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Research Article

Optimal methods for the preparation of fungal mycelium for examination with the scanning electron microscope

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Abstract

Introduction: Plant-pathogenic fungi are considered a serious threat to world food security, causing spoilage in plant products and food poisoning. One of the ways to identify fungi is to examine their mycelium using a scanning electron microscope (SEM). In order to take high-resolution microscopic images and obtain the surface properties of mushroom mycelium samples, it is very important to prepare the samples, i.e. the method of dehydrating and drying them. **Materials and Methods:** In this research, the effect of two dehydration methods and two drying methods on the mycelia of two fungi, *Rhizoctonia solani* and *Rhizopus stolonifer*, were compared to study them with SEM. **Results:** Microscopic observations showed that for *R. stolonifer*, dehydration with 100% ethanol and drying and for *R. solani* at room temperature and dehydration with 50% ethanol and the freezing method produce a smooth and high-resolution image compared to other methods. **Conclusion:** Dehydration of fungal mycelium with 50% to 100% ethanol and drying at room temperature or freezing are the optimal methods to prepare them for examination with SEM and produce a smooth and high-resolution image, and the surface features of their mycelium are preserved and well-defined.

Key words: Ethanol, *Rhizoctonia*, *Rhizopus*

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مقاله پژوهشی

روشهای بهینه آماده‌سازی میسیلیوم قارچها برای مطالعه با میکروسکوپ الکترونی روبشی

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چکیده

مقدمه: قارچهای بیمارگرگیاهی با ایجاد فساد در محصولهای گیاهی و مسمومیت‌های غذایی تهدیدی جدی برای امنیت غذایی جهان محسوب می‌شوند. یکی از روشهای شناسایی قارچها مطالعه میسیلیوم آنها با میکروسکوپ الکترونی روبشی (SEM) است. برای گرفتن تصاویر میکروسکوپی با وضوح بالا و حفظ ویژگی‌های سطحی نمونه‌های میسیلیوم قارچها، آماده‌سازی نمونه‌ها یعنی روش آبگیری و خشک کردن آن‌ها بسیار مهم است. **مواد و روش‌ها:** تاثیر دو روش آبگیری و دو روش خشک کردن میسیلیوم دو قارچ *Rhizoctonia solani* و *Rhizopus stolonifer* برای مطالعه آنها با میکروسکوپ الکترونی روبشی در این پژوهش، مقایسه شدند. **یافته‌ها:** مشاهده‌های میکروسکوپی نشان دادند که در *R. stolonifer* آبگیری با اتانول ۱۰۰ درصد و خشک کردن و در *R. solani* در دمای اتاق و آبگیری با اتانول ۵۰ در صد و روش انجماد نسبت به سایر روش‌ها تصویر صاف و با وضوح بالا تولید می‌کنند. **نتیجه‌گیری:** آبگیری میسلیوم قارچها با اتانول ۵۰ تا ۱۰۰ درصد و خشک کردن در دمای اتاق یا منجمد کردن آن، روشهای بهینه برای آمادهسازی آنها برای مطالعه با SEM هستند و تصویر صاف و با وضوح بالا تولید میکنند و ویژگی‌های سطحی میسلیوم آنها حفظ شده و به خوبی قابل رویت است.

واژگان کلیدی: اتانول، *Rhizoctonia*، *Rhizopus*

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Introduction مقدمه

Plant protection against diseases is related to research on plant pathogens, especially plant pathogenic fungi (Crous et al. 2015). Filamentous fungi are one of the most economically destructive plant pathogens and cause threatening infections in humans (Harris et al. 2005). The first convincing evidence that fungi cause plant disease was in the case of *Phytophthora infestans* (Berkeley 1948). The scientific names of plant pathogenic fungi provide a piece of information to the accumulated knowledge about the biology, distribution, ecology, host range, and control, as well as the risks associated with the fungal pathogen. Mischaracterization can lead to unnecessary control measures or, more importantly, no control measures being taken to control potentially devastating pathogens (Crous et al. 2015). Therefore, there is a need for more accuracy to examine the morphology and precise identification of fungal pathogens, so that on the one hand it leads to their correct naming, and on the other hand, when applying control treatments, the obtained results can be presented with more confidence.

Rhizopus stolonifer (Ehrenb.:Fr.) Vuill. is the cause of rhizopus rot disease in various fruits and vegetables. This fungus infects ripe fruits at the post-harvest stage and damaged and wounded growing fruits (Hernandez-Lauzardo et al. 2008). *Rhizoctonia solani*, the most important species of the genus *Rhizoctonia*, is a terrestrial plant pathogen with significant diversity in morphology, has multiple hosts, and is an invasive pathogen that has become a global threat (Ali et al. 2017, Ajayi-Oyetunde and Bradley 2018).

Various tools for studying filamentous fungi include genome sequencing, electron tomography, and advanced live-cell imaging with high resolution (Harris et al. 2005). In 1840, microscopes were actively used, and in the early 20th century, the shape, color, and size of spores were emphasized in the classification and identification of fungi (Crous et al. 2015). Scanning Electron Microscope (SEM) is one of the most widely used tools for investigating the morphology of microstructures and properties of chemical compounds (Zhou et al. 2006), which emerged in the mid-1960s, and it was an important period for the systematics of fungi due to the isolation and identification of similar pathogens and the distinction between species (Crous et al. 2015). Microscopic analysis is generally used to identify microorganisms and specifically to classify species (Venkatesh Babu et al. 2018). According to the way of image creation in SEM, its images have a specific three-dimensional appearance and are useful for displaying the surface structure of the desired samples with high resolution (Pathan et al. 2010). Microorganisms, including bacteria, fungi, and microalgae, are composed primarily of water, which limits imaging by SEM (Collins et al. 1993). Therefore, sample preparation requires that microorganisms be fixed, frozen, or dehydrated and covered with a conductive film prior to imaging in a high vacuum environment (Collins et al. 1993) because the samples are distorted in the high vacuum environment due to the removal of water (Pathan et al. 2010). In addition, there is a possibility that the biological samples will be mechanically damaged during the preparation stage, and the morphological information will be disturbed and the presentation of the results will be wrong (Collins et al. 1993).

There are many techniques for dehydrating biological samples with the aim of minimizing cell distortion and preserving maximum original shape and structure including air drying, freeze drying (lyophilization), critical point drying, and various types of chemical stabilization operations before dehydrating the samples (Pathan et al. 2010). On the other hand, new/modified techniques are constantly being developed for sample preparation due to the diversity in tissue types, shape, structure, and composition of biological tissues, and diversity in individual skills and equipment in different laboratories for imaging by SEM. This study investigated the common techniques used in the past with a few changes for the preparation of fungal mycelium tissue samples with the aim of identifying, introducing, and choosing the best method for taking high-resolution SEM images while preserving the surface characteristics of the samples and preserving the structure and natural dimensions of the samples.

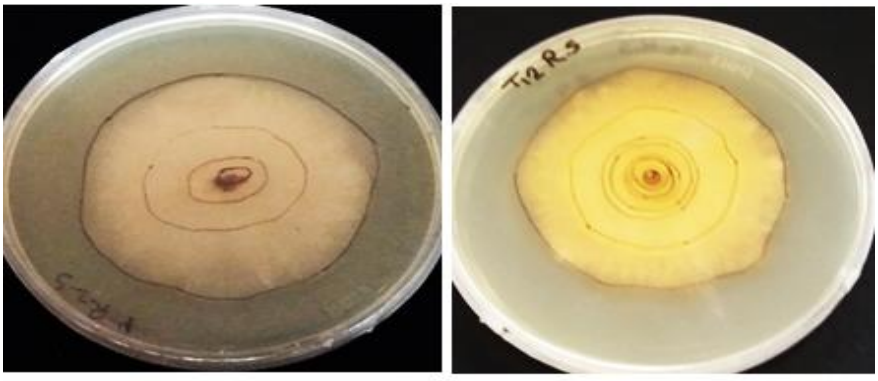
Materials and Methods مواد و روش‌ها

Studied fungi

This study was conducted on SEM imaging of fungal strains of *Rhizoctonia solani* AG4-HG II and *Rhizopus stolonifer* grown in Petri dishes containing potato dextrose agar (PDA) in the central laboratory of the Ferdowsi University of Mashhad in 2018 (Figure 1). The strain of *R. solani* was obtained from the Department of Plant Protection, which was previously identified by Pourmehdi and Taheri (2015) (Pourmahdi and Taheri 2015). *R. stolonifer* strain was extracted and purified from infected strawberry fruits with symptoms of rhizopus rot disease and confirmed through mycological taxonomy and light microscopy. Then, fungal strains were incubated at 25 ±3°C.

Sample preparation steps until imaging by SEM

A sampling of two-day-old *R. solani* and one-day-old *R. stolonifer* strains cultivated on PDA was done without applying any treatment after checking with a stereomicroscope.



Rhizopus stolonifer *Rhizoctonia solani*

شکل ۱. قارچهای کشت شده روی محیط PDA برای مطالعه با میکروسکوپ الکترونی روبشی.

Figure 1. Fungi cultivated on PDA medium for studying with scanning electron microscope.

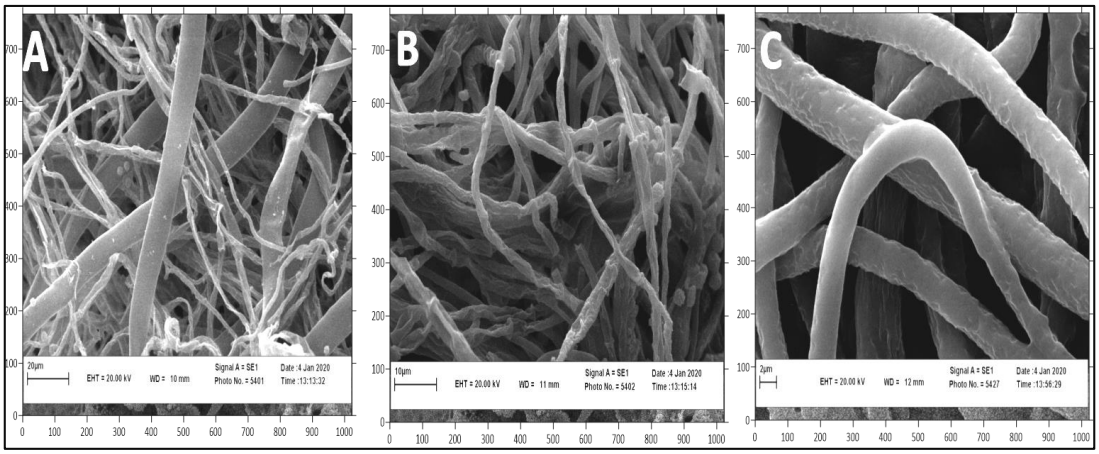
The three mm diameter mycelial disks were taken from the margin of Petri dishes containing fungal strains. Because stabilizing solutions are not able to quickly penetrate thicker samples. The samples were fixed. The water of the fungal samples was taken, and this stage is known as the dehydration stage. Then the samples were covered with a layer of palladium-gold metals using an ion sputter-cutter (gold-palladium using an ion sputter-cutter (SC7620). The samples were attached to a stable frame and prepared for imaging. The imaging of fungal mycelium samples was done by Philips LEO 1450 VP scanning electron microscope, Germany, located in the central laboratory of Ferdowsi University of Mashhad.

Preparation methods of fungal samples

The preparation method of fungal hyphae for SEM studies was carried out following a previously published method with slight modifications (Liu et al. 2017). The fungal mycelial disks (3 mm diameter) were kept in 2.5% glutaraldehyde in phosphate-buffered saline for 12 h at 4 °C. Then the samples were washed with sterile distilled water for 20 min. The dehydrating stage was done in 2 models (dehydrating to 100% ethanol and dehydrating to 50% ethanol) and the drying stage was done in two models (air drying and freeze drying). Thus: A. The samples were dehydrated in 30%, 50%, 70%, 80%, 90%, and 95% ethanol for 10 minutes and for 100% ethanol for 30 minutes and dried in air. Dehydration of the samples was done gradually and slowly, so the ethanol is gradually replaced with the water of the samples from a lower percentage so that the samples are accompanied by minimum shrinkage and maximum surface characteristics of the samples. B. The samples were dehydrated in 30%, 50%, 70%, 80%, 90%, and 95% ethanol for 10 minutes and for 100% ethanol for 30 minutes and dried with an F10 model Freeze dryer manufactured by Pishtaz Equipment Company at a temperature of -48°C under a pressure of 0.70 millibars for 24 hours. C. The samples were dehydrated to 50% ethanol and dried by a freeze-dryer under the mentioned conditions. The fixation, drying/dehydrating steps of the samples should be performed as carefully as possible to minimize shrinkage while ensuring that cellular structures are preserved as close to the natural state as possible. After drying, the samples were glued on special SEM stubs with double-sided adhesives, and the final stage of coating with gold-palladium particles was done in a vacuum, and then the fungal mycelium samples were photographed by a scanning electron microscope.

Results **یافته‌ها**

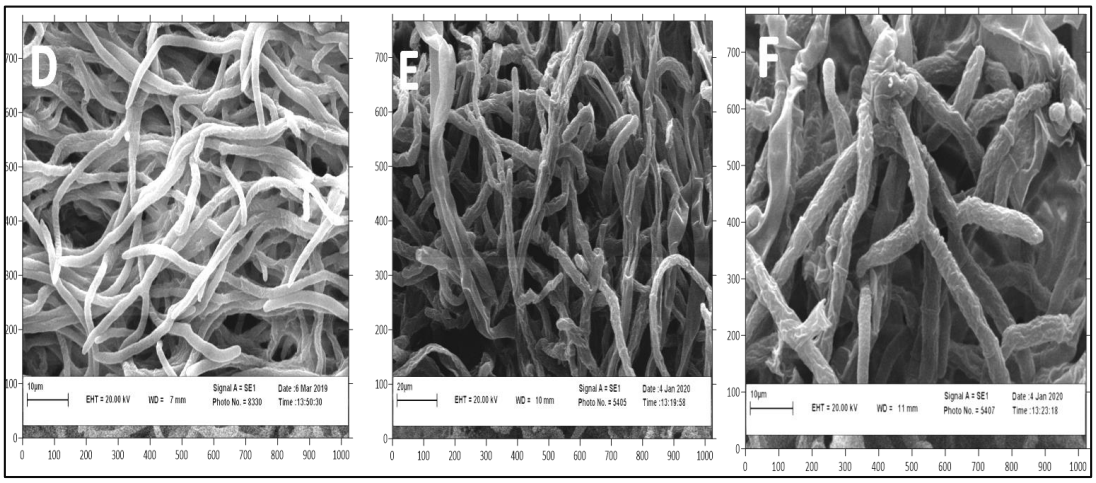
The shrinkage rate of fungal mycelium without applying any treatment was investigated after SEM imaging to introduce and choose the best method of dehydrating and drying fungal mycelial disks. The effects of two different methods of dehydration (dehydration to 100% ethanol and dehydration to 50% ethanol) and drying (drying in the air and using a freeze dryer) on the mycelium sample of two fungal strains of *R. stolonifer* and *R. solani* were compared. Microscopic observations showed significant differences between these two fungal strains. Microscopic images obtained from the fungal strain



شکل ۲. اثر نحوه آبگیری و خشک کردن نمونه‌های قارچی *R. stolonifer* برای تهیه تصاویر میکروسکوپ الکترونی روبشی. A: آبگیری تا اتانول ۵۰ درصد و خشک کردن با فریزدرایر، B: آبگیری تا اتانول ۱۰۰ درصد و خشک کردن بوسیله فریزدرایر، و C: آبگیری تا اتانول ۱۰۰ درصد و خشک کردن در هوا.

Figure 2. The effect of dehydrating and drying of mecillium samples of *R. stolonifer* for imaging by SEM. A: Dehydration to 50% ethanol and drying with a freeze-dryer, B: Dehydration to 100% ethanol along with freeze-drying, C: Dehydration to 100% ethanol and air-drying.

samples of *R. stolonifer* showed the maximum shrinkage and loss of surface characteristics of the samples in the technique of dehydration to 100% ethanol drying with a freeze dryer (Figure 2, B) followed by dehydration to 50% ethanol and drying with a freeze dryer (Figure 2, A) and finally dehydrating to 100% ethanol and drying in the air



شکل ۳. اثر نحوه آبگیری و خشک کردن نمونه‌های قارچی *R. stolonifer* برای تهیه تصاویر میکروسکوپ الکترونی روبشی. D: آبگیری تا اتانول ۵۰ درصد و خشک کردن با فریزدرایر، E: آبگیری تا اتانول ۱۰۰ درصد و خشک کردن بوسیله فریزدرایر، و F: آبگیری تا اتانول ۱۰۰ درصد و خشک کردن در هوا.

Figure 3. The effect of dehydrating and drying of mecillium samples of *R. solani* for imaging by SEM. D: Dehydration to 50% ethanol and drying with a freeze-dryer, E: Dehydration to 100% ethanol along with freeze-drying, F: Dehydration to 100% ethanol and air-drying.

(Figure 2, C). While the microscopic images of the *R. solani* fungal strain samples showed that the maximum shrinkage and wrinkling were observed in the samples dehydrated to 100% ethanol and dried by a freeze dryer (Fig. 3, E), which causes the loss of the surface and structural characteristics of the samples, followed by in the samples dehydrated to 100% ethanol and dried in the air (Figure 3, F), and then in the samples that were dehydrated to 50% ethanol and dried by a freeze dryer (Figure 3, D). Therefore, dehydrating to 100% ethanol and using a freeze dryer caused maximum mycelium shrinkage in both fungal strains. SEM images of fungal mycelium in these two different strains showed that the method of dehydrating and drying had an effect on the high-resolution image or the shrinkage of the tissue of the biological sample.

Discussion بحث

The microscopic images obtained from the preparation of the samples by dehydrating to 100% ethanol and drying with a freeze-dryer were associated with maximum wrinkling and shrinkage, which were in contrast with previous reports of Liu et al. (2017) and Vakili-Ghartavol et al. (2022) who observed SEM images of the control samples were smooth and with minimal shrinkage by dehydrating to 100% ethanol and drying with a freeze-dryer. According to the differences observed in the microscopic images, the method of dehydrating and drying the fungal mycelium samples depended on the fungal strain, which showed significant changes in the structure and surface characteristics of the fungal mycelium. It seemed that in the structure of the fungal mycelium, the composition of the cell wall is different between the strains, which causes these differences. There was evidence that the preparation method of samples for SEM imaging caused significant changes in the dimensions of fungal spores. The quality and resolution of images in SEM imaging were directly dependent on the three critical factors of fixation, post-fixation, and dehydration (Venkatesh Babu et al. 2018). For example, Air-dried spores were significantly smaller than frozen spores and fresh spores (Beckett et al. 1984). Also, volume changes were observed in mouse embryonic tissue after stabilization through dehydration and drying to the critical point (Boyde and Maconnachie 1979). The morphology of *Sordaria humana* was examined with SEM in critical point-dried, air-dried, vacuum-dried, freeze-dried, partially frozen–hydrated, hydrated, and fully frozen-hydrated states after treatment, and the observations showed that the appearance of its tissue structure was dependent on the preparation method and the operating conditions used in the microscope, and all the sample preparation methods caused secondary changes and provided wrong reports. However, the frozen-hydrated method showed the best images (Read et al. 1983). In one study, 5 preparation methods (no treatment, glutaraldehyde in phosphate buffer method and dehydration with ethanol, glutaraldehyde in phosphate buffer method and dehydration with tetroxyde osmium, flash freezing with liquid nitrogen and flash freezing and drying with a freeze dryer) fungal samples of *Curvularia lunata* were examined for imaging by SEM microscope and the findings of this study showed that the preparation methods affected the morphology of the fungal

sample (Venkatesh Babu et al. 2018).

Therefore, careful preparation of samples for SEM is essential to ensure that the difference observed between control and treated cells is due to the effect of the treatment and not to the preparation method (Burt 2004, Ebrahimi and Khosravi-Darani 2013). Dimensional changes during sample preparation are likely to be related to changes in shape and relative relationships between organelles, cells, and tissues of different compositions, Therefore, all those who interpret microscopic images for biological samples should pay attention to this point. Also, there are different methods for preparing microscopic images that may provide different results as the effect of treatment. Therefore, it is preferred to introduce the best sample preparation method, so there is a need to investigate and introduce a specific technique that enables the imaging of hydrated biological samples with minimal manipulation.

Conclusion نتیجه‌گیری

In the present study, several methods of dehydrating and drying fungal mycelium for imaging by SEM were investigated. The choice of a suitable method for the study of fungal mycelium depends on characteristics such as the structure of the examined tissue, the surface characteristics of the samples, the availability of preparation equipment, and the capabilities of the electron microscope. The results showed that in the *R. stolonifer* strain, dehydration to 100% ethanol and drying in air, while in the *R. solani* strain, dehydration to 50% ethanol and freeze-drying resulted in high-resolution microscopic images with minimum wrinkling and shrinkage and was associated with maintaining the maximum surface characteristics of the samples and their structural integrity. The microscopic images obtained from the method of dehydrating to 100% ethanol and drying with a freeze dryer were associated with maximum shrinkage for both fungal strains. The findings of this preliminary study can be promoted to encourage and reflect on other researchers and users of SEM, for the purpose of research to optimize preparation methods and find better options for their specific purposes.

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