Studies on Sporopollenin Structure during Pollen Development

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To clarify whether the structure of sporopollenin varies during pollen development FTIR and ^{13}C NMR spectroscopic analyses of *Tulipa* and *Cucurbita* sporopollenin from three different developmental stages were carried out.

The spectra of sporopollenin obtained from an early, a middle postmeiotic and the mature stage have been compared. The assumption that sporopollenin mainly consists of long chain aliphatics with varying amounts of aromatics is corroberated by our results.

Only slight changes in sporopollenin structure could be recognized during pollen wall development of *Cucurbita maxima*. In contrast to this the spectra of *Tulipa* sporopollenin of the three developmental stages exhibited significant differences concerning the aliphatic and aromatic components.

Introduction

Sporopollenin is a biopolymer which is substantially involved in the formation of the outer pollen and spore walls. Because of its high resistance towards nonoxidative chemical, physical, and biological degradation, neither its definite chemical structure nor the involved biochemical pathways have been clarified in detail yet.

As the macromolecule is insoluble in most common solvents, solid state analyses like NMR and FTIR spectroscopy are suitable techniques to study structures of sporopollenins (Guilford *et al.*, 1988; Espelie *et al.*, 1989; Hemsley *et al.*, 1992, 1993; Wilmesmeier *et al.*, 1993).

The results of those spectroscopic investigations, concerning sporopollenin fractions of different systematic origin, revealed far-reaching similarities of characteristic signals. At present it is well accepted that sporopollenin is mainly composed of long chain aliphatics with a varying amount of aromatics. Contributions of single chemical groups to the entire molecule however, were found to differ (Hemsley *et al.*, 1992, 1993; Wilmesmeier *et al.*, 1993).

Therefore to our current state of knowledge, sporopollenin is considered to represent a group of closely related macromolecules, showing significant variations due to their diverging phylogenetic origins.

Even the resistant biopolymer of some algal walls is considered as sporopollenin, regarding chemical properties and spectroscopic data (Guilford *et al.*, 1988; Derenne *et al.*, 1992).

Although sporopollenins of different systematic origin were subject of intensive NMR- and FTIR-spectroscopic analyses investigations comparing sporopollenin of different developmental stages are still missing.

Investigations by electronmicroscopy (Heslop-Harrison, 1968; Southworth, 1983; Blackmore and Barnes, 1990), histochemistry (Southworth, 1973), UV and fluorescence spectroscopy, respectively (Willemse, 1972; Southworth, 1983) indicate, that the chemical structure of sporopollenin changes during the various stages of exine development.

An increase in resistance towards acetolysis in the course of maturation (Heslop-Harrison, 1968) and changes in stainability and UV absorption (Heslop-Harrison, 1968; Echlin, 1969; Southworth, 1974) have been reported.

FTIR- and NMR-spectroscopic techniques were used to obtain clear data about variations on the molecular level that occur during sporopollenin development. To account for developmental as well as taxonomical differences sporopollenin fractions obtained from anthers of different plant

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taxa (*Tulipa* cv. "Apeldoorn", monocotyledonae and *Cucurbita maxima*, dicotyledonae) each at three different developmental stages were investigated.

Material and Methods

Plant material

Androecia of *Cucurbita maxima* var. "Gelber Zentner" cultivated in a greenhouse of the Botanical garden, Münster, and anthers of *Tulipa* cv. "Apeldoorn" (Nebelung, Münster, BRD), grown outside, were used.

Characterization of the three investigated developmental stages:

1. *Cucurbita maxima*

Stage **I:**
- Anthers 4.0–5.5 mm in length.
- Mononuclear microspores just after tetrad disruption, with exine already patterned and nonvacuolated cytoplasm.

Stage **II:**
- Anthers 7.0–9.0 mm in length.
- Pollen grains just after mitosis I; exine showing characteristic patterns (spinulae); tapetum cells not degenerated, no fibers formed in the anther wall.

Stage **III:**
- Mature pollen after anther dehiscence.

2. *Tulipa* cv. "Apeldoorn"

Stage **I:**
- Anthers 11.0 mm in length.
- Microspores immediatly after tetrad disruption with a thin exine.

Stage **II:**
- Anthers 13.0 mm in length.
- Mononuclear microspores with a clearly developed exine.

Stage **III:**
- Mature pollen after anther dehiscence.

Enrichment of the sporopollenin fractions

Cucurbita maxima

Androecia were cut into cross sections and pollen was washed out by a sharp jet of buffer (see Gubatz and Wiermann, 1993).

Tulipa

Anthers of the first and the second investigated developmental stage were decapitated and the loculus material was squeezed out according to Rittscher and Wiermann (1988a). Mature pollen was collected from dehisced anthers by trapping with a broad needle.

Sporopollenin was enriched from pollen by successive exhaustive extractions with methanol, chloroform, acetone, methanol, ethylene glycol monomethyl ether, and double-distilled water. The remaining solid was washed with 5 % HCl once and with double-distilled water until pH 7 was reached. The extracted material was then subsequently treated with phosphoric acid according to Rittscher and Wiermann (1988a).

The samples were lyophilized and stored in a desiccator until examination.

^{13}C CP/MAS NMR and FTIR spectroscopy

Spectroscopic analyses were carried out as previously described (Wilmesmeier *et al.*, 1993).

FTIR spectroscopy was carried out using a Nicolet 5 DXC FTIR spectrometer with Omnic 1.2 software from Nicolet Instrument Coorporation (Offenbach, Germany).

Results

Results of ^{13}C NMR and FTIR spectroscopy of mature sporopollenin

As reported (Hemsley *et al.*, 1992; Wilmesmeier *et al.*, 1993) the spectra of sporopollenins of different plant taxa exhibit wide similarities, nonetheless some differences are obvious.

This is confirmed by ^{13}C CP/MAS NMR and FTIR spectra of mature *Cucurbita* and *Tulipa* sporopollenin shown in Figs. 1a–1b, III and Figs. 2a–2b, III, respectively (assignments see Table I).

The NMR spectra of mature sporopollenin are dominated by aliphatic groups (signal 7 in Fig. 1a, III and Fig. 2a, III). The spectrum of *Tulipa* sporopollenin (Fig. 2a, III) shows strong signals attributed to aromatic groups as well (signals 4 and 5). These results are confirmed by the FTIR analyses (Fig. 1b, III and 2b, III). Signals C, H and I represent aliphatic groups and signals G and O indicate aromatic structures. Moreover, NMR signal 2 and

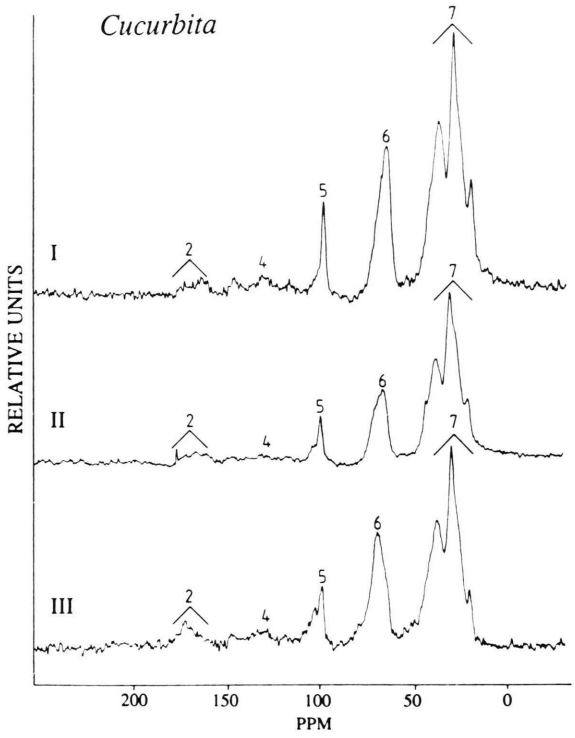

Fig. 1a. ^{13}C CP/MAS NMR spectra of three developmental stages of *Cucurbita* sporopollenin.

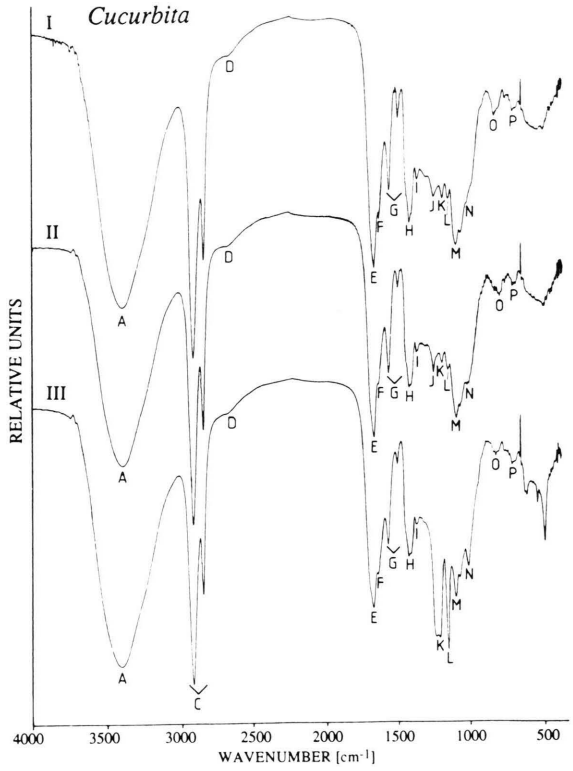

Fig. 1b. FTIR spectra of three developmental stages of *Cucurbita* sporopollenin.

FTIR signals D and E account for a small amount of carbonyl- and carboxyl functions respectively.

Comparative analyses of sporopollenin spectra obtained from pollen of three different developmental stages

Cucurbita

^{13}C CP/MAS NMR and FTIR spectra of the three developmental stages of *Cucurbita* sporopollenin are shown in Figs. 1a and 1b, respectively.

FTIR spectra of *Cucurbita* sporopollenin of different developmental stages (Fig. 1b) only show slight variations. Signals L, M, and N attributed to ether and partly to ester bonds, increase slightly from stage II to III.

Peaks characteristic of carbonyl and carboxyl groups (D, E, F) show no significant changes during pollen development (see also the NMR signals 6 and 4 in Fig. 1a).

According to the NMR spectra a decrease in aromatic groups (signals 4 and 5) occurs between stage I and III. Signal 4 can be detected in stage I only, signal 5 – which is also attributed to alkene groups – exhibits a side peak at 105–110 ppm at later stages of development, indicating a qualitative change in molecular structure.

Signal 7 is subdivided into three peaks in all investigated stages of development. There is neither a decrease in their height, nor a proportional change detectable. The occurrence of three distinct aliphatic peaks is an indication of CH_3-, CH_2-, and CH-groups occurring simultaneously.

Tulipa

^{13}C CP/MAS and FTIR NMR spectra of the three different developmental stages of *Tulipa* sporopollenin are shown in Figs. 2a and 2b.

In contrast to *Cucurbita* spectra of *Tulipa* sporopollenin show differences.

Table I. Assignments of the ^{13}C CP/MAS NMR and FTIR signals marked in Figs. 1 and 2. For further information concerning band origins in FTIR spectra and carbon type in NMR spectra see Wilmesmeier *et al.* (1993). To facilitate a direct comparison of our results with those published by Wilmesmeier *et al.* (1993) signals 1 and 3 are listed in Table I although they do not appear in the NMR spectra presented here (Figs. 1a and 2a).

No. in NMR spectra	Chemical shift [ppm]	Classification	Letter in FTIR spectra	Wave number [cm^{-1}]
1	200–210	Carbonyl-	D	3000–2500
2	170–180	Carboxyl-	E	1700–1800
	160–170	groups	F	1640
2	170–180	Ester	K	1200
	160–170		L	1165
3	145		B	3050–3030
4	130	Aromatics	G	1610; 1580; 1517
5	105		O	830
			L	1165
6	60–75	Ether	M	1110
			N	1010
			C	2930–2860
7	30	Aliphatics	H	1440
	15		J	1380
			P	720

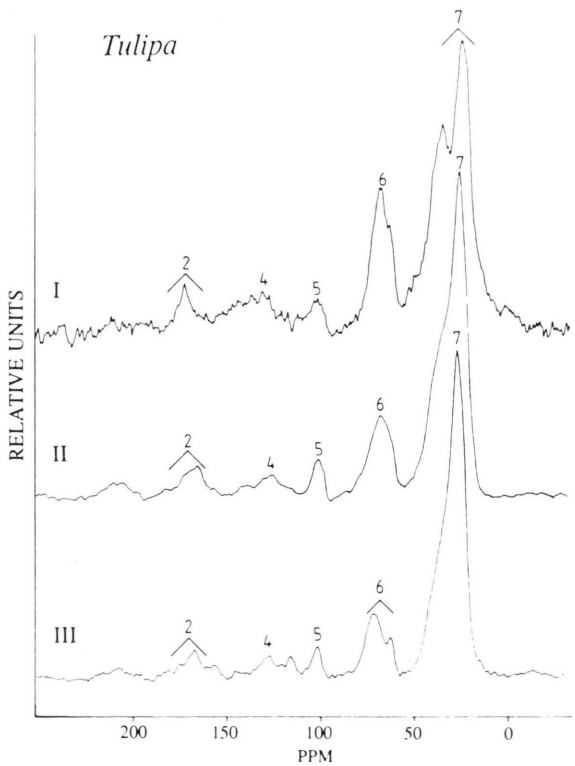

Fig. 2a. ^{13}C CP/MAS NMR spectra of three developmental stages of *Tulipa* sporopollenin.

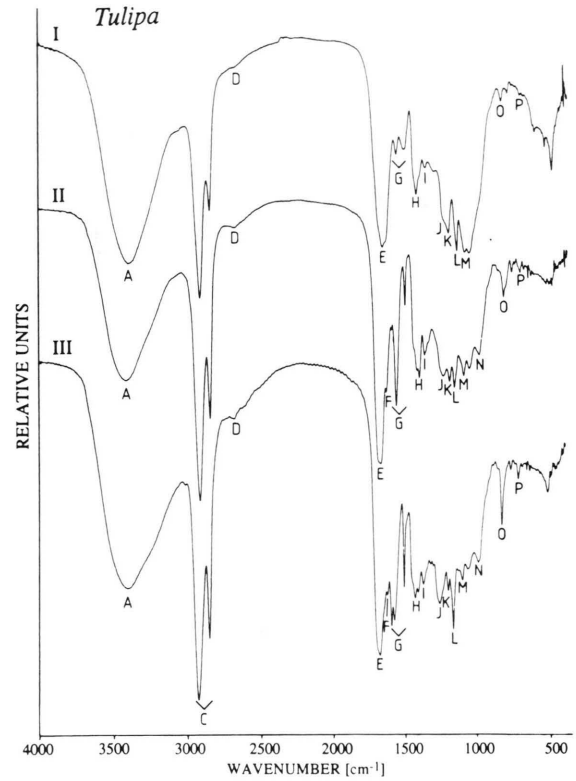

Fig. 2b. FTIR spectra of three developmental stages of *Tulipa* sporopollenin.

At stage I CH_3- and CH_2-groups present in the sporopollenin molecule are represented by a double peak in the NMR spectrum (Fig. 2a signal 7). In the spectra of the mature polymer these aliphatic peaks overlap, resulting in one prominent peak.

Furthermore an increase in the intensity of the aliphatic peaks is observed. This finding is confirmed by the results of the FTIR spectra (signals C, H and I in Fig. 2b).

Signals which represent aromatic groups (G, O) also show a continuous increase in intensity from stage I to III. This result is supported by NMR-spectroscopy (signal 4).

Concerning the signals indicating either ether or carbonyl and carboxyl functions, no differences between the three stages of development were found, revealing a constant contribution of these groups to the molecular structure of sporopollenin.

Discussion

Analysis of the ^{13}C-NMR-and FTIR-spectra of mature sporopollenin derived from mature *Tulipa* and *Cucurbita* pollen shows that the molecule predominantly consists of aliphatic structures. Varying amounts of aromatics were detected, depending on different origin. These results are in good accordance with those published earlier (Guilford *et al.*, 1988; Espelie *et al.*, 1989; Hemsley *et al.*, 1992; 1993; Wilmesmeier *et al.*, 1993).

NMR spectra of sporopollenin revealing CH_3-, CH_2-, and CH-groups as distinct peaks have been previously published (Hemsley *et al.*, 1992; 1993). Actually aliphatic signals are frequently found to dominate the NMR spectra of sporopollenin, but they often overlap, exhibiting only one broad signal (Guilford *et al.*, 1988; Espelie *et al.*, 1989). Hemsley *et al.* (1993) supposed that broad bases of signals result from small variations in the chemical environment, indicating variations in adjacent parts of the surrounding chemical structure.

After histochemical investigations an aliphatic component in the structure of sporopollenin has been postulated by Southworth (1973). The good stainability with OsO_4 and the decrease in absorption at 280 nm during exine formation support these findings and suggest that parts of the aliphatic chains are unsaturated. On the basis of the spectra presented here a definite confirmation of these results is not possible.

On the basis of histochemical results Southworth (1973) proposed that carbonyl and carboxylic groups are present in sporopollenin. Their occurrence has later been proved by Guilford *et al.* (1988). Our results correspond well with these findings.

The presence of ether bonds postulated by Shaw and Yeadon (1966) has already been corroborated by the NMR spectra of several sporopollenin samples (Guilford *et al.*, 1988; Wilmesmeier *et al.*, 1993).

Mature sporopollenin of *Tulipa* and *Cucurbita* show far-reaching similarities, however, the results of the spectroscopic analyses indicate that the course of sporopollenin maturation is different in both species.

The spectra of *Cucurbita* sporopollenin reveal only small changes in the characteristic signals. Only an increase of ether bonds and a qualitative change in the molecular structure of aromatic and/or alkene groups are detectable.

The macromolecule might probably undergo only a few structural changes during pollen development. The characteristic exine structures of *Cucurbita* pollen, the macrospines, are already visible at stage I, indicating a far-reaching differentiation at this very early developmental stage.

In *Cucurbita* sporopollenin appears very early in its final structure. In this context the extreme different duration of anther development in both investigated species has to be emphasized. It takes up to six weeks in *Cucurbita* and about six months in *Tulipa*.

According to their different staining properties and acetolysis resistance Dickinson (1976) distinguishes two polymers occurring during pollen development: protosporopollenin, which he proposes to be derived from the microspore itself and the tapetum-derived sporopollenin in the common sense.

In contrast to *Cucurbita* for *Tulipa* an increase of aromatic signals accompanied by overlapping of aliphatic signals during the sporopollenin maturation could be demonstrated. A possible increase of aromatic compounds in the sporopollenin molecule has been postulated by Southworth (1973, 1974) for pollen development in *Lilium*.

Studies on sporopollenin biosynthesis using a tracer technique proved that [U-^{14}C]phenylalanine is entirely incorporated into the sporopollenin molecule (Gubatz and Wiermann, 1993). Moreover, results of degradation experiments, exhibited *p*-hydroxybenzoic acid to be the major labeled degradation product (Rittscher and Wiermann, 1988b; Gubatz and Wiermann, 1992). These results correlate well with our findings that aromatics are involved in the exine formation of *Tulipa* pollen.

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Blackmore S. and Barnes S. H. (1990), Pollen wall development in angiosperms. In: Microspores Evolution and Ontogeny (S. Blackmore and R. B. Knox, eds.). Academic Press, London, San Diego, New York, Boston, Sydney, Tokyo, Toronto.

Derenne S., Largeau C., Berkaloff C., Rousseau B., Wilhelm C. and Hatcher P. G. (1992), Non-hydrolysable macromolecular constituents from outer walls of *Chlorella fusca* and *Nanochlorum eucaryotum*. Phytochemistry **31(6)**, 1923–1929.

Dickinson H. G. (1976), Common factors in exine deposition. In: The Evolutionary Significance of the Exine (I. K. Ferguson and J. Muller, eds.). Academic Press, London.

Echlin P. and Godwin H. (1969), The ultrastructure and ontogeny of pollen in *Helleborus foetidus* L. III. The formation of the pollen grain wall. J. Cell Sci. **5**, 459–477.

Espelie K. E., Loewus F. A., Pugmire R. J., Woolfenden W. R., Baldi B. G. and Given P. H. (1989), Structural analysis of *Lilium longiflorum* sporopollenin by ^{13}C NMR spectroscopy. Phytochemistry **28(3)**, 751–753.

Gubatz S. and Wiermann R. (1992), Studies on sporopollenin biosynthesis in *Tulipa* anthers. III: Incorporation of specificially labeled [^{14}C]phenylalanine in comparison to other precursors. Bot. Acta **105(6)**, 407–413.

Gubatz S. and Wiermann R. (1993), Studies on sporopollenin biosynthesis in *Cucurbita maxima* I: The substantial labeling of sporopollenin from *Cucurbita maxima* after application of [^{14}C]phenylalanine. Z. Naturforsch. **48c**, 10–15.

Guilford W. J., Schneider D. M., Labowitz J. and Opella S. J. (1988), High resolution solid state ^{13}C NMR spectroscopy of sporopollenins from different plant taxa. Plant Physiol. **86**, 134–136.

Hemsley A. R., Chaloner W. G., Scott A. C. and Groombridge C. J. (1992), Carbon-13 solid-state nuclear magnetic resonance of sporopollenins from modern and fossile plants. Ann. Bot. **69**, 545–549.

Hemsley A. R., Barbie P. J., Chaloner W. G. and Scott A. C. (1993), The composition of sporopollenin and its use in living and fossil plant systematics. Grana **Suppl. 1**, 2–11.

Heslop-Harrison J. (1968), Pollen wall development. Science **161**, 230–237.

Rittscher M. and Wiermann R. (1988a), Studies on sporopollenin biosynthesis in *Tulipa* anthers I: The development of an appropriate application method for radiolabeled precursors. Sex. Plant Reprod. **1**, 125–131.

Rittscher M. and Wiermann R. (1988b), Studies on sporopollenin biosynthesis in *Tulipa* anthers II. Incorporation of precursors and degradation of the radiolabeled polymer. Sex. Plant Reprod. **1**, 132–139.

Shaw G. and Yeadon A. (1966), Chemical studies on the constitution of some pollen and spore membranes. J. Chem. Soc. **(C)**, 16–23.

Southworth D. (1973), Cytochemical reactivity of pollen walls. J. Histochem. Cytochem. **21(1)**, 79–80.

Southworth D. (1974), Solubility of pollen exines. Am. J. Bot. **61(1)**, 36–44.

Southworth D. (1983), Exine development in *Gerbera jamesonii* (Asteraceae: Mutisieae). Am. J. Bot. **70(7)**, 1038–1047.

Willemse M. T. M. (1972,) Changes in the autofluorescence of the pollen wall during microsporogenesis and chemical treatments. Acta Bot. Neerl. **21(1)**, 1–16.

Wilmesmeier S., Steuernagel S. and Wiermann R. (1993), Comparative FTIR and ^{13}C CP/MAS NMR spectroscopic investigations on sporopollenin of different systematic origin. Z. Naturforsch. **48c**, 697–701.