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STERILITY OF *MISCANTHUS* ×*GIGANTEUS* RESULTS FROM HYBRID INCOMPATIBILITY

ANETA SŁOMKA[1*], ELŻBIETA KUTA[1], AGNIESZKA PŁAŻEK[2], FRANCISZEK DUBERT[3], IWONA ŻUR[3], EWA DUBAS[3], PRZEMYSŁAW KOPEĆ[2], GRZEGORZ ŻUREK[4]

[1]*Department of Plant Cytology and Embryology, Jagiellonian University, Grodzka 52, 31-044 Cracow, Poland*
[2]*Department of Plant Physiology, University of Agriculture, Podłużna 3, 30-239 Cracow, Poland*
[3]*Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Cracow, Poland*
[4]*Department of Grasses, Leguminous and Energy Plants, Institute of Plant Breeding and Acclimatization, Radzików, 05-870 Błonie, Poland*

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Miscanthus ×*giganteus* Greef et Deu. (Poaceae), a hybrid of *Miscanthus sinensis* and *M. sacchariflorus* native to Japan, is an ornamental and a highly lignocellulosic bioenergy crop, cultivated in the European Union as an alternative source of energy. This grass reproduces exclusively vegetatively, by rhizomes or via expensive in vitro micropropagation. The present study was aimed at finding the barriers that prevent sexual seed production, based on detailed embryological analyses of the whole generative cycle, including microsporogenesis, pollen viability, megasporogenesis, female gametophyte development, and embryo and endosperm formation. Sterility of *M.* ×*giganteus* results from abnormal development of both male and female gametophytes. Disturbed microsporogenesis (laggard chromosomes, univalents, micronuclei) was further highlighted by low pollen staining. The frequency of stainable pollen ranged from 13.9% to 55.3% depending on the pollen staining test, and no pollen germination was observed either in vitro or in planta. The wide range of pollen sizes (25.5–47.6 μm) clearly indicated unbalanced pollen grain cytology, which evidently affected pollen germination. Only 9.7% of the ovules developed normally. No zygotes nor embryos were found in any analyzed ovules. Sexual reproduction of *M.* ×*giganteus* is severely hampered by its allotriploid (2n=3x=57) nature. Hybrid sterility, a strong postzygotic barrier, prevents sexual reproduction and, therefore, seed formation in this taxon.

Key words: *Miscanthus* ×*giganteus*, allotriploid, pollen viability, female gametophyte development, sterility.

INTRODUCTION

Miscanthus ×*giganteus* (= *M. ogiformis* Honda; Poaceae, tribe Andropogoneae, subtribe Sacchnrineae), a grass and a highly lignocellulosic bioenergy perennial crop with a C4 photosynthetic system, is cultivated all over the world as an ornamental plant and also as an alternative source of energy. The grass is a natural interspecific triploid hybrid (2n=3x=57) originated from crosses between diploid *M. sinensis* (2n=2x=38) and allotetraploid *M. saccachriflorus* (2n=4x=76). The allotriploid origin of this grass has been confirmed based on characters including micromorphological (stomata cell size), cytogenetic (genome size, chromosome number and structure) and molecular (DNA sequencing, AFLP) traits (Linde-Laursen, 1993; Lafferty and Lelley, 1994; Hodkinson et al., 2002; Rayburn et al., 2009). Its potential as an economically important crop has not been fully harnessed due to infertility (Chou, 2009).

Commercially available plants are *M.* ×*giganteus* clones sharing the same haplotype, as confirmed by chloroplast DNA markers (de Cesare et al., 2010), and are partly or totally sterile due to disturbed microsporogenesis (Adati and Mitsuishi, 1955; Linde-Laursen, 1993; Lafferty and Lelley, 1994). The lack of regular chromosome pairing in

*e-mail: a.slomka@iphils.uj.edu.pl

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meiosis led to reduced pollen stainability ranging from 0% staining pollen (Linde-Laursen, 1993) to ~50.6% stainable pollen grains (Adati, 1958; the author named this allotriploid *M. sacchariflorus*). The plants are totally (Lafferty and Lelley, 1994) or partly infertile, producing seeds with very low frequency (Linde-Laursen, 1993). The genetic identity of clones obtained mainly via rhizomes translates to high uniformity of morphological, physiological and anatomical characters, which presents a risk for agriculture, hence the imperative to create other sources of plant diversity (Lewandowski et al., 2003). In the last decade, recently established populations of *M.* ×*giganteus* that could serve as a new gene source have been found in sympatric populations of its parents in Japan (Nishiwaki et al., 2011). An alternative pathway for obtaining genetic differentiation employs biotechnological methods to modify existing genotypes by transformation or by plant multiplication in vitro via organogenesis or somatic embryogenesis (Lewandowski, 1999; Płażek and Dubert, 2010 and lit. cited therein). Somaclonal variation induced in regenerated *M.* ×*giganteus* plants by culture conditions could represent new genotypes (Seok Kim et al., 2010). *Miscanthus* ×*giganteus* plant fertility is increased by doubling the chromosome number in vitro and ex vitro to obtain stable hexaploid plants (Głowacka et al., 2009, 2010; Yeon Yu et al., 2009). The obtained derivatives have higher pollen viability and produce more seeds than the parents (Głowacka et al., 2010). The usefulness of newly obtained genotypes as biofuel material is now being investigated. Another technique that could be applied to increase the genetic diversity of *M.* ×*giganteus* is polyploid-to-monoploid reduction by haploidization followed by breeding among monoploid plants in which it is simpler to analyze segregation ratios and inheritance of desirable quantitative traits. Finally, a polyploid condition can be restored through sexual polyploidization or colchicine treatment. This technique gives good results mainly in autopolyploids but is also possible in allopolyploids producing euploid gametes (Khan et al., 2009 and lit. cited therein). Potentially, in allotriploid *M.* ×*giganteus* both aneupoid and euploid (1×, 2×, 3×) gametes can be formed. Reduced ploidy level (haploidization) could be obtained by backcrossing or by in vitro andro- or gynogenesis in culture of microspores (or uninucleate pollen grains) and unfertilized ovules (or ovaries, pistils), respectively. Both in vitro techniques require production of viable pollen and female gametophytes with functioning haploid elements (egg, synergids, antipodes). A detailed assessment of *M.* ×*giganteus* fertility is indispensable to any attempts at androgenesis, gynogenesis or backcrossing. Allotriploid *M.* ×*giganteus* might prove as useful for crop improvement provided that it can be brought to an adequate level of fertility.

In this work we (i) reassessed previous divergent results on pollen viability based on histochemical tests adding more sensitive viability tests, and germination of pollen grains on stigmas and on artificial media in order to determine the possible role of pollen as the male genome donor in reproduction; and (ii) analyzed female gametophyte development and embryo formation, not previously investigated in *M.* ×*giganteus* and other *Miscanthus* species. Both tasks yielded information about *M.* ×*giganteus* fertility and consequently about its potential in andro- and gynogenesis or backcrossing and as a bridge genotype for other breeding purposes in the genus *Miscanthus*.

MATERIALS AND METHODS

PLANT MATERIAL

Miscanthus ×*giganteus* rhizomes were obtained from the Institute of Plant Breeding and Acclimatization in Radzików near Warsaw (Poland). The plants were cultivated from September to August in a glasshouse in 15 dm^3 pots filled with commercial soil (pH=5.8) at 25°C and 65% humidity in daylight supplemented according to need with light at 400 $\mu mol\ m^{-2}\ s^{-1}$ from AgroPhilips lamps. In the winter a 12 h photoperiod was kept, while from the spring only natural light length was maintained. Fresh and fixed inflorescences at different developmental stages were analyzed.

POLLEN CHARACTERISTICS

Pollen size and stainability

For acetocarmine, lactophenol blue and Alexander pollen stainability tests, six inflorescences (one from six different plants) with freshly opened flowers were fixed in acetic alcohol (glacial acetic acid and 96% ethanol, v/v 1:3). Seven thousand pollen grains isolated from 14 flowers were analyzed. The pollen stainability of each flower was estimated from three applied tests. Anthers from each flower were separated onto three glass slides, and their pollen grains were stained separately with acetocarmine, Alexander dye or lactophenol blue. For fluorescein diacetate (FDA) staining, pollen from non-fixed, newly opened flowers (from the same plants as for the other tests) was used immediately.

Acetocarmine stain (1%) was used for the acetocarmine test (Singh, 2003). Cytoplasm of viable pollen stains red, and nonviable pollen remains transparent.

Alexander's dye is a mixture of malachite green staining the cellulose of pollen walls green, and acid fuchsin staining the pollen protoplast red (Singh, 2003). Viable pollen grains appear purple, and non-viable pollen are green.

FDA dye was prepared according to Dafni (1992) [2 ml 20% saccharose in distilled water with several drops of FDA stock solution (2 mg FDA/1 ml acetone)]. Stained pollen was kept in a humid chamber for 0.5 h at 24°C. This technique is based on FDA entering the vegetative cell where it is hydrolysed by esterase to a fluorescein. Viable pollen fluoresces yellow-green.

Pollen stainability results were compared with ANOVA and the Tukey post hoc test in Statistica 7.0.

Pollen size was measured from 500 stainable (acetocarmine test) pollen grains with a calibrated eyepiece micrometer.

Pollen germination

Sixty opened flowers were cross-pollinated by hand. Pistils were fixed in acetic alcohol (v/v 1:3) 1, 3, 8 and 24 h after pollination. For clearing, pistils were placed in 0.1 M NaOH for 0.5 h at 40°C and after rinsing with distilled water were stained with 0.01% toluidine blue for 15 min to suppress autofluorescence, then stained with 0.1% aniline blue diluted in 0.15 M K_2HPO_4 for 1 h (Wędzony, 1996). Pollen tubes exhibit light blue fluorescence.

For pollen germination on medium, the micro- and macroelement content of the medium followed Yuanjie et al. (2009), who applied it for *Miscanthus sinensis* (one of the parental species of *M.* ×*giganteus*). The medium contained 10 μg H_3BO_3, 30 μg $Ca(NO_3)_2 \cdot 4H_2O$, 20 μg $MgSO_4 \cdot 7H_2O$ and 10 μg KNO_3, dissolved in 1 ml distilled water. Different sucrose concentrations were tested, adjusted to 15%, 20%, 25% or 30% by adding the amount required to the 1 ml water solution of micro-and macroelements. Pollen isolated from 40 flowers was tested for germinability in a humid chamber at 23/24°C and 18/19°C for 3 h, as recommended by Yuanjie et al. (2009).

FEMALE GAMETOPHYTE DEVELOPMENT AND EMBRYO FORMATION

Paraffin method

83 flowers at different stages of development collected from six plants were fixed in FAA (ethyl alcohol, glacial acetic acid, formaldehyde, distilled water, 10:1:2:7 v/v) for 24–48 h and then stored in 70% ethanol at 4°C until used. Fixed flowers were dehydrated in a graded ethanol series (30%, 50%, 70%, 96%, 100%, 100%, 100%), embedded in paraffin and sectioned 10 μm thick on a rotary microtome (Reichert), transferred to glass slides, stained with Heidenhain's hematoxylin combined with alcian blue, and mounted in Entellan (Aldrich).

Clearing method

63 flowers at different stages of development were fixed in FAA (as above) and stored in 70% ethanol at 4°C until use. Ovules dissected from the pistils (mature stages) and whole pistils (young stages) were dehydrated in 70%, 96% and 100% ethanol for 1 h each, and cleared in methyl salicylate by placing them in increasing concentrations of methyl salicylate diluted in 100% ethanol (1:3, 1:1, 3:1 v/v) for 1.5 h each. Cleared ovules were stored in methyl salicylate in vials. The preparations were made according to Herr (1971).

MICROSCOPY

All examinations were made with a Nikon E80i microscope with bright-field illumination (ovule sections, pollen stained with acetocarmine, Alexander dye, lactophenol blue), Nomarski interference contrast (cleared ovules), fluorescence filter B-2A (pollen stained with FDA) or filter UV-2A (pollen germination on stigma and on medium). Photographs were taken with a Zeiss Axio Cam MRe digital camera coupled with Zeiss Axio Vision 3.0 software.

RESULTS

MICROSPOROGENESIS, POLLEN STAINABILITY AND GERMINATION, DEVELOPMENT OF MALE GAMETOPHYTE

Microsporogenesis was disturbed in 12.5% of 127 analyzed microsporocytes. Laggard chromosomes and micronuclei occured at the first and second meiotic divisions (Fig. 1a–f), leading to abnormal tetrad formation and finally to reduced pollen stainability, to formation of cytologically unbalanced pollen grains differing in size, and to lack of pollen germination.

Pollen stainability was test-dependent. 55% of the pollen grains stained in acetocarmine, Alexander dye and lactophenol blue, reagents which detect only the presence of cytoplasm in cells (Tab. 1, Fig. 1g–i). Staining with FDA, a reagent detecting the enzymatic activity of pollen grains, the results were significantly different ($p<0.05$); the mean value of pollen stainability in FDA did not exceed 13.9% (Tab. 1). Significant variation of pollen size resulting from disturbed meiosis was clearly visible in all tests (Fig. 1g–i). Pollen diameter ranged from 25.5 to 47.6 μm; 34 μm was the most frequent diameter (21.4%) (Fig. 2). Giant pollen reaching 42.5 μm were fairly frequent (7.1%). Normal development of the

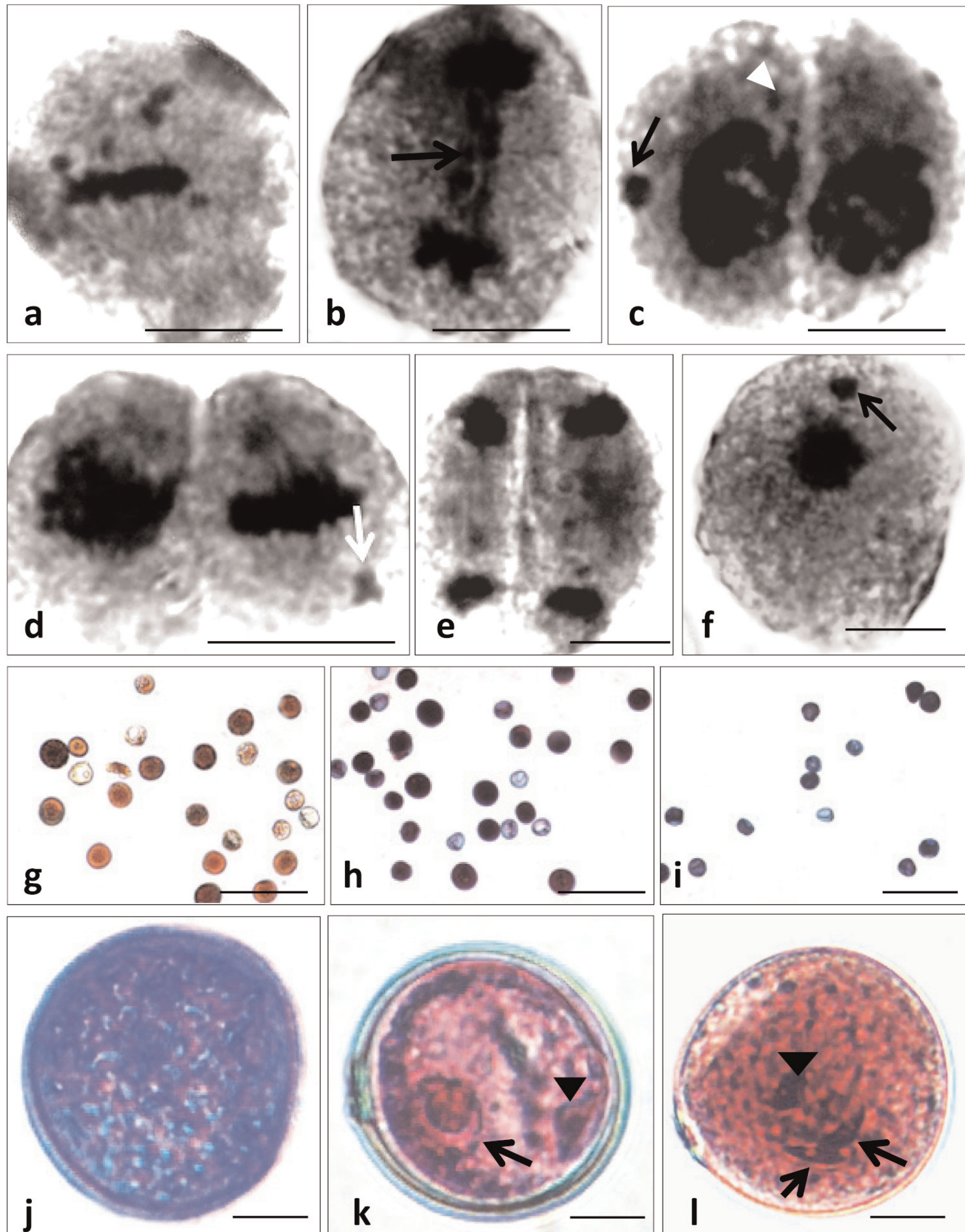

Fig. 1. *Miscanthus ×giganteus*. Microsporogenesis and microspore (**a–f**), pollen viability (**g–i**), male gametophyte development (**j–l**). (**a**) Metaphase I with univalents scattered along the spindle, (**b**) Telophase I with laggard chromosomes (arrow), (**c**) Diad with micronucleus (arrow) and single chromosome (arrowhead), (**d**) Metaphase II with chromosomes located behind metaphase plate (arrow), (**e**) Telophase II with chromatids scattered along the spindle, (**f**) Microspore with micronucleus (arrow), (**g–i**) Viable (stainable) and non-viable (non-stainable) empty pollen grains differing in size, treated with acetocarmine (**g**), Alexander dye (**h**), lactophenol blue (**i**), (**j**) Normal microspore, (**k**) 2-nucleate pollen grain with vegetative (arrow) and generative (arrowhead) nuclei, (**l**) 3-nucleate pollen grain with vegetative nucleus (arrowhead) and two sperm cells (arrows). Bars in a–f, j–l = 10 μm, in g–i = 100 μm.

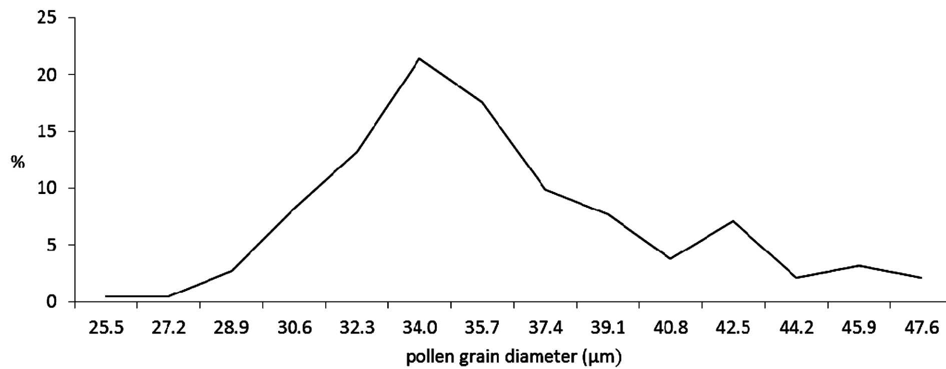

Fig. 2. Diagram of pollen size distribution in *Miscanthus ×giganteus*.

male gametophyte starting from the uninucleate microspore and leading to 3-celled pollen grains (Fig. 1j–l) occured in 10% of acetocarmine-stained pollen.

Pollen viability was not correlated with pollen germination. None of the pollen grains germinated either on any artificial media used or on stigmas after cross-pollination by hand.

TABLE 1. Frequency of stainable pollen grains of *Miscanthus ×giganteus* in different staining tests

Plant no./ flower no.	Frequency of stainable pollen grains (%)			
	Aceto-carmine	Alexander	Lactophenol blue	Fluorescein diacetate (FDA)
1/1	57.7	56.1	64.0	14.0
1/2	57.9	57.1	63.2	14.0
2/1	49.4	43.2	48.3	13.3
2/2	51.4	53.2	49.3	13.3
2/3	54.5	65.0	51.5	17.2
3/1	54.5	62.0	51.9	17.2
3/2	51.7	59.0	55.1	15.2
4/1	52.9	53.0	55.0	12.2
4/2	48.4	43.4	51.5	11.3
5/1	51.0	54.0	56.0	15.2
5/2	62.0	52.3	57.8	14.3
6/1	66.1	46.2	52.7	11.0
6/2	42.7	51.3	49.3	10.9
6/3	68.0	75.0	69.0	16.1
Mean	54.9^{a}	55.1^{a}	55.3^{a}	13.9^{b}
Standard deviation	6.7	8.2	6.0	2.0

Values with different letters differ significantly at $p<0.05$ by ANOVA followed by the Tukey post hoc test

MEGASPOROGENESIS, FEMALE GAMETOPHYTE DEVELOPMENT

Regular megasporogenesis and normal female gametophyte development according to the monosporous Polygonum type were found in only 9.7% of the analyzed ovules (Fig. 3a–d). One megaspore mother cell (Fig. 3a) underwent meiotic division and gave rise to a linear megaspore tetrad. Three of the four megaspores degenerated, and the chalazal megaspore developed into a 1-nucleate female gametophyte (Fig. 3b). Three successive mitoses led to the formation of a 2-nucleate (Fig. 3c), 4-nucleate and finally 8-nucleate female gametophyte. The 7-celled (8-nucleate) female gametophyte consisted of two synergids, an egg cell, three antipodes and the central cell with 2 polar nuclei, the latter fused, forming a secondary nucleus (Fig. 3d).

In 90.3% of the ovules, megasporogenesis and female gametophyte development were severely disturbed or else deviated from what is described as typical for grasses (Fig. 3e–k). Abnormal development was usually associated with degeneration processes observed in the early stages of ovule development, before megasporogenesis (Fig. 3e), shortly after female meiosis, and in more advanced stages of female gametophyte development, leading to degeneration of young and older gametophytes and cells in the mature female gametophyte (Fig. 3f–h). Enlarged somatic cells of the nucellus were observed in several ovules. They accompanied the abnormal and degenerating sexual female gametophytes (3i–k). Multiple female gametophyte-like structures probably originated from enlarged nucellus cells and therefore could be identified as aposporous female gametophyte initials (Fig. 3i–k). The stage of their development was difficult to determine because they began to degenerate very early. Single unorganized female gametophytes and also incompletely developed ones forming only two polar nuclei or else an egg cell with synergids and one polar nucleus were observed. No embryos or endosperm were noted in any of the analyzed ovules. Plants did not set seeds at all.

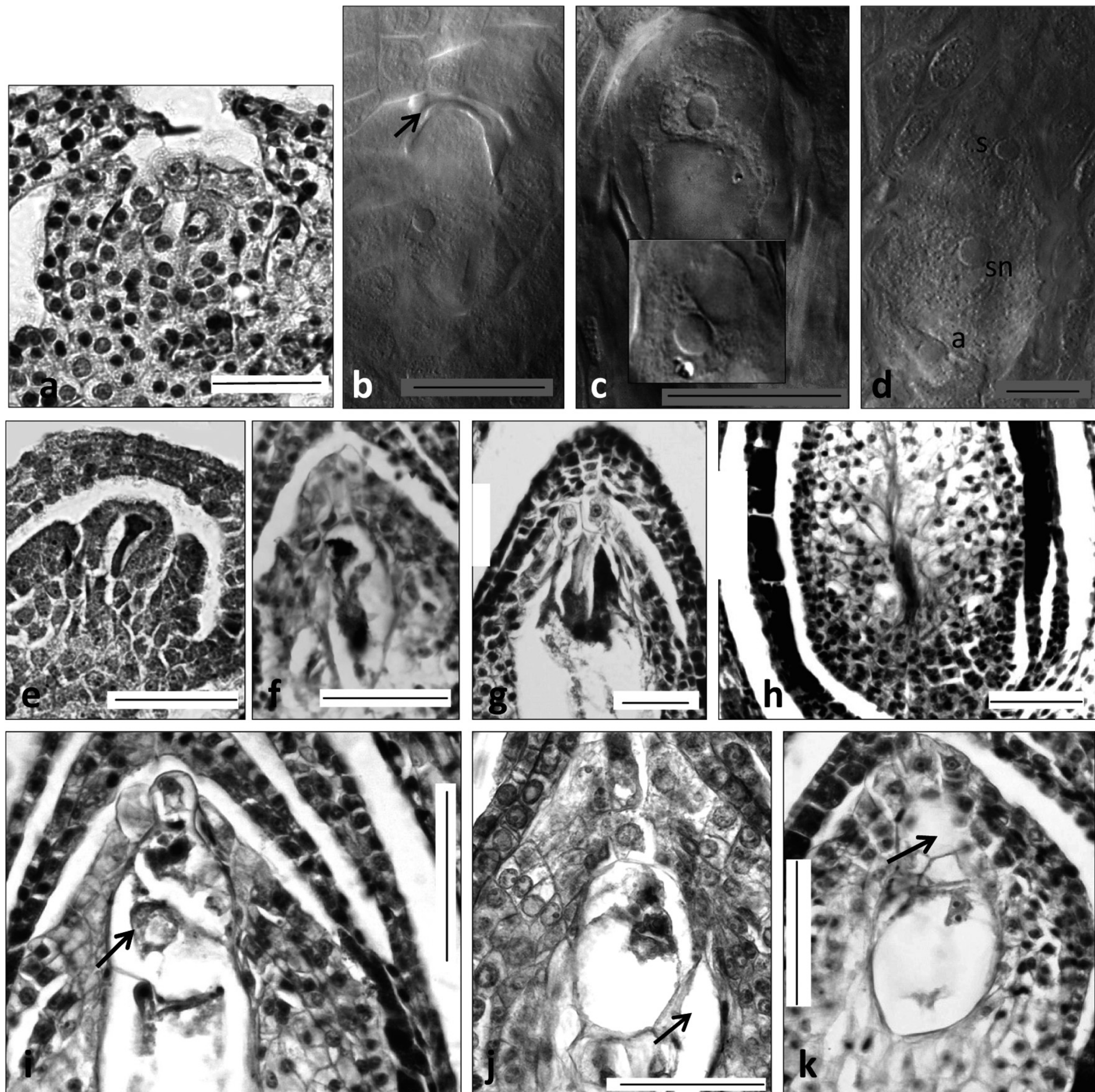

Fig. 3. *Miscanthus ×giganteus*. Processes in ovules. (**a–d**) Normal development of female gametophyte: (**a**) One mega – spore mother cell, (**b**) 1-nucleate female gametophyte with remnants of degenerated megaspores (arrow), (**c**) 2-nucleate female gametophyte, (**d**) 7-celled mature female gametophyte; one antipodal cell (a) secondary nucleus (sn) and synergid (s) visible, (**e–h**) Degeneration at different stages of female gametophyte development: (**e**) Degenerated megaspore mother cell, (**f**) Degenerated elements of female gametophyte, (**g**) Degenerated elements of egg apparatus, (**h**) Remnants of mature female gametophyte, (**i–k**) Enlarged somatic cells (arrows) accompanying abnormal and degenerating sexual female gametophytes. Bars in a–e = 50 µm, in f–k = 100 µm.

DISCUSSION

Triploids with an odd-numbered chromosome complement are sterile or their fertility is highly reduced due to a genetic imbalance and disturbed chromosome pairing in male and female meiosis. Usually they reproduce clonally by rhizomes or micropropagules and are characterized by low genetic

diversity. However, triploids could produce some fertile gametes which might allow these cytotypes to be used to produce higher polyploids (tetraploids, pentaploids, hexaploids) or aneuploids in economically important plants such as *M. sinensis* (Rounsaville et al., 2011), melon, blueberry, elm, aspen and poplar (Wang et al., 2010 and lit. cited therein).

The wide range of pollen sizes in *M. ×giganteus* may indicate that the pollen grains represent different levels of ploidy (euploid and aneuploid). This is supported by the finding that when a plant produces mainly reduced pollen grains, the diameter distribution curve usually resembles a normal distribution (Tondini et al., 1993) unlike the curve obtained for *M. ×giganteus*. The stainability of pollen (despite their size) estimated by the acetocarmine test was moderate (54.9%) as compared with the pollen viability results for other allotriploids, ranging from 10% in *Cucumis hytivus* up to 90.3% in *Populus tremula* (Wang et el., 2010), and is in accord with results reported for *M. ×giganteus* by Adati (1958). In our study the pollen stainability did not differ significantly between the acetocarmine test and the other two tests that stain pollen grain cytoplasm (~55%), but differed significantly from the FDA test (13.9%). The latter is the most sensitive of the four tests used, and showed the most significant reduction of pollen stainability. These data make it evident that the frequency of stainable pollen strongly depends on the test applied, so caution is required in its assessment (Słomka et al., 2010). Furthermore, pollen stainability is not an indication of pollen's potential for germination or cytologically balanced pollen, as pollen grains in different size are also stainable. The pollen of *M. ×giganteus* showed no germinability, neither on media nor in planta (on stigmas after artificial cross-pollination), dramatically reducing the usefulness of this taxon as a donor plant in various crossing experiments. However, there is an opportunity to use it for further manipulations with the help of in vitro culture. 13.9% of the stainable, regular-shaped pollen cultured on media supplemented with growth regulators and under special conditions could switch from the gametophytic to the sporophytic pathway and developed androgenic embryos, being a source for production of viable homozygous, doubled haploids.

Triploid sterility, with some exceptions such as the female-fertile allotriploid *Lilium lankongense* (Proscevičius et al., 2007), results also from abnormal female gametophyte development (Rousaville et al., 2011). In *M. ×giganteus* the female gametophytes sporadically reach the mature, properly organized stage. Usually they were abnormally organized and began to degenerate very early. This pattern is a typical phenomenon in hybrids, segmental allopolyploids arising from interspecific crosses between closely related species, resulting from intragenomic conflicts – hybrid incompatibility (Moyle and Nakazato, 2008). In *M. ×giganteus* such interaction of genes arises from the close relationship between its parents as *Miscanthus sinensis* (diploid) and *M. sacchariflorus* (allotetraploid containing the *M. sinensis* genome) share a common genome (Hodkinson et al., 2002; Rayburn et al., 2009). Ovules with normal mature female gametophytes might serve as a source for production of haploids by way of gynogenesis in culture. The unreduced female gametophytes that accompanied the sexual ones also potentially can undergo development. These structures resembling unorganized aposporous and also incomplete monopolar female gametophytes without antipodes, with polar nuclei and an egg cell and a synergid, or with polar nuclei only (Bhanwra, 1988; Ma and Huang, 2007; Guohua et al., 2009) observed in *M. ×giganteus* may be interpreted as a tendency toward apomixis (apospory). Although apomixis could have been inherited from *M. sinensis* (Chou et al., 2000), apomictic female gametophytes were arrested at an early stage of development or else degenerated and did not develop embryos. The tendency probably is blocked by the triploid and hybrid nature of *M. ×giganteus*, as in the triploid hybrid *Paspalum* in which apospory was inherited from the apomictic 4x parent but was not strong enough to prevent seed sterility (Carlos et al., 2004).

It is evident from the present embryological study that (1) *M. ×giganteus* is sterile due to hybrid incompatibility, resulting in the failure of normal male and female gametophyte development and embryo formation, and therefore is not good material for crossing experiments; (2) New genotypes might be produced by way of andro- and gynogenesis using in vitro techniques to culture viable microspores, uninucleate pollen, or unpollinated ovules even if they arise with very low frequency.

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