

# Effect of n-butanol and cold pretreatment on the cytoskeleton and the ultrastructure of maize microspores when cultured in vitro

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## Introduction

Production of double haploid (DH) plants is a valuable tool in conventional breeding. This technique reduces the time needed for the development of new, improved varieties. Developing microspores of higher plants possess the ability to switch their default gametophytic developmental program to the sporophytic pathway under certain circumstances. This process, called androgenesis can be induced in isolated microspore and anther cultures. Androgenesis is the most widely used method for double haploid production, yielding diploid homozygous plants in large number and with high genetic variability (Reed et al. 2004). Haploid plants of more than 200 species were produced using androgenesis so far (Dunwell 2010). For the in vitro induction of the androgenetic process, immature anthers or isolated microspores at the mid- or late unicellular developmental stage are transferred to culture (Maluszynski et al. 2003). First pollen mitosis (PM I) is an important milestone in pollen development. Although androgenic induction of late bicellular pollen is reported from *Brassica napus* (Binarova et al. 1997), the developing microspores of most studied species lose their totipotency around PM I (Maraschin et al. 2005b). Gene expression show significant alterations at this time. A new subset of genes required for pollen development become active, including the genes involved in starch synthesis, and other genes expressed exclusively in pollen grains (Mascarenhas 1990). In order to alter the developmental program of the microspores and divert them towards the embryogenic path, a wide range of treatments are used in anther and microspore cultures, including heat, cold, starvation, and chemical agents (e.g. colchicine, mannitol) (Shariatpanahi et al. 2006; Islam and Tuteja 2012). Stress generated by these treatments is a necessary trigger for the embryogenic induction of immature microspores (Soriano et al. 2013).

The ability to respond with the desired embryogenic induction to the applied pretreatments is highly genotype-dependent in maize (Kuo et al. 1986) and many other crop plants, such as wheat (Barnabás et al. 2001), apple (Höfer 2004), tomato (Zagorska et al. 1998) and pepper (Mitykó et al. 1995). While the germplasm-dependent manner of the androgenic response is well known, genetic background of the androgenic developmental switch remained unclear so far. Untreated microspores of barley show strong expression of cell division related genes before PM I, while the amount of transcripts related with starch and energy production increased highly after the division (Maraschin et al. 2006). In stress-treated androgenic microspores, transcription of genes involved in pollen development were downregulated, while genes related with protein and starch catabolism, stress response, and the inhibition of programmed cell death were expressed at a higher level compared to the binuclear pollen stage. Expression of endosperm and embryo-specific genes in multicellular structures derived from maize microspores was confirmed by Massonneau et al. (2005). The authors found that both endosperm-specific (ESR-2 and AE-3) and embryo-specific (LTP2, OCL-1 and OCL-3) genes were expressed in the early, exine-surrounded stage of development, around after 7 days of culture. Later, from the 12<sup>th</sup> day of culture, only the transcripts of embryo-specific genes were detected from multicellular structures, embryo-like structures, and embryo bowls. Callus-like structures expressed neither endosperm nor embryo-specific genes. Involvement of epigenetic changes in androgenetic induction of microspores in *Brassica napus* confirmed recently (Solís et al. 2012).

First structural sign of embryogenic induction is the dedifferentiation of the cytoplasm. This process involves a decrease in the number of organelles, lipid bodies, starch granules and ribosomes.

Degradation and recycling of these cytoplasmic components through two different pathways: the ubiquitin-26S proteasome system and autophagy (Alché et al. 2000; Maraschin et al. 2005b). In the next step, the nucleus moves from the periphery to the center of the microspore. Simultaneously, the vacuole is fragmented by cytoplasmic strands, creating the so-called star-like structure (reviewed by Touraev et al. 1997). This balanced distribution of cytoplasmic elements supports the symmetric division of the microspore, which is generally considered as a marker of embryogenic induction (Soriano et al. 2013).

Cytoskeleton is an essential cell compartment for the successful completion of most of the above-mentioned structural changes. The plant cytoskeleton is a dynamic filamentous network, consisting of actin filaments and microtubules associated by various proteins (Collings 2008; Petrášek and Schwarzerová 2009). This complex system has diverse roles in the life of a cell: takes part in the formation of cell shape, sets up and maintains cell polarity, transports organelles, and coordinates cell division (Kost and Chua 2002). New functions of the cytoskeleton were found recently, such as phytohormone signalization (Lanza et al. 2012) and programmed cell death (Smertenko and Franklin-Tong 2011). Nevertheless, there is a lack of information on the relation of the plant cytoskeleton and abiotic stresses, although the effects of salt and osmotic stresses (Wang et al. 2011) and heat stress (Malerba et al. 2010) are studied in some types of vegetative cells.

Improvement of androgenic response in microspore and anther cultures can be achieved primarily by the enhancement of embryogenic induction frequency, which can be carried out by various pretreatments, such as heat, cold, starvation and chemical agents (reviewed by Zorinians et al. 2005 and Shariatpanahi et al. 2006). In the case of maize microspore and anther culture, tassels are usually pretreated by cold, placing the tassels at around 7 °C in the dark for 7-21 days (Gaillard et al. 1991; Barnabás 2003). Besides this, androgenic induction was successfully triggered by chemical agents such as colchicine (Obert and Barnabás 2004), and 2-HNA (2-hydroxynicotinic acid, Zheng et al. 2003).

Application of a primary alcohol, *n*-butanol (or 1-butanol) is reported to elevate the proportion of embryogenic microspores in wheat anther culture (Soriano et al. 2008; Broughton 2011). *N*-butanol decreases the production of a signaling phospholipid, phosphatidic acid (PA), catalysed by phospholipase D (EC 3.1.4.4, PLD) (Liscovitch et al. 2000). Phosphatidic acid antagonist effect of *n*-butanol is not selective to PLD isoforms, as it diverts the transphosphatidylation reaction step, typical of all PLD isoenzymes (Motes et al. 2005). In the absence of *n*-butanol, phospholipase D isoenzymes hydrolyze different phospholipids at the terminal phosphodiester bond, yielding the former head group molecule of the hydrolyzed membrane lipid (e.g. choline, ethanolamine or glycerol) and different PA species (Wang et al. 2014a). These PA molecule types may have various target enzymes (Wang et al. 2014b), regulating diverse functions of cells, such as response to abscisic acid and reactive oxygen species, programmed cell death, membrane trafficking and cytoskeletal dynamics (Hong et al. 2010; Pleskot et al. 2013). When *n*-butanol is present, PLD transfers the phosphatidyl group to butanol, instead of -OH group originated from water. This results in the production of phosphatidylbutanol instead of PA, reducing the amount of PA generated in the cell (Munnik et al. 1995; Wang et al. 2014a). This triggered reversible microtubule depolymerization and/or the release of cortical microtubules from the plasma membrane in Arabidopsis BY-2 cell culture (Dhonukshe et al. 2003; Hirase et al. 2006). Our previous results indicated that *n*-butanol increases androgenic response in maize microspore and anther cultures (Földesiné Füredi et al. 2011; Földesiné Füredi et al. 2012). In order to link this androgenesis-enhancing feature to the previously demonstrated microtubule-disrupting effect, cytoskeleton of *n*-butanol-treated maize microspores were studied using fluorescent probes by confocal laser scanning microscopy. Moreover, cytoskeleton-altering effects of two potentially embryogenic treatments (cold pretreatment and *n*-butanol treatment) were compared. Possible ultrastructural alterations of microspores or microspore derived structures caused by *n*-butanol treatment are studied for the first time.

## Materials and methods

### *Plant material*

Plants of a single cross maize hybrid line (A 18) were used as donor plants for androgenic anther culture, according to (Földesiné Füredi et al. 2012). Briefly, plants were grown in phytotron growth chambers using a climatic program designed for growing maize (Tischner et al., 1997). After cold pretreatment (7 °C for 10 days) in the dark, immature anthers containing microspores at the late uninuclear developmental stage were placed on a modified liquid YP medium (Genovesi and Collins 1982) supplemented with 0.1 mg l<sup>-1</sup> 2,3,5-triiodobenzoic acid, 500 mg l<sup>-1</sup> casein hydrolysate, and 120 g l<sup>-1</sup> sucrose at pH 5.8. To investigate the effect of *n*-butanol, this chemical agent was added to the medium at a final concentration of 0.2%, or omitted. After 6 hours, treated anthers were transferred to *n*-butanol free induction medium. Experiments were performed either on cold pretreated and untreated control anthers, in order to distinguish the effect of cold pretreatment and *n*-butanol treatment. Anthers of all treatments (1500 anthers per treatment) were cultured at 29 °C in the dark for 28 days. On the 28<sup>th</sup> day, androgenic induction and embryo/callus ratio was determined using a stereomicroscope.

### *Treatment, sample collection*

In order to study the effect of cold pretreatment and *n*-butanol treatment on actin and microtubule cytoskeleton elements, microspores were obtained from anthers by mechanical isolation. Anthers were placed in 1 ml of culture medium and gently agitated to release microspores to the medium. Suspension was filtered through a 100 µm nylon mesh filter to remove debris from anther walls. Microspores from *n*-butanol treated anthers were isolated using culture medium supplemented with 0.2% *n*-butanol. Untreated control, cold pretreated and *n*-butanol treated microspores were immediately processed by fixation and cytoskeleton labeling. To investigate the recovery of cytoskeleton, *n*-butanol was removed from a portion of treated microspores by washing two times with culture medium. After 30 minutes in culture medium, these microspores were fixed and labeled as well. Anthers from all treatments were collected for light and electron microscope studies on the first, third, seventh and fourteenth days of culture.

### *Actin and microtubule labeling*

Labeling of actin filaments was carried out according to Lovy-Wheeler et al (2005) with slight modifications. Microspores were fixed for 30 minutes in a buffer containing 100 mM PIPES, 5 mM MgSO<sub>4</sub>, 0.5 mM CaCl<sub>2</sub>, 7.5% sucrose, 0.05% Triton X-100 and 2% paraformaldehyde as fixative, at pH 9.0. Infiltration of the fixation buffer was facilitated by vacuum for 10 minutes. The fixative was washed out with the above buffer containing 10 mM EGTA at pH 7, then F-actin was stained with 6.6 µM rhodamine phalloidin (Sigma) for 30 min.

Visualization of microtubules was performed by indirect immunofluorescence. Microspores were fixed in PEM buffer (100 mM PIPES, 10 mM EGTA, 10 mM MgSO<sub>4</sub>, 7.5% sucrose, pH 7.4) supplemented with and 4% paraformaldehyde. Proteinase inhibitor cocktail (32 µg ml<sup>-1</sup> benzamidine HCl, 2 µg ml<sup>-1</sup> phenanthroline monohydrate, 20 µg ml<sup>-1</sup> aprotinin, 20 µg ml<sup>-1</sup> leupeptin, 20 µg ml<sup>-1</sup> pepstatin, final concentrations) was added to buffers during fixation, digestion of microspore wall, and incubation with primary and secondary antibodies. After fixation, microspores were washed three times with PEM buffer. Microspore wall was partially digested with 10 mg ml<sup>-1</sup> β-glucuronidase in PEM buffer at room temperature for 90 min (Simmonds and Keller 1999). After 60 min of digestion, DMSO and Nonidet P 40 substitute (Sigma) were added to the mixture at 5% and 0.1% final concentrations, respectively. Microspores were pelleted and suspended in 1% Triton x-100. After washing three more times, microspores were transferred to PBS buffer by changing PEM buffer to PBS gradually in three steps. Blocking, incubation and washing of unbound antibodies were performed in PBS buffer containing 1% bovine serum albumin (BSA). Primary and secondary antibodies were monoclonal anti-α-tubulin antibody produced in mouse (Sigma T9026) and anti-mouse IgG (whole molecule) - FITC antibody produced in goat (Sigma F0257), respectively. Both

antibodies were used at 1:200 dilution, with 60 min incubation at room temperature. Actin and microtubule filaments were imaged using a Leica TCS SP8 confocal laser-scanning microscope (Leica, Germany).

#### *Light- and transmission electron microscopy*

Immature anthers containing microspores were fixed in PEM buffer containing 7.5% sucrose and 4% glutaraldehyde for 4 hours at pH 7.2. Fixative was vacuum infiltrated until anthers sank in vials. Anthers were rinsed three times, 30 min each in PEM buffer and postfixed for 3 h at 4 °C in 1% (w/v) osmium tetroxide (OsO<sub>4</sub>) in Milli-Q water. After washing in Milli-Q water, the samples were dehydrated through a gradient series of ethanol, infiltrated with epoxy resin according to Spurr (1969). Resin blocks were polymerized for 48 h at 60 °C. Semi-thin (1 µm) and ultra-thin (70 nm) sections were made using an Ultracut E microtome (Reichert-Jung GmbH, Heidelberg, Germany). For light microscopy, semi-thin sections were collected on glass slides, stained with toluidine blue (0.5% toluidine blue in 0.1% sodium carbonate buffer at pH 11.1) or auramine O (0.001% in 50 mM Tris-HCl buffer at pH 7.5). For transmission electron microscopy, ultra-thin sections were mounted on Formvar-coated (SPI-Chem, West Chester, PA, USA) 100-mesh nickel grids. Sections were contrasted with 3% (w/v) aqueous uranyl acetate and 0.08% (w/v) lead citrate. The sections were examined using a Philips Morgagni 268D electron microscope at 80 kV accelerating voltage.

#### *Quantitative evaluation of fluorescent signals*

Quantification of cytoskeletal changes following cold pretreatment and the introduction of *n*-butanol were determined by the methods of Higaki and others (2010), using the image processing program ImageJ (<http://rsbweb.nih.gov/ij/>). Density of cytoskeletal elements, using the parameter “occupancy” was determined from three replicates, using pictures of 50 cells per treatment. All data were pooled means from the replicates and were statistically evaluated using ANOVA (SSPS for Windows, version 10.0).

## **Results**

#### *Pretreatment-dependent induction of maize microspores*

The applied various pretreatments triggered significantly different levels of microspore induction (Table 1). Anthers of highly responsive A-18 maize genotype used in our experiments yielded embryos and calli at low frequency in culture medium without cold pretreatment or the application of *n*-butanol. Induction of calli was more than twice prevalent than of embryos in this case. Application of 0.2% *n*-butanol without cold pretreatment elevated the percentage of responding microspores about six times. This treatment yielded a nearly equal amount of embryos and calli per 100 anthers. Number of embryos was even higher when cold pretreatment was used alone. Highest embryo induction was achieved when *n*-butanol treatment was applied after the 10-day cold pretreatment, yielding 20.95 embryos per 100 anthers on average. Cold pretreatment alone and combined cold and *n*-butanol treatment resulted in the highest embryo/callus ratio, inducing more than twice as many embryos than calli (Table 1).

Table 1. Effects of various pretreatments on the embryo and callus induction in maize anther culture. Values indicate the number of induced structures per 100 anthers.

Treatment	Embryo induction	Callus induction	Embryo/callus ratio
no treatment (control)	0.52±0.33 <sup>a</sup>	1.02±0.32 <sup>a</sup>	0.43
0.2% <i>n</i> -butanol	5.00±2.32 <sup>b</sup>	5.19±1.93 <sup>b</sup>	0.96
cold pretreatment	11.90±3.59 <sup>c</sup>	5.53±1.84 <sup>b</sup>	2.15
cold pretreatment + 0.2% <i>n</i> -butanol	20.95±4.73 <sup>d</sup>	9.86±2.09 <sup>c</sup>	2.12

Different letters within a column show significant differences between mean values at the  $P < 0.005$  level of probability

#### *Effect of various pretreatments on cytoskeletal elements*

Cold pretreatment resulted in a significantly elevated amount of actin filaments (Fig. 1 a-b, d), which was confirmed by the quantitative analysis of fluorescence signal intensities from the rhodamine phalloidin labeled microspores (Fig. 2.). However, evenly distributed structure of cortical actin network did not change during the pretreatment; neither the production of actin cables was observed. Exposure to cold did not affected the structure nor the density of microtubule cytoskeleton in the microspores compared to untreated control (Fig. 1 e-f, h). In contrast with cold pretreatment, actin microfilaments remained similar to the control after the supplementation of culture medium with 2 mM *n*-butanol for 6 hours. This treatment caused the depolymerization of microtubules, which resulted in the total disappearance of cortical microtubule cytoskeleton (Fig. 1 a, c, d). Interestingly, dense networks of MTs surrounding the nucleus and the aperture remained largely intact, indicating that *n*-butanol does not affect all kinds of microtubule arrays uniformly (Fig. 1 g, i). After the removal of *n*-butanol, the original structure of cortical microtubule cytoskeleton substantially recovered in 30 minutes (Fig. 1 j). Actin cytoskeleton did not change significantly following the chemical treatment (Fig. 1 e, g, h). Quantification of fluorescent signals from specifically labeled cytoskeletal elements verified that cold pretreatment selectively increased the amount of actin microfilaments, while *n*-butanol reversibly depolymerized the cortical microtubules of maize microspores (Fig. 2.).

#### *Alterations during the early development of microspore derived structures*

The early development (3-14 days) of MDSs originated from different treatments was studied in order to evaluate the effects of the applied *n*-butanol treatment on the embryogenic process. Since cold pretreatment is necessary for microspore embryogenesis in most maize genotypes, cold pretreated anthers were taken as control in our structural studies. Microspore derived structures were taken from cold pretreated anthers and cold pretreated anthers supplemented with *n*-butanol at the end of treatments and after 3, 7 and 14 days of culture and studied by histological methods. Histological study of structures collected after 3 and 7 days of culture indicated that *n*-butanol slowed the development of embryogenic microspores. During the first seven days, microspores treated with *n*-butanol performed fewer divisions compared to cold pretreated control (Fig. 3 a-b, d-e). During this period, both *n*-butanol treated and untreated MDSs showed two differently organized domains similar to that previously reported by Testillano et al (2002). The smaller, probably embryogenic domain contained small cells with densely stained cytoplasm, while the other, probably not embryogenic group of cells (mentioned as endosperm-like domain by Testillano and coworkers) consisted of large, highly vacuolated cells. By the 14<sup>th</sup> day of culture, vast majority of the MDSs reached the proembryo developmental stage, regardless of the applied treatment (Fig. 3 c, f). Although the majority of the activated microspores followed the above-mentioned developmental process, both studied treatments triggered the formation of MDSs containing numerous, distinct clusters of cells (Fig. 3 g). Two types of clusters were distinguished, showing structural correspondence with the embryogenic and endosperm-like domains of the young microspore-derived structures. The application of a fluorescent dye, auramine-o enabled the study of the newly synthesized cell walls at the light microscopic level. Although auramine-o is known for its specificity for the sporopollenin exine in intact pollen grains (Lalonde et al. 1997; Nishikawa et al. 2005), in the case of microspores embedded in Spurr's resin the

dye stained specifically the intine and the new, inner cell walls instead of the exine layer. This study revealed the presence of incomplete and irregular cell walls in the case of *n*-butanol-treated cells (Fig. 3 h-i), which were further investigated by transmission electron microscopy.

#### *Ultrastructural attributes of microspore derived structures originated from different treatments*

Transmission electron microscopic study of cultured microspores revealed differences at the ultrastructural level triggered by *n*-butanol treatment. At the third day of culture, microspores following the gametophytic (Fig 4 a) and sporophytic (Fig. 4 b) developmental pathway were clearly distinguishable in both control and *n*-butanol treated samples. Cytoplasm of microspores successfully induced by either cold or *n*-butanol pretreatment was less dense and contained significantly less organelles; starch granules were totally absent (Fig. 4 c, d). This phenomenon is the result of the so-called cytoplasmic cleaning (Seguí-Simarro and Nuez 2008), dedifferentiation of cytoplasm through the disassembly of organelles, which is generally considered as a necessary prerequisite of microspore embryogenesis. Autophagy-related structures, such as phagophores and autophagosomes were also observed in these activated microspores, on the third day of culture (Fig 4 d, f, g). Cytoplasmic cleaning and autophagy were both typical of cold treated control and *n*-butanol treated androgenic microspores. Frequent occurrence of incomplete and severely irregular cell walls was observed only in *n*-butanol-treated microspores, from the first, mostly symmetric cell division (Fig. 4.). Irregularities, such as curved, wavy appearance of cell walls, the appearance of extremely thickened wall segments and the formation of inclusions embedded in cell wall material containing cytoplasmic elements, e.g. as organelles (Fig. 4 f). Nevertheless, irregular or incomplete cell walls were only sparsely observed in *n*-butanol-treated MDSs sampled on the 7<sup>th</sup> and 14<sup>th</sup> days of culture. Autophagy-related structures, such as phagophores (Fig. 5 a), phagosomes (Fig. 5 a inset), intravacuolar autophagic bodies (Fig. 5 b), intravacuolar deposits of autophagic bodies with digested material (Fig. 5 c), and small vesicles probably containing digested material to be excreted (Fig. 5 d) were still visible in *n*-butanol treated MDSs after 14 days of culture. Contrarily, these structures were absent in cold treated control. On the 14<sup>th</sup> day of culture, signs of programmed cell death (PCD), such as highly condensed degenerating nuclei, deconstruction of cytoplasm and organelles were observed in both *n*-butanol treated and control MDSs (Fig. 5 d-e). These microspore-derived structures contained several dividing cell groups, covered by degenerating cells and debris (Figs. 3 g, 5 e).

## **Discussion**

### *N-butanol: an effective embryogenesis-enhancing agent*

While long exposures to *n*-butanol effectively slows or even blocks growth and development in *Arabidopsis* seedlings (Gardiner et al. 2003; Motes et al. 2005), shorter treatments with similar concentration resulted in elevated androgenic induction in wheat (Soriano et al. 2008; Broughton 2011), maize (Földesiné Füredi et al. 2011) and barley (Castillo et al. 2014) microspore and anther cultures. As reported in barley microspore culture, *n*-butanol may act in a genotype- and pretreatment-dependent manner (Castillo et al. 2014). In these experiments, *n*-butanol increased the number of embryos and green plants in low-responding cultivars, but not in the studied, medium and high responding cultivars. Moreover, this beneficial effect of *n*-butanol occurred only in the case of anthers pretreated with mannitol. Cold pretreated microspores from low-responding genotypes did not showed increased embryo number following the *n*-butanol treatment. In our system, *n*-butanol elevated the number of embryos when used alone or in combination with cold pretreatment, and increased the embryo/callus ratio, when used without cold pretreatment. This underlines that induction of androgenesis is an intricate process, depending on multiple factors.

Although beneficial effect of *n*-butanol on microspore embryogenesis is well proven in different species, it was not yet confirmed if elevated embryogenic response is related to changes in cytoskeletal structure. Our studies demonstrated that *n*-butanol concentration effectively used to

elevate androgenic induction in maize anther culture disrupted the cortical microtubules in microspores as well. We also found that microtubule-depolymerizing effect of *n*-butanol was reversible in maize microspores, similarly to previous reports published by other researchers, concerning cell cultures and vegetative organs (Dhonukshe et al. 2003; Gardiner et al. 2003; Hirase et al. 2006).

#### *Effect of n-butanol on cortical microtubule array*

Effect of *n*-butanol on microtubule network is very similar to that of colchicine, decreasing the amount of cortical microtubules in the cells. Colchicine, unlike *n*-butanol, acts directly on microtubules, as it binds to  $\beta$ -tubulin, blocking tubulin polymerization. In the absence of newly polymerized microtubules, cell polarity can no longer be maintained (Siegrist and Doe 2007). After the removal of colchicine, a new, evenly distributed microtubule network appears, enabling a symmetric cell division. Colchicine without cold pretreatment triggered embryogenesis in maize anther culture similarly to cold pretreated control (Obert and Barnabás 2004), but when applied following the cold pretreatment, number of embryos did not elevated as considerably as after the application of *n*-butanol in present study. Colchicine, like *n*-butanol in our previous study (Földesiné Füredi et al. 2012) significantly elevated the proportion of symmetric cell divisions in tobacco (Eady et al. 1995) and wheat (Szakács and Barnabás 1995). Eady and coworkers (1995) reported the activation of vegetative cell-specific tomato promoter *lat52* in daughter cells originated from colchicine-triggered symmetric cell division of tobacco microspores. This points out that division symmetry can affect gene expression and may modify the developmental fate of the daughter cells (Eady et al. 1995). Authors of the article suggest that gene expression changing effect of altered division symmetry may be done by changing the uneven distribution of expression factors and/or inhibitors in daughter cells. Despite the similar effects on microtubule cytoskeleton, colchicine and *n*-butanol may have different influence on microspore induction. In the case of *n*-butanol, androgenesis may be aided many other ways besides the alteration of division symmetry, as the decrease of all signaling PA species influence various signal pathways.

*N*-butanol treatment applied without cold pretreatment successfully induced embryos and calli in our experiments, however, with lower frequency compared to cold pretreated anthers (Table 1). The embryo/callus ratio was different between these two treatments as well. *N*-butanol induced the same amount of embryos and calli, while embryo induction was twice as frequent as callus induction when cold pretreatment was used alone (Table 1). Although application of *n*-butanol following cold pretreatment did not elevated further the embryo/callus ratio, this combined treatment resulted in a higher number of responding microspores compared to cold treatment alone. These results suggest that cytoskeletal reorganization induces developmental program shift from the gametophytic to the sporophytic pathway, but gene expression changes or epigenetic alterations induced by cold stress (Solís et al. 2012) ensure a more efficient induction of embryogenesis instead of generating callus.

#### *Cold pretreatment alters actin filament network of maize microspores*

The hypothesis that cytoskeleton has an important role in embryogenic induction leads to the question: How does cold pretreatment essential to microspore embryogenesis change the structure of actin and/or microtubule network? Our confocal laser scanning microscopic studies revealed that only actin filament network of maize microspores was altered after the applied cold pretreatment, while microtubule cytoskeleton remained unchanged. The fact that cold pretreatment triggered both the increase of actin amount and the efficient induction of embryogenesis, suggests that actin cytoskeleton is involved in cytoplasm reorganization leading to or involved in the induction of embryogenic microspores. This conception is supported by the results of Sheahan et al. (2004), who documented the crucial role of actin cytoskeleton in organelle partitioning and redistribution of tobacco protoplasts. Similarly to our results, elevation of a certain actin isoform during cold pretreatment of maize microspores was observed by proteomic methods (Uváčková et al. 2012), although less than in our experiment.

This raises the question, what mechanism links low temperature exposition to the redistribution of actin network. It is proven that activity of phospholipase D increased after cold exposure (Ruelland et al. 2002), leading to the elevation of intracellular PA levels. In Arabidopsis, phosphatidic acid negatively affected the actin-binding ability of a heterodimeric capping protein (CP) that binds actin filaments at the barbed ends (Huang et al. 2006; Pleskot et al. 2012). This regulatory protein decreases filament length and annealing frequency through the lowering of dynamic activity at filament ends. Therefore, elevated levels of PA expectedly increase the amount of actin in the cells. Indeed, exogenous PA is reported to enhance the amount of filamentous F-actin in Arabidopsis and tobacco suspension cultures and Arabidopsis epidermal cells (Huang et al. 2006; Pleskot et al. 2010; Li et al. 2012). As recently discovered, phosphatidic acid has a regulating role in the case of microtubules as well. The binding of exogenously added PA to a microtubule associated protein MAP65-1, elevates the microtubule polymerization and bundling activity of this regulatory protein, stabilizing microtubule cytoskeleton during salt stress in Arabidopsis (Zhang et al. 2012). Nevertheless, microtubule content did not elevated further, when various PA species were added to control cells, suggesting that MAP65-1 may have a stress-related role. These data from literature altogether offer explanation to our findings concerning the effects of cold pretreatment on actin and microtubule cytoskeleton. Increased amount of actin filaments is probably the result of cold-induced PLD activation and subsequent elevation of PA content in cells. Unchanged microtubule cytoskeleton can be explained by the same factors: high level of PA might neutralized the eventual microtubule disassembly induced by cold stress through the activity of regulator proteins like MAP65-1. Early cold-induced disruption of microtubules and subsequent formation of new filaments were observed in cold tolerant wheat genotypes (Abdrakhamanova et al. 2003), suggesting the dynamic behavior of microtubules during long-term cold exposure. Besides this, cold stress sensitivity of microtubule network in microspores may show developmental stage dependency according to recent publications (De Storme et al. 2012; Barton et al. 2014), in which cold treatment only affected microtubular organization during the telophase of meiosis.

#### *N-butanol triggers abnormal formation of cell walls*

One of the most conspicuous structural alterations caused by the *n*-butanol treatment is the appearance of abnormal cell walls. Although cell plate, predecessor of the new cell wall is synthesized during the cytokinesis, the phragmoplast, a microtubular structure required for this process, appears in the telophase of the cell division. The phragmoplast array contains two sets of parallel microtubules, with their plus ends facing each other at the site of the prospective cell plate and subsequent cell wall. The role of the phragmoplast is to drive the vesicles containing newly synthesized cell wall material from the cytoplasm to the site of cell plate construction (Liu et al. 2011). As the exact location of the forming cell plate is determined by the plus ends of MTs involved in the phragmoplast, various events inducing dissociation or depolymerization of microtubules during the telophase are likely change the location and/or the structure of the new cell wall. Application of colchicine triggered abnormal cell plate formation in oat roots (Holmsen and Hess 1985), leading to the synthesis of branching, wavy cell plates. Colchicine altered the orientation of otherwise normally structured cell plates in *Funaria* as well (Schmiedel et al. 1981). In our experiments, we observed irregular cell walls very similar to the colchicine treated samples of Holmsen and Hess (1985), which underlines the parallel effect of colchicine and *n*-butanol. Although the suggested polarity-ceasing effect of *n*-butanol is considered to take effect before the onset of PM I, it is expected that microspores already commenced mitosis at the beginning of the *n*-butanol treatment may show altered cell walls due to the presence of *n*-butanol during telophase and cytokinesis. Moreover, removal of *n*-butanol at the end of treatment may be slow or uneven from intact anthers, leading to considerably different local concentrations. This is supported by the observation that microspore derived structures in anthers frequently appeared in clusters (data not shown).

Although literature data suggests that different arrays of microtubules are main factors involved in the establishment of division plane and cell plate (Rasmussen et al. 2013), it is proven that depolymerization of actin filaments during cell division alters division symmetry in vegetative cells (Liu and Palevitz 1992; Eleftheriou and Palevitz 1992) and even in microspores, inducing



embryogenesis (Gervais et al. 2000). To sum up literature data, it can be said that cooperation of microtubule and actin arrays during telophase and cytokinesis is necessary for the formation of normal cell walls.

#### *Autophagy sustained selectively in *n*-butanol treated samples*

Cytoplasmic cleaning is the essential part of the dedifferentiation of cytoplasm, taking place in cultured microspores. This process, together with gene expression changes lead to the totipotency and to the embryogenic induction of microspores (Seguí-Simarro and Nuez 2008). Corral-Martínez and coworkers (2013) found evidence for the involvement of autophagy in the early development of embryogenic microspore derived structures in *Brassica*, pointing out that the autophagic process and a subsequent excretion of digested cell compartments are elemental parts of androgenic induction. By the application of monodansylcadaverine (MDC), a fluorescent amine specifically staining autophagosomes, the authors demonstrated that autophagy was present only in embryogenic microspores. Neither non-embryogenic structures, nor microspore derived embryos after only 4 days of development were positive to MDC staining, suggesting that autophagy only takes part in the very first step of androgenesis, the dedifferentiation of cytoplasm. In our system, large autophagosomes containing cytoplasm and organelles were observed, which confirmed that macroautophagy took place in embryogenic microspores and MDSs (Li and Vierstra 2012). Autophagy-related structures were observed in cold treated control and *n*-butanol treated samples on the third day of culture. Moreover, microspore derived structures from *n*-butanol treated samples showed both initial- and progressed-stage autophagy after 7 and even 14 days of development as well. Although slowed development of *n*-butanol treated MDSs could explain this, MDSs from cold treated control and *n*-butanol treated samples show parallel development from the 7<sup>th</sup> day of culture, making this alternative unlikely. Prolonged existence of autophagic processes may contribute to the elevated embryo induction observed after *n*-butanol treatment, enabling the formation of new, potentially embryogenic cells during the later phase of anther culture, which is generally not yields new embryos. According to Varnier et al (2009), programmed cell death during microspore embryogenesis is a significant factor that lowers the number of embryos and regenerated plants. In our experiments, clear signs of programmed cell death were observed in both cold-treated control and *n*-butanol treated cultures at the 14<sup>th</sup> day of culture.

Gene expression study of maize microspore-derived structures demonstrated the parallel expression of endosperm- and embryo specific genes in 7-day stage structures (Massonneau et al. 2005). The same study revealed that only the embryo specific genes expressed in MDSs on the 15<sup>th</sup> day of culture. This indicates that genetic program of endosperm-like domain in developing embryos changes, or more likely, that these cells die by this time. Ultrastructural study of the developing structures showed that cell domains containing large vacuoles could be found only sparsely in MDSs on the 14<sup>th</sup> day of culture. The disappearance of these domains in MDSs overlapped with the onset of PCD, suggesting that mainly non-embryogenic cells of endosperm-like domains underwent PCD. Remaining cells of MDSs performed additional divisions, forming numerous, actively dividing cell clusters, resembling the structural and cytological features of younger-stage proembryos (Figs 3 g, 5 e). PCD of cells within developing proembryos were found in barley microspore culture as well, starting five days after the start of culture (Maraschin et al. 2005a). The authors considered that programmed cell death might be a developmental feature, required for the transition from a multicellular structure to a proembryo. Coexistence of autophagy and programmed cell death of non-embryogenic cells in *n*-butanol treated samples may elevate the final number of embryos.

Present study revealed the different effects of cold pretreatment and the *n*-butanol treatment on the cytoskeleton of maize microspores. However, further studies are needed to find out whether the enhanced embryogenesis triggered by *n*-butanol is based exclusively on its cytoskeleton-altering effect or it is aided by the influence of phosphatidic acid-dependent signaling pathways as well.

#### **Acknowledgements**

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## Figures

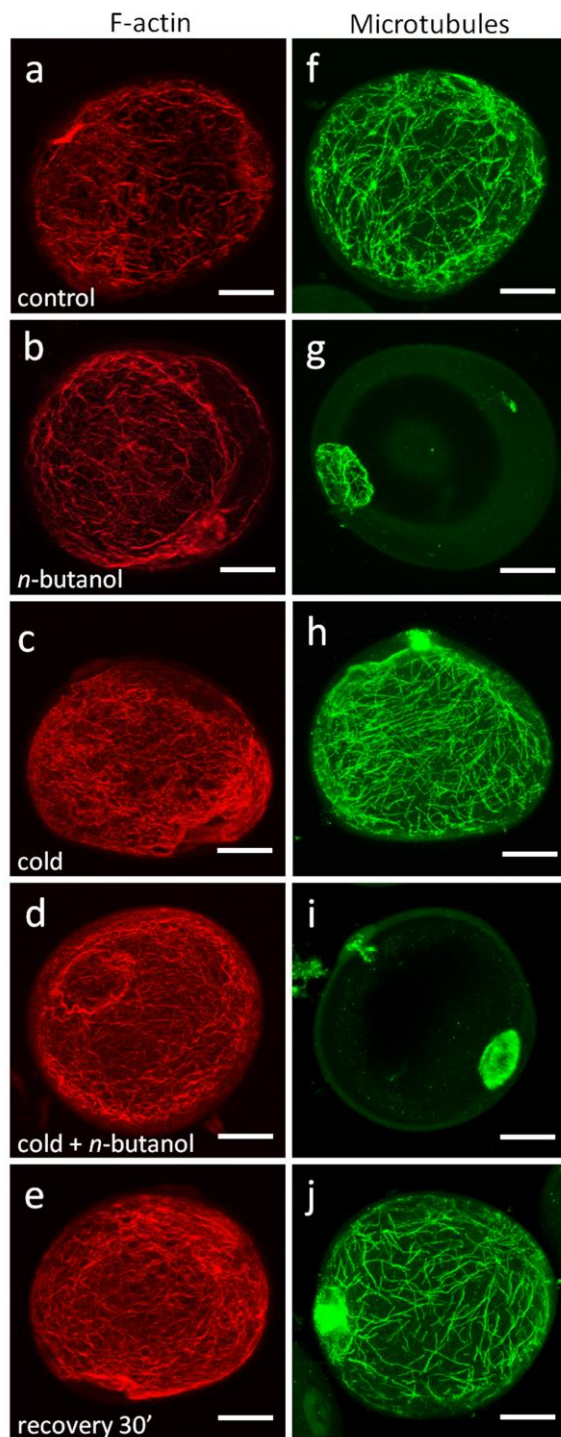


Figure 1. Effects of cold pretreatment and 0.2% *n*-butanol treatment on the cytoskeletal organization of maize microspores. (a)-(e): F-actin microfilaments visualized by rhodamine phalloidin, (f)-(j): microtubules labeled with indirect immunofluorescence. Treatments: control (a, f), 0.2% *n*-butanol for 6 hours without cold pretreatment (b, g), cold pretreatment (c, h), cold pretreatment followed by *n*-butanol treatment (d, i), recovery of cold- and *n*-butanol treated microspores 30 minutes after the removal of *n*-butanol (e, j). Cold pretreatment led to the elevation of F-actin amount (c-e) compared to control (a). *N*-butanol treatment triggered the depolymerization of cortical microtubules in both cold



untreated control (g) and cold treated (i) microspores, while F-actin network of these microspores (b and d) were unaffected. Bars represent 10  $\mu\text{m}$ .

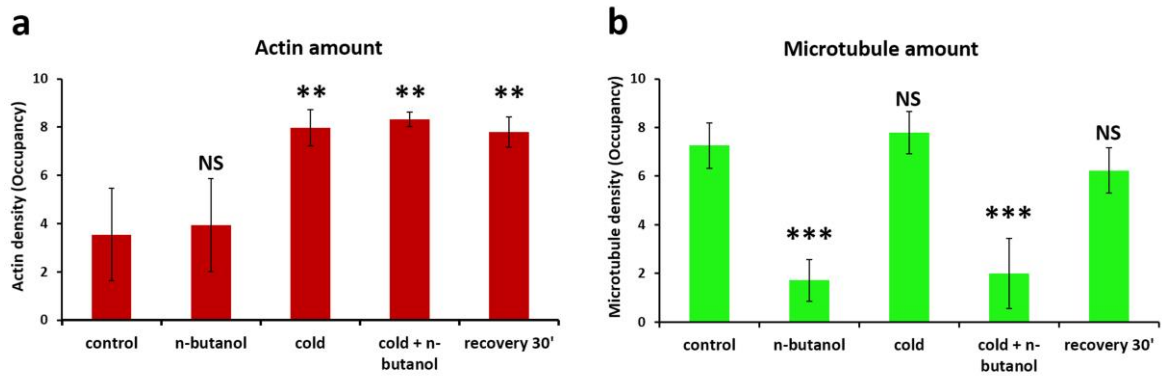


Figure 2. Changes in actin (a) and microtubule (b) network densities triggered by the applied treatments. (a): *n*-butanol treatment did not alter the amount of F-actin, while cold pretreatment elevated its occupancy nearly twofold. (b): application of *n*-butanol significantly decreased the density of cortical microtubules, in contrast with cold pretreatment, which had no effect on it. After the removal of *n*-butanol, amount of microtubules increased to the level similar to control microspores. Asterisks show significant difference between the value and the untreated control, evaluated by analysis of variance. \*\* $P < 0.005$ , \*\*\*  $P < 0.0005$

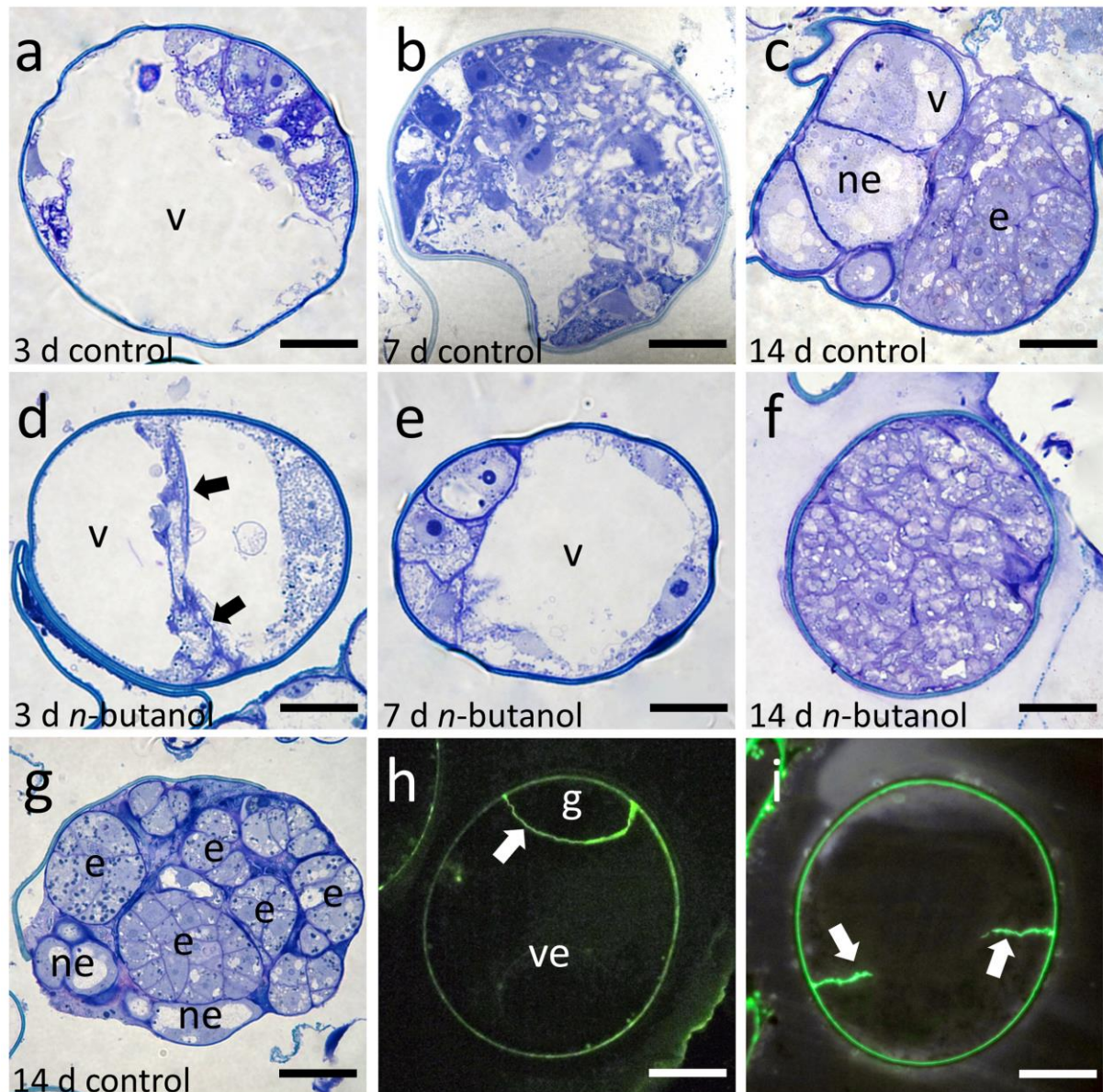


Figure 3. Altered early development of microspore-derived structures after *n*-butanol treatment. Semithin sections stained with toluidine blue. (a)-(c): cold-treated control after 3, 7 and 14 days of culture. (d)-(f): *n*-butanol treated microspores and MDSs after 3, 7 and 14 days of culture. Note that *n*-butanol treated microspores performed fewer divisions during the first seven days compared to control. (g): microspore derived structure showing numerous, embryogenic and non-embryogenic cell clusters, surrounded by debris originated from dead cells. (h)-(i): Sections of microspores after 3 days of culture, stained with auramine-O. (h): Microspore following the gametophytic developmental pathway, containing a generative and a vegetative cell. (i): Symmetrically divided microspore after *n*-butanol treatment, with incomplete cell wall. Note the free ends of cell wall (arrows). *v* vacuole, *e* embryogenic domain, *ne* non-embryogenic domain, *ve* vegetative cell, *g* generative cell, *arrows* cell walls. Bars represent 10  $\mu$ m.

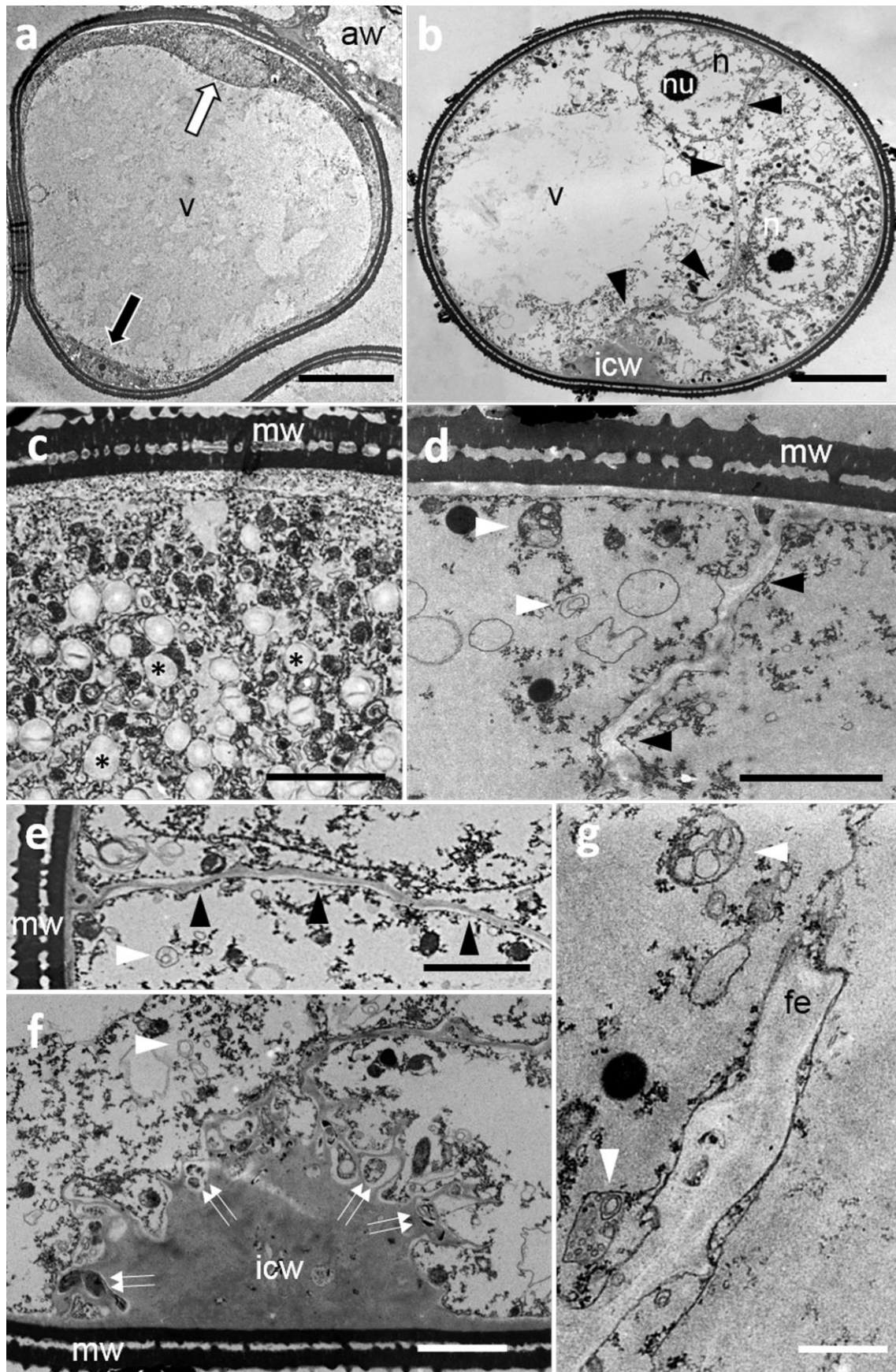


Figure 4. Ultrastructure of microspores on the third day of culture. (a) cold treated control bicellular microspore following the gametophytic developmental pathway. (b) n-butanol treated microspore,

showing the signs of embryogenic activation: symmetric division, uniform nuclei in daughter cells, cytoplasmic cleaning. (c) cytoplasm structure of microspore shown in (a). Note the considerable amount of starch granules labeled by asterisks. (d) cytoplasm structure of embryogenic microspore shown in (b). Note the progressed cytoplasmic cleaning and the presence of autophagosomes (white arrows). (e) normally developed cell wall from an *n*-butanol treated microspore. (f) irregular cell wall from an *n*-butanol treated microspore shown in (b). Note the considerably thickened part of the wall with cytoplasm-containing inclusions (double arrow). (g) free end of incomplete cell wall from an *n*-butanol treated microspore. *cw* cell wall, *mw* microspore wall, *v* vacuole, *n* nucleus, *nu* nucleolus, *aw* anther wall, *fe* free end of cell wall, *white arrow* vegetative nucleus, *black arrow* generative nucleus, *white arrowhead* autophagy-related structures, *black arrowhead* cell wall, *double arrow* inclusions in cell wall containing cytoplasmic elements, *asterisk* starch granule. Bars: (a)-(b):10  $\mu\text{m}$ ; (c),(e),(f): 3  $\mu\text{m}$ ; (d): 2  $\mu\text{m}$ ; (g): 500 nm.



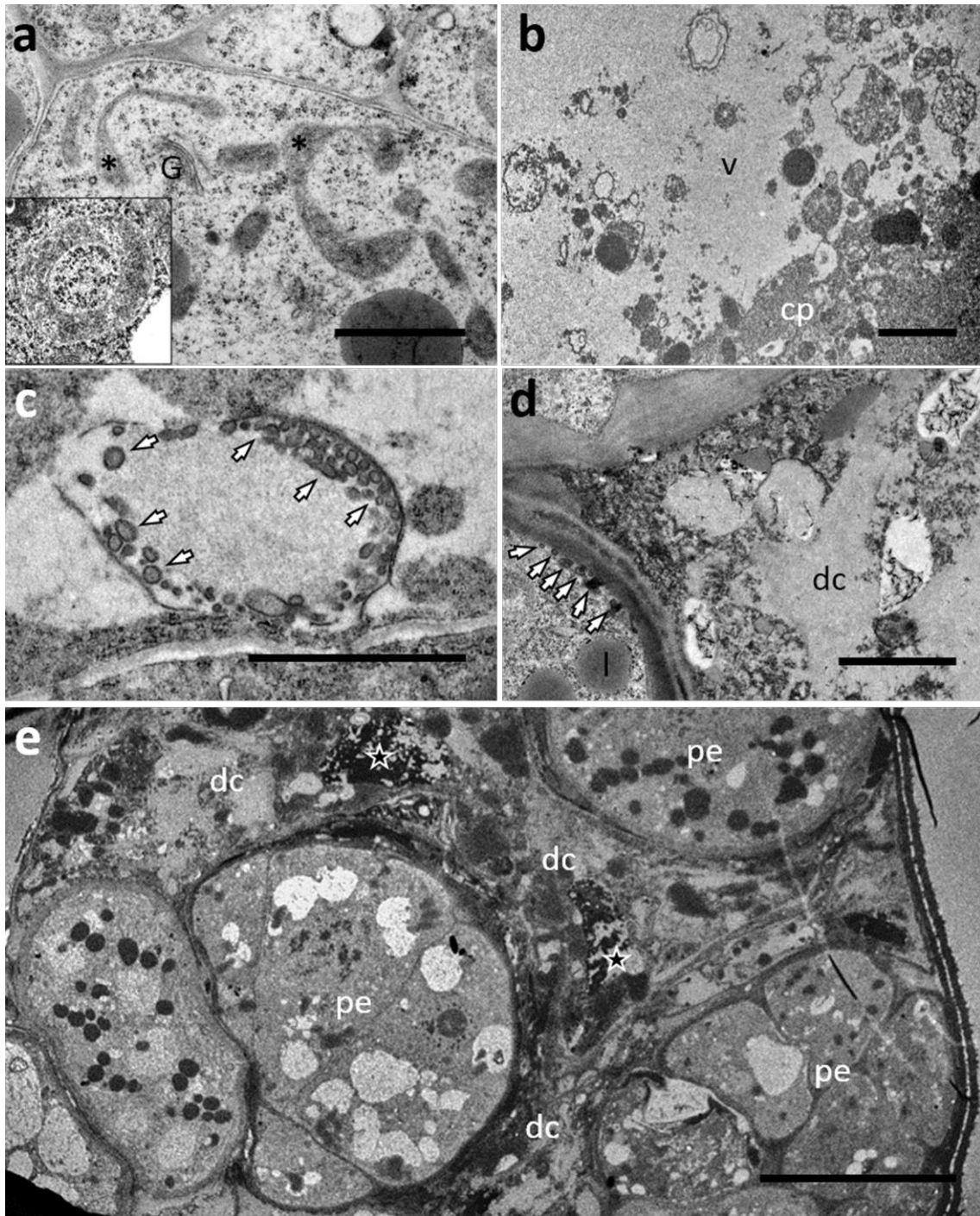


Figure 5. Autophagy and programmed cell death in microspore derived structures after 14 days of culture. (a)-(d) *n*-butanol treated MDSs, (e) cold pretreated control MDSs. (a) phagophores engulfing cytoplasm containing organelles. Inset: autophagosome. (b) intravacuolar autophagic bodies, (c) intravacuolar deposit of autophagic bodies with digested material (arrows), (d) coexistence of autophagy and programmed cell death in *n*-butanol treated MDSs. Note the small vesicles probably digested material to be excreted (arrows) and the neighboring, degenerating cell (dc) committing programmed cell death. (e) cold pretreated control microspore derived structure containing multiple proembryo-like structures (pe). dc degenerating cells, l lipid body, G Golgi apparatus, v vacuole, cp cytoplasm, asterisk phagophore, star highly condensed, degenerating nucleus. Bars: (a)-(b): 2 μm, (c)-(d): 5 μm, (e): 10 μm