INTRODUCTION

Considering the crucial role of the male gamete in double fertilization, it is remarkable that research on the physiology of sperm cells in flowering plants got under way only recently. According to Mendelian genetics, offspring receive one nucleus of the male gamete and one from the female gamete. The effect of DNA-containing cytoplasmic organelles in the male gamete (namely...
mitochondria and plastids) on the cytoplasmic organization of the offspring depends on the presence and quantity of these organelles in the sperm.

This review treats the function, isolation, and characterization of sperm cells, therefore summarizing research conducted mainly since 1984. Literature concerning pollen development (26), physiology (31, 32), incompatibility (9, 45), and the isolation and characterization of generative cells (30, 49) has been reviewed elsewhere.

DEVELOPMENTAL CONTEXT OF SPERM CELLS

The sperm of angiosperms are cellular descendants of meiotic divisions occurring in the anther of the flower. Meiosis in each meiocyte forms four genetically dissimilar microspores. Each microspore undergoes a mitotic division to form a large vegetative cell and a smaller, reproductive generative cell. Together these cells form the pollen grain or, strictly speaking, the microgametophyte. Subsequently, the generative cell migrates into the vegetative cell and becomes an elongated, spindle-shaped, or even filiform cell, depending on the species; each generative cell retains its own plasma membrane and is, in turn, enveloped by the vegetative cell membrane (26). The generative cell undergoes a second mitotic division to form the two sperm.

Depending on the timing of generative cell mitosis the two sperm cells may be formed before germination of the pollen (tricellular) or after pollen germination (bicellular). Of 243 families surveyed (11), bicellular pollen was present in 137 families (56%), tricellular pollen in 55 families (23%), and both types of pollen in 51 families (21%). Bicellular pollen is typically regarded as the ancestral condition in most families, tricellular pollen as the derived condition (11). Most of the research reviewed here concerns sperm cells isolated from pollen grains in tricellular species. Bicellular species must be cultured in order to grow pollen tubes, trigger mitosis, and obtain sperm cells.

Structural Characterization

Flowering plant sperm are structurally simple, apparently nonmotile cells that contain a normal complement of cellular organelles, including heritable cytoplasmic organelles such as mitochondria and, in some plants, plastids (10, 19). In contrast to animal species, competition in angiosperms is a property of the gametophyte (pollen grain and tube) and not the gamete; once this competition is completed, both sperm cells contribute to the formation of the embryo during double fertilization: One sperm cell fuses with the egg to produce the embryo, the other fuses with the central cell to produce the nutritive endosperm.

The sperm cells are typically connected to one another and physically
Table 1  Summary of the fertilization characteristics of the sperm cells in angiosperms

<table>
<thead>
<tr>
<th>Pollen type and species</th>
<th>Methoda</th>
<th>Morphologyb</th>
<th>Functional sperm</th>
<th>Sample</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Tricellular pollen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocotyledons:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hordeum vulgaris</td>
<td>Recon</td>
<td>I</td>
<td>—</td>
<td>n = 5</td>
<td>40</td>
</tr>
<tr>
<td>Zea mays</td>
<td>Recon</td>
<td>D</td>
<td>B+ (3, 55)</td>
<td>n = 1, 1, 1</td>
<td>37, 56, 57</td>
</tr>
<tr>
<td>Dicotyledons:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brassica campestris</td>
<td>Recon</td>
<td>MtD</td>
<td>—</td>
<td>n = 5</td>
<td>36</td>
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<tr>
<td>Brassica oleracea</td>
<td>Recon</td>
<td>MtD</td>
<td>—</td>
<td>n = 3</td>
<td>36</td>
</tr>
<tr>
<td>Euphorbia dulcis</td>
<td>Recon</td>
<td>MtD</td>
<td>—</td>
<td>n = 1</td>
<td>43</td>
</tr>
<tr>
<td>Gerbera jamesonii</td>
<td>Recon</td>
<td>MtD</td>
<td>—</td>
<td>n = 1</td>
<td>48</td>
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<tr>
<td>Plumbago zeylanica</td>
<td>Recon</td>
<td>MtPD</td>
<td>P+ (60)</td>
<td>n = 11</td>
<td>59</td>
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<td>Spinacia oleracea</td>
<td>Recon</td>
<td>MtD</td>
<td>—</td>
<td>n = 7</td>
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<td>Bicellular pollen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocotyledons:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gladiolus gandavensis</td>
<td>ImAn</td>
<td>D</td>
<td>—</td>
<td>n = 6</td>
<td>66</td>
</tr>
<tr>
<td>Dicotyledons:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>Recon</td>
<td>I</td>
<td>—</td>
<td>n = 9</td>
<td>H.-S. Yu, personal communication</td>
</tr>
<tr>
<td>Petunia hybrida</td>
<td>Recon</td>
<td>I</td>
<td>—</td>
<td>n = 1</td>
<td>77</td>
</tr>
<tr>
<td>Rhododendron spp.</td>
<td>ImAn</td>
<td>D</td>
<td>—</td>
<td>n = 7</td>
<td>66</td>
</tr>
</tbody>
</table>

a  ImAn: image analysis of nuclear fluorochromatic patterns; Recon: serial TEM and 3-D reconstruction
b  I: isomorphic; D: dimorphic (condition of organellar DNA unknown); MtD: mitochondrial dimorphism; MtPD: mitochondrial and plastid dimorphism
c  B+: sperm cell containing B-chromosomes; P+: sperm cell containing most plastids

associated with the vegetative nucleus, transported within the pollen tube as a "linked unit" (64) termed the "male germ unit" (14). The linkage of the sperm cells increases the effectiveness of gamete delivery, reduces heterofertilization, and ensures nearly simultaneous transmission of the two sperm cells into the receptive embryo sac (64). This association is present in most species of flowering plants studied to date (24) or is formed soon after pollen germination (41).

**Sperm Dimorphism and Preferential Fertilization**

Although the association between one sperm cell and the vegetative nucleus imposes a degree of polarity, strong cellular differences may also occur between the two sperm, including differences in the content of heritable organelles termed cytoplasmic heterospermy, and differences in the nucleus termed nuclear heterospermy (60). Although cytoplasmic heterospermy may be relatively common in flowering plants (Table 1), nuclear differences have been reported only in *Zea mays*, in which B-chromosomes frequently undergo nondisjunction during generative cell mitosis (3). The most common form of
cytoplasmic dimorphism is mitochondrial inequality: Sperm cells associated with the vegetative nucleus usually contain more mitochondria than the other cell. The most extreme form examined to date is found in *Plumbago zeylanica*, where both plastid and mitochondrial content differ (10, 59).

 Preferential fertilization, in which one sperm cell has a greater likelihood of fusing with the egg, may also occur in species where the sperm cells differ (Table 1). In *Plumbago*, fusion between the egg and the plastid-rich sperm cell occurs in 94% of the cases examined (60), selectively transmitting male plastids into the zygote (58). Transmission of male mitochondria into both the egg and central cell, however, occurs at a nearly constant 1:1000 ratio of male:female mitochondria (62), because the differences in mitochondrial content in the egg and central cell match those of the dimorphic sperm cells. Whether this ratio represents the maximum permissible dose of sperm mitochondria to prevent sperm transmission of mitochondrial DNA or the minimum needed to make recombination of mitochondrial DNA possible is unclear (62).

 Preferential fertilization (termed meiotic drive by geneticists) also occurs in *Zea*, in which the sperm cell containing one set of extra B-chromosomes fuses with the egg about 65% of the time (2, 3, 55). Rather than simply providing a marker for identifying the sperm cell destined to fuse with the egg, however, B-chromosomes appear to confer a selective advantage to the sperm cell that contains them (2). It is interesting that preferentiality may be eliminated by introducing excessive B-chromosomes into the sperm or by introducing a specific B-chromosome (TB-9b) into the egg. This observation suggests that maternal discrimination is active only under relatively strict conditions (2).

 Maternal control of fertilization is also suggested in a mutant line of barley in which seeds are produced containing normal endosperm but haploid embryos (38). One sperm cell apparently fuses with the central cell regardless of pollen source whereas the other remains unfused (38). Research on the mechanism of fertilization in normal lines of barley indicates that the zygote of plants with uniparental, maternal cytoplasmic inheritance are cytoplasmically restrictive: Male cytoplasmic organelles may be shed by the separation or pinching off of cellular folds during maturation of the sperm (40) and the sperm cytoplasm excluded from the egg, remaining outside of the cell (39). However, the central cell in the same plant may be cytoplasmically permissive, allowing sperm organelles to be transmitted into the central cell (39). Plants with biparental inheritance appear to be cytoplasmically permissive during the entire double fertilization process (58, 60).

**ISOLATION OF SPERM CELLS**

Sperm cell isolation was first reported in detail by Cass, who placed pollen grains of barley in a Brewbaker-Kwack (BK) pollen germination medium (1)
with 20% sucrose (4), obtaining release of the sperm cells by osmotic shock and observing the behavior of the living cells using Nomarski differential interference contrast microscopy. Over 30 min, the originally paired sperm cells separated, lost their spindle shape, and became ellipsoidal to spheroidal. Alternative media, such as filtrates of ovular material, were also examined but there were no specific benefits or effects on sperm cell appearance or behavior (4).

**Technical Details and Protocols**

Numerous isolation protocols have emerged (Table 2), including some modified for collection of sperm cells en masse (Table 3). Sperm cell release has been conducted using two general techniques: osmotic shock, or direct physical separation by grinding or applied pressure. The pollen cytoplasm and gametes are usually incubated for an additional 10–20 min to allow the gametes to separate from vegetative cell cytoplasm, and then pollen cell walls are removed by filtration. A nylon mesh two thirds to one half the average diameter of the pollen efficiently captures the pollen walls. En masse isolation of sperm cells involves either centrifugation on a discontinuous gradient or filtration using a polycarbonate (e.g. Nuclepore) filter. Details of the procedures used and results are given in Table 2.

Isolation of sperm cells using osmotic shock relies on the greater sensitivity of the vegetative cell membrane to changes in the milieu. In some cases, the shock of entering an aqueous medium from a nearly desiccated state is sufficient to rupture pollen grains regardless of the osmotic concentration used (29), but sperm cells may be surprisingly intact (61). After prehydration (for example, exposure in a humid chamber), pollen is more gradually introduced to a hydrated state, possibly enhancing viability of the isolated sperm cells (67). Pollen grains may then be subjected to an abrupt change in osmotic concentration (80) or pH (54) or lysed in other ways.

The isolation of sperm cells can also be accomplished by using physical stress to break the pollen plasma membrane. Methods used include glass tissue homogenizers (6, 67, 68) or the pressure of a glass roller applied to a smooth glass surface (71–73). Clearance height in the homogenizer is not critical, because the resistant exines will set the minimum. Isotonic or slightly hypertonic media are best for the isolation of the cells (68).

The most frequently used media for sperm isolation are modifications of the BK medium (1)—e.g. 100 ppm H$_3$BO$_3$, 300 ppm Ca(NO$_3$)$_2$·4H$_2$O, 200 ppm MgSO$_4$·7H$_2$O, 100 ppm KNO$_3$, and 10% sucrose. Other successfully applied media include the Roberts medium, which is a Tris-buffered modification of BK medium (52); a modified K3 Kao protoplast culture medium containing BK macronutrients, plus micronutrients, coenzymes, vitamins, and hormones (44); and the RY-2 protoplast medium used for plantlet regeneration (79).
<table>
<thead>
<tr>
<th>Pollen type and species</th>
<th>Medium*</th>
<th>Procedure</th>
<th>Evaluation technique*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tricellular Pollen:</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Monocotyledons:</td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>BKS 30</td>
<td>OS</td>
<td>DIC</td>
<td>4</td>
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<tr>
<td><em>Lolium perenne</em></td>
<td>RY-2 20</td>
<td>OS/G</td>
<td>FCR+, DAPI+, PCM</td>
<td>75</td>
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<tr>
<td><em>Secale cereale</em></td>
<td>BKS +d</td>
<td>GH</td>
<td>FCR+, Ho+</td>
<td>80</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>BKS 30</td>
<td>OS/G</td>
<td>FCR+, DAPI+, EtB+</td>
<td>35</td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>BKS 20</td>
<td>OS</td>
<td>FCR+, DAPI+</td>
<td>69</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>NA</td>
<td>OS</td>
<td>DAPI+, PCM</td>
<td>6</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>BKS 15</td>
<td>OS/G</td>
<td>FCR+, PCM, TEM</td>
<td>35</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>BKS 15</td>
<td>OS</td>
<td>FCR+, DAPI+, TEM, SEM</td>
<td>15</td>
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<tr>
<td><em>Zea mays</em></td>
<td>BKS 15</td>
<td>OS</td>
<td>FCR+, DAPI+, PCM</td>
<td>34</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>BKS 15 +</td>
<td>OS</td>
<td>FCR+, PCM</td>
<td>53</td>
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<tr>
<td><em>Zea mays</em></td>
<td>BKS 15</td>
<td>OS</td>
<td>EvB-, DIC, PCM, TEM, ABB, CBB</td>
<td>5</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>BKS/K3 30/15%</td>
<td>2-step OS/G</td>
<td>FCR+, Ho+</td>
<td>80</td>
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<tr>
<td><strong>Dicotyledons:</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Ambrosia sp.</em></td>
<td>BKS</td>
<td>OS</td>
<td>FCR+, EtB+, PCM</td>
<td>33</td>
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<td><em>Artemesia sp.</em></td>
<td>BKS</td>
<td>OS</td>
<td>FCR+, EtB+, PCM</td>
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<tr>
<td><em>Bellis sp.</em></td>
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<td>OS</td>
<td>FCR+, EtB+, PCM</td>
<td>33</td>
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<tr>
<td><em>Beta vulgaris</em></td>
<td>20 +g</td>
<td>OS</td>
<td>FCR ±, PI−, Ho, DIC</td>
<td>46</td>
</tr>
<tr>
<td><em>Brassica campestris</em></td>
<td>NA</td>
<td>GH</td>
<td>Ho+, PCM</td>
<td>6</td>
</tr>
<tr>
<td><em>Brassica campestris</em></td>
<td>M+T*h</td>
<td>GH</td>
<td>Ho+, PCM</td>
<td>23</td>
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<tr>
<td><em>Brassica napus</em></td>
<td>BKS 12.5</td>
<td>GH</td>
<td>FCR+, PCM</td>
<td>33</td>
</tr>
<tr>
<td><em>Brassica napus</em></td>
<td>RM+d</td>
<td>GH</td>
<td>FCR+, Ho+</td>
<td>80</td>
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<tr>
<td><em>Brassica oleracea</em></td>
<td>BKS 15</td>
<td>OS/G</td>
<td>FCR+, DAPI+, EtB+</td>
<td>35</td>
</tr>
<tr>
<td><em>Gerbera jamesonii</em></td>
<td>M-S+c</td>
<td>GH</td>
<td>FCR−, SEM, TEM</td>
<td>67</td>
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</tbody>
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### SPERM CELLS OF FLOWERING PLANTS

<table>
<thead>
<tr>
<th>Species</th>
<th>Medium Code</th>
<th>Culture Method</th>
<th>Staining</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gerbera jamesonii</td>
<td>S 1 M&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GH</td>
<td>EvB&lt;sup&gt;b&lt;/sup&gt;, DAPI&lt;sup&gt;b&lt;/sup&gt;, DIC, SEM</td>
<td>68</td>
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<tr>
<td>Impatiens sp.</td>
<td>S 20% +&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>DIC</td>
<td>46</td>
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<tr>
<td>Plumbago zeylanica</td>
<td>BKS 5-50</td>
<td>OS</td>
<td>EvB&lt;sup&gt;b&lt;/sup&gt;, FCR&lt;sup&gt;b&lt;/sup&gt;, Ho&lt;sup&gt;b&lt;/sup&gt;+, DIC</td>
<td>64</td>
</tr>
<tr>
<td>Plumbago zeylanica</td>
<td>20</td>
<td>OS</td>
<td>FCR&lt;sup&gt;b&lt;/sup&gt;+, EtB&lt;sup&gt;b&lt;/sup&gt;+, PCM</td>
<td>61</td>
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<tr>
<td>Senecio sp.</td>
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<td>OS</td>
<td>FCR&lt;sup&gt;b&lt;/sup&gt;+, PCM</td>
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<td>Spinacia oleracea</td>
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<td>P</td>
<td>FCR&lt;sup&gt;b&lt;/sup&gt;+, PCM, TEM</td>
<td>71, 72</td>
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<tr>
<td>Spinacia oleracea</td>
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<td>FCR&lt;sup&gt;b&lt;/sup&gt;+, PCM, SEM</td>
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<td>OS</td>
<td>FCR&lt;sup&gt;b&lt;/sup&gt;+, EtB&lt;sup&gt;b&lt;/sup&gt;+, PCM</td>
<td>33</td>
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### Bicellular Pollen

**Monocotyledons:**

<table>
<thead>
<tr>
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<th>Medium Code</th>
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<th>Staining</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lilium longiflorum</td>
<td>BKS 10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Enz&lt;sub&gt;i&lt;/sub&gt;, GH</td>
<td>My&lt;sup&gt;d&lt;/sub&gt;+, PCM</td>
<td>67</td>
</tr>
<tr>
<td>Gladiolus grandavensis</td>
<td>semi-vivo&lt;sup&gt;k&lt;/sup&gt;</td>
<td>OS&lt;sup&gt;k&lt;/sup&gt; or Enz&lt;sub&gt;i&lt;/sub&gt;</td>
<td>DAPI&lt;sup&gt;d&lt;/sup&gt;+, EtB, Ho, SEM</td>
<td>66</td>
</tr>
</tbody>
</table>

**Dicotyledons:**

<table>
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<tr>
<th>Species</th>
<th>Culture Method</th>
<th>Staining</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhododendron spp.</td>
<td>semi-vivo&lt;sup&gt;k&lt;/sup&gt;</td>
<td>OS&lt;sup&gt;k&lt;/sup&gt; or Enz&lt;sub&gt;i&lt;/sub&gt;</td>
<td>DAPI&lt;sup&gt;d&lt;/sup&gt;+, EtB, Ho, SEM</td>
</tr>
</tbody>
</table>

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<sup>a</sup> BKS: Brewbaker-Kwack pollen growth medium, plus sucrose (in %) (1); K3: modified Kao protoplast culture medium (44); M-S: mannitol-sucrose; M-T: mannitol-Tris; RM: Roberts medium (52); S: sucrose (in %); RY-2: medium (79); V: vitamins

<sup>b</sup> G: grinding; GH: glass homogenizer; OS: osmotic shock; P: pressure

<sup>c</sup> +: positive (dye incorporated); -: negative (dye excluded); ABB: Aniline blue black; CB: Coomassie brilliant blue; DAPI: 4,6-diamidino-2-phenylindole (8); DIC: differential interference contrast microscopy; EtB: ethidium bromide (22); EvB: Evans blue (17); FCR: fluorochromatic reaction using fluorescein diacetate (21); H: Hoechst 33258 (22); My: mytomycin (8); PCM: phase contrast microscopy; PI: propidium iodide (25)

<sup>d</sup> 5% sucrose, 8% sorbitol, 0.3% potassium dextran sulfate, 10 μg ml<sup>-1</sup> fluorescein diacetate

<sup>e</sup> Vitamins used: 0.5 mg ml<sup>-1</sup> nicotinic acid, 0.5 mg ml<sup>-1</sup> pyridoxine, 0.1 mg ml<sup>-1</sup> thiamine HCl, 2 mg ml<sup>-1</sup> glycine

<sup>f</sup> Sperm stored in 5% sucrose, 8% sorbitol, 0.5% potassium dextran sulfate, 10 μg ml<sup>-1</sup> fluorescein diacetate; K3: macronutrients

<sup>g</sup> 1 M mannitol, 10 mM Tris pH 7.5, 3 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 10 mM NaCl

<sup>h</sup> 0.4 M mannitol, 10 mM Tris pH 7.5, 3 mM CaCl<sub>2</sub>, 1.5 mM MgCl<sub>2</sub>, 10 mM NaCl

<sup>i</sup> 1 M sucrose, 2.1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1.6 mM H<sub>2</sub>BO<sub>3</sub>; pollen 20 mg 0.2 ml<sup>-1</sup>; grinding medium: 1 M mannitol, 0.2 M sucrose, 10 mM HEPES buffer (pH 7.2), 0.3-1.0% bovine serum albumin, 0.3% polyvinylpyrrolidone, 10 mM cysteine, 2.1 mM Ca(NO<sub>3</sub>)<sub>2</sub>

<sup>j</sup> Pollen tubes grown in vitro; enzymes: 2% cellulysin, 11 units mg<sup>-1</sup> pectinase for 2 hr at end of in vitro pollen tube growth

<sup>k</sup> Styles implanted in BKS 12 plus 0.6% agar for 24 hr in the dark until pollen tubes emerge (8 hr Gladiolus, 24 hr Rhododendron), then incubated in drop of 5 or 7.5% sucrose in medium for OS, or in enzymes<sup>b</sup>

<sup>l</sup> Enzymes: 0.5% macerozyme R-10 (Serva), 1% cellulase (Onozuka R-10, Serva) in BKS medium
Table 3  Summary of methods for collecting sperm cells en masse.

<table>
<thead>
<tr>
<th>Pollen type and species</th>
<th>Centrifugation*</th>
<th>Method*</th>
<th>Concentrationb</th>
<th>Viabilityc</th>
<th>Half-Lifed</th>
<th>Yeildse</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricellular Pollen:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocotyledon:</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Lolium perenne</td>
<td>15,000 × g 30 min</td>
<td>P (0/*/30%)</td>
<td>NA</td>
<td>PCM+</td>
<td>overnight</td>
<td>2%</td>
<td>75</td>
</tr>
<tr>
<td>* Triticum aestivum</td>
<td>NA</td>
<td>Sor (20/40%)</td>
<td>NA</td>
<td>most</td>
<td>15 min</td>
<td>NA</td>
<td>69</td>
</tr>
<tr>
<td>* Zea mays</td>
<td>NA</td>
<td>NDC (1.07 g ml⁻¹) Su*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>* Zea mays</td>
<td>9,000 × g 40 min</td>
<td>P (15*/40%)²</td>
<td>3 × 10⁶</td>
<td>90% FCR</td>
<td>20 hr</td>
<td>20%</td>
<td>15</td>
</tr>
<tr>
<td>* Zea mays</td>
<td>55 × g 3 min</td>
<td>BKS (15/*30)³</td>
<td>1.5 × 10⁶</td>
<td>&gt;50% EvB</td>
<td>3 hr</td>
<td>30%</td>
<td>5</td>
</tr>
<tr>
<td>Dicotyledons:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Beta vulgaris</td>
<td>3,000 rpm 15 min</td>
<td>P (20/30*/50%)</td>
<td>7.3 × 10⁶</td>
<td>30% F-PI</td>
<td>NA</td>
<td>NA</td>
<td>46</td>
</tr>
<tr>
<td>* Brassica campestris</td>
<td>NA</td>
<td>NDC (1.22 g ml⁻¹) Pt*</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>* Brassica campestris</td>
<td>800 × g 10 min P*</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>* Gerbera jamesonii</td>
<td>850 × g P*</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>* Gerbera jamesonii</td>
<td>850 × g 10 min P*</td>
<td>—</td>
<td>2 × 10⁵</td>
<td>NA</td>
<td>NA</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>* Plumbago zeylanica</td>
<td>8,000 × g 8 min</td>
<td>S (30%) Pt*</td>
<td>8.8 × 10⁶</td>
<td>most EvB</td>
<td>8 hr</td>
<td>60%</td>
<td>61</td>
</tr>
<tr>
<td>* Spinacia oleracea</td>
<td>13,000 × g 30 min</td>
<td>P (*/10/30/50%)</td>
<td>4 × 10⁶</td>
<td>90% F</td>
<td>18 hr</td>
<td>5-10%</td>
<td>71</td>
</tr>
<tr>
<td>* Spinacia oleracea</td>
<td>13,000 × g 40 min</td>
<td>P (20%*)</td>
<td>1 × 10⁶</td>
<td>90% F</td>
<td>30 hr i</td>
<td>5-10%</td>
<td>72, 73</td>
</tr>
<tr>
<td>Bicellular Pollen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocotyledon:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Gladiolus gandavensis</td>
<td>omitted</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>60%</td>
<td>66</td>
</tr>
<tr>
<td>Dicotyledon:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>* Rhododendron spp.</td>
<td>omitted</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>66</td>
<td></td>
</tr>
</tbody>
</table>

* Media for centrifugation: BKS: Brewbaker-Kwack pollen growth medium, plus sucrose (in %); NA: not available; P: Percoll gradient (discontinuous gradient, in %); S: sucrose pad (in %); Sor: sorbitol gradient (discontinuous gradient, in %); —: medium replaced by a 1 μm polycarbonate filter. Recovery location for sperm cells is indicated by asterisk (*) next to appropriate layer. Pt: pellet or Su: supernatant.

b  Sperm cells per milliliter

² Percentage viability as indicated by: EvB: Evans blue (−); FCR: fluorescein diacetate (+); F-PI: FCR (+) propidium iodide (−)

d  Viability half-life

e  Percentage recovery compared to the number of pollen grains treated × 2 sperm per pollen grain
Because few of the cited reports use parallel methodology, it is difficult to conclude which, if any, of the media is ideal for most angiosperms; but the life expectancy of isolated sperm cells seems strongly improved by complex media (Tables 2 and 3) and particularly by the addition of vitamins (53). As protocols are refined, a distinction will likely be made between isolation media (designed to optimize recovery) and storage or culture media (designed to extend sperm cell viability).

**Assessment of Sperm Cell Quality**

Sperm cells have been assessed for (a) structural intactness, (b) membrane integrity, (c) enzyme activity, and (d) respiratory activity. All of these aspects are appropriate; perhaps most crucial would be an assay of the cell’s ability to effect fertilization, which is not currently feasible.

Techniques to assay structural intactness include transmission (6, 15, 42, 68, 73, 74) and scanning electron microscopy (66, 68), which can be used to assess membrane structure and cell shape; intramembrane particle distribution can be inferred from freeze-fracture replicas (74). Integrity of membranes can be assessed using the exclusion of polar dyes such as Evans blue (17) or propidium iodide (25), both of which are rapidly absorbed into dead cells (Table 2). Enzyme activity is readily demonstrated using the so-called fluorochromatic reaction (21) in which fluorescein diacetate is cleaved by cellular esterase. Fluorescein diacetate is easily absorbed by living cells but becomes fluorescent only if esters are cleaved from the fluorescein molecule and the cell is intact enough to retain the polar dye. Respiratory activity has been demonstrated using an assay for ATP involving cleavage by luciferin yielding measurable luminescence. The results of this method roughly correspond to those using fluorescein diacetate (54) but ATP is still evident even when the cells are no longer viable according to the previous method.

During isolation, the sperm cells commonly assume an ellipsoidal to spheroidal shape and lose their microtubules (5, 15, 73). A comparable loss of microtubules has been described in detail using anti-tubulin immunofluorescence of freshly isolated generative cells in bicellular plants (70). These changes appear comparable to those observed in isolated generative cells (70). An exception to this is the isolation of sperm cells in *Gerbera*, in which the native shape is retained (68). Spheroidal sperm cells are not typically seen in young growing pollen tubes in vivo, but a significant rounding of these cells seems characteristic as pollen tubes near the ovule (38, 58, 64, 65). Although spheroidal sperm cells obtained through isolation are artifactual, this feature is common in somatic plant protoplasts, typically without impairing cell quality.

Isolated sperm cells lose the plasma membrane of the vegetative cell that usually surrounds them in vivo (26). This exposes the true surface of the
sperm cells (15, 64) and makes it possible to characterize the plasma membrane. In vivo, the same modification of the surface of the sperm cell occurs once sperm cells are discharged into the embryo sac (38, 58, 65) and is postulated to be a prerequisite for sperm fusion in angiosperms (58).

A significant remaining question about the condition of isolated sperm cells is whether they are completely mature in the pollen grain or whether essential gene products remain to be transcribed or translated during pollen tube growth prior to fertilization (66). Given the vast range of times between pollination and gametic fusion in different angiosperms (20 min to several months), these plants likely vary in this respect.

PHYSIOLOGICAL CHARACTERIZATION OF SPERM CELLS

Immunological Characterization

Hybridoma antibodies have been elicited to an inoculum of intact sperm cells of Brassica (23) and Plumbago (47) to generate antibody libraries to surface epitopes. Intact cells were used because of the small number of sperm cells available and the technical problems of preparing isolated plasma membranes. Depending on how long sperm cells remain intact in the inoculum, whole cells would be likely to elicit some antibodies to surface compounds. Initial attempts were partially successful, but techniques for retaining integrity of the sperm cells during inoculation and for increasing the sensitivity of screening for surface-specific epitopes will be needed before it is possible to produce a useful monoclonal antibody library for recognition studies.

The only detailed report on antibodies elicited to isolated sperm, in Plumbago (47), indicates the feasibility of this approach. Sperm antigens are effective in eliciting antibody-producing hybridoma lines to a wide spectrum of different epitopes in which pollen wall compounds, including allergens, are not immunodominant. The secreting lines were specific for epitopes in the sperm-enriched (23%), cytoplasmic-particulate (4%), and water-soluble fractions (8%). Antibody-producing lines were mainly IgM (61%), reflecting the high glycoprotein content of the cells (47). Identification of a recognition factor from this approach is complicated by the need to develop an appropriate functional assay. Further research in this area is warranted if specific recognition compounds occur as in pollen tube–pistil interactions (9, 45).

Polypeptide/Protein Characterization

Biochemical characterization of the proteins of male gametes using SDS-PAGE has been conducted on a small number of species: Brassica, Gerbera
(27), Plumbago (18), and Zea (P. Roeckel, C. Dumas, personal communication). These results indicate that unique polypeptide bands are readily identified in sperm fractions of higher plants. In order to reduce this effect, presence/absence comparisons with possible contaminants were conducted in Plumbago (18).

Two-dimensional gel electrophoresis (IEF/SDS-PAGE) was conducted to compare polypeptide heterogeneity in P. zeylanica (18). The sperm-enriched fraction and two selected contaminant fractions were examined. Cytoplasmic particulates pelleted at 100,000 × g constituted the first fraction; water soluble molecules collected from the supernatant formed the second fraction. The sperm-enriched fraction contained the most polypeptide spots (515 from Mr 33,000 to 205,000). The cytoplasmic-particulate fraction yielded 427 spots, and the water-soluble fraction had 285. One quarter of the spots were found in all fractions, about half were found in two fractions, and one quarter were found in a single fraction. Of the latter group, 51.9% of the polypeptides were unique to the sperm-enriched fraction, 40.4% to the water-soluble fraction, and 2% to the cytoplasmic-particulate fraction. Polypeptides of sufficient molecular weight to be involved in recognition (Mr >70,000) and >200,000) were present in the sperm-enriched fraction (18). Some of the differences between polypeptides undoubtedly result from generational differences of gene expression between the sporophyte and gametophyte (32). Although pollen wall proteins are synthesized by the sporophyte, gametophytic gene products may control gametic recognition, fusion, and fertilization.

No studies of developmental polypeptide changes in the male gametic line are currently available. Polypeptide changes in developing pollen and microspores of Zea (16), Triticum (76), and Brassica (13) indicate that developmental changes do occur. In Triticum, 11 new bands were detected in SDS-PAGE and IEF gels and 4 bands were lost during pollen maturation, indicating a small number of stage-specific changes. Changes during anther development in Zea (12) reflect similar stage-specific changes in the proteins and activities of specific enzymes. With further research, considerable progress in identifying sperm-specific polypeptides may be expected.

**Elemental Characterization**

Energy-dispersive X-ray analysis of pollen components indicates that sperm cells contain significant quantities of carbon, oxygen, phosphorus, calcium, and potassium, with smaller amounts of magnesium, silicon, manganese, and detectable levels of chromium. Although mostly similar, the dimorphic sperm of Plumbago exhibit small differences in Kα lines of calcium, potassium, and phosphorus (63). Other studies of pollen have indicated similar elemental
composition in the pollen tube tip (50). The surfaces of pollen grains in different species, however, display significant elemental differences (7).

**Molecular Characterization**

Conflicting information is available regarding the synthesis of RNA in sperm cells. In the tricellular pollen of *Secale cereale*, [5-3H]uridine was incorporated into both the sperm and vegetative nuclei during in vitro pollen tube growth (20). The types of RNA synthesized by the sperm nuclei were not determined. However, in the bicellular pollen of *Hyoscyamus niger*, exposure to short pulses of [5-3H]uridine did not detect incorporation (51). Determination of whether RNA synthesis is related to the timing of sperm cell formation will require additional research.

Translational products within sperm cells have been obtained by means of in vitro translation of isolated RNA species using [35S]methionine, combined with electrophoresis and radioautography (P. Roeckel and C. Dumas, personal communication). These studies demonstrate that translatable mRNA pools exist for a number of polypeptides in the sperm cells of corn; their identity has not yet been fully determined. The use of stage-specific promoters (e.g. 73a) and reporter genes in combination with particle-gun transformation to elicit transgenic plants seems to be a promising approach to understanding the developmental processes underlying sperm differentiation. Adaptation and application of existing molecular biological methods to sperm cell biology are critically needed.

**CONCLUSIONS AND OVERVIEW**

Sperm cells have only recently been isolated and used in basic and applied science. Living sperm cells have been isolated in many species of flowering plants; now key methodological improvements will be required if we are to extend the useful lifespan of these cells. Further research involving immunological characterization, cell sorting, cell culture, radiolabeling, gene expression, and molecular biology of the sperm cells will require basic knowledge about the maintenance and storage of sperm cells.

After these technical problems are solved, sperm cells could play a unique role in the developing field of reproductive-cell engineering. Because the cytoplasm of some sperm cells lacks plastids, it could be used to produce fusion hybrids with selected cytoplasmic constitutions. Sperm cells are naturally produced, haploid cells that may be a logical substitute for somatic protoplasts in specific instances or in germ plasm storage. Recently, Kranz and coworkers have combined the isolated egg and sperm cells of *Zea* using
electrofusion to produce a microcallus (28). As problems coping with gametic cells are resolved, in vitro fusion of male and female gametes of higher plants ex ovulo may help us to model and thus better understand fertilization in angiosperms.

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