Pollen viability and membrane lipid composition

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Stellingen

1 De factoren die de transitietemperatuur van de membraanlipiden beïnvloeden, bepalen de levensduur van pollen.
dit proefschrift

2 Pollenveroudering wordt veroorzaakt door vrije radicalen.
dit proefschrift

3 Bij de snelheid van pollenbuisgroei spelen behalve het cytoskelet ook de membraanlipiden een rol.
Steer MW & Steer JM (1989) New Phytol 111: 323-358; dit proefschrift

4 Jain en Shivanna gaan er ten onrechte van uit dat het voorkomen van lysolipiden in lipidextracten van verouderd pollen een isoleringsartefact is.
Jain A & Shivanna KR (1989) Phytochem 28: 999-1002; dit proefschrift

5 Uitspraken omtrent de chemische samenstelling van biologische systemen dienen gerelateerd te worden aan hun fysiologische betekenis
Caffrey M et al (1987) Biochim Biophys Acta 921: 124-134; Gunarsekaran M & Andersen WR (1973) Res Com Chem Pathol Pharmacol 6: 633-642

6 Aguegia & Fatokum houden bij de bepaling van geschikte bewaarcondities voor *Xanthosoma sagittifolium* L. pollen geen rekening met het optreden van imbibitieschade bij herbevochtiging.
Aguegia A & Fatokum CA (1988) Euphytica 39: 195-198; Hoekstra FA, Van der Wal EW (1988) J Plant physiol 133: 257-262

7 Het is onwaarschijnlijk dat de door Maguire et al. geconstateerde verandering in phospholipidenasymmetrie in de erythrocytenmembraan ten gevolge van infectie met de malaria parasiet ook *in vivo* optreedt.
Maguire PA, Prudhomme J, Sherman IW, Parasitology (1991) 102: 179-186

8 Met de stelling dat elke vooruitgang zijn eigen achterlijkheid schept, geeft Kellendonk aan dat literatuur en natuurwetenschappen meer overeenkomen dan vaak wordt gedacht.

Kellendonk F, (1987) Idolen. *In*: De veren van de zwaan, Meulenhof Nederland bv, Amsterdam, pp 149-164

9 De uitspraak 'The scientist wants to discover a cause for everything' is in tegenspraak met de inhoud van 'Fantasia of the Unconscious'.

Lawrence DH, (1922) Fantasia of the Unconscious, Penguin Books Ltd, Harmondworth, Middlesex, England

10 Positieve discriminatie maakt negatief onderscheid.

Stellingen behorende bij het proefschrift 'Pollen viability and membrane lipid composition', DGJL van Bilsen

Wageningen, 4 oktober 1993

Nawoord

Voor het schrijven van een proefschrift is meer nodig dan wetenschap alleen. Allen die mij, op welke wijze dan ook, in de afgelopen jaren hierbij hebben geholpen wil ik op deze plek van harte bedanken voor hun interesse en hun steun.

Curriculum vitae

De schrijfster van dit proefschrift werd op 26 juni 1963 in Maastricht geboren. Na het behalen van het gymnasium ß diploma in 1981, werd aan de Rijksuniversiteit Utrecht begonnen met de studie scheikunde. Het kandidaatsexamen (S2) werd in januari 1985 behaald, het doctoraalexamen met als hoofdfvak bio-organische chemie (vakgroep Organische Chemie) en als bijvak biochemie (vakgroep Biochemie, thans Biochemie van Lipiden), in mei 1987. Van januari 1987 tot oktober 1987 werkte zij bij de vakgroep Biochemie. In november 1987 werd als wetenschappelijk onderzoeksmedewerkster in dienst van BION (ZWO) bij de vakgroep Plantenfysiologie (Landbouwuniversiteit Wageningen) begonnen met het onderzoek dat tot dit proefschrift heeft geleid. Sinds december 1992 werkt zij in de sectie Bakery Products van het Unilever Research Laboratorium te Vlaardingen.

Voor Marc

Aan mijn ouders

Contents

Abbreviations

ANS	8-anilino-1-naphtalene sulfonic acid
CF	5(6)-carboxyfluorescein
$CHCl_3$	chloroform
DSC	differential scanning calorimetry
FFA	free fatty acid
FTIR	Fourier transform infrared spectroscopy
HAc	acetic acid
HPTLC	high performance thin layer chromatography
LPC	lysophosphatidylcholine
LPE	lysophosphatidylethanolamine
LPL	lysophospholipid
LUV	large unilamellar vesicles
MeOH	methanol
MLV	multilamellar vesicles
NL	neutral lipid
PC	phosphatidylcholine
PL	phospholipid
TLC	thin layer chromatography
16:0-LPC	16:0-*sn*-1-palmitoyl-lysophosphatidylcholine
16:0	palmitic acid
18:0	stearic acid
18:1	oleic acid
18:2	linoleic acid
18:3	linolenic acid

1. General introduction

Pollen

Pollen is the male gametophyte of seed plants. Pollen grains develop in the anthers of flowering plants. A mature pollen grain consists of a vegetative cell in which one or two generative cells are embedded. During the development in the anther, most pollen becomes desiccation tolerant (Hoekstra and Van Roekel, 1988; Hoekstra et al., 1989a). After maturation in the anther is completed, the pollen is usually shed in a partly desiccated condition. Upon arrival on a compatible pistil, pollen germination starts. First the dry grain takes up water and swells, the vegetative cell gets activated and grows out to produce the pollen tube. Visibility of the pollen tube is the criterion for germination. The pollen tube penetrates into the pistil and further elongates until it reaches the ovary. Through the pollen tube the two sperm cells, derived from the generative cell(s), are transported to the female gametophyte. One sperm cell fuses with the egg cell, thus generating the zygote. The other sperm cell fuses with the central cells which will further develop into the endosperm. This double fertilization is the first step of seed development (Shivanna and Johri, 1985; Stanley and Linskens, 1974).

Mature pollen of angiosperms is either bi- or tricellular (formerly called bi-and trinuclear). The bicellular type contains one generative and one vegetative cell; the generative one undergoes a second mitosis after pollen tube growth has already started, the rate of its metabolism and germination are relatively slow. The tricellular type contains one vegetative and two sperm cells; the second mitosis has already occurred during development in the anther and the highly active pollen readily germinates (Hoekstra and Bruinsma, 1975b; Knox, 1984). In general, metabolic activity is much higher in tricellular pollen species than in the bicellular ones. The higher metabolic activity is associated with a shorter lag time and a faster pollen tube growth upon rehydration, but is also correlated with a higher vulnerability resulting in a shorter life span (Hoekstra, 1979). In seeds metabolic activity and longevity are related (Benson, 1990). Probably metabolic activity is also directly related to pollen longevity and might thereby play a key role in pollen viability.

Pollen viability

Functional pollen that retains its viability until its transfer to the stigma and succesful completion of pollen tube growth into the pistil are necessary for fertilization and seed development. *In vivo*, the pollen viability can be monitored as the degree of seed set after pollination (Matthews and Bramlett, 1983; Smith et al., 1990). Viability assays *in vitro*, *i.e.* in sugar-containing, artificial media, can be performed by determining pollen germination (Hoekstra and Bruinsma, 1975a). For plant reproductive biology and plant breeding purposes, pollen is often stored for prolonged time (Knox and Singh, 1987; Smith et al., 1990; Stanley and Linskens, 1974; Toriyama et al., 1988). During storage a severe loss of pollen viability can occur (Al-Helal et al., 1988; Matthews and Bramlett, 1983; Pfundt, 1910; Shivanna and Johri, 1985). Usually a decline in viability coincides with an increase in leakage of endogenous solutes, *e.g.* K^+, into the surrounding germination medium (Hoekstra, 1986; Hoekstra and Van Roekel, 1988; Hoekstra et al., 1992). The rate of this loss of viability is related to storage conditions such as humidity and temperature. For each species optimal conditions are different, and under identical conditions longevity can vary widely among different species (Pfundt, 1910; Shivanna and Johri, 1985). In general, storage at low humidity and low temperature is favourable to extend longevity of the pollen, because these conditions drastically reduce metabolism and the rate of degradation processes (Pfundt, 1910; Shivanna and Johri, 1985; Weaver and Timm, 1988; Yates and Sparks, 1990).

As stated, leakage of endogenous K^+ increases when pollen viability decreases (Hoekstra, 1986). The leakage of cellular components usually points to problems at the membrane level (Hoekstra et al., 1989b). The aim of this study was to investigate the mechanism underlying the decline in pollen viability during storage, especially in relation to the condition of the pollen membranes. Pollen was also used because it may serve as a model system for other dry cells, organs and organisms, *e.g.* seeds.

Membranes in the dry condition

Membranes are important parts of every cell. They fence off the cell from its environment and provide the necessary compartmentalization that is required for optimal functioning of the cell. Because of their selective permeability they allow the transport of a

restricted set of compounds between cellular compartments or the environment, and chemical signals and energy are transduced through their interfaces. Thereby, the membranes provide the conditions for the functioning of all molecules that are associated with transport and signal and energy transduction. The average membrane has a thickness of about 5 nm and contains about equal amounts of protein and lipid molecules. In the lipid moiety, the phospholipids play the important role. Their general structure is formed by a diacylglycerol whose free primary hydroxyl group is phosphorylated. The variety of residues that are linked to this phosphate group reflects the various molecular forms of phospholipids (Evans and Graham, 1989). Apart from the polar membrane lipids, pollen may contain large amounts of neutral storage lipids in the form of lipid bodies (Shivanna and Johri, 1985).

Due to their amphipatic character, phospholipids tend to aggregate in ordered, usually lamellar bilayer structures (Fig. 1). This phospholipid bilayer structure is hydrophobic (non-polar) in the acyl chain region and hydrophilic (polar) at the headgroup region, and forms an integral part of biological membranes, the polar headgroups lining the aqueous medium at both sides. Liposomes, composed of synthetic or natural lipids, are often used as model membranes to study the lipidic part of membranes. Phospholipid bilayers exist in two mesomeric crystalline forms: a solid state and a fluid state. In the fluid state, the hydrocarbon chains have a high flexibility and rotation, whereas in the gel state the intramolecular distance between the head groups is reduced and the C-C bonds of the acyl chains are tightly packed and rigid. At the lipid phase transition temperature (T_m) the bilayer changes from the solid state, the gel phase, to the fluid state, the liquid crystalline phase (Chapman et al., 1967; Evans and Graham, 1989; Hauser et al., 1981). The temperature at which pure hydrated phospholipids undergo a gel to liquid crystalline phase transition (T_m) rises considerably during dehydration. In some pure phospholipids and isolated membranes T_m rises as much as 70°C. Consequently, a fully hydrated phospholipid that is in liquid crystalline phase might very well undergo a transition to gel phase when it is dried at the same temperature, followed by a transition back to liquid crystalline when it is rehydrated (Crowe et al., 1987). The coexistance of gel phase and liquid crystalline phase at the transition temperature causes the leakage of entrapped solutes which accompanies phospholipid phase transitions. When hydrated phospholipid bilayers are heated through their T_m leakage occurs (Blok et al., 1975) as well as when dry gel phase liposomes are

rehydrated (Crowe et al., 1985).

Drying of pollen is usually achieved through a direct evaporation of cellular water in the presence of dehydrating agents or on air. Drying is generally accompanied by a membrane transition from the liquid crystalline state to the gel phase (Fig.1). Because this transition occurs in the presence of only limited amounts of water, there will be hardly any leakage of intracellular components. When the dry pollen is immersed in water transition from the gel phase back to the liquid crystalline phase occurs. Under this condition, the phase transition proceeds in excess water and is accompanied by leakage of cellular solutes. This process is a major cause of damage when desiccation-tolerant pollen is rehydrated (Crowe et al., 1989b). However, when the right precautions are undertaken to evade a phase transition in excess water, imbibitional damage can be avoided and the pollen

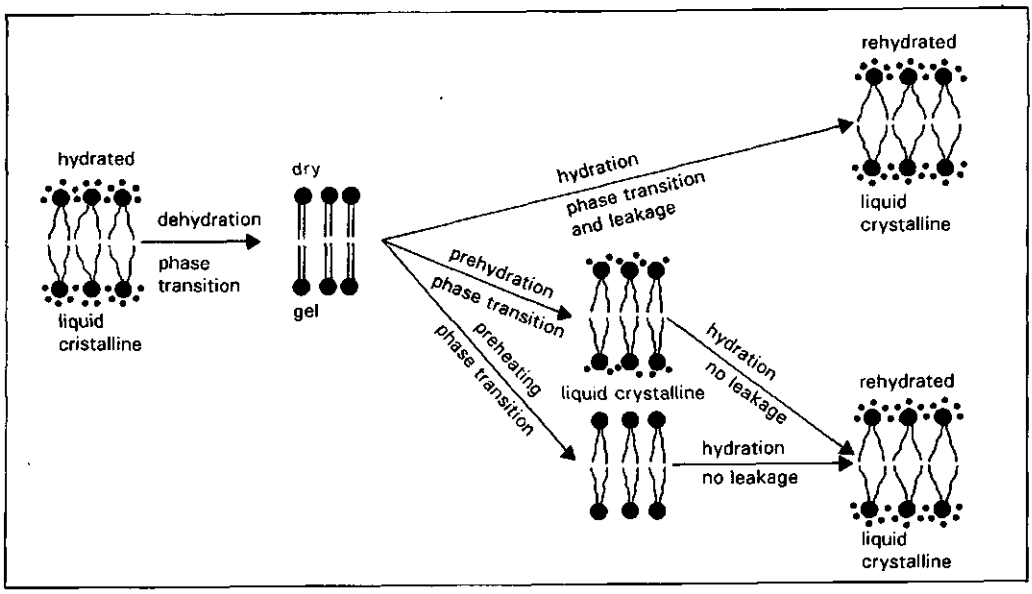

Figure 1. Diagram of events which may occur in membranes of pollen during dehydration and rehydration at room temperature. The hydrated membranes are in liquid crystalline phase and after dehydration the membranes are in gel phase. During rehydration the membranes return from the gel phase to the liquid crystalline phase. Without the pretreatments, preheating or prehydration in water vapor, this membrane phase transition occurs in excess water and is thus accompanied by leakage of intracellular solutes. Due to the pretreatments this phase transition occurs in a (semi-dry) state, before excess water is present and leakage of intracellular solutes is prevented (adapted after Crowe et al., 1989b)

remains viable upon rehydration (Crowe et al., 1989a). This can be achieved by either a prehydration in water vapor, which decreases the average transition temperature, or rehydration at a temperature above the gel to liquid crystalline phase transition (Fig. 1). Still, even when imbibitional damage is minimized, leakage of endogenous K^+ increases upon storage, indicating that the membrane integrity of the pollen deteriorates during storage (Hoekstra et al., 1992).

Membranes and cellular aging

Similarly as pollen, seeds show an enhanced leakage of K^+ as their vitality declines (Wilson and McDonald, 1986). In seeds loss of viability is partly attributed to membrane damage caused by lipid peroxidation (Benson, 1990; Buchvarov and Gantchev, 1984; Gidrol et al., 1989; Harman and Mattick, 1976; Pukacka and Kuiper, 1988). The membrane lipids contain unsaturated bond systems, which are electron-rich and thus provide ideal targets for free radical and $^1O_2$ attack. The lipid peroxidation process includes four separate phases: initiation, propagation, termination and decomposition. The initiation consists of the reaction of a free radical with a double bond in one of the lipid acyl chains, giving a lipid radical and a hydrogen radical. The initial fatty acid radical undergoes an intramolecular rearrangement in which the double bond system forms a conjugated diene. This rearranged lipid radical reacts with molecular oxygen, to form a peroxide radical. The peroxide radical can then abstract a hydrogen radical from another lipid, thus initiating a chain reaction (propagation). These chain reactions are terminated by the interaction of two lipid radicals (termination). The radicals and hydroperoxides that are generated during the process are not always stable. Their decomposition produces a whole range of breakdown products, which are often cytotoxic. At the phospholipid level the major antioxidant guarding against oxidative stress is vitamin E (α-tocopherol), which is embedded in the lipid bilayer and scavenges the free radicals present in that region. Enzymes also partake in the defense against oxidative damage at the lipid membrane level. To prevent oxidation, superoxide dismutase catalyses the dismutation of the superoxide radical into hydrogen peroxide and oxygen. Hydrogen peroxide is further broken down into oxygen and water by catalase. Gluthatione, a sulfur containing tripeptide, detoxifies membranes that contain

peroxidized lipids by converting them into lipid hydroxides. These lipid hydroxides are removed from the membranes through excision by phospholipase A_2 (Benson, 1992; Caffrey et al., 1987).

Lipid peroxidation is generally accepted as a senescence-promoting factor (Benson, 1990; Leshem, 1988; Platt-Aloia, 1988). Pollen lipids contain high levels of esterified polyunsaturated fatty acids, mainly linoleic (18:2) and linolenic (18:3) acid, in their polar and neutral lipid fractions (Caffrey et al., 1987; Evans et al., 1987; Hoekstra, 1986; Hoekstra and Van Roekel, 1988; Sekiya et al., 1990; Seppänen et al.,1989; Toriyama et al., 1988). These are known to be very susceptible to lipid peroxidation (Benson, 1990; Chan, 1987). A striking correlation was found between pollen longevity and the linolenic acid content in their total lipids: pollen species with short longevity tend to have a high linolenic acid content and vice versa. Furthermore the time required between the immersion of the pollen in the germination medium and the appearance of the first pollen tubes (the lag period) is also negatively correlated with linolenic acid (Hoekstra, 1986). This suggests that lipid peroxidation is involved in the aging process. In the dry state, free radicals are immobilized in pollen and seeds. Upon rehydration they are liberated, but their exact contribution to aging processes during dry storage is yet unclear (Priestley et al., 1985). There is sofar no direct evidence that lipid peroxidation is involved in the aging of pollen.

Phospholipids may decompose into free fatty acids and the remaining lysophospholipids. During storage of seeds these deesterification products can accumulate (McKersie et al., 1988; Nakayama et al., 1981). Because of this change in membrane lipid composition, the membranes of aged seeds have undergone lateral phase separations which result in the formation of coexisting gel phase and liquid crystalline phase domains (McKersie et al., 1988). In contrast to the reversible phase transition of phospholipid domains that is solely the result of drying (Hoekstra et al., 1989b), the effects of a phase transition resulting from irreversible changes in lipid composition might not be reversed as easily by prehydration. Decline in pollen viability might be attributed to a similar mechanism, involving the deesterification of phospholipids and phase separation during storage which, then, through a loss of membrane integrity results in imbibitional damage upon rehydration.

Outline of the research

Is there any evidence for a deterioration of membrane lipids during storage of pollen? So far most reports on pollen viability are not concerned with changes in phospholipid composition and their implications for membrane integrity. There are reports on the effects on germination of humidity and temperature during storage (Al-Helal et al., 1988; Knox and Singh, 1987; Matthews and Bramlett, 1983; Polito and Luza, 1988; Watanabe and Takahashi, 1989), on the storage of pollen in organic solvents (Iwanami et al., 1988; Jain and Shivanna 1988a; Jain and Shivanna 1988b), and on the effect of oxidative stress on pollen tube growth (Feder and Shrier, 1990). Few papers report on the loss of viability owing to a degradation of total membrane phospholipids, but the authors do not attribute any role to specific degradation products such as free fatty acids or lysophospholipids (Jain and Shivanna, 1989; Shivanna and Heslop-Harrison, 1981). To gain more insight in the mechanism involved in the loss of integrity of the pollen membrane during storage a detailed study of changes in phospholipid composition during storage and their implication for membrane integrity is necessary. In this thesis the changes in lipid composition during storage of pollen from several bicellular species, each having a different linolenic acid content, have been monitored, and implications for membrane integrity and membrane phase behaviour during rehydration have been investigated. Because of their extreme vulnerability and sensitivity tricellular species were not used (Hoekstra and Bruinsma, 1975a).

Germination, K^+ leakage and phospholipid composition of *Typha latifolia* L. pollen were monitored during storage in air at 40 and 75% relative humidity and 24°C. The fatty acid composition of the pollen phospholipids prior and after aging treatment was determined, to study whether lipid peroxidation and/or phospholipase A_2 were involved. To investigate whether age-related changes in lipid composition increase membrane permeability, liposome studies were performed with extracted pollen lipids (Chapter 2).

Fourier transform infrared spectroscopy can be used to monitor membrane phase behaviour *in vivo* and *in vitro* (Hoekstra et al., 1991). This technique was performed on isolated membranes and whole *Typha* pollen, to study whether aging changed membrane phase behaviour. The time course of the leakage of endogenous K^+ from aged, reimbibed pollen was monitored in relation to its phase behaviour. Leakage and phase behaviour were

also studied in liposomes whose composition mimicked that of aged pollen (Chapter 3).

To assess whether phospholipid degradation is a common feature during pollen storage, regardless of the endogenous linolenic acid content, two other bicellular species were studied. For this purpose pollen from *Papaver rhoeas* L. and *Narcissus poeticus* L. were chosen, because these pollen species have high levels of linolenic acid. Germination, K^+ leakage and phospholipid composition were monitored during storage. Pollen was stored at 40 and 75% relative humidity and 24°C (Chapter 4).

Since the longevity of pollen is correlated with its linolenic acid content (Hoekstra, 1986), attempts were undertaken to manipulate the level of linolenic acid *in vivo* by catalytic hydrogenation, using Pd-alizarine as the catalyst (Chapter 5).

Not only longevity, but also the start of pollen tube growth is suggested to be related to the linolenic acid content (Hoekstra, 1986). To study whether high levels of linolenic acid enhance pollen tube growth rate, *Arabidopsis thaliana* (L.) Heynh. mutants with altered lipid composition were used (Chapter 6).

Results are discussed and conclusions drawn with regard to the implications of membrane deterioration during pollen storage for biological and plant breeding purposes (Chapter 7).

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2. Decreased Membrane Integrity in Aging *Typha latifolia* L. Pollen: Accumulation of Lysolipids and Free Fatty Acids

Van Bilsen DGJL, Hoekstra FA (1992) Plant Physiology 101: 675-682

ABSTRACT

Aging of cattail (*Typha latifolia* L.) pollen was studied at 24°C under conditions of 40% and 75% relative humidity (RH). The decline of viability coincides with increased leakage at imbibition; both processes develop much faster at the higher humidity condition. During aging phospholipids are deesterified and free fatty acids (FFAs) and lysophospholipids (LPLs) accumulate, again, much more rapidly at 75% RH than at 40% RH. The fatty acid composition of the remaining phospholipids hardly changes during aging. This suggests limited involvement of lipid peroxidation in the degradation process. Tests with phospholipase A_2 revealed that the saturated fatty acids occur at the *sn*-1 position of the glycerol backbone of the phospholipids. The fatty acid composition of the LPLs is similar to that of the phospholipids from which they were formed, indicating that the deesterification occurs at random. This favors involvement of free radicals instead of phospholipases in the deesterification process.

Liposome studies were carried out to characterize components in the lipid fraction that might account for the leakage associated with aging. Entrapped carboxyfluorescein leaked much more from liposomes when they were partly made up from total lipids from aged pollen than from non-aged pollen. The components causing the leakage were found in both the polar and the neutral lipid fractions. Further purification and subsequent interchanging of the FFAs and LPLs between extracts from aged and non-aged pollen, revealed that in neutral lipid extracts the FFAs are entirely responsible for the leakage, while in the phospholipid fraction the LPLs are largely responsible for the leakage. The leakage from the liposomes is not caused by fusion. We suggest that the observed loss of viability and increased leakage during aging are due to the non-enzymatic accumulation of FFAs and LPLs in the pollen membranes.

For plant breeding studies and germplasm conservation, long term storage of pollen is often required. The applied storage conditions can have a large effect on pollen viability (Pfundt, 1910; Hoekstra and Bruinsma, 1975; Shivanna et al., 1991). Particularly at elevated temperatures and humidities, longevity is much reduced. This loss of viability may coincide with an increased leakage of soluble cytosolic components from the pollen upon rehydration and with increased endogenous levels of FFAs, which are typical signs of reduced membrane integrity (Hoekstra, 1986; McKersie et al., 1988).

Senescence of plant cells and organs is generally associated with lipid peroxidation processes in which polyunsaturated fatty acids are preferentially degraded (Benson, 1990). Toxic products of this degradation accumulate intracellularly, and gel phase domains are formed in the membranes (Barber and Thompson, 1980; Benson, 1990; Yao et al., 1991). Pollen species are rich sources of polyunsaturated fatty acids (Hoekstra, 1986; Hoekstra et al., 1989). As much as 70% of the total fatty acids of their PLs and storage lipids may consist of linoleic and linolenic acid, which renders pollen lipids favorable substrates for peroxidation reactions (Hoekstra, 1986; Hoekstra et al., 1989; Benson, 1990). Free radicals not only affect the degree of unsaturation of PLs, but can also cause a deesterification of fatty acids from the glycerol backbone of the PL *in vitro* (Niehaus, 1978; McKersie et al., 1988). These *in vitro* deesterification studies mimic the membrane changes observed *in situ* in stored seeds, from which McKersie et al. (1988) concluded that free radical-mediated deesterification also occurs during natural aging of seeds. Similarly as in seeds, free radicals were demonstrated in *Typha latifolia* pollen, in which they are trapped in the dry state (Priestley et al., 1985). This suggests that free radical activity might indeed play a role in the membrane degradation observed during aging of pollen.

PLs can also be deesterified enzymatically. Phospholipase A_2 deesterifies PL by cleavage of the ester bond at the *sn*-2 position of the glycerol backbone and thus generates FFAs and LPLs (Rosenberg, 1990). In liposomes composed of unsaturated PL, phospholipase A_2 activity is enhanced by lipid peroxidation, implying a possible activation of the enzyme as a consequence of the peroxidation (Sevanian et al., 1988). Moreover, treatment with exogenous phospholipase A_2 and phospholipase D simulates the observed lipid degradation and the changes in the lipid-protein interaction in membranes from senescent cotyledons of bean seedlings (Duxbury et al., 1991). However, at low water content, enzyme activity should be suppressed, and the diffusion of the enzyme and its substrates is restricted

(McKersie et al., 1988).

To determine whether the enhanced membrane permeability of pollen after dry (semidry) aging is caused by PL deesterification and/or peroxidation, lipids from fresh and aged cattail pollen were extracted and analyzed. To evaluate whether the altered phospholipid composition may affect bilayer permeability we performed liposome studies involving lipids from aged and non-aged pollen. Evidence is presented here that the FFA and LPL generated during storage are the major components responsible for the increased membrane permeability.

MATERIALS AND METHODS

Plant Material and Treatments

The collection and handling of *Typha latifolia* L. pollen were performed as previously described (Hoekstra et al., 1991). Dry pollen was incubated at 24°C, either in an atmosphere of 40% RH (corresponding to a moisture content of 0.06 g H_2O g^{-1} dry matter) or in an atmosphere of 75% RH (moisture content 0.17 g H_2O g^{-1} dry matter). Equilibrium moisture contents were reached within 24 h of exposure at these RHs. Germination and leakage of cellular K^+ were determined as described elsewhere (Hoekstra et al., 1992). Pollen was prehydrated in water vapor at 24°C for one h before germination.

Lipid Analysis

Total lipid extracts from the pollen were prepared as described elsewhere (Hoekstra et al., 1989; Hoekstra et al., 1991). Prior to lipid extraction pollen was washed with hexane to remove hydrocarbons from the pollen wall. Lipids were separated into a neutral and a polar fraction by standard methods (Hoekstra et al., 1989). PLs were separated into their different subclasses by 2-dimensional chromatography on preactivated HPTLC-plates (Broekhuyse, 1969). The plates were first developed in $CHCl_3$:MeOH:NH_4OH:H_2O (90:54:5.5:5.5, v/v/v/v) and subsequently in $CHCl_3$:MeOH:HAc:H_2O (90:40:12:2, v/v/v/v).

The individual PLs were visualized with iodine vapor, scraped from the plate, and oxidized by 60% perchloric acid for 30 min at 180°C, and the inorganic phosphorus was measured spectrophotometrically at 797 nm after formation of an ammonium molybdate complex (Böttcher et al., 1961). The NLs were separated on TLC-plates using hexane:diethylether:HAc (80:20:1, v/v/v) as the developing solvent and heptadecanoic acid as the internal standard (Hoekstra et al., 1989). After the plates were sprayed with 0.1% ANS in MeOH and inspection with UV-light the FFA-band was scraped off. After extraction, the FFAs were methylated using freshly prepared diazomethane in diethylether (Hoekstra et al., 1989). Fatty acid methylesters were routinely analyzed on a Shimadzu GC8A GC, equipped with a 30 m J&W DB225 megabore column (J&W Scientific, Folsom CA), coupled to a Spectra Physics SP4100 integrator. Identification was by comparing with standards (Sigma, St Louis, MO) and GC-MS analysis. GC-MS spectra were recorded on a Hewlett Packard 5970B GC-MS system equipped with a CP-Sil-88 fused silica column (Chrompack, 50 m x 0.25 mm x 0.20 μm film thickness). Electron impact spectra were obtained at 70 eV electron energy. Peroxidation of polyunsaturated fatty acids was determined by monitoring diene specific absorption at 235 nm (Chan, 1987).

Membrane Isolation

Microsomal membranes from control and aged pollen were obtained as follows. Pollen was prehydrated in water vapor at 4°C for one h to minimize imbibitional damage and increase the yield of membranes. The pollen was then ruptured in an ice-cooled French pressure cell at approximately 15,000 p.s.i. in a 10 mM TES buffer containing 0.1 mM sodium-EDTA and 0.1 M mannitol, pH 7.4 (buffer I). The homogenate was centrifuged at 800*g* for 10 min to remove unruptured pollen and pollen wall debris. The supernatant was centrifuged at 10,000*g* for 10 min. The resulting supernatant was centrifuged at 100,000*g* for 60 min at 4°C. The membrane pellet was then washed with 10 mM TES, 0.1 mM Na-EDTA, pH 7.4 (buffer II) and recentrifuged at 100,000*g* for 60 min. After centrifugation the membrane pellet was resuspended in a small volume of buffer II and passed over a Sephadex G50 column to remove adhering carbohydrates (Hoekstra et al., 1991). The isolated membrane fraction was then lyophilized and stored at -80°C until further use.

PL Purification

Pollen PLs were also subfractionated by HPLC (Kaitaranta and Bessman, 1981). They were loaded on a semipreparative HPLC-column (15 cm length, 9.4 mm diameter, Sperisorb S5W, Merck, Darmstadt, Germany) connected to a Spectra Physics isochrom isocratic pump. Elution was with CH_3CN:MeOH:H_2O (80:15:6.5, v/v/v) at approximately 340 p.s.i. Eluting PLs were detected with a Spectra Physics SP770 UV detector at 213 nm and a Spectra Physics SP8430 refractive index detector. Subfractions obtained were freed from the eluents by rotary evaporation and subsequent lyophilization. The subfractions were then resuspended in $CHCl_3$ and stored at -80°C until use. PL content was determined by phosphorus analysis (Böttcher et al., 1961). Purity was verified by one-dimensional TLC using $CHCl_3$:MeOH:HAc (60:25:8, v/v/v) as the developing solvent (Hoekstra et al., 1989).

Larger quantities of PC from nonaged pollen were purified as follows: a total lipid extract from 2 g of pollen was loaded on a BioSil A column (18 cm length, 16 mm diameter), that was prewashed with 110 mL $CHCl_3$. The NLs were eluted by washing the column with 150 mL of $CHCl_3$, pigments were subsequently removed with acetone (100 mL). The polar lipids were subfractionated with MeOH (200 mL). Subfractions were analyzed on TLC (Hoekstra et al., 1989), and those containing pure PC were combined. The MeOH was evaporated, and the isolated PC was stored in $CHCl_3$ at -80°C until further use. PL content was determined by phosphorus analysis (Böttcher et al., 1961).

Phospholipase A_2 (from porcine pancreas, 700 U/mg protein, catalog No. 161454, Boehringer, Mannheim, Germany) incubation of purified pollen PC was as described by Brockerhoff (1975).

Liposome Studies

PLs were purified as described above. The $CHCl_3$ was evaporated in a stream of nitrogen and after additional drying under vacuum for 1 h, 0.1 mM sodium-EDTA in 10 mM Tes buffer (pH 7.4) was added (0.5 mg lipid/mL buffer). After three cycles of freezing and thawing and intermittant vortexing, LUV (with an average diameter between 80-100 nm, on account of the trapped volumes, using the calibration curve of Deamer and Uster [1983])

were prepared by rapid extrusion through two stacked polycarbonate filters (100 nm pore size; Nuclepore Corp, Pleasanton CA) at 500 p.s.i. (five times). The LUV were composed of 50% *Typha* pollen-PC and 50% lipids, isolated as described above. For leakage studies the hydrophylic dye 5(6)-CF, purified according to the procedure of Klausner et al. (1981), was encapsulated at 200 mM during the preparation of the vesicles. Leakage of entrapped CF was recorded essentially as described elswehere (Hoekstra et al., 1989). The excitation wavelenght was 492 nm and the emission wavelength was 520 nm. Vesicle fusion was estimated by resonance energy transfer between the fluorescent probes *N*-(7-nitro-2,1,3-benzoxadiazyl-4-yl)phosphatidylethanolamine (donor) and *N*-(lissamine rhodamine B sulfonyl)phosphatidylcholine (acceptor) incorporated in the bilayer (Hoekstra, 1982; Deleers et al., 1987). The dyes were added separately via $CHCl_3$ to two similar lipid preparations at 0.5 mol percent each. After the production of LUV the donor and acceptor vesicles were combined in a 1:1 ratio. The excitation wavelenght was 450 nm and the emission wavelength was 590 nm.

Chemicals

All organic solvents, HPTLC and TLC plates (Kieselgel 60) were purchased from Merck (Darmstadt, Germany), CF came from Serva (Heidelberg, Germany), heptadecanoic acid was obtained from Fluka (Buchs, Switzerland), ANS and diheptadecanoyl-PC were from Sigma (St Louis, MO), SEP-PAK silica cartridges were obtained from Waters (Millipore Corp, Milford, MA), BioSil A was from Bio-Rad (Richmond, CA). Di-(1-pyrenedecanoyl)-L-α-phosphatidylcholine was a generous gift of Dr E.H.W. Pap from the Department of Biochemistry, Agricultural University Wageningen, The Netherlands.

RESULTS

Viability

Figure 1 shows the effect of internal moisture content of the pollen (0.06 and 0.17 g H_2O g^{-1} dry matter corresponding to exposure to 40 and 75% RH, respectively) during storage on viability and leakage of endogenous K^+ upon reimbibition. In contrast to dry storage, incubation at elevated moisture content considerably reduced longevity. Reduction of viability was always accompanied by an increase in leakage of endogenous K^+ from the grains, which was measured over the first 5 min of imbibition. Increased leakage of endogenous solutes is indicative of decreased membrane integrity (McKersie et al., 1988; Hoekstra et al., 1989).

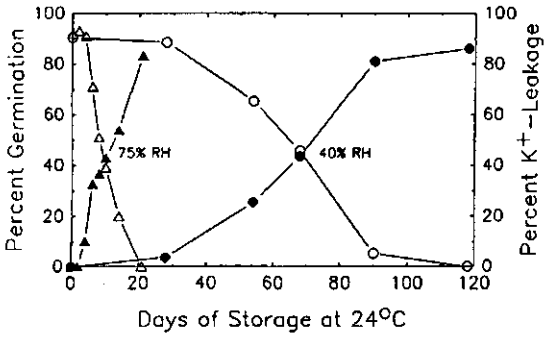

Figure 1. Effect of pollen storage at two RHs (24°C) on viability (open symbols) and leakage of endogenous K^+ (closed symbols). Leakage of K^+ was measured over the first 5 min of imbibition. RHs of 40% and 75% give internal moisture contents of 0.06 and 0.17 g H_2O g^{-1} dry matter, respectively.

Lipid Degradation

Loss of viability through membrane deterioration is often attributed to lipid peroxidation and the subsequent formation of toxic peroxidation products (Barber and Thompson, 1980; Benson, 1990). However, no significant increase in diene-specific absorption was observed in lipid extracts from aged pollen (data not shown), and no large selective loss of polyunsaturated fatty acids occurred during storage (Table I).

Table I. *Fatty acid composition of PLs isolated from* Typha *pollen, during dry (semidry) aging at 24°C*

Moisture contents were 0.06 and 0.17 g of H_2O g^{-1} dry weight. Average of data of 2 or more extractions.

Storage and Aging Condition	PL Fatty Acid Composition				
	16:0	18:0	18:1	18:2	18:3
	mol percent				
Control (no aging)	28.8	0.9	0.6	62.5	7.2
0.06 g of H_2O g^{-1} dry wt					
50% of iv[a] (70 d)	30.5	0.2	0.4	62.1	6.9
0% of iv (120 d)	29.0	0.8	0.8	62.0	7.3
0.17 g of H_2O g^{-1} dry wt					
50% of iv (10 d)	31.3[b]	0.7	0.9	59.0[b]	8.4
0% of iv (18 d)	35.7[c]	1.1	1.1	56.1[c]	6.0

[a] Abbreviation: iv, initial viability (92%). The number of days to reach reduced viability are in parenthesis.

[b] Significantly different from the control at P=0.025 and [c] at P=0.001, Wilcoxon rank sum test.

This is an indication that another mechanism of lipid degradation, different from peroxidation operates during (semi-)dry aging of pollen. In seeds, loss of viability coincides with the accumulation of free (*i.e.* unesterified) fatty acids (McKersie et al., 1988). To determine whether PL deesterification occurred during storage, the phospholipid content and that of the breakdown products (FFA and LPL) were monitored during storage (Fig. 2). The PL content of the pollen remained stable (at 0.06 g H_2O g^{-1} dry weight) or declined (at 0.17 g H_2O g^{-1} dry weight) during storage, whereas the FFA content increased under both conditions. There was also a significant accumulation of LPL during storage. At the higher humidity the accumulated LPL were further degraded.

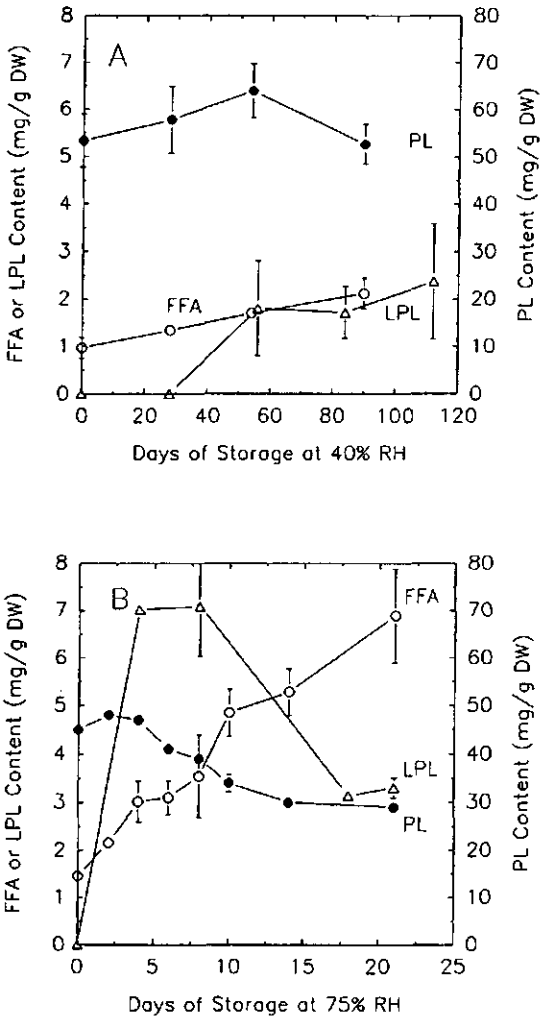

Figure 2. Effect of pollen storage at the two internal moisture levels (24°C) on the contents of total PL, FFA and LPL. A, At 40% RH, B at 75% RH. Average of data of 2 or more extractions; error bars are SD. DW dry weight.

Origin and Localisation of the Degradation Products

In an attempt to demonstrate that the FFAs and LPLs as found in total lipid extracts do occur in the membranes, lipid extracts from pollen and from isolated pollen membranes

Table II. *Composition of PL extracted from intact* Typha *pollen grains and from membranes isolated from them*

Pollen was either untreated or aged for 18 d in 75% RH at 24°C. The amount of FFA is also given. Each value is the average of four HPTLC separations and phosphorus determinations.

Source	Phospholipids								FFA/PL
	LPC[a]	PC	LPE	PE	PI/PS	PA	PG	CL	
	mol percent								*mg/mg*
Pollen									
control	0.0	63.2	0.0	18.3	7.8	1.0	7.0	2.7	0.033
aged	10.5[b]	37.9[b]	5.2[b]	14.7[b]	10.4	8.6[b]	5.5[b]	7.1[b]	0.203
Membranes									
control	1.8	57.9	1.0	17.9	6.3	3.5	6.6	5.1	0.036
aged	8.5[c]	49.2[c]	3.7[c]	16.8[c]	8.5[c]	4.2	4.5[c]	4.4	0.204

[a] Abbreviations: LPC, lysophosphatidylcholine; PC, phosphatidylcholine; LPE, lysophosphatidylethanolamine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin.

[b] Significantly different from the control at $P = 0.005$.

[c] $P = 0.05$, Wilcoxon rank sum test.

were compared (Table II). The increases in LPC and LPE in the aged pollen are at the expense of PC and PE. The accumulation of FFA and the LPL in the pollen coincides with that in the membranes. To ascertain that formation of LPL in the aged pollen is a true aging phenomenon and not an isolation artifact, the aged pollen was homogenized and extracted in the presence of di-(1-pyrenedecanoyl)-L-α-phosphatidylcholine (25 nmol for 30 mg pollen). After two-dimensional chromatography the HPTLC-plates were monitored for fluorescent spots. No fluorescence was detected in the LPC spot, nor was there any fluorescence at the solvent front indicative of the presence of FFA generated during the extraction procedure.

Mode of Deesterification

As shown in Table II, the main polar product of PL deesterification is LPC. To study the mechanism of this deesterification, the fatty acid distribution of PC had to be determined. For this purpose, PC was incubated with phospholipase A_2, which deesterifies acyl chains at the *sn*-2 position of the glycerol backbone (Rosenberg, 1990). In the resulting LPC, the mol % of 16:0 was considerably increased and that of 18:2 decreased, as compared to the mol % of these two fatty acids in the original PC (Table III).

Table III. *Fatty acid composition of pollen PC and LPC*

Pollen PC (from untreated pollen) was either analyzed directly or after treatment with phospholipase A_2 to produce LPC that was purified on TLC, scraped off, and methylated. The LPC (theoretical) was calculated on the basis of a hypothetical phospholipase A_2 treatment, assuming the fatty acids 16:0, 18:0 and 18:1 at the *sn*-1 position and 18:3 at the *sn*-2 position. The endogenous LPC was formed after 18 d of aging at 75% RH (24°C). Data are average of 2 determinations.

	Fatty acids				
Source	16:0	18:0	18:1	18:2	18:3
	mol percent				
PC (non-aged)	28.5	0.7	0.2	63.8	6.7
LPC (aging)	31.5	1.6	1.3	61.8	3.8
LPC (PLase A_2)	55.7	0.6	0.0	43.5	0.1
LSD (P=0.05)	7.7	0.5	0.1	6.3	0.9
LPC (theoretical)	57.1	1.4	0.4	41.1	0.0

Assuming that the fatty acids, 16:0, 18:0 and 18:1 are at the *sn*-1 position and that 18:3 is entirely at the *sn*-2 position (*e.g.* Brown et al., 1987), the theoretical composition of *sn*-1-LPC is very similar to that found after phospholipase A_2 incubation. This confirms the preference of 16:0 in pollen PC for the *sn*-1 position. Table III further shows that the LPC formed during aging has a fatty acid composition that is very similar to that of PC. Deesterification of acyl chains during aging predominantly occurs at random at both the *sn*-1

and *sn*-2 positions.

Leakage and Fusion Studies

To study whether the accumulation of LPL and FFA could account for the changed bilayer permeability during aging, liposome studies were undertaken. The liposomes were composed of 50% purified pollen PC and 50% other lipids. The presence of a polar lipid, 50% PC in this case, is a prerequisite for the stability of liposomes containing NL, because NL alone does not permit formation of liposomes. Figure 3A shows that liposomes containing total lipids extracted from aged pollen leak considerably faster than those containing total lipids from control pollen. As a reference, liposomes composed solely of *Typha* pollen PC are very stable. After separation of the crude extract into a neutral and a polar fraction, liposomes were prepared for similar studies. When the liposomes contain neutral or polar lipids from aged pollen, they leak, whereas they leak considerably less so in the controls (Figs. 3B and C). This demonstrates that the compounds responsible for the leakage in Figure 3A do occur in both lipid fractions.

Purification of the lipid extracts into their respective subclasses may reveal whether FFAs and LPLs are specifically involved in the increased leakage of entrapped solutes. For that purpose NLs from aged and control pollen were purified on TLC, and their FFA fractions were interchanged. Figure 4A shows that the LUV containing control NLs supplied with FFAs from aged pollen, leak CF similary as the LUV from aged NLs in figure 3B. Alternatively, LUV containing aged NLs from which the FFAs were exchanged for the small amount of FFAs in control NLs, exhibit an excellent retention (Fig. 4B). The increased leakage of entrapped CF is clearly related to the increased FFA content of the aged pollen. Similarly the PL from aged and control pollen were separated into their subclasses by HPLC and then recombined after interchange of their LPL. Subsequently, LUV were prepared of these purified, recombined pollen PLs, and leakage of entrapped CF was recorded (Fig. 4, C and D). The rate of leakage was enhanced when the PLs from control pollen were combined with the LPLs from aged pollen (Fig. 4C). When PLs purified from aged pollen were recombined with the small amount of LPLs from control pollen leakage was suppressed (Fig. 4D), which indicates that leakage depends on the presence of LPL in the phospholipid

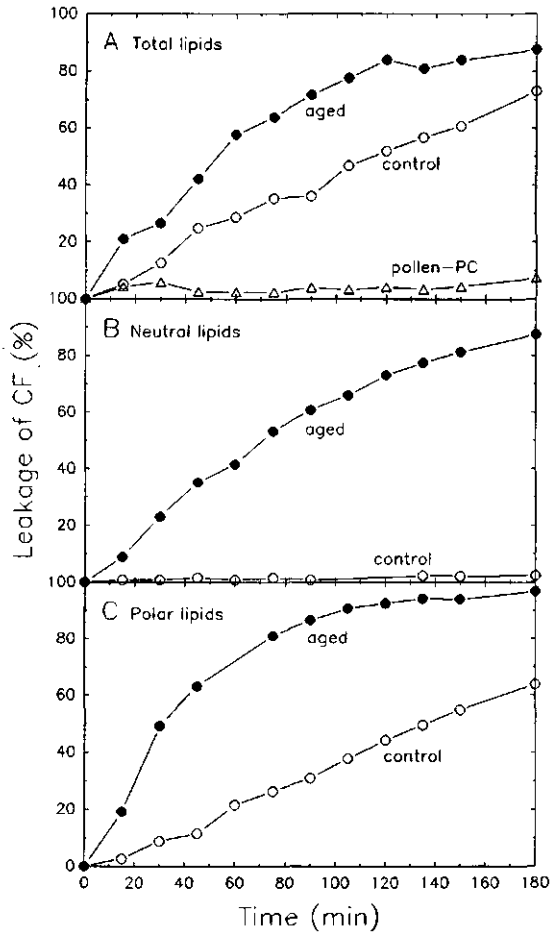

Figure 3. Leakage of entrapped CF from LUV composed of *Typha* pollen lipids. The LUV were produced from pollen-PC and pollen lipid mixtures, in the ratio 1:1 (weight percentage). The lipid mixtures (50% of the LUV) are: A, total lipid extract from aged pollen or from control pollen, and pollen-PC as a reference; B, NL lipid extract from aged pollen or from control pollen; C, polar lipid extract from aged pollen or from control pollen. Aging treatment was for 18 d at 24°C in 75% RH. Pairs of aged and control leakage curves were repeated three times. The differences between the curves in A,B and C are significant at P=0.001 (sign test for paired comparisons).

fraction.

Leakage of entrapped solutes is often related to fusion, especially for liposomes containing LPLs or FFAs (Van Echteld et al., 1981; McKersie et al., 1989). To verify

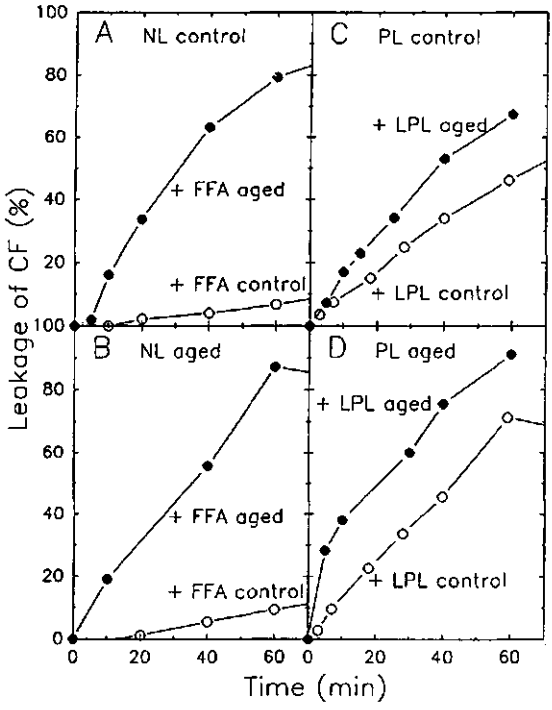

Figure 4. Leakage of entrapped CF from LUV composed of purified *Typha* pollen lipids. The LUV were composed of 50 % pollen-PC and 50 % purified pollen lipids. A and B, Purified NLs from control or aged pollen before and after exchange of their FFAs. D and C, Purified PLs from control or aged pollen before and after exchange of their LPL. Aging treatment was for 18 d at 24°C in 75% RH. Triplicate testing of pairs of aged and control vesicles. Curves are significantly different at P=0.01 (sign test for paired comparisons).

whether the observed leakage was the result of vesicle fusion, resonance energy transfer studies were performed (Fig. 5). No fusion was detected in liposomes composed of lipids from aged pollen. To ascertain that this observation was not caused by a malfunctioning of the resonance energy transfer assay, fusion was also monitored in the presence of Al^{3+}, which is very fusogenic (Deleers et al.,1987). In the presence of Al^{3+} fusion occurred within 10 min.

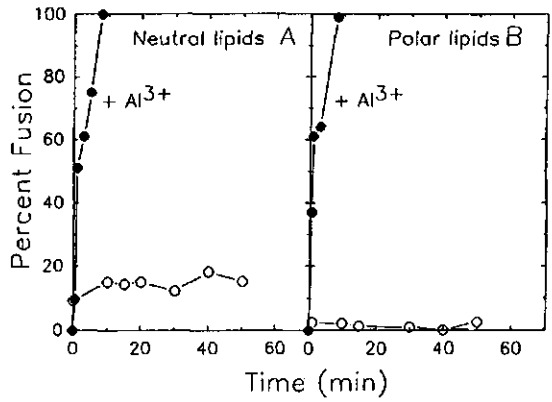

Figure 5. Fusion of LUV composed of 50 % pollen-PC and 50 % pollen lipid mixtures. A, NLs from aged pollen; B, polar lipids from aged pollen, purified as in Figure 3B and C. Vesicle fusion was estimated at 590 nm, by resonance energy transfer between the fluorescent probes *N*-NBD-PE and *N*-Rh-PE, incorporated in different LUV populations. The fusogenic agent, Al^{3+}, was added to verify the proper functioning of the resonance energy transfer assay.

DISCUSSION

As expected, storage of *T. latifolia* pollen at elevated moisture content accelerated its aging (Pfundt, 1910; Hoekstra and Bruinsma, 1975; Hoekstra, 1986; Shivanna et al., 1991). The extremely rapid leakage of endogenous K^+ from aged pollen, within the first 5 min of imbibition is an indication that membrane integrity is impaired. Aging experiments with seeds at elevated humidities and temperatures also show a rapid decline of viability and loss of membrane integrity that is often accompanied by peroxidation of polyunsaturated fatty acids (Benson, 1990). Thus, significant changes in fatty acid composition are generated (Benson, 1990). However, in the present aging experiments with pollen at 40% RH, no significant decrease in mol % of linoleic acid occurred, whereas at 75% RH only a slight decrease of linoleic acid and increase of palmitic acid were observed (Table I). Apparently, peroxidation of polyunsaturated fatty acids is not the main degradation process in membranes of aging cattail pollen.

The extractions of lipids from the aging pollen in Tables I and II were done directly from the dry pollen without preceding addition of water or isolation of membranes. Such

procedure provides the best estimate of the actual status of lipid degradation *in situ*, and enabled us to conclude that pollen PL are deesterified during dry aging. Use of a fluorescent dipyrene-PC probe during crushing of the pollen and subsequent lipid isolation verified that the deesterification process developed during aging, and did not occur during the isolation procedure. Analyses of lipid changes in membranes isolated from the aged material might be less reliable due to the possibility of further degradation during the isolation procedures. Nevertheless there is no indication that the large amounts of LPLs and FFAs found in isolated membranes of the aged pollen (Table II) do not represent the accumulation of these compounds in membranes *in situ*. As to the question whether the mechanism of lipid degradation differs at the two humidity conditions, the following remarks can be made. At 75% RH there is a large decline in total PL content and a 5-fold increase in FFA content during the gradual loss of vitality (Fig. 2). In contrast, the content of PLs does not significantly decline at the dryer storage condition of 40% RH, and the FFA content only doubles. During the large PL degradation at 75% RH, LPLs rapidly accumulate and level off in the course of the incubation. Apparently, a further decomposition of LPL occurs at the higher RH. At 75% RH triglycerides were also degraded (data not shown), which indicates the breakdown of storage lipids as well. Therefore, relatively small differences in storage conditions may have a large effect on the the rate of lipid degradation and on the degree to which the degradation process progresses.

Also in seeds aging leads to PL deesterification (Senaratna et al., 1988). In smooth microsomal membranes isolated from rehydrated, naturally aged soybean seeds, the PL content was decreased with only minor changes in fatty acid composition, and the FFA content was increased (McKersie et al., 1988; Senaratna et al., 1988). No LPL were found, which is a general observation in published work on seed aging (Thompson, 1988). Problems with the isolation of these strongly polar LPL from oily seeds might account for their seeming absence upon aging, because work in Japan on storage at room temperature of soybean seeds having 13% moisture content revealed an increase in LPL content (Nakayama et al., 1981). These authors made lipid extractions directly from the aged seeds without previous rehydration (Nakayama et al., 1981), which excludes possible decomposition of the LPLs during rehydration or membrane isolation. It is possible that aging at elevated moisture contents allows for a rapid decomposition of LPL as is also suggested by our data. This contributes further to the opinion that the observed lipid degradation depends on the applied

aging treatment (Priestley et al., 1985).

Phospholipase A_2 is generally involved in the formation of LPC *in situ* (Sevanian et al., 1988; Rosenberg, 1990). We show here, on the account of the fatty acid composition of the LPC and its presumed precursor, PC, that phospholipase A_2 or A_1 can not be responsible for the deesterification during pollen aging. Similarly as in PLs of most other organisms, in pollen PC the saturated palmitic acid is present exclusively at the *sn*-1 position (Table III). Phospholipase A_2 activity would thus result in a significant increase of palmitic acid in the LPC, and phospholipase A_1 in a significant decrease (Sevanian et al., 1988; Rosenberg, 1990). No such increase or decrease was observed, because the fatty acid composition of LPC from aged pollen is very similar to that of non-aged PC (Table III). Alternatively, phospholipase B could have been active because it can act on both the *sn*-1 and the *sn*-2 position of the glycerol backbone and can degrade PL as well as LPL (Rosenberg, 1990). Because the aforementioned enzymes have not convincingly been demonstrated in higher plants the typical plant PL degrading enzyme, lipolytic acyl hydrolase, may be involved, yielding FFA in a non-specific manner (Paliyath and Thompson, 1987). However, enzyme activity is not very likely at all at the low water content in the present experiments, since metabolic activity and respiration are strongly suppressed (Hoekstra and Bruinsma, 1975; McKersie et al., 1988; Benson, 1990). For the same reasons, lipid deesterification in aged seeds is attributed to free radicals rather than to phospholipase activity (McKersie et al., 1988).

The liposome studies show that PL bilayer permeability is increased by the addition of FFAs or LPLs that were produced during dry aging of pollen. This applies to the relative proportion of these two compounds as they occur in the lipid extracts. Results from model membrane studies show that fusion can be the cause of the leakage, *e.g.* from DPPC vesicles that contained palmitic acid (McKersie et al., 1989) or palmitoyl-LPC (Poole et al., 1970; Van Echteld et al., 1981); however, when the unsaturated oleic acid was used instead, the increased leakage of entrapped solutes was not due to fusion (McKersie et al., 1989). Figure 5 demonstrates that the leakage is not the result of fusion between the vesicles. The liposomes composed of the aged pollen lipids contain complex mixtures of saturated and polyunsaturated FFAs or LPLs. Apparently these mixed FFAs and LPLs enhance leakage, but they do not induce vesicle fusion. Leakage is proportional to the amounts of FFAs or LPLs included in the liposomes (Poole et al., 1970; Van Echteld et al., 1981; McKersie et

al., 1989).

During extraction and subsequent purification of the pollen lipids the FFA derived from membrane lipids and storage lipids get mixed up. The amount of FFA used in our liposome studies, therefore, may have been too high in relation to the actual amount present in the membranes. Yet, the ratio of FFA to PL in the total pollen extract as compared to that in the isolated membranes is very similar. Analysis of the isolated microsomal membranes also revealed some contamination by triglycerides which might have contained some FFA. This obstructs an exact determination of the precise amounts of FFA originating from membrane lipid degradation.

There is no direct quantitative relation between the rate of leakage from the liposomes and the rate of leakage from aged pollen upon imbibition. The aged pollen grains leak most of their endogenous K^+ within the first 5 min after imbibition (Fig. 1), whereas the leakage of CF from the liposomes is much slower (Figs 3 and 4). This suggests that FFA and LPL have a more destabilizing effect on bilayer integrity in the dry (semidry) pollen than in the fully hydrated liposomes. Therefore, studies are in progress on the effect of these two compounds on the behavior of dry membranes.

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3. Lateral Phase Separation in Membranes of Aging Dry Pollen Causes Imbibitional Leakage

Van Bilsen DGJL, Hoekstra FA, Crowe LM, Crowe JH, Plant Physiology, in press

ABSTRACT

Aging of dry pollen has earlier been shown to coincide with increases of free fatty acids and lysophospholipids. These compounds reduce the integrity of hydrated liposomes made from isolated pollen phospholipids, but do not lead to their total destruction. However, a massive, instantaneous leakage occurs upon imbibition of dry cattail pollen (*Typha latifolia* L.) that has aged to the point of complete loss of viability. To resolve the apparent discrepancy in stability between hydrated and dry membranes, the lyotropic phase behavior of liposomes containing lysophospholipid (12 mol percent) was studied with differential scanning calorimetry and Fourier transform infrared spectroscopy. In the two liposome systems studied, dehydration caused phase separation of the lipids.

Fourier transform infrared data on phase behavior of isolated membranes from aged and control pollen and of membranes *in situ* indicate that aging, probably through the presence of lysolipids and free fatty acids, broadens and extends the phase transition stepwise into the higher temperature range, which may indicate a phase separation. We suggest that lateral phase separation is responsible for the massive leakage of endogenous solutes and death which occur when aged pollen is imbibed.

In an atmosphere of 40% RH *Typha latifolia* pollen lives approximately 120 d at 24°C (Van Bilsen and Hoekstra, 1993). At the higher humidity of 75%, aging is considerably accelerated, and viability is lost within 18 d. In general, dry and semidry aging exhibit the same characteristics: (a) lipid deesterification, resulting in the accumulation of LPLs and FFAs; (b) decreased PL content; (c) insignificant lipid peroxidation; (d) increased leakage of endogenous K^+ upon reimbibition coincident with loss of viability (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). Also, in several other pollen species, loss of viability during storage has been shown to coincide with deesterification of membrane lipids (Van Bilsen et al., submitted). The impact of the observed changes in lipid composition on bilayer permeability has been studied in detail in liposomes composed of pollen lipids (Van Bilsen and Hoekstra, 1993). An increased leakage from the liposomes due to the products formed during aging was observed. However, this moderate reduction in liposomal integrity cannot explain the instantaneous, massive leakage that occurs upon imbibition of aged pollen (Hoekstra and Van Roekel, 1985).

Also, in aging seeds membrane phospholipids deteriorate, and FFA (McKersie et al., 1988) and LPL (Nakayama et al., 1981) accumulate. This is accompanied by an increase in membrane microviscosity and leakage of cytoplasmic solutes, which is indicative of reduced membrane integrity (McKersie et al., 1988).

In general, an increased leakage of cellular solutes from pollen is caused by problems at the membrane level (Hoekstra and Van der Wal, 1988; Sack et al., 1988; Crowe et al., 1989b; Crowe et al., 1989c; Hoekstra et al., 1989; Hoekstra et al., 1992). Thus, a membrane phase transition from gel to liquid crystalline was suggested to be responsible for the leakage and death of initially viable, dry pollen during imbibition in liquid medium (Hoekstra and Van der Wal, 1988; Crowe et al., 1989c; Hoekstra et al., 1992). Fourier transform infrared spectroscopic measurements on intact pollen have indicated that the membrane phase transition temperature rises during drying, to exceed room temperature (Crowe et al., 1989b; Crowe et al., 1989c; Hoekstra et al., 1992). This means that membrane phospholipids in dry pollen are at least partially in gel phase at room temperature, but entirely so at low temperature. That the phase change from liquid crystalline to gel phase during drying does not lead to leakage and death has been attributed to the lack of free water for solute transport under those conditions (Hoekstra, 1992). The situation during imbibition is entirely different in that ample water is then available for solute leakage during the reverse

transition back to the liquid crystalline phase. Imbibitional damage can be prevented by treatments or conditions that return the phospholipids to the liquid crystalline state prior to imbibition, such as preheating at imbibition and pretreatment in humid air (Hoekstra, 1984; Hoekstra and Van der Wal, 1988; Crowe et al., 1989a). But even when carefully prehydrated in water vapor, aged pollen still shows decreased germination (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). This suggests that aging may have irreversibly changed membrane phase properties.

Pollen aged under dry or semidry conditions always contains LPL and FFA. In contrast to the situation in the liquid crystalline state, mixtures of LPC and various PCs are immiscible when the PC is in the gel state (Van Echteld et al., 1980). This may occur during production of the LPLs in aging dry pollen. Thus, the newly formed LPLs may induce a lateral phase separation in the dry membranes, which may cause irreversible damage during imbibition. The effect of the FFA, palmitic acid, on the behavior of dipalmitoyl-PC liposomes during drying and rehydration has already been studied in detail (Crowe et al., 1989e). Apart from a phase separation in the dry liposomes, palmitate induces fusion between the liposomes during rehydration, which is responsible for leakage of solutes from them.

In this paper we report on the behavior of LPL in dry and hydrated model membrane systems in the ratio in which they occur in aging pollen membranes. These systems were studied with DSC and FTIR and are compared with membrane behavior *in situ* as studied by FTIR. We present evidence that extensive phase separation occurs during aging due to lipid compositional changes, which results in imbibitional leakage and death.

MATERIALS AND METHODS

Plant Material and Treatments

Collecting and handling of *Typha latifolia* L. pollen were performed as previously described (Hoekstra et al., 1991). For aging purposes pollen was incubated at 24°C, in an atmosphere of 75% RH produced by a saturated NaCl solution (final moisture content 0.17 g H_2O g^{-1} dry matter). Germination and leakage of cellular K^+ were determined as described

elsewhere (Hoekstra et al., 1992). Pollen was prehydrated in water vapor at 24°C for one h before germination and leakage experiments.

Membrane Isolation

Membranes from control and aged pollen were obtained as described previously (Hoekstra et al., 1991; Van Bilsen and Hoekstra, 1993). The isolated membrane fraction was lyophilized and stored at -80°C until further use.

Liposome studies

All purchased lipids were used without further purification. For the PC/LPC mixtures the lipids were dissolved in $CHCl_3$:MeOH (1:1, v/v) to ensure a homogeneous distribution of the LPC. The solvents were evaporated in a stream of nitrogen, and after additional drying under vacuum for 1 h, buffer (10 mM Tes, 0.1 mM EDTA, pH 7.4) was added. Multilamellar vesicles for FTIR and DSC measurements were prepared by vortexing of the lipids in buffer. A portion of the freshly prepared MLV was lyophilized to determine the transition temperatures of the dry material. All dried samples were handled under dry nitrogen to prevent rehydration on exposure to air.

Infrared spectroscopy

Infrared spectra were recorded on a Perkin-Elmer 1750 Fourier transform infrared spectrometer, and data were acquired and analyzed with a Perkin Elmer 7500 data station using Perkin-Elmer software. Each spectrum was the average of 10 scans at each temperature in the infrared region 3000 cm^{-1} to 900 cm^{-1}. The gel-to-liquid-crystalline phase transition was monitored by observing the shift with temperature in the infrared absorption bands attributed to the CH_2 symmetric stretch (at 2850 cm^{-1}) of the phospholipid acyl chains. Where necessary samples were lyophilized prior to recording the infrared spectra, removed from the

lyophilizer under vacuum and sandwiched between two rectangular BaF_2 windows under dry nitrogen to avoid rehydration and oxidation as much as possible (Hoekstra et al., 1991).

Differential scanning calorimetry

Phase transitions were measured with a Hart Series 7077 high sensitivity differential scanning calorimeter, assisted by an IBM PC-XT data station and Hart Scientific (Provo, UT) software (Crowe et al., 1989e). Samples were scanned from -30 to 90°C or higher at a rate of 20°C h^{-1}. This calorimeter has 3 sample cells, and up to 3 samples can be run simultaneously for direct comparison. Dry samples were loaded into the sample pans and sealed in a glove box flushed with dry N_2.

Chemicals

All organic solvents were purchased from Merck (Darmstadt, Germany), 16:0-LPC from Serva (Heidelberg, Germany), egg-PC from Avanti Polar Lipids (Birmingham, AL).

RESULTS

Imbibitional Leakage of Aged Pollen

Figure 1 shows that within the first minute of imbibition virtually all endogenous K^+ has leaked from aged pollen grains. In control pollen (germination capacity 90%) the leakage was slower and did not exceed 46% of the total amount of extractable K^+. Care was taken not to cause injury during the change from the dry to the hydrated state, by carefully rehydrating the pollen from the vapor phase prior to imbibition. However, prehumidification did not reduce K^+ leakage from the aged pollen (data of leakage from nonprehumidified aged pollen not shown). This indicates that possible aging-related phase separations in the membranes are not properly reversed by this pretreatment.

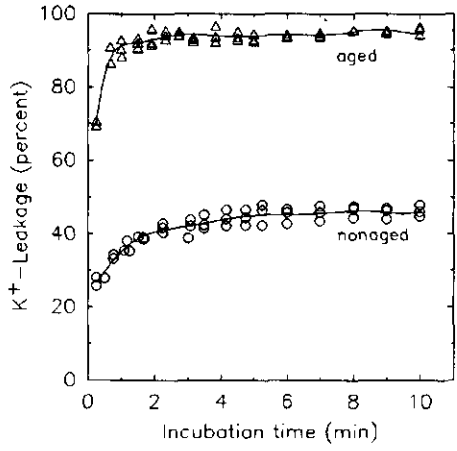

Figure 1. Leakage of endogenous K^+ from aged and nonaged *T. latifolia* pollen. Pollen was exposed for 1 h to humid air at 24°C prior to immersion in liquid medium. Aging was achieved by storage at 75% RH and 24°C for 18 d (zero percent germination). Leakage was monitored during 10 min after immersion of the pollen grains in K^+-less germination medium.

Influence of LPL on Phase Behavior of Liposomes

In order to understand the massive leakage from the aged grains during rehydration, liposome studies were undertaken, with special reference to the effects of LPL on the melting behavior and miscibility of the lipids in the hydrated and dehydrated condition. The gel-to-liquid crystalline phase transition of hydrated 16:0-*sn*1-LPC, which is a naturally occurring LPC in aging cattail pollen [in addition to 18:2-*sn*-1-LPC and 18:2-*sn*-2-LPC (Van Bilsen and Hoekstra, 1993)], was determined with DSC and FTIR. With both methods, T_m for this phase change in the hydrated micelles was seen to occur at 2°C, in accordance with previous results with DSC (Van Echteld et al., 1980, 1981). In the dry micelles, T_m was seen with DSC to rise to 94°C. An attempt to measure the same transition temperature with FTIR indicated that up to 75°C the transition had not yet occurred, but due to instrumental limitations scanning at higher temperatures was not possible.

The phase transition of fully hydrated liposomes composed of DPPC (Fig. 2) or egg-PC (Fig. 3) was not affected after the addition of 12 mol% LPC, which is the amount of LPL occurring during aging.

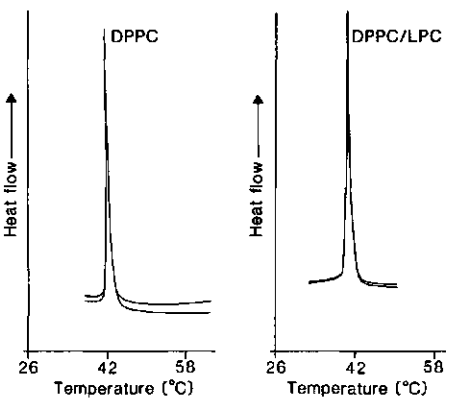

Figure 2. Two sequential differential scanning calorimetry heating scans of hydrated MLV composed of pure DPPC and a DPPC/LPC mixture (12 mol% 16:0 *sn*-1-LPC). Both samples were run simultaneously.

In general, larger amounts of LPC are required to generate a detectable phase separation in excess water (Van Echteld et al., 1980, 1981). The phase behavior of hydrated egg-PC was studied by FTIR spectroscopy and not by DSC, because the thermotropic phase transition of

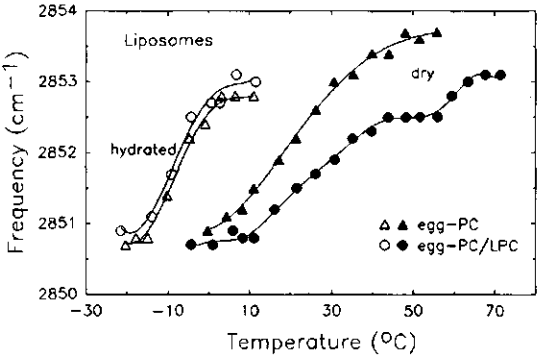

Figure 3. Frequency *versus* temperature plots (FTIR) of hydrated and dried MLV composed of egg-PC and an egg-PC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). Triangles: egg-PC; circles: egg-PC/LPC. The data points represent vibrational frequencies of the symmetric CH_2 band. In the presence of LPC, phase transition of the dried vesicles is stepwise and broadened, indicative of phase separations.

the lipid lies in the region of the ice endotherm. Drying evokes large changes in phase behavior. Using DSC, increased T_ms were found for dried DPPC and egg-PC liposomes as compared to the hydrated controls (Figs 4 and 5). The elevated T_ms were established at approximately 72°C and 44°C for dry DPPC and dry egg-PC, respectively. For DPPC this is less than reported (Kodama et al., 1985; LM Crowe and Crowe, 1988), which may be attributed to some residual water in these dried liposomes.

During drying of mixtures of these phospholipids with LPC lateral phase separation occurred (Figs 4 and 5). Dried liposomes composed of DPPC and LPC exhibited two phase

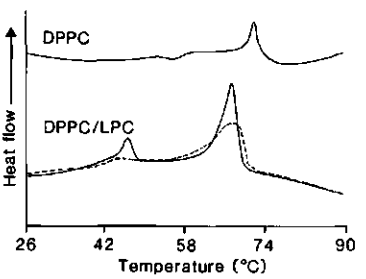

Figure 4. Differential scanning calorimetry scans of dried MLV composed of pure DPPC and a DPPC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). The dotted curve represents the second scan of the DPPC/LPC mixture. The two samples were run simultaneously.

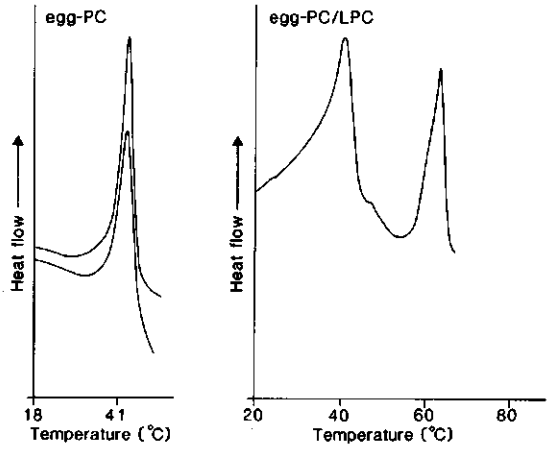

Figure 5. Differential scanning calorimetry scans of dried MLV composed of egg-PC (2 scans) and an egg-PC/LPC mixture (12 mol% 16:0-*sn*-1-LPC). The two samples were run simultaneously.

transitions (Fig. 4), indicating the separate melting of two different lipid domains. When this phase separated sample was heated for a short period above its highest transition, then rapidly cooled and scanned again, its lowest transition had vanished (Fig. 4, dotted line), which is indicative of remixing of the components. In dry egg-PC having a T_m below that of dry 16:0-LPC, the presence of LPC also caused a dehydration-dependent phase separation (Fig. 5). In addition, FTIR spectra were run on these dried egg-PC/LPC vesicles (Fig. 3). The wavenumber *versus* temperature plots of the dry egg-PC/LPC mixture is stepwise and broadened as compared to the dried egg-PC alone, indicative of the destabilizing effect of LPC on dried phospholipid bilayers. The contribution of the phase separated LPC to the wavenumber shift of the lipid mixture at temperatures over 60°C is clearly visible in Figure 3.

Phase Separation in Isolated Pollen Membranes

In order to demonstrate phase separation in membranes of aged cattail pollen, microsomal membranes were isolated after 0 and 18 d at 75% RH and 24°C, and then lyophilized. Pollen viability was completely lost after the semidry storage. It was earlier

shown that the membranes isolated from aged pollen contain LPL, and that LPL accumulation was not an isolation artefact (Van Bilsen and Hoekstra, 1993). Fourier transform IR spectroscopy is particularly suitable for the estimation of lipid phase transitions in complex biological systems (Casal and Mantsch, 1984). Employing this method, microsomal membranes isolated from viable (control) pollen showed a sharp phase transition around 42°C when dry (Fig. 6). The membranes isolated from aged pollen exhibit stepwise phase transitions, also at higher temperature. The overall temperature range over which these phase transitions proceed is broadened in membranes from aged pollen.

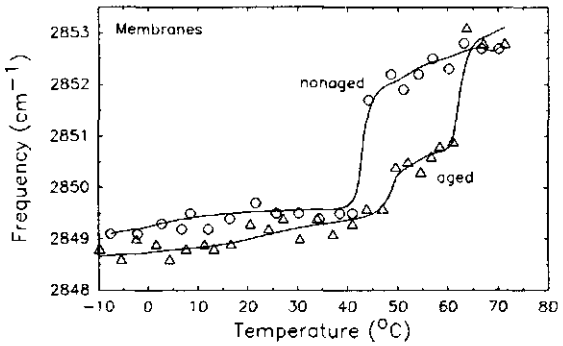

Figure 6. Frequency *vs* temperature plots (FTIR) of dried microsomal membranes isolated from nonaged and aged (18 d, 75% RH at 24°C) cattail pollen. Circles: membranes from nonaged pollen; Triangles: membranes from aged pollen. The data points represent vibrational frequencies of the symmetric CH_2 band.

Phase Separation in Membranes of Intact Pollen

To examine whether membrane phase separation also occurs *in situ* during aging, FTIR spectra of control and aged pollen were made. Before the measurements the moisture contents of the two pollen samples were adjusted to about 8%, representing the water content after exposure for 2 d to 50% RH over a saturated $Ca(NO_3)_2$ solution. In Figure 7 the wavenumber *versus* temperature plots for control and aged pollen are shown. Whereas the nonaged pollen has a discrete wavenumber shift, giving a T_m of approximately 10°C, it is not possible to estimate T_m of pollen after 18 d of storage at 75% RH. The phase transition in the aged pollen is broader and more gradual than in the control pollen.

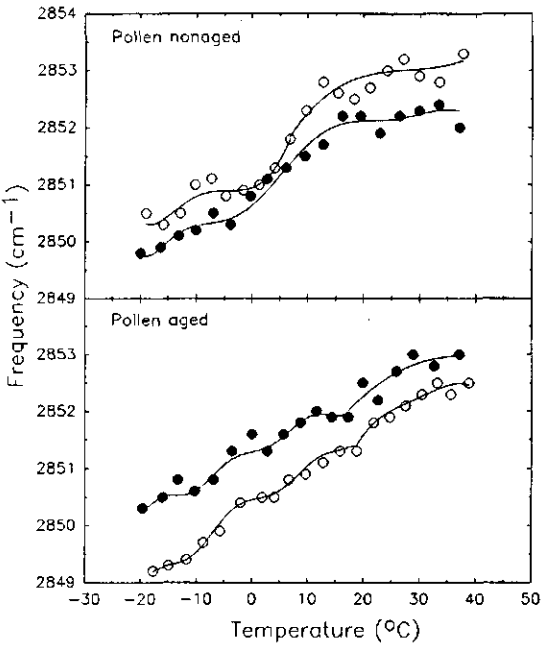

Figure 7. Frequency *vs* temperature plots (FTIR) of dry, nonaged and aged cattail pollen. Aging was achieved by storage at 75% RH and 24°C for 18 d. The two curves in each graph represent different pollen samples both equilibrated at 50% RH for 2 d before the measurements. The data points represent vibrational frequencies of the symmetric CH_2 band.

DISCUSSION

Drying of hydrated nonaged cattail pollen causes the T_m of its membranes to exceed room temperature, which leads to gel phase domains in the dry state (Crowe et al., 1989c; Hoekstra et al., 1992). Considerable leakage occurs when this dry pollen is rehydrated in germination medium. However, imbibitional leakage can be minimized by pretreatment in humid air, suggesting that possible phase separations can be reversed by this treatment (Hoekstra and Van der Wal, 1988; Crowe et al., 1989c). The fact that K^+ always leaks from the pollen for about 40 percent of the total of K^+ present has been attributed to its occurrence on the pollen wall (Hoekstra et al., 1992). In contrast, prehumidifying could not prevent the complete leakage of K^+ from the aged grains within the first min after immersion in germination medium. In the light of the extensive phase separation observed in dry model

membranes containing 16:0-LPC and in aged pollen and its membranes, the severe leakage of K^+ suggests that even after prehumidification this phase separation is not readily reversed.

Imbibitional damage of viable dry pollen can also be decreased by preheating during imbibition (*e.g.* at 35°C), allowing for the melting and remixing of membrane components before the pollen is imbibed (Hoekstra et al., 1992). For aged pollen, preheating to even higher temperatures, at which full melting and mixing occurs, might be a strategy to improve germination (*cf.* the dotted curve of the second scan in Fig. 4). In this respect, dry pollen generally is remarkably resistant to heating at high temperatures (Marcucci et al., 1982).

For the exact determination of transition temperatures of pure lipids DSC is more accurate than FTIR spectroscopy. However, when the thermotropic transition of water interferes with that of lipids, such as in hydrated egg-PC, supercooling may be necessary (Van Echteld et al., 1981). In contrast, melting of water does not interfere with the wavenumber shift of the CH_2 stretching bands. Fourier transform IR spectroscopy is particularly suitable for the estimation of lipid phase transitions in complex biological systems (Casal and Mantsch, 1984). Therefore, FTIR enables direct comparison of lipid phase behavior in model systems with that in isolated membranes and intact cells (Crowe et al., 1989d; Hoekstra et al., 1989).

With respect to dehydration, pure 16:0-LPC exhibits the same characteristics as DPPC and egg-PC, *i.e.* T_m increases considerably (JH Crowe and Crowe, 1988). In hydrated MLV phase behavior does not change as a result of the presence of 12 mol percent 16:0-LPC. In dry MLV the effect of the lysolipid is of a totally different nature. Both the DSC scans and the FTIR data indicate that in the presence of 16:0-LPC the MLV undergo a dehydration dependent phase separation, irrespective of whether T_m of the phospholipid is higher (DPPC) or lower (egg-PC) than the T_m of the lysolipid. This is reflected by the two melting isotherms in the DSC-scans and the broadened and stepwise change in the vibrational frequency of the symmetric CH_2 stretching bands. About the exact nature of these domains we can only speculate. Lysophospholipids can form bilayers with interdigitated acyl chains, but the formation of this type of lysolipid domains involves large differences in bilayer thickness (Hauser et al., 1981). We suggest that for the egg-PC/LPC mixture the first endotherm reflects the melting of egg-PC domains, and the second endotherm the melting of a mixture preferentially composed of 16:0-LPC. Because the temperature for the higher transition is much lower than that of pure dry 16:0-LPC it cannot be attributed to the melting of pure

16:0-LPC domains. In the case of the DPPC/LPC mixture the lower transition corresponds to the melting of domains preferentially consisting of LPC with possibly traces of DPPC. The higher transition corresponds with the melting of DPPC domains. Apparently LPC is not only immiscible in the gel phase of both tested PCs, but also in the dry liquid crystalline phase the components are not homogenously mixed.

The FTIR plots of the wavenumber shift *versus* temperature indicate that in both isolated membranes and intact pollen aging causes an upward extention of the phase transition. This is compatible with the suggestion that phase separated domains of LPL will be at the higher temperature side of the transition, because the natural phospholipids in cattail pollen are of the unsaturated type (Hoekstra et al., 1991) having a hydrated T_m around -9°C and a dehydrated T_m around 32°C.

We conclude that LPL formed by phospholipid deesterification during dry and semidry storage of pollen are a major cause of imbibitional leakage and death. They are able to do so by their property of causing lateral phase separation in the dry membrane. We may consider aging-induced leakage at imbibition as a special case of imbibitional injury.

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4. Declining viability and lipid degradation during pollen storage

Van Bilsen DGJL, Van Roekel GC, Hoekstra FA, submitted to Sexual Plant Reproduction

ABSTRACT

Declining viability of pollen during storage at 24°C in atmospheres of 40% RH and 75% RH was studied, with special emphasis on lipid changes. Pollen of *Papaver rhoeas* L. and *Narcissus poeticus* L., characterized by a high linolenic acid content, were compared with the low linolenic acid containing *Typha latifolia* L. pollen, on which we reported in Plant Physiol 101:675-681. The rationale behind this was to solve the question of whether lipid peroxidation is involved in the rapid viability loss and reduced membrane integrity of the unsaturated-lipid pollen types, particularly. Viability and membrane integrity degraded more rapidly at 75% RH than at 40% RH. All pollen species showed deesterification of acyl chains of lipids but no detectable peroxidation, at both RHs. Considerable amounts of lipid-soluble antioxidants were detected that did not degrade during storage. Free fatty acids and lysophospholipids were formed during storage, the effects of which on membranes are discussed. These degradation products were very prominent in the short-lived *Papaver* pollen. The loss of viability thus coincided with phospholipid deesterification. A significant decrease of the phospholipid content occurred at 75% RH, but not at 40% RH. Based on compositional analyses of phospholipids and newly formed free fatty acids, it was concluded that the deesterification of acyl chains from the lipids occurred at random. We suggest, due to the low water content of the pollen, that free radicals rather than unspecific acyl hydrolases are involved in the deesterification process.

Longevity of partially hydrated pollen and seeds is generally determined by their rate of metabolic activity (Hoekstra and Bruinsma, 1975; Benson, 1990; Shivanna et al., 1991). Respiration of pollen is strongly restricted when pollen is in equilibrium with air of low RH, and longevity is considerably increased (Hoekstra and Bruinsma, 1975). But even in the dry state, large differences in longevity between pollen of different species exist (Pfundt, 1910). A striking correlation between pollen longevity and linolenic acid content in pollen lipids was observed (Hoekstra, 1986; Hoekstra, 1992). Pollen having a high linolenic acid (18:3) content tends to have a short longevity and vice versa. This correlation suggests that peroxidation of polyunsaturated fatty acids might play a role in limiting pollen longevity (Chan, 1987; Benson, 1990). Senescence of pollen and seeds during (semi-)dry storage was also reported to coincide with deesterification of phospholipids (Nakayama et al., 1981; McKersie et al., 1988; Van Bilsen and Hoekstra, 1993). The resulting accumulation of LPLs and FFAs (*i.e.* unesterified) in the membranes matches the loss of membrane integrity *in situ*. When these PL degradation products are incorporated in liposomes, they cause an increased bilayer permeability (Van Bilsen and Hoekstra, 1993). Sofar, reports on the occurrence of LPLs and FFAs during aging are scarce, for instance on soybean seeds, (Nakayama et al., 1981) and pollen of *Typha latifolia* L. (Van Bilsen and Hoekstra, 1993).

Seeds usually remain viable for extended periods of time. To accelerate aging and possible changes in membrane composition associated with aging, often conditions of high temperature and RH are applied (Priestley and Leopold, 1979; Buchvarov and Gantchev, 1984; Wilson and McDonald, 1986; Pukacka and Kuiper, 1988; McKersie et al., 1988). This procedure is called accelerated aging. Unfortunately, the biochemical changes observed during accelerated aging are not always similar to those seen during natural (dry) aging (Priestley and Leopold, 1983; Buchvarov and Gantchev, 1984; Priestley et al., 1985). For *Typha* pollen the characteristics of membrane deterioration are generally similar during storage both at 40% RH and 75% RH (24°C) (Van Bilsen and Hoekstra, 1993). However, LPLs decompose further during storage at 75% RH, which is less pronounced at 40% RH. Furthermore, at 75% RH a slight preferential degradation of polyunsaturated fatty acids occurs, which is not observed at 40% RH.

To study the extent to which peroxidation and/or deesterification of fatty acids are involved in the degradation of membrane lipids during dry storage of pollen, we have monitored the lipid composition of two pollen species that are characterized by a much higher

linolenic acid (18:3) content than present in *Typha* pollen, on which we have reported earlier (Van Bilsen and Hoekstra, 1993)

In this paper we describe the results of such experiments using *Papaver rhoeas* L. and *Narcissus poeticus* L. pollen stored at 24°C in an atmosphere of 40% and 75% RH.

MATERIALS AND METHODS

Plant Material and Treatments

Collecting, handling and storage of *Typha latifolia* L. pollen were performed as described earlier (Hoekstra et al., 1991). Closed anthers from *Narcissus poeticus* L., *Papaver rhoeas* L. and *Nicotiana tabacum* L. cv. Mont Calme Brun plants, that were grown in the laboratory garden, were collected at anthesis and allowed to open on the laboratory bench. After sieving and further drying over silica gel for a few hours (to below 5% water on a fresh weight basis), pollen was stored at -20°C until further use. Aging was brought about by incubating the pollen at 24°C in an atmosphere of 40% RH, produced by a flow of air that was previously saturated with water vapour and then led through a cooling tower to remove a fraction of the water vapour. Alternatively, pollen was incubated at 24°C in an atmosphere of 75% RH, produced in a closed container above a saturated NaCl solution.

Germination *in vitro* was performed on solid media (0.6% Difco agar) in small plastic Petri dishes containing 0.2 M sucrose, 0.8 mM H_3BO_3, 0.7 mM $Ca(NO_3)_2.4H_2O$, 0.4 mM $MgSO_4.7H_2O$, and 0.5 mM KNO_3 in 2 mM Na-phosphate-citrate buffer pH 5.9. Before the pollen was placed on this medium, it was imbibed for 5 min in liquid medium containing 0.3 M sucrose, and twice the concentration of salts as in the solid medium, but without KNO_3. Pollen was then rapidly filtered, and K^+ in a fraction of the pollen and the medium was determined by flame photometry, essentially as described by Hoekstra et al. (1992). Pollen was prehydrated in water vapour at room temperature for one hour before imbibition in germination medium.

Prior to all treatments pollen was washed three times with hexane to remove apolar hydrocarbons and carotene from the pollen wall, and the adhering hexane was removed by evaporation. This was done to allow for a synchronous rehydration of the pollen in the liquid

germination medium. This treatment does not impair viability (Iwanami and Nakamura, 1972).

Antioxidant assay

The lipid-soluble antioxidant activity of the hexane-washed pollen was determined by measuring the extent of inhibition by a lipid extract of the O_2 uptake that results from Fe^{2+}-stimulated linoleic acid oxidation. Briefly, in a Clark type oxygen electrode the following solutions were pipetted: 1 mL of a 20 mM KH_2PO_4 buffer pH 5.6, 0.2 mL 10% Tween 20, 1 mL of a freshly prepared solution of 0.5 mM $FeSO_4$ in 1 mM Na-EDTA, and 0.1 mL of a freshly prepared solution of 0.2M linoleic acid in ethanol. Before closing the cell of the Clark electrode the solution was vigorously stirred for 1 min. A fraction of the total lipid extract from pollen was taken up in ethanol, and after a constant O_2 uptake was established, an aliquot of the extract was injected through a slit in the Clark cell. The linear O_2 uptake was reduced proportionally to the amount of antioxidants present in the extract. α-Tocopherol was used as a standard. The quantity of antioxidants present in the extract was expressed as α-tocopherol equivalents. A reasonably straight calibration curve is obtained when plotting values of the ratio, VO_2 initial/VO_2 inhibited, versus the α-tocopherol concentration applied, with the best reliability at approximately 50% inhibition.

Lipid analysis

Total lipid extracts from pollen were prepared as described elsewhere (Hoekstra et al., 1989; Hoekstra et al., 1992; Van Bilsen and Hoekstra, 1993). The total lipid extracts obtained were separated in a neutral and a polar lipid fraction by passing them over a SEP-PAK silica cartridge (Waters Associates Inc., Milford, MA, USA, cat. # 51900). The NLs were eluted with 20 mL $CHCl_3$, followed by elution with 30 mL MeOH for the polar lipids. Total PL and FFA contents were determined by homogenizing the pollen and extracting the lipids in the presence of known amounts of diheptadecanoic phosphatidylcholine and heptadecanoic acid as internal standards, respectively, and subsequent quantification by GC

of the fatty acid methylesters derived from them. PLs were separated into their different subclasses by 2-dimensional chromatography on preactivated HPTLC-plates as described elsewhere (Van Bilsen and Hoekstra, 1993). The individual PLs were visualized with iodine vapor, scraped from the plate, oxidized in 60% perchloric acid (30 min, 180°C), and the inorganic phosphorus was measured spectrophotometrically at 797 nm after formation of an ammonium molybdate complex (Böttcher et al., 1961). The NLs were separated by TLC-chromatography using hexane/diethylether/acetic acid (80/20/1, v/v) as eluents (Hoekstra and Van Roekel, 1988). After visualisation with 0.1% ANS in MeOH the FFA-band was scraped from the plate, the FFAs were re-extracted (Hoekstra and Van Roekel, 1988) and methylated using freshly prepared diazomethane in MeOH/diethylether (1/4, v/v) (Van Bilsen and Hoekstra, 1993).

Fatty acid methylesters were analyzed on a Shimadzu GC8A GC, equipped with a 30 m J&W DB225 megabore column (J&W Scientific, Folsom CA, USA), coupled to a Spectra Physics SP4100 integrator. Identification was by comparing with standards (Sigma, St louis, MO, USA) and GC-MS. GC-MS spectra were recorded on a Hewlett Packard 5970B GC-MS system equipped with a CP-Sil-88 fused silica column (Chrompack, 50 m x 0.25 mm x 0.20 μm film thickness). Electron impact spectra were obtained at 70 eV electron energy.

Chemicals

All organic solvents, HPTLC and TLC plates (kieselgel 60) were purchased from Merck (Darmstadt, Germany), ANS from Sigma (St Louis, MO, USA), heptadecanoic acid, diheptadecanoyl phosphatidylcholine from Fluka (Buchs, Switzerland) and agar from Difco (Detroit, Michigan, USA).

RESULTS

Germination and leakage during storage

Pollen of two plant species, *Papaver rhoeas* L. and *Narcissus poeticus* L., were stored at 24°C under two conditions of RH, and the decline of viability was assessed at intervals. At 75% RH, giving water contents of approximately 15% (on a fresh weight basis) for both *Papaver* and *Narcissus* pollen, the loss of viability occurred in a few days (Figs 1 and 2). This was accompanied by an increased loss of endogenous K^+ that was recovered from K^+-less germination medium after the first 5 min of imbibition. Life spans of the two pollen species at 40% RH, giving moisture contents of approximately 7-8%, were much longer. Under these conditions viability loss in figures 1 and 2 also coincided with reduced retention of endogenous K^+. In the case of dry aging (at 40% RH) of *Narcissus* pollen it was noticed that the loss of viability occasionally preceded the increase of the K^+-leakage (data not shown). However, elevated K^+-leakage and lack of swelling ability were always tightly linked. As compared to the relatively long life span of *Typha* pollen, which is often longer than 100 days at 40% RH (Hoekstra, 1992; Van Bilsen and Hoekstra, 1993), the present species are relatively short-lived under both conditions of RH. The apparent linkage of life span with membrane integrity urged for an inspection of the membrane lipids and the aging-related changes in their composition.

Qualitative and quantitative lipid changes with storage

Table I reports on the extent of fatty acid unsaturation in PLs of nonaged *Papaver* and *Narcissus* pollen, with that of *Typha* pollen included for comparison (*cf* Van Bilsen and Hoekstra, 1993). Minor fatty acids of only few tenth of a percent of the total have been left out. *Papaver* and *Narcissus* pollen have considerably more poly-unsaturated acyl chains in their PLs than *Typha*, which is evident from their higher average number of double bonds per acyl chain. Acyl chains with more than one double bond are considerably more sensitive to peroxidation than their saturated or mono-unsaturated counterparts (Schaich, 1980), which might explain the increased membrane permeability during aging of these species.

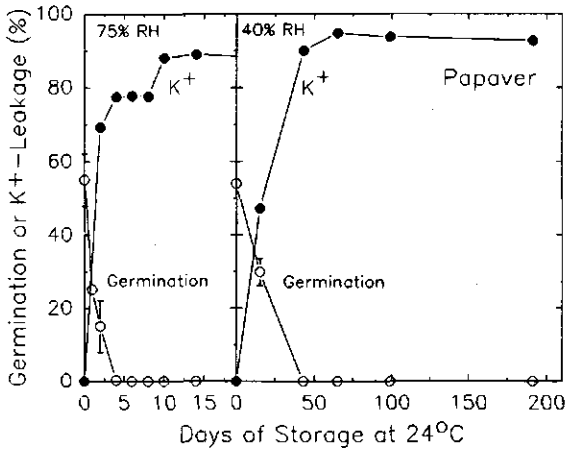

Figure 1. Percent germination and leakage of endogenous K^+ from imbibing grains in the course of storage of *Papaver* pollen at 40% RH and 75% RH. Germination and leakage data are from the same pollen sample. Germination percentages are average of data of 2 to 4 replications (±SD); K^+-leakage percentages are from single extractions.

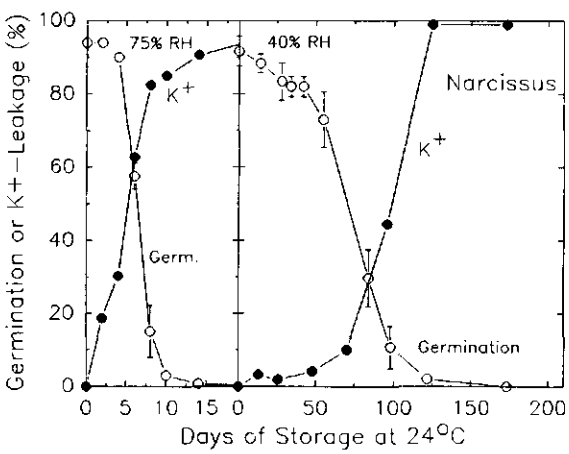

Figure 2. As in Fig. 1, but for *Narcissus* pollen.

Table I. *Phospholipid fatty acid composition of nonaged pollen of* Papaver, Narcissus *and* Typha. Average of data from 6 extractions.

Species	Fatty Acid Composition (mol% ± SD)					Number of double bonds per FA
	16:0	18:0	18:1	18:2	18:3	
Papaver	30.5±1.9	0.5±0.1	2.8±0.8	18.4±0.9	47.8±1.7	1.83
Narcissus	22.2±1.5	0.5±0.0	1.7±0.1	29.5±3.1	46.1±2.0	1.99
Typha	28.4±1.3	0.9±0.2	0.6±0.2	63.5±1.1	6.6±0.8	1.48

To investigate whether this is the case, the change in the average number of double bonds per acyl chain during storage at 75% RH was followed (Fig. 3). No considerable loss of double bonds was noticed in the acyl chains of the PLs, despite the large proportion of linolenic acid in the two pollen species. This was also true for the NL fraction (Fig. 3), indicative of an essentially unchanged degree of acyl chain unsaturation. An explanation for this was sought in the possible occurrence of lipid-soluble antioxidants. Inspection of the amount and stability of such antioxidants learned that particularly large amounts of these compounds occur in the two unsaturated pollen species, which do not seem to degrade

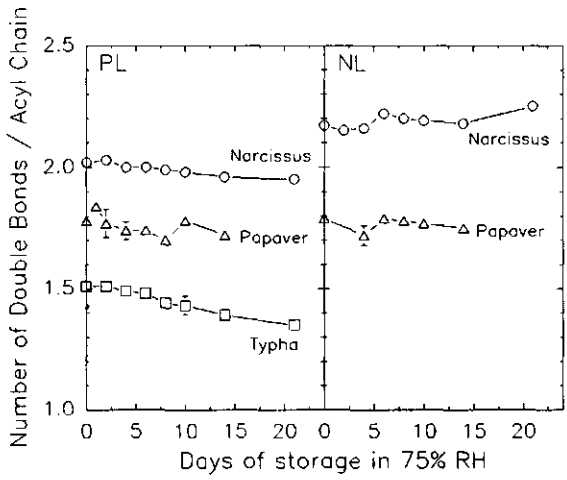

Figure 3. Number of double bonds per acyl chain in PLs and NLs in the course of storage of pollen at 24°C in an atmosphere of 75% RH. Average of data from duplicate extractions (±SD). In the case that error bars are not visible, they are smaller than the symbol size.

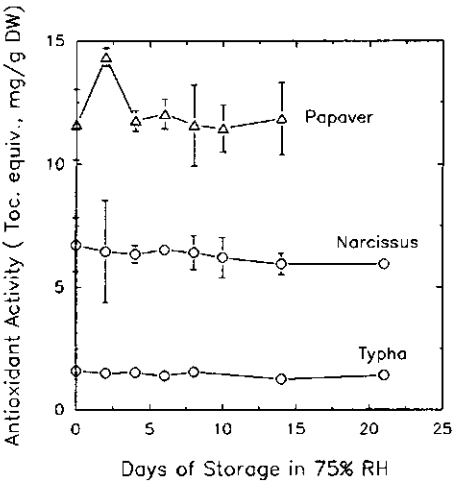

Figure 4. Antioxidant levels in pollen of *Papaver*, *Narcissus*, and *Typha* in the course of storage at 75% RH and 24°C, expressed as α-tocopherol equivalents. Values (±SD) are averages of data from 2 to 3 extractions.

much during aging (Fig. 4). The foregoing data indicate that a mechanism other than lipid peroxidation operates during this semidry storage.

We previously reported for *Typha* pollen, that viability loss coincides with a deesterification of membrane lipids. To study whether this is a general feature among pollen species, PL and FFA contents were monitored during storage at 75% RH. The loss of germination capacity clearly coincides with the loss of PLs and accumulation of FFAs (Fig. 5). The expectation was that during aging under conditions drier than 75% RH, lipid changes might be slower, but not necessarily different. To test this, PL contents and FFA accumulation were followed at 40% RH (Table II). Although less pronounced, FFA levels were increased after few weeks of dry storage. Changes in the PL contents were not statistically significant. With an unchanged PL content, preferential losses of polyunsaturated fatty acids are not likely and were, accordingly, not observed (Fig. 6). The foregoing behaviour is also true for another species (*Nicotiana tabacum*; data not shown). If the FFAs originate from the PLs one would expect the occurrence of LPLs. This would be in accordance with earlier results of dry and semidry aging of *Typha* pollen (Van Bilsen and Hoekstra, 1993).

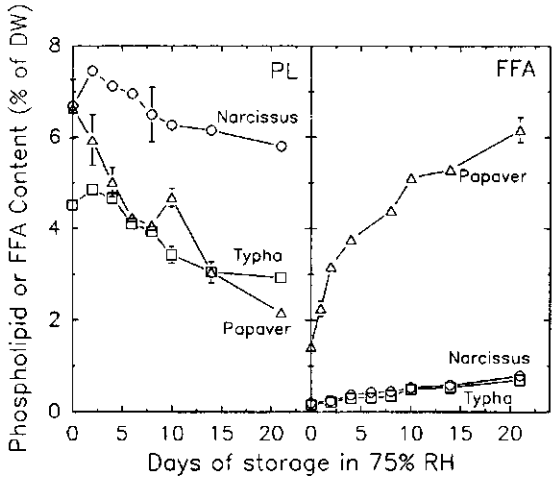

Figure 5. Change in phospholipid and free fatty acid contents in the course of storage of pollen at 24°C in an atmosphere of 75% RH. Values (±SD) are average of data from two extractions.

Table II. *Effect of storage at 24°C in an atmosphere of 40% RH on phospholipid and free fatty acid contents.* Values (±SD) are averages of data from 3 or more extractions.

Species/age		PL-Content (± SD) (% of DW)	FFA-Content (± SD) (% of DW)
Papaver	nonaged	5.25 (0.07)	0.48 (0.03)
	aged (55d)	5.33 (0.66)	1.27 (0.07)
Narcissus	nonaged	7.36 (1.02)	0.14 (0.03)
	aged (84d)	6.86 (0.72)	0.30 (0.03)
Typha	nonaged	5.32 (0.47)	0.12 (0.01)
	aged (130d)	5.38 (0.36)	0.22 (0.06)

Figure 7, showing the compositional changes of the different PLs during storage at 40% RH, indicates that this is indeed the case. At the onset of viability loss, LPC appears, followed shortly thereafter by the appearance of LPE. PI/PS was particularly degraded, but their lysoderivatives were not recovered. This is most likely due to the preference of these

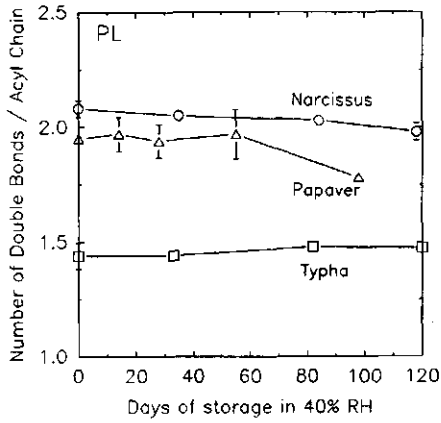

Figure 6. Number of double bonds per acyl chain in phospholipids in the course of storage of pollen at 24°C in an atmosphere of 40% RH. Average of data from 3 or more extractions (±SD). In the case that error bars are not visible, they are smaller than the symbol size.

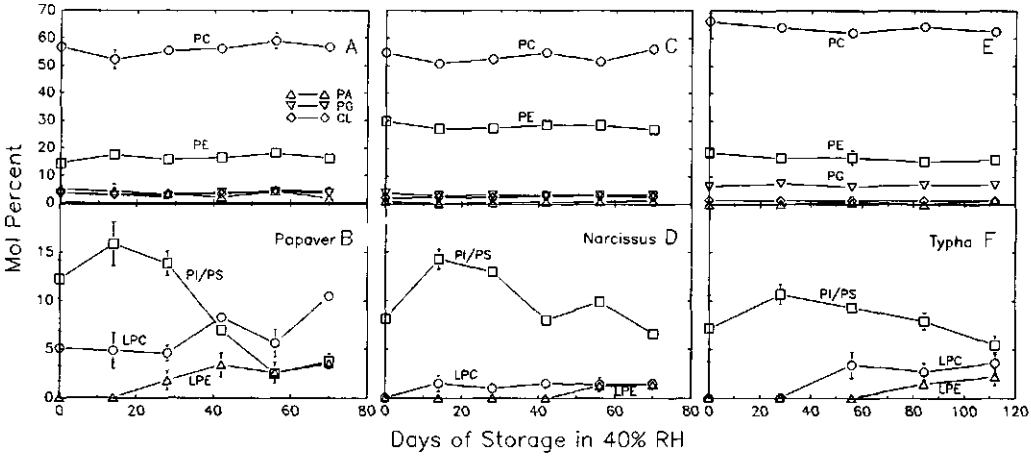

Figure 7. Change in phospholipid composition of *Papaver* (A,B), *Narcissus (C,D)* and *Typha* (E,F) pollen stored at 40% RH and 24°C. Each value (±SD) is the average of data from 4 HPTLC separations and phosphorus determinations. PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin.

lysoderivatives for the more polar phase during extraction, which was discarded. Because PI/PS were only minor components, compared with PC and PE, the recovery of their lyso-

derivates was not further pursued.

Fatty acids are usually esterified to the glycerol backbone of the PL in a specific manner, the unsaturated types at the *sn*-2 position and the saturated ones at the *sn*-1 position. This was also established for the PLs in *Typha* pollen. In an attempt to unravel the mechanism of deesterification, *i.e.* whether deesterification occurs at random or at a specific C-atom of the glycerol backbone, the fatty acid composition of the FFA pool after a certain period of aging at 40% RH was determined (Table III). Comparison with the fatty acid composition of PLs in Table I learns that the compositions are fairly similar in the case of *Papaver* and *Narcissus* pollen, and deviating in the case of *Typha* pollen.

Table III. *Free fatty acid composition in pollen of the plant species,* Papaver, Narcissus *and* Typha, *aged at 24°C in an atmosphere of 40% RH.*

Average of data from 4 or more extractions.

Species/age (days)	Fatty Acid Composition (mol% ± SD)					FFA-Content (±SD) (% of DW)
	16:0	18:0	18:1	18:2	18:3	
Papaver (63)	31.5±1.9	1.1±0.5	5.3±0.1	18.0±0.4	44.1±1.2	1.34±0.12
Narcissus (63)	27.3±4.3	5.2±1.6	1.4±1.1	21.6±1.3	44.4±3.2	0.26±0.04
Typha (82)	44.6±5.6	0.3±0.6	5.1±1.5	43.7±6.8	6.3±1.7	0.17±0.02

DISCUSSION

The results shown in Figs 1 and 2 confirm the widely observed phenomenon that longevity is much reduced when pollen is stored at elevated RH (Pfundt, 1910; Hoekstra and Bruinsma, 1975; Van Bilsen and Hoekstra, 1993). It was reported earlier for a number of pollen species that viability loss during storage is matched by an increased leakage of solutes from the grains at imbibition (Hoekstra and Van Roekel, 1985). Such increased leakage is indicative of increased plasma membrane permeability. Therefore, changes in membrane composition are most likely involved. Although there may be deteriorative processes during

storage which reduce germinability ahead of any change in membrane permeability, we may assume that the inability to swell curtails germination capacity altogether.

Pollen species that are rich in poly-unsaturated linolenic acid (18:3) in their lipids tend to have shorter longevities than those having linoleic acid (18:2) as the major fatty acid (Hoekstra, 1986). This is also true for the two pollen species in this paper (Figs 1 and 2), which are relatively short-lived as compared to *Typha* pollen (*cf.* Van Bilsen and Hoekstra, 1993). Linolenic acid is about fourfold more sensitive to peroxidation than linoleic acid (Schaich, 1980), which strongly suggests that lipid peroxidation may be involved in the viability loss of these linolenic acid-rich pollen species. However, during dry storage of *Typha* pollen - a linoleic acid-rich type - no evidence for extensive peroxidation was found, and the extent of unsaturation remained unchanged (Van Bilsen and Hoekstra, 1993). Instead, another type of lipid breakdown - deesterification of the acyl chains - was observed. In the present paper we have used the linolenic acid-rich *Papaver* and *Narcissus* pollen (Table I), expecting to observe concurrence of lipid peroxidation with increased membrane permeability during storage. However, at both RHs, the high degree of fatty acid unsaturation was maintained (Figs 3 and 6), which is evidence for the lack of extensive peroxidation. Similarly as in *Typha* pollen, the deesterification coincided with the loss of viability, leading to the accumulation of FFAs (Fig. 5; Table II) and LPLs (Fig. 7), and to the loss of PLs, much more at 75% RH than at 40% RH (Fig.5; Table II). The occurrence of LPLs is not an artifact from the lipid isolation and purification (Van Bilsen and Hoekstra, 1993). *Papaver* pollen, particularly, accumulated large amounts of FFAs (Fig. 5; Table II) and LPLs (Fig. 7), and is very short-lived.

α-Tocopherol-like antioxidants prevent peroxidation of poly-unsaturated fatty acids *in vitro* and *in vivo* (Chan, 1987; Benson, 1990), but they are unable to prevent deesterification of acyl chains *in vitro* (McKersie et al., 1990). This may also apply for intact pollen, since it contains a sometimes considerable and stable amount of antioxidants during dry and semidry storage (Fig. 4), has extensive deesterification, but lacks peroxidation.

If peroxidation is not involved in the lipid degradation, how could we explain the tendency of linolenic acid-rich pollen species to be generally short-lived? It was reported earlier, that the linolenic acid-rich pollen species tend to rapidly protrude pollen tubes (Hoekstra, 1986). This may reflect a higher general level of development and metabolism, which does not allow for an extended storage longevity. The correlation would then be of

a non-causal nature.

Senescence of hydrated plant organs is often associated with leakage of intracellular solutes and, thus, with increased membrane permeability (Faragher et al., 1987). The decreased PL:sterol ratio, mainly through the loss of PL, is held responsible for the increased permeability of these membranes (McKersie et al. 1978; Senaratna et al., 1984). During pollen storage at 75% RH a considerable loss of PLs is observed, but not at 40% RH (Fig. 5). This is a major difference between aging at low and high RH. Since aged pollen under both RH regimes showed extensive leakage upon imbibition, there must be another factor controlling membrane integrity than a reduced PL:sterol ratio, in the 40% RH-aged material, particularly.

The products of deesterification, FFAs and LPLs, as they are produced in *Typha* pollen during dry and semidry storage, have been examined in detail as to their effects on liposomes composed of *Typha* pollen lipids (Van Bilsen and Hoekstra, 1993). Both LPLs and FFAs cause a moderate leakage from liposomes in suspension. However, upon imbibition of dry aged pollen an instantaneous, massive leakage occurs. This may be due to the behaviour of FFAs and LPLs in dry bilayers, in which they can cause lateral phase separation (Crowe et al., 1989; Van Bilsen et al., in press). Problems with the remixing of the phase-separated domains during rehydration may cause the leakage. There is some evidence that lateral phase separation occurs, also *in situ*. Employing Fourier transform IR spectroscopy, a complex melting behaviour of lipids was observed in dried membranes isolated from aged *Typha* pollen, which was interpreted to mean that a phase separation had occurred (Van Bilsen et al., in press). Probing dry aged pollen, a similar conclusion could be reached.

The position of the fatty acids in the glycerol backbone of a PL is usually not random, the unsaturated types preferentially occupying the *sn*-2 position and the saturated types the *sn*-1 position (Rosenberg, 1990). Using phospholipase A_2, this was also established for *Typha* pollen (Van Bilsen and Hoekstra, 1993) and most likely also applies for other pollen. The FFAs from *Papaver* and *Narcissus* pollen, generated during storage, have a fatty acid distribution that resembles that of their PLs (*cf.* Tables I and III). This would mean that no stereospecific cleavage of the acyl chains occurs, which excludes the involvement of the phospholipase A types. Although it may point to the activity of unspecific lipid acyl hydrolases (Galliard, 1980), because of the low water content enzymic degradation seems unlikely (McKersie et al., 1988). An at random deesterification, mediated by free radicals

as proposed by Niehaus (1978) is more likely. Such mechanism was earlier suggested to operate in aging seeds as well (McKersie et al., 1988). The FFAs from *Typha* had a significantly lower linoleic acid content than the PLs. This might be due to the small amounts that are produced, or it may be degraded by peroxidation because of insufficient protection by the relatively small amount of antioxidants.

We conclude that deesterification of PLs in pollen during dry and semidry aging is a general phenomenon, irrespective of the degree of unsaturation of the acyl chains of the lipids. The products formed, FFAs and LPLs, are usually formed temporally ahead of the decline of viability. The initially low viability of the *Papaver* pollen used, may be explained in this respect by the presence, right from the onset of the storage treatment, of certain amounts of FFAs and lysophosphatidylcholine.

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5. Lipid unsaturation and pollen viability and germination

I: Catalytic hydrogenation of pollen lipids

Van Bilsen DGJL, Hoekstra FA, in prep.

ABSTRACT

The level of the unsaturated fatty acid, linolenic acid, in the lipids of pollen species has been shown to correlate negatively with longevity and positively with start of tube growth. We investigated the possibility of *in situ* saturation by catalytic hydrogenation of unsaturated fatty acids using the sulphonated alizarine derivative, $Pd(QS)_2$, in an attempt to demonstrate a causal relationship between these parameters.

Hydrogen bubbling in the presence of the catalyst was performed on extracted phospholipids, pollen fragments and whole pollen grains from a number of plant species. Phospholipid fatty acid composition was monitored before and after the treatment. Whereas hydrogenation of extracted phospholipids was successful, no change in fatty acid composition was achieved with *in situ* treatment of whole pollen, even when membrane permeability was artificially increased. In fragmented pollen hydrogenation of the unsaturated fatty acids was also difficult. This suggests an inhibition of the catalytic reaction, rather than lack of penetration of the catalyst, as the primary cause of the failure to achieve catalytic hydrogenation. Thus, *in situ* treatment of pollen with hydrogen in the presence of $Pd(QS)_2$ is not a suitable method to hydrogenate polyunsaturated fatty acids in the attempt to extend pollen longevity and postpone tube growth.

Pollen life span depends upon the integrity of its membranes, more precisely upon its phospholipid composition (Hoekstra, 1986; Van Bilsen and Hoekstra, 1993; Van Bilsen et al., in press; Van Bilsen et al., submitted). During (semi-)dry storage at room temperature a deesterification of phospholipids takes place, which leads to a changed membrane composition (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). Some newly formed lipid components are more prone to lateral phase separation, which is considered to be the main cause of viability loss during storage (Van Bilsen et al., in press). Among pollen species a negative correlation was established between the level of linolenic acid (18:3) in their phospholipids and longevity (Hoekstra, 1986; Van Bilsen et al., submitted). Species having linolenic acid as the major fatty acid show a faster breakdown of membrane phospholipids during storage than those having mainly linoleic acid (18:2). These findings suggest that the higher reactivity of the polyunsaturated linolenic acid towards free-radical reactions (Chan, 1987; Schaich, 1980) is responsible for the rapid loss of membrane integrity in the 18:3 species. However, the expected preferential degradation of the most unsaturated fatty acids does not occur during dry storage, suggesting that other mechanisms of lipid breakdown might play a role during aging (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted).

Storage at low moisture contents (0.05 g H_2O g^{-1} dry matter) preserves viability better than does storage at elevated moisture contents (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). However, very dry pollen has an increased risk of imbibitional damage on rehydration due to the formation of gel phase domains in the dry state (Crowe et al., 1989; Hoekstra et al., 1992a). High levels of linolenic acid in phospholipids as they occur, for instance, in *Impatiens glandulifera* Royle pollen, prevent formation of gel phase domains with drying because of their low intrinsic transition temperature (T_m). In this way, this pollen is insensitive to imbibitional stress, particularly at chilling temperature (Hoekstra et al., 1992b). But as a consequence, dry pollen of *Impatiens* has a short life span, 2 weeks at room temperature (Hoekstra, 1986). In contrast, *Typha latifolia* L. pollen, with linoleic acid as the major fatty acid, is very sensitive to imbibitional stress because of a phase change from the gel to the liquid crystalline phase during rehydration (Crowe at al., 1989; Hoekstra et al., 1992a), but has a life span of approximately 18 weeks when stored dry at room temperature (Hoekstra, 1986; Hoekstra, 1992). These observations suggest a positive correlation between the

membrane T_m and pollen longevity.

The number of unsaturated fatty acids in the membrane lipids clearly determines membrane fluidity (Evans and Graham, 1989; Hoekstra et al., 1992b). However, less evident is the causal relationship between extent of unsaturation and pollen longevity. To clarify this relationship, reduction of the number of unsaturated fatty acids in the membranes would be helpful. Reduction of double bonds can be achieved by a direct chemical approach using catalytic hydrogenation (Vigh et al., 1984; Vigh et al., 1985a; Vigh et al., 1985b; Vigh et al., 1985c; Vigh et al., 1987; Vigh et al., 1988), or by a genetic approach using site-directed mutagenesis affecting desaturases (Browse et al., 1989; Browse et al., 1990). Catalytic hydrogenation has been succesfully applied on synthetic phospholipids (Vigh et al., 1987), membrane lipids in cell organelles (Vigh et al., 1985c; Horváth et al., 1986; Gombos et al., 1988) and cell suspensions (Horváth et al., 1986; Vigh et al., 1984; Vigh et al., 1985a; Vigh et al.,1985b; Vigh et al., 1988) from various species to study membrane fluidity in relation to thermal stability, chilling susceptibility, or lipid polymorphism. Site-directed mutagenesis has been used to study lipid metabolism in *Arabidopsis thaliana* L. Heynh. (Browse et al., 1989; Browse et al., 1990) and the relation between fatty acid composition and pollen tube growth in the same species (Van Bilsen et al., in prep). Whereas the effects of site-directed mutagenesis are irreversible and may affect other tissue apart from pollen (Browse et al., 1989; Browse et al., 1990; Van Bilsen et al., in prep), cells can fully recover from *in vivo* hydrogenation when carried out under mild conditions (Vigh et al., 1988). As a means to prolong pollen life span, catalytic hydrogenation seems thus more suitable than site-directed mutagenesis.

We have subjected extracted phospholipids, pollen fragments and intact pollen from several species to homogeneous catalytic hydrogenation, using the sulphonated alizarine derivative $Pd(QS)_2$, and monitored the viablity of the pollen and the fatty acid composition of the phospholipids before and after the treatment.

MATERIALS AND METHODS

Pollen sources and treatments

Collection, handling and storage of *Typha latifolia* L. pollen was performed as described earlier (Hoekstra et al., 1991; Hoekstra et al., 1992b). Closed mature anthers from *Impatiens glandulifera* Royle, *Narcissus poeticus* L. and *Papaver rhoeas* L. were collected and allowed to open on the laboratory bench. The plants were grown at the laboratory garden. Flowering parts from *Alnus glutinosa* L. and *Secale cereale* L., and the male inflorescences from *Zea mays* L. were collected, put on vases and allowed to shed their pollen on the laboratory bench. *Secale* and *Zea* were grown at the Department of Plant Breeding of the Wageningen Agricultural University, and *Alnus* inflorescences were collected from field populations in The Netherlands. After sieving and further drying over silica gel (to below 5% water on a fresh weight basis), pollen was stored at -20°C until further use.

Catalyst, hydrogenation buffers and hydrogenation protocols

The catalyst, $Pd(QS)_2$ (QS = sulfonated alizarine: $C_{14}H_6O_7NaS$), was preactivated immediately before use. A stock solution of the catalyst at 2.5 mg per mL was prepared in the appropriate hydrogenation buffer. Preactivation consisted of bubbling hydrogen through the solution for 30 min, followed by vigorous shaking until the green stage of the reduced complex was formed. Subsequently, hydrogen was replaced with air by continuous stirring in an open vial until the complex achieved a dark red colour (Dr L. Vigh, personal communication). Hydrogenation solutions consisted of 0.33 M sorbitol, 10 mM NaCl, 2 mM Na-EDTA, 5 mM $MgCl_2$, 1 mM $MnCl_2$, in 30 mM phosphate buffer (Thomas et al., 1986), at three different pH values, 3.80, 5.55 or 6.50. Alternatively, pollen germination medium was used as hydrogenation buffer (1.6 mM H_3BO_4, 1.4 mM $Ca(NO_3)_2.4H_2O$, 0.8 mM $MgSO_4.7H_2O$, 1 mM KNO_3 and 0.4 M sucrose in 2 mM Na-phosphate-citrate buffer, pH 4.5). All these buffers were made oxygen-free by sonication and bubbling with hydrogen prior to use. After addition of an aliquot of the catalyst stock

solution to the different oxygen-free suspensions, hydrogen was applied in a constant stream for the desired incubation time. Incubations were terminated by removal of the hydrogen stream, directly followed by lipid extraction according to the method of Bligh and Dyer (Bligh and Dyer, 1959) or, in the case of intact pollen, according to earlier described methods (Hoekstra et al., 1991). The lipids were dried over anhydrous Na_2SO_4 before methylation and subsequent identification by gas chromatography. Controls were incubated without the catalyst. Hydrogenations were carried out at room temperature.

Hydrogenation of extracted pollen phospholipids

Phospholipids were extracted from *Typha* and *Impatiens* pollen as described elsewhere (Hoekstra et al., 1989). Small unilamellar vesicles (SUV) were prepared by sonication of the phospholipid in a round type bath sonicator (Model G1125SP1T, Laboratory Supplies Co., Hicksville N.Y). Lipid content was determined by the method of Böttcher et al. (Böttcher et al., 1961). The ratio of catalyst *vs* phospholipids (w/w) was 1.4 for *Impatiens* and 1.1 for *Typha*.

Hydrogenation of crushed *Typha* pollen

Pollen was immersed in bidestilled water (25 mL) and directly frozen at -30°C in a precooled X-press (AB-Biox, Järfälla, Sweden). The frozen pollen was crushed at approximately 15.000 p.s.i. in the X-press. The frozen material was either directly lyophilized, or first thawed, then centrifuged at 100,000 x *g* for 45 minutes, and the pellet obtained was lyophilized (purification). The crude fragments were suspended in degassed hydrogenation buffer at pH 6.5 or 3.8 at a catalyst *vs* phospholipid ratio of 1.7 [w/w, based on lipid phosphorus content (Böttcher et al., 1961) after a quantitative lipid extraction according to Bligh and Dyer (Bligh and Dyer, 1959)]. The partially purified fragments were suspended in degassed hydrogenation buffer, pH 6.5, at a catalyst *vs* phospholipid ratio of 0.2 or 4.0.

Hydrogenation of whole pollen

Pollen samples from *Impatiens* and *Typha* were carefully prehydrated in water vapor at 20°C prior to complete immersion in hydrogenation buffers at pH values of 3.8, 5.55 and 6.5. *Typha* pollen was also incubated in the buffers in the presence of 10% DMSO (v/v; at pH 3.8 and 6.5). Aging of *Typha* pollen was achieved by storage for 18 days at 75 % RH and 24°C. The aged pollen was directly immersed in the above mentioned DMSO (10% v/v) buffers at pH = 3.8 and 6.5, without prehumidification. Preactivated catalyst stock solution was added to the pollen in a ratio of 1 mg of the catalyst per 25 mg of pollen.

Pollen from *Secale*, *Alnus*, *Zea*, *Narcissus* and *Papaver* were prewashed with hexane to remove hydrocarbons from the pollen wall, and the excess hexane was removed by evaporation (Hoekstra et al., 1989; Hoekstra et al., 1991). The pollen was then directly immersed in hydrogenation buffer, pH 6.5, without prehydration in water vapor (1 mg catalyst per 25 mg pollen). Pollen of *Narcissus* and *Papaver* were also incubated at elevated hydrogen pressure (2.5 atm; 1 mg catalyst per 25 mg pollen; hydrogenation buffer, pH 6.5).

Analysis of phospholipid fatty acid composition

Fatty acid composition of phospholipids was determined as described earlier (Hoekstra and Van Roekel, 1988). The methylated fatty acids were analyzed on a J&W DB225 megabore column (J&W Scientific, Folsom CA, USA, length 30 m, diameter 0.54 mm), attached to a Shimadzu GC8A gaschromatograph, coupled to a Spectra Physics SP4100 integrator and identified by comparison with known standards (Sigma, St Louis, MO).

Catalyst

$Pd(QS)_2$ was a generous gift from Dr Laszlo Vigh, Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Science, Szeged, Hungary.

RESULTS

Phospholipids extracted from *Typha* and *Impatiens* pollen were easily saturated during hydrogen bubbling in the presence of $Pd(QS)_2$, as can be deduced from the fatty acid composition of control and hydrogenated vesicles (Table I). The difference in pH between the two buffers used did not influence the hydrogenation efficiency. In the germination medium (Hoekstra et al., 1992a) hydrogenation of the liposomes was also successful.

Attempts to saturate the phospholipids of *Impatiens* and *Typha* pollen *in situ* were not successful (Table II). To enhance membrane permeability, *Typha* pollen was incubated in the presence of DMSO or directly immersed in the buffer without prehumidification (aged material). These treatments failed to improve conversion of

Table I. *Fatty acid composition of small unilamellar vesicles, composed of phospholipids extracted from* Impatiens *and* Typha *pollen, after treatment with* $Pd(QS)_2$ *and hydrogen in hydrogenation buffer or germination medium at room temperature for various times at two different pHs.*

The ratio of catalyst *vs* phospholipids (w/w) was 1.4 for *Impatiens* and 1.1 for *Typha*.

Pollen	Treatment and pH of Buffer or Medium	Reaction Time	Phospholipid Fatty Acid Composition				
			16:0	18:0	18:1	18:2	18:3
		min	*mol percent*				
Impatiens	Buffer -$Pd(QS)_2$, pH 6.50	10	29.6	1.0	1.0	4.9	63.5
	Buffer +$Pd(QS)_2$, pH 6.50	5	30.8	23.1	12.6	2.9	30.5
	Buffer +$Pd(QS)_2$, pH 6.50	10	30.3	50.6	17.0	1.0	1.0
	Buffer +$Pd(QS)_2$, pH 5.55	10	31.7	46.0	17.4	3.9	1.0
	Medium +$Pd(QS)_2$, pH 4.50	5	31.5	47.4	15.8	4.8	0.5
	Medium +$Pd(QS)_2$, pH 4.50	10	30.8	50.3	18.9	0.0	0.0
Typha	Control -$Pd(QS)_2$, pH 6.50	15	32.1	1.0	4.9	56.1	5.9
	Buffer +$Pd(QS)_2$, pH 6.50	15	31.4	68.6	0.0	0.0	0.0
	Buffer +$Pd(QS)_2$, pH 5.55	15	32.4	67.6	0.0	0.0	0.0

polyunsaturated phospholipids into saturated ones. Even in aged *Typha* pollen, used because of its high membrane permeability, phospholipids were apparently not saturated. In both species tested, saturation was never observed. This implies that some inactivation or inhibition of the catalyst, rather than insufficient membrane permeability, has prevented hydrogenation.

Table II. *Fatty acid composition of phospholipids extracted from* Impatiens *and* Typha *pollen after incubation of whole pollen under hydrogenating conditions in the presence of* $Pd(QS)_2$ *catalyst at room temperature.*

Pollen was carefully prehumidified, prior to complete immersion in the appropriate buffer or germination medium, except for the aged *Typha* pollen. Pollen was incubated with 1 mg of catalyst per 25 mg pollen, which corresponds with catalyst *vs* phospholipid ratios of approx. 0.6-1.0 (w/w).

Pollen Source	Buffers	Reaction Time	Phospholipid Fatty Acid Composition				
			16:0	18:0	18:1	18:2	18:3
		min	*mol percent*				
Impatiens	Control, Buffer -$Pd(QS)_2$	10	28.9	0.9	0.9	3.6	65.7
	Buffer, pH 6.50	10	30.2	1.0	1.0	4.0	63.8
	Buffer, pH 6.50	25	30.2	1.0	1.0	4.1	63.9
	Buffer, pH 5.55	25	28.1	1.0	1.0	4.0	65.9
	Medium, pH 4.50	25	28.7	1.0	1.0	4.1	65.3
Typha	Control, Buffer -$Pd(QS)_2$	45	31.1	0.7	3.8	57.7	6.7
	Buffer, pH 6.50	45	30.1	1.2	5.1	57.4	6.1
	Buffer, pH 6.50 + DMSO	45	36.6	1.0	3.9	53.5	5.0
	Buffer, pH 5.55	45	32.8	1.0	4.0	56.2	6.1
	Buffer, pH 3.80	45	30.6	1.0	4.0	58.3	6.1
	Buffer, pH 3.80 + DMSO	45	32.5	1.0	4.0	56.6	6.0
Typha, aged	Control, Buffer -$Pd(QS)_2$	45	34.7	1.1	3.8	54.6	5.8
	Buffer, pH 6.50 + DMSO	45	36.6	1.0	3.9	53.5	5.0
	Buffer, pH 5.55 + DMSO	45	32.5	1.0	4.0	56.6	6.0

When *Typha* pollen was washed after 45 min in hydrogenation buffer in the presence of H_2 and catalyst, and the buffer changed for germination medium, a considerable number of cells germinated. In contrast, *Impatiens* pollen was severely damaged by the prehydration in humid air at 20°C. Prehydration at 2°C could overcome that problem, but because H_2 bubbling at room temperature proper was detrimental, the hydrogenation procedure was also tried at 2°C (data not shown). Although pollen remained viable under those conditions, even liposomes from *Impatiens* pollen phospholipid did not become saturated after 20 min under hydrogenation conditions at 2°C.

To study whether the presence of certain inhibitory agents might have interfered with the treatment, crude and partially purified fragments from *Typha* pollen were subjected to hydrogenation. Their fatty acid composition is shown in Table III. In the crude fragments no change in fatty acid composition was observed.

Table III. *Fatty acid composition of phospholipids extracted from crushed* Typha *pollen before and after incubation of pollen fragments under hydrogenating conditions in the presence of various amounts of* $Pd(QS)_2$ *catalyst.*

All incubations were carried out in hydrogenation buffer, pH 6.5 at room temperature.

Pollen fragments	Catalyst concentration	Reaction time	Phospholipid Fatty Acid Composition				
			16:0	18:0	18:1	18:2	18:3
	mg/mg phospholipid	*min*	*mol percent*				
Purified	0.0	15	31.5	1.0	3.7	57.4	6.4
	0.4	15	36.3	1.0	4.0	52.7	6.0
	0.4	45	32.5	1.0	4.9	55.6	6.0
	2.0	15	33.5	1.0	5.9	53.6	6.0
	2.0	45	28.3	1.0	55.6	9.0	6.0
Crude	0.0	15	35.5	0.9	3.7	53.8	6.2
	1.7	15	33.5	1.0	3.9	55.6	6.0
	1.7	45	30.6	1.0	5.0	57.3	6.1

Phospholipids fromthe partially purified fragments were partly saturated, only after prolonged incubation times and in the presence of an elevated amount of $Pd(QS)_2$. This indicates that the lack of hydrogenation, observed in intact pollen, may be caused by an inhibition or inactivation of the catalyst.

To examine whether the inability to saturate phospholipids in crude crushed and whole *Typha* pollen is characteristic for this species only, several other pollen species were subjected to catalytic hydrogenation. Pollen from the species listed in Table IV were used. Membrane permeability was increased by omitting the prehydration procedure prior

Table IV. *Fatty acid composition of phospholipids extracted from intact pollen of* Zea, Papaver, Alnus, Narcissus *and* Secale, *after hydrogenation in hydrogenation buffer, pH 6.5, at room temperature for 30 min.*

Pollen was incubated in the presence of 1 mg $Pd(QS)_2$ per 25 mg pollen, corresponding with catalyst *vs* phospholipid ratios of approx. 0.6-1.0 (w/w).

Pollen Source	Treatment	Hydrogen Pressure	Phospholipid Fatty Acid Composition			
			16:0	18:0 + 18:1	18:2	18:3
		atm	*mol percent*			
Alnus	Control	1.0	19.1	13.5	39.9	27.5
	+ $Pd(QS)_2$	1.0	20.6	14.1	38.7	26.6
Narcissus	Control	1.0	21.1	1.9	31.0	45.9
	+ $Pd(QS)_2$	1.0	21.8	2.0	34.0	42.3
	+ $Pd(QS)_2$	2.5	20.5	1.8	32.8	45.0
Papaver	Control	1.0	27.3	1.0	26.1	45.7
	+ $Pd(QS)_2$	1.0	28.9	2.0	25.6	43.6
	+ $Pd(QS)_2$	2.5	27.6	1.6	26.5	44.3
Secale	Control	1.0	17.1	2.0	5.9	75.0
	+ $Pd(QS)_2$	1.0	21.3	14.6	6.9	57.2
Zea	Control	1.0	38.5	1.9	5.7	53.9
	+ $Pd(QS)_2$	1.0	36.5	1.9	6.7	54.9

to immersion of the dry pollen into the hydrogenation buffer. Possible interference of hydrocarbons present on the pollen wall was prevented by a hexane wash prior to the incubations (Hoekstra et al., 1991; Hoekstra et al., 1992a). Incubations of *Narcissus* and *Papaver* pollen were also carried out at elevated hydrogen pressure. As is evident from the results shown in Table IV, only in *Secale* pollen a slight change of fatty acid composition in the direction of more saturation was observed. However, this pollen was non-viable because it is not desiccation-tolerant. In none of the viable species tested any significant change in fatty acid composition was detected after incubation. Elevated hydrogen pressures did not have any effect.

DISCUSSION

An interesting correlation has been estabished between linolenic acid content and pollen longevity, on the one hand, and linolenic acid content and pollen tube growth rate, on the other hand (Hoekstra, 1986; Hoekstra, 1992). Low levels of linolenic acid correlate positively with a long life span and high levels of linolenic acid correlate positively with rapid tube growth, thus revealing the dualistic role linolenic acid seems to play in pollen physiology in general (Hoekstra, 1986; Van Bilsen et al., submitted). This would mean that a modification of the fatty acids *in situ* may give important information about the role of membrane fluidity on pollen longevity and pollen germination in general. Because catalytic hydrogenation, using the sulphonated alizarine derivative, $Pd(QS)_2$, has been successfully applied on several plant membrane systems to study the role of membrane fluidity in various membrane-related processes (Vigh et al., 1984; Vigh et al., 1985a, Vigh et al., 1985b; Vigh et al., 1988; Horváth et al., 1986), it was also applied on pollen.

To determine the experimental conditions required for optimal hydrogenation, liposomes from pollen phospholipids were incubated with $Pd(QS)_2$ under different conditions. The observed fast hydrogenation of phospholipids extracted from *Typha* and *Impatiens* pollen was in accordance with previous reports on catalytic hydrogenation using $Pd(QS)_2$ (Vigh et al., 1984; Vigh et al., 1985a; Vigh et al., 1985b; Vigh et al., 1985c, Vigh et al., 1987; Vigh et al., 1988). This also shows that although procedures and

incubation conditions were slightly different from those reported, the treatment was effective: polyunsaturated fatty acids were converted to saturated ones.

For whole, intact pollen the procedure was not effective. The liposome studies show that this is not due to the experimental conditions applied. For several reasons hydrogenation might fail in intact pollen. Lack of penetration of the rather polar catalyst (MW 789) into the cytosol is one of them. If the catalyst does not penetrate into the pollen, only the outer layer of the plasma membrane can be saturated. This is such a small fraction of the total phospholipid content, that a change in its fatty acid composition may not be detected. To enhance the possibility of penetration of the catalyst, treatments that increase membrane permeability were applied. A lowering of the pH of the hydrogenation buffer was one of them. The liposome studies showed that this did not appreciably reduce the hydrogenation efficiency. Another possibility to enhance membrane permeability was to artificially induce imbibitional damage by omitting prehydration of dry pollen before immersion (Crowe et al., 1989; Hoekstra et al., 1992a) or using aged pollen grains whose membranes have become leaky (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., in press; Van Bilsen et al., submitted). But even when the pollen was treated in such ways that plasma membrane permeability was increased to facilitate penetration of the catalyst into the cells, phospholipids were not saturated. This suggests that a proper functioning of the catalyst was inhibited rather than lack of penetration.

Inhibition of the catalyst could be ascribed to cytoplasmic solutes or lipid bodies. Therefore, pollen fragments were incubated. The crude fragments were not saturated. Removal of part of the cytosol content improved hydrogenation, but prolonged incubation times and an elevated amount of catalyst were required to partly saturate the phospholipids of the thus purified fragments. This suggests that certain components in the cytoplasm of the *Typha* pollen interfered with the catalyst and prevented its functioning.

To test whether this interference was restricted to *Typha*, other species were also incubated with the catalyst. The pollen wall contains several hydrocarbons that might interfere with the catalyst. These were removed by a (non-destructive) hexane wash, to prevent a possible immobilisation of the catalyst onto the pollen wall. To enhance penetration all dry pollen species were directly immersed into the buffers. But even then, only in the desiccation-sensitive pollen of *Secale*, some saturation was achieved. In all

viable species tested the $Pd(QS)_2$ was not effective. It thus seems that $Pd(QS)_2$ can not function in pollen, because pollen contain inhibitory or inactivating substances. Other catalysts to hydrogenate unsaturated fatty acids without reducing viability are not known. We conclude that this catalyst is unfit to saturate lipids in pollen to study the relation between phospholipid fatty acid composition, and pollen viability and germination.

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6. Lipid unsaturation and pollen viability and germination II: Pollen tube growth and linolenic acid content in lipids of mutants of *Arabidopsis thaliana* L. Heynh.

Van Bilsen DGJL, Van Roekel GC, Hoekstra FA, in prep.

ABSTRACT

Pollen tube growth rate of *Arabidopsis thaliana* L. Heynh. mutants with altered lipid composition was studied and compared with wild type. In pollen from the *fad*2 line, which has a deficiency in the eukaryotic pathway of lipid biosynthesis, a decreased level of linolenic acid coincides with a decreased pollen tube growth rate. Pollen tube length is markedly reduced. The lag times between immersion in germination medium and onset of tube emergence were not affected. Grown under standardized conditions the plants from this mutant line are smaller than the wild type plants.

Pollen tube growth was not affected in pollen from the mutant lines LK3, JB25 and JB60 wich have alterations in the prokaryotic pathway of lipid biosynthesis, mainly localized in the chloroplast. Linolenic acid content of pollen of these mutants was not less than in wild type pollen.

These results confirm that high levels of linolenic acid in pollen are associated with rapid pollen tube growth and show that the eukaryotic pathway of lipid biosynthesis is predominant in pollen.

Pollen contain large amounts of polyunsaturated fatty acids in their lipids, predominantly linolenic and linoleic acid (Hoekstra, 1986; Hoekstra et al., 1992b; Evans et al., 1987, 1988; Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). In pollen species with high levels of linolenic acid, a tendency for a short lag period and rapid tube growth was observed (Hoekstra, 1986). The lag period is the time between imbibition in germination medium and first emergence of pollen tubes. Longer lag periods and slow tube growth usually occur in pollen containing relatively low levels of linolenic acid. Therefore, a correlation between tube growth rate and phospholipid fatty acid composition has previously been suggested (Hoekstra, 1986, 1992).

The thermotropic lipid phase transitions of pollen membranes are dependent on the degree of fatty acid unsaturation (Hoekstra et al.,1992a, 1992b). In pollen species containing predominantly linoleic acid, the membrane transition temperature is higher than that of pollen species containing high levels of linolenic acid. The observed relation between high linolenic acid content and rapid pollen tube growth suggests that high membrane fluidity and rapid tube growth rates may be causally linked. Extensive fusion of secretory vesicles with the plasma membrane in the tip of the pollen tube is involved in tube extension (Picton and Steer, 1985; Dorne et al., 1988; Steer and Steer, 1989). The fluidity of the plasma membrane and secretory vesicles might thus influence the rate at which pollen tubes grow. However, differences in rate of tube growth between pollen species may also be caused by other factors than membrane lipid composition. The duration of the lag phase and the rate of tube growth, for instance, are correlated with *de novo* protein synthesis and elevated metabolic activity, wich can vary widely among different pollen species (Hoekstra and Bruinsma, 1975; Çapková et al., 1987). To study the sole effect of lipid composition on pollen germination, these other factors have to be excluded. Mutant lines from *Arabidopsis thaliana* L. Heynh., exhibiting an altered fatty acid composition (Browse et al., 1985, 1989, 1990; Kunst et al., 1988; Lemieux et al., 1990; Miquel and Browse, 1990) enable such studies.

The eukaryotic pathway of lipid biosynthesis, which is localized in the endoplasmatic reticulum, is considered the major contributing pathway in pollen and seeds. In contrast, the prokaryotic pathway, localized in the chloroplast, is predominant in most other plant organs, but it may also be involved (Evans et al., 1990).

This paper reports on the correlation between rate of tube growth and fatty acid composition of total lipids of pollen from wild type *Arabidopsis* and from the mutant lines

LK3, JB25, JB60 and *fad*2, exhibiting deficiencies in either the prokaryotic or the eukaryotic pathway. The *fad*2 line is deficient in the endoplasmatic reticulum 16:0/18:1-phosphatidylcholine desaturase wich is part of the eukaryotic pathway (Lemieux et al., 1990; Miquel and Browse, 1990; Somerville et al., 1991). From the other lines used, exhibiting mutations in the prokaryotic pathway of lipid biosynthesis, JB60 lacks *trans*-hexadecanoic acid (Browse et al., 1985), JB25 chloroplast glycerol-3-phosphate acyltransferase activity (Kunst et al., 1988), and LK3 is deficient in the chloroplast 16:1/18:1 desaturase (Browse et al., 1989).

MATERIALS AND METHODS

Plant material and growing conditions

The lines of *Arabidopsis thaliana* L. Heynh. described here originate from the Columbia wild type. The methods for production and selection of the mutant lines were described previously: the JB25 line was described by Kunst et al. (1988), the JB60 line by Browse et al. (1985), the LK3 line by Browse et al. (1989), and the *fad*2 line by Lemieux et al. (1990) and by Somerville et al. (1991). Seeds of wild type and mutants of *Arabidopsis* were a generous gift of Dr C. Somerville, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan. Seeds were imbibed at 4°C in water for 4 days in the dark to break dormancy and subsequently transferred to 25°C in the light to start germination. The seedlings were transferred to soil after 7-8 days storage at 25°C. Plants were grown in the greenhouse of the Department of Genetics of the Wageningen Agricultural University. Wild type and *fad*2 were also grown in a growth cabinet at 20.5°C, 70% relative humidity and 16 hrs of fluorescent light.

Pollen collecting and germination tests

Pollen was collected from freshly opened flowers with a capillary pipette, equiped with a fine gauze wich retained the pollen. The pipette was connected to a vacuum pump.

Visible amounts of pollen were collected and directly used for methylation. For germination tests, freshly opened flowers were picked and dipped onto the germination medium, wich consisted of 1.0 g H_3BO_4, 3.0 g $Ca(NO_3)_2.4H_2O$, 2.0 g $MgSO_4.7H_2O$, 1.0 g KNO_3, 13.7 g sucrose and 600 mg agar (Difco) in 100 ml 2 mM Na-phosphate-citrate buffer, pH 6.0.

Fatty acid analysis of pollen lipids

Fatty acid composition was determined as described earlier (Hoekstra and Van Roekel, 1988). Pollen lipids were methylated *in situ* without previous extraction. Pollen was dispersed in 3 mL 0.2 M KOH in methanol and methylated at 70°C for 15 minutes. The methylated fatty acids were washed with 1 mL saturated NaCl and extracted with 2 mL hexane. After drying over anhydrous Na_2SO_4, fatty acid methylesters were analyzed by gas chromatography and GC-MS. Gas chromatography was done as described elsewhere (Van Bilsen and Hoekstra, 1993). Identification was by comparing with standards (Sigma, St louis, MO) and verification on GC-MS. GC-MS spectra of the methylated fatty acids were recorded as described earlier (Van Bilsen and Hoekstra, 1993).

RESULTS

Fatty acid composition of pollen lipids

Table I shows that the *fad*2 line has pollen with a decreased amount of linolenic acid in the total lipid fraction and an increasd amount of oleic acid. On the contrary, the other mutant lines had a higher 18:3 fatty acid content than the wild type. In the *fad*2 line it is evident that the deficiency in the eukaryotic pathway, localized in the endoplasmatic reticulum (Lemieux et al., 1990; Miquel and Browse, 1990), is expressed in the pollen. This line is therefore suitable for studying the effect of a reduced linolenic acid content in the pollen on the rate of tube growth. The fact that the linolenic acid content in the pollen of the other lines that are deficient in the prokaryotic pathway is not reduced, suggests that in pollen lipid biosynthesis mainly desaturases from the eukaryotic pathway are involved (Evans et al., 1990).

Table I. *Fatty acid composition of pollen lipids from wild type and mutants of* Arabidopsis thaliana *L. Heynh., methylated in situ.*

The *fad*2 line is deficient in the 16:0/18:1 phosphatidylcholine desaturase, that is part of the eukaryotic pathway of lipid biosynthesis and is located in the endoplasmatic reticulum. The other mutant lines have alterations in the prokaryotic pathway, localized in the chloroplast.

	Fatty Acid Composition					
	16:0	16:1	18:0	18:1	18:2	18:3
	mol percent					
Wild type	26.5	tr	1.9	12.4	26.3	32.9
*fad*2	31.8	tr	6.0	29.8	15.7	16.8
LK3	37.0	tr	1.2	6.8	11.7	43.3
JB25	37.4	tr	1.3	7.3	14.8	39.2
JB60	27.0	tr	1.6	7.3	12.4	51.8

Pollen germination behaviour

The lag times between the immersion in the germination medium and the onset of tube elongation was approximately 90 min for all mutant and wild type pollen. With respect to the rate of tube elongation the *fad*2 line behaved differently from the wild type. Pollen tubes from the *fad*2 line were much shorter than those from the wild type (Fig. 1). Wild type tubes were smoother and thinner than the *fad*2 tubes, the latter seem to have been hampered during their growth. Especially in the area at the tip, the difference is striking, the wild type pollen tubes have a rather narrow tip, in comparison with that of the *fad*2 pollen (Fig. 1). This confirms that there is a direct correlation between pollen linolenic acid content and pollen tube growth (Hoekstra and Van Roekel, 1988). For the other mutant lines tube growth rates were similar to those of the wild type pollen.

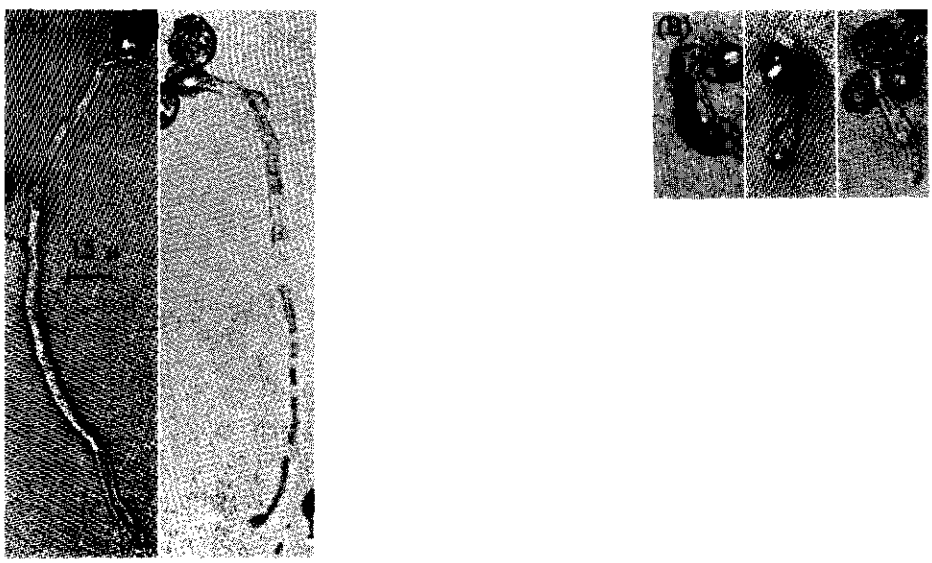

Figure 1. Pollen from wild type (A) and *fad*2 line (B) of *Arabidopsis thaliana* L. Heynh. Pollen was dipped onto the germination medium directly from the flowers.

Plant growth rate

At 20.5°C plant growth rate was also decreased in the *fad*2 mutant (Fig. 2). The *fad*2 line is chilling-sensitive (Lemieux et al., 1990; Miquel and Browse, 1990, Somerville et al., 1992). Under greenhouse conditions the other lines all had the same growth rate as the wild type (not shown).

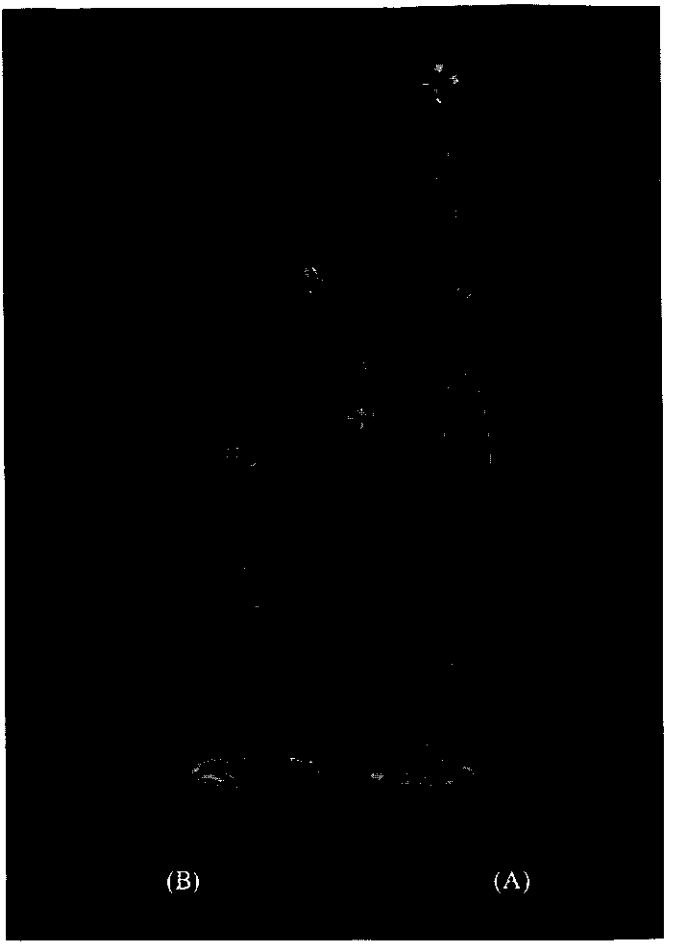

Figure 2. Wild type (A) and *fad*2 line (B) plants of *Arabidopsis thaliana* L. Heynh. Plants were grown at 70% relative humidity, 16 hours of fluorescent light/day and 20.5°C. After 35 days under these conditions the *fad*2 plants are smaller than the wild type.

DISCUSSION

Many aspects of pollen tube elongation have been studied, both *in vitro* and *in vivo* (Hoekstra and Bruinsma, 1975; Hoekstra, 1979; Picton and Steer, 1985; Çapková et al., 1987; Dorne et al., 1988; Steer and Steer, 1989). Previous reports indicated that linolenic acid might play an important role in the pollen germination proces (Hoekstra, 1986; Hoekstra et al., 1992a, 1992b). The present observation that the reduced linolenic acid content of the *fad*2 pollen coincides with a reduced rate of tube elongation, supports the hypothesis that the rate of fusion of the secretory vesicles with the plasma membrane of the vegetative cell determines the rate of pollen tube elongation. When germinating pollen is transferred to a medium that contains an inhibitor, a notable accumulation of secretory vesicles occurs at the tube tip (Picton and Steer, 1985). It is tempting to suggest that the bulky features of the *fad*2 pollen tubes are also caused by an accumulation of secretory vesicles at the tube tip.

The cytoskeleton is also involved in the extension of the pollen tube (Steer and Steer, 1989). Since the only difference between wild type and *fad*2 line is the activity of their endoplasmatic reticulum 16:0/18:1-PC desaturase, it is more likely that the observed differences in growth rate among wild type and *fad*2 pollen are caused by a different fluidity of the membranes involved than by differences in the cytoskeleton. On the other hand, the reason for the lack of difference in tube growth between wild type and the other mutants, that have an increased linolenic acid content, may be that the increased membrane fluidity is no longer limiting pollen tube growth.

The similar lag times of mutant and wild type pollen suggest that the linolenic acid content is not involved in the duration of the lag phase. It has been reported before that the metabolic state of the pollen plays a role in the germination proces (Hoekstra and Bruinsma, 1975; Hoekstra, 1979), the duration of the lag phase being determined by the extend to which pollen is metabolically developed at maturity. High metabolic state and high levels of linolenic acid could thus be independent modes towards rapid germination (Hoekstra, 1986).

In plant breeding studies, pollen plays an important role. The correlation between gametophytic (pollen) and sporophytic (seed) generations for polyunsaturated fatty acids in oilseed rape, *Brassica napus* L., supports the concept of selective gene transfer through pollen selection (Evans et al., 1988; 1990). Thus, pollen may be involved in the selection for lipid quality, with the restriction that the alterations in the lipid composition have to occur

in the eukaryotic pathway, which allows them to be expressed in the pollen. When the altered lipid composition is the result of a mutation in the prokaryotic pathway, such as observed in the LK3, JB25 and JB60 lines, the pollen of these plants are useless for pollen selection.

It is concluded that up to a certain limit the linolenic acid content of pollen lipids determines the quality and growth rate of the germinated pollen.

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7. General discussion

Pollen aging and loss of viability clearly correlates with changes in the membrane lipids during storage. In (semi-)dry pollen, stored at room temperature, phospholipids are deesterified *in situ* and thus the membrane composition changes. This deesterification is a random process, resulting in the accumulation of lysophospholipids and free fatty acids. As a consequence the membrane phase behaviour of aged pollen is different from that of non-aged pollen. In aged pollen, the overall membrane transition temperature has risen and phase separation has occurred. Rehydration is then accompanied by imbibition damage, endogenous solutes leak into the surrounding medium and viability is lost (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., in press; Van Bilsen et al., submitted).

Enzymes or free radicals

In pollen the main pathway for lipid biosynthesis is the eukaryotic pathway (Evans et al., 1990; McKersie et al., 1990; Van Bilsen and Hoekstra, in prep). Phospholipids generated through this pathway contain a 16-carbon atom fatty acid at the *sn*-1 position and an 18-carbon atom fatty acid at the *sn*-2 position or 18-carbon atom fatty acids at both positions (Browse et al., 1990). No change in phospholipid fatty acid composition is observed during aging. The fatty acid composition of the accumulating lysolipids and free fatty acids is similar to the phospholipid fatty acid composition of the non-aged pollen. This indicates that both the *sn*-1 and the *sn*-2 positions are deesterified at random (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., in press; Van Bilsen et al., submitted).

Pollen and seeds show similarities when aging is considered, but a major drawback when comparing the work on aged seeds is the variety of aging treatments, extraction procedures and analytical methods used. A slight difference in aging treatment or extraction procedures can already give large differences in the amount and type of degradation products that are detected (Nakayama et al., 1981; Priestley et al., 1985; Benson, 1990; Van Bilsen and Hoekstra 1993; Van Bilsen et al., submitted). This has made it very difficult to correlate the work on the role of free radicals in stored seeds. Moreover, different seed parts exhibit different sensitivities to free radicals (Benson 1990). The work by McKersie and Senaratna on isolated soybean seeds correlates well with the present work on pollen. They observed a similar type of lipid degradation under semi-dry conditions in soybean seed axes, wich they

correlated with free-radical activity rather than enzyme activity (McKersie et al., 1988; McKersie et al., 1990; Senaratna et al., 1988).

The hypothesis favouring a free radical mediated degradation is further supported by the suppressed respiration and the immobilisation of cellular phosphorus at low water content (Hoekstra and Bruinsma, 1975; Caffrey et al., 1987). On the one hand, enzyme activity is unlikely already from an energetic point of view (Dawson et al., 1985). On the other hand the same conditions that suppress enzyme activity also suppress free radical activity (Priestley et al., 1985; Benson, 1990). Moreover, one should also consider that an overall low water content does not exclude an inhomogeneous distribution of this water in the pollen. But even when the membranes are partially hydrated (Van Bilsen and Hoekstra, 1993), or high amounts of linolenic acid are present in the dry pollen (Van Bilsen et al., submitted), membrane packing is very tight compared with the fully hydrated state (Crowe et al., 1989; Hoekstra et al., 1992b). Such tightly packed bilayers are almost resistent to enzyme attack (Dawson et al., 1985). Thus, low metabolic activity, bilayer geometrics and the observed conservation of the initial fatty acid composition all support the concept of free-radical mediated degradation as proposed by Mckersie et al. (1988).

Membrane fluidity

Pollen aging does not result in a selective loss of polyunsaturated fatty acids, suggesting that the high vulnerabality to free radicals of linolenic acid itself is not the key factor determining pollen life span (Chan, 1987; Van Bilsen and Hoekstra 1993a; Van Bilsen et al., submitted). On comparison with linoleic acid the extra double bond in linolenic acid is closer to the hydrophobic core of the membrane, the distance between the ester bond and the first double bond is the same for both (Senaratna and McKersie, 1986). In the pollen species studied, the degradative activity is focused on the region of the ester bonds, so it seems that the partially dry state and the presence of tocopherol-like antioxidantia provide enough protection to ensure conservation of the degree of unsaturation during storage at low water content (Chan, 1987; McKersie et al., 1988; McKersie et al., 1990; Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted).

Apparently, the ester bonds are not sufficiently protected towards degradation during

storage (Van Bilsen and Hoekstra, 1993a; Van Bilsen et al., submitted; Van Bilsen et al., in press). Most likely the lone pairs of the oxygen atoms partaking in the ester bond act as pseudo double bonds and react *e.g.* with superoxide radicals. After initiation by reacting with a free-radical moiety the process might propagate as described for other radical-activated bonds, eventually leading to the cleavage of the ester bond (Chan, 1987). An analogous sequence has been proposed for the membrane degradation observed in aging seeds, a process which is, apart from the accumulation of free fatty acids, very similar to the aging of pollen membranes (Senaratna and McKersie, 1986).

Membrane fluidity is directly proprotional to the level of polyunsaturated fatty acids in the membrane lipids. A high level of polyunsaturated fatty acids gives a high membrane fluidity (Small, 1986; Hoekstra et al., 1992b). In the pollen this has no direct consequence for lipid peroxidation, but it evidently increases the speed of the deesterification process (Van Bilsen et al., submitted). Thus not an enhaced peroxidation of polyunsaturated fatty acids, but an increased memebrane fluidity accelerates pollen aging during storage.

Pollen storage

It has already been shown by Pfundt that pollen viability depends on such storage conditions as relative humidity and temperature (Pfundt, 1910). At room temperature storage at 40% relative humidity (which corresponds with ca 0.06 g H_2O g^{-1} dry weight) is most favourable to enhance longevity (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). At lower water content the possible formation of non-bilayer structures in the membranes, which severely complicate rehydration, has to be considered (Hoekstra et al., 1992a). Storage at 75% relative humidity (0.17 g H_2O g^{-1} dry weight) accelerates aging rapidly and thus shortens pollen life span (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted). Another factor influencing pollen life span is the linolenic acid content of the membrane lipids (Van Bilsen et al., submitted). Both elevated water content and high levels of linolenic acid affect the pollen membrane during storage. Both factors decrease the transition temperature (T_m) of the membrane phospholipids (Crowe et al., 1989; Hoekstra et al., 1992b). Since a decrease in T_m increases the susceptibility to lipid deesterification (Van Bilsen and Hoekstra, 1993; Van Bilsen et al., submitted), the T_m of the pollen membrane is thus an important factor.

Assuming that the above described free radical mediated degradation mechanism is the pathway for membrane degradation occurring in aging pollen, the key to the variety in life span has to be the accessability of the ester bond. Pollen membranes that contain mainly linoleic acid are in the dry, gel state more tightly packed than membranes that are in a liquid crystalline state, due to partial hydration or because their main fatty acid is linolenic acid (Crowe et al., 1989; Hoekstra et al., 1992b; Van Bilsen and Hoekstra 1993; Van Bilsen et al., submitted). A more losely packed glycerol region is easier reached by, for instance superoxide radicals, so it is more receptive to the initiation step. Once initiated, the slightly enhanced mobility of the phospholipids in dry or partially hydrated liquid crytalline bilayers will also fasten radical propagation towards other phospholipids. Thus the presence of linolenic acid has a twofold negative effect on longevity.

High levels of linolenic acid are hazardous for long term pollen survival, but there is also a positive contribution to viability: imbibition damage is less likely to occur in species with low membrane phase transition temperatures and tube elongation is faster (Hoekstra et al., 1992b; Van Bilsen et al., in press). When the pollen is transferred towards the stigma directly after dehiscence, high levels of linolenic acid are an advantage because they then enhance viability. There is only minor imbibition damage and, once fully rehydrated, tube elongation is very fast. This is advantageous to ensure the survival of the species (Hoekstra and Bruinsma, 1975; Hoekstra 1979; Van Bilsen et al., in prep). For long term storage, such as is sometimes required for plant breeding, pollen with high levels of linolenic acid in their phospholipids are at risk, because of the increased susceptibility towards lipid deesterification and the subsequent membrane phase separation which is responsible for large imbibition damage upon rehydration (Crowe et al., 1989; Knox and Singh, 1987; Mulcahy et al., 1986; Van Bilsen and Hoekstra 1993; Van Bilsen et al., in press). In some species, containing mainly linoleic acid, the reduced sensivity towards imbibition damage is achieved by augmentation of intracellular sucrose (Hoekstra et al., 1992b). With respect to long term storage this type of adaptation towards the avoidance of imbibition damage facilitates handling.

Homogeneous catalytic hydrogenation has been aplied to reduce the level of polyunsaturated fatty acids *in situ* aiming to simplify storage conditions, but unfortunately this was not successful (Van Bilsen and Hoekstra, in prep). Since a chemical modulation of the endogenous level of polyunsaturated fatty acids turned out to be not possible and genetic

modification has its drawbacks on pollen tube growth, other options to prolong life span have to be considered (Van Bilsen et al., in prep). Storage in organic solvent has been proposed as a means to protect pollen from degradation (Jain and Shivanna, 1988). Caution has to be taken when applying this, polar solvents will always extract membrane lipids, which in turn reduces membrane integrity and thereby decreases viability (Jain and Shivanna, 1988; Evans et al., 1990; Van Bilsen and Hoekstra, 1993; Van Bilsen et al., in press; Van Bilsen et al., submitted). If pollen is stored in organic solvents, apolar liquids such as hexane are preferable. Generally all conditions that prevent oxidative membrane damage are suitable to enhance longevity. One of the obvious means is dry storage at room temperature, providing the pollen is dehydration-tolerant. However, for species with high levels of linolenic acid this may not be sufficient and in those cases an additional lowering of storage temperature and/or the replacement of air by nitrogen is neccessary. Since all evidence presented in this thesis points towards a correlation between viability and the T_m of the membrane phospholipids, Fourier transform infrared spectroscopy is an important tool for designing storage conditions of various pollen species with respect to their longevity, for instance in plant breeding studies (Hoekstra et al., 1992b; Van Bilsen et al., in press). The method is non-destructive, has successfully been applied on various species and can be used not only as an aid in designing long term storage conditions but also for monitoring viability at designed intervals during storage (Crowe et al., 1989; Hoekstra et al., 1992b; Van Bilsen et al., in press).

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Summary

In this thesis membrane lipid composition is studied in relation to pollen viability during storage. Chapter 1 reviews pollen viability, membranes in the dry state and membrane changes associated with cellular aging. This chapter is followed by a study of age-related changes in phospholipid composition in *Typha latifolia* L. pollen.

Typha pollen was stored at room temperature, with internal moisture contents of respectively 0.06 and 0.15 g H_2O g^{-1} dry weight. In the course of the storage period, imbibition was accompanied by increased leakage of endogenous K^+ and germination declined. Simultaneously, phospholipids were deesterified *in situ*, leading to the accumulation of lysophospholipids and free fatty acids in the membranes. Viability declined much faster at the higher internal moisture content. An analysis of the fatty acid composition of the pollen phospholipids after aging indicated that the membrane degradation was not mediated by phospholipase A_2. When stored with 0.06 g H_2O g^{-1} dry weight, the level of polyunsaturated fatty acids remained constant, at the higher internal moisture content a slight decline in the level of linolenic acid content was found. The observed phospholipid degradation could be mediated by an unspecific lipid acyl hydrolase, but free-radical activity is more likely because of the low metabolic activity at the studied moisture levels. The pollen lipids were purified for liposome studies. These revealed that the lysophospholipids and the free fatty acids, accumulated during aging, enhanced the leakage of entrapped solutes from the liposomes (Chapter 2).

The phase behaviour of liposomal membranes containing lysophospholipids was studied after drying, to investigate the effect of these compounds in dried and in reimbibed phospholipid bilayers. Liposome studies, using Differential scanning calorimetry and Fourier transform infrared spectroscopy, showed that the lysophospholipids caused a dehydration dependent lateral phase separation. Membrane phase behaviour was also studied in *Typha* pollen and isolated pollen membranes. After aging the pollen membranes also exhibited lateral phase separation. The phase separation clearly coincided with the above described deesterification of membrane lipids *in situ* after aging, and caused an extremely fast leakage of endogenous K^+ upon imbibition (Chapter 3).

Two other pollen species were studied to assess whether the observed phospholipid deesterification is a general characteristic of pollen aging. In the course of storage with an internal moisture level 0.06 g H_2O g^{-1} dry weight, germination declined and the leakage of endogenous K^+ upon imbibition was increased in pollen from *Papaver rhoeas* L. and *Narcissus poeticus* L. At the same time phospholipid deesterification was observed in these species and lysophospholipids and free fatty acids accumulated. The degradation was again accelerated when the pollen was stored with an internal moisture content of 0.15 g H_2O g^{-1} dry weight. In pollen species with high levels of linolenic acid such as *Papaver* and *Narcissus* the deesterification occurred at a higher rate than in pollen species such as *Typha* in which linoleic acid is predominant. However, no selective loss of polyunsaturated fatty acids was observed. The degradation of the phospholipids in the pollen during dry storage was again most likely free radical-mediated (Chapter 4).

Although there was no large preferential degradation of linolenic acid, longevity was somehow correlated with the linolenic acid content. To study whether membrane fluidity in general determines longevity, attempts were undertaken to manipulate the level of linolenic acid *in situ* . Unfortunately catalytic hydrogenation, using Pd-alizarine as the catalyst, failed to result in saturation of the phospholipids in intact pollen, owing to an inhibition of the activity of the catalyst in viable pollen (Chapter 5).

Because catalytic hydrogenation was unsuccessful, an *in situ* modification of the level of linolenic acid during the germination process was not possible. So, to study the role of linolenic acid in relation to pollen germination, *Arabidopsis thaliana* L. Heynh. mutants exhibiting desaturase-deficiencies were used. Pollen tube growth rate was severely decreased in pollen from the mutant *fad2*, in which the activity of the 16:0/18:1 desaturase located in the endoplasmatic reticulum is suppressed. In pollen from this mutant line the level of polyunsaturated fatty acids was decreased, confirming the correlation between membrane fluidity and pollen tube growth rate. In the other mutants in which chloroplast located enzymes were affected, pollen tube growth rate was unaffected (Chapter 6).

The results from these studies show that with respect to aging and storage of pollen, the key factor in controling viability is the fluidity of the phospholipid bilayer. When the pollen membranes are in the high fluidity liquid crystalline phase during storage, aging is much more rapid than when the membranes are in the low fluidity gel phase. So for storage gel phase is preferable, but for rapid tube growth, on the contrary the liquid crystalline phase

is required. Thus for both proper storage and germination conditions, membrane fluidity has to be carefully monitored, for instance with the aid of Fourier transform infrared spectroscopy (Chapter 7).

Samenvatting

In dit proefschrift worden de veranderingen die optreden in de pollenmembraan tijdens bewaring beschreven. Stuifmeel veroudert tijdens bewaring. Dit blijkt o.a. uit de vitaliteitsdaling die optreedt wanneer pollen langere tijd wordt bewaard onder semi-droge condities. Deze vitaliteitsdaling gaat gepaard met een toegenomen lekkage van cellulaire componenten wanneer het pollen wordt herbevochtigd. Hieruit blijkt dat de celmembraan ten gevolge van het verouderingsproces zijn barrière-functie verliest.

Tijdens veroudering verandert de fosfolipidensamenstelling van de membranen van *Typha latifolia* L. (Grote Lisdodde) pollen. Er treedt een vetzuurafsplitsing op (de-esterificatie), waardoor in de membraan vrije vetzuren en lysofosfolipiden gevormd worden. Naarmate het pollen bij een hoger vochtgehalte bewaard wordt veroudert het sneller. Uit de analyse van de vetzuursamenstelling na veroudering blijkt dat de de-esterificatie niet het gevolg is van fosfolipase A_2 aktiviteit. Als het pollen met een intern vochtgehalte van 0,06 g water per g drooggewicht wordt bewaard, blijft het gehalte aan meervoudig onverzadigde vetzuren constant. Wordt het pollen bij een iets hoger vochtgehalte bewaard (0,15 g water per g drooggewicht), dan wordt een kleine afname in de hoeveelheid meervoudig onverzadigde vetzuren waargenomen. De waargenomen de-esterificatie kan veroorzaakt zijn door een aspecifieke lipolytische acylhydrolase, maar het is waarschijnlijker dat de afbraak optreedt onder invloed van vrije radicalen, omdat bij dergelijk lage interne vochtgehaltes de metabolische aktiviteit van het stuifmeel erg laag is. Als de nieuw gevormde componenten ingebouwd worden in modelmembranen (liposomen) die gemaakt zijn van lipiden uit vers pollen, vertonen deze liposomen een verhoogde lekkage, zoals liposomen van lipiden uit oud pollen. Vice versa geldt ook dat het weglaten van deze componenten uit liposomen van oud pollen de lekkage terugdringt. Hieruit blijkt dat de gevormde vrije vetzuren en lysofosfolipiden de doorlaatbaarheid van gehydrateerde liposomen verhogen. Dit geeft aan dat ze een rol spelen bij de eerder genoemde toename in de lekkage van cellulaire componenten (Hoofdstuk 2).

Omdat het pollen semi-droog bewaard wordt, is ook het gedrag van semi-droge (model)systemen bestudeerd. Na drogen vindt in aanwezigheid van lysofosfolipiden in

liposomen een opsplitsing (fasescheiding) plaats van de voorheen homogeen gemengde fosfolipiden. Een fasescheiding wordt na veroudering ook waargenomen in geïsoleerde celmembranen en in het pollen zelf. Dergelijke fasescheidingen zorgen voor interne breukvlakken in de membraan. Langs deze kieren kan de celinhoud bij herbevochtiging naar buiten lekken, hetgeen meestal gepaard gaat met de dood van de cel (Hoofdstuk 3).

Ook bij twee ander pollensoorten, nl. *Papaver rhoeas* L. (Klaproos) en *Narcissus poeticus* L. (Witte Narcis) gaan veroudering en de afbraak van fosfolipiden samen. Werderom verloopt de opeenhoping van vrije vetzuren en lysofosfolipiden sneller naarmate het pollen met een hoger intern vochtgehalte bewaard wordt. Beide soorten bevatten een aanzienlijk hogere hoeveelheid meervoudig onverzadigde vetzuren dan Lisdoddestuifmeel. Toch is ook bij deze twee soorten geen significante daling in de hoeveelheid meervoudig onverzadigde vetzuren waargenomen bij veroudering. Ook in Klaproos en in Witte Narcis is veroudering waarschijnlijk het gevolg van een door vrije radikalen veroorzaakte de-esterificatie van fosfolipiden (Hoofdstuk 4).

Hoewel de afbraak van meervoudig onverzadigde vetzuren dus geen rol speelt bij pollenveroudering, is toch de levensduur van pollen met een hoog gehalte aan meervoudig onverzadigde vetzuren korter. Omdat de membraanvloeibaarheid ook bepaald wordt door het gehalte aan meervoudig onverzadigde vetzuren, is geprobeerd dit gehalte via chemische weg te manipuleren om zo inzicht te verkrijgen in de rol van de membraanvloeibaarheid bij het verouderingsproces. Helaas was het niet mogelijk door middel van een chemische modificatie de vetzuursamenstelling in het pollen te veranderen: de hierbij benodigde katalysator werd in intact stuifmeel geremd (Hoofdstuk 5).

In pollensoorten met een hoog gehalte aan meervoudig onverzadigde vetzuren verloopt de pollenbuisgroei in het algemeen sneller dan in soorten met een lage hoeveelheid meervoudig onverzadigde vetzuren. Om inzicht te verkrijgen in de relatie tussen de membraanvloeibaarheid en de snelheid van pollenbuisgroei, werden *Arabidopsis thaliana* L. Heynh. (Zandraket) mutanten die een veranderde vetzuursamenstelling hebben, bestudeerd. In het stuifmeel van de zgn. *fad2* lijn is de hoeveelheid meervoudig onverzadigde vetzuren lager dan in het wild-type. Pollen van deze mutant vertoont een aanzienlijk vertraagde pollenbuisgroei, hetgeen bevestigt dat membraanvloeibaarheid en de snelheid van pollenbuisgroei eveneens gerelateerd zijn (hoofdstuk 6).

De in dit proefschrift beschreven experimenten en hun resultaten geven aan, dat de

membraansamenstelling een grote rol speelt bij de vitaliteit van stuifmeel. De vorming van de reeds genoemde afbraakprodukten in de membraan heeft een grote verandering in de fysiche toestand van de membraan tot gevolg en bepaalt hierdoor de vitaliteit van het pollen. Tevens is gebleken dat de membraanvloeibaarheid van cruciaal belang is voor zowel de levensduur van het pollen, als de snelheid van pollenbuisgroei. Een hoge membraanvloeibaarheid is sterk nadelig voor de levensduur van het pollen, maar daarentegen juist noodzakelijk voor een efficiënte bevruchting. Het is daarom noodzakelijk de membraanvloeibaarheid tijdens opslag en bevruchtingexperimenten nauwlettend in het oog te houden, bijvoorbeeld door middel van de in dit proefschrift beschreven Fourier-transform-infraroodmetingen.

Nawoord

Voor het schrijven van een proefschrift is meer nodig dan wetenschap alleen. Allen die mij, op welke wijze dan ook, in de afgelopen jaren hierbij hebben geholpen wil ik op deze plek van harte bedanken voor hun interesse en hun steun.

Curriculum vitae

De schrijfster van dit proefschrift werd op 26 juni 1963 in Maastricht geboren. Na het behalen van het gymnasium ß diploma in 1981, werd aan de Rijksuniversiteit Utrecht begonnen met de studie scheikunde. Het kandidaatsexamen (S2) werd in januari 1985 behaald, het doctoraalexamen met als hoofdfvak bio-organische chemie (vakgroep Organische Chemie) en als bijvak biochemie (vakgroep Biochemie, thans Biochemie van Lipiden), in mei 1987. Van januari 1987 tot oktober 1987 werkte zij bij de vakgroep Biochemie. In november 1987 werd als wetenschappelijk onderzoeksmedewerkster in dienst van BION (ZWO) bij de vakgroep Plantenfysiologie (Landbouwuniversiteit Wageningen) begonnen met het onderzoek dat tot dit proefschrift heeft geleid. Sinds december 1992 werkt zij in de sectie Bakery Products van het Unilever Research Laboratorium te Vlaardingen.

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