

English Version

Abstract

For protecting 'Itália' against *Botrytis cinerea* in the post harvest it was evaluated *in vivo* direct and indirect effect of water based extracts of *Agaricus blazei* and *Lentinula edodes* treating grape bunches before or after inoculating the pathogen. Two trials were performed: 1) grape bunches were inoculated before and after 4 h they were sprayed with different concentrations of extracts from basidiocarps (0.0; 2.5; 5.0; 10.0; 20.0 or 40.0%); 2) bunches were sprayed or not with *A. blazei* extracts (5,0%) 24, 48, 72 or 96 h before pathogen inoculation. For inoculation, in each bunch, 10 berries were injured with a ± 2 mm deep sting and sprayed with a conidial suspension ($\pm 10^5$ conidia mL⁻¹). After treatment, bunches were kept at 25 ± 1 °C/80-90% RH and daily evaluated for incidence and severity of rottenness. The *in vitro* effect of *A. blazei* e *L. edodes* extracts for pathogen control were evaluated in order to verify if these agents have a direct effect over mycelia growth and *B. cinerea* conidia germination. Results show that *A. blazei* e *L. edodes* extracts did not control rottenness caused by *B. cinerea* in 'Italia' grapes when sprayed before or after fungus inoculation. In the *in vitro* trials, both basidiocarps extracts stimulated *B. cinerea* conidia germination at the tested concentrations; regarding mycelia growth, *L. edodes* had negative, while *A. blazei* had positive effect.

Key words: *Vitis vinifera*; table grapes; *Botrytis cinerea*

Use of *Agaricus blazei* and *Lentinula edodes* extracts for post-harvest control of gray mold in 'Itália' grape

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Introduction

The development of fungus, especially *B. cinerea*, during the storage and transport of table grapes is responsible for significant losses post-harvest in all the productive regions of the world (BULIT and DUBOS, 1990; DIAS et al., 1998; ZAHAVI et al., 2000). Even the best phytosanitary treatment effected in field is not enough to dispense its use after the harvest (ZOFFOLI et al., 1999). For this reason, SO₂ is used on the control of rottenness on the post-harvest of table grapes combined to the storage under low temperatures (BULIT e DUBOS, 1990; ZOFFOLI et al., 1999; ZAHAVI et al., 2000). However, depending on the cultivar, temperature and storage humidity, among other factors, SO₂ may present differences concerning the efficiency and level of waste, which limit of tolerance is 10 µg.g⁻¹ (FDA, 2003). It is still necessary to consider the increasing concern of the consumers on the waste

of SO₂ in fresh grapes and their phytotoxic potential (LYDAKIS e AKED, 2003).

In this sense, the emphasis in protection of fruit post-harvest against rottenness has been changed from the use of chemical products to alternative techniques of control, which ensure the safety of the product and do not endanger the health of people (ROMANAZZI et al., 2002), minimizing or substituting the use of fungicides in fruit.

There are works which mention compounds that come from *L. edodes*, usually known as "Shiitake", with action over plant and animal pathogens. Piccinin (2000) demonstrated that the filtrate of the pileus of *L. edodes* has bacteriostatic effect, while the filtrate of the stipe and the mycelia growth were bactericides over *Xanthomonas campestris* pv. *Passiflorae*. Ishikawa et al. (2001) also verified the bacteriostatic effect of 35 isolates of *L. edodes* in the growth of *Bacillus subtilis*. Preparations obtained by *L. edodes* reduced the number of injuries caused by *Tobacco mosaic virus*

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(TMV) in half-leaves of tobacco and caused total inhibition of the growth of the bacteria *X. campestris* pv. *passiflorae*, *in vitro*; however, when autoclaves, the filtrates lost completely the activity which inhibits the multiplication of the bacteria (TONUCCI and PASCHOLATI, 2003).

Concerning the effect over pathogenic fungus, the extracts obtained with the mycelial growth of *L. edodes*, incorporated by the cultivation at 100, 200 and 300 $\mu\text{L mL}^{-1}$, inhibited the development *in vitro* of the fungus *Helminthosporium* sp., *Fusarium solani* and *Phomopsis sojae* (SASAKI, 1997). Piccinin (2000) showed that the filtrate obtained by the vegetative growth of *L. edodes*, as well as the extracts obtained by pileum and stipe of the mushroom, reduce significantly the mycelial growth of *Exserohilum turcicum* and *Colletotrichum sublineolum* from the concentration of 1% (v/v) in the means of cultivation. At 2%, the preparations reduced the sporulation of the pathogens. Di Piero (2003) verified that *L. edodes* reduces more than 50% of the anthracnose severity caused by *Colletotrichum lagenarium* in cucumber plants maintained in green house, when filtrates from basidiocarp at 20% were applied in the host, besides introducing local and systemic accumulation of peroxidase and chitinase, however not of β -1,3-glucanase.

By its turn, researches involving the mushroom *A. blazei* and its possible pathologic use are beginning. It was found that filtrates of *A. blazei* caused reduction of 80% in the incidence of the disease caused by passion fruit woodiness virus (PWV), however, it was noted only local protection, probably antivirus substances produced by the mushroom affected the infectivity of PWV (DI PIERO and PASCHOLATI, 2002). Extracts of *A. blazei*, in different concentrations and inoculation of four days, showed only a local effect in the reduction of the anthracnose severity in cucumbers (DI PIERO, 2003). In 2001 and 2002, Fiori-Tutida et al. verified the positive effect of the crude extract of *L. edodes* in the inhibition of the germination of spores of *Puccinia recondita* f. sp. *tritici*. However, the crude extract obtained by *A. blazei* did not inhibit the germination of spores of *Bipolaris sorokiniana*; however, both extracts were capable to induce the accumulation of

phytoalexins in soybean cotyledons.

Silva et al. (2007) verified that isolates of *L. edodes* and of *A. blazei* did not provide inhibitory effects direct on the growth of the bacteria *Ralstonia solanacearum*, causal agent of the bacterial wilt in tomato. However, in greenhouse, it was verified that plants treated two days before the inoculation with the isolate LE-96/17 of *L. edodes* in the concentration of 10% (v/v) had significant reduction in the occurrence of wilt. Moreover, LE-96/17 and ABL-26 of *Agaricus blazei* caused increase in the activity of peroxidase and, based on the results, the authors conclude that the mushroom *L. edodes* presents potential to reduce the bacteria wilt, probably, through the induction of resistance.

As for quiescent infection, as the one caused by *B. cinerea*, in which the inoculum come from the pre-harvest period, before that a post-harvest treatment can be applied, antagonist microorganisms may be promising as "live fungicides" in the control of diseases (WILSON et al., 1991; KOOMEN e JEFFRIES, 1993).

For this reason, the goal of this work was to evaluate the healing and protective effect of the extracts of the basidiocarps *L. edodes* and *A. blazei* in 'Itália' grape against *B. cinerea* in post-harvest. The *in vitro* effects of the extracts over the mycelial growth and the germination of the conidia of the fungus were also investigated.

Material and methods

Inoculation of *Botrytis cinerea* in bunches of 'Itália' grape

The pathogen of interest (*B. cinerea*) was isolated by grapes with symptoms of gray mold, coming from vineyards of the state of São Paulo. For inoculation, in each branch of grape 10 berries were injured, with a hole per bag of ± 2 mm of depth, with the aid of a microsyringe (Hamilton Co., Reno, Nevada, 100 μL). After performing the experiment, the branches were inoculated by spraying the spore suspension, in the concentration of $\pm 10^5$ conidia mL^{-1} , in accordance with methodology described by Romanazzi et al. (2002), with some modifications.

Spraying of bunches with the extracts of the basidiocarps

Samples of the dry powder of the basidiocarp of the mushroom *Agaricus blazei* (Murril) ss. Heinem (lineage ABL 29/99) and *Lentinula edodes* (Berk.) Pegler (lineage LE 96/17) were given by the Mushroom Production Center of the Faculty of Agricultural Science/UNESP, Botucatu campus/SP. To obtain the aqueous extracts, the dry powder of the mushroom basidiocarp was suspended in distilled water (14 mL g⁻¹) and, after 24 h of incubation at 10°C, samples were filtered at vacuum, using filter paper Whatman n° 1 and centrifuged at 20.000 g for 25 minutes. Then, the supernatant was filtered in membrane of the type Millipore (pore diameter = 0,2 µm), under aseptic conditions. The sterile filtrates (concentrated aqueous extracts) were stored in refrigerator at 10 °C and used for the dilutions. Aqueous extract at 10% v/v (10 mL of concentrated extract + 90 mL of distilled water) represents: [(1000 mg 14 mL⁻¹). 10%] = 7,15 mg of the dry powder of basidiocarp mL⁻¹.

In order to evaluate the direct effect (healing) of the extracts over the pathogens, in the first experiment, branches of ripe 'Itália' grape (>15 °Brix), coming from the municipality of São Miguel Arcanjo/SP, were inoculated, as described before and, after four hours, sprayed with different concentration of the basidiocarp extracts (0.0; 2.5; 5.0; 10.0; 20.0 or 40.0%). With the goal to evaluate the potential of the extract of *A. blazei* to protect branches of grape against *B. cinera*, on the second experiment, the branches were or not sprayed (5.0%) 24, 48, 72 or 96 h before the fungus inoculation. After the inoculation, the branches were packed in cardboard boxes at 25±1 °C/80-90% RH for a period of 6 and 4 days in the first and second experiment, respectively, to the disease evaluation.

Evaluations of incidence (number of berries infected) and hardness (percentage of the berry area infected, determined through grading scale) were performed daily in the 10 berries inoculated by brunch. The grading scale adopted to evaluate the hardness of the rot ranged from 1 to 6 and was elaborated based on the area of the injury, corresponding to approximately <0.2; 0.5; 1.0; 2.0 and >3.0 cm² of the area of the berry injured, which equals

to 2, 5, 10, 20, 30 and 50% of the area, respectively. The results were expressed in disease index calculated by the formula: $DI(\%) = \{[(n_1 \times 1) + \dots + (n_6 \times 6)] \times (6 \times N)^{-1}\} \times 100$, in which $n_{1...6}$ = number of berries infected with the respective grade and N = total number of inoculated berries.

The experimental design was completely randomized, with 10 replications and a brunch of grapes as the experimental unit. In order to evaluate the second experiment, it was adopted the factorial arrangement (2x4). The data obtained were submitted to analysis of variance and the measures compared by Tukey test at 5% of probability. For the statistic analysis, the means were transformed in $\sqrt{x+0.5}$. Besides the mean comparison, analysis of regression was effected to verify the relation between doses and disease index.

In vitro effect of the extracts of *Agaricus blazei* and *Lentinula edodes* over *Botrytis cinerea*

To evaluate the effect of the mushroom extracts in the germination of the *B. cinera* conidia, polystyrene plates containing half Agar-Water culture were divided in four quadrants, in which each quadrant received a drop of spore suspension ($\pm 10^5$ conidia mL⁻¹), added from the aqueous extracts of the basidiocarp at 0.0; 2.5; 5.0; 10.0; 20.0 or 40.0%. Each parcel was represented by a plate, and it was used ten replications per treatment, which were maintained in dark at ±22 °C until the germination of the conidia. In the evaluation, it was counted 50 conidia per quadrant, after 8, 24, 32, 48 and 56 h, through the observation in optic microscope. Conidia were considered germinated when the germ tube presented size equal or superior to the conidia diameter (MERCIER et al., 2001).

Aiming to verify the effect of the mushrooms in the mycelial growth of the pathogen, the aqueous extracts of basidiocarps were incorporated in mean Potato-Dextrose-Agar (PDA) with add of antibiotic (chlortetracycline - 100 µg mL⁻¹ + chloramphenicol 100 µg mL⁻¹) at 45° C, in the proportion of 0.0; 2.5; 5.0; 10.0; 20.0 or 40.0% and poured in polystyrene plates. Mycelium discs with 3 mm of diameter, took from the border of colonies with 3 days of cultivation, were transferred to the center of the plates which were maintained at ±22 °C under light alternating

light (12 h). The mycelial growth of the fungus was determined daily, measuring the diameter of the colony in two opposed directions, until the colony of one of the treatments achieve the border of the plate (TERRY e JOYCE, 2000). A number of seven and six replications, respectively for *A. blazei* and *L. edodes*, was used.

Results and discussion

When observing Figure 1, it can be verified that the different concentration of the extract of mushrooms *A. blazei* or *L. edodes* applied to 'Itália' grape, after 4 h of inoculation with *B. cinera*, did not

differ statistically in disease index. However, it was verified the lower values of occurrence of rottenness in the brunches treated with the extract of *A. blazei* at 5.0% during the days of evaluation. This reduction was observed for the extract of the mushroom *L. edodes* only in the highest concentration (40.0%). The extract of *A. blazei* in the concentration to 20.0% was more efficient then *L. edodes* in the reduction of the gray mold.

Extracts of *L. edodes* and *A. blazei* provided local protection in passion fruit against woodiness virus (PWV) (DI PIERO, 2003). The author affirms that the extract of these two mushrooms can activate mechanisms of vegetal defense against the passion

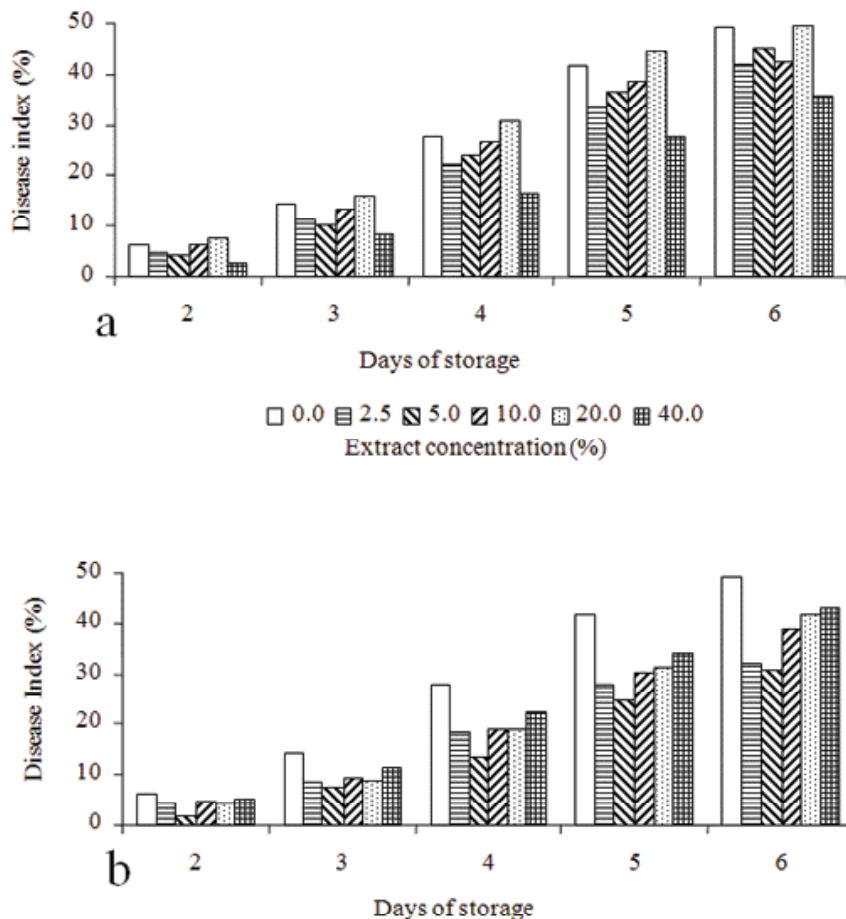


Figure 1. Index of disease caused by *Botrytis cinerea* in 'Itália' grape brunches, inoculated and after 4 h treated with different concentrations of the extract of *Agaricus blazei* (a) or *Lentinula edodes* (b). Averages did not differ statistically (Tukey $P \leq 0,05$). Original data were transformed to $\sqrt{x+0,5}$, for statistic analysis.

fruit woodiness virus (PWV), but not in sufficient magnitude to protect a species of economic interest that is systematically invaded by the virus. At the same way that it was obtained on the pathosystem studied, the author observed that isolates of *L. edodes* provided a lower protection than that provided by isolates of *A. blazei* in passion fruit. Moreover, the protection was dependent on the concentration of the extract and on the time interval between the treatment and the inoculation; extract concentration of *L. edodes* inferior to 40.0% was not efficient to reduce the virus incidence; data similar to those obtained in this work.

The results of the disease index in grape branches treated with extract from the mushroom *A. blazei* (5.0%), 24, 48, 72 or 96 hours before the inoculation of branches with *B. cinerea* (second experiment) are presented in Table 1.

It can be verified that the time between the treatment and the inoculation had higher influence over the index of disease caused by *B. cinerea* than the factor treatment (with or without extract). Regarding the factor treatment, it can be verified that the application of aqueous extract of *A. blazei* did not result in efficient protection of grape against the pathogen, considering than when applied 24, 48 or 96 h before the inoculation, the disease index did not differ statistically from the respective controls (without extract) and, still, when applied 72 hours before the inoculation, the disease index was significantly higher than the control. However, grape branches treated with *A. blazei* 96 h before the inoculation present average values of disease index significantly lower than branches treated 72 before the inoculation with *B. cinerea*.

The results obtained by Camili et al. (2007), when evaluating the effect of chitosan solution, in the concentration of 1.5 and 2.0% (v/v), applied before the inoculation with *B. cinerea*, corroborate with those presented in this work, since they do not obtain significant answer of the development of the gray mold in 'Itália' grape; however, other authors affirm that chitosan activate responses of defense in the vegetal tissue.

Therefore, new researches must be conducted aiming to better explore the viability of the use of basidiocarp extracts from *L. edodes* and *A. blazei*, once

they have shown satisfactory results in other vegetal species. Maybe not only the use of the crude extract, but also fractions obtained by these extracts may be used isolated, or combined with other methods of control of gray mild in grape, according to the results obtained by Di Piero et al. (2006), who, when fractionating the crude aqueous extract of basidiocarps of *L. edodes*, obtained positive results in the reduction of anthracnose in cucumber cotyledons.

In a significant way, the crude extracts of both basidiocarps stimulated the germination of the conidia in all the concentrations studied (Figure 2), after 8 h of submission to the treatment. Similar data were verified by Piccinin (2000), who verified, when using preparations of filtrate of basidiocarp autoclaved of *L. edodes*, that there was a stimulus on the germination of spores of *Colletotrichum sublineolum*. Tutida et al. (2007) also verified that the crude aqueous extracts of both mushrooms did not have significant effect on the germination of spores of *B. sorokiniana*.

According to Di Piero (2003) the extract of the basidiocarps, mainly those of *A. blazei*, stimulate the germination *in vitro* of spores of *C. lagenarium*, and caused an increase in the length of the germ tube of these spores in relation to those which germinated in the control treatment. In addition to that, the isolates LE 95/01 and LE 99/22 of *L. edodes*, tested at 10.0% (v/v), did not present effect *in vitro* over *Xanthomonas campestris* pv. *vesicatoria*, when it was applied on the concentration of 0.15 or 0.05 units of absorbance (U.A.), while the isolates ABL 97/11 and mainly ABL 99/28 of *A. blazei* stimulate the bacterial growth in both concentrations, showing that, in these situations, the nutritional aspect of the basidiocarp extracts surpass any antibiotic compound which could be present in them.

Adversely, positive results were obtained by Fiori-Tutida et al. (2007), in which extracts of mushrooms reduced the germination of spores of *P. recondita* f. sp. *tritici*, with emphasis to the isolate LE 96/17 of *L. edodes* which presented inhibition of approximately 52.4%. Moreover, Fiori-Tutida et al. (2001; 2002) verified the effect of the crude extract of *L. edodes* in the inhibition of the spore germination of *Puccinia recondita* f. sp. *tritici*; however, the crude extract obtained by *A. blazei* did not inhibit

Table 1. Effect of the extract of *Agaricus blazei* (5.0%), over the disease index (%) in 'Itália' grape brunches, inoculated with *Botrytis cinerea* after the treatment and stored at 25±1 °C / 80-90% RH.

		Time interval between treatment and inoculation ^z					
		Treatment	24 h	48 h	72 h	96 h	Mean
2 ^x	With Agaricus		10.17 ^y Aa	9.84 ABa	12.84 Aa	5.30 Ba	** 9.53 a
	Without Agaricus		12.00 Aa	10.00 ABa	6.83 Bb	7.00 ABa	* 8.96 a
			Ns	Ns	**	Ns	
	Mean		11.08 A	9.92 A	9.83 A	6.15 B	
			**				
		C.V. (%)	23.76				
			24 h	48 h	72 h	96 h	Mean
3	With Agaricus		22.00 ABa	22.35 ABa	32.67 Aa	14.67 Ba	** 22.92 a
	Without Agaricus		30.00 Aa	23.67 Aa	19.34 Ab	17.67 Aa	ns 22.67 a
			Ns	Ns	**	Ns	
	Mean		26.00 A	23.01 AB	26.00 A	16.17 B	
			*				
		C.V. (%)	25.32				
			24 h	48 h	72 h	96 h	Mean
4	With Agaricus		41.83 ABa	48.83 ABa	57.67 Aa	29.00 Ba	** 44.33 a
	Without Agaricus		49.50 Aa	43.33 Aa	34.83 Ab	34.17 Aa	ns 40.46 a
			Ns	Ns	**	Ns	
	Mean		45.67 A	46.08 A	46.25 A	31.58 A	
			*				
		C.V. (%)	22.42				

^x Days of storage

^y Average of ten replications. Means followed by the same uppercase letter in line, and lowercase in column do not differ statistically (Tukey $P \leq 0,05$). Original data were transformed in $\sqrt{x+0,5}$, for statistic analysis.

^z Significance of the F test of the analysis of variance to the effect of the treatment over the disease index, n.s. = non significant; *,** = significant at 5 and 1% of probability, respectively.

Interaction = **, * and *, for 2, 3 and 4 days of storage, respectively.

the germination of spores of *B. sorokiniana*, even though both extracts have been capable to induce the accumulation of phytoalexins in soybean cotyledons.

The evaluation of the effect of the basidiocarps extracts over the mycelia growth of *B. cinerea* revealed that *A. blazei*, as in the test of conidia germination, stimulates mycelia growth of the pathogen in doses superior to 2.5% in a significant way, however, with 96 h all the treatments were similar to the control (Figure 3a). One hypothesis to explain the stimulus,

both in conidia germination and in mycelial growth would be the presence or not only of proteins of high biological value found in the composition of mushrooms, but also of vitamins and chemical elements as phosphorus, magnesium, calcium, iron, among others (URBEN and OLIVEIRA, 1998).

Consequently, *L. edodes* was efficient to retard the mycelial growth of *B. cinerea* in the dose equal or superior to 10.0%, but the effect of the concentration have become less significant over time, once, 96 h

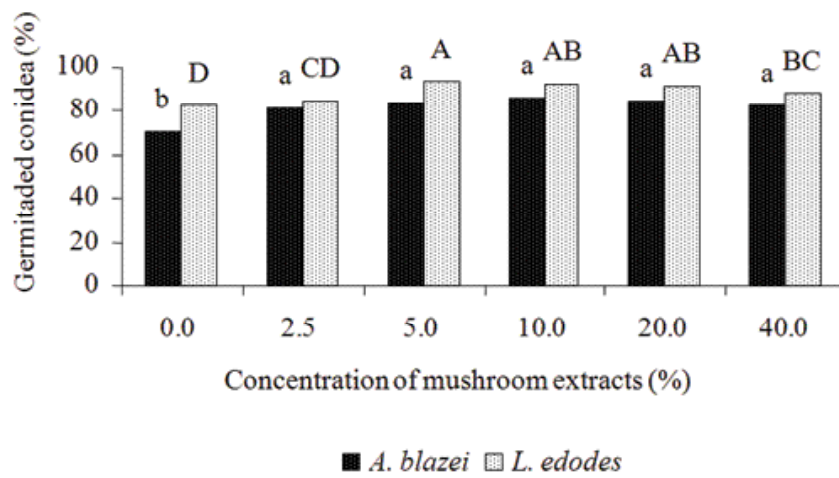


Figure 2. Effect of different concentrations of mushroom extracts, *in vitro*, over the germination of conidia of *Botrytis cinerea*, after 8 hours. Means followed by the same lowercase letter to *Agaricus blazei* and uppercase letter for *Lentinula edodes*, do not differ significantly (Tukey $P \leq 0,05$).

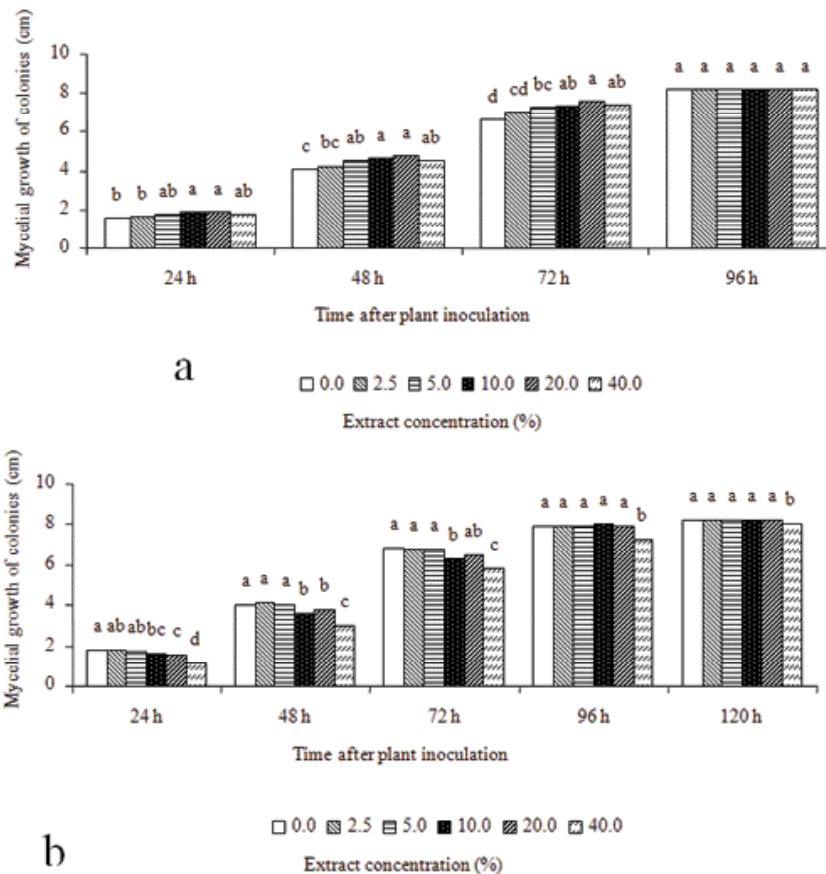


Figure 3. Effect of different concentrations of extracts from *Agaricus blazei* (a) and *Lentinula edodes* (b) over the mycelial growth of *Botrytis cinerea*. Means followed by the same letter do not differ significantly (Tukey $P \leq 0,05$).

after the plant inoculation, only the concentration of 40.0% differed from the control (Figure 4b)

These results are in accordance with those presented by Di Piero (2003), in which aqueous extracts of *L. edodes*, incorporated to PDA at 5.0% reduced the mycelial growth of *C. lagenarium*, however, the effect although statically significant, was lowly visible. At 10% there was no effect of the extracts of *L. edodes*, as well as of *A. blazei* over the mycelial growth of the phytopathogen. To Fiori-Tutida et al. (2007) the crude aqueous extract of both mushrooms did not have significant effect on the mycelial growth of *B. sorokiniana*.

It is verified, in a general way, that the extract of *A. blazei* did not inhibit the mycelial growth and the germination of conidia of *B. cinerea* *in vitro*, however, the results obtained in experience 2 *in vivo*, in which the extract of the basidiocarp was sprayed in 'Itália' grape branches showed disease

control, although non significant, when applied 96 hours before the branches were inoculated with the pathogen. This indicates a possible activation of resistance mechanisms in the fruit tissue.

Conclusion

Extracts of the mushrooms *Agaricus blazei* and *Lentinula edodes* did not control the rot caused by *B. cinerea* in 'Itália' grape branches when applied after the inoculation. While the extract of *A. blazei*, at 5.0%, applied 96 h before the inoculation of grapes with *B. cinerea*, reduces lightly the rot severity, even though not statistically significant. *In vitro*, both extracts of the basidiocarps stimulated the germination of conidia and of *B. cinerea* in the tested concentrations; concerning mycelial growth, *L. edodes* retards, while *A. blazei* stimulates.

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