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RESEARCH ARTICLE

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Comparative Study of Mechanical and Biological Pretreatment for Releasing Spores of Black Truffle *Tuber aestivum*

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ABSTRACT

It is well known that the number of true truffles in the wild is decreasing. The aim of the study was to develop an effective, simple and affordable method of asci disruption to release black truffle spores. It was shown that the spore release can be achieved by different ways, such as mechanical or biological destruction. Mechanical homogenization of fruiting bodies using an immersion blender in tandem with a ball mill was shown to be effective and led to destruction of at least 85% of asci and release of spores. Also, the first approach we applied was the biological method of spore activation performed by African and grape snails. As a result of digestion of truffle fruiting bodies, the spores not only lost their protective shells, but also changed their morphology, which promoted their germination *in vitro*. The spores obtained using these two methods are capable of forming mycelial hyphae on nutrient media. The results of our study can be used to prepare inoculum of *Tuber* spp. and to obtain their pure cultures in agriculture.

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Black truffle; *Tuber* sp.; spores; asci; gastropods; digestion

1. Introduction

True truffles (*Tuber* spp.) are marsupial fungi whose fruiting bodies are formed underground and build associations with the roots of certain tree species (oak, pine, hazelnut, etc.) [1]. This symbiosis allows truffles to obtain essential nutrients from plants. In turn, truffles facilitate water and mineral absorption from soil for plant growth [2]. These fungi are poorly studied organisms, and little attention has been paid to their biotechnological importance and agriculture. The relevance of this study is supported by several facts [3]. First, the number of truffles in the wild is significantly decreasing. Second, truffle spores are often absent in truffle seed material sold by dishonest suppliers. Third, extracts of *Tuber* spp. are often characterized by biological activity, such as antimicrobial, cytotoxic, anticancer, and antiproliferative, and are of great promising interest in pharmaceutical and cosmeceutical industries [4–6].

Since truffles are representatives of rare fungi, and often listed as protected species, collecting them in the wild for commercial purposes can be environmentally hazardous. The development of methods for truffle cultivation under controlled laboratory conditions is one of the promising areas of research. However, isolation of pure mycelium or truffles is a non-typical and non-trivial task. The problem is a complex of truffles symbionts that include bacteria, fungi, invertebrates, microalgae and other organisms [7–11]. They actively colonize truffles at all stages of their life cycle, which makes it difficult to obtain a pure culture. The little studied biochemical interactions between truffles and symbiotic microorganisms also play an important role in the process of mycelium- or truffle-formation [12, 13].

It is well known that truffles are a unique and valuable product. Truffles are in great demand in gastronomy due to their flavors and aromas. These fungi are rare and limited in their availability due to their specific requirements for climate and soil conditions [14, 15]. Due to their high cost and rarity, truffles have become a subject of interest for agriculture [16]. A great amount of research is aimed at optimization and improvement of truffle cultivation methods to make truffles more available and commercially viable [17]. For example, patent KZ 23874A4 [18] is known for production of protein product and mycelium from truffles. Homogenized truffles are used as a mother culture, which is placed

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in a fermenter and cultivated until the biomass growth. However, this invention does not take into account the numerous symbiont fungi of truffles. This leads to growth of symbiotic microorganisms, but not *Tuber* sp [19–21].

Currently, there are different methods allowing production of truffle spore suspension, but they are often inefficient and require a significant amount of time, resources and specific components. The method of host plant seedlings mycorrhization with truffle spore suspension is widespread [22, 23]. The problem, however, is that the successfully established symbiotic relationships between plants and truffles can be unstable due to contamination of host plants with pathogens and other symbiont fungi. Each truffle is known to be home to multiple mycelial fungi [24–28]. In addition, not all host plants can be successfully mycorrhized with truffles, which limits the choice of species and varieties for truffle cultivation [29]. In the absence of molecular control, plants can be mycorrhized with either truffle fungi or truffle-associated phytopathogenic fungi, or both. Also, there are many patents that describe adding chemical or bioactive components to the truffle suspension. For example, patent CN 108934768B [30]. This invention describes activation of *Tuber melanosporum* spores using moisture-retaining agents, gibberellin, forchlorfenuron, and so on. The disadvantage of this method is the large number of components added to the truffle spore inoculum. It should be noted that hormones and growth factors are expensive agents. Incorporating such substances into the suspension makes the process much more complicated and expensive.

A lot of studies and inventions neglect the crucial point ensuring germination of truffle spores. One of the most important conditions for truffle reproduction in the wild is the release of spores from asci, which serve as protective shells making spores safe from negative influences. This is also where the truffle spores mature actively. When the mature spores are released from asci, they germinate and mycorrhiza is formed, which then develops into the fruiting body. However, most information sources suggest that grinding the fruiting body of truffle with an immersion blender is sufficient to obtain spore suspension. The obtained substance is then inoculated to the roots of a host plant to ensure mycorrhiza formation. However, none of these methods address the extraction of mature truffle spores from asci.

In nature, truffle spores are often spread by animals because their digestive enzymes are able to break asci open and thus release the spores into the environment [31]. Mature spores without asci germinate faster and form mycorrhiza more efficiently, which is necessary for productive truffle cultivation. Slugs, arthropods or small rodents are known to be sufficient for digestion of truffle asci [32]. However, we have not found any information on digestion of truffle asci by gastropods, or development of a method for mechanical destruction of asci as a pretreatment for activation of truffle spores.

Therefore, the aim of this study was to develop a method for destroying asci to release *Tuber aestivum* spores, creating an inoculum with activated black truffle spores ready for germination.

2. Material and methods

2.1. Sampling

The fruiting bodies of black truffle *T. aestivum* (Figure 1) were collected in coniferous forest near the city of Krasnodar, Russia ($N=12$). The taxonomic affiliation of these specimens was confirmed by phylogenetic analysis detailed in the Supplementary Materials (Supplementary Figures 1 and 2, Supplementary Table 1). These fungi had a globular shape and were covered with a rough, dense, pyramidal peridium of black color. The average size of the fruiting bodies was 5 cm. The average weight of the fungi was about 15 g.

Figure 1. The fruit body of the black summer truffle *Tuber aestivum*.

Inside the black truffle fruiting body there was a gray, brown or cream-colored gleba. A distinctive feature of the truffle gleba was the presence of a marbled pattern formed by numerous dense sinuous veins of white color.

These fungi were delivered from Krasnodar (south of Russia) to Irkutsk (Siberia, Russia) in plastic containers with soil and rice to prevent rotting. The truffles were transported under thermostatic conditions at 6–10 °C. Before the experiment, the samples were stored at the temperature of −20 °C.

2.2. Mechanical method of truffle spore release

We carried out a series of experiments involving different methods of asci mechanical destruction. For this purpose, a section of the *T. aestivum* fruiting body was excised with a sterile scalpel, transferred into a microtube, and homogenized using different methods after adding 1 ml of sterile distilled water.

Finally, the fungi samples were homogenized using BABRx1 laboratory vibration ball mill (LLC Mycotech, Russia) through intense shaking with steel balls at 3000 rpm. The samples underwent 4 homogenizations for 3 min each. During breaks, the samples were cooled, and microscopy of the obtained material was performed.

Then, we added up to 200 ml of distilled water to the whole fruiting body of a truffle and used the immersion blender for homogenization. A small fraction of the truffle fruiting body was sampled and put into test tubes. One sample was immediately sent for microscopic analysis, and metal balls were added to the second sample, after which four-fold grinding was conducted. Further, the spore suspension underwent purification and microscopy.

2.3. Digestion of truffle asci using the gastrointestinal tract of gastropods

For our experiments, we selected adult, healthy and active gastropods of the genera *Achatina* sp. (African snail) or *Helix* sp. (grape snail). The age of the gastropods used in the experiments was no more than three months. To prepare experiments, the gastropods were fed with only boiled potatoes for seven days to completely clean their digestive tract from food and plant polymers of unclear etiology. As part of regular sanitation and hygiene measures, the molluscs were washed with warm water and kept in aquariums or plastic containers. The aquarium for gastropods was preliminarily rinsed with hot water and sterilized. A special sterile coconut fiber mat was placed on the bottom of the aquarium. The gastropods were also provided with constant access to fresh (clean or sterile) water and natural air circulation. Before the experiment, gastropods underwent intestinal cleansing from potato residues. To do this, the gastropods were not given any food for three–four days.

Then, the mature (dense and firm) fruiting body of black truffles of the genus *T. aestivum* was ground with a blender and mixed with boiled potatoes in a ratio of 1:5 (truffle to potato) (Figure 2). Gastropod excrements were collected and used for the next steps of our study.

2.4. Purification of spore suspension by centrifugation

Truffle spores were isolated from excrements of gastropods and then purified. To purify the truffle spore suspension from hyphae debris and its symbionts, it was centrifuged for 5 min at the rate of 12,000 rpm to separate the precipitate from the supernatant. The samples were washed five times in solutions of sterile water or nutrient media to purify them. As a final step, the spore suspension was resuspended in a 3% hydrogen peroxide solution for 1 min and washed three times with sterile water, resulting in a highly purified sterile suspension of truffle spores.

Figure 2. Gastropods of genus *Achatina* sp. eat boiled potatoes with black truffle.

2.5. Detection of truffles spores' germination

The germination of truffle spores was estimated on glass slides that were cleaned from dust and exposed to 70% alcohol solution for 15–20 min. The slides were then washed with distilled water, dried and autoclaved to sterilize. A drop of agarized nutrient media was loaded to glass slides. Homogenate containing truffle spores was applied to solidified nutrient media including Sabouraud's medium, Getchinson's medium, potato-dextrose agar, and Chapek's medium. The slides were placed in sterile Petri dishes, which were then kept under thermostatic conditions at 18–30 °C for two weeks to allow the spores to germinate and form fungal hyphae. Microscopy of truffle spores was performed immediately after homogenization or digestion and on the 7th, 10th, and 14th day of the experiment.

2.6. Statistical analysis

The experiments were conducted in 12 replicates. More than 100 microbiological inoculations of spore suspension on slides were performed. The inoculated spore suspension was analyzed, and changes in spore and asci morphology were observed using the light microscope «Micromed 2» (Model 3–20, Micromed, Russia).

Each experiment was analyzed in three microscopic fields. In order to generate tables and graphs, we focused on the number of spores observed within the microscope field of view at a 10× magnification. The spore counts and sizes were analyzed using ImageJ software (National Institutes of Health, v1.54h). Reliability was assessed using the Mann–Whitney *U*-criterion with Bonferroni correction. For statistical analysis, we used the program Past 4.10 (Natural History Museum, Norway). The total number of spores in the microscope field of view was taken for 100%, from which the percentage of damaged spores was calculated for each of the 12 repetitions. Graphs were plotted using the average percentage ratio and the overall standard deviation.

3. Results

3.1. Mechanical destruction of truffle spores

The light micrographs taken during the study allow us to compare the efficiency of asci destruction using different methods of mechanical grinding. The micrographs show asci with truffle spores during primary homogenization (Figure 3a) and after different homogenization methods (Figure 3b–d).

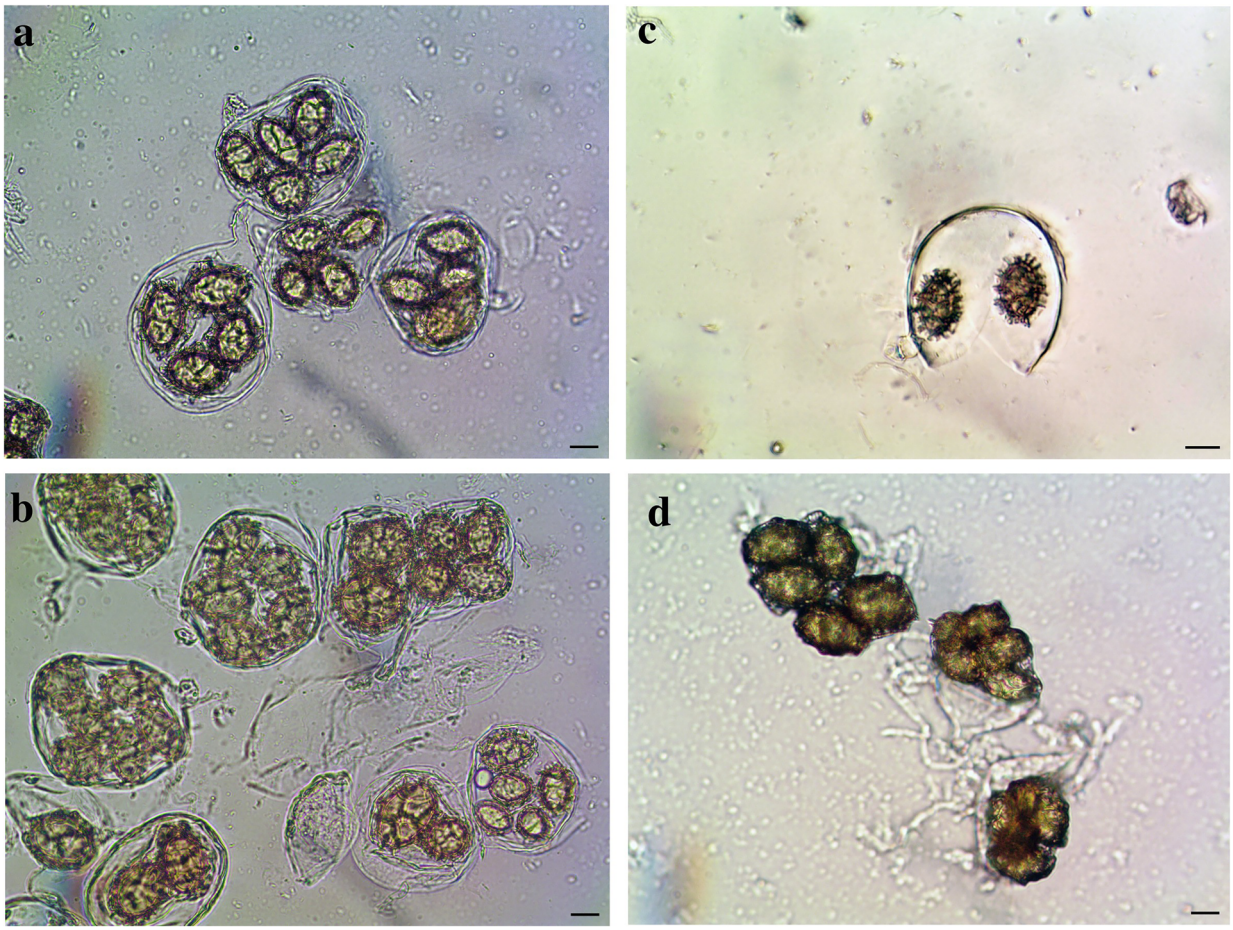

Figure 3. Representative light micrographs before (a) and after homogenization with immersion blender (b), vibrating ball mill (c) and immersion blender in tandem with ball mill (d). Scale bars = 10 µm.

The highest degree of disruption of truffle asci was achieved by a combination of two homogenization methods – homogenization using the immersion method in tandem with a vibrating ball mill. Tables with data on the number of asci disrupted and spores released as a result of using different homogenization methods are presented in the Supplementary Materials (Supplementary Tables 2 and 3). This technique resulted in the disruption of 86.4 ± 6.4% (Figure 4a) of asci and the release of 70.9 ± 7.8% ($p < 0.01$) of spores (Figure 4b). Based on the microscopic analysis, this method was the most effective in disrupting asci and releasing spores compared to other methods.

Experiments on germination of black truffles allowed us to obtain light micrographs of mechanically disintegrated spore samples. Figure 5a shows the spores after 7-day of germination under thermostat conditions. Based on the obtained light micrographs, we could observe that after 7-day exposure at 22–24°C, the spores increased in size and changed their morphology. Figure 5b shows the spores after two weeks of germination under stable temperature 22–24°C. After two weeks, spores did not show further increase in size, but we noted germination of fungal hyphae on agar.

3.2. Biological destruction of truffle spores

During the experiment on feeding gastropods with truffle fruiting bodies, the following micrographs were taken. Light micrographs of truffle asci before (Figure 6a) and after digestion of *Tuber* sp. truffles by gastropods of the genera *Achatina* sp. (Figure 6b) and *Helix* sp. (Figure 6c). Single spores of truffles were detected in mollusc excrements. The number of single spores obtained with this method increases significantly from 7 ± 2% to 90.2 ± 4.6% ($p < 0.01$), as shown in Figure 7.

It was shown that the asci are destroyed and spores are released under the influence of enzymes in the digestive tract of gastropods. Spores digested by gastropods *Achatina* sp. were found to swell by 57.2 ± 23.1% during digestion in the digestive tract of molluscs (Figure 8). Truffle spores changed their morphology after passing through the gastrointestinal tract of gastropods. Spores became rounder and more friable, and their reticular pattern changed. The data obtained in this experiment are presented in the Supplementary Materials (Supplementary Table 4 and 5).

Experiments with black truffle germination allowed obtaining light micrographs of biologically

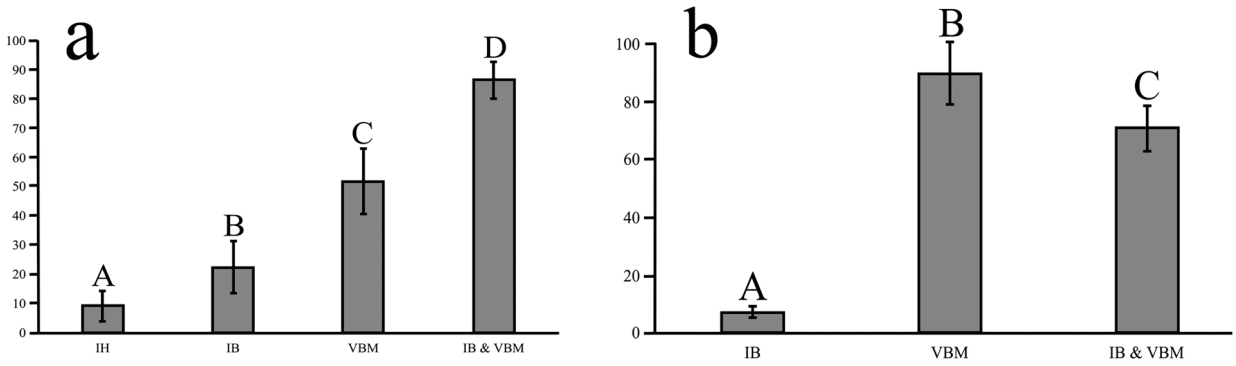

Figure 4. The percentage of broken asci (a) and free spores (b) in a truffle sample using various homogenization methods.

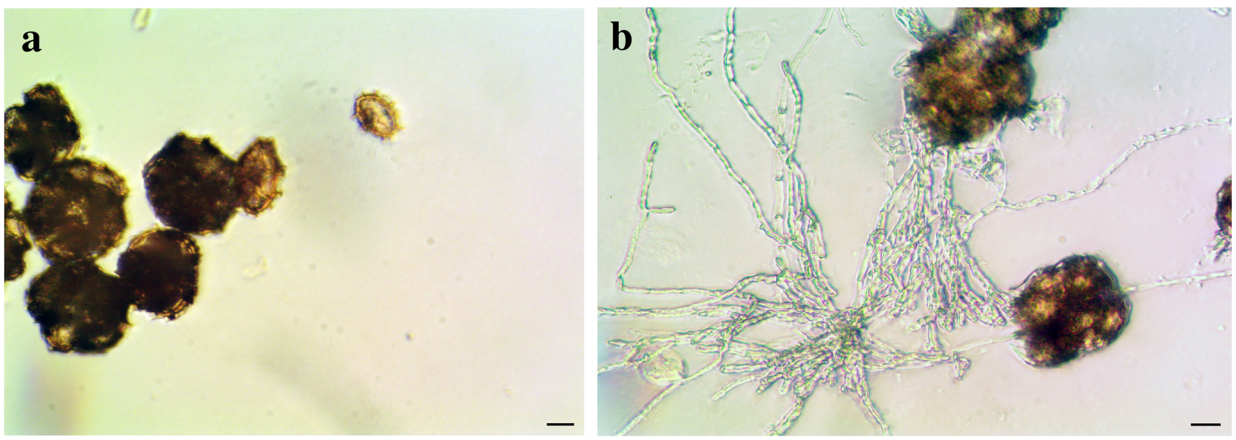

Figure 5. Representative light micrographs of spore suspension obtained after homogenization on the 7th (a) and 14th (b) day. Scale bars = 10 µm.

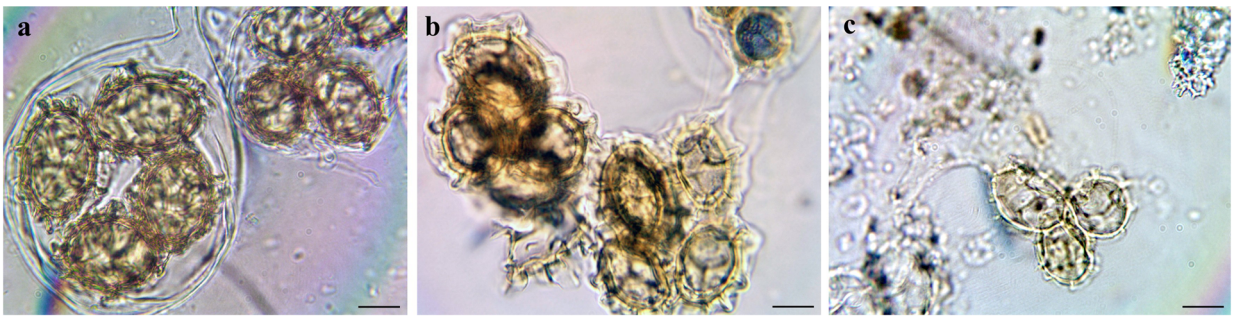

Figure 6. Representative light micrographs of truffle asci before (a) and after digestion by gastropods of the genera *Achatina* sp. (b) and *Helix* sp. (c). Scale bars = 10 µm.

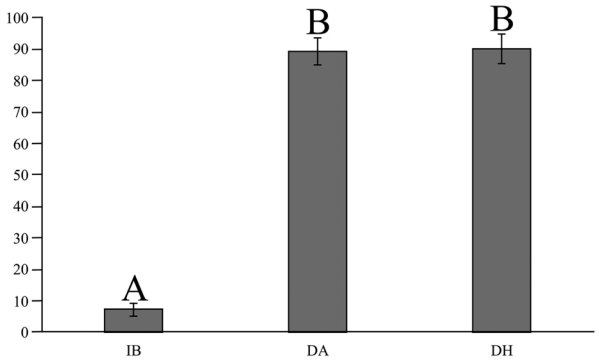

Figure 7. The percentage of free spores in a truffle sample after its passage through the digestive tract of gastropods of the genera *Achatina* sp. and *Helix* sp.

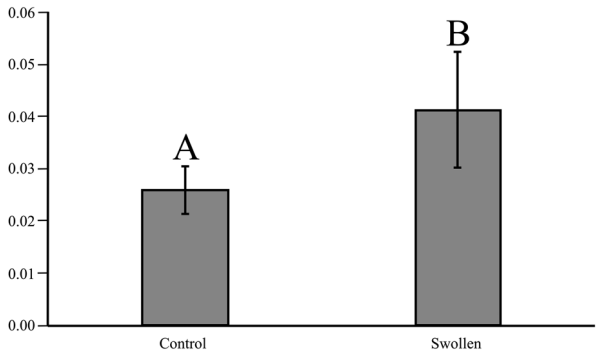

Figure 8. The difference in size (mm) between spores homogenized by a ball mill and spores passed through the digestive tract of the gastropod *Achatina* sp.

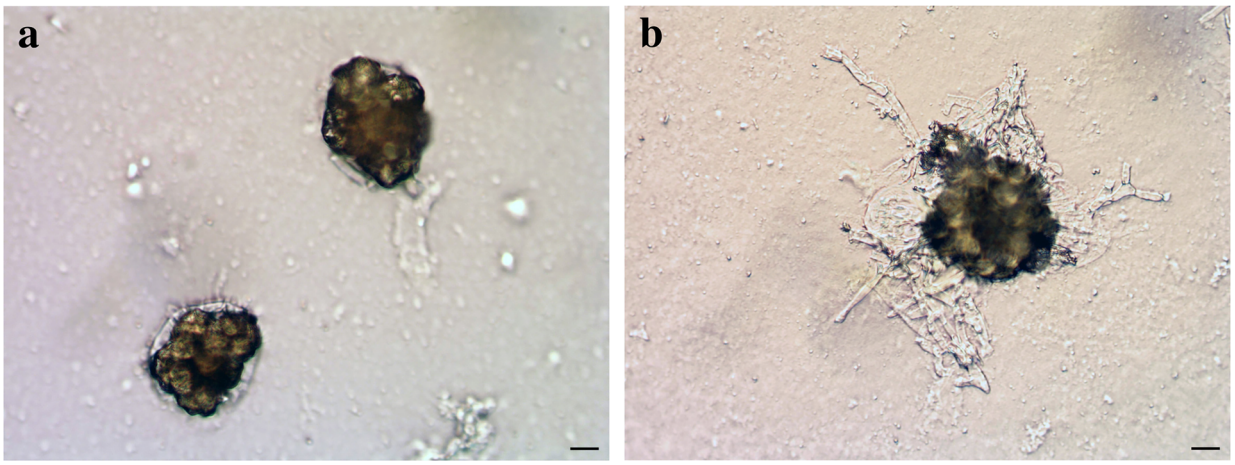

Figure 9. Representative light micrographs of spore suspension obtained after digestion by gastropods on day 7 (a) and 14 (b). Scale bars = 10 µm.

disintegrated spore samples (Figure 9). Images were obtained for nutrient medium Getchinson, potato-dextrose agar and Chapek's medium, indicating that the medium composition is not a determinant for spore germination. It was shown that the spores did not form mycelium after one week. The spores remained voluminous and friable for up to 7 days, as depicted in Figure 9a. After two weeks, the spores developed into a network of fungal hyphae, as illustrated in Figure 9b. The spores germinated on glass slides incubated at 18–22 °C and increased 4-fold in size by day 14 of the experiment. However, they did not germinate (no visual traces of spore germination were found). The spores germinating on glass slides incubated at 26–30 °C did not change their morphology during the entire time of the experiments.

3.3. Physiological peculiarities of gastropods fed with truffles

The first experiments on feeding gastropods with black truffle fruiting bodies were partially unsuccessful. First

of all, when preparing the molluscs for truffle diet, we cleared fibers and other components that interfered with microscopy from their gastrointestinal tracts. This was achieved by means of prolonged starvation. Varying the timing of snail gut cleansing, we determined the best duration for that. A fasting period of less than 3-day before the experiment was not enough for cleansing, and excrement microscopy in this case revealed the excess of fibers. A cleaning period of 5-day was not suitable because during visual examination, the gastropods were very sluggish and hibernated. Thus, 4-day of cleaning a snail gastrointestinal tract was the optimal time.

When the molluscs were fed with only truffles without any additives, it resulted in their starvation and their physiological condition deteriorated. Feeding gastropods with truffles mixed with mashed potatoes in a ratio of 1:10 to 1:100 made it difficult to detect truffle spores due to their low concentration. Thus, the optimal ratio between truffles and mashed potatoes was 1:5.

4. Discussion

Truffles are a little studied group of organisms. These fungi are becoming increasingly rare in their natural habitat, indicating that their numbers are declining in the wild. Methods for truffle cultivation and artificial reproduction are few and suitable only for several species of the fungi. The main objective of this study was to develop a method of activating truffle spores by destroying the asci in order to optimize the methods for obtaining pure truffle cultures and their cultivation. Here, we tested mechanical and biological pretreatment of asci destruction related to spore germination *in vitro*.

The developed method has a number of advantages due to cheaper processes for obtaining spore suspensions of truffles. The mechanical method of spore activation makes it possible to better control the quantity of truffle spores and their distribution in the inoculum to be placed to the desired areas or plant seedlings. Microscopy of samples at all stages of the experiment revealed that mechanical breakdown of asci with the immersion blender and ball mill led to destruction of at least 85% of the asci and the release of spores. This is useful for establishing truffle plantations and controlling the truffle cultivation process. In addition, the mechanical method of spore activation does not require the use of rare or difficult-to-obtain chemical reagents, such as expensive plant hormones.

The results of our study have shown that gastropods of the genera *Achatina* sp. and *Helix* sp. are capable of consuming truffles. In times when food with a high nutritional value like boiled potatoes are scarce, these gastropods can eat these fungi and digest protective shells of their spores. Here we demonstrated that truffle spores change both their volume and morphology under the action of digestive enzymes in gastropods. Spores become more friable, the spore reticular pattern changes, etc. This suggests that these enzymes prepare the spores for germination in soil and promote their germination. The developed method has several advantages in terms of obtaining spore suspensions of truffles. Gastropods are affordable and widely distributed organisms. Keeping molluscs does not require much space and special care, which reduces the cost of obtaining activated spore suspension and further cultivation of truffles. This method of spore activation is environmentally safe and sustainable, as it does not require the application of rare or hard-to-find chemical reagents, rare plant extracts, etc., which reduces its environmental impact.

In the wild, truffle spores are spread by mycophagous animals. Mycophagous animals are the animals that can recognize edible mushrooms and distinguish them from poisonous ones. The digestive tract of such animals must have a special enzyme for efficient digestion of mushroom biomass. Mycophagous animals mainly include vertebrates such as wild boars [33], rodents such as flying squirrels [34], porcupines [35], and others. In addition to this, some invertebrates are also known to be mycophagous: some insects [36], slugs [37], and so on. There are several reasons why animals consume truffles as food. For instance, the nutrient composition of truffles supplements the plant-based diet of these animals and can compensate for deficiencies in, for example, essential amino acids, vitamin D, and micronutrients [38, 39]. Truffles are also known to live in habitats such as dense forests where other food sources of high nutritional quality are either scarce or highly seasonal [32]. Thanks to the digestive enzymes of these animals, the spores of the truffle fungi lose their protective shells and are released into the environment. In addition, mature spores without asci have been shown to germinate faster and form mycorrhiza more efficiently, which is essential for productive truffle cultivation [31, 40].

Comparing the results obtained with different methods of activation of *T. aestivum* spores, it can be concluded that the digestion of fruiting bodies by molluscs is optimal. The results remained predictable regardless of the genus of gastropods used. The number of released spores did not vary when using gastropods of the genus *Achatina* sp. The same was true for the genus *Helix* sp. Passing through the digestive tract of gastropods led to an increase in spore volume and changes in their morphology. In contrast, mechanical disruption left the spores unchanged from their original state within the asci. It is known from the literature, that the digestive juice of gastropods, in particular the genus *Helix* sp., is capable of lysing fungal cell walls. This is due to the large number of different

enzymes that are present in the digestive juice that can act on substrates such as chitin, mannans, and glucans [41, 42]. The release of spores from truffle asci as they pass through the digestive tract of molluscs may be due to the presence of a radula consisting of a series of closely spaced, numerous teeth [37,43]. Microscopic analysis of spores that have passed through the gastropod digestive tract showed that these spores begin to form mycelium by the 7th day of germination. Interestingly, after mechanical homogenization on the 7th day of germination, the spores remain swollen without forming hyphae. It should be noted that both methods are effective, but the biological method showed that the digestive juice of gastropods may have a role in accelerating the spore germination process.

Resulting from the experiments, two of the most environmentally friendly methods for truffle spore production have been developed. We demonstrated that homogenization of fruiting bodies using an immersion blender in tandem with a vibratory ball mill was effective for destroying asci and releasing spores. Also, this study for the first time demonstrated the possibility of using gastropods for digestion of truffle fruiting bodies. Over the digestion time, spores lost their protective shells and changed their morphology, which favored their germination. Positive effects of mechanical and biological pretreatments were confirmed by germination of truffle spores *in vitro* on agarized nutrient media and by microscopic observations. The use of this method can be helpful to ensure the more efficient and rapid germination of truffle fungi spores on different substrates under laboratory-controlled conditions. The developed methods can be applied in mushroom farming and be helpful in the establishment of black truffle production.

Disclosure statement

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