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Cell architecture during the morphogenesis of coenocytic alga *Vaucheria sessilis*. II. Organization of microfilament system in the course of germination

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Summary

The architecture of microfilaments during the morphogenesis of *Vaucheria sessilis* (Vauch.) D.C. was investigated by fluorescence microscopy of (un)fixed cells using fluorescein-labelled phalloidin (FITS) and differential interference contrast (D.I.C.). The organization of F-actin system varies during aplanospore germination. In intact aplanospore, the arrangement of short fine microfilaments is irregular. Cortical girding band lies beneath the cell membrane and persists during germination. Fine microfilaments rearrange themselves into thick bundles which gradually reorient in axial direction. Cortical band, which is typical for vegetative thallome, is forming *de novo* in the secondary projection. At the same time, dense and fine actin network appears in the apical zone. Final phase of germination (formation of the vegetative branch) is characterized by active migration of organelles, and by formation of an apical cluster of nuclei. This process is accompanied by the reorganization of apical microfilament ring into a honeycomb-like network. Controversies with F-actin structure, revealed by many scientists who examined apical growth, can be explained by an extremely dynamic organization of microfilaments in the tip-growing cells. Moreover, the pattern of F-actin architecture strongly depends on the physiological status of the cell.

Key words: microfilaments, cytoskeleton, germination, morphogenesis, *Vaucheria*, apical growth

Introduction

The cytoskeleton of algae is considered to be composed of microfilaments and microtubules. Multiple functions have been assigned to these cytoskeletal elements. These include a certain role in intracellular motility, mitosis and cytokinesis, internal spatial organization, and maintenance of cell shape. One of key questions in modern cell biology is the problem of morphogenetical functions governed by cytoskeleton. In last decades, the elaboration of indirect immunofluorescence methods proved the significance of microtubules in the plant cell morphogenesis. The latter determine both the divisional plane and the position of cellulose microfibrills within cell wall (Cyr, 1994). However, functions of cytoskeletal systems in the tip-growing cells have yet to be determined. The organization of cytoskeleton in such cells varies from one in the cells which expand by diffuse growth. Microtubules and actin filaments in the tip-growing cells extend axially, and mainly distribute themselves throughout cortex and endoplasm (Cai et al., 1997). The effect of treatment with microtubule-depolymerizing agents indicates that microtubules are not essential for tip growth. All subsequent processes involving the maintenance of cytoplasmic architecture, polarity, and intracellular motility are entirely dependent on actin cytoskeleton (Menzel, 1994). Treatment with actin antagonists rapidly arrests tip extension that also indicates a fundamental role of this cytoskeletal element (Kropf et al., 1998). Therefore, the architecture of microfilament system in apical zone has been thoroughly investigated, and published data revealed some controversies with F-actin structure. Two different patterns of actin filaments have been observed, depending on the object of study and methods used, namely dense network (Derksen et al., 1995) and the sparse randomly oriented filaments (Miller et al., 1996). At present, it seems that F-actin struc-

ture localized within the apex may be variable. However, most of the previous studies did not examine the polymorphism of cytoskeleton at different stages of growth.

The objective of this research is to ascertain the organization of microfilaments during the germination of aplanospores in siphonaceous alga *Vaucheria sessilis*, and to demonstrate the dynamics of F-actin system throughout the life cycle of tip-growing cells.

Material and Methods

Culture procedures

The object of investigation was *Vaucheria sessilis* (Vauch.) D.C. CALU 1024. Cultures were maintained in standard mineral medium: 66.7 mg/L K_2HPO_4, 33.3 mg/L $MgSO_4 \cdot 7H_2O$, 100 mg/L KNO_3, 1 mL/L microelement mixture (0.022 g/L $ZnSO_4 \cdot 7H_2O$, 1.81 g/L $MnSO_4$, 0,79 g/L $CuSO_4 \cdot 5H_2O$, 2.63 g/L $NaBO_3 \cdot 4H_2O$, 1 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 9.3 g/L $FeSO_4 \cdot 7H_2O$, 1.2 g/L $CaCl_2$, 0.02 g/L $Co(NO_3)_2 \cdot H_2O$, 10 g/L Na_2EDTA). The medium additionally contained 0.1% soil extract and was solidified with 1.5% Bacto agar (Difco). Cultures were kept under continuous illumination of 10^3 lux.

Aplanosporogenesis was synchronized by alternating 24h light-dark periods in liquid medium. In the end of dark period, the aplanospores were collected with a Pasteur pipette, with visual control under a Biolam10 binocular.

Microscopic observations

A Leica microscope equipped with Nomarski differential interference contrast (D.I.C.) optics was used for the examination of F-actin in untreated thallomes. Photoimages were obtained with an optical-computer system Leica DMRXA.

Destruction of cytoskeleton

The targeted desintegration of cytoskeleton was performed with colchicine (Sigma Chemical Co., MO, USA), phalloidin (Sigma Chemical Co., MO, USA), and cytochalasin D (Sigma Chemical Co., MO, USA). Colchicine stock solution (5 mg mL^{-1}) was prepared in 100% dimethylsulfoxide (DMSO). Phalloidin was stored as methanolic solution (500 mg mL^{-1}), cytochalasin D (1 mg mL^{-1}) was kept in 100% DMSO. The material was correspondingly incubated with colchicine (50 mg mL^{-1}, 60 min), phalloidin (2–10 mg mL^{-1}, 30 min), or cytochalasin D (10 mg ml^{-1}, 30–60 min). The above concentations of colchicine and cytochalasin D were introduced into culture media.

Actin labelling

Stock solution of the fluorescein-labelled phalloidin (FITC) (Sigma, 2mg mL^{-1}) was prepared in 100% methanol. The material was rinsed for 15 min in 0,2 M phosphate-buffered saline (PBS) containing 10 mM EGTA, 100 mM $MgSO_4$, and 1% DMSO. After that, the cells were transferred to PBS-buffer containingh FITC-phalloidin (final concentration 20 mg mL^{-1}). Incubations were carried out for 2h at room temperature in the dark. Cells were rinsed in PBS before the examination under optical-computer system Leica DMRXA (L4 Filter). Methanol or DMSO in administered concentrations make no effect on cytoskeleton of *Vaucheria* (Peat and Oliveira, 1994).

Results

Morphological pecularities of the different stages of germination were described in our previous study (Gavrilova and Rudanova, 1999). Two systems of the microfilaments in intact aplanospore were detected, namely, a broad girding cortical band beneath the cell membrane, and fine, short filaments loosely scattered throughout the volume of the cell (Figs 1, 7a). Cortical band consists of long, parallel actin cables (Fig. 1c). Visible length of fine filaments is 2–4 μm, and they expand in different directions between organelles. The FITC-phalloidin staining revealed numerous bright fluorescent loci (Fig. 1a) without a defined structure. The methods used did not reveal the coordination between fine filaments and actin loci.

The stage of hyaline cap formation is very short, and D.I.C. microscopy did not reveal any changes in the organization of microfilament. In resemblance with the preceding stage, cortical band, fine filaments, and actin loci are also present (Fig. 2). Moreover, intense and diffuse fluorescence was also observed, mainly in the association with the zone of hyaline cap (Figs 2, 7b). The domains with diffuse fluorescence probably possess a complex network consisting of significantly more delicate F-actin filaments.

Strong reorganization of microfilaments occured at the stage of formation of primary projection. Fine filaments rearranged themselves into parallel fibers associated in thick bundles 5–25 μm length, 0,5–0,9 μm width (Fig. 3). The bright fluorescent loci disappeared. Thick bundles show bending and demonstrate a network with interconnecting strands (Figs 3a, 3b, 7c). In the zone of primary projection no specific structure was detected.

Cytoskeleton pattern typical of the vegetative thallome begins to form in the secondary projection during the germination phase 3. Numerous short, axially aligned F-actin fibers 2–4 μm length are visible in secondary projection (Figs 4d, 7d). Very fine fibers often bend, and therefore the FITC-phalloidin labelling demonstrates only a thin unregular network (Fig. 4a). The latter becomes denser in apical zone, and an intense diffuse fluorescence can be seen in the apex (Figs 4a, 4b). The onset of formation of cortical band can be detected by means of both fluorescence and D.I.C. microscopy (Figs 4a, 4d). Cortical band does not expand continuously, and the peripheral fluorescence is interrupted. Girding band is still present beneath the cell membrane of aplanospore, although it is destroyed at the base of secondary projection and does not reveal

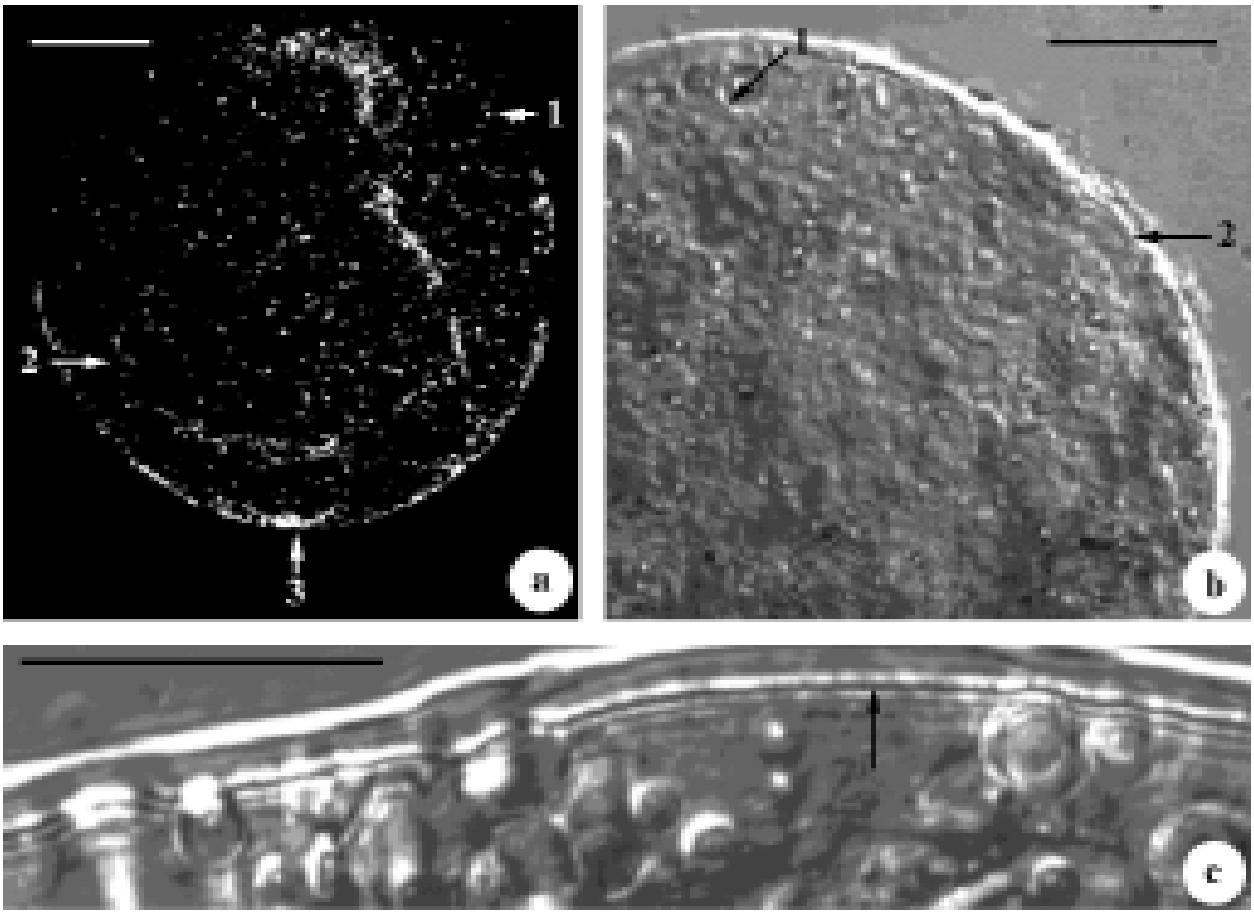

Fig. 1. Phase 0 (intact aplanospore). **a** – FITC-phalloidin staining. Numerous bright fluorescented loci are scattered througthout volume of aplanospore (1). Position of short, very fine microfilaments is unregulated (2). Cortical girding band is presented (3). **b** – D.I.C. optic. Note fine separate fibers between organells (1), and cortical band (2). **c** – D.I.C. optic. Broad girding cortical band of microfilaments is arrowed. Scale bar: 10μm.

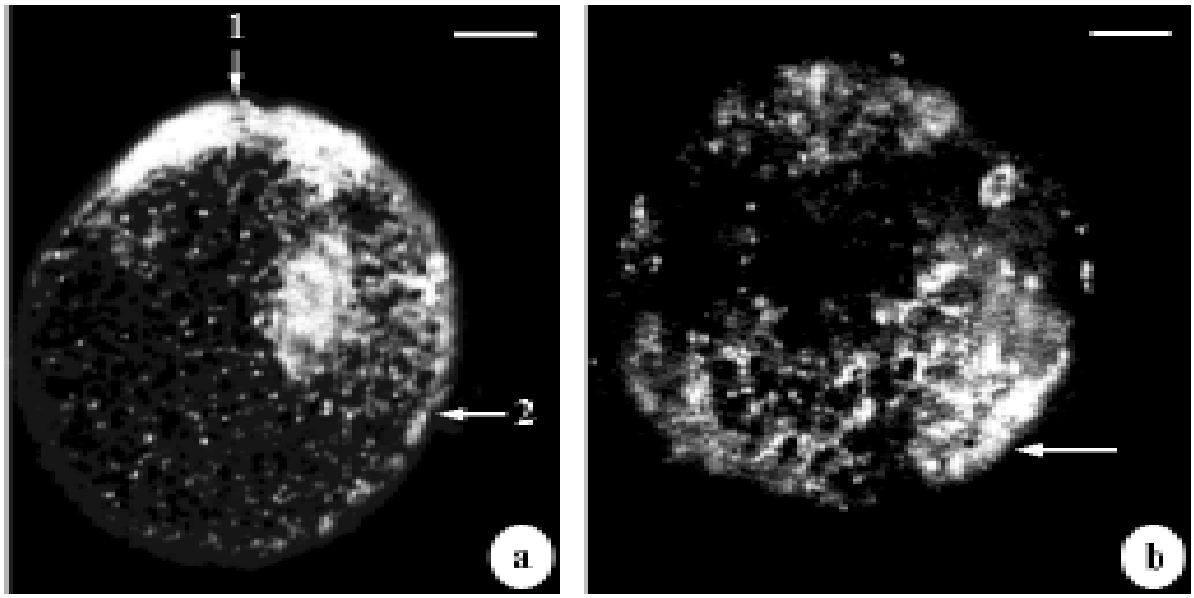

Fig. 2. Phase 1 of germination (formation of hyaline cap). **a** – FITC-phalloidin staining. Domain with diffuse fluorescence is observed in place of formation of hyaline cap (1). Cortical band persists beneath cell membrane (2). **b** – FITC-phalloidin staining. Zone of hyaline cap is arrowed. Notice brigthly fluorecsent area without defined structure. Scale bar: 10μm.

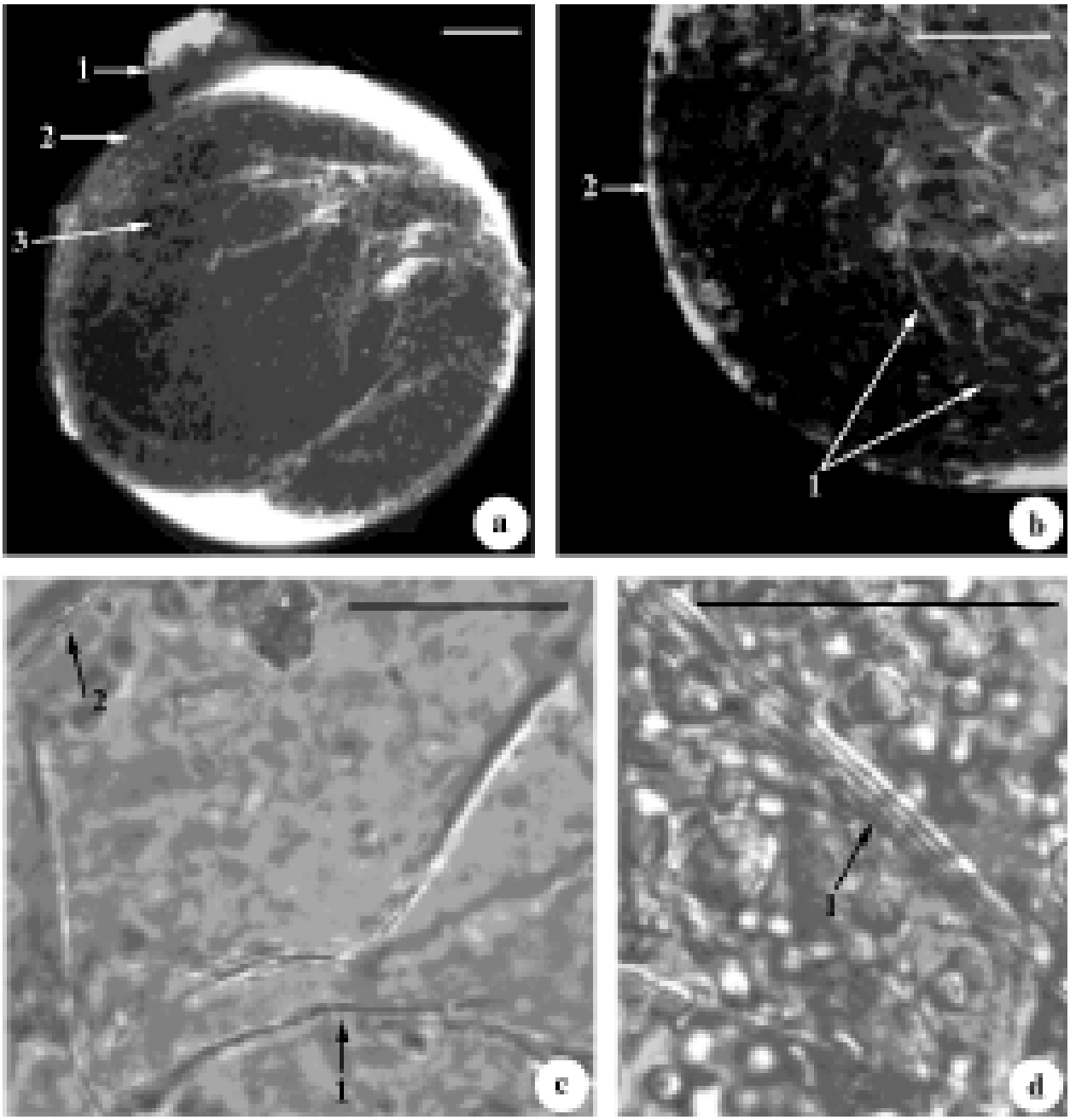

Fig. 3. Phase 2 of germination (formation of primary projection). **a** – FITC-phalloidin staining. Notice forming primary projection (1), cortical band (2), long, thick bundles in the aplanospore (3). **b** – FITC-phalloidin staining. Thick microfilament bundles extending in different directions (1), cell membrane is girded by the cortical band (2). **c** – D.I.C. optic. Thick bundles are winding (1), cortical band is presented (2). **d** – D.I.C. optic. Microfilament bundles consist of several parallel fibers (1). Scale bar: 10μm.

any interaction with forming cortical band of the branch. Thick F-actin bundles in aplanospore become transformed into thin straight fibers 5–20 μm length (Fig. 4c). The number of fibers increases during the germination phase 3, and fibers get oriented predominatingly in the direction of growth (Figs 4c, 7d).

Germination phase 4 represents the stage of formation of vegetative branch, and it is characterized by an active migration of organelles and the formation of apical cluster of nuclei (Gavrilova and Rudanova, 1999). In this period the building of the cortical microfilament system becomes complete (Figs 5a, 5b, 5c). Like in all previous stages, cortical band does not have visible connections with the cortical system of former aplanospore (Fig. 5a). The aggregation of microfilaments into cortical cables obviously occurred *de novo* in the forming thallome, not as a prolongation of girding band. The axially aligned F-actin fibers increase in number, expand between organelles, and compose a fine network in the forming branch (Figs 5b, 5d). The apical dense network transforms into a ring of

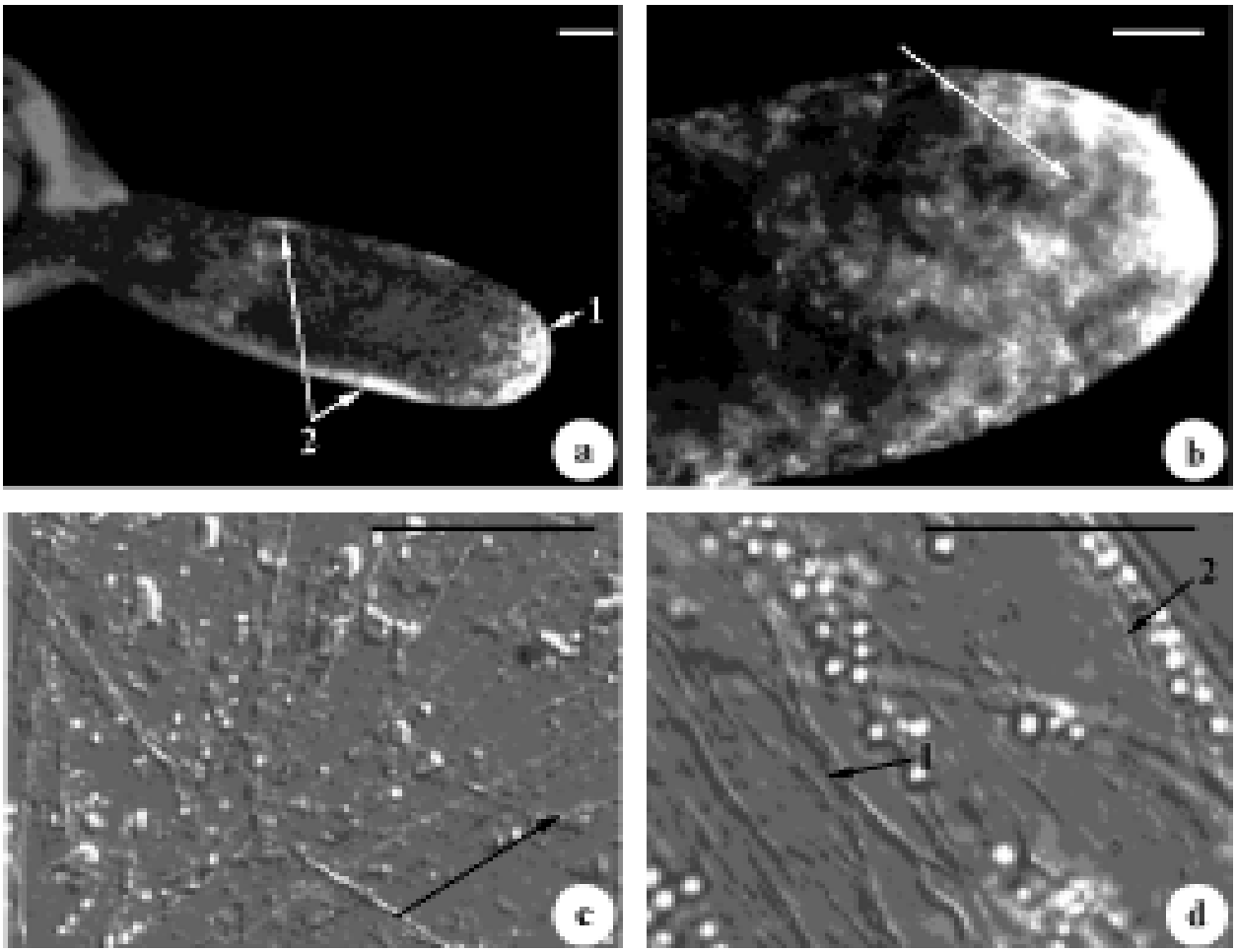

Fig. 4. Phase 3 of germination (formation of secondary projection). **a** – FITC-phalloidin staining. Notice the formation of a cortical band in the secondary projection (2), and bright fluorescence of the apical zone (1). **b** – FITC-phalloidin staining. Apical zone of secondary projection with a dense net of fine fibers (arrow). **c** – D.I.C. optic. Fragment of aplanospore. The direction of growth of the secondary projection is arrowed. Thin straight fibers are orienting predominatingly in direction of filament. **d** – D.I.C. optic. Fragment of secondary projection with thin, winding fibers of microfilaments (1), and forming cortical band (2). Scale bar: 10μm.

fibers which delimits the apical zone (Figs 6a, 6b, 7e). The apical ring is destroyed concomittantly with the decrease of growth rate and the appearance of apical cluster of nuclei (Figs 6c, 7f). The end of germination is characterized by the formation of stable honeycomb-like net in apical zone, that occupies 50–70 μm of the tip length (Figs 6d, 6e, 6f, 7g).

Different anti-cytoskeleton agents were used for the elucidation of significance of the different cytoskeleton systems in germination. However, all drugs (colchicine, cytochalasine D, phalloidin) applied at the early stage of germination caused a rapid arrest of morphogenesis. Growth can be restored after the drug was removed (in case of colchicine and cytochalasin D). The treatment of aplanospores with anti-cytoskeleton agents at the germination stages 3–4 provokes the delay in development, and pronounced inhibition of growth rate of the forming branch (Table 1). The destruction of drugs after 7 days, or their removement from the medium caused recovery of the growth rate (Table 1).

Discussion

A pecularity of apical growth consists in crucial role of microfilaments in the morphogenesis. Anti-actin drugs are known to dramatically disturb growth processes. In our experiments cytochalasin D, which is known to provoke the destroyment of cortical actin fibrills of *Vaucheria* and prevent cytoplasmic flow (Peat and Oliveira, 1994), demonstrated significant inhibition of the germination. Microfilaments are involved not only in the maintenance of tip growth , but also in the localization of apical zone. F-actin accumulates in diverse patterns at the sites of tip initiation (Blatt et al., 1980; Anderson and Soll, 1986; Kropf et al., 1989; Quadar and Schepf, 1989). The areas of intense diffuse fluorescense of actin were detected in this study at the first stage of germination of *Vaucheria sessilis*. The importance of actin reticulation and its rearrangement for the establishment of tip zone was prooved by phalloidin treatment. It is supposed that phalloidin influences spatial reorientation of actin fibers and does not

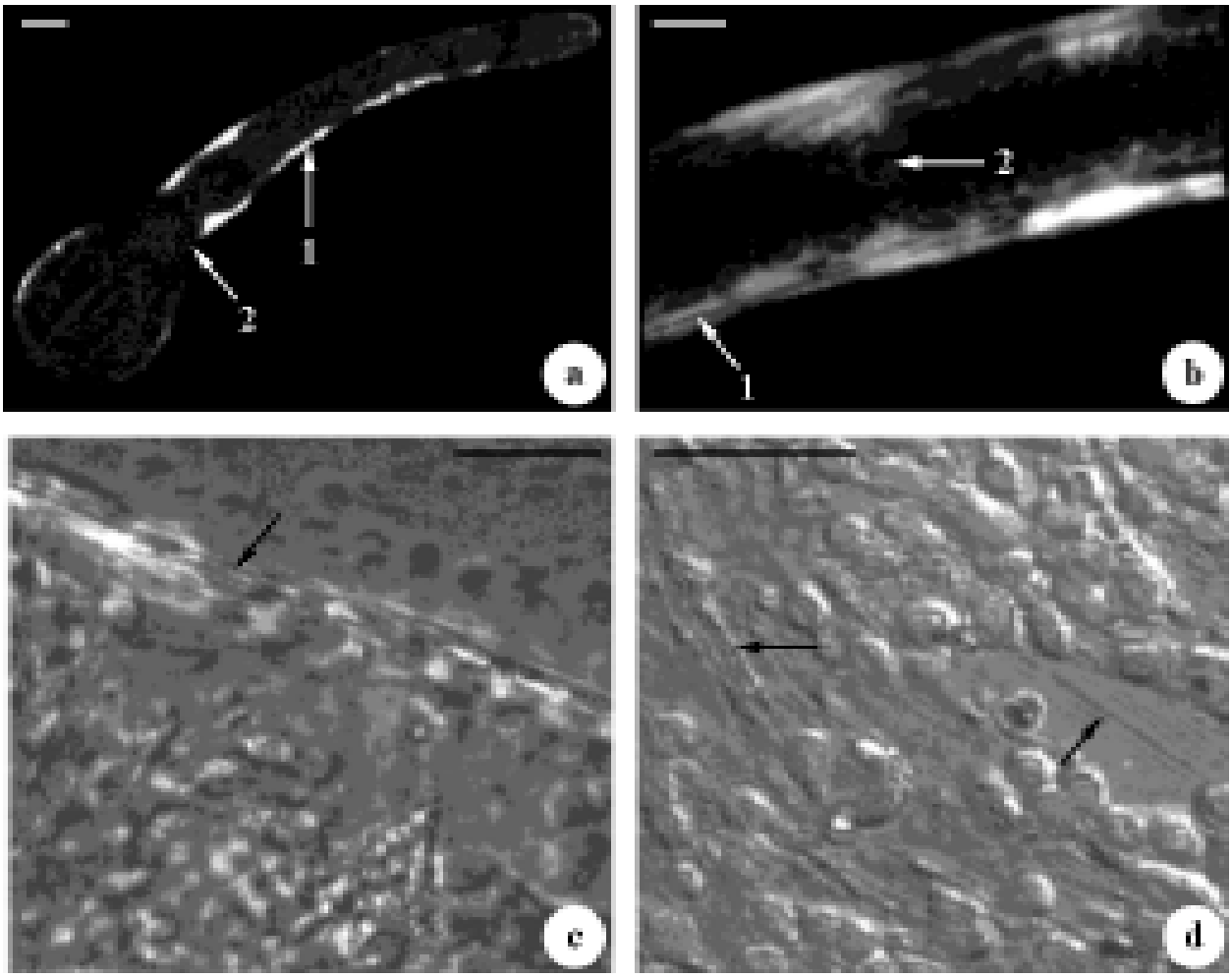

Fig. 5. Phase 4 of germination (formation of vegetative branch). **a** – FITC-phalloidin staining. Notice cortical band (1), absence of cortical layer in the base of the branch (2). **b** – FITC-phalloidin staining. Fragment of the branch with pronounced cortical band (1), and a fine net of fibers (2). **c** – D.I.C. optic. Fragment of cortical band in the branch (arrow). **d** – D.I.C. optic. Fine fibers between organells in the branch (arrow). Scale bar: 10μm.

prevent the motility based on myosin motors (Dancker et al., 1975). Similarly with cytochalasin D, phalloidin caused an arrest of the germination. The same effect was produced by colchicine which inhibited the process of microtubule assembly. Even in high concentrations it did not influence the structure of cortical actin fibers and cytoplasmic movement (Blatt et al., 1980; Peat and Oliveira, 1994). Colchicine also is known to be an extremely potent antimitotic drug in the case of algae (Gunning and Hardham, 1982). As it was previously demonstrated, the continuing division of nuclei represents an important attribute of germination (Gavrilova and Rudanova, 1999), hence the inhibition of mitosis seems to provoke the arrest of germination.

In the course of germination, actin exists in three different stuctural forms, namely, cortical cables, reticulated fibers, and dense plaques. Bright-fluorescent phalloidin-positive loci were detected in oomycetes (Bachewich and Heath, 1998), in moss protonemae (Quader and Schnepf, 1989), and in the coenocitic algae *Caulerpa* (Menzel, 1987) and *Vaucheria longicaulis* (Peat and Oliveira, 1994). These structures, termed patches or plaques, were carefully investigated in growing yeasts. Previous studies show correlations between the rearrangement of patches and actin cables: if the patches are clustered, the cables are well pronounced and orient themselves along the the axis of mother bud (Kilmartin and Adams, 1984), however, in cells with the depolarized, randomly distributed patches the cables are usually unobserved (Chowdhury et al., 1992; Karpova et al., 1998). The plaques remained unchanged after an anti-actin drug treatment (Peat and Oliveira, 1994; Bachewich and Heath, 1998; Karpova et al., 1998). Direct contacts between the patches and actin arrays were detected during drug application, as well as in the forming apical zones. In the case of *Vaucheria longicaulis*, phalloidin-positive loci are considered as the organizing centers for microfilaments which irradiate from the foci localized in apical zone of the vegetative thallome and "contract into focus" after the treatment with cytochalasin (Peat and Oliveira, 1994). In the present study, no direct assosiation

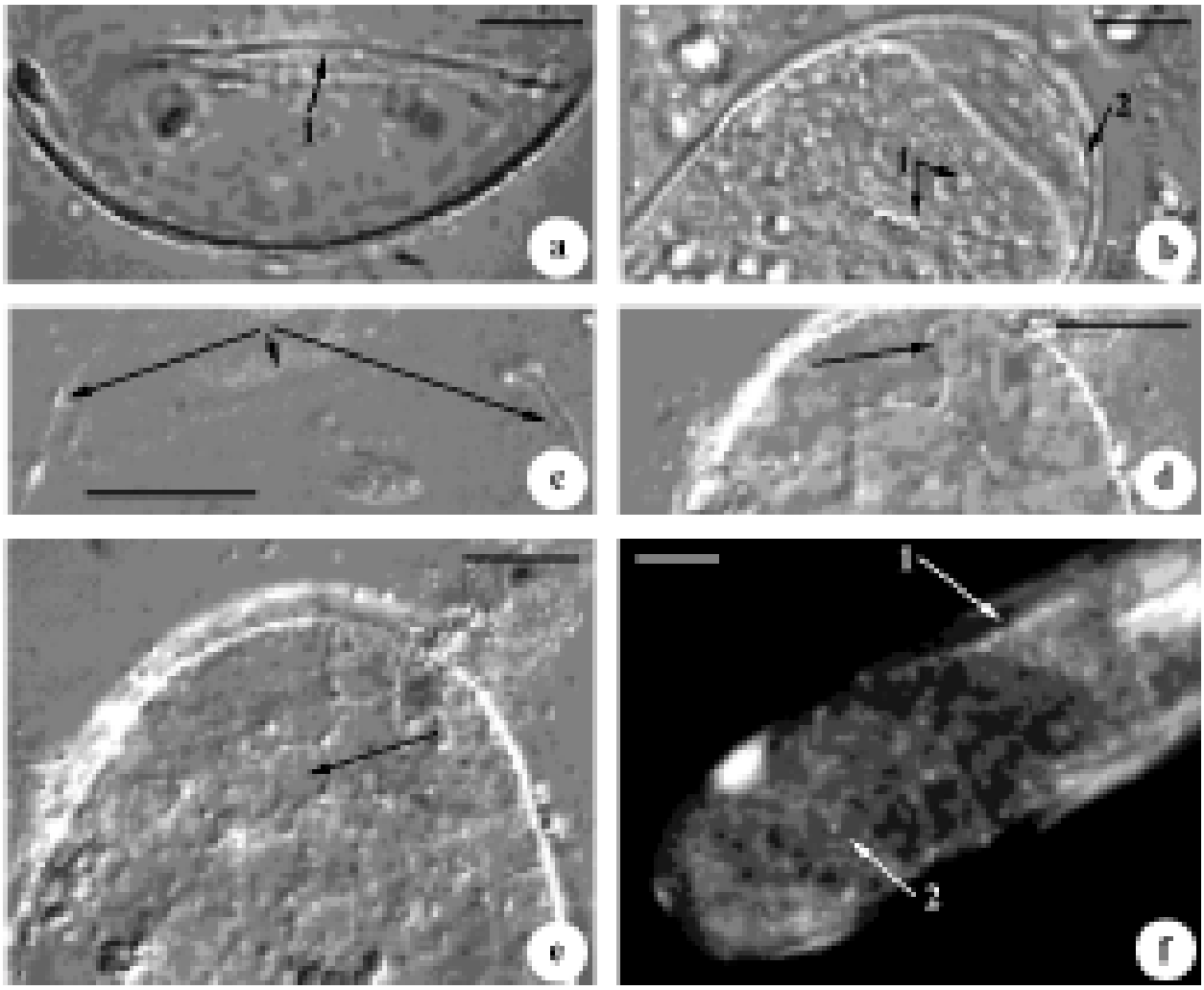

Fig. 6. Phase 4 of germination (apical zone of vegetative branch). **a**, **b**, **c** – D.I.C. optic. Apical ring of microfilaments. **d** – D.I.C. optic. Rearrangement of apical ring into honeycomb-like net. **e** – D.I.C. optic. Honeycomb-like net. **f** – FITC-phalloidin staining. Honeycomb-like net (2) and cortical band (1). Scale bar: 10μm.

between actin fibers and plaques was detected, however, the disappearence of phalloidin-positive loci correlates with the rearrangement of fine actin fibers into thick bundles alligned axially. Obviously, the correlation between actin patches and spatial architecture of actin bundles represents a common feature of tip growth, although the mechanisms of their interaction remain obscure.

The architecture of F-actin in vegetative branches was investigated in *Vaucheria longicaulis*. Staining with phalloidin revealed cortical F-actin bundles and a net of fine fibrillar actin distributed in endoplasm. This net disappears after fixation with aldehyde. In the apical zone F-actin was generally organized in dense focal masses, from which microfilament fibers seem to radiate (Peat and Oliveira, 1994). *V. sessilis* demonstrated a similar pattern of the microfilament architecture. A cortical band begins to organize in the secondary projection simultaneously in several zones, without contacting with cortical microfilaments of the aplanospore. This organization pattern suggests a complex composition of cables. Multiple sites of formation of cortical bands, as well as the absence of polar elongation might indicate that cables contain numerous filaments, that these filaments have different lengths, and that the ends of filaments are not mutually aligned (Karpova et al., 1998). Fine actin net in the endoplasm reveales a close association with organelles. This peculiarity of the tip growing algae has not been detected in filamentous fungi (Vasilyev, 1996). At the same time, actin net participates in the movement of chloroplats in siphonaceous algae (Blatt and Brigs, 1980; Menzel and Schliwa, 1986).

The organization of microfilaments in apical zone revealed a high variability, namely, dense network, apical ring, and honeycomb-like net. The pattern of actin architecture in tips of *V. longicaulis* differed from that of *V. sessilis*. However, radial arrays of actin in *V. longicaulis* can form as the result of insure during sample preparation (the branch was dissected in 1 mm pieces prior to fixation). Besides, a dense actin filament network observed at the start of vegetative branch formation in *V. sessilis* could

Table 1. Influence of anticytoskeleton agents on growth of young branches of *Vaucheria sessilis*

variant	2 days		5 days		7 days	
	L	S	L	S	L	S
normal growth	2520±390	1260	5063± 897	847	7421± 2012	1189
growth with colchicine	140± 30	70	561± 84	140	637± 145	538
growth with phalloidin	490± 125	245	1108± 324	206	1403±105	148
growth with cytochalasin D	100± 45	50	1647±270	160	3466±881	909

L – length of branches (μm); S – growth rate per day (μm · d^{-1}).

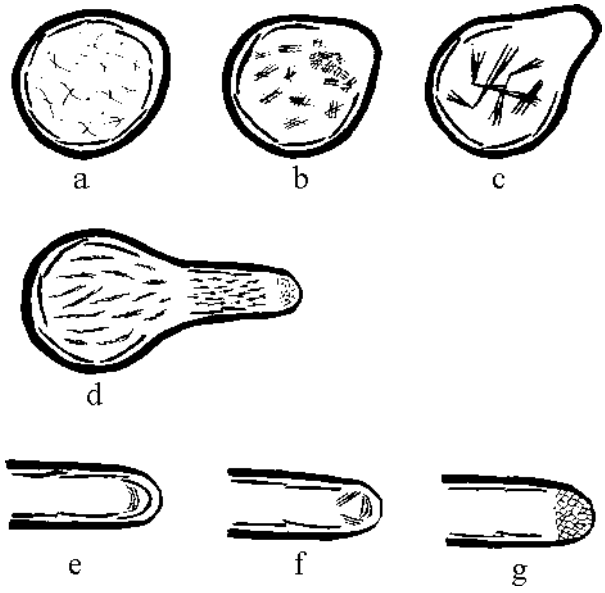

Fig.7. Scheme of rearrangement of microfilament system in course of germination. **a** – Phase 0 (intact aplanospore). **b** – Phase 1 of germination (formation of hyaline cap). **c** – Phase 2 of germination (formation of primary projection). **d** – Phase 3 of germination (formation of secondary projection). **e**, **f**, **g** – Phase 4 of germination (apical zone of young vegetative branch).

reveal the same type of F-actin (if a higher-resolving microscope will be used). In all cases, the presence of a dense actin network corresponds to the physiological status specified by a necessity of structural reinforcement of cell cortex. The transversal ring of cortical F-actin in apical zone was detected in fern protonemae and algal rhizoids which lack a dense net of F-actin in their tips (Kagawa et al, 1992; Henry et al., 1996; Kropf et al., 1998). It was assumed that the cortical ring is associated with the adhesion of plasma membrane to the cell wall. Although adhesion to the extracellular matrix is known to be involved in signal transduction in animal cells, the role it plays in apical growth is not clear.

The honeycomb-like net seems to be the most stable spatial pattern of the organization of actin in the tip, however, the density of this net can vary. The above variability can be associated with hypothetical functioning of microfilaments: in the regulation of the position of terminal cellulose-synthesizing complexes (TC) in tip-growing cells (Kobayashi et al., 1988; Kropf et al., 1989). The density of TC decreases from the tip towards basal regions (Tsekos, 1999). High density of TC in the tip region is consistent with the assumption that most of cellulose microfibrills are synthesized and secreted within the apex where the bulk cell wall growth and synthesis occur. In agreement with this idea, all deviations in growth rate and cell wall synthesis would correlate with the position and amount of TC, and, correspondingly, with microfilament architecture.

Controversies about the structure of F-actin which have been revealed in the previous studies (Peat and Oliveira, 1994; Derksen et al., 1995; Miller et al., 1996) can be explained by an extremely dynamic organization of the microfilaments in tip-growing cells. Moreover, the pattern of F-actin architecture seems to depends strongly on physiological status of the cell.

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