolysis products (**44** and **50**) are detected which are characteristic for the *Nelumbo* sample. The peak shape [Fig. 2(b)] and their mass spectra, with the main fragments *m*/*z* 57, 60, 73 and 98, imply a structure similar to that of the anhydrosugar levoglucosan (**53**). Based on comparison with literature data [20–23] these two compounds are tentatively identified as levogalactosan (**44**) and levomannosan (**50**).

In-source pyrolysis-mass spectrometer (DTMS)

In-source PyMS data of the *Nelumbo* sample were obtained under low voltage EI [Fig. 3(a)] and

Table 1. Pyrolysis products detected in the CuPy-GC(-MS) traces (Fig. 2) from the water lily propagules of *Nymphaea caerulea* and *Nelumbo nucifera*

No.	Compound name	Structure/Type	M^+
1	hydroxypropanone	PS	74
2	acetic acid	PS	60
3	unknown	PS	84
4	toluene		92
5	(2*H*)-furan-3-one	PS	84
6	3-furaldehyde	PS	96
7	2,4-pentadienal	PS	82
8	2-furaldehyde	PS	96
9	4-methyltetrahydrofuran-3-one	PS	96
10	2-hydroxymethylfuran	PS	98
11	(5*H*)-furan-2-one	PS	84
12	unknown	PS	98
13	2,3-dihydro-5-methylfuran-2-one	PS	98
14	5-methyl-2-furaldehyde	PS	110
15	4-hydroxy-5,6-dihydro-(2*H*)-pyran-2-one	PS	114
16	5,6-dihydropyran-2,5-dione	PS	112
17	phenol	P	94
18	2-hydroxy-3-methyl-2-cyclopenten-1-one	PS	112
19	unknown	PS	114
20	2-methylphenol	P—C	108
21	2-methoxyphenol	G	124
22	3- and 4-methylphenol	P—C	108
23	5-hydroxymethyl-2-tetrahydro-furaldehyde-3-one	PS	144
24	2,4-dimethylphenol	C—P—C	122
25	4-ethylphenol	P—C—C	122
26	4-methyl-2-methoxylphenol	G—C	138
27	1,2-benzenediol	Ca	110
28	5-hydroxymethyl-2-furaldehyde	PS	124
29	4-ethenylphenol	P—C=C	120
30	unknown	PS	
31	3-methyl-1,2-benzenediol	Ca—C	124
32	4-ethyl-2-methoxyphenol	G—C—C	152
33	unknown	PS	
34	4-methyl-1,2-benzenediol	Ca—C	124
35	4-ethenyl-2-methoxyphenol	G—C=C	150
36	2,6-dimethoxyphenol	S	154
37	propenylphenol	P—C=C—C	134
38	2-methoxy-4-(1-propenyl)phenol	G—C—C=C	164
39	2-methoxy-4-propylphenol	G—C—C—C	166
40	4-formyl-2-methoxyphenol	G—CHO	152
41	unknown	PS	126
42	C_2-1,2-benzenediol	Ca—C_2	138
43	2-methoxy-4-(2-(*Z*)-propenyl)phenol	G—C=C—C	164
44	anhydrosugar (levogalactosan ?)	PS	162
45	2,6-dimethoxy-4-methylphenol	S—C	168
46	2-methoxy-4-(2-(*E*)-propenyl)phenol	G—C=C—C	164
47	4-ethanal-2-methoxyphenol	G—C—CHO	166
48	4-($C_{3:2}$)-2-methoxyphenol	G—$C_{3:2}$	162
49	4-acetyl-2-methoxyphenol	G—CO—C	166
50	anhydrosugar (levomannosan ?)	PS	162
51	4-($C_{3:2}$)-2-methoxyphenol	G—$C_{3:2}$	162
52	C_3-1,2-benzenediol	Ca—C_3	152
53	1,6-anhydro-β-D-glucopyranose (levoglucosan)	PS	162
54	4-carboxy-2-methoxyphenol methyl ester	G—$COOCH_3$	182
55	2-methoxy-4-(2-propanone)phenol	G—C—CO—C	180
56	2,6-dimethoxy-4-ethylphenol	S—C—C	182
57	2,6-dimethoxy-4-ethenylphenol	S—C=C	180
58	2-methoxy-4-(3-propanone)phenol	G—CO—C—C	180
59	2,6-dimethoxy-4-(1-propenyl)phenol	S—C—C=C	194

Table 1.—*Continued*

No.	Compound name	Structure/Type	M^+
60	4-carboxy-2-methoxyphenol	G—COOH	168
61	2,6-dimethoxy-4-propylphenol	S—C—C—C	196
62	2,6-dimethoxy-4-formylphenol	S—CHO	182
63	2-methoxy-4(1-hydroxypropyl)phenol	G—C—C—COH	182
64	2,6-dimethoxy-4-(2-(*Z*)-propenyl)phenol	S—C=C—C	194
65	2,6-dimethoxy-4-(2-(*E*)-propenyl)phenol	S—C=C—C	194
66	2-methoxy-4-(2-(*E*)-propenal)phenol	G—C=C—CHO	178
67	4-acetyl-2,6-dimethoxyphenol	S—CO—C	196
68	2,6-dimethoxy-4-(2-propanone)phenol	S—C—CO—C	210
69	2,6-dimethoxy-4-(2-(*E*)-propenal)phenol	S—C=C—CHO	208
70	1,2-di(4-hydroxy-3-methoxyphenyl)ethene; (α,β-diguaiacylethene)	G—C=C—G	272

P = phenol; G = 2-methoxyphenol (Guaiacyl); S = 2,6-dimethoxyphenol (Syringyl); Ca = 1,2-benzenediol (catechol); PS = polysaccharide.

ammonia CI conditions [Fig. 3(b)]. The mass peak distribution in Fig. 3(a) is typical for hexose polysaccharides [24]. This identification was confirmed by the ammonia CI data shown in Fig. 3(b). A high intensity m/z 180, identified as an 1,6-anhydrohexose $[M+NH_4]^+$, and corresponding ions indicative of further dehydrated anhydrohexosesugars at m/z 162 and 144 [24–26] are observed. Ions indicative of anhydrohexosesugar oligomers are present at m/z 342 and 504 [27], which is an indication for the linear nature of the polymer chain. Low intensity anhydropentose sugars ions are observed at m/z 132 and 150. The corresponding EI ions are observed at m/z 144. Ions at m/z 146 and 164 in Fig. 3(b) suggest the presence of deoxysugars. The pentose and deoxysugars can be interpreted as indicative for xyloglycan, a polysaccharide which is normally closely associated with cellulose microfibrils.

Ions indicative of methoxy phenolic compounds at m/z 152, 164 and 178 are observed at low intensity and do not show a typical lignin pattern [28]. Experiments with reactive Py-MS using tetramethylammoniumhydroxide as a transalkylation reagent, confirmed the polysaccharide nature of the sample but did not reveal new aspects.

Solid-state ^{13}C *NMR of* Nelumbo *sample*

The NMR spectrum of the *Nelumbo* [Fig. 4(a)] is dominated by resonances indicating polysaccharides, at 65 ppm (indicating C-6 carbons of polysaccharides), at 72 ppm (indicating C-2, 3, and 5 carbons of polysaccharides), at 90 ppm (indicating C-4 carbons of crystalline polysaccharides) and at 105 ppm (indicating C-1 carbons of polysaccharides). This latter resonance may also indicate nonprotonated aromatic carbons in tannins [29–31], or C-2 and C-6 in dimethoxyphenols [32]. In addition, several other peaks are present at *ca* 56 ppm (indicating methoxyl carbons), 115 ppm (indicating protonated aromatic carbons), 130 ppm (indicating carbon-substituted aromatic carbons), 145 ppm (indicating O-aryl carbons in dihydric phenols; e.g. 1,2-benzenediols), 153 ppm (indicating O-aryl carbons in monohydric phenols) and 175 ppm (carboxyl carbons) indicating, perhaps, the carboxyls of uronic acid-based sugars. However, these are only very minor peaks when compared with those indicating polysaccharides. The dipolar dephasing spectrum [dephasing delay of 60 μsec; Fig. 4(b)] is dominated by resonances at 72, 106, 130, 145, 153 and 175 ppm. The presence of carboxyl (175 ppm) and the oxygen-substituted carbons of mono-, di- and trihydric phenols of tannins (130, 145 and 153 ppm) are expected in the dipolar dephased spectrum. The peak at 72 ppm for polysaccharides is not expected because it should normally dephase away at a dipolar dephasing time of 60 μsec. This could be due to the fact that the polysaccharides in *Nelumbo* are not common polysaccharides as already indicated by the pyrolysis data. The peak at 106 ppm could be from tannins [29] or from ketals [33] associated with certain polysaccharides. Considering that intense peaks are observed for phenolic units present in tannins, the major portion of the peak at 106 ppm is likely to be from these latter compounds.

DISCUSSION

Biomacromolecule in Nymphaea *sclerotesta*

The sclerotic testa of the water lily analyzed is composed of a native angiosperm lignin-cellulose complex as revealed by the presence of their specific pyrolysis (CuPy-GC) products [22, 34, 35]. Most polysaccharide products (**1**, **2**, **5**, **6**, **8**, **10**, **11**, **14**, **28** and **53**) encountered in these pyrolysates are likely derived from cellulose [36], the main constituent of the holocellulose in the lignin-cellulose complex. In addition, 4-hydroxy-5,6-dihydro-(2*H*)-pyran-2-one (**15**), is considered as a xylan marker, a hemicellulose constituent which is the other component of the holocellulose [22, 36].

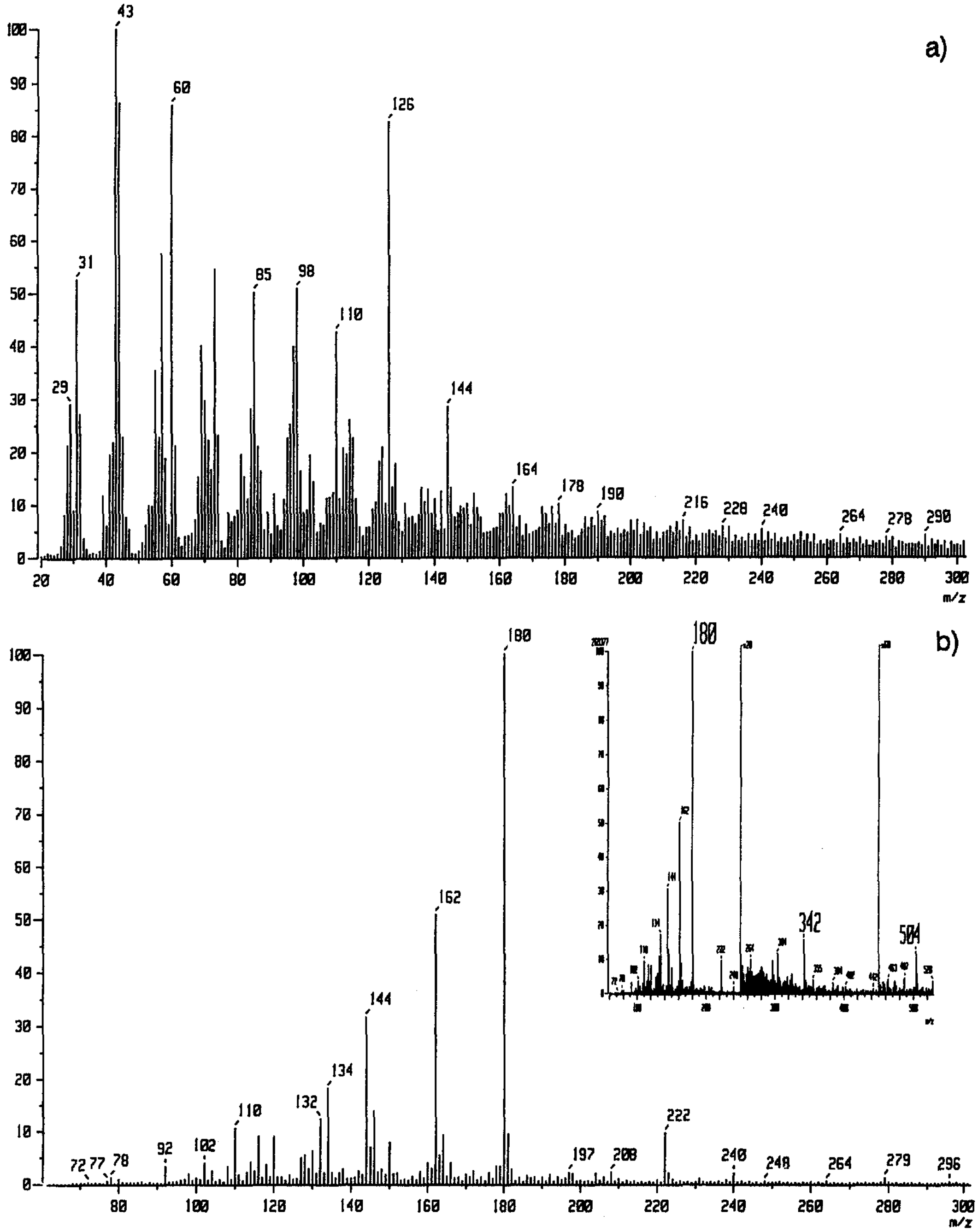

Fig. 3. Py-EIMS (a) and Py-CIMS (b) spectra of insoluble wall material of *Nelumbo nucifera*.

Biomacromolecule in sclerotic layers of Nelumbo

In contrast to the pyrolysate (CuPy-GC) of the testa of *Nymphaea* [Fig. 2(a)], the pyrolysate of the *Nelumbo* fruit wall plus testa, is dominated by polysaccharide pyrolysis products (**1**, **2**, **3**, **8**, **10**, **11**, **13**, **18**, **19**, **28**, **44** and **50**), in addition to 1,2-benzenediols [**27** and **34**; Fig. 2(b)]. Polysaccharides, in particular hexosans, are also evident from the Py-CIMS, i.e. *m*/*z* 504, 342, 222, 180, 162 and 144 [Fig. 3(b)]. This signature is usually assigned to β(1,4)-glucans, because they are the most common in plant cell walls. However, based on CuPy-GC and NMR data in which such signature is absent, this type of polysaccharide is rather unlikely. The small contributions of pentoses and deoxysugars (fucose?) suggest the presence of another plant polysaccharide, such as a xyloglucan. The ion *m*/*z* 110 in the Py-EIMS spectrum may indicate 1,2-benzenediols moieties. However, it should be noted that this ion is often found in polysaccharide Py-EIMS data. Evidence of polysaccharides and 1,2-benzenediols is also recognized by their characteristic resonances in the NMR spectrum, *viz.*, 65, 72, 90 and 105 ppm for polysaccharides [Fig. 4(a)] and 115 , 130 and 145 ppm

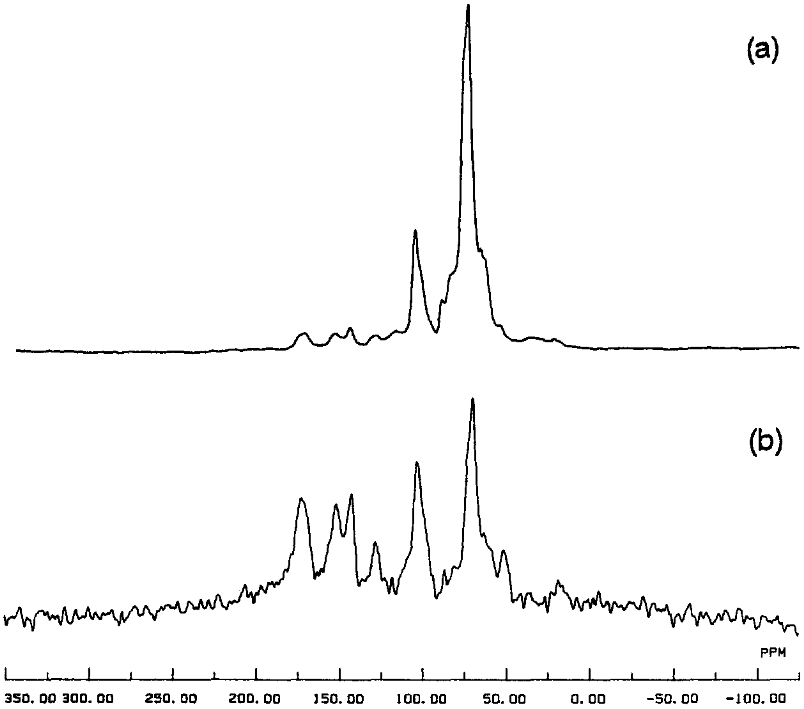

Fig. 4. Solid-state ^{13}C NMR spectra of propagule wall of *Nelumbo nucifera*. (a) conventional spectrum; (b) 60 μsec dipolar dephased spectrum.

for the aromatic carbons in 1,2-benzenediol-moieties [Fig. 4(b)]. From the NMR and Py-MS spectra it is obvious that the contribution of the latter moieties is low when compared with that of the polysaccharides.

Although, most of the polysaccharide pyrolysis products detected in the CuPy trace are recognized in pyrolysates of cellulose [36], their distribution pattern in this sample is very different [cf. Fig 2(a) and (b)]. Moreover, the anhydrosugar, levoglucosan (**53**) and the xylan marker (compound **15**) are absent, suggesting the virtual absence of both cellulose and xylan hemicelluloses. However, this latter observation seems partly inconsistent because several other characteristic compounds in the pyrolysate [Fig. 2(a)], such as 2,3-dihydro-5-methylfuran-2-one (**13**), 5,6-dihydropyran-2,5-one (**16**) and 2-hydroxy-3-methyl-2-cyclopenten-1-one (**18**) are relatively important and are also mentioned as specific xylan markers [28]. These results indicate that the (structural) polysaccharide precursor in this sample is different from that recognized in typical lignin-cellulose complexes. A distinctly different polysaccharide composition is further substantiated by the fact that the dipolar dephased NMR spectrum [Fig. 4(b)] still shows a strong resonance at 72 ppm which would have been absent in the case of a common lignin-cellulose complex [33]. The recognition of two different anhydrosugars (probably levogalactosan **44** and levomannosan **50**) in the CuPy-GC trace leads us to suggest that the polyhexose building units are galactose and mannose instead of glucose. An abundant contribution from galactose appears in good agreement with data on the neutral polysaccharides of whole *Nelumbo* fruits [37].

Evidence of methoxyphenols, indicating lignin, is virtually absent from both the CuPy-GC trace and the Py-MS spectra. Using mass chromatography only traces of 2-methoxyphenol (**21**) and 4-ethenyl-2-methoxyphenol (**35**) were detected in the CuPy-GC trace. However, the absence of characteristic lignin monomers in the Py-EIMS spectrum implies that the methoxylphenols detected in the CuPy-GC trace are most likely derived from a source other than lignin. Furthermore, the characteristic resonances for methoxyphenols in the NMR spectrum are minor and can also, alternatively, be explained by the presence of the alkylphenols and 1,2-benzenediols. Based on these observations, in combination with the absence of the specific pyrolysis markers for cellulose, we believe that the sclerotic layers of the *Nelumbo* do not contain lignin-cellulose.

The only other relatively abundant pyrolysis proucts in the Py-GC trace, apart from the polysaccharide pyrolysis products, are 1,2-benzenediol (**27**) and 4-methyl-1,2-benzenediol (**34**). These products are often recognized in pyrolysates of extant lignin-containing tissues but in small amounts only [22, 23]. Their abundance in the pyrolysate of the sclerotic layer of *Nelumbo* is therefore rather surprising. Recent studies using tannin monomers, (catechin = flavan-3-ol) and condensed tannins showed that upon flash pyrolysis only two major products were detected, *viz*., 1,2-benzenediol and 4-methyl-1,2-benzenediol [38–40]. Thus, tannin-moieties could be the source for the benzenediols detected. Additional evidence for this suggestion is found in the dipolar dephasing NMR spectrum [Fig. 4(b)], which shows an intense signal at

106 ppm indicating a contribution from tannins [30, 31]. Moreover, tannins could also explain the resonances at 130, 145 and 153 ppm in the NMR spectrum [Fig. 4(b)] [29–31]. With respect to the Py-MS data, the only evidence which could indicate tannins is the mass fragment m/z 110 representing 1,2-benzenediol. In view of this, it is interesting to note that Shen and co-workers [41] suggested that insoluble forms of proanthocyanidins are bound covalently to polysaccharide structures in the developing cellular matrix. These proanthocyanidins-polysaccharide complexes could possibly serve as an alternative for the lignin-celluloses as proposed by Bate-Smith and Lerner [42].

Based on the above evidence, we propose that the sclerotic propagule wall of *Nelumbo* is composed of a complex of predominantly polysaccharides, with mainly galactose and mannose units, and insoluble tannins, which plays the same structural role as lignin-cellulose in other strengthening tissues.

Chemosystematic implications

As mentioned in the introduction, water lily genera are now subdivided into three families based mainly on morphological criteria. However, morphological evidence from some fossil seeds, e.g. the extinct *Sabrenia*, shows that these genera are somewhat intermediate in position [8], hence, questioning the subdivision. For a critical phylogenetic review the reader is referred to Moseley and others [43]. The separate taxonomical status of *Nelumbo* is supported by the propagule morphology and anatomy based on the unspecialized testa and the fact that the propagules of this genus are single-seeded fruits, whereas the propagules from the other water lilies are seeds [cf. 8, 18].

To date, chemosystematic data to substantiate the subdivision of the water lilies are few and only concentrate on soluble constituents. The Nymphaeaceae *s.s.* contain specific sequiterpene alkaloids, in addition to gallo- and ellagitannins (hydrolyzable tannins) accompanied by trihydroxylated flavonoids [4, 12]. In contrast, the Cabombaceae do not produce alkaloids, but gallo- and ellagitannins occur in *Brasenia* [2]. The Nelumbonaceae are characterized by the presence of benzylisoquinoline alkaloids and nonacosan-10-ol, in addition to flavonols, flavones and proanthocyanides, suggesting an affinity closer to the Ranunculiflorae, rather than the Nymphaeales [3, 12, 44].

Our chemical data, as revealed by pyrolysis and NMR, on the insoluble constituents of the sclerotic propagule wall of the *Nelumbo* showed the presence of a polysaccharide-tannin complex which is profoundly different from the macromolecular constituents, lignin-cellulose, present in other sclerotic propagule walls, testae, of water lilies [cf. *Nymphaea caerulea*, herein Fig. 2(a); *Nymphaea ampla*; *Nuphar advena*, *Brasenia schreberi*; in ref. [18]]. These data, therefore, support the systematic distinction of this genus in the separate family Nelumbonaceae as indicated by propagule morphology.

Bias in fossil record

The exceptional longevity of *Nelumbo* fruits is well known [14–17]. The most significant factor contributing to the longevity is believed to be the water- and air-tight architecture of the thick fruit wall [14, 17]. In addition, the presence of extremely well-preserved polyunsaturated fatty acids [17], robust proteins [14] and the anoxic depositional environment in which the ancient, Holocene, fruits were deposited, are considered other factors assisting to the longevity of these fruits. In view of this, it is even more surprising that no fossil propagules, older than the Holocene, have been found.

To data, all modern and fossil water lily propagule walls, i.e. seed coats, studied, contain lignin-cellulose, in the case of extant samples or modified lignin, in the case of fossil samples [16]. Moreover, sclerotic seed coat and/or fruit wall tissues of different plant families which have been preserved in the fossil record revealed (modified) lignin as their macromolecular constituent [19, 45–48]. This clearly indicates that the presence of lignin in extant sclerotic propagule walls is a prerequisite for the preservation of these tissues in the fossil record. Cellulose, and, most probably, other polysaccharides, are shown not to be essential for these entities to be morphologically preserved [19, 45–48]. We believe that the distinct chemical composition of the tough outer wall of *Nelumbo*, containing a tannin-polysaccharide complex, is an additional factor in favour of prolonged longevity. With respect to the preservation potential of such a distinctive chemical complex, tannins have been reported to be preserved in fossil plant remains [29]. However, the mere fact that the macromolecular constituents in the propagule walls of *Nelumbo* are primarily composed of polysaccharides, which are normally lost upon fossilization, with only a relatively small contribution of tannins and are devoid of lignin, is considered to explain the absence of the propagules of the genus *Nelumbo* in the fossil record despite their high physical resistance.

EXPERIMENTAL

Sample description. The samples studied are the sclerotic (i.e., hardened and thickened) propagule walls of *Nymphaea caerulea* Savign and *Nelumbo nucifera* Gaertn. The propagule wall of the *Nymphaea* is a seed coat which is composed of a sclerotic outer seed coat layer (testa) and a translucent inner seed coat layer (tegmen); only the testa was analyzed. In contrast, the propagule wall of *Nelumbo* is that of a single seeded fruit, composed of a firmly attached fruit wall and seed coat, which was analyzed as a whole. Detailed refs concerning these species can be found elsewhere [3, 4, 8]. Both samples are taken from the authors comparative collection (MEC) and were identified by comparison with herbarium material.

Scanning electron microscopy. Samples were mounted onto cover slips on stubs using 'Bostic' adhesive, coated with gold in a Polaron sputter coating unit and examined under a Philips 501B SEM at 30 kV.

Extraction. Testae of the *Nymphaea* were left intact to allow sample handling, whereas the *Nelumbo* fruit wall was powdered prior to extraction. Both samples were extracted ultrasonically with MeOH ($\times 3$) and CH_2Cl_2 ($\times 3$). The residues were dried in a vacuum stove at 30°.

Curie-point pyrolysis. Curie-point pyrolysis-gas chromatography (CuPy-GC) analyses were performed using a FOM-4LX unit for pyrolysis. The samples were pressed on a ferromagnetic wire. The Curie temp was 610°. The pyrolysis time was 10 sec. The interface and injector temps were set at 200°. The gas chromatograph, equipped with a cryogenic unit, was programmed from 0° (5 min) to 300° (10 min) at a rate of 3° min^{-1}. Sep was achieved using a fused-silica capillary column (25 m $\times$ 0.32 mm) coated with CP Sil-5 (film thickness 0.4 μm). He was used as the carrier gas. Detector temp. was set at 330°. CuPy-GC-MS analyses were performed using the same equipment and conditions as described above for the CuPy-GC. The gas chromatograph was connected to a VG Autospec Ultima operated at 70 eV with a mass range m/z 45–800 and a cycle time of 1.7 sec. The C_0-C_2 alkylphenol and C_0-C_1 alkylbenzenediol pyrolysis products were identified by comparison of their relative R_ts and MS with those of authentic standards. All other compounds were identified by comparison of their RR_ts and MS with those of compounds reported in the lit. [20, 22, 35, 36, 49].

Direct temperature resolved mass spectrometry (DTMS). The in-source mass spectrometric exps were performed on a double-focusing mass spectrometer using a direct insertion probe equipped with a resistively heatable Pt-Rh (9:1) filament (diam. 100 μm). The probe filament was temp-programmed to heat at a rate of 1 A min^{-1} corresponding to *ca* 16° sec^{-1}. Ions were generated by 16 eV EI ionization and extracted at 10 kV. The mass spectrometer was scanned from m/z 20–1000 at a cycle time of 1 scan sec^{-1} at a resolution of 3000. TMAH spiking for transmethylation took place by addition of 10 μl of a 2.5% aq. soln of tetramethylammoniumhydroxide to the dried plant particles on the filament. NH_3 CI was performed at a pressure of 20 Pa with an ionization energy of 250 eV. Data acquisition and processing was performed on-line.

Solid state ^{13}C NMR. Spectra were recorded at a field strength of 2.35 Tesla. A 1 msec contact time and a 1 sec delay were used for each of *ca* 100 000 acquisitions obtained by the method of cross-polarization with magic angle spinning ($\sim$3.5 kHz). The dipolar-dephasing spectrum was acquired using a dephasing delay of 60 μsec; further details can be obtained from ref. [50]. Interpretation of the different shifts are based on data reported in the lit. [29, 30, 50].

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