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DISTRIBUTION OF MICROTUBULES DURING REGULAR AND DISTURBED MICROSPOROGENESIS AND POLLEN GRAIN DEVELOPMENT IN *GAGEA LUTEA* (L.) KER.-GAW.

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The microtubular cytoskeleton in dividing microsporocytes and developing pollen grains of *Gagea lutea* (L.) Ker.-Gaw. (Liliaceae) was investigated with a modified indirect immunofluorescence method. Meiotic and mitotic stages were identified by DAPI staining. The microtubular cytoskeleton was compared in plants originating from natural localities and others grown in the laboratory. In natural conditions, microsporocytes and pollen grains of wild early-spring *Gagea lutea* plants are subjected to abiotic factors including cold exposure and lack of water. The persistent influence of these factors can disturb microtubular cytoskeleton functioning. The following disturbances were observed in the course of microsporogenesis and pollen development: abnormal chromosome configurations in the metaphase of meiosis I; abnormally divided dyads with irregular, radial microtubule systems around the nuclei; the formation of differently sized microspores with irregular shapes, and irregular division; and the formation of pollen grains with vacuoles abnormal for their development stage. Similar kinds of disturbances were observed after 1.5 months of cold treatment (4°C) and drying in the laboratory. These abiotic factors simulated in laboratory conditions caused more disturbances in the course of microsporogenesis and produced more frequent defective pollen grains than in the sample that had experienced cold and drying in natural conditions.

Key words: *Gagea lutea*, microtubular cytoskeleton, microsporogenesis, disturbances, abiotic factors.

INTRODUCTION

Microtubules are major components of all eukaryotic cells, playing a basic role in processes such as chromosome segregation, cellulose deposition, vesicle transport, cell motility, maintenance of cell shape, and cell expansion (Waldin et al., 1992). Because of their great importance in the cell, microtubules have been studied intensively by different methods (for references, see: Vesk et al., 1996). The division of meiotic cells in plants involves microtubule configurations, most of which are similar to those present in somatic tissues. Three microtubular configurations occur in meiotic plant cells during microsporogenesis: the microtubular system in the cortical cytoplasm and at the nuclear envelope; metaphase I and II spindles; and phragmoplasts after the first and second meiotic divisions. These configurations have been the subject of many detailed investigations (van Lammeren et al., 1985; Hogan, 1987; Brown and Lemmon, 1988b, 1991a, 1996, 2000; Traas et al., 1989; Liu et al., 1993; Genualdo et al., 1998; Giełwanowska et al., 2003, 2005). They appear regularly during

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typical meiosis completed with successive as well as simultaneous cytokinesis.

In the meiocytes of the successive type of cytokinesis (sporo- and microsporocytes), microtubules forming the phragmoplast develop in the equatorial region following meiosis I; in the middle plane, the cell plate is set up to divide the binucleate meiocyte (Brown and Lemmon, 199la). After meiosis, the final quadripartitioning into four microspores takes place with the formation of microtubule arrays that interconnect the nuclei in both halves of the dyad. In general, the planes of division in micro- and sporogenesis depend on the position of the nuclei, which define cytoplasmic domains via a radial system of microtubules (Brown and Lemmon, 1988a,b, 1991a,b, 1996). The organization of microtubules during pollen grain development resembles the organization found during mitotic division, although the division into a two-cellular pollen grain is asymmetric.

Microsporogenesis and pollen grain development have been at the center of interest of biologists because generative reproduction in plants depends on proper pollen structure and function. Proper organization of the microtubular cytoskeleton during meiotic and mitotic divisions is essential for the formation of high-quality, viable pollen grains. Disturbances during both processes decrease the chance of effective pollination and fertilization of maternal plants.

In this paper we report on the distribution of microtubules (microtubular cytoskeleton elements) formed during regular microsporogenesis and pollen grain development in *Gagea lutea*, and describe disturbances in the course of both these processes. We also discuss the influence of abiotic factors such as variable temperature (cold treatment) and periodic lack of water on the microtubular cytoskeleton during these processes, which are so significant for plant reproduction.

MATERIALS AND METHODS

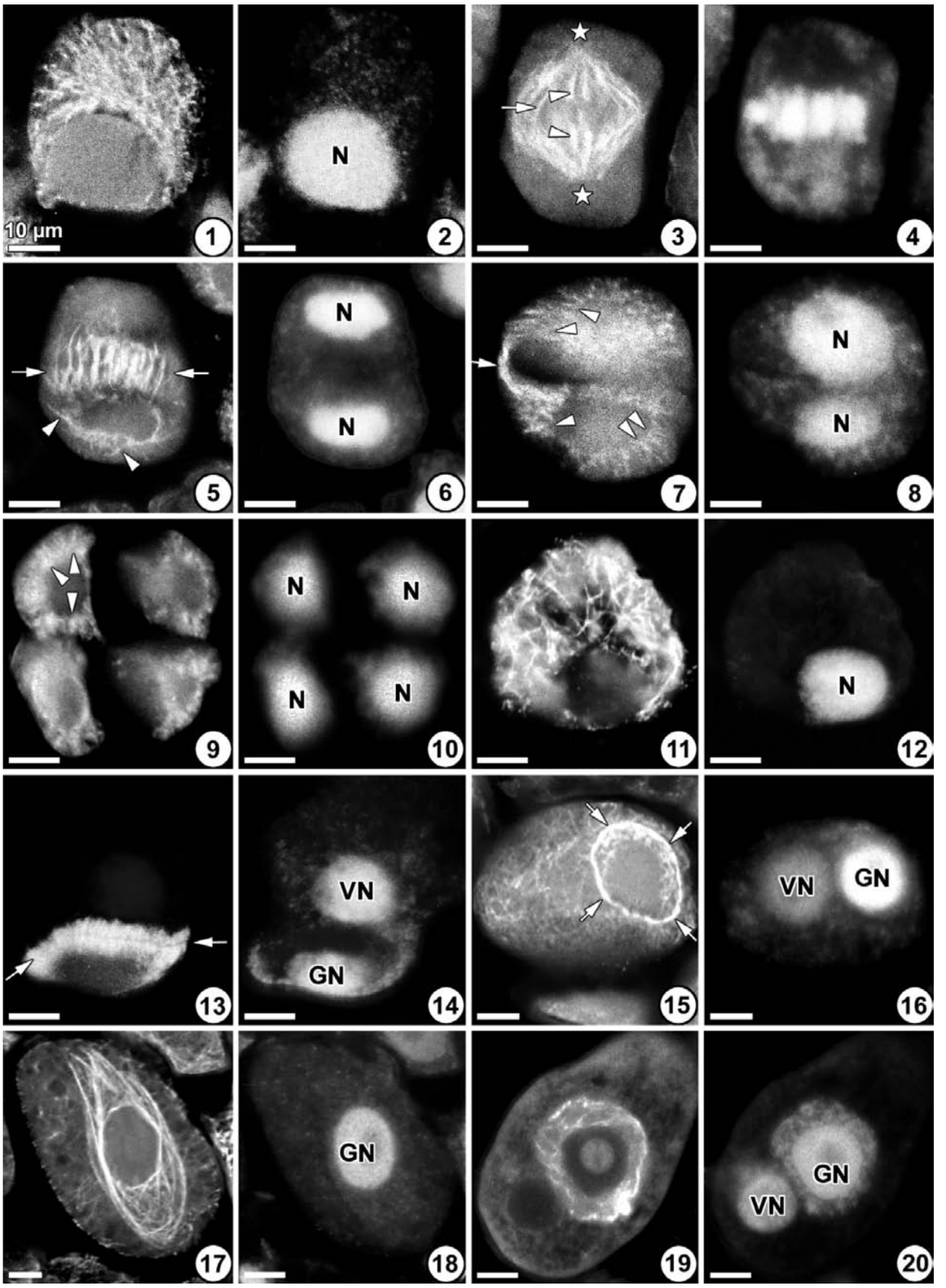
PLANT MATERIAL

Plants of *Gagea lutea* (L.) Ker.-Gaw. (Liliaceae) growing in natural habitats in Gdańsk and Stalowa Wola were used in the study. Flower buds were collected in winter months (December and January), and 75 *G. lutea* plants were transplanted to pots with soil and transported to the laboratory. These plants were kept refrigerated at 4°C and not watered for 1.5 months during the winter. After that period of cold and drying, the plants were cultivated in laboratory conditions at room temperature.

IMMUNOLOCALIZATION OF β-TUBULIN IN SECTIONED MATERIAL

Anthers were excised from flower buds and fixed immediately in 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.25% glutaraldehyde in microtubule stabilizing buffer (MSB) for 4 h at room temperature. The MSB consisted of 50 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]), 10 mM EGTA (ethylene glycol-bis[β-aminoethyl ether] N,N,N',N'-tetraacetic acid), and 1 mM $MgCl_2$, pH 6.8. The fixed anthers were processed according to the procedure of Vitha et al. (1997, 2000). After fixation and three rinses in MSB, they were dehydrated in a graded ethanol series containing 10 mM dithiothreitol (DTT, Sigma) (Brown et al., 1989) to minimize the background of cytoplasm. Then the plant material was infiltrated with Steedman's Wax: polyethylene glycol 400 distearate (Aldrich) and cetyl alcohol (Sigma) in a 9:1 (w/w) proportion. After polymerization of the wax, 10 μm sections were made from the embedded anthers and stretched on a small drop of distilled water on microscope slides coated with Mayer's egg albumen. The sections were dried overnight, dewaxed in ethanol, rehydrated in an ethanol-PBS series, washed in PBS

Figs. 1–20. Regular microsporogenesis and pollen grain development in *Gagea lutea*. Microtubules visualized by indirect immunofluorescence with a fluorescence microscope. **Figs. 1, 2**. Matching pair of prophase I microsporocytes. **Fig. 1**. Arrays of microtubules in the cytoplasm. **Fig. 2**. Nucleus (N) after DAPI staining. **Figs. 3, 4**. Metaphase I. **Fig. 3.** Short kinetochore (arrowheads) and thinner, longer interzonal microtubules (arrow) in the bipolar spindle (asterisks). **Fig. 4.** Compact chromosome plate after DAPI staining. **Figs. 5, 6**. Matching pair of telophase I microsporocytes. **Fig. 5.** Microtubules of a phragmoplast (arrows), with system of radial microtubules (arrowheads) around the nucleus. **Fig. 6**. Two flattened nuclei (N) stained with DAPI. **Figs. 7, 8**. Dyad. **Fig. 7**. System of radial microtubules (arrowheads) around the nuclei: at left, narrow part of phragmoplast (arrow). **Fig. 8**. Nuclei (N) after DAPI staining. **Figs. 9, 10**. Tetrad of microspores. **Fig. 9**. System of radial microtubules inside each of four microspores (arrowheads in one of the cells). **Fig. 10**. Four nuclei (N) of the microspores stained with DAPI. **Figs. 11, 12**. Matching pair of released microspores. **Fig. 11**. Abundant microtubular network extends through the cytoplasm. **Fig. 12**. Nucleus (N) after DAPI staining. **Figs. 13, 14**. Matching pair of dividing pollen grains. **Fig. 13**. Microtubular phragmoplast between generative (GN) and vegetative (VN) nuclei, which are visible after DAPI staining in **Fig. 14**. Arrows point to the dark line of the new cell plate forming between the vegetative and generative cells. **Figs. 15, 16**. Two-cellular pollen grain. **Fig. 15**. Dense layer of thick microtubules gathered in generative cell (arrows): a subtle microtubular net is visible in the cytoplasm of the vegetative cell. **Fig. 16**. Vegetative (VN) and generative (GN) nuclei after DAPI staining. **Figs. 17, 18**. Two-cellular pollen grain older than in Fig. 15. **Fig. 17**. Thick strings of microtubules in generative cell. **Fig. 18**. Generative nucleus (GN) after DAPI staining (vegetative nucleus is present in another section). **Figs. 19, 20**. Mature pollen grain. **Fig. 19**. Microtubules gathered in generative cell. **Fig. 20**. Vegetative (VN) and generative (GN) nuclei of the pollen grain shown in Fig. 19.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and preincubated in PBS with 0.1% BSA for 30 min. The samples were incubated overnight at 4˚C with mouse anti-β-tubulin monoclonal antibody (Amersham) diluted 1:200 in PBS. Then the sections were rinsed three times in PBS and incubated for 4 h in secondary Alexa 488-conjugated anti-mouse antibody (Molecular Probes) diluted 1:800 in PBS. The slides were rinsed in PBS, and the nuclei stained with 1 µg/ml 4',6'-diamidino–2-phenylindole dihydrochloride (DAPI) in PBS. Then the sections were treated with 0.01% toluidine blue to diminish cell wall autofluorescence and mounted in antifading solution (Citifluor, Agar). In control experiments conducted in a similar manner and omitting the first antibody, no tubulin staining was detected.

FLUORESCENCE MICROSCOPY

Fluorescence was observed with a Nikon Eclipse E 800 epifluorescence microscope, using a B–1E filter (EX 470–490 nm, DM 505, BA 520–560) to observe tubulin labeled with Alexa 488, and a UV–2A filter (EX 330–380, DM 400, BA 420) to examine DAPI-stained nuclei. Photographs were taken on Kodak T-max film, ASA 400. Image processing was done with Adobe Photoshop.

LIGHT MICROSCOPY

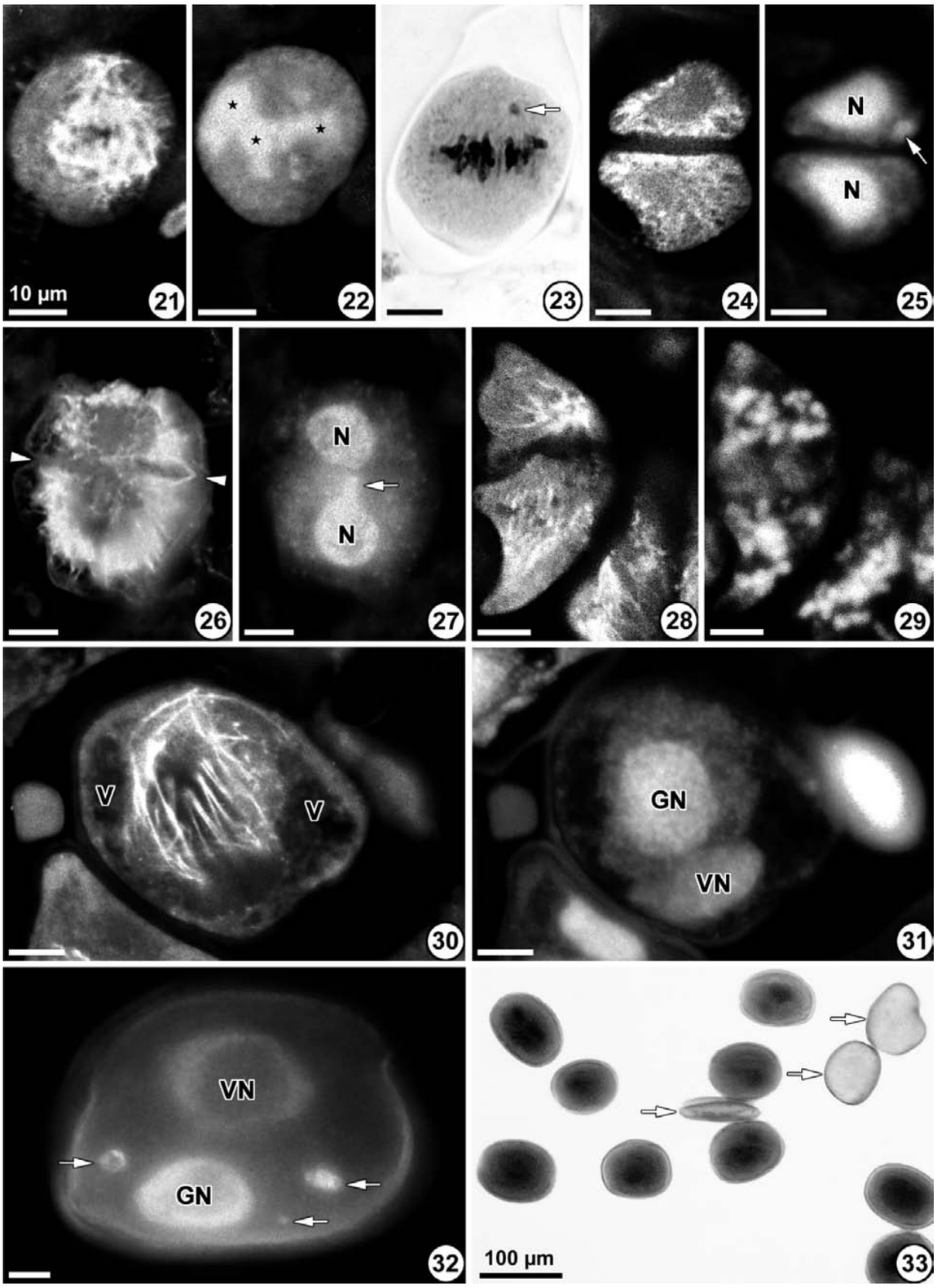
Acetocarmine stain was used for visualization of chromosomes. Alexander's method was applied to differentiate fertile and sterile pollen grains (Dafni, 1992). Sterile pollen grains were counted with a hemacytometer.

RESULTS

In *Gagea lutea* plants growing in natural conditions, 70% of the pollen grains underwent normal microsporogenesis and pollen grain development. Meiosis begins in September, but we observed the meiocytes at different stages of development during autumn and winter months. At prophase of meiosis I, microtubules are dispersed throughout the cytoplasm of the microsporocyte. At the stage when the meiotic cell is highly polarized and the nucleus occupies one of its poles, the microtubular network is distributed asymmetrically (Figs. 1, 2). The microtubules form arrays scattered randomly in the cytoplasm, but near the nuclear envelope they gather in a thin, compact layer. Such a layer only occurs close to the nuclear envelope adjacent to the cytoplasmic region of the microsporocyte. During the metaphase of meiosis I, a short kinetochore and thinner, longer interzonal microtubules form the bipolar spindle (Fig. 3), which acts during anaphase. The compact chromosome plate occupies the equatorial plane of the microsporocyte (Fig. 4). At the end of meiosis I, the arrays of microtubules extend into the internuclear region from the sister nuclei, and a conspicuous phragmoplast is formed (Fig. 5) between the telophase nuclei (Fig. 6). The phragmoplast disappears after the cell plate dividing the meiocyte into two separate cells is established. Following microsporogenesis, microtubules appear at the nuclear envelopes of the dyad (Figs. 7, 8) and, starting from prophase of meiosis II, they behave in the same way as in the successive stages of meiosis I. Meiosis II is completed with the formation of a microspore tetrad (Figs. 9, 10). In the separated young microspores a system of radial microtubules develops around each of the nuclei.

The young released microspore is spherical; later its shape changes. Most of its volume is occupied by cytoplasm in which the clear microtubule arrays form a net (Fig. 11). The nucleus of the microspore is in a peripheral position close to the cell wall (Fig. 12). In this asymmetrical position the microspore nucleus divides mitotically. At the end of the mitosis a conspicuous phragmoplast is set up between the generative and vegetative nuclei (Figs. 13, 14). The central part of the phragmoplast disintegrates, and the cell wall appears in place of the former phragmoplast, dividing the pollen grain into two asymmetric cells. In the two-cellular pollen grain, microtubules are observed in abundance in the generative cell, whereas in the cytoplasm of the vegetative cell a subtle microtubular net is visible (Fig. 15). At this stage, the generative and vegetative nuclei

Figs. 21–33. Disturbances in the course of microsporogenesis and pollen grain development in *Gagea lutea*. Microtubules visualized by indirect immunofluorescence. **Figs. 21–23**. Metaphase I microsporocyte. **Fig. 21**. Irregular spindle. **Fig. 22**. Chromosomes (asterisks; visible after DAPI staining) scattered irregularly in the cytoplasm. **Fig. 23**. Chromosomes stained with acetocarmine, one outside the chromosome plate (arrow). **Figs. 24–27**. Dyad. **Fig. 24**. Differently sized cells with systems of radiating microtubules. **Fig. 25**. Irregular-shaped nuclei (N) and micronucleus in one of the dyad cells (arrow). **Fig. 26**. Irregularly distributed microtubules in the radial systems around the dyad nuclei, and incomplete cell wall between two post-telophase I nuclei (arrowheads). **Fig. 27**. Dyad nuclei (N) after DAPI staining, with remnants of chromosome bridge in the central part of the microsporocyte (arrow). **Figs. 28, 29**. Three microspores of the tetrad. **Fig. 28**. Mitotic spindles in the microspores. **Fig. 29**. Chromosome plates visible after DAPI staining. **Figs. 30, 31**. Matching pair of two-cellular pollen grains. **Fig. 30**. Extended microtubular system and two vacuoles (V) in generative cell cytoplasm. **Fig. 31**. Vegetative (VN) and generative (GN) nuclei after DAPI staining. **Fig. 32**. Two-cellular pollen grain older than in Fig. 31 after DAPI staining, with vegetative (VN) and generative (GN) nuclei visible, and three micronuclei (arrows). **Fig. 33**. Mature pollen grains stained by Alexander's method. Non-aborted pollen grains are dark, and aborted ones light (arrows).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are the same size, although the DNA of the generative cell gives stronger fluorescence after DAPI staining (Fig. 16). In the further course of pollen grain development, the generative cell moves from the periphery towards the pollen grain center. At this stage, occasionally thick and long arrays of microtubules form a spindle-shaped structure inside the generative cell: in the cytoplasm of the vegetative cell a subtle microtubular net is barely visible (Figs. 17, 18). In the mature pollen grain, usually the generative cell is almost spherical, with an extensive system of cortical microtubules (Fig. 19). The microtubules are gathered mainly in the generative cell. The vegetative nucleus is smaller than the generative one (Fig. 20).

In the anthers of plants growing in natural conditions, up to 30% of the pollen grains develop abnormally as the viable pollen grains form. The disturbances can occur in the course of microsporogenesis or pollen grain development. The first disturbance observed was at metaphase of meiosis I. Figure 21 shows an atypical meiotic spindle with scattered microtubule bundles. Instead of fusiform shape, the spindle assumes an irregular form without typical poles. In a microsporocyte with such a spindle, the chromosomes do not form a regular metaphase plate (Fig. 22). Chromosomes can occur outside the metaphase plate (Fig. 23), resulting in the appearance of micronuclei during further stages of microsporogenesis (Figs. 24, 25).

After the first meiotic division, irregularly distributed microtubules were observed in radial systems around the dyad nuclei (Fig. 26). In such a dyad, the cell wall between two post-telophase I nuclei is incomplete, and there are remnants of chromosome bridges between the nuclei (Fig. 27).

Pollen mitosis can occur in tetrad microspores. All four microspores in a tetrad can divide synchronously (Figs. 28, 29), but sometimes four different mitotic stages can be found in one tetrad. The newly formed microspores in one tetrad can be irregular in shape and can differ in size.

In the young, two-cellular pollen grain, thick arrays of microtubules surround the generative nucleus. Large vacuoles are formed in the peripheral parts of the pollen grain (Fig. 30). The aberrant size of these vacuoles is shown in Figure 31.

The cytoskeleton disturbances described above influence the structure of the mature pollen grain. Micronuclei were observed in some pollen grains (Fig. 32), varying in number from one to seven. In most cases, the disturbances during microsporogenesis and pollen grain development lead to the production of a sterile pollen grain (Fig. 33).

In the distribution of microtubules during both regular and disturbed microsporogenesis and pollen grain development, there were no observed differences between *Gagea lutea* plants originating from natural habitats in Gdańsk and Stalowa Wola.

The same kinds of disturbances as described above were also observed after 1.5 months of cold (4˚C) and drying in laboratory conditions. Among the pollen grains developing in laboratory conditions, 50% were aborted. The remaining pollen grains had undisturbed development.

DISCUSSION

Two well-known processes typical for the majority of angiosperms lead to the formation of mature pollen grains: microsporogenesis and pollen grain development. Both of them also lead to the production of mature pollen grains in *G. lutea*. In this species, microsporogenesis is accomplished by successive cytokinesis. Such a type of cytokinesis, with cell walls assembled after both telophase I and telophase II, is typical for most Monocotyledones, Bryophyta, Pteridophyta and Gymnospermae. In *G. lutea* growing in natural conditions, in 70% of the cases there are no disturbances of these two processes – successive meiotic division and pollen grain development. In both microsporogenesis and pollen development, the microtubular cytoskeleton plays a fundamental role in forming spindles, phragmoplasts and microtubular networks in the cortical cytoplasm and at the surface of the nuclear envelope (Hogan, 1987; Busby and Gunning, 1988; Brown and Lemmon, 1988b, 1991a, 1996, 2000; van Lammeren et al., 1985; Staiger and Cande, 1990, 1991; Staiger and Lloyd, 1991; Liu et al., 1993; Genualdo et al., 1998; Shimamura et al., 1998; Giełwanowska et al., 2003). The changes in the microtubular cytoskeleton during the regular meiotic and mitotic divisions leading to two-cellular pollen grains in *G. lutea* are typical for most plants and do not require discussion here (for references, see: Otegui and Staehelin, 2000).

Thirty percent of pollen grains in the *G. lutea* anther are sterile. The disturbances leading to this phenomenon appeared during microsporogenesis and pollen grain development. The following kinds of disturbances in the course of microsporogenesis were observed: abnormal chromosome configurations in the metaphase of meiosis I; abnormally divided dyads with irregular, radial microtubule systems around the nuclei; and the formation of microspores irregular in shape and differing in size; and irregularity of division. Vacuoles appeared during the formation of the pollen grain, but that is abnormal for this developmental stage. Undoubtedly the observed disturbances are directly linked to the functioning of the microtubular cytoskeleton. In undisturbed divisions, the configurations of the microtubular cytoskeleton rearrange according to each phase of the plant cell meiotic and mitotic cycle (Asada and Collins, 1997). Microtubules forming the cytoskeleton are very dynamic polymers of α- and β-tubulin heterodimers, and they operate in all

key developmental events, including cell division, growth and differentiation (Marc, 1997). Disturbances affecting the cytoskeleton might result in disturbed meiotic or mitotic divisions and abnormal chromosome segregation.

Gagea lutea is an early-spring monocotyledonous plant. In Poland's climate, dividing microsporocytes and developing pollen grains of wild early-spring plants are subjected to abiotic factors in winter, which is the time of microsporogenesis and pollen grain development in *G. lutea*. Among these factors are variable temperature (cold exposure) and periodic lack of water. Both stresses act simultaneously. The persistent influence of these factors, which are natural stresses, can bring about disturbances in microtubular cytoskeleton functioning. It is believed that depolymerization of microtubules induced by lowering temperature causes the loss of subunits or small fragments from their ends (for references, see: Schliwa, 1986). If microtubules are depolymerized in key configurations during microsporogenesis or pollen grain development, both processes can be disturbed in ways observed in *G. lutea*. Depending on its intensity and duration, low temperature generally impairs the metabolic activity, growth and viability of plant cells. Biomembranes usually become more rigid at low temperatures, and more energy is required to activate biochemical processes. All this can disturb both meiotic and mitotic divisions. On the other hand, the influence of an abiotic factor like low temperature cannot be judged without considering periodic lack of water. At low temperatures, plants cannot take up enough water to cover their requirements. The strain imposed on the water balance by winter conditions may cause damage due to desiccation as well as to disturbed meiosis and mitosis (for references, see: Larcher, 2003).

Disturbances similar to those in the anther of wild-growing *G. lutea* were observed in plants exposed to 1.5 months of cold (4˚C) and drying in the laboratory. They had more disturbances in the course of microsporogenesis and more defective pollen grains than plants exposed to those abiotic factors in natural conditions. The plants in the laboratory probably were subjected to a more restrictive regime than those growing in the natural environment.

Proper pollen grain development depends not only on microtubular cytoskeleton functioning. For example, in many plant species, male sterility has been found to be caused by distortions in tapetum and endothecium functioning, or reduction of anthers. Additionally, male sterility can be governed in genetic, cytoplasmic, or cytoplasmic-genetic ways (Klein, 2000). The development of the male gametophyte can be disturbed at all steps. These points help explain the high percentage of abnormal pollen grains (50%) derived in laboratory conditions. In an investigation of *G. lutea* pollen grain germination, Zhang et al. (1995) found that only about 30% of pollen grains were viable and germinated in the pollen tubes successfully; this low germination percentage was not unexpected, since a large amount (even more than 50%) of abnormal, shrunken pollen grains were present in the samples they investigated.

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