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# Volatile organic compounds emitted by mycoparasitic fungi *Hypomyces perniciosus* and *Cladobotryum mycophilum* suppress the growth of *Agaricus bisporus*

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*Hypomyces perniciosus* and *Cladobotryum mycophilum* are mycoparasitic fungi infecting *Agaricus bisporus* and causing wet bubble and cobweb diseases, respectively. In this work, the role of volatile organic compounds (VOCs) emitted by these fungal mycoparasites in the suppression of *A. bisporus* was investigated.

The VOCs of *H. perniciosus* and *C. mycophilum* effectively reduced the mycelial growth of *A. bisporus* by 60% and 73% after 5 days of incubation, respectively, compared to that of the control as assessed by the two-sealed-base-plates assay. Further, the VOCs of *H. perniciosus* and *C. mycophilum* were collected in a headspace solid-phase microextraction procedure, and their components analysed by means of gas chromatography-mass spectrometry. Ethanol was identified as the major volatile component in *H. perniciosus* and *C. mycophilum*. Ethanol vapour significantly retarded the growth of *A. bisporus* in an in vitro assay. The results of this study suggest that ethanol produced by *H. perniciosus* and *C. mycophilum* could be involved in the suppression of *A. bisporus*.

**Key words:** antifungal, cobweb, ethanol, wet bubble, white button mushroom.

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*Hypomyces perniciosus* a *Cladobotryum mycophilum* jsou mykoparazitické houby, které infikují *Agaricus bisporus* a způsobují symptomy známé jako mokrá hniloba a pavučinová plíseň. V této

práci byla zkoumána role těkavých organických látek, vylučovaných těmito mykoparazity, v potlačení růstu *A. bisporus*.

Těkavé látky z *H. perniciosus* a *C. mycophilum* účinně inhibují růst mycelia *A. bisporus*; metodou dvou přiložených spodních misek byla zjištěna redukce o 60 %, respektive 73 % po 5 dnech od inkubace ve srovnání s kontrolní kulturou. Dále byly těkavé látky z kultur obou mykoparazitů shromážděny procesem mikroextrakce na tuhou fázi a jejich složky následně analyzovány plynovou chromatografií s hmotnostní spektrometrií. Jako hlavní těkavá složka byl u obou druhů zjištěn etanol, jehož pára v pokusu in vitro zřetelně brzdila růst *A. bisporus*. Výsledky studie naznačují, že etanol produkovaný druhy *H. perniciosus* a *C. mycophilum* se může podílet na potlačení růstu žampionů.

## INTRODUCTION

Button mushroom [*Agaricus bisporus* (Lange) Imbach] is one of the most popular and commercially cultivated edible mushrooms worldwide. Diseases, pests and nematodes are major constraints in the commercial production of *A. bisporus*. Several fungal pathogens including *Hypomyces perniciosus* Magnus [*Mycogone perniciosa* (Magnus) Delacroix], *Lecanicillium fungicola* (Preuss) Zare et W. Gams [*Verticillium fungicola* (Preuss) Hassebrauk], *Cladobotryum mycophilum* (Oudem.) W. Gams et Hoozem and *Trichoderma aggressivum* Samuels et W. Gams infect *A. bisporus* and cause various diseases (Berendsen et al. 2010, Gea et al. 2021). The species *H. perniciosus* and *C. mycophilum* cause wet bubble and cobweb diseases, respectively, in white button mushroom and drastically reduce its yield and quality (Gea et al. 2021). The appearance of whitish cobweb-like mycelial growth on the fruitbodies and casing soil, brown or black lesions on the fruitbodies, and discolouration and decay of fruitbodies are the typical symptoms of cobweb disease (Back et al. 2010). Yield losses of 6–38% due to cobweb disease in different strains of *A. bisporus* were reported in a study under conditions of artificial inoculation (Muhammad et al. 2019). *Hypomyces perniciosus* infection on *A. bisporus* induces deformation of fruitbodies and exudation of brown-coloured liquid (Fletcher et al. 1995, Fu et al. 2016).

It is important to understand the mechanisms of pathogenesis in mycoparasites to identify and develop new resistant strains of *A. bisporus* and to devise appropriate control measures. The production of cell wall degrading enzymes and toxins by *Trichoderma aggressivum*, the cause of green mould disease, in an antagonistic interaction with *A. bisporus* has been documented (Krupke et al. 2003, Guthrie et Castle 2006, Abubaker et al. 2013). By genome sequencing, Xu et al. (2020) described many genes associated with pathogenicity and virulence in *Cladobotryum dendroides*. Amey et al. (2003) demonstrated the role of β-1,6-glucanase produced by *Lecanicillium fungicola*, which causes dry bubble disease in button mushroom, in the infection of *A. bisporus*. Li et al. (2019), while analysing the genome of a highly virulent HP10 strain of *H. perniciosus*, identified certain

genes encoding protein kinases, carbohydrate-active enzymes, peptidases, cytochrome P450, and secondary metabolites which are essential for adaptation to harsh environments and mycoparasitism. Huang et al. (2014) reported *A. bisporus* growth inhibition and formation of mycelial knots and breakages by volatile compounds of *H. perniciosus*. Al-Harrasi et al. (2021) reported the presence of phenolic acids, mainly syringic acid, in culture filtrate of *H. perniciosus*, which induced electrolyte leakage from *A. bisporus* mycelium.

In this study, the antifungal effect of volatile organic compounds (VOCs) produced by *H. perniciosus* and *C. mycophilum* on *A. bisporus* was evaluated. Further, the composition of VOCs of the above fungal mycoparasites was determined using headspace solid-phase microextraction (HS-SPME) gas chromatography coupled to mass spectrometry (GC-MS).

## MATERIAL AND METHODS

Fungal mycoparasites. Cultures of mycoparasitic fungi, viz. *Hypomyces perniciosus* (culture ID: VLH1001; GenBank accession number MZ149255) and *Cladobotryum mycophilum* (culture ID: VLC1002; GenBank accession number OL342770) originally isolated from diseased white button mushroom were obtained from the Department of Plant Sciences, Sultan Qaboos University and used in this study. Until use, the fungal cultures were maintained on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, UK) medium at 4 °C.

*Agaricus bisporus* culture. A pure culture of *Agaricus bisporus* was obtained from fresh mushroom fruitbodies collected from the Gulf Mushroom Products Company, Barka, Sultanate of Oman. The mushroom was surface sterilised with 70% ethanol by using a sterile cotton swab and cut into halves longitudinally by using a sterile scalpel. A small portion of the tissue connecting the cap and stipe was transferred aseptically onto PDA medium and incubated at 25 °C for 5–7 days. The fungus was purified using the hyphal tip culture method and identified based on morphological characters and confirmed by ribosomal RNA-encoding DNA (rDNA) internal transcribed spacer (ITS) sequence analysis (White et al. 1990). The culture was maintained on PDA medium and stored at 4 °C.

Testing production of antifungal volatile compounds by mycoparasites. The antifungal effect of VOCs against *A. bisporus* emitted by the mycoparasites was tested by using the two-sealed-base-plates assay as described by Li et al. (2015). The bottom plates of two Petri dishes containing sterilised PDA medium were used for this assay. A mycelial disc (6 mm diameter) of *A. bisporus* taken from a 7-day-old PDA culture by using a sterilised cork borer was transferred to the centre of a PDA plate. In the same manner, a mycelial disc (6 mm diameter) of *H. perniciosus* / *C. mycophilum* was placed on another PDA plate. Both the inoculated plates were placed face-to-face and wrapped with two layers of parafilm and incubated at 27 °C. The diameter mycelial growth of *A. bisporus* was measured and the inhibition percentage was determined after 5 days of incubation. A non-inoculated PDA plate paired with a PDA plate inoculated with a mycelial disc of *A. bisporus* was used as control. Each treatment included six replicates.

Analysis of volatile compounds. *Hypomyces perniciosus* and *Cladobotryum mycophilum* were cultured in 10 ml of sterilised potato dextrose broth in 25-ml glass vials for 72 h at 27 °C. The volatile compounds released by the mycoparasites were collected separately by using the headspace solid-phase microextraction technique as described by Jayakumar et al. (2021) and analysed

with GC-MS using a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corporation, Kyoto, Japan) fitted with a Rtx-5MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness), coupled to a Shimadzu QP-2010 ultra-mass spectrometer. The runtime was 32 minutes 54 seconds. The National Institute of Standards and Technology (NIST) 2011 Version 2.3 and Wiley 9th edition (Wiley) mass spectrum libraries were used for identification of the compounds.

Testing antifungal activity of ethanol against *A. bisporus*. A 6-mm diameter mycelial plug of *A. bisporus* obtained from a 7-day-old PDA culture was placed in the centre of a PDA plate. Sterile filter paper discs (Whatman no. 1; 6-mm diameter) containing 99.9% pure ethanol (0, 50 and 100 µl) were placed on the inside of the lid. The plates were sealed carefully with two layers of parafilm and incubated at 27 °C. After 3 days of incubation, the radial growth of the fungus was measured using a ruler. Each treatment included six replicates.

Statistical analysis. The data were analysed using analysis of variance (ANOVA), and mean separation was performed using Duncan's multiple range test (DMRT) at the 5% level (SAS v8; SAS Institute, Cary, NC, USA).

## RESULTS

The two-sealed-base-plates assay revealed that the VOCs emitted by *H. perniciosus* and *C. mycophilum* inhibited the growth of *A. bisporus* and showed 60% and 73% reduction, respectively, in mycelial growth after 5 days of incubation compared to that of the control ($P < 0.05$) (Tab. 1; Fig. 1).

**Tab. 1.** Inhibition of *Agaricus bisporus* by volatile compounds produced by *Hypomyces perniciosus* and *Cladobotryum mycophilum*.
Data were recorded after 5 days of incubation. Data are the means of six replications.
Means within a column followed by a different letter are significantly different at $P = 0.05$.

| Treatment | Growth diameter of *A. bisporus* (cm) | % reduction |
|---|---|---|
| *Hypomyces perniciosus* | 3.36 b | 59.7 |
| *Cladobotryum mycophilum* | 2.25 b | 73.0 |
| Control | 8.33 a | – |

The analysis of headspace microbial VOCs by GC-MS revealed that the major VOCs released by *H. perniciosus* were ethanol (52.7%) and acetone (11.8%) (Tab. 2; Fig. 2A). Ethanol (45.5%), 2-heptanone (13.9%) and 5-hydroxy-8a-methyloctahydro-1(2H)-naphthalenone (11.3%) were the major VOCs produced by *C. mycophilum* (Tab. 3; Fig. 2B). In the headspace of non-inoculated PDA medium (control), butanal, 3-methyl- (28.47%), ethanol (16.7%), butanal, 2-methyl- (15.94%) and pentanal, 2-methyl- (11.98%) were detected (Tab. 4; Fig. 2C).

The results of this study also indicated that vapour from pure ethanol at a dose of 50 µl/disc and 100 µl/disc showed 24.4% and 40.3% inhibition, respectively, of mycelial growth of *A. bisporus* in an in vitro assay (Tab. 5; Fig. 3).

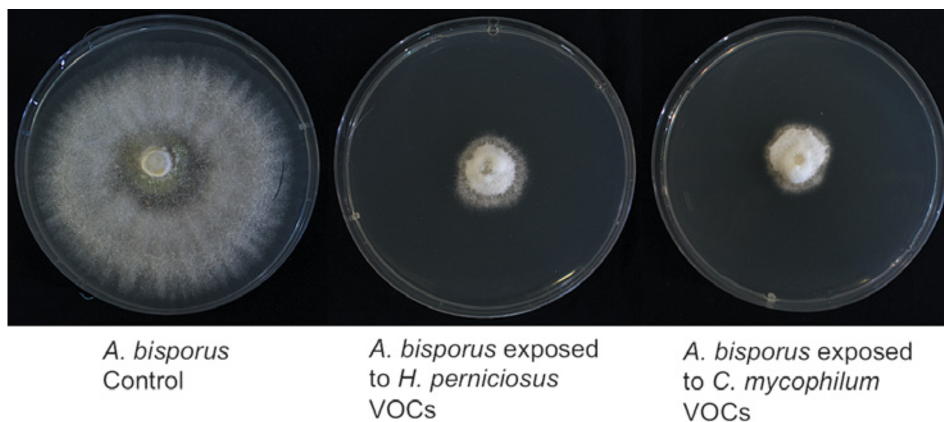
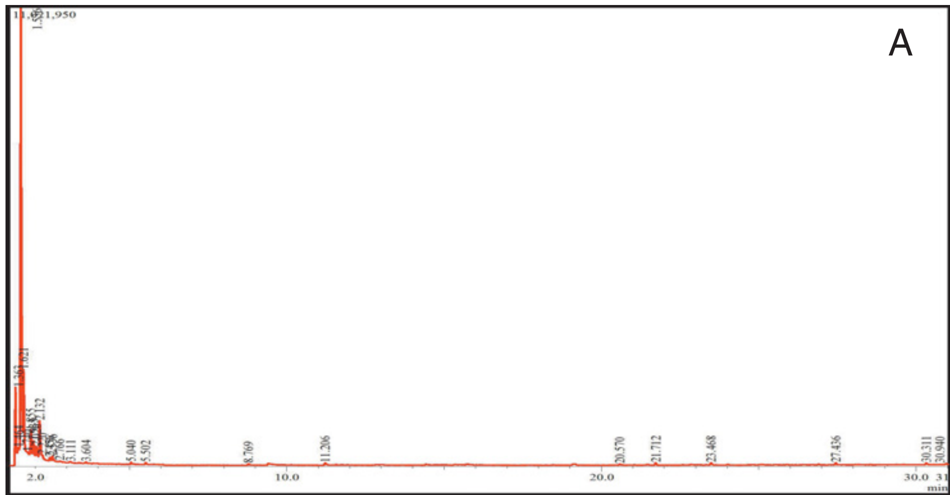



**Fig. 1.** Inhibition of mycelial growth of *Agaricus bisporus* by volatile compounds of *Hypomyces perniciosus* and *Cladobotryum mycophilum* as assessed by means of the two-sealed-base-plates method.

**Tab. 2.** Volatile compounds produced by *Hypomyces perniciosus*.

| Compound | Retention time (min) | Peak area | Peak area % |
|---|---|---|---|
| Carbamic acid, monoammonium salt | 1.363 | 4170918 | 7.61 |
| Cyclobutanol | 1.464 | 1600856 | 2.92 |
| Ethanol | 1.536 | 28886399 | 52.72 |
| Acetone | 1.621 | 6486859 | 11.84 |
| 1,2-Propanediol, 3-chloro- | 1.740 | 1468981 | 2.68 |
| Silanol, trimethyl- | 1.855 | 2523484 | 4.60 |
| Acetic acid | 1.935 | 1764380 | 3.22 |
| 2-Butanone | 2.026 | 1582399 | 2.89 |
| Ethyl acetate | 2.132 | 3260453 | 5.95 |
| 1-Propanol, 2-methyl- | 2.220 | 884804 | 1.61 |
| Butanal, 3-methyl- | 2.456 | 513999 | 0.94 |
| 1-Butanol | 2.536 | 390052 | 0.71 |
| Silanediol, dimethyl- | 2.766 | 51578 | 0.09 |
| 2,4-Dimethylfuran | 3.111 | 62149 | 0.11 |
| Disulfide, dimethyl | 3.604 | 80897 | 0.15 |
| Undecane, 4,4-dimethyl- | 5.040 | 109950 | 0.20 |
| 2,4-Dimethyl-1-heptene | 5.502 | 67002 | 0.12 |
| Benzaldehyde | 8.769 | 96647 | 0.18 |
| Benzaldehyde, 2-hydroxy- | 11.206 | 170959 | 0.31 |
| .alpha.-Cubebene | 20.570 | 53285 | 0.10 |
| 1,5,9,11-Tridecatetraene, 12-methyl-, (E,E)- | 21.712 | 131125 | 0.24 |
| Ethyl 3,4,5-tris[(trimethylsilyl)oxy]benzoate | 23.468 | 186568 | 0.34 |
| Unidentified | 27.436 | 109518 | 0.20 |
| 2,4-Diphenyl-4-methyl-1-pentene | 30.311 | 88855 | 0.16 |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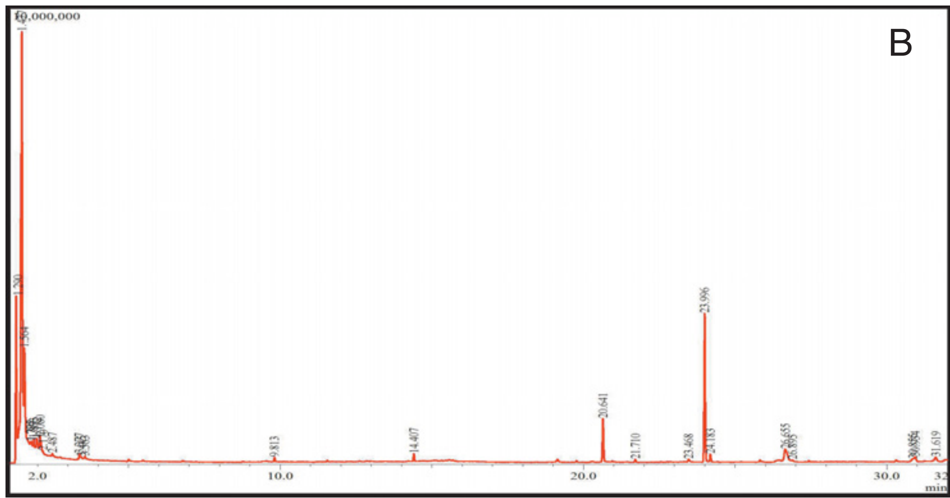
B
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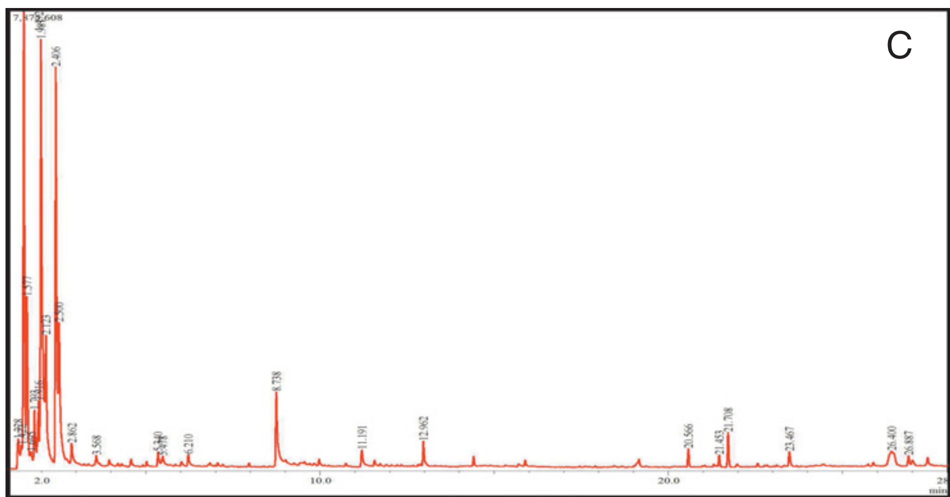
C
2.406
1.577
2.123
2.530
2.862
3.568
6.210
8.738
11.191
12.962
20.566
21.453
21.708
23.467
26.400
26.887
2.0
10.0
20.0
min

◀ **Fig. 2.** Chromatogram of volatile compounds in the headspace of nutrient broth inoculated with *Hypomyces perniciosus* (**A**) and *Cladobotryum mycophilum* (**B**) and non-inoculated nutrient broth (control) (**C**).

**Tab. 3.** Volatile compounds produced by *Cladobotryum mycophilum*.

| Compound | Retention time (min) | Peak area | Peak area % |
|---|---|---|---|
| Carbon dioxide | 1.290 | 7739532 | 10,00 |
| Ethanol | 1.477 | 35250058 | 45.53 |
| 2-Heptanone | 1.564 | 10747948 | 13.89 |
| Ethanethioamide | 1.775 | 824369 | 1.07 |
| Silanol, trimethyl- | 1.797 | 1055049 | 1.36 |
| Acetic acid | 1.873 | 1451491 | 1.88 |
| 2-Butanone | 1.972 | 1871488 | 2.42 |
| Ethyl acetate | 2.080 | 1774562 | 2.29 |
| 1-Propanol, 2-methyl- | 2.170 | 223941 | 0.29 |
| 1-Butanol | 2.487 | 144479 | 0.19 |
| 1-Butanol, 3-methyl- | 3.377 | 304849 | 0.39 |
| Sulphuric acid dibutyl ester | 3.445 | 174301 | 0.23 |
| Disulfide, dimethyl | 3.563 | 201327 | 0.26 |
| Cyclotetrasiloxane, octamethyl- | 9.813 | 207385 | 0.27 |
| Cyclopentasiloxane, decamethyl- | 14.407 | 373605 | 0.48 |
| (+)-.alpha.-Longipinene | 20.641 | 2527134 | 3.27 |
| trans-.alpha.-Bergamotene | 21.710 | 165065 | 0.21 |
| Ethyl 3,4,5-tris[(trimethylsilyl)oxy]benzoate | 23.468 | 167191 | 0.22 |
| 5-Hydroxy-8a-methyloctahydro-1(2H)-naphthalenone | 23.996 | 8765706 | 11.33 |
| 3-Aminomethyl-3,5,5-trimethylcyclohexanol | 24.183 | 461643 | 0.60 |
| Mandelic acid, 3,4-dihydroxy, tetrakis-TMS | 26.655 | 1828468 | 2.36 |
| Unidentified | 26.895 | 157092 | 0.20 |
| Unidentified | 30.855 | 287287 | 0.37 |
| Oxazepam, 2TMS derivative | 30.934 | 349338 | 0.45 |
| 1-Hexacosanol | 31.619 | 340352 | 0.44 |

**Tab. 4.** Volatile compounds detected in the headspace of non-inoculated nutrient broth (control).

| Compound | Retention time (min) | Peak area | Peak area % |
|---|---|---|---|
| l-Alanine ethylamide, (S)- | 1.270 | 1224824 | 1.55 |
| Ethanol | 1.451 | 13201807 | 16.70 |
| Pentanal, 2-methyl- | 1.538 | 9466419 | 11.98 |
| Methane, oxybis[chloro- | 1.680 | 1101415 | 1.39 |
| Propanal, 2-methyl- | 1.753 | 4875736 | 6.17 |
| Pentane, 2,4-dimethyl- | 1.935 | 5007484 | 6.33 |
| Trichloromethane | 2.077 | 885832 | 1.12 |
| Cyclopentane, methyl- | 2.160 | 379751 | 0.48 |
| Butanal, 3-methyl- | 2.368 | 22502861 | 28.47 |

▶

| Compound | Retention time (min) | Peak area | Peak area % |
|---|---|---|---|
| Butanal, 2-methyl- | 2.457 | 12600669 | 15.94 |
| Disulfide, dimethyl | 3.537 | 689520 | 0.87 |
| Toluene | 3.907 | 2039858 | 2.58 |
| Benzaldehyde | 8.721 | 1053962 | 1.33 |
| Unidentified | 10.534 | 247788 | 0.31 |
| Hexadecane | 10.850 | 593401 | 0.75 |
| Benzeneacetaldehyde | 11.185 | 235525 | 0.30 |
| Decane, 2,2,6-trimethyl- | 11.435 | 392681 | 0.50 |
| Heptane, 4-ethyl-2,2,6,6-tetramethyl- | 11.716 | 204122 | 0.26 |
| Heptalene, 7,7'-dihydro-6,6'-bis(trimethylsilyl)methyl- | 14.396 | 528132 | 0.67 |
| 1-Decanol | 17.776 | 208022 | 0.26 |
| 1-Decanol, 2-hexyl- | 18.137 | 194014 | 0.25 |
| Heptane, 2,2,4,6,6-pentamethyl- | 19.149 | 815526 | 1.03 |
| Bicyclo[5.2.0]nonane, 2-methylene-4,8,8-trimethyl-4-vinyl- | 21.708 | 286233 | 0.36 |
| Propanoic acid, 2,2-dimethyl-, 2-ethylhexyl ester | 25.669 | 313401 | 0.40 |

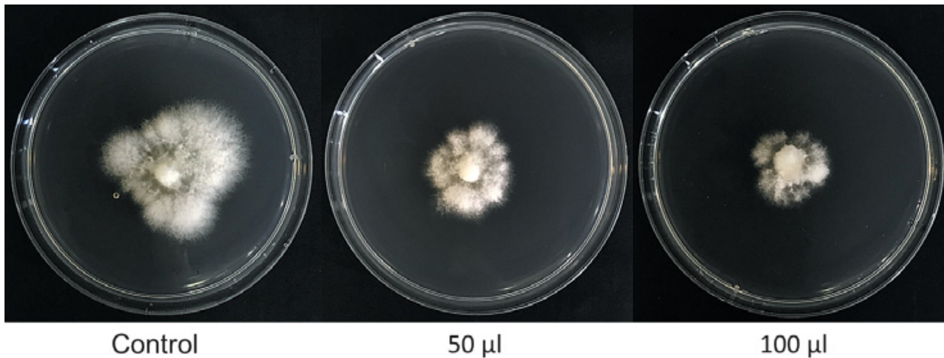


**Fig. 3.** Inhibition of *Agaricus bisporus* by vapour from pure ethanol.

**Tab. 5.** Inhibition of *Agaricus bisporus* by ethanol vapour.
Data were recorded after 3 days of incubation. Data are the means of six replications.
Means within a column followed by a different letter are significantly different at $P = 0.05$.

| Ethanol vapour treatment (µl/disc) | Growth diameter of *A. bisporus* (cm) | % reduction |
|---|---|---|
| 50 µl | 2.95 b | 24.4 |
| 100 µl | 2.33 c | 40.3 |
| Control | 3.90 a | – |

The source data for Tables 1 and 5 are available in Mendeley Data, V1, DOI: https://doi.org/10.17632/dvh84sntzz.1.

## DISCUSSION

The production of antifungal volatiles by antagonistic fungi is a common phenomenon (Yalage Don et al. 2020, Speckbacher et al. 2021). For instance, Li et al. (2015) demonstrated the production of antifungal VOCs by *Ceratocystis fimbriata* and their effectiveness in the control of peach brown rot (caused by *Monilinia fructicola*) and citrus green mould (caused by *Penicillium digitatum*). The antifungal activity of ethanol against various plant pathogenic fungi has been documented in other studies. The efficacy of ethanol fumigation in the control of *Botrytis cinerea* in grapes under postharvest storage conditions has been described (Mlikota Gabler et al. 2004, Chervin et al. 2005). Ethanol in combination with $CaCl_2$ (Chervin et al. 2005) and $C_6H_7KO_2$ (Guha et Basak 2013) has been reported to control *B. cinerea* more effectively. Mlikota Gabler et al. (2004) reported that the germination of conidia of *Alternaria alternata* was inhibited when exposed to ethanol. Yalage Don et al. (2020), when studying VOCs produced by the biocontrol agent *Aureobasidium pullulans* for antagonistic actions against *Alternaria alternata* and *Botrytis cinerea*, found that ethanol and 2-phenylethanol were the key inhibitory VOCs. Speckbacher et al. (2021) reported the production of ethanol, acetone and 2-heptanone by the mycoparasitic fungus *Trichoderma atroviride* during co-cultivation with its host *Fusarium oxysporum*. More release of 2-heptanone by *T. atroviride* was reported when co-cultured with *F. oxysporum* as compared to its axenic culture. The antifungal activity of ethanol against fungal pathogens has been attributed to disruption of the fungal membrane structure and cellular metabolic dysfunction (Dantigny et al. 2005, Alpha et al. 2015). The enhanced release of ethanol by both *H. perniciosus* and *C. mycophilum* and its direct antifungal properties in this study suggest its role in mycoparasitism towards *A. bisporus*.

Mushrooms are also known to produce VOCs with biological activities (Pinho et al. 2008, Ouzouni et al. 2009). The production of antifungal VOCs by a few edible mushrooms has been reported. For instance, Oka et al. (2015) reported the inhibitory activity of volatile compounds of the edible mushroom *Hypsizygus marmoreus* against *Alternaria brassicicola*. The volatile compound was identified as 2-methylpropanoic acid 2,2-dimethyl-1-(2-hydroxy-1-methylethyl)propyl ester. Petrovic et al. (2013) reported that the volatile compounds of *Laetiporus sulphureus* suppressed the growth of *Aspergillus flavus*. Nishino et al. (2013) demonstrated the antifungal activity of 1-phenyl-3-pentanone, a volatile compound from *Mycoleptodonoides aitchisonii*, against some phytopathogenic fungi. However, studies on volatile antimicrobials of fungi pathogenic to edible mushrooms are limited. To our knowledge, this is the first detailed study demonstrating the production of antifungal VOCs, more specifically ethanol, by *H. perniciosus* and *C. mycophilum*. Further research is needed to understand the relationship

between the ability to produce ethanol and the virulence of mycoparasitic fungi, and to study the morphological changes in *A. bisporus* upon exposure to VOCs of mycoparasitic fungi by using scanning electron microscopy.

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