Instantaneous change in hyphal diameter in basidiomycete fungi

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Abstract:

Under certain conditions, fungi are capable to fast (down to seconds) change the size of their cells. For example, it is known that the cells of many species of yeasts under hyperosmosis instantly and reversibly shrink entirely, without plasmolysis, with a decrease in volume of up to 40% or more. There is limited data that filamentous fungi can also instantly change the diameter of their unspecialized hyphae. This property is fundamental and is a powerful tool for survival and preservation of the physiological functions of the fungus in sharply changing external conditions, but requires detailed study. In this work, which is a large-scale (more than 50,000 cells measured) light microscopic study, the ability of three unrelated basidiomycete species to fast change the diameter of their hyphae under the influence of various factors was analyzed. It was found, for example, that all three fungi respond similarly to moderate hyperosmotic shock and shrink by an average of 12-14% in diameter, but inhibitors of actin assembly can cause both expansion and shrinkage of hyphae, or have no effect on a fungus. Combining the current results with those previously obtained in the study of macroinvagination systems of the fungal plasma membrane, different approaches for basidiomycetes to respond to sudden stress changes were proposed. The previously presented curtain model has also been advanced. The model describes a general mechanism for the rapid regulation of hyphal sizes, which involves the elastic properties of the cell wall, adhesion of the plasma membrane to it, regulation of turgor, a system of actin cables that regulate the tension of the plasma membrane and cell size, as well as a complex system of macroinvaginations of the plasma membrane, ensuring constant integrity and functionality of the latter.

Keywords: hyphal shrinkage, plasmolysis, F-actin, hyperosmotic stress, turgor pressure, curtain model

1. Introduction

Fungi are extremely flexible organisms that quickly conform to their environment. One expression of fungal flexible is the ability to change the thickness of their hyphae (cell size in the case of yeast). Changes in hyphal diameters can occur over tens of minutes or hours during hyphal growth. For example, xylotrophic basidiomycetes, when inoculated from a rich nutrient medium to a medium without a nitrogen source, switch to in economical mode – the average thickness of *de novo* grown hyphae decreases sharply (Mazheika et al., 2020a). Another example can be found in the recent work of a group led by Prof. N. Takeshita, which showed that some fungi, for example, *Aspergillus nidulans* with an average hyphal diameter of 2-3 µm, easily grow through a 1 µm-width channel of microfluidic device (Fukuda et al., 2021). One more example is the formation of giant yeast cells in *Cryptococcus neoformans* during pathogenesis to evade the host immune response and macrophage attacks (Zaragoza et al., 2010).

Changes in the diameters of fungal hyphae/cells can occur within tens of minutes, not necessarily accompanied by growth, due to a rapid regulatory response leading to changes in gene expression and physiological restructuring of the fungal cell. Thus, during hyperosmotic shock in yeast and *Neurospora crassa*, as described in more detail below, turgor pressure drops and the size of hyphae/cells decreases (Slaninova et al., 2000; Lew, Nasserifar, 2009). This activates the CWI (Cell Wall Integrity) pathway, which also leads to the initiation of the HOG (High Osmolarity Glycerol) pathway (de Oliveira et al., 2021). As a result, in less than half an hour the synthesis of glycerol begins in the fungal cell, which, together with the regulation of ionic uptake, in about an hour can allow the hyphae/cells to restore turgor pressure and their initial size (Lew, Nasserifar, 2009; Ene et al., 2015).

Finally, the change in hyphae/cell diameter can occur instantly: within seconds, to a maximum of several minutes, due to the potential plasticity and elasticity of the cell wall, and other properties of the fungal cell. An example is the constricting rings of nematophagous fungi, which are capable of significantly increasing the diameter of the cells of ring in a few seconds and thus trapping the nematode (Chen et al., 2022; Panstruga et al., 2023). Fundamentally important for biology and for this article is another phenomenon: back in the 70s of the last century, it was noticed that hyperosmotic shock could cause a sharp but reversible shrinkage of cells in *Saccharomyces cerevisiae* and other yeasts with a loss of volume up to 40% or more (Kopecká et al., 1973; Morris et al., 1986). Various properties of instantaneous changes in the size of fungal cells are given in the Table 1. For comparison, the Table 1 includes information about plant cells, which, despite the presence of a cell wall, respond to osmotic shock differently from fungi in some respects. The Table 1 shows that the issue of instantaneous changes in the thickness of hyphae in filamentous fungi has been studied fluently and on a very small number of species. Filamentous basidiomycetes are almost completely unstudied in this matter. Although it is from these fungi, especially xylotrophic and mycorrhizal ones, knowing their amazing ability to utilize of

substrates, unique intracellular structural adaptations, etc., original approaches to overcoming osmotic and other stresses could be expected (Sheldrake, 2020; Mazheika et al., 2020a, 2022).

The Table 1 demonstrates that instantaneous shrinkage of cells in a hyperosmotic environment leads to the formation (or increase in the number and size) of invaginations of the plasma membrane. This behavior of the plasma membrane is quite predictable: with a sharp decrease in the cell surface, the excess membrane must be temporarily removed without compromising integrity and functionality of the plasma membrane (Kopecká et al., 1973; Oparka, 1994). For this, special structures are needed – invaginations of the plasma membrane or, more precisely, macroinvaginations, since their sizes usually exceed, for example, clathrin-coated endocytic pits. The plasma membrane of a fungus can be vaguely compared with accordion bellows, and in some cases, such as in the constricting rings of *Arthrobotrys dactyloides*, the similarity is more great (Chen et al., 2023). The question of whether the macroinvaginations of the plasma membrane are endocytic structures (whether they are scissored from the membrane and what is their further fate in the cytoplasm) has not had a clear answer among biologists for a long time (Oparka, 1994; Mazheika, Kamzolkina, 2021). Previously, the authors of this paper associated the macroinvaginations with endocytosis and divided fungi into two categories: with classical (patch-type) endocytosis with small endocytic pits, and with macrovesicular endocytosis, which is characterized by a larger range of sizes of primary invaginations of the plasma membrane (Mazheika et al., 2020a, b). However, recent detailed study of some species of wood-decaying basidiomycetes using time-lapse video and Z-stacks fluorescent microscopic technologies changed our view of the problem (detailed information in Mazheika et al., 2022). It turned out that the scission of macroinvaginations from the plasma membrane a phenomenon that exists, but not frequent in these fungi. At the same time, these macroinvaginations constitute an extremely dynamic complex system of various types of macroinvaginations that constantly exists in hyphal cells (at least when the mycelium is immersed in the liquid phase). The number of macroinvaginations per cell and the representation of different types of them depend on various factors. The main “workhorse” is small, dense, complexly organized invaginations, the number of which can reach several dozen per cell, and which correspond to classic fungal lomasomes (Brushaber, Jenkins, 1971; Mazheika, Kamzolkina, 2021). An example of other type of macroinvaginations is unfolded tubular invaginations and if in *R. solani* these structures rarely exceed 5 µm in length, then in *S. hirsutum* thick tubes can be formed, more than 30 µm in length, and extending deep into the cytoplasm of the hyphae. However, there are much fewer such large macroinvaginations than lomasomes in cells of xylotrophs, and, importantly, they develop only under certain stressful conditions (for example, during prolonged observation of a microscopic specimen). In result in a microscopic specimen, when using a fluorescent tracer of endocytosis of the FM4-64 type and working in one focal plane, increasingly larger macroinvaginations appear sequentially, which imitates the endocytosis path from primary pits to vacuoles-lysosomes. Accordingly, filamentous fungi better to be divided not according to the type of endocytosis but according to the types of macroinvagination systems. Ascomycetes and *R. solani* have a

relatively simple such system: lomasomes are simpler and smaller than those of xylotrophic basidiomycetes, and other tubular and lamellar invaginations rarely exceed a few microns in size. Xylotrophic basidiomycetes and, most likely, representatives of some other groups of basidiomycetes, have the system of macroinvaginations that exceed the previous group in both structural complexity and possible size.

It is obvious that the ability to instantly change the diameter of hyphae and the ability to form macroinvaginations of the plasma membrane are closely related and represent a single mechanism that is a powerful tool for the rapid adaptation of the fungus to various changes occurring in the substrate. Therefore, this work is a logical continuation of our previous studies of the fungal macroinvagination systems (Mazheika et al., 2020b, 2022). The goal of this work is a light microscopic quantitative and qualitative analysis using a large number of fungal cells (several tens of thousands) of the ability of three species of basidiomycetes from different systematic and ecological groups to change the thickness of their hyphae in response to various factors. There are inhibitors of F-actin assembly among the factors. The results of the analysis of the influence of these inhibitors, together with other results, can help advance the curtain model we previously proposed, according to which actin cables play an important role in maintaining cell protoplast shape and regulating plasma membrane tension in basidiomycetes (Mazheika et al., 2020b).

Table 1. Some properties of fungal and plant cells in the first minutes of hyperosmotic shock

	Yeast	Filamentous ascomycetes	Filamentous basidiomycetes	Higher Plants
Rapid change in cell size	For at least one and a half dozen species of yeast, both ascomycete and basidial, rapid (less than 1 min) cell shrinkage has been shown. For example, 0.5 M NaCl shrinks *Saccharomyces cerevisiae* cells by approximately 40% by volume, 2.5 M – up to 60-70% (will depend on various conditions, for example, on the carbon source in the nutrient medium).	We know of only one work with a detailed analysis of initial changes in the hyphal size, turgor, membrane polarization, etc. in hyperphase in *Neurospora crassa*. Lew and Nasserifar (2009) showed that *N. crassa* hyphae in 0.55 M sucrose shrink rapidly within about one min. The volume decreases by approximately 40%, which corresponds to a decrease in hyphal diameter of approximately 20-25%.	Our recent paper (Mazheika et al., 2020b) showed that 0.6 M sorbitol causes shrinkage of non-apical hyphae of *Rhizoctonia solani* by approximately 15%.	Probably, under different conditions and in different cells, everything can be different, especially since the elastic modulus of the plant cell wall varies greatly. But Morris et al. (1986) states that in plants the cell wall does not change shape and size in the hyperphase, even during plasmolysis.
Changes in cell wall thickness and structure	Along with the compression of the entire cell, an instant thickening of the cell wall occurs. More precisely, the inner chitin-glucan layer becomes compacted and thickened, and it creeps onto the outer mannan layer. Therefore, the total thickness of the cell wall does not necessarily change in principle. For example, in	No data. There are no noticeable changes in wall thickness in light microscopic photographs in Lew and Nasserifar 2009; TEM analysis is required. By analogy with yeast, structural rearrangements should occur in the cell wall and the elasticity modulus should change. However, since longitudinal shrinkage	There are no exact data, but Mazheika et al. (2020) showed loosening of the inner layer of the calcofluor-labeled cell wall in *R. solani* in hyperphase.	See above.

	Candida albicans grown on glucose as a carbon source in 1M NaCl, the total wall thickness remains almost unchanged, but the thickness of the chitin-glucan layer increases by about 1.5 times. On lactate, the inner layer increases almost 3 times, the total thickness – almost 1.5 times.	in hyphae can be neglected, the wall is compacted only in the cross section – this has less effect on the wall thickness.		
Plasmolysis	No, but the vacuoles decrease in size	There are no exact data, but, again, plasmolysis is not visible in the photographs in Lew and Nasserifar 2009 and other works. In addition, in mycology there is a paradigm that the plasma membrane in fungi is firmly attached to the cell wall through focal adhesion sites and GPI proteins, which makes plasmolysis difficult. Indirect confirmation is the overestimation of the turgor value by the method of primary (incipient) plasmolysis compared to direct methods of measuring turgor pressure (for example, for *N. crassa* the difference can be about 3 times).	In *R. solani*, mild plasmolysis was observed in only 16% of cells at 0.6 M sorbitol. Most often it was convex plasmolysis with slight detachment of the protoplast from the septa. However, in this work (Mazheika et al., 2020b) there is an important nuance: the mycelium was microscoped after 15 minutes of preincubation in sorbitol, in other words, the first minutes of osmotic shock were beyond observation.	Plasmolysis in hyperphase is a common occurrence (but not always fast – it may take tens of minutes). Oparka (1994) distinguishes different types of plasmolysis, but two main ones: concave and convex. With any type of plasmolysis, the protoplast remains connected to the cell wall at focal adhesion sites through the thin Hechtian strands and the wider Hechtian reticulum. The cortical endoplasmic reticulum plays an important role in the attachment of the protoplast to the cell wall. It is contained in the Hechtian strands and Hechtian reticulum.
Formation of plasma membrane invagination s	Relatively deep invaginations of the plasma membrane are formed (in addition to eisosomes, which also become some deeper). Another option: existing tubular/lamellar invaginations become deeper in the hyperphase. These invaginations are often labeled with cell wall probes such as calcofluor – apparently the wall layer adjacent to the periplasmic space is loosened and drawn into the plasma membrane invaginations	Bitsikas et al. (2011) indicate the size of invaginations of the plasma membrane of *Aspergillus nidulans* under hyperosmotic conditions as 0.5-2.3 µm.	On this issue, our group experienced a sharp change in vision of the problem (Mazheika et al., 2022). Most of the membrane structures that we previously mistook for endocytic structures turned out to be macroinvaginations of the plasma membrane. Accordingly, among basidiomycetes there are fungi with relatively simple macroinvaginations (small vesicular or tubes/lamellae up to 5 µm long), such as in *R. solani*, and with very complex ones (lomasomes with secondary invaginations), or large ones (thick tubes extending tens of microns deep into the cytoplasm). Such macroinvaginations are in xylotrophic basidiomycetes.	In the hyperphase during plasmolysis, the plasma membrane forms multiple invaginations.

Remodeling of the actin cytoskeleton	Rapid disassembly of the actin cytoskeleton occurs. It is possible that such disassembly in *S. cerevisiae* is regulated by the CWI mechanoreceptor Wsc1, an integrin-like protein that links actin cables with the plasma membrane, and the cell wall through adapter proteins.	There is direct evidence in the work of Bitsikas et al. (2011) – in *A. nidulans*, during hyperosmotic shock, disassembly of F-actin occurs. The same authors point out that the effect of hyperphase is similar to the treatment of mycelium with latrunculin B. Then, perhaps, in many filamentous ascomycetes, the hyperphase disassembles actin cables, because there are papers in which the photos show that treatment of mycelium with actin assembly inhibitors leads to an increase in the number and size of plasma membrane invaginations.	No data. However, actin assembly inhibitors affect the formation, number and size of some types of macroinvaginations: in some cases in *Stereum hirsutum*, hyperosmosis and latrunculin A act very similarly.	Rearrangements of the actin cytoskeleton are not so significant. Hechtian strands contain actin strands connected to focal adhesion site proteins.
References	Gray, 1945; Kopecká et al., 1973; Morris et al., 1986; Slaninova et al., 2000; Ene et al., 2015; Palmer et al., 2015; Elhasi, Blomberg, 2019; Lenardon et al., 2020; Gow, Lenardon, 2023	Yamashita and May, 1998; Heath, Steinberg, 1999; Lew et al., 2004; Upadhyay and Shaw, 2008; Lew, Nasserifar, 2009; Lewis et al., 2009; Bitsikas et al., 2011; Walker, White, 2017; Lew, 2019	Mazheika et al., 2020b; Mazheika et al., 2022	Morris et al., 1986; Oparka, 1994; Cheng et al., 2017; Lew, 2019

2. Material and Methods

2.1. *Strains and culture conditions*

Wild-type strains of three species of basidiomycetes were used: *Stereum hirsutum* (Willd.) Pers. (Mazheika et al., 2020a); *Rhizoctonia solani* J.G. Kühn (Kamzolkina et al., 2017; Mazheika et al., 2020b); *Coprinus comatus* (O.F. Müll.) Pers. (Mazheika et al., 2020a). Mycelium was grown and stored on malt agar with 1.5% agar (Panreac, Spain; Mazheika et al., 2020a). To prepare microscopic samples, the material was grown on the same malt agar in 9 cm Petri dishes, but the nutrient medium was covered with a cellophane disk (Mazheika et al., 2022). Colonies 4.5 days old were most often used (colonies should not reach the edge of the dish: *R. solani* and *S. hirsutum* were inoculated to the edge of the dish, *C. comatus* in the center).

2.2. *Preparation of light microscopic samples*

To prepare the microscopic slides, a piece of cellophane with the marginal mycelium of the colony was cut out with a blade so that the marginal mycelium and a couple of mm of mycelium with secondary growth were included in the sample. The size of such a cellophane piece is approximately 7 x 10 mm. The material was placed on a glass slide and mounted with 30 µl of malt medium without additives (main control, Malt) or with various additives. In all cases, the same nutrient medium was used on which the

mycelium was grown with the addition of agar. The exception is the experiments with protease K – there the malt medium for mounting the slides was brought to pH 8 with KOH (in the usual malt medium the pH is about 5.5).

Various options for mounting slides and experimental conditions are described in Table 2.

Immediately after preparing the slide, it was placed as quickly as possible under a microscope Imager M1 (Zeiss, Germany) with a Hamamatsu 1394 ORCA-ERA (Japan) camera. Samples were photographed in the light transmission mode with x20 lens and constant shutter speed 100 ms with adjusting light intensity. Photographs were taken for 25 minutes, divided into two observation time intervals of 0-10 minutes and 10-25 minutes. Approximately 40 to 80 fields of view were photographed at each interval. The fields of view were not chosen randomly: photographs were taken mainly so as not to fall on the very edge of the colony with a large number of apical hyphae, but they were not photographed in the basal zones with a dense plexus of hyphae. They were choosen also so that in the field of view there were septa or septa with clamp connections.

For each mycelium treatment option, at least three biological replicates were set (a repeat was considered to be a sample taken from a separate Petri dish with mycelium, grown in most cases in different batches).

2.3. *Processing of results, computer programs used*

ImageJ 1.54d (Schneider et al., 2012) was used to measure hyphal diameters using the Straight tool and the Measure function. The hyphae located in a certain focal section were measured: the focus during shooting was adjusted so that the thickness of the cell wall was minimal, but with maximum contrast, while the cell diameter was maximum. Cells were measured on the outer clear contrast boundaries of the cell wall. When selecting measurement sites, we were guided by the following rules: apical hyphae were not measured (the tip of the hyphae should not be visible in the field of view); severely uneven, swollen, etc. hyphae were not measured. Only hyphae were measured that have: septa in *R. solani*; complex clamp connections with at least two connections (usually the hyphae with one connection in this species are very thin) in *S. hirsutum*; in *C. comatus* – regular clamp connections (in this species, all clamp connections have one connection; see Fig. 1A-C). Measurements were taken at a distance from 20 µm to several hundred µm from the septum or clamp connection. If the hypha has different thickness along its length, it was measured at the widest point.

Data were then transferred to Excel (Microsoft, USA), Origin Pro 7.0 (OriginLab, USA) and Statistica 6 (StatSoft, USA). Scatter plots were generated in Origin Pro. All statistical calculations were performed in Statistica 6. Paired samples were compared with each other using the Whitney-Mann test (U-test); three or more samples were compared using the nonparametric Kruskal-Wallis ANOVA (H-test). In dynamic experiments, the dependence of hyphal diameter on time was assessed using Spearman correlations (r_s). Differences in the average diameters of hyphae (D) between the control and the sample

were assessed as a percentage using the following formula $_{\Delta}D = 100 - (D_1 \times 100)/D_2$, where D_1 and D_2 are the average diameters of the hyphae in the control and sample, with a smaller number being put in numerator, the larger one goes into the denominator, but if the average diameter of the sample is greater than the control (hyphal expansion), the "-" sign was placed in front of the $_{\Delta}D$ value. In experiments with establishing a control scatter of hyphal diameters, $_{\Delta}D$ was found between the extreme values of D among three samples (Table 2).

Images and videos were processed and generated in Photoshop CS (Adobe Systems, USA) and ImageJ 1.54d.

Table 2. Description of various processing/mounting methods used to study the influence of different conditions on the instant change in diameter of fungal hyphae[a]

Treatment method	Method description
Scatter of control	Calculation of internal scatter D[b] in control. Three samples were cut out from one Petri dish with a 4.5-day-old fungal colony. Samples were cut equidistant from each other and not near the edge of the dish (if the inoculum was placed at the edge). The slides were mounted with malt medium (Malt). The minimum and maximum D out of three were found, and calculated $_{\Delta}D$ between them. At least 3 independent experiments were carried out, the sum of the mean $_{\Delta}D$ and SD was calculated, and rounded to the nearest higher half-unit. It was assumed that all $_{\Delta}D$ less than this value mean no change in the thickness of the hyphae
0.6 M sorbitol	Samples (Sorb) were mounted with malt medium with added sorbitol (Sigma-Aldrich, USA) at a final concentration of 0.6 M. Control (Malt) – slides mounted with malt medium
Preincubation	In this series of experiments, the usual control (Malt) and a sample with 0.6 M sorbitol (Sorb) were first cut out from the Petri dish and analyzed. Then the cellophane disc with the fungal colony was transferred to a fresh Petri dish without malt agar and 15 ml of malt medium was poured. After 45 min of incubation in the dark at room temperature, another sample (Malt.Pred) was cut out and the slide was mounted with the same malt medium. After 75 minutes, the fourth sample (Sorb.Pred) was cut out and mounted with malt medium with sorbitol
1% Triton X-100	To test the effect of the detergent, Triton X-100 (Sigma-Aldrich) was added to the malt medium (X100) to a final concentration of 1% v/v (from 10% water stock), and the corresponding amount of water was added to the control malt medium (Malt). These experiments also analyzed samples mounted with malt medium with sorbitol (Sorb) and samples containing both Triton X-100 and sorbitol (X100-Sorb)
1 mg/ml protease K **2.3 mg/ml BSA**	To mount the slides, Proteinase K (Medgen, Russia, 30ea) stock (see below) was added to the malt medium adjusted to pH 8 to a final concentration of 1 mg/ml (ProtK). The buffer without proteinase K was added to the control (Malt). A second control (BSA) was also used: instead of proteinase K, an equimolar amount of BSA (bovine serum albumin; AppliChem, Germany) was added (final concentration 2.3 mg/ml). 20 mg/ml stock of Proteinase K was stored at -20^0 in the following buffer: 0.1 M Tris-HCl pH 8 with 1.5 mM $CaCl_2$ (Chimmed, Russia) and 50% v/v glycerol (Chimmed).
Water	Here the sample was mounted with distilled water (Water), the control was malt medium (Malt)
Mechanical stress	The sample (Stress) was placed on a slide, mounted with malt medium, covered with a coverslip, and then lightly tapped on the coverslip with a plastic tube. Immediately analyzed using a microscope. Control – with malt medium (Malt)
LatA, CytD	The F-actin assembly inhibitors latrunculin A (LatA; Santa Cruz Biotechnology, USA) and cytochalasin D (CytD; Enzo, Switzerland) were added to malt medium from 2.5 mM and 5 mM stocks in DMSO (dimethyl sulfoxide; Sigma-Aldrich, USA), respectively, to final concentrations of 50 to 200 µM. Controls (Malt) were supplemented with the appropriate amount of DMSO
Dynamic changes	In the dynamic experiments, the sample was mounted with malt medium with 0.6 M sorbitol, the slide was quickly installed in the microscope, a suitable field of view was found, and photographs began to be taken at intervals of 60 seconds. Since changes and loss of focus could occur in the slide over time, before each photograph the focus was carefully adjusted, focusing on predetermined marker hyphae. When processing the data, from 3 to 6 non-apical hyphae were selected in each sample, three points distant from each other were taken on each hypha, and their diameters were measured. For each measurement point, the Spearman correlation coefficient was found between hyphal diameter and observation time. It was assumed that the hypha at a given point directionally expands or shrinks at $r > 0.4$ and $r < -0.4$, $p < 0.05$, respectively

[a] The Table is made in the same style as Tables 3-5, containing the results of using the methods described here

[b] D – average diameter of hyphae in the sample, μm; $_{\Delta}D$ (Malt/Sorb) – how much the average diameter of hyphae in the sample (here – Sorb) differs from the control (Malt), in %. If $_{\Delta}D < 0$, this means that the hyphae in the sample are expanding relative to the control. If $_{\Delta}D > 0$ – shrink (see Material and methods)

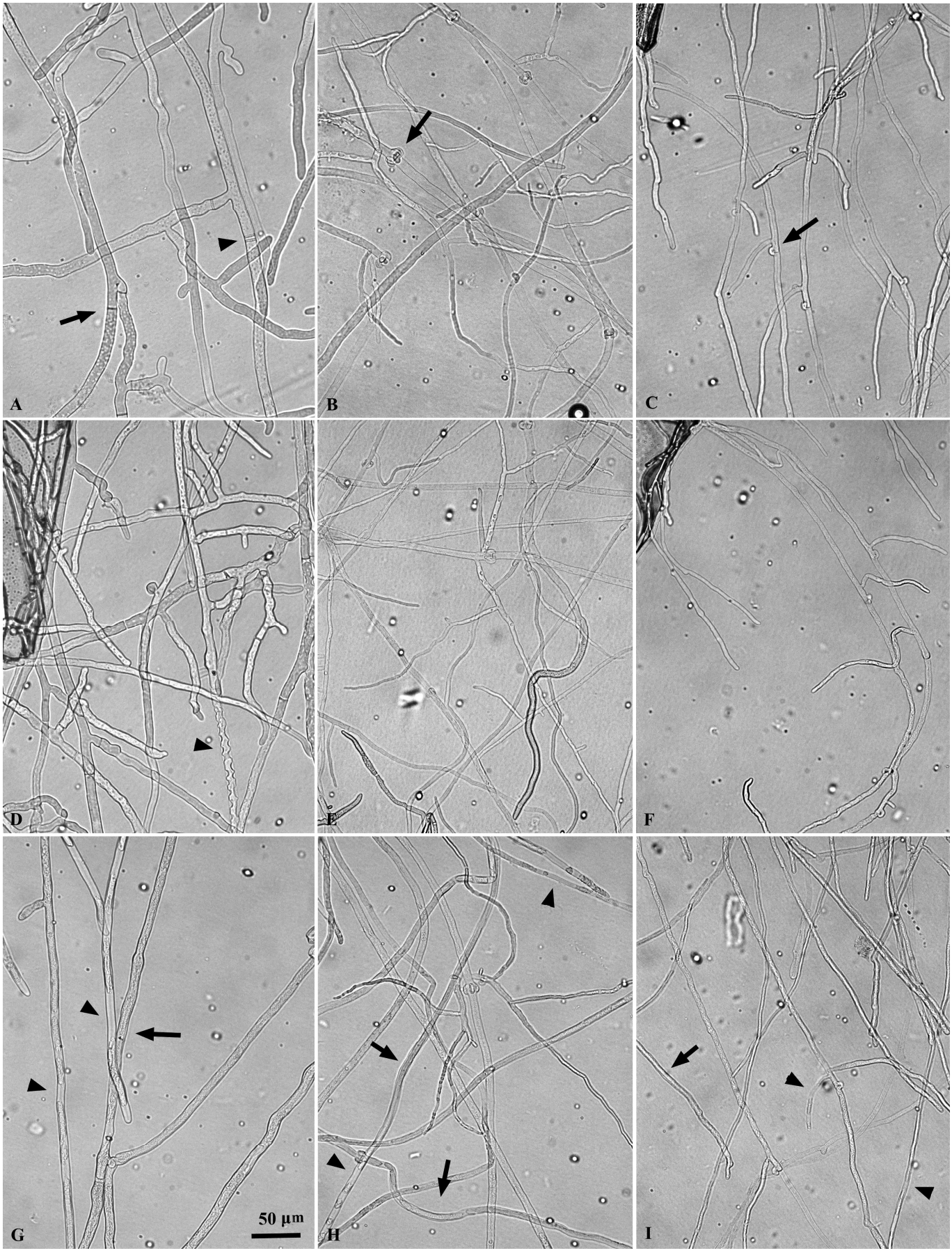


Fig. 1. Bright-field microscopic photographs of the mycelium of the three studied fungi under different processing conditions. A-C – control mycelium of *R. solani* (A), *S. hirsutum* (B) and *C. comatus* (C). The slides are mounted with malt medium. The arrows indicate the septum without a clamp connection in *R. solani*, the septum with a complex clamp connection in *S. hirsutum*, and the septum with a regular clamp connection in *C. comatus*. The arrowhead shows a double

septum in *R. solani*. D-F – microscopic slides are mounted by the medium with 0.6 M sorbitol. Even without measurements, it is clear that the hyphae, on average, become narrower relative to the control. At the same time, *S. hirsutum* (E) and *C. comatus* (F) have practically no other morphological changes and plasmolysis. In *R. solani* (D), concave plasmolysis can be observed in some cells (arrowhead) in the first 10 minutes after preparation of the slide. G-I – mycelium treatment with 1% Triton X-100. In all three fungi, shrinkage of the hyphae and convex plasmolysis (arrows) can be observed with splitting of the protoplast into parts (arrowhead) in some cells.

3. Results

3.1. *Results underlying all other results obtained in the study*

The main results obtained in this study are displayed in Tables 3-5. During the work, about 55,000 cell diameters in the three studied fungal species were measured. The average diameter of hyphae decreases in the order: *R. solani*, *S. hirsutum* and *C. comatus* (Tables 3-5). The growth rate of the mycelium also decreases in this order (data not shown). It is important to pay attention to the following underlying results: A) change in hyphal diameters when mounting slides with hyperosmotic medium, medium with detergent or Protease K, medium with actin assembly inhibitors, etc. happens very fast (within the first few minutes or faster). With our methodological approaches, such rapid expansion or shrinkage of hyphae is difficult to record directly due to the presence of a time interval between preparing the specimen and placing it under the microscope (about 1.5-2 min). Therefore, conclusions about rapid shrinkage/expansion are made indirectly based on: the comparison of D (at $|_{\Delta}D| > 4.5\%$) in the intervals of 0-10 min and 10-25 min – on average among the three fungi studied, only in 24.3% of slides D (0-10min) ≠ D (10-25min), which indicates a rapid change in D in first minutes and then its preservation within 25 minutes in the most samples; dynamic experiments in which there is no general tendency to a gradual change in hyphal diameters over the course of an hour after the shrinkage in sorbitol (Tables 3-5). B) Once shrinked (expanded), the hyphae generally remain in this state, without returning to their original state (at least for an hour). Although in a quarter of all studied samples there is a tendency towards a some gradual change in D: D (0-10min) ≠ D (10-25min), p (U-test) < 0.05; but not necessarily towards control D. C) Hyphae shrink or expand as a whole, together with the cell wall. Plasmolysis (detachment of the protoplast from the cell wall) rarely occurs but there are some exceptions: *R. solani*, in which reversible concave plasmolysis occurs in the first minutes in the hyperphase (Tables 1, 4; Fig. 1D); similar effect to *R. solani* in *S. hirsutum* after one hour preincubation in the liquid medium (Table 3); and treatment with a detergent, which induces irreversible convex plasmolysis in all fungi (Tables 3-5; Fig. 1G-I). However, in all cases, and even under the action of the powerful detergent, plasmolysis does not occur in all hyphal cells. D) D was assessed in the work – the average diameter of certain hyphae in the sample. Accordingly, the changing D is an indicator of the trend of changes in the thickness of hyphae, while individual hyphae and individual sections of hyphae can behave differently – not change or go against the general trend. This is clearly seen in the dynamic experiments, when neighboring sections of the same hypha can have

different trends: one gradually expands within an hour after being placed in the hyperphase; the other narrows (Table 3). In addition, for example, Fig. 2A shows that the mycelium of *S. hirsutum*, placed in the hyperphase after preincubation in malt medium, on average shrinks relative to its control, but the sampling spread increases – hyphae appear that are even wider than the control hyphae. E) The D of the hyphae before placing the mycelium in a microscopic slide is not known: in reality, we evaluate and compare the D of the mycelium for 1.5-2 minutes or less located in a thin layer of liquid between the slide and the cover glass. Mycelium growing in a Petri dish on cellophane on malt agar, in conditions limited by the presence of free moisture, may differ in D from the microscopic sample.

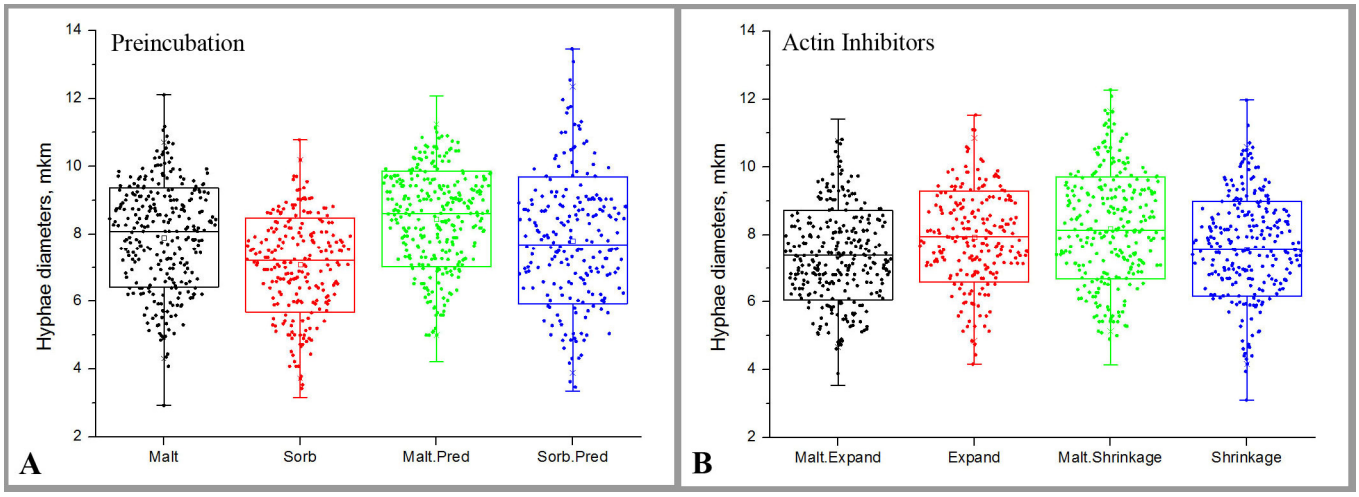
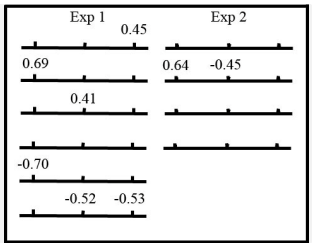


Fig. 2. Combined diagrams of the influence of preincubation in malt medium (A) and inhibitors of F-actin assembly (B) on the diameter of *S. hirsutum* hyphae. A – samples from two experiments were combined. For the experimental conditions, see Table 2. It can be seen that preincubation of the mycelium in the liquid medium leads to a slight expansion of the hyphae (Malt.Pred) relative to the usual control (Malt). Mounting samples, both without preincubation (Sorb) and with preincubation (Sorb.Pred), in sorbitol leads to shrinkage of the mycelium. However, the preincubated mycelium shrinks less on average, and its sampling spread is greater than that of the controls and Sorb. B – out of 9 experiments with different concentrations of LatA and 100 µM CytD (see Table 2), this diagram combines 4 experiments (there is no correlation between the dose of the inhibitor and the response to it): two with mycelia expansion under the influence of inhibitors, two with shrinkage. It can be seen that the mycelium is expanding (Expand), in which the control (Malt.Expand) is on average narrower in diameter than in the control (Malt.Shrinkage) to the shrinking mycelium (Shrinkage). The values of box and whiskers in the diagram columns are shown in the scheme in Fig. 3.

Table 3. The influence of different conditions for preparing microscopic slides on the change in the average diameters of *S. hirsutum* hyphae

Treatment method[a]	Number of independent experiments (exp) with the same result	Comparison of the 0-10 and 10-25 min intervals	Morphological changes	Verbal description of the result
Scatter of control	4 exp with $_{\Delta}D$ (Malt/Malt)[b] from 3.4 to 4.3%, $p < 0.01$[c] Mean $_{\Delta}D$ + SD = 4.1% (all $_{\Delta}D$ < 4.5% are	In 4 out of 12 slides D (0-10min) < D (10-25min), $p < 0.05$	No	The change in the average diameters of hyphae of samples taken from one Petri dish does not exceed 4.3%

	accepted as no changes)[d]			
0.6 M sorbitol	9 exp with $_{\Delta}$D (Malt/Sorb) from 8.2 to 16.5%, $p < 0.01$ Mean $_{\Delta}$D = 11.6%, SD = 2.6	In 3 (2 Malt, 1 Sorb) out of 18 slides D (0-10min) > D (10-25min), $p < 0.05$	Plasmolysis was observed in only one experiment out of 9	When the slides are mounted with sorbitol, the hyphae sharply shrink, by an average of 11.6% relative to the control
Preincubation	In 2 exp: $_{\Delta}$D (Malt/Malt-Pred) = -5.5 and -7.5%, $p < 0.01$; $_{\Delta}$D (Malt/Sorb-Pred) < 4.5%; $_{\Delta}$D (Malt-Pred/Sorb-Pred) = 7 and 7.7%	In 1 (Malt-Pred) out of 12 slides D (0-10min) < D (10-25min), $p < 0.01$	Sorb-Pred has concave-type plasmolysis in the first 5-10 minutes	If the mycelium is preincubated for about an hour in malt medium, the hyphae expand slightly relative to the control (by an average of 6.5%). In the preincubated sample mounted with sorbitol, hyphae shrink (on average by 7.4%), but not as strong as without preincubation. In fact, the mycelium returns to a state of control
	In 1 exp: all $_{\Delta}$D < 4.5%		Sorb-Pred does not have plasmolysis	
1% Triton X-100	3 exp with $_{\Delta}$D (Malt/X100) from 7.2 to 15.5%, $p < 0.01$ Mean $_{\Delta}$D = 12.5%, SD = 4.6 All $_{\Delta}$D (X100/X100-Sorb) < 4.5% Mean $_{\Delta}$D (Malt/Sorb) in these exp = 11.7%, SD = 1.7 (no statistical difference with $_{\Delta}$D (Malt/X100))	All D (6 slides) are not statistically different	Strong and irreversible convex plasmolysis occurs with the splitting of the protoplast into parts	When the slides are mounted with medium containing 1% Triton X-100, a sharp shrinkage of the hyphae occurs by an average of 12.5% relative to the control. However, if the morphological differences between the shrinkage in sorbitol and Triton are significant, then there is no quantitative difference (including, there is no difference between D (X100) and D (X100-Sorb))
1 mg/ml protease K **2.3 mg/ml BSA**	3 exp with $_{\Delta}$D (Malt/ProtK) = 4.4%, 5.2% and 15.9%, $p < 0.01$ Mean $_{\Delta}$D = 8.5%, SD = 6.4 All $_{\Delta}$D (Malt/BSA) < 4.5%	In 2 (ProtK and BSA) out of 9 slides D (0-10min) < D (10-25min), $p < 0.05$	Plasmolysis is not observed	In the presence of active protease K, the mycelium shrinks, but on average less than in the sorbitol or detergent. Equimolar amounts of BSA had no effect on hyphal diameter.
Water	In 1 exp: $_{\Delta}$D (Malt/Water) = 4.9%, $p < 0.01$ In 2 exp: all $_{\Delta}$D < 4.5%	In 2 (2 Malt) out of 7 slides D (0-10min) ≠ D (10-25min)[e], $p < 0.05$	Plasmolysis is not observed, but over time, the number of highly vacuolated hyphae increases	Water as a mounting medium for slides has little effect on the diameter of the hyphae
Mechanical stress	In 2 exp: $_{\Delta}$D (Malt/Stress) = 4.8% and 6.9%, $p < 0.05$ In 1 exp: $_{\Delta}$D < 4.5%	All D (6 slides) are not statistically different	Severe hyphal damage and high vacuolization can be observed	Mechanical damage to the mycelium leads to slight shrinkage of the hyphae
LatA, CytD	In 5 exp: all $_{\Delta}$D < 4.5% In 2 exp: $_{\Delta}$D (Malt/LatA) = 7.2%, $p < 0.01$ In 2 exp: $_{\Delta}$D (Malt/LatA or CytD) = -6.0% and -6.6%, $p < 0.01$	In 4 (3 Malt, 1 LatA) out of 13 slides D (0-10min) ≠ D (10-25min), $p < 0.05$	Plasmolysis is not observed	Here, 50, 100, and 200 µM concentrations of LatA and 100 µM CytD were used. Addition of the inhibitors can lead to different results regardless of the dose (Spearman correlation = 0.07, $p > 0.05$): no effect on the diameter of the hyphae, slightly shrinking the hyphae, or slightly expanding them.
Dynamic changes	In 2 exp[f]:	In slides mounted with 0.6 M sorbitol there is no directional trend in the change in the diameter[g] of hyphae for one hour. The hyphae either do not change their diameter (or change it back and forth), or individual sections of the hyphae directionally expand or shrink.		

		
General information	20,146 hyphal diameters measured in *S. hirsutum*. The average diameter of hyphae in the control (Malt) is 7.9 µm[h], SD = 0.5. The smallest diameter in the control under our conditions is 2.9 µm; the largest is 12.7 µm	

[a] The conditions for preparing microscopic slides are described in Table 2

[b] See Table 2 and Materials and Methods for the symbol description

[c] p of U-test when comparing diameters of sample and control

[d] We accept $_{\Delta}D$ as unchanged for $_{\Delta}D < 4.5\%$, even if p(U-test) < 0.05

[e] D (0-10min) ≠ D (10-25min) means that in some slides there is a tendency for hyphae to expand over time, in others – to shrink

[f] See Table 2 and Materials and Methods for more details. The figure schematically shows 6 hyphae analyzed in the first experiment, and 4 hyphae in the second. Short transverse lines – three points of diameter measurement on each hypha for an hour with an interval of 1 minute. The number above these lines is the Spearman correlation coefficient for the dependence of hyphal diameter on time. If there is no number, then it is < 0.4

[g] It is important to understand that the hyphae here have already shrunk in the first minutes after being placed in sorbitol; the experiment examines the further dynamics of hyphae diameters over the course of an hour

[h] In this case, the average diameter, as well as the minimum and maximum, are not common indicators for the mycelium of the entire colony, since we measured the diameters based on certain principles (see Materials and Methods)

3.2. *The influence of various factors on changes in the diameter of hyphae in three studied species of fungi*

Quantitative and descriptive results of this study are shown in Tables 3-5. In this section, the reaction (change D) of three species of basidiomycetes belonging to different systematic and ecological-trophic groups to different methods of preparing microscopic samples is compared. Comparative results are considered in order and in accordance with Tables 3-5.

For further work, it was necessary to establish the value of the control scatter D (see Table 2) in order to accept the minimum value of $_{\Delta}D$, below which changes in the size of the hyphae can be neglected. It turned out that, despite different growth rates and different average thickness of hyphae in the three species of fungi, their control spread of D is very close (Fig. 3A). For all three fungi, control mean $_{\Delta}D$ + SD = 4.0-4.1%, and maximum control $_{\Delta}D$ = 3.9-4.3% (Tables 3-5). Accordingly, we accepted $_{\Delta}D = 4.5\%$ for all three fungi as a value below which, even at p (U-test) < 0.01, the change in hyphal diameter should be neglected.

Mounting the slides with the hypertonic medium based on 0.6 M sorbitol in all three fungi causes sharp shrinkage of the hyphae (Fig. 3B). In only one experiment out of seven, *R. solani* has $_{\Delta}D < 4.5\%$. The degree of shrinkage in the three fungi is close: on average, *S. hirsutum* shrinks the least (mean $_{\Delta}D$ = 11.6%; Table 3), and *R. solani* shrinks the most (mean $_{\Delta}D$ = 14.3%; Table 4). In *S. hirsutum* and *C. comatus*, hyphae in sorbitol shrink without plasmolysis (only in one experiment out of nine in *S. hirsutum*

was concave plasmolysis observed). In *R. solani*, the situation is somewhat different: in the first minutes after mounting a slide, concave plasmolysis occurs in some cells (the incidence of plasmolysis varies from experiment to experiment), which then disappears (more details in Table 4; Fig. 1D; Video S1).

For *S. hirsutum*, an additional variant of experiment was carried out with preincubation of the mycelium in malt medium before mounting the slides. It turned out that such preincubation affects the behavior of mycelium in microscopic samples. Preincubation results in some hyphal expansion relative to normal controls, and mounting of preincubated mycelium in sorbitol reduces mean hyphal shrinkage and increases the variation in such shrinkage (see Fig. 2A and Table 3). In addition, in slides mounted with sorbitol after preincubation in malt medium, concave plasmolysis occurs, which is extremely rare without preincubation (Table 3).

Triton X-100 added to mounting medium produces an effect quantitatively very similar to that of sorbitol. In all three fungi, the hyphae shrink sharply by about 11-13% (there is no statistical difference with shrinkage in sorbitol; Fig. 3C). However, unlike sorbitol, Triton X-100 induces convex plasmolysis in some cells with protoplast splitting in all three fungi (see Fig. 1H-I and the characteristics of plasmolysis in each species in Tables 3-5).

The effect of Proteinase K on D of the studied fungi is not as strong and uniform as the effects of sorbitol and Triton X-100 (Fig. 3D). Proteinase K has the strongest effect on *S. hirsutum*: it leads to hyphal shrinkage almost like in sorbitol (Table 3). However, the enzyme has little effect on the change in the diameter of the hyphae of *R. solani* and *C. comatus* (Tables 4 and 5). In experiments with Proteinase K, BSA was used as an additional control to exclude the non-enzymatic effect of Proteinase K on fungal D. In *S. hirsutum* and *R. solani*, BSA does not affect changes in hyphal diameter. However, unexpectedly, in *C. comatus*, in two of three experiments, BSA caused a change in hyphal diameter (Table 5).

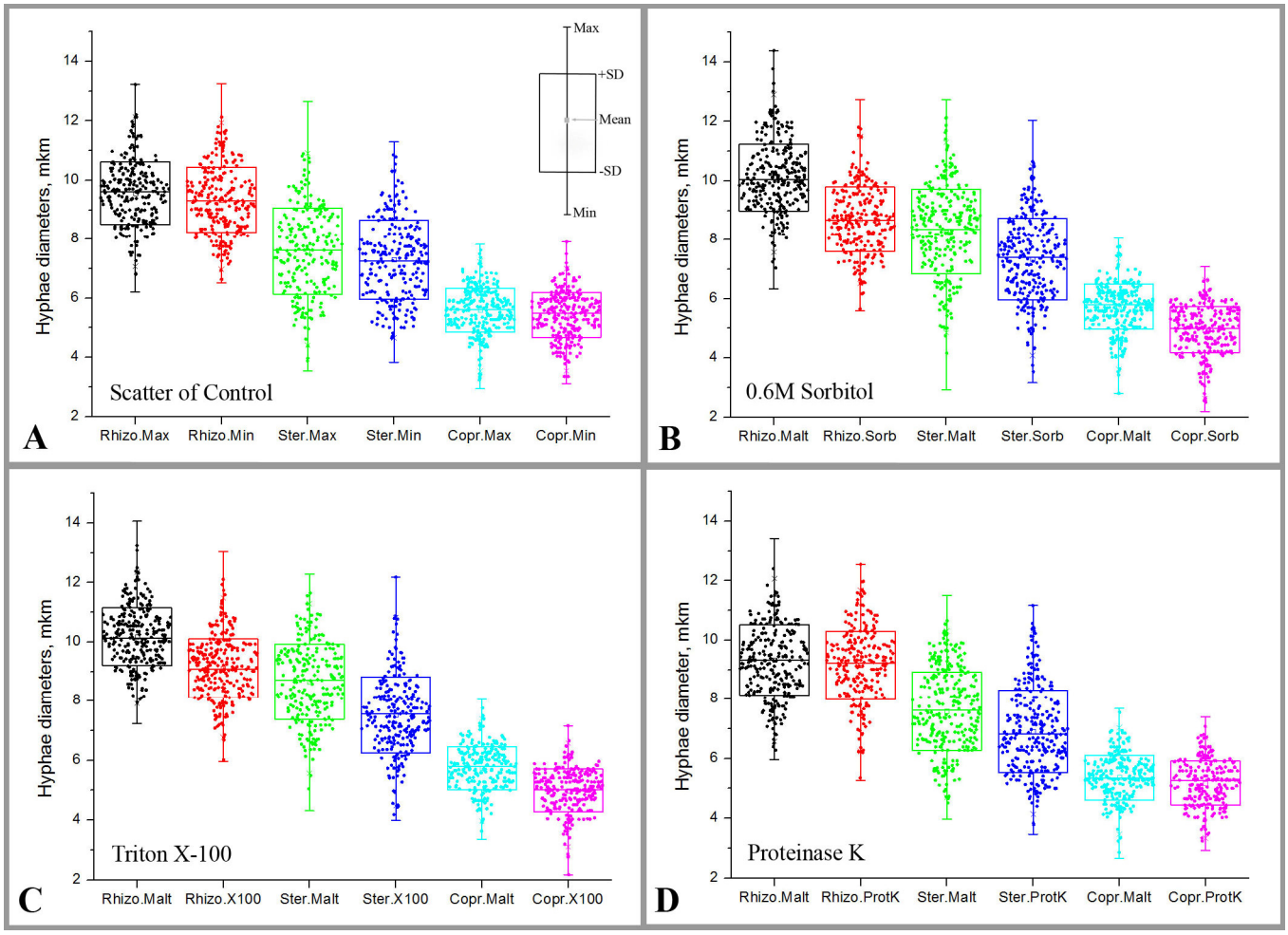


Fig. 3. Combined diagrams of the influence of three factors on the instantaneous change in the diameters of hyphae in the three studied fungi, as well as, for comparison, a diagram of the internal scatter of diameters in the control (A). Each column of the chart combines samples from 3-9 experiments together. Each sample is the sum of samples from two time intervals 0-10 min and 10-25 min. Detailed information is contained in Tables 2-5. Each factor is characterized by two columns: control (Malt) and sample (Sorb, X100 and ProtK). The diagrams show that mounting microscopic slides with 0.6 M sorbitol (Sorb; B) or Triton X-100 (X100; C) leads to pronounced shrinkage of hyphae relative to the control (Malt). *R. solani* and *S. hirsutum* each have one outlier experiment excluded in panel B. The effect of proteinase K (D) is not so pronounced: only *S. hirsutum* has a general tendency to shrink hyphae (for the other two fungi, see Tables 4 and 5). Species designations: *R. solani* – Rhizo; *S. hirsutum* – Ster; *C. comatus* – Copr.

Distilled water, as a mounting medium, and which can be considered as a hypotonic medium, has little effect on the change in hyphal diameters. It has almost no effect on *C. comatus* and *S. hirsutum*; in *R. solani*, in one experiment out of three, it caused the expansion of hyphae by 7% (Tables 3-5). Moreover, in the vast majority of experiments with water there is no difference between D (0-10 min) and D (10-25 min), which indicates that water does not cause not only an immediate change in D, but also a gradual one.

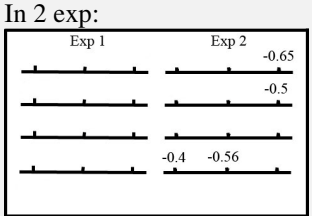
Another additional variant of the experiment was carried out on *S. hirsutum* – mechanical stress. Tapping the cover glass of the slide caused a slight shrinkage of the hyphae in *S. hirsutum* relative to the control (see Table 3). However, damage and crowding of hyphae can lead to unreliability in measuring hyphal diameters.

Inhibitors of F-actin assembly, LatA and CytD, have the most ambiguous effect of all the factors on the fungi studied. They do not have any effect on the diameter of the hyphae of *R. solani* and *C. comatus*. However, on *S. hirsutum* they act as follows: A) the effect of inhibitors on D is dose-independent: r_s = 0.07 for the dependence of $_{\Delta}D$ on the concentration of the inhibitor. B) In five of nine experiments, the inhibitors did not affect the diameters of hyphae; in two experiments, they caused expansion of hyphae by 6-7%, in two – shrinkage by 6-7%.

All the experiments described above deal with averages values among the numerous of hyphae. Changes in the diameters of individual hyphae within an hour after preparing the microscopic specimen with sorbitol were analyzed in dynamic experiments (Table 2). It turned out: A) none of the 25 individual hyphae of three species of fungi has a general tendency to expand or shrink after shrinkage in sorbitol – a gradual change in diameter can occur only in one or two points out of three selected on each hypha, but never in all three. B) Only in 17-27%, depending on the species of fungus, of the points selected on the hyphae, a gradual change in diameter occurs. At the remaining points, either no changes occur within an hour, or they occur back and forth, and therefore cannot be described by linear Spearman correlation. C) There is a difference in dynamic experiments between the three fungal species: *S. hirsutum* has the most hyphal sections with a tendency to gradually change in diameter (about 27%), and half of them tend to expand, half to shrink. In *R. solani*, only about 17% of the hyphal sections tend to change – all become narrower. In *C. comatus*, 19% of points are variable – on the contrary, all tend to expand (see Tables 3-5).

Table 4. The influence of different conditions for preparing microscopic slides on the change in the average diameters of *R. solani* hyphae[a]

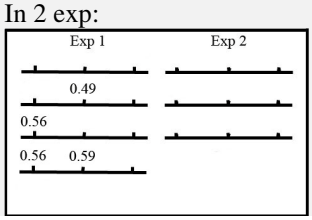
Treatment method	Number of independent experiments (exp) with the same result	Comparison of the 0-10 and 10-25 min intervals	Morphological changes	Verbal description of the result
Scatter of control	3 exp with $_{\Delta}D$ (Malt/Malt) from 0.5 to 4.3%, $p < 0.01$ Mean $_{\Delta}D$ + SD = 4.0% (all $_{\Delta}D < 4.5\%$ are accepted as no changes)	In 1 out of 9 slides D (0-10min) < D (10-25min), $p < 0.01$	No	The change in the average diameters of hyphae of samples taken from one Petri dish does not exceed 4.3%
0.6 M sorbitol	6 exp with $_{\Delta}D$ (Malt/Sorb) from 10.5 to 17.5%, $p < 0.01$ Mean $_{\Delta}D$ = 14.3%, SD = 2.4 In 1 exp: $_{\Delta}D < 4.5\%$	In 4 (1 Malt, 3 Sorb) out of 14 slides D (0-10min) ≠ D (10-25min), $p < 0.05$ In all Sorb D (0-10min) > D (10-25min); in Malt D (0-10min) < D (10-25min)	In the first 8 minutes there is strong concave plasmolysis, less often in apical cells. Then it disappears, leaving only convex plasmolysis in some cells. 20 minutes later it disappears too (all this is clearly visible in Video S1)	When the slides are mounted with sorbitol, the hyphae sharply shrink, by an average of 14.3% relative to the control
1% Triton X-100	3 exp with $_{\Delta}D$ (Malt/X100) from 10.3 to 11.3%, $p < 0.01$	In 1 (X100-Sorb) out of 6 slides D (0-	Strong and irreversible	When the slides are mounted with medium containing 1% Triton X-100, a

	Mean $_{\Delta}D$ = 10.8%, SD = 0.5 All $_{\Delta}D$ (X100/X100-Sorb) < 4.5% Mean $_{\Delta}D$ (Malt/Sorb) in these exp = 12.2% (excluding one experiment in which $_{\Delta}D$ < 4.5%)	10min) > D (10-25min), p < 0.01	convex plasmolysis occurs with the splitting of the protoplast into parts. More expressed in apical cells	sharp shrinkage of the hyphae occurs by an average of 10.8% relative to the control. From the reaction to sorbitol, the reaction of the mycelium to the detergent differs slightly quantitatively and in different types of response plasmolysis
1 mg/ml protease K 2.3 mg/ml BSA	1 exp with $_{\Delta}D$ (Malt/ProtK) = 5.0%, p < 0.01 All $_{\Delta}D$ (Malt/BSA) < 4.5% In 2 exp: $_{\Delta}D$ < 4.5%	In 5 (ProtK, 2 BSA and 2 Malt) out of 10 slides D (0-10min) < D (10-25min), p < 0.01	There are rare cells with concave plasmolysis	Proteinase K has little effect on hyphal diameters. Equimolar amounts of BSA had no effect on hyphal diameter.
Water	In 1 exp: $_{\Delta}D$ (Malt/Water) = -7.0%, p < 0.01 In 2 exp: all $_{\Delta}D$ < 4.5%	In 1 (Malt) out of 6 slides D (0-10min) < D (10-25min), p < 0.05	Plasmolysis is not observed, but at the beginning of incubation in water, the hyphae are less even in thickness along the long axis	Water as a mounting medium for slides has little effect on the diameter of the hyphae, but if it influences, the hyphae expands
LatA	In 4 exp: all $_{\Delta}D$ < 4.5% (here 3 exp with 50 µM and 1 exp with 100 µM LatA)	In 4 (Malt, 3 LatA) out of 5 slides D (0-10min) < D (10-25min), p < 0.05	Plasmolysis is not observed	LatA does not affect hyphal diameters
Dynamic changes	In 2 exp: 	In slides mounted with 0.6 M sorbitol the hyphae either do not change their diameter (or change it back and forth) in one hour, or individual sections of the hyphae directionally shrink		
General information	17,832 hyphal diameters measured in *R. solani*. The average diameter of hyphae in the control (Malt) is 9.7 µm, SD = 0.4. The smallest diameter in the control under our conditions is 6.0 µm; the largest is 14.4 µm			

[a] The Table is designed in the same style and with the same symbols as the Table 3 for *S. hirsutum*, so see explanations in Table 3 and the slide processing conditions in Table 1

Table 5. The influence of different conditions for preparing microscopic slides on the change in the average diameters of *C. comatus* hyphae[a]

Treatment method	Number of independent experiments (exp) with the same result	Comparison of the 0-10 and 10-25 min intervals	Morphological changes	Verbal description of the result
Scatter of control	3 exp with $_{\Delta}D$ (Malt/Malt) from 1.6 to 3.9%, p < 0.01 Mean $_{\Delta}D$ + SD = 4.1% (all $_{\Delta}D$ < 4.5% are accepted as no changes)	In 2 out of 9 slides D (0-10min) < D (10-25min), p < 0.05	No	The change in the average diameters of hyphae of samples taken from one Petri dish does not exceed 3.9%

0.6 M sorbitol	5 exp with $_{\Delta}$D (Malt/Sorb) from 5.6 to 15.4%, $p < 0.01$ Mean $_{\Delta}$D = 11.9%, SD = 4.0 (in 1 exp there is obvious outlier – 5.6%, without taking it into account mean $_{\Delta}$D = 13.5%)	All D (10 slides) are not statistically different	Plasmolysis is not observed	When the slides are mounted with sorbitol, the hyphae sharply shrink, by an average of 11.9% (13.5%) relative to the control
1% Triton X-100	3 exp with $_{\Delta}$D (Malt/X100) from 12.3 to 14.8%, $p < 0.01$ Mean $_{\Delta}$D = 13.1%, SD = 1.4 All $_{\Delta}$D (X100/X100-Sorb) < 4.5%	In 4 (2 X100 and 2 X100-Sorb) out of 7 slides D (0-10min) ≠ D (10-25min), $p < 0.01$	There is convex-type plasmolysis, in some cells the protoplast completely separates away from the cell walls. But in general, plasmolysis and protoplast split are not as pronounced as in the other fungi	When the slides are mounted with medium containing 1% Triton X-100, a sharp shrinkage of the hyphae occurs by an average of 13.1% relative to the control. However, if the morphological differences between the shrinkage in sorbitol and Triton are significant, then there is no quantitative difference (including, there is no difference between D (X100) and D (X100-Sorb))
1 mg/ml protease K 2.3 mg/ml BSA	1 exp with $_{\Delta}$D (Malt/ProtK) = 8.8%, $p < 0.01$ In 2 exp: $_{\Delta}$D (Malt/ProtK) < 4.5% 1 exp with $_{\Delta}$D (Malt/BSA) < 4.5% 1 exp with $_{\Delta}$D (Malt/BSA) = 7.2%, $p < 0.01$ 1 exp with $_{\Delta}$D (Malt/BSA) = -6.5%, $p < 0.01$	In 3 (ProtK, BSA and Malt) out of 9 slides D (0-10min) ≠ D (10-25min), $p < 0.05$	Plasmolysis is not observed	Proteinase K has not regular effect on hyphal diameters – only in one experiment out of three are the hyphae significantly shrinked. More unusually, here BSA has an effect on hyphal thickness, leading to slight expansion of the hyphae in one case and shrinkage in another
Water	In 3 exp: all $_{\Delta}$D (Malt/Water) < 4.5%	In 1 (Water) out of 6 slides D (0-10min) < D (10-25min), $p < 0.01$	Plasmolysis is not observed	Water as a mounting medium for slides has not any effect on the diameter of the hyphae
LatA	In 3 exp: all $_{\Delta}$D (Malt/LatA) < 4.5% (here all exp with 50 µM LatA)	All D (3 slides) are not statistically different	Plasmolysis is not observed	LatA does not affect hyphal diameters
Dynamic changes	In 2 exp:  	In slides mounted with 0.6 M sorbitol the hyphae either do not change their diameter (or change it back and forth) in one hour, or individual sections of the hyphae directionally expand		
General information	17,072 hyphal diameters measured in *C. comatus*. The average diameter of hyphae in the control (Malt) is 5.6 µm, SD = 0.2. The smallest diameter in the control under our conditions is 2.7 µm; the largest is 8.1 µm			

[a] The Table is designed in the same style and with the same symbols as the Table 3 for *S. hirsutum*, so see explanations in Table 3 and the slide processing conditions in Table 2

4. Discussion

4.1. *Discrepancies between changes in hyphal diameter and the dynamics of macroinvagination formation*

In our previous work, we studied the influence of practically the same factors that are considered in this study on the system of macroinvaginations of *S. hirsutum* (Mazheika et al., 2022). Even earlier, the effect of F-actin assembly inhibitors on the macroinvagination system of *R. solani* was analyzed (Mazheika et al., 2020b). Now it is possible to combine current results with previously obtained ones and draw new conclusions.

In some cases, there is a discrepancy between changes in the thickness of *S. hirsutum* hyphae and changes in the macroinvagination system. Thus, in *S. hirsutum*, hyperosmotic conditions cause a sharp shrinkage of the hyphae by an average of 12%; an hour's preincubation in liquid medium slightly expands the hyphae and reduces the effect of hyperphase on mycelium; inhibitors of actin assembly may either slightly expand or shrink or have no effect on hyphae. At the same time, all of these factors increase the number of lomasomes (glomeruli and pendants; see Mazheika et al., 2022) in *S. hirsutum* cells on average by 2-3 times. Similarly, the listed factors increase the frequency of occurrence of some other macroinvaginations, hollow and larger in size than lomasomes – vesicles and tubes – but not as definitely as in the case of lomasomes (see Mazheika et al., 2022). According to the logic outlined in the Introduction, hyphal shrinkage should lead to an increase in the number and/or size of macroinvaginations, expansion – to decrease. However, this principle only works in the case of 0.6 M sorbitol and in some cases when using actin assembly inhibitors.

In order to understand the detected discrepancy, it is necessary to indicate the position of lomasomes among other fungal macroinvaginations. In the complex system of macroinvaginations of *S. hirsutum*, lomasomes are the most numerous and serve as the main tool for creating and consuming the membrane pool. Lomasomes consist of very thin tubes or lamellae, both primary invaginations of the plasma membrane and secondary invaginations of tubes swollen into vesicles; the putative mechanisms of their formation are described in Mazheika et al. (2022). They are apparently always present in the hyphae in the required quantity (at least in the mycelium immersed in liquid), in contrast to other macroinvaginations that appear in cells under certain conditions. Probably, the permanent presence of lomasomes provides the fungus with an initial level of protection from sudden changes in cell size – a kind of airbag; the remaining macroinvaginations enhance protection in addition to lomasomes or, like thick tubes, appear under specific stress conditions.

It can be assumed that the osmolarity of the cytoplasm of hyphae growing on cellophane on a solid medium is on average higher than that of mycelium growing in a liquid medium of the same composition (although it may depend on the humidity and freshness of Petri dishes with agar medium). With a relatively long preincubation (one hour, but not 12) in an excess of liquid nutrient medium, the fungus

begins to pump water into the cells and the hyphae expand slightly on average. At the same time, an hour interval is enough to begin *de novo* membrane synthesis or use endomembranes – the area of the plasma membrane grows, outpacing the general expansion of hyphae. A non-equilibrium process arises: the formation of lomasomes accelerates, along with an increase in the number of lomasomes, the number of plaques (the result of the elimination of lomasomes and other macroinvaginations; see Mazheika et al., 2022) increases – all this indicates that the fungus is trying to find a balanced state for its plasma membrane in changed conditions.

The possible nature of the discrepancy in the case of using LatA and CytD is explained in p. 4.3.

4.2. *Two approaches to preserving the functionality of mycelium in changing conditions*

1% Triton X-100 served as a kind of positive control in our study. At this concentration of nonionic detergent, permeabilization of the plasma membrane occurs – turgor is reset to zero; the bonds of membrane lipids with proteins of focal adhesion sites and GPI proteins are disrupted – the plasma membrane separates from the cell wall; finally, the actin cytoskeleton may also lose its functions. In other words, the main factors influencing the rigidity of the cell wall are removed, and the elasticity modulus tends to a minimum (Lew, 2019). In turn, this can mean that the shrinkage of hyphae in Triton X-100 is approaching the maximum possible (although it cannot be ruled out that there may be conditions with greater cell shrinkage). Proteinase K also leads to permeabilization of the plasma membrane and destruction of focal adhesion sites, but unlike Triton X-100, it acts not on lipids, but on proteins. However, its effect on cells is much weaker than 1% Triton X-100. Moreover, due to differences in proteins, access to them or something else, the proteinase affects the thickness of hyphae differently in different species of fungi. 0.6 M sorbitol leads to hyphal shrinkage very quantitatively close to the effect of Triton X-100, which may indicate not only a release of turgor pressure (without permeabilization of the plasma membrane), but also a reorganization of the actin cytoskeleton, as occurs in hyperphase in yeast and *A. nidulans* (Table 1).

There is an important difference between the shrinkage of hyphae under hyperosmotic conditions in the basidiomycetes studied here and the shrinkage of *N. crassa* studied by Lew and Nasserifar (2009). If *N. crassa*, after an osmotic shock for about an hour, almost restores the original turgor pressure and hyphal diameter, then the basidiomycetes do not return to the original hyphal thickness within an hour and do not even have a tendency to do so. Here they are closer in behavior to oomycetes (Lew et al., 2004; Walker et al., 2006). It can be assumed that basidiomycetes do not tend to expend energy and other resources to create a certain turgor pressure in a changing environment. Rather, they try to maintain the functionality of their cells (see below) with minimal turgor, and leave the increase in turgor pressure for later, when external conditions have stabilized. Turgor pressure, rather, is not important in its absolute value (with the exception of apical and some specialized cells), what is more important is its gradient

along the length of the hyphae, which ensures mass flow transport, and this does not require high absolute pressure values (Lew, 2019; Herman, Bleichrodt, 2022).

There is variation in the response to osmotic shock among the fungi studied. While all three fungi are similar in terms of the degree of hyphae shrinkage and the irreversibility of such change, they are not similar in terms of the severity of plasmolysis. Under the influence of sorbitol, *S. hirsutum* and *C. comatus* usually do not undergo plasmolysis, while in *R. solani*, pronounced, rapidly reversible concave plasmolysis may occur in the first 10 minutes. There may be a relationship between the type of macroinvagination system and the response to osmotic shock. Therefore, *S. hirsutum*, which has a more complex system, copes with the osmotic effect more easily with its help: it manages to dynamically regulate the size of the protoplast in accordance with the entire cell shrinking. Thus, *S. hirsutum*, under changing external osmotic conditions, maintains its cells in the highest possible functional state. *R. solani*, which is a parasite/endobiont of living plant tissues, usually experiences sharp changes in substrate osmolarity less frequently in nature and has larger cells, and the simpler system of macroinvaginations. This system does not always cope with sudden changes in hyphal size, so *R. solani* uses a different approach to maintain the functionality of its cells. The fungus sacrifices the first minutes in a hyperosmotic environment, losing functionality: the protoplast is first partially detached from the cell wall and shrinks regardless of the latter. Then a slight regulation of turgor and the work of the macroinvagination system adjust the protoplast to the cell wall, and the cells restore or partially restore their functionality.

It should be noted that the two described approaches to restoring cell functions are not strictly related to the type of macroinvagination system in the basidiomycete. Thus, under certain conditions (in our case, preincubation in a liquid medium before sorbitol) *S. hirsutum* begins to behave similar to *R. solani*: it also triggers rapid reversible concave plasmolysis.

4.3. *The role of actin in changing the size of hyphae and the updated curtain model*

One of our previous works (Mazheika et al., 2020b) proposed the curtain model, according to which in non-apical hyphal cells the tension of the plasma membrane is regulated not only by turgor pressure, but also by actin cables. This model was constructed based on the results of the action of actin assembly inhibitors on the macroinvaginations (at that time we described them as endocytic structures) of *R. solani*, in which F-actin disruption increases the number and size of parietal invaginations, and stimulates the formation of tubular invaginations of the plasma membrane. In fact, the effect of the inhibitors on *R. solani* is similar to their effect on xylotrophs, but in *R. solani* the cell diameter does not change, and in xylotrophs the formation of large tubes (which *R. solani* does not have) does not depend on actin assembly inhibitors (Mazheika et al., 2022).

The results described above, and other results of the work, indicate that, on the one hand, the role of actin in the cells of different species of basidiomycetes may differ to some extent, on the other hand, the

tension of the plasma membrane is not the one important function of actin cables in the fungal cell described by the curtain model.

It can be assumed that the system of cortical actin cables in basidiomycetes performs three main functions. However, first of all, it is necessary to indicate that the actin cable system is not a skeletal or frame structure in the usual sense. This system is extremely dynamic: formin-dependent bundles of F-actin filaments rapidly grow, cross-linked (Berepiki et al., 2011), disassembled, and new ones are nucleated (see Video 9 in Wernet et al., 2023).

The first function of actin cables, as mentioned above, is to regulate the tension of the plasma membrane in the absence of pronounced turgor pressure. Actin cables, by moving focal adhesion sites or anchor proteins along the inner layer of the cell wall, like moving a curtain on a curtain rod, stretch or relax the plasma membrane, pressing it against the inside of the cell wall. The second function follows from the results of the present study: in *S. hirsutum*, actin also regulates the diameter of hyphae. It can be assumed that actin cables, if necessary, restrain/slow down the shrinkage or expansion of hyphae (they can, on the contrary, themselves take part in changing the diameter of cells, but this cannot be shown with our methodological approaches). It is possible that in *R. solani* the first function of actin prevails, since in the fungus there is no relation between F-actin assembly and hyphal thickness, while in *S. hirsutum* – the second or both functions are combined. Then the discrepancy between the expansion of hyphae in *S. hirsutum* and the increase in the number of lomasomes when treated with actin assembly inhibitors can be explained. In the case when actin cables keep cells from expanding, they simultaneously create tension on the plasma membrane – when actin is disassembled, the cells some expand, but the tension on the membrane drops, and a nonequilibrium process of formation of macroinvaginations occurs.

Finally, actin cables have a third proposed function. Actin is not directly involved in the formation of macroinvaginations, otherwise they could not form in the presence of actin assembly inhibitors. Actin acts indirectly here: in the absence of inhibition of F-actin assembly, it weakens, for example, the tension of the plasma membrane, general or local, then BAR proteins and dynamin-like proteins promote the invagination of a very thin tube or lamella, which then turn into complex lomasomes or small vesicles. Another way, on the contrary, occurs through actin inactivation, which can occur not only artificially when treated with actin assembly inhibitors, but also in the native state: actin can be disassembled/reorganized during osmotic shock or mechanical stress (or for other causes). The first is shown in the literature (Table 1) and indirectly in this work, the second – both in this study and earlier: we have shown that mechanical damage to the hyphae leads to a slight change in their thickness and to the acceleration of the formation of large hollow macroinvaginations. Actually, this is how we explain the sequential appearance over time in a microscopic specimen labeled with styryl fluorescent of increasingly larger macroinvaginations: mechanical pressure of the cover slip and some drying of the specimen leads to actin reorganization and loss of control by actin, BAR proteins, etc. over the formation of invaginations. In result huge tubes are formed that can swell into large vacuole-like vesicles (but actin

disassembly alone is not enough for this effect, otherwise thick tubes would form faster under the action of actin inhibitors, but this does not happen). It follows that actin not only participates indirectly through the tension of the plasma membrane in the formation of macroinvaginations, but also possibly controls the width of the neck of large macroinvaginations (not lomasomes), since pinching proteins (BAR proteins, dynamin) cannot control invagination tubes with a wide width neck.

Unlike most cells of land plants, which are protected by integumentary, protective and mechanical tissues from external conditions, fungal cells of substrate hyphae are in direct contact with the substrate. To a certain extent, the fungus can resist changes occurring in the substrate through the synthesis of hydrophobins, the formation of external capsules/matrices, melanization of the cell wall, changes in the permeability of its membrane, etc. However, all these protective mechanisms and structures cannot preserve the active living hypha's functionality during sudden and strong changes in external conditions. Therefore, fungi took a different path: not to swim against the current, but to be carried away by the current until they reached a quiet backwater. At the same time, it is important not to lose functionality, to continue metabolic processes, nutrition, secretion, growth, etc. with minimal lags during changes in external conditions. Mechanisms for preserving the functionality of fungal cells, among other things, is the ability of fungi to quickly change the diameter of their hyphae in response to various stress conditions, primarily to changes in the osmolarity of the liquid phase of the substrate. This mechanism involves elasticity and other properties of the cell wall, turgor regulation mechanisms, plasma membrane macroinvagination systems, adhesion of the plasma membrane to the cell wall, and the actin cable system. However, our knowledge about the named physiological processes and structures that are of fundamental importance in the life of fungi remains extremely ephemeral. Therefore, extensive, high-quality microscopic studies analyzing the dynamics of fluorescently labeled actin cables in basidiomycetes (as the group of fungi with the most complex behavior in rapid changes in cell size) is needed first. Then, it is necessary to search for proteins that ensure adhesion of the plasma membrane to the cell wall and to study connections between them and actin. Finally, it is important to analyze the dynamics of the distribution of turgor pressure and tension of the plasma membrane along the fungal hypha (using, for example, fluorescent mechanosensors of tension) so that the curtain model turns from a general theoretical one into a model that describes the real complex organism of a filamentous fungus.

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Caption for supplementary Video

Video S1. Time-lapse video obtained in a experiment of dynamic changes in *R. solani*. The microscopic slide is mounted with malt medium with 0.6 M sorbitol. The same field of view was snapped at 1-min intervals for an hour. It can be seen that concave plasmolysis occurs in some cells in the first 8 minutes. Then it disappears; only a small convex plasmolysis remains near individual septa. After 20 minutes it disappears too. Scale bar 20 µm.