

In vitro* antimicrobial activity of aqueous extracts from *Lentinula edodes* isolates against *Colletotrichum sublineolum* and *Xanthomonas axonopodis* pv. *passiflorae

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Data de chegada: 01/04/2014. Aceito para publicação em: 19/12/2014.

10.1590/0100-5405/1995

ABSTRACT

Tonucci-Zanardo, N.M.; Pascholati, S. F., Di Piero, R.M. ***In vitro* antimicrobial activity of aqueous extracts from *Lentinula edodes* isolates against *Colletotrichum sublineolum* and *Xanthomonas axonopodis* pv. *passiflorae*** *Summa Phytopathologica*, v.41, n.1, p.13-20, 2015.

The aim of this study was to evaluate the antimicrobial activity of aqueous extracts from fruiting bodies of different isolates of *Lentinula edodes* on the pathogens *Colletotrichum sublineolum*, the causal agent of anthracnose in sorghum, and *Xanthomonas axonopodis* pv. *passiflorae*, the causal agent of bacterial spot in passion fruit. Results showed that the aqueous extracts from isolates LE JAB-K and LE 95/01 significantly reduced *C. sublineolum* spore germination, while the isolate LE 96/22 was the only one to inhibit the pathogen mycelial growth. However, all *L. edodes* isolates showed inhibitory effect on *C. sublineolum* appressorium formation. Regarding *X. axonopodis* pv. *passiflorae*, the aqueous extracts

from all *L. edodes* isolates significantly reduced the *in vitro* multiplication of the bacterium. However, antimicrobial activity was lost when the extracts were autoclaved, demonstrating their thermolabile property. The aqueous extract from isolate LE 96/22 was also partially purified by anion exchange chromatography and fraction V exhibited high inhibitory activity on the *in vitro* mycelial growth of *C. sublineolum*, while the multiplication of *X. axonopodis* pv. *passiflorae* was inhibited by fractions IV, V and VII. Thus, *L. edodes* isolates were shown to produce compounds exhibiting antifungal and antibacterial activities against phytopathogens, which are mainly concentrated in fraction V.

Additional keywords: mushroom, anthracnose, bacterial spot, sorghum, passion fruit

RESUMO

Tonucci-Zanardo, N.M.; Pascholati, S. F., Di Piero, R.M. **Atividade antimicrobiana *in vitro* de extratos aquosos de isolados de *L. edodes* contra *Colletotrichum sublineolum* e *Xanthomonas axonopodis* pv. *passiflorae***. *Summa Phytopathologica*, v.41, n.1, p.13-20, 2015.

O objetivo deste estudo foi avaliar a atividade antimicrobiana dos extratos aquosos de corpos de frutificação de diferentes isolados de *Lentinula edodes* sobre os patógenos *Colletotrichum sublineolum*, agente causal da antracnose em sorgo e em *Xanthomonas axonopodis* pv. *passiflorae*, agente causal da mancha bacteriana em maracujá. Os resultados mostraram que os extratos aquosos dos isolados LE JAB - K e LE 95/01 reduziram significativamente a germinação de esporos de *C. sublineolum*, enquanto que o isolado LE 96/22 foi o único a inibir o crescimento micelial do patógeno. No entanto, todos os isolados de *L. edodes* mostraram efeito inibitório na formação de apressórios por *C. sublineolum*. Quanto a *X. axonopodis* pv. *passiflorae*, os extratos aquosos obtidos de todos

os isolados do cogumelo reduziram significativamente a multiplicação *in vitro* da bactéria. No entanto, a atividade antimicrobiana foi perdida quando os extratos foram autoclavados demonstrando assim a propriedade termolábil dos mesmos. O extrato aquoso do isolado LE 96/22 também foi parcialmente purificado por cromatografia de troca aniônica e a fração V exibiu elevada atividade inibitória sobre o crescimento micelial *in vitro* de *C. sublineolum*, enquanto que a multiplicação de *X. axonopodis* pv. *passiflorae* foi inibida pelas frações IV, V e VII. Desta forma, demonstrou-se que os isolados de *L. edodes* produzem compostos que apresentam atividade antifúngica e antibacteriana contra fitopatógenos, que estão principalmente concentrados na fração V.

Palavras-chave adicionais: cogumelo, antracnose, mancha bacteriana, sorgo, maracujá.

Lentinus edodes (Berk.) Pegler (= *Lentinula edodes* (Berk.) Singer) or shiitake, as it is popularly known, is a basidiomycete with nutritional, therapeutic and medicinal properties. *L. edodes* contains proteins, lipids (primarily linoleic acid), carbohydrates, fibers, minerals, vitamins B1, B2 and C, and ergosterol (25). Furthermore, researchers have shown that *L. edodes* produces several compounds with antithrombotic and anticholesterol effects, besides antifungal, antibacterial and antiviral activities (9). Several studies demonstrated the therapeutic activity of polysaccharides (glucans and heterogalactans) and glucanproteins

present in the vegetative mycelium or fruiting bodies of the fungus (14). The lentinan, a β -(1 \rightarrow 3)-D-glucan extracted from *L. edodes*, showed antitumor, antibacterial, antiviral and anticlotting activities (7).

The cotinellin isolated from *L. edodes* exhibited antibiotic activity against the pathogens *Candida albicans*, *Staphylococcus aureus* and *Bacillus subtilis* (1). A compound isolated from the fruiting body of *L. edodes* had fungistatic effect against *C. albicans* (15). Lenthionine, a substance obtained from the mycelial culture filtrate of *L. edodes*, exhibited inhibitory activity on *Streptococcus pyogenes*, *S. aureus*,

B. megaterium and *C. albicans* (5). In addition, there are reports demonstrating the production of metabolites by *L. edodes* with antibiotic activity against several phytopathogens (23), and disease control was demonstrated in several crops such as sorghum, passion fruit (19) and cucumber (3). The mycelial culture filtrate of *L. edodes* was also observed to cause inhibitory effects on *Helminthosporium* spp. and *H. euphorbiae* (22). The leached of mycelium from *L. edodes* also presented inhibitory activity on several species of phytopathogenic bacteria such as *Pseudomonas syringae* pv. *glycinea*, *P. syringae* pv. *tabaci*, *Xanthomonas campestris* pv. *glycines* and *Erwinia amylovora* (16).

The fungus *Colletotrichum sublineolum* (P. Henn. Kabat et Bub.) causes anthracnose, one of the main diseases of sorghum crop in Brazil. It can cause losses of up to 88% in grain yield and drastically affect the quality of the produced seed. The pathogen infects leaves, stems, flowers and seeds of sorghum at any age. Symptoms on the leaves begin with small round lesions of staining purple that evolve and result in a large necrotic leaf area (17).

The bacterial spot in yellow passion fruit plants is caused by *Xanthomonas axonopodis* pv. *passiflorae* (Pereira) Dye. The bacterium is rod-shaped, 0.5 x 1.5 µm, Gram-negative and aerobic, which does not produce spores or capsules and has a polar flagellum. It is important in Brazil due to its occurrence in all producing regions, the high susceptibility of economically important cultivars and the difficulty to control it (4).

Chemical control has been one of the main attempts to reduce the losses caused by these diseases. Problems associated with the selection of pathogen isolates resistant to fungicides (2) and the public general perception of the potential impact of traditional control practices on the health and the environment led to an increased demand for residue-free chemical products. Consequently, both farmers and researchers started to consider the use of alternative methods to control diseases (21). Therefore, in the present study, we investigated the *in vitro* antimicrobial effects of aqueous extracts from fruiting bodies of four isolates of *L. edodes* and their partially purified fractions against *C. sublineolum* and *X. axonopodis* pv. *passiflorae*.

MATERIALS AND METHODS

Phytopathogens

C. sublineolum was isolated from symptomatic leaves of sorghum and grown in oat-agar medium at 21 °C, under near U.V. light (10). *X. axonopodis* pv. *passiflorae* was isolated from the leaves of passion fruit plants (*Passiflora edulis* f. *flavicarpa*) showing systemic symptoms of the disease based on a modification of the method of Malavolta et al. (13). The leaves were cut into small pieces and received a drop of distilled water to observe the bacterial run under an optical microscope. The bacteria were transferred to nutrient agar medium and maintained at 28 °C in the dark.

Obtaining aqueous extracts from fruiting bodies of *L. edodes*

The fruiting bodies were obtained after the mushroom cultivation in *Eucalyptus grandis* and *E. saligna* logs kept under a shaded house at the Department of Plant Production, Faculty of Agricultural Sciences/UNESP/Botucatu/SP (18, 20).

The dehydrated powder from fruiting bodies of *L. edodes* isolates LE JAB-K, LE 95/01, LE 96/17 and LE 96/22 received sterile distilled water (1 g powder in 14 mL of distilled water). The mixture was incubated at 4 °C for 24 h. Afterwards, the extract was filtered through

Whatman # 4 filter paper by using a Buchner funnel, centrifuged (20.000g/ 25 min at 4 °C) and the supernatant was filtered through a Millipore membrane (0.2 µm) and stored at 5 °C until used. Depending on the experimental requirements, part of the aqueous extract was autoclaved at 121 °C for 15 min and stored in a refrigerator.

Effect of the aqueous extracts on conidial germination, appressorium formation and mycelial growth of *C. sublineolum*

Colletotrichum sublineolum was isolated from symptomatic leaves of sorghum. Segments of plant tissue (approximately 8 mm²) were placed in a solution of commercial sodium hypochlorite (three parts water to one part hypochlorite; v/v). After 5 min, the tissue was washed three times in distilled water to remove excess hypochlorite. Then, five segments of the tissue were placed on the surface of the water-agar culture medium inside Petri dishes and kept at 23 °C under continuous light for 10 days. After this period, mycelial disks were transferred to oatmeal agar medium to obtain pure cultures of the fungus.

ELISA plates were used to evaluate spore germination and appressorium formation of *C. sublineolum*. Each well (eight per treatment) received 40 µL of conidial suspension (10⁵ conidia per mL) and 40 µL of aqueous extract. *L. edodes* aqueous extracts were used at the final concentrations of 50% (v/v). Control consisted of sterile distilled water plus conidial suspension, and eight replicates for each treatment were evaluated. The plates were maintained in the dark at 23 °C for 20 h and, after that period, 20 µL of lactophenol were added to each well to stop spore germination. Spore germination and appressorium formation were evaluated by using an optical microscope. At least 100 spores were counted per replicate.

To evaluate the effect of aqueous extracts on the mycelial growth of *C. sublineolum*, 0.5 mL of the extracts were added to the surface of potato dextrose agar (PDA) in Petri dishes and spread with the aid of a Drigalsky loop. The control plate received sterile distilled water on the surface of the PDA medium in Petri dishes instead of aqueous extracts from the isolates. After 24 h, a 6mm-diameter agar plug containing mycelium of *C. sublineolum* was transferred to the dishes (six Petri dishes per treatment) which were maintained under fluorescent light at 26 °C. At every three days, the mycelial growth of the pathogen was measured by calculating the average of two diameters of colonies at right angles for each plate. In this experiment, the isolate LE JAB-K was not used because of the low availability of the fruiting body powder.

Effects of the aqueous extracts on *X. axonopodis* pv. *passiflorae* multiplication

In plastic conical test tubes, the aqueous extracts from fruiting bodies (original concentration of 1 g powder in 14 mL of distilled water) of the different isolates were mixed with distilled water to obtain the final concentrations of 5, 10, 15 or 20% (v/v). Control treatment was represented by water. Then, the tubes showing volume of 9 mL of extracts received 1 mL of the bacterial suspension adjusted to 80% transmittance at 680 nm in a spectrophotometer. The suspensions were homogenized and incubated in the darkness for 24 h at 30 °C. After incubation, 250 µL of the suspensions were spread on nutrient agar medium, and the plates (five per treatment) were maintained at 30 °C in the darkness for 72 hours. After 3 days, bacterial growth was evaluated by adding 20 mL of distilled water to each plate and the transmittance of bacterial suspensions was determined at 680 nm. Transmittance values were transformed into absorbance values to express bacterial growth. In another assay, the aqueous extracts at 20% (v/v) were autoclaved during 15 min at 121 °C. These autoclaved aqueous extracts were used as described above, but the isolate LE 96/17 was not used because it

was no longer available.

Partial purification of the fruiting body fractions by anion exchange chromatography

A sample (75 mL) of the aqueous extract from isolate LE 96/22, containing 4.6 mg of protein and 1.583 mg of carbohydrate, was lyophilized, resuspended in 5 mL distilled water and filtered through a Millipore membrane (0.2 μm). The sample was subjected to anion exchange chromatography (AEC) in a column (2.5 x 15 cm) filled with DEAE-Cellulose equilibrated with 25 mM sodium phosphate buffer (pH 6.0). The unbound compounds were eluted with the buffer at a flow rate of 2.5 mL $\cdot\text{min}^{-1}$, while the bounded compounds were eluted by using a linear gradient of NaCl (0 to 1 M). The collected fractions were pooled according to their profiles at 280 nm, dialyzed in distilled water and concentrated to 9 mL with polyethylene glycol 20,000. The fractions were used for the bioassays with *C. sublineolum* and *X. axonopodis*.

Effects of fractions obtained through AEC on *C. sublineolum* and *X. axonopodis*

The experiment regarding conidial germination and appressorium formation was performed by using Elisa plates, where each well (eight per treatment) received 40 μl of spore suspensions of *C. sublineolum* (10^5 spores mL $^{-1}$) and 40 μl of the aqueous fractions from isolate LE 96/22 and were kept at 26 °C in the absence of light for 20 h. After this period, 20 μL of lactophenol were added to each well to stop spore germination. The spore germination and the appressorium formation were evaluated by using an optical microscope, and at least 100 spores were counted per replicate.

To evaluate the effect on mycelial growth, the fractions obtained by AEC were filtered through Millipore filters (0.2 μm), and 100 μL aliquots were spread onto PDA medium inside 5.0-cm diameter Petri dishes. After 24 h, the dishes received a 0.3-cm diameter agar plug with mycelium of *C. sublineolum* and were kept at 26 °C under constant fluorescent light. The diameter of the mycelial growth of colonies was evaluated at every three days based on the average between two

opposite measurements until the colony in the control plates reached the borders. Each treatment consisted of five replicates.

To evaluate the effect of fractions on *X. axonopodis* pv. *passiflorae*, test tubes containing 0.9 mL of each fraction received 0.1 mL of the bacterial suspension with 80% transmittance at 680 nm. Control treatment was represented by water. The tubes were maintained in the darkness during 24 h at 30 °C. Afterwards, aliquots of 200 μl of each tube were spread onto nutrient agar medium and the plates maintained in the darkness at 30 °C. After 2 days, each plate received 20 mL distilled water and the transmittance of the bacterial suspension was determined at 680 nm in a spectrophotometer. The transmittance values were transformed into absorbance to express the bacterial growth. Experimental design was completely randomized, with five replicates, where each Petri dish was considered an experimental unit. Data were subjected to analysis of variance and means were compared by using Tukey's test.

RESULTS AND DISCUSSION

The fruiting body and the mycelium of *L. edodes* have compounds with antimicrobial activity. In this study, we observed the *in vitro* inhibitory effects of aqueous extracts from fruiting bodies of *L. edodes* on spore germination, appressorium formation and mycelial growth of *C. sublineolum*, as well as on the multiplication of *X. axonopodis* pv. *passiflorae*.

C. sublineolum spore germination was significantly inhibited by the aqueous extracts from isolates LE JAB-K and LE 95/01, LE 96/17, but not by the extract from isolate LE 96/22 (Figure 1). In contrast, the aqueous extracts from all isolates significantly reduced *C. sublineolum* appressorium formation (Figure 2), and only the aqueous extract from isolate LE 96/22 reduced mycelial growth (Figure 3). Maki (12) also observed differences in the antimicrobial activity among different isolates of *L. edodes* against *C. albicans*. Similar effects were also observed for aqueous extracts from fruiting bodies of *L.*

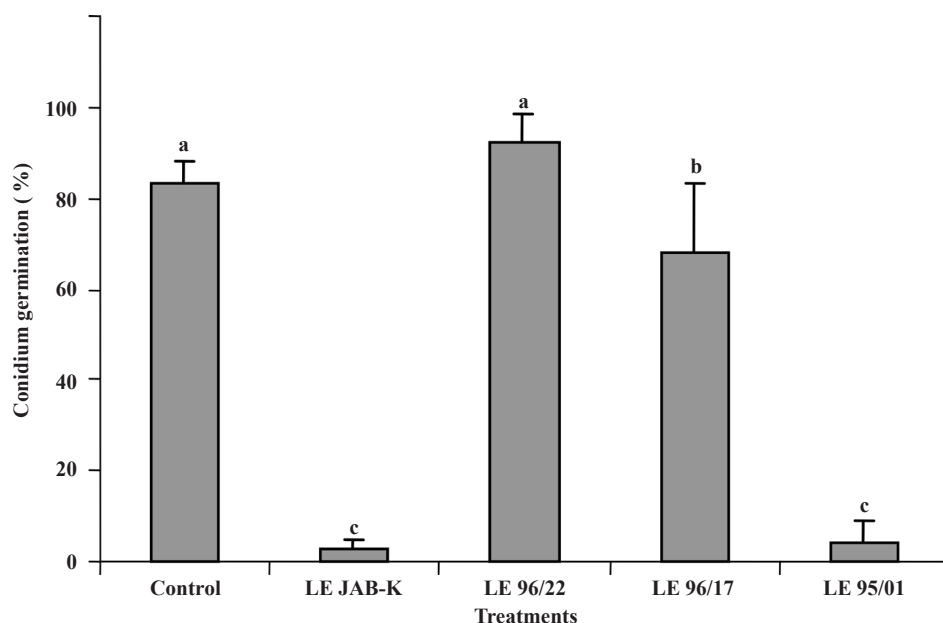


Figure 1. Effects of aqueous extracts from fruiting bodies of the isolates LE JAB-K, LE 96/22, LE 96/17 and LE 95/01 of *Lentinula edodes* on conidium germination of *Colletotrichum sublineolum*. Values are the means of three replicates (\pm SD). Means followed by the same letter do not differ according to Tukey's test at 5%.

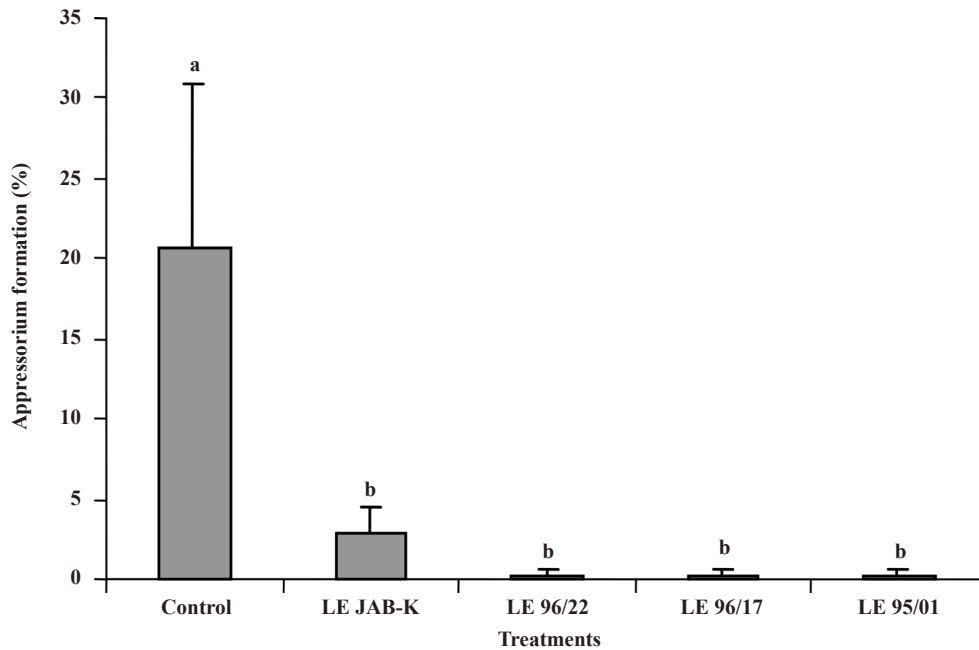


Figure 2. Effects of aqueous extracts from fruiting bodies of the isolates LE JAB-K, LE 96/22, LE 96/17 and LE 95/01 of *Lentinula edodes* on appressorium formation of *Colletotrichum sublineolum*. Values are the means of three replicates (\pm SD). Means followed by the same letter do not differ according to Tukey's test at 5%.

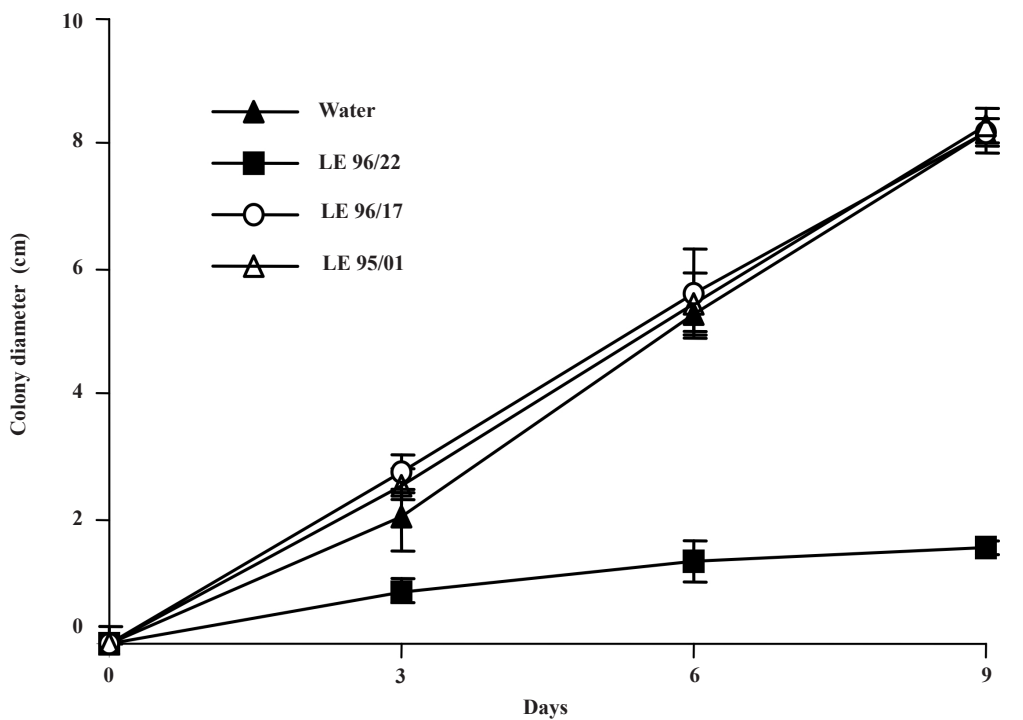


Figure 3. Effects of aqueous extracts from fruiting bodies of the isolates LE 96/22, LE 96/17 and LE 95/01 of *Lentinula edodes* on mycelial growth of *Colletotrichum sublineolum*. Values are the means of six replicates (\pm SD).

edodes, isolate "Piracicaba", which reduced *in vitro* spore germination, appressorium formation and mycelial growth of *C. sublineolum* (19). The appressorium is an important fungal structure during the process of penetration through the leaf surface; therefore, inhibition of spore germination and mainly appressorium formation is an important measure to control plant diseases (11).

Since isolate LE 96/22 was capable of inhibiting *C. sublineolum*

mycelial growth and appressorium formation, it was subjected to anion exchange chromatography to separate the compounds exhibiting inhibitory activity (Figure 4). Peak V reduced the mycelial growth of *C. sublineolum*, but peaks I, II, III, IV and VII did not show any inhibitory effects (Figure 5). Similar results were obtained by using aqueous extracts from fruiting bodies of isolates LE 95/01 and LE 96/22 of *L. edodes*, which stimulated the germination of *Colletotrichum*

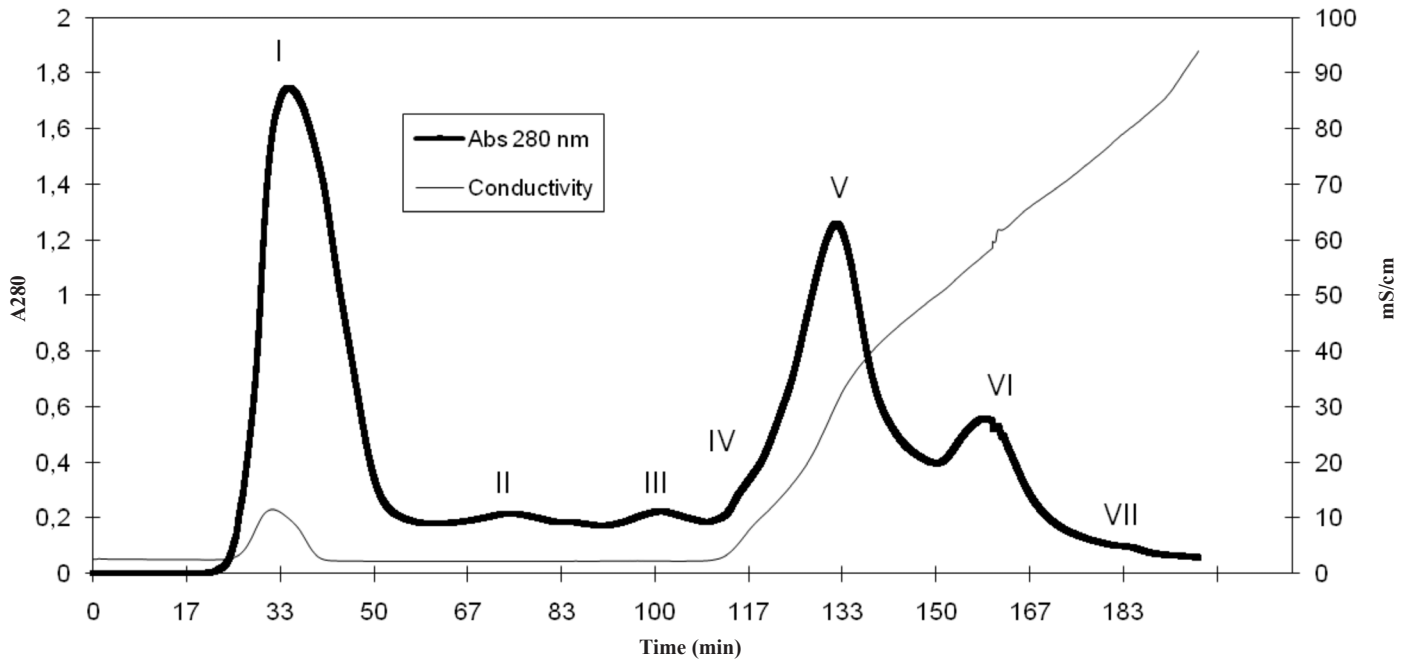


Figure 4. Anion exchange chromatography of crude aqueous extract from isolate LE 96/22 of *Lentinula edodes*. The chromatography was performed in column filled with DEAE-Cellulose equilibrated with 25 mM phosphate buffer (pH 6.0), at a flow rate of 2.5 mL.min⁻¹, and the bounded material was eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer. The fractions were pooled as peaks I, II, III, IV, V, VI and VII.

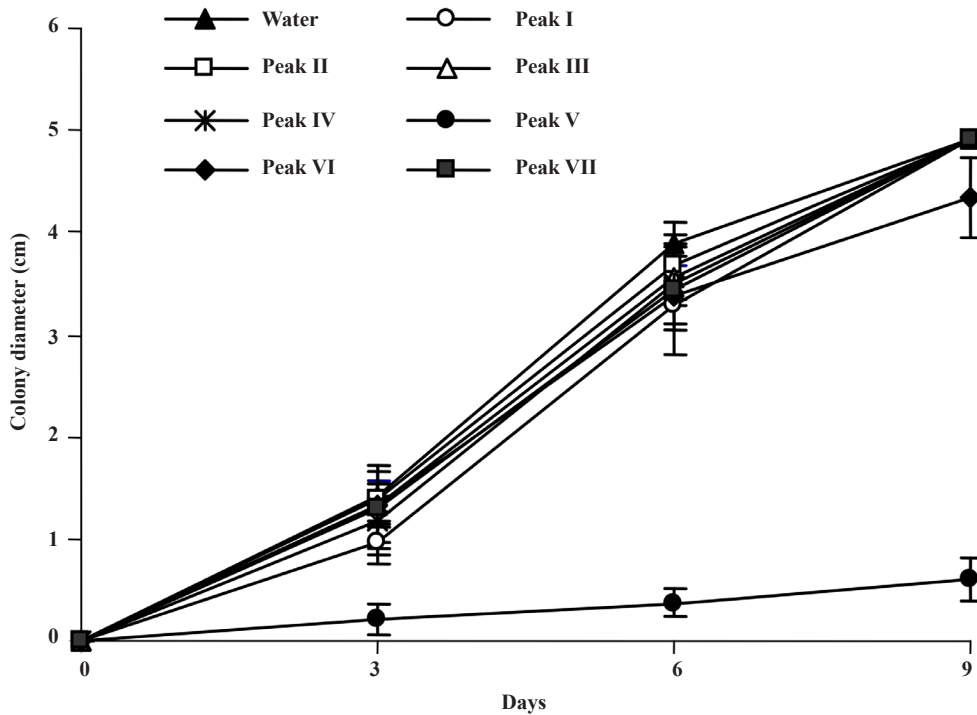


Figure 5. Effect of the pooled fractions of *Lentinula edodes* (isolate LE 96/22), obtained by anion exchange chromatography, on the mycelial growth of *Colletotrichum sublineolum*. The bars represent the standard deviation of the means. Values are the means of five replicates (\pm SD).

lagenarium conidia (3). The fractions obtained after AEC did not show any inhibitory effects on appressorium formation when compared to the crude aqueous extract from isolate LE 96/22 (data not shown). Activity loss after AEC can be explained by the low recovery of bioactive

components from the extract due to the sample preparation conditions.

X. axonopodis multiplication was significantly reduced by all aqueous extracts (Figure 6). The antibiotic effect of *L. edodes* on *X. axonopodis* increased as the concentration of the aqueous extracts

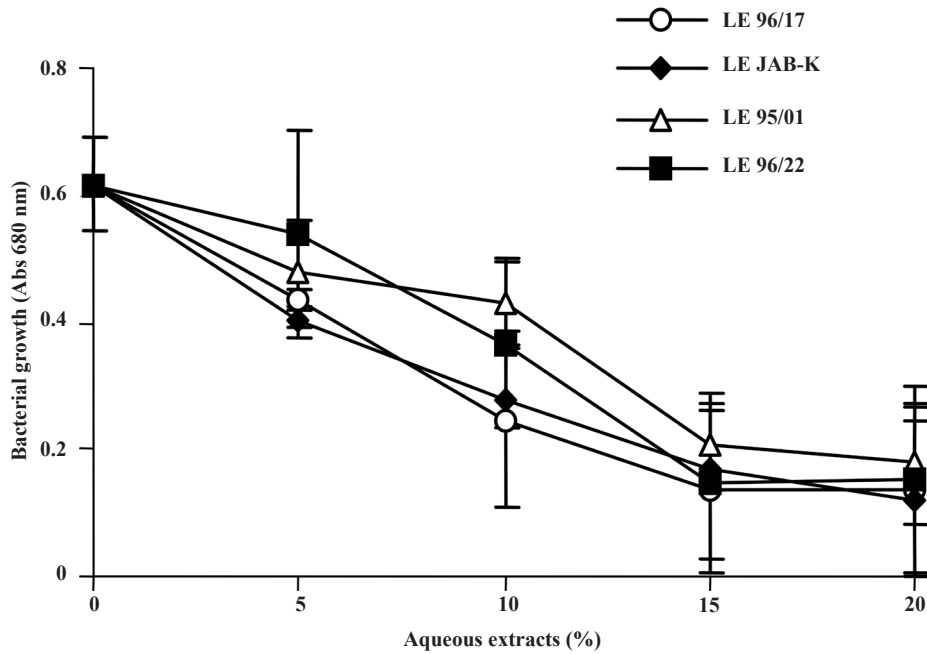


Figure 6. Effects of aqueous extracts from fruiting bodies of *Lentinula edodes*, at different concentrations, on the *in vitro* multiplication of *Xanthomonas axonopodis* pv. *passiflorae*. Values are the means of five replicates (\pm SD). Significant effect of concentration was observed based on the F test at 5%

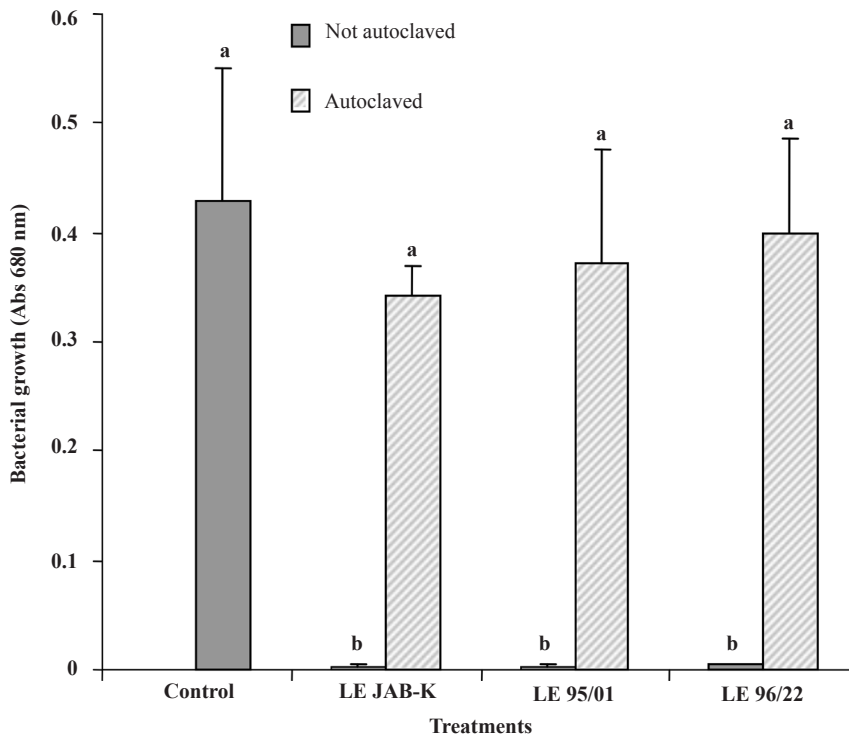


Figure 7. Effect of autoclaving the aqueous extracts (20%) from fruiting bodies of the isolates LE JAB-K, LE 95/01 and LE 96/22 of *Lentinula edodes* on the *in vitro* multiplication of *Xanthomonas axonopodis* pv. *passiflorae*. Values are the means of three replicates (\pm SD). Means followed by the same letter do not differ according to Tukey's test at 5%

increased up to 15%. Piccinin (19) reported that the aqueous extracts from the fruiting body of *L. edodes*, isolate "Piracicaba", exhibited a bacteriostatic effect on *X. axonopodis*, while the aqueous extract from the stipe exhibited a bactericidal effect.

The aqueous extracts from fruiting bodies that were subjected to autoclaving lost their inhibitory activities on bacterial multiplication

(Figure 7). This indicates that those extracts have bioactive substances which are thermolabile. According to Hirasawa (6), the aqueous extract from *L. edodes* was thermolabile when heated up to 60 °C for 30 min, reducing by 60% its antibacterial activity against *Streptococcus mutans* and *Prevotella intermedia*, whereas heating of up to 100 °C for 5 min led to complete loss of its antibiotic activity. On the other hand, *L.*

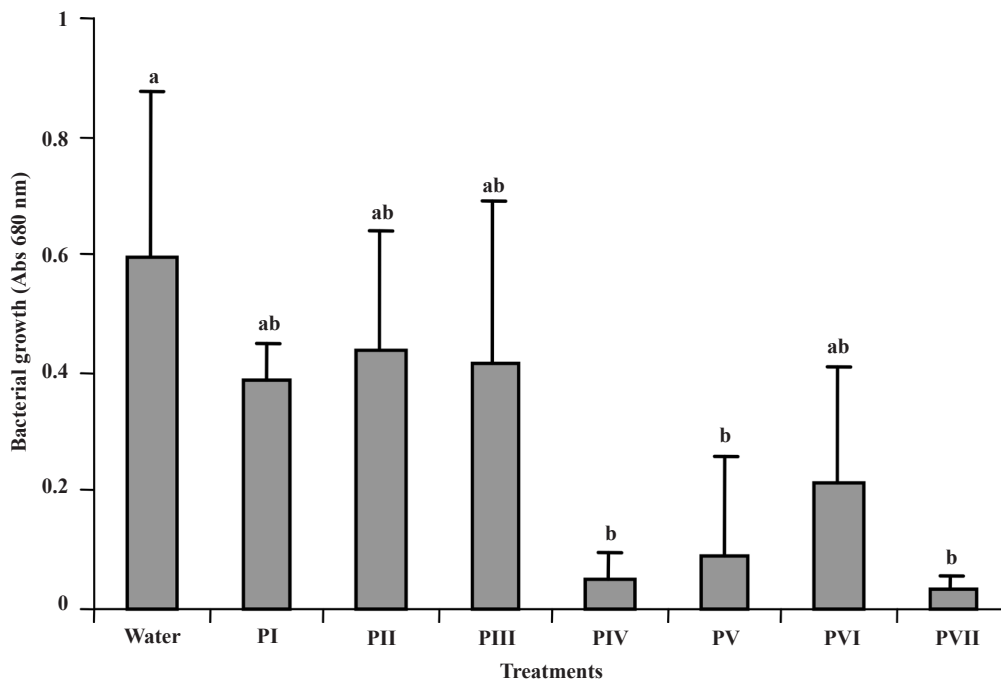


Figure 8. Effect of the pooled fractions (peaks I to VII) of *Lentinula edodes* (isolate LE 96/22), obtained by anion exchange chromatography, on the *in vitro* multiplication of *Xanthomonas axonopodis* pv. *passiflorae*. Values are the means of five replicates (\pm SD). Means followed by the same letter do not differ according to Tukey's test at 5%.

edodes culture filtrate did not lose its antibacterial activity against *Bacillus subtilis* when subjected to 100 °C for 10 min, but 20, 30 and 60-min heating resulted in 43, 72 and 100% reduction in the activity, respectively (8).

The fractions of aqueous extracts from fruiting bodies of isolate LE 96/22 also exhibited antibiotic activity on *X. axonopodis* multiplication *in vitro*, since peaks IV, V and VII significantly reduced bacterial growth (Figure 8).

In the present study, peak V for the aqueous extract from isolate LE 96/22 of basidiomycete *L. edodes* showed strong antibacterial and antifungal activities. Basidiomycetes as a source of antimicrobial substances have been widely explored. The discovery of strobirulins, produced by the mushroom *Strobilurus tenacellus*, is one of the most important examples of this type of investigation (24).

The use of fungicides is known to be the main control method for plant diseases but the emergence of resistant fungal isolates, the costs of chemical control and the safety for the environment are important issues to be taken into consideration. Therefore, the search for new products, preferably of low toxicity to humans and to the environment, is necessary.

CONCLUSION

Aqueous extracts from *L. edodes* isolates have compounds exhibiting antifungal and antibacterial activities against plant pathogens.

ACKNOWLEDGMENTS

To Dr. Augusto Ferreira da Eira for supplying the *L. edodes* isolates. This study was supported in part by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and by CNPq (Conselho Nacional Científico Tecnológico).

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