Current Research in Environmental & Applied Mycology 7(2): 94–102 (2017) ISSN 2229–2225

www.creamjournal.org

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Doi 10.5943/cream/7/2/6

Antagonistic role of hypha and cell-free culture filtrates of medicinal mushrooms to *Verticillium* sp. and *Pythium* sp. fungal pathogens

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Owaid MN 2017 – Antagonistic role of hypha and cell-free culture filtrates of medicinal mushrooms to *Verticillium* sp. and *Pythium* sp. fungal pathogens. Current Research in Environmental & Applied Mycology 7(2), 94–102, Doi 10.5943/cream/7/2/6

Abstract

The aim of this experiment is to study the effects of interspecific interactions of three medicinal mushrooms (*Coriolus versicolor, Hericium erinaceus* and *Lentinula edodes*) on the growth of two fungal pathogens (*Verticillium* sp. and *Pythium* sp.) using dual culture plate assay. In single cultures in PDA plates, overgrowth of mushrooms was completed after 5, 11, and 12 days for *C. versicolor, L. edodes*, and *H. erinaceus* respectively. Generally, *C. versicolor* presented the fastest mycelial growth in dual culture after incubation for ten days. From another side, the best significant (p<0.01) inhibition was 67.13% and 65.28% by hyphal of *C. versicolor* toward *Verticillium* sp. and *Pythium* sp. respectively. While, the lower inhibition activity (26.76%) was recorded on *Verticillium* sp. plate by mycelia of *H. erinaceus* during 5 days. After 15 days, metabolites of fungi showed only on edges of *L. edodes* mushroom colonies in form shining golden drops. The solid medium of cell-free culture filtrate of *C. versicolor* exhibited best inhibition percentage of 6.67% against *Verticillium* sp. fungus significantly (p<0.01). While, *Pythium* sp. recorded the lower inhibition percentage on solid medium of *L. edodes* filtrate (2.27%). In general, *Pythium* sp. was more sensitive than *Verticillium* sp. on the solid media of cell–free culture filtrate of all mushrooms.

Key words – Dual culture – mycelial growth – *Coriolus versicolor* – *Hericium erinaceus* – *Lentinula edodes*

Introduction

Mushroom has been used as a precious food source and natural medicine in the ancient world (Wasser & Weis 1999). Generally, mycelia of medicinal mushroom and its cell–free culture filtrate are using as antimicrobial factors (Owaid et al. 2015). As a commercial mushroom, Shiitake mushroom and other medicinal mushrooms can be cultivated on cellulosic matters because it can degrade some organic matters, such as wheat straw and wood chips (Van Kuijk et al. 2016).

The medicinal mushrooms have inhibitory effect against microbes and fungi. Also, some fungal pathogens have negative effect toward cultivation of some important mushrooms. The shiitake mushroom *Lentinula edodes* is considered as antioxidant agent and good source of mineral elements and has efficient levels of carbohydrates, proteins, lipids, and crude fibers (Jang et al. 2015). Extracts of mycelia and biomass of *Coriolus versicolor* have inhibitory effect against drug–

Submitted 08 March 2017, Accepted 11 May 2017, Published 23 May 2017
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resistant bacteria (Zaidi et al. 2013, Duvnjak et al. 2016). and spent mushroom substrates of *Lentinula edodes* have antibacterial activity against Gram positive and Gram negative pathogenic bacteria (Zepeda-Bastida et al. 2016). Furthermore, exopolysaccharides of *Coriolus versicolor* exhibited strong antibacterial activities (Duvnjak et al. 2016).

Of species of *Verticillium* sp., *Verticillium fungicola* effects against fruiting initiation of *Agaricus bisporus* (Largeteau & Savoie 2008). Chaudhary & Tripathi (2016) studied the interaction between some wild fungi and mycoparasitic fungi, such as *Fusarium* sp., *Pythium* sp. and *Aspergillus* sp. in dual cultures plate assay that showed the deadlock with mycelial contact at 40% against fungi. *Hericium erinaceus* has also shown anti-fungal activity against the mold *Aspergillus niger*. Whereas, *L. edodes* considers a promising medicinal mushroom and it controls on infections by *Aspergillus parasiticus* and the aflatoxin production (Reverberi et al. 2005).

Changes in colony color of pairs of fungi in dual cultures due to diffusion of metabolites of *Aspergillus parasiticus*, which was stimulated by *Lentinula edodes* and this pigmentation occurred by phenoloxidase or peroxidase activities of mycelia (Li 1981, 1983). Some colonies interactions between plant pathogenic fungi and mushrooms in dual culture plate assay show synergistic intermingling growth without antagonistic effects (Parani & Eyini 2016). Bhadra et al. (2014) used Bavistin and Diathane M–45 fungicides to inhibit the growth of plant pathogenic fungi compared with *Trichoderma* sp. in dual cultures. Some studies reported inhibition of plant pathogenic fungi by mycelia of *Pleurotus* spp. (Borhani et al. 2011) and *Flammulina velutipes* (Angelini et al. 2008) in dual culture method.

Fungal interspecific interactions can change the function of mycelia, such as mycelia colonization, distribution of nutrients within mycelia and respiration. The changes in laccase due to the interaction of *Pleurotus ostreatus* mushroom with *Trichoderma longibrachiatum* are linked to defense reaction of *P. ostreatus* as they are associated with the produced brown lines at contact zones which limit the progression of *Trichoderma* sp. (Velazquez-Cedeno et al. 2007). The antagonism index is composed from deadlock with mycelial contact, deadlock at a distance, overgrowth without initial deadlock, partial replacement after initial deadlock with contact, complete replacement after initial deadlock with contact, partial replacement after initial deadlock at a distance and complete replacement after initial deadlock at a distance (Chaudhary & Tripathi 2016).

This work was achieved to check the sensitivity of some fungal pathogens by mycelia and cell-free culture filtrates of some famous medicinal mushrooms namely *Coriolus versicolor*, *Hericium erinaceus* and *Lentinula edodes*. This is important to use spent mushroom substrates of these mushrooms as a biocontrol against pathogens of plants and mushrooms in the organic systems in mushroom farms and agricultural fields. Thus this study seeks to access the antifungal activity against two pathogenic fungi *Verticillium* sp., and *Pythium* sp.

Materials & Methods

Strains of fungi

Three medicinal mushroom species were investigated in the current study namely *Coriolus versicolor*, *Hericium erinaceus* and *Lentinula edodes* which were obtained from Mushroom Box Co., UK. They were subcultured on potato dextrose agar (PDA) medium and stored at 25±1 °C until the use. Also, two varieties of fungal pathogens viz., *Pythium* sp. and *Verticillium* sp., grown on roots of Cress plant (*Lepidium sativum*), were obtained from Plant and Fungal Pathology Lab in College of Science, University of Anbar, Iraq.

The colonies interaction between pathogenic fungi and mushrooms

Antifungal activity of mycelia of medicinal mushrooms was investigated toward two pathogenic fungi on PDA using dual culture plate assay. PDA medium was autoclaved for 15 min at 121°C and poured within Petri dishes (9 cm). Culture plugs measuring 5 mm were made in Petri dishes of the stock cultures for both fungus and mushroom. A culture plug of 7–day–old mushroom

was placed on 3 cm away from the culture plug of the pathogenic fungus separately. Afterwards, inoculated plates were incubated for 7 days at 25±1°C. Those plates were checked and measured of zone of inhibition and recorded by comparing with the control. The number of days taken for growth completion over the mycelia of pathogens in plate was recorded (Owaid et al. 2017).

Collection of cell-free culture filtrate

Medicinal mushrooms were cultured in 50 ml of potato dextrose broth (PDB) using 10–day–old 5 mm fungal plug followed by incubation for 20 days at 25±1 °C and observed daily. The liquid cultures of mushrooms were filtered through Whatman No. 1 filter paper twice and pH adjusted to 7 with HCl (1N). The cell–free culture filtrate is used to test sensitivity of pathogenic fungi (Owaid et al. 2017).

Percentage inhibition of mycelial growth (PIMG) and biomass weight (PIBW)

Mushroom's cell-free culture filtrates were diluted to 50% (v/v) with potato dextrose broth (PDB), separately. Agar (1.5%) was added, dissolved, and autoclaved at 121 °C for 25 min. Fresh PDA plates were used as a control. The 7–day–old cultures of two pathogenic fungi were placed in the center of plates and incubated at 25±1 °C for ten days to detect the inhibitory action by measuring the radial growth of the fungal pathogens on the solid media of cell–free culture filtrate (R2) and the radial growth on fresh PDA plate as a control (R1). The PIMG was calculated by using following equation: PIMG = {(R1–R2)/R1}*100.

Determination of the percentage inhibition of biomass weight (PIMW) of the pathogenic fungi was done by diluting cell–free culture filtrates to 50% (v/v) with PDB, separately. After autoclaving, inoculation of pathogenic fungi was achieved using 50 ml of the new liquid medium in 250 ml of the conical flask and incubated at 25±1 °C for ten days, while PDB alone was used as a control. Afterwards, the mycelia were collected using filtering by Whatman No. 1 filter paper, dried and weighed. PIBW was assumed using the previous equation (Owaid et al. 2017).

Statistical analysis

The data have been expressed by their mean values. The results are collected in triplicates and subjected to one way and two ways analysis of variance (ANOVA) by SAS program (Version 9.0, SAS Institute Inc., USA). The significance of variance was determined according to Duncan's Multiple Range Test (DMRT). The value ≤ 0.01 was considered to be statistically significant.

Results

Properties of culture filtrates and mycelial growth of mushrooms

Characteristics of biomass, cell–free culture filtrate and mycelial growth of three medicinal mushrooms exhibited in (Table 1). The mycelial growth rate of *C. versicolor* (day 4th) was 67 mm/day as a higher average significantly ($p<0.01$), followed *L. edodes* and *H. erinaceus* of 25 and 13.5 mm/day respectively. Also, the growth rate is increasing with time (day 5th) at averages 85, 33 and 23 mm for *C. versicolor*, *L. edodes*, and *H. erinaceus*, respectively. Overgrowth of PDA plates was completed after 5, 11, and 12 days by *C. versicolor, L. edodes,* and *H. erinaceus,* respectively. *H. erinaceus* has a long time to overgrow on PDA significantly ($p<0.01$). Likewise, (Fig 1) exhibited that the mushroom *C. versicolor* has a rapid mycelial growth compared with the others.

In broth culture, all medicinal mushroom species changed pH of broth from 7 to approx. 5-6, see (Table 1). Furthermore, the cell–free culture filtrate of the mushroom *L. edodes* exhibited a higher concentration of hydrogen (pH) at average 6.09 significantly ($p<0.01$), then decreased to 5.25 and 4.99 for broth cultures of *H. erinaceus* and *C. versicolor* respectively. From another aspect, in broth culture sized 100 ml of PDB; the best–dried biomass formation was 0.42 g with *C. versicolor*, followed by *H. erinaceus* and *L. edodes* of 0.20 and 0.12 g respectively.

Table 1 Properties of medicinal mushrooms in solid and broth media.

Medicinal mushrooms	*In Potato dextrose broth*		*In Potato dextrose agar*		
	pH of the cell-free culture filtrate	**The dried biomass (g/100ml)**	**Mycelial growth rate in 4 days**	**Mycelial growth rate in 5 days**	**Overgrowth on PDA plates (days)**
L. edodes	0.12±0.01^{c}	6.09±0.02^{a}	25±0.57^{b}	33±0.57^{b}	**11±0.0^{b}**
H. erinaceus	0.20±0.0^{b}	5.25±0.02^{b}	13.5±0.28^{c}	23.5±0.28^{c}	**12±0.0^{a}**
C. versicolor	0.42±0.02^{a}	4.99±0.07^{c}	67±0.57a	85±0^{a}	**5±0.0^{c}**
The mean	**0.24±0.02**	**5.44±0.08**	**35.16±0.86**	**47.16±0.64**	p<0.01

Legend: ±MSD: Mean of standard deviation.

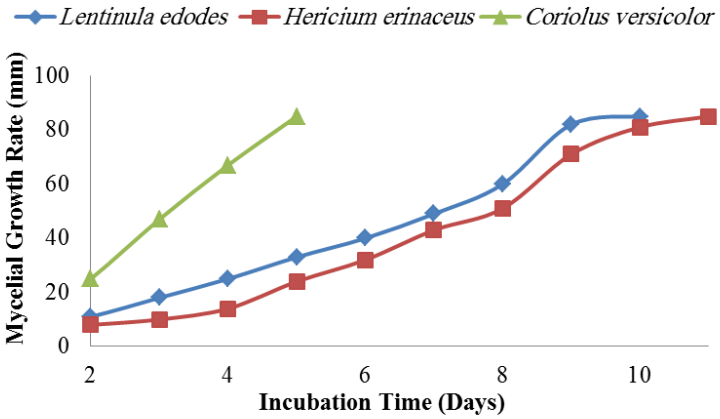


Fig 1 – Comparison study of mycelia growth rate (MGR) of three medicinal mushrooms.

Growth of pathogenic fungi in Dual cultures

The mycelial growth rate of pathogenic fungi *Verticillium* sp. and *Pythium* sp., showed in (Table 2), on PDA plates in dual cultures together with three medicinal mushrooms separately. The higher growth of *Verticillium* sp. and *Pythium* sp. has been showed at average 56.50 mm/d on PDA plates (control) in alone (single) cultures after 4 days. Culturing hypha of medicinal mushrooms with pathogens in the same plate showed inhibitory effects to each pathogenic fungus (in the dual culture). The growth of pathogenic fungi significantly (p<0.01) decreased by mycelia of *H. erinaceus* and *L. edodes* of average 48.66 mm/d and 41.66 mm/d respectively. While the lower growth observed of 23.66 mm/d and 22.66 mm/d for the growth of *Verticillium* sp. and *Pythium* sp. respectively on plates of *C. versicolor* mycelia (in dual culture). *Coriolus versicolor* was stronger one against pathogenic fungi in this experiment, followed by *L. edodes* and *H. erinaceus*, respectively. In day 4 of incubation, the fungal pathogen *Verticillium* sp. is more sensitive than the isolate *Pythium* sp. at averages 42.41 mm/d and 42.58 mm/d respectively.

Table 2 Mycelial growth of fungal pathogens in dual cultures in 4 days (mm).

The fungal pathogen	*Alone culture*	*Dual cultures with mycelia of mushrooms:*			*Mean of fungal pathogens*
		L. edodes	***H. erinaceus***	***C. versicolor***	
***Verticillium* sp.**	56.00±0^{a}	42.66±1.33^{d}	47.33±0.66^{c}	23.66±0.88^{e}	**42.41^{a}**
***Pythium* sp.**	57.00±0^{a}	40.66±0.66^{d}	50.00±1.15^{b}	22.66±0.66^{e}	**42.58^{a}**
Mean of mushrooms	**56.50^{a}**	**41.66^{c}**	**48.66b**	**23.16^{d}**	p<0.01

Legend: ±MSD: Mean of standard deviation.

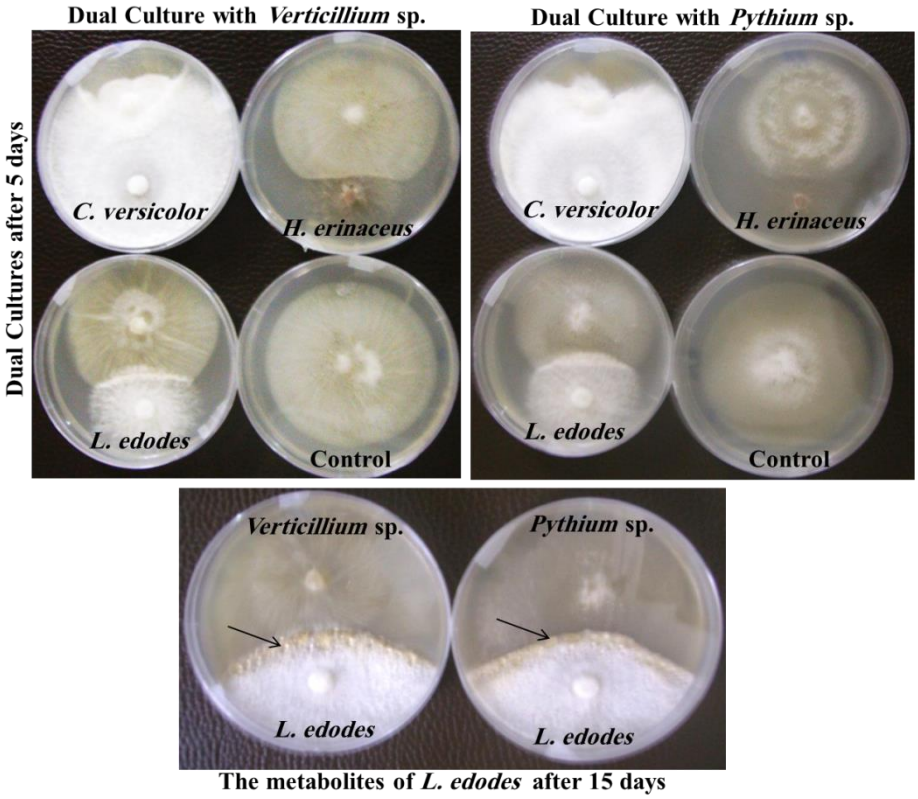
Inhibition activity of medicinal mushrooms mycelia against growth of *Verticillium* sp. and *Pythium* sp. has been studied in dual culture method after 5 days, was showed in (Table 3) and (Figs 2). Generally, the medicinal mushroom *C. versicolor* has a higher inhibitory effect (66.2%) against the studied pathogenic fungi. The best significant (p<0.01) inhibition was 67.13% and 65.28% by hypha of *C. versicolor* toward *Verticillium* sp. and *Pythium* sp. respectively. The moderate inhibition exhibited by mycelia of *L. edodes* (44.44% and 39.43%) against *Pythium* sp.

and *Verticillium* sp. respectively. While the lower inhibition activity was (26.76%) recorded on *Verticillium* sp. plate by mycelia of *H. erinaceus* within 5 days. After 15 days, only in dual culture of *L. edodes*, metabolites of fungi showed on edges of mushroom colonies in form shining golden drops of liquid. The finding of fungal pathogens together with *L. edodes* in PDA plate induce mycelia of this pathogen for secretion these secondary metabolism substances. The role of these metabolites is unknown and need more studies under electron microscope and biochemical tests for indication the mechanism of work. This mushroom could not overgrow on the fungal pathogens opposite overgrowth of other mushrooms on the pathogens in this study. But in broth culture, this mushroom could produce metabolites resistance to those pathogens. Pathogens invaded into territory of *L. edodes* then stopped and secreted metabolites both formed antithetic line with *H. erinaceus*. Fungal pathogens were slightly invaded or formed deadlock with *C. versicolor* which has fast overgrowth on pathogens.

Table 3 Inhibition percentage of fungal pathogens by mushroom hypha in dual cultures after 5 days.

Pathogenic Fungi	**Mycelia of Medicinal mushrooms**			**Mean**
	L. edodes	***H. erinaceus***	***C. versicolor***	
***Verticillium* sp.**	39.43±1.40^{c}	26.76±2.15^{d}	67.13±0.93^{a}	**44.44^{a}**
***Pythium* sp.**	44.44±0.00^{b}	29.63±0.92^{d}	65.28±1.39^{a}	**46.70^{a}**
Mean	**41.43^{b}**	**28.19^{c}**	**66.20^{a}**	p<0.01

Legend: ±MSD: Mean of standard deviation.



Figs 2 – Dual cultures between the fungal pathogens and three medicinal mushrooms.

Influence of cell-free culture filtrates of mushrooms against fungal pathogens in the agar media

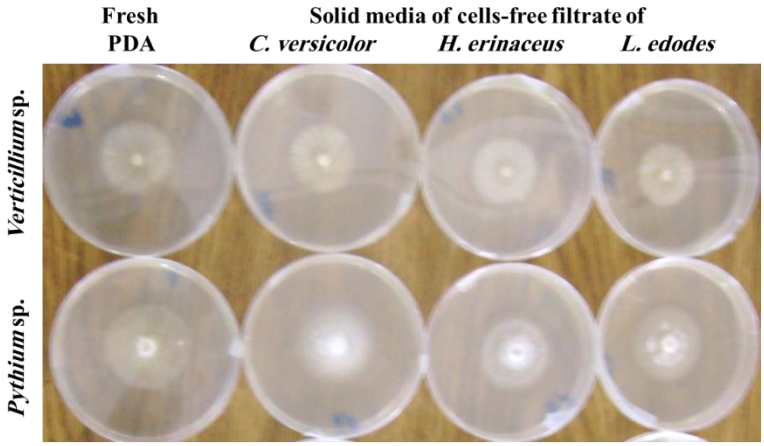
Inhibitory actions of filtrates of mushrooms after disposing of the fresh biomass (mycelia/cells) in their solid media to *Verticillium* sp. and *Pythium* sp. after 3 days were shown in (Table 4) and (Figs 3). The solid medium of cell-free culture filtrate of *C. versicolor* exhibited best inhibition percentage of 6.67% against the fungal pathogen *Verticillium* sp. significantly (p<0.01).

The inhibitory effect decreased to 5.33% and 4.55% on the media of cell-free culture filtrates of *L. edodes* and *C. versicolor* against *Verticillium* sp. and *Pythium* sp., respectively. The lower inhibition was recorded for the pathogen *Pythium* sp. on solid media of *L. edodes* filtrate (2.27%). Also, the solid media of *H. erinaceus* showed an inhibitory potent of mean 3.37% against the studied pathogenic fungi after 3 days. Furthermore, the fungal pathogen *Pythium* sp. was more sensitive than *Verticillium* sp. with inhibition percentage 3.55% and 5.11% respectively on agar media of filtrates of all medicinal mushrooms in this investigation, in significant (p<0.01).

Table 4 Inhibition activity of the solid media of cell-free culture filtrate of medicinal mushrooms toward fungal pathogens after 3 days (%).

The fungal pathogen	**Solid media of cell-free culture filtrate of medicinal mushrooms**			**Mean of fungal pathogens**
	L. edodes	***H. erinaceus***	***C. versicolor***	
***Verticillium* sp.**	5.33±0.77^{ab}	3.33±1.15^{bc}	6.67±0.00^{a}	**3.55^{b}**
***Pythium* sp.**	2.27±0.00^{c}	3.41±0.65^{bc}	4.55±0.00^{ab}	**5.11^{a}**
Mean of mushroom filtrates	**4.10^{b}**	**3.37^{b}**	**5.61^{a}**	p<0.01

Legend: ±MSD: Mean of standard deviation.



Figs 3 – Mycelia growths of fungal pathogens in soild media of cell-free culture filtrate of three medicinal mushrooms.

Discussion

The relationship between fungal pathogen and mushroom indicates the invasive nature of the hyphae of the mycoparasites through penetration of the hyphal mushroom, then degeneration and death of the invaded mycelia (Jeffries & Young 1994). The specify interaction between the fungal pathogens and *L. edodes* leads to laccase production in extracellular (Savoie et al. 1998), may be reflects on the dark color of the colony as shown in (Figs 2). *C. versicolor* and *L. edodes* showed overgrowth without initial deadlock or partial replacement after initial deadlock at contact, which agreed with Badalyan et al. (2004). The strength of mycelial mushroom to overgrow and cover mycelia of fungal pathogens is variant according to mushroom species. That appeared firstly at incubation time, mycelia of *L. edodes* was slightly invaded or build deadlock against fungal pathogens (Lee et al. 2008). The repulsion take places between various fungal colonies when pairs in dual cultures, which shows cellular destroying on both sides (Dekan 1983). Recently, by Scanning Electron Microscopy images, researchers could determine the dark zones between the fungi in dual cultures due to form compounds of melanin that protect hyphal cells and they become resistant to attack fungal pathogens. Formation of melanin is a part of the defensive response toward fungal pathogens that helps mushrooms to adapt to the environmental stress (Lee et al. 2008).

There are limited data about inducing mycelia for metabolites synthesis in mycelial interaction, thus we tested those mushrooms against some locally fungal pathogens that may be

used in biotechnology of inducing production metabolites in the future. The understanding of this specific interaction may be a useful term for biocontrol of green mold diseases during *L. edodes* cultivation and needs new investigations. All results agree with results of Chaudhary & Tripathi (2016) who mentioned that medicinal mushrooms produce metabolites with antifungal characteristics. From another side, Owaid et al. (2017) investigated the antifungal activities of four *Pleurotus* species toward *Pythium* sp., *Trichoderma harzianum*, and *Verticillium* sp. on PDA using dual cultures; and the highest sensitivity was recorded with *Verticillium* sp. fungus, while, mycelia of some mushrooms didn't overgrow the mycelia of *Verticillium* sp. fungus.

However, the measurement of the level of susceptibility is difficult because it influences by many factors and needs to have very well controlled conditions of cultivation or to increase dramatically the number of replications of the experiment (Savoie & Largeteau 2004). Production of antibiotic by *Agaricus bisporus* mushroom has been suggested as one of the factors of resistance to *Verticillium fungicola* in a similar method as in this work (Goulas 1987) because of fungistatic effects on the growth of commercial mushrooms in the mushroom farms.

Metabolites of *Pleurotus* spp. mycelia have a good role against pythopathogenic fungi which have a lower sensitive, according to the genetic properties of mushrooms which produced various metabolites in broth cultures (Owaid et al. 2017). Antifungal factors, such as chitinase and protease in the cell–free culture filtrate of medicinal mushrooms could control fungal pathogens in broth cultures (Hassan et al. 2011). *Verticillium* sp. mycelia had the highest inhibition zone in cell–free culture filtrate of oyster mushroom (Owaid et al. 2017).

Sterilization of cell–free culture filtrates of medicinal mushrooms by the autoclaving process leads to change structures of proteins. High pressure thermal in this process results reversible or irreversible, partial or complete enzyme inactivation in the fungal broth. Thus, cell–free culture filtrates of mushrooms showed variable inhibitory effects against *Verticillium* sp. and *Pythium* sp. as referred by Owaid et al. (2017). The reason of that may be return to enhancing or retarding enzymatic reactions by pressure and fungal proteins become less resistance to enzymatic depolymerisation while the polysaccharides stay sable by pressure (Ludikhuyze et al. 2001).

Conclusion

The aim of this work is to investigate the effects of interspecific interactions of three medicinal mushrooms viz., *Coriolus versicolor, Hericium erinaceus* and *Lentinula edodes* on the growth of two fungal pathogens namely *Verticillium* sp. and *Pythium* sp. using dual culture plate assay. In single cultures in PDA plates, overgrowth of mushrooms was completed after 5, 11, and 12 days for *C. versicolor*, *L. edodes*, and *H. erinaceus*, respectively. Generally, *C. versicolor* presented the fastest mycelial growth in dual culture after incubation for ten days and inhibited growth of *Verticillium* sp. and *Pythium* sp. significantly ($p<0.01$) at percentages 67.13% and 65.28%, respectively. After 15 days, metabolites of fungi showed only on edges of *L. edodes* mushroom colonies in form shining golden drops. The solid medium of cell–free culture filtrate of *C. versicolor* exhibited best inhibition percentage of 6.67% against *Verticillium* sp. fungus significantly ($p<0.01$). Generally, *Pythium* sp. was more sensitive than *Verticillium* sp. on the solid media of cell–free culture filtrate of all mushrooms.

Acknowledgements

The author thanks Department of Biology, College of Science for obtaining the fungal pathogens and helping to achieve this work in the Plant and Fungal Pathology Labour in University of Anbar, Iraq.

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