Isolation and characterization of *Spinacia oleracea* L. sperm cells

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Isolation and characterization of *Spinacia oleracea* L. sperm cells

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CONTENTS

Chapter 1: GENERAL INTRODUCTION

1.1 Research objective

In angiosperm plants, the male gametophyte (the pollen grain and tube) produces two sperm cells, which are transported to the female gametophyte (the embryo sac). One sperm cell will fuse with the egg cell, while the other will fuse with the central cell. Many aspects of the gamete transfer and fusion mechanism are still unclear. The gametes, both male and female, are specialized cells with the natural capacity to fuse in a well determined way. The fusion products are intended to regenerate into new individuals. There are indications that fusion of male and female gametes is not a random process. For some species preferential fertilization has been demonstrated. This means that one sperm cell is predetermined to fuse with the egg cell, while the other fuses with the central cell. Basic knowledge of gametes is of great importance for both traditional plant breeding, as well as for modern biotechnological methods and gene manipulation. To use gametes in biotechnology, it is essential that cell biology, developmental processes and cell characteristics are well understood. Furthermore, is it essential that gametes can be isolated in viable conditions.

In this project the characteristics of isolated sperm cells of *Spinacia oleracea* have been studied. A method is developed to isolate sperm cells in large quantities. The availability of isolated cells opens possibilities to investigate characteristics which can not be examined when the cells are still inside the pollen grain. Some characteristics of the isolated sperm cells are compared with those of the sperm cells in the natural environment inside the pollen grain. In chapter 2 the ultrastructural details of in-situ sperm cells are presented, as a reference for the ultrastructural analysis of the isolated sperm cells. In chapter 3, the method to isolate *Spinacia oleracea* sperm cells is presented, together with the analysis of the conditions under which the sperm cells stay viable after isolation. The analysis of mitochondrial numbers of isolated sperm cells is reported in chapter 4 and are discussed in relation to possible sperm cell dimorphism. In chapter 5, the ultrastructural analysis of the isolated sperm cell is presented with emphasis on the cytoplasmic composition and plasma membrane characteristics. All these results contribute to a better understanding of the characteristics of sperm cells and can open new possibilities for the study of the fertilization process in higher plants and open possibilities in applying these cells in biotechnology and plant breeding.

1.2 The male gamete

Approximately 70% of the angiosperm plant species have bicellular pollen grains, which consist of a large vegetative cell and a small generative cell. The remaining 30% of the plant species have tricellular pollen grains, composed of a vegetative

cell and two sperm cells. The two sperm cells are derived from the generative cell by a mitotic division (Knox 1984a). After its development and maturation, the pollen grain is transported to the stigma. Following this pollination, the vegetative cell germinates and produces a pollen tube. The stage from pollination to fusion of the gametes is termed the progamic phase. The pollen tube functions as a pathway for the sperm cells to reach the female gametophyte, where fusion takes place. One sperm cell fuses with the egg cell, which will result in the zygote. The other sperm cell fuses with the central cell, leading to the development of the endosperm. This process of double gamete fusion is called the double fertilization.

Pollen grain development

The formation of pollen grains is a two phase process. It starts with the microsporogenesis, leading to the formation of microspores. During the subsequent microgametogenesis, the microspores develop into pollen grains (Heslop-Harrison 1971, Shivanna and Johri 1985). The sporogenous tissue of the anther differentiates into pollen mother cells. These cells undergo meiosis, resulting in four microspores arranged in a tetrad. The four haploid microspores are enveloped by a thick callosic wall. This callosic wall is broken down and the four cells of one tetrad are released as four individual uni-nucleate microspores.

The microspores undergo an unequal mitotic division, resulting in a small generative cell and a large vegetative cell. The generative cell has a lens shape and is attached to the peripheral wall of the vegetative cell. During maturation the generative cell completely detaches from the peripheral wall and becomes completely surrounded by the vegetative cell. At this stage, the generative cell is spindle shaped in most species. The function of the spindle-shape is yet unknown.

The generative cell divides mitotically into two sperm cells. This second mitotic division can take place either in the pollen grain or in the pollen tube. The generative cell or the two sperm cells are enclosed by their own plasma membrane. Additionally, the plasma membrane of the vegetative cell surrounds the generative cell or the sperm cell pair. The space between these two membranes is very narrow and can contain wall components or other material. In this way the special situation of an intact cell (or pair of cells), completely surrounded by another cell, is established.

The vegetative cell has a thick peripheral wall, consisting of layers. The two major layers, termed the exine (made of sporopollenin) and the intine (made of polysaccharides) are well discernable. Each of the major layers can be composed of sublayers. The wall can have one or more apertures. In these apertures the exine is thin or not present and the intine can reach the outer surface. Also callose can be present at the apertural regions. One of the apertures functions as the starting point for the pollen tube to emerge. The exine can have cavities, filled with proteins and other components. These components may have a number of functions, of which an important one is the function in the recognition reaction between the pollen grain and the stigma.

When the mature pollen grain has arrived on a compatible pistil, the germination can start. First the pollen grain will take up water and swells. The vegetative cell is activated and starts to grow. It produces a pollen tube which penetrates the pistil. This can either be inter-cellular or through an open stylar canal. This pollen tube grows towards the female gametophyte and transports the sperm cells to the female gametes.

Vegetative cell cytoplasm

For many bicellular as well as tricellular species the ultrastructure of pollen grains is well described (Heslop-Harrison 1971, Knox 1984a, 1984b, Shivanna and Johri 1985, Stanley and Linskens 1974). The large vegetative cell contains numerous mitochondria, plastids, dictyosomes, endoplasmatic reticulum (ER), ribosomes and one nucleus. Frequently, numerous vesicles are present in the mature vegetative cell. The nucleus is often highly lobed and has many nuclear pores. The cell can contain large amounts of reserve substances, like starch or lipids (Baker and Baker 1983). These reserve substances are used during activation of the grain and subsequent pollen tube growth. Mitochondria generally are abundant and have many cristae. This is an indication for a high metabolic potential of the pollen grain. Dictyosomes can be highly active, producing different types of vesicles (Heslop-Harrison 1987). These vesicles contain polysaccharides and are a storage of wall precursors and plasma membrane components for the new to form pollen tube. The endoplasmatic reticulum is covered with ribosomes which can form new proteins. According to Mascarenhas et al. (1986) 15% of the genes expressed in the pollen are not expressed in the vegetative tissue, therefore, 15% is pollen specific. Some of these pollen specific proteins may be related to gamete recognition during the fusion process as already pointed out by Knox et al. (1988).

Sperm cells

In general, sperm cells are small. They contain a limited amount of cytoplasm. The major part of the cell is occupied by the condense nucleus. Organelles like mitochondria, ER, ribosomes, vacuoles, dictyosomes and vesicles are found in almost all species. Sometimes lipid droplets are present. Plastids are rare in sperm cells. In the majority of angiosperm species, there is a purely maternal inheritance of plastids (Hagemann 1983). In most species of the Gramineae, the sperm cells initially have plastids, but these are lost during pollen tube growth and fusion of the gametes (Hagemann and Schröder 1984). Corriveau and Coleman (1988) have shown that in only 14% of 235 species which they have investigated, biparental inheritance of plastids occurred.

With conventional electron microscopical techniques, based on chemical fixation with glutar-aldehyde, a narrow electron translucent space in between the plasma membrane of the sperm cells and the plasma membrane of the vegetative cell can be seen. This suggests that no cell wall mate-

rial is present. However, when freeze fixation and freeze-substitution is used, the presence of electron dense material is shown in between this narrow space (Cresti et al. 1987). By applying monoclonal antibodies, the presence of arabino-galactan glycoproteins (AGP) in this inter-membrane space is evidenced (Van Aelst and Van Went 1992). After their formation the two sperm cells remain connected to each other, forming a spindle shaped pair with tail-like extensions. These tail-like extensions are filled with cytoplasm and can be very thin and long. This is established by three-dimensional reconstruction using serial sections. In this way it is shown that in most species one or both tail-like extension are closely associated with the vegetative nucleus. The curling tail-like extensions are positioned in deep invaginations of the vegetative nucleus. Dumas et al. (1984) termed this complex of vegetative nucleus associated with the two sperm cells the male germ unit (MGU). Such a MGU is found in most tricellular species.

Using the technique of three dimensional reconstruction an other important aspect of sperm cells comes forward. In several species the two sperm cells of one pollen grain proved to be cytological different. The best example of this sperm cell dimorphism is *Plumbago zeylanica*. In this species, one sperm cell contains many mitochondria and few plastids while the other sperm cell contains many plastids and few mitochondria (Russell 1984). In other species the volume of the sperm cells is different. In *Brassica campestris* and in *B. oleracea*, McConchie et al. (1987b) report that the sperm cell associated with the vegetative nucleus is significantly bigger than the unattached sperm cell. They also report differences in cell surface, nuclear volume, cytoplasmic volume and mitochondria content between the two sperm cells of one pollen grain. This phenomenon of dimorphism of sperm cells is highly interesting in relation to the understanding of the fertilization process and the mechanisms of gamete recognition. Russell (1985) has been able to correlate sperm cell dimorphism to specific functioning of the sperm cells during the fertilization process. In *Plumbago zeylanica*, he reports that the sperm cell with the highest amount of plastids fuses with the egg cell to form the zygote, while the sperm cell with the highest amount of mitochondria fuses with the central cell to form the endosperm. This phenomenon is termed preferential fertilization. The theory of preferential fertilization is also sustained by genetical investigations (Roman 1948).

1.3 Isolated male gamete

One of the earliest attempts to isolate sperm cells from higher plants dates from 19 years ago (Cass 1973). In this work on *Hordeum vulgare*, the ultrastructure of the pollen grain and the sperm cells is presented. It is shown that the sperm cells become spherical in shape after release from the pollen grain. It lasted, however, until 1984 before possible biotechnological applications were clearly defined (Dumas et al. 1984, Wilms and Keijzer 1985, McConchie and Knox 1986). In 1986 a mass isolation of living male gametes was achieved in *Plumbago zeylanica* (Russell 1986). Since then, other laboratories per-

formed similar studies and reported mass isolations in a number of other species (summarized in Russell 1990, Roeckel et al. 1990, Russell et al. 1990, Theunis et al. 1991). Recently, the first electrofusions between isolated male and female gametes have been accomplished (Kranz et al. 1991a).

Methods of isolation

Two main principles are used to liberate generative cells or sperm cells from pollen grains or pollen tubes:

1. The osmotic bursting technique, with or without an enzyme pretreatment.
2. Grinding or squashing of the material.

Solutions of different osmotic values, ranging from pure water (Keijzer et al. 1986) to 20% sucrose (Russell 1986), have been used to induce bursting of pollen grains. In some species prehydration of the pollen is necessary to allow many pollen grains to burst (Nielsen and Olesen 1988a). Small grains, however, can resist the incubation, even in pure water. Enzymes used for pretreatment are mixtures of pectinase, cellulase and pectolyase for pollen grains (Zhou et al. 1990) and maceroenzyme R-10 and cellulase for pollen tubes (Shivanna et al. 1988). The great advantage of the osmotic bursting technique is the simultaneously breaking of a large proportion of grains or tubes, resulting in a high yield of generative cells or sperm cells. In *Plumbago zeylanica* a yield as high as 75% has been reported (Russell 1986). The method is therefore especially suitable for species producing small amounts of pollen. A negative aspect of this technique is the swelling and possible bursting of the isolated cells in the hypotonic incubation medium.

The second technique of grinding or squashing is done with tissue homogenizers of glass, electric blenders, or glass rollers. The grinding or squashing can be carried out in any chosen medium with an optimal osmotic value for the isolated cells. In this way, the risk of osmotic bursting of the isolated cells can be avoided. This method has been proven to give lower yields of isolated cells, because only a limited portion of the pollen grains is actually broken. The technique is difficult to standardize, because of the variable dimensions of tissue homogenizers and the forces used. In order to obtain an enriched fraction of isolated generative cells or sperm cells, the mixture of unbroken pollen grains, pollen grain walls, vegetative cytoplasm, vegetative nuclei and isolated cells is usually filtered with nylon filters and further purified by centrifugation on a sucrose gradient or a percoll gradient (Theunis et al. 1991). For structural and biochemical research, both fixation media as well as preserving media are used during isolation. For physiological studies a medium preserving the viability is a prerequisite. In many experiments the cell integrity is checked just after isolation and mostly viable cells are isolated. By using adequate storage conditions, the lifespan of the isolated cells can be expanded considerably (Russell 1991, Theunis et al. 1991).

Structural characteristics

The physical connection between the two sperm cells, which exists in most species, generally disappears after release from the pollen grain or pollen tube (e.g. *Plumbago zeylanica*, Russell 1986). Only in *Rhododendron* spp and *Gladiolus gandavensis* it has been reported that a portion of the sperm cells remains in pairs (Shivanna et al. 1988). In general, sperm cells change from spindle shaped inside the grain or tube to spherical after isolation. Only in *Gerbera jamesonii* Southworth and Knox (1989) found that the sperm cells remain in their original spindle-shape after isolation. An association between the sperm cells and the vegetative nucleus is seldomly retained after isolation.

Several different histological staining techniques have been used to check the presence of a wall or wall-like material around the isolated sperm cells. Matthys-Rochon et al. (1988) used Calcofluor White and a Periodic Acid Thiocarbo-hydrazide Silver Proteinate (PATAg) staining in *Zea mays*, but they did not find any wall-like material. Only Tanaka (1988) and Tanaka et al. (1989) showed the presence of a cell wall by using Aniline Blue and Calcofluor White around isolated generative cells of *Lilium longiflorum*, *Tulipa gesneriana* and *Trillium kamtschaticum*, but only in the first period after isolation. This layer disappeared after treatment with wall degrading enzymes.

The presence and constitution of a microtubular cytoskeleton has been studied in isolated cells by means of immunolabelling and fluorescence microscopy. It appears that the bundles of microtubules are lost after the cells have become spherical (Theunis et al. 1992), or that their orientation is altered in a mesh like pattern (Zee and Aziz-Un-Nisa 1991).

The ultrastructure of isolated sperm cells has been studied in several species using both transmission electron microscopy (TEM) as well as scanning electron microscopy (SEM) (Theunis et al. 1991). With TEM it is demonstrated that the sperm cells are spherical and that they are only surrounded by the sperm cell plasma membrane. The vegetative plasma membrane and the inter-membranous material, which envelope the sperm cells in the native state inside the pollen, are apparently lost during the isolation procedure. SEM studies have been used to show the surface of the outer membrane of the isolated cells and its conditions.

Biochemical characteristics

Protein analysis of isolated sperm cells is of recent times and only few reports are available. In *Plumbago zeylanica*, approximately 13.5% of the selected polypeptides were unique to the fraction containing the sperm cells and vegetative nuclei (Geltz and Russell 1988). Knox et al. (1988) found specific proteins in extracts of sperm cells of both *Gerbera jamesonii* and *Brassica campestris*.

In order to explore and localize proteins of isolated sperm cells, antibodies against sperm cells have been produced. Hough et al. (1986) obtained five specific antibodies against isolated sperm cell antigens of *Brassica campestris*, while Pennell et al. (1987) obtained antibodies against the sperm cell rich fraction of *Plumbago*

zeylanica.

The main problem for biochemical investigations is the availability of sufficient quantities of pure, clean gametic cells, or cell constituents.

Chapter 2: STUDIES OF THE MATURE POLLEN OF *SPINACIA OLERACEA* AFTER FREEZE SUBSTITUTION AND OBSERVED WITH CLSM

Abstract

The three-dimensional configuration of the nuclei of the trinucleate pollen grain of *Spinacia oleracea* L. has been examined with means of confocal laser scanning fluorescence microscopy (CLSM). It shows the presence of a male germ unit (MGU) in which all three nuclei are mostly positioned in the periphery of the pollen grain. After freeze fixation and freeze substitution, the ultrastructure is better preserved than with the standard chemical fixation. It shows the presence inside the pollen grain of mitochondria, dictyosomes, large starch containing plastids, endoplasmic reticulum (ER), vacuoles and the MGU. In the sperm cells also mitochondria, vesicles, dictyosomes and ER is found. No microtubules were found in the grain and only very few inside the sperm cells. This is in contrast with earlier published results where fluorescent labelled antibodies were used.

Key words

Spinacia oleracea, pollen, freeze substitution, sperm cells, male germ unit, MGU, ultrastructure.

Introduction

Pollen grains of *Spinacia oleracea* are trinucleate. At time of dehiscence the large vegetative cell contains two sperm cells (Wilms and Van Aelst 1983). The three nuclei can be stained with fluorochromes and observed with a confocal laser scanning fluorescence microscope (CLSM). In this way it is possible to observe optical sections. The spatial relation between the two sperm cell nuclei and the vegetative nucleus can be observed and a three-dimensional image of the mature pollen grain is obtained. With normal fluorescence microscopy this would be impossible.

In order to get information on the ultrastructure of the mature grain, it is necessary to fix the material. In general, pollen grains are difficult to fix with the standard chemical procedures. Most species have pollen grains with low water content (2 to 20%) except grasses which can have a water content of up to 60% (Stanley and Linskens 1974). This, in combination with the thickness of the pollen wall, is a barrier for fast penetration of the fixation chemicals. Another aspect of the *Spinacia oleracea* pollen grain which makes fixation difficult, is the high concentration of starch grains. As also found in other species, starch grains hinder the fixation process. Difficulties can be partially overcome by using freeze fixation and freeze substitution (Lancelle et al. 1987a) in which the first physical preservation (freezing) is followed by a very slow chemical fixation.

In order to observe the ultrastructure of *Spinacia oleracea* pollen grains, the freeze substitution technique was applied and the

results were compared with previous results obtained with classically fixed material (Wilms and Van Aelst 1983).

Material and Methods

Plants of *Spinacia oleracea* L. cv pre-vital, were grown in the greenhouse of the botanical garden of the University of Siena (Italy). Pollen grains were collected from freshly opened flowers and checked for viability with fluorescein diacetate (FDA) (Heslop-Harrison et al. 1984). Samples were used, only when 90% or more pollen grains were FDA positive.

For staining of nuclei, the mature pollen grains of *Spinacia oleracea* were fixed for 15 minutes in acetic-alcohol (acetic acid:ethanol = 1:3), washed twice in water and mounted in 1 μg/ml ethidium bromide or 5μg/ml propidium iodide. Observations were made with confocal laser fluorescence microscopy (BIORAD MRC500).

Pollen grains were prehydrated for 1 h in 100% relative humidity and were placed on formvar coated loops in a Brewbaker and Kwack medium (Brewbaker and Kwack 1963) containing 20% sucrose. After removing as much medium as possible, the material was frozen in liquid propane, using a propane plunger or high pressure freezing (Reichert HPM 010). After freezing, they were stored in a methanol solution containing 2% osmium tetroxide (OsO_4), at -80°C for 30 h. After slowly raising the temperature to 20°C in 17 h, the material was washed several times in fresh methanol without OsO_4 and infiltrated with Spurr's resin. Observations were made with a Zeiss EM 9A or a Jeol 100 B electron microscope.

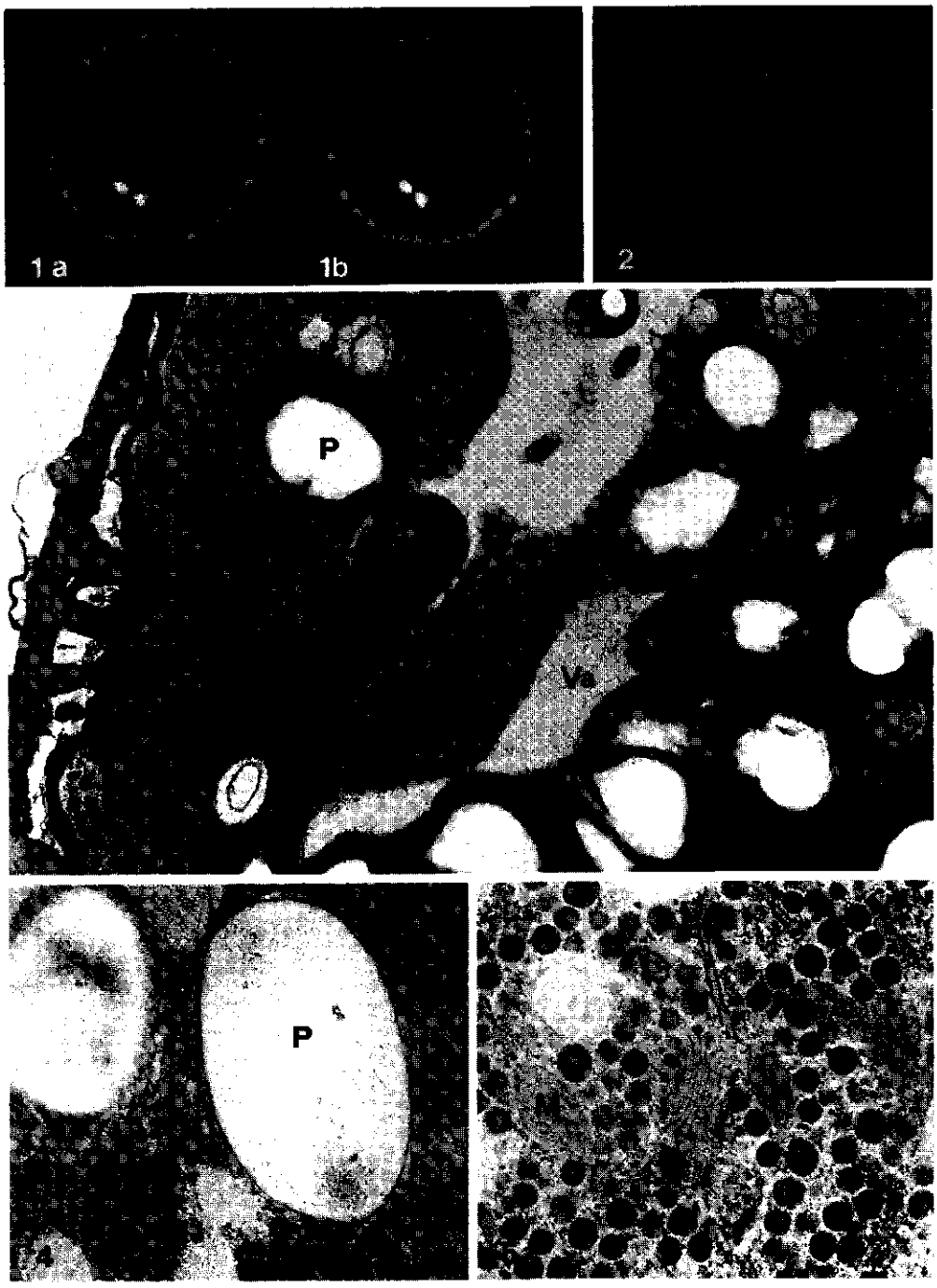
Fig. 1a-b. Stereo pair of mature pollen grain of *Spinacia oleracea* stained with 5 μg/ml propidium iodide and observed with CLSM. The two sperm cell nuclei are located at the periphery of the grain, in close vicinity of the vegetative nucleus, thus forming a male germ unit (MGU). 1,500 X

Fig. 2. CLSM image of mature pollen grain of *Spinacia oleracea* stained with 1 μg/ml ethidium bromide. As a result of the a-specific staining of the exine, the structure of the pollen grain wall becomes observable. The hexagonal distribution of the germ pores is clearly visible. 1,500 X

Fig. 3. Overview of the ultrastructure of the pollen grain. (I=intine, E=exine, P=plastid with starch, Va=vacuole, Vi=vesicle, M=mitochondria, arrow=micropore in exine layer). 18,500 X

Fig. 4. Detail of plastid full of starch. Only the outer membrane is visible. (P=plastid). 36,500 X

Fig. 5. Detail of a dictyosome and adjacent vesicles. (M=mitochondria, Vi=vesicle, ER=endoplasmic reticulum). 36,500 X


1 a
1b
2
P
Va
P

Results

The pollen grain

The CLSM images clearly show the trinucleate state of the pollen grain (Fig. 1). The two sperm nuclei and therefore, the two sperm cells are very close together. The vegetative nucleus is positioned close to this sperm cell pair. The sperm cells are usually located in the periphery of the pollen grain (Fig. 1). The vegetative nucleus is located centripetally to the sperm cells but hardly ever in the center of the grain.

The wall of the pollen grain consist of an intine layer and a tectate exine layer. Many small protruding exine spikes are visible (Figs 2, 3). Inside this exine layer many hollow areas (lumina) are present (Fig. 3). The exine layer shows many germination pores. Here, the less thick intine layer reaches the surface. These germination pores have a hexagonal distribution on the pollen grain surface (Fig. 2). Inside the lumina a substance is found, which is in contact with the outside by means of small channels inside the exine layer (micropores) (Fig. 3). This material does, however, not glue the pollen together. At time of dehiscence the pollen grains are individual and are very easily spread by the wind.

The most conspicuous characteristic of the cytoplasm is the large amount of large plastids, which are completely filled with starch (Figs 3, 4). They are present throughout the pollen grain but, are absent in the periphery of the grain (Fig. 3). Only the outer membrane of the plastid is visible (Fig. 4).

The dictyosomes consist of 4-6 cisternae, and appear to be active in producing vesicles (Fig. 5). Numerous vesicles are also present in the cytoplasm. The vesicles and also the cisterns of the dictyosomes are highly electron dense.

The mitochondria are oval to round. They

Fig. 6. Nucleus region of the sperm cell. Long tail-like extensions are wrapped around the cell. (SN=sperm cell nucleus, T=tail-like extension, P=plastid, Vi=vesicle). 36,000 X

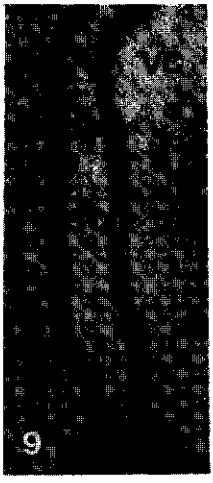
Fig. 7. Detail of circular structures (arrow) close to the pollen grain wall, composed of two membrane layers, with a granular space between them. (E=exine, I=intine, M=micropore). 36,000 X

Fig. 8. Two sperm cells connected to each other forming a pair. They are surrounded by the vegetative cell membrane. No vegetative membrane is visible between the two sperm cells. (SC=sperm cell). 25,000 X

Fig. 9. Detail of sperm cell plasma membrane and vegetative cell membrane, visible in a tail-like extension of the sperm cell. (SC=sperm cell, VC=vegetative cell). 101,000 X

Fig. 10. Detail of the tail-like extensions of the sperm cells penetrating the vegetative nucleus. (T=tail-like extension, VN=vegetative nucleus, SC=sperm cell). 36,000 X


SC



are rather small, with a length of approximately 0.5 μm and a width of approximately 0.3 μm (Figs 3, 5), and show a low contrast with the cytoplasm. The many cristae are barely visible.

The endoplasmic reticulum (ER) occurs throughout the pollen grain as short single elements or as a cistern. They are mostly linear, but can also have a bent shape or can even have a round configuration (Figs 3, 4, 5). Many ribosomes are attached to the ER (Figs 3, 4, 5). Around the sperm cell pair and the vegetative nucleus more layers of endoplasmic reticulum are found (Fig. 6, 10). Many free ribosomes are also present throughout the cytoplasm.

Despite a rather good preservation of the fine structures of the cytoplasm neither microtubules nor microfilaments could be observed in the pollen grain.

Frequently, peculiar circular to tube-like structures are observed in the periphery of the pollen grain (Fig. 7). They consist of two membrane layers, separated by a space containing granular electron dense material. Occasionally, this structure is fused with the plasma membrane of the pollen grain.

Some vacuoles with peculiar shapes seem to fill the spaces between the organelles. The size of these vacuoles varies considerable, as do the number of vacuoles within one pollen grain. The total volume of the vacuoles in one pollen grain can be substantial.

The vegetative nucleus is mostly homogeneous in structure and highly lobed.

The sperm cell

The mature pollen grain is divided in two sperm cells and a vegetative cell (Fig. 1). Each sperm cell is enclosed by a sperm cell plasma membrane. Together they are surrounded by a plasma membrane of the vegetative cell, thus connected and forming a pair of sperm cells (Fig. 8). The two plasma membranes are separated by a very small space (Fig. 9). No vegetative plasma membrane is seen at the point of bordering between the two sperm cells (Fig. 8).

The major part of the sperm cell consist of the nucleus, surrounded by a thin layer of cytoplasm (Fig. 6). The structure of the nucleus is heterochromatic. Along one side, the cell is flat and connected to the other sperm cell. At the other end, the sperm cell forms a long tail-like extension (Fig. 6). This tail-like extension can wrap around the sperm cell itself (Fig. 6) or can penetrate the vegetative nucleus (Fig. 10). Mitochondria, ribosomes, short cisterns of ER and vesicles can be found throughout the sperm cell, also inside the extension (Fig. 6). Although the freeze substitution technique was applied, only few microtubules and no microtubular bundles have been seen. Microfilaments were not observed.

Discussion

The advantages of CLSM in studying pollen grains are a higher resolution and the possibility of serial optical sectioning, allowing a three dimensional imaging (Brakenhoff et al. 1985). Its use has enabled us to observe the configuration of the

two sperm cells and the vegetative nucleus in numerous pollen grains of *Spinacia oleracea*. Together with the ultrastructural information we can conclude that the vegetative nucleus and the two sperm cells are organized in a male germ unit (MGU) (Dumas et al. 1984). Also Wilms and Van Aelst (1983) have reported a connection between the sperm cell pair and the vegetative nucleus. Only by using the CLSM the peripheral position of the MGU inside the pollen grain becomes clear. This peripheral position may be related to the pollen germination. A correlation could be possible between the position of the male germ unit and the place where the pollen tube is formed.

The ultrastructural studies reveal a tectate pollen grain, with a large amount of substance in the lumina of the exine layer. This substance can probably cause an allergenic reaction in some people (including one of the present authors) as proposed by Knox et al. (1970).

The cytoplasm of the mature pollen grain of *Spinacia oleracea* is similar as described by Wilms and Van Aelst (1983). A striking phenomenon is the large amount of starch, as is also described for other species (Stanley and Linskens 1974). The presence of this large amount of reserve substances could enable the pollen tube to grow autotrophic and facilitate a fast fertilization. This is in contrast with the findings of Mulcahy and Mulcahy (1983). They suggest that binucleate pollen shows a first growth step which is autotrophic nourished and a heterotrophic nourished second step. While trinucleate pollen grains are supposed to have only a heterotrophic step. An other possible function of the high starch content in *Spinacia oleracea* could be the stabilization of the osmotic pressure during the germination.

No microtubules are found in the vegetative cell of *Spinacia oleracea*. This is in agreement with the negative results from anti-tubulin labelling of sectioned mature pollen grains in this species (Theunis and Wilms 1988).

In the freeze substituted material small golgi vesicles are visible as independent round structures and seem to contain an electron dense substance. The vesicles have a possible function during the pollen tube growth. Standard chemical fixation, however, shows golgi vesicles as electron translucent structures, thus without any visible content (Wilms and Van Aelst 1983). After standard chemical fixation, these vesicles are often fused, to form branching structures of vesicles. Likely, this is artificial, due to inadequate fixation. By using freeze substitution, this fusion of vesicles is overcome. This clearly shows the advantages of the freeze substitution technique.

The cytoplasm of the sperm cells contains a normal set of organelles as also reported by Wilms and Van Aelst (1983) and Theunis (1990). Plastids are lacking and ER is sparse. Microtubules in the sperm cells are hardly present, although their presence has been shown by means of monoclonal antibody labelling, in the periphery of the sperm cell inside the pollen grain (Theunis and Wilms 1988). With standard chemical fixated material, ultra-

structurally, microtubules in the sperm cells have been shown in only a few species (Cresti et al. 1990, Dumas et al. 1985, McConchie et al. 1987a, 1987b, Yu et al. 1989, Zhu et al. 1980). The lack of microtubules in sperm cells, in ultrastructural observations, after standard chemical fixed material, could be due to breakdown of the microtubules during the rather slow chemical fixation. The lack of microtubules even after freeze substitution is more difficult to explain. In freeze substitution, a chemical fixation is preceded by physical fixation by freezing in liquid propane (Lancelle et al. 1987a) and takes place at very low temperatures. In this way the physical fixation is being replace by a chemical one and therefore, a better fixation can be achieved with also better fixation of the microtubules (Lancelle et al. 1987b, Cresti et al. 1987). The lack of microtubular bundles even after freeze substitution shows that the chemical fixation at low temperatures does only partially prevent the breakdown of the microtubules. Also in other reports, in which freeze substitution is used, probably not all the microtubules are fixed.

After conventional chemical fixation, the distance between the vegetative plasma membrane and the sperm cell plasma membrane is very irregular, giving the impression that numerous plasmodesmata connect the vegetative cell and the sperm cells. After freeze substitution, however, no such space is seen and it is hence likely to be an artifact of the classical fixation method. Also the presence of plasmodesmata is therefore unlikely.

The internal structures of the mitochondria are poorly stained and show a low contrast with the background. In classically fixed material this contrast is much higher and gives therefore more detailed information. The circular structures in the periphery of the pollen grain, containing granular material, can be very thin ends of the tail of the *Spinacia oleracea* sperm cells. Also in *Rhododendron* (Theunis et al. 1985) it was shown that cell wall like material is present in the end of the tail of the generative cell which is connected to the intine. A three dimensional reconstruction of such endings is needed to show whether they are part of the sperm cell and will require analysis of freeze substituted material.

In conclusion we can say that freeze substituted material gives a better ultrastructural image, partially because of the velocity of fixation. This overcomes changes of the ultrastructure during fixation. However, since sometimes low contrast is obtained, the use of classically fixed material can help to complete the studies.

Acknowledgment

The authors wish to thank dr E.S. Pierson for helpful discussion. This research was partially supported by The Commission of the European Communities with grant nr B/BIOT 900032 and B/BIOT 900032-001 in the BRIDGE project and partially by the National Research Council of Italy, special project RAISA sub project N. 2, paper N. 152

Chapter 3: ISOLATION OF SPERM CELLS FROM MATURE POLLEN GRAINS OF *SPINACIA OLERACEA*

Abstract

A technique has been developed to isolate sperm cells from mature pollen of *Spinacia oleracea*. By squashing the pollen grains in a 25% sucrose solution and subsequent centrifugation on a percoll layer it is possible to isolate the sperm cells in high numbers. All steps were carried out at 4°C. In this way the isolated sperm cells are kept alive for several hours. Several other storage conditions were examined.

Key words

Sperm cell, isolation, pollen, *Spinacia oleracea*.

Introduction

In contrast to animal gametes, plant gametes are not free-living cells. In plants, the female gamete is located inside the embryo sac, which is surrounded by nucellar cells and integuments. Male gametes are even more excluded from the outer world; the generative cell or the two sperm cells are situated inside a vegetative cell and completely enclosed by this cell. The generative cell divides in two sperm cells, either within the pollen grain or within the pollen tube.

Mature pollen grains of *Spinacia oleracea* are tricellular (Wilms and Van Aelst 1983). The sperm cells are connected to each other and have long tail-like extensions which are in close association with the vegetative nucleus. Each sperm cell is surrounded by a plasma membrane and together they are enclosed within the vegetative plasma membrane. In between the generative and vegetative plasma membranes there is a narrow electron transparent space. The sperm cells contain mitochondria, endoplasmatic reticulum, dictyosomes and vesicles (Wilms and Van Aelst 1983). A close association between the sperm cells and the vegetative nucleus, either in the pollen grain or later in the pollen tube, is observed in many other species. Dumas et al. (1984, 1985) named this association the male germ unit (MGU), indicating that it is a single transmitting unit during reproduction. In order to investigate the structural and functional characteristics of sperm cells in more detail, the sperm cells have to be isolated out of the pollen grains. With these isolated haploid cells, it will be possible to study several cell characteristics in more detail, including cell metabolism, which is not possible when the sperm cells are inside a pollen grain. It is also possible to study the effect of external influences on sperm cells, without the interference of and interaction with the vegetative cell. In this way, it is possible to manipulate sperm cells to a certain extend. In the recent past many efforts have been started to isolate sperm cells. Several species have been tried: *Beta*

(Nielsen and Olesen 1988b), *Brassica* (Matthys-Rochon et al. 1987, Hough et al. 1986), *Gerbera* (Southworth 1986), *Hordeum* (Jensen et al. 1986), *Plumbago* (Russell 1986), *Triticum* (Matthys-Rochon et al. 1987), *Zea* (Matthys-Rochon et al. 1987, Dupuis et al. 1987, Cass and Fabi 1988, Roeckel et al. 1988). Only in *Zea* and *Plumbago* the sperm cells are isolated in large quantities and appear to be vital after the isolation procedure.

In the future, when egg cells are isolated as well, it might become possible to achieve in vitro fertilization of plant gametes. This may overcome incompatibility and incongruency barriers and can open new perspectives to molecular biologists and plant breeders for new breeding methods in plants. Furthermore, it may provide a system to study the process of fertilization, which is very difficult to observe and manipulate in vivo.

Another application of isolated sperm cells lies within the field of protoplast fusion and culture. Since sperm cells have a "natural capacity" for cell fusion, it is very likely that isolated sperm cells could be used for this purpose in protoplast experiments, especially in species in which protoplast fusion and regeneration is difficult to achieve with "classical" techniques.

In this paper results are shown of isolation experiments of sperm cells of *Spinacia oleracea* and the behaviour in isolation medium and under different storage conditions.

Material and Methods

Plant material

Fresh pollen was collected from just opened flowers of *Spinacia oleracea L.*, cv Pre-vital, grown in the greenhouse. Viability as standard for pollen quality, was tested with fluorescein diacetate (FDA) (Heslop-Harrison et al. 1984). Only pollen samples containing more than 90% viable pollen were used for the isolation procedure.

Isolation procedure

For each experiment approximately 200-300 mg of fresh pollen was prehydrated in humid air (100% RH) for 1 h. Pollen was suspended in Brewbaker and Kwack medium (Brewbaker and Kwack 1963), containing 0.73 M sucrose (BKS 25) or another sucrose concentration if mentioned. In this way a homogeneous cell suspension was obtained. The suspended pollen grains were broken by squashing them with a glass roller on an abraded glass surface (Theunis et al. 1988). In this way the sperm cells were released.

Unbroken pollen grains and broken pollen grain walls prevent sperm cells to be broken as well. By filtration over a 25 μm filter, most unbroken pollen grains, wall fragments and cytoplasmic debris were excluded. The filtrate consisting of a mixture of cytoplasmic material and sperm cells was layered on top of a 20% percoll layer in BKS 25 and centrifuged for 40 minutes at 13000 g. The layer on top of the 20% percoll in BKS 25 was extracted. All steps were done at a temperature of 4°C.

Cytological analysis and measurements

Sperm cells and pollen grains were examined with a phase contrast Leitz dialux microscope.
Viability of pollen grains and of sperm cells was checked with FDA test (Heslop-Harrison et al. 1984).
Diameter measurements were carried out with a MOP 30 computer (Kontron GMBH) with magnetic digitizing tablet. Volumes were calculated with a MOP 30 computer on basis of the measured minimum and maximum diameters of each cell.

Histological staining

In order to check for cell wall material, the isolated sperm cells were stained for several cell wall components.
Calcofluor white MR2 to check for cellulose, Analine blue to check for callose and periodic acid Schiff's reaction (PAS) to check for carbohydrates, were applied according to the methods used by Heslop-Harrison (1979).

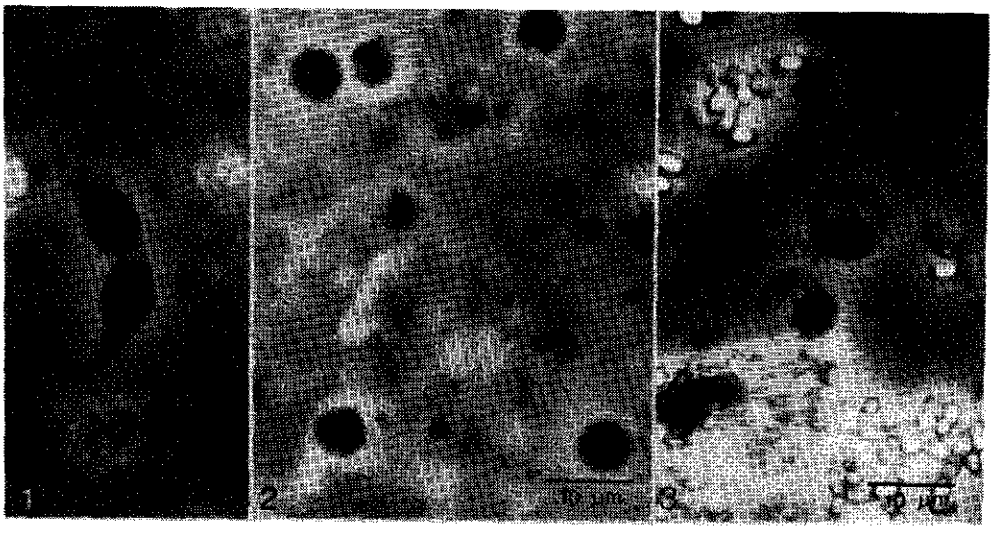


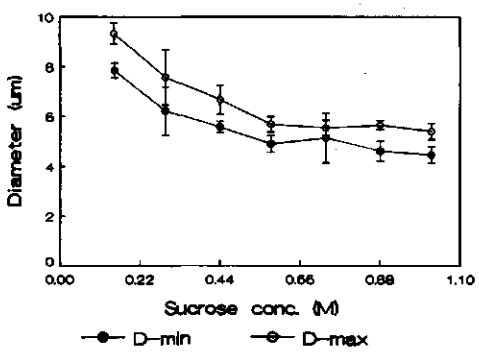
Fig. 1. Spindle shaped sperm cells still connected, after squashing in BK medium with 0.73 M sucrose (BKS 25).
Fig. 2. Sperm cells released after squashing in BK medium with 0.73 M sucrose (BKS 25). The sperm cells are spherical and no longer connected.
Fig. 3. Sperm cells released after squashing in BK medium with 0.1 M sucrose (BKS 5). The sperm cells are spherical, swollen and no longer connected. Sperm cell organelles are visible within a distinct sperm cell plasma membrane.

Results

The first step in isolating sperm cells from mature pollen grains is to break the pollen grain wall and to release the sperm cell into an isolation medium. *Spinacia oleracea* sperm cells can be released from their pollen grain by squashing the pollen grain. The pollen grain wall breaks and the vegetative cytoplasm including the two sperm cells, the vegetative nucleus and the numerous starch grains is pushed outside. Immediately after the gentle squashing, some of the released sperm cells are still connected and spindle shaped, with tail-like extensions (Fig. 1). Most of the sperm cells, however, become spherical and separated from each other after being released into the isolation medium (Fig. 2), but even in the spherical form some of the sperm cells still appear to be connected.

The shape of the sperm cells is not affected by the sucrose concentration of the isolation medium (Fig. 3). Measuring the quotient of the minimal and maximal diameters in different sucrose concentrations, gives similar numbers, indicating a constant oval to round shape of the isolated sperm cells (Fig. 4).

The volume of the isolated sperm cells, however, is highly influenced by the sucrose concentration in the isolation medium (Fig. 5). If the pollen grains are squashed in a sucrose concentration of approximately 1.0 M in the BK medium, the sperm cells are very small, approximately 5 μm and the volume is approximately 70 μm^3. They appear to be very condense when observed with phase contrast microscopy. Cell membranes are not distinguishable and no organelles can be seen. If the sucrose concentration is decreased, this situation is maintained until the sucrose concentration is as low as 0.6 M (Fig. 5). At that stage, the sperm cells start to swell. Decreasing the sucrose concentration even further, makes the sperm cells swell more. The sperm cell



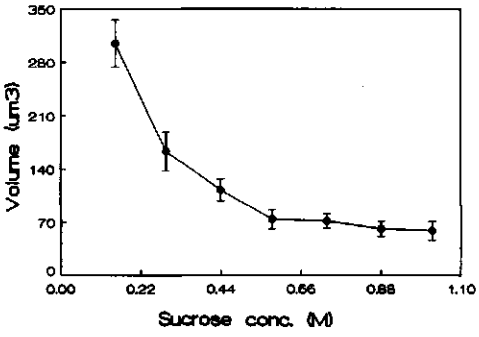


Fig. 4. Effect of different sucrose concentrations on minimal and maximal diameters of isolated sperm cells.

Fig. 5. Effect of different sucrose concentrations on volume of isolated sperm cells.

plasma membrane and the nucleus become distinct and organelles are clearly visible (Fig. 3). At the 0.1 M sucrose level, the sperm cell diameter is approximately 8 μm and the volume is increased up to approximately 300 μm^3. At this low sucrose concentration many free sperm cell nuclei are visible, indicating bursting of the sperm cells.

The BK medium supplemented with 25% sucrose was chosen as isolation medium in order to reduce the damage caused by squashing the pollen grain and possible damage of the sperm cells caused by swelling during the initial steps of the isolation procedure. In this medium the volume of the sperm cells is the smallest it can be and the surface of the plasma membrane is in its smallest configuration.

The second step in the isolation procedure is to separate the sperm cells in the isolation media from pollen grain remnants and subsequently from the rest of the cytoplasmic material. This is achieved by filtering

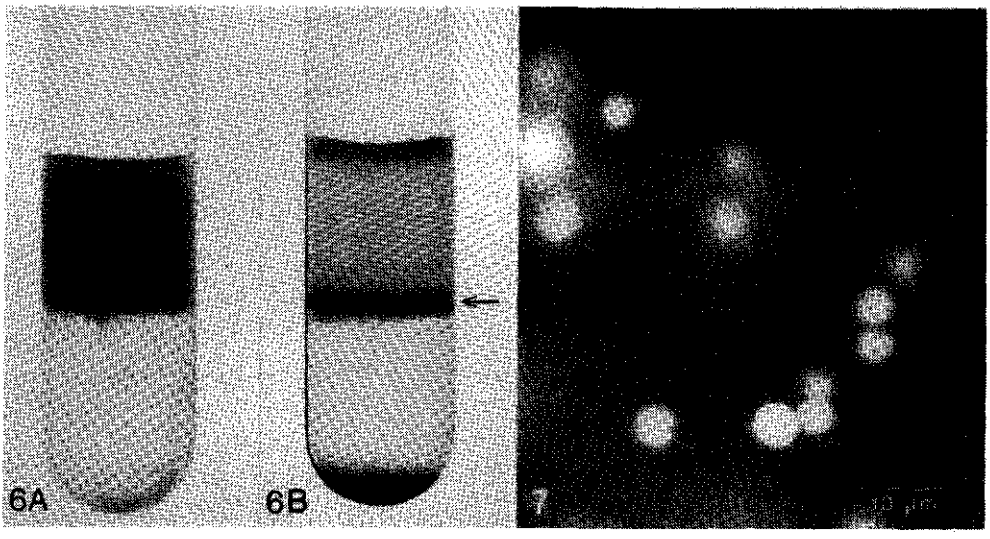


Fig. 6. Squashed pollen grains of *Spinacia oleracea* in BKS 25 on top of a 20% percoll layer in BKS 25.

A: Before centrifugation.

B: After centrifugation. Arrow indicates the sperm cell band on top of the percoll layer.

Fig. 7. Positive FDA staining of sperm cells after the isolation procedure (arrows).

the squashed material on a 25 μm nylon filter. In this way, unbroken pollen grains, pollen grain walls and cytoplasmic debris are excluded. After this filtration step, the material is centrifuged on a 20% percoll layer in BKS 25. This results in a separation of cell parts (Fig. 6). The large cytoplasmic organelles and the remaining small pollen grains and wall fragments are collected at the bottom of the tube. The sperm cells are gathered in a narrow band on top of the 20% percoll layer, together with small cytoplasmic organelles. The sperm cell band is extracted and used for further examination.

After the isolation procedure, the sperm cells are spherical and appear intact with an average diameter of 5 μm. With this method a yield of approximately 5% to 10% can be achieved which results in approximately 4 million sperm cells per millilitre.

Viability

Immediately after isolation, more than 90% of the isolated sperm cells is FDA positive, indicating the viability of the cells (Fig. 7). For various storage conditions the viability time was examined (viability time is defined as the storage time at which 50% of the sperm cells is FDA positive). If kept at room temperature after 10 h of storage, 50% of the isolated sperm cells is FDA positive (Fig. 8). Storage of the isolated sperm cells at 0°C increases this viability time with 10 h. Adding of 1% vitamin C to the storage medium increases the viability time with another 10 h, up to 30 h. The addition of 0.1 M ATP to the storage medium did not influence the viability time, neither at 0°C nor at 22°C storage temperature.

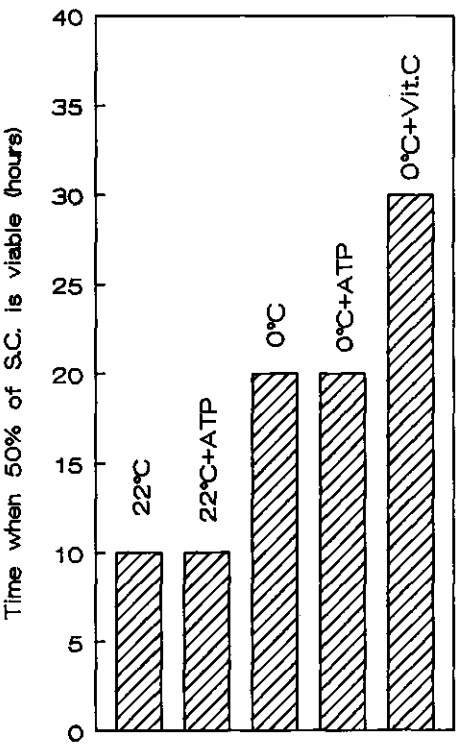


Fig. 8. Viability of isolated sperm cells of *Spinacia oleracea* under different storage conditions

Cytochemical staining

After isolation, the free sperm cells do not give any positive staining with Calcofluor white MR2 for cellulose, Analine blue for callose, or with the PAS reaction for carbohydrates.

Discussion

In mature *Spinacia oleracea* pollen grains, the gametes are completely surrounded by the vegetative cell. To isolate the gametes, the pollen grain has to be broken without damaging the gametes.

The osmotic shock method developed for *Plumbago* (Russell 1986) and *Zea* (Matthys-Rochon et al. 1987, Dupuis et al. 1987, Cass and Fabi 1988, Roeckel et al. 1988) did not work on *Spinacia oleracea*. Even in pure water only a small number of the pollen grains bursts. The pollen grain wall is possibly too strong. For this reason a physical way of breaking the pollen grain is to be preferred. A squashing technique was developed for breaking the pollen grains using a glass roller for pressing the pollen grains against a glass plate with an abraded surface. By using the squashing technique, the cytoplasmic content, including the two sperm cells, are pushed out of the pollen grain, leaving an empty or partially emptied pollen grain behind (Theunis et al. 1988). The breaking of the pollen grains is at random. Not all pollen grains, however, are broken. A high percentage remains intact. Large amounts of pollen grains are needed to isolate great numbers of sperm cells. The used method, however, is very reproducible and *Spinacia* pollen grains are very easy to collect.

The sperm cells in the pollen grain are spindle shaped and have long, tail-like extensions (Wilms and Van Aelst 1983, Wilms et al. 1986). The two sperm cells of a pollen grain are connected to each other and associated with the vegetative nucleus.

Squashing the pollen grain is the first step in the isolation procedure. Just after squashing, some of the sperm cells are still in pairs and are still spindle shaped, as found in the pollen grain. Most sperm cells, however, are single and have a spherical shape. The close association with the vegetative nucleus is lost during squashing of the pollen grains. This indicates that the close association between the sperm cells and the vegetative nucleus as reported by Wilms et al. (1986), is not based on a firm binding between the two.

The volume of the isolated sperm cells is strongly depending on the osmotic pressure of the isolation medium. This shows that the semi-permeability of the plasma membrane is maintained. This is another proof that the isolated sperm cells are viable. The diameter of the isolated sperm cells varies from 4 to 9 μm with different sucrose concentrations. Also the surface area and volume of the isolated sperm cells are influenced by different sucrose concentrations. In a low sucrose concentration (0.1 M), the volume of the sperm cell is approximately four and a half times as large as the volume of the sperm cell when it is in its smallest condition. The surface area is about twice and a half times as large. In spite of this increase in size the cells stay intact, indicating the flexible character of the plasma membrane. Not all sperm cells do resist the osmotic pressure of the 0.1 M sucrose medium, since also free sperm nuclei can be seen in the isolation medium. This is a strong indication that one should be careful in applying osmotic shock techniques to break pollen

grain walls (Cass and Fabi 1988, Russell 1986, Dupuis et al. 1987, Nielsen and Olesen 1988b). Also the released sperm cells can burst by this osmotic shock.

During the second step, the subsequent centrifugation on the 20% percoll layer, the spindle-shape disappears and the sperm cells become spherical. Also the connection between the two sperm cells disappears during the isolation procedure, only a few remain connected. Similar results are reported for *Zea* and *Brassica* (Dupuis et al. 1987, Matthys-Rochon et al. 1987). In these species the isolated sperm cells also become spherical and separate, soon after release.

In a previous communication we described the presence of microtubules inside the sperm cells of *Spinacia oleracea* (Theunis and Wilms 1988). The microtubular cytoskeleton is presumably involved in the shaping of the sperm cells. The change in shape of the sperm cell could be due to the breakdown of the cytoskeleton, as has also been suggested by Tanaka (1988). Future research has to support this idea.

The FDA test (Heslop-Harrison et al. 1984) is regarded a good test for establishing viability. In *Spinacia oleracea* most of the freshly isolated sperm cells are FDA positive, indicating their viability. A large percentage of the isolated cells looses its viability during storage. This loss of viability is probably caused by the loss of their natural surroundings. Furthermore, the absence of the vegetative plasma membrane implies, that the sperm cells become in direct contact with the degenerative cytoplasm of the vegetative cell and its enzymatic degrading activity. Storing of the sperm cells at 0°C instead of room temperature results in doubling of the sperm cell lifespan. Possibly, the lower temperature slows down the metabolism of the sperm cell, resulting in delayed exhausting of the cell. The lower temperature will also slow down the possible degrading activity in the medium. Vitamin C is known to act as anti-oxidant and therefor could have an inhibition effect on degradation in the storage medium. The results of addition of vitamin C to the medium indicates the latter.

A third possibility of the rapid decrease of viability, is the loss of an energy supply. The loss of the surrounding pollen grain cytoplasm might result in the loss of possible transfer systems between the sperm cell and the vegetative cell. Since the sperm cells lack plastids and storage substances, they must depend on the surrounding vegetative cell for their maintenance. The lifespan, however, is not prolonged when 0.1M ATP is added to the storage medium. This implies that loss of viability is not an energy depending problem.

The conditions to keep isolated sperm cells viable for a longer period are still to be improved. A possible way of keeping them viable, is making use of cryo-preservation as also suggested by Cass and Fabi (1988). The sperm cells are very small in diameter and will most probably survive the freezing without any damage caused by ice crystals (Van Aelst et al. 1989). Also the high sucrose concentration of the isolation medium could be an advantage in the cryo-preservation.

Many of the changes the sperm cell undergoes are, however, very similar to the changes the sperm cell undergoes in the synergid during fertilization (Mogensen

1982). They become spherical, separate, loose their vegetative plasma membrane and are surrounded by degenerating cytoplasm of the synergid.
After using staining techniques for cell wall compounds, no cell wall was found around isolated sperm cells. Therefore, they are "true protoplast" and can be used in experiments were protoplasts are needed. Especially the potential fusing capacity could play an important role in future fusion experiments. The fact that sperm cells have a haploid genome, could be of advantage in in vitro culture experiments (McConchie and Knox 1986). Other use of isolated sperm cells could lay in the fusion with isolated female gametes, in order to achieve an in vitro fertilization (Keijzer et al. 1988) and interspecific hybridization.

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Chapter 4: NUMBERS OF MITOCHONDRIA IN ISOLATED SPERM CELLS OF *SPINACIA OLERACEA* L.

Abstract

Sperm cells of *Spinacia oleracea* are isolated in large quantities of individual cells as well as in low quantities of sperm cell pairs. In order to establish the occurrence of sperm cell dimorphism with respect to mitochondrial content, mitochondrial numbers of both individual sperm cells and sperm cell pairs are analyzed. The mitochondrial numbers are counted by using UV fluorescence microscopy and specific labelling of the mitochondria with $DiOC_6(3)$. A variation of 2 to 25 mitochondria per sperm cell has been found, with and average of 12. Statistical analysis of the data obtained from individual sperm cells indicates the presence of two populations of sperm cells. One with an average number of 10 mitochondria per sperm cell and a second one with an average number of 17. However, the analysis of sperm cell pairs shows that within one pair there is no significant difference in the number of mitochondria of the two sperm cells. Therefore, we conclude that in *Spinacia oleracea* there is no sperm cell dimorphism with respect to the number of mitochondria.

Key words

Sperm cell, dimorphism, mitochondria, quantitative analysis, *Spinacia oleracea*

Introduction

In angiosperm plants the fusion of the egg cell with a sperm cell is accompanied by the fusion of the central cell with a second sperm cell, the so-called double fertilization. Both sperm cells are delivered in the embryo sac by the pollen tube and are formed by mitosis of the generative cell. For a number of species it has been reported that the two sperm cells within one pollen grain or tube are not identical (Russell 1984, McConchie et al. 1987a, 1987b, Wilms 1986). The most striking differences were found in *Plumbago zeylanica* (Russell 1984) were one sperm cell contains numerous mitochondria and only few plastids, whereas the second sperm cell has many plastids and only few mitochondria. In other species differences in shape, volume, surface area of nucleus, size of nucleus and numbers of mitochondria and plastids were found, but less striking than in *Plumbago zeylanica*. Such sperm cell dimorphism can be related to preferential fertilization. Some cytological and genetical evidences have been found to support the existence of preferential fertilization (Russell 1985, Roman 1948). In *Plumbago zeylanica* seventeen fusions between sperm cells and egg cells have been analyzed ultrastructurally (Russell 1985). In sixteen of these fusions the sperm cells with the low number of mitochondria fuses with the egg cell, while the sperm cell with the high number of mito-

chondria fused with the central cell.

For *Spinacia oleracea*, Wilms (1986) reported a difference in the number of mitochondria of the two sperm cells in the pollen grain. The conclusion was based on quantitative analysis of the sperm cell content, using serial sectioning and 3-dimensional reconstruction. This technique is very laborious and time consuming, so that only few cells were analyzed. Presently, techniques are available to isolate large numbers of living sperm cells (Theunis and Van Went 1989), as well as vital staining methods for mitochondria (Matzke and Matzke 1986, Wu 1987, Vannini et al. 1988). The use of these mitochondrial staining methods enabled us to analyze the mitochondrial numbers in a large quantity of isolated sperm cells and isolated sperm cell pairs. These numbers show whether a dimorphism in *Spinacia oleracea* exists between the sperm cells of one pollen grain. The results are reported here.

Material and Methods

Plants from *Spinacia oleracea* cv Pre-vital were grown in a greenhouse. For all experiments fresh pollen was collected and controlled with FDA (Heslop-Harrison et al. 1984) for viability. Only samples with more than 90% positive FDA fluorescence were used. Large numbers of individual sperm cells were isolated as described by Theunis and Van Went (1989). Sperm cell pairs were isolated by gentle squashing of the mature pollen grains under a cover slip, in the same isolation medium as mentioned in Theunis and Van Went (1989).

To investigate the mitochondrial numbers, the fluorochromatic dye 3,3' dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Sigma USA) was used in a concentration of 0.1 μg/ml isolation medium to label the mitochondria (Matzke and Matzke 1986). No wash step was required to remove the unbounded $DiOC_6(3)$. The sperm cells were examined with UV-fluorescence microscopy and confocal laser scanning fluorescent microscopy (CLSM).

In addition, experiments were carried out to examine mitochondrial numbers with UV fluorescence microscopy after staining with 10 mg/ml Rhodamine 123 (Sigma USA) (Vannini et al. 1988, Wu 1987).

Mitochondrial numbers of individual sperm cells were statistically analyzed using the method of maximum likelihood (MacLachan and Basford 1987). The calculated probability of the existence of one population of sperm cells were compared with the calculated probability of the existence of two populations of sperm cells.

Results

Labelling of mitochondria

The use of the fluorochromatic dye $DiOC_6(3)$ results in a good labelling of the mitochondria. A final concentration of 0.1 μg/ml gives the best results. With this low concentration of label, the mitochondria inside the sperm cell show a yellow-green fluorescence. The background fluorescence is very low and a rinse step is not required. With this dye it is possible to observe the individual mitochondria inside the sperm cell (Fig. 1). If higher concentrations are used, also the nuclear

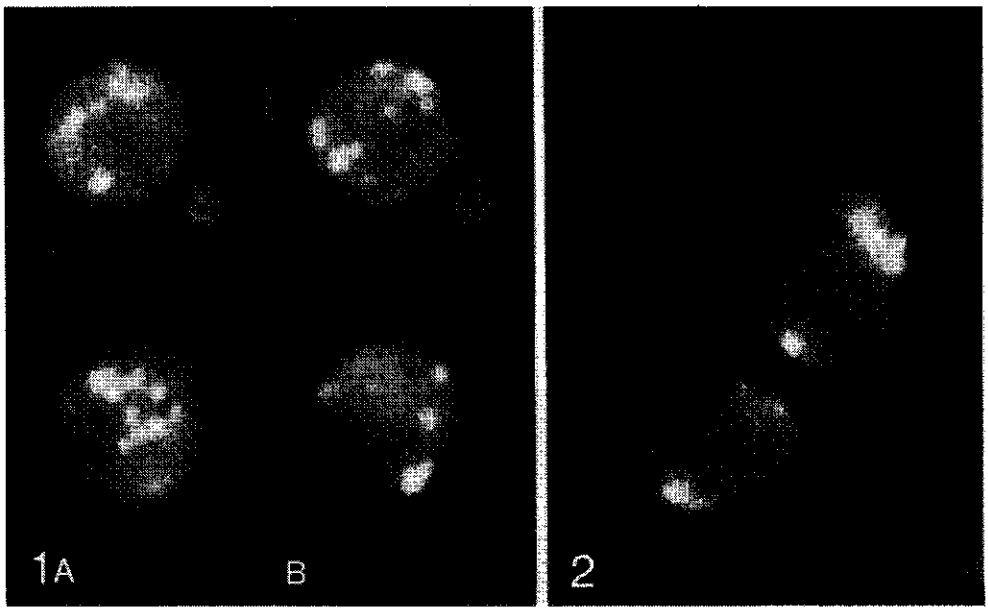


Fig. 1a-b. Isolated sperm cell of *Spinacia oleracea* stained with 0.1 μg/ml $DiOC_6(3)$. (a and be are photographs of the same cell at different focus levels).
Fig. 2. Isolated sperm cell pair of *Spinacia oleracea* stained with 0.1 μg/ml $DiOC_6(3)$.

envelope, the plasma membrane and the ER cisterns become fluorescing.
Apart from analysis with UV fluorescence microscopy, we attempted to use confocal laser scanning microscopy for optical 3-dimensional reconstruction. The movement of the cells and the slight cytoplasmic movement inhibited, however, the functioning of the optical scanning device.
Labelling of the mitochondria with Rhodamine 123 appeared to be unsatisfactory. At a concentration of 10 mg/ml Rhodamine 123 sufficiently labels the mitochondria, but the high background fluorescence requires a washing step during which many sperm cells are lost. Moreover, this rinsing step does not completely eliminates the background fluorescence. An a-specific staining of the cytoplasm remains. Lower concentrations of Rhodamine 123 are not advisable because of the low fluorescence of the mitochondria, even after 5 h of incubation.

Mitochondria in unpaired sperm cells

With the isolation method of Theunis and Van Went (1989) only loose sperm cells are obtained. During the isolation procedure the paired configuration of the

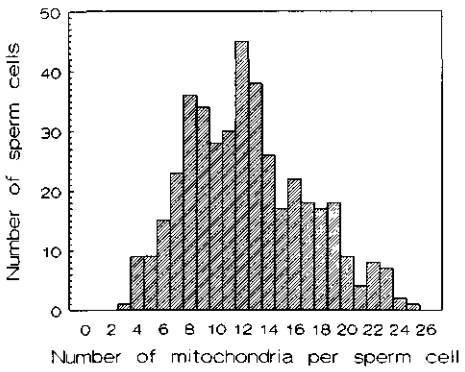


Fig. 3. Graph of frequency distribution of individual isolated sperm cells of *Spinacia oleracea* in relation to their number of mitochondria.

sperm cells is lost. The sperm cell mitochondria appear to be spherical and similar in size. They usually are randomly distributed, but also clusters of 3 to 10 mitochondria are found (Fig. 2). Sometimes two to four of such clusters are present. By passing carefully from one optical plane to the other it is possible to observe the individual mitochondria and the number of mitochondria per sperm cell can be counted. In some cases the clustering of mitochondria makes a good observation of the individual mitochondria difficult, but in general their number could be established accurately. In this way, 417 isolated sperm cells of *Spinacia oleracea* are analyzed (Fig. 3).

The number of mitochondria per sperm cell varies from 2 to 25 and the average number of mitochondria is 12.4 $\pm$ 4.6. Fig. 3 shows the number of sperm cells with a specific number of mitochondria. The data distribution suggests that there might be a mixture of two populations of sperm cells, each with a different average number of mitochondria. Computer simulation of maximum likelihood (MacLachan and Basford 1987) shows that the calculated probability of the existence of one population with a high variation is lower than the calculated probability of the existence of two populations. The existence of one population with a wide variation in the number of mitochondria can however, not be ruled out by this method. With this method, the most likely average mitochondrial numbers are 10.3 mitochondria for one group of sperm cells and 17.8 mitochondria for another group of sperm cells.

Mitochondria in pairs of sperm cells

If pollen grains of *Spinacia oleracea* are gently squashed, the sperm cells are released from the pollen grains in their original paired configuration. Most of the sperm cell pairs remain intact sufficiently long to enable the counting of the mitochondria in each of the paired sperm cells. In this way, the number of mitochondria in sperm cells of the same pair can be compared directly.

Using the $DiOC_6(3)$ staining as described before, 52 sperm cell pairs are analyzed. For each pair, the sperm cell with the lowest number of mitochondria is arbitrarily called SC1 and the other SC2 (Fig. 4). The dots in fig. 4 represent sperm cell pairs and their positions are determined by the number of mitochondria in the paired cells. It is evident that there is a great

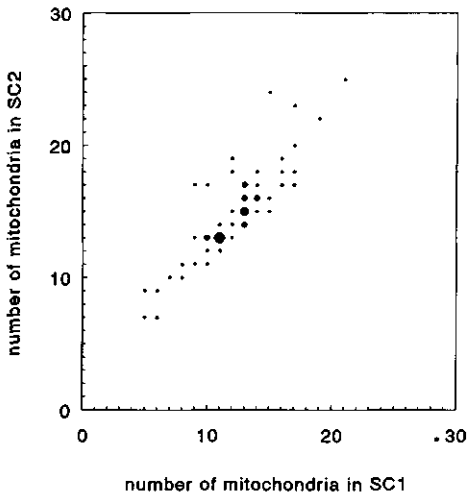


Fig. 4. Graph of number of mitochondria in both sperm cells of one pair. The sperm cell with the lowest number of mitochondria is termed SC1, the sperm cell with the highest number of mitochondria is termed SC2.

variation in the number of mitochondria per sperm cell. The number is varying from 5 to 25, but within sperm cell pairs the numbers of mitochondria are fairly equal. The greatest difference we found between the two sperm cells of one pair was nine mitochondria. Table 1 shows the average numbers of mitochondria of the SC1's, SC2's and their differences.

	SC1	SC2	total	Difference per pair
average	12.2	15.0	27.3	2.8
std	3.4	3.9	7.0	1.9

Table 1. Average number of sperm cells in the sperm cells of one pair.

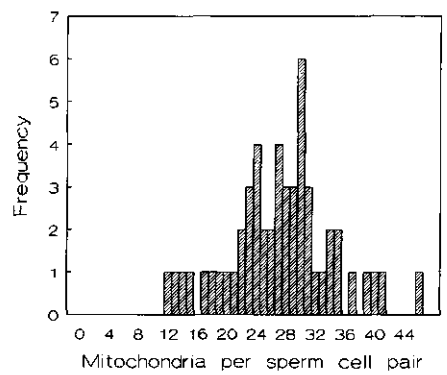


Fig. 5. Graph of frequency distribution of isolated sperm cell pairs of *Spinacia oleracea* in relation to the number of mitochondria of both sperm cells added together.

The average number of mitochondria in SC1 is 12.2 $\pm$ 3.4 and the average number of mitochondria in SC2 is 15.0 $\pm$ 3.9. The average difference found between the two sperm cells is 2.8 mitochondria. This difference, however, is not significant. Also with the method of maximum likelihood, these data do not support the idea of two populations of sperm cells.

Table 1 also shows the average total number of mitochondria per pair (27.3 $\pm$ 7.0). The actual number per pair varies from 12 to 46 mitochondria (Fig. 5). The frequency distribution of these number is comparable to that shown in Fig. 3 for mitochondrial numbers of unpaired sperm cells.

Discussion

The cationic fluorescent cyanine dye, $DiOC_6(3)$, is useful for studying the relative membrane potentials of plant mitochondria in living cells (Matzke and Matzke 1986). It can be used in low concentrations, of 0.1 μg/ml.

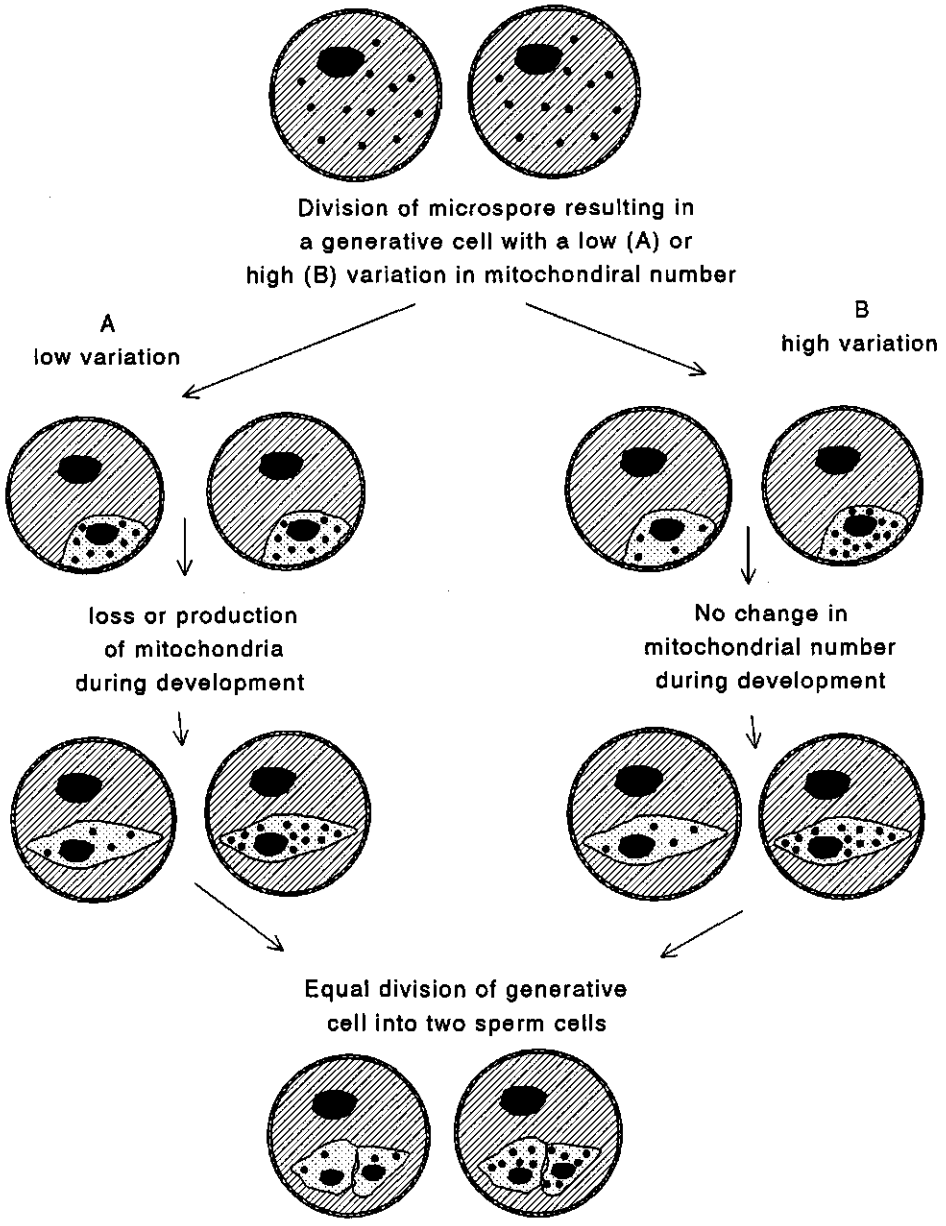
In our result the first visible fluorescence is located in the mitochondria. Ultrastructural analysis of isolated sperm cells of *Spinacia oleracea* (Theunis 1990) show that the mitochondria are spherical and located in the periphery of the cell. This is in accordance with the configuration we find after staining with $DiOC_6(3)$. If used in higher concentrations, also other membranes will be stained, like ER and the nuclear envelope (Terasaki et al. 1984). The right concentration has to be experimentally established and is different for every cell type. Together with the characteristics of $DiOC_6(3)$ and the findings reported by Matzke and Matzke (1986) we conclude that $DiOC_6(3)$ is a very specific stain for mitochondria in isolated sperm cells. The stain, however, functions only with viable cells (Matzke and Matzke 1986) and in our case is toxic to the isolated cells. After 30 min of incubation the labelling is less prominent and a higher background staining occurs. Therefore, the Rhodamine 123 fluorescent dye has been applied (Vannini et al. 1988, Wu et al. 1987). The aspecific staining of the background is, however, to high to analyze the number of mitochondria. Even after a rinsing step, which causes loss or damages of the small and fragile sperm cells, this background staining is to high.

The spherical mitochondria are clustered in 2 to 3 groups, but also individual mitochondria are found. The mitochondria appear to be similar in size and not branching. No such reticulate network of mitochondria is found, like was observed in sperm cells of *Zea mays* (McConchie et al. 1987a, Mogensen et al. 1990). After morphometric analysis of thin sections, Theunis (1990) found that the total volume of mitochondria inside the isolated sperm cell is 2.5% of the total volume of the cell. Together with the great variation we find in the number of mitochondria and their similar size, we conclude that a rather big variation exist in total mitochondrial volume per sperm cell. It is, however, still a low mitochondrial volume, one of the lowest found in plant cells. Higher plants can have 20% of the total cell volume occupied by mitochondria (Douce 1985). This means that the sperm cells are highly dependent on the vegetative cytoplasm in regard to their metabolic activity as was already mentioned by McConchie et al. (1987b).

In order to discuss the results in the light of dimorphism of sperm cells, first we have to establish what we mean by dimorphism. Sperm cell dimorphism exists when the two sperm cells of one pollen grain or tube are different in a certain morphological characteristic and furthermore, that this difference is statistically significant.

In previous work of Russell (1984) it is clear that in *Plumbago zeylanica* the sperm cells from one pollen grain are dimorphic, having respectively 39.81 and 256.18 mitochondria (22 cells counted). In *Brassica campestris* (McConchie et al. 1987b) one sperm cell has an average of 23.43 mitochondria (with an range of 10-34)

Fig. 6. Schematic drawing showing possible mechanism, leading to a high variation of number of mitochondria in sperm cells.



and the second sperm cell has an average of 6.43 mitochondria (with na range of 4-11) (5 pairs counted). In this example, the sperm cells are dimorphic, as also conclude the authors. Their result on *Brassica oleracea*, however, with an average of 13.2 mitochondria (with a variation of 11-15) for one sperm cell and of 9.8 mitochondria (with a range of 6-14) for the other sperm cell, can not be termed dimorphic (7 pairs counted). In *Spinacia oleracea* comparable numbers were found. Wilms (1986) reported an average of 20.3 with an variation of 9 to 22 for sperm cell one and an average of 24.7 with a variation of 12 to 42 for the other sperm cell (7 pairs counted). Such a difference may be an indication for dimorphism.

When sperm cells are isolated as described by Theunis and van Went (1989) a large number of individual cells can be analyzed. The use of isolated sperm cells enables the analysis of many hundreds of sperm cells which is a great advantage in comparison to the counting of mitochondria based on serial reconstruction of ultra-thin sections (Wilms 1986, McConchie et al. 1987a, 1987b, Russell 1984). If the numbers of mitochondria of isolated, individual sperm cells are counted, two populations of sperm cells seem to be mixed together. With the method of maximum likelihood in unravelling mixtures of populations (MacLachan and Basford 1987) the average numbers of mitochondria are 10.3 for one group of sperm cells and 17.9 for the other group of sperm cells. The analysis of pairs of sperm cells however give different result. The possible difference between the two sperm cells of one pair, should be much easier to detect with isolated sperm cell pairs than with isolated, individual sperm cells. No significant difference, however, can be shown. Therefore, it is more likely that also for the individual sperm cells not two populations exists, but that in *Spinacia oleracea* all sperm cells form just one population with a great range of variation in number of mitochondria. In *Spinacia oleracea* no dimorphism exists if considered the number of mitochondria. The variation between sperm cells (2 to 25 mitochondria) is much bigger than the variation between the sperm cells of one pair.

The variation between sperm cell is very high and ranges from 2 to 25. Also when the mitochondrial numbers of the sperm cell of one pair are added, a great variation exists from 12 to 46 mitochondria. To explain this high variation, two aspects have to be taken into account: 1 cell division, 2 the possibility of organelle extrusion/breakdown and multiplication. After microspore division, a small variation of the number of mitochondria in the generative cell can exist (Fig. 6 A), or a big variation can exists (Fig. 6 B). During the subsequent development of the generative cell, mitochondria can be extruded, as earlier described by Mogensen (1988), Mogensen and Rusche (1985) or be multiplied. This will give rise to a great variation. In fig. 6 this is schematically shown under A. If no such extrusion or multiplication exists (Fig. 6 B), the variation of the generative cell will be reflected in the sperm cells. In *Spinacia oleracea* the division of the generative cell must be equal since we find a clear correlation between the sperm cell of one pair. If extrusion takes place, the variation in the number of

mitochondria will only become bigger during sperm cell development in the pollen tube. Further research is necessary to clarify the existence of this high mitochondrial variation.

Acknowledgements

A.C. van Aelst is thanked for the technical help. A. Otten (mathematical department) is thanked for his help in the mathematical analysis of the data.

Chapter 5: FREEZE-FRACTURE STUDIES ON ISOLATED SPERM CELLS OF *SPINACIA OLERACEA* L.

Abstract

With freeze-fracturing sperm cells appear to be fractured preferentially through the plasma membranes. Only few fracture planes through the cytoplasm are found. Both the PF as well as the EF face of the sperm cell plasma membranes show a slightly undulating surface and contain intra-membrane particles. The particle distribution is irregular and does not show any clustering. The EF face of the plasma membrane contains approximately three times more particles per μm^2 than the PF face.

Key words

Spinacia oleracea, isolation, sperm cell membrane, intra-membrane particles, freeze-fracture

Introduction

Angiosperm plant sperm cells are specialised to fuse in the embryo sac with the egg cell and central cell. It is likely that specific surface recognition factors and a specific physico-chemical character related to the fusion capacity is present in the sperm cell membrane, or membrane areas. Such specific factors or characteristics could be expressed in the number, size and distribution of the intra-membrane particles (IMP's) of the sperm cell plasma membrane. The number, distribution and size of the IMP's can be established with freeze-fracturing. In pollen grains and pollen tubes the sperm cells are tightly enclosed within the plasma membrane of the vegetative cell (Cresti et al. 1987, Southworth et al. 1989a, 1989b, Van Aelst et al. 1989). Freeze-fracturing of pollen grains and pollen tubes preferentially results in fracture planes of the vegetative plasma membrane which surrounds the sperm cells (Southworth et al. 1989a, 1989b). For *Spinacia oleracea* L., a tricellular pollen species, a technique was developed to isolate sperm cells from pollen grains (Theunis et al. 1988). These isolated sperm cells are intact and alive. They have lost the surrounding vegetative plasma membrane and became spherical. (Theunis and Van Went 1989). In this paper we present the results of a freeze-fracture study of these isolated *Spinacia oleracea* sperm cells.

Materials and Methods

Sperm cells of *Spinacia oleracea* L. c.v. Pre-vital were isolated from mature pollen grains with the technique described by Theunis et al. (1988) and Theunis and Van Went (1989). Viability of the isolated sperm cells was tested with FDA (Heslop-Harrison et al. 1984). The sperm cells were isolated and freeze-fractured in the isolation medium containing 25% sucrose.

Small droplets of medium with sperm cells were mounted on specimen carriers and quickly frozen by plunging into liquid propane. Freeze-fracturing was carried out with a BAF 400 (Balzers). The replica's were cleaned with chromic acid and sodium hypochlorite and observed in a Philips 301. Density of the IMP's was measured with a Contron MOP-30 image analyzer (Zeiss). The calculations are based on 15 measurements per EF and PF face of 5 different cells. The freeze-fracture nomenclature follows Branton et al.(1975).

Results

The isolated living sperm cells are spherical in shape (Fig. 1). Only incidentally transverse fracture planes through whole sperm cells were found. In these transverse fractures the nuclear membrane with nuclear pores is present. Around the nucleus a relatively small layer of cytoplasm is visible in which some organelles are observable (Fig. 2). Most of the fracture planes of sperm cells follow the sperm cell plasma membrane, exposing either the PF or EF face (Figs 3, 4, 5, 6). No remnants of the vegetative cell plasma membrane were found. The freeze-fracture replica's show relatively large areas of the plasma membrane of the sperm cells representing either the EF (Fig. 3) or PF (Fig. 4) plane of the membrane. The plasma membranes are without perforations or ruptures. A considerable percentage of EF areas (Fig. 5) and PF areas (Fig. 6) shows a slightly undulating surface. The IMP distribution on both the EF (Fig. 5) and PF (Fig. 6) is irregularly. No pattern or clustering of the IMP's can be found on the EF or PF face. The EF face of the cell plasma membrane contains approximately three times more particles than the PF face. On the PF face the density of IMP's

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Fig. 1. Free sperm cells (arrowheads) and remnants of the vegetative cell of *Spinacia oleracea* L. in the isolation medium containing 25% sucrose. The sperm cells have lost their paired configuration and have become spherical. 700 X.

Figs 2-7. The arrow in each photograph indicates the direction of the shadow.

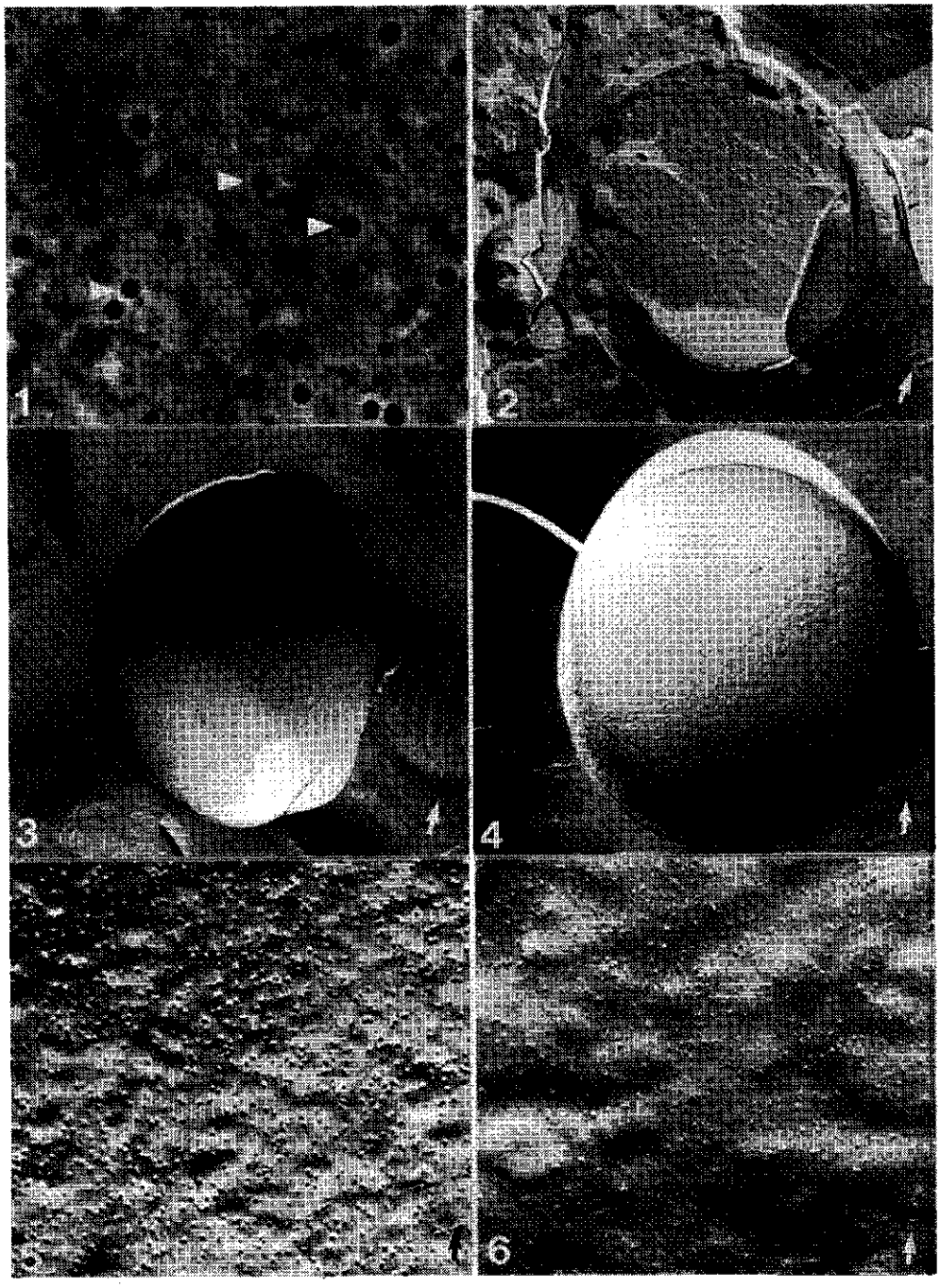
Fig. 2. Transverse fractured sperm cell of *Spinacia oleracea* L. In the centre of the cell the fractured nucleus is visible. Arrowheads point to the sperm cell plasma membrane. A few organelles are present in the cytoplasm. 14,500 X.

Fig. 3. The EF face of the *Spinacia oleracea* L. sperm cell plasma membrane 15,000 X.

Fig. 4. The PF face of the *Spinacia oleracea* L. sperm cell plasma membrane 19,500 X.

Fig. 5. Detail of the EF face of the *Spinacia oleracea* L. sperm cell plasma membrane. The undulating surface contains approximately 2088 IMP/μm^2. 120,000 X

Fig. 6. Detail of the PF face of the *Spinacia oleracea* L. sperm cell plasma membrane. The undulating surface contains approximately 719 IMP/μm^2. 120,000 X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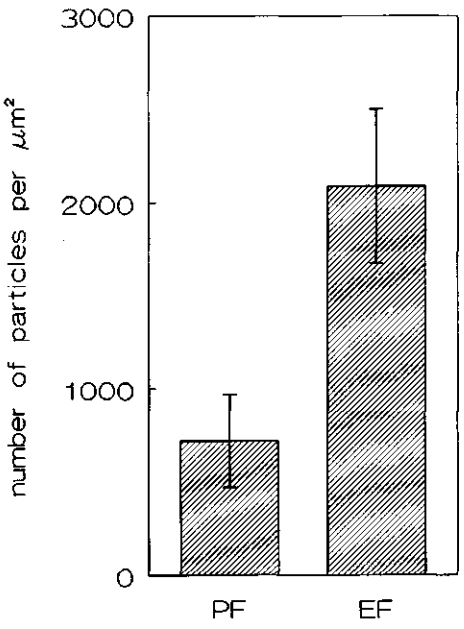

Fig. 7. Density of the IMP's in the plasma membrane (EF and PF face) of the isolated sperm cell of *Spinacia oleracea* L.

is 719 per μm^2 (± 250). On the EF face, the density of IMP's is 2088 per μm^2 (± 413) (Fig. 7).

Discussion

Freeze-fracturing of isolated sperm cells results predominantly in fracture planes of the sperm cell plasma membranes. This will be due to the small size of the sperm cells, the spherical shape and the absence of the vegetative plasma membrane which surrounds the sperm cell in-vivo (Theunis and Van Went 1989). In contrast, freeze-fracturing of sperm cells, still within the pollen grain or tube, results mainly in fracture planes of the surrounding vegetative plasma membrane (Southworth 1989a, 1989b). This clearly demonstrates the advantage of the use of isolated sperm cells for studies of the sperm cell plasma membrane.

The medium in which the sperm cells are isolated and freeze-fractured contains 25% sucrose. This percentage of sucrose keeps the sperm cells in their most condensed volume (Theunis and Van Went 1989). This relatively high percentage of sucrose also operates as an adequate cryo-protectant during the fast-freezing (Franks 1986) of the sperm cells. It is likely that in this shrunken condition an excessive amount of plasma membrane is present in relation to the cell volume which results in the undulated surface of the sperm cell plasma membrane.

Generally, in plant cell plasma membranes the PF face contains more IMP's than the EF face (Wilkinson and Northcote 1980, Platt-Aloia et al.1986, Kroh and Knuiman 1985, Kerhoas et al. 1987). This is in contrast with our results found in *Spinacia oleracea* sperm cells, where the EF face contains significantly more IMP's per μm^2 than the PF face. Furthermore, in somatic plant cells the IMP's on the PF face can be arranged in hexagonal arrays (Wilkinson and Northcote 1980, Kroh and Knuiman 1985), honeycomb pattern (Emons et al. 1988) or rosettes (Emons 1985, Schnepf 1985). It has been stated that a specific distribution can be induced by high sucrose concentrations (Emons et al. 1988) or can result from plasmolysis (Wilkinson and Northcote 1980, Emons 1985). Our results do not support these postulations. In spite of the high sucrose content of the medium

and the shrunken condition of the isolated sperm cells, no honeycomb or hexagonal pattern of IMP distribution could be found in the plasma membrane of the isolated sperm cells. Our results indicate that both the IMP number and distribution in the plasma membranes of isolated sperm cells clearly differ from plasma membranes of somatic plant protoplasts.

The IMP arrangement of the spinach sperm cells inside the pollen grain is not tested. No other freeze-fracture data about sperm cell plasma membranes are available. Only very few data about freeze-fractured generative cell plasma membranes are reported (Emons et al. 1988, Southworth 1989a, 1989b).

In order to relate this specific plasma membrane characteristic to the specialized fusion function of plant gametes, further freeze-fracture studies will be focused on plasma membrane differentiation during generative cell and sperm cell development.

Acknowledgement

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Chapter 6: ULTRASTRUCTURAL ANALYSIS OF SPERM CELLS OF *SPINACIA OLERACEA* ISOLATED FROM MATURE POLLEN GRAINS

Abstract

In isolated condition, the sperm cells of *Spinacia oleracea* are no longer arranged in pairs as in the pollen grain. The vegetative plasma membrane, which surrounds a sperm cell pair in a mature pollen grain, is lost during the isolation procedure. The sperm cells become spherical in shape.
The isolated sperm cell is surrounded by an intact plasma membrane. The heterochromatic or euchromatic sperm cell nucleus is located in the center of the cell. Mitochondria are round to oval and have distinct cristae. Often they are clustered in groups of 5 to 10 mitochondria. Dictyosomes are present in the cytoplasm and consist of 4 to 5 cisterns. Endoplasmatic reticulum is mostly situated at the periphery of the sperm cell, as single cisterns very near the plasma membrane.
From diameters of sectioned sperm cells in electron micrographs, it is possible to calculate the average diameter of the whole sperm cell (method according to Weibel 1979, 1980). This average diameter is 3.66 μm with a variation of 3.0 μm to 4.2 μm, resulting in an average volume of 25.6 μm^3. The nuclear volume is 12.8 μm^3 (50.0% of the whole cell) and the mitochondrial volume is 0.7 μm^3 (2.5% of the whole cell). The frequency distribution of the diameters of the isolated sperm cells shows only one peak with a normal distribution, indicating that there is no dimorphism in volume.

Key words

Spinacia oleracea, sperm cell ultrastructure, morphometric analysis

Introduction

In *Spinacia oleracea* the sperm cells are formed during pollen grain maturation (Wilms and Van Aelst 1983). The two sperm cells are linked to each other, forming a pair which is surrounded by a plasma membrane of the vegetative cell. The sperm cell pair in the mature pollen grain has a spindle shaped configuration. After pollination, the sperm cells are transported to the embryo sac through the pollen tube and discharged in the degenerating synergid (Wilms 1981). In the synergid the two sperm cells are separate from each other and they are spherical. Russell (1984) reported that in *Plumbago* the two sperm cells of one pollen tube are dimorphic, with one sperm cell having most of the plastids and the other sperm cell having most of the mitochondria. It is further shown that in *Plumbago* the sperm cell with most of the plastids usually fuses with the egg cell (Russell 1985), while the sperm cell with most of the mitochondria fuses with the central cell. This indicates a predetermination of the two sperm cells. In no other species examined, a clear dimorphism like in *Plumbago* is found. In *Spinacia oleracea*, however, there is an indication of dimorphism between the sperm

cells of one pollen grain in regard to the number of mitochondria per sperm cell (Wilms 1986).

In order to examine the sperm cell in more detail and to get knowledge of the fertilization process, many efforts have been undertaken to isolate sperm cells out of their natural surrounding pollen grains or pollen tubes (Hough et al. 1986, Russell 1986, Dupuis et al. 1987, Cass en Fabi 1988, Matthys-Rochon et al. 1988, Nielsen and Olesen 1988a, Shivanna et al. 1988, Southworth and Knox 1988a, 1989). Also for sperm cells of *Spinacia oleracea* an isolation technique is developed (Theunis and Van Went 1989). Sperm cells can be isolated in large quantities and are very suitable for membrane studies (Van Aelst et al. 1990) and quantitative ultrastructural analysis. In this study, isolated sperm cells of *Spinacia oleracea* are examined in large quantities and a morphometric analysis is carried out in order to get more information about sizes and volumes of sperm cells and to establish a possible dimorphism between sperm cells.

Material and Methods

Plant material and fixation

Plants of *Spinacia oleracea* L. cv Pre-vital, were grown in a greenhouse. Pollen were collected from just opened flowers and their viability was checked with fluorescein diacetate (FDA) (Heslop-Harrison et al. 1984). Pollen samples were used for isolation only if 90% or more pollen were FDA positive.

Sperm cells were isolated from these mature pollen grains with the technique described by Theunis and Van Went (1989). The viability of isolated sperm cells was checked with FDA. Only samples with 95% of the cells FDA positive were used for fixation.

Isolated sperm cells were fixed for 30 min in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) supplemented with 12% sucrose at 4°C. They were then rinsed twice in 0.1 M phosphate buffer containing 20% sucrose (pH 7.0) and post fixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.0) supplemented with 15% sucrose. After rinsing twice in 0.1 M phosphate buffer (pH 7.0) containing 20% sucrose, the fixed sperm cells were dehydrated in an ethanol epoxy propane series. Dehydration was performed by slowly dripping 100% ethanol in the suspension of fixed sperm cells. Ethanol was slowly replaced by epoxy propane. During the procedure the sperm cells were centrifuged, if necessary, at 4000 rpm. After embedding in Spurr's resin, the material was ultrathin sectioned with a LKB microtome and examined with a Philips 301 transmission electron microscope (TEM).

Morphometric analysis

Sperm cells were photographed at a magnification of 5500 times and printed with a final magnification of 20000 times. From these electron micrographs the section areas of 270 sperm cells were measured with use of computer aided morphometrics. These section areas were used to calculate the profile diameters. From these profile diameters it is possible to calculate the actual sperm cell diame-

ters, because of the spherical shape of the sperm cells. We used the by Weibel (Weibel 1979, 1980) adapted unfolding method of Cruz-Orive to determine the real frequency distribution of the sperm cell diameters. The average relative volumes of nuclei, mitochondria and dictyosomes, in relation to the cell volume was determined by use of the same computer morphometrics; the section areas of these organelles in the 270 sperm cells, were added and divided by the total section area of the same 270 sperm cells.

Results

Ultrastructure

After the isolation process the sperm cells have lost their paired configuration (Fig. 1) and are completely spherical. The vegetative plasma membrane which surrounds a sperm cell pair in the mature pollen grain in *Spinacia oleracea* is no longer present (Figs 2-6).

The sperm cells show an intact plasma membrane. Only occasionally sperm cells with small parts of their plasma membranes missing, are found. The sperm cell nucleus is located in the center of the cell and occupies a considerable portion of its volume. The structure of the nucleus can be euchromatic (Fig. 2) or heterochromatic (Fig. 3). Sometimes a nucleolus can be observed (Fig. 4). The nucleus usually is spherical to oval in shape (Fig. 3, 6), but sometimes irregular shapes can be observed (Fig. 5). If the nucleus is only grazed, nucleopores can be seen. Mitochondria are spherical to oval in shape and have distinct cristae (Fig. 3). Often they are clustered in groups of 5 to 10 mitochondria and more than one of these clusters can be present in the sperm cell cytoplasm. Dictyosomes are also present and consist of 4 to 5 cisterns (Fig. 6). Some small vesicles can be seen in association with these dictyosomes. Only small vacuoles are observed in the sperm cells. Furthermore, the cytoplasm contains many ribosomes and ribosomes organized in polysomes (Fig. 2). Endoplasmatic reticulum is present. It is usually situated at the periphery of the sperm cell, as single cisterns very near the plasma membrane of the sperm cell (Fig. 6) and is often dilated. Only rarely ribosomes are seen to be associated with the ER. The isolated sperm cells did not show a microtubular cytoskeleton. Occasionally, some sperm cells are found with small lipid droplets attached to the outside of the plasma membrane (Fig. 5). Since remnants of the cytoplasm of the vegetative cell can not be completely excluded in the isolation procedure, numerous vesicles, membranous structures, ribosomes and other cytoplasmic structures are observed in the surrounding of the isolated sperm cell (Fig. 1).

Morphometric analysis

The frequency distribution of the diameters of the 270 sperm cell profiles in the sections is shown in Fig. 7A. The average profile diameter is of course smaller than the average diameter of the intact cell,

because not every section is through the exact middle of the cell. The diameter of the profiles varies between 0 μm to 4.5 μm, with an average of 3.27 μm (Fig. 7A). From these profile diameters, it is calculated that the average diameter of an intact sperm cell is 3.66 μm with a normally distributed variation between 3.0 μm to 4.2 μm (method according to Weibel 1979, 1980) (Fig. 7B).

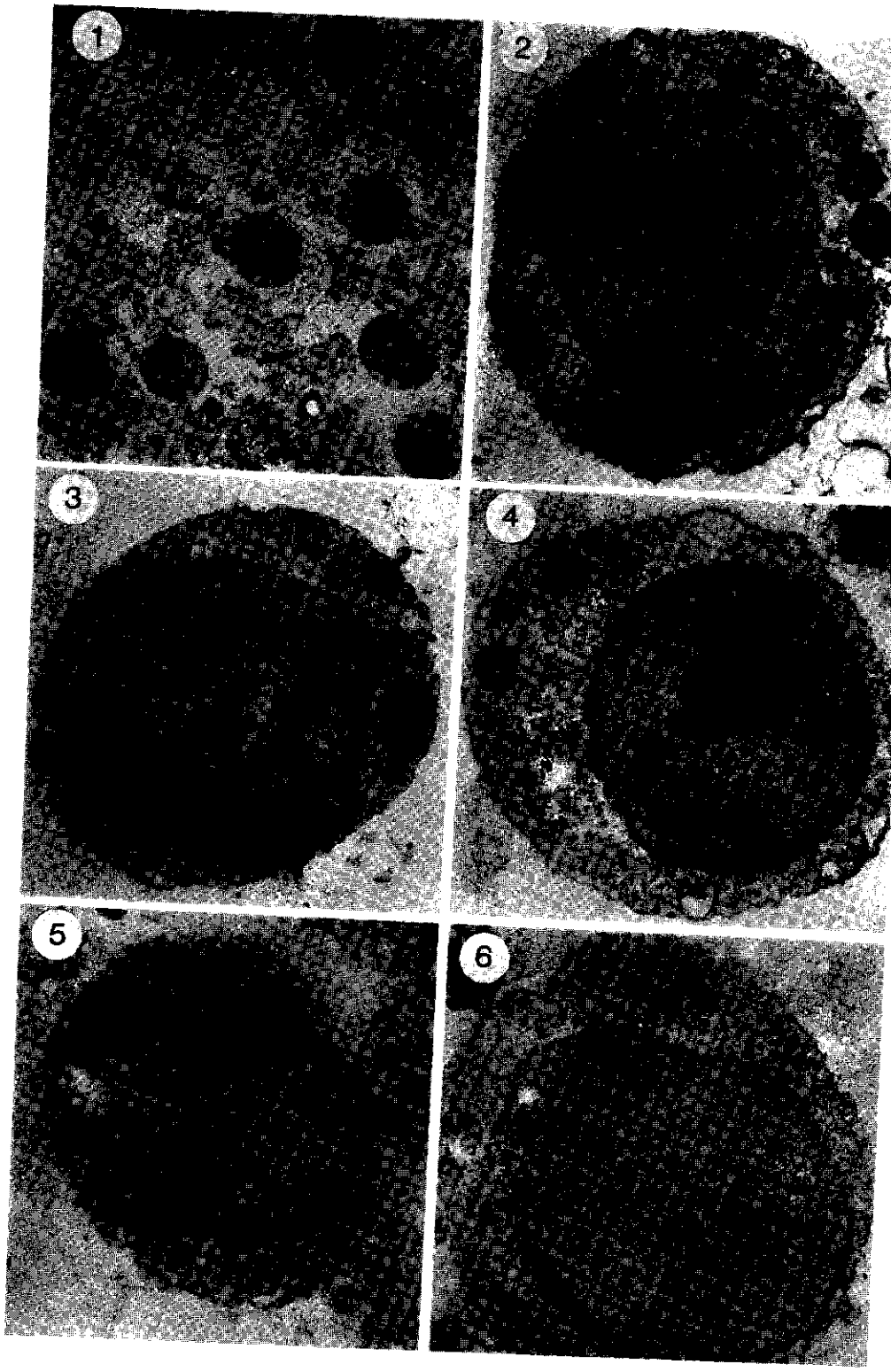
For measurements of the various organelles a different technique is applied. The section areas of the nuclei takes 50.0 % of the section areas of the cells. Because sections are taken at random, it is possible to extrapolate this area percentage to volume percentage. Since the average cell volume is 25.6 μm^3, the average nuclear volume will be 12.8 μm^3. If one considers the nucleus as a perfect sphere, the average nuclear diameter is calculated to be 2.9 μm^3. In the same way, mitochondria are found to occupy 2.5% of the volume of the sperm cells, with an average mitochondrial volume per cell of 0.7 μm^3. The section areas of the dictyosomes take 0.6% in relation to the section area of the complete cell, which results in an average dictyosomes volume of 0.2 μm^3 per cell.

	Volume (% of whole cell)	Average volume (μm^3)
cell	100%	25.6
nucleus	50.0%	12.8
mitochondria	2.5%	0.7
dictyosomes	0.6%	0.2

Table 1. Average relative volume and average absolute volume of the isolated sperm cells of *Spinacia oleracea* and its nucleus, total mitochondria and total dictyosomes.

Figs 1-6. Isolated sperm cells of *Spinacia oleracea* which have lost their paired configuration, the surrounding vegetative plasma membrane and their tail-like structures.

Fig. 1. Isolated sperm cells surrounded by remnants of the vegetative cytoplasm (6,000 X).
Fig. 2. Isolated sperm cell with mitochondria with distinct cristae and an euchromatic nucleus in the center of the cell (18,000 X).
Fig. 3. Isolated sperm cell with a group of clear mitochondria and a heterochromatic nucleus (18,000 X).
Fig. 4. Isolated sperm cell with a round nucleus containing a clear nucleolus (18,000 X).
Fig. 5. Isolated sperm cell with an irregular c-shaped euchromatic nucleus (18,000 X).
Fig. 6. Isolated sperm cell with clear dictyosomes and endoplasmatic reticulum near the plasma membrane (18,000 X).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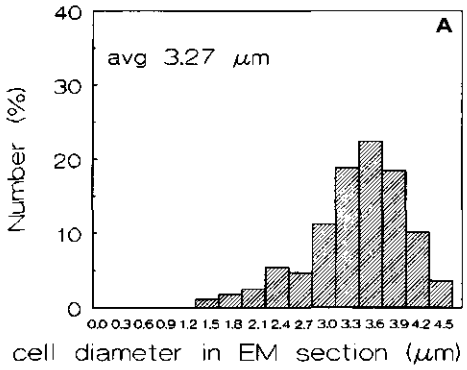

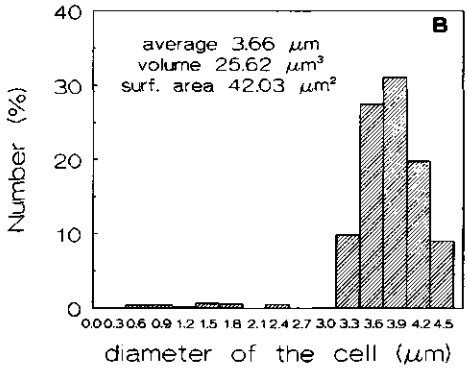

Fig. 7A. Frequency distribution of the profile diameters of the isolated sperm cells of *Spinacia oleracea* in the electron micrographs, with an average diameter of 3.27 μm. At random, 270 sperm cells have been measured.

Fig. 7B. Frequency distribution of the diameters of the isolated sperm cells of *Spinacia oleracea* as calculated out of the data from fig. 7A (according to the method of Weibel 1979, 1980). The average diameter of the isolated sperm cell is 3.66 μm. Only one peak with a normal distribution can be seen.

Discussion

Ultrastructure

At present only few ultrastructural studies of isolated plant sperm cells have been published (Cass and Fabi 1988, Matthys-Rochon et al. 1988, Wagner et al. 1989). Adequate fixation and analysis proved to be difficult. The method we developed for the chemical fixation of isolated sperm cells for transmission electron microscopy resulted in a good preservation of the sperm cell ultrastructure. We found that the isolated sperm cells are very sensitive to osmotic pressure changes. The osmotic pressure of the subsequent fixation media must be kept within a narrow range. Balancing the osmotic pressure is achieved by adding a specific amount of osmoticum (sucrose) to each step.

The isolated sperm cells contain a similar set of organelles as the sperm cells inside the pollen grains (Wilms and Van Aelst 1983). Microtubules, however, could not be observed. The nucleus can be heterochromatic or euchromatic. This indicates that not all the sperm cells (and pollen grains used) were in the same developmental stage and that there was a difference in metabolic activity, although the pollen grains used were of newly opened anthers. The shape of the nucleus was mostly spherical, which was different from the elongated shape inside the grain (Wilms and Van Aelst 1983). The former connection-site with the other sperm cell of one pair could not be determined, because of their separation and the rounding of the sperm cells.

The distribution of the mitochondria cor-

responds with the distribution of the mitochondria in the half spindle shaped sperm cells inside the pollen grain. The mitochondrial clusters originating from the tail-like extensions may stay in clusters, while the separate mitochondria from the main body remain separate in the isolated sperm cell as well. The dilated endoplasmatic reticulum (ER) was also seen in *Zea* (Cass and Fabi 1988, Wagner et al. 1989). The few ER layers present with only few ribosomes in contact with it, indicates a low synthesis activity. Also dictyosomes present in the cell were not very active. Only in a few occasions vesicles were seen near dictyosomes.

The ultrastructural observations revealed the absence of the vegetative plasma membrane around the isolated sperm cells. Also in *Zea* (Cass and Fabi 1988, Wagner et al. 1989) and *Brassica* (Matthys-Rochon et al. 1988) the absence of the vegetative plasma membrane was reported. The separation of the sperm cell from the surrounding pollen cytoplasm, which may have a possible stabilizing function, could be a reason for this loss of the vegetative plasma membrane. Also degradation of the plasma membrane in combination with rinsing during the isolation procedure could be a reason for the absence of the vegetative plasma membrane around the sperm cells.

An explicit change of the isolated sperm cells of *Spinacia oleracea*, compared to the sperm cell still inside the pollen grain, was the change in shape. They became from half spindle shaped in vivo, to round shaped when isolated. The fixation method probably did not affect the shape of the sperm cells because the rounding of the cell took place already during the isolation procedure. Similar changes in shape were also found for isolated sperm cells of most other plant species (*Beta*; Nielsen and Olesen 1988a, *Brassica*; Matthys-Rochon et al. 1988, Hough et al. 1986, *Plumbago*; Russell 1986, *Zea*; Dupuis et al. 1987, Cass and Fabi 1988). Only in *Gerbera* (Southworth and Knox 1988, 1989) the isolated sperm cells stayed in their original spindle-shape. The isolated sperm cells did not show a microtubular cytoskeleton. It could be that the rounding of the sperm cells is caused by breakdown of the microtubular cytoskeleton, as was also suggested by Tanaka (1988) for isolated generative cells of *Lilium*. Also the separation of the sperm cell from the surrounding pollen cytoplasm could be a reason for the loss of the original shape. Especially the loss of the vegetative plasma membrane may cause the sperm cells to become round. Attempts to isolate in a medium in which the original shape was preserved have thus far failed.

Morphometric Analysis

The average diameter of the isolated sperm cells of *Spinacia oleracea* was in the same order of magnitude as that reported for isolated sperm cells of other species (Table 2), although the sizes of the pollen grains were very different in these species. The frequency distribution of the sphere sizes of the isolated sperm cells showed that there was one population with a normal distribution. There was no dimorphism

Plant species	diameter of S.C.	Reference
Zea mays	6.95 ± 2.2 μm	(Cass and Fabi 1988).
Zea mays	10 μm	(Dupuis et al. 1987).
Zea mays	7.66 ± 0.9 μm	(Wagner et al. 1989).
Gerbera jamesonii	w=3-5 μm, l=20-35 μm	(Southworth and Knox 1989).
Rhododendron spec.	3-4 μm	(Shivanna et al. 1989).
Gladiolus gandavensis	2-3 μm	(Shivanna et al. 1989).
Beta vulgaris	4.3 μm	(Nielsen and Olesen 1988).
Spinacia oleracea	3.66 μm	(This paper)

Table 2. Diameters of isolated sperm cells of different plant species.

with respect to cell volume.

The method used for measuring the organelles resulted in an average volume of organelles per cell volume. However, it gave no information about possible differences of volumes of organelles between the two sperm cells of one pair. Therefore, it was impossible to establish if organelle dimorphism occurs within the two sperm cells, as suggested by Wilms (1986). The data, however, elucidated some aspects of the sperm cell characteristics. The sperm cell is not merely a big nucleus with a thin shell of plasma, but 50% of the cell consisted of cytoplasm which may be transferred to the next generation and has to be considered in the fertilization process. The mitochondrial volume of 2.5% of the total sperm cell volume in *Spinacia oleracea*, was comparable with 5.9% found in *Zea mays* (Wagner et al. 1989). According to Douce (1985), in plant cells in general, about one fifth of the cell volume consists of mitochondria. This shows that sperm cells have a rather low mitochondria content. This is in accordance with the presumed low metabolic activity of the sperm cells. The 0.6% dictyosome volume is rather low, but a comparison with other plant cells in general was not possible because of the great variation between various cell types and developmental stages.

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Chapter 7: GENERAL DISCUSSION

7.1 Isolation methods

The isolation of male gametes is a two step process. The first step is to open the surrounding vegetative cell (the pollen grain or the pollen tube) in order to liberate the sperm cells. The second step is to separate the released gametes from the vegetative cytoplasm.

The breaking of the vegetative cell

In general, the techniques used to open the pollen grain or pollen tube can be arranged in two groups:

a: The "osmotic shock" method, which is based on osmotic shock treatment

b: The "breaking" method, which is based on the physical breaking of the pollen grain or pollen tube.

With the osmotic shock method, the vegetative cell is bursted in a hypotonic medium. Large quantities of sperm cells or generative cells can be liberated, more or less simultaneously. It is a method which can be easily standardized and which is highly reproducible. Species with small as well as species with large pollen grains can be used, although large pollen grains tend to burst easier. The thickness and character of the pollen grain wall strongly influences the success of the technique. In some species the osmotic shock method does not give good results. Most probably because in such species the pollen grain wall is too strong or too tough to be broken in this way. Sometimes a pretreatment of the material is required to make the osmotic shock method efficient, like prehydration (Nielson and Olesen 1988a, 1988b). Shivanna et al. (1988) have applied the osmotic shock method on pollen tubes which previously had been treated with enzymes to soften the cell wall. In some occasions, the medium requires a specific pH to get a higher yield (Van der Maas and Zaal 1990). When the osmotic shock method is used, both a strong or a weak osmotic shock can be applied, resulting respectively in a higher or a smaller number of broken pollen grains. The osmotic condition of the bursting medium however can have a negative effect on the liberated sperm cells. The low osmotic value of the bursting medium can cause the bursting of the liberated sperm cells. Therefore, an optimal osmotic shock is selected based on an equilibrium between the shock necessary for high numbers of bursting pollen grains and the shock with least damage to the liberated cells (table 1).

With the "breaking" method, a physical force is used to break the pollen grains or tubes. This can be done with tissue homogenizers, electric blenders or glass rollers. In this way, the pollen grains are squashed or completely broken, causing the vegetative cytoplasm containing the sperm cells to come out of the pollen grain. It is possible to chose the medium to have an optimal osmotic value for the isolated cells. On the other hand, any medium can be chosen, depending on the requirements

related to the intended use of the liberated cells. The medium can even be pure oil. In this way, the isolated cells can be released in their original shape.

In general, large pollen grains are easy to break, but small and therefore more resistant pollen grains can also be used. Although no osmotic shock is required to burst the cells, the osmotic value of the medium may help to break the pollen grains or tubes. In a medium with a low osmotic value, the breaking method functions better than in a medium with a high osmotic value (table 1).

	Osmotic shock	Breaking
*Percentage breaking	high	low
*Larger pollen are easier to break	yes	yes
*Any medium can be chosen	no	yes
*Original shape	no	yes
*Viability	high	high
*Possibility to purify	yes	yes
*Reproducibility	high	low
*Difficult to standardize	no	yes
*Final amount of gametes	high	low
*Yield as % of pollen grains	high	low

Table 1: Characteristics of the osmotic shock technique versus the physical breaking technique.

Both techniques have disadvantages (table 1). The main problem with the osmotic shock technique is the possibility of bursting of the isolated sperm cells. The main problem of the squashing technique is the low yield and the laborious work.

For some species, no choice between the osmotic shock technique or the breaking technique is possible, because only one of the two techniques give results at all. With pollen grains of *Spinacia oleracea*, the osmotic shock technique does not work. Most likely, the pollen grains are to small and the pollen grain wall is to resistant to be broken by the osmotic shock. For this reason the squashing technique was developed and applied.

The purification

The second step in the isolation procedure, is to separate the liberated cells from the vegetative cytoplasm. The mixture of unbroken pollen grains, wall fractions, vegetative cytoplasm, vegetative nuclei and isolated cells are usually filtered on nylon filters. In this way, the larger parts like unbroken pollen grains and wall fractions are excluded. Further purification can be done manually (Kranz et al. 1991a) or by centrifugation on a sucrose gradient (Russell 1986, Cass and Fabi 1988) or a percoll gradient (Dupuis et al. 1987, Nielsen and Olesen 1988a, 1988b, Tanaka 1988, Theunis and Van Went 1989). If collected manually, the isolated sperm cell fraction will be very pure and hardly contaminated, but the number of cells will be very low (Kranz et al. 1991a). When purified on a percoll or sucrose gradient, the fraction containing the sperm cells is

contaminated with small vegetative organelles, proteins and fragments of the pollen grain. Concentration of isolated cells, in number of cells per ml medium, varies from $8.8x10^6$ for *Plumbago zeylanica* (Russell 1986), $4x10^6$ for *Spinacia oleracea* (Theunis and Van Went 1989), $1.5x10^6$-$3x10^6$ for *Zea mays* (Dupuis et al. 1987, Cass and Fabi 1988), $0.7x10^6$ for *Beta vulgaris* (Nielsen and Olesen 1988a, 1988b) and $0.02x10^6$ for *Gerbera jamesonii* (Southworth and Knox 1989).

The significance of both isolation methods in gamete research.

The choice of isolation method is important for the use of isolated cells in studies in the field of biotechnology, cell-biology, physiology, histology, biochemistry, immunology, morphology and cytology. Apart from the plant species used, the character and the future aims of the research determine which of the two methods is to be preferred (table 2).

	"Osmotic" shock	"Breaking"
Biotechnology	+++	+++
Cell biology	+++	++
-Fusion exp.	++	+++
Morphology	+	+++
Cytology	++	+++
Physiology	+++	+++
-Viability	+++	++
Biochemistry	+++	+
Immunology	+++	++
Histology	+	++

Table 2: Applicability of the "osmotic shock" techniques and the "breaking" technique in different fields of research.
+++= Technique useful in many studies in this field
++ = Technique useful in some studies in this field
+ = Technique useful in few studies in this field.

The "breaking" method has advantages especially in morphological, cytological, histological and in some physiological studies. The two main advantages are the possibility to chose any medium for the breaking and the possibility to isolate the sperm cells in their original shape. For these reasons, morphological studies of the shape of isolated sperm cells and its variations are only possible when the "breaking" method is applied (Theunis et al. 1992). By using different sucrose concentrations when the pollen grains are broken, the initial swelling and shrinking of the cells can be visualized.

The free choice of a medium gives the possibility to isolate the cells in medium which immediately fixes the cells. With these direct fixations, better morphological and cytological results can be obtained. It also enables more precise histological experiments.

A possibility in physiology, is to test different types of osmotica and additives to improve the viability of the cells, before any damage caused by an osmotic shock

has happened.
Also the "osmotic shock" method has its advantages above the breaking method. The main advantages are the high yield and the higher final numbers of gametes possible to isolate. This is especially important for biochemical, molecular biological or immunological studies. For the detection and further analyses of sperm cell specific proteins high numbers of cells are required (Geltz and Russell 1998). The same stands for analyses of enzymes, RNA and DNA. For these studies, however, the purification is still a problem. At present, purification results in sperm cell-rich fractions, contaminated with small vegetative organelles, proteins and other components. More attention should therefore be directed to the purification step of the isolation procedure. In immunological studies, when antibodies are produced against sperm cell proteins, high numbers of cells are necessary (Hough et al. 1986, Pennell et al. 1987). On the other hand, when antibodies have already been elicited, the testing of these antibodies can be done on small numbers of sperm cells. For these analyses the method of "breaking" is more useful, or even testing on in-situ sperm cells.
For other experiments in the fields of biotechnology and cell biology both methods can be used, because neither high numbers nor originally shaped sperm cells are necessary. For some cell-biological studies, when in vitro regeneration is aimed, the higher amounts of isolated cells achieved with the osmotic shock method are an advantage.
All these above mentioned considerations are general remarks. For some species there is no choice possible, because only one of the two methods gives results at all. It gives, however, an indication of the aspects which have to be considered in making a right choice of the isolation technique to use.

7.2 The isolated sperm cell

In this part of the discussion, important characteristics of isolated sperm cells will be discussed. Especially those which have a general significance or which have also been observed in a number of other species. Separately, the importance of dimorphism will be discussed in relation to the role of preferential fertilization. The observations on *Spinacia oleracea* which have been presented and discussed in the previous chapters will only be mentioned in short.

The sperm cell shape

Generally, in most species, the in-situ sperm cells are spindle shaped and have tail-like extensions (Russell et al. 1990). The spindle-shape is lost during or just after isolation. The same is found in *Spinacia oleracea* (Theunis et al. 1991). The isolated cells become spherical in form. The rounding does not seem to be related to the osmotic potential of the isolation medium since in both hypertonic as well as hypotonic media the cells become spherical. The time involved in this change, however, can be influenced by the osmotic value of the isolation medium (Theunis et al. 1992). Also the size of the sperm cells can change according to the osmotic value of the isolation medium.

Tanaka (1988) suggests that the change in shape is a result of the breakdown of the microtubular cytoskeleton. Theunis et al. (1992) have shown that in generative cells of *Nicotiana tabacum* the microtubular cytoskeleton is still present just after isolation, when these generative cells are still spindle shaped. When the cells have become spherical, however, the microtubules have disappeared. Most likely, the cytoskeleton is responsible or partially responsible for the maintenance of the spindle-shape. Another factor possibly involved in the loss of the spindle-shape can be the disappearance of the vegetative plasma membrane, which together with the vegetative cytoplasm could have an upholding force. After the loss of the vegetative plasma membrane, the "wall material" which is possibly present around the sperm cell pair can subsequently easily disappear. Whether wall-like material is present around the sperm cells is not clearly established. It is clear, however, that some material is present, but whether this can have a function in the shaping of the cell, still has to be studied.

On the other hand, the loss of the spindle-shape might be a natural reaction on the environmental changes. Also in nature at time of fertilization, when the sperm cells are released from the pollen tube, they adapt a spherical configuration (Mogensen 1982, Russell 1982, 1983, Wilms 1981). Therefore, in theory, the rounding of the sperm cells after isolation may be a natural reaction, which proceeds the fusion process.

If this assumption is correct, then the spindle-shape must have a function at an earlier time. The following possible functions can be directed to the spindle-shape:

1 The diameter of a spindle-shape is smaller than that of a spherical cell and therefore, a pollen tube with a smaller diameter is sufficient for transport of the gametes.

2 The spindle-shape gives a larger surface area, which can be useful in communication and transport.

3 The spindle-shape is better adapted to the cytoplasmic streaming in the pollen tube.

4 Because of the presence of tail-like extensions, a physical connection of the sperm cell with the vegetative nucleus is possible. If this connection is necessary either for communication between the two cells, or for transportation as a unit through the tube, a spindle shaped cell will be easier connected to the vegetative nucleus, than a spherical one.

Viability

Under natural conditions, when the sperm cells are inside the pollen grain, their lifetime can be very long. Pollen grains can be stored for long periods with the sperm cells surviving this storage. When isolated, the male gametes do not endure very long. As soon as sperm cells are released from their surrounding vegetative cell, some of them loose their viability. Therefore, always a mixture of isolated viable cells and dead cells is present. The

percentage of viable cells decreases in time. Most reports, when lifetime or storage times are mentioned, are unclear about the percentage of viable cells. Most likely, when data concerning storage time are reported, this implies the time when no viable sperm cells are present anymore. Stored isolated sperm cells are found to remain viable from 15 min (Szakács and Barnabás 1990) to 72 h (Yang and Zhou 1989). The lifetime can be expanded by adding specific components to the storage medium, like vitamin C and vitamin E, but the duration of lifetime can not be extended more than a few days (Theunis et al. 1991). Also lowering of the temperature expands the lifetime. Furthermore, a depletion of energy has been considered to be the cause of death. The addition of ATP to the storage medium did, however, not lead to an expansion of the lifespan.

Under the present conditions, the isolated cells start to degrade soon after isolation. The storage conditions are not specific enough to keep the cells viable or to ensure cell division and further development. Emphasis should be put on adjusting media for storage conditions. Storage by means of freezing could be a possible way (Cass and Fabi 1988).

Under natural conditions, when fertilization is about to take place, the sperm cells are released from the pollen tube into the penetrated synergid. They become spherical and loose the surrounding vegetative plasma membrane (Mogensen 1988, Russell 1982, 1983). Soon after this release the male and female gametes fuse. Therefore, the sperm cells which are discharged in the synergid, only have to stay viable for a short period. The fusion products start to develop shortly after completion of the fusion. The isolated sperm cells can be compared with sperm cells released into the synergid. Also they are freed from their natural surrounding and are liberated in a mass of degenerating cytoplasm. Therefore, the relative short lifespan of isolated sperm cells could be the natural lifespan of the sperm cells when they are released out of the pollen tube in the synergid. Whether their is a relation between the lifespan of isolated cells and the speed of the fertilization process is an interesting subject for research.

7.3 Sperm cell dimorphism

In a number of plant species, ultrastructural research of sperm cells has shown that the two sperm cells of one pollen grain or tube are morphological different. This phenomenon is termed "sperm cell dimorphism". In general, one can speak of sperm cell dimorphism if a morphological characteristic is different between the two sperm cell of one pollen grain or tube and that this difference is statistically significant. Several types of characteristics can be dimorphic: shape and size of the cells and number of organelles. Differences in number of organelles can have a direct effect on the next generation. The presence of DNA in male and female organelles makes their cytoplasmic inheritance important for genetical aspects of the next generation. The two different types of zygotes, which are formed when sperm cell dimorphism is combined with at random gamete fusion, can develop in different ways and lead to different populations of individuals

	mitochondria (number)		plastids (number)		surface area (μm^2)		volume (μm^3)		reference
	SC1	SC2	SC1	SC2	SC1	SC2	SC1	SC2	
Brassica campestris (7)	6.4	23.4 **	0	0	35.5	40.8 *	9.0	10.8 **	McConchie et al. 1987b
Brassica oleracea (5)	9.8	13.2 *	0	0	46.2	65.1 *	14.8	17.5 **	McConchie et al. 1987b
Gladiolus gandavensis (?)					2.4	4.1 **	2.7	5.8 **	Shivanna et al. 1988
Hordeum vulgare (1)	36	24	0	0	99.1	112.5	61.6	67.9	Mogensen and Rusche 1985
Hordeum vulgare (1)	31	32	0	0	65.9	84.4	51.0	56.4	Mogensen and Rusche 1985
Plumbago zeylanica (22)	39.8	256.2 ***	24.3	0.5 ***	84.7	147.9 ***	48.9	69.5 ***	Russell 1984
Spinacia oleracea (7)	20.3	24.7 NS							Wilms 1986
Rhododendron laetum (1)	80	92	24	28	145.1	175.4	94.8	101.3	Taylor et al. 1989
Rhododendron macgregoriae (6)	60.5	93.7 NS	11.8	20.2 NS	65.3	99.4 **	33.3	42.5 NS	Taylor et al. 1989
Rhododendron spec (?)					5.3	7.4 **	8.8	12.8 **	Shivanna et al. 1988
Zea mays (1)					232.0	264.7	60.8	73.4	Rusche 1988
Zea mays (1)	5	14			313.2	366.2	209.7	234.5	Rusche and Mogensen 1988
egg cell of:									
Plumbago zeylanica (6)	39900		730		38600		543000		Russell 1987

Table 3: Results of quantitative analysis of morphological differences between sperm cells of one pollen grain in different species. In brackets is the number of sperm cells which have been analyzed. The rate of significance between the different sperm cells according to the authors is given as follows: * = $P<0.05$; ** = $P<0.01$; *** = $P<0.001$; NS = not significant. As comparison is given the corresponding numbers of egg cells of *Plumbago zeylanica*.

in the next generation. On the other hand, dimorphism is sometimes combined with preferential fertilization; when already is predetermined which of the two sperm cells fuses with the egg cell and which withthe central cell. In this case only one type of zygote will be formed. It is a method by which preferentially biparental or maternal inheritance of organelles is guaranteed.

In a number of species, sperm cell dimorphism has been quantified. The differences in mitochondrial content, plastid content, volume and surface area of cells are listed in table 3. Most of these results have been obtained by applying serial sectioning and 3-dimensional reconstruction of in situ sperm cells. Other forms of dimorphism, which are not listed in table 3, are the existence of a close association of one of the two sperm cells with the vegetative nucleus and the difference in shape between the two sperm cells. This has been found in many species (Russell et al. 1990).

From table 3 it is clear that one of the most distinct examples of sperm cell dimorphism is found in *Plumbago zeylanica*. In this species, one sperm cell contains, as average, 256.18 mitochondria (range 154-311) while the other contains only 39.81 mitochondria (range 22-52) (Russell 1984). In some other species, the significance is not very high. In *Brassica oleracea* for example, it has been found that one sperm cell has an average of 13.2 mitochondria (range 11-15) while the other sperm cell of the same pair has an average of 9.8 mitochondria (range 6-14) (table 3). Only 5 pollen grains have been analyzed in this example and this is (probably) not sufficient to prove dimorphism. Other conclusions are even based on one observation, which can never be a basis to state the existence of dimorphism of that character.

In *Spinacia oleracea* Wilms (1986) has reported a slight form of sperm cell dimorphism with respect to number of mitochondria in the sperm cell. He concluded that the number of pollen grains which has been analyzed is not sufficient to definitely confirm the existence of dimorphism. The present results clearly show that in *Spinacia oleracea* no such dimorphism in numbers of mitochondria is present. Nor any dimorphism in volume and size of the sperm cells, mitochondrial content or other organelle content could be evidenced. Neither any difference in number and distribution of membrane bound particles in sperm cells could be demonstrated. The two sperm cells from one pollen grain are morphologically identical.

It is very understandable that in most quantitative studies of dimorphism, so few pollen grains are investigated. The serial sectioning and 3-dimensional reconstruction, until now necessary for this research, are difficult and time consuming. The use of isolated sperm cells, however, gives the opportunity to investigate large numbers of sperm cells. It makes the statistical calculations more reliable. More methods should be developed in which isolated sperm cells are used to investigate dimorphism and its significance in fertilization.

The existence of dimorphism in combination with preferential fertilization, makes sperm cell research even more important, especially for biotechnology and cell-biology. It gives large possibilities for

genetical engineering and regulation of the transport of mitochondria, plastids or other organelles to the next generation.

7.4 Fusion products; Future research

For cell fusion experiments and in vitro fertilization the availability of both male as well as female gametes is necessary. Therefore, not only male gametes have been isolated, but also female gametes have been subject of isolation experiments (Theunis et al. 1991). Recent research has led to the availability of both isolated male and female gametes and the first in vitro fusion experiments have been carried out. Fusion products of isolated gametes of *Lilium longiflorum* are obtained by using electro-fusion (Ueda et al. 1990). No karyogamy occurs, nor does any regeneration take place. In *Zea mays* electro-fusion of isolated sperm cells with isolated egg cells is recently achieved (Kranz et al. 1991a). The obtained fusion products form minicalli in a culture medium containing *Zea mays* feeder cells, whereas unfertilized egg cells do not divide. Fusion of isolated sperm cells with isolated synergids, central cells and mesophyllic cytoplasts has also been realized (Kranz et al. 1991b).
The fact that recently fusion of isolated gametes has been achieved proves that these cells can be used for artificial fertilization. It still has to be established, whether generative cells have the same potencies as sperm cells to fuse with gametophytic cells into a product with regeneration capacities. In addition, the consequences of dimorphism of the two sperm cells of one pair and preferential fertilisation have to be considered in this context. It may be questioned whether sperm cells formed in pollen tubes grown in vitro or in semi- vivo have the same properties as sperm cells from pollen tubes grown in the style. Important for manipulation could be the maturation stage of the sperm cells. Also, it still has to be kept in mind, that isolated sperm cells differ in some characteristics with the in situ sperm cells. The question remains, whether isolated sperm cells have the same properties as the sperm cells inside the vegetative cell. Original ideas have been formulated about the use of isolated gametes in biotechnology: insertion of external genetic material by means of micro-injection of DNA fragments or micronuclei, induction of genetic transformations, transmission of organelles, routine in vitro fertilization, culture of isolated gametic cells and gametic fusion products, toxicological tests. Modern microscopical techniques, like rapid freeze fixation and freeze substitution, immunogold labelling, in situ-hybridization, video enhanced contrast microscopy, confocal laser scanning microscopy in combination with microinjection, will certainly be further applied for a better understanding of the mechanisms of fertilization and embryological development. Biotechnological engineering on angiosperm gametes and in vitro fertilization in plants will indeed come of age in the near future.

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SUMMARY

Gametes are specialized cells with the natural capacity to fuse in a well determined way. The fusion products are intended to develop into new individuals. Basic knowledge of gametes is of great importance for both traditional plant breeding as well as for modern biotechnology and gene manipulation. For applications in these fields, more knowledge is necessary of the characteristics of gametes and the mechanisms involved in the process of gamete recognition and fusion. Isolated gametes form ideal material to investigate this. The present study was focused on the isolation and characterization of the male gametes, the sperm cells.

In chapter 1 an introduction is given. The current information we have on in situ sperm cells, and on the subject of sperm cell isolation, is summarized in this chapter.

In chapter 2 the ultrastructure is described of the pollen grains of *Spinacia oleracea* and the sperm cell pair therein. The pollen grain is trinucleate and consists of a vegetative cell and two sperm cells. The pollen grain wall is tectate, with many germination pores, which have a hexagonal distribution. The vegetative nucleus together with the sperm cells are located in the periphery of the pollen grain and are organized in a "male germ unit". The cytoplasm of the vegetative cell contains vacuoles and electron dense vesicles. The mitochondria have a size of 0.3 μm to 0.5 μm. The ER is often organized in single elements, and bears ribosomes. The plastids are filled with starch and only the outer membrane is visible. The high amount of starch may be used in an autotrophic way of germination, or for osmotic stabilization during germination. Microtubules are not found in the vegetative cytoplasm.

The sperm cell inside the pollen grain contains a heterochromatic nucleus, mitochondria, dictyosomes, and ER. The two sperm cells are attached to each other. They form a pair which is surrounded by a vegetative plasma membrane. Only a few microtubules have been shown in the sperm cell cytoplasm. In previous studies, microtubules have been clearly demonstrated inside the sperm cells. Therefore, it was concluded that the used method of freeze substitution does not completely stop the breakdown of microtubules.

In order to release the sperm cells, the vegetative cells has to be opened. The osmotic shock method, which has been used in some species, does not work for pollen grains of *Spinacia oleracea*. Even in pure water only a small percentage of the pollen grains bursts. For this reason, a mechanical method has been developed, using a glass roller to squash large quantities of pollen grains. This method is described in chapter 3.

Because of the squashing of the grain, the sperm cells are released from the pollen grain together with most of the vegetative cytoplasm. Since physical breaking is applied, any medium can be chosen in which the breaking is performed. After the squashing, the mixture of pollen grains, free sperm cells, vegetative nuclei, vegetative organelles, and pollen grain fragments is filtered over a 25 μm nylon filter. Subsequently, the filtrate is

centrifuged on a 20% percoll layer for further elimination of small debris. With this method, a fraction is obtained which contains numerous sperm cells, but which is still contaminated with small vegetative organelles, and small pollen fragments. The yield is approximately 5-10% with a final concentration of $4x10^6$ sperm cells/ml.

The free sperm cells are elongated just after squashing, but become spherical after a short time. The originally paired sperm cells separate. The close association of the sperm cell pair with the vegetative nucleus is not maintained during squashing and is therefore, not a firm binding. The sucrose concentration of the medium does not influence the change in shape of the free sperm cells. The volume, however, is influenced by the osmotic value of the medium. The 25% sucrose concentration was chosen for the rest of the experiments in order to avoid damage caused by osmotic swelling. The diameter of the isolated sperm cells can vary from 4 μm to 9 μm, depending on the sucrose concentration.

Immediately after isolation, more than 90% of the sperm cells is viable (tested with the fluorescein diacetate test). Soon after isolation, however, some of the cells loose their viability. After 18 h, only 50% of the isolated cells is still viable. Storage of the isolated sperm cells at low temperatures (0°C) doubles the lifespan. Addition of 1% vitamin C also enlarges the lifespan. It is concluded that depletion of energy is not the cause of the loss of viability, since addition of 0.1 M ATP makes no difference for the lifespan.

With histochemical tests, using calcofluor white MR2 for cellulose, analine blue for callose, and the PAS reaction for carbohydrates, no cell wall material was observed around the isolated sperm cells. This indicates that the cells are true protoplasts.

In chapter 4 the results are presented of the analysis of the numbers of mitochondria in isolated sperm cells. To visualize the mitochondria, two staining methods have been used. The $DiOC_6(3)$ staining (in a concentration of 0.1 μg/ml) gives better results with less background staining, than the Rhodamine 123 staining.

The analysis was carried out on individual sperm cells, as well as on sperm cell pairs. If individual sperm cells were used, two populations of sperm cells seemed to be present, with an average of respectively 10.3 and 17.8 mitochondria per sperm cell. However, by counting the mitochondria in sperm cell pairs, it is found that there is only one population of sperm cells. The average is 12.4 $\pm$ 4.6 mitochondria per sperm cell. The number of mitochondria per sperm cell varies from 2 to 25, which is a high variation. This high variation can be explained in two ways. It is possible that already after the division of the microspore, a high variation exists in number of mitochondria per generative cell. The second explanation can be, that during the development of the generative cell and/or sperm cells, mitochondria are produced or lost.

With the technique of freeze-fracturing, the plasma membranes of the isolated sperm cells were examined, including the intra-membrane particles (IMP's). In chapter 5 the results are presented. Also with this method, no remnants of the vegetative plasma membrane were found around the sperm cells. Only incidentally, transverse fracture planes through whole sperm cells are found. Most of the fracture planes of sperm cells follow the sperm cell plasma membrane exposing either the PF or the EF face. Neither the ES or the PS faces

are found. Both the PF as well as the EF face show IMP's. These IMP's are randomly distributed, and no pattern can be recognized. The PF face has a density of 719 IMP's/μm^2. The EF face has a density of 2088 of IMP's/μm^2. Evidently, the EF half of the sperm cell membrane contains approximately 3 times more IMP's than the PF half. For sporophytic protoplast it has been reported that the PF half contains more IMP's than the EF half. The specific IMP distribution in the sperm cell plasma membrane may be related to the process of gamete recognition and subsequent fusion. With respect to IMP's density, only one type of sperm cells was observed and therefore for this character, no dimorphism could be established.

Morphometrical and ultrastructural characterization of the isolated sperm cells has been reported in chapter 6. Experiments with various fixations demonstrate that sperm cells are fragile, and difficult to fixate. The osmotic value of the fixation media appear to be of great importance. Also with this method it is clear that the isolated sperm cells are separate and completely spherical. The surrounding vegetative plasma membrane has disappeared. No cell wall material is observed. The sperm cell contains a large nucleus which can be either heterochromatic or euchromatic. The mitochondria are spherical and frequently appear to be clustered in groups of 5 to 10 mitochondria, but also individual mitochondria have been observed. The dictyosomes have 4 to 5 cisterns with associated small vesicles. Small vacuoles are present. The endoplasmatic reticulum is sparse, and often dilated. Ribosomes are sometimes grouped in polysomes. No microtubules have been observed. From these observations it is clear that isolated cells contain a similar set of organelles as the in situ sperm cells. After measuring the section diameters, the average diameter of the complete cell is calculated to be 3.66 μm with the used fixation conditions. From surface area's in the sections is calculated that 50% of the sperm cell is occupied by its nucleus, 2.5% of the cell is mitochondria, and 0.6% of the cell is dictyosome. The ultrastructural analysis did not give any indication that in *Spinacia oleracea* sperm cell dimorphism in regard of sperm cell size, exists.

In chapter 7 the results of the present thesis are discussed in general sense and in a broader context. Results which have already been discussed in previous chapters are not further discussed in this general discussion.

The isolation technique of "physical breaking", developed for *Spinacia oleracea* is compared with the "osmotic shock" technique, used for other species. The advantages and disadvantages of both techniques are presented and discussed. The usefulness of the two techniques in further research of isolated gametes are explained. Preferences for one of the two technique clearly depends on the aims of the further research.

The general discussion highlights the two major phenomena sperm cells show when isolated: the changing of a spindle shaped cell to a spherical cell, and the loss of viability. The change in shape occurs in all species observed thus far. Likely, the change in shape is a natural process which also occurs during fertilization. The loss of viability can be

slowed down with low temperature, and anti-oxidantia. During the natural fertilization process, extended viability is not necessary, since the free sperm cells fuse rapidly with the female partners. So also for this character it may be a natural phenomenon.
Sperm cell dimorphism, which has been reported in some species, is not found in *Spinacia oleracea*. The isolated sperm cells form good material to study dimorphism because of the large quantities of cells which can be analyzed.

SAMENVATTING

Gameten zijn gespecialiseerde cellen met een natuurlijke capaciteit om volgens een geordend patroon te fuseren. Het fusie produkt ontwikkelt zich tot een nieuw individu. Basis kennis van gameten is van zeer groot belang voor zowel traditionele plantenveredeling als voor moderne biotechnologie en gen manipulatie. Voor toepassingen in deze gebieden is meer kennis noodzakelijk van de eigenschappen van gameten en de mechanismen die betrokken zijn bij het proces van gameet herkenning en fusie. Geïsoleerde gameten vormen ideaal materiaal om dit te onderzoeken. Deze studie is toegespitst op de isolatie en de karakterisering van mannelijke gameten, de spermacellen.

Hoofdstuk 1 is een introductie. De beschikbare informatie over in situ spermacellen en spermacel isolatie wordt samengevat in dit hoofdstuk.

In hoofdstuk 2 is de ultrastructuur beschreven van de pollenkorrels van *Spinacia oleracea*. De pollenkorrel is tri-nucleaat en bestaat uit een vegetatieve cel en twee spermacellen. De wand van de pollenkorrel is tectaat, met vele kiemporen die hexagonaal verdeeld zijn. De vegetatieve nucleus is samen met de spermacellen gelokaliseerd in de periferie van de pollenkorrel. De spermacellen vormen een paar en zijn met de vegetatieve kern georganiseerd in een "male germ unit". Het cytoplasma van de vegetatieve cel bevat vacuoles en elektronen dichte vesicles. De mitochondriën hebben een grootte van 0,3 μm tot 0,5 μm. Het endoplasmatisch reticulum (ER) is meestal georganiseerd in afzonderlijke elementen en draagt ribosomen. De plastiden zijn gevuld met zetmeel en slechts het buitenste membraan is zichtbaar. Het hoge gehalte aan zetmeel zou gebruikt kunnen worden voor een autotrofische kieming of voor osmotische stabilisatie gedurende de kieming. Microtubuli zijn niet gevonden in het vegetatieve cytoplasma. De spermacellen in de pollenkorrel bevatten een heterochromatische kern, mitochondriën, dictyosomen en ER. De twee spermacellen en vormen een paar wat omgeven is door een vegetatief plasmamembraan. Slechts enkele microtubuli zijn gevonden in het spermacel cytoplasma. In eerdere studies zijn microtubuli duidelijk aangetoond in de spermacellen. Daarom wordt geconcludeerd dat de gebruikte methode van vries-substitutie, in vergelijking met de klassieke fixatie methoden, de afbraak van microtubuli niet geheel voorkomt.

Om spermacellen te isoleren is in eerdere studies gebruik gemaakt van een osmotische shock, om de vegetatieve cel te openen. Deze methode werkt niet bij pollenkorrels van *Spinacia oleracea*. Zelfs in puur water knapt slechts een klein gedeelte van de pollenkorrels. Daarom is een mechanische methode ontwikkeld, waarbij gebruik gemaakt wordt van een glazen roller waarmee grote hoeveelheden pollenkorrels gebroken kunnen worden. Deze methode is beschreven in hoofdstuk drie.

Door het samenpersen van de korrel komt het grootste gedeelte van het vegetatieve cytoplasma met de spermacellen naar buiten. Vanwege het toepassen van een fysieke breekmethode, kan elk willekeurig medium gekozen worden. Na het samenpersen van de

pollen korrels wordt het mengsel van pollenkorrels, vrije spermacellen, vegetatieve nuclei, vegetatieve organellen en pollenkorrelfragmenten gefiltreerd over een 25 μm nylon filter. Vervolgens is dit filtraat gecentrifugeerd op een 20% percoll laag voor verdere opsplitsing. Door deze methode wordt een fractie verkregen die talrijke spermacellen bevat, maar ook kleine vegetatieve organellen en kleine pollenkorrelfragmenten. Met deze methode wordt een opbrengst bereikt van ongeveer 5 tot 10% met een uiteindelijke concentratie van $4x10^6$ spermacellen/ml.

Net na de isolatie zijn de vrije spermacellen langgerekt, maar na een korte tijd worden ze rond. Het spermacelpaar splitst. De nauwe verbintenis tussen het spermacelpaar en de vegetatieve nucleus wordt verbroken. De sucrose concentratie van het medium is niet van invloed op de vormverandering van de vrije spermacellen. Het volume daarentegen wordt wel door de osmotische waarde van het medium beïnvloed. Een medium met een concentratie van 25% sucrose is daarom uiteindelijk gekozen voor de rest van de experimenten, om schade door zwelling van de spermacellen te voorkomen. De diameter van de geïsoleerde spermacellen varieert van 4 μm tot 9 μm, afhankelijk van de sucrose concentratie.

Onmiddellijk na isolatie is meer dan 90% van de spermacellen vitaal (getest met de fluoresceine diacetaat test). Sommige cellen verliezen echter snel na de isolatie hun vitaliteit. Na 18 uur is nog slechts 50% van de geïsoleerde cellen vitaal. Opslag van de geïsoleerde spermacellen bij lage temperatuur (0°C) verdubbeld de levensduur. Ook toevoeging van 1% vitamine C vergroot de levensduur. Omdat toevoeging van 0,1 M ATP geen verschil veroorzaakt in levensduur, mag geconcludeerd worden dat gebrek aan energie niet de oorzaak is van het verlies van vitaliteit.

Met histochemische testen, waarbij gebruik gemaakt werd van calcofluor white MR2 voor cellulose, analine blauw voor callose en de PAS reactie voor carbohydraten, kon de aanwezigheid van celwand materiaal niet worden vastgesteld. Dit geeft aan dat geïsoleerde spermacellen echte protoplasten zijn.

In hoofdstuk 4 worden de resultaten gepresenteerd van de analyse van de aantallen mitochondriën van geïsoleerde spermacellen. Om de mitochondriën zichtbaar te maken, werden twee kleuringsmethoden toegepast. De $DiOC_6(3)$ kleuring (in een concentratie van 0,1 μg/ml) geeft betere resultaten met minder achtergrond kleuring dan de Rhodamine 123 kleuring. De analyse werd uitgevoerd op individuele spermacellen maar ook op paartjes van spermacellen. Bij de analyse van de individuele spermacellen leken twee populaties te bestaan, met respectievelijk 10,3 en 17,8 mitochondriën per spermacel. Bij analyse van mitochondriën in spermacelparen kwam echter naar voren dat slechts één populatie aanwezig is. Het gemiddeld aantal mitochondriën per spermacel is 12,4 $\pm$ 4,6. Het aantal mitochondriën kan variëren van 2 tot 25 per spermacel. De aanwezigheid van deze grote variatie kan op twee manieren verklaard worden. Het is mogelijk dat reeds een grote variatie bestaat na de deling van de microspore. Een tweede verklaring kan zijn dat gedurende de ontwikkeling van de generatieve cel en/of de spermacellen mitochondriën geproduceerd worden, of dat mitochondriën verloren gaan.

Met behulp van de vries-breek techniek is het plasmamembraan van geïsoleerde spermacellen onderzocht. In hoofdstuk 5 worden daarvan de resultaten gepresenteerd. Ook met deze methode werden geen restanten van het vegetatieve plasmamembraan rond de spermacellen gevonden. Slechts enkele keren werden transversale breukvlakken door hele spermacellen aangetroffen. De meeste breukvlakken volgen het spermacel plasmamembraan waardoor of de PF zijde of de EF zijde zichtbaar wordt. Noch de ES noch de PS zijde zijn aangetroffen. Zowel de PF als de EF zijde tonen inter-membraanpartikels (IMP's). Deze IMP's zijn willekeurig gerangschikt zonder bijzondere patronen. De PF zijde heeft een dichtheid van 719 IMP's/μm^2. De EF zijde heeft een dichtheid van 2088 IMP's/μm^2. Klaarblijkelijk bevat de EF zijde ongeveer 3 maal zo veel IMP's als de PF zijde, terwijl voor sporofytische protoplasten is gerapporteerd dat de PF zijde meer IMP's bevat dan de EF zijde. Deze specifieke IMP distributie in spermacel plasmamembranen zou gerelateerd kunnen zijn aan de gameet herkenning en fusie. Ten opzichte van IMP dichtheid werd slechts één type cellen gevonden, waaruit blijkt dat geen dimorfisme van IMP dichtheid aanwezig is.

Morfometrische en ultrastructurele karakterisering van de geïsoleerde spermacellen zijn beschreven in hoofdstuk 6. Experimenten met verschillende fixaties, geven aan dat spermacellen fragiel zijn en moeilijk te fixeren. De osmotische waarde van het fixatie medium is van groot belang.

De geïsoleerde spermacellen zijn los van elkaar en geheel rond. Het vegetatieve plasmamembraan rond de spermacellen is verdwenen. Celwand materiaal is niet gevonden. De spermacellen bevatten een grote nucleus die zowel heterochromatisch als euchromatisch kan zijn. De mitochondriën zijn rond en komen vaak in klusters voor van 5 tot 10, echter individuele mitochondriën zijn ook waargenomen. De dictyosomen bestaan uit 4 tot 5 cisternen met geassocieerde kleine vesicles. Kleine vacuoles zijn aanwezig. Endoplasmatisch reticulum (ER) is schaars en de cisternen hiervan zijn vaak gezwollen. Ribosomen zijn soms gegroepeerd in polysomen. Microtubuli zijn niet waargenomen. Uit deze observaties is duidelijk dat geïsoleerde spermacellen een vergelijkbaar set organellen bevatten als de spermacellen in situ. Na metingen van coupe diameters, is berekend dat de diameter van de spermacel 3,66 μm is, onder de gebruikte fixatie conditie. Uit de oppervlakten van coupes is berekend dat 50% van het spermacelvolume bestaat uit kern, 2,5% uit mitochondriën en 0,6% uit dictyosomen. De ultrastructurele analyse van de spermacel grootte geeft geen aanwijzingen dat spermacel dimorfisme bestaat in *Spinacia oleracea*.

In hoofdstuk 7 worden de resultaten van dit proefschrift bediscussieerd en in een breder kader geplaatst. Resultaten die al eerder uitvoerig zijn behandeld, worden niet verder bediscussieerd in deze algemene discussie.

De isolatie techniek m.b.v (fysiek breken) ontwikkeld voor *Spinacia oleracea* wordt vergeleken met de "osmotische shock" techniek, die op andere soorten is toegepast. De voor- en nadelen van beide technieken worden met elkaar vergeleken. De gebruikswaarde

van de twee technieken voor verder onderzoek van geïsoleerde gameten wordt toegelicht. Voorkeur voor of de ene of de andere techniek is duidelijk afhankelijk van het doel van het verdere onderzoek. In de algemene discussie worden de twee belangrijkste kenmerken van geïsoleerde spermacellen nader besproken; de verandering van spoelvormige in rond cellen en het verlies van vitaliteit. De verandering van vorm is waarschijnlijk een natuurlijk proces wat ook plaatsvindt tijdens de bevruchting. Het verlies van vitaliteit kan afgeremd worden door lage temperaturen en door anti-oxidantia. Gedurende de bevruchting is geen lange levensduur vereist. Dus ook deze eigenschap is misschien een natuurlijk fenomeen.
Spermacel dimorfisme is in verschillende plantensoorten aangetoond. Het is echter niet aanwezig in *Spinacia oleracea*. Geïsoleerde spermacellen vormen ideaal materiaal om spermacel dimorfisme te onderzoeken omdat grote hoeveelheden cellen kunnen worden geanalyseerd.

CURRICULUM VITAE

Cornelis Hubertus Theunis werd geboren op 9 februari 1961 te Venray. Aldaar bezocht hij het Boschveld College waar in 1979 hij het diploma Atheneum B behaalde. In dat zelfde jaar begon hij met de studie biologie aan de landbouwuniversiteit in Wageningen. Het kandidaatsdiploma werd gehaald in 1986. De doctoraalstudie omvatte het verzwaard hoofdvak plantencytologie en -morfologie en de bijvakken plantenfysiologie en pedagogiek en didactiek. De praktijktijd bracht hij door aan de Universiteit van Melbourne in Australië, en het schoolhospitium werd gedaan aan het stedelijk lyceum in Zutphen. In maart 1987 behaalde hij het ingenieursdiploma Biologie.
Van maart 1987 tot januari 1990 heeft hij onderzoek verricht bij de vakgroep plantencytologie en -morfologie van de landbouwuniversiteit te Wageningen. Van maart 1990 tot september 1991 werd dit onderzoek voortgezet bij de vakgroep omgevingsbiologie van de Universiteit van Siena in Italië. Dit onderzoek resulteerde in dit proefschrift.