

Inhibitory activity of alcoholic extracts of edible fungi against *Rhizoctonia solani*

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Abstract

Fungal diseases represent one of the causes of annual crop losses. *Rhizoctonia solani* is a pathogenic fungus with worldwide distribution associated with root and tuber diseases of different crops; it causes important economic losses in perennial and annual plants, including almost all horticultural crops. To mitigate losses due to fungus, fungicides of synthetic origin have been used; however, many of these substances are associated with carcinogenicity and are toxic to the environment. Edible macromycete mushrooms are a source of antifungal compounds to control diseases in agricultural crops. This work evaluated the antifungal activity of extracts of edible mushrooms (*Lactarius deliciosus*, *Ustilago maydis*, *Amanita jacksonii* and *Amanita rubescens*) against the phytopathogen *R. solani* by means of three techniques: diffusion of wells, discs and plate dilution. The results show that with the diffusion of wells, no effect was observed on the growth of *R. solani* with the alcoholic extracts and their dilutions. While with disc diffusion, a qualitatively slower growth was observed compared to the controls of *R. solani* with the ethanolic extract of *A. jacksonii* and in its 1:1 dilution of methanol-water. In the plate dilution, it was found that the 1:1 ethanol-water dilution of *L. deliciosus* had 88% inhibition on the growth of *R. solani*, followed by the ethanol extract of *L. deliciosus* (65%), methanol extract from *U. maydis* (63%) and methanol extract from *L. deliciosus* (57%). Therefore, the extracts and the dilution must be studied in greater depth, by being tested in an *in vivo* system and identifying the secondary metabolites present.

Keywords: *Lactarius*, *Ustilago*, phytopathogen.

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Introduction

Fungal diseases represent one of the main causes of crop losses annually, causing 60% of them during production and postharvest (Sommer *et al.*, 2002). *Rhizoctonia solani* is a worldwide pathogenic causative agent associated with root and tuber diseases of different crops that causes important economic losses. The families and crops where this phytopathogen have been found are mentioned below. Poaceae or Gramineae: rice, wheat, barley, oats; Fabaceae: soybeans, peanuts, dried beans, alfalfa, chickpeas, lentils, peas; Solanaceae: tobacco and potato; Amaranthaceae: sugar beet; Brassicaceae: rapeseeds; Rubiaceae: coffee; Malvaceae: cotton and Asteraceae: lettuce (Ajayi and Bradley, 2018).

This necrotrophic pathogen (requires first killing the cells of its host and then taking the nutrients) is one of the most destructive found in the soil (Khaledi *et al.*, 2015), among the diseases they commonly cause is the so-called damping-off of seedlings and root rot (Arcos and Zúñiga, 2015). The symptoms caused by the pathological complex of *R. solani* are observed in the underground and aerial parts of plants in two phases: 1) stem canker, which is the infection of growing plants; and 2) black crust or tuber infection with the formation of sclerotia. The 'black crust' is the most obvious sign of disease caused by *R. solani* and is characterized by black sclerotia of irregular sizes in the tuber, which, in a state of severity, can deform and crack it (Hide *et al.*, 1973; Frank and Leach, 1980; Tsrör, 2010).

To counteract the effects of this phytopathogen, fungicides belonging to different chemical groups with different active ingredients have been used. Four groups have been mainly reported: 1) quinone outside inhibitors (Qol) and active ingredients such as azoxystrobin, pyraclostrobin and trifloxystrobin; 2) succinate dehydrogenase inhibitors (SDHI) and active ingredients such as carboxin, penflufen and sedaxane; 3) demethylation inhibitors (DMI) and active ingredients such as penconazole and prothioconazole; and 4) inhibitors of phenylpyrroles (PP) with the active ingredient fludioxonil (Ajayi and Bradley, 2018).

Nevertheless, the Fungicide Resistance Action Committee (FRAC) stated that the risk of developing resistance to fungicides is high since they act at a single site within a metabolic pathway of the phytopathogen, so the dependence on these chemical fungicides for its control increases the risk that these organisms are resistant to these substances (FRAC, 2017). Although the use of broad-spectrum fungicides helps in the control of diseases generated by *R. solani*, these substances are highly toxic and have caused serious environmental and health problems (associated with carcinogenicity) (Patiño *et al.*, 2020).

The strategies for the control of this phytopathogen are limited because of the high survival rates due to the formation of sclerotia, other natural and less toxic alternatives have been sought. Such is the case of plant extracts with antifungal activity, an example is the work of Al-Askar and Rashad (2010), who test hydroalcoholic extracts of four medicinal plants: cinnamon (*Cinnamomum verum* Presl.), anise (*Pimpinella anisum* L.), black cumin (*Nigella sativa* L.) and cloves (*Syzygium aromaticum* L. Merr. and Perry) against *R. Solani* in peas (*Pisum sativum* L.).

They found that clove extract at 1% completely inhibited radial fungal growth; while for the other hydroalcoholic extracts (4%) of plants, an inhibition of 90.5 to 100% was observed. The authors mention that the antifungal effect of the hydroalcoholic clove extract can be attributed to eugenol, the main active compound of the essential oil of this plant, which can form bonds in the active sites of the enzymes by deactivating them. In other research, Rafiq *et al.* (2021) evaluated the antifungal potential of methanolic extracts of leaves of safflower (*Carthamus oxycantha*) against *R. solani*, finding 27 to 37% inhibition in the growth of the phytopathogenic fungus. The observed effects were attributed to 95 compounds found in methanolic extracts, among which a derivative of D-ribofuranose, phthalate and, to a lesser extent, some fatty acids stand out.

While in the case of macromycete fungi, it has been reported (Patiño *et al.*, 2020) that these organisms possess a potential source of antifungal compounds that could be used to control pathogens in plants and humans. Such is the case of metabolites from ethanolic extracts of *Amanita rubescens*, which showed antibacterial activity and antioxidant activity against *Candida albicans*, *Streptococcus agalactiae* and *Staphylococcus aureus* (Martínez *et al.*, 2021). Another example is glycolipids (biosurfactants) obtained from *Ustilago maydis*, which have shown antimicrobial activity against *Salmonella enterica* and *S. aureus* (Cortes *et al.*, 2011).

In the case of methanolic extracts of *Lactarius* sp., their antitumor and antimicrobial activity against *S. aureus* has been reported, attributed to metabolites such as sesquiterpenes, steroids, nitrogenous compounds and other secondary metabolites (Feussi *et al.*, 2017). Doğan and Akbaş (2013) report the antimicrobial activity of chloroform, acetone and methanol extracts of *Amanita caesarea* against four gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Streptococcus pyogenes*), five gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Salmonella enteritidis*) and a yeast (*Candida albicans*) by the microdilution method, finding a high inhibitory effect for *C. albicans* at a concentration of 4.8 µg mL of acetone extract, following the scale of Craig (1998).

In addition, Doğan and Akbaş (2013) report that the methanolic extract presented the maximum antimicrobial effect against *Staphylococcus pyogenes* and *E. coli* (MIC values, 78 and 156 mg mL⁻¹, according to Craig's scale), attributing this effect to diterpenes, triterpenes and polysaccharides. The most used solvents to perform the extraction of metabolites in fungi are water, ethanol, methanol, acetone and ethyl acetate (Elbatrawy *et al.*, 2015), which was a point to consider when making the extracts; in the same way it was sought that the chosen solvents were easily accessible and handled by producers in the agricultural sector, in order to seek their applicability in the future.

It is worth mentioning that reports on the use of macromycete fungi extracts for the control of phytopathogens are scarce (Patiño *et al.*, 2020; Volcão *et al.*, 2021). Reinoro *et al.* (2013) proved that extracts of macromycete fungi native to Chile, by means of the disc diffusion technique, have active biological activity against phytopathogenic fungi.

The extract of *Mycena hyalinotricha* showed greater antifungal activity in the growth of *Penicillium notatum*, *Ceratocistys pilifera* and *Rhizoctonia solani* as it formed inhibition halos. The authors concluded that edible fungi extracts are good sources of antifungal active compounds and suggest the need to further investigate the potential of these metabolites in the agroforestry

industry as a control of phytopathogens. Another study that explores the effect of active metabolites of edible fungi against phytopathogens is reported by Priya *et al.* (2019), who studied eight species of fungi, of which *Lentinus edodes* or shiitake fungus, *Schizophyllum commune*, *Coprinus comatus* and *Volvariella volvaceae* are edible, against the growth of the phytopathogenic fungus *Colletotrichum capsici*.

They found that *L. edodes* showed the maximum antifungal activity by inhibiting the mycelial growth of *C. capsici* by 45.55% with a maximum inhibition zone of 4.86 mm. The identification and study of bioactive compounds secreted by edible macromycetes are necessary in order to develop broad-spectrum fungicides against plant pathogens. Therefore, the present research aimed to test the antifungal effect of alcoholic extracts (methanol and ethanol) of four edible fungi (*Lactarius deliciosus*, *Ustilago maydis*, *Amanita jacksonii* and *Amanita rubescens*) against the phytopathogen *R. solani* through three methodologies.

Materials and methods

Obtaining edible fungi

The edible fungi were obtained in the local markets of the city of Xalapa, Veracruz in the period of July-August 2021. Subsequently, the specimens were taken to the Laboratory of Microbial Biotechnology of the Institute of Applied Chemistry to be cleaned and cut.

Obtaining hydroalcoholic extracts

Three hundred thirty grams of the fungal material of each respective fungus were weighed, then cut into fragments of approximately 4 cm x 4 cm, then the fragments were placed in an airtight glass bottle of 3 L capacity. Six containers with fungal material were established, 350 ml of methanol were added to three of them and 350 ml of ethanol were added to the other three containers. The containers with fungal material and solvents were left to macerate for five days at room temperature and after this time, they were filtered by gravity with filter paper of medium filtration, 8 µm pore, then the supernatant obtained from each respective fungus and solvent was placed in an Erlenmeyer flask of 500 ml.

The liquid obtained in each treatment was evaporated in a rotary vapor to obtain the concentrated extract, which regularly presents pasty-oily consistency. The three methanolic extracts and the three ethanolic extracts were established as stock solutions and from these, the hydroalcoholic extracts (treatments) were prepared with the following proportions: ethanol-water 1:1, methanol-water 1:1, ethanol-water 3:7 and methanol-water 3:7 (all in triplicate).

Antifungal activity of alcoholic extracts

Disc diffusion

Petri dishes were prepared with potato dextrose agar (PDA) medium, then filter paper discs (medium filtration, 8 µm pore) with 0.5 mm diameter were cut and sterilized in autoclave at 120 lb pressure for 20 min. Subsequently, under aseptic conditions, the filter paper discs (four

discs for each alcoholic extract) were impregnated with 150 µl of each alcoholic extract and left to stand until the disc completely absorbed the extract. Then the Petri dishes with PDA were inoculated with a disc of the fungus *R. solani*, which was placed in the center of the dish with culture medium.

Four discs impregnated with each extract were placed equidistant to the disc with mycelium of *R. solani*. *R. solani* was isolated from the rhizosphere of poblano pepper (*Capsicum annuum* L.) and is part of the strain collection of the Department of Phytopathology of the Colegio de Postgraduados Montecillo Campus, State of Mexico. Treatments included a control of sterile distilled water, a methanol control and an ethanol control. The dishes were incubated at 28 °C for three days, after the incubation time it was observed if there was inhibition on the growth of *R. solani* and the diameter of inhibition around the disc impregnated with the extract was measured in millimeters with a ruler, all treatments were carried out in triplicate.

Well diffusion

Petri dishes were prepared with PDA medium, and three holes were made in the agar with the help of a punch of 0.5 mm in diameter. One hundred microliters of each alcoholic extract (ethanol-water 1:1, methanol-water 1:1, ethanol-water 3:7 and methanol-water 3:7) were added to each well, then a disc of *R. solani* with fresh mycelium was placed in the center of each dish (Cruz *et al.*, 2010). Treatments included a control of sterile distilled water, a methanol control and an ethanol control (all in triplicate). Subsequently, the dishes were incubated at 28 °C for three days, after the incubation time it was observed if there was inhibition on the growth of *R. solani* close to the well, to measure the inhibition halo, it was measured in millimeters.

Plate dilution

Determination of the concentration of a solution of *R. solani* mycelium fragments

One hundred milliliters of potato dextrose broth were prepared, then the culture medium was inoculated with a 7 mm diameter disc of fresh mycelium of *R. solani*. It was incubated for four days at 28 °C, after this time the fungal solution was filtered through a sterile filter system (plastic column packed with cotton), to allow the passage of the smallest fragments of mycelium. The concentration of the mycelium fragments of the filtered liquid was estimated using a Neubauer chamber.

Once the concentration of the solution of fungal mycelium fragments (2×10^7 mycelium fragments ml^{-1} , 1×10^4 CFU) was known, the antifungal activity was determined by the plate microdilution method using the method described by Casaril *et al.* (2011); Ren *et al.* (2014) with modifications. In each well of a 96-well plate, the following were added: 100 µl of potato dextrose broth, 50 µl of the respective alcoholic extract of edible fungi (ethanol-water 1:1, methanol-water 1:1, ethanol-water 3:7 and methanol-water 3:7) and 50 µl of the solution of mycelium fragments of *R. solani*. Treatments included a control of sterile distilled water, a methanol control, an ethanol control, a control of potato dextrose broth, and a control of potato dextrose, all with solution of *R. solani* mycelium fragments (all in triplicate).

Subsequently, the dishes were incubated at room temperature in a horizontal stirrer at 400 rpm for four days, after this time the absorbance was read at 595 nm with a microplate reader. Antifungal activity was measured by comparing the optical densities of wells containing the same extracts and controls. The percentage of fungal growth inhibition was calculated using the following equation: $\% \text{ inhibition} = [A_F - (A_{FE} - A_E)] / A_F \times 100$. Where: A_F is the absorbance of the fungal control without the addition of the extract; A_{FE} is the absorbance of the mixture (alcoholic extract and suspension of mycelium fragments), and A_E is the absorbance of the corresponding extract.

Statistical analysis

For the plate dilution technique, the bioassay was established under a completely randomized experimental design with three repetitions. The data obtained were performed an analysis of variance Anova and the mean comparison test (Tukey, $\alpha = 0.05$) with the statistical program SAS Institute (2020).

Results and discussion

Antifungal activity of hydroalcoholic extracts by well diffusion, disc diffusion and plate dilution techniques

Disc diffusion

In the results obtained with the disc diffusion method, it was observed that the fungus in some treatments such as ethanolic extract of *A. jacksonii* in its 1:1 methanol-water dilution, but also the extract of *A. rubescens* in the 3:7 ethanol-water dilution and 1:1 methanol-water dilution; extract of *U. maydis* 3:7 methanol-water dilution and of *L. Deliciosus* 3:7 ethanol-water dilution; grew slower and less abundantly compared to the controls (Figure 1).

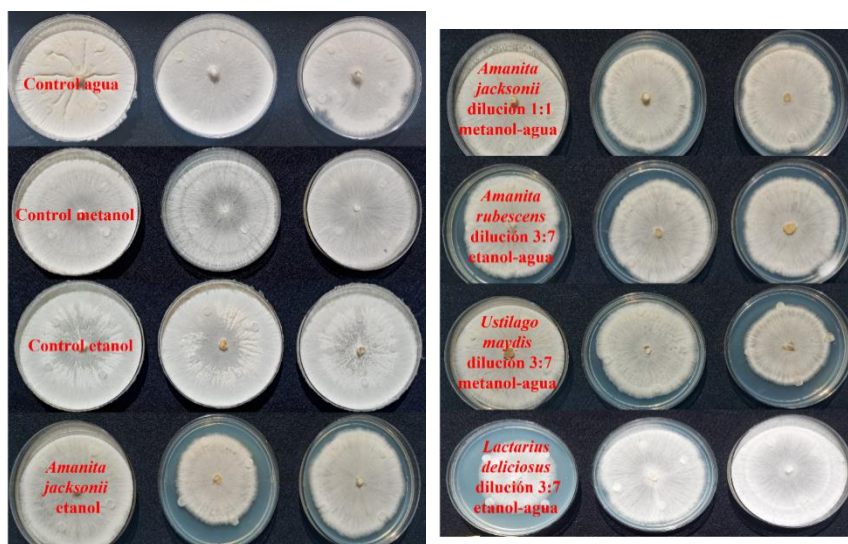


Figure 1. Alcoholic extract and dilutions of *Amanita jacksonii* against *Rhizoctonia solani* at 28 °C for three days of incubation.

It was qualitatively observed that *R. solani* grew more slowly in the extract of *A. rubescens* in the 3:7 ethanol-water dilution, where the fungus did not grow homogeneously and in the entire surface of the PDA as in the case of the controls. While the metabolic profile of each fungus is unique (Shen *et al.*, 2017), the observation of the impact on growth and development of *R. solani* could be due to metabolites reported in alcoholic extracts of edible fungi, such as low molecular weight phenolic compounds that have antimicrobial activity (Alves *et al.*, 2013).

Another possibility is that a minimal percentage of polar metabolites of phenolic nature were released in the alcoholic extracts of some macromycetes and could diffuse on the agar showing a slower growth in *R. solani* with respect to the controls (Naczki and Shahidi, 2006). In addition, disc diffusion depends directly on the diffusion capacity of the active substances present in the extracts, which can cause low mobility of metabolites in the agar if they have low solubility or high molecular mass.

Well diffusion

No effect on *R. solani* growth was observed with the alcoholic extracts and their dilutions (Figure 2), as *R. solani* grew in and out of the wells with extracts. These results could be related to the molecular weight of the compounds present in the extract, since if the molecular weight of the compounds is very high or if they have low solubility, their mobility through the agar will be difficult (non-diffusible), this could be a reason why no inhibitory effect from the alcoholic extracts and their dilutions was observed (Ren *et al.*, 2014; Martínez *et al.*, 2021).

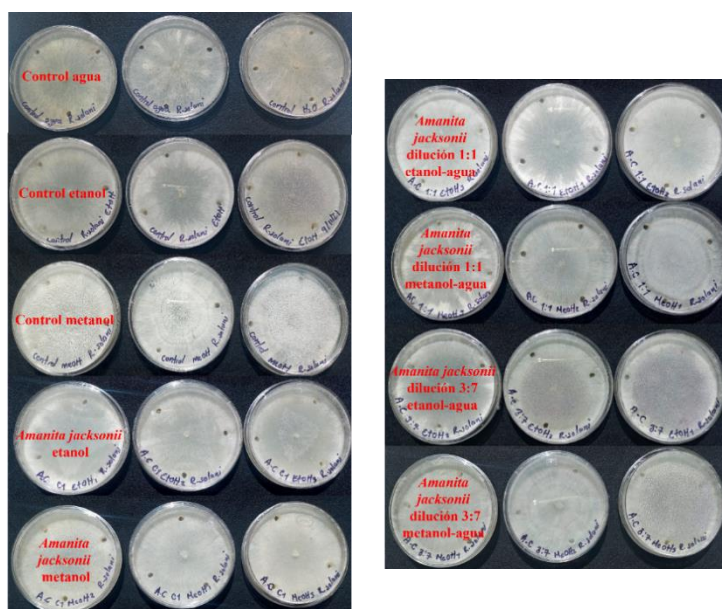


Figure 2. Dilutions of alcoholic extracts of *Lactarius deliciosus*, *Ustilago maydis*, *Amanita jacksonii* and *Amanita rubescens* against *Rhizoctonia solani* at 28 °C for three days of incubation.

Although it is not known what type of metabolites are present in the extracts of the edible fungi tested, it should be noted that the fungal material was used fresh and due to the type of solvents used (methanol, ethanol and water), it was sought to drag, in the extracts, polar metabolites such as polyphenols, sugars, alkaloids, saponins, glycosides and some proteins from macromycete fungi (Naczki and Shahidi, 2006; Akpi *et al.*, 2017). The presence or absence of these metabolites in the extracts will depend on the environment in which the fungus develops and the different sources of nutrients available during its growth (Shen *et al.*, 2017).

There may also be the possibility that the polar compounds present in the alcoholic extracts of edible fungi did not have inhibitory activity against *R. solani* in the well diffusion technique. On the other hand, it has been reported that, for the genus *Amanita*, peptides are the main constituents of polar extracts, these metabolites have greater antimicrobial activity in liquid medium or broth than in solid media such as agar, because they have low solubility (Li and Oberlies, 2005).

Plate dilution

The results indicate that the greatest inhibitory activity was shown by the 1:1 ethanol-water dilution of *L. deliciosus* (88%), while the lowest inhibitory activity was presented by the ethanolic extract of *A. rubescens* (0.5%) (Figure 3). These results differ from those found in well and disc diffusion techniques. This behavior can be explained by what is reported in the literature (Ren *et al.*, 2014; Balouiri *et al.*, 2016), as they mention that, in the plate dilution technique, the metabolites present in the extracts tend to diffuse better in liquid media than in solid media, such is the case of the potato dextrose broth that was used in this technique.

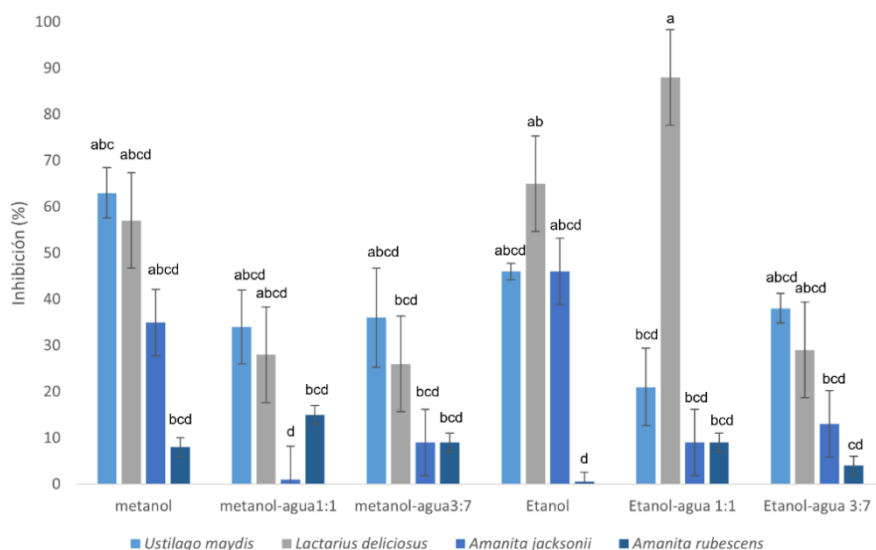


Figure 3. Alcoholic extracts and dilutions of edible fungi with inhibitory activity on the growth of *Rhizoctonia solani*. Mean \pm standard error, n= 3.

One of the advantages of using this technique is its ability to identify antimicrobial activity and quantify it accurately, since the results are not affected by the growth rate of different microorganisms (Morales *et al.*, 2013). This is a possible reason that there are stronger

inhibitions with greater effectiveness. The inhibitory activity shown by the ethanolic extract and the 1:1 ethanol-water dilution of *L. deliciosus* in the growth of *R. solani* can be attributed to the presence of phenolic compounds contained in the ethanolic extract of this edible fungus (Zavastin *et al.*, 2015).

L. deliciosus has proven antifungal activity as reported by Volcão *et al.* (2021), who evaluated the antifungal activity of the hydroalcoholic extract obtained from *L. deliciosus* against the phytopathogen *Monilinia fruticola* (Wint.), they report that the extract inhibited fungal growth and reduced mycelial development of the phytopathogen at a concentration of 1.25 mg L⁻¹. On the other hand, the processing of the fungal material (fresh or dried), the extraction time and the type of solvent should also be considered, which will influence the antimicrobial activity of the extracts (Adhikari *et al.*, 2018).

Among the most used solvents to perform the extraction of metabolites in fungi are water, ethanol, methanol, acetone and ethyl acetate (Elbatrawy *et al.*, 2015). In the results found in this study, a 1:1 ethanol-water dilution of *L. deliciosus* was the one that presented greater antifungal activity, even greater than those extracts that did not have any dilution and were only the solvent, a possible explanation for this behavior is reported by Yim *et al.* (2012), who point out that the best solvent for the extraction of phenolic compounds from fungi is water since it does not break glycosidic bonds, which indicates the presence of free phenols. In the 1:1 dilution, the presence of water favored the extraction of free phenols and therefore it was possible to quantify the highest antifungal activity of this extract.

Conclusions

Regarding disc diffusion, in some treatments it was observed that the fungus did not grow homogeneously as in the controls, which qualitatively suggests slow growth of *R. solani*; however, more evidence and studies are needed to affirm that with this methodology there is some quantifiable inhibitory effect. While in the disc diffusion method, no inhibition of the growth of *R. solani* was found. Nevertheless, the results obtained by the plate dilution technique are considered reliable, since the alcoholic extracts (and dilutions) had a direct contact in liquid medium with *R. solani* in the microplate wells.

Therefore, the 1:1 ethanol-water dilution of *L. deliciosus*, ethanol extract of *L. deliciosus*, methanol extract of *U. maydis* and methanol extract of *L. deliciosus* should be studied in greater depth, by testing them in an *in vivo* system and identifying the metabolites present.

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