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Functional aspects of mature seed coat of the *Cannaceae*

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Key words: *Cannaceae, Canna.*–PyMS, PyGCMS, histochemistry, macromolecules, germination, imbibition.

Abstract: *Cannaceae* seeds have been analysed regarding seed coat structure, germination and macromolecular composition of the seed coats. Data of several mass spectrometric techniques were combined with those of microscopic and histochemical techniques to acquire insight into the functions of the seed coat. *Cannaceae* seeds have an exotestal layer of Malpighian cells with a hydrophobic and a hydrophilic part. The hydrophobic part is mainly responsible for the impermeability of the seed and contains silica, callose, lignin as water repellent substances. Water can only enter the seed after a certain temperature-induced opening of an imbibition lid. During imbibition the hydrophilic part of the Malpighian cells swells and the seed coat ruptures due to differences in pressure in the upper and lower part of the Malpighian cells.

Within the order *Zingiberales* there are still unsolved questions about the relations between seed coat structure, macromolecular composition and germination. Because there is a dearth of reliable data on the macromolecular components, the functional interpretation of seed coat has remained an almost unexplored field of research. The family *Cannaceae* has a somewhat distinct position within this order, having seeds with an exotesta of Malpighian cells, a long life time, and germination with an imbibition lid. Most taxa of the Zingiberales have an endotestal seed coat and germination with a seed lid.

Cannaceae are indigenous at moist and humus-rich sites in the tropical and subtropical parts of South and Central America and the West Indies. The only genus within this family, *Canna*, is nowadays widely distributed, and various cultivars (most of hybrid origin) are well known as ornamentals. The family has a very distinct position within the *Zingiberales* and the closest relationships of *Cannaceae* are considered so be with *Marantaceae* (TOMLINSON 1962, DAHLGREN & al. 1985, KRESS 1990). The ovule is anatropous, bitegmic, and crassinucellar, with the micropyle formed by the inner integument (DAVIS 1966).

Seeds of the *Cannaceae* are able to survive extremely long dormant periods, as proven by SIVORI & al. (1968) and LERMAN & CIGLIANO (1971) with about 600 years old, viable seeds collected from an archaeological site in Argentina.

The seed structure as well as some functional relationship with germination are described in detail by GROOTJEN & BOUMAN (1988). The seeds are ovoid, rather smooth, black, pachychalazal, and provided with a rudimentary micropylar collar. In the fruit, the seeds are embedded in loose hairs, which develop from the funicle and are often regarded as a true aril. When the seeds are shed, these hair tufts remain on the capsule wall. The seed coat is formed mainly out of the chalaza (NETOLITZKY 1926, GROOTJEN & BOUMAN 1988). The chalazal part can be distinguished from the integumentary part by the presence of stomata in the exotesta. GROOTJEN & BOUMAN (1988) described four different layers in the seed coat. The exotesta consists of Malpighian cells, with a typical light line. This palisade layer is followed by a second layer of subepidermal cells and a third vascularised layer initially composed of large tangentially stretched and strongly vacuolised cells. The innermost layer consists of two tanniniferous thin-walled cell layers. Seed coats with Malpighian cells are rare within the monocotyledons. This layer is more common in dicotyledonous families, where it occurs in the outer layer of the testa of *Leguminosae*, *Rhamnaceae*, the inner layer of the testa of *Geraniaceae* and the outer layer of the tegmen of *Cistaceae*, *Malvaceae*, *Tiliaceae* and *Euphorbiaceae* (BOESEWINKEL & BOUMAN 1995). An imbibition lid with a preformed rupture layer has developed at the raphal part of the seed. In the mature seed the raphal area is recognisable as a small elevation close to the hilar-micropylar slit (GROOTJEN & BOUMAN 1988).

In the present study insights into the chemical composition and functions of the seed coat of *Cannaceae* will be acquired by studying pyrolysis mass spectrometric (PyMS) data and pyrolysis gas chromatography mass spectrometric (PyGCMS) data. These data will be compared with data on seed structure obtained with scanning electron microscopy (SEM) and light microscopy combined with histochemical techniques.

Material and methods

Plant material. Mature seeds of *Canna indica* L., *C. flaccida* SALISB., *C. tuerckheimii* KRAENZLIN, *C. lutea* MILL., *C. jaegeriana* URB. and *C. glauca* L. used in this study have been collected via botanical gardens all over the world and by Prof. Dr P. J. M. MAAS, University of Utrecht.

Germination. For germination, seeds were placed in petri dishes on a wet filter or in water. The petri dishes were placed at 25 °C, 40 °C, 50 °C or at 80 °C. Alternatively seeds were put under dry conditions at 25 °C, 40 °C or 50 °C. All petri dishes were kept in the dark for the first 24 hours and afterwards kept at 25 °C under normal day and night conditions. Water was added to the dry petri dishes after the first 24 hours.

Mass spectrometry. Sample preparation. The seed coat of several *Canna* species was isolated and homogenised in a few drops of water in a glass mortar with a glass pestle. Homogenised seed coat material was extracted with a hexane/dichloromethane mixture (1:1 v/v), and centrifuged after three hours. The pellet was suspended and extracted with an ethanol/acetone mixture (1:1 v/v), and was finally washed three times in deionised water. The homogenised and extracted seed coat material was suspended in a few drops of water.

Transmethylation. For methylation of phenolic hydroxyl and carboxyl groups, a small droplet of 2.5% (w/v) tetramethylammonium (TMAH) was added to the wet sample on the filament of the direct insertion probe prior of pyrolysis mass spectrometry (PyMS) and pyrolysis gas chromatography mass spectrometry (PyGCMS) experiments.

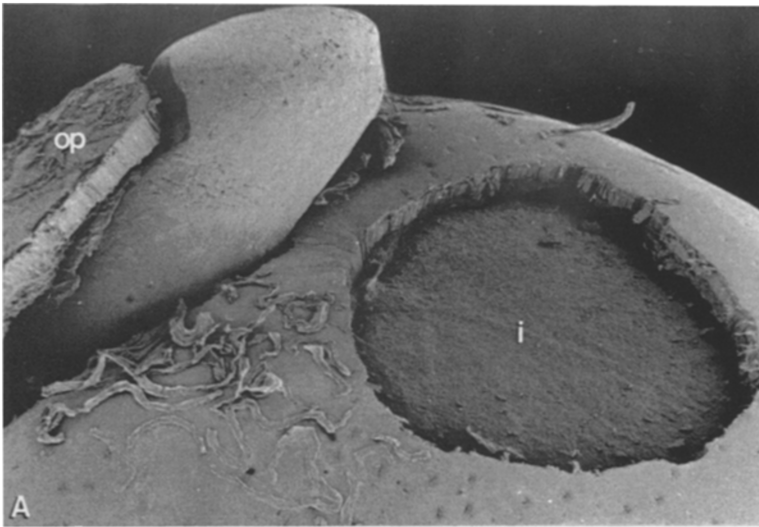
Pyrolysis mass spectrometry. A small drop of a plant material suspension of the homogenised and unextracted or extracted seed coat was placed on a Pt/Rh (9:1; ϕ 100 μm) filament of the direct insertion probe for in-source pyrolysis mass spectrometry using a JEOL DX-303 double focusing mass spectrometer. The wire was inserted into the evacuated ion source, after drying the sample. The ion source temperature was 180 °C. The wire was resistively heated with 16 °C s^{-1} to a final temperature of 800 °C with a rate of 1 amp min^{-1}. Compounds, formed upon desorption or pyrolysis, were ionised by 16 eV electron ionisation (EI) and ammonia chemical ionisation (CI) PyMS. The scan cycle time was 1 s and the ions were analysed over a selected mass range of m/z 20–1000.

Curie-point pyrolysis gas chromatography mass spectrometry. Curie-point pyrolysis gas chromatography mass spectrometry (PyGCMS) under (70 eV) EI conditions was conducted on a Finnigan INCOS 50 quadrupole mass spectrometer connected to a HP 5890 series II gas chromatograph. About 5 μg of a suspension of the sample was applied to a ferromagnetic wire with a Curie-point temperature of 610 °C. The pyrolysis products were injected into a 25 m CPSILS-5 CB fused silica capillary column (i.d. 0.32 mm; film thickness 1.2 μm using He as carrier gas, flow 30 cm s^{-1}). The GC oven was kept at 30 °C during pyrolysis and was subsequently programmed to 320 °C at a rate of 6 °C min^{-1}. The compounds were ionised at 70 eV electron impact energy. The scan cycle time was 0.5 s with a selected mass range of m/z 20–500.

Thin layer Röntgen diffraction analyses. The outer part of the Malpighian layer of the *Cannaceae* seed coat was ashed or grinded and analysed on silica in a thin layer Philips horizontal Röntgen diffraction meter PW 1380 in combination with an analyses system PW 1375. The CuK α radiation was 1.65 kW (HV generator PW 1011). The divergention slit was 0.25°. As goniometer a PW 1371 and a flat LiF monochromator crystal were used. The standard theta value was 1°.

Scanning electron microscopy. Mature seeds were sectioned transversely, coated with a thin layer of gold/palladium and examined with an ISI-DS130 Dual Stage scanning electron microscope (9 kV), fitted with an ISI-Robinson Detector RBSE 130R (19 kV) for backscatter imaging. Furthermore, for silica analysis, seeds were sectioned transversely, fixed on aluminium stubs cemented with carbon cement and air dried under low humidity at room temperature. The specimens were carbon coated and examined in a Cambridge Stereoscan 150 (at 20 kV) fitted with a backscatter detector (KE Developments, Cambridge, England) and a LINK energy dispersive X-ray analyser. The presence of silica was detected with the reference X-ray spectra diagrams. The specific peak (1,74 KeV) for silica in the spectrum was isolated and the spectrum pulses were simultaneous with the scanning of the sample carried back so that an element distribution image of silica could be created (X-ray mapping).

Light microscopy and histochemistry. Hand cut sections were stained for lignin with natriumhypochlorite, phloroglucinol/HCl and lignin pink; for cuticle (neutral fats and fatty acids) with Sudan III, Sudan IV and Sudan black B; for 'cutin' with the azure B method; for unsaturated acidic waxes with the auramine O method; for lipids with magdala red and Nile blue sulphate; for suberin with the phosphate buffer method and the cyanin/potassium method; for protein with Coomassie brilliant blue; for condensed tannins with the nitroso reaction and vanillin/HCl; for hydrolysable tannins with ferric chloride; for pectin with ruthenium red; for cellulose with chlor-zinc-iodide and toluidine blue; for mucilage with alcian blue; for callose with aniline blue and the soda method (Gahan 1984, Krisnamurthy 1988). Sections for staining with ruthenium red and for the cyanin/potassium method were also pre-treated with Eau de Javelle (potassium hypochlorite).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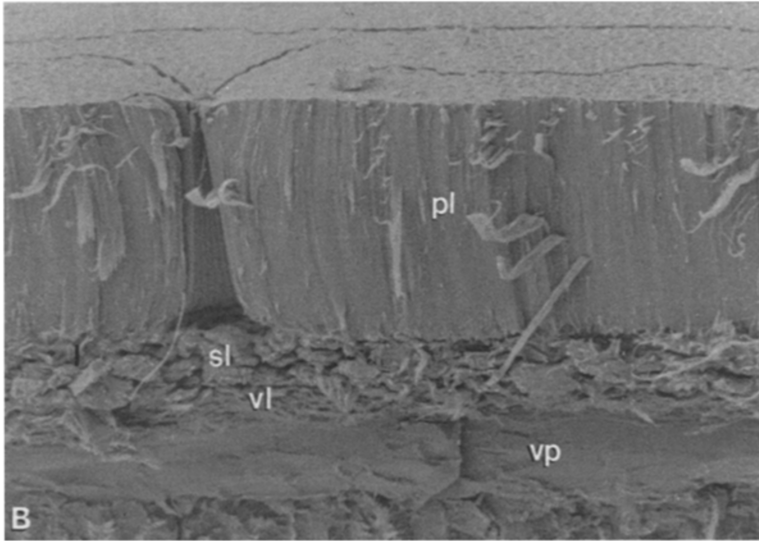
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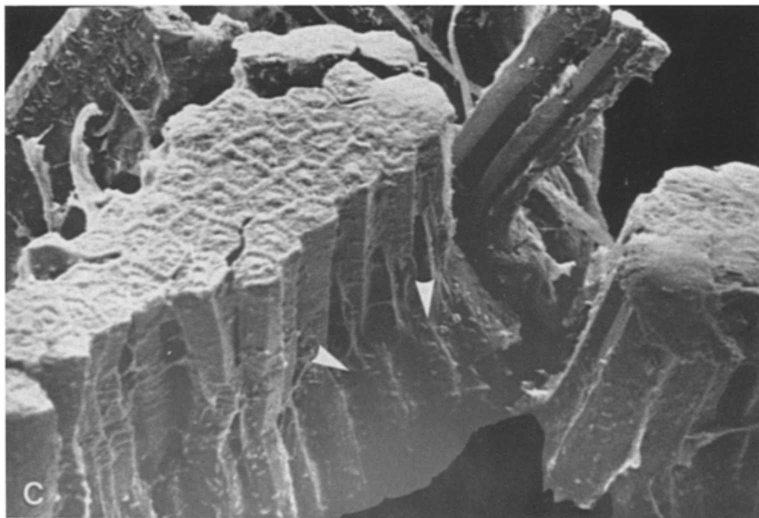
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Results

Imbibition and germination. Air-dried intact seeds placed on a wet filter paper or in water at 40–50 °C overnight opened their imbibition lid after 24 hours in the dark (Fig. 1A). Water enters due to the opened imbibition lid (raphal scar) (Fig. 1A) and the seed coat splits in between the second subepidermal and the compressed vascularised layer (Fig. 1B), and concurrently the exotesta looses its hardness when the Malpighian cells swell. The Malpighian cells did not increase in length but the cells were somewhat swollen beneath the light line (Fig. 1C). Imbibition caused the seed coat to rupture. During the imbibition the upper two cell layers of the vascularised layer released partly with the palisade layer. After three following days at 25 °C the germination lid was lifted due to the pressure of the root.

Opening of the imbibition lid does not always imply that the seed will germinate. In some cases the seed germinated after a year or did not germinate at all during the experiment. The imbibition lid remained closed if placed in an atmosphere of 100% r.h. at only 25 °C during three weeks. Seeds incubated at 25 °C, 40 °C or 50 °C in a dry atmosphere kept their imbibition lid closed, but a few hours after putting them in a wet atmosphere at 25 °C, the seeds with the incubation temperature of 50 °C opened their imbibition lid. No germination occurred if the seeds were exposed to a temperature of 80 °C for the first 24 hours, although the imbibition lid opened.

Macromolecules. The evidence for the presence of macromolecular components in the biological tissues has to come from a combination of separation and ionisation techniques (Graven & al. 1996). All the measured species of *Cannaceae* gave an identical chemical 'fingerprint'. The Py (EI) MS overall spectrum of the seed coat of *Canna indica* is shown in Fig. 2. The spectrum contains markers for a volatile fraction and a non-extractable macromolecular fraction. The volatile lipid fraction is evaporated in an early stage of the temperature ramp whereas non extractable thermolysis products of for instance lignin appear at higher temperature (Fig. 3).

A relatively low abundance of a volatile fraction of lipids is thermally desorbed in an early stage of the temperature ramp. This fraction contains fatty acids (*m/z* 228, $C_{14:0}$; 242, $C_{15:0}$; 256, $C_{16:0}$; 264, $C_{18:1}$ [M-H_2O]; 284, $C_{18:0}$), sterols (*m/z* 368–386, C_{27}; 382–400, C_{28}; 396–414, C_{29}), diglycerides (*m/z* 550, $C_{16,16}$[M-H_2O]; 576, $C_{16,18}$[M-H_2O]; 602, $C_{18,18}$[M-H_2O]), triglycerides (*m/z* 830, $C_{16,16,18}$; 859, $C_{16,18,18}$[M-H_2O]; 884, $C_{18,18,18}$[M-H_2O]), and for aliphatic wax esters (*m/z* 620, C_{42}; 648, C_{44}; 676, C_{46}).

Biopolymer materials, such as lignin, are pyrolysed at higher temperatures. Evidence for lignin in the mass spectrum of *Cannaceae* can be observed as the monomeric pyrolysis products at *m/z* 124, 137, 138, 150, 152, 164, 166 and 180. However, the contribution of lignin is rather low, but it is not extractable as been shown in Fig. 4. The presence of lignin is confirmed by PyGCMS as shown in Fig. 5 and Table 1.

Fig. 1. *A* Germinating seed of *Canna indica* with opened imbibition lid (i) and opened reduced operculum (op) (approx. × 30). *B* Longitudinal section of *Canna* mature seed coat (approx. × 245); *pl* palisade or Malpighian cell layer, *sl* subepidermal layer, *vl* vascularised layer, *vp* vegetable polyphenolic tannin layer. *C* Malpighian cells are swollen beneath the light line (approx. × 330)

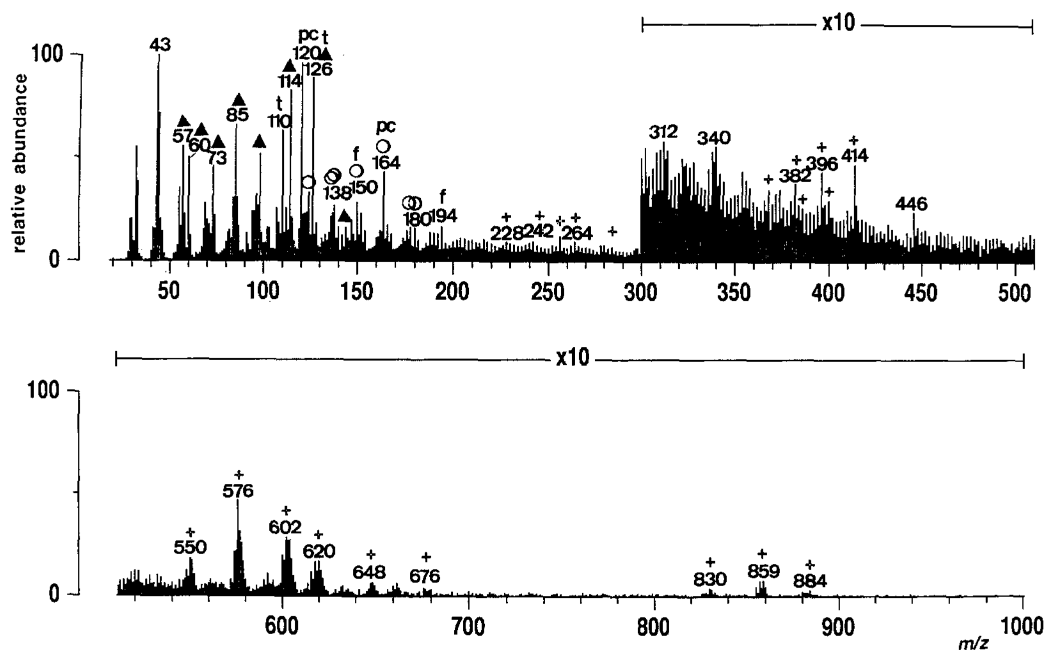

Fig. 2. Pyrolysis (EI) mass spectrum of the mature seed coat of *Canna*. ○ guaiacyl lignin; ▲ polysaccharides; + lipids, *f* ferulic acid, *pc* *p*-coumaric acid, *t* vegetable polyphenol markers

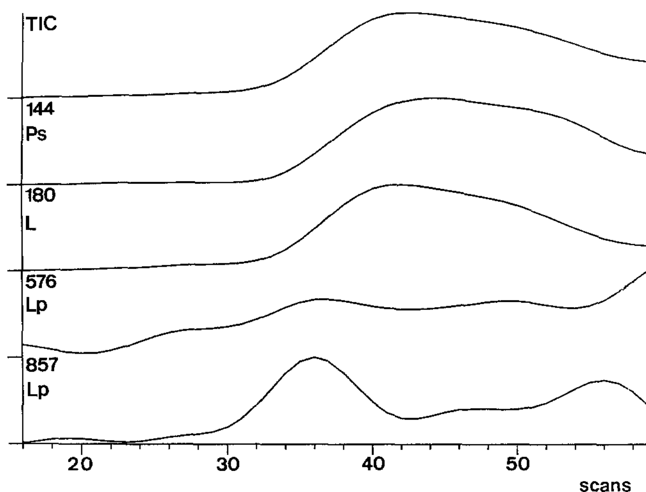

Fig. 3. Total ion current and mass chromatograms of the total seed coat of *Canna indica*. The ions of the mass chromatogram comprise markers of several compound classes as polysaccharides (Ps), guaiacyl lignin (G), diglyceridic fragments and triglycerides (Lp)

The spectra of the extracted and non-extracted seed coat samples show a high contribution of pyrolysis products indicative of the presence of polysaccharides. This is shown by the fragment peaks *m/z* 43, 57, 60, 73, 126 and 144 for hexose sugars, and *m/z* 58, 85 and 114 for pentose sugars. The presence of polysaccharides is confirmed with ammonia chemical ionisation as shown in Fig. 6. This figure contains mass peaks for ammoniated hexose sugar monomers (*m/z* 144, 162, 180, 222, 240), dimers (*m/z* 324, 342), trimers (*m/z* 402, 504) and tetramers (*m/z* 666)

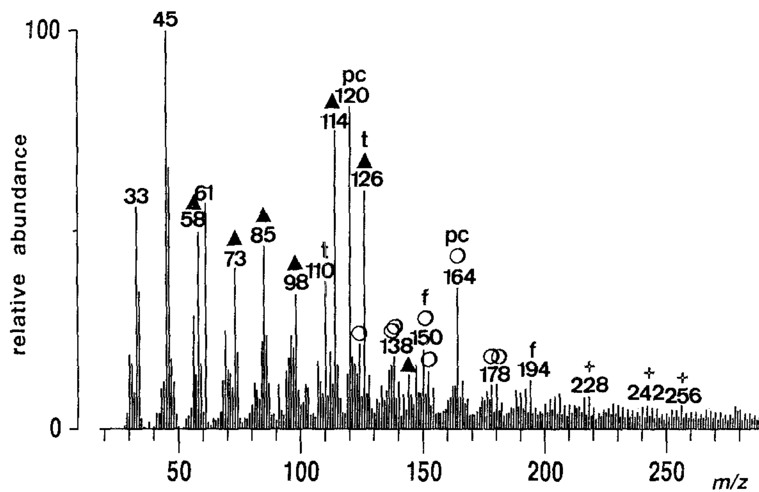

Fig. 4. Pyrolysis (EI) mass spectrum of the seed coat of *Canna* after extraction with hexane dichloromethane and acetone/ethanol. ○ guaiacyl lignin, ▲ polysaccharides, + lipids, *f* ferulic acid, *pc* *p*-coumaric acid, *t* vegetable polyphenol markers

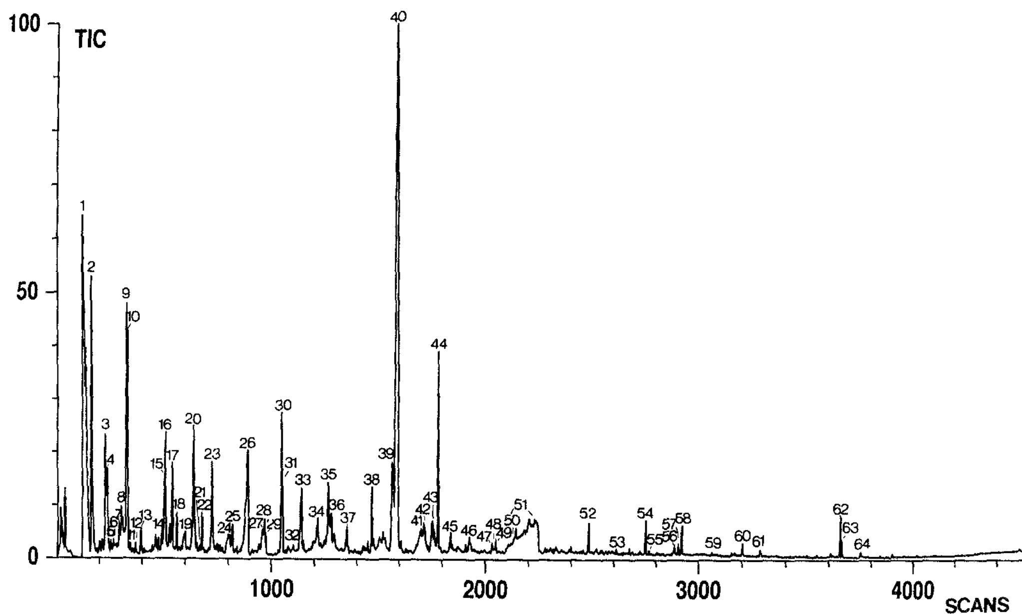

Fig. 5. Total ion chromatogram of a pyrolysis (EI) gaschromatography mass spectrometric run of a solvent extracted seed coat of *Canna indica*

thereof, which are prominent for cellulose, and ammoniated pentose sugar monomers (*m/z* 132, 150) and dimers (*m/z* 264, 282), which are prominent in hemicelluloses (Pouwels & Boon 1990). The abundance of trimeric and tetrameric anhydrosugars in the pyrolysates is low due to the presence of anorganic matter in the seed coat of *Cannaceae*, as indicated by *m/z* 134 (Scheijen & Boon 1989).

Table 1. PyGCMS data of the extracted *Canna indica* seed coat. Identified peaks from a chromatogram after extraction of the seed coat sample. From every peak the m/z of the molecular ion is given ($M^{+\bullet}$). *PS* polysaccharide; *L* lignin; *Lp* lipid, *pc* *p*-coumaric acid; *f* ferulic acid; *t* vegetable polyphenol markers

Peak nr.	Compound	Origin	Scan	$M^{+\bullet}$
1	carbon dioxide		132	44
2	propanedialdehyde	PS	160	72
3	2,3-butadione	PS	223	86
4	2-butanone	PS	235	72
5	2-methylfuran	PS	250	82
6	3-methylfuran	PS	263	82
7	2-butanal (cis)	PS	290	70
8	2-butanal (trans)	PS	299	70
9	acetic acid		324	60
10	hydroxypropanone	PS	331	74
11	1-pentene-3,4-dione	PS	345	98
12	2,3-pentanedione	PS	367	100
13	tetrahydrofuran-3-one	PS	392	86
14	(3H)-furan-2-one	PS	489	84
15	3-hydroxypropanal	PS	502	74
16	1-hydroxybutane-2-one	PS	507	88
17	pyruvic acid methyl ester	PS	539	102
18	(2H)-furan-3-one	PS	560	84
19	3-furaldehyde	PS	591	96
20	2-furaldehyde	PS	640	96
21	2-methyl-2,3-dihydrofuran-3-one	PS	676	98
22	(4)-methyltetrahydrofuran	PS	677	100
23	1-acetyl oxypropane-2-one	PS	724	116
24	(5H)-furan-3-one	PS	800	84
25	2-methyl-2-cyclopentene	PS	818	96
26	2,3-dihydro-5-methylfuran-2-one	PS	877	98
27	5-methyl-2-furaldehyde	PS	958	110
28	3-methyltetrahydrofuran-2,3-dione	PS	967	114
29	2-hydroxy-3-methyl-2-cyclopentene-1-one	PS	975	112
30	phenol		1047	94
31	4-hydroxy-5,6-dihydride-(2H)-pyran-2-one	PS	1054	114
32	3-hydroxy-2-methyl-2-cyclopentene-1-one	PS	1103	112
33	2-hydroxy-3-methyl-cyclopentene-1-one	PS	1136	112
34	2-methylphenol		1214	108
35	3- or 4-methylphenol		1265	108
36	guaiacol	L	1280	124
37	dimethylpyranone	PS	1353	126
38	4-ethylphenol		1474	122
39	dihydroxybenzene	t	1564	110
40	vinylphenol	L	1588	120 pc
41	guaiacylethane	L	1697	152
42	guaiacylethane	L	1713	152
43	methyldihydroxybenzene	t	1751	124

Table 1 (continued)

44	4-vinylguaiacol	L	1780	150f
45	syringol	L	1838	154
46	guaiacolaldehyde	L	1925	152
47	4-methylsyringol	L	2031	168
48	guaiacolethanol	L	2042	166
49	4-(trans-2-propenyl) guaiacol	L	2048	164
50	guaiacolethanon (acetylguaiacol)	L	2096	166
51	levoglucosan	PS	2100–2250	162
52	4-(prop-1-enyl) syringol	L	2482	194
53	$C_{14:0}$ fatty acid	Lp	2611	228
54	phtalaat	artefact	2751	(149)
55	$C_{15:0}$ fatty acid	Lp	2768	242
56	phtalaat	artefact	2888	(149)
57	unknown		2906	236
58	$C_{16:0}$ fatty acid	Lp	2922	256
59	$C_{17:0}$ fatty acid	Lp	3061	270
60	$C_{18:0}$ fatty acid	Lp	3202	284
61	unknown		3282	260
62	C alkane		3652	352
63	phtalaat	artefact	3662	(149)
64	3-methoxy-4,4-hydroxy stilbene (trans)	L	3751	272

The EI mass spectrum of *Cannaceae* has a relatively abundant *m/z* 120 and 150 indicative for *p*-coumaric acid and ferulic acid which is typical for monocotyledonous plants (Boon 1989). Mass peaks for ester bound (*m/z* 120) and ether bound (*m/z* 164) *p*-coumaric acid, and ester bound (*m/z* 150) and ether bound (*m/z* 194) ferulic acid are shown in Figs. 2 and 4. The quasimolecular ions *m/z* 121 (vinylphenol) and *m/z* 151 (vinylguaiacol) in the ammonia CI spectrum (Fig. 5) also point to the presence of these phenolic acids. The identity of these phenolic acids was confirmed by PyGCMS seed coat samples in the presence of TMAH. This spectrum also shows dimers of these phenolic acids. A mass chromatogram of TMAH methylated *p*-coumaric acid (*m/z* 192) and ferulic acid (*m/z* 222) and their dimers is inserted in Fig. 7 (Mulder & al. 1992).

The relatively high masses *m/z* 94, 110 and 126 in the Figs. 2 and 4 may indicate the presence of vegetable polyphenols, which appear in later stage of the temperature ramp.

Histochemical observations of the mature seed coat. The general structure of the seed coat is in agreement with the description of Grootjen & Bouman (1988). The top of each Malpighian cell is filled with a single more or less globular silica body (Fig. 8A-D). It was not possible to demonstrate the presence of the silica as amorphous or crystalline with the used techniques. This is probably due to the very small bodies in the seed coat. The cell walls above the light line stain positively with lignin pink and phloroglucinol/HCl, indicating the presence of lignin in this area.

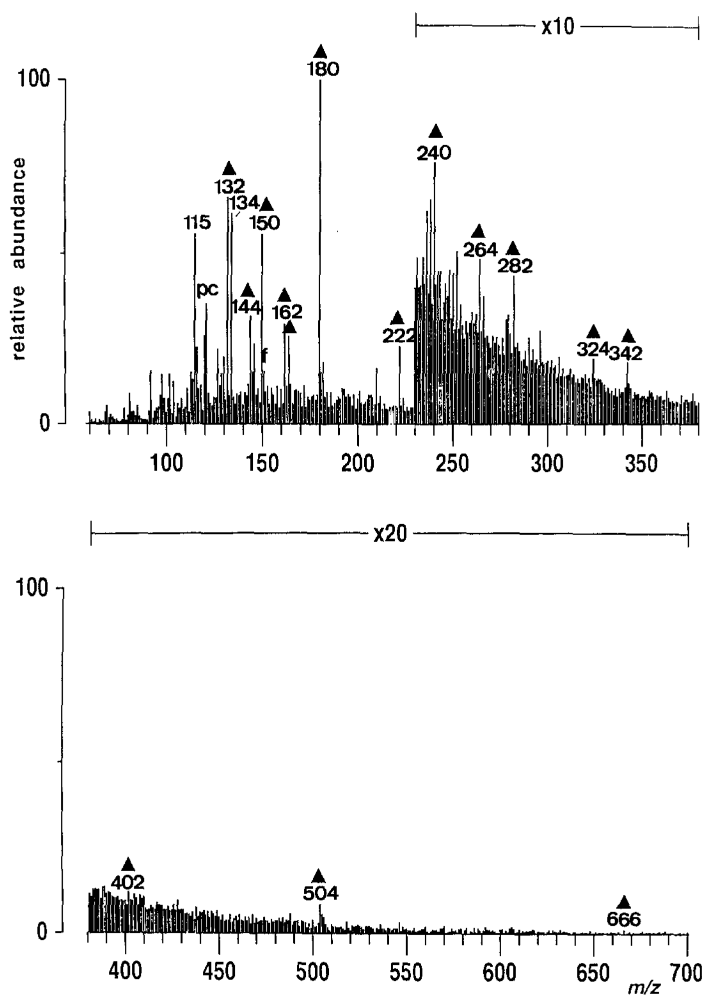

Fig. 6. Pyrolysis (ammonia CI) mass spectrum of the extracted seed coat of *Canna*. ▲ polysaccharides, *f* ferulic acid, *pc* *p*-coumaric acid

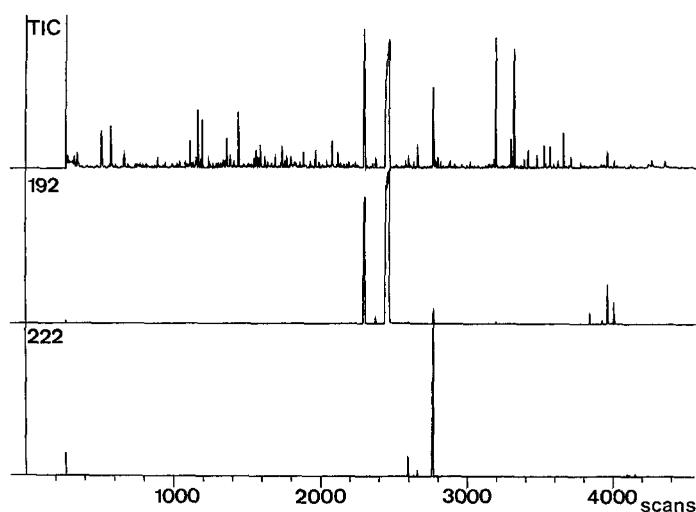

Fig. 7. Py (EI) GCMS partial mass chromatogram of the TMAH treated samples of the extracted seed coat of *Canna* for *m*/*z* 192 (*p*-coumaric acid) and *m*/*z* 222 (ferulic acid)

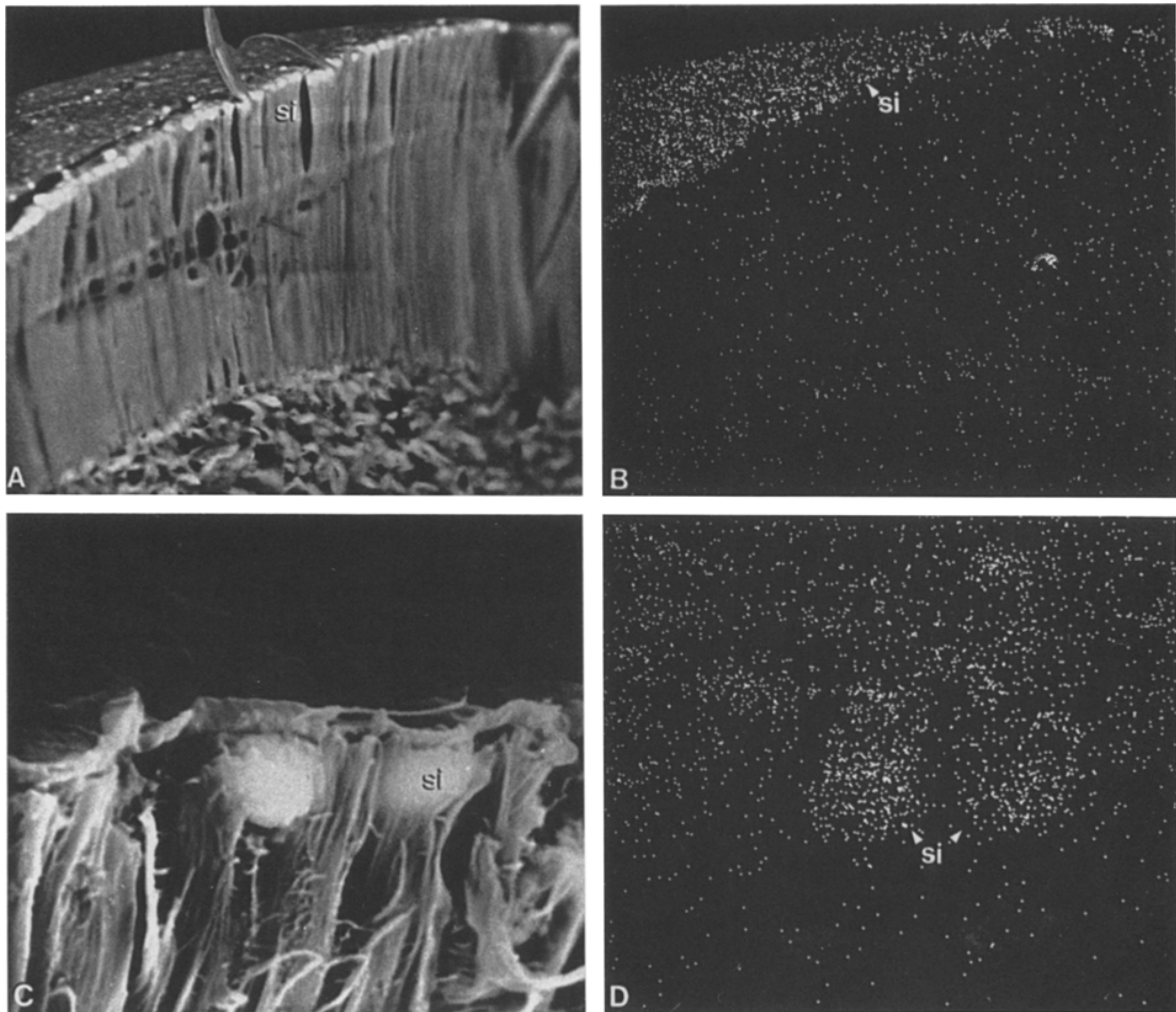

Fig. 8. Backscatter image and bright dot mapping of the silica bodies (si) in top of each Malpighian cell of *Cannaceae* seed coat (A, B approx. × 350; C, D approx, × 2450)

Below the light line the Malpighian cells stain more intensively for cellulose. In the cell lumen beneath the light line some vegetable polyphenol like material is present. The histochemical tests with aniline blue and the soda method indicated the presence of callose in the Malpighian cells above the light line. A thin cuticle covers the palisade layer, and stains positively with Nile blue sulphate, Sudan III, Sudan IV and auramine O. The bright greenish yellow fluorescence, due to the Auramine O, could be seen at the light line and the primary wall of the vascularised and the S1 layer of secondary wall of the 'tannin' layer. The cyanin stain indicated the presence of lipid components in the primary wall of the vascularised layer and in the S1 layer of the secondary wall of the vegetable polyphenol layer. All the cell walls contain

some protein. The light line disappears when treated with Eau de Javelle. The Eau de Javelle destroys vegetable polyphenols and makes lignified cell walls loose their staining ability (KRISNAMURTHY 1988). After this treatment pectin could be detected with ruthenium red in the Malpighian cells.

Discussion

The seeds of *Cannaceae* are equipped with a special mechanism allowing them to survive long periods of unfavourable conditions. The seed coat of *Canna* is very hard and impermeable to water. The mechanical strength and water restriction is assigned to the exotestal layer of Malpighian cells, which acts as a physical barrier to imbibition. If the seeds do not imbibe water they remain hard and unswollen. Seeds are able to imbibe water after artificial filing of the seed coat or after an imbibition lid has been released, but its mechanism is still unknown (GROOTJEN & BOUMAN 1988). In nature the release of an imbibition lid may be induced by heat treatment caused by solar radiation and/or microclimatical changes. An incubation temperature of 50 °C seems to be sufficient to open the imbibition lid. The combination of an impermeable palisade layer and an imbibition lid is the water regulating mechanism in *Cannaceae* seeds. The stomata on the integumentary part of the seed do not play any role during the imbibition of the seed (GROOTJEN & BOUMAN 1988). When the imbibition lid has opened, the water moves in between the vascularised layers and entering the Malpighian layer from the lower part. Water can freely pass through the network of cellulose fibrils beneath the light line. As a result, the lower part of the exotesta swells up and the seed coat ruptures.

The upper part of the Malpighian cells is responsible for the water impermeability of the seed coat of *Canna*. According to the histochemical data, the upper part is impregnated with callose and lignin components and the light line with lipid material, whereas the lower part of the Malpighian cells mainly consists of cellulose. This tightly packed network in the Malpighian cells above the light line acts as an effective barrier against water entry.

Members of the *Cannaceae* are known for the extreme longevity of their seeds. Most genera with long living seeds have been reported to have seed coats with Malpighian cells (BOESEWINKEL & BOUMAN 1995). Information on hard seed coats with a palisade or Malpighian layer has come from studies of the *Leguminosae* and *Malvaceae*. In these taxa special devices are present which enable seeds to imbibe water preceding germination. Seed coats only become permeable after release or splitting of a special region of the Malpighian layer.

In many legumes a hilar groove functions as a hygroscopical activating valve permitting moisture to be lost from the drying seed (TRAN & CAVANAGH 1984). Rupturing or lifting of the so-called lens permits water entry. In *Malvaceae* the raphal tissue releases from the seed and exposes the chalazal area with a median cleft. Only raising of a blister, containing a region of the palisade layer, induces imbibition and germination (EGLEY & PAUL 1981). The seed dormancy in legume seeds is usually considered to be effected through the physical restriction of water uptake by the impermeable outer part of the epidermal layer of the Malpighian cells (WERKER 1980/1981, TRAN & CAVANAGH 1984, GOPINATHAN & BABU 1985, SERRATO-VALENTI & al. 1992).

Malpighian layers in seed coats are able to exclude water by water repellent macromolecules. Previous theories assumed that the light line is the source of impermeability. However, it has been demonstrated that the light line is due to a change of the orientation and density of the cellulose fibrils (GROOTJEN & BOUMAN 1988), or a juxtaposition of different macromolecular components in the Malpighian cell walls (HAMLY 1935, SPURNY 1973, MANNING & VAN STADEN 1985). In *Malvaceae* the light line is even situated in the hydrophilic part of the Malpighian cells. According to VAN STADEN & al. (1989) the chemical and physical conditions responsible for producing the light line are not alone responsible for the palisade impermeability. In *Cannaceae* the light line is due to a juxtaposition of macromolecular components above and around the light line. In *Cannaceae* the light line is also visible with SEM and disappears after treatment with strong oxidants, which suggests that the light line is not only a purely optical phenomenon.

The Malpighian cells consist of a hydrophilic and a hydrophobic part. The hydrophobic part is responsible for the exclusion of water. Macromolecular components in the hydrophobic part such as lignin, suberin, callose and silica, enable water to be excluded. The hydrophobic part can either be the upper layer of the Malpighian cells as in exotestal seeds, or the lower part as in exotegmic seeds (*Malvaceae*). The relative position of the hydrophilic and hydrophobic parts seems to be related to the way in which the water enters and moves in the seed coat. In legumes and *Cannaceae* the water is conducted by the vascularised testa and subsequently absorbed by the lower, hydrophilic part of the exotestal Malpighian layer. In *Malvaceae* water enters the chalazal region, and is absorbed by the upper part of the exotegmic layer, via the inner tangential walls of the endotesta. This area swells while the lignified 'twisted' part does not change (SERRATO-VALENTI & al. 1992). A difference of pressure arises and leads to rupturing of the Malpighian layer.

In the *Cannaceae* callose makes the palisade layer impermeable, but also the lignin causes a more tightly packed network. Also the hydrophobic part of the Malpighian cells in seeds of the *Leguminosae* contain suberin and callose. Callose, a polymer of ß-1,3-D-glucose, often occurs temporarily as, e.g., a blocking element in sieve plates, in the walls of micro- and megaspore tetrads, and as extra cell thickening in pollen tubes and as closing element of them. Callose occurrence has been reported in the impermeable seed coat of *Trifolium subterraneum* (*Papilionaceae*) (BHALLA & SLATTERY 1984), *Gleditsia triacanthos* (*Caesalpiniaceae*) (BEVILACQUA & al. 1985), *Sesbania punicea* (CAV.) BENTH. (*Papilionaceae*) (BEVILACQUA & al. 1987), *Melilotus alba* MEDIK. (*Papilionaceae*) (COE & MARTIN 1920, BEVILACQUA & al. 1989), and of *Gasteria verrucosa* (MILL.) HAW. (*Asphodelaceae*) (WITTICH & GRAVEN 1995). It is regarded as an important factor for water exclusion and hence for the seed coat imposed dormancy of these seeds (BEVILACQUA & al. 1989). In seeds of *Melilotus alba*, a combination of lipids and callose prevents both apoplastic and symplastic water movement from the environment to the inner side of the seed (BEVILACQUA & al. 1989).

GROOTJEN & BOUMAN (1988) reported a strong reaction of Sudan IV in the palisade layer, especially beneath the light line, whereas neither lignin nor pectin were visible through specific staining. The histochemical results presented in this paper do not confirm the results of GROOTJEN & BOUMAN (1988).

Several of our tests on lipids are negative. This is in accordance to the very low abundance of lipids in the PyMS spectra. Only the Auramine O test indicates the presence of some lipid material at the location of the light line. Furthermore, pectin and lignin above the light line are demonstrated. The presence of these compounds in the seed coat of *Cannaceae* is also confirmed by the PyMS and PyGCMS results. The latter methods are more reliable in demonstrating macromolecular components than histochemical methods. The negative results of different staining methods are not only due to the absence of the specific compounds but also may be the result of the interference of the various macromolecular components hindering the stains to enter the reactive sites in the walls.

The silica in the top of the Malpighian cells of *Cannaceae* has several mixed functions. Although it was not possible to get a clear insight into the chemical appearance of the silica with the used techniques. Silica occurs in nature in crystalline and amorphous forms. As argued before it may play a role in the water regulation of the seed coat. It provides support in addition to that provided by the cellulose, lignin and the hard chalazosperm, and silica could provide both mechanical support and protection against attack caused by pathogens, insects and other predators (LANNING & ELEUTERIUS 1992). In *Schoenus nigricans*, silicification has an ecological benefit for protection against predatory larvae (ERNST & VAN DER HAM 1988). On the other hand it hampers the rapid germination of the highly silicified nutlets and therefore enhances survival in the seed bank (ERNST & al. 1995). The occurrence and locations of crystals is specific and useful in taxonomic classification. In the families of the *Zingiberales* silica occurs as deposit in exotestal or endotestal cells (GRAVEN & al. 1996).

Cannaceae seeds combine several defence systems in its seed coat. Next to the mechanical protection by the Malpighian layer, also vegetable polyphenols may form a barrier against certain harmful insects, fungi and other pathogens (SWAIN 1965). In *Canna* for example the innermost tanniniferous layer of the seed coat consists of two layers of large thin-walled cells filled with vegetable polyphenols ('tannins') as indicated by the peaks m/z 94, 110 and 126 (Figs. 2 and 4). Lignins have been reported to contain only negligible amounts of catechol (m/z 110). It is reasonable to use catechol as a diagnostic fragment for polyphenols (GALLETI & REEVES 1992).

The vegetable polyphenol layer may play a role in the dormancy of the seed after the release of the imbibition lid when environmental conditions change unfavourable. In our experiments, some of the seeds in the experiments were able to germinate a year after opening of the imbibition lid showing that the seeds of the *Cannaceae* have a secondary dormancy. Although the first defence line has passed and the seeds can imbibe water, still no germination occurs during unfavourable conditions. Vegetable polyphenols in seeds appear to be associated with maintaining dormancy (CHALKER-SCOTT & KRAHMER 1989). For instance, the real cause of dormancy in *Cornus* (*Cornaceae*) seed is the high level of vegetable polyphenols in the seed coat restricting the embryo development (GUAN 1990). Polyphenols may influence gas exchange and the permeability of water. The phenolic substances in the coat of *Cynoglossum officinale* (*Boraginaceae*) inhibit seed germination by controlling O_2 availability to the embryo. The O_2 uptake of *Cynoglossum officinale* increased approximately six-fold upon removal of the strongly phenolic coat (QI & al. 1993).

The removal of the coat resulted in nearly complete germination of innately dormant seeds. Furthermore it can protect the embryo and endosperm during the imbibition period against environmental stress and disease. The cell walls of the vegetable polyphenol layer contain fatty components as shown by the potassium/cyanin test and Auramine O, indicating the presence of suberin-like material. Fatty acids could be detected with PyMS and PyGCMS, after extraction with hexane/dichloromethane and acetone/ethanol. These fatty acids are cell wall bound and may be involved with the very thin cuticle, which covers the seed. Phenolic acids are considered to be a compound group which cross-links hemicellulose or links hemicellulose to lignin (FORD & HARTLEY 1990; HARTLEY & al. 1990 a,b; MULDER & al. 1992). It is difficult to detect phenolic acids in biological matrices with histochemical analysis, as it is easily demonstrated by several PyMS and PyGCMS techniques (MULDER & al. 1992).

The PyMS spectrum of *Canna* in our experiments is dominated by the phenolic acids *p*-coumaric acid and ferulic acid. WEHLING & al. (1989) showed that *p*-coumaric acid is a very genuine structural unit in the sporopollenin skeleton and that it plays an important role in the resistance of the sporopollenin. The seed coat of *Canna* is probably more heavily impregnated with phenolic acids than with lignin, to resist degradation and to survive long periods. Dimers of the phenolic acids also contribute to the hardening of the cell wall due to oxidative coupling of the carbohydrate esters of the phenolic acids (FRY & MILLER 1989, ISHII 1991).

The very thin cuticle on the outer side of seed is probably of minor importance for the water impermeability of the seed coat. A thin cuticle also occurs on the Malpighian cell layer of the leguminosous seeds (NETOLITZKY 1926, WERKER & al. 1973, GUTTERMAN 1978). The cuticle of this seed has been considered to the cause of impermeability. However artificial removal of the outer cuticle in *Caesalpinaceae* did not render the seed permeable to water (BALLARD 1973).

The seed coat of *Canna* is both anatomically and chemically well differentiated. The mainly polysaccharide PyMS spectrum with the high contributions of phenolic acids gives an unique chemical fingerprint of the seed coat of *Cannaceae*, which makes it distinguishable from other families (GRAVEN & al. 1996, GRAVEN unpubl).

It is remarkable that the monocotyledonous family of the *Cannaceae* has Malpighian cells, whereas these occur mainly in dicotyledonous families. A remarkable feature of the *Cannaceae* within the *Zingiberales* is the presence of an imbibition lid and the rupturing of the exotesta during imbibition. This does not occur in other members of the order.

The differences in seed structure between *Cannaceae* and other members of the *Zingiberales* seem to be the result of adaptation of *Canna* to alternative dispersal and germination strategies (GROOTJEN & BOUMAN 1988).

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