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Original article

Evaluation of the infection process by *Lecanicillium fungicola* in *Agaricus bisporus* by scanning electron microscopy

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ABSTRACT

Background: *Lecanicillium fungicola* causes dry bubble disease in *Agaricus bisporus* mushrooms leading to significant economic losses in commercial production.
Aims: To monitor the infection process of *L. fungicola* in Brazilian strains of *A. bisporus.*
Methods: The interaction between the mycelium of *L. fungicola* (LF.1) and three strains of *A. bisporus* (ABI 7, ABI 11/14 and ABI 11/21) was studied. Electron microscopy and X-ray microanalyses of vegetative growth and basidiocarp infection were evaluated.
Results: Micrographs show that the vegetative mycelium of the Brazilian strains of *A. bisporus* is not infected by the parasite. The images show that the pathogen can interlace the hyphae of *A. bisporus* without causing damage, which contributes to the presence of *L. fungicola* during the substrate colonization, allowing their presence during primordial formation of *A. bisporus*. In the basidiocarp, germ tubes form within 16 h of infection with *L. fungicola* and the beginning of penetration takes place within 18 h, both without the formation of specialized structures.
Conclusions: Scanning electron microscopy enabled the process of colonization and reproduction to be observed within the formation of phialides, conidiophores and verticils of *L. fungicola*. The formation of calcium oxalate crystals by the pathogen was also visible using the X-ray microanalysis, both at the hyphae in the Petri plate and at basidiocarp infection site.

Evaluación del proceso de infección por *Lecanicillium fungicola* en *Agaricus bisporus* por microscopia electrónica de barrido

Palabras clave:
Microscopia electrónica
Seta
Microanálisis de rayos X
Micopatógeno

RESUMEN

Antecedentes: *Lecanicillium fungicola* es el agente causal de la enfermedad de la mole seca en *Agaricus bisporus*, responsable de importantes pérdidas económicas en la producción comercial de esta seta.
Objetivos: Comprobar el proceso de infección de *L. fungicola* en cepas brasileñas de *A. bisporus.*
Métodos: Se estudió la interacción entre el micelio de *L. fungicola* (LF.1) y tres cepas de *A. bisporus* (ABI 7, ABI 11/14 y ABI 11/21). Se evaluaron mediante microscopia electrónica y microanálisis de rayos X el crecimiento vegetativo y la infección de los basidiocarpos.
Resultados: Las micrografías muestran que el micelio vegetativo de las cepas brasileñas de *A. bisporus* no resultó afectado por la infección del parásito. Las imágenes muestran también cómo el agente patógeno puede entrelazar las hifas de *A. bisporus* sin causar daños, lo que contribuye a la perpetuación de *L. fungicola* durante la colonización del sustrato y durante la formación de los primordios de *A. bisporus.* En el basidiocarpo, los tubos germinales se forman después de 16 h de la infección con *L. fungicola* y el comienzo de la penetración tiene lugar tras 18 h, sin formación de estructuras especializadas.

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Conclusiones: La microscopia electrónica permite observar el proceso de colonización y reproducción con la formación de fiálides, conidióforos y verticilos de *L. fungicola*. La formación de cristales de oxalato de calcio por parte del agente patógeno también fue visible mediante el microanálisis por rayos X, tanto en la infección de las hifas en placa de Petri como en la de los basidiocarpos.

Lecanicillium fungicola (Preuss) Zare and Gams [synonyms: *Verticillium fungicola* (Preuss) Hassebrauk, *Verticillium malthousei* (Preuss) Ware] is the etiological agent of the disease known as dry bubble (Zare and Gams[24]) in commercial mushroom (*Agaricus bisporus*) production, and contributes to major economic losses.[1] Symptoms vary from small brown necrotic lesions on the basidiocarp to stem peeling/bending or splitting of the stipe, and shapeless and undifferentiated non-necrotic masses in the mushroom tissue.[14] Control of the disease depends on exhaustive sanitation and hygiene and on the use of fungicides. Fungicide resistance is common.[10,18]

L. fungicola infection seems to occur first by adhesion of hydrophobins present in the wall of both fungi. After the initial connection, the fungus can grow inter- and intracellularly, due to weakening of the cell wall of *A. bisporus* by the action of lytic enzymes and mechanical pressure of the pathogen.[6] The infection occurs when the mushrooms develop mature sporocarps prior to or after harvest.[15] Observation of them suggests that the vegetative mycelium is not affected. Some microscopic and biochemical studies have been published to clarify the process of infection, but this is not fully elucidated.[2,6,24]

There is some variability in relation to virulence between groups of *L. fungicola*.[12] Two varieties of *L. fungicola* affect cultures of *A. bisporus*: the *aleophilum* variety, which affects mushroom culture in Canada and in the USA,[7] and the *fungicola* variety, responsible for the disease in Britain, the Netherlands, Spain and France.[12] In Brazil, the variety that affects the production of *A. bisporus* has not been identified.

The *L. fungicola* infection process has been analyzed by biochemical and molecular methods.[7,15] However, its visualization is only possible through morphological studies. Scanning electron microscopy has been an important tool in studies related to the pathogen/host interaction, i.e. adhesion, germination, penetration, colonization and reproduction of pathogens.[4] In addition, morphological studies are prerequisites in the development of control strategies.

This study was conducted in order to monitor the infection process of *L. fungicola* in three strains of *A. bisporus*, both in the vegetative phase and at the stage of basidiocarps, by scanning electron microscopy.

Material and methods

Hyphal interaction

To study the interaction along the vegetative stage, mycelia of *L. fungicola* (LF.1) and three strains of *A. bisporus* (ABI 7, ABI 11/14 and ABI 11/21) from the mycology collection of the Edible Mushrooms Laboratory at the Federal University of Lavras (UFLA) were used. The strains used were maintained in subcultures (Potato Dextrose Agar – PDA; Difco, Lawrence, USA), and were isolated directly from the basidioma, according to Zied et al.[25] The *L. fungicola* strain was isolated from diseased mushrooms collected by growers in the city of Mogi das Cruzes, Sao Paulo State (Brazil). The strains of *A. bisporus* are described in the manuscript presented by Zied et al.[26]

Discs (0.5 cm in diam.) containing inoculum of the fungi (*A. bisporus* strains and *L. fungicola*) were transferred to a Petri dish (8 cm in diameter) with PDA on opposite sides and maintained at $25 \pm 0.5\,^{\circ}C$ in a controlled chamber. Due to the difference in the growth rate of the two fungi strains, *A. bisporus* was inoculated 10 days before *L. fungicola*. Five 1 cm^2 samples were taken at the interface of the colonies on days 1, 3, 5 and 10. The collection time was determined from tests developed previously by the authors.

Cultivation of mushrooms and inoculation of L. fungicola

For the infection studies of *L. fungicola* in mushrooms the same strains described aforementioned were used. Commercially prepared, pasteurized and conditioned compost was obtained from "Sítio dos Micélios" (Barbacena, MG, Brazil) and inoculated with the strains of *A. bisporus* and maintained at $25 \pm 1\,^{\circ}C$ for 20 days, until the total colonization of the substrate. In order to induce basidiocarps formation, a casing layer containing dystrophic red latosol (Oxisol) and coal, in the ratio 1:2, and calcitic limestone (5%) was used. After the addition of the casing layer, the substrates were incubated at $20 \pm 1\,^{\circ}C$ and 90% humidity in mushroom-growing rooms (Universidade Federal de Lavras), following the methodology presented by Pardo-Giménez et al.[20] After the onset of the primordium (mushrooms with a diam. of approximately 2.5 cm), 5 μl of a *L. fungicola* (1×10^4 ml^{-1}) spore suspension were inoculated at different points (0.5 cm apart) on the surface of the pileus. The spore suspension was obtained by adding distilled and autoclaved water into a pure culture of the pathogen, and then rubbing was carried out with a Drigalsky handle, following the methodology described by Zied et al.[26] Five 1 cm^2 samples of the infected mushrooms were taken at 12, 14, 16, 18, 20, 30, 40, 50, 70 and 140 h. The collection time was determined from previous tests presented by Zied et al.[26] For each time interval, 30 mushrooms were used, and five samples were taken from each one, making a total of 150 samples.

Scanning electron microscopy

All collected samples were pre-fixed in modified Karnovsky fixer (2.5% glutaraldehyde, 2.5% formaldehyde in 0.05 M phosphate buffer, pH 7.2) for a period of, at least, 24 h at $4\,^{\circ}C$. Some mushroom samples infected with the pathogen were removed from the pre-fixative and transferred to 30% glycerol for 30 min; then the samples were immersed in liquid nitrogen and fractured with a scalpel on a metal plate. After fixation, the standard protocol for scanning electron microscopy described by Bozzola and Russell,[3] with modifications, was used.

Samples were washed three times in 0.05 M phosphate buffer for 10 min each, and dehydrated in acetone gradient (25%, 50%, 75% and 90%, and three times 100%). After dehydration, samples were taken to a Balzers CPD 030 critical point dryer device to replace acetone with CO_2 and to complement drying. Specimens were mounted on aluminum brackets (stubs) with carbon tape on aluminum foil paper, covered with gold in a Balzers SCD 050 evaporator and then observed in a LEO EVO 40 XVP scanning electron