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MICROTUBULAR CYTOSKELETON DURING MICROSPOROGENESIS OF *DACTYLORHIZA MAJALIS* (RCHB.) HUNT ET SUMMERH.

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Changes in the microtubular cytoskeleton of *Dactylorhiza majalis* (Rchb.) Hunt et Summerh. (Orchidaceae) during microsporogenesis were investigated by the immunofluorescence method. *Dactylorhiza* microsporocytes in microsporangia divide into tetrads after a regular meiosis, which is completed with simultaneous cytokinesis. Three typical configurations of the microtubular cytoskeleton appear during microsporogenesis: the microtubular system in the cytoplasm and at the nuclear envelope, meiotic spindles, and phragmoplasts. Microtubules of the early prophase I microsporocyte are dispersed throughout the cortical cytoplasm, and later the arrays of microtubules are visible at the nuclear envelope. During metaphase I, the microtubules form the spindle which also acts during anaphase I. At telophase I, interzonal microtubules of the first meiotic spindle disappear, and new microtubular arrays extend from the nuclei towards the equatorial plane of the microsporocyte. There, these microtubules form the phragmoplast, which disintegrates before the second meiotic division. During the second meiotic division, the microtubular cytoskeleton repeats the configurations from the first meiotic division. The microtubular arrays emanating from the telophase II nuclei form interconnections of all non-sister and sister nuclei. During the formation of the cell plates between the future microspores, these microtubular arrays disappear. The results support the view that cytoskeletal configurations participate in the formation of the nuclear-cytoplasmic domains of the dividing microsporocyte in its transition from mononucleate microsporocyte to undivided dyad, and to four microspore domains after the second meiotic division.

Key words: *Dactylorhiza*, microtubular cytoskeleton, microsporogenesis.

INTRODUCTION

The microtubular cytoskeleton plays a significant role in the majority of important cellular processes of all eukaryotic organisms. Except for its "skeletal" function for cells, it takes part in chromosome separation, morphogenesis and motility. The rapid assembly and disassembly of microtubules allows the cytoskeleton to form different configurations during cell divisions. Generally, similar microtubule configurations occur in both somatic tissues and dividing meiotic cells – the microtubular system in the cortical cytoplasm and at the nuclear envelope, metaphase spindles, and phragmoplasts. Unlike in vegetative cell division, however, meiotic cells lack a preprophase band of microtubules. The first three microtubular configurations appear regularly in meiotically dividing plant cells, with simultaneous as well as successive cytokinesis (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; Yang and Ma, 2001; Giełwanowska et al., 2003; Bohdanowicz at al., 2005).

In sporo- and microsporocytes completing meiosis with the simultaneous type of cytokinesis, microtubules forming the conspicuous phragmoplast may develop in the equatorial region following meiosis I, but no cell plate is set up to divide the binucleate meiocyte. Usually, the phragmoplast expands centrifugally as a ring of co-aligned microtubules in the equatorial plane of the meiotic cell (Brown and Lemmon, 199la). The

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establishment of division planes for quadripartitioning begins after meiosis with the formation of microtubule arrays which interconnect all four of the non-sister as well as sister nuclei. According to Brown and Lemmon (1988a,b, 1991a,b, 1996) the planes of division in micro- and sporogenesis depend on the deposition of nuclei, which define the cytoplasmic domains via a radial system of microtubules. Recent experiments with mutants of *Arabidopsis thaliana* (Yang et al., 2003) indicate that the gene *TES* (tetraspore) is involved in the formation of the radial arrays of microtubules. *TES* encodes a kinesin required to establish the spore domains.

The Orchidaceae family is undoubtedly the largest and most heterogeneous of all families of flowering plants, consisting of up to 35,000 species. This huge systematic group is characterized by very high diversity of different processes, including the events during microsporogenesis and pollen grain development that lead to the formation of pollen grains. Because the Orchidaceae family is so large, the meiotic events (including cytokinesis) in the particular orchid subfamilies and species are not completely known. In this paper we focus on microsporogenesis and show the distribution of microtubules (microtubular cytoskeleton elements) formed during this process in the *Dactylorhiza* orchid.

MATERIAL AND METHODS

PLANT MATERIAL

Young flower buds of *Dactylorhiza majalis*. (Rchb.) Hunt et Summerh. (Orchidaceae) were collected from a large natural population in a wet meadow near Olsztyn, Poland.

FLUORESCENCE MICROSCOPY

Microsporangia were isolated from young anthers. After determining developmental stages from acetocarmine squash preparations, material was fixed for 24 h in 4% paraformaldehyde in microtubule-stabilizing buffer (MSB) containing 50 mM Pipes, 10 mM EGTA and 5 mM $MgSO_4$ (pH 6.2) at room temperature. After fixation, microsporangia were rinsed in buffer, dehydrated, embedded in PEG, sectioned, and treated for indirect immunofluorescence according to the method described by van Lammeren et al. (1985). Briefly, sections were mounted on 3 Aminopropyltrimethoxysilane (Sigma-Aldrich Co.) coated slides and washed three times (5 min each) in phosphate-buffered saline (PBS). Then they were treated with 0.1 M NH_4Cl and blocked with 0.1% bovine serum albumin (BSA) in PBS for 45 min. Subsequently, cells were incubated in a humid chamber for 90 min at 37˚C with monoclonal anti-mouse-β-tubulin (Sigma-Aldrich) diluted 1:100 in 0.1% BSA in PBS. After washing with PBS and with the blocking buffer, incubation with the secondary antibody was carried out for 90 min at 37˚C. The secondary antibody was conjugated with FITC (Sigma-Aldrich) diluted 1:300 in PBS. Controls showed no tubulin fluorescence. To stain DNA in the nuclei and organelles, 4',6-diamidino–2-phenylindole dihydrochloride (DAPI) was added to the sections. The sections were observed with an epifluorescence microscope (Nikon Optiphot II). There were several preparations of the examined stages. The images of selected microsporocyte stages were recorded on Kodak TMAX–400 film.

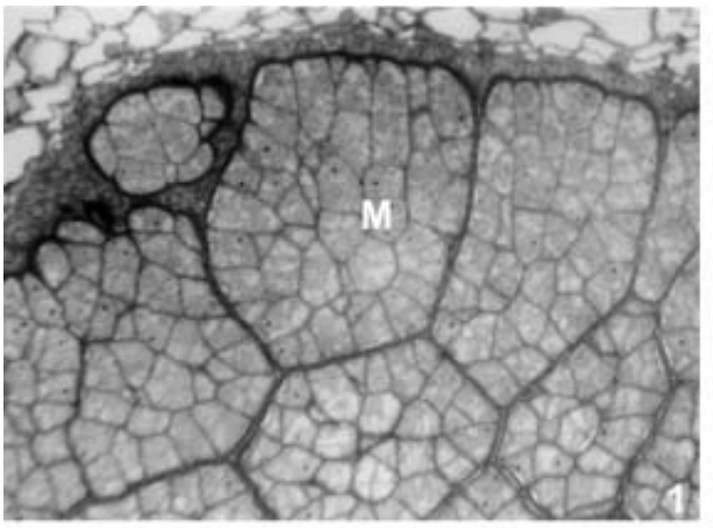
LIGHT MICROSCOPY

For bright field microscopy, microsporangia were fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). Specimens were washed three times in 0.05 M sodium cacodylate buffer and postfixed in 1% osmium tetroxide. Then the samples were dehydrated in a graded ethanol and acetone series followed by propylene oxide, infiltrated, and embedded in Spurr's resin (Spurr, 1969). The material was sectioned at 2 μm and stained with 1% toluidine blue. To visualize whole massulae, the content of the microsporangium was pressed out slightly.

RESULTS

In *Dactylorhiza*, microsporogenesis occurs in a manner typical of the majority of orchids and angiosperms, in which meiosis is completed with a simultaneous type of cytokinesis. Microsporocytes in the microsporangium form groups called massula (Fig. 1). In a single massula, all microsporocytes form one group during the whole process of meiotic division. Each single massula is surrounded with a thick wall; all massulae in the microsporangium are enveloped by tapetal cells. Even after the microsporangium is squashed, the microsporocytes in the massula stay together, joined by elastoviscin strands (Fig. 2). The microsporocytes in the massula are at the same developmental stage before

Figs. 1–5. Massulae in microsporangium of *Dactylorhiza majalis*. **Fig. 1.** Preprophase microsporocytes in microsporangium gathered in massulae (M). Semithin transversal section after toluidine blue staining. × 150. **Fig. 2.** Squash preparation of microsporangium, light microscopy. Massulae (M) joined with viscin strands (arrows). × 40. **Fig. 3.** Massulae (M) in microsporangium visible, fluorescence microscopy. Transversal section. Microtubules visualized by indirect immunofluorescence. × 80. **Figs. 4, 5.** Fragment of massula with matching pair of prophase I microsporocytes. × 1000. **Fig. 4.** Short microtubules distributed in the cytoplasm, visualized by indirect immunofluorescence. Some microtubules are gathered at the nucleus. **Fig. 5.** DAPI staining.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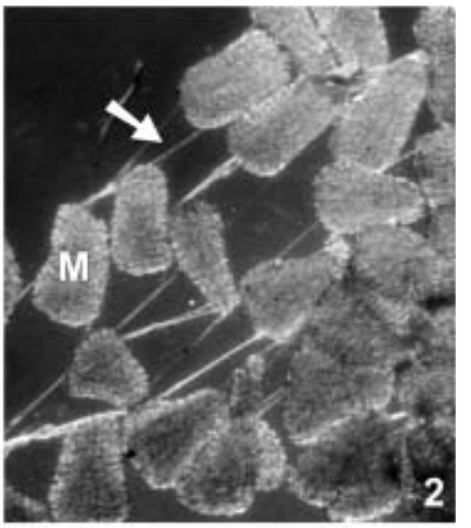
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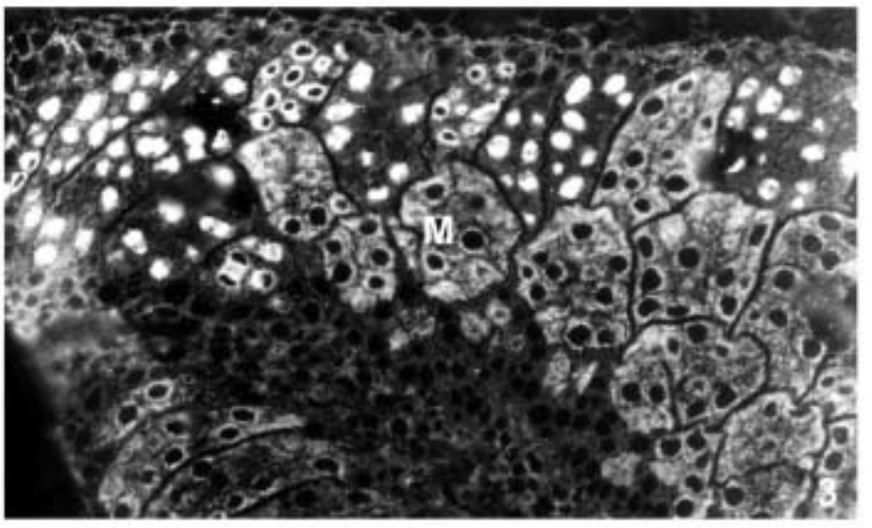
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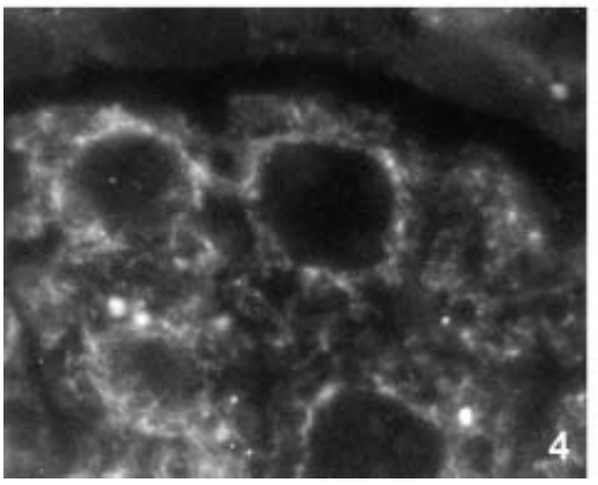
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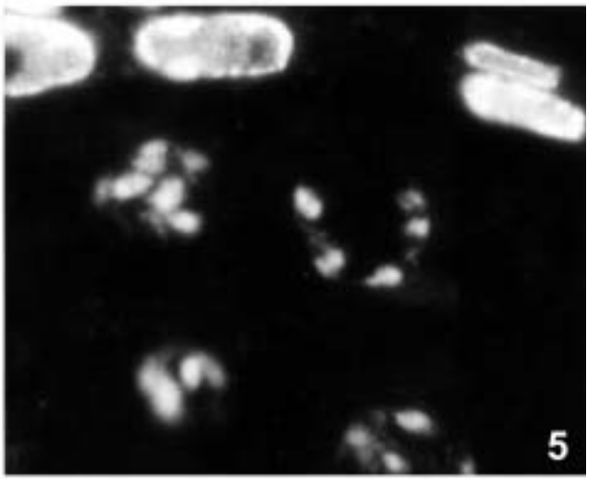
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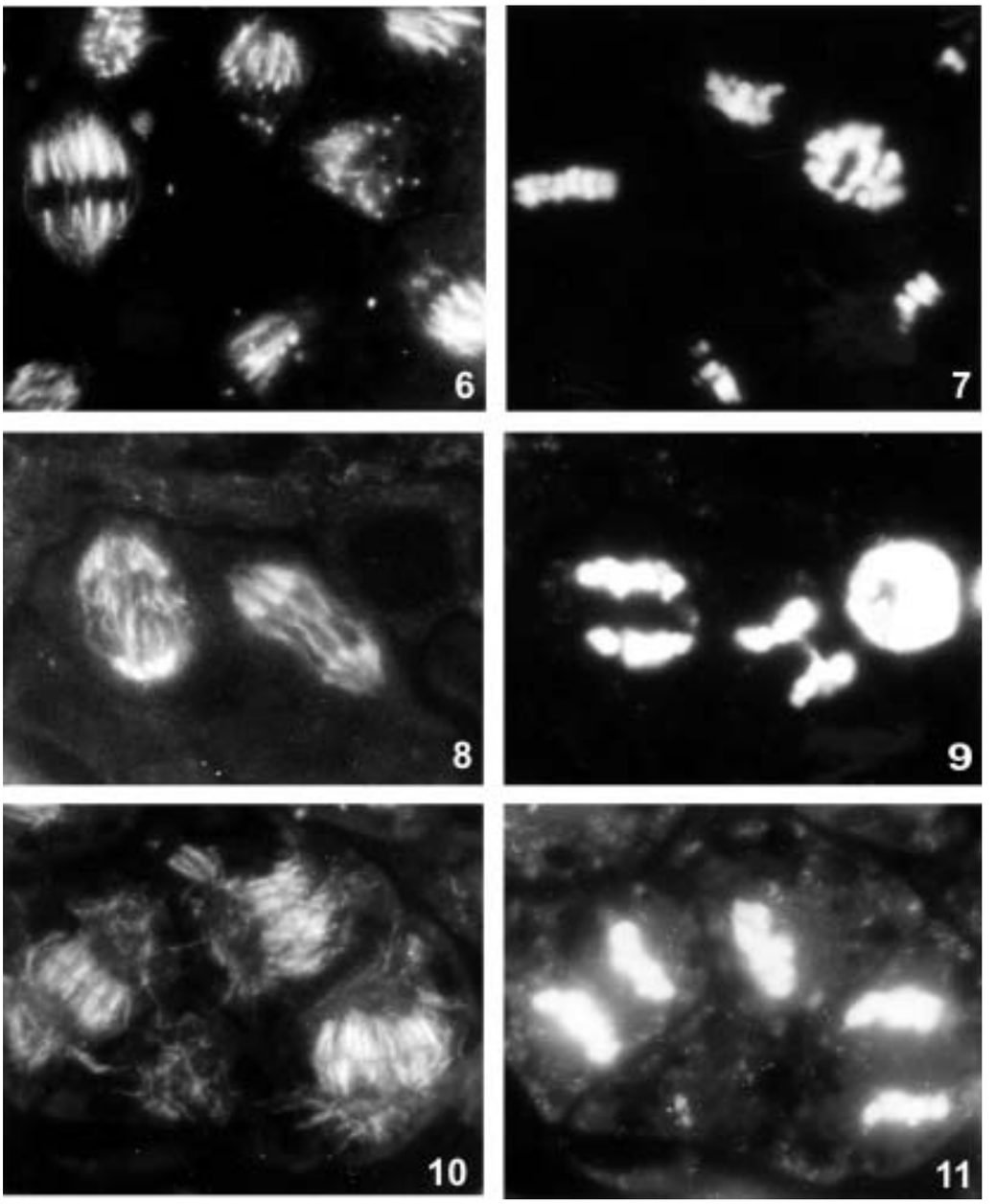
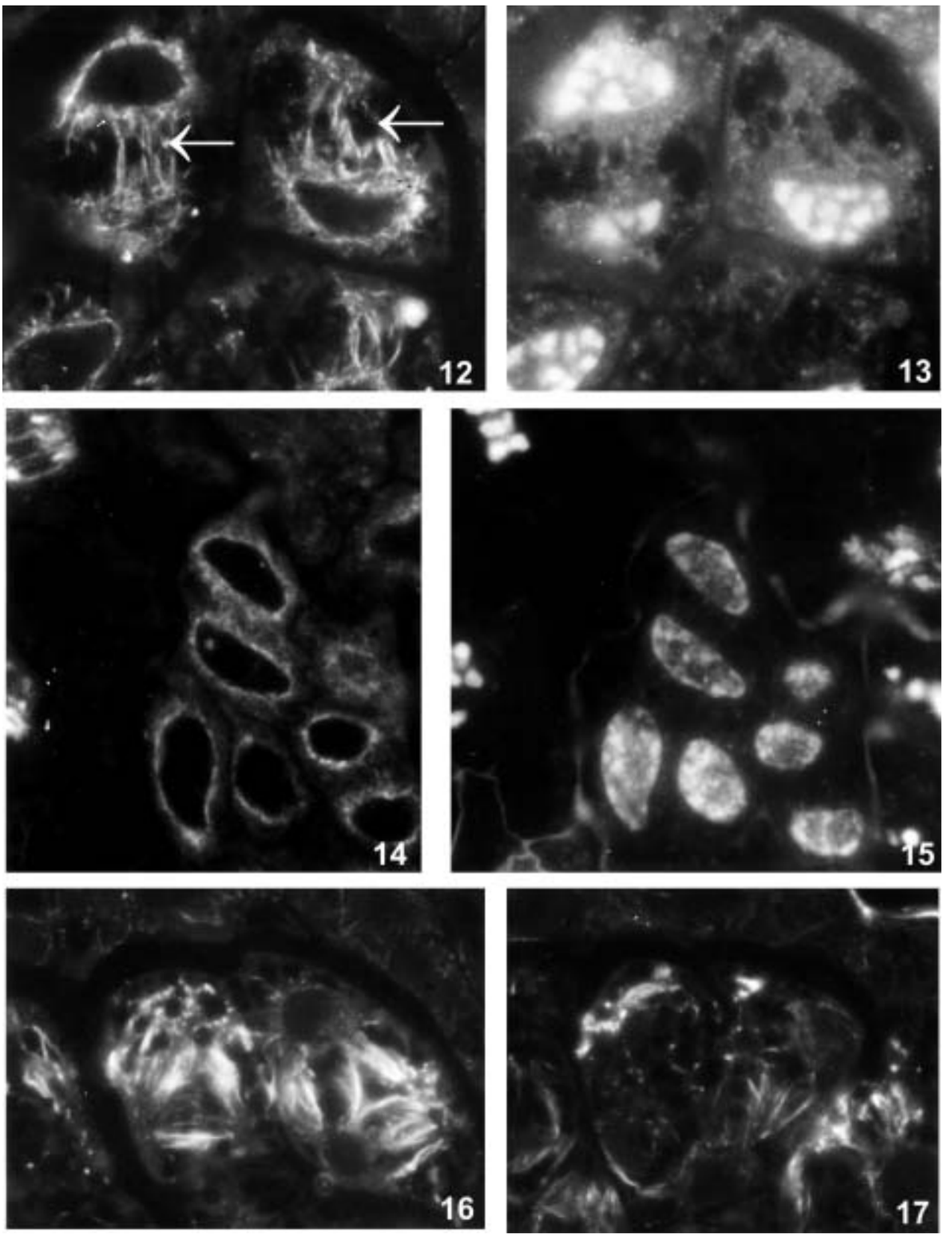


Figs. 6–11. First meiotic division during microsporogenesis in *Dactylorhiza majalis*. × 1000. **Figs. 6, 8, 10.** Microtubules visualized by indirect immunofluorescence. **Figs. 7, 9, 11.** Chromosomes after DAPI staining. **Figs. 6, 7.** Metaphase I. **Fig. 6.** Kinetochore and interzonal microtubules of spindles; most spindles sectioned obliquely. **Figs. 8, 9.** Matching pair of anaphase I microsporocytes. **Fig. 8.** Short kinetochore and thinner, longer interzonal microtubules. **Fig. 10–11.** Microtubules of phragmoplast extending between the flattened post-telophase I nuclei visible in Fig. 11.

Figs. 12–17. Second meiotic division in *Dactylorhiza majalis*. **Figs. 12, 14, 16, 17.** Microtubules visualized by indirect immunofluorescence. **Figs. 12, 13.** Microsporocytes in interkinesis. × 1500. **Fig. 12.** Remnants of phragmoplasts (arrows) visible in central part of microsporocytes. **Fig. 13.** The same microsporocytes as in Fig. 12, with nuclei after DAPI staining. **Figs. 14, 15.** Prophase II. × 1300. **Fig. 14.** Microtubules around surface of nuclei. In left upper corner, fragment of metaphase spindle in neighboring massulae. **Fig. 15.** The same microsporocytes as shown in Fig. 14, with nuclei and chromosomes after DAPI staining. **Fig. 16.** Two tetrad nuclei in microsporocyte. Microtubular systems between sister and non-sister nuclei. × 1400. **Fig. 17.** Disappearing phragmoplast between tetrad nuclei (older stage than in Fig. 16). × 1600.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and at the beginning of the meiosis. Later, gradual differentiation of the development stages in the neighboring massulae occurs (Fig. 3).

As the fluorescence observations indicate, all microtubules of the prophase I microsporocytes are distributed at random throughout the cytoplasm of the slightly elongated cell. At late prophase I, the fluorescence and the number of short microtubule arrays gradually increase in the perinuclear zone (Fig. 4). After DAPI staining of such a section, condensed chromosomes are visible (Fig. 5). The cells of tapetal tissue do not show any microtubular structures at this time. At prometaphase, when kinetochore microtubules are formed, almost all the cytoplasmic fluorescence disappears. During metaphase I, tubulin is present at the kinetochores, and with the interzonal elements it forms the metaphase spindle (Fig. 6) holding the chromosome plate compact (Fig. 7). At the end of anaphase I, the system of interzonal microtubules of the spindle (Fig. 8) is visible between two groups of chromosomes (Fig. 9). At telophase I, when two sister nuclei are situated at the ends of the meiotic cell, the interzonal microtubules seem to become enriched with newly formed polar microtubules.

After completion of the first meiotic division, the arrays of microtubules emanating from the opposing nuclear envelopes of the sister nuclei extend into the internuclear region. In this way, centrifugal expansion of the phragmoplast takes place in the equatorial region of the cell, between the post-telophase I nuclei (Figs. 10, 11). The phragmoplast expands quickly and fills up even the periphery of the microsporocyte. Then the phragmoplast disintegrates during interkinesis (Figs. 12, 13), while all microtubules are concentrated as a thin, compact layer adjacent to the nuclear envelopes of the elongated, prophase II nuclei (Figs. 14, 15).

During metaphase and anaphase II, tubulin fluoresces only in the meiotic spindles. At the end of telophase II, the interzonal microtubules behave in the same way as at the corresponding stage of the first meiotic division. They gradually diminish and disappear. Then, columnar arrays of interzonal-like microtubules appear from the microtubules proliferating at the proximal surfaces of the reforming tetrad nuclei.

Finally, a prominent nuclear-based phragmoplast, arranged of microtubule arrays emanating equally from the four tetrahedrally arranged nuclei, interconnects all nuclei of the newly formed tetrad (Fig. 16). In the older microspore tetrad (Fig. 17), the phragmoplast microtubules disappear and the cell plates between the tetrad nuclei are simultaneously set up.

DISCUSSION

In general, our observations of the meiotically dividing *Dactylorhiza* microsporocytes agree with earlier findings concerning microtubule distribution during sporo- or microsporogenesis in different well-studied plants (for references see the Introduction). The fundamental changes of the microtubular cytoskeleton, from meiotic prophase to tetrad formation, are very similar in various taxonomic groups, indicating that the micro-tubule organization during both meiotic divisions in *Dactylorhiza* is very typical and common.

During early prophase of the first meiotic division, short microtubule arrays form a reticulate pattern of distribution. Generally the microtubule arrays seem to be scattered randomly in the microsporocyte cytoplasm. The microtubular system in the cortical cytoplasm of late prophase I microsporocytes in different plants is also built of a subtle net of microtubules. However, starting from zygotene of prophase I, microtubule arrays thicker than in the earlier phases are dispersed randomly in the cytoplasm until the end of meiotic prophase. Such a distribution of microtubules is observed also in all massulae comprising the microsporangium of *Dactylorhiza*. A similar schedule of microtubule distributions is common, and has been described previously in, for example, the microsporocytes of *Gasteria* (van Lammeren et al., 1985). A comparable distribution of microtubules was also reported in the megaspore mother cells of *Gasteria* during the premeiotic phase (Bednara et al., 1988). Besides being found in *Gasteria verrucosa* microsporocytes and megasporocytes, a similar microtubular net has been observed in sporo- and microsporocytes of many different species belonging to various taxonomic groups, such as *Conocephalum japonicum* (*Bryophyta*) (Shimamura et al., 1998), *Ophioglossum vulgatum* (*Ophioglossaceae*) (Giełwanowska et al., 2003), *Equisetum hyemale* (*Equisetaceae*) (Bednara et al., 1995), and also *Zea* (Staiger and Cande, 1990), *Lilium* (Sheldon and Dickinson, 1986; Tanaka, 1991) and *Gagea lutea* (Bohdanowicz et al., 2005) from Angiospermae. The function of the microtubular net at prophase I clearly seems to be skeletal. In detailed studies of changes in the pattern of organization of microtubules during microspore formation in rice, Xu and Ye (1998) found differences in the thickness of microtubular bundles between the microsporocyte and the mature microspore. They suggested that shorter and thicker microtubule bundles are needed to maintain the shape of the microsporocyte, which is surrounded by a wall which is thinner and more plastic than that of the mature microspore.

At the end of prophase and during prometaphase of the first meiotic division, the short microtubules gather at the nucleus. This process is connected with the start of kinetochore microtubule formation at the condensed chromosomes. All microtubules in the microsporocyte, previously scattered in the cytoplasm, are engaged in the formation of the metaphase spindle. At the same time, the microtubules disappear from the cytoplasm of microsporocytes (Bednara et al., 1988; Xu

and Ye, 1998) as well as homosporous fern sporocytes (Giełwanowska et al., 2003). During anaphase to telophase I, the interzonal microtubules of the meiotic spindle remain intact, but disintegrate completely before the first meiotic division is completed. A similar process has been found in *Equisetum* and *Ophioglossum* sporocytes (Bednara et al., 1995; Giełwanowska et al., 2003).

Before the beginning of the second meiotic division, the extended, post-telophase I phragmoplast between the two nuclei disintegrates. As meiosis progresses, the behavior of the microtubular cytoskeleton is very similar to that during the first meiotic division, and all configurations are copied. At early telophase II, the microtubules start to form the phragmoplast. The large phragmoplast extends between sister and non-sister post-telophase II nuclei. In *Dactylorhiza* microsporogenesis completed with simultaneous cytokinesis, the two sister nuclei remain in one cell after the first meiotic division. The nuclei are divided by the phragmoplast into two compartments or domains in the meiotic cell. After the second meiotic division, four compartments can be clearly distinguished. Four newly formed nuclei of the future microspores are separated by the microtubular system. Similarly, during meiotic interkinesis, the cell of the slipper orchid (*Cypripedium californicum*) has two domains defined by the equatorial organelle layer and by the radial microtubule system (Brown and Lemmon, 1996; Pickett-Heaps et al., 1999). As the latter authors suggest, "the establishment of such domains and their integrity can play a fundamental role in the second meiotic division." In both dyad domains (or compartments), the second meiotic division takes place independently and simultaneously on both sides of the microsporocyte.

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