El Colegio de la Frontera Sur

Microorganismos presentes en la micósfera de especies de *Pleurotus* y su efecto en cepas del género *Trichoderma.*

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Con orientación en Biotecnología Ambiental

Por

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Dedicatoria

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Resumen

El cultivo de hongos comestibles es una actividad que trae beneficios para los productores a nivel mundial, debido a periodos cortos de cultivo y al aprovechamiento de residuos agroindustriales como sustratos para su crecimiento. Sin embargo, la producción se ha visto afectada por plagas y enfermedades como la del moho verde ocasionada por *Trichoderma* spp., que dañan severamente los cultivos. La utilización de productos químicos y fungicidas pueden tener resultados eficaces, pero también ocasionar daños a la salud humana y problemas ambientales si se utilizan inadecuadamente. Como alternativa se han probado compuestos naturales y microorganismos como agentes de control biológico. El objetivo de este trabajo fue caracterizar la interacción de los microorganismos presentes en el sustrato previo al cultivo y en la micósfera de *Pleurotus* spp., con cepas del género *Trichoderma* causantes de la enfermedad del moho verde. Para ello, se aislaron microorganismos del proceso de pasteurización del sustrato y cultivo de tres especies de *Pleurotus* (*P. ostreatus, P. djamor, P. pulmonarius*). Se realizaron pruebas de confrontación *in vitro* contra tres cepas de *Trichoderma* spp. y cinco cepas de hongos de interés comercial. El 31 % de las cepas aisladas produjo un efecto positivo, aunque variable en la inhibición según la cepa de *Trichoderma* spp. confrontada. En las confrontaciones triples (*Trichoderma* sp. vs antagonista vs *Pleurotus* sp) se encontraron tres microorganismos capaces de inhibir el crecimiento de *Trichoderma* sp con efecto mínimo sobre *P. ostreatus* (disminución de 2-8 % del crecimiento). Estos microorganismos fueron identificados con el marcador molecular 16S rDNA como *Bacillus cereus, B. subtilis*, y una tercer cepa *Bacillus* sp.

Palabras clave: cultivo de hongos, hongos comestibles, antagonismo, control biológico, enfermedades fúngicas, moho verde.

Capítulo 1. Introducción

El cultivo de hongos comestibles es una actividad que beneficia la economía de muchos productores a nivel nacional y mundial. La demanda de este producto ha aumentado en los últimos años, debido a factores como el creciente aumento de la población, cambios en los hábitos alimenticios, las características nutritivas de los hongos, etc. (Sánchez 2010; Sánchez y Royse 2017; Gea et al. 2021). El cultivo de hongos comestibles constituye un verdadero sistema de producción-consumo, pues se trata de procesos biotecnológicos aplicados que pueden desarrollarse a pequeña y gran escala para la generación de alimentos, suplementos, enzimas y productos metabólicos con amplio potencial de utilización en la industria (Sánchez et al. 2007). La producción de hongos comestibles está asociada a la micósfera, es decir al microambiente donde crecen y se desarrollan la setas cultivadas (Sánchez y Royse 2017). Propiedades del sustrato como la humedad, la proporción carbono-nitrógeno, el pH y factores como la cantidad de inóculo, los agentes antimicrobianos y las interacciones entre organismos en el sustrato (Avendaño-Hernández y Sánchez 2013; Bellettini et al. 2018; Barba et al. 2019) influyen de manera significativa. Sin embargo, algunas enfermedades fúngicas y/o bacterianas, favorecidas por condiciones no adecuadas en el cultivo (como temperaturas cálidas y humedad del sustrato, entre otras) afectan la producción generando pérdidas económicas (Hoa y Wang 2015; Bellettini et al. 2018).

La enfermedad del moho verde ocasionada por *Trichoderma* spp (*T. asperellum, T. atroviride, T. citrinoviride, T. harzianum, T. longibrachiatum, T. pleurotum, T. pleuroticola, T. virens*) (Colavolpe et al. 2014; Bellettini et al. 2018) es de las más graves en el cultivo de hongos comestibles. Estas especies de hongos saprófitos crecen con gran facilidad y son competidoras del espacio y los recursos nutricionales con otros hongos (Velázquez-Cedeño et al. 2008; Infante et al. 2009; Romero-Arenas et al. 2009; Roberti et al. 2019). Para contrarrestarla se han utilizado diversos productos químicos como el uso de fungicidas procloraz y metrafenona (Gea 2001; Luković et al. 2020) y la utilización de hidróxido de calcio (Velázquez-Cedeño et al. 2008). También se han estudiado una gran variedad de compuestos naturales y microorganismos como agentes de biocontrol

eficaces contra *Trichoderma* spp (Gbolagade 2006; Velázquez-Cedeño et al*.* 2007; Kim et al. 2008; Saju y Sinhu 2011; Kosanovic´ et al. 2013; Gupta y Vakhlu 2015; Sjaarda et al*.* 2015; Roberti et al*.* 2019; Stanojevic´ et al. 2019).

Por lo que en este estudio se plantea como pregunta de investigación si en la micósfera donde se cultivan hongos comestibles hay microrganismos con capacidad antagonista para el crecimiento de cepas del género *Trichoderma*. Puesto que en investigaciones anteriores se ha estudiado el efecto de algunos organismos asociados a la micósfera de hongos de interés comercial (bacterias, hongos, nemátodos o protozoarios), con efectos promotores o inhibidores en su desarrollo (Mata et al. 2017; Sánchez y Royse 2017). Teniendo como hipótesis que existen microorganismos que inhiben el crecimiento de cepas del género *Trichoderma,* el objetivo de este trabajo fue caracterizar la interacción de los microorganismos presentes en el sustrato previo al cultivo y en la micósfera de *Pleurotus* spp., con cepas del género *Trichoderma* causantes de la enfermedad del moho verde, para conocer aquellos que actúen como antagonistas del moho verde y que a su vez pudieran tener efectos promotores del crecimiento del hongo de interés. Esto permitirá desarrollar más adelante estrategias de control biológico contra la enfermedad del moho verde.

Después del capítulo 1 (Introducción), este trabajo cuenta con dos capítulos adicionales. El capítulo 2 consiste en un artículo científico titulado “Microorganismos presentes en la micósfera de especies *Pleurotus* y su efecto en cepas del género *Trichoderma”*, mientras que el capítulo 3 integra las conclusiones de este estudio y perspectivas para futuras investigaciones.

Capítulo 2. Artículo: Microorganismos presentes en la micósfera de especies de *Pleurotus* y su efecto en cepas del género *Trichoderma.*

Microorganisms present in the mycosphere of *Pleurotus* species and their effect on strains of the genus *Trichoderma.*

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Microorganisms present in the mycosphere of *Pleurotus* species and their effect on strains of the genus *Trichoderma.*

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Abstract

The interactions among microorganisms that are associated with the mycospheres of edible mushrooms are of great importance. Some of them can directly intervene in the development of the fungus of interest, in addition to acting as inhibitors of other pathogenic fungi that cause disease in crops. The objective of this work was to isolate and characterize microorganisms from the mycospheres of *Pleurotus* spp. to identify effective antagonists for controlling green mold disease caused by *Trichoderma* species. For this purpose, microorganisms were isolated from the substrate pasteurization process and cultivation of three *Pleurotus* species. Subsequently, *in vitro* confrontation tests were carried out against *Trichoderma* spp. strains and five strains of edible fungi of commercial interest. It was found that 31 % of the isolated strains produced some degree of inhibition of *Trichoderma* spp. When performing triple confrontations (e.g., *Trichoderma* sp. vs antagonist vs *Pleurotus* sp.), three microorganisms were found to be capable of inhibiting the growth of *Trichoderma* spp. with a minimal effect on *P. ostreatus* (e.g., growth decrease of 2-8 %). They were identified as *Bacillus cereus*, *B. subtilis*, and a third strain of *Bacillus* sp. based on 16S rDNA gene analysis.

Key words: biological control, edible mushrooms, green mold, antagonists, *Bacillus* sp.

1 Introduction

There are diverse interactions occurring among organisms that cohabit an ecosystem. These relationships are complex since they can be beneficial for all parts involved or only for some of them, as well as not having any effect or influence on each other. Mushrooms are not exempt from these relationships, which occur precisely in the mycosphere -the microenvironment where they develop- (Sánchez and Royse, 2017). In the mycosphere, microorganisms influence the nutrient cycling and can have promoting or inhibiting effects on fungi development. In this sense, the most studied microorganisms have been those that directly benefit fungal growth, such as siderophore-secreting microorganisms (Singh et al., 2000; Sahu and Sindhu, 2011), nitrogen fixers and antibiotic producers with the capacity to inhibit the development of other microorganisms (Sánchez and Royse, 2017).

In the cultivation of edible mushrooms and others of commercial interest, pests and diseases that occur during their development cycle are a relevant issue because they generate economic losses by reducing productivity. The fungi of the genus *Trichoderma* represent the main contaminants of the substrate that affect the cultivation of *Pleurotus* spp. (Hatvani et al., 2006; Colavolpe et al., 2014), which are possibly the most harmful antagonists since they can cause a production loss of more than 77 % (Gea, 2001).

These saprophytic species, which proliferate in soils with different amounts of organic matter, can grow in conditions that are unfavorable to other mushrooms and can colonize substrates. They are direct competitors for space and nutritional resources of other mushrooms (Infante et al., 2009; Romero-Arenas et al., 2009).

Some authors have mentioned that the capacity of a species to defend itself from the attacks of antagonistic organisms results from the resistance of the strain and the type of substrate in which it is cultivated. In the case of the *Pleurotus* genus, it has been observed that the mycelium forms large amounts of hyphae with aerial growth in the contact zone with those of the *Trichoderma* genus, where a dense mycelial barrier is observed that retards the growth of green mold (Mata et al., 2017). Microorganisms have also been found to be involved in inhibiting the growth of *Trichoderma* spp., such as the gram-positive bacteria of the genus *Bacillus*, for example the growth inhibition of *T. harzianum*

by *Bacillus polymyxa* (Velázquez-Cedeño et al., 2007; 2008). However, this effort is not sufficient to stop the advancement of the parasitic fungus, which invariably ends up colonizing the strains of *Pleurotus* spp.

Gea et al. (2021) reviewed fungal diseases in edible mushroom cultivation and pointed out that the microbiome selected and isolated from mushroom substrates can be effectively reintroduced during cultivation as a biological control agent (BCA) to control green mold. For this reason, it is of particular importance to find natural ways or strategies that avoid or diminish the damage caused by species of the genus *Trichoderma* during the cultivation of edible mushrooms *Pleurotus* spp.

The objective of this work was to study the interaction of microorganisms present in the mycosphere of *Pleurotus* spp. with pathogenic strains of the genus *Trichoderma* to isolate possible antagonists to further develop biological control strategies against green mold disease that is suffered by the edible species of the genus *Pleurotus.*

2 Materials and Methods

2.1 Biological Material

Eight strains that were provided from the mycological collection of El Colegio de la Frontera Sur at Tapachula, Chiapas, Mexico were used. They were *Pleurotus ostreatus* ECS-1123, *Pleurotus djamor* ECS-123, *Pleurotus pulmonarius* ECS-192, *Lentinula edodes* ECS-401, *Auricularia fuscosuccinea* ECS-210, *Trichoderma atroviride* ECS-616, *Trichoderma* spp. ECS-622 and ECS-613. Reactivation of the strains was performed in Petri plates with malt extract (MEA) incubated at 25 °C until the mycelium covered the entire surface of the plate.

2.2 Culture cycle and sample collection

Isolation of the microorganisms was performed from three subsequent batches of *Pleurotus* spp. cultivation (batch A: *P. ostreatus* ECS-1123, batch B: *P. djamor* ECS-123 and batch C: *P. pulmonarius* ECS-196) on a substrate that was composed of a mixture of Pangola grass *Digitaria eriantha*, coffee husks and corn cobs with humidities of 65.3 % (batch A), 63.2 % (batch B), and 66 % (batch C). Substrate pasteurization was performed

by self-heating according to the methodology described by Avendaño-Hernández and Sánchez (2013) and Morales and Sánchez (2017).

Samples were taken from the initial substrate (prior to the pasteurization process), 30 hours after starting the process (time of substrate removal), at the end of pasteurization (which also corresponds to the spawning day or day zero), during colonization (days 8 and 15 after spawning) and at the first flush (24 days after spawning). For incubation and fruiting, guidelines described by Zadrazil and Kurtzman (1982) were followed.

2.3 Isolation and characterization of microorganisms

Isolation and characterization of the microorganisms were performed according to the serial dilution methodology used by Torres et al. (2016) and Díaz-Martínez et al. (2019).

A 10 g sample of substrate was taken (from three different locations of a batch in the pasteurization process and from different bags during the spawning and colonization of the cultured strains) and was suspended in 90 ml of sterile water and serial dilutions up to 10^{-7} were prepared. Subsequently, 100 µL of these dilutions were taken and spread on malt extract agar (MEA), Luria-Bertani (LB) and Sabouraud (SAB) culture media to isolate microorganisms. They were incubated at 25 °C for 48 hours. The colonies were selected according to their morphology and abundance, attempts were made to take representatives of all forms present, and successive cultures were made until the purity of the isolates was confirmed (cross streaking method). The isolated microorganisms were characterized by Gram staining and by their micro- and macroscopic morphologies.

2.4 *In vitro* confrontation tests

Confrontation tests were carried out between the isolated microorganisms and the three strains of *Trichoderma* spp. under study. For this purpose, a 5 mm diameter disk of fungal mycelium was inoculated at one end of a Petri plate with malt extract against a loop of similar diameter with each isolated microorganism at the other end, which was followed by incubation for seven days at 24 °C. The tests were performed in triplicate.

Subsequently, the microorganisms that caused growth inhibition of *Trichoderma* spp. strains were tested against *P. ostreatu*s ECS-1123, *P. djamor* ECS-123, *P. pulmonarius*

ECS-192, *L. edodes* ECS-401, and *A*. *fuscosuccinea* ECS-210 to determine the types of interactions among them (Quiroz-Sarmiento et al., 2008).

Once the microorganisms with greater inhibitory effects on *Trichoderma* spp. were determined, which allowed better growth of the edible strains of interest (only five microorganisms), a third triple confrontation test was carried out, as described below:

For this purpose, test tubes (18 cm long) were used. Previously sterilized Pangola grass was used as substrate with a humidity of 36±2 %. Subsequently, the substrate was inoculated with a solution of the selected microorganisms ($1x10^5$ CFU g^{-1}) to obtain a humidity of 65 % (Torres et al*.,* 2016; Díaz-Martínez et al., 2019). A 5 mm disc four days old of *Trichoderma* sp. ECS-622 and four sorghum grains previously colonized by *P. ostreatus* ECS-1123 were aseptically placed at the bottom of a sterile test tube, and then 10 grams of the sterile substrate were added, so that the tubes contained sterile grass, under the following conditions 1) inoculated with each microorganism with inhibitory potential of *Trichoderma* sp., 2) control without microorganisms, 3) with *Trichoderma* sp. ECS-622, 4) with *P. ostreatus* ECS-1123, 5) dual confrontation of *Trichoderma* sp. ECS-622 against *P ostreatus*. ECS-1123 and 6) triple confrontation: each microorganism plus *Trichoderma* sp. ECS-622 and *P. ostreatus* ECS-1123. The evaluation was carried out over a period of 20 days.

2.5 Variables to measure

To evaluate the inhibition levels that were caused by the isolated microorganisms on the *Trichoderma* spp. strains, the separation distances (in cm) between the colonies of *Trichoderma* spp. and those of the evaluated microorganisms were recorded after seven days of interaction in Petri plates.

To determine the effects on the growth of the isolated antagonistic microorganisms on the edible mushroom strains, the diameters of the latter were measured after seven days of confrontation on Petri plates, and significant differences from the control grown in the absence of the antagonist were observed.

To determine the effects of a triple confrontation on the growth of *P. ostreatus* ECS-1123 in test tubes, the mycelial growth measurements were plotted against time (cm/day), and

a regression line was calculated for each treatment. The slope of each line was defined as the linear extension rate (LER).

2.6 Identification of isolated strains

The microorganisms that showed clear inhibitory effects on *Trichoderma* spp. strains and allowed growth of the fungal strains of edible interest in the triple confrontation tests were identified molecularly, according to the following protocol:

DNA extraction was performed with the AquaPure Genomic DNA Isolation Kit (Cat No. 732-6340, Bio-Rad, CA. USA). At the end of the procedure, the DNA was suspended in 50 µl of elution buffer.

The total nucleic acids extracted were visualized by electrophoresis on 1 % agarose gels, in which 5 µl of a total of 50 µl of extraction was used. A 1:20 dilution of all samples was made, and 1 µl was used to perform PCR using the oligonucleotides fD1 and rD1 (targeting the 16S ribosomal gene), which have the sequences of fD1:5'-AGAGAGTTTGATCCTGGCTCAG-3' and rD1:5'-AAGGAGGAGGTGATCCAGCC-3', as described by Weisburg, et al. (1991).

PCRs were carried out with a final volume of 20 µl using PCR Master Mix 2X, Promega brand (Cat. No. M7502. Wisconsin, USA) under the following conditions: one cycle at 95 °C for 5 min, 30 cycles starting at 95 °C for 30 s (denaturation), 30 s at 53 °C (alignment), 72 °C for 90 s (extension), and then a final cycle of 72 °C for 5 min (final extension).

The PCR results were analyzed on 1 % agarose gels. The amplified DNA products except for a single PCR product with approximately 1500 bp were purified using the DNA Clean and Concentrator kit (Zymo Research, CA, USA). All purified products were verified by 1 % agarose gel electrophoresis (90 V for 30 min) in 1x buffer solution of Tris, borate and EDTA (TBE) using 3 of 40 µl of reaction to load the gel using GeneRuler 1 kb as a molecular weight marker. After electrophoresis, the fragments were visualized with ultraviolet light to confirm their degrees of purity and to estimate their concentrations.

2.7 Sequencing and identity assignment

The PCR products were sent for Sanger sequencing to the DNA Synthesis and Sequencing Unit, Institute of Biotechnology, UNAM. The sequences obtained were reviewed and compared with the electropherograms obtained to determine the cleanliness and reliability of the signals. Subsequently, the sequences were analyzed by means of the GenBank databases and by using the BLAST (Basic Local Alignment Search Tool) algorithm to search for identity with other DNA sequences.

2.8 Statistical design

For the confrontation tests, a completely randomized single-factor design with three replications was used. The data obtained were subjected to one-way analysis of variance (ANOVA), and a mean comparison test (Tukey's test, $P \leq 0.05$) was performed using the R statistical package (The R Project for Statistical Computing) version 4.0.4.

3 Results

3.1 Isolation of microorganisms

A total of 189 microbial isolates were obtained from different stages of the substrate pasteurization process, at 8 and 15 days of substrate colonization, and during the first harvest (24 days). The different isolated colonies presented variations in coloration (e.g., beige, clear or yellow) and were generally circular, filamentous or punctate, with umbilicate or convex elevations. Some colonies presented creamy consistencies.

The microorganisms that were identified microscopically by Gram staining corresponded to 68 % bacillary forms and 32 % cocci. No fungi or yeasts were reported in the isolates. These microorganisms exhibited gram-negative (60 %) and gram-positive (40 %) reactions. The microbial population densities in the different analyzed batches varied with respect to the sampling times during the different stages evaluated. Figure 1 shows the populations that were observed in the three media used for the isolation of the microorganisms analyzed (e.g., Sabouraud, Luria Bertani and malt extract agar).

The total populations of culturable microorganisms ranged from $2x10^4$ to $5x10^7$ CFU g^{-1} in the three cultured batches that were sampled. In addition, variations in the population

densities of the microorganisms were observed that depended on the batch and stage of the process from which they were isolated. Figure 1A shows that a growth trend was observed as the stage of the process progressed. In Batch A**,** a greater number of microorganisms isolated in the first harvest and a greater presence in the Sabouraud medium was observed.

However, for the microorganisms isolated from batch B, variable behavior was observed (see Figure 1B), and the largest quantity of microorganisms isolated corresponded to the removal process stage on Luria-Bertani medium and at eight days after spawning for microorganisms with growth on malt extract agar. Figure 1C shows that most of the isolated microorganisms from batch C at each stage of the process were on Luria-Bertani medium, with the highest quantity of colonies isolated eight days after spawning.

3.2 Confrontation tests

3.2.1 Confrontation against strains of *Trichoderma* species

The confrontation experiments generated a wide range of responses, from no inhibition to an inhibitory halo with a diameter of 3.23 cm (*Trichoderma* sp. ECS-613, *Trichoderma atroviride* ECS-616, lot A).

Table 1 shows the total microorganisms that were isolated for each of the evaluated batches. On average, 63 isolates were obtained for each batch, 31 % of which showed antagonistic effects against the *Trichoderma* strains evaluated. Only a small percentage of these microorganisms exhibited inhibition distances greater than two centimeters in the confrontation tests; batch B presented the highest percentage (8.77 %), which was followed by batch A (7.81 %) and batch C (4.41 %).

The statistical analysis indicated significant differences among each of the evaluated treatments (isolated microorganisms vs. *Trichoderma* strains); they also exhibited variations in their antagonistic effects on each of the *Trichoderma* strains confronted.

Tables 2, 3 and 4 list the isolated microorganisms that produced some degree of inhibition in the growth of some strains of the genus *Trichoderma* that were used (e.g., *Trichoderma* sp. ECS-613, *Trichoderma atroviride* ECS-616 or *Trichoderma* sp. ECS-622). It can be observed that the strain *Trichoderma* sp. ECS-613 was the most sensitive at the time of

confrontation with the isolated microorganisms of batches A and B (Tables 1 and 2), since the analysis showed an inhibition greater than 2.4 cm in most of the confrontations, while the strain *Trichoderma* sp. ECS-622 was the most resistant in these confrontations, so the inhibition distances were smaller in comparison with the two remaining strains. On the other hand, the confrontation tests against the microorganisms that were isolated from batch C (Table 3) showed that the strain *Trichoderma* sp. ECS-622 was the most sensitive at the time of the tests, while *Trichoderma atroviride* ECS-616 and *Trichoderma* sp. ECS-613 presented greater resistances since 61 % of the microorganisms evaluated did not present any inhibitory effect on them.

3.2.3 Confrontation against strains of commercial interest

The confrontation experiments of the isolated microorganisms against the *Trichoderma* sp. strains enabled the selection of those that presented good results in terms of inhibition of the *Trichoderma* sp. strains (e.g., distances greater than 2.5 cm). Significant differences were observed among the treatments when confronting these microorganisms against the *P. ostreatus* ECS-1123, *P. djamor* ECS-123, *P. pulmonarius* ECS-196, *A. fuscosuccinea* ECS-210 and *L. edodes* ECS-401 strains. The *P. ostreatus, P. djamor* and *P. pulmonarius* strains exhibited good growth diameters in most of the confrontations (e.g., between 50 and 90 % of the total diameter of the Petri dish (4.9 cm)), and only 1 % exhibited growth similar to the control. The strains of *L. edodes* and *A. fuscosuccinea* presented regular growth diameters in most of the confrontations (between 50 and 80 %). Table 5 shows that although these microorganisms did not permit greater growth than the control, some of them did promote normal growth of the strains of interest, while others retarded it to the point of inhibiting their growth.

Among the microorganisms from batch A, strain 1LCR allowed good growth of the edible fungal strains that were evaluated, with growth of *P. pulmonarius* ECS-196 and *P. ostreatus* ECS-1123 that was equal to the control and good growth for *P. djamor* ECS-123, *A. fuscosucccinea* ECS-210 and *L. edodes* ECS-401. In the case of strain 1LD8, normal growth was observed for strain *P. pulmonarius* ECS-196, and good growth was observed for *L. edodes* ECS-401 and *P. ostreatus* ECS-1123, which was greater than 4 cm in diameter. On the other hand, strains 1LDI and 1SDI exerted a lower inhibitory effect

on the growth of the strains evaluated in the confrontation, since diameters of less than 2.8 cm were found. In the presence of strain 1SDI, *P. pulmonarius* ECS-196 did not grow at all.

The edible fungal strains of interest that were evaluated in the confrontation tests against the microorganisms from batch B showed growth diameters of less than three centimeters for most of the strains evaluated. Only strain 2LASi allowed growth greater than 3 cm for *P. djamor* ECS-123 and *P. ostreatus* ECS-1123; in contrast, 2LACo did not allow growth of the *P. djamor* ECS-123 strain.

On the other hand, it was observed that the microorganisms that were evaluated from batch C allowed growth distances that were greater than two centimeters. Although none presented growth equal to or greater than the control, it is highlighted that strain 3LAI allowed growth that was very similar to that of the control for *P. pulmonarius* ECS-196. Strain 3LFSi showed smaller growth distances for each confrontation than the other strains evaluated.

3.2.4 Triple confrontation

Based on the results obtained in the previously mentioned confrontations, five microorganisms were chosen from the three batches and were used to carry out a third group of *in vitro* confrontation tests (i.e., simultaneous triple confrontations of *P. ostreatus* against *Trichoderma* sp. ECS-622 and each of the five microorganisms selected for the study).

It is necessary to emphasize that in these tests, *Trichoderma* sp. ECS-622 was able to grow in the presence of bacteria 1LCR and 1LD8, although these microorganisms inhibited growth during the first days of confrontation (2 y 3 days respectively). Treatments 2LDCo, 3LCI and 3LCSi did not allow *Trichoderma* sp. ECS-622 growth at twenty days of evaluation. In contrast, *P. ostreatus* ECS-1123 was able to grow in the presence of the three antagonistic bacteria (e.g., 2LDCo, 3LCI and 3LCSi), although the growth amounts were lower than that presented by the same strain (ECS-1123) when cultivated alone (2-8 % lower). The growth amounts of *Trichoderma* sp. ECS-622 were observed from day two for both the control, with only *Trichoderma* sp. ECS-622, and for the dual control *P.*

ostreatus+*Trichoderma* sp. ECS-622. This was indicated by the characteristic green coloration of the *Trichoderma* spp. spores. On the other hand, the control that contained only grass and the controls that contained only the microorganisms under study did not present cross-contamination (Figure 2).

Table 6 presents the linear extension rates of *P. ostreatus* ECS-1123 during the triple confrontations. The growth amounts of *P. ostreatus* were variable in each treatment, and inhibition rates of 2 % for the confrontation with strain 2LDCo, 3 % with strain 3LCSi, 8 % with strain 3LCI, and 11 % and 26 % for strains 1LCR and 1LD8, respectively, were observed.

With respect to the evaluated strains, those presenting inhibitory effects on *Trichoderma* ECS-622 and allowing growth of *P. ostreatus* ECS-1123, according to the genetic analysis, were determined to belong to the genus *Bacillus* by comparing the sequences that were analyzed in the GenBank database (NCBI Blast) (Table 7). Strain 3LCSi was characterized as *B. cereus* with 98.51 % identity at the DNA level (16S) and 3LCI has 97 % identity with *B. subtilis* strains. In addition, although two purifications were conducted, it was not possible to identify strain 2LDCo at the species level. However, it was determined to belong to the genus *Bacillus* by its taxonomic characteristics (e.g., Gram staining and microscopy) and identity at the DNA level (16S rDNA), which corresponded to 100 % in a region of 373 base pairs (bp) for *B. cereus* and *B. thuringiensis.*

4 Discussion

4.1 Microbial population of the mycosphere

In the present work, the microbial populations that were associated with three batches under cultivation of *Pleurotus* spp. were analyzed to determine their responses to the presence of *Trichoderma* spp. (e.g., a fungus causing green mold disease). A total of 189 microorganisms were isolated from the pasteurization process and from the mycospheres during the cultivation of three *Pleurotus* species. The total populations of culturable microorganisms ranged from $2x10^4$ to $5x10^7$ CFU g^{-1} for the different sampling days. These results agree with studies that have been performed on similar substrates with similar pasteurization and culture conditions, for which the microbial populations have

been found to range from $8.51x10^5$ to $1.34x10^6$ CFU g^{-1} (Torres et al., 2016) and from $2.6x10^4$ to $6.5x10^8$ CFU g^{-1} (Díaz-Martínez et al., 2019). Additionally, these populations were associated with the selectivity of the culture media in which they were isolated, as they were selective for the growth of certain groups of microorganisms: Luria Bertani for bacterial growth, malt extract and Sabouraud for fungal and yeasts cultures. The absence of fungi and yeasts in the isolates could be related to the incubation times (in the petri plate) since they vary according to the nature of the sample and the species. In this study, the incubation time evaluated was 48 hours, while most fungi and yeasts last more than 48 hours to grow (Bosshard, 2011). In addition, it has been described that a succession in microbial communities is present during the pasteurization process and fungal cultivation that influences the bioconversion of cellulose, which provide a nutrient-rich substrate that benefits mushrooms in their growth (Velázquez-Cedeño et al., 2006, 2008; Zhang et al., 2014; Milijašević-Marčić et al*.,* 2016). This microbial succession is related to nutrient availability, pH, substrate moisture and mainly to the variations in temperature profiles throughout the pasteurization process (Castañeda and Leal, 2007; Camacho et al*.,* 2014; Sánchez et al*.,* 2017). Stölzer and Grabbe (1991) and Mata et al. (2017) indicated that a pasteurization treatment at 65 °C eliminated most of the contaminating fungi that could be present in the substrate, which provided ideal conditions for the development of thermophilic bacteria with the inhibitory capacity against contaminating fungi without damaging the mycelium of *Pleurotus* spp. strains.

The variations in the number of microorganisms that were isolated during the culture cycle of the strains (from spawning) in the different batches were attributed to the symbiotic relationship that occurs in the mycosphere among microorganisms and *Pleurotus* strains. By using three strains from different *Pleurotus* species (i.e., *P. ostreatus, P. djamor* and *P. pulmonarius*), the type of microorganism that could be isolated would be different since the fungal hyphae can excrete enzymes and metabolites, in addition to volatile compounds, that are specific of each strain. They provide ideal conditions for the different bacteria present in the mycosphere to benefit in their development or be inhibited (Beltrán-García et al*.,* 1997; Mata et al., 2017; Sánchez and Royse, 2017; Barba et al., 2019;).

4.2 Confrontation- antagonism.

Among the isolated microorganisms, 31 % exhibited inhibitory effects when confronted with strains of *Trichoderma* spp. and approximately 5 % showed inhibition distances that were greater than two centimeters for the three strains. This relatively high incidence of microorganisms with inhibitory effects on *Trichoderma* spp. could explain in part why few cases of green mold contamination have been reported when pasteurizing substrates by self-heating (Avendaño-Hernandez and Sanchez, 2013; Colmenares-Cruz et al*.,* 2017; Morales and Sanchez, 2017). In addition, previous research has demonstrated that microorganisms that were extracted from the mycospheres of edible mushrooms exhibited zones of inhibition against *T. harzianum, T. koningii, T. viridescens* and *T. aggressivum* species, which are similar to the strains reported in this study (Kim et al., 2008; Milijašević-Marčić et al*.,* 2016). In the confrontation tests against the mushroom strains of commercial interest, it was observed that some of these microorganisms presented a complete inhibitory effect on some of the strains studied (1 % of the total microorganisms evaluated). These growth amounts of edible fungal strains in the presence of the evaluated microorganisms could be related to their antimicrobial properties. Previous research on protein extracts of fungi, such as *Auricularia* sp. and *A. bisporus,* has shown inhibitory effects against some pathogens such as *Pseudomonas* sp. and *Bacillus* sp. (Oli et al., 2020).

In the triple confrontation tests in tubes with sterile substrates (e.g., microorganism vs *Trichoderma* ECS-622 vs *P. ostreatus* ECS-1123), the total inhibition of *Trichoderma* sp. ECS-622 and growth of *P. ostreatus* ECS-1123 were observed, although with a small percentage of inhibition (2-8 %) with respect to the control, *P. ostreatus.* These microorganisms with antagonistic effects on *Trichoderma* species were identified in the genetic analysis within the genus *Bacillus*. This situation suggests the possibility of using these bacteria in biological control strategies by inhibiting *Trichoderma* sp. and exhibiting a minimal effect on mushrooms that are cultivated. There have been studies showing that the use of antagonistic bacteria of the genus *Bacillus* can inhibit the growth of mycopathogenic fungi, such as *Trichoderma* sp. and *Rhizoctonia solani,* in the cultivation of edible mushrooms, such as *Agaricus bisporus*, *P. ostreatus, Flammulina velutipes* and

Lentinus edodes (Savoie et al*.,* 2001; Kim et al*.,* 2008; Velázquez-Cedeño et al*.,* 2008; Nagy et al., 2012; Kosanović et al*.,* 2013; Milijašević-Marčić et al., 2016; Potočnik et al*.,* 2019).

The inhibitory potential against contaminating fungi is related to the different mechanisms of action that these bacteria can perform. It has been reported that the synergism of multiple mechanisms of action will provide greater capability of suppressing the growth of green mold (Chittihunsa et al*.,* 2007; Pandin et al*.,* 2018) and the production and secretion of compounds with antifungal activity (Sanchez and Royse, 2017; Gea et al*.,* 2021). Bacteria of the genus *Bacillus* may employ more than one biological control mechanism (Saha et al*.,* 2012; Stanojević et al*.,* 2019 Villa-Rodríguez et al*.,* 2019; Zhou et al*.,* 2021), like the production of hydrolytic enzymes such as protease involved in fungal cell wall lysis in synergy with chitinase and β-1,3-glucanase (Gupta and Vakhlu, 2015) and surfactin production and biofilm formation (Crane y Bergstrom, 2014), phytohormones and volatile compounds such as aldehydes, pyrazines, hydrocarbons, and ketones (Beltran-García et al*.,* 1997; Khabbaz et al., 2015). *Bacillus* spp can also secrete a series of antimicrobial lipopeptide substances, which can cause formation of pores in the cell membrane as a mechanism of action (Liu et al., 2007). Antibiosis is the mode of antagonism that is generally observed with *Bacillus* spp., most produce different antibiotics (e.g., bacillomycin, fengicin, mycosubtilin and zwittermicin) that are effective in suppressing the growth of pathogens (Kim et al*.,* 2008).

4.3 Identification of the strains of interest.

Strain 3LCI, which is effective against the inhibition of *Trichoderma* ECS-622, has an identity percentage of 97.1 % with *Bacillus subtilis*. This microorganism has been reported to be efficient in the antagonism of *Trichoderma* spp., which makes it a good candidate as a biocontrol agent for green mold disease since it produces antibiotics with selective antagonistic capacities. Furthermore, it induces the production of laccases in *Pleurotus* sp. as a biological response to *Trichoderma* spp. antagonism (Velázquez-Cedeño et al., 2008; Nagy et al., 2012; Kosanovic et al., 2013). Strain 3LCSi, has an identity percentage of 99 % with *Bacillus cereus,* which has been reported as an effective biocontrol agent against plant pathogenic fungi of the genera *Phytophthora* and *Fusarium* (Wen-Teish et

al., 2007). The inhibitory action of *B. cereus* is related to the production of antifungal hydrolytic enzymes, which provides a competitive advantage to *P. ostreatus* over *Trichoderma* sp.

The 16s rDNA amplified fragment from the strain 2LDCo has 100 % identity with two species of *Bacillus*, so it was not possible to identify it precisely. DNA sequencing analysis shows that it could be *B. cereus* or *B. thuringiensis.* Both bacterial species have been identified as potential antagonists of *Trichoderma* sp. (Wen-Teish et al., 2007; Kim et al., 2008). Therefore, biotechnological applications to mushroom crops for human consumption would be beneficial.

5 Conclusion

It was found that 31 % of the microorganisms that were isolated from the mycospheres of three strains of *Pleurotus* sp. exhibited antagonistic effects against the strains of *Trichoderma* spp. Strains 2LDCo, 3LCI and 3LCSI belonging to the genus *Bacillus* inhibited 100 % of the growth of *Trichoderma* ECS-622 and permitted the growth of *Pleurotus ostreatus* ECS-1123 with a relatively small percentage of inhibition (2 to 8 %). Therefore, it is important to continue with deeper studies to know the mechanism of action on the practical uses of these isolates to establish successful biocontrol strategies for *Trichoderma* spp.

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7 Conflict of interest.

The authors declare that there is no personal or financial conflict of interest in the completion of this paper.

8 Author contributions

ACC designed the experiment, performed the experiment, writing – original draft, review & editing. EDG and KGN provided supervision, writing - review & editing. JES conceptualized and designed the experiment, and provided supervision, financing, writing - review & editing.

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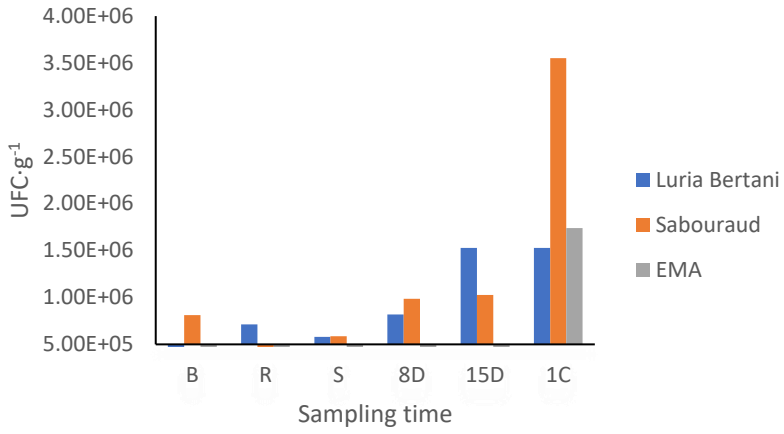
Captions to Figures and Tables

Figure 1. Population densities (CFU g^{-1}) of total microorganisms during and after substrate pasteurization of batch A) used to cultivate *P. ostreatus* ECS-1123, batch B): used to cultivate *P. djamor* ECS-123 and batch C): used to grow *P. pulmonarius* ECS-196. B: beginning of pasteurization, R: turning of substrate, S: end of pasteurization (time of spawning *Pleurotus* spp.), 8D: at 8 days of mycelial growth,15D: at 15 days of mycelial growth and 1C: first flush.

Figure 2. Simultaneous triple confrontations of *Trichoderma* sp ECS-0622 and *P. ostreatus* ECS-1123 against each of the five selected microorganisms at twenty days of incubation. 1LCR and 1LD8 correspond to batch A, 2LDCo corresponds to batch B and 3LCI and 3LCSi corresponds to the batch C culture. Incubation temperature 22 °C, substrate: Pangola grass

Figure 1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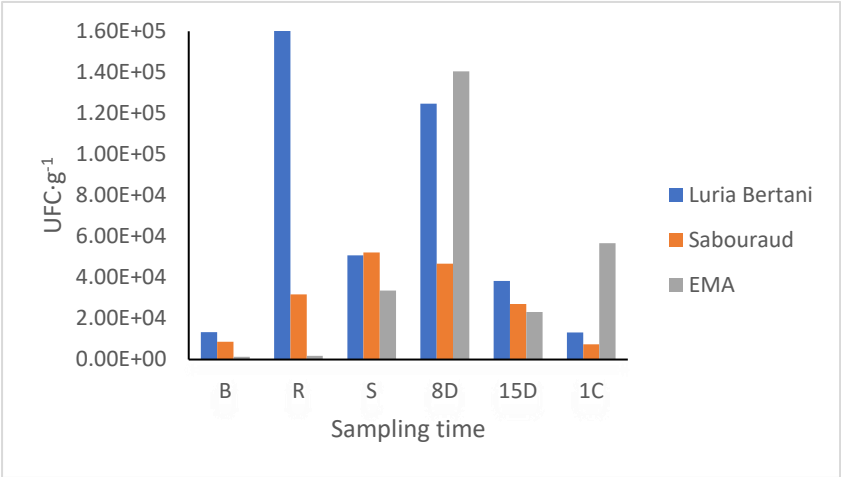
UFC·g-1
B
R
S
8D
15D
1C
Sampling time
Luria Bertani
Sabouraud
EMA

A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B
R
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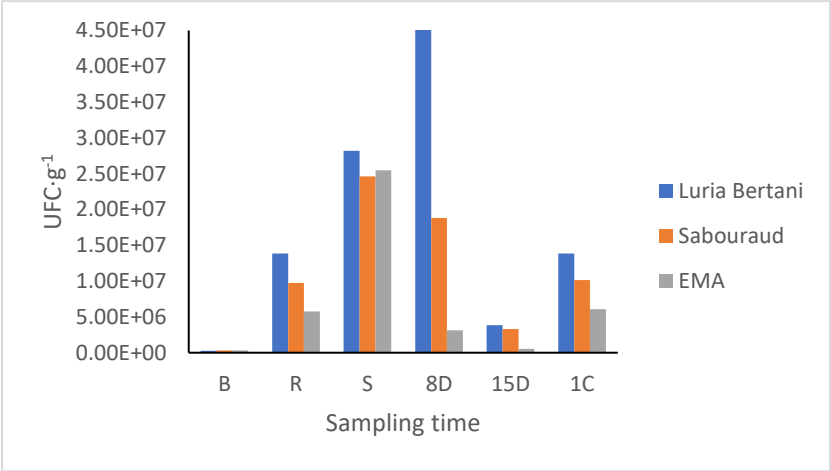
8D
15D
1C
Sampling time
Luria Bertani
Sabouraud
EMA

B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0.00E+00
UFC·g-1
B
R
S
8D
15D
1C
Sampling time
Luria Bertani
Sabouraud
EMA

C

Figure 2

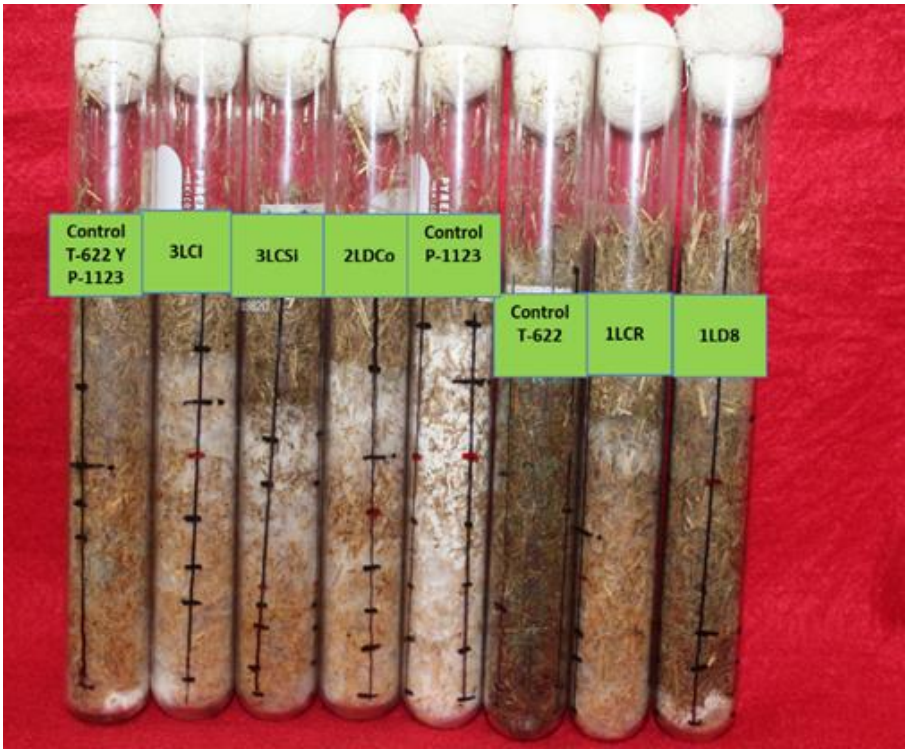
Control
T-622 Y
P-1123
3LCI
3LCSI
2LDCo
Control
P-1123
Control
T-622
1LCR
1LD8

Table 1. Counts of isolated microorganisms and their inhibition capacities of *Trichoderma* spp.

	Batch A	**Batch B**	**Batch C**	**Average**
Total Microorganisms isolated	64 (100 %)	57 (100 %)	68 (100 %)	63 (100 %)
Number of microorganisms with certain inhibition to *Trichoderma* spp.	22 (34.37 %)	16 (28 %)	25 (36.76 %)	19.6 (31.1 %)
Number of microorganisms with an inhibition distance >2 cm in the three *Trichoderma* spp. strains.	4 (7.81 %)	3 (8.77 %)	3 (4.41 %)	3.3 (4.9 %)

Table 2. Inhibition distances (cm) among three strains of *Trichoderma* spp. And different microorganisms that were isolated from batch A and were used to cultivate *Pleurotus ostreatus* ECS-1123. Observations made at 7 days of confrontation.

Microorganism	***Trichoderma* sp ECS-613**	***Trichoderma atroviride* ECS-616**	***Trichoderma* sp ECS-622**
Control	0 g	0 e	0 g
1LAI	3.23±0.23 a	2.8±0.17 ab	2±0 abc
1LCI	3.13±0.11 ab	2.5±0.55 ab	1.83±0.23 abcde
1LCR	3.06±0.28 ab	2.46±0.23 ab	1.56±0.40 bcdef
1LBR	3±0.17 ab	0 e	1.16±0.15 ef
1LASi	2.76±0.23 abc	0 e	2.13±0.35 ab
1LC15	2.73±0.20 abc	2.16±0.28 bc	1.7±0.26 bcdef
1LDI	2.66±0.57 abc	2.56±0.05 ab	2.23±0.20 ab
1LAR	2.56±0.15 abcd	2.83±0.05 ab	1.16±0.28 ef
1LB15	2.5±0 abcd	3.23±0.32 a	1.9±0.17 abcd
1LD8	2.5±0 abcd	2.03±0.28 bc	1.93±0.05 abcd
1LB8	2.46±0.11 abcde	2.4±0.26 ab	2±0 abc
1SDI	2.33±0.11 abcde	2.33±0.46 abc	2±0 abc
1SA15	2.3±0 abcde	2.2±0.17 bc	1.3±0.17 def
1LA15	2.16±0.20 abcde	2.5±0.1 ab	1±0 f
1LDSi	2.13±0.35 bcde	1.83±0.49 bc	1.1±0.17 f
1LBI	1.8±0.2 cde	2.13±0.28 bc	1.36±0.11 cdef
1LC8	1.53±0.15 def	2.13±0.46 bc	1.36±0.32 cdef
1EAI	1.4±0 ef	1.36±0.25 cd	1.03±0.28 f
1EBR	0 g	1.13±0.05 de	1±0 def
1LBSi	0 g	0 e	2.06±0.11 abc
1SCR	0 g	2.56±0.15 ab	2.5±0 a
		p	
	< 2.2e-16	**< 2.2e-16**	**1.42E-15**

* Equal letters in the same column indicate no significant difference between treatment and control (p=0.05). Petri dish diameter 4.9 cm. The mean corresponds to three replicates.

Table 3 Inhibition distances (cm) among three strains of *Trichoderma* spp. And different microorganisms that were isolated from batch B and were used to cultivate *Pleurotus djamor* ECS-123. Observations made at 7 days of confrontation.

Microorganism	*Trichoderma* sp ECS-613	*Trichoderma atroviride* ECS-616	*Trichoderma* sp ECS-622
Control	0 g	0 f	0 i
2SCI	3.03±0.15 a	2.30±0 abcd	1.46±0.11 f
2LASi	2.70±0ab	2.30±0 abcd	2.56±0.05 b
2LGCo	2.70±0.2 ab	2.43±0.11 abcd	1.70±0 ef
2ECI	2.66±0.11 ab	2.66±0.05 ab	2.46±0.11 b
2LCI	2.60±0.34 abc	2.46±0.35 abcd	0.46±0.05 h
2LD15	2.56±0.05 abc	2.06±0.15 de	1.86±0.05 de
2SD15	2.53±0.23 abc	2.76±0.28 a	1.06±0.11 g
2SCCo	2.50±0.17 bc	2.13±0.05 bcde	1.66±0.05 ef
2LE15	2.40±0.10 bcd	2.10±0.0 cde	1.86±0.20 de
2LECo	2.40±0.10 bcd	2.63±0.35 abc	2.56±0.11 b
2LCCo	2.30±0.0 bcd	0 f	2.10±0.1 cd
2LCR	2.30±0.0 bcd	0 f	2.33± 0.11 bc
2LACo	2.13±0.05 cde	1.73±0.05 e	2.56±0.11 b
2LDCo	1.93±0.28 de	1.66±0.20 e	3.20±0.1 a
2LBCo	1.63±0.05 e	2.50±0.43 abcd	0.60±0.10 h
2LC15	1±0 f	0 f	1±0 g
		p	
	< 2.2e-16	**< 2.2e-16**	**< 2.2e-16**

*** Equal letters in the same column indicate no significant difference between treatment and control (p=0.05). Petri dish diameter 4.9 cm. The mean corresponds to three replicates.**

Table 4 Inhibition distances (cm) among three strains of *Trichoderma* spp. And different microorganisms that were isolated from batch C and were used to cultivate *Pleurotus pulmonarius* ECS-196. Observations made at 7 days of confrontation.

Microorganism	***Trichoderma* sp ECS-613**	***Trichoderma atroviride* ECS-616**	***Trichoderma* sp ECS-622**
Control	0 g	0 d	0 i
3LE8	2.93±0.11 a	2.86±0.11 a	2.56±0.11 abc
3LCSi	2.46±0.15 b	2.33±0.05 ab	2.1±0.5 abcde
3LAI	2.2±0 bc	2.1±0.17 ab	1.83±0.15 defg
3LCI	2.03±0.11 cd	2.33±0.28 ab	2.06±0.11 bcdef
3LBR	1.96±0.23 cde	0 d	2.7±0.1 ab
3LFSi	1.66±0.28 def	1.83±0.11 b	1.83±0.15 efg
3LDR	1.63±0.05 ef	2.16±0.15 ab	1.8±0 efg
3LESi	1.43±0.37 f	0 d	2.76±0.25 a
3EC15	1.33±0.28 f	1.6±0.17 bc	1.96±0.15 cdef
3EA8	0 g	0 d	1.93±0.11 cdef
3ECSi	0 g	0 d	1.76±0.25 efg
3ED15	0 g	0 d	1.53±0.05 efgh
3LAR	0 g	2.03±0.45 ab	2.5±0.43 abcd
3LB8	0 g	0 d	2.13±0.11 abcde
3LBCo	0 g	0 d	1.96±0.15 cdef
3LCR	0 g	0.85±0. 93 cd	1.83±0.25 efg
3LD8	0 g	0 d	1±0 h
3LDCo	0 g	0 d	1.4±0.17 fgh
3LECo	0 g	0 d	1.83±0.57 efg
3SB15	0 g	0 d	1.9±0 cdef
3SC15	0 g	0 d	1.5±0 efgh
3SC8	0 g	0 d	2.43±0.11 abcd
3SCR	0 g	0 d	1.2±0 gh
3SF8	0 g	0 d	1.5±0 efgh
		p	
	< 2.2e-16	**< 2.2e-16**	**< 2.2e-16**

* Equal letters in the same column indicate no significant difference between treatment and control (p=0.05). Petri dish diameter 4.9 cm. The mean corresponds to three replicates.

Table 5 Growth diameters (cm) of five strains of edible fungi at 7 days of plate confrontation with different microorganisms isolated from *Pleurotus* spp. culture batches.

Lot	m.o.	*Pleurotus djamor* ECS-123	*Pleurotus pulmonarius* ECS-196	*Auricularia fuscosuccinea* ECS-210	*Lentinula edodes* ECS-401	*Pleurotus ostreatus* ECS-1123
A: cultivo de *P. ostreatus* ECS-1123	control	4.9±0 a	4.9±0 a	4.9±0 a	4.9±0 a	4.9±0 a
	1LCR	3.56±0.05 b	4.9±0 a	3.80±0.10 bc	3.63±0.2 c	4.9±0 a
	1LB15	3.46±0.20 bc	3.56±0.20 bc	2.13±0.11 f	2.13±0.15 d	3.26±0.11 d
	1LAI	3.20±0.20 bcd	3.23±0.11 c	2.16±0.05 f	2.13±0.05 d	2.66±0.11 e
	1LD8	3.13±0.11 cd	4.9±0 a	3.93±0.15 b	4.36±0.11 b	4.76±0.05 a
	1LA15	3.10±0.20 cd	2.86±0.11 d	2.10±0.34 f	2.16±0.15 d	3.93±0.15 bc
	1LCI	3.10±0.10 cd	0 e	3.10±0 de	3.30±0 c	4.26±0.15 b
	1LAR	3.00±0.17 d	3.33±0.11 c	2.33±0.58 f	3.60±0.30 c	3.80±0.26 c
	1LB8	3.00±0.10 d	3.43±0.11 c	3.23±0.20 cd	4.20±0.10 b	3.83±0.15 bc
	1LC15	2.96±0.05 d	3.80±0.26 b	2.43±0.15 ef	3.56±0.15 c	3.93±0.15 bc
	1LDI	2.46±0.05 e	2.86±0.05 d	1.86±0.23 f	2.26±0.11 d	2.56±0.25 e
	1SDI	2.13±0.05 e	0 e	1.90±0.10 f	2.23±0.11 d	2.60±0.20 e
				p		
		3.49E-16	**< 2.2e-16**	**4.63E-14**	**< 2.2e-16**	**< 2.2e-16**
B: cultivo de *P. djamor* ECS-123	2LASi	3.26±0. 05 b	2.53±0.11 bc	2.10±0.30 cde	2.53±0.05 b	3.63±0.11 b
	2LGCo	2.86±0.11 c	2.93±0.15 b	2.50±0.20 bc	2.06±0.05 def	2.40±0.20 cd
	2LDCo	2.66±0.11 cd	2.63±0.23 bc	2.63±0.05 b	2.16±0.11 cde	2.53±0.05 cd
	2LD15	2.50±0 de	2.90±0.10 b	2.43±0.11 bcd	2.23±0.11 cd	2.36±0. 05 cd
	2SCI	2.36±0.05 ef	2.86±0.05 b	1.93±0.15 de	2.03±0.11 def	2.30±0 d
	2SCCo	2.26±0.05 efg	2.00±0.10 d	2.00±0.17 cde	1.93±0.15 ef	2.26±0.28 d
	2LECo	2.20±0.10 fgh	2.76±0.11 bc	2.46±0.20 bc	2.40±0.10 bc	2.33±0.05 cd
	2ECI	2.10±0.10 gh	2.76±0.23 bc	2.16±0.11 bcde	2.10±0 def	2.73±0.11 c
	2SE15	2.00±0.17 hi	2.96±0.15 b	1.80±0.17 e	1.90±0 f	2.23±0.20 d
	2SD15	1.80±0.1 i	2.83±0.25 b	1.93±0.20 de	1.93±0. 05 ef	2.33±0.05 cd
	2LACo	0 j	2.33±0.05 cd	2.30±0.17 bcde	2.20±0 cd	2.53±0.15 cd
				p		
		< 2.2e-16	**4.02E-15**	**2.68E-15**	**< 2.2e-16**	**< 2.2e-16**
C: cultivo de *P. pulmonarius* ECS-196	3LAI	3.53±0.20 b	4.83±0.11 a	3.53±0.11 b	3.43±0.20 b	3.63±0.25 b
	3LCI	3.13±0.11 bc	3.96±0.20 c	2.43±0.15 d	3.36±0.11 b	3.33±0.05 bc
	3LCSi	2.96±0.05 c	4.43±0.11 b	2.86±0.05 c	3.46±0.05 b	3.00±0.10 c
	3LE8	2.86±0.05 cd	3.76±0.05 c	2.33±0.23 d	2.70±0.17 c	3.33±0.23 bc
	3LFSi	2.46±0.32 d	3.73±0.23 c	2.23±0.11 d	2.53±0.25 c	3.20±0.36 bc
				p		
		9.61E-09	**5.11E-07**	**8.21E-11**	**7.41E-09**	**1.42E-06**

* Equal letters in the same column indicate no significant difference between treatment and control (p=0.05). Petri dish diameter 4.9 cm. The mean corresponds to three replicates.

Table 6. Linear extension rates (cm/day) of *Pleurotus ostreatus* ECS-1123 in confrontations with *Trichoderma* sp ECS-622 and simultaneously with each of the microorganisms isolated from the mycosphere and selected from the preceding tests (incubation for 20 days at 22 °C, substrate: Pangola grass at 65 % humidity).

Treatment	Linear extension rate (cm/day)	Percentage growth (%) of *P. ostreatus* ECS-1123	Growth of *Trichoderma* sp ECS-622
CONTROL_*Trichoderma* sp ECS-622 alone	0.85± 0.082 a	----------	Yes
CONTROL_*P ostreatus* ECS-1123 alone	0.63± 0.026b	100	No
P. ostreatus in presence of:			
1LCR	0.47± 0.017 c	74	Yes
1LD8	0.56± 0.011 bc	89	Yes
2LDCo	0.62± 0.05 b	98	No
3LCI	0.58± 0.015 b	92	No
3LCSi	0.61± 0.012b	97	No

*** Equal letters indicate that there are no significant differences between treatment and control (p=0.05). The mean value corresponds to three replicates.**

Table 7. Genetic affiliations of the studied strains according to comparative analyses based on 16S rDNA sequences. Access BLAST May 2021

Sample	Taxonomic characteristics observed	BLAST Species Identity	Region in the 16S gene (% Identity)	No. of access of the closest relative
3LCSi	Gram-positive, bacilli-shaped microorganisms. They form circular colonies of creamy consistency and convex elevation.	*Bacillus cereus*	2713693- 2715156 (1452 pb, Identity 99 %)	CP053954.1
3LCI	Gram-positive, bacillus-shaped microorganisms. They form irregular colonies with membrane-like terminations of creamy consistency and flat elevation.	*Bacillus subtilis*	Region 3- 1445 (1404 pb, Identity 97 %)	MN493777.1
2LDCo	Gram-positive bacilli-shaped microorganisms. It forms irregular circular colonies of crusty consistency, without elevation.	*Bacillus* sp	*B. cereus; region 1117-1447 (*373 pb, Identity 100 %)	MT642947.1
			*B. thuringensis; region 1080-1440 (*373 pb, Identity 100 %)	MT598028.1

Capítulo 3. Conclusiones

La importancia de conocer la interacción entre un macromiceto que se cultiva y los microorganismos presentes en su micósfera deriva eventualmente en el aprovechamiento de potenciales antagonistas de hongos contaminantes que le compiten por espacio y nutrientes y que terminan afectando su desarrollo y producción Así como de otros microorganismos que estimulan el crecimiento del hongo de interés. En este estudio se encontraron microorganismos con efecto inhibitorio del crecimiento de cepas de *Trichoderma* spp., contaminantes causantes de la enfermedad del moho verde en el cultivo de setas comestibles del género *Pleurotus.*

La caracterización morfológica y molecular de los microorganismos aislados, permitió su identificación como especies pertenecientes al género *Bacillus.* Las cuales previamente han sido reportadas por el efecto inhibitorio hacia cepas de *Trichoderma* spp.*,* por lo que se confirma su posible potencial como organismos útiles en estrategias de control biológico. En este trabajo se demostró que las cepas pertenecientes al género *Bacillus* pueden inhibir 100% del crecimiento de *Trichoderma* sp. en pruebas de confrontación *in vitro*. Esto se lleva a cabo sin perjudicar el crecimiento del *Pleurotus* sp. Por lo tanto, los aislados de *Bacillus* spp. pueden ser considerados en aplicaciones como antagonistas eficaces contra los patógenos de la enfermedad del moho verde causada por *Trichoderma* spp.

La identificación mediante rRNA 16S indicó que los tres aislados corresponden a *B. subtilis, B. cereus* y *Bacillus sp.* Dado que se ha reportado a *B. cereus* como agente patógeno oportunista para humanos, se hace necesario confirmar la identificación con herramientas de diagnóstico más precisas y avanzadas, que confirmen la identidad de las cepas aisladas (Bottone 2010; Messelhäußer y Ehling-Schulz 2018). Esto particularmente porque, estudios previos indican que el análisis mediante rRNA 16S no refleja la diversidad de grupos bacterianos estrechamente relacionados*.* Recientemente, la tipificación de secuencias de múltiples locus (MLST), ha utilizada con éxito para estudiar la diversidad filogenética del género *Bacillus* debido a su enfoque estandarizado, altamente inequívoco y reproducible, ya que caracteriza cepas bacterianas utilizando fragmentos internos de múltiples genes domésticos (Le et al. 2019).

La confirmación de la identidad de las especies aisladas, así como la elucidación del (los) mecanismo(s) de acción que presentan las cepas seleccionadas, permitirán un mejor entendimiento y aprovechamiento de las estrategias de biocontrol. Así también, para explorar el posible uso práctico de los aislados bacterianos antagonistas encontrados y su posible inocuidad para el hongo comestible y los seres humanos.

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